

2013

USP 36

THE UNITED STATES PHARMACOPEIA

NF 31

THE NATIONAL FORMULARY

Volume 1

*By authority of the United States Pharmacopeial Convention
Prepared by the Council of Experts and its Expert Committees*

Official from May 1, 2013

The designation on the cover of this publication, "USP NF 2013," is for ease of identification only. The publication contains two separate compendia: *The United States Pharmacopeia*, Thirty-Sixth Revision, and *The National Formulary*, Thirty-First Edition.

THE UNITED STATES PHARMACOPEIAL CONVENTION
12601 Twinbrook Parkway, Rockville, MD 20852

SIX-MONTH IMPLEMENTATION GUIDELINE

The *United States Pharmacopeia–National Formulary* and its supplements become official **six months** after being released to the public. The *USP–NF*, which is released on November 1 of each year, becomes official on May 1 of the following year. This six-month implementation timing gives users more time to bring their methods and procedures into compliance with new and revised *USP–NF* requirements.

The table below describes the official dates of the *USP–NF* and its supplements. The 2011 *USP 35–NF 30*, and its supplements, *Interim Revision Announcements (IRAs)* and *Revision Bulletins* to that edition, will be official until May 1, 2013, at which time the *USP 36–NF 31* becomes official.

Publication	Release Date	Official Date	Official Until
<i>USP 36–NF 31</i>	November 1, 2012	May 1, 2013	May 1, 2014 (except as superseded by supplements, <i>IRAs</i> , and <i>Revision Bulletins</i>)
<i>First Supplement to the USP 36–NF 31</i>	February 1, 2013	August 1, 2013	May 1, 2014 (except as superseded by <i>Second Supplement</i> , <i>IRAs</i> , and <i>Revision Bulletins</i>)
<i>Second Supplement to the USP 36–NF 31</i>	June 1, 2013	December 1, 2013	May 1, 2014 (except as superseded by <i>IRAs</i> and <i>Revision Bulletins</i>)
<i>USP 37–NF 32</i>	November 1, 2013	May 1, 2014	May 1, 2015 (except as superseded by supplements, <i>IRAs</i> , and <i>Revision Bulletins</i>)

The table below gives the details of the *IRAs* that will apply to *USP 36–NF 31*.

IRA	PF Posting Date	Comment Due Date	IRA Posting Date	IRA Official Date
39(1)	January 2, 2013	March 31, 2013	May 31, 2013	July 1, 2013
39(2)	March 1, 2013	May 31, 2013	July 26, 2013	September 1, 2013
39(3)	May 1, 2013	July 31, 2013	September 27, 2013	November 1, 2013
39(4)	July 2, 2013	September 30, 2013	November 29, 2013	January 1, 2014
39(5)	September 4, 2013	November 30, 2013	January 31, 2014	March 1, 2014
39(6)	November 1, 2013	January 31, 2014	March 28, 2014	May 1, 2014

Revision Bulletins published on the USP website become official on the date specified in the *Revision Bulletin*.

NOTICE AND WARNING

Concerning U.S. Patent or Trademark Rights—The inclusion in *The United States Pharmacopeia* or in the *National Formulary* of a monograph on any drug in respect to which patent or trademark rights may exist shall not be deemed, and is not intended as, a grant of, or authority to exercise, any right or privilege protected by such patent or trademark. All such rights and privileges are vested in the patent or trademark owner, and no other person may exercise the same without express permission, authority, or license secured from such patent or trademark owner.

Concerning Use of USP or NF Text—Attention is called to the fact that *USP* and *NF* text is fully copyrighted. Authors and others wishing to use portions of the text should request permission to do so from the Secretary of the USPC Board of Trustees.

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Mission and Preface

USP 36–NF 31 and Supplements

This section provides background information on the United States Pharmacopeial Convention (USP), as well as general information about the 36th revision of the *United States Pharmacopeia* (USP 36) and the 31st edition of the *National Formulary* (NF 31) and its Supplements. Unless otherwise noted, the text in USP 36–NF 31 is official May 1, 2013, the text in the *First Supplement* to USP 36–NF 31 is official August 1, 2013, and the text in the *Second Supplement* to USP 36–NF 31 is official December 1, 2013.

MISSION STATEMENT

USP–NF is published in continuing pursuit of the mission of USP: *To improve the health of people around the world through public standards and related programs that help ensure the quality, safety, and benefit of medicines and foods.*

HISTORY

On January 1, 1820, 11 physicians met in the Senate Chamber of the U.S. Capitol building to establish a pharmacopeia for the United States. These practitioners sought to create a compendium of the best and most fully established medicines, give them useful names, and provide recipes for their preparation. Nearly a year later, on December 15, 1820, the first edition of *The Pharmacopoeia of the United States* was published. Over time, the nature of the *United States Pharmacopeia* (USP) changed from being a compendium of recipes to a compendium of documentary standards for identity and quality that typically involve reference materials used as comparison standards in specified tests and assays. The publishing schedule of USP also changed over time. From 1820 to 1942, USP was published at 10-year intervals; from 1942 to 2000, at five-year intervals; and beginning in 2002, annually.

In 1888, the American Pharmaceutical Association published the first *National Formulary* under the title *The National Formulary of Unofficial [sic] Preparations* (NF). Both USP and NF were recognized in the Federal Food and Drugs Act of 1906 and again in the Federal Food, Drug, and Cosmetic Act of 1938 (FD&C Act). In 1975, USP acquired the *National Formulary* (NF), which now contains excipient standards that also call for reference materials. USP continues to develop USP and NF, through the work of the Council of Experts, into compendia that provide standards for articles based on advances in analytical and metrological science. As these and allied sciences evolve, so do USP and NF.

CONTENT OF USP–NF

USP–NF contains official substance (ingredient) and preparation (product) monographs for official articles recognized in USP–NF. The terms *official substance*, *official preparation*, and *official article* are defined in the *General Notices and Requirements* (General Notices). With few exceptions, all articles for which monographs are provided in USP–NF are legally

marketed in the United States or are contained in legally marketed articles.

A USP–NF monograph for an official substance or preparation may consist of various components, including the article's name; definition; packaging, storage, and other requirements; and a specification. The specification consists of a series of universal tests (description, identity/identification, impurities, assay) and specific tests, one or more analytical procedures for each test, and acceptance criteria. Ingredients are defined as either drug substances or excipients. An excipient is any component, other than the active substance(s), intentionally added to the formulation of a dosage form. Excipients are not necessarily inert. Drug substances and excipients may be synthetic, semi-synthetic, drawn from nature (natural source), or manufactured using recombinant technology. Drugs that consist of larger molecules and mixtures requiring a potency test are usually referred to as biologicals or biotechnological articles.

General chapters provide frequently cited procedures, sometimes with acceptance criteria, in order to compile into one location repetitive information that appears in many monographs. New and revised monographs and general chapters and obsolete matter deleted from this edition are indicated in the *Admissions* section.

USP–NF Organization—USP–NF is printed as a three-volume set. *Volume 1* includes front matter (*Mission and Preface*, *People*, *Governance* pages and websites, and *Admissions/Annotations*). It also includes *USP General Notices*, general chapters, dietary supplement general chapters, *Reagents*, *Reference Tables*, *dietary supplement monographs*, *NF Admissions*, *Excipients*, and *NF monographs*. *Volume 2* includes USP monographs A–I, and *Volume 3* includes USP monographs J–Z. To facilitate convenient use and reference, all three volumes include the full index, as well as the *USP General Notices* and the *Guide to General Chapters*. General chapters specific to dietary supplements are included in numerical order with the rest of the general chapters in USP. Excipient monographs usually are presented in NF but also may appear in USP with suitable cross-referencing when they are also drug substances. The *Excipients* section (*Volume 1*) presents a tabulation of excipients by functional category.

Revisions to USP–NF—USP–NF is continuously revised. Revisions are presented annually as *Standard Revisions* in USP–NF and in twice-yearly *Supplements*, and as *Accelerated Revisions* on USP's website [*Errata*, *Interim Revision Announcements* (IRAs), and *Revision Bulletins*].

Standard Revisions—USP's Standard Revision Process calls for publication of a proposed revision in the *Pharmacopeial Forum* (PF) for a 90-day notice and comment period and, after the revision is approved by the relevant USP Expert Committee, publication in the next USP–NF or *Supplement*, as applicable.

Accelerated Revisions—The Accelerated Revision process is used to make revisions to USP–NF official more quickly than through USP's *Standard Revisions* process. Accelerated Revisions, which include *Errata*, IRAs, and *Revision Bulletins*, are posted on USP's website, do not always require notice and comment, and allow for a revision to become official prior

to the next *USP–NF* or *Supplement*. See the *USP Guideline on Use of Accelerated Processes for Revisions to the USP–NF*, which is posted on USP’s website.

Errata—An Erratum/Errata is content erroneously published in a USP publication that does not accurately reflect the intended official or effective requirements as approved by the Council of Experts. These typically are changes that do not have a broad impact on the standards. Errata are not subject to public comment and are communicated to the stakeholders by posting in the “New Official Text” section of USP’s website. As of *USP 36–NF 31* errata will no longer be published in the *USP–NF* and *Supplement* print products. Errata become official on the first day of the month following their posting to the USP website. Errata are incorporated into the next available *USP–NF* or *Supplement* and are tagged when printed as described below.

Interim Revision Announcements (IRAs)—An IRA appears in *PF* first as a *Proposed Interim Revision Announcement* with a 90-day comment period. If there are no significant comments, the IRA becomes official in the “New Official Text” section of USP’s website, with the official date indicated. IRAs are incorporated into the next available *USP–NF* or *Supplement*.

Revision Bulletins—If circumstances require rapid publication of official text, a revision or postponement may be published through a *Revision Bulletin*. *Revision Bulletins* are posted on USP’s website with the official date indicated. *Revision Bulletins* are incorporated into the next available *USP–NF* or *Supplement*.

Pharmacopeial Forum (PF)—The *PF* is USP’s official publication for public notice and comment. Proposals for revision are presented in the *In-Process Revision* or the *Proposed Interim Revision Announcement* (see above) sections and represent draft revisions that are expected to advance to official status pending final review and approval by the relevant Expert Committee.

On January 3, 2011, *PF* transitioned to an online-only publication that is available free of charge. The print version is no longer available. The new online-only *PF* includes proposed changes and additions to the *USP–NF*, including *Stage 4 Harmonization*, and *Stimuli* articles for which USP is seeking public comments. All proposals, including IRAs, will have a 90-day comment period. Other information that was contained in *PF*, including official text (final IRAs) is now published solely on USP’s website or moved into other USP publications.

This change to make *PF* freely available will help facilitate open and public participation when revisions are proposed to the *USP–NF*.

Supplements—*Supplements* to *USP–NF* follow a standard schedule each year: the *First Supplement* is published in February and becomes official August 1. The *Second Supplement* is published in June and becomes official December 1. Users of USP print products must retain *Supplements* and check the “New Official Text” section of USP’s website in order to have up-to-date official text. The *USP–NF* online version is updated with each *Supplement* or annual revision. Each time a new edition or *Supplement* is released during the subscription period, a new electronic version is issued. The *Index* in each *Supplement* is cumulative and includes citations to the annual revision and, for the *Second Supplement*, citations to the *First Supplement*. The contents of the two *Supplements* are integrated into the annual edition of the following year, along with new official revisions that have been adopted since the *Second Supplement* to the previous compendia.

USP–NF Spanish Edition—In 2006, USP began providing a Spanish edition of *USP–NF*. Maintenance of this edition follows the same revision approaches as the English edition.

USP Reference Standards—When approved for use as a comparison standard as a component of a USP monograph or other compendial procedure, use of USP Reference Standards promotes uniform quality of drugs and supports reliability and consistency by those performing compliance test-

ing and other users of *USP–NF*, including manufacturers, buyers, and regulatory authorities. The *USP Catalog*, which lists the collection of USP Reference Standards, can be accessed on USP’s website (www.usp.org). The listing identifies new items, replacement lots, lots of a single item that are simultaneously official, lots deleted from official status, and a preview of items eventually to be adopted. Purchase order information is included, and the names of distributors who can facilitate international availability of these items are suggested. This program benefits from the widespread voluntary contribution of suitable materials and test data from pharmaceutical manufacturers. USP advances this material via careful characterization studies and collaborative testing, followed by review and approval of the compendial use of the reference material by Expert Committees of the Council of Experts.

Symbols—Symbols identify the beginning and end of each revision, or nonharmonized text. The following table summarizes the types of symbols and the associated subscripts used in USP publications:

Revision Type	Symbol	Subscript
Interim Revision Announcement	● new text ● (IRA 1-Jul-2013)	(IRA 1-Jul-2013)*
Revision Bulletin	● new text ● (RB 1-Jan-2013)	(RB 1-Jan-2013)*
Text deletion	● (IRA 1-Jul-2013) OR ■ 1S (USP36) OR ▲▲ (USP36)	(IRA 1-Jul-2013)* 1S (USP36)* USP36**
Adopted in Supplement	■ new text ■ 1S (USP36)	1 or 2S (USP annual edition)*
Adopted in USP–NF	▲ new text ▲ (USP36)	USP annual edition**
Harmonization	◆ indicates residual national text or nonharmonized text	
Errata	● new text ● (ERR 1-Jul-2012)	(ERR 1-Jul-2012)

* A subscript number or date indicates the IRA, Revision Bulletin, or Supplement in which the revision first appeared.

** An example of a revision that was officially adopted in the *USP–NF* would be ▲ (USP36).

The following table shows symbols and official dates for IRAs and Supplements to *USP 36–NF 31*.

IRAs and Supplements to USP 36–NF 31 Official Dates and Symbols			
Supplement	Proposed IRA	Official Date	Symbols
1	39(1)	July 1, 2013	● and ● (IRA 1-Jul-2013)
		Aug. 1, 2013	■ and ■ 1S (USP36)
	39(2)	Sept. 1, 2013	● and ● (IRA 1-Sep-2013)
2	39(3)	Nov. 1, 2013	● and ● (IRA 1-Nov-2013)
		Dec. 1, 2013	■ and ■ 2S (USP36)
	39(4)	Jan. 1, 2014	● and ● (IRA 1-Jan-2014)
	39(5)	Mar. 1, 2014	● and ● (IRA 1-Mar-2014)
	39(6)	May 1, 2014	● and ● (IRA 1-May-2014)

Commentary—In accordance with USP’s *Rules and Procedures of the Council of Experts*, USP publishes all proposed revisions to *USP–NF* for public review and comment in the *PF*, USP’s bimonthly online journal for public notice and comment. After comments are considered and incorporated as the Expert Committee deems appropriate, the proposal may advance to official status or be republished in *PF* for further notice and comment, in accordance with the *Rules and Procedures*. In cases when proposals advance to official status without republication in *PF*, a summary of comments received and the appropriate Expert Committee’s responses are published in the *Commentary* section of the USP website at the time the revision is published.

The *Commentary* is not part of the official text and is not intended to be enforceable by regulatory authorities. Rather, it explains the basis of the Expert Committee's response to public comments. If there is a difference between the contents of the *Commentary* and the official text, the official text prevails. In case of a dispute or question of interpretation, the language of the official text, alone and independent of the *Commentary*, shall prevail.

Chemical Names and CAS Registry Numbers—Chemical subtitles given in the monographs are index names used by the Chemical Abstracts Service (CAS) of the American Chemical Society. They are provided only in monographs in which the titles specify substances that are definable chemical entities. The first subtitle is the inverted form of the systematic chemical name developed by CAS for the purpose of the Collective Index (CI). The second subtitle, given in uninverted form, is a preferred IUPAC name (PIN) sanctioned and used by the International Union of Pure and Applied Chemistry (IUPAC). Preferred IUPAC names are also used by the World Health Organization (WHO). Occasionally a third subtitle is supplied for historical reasons or when the synonym uses an alternative, but equivalent, naming convention. Monographs with chemical subtitles also generally carry CAS registry numbers. These bracketed numbers function independently of nomenclature as invariant numerical designators of unique, unambiguous chemical substances in the CAS registry and thus are convenient and widely used.

Print and Electronic Presentations—All USP–NF publications are available in print form (with the exception of the *Pharmacopeial Forum* and *Accelerated Revisions*, discussed above, which are posted on USP's website until incorporation into the next USP–NF or *Supplement*). In addition, USP–NF and its two annual *Supplements* are available in USB flash drive and online versions. The USB flash drive version makes USP–NF accessible to users on their computer hard drives. The online format allows individual registered users to access the online format through the Internet. Both electronic formats provide access to official USP–NF content, along with extensive search options. The electronic formats are cumulatively updated to integrate the content of *Supplements*. A searchable electronic version of the *USP Dictionary* also is available.

USP GOVERNING, STANDARDS-SETTING, AND ADVISORY BODIES

USP's governing, standards-setting, and advisory bodies include the USP Convention, the Board of Trustees, the Council of Experts and its Expert Committees, Expert Panels (formerly known as Advisory Panels), and staff. Additional volunteer bodies include Stakeholder Forums, Project Teams, and Advisory Groups, which act in an advisory capacity to provide input to USP's governing, standards-setting, and management bodies.

USP Convention—The composition of the USP Convention membership is designed to ensure a global representation from all sectors of health care, with an emphasis on practitioners, given USP's practitioner heritage (see the *History* section). Voting Delegates of Convention member organizations elect USP's President, Treasurer, other members of the Board of Trustees, and the Council of Experts. They also adopt resolutions to guide USP's strategic direction and amend USP's Bylaws. Convening on a 5-year cycle, the last meeting of the USP Convention occurred in April 2010 in Washington, DC. A listing of all current Voting Delegates of the USP Convention is included in the *People* section.

Board of Trustees—USP's Board of Trustees is responsible for the management of the business affairs, finances, and property of USP. During its 5-year term, the Board defines USP's strategic direction through its key policy and operational decisions. A listing of the members of the 2010–2015 Board of Trustees is included in the *People* section.

Council of Experts—The Council of Experts is the standards-setting body of USP. For the 2010–2015 cycle it is composed of 22 members, elected to 5-year terms by USP's Convention, each of whom chairs an Expert Committee. These Chairs in turn elect the members of their Expert Committees. The Expert Committees are responsible for the content of USP's official and authorized publications (see *Figure 1*). The Executive Committee of the Council of Experts includes all Expert Committee Chairs and provides overall direction, is an appeals body, and performs other functions that support the Council of Experts' operations.

Expert Panels to the Council of Experts—The Chair of the Council of Experts may appoint Expert Panels to assist the Council of Experts by providing advisory recommendations to particular Expert Committees in response to a specific charge consistent with the Expert Committee's Work Plan. Expert Panels are continuously formed; their topics and membership appear in the *People* section.

Stakeholder Forums and Project Teams—USP has formed several domestic and international Stakeholder Forums and Project Teams to exchange information on USP's standards-setting activities. Stakeholder Forums may form Project Teams to work on selected topics. The following lists the current USP Stakeholder Forums.

North American Stakeholder Forums (United States and Canada)

- Prescription/Nonprescription
- Dietary Supplements
- Food Ingredients
- Veterinary Drugs

International Stakeholder Forums

- India
- Mexico
- Brazil
- Others

USP also conducts Scientific and Standards Symposia (formerly Annual Scientific Meetings) in the United States, India, China, Latin America, Middle East/North Africa, and other regions of the world.

Staff—USP maintains a staff of over 700 scientists, professionals, and administrative personnel at its Rockville, Maryland, headquarters and throughout the world, including an account management office in Basel, Switzerland, and laboratory facilities in Hyderabad, India; Shanghai, China; and São Paulo, Brazil.

RULES AND PROCEDURES

Governing Documents—USP–NF standards are recognized widely because they are authoritative and science-based and are established by a transparent and credible process. See the *Articles of Incorporation* section in this book; the *Bylaws* and the *Rules and Procedures of the Council of Experts* are available on USP's website (www.usp.org). Collectively, these documents serve USP volunteers and staff as the governing principles for USP's standards-setting activities.

Conflicts of Interest—USP's Conflict of Interest provisions require all members of the Council of Experts, its Expert Committees, Expert Panels, Board of Trustees, and key staff to disclose financial or other interests that may interfere with their duties as USP volunteers. Members of the Board of Trustees, Council of Experts, and its Expert Committees are required to serve USP as individual experts and not serve any outside interest, and are not allowed to take part in the final discussion or vote on any matter in which they have a conflict of interest or the appearance of a conflict of interest. Members of advisory Expert Panels may participate and vote, so long as any notable interests and conflicts have been adequately and promptly disclosed and are communicated to the relevant Expert Committee along with any Expert Panel recommendations.

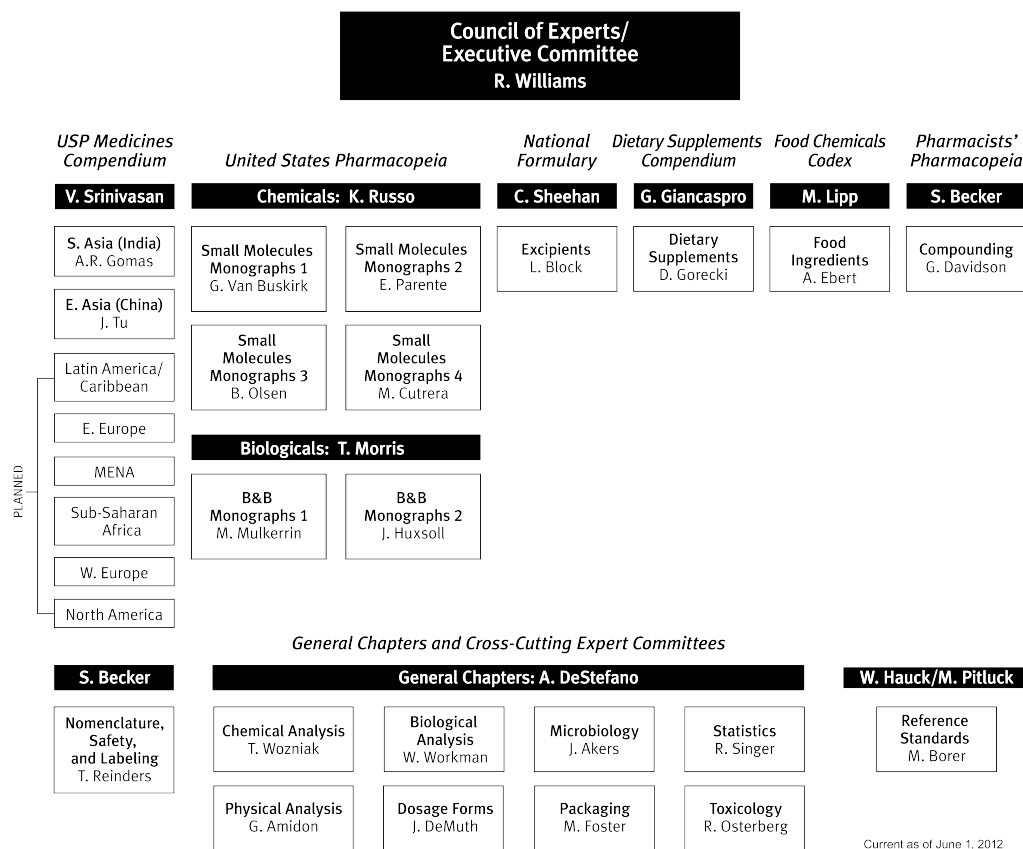


Figure 1. 2010–2015 USP Council of Experts.

Confidentiality and Document Disclosure—Members of the Council of Experts, Expert Committees, and Expert Panels sign confidentiality agreements, in keeping with USP's Confidentiality Policy and the confidentiality provisions of the *Rules and Procedures of the Council of Experts*. The USP Document Disclosure Policy, available on USP's website, contributes to the transparency of the standards-setting process by making information available to the public, yet provides protection to manufacturers and others who submit confidential information to USP.

Authority for Publication—*USP–NF* is published in accordance with Article II, Purposes, of the USP Bylaws, which states, "The purposes for which the Convention is formed are as set forth in the Articles of Incorporation and include developing and disseminating public standards for medicines and other articles, and engaging in related public health programs."

USP–NF REVISION PROCESS

Public Participation—Although USP's Council of Experts is the ultimate decision-making body for *USP–NF* standards, these standards are developed by an exceptional process of public involvement and substantial interaction between USP and its stakeholders, both domestically and internationally. Participation in the revision process results from the support of many individuals and groups and also from scientific, technical, and trade organizations.

Requests for Revision of the *USP–NF*, whether new monographs or general chapters or those needing updating, contain information submitted voluntarily by manufacturers and other interested parties. At times USP staff and Expert Committees may develop information to support a *Request for Revision*. USP has prepared a document titled *Guideline for*

Submitting Requests for Revision to USP–NF (available at www.usp.org; search on "Submission Guidelines"). Via *PF*, USP solicits and encourages public comment on these revision proposals. Comments received are considered by the Expert Committees, who determine whether changes should be made to the proposed revisions based on such comments. Proposed standards are finalized when Expert Committees vote to make them official text in *USP–NF*. Thus, the USP standards-setting process gives those who manufacture, regulate, and use therapeutic products the opportunity to comment on the development and revision of *USP–NF* standards. *Figure 2* shows the public review and comment process and its relationship to standards development.

Working with the Food and Drug Administration (FDA)—As specified in U.S. law, USP works with the Secretary of the Department of Health and Human Services in many ways. The principal agency in the Department for this work is the Food and Drug Administration. The FDA Liaison Program allows FDA representatives to participate in Expert Committee and Expert Panel meetings, enabling interactions between FDA scientific staff and Expert Committees. Staff in the FDA Centers who are responsible for review of compendial activities provide specific links and opportunities for exchange of comments. Dr. Paul Seo in the Center for Drug Evaluation and Research provides a primary compendial point of contact between FDA and USP.

LEGAL RECOGNITION

Recognition of *USP–NF*—*USP–NF* is recognized by law and custom in many countries throughout the world. In the United States, the FD&C Act defines the term "official compendium" as the official *USP*, the official *NF*, the official *Homeopathic Pharmacopeia of the United States*, or any supple-

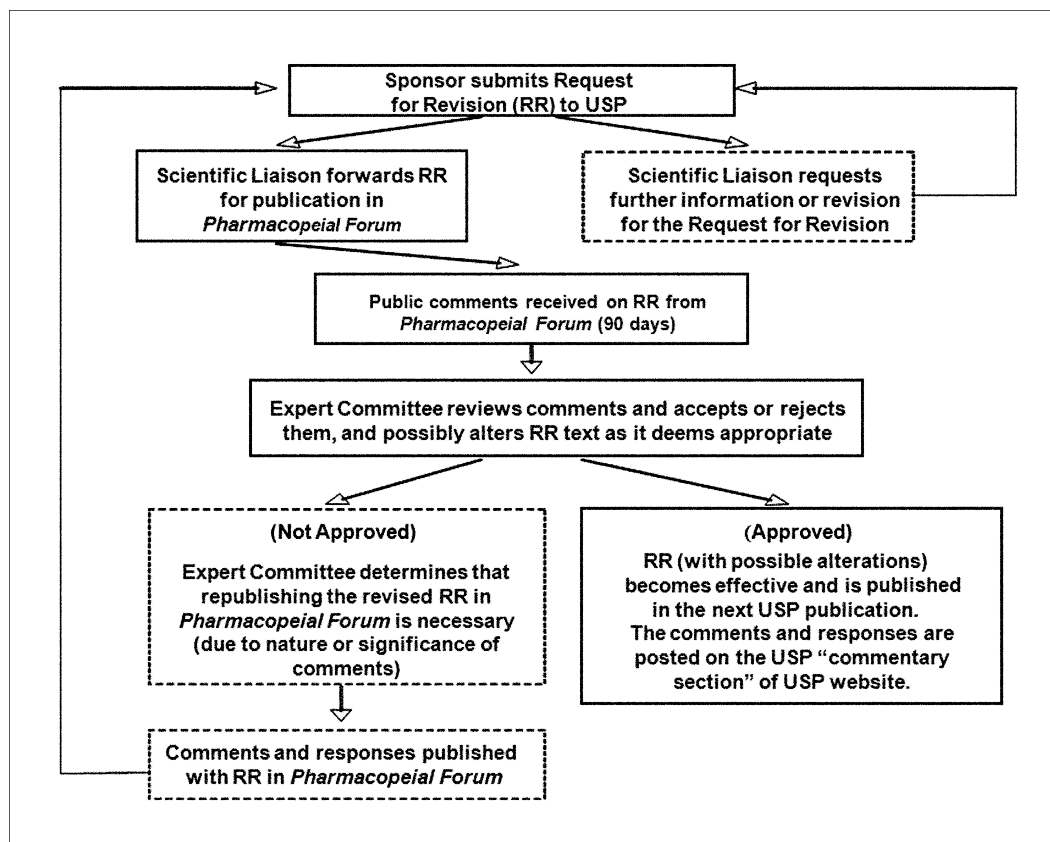


Figure 2. USP's standards-setting public review and comment process.

ment to them. As noted below (and in *General Notices* section 2.30), *USP–NF* standards play a role in the adulteration and misbranding provisions of the FD&C Act (which apply as well to biologics, a subset of drugs, under the Public Health Service Act). USP has no role in enforcement of these or other provisions that recognize *USP–NF* standards, which is the responsibility of FDA and other government authorities in the United States and elsewhere.

Under the relevant FD&C Act provisions, a drug will be deemed misbranded unless its label bears to the exclusion of any other nonproprietary name the “established” name, which ordinarily is the compendial name (see discussion of *Nomenclature*, below). A drug with a name recognized in *USP–NF* must comply with the identity/identification requirements of its monograph, or be deemed adulterated, misbranded, or both. Drugs also must comply with compendial standards for strength, quality, and purity (tests for assay and impurities), unless labeled to show all respects in which the drugs differ. FDA requires that names for articles that are not official must be clearly distinguished and differentiated from any name recognized in an official compendium. Drugs with a name recognized in *USP–NF* also will be considered misbranded unless they meet compendial standards for packaging and labeling.

Drugs—USP's goal is to have substance and preparation (product) monographs in *USP–NF* for all FDA-approved drugs, including biologics, and their ingredients. USP also develops monographs for legally marketed therapeutic products not approved by FDA, e.g., pre-1938 drugs, over-the-counter (OTC) drugs marketed under FDA's OTC Monograph system, dietary supplements, and compounded preparations. Although submission of information needed to develop a monograph by the Council of Experts is voluntary, compliance with a *USP–NF* monograph, if applicable, is mandatory.

Biologics—In the United States, all biologics are considered a subset of drugs, whether they are approved by FDA under the FD&C Act (and receive a new drug application [NDA]) or under the Public Health Service Act (PHS Act, where they receive a biologics license application [BLA]). As a result, all PHS Act biologics are subject to the drug regulatory requirements of the FD&C Act, which means they are required to comply with the adulteration and misbranding provisions of the FD&C Act, including *USP–NF* compendial requirements. This is equally so for biologics approved under the longstanding PHS Act “351(a)” pathway, as well as the new “351(k)” pathway for biosimilars added by the 2010 healthcare reform legislation (Biologics Price Competition and Innovation Act, Title VII, Subtitle A of the Patient Protection and Affordable Care Act, Public Law 111-148).

Medical Devices—Section 201(h) of the FD&C Act defines a device as an instrument, apparatus, similar article, or component thereof recognized in *USP–NF*. Section 502(e) of the FD&C Act defines the established name of a device in the absence of an FDA designation of the official name as the official title in an official compendium. Despite these statutory provisions, there is no comparable recognition of USP's role in establishing compendial standards for medical devices as exists for drugs and biologics. Under authority granted by the Food and Drug Administration Modernization Act of 1997, the Center for Devices and Radiological Health recognizes national and international standards, including some *USP* tests and assays, for medical devices.

Dietary Supplements—The Dietary Supplement Health and Education Act of 1994 amendments to the FD&C Act provide that a dietary supplement may be deemed a misbranded food if it is covered by the specifications of an official compendium (e.g., *USP–NF*), is represented as conforming to the specifications of an official compendium, and fails to so conform. This contrasts with pharmaceutical products, wherein conformance to applicable compendial stan-

dards is mandatory, whether or not the product claims to conform.

Compounded Preparations—Compounding means the preparation, mixing, assembling, altering, packaging, and labeling of a drug or device or other article, as the result of a practitioner's order or in anticipation of such an order based on routine, regularly observed prescribing patterns. USP provides both general chapters and monographs for compounded preparations. Compounded preparation monographs include formulas (ingredients and quantities), specific directions to correctly compound the particular preparation, packaging and storage information, labeling information, pH, beyond-use dates based on stability studies, and detailed assays (majority of monographs). Standards in *USP–NF* for compounded preparations may be enforced by both the states (as pharmacy practice/compounding is traditionally regulated by state boards of pharmacy), and FDA (as compounded preparations subject to FDA regulation as drugs remain subject to the adulteration and misbranding provisions of the FD&C Act, which require conformance to *USP–NF* standards).

Nomenclature—USP, as a member of the United States Adopted Names (USAN) Council, works to determine names for drug and biological substances. USP's authority to develop official nonproprietary names is identified in the misbranding provision of the FD&C Act, section 502(e) (see also FDA's policy on established names set forth in 21 CFR 299.4). Under both USP rules, and applicable federal law, official names mean the official title of an article recognized in *USP* or *NF*, which is determined when a monograph for the article is published, including the article's name in the monograph title. USP Expert Committees may not complete work on an applicable monograph until after FDA has licensed a drug or biologic, or USAN has designated a name. FDA-approved nonproprietary names are considered by FDA and the courts to be interim names that exist only unless and until USP designates a name. Congress in 1962 gave FDA the authority to change a USP-designated name; in the event FDA finds a USP name to be unduly complex or not useful for some other reason, the agency may conduct notice and comment rulemaking under section 508 of the FD&C Act, and designate a different official name for use in *USP* and *NF*. In contrast to USP's role in designating nonproprietary names, the designation of proprietary (brand) names is solely the responsibility of FDA, working with applicants.

The USP Nomenclature Expert Committee, the predecessor to the 2010–2015 Nomenclature, Safety, and Labeling (NSL) Expert Committee, was formed in 1986 to create appropriate established names for dosage forms and combination drug products, and to develop naming policies. Today, the NSL Expert Committee coordinates its work with the USAN Council, and in the great majority of cases retains the existing name given by USAN or FDA. The NSL also establishes the Pronunciation Guide, which is used by USAN.

The USAN Council began in 1961 by providing ingredient names for drugs prior to their marketing. USP participates in this activity, together with the American Medical Association, the American Pharmacists Association, and FDA. The Council's output is incorporated into the *USP Dictionary of USAN and International Drug Names* (see *USP Dictionary*, below).

HARMONIZATION ACTIVITIES

Pharmacopeial Discussion Group—USP harmonizes pharmacopeial excipient monographs and general chapters through the Pharmacopeial Discussion Group (PDG), which

includes representatives from the European, Japanese, and United States pharmacopeias, and WHO (as an observer). According to the PDG definition, "a pharmacopeial general chapter or other pharmacopeial document is harmonized when a pharmaceutical substance or product tested by the document's harmonized procedure yields the same results, and the same accept/reject decision is reached." General information chapter <1196>, *Pharmacopeial Harmonization*, provides (1) the PDG Policy Statement, (2) the PDG Working Procedures and a definition of each stage of harmonization, (3) a discussion, (4) a status report, and (5) a glossary. More information regarding PDG is available on USP's website.

OTHER USP PUBLICATIONS

Chromatographic Columns—This comprehensive reference, previously titled *Chromatographic Reagents*, provides detailed information needed to conduct chromatographic procedures found in *USP–NF*. *Chromatographic Columns* lists the brand names of the column reagents cited in every proposal for new or revised gas- or liquid-chromatographic analytical procedures that have been published in *PF* since 1980. *Chromatographic Columns* also helps to track which column reagents were used to validate analytical procedures that have become official. The branded column reagents list is updated bimonthly and maintained on USP's website.

USP Dictionary—The *USP Dictionary of USAN and International Drug Names* provides in a single volume the most up-to-date United States Adopted Names of drugs; official *USP–NF* names; nonproprietary, brand, and chemical names; graphic formulas; molecular formulas and weights; CAS registry numbers and code designations; drug manufacturers; and pharmacologic and therapeutic categories. The *Dictionary* helps to ensure the accuracy of the following: product labeling; reports, articles, and correspondence; FDA regulatory filings; and pharmaceutical package inserts. It is published annually. (See *Nomenclature*.)

USP Dietary Supplements Compendium—The *Dietary Supplements Compendium* combines, in a single volume, *USP–NF* standards for dietary supplements, standards and information from the *Food Chemicals Codex*, regulatory and industry documents, and other tools and resources. It is published every 2 years, as a hardcover print edition.

Food Chemicals Codex—The *Food Chemicals Codex* (FCC) is a compendium of internationally recognized monograph standards and tests for the purity and quality of food ingredients, e.g., preservatives, flavorings, colorings, and nutrients. FCC is published every 2 years with supplements every 6 months, and is available in print and electronic formats. Proposed revisions to FCC are available for public viewing and comment through the *FCC Forum*. The *FCC Forum* can be accessed for free at forum.foodchemicalscodex.org.

USP Medicines Compendium—The *USP Medicines Compendium* (MC) is an online compendium that includes monographs, general chapters, and reference materials for suitable chemical and biological medicines and their ingredients approved by national regulatory authorities. The purpose of the MC is to help ensure that these medicines are of good quality by providing up-to-date, relevant public standards and reference materials. MC standards are available to manufacturers, purchasers, national regulatory authorities, and others to ensure conformity of a medicine to MC standards through testing. The MC does not include standards for foods or for traditional medicines/dietary supplements. The MC is available at www.usp-mc.org.

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USP 36

**THE UNITED STATES
PHARMACOPEIA**

Official from May 1, 2013

THIRTY-SIXTH REVISION

Admissions

Articles Admitted to *USP 36* by Supplement

First Supplement (August 1, 2012)

GENERAL CHAPTERS

⟨1032⟩ Design and Development of Biological Assays

⟨1033⟩ Biological Assay Validation

⟨1034⟩ Analysis of Biological Assays

⟨1105⟩ Immunological Test Methods—Surface Plasmon Resonance

⟨1644⟩ Theory and Practice of Electrical Conductivity Measurements of Solutions

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Anagrelide Capsules

Celecoxib

Cetirizine Hydrochloride Tablets

Esomeprazole Magnesium Delayed-Release Capsules

Estazolam

Estazolam Tablets

Felbamate

Felbamate Oral Suspension

Felbamate Tablets

Lamotrigine Tablets for Oral Suspension

Montelukast Sodium

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Tacrolimus Capsules

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Second Supplement (December 1, 2012)

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Diosmin

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 Duloxetine Delayed-Release Capsules
 Esmolol Hydrochloride
 Fenofibrate Tablets
 Fosfomycin Tromethamine

Goserelin Acetate
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 Olmesartan Medoxomil
 Sildenafil Citrate
 Sumatriptan Injection
 Venlafaxine Hydrochloride Extended-Release Capsules
 Voriconazole

New Articles Appearing in *USP 36* That Were Not Included in *USP 35* Including Supplements

[NOTE—The articles included in this list are noted in the book with the following symbols ▲ ▲ *USP36*. This applies to new articles as well as sections of existing items that have been revised.]

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⟨17⟩ Prescription Container Labeling

⟨1761⟩ Applications of Nuclear Magnetic Resonance Spectroscopy

⟨1197⟩ Good Distribution Practices for Bulk Pharmaceutical Excipients

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Black Pepper
 Powdered Black Pepper
 Powdered Black Pepper Extract
 Melatonin Tablets
 Oil-Soluble Vitamins Oral Solution

Oil-Soluble Vitamins With Minerals Capsules
 Oil-Soluble Vitamins With Minerals Oral Solution
 Oil-Soluble Vitamins With Minerals Tablets
meso-Zeaxanthin
meso-Zeaxanthin Preparation

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 Amiodarone Hydrochloride Oral Suspension
 Amlodipine Oral Suspension
 Chloroquine Phosphate Oral Suspension
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 Dapsone Oral Suspension
 Didanosine Delayed-Release Capsules
 Doxycycline Tablets
 Enalapril Maleate Oral Suspension
 Hydrochloric Acid Injection
 Iron, Carbonyl
 Isradipine Oral Suspension
 Lisinopril Oral Suspension

Omeprazole Oral Suspension
 Pentoxifylline Oral Suspension
 Phenobarbital Oral Suspension
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 Pyrazinamide Oral Suspension

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 Rifabutin Oral Suspension
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 Sodium Phenylbutyrate Oral Suspension
 Sotalol Hydrochloride Oral Suspension
 Spironolactone and Hydrochlorothiazide Oral Suspension
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ANNOTATED LIST

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Page citations refer to the pages of USP 36. Note—In the table below, if a section is new or if a subsection is added to or deleted from an existing section, it is labeled as such in parentheses after the section or subsection name. Items on this list that appear without the designation “new”, “added”, or “deleted” are items in which changes have been made to existing official text.

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GENERAL NOTICES AND REQUIREMENTS

The *General Notices and Requirements* section (the *General Notices*) presents the basic assumptions, definitions, and default conditions for the interpretation and application of the *United States Pharmacopeia* (USP) and the *National Formulary* (NF).

Requirements stated in these *General Notices* apply to all articles recognized in the USP and NF (the “compendia”) and to all general chapters unless specifically stated otherwise. Where the requirements of an individual monograph differ from the *General Notices* or a general chapter, the monograph requirements apply and supersede the requirements of the *General Notices* or the general chapter, whether or not the monograph explicitly states the difference.

1. TITLE AND REVISION

The full title of this publication (consisting of three volumes and including its *Supplements*), is *The Pharmacopeia of the United States of America*, Thirty-Sixth Revision and the *National Formulary*, Thirty-First Edition. These titles may be abbreviated to USP 36, to NF 31, and to USP 36–NF 31. The *United States Pharmacopeia*, Thirty-Sixth Revision, and the *National Formulary*, Thirty-First Edition, supersede all earlier revisions. Where the terms “USP,” “NF,” or “USP–NF” are used without further qualification during the period in which these compendia are official, they refer only to USP 36, NF 31, and any *Supplement(s)* thereto. The same titles, with no further distinction, apply equally to print or electronic presentation of these contents. Although USP and NF are published under one cover and share these *General Notices*, they are separate compendia.

This revision is official beginning May 1, 2013, unless otherwise indicated in specific text.

Supplements to USP and NF are published periodically.

Interim Revision Announcements are revisions to USP and NF that are published on the USP website. *Interim Revision Announcements* contain official revisions and their effective dates. Announcements of the availability of new USP Reference Standards and announcements of tests or procedures that are held in abeyance pending availability of required USP Reference Standards are also available on the “New Official Text” tab of USP’s website.

Revision Bulletins are revisions to official text or postponements that require expedited publication. They are published on the USP website and generally are official immediately unless otherwise specified in the *Revision Bulletin*.

Errata are corrections to items erroneously published that have not received the approval of the Council of Experts and that do not reflect the official requirements. *Errata* are effective upon publication.

2. OFFICIAL STATUS AND LEGAL RECOGNITION

2.10. Official Text

Official text is text contained in USP and NF, including monographs, general chapters, and these *General Notices*. Revisions to official text are provided in *Supplements*, *Interim Revision Announcements*, and *Revision Bulletins*. General chapters numbered from 1000 to 1999 are considered interpretive and are intended to provide information on, give definition to, or describe a particular subject. They contain no mandatory requirements applicable to any official article unless specifically referenced in *General Notices*, a monograph, or a general chapter numbered below 1000. General chapters numbered above 2000 apply only to articles that are

intended for use as dietary ingredients and dietary supplements.

2.20. Official Articles

An *official article* is an article that is recognized in USP or NF. An article is deemed to be recognized and included in a compendium when a monograph for the article is published in the compendium and an official date is generally or specifically assigned to the monograph.

The title specified in a monograph is the *official title* for such article. Other names considered to be synonyms of the official titles may not be used as substitutes for official titles.

Official articles include both *official substances* and *official products*. An *official substance* is a drug substance, excipient, dietary ingredient, other ingredient, or component of a finished device for which the monograph title includes no indication of the nature of the finished form.

An *official product* is a drug product, dietary supplement, compounded preparation, or finished device for which a monograph is provided.

2.30. Legal Recognition

The USP and NF are recognized in the laws and regulations of many countries throughout the world. Regulatory authorities may enforce the standards presented in the USP and NF, but because recognition of the USP and NF may vary by country, users should understand applicable laws and regulations. In the United States under the Federal Food, Drug, and Cosmetic Act (FDCA), both USP and NF are recognized as official compendia. A drug with a name recognized in USP–NF must comply with compendial identity standards or be deemed adulterated, misbranded, or both. See, e.g., FDCA § 501(b) and 502(e)(3)(b); also FDA regulations, 21 CFR § 299.5(a&b). To avoid being deemed adulterated, such drugs must also comply with compendial standards for strength, quality, and purity, unless labeled to show all respects in which the drug differs. See, e.g., FDCA § 501(b) and 21 CFR § 299.5(c). In addition, to avoid being deemed misbranded, drugs recognized in USP–NF must also be packaged and labeled in compliance with compendial standards. See FDCA § 502(g).

A dietary supplement represented as conforming to specifications in USP will be deemed a misbranded food if it fails to so conform. See FDCA § 403(s)(2)(D).

Enforcement of USP standards is the responsibility of FDA and other government authorities in the U.S. and elsewhere. USP has no role in enforcement.

3. CONFORMANCE TO STANDARDS

3.10. Applicability of Standards

Standards for an article recognized in a USP compendium are expressed in the article’s monograph, applicable general chapters, and *General Notices*. Unless specifically exempted elsewhere in a compendium, the identity, strength, quality, and purity of an article are determined by the official tests, procedures, and acceptance criteria, whether incorporated in the monograph itself, in the *General Notices*, or in the applicable general chapters. Early adoption of revised standards is allowed. Where revised standards for an existing article have been published as final approved “official text” (as approved in section 2.10) but are not yet official (six months after publication, unless otherwise specified; see “official date,” section 2.20) compliance with the revised standard shall not preclude a finding or indication of conformance with USP official standards, unless USP specifies

otherwise by prohibiting early adoption in a particular standard.

The standards in the relevant monograph, general chapter(s), and *General Notices* apply at all times in the life of the article from production to expiration. The manufacturer's specifications, and good manufacturing practices generally (including, e.g., Quality by Design initiatives), are developed and followed to ensure that the article will comply with compendial standards until its expiration date, when stored as directed. Thus, any official article is expected to meet the compendial standards if tested, and any official article actually tested as directed in the relevant monograph must meet such standards to demonstrate compliance.

At times, compendial standards take on the character of statistical procedures, with multiple units involved and perhaps a sequential procedural design to allow the user to determine that the tested article meets or does not meet the standard. The similarity to statistical procedures may seem to suggest an intent to make inference to some larger group of units, but in all cases, statements about whether the compendial standard is met apply only to the units tested. Repeats, replicates, statistical rejection of outliers, or extrapolations of results to larger populations, as well as the necessity and appropriate frequency of batch testing, are neither specified nor proscribed by the compendia. Frequency of testing and sampling are left to the preferences or direction of those performing compliance testing, and other users of *USP-NF*, including manufacturers, buyers, or regulatory authorities.

Official products are prepared according to recognized principles of good manufacturing practice and from ingredients that meet *USP* or *NF* standards, where standards for such ingredients exist (for dietary supplements, see section 3.10.20).

Official substances are prepared according to recognized principles of good manufacturing practice and from ingredients complying with specifications designed to ensure that the resultant substances meet the requirements of the compendial monographs.

3.10.10. Applicability of Standards to Drug Products, Drug Substances, and Excipients

The applicable *USP* or *NF* standard applies to any article marketed in the United States that (1) is recognized in the compendium and (2) is intended or labeled for use as a drug or as an ingredient in a drug. The applicable standard applies to such articles whether or not the added designation "*USP*" or "*NF*" is used. The standards apply equally to articles bearing the official titles or names derived by transposition of the definitive words of official titles or transposition in the order of the names of two or more active ingredients in official titles, or where there is use of synonyms with the intent or effect of suggesting a significant degree of identity with the official title or name.

3.10.20. Applicability of Standards to Medical Devices, Dietary Supplements, and Their Components and Ingredients

An article recognized in *USP* or *NF* shall comply with the compendial standards if the article is a medical device, component intended for a medical device, dietary supplement, dietary ingredient, or other ingredient that is intended for incorporation into a dietary supplement, and is labeled as conforming to the *USP* or *NF*.

Generally, dietary supplements are prepared from ingredients that meet *USP*, *NF*, or *Food Chemicals Codex* standards. Where such standards do not exist, substances may be used in dietary supplements if they have been shown to be of acceptable food grade quality using other suitable procedures.

3.20. Indicating Conformance

A drug product, drug substance, or excipient may use the designation "*USP*" or "*NF*" in conjunction with its official title or elsewhere on the label only when (1) a monograph is provided in the specified compendium and (2) the article

complies with the identity prescribed in the specified compendium.

When a drug product, drug substance, or excipient differs from the relevant *USP* or *NF* standard of strength, quality, or purity, as determined by the application of the tests, procedures, and acceptance criteria set forth in the relevant compendium, its difference shall be plainly stated on its label.

When a drug product, drug substance, or excipient fails to comply with the identity prescribed in *USP* or *NF* or contains an added substance that interferes with the prescribed tests and procedures, the article shall be designated by a name that is clearly distinguishing and differentiating from any name recognized in *USP* or *NF*.

A medical device, dietary supplement, or ingredient or component of a medical device or dietary supplement may use the designation "*USP*" or "*NF*" in conjunction with its official title or elsewhere on the label only when (1) a monograph is provided in the specified compendium and (2) the article complies with the monograph standards and other applicable standards in the compendium.

The designation "*USP*" or "*NF*" on the label may not and does not constitute an endorsement by *USP* and does not represent assurance by *USP* that the article is known to comply with the relevant standards. *USP* may seek legal redress if an article purports to be or is represented as an official article in one of *USP*'s compendia and such claim is determined by *USP* not to be made in good faith.

The designation "*USP-NF*" may be used on the label of an article provided that the label also bears a statement such as "*Meets NF standards as published by USP,*" indicating the particular compendium to which the article purports to apply.

When the letters "*USP*," "*NF*," or "*USP-NF*" are used on the label of an article to indicate compliance with compendial standards, the letters shall appear in conjunction with the official title of the article. The letters are not to be enclosed in any symbol such as a circle, square, etc., and shall appear in capital letters.

If a dietary supplement does not comply with all applicable compendial requirements but contains one or more dietary ingredients or other ingredients that are recognized in *USP* or *NF*, the individual ingredient(s) may be designated as complying with *USP* or *NF* standards or being of *USP* or *NF* quality provided that the designation is limited to the individual ingredient(s) and does not suggest that the dietary supplement complies with *USP* standards.

4. MONOGRAPHS AND GENERAL CHAPTERS

4.10. Monographs

Monographs set forth the article's name, definition, specification, and other requirements related to packaging, storage, and labeling. The specification consists of tests, procedures, and acceptance criteria that help ensure the identity, strength, quality, and purity of the article. For general requirements relating to specific monograph sections, see section 5, *Monograph Components*.

Because monographs may not provide standards for all relevant characteristics, some official substances may conform to the *USP* or *NF* standard but differ with regard to nonstandardized properties that are relevant to their use in specific preparations. To assure interchangeability in such instances, users may wish to ascertain functional equivalence or determine such characteristics before use.

4.10.10. Applicability of Test Procedures

A single monograph may include several different tests, procedures, and/or acceptance criteria that reflect attributes of different manufacturers' articles. Such alternatives may be presented for different polymorphic forms, impurities, hydrates, and dissolution cases. Monographs indicate the tests, procedures, and/or acceptance criteria to be used and the required labeling.

A test in a monograph may contain and require multiple procedures. However, multiple procedures may be included in particular monographs specifically for the purpose of assuring the availability of an appropriate procedure for a par-

ticular product. In such cases, a labeling statement to indicate the appropriate application of the procedure(s) will be included in the monograph. A labeling statement is not required if Test 1 is used.

4.10.20. Acceptance Criteria

The acceptance criteria allow for analytical error, for unavoidable variations in manufacturing and compounding, and for deterioration to an extent considered acceptable under practical conditions. The existence of compendial acceptance criteria does not constitute a basis for a claim that an official substance that more nearly approaches 100 percent purity "exceeds" compendial quality. Similarly, the fact that an article has been prepared to tighter criteria than those specified in the monograph does not constitute a basis for a claim that the article "exceeds" the compendial requirements.

An official product shall be formulated with the intent to provide 100 percent of the quantity of each ingredient declared on the label. Where the minimum amount of a substance present in a dietary supplement is required by law to be higher than the lower acceptance criterion allowed for in the monograph, the upper acceptance criterion contained in the monograph may be increased by a corresponding amount.

The acceptance criteria specified in individual monographs and in the general chapters for compounded preparations are based on such attributes of quality as might be expected to characterize an article compounded from suitable bulk drug substances and ingredients, using the procedures provided or recognized principles of good compounding practice, as described in these compendia.

4.20. General Chapters

Each general chapter is assigned a number that appears in angle brackets adjacent to the chapter name (e.g., *Chromatography* (621)). General chapters may contain the following:

- Descriptions of tests and procedures for application through individual monographs,
- Descriptions and specifications of conditions and practices for pharmaceutical compounding,
- General information for the interpretation of the compendial requirements,
- Descriptions of general pharmaceutical storage, dispensing, and packaging practices, or
- General guidance to manufacturers of official substances or official products.

When a general chapter is referenced in a monograph, acceptance criteria may be presented after a colon.

Some chapters may serve as introductory overviews of a test or of analytical techniques. They may reference other general chapters that contain techniques, details of the procedures, and, at times, acceptance criteria.

5. MONOGRAPH COMPONENTS

5.10. Molecular Formula

The use of the molecular formula for the active ingredient(s) named in defining the required strength of a compendial article is intended to designate the chemical entity or entities, as given in the complete chemical name of the article, having absolute (100 percent) purity.

5.20. Added Substances

Added substances are presumed to be unsuitable for inclusion in an official article and therefore prohibited, if: (1) they exceed the minimum quantity required for providing their intended effect; (2) their presence impairs the bioavailability, therapeutic efficacy, or safety of the official article; or (3) they interfere with the assays and tests prescribed for determining compliance with the compendial standards.

The air in a container of an official article may, where appropriate, be evacuated or be replaced by carbon dioxide, helium, argon, or nitrogen, or by a mixture of these gases. The use of such gas need not be declared in the labeling.

5.20.10. Added Substances, Excipients, and Ingredients in Official Substances

Official substances may contain only the specific added substances that are permitted by the individual monograph. Where such addition is permitted, the label shall indicate the name(s) and amount(s) of any added substance(s).

5.20.20. Added Substances, Excipients, and Ingredients in Official Products

Suitable substances and excipients such as antimicrobial agents, pharmaceutical bases, carriers, coatings, flavors, preservatives, stabilizers, and vehicles may be added to an official product to enhance its stability, usefulness, or elegance, or to facilitate its preparation, unless otherwise specified in the individual monograph.

Added substances and excipients employed solely to impart color may be incorporated into official products other than those intended for parenteral or ophthalmic use, in accordance with the regulations pertaining to the use of colors issued by the U.S. Food and Drug Administration (FDA), provided such added substances or excipients are otherwise appropriate in all respects. (See also *Added Substances under Injections* (1).)

The proportions of the substances constituting the base in ointment and suppository products and preparations may be varied to maintain a suitable consistency under different climatic conditions, provided that the concentrations of active ingredients are not varied and provided that the bioavailability, therapeutic efficacy, and safety of the preparation are not impaired.

5.20.20.1. In Compounded Preparations

Compounded preparations for which a complete composition is given shall contain only the ingredients named in the formulas unless specifically exempted herein or in the individual monograph. Deviation from the specified processes or methods of compounding, although not from the ingredients or proportions thereof, may occur provided that the finished preparation conforms to the relevant standards and to preparations produced by following the specified process.

Where a monograph for a compounded preparation calls for an ingredient in an amount expressed on the dried basis, the ingredient need not be dried before use if due allowance is made for the water or other volatile substances present in the quantity taken.

Specially denatured alcohol formulas are available for use in accordance with federal statutes and regulations of the Internal Revenue Service. A suitable formula of specially denatured alcohol may be substituted for Alcohol in the manufacture of official preparations intended for internal or topical use, provided that the denaturant is volatile and does not remain in the finished product. A preparation that is intended for topical application to the skin may contain specially denatured alcohol, provided that the denaturant is either a usual ingredient in the preparation or a permissible added substance; in either case the denaturant shall be identified on the label of the topical preparation. Where a process is given in the individual monograph, any preparation compounded using denatured alcohol shall be identical to that prepared by the monograph process.

5.20.20.2. In Dietary Supplements

Additional ingredients may be added to dietary supplement products provided that the additional ingredients: (1) comply with applicable regulatory requirements; and (2) do not interfere with the assays and tests prescribed for determining compliance with compendial standards.

5.30. Description and Solubility

Only where a quantitative solubility test is given in a monograph and is designated as such is it a test for purity.

A monograph may include information regarding the article's description. Information about an article's "description and solubility" also is provided in the reference table *Description and Relative Solubility of USP and NF Articles*. The reference table merely denotes the properties of articles that comply with monograph standards. The reference table is

intended primarily for those who use, prepare, and dispense drugs and/or related articles. Although the information provided in monographs and the information in the reference table may indirectly assist in the preliminary evaluation of an article, it is not intended to serve as a standard or test for purity.

The approximate solubility of a compendial substance is indicated by one of the following descriptive terms:

Descriptive Term	Parts of Solvent Required for 1 Part of Solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1,000
Very slightly soluble	From 1,000 to 10,000
Practically insoluble, or Insoluble	Greater than or equal to 10,000

5.40. Identity

A compendial test titled *Identity* or *Identification* is provided as an aid in verifying the identity of articles as they are purported to be, e.g., those taken from labeled containers, and to establish whether it is the article named in *USP–NF*. The *Identity* or *Identification* test for a particular article may consist of one or more procedures. When a compendial test for *Identity* or *Identification* is undertaken, all requirements of all specified procedures in the test must be met to satisfy the requirements of the test. Failure of an article to meet all the requirements of a prescribed *Identity* or *Identification* test (i.e., failure to meet the requirements of all of the specified procedures that are components of that test) indicates that the article is mislabeled and/or adulterated.

5.50. Assay

Assay tests for compounded preparations are not intended for evaluating a compounded preparation before dispensing, but instead are intended to serve as the official test in the event of a question or dispute regarding the preparation's conformance to official standards.

5.50.10. Units of Potency (Biological)

For substances that cannot be completely characterized by chemical and physical means, it may be necessary to express quantities of activity in biological units of potency, each defined by an authoritative, designated reference standard.

Units of biological potency defined by the World Health Organization (WHO) for International Biological Standards and International Biological Reference Preparations are termed International Units (IU). Monographs refer to the units defined by USP Reference Standards as "USP Units." For biological products, units of potency are defined by the corresponding U.S. Standard established by FDA, whether or not International Units or USP Units have been defined (see *Biologics* <1041>).

5.60. Impurities and Foreign Substances

Tests for the presence of impurities and foreign substances are provided to limit such substances to amounts that are unobjectionable under conditions in which the article is customarily employed (see also *Impurities in Official Articles* <1086>).

Nonmonograph tests and acceptance criteria suitable for detecting and controlling impurities that may result from a change in the processing methods or that may be introduced from external sources should be employed in addition to the tests provided in the individual monograph, where the presence of the impurity is inconsistent with applicable good manufacturing practices or good pharmaceutical practice.

5.60.10. Other Impurities in USP and NF Articles

If a *USP* or *NF* monograph includes an assay or organic impurity test based on chromatography, other than a test for residual solvents, and that monograph procedure does not detect an impurity present in the substance, the amount and identity of the impurity, where both are known, shall be stated in the labeling (certificate of analysis) of the official substance, under the heading *Other Impurity(ies)*.

The presence of any unlabeled other impurity in an official substance is a variance from the standard if the content is 0.1% or greater. The sum of all *Other Impurities* combined with the monograph-detected impurities may not exceed 2.0% (see *Ordinary Impurities* <466>), unless otherwise stated in the monograph.

The following categories of drug substances are excluded from *Other Impurities* requirements:

- fermentation products and semi-synthetics derived therefrom,
- radiopharmaceuticals,
- biologics,
- biotechnology-derived products,
- peptides,
- herbals, and
- crude products of animal or plant origin.

Any substance known to be toxic shall not be listed under *Other Impurities*.

5.60.20. Residual Solvents in USP and NF Articles

All *USP* and *NF* articles are subject to relevant control of residual solvents, even when no test is specified in the individual monograph. If solvents are used during production, they must be of suitable quality. In addition, the toxicity and residual level of each solvent shall be taken into consideration, and the solvents limited according to the principles defined and the requirements specified in *Residual Solvents* <467>, using the general methods presented therein or other suitable methods.

5.70. Performance Tests

Where content uniformity determinations have been made using the same analytical methodology specified in the Assay, with appropriate allowances made for differences in sample preparation, the average of all of the individual content uniformity determinations may be used as the Assay value.

5.80. USP Reference Standards

USP Reference Standards are authentic specimens that have been approved as suitable for use as comparison standards in *USP* or *NF* tests and assays. (See *USP Reference Standards* <11>.) Where a procedure calls for the use of a compendial article rather than for a USP Reference Standard as a material standard of reference, a substance meeting all of the compendial monograph requirements for that article shall be used. If any new *USP* or *NF* standard requires the use of a new USP Reference Standard that is not yet available, that portion of the standard containing the requirement shall not be official until the specified USP reference material is available.

Unless a reference standard label bears a specific potency or content, assume the reference standard is 100.0% pure in the official application. Unless otherwise directed in the procedure in the individual monograph or in a general chapter, USP Reference Standards are to be used in accordance with the instructions on the label of the Reference Standard.

6. TESTING PRACTICES AND PROCEDURES

6.10. Safe Laboratory Practices

In performing compendial procedures, safe laboratory practices shall be followed, including precautionary measures, protective equipment, and work practices consistent with the chemicals and procedures used. Before undertaking any procedure described in the compendia, the analyst should be aware of the hazards associated with the chemicals and the techniques and means of protecting against them. These compendia are not designed to describe such hazards or protective measures.

6.20. Automated Procedures

Automated and manual procedures employing the same basic chemistry are considered equivalent.

6.30. Alternative and Harmonized Methods and Procedures

Alternative methods and/or procedures may be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction, or in other special circumstances. Such alternative procedures and methods shall be validated as described in the general chapter *Validation of Compendial Procedures* (1225) and must be shown to give equivalent or better results. Only those results obtained by the methods and procedures given in the compendium are conclusive.

Alternative procedures should be submitted to USP for evaluation as a potential replacement or addition to the standard (see section 4.10, *Monographs*).

Certain general chapters contain a statement that the text in question is harmonized with the corresponding text of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia* and that these texts are interchangeable. Therefore, if a substance or preparation is found to comply with a requirement using an interchangeable method or procedure from one of these pharmacopoeias, it should comply with the requirements of the *USP*. When a difference appears, or in the event of dispute, only the result obtained by the method and/or procedure given in the *USP* is conclusive.

6.40. Dried, Anhydrous, Ignited, or Solvent-Free Basis

All calculations in the compendia assume an "as-is" basis unless otherwise specified.

Test procedures may be performed on the undried or unignited substance and the results calculated on the dried, anhydrous, or ignited basis, provided a test for *Loss on Drying*, or *Water*, or *Loss on Ignition*, respectively, is given in the monograph. Where the presence of moisture or other volatile material may interfere with the procedure, previous drying of the substance is specified in the individual monograph and is obligatory.

The term "solvent-free" signifies that the calculation shall be corrected for the presence of known solvents as determined using the methods described in *Residual Solvents* (467) unless a test for limit of organic solvents is provided in the monograph.

The term "previously dried" without qualification signifies that the substance shall be dried as directed under *Loss on Drying* (731) or *Water Determination* (921) (gravimetric determination).

Where drying in vacuum over a desiccant is directed, a vacuum desiccator, a vacuum drying pistol, or other suitable vacuum drying apparatus shall be used.

6.40.10. Ignite To Constant Weight

"Ignite to constant weight" means that ignition shall be continued at $800 \pm 25^\circ$, unless otherwise indicated, until two consecutive weighings, the second of which is taken after an additional period appropriate to the nature and quantity of the residue, do not differ by more than 0.50 mg per g of substance taken.

6.40.20. Dried To Constant Weight

"Dried to constant weight" means that drying shall be continued until two consecutive weighings, the second of which is taken after an additional drying period appropriate to the nature and quantity of the residue, do not differ by more than 0.50 mg per g of substance taken.

6.50. Preparation of Solutions

6.50.10. Filtration

Where a procedure gives direction to "filter" without further qualification, the liquid shall be passed through suitable filter paper or equivalent device until the filtrate is clear. Due to the possibility of filter effects, the initial volumes of a filtrate may be discarded.

6.50.20. Solutions

Unless otherwise specified, all solutions shall be prepared with Purified Water. Solutions for quantitative measures shall

be prepared using accurately weighed or accurately measured analytes (see section 8.20, *About*).

An expression such as "(1 in 10)" means that 1 part *by volume* of a liquid shall be diluted with, or 1 part *by weight* of a solid shall be dissolved in, a sufficient quantity of the diluent or solvent to make the volume of the finished solution 10 parts *by volume*. An expression such as "(20:5:2)" means that the respective numbers of parts, by volume, of the designated liquids shall be mixed, unless otherwise indicated.

6.50.20.1. Adjustments to Solutions

When a specified concentration is called for in a procedure, a solution of other normality or molarity may be used, provided that allowance is made for the difference in concentration and that the change does not increase the error of measurement.

Unless otherwise indicated, analyte concentrations shall be prepared to within ten percent (10%) of the indicated value. In the special case in which a procedure is adapted to the working range of an instrument, solution concentrations may differ from the indicated value by more than ten percent (10%), with appropriate changes in associated calculations. Any changes shall fall within the validated range of the instrument.

When adjustment of pH is indicated with either an acid or base and the concentration is not indicated, appropriate concentrations of that acid or base may be used.

6.50.20.2. Test Solutions

Information on Test Solutions (TS) is provided in the *Test Solutions* portion of the *Reagents, Indicators, and Solutions* section of the *USP-NF*. Use of an alternative Test Solution or a change in the Test Solution used may require validation.

6.50.20.3. Indicator Solutions

Where a procedure specifies the use of an indicator TS, approximately 0.2 mL, or 3 drops, of the solution shall be added unless otherwise directed.

6.60. Units Necessary to Complete a Test

Unless otherwise specified, a sufficient number of units to ensure a suitable analytical result shall be taken.

6.60.10. Tablets

Where the procedure of a Tablet monograph directs to weigh and finely powder not fewer than a given number of Tablets, a counted number of Tablets shall be weighed and reduced to a powder. The portion of the powdered Tablets taken shall be representative of the whole Tablets and shall, in turn, be weighed accurately.

6.60.20. Capsules

Where the procedure of a Capsule monograph gives direction to remove, as completely as possible, the contents of not fewer than a given number of the Capsules, a counted number of Capsules shall be carefully opened and the contents quantitatively removed, combined, mixed, and weighed accurately. The portion of mixed Capsules contents taken shall be representative of the contents of the Capsules and shall, in turn, be weighed accurately.

6.70. Reagents

The proper conduct of the compendial procedures and the reliability of the results depend, in part, upon the quality of the reagents used in the performance of the procedures. Unless otherwise specified, reagents conforming to the specifications set forth in the current edition of *Reagent Chemicals* published by the American Chemical Society (ACS) shall be used. Where such ACS reagent specifications are not available or where the required purity differs, compendial specifications for reagents of acceptable quality are provided (see the *Reagents, Indicators, and Solutions* section of the *USP-NF*). Reagents not covered by any of these specifications should be of a grade suitable to the proper performance of the method of assay or test involved.

Listing of these reagents, including the indicators and solutions employed as reagents, in no way implies that they have therapeutic utility; furthermore, any reference to *USP* or *NF* in their labeling shall include also the term "reagent"

or “reagent grade.” USP may supply reagents if they otherwise may not be generally commercially available.

6.80. Equipment

Unless otherwise specified, a specification for a definite size or type of container or apparatus in a procedure is given solely as a recommendation. Other dimensions or types may be used if they are suitable for the intended use.

6.80.10. Apparatus for Measurement

Where volumetric flasks or other exact measuring, weighing, or sorting devices are specified, this or other equipment of at least equivalent accuracy shall be employed.

6.80.10.1. Pipet

Where a pipet is specified, a suitable buret may be substituted. Where a “to contain” pipet is specified, a suitable volumetric flask may be substituted.

6.80.10.2. Light Protection

Where low-actinic or light-resistant containers are specified, either containers specially treated to protect contents from light or clear containers that have been rendered opaque by application of a suitable coating or wrapping may be used.

6.80.20. Instrumental Apparatus

An instrument may be substituted for the specified instrument if the substitute uses the same fundamental principles of operation and is of equivalent or greater sensitivity and accuracy. These characteristics shall be qualified as appropriate. Where a particular brand or source of a material, instrument, or piece of equipment, or the name and address of a manufacturer or distributor, is mentioned (ordinarily in a footnote), this identification is furnished solely for informational purposes as a matter of convenience, without implication of approval, endorsement, or certification.

6.80.20.1. Chromatographic Tubes and Columns

The term “diameter” refers to internal diameter (ID).

6.80.20.2. Tubing

The term “diameter” refers to outside diameter (OD).

6.80.20.3. Steam Bath

Where use of a steam bath is directed, use actively flowing steam or another regulated heat source controlled at an equivalent temperature.

6.80.20.4. Water Bath

A water bath requires vigorously boiling water unless otherwise specified.

7. TEST RESULTS

7.10. Interpretation of Requirements

Analytical results observed in the laboratory (or calculated from experimental measurements) are compared with stated acceptance criteria to determine whether the article conforms to compendial requirements.

The reportable value, which often is a summary value for several individual determinations, is compared with the acceptance criteria. The reportable value is the end result of a completed measurement procedure, as documented.

Where acceptance criteria are expressed numerically herein through specification of an upper and/or lower limit, permitted values include the specified values themselves, but no values outside the limit(s). Acceptance criteria are considered significant to the last digit shown.

7.10.5. Nominal Concentrations in Equations

Where a “nominal concentration” is specified, calculate the concentration based on the label claim. In assay procedures, water correction is typically stated in the Definition and on the label of the USP Reference Standard. For other procedures, correction for assayed content, potency, or both is made prior to using the concentration in the equation provided in the monograph.

7.10.10. Equivalence Statements in Titrimetric Procedures

The directions for titrimetric procedures conclude with a statement of the weight of the analyte that is equivalent to each mL of the standardized titrant. In such an equivalence statement, the number of significant figures in the concentration of the titrant should be understood to correspond to the number of significant figures in the weight of the analyte. Corrections to calculations based on the blank determination are to be made for all titrimetric assays where appropriate (see *Titrimetry* (541)).

7.20. Rounding Rules

The observed or calculated values shall be rounded off to the number of decimal places that is in agreement with the limit expression. Numbers should not be rounded until the final calculations for the reportable value have been completed. Intermediate calculations (e.g., slope for linearity) may be rounded for reporting purposes, but the original (not rounded) value should be used for any additional required calculations. Acceptance criteria are fixed numbers and are not rounded.

When rounding is required, consider only one digit in the decimal place to the right of the last place in the limit expression. If this digit is smaller than 5, it is eliminated and the preceding digit is unchanged. If this digit is equal to or greater than 5, it is eliminated and the preceding digit is increased by 1.

8. TERMS AND DEFINITIONS

8.10. Abbreviations

- RS refers to a USP Reference Standard.
- CS refers to a Colorimetric Solution.
- TS refers to a Test Solution.
- VS refers to a Volumetric Solution that is standardized in accordance with directions given in the individual monograph or in the *Reagents, Indicators, and Solutions* section of USP–NF.

8.20. About

“About” indicates a quantity within 10%.

If the measurement is stated to be “accurately measured” or “accurately weighed,” follow the statements in the gen-

Illustration of Rounding Numerical Values for Comparison with Requirements			
Compendial Requirement	Unrounded Value	Rounded Result	Conforms
Assay limit $\geq 98.0\%$	97.96%	98.0%	Yes
	97.92%	97.9%	No
	97.95%	98.0%	Yes
Assay limit $\leq 101.5\%$	101.55%	101.6%	No
	101.46%	101.5%	Yes
	101.45%	101.5%	Yes
Limit test $\leq 0.02\%$	0.025%	0.03%	No
	0.015%	0.02%	Yes
	0.027%	0.03%	No
Limit test ≤ 3 ppm	3.5 ppm	4 ppm	No
	3.4 ppm	3 ppm	Yes
	2.5 ppm	3 ppm	Yes

eral chapters *Volumetric Apparatus* (31) and *Weights and Balances* (41), respectively.

8.30. Alcohol Content

Percentages of alcohol, such as those under the heading *Alcohol Content*, refer to percentage by volume of C_2H_5OH at 15.56°. Where a formula, test, or assay calls for alcohol, ethyl alcohol, or ethanol, the *USP* monograph article Alcohol shall be used. Where reference is made to " C_2H_5OH ," absolute (100 percent) ethanol is intended. Where a procedure calls for dehydrated alcohol, alcohol absolute, or anhydrous alcohol, the *USP* monograph article Dehydrated Alcohol shall be used.

8.40. Atomic Weights

Atomic weights used in computing molecular weights and the factors in the assays and elsewhere are those established by the IUPAC Commission on Atomic Weights and Isotopic Abundances.

8.50. Blank Determinations

Where it is directed that "any necessary correction" be made by a blank determination, the determination shall be conducted using the same quantities of the same reagents treated in the same manner as the solution or mixture containing the portion of the substance under assay or test, but with the substance itself omitted.

8.60. Concomitantly

"Concomitantly" denotes that the determinations or measurements are to be performed in immediate succession.

8.70. Desiccator

The instruction "in a desiccator" indicates use of a tightly closed container of suitable size and design that maintains an atmosphere of low moisture content by means of a suitable desiccant such as anhydrous calcium chloride, magnesium perchlorate, phosphorus pentoxide, or silica gel. See also section 8.220, *Vacuum Desiccator*.

8.80. Logarithms

Logarithms are to the base 10.

8.90. Microbial Strain

A microbial strain cited and identified by its ATCC catalog number shall be used directly or, if subcultured, shall be used not more than five passages removed from the original strain.

8.100. Negligible

"Negligible" indicates a quantity not exceeding 0.50 mg.

8.110. NLT/NMT

"NLT" means "not less than." "NMT" means "not more than."

8.120. Odor

"Odorless," "practically odorless," "a faint characteristic odor," and variations thereof indicate evaluation of a suitable quantity of freshly opened material after exposure to the air for 15 minutes. An odor designation is descriptive only and should not be regarded as a standard of purity for a particular lot of an article.

8.130. Percent

"Percent" used without qualification means:

- For mixtures of solids and semisolids, percent weight in weight;
- For solutions or suspensions of solids in liquids, percent weight in volume;
- For solutions of liquids in liquids, percent volume in volume;
- For solutions of gases in liquids, percent weight in volume.

For example, a 1 percent solution is prepared by dissolving 1 g of a solid or semisolid, or 1 mL of a liquid, in sufficient solvent to make 100 mL of the solution.

8.140. Percentage Concentrations

Percentage concentrations are expressed as follows:

- *Percent Weight in Weight* (w/w) is defined as the number of g of a solute in 100 g of solution.

- *Percent Weight in Volume* (w/v) is defined as number of g of a solute in 100 mL of solution.
- *Percent Volume in Volume* (v/v) is defined as the number of mL of a solute in 100 mL of solution.

8.150. Pressure

Pressure is determined by use of a suitable manometer or barometer calibrated in terms of the pressure exerted by a column of mercury of the stated height.

8.160. Reaction Time

Reaction time is 5 minutes unless otherwise specified.

8.170. Specific Gravity

Specific gravity is the weight of a substance in air at 25° divided by the weight of an equal volume of water at the same temperature.

8.180. Temperatures

Temperatures are expressed in centigrade (Celsius) degrees, and all measurements are made at 25° unless otherwise indicated. Where moderate heat is specified, any temperature not higher than 45° (113° F) is indicated.

8.190. Time

Unless otherwise specified, rounding rules, as described in section 7.20, *Rounding Rules*, apply to any time specified.

8.200. Transfer

"Transfer" indicates a quantitative manipulation.

8.210. Vacuum

"Vacuum" denotes exposure to a pressure of less than 20 mm of mercury (2.67 kPa), unless otherwise indicated.

8.220. Vacuum Desiccator

"Vacuum desiccator" indicates a desiccator that maintains a low-moisture atmosphere at a reduced pressure of not more than 20 mm of mercury (2.67 kPa) or at the pressure designated in the individual monograph.

8.230. Water

8.230.10. Water as an Ingredient in an Official Product

As an ingredient in an official product, water meets the requirements of the appropriate water monograph in *USP* or *NF*.

8.230.20. Water in the Manufacture of Official Substances

When used in the manufacture of official substances, water may meet the requirements for drinking water as set forth in the regulations of the U.S. Environmental Protection Agency (potable water).

8.230.30. Water in a Compendial Procedure

When water is called for in a compendial procedure, the *USP* article Purified Water shall be used unless otherwise specified. Definitions for *High-Purity Water* and *Carbon Dioxide-Free Water* are provided in *Containers—Glass* (660). Definitions of other types of water are provided in *Water for Pharmaceutical Purposes* (1231).

8.240. Weights and Measures

In general, weights and measures are expressed in the International System of Units (SI) as established and revised by the *Conférence générale des poids et mesures*. For compendial purposes, the term "weight" is considered to be synonymous with "mass."

Molality is designated by the symbol *m* preceded by a number that represents the number of moles of the designated solute contained in 1 kilogram of the designated solvent.

Molarity is designated by the symbol *M* preceded by a number that represents the number of moles of the designated solute contained in an amount of the designated solvent that is sufficient to prepare 1 liter of solution.

Normality is designated by the symbol *N* preceded by a number that represents the number of equivalents of the designated solute contained in an amount of the designated solvent that is sufficient to prepare 1 liter of solution.

Symbols commonly employed for SI metric units and other units are as follows:

Bq = becquerel	dL = deciliter
kBq = kilobecquerel	L = liter
MBq = megabecquerel	mL = milliliter ^c
GBq = gigabecquerel	μL = microliter
Ci = curie	Eq = gram-equivalent weight
mCi = millicurie	mEq = milliequivalent
μCi = microcurie	mol = gram-molecular weight (mole)
nCi = nanocurie	Da = dalton (relative molecular mass)
Gy = gray	mmol = millimole
mGy = milligray	Osmol = osmole
m = meter	mOsmol = milliosmole
dm = decimeter	Hz = hertz
cm = centimeter	kHz = kilohertz
mm = millimeter	MHz = megahertz
μm = micrometer (0.001 mm)	V = volts
nm = nanometer ^a	MeV = million electron volts
kg = kilogram	keV = kilo-electron volt
g = gram	mV = millivolt
mg = milligram	psi = pounds per square inch
μg; mcg = microgram ^b	Pa = pascal
ng = nanogram	kPa = kilopascal
pg = picogram	g = gravity (in centrifugation)
fg = femtogram	

^a Previously the symbol mμ (for millimicron) was used.

^b The symbol μg is used in the *USP* and *NF* to represent micrograms, but micrograms may be represented as "mcg" for labeling and prescribing purposes. The term "gamma," symbolized by γ, frequently is used to represent micrograms in biochemical literature.

^c One milliliter (mL) is used herein as the equivalent of one cubic centimeter (cc).

9. PRESCRIBING AND DISPENSING

9.10 Use of Metric Units

Prescriptions for compendial articles shall be written to state the quantity and/or strength desired in metric units unless otherwise indicated in the individual monograph (see also *Units of Potency*, section 5.50.10 above). If an amount is prescribed by any other system of measurement, only an amount that is the metric equivalent of the prescribed amount shall be dispensed. Apothecary unit designations on labels and labeling shall not be used.

9.20 Changes in Volume

In the dispensing of prescription medications, slight changes in volume owing to variations in room temperatures may be disregarded.

10. PRESERVATION, PACKAGING, STORAGE, AND LABELING

10.10. Storage Under Nonspecific Conditions

If no specific directions or limitations are provided in the *Packaging and Storage* section of an individual *USP* monograph or in the labeling of an article recognized in *USP*, the conditions of storage shall include storage at controlled room temperature, protection from moisture, and, where necessary, protection from light. Such articles shall be protected from moisture, freezing, and excessive heat, and, where necessary, from light during shipping and distribution. Drug substances are exempt from the requirements in this paragraph.

Regardless of quantity, where no specific storage directions or limitations are provided in an individual *NF* monograph or stated in the labeling of an article recognized in *NF*, the conditions of storage and distribution shall include protection from moisture, freezing, excessive heat, and, where necessary, from light.

10.20. Containers

The container is that which holds the article and is or may be in direct contact with the article. The immediate container is that which is in direct contact with the article at all times. The closure is a part of the container.

Before being filled, the container should be clean. Special precautions and cleaning procedures may be necessary to ensure that each container is clean and that extraneous matter is not introduced into or onto the article.

The container does not interact physically or chemically with the article placed in it so as to alter the strength, quality, or purity of the article beyond the official requirements.

The compendial requirements for the use of specified containers apply also to articles as packaged by the pharmacist or other dispenser, unless otherwise indicated in the individual monograph.

10.20.10. Tamper-Evident Packaging

The container or individual carton of a sterile article intended for ophthalmic or otic use, except where extemporaneously compounded for immediate dispensing on prescription, shall be so sealed that the contents cannot be used without obvious destruction of the seal.

Articles intended for sale without prescription are also required to comply with the tamper-evident packaging and labeling requirements of the FDA where applicable.

Preferably, the immediate container and/or the outer container or protective packaging used by a manufacturer or distributor for all dosage forms that are not specifically exempt is designed so as to show evidence of any tampering with the contents.

10.20.20. Light-Resistant Container

A light-resistant container (see *Light Transmission Test* under *Containers—Performance Testing* (671)) protects the contents from the effects of light by virtue of the specific properties of the material of which it is composed, including any coating applied to it. Alternatively, a clear and colorless or a translucent container may be made light-resistant by means of an opaque covering, in which case the label of the container bears a statement that the opaque covering is needed until the contents are to be used or administered. Where it is directed to "protect from light" in an individual monograph, preservation in a light-resistant container is intended.

Where an article is required to be packaged in a light-resistant container, and if the container is made light-resistant by means of an opaque covering, a single-use, unit-dose container or mnemonic pack for dispensing may not be removed from the outer opaque covering before dispensing.

10.20.30. Well-Closed Container

A well-closed container protects the contents from extraneous solids and from loss of the article under the ordinary or customary conditions of handling, shipment, storage, and distribution.

10.20.40. Tight Container

A tight container protects the contents from contamination by extraneous liquids, solids, or vapors; from loss of the article; and from efflorescence, deliquescence, or evaporation under the ordinary or customary conditions of handling, shipment, storage, and distribution; and is capable of tight reclosure. Where a tight container is specified, it may be replaced by a hermetic container for a single dose of an article.

A gas cylinder is a metallic container designed to hold a gas under pressure. As a safety measure, for carbon dioxide, cyclopropane, helium, nitrous oxide, and oxygen, the Pin-Index Safety System of matched fittings is recommended for cylinders of Size E or smaller.

[NOTE—Where packaging and storage in a *tight container* or a *well-closed container* is specified in the individual monograph, the container used for an article when dispensed on prescription meets the requirements under *Containers—Performance Testing* (671).]

10.20.50. Hermetic Container

A hermetic container is impervious to air or any other gas under the ordinary or customary conditions of handling, shipment, storage, and distribution.

10.20.60. Single-Unit Container

A single-unit container is one that is designed to hold a quantity of drug product intended for administration as a single dose or a single finished device intended for use promptly after the container is opened. Preferably, the immediate container and/or the outer container or protective packaging shall be so designed as to show evidence of any tampering with the contents. Each single-unit container shall be labeled to indicate the identity, quantity and/or strength, name of the manufacturer, lot number, and expiration date of the article.

10.20.70. Single-Dose Container

A single-dose container is a single-unit container for articles intended for parenteral administration only. A single-dose container is labeled as such. Examples of single-dose containers include prefilled syringes, cartridges, fusion-sealed containers, and closure-sealed containers when so labeled. (See also *Containers for Injections* under *Injections* (1).)

10.20.80. Unit-Dose Container

A unit-dose container is a single-unit container for articles intended for administration by other than the parenteral route as a single dose, direct from the container.

10.20.90. Unit-of-Use Container

A unit-of-use container is one that contains a specific quantity of a drug product and that is intended to be dispensed as such without further modification except for the addition of appropriate labeling. A unit-of-use container is labeled as such.

10.20.100. Multiple-Unit Container

A multiple-unit container is a container that permits withdrawal of successive portions of the contents without changing the strength, quality, or purity of the remaining portion.

10.20.110. Multiple-Dose Container

A multiple-dose container is a multiple-unit container for articles intended for parenteral administration only. (See also *Containers for Injections* under *Injections* (1).)

10.20.120. Requirements under the Poison Prevention Packaging Act (PPPA)

This act (see the website, www.cpsc.gov/businfo/pppa.html) requires special packaging of most human oral prescription drugs, oral controlled drugs, certain non-oral prescription drugs, certain dietary supplements, and many over-the-counter (OTC) drug preparations in order to protect the public from personal injury or illness from misuse of these preparations (16 CFR § 1700.14).

The immediate packaging of substances regulated under the PPPA shall comply with the special packaging standards (16 CFR § 1700.15 and 16 CFR § 1700.20). The PPPA regulations for special packaging apply to all packaging types including reclosable, nonclosable, and unit-dose types.

Special packaging is not required for drugs dispensed within a hospital setting for inpatient administration. Manufacturers and packagers of bulk-packaged prescription drugs do not have to use special packaging if the drug will be repackaged by the pharmacist. PPPA-regulated prescription drugs may be dispensed in non-child-resistant packaging upon the request of the purchaser or when directed in a legitimate prescription (15 U.S.C. § 1473).

Manufacturers or packagers of PPPA-regulated OTC preparations are allowed to package one size in non-child-resistant packaging as long as popular-size, special packages are also supplied. The non-child-resistant package requires special labeling (16 CFR § 1700.5).

Various types of child-resistant packages are covered in ASTM International Standard D-3475, *Standard Classification of Child-Resistant Packaging*. Examples are included as an aid in the understanding and comprehension of each type of classification.

10.30. Storage Temperature and Humidity

Specific directions are stated in some monographs with respect to the temperatures and humidity at which official articles shall be stored and distributed (including the shipment of articles to the consumer) when stability data indicate that storage and distribution at a lower or a higher temperature and a higher humidity produce undesirable results. Such directions apply except where the label on an article states a different storage temperature on the basis of stability studies of that particular formulation. Where no specific storage directions or limitations are provided in the individual monograph, but the label of an article states a storage temperature that is based on stability studies of that particular formulation, such labeled storage directions apply. The conditions are defined by the following terms.

10.30.10. Freezer

"Freezer" indicates a place in which the temperature is maintained thermostatically between -25° and -10° (-13° and 14° F).

10.30.20. Cold

Any temperature not exceeding 8° (46° F) is "cold." A "refrigerator" is a cold place in which the temperature is maintained thermostatically between 2° and 8° (36° and 46° F).

10.30.30. Cool

Any temperature between 8° and 15° (46° and 59° F) is "cool." An article for which storage in a *cool place* is directed may, alternatively, be stored and distributed in a *refrigerator*, unless otherwise specified by the individual monograph.

10.30.40. Controlled Cold Temperature

"Controlled cold temperature" is defined as temperature maintained thermostatically between 2° and 8° (36° and 46° F), that allows for excursions in temperature between 0° and 15° (32° and 59° F) that may be experienced during storage, shipping, and distribution such that the allowable calculated mean kinetic temperature is not more than 8° (46° F). Transient spikes up to 25° (77° F) may be permitted if the manufacturer so instructs and provided that such spikes do not exceed 24 hours unless supported by stability data or the manufacturer instructs otherwise.

10.30.50. Room Temperature

"Room temperature" indicates the temperature prevailing in a working area.

10.30.60. Controlled Room Temperature

"Controlled room temperature" indicates a temperature maintained thermostatically that encompasses the usual and customary working environment of 20° to 25° (68° to 77° F); that results in a mean kinetic temperature calculated to be not more than 25° ; and that allows for excursions between 15° and 30° (59° and 86° F) that are experienced in pharmacies, hospitals, and warehouses. Provided the mean kinetic temperature remains in the allowed range, transient spikes up to 40° are permitted as long as they do not exceed 24 hours. Spikes above 40° may be permitted if the manufacturer so instructs. Articles may be labeled for storage at "controlled room temperature" or at "up to 25° ", or other wording based on the same mean kinetic temperature. The mean kinetic temperature is a calculated value that may be used as an isothermal storage temperature that simulates the nonisothermal effects of storage temperature variations.

An article for which storage at *controlled room temperature* is directed may, alternatively, be stored and distributed in a *cool place*, unless otherwise specified in the individual monograph or on the label.

10.30.70. Warm

Any temperature between 30° and 40° (86° and 104° F) is "warm."

10.30.80. Excessive Heat

"Excessive heat" means any temperature above 40° (104° F).

10.30.90. Protection From Freezing

Where, in addition to the risk of breakage of the container, freezing subjects an article to loss of strength or potency, or to destructive alteration of its characteristics, the container label bears an appropriate instruction to protect the article from freezing.

10.30.100. Dry Place

The term "dry place" denotes a place that does not exceed 40% average relative humidity at *Controlled Room Temperature* or the equivalent water vapor pressure at other temperatures. The determination may be made by direct measurement at the place or may be based on reported climatic conditions. Determination is based on not less than 12 equally spaced measurements that encompass either a season, a year, or, where recorded data demonstrate, the storage period of the article. There may be values of up to 45% relative humidity provided that the average value is 40% relative humidity.

Storage in a container validated to protect the article from moisture vapor, including storage in bulk, is considered storage in a dry place.

10.40. Labeling

The term "labeling" designates all labels and other written, printed, or graphic matter upon an immediate container of an article or upon, or in, any package or wrapper in which it is enclosed, except any outer shipping container. The term "label" designates that part of the labeling upon the immediate container.

A shipping container containing a single article, unless such container is also essentially the immediate container or the outside of the consumer package, is labeled with a minimum of product identification (except for controlled articles), lot number, expiration date, and conditions for storage and distribution.

Articles in these compendia are subject to compliance with such labeling requirements as may be promulgated by governmental bodies in addition to the compendial requirements set forth for the articles.

10.40.10. Amount of Ingredient Per Dosage Unit

The strength of a drug product is expressed on the container label in terms of micrograms or milligrams or grams or percentage of the therapeutically active moiety or drug substance, whichever form is used in the title, unless otherwise indicated in an individual monograph. Both the active moiety and drug substance names and their equivalent amounts are then provided in the labeling.

Official articles in capsule, tablet, or other unit dosage form shall be labeled to express the quantity of each active ingredient or recognized nutrient contained in each such unit; except that, in the case of unit-dose oral solutions or suspensions, whether supplied as liquid preparations or as liquid preparations that are constituted from solids upon addition of a designated volume of a specific diluent, the label shall express the quantity of each active ingredient or recognized nutrient delivered under the conditions prescribed in *Deliverable Volume* (698). Official drug products not in unit dosage form shall be labeled to express the quantity of each active ingredient in each milliliter or in each gram, or to express the percentage of each such ingredient (see 8.140., *Percentage Concentrations*), except that oral liquids or solids intended to be constituted to yield oral liquids may, alternatively, be labeled in terms of each 5-mL portion of the liquid or resulting liquid. Unless otherwise indicated in a monograph or chapter, such declarations of strength or quantity shall be stated only in metric units. See also 5.50.10., *Units of Potency (Biological)*.

10.40.20. Use of Leading and Terminal Zeros

To help minimize the possibility of errors in the dispensing and administration of drugs, the quantity of active ingredient when expressed in whole numbers shall be shown without a decimal point that is followed by a terminal zero (e.g., express as 4 mg [not 4.0 mg]). The quantity of active ingredient when expressed as a decimal number smaller than 1

shall be shown with a zero preceding the decimal point (e.g., express as 0.2 mg [not .2 mg]).

10.40.30. Labeling of Salts of Drugs

It is an established principle that official articles shall have only one official title. For purposes of saving space on labels, and because chemical symbols for the most common inorganic salts of drugs are well known to practitioners as synonymous with the written forms, the following alternatives are permitted in labeling official articles that are salts: HCl for hydrochloride; HBr for hydrobromide; Na for sodium; and K for potassium. The symbols Na and K are intended for use in abbreviating names of the salts of organic acids, but these symbols are not used where the word Sodium or Potassium appears at the beginning of an official title (e.g., Phenobarbital Na is acceptable, but Na Salicylate is not to be written).

10.40.40. Labeling Vitamin-Containing Products

The vitamin content of an official drug product shall be stated on the label in metric units per dosage unit. The amounts of vitamins A, D, and E may be stated also in USP Units. Quantities of vitamin A declared in metric units refer to the equivalent amounts of retinol (vitamin A alcohol). The label of a nutritional supplement shall bear an identifying lot number, control number, or batch number.

10.40.50. Labeling Botanical-Containing Products

The label of an herb or other botanical intended for use as a dietary supplement bears the statement, "If you are pregnant or nursing a baby, seek the advice of a health professional before using this product."

10.40.60. Labeling Parenteral And Topical Preparations

The label of a preparation intended for parenteral or topical use states the names of all added substances (see 5.20., *Added Substances, Excipients, and Ingredients* and see *Labeling under Injections* (1)), and, in the case of parenteral preparations, also their amounts or proportions, except that for substances added for adjustment of pH or to achieve isotonicity, the label may indicate only their presence and the reason for their addition.

10.40.70. Labeling Electrolytes

The concentration and dosage of electrolytes for replacement therapy (e.g., sodium chloride or potassium chloride) shall be stated on the label in milliequivalents (mEq). The label of the product shall indicate also the quantity of ingredient(s) in terms of weight or percentage concentration.

10.40.80. Labeling Alcohol

The content of alcohol in a liquid preparation shall be stated on the label as a percentage (v/v) of C₂H₅OH.

10.40.90. Special Capsules and Tablets

The label of any form of Capsule or Tablet intended for administration other than by swallowing intact bears a prominent indication of the manner in which it shall be used.

10.40.100. Expiration Date and Beyond-Use Date

The label of an official drug product or nutritional or dietary supplement product shall bear an expiration date. All articles shall display the expiration date so that it can be read by an ordinary individual under customary conditions of purchase and use. The expiration date shall be prominently displayed in high contrast to the background or sharply embossed, and easily understood (e.g., "EXP 6/08," "Exp. June 08," or "Expires 6/08"). [NOTE—For additional information and guidance, refer to the Consumer Healthcare Products Association's *Voluntary Codes and Guidelines of the Self-Medication Industry*.]

The monographs for some preparations state how the expiration date that shall appear on the label shall be determined. In the absence of a specific requirement in the individual monograph for a drug product or nutritional supplement, the label shall bear an expiration date assigned for the particular formulation and package of the article, with the following exception: the label need not show an expiration date in the case of a drug product or nutritional supplement packaged in a container that is intended for sale

without prescription and the labeling of which states no dosage limitations, and which is stable for not less than 3 years when stored under the prescribed conditions.

Where an official article is required to bear an expiration date, such article shall be dispensed solely in, or from, a container labeled with an expiration date, and the date on which the article is dispensed shall be within the labeled expiry period. The expiration date identifies the time during which the article may be expected to meet the requirements of the compendial monograph, provided it is kept under the prescribed storage conditions. The expiration date limits the time during which the article may be dispensed or used. Where an expiration date is stated only in terms of the month and the year, it is a representation that the intended expiration date is the last day of the stated month. The beyond-use date is the date after which an article shall not be used. The dispenser shall place on the label of the prescription container a suitable beyond-use date to limit the patient's use of the article based on any information supplied by the manufacturer and the *General Notices*. The beyond-use date placed on the label shall not be later than the expiration date on the manufacturer's container.

For articles requiring constitution before use, a suitable beyond-use date for the constituted product shall be identified in the labeling.

For all other dosage forms, in determining an appropriate period of time during which a prescription drug may be retained by a patient after its dispensing, the dispenser shall take into account, in addition to any other relevant factors, the nature of the drug; the container in which it was packaged by the manufacturer and the expiration date thereon; the characteristics of the patient's container, if the article is repackaged for dispensing; the expected storage conditions to which the article may be exposed; any unusual storage conditions to which the article may be exposed; and the expected length of time of the course of therapy. The dispenser shall, on taking into account the foregoing, place on the label of a multiple-unit container a suitable beyond-use date to limit the patient's use of the article. Unless otherwise specified in the individual monograph, or in the absence of stability data to the contrary, such beyond-use date shall be not later than (a) the expiration date on the manufacturer's container, or (b) 1 year from the date the drug is dispensed, whichever is earlier. For nonsterile solid and liquid dosage forms that are packaged in single-unit and unit-dose containers, the beyond-use date shall be 1 year from the date the drug is packaged into the single-unit or unit-dose container or the expiration date on the manufacturer's container, whichever is earlier, unless stability data or the manufacturer's labeling indicates otherwise.

The dispenser shall maintain the facility where the dosage forms are packaged and stored, at a temperature such that the mean kinetic temperature is not greater than 25°. The

plastic material used in packaging the dosage forms shall afford better protection than polyvinyl chloride, which does not provide adequate protection against moisture permeation. Records shall be kept of the temperature of the facility where the dosage forms are stored, and of the plastic materials used in packaging.

10.40.100.1. Compounded Preparations

The label on the container or package of an official compounded preparation shall bear a beyond-use date. The beyond-use date is the date after which a compounded preparation is not to be used. Because compounded preparations are intended for administration immediately or following short-term storage, their beyond-use dates may be assigned based on criteria different from those applied to assigning expiration dates to manufactured drug products.

The monograph for an official compounded preparation typically includes a beyond-use requirement that states the time period following the date of compounding during which the preparation, properly stored, may be used. In the absence of stability information that is applicable to a specific drug and preparation, recommendations for maximum beyond-use dates have been devised for nonsterile compounded drug preparations that are packaged in tight, light-resistant containers and stored at controlled room temperature unless otherwise indicated (see *Stability Criteria and Beyond-Use Dating under Stability of Compounded Preparations* in the general test chapter *Pharmaceutical Compounding—Nonsterile Preparations* (795)).

10.50. Guidelines for Packaging and Storage Statements in USP–NF Monographs

In order to provide users of the *USP* and *NF* with proper guidance on how to package and store official articles, every monograph in the *USP* and *NF* shall have a packaging and storage specification.

For the packaging portion of the statement, the choice of containers is given in this section 10, *Preservation, Packaging, Storage, and Labeling*, and includes *Light-Resistant Container*, *Well-Closed Container*, *Tight Container*, *Hermetic Container*, *Single-Unit Container*, *Single-Dose Container*, *Unit-Dose Container*, and *Unit-of-Use Container*. For most preparations, the choice is determined by the container in which it shall be dispensed (e.g., tight, well-closed, hermetic, unit-of-use, etc.). For drug substances, the choice would appear to be tight, well-closed, or, where needed, a light-resistant container. For excipients, given their typical nature as large-volume commodity items, with containers ranging from drums to tank cars, a well-closed container is an appropriate default. Therefore, in the absence of data indicating a need for a more protective class of container, the phrase "Preserve in well-closed containers" should be used as a default for excipients.

Chart Guide

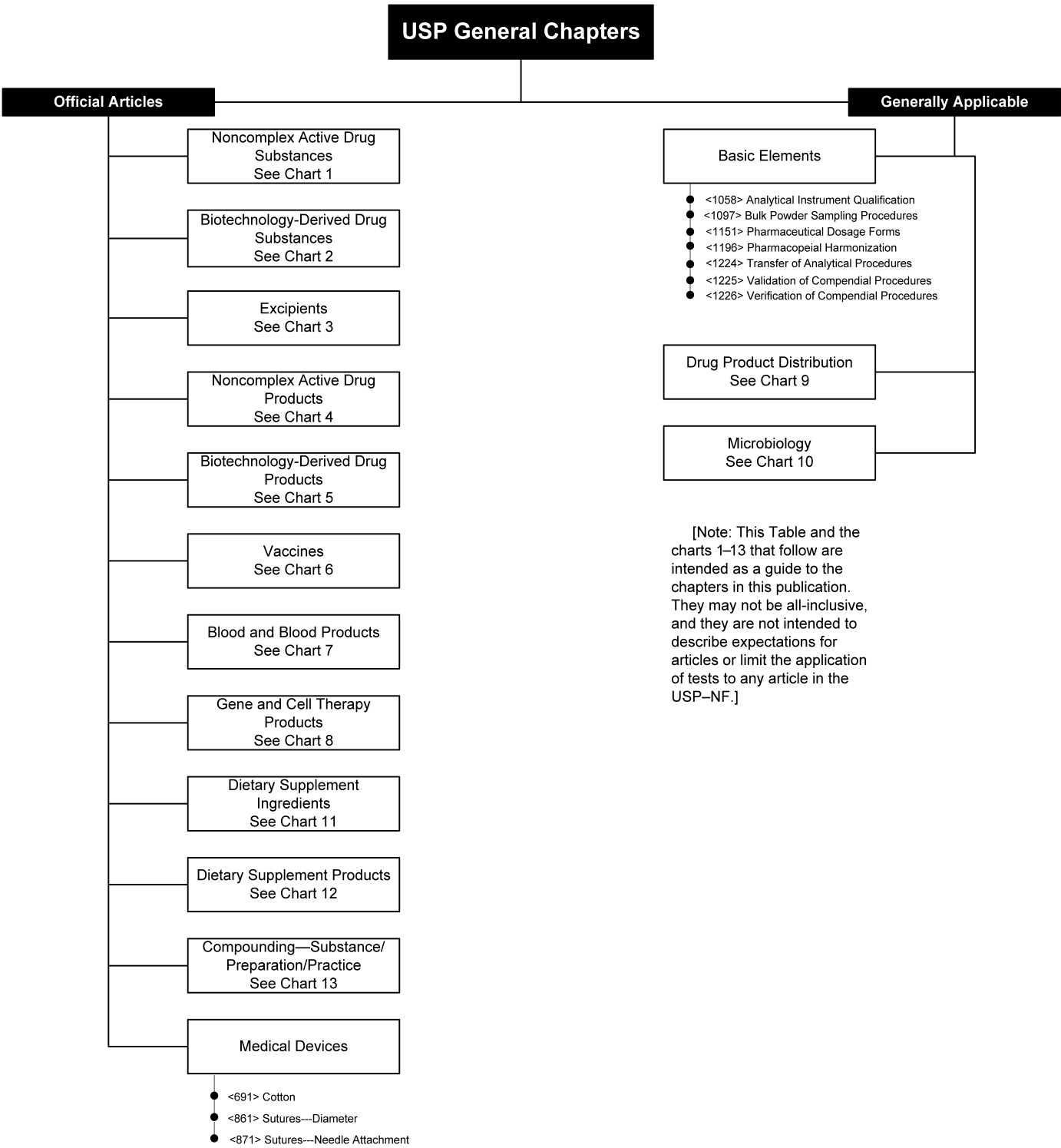


Chart 1. Noncomplex Active Drug Substances

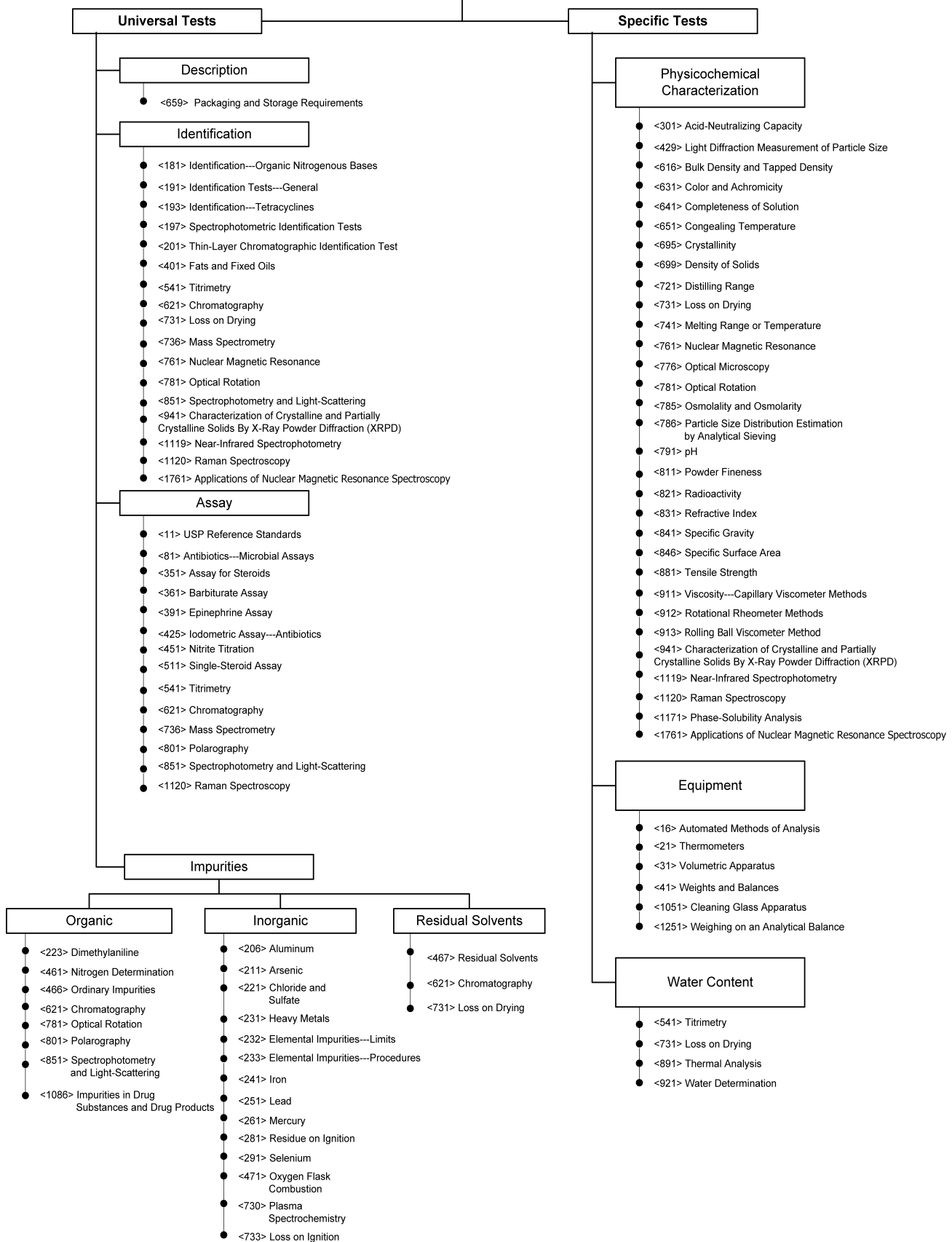


Chart 2. Biotechnology-Derived Drug Substances



Chart 3. Excipients

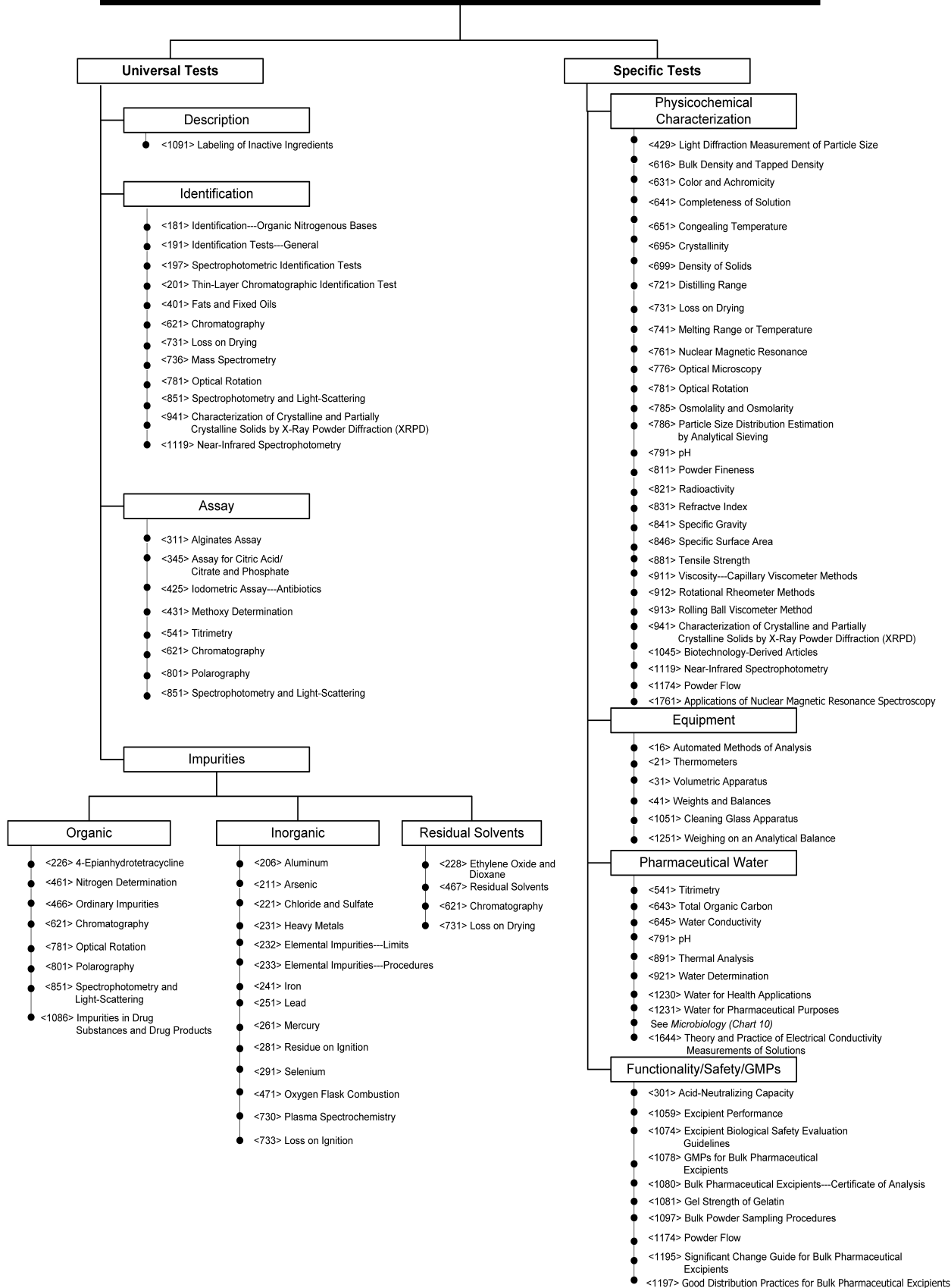


Chart 4. Noncomplex Active Drug Products

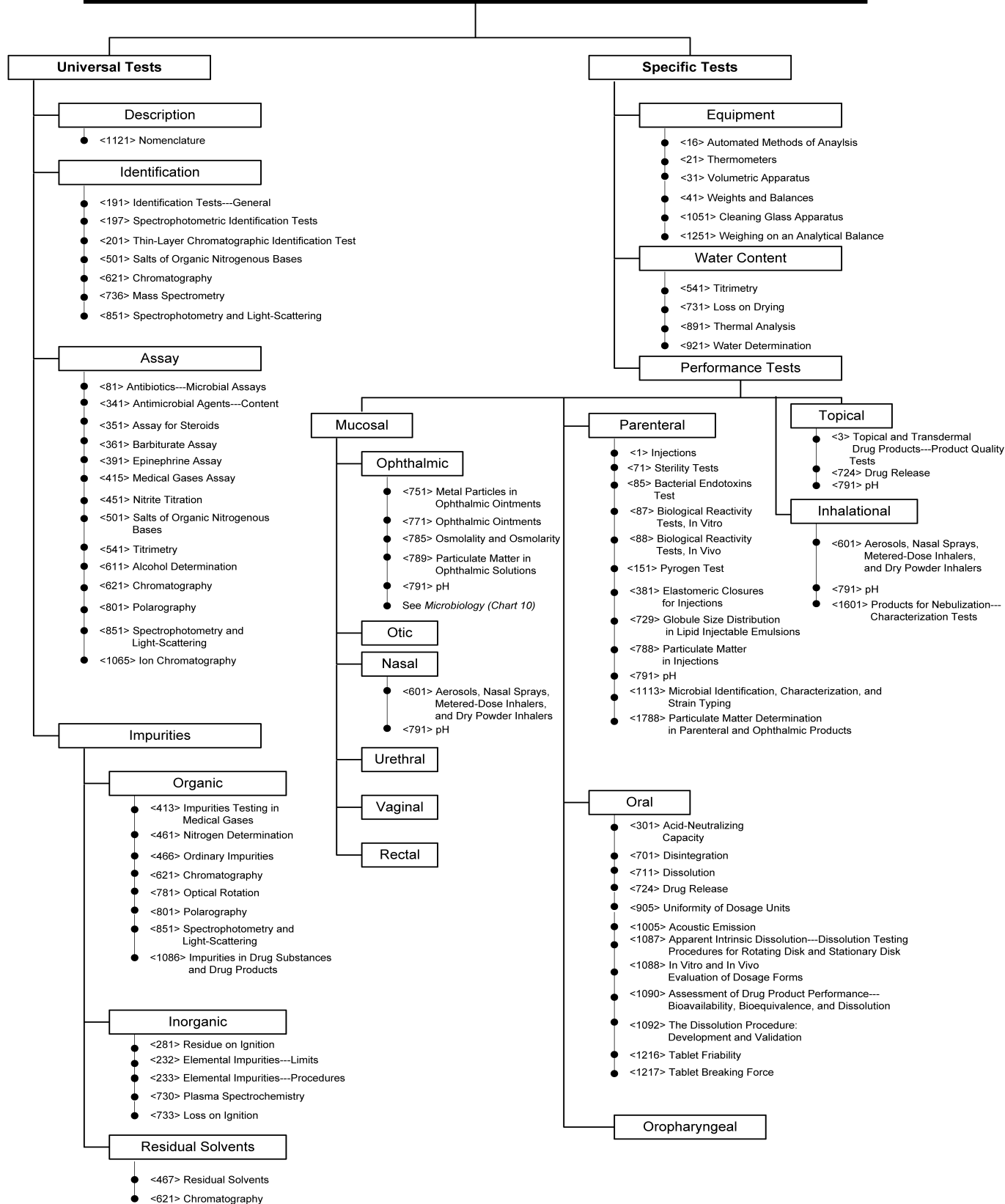


Chart 5. Biotechnology-Derived Drug Products

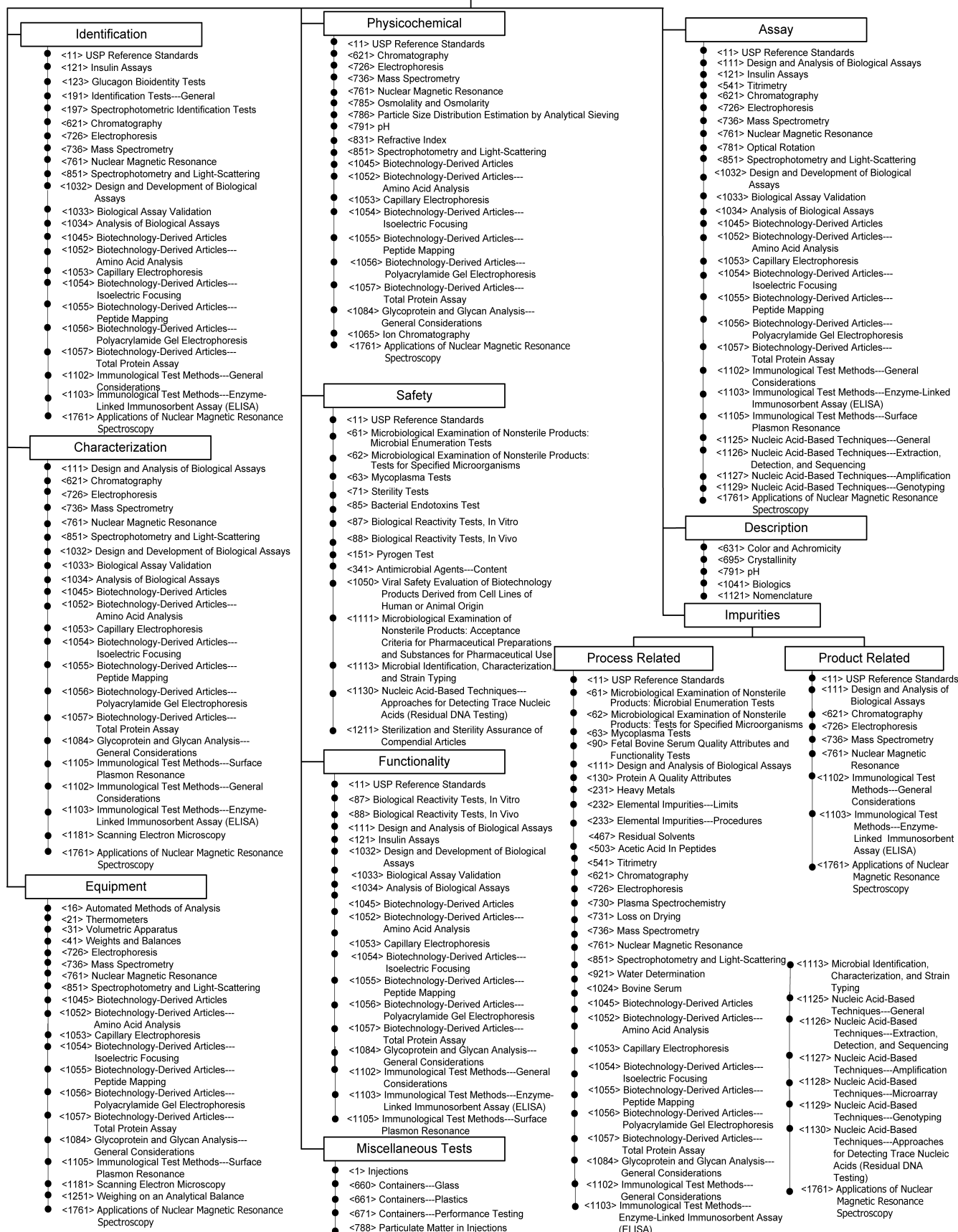


Chart 6. Vaccines

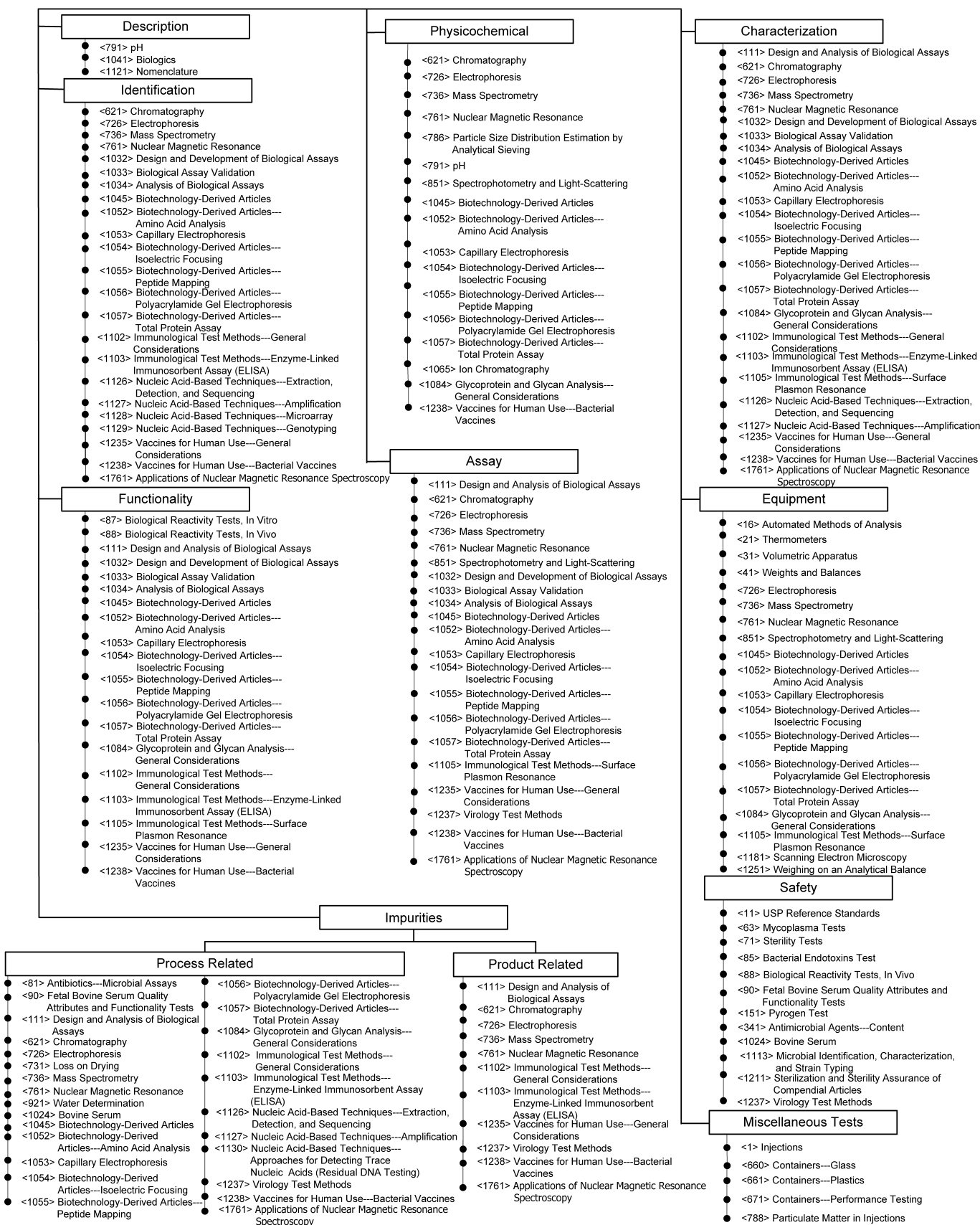


Chart 7. Blood and Blood Products

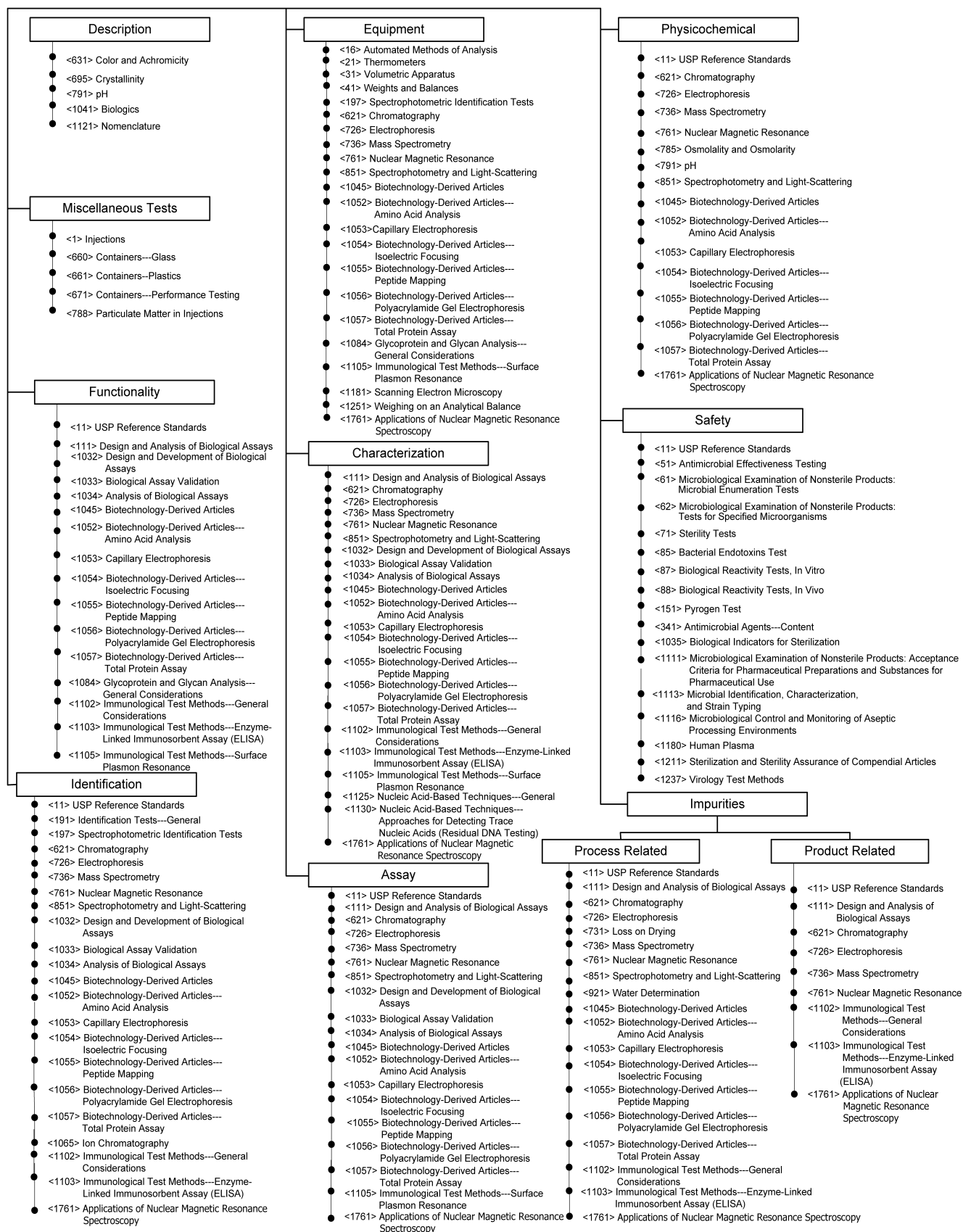


Chart 8. Gene and Cell Therapy Products

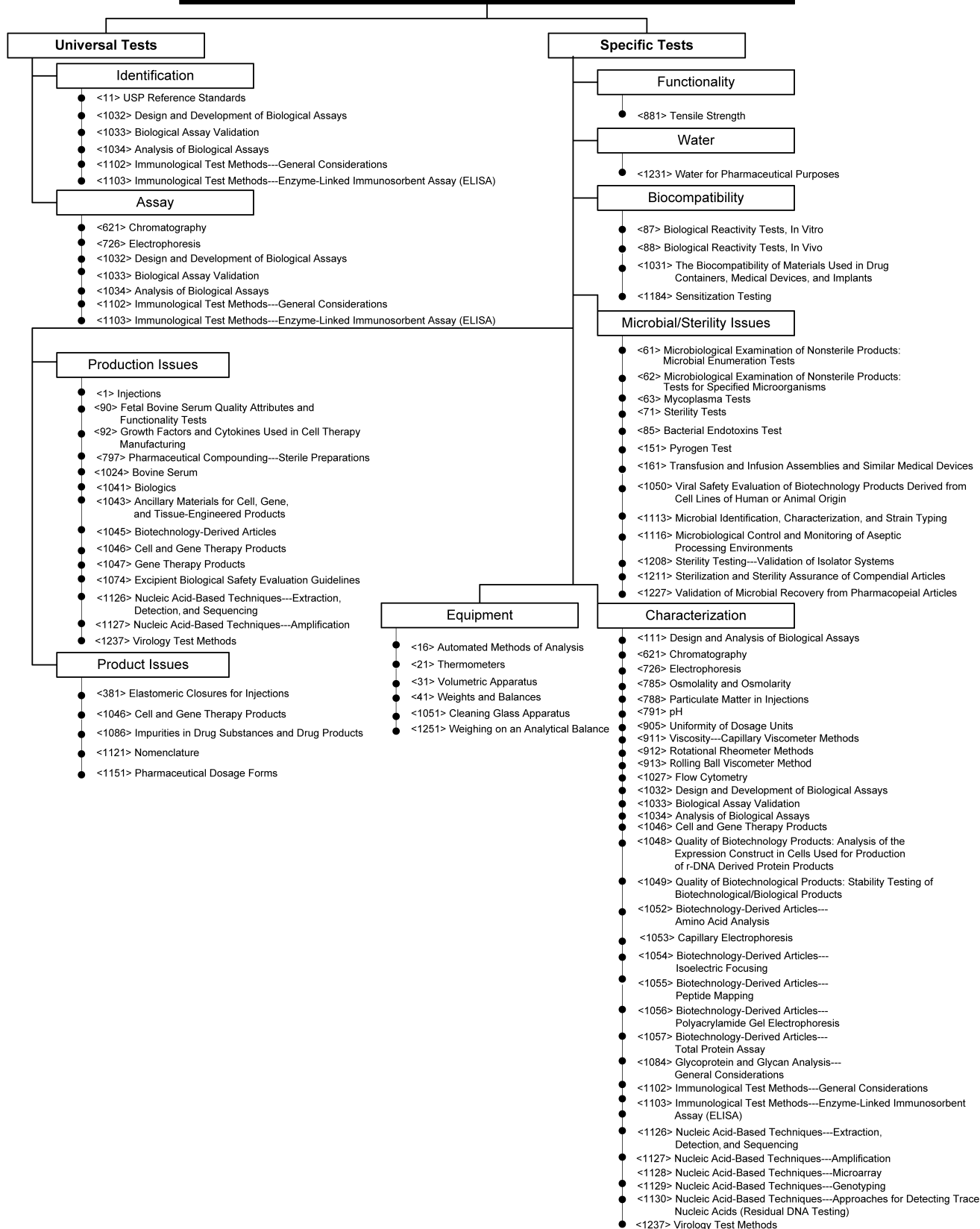


Chart 9. Drug Product Distribution

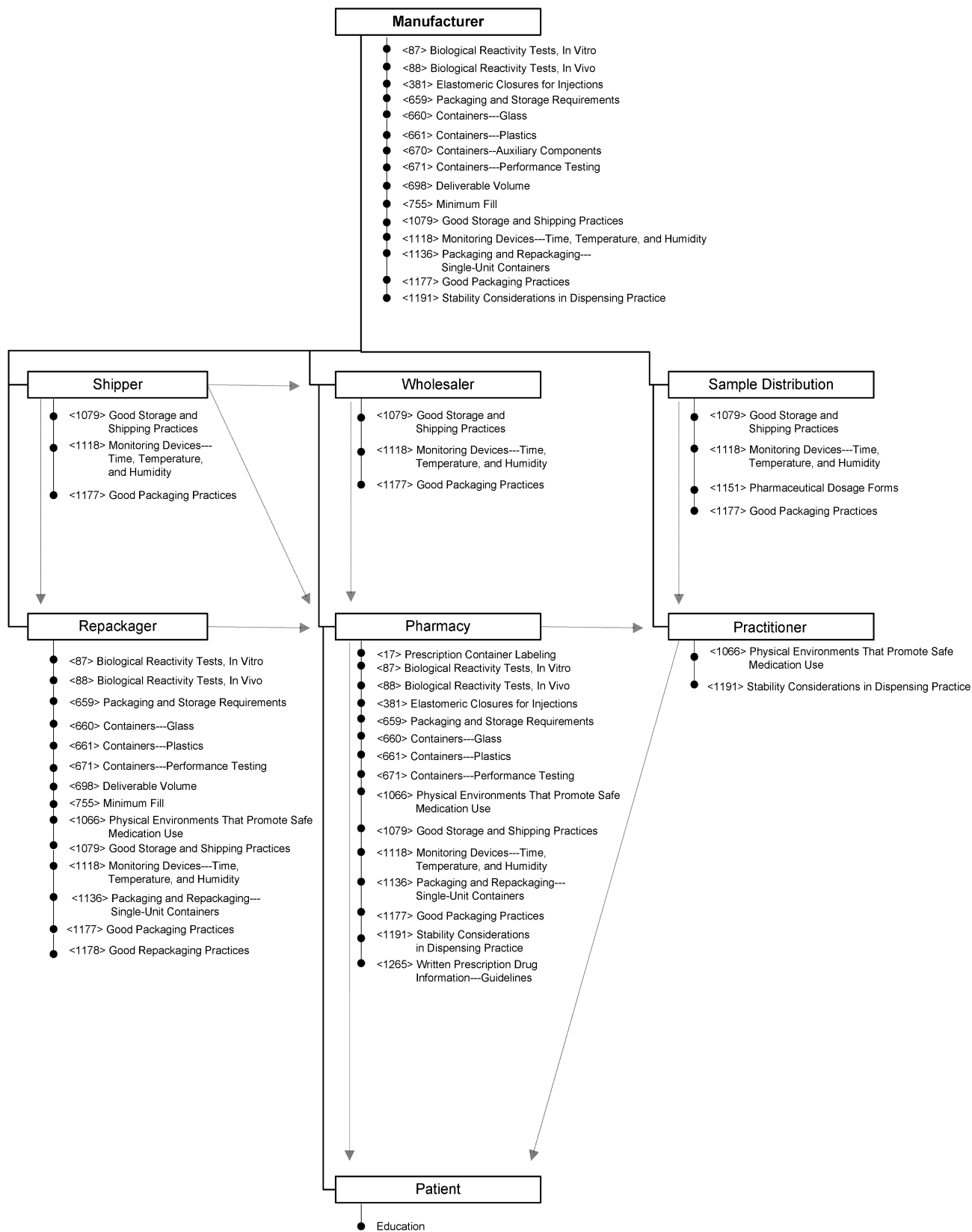


Chart 10. Microbiology

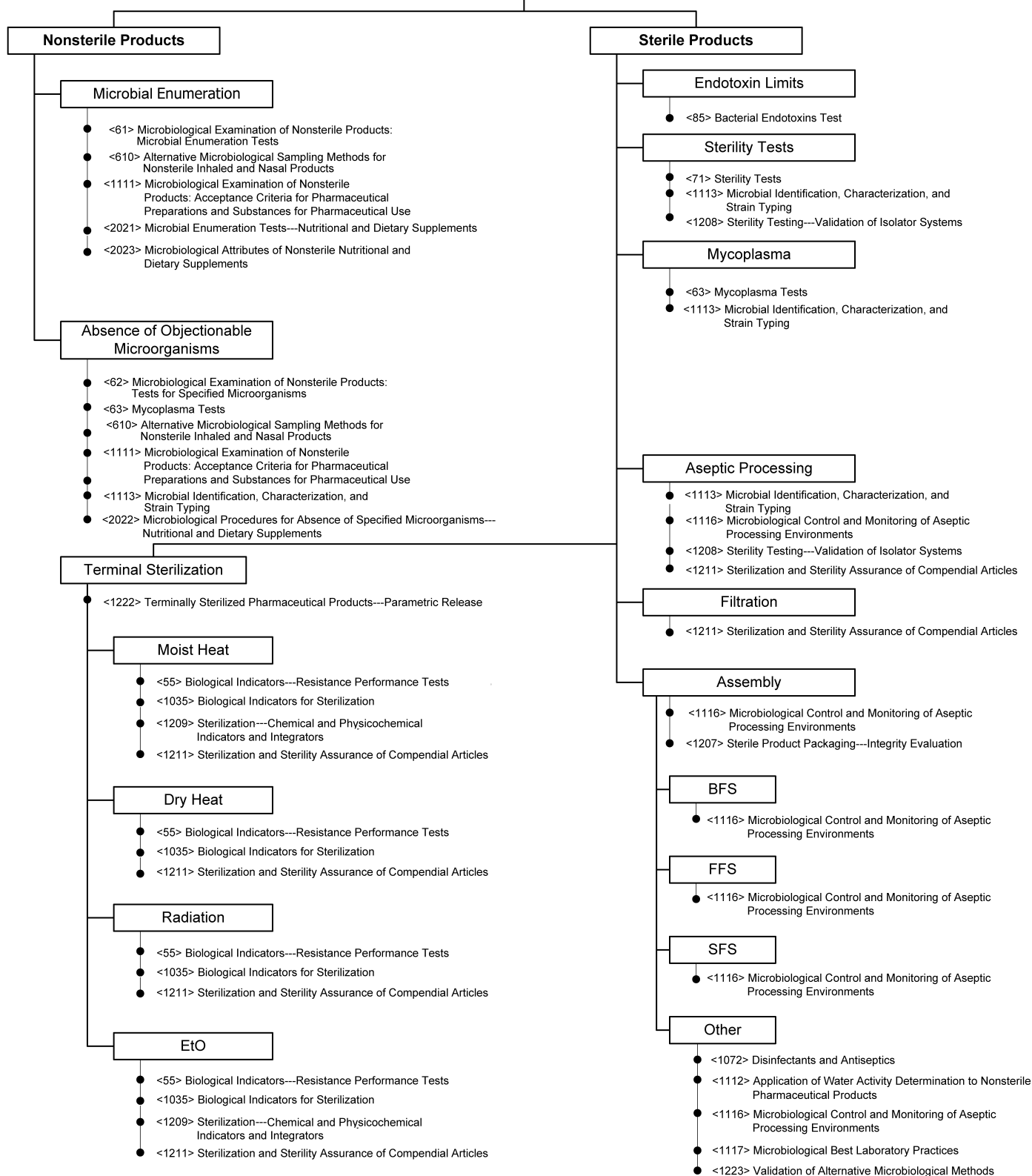


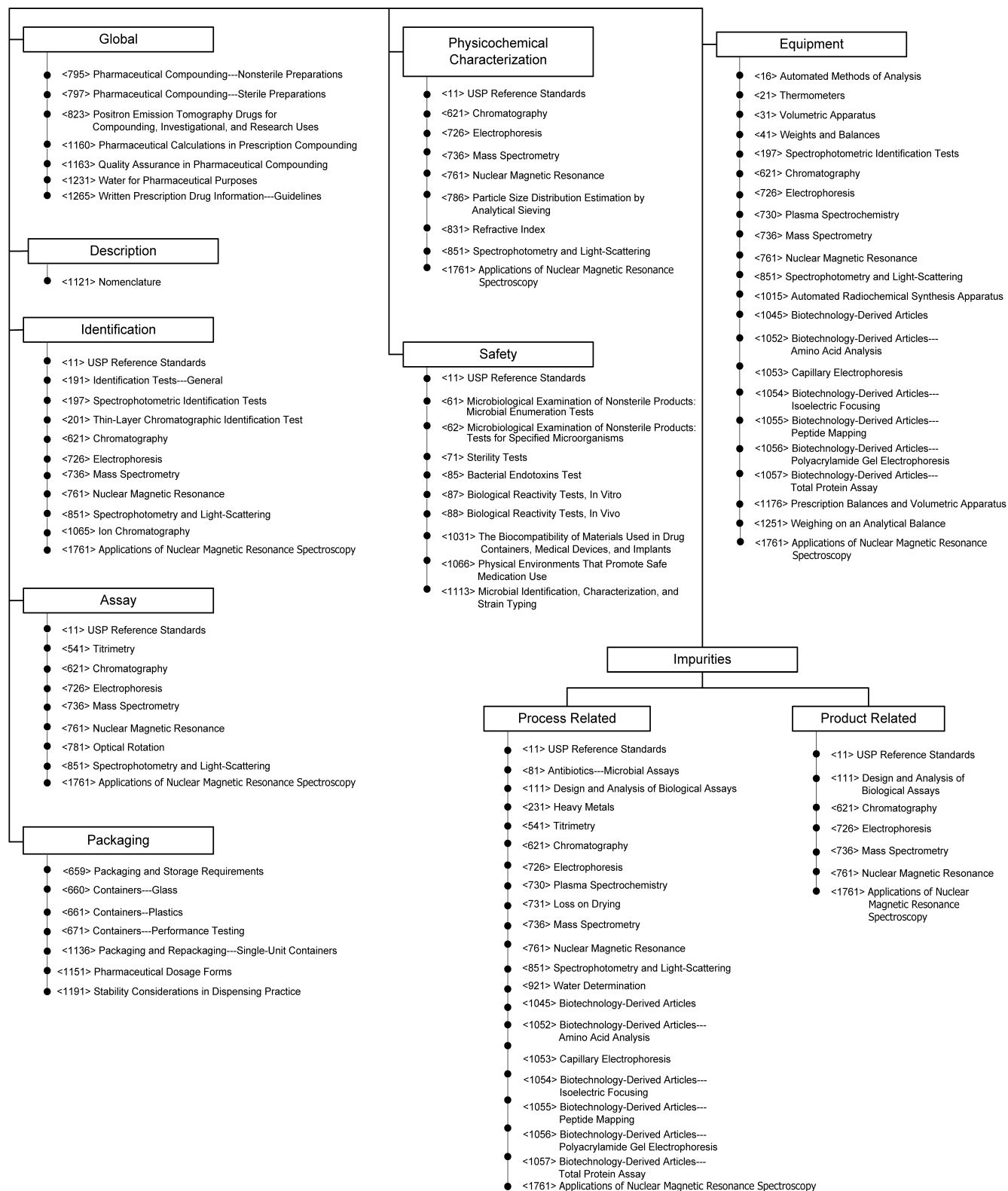
Chart 11. Dietary Supplement Ingredients



Chart 12. Dietary Supplement Products



Chart 13. Compounding—Substance/Preparation/Practice



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(For complete alphabetical list of all general chapters in this Pharmacopeia, see under “General chapters” in the index.)

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General Chapters

General Tests and Assays

General Requirements for Tests and Assays

〈1〉 INJECTIONS

INTRODUCTION

Parenteral articles are preparations intended for injection through the skin or other external boundary tissue, rather than through the alimentary canal, so that the active substances they contain are administered, using gravity or force, directly into a blood vessel, organ, tissue, or lesion. Parenteral articles are prepared scrupulously by methods designed to ensure that they meet Pharmacopeial requirements for sterility, pyrogens, particulate matter, and other contaminants, and, where appropriate, contain inhibitors of the growth of microorganisms. An Injection is a preparation intended for parenteral administration and/or for constituting or diluting a parenteral article prior to administration.

NOMENCLATURE AND DEFINITIONS

Nomenclature¹

The following nomenclature pertains to five general types of preparations, all of which are suitable for, and intended for, parenteral administration. They may contain buffers, preservatives, or other added substances.

1. *[DRUG] Injection*—Liquid preparations that are drug substances or solutions thereof.

¹ This nomenclature has been adopted by the USP Drug Nomenclature Committee for implementation by supplemental revisions of USP 34–NF 29. For currently official monograph titles in the form *Sterile [DRUG]* that have not yet been revised, the following nomenclature continues in use in this Pharmacopeia: (1) medicaments or solutions or emulsions thereof suitable for injection, bearing titles of the form *[DRUG] Injection*; (2) dry solids or liquid concentrates containing no buffers, diluents, or other added substances, and which, upon the addition of suitable solvents, yield solutions conforming in all respects to the requirements for Injections, and which are distinguished by titles of the form *Sterile [DRUG]*; (3) preparations the same as those described under (2) except that they contain one or more buffers, diluents, or other added substances, and which are distinguished by titles of the form *[DRUG] for Injection*; (4) solids which are suspended in a suitable fluid medium and which are not to be injected intravenously or into the spinal canal, distinguished by titles of the form *Sterile [DRUG] Suspension*; and (5) dry solids which, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for Sterile Suspensions, and which are distinguished by titles of the form *Sterile [DRUG] for Suspension*.

2. *[DRUG] for Injection*—Dry solids that, upon the addition of suitable vehicles, yield solutions conforming in all respects to the requirements for *Injections*.
3. *[DRUG] Injectable Emulsion*—Liquid preparations of drug substances dissolved or dispersed in a suitable emulsion medium.
4. *[DRUG] Injectable Suspension*—Liquid preparations of solids suspended in a suitable liquid medium.
5. *[DRUG] for Injectable Suspension*—Dry solids that, upon the addition of suitable vehicles, yield preparations conforming in all respects to the requirements for *Injectable Suspensions*.

Definitions

BIOLOGICS

The Pharmacopeial definitions for sterile preparations for parenteral use generally do not apply in the case of the biologics because of their special nature and licensing requirements (see *Biologics* 〈1041〉).

INGREDIENTS

Vehicles and Added Substances

Aqueous Vehicles—The vehicles for aqueous Injections meet the requirements of the *Pyrogen Test* 〈151〉 or the *Bacterial Endotoxins Test* 〈85〉, whichever is specified. *Water for Injection* generally is used as the vehicle, unless otherwise specified in the individual monograph. Sodium chloride may be added in amounts sufficient to render the resulting solution isotonic; and *Sodium Chloride Injection*, or *Ringer's Injection*, may be used in whole or in part instead of *Water for Injection*, unless otherwise specified in the individual monograph. For conditions applying to other adjuvants, see *Added Substances* in this chapter.

Other Vehicles—Fixed oils used as vehicles for nonaqueous Injections are of vegetable origin, are odorless or nearly so, and have no odor suggesting rancidity. They meet the requirements of the test for *Solid paraffin* in *Mineral Oil*, the cooling bath being maintained at 10°, have a *Saponification Value* between 185 and 200 (see *Fats and Fixed Oils* 〈401〉), have an *Iodine Value* between 79 and 141 (see *Fats and Fixed Oils* 〈401〉), and meet the requirements of the following tests.

Unsaponifiable Matter (see *Fats and Fixed Oils* 〈401〉): not more than 1.5%.

Acid Value (see *Fats and Fixed Oils* 〈401〉): not more than 0.2.

Peroxide Value (see *Fats and Fixed Oils* 〈401〉): not more than 5.0.

Water, Method Ic (921): not more than 0.1%.

Limit of Copper, Iron, Lead, and Nickel—[NOTE—The test for nickel is not required if the oil has not been subjected to hydrogenation, or a nickel catalyst has not been used in processing.] Proceed as directed in the section *Trace Metals in Fats and Fixed Oils* (401). Not more than 1 ppm of copper is found; not more than 1 ppm of iron is found; not more than 1 ppm of lead is found; and not more than 1 ppm of nickel is found.

Synthetic mono- or diglycerides of fatty acids may be used as vehicles, provided they are liquid and remain clear when cooled to 10° and have an *Iodine Value* of not more than 140 (see *Fats and Fixed Oils* (401)).

These and other nonaqueous vehicles may be used, provided they are safe, in the volume of Injection administered, and also provided they do not interfere with the therapeutic efficacy of the preparation or with its response to prescribed assays and tests.

Added Substances—Suitable substances may be added to preparations intended for injection to increase stability or usefulness, unless proscribed in the individual monograph, provided they are harmless in the amounts administered and do not interfere with the therapeutic efficacy or with the responses to the specified assays and tests. No coloring agent may be added, solely for the purpose of coloring the finished preparation, to a solution intended for parenteral administration (see also *Added Substances in General Notices and Antimicrobial Effectiveness Testing* (51)).

Observe special care in the choice and use of added substances in preparations for injection that are administered in a volume exceeding 5 mL. The following maximum limits prevail unless otherwise directed: for agents containing mercury and the cationic, surface-active compounds, 0.01%; for chlorobutanol, cresol, phenol, and similar types of substances, 0.5%; and for sulfur dioxide, or an equivalent amount of the sulfite, bisulfite, or metabisulfite of potassium or sodium, 0.2%.

A suitable substance or mixture of substances to prevent the growth of microorganisms must be added to preparations intended for injection that are packaged in multiple-dose containers, regardless of the method of sterilization used, unless one of the following conditions prevails: (1) there are different directions in the individual monograph; (2) the substance contains a radionuclide with a physical half-life of less than 24 hours; and (3) the active ingredients are themselves antimicrobial. Such substances are used in concentrations that will prevent the growth of or kill microorganisms in the preparations for injection. Such substances also meet the requirements of *Antimicrobial Effectiveness Testing* (51) and *Antimicrobial Agents—Content* (341). Sterilization processes are used even though such substances are used (see also *Sterilization and Sterility Assurance of Compendial Articles* (1211)). The air in the container may be evacuated or be displaced by a chemically inert gas. Where specified in a monograph, information regarding sensitivity of the article to oxygen is to be provided in the labeling.

LABELS AND LABELING

Labeling

NOTE—See definitions of “label” and “labeling” in section 10.40 *Labeling* under section 10. *Preservation, Packaging, Storage, and Labeling of the General Notices and Requirements*.

The label states the name of the preparation; in the case of a liquid preparation, the percentage content of drug or amount of drug in a specified volume; in the case of a dry preparation, the amount of active ingredient; the route of administration; a statement of storage conditions and an expiration date; the name and place of business of the manufacturer, packer, or distributor; and an identifying lot num-

ber. The lot number is capable of yielding the complete manufacturing history of the specific package, including all manufacturing, filling, sterilizing, and labeling operations.

Where the individual monograph permits varying concentrations of active ingredients in the large-volume parenteral, the concentration of each ingredient named in the official title is stated as if part of the official title, e.g., Dextrose Injection 5%, or Dextrose (5%) and Sodium Chloride (0.2%) Injection.

The labeling includes the following information if the complete formula is not specified in the individual monograph: (1) In the case of a liquid preparation, the percentage content of each ingredient or the amount of each ingredient in a specified volume, except that ingredients added to adjust to a given pH or to make the solution isotonic may be declared by name and a statement of their effect; and (2) in the case of a dry preparation or other preparation to which a diluent is intended to be added before use, the amount of each ingredient, the composition of recommended diluent(s) [the name(s) alone, if the formula is specified in the individual monograph], the amount to be used to attain a specific concentration of active ingredient and the final volume of solution so obtained, a brief description of the physical appearance of the constituted solution, directions for proper storage of the constituted solution, and an expiration date limiting the period during which the constituted solution may be expected to have the required or labeled potency if it has been stored as directed.

Containers for Injections that are intended for use as dialysis, hemofiltration, or irrigation solutions and that contain a volume of more than 1 L are labeled to indicate that the contents are not intended for use by intravenous infusion.

Injections intended for veterinary use are labeled to that effect.

The container is so labeled that a sufficient area of the container remains uncovered for its full length or circumference to permit inspection of the contents.

STRENGTH AND TOTAL VOLUME FOR SINGLE- AND MULTIPLE-DOSE INJECTABLE DRUG PRODUCTS

For single-dose and multiple-dose injectable drug products, the strength per total volume should be the primary and prominent expression on the principal display panel of the label, followed in close proximity by strength per mL enclosed by parentheses. For containers holding a volume of less than 1 mL, the strength per fraction of a mL should be the only expression of strength. Strength per single mL should be expressed as mg/mL, not mg/1 mL.

The following formats are acceptable for contents of greater than 1 mL:

Total strength/total volume: 500 mg/10 mL

Strength/mL: 50 mg/mL

or

Total strength/total volume: 25,000 Units/5 mL

Strength/mL: 5,000 Units/mL

The following format is acceptable for contents of less than 1 mL: 12.5 mg/0.625 mL

There are, however, some exceptions to expressing strength per total volume. In certain cases, the primary and prominent expression of the total drug content per container would not be effective in preventing medication errors (e.g., insulin). An example is the use of lidocaine or other similar drugs used as a local anesthetic where the product is ordered and administered by percentage (e.g., 1%, 2%) or a local anesthetic in combination with epinephrine that is expressed as a ratio (e.g., 1:100,000). In such cases, the total strength should be expressed: for example, 1% (100 mg/10 mL). Dry solids, which need to be reconstituted, should follow the same format, with the exception that only the total strength of the drug should be listed, not the strength/total volume or strength/mL.

Aluminum in Large-Volume Parenterals (LVPs), Small-Volume Parenterals (SVPs), and Pharmacy Bulk Packages (PBPs) Used in Total Parenteral Nutrition (TPN) Therapy

- (a) The aluminum content of LVPs used in TPN therapy must not exceed 25 µg per L (µg/L).
- (b) The package insert of LVPs used in TPN therapy must state that the drug product contains no more than 25 µg of aluminum per L. This information must be contained in the "Precautions" section of the labeling of all LVPs used in TPN therapy.
- (c) If the maximum amount of aluminum in SVPs and PBPs is 25 µg per L (µg/L) or less, instead of stating the exact amount of aluminum that each contains, as in paragraph (d), the immediate container label for SVPs and PBPs used in the preparation of TPN parenterals (with exceptions as noted below) may state: "Contains no more than 25 µg/L of aluminum". If the SVP or PBP is a lyophilized powder, the immediate container label may state the following: "When reconstituted in accordance with the package insert instructions, the concentration of aluminum will be no more than 25 µg/L".
- (d) The maximum level of aluminum at expiry must be stated on the immediate container label of all SVPs and PBPs used in the preparation of TPN parenterals and injectable emulsions. The aluminum content must be stated as follows: "Contains no more than ___ µg/L of aluminum". The immediate container label of all SVPs and PBPs that are lyophilized powder used in the preparation of TPN solutions must contain the following statement: "When reconstituted in accordance with the package insert instructions, the concentration of aluminum will be no more than ___ µg/L." This maximum amount of aluminum must be stated as the highest one of the following three levels:
 - (1) The highest level for the batches produced during the last three years
 - (2) The highest level for the latest five batches
 - (3) The maximum level in terms of historical levels, but only until completion of production of the first five batches after July 26, 2004.

The package insert for all LVPs, SVPs, and PBPs used in the preparation of TPN products must contain a warning statement. This warning must be contained in the "Warning" section of the labeling and must state the following: "WARNING: This product contains aluminum that may be toxic. Aluminum may reach toxic levels with prolonged parenteral administration if kidney function is impaired. Premature neonates are particularly at risk because their kidneys are immature, and they require large amounts of calcium and phosphate solutions that contain aluminum. Research indicates that patients with impaired kidney function, including premature neonates, who receive parenteral levels of aluminum at greater than 4 to 5 µg per kg per day accumulate aluminum at levels associated with central nervous system and bone toxicity. Tissue loading may occur at even lower rates of administration of TPN products."

PACKAGING

Containers for Injections

Containers, including the closures, for preparations for injections do not interact physically or chemically with the preparations in any manner to alter the strength, quality, or purity beyond the official requirements under the ordinary or customary conditions of handling, shipment, storage,

sale, and use. The container is made of material that permits inspection of the contents. The type of glass preferable for each parenteral preparation is usually stated in the individual monograph. Unless otherwise specified in the individual monograph, plastic containers may be used for packaging injections (see *Containers—Plastics* (661)).

For definitions of single-dose and multiple-dose containers, see sections 10.20.70 and 10.20.110, respectively, in the *General Notices and Requirements*. Containers meet the requirements in *Containers—Glass* (660) and *Containers—Plastics* (661).

Containers are closed or sealed in such a manner as to prevent contamination or loss of contents. Validation of container integrity must demonstrate no penetration of microbial contamination or chemical or physical impurities. In addition, the solutes and the vehicle must maintain their specified total and relative quantities or concentrations when exposed to anticipated extreme conditions of manufacturing and processing, and storage, shipment, and distribution. Closures for multiple-dose containers permit the withdrawal of the contents without removal or destruction of the closure. The closure permits penetration by a needle and, upon withdrawal of the needle, closes at once, protecting the container against contamination. Validation of the multiple-dose container integrity must include verification that such a package prevents microbial contamination or loss of product contents under anticipated conditions of multiple entry and use.

Piggyback containers are usually intravenous infusion containers used to administer a second infusion through a connector of some type or an injection port on the administration set of the first fluid, thereby avoiding the need for another injection site on the patient's body. Piggyback containers are also known as secondary infusion containers.

Potassium Chloride for Injection Concentrate

The use of a black closure system on a vial (e.g., a black flip-off button and a black ferrule to hold the elastomeric closure) or the use of a black band or series of bands above the constriction on an ampul is prohibited, except for *Potassium Chloride for Injection Concentrate*.

Neuromuscular Blocking and Paralyzing Agents

All injectable preparations of neuromuscular blocking agents and paralyzing agents must be packaged in vials with a cautionary statement printed on the ferrules or cap overseals. Both the container cap ferrule and the cap over-seal must bear in black or white print (whichever provides the greatest color contrast with the ferrule or cap color) the words: "Warning: Paralyzing Agent" or "Paralyzing Agent" (depending on the size of the closure system). Alternatively, the over-seal may be transparent and without words, allowing for visualization of the warning labeling on the closure ferrule.

Containers for Sterile Solids

Containers, including the closures, for dry solids intended for parenteral use do not interact physically or chemically with the preparation in any manner to alter the strength, quality, or purity beyond the official requirements under the ordinary or customary conditions of handling, shipment, storage, sale, and use.

A container for a sterile solid permits the addition of a suitable solvent and withdrawal of portions of the resulting solution or suspension in such manner that the sterility of the product is maintained.

Where the *Assay* in a monograph provides a procedure for the *Sample solution*, in which the total withdrawable contents are to be withdrawn from a single-dose container

with a hypodermic needle and syringe, the contents are to be withdrawn as completely as possible into a dry hypodermic syringe of a rated capacity not exceeding three times the volume to be withdrawn and fitted with a 21-gauge needle not less than 2.5 cm (1 inch) in length, with care being taken to expel any air bubbles, and discharged into a container for dilution and assay.

Container Content

Each container of an injection contains sufficient excess to allow withdrawal of the labeled quantity of drug. Such withdrawal shall be performed according to labeled directions, if provided.

DETERMINATION OF VOLUME OF INJECTION IN CONTAINERS

This section is harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. These pharmacopoeias have undertaken not to make any unilateral change to this harmonized section. A portion of the present text (see below) is national *USP* text, and therefore not part of the harmonized text; it is marked with symbols (♦) to specify this fact.

Suspensions and emulsions must be shaken before withdrawal of the contents and before the determination of the density. Oily and viscous preparations may be warmed according to the instructions on the label, if necessary, and thoroughly shaken immediately before removing the contents. The contents are then cooled to 20°–25°C before measuring the volume. ♦Sterile solid formulations must be constituted according to labeled directions before removing the contents. Contents are then to be measured following the procedures for suspensions, emulsions, or solutions, as appropriate.♦

Single-Dose Containers—Select 1 container if the volume of the container is 10 mL or more, 3 containers if the nominal volume is more than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. Take up individually the total contents of each container selected into a dry syringe of a capacity not exceeding three times the volume to be measured and fitted with a 21-gauge needle not less than 2.5 cm (1 inch) in length. Expel any air bubbles from the syringe and needle, and then discharge the contents of the syringe, without emptying the needle, into a standardized, dry cylinder (graduated to contain rather than to deliver the designated volumes) of such size that the volume to be measured occupies at least 40% of its graduated volume. Alternatively, the volume of the contents in mL may be calculated as the mass, in g, divided by the density. For containers with a nominal volume of 2 mL or less, the contents of a sufficient number of containers may be pooled to obtain the volume required for the measurement, provided that a separate, dry syringe assembly is used for each container. The contents of containers holding 10 mL or more may be determined by means of opening them and emptying the contents directly into the graduated cylinder or tared beaker.

The volume is not less than the nominal volume in the case of containers examined individually or, in the case of containers with a nominal volume of 2 mL or less, is not less than the sum of the nominal volumes of the containers taken collectively.

Multi-Dose Containers—For Injections in multiple-dose containers labeled to yield a specific number of doses of a stated volume, select 1 container, and proceed as directed for single-dose containers, using the same number of separate syringe assemblies as the number of doses specified. The volume is such that each syringe delivers not less than the stated dose.

Injections in Cartridges or Prefilled Syringes—Select 1 container if the volume is 10 mL or more, 3 containers if the nominal volume is more than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. If necessary, fit the containers with the accessories required for their use (needle, piston, syringe) and transfer the entire contents of each container without emptying the needle into a dry tared beaker by slowly and constantly depressing the piston. Determine the volume in mL, calculated as the mass, in g, divided by the density.

The volume measured for each of the containers is not less than the nominal volume.

Large-Volume Intravenous Solutions—For intravenous solutions, select 1 container. Transfer the contents into a dry measuring cylinder of such a capacity that the volume to be determined occupies at least 40% of the nominal volume of the cylinder. Measure the volume transferred.

The volume is not less than the nominal volume.

Labeling on Ferrules and Cap Overseals

Healthcare practitioners using injectable products must be able to easily see and act on labeling statements that convey important safety messages critical for the prevention of imminent life-threatening situations. These cautionary labeling statements must be simple, concise, and devoid of nonessential information. Products that do not require cautionary statements should be free of information, so that those with cautionary statements are immediately apparent. Accomplishing this requires a systematic approach to labeling of injectable products, and one that assures that the ferrule and cap overseal—an area of these products that is highly visible to practitioners as they use these medicines—is reserved for critical safety messages. Accordingly:

1. Only cautionary statements may appear on the top (circle) surface of the ferrule and/or cap overseal of a vial containing an injectable product. The cautionary statement should appear on both the ferrule and cap but may appear solely on the ferrule if the cap overseal is transparent and the cautionary statement beneath the cap is readily legible. A cautionary statement is one intended to prevent an imminent life-threatening situation and may include instructional statements that provide potency or other safety-related instructions if warranted. Examples of such statements include but are not limited to: "Warning—Paralyzing Agent" and "Dilute Before Using." The cautionary statement should be printed in a contrasting color and clearly visible under ordinary conditions of use.
2. If no cautionary statement is necessary, the top surface of the vial, including the ferrule and cap overseal, must remain blank.
3. Other statements or features including but not limited to identifying numbers or letters, such as code numbers, lot numbers, company names, logos, or product names, etc., may appear on the side (skirt) surface of the ferrule on vials containing injectable products but not on the top (circle) surface of the ferrule or cap overseal. The appearance of such statements or features on the skirt surface of the ferrule should not detract from, or interfere with, the cautionary statement on the top surface.

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Packaging and Storage

The volume of injection in single-dose containers provides the amount specified for parenteral administration at one time and in no case is more than sufficient to permit the withdrawal and administration of 1 L.

Preparations intended for intraspinal, intracisternal, or peridural administration are packaged only in single-dose containers.

Unless otherwise specified in the individual monograph, a multiple-dose container contains a volume of Injection sufficient to permit the withdrawal of not more than 30 mL.

The following injections are exempt from the 1-L restriction of the foregoing requirements relating to packaging:

1. Injections packaged for extravascular use as irrigation solutions or peritoneal dialysis solutions
2. Injections packaged for intravascular use as parenteral nutrition or as replacement or substitution fluid to be administered continuously during hemofiltration

Injections packaged for intravascular use that may be used for intermittent, continuous, or bolus replacement fluid administration during hemodialysis or other procedures, unless excepted above, must conform to the 1-L restriction.

Injections labeled for veterinary use are exempt from packaging and storage requirements concerning the limitation to single-dose containers and the limitation on the volume of multiple-dose containers.

Change to read:

FOREIGN AND PARTICULATE MATTER

▲ Articles intended for parenteral administration shall be prepared in a manner designed to exclude particulate matter as defined in *Particulate Matter in Injections* (788) and other foreign matter as appropriate for the dosage form.▲ ^{USP36} Each final container of all parenteral preparations shall be inspected to the extent possible for the presence of observable foreign and particulate matter (hereafter termed “visible particulates”) in its contents. The inspection process shall be designed and qualified to ensure that every lot of all parenteral preparations is essentially free from visible particulates. Qualification of the inspection process shall be performed with reference to particulates in the visible range of a type that might emanate from the manufacturing or filling process. Every container whose contents show evidence of visible particulates shall be rejected. The inspection for visible particulates may take place when inspecting for other critical defects, such as cracked or defective containers or seals, or when characterizing the appearance of a lyophilized product.

Where the nature of the contents or the container-closure system permits only limited capability for the inspection of the total contents, the 100% inspection of a lot shall be supplemented with the inspection of constituted (e.g., dried) or withdrawn (e.g., dark amber container) contents of a sample of containers from the lot.

All large-volume Injections for single-dose infusion and small-volume Injections are subject to the light obscuration or microscopic procedures and limits for subvisible particulate matter set forth in *Particulate Matter In Injections* (788), unless otherwise specified in the individual monograph. An article packaged as both a large-volume and a small-volume Injection meets the requirements set forth for small-volume Injections where the container is labeled as containing 100 mL or less, if the individual monograph states a test for *Particulate Matter in Injections* (788); it meets the requirements set forth for large-volume Injections for single-dose infusion where the container is labeled as containing more than 100 mL.

▲▲ ^{USP36}

STERILITY

Sterility Tests—Preparations for injection meet the requirements in *Sterility Tests* (71).

CONSTITUTED SOLUTIONS

Dry solids from which constituted solutions are prepared for injection bear titles of the form *[DRUG] for Injection*. Because these dosage forms are constituted at the time of use by the healthcare practitioner, tests and standards pertaining to the solution as constituted for administration are not included in the individual monographs on sterile dry solids or liquid concentrates. However, in the interest of assuring the quality of injection preparations as they are actually administered, the following nondestructive tests are provided for demonstrating the suitability of constituted solutions when they are prepared just prior to use.

Completeness and Clarity of Solution—Constitute the solution as directed in the labeling supplied by the manufacturer for the sterile dry dosage form.

A: The solid dissolves completely, leaving no visible residue as undissolved matter.

B: The constituted solution is not significantly less clear than an equal volume of the diluent or of Purified Water contained in a similar vessel and examined similarly.

Particulate Matter—Constitute the solution as directed in the labeling supplied by the manufacturer for the sterile dry dosage form: the solution is essentially free from particles of foreign matter that can be observed on visual inspection.

<3> TOPICAL AND TRANSDERMAL DRUG PRODUCTS —PRODUCT QUALITY TESTS

INTRODUCTION

Topically applied drug products fall into two general categories: those applied to achieve local action and those applied to achieve systemic effects after absorption through the skin into the blood circulation. Local action can occur at or on the surface of the application site (e.g., stratum corneum, ocular epithelium), in the underlying tissues (e.g., epidermis and/or dermis) and on subcutaneous tissues (e.g., muscle or joint).

Topically applied drug products include, but are not restricted to creams, gels, ointments, pastes, suspensions, lotions, foams, sprays, aerosols, solutions, and transdermal delivery systems (TDS, also known as patches). The definitions and descriptions of these dosage forms, and brief information on their composition and/or manufacturing process can be found in *Pharmaceutical Dosage Forms* (1151).

Procedures and acceptable criteria for testing topically applied drug products can be divided into those that assess general product quality attributes and those that assess product performance. The product quality attributes include the following: description, identification, assay (strength), impurities, physicochemical properties, uniformity of dosage units, water content, pH, apparent viscosity, microbial limits, antimicrobial preservative content, antioxidant content, sterility, if applicable, and other tests that may be product specific. Product performance testing assesses drug release and other attributes that affect drug release from the finished dosage form.

Although most topically applied drug products are semisolids, liquids, or suspensions, TDS are physical devices that are applied to the skin and vary in their composition and

method of fabrication. TDS release their active ingredients by different mechanisms. They can be passive or active. This chapter covers only the tests related to passive TDS.

PRODUCT QUALITY TESTS FOR TOPICALLY APPLIED DRUG PRODUCTS

Universal Tests

Universal tests (see *ICH Guidance Q6A—Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances*, available at www.ich.org) are listed below and are applicable to all topically applied drug products.

Description: A qualitative description of the drug product should be provided. The acceptance criteria should include the final acceptable appearance of the finished dosage form and packaging. A visual examination should identify changes in color, adhesive migration (i.e., cold flow) for TDS, separations, crystallization, etc., that are specific to the drug product. The description should specify the content or the label claim of the article. This is not a compendial test but is part of the manufacturer's specification for the drug product.

Identification: Identification tests are discussed in *General Notices and Requirements*, 5.40. Identification tests should establish the identity of the drug or drugs present in the article and should discriminate between compounds of closely related structures that are likely to be present. Identity tests should be specific for the drug substance(s) (e.g., infrared spectroscopy). Near infrared (NIR) or Raman spectrophotometric methods also could be acceptable for the identification of the drug product (see *Near-Infrared Spectrophotometry* {1119} and *Raman Spectroscopy* {1120}). Identification solely by a single chromatographic retention time is not specific.

Assay: A specific and stability-indicating test should be used to determine the strength (content) of the drug product. In cases when the use of a nonspecific assay (e.g., *Titrimetry* {541}) is justified, other supporting analytical procedures should be used to achieve overall specificity.

Impurities: Process impurities, synthetic by-products, impurities associated with the adhesive (e.g., residual monomers), residual solvents (see *Residual Solvents* {467}), heavy metals (see *Heavy Metals* {231}), and other inorganic and organic impurities may be present in the drug substance and excipients used in the manufacture of the drug product and should be assessed and controlled. Impurities arising from the degradation of the drug substance and those arising during the manufacturing process of the drug product should also be assessed and controlled.

Specific Tests

In addition to the universal tests listed above, the following specific tests should be considered on a case-by-case basis.

Uniformity of Dosage Units: This test is applicable for TDS and for dosage forms packaged in single-unit containers (see *Uniformity of Dosage Units* {905}).

Water Content: A test for water content should be included when appropriate (see *Water Determination* {921}). This test is generally formulation dependent. Therefore, it is not included in the compendial drug product monograph but is part of the manufacturer's specification for the drug product.

Microbial Limits: Microbial examination of nonsterile drug products is performed according to the methods given in general chapters *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* {61} and *Microbiologi-*

cal Examination of Nonsterile Products: Tests for Specified Microorganisms {62}, unless the formulation itself is demonstrated to have antimicrobial properties. Acceptance criteria for nonsterile pharmaceutical products based on total aerobic microbial count (TAMC) and total combined yeasts and molds count (TYMC) are given in *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* {1111}.

Antimicrobial Preservative Content: Acceptance criteria for antimicrobial preservative content in multidose products should be established. They should be based on levels of antimicrobial preservative necessary to maintain the product's microbiological quality at all stages throughout its proposed usage and shelf life (see *Antimicrobial Effectiveness Testing* {51}).

Antioxidant Content: If antioxidants are present in the drug product, tests of their content should be established unless oxidative degradation can be detected by another test method such as impurity testing. Acceptance criteria for antioxidant content should be established. They should be based on the levels of antioxidant necessary to maintain the product's stability at all stages throughout its proposed usage and shelf life.

Sterility: Depending on the use of the dosage form (e.g. ophthalmic preparations, products that will be applied to open wounds or burned areas), sterility of the product should be demonstrated as appropriate (see *Sterility Tests* {71}).

pH: When applicable, topically applied drug products should be tested for pH at the time of batch release and at designated stability time points for batch-to-batch monitoring. Because some topically applied drug products contain very limited quantities of water or aqueous phase, pH measurements may not always be warranted.

This test is generally formulation dependent. Therefore, it is not included in the compendial drug product monograph but is part of the manufacturer's specification for the drug product.

Particle Size: The particle size of the active drug substance(s) in topically applied drug products is usually determined and controlled at the formulation development stage.

However, topically applied drug products should be examined for evidence of particle size alteration (i.e., appearance of particles, changes in particle form, size, shape, habit, or aggregation) of the active drug substance that may occur during the course of product processing and storage. Such examinations should be conducted at the time of batch release and at designated stability test time points for batch-to-batch monitoring because changes that are visually (macro- and microscopically) observable would likely compromise the integrity and/or performance of the drug product. These types of testing are generally formulation dependent. Therefore, such tests are not included in compendial monographs but are part of the manufacturer specification for the drug product.

SPECIFIC TESTS FOR OPHTHALMIC DRUG PRODUCTS

Ophthalmic dosage forms must meet the requirements of *Sterility Tests* {71}. If the specific ingredients used in the formulation do not lend themselves to routine sterilization techniques, ingredients that meet the sterility requirements described under *Sterility Tests* {71}, along with aseptic manufacture, may be used. Multiple-use ophthalmic preparations must contain a suitable substance or mixture of substances to prevent growth of, or to destroy, microorganisms accidentally introduced during the use of the product (see *Added Substances* under *Ophthalmic Ointments* {771}), unless otherwise directed in the individual monograph or unless the formula itself is bacteriostatic and/or the delivery system promotes bacteriostasis. The finished ophthalmic preparation must be free from large particles and must meet the

requirements for *Leakage* and for *Metal Particles* under *Ophthalmic Ointments* <771>. The immediate containers for ophthalmic preparations shall be sterile at the time of filling and closing. It is mandatory that the immediate containers for ophthalmic preparations be sealed and tamper-proof so that sterility is ensured at the time of first use.

SPECIFIC TESTS FOR TOPICALLY APPLIED SEMISOLID DRUG PRODUCTS

Apparent Viscosity

Viscosity is a measure of a formulation's resistance to flow and is an assessment of the rheological properties of the dosage form (e.g., semisolid dosage form). Because only Newtonian fluids possess a measurable viscosity that is independent of shear rate, semisolid pharmaceutical dosage forms which are non-Newtonian products exhibit an apparent viscosity.

The apparent viscosity of semisolid drug products should be tested at the time of batch release and initially at designated stability test time-points to set specifications for batch-to-batch and shelf life monitoring. Measurement procedures should be developed as outlined in *Viscosity* <911>. For semisolids that show thixotropy and/or irreversible changes in viscosity after shearing, specific attention should be given to sample preparation procedures to minimize variability in the measurement of apparent viscosity caused by variable shear histories (e.g., mixing speed and temperature, filling operation, sample handling). Furthermore, for some products, it may be warranted to have apparent viscosity specifications at more than one set of conditions (e.g., bulk in-process stage, final packaged product, high and low shear rates, different temperatures).

Apparent viscosity specifications based on data obtained during product development and shelf life testing should be established for batch release and throughout their proposed shelf life.

The apparent viscosity test is formulation and/or process dependent. Therefore, it is not included in compendial drug product monographs but is part of the manufacturer's specification for the drug product. Furthermore, the specifications for apparent viscosity of semisolid dosage forms at batch release and during stability testing may be different. Although the apparent viscosity of the finished drug product at the time of batch release must conform to the product development specifications, for stability testing, the apparent viscosity specifications for the drug product should be based on statistical assessment of the product over its shelf life.

Uniformity in Containers

Topically applied semisolid drug products may show physical separation during manufacturing processes and during their shelf life. To ensure the integrity of the drug product, it is essential to evaluate the uniformity of the finished product at the time of batch release and throughout its assigned shelf life.

PRODUCTS PACKAGED IN TUBES

Within-tube content uniformity can be assessed in the following manner.

Carefully remove or cut off the bottom tube seal and make a vertical cut from the bottom to the top of the tube. Carefully cut the tube around the upper rim, open the two flaps and lay the flaps open to expose the product.

Inspect the product visually for the presence of phase separation, change in physical appearance and texture, and other properties described in the product test for *Descrip-*

tion. If there is no observable phase separation or change in physical appearance and texture, and if the product meets the *Description* acceptance criteria, proceed as described below. If the product exhibits phase separation and/or change in physical appearance or texture, the product fails the tube content uniformity test.

The procedures describe below can be modified depending on the sensitivity of the quantitative procedure used to assay the drug substance(s) present in the formulation.

For Multiple-Dose Products That Contain 5 g or More:

Procedure 1—

1. Using a single tube, after visually inspecting the product remove an appropriate amount of product from the top, middle, and bottom portions of the tube. The sample size should be sufficient for at least one assay determination of the active ingredient(s). Carry out the assay test for the active ingredient(s) in each portion of the product, and evaluate the test results using *Acceptance Criteria A*.
2. If the product fails *Acceptance Criteria A*, test 3 additional tubes from the same batch following step 1 described above, and evaluate all 12 test results using *Acceptance Criteria B*.

Procedure 2—

1. Using two tubes, after visually inspecting the product, remove an appropriate amount of product from the top, middle, and bottom portions of each tube. The sample size should be sufficient for at least one assay determination of the active ingredient(s). Carry out the assay test for the active ingredient(s) in each portion of the tube, and evaluate the test results using *Acceptance Criteria A*.
2. If the product fails *Acceptance Criteria A*, test 2 additional tubes from the same batch following step 1 described above, and evaluate all 12 test results using *Acceptance Criteria B*.

For Multiple-Dose Products That Contain Less Than 5 g of Product:

1. Test the top and bottom portions of 2 tubes using *Procedure 1* or *Procedure 2* as described above. Evaluate the test results using *Acceptance Criteria A*.
2. If the product fails *Acceptance Criteria A*, test 2 additional tubes from the same batch following step 1 described above, and evaluate all 8 test results using *Acceptance Criteria B*.

Tube (Container) Content Uniformity Test Acceptance Criteria: In determining the relative standard deviation (RSD) from multiple tubes, first determine the variance from the three measurements for each tube and average across the tubes. The RSD is calculated using this average variance.

Acceptance Criteria A—All assay results are within the range of 90%–110% of the product label claim and the RSD is NMT 6% or as specified in the product specification or in the compendial monograph. If the RSD is greater than 6%, use *Acceptance Criteria B*.

Acceptance Criteria B—No assay result is outside the range of 90%–110% of the product label claim and the RSD of the 12 assay results is NMT 6% or as specified in the product specification or in the compendial monograph.

PRODUCTS PACKAGED IN CONTAINERS OTHER THAN TUBES

For semisolid products packaged in a container other than a tube when the sampling method presented above cannot be used, other sampling methods are acceptable, such as the one described below for a jar.

1. Select a suitable syringe of sufficient length to extend to the bottom of the container.
2. Remove and set aside the syringe plunger and cut off the bottom of the syringe barrel. Sampling should take place from a location to the left/right of the mid-line of the jar surface to preserve an undisturbed

region on the other side for any additional investigation (See Figure 1).

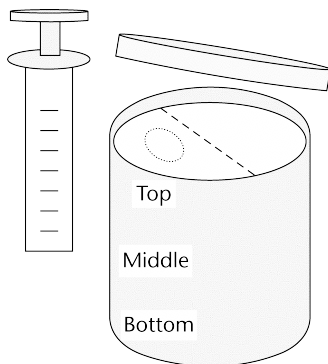


Figure 1. Sampling from a jar container.

3. Slowly push the syringe barrel into the container until it reaches the bottom. Then, twist the syringe barrel containing the sample core, and remove the syringe from the container.
4. Insert the syringe plunger into the barrel and carefully extrude the sample core onto a clean surface in three equal portions to represent the top, middle, and bottom portions of the container.
5. Remove an appropriate sample representative of the middle section of the top, middle, and bottom portions of the container samples, and test according to the instructions outlined in *Products Packaged in Tubes*.

SPECIFIC TESTS FOR TRANSDERMAL DELIVERY SYSTEMS

TDS or patches are formulated with an adhesive layer to ensure intimate contact with the skin to allow the delivery of the desired dose of drug. Adhesives in TDS must permit easy removal of the release liner before use, must adhere properly to human skin upon application, must maintain adhesion to the skin during the prescribed period of use, and must permit easy removal of the TDS at the end of use without leaving a residue or causing damage to the skin or other undesirable effect(s). Additionally, adhesives must be able to maintain the performance of the TDS throughout the shelf life of the drug product.

Three types of TDS adhesion tests are generally used: peel adhesion test (from a standard substrate), release liner peel test, and tack test.

Acceptance criteria are product specific and defined to assure that adhesion of each batch of TDS are within the range defined by the product design and are consistent between batches based on the product development specifications or statistical assessment of multiple product batches over the product's shelf life.

Peel Adhesion Test

This test measures the force required to remove (peel away) a TDS attached to a standard substrate surface (e.g., polished stainless steel). The TDS is applied to the substrate using specified techniques for application and is conditioned at a specified temperature and time. Then, the TDS is peeled away from the substrate with an instrument that allows control of peel angle (e.g., 90 or 180 degrees) and peel rate (e.g., 300 mm/min), and the peel force is recorded. This procedure is repeated using a minimum of five independent samples. The product fails the test if the mean peel force is outside the acceptable range determined during product development and/or based on statistical as-

essment of multiple product batches over the product's shelf life.

Release Liner Peel Test

This test measures the force required to separate the release liner from the adhesive layer of the TDS. The test is performed with a finished product sample. The test sample is conditioned using specific procedures (temperature and time). Then the release liner is pulled away from the TDS with an instrument that allows for control of peel angle (e.g., 90 or 180 degrees) and peel rate, and the peel force is recorded. This procedure is repeated using a minimum of five independent samples. The product fails the test if the mean peel force is outside the acceptable range determined during product development and/or based on statistical assessment of multiple product batches over the product's shelf life.

Tack Test

Several methods of tack tests have been developed. Examples include the probe tack method and the rolling ball method. It is up to the TDS manufacturer to decide which one is more appropriate for each drug product.

PROBE TACK METHOD

This test measures the force required to separate the tip of the test probe from the adhesive layer of the TDS. This test uses an instrument designed to create a bond between the tip of the stainless steel test probe of defined geometry and the TDS using a controlled force (light pressure) and specified test conditions (i.e., rate, contact time, contact pressure, temperature). Then, while controlling the rate of probe removal, the test measures the profile of force required to separate the probe tip from the TDS and the maximum force required to break the bond (tack). This procedure is repeated using a minimum of five independent samples. The product fails the test if the mean test result [force profile(s) and/or tack] is outside the acceptable range determined during product development and/or based on statistical assessment of multiple product batches over the product's shelf life.

ROLLING BALL METHOD

This test measures the distance travelled by a defined ball on the adhesive layer of the TDS under defined conditions, as a parameter dependent on the tack properties of the adhesive layer. This test uses a setup designed to roll a ball (with defined material, weight, size, and surface) from a ramp (with defined angle and length) onto the adhesive layer (with defined orientation) under specified test conditions (temperature) (see ASTM D3121 for more details). The distance traveled by the ball on the adhesive layer is measured using a suitable measuring device. This procedure is repeated using a minimum of five independent samples. The product fails the test if the mean distance travelled is outside the acceptable range determined during product development and/or based on statistical assessment of multiple product batches over the product's shelf life.

Leak Test

This test is applicable only to form-fill-seal (reservoir or pouched)-type TDS. Form-fill-seal TDS must be manufactured with zero tolerance for leaks because of their potential for dose dumping if leaking occurs.

In-process control methods to examine TDS for leakers or potential leakers are needed and require considerable development on the part of TDS manufacturers.

IN-PROCESS TESTING

During the manufacturing process, the presence of leakage, or potential for leakage, because of TDS perforation, cuts, and faulty seals resulting from failures such as air bubbles, gel splash, or misalignment of a TDS's backing and release liner layers, must be examined. Unless automated process analytical technology is implemented, in-process testing to identify these defects should be performed using the following test procedures:

Visual Inspection:

1. A specified number of TDS, defined based on the batch size, should be randomly examined.
2. Each sampled TDS should be thoroughly visually inspected for leakage.
3. The product fails if any of the TDS examined is detected with a leak.

Seal Integrity:

Transdermal system seals should be stress tested to ensure that the application of pressure does not force seals to open, thereby leading to leakage.

1. A specified number of TDS, defined based on the batch size, should be randomly examined.
2. Each sampled TDS should be thoroughly visually inspected for leakage.
3. Each sampled TDS is placed on a hard, flat surface and overlaid with a weight so that it is subjected to 13.6 kg. The weight should be left in place for 2 minutes. Upon removal of the weight, the TDS should be visually inspected for leakage.
4. The product fails if the number of TDS detected with a leak is greater than the acceptable limit established by the manufacturer.

Packaged Product Testing:

TDS may leak after they have been individually placed in the primary packaging material as a result of the packaging operation itself or by user opening of the packaging. Therefore, TDS should be tested for leakage after they have been manufactured and packaged in their primary packaging material.

1. A specified number of TDS, defined based on the batch size, should be randomly tested after they have been placed in their primary packaging material.
2. The sampled TDS should be removed from their packaging and thoroughly visually inspected for leakage.
3. Each sampled TDS should then be uniformly wiped with a solvent-moistened swab. Both the backing side and the release liner side of the TDS should be wiped. The inside surface of the pouch should also be wiped. The swab(s) is (are) then extracted and assayed for the drug.
4. The product fails if the total amount of drug from the TDS, and the corresponding pouch, exceed the acceptable limit established by the manufacturer.

supplements, food ingredients, impurities, degradation products, reagents, and performance verification standards). When approved as suitable for use as comparison standards for documentary tests or assays (i.e., as a monograph component) in the *United States Pharmacopeia (USP)* or *National Formulary (NF)*, USP RS also assume official status and legal recognition in the United States. Assessment of the suitability for use in other applications rests with the user. Official USP RS are primary standards in jurisdictions that so recognize them as such and, when appropriate, are calibrated relative to international reference materials such as those provided by the World Health Organization. USP RS are never intended for therapeutic use. USP's RS are provided for legal metrology purposes and can help ensure comparability of results and traceability to *Système International d'Unités (SI)* units whether certified or not. USP RS are Reference Materials as defined in the *International Vocabulary of Metrology—Basic and General Concepts and Associated Terms (VIM)*: 3rd Edition 2007.

TYPES OF REFERENCE STANDARDS

Reference Standards for USP or NF Articles

Reference Standards for official articles in *USP* or *NF* are provided as pure materials or as mixtures of chemicals reflective of the corresponding drug substances or excipients. The use of these materials is specified in the article's monograph, and these materials generally are necessary for use in the *Assay* and/or the *Identification* tests. The suitability of a USP RS for uses outside those specified in a monograph is the responsibility of the user. The property value or calculation value of the Reference Standard is stated on the label and should be included in calculations used in the monograph and applicable general chapters. For Reference Standards that do not bear a property value or calculation value on the label or in accompanying documentation, assume the Reference Standard is 100.0% pure for compendial quantitative applications.

Impurity Reference Standards

Reference Standards for impurities may include the following:

- Organic impurities that may arise either during the manufacturing process or during the shelf-life storage of an article and may include starting materials, intermediates, by-products, reagents, catalysts, and/or degradation products.
- Inorganic impurities that normally result from a synthesis process and may include reagents, catalysts, heavy metals, or inorganic salts
- Residual solvents that may be either inorganic or organic liquids that are used to prepare solutions or suspensions during the synthesis of an article

Impurity Reference Standards may be presented as purified single-component materials or as mixtures of more than one impurity. Other options for controlling impurities may include presenting the official article with a labeled impurity content; using relative chromatographic retention times and response factors; or providing theoretical values such as UV absorptivities at selected wavelengths.

In earlier editions of the compendium, impurities were designated by their chemical names. For ease of indexing and searching, these have been gradually replaced with the designation "X related compound Y RS", where X is the name of the official article, and Y is a sequential alphabetical letter. The assignment of this letter does not necessarily match the naming schemes of other compendia. Reference Standard impurity mixtures may also be designated by their intended use, such as "X System Suitability RS". The con-

<11> USP REFERENCE STANDARDS

Reference Standards provided by the United States Pharmacopeial Convention (USP Reference Standards, or RS) are highly characterized specimens reflective of specified drugs and foods (drug substances, biologics, excipients, dietary

ventional names and the chemical names are reproduced in the catalog and on the RS product label.

Certified Reference Materials

USP's Certified Reference Materials (CRMs) are Reference Standards that provide certified property values with associated uncertainties and metrological traceability, in accordance with International Organization for Standardization (ISO) Guides 30–35. Correct use of these CRMs support traceability of results to SI units and comparability of procedures.

USP Reference Standards for Biologicals

USP provides RS for biologic drugs and ancillary materials. For historical and other reasons, and as noted in Section 5.50.10 *Units of Potency (Biological)* in the *General Notices and Requirements*, USP RS for biologicals may diverge in unitage, by definition, or otherwise from other internationally recognized standards. Unless so noted in the documentary standard, international reference standards generally are not interchangeable and the USP RS is required in the tests and assays of USP–NF.

NF Reference Standards

Reference Standards currently labeled as “NF Reference Standards” are intended to be designated and labeled as “USP Reference Standards” pursuant to the consolidation of USP and NF within the USP as of January 2, 1975. Where a USP Reference Standard is called for, the corresponding substance labeled as an “NF Reference Standard” may be used.

Transition of Authentic Substances to USP Reference Standards

Previously, highly characterized reference materials not required for use in a USP–NF monograph or general chapter were developed by USP as a service and were distributed as Authentic Substances (AS). AS typically are highly characterized chemicals that are collaboratively tested and made available as a service primarily to analytical, clinical, pharmaceutical, and research laboratories. Such materials may be used for identification, method development, evaluation of method performance, or other applications as found suitable and validated by the user. USP will no longer introduce materials labeled “Authentic Substances.” All reference materials released, whether or not required for use in a USP–NF monograph or general chapter, will be “USP Reference Standards.”

Authentic Visual References

Authentic Visual References are USP Reference Standards, but unlike chemical reference materials, Authentic Visual References (AVR) are not used in chemical analyses. Instead, AVR are visual images used by analysts to compare certain test articles to ensure that they meet compendial requirements. AVR are incorporated by reference into the monograph.

USP Performance Verification Test Standards

These materials are provided to analyze and where appropriate to facilitate adjustment of the operation of an instrument to ensure that the results obtained are accurate and/or precise or otherwise give acceptable results. The use of these Reference Standards is generally described in associated general test chapters and allied information.

APPLICATIONS OF USP REFERENCE STANDARDS

Official applications of USP RS are specified in USP–NF monographs and general chapters. They include the following:

- quantitative uses in assays for drug substances and formulations, limit tests, or blanks and controls
- qualitative uses, (e.g., identification tests, system suitability tests, or chromatographic peak markers)
- method-specific uses, (e.g., performance verification standards, AVR, melting point standards, and the particle count set)

As described above, USP also provides Authentic Substances, not specified for use in a USP monograph or general chapter, which are used at the user's discretion.

PACKAGING

The amount of material per individual USP RS container depends on the compendial application of the standard and is generally sufficient for several replicates. Some standards (mainly materials with significant handling requirements or materials that are available only in small amounts) are provided in single-use containers. Such single-use products generally are lyophilized, and their content is labeled in mass or activity units per container. If so labeled, the content of the container should be reconstituted in its entirety without any additional weighing. Instructions for reconstitution are given either on the label or in the monographs where the standard is used.

LABELING

The label text provides all the information needed for the correct storage and use of the USP RS in monograph applications. The label includes directions for use, safety warnings, required information for controlled substances, and a property value or calculation value for standards with quantitative applications. For performance verification standards, acceptance ranges are provided. Where necessary, USP RS are accompanied by additional documentation such as Technical Data Sheets or Typical Chromatograms.

Unless otherwise directed in the procedure in the individual monograph or in a general chapter, USP RS should be used in accordance with the instructions on the label of the Reference Standard. Material Safety Data Sheets for all USP reference materials are available on the USP Web site.

Although USP RS undergo retesting on a predefined schedule to determine continued suitability for use, USP RS do not carry an expiration date on the label. A lot of USP RS may be used in its official applications as long as it is listed as “Current Lot” in the current USP Reference Standards Catalog or has not reached its Valid Use Date. Upon depletion, the lot is designated in the catalog as “Previous Lot” and a “Valid Use Date” is assigned. USP publishes the Catalog of Reference Standards bimonthly. The most current version of the catalog can be found on the USP Web site at www.usp.org. The user is responsible for ascertaining before use that the USP RS lot of interest currently carries official status, either as a “Current Lot” or as a “Previous Lot” within the Valid Use Date.

PROPER USE

Many compendial tests and assays are based on comparison of a test specimen with a USP RS. In such cases, measurements are made on preparations of both the test specimen and the Reference Standard. Where it is directed that a Standard solution or a Standard preparation be prepared for a quantitative determination by stepwise dilution or otherwise, it is intended that the Reference Standard substance

be accurately weighed (see *Weights and Balances* <41> and *Volumetric Apparatus* <31>). Due account should also be taken of the potential errors associated with weighing small masses (see also Section 6.50.20.1 *Adjustments to Solutions* in the *General Notices and Requirements*). Reference Standards that are defined on a content-per-container basis are an exception, as noted above.

USP RS instructions for use include the following:

- **As Is:** Use without any prior treatment or correction for volatiles. This is the preferred option, and is selected whenever valid data indicate that the volatiles content is constant over time.
- **Dry Before Use:** Use immediately after drying under stated conditions. Drying should not be performed in the original container. A portion of the material should be transferred to a separate drying vessel.
- **Determine Water Content Titrimetrically At Time of Use:** Use with a correction for the water content or the loss on drying, determined on a separate portion of material. Where the titrimetric determination of water is required at the time a Reference Standard is to be used, proceed as directed for *Method I* under *Water Determination* <921>. Instrumental or microanalytical methods are acceptable for this purpose. When using typical amounts (about 50 mg of the Reference Standard), titrate with a 2- to 5-fold dilution of the reagent. Where the determination of the loss on drying on a separate portion of USP RS is required, proceed as directed on the label. Sample sizes smaller than those required in the general test chapter *Loss on Drying* <731> may be used for a USP RS provided that the user can obtain a sufficiently accurate result.

Whenever the labeled directions for use require drying or a correction for volatiles, it should be performed at the time of use. Further experimental details should be controlled by the user's Standard Operating Procedures and good laboratory practices.

STORAGE

USP RS should be stored in the packaging configuration provided by USP (e.g., vials that are packaged in hermetically sealed bags). When special storage conditions are specified, label directions should be followed. Unopened vials should be stored as indicated on the label. The user is responsible for ensuring that the contents of opened vials continue to be suitable for their intended use and that value assignment and uncertainty information are maintained.

Apparatus for Tests and Assays

<16> AUTOMATED METHODS OF ANALYSIS

Where a sufficiently large number of similar units are to be subjected routinely to the same type of examination, automated methods of analysis may be far more efficient and precise than manual methods. Such automated methods have been found especially useful in testing the content uniformity of tablets and capsules and in facilitating methods requiring precisely controlled experimental conditions. Many

manufacturing establishments, as well as the laboratories of regulatory agencies, have found it convenient to utilize automated methods as alternatives to Pharmacopeial methods (see *Procedures under Tests and Assays* in the *General Notices and Requirements*). In addition, the detection system and calculation of results for automated methods are often computerized.

Before an automated method for testing an article is adopted as an alternative, it is advisable to ascertain that the results obtained by the automated method are equivalent in accuracy and precision to those obtained by the prescribed Pharmacopeial method, bearing in mind the further principle stated in the *General Notices and Requirements* that "where a difference appears, or in the event of dispute, only the result obtained by the procedure given in this Pharmacopeia is conclusive."

It is necessary to monitor the performance of the automated analytical system continually by assaying standard preparations of known composition frequently interspersed among the test preparations. Where immiscible solvents are employed in the automated apparatus for rapid extractions, they are often separated for analysis before complete extraction is attained, and the chemical reactions utilized in automated methods rarely are stoichiometric. Both the accuracy and the precision of the determinations depend upon precise adjustment of the equipment, so maintained that all standard and test preparations are exposed to identical physical and chemical manipulations for identical time intervals. Excessive variability in the response of the standard preparations indicates that the analytical system is malfunctioning and that the test results are therefore invalid. However, where automated systems are shown to operate reliably, the precision of the automated method may surpass that of the manual procedure employing the same basic chemistry.

Many of the manual methods given in this Pharmacopeia can be adapted for use in automated equipment incorporating either discrete analyzers or continuous flow systems and operating under a variety of conditions. On the other hand, an analytical scheme devised for a particular automated system may not be readily transposable for use either in a manual procedure or in other types of automated equipment.

The apparatus required for manual methods is, in general, less complicated than the apparatus of automated systems, even those systems used for the direct automated measurement of a single analyte (i.e., the substance being determined or analyzed for) in a binary mixture. However, because of their versatility, automated systems designed for the rapid determination of a specified substance often can be readily modified by the addition of suitable modules and accessories to permit the determination of one or more additional substances in a dosage form. Such extended systems have been utilized, for example, in the automated analysis of articles containing both estrogens and progestogens.

The accompanying pertinent diagrams represent examples of automated methods. Diagrams for official methods are reproduced here rather than in the individual monographs. The descriptions of the procedural details in these methods exemplify the general approach in automated analysis applicable to dosage forms. It should be noted that the diagrams, with many minutiae, are an indispensable part of the directions for conducting the analysis.

DIAGRAMS

The diagrams shown below are arranged in alphabetic order by the name of the drug first mentioned, where the diagram is for a procedure for a specific article. Where there is no procedure in this chapter for a particular diagram, reference is to be made to the named monograph.

ANTIBIOTICS—HYDROXYLAMINE ASSAY

The following procedure is applicable for the assay of those Pharmacopeial antibiotics, such as cephalosporins and penicillins, that possess the beta-lactam structure.

Apparatus—Automatic analyzer consisting of (1) a liquid sampler, (2) a proportioning pump, (3) suitable spectrophotometers equipped with matched flow cells and analysis capability at 480 nm, (4) a means of recording spectrophotometric readings, and/or computer for data retrieval and calculation, and (5) a manifold consisting of the components illustrated in the accompanying pertinent diagram.

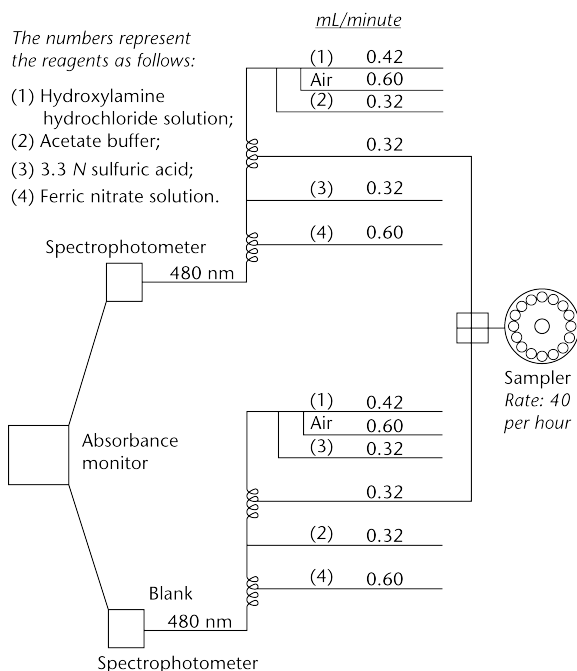


Diagram for Automated Hydroxylamine Assay for Antibiotics

Reagents—

Hydroxylamine Hydrochloride Solution—Dissolve 20 g of hydroxylamine hydrochloride in 5 mL of polyoxyethylene (23) lauryl ether solution (1 in 1000), and add water to make 1000 mL.

Acetate Buffer—Dissolve 173 g of sodium hydroxide and 20.6 g of sodium acetate in water to make 1000 mL. Dilute 75 mL of this solution with water to 500 mL, and mix.

Ferric Nitrate Solution—Suspend 233 g of ferric nitrate in about 600 mL of water, add 2.8 mL of sulfuric acid, stir until the ferric nitrate is dissolved, add 1 mL of polyoxyethylene (23) lauryl ether, dilute with water to 1000 mL, and mix.

USP Reference Standards (11)—Use the USP Reference Standard as directed in the individual monograph.

Standard Preparation—Unless otherwise directed in the individual monograph, dissolve an accurately weighed quantity of the USP Reference Standard in water, and quantitatively dilute with water to obtain a solution having a known concentration of about 1 mg per mL.

Assay Preparation—Unless otherwise directed in the individual monograph, using the specimen under test, prepare as directed under *Standard Preparation*.

Procedure—With the sample line pumping water, the other lines pumping their respective reagents, and the spectrophotometer set at 480 nm, standardize the system until a steady absorbance baseline has been established. Transfer portions of the *Standard Preparation* and the *Assay Preparation* to sampler cups, and place in the sampler. Start the

sampler, and conduct determinations of the *Standard Preparation* and the *Assay Preparation* typically at the rate of 40 per hour, using a ratio of about 2:1 for sample and wash time. Calculate the potency by the formula given in the individual monograph, in which C is the concentration, in mg per mL, of USP Reference Standard in the *Standard Preparation*; P is the potency, in μg per mg, of the USP Reference Standard; and A_U and A_S are the absorbances, corrected for the absorbances of the respective blanks, of the solutions from the *Assay Preparation* and the *Standard Preparation*, respectively.

ASSAY FOR ASCORBIC ACID

The following procedure is applicable for the assay of ascorbic acid in Pharmacopeial multivitamin-minerals combination products (solid and liquid dosage forms) that contain components that interfere in other methods of assay.

Apparatus—Automatic analyzer consisting of (1) a liquid sampler; (2) a proportioning pump; (3) a suitable fluorimeter equipped with a flow cell and filters: primary—335 nm, and secondary—426 nm; (4) a means of recording fluorimeter readings; and (5) a manifold consisting of the components illustrated in the accompanying pertinent diagram.

Reagents—

Extracting Solution—Dissolve 600 g of metaphosphoric acid in 1200 mL of water. Add 400 mL of glacial acetic acid, dilute with water to 2000 mL, and mix.

Dilute Extracting Solution—Dissolve 60 g of metaphosphoric acid in 1200 mL of water. Add 160 mL of glacial acetic acid, dilute with water to 2000 mL, and mix.

Surfactant Solution—Prepare a 30% solution of polyoxyethylene (23) lauryl ether by melting 150 g in a container on a steam bath and slowly adding approximately 250 mL of water with continuous stirring. Cool and dilute with water to make 500 mL.

Wash Solution—Add 1 mL of *Surfactant Solution* to 3000 mL of *Dilute Extracting Solution*, and mix.

Carbon Extraction Solution—Dissolve 60 g of metaphosphoric acid in 1200 mL of water. Add 160 mL of glacial acetic acid, and mix. Add 33 g of activated charcoal powder, mix, and dilute with water to 2000 mL. Continually mix the solution at a rate that maintains homogeneity.

Sodium Acetate Solution—Dissolve 500 g of sodium acetate trihydrate in water to make 1000 mL, mix, and filter.

Phenylenediamine Solution—Dissolve 200 mg of *o*-phenylenediamine dihydrochloride in water to make 1000 mL, and mix. Prepare fresh daily.

USP Reference Standards (11)—USP Ascorbic Acid RS.

Standard Stock Solution—Dissolve an accurately weighed quantity of USP Ascorbic Acid RS in *Dilute Extracting Solution* to obtain a solution having a known concentration of about 0.1 mg per mL.

Standard Preparations—Transfer 10.0, 20.0, 30.0, 40.0, and 50.0 mL of *Standard Stock Solution* to separate 100-mL volumetric flasks, dilute the contents of each flask with *Carbon Extraction Solution* to volume, mix, and filter to obtain *Standard Preparations A, B, C, D, and E* having known concentrations of 10 μg , 20 μg , 30 μg , 40 μg , and 50 μg of USP Ascorbic Acid RS per mL, respectively.

Assay Preparation—

For Liquid Preparations—Transfer an accurately measured volume of the liquid preparation, equivalent to 150 mg of ascorbic acid, to a 100-mL volumetric flask. Add 10 mL of *Extracting Solution* and 6 mL of glacial acetic acid. Dilute with water to volume, and mix. Transfer 2.0 mL of this solution to a 100-mL volumetric flask, dilute with *Carbon Extraction Solution* to volume, mix, and filter.

For Tablet Preparations—Weigh and finely powder not fewer than 20 Tablets. Transfer an accurately weighed quan-

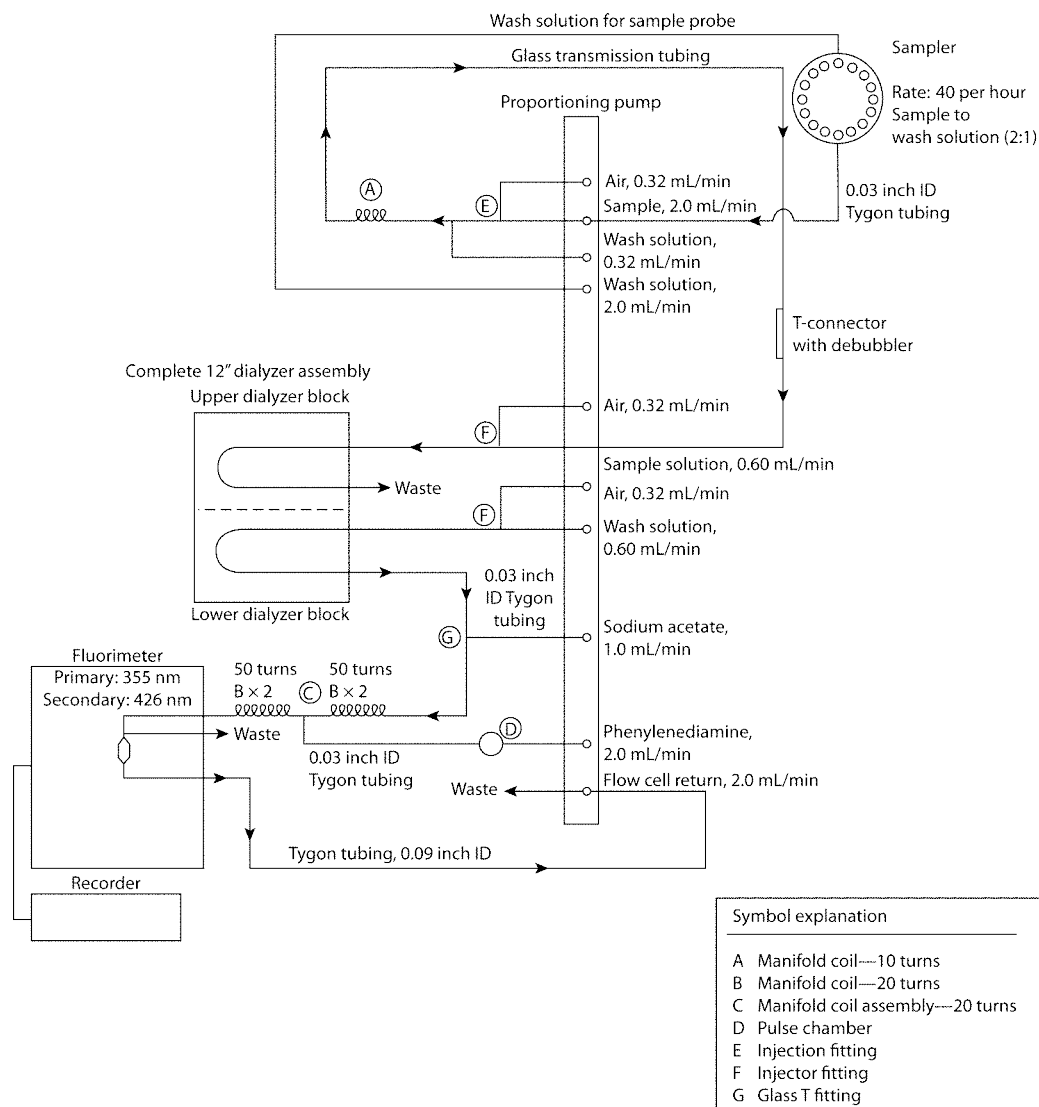


Diagram for Automated Ascorbic Acid

tity of the powder, equivalent to about 250 mg of ascorbic acid, to a 250-mL volumetric flask. Add 25 mL of *Extracting Solution*, 15 mL of glacial acetic acid, and about 100 mL of water, and swirl to mix. Heat for 15 minutes in a 70° water bath, swirling after about 7 minutes. Cool, and dilute with water to volume. Transfer 2.0 mL of this solution to a 100-mL volumetric flask, dilute with *Carbon Extracting Solution* to volume, mix, and filter.

For Capsule Preparations—Empty the contents, if necessary by cutting open with a sharp blade, of not fewer than 20 Capsules in a suitable container, and mix thoroughly. Transfer a portion of the capsule contents, equivalent to about 250 mg of ascorbic acid, to a 250-mL volumetric flask, and proceed as directed for Tablets above, beginning with "Add 25 mL of."

Procedure—With the sample line pumping the *Wash Solution*, the other lines pumping their respective reagents,

and the fluorimeter equipped with proper filters, standardize the system by pumping until a steady baseline has been established. Transfer portions of the *Standard Preparations* and the *Assay Preparation* to sample cups, and place in the sampler. Start the sampler, and conduct determinations of each *Standard Preparation* and the *Assay Preparation* at the rate of 40 per hour, using a ratio of about 2:1 for sample and wash time. Derive a standard response line by plotting the respective *Standard Preparation* concentration (10.0, 20.0, 30.0, 40.0, and 50.0 µg per mL) versus transmittance. From the measured transmittance and the standard response line, determine the ascorbic acid concentration, *C*, in µg per mL, of the *Assay Preparation*. Calculate the quantity, in mg, of $C_6H_8O_6$ in the portion of liquids, tablets, or capsule contents taken by the appropriate formula:

For Liquids: $5C/V$ in which V is the volume, in mL, of liquid preparation taken to prepare the Assay Preparation.

For Tablets or Capsules: 12.5C.

ASSAY FOR IODIDE

Apparatus—Automatic analyzer consisting of (1) a liquid sampler, (2) a proportioning pump, (3) a heating bath, (4) a suitable colorimeter equipped with a 2.0- × 50-mm flow cell and analysis capability at 420 nm, (5) a means of recording colorimetric readings, and (6) a manifold consisting of the components illustrated in the accompanying pertinent diagram.

Reagents—

Acetic Acid Carrier Solution—Transfer 3.0 mL of glacial acetic acid to a 2000-mL volumetric flask containing about 800 mL of water. Add 2 mL of polyoxyethylene (23) lauryl ether, and dilute with water to volume.

Surfactant Solution—Prepare a 30% solution of polyoxyethylene (23) lauryl ether by melting 150 g in a container on a steam bath and slowly adding approximately 250 mL of water with continuous stirring. Cool, and dilute with water to make 500 mL.

Arsenious Acid Solution—Transfer 19.6 g of arsenic trioxide and 14.0 g of sodium hydroxide to a 2000-mL volumetric flask. Add about 150 mL of water, and dissolve with stirring. Dilute with water to a volume of about 800 mL, and add 66 mL of sulfuric acid. Cool to room temperature. Transfer 50.0 g of sodium chloride to the solution, and mix to dissolve. Add 2 mL of *Surfactant Solution*, dilute with water to volume, mix, and filter.

Ceric Ammonium Sulfate Solution—Transfer 12.65 g of ceric ammonium sulfate to a 1000-mL volumetric flask. Add about 700 mL of water followed by 100 mL of sulfuric acid,

swirling to mix. Heat to dissolve, and cool to room temperature. Add 1 mL of *Surfactant Solution*, dilute with water to volume, mix, and filter.

3% Acetic Acid Solution—Transfer 30 mL of glacial acetic acid to a 1000-mL volumetric flask containing about 300 mL of water. Dilute with water to volume, and mix.

Standard Preparations—

Standard Stock Solution—Transfer an accurately weighed quantity of 1.3080 g of potassium iodide, previously dried for 24 hours at 105°, to a 1000-mL volumetric flask. Dilute with water to volume, and mix to obtain a solution having an iodide concentration of 1000 µg per mL.

Intermediate Standard Solution—Quantitatively dilute a suitable volume of *Standard Stock Solution* with water to obtain a solution having an iodide concentration of 1 µg per mL.

Working Standard Preparations—Transfer 2.0, 4.0, 6.0, 8.0, and 10.0 mL of *Intermediate Standard Solution* to separate 100-mL volumetric flasks. Add 5 mL of 3% *Acetic Acid Solution*. Dilute the contents of each flask with water to volume, and mix to obtain *Standard Preparations A, B, C, D, and E* having known iodide concentrations of about 0.02 µg per mL, 0.04 µg per mL, 0.06 µg per mL, 0.08 µg per mL, and 0.1 µg per mL, respectively.

Assay Preparation—

For Liquid Preparations—Transfer an accurately measured volume of the liquid preparation, equivalent to 16 µg of iodide, to a 200-mL volumetric flask. Add 10 mL of 3% *Acetic Acid Solution* to dissolve, dilute with deionized water to volume, mix, and filter. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, add 5.0 mL of 3% *Acetic Acid Solution*, dilute with deionized water to volume, mix, and filter to obtain a solution having an iodide concentration of about 0.08 µg per mL.

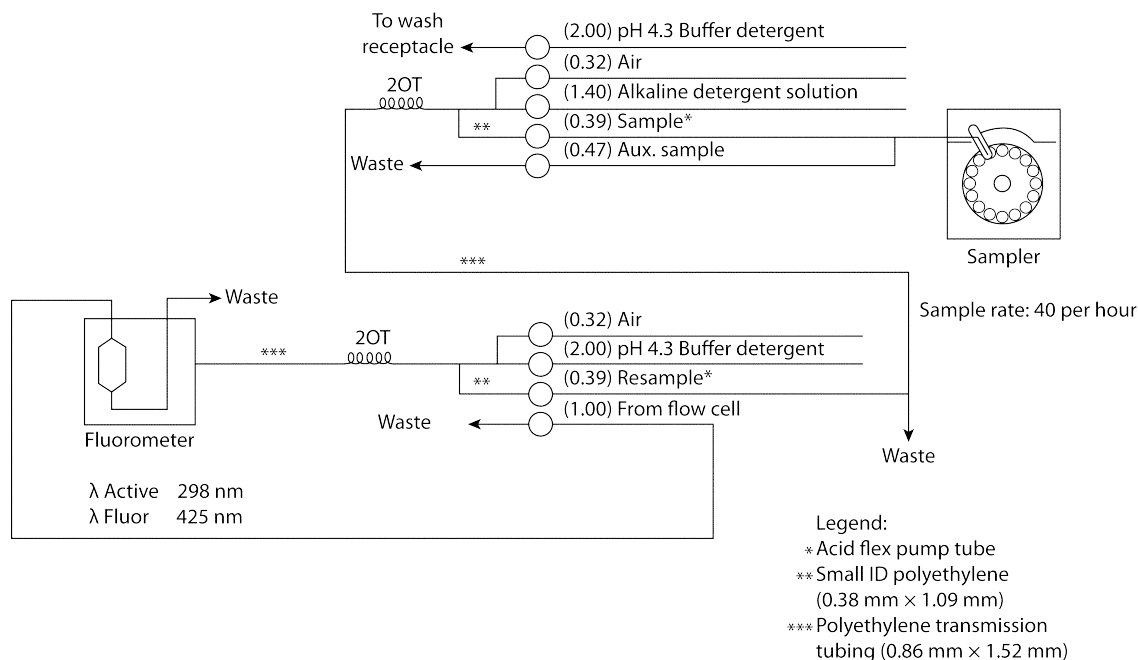


Diagram for Automated Aspirin Determinative Step of the Dissolution Test for Aspirin, Alumina, and Magnesium Oxide Tablets

For Tablet Preparations—Weigh and finely powder not fewer than 20 Tablets. Transfer an accurately weighed quantity of the powder, equivalent to about 250 μg of iodide, to a 250-mL volumetric flask. Add 100 mL of 1 N hydrochloric acid, and mix with the aid of sonication for 30 minutes. Dilute with water to volume, mix, and filter. Transfer 8.0 mL of the filtered solution to a 100-mL volumetric flask, add 5 mL of 3% Acetic Acid Solution, dilute with water to volume, and mix to obtain a solution having an iodide concentration of about 0.08 μg per mL.

For Capsule Preparations—Empty the contents, if necessary by cutting open with a sharp blade, of not fewer than 20 Capsules into a suitable container, and mix thoroughly. Transfer a portion of the capsule contents, equivalent to about 250 μg of iodide, to a 250-mL volumetric flask and proceed as directed for Tablets above, beginning with "Add 100 mL of."

Procedure—With the sample line pumping the Acetic Acid Carrier Solution, the other lines pumping their respective reagents, and the colorimeter equipped with 420-nm filters, standardize the system until a steady baseline has been established. Transfer portions of the *Standard Preparation* and the *Assay Preparation* to the sampler cups, and place in the sampler. Start the sampler, and conduct determinations of each *Standard Preparation* and the *Assay Preparation* at the rate of 30 per hour, using a ratio of about 1:4 for sample and wash time. Derive a standard response line by plotting the respective *Standard Preparation* concentration (0.02, 0.04, 0.06, 0.08, and 0.10 μg per mL) versus absorbance. [NOTE—This is an indirect absorbance relationship: the greater the iodide amount, the less the absorbance.] From the measured transmittance and the standard response line, determine the iodide concentration, C , in μg per mL, of the *Assay Preparation*. Calculate the quantity, in

μg , of iodide in the portion of liquids, tablets, or capsules contents taken by the formula:

For Liquids: $2000C/V$ in which V is the volume, in mL, of the liquid preparation taken to prepare the *Assay Preparation*.

For Tablets and Capsules: $3125C$.

CONTENT UNIFORMITY OF NITROGLYCERIN TABLETS

This is not to be considered as the official method. It is detailed here for further illustration of descriptions of automated methods.

Apparatus—Automatic analyzer consisting of (1) a liquid sampler, (2) a proportioning pump, (3) a heating bath, (4) a suitable spectrophotometer equipped with a 5-mm flow cell and analysis capability at 545 nm, (5) a means of recording spectrophotometric readings, and (6) a manifold consisting of the components illustrated in the accompanying pertinent diagram.

Reagents—

1 Percent Strontium Hydroxide Solution—Dissolve 20.0 g of strontium hydroxide [$\text{Sr}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$] in 1800 mL of carbon dioxide-free water, heating if necessary. Cool to room temperature, dilute with carbon dioxide-free water to 2000 mL, and mix. Allow to stand overnight, and filter. Store the clear solution in tightly closed containers, protected from carbon dioxide.

0.3 Percent Procaine Hydrochloride Solution—Dissolve 3.0 g of procaine hydrochloride in water to make 1000 mL.

0.1 Percent *N*-(1-Naphthyl)ethylenediamine Dihydrochloride Solution—Dissolve 1.0 g of *N*-(1-naphthyl)ethylenediamine

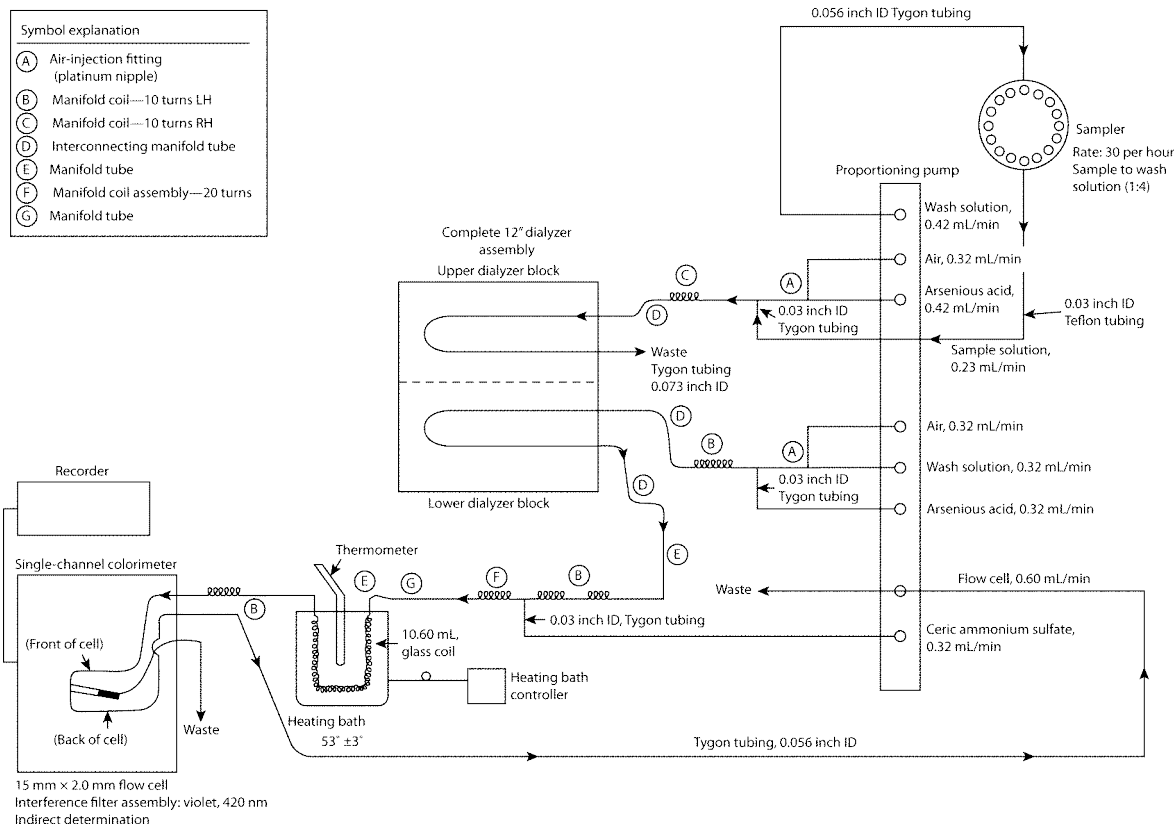


Diagram for Automated Iodide Assay

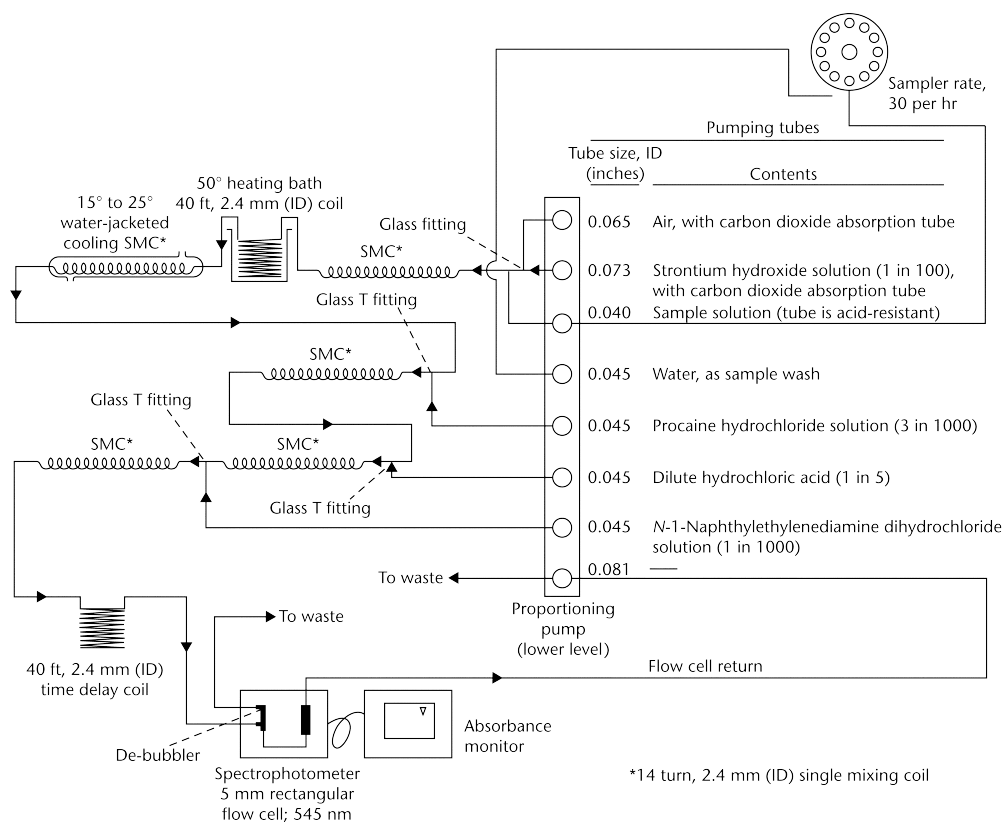


Diagram for Automated Assay for Nitroglycerin Tablets

dihydrochloride in water to make 1000 mL. Prepare fresh each week.

Standard Preparation—Dissolve an accurately weighed portion of 10% nitroglycerin-beta-lactose absorbate, previously standardized, in water, and dilute quantitatively and stepwise with water to obtain a solution having a known concentration of about 30 µg per mL.

Test Preparation—Dissolve 1 Nitroglycerin Tablet in water to obtain a solution having a concentration of about 30 µg of nitroglycerin per mL.

Procedure—With the sample line pumping water, the other lines pumping their respective reagents, and the spectrophotometer set at 545 nm, standardize the system by pumping until a steady absorbance baseline has been established. Transfer portions of the *Standard Preparation* and the *Test Preparation* to sampler cups, and place in the sampler. Start the sampler, and conduct determinations of the *Standard Preparation* and the *Test Preparation* at a rate of 30 per hour, using a ratio of 1:1 for sample and wash time. First,

run two standards, discarding the first value, then continue the run using one standard after each five samples, recording the absorbance values. Calculate the quantity, in mg, of $C_3H_5N_3O_9$ in the Tablet taken by the formula:

$$(T/D)C(A_U/A_S)$$

in which T is the labeled quantity, in mg, of nitroglycerin in the Tablet; D is the concentration, in µg per mL, of nitroglycerin in the solution from the Tablet, based on the labeled quantity per Tablet and the extent of dilution; C is the concentration, in µg per mL, of nitroglycerin in the *Standard Preparation*; A_U is the absorbance of the *Test Preparation*; and A_S is the average of the absorbances of the two *Standard Preparations* that bracket the *Test Preparation*.

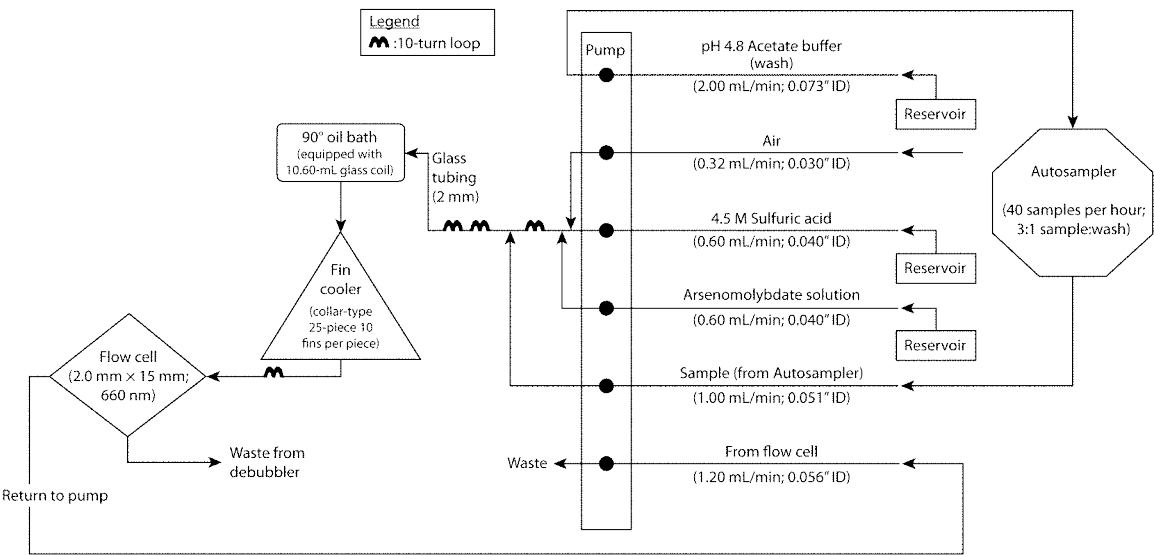


Diagram of Dissolution Test Method for Erythromycin Ethylsuccinate Tablets Labeled as Chewable

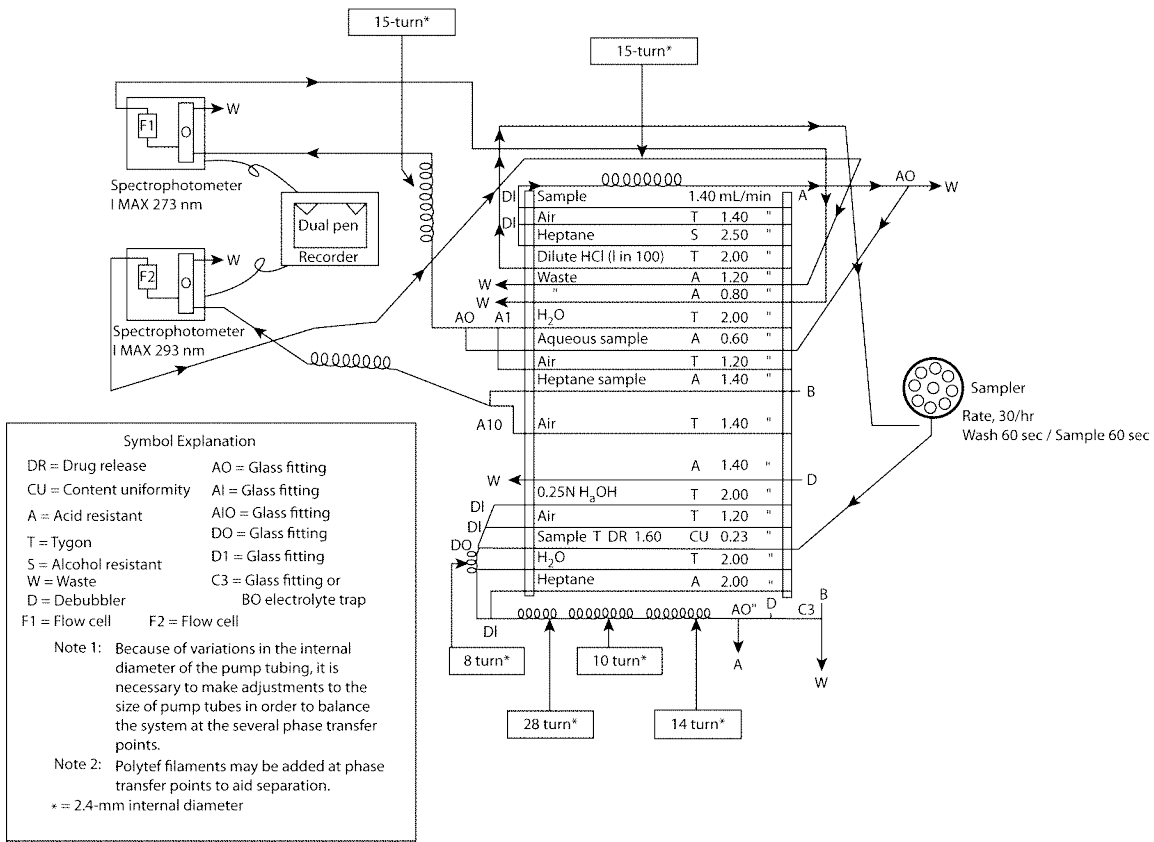


Diagram for Automated Drug Release and Content Uniformity Test for Propranolol Hydrochloride and Hydrochlorothiazide Extended-Release Capsules

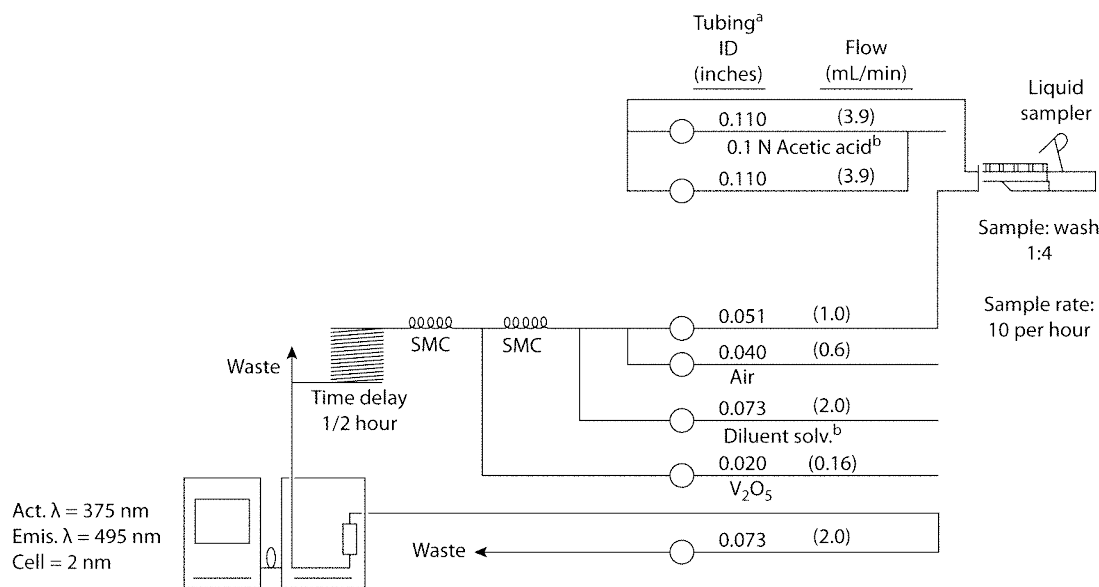


Diagram for Automated Dissolution and Content Uniformity Test for Reserpine Tablets

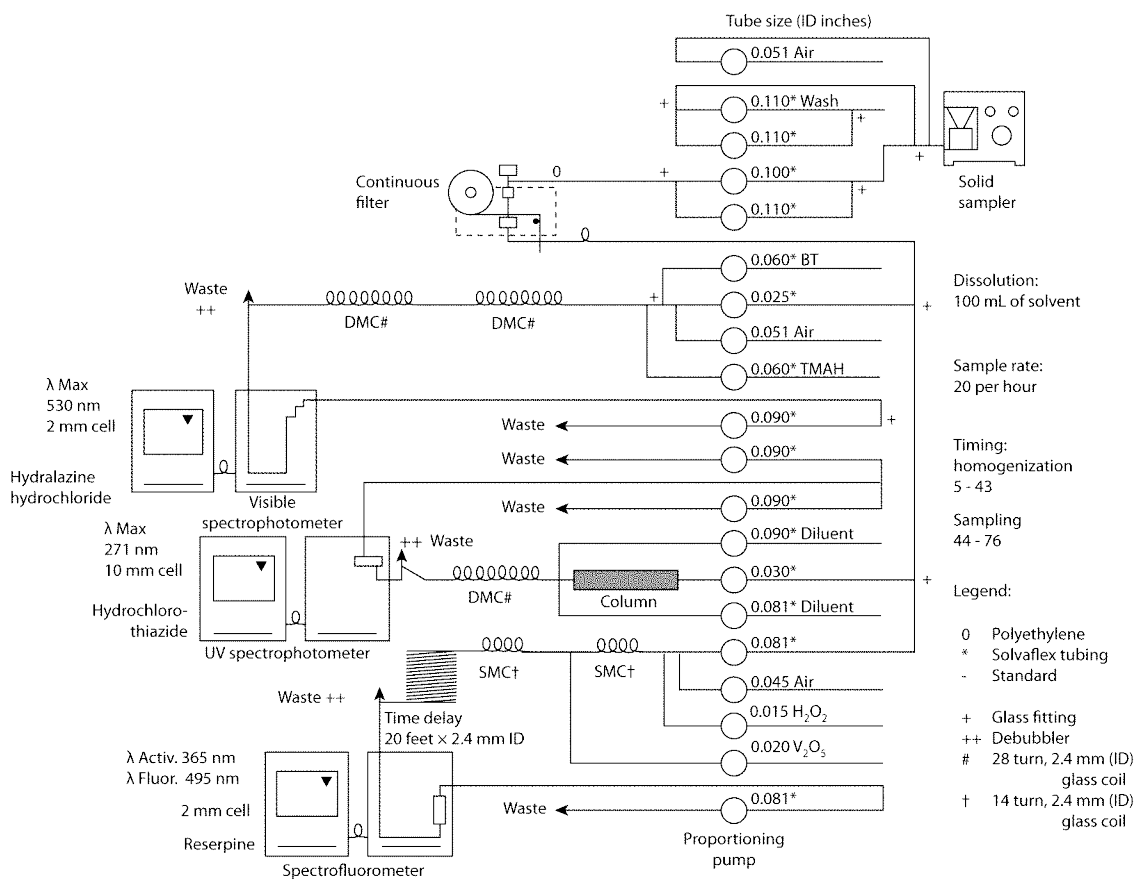


Diagram for Automated Content Uniformity Test for Reserpine, Hydralazine Hydrochloride, and Hydrochlorothiazide Tablets

Add the following:

▲<17> PRESCRIPTION CONTAINER LABELING

INTRODUCTION

Medication misuse has resulted in more than 1 million adverse drug events per year in the United States. Patients' best source (and often only source) of information regarding the medications they have been prescribed is on the prescription container label. Although other written information and oral counseling sometimes may be available, the prescription container label must fulfill the professional obligations of the prescriber and pharmacist. These obligations include giving the patient the most essential information needed to understand how to safely and appropriately use the medication and to adhere to the prescribed medication regimen.

Inadequate understanding of prescription directions for use and auxiliary information on dispensed containers is widespread. Studies have found that 46% of patients misunderstood one or more dosage instructions, and 56% misunderstood one or more auxiliary warnings. The problem of misunderstanding is particularly troublesome in patients with low or marginal literacy and in patients receiving multiple medications that are scheduled for administration using unnecessarily complex, nonstandardized time periods. In one study, patients with low literacy were 34 times more likely to misinterpret prescription medication warning labels. However, even patients with adequate literacy often misunderstand common prescription directions and warnings. In addition, there is great variability in the actual auxiliary warning and supplemental instructional information applied by individual practitioners to the same prescription. The specific evidence to support a given auxiliary statement often is unclear, and patients often ignore such information. The essential need for, and benefit of, auxiliary label information (both text and icons) in improving patient understanding about safe and appropriate use of their medications vs. explicit simplified language alone require further study.

Lack of universal standards for labeling on dispensed prescription containers is a root cause for patient misunderstanding, nonadherence, and medication errors. On May 18, 2007, the USP Safe Medication Use Expert Committee established an Advisory Panel to: 1) determine optimal prescription label content and format to promote safe medication use by critically reviewing factors that promote or distract from patient understanding of prescription medication instructions and 2) create universal prescription label standards for format/appearance and content/language.

In November 2009, the Health Literacy and Prescription Container Labeling Advisory Panel presented its recommendations to the Safe Medication Use Expert Committee, which then requested that USP develop patient-centered label standards for the format, appearance, content, and language of prescription medication instructions to promote patient understanding. These recommendations form the basis of this general chapter.

Note—These standards do not apply when a prescription drug will be administered to a patient by licensed personnel who are acting within their scope of practice.

PRESCRIPTION CONTAINER LABEL STANDARDS TO PROMOTE PATIENT UNDERSTANDING

Organize the prescription label in a patient-centered manner: Information shall be organized in a way that best reflects how most patients seek out and understand medication instructions. Prescription container labeling should feature only the most important patient information needed for safe and effective understanding and use.

Emphasize instructions and other information important to patients: Prominently display information that is critical for patients' safe and effective use of the medicine. At the top of the label specify the patient's name, drug name (spell out full generic and brand name) and strength, and explicit clear directions for use in simple language.

The prescription directions should follow a standard format so the patient can expect that each element will be in a regimented order each time a prescription is received.

Other less critical but important content (e.g., pharmacy name and phone number, prescriber name, fill date, refill information, expiration date, prescription number, drug quantity, physical description, and evidence-based auxiliary information) should not supersede critical patient information. Such less critical information should be placed away from dosing instructions (e.g., at the bottom of the label or in another less prominent location) because it distracts patients, which can impair their recognition and understanding.

Simplify language: Language on the label should be clear, simplified, concise, and familiar, and should be used in a standardized manner. Only common terms and sentences should be used. Do not use unfamiliar words (including Latin terms) or medical jargon.

Use of readability formulas and software is not recommended to simplify short excerpts of text like those on prescription labels. Instead, use simplified, standardized sentences that have been developed to ensure ease of understanding the instructions correctly (by seeking feedback from samples of diverse consumers).

Give explicit instructions: Instructions for use (i.e., the SIG or signatur) should clearly separate the dose itself from the timing of each dose in order to explicitly convey the number of dosage units to be taken and when (e.g., specific time periods each day such as morning, noon, evening, and bedtime). Instructions shall include specifics on time periods. Do not use alphabetic characters for numbers. For example, write "Take 2 tablets in the morning and 2 tablets in the evening" rather than "Take two tablets twice daily").

Whenever available, use standardized directions (e.g., write "Take 1 tablet in the morning and 1 tablet in the evening" if the prescription reads b.i.d.). Vague instructions based on dosing intervals such as twice daily or 3 times daily, or hourly intervals such as every 12 hours, generally should be avoided because such instructions are implicit rather than explicit, they may involve numeracy skills, and patient interpretation may vary from prescriber intent. Although instructions that use specific hourly times (e.g., 8 a.m. and 10 p.m.) may seem to be more easily understood than implicit vague instructions, recommending dosing by precise hours of the day is less readily understood and may present greater adherence issues due to individual lifestyle patterns, e.g., shift work, than more general time frames such as in the morning, in the evening, after breakfast, with lunch, or at bedtime. Consistent use of the same terms should help avoid patient confusion.

Ambiguous directions such as "take as directed" should be avoided unless clear and unambiguous supplemental instructions and counseling are provided (e.g., directions for use that will not fit on the prescription container label). A clear statement referring the patient to such supplemental materials should be included on the container label.

Include purpose for use: If the purpose of the medication is included on the prescription, it should be included on the prescription container label unless the patient prefers that it not appear. Always ask patients their preference when prescriptions are submitted for filling. Confidentiality and FDA approval for intended use (e.g., labeled vs. off-label use) may limit inclusion of the purpose on labels. Current evidence supports inclusion of purpose-for-use language in clear, simple terms (e.g., “for high blood pressure” rather than “for hypertension”).

Limit auxiliary information: Auxiliary information on the prescription container label should be evidence-based in simple explicit language that is minimized to avoid distracting patients with nonessential information. Most patients, particularly those with low literacy, pay little attention to auxiliary information. The information should be presented in a standardized manner and should be critical for patient understanding and safe medication use (e.g., warnings and critical administration alerts). Icons are frequently misunderstood by patients. In addition, icons that provide abstract imagery for messages that are difficult to visually depict may be ineffective at improving understanding compared with simplified text alone. Use only icons for which there is adequate evidence, through consumer testing, that they improve patient understanding about correct use. Evidence-based auxiliary information, both text and icons, should be standardized so that it is applied consistently and does not depend on individual practitioner choice.

Address limited English proficiency: Whenever possible, the directions for use on a prescription container label should be provided in the patient’s preferred language. Otherwise there is a risk of misinterpretation of instructions by patients with limited English proficiency, which could lead to medication errors and adverse health outcomes. Additionally, whenever possible, directions for use should appear in English as well, to facilitate counseling; the drug name shall be in English so that emergency personnel and other intermediaries can have quick access to the information.

Translations of prescription medication labels should be produced using a high-quality translation process. An example of a high-quality translation process is:

- Translation by a trained translator who is a native speaker of the target language
- Review of the translation by a second trained translator and reconciliation of any differences
- Review of the translation by a pharmacist who is a native speaker of the target language and reconciliation of any differences
- Testing of comprehension with target audience

If a high-quality translation process cannot be provided, labels should be printed in English and trained interpreter services used whenever possible to ensure patient comprehension. The use of computer-generated translations should be limited to programs with demonstrated quality because dosage instructions can be inconsistent and potentially hazardous. Standardized translated instructions and technology advances are needed to ensure the accuracy and safety of prescription container labeling for patients with low English proficiency.

Improve readability: Labels should be designed and formatted so they are easy to read. Currently no strong evidence supports the superiority in legibility of serif vs. sans serif typefaces, so simple uncondensed fonts of either type can be used.

Optimize typography by using the following techniques:

- High-contrast print (e.g., black print on white background).
- Simple, uncondensed familiar fonts with sufficient space within letters and between letters (e.g., Times Roman or Arial).
- Sentence case (i.e., punctuated like a sentence in English: initial capital followed by lower-case words except proper nouns).

- Large font size (e.g., minimum 12-point Times Roman or 11-point Arial) for critical information. Note that point size is not the actual size of the letter, so 2 fonts with the same nominal point size can have different actual letter sizes. X-height, the height of the lower-case x in typeface, has been used as a more accurate indicator of apparent size than point size. For example, for a given point size, the x-height and apparent size of Arial are actually bigger than those for Times Roman. Do not use type smaller than 10-point Times Roman or equivalent size of another font. Older adults, in particular, have difficulty reading small print.
 - Adequate white space between lines of text (25%–30% of the point size).
 - White space to distinguish sections on the label such as directions for use vs. pharmacy information.
 - Horizontal text only.
- Other measures that can also improve readability:
- If possible, minimize the need to turn the container in order to read lines of text.
 - Never truncate or abbreviate critical information.
 - Highlighting, bolding, and other typographical cues should preserve readability (e.g., high-contrast print and light color for highlighting) and should emphasize patient-centric information or information that facilitates adherence (e.g., refill ordering).
 - Limit the number of colors used for highlighting (e.g., no more than one or two).
 - Use of separate lines to distinguish when each dose should be taken.

Address visual impairment:

- Provide alternative access for visually impaired patients (e.g., tactile, auditory, or enhanced visual systems that may employ advanced mechanics of assistive technology).

▲ USP36

<21> THERMOMETERS

Temperature reading devices suitable for Pharmacopeial tests conform to specifications that are traceable to a NIST standard. Temperature reading devices may be of the liquid-in-glass type or an analog or digital temperature indicator type, such as a resistance temperature device, thermistor, or thermocouple.

An analog or digital temperature indicator consists of a temperature probe, which houses a sensor. The probe is attached to a meter capable of translating a signal in ohms or millivolts into a temperature reading. The temperature probe portion of the analog or digital temperature indicator that is submerged in the medium whose temperature is being measured must be made of inert material. Standardization of analog and digital temperature indicator devices is performed on an established testing frequency with a temperature standard traceable to NIST. In the selection of a temperature reading device, careful consideration of the condition under which it is to be used is essential.

Liquid-in-glass thermometers may be standardized for total immersion, partial immersion, or full immersion. Insofar as practicable, each thermometer should be employed according to the condition of immersion under which it was standardized. Standardization of thermometers is performed on an established testing frequency with a temperature standard traceable to NIST. Refer to the current issue of ASTM standards E1. Standardization of liquid-in-glass thermometers for total immersion involves immersion of the thermometer to the top of the liquid column, with the re-

remainder of the stem and the upper expansion chamber exposed to ambient temperature. Standardization for partial immersion involves immersion of the thermometer to the indicated immersion line etched on the front of the thermometer, with the remainder of the stem exposed to ambient temperature. Standardization for full immersion involves immersion of the entire thermometer, with no portion of the stem exposed to ambient temperature. For use under other conditions of immersion, an emergent stem correction is necessary to obtain correct temperature readings.

<31> VOLUMETRIC APPARATUS

Most of the volumetric apparatus available in the United States is calibrated at 20°, although the temperatures generally prevailing in laboratories more nearly approach 25°. To minimize volumetric error, the temperature should be the same for the volumetric apparatus, the material being prepared, the solvents being used to prepare the volumetric solutions, the area in which they are prepared, and the final volume adjustment.

Use—To attain the degree of precision required in many Pharmacopeial assays involving volumetric measurements and directing that a quantity be “accurately measured,” the apparatus must be chosen and used with care. A buret should be of such size that the titrant volume represents not less than 30% of the nominal volume. Where less than 10 mL of titrant is to be measured, a 10-mL buret or a microburet generally is required.

The design of volumetric apparatus is an important factor in assuring accuracy. For example, the length of the graduated portions of graduated cylinders should be not less than five times the inside diameter, and the tips of burets and pipets should restrict the outflow rate to not more than 500 µL per second.

Standards of Accuracy—The capacity tolerances for volumetric flasks, transfer pipets, and burets are those accepted by the National Institute of Standards and Technology (Class A),¹ as indicated in the accompanying tables. Use Class A volumetric apparatus unless otherwise specified in the individual monograph. For plastic volumetric apparatus the accepted capacity tolerances are Class B.²

The capacity tolerances for measuring (i.e., “graduated”) pipets of up to and including 10-mL capacity are somewhat larger than those for the corresponding sizes of transfer pipets, namely, 10, 20, and 30 µL for the 2-, 5-, and 10-mL sizes, respectively.

Transfer and measuring pipets calibrated “to deliver” should be drained in a vertical position and then touched against the wall of the receiving vessel to drain the tips. Volume readings on burets should be estimated to the nearest 0.01 mL for 25- and 50-mL burets, and to the nearest 0.005 mL for 5- and 10-mL burets. Pipets calibrated “to contain” are called for in special cases, generally for measuring viscous fluids like syrups; however, a volumetric flask may be substituted for a “to contain” pipet. In such cases, the pipet or flask should be washed clean, after draining, and the washings added to the measured portion.

¹ See ASTM 288-06, ASTM E287-02, ASTM E1189-00, and ASTM E969-02.

² See ASTM E 288, Fed. Spec. NNN-F-289, and ISO Standard 384.

Volumetric Flasks

Designated volume, mL	10	25	50	100	250	500	1000
Limit of error, mL	0.02	0.03	0.05	0.08	0.12	0.20	0.30
Limit of error, %	0.20	0.12	0.10	0.08	0.05	0.04	0.03

Transfer Pipets

Designated volume, mL	1	2	5	10	25	50	100
Limit of error, mL	0.006	0.006	0.01	0.02	0.03	0.05	0.08
Limit of error, %	0.60	0.30	0.20	0.20	0.12	0.10	0.08

Burets

Designated volume, mL	10 (“micro” type)	25	50
Subdivisions, mL	0.02	0.1	0.1
Limit of error, mL	0.02	0.03	0.05

<41> WEIGHTS AND BALANCES

The intent of this section is to bring the requirements for weights into conformity with American National Standard ANSI/ASTM E617, “Laboratory Weights and Precision Mass Standards.” This standard is incorporated by reference and should be consulted for full descriptions and information on the tolerances and construction of weights.¹

Pharmacopeial tests and assays require balances that vary in capacity, sensitivity, and reproducibility. Unless otherwise specified, when substances are to be “accurately weighed” for Assay, the weighing is to be performed with a weighing device whose measurement uncertainty (random plus systematic error) does not exceed 0.1% of the reading. Measurement uncertainty is satisfactory if three times the standard deviation of not less than ten replicate weighings divided by the amount weighed, does not exceed 0.001. Unless otherwise specified, for titrimetric limits tests, the weighing shall be performed to provide the number of significant figures in the weight of the analyte that corresponds to the number of significant figures in the concentration of the titrant.

The class designations below are in order of increasing tolerances.

Class 1.1 weights are used for calibration of low-capacity, high-sensitivity balances. They are available in various denominations from 1 to 500 mg. The tolerance for any denomination in this class is 5 µg. They are recommended for calibration of balances using optical or electrical methods for accurately weighing quantities below 20 mg.

Class 1 weights are designated as high-precision standards for calibration. They may be used for weighing accurately

¹ Copies of ASTM Standard E 617-81 (Reapproved 1985) may be obtained from the American Society for Testing and Materials, 1916 Race Street, Philadelphia, PA 19103.

quantities below 20 mg. (For weights of 10 g or less, the requirements of class 1 are met by *USP XXI* class M.)
Class 2 weights are used as working standards for calibration, built-in weights for analytical balances, and laboratory weights for routine analytical work. (The requirements of class 2 are met by *USP XXI* class S.)²
Class 3 and class 4 weights are used with moderate-precision laboratory balances. (Class 3 requirements are met by *USP XXI* class S-1; class 4 requirements are met by *USP XXI* class P.)²
A weight class is chosen so that the tolerance of the weights used does not exceed 0.1% of the amount weighed. Generally, class 2 may be used for quantities greater than 20 mg, class 3 for quantities of greater than 50 mg, and class 4 for quantities of greater than 100 mg. Weights should be calibrated periodically, preferably against an absolute standard weight.

Microbiological Tests

(51) ANTIMICROBIAL EFFECTIVENESS TESTING

Antimicrobial preservatives are substances added to non-sterile dosage forms to protect them from microbiological growth or from microorganisms that are introduced inadvertently during or subsequent to the manufacturing process. In the case of sterile articles packaged in multiple-dose containers, antimicrobial preservatives are added to inhibit the growth of microorganisms that may be introduced from repeatedly withdrawing individual doses.
Antimicrobial preservatives should not be used as a substitute for good manufacturing practices or solely to reduce the viable microbial population of a nonsterile product or control the presterilization bioburden of multidose formulations during manufacturing. Antimicrobial preservatives in compendial dosage forms meet the requirements for *Added Substances* under *Ingredients and Processes* in the *General Notices*.
All useful antimicrobial agents are toxic substances. For maximum protection of patients, the concentration of the preservative shown to be effective in the final packaged product should be below a level that may be toxic to human beings.

The concentration of an added antimicrobial preservative can be kept at a minimum if the active ingredients of the formulation possess an intrinsic antimicrobial activity. Antimicrobial effectiveness, whether inherent in the product or whether produced because of the addition of an antimicrobial preservative, must be demonstrated for all injections packaged in multiple-dose containers or for other products containing antimicrobial preservatives. Antimicrobial effectiveness must be demonstrated for multiple-dose topical and oral dosage forms and for other dosage forms such as ophthalmic, otic, nasal, irrigation, and dialysis fluids (see *Pharmaceutical Dosage Forms* (1151)).
This chapter provides tests to demonstrate the effectiveness of antimicrobial protection. Added antimicrobial preservatives must be declared on the label. The tests and criteria for effectiveness apply to a product in the original, unopened container in which it was distributed by the manufacturer.

² Note that the designations S and P no longer designate weight classes but rather weight grades, that is, design limitations such as range of density of materials, surface area, surface finish, corrosion resistance, and hardness.

PRODUCT CATEGORIES

For the purpose of testing, compendial articles have been divided into four categories (see *Table 1*). The criteria of antimicrobial effectiveness for these products are a function of the route of administration.

Table 1. Compendial Product Categories

Category	Product Description
1	Injections, other parenterals including emulsions, otic products, sterile nasal products, and ophthalmic products made with aqueous bases or vehicles.
2	Topically used products made with aqueous bases or vehicles, nonsterile nasal products, and emulsions, including those applied to mucous membranes.
3	Oral products other than antacids, made with aqueous bases or vehicles.
4	Antacids made with an aqueous base.

TEST ORGANISMS

Use cultures of the following microorganisms¹: *Candida albicans* (ATCC No. 10231), *Aspergillus niger* (ATCC No. 16404), *Escherichia coli* (ATCC No. 8739), *Pseudomonas aeruginosa* (ATCC No. 9027), and *Staphylococcus aureus* (ATCC No. 6538). The viable microorganisms used in the test must not be more than five passages removed from the original ATCC culture. For purposes of the test, one passage is defined as the transfer of organisms from an established culture to fresh medium. All transfers are counted. In the case of organisms maintained by seed-lot techniques, each cycle of freezing, thawing, and revival in fresh medium is taken as one transfer. A seed-stock technique should be used for long-term storage of cultures. Cultures received from the ATCC should be resuscitated according to directions. If grown in broth, the cells are pelleted by centrifugation. Resuspend in 1/20th the volume of fresh maintenance broth, and add an equal volume of 20% (v/v in water) sterile glycerol. Cells grown on agar may be scraped from the surface into the 10% glycerol broth. Dispense small aliquots of the suspension into sterile vials. Store the vials in liquid nitrogen or in a mechanical freezer at no more than -50°. When a fresh seed-stock vial is required, it may be removed and used to inoculate a series of working cultures. These working cultures may then be used periodically (each day in the case of bacteria and yeast) to start the inoculum culture.

MEDIA

All media used in the test must be tested for growth promotion. Use the microorganisms indicated above under *Test Organisms*.

PREPARATION OF INOCULUM

Preparatory to the test, inoculate the surface of a suitable volume of solid agar medium from a recently revived stock culture of each of the specified microorganisms. The culture conditions for the inoculum culture are described in *Table 2* in which the suitable media are Soybean-Casein Digest or Sabouraud Dextrose Agar Medium (see *Microbial Enumeration Tests* (61) and *Tests for Specified Microorganisms* (62)).
To harvest the bacterial and *C. albicans* cultures, use sterile saline TS, washing the surface growth, collecting it in a suitable vessel, and adding sufficient sterile saline TS to obtain a microbial count of about 1 × 10⁸ colony-forming units

¹ Available from American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 (<http://www.atcc.org>).

Table 2. Culture Conditions for Inoculum Preparation

Organism	Suitable Medium	Incubation Temperature	Inoculum Incubation Time	Microbial Recovery Incubation Time
<i>Escherichia coli</i> (ATCC No. 8739)	Soybean–Casein Digest Broth; Soybean–Casein Digest Agar	32.5 ± 2.5°	18 to 24 hours	3 to 5 days
<i>Pseudomonas aeruginosa</i> (ATCC No. 9027)	Soybean–Casein Digest Broth; Soybean–Casein Digest Agar	32.5 ± 2.5°	18 to 24 hours	3 to 5 days
<i>Staphylococcus aureus</i> (ATCC No. 6538)	Soybean–Casein Digest Broth; Soybean–Casein Digest Agar	32.5 ± 2.5°	18 to 24 hours	3 to 5 days
<i>Candida albicans</i> (ATCC No. 10231)	Sabouraud Dextrose Agar; Sabouraud Dextrose Broth	22.5 ± 2.5°	44 to 52 hours	3 to 5 days
<i>Aspergillus niger</i> (ATCC No. 16404)	Sabouraud Dextrose Agar; Sabouraud Dextrose Broth	22.5 ± 2.5°	6 to 10 days	3 to 7 days

(cfu) per mL. To harvest the cells of *A. niger*, use sterile saline TS containing 0.05% of polysorbate 80, and add sufficient sterile saline TS to obtain a count of about 1×10^8 cfu per mL.

Alternatively, the stock culture organisms may be grown in a suitable liquid medium (i.e., Soybean–Casein Digest Broth or Sabouraud Dextrose Broth) and the cells harvested by centrifugation, then washed and resuspended in sterile saline TS to obtain a microbial count of about 1×10^8 cfu per mL. [NOTE—The estimate of inoculum concentration may be performed by turbidimetric measurements for the challenge microorganisms. Refrigerate the suspension if it is not used within 2 hours.]

Determine the number of cfu per mL in each suspension, using the conditions of media and microbial recovery incubation times listed in Table 2 to confirm the initial cfu per mL estimate. This value serves to calibrate the size of inoculum used in the test. The bacterial and yeast suspensions are to be used within 24 hours of harvest, but the fungal preparation may be stored under refrigeration for up to 7 days.

PROCEDURE

The test can be conducted either in five original containers if sufficient volume of product is available in each container and the product container can be entered aseptically (i.e., needle and syringe through an elastomeric rubber stopper), or in five sterile, capped bacteriological containers of suitable size into which a sufficient volume of product has been transferred. Inoculate each container with one of the prepared and standardized inoculum, and mix. The volume of the suspension inoculum used is between 0.5% and 1.0% of the volume of the product. The concentration of test microorganisms that is added to the product (Categories 1, 2, and 3) are such that the final concentration of the test preparation after inoculation is between 1×10^5 and 1×10^6 cfu per mL of the product. For Category 4 products (antacids) the final concentration of the test preparation after inoculation is between 1×10^3 and 1×10^4 cfu per mL of the product.

The initial concentration of viable microorganisms in each test preparation is estimated based on the concentration of microorganisms in each of the standardized inoculum as determined by the plate-count method.

Incubate the inoculated containers at $22.5 \pm 2.5^\circ$. Sample each container at the appropriate intervals specified in Table 3. Record any changes observed in appearance at these intervals. Determine by the plate-count procedure the number of cfu present in each test preparation for the applicable intervals (see Procedure under Microbial Enumeration Tests <61> and Tests for Specified Microorganisms <62>). Incorporate an inactivator (neutralizer) of the specific antimicrobial in the plate count or in the appropriate dilution prepared for

plating. These conditions are determined in the validation study for that sample based upon the conditions of media and microbial recovery incubation times listed in Table 2. Using the calculated concentrations of cfu per mL present at the start of the test, calculate the change in \log_{10} values of the concentration of cfu per mL for each microorganism at the applicable test intervals, and express the changes in terms of log reductions.

CRITERIA FOR ANTIMICROBIAL EFFECTIVENESS

The requirements for antimicrobial effectiveness are met if the criteria specified under Table 3 are met (see Significant Figures and Tolerances under General Notices). No increase is defined as not more than 0.5 \log_{10} unit higher than the previous value measured.

Table 3. Criteria for Tested Microorganisms

For Category 1 Products	
Bacteria:	Not less than 1.0 log reduction from the initial calculated count at 7 days, not less than 3.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast and Molds:	No increase from the initial calculated count at 7, 14, and 28 days.
For Category 2 Products	
Bacteria:	Not less than 2.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast and Molds:	No increase from the initial calculated count at 14 and 28 days.
For Category 3 Products	
Bacteria:	Not less than 1.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast and Molds:	No increase from the initial calculated count at 14 and 28 days.
For Category 4 Products	
Bacteria, Yeast, and Molds:	No increase from the initial calculated count at 14 and 28 days.

(55) BIOLOGICAL INDICATORS— RESISTANCE PERFORMANCE TESTS

TOTAL VIABLE SPORE COUNT

For paper carrier biological indicators, remove three specimens of the relevant biological indicators from their original individual containers. Disperse the paper into component fibers by placing the test specimens in a sterile 250-mL cup of a suitable blender containing 100 mL of chilled, sterilized *Purified Water* and blending for a time known to be adequate to achieve a homogeneous suspension. It is not unusual for blending times of 15 minutes or more to be required for optimal recovery. Transfer a 10-mL aliquot of the suspension to a sterile, screw-capped 16- × 125-mm tube. For *Biological Indicator for Steam Sterilization, Paper Carrier*, heat the tube containing the suspension in a water bath at 95° to 100° for 15 minutes (heat shock), starting the timing when the temperature reaches 95°. For *Biological Indicator for Dry-Heat Sterilization, Paper Carrier*, and for *Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier*, heat the tube containing the suspension in a water bath at 80° to 85° for 10 minutes, starting the timing when the temperature of the spore suspension reaches 80°. Cool rapidly in an ice-water bath at 0° to 4°. Transfer two 1-mL aliquots to suitable tubes, and make appropriate serial dilutions in sterilized *Purified Water*, the dilutions being selected as calculated to yield preferably 30 to 300 colonies, but not less than 6, on each of a pair of plates when treated as described below. Where the biological indicator has a low spore concentration, it may be necessary to modify the dilution series and to use more plates at each dilution. Prepare a separate series of plates for each aliquot. Place 1.0 mL of each selected dilution in each of two 15- × 100-mm Petri dishes. Within 20 minutes, add to each plate 20 mL of *Soybean-Casein Digest Agar Medium* that has been melted and cooled to 45° to 50°. Swirl to attain a homogeneous suspension, and allow it to solidify. Incubate the plates in an inverted position at 55° to 60° for *Biological Indicator for Steam Sterilization, Paper Carrier*, and at 30° to 35° for *Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier* and for *Biological Indicator for Dry-Heat Sterilization, Paper Carrier* or at the optimal recovery temperature specified by the manufacturer. Examine the plates after 24 and 48 hours, recording for each plate the number of colonies; and use the number of colonies observed after 48 hours to calculate the results. Calculate the average number of spores per specimen from the results, using the appropriate dilution factor. The test is valid if the log number of spores per carrier at 48 hours is equal to or greater than the log number after 24 hours in each case. For *Biological Indicator for Steam Sterilization, Self-Contained*, aseptically remove the three carriers from the container, and proceed as directed for *Biological Indicator for Steam Sterilization, Paper Carrier*.

For *Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Nonpaper Carriers*, aseptically remove the three carriers from their original packaging or container. Place each carrier in a suitable sterile container containing 100 mL of chilled *Purified Water*, and sonicate or shake on a reciprocal shaker for an appropriate time. Fifteen minutes or more may be required for optimal recovery. A previous study should be conducted that ensures that the recovery method results in at least 50% to 300% recovery of the labeled spore viable count. Transfer a 10-mL aliquot of the suspension to a sterile, screw-capped 16- × 125-mm tube. Heat the tubes containing suspensions of *Bacillus atrophaeus*, *Bacillus subtilis*, and *Bacillus coagulans* at 80° to

85° for 10 minutes. Heat the tubes containing a suspension of *Geobacillus stearothermophilus* at 95° to 100° for 15 minutes. Start the timing when the lowest temperature of the stated temperature ranges is reached. Cool rapidly in an ice-water bath at 0° to 4°. Transfer two 1-mL aliquots to suitable tubes, and make appropriate serial dilutions in *Purified Water*. The selected dilutions should be those that will preferably yield 30 to 300 colonies but not fewer than 6 on each pair of plates when treated as described below. When the biological indicator has a low spore concentration, it may be necessary to modify the dilution series and to use more plates at each dilution. Prepare a separate series of plates for each aliquot. Place 1.0 mL of each selected dilution in each of two 15- × 100-mm Petri dishes. Within 20 minutes add the aliquot to each plate containing 20 mL of agar that has been melted and cooled to between 45° and 50°. Swirl to attain a homogeneous suspension.

For *G. stearothermophilus*, *B. atrophaeus*, *B. subtilis*, and *B. coagulans*, use *Soybean-Casein Digest Agar Medium* and incubate the plates in an inverted position aerobically at the following respective temperatures for each microorganism: 55° to 60°, 30° to 35°, and 48° to 52°, or at the optimum temperature specified by the biological indicator manufacturer. Examine the plates after 24 and 48 hours. Record the number of colonies observed on each plate. Calculate the average number of spores per carrier from the results, using the appropriate dilution factor. The test is valid if the log number of spores per carrier at 48 hours is equal to or greater than the log number after 24 hours in each case.

For *Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Liquid Spore Suspensions*, using *G. stearothermophilus*, *B. atrophaeus*, *B. subtilis*, and *B. coagulans* as biological indicators, prepare an appropriate serial dilution of the original spore suspension in chilled *Purified Water* contained in a sterile, screw-capped 16- × 125-mm tube, and proceed with the viable spore count procedures specified under *Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Nonpaper Carriers*.

D-VALUE DETERMINATION

Conduct all the tests described in this section under aseptic conditions, using sterilized equipment for nonthermophilic microorganisms. D-value determination for *G. stearothermophilus* and *B. coagulans* can be performed in a controlled but unclassified environment.

Apparatus

The test equipment for the determination of microbial resistance is described in substantial detail in ISO 18472, *Sterilization of Health Care Products—Biological and Chemical Indicators—Test Equipment*.¹ The details of individual Biological Indicator Evaluation Resistometers (BIERs) vary with the specifics of their design and the particular sterilization process in conjunction with which they are used. Provided that the performance of the BIER vessel meets the requirements of the ISO standard for exposure of the biological indicator, design differences are acceptable.

Procedure

Carry out the tests for D value at each of the applicable sets of sterilization conditions for which the packaged biological indicator under test is labeled for use. Take a sufficient number of groups of specimens of biological indicators in their original individual containers, each group consisting of not less than 5 specimens. The number of groups provides a range of observations from not less than one labeled

¹ ANSI/ AAMI/ ISO 18472:2006, *Sterilization of Health Care Products—Biological and Chemical Indicators—Test Equipment*. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA 22201-4795

D value below the labeled survival time through not less than one labeled D value above the labeled kill time. Place each group on a separate suitable specimen holder that permits each specimen to be exposed to the prescribed sterilizing condition at a specific location in the sterilizing chamber of the BIER. Check the BIER apparatus for operating parameters using specimen holders without specimens. Select a series of sterilizing times in increments from the shortest time for the specimens to be tested. The differences in sterilizing times over the series are as constant as feasible, and the difference between adjacent times is no greater than 75% of the labeled D value.

Test procedures for the use of BIER vessels for the evaluation of microbial resistance are defined in a series of ISO standards under the 11138 series.^{2, 3, 4, 5} The appropriate standard should be followed for the biological indicator. The test methods and carriers used with the BIER may be adapted to the specifics of the biological indicator. The method and apparatus used for paper carriers may differ from those for other carriers and will be substantially different from those used for suspensions of biological indicators.

The D-value exposure conditions for alternative material carriers are the same as the conditions used to determine the D value for paper carriers. If the manufacturer's label permits usage of the biological indicator carrier with multiple sterilization methods, then data on D value, survival time, and kill time will need to be provided by the manufacturer for each sterilization method. It is possible that biological indicators inoculated onto carriers other than paper will be used for gaseous or vapor sterilization/decontamination methods such as vapor phase hydrogen peroxide and chlorine dioxide.

Standard physical conditions for the evaluation of biological indicators for use with vapor phase hydrogen peroxide or chlorine dioxide have not been defined. In the case of chlorine dioxide, concentration of the gas, relative humidity, and temperature are critical process control conditions that can be accurately measured. The manufacturer of biological indicators marketed for use with chlorine dioxide should state the conditions under which the D-value determination was conducted so that the user can at least discern the resistance of a lot of biological indicators as compared to their own anticipated use conditions. The situation with vapor phase hydrogen peroxide is a more complex one. Various equipment manufacturers have proposed different decontamination or sterilization conditions. Thus, there is no standard process for the conduct of vapor phase hydrogen peroxide or surface sterilization. It follows, then, that there are no industry standard biological indicator evaluation methods for vapor hydrogen peroxide, and it has been reported that there may not be a direct correlation between vapor concentration and rate or even effectiveness of biological indicator inactivation. Additionally, it is difficult to accurately assess relative humidity, which is often defined as a critical process parameter, in the presence of vapor hydrogen peroxide. For these reasons it is more reasonable to consider resistance of biological indicators to be a relative or comparative measure from the manufacturer rather than a true D value. It follows that, depending upon equipment and processes employed, it may be impossible for an end user to duplicate the biological indicator resistance tests performed by the manufacturer.

² ANSI/AAMI/ISO 11138-1:2006, Sterilization of health care products—Biological indicators—Part 1: General requirements, 2nd ed. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA.

³ ANSI/AAMI/ISO 11138-2:2006, Sterilization of health care products—Biological indicators—Part 2: Biological indicators for ethylene oxide sterilization processes, 3rd ed. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA.

⁴ ANSI/AAMI/ISO 11138-3:2006, Sterilization of health care products—Biological indicators—Part 3: Biological indicators for moist heat sterilization processes. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA.

⁵ ANSI/AAMI/ISO 11138-4:2006, Sterilization of health care products—Biological indicators—Part 4: Biological indicators for dry heat sterilization processes. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA.

For *Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Liquid Spore Suspensions*, conduct D-value determinations for each of the microorganisms that are provided as a liquid spore crop suspension. The test is conducted using appropriate serial dilutions predicated upon the stated spore titer of the suspension in *Purified Water* in a sterile tube.

Where the suspension is placed on or in a substrate such as an elastomeric closure or formulated product, its resistance may differ from that determined in *Purified Water*. That difference may be significant to the usage of the biological indicators and appropriate measurements made prior to use in sterilization validation activities.

Recovery

After completion of the sterilizing procedure for *Biological Indicator for Dry-Heat Sterilization, Paper Carrier*; *Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier*; or *Biological Indicator for Steam Sterilization, Paper Carrier*, whichever is applicable, and within a noted time not more than 4 hours, aseptically remove and add each strip to a suitable medium (see *Media* under *Sterility Tests* (71)) to submerge the biological indicator completely in a suitable tube. For each *Biological Indicator for Steam Sterilization, Self-Contained* specimen, the paper strip is immersed in the self-contained medium according to manufacturers' instructions, within a noted time not more than 4 hours. Incubate each tube at the optimal recovery temperature specified by the manufacturer. Observe each inoculated medium-containing tube at appropriate intervals for a total of 7 days after inoculation. (Where growth is observed at any particular observation time, further incubation of the specimen(s) concerned may be omitted.) Note the number of specimens showing no evidence of growth at any time.

For *Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Nonpaper Carriers*, recovery of spores from the biological indicator carriers will follow recovery procedures described in the procedures under *Total Viable Spore Count*. D-value determination methods for paper carrier biological indicators may be used to calculate the D value for nonpaper carriers. Incubation conditions for the microorganisms that may be used for nonpaper biological indicators are described in the *Total Viable Spore Count* section.

For *Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Liquid Spore Suspensions*, the method of recovery following sterilization exposure conditions are those methods described in the *Total Viable Spore Count* section for liquid suspensions, and when a dry heat D-value determination is made from *B. atrophaeus* suspensions, the same recovery procedures as described under *Biological Indicator for Steam Sterilization, Paper Carrier* are followed.

Where *C. sporogenes* is used as a biological indicator, methods for preparation, inoculation, and recovery methods and media must be adapted to accommodate the use of this anaerobic sporeformer.

Calculation

The determination of D values of biological indicators can be performed using the Limited Spearman-Kärber, Survival Curve Method or Stumbo-Murphy-Cochran procedures.^{6, 7, 8}

⁶ Pflug, I.J. *Syllabus for an Introductory Course in the Microbiology and Engineering of Sterilization Processes*, 4th ed. St. Paul, MN: Environmental Sterilization Services, 1980.

⁷ Pflug, I.J., and G.M. Smith. The Use of Biological Indicators for Monitoring Wet-Heat Sterilization Processes, in *Sterilization of Medical Products*, ed. E.R.L. Gaughran and K. Kereluk. New Brunswick, NJ: Johnson and Johnson, 1977, 193-230.

⁸ Holcomb, R.G., and I.J. Pflug. The Spearman-Kärber Method of Analyzing Quantal Assay Microbial Destruction Data, in *Microbiology and Engineering Sterilization Processes*, ed. I.J. Pflug. St. Paul, MN: Environmental Sterilization Services, 1979.

It is preferable to use the same method as that defined by the biological indicator manufacturer to determine D values. The use of a different method can result in differences that are more an artifact of the method than a variation in the performance of the biological indicator.

Survival Time and Kill Time

Take two groups, each consisting of 10 specimens of the relevant biological indicator, in their original, individual containers. Place the specimens of a group in suitable specimen holders that permit each specimen to be exposed to the sterilizing conditions at a specific location in the BIER chamber.

Expose the specimens for the required survival time, enter the chamber, and remove the holder(s) containing the 10 specimens. Repeat the above procedure immediately, or preheat if a substantial interval has elapsed, so as to subject the second holder(s) containing 10 specimens similarly to the first conditions, but for the required kill time.

The *Survival time and kill time* for all monographed biological indicators is described in the official monograph under the heading for each.

(61) MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: MICROBIAL ENUMERATION TESTS

INTRODUCTION

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi that may grow under aerobic conditions.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

The methods are not applicable to products containing viable microorganisms as active ingredients.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopeial method has been demonstrated.

GENERAL PROCEDURES

Carry out the determination under conditions designed to avoid extrinsic microbial contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any microorganisms that are to be revealed in the test.

If the product to be examined has antimicrobial activity, this is, insofar as possible, removed or neutralized. If inactivators are used for this purpose, their efficacy and their absence of toxicity for microorganisms must be demonstrated.

If surface-active substances are used for sample preparation, their absence of toxicity for microorganisms and their compatibility with any inactivators used must be demonstrated.

ENUMERATION METHODS

Use the *Membrane Filtration* method or one of the *Plate-Count Methods*, as directed. The *Most-Probable-Number (MPN) Method* is generally the least accurate method for microbial counts; however, for certain product groups with very low bioburden, it may be the most appropriate method.

The choice of a method is based on factors such as the nature of the product and the required limit of microorganisms. The method chosen must allow testing of a sufficient sample size to judge compliance with the specification. The suitability of the chosen method must be established.

GROWTH PROMOTION TEST, SUITABILITY OF THE COUNTING METHOD AND NEGATIVE CONTROLS

General Considerations

The ability of the test to detect microorganisms in the presence of product to be tested must be established.

Suitability must be confirmed if a change in testing performance or a change in the product that may affect the outcome of the test, is introduced.

Preparation of Test Strains

Use standardized stable suspensions of test strains or prepare as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in *Table 1*.

Use *Buffered Sodium Chloride–Peptone Solution pH 7.0* or *Phosphate Buffer Solution pH 7.2* to make test suspensions; to suspend *A. brasiliensis* spores, 0.05% of polysorbate 80 may be added to the buffer. Use the suspensions within 2 hours, or within 24 hours if stored between 2° and 8°. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *A. brasiliensis* or *B. subtilis*, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2° to 8° for a validated period of time.

Negative Control

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of microorganisms. A negative control is also performed when testing the products as described under *Testing of Products*. A failed negative control requires an investigation.

Growth Promotion of the Media

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from the ingredients described.

Inoculate portions/plates of *Soybean–Casein Digest Broth* and *Soybean–Casein Digest Agar* with a small number (not more than 100 cfu) of the microorganisms indicated in *Table 1*, using a separate portion/plate of medium for each. Inoculate plates of *Sabouraud Dextrose Agar* with a small number (not more than 100 cfu) of the microorganisms indicated in *Table 1*, using a separate plate of medium for each. Incubate according to the conditions described in *Table 1*.

Table 1. Preparation and Use of Test Microorganisms

Microorganism	Preparation of Test Strain	Growth Promotion		Suitability of Counting Method in the Presence of Product	
		Total Aerobic Microbial Count	Total Yeasts and Molds Count	Total Aerobic Microbial Count	Total Yeasts and Molds Count
<i>Staphylococcus aureus</i> such as ATCC 6538, NCIMB 9518, CIP 4.83, or NBRC 13276	Soybean–Casein Digest Agar or Soybean–Casein Digest Broth 30°–35° 18–24 hours	Soybean–Casein Digest Agar and Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days		Soybean–Casein Digest Agar/MPN Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days	
<i>Pseudomonas aeruginosa</i> such as ATCC 9027, NCIMB 8626, CIP 82.118, or NBRC 13275	Soybean–Casein Digest Agar or Soybean–Casein Digest Broth 30°–35° 18–24 hours	Soybean–Casein Digest Agar and Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days		Soybean–Casein Digest Agar/MPN Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days	
<i>Bacillus subtilis</i> such as ATCC 6633, NCIMB 8054, CIP 52.62, or NBRC 3134	Soybean–Casein Digest Agar or Soybean–Casein Digest Broth 30°–35° 18–24 hours	Soybean–Casein Digest Agar and Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days		Soybean–Casein Digest Agar/MPN Soybean–Casein Digest Broth ≤100 cfu 30°–35° ≤3 days	
<i>Candida albicans</i> such as ATCC 10231, NCPF 3179, IP 48.72, or NBRC 1594	Sabouraud Dextrose Agar or Sabouraud Dextrose Broth 20°–25° 2–3 days	Soybean–Casein Digest Agar ≤100 cfu 30°–35° ≤5 days	Sabouraud Dextrose Agar ≤100 cfu 20°–25° ≤5 days	Soybean–Casein Digest Agar ≤100 cfu 30°–35° ≤5 days MPN: not applicable	Sabouraud Dextrose Agar ≤100 cfu 20°–25° ≤5 days
<i>Aspergillus brasiliensis</i> such as ATCC 16404, IMI 149007, IP 1431.83, or NBRC 9455	Sabouraud Dextrose Agar or Potato–Dextrose Agar 20°–25° 5–7 days, or until good sporulation is achieved	Soybean–Casein Digest Agar ≤100 cfu 30°–35° ≤5 days	Sabouraud Dextrose Agar ≤100 cfu 20°–25° ≤5 days	Soybean–Casein Digest Agar ≤100 cfu 30°–35° ≤5 days MPN: not applicable	Sabouraud Dextrose Agar ≤100 cfu 20°–25° ≤5 days

For solid media, growth obtained must not differ by a factor greater than 2 from the calculated value for a standardized inoculum. For a freshly prepared inoculum, growth of the microorganisms comparable to that previously obtained with a previously tested and approved batch of medium occurs. Liquid media are suitable if clearly visible growth of the microorganisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Suitability of the Counting Method in the Presence of Product

PREPARATION OF THE SAMPLE

The method for sample preparation depends on the physical characteristics of the product to be tested. If none of the procedures described below can be demonstrated to be satisfactory, a suitable alternative procedure must be developed.

Water-Soluble Products—Dissolve or dilute (usually a 1 in 10 dilution is prepared) the product to be examined in *Buffered Sodium Chloride–Peptone Solution pH 7.0*, *Phosphate Buffer Solution pH 7.2*, or *Soybean–Casein Digest Broth*. If necessary, adjust to a pH of 6 to 8. Further dilutions, where necessary, are prepared with the same diluent.

Nonfatty Products Insoluble in Water—Suspend the product to be examined (usually a 1 in 10 dilution is pre-

pared) in *Buffered Sodium Chloride–Peptone Solution pH 7.0*, *Phosphate Buffer Solution pH 7.2*, or *Soybean–Casein Digest Broth*. A surface-active agent such as 1 g per L of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If necessary, adjust to a pH of 6 to 8. Further dilutions, where necessary, are prepared with the same diluent.

Fatty Products—Dissolve in isopropyl myristate sterilized by filtration, or mix the product to be examined with the minimum necessary quantity of sterile polysorbate 80 or another noninhibitory sterile surface-active reagent heated, if necessary, to not more than 40° or, in exceptional cases, to not more than 45°. Mix carefully and if necessary maintain the temperature in a water bath. Add a sufficient quantity of the prewarmed chosen diluent to make a 1 in 10 dilution of the original product. Mix carefully, while maintaining the temperature for the shortest time necessary for the formation of an emulsion. Further serial 10-fold dilutions may be prepared using the chosen diluent containing a suitable concentration of sterile polysorbate 80 or another noninhibitory sterile surface-active reagent.

Fluids or Solids in Aerosol Form—Aseptically transfer the product into a membrane filter apparatus or a sterile container for further sampling. Use either the total contents or a defined number of metered doses from each of the containers tested.

Transdermal Patches—Remove the protective cover sheets ("release liners") of the transdermal patches and place them, adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with a suitable sterile

porous material (e.g., sterile gauze) to prevent the patches from sticking together, and transfer the patches to a suitable volume of the chosen diluent containing inactivators such as polysorbate 80 and/or lecithin. Shake the preparation vigorously for at least 30 minutes.

INOCULATION AND DILUTION

Add to the sample prepared as directed above and to a control (with no test material included) a sufficient volume of the microbial suspension to obtain an inoculum of not more than 100 cfu. The volume of the suspension of the inoculum should not exceed 1% of the volume of diluted product.

To demonstrate acceptable microbial recovery from the product, the lowest possible dilution factor of the prepared sample must be used for the test. Where this is not possible due to antimicrobial activity or poor solubility, further appropriate protocols must be developed. If inhibition of growth by the sample cannot otherwise be avoided, the aliquot of the microbial suspension may be added after neutralization, dilution, or filtration.

NEUTRALIZATION/REMOVAL OF ANTIMICROBIAL ACTIVITY

The number of microorganisms recovered from the prepared sample diluted as described in *Inoculation and Dilution* and incubated following the procedure described in *Recovery of Microorganisms in the Presence of Product*, is compared to the number of microorganisms recovered from the control preparation.

If growth is inhibited (reduction by a factor greater than 2), then modify the procedure for the particular enumeration test to ensure the validity of the results. Modification of the procedure may include, for example,

- (1) An increase in the volume of the diluent or culture medium;
- (2) Incorporation of a specific or general neutralizing agents into the diluent;
- (3) Membrane filtration; or
- (4) A combination of the above measures.

Neutralizing Agents—Neutralizing agents may be used to neutralize the activity of antimicrobial agents (see Table 2). They may be added to the chosen diluent or the medium preferably before sterilization. If used, their efficacy and their absence of toxicity for microorganisms must be demonstrated by carrying out a blank with neutralizer and without product.

Table 2. Common Neutralizing Agents/Methods for Interfering Substances

Interfering Substance	Potential Neutralizing Agents/Method
Glutaraldehyde, mercurials	Sodium hydrogen sulfite (Sodium bisulfite)
Phenolics, alcohol, aldehydes, sorbate	Dilution
Aldehydes	Glycine
Quaternary ammonium compounds (QACs), parahydroxybenzoates (parabens), bis-biquanides	Lecithin
QACs, iodine, parabens	Polysorbate
Mercurials	Thioglycollate
Mercurials, halogens, aldehydes	Thiosulfate
EDTA (edetate)	Mg or Ca ions

If no suitable neutralizing method can be found, it can be assumed that the failure to isolate the inoculated organism is attributable to the microbicidal activity of the product.

This information serves to indicate that the article is not likely to be contaminated with the given species of the microorganism. However, it is possible that the product inhibits only some of the microorganisms specified herein, but does not inhibit others not included among the test strains or those for which the latter are not representative. Then, perform the test with the highest dilution factor compatible with microbial growth and the specific acceptance criterion.

RECOVERY OF MICROORGANISMS IN THE PRESENCE OF PRODUCT

For each of the microorganisms listed, separate tests are performed. Only microorganisms of the added test strain are counted.

Membrane Filtration—Use membrane filters having a nominal pore size not greater than 0.45 µm. The type of filter material is chosen in such a way that the bacteria-retaining efficiency is not affected by the components of the sample to be investigated. For each of the microorganisms listed, one membrane filter is used.

Transfer a suitable quantity of the sample prepared as described under *Preparation of the Sample, Inoculation and Dilution*, and *Neutralization/Removal of Antimicrobial Activity* (preferably representing 1 g of the product, or less if large numbers of cfu are expected) to the membrane filter, filter immediately, and rinse the membrane filter with an appropriate volume of diluent.

For the determination of total aerobic microbial count (TAMC), transfer the membrane filter to the surface of the *Soybean–Casein Digest Agar*. For the determination of total combined yeasts and molds count (TYMC), transfer the membrane to the surface of the *Sabouraud Dextrose Agar*. Incubate the plates as indicated in Table 1. Perform the counting.

Plate-Count Methods—Perform plate-count methods at least in duplicate for each medium, and use the mean count of the result.

Pour-Plate Method—For Petri dishes 9 cm in diameter, add to the dish 1 mL of the sample prepared as described under *Preparation of the Sample, Inoculation and Dilution*, and *Neutralization/Removal of Antimicrobial Activity* and 15 to 20 mL of *Soybean–Casein Digest Agar* or *Sabouraud Dextrose Agar*, both media maintained at not more than 45°. If larger Petri dishes are used, the amount of agar medium is increased accordingly. For each of the microorganisms listed in Table 1, at least two Petri dishes are used.

Incubate the plates as indicated in Table 1. Take the arithmetic mean of the counts per medium, and calculate the number of cfu in the original inoculum.

Surface-Spread Method—For Petri dishes 9 cm in diameter, add 15 to 20 mL of *Soybean–Casein Digest Agar* or *Sabouraud Dextrose Agar* at about 45° to each Petri dish, and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example, in a laminar-airflow cabinet or in an incubator. For each of the microorganisms listed in Table 1, at least two Petri dishes are used. Spread a measured volume of not less than 0.1 mL of the sample, prepared as directed under *Preparation of the Sample, Inoculation and Dilution*, and *Neutralization/Removal of Antimicrobial Activity* over the surface of the medium. Incubate and count as directed for *Pour-Plate Method*.

Most-Probable-Number (MPN) Method—The precision and accuracy of the *MPN Method* is less than that of the *Membrane Filtration* method or the *Plate-Count Method*. Unreliable results are obtained particularly for the enumeration of molds. For these reasons, the *MPN Method* is reserved for the enumeration of TAMC in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least three serial 10-fold dilutions of the product as described for *Preparation of the Sample, Inoc-*

ulation and Dilution, and Neutralization/Removal of Antimicrobial Activity. From each level of dilution, three aliquots of 1 g or 1 mL are used to inoculate three tubes with 9 to 10 mL of *Soybean-Casein Digest Broth*. If necessary a surface-active agent such as polysorbate 80, or an inactivator of antimicrobial agents may be added to the medium. Thus, if three levels of dilution are prepared, nine tubes are inoculated.

Incubate all tubes at 30° to 35° for not more than 3 days. If reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth or in *Soybean-Casein Digest Agar* for 1 to 2 days at the same temperature, and use these results. From *Table 3*, determine the most probable number of microorganisms per g or mL of the product to be examined.

Table 3. Most-Probable-Number Values of Microorganisms

Observed Combinations of Numbers of Tubes Showing Growth in Each Set			MPN per g or per mL of Product	95% Confidence Limits
Number of g or mL of Product per Tube				
0.1	0.01	0.001		
0	0	0	<3	0–9.4
0	0	1	3	0.1–9.5
0	1	0	3	0.1–10
0	1	1	6.1	1.2–17
0	2	0	6.2	1.2–17
0	3	0	9.4	3.5–35
1	0	0	3.6	0.2–17
1	0	1	7.2	1.2–17
1	0	2	11	4–35
1	1	0	7.4	1.3–20
1	1	1	11	4–35
1	2	0	11	4–35
1	2	1	15	5–38
1	3	0	16	5–38
2	0	0	9.2	1.5–35
2	0	1	14	4–35
2	0	2	20	5–38
2	1	0	15	4–38
2	1	1	20	5–38
2	1	2	27	9–94
2	2	0	21	5–40
2	2	1	28	9–94
2	2	2	35	9–94
2	3	0	29	9–94
2	3	1	36	9–94
3	0	0	23	5–94
3	0	1	38	9–104
3	0	2	64	16–181
3	1	0	43	9–181
3	1	1	75	17–199
3	1	2	120	30–360
3	1	3	160	30–380
3	2	0	93	18–360
3	2	1	150	30–380
3	2	2	210	30–400
3	2	3	290	90–990
3	3	0	240	40–990
3	3	1	460	90–1980
3	3	2	1100	200–4000
3	3	3	>1100	

RESULTS AND INTERPRETATION

When verifying the suitability of the *Membrane Filtration* method or the *Plate-Count Method*, a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control defined in *Inoculation and Dilution* in the absence of product must be obtained. When verifying the suitability of the *MPN Method*, the calculated value from the inoculum must be within 95% confidence limits of the results obtained with the control.

If the above criteria cannot be met for one of more of the organisms tested with any of the described methods, the method and test conditions that come closest to the criteria are used to test the product.

TESTING OF PRODUCTS

Amount Used for the Test

Unless otherwise directed, use 10 g or 10 mL of the product to be examined taken with the precautions referred to above. For fluids or solids in aerosol form, sample 10 containers. For transdermal patches, sample 10 patches.

The amount to be tested may be reduced for active substances that will be formulated in the following conditions: the amount per dosage unit (e.g., tablet, capsule, injection) is less than or equal to 1 mg, or the amount per g or mL (for preparations not presented in dose units) is less than 1 mg. In these cases, the amount of sample to be tested is not less than the amount present in 10 dosage units or 10 g or 10 mL of the product.

For materials used as active substances where the sample quantity is limited or batch size is extremely small (i.e., less than 1000 mL or 1000 g), the amount tested shall be 1% of the batch unless a lesser amount is prescribed or justified and authorized.

For products where the total number of entities in a batch is less than 200 (e.g., samples used in clinical trials), the sample size may be reduced to two units, or one unit if the size is less than 100.

Select the sample(s) at random from the bulk material or from the available containers of the preparation. To obtain the required quantity, mix the contents of a sufficient number of containers to provide the sample.

Examination of the Product

MEMBRANE FILTRATION

Use a filtration apparatus designed to allow the transfer of the filter to the medium. Prepare the sample using a method that has been shown to be suitable as described in *Growth Promotion Test and Suitability of the Counting Method*, transfer the appropriate amount to each of two membrane filters, and filter immediately. Wash each filter following the procedure shown to be suitable.

For the determination of TAMC, transfer one of the membrane filters to the surface of *Soybean-Casein Digest Agar*. For the determination of TYMC, transfer the other membrane to the surface of *Sabouraud Dextrose Agar*. Incubate the plate of *Soybean-Casein Digest Agar* at 30° to 35° for 3 to 5 days and the plate of *Sabouraud Dextrose Agar* at 20° to 25° for 5 to 7 days. Calculate the number of cfu per g or per mL of product.

When examining transdermal patches, separately filter 10% of the volume of the preparation described for *Preparation of the Sample* through each of two sterile filter membranes. Transfer one membrane to *Soybean-Casein Digest Agar* for TAMC and the other membrane to *Sabouraud Dextrose Agar* for TYMC.

PLATE-COUNT METHODS

Pour-Plate Method—Prepare the sample using a method that has been shown to be suitable as described in *Growth Promotion Test and Suitability of the Counting Method*. Prepare for each medium at least two Petri dishes for each level of dilution. Incubate the plates of *Soybean–Casein Digest Agar* at 30° to 35° for 3 to 5 days and the plates of *Sabouraud Dextrose Agar* at 20° to 25° for 5 to 7 days. Select the plates corresponding to a given dilution and showing the highest number of colonies less than 250 for TAMC and 50 for TYMC. Take the arithmetic mean per culture medium of the counts, and calculate the number of cfu per g or per mL of product.

Surface-Spread Method—Prepare the sample using a method that has been shown to be suitable as described in *Growth Promotion Test and Suitability of the Counting Method*. Prepare at least two Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of cfu, proceed as directed for the *Pour-Plate Method*.

MOST-PROBABLE-NUMBER METHOD

Prepare and dilute the sample using a method that has been shown to be suitable as described in *Growth Promotion Test and Suitability of the Counting Method*. Incubate all tubes for 3 to 5 days at 30° to 35°. Subculture if necessary, using the procedure shown to be suitable. Record for each level of dilution the number of tubes showing microbial growth. Determine the most probable number of microorganisms per g or mL of the product to be examined from Table 3.

Interpretation of the Results

The total aerobic microbial count (TAMC) is considered to be equal to the number of cfu found using *Soybean–Casein Digest Agar*; if colonies of fungi are detected on this medium, they are counted as part of TAMC. The total combined yeasts and molds count (TYMC) is considered to be equal to the number of cfu found using *Sabouraud Dextrose Agar*; if colonies of bacteria are detected on this medium, they are counted as part of TYMC. When the TYMC is expected to exceed the acceptance criterion due to the bacterial growth, *Sabouraud Dextrose Agar* containing antibiotics may be used. If the count is carried out by the *MPN Method*, the calculated value is TAMC.

When an acceptance criterion for microbiological quality is prescribed, it is interpreted as follows:

- 10^1 cfu: maximum acceptable count = 20;
 - 10^2 cfu: maximum acceptable count = 200;
 - 10^3 cfu: maximum acceptable count = 2000;
- and so forth.

The recommended solutions and media are described in *Tests for Specified Microorganisms (62)*.

(62) MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: TESTS FOR SPECIFIED MICROORGANISMS

INTRODUCTION

The tests described hereafter will allow determination of the absence of, or limited occurrence of, specified microorganisms that may be detected under the conditions described.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopeial method has been demonstrated.

GENERAL PROCEDURES

The preparation of samples is carried out as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests (61)*.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralized as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests (61)*.

If surface-active substances are used for sample preparation, their absence of toxicity for microorganisms and their compatibility with any inactivators used must be demonstrated as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests (61)*.

GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA, SUITABILITY OF THE TEST AND NEGATIVE CONTROLS

The ability of the test to detect microorganisms in the presence of the product to be tested must be established. Suitability must be confirmed if a change in testing performance or a change in the product that may affect the outcome of the test is introduced.

Preparation of Test Strains

Use standardized stable suspensions of test strains as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot.

AEROBIC MICROORGANISMS

Grow each of the bacterial test strains separately in containers containing *Soybean–Casein Digest Broth* or on *Soybean–Casein Digest Agar* at 30° to 35° for 18 to 24 hours. Grow the test strain for *Candida albicans* separately on *Sabouraud Dextrose Agar* or in *Sabouraud Dextrose Broth* at 20° to 25° for 2 to 3 days.

<i>Staphylococcus aureus</i>	such as ATCC 6538, NCIMB 9518, CIP 4.83, or NBRC 13276
<i>Pseudomonas aeruginosa</i>	such as ATCC 9027, NCIMB 8626, CIP 82.118, or NBRC 13275
<i>Escherichia coli</i>	such as ATCC 8739, NCIMB 8545, CIP 53.126, or NBRC 3972
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium or, as an alternative,	such as ATCC 14028
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Abony	such as NBRC 100797, NCTC 6017, or CIP 80.39
<i>Candida albicans</i>	such as ATCC 10231, NCPF 3179, IP 48.72, or NBRC 1594

Use *Buffered Sodium Chloride–Peptone Solution pH 7.0* or *Phosphate Buffer Solution pH 7.2* to make test suspensions. Use the suspensions within 2 hours or within 24 hours if stored at 2° to 8°.

CLOSTRIDIA

Use *Clostridium sporogenes* such as ATCC 11437 (NBRC 14293, NCIMB 12343, CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.3). Grow the clostridial test strain under anaerobic conditions in *Reinforced Medium for Clostridia* at 30° to 35° for 24 to 48 hours. As an alternative to preparing and then diluting down a fresh suspension of vegetative

cells of *Cl. sporogenes*, a stable spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2° to 8° for a validated period.

Negative Control

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of microorganisms. A negative control is also performed when testing the products as described under *Testing of Products*. A failed negative control requires an investigation.

Growth Promotion and Inhibitory Properties of the Media

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Verify suitable properties of relevant media as described in *Table 1*.

Test for Growth-Promoting Properties, Liquid Media—Inoculate a portion of the appropriate medium with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Clearly visible growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Test for Growth-Promoting Properties, Solid Media—Perform *Surface-Spread Method* (see *Plate-Count Methods* under *Microbiological Examination of Nonsterile Products: Mi-*

Table 1. Growth Promoting, Inhibitory, and Indicative Properties of Media

Test/Medium	Property	Test Strains
<i>Test for bile-tolerant Gram-negative bacteria</i>		
Enterobacteria Enrichment Broth Mossel	Growth promoting	<i>E. coli</i>
		<i>P. aeruginosa</i>
	Inhibitory	<i>S. aureus</i>
Violet Red Bile Glucose Agar	Growth promoting + Indicative	<i>E. coli</i>
		<i>P. aeruginosa</i>
<i>Test for Escherichia coli</i>		
MacConkey Broth	Growth promoting	<i>E. coli</i>
	Inhibitory	<i>S. aureus</i>
MacConkey Agar	Growth promoting + Indicative	<i>E. coli</i>
<i>Test for Salmonella</i>		
Rappaport Vassiliadis Salmonella Enrichment Broth	Growth promoting	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium or
		<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Abony
	Inhibitory	<i>S. aureus</i>
Xylose Lysine Deoxycholate Agar	Growth promoting + Indicative	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium or
		<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Abony
<i>Test for Pseudomonas aeruginosa</i>		
Cetrimide Agar	Growth promoting	<i>P. aeruginosa</i>
	Inhibitory	<i>E. coli</i>
<i>Test for Staphylococcus aureus</i>		
Mannitol Salt Agar	Growth promoting + Indicative	<i>S. aureus</i>
	Inhibitory	<i>E. coli</i>
<i>Test for Clostridia</i>		
Reinforced Medium for Clostridia	Growth promoting	<i>Cl. sporogenes</i>
Columbia Agar	Growth promoting	<i>Cl. sporogenes</i>
<i>Test for Candida albicans</i>		
Sabouraud Dextrose Broth	Growth promoting	<i>C. albicans</i>
Sabouraud Dextrose Agar	Growth promoting + Indicative	<i>C. albicans</i>

Microbial Enumeration Tests (61)), inoculating each plate with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Test for Inhibitory Properties, Liquid or Solid Media—Inoculate the appropriate medium with at least 100 cfu of the appropriate microorganism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test microorganism occurs.

Test for Indicative Properties—Perform *Surface-Spread Method* (see *Plate-Count Methods* under *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61)), inoculating each plate with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

Suitability of the Test Method

For each new product to be tested perform sample preparation as described in the relevant paragraph under *Testing of Products*. At the time of mixing, add each test strain in the prescribed growth medium. Inoculate the test strains individually. Use a number of microorganisms equivalent to not more than 100 cfu in the inoculated test preparation.

Perform the test as described in the relevant paragraph under *Testing of Products* using the shortest incubation period prescribed.

The specified microorganisms must be detected with the indication reactions as described under *Testing of Products*.

Any antimicrobial activity of the product necessitates a modification of the test procedure (see *Neutralization/Removal of Antimicrobial Activity* under *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61)).

For a given product, if the antimicrobial activity with respect to a microorganism for which testing is prescribed cannot be neutralized, then it is to be assumed that the inhibited microorganism will not be present in the product.

TESTING OF PRODUCTS

Bile-Tolerant Gram-Negative Bacteria

Sample Preparation and Pre-Incubation—Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), but using *Soybean-Casein Digest Broth* as the chosen diluent, mix, and incubate at 20° to 25° for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 hours but not more than 5 hours).

Test for Absence—Unless otherwise prescribed, use the volume corresponding to 1 g of the product, as prepared in *Sample Preparation and Pre-Incubation*, to inoculate *Enterobacteria Enrichment Broth Mossel*. Incubate at 30° to 35° for 24 to 48 hours. Subculture on plates of *Violet Red Bile Glucose Agar*. Incubate at 30° to 35° for 18 to 24 hours.

The product complies with the test if there is no growth of colonies.

Quantitative Test—

Selection and Subculture—Inoculate suitable quantities of *Enterobacteria Enrichment Broth Mossel* with the preparation

as directed under *Sample Preparation and Pre-Incubation* and/or dilutions of it containing respectively 0.1 g, 0.01 g, and 0.001 g (or 0.1 mL, 0.01 mL, and 0.001 mL) of the product to be examined. Incubate at 30° to 35° for 24 to 48 hours. Subculture each of the cultures on a plate of *Violet Red Bile Glucose Agar*. Incubate at 30° to 35° for 18 to 24 hours.

Interpretation—Growth of colonies constitutes a positive result. Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result. Determine from *Table 2* the probable number of bacteria.

Table 2. Interpretation of Results

Results for Each Quantity of Product			
0.1 g or 0.1 mL	0.01 g or 0.01 mL	0.001 g or 0.001 mL	Probable Number of Bacteria per g or mL of Product
+	+	+	more than 10 ³
+	+	–	less than 10 ³ and more than 10 ²
+	–	–	less than 10 ² and more than 10
–	–	–	less than 10

Escherichia coli

Sample Preparation and Pre-Incubation—Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), and use 10 mL or the quantity corresponding to 1 g or 1 mL, to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean-Casein Digest Broth*, mix, and incubate at 30° to 35° for 18 to 24 hours.

Selection and Subculture—Shake the container, transfer 1 mL of *Soybean-Casein Digest Broth* to 100 mL of *MacConkey Broth*, and incubate at 42° to 44° for 24 to 48 hours. Subculture on a plate of *MacConkey Agar* at 30° to 35° for 18 to 72 hours.

Interpretation—Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.

The product complies with the test if no colonies are present or if the identification tests are negative.

Salmonella

Sample Preparation and Pre-Incubation—Prepare the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), and use the quantity corresponding to not less than 10 g or 10 mL to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean-Casein Digest Broth*, mix, and incubate at 30° to 35° for 18 to 24 hours.

Selection and Subculture—Transfer 0.1 mL of *Soybean-Casein Digest Broth* to 10 mL of *Rappaport Vassiliadis Salmonella Enrichment Broth*, and incubate at 30° to 35° for 18 to 24 hours. Subculture on plates of *Xylose Lysine Deoxycholate Agar*. Incubate at 30° to 35° for 18 to 48 hours.

Interpretation—The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centers. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

Pseudomonas aeruginosa

Sample Preparation and Pre-Incubation—Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean–Casein Digest Broth*, and mix. When testing transdermal patches, filter the volume of sample corresponding to one patch of the preparation (see *Transdermal Patches* under *Preparation of the Sample* in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61)) through a sterile filter membrane, and place in 100 mL of *Soybean–Casein Digest Broth*. Incubate at 30° to 35° for 18 to 24 hours.

Selection and Subculture—Subculture on a plate of *Cetrimide Agar*, and incubate at 30° to 35° for 18 to 72 hours.

Interpretation—Growth of colonies indicates the possible presence of *P. aeruginosa*. This is confirmed by identification tests.

The product complies with the test if colonies are not present or if the confirmatory identification tests are negative.

Staphylococcus aureus

Sample Preparation and Pre-Incubation—Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean–Casein Digest Broth*, and homogenize. When testing transdermal patches, filter the volume of sample corresponding to one patch of the preparation (see *Transdermal Patches* under *Preparation of the Sample* in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61)) through a sterile filter membrane, and place in 100 mL of *Soybean–Casein Digest Broth*. Incubate at 30° to 35° for 18 to 24 hours.

Selection and Subculture—Subculture on a plate of *Mannitol Salt Agar*, and incubate at 30° to 35° for 18 to 72 hours.

Interpretation—The possible presence of *S. aureus* is indicated by the growth of yellow or white colonies surrounded by a yellow zone. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

Clostridia

Sample Preparation and Heat Treatment—Prepare a sample using a 1 in 10 dilution (with a minimum total volume of 20 mL) of not less than 2 g or 2 mL of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61). Divide the sample into two portions of at least 10 mL. Heat one portion at 80° for 10 minutes, and cool rapidly. Do not heat the other portion.

Selection and Subculture—Use 10 mL or the quantity corresponding to 1 g or 1 mL of the product to be examined of both portions to inoculate suitable amounts (de-

termined as described under *Suitability of the Test Method*) of *Reinforced Medium for Clostridia*. Incubate under anaerobic conditions at 30° to 35° for 48 hours. After incubation, make subcultures from each container on *Columbia Agar*, and incubate under anaerobic conditions at 30° to 35° for 48 to 72 hours.

Interpretation—The occurrence of anaerobic growth of rods (with or without endospores) giving a negative catalase reaction indicates the presence of *Clostridia*.

This is confirmed by identification tests. The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

Candida albicans

Sample Preparation and Pre-Incubation—Prepare the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), and use 10 mL or the quantity corresponding to not less than 1 g or 1 mL, to inoculate 100 mL of *Sabouraud Dextrose Broth*, and mix. Incubate at 30° to 35° for 3 to 5 days.

Selection and Subculture—Subculture on a plate of *Sabouraud Dextrose Agar*, and incubate at 30° to 35° for 24 to 48 hours.

Interpretation—Growth of white colonies may indicate the presence of *C. albicans*. This is confirmed by identification tests.

The product complies with the test if such colonies are not present or if the confirmatory identification tests are negative.

RECOMMENDED SOLUTIONS AND CULTURE MEDIA

NOTE—This section is given for information.

The following solutions and culture media have been found satisfactory for the purposes for which they are prescribed in the test for microbial contamination in the Pharmacopeia. Other media may be used provided that their suitability can be demonstrated.

Stock Buffer Solution—Transfer 34 g of potassium dihydrogen phosphate to a 1000-mL volumetric flask, dissolve in 500 mL of Purified Water, adjust with sodium hydroxide to a pH of 7.2 ± 0.2 , add Purified Water to volume, and mix. Dispense in containers, and sterilize. Store at a temperature of 2° to 8°.

Phosphate Buffer Solution pH 7.2—Prepare a mixture of Purified Water and *Stock Buffer Solution* (800:1 v/v), and sterilize.

Buffered Sodium Chloride–Peptone Solution pH 7.0	
Potassium Dihydrogen Phosphate	3.6 g
Disodium Hydrogen Phosphate Dihydrate	7.2 g (equivalent to 0.067 M phosphate)
Sodium Chloride	4.3 g
Peptone (meat or casein)	1.0 g
Purified Water	1000 mL

Sterilize in an autoclave using a validated cycle.

Soybean–Casein Digest Broth	
Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean	3.0 g
Sodium Chloride	5.0 g
Dibasic Hydrogen Phosphate	2.5 g

Soybean–Casein Digest Broth	
Glucose Monohydrate	2.5 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25° . Sterilize in an autoclave using a validated cycle.

Soybean–Casein Digest Agar	
Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25° . Sterilize in an autoclave using a validated cycle.

Sabouraud Dextrose Agar	
Dextrose	40.0 g
Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1)	10.0 g
Agar	15.0 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25° . Sterilize in an autoclave using a validated cycle.

Potato Dextrose Agar	
Infusion from potatoes	200 g
Dextrose	20.0 g
Agar	15.0 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25° . Sterilize in an autoclave using a validated cycle.

Sabouraud Dextrose Broth	
Dextrose	20.0 g
Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1)	10.0 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is 5.6 ± 0.2 at 25° . Sterilize in an autoclave using a validated cycle.

Enterobacteria Enrichment Broth Mossel	
Pancreatic Digest of Gelatin	10.0 g
Glucose Monohydrate	5.0 g
Dehydrated Ox Bile	20.0 g
Potassium Dihydrogen Phosphate	2.0 g
Disodium Hydrogen Phosphate Dihydrate	8.0 g
Brilliant Green	15 mg
Purified Water	1000 mL

Adjust the pH so that after heating it is 7.2 ± 0.2 at 25° . Heat at 100° for 30 minutes, and cool immediately.

Violet Red Bile Glucose Agar	
Yeast Extract	3.0 g
Pancreatic Digest of Gelatin	7.0 g
Bile Salts	1.5 g
Sodium Chloride	5.0 g
Glucose Monohydrate	10.0 g
Agar	15.0 g
Neutral Red	30 mg
Crystal Violet	2 mg
Purified Water	1000 mL

Adjust the pH so that after heating it is 7.4 ± 0.2 at 25° . Heat to boiling; do not heat in an autoclave.

MacConkey Broth	
Pancreatic Digest of Gelatin	20.0 g
Lactose Monohydrate	10.0 g
Dehydrated Ox Bile	5.0 g
Bromocresol Purple	10 mg
Purified Water	1000 mL

Adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25° . Sterilize in an autoclave using a validated cycle.

MacConkey Agar	
Pancreatic Digest of Gelatin	17.0 g
Peptones (meat and casein)	3.0 g
Lactose Monohydrate	10.0 g
Sodium Chloride	5.0 g
Bile Salts	1.5 g
Agar	13.5 g
Neutral Red	30.0 mg
Crystal Violet	1 mg
Purified Water	1000 mL

Adjust the pH so that after sterilization it is 7.1 ± 0.2 at 25° . Boil for 1 minute with constant shaking, then sterilize in an autoclave using a validated cycle.

Rappaport Vassiliadis Salmonella Enrichment Broth	
Soya Peptone	4.5 g
Magnesium Chloride Hexahydrate	29.0 g
Sodium Chloride	8.0 g
Dipotassium Phosphate	0.4 g
Potassium Dihydrogen Phosphate	0.6 g
Malachite Green	0.036 g
Purified Water	1000 mL

Dissolve, warming slightly. Sterilize in an autoclave using a validated cycle, at a temperature not exceeding 115° . The pH is to be 5.2 ± 0.2 at 25° after heating and autoclaving.

Xylose Lysine Deoxycholate Agar	
Xylose	3.5 g
L-Lysine	5.0 g
Lactose Monohydrate	7.5 g
Sucrose	7.5 g
Sodium Chloride	5.0 g
Yeast Extract	3.0 g
Phenol Red	80 mg
Agar	13.5 g
Sodium Deoxycholate	2.5 g
Sodium Thiosulfate	6.8 g
Ferric Ammonium Citrate	0.8 g
Purified Water	1000 mL

Adjust the pH so that after heating it is 7.4 ± 0.2 at 25° . Heat to boiling, cool to 50° , and pour into Petri dishes. Do not heat in an autoclave.

Cetrimide Agar	
Pancreatic Digest of Gelatin	20.0 g
Magnesium Chloride	1.4 g
Dipotassium Sulfate	10.0 g
Cetrimide	0.3 g
Agar	13.6 g
Purified Water	1000 mL
Glycerol	10.0 mL

Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilization it is 7.2 ± 0.2 at 25° . Sterilize in an autoclave using a validated cycle.

Mannitol Salt Agar	
Pancreatic Digest of Casein	5.0 g
Peptic Digest of Animal Tissue	5.0 g
Beef Extract	1.0 g
D-Mannitol	10.0 g
Sodium Chloride	75.0 g
Agar	15.0 g
Phenol Red	0.025 g
Purified Water	1000 mL

Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilization it is 7.4 ± 0.2 at 25° . Sterilize in an autoclave using a validated cycle.

Reinforced Medium for Clostridia	
Beef Extract	10.0 g
Peptone	10.0 g
Yeast Extract	3.0 g
Soluble Starch	1.0 g
Glucose Monohydrate	5.0 g
Cysteine Hydrochloride	0.5 g
Sodium Chloride	5.0 g
Sodium Acetate	3.0 g
Agar	0.5 g
Purified Water	1000 mL

Hydrate the agar, and dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is about 6.8 ± 0.2 at 25° . Sterilize in an autoclave using a validated cycle.

Columbia Agar	
Pancreatic Digest of Casein	10.0 g
Meat Peptic Digest	5.0 g
Heart Pancreatic Digest	3.0 g
Yeast Extract	5.0 g
Maize Starch	1.0 g
Sodium Chloride	5.0 g
Agar, according to gelling power	10.0–15.0 g
Purified Water	1000 mL

Hydrate the agar, and dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is 7.3 ± 0.2 at 25° . Sterilize in an autoclave using a validated cycle. Allow to cool to 45° to 50° ; add, where necessary, gentamicin sulfate corresponding to 20 mg of gentamicin base, and pour into Petri dishes.

<63> MYCOPLASMA TESTS

INTRODUCTION

The genus *Mycoplasma* represents a group of minute bacteria which have no cell walls. The genus comprises more than 120 species. They are the smallest self-replicating prokaryotic organisms. The cells vary in size and morphology

and cannot be Gram stained, but impressions of colonies on solid agar can be stained with methylene blue or equivalent stain. *Mycoplasma* are parasites and commensals, and some may be pathogenic to a variety of animal and plant hosts. In humans, *Mycoplasma* are usually surface parasites that colonize the epithelial lining of the respiratory and urogenital tracts. *Mycoplasma* are common and may cause serious contamination in cell and/or tissue cultures used to generate compendial articles. They may also cause contamination of filtered sterilized soybean casein digest broth. A cell culture infection may persist for an extended period of time without causing apparent cell damage. Infection of cells in a culture can affect nearly every pathway of cell metabolism, including alteration of the cells' phenotypical characteristics and normal growth. The presence of *Mycoplasma* species does not always result in turbid growth in cultures or visible alteration of the cells.

Testing for *Mycoplasma* is a necessary quality control requirement to assure reliably pure biotechnological products and allied materials used to generate these products. This general test chapter describes two methods required to detect *Mycoplasma* contamination of test articles, tissues and/or cell cultures used to produce test articles, digest broth, or any other material in which *Mycoplasma* contamination is suspected. These are: (A) the agar and broth media procedure and (B) the indicator cell culture procedure. These tests require careful aseptic technique and suitable laboratory conditions. In order to ensure appropriate testing and interpretation of results, personnel should be properly trained and qualified. A validated nucleic acid amplification technique (NAT) or an enzymatic activity based method may be used to detect *Mycoplasma*, provided such a method is shown to be comparable to both methods (A) and (B). Alternative methods must be suitably validated. Validation requirements for alternate methods will not be addressed in this chapter.

CULTURE METHOD

Choice of Media

The test is carried out using a sufficient number of both solid and liquid media to ensure growth in the chosen incubation conditions of small numbers (approximately 100 colony-forming units, cfu; or 100 color-changing units, ccu) of *Mycoplasmas* that may be present in the test article/material. Liquid media must contain phenol red. The range of media chosen is shown to have satisfactory nutritive properties for at least the microorganisms shown in *Quality Control Test Strain Organisms* (below). The nutritive properties of each new batch of medium are verified for the appropriate microorganisms in the list. When testing for *Mycoplasmas* include in each test at least two known *Mycoplasma* species or strains (listed in *Quality Control Test Strain Organisms*) as positive controls, one of which should be a dextrose fermenter (i.e., *M. pneumoniae* or equivalent species and strain) and one of which should be an arginine hydrolyzer (i.e., *M. orale* or equivalent species and strain). Only when testing insect cell lines should one include a *Spiroplasma* control strain (e.g., *S. citri* ATCC 29747, *S. melliferum* ATCC 29416, or equivalent species and strains). Additionally, these strains may be a little more fastidious in their nutritional requirements. They require lower incubation temperatures (as do insect cell lines).

Quality Control Test Strain Organisms

Positive control cultures should be not more than 15 passages from isolation. *Mycoplasma* species or strains suitable for use are listed below:

- *Acholeplasma laidlawii* (vaccines and/or cell-derived materials/cultures for human and veterinary use when an antibiotic has been used during production)
- *M. gallisepticum* (when avian material has been used during production or when the vaccine or cell culture is intended for use in poultry)
- *M. hyorhinis* (nonavian veterinary vaccines or cell cultures)
- *M. orale* (vaccines for human and veterinary use)
- *M. pneumoniae* (vaccines or cell banks for human use) or another suitable species of D-glucose fermenter such as *M. fermentans*
- *M. synoviae* (when avian material has been used during production or when the vaccine or cell bank is intended for use in poultry)

The test strains may be field isolates that have undergone a limited number of subcultures (not more than 15), are stored frozen (-20° or lower) or freeze-dried, and are identified as being of the required species by comparison with type cultures, for example, those shown in Table 1.

Incubation Conditions

Incubate liquid media in tightly stoppered containers at $36 \pm 1^{\circ}$. Incubate solid media in microaerophilic conditions (hydrogen atmosphere containing $< 0.5\%$ oxygen and/or nitrogen containing $5\%–10\%$ carbon dioxide in nitrogen). Sufficient humidity should be available to prevent desiccation of the agar surface at $36 \pm 1^{\circ}$.

Nutritive Properties

Carry out the test for nutritive properties for each new batch of medium. Inoculate the chosen media with the appropriate test microorganisms; use not more than 100 cfu per plate containing at least 9 mL of solid media and per 100-mL container of liquid medium; use a separate plate and container for each species of microorganism. Incubate the media and make subcultures from 0.2 mL of liquid medium to solid medium at the specified intervals (see below under *Test for Mycoplasma in the Test Article/Material*). The solid medium complies with the test if a count within a 0.5-log unit range of the inoculate amount is found for each test microorganism. The liquid medium complies with the test if growth is found on agar plates subcultured from the broth, for at least 1 subculture for each test microorganism. The use of a microscope at $100\times$ or greater may be helpful.

Inhibitory Substances

The test for inhibitory substances is carried out once for a given product and is repeated whenever there is a change in production method that may affect the detection of Mycoplasma. To demonstrate absence of inhibitory substances, carry out the test for nutritive properties in the presence and absence of the test article/material. If growth of a test microorganism occurs more than 1 subculture sooner in the absence of the test article/material than in its presence, inhibitory substances are present. The same is true if plates directly inoculated with the test article/material are

not within a 0.5-log unit range of the number of colonies of those inoculated without the test article/material. In both cases, inhibitory substances must be neutralized or their effect otherwise countered, by an appropriate method, for example, by passage in substrates not containing inhibitors or dilution in a larger volume of medium, before the test. If dilution is used, larger medium volumes may be used or the inoculum's volume may be divided among several 100-mL flasks. The effectiveness of the neutralization or other process is checked by repeating the test for inhibitory substances after neutralization.

Test for Mycoplasma in the Test Article/Material

Inoculate no less than 10 mL of the test article/material per 100 mL of each liquid medium. If a significant pH change occurs upon the addition of the test article/material, the liquid medium is restored to its original pH value by the addition of a sterile solution of either sodium hydroxide or hydrochloric acid. Inoculate 0.2 mL of the test article/material on each plate of each solid medium. Incubate liquid media for 20–21 days. Incubate solid media for not less than 14 days, except those plates corresponding to the 20–21 day subculture, which are incubated for 7 days. Concurrently, incubate an uninoculated 100-mL portion of each liquid medium and agar plate, as a negative control. On days 2–4 after inoculation, subculture each liquid medium by inoculating 0.2 mL on at least 1 plate of each solid medium. Repeat the procedure between days 6 and 8, again between days 13 and 15, and again between days 19 and 21 of the test. Observe the liquid media every 2 or 3 days and if a color change occurs, subculture. If a liquid medium shows bacterial or fungal contamination, the test is invalid. The test is valid if at least 1 plate per medium and per inoculation day can be read. Include in the test positive controls prepared by inoculation of not more than 100 cfu of at least 1 test microorganism on agar medium or into broth medium. Where the test for Mycoplasmas is carried out regularly, it is recommended to use the test microorganisms in regular rotation. The test microorganisms used are those listed under *Choice of Media*. Incubate broths and plates in a humidified atmosphere with microaerophilic conditions ($5\%–10\% \text{ CO}_2$).

Interpretation of Results

At the end of the prescribed incubation period, examine all inoculated solid media for the presence of Mycoplasma colonies. The product complies with the test if growth of typical Mycoplasma colonies has not occurred. The product does not comply with the test if growth of typical Mycoplasma colonies has occurred on any of the solid media. The test is invalid if 1 or more of the positive controls do not show growth of Mycoplasmas on at least 1 subculture plate. The test is invalid if 1 or more of the negative controls show growth of Mycoplasmas. If suspect colonies are observed, use a suitable validated method to determine whether they are due to Mycoplasmas.

Table 1. Type Cultures for Identifying Field Isolates Used as Test Strains

Test Organism	NCTC Number	CIP Number	ATCC Number
<i>A. laidlawii</i>	NCTC 10116	CIP 75.27	ATCC 23206
<i>M. gallisepticum</i>	NCTC 10115	CIP 104967	ATCC 19610
<i>M. fermentans</i>	NCTC 10117	CIP 105680	ATCC 19989
<i>M. hyorhinis</i>	NCTC 10130	CIP 104968	ATCC 17981
<i>M. orale</i>	NCTC 10112	CIP 104969	ATCC 23714
<i>M. pneumoniae</i>	NCTC 10119	CIP 103766	ATCC 15531
<i>M. synoviae</i>	NCTC 10124	CIP 104970	ATCC 25204

Recommended Solutions and Media for the Culture Method

NOTE—This section is provided for information.

SOLUTIONS

Beef Heart Infusion Broth

Beef heart (for preparation of the infusion)	500 g
Peptone	10 g
Sodium chloride	5 g
Distilled water	to 1000 mL

Essential Vitamins

Biotin	100 mg
Calcium pantothenate	100 mg
Choline chloride	100 mg
Folic acid	100 mg
<i>D</i> -Inositol	200 mg
Nicotinamide	100 mg
Pyridoxal hydrochloride	100 mg
Riboflavine	10 mg
Thiamine hydrochloride	100 mg
Distilled water	to 1000 mL

Agar, Purified

A highly refined agar for use in microbiology and immunology, prepared by an ion-exchange procedure that results in a product having superior purity, clarity, and gel strength. It contains the following ingredients:	
Water	12.2%
Ash	1.5%
Acid-insoluble ash	0.2%
Chlorine	0
Phosphate (calculated as P ₂ O ₅)	0.3%
Total nitrogen	0.3%
Copper	8 ppm
Iron	170 ppm
Calcium	0.28%
Magnesium	0.32%

Hanks' Balanced Salt Solution (modified)

Sodium chloride	6.4 g
Potassium chloride	0.32 g
Magnesium sulphate heptahydrate	0.08 g
Magnesium chloride hexahydrate	0.08 g
Calcium chloride, anhydrous	0.112 g
Disodium hydrogen phosphate dihydrate	0.0596 g
Potassium dihydrogen phosphate, anhydrous	0.048 g
Distilled water	to 800 mL

Brain Heart Infusion

Calf-brain infusion	200 g
Beef-heart infusion	250 g
Proteose peptone	10 g
Glucose monohydrate	2 g
Sodium chloride	5 g
Disodium hydrogen phosphate, anhydrous	2.5 g
Distilled water	to 1000 mL

PPLO Broth

Beef-heart infusion	50 g
Peptone	10 g
Sodium chloride	5 g
Distilled water	to 1000 mL

MEDIA

The following media are recommended. Other media may be used, provided they meet the criteria given in the sections *Choice of Culture Media*, *Incubation Conditions*, *Nutritive Properties*, and *Inhibitory Substances*.

Hayflick Media (Recommended for the general detection of Mycoplasmas)

Liquid Medium	
Beef heart infusion broth	90.0 mL
Horse serum (unheated)	20.0 mL
Yeast extract (250 g/L) (fresh yeast extract is recommended)	10.0 mL
Phenol red (0.6 g/L solution)	5.0 mL
Penicillin (20,000 IU/mL)	0.25 mL
Deoxyribonucleic acid (2 g/L solution)	1.2 mL
Adjust to a pH of 7.8	
Solid Medium	
Prepare as described above replacing beef heart infusion broth by beef heart infusion agar containing 15 g/L of agar.	

Frey Media (Recommended for the detection of *M. synoviae*)

Liquid Medium	
Beef heart infusion broth	90.0 mL
Essential vitamins	0.025 mL
Glucose monohydrate (500 g/L solution)	2.0 mL
Swine serum (inactivated at 56° for 30 min)	12.0 mL
β -Nicotinamide adenine dinucleotide (10 g/L solution)	1.0 mL
Cysteine hydrochloride (10 g/L solution)	1.0 mL
Phenol red (0.6 g/L solution)	5.0 mL
Penicillin (20,000 IU/mL)	0.25 mL
Mix the solutions of β -nicotinamide adenine dinucleotide and cysteine hydrochloride and after 10 min add to the other ingredients. Adjust to a pH of 7.8.	
Solid Medium	
Beef heart infusion broth	90.0 mL
Agar, purified	1.4 g
Adjust to pH 7.8, sterilize by autoclaving then add:	
Essential vitamins	0.025 mL
Glucose monohydrate (500 g/L solution)	2.0 mL

Frey Media
(Recommended for the detection of *M. synoviae*) (Continued)

Swine serum (unheated)	12.0 mL
β -Nicotinamide adenine dinucleotide (10 g/L solution)	1.0 mL
Cysteine hydrochloride (10 g/L solution)	1.0 mL
Phenol red (0.6 g/L solution)	5.0 mL
Penicillin (20,000 IU/mL)	0.25 mL

Friis Media
(Recommended for the detection of nonavian Mycoplasmas)

Liquid Medium	
Hanks' balanced salt solution (modified)	800 mL
Distilled water	67 mL
Brain heart infusion	135 mL
PPLO Broth	248 mL
Yeast extract (170 g/L)	60 mL
Bacitracin	250 mg
Meticillin	250 mg
Phenol red (5 g/L)	4.5 mL
Horse serum	165 mL
Swine serum	165 mL
Adjust to a pH of 7.40–7.45	
Solid Medium	
Hanks' balanced salt solution (modified)	200 mL
DEAE-dextran	200 mg
Agar, purified	15.65 g
Mix well and sterilize by autoclaving. Cool to 100°. Add to 1740 mL of Liquid Medium as described above.	

INDICATOR CELL CULTURE METHOD

Cell cultures are stained with a fluorescent dye that binds to DNA. Mycoplasmas are detected by their characteristic particulate or filamentous pattern of fluorescence on the cell surface and, if contamination is heavy, in surrounding areas. Mitochondria in the cytoplasm may be stained but are readily distinguished from Mycoplasmas. For viral suspensions, if the interpretation of results is affected by marked cytopathic effects, neutralize the virus using a specific antiserum that has no inhibitory effects on Mycoplasmas, or use a cell culture substrate that does not allow growth of the virus. To demonstrate the absence of inhibitory effects of serum, carry out the positive control tests in the presence and absence of the antiserum.

Verification of the Substrate

Use Vero cells or equivalent cell culture (for example, the production cell line) that is equivalent in effectiveness for detecting Mycoplasmas. Test the effectiveness of the cells to be used by applying the procedure shown below and inoculating not more than 100 cfu or ccu microorganisms of suitable reference strains of *M. hyorhinis* and *M. orale*. The cells are suitable if both reference strains are detected. The indicator cells must be subcultured without an antibiotic before use in the test.

Test Method

NOTE—The following is provided for information.

SOLUTIONS

Phosphate Buffered Saline—

2.0 M Monobasic Potassium Phosphate—Dissolve 13.61 g of anhydrous monobasic potassium phosphate in 50 mL of water.

2.0 M Dibasic Potassium Phosphate—Dissolve 17.42 g of anhydrous dibasic potassium phosphate in 50 mL of water.

Phosphate Buffered Saline Solution (pH 7.4)—Combine 3.6 mL of 2.0 M Monobasic Potassium Phosphate, 16.4 mL of 2.0 M Dibasic Potassium Phosphate, 8 g of sodium chloride, and 1 L of water. Mix thoroughly. Adjust the pH if necessary.

Bisbenzimidazole Stock Solution—Dissolve 5 mg of bisbenzimidazole in water, and dilute with the same solvent to 100 mL. Store in the dark.

Bisbenzimidazole Working Solution—Immediately before use, dilute 100 μ L of Bisbenzimidazole Stock Solution with Phosphate Buffered Saline Solution (pH 7.4) to 100 mL.

Phosphate-Citrate Buffer Solution pH 5.5—Mix 56.85 mL of a 28.4-g/L solution of anhydrous disodium hydrogen phosphate and 43.15 mL of a 21-g/L solution of citric acid.

METHOD

- Seed the indicator cell culture at a suitable density (for example, 2×10^4 to 2×10^5 cells/mL, 4×10^3 to 2.5×10^4 cells/cm²) that will yield confluence after 3 days of growth. Inoculate 1 mL of the product to be examined into the cell culture vessel, and incubate at $36 \pm 1^\circ$.
- After at least 3 days of incubation, when the cells have grown to confluence, make a subculture on cover slips in suitable containers or on some other surface (for example, chambered slides) suitable for the test procedure. Seed the cells at low density so that they reach 50% confluence after 3–5 days of incubation. Complete confluence impairs visualization of Mycoplasmas after staining and must be avoided.
- Remove the medium and rinse the indicator cells with phosphate buffered saline, pH 7.4, then add a suitable fixing solution (a freshly prepared mixture of 1 volume of acetic acid, glacial, TS and 3 volumes of methanol, is suitable when bisbenzimidazole is used for staining).
- Remove the fixing solution and wash the cells with sterile Purified Water. Dry the slides completely if they are to be stained more than 1 hour later (particular care is needed for staining of slides after drying owing to artifacts that may be produced).
- Add a suitable DNA stain and allow standing for a suitable time (bisbenzimidazole working solution and a standing time of 10 minutes are suitable).
- Remove the stain and rinse the monolayer with Purified Water.
- Mount each coverslip, where applicable (a mixture of equal volumes of glycerol and Phosphate-Citrate Buffer Solution pH 5.5 is suitable for mounting). Examine by fluorescence (for bisbenzimidazole stain a 330 nm/380 nm excitation filter and an LP 440 nm barrier filter are suitable) at 400 \times magnification or greater.
- Compare the microscopic appearance of the test cultures with that of the negative and positive controls, examining for extranuclear fluorescence. Mycoplasmas produce pinpoints or filaments over the indicator cell cytoplasm. They may also produce pinpoints and filaments in the intercellular spaces. Multiple microscopic fields are examined according to the protocol established during validation.

Interpretation of Results

The product to be examined complies with the test if fluorescence typical of Mycoplasmas is not present. The test is invalid if the positive controls do not show fluorescence typical of Mycoplasmas. The test is invalid if the negative controls show fluorescence typical of Mycoplasmas.

<71> STERILITY TESTS

♦Portions of this general chapter have been harmonized with the corresponding texts of the European Pharmacopeia and/or the Japanese Pharmacopeia. Those portions that are not harmonized are marked with symbols (♦♦) to specify this fact.♦

These Pharmacopeial procedures are not by themselves designed to ensure that a batch of product is sterile or has been sterilized. This is accomplished primarily by validation of the sterilization process or of the aseptic processing procedures.

The test is applied to substances, preparations, or articles which, according to the Pharmacopeia, are required to be sterile. However, a satisfactory result only indicates that no contaminating microorganism has been found in the sample examined under the conditions of the test.

PRECAUTIONS AGAINST MICROBIAL CONTAMINATION

The test for sterility is carried out under aseptic conditions. In order to achieve such conditions, the test environment has to be adapted to the way in which the sterility test is performed. The precautions taken to avoid contamination are such that they do not affect any microorganisms that are to be revealed in the test. The working conditions in which the tests are performed are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.

CULTURE MEDIA AND INCUBATION TEMPERATURES

Media for the test may be prepared as described below or equivalent commercial media may be used provided that they comply with the requirements of the *Growth Promotion Test of Aerobes, Anaerobes, and Fungi*.

The following culture media have been found to be suitable for the test for sterility. *Fluid Thioglycollate Medium* is primarily intended for the culture of anaerobic bacteria. However, it will also detect aerobic bacteria. *Soybean–Casein Digest Medium* is suitable for the culture of both fungi and aerobic bacteria.

Fluid Thioglycollate Medium

L-Cystine	0.5 g
Sodium Chloride	2.5 g
Dextrose Monohydrate/Anhydrous	5.5/5.0 g
Agar	0.75 g
Yeast Extract (water-soluble)	5.0 g
Pancreatic Digest of Casein	15.0 g
Sodium Thioglycollate	0.5 g

Fluid Thioglycollate Medium (Continued)

or Thioglycolic Acid	0.3 mL
Resazurin Sodium Solution (1 in 1000), freshly prepared	1.0 mL
Purified Water	1000 mL

pH after sterilization: 7.1±0.2.

Mix the L-cystine, agar, sodium chloride, dextrose, yeast extract, and pancreatic digest of casein with the purified water, and heat until solution is effected. Dissolve the sodium thioglycollate or thioglycolic acid in the solution and, if necessary, add 1 N sodium hydroxide so that, after sterilization, the solution will have a pH of 7.1 ± 0.2. If filtration is necessary, heat the solution again without boiling, and filter while hot through moistened filter paper. Add the resazurin sodium solution, mix, and place the medium in suitable vessels that provide a ratio of surface to depth of medium such that not more than the upper half of the medium has undergone a color change indicative of oxygen uptake at the end of the incubation period. Sterilize using a validated process. If the medium is stored, store at a temperature between 2° and 25° in a sterile, airtight container. If more than the upper one-third of the medium has acquired a pink color, the medium may be restored once by heating the containers in a water-bath or in free-flowing steam until the pink color disappears and by cooling quickly, taking care to prevent the introduction of nonsterile air into the container. Do not use the medium for a longer storage period than has been validated.

Fluid Thioglycollate Medium is to be incubated at 30°–35°. For products containing a mercurial preservative that cannot be tested by the membrane filtration method, *Fluid Thioglycollate Medium* incubated at 20°–25° may be used instead of *Soybean–Casein Digest Medium* provided that it has been validated as described in *Growth Promotion Test of Aerobes, Anaerobes, and Fungi*. Where prescribed or justified and authorized, the following alternative thioglycollate medium might be used. Prepare a mixture having the same composition as that of the *Fluid Thioglycollate Medium*, but omitting the agar and the resazurin sodium solution. Sterilize as directed above. The pH after sterilization is 7.1 ± 0.2. Heat in a water bath prior to use and incubate at 30°–35° under anaerobic conditions.

Soybean–Casein Digest Medium

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean Meal	3.0 g
Sodium Chloride	5.0 g
Dibasic Potassium Phosphate	2.5 g
Dextrose Monohydrate/Anhydrous	2.5/2.3 g
Purified Water	1000 mL

pH after sterilization: 7.3±0.2.

Dissolve the solids in the Purified Water, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with 1 N sodium hydroxide so that, after sterilization, it will have a pH of 7.3 ± 0.2. Filter, if necessary to clarify, dispense into suitable containers, and sterilize using a validated procedure. Store at a temperature between 2° and 25° in a sterile well-closed container, unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated.

Soybean–Casein Digest Medium is to be incubated at 22.5 ± 2.5°.

♦Media for Penicillins or Cephalosporins

Where sterility test media are to be used in the *Direct Inoculation of the Culture Medium* method under *Test for Sterility of the Product to be Examined*, modify the preparation

of *Fluid Thioglycollate Medium* and the *Soybean–Casein Digest Medium* as follows. To the containers of each medium, transfer aseptically a quantity of β -lactamase sufficient to inactivate the amount of antibiotic in the specimen under test. Determine the quantity of β -lactamase required to inactivate the antibiotic by using a β -lactamase preparation that has been assayed previously for its penicillin- or cephalosporin-inactivating power. [NOTE—Supplemented β -lactamase media can also be used in the membrane filtration test.]

Alternatively (in an area completely separate from that used for sterility testing), confirm that an appropriate amount of β -lactamase is incorporated into the medium, following either method under *Method Suitability Test*, using less than 100 colony-forming units (cfu) of *Staphylococcus aureus* (see *Table 1*) as the challenge. Typical microbial growth of the inoculated culture must be observed as a confirmation that the β -lactamase concentration is appropriate.♦

Table 1. Strains of the Test Microorganisms Suitable for Use in the Growth Promotion Test and the Method Suitability Test

Aerobic bacteria	
<i>Staphylococcus aureus</i>	ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518, NBRC 13276
<i>Bacillus subtilis</i>	ATCC 6633, CIP 52.62, NCIMB 8054, NBRC 3134
<i>Pseudomonas aeruginosa</i> *1,♦	ATCC 9027, NCIMB 8626, CIP 82.118, NBRC 13275
Anaerobic bacterium	
<i>Clostridium sporogenes</i> *2,♦	ATCC 19404, CIP 79.3, NCTC 532 or ATCC 11437, NBRC 14293
Fungi	
<i>Candida albicans</i>	ATCC 10231, IP 48.72, NCPF 3179, NBRC 1594
<i>Aspergillus brasiliensis</i> (<i>Aspergillus Niger</i>)	ATCC 16404, IP 1431.83, IMI 149007, NBRC 9455

*1 An alternative microorganism is *Kocuria rhizophila* (*Micrococcus luteus*) ATCC 9341.♦

*2 An alternative to *Clostridium sporogenes*, when a nonspore-forming microorganism is desired, is *Bacteroides vulgatus* (ATCC 8482).♦

The media used comply with the following tests, carried out before, or in parallel, with the test on the product to be examined.

Sterility

Incubate portions of the media for 14 days. No growth of microorganisms occurs.

Growth Promotion Test of Aerobes, Anaerobes, and Fungi

Test each lot of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Suitable strains of microorganisms are indicated in *Table 1*.

Inoculate portions of *Fluid Thioglycollate Medium* with a small number (not more than 100 cfu) of the following microorganisms, using a separate portion of medium for each of the following species of microorganism: *Clostridium sporogenes*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. ♦Inoculate portions of alternative thioglycollate medium with a small number (not more than 100 cfu) of *Clostridium sporogenes*.♦ Inoculate portions of *Soybean–Casein Digest Medium* with a small number (not more than 100

cfu) of the following microorganisms, using a separate portion of medium for each of the following species of microorganism: *Aspergillus brasiliensis*, *Bacillus subtilis*, and *Candida albicans*. Incubate for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot.

The media are suitable if a clearly visible growth of the microorganisms occurs.

♦DILUTING AND RINSING FLUIDS FOR MEMBRANE FILTRATION

Fluid A

PREPARATION

Dissolve 1 g of peptic digest of animal tissue in water to make 1 L, filter or centrifuge to clarify, if necessary, and adjust to a pH of 7.1 ± 0.2 . Dispense into containers, and sterilize using a validated process.

PREPARATION FOR PENICILLINS OR CEPHALOSPORINS

Aseptically add to the above *Preparation*, if necessary, a quantity of sterile β -lactamase sufficient to inactivate any residual antibiotic activity on the membranes after the solution of the test specimen has been filtered (see *Media for Penicillins or Cephalosporins*).

Fluid D

To each L of *Fluid A* add 1 mL of polysorbate 80, adjust to a pH of 7.1 ± 0.2 , dispense into containers, and sterilize using a validated process. Use this fluid for articles containing lecithin or oil, or for devices labeled as "sterile pathway."

Fluid K

Dissolve 5.0 g of peptic digest of animal tissue, 3.0 g of beef extract, and 10.0 g of polysorbate 80 in water to make 1 L. Adjust the pH to obtain, after sterilization, a pH of 6.9 ± 0.2 . Dispense into containers, and sterilize using a validated process.♦

METHOD SUITABILITY TEST

Carry out a test as described below under *Test for Sterility of the Product to be Examined* using exactly the same methods, except for the following modifications.

Membrane Filtration

After transferring the content of the container or containers to be tested to the membrane, add an inoculum of a small number of viable microorganisms (not more than 100 cfu) to the final portion of sterile diluent used to rinse the filter.

Direct Inoculation

After transferring the contents of the container or containers to be tested (for catgut and other surgical sutures for veterinary use: strands) to the culture medium, add an inoc-

ulum of a small number of viable microorganisms (not more than 100 cfu) to the medium.

In both cases use the same microorganisms as those described above under *Growth Promotion Test of Aerobes, Anaerobes, and Fungi*. Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

If clearly visible growth of microorganisms is obtained after the incubation, visually comparable to that in the control vessel without product, either the product possesses no antimicrobial activity under the conditions of the test or such activity has been satisfactorily eliminated. The test for sterility may then be carried out without further modification.

If clearly visible growth is not obtained in the presence of the product to be tested, visually comparable to that in the control vessels without product, the product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. Modify the conditions in order to eliminate the antimicrobial activity, and repeat the *Method Suitability Test*.

This method suitability is performed (a) when the test for sterility has to be carried out on a new product; and (b) whenever there is a change in the experimental conditions of the test. The method suitability may be performed simultaneously with the *Test for Sterility of the Product to be Examined*.

TEST FOR STERILITY OF THE PRODUCT TO BE EXAMINED

◆Number of Articles to Be Tested

Unless otherwise specified elsewhere in this chapter or in the individual monograph, test the number of articles specified in *Table 3*. If the contents of each article are of sufficient quantity (see *Table 2*), they may be divided so that equal appropriate portions are added to each of the specified media. [NOTE—Perform sterility testing employing two or more of the specified media.] If each article does not contain sufficient quantities for each medium, use twice the number of articles indicated in *Table 3*.◆

Table 2. Minimum Quantity to be Used for Each Medium

Quantity per Container	Minimum Quantity to be Used (unless otherwise justified and authorized)
<i>Liquids</i>	
Less than 1 mL	The whole contents of each container
1–40 mL	Half the contents of each container, but not less than 1 mL
Greater than 40 mL, and not greater than 100 mL	20 mL
Greater than 100 mL	10% of the contents of the container, but not less than 20 mL
<i>Antibiotic liquids</i>	1 mL
<i>Insoluble preparations, creams, and ointments to be suspended or emulsified</i>	Use the contents of each container to provide not less than 200 mg
<i>Solids</i>	
Less than 50 mg	The whole contents of each container
50 mg or more, but less than 300 mg	Half the contents of each container, but not less than 50 mg
300 mg–5 g	150 mg
Greater than 5 g	500 mg
Catgut and other surgical sutures for veterinary use	3 sections of a strand (each 30-cm long)

Table 2. Minimum Quantity to be Used for Each Medium (Continued)

Quantity per Container	Minimum Quantity to be Used (unless otherwise justified and authorized)
◆Surgical dressing/cotton/gauze (in packages)	100 mg per package
Sutures and other individually packaged single-use material	The whole device
Other medical devices	The whole device, cut into pieces or disassembled◆

Table 3. Minimum Number of Articles to be Tested in Relation to the Number of Articles in the Batch

Number of Items in the Batch*	Minimum Number of Items to be Tested for Each Medium (unless otherwise justified and authorized)**
<i>Parenteral preparations</i>	
Not more than 100 containers	10% or 4 containers, whichever is the greater
More than 100 but not more than 500 containers	10 containers
More than 500 containers	2% or 20 containers, whichever is less
◆For large-volume parenterals	2% or 10 containers, whichever is less
<i>Antibiotic solids</i>	
Pharmacy bulk packages (<5 g)	20 containers
Pharmacy bulk packages (≥5 g)	6 containers
Bulks and blends	See <i>Bulk solid products</i> ◆
<i>Ophthalmic and other noninjectable preparations</i>	
Not more than 200 containers	5% or 2 containers, whichever is the greater
More than 200 containers	10 containers
If the product is presented in the form of single-dose containers, apply the scheme shown above for preparations for parenteral use.	
Catgut and other surgical sutures for veterinary use	2% or 5 packages, whichever is the greater, up to a maximum total of 20 packages
◆Not more than 100 articles	10% or 4 articles, whichever is greater
More than 100, but not more than 500 articles	10 articles
More than 500 articles	2% or 20 articles, whichever is less◆
<i>Bulk solid products</i>	
Up to 4 containers	Each container
More than 4 containers, but not more than 50 containers	20% or 4 containers, whichever is greater
More than 50 containers	2% or 10 containers, whichever is greater

*If the batch size is unknown, use the maximum number of items prescribed.

**If the contents of one container are enough to inoculate the two media, this column gives the number of containers needed for both the media together.

The test may be carried out using the technique of *Membrane Filtration* or by *Direct Inoculation of the Culture Medium* with the product to be examined. Appropriate negative controls are included. The technique of membrane filtration

is used whenever the nature of the product permits; that is, for filterable aqueous preparations, for alcoholic or oily preparations, and for preparations miscible with, or soluble in, aqueous or oily solvents, provided these solvents do not have an antimicrobial effect in the conditions of the test.

Membrane Filtration

Use membrane filters having a nominal pore size not greater than 0.45 μm , in which the effectiveness to retain microorganisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily, and weakly alcoholic solutions; and cellulose acetate filters, for example, are used for strongly alcoholic solutions. Specially adapted filters may be needed for certain products (e.g., for antibiotics).

The technique described below assumes that membranes about 50 mm in diameter will be used. If filters of a different diameter are used, the volumes of the dilutions and the washings should be adjusted accordingly. The filtration apparatus and membrane are sterilized by appropriate means. The apparatus is designed so that the solution to be examined can be introduced and filtered under aseptic conditions: it permits the aseptic removal of the membrane for transfer to the medium, or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

AQUEOUS SOLUTIONS

If appropriate, transfer a small quantity of a suitable, sterile diluent such as ♦*Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*)♦ onto the membrane in the apparatus and filter. The diluent may contain suitable neutralizing substances and/or appropriate inactivating substances, for example, in the case of antibiotics.

Transfer the contents of the container or containers to be tested to the membrane or membranes, if necessary, after diluting to the volume used in the *Method Suitability Test* with the chosen sterile diluent, but using not less than the quantities of the product to be examined prescribed in *Tables 2* and *3*. Filter immediately. If the product has antimicrobial properties, wash the membrane not less than three times by filtering through it each time the volume of the chosen sterile diluent used in the *Method Suitability Test*. Do not exceed a washing cycle of five times 100 mL per filter, even if during method suitability it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. Transfer the whole membrane to the culture medium or cut it aseptically into two equal parts, and transfer one half to each of two suitable media. Use the same volume of each medium as in the *Method Suitability Test*. Alternatively, transfer the medium onto the membrane in the apparatus. Incubate the media for not less than 14 days.

SOLUBLE SOLIDS

Use for each medium not less than the quantity prescribed in *Tables 2* and *3* of the product dissolved in a suitable solvent, such as the solvent provided with the preparation, Sterile Water for Injection, sterile saline, or a suitable sterile solution such as ♦*Fluid A* (*Diluting and Rinsing Fluids for Membrane Filtration*)♦, and proceed with the test as described above for *Aqueous Solutions* using a membrane appropriate to the chosen solvent.

OILS and OILY SOLUTIONS

Use for each medium not less than the quantity of the product prescribed in *Tables 2* and *3*. Oils and oily solutions of sufficiently low viscosity may be filtered without dilution through a dry membrane. Viscous oils may be diluted as necessary with a suitable sterile diluent such as isopropyl

myristate shown not to have antimicrobial activity in the conditions of the test. Allow the oil to penetrate the membrane by its own weight, and then filter, applying the pressure or suction gradually. Wash the membrane at least three times by filtering through it each time about 100 mL of a suitable sterile solution such as ♦*Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*)♦ containing a suitable emulsifying agent at a concentration shown to be appropriate in the *Method Suitability Test*, for example polysorbate 80 at a concentration of 10 g per L ♦(*Fluid K*)♦. Transfer the membrane or membranes to the culture medium or media, or vice versa, as described above for *Aqueous Solutions*, and incubate at the same temperatures and for the same times.

OINTMENTS and CREAMS

Use for each medium not less than the quantities of the product prescribed in *Tables 2* and *3*. Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1% in isopropyl myristate as described above, by heating, if necessary, to not more than 40°. In exceptional cases it may be necessary to heat to not more than 44°. Filter as rapidly as possible, and proceed as described above for *Oils and Oily Solutions*.

♦PREFILLED SYRINGES

For prefilled syringes without attached sterile needles, expel the contents of each syringe into one or two separate membrane filter funnels or into separate pooling vessels prior to transfer. If a separate sterile needle is attached, directly expel the syringe contents as indicated above, and proceed as directed for *Aqueous Solutions*. Test the sterility of the needle, using *Direct Inoculation* under *Method Suitability Test*.

SOLIDS FOR INJECTION OTHER THAN ANTIBIOTICS

Constitute the test articles as directed on the label, and proceed as directed for *Aqueous Solutions* or *Oils and Oily Solutions*, whichever applies. [NOTE—If necessary, excess diluent can be added to aid in the constitution and filtration of the constituted test article.]

ANTIBIOTIC SOLIDS FOR INJECTION

Pharmacy Bulk Packages, <5 g—From each of 20 containers, aseptically transfer about 300 mg of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*), and mix; or constitute, as directed in the labeling, each of 20 containers and transfer a quantity of liquid or suspension, equivalent to about 300 mg of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A*, and mix. Proceed as directed for *Aqueous Solutions* or *Oils and Oily Solutions*, whichever applies.

Pharmacy Bulk Packages, ≥ 5 g—From each of 6 containers, aseptically transfer about 1 g of solids into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A*, and mix; or constitute, as directed in the labeling, each of 6 containers and transfer a quantity of liquid, equivalent to about 1 g of solids, into a sterile 500-mL conical flask, dissolve in about 200 mL of *Fluid A*, and mix. Proceed as directed for *Aqueous Solutions*.

ANTIBIOTIC SOLIDS, BULKS, and BLENDS

Aseptically remove a sufficient quantity of solids from the appropriate amount of containers (see *Table 2*), mix to obtain a composite, equivalent to about 6 g of solids, and

transfer to a sterile 500-mL conical flask. Dissolve in about 200 mL of *Fluid A*, and mix. Proceed as directed for *Aqueous Solutions*.

STERILE AEROSOL PRODUCTS

For fluid products in pressurized aerosol form, freeze the containers in an alcohol-dry ice mixture at least at -20° for about 1 hour. If feasible, allow the propellant to escape before aseptically opening the container, and transfer the contents to a sterile pooling vessel. Add 100 mL of *Fluid D* to the pooling vessel, and mix gently. Proceed as directed for *Aqueous Solutions* or *Oils and Oily Solutions*, whichever applies.

DEVICES WITH PATHWAYS LABELED STERILE

Aseptically pass not less than 10 pathway volumes of *Fluid D* through each device tested. Collect the fluids in an appropriate sterile vessel, and proceed as directed for *Aqueous Solutions* or *Oils and Oily Solutions*, whichever applies.

In the case of sterile, empty syringes, draw sterile diluent into the barrel through the sterile needle, if attached, or through a sterile needle attached for the purpose of the test, and express the contents into a sterile pooling vessel. Proceed as directed above.♦

Direct Inoculation of the Culture Medium

Transfer the quantity of the preparation to be examined prescribed in *Tables 2* and *3* directly into the culture medium so that the volume of the product is not more than 10% of the volume of the medium, unless otherwise prescribed.

If the product to be examined has antimicrobial activity, carry out the test after neutralizing this with a suitable neutralizing substance or by dilution in a sufficient quantity of culture medium. When it is necessary to use a large volume of the product, it may be preferable to use a concentrated culture medium prepared in such a way that it takes into account the subsequent dilution. Where appropriate, the concentrated medium may be added directly to the product in its container.

OILY LIQUIDS

Use media to which have been added a suitable emulsifying agent at a concentration shown to be appropriate in the *Method Suitability Test*, for example polysorbate 80 at a concentration of 10 g per L.

ointments and Creams

Prepare by diluting to about 1 in 10 by emulsifying with the chosen emulsifying agent in a suitable sterile diluent such as ♦*Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*).♦ Transfer the diluted product to a medium not containing an emulsifying agent.

Incubate the inoculated media for not less than 14 days. Observe the cultures several times during the incubation period. Shake cultures containing oily products gently each day. However, when *Fluid Thioglycollate Medium* is used for the detection of anaerobic microorganisms, keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

CATGUT and OTHER SURGICAL SUTURES FOR VETERINARIAN USE

Use for each medium not less than the quantities of the product prescribed in *Tables 2* and *3*. Open the sealed package using aseptic precautions, and remove three sections of the strand for each culture medium. Carry out the test on three sections, each 30-cm long, which have been cut off from the beginning, the center, and the end of the strand. Use whole strands from freshly opened cassette packs. Transfer each section of the strand to the selected medium. Use sufficient medium to cover adequately the material to be tested (20 mL to 150 mL).

♦SOLIDS

Transfer a quantity of the product in the form of a dry solid (or prepare a suspension of the product by adding sterile diluent to the immediate container), corresponding to not less than the quantity indicated in *Tables 2* and *3*. Transfer the material so obtained to 200 mL of *Fluid Thioglycollate Medium*, and mix. Similarly, transfer the same quantity to 200 mL of *Soybean-Casein Digest Medium*, and mix. Proceed as directed above.

PURIFIED COTTON, GAUZE, SURGICAL DRESSINGS, and RELATED ARTICLES

From each package of cotton, rolled gauze bandage, or large surgical dressings being tested, aseptically remove two or more portions of 100- to 500-mg each from the innermost part of the sample. From individually packaged, single-use materials, aseptically remove the entire article. Immerse the portions or article in each medium, and proceed as directed above.

STERILE DEVICES

Articles can be immersed intact or disassembled. To ensure that device pathways are also in contact with the media, immerse the appropriate number of units per medium in a volume of medium sufficient to immerse the device completely, and proceed as directed above. For extremely large devices, immerse those portions of the device that are to come into contact with the patient in a volume of medium sufficient to achieve complete immersion of those portions.

For catheters where the inside lumen and outside are required to be sterile, either cut them into pieces such that the medium is in contact with the entire lumen or fill the lumen with medium, and then immerse the intact unit.♦

OBSERVATION AND INTERPRETATION OF RESULTS

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 mL) of the medium to fresh vessels of the same medium, and then incubate the original and transfer vessels for not less than 4 days.

If no evidence of microbial growth is found, the product to be examined complies with the test for sterility. If evidence of microbial growth is found, the product to be examined does not comply with the test for sterility, unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product to be examined. The test

may be considered invalid only if one or more of the following conditions are fulfilled:

- The data of the microbiological monitoring of the sterility testing facility show a fault.
- A review of the testing procedure used during the test in question reveals a fault.
- Microbial growth is found in the negative controls.
- After determination of the identity of the microorganisms isolated from the test, the growth of this species (or these species) may be ascribed unequivocally to faults with respect to the material and/or the technique used in conducting the sterility test procedure.

If the test is declared to be invalid, it is repeated with the same number of units as in the original test. If no evidence of microbial growth is found in the repeat test, the product examined complies with the test for sterility. If microbial growth is found in the repeat test, the product examined does not comply with the test for sterility.

APPLICATION OF THE TEST TO PARENTERAL PREPARATIONS, OPHTHALMIC, AND OTHER NONINJECTABLE PREPARATIONS REQUIRED TO COMPLY WITH THE TEST FOR STERILITY

When using the technique of membrane filtration, use, whenever possible, the whole contents of the container, but not less than the quantities indicated in *Table 2*, diluting where necessary to about 100 mL with a suitable sterile solution, such as ♦*Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration*).♦

When using the technique of direct inoculation of media, use the quantities shown in *Table 2*, unless otherwise justified and authorized. The tests for bacterial and fungal sterility are carried out on the same sample of the product to be examined. When the volume or the quantity in a single container is insufficient to carry out the tests, the contents of two or more containers are used to inoculate the different media.

MINIMUM NUMBER OF ITEMS TO BE TESTED

The minimum number of items to be tested in relation to the size of the batch is given in *Table 3*.

Biological Tests and Assays

(81) ANTIBIOTICS—MICROBIAL ASSAYS

Introduction and General Information

The activity (potency) of antibiotics can be demonstrated by their inhibitory effect on microorganisms under suitable conditions. A reduction in antimicrobial activity may not be adequately demonstrated by chemical methods. This chapter summarizes procedures for the antibiotics recognized in the *United States Pharmacopeia (USP)* for which the microbiological assay is the standard analytical method.

Two general techniques are employed: the cylinder-plate (or plate) assay and the turbidimetric (or tube) assay. *Table 7* lists all the antibiotics that contain microbial assays and specifies the type of assay (cylinder-plate or turbidimetric).

Table 1

Antibiotic	Type of Assay
Amphotericin B	Cylinder-plate
Bacitracin	Cylinder-plate
Bleomycin	Cylinder-plate
Capreomycin	Turbidimetric
Carbenicillin	Cylinder-plate
Chloramphenicol	Turbidimetric
Chlortetracycline	Turbidimetric
Cloxacillin	Cylinder-plate
Colistimethate	Cylinder-plate
Colistin	Cylinder-plate
Dihydrostreptomycin	Cylinder-plate
	Turbidimetric
Erythromycin	Cylinder-plate
Gentamicin	Cylinder-plate
Gramicidin	Turbidimetric
Nafcillin	Cylinder-plate
Natamycin	Cylinder-plate
Neomycin	Cylinder-plate
	Turbidimetric
Novobiocin	Cylinder-plate
Nystatin	Cylinder-plate
Oxytetracycline	Turbidimetric
Paromomycin	Cylinder-plate
Penicillin G	Cylinder-plate
Polymyxin B	Cylinder-plate
Sisomicin	Cylinder-plate
Tetracycline	Turbidimetric
Thiostrepton	Turbidimetric
Troleandomycin	Turbidimetric
Tylosin	Turbidimetric
Vancomycin	Cylinder-plate

[NOTE—Perform all procedures under conditions designed to avoid extrinsic microbial contamination. Take adequate safety precautions while performing these assays because of possible allergies to drugs and because live cultures of organisms are used in the procedures.]

Cylinder-plate assay: The cylinder-plate assay depends on diffusion of the antibiotic from a vertical cylinder through a solidified agar layer in a Petri dish or plate. The growth of the specific microorganisms inoculated into the agar is prevented in a circular area or *zone* around the cylinder containing the solution of the antibiotic.

Turbidimetric assay: The turbidimetric assay depends on the inhibition of growth of a microorganism in a uniform solution of the antibiotic in a fluid medium that is favorable to the growth of the microorganism in the absence of the antibiotic.

Units and Reference Standards: The potency of antibiotics is designated in either units (U) or µg of activity. In each case the unit or µg of antibiotic activity was originally established against a United States Federal Master Standard for that antibiotic. The corresponding USP Reference Standard is calibrated in terms of the master standard.

Originally, an antibiotic selected as a reference standard was thought to consist entirely of a single chemical entity and was therefore assigned a potency of 1000 µg/mg. In several such instances, as the manufacturing and purification methods for particular antibiotics became more advanced, antibiotics containing more than 1000 µg of activity/mg became possible. Such antibiotics had an activity equivalent to

a given number of μg of the original reference standard. In most instances, however, the μg of activity is exactly equivalent numerically to the μg (weight) of the pure substance. In some cases, such as those listed below, the μg of activity defined in terms of the original master standard is equal to a unit:

1. Where an antibiotic exists as the free base and in salt form and the μg of activity has been defined in terms of one of these forms
2. Where the antibiotic substance consists of a number of components that are chemically similar but differ in antibiotic activity
3. Where the potencies of a family of antibiotics are expressed in terms of a reference standard consisting of a single member which, however, might itself be heterogeneous

Do not assume that the μg of activity corresponds to the μg (weight) of the antibiotic substance.

Apparatus: Labware used for the storage and transfer of test dilutions and microorganisms must be sterile and free of interfering residues (see *Cleaning Glass Apparatus* (1051)). Use a validated sterilization method, such as dry heat, steam, or radiation; or use sterile, disposable labware.

Temperature control: Thermostatic control is required in several stages of a microbial assay: when culturing a microorganism and preparing its inoculum, and during incubation in plate and tube assays. Refer to specific temperature requirements below for each type of assay.

Test organisms: The test organism for each antibiotic is listed in *Table 3* for the cylinder-plate assay and *Table 8* for the turbidimetric assay. The test organisms are specified by the American Type Culture Collection (ATCC) number.

In order to ensure acceptable performance of test organisms, store and maintain them properly. Establish the specific storage conditions during method validation or verification. Discard cultures if a change in the organism's characteristics is observed.

Prolonged storage: For prolonged storage, maintain test organisms in a suitable storage solution such as 50% fetal calf serum in broth, 10%–15% glycerol in tryptic soy broth, defibrinated sheep blood, or skim milk. Prolonged-storage cultures are best stored in the freeze-dried state; temperatures of -60° or below are preferred; temperatures below -20° are acceptable.

Primary cultures: Prepare primary cultures by transferring test organisms from prolonged-storage vials onto appropriate media, and incubate under appropriate growth conditions. Store primary cultures at the appropriate temperature, usually 2° – 8° , and discard after three weeks. A single primary culture can be used to prepare working cultures only for as many as seven days.

Working cultures: Prepare working cultures by transferring the primary culture onto appropriate solid media to obtain isolated colonies. Incubate working cultures under appropriate conditions to obtain satisfactory growth for preparation of test inocula. Prepare fresh working cultures for each test day.

Uncharacteristic growth or performance of a test organism: Use new stock cultures, primary cultures, or working cultures when a test organism shows uncharacteristic growth or performance.

Assay designs: Suitable experimental designs are key to increasing precision and minimizing bias. Control of the incubation parameters, temperature distribution and time, is critical for minimizing bias; it can be accomplished by staging the plates and racks as described for each assay.

Cylinder-plate assay: The comparisons are restricted to relationships between zone diameter measurements within plates, excluding the variation between plates. Individual plate responses are normalized on the basis of the relative zone size of the standard compared to the mean zone size of the standard across all plates.

Turbidimetric assay: To avoid systematic bias, place replicate tubes randomly in separate racks so that each rack contains one complete set of treatments. The purpose of this configuration is to minimize the influence of temperature distribution on the replicate samples. The turbidimetric assay, because of the configuration of the samples in test tube racks, is sensitive to slight variations in temperature. The influence of temperature variation can also be decreased by ensuring proper airflow or heat convection during incubation. At least three tubes for each sample and standard concentration (one complete set of samples) should be placed in a single rack. The comparisons are restricted to relationships between the observed turbidities within racks.

Potency considerations: Within the restrictions listed above, the recommended assay design employs a five-concentration standard curve and a single concentration of each sample preparation.

For the cylinder-plate assay, each plate includes only two treatments, the reference treatment (median level standard, i.e., S_3) and one of the other four concentrations of the standard (S_1 , S_2 , S_4 , and S_5) or the sample (U_3). The concentration of the sample is an estimate based on the target concentration. The sample should be diluted to give a nominal concentration that is estimated to be equivalent to the median reference concentration (S_3) of the standard. The purpose of diluting to the median reference concentration is to ensure that the sample result will fall within the linear portion of the standard curve. The test determines the relative potency of U_3 against the standard curve. The sample (U_3) should have a relative potency of about 100%. The final potency of the sample is obtained by multiplying the U_3 result by the dilution factor.

An assay should be considered preliminary if the computed potency value of the sample is less than 80% or more than 125%. In this case, the results suggest that the sample concentration assumed during preparation of the sample stock solution was not correct. In such a case, one can adjust the assumed potency of the sample on the basis of the preliminary potency value and repeat the assay. Otherwise, the potency will be derived from a portion of the curve where the standard and sample responses will likely not be parallel.

Microbial determinations of potency are subject to inter-assay as well as intra-assay variables; therefore two or more independent assays are required for a reliable estimate of the potency of a given sample. Starting with separately prepared stock solutions and test dilutions of both the standard and the sample, perform additional assays of a given sample on a different day. The mean potency should include the results from all the valid independent assays. The number of assays required in order to achieve a reliable estimate of potency depends on the variability of the assay and the required maximum uncertainty for the potency estimate. The latter is assessed by the width of the confidence interval (refer to *Calculations, Confidence limits and combinations of assay calculations*). The combined result of a series of smaller, independent assays spread over a number of days is a more reliable estimate of potency than one from a single large assay with the same total number of plates or tubes. Note that additional assays or lower variability allows the product to meet tighter specification ranges. Reducing assay variability achieves the required confidence limit with fewer assays.

Cylinder-Plate Method

Temperature control: Use appropriately qualified and calibrated equipment to obtain the temperature ranges specified in *Table 3*.

Apparatus

Plates: Glass or disposable plastic Petri dishes (approximately 20 × 100 mm or other appropriate dimensions) with lids

Cylinders: Stainless steel or porcelain cylinders; 8 ± 0.1-mm o.d.; 6 ± 0.1-mm i.d.; 10 ± 0.1-mm high. [NOTE—Carefully clean cylinders to remove all residues; occasional cleaning in an acid bath, e.g., with about 2 N nitric acid or with chromic acid (see *Cleaning Glass Apparatus* (1051)) is required.]

Standard solutions: To prepare a stock solution, dissolve a suitable quantity of the USP Reference Standard of a given antibiotic, or the entire contents of a vial of USP Reference Standard, where appropriate, in the solvent specified in *Table 2*; and dilute to the specified concentration. Store at 2°–8°, and use within the period indicated. On the day of the assay, prepare from the stock solution five or more test dilutions, in which the successive solutions increase stepwise in concentration, usually in the ratio of 1:1.25. Use the final diluent specified such that the median has the concentration suggested in *Table 2*.

Sample solutions: Assign an assumed potency per unit weight or volume to the sample. On the day of the assay prepare a stock solution in the same manner specified for the USP Reference Standard (*Table 2*). Dilute the sample

stock solution in the specified final diluent to obtain a nominal concentration equal to the median concentration of the standard (S_3).

Inocula: Suspend the test organism from a freshly grown slant or culture in 3 mL of sterile saline TS. Glass beads can be used to facilitate the suspension. Spread the saline suspension onto the surface of two or more agar plates (covering the entire surface) or onto the surface of a Roux bottle containing 250 mL of the specified medium (see *Table 3*).

Incubate for the specified time and at the temperature as specified in *Table 3*, or until growth is apparent.

After incubation, harvest the organism from the plates or Roux bottle with approximately 50 mL of sterile saline TS (except use *Medium 34* for bleomycin; see the section *Media and Solutions*), using a sterile bent glass rod or sterile glass beads. Pipet the suspension into a sterile glass container. This is the harvest suspension.

Dilute an appropriate amount of the harvest suspension with sterile saline TS. Using the UV-visible spectrophotometer, measure % transmittance at 580 nm. The target value is approximately 25% transmittance at 580 nm. This value is used to standardize the harvest suspension volume added to the seed layer agar.

Starting with the suggested volumes indicated in *Table 3*, determine during method verification the proportions of

Table 2

Antibiotic	Stock Solution					Test Dilution	
	Initial Solvent	Initial Concentration	Further Diluent	Final Concentration	Use Within	Final Diluent	Median Concentration (S_3) ^{a,b}
Amphotericin B ^{c,d}	Dimethyl sulfoxide	—	—	1 mg/mL	Same day	B.10 ^e	1 µg/mL
Bacitracin ^f	0.01 N hydrochloric acid	—	—	100 U/mL	Same day	B.1 ^e	1 U/mL
Bleomycin	B.16 ^e	—	—	2 U/mL	14 days	B.16 ^e	0.04 U/mL
Carbenicillin	B.1 ^e	—	—	1 mg/mL	14 days	B.1 ^e	20 µg/mL
Cloxacillin	B.1 ^e	—	—	1 mg/mL	7 days	B.1 ^e	5 µg/mL
Colistemetate ^c	Water	10 mg/mL	B.6 ^e	1 mg/mL	Same day	B.6 ^e	1 µg/mL
Colistin	Water	10 mg/mL	B.6 ^e	1 mg/mL	14 days	B.6 ^e	1 µg/mL
Dihydrostreptomycin ^g	B.3 ^e	—	—	1 mg/mL	30 days	B.3 ^e	1 µg/mL
Erythromycin	Methanol	10 mg/mL	B.3 ^e	1 mg/mL	14 days	B.3 ^e	1 µg/mL
Gentamicin	B.3 ^e	—	—	1 mg/mL	30 days	B.3 ^e	0.1 µg/mL
Nafcillin	B.1 ^e	—	—	1 mg/mL	2 days	B.1 ^e	2 µg/mL
Natamycin	Dimethyl sulfoxide	—	—	1 mg/mL	Same day	B.10 ^e	5 µg/mL
Neomycin ^g	B.3 ^e	—	—	1 mg/mL	14 days	B.3 ^e	1 µg/mL
Novobiocin	alcohol	10 mg/mL	B.3 ^e	1 mg/mL	5 days	B.6 ^e	0.5 µg/mL
Nystatin ^{c, h}	Dimethylformamide	—	—	1000 U/mL	Same day	B.6 ^e	20 U/mL
Paromomycin	B.3 ^e	—	—	1 mg/mL	21 days	B.3 ^e	1 µg/mL
Penicillin G	B.1 ^e	—	—	1000 U/mL	4 days	B.1 ^e	1 U/mL
Polymyxin B ⁱ	Water	—	B.6 ^e	10,000 U/mL	14 days	B.6 ^e	10 U/mL
Sisomicin	B.3 ^e	—	—	1 mg/mL	14 days	B.3 ^e	0.1 µg/mL
Vancomycin	Water	—	—	1 mg/mL	7 days	B.4 ^e	10 µg/mL

^aIt is acceptable to adjust the median concentration to optimize zone sizes if the data remain in the linear range.

^bµg in this column refers to µg of activity.

^cPrepare the USP Reference Standard and sample test dilutions simultaneously.

^dFurther dilute the stock solution with dimethyl sulfoxide to give concentrations of 12.8, 16, 20, 25, and 31.2 µg/mL before making the test dilutions. The test dilution of the sample should contain the same amount of dimethyl sulfoxide as the test dilutions of the USP Reference Standard.

^eThe letter B refers to buffer. See *Media and Solutions, Buffers* for a description of each buffer listed in this table.

^fEach of the standard test dilutions should contain the same amount of hydrochloric acid as the test dilution of the sample.

^gThe turbidimetric assay can be used as an alternative procedure.

^hFurther dilute the stock solution with dimethylformamide to give concentrations of 256, 320, 400, 500, and 624 U/mL before making the test dilutions. Prepare the standard test dilutions simultaneously with test dilutions of the sample to be tested. The test dilution of the sample should contain the same amount of dimethylformamide as the test dilutions of the standard. Use low-actinic glassware.

ⁱPrepare the stock solution by adding 2 mL of water for each 5 mg of the USP Reference Standard.

Table 3

Antibiotic	Test Organism	ATCC ^a Number	Incubation Conditions			Suggested Inoculum Composition	
			Medium ^b	Temperature (°)	Time	Medium ^b	Amount (mL/100 mL)
Amphotericin B	<i>Saccharomyces cerevisiae</i>	9763	19	29–31	48 h	19	1.0
Bacitracin	<i>Micrococcus luteus</i>	10240	1	32–35	24 h	1	0.3
Bleomycin	<i>Mycobacterium smegmatis</i>	607	36	36–37.5	48 h	35	1.0
Carbenicillin ^c	<i>Pseudomonas aeruginosa</i>	25619	1	36–37.5	24 h	10	0.5
Cloxacillin	<i>Staphylococcus aureus</i>	29737	1	32–35	24 h	1	0.1
Colistimethate	<i>Bordetella bronchiseptica</i>	4617	1	32–35	24 h	10	0.1
Colistin	<i>Bordetella bronchiseptica</i>	4617	1	32–35	24 h	10	0.1
Dihydrostreptomycin	<i>Bacillus subtilis</i>	6633	32	32–35	5 days	5	As required
Erythromycin	<i>Micrococcus luteus</i>	9341	1	32–35	24 h	11	1.5
Gentamicin	<i>Staphylococcus epidermidis</i>	12228	1	32–35	24 h	11	0.03
Nafcillin	<i>Staphylococcus aureus</i>	29737	1	32–35	24 h	1	0.3
Neomycin	<i>Staphylococcus epidermidis</i>	12228	1	32–35	24 h	11	0.4
Novobiocin	<i>Staphylococcus epidermidis</i>	12228	1	32–35	24 h	1	4.0
Nystatin	<i>Saccharomyces cerevisiae</i>	2601	19	29–31	48 h	19	1.0
Paromomycin	<i>Staphylococcus epidermidis</i>	12228	1	32–35	24 h	11	2.0
Penicillin G	<i>Staphylococcus aureus</i>	29737	1	32–35	24 h	1	1.0
Polymyxin B	<i>Bordetella bronchiseptica</i>	4617	1	32–35	24 h	10	0.1
Sisomicin	<i>Staphylococcus epidermidis</i>	12228	1	32–35	24 h	11	0.03
Vancomycin	<i>Bacillus subtilis</i>	6633	32	32–35	5 days	8	As required

^a American Type Culture Collection, 10801 University Boulevard, Manassas VA 20110-2209 (<http://www.atcc.org>)

^b See *Media and Solutions, Media*.

^c Use 0.5 mL of a 1:25 dilution of the stock suspension/100 mL of *Medium 10*.

stock suspension to be added to the inoculum medium that result in satisfactory zones of inhibition of approximately 14–16 mm in diameter for the median concentration of the standard (S_3). [NOTE—Zone sizes that are outside the 11 to 19-mm range are not desirable, because these contribute to assay variability.] If the dilution percentage transmittance is above 25%, a ratio may be used to normalize the addition of organism to the seed layer. The normalization factor can be determined by dividing the percentage transmittance obtained from the dilution by 25. This ratio can then be multiplied by the suggested inoculum amount to obtain the volume (mL) of harvest suspension that needs to be added to the seed layer. Adjust the quantity of inoculum on a daily basis, if necessary, to obtain an optimum concentration–response relationship.

Alternatively, determine during method verification the proportion of harvest suspension to be incorporated into the inoculum, starting with the volumes indicated in *Table 3*, that result in satisfactory demarcation of the zones of inhibition of about 14–16 mm in diameter for the median concentration of the standard (S_3) and giving a reproducible concentration–response relationship. Prepare the inoculum by adding a portion of stock suspension to a sufficient amount of agar medium that has been melted and cooled to 45°–50°. Swirl the mixture without creating bubbles in order to obtain a homogeneous suspension.

Analysis: Prepare the base layer for the required number of assay Petri plates, using the medium and volume shown in *Table 4*. Allow it to harden into a smooth base layer of uniform depth. Prepare the appropriate amount of seed layer inoculum (*Table 5*) as directed for the given antibiotic (*Table 3*) with any adjustments made based on the preparatory trial analysis. Tilt the plate back and forth to spread the inoculum evenly over the base layer surface, and allow it to harden.

Table 4 (base layer)

Antibiotic	Medium ^a	Target Volume (mL)
Amphotericin B ^b	—	—
Bleomycin	35	10
Carbenicillin	9	21
Colistimethate	9	21
Colistin	9	21
Dihydrostreptomycin	5	21
Erythromycin	11	21
Gentamicin	11	21
Neomycin	11	21
Nystatin ^b	—	—
Paromomycin	11	21
Polymyxin B	9	21
Sisomicin	11	21
Vancomycin	8	10
All others	2	21

^a See *Media and Solutions, Media*.

^b No base layer is used.

[NOTE—The base layer may be warmed to facilitate a uniform seed layer.]

Table 5 (seed layer)

Antibiotic	Medium ^a	Target Volume (mL)
Amphotericin B	Refer to <i>Table 3</i>	8
Bleomycin		6
Nystatin		8
All others		4

^a See *Media and Solutions, Media*.

Drop six assay cylinders on the inoculated surface from a height of 12 mm, using a mechanical guide or other device to ensure even spacing on a radius of 2.8 cm, and cover the

plates to avoid contamination. Fill the six cylinders on each plate with dilutions of antibiotic containing the test levels (S_1 – S_5 and U_3) specified in the following paragraph. Incubate the plates as specified in *Table 6* for 16–18 h, and remove the cylinders. Measure and record the diameter of each zone of growth inhibition to the nearest 0.1 mm.

Table 6

Antibiotic	Incubation Temperature (°)
Amphotericin B	29–31
Carbenicillin	36–37.5
Colistimethate	36–37.5
Colistin	36–37.5
Dihydrostreptomycin	36–37.5
Gentamicin	36–37.5
Neomycin	36–37.5
Novobiocin	34–36
Nystatin	29–31
Paromomycin	36–37.5
Polymyxin B	36–37.5
Sisomicin	36–37.5
Vancomycin	36–37.5
All others	32–35

The standards (S_1 – S_5) and a single test level of the sample (U_3) corresponding to S_3 of the standard curve, as defined in *Standard solutions* and *Sample solutions* will be used in the assay. For deriving the standard curve, fill alternate cylinders on each of three plates with the median test dilution (S_3) of the standard and each of the remaining nine cylinders with one of the other four test dilutions of the standard. Repeat the process for the three test dilutions of the standard. For the sample, fill alternate cylinders on each of three plates with the median test dilution of the standard (S_3), and fill the remaining nine cylinders with the corresponding test dilution (U_3) of the sample.

Turbidimetric Method

Temperature control: Use appropriately qualified and calibrated equipment to obtain the temperature ranges specified in *Table 8*. [NOTE—Temperature control can be achieved using either circulating air or water. The greater heat capacity of water lends it some advantage over circulating air.]

Spectrophotometer: Measuring absorbance or transmittance within a fairly narrow frequency band requires a suitable spectrophotometer in which the wavelength can be varied or restricted by use of 580-nm or 530-nm filters. Alternatively, a variable-wavelength spectrophotometer can be used and set to a wavelength of 580 nm or 530 nm.

The instrument may be modified as follows:

1. To accept the tube in which incubation takes place (see *Apparatus* below)
2. To accept a modified cell fitted with a drain that facilitates rapid change of contents
3. To contain a flow cell for a continuous flowthrough analysis

Autozero the instrument with clear, uninoculated broth prepared as specified for the particular antibiotic, including the same amount of test dilution (including formaldehyde if specified) as found in each sample.

Either absorbance or transmittance can be measured while preparing inocula.

Apparatus: Glass or plastic test tubes, e.g., 16 × 125 mm or 18 × 150 mm. [NOTE—Use tubes that are relatively uniform in length, diameter, and thickness and substantially free from surface blemishes and scratches. In the spectrophotometer, use matched tubes that are free from scratches or blemishes. Clean tubes thoroughly to remove all antibiotic residues and traces of cleaning solution. Sterilize tubes before use.]

Table 7

Antibiotic	Stock Solution					Test Dilution	
	Initial Solvent	Initial Concentration	Further Diluent	Final Stock Concentration	Use Within	Final Diluent	Median Concentration (S_3) ^a
Capreomycin	Water	—	—	1 mg/mL	7 days	Water	100 µg/mL
Chloramphenicol	Alcohol	10 mg/mL	Water	1 mg/mL	30 days	Water	2.5 µg/mL
Chlortetracycline	0.01 N hydrochloric acid	—	—	1 mg/mL	4 days	Water	0.06 µg/mL
Dihydrostreptomycin ^b	Water	—	—	1 mg/mL	30 days	Water	30 µg/mL
Gramicidin	Alcohol	—	—	1 mg/mL	30 days	Alcohol	0.04 µg/mL
Neomycin ^{b,d}	B.3 ^c	—	—	100 µg/mL	14 days	B.3 ^c	1.0 µg/mL
Oxytetracycline	0.1 N hydrochloric acid	—	—	1 mg/mL	4 days	Water	0.24 µg/mL
Tetracycline	0.1 N hydrochloric acid	—	—	1 mg/mL	1 day	Water	0.24 µg/mL
Thiostrepton	Dimethyl sulfoxide	—	—	1 U/mL	Same day	Dimethyl sulfoxide	0.80 U/mL
Troleandomycin	Isopropyl alcohol and water (4:1)	—	—	1 mg/mL	Same day	Water	25 µg/mL
Tylosin	Methanol	10 mg/mL	B.16 ^c	1 mg/mL	30 days	Methanol and B.3 ^c (1:1)	4 µg/mL

^a µg in this column refers to µg of activity.

^b The cylinder-plate assay can be used as an alternative procedure.

^c The letter B refers to buffer. See *Media and Solutions, Buffers* for a description of each buffer listed in this table.

^d Dilute the 100-µg/mL stock solution with *Buffer B.3* to obtain a solution having a concentration equivalent to 25 µg/mL of neomycin. To separate 50-mL volumetric flasks add 1.39, 1.67, 2.00, 2.40, and 2.88 mL of this solution. Add 5.0 mL of 0.01 N hydrochloric acid to each flask, dilute with *Buffer B.3* to volume, and mix to obtain solutions having concentrations of 0.69, 0.83, 1.0, 1.2, and 1.44 µg/mL of neomycin. Use these solutions to prepare the standard response line.

Standard solutions: To prepare a stock solution, dissolve a quantity of the USP Reference Standard of a given antibiotic or the entire contents of a vial of USP Reference Standard, where appropriate, in the solvent specified in *Table 7*, and dilute to the required concentration. Store at 2°–8°, and use within the period indicated. On the day of the assay, prepare from the stock solution five or more test dilutions, the successive solutions increasing stepwise in concentration, usually in the ratio of 1:1.25. [NOTE—It may be necessary to use smaller ratios for the successive dilutions from the stock solution for the turbidimetric assay.] Use the final diluent specified such that the median level of the standard (S_3) has the concentration suggested in *Table 7*.

Sample solutions: Assign an assumed potency per unit weight or volume to the unknown, and on the day of the assay prepare a stock solution in the same manner specified for the USP Reference Standard (*Table 7*). Dilute the sample stock solution in the specified final diluent at a nominal concentration equal to the median concentration of the standard (S_3) as specified in *Table 7*.

Inocula: Suspend the test organism from a freshly grown slant or culture in 3 mL of sterile saline TS. Glass beads can be used to facilitate the suspension. *Enterococcus hirae*

(ATCC 10541) and *Staphylococcus aureus* (ATCC 9144) are grown in a liquid medium, not on agar. Spread the saline suspension onto the surface of two or more agar plates (covering the entire surface) or onto the surface of a Roux bottle containing 250 mL of the specified medium (see *Table 8*). Incubate at the time and temperature specified in *Table 8*, or until growth is apparent.

After incubation, harvest the organism from the plates or Roux bottle with approximately 50 mL of sterile saline TS, using a sterile bent glass rod or sterile glass beads. Pipet the suspension into a sterile glass bottle. This is the harvest suspension.

Determine during method verification the quantity of harvest suspension that will be used as the inoculum, starting with the volume suggested in *Table 8*. Prepare also an extra S_3 as a test of growth. Incubate the trial tests for the times indicated in *Table 11*. Adjust the quantity of inoculum daily, if necessary, to obtain the optimum concentration–response relationship from the amount of growth of the test organism in the assay tubes. At the completion of the specified incubation periods, tubes containing the median concentration of the standard should have absorbance values as specified in *Table 9*. Determine the exact duration of incubation

Table 8

Antibiotic	Test Organism	ATCC ^a Number	Incubation Conditions			Suggested Inoculum Composition	
			Medium ^b	Temperature (°)	Time	Medium ^b	Amount (mL/100 mL)
Capreomycin	<i>Klebsiella pneumoniae</i>	10031	1	36–37.5	16–24 h	3	0.05
Chloramphenicol	<i>Escherichia coli</i>	10536	1	32–35	24 h	3	0.7
Chlortetracycline	<i>Staphylococcus aureus</i>	29737	1	32–35	24 h	3	0.1
Dihydrostreptomycin	<i>Klebsiella pneumoniae</i>	10031	1	36–37.5	16–24 h	3	0.1
Gramicidin	<i>Enterococcus hirae</i>	10541	3	36–37.5	16–18 h	3	1.0
Neomycin	<i>Klebsiella pneumoniae</i>	10031	1	36–37.5	16–24 h	39	2
Oxytetracycline	<i>Staphylococcus aureus</i>	29737	1	32–35	24 h	3	0.1
Tetracycline	<i>Staphylococcus aureus</i>	29737	1	32–35	24 h	3	0.1
Thiostrepton	<i>Enterococcus hirae</i>	10541	40	36–37.5	18–24 h	41	0.2
Troleandomycin	<i>Klebsiella pneumoniae</i>	10031	1	36–37.5	16–24 h	3	0.1
Tylosin	<i>Staphylococcus aureus</i>	9144	3	35–39	16–18 h	39	2–3

^aAmerican Type Culture Collection, 10801 University Boulevard, Manassas VA 20110-2209 (<http://www.atcc.org>)

^bSee *Media and Solutions, Media*.

Table 9

Antibiotic	Absorbance, NLT (a.u.)
Capreomycin	0.4
Chlortetracycline	0.35
Gramicidin	0.35
Tetracycline	0.35
All others	0.3

by observing the growth in the reference concentration (median concentration) of the standard (S_3).

Analysis: On the day of the assay, prepare the necessary concentration of antibiotic by dilution of stock solutions of the standard and of each sample as specified under *Standard solutions* and *Sample solutions*. Prepare five test levels, each in triplicate, of the standard (S_1 – S_5) and a single test level (U_3), also in triplicate, of up to 20 samples corresponding to S_3 (median concentration) of the standard.

Table 10

Antibiotic	Volume of Test Dilution (mL)	Volume of Inoculum (mL)
Gramicidin	0.10	9.0
Thiostrepton	0.10	10.0
Tylosin	0.10	9.0
All others	1.0	9.0

Place the tubes in test tube racks or other carriers. Include in each rack 1–2 control tubes containing 1 mL of the inoculum medium (see *Table 8*) but no antibiotic. Add the volumes of the standard and sample test dilutions as indicated in *Table 10*. Randomly distribute one complete set, including the controls, in a tube rack. Add the volume of inoculum specified in *Table 10* to each tube in the rack in turn, and place the completed rack immediately in an incubator or a water bath maintained at the temperature specified in *Table 8* and for the time specified in *Table 11*.

Table 11

Antibiotic	Incubation Time (h)
Capreomycin	3–4
Chloramphenicol	3–4
Cycloserine	3–4
Dihydrostreptomycin	3–4
Streptomycin	3–4
Troleandomycin	3–4
Tylosin	3–5
All others	4–5

After incubation, immediately inhibit the growth of the organism by adding 0.5 mL of dilute formaldehyde to each tube, except for tylosin. For tylosin, heat the rack in a water bath at 80°–90° for 2–6 min or in a steam bath for 5–10 min, and bring to room temperature. Read absorbance or transmittance at 530 or 580 nm, analyzing one rack at a time.

Media and Solutions

The media required for the preparation of test organism inocula are made from the ingredients listed herein. Minor modifications of the individual ingredients are acceptable; and reconstituted dehydrated media can be substituted, provided that the resulting media possess equal or better growth-promoting properties and give a similar standard curve response.

Media: Dissolve the ingredients in water to make 1 L, and adjust the solutions with either 1 N sodium hydroxide or 1 N hydrochloric acid as required, so that after steam sterilization the pH is as specified.

Medium 1

Peptone	6.0 g
Pancreatic digest of casein	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Water	1000 mL
pH after sterilization	6.6 ± 0.1

Medium 2

Peptone	6.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Agar	15.0 g
Water	1000 mL
pH after sterilization	6.6 ± 0.1

Medium 3

Peptone	5.0 g
Yeast extract	1.5 g
Beef extract	1.5 g
Sodium chloride	3.5 g
Dextrose	1.0 g
Dibasic potassium phosphate	3.68 g

Medium 3 (Continued)

Monobasic potassium phosphate	1.32 g
Water	1000 mL
pH after sterilization	7.0 ± 0.05

Medium 4

Peptone	6.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Water	1000 mL
pH after sterilization	6.6 ± 0.1

Medium 5

Peptone	6.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Agar	15.0 g
Water	1000 mL
pH after sterilization	7.9 ± 0.1

Medium 8

Peptone	6.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Agar	15.0 g
Water	1000 mL
pH after sterilization	5.9 ± 0.1

Medium 9

Pancreatic digest of casein	17.0 g
Papaic digest of soybean	3.0 g
Sodium chloride	5.0 g
Dibasic potassium phosphate	2.5 g
Dextrose	2.5 g
Agar	20.0 g
Water	1000 mL
pH after sterilization	7.2 ± 0.1

Medium 10

Pancreatic digest of casein	17.0 g
Papaic digest of soybean	3.0 g
Sodium chloride	5.0 g
Dibasic potassium phosphate	2.5 g
Dextrose	2.5 g
Agar	12.0 g
Water	1000 mL
Polysorbate 80 (added after boiling the medium to dissolve the agar)	10 mL
pH after sterilization	7.2 ± 0.1

Medium 11

Peptone	6.0 g
Pancreatic digest of casein	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Dextrose	1.0 g
Agar	15.0 g
Water	1000 mL
pH after sterilization	8.3 ± 0.1

Medium 13

Peptone	10.0 g
Dextrose	20.0 g
Water	1000 mL
pH after sterilization	5.6 ± 0.1

Medium 19

Peptone	9.4 g
Yeast extract	4.7 g
Beef extract	2.4 g
Sodium chloride	10.0 g
Dextrose	10.0 g
Agar	23.5 g
Water	1000 mL
pH after sterilization	6.1 ± 0.1

Medium 32

Peptone	6.0 g
Pancreatic digest of casein	4.0 g
Yeast extract	3.0 g
Beef extract	1.5 g
Manganese sulfate	0.3 g
Dextrose	1.0 g
Agar	15.0 g
Water	1000 mL
pH after sterilization	6.6 ± 0.1

Medium 34

Glycerol	10.0 g
Peptone	10.0 g
Beef extract	10.0 g
Sodium chloride	3.0 g
Water	1000 mL
pH after sterilization	7.0 ± 0.1

Medium 35

Glycerol	10.0 g
Peptone	10.0 g
Beef extract	10.0 g
Sodium chloride	3.0 g
Agar	17.0 g
Water	1000 mL
pH after sterilization	7.0 ± 0.1

Medium 36

Pancreatic digest of casein	15.0 g
Papaic digest of soybean	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1000 mL
pH after sterilization	7.3 ± 0.1

Medium 39

Peptone	5.0 g
Yeast extract	1.5 g
Beef extract	1.5 g
Sodium chloride	3.5 g
Dextrose	1.0 g
Dibasic potassium phosphate	3.68 g
Monobasic potassium phosphate	1.32 g
Water	1000 mL
pH after sterilization	7.9 ± 0.1

Medium 40

Yeast extract	20.0 g
Polypeptone	5.0 g
Dextrose	10.0 g
Monobasic potassium phosphate	2.0 g
Polysorbate 80	0.1 g
Agar	10.0 g
Water	1000 mL
pH after sterilization	6.7 ± 0.2

Medium 41

Pancreatic digest of casein	9.0 g
Dextrose	20.0 g
Yeast extract	5.0 g
Sodium citrate	10.0 g
Monobasic potassium phosphate	1.0 g
Dibasic potassium phosphate	1.0 g
Water	1000 mL
pH after sterilization	6.8 ± 0.1

Solutions

Buffers: Prepare as directed in *Table 12*, or by other suitable means. The buffers are sterilized after preparation; the pH specified in each case is the pH after sterilization.

Other solutions: See *Reagents, Indicators, and Solutions*.

Water: Use Purified Water.

Saline: Use saline TS.

Dilute formaldehyde: Formaldehyde solution and water (1:3)

Calculations

Introduction: Antibiotic potency is calculated by interpolation from a standard curve using a log-transformed straight-line method with a least-squares fitting procedure (see below for calculation details). The analyst must consider three essential concepts in interpreting antibiotic potency results:

Table 12. Buffers

Buffer	Concentration of Dibasic Potassium Phosphate (g/L)	Concentration of Monobasic Potassium Phosphate (g/L)	Volume of 10 N Potassium Hydroxide (mL)	pH after Sterilization ^a
Buffer B.1 (1%, pH 6.0)	2	8	—	6.0 ± 0.05
Buffer B.3 (0.1 M, pH 8.0)	16.73	0.523	—	8.0 ± 0.1
Buffer B.4 (0.1 M, pH 4.5)	—	13.61	—	4.5 ± 0.05
Buffer B.6 (10%, pH 6.0)	20	80	—	6.0 ± 0.05
Buffer B.10 (0.2 M, pH 10.5)	35	—	2	10.5 ± 0.1
Buffer B.16 (0.1 M, pH 7.0)	13.6	4	—	7.0 ± 0.2

^aAdjust the pH with 18 N phosphoric acid or 10 N potassium hydroxide.

1. Biological concentration–response relationships generally are not linear. The antibiotic potency method allows fitting the data to a straight line by evaluating a narrow concentration range where the results approach linearity. The assay results can be considered valid only if the computed potency is 80%–125% of that assumed in preparing the sample stock solution. When the calculated potency value falls outside 80%–125%, the result for the sample may fall outside the narrow concentration range where linearity has been established. In such a case, adjust the assumed potency of the sample accordingly, and repeat the assay to obtain a valid result.
2. The most effective means of reducing the variability of the reportable value (the geometric mean potency across runs and replicates) is through independent runs of the assay procedure. The combined result of a series of smaller, independent assays spread over a number of days is a more reliable estimate of potency than that from a single large assay with the same total number of plates or tubes. Three or more independent assays are required for antibiotic potency determinations.
3. The number of assays needed in order to obtain a reliable estimate of antibiotic potency depends on the required specification range and the assay variability. The confidence limit calculation described below is determined from several estimated log potencies that are approximately equal in precision. If the value calculated for the width of the confidence interval, W , is too wide, no useful decision can be made about whether the potency meets its specification.

The laboratory should predetermine in its standard operating procedures a maximum acceptable value for the confidence interval width. This maximum value should be determined during development and confirmed during validation or verification. If the calculated confidence interval width exceeds this limit, the analyst must perform additional independent potency determinations to meet the limit requirement. Note that the decision to perform additional determinations does not depend on the estimated potency but only on the uncertainty in that estimate as determined by the confidence interval width. Assay variability has a greater impact on the calculated confidence limit than does the number of independent potency determinations. As a result, the analyst should first consider decreasing variability to the extent possible before conducting potency determinations.

The following sections describe the calculations for determining antibiotic potency as well as for performing the confidence limit calculation. Methods for calculating standard error are also shown in order to allow estimates of assay variance. Where logarithms are used, any base log is acceptable. *Appendix 1* provides formulas for hand calculations applicable when the concentrations are equally spaced in the log scale. Alternative statistical methods may be used if appropriately validated.

Cylinder-plate assay: This section details analysis of the sample data and determination of the potency of an unknown, using the cylinder-plate assay.

Sample data: *Table 13* shows the data from one assay that will be used as an example throughout this section. For each of the 12 plates, zones 1, 3, and 5 are the reference concentration and the other three zones are for one of the other four concentrations, as shown. Other columns are needed for calculations and are explained below.

Step 1: Perform initial calculations and variability suitability check.

For each set of three plates, average the nine reference values and average the nine standard values.

Example (see *Table 13*)

$$15.867 = \bar{X} \\ (16.1, 15.6, \dots, 15.8)$$

$$14.167 = \bar{X}(14.6, 14.1, \dots, 14.8)$$

For each set of three plates determine the standard deviation of the nine reference values and the standard deviation of the nine standard values. For each standard deviation, determine the corresponding relative standard deviation.

Example (see *Table 13*)

$$0.200 = \sigma(16.1, \dots, 15.8)$$

$$1.3\% = (0.200/15.867) \times 100$$

$$0.324 = \sigma(14.6, \dots, 14.1)$$

$$2.3\% = (0.324/14.167) \times 100$$

For a variability suitability criterion, each laboratory should determine a maximum acceptable value for the relative standard deviation. If any of the eight relative standard deviations (four for the reference and four for the standard) exceed this predetermined maximum, the assay data are not suitable and should be discarded. [NOTE—The suggested limit for relative standard deviation is NMT 10%.]

Step 2: Perform a plate-to-plate variation correction.

This correction is applied to convert the average zone measurement obtained for each concentration to the value it would be if the average reference concentration measurement for that set of three replicate plates were the same as the value of the correction point:

$$\bar{X}_C = \bar{X}_S - (\bar{X}_R - P)$$

\bar{X}_C = corrected standard mean

\bar{X}_S = original standard mean

\bar{X}_R = reference mean

P = correction point

Example: For the first set of three plates in *Table 13* (S_1), the correction is:

$$14.022 = 14.167 - (15.867 - 15.722) = 14.167 - 0.145$$

Step 3: Determine the standard curve line.

Generate the standard curve line by plotting the corrected zone measurements versus the log of the standard concentration values. Calculate the equation of the standard curve line by performing a standard unweighted linear regression

on these values, using appropriate software or the manual calculations of *Appendix 1*. [NOTE—Use either the natural log or the base 10 log to plot the standard curve and determine the regression equation; both provide the same final test result.] Each laboratory should determine a minimum value of the coefficient of determination ($\%R^2$) for an acceptable regression. The regression is acceptable only if the obtained $\%R^2$ exceeds this predetermined value. [NOTE—The suggested limit for the percentage coefficient of determination is NLT 95%.]

Table 13. Sample Data (Cylinder-Plate Assay)

Standard	Concentration (U/mL)	Plate replicate	Reference (S ₁)					Sample							Corrected Mean (mm)
			Zone 1 (mm)	Zone 3 (mm)	Zone 5 (mm)	Mean (mm)	SD	%RSD	Zone 2 (mm)	Zone 4 (mm)	Zone 6 (mm)	Mean (mm)	SD	%RSD	
S ₁	3.20	1	16.1	15.6	15.8	15.867	0.200	1.3	14.6	14.1	13.5	14.167	0.324	2.3	14.022
		2	16.0	15.9	16.2				14.5	14.1	14.4				
		3	15.7	15.7	15.8				14.0	14.2	14.1				
S ₂	4.00	1	15.8	15.6	15.5	15.567	0.158	1.0	14.7	15.1	14.8	14.833	0.265	1.8	14.989
		2	15.7	15.5	15.6				14.7	14.9	15.2				
		3	15.7	15.4	15.3				14.8	15.0	14.3				
S ₄	6.25	1	15.6	15.8	16.0	15.789	0.169	1.1	16.6	16.8	16.3	16.578	0.233	1.4	16.511
		2	15.8	15.6	15.7				16.6	16.5	16.2				
		3	16.1	15.7	15.8				16.9	16.5	16.8				
S ₅	7.8125	1	15.6	15.6	15.5	15.667	0.141	0.9	17.3	17.0	17.0	17.167	0.224	1.3	17.222
		2	15.6	15.7	15.5				17.3	17.4	17.2				
		3	15.9	15.8	15.8				17.3	17.3	16.7				
U ₃	unknown	1	15.7	15.8	15.7	15.722 ^a	0.179	1.1	15.3	15.8	15.7	15.478	0.307	2.0	15.522
		2	15.9	15.7	15.7				15.8	15.8	15.5				
		3	15.5	15.8	15.3				15.2	15.1	15.1				

^aThis is the value of the overall reference mean, referred to as the "correction point" below.

Example: Table 14 summarizes the portion of Table 13 needed for this part of the calculation.

Table 14

Standard Set	Corrected Zone Measurements (mm)	Concentration (U/mL)
S_1	14.022	3.2
S_2	14.989	4.0
Reference (S_3)	15.722	5
S_4	16.511	6.25
S_5	17.222	7.8125

Linear regression results**Standard curve line:**

$$Z = [3.551 \times \ln(C)] + 9.978$$

Z = corrected zone measurement

C = concentration

$\%R^2 = 99.7$

Sample potency determination: To estimate the potency of the unknown sample, average the zone measurements of the standard and the zone measurements of the sample on the three plates used. Correct for plate-to-plate variation using the correction point determined above to obtain a corrected average for the unknown, \bar{U} . [NOTE—An acceptable alternative to using the correction point is to correct using the value on the estimated regression line corresponding to the log concentration of S_3 .] Use the corrected average zone measurement in the equation of the standard curve line to determine the log concentration of the sample, L_U , by:

$$L_U = (\bar{U} - a)/b$$

a = intercept of the regression line

b = slope of the regression line

To obtain the potency of the unknown, take the antilog of L_U and multiply the result by any applicable dilution fac-

tor. This value can also be expressed as a percentage of the reference concentration value.

Example: Corrected sample zone measurement (Table 13) = 15.522

Natural log of the sample concentration:

$$L_U = (15.522 - 9.978)/3.551 = 1.561$$

Sample concentration:

$$C_U = e^{1.561} = 4.765$$

Percentage of reference concentration:

$$\text{Result} = (4.765/5.000) \times 100 = 95.3\%$$

Turbidimetric assay: This section details analysis of the sample data and determination of the potency of an unknown using the turbidimetric assay. The method assumes that the tubes are randomly distributed within the heat block or other temperature control device. If the device has a temperature profile that is not uniform, a randomized blocks design is preferred. In such a design, the rack is divided into areas (*blocks*) of relatively uniform temperature and at least one tube of each Standard concentration and of each unknown is placed in each area. The data analysis of a randomized block design is different from the following.

Sample data: Table 15 shows the data from one assay that will be used for an example throughout this section. Other columns are needed for calculations and are explained below.

Step 1: Perform initial calculations and variability suitability check.

For each concentration (including the sample), average the three absorbance values.

Example: See S_1 in Table 15.

$$0.8487 = \bar{X}(0.8545, 0.8422, 0.8495)$$

For each concentration, determine the standard deviation of the three readings and a combined standard deviation for all the concentrations.

Table 15. Sample Data (Turbidimetric Assay)

Standard	Concentration (μg/mL)	Replicate	Absorbance (a.u.)	Average (a.u.)	Standard Deviation
S_1	64	1	0.8545	0.8487	0.0062
		2	0.8422		
		3	0.8495		
S_2	80	1	0.8142	0.8269	0.0125
		2	0.8273		
		3	0.8392		
S_3	100	1	0.6284	0.6931	0.0640
		2	0.6947		
		3	0.7563		
S_4	125	1	0.6933	0.6827	0.0119
		2	0.6850		
		3	0.6699		
S_5	156	1	0.5299	0.5465	0.0272
		2	0.5779		
		3	0.5316		
U_3	unknown	1	0.7130	0.7430	0.0460
		2	0.7960		
		3	0.7201		

Example: See S_1 in Table 15.

$$0.0125 = SD(0.8545, 0.8422, 0.8495)$$

The combined value is calculated by taking the square root of the average of the five variances:

$$0.0325 = \{[(0.0062)^2 + (0.0125)^2 + (0.0640)^2 + (0.0119)^2 + (0.0272)^2]/5\}^{1/2}$$

For a variability suitability criterion, each laboratory should determine a maximum acceptable combined standard deviation. If the combined standard deviation exceeds this predetermined maximum, the assay data are not suitable and should be discarded. [NOTE—The suggested limit for the combined standard deviation is NMT 10% of the average absorbance value across the five concentrations.] If the number of replicates per concentration is at least five, then a relative standard deviation can be computed for each concentration after checking for outliers and compared to a maximum acceptable relative standard deviation. [NOTE—The suggested limit for the relative standard deviation is NMT 10%.]

Step 2: Determine the standard curve line.

Generate the standard curve line by plotting the average absorbance values versus the log of the standard concentration values. Calculate the equation of the standard curve line by performing an unweighted linear regression on these values using appropriate software or the manual calculations of Appendix 1. [NOTE—Use either the natural log or the base 10 log to plot the standard curve and determine the regression equation; both provide the same final test result.] Each laboratory should determine a minimum value of the percentage coefficient of determination ($\%R^2$) for an acceptable regression. The regression is acceptable only if the $\%R^2$ value obtained exceeds this predetermined value. [NOTE—The suggested limit for the percentage coefficient of determination is NLT 90%.]

Example: Table 16 summarizes the portion of Table 15 needed for this part of the calculation.

Table 16

Set of Standards	Average Absorbance Values (a.u.)	Concentration ($\mu\text{g/mL}$)
S_1	0.8487	64
S_2	0.8269	80
S_3	0.6931	100
S_4	0.6827	125
S_5	0.5465	156

Linear regression results

Standard curve line:

$$\text{Absorbance} = 2.2665 - [0.7735 \times \log_{10}(\text{concentration})]$$

$$\%R^2 = 93.0\%$$

Sample potency determination: To estimate the potency of the unknown sample, average the three absorbance measurements to obtain an average for the unknown, \bar{U} . Use this average measurement in the equation of the standard curve line to determine the log concentration of the unknown sample, L_U , by:

$$L_U = (\bar{U} - a)/b$$

a = intercept of the regression line

b = slope of the regression line

To obtain the potency of the unknown, take the antilog of L_U and multiply the result by any applicable dilution factor. This value can also be expressed as a percentage of the reference concentration value.

Example: Average sample absorbance (Table 15) = 0.7430.

$$\log_{10}(C_U) = (0.7430 - 2.2665)/(-0.7735) = 1.9696$$

$$C_U = 10^{1.9696} = 93.2$$

$$\text{Percentage of reference concentration} = (93.2/100.0) \times 100 = 93.2\%$$

C_U = concentration of the sample

Confidence limits and combination of assays calculations: Because of interassay variability, three or more independent determinations are required for a reliable estimate of the sample potency. For each independent determination, start with separately prepared stock solutions and test dilutions of both the Standard and the sample, and repeat the assay of a given sample on a different day.

Given a set of at least three determinations of the unknown potency, use the method of Appendix 2 to check for any outlier values. This determination should be done in the log scale.

To obtain a combined estimate of the unknown potency, calculate the average, M , and the standard deviation of the accepted log potencies. [NOTE—Use either the natural log or the base 10 log.] Determine the confidence interval for the potency as follows:

$$\text{antilog}[M - t(0.05, N - 1) \times SD/\sqrt{N}], \text{antilog}[M + t(0.05, N - 1) \times SD/\sqrt{N}]$$

M = average

SD = standard deviation

N = number of assays

$t(0.05, N-1)$ = the two-sided 5% point of a Student's t -distribution with $N-1$ degrees of freedom

NOTE—The t value is available in spreadsheets, statistics texts, and statistics software.

$$W = \text{antilog}\{[t(0.05, N - 1) \times SD/\sqrt{N}]\}$$

W = half-width of the confidence interval

Compare the half-width of the confidence interval to a predetermined maximum acceptable value. If the half-width is larger than the acceptance limit, continue with additional assays.

Example: Suppose the sample is assayed four times, with potency results in the natural log scale of 1.561, 1.444, 1.517, and 1.535. Then:

$$N = 4$$

$$M = \bar{X}(1.561, 1.444, 1.517, 1.535) = 1.514$$

$$SD = \sigma(1.561, 1.444, 1.517, 1.535) = 0.050$$

$$t = 3.182$$

The confidence interval in the log scale is

$$1.514 \pm (3.182 \times 0.050/\sqrt{4}) = (1.434, 1.594)$$

Taking antilogs, the estimated potency is

$$e^{1.514} = 4.546$$

with a 95% confidence interval for the potency of $e^{1.434}$, $e^{1.594} = (4.197, 4.924)$.

The confidence interval half-width to compare to an acceptance value is the ratio $4.924/4.546 = 1.083$.

Appendix 1. Formulas for Manual Calculations of Regression and Sample Concentration

If the concentrations are equally spaced in the logarithmic scale, the calculations can be performed using the following formula. Let:

\bar{S}_k = mean corrected zone measurement (cylinder-plate assay) or average absorbance value (turbidimetric assay) for standard set k

$k = 1, 2, 3, 4, 5$

\bar{S} = mean of the five \bar{S}_k values

L_k = logarithm of the k th concentration. [NOTE—Use either the natural log or the base 10 log. Slope of the regression line is calculated by:]

$$b = (Y_{\text{high}} - Y_{\text{low}})/(X_{\text{high}} - X_{\text{low}})$$

$$Y_{\text{high}} = 1/5(3\bar{S}_5 + 2\bar{S}_4 + \bar{S}_3 - \bar{S}_1)$$

$$Y_{\text{low}} = 1/5(3\bar{S}_1 + 2\bar{S}_2 + \bar{S}_3 - \bar{S}_5)$$

$$X_{\text{high}} = L_5$$

$$X_{\text{low}} = L_1$$

Combine and simplify to:

$$b = (4\bar{S}_5 + 2\bar{S}_4 - 2\bar{S}_2 - 4\bar{S}_1)/[5(L_5 - L_1)]$$

The log of the concentration of the sample is found using:

$$L_U = L_{\text{reference}} + [(\bar{U} - \bar{S})/b]$$

For example, using the data for the cylinder-plate assay in Table 13 and natural logarithms:

$$b = [(4 \times 17.222) + (2 \times 16.511) - (2 \times 14.989) - (4 \times 14.020)]/[5\{\ln(7.81) - \ln(3.2)\}] = 3.551$$

$$\bar{S} = (14.020 + 14.989 + 15.722 + 16.511 + 17.222)/5 = 15.693$$

$$\text{Natural log of sample concentration} = \ln(5) + [(15.522 - 15.693)/3.551] = 1.561$$

$$\text{Sample concentration} = e^{1.561} = 4.765$$

Appendix 2. Procedure for Checking for Outliers; Rejection of Outlying or Aberrant Measurements

A measurement that is clearly questionable because of a failure in the assay procedure should be rejected, whether it

is discovered during the measuring or tabulation procedure. The arbitrary rejection or retention of an apparently aberrant measurement can be a serious source of bias. In general, the rejection of measurements solely on the basis of their relative magnitudes is a procedure that should be used sparingly.

Each suspected potency measurement, or outlier, may be tested against the following criterion. This criterion is based on the variation within a single group of supposedly equivalent measurements from a normal distribution. On average, it will reject a valid observation once in 25 trials or once in 50 trials. Designate the measurements in order of magnitude from y_1 to y_N , where y_1 is the candidate outlier, and N is the number of measurements in the group. Compute the relative gap by using Table A2-1, *Test for Outlier Measurements*, and the formulas below:

When $N = 3$ to 7:

$$G_1 = (y_2 - y_1)/(y_N - y_1)$$

When $N = 8$ to 10:

$$G_2 = (y_2 - y_1)/(y_{N-1} - y_1)$$

When $N = 11$ to 13:

$$G_3 = (y_3 - y_1)/(y_{N-1} - y_1)$$

If G_1 , G_2 , or G_3 , as appropriate, exceeds the critical value in Table A2-1, *Test for Outlier Measurements*, for the observed N , there is a statistical basis for omitting the outlier measurement(s).

Example: Estimated potencies of sample in log scale = 1.561, 1.444, 1.517, 1.535.

Check lowest potency for outlier:

$$G_1 = (1.517 - 1.444)/(1.561 - 1.444) = 0.624 < 0.889$$

Therefore 1.444 is not an outlier.

Check highest potency for outlier:

$$G_1 = (1.561 - 1.535)/(1.561 - 1.444) = 0.222 < 0.889$$

Therefore 1.561 is not an outlier.

Outlier potencies should be marked as outlier values and excluded from the assay calculations. NMT one potency can be excluded as an outlier.

Table A2-1. Test for Outlier Measurements

In samples from a normal population, gaps equal to or larger than the following values of G_1 , G_2 , and G_3 occur with a probability $P = 0.01$, when outlier measurements can occur only at one end; or with $P = 0.02$, when they may occur at either end.					
N	3	4	5	6	7
G_1	0.987	0.889	0.781	0.698	0.637
N	8	9	10		
G_2	0.681	0.634	0.597		
N	11	12	13		
G_3	0.674	0.643	0.617		

(85) BACTERIAL ENDOTOXINS TEST

♦Portions of this general chapter have been harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. Those portions that are not harmonized are marked with symbols (♦) to specify this fact.♦

The Bacterial Endotoxins Test (BET) is a test to detect or quantify endotoxins from Gram-negative bacteria using amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*).

There are three techniques for this test: the gel-clot technique, which is based on gel formation; the turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and the chromogenic technique, based on the development of color after cleavage of a synthetic peptide-chromogen complex. Proceed by any of the three techniques for the test. In the event of doubt or dispute, the final decision is made based upon the gel-clot limit test unless otherwise indicated in the monograph for the product being tested. The test is carried out in a manner that avoids endotoxin contamination.

APPARATUS

Depyrogenate all glassware and other heat-stable materials in a hot air oven using a validated process.*¹ A commonly used minimum time and temperature is 30 min at 250°. If employing plastic apparatus, such as microplates and pipet tips for automatic pipettors, use apparatus that is shown to be free of detectable endotoxin and does not interfere in the test. [NOTE—In this chapter, the term “tube” includes any other receptacle such as a microtiter well.]

REAGENTS AND TEST SOLUTIONS

Amoebocyte Lysate—A lyophilized product obtained from the lysate of amoebocytes (white blood cells) from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). This reagent refers only to a product manufactured in accordance with the regulations of the competent authority. [NOTE—*Amoebocyte Lysate* reacts to some β -glucans in addition to endotoxins. *Amoebocyte Lysate* preparations that do not react to glucans are available: they are prepared by removing the G factor reacting to glucans from *Amoebocyte Lysate* or by inhibiting the G factor reacting system of *Amoebocyte Lysate* and may be used for endotoxin testing in the presence of glucans.]

Water for Bacterial Endotoxins Test (BET)—Use Water for Injection or water produced by other procedures that

*¹ For a validity test of the procedure for inactivating endotoxins, see *Dry-Heat Sterilization under Sterilization and Sterility Assurance of Compendial Articles* (1211). Use *Lysate TS* having a sensitivity of not less than 0.15 Endotoxin Unit per mL.♦

shows no reaction with the lysate employed, at the detection limit of the reagent.

Lysate TS—Dissolve *Amoebocyte Lysate* in *Water for BET*, or in a buffer recommended by the lysate manufacturer, by gentle stirring. Store the reconstituted lysate, refrigerated or frozen, according to the specifications of the manufacturer.

PREPARATION OF SOLUTIONS

Standard Endotoxin Stock Solution—A *Standard Endotoxin Stock Solution* is prepared from a USP Endotoxin Reference Standard that has been calibrated to the current WHO International Standard for Endotoxin. Follow the specifications in the package leaflet and on the label for preparation and storage of the *Standard Endotoxin Stock Solution*. Endotoxin is expressed in Endotoxin Units (EU). [NOTE—One USP Endotoxin Unit (EU) is equal to one International Unit (IU) of endotoxin.]

Standard Endotoxin Solutions—After mixing the *Standard Endotoxin Stock Solution* vigorously, prepare appropriate serial dilutions of *Standard Endotoxin Solution*, using *Water for BET*. Use dilutions as soon as possible to avoid loss of activity by adsorption.

Sample Solutions—Prepare the *Sample Solutions* by dissolving or diluting drugs using *Water for BET*. Some substances or preparations may be more appropriately dissolved, or diluted in other aqueous solutions. If necessary, adjust the pH of the solution to be examined (or dilution thereof) so that the pH of the mixture of the lysate and *Sample Solution* falls within the pH range specified by the lysate manufacturer, usually 6.0–8.0. The pH may be adjusted by use of an acid, base, or suitable buffer as recommended by the lysate manufacturer. Acids and bases may be prepared from concentrates or solids with *Water for BET* in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

DETERMINATION OF MAXIMUM VALID DILUTION (MVD)

The maximum valid dilution is the maximum allowable dilution of a specimen at which the endotoxin limit can be determined. Determine the MVD from the following equation:

$$\text{MVD} = (\text{endotoxin limit} \times \text{concentration of Sample Solution}) / (\lambda)$$

Endotoxin Limit—The endotoxin limit for parenteral drugs, defined on the basis of dose, equals K/M^{*2} , where K

*² K is 5 USP-EU/kg of body weight for any route of administration other than intrathecal (for which K is 0.2 USP-EU/kg of body weight). For radiopharmaceutical products not administered intrathecally, the endotoxin limit is calculated as 175 EU/V, where V is the maximum recommended dose in mL. For intrathecally administered radiopharmaceuticals, the endotoxin limit is obtained by the formula 14 EU/V. For formulations (usually anticancer products) administered on a per square meter of body surface, the formula is K/M , where $K = 100$ EU/m² and M is the maximum dose/m².♦

is a threshold pyrogenic dose of endotoxin per kg of body weight, and M is equal to the maximum recommended bolus dose of product per kg of body weight. When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period. The endotoxin limit for parenteral drugs is specified in the individual monograph in units such as EU/mL, EU/mg, EU/Unit of biological activity, etc.

Concentration of Sample Solution—

mg/mL: in the case of endotoxin limit specified by weight (EU/mg);

Units/mL: in the case of endotoxin limit specified by unit of biological activity (EU/Unit);

mL/mL: when the endotoxin limit is specified by volume (EU/mL).

λ : the labeled sensitivity in the *Gel-Clot Technique* (EU/mL) or the lowest concentration used in the standard curve for the *Turbidimetric Technique* or *Chromogenic Technique*.

GEL-CLOT TECHNIQUE

The gel-clot technique is used for detecting or quantifying endotoxins based on clotting of the lysate reagent in the presence of endotoxin. The minimum concentration of endotoxin required to cause the lysate to clot under standard conditions is the labeled sensitivity of the lysate reagent. To ensure both the precision and validity of the test, perform the tests for confirming the labeled lysate sensitivity and for interfering factors as described in *Preparatory Testing*, immediately below.

Preparatory Testing

Test for Confirmation of Labeled Lysate Sensitivity—

Confirm in four replicates the labeled sensitivity, λ , expressed in EU/mL of the lysate prior to use in the test. The test for confirmation of lysate sensitivity is to be carried out when a new batch of lysate is used or when there is any change in the test conditions that may affect the outcome of the test. Prepare standard solutions having at least four concentrations equivalent to 2λ , λ , 0.5λ , and 0.25λ by diluting the USP Endotoxin RS with *Water for BET*.

Mix a volume of the *Lysate TS* with an equal volume (such as 0.1-mL aliquots) of one of the *Standard Endotoxin Solutions* in each test tube. When single test vials or ampuls containing lyophilized lysate are used, add solutions directly to the vial or ampul. Incubate the reaction mixture for a constant period according to the directions of the lysate manufacturer (usually at $37 \pm 1^\circ$ for 60 ± 2 min), avoiding vibration. To test the integrity of the gel, take each tube in

turn directly from the incubator, and invert it through about 180° in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if an intact gel is not formed. The test is considered valid when the lowest concentration of the standard solutions shows a negative result in all replicate tests.

The endpoint is the smallest concentration in the series of decreasing concentrations of standard endotoxin that clots the lysate. Determine the geometric mean endpoint by calculating the mean of the logarithms of the endpoint concentrations of the four replicate series and then taking the antilogarithm of the mean value, as indicated in the following formula:

$$\text{geometric mean endpoint concentration} = \text{antilog} (\Sigma e/f)$$

where Σe is the sum of the log endpoint concentrations of the dilution series used, and f is the number of replicate tubes. The geometric mean endpoint concentration is the measured sensitivity of the lysate (in EU/mL). If this is not less than 0.5λ and not more than 2λ , the labeled sensitivity is confirmed and is used in tests performed with this lysate.

Test for Interfering Factors—Usually prepare solutions (A–D) as shown in *Table 1*, and perform the inhibition/enhancement test on the *Sample Solutions* at a dilution less than the MVD, not containing any detectable endotoxins, operating as described for *Test for Confirmation of Labeled Lysate Sensitivity*. The geometric mean endpoint concentrations of *Solutions B* and *C* are determined using the formula described in the *Test for Confirmation of Labeled Lysate Sensitivity*. The test for interfering factors must be repeated when any condition changes that is likely to influence the result of the test.

The test is considered valid when all replicates of *Solutions A* and *D* show no reaction and the result of *Solution C* confirms the labeled sensitivity.

If the sensitivity of the lysate determined in the presence of *Solution B* is not less than 0.5λ and not greater than 2λ , the *Sample Solution* does not contain factors that interfere under the experimental conditions used. Otherwise, the *Sample Solution* to be examined interferes with the test.

If the sample under test does not comply with the test at a dilution less than the MVD, repeat the test using a greater dilution, not exceeding the MVD. The use of a more sensitive lysate permits a greater dilution of the sample to be examined, and this may contribute to the elimination of interference.

Interference may be overcome by suitable treatment such as filtration, neutralization, dialysis, or heating. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described

Table 1. Preparation of Solutions for the Inhibition/Enhancement Test for Gel-Clot Techniques

Solution	Endotoxin Concentration/ Solution to Which Endotoxin Is Added	Diluent	Dilution Factor	Endotoxin Concentration	Number of Replicates
A ^a	None/ <i>Sample Solution</i>	—	—	—	4
B ^b	2λ / <i>Sample Solution</i>	<i>Sample Solution</i>	1	2λ	4
			2	1λ	4
			4	0.5λ	4
			8	0.25λ	4
C ^c	2λ / <i>Water for BET</i>	<i>Water for BET</i>	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D ^d	None/ <i>Water for BET</i>	—	—	—	2

^a *Solution A*: A *Sample Solution* of the preparation under test that is free of detectable endotoxins.

^b *Solution B*: Test for interference.

^c *Solution C*: Control for labeled lysate sensitivity.

^d *Solution D*: Negative control of *Water for BET*.

above using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

Limit Test

Procedure—Prepare *Solutions A, B, C, and D* as shown in *Table 2*, and perform the test on these solutions following the procedure above for *Preparatory Testing, Test for Confirmation of Labeled Lysate Sensitivity*.

Table 2. Preparation of Solutions for the Gel-Clot Limit Test

Solution*	Endotoxin Concentration/ Solution to Which Endotoxin Is Added	Number of Replicates
A	None/Diluted <i>Sample Solution</i>	2
B	2λ/Diluted <i>Sample Solution</i>	2
C	2λ/ <i>Water for BET</i>	2
D	None/ <i>Water for BET</i>	2

* Prepare *Solution A* and the positive product control *Solution B* using a dilution not greater than the MVD and treatments as described for the *Test for Interfering Factors* in *Preparatory Testing*. The positive control *Solutions B* and *C* contain the *Standard Endotoxin Solution* at a concentration corresponding to twice the labeled lysate sensitivity. The negative control *Solution D* consists of *Water for BET*.

Interpretation—The test is considered valid when both replicates of *Solutions B* and *C* are positive and those of *Solution D* are negative. When a negative result is found for both replicates of *Solution A*, the preparation under test complies with the test. When a positive result is found for both replicates of *Solution A*, the preparation under test does not comply with the test.

When a positive result is found for one replicate of *Solution A* and a negative result is found for the other, repeat the test. In the repeat test, the preparation under test complies with the test if a negative result is found for both replicates of *Solution A*. The preparation does not comply with the test if a positive result is found for one or both replicates of *Solution A*. However, if the preparation does not comply with the test at a dilution less than the MVD, the test may

be repeated using a greater dilution, not exceeding the MVD.

Quantitative Test

Procedure—The test quantifies bacterial endotoxins in *Sample Solutions* by titration to an endpoint. Prepare *Solutions A, B, C, and D* as shown in *Table 3*, and test these solutions by following the procedure in *Preparatory Testing, Test for Confirmation of Labeled Lysate Sensitivity*.

Calculation and Interpretation—The test is considered valid when the following three conditions are met: (1) Both replicates of negative control *Solution D* are negative; (2) Both replicates of positive product control *Solution B* are positive; and (3) The geometric mean endpoint concentration of *Solution C* is in the range of 0.5λ to 2λ.

To determine the endotoxin concentration of *Solution A*, calculate the endpoint concentration for each replicate by multiplying each endpoint dilution factor by λ. The endotoxin concentration in the *Sample Solution* is the endpoint concentration of the replicates. If the test is conducted with a diluted *Sample Solution*, calculate the concentration of endotoxin in the original *Sample Solution* by multiplying by the dilution factor. If none of the dilutions of the *Sample Solution* is positive in a valid assay, report the endotoxin concentration as less than λ (if the diluted sample was tested, report as less than λ times the lowest dilution factor of the sample). If all dilutions are positive, the endotoxin concentration is reported as equal to or greater than the greatest dilution factor multiplied by λ (e.g., initial dilution factor times eight times λ in *Table 3*).

The preparation under test meets the requirements of the test if the concentration of endotoxin in both replicates is less than that specified in the individual monograph.

PHOTOMETRIC QUANTITATIVE TECHNIQUES

Turbidimetric Technique

This technique is a photometric assay measuring increases in reactant turbidity. On the basis of the particular assay principle employed, this technique may be classified as either an endpoint-turbidimetric assay or a kinetic-turbidimet-

Table 3. Preparation of Solutions for the Gel-Clot Assay

Solution	Endotoxin Concentration/ Solution to Which Endotoxin Is Added	Diluent	Dilution Factor	Endotoxin Concentration	Number of Replicates
A ^a	None/ <i>Sample Solution</i>	<i>Water for BET</i>	1	—	2
			2	—	2
			4	—	2
			8	—	2
B ^b	2λ/ <i>Sample Solution</i>	—	1	2λ	2
C ^c	2λ/ <i>Water for BET</i>	<i>Water for BET</i>	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D ^d	None/ <i>Water for BET</i>	—	—	—	2

^a *Solution A*: *Sample Solution* under test at the dilution, not to exceed the MVD, with which the *Test for Interfering Factors* was completed. Subsequent dilution of the *Sample Solution* must not exceed the MVD. Use *Water for BET* to make a dilution series of four tubes containing the *Sample Solution* under test at concentrations of 1, 1/2, 1/4, and 1/8 relative to the concentration used in the *Test for Interfering Factors*. Other dilutions up to the MVD may be used as appropriate.

^b *Solution B*: *Solution A* containing standard endotoxin at a concentration of 2λ (positive product control).

^c *Solution C*: Two replicates of four tubes of *Water for BET* containing the standard endotoxin at concentrations of 2λ, λ, 0.5λ, and 0.25λ, respectively.

^d *Solution D*: *Water for BET* (negative control).

ric assay. The endpoint-turbidimetric assay is based on the quantitative relationship between the concentration of endotoxins and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period. The kinetic-turbidimetric assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance or transmission of the reaction mixture, or the rate of turbidity development. The test is carried out at the incubation temperature recommended by the lysate manufacturer (which is usually $37 \pm 1^\circ$).

Chromogenic Technique

This technique is an assay to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with lysate. On the basis of the particular assay principle employed, this technique may be classified as either an endpoint-chromogenic assay or a kinetic-chromogenic assay. The endpoint-chromogenic assay is based on the quantitative relationship between the concentration of endotoxins and the release of chromophore at the end of an incubation period. The kinetic-chromogenic assay is a method to measure either the time (onset time) needed to reach a predetermined absorbance of the reaction mixture, or the rate of color development. The test is carried out at the incubation temperature recommended by the lysate manufacturer (which is usually $37 \pm 1^\circ$).

Preparatory Testing

To assure the precision or validity of the turbidimetric and chromogenic techniques, preparatory tests are conducted to verify that the criteria for the standard curve are valid and that the sample solution does not interfere with the test. Validation for the test method is required when conditions that are likely to influence the test result change.

Assurance of Criteria for the Standard Curve—The test must be carried out for each lot of lysate reagent. Using the *Standard Endotoxin Solution*, prepare at least three endotoxin concentrations within the range indicated by the lysate manufacturer to generate the standard curve. Perform the assay using at least three replicates of each standard endotoxin concentration according to the manufacturer's instructions for the lysate (volume ratios, incubation time, temperature, pH, etc.). If the desired range is greater than two logs in the kinetic methods, additional standards should be included to bracket each log increase in the range of the standard curve. The absolute value of the correlation coefficient, r , must be greater than or equal to 0.980 for the range of endotoxin concentrations set up.

Test for Interfering Factors—Select an endotoxin concentration at or near the middle of the endotoxin standard curve. Prepare *Solutions A, B, C, and D* as shown in *Table 4*. Perform the test on *Solutions A, B, C, and D* at least in dupli-

cate, according to the instructions for the lysate employed, for example, concerning volume of *Sample Solution* and *Lysate TS*, volume ratio of *Sample Solution* to *Lysate TS*, incubation time, etc.

The test is considered valid when the following conditions are met.

1. The absolute value of the correlation coefficient of the standard curve generated using *Solution C* is greater than or equal to 0.980.
2. The result with *Solution D* does not exceed the limit of the blank value required in the description of the lysate reagent employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution, if any (*Solution A, Table 4*), from that containing the added endotoxin (*Solution B, Table 4*). In order to be considered free of factors that interfere with the assay under the conditions of the test, the measured concentration of the endotoxin added to the *Sample Solution* must be within 50%–200% of the known added endotoxin concentration after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is out of the specified range, the *Sample Solution* under test is considered to contain interfering factors. Then, repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the *Sample Solution* or diluted *Sample Solution* not to exceed the MVD may be eliminated by suitable validated treatment such as filtration, neutralization, dialysis, or heat treatment. To establish that the chosen treatment effectively eliminates interference without loss of endotoxins, perform the assay described above, using the preparation to be examined to which Standard Endotoxin has been added and which has then been submitted to the chosen treatment.

Test Procedure

Follow the procedure described for *Test for Interfering Factors* under *Preparatory Testing*, immediately above.

Calculation

Calculate the endotoxin concentration of each of the replicates of *Solution A*, using the standard curve generated by the positive control *Solution C*. The test is considered valid when the following three requirements are met.

1. The results of the control *Solution C* comply with the requirements for validation defined for *Assurance of Criteria for the Standard Curve* under *Preparatory Testing*.
2. The endotoxin recovery, calculated from the concentration found in *Solution B* after subtracting the concentration of endotoxin found in *Solution A*, is within the range of 50%–200%.

Table 4. Preparation of Solutions for the Inhibition/Enhancement Test for Photometric Techniques

Solution	Endotoxin Concentration	Solution to Which Endotoxin Is Added	Number of Replicates
A ^a	None	<i>Sample Solution</i>	Not less than 2
B ^b	Middle concentration of the standard curve	<i>Sample Solution</i>	Not less than 2
C ^c	At least three concentrations (lowest concentration is designated λ)	<i>Water for BET</i>	Each not less than 2
D ^d	None	<i>Water for BET</i>	Not less than 2

^a *Solution A*: The *Sample Solution* may be diluted not to exceed MVD.

^b *Solution B*: The preparation under test at the same dilution as *Solution A*, containing added endotoxin at a concentration equal to or near the middle of the standard curve.

^c *Solution C*: The standard endotoxin at the concentrations used in the validation of the method described for *Assurance of Criteria for the Standard Curve* under *Preparatory Testing* (positive controls).

^d *Solution D*: *Water for BET* (negative control).

- The result of the negative control *Solution D* does not exceed the limit of the blank value required in the description of the lysate employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

Interpretation

In photometric assays, the preparation under test complies with the test if the mean endotoxin concentration of the replicates of *Solution A*, after correction for dilution and concentration, is less than the endotoxin limit for the product.

(87) BIOLOGICAL REACTIVITY TESTS, IN VITRO

The following tests are designed to determine the biological reactivity of mammalian cell cultures following contact with the elastomeric plastics and other polymeric materials with direct or indirect patient contact or of specific extracts prepared from the materials under test. It is essential that the tests be performed on the specified surface area. When the surface area of the specimen cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic or other material for every mL of extraction fluid. Exercise care in the preparation of the materials to prevent contamination with microorganisms and other foreign matter.

Three tests are described (i.e., the *Agar Diffusion Test*, the *Direct Contact Test*, and the *Elution Test*).^{*} The decision as to which type of test or the number of tests to be performed to assess the potential biological response of a specific sample or extract depends upon the material, the final product, and its intended use. Other factors that may also affect the suitability of sample for a specific use are the polymeric composition; processing and cleaning procedures; contacting media; inks; adhesives; absorption, adsorption, and permeability of preservatives; and conditions of storage. Evaluation of such factors should be made by appropriate additional specific tests before determining that a product made from a specific material is suitable for its intended use.

USP Reference Standards (11)—*USP High-Density Polyethylene RS. USP Positive Bioreaction RS.*

Cell Culture Preparation—Prepare multiple cultures of L-929 (ATCC cell line CCL 1, NCTC clone 929) mammalian fibroblast cells in serum-supplemented minimum essential medium having a seeding density of about 10^5 cells per mL. Incubate the cultures at $37 \pm 1^\circ$ in a humidified incubator for not less than 24 hours in a $5 \pm 1\%$ carbon dioxide atmosphere until a monolayer, with greater than 80% confluence, is obtained. Examine the prepared cultures under a microscope to ensure uniform, near-confluent monolayers. [NOTE—The reproducibility of the *In Vitro Biological Reactivity Tests* depends upon obtaining uniform cell culture density.]

Extraction Solvents—*Sodium Chloride Injection* (see monograph—use Sodium Chloride Injection containing 0.9% of NaCl). Alternatively, serum-free mammalian cell culture media or serum-supplemented mammalian cell culture media may be used. Serum supplementation is used when extraction is done at 37° for 24 hours.

^{*} Further details are given in the following publications of the American Society for Testing and Materials, 1916 Race St., Philadelphia, PA 19103: "Standard Test Method for Agar Diffusion Cell Culture Screening for Cytotoxicity," ASTM Designation F 895-84; "Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices," ASTM Designation F 813-83.

Apparatus—

Autoclave—Employ an autoclave capable of maintaining a temperature of $121 \pm 2^\circ$, equipped with a thermometer, a pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about 20° , but not below 20° , immediately following the heating cycle.

Oven—Use an oven, preferably a mechanical convection model, that will maintain operating temperatures in the range of 50° to 70° within $\pm 2^\circ$.

Incubator—Use an incubator capable of maintaining a temperature of $37 \pm 1^\circ$ and a humidified atmosphere of $5 \pm 1\%$ carbon dioxide in air.

Extraction Containers—Use only containers, such as ampuls or screw-cap culture test tubes, or their equivalent, of Type I glass. If used, culture test tubes, or their equivalent, are closed with a screw cap having a suitable elastomeric liner. The exposed surface of the elastomeric liner is completely protected with an inert solid disk 50 to 75 μm in thickness. A suitable disk can be fabricated from polytetrafluoroethylene.

Preparation of Apparatus—Cleanse all glassware thoroughly with chromic acid cleansing mixture and, if necessary, with hot nitric acid followed by prolonged rinsing with Sterile Water for Injection. Sterilize and dry by a suitable process containers and devices used for extraction, transfer, or administration of test material. If ethylene oxide is used as the sterilizing agent, allow not less than 48 hours for complete degassing.

Procedure—

Preparation of Sample for Extracts—Prepare as directed in the *Procedure under Biological Reactivity Tests, In Vivo* (88).

Preparation of Extracts—Prepare as directed for *Preparation of Extracts in Biological Reactivity Tests, In Vivo* (88) using either Sodium Chloride Injection (0.9% NaCl) or serum-free mammalian cell culture media as *Extraction Solvents*. [NOTE—If extraction is done at 37° for 24 hours in an incubator, use cell culture media supplemented by serum. The extraction conditions should not in any instance cause physical changes, such as fusion or melting of the material pieces, other than a slight adherence.]

Agar Diffusion Test

This test is designed for elastomeric closures in a variety of shapes. The agar layer acts as a cushion to protect the cells from mechanical damage while allowing the diffusion of leachable chemicals from the polymeric specimens. Extracts of materials that are to be tested are applied to a piece of filter paper.

Sample Preparation—Use extracts prepared as directed or use portions of the test specimens having flat surfaces not less than 100 mm² in surface area.

Positive Control Preparation—Proceed as directed for *Sample Preparation*.

Negative Control Preparation—Proceed as directed for *Sample Preparation*.

Procedure—Using 7 mL of cell suspension prepared as directed under *Cell Culture Preparation*, prepare the monolayers in plates having a 60-mm diameter. Following incubation, aspirate the culture medium from the monolayers, and replace it with serum-supplemented culture medium containing not more than 2% of agar. [NOTE—The quality of the agar must be adequate to support cell growth. The agar layer must be thin enough to permit diffusion of leached chemicals.] Place the flat surfaces of *Sample Preparation*, *Negative Control Preparation*, and *Positive Control Preparation* or their extracts in an appropriate extracting medium, in duplicate cultures in contact with the solidified agar surface. Use no more than three specimens per prepared plate. Incubate all cultures for not less than 24 hours at $37 \pm 1^\circ$, prefer-

ably in a humidified incubator containing $5 \pm 1\%$ of carbon dioxide. Examine each culture around each *Sample*, *Negative Control*, and *Positive Control*, under a microscope, using a suitable stain, if desired.

Interpretation of Results—The biological reactivity (cellular degeneration and malformation) is described and rated on a scale of 0 to 4 (see Table 1). Measure the responses of the cell cultures to the *Sample Preparation*, the *Negative Control Preparation*, and the *Positive Control Preparation*. The cell culture test system is suitable if the observed responses to the *Negative Control Preparation* is grade 0 (no reactivity) and to the *Positive Control Preparation* is at least grade 3 (moderate). The *Sample* meets the requirements of the test if the response to the *Sample Preparation* is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed.

Table 1. Reactivity Grades for Agar Diffusion Test and Direct Contact Test

Grade	Reactivity	Description of Reactivity Zone
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen
3	Moderate	Zone extends 0.5 to 1.0 cm beyond specimen
4	Severe	Zone extends greater than 1.0 cm beyond specimen

Direct Contact Test

This test is designed for materials in a variety of shapes. The procedure allows for simultaneous extraction and testing of leachable chemicals from the specimen with a serum-supplemented medium. The procedure is not appropriate for very low- or high-density materials that could cause mechanical damage to the cells.

Sample Preparation—Use portions of the test specimen having flat surfaces not less than 100 mm² in surface area.

Positive Control Preparation—Proceed as directed for *Sample Preparation*.

Negative Control Preparation—Proceed as directed for *Sample Preparation*.

Procedure—Using 2 mL of cell suspension prepared as directed under *Cell Culture Preparation*, prepare the monolayers in plates having a 35-mm diameter. Following incubation, aspirate the culture medium from the cultures, and replace it with 0.8 mL of fresh culture medium. Place a single *Sample Preparation*, a *Negative Control Preparation*, and a *Positive Control Preparation* in each of duplicate cultures. Incubate all cultures for not less than 24 hours at $37 \pm 1^\circ$ in a humidified incubator containing $5 \pm 1\%$ of carbon dioxide. Examine each culture around each *Sample*, *Negative Control*, and *Positive Control Preparation*, either visually or under a microscope, using a suitable stain, if desired.

Interpretation of Results—Proceed as directed for *Interpretation of Results* under *Agar Diffusion Test*. The *Sample* meets the requirements of the test if the response to the *Sample Preparation* is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed.

Elution Test

This test is designed for the evaluation of extracts of polymeric materials. The procedure allows for extraction of the specimens at physiological or nonphysiological temperatures for varying time intervals. It is appropriate for high-density materials and for dose-response evaluations.

Sample Preparation—Prepare as directed in *Preparation of Extracts*, using either Sodium Chloride Injection (0.9% NaCl) or serum-free mammalian cell culture media as *Extraction Solvents*. If the size of the *Sample* cannot be readily measured, a mass of not less than 0.1 g of elastomeric material or 0.2 g of plastic or polymeric material per mL of extraction medium may be used. Alternatively, use serum-supplemented mammalian cell culture media as the extracting medium to simulate more closely physiological conditions. Prepare the extracts by heating for 24 hours in an incubator containing $5 \pm 1\%$ of carbon dioxide. Maintain the extraction temperature at $37 \pm 1^\circ$, because higher temperatures may cause denaturation of serum proteins.

Positive Control Preparation—Proceed as directed for *Sample Preparation*.

Negative Control Preparation—Proceed as directed for *Sample Preparation*.

Procedure—Using 2 mL of cell suspension prepared as directed under *Cell Culture Preparation*, prepare the monolayers in plates having a 35-mm diameter. Following incubation, aspirate the culture medium from the monolayers, and replace it with extracts of the *Sample Preparation*, *Negative Control Preparation*, or *Positive Control Preparation*. The serum-supplemented and serum-free cell culture media extracts are tested in duplicate without dilution (100%). The Sodium Chloride Injection extract is diluted with serum-supplemented cell culture medium and tested in duplicate at 25% extract concentration. Incubate all cultures for 48 hours at $37 \pm 1^\circ$ in a humidified incubator preferably containing $5 \pm 1\%$ of carbon dioxide. Examine each culture at 48 hours, under a microscope, using a suitable stain, if desired.

Interpretation of Results—Proceed as directed for *Interpretation of Results* under *Agar Diffusion Test* but using Table 2. The *Sample* meets the requirements of the test if the response to the *Sample Preparation* is not greater than grade 2 (mildly reactive). Repeat the procedure if the suitability of the system is not confirmed. For dose-response evaluations, repeat the procedure, using quantitative dilutions of the sample extract.

Table 2. Reactivity Grades for Elution Test

Grade	Reactivity	Conditions of all Cultures
0	None	Discrete intracytoplasmic granules; no cell lysis
1	Slight	Not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules; occasional lysed cells are present
2	Mild	Not more than 50% of the cells are round and devoid of intracytoplasmic granules; no extensive cell lysis and empty areas between cells
3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed
4	Severe	Nearly complete destruction of the cell layers

(88) BIOLOGICAL REACTIVITY TESTS, IN VIVO

The following tests are designed to determine the biological response of animals to elastomerics, plastics, and other polymeric material with direct or indirect patient contact, or

by the injection of specific extracts prepared from the material under test. It is essential to make available the specific surface area for extraction. When the surface area of the specimen cannot be determined, use 0.1 g of elastomer or 0.2 g of plastic or other material for every mL of extraction fluid. Also, it is essential to exercise care in the preparation of the materials to be injected or instilled to prevent contamination with microorganisms and other foreign matter. Three tests are described. The *Systemic Injection Test* and the *Intracutaneous Test* are used for elastomeric materials, especially to elastomeric closures for which the appropriate *Biological Reactivity Tests, In Vitro* (87) have indicated significant biological reactivity. These two tests are used for plastics and other polymers in addition to a third test, the *Implantation Test*, to test the suitability of these materials intended for use in fabricating containers and accessories thereto, for use in parenteral preparations, and for use in medical devices, implants, and other systems.

These three tests are applied to materials or medical devices, if there is a need for classification of plastics and other polymers based on in vivo biological reactivity testing.

For the purpose of this chapter, these definitions apply: the *Sample* is the specimen under test or an extract prepared from such a specimen. A *Blank* consists of the same quantity of the same extracting medium that is used for the extraction of the specimen under test, treated in the same manner as the extracting medium containing the specimen under test. A *Negative Control*¹ is a specimen that gives no reaction under the conditions of the test.

CLASSIFICATION OF PLASTICS

Six Plastic Classes are defined (see *Table 1*). This classification is based on responses to a series of in vivo tests for which extracts, materials, and routes of administration are specified. These tests are directly related to the intended end-use of the plastic articles. The choice of extractants is representative of the vehicles in preparations with which the plastics are likely to be in contact. The *Table 1* classification facilitates communication among suppliers, users, and manufacturers of plastics by summarizing the tests to be performed for containers for injections and medical devices if a need for classification exists.

With the exception of the *Implantation Test*, the procedures are based on the use of extracts that, depending on the heat resistance of the material, are prepared at one of

three standard temperatures: 50°, 70°, and 121°. Therefore, the class designation of a plastic must be accompanied by an indication of the temperature of extraction (e.g., IV-121°, which represents a class IV plastic extracted at 121°, or I-50°, which represents a class I plastic extracted at 50°).

Plastics may be classified as USP Plastic Classes I–VI only on the basis of the response criteria prescribed in *Table 1*.

This classification does not apply to plastics that are intended for use as containers for oral or topical products, or that may be used as an integral part of a drug formulation. *Table 1* does not apply to natural elastomers, which are to be tested in Sodium Chloride Injection and vegetable oils only.

The *Systemic Injection Test* and the *Intracutaneous Test* are designed to determine the systemic and local, respectively, biological responses of animals to plastics and other polymers by the single-dose injection of specific extracts prepared from a *Sample*. The *Implantation Test* is designed to evaluate the reaction of living tissue to the plastic and other polymers by the implantation of the *Sample* itself into animal tissue. The proper preparation and placement of the specimens under aseptic conditions are important in the conduct of the *Implantation Test*.

These tests are designed for application to plastics and other polymers in the condition in which they are used. If the material is to be exposed to any cleansing or sterilization process prior to its end-use, then the tests are to be conducted on a *Sample* prepared from a specimen preconditioned by the same processing.

Factors such as material composition, processing and cleaning procedures, contacting media, inks, adhesives, absorption, adsorption and permeability of preservatives, and conditions of storage may also affect the suitability of a material for a specific use. Evaluation of such factors should be made by appropriate additional specific tests to determine the suitability of a material for its intended use.

USP Reference Standards (11)—USP High-Density Polyethylene RS.

Extracting Media—

SODIUM CHLORIDE INJECTION (see monograph). Use Sodium Chloride Injection containing 0.9% of NaCl.

1 IN 20 SOLUTION OF ALCOHOL IN SODIUM CHLORIDE INJECTION.

POLYETHYLENE GLYCOL 400 (see monograph).

VEGETABLE OIL— Use freshly refined Sesame Oil (see monograph) or Cottonseed Oil (see monograph) or other suitable vegetable oils.

Table 1. Classification of Plastics

Plastic Classes ^a						Tests to be Conducted			
I	II	III	IV	V	VI	Test Material	Animal	Dose	Procedure ^b
x	x	x	x	x	x	Extract of <i>Sample</i> in Sodium Chloride Injection	Mouse	50 mL/kg	A (iv)
x	x	x	x	x	x		Rabbit	0.2 mL/animal at each of 10 sites	B
	x	x	x	x	x	Extract of <i>Sample</i> in 1 in 20 Solution of Alcohol in Sodium Chloride Injection	Mouse	50 mL/kg	A (iv)
	x	x	x	x	x		Rabbit	0.2 mL/animal at each of 10 sites	B
		x		x	x	Extract of <i>Sample</i> in Polyethylene Glycol 400	Mouse	10 g/kg	A (ip)
				x	x		Rabbit	0.2 mL/animal at each of 10 sites	B
		x	x	x	x		Mouse	50 mL/kg	A (ip)
			x	x	x	Extract of <i>Sample</i> in Vegetable Oil	Rabbit	0.2 mL/animal at each of 10 sites	B
			x		x	Implant strips of <i>Sample</i>	Rabbit	4 strips/animal	C
			x		x	Implant <i>Sample</i>	Rat	2 <i>Samples</i> /animal	C

^a Tests required for each class are indicated by "x" in appropriate columns.

^b Legend: A (ip)—Systemic Injection Test (intraperitoneal); A (iv)—Systemic Injection Test (intravenous); B—Intracutaneous Test (intracutaneous); C—Implantation Test (intramuscular or subcutaneous implantation).

¹ USP High-Density Polyethylene RS.

DRUG PRODUCT VEHICLE (where applicable).

WATER FOR INJECTION (see monograph).

NOTE—The Sesame Oil or Cottonseed Oil or other suitable vegetable oil meets the following additional requirements. Obtain, if possible, freshly refined oil. Use three properly prepared animals, and inject the oil intracutaneously in a dose of 0.2 mL into each of 10 sites per animal, and observe the animals at 24, 48, and 72 hours following injection. Rate the observations at each site on the numerical scale indicated in *Table 2*. For the 3 rabbits (30 injection sites), at any observation time, the average response for erythema is not greater than 0.5 and for edema is not greater than 1.0, and no site shows a tissue reaction larger than 10 mm in overall diameter. The residue of oil at the injection site should not be misinterpreted as edema. Edematous tissue blanches when gentle pressure is applied.

Table 2. Evaluation of Skin Reactions

Erythema and Eschar Formation	Score
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet-redness) to slight eschar formation (injuries in depth)	4
Edema Formation ^a	Score
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

^a Excludes noninflammatory (mechanical) edema from the blank or extraction fluid.

Apparatus—The apparatus for the tests includes the following.

AUTOClave—Use an autoclave capable of maintaining a temperature of $121 \pm 2.0^\circ$, equipped with a thermometer, a pressure gauge, a vent cock, a rack adequate to accommodate the test containers above the water level, and a water cooling system that will allow for cooling of the test containers to about, but not below, 20° immediately following the heating cycle.

OVEN—Use an oven, preferably a forced-circulation model, that will maintain operating temperatures of 50° or 70° within $\pm 2^\circ$.

EXTRACTION CONTAINERS—Use only containers, such as ampuls or screw-cap culture test tubes, of Type I glass. If used, culture test tubes are closed with screw caps having suitable elastomeric liners. The exposed surface of the elastomeric liner is completely protected with an inert solid disk 0.05 mm to 0.075 mm in thickness. A suitable disk may be fabricated from a polytetrafluoroethylene resin.

Preparation of Apparatus—Cleanse all glassware thoroughly with chromic acid cleansing mixture, or if necessary with hot nitric acid, followed by prolonged rinsing with water. Clean cutting utensils by an appropriate method (e.g., successive cleaning with acetone and methylene chloride) prior to use in subdividing a specimen. Clean all other equipment by thorough scrubbing with a suitable detergent and prolonged rinsing with water.

Render containers and equipment used for extraction, and in transfer and administration of test material, sterile and dry by a suitable process. [NOTE—If ethylene oxide is used as the sterilizing agent, allow adequate time for complete degassing.]

Procedure—

PREPARATION OF SAMPLE—Both the *Systemic Injection Test* and the *Intracutaneous Test* may be performed using the same extract, if desired, or separate extracts may be made for each test. Select and subdivide into portions a *Sample* of the size indicated in *Table 3*. Remove particulate matter, such as lint and free particles, by treating each subdivided *Sample* or *Negative Control* as follows: place the *Sample* into a clean, glass-stoppered, 100-mL graduated cylinder of Type I glass, and add about 70 mL of *Water for Injection*. Agitate for about 30 seconds, and drain off the water. Repeat this step, and dry those pieces prepared for the extraction with *Vegetable Oil* in an oven at a temperature not exceeding 50° . [NOTE—Do not clean the *Sample* with a dry or wet cloth or by rinsing or washing with an organic solvent, surfactant, etc.]

PREPARATION OF EXTRACTS—Place a properly prepared *Sample* to be tested in an extraction container, and add 20 mL of the appropriate extracting medium. Repeat these directions for each extracting medium required for testing. Also, prepare one 20-mL blank of each medium for parallel injections and comparisons. Extract by heating in an autoclave at 121° for 60 minutes, in an oven at 70° for 24 hours, or at 50° for 72 hours. Allow adequate time for the liquid within the container to reach the extraction temperature. [NOTE—The extraction conditions should not in any instance cause physical changes such as fusion or melting of the *Sample* pieces, which result in a decrease in the available surface area. A slight adherence of the pieces can be tolerated. Always add the cleaned pieces individually to the extracting medium. If culture tubes are used for autoclave extractions

Table 3. Surface Area of Specimen To Be Used^a

Form of Material	Thickness	Amount of Sample for each 20 mL of Extracting Medium	Subdivided into
Film or sheet	<0.5 mm	Equivalent of 120 cm ² total surface area (both sides combined)	Strips of about 5 × 0.3 cm
	0.5–1 mm	Equivalent of 60 cm ² total surface area (both sides combined)	
Tubing	<0.5 mm (wall)	Length (in cm) = 120 cm ² /(sum of ID and OD circumferences)	Sections of about 5 × 0.3 cm
	0.5–1 mm (wall)	Length (in cm) = 60 cm ² /(sum of ID and OD circumferences)	
Slabs, tubing, and molded items	>1 mm	Equivalent of 60 cm ² total surface area (all exposed surfaces combined)	Pieces up to about 5 × 0.3 cm
Elastomers	>1 mm	Equivalent of 25 cm ² total surface area (all exposed surfaces combined)	Do not subdivide ^b

^a When surface area cannot be determined due to the configuration of the specimen, use 0.1 g of elastomer or 0.2 g of plastic or other polymers for every 1 mL of extracting fluid.

^b Molded elastomeric closures are tested intact.

with *Vegetable Oil*, seal screw caps adequately with pressure-sensitive tape.]

Cool to about room temperature but not below 20°, shake vigorously for several minutes, and decant each extract immediately, using aseptic precautions, into a dry, sterile vessel. Store the extracts at a temperature between 20° and 30°, and do not use for tests after 24 hours. Of importance are the contact of the extracting medium with the available surface area of the plastic and the time and temperature during extraction, the proper cooling, agitation, and decanting process, and the aseptic handling and storage of the extracts following extraction.

SYSTEMIC INJECTION TEST

This test is designed to evaluate systemic responses to the extracts of materials under test following injection into mice.

Test Animals—Use healthy, not previously used albino mice weighing between 17 and 23 g. For each test group use only mice of the same source. Allow water and food, commonly used for laboratory animals and of known composition, ad libitum.

Procedure—[NOTE—Agitate each extract vigorously prior to withdrawal of injection doses to ensure even distribution of the extracted matter. However, visible particulates should not be injected intravenously.] Inject each of the five mice in a test group with the *Sample* or the *Blank* as outlined in Table 4, except to dilute each g of the extract of the *Sample* prepared with *Polyethylene Glycol 400*, and the corresponding *Blank*, with 4.1 volumes of *Sodium Chloride Injection* to obtain a solution having a concentration of about 200 mg of polyethylene glycol per mL.

Table 4. Injection Procedure—Systemic Injection Test

Extract or Blank	Dose per kg	Route ^a	Injection Rate (μL per second)
Sodium Chloride Injection	50 mL	IV	100
1 in 20 solution of Alcohol in Sodium Chloride Injection	50 mL	IV	100
Polyethylene Glycol 400	10 g	IP	—
Drug product vehicle (where applicable)	50 mL	IV	100
	50 mL	IP	—
Vegetable Oil	50 mL	IP	—

^a IV = intravenous (aqueous sample and blank); IP = intraperitoneal (oleaginous sample and blank).

Observe the animals immediately after injection, again 4 hours after injection, and then at least at 24, 48, and 72 hours. If during the observation period none of the animals treated with the extract of the *Sample* shows a significantly greater biological reactivity than the animals treated with the *Blank*, the *Sample* meets the requirements of this test. If two or more mice die, or if abnormal behavior such as convulsions or prostration occurs in two or more mice, or if a body weight loss greater than 2 g occurs in three or more mice, the *Sample* does not meet the requirements of the test. If any animals treated with the *Sample* show only slight signs of biological reactivity, and not more than one animal shows gross symptoms of biological reactivity or dies, repeat the test using groups of 10 mice. On the repeat test, all 10 animals treated with the *Sample* show no significant biological reactivity above the *Blank* animals during the observation period.

INTRACUTANEOUS TEST

This test is designed to evaluate local responses to the extracts of materials under test following intracutaneous injection into rabbits.

Test Animals—Select healthy, thin-skinned albino rabbits with fur that can be clipped closely and skin that is free from mechanical irritation or trauma. In handling the animals, avoid touching the injection sites during observation periods, except to discriminate between edema and an oil residue. [NOTE—Rabbits previously used in unrelated tests, such as the *Pyrogen Test* (151), and that have received the prescribed rest period, may be used for this test provided that they have clean, unblemished skin.]

Procedure—[NOTE—Agitate each extract vigorously prior to withdrawal of injection doses to ensure even distribution of the extracted matter.] On the day of the test, closely clip the fur on the animal's back on both sides of the spinal column over a sufficiently large test area. Avoid mechanical irritation and trauma. Remove loose hair by means of vacuum. If necessary, swab the skin lightly with diluted alcohol, and dry the skin prior to injection. More than one extract from a given material can be used per rabbit, if it is determined that the test results will not be affected. For each *Sample* use two animals and inject each intracutaneously, using one side of the animal for the *Sample* and the other side for the *Blank*, as outlined in Table 5. [NOTE—Dilute each g of the extract of the *Sample* prepared with *Polyethylene Glycol 400*, and the corresponding *Blank*, with 7.4 volumes of *Sodium Chloride Injection* to obtain a solution having a concentration of about 120 mg of polyethylene glycol per mL.]

Table 5. Intracutaneous Test

Extract or Blank	Number of Sites (per animal)	Dose (μL per site)
Sample	5	200
Blank	5	200

Examine injection sites for evidence of any tissue reaction such as erythema, edema, and necrosis. Swab the skin lightly, if necessary, with diluted alcohol to facilitate reading of injection sites. Observe all animals at 24, 48, and 72 hours after injection. Rate the observations on a numerical scale for the extract of the *Sample* and for the *Blank*, using Table 2. Reclip the fur as necessary during the observation period. The average erythema and edema scores for *Sample* and *Blank* sites are determined at every scoring interval (24, 48, and 72 hours) for each rabbit. After the 72 hour scoring, all erythema scores plus edema scores are totalled separately for each *Sample* and *Blank*. Divide each of the totals by 12 (2 animals × 3 scoring periods × 2 scoring categories) to determine the overall mean score for each *Sample* versus each corresponding *Blank*. The requirements of the test are met if the difference between the *Sample* and the *Blank* mean score is 1.0 or less. If at any observation period the average reaction to the *Sample* is questionably greater than the average reaction to the *Blank*, repeat the test using three additional rabbits. The requirements of the test are met if the difference between the *Sample* and the *Blank* mean score is 1.0 or less.

IMPLANTATION TEST

The implantation test is designed for the evaluation of plastic materials and other polymeric materials in direct contact with living tissue. Of importance are the proper preparation of the implant strips and their proper implantation under aseptic conditions. The intramuscular implantation test requires healthy adult rabbits. The test specimens are placed into needles as the delivery system for implantation. Although most materials lend themselves readily to this

method, there are a number of materials that are unsuitable for intramuscular implantation. For materials with physical characteristics unsuitable for routine intramuscular implantation, the subcutaneous rat implantation model is a viable alternative.

Intramuscular Implantation in Rabbits

Prepare for implantation 8 strips of the *Sample* and 4 strips of USP High-Density Polyethylene RS. Each strip should measure not less than 10 × 1 mm. The edges of the strips should be as smooth as possible to avoid additional mechanical trauma upon implantation. Strips of the specified minimum size are implanted by means of a hypodermic needle (15- to 19-gauge) with intravenous point and a sterile trocar. Use either presterilized needles into which the sterile plastic strips are aseptically inserted, or insert each clean strip into a needle, the cannula and hub of which are protected with an appropriate cover, and then subjected to the appropriate sterilization procedure. [NOTE—Allow for proper degassing if agents such as ethylene oxide are used.]

Test Animals—Select healthy, adult rabbits weighing not less than 2.5 kg, and with paravertebral muscles that are sufficiently large in size to allow for implantation of the test strips. Do not use any muscular tissue other than the paravertebral site. The animals must be anesthetized with a commonly used anesthetic agent to a degree deep enough to prevent muscular movements, such as twitching.

Procedure—Perform the test in a clean area. On the day of the test or up to 20 hours before testing, clip the fur of the animals on both sides of the spinal column. Remove loose hair by means of vacuum. Swab the skin lightly with diluted alcohol, and dry the skin prior to injection.

Implant four strips of the *Sample* into the paravertebral muscle on one side of the spine of each of two rabbits, 2.5–5 cm from the midline and parallel to the spinal column, and about 2.5 cm apart from each other. In a similar fashion implant two strips of USP High-Density Polyethylene RS in the opposite muscle of each animal. Insert a sterile stylet into the needle to hold the implant strip in the tissue while withdrawing the needle. If excessive bleeding is observed after implantation of a strip, place a duplicate strip at another site.

Keep the animals for a period of not less than 120 hours, and sacrifice them at the end of the observation period by administering an overdose of an anesthetic agent or other suitable agents. Allow sufficient time to elapse for the tissue to be cut without bleeding. Examine macroscopically the area of the tissue surrounding the center portion of each implant strip. Use a magnifying lens and auxiliary light source. Observe the *Sample* and *Control* implant sites for hemorrhage, necrosis, discolorations, and infections, and record the observations. Measure encapsulation, if present, by recording the width of the capsule (from the periphery of the space occupied by the implant *Control* or *Sample* to the periphery of the capsule) rounded to the nearest 0.1 mm. Score encapsulation according to *Table 6*.

Table 6. Evaluation of Encapsulation in the Implantation Test

Capsule Width	Score
None	0
Up to 0.5 mm	1
0.6–1.0 mm	2
1.1–2.0 mm	3
Greater than 2.0 mm	4

Calculate the differences between average scores for the *Sample* and *Control* sites. The requirements of the test are met if the difference does not exceed 1.0, or if the difference between the *Sample* and *Control* mean scores for more than one of the four implant sites does not exceed 1 for any implanted animal.

Subcutaneous Implantation in Rats

Prepare for implantation 10 sample specimens and 10 control specimens. The size and shape of the control specimens shall be as similar to that of the test specimens as practically possible. For example, specimens made of sheeting material shall be 10–12 mm in diameter and from 0.3 to 1 mm in thickness. The edges of the specimens should be as smooth as possible to avoid additional mechanical trauma upon implantation.

Test Animals—Select healthy rats weighing between 225 and 350 g at the time of implantation.

Procedure—Perform the test in a clean area. Anesthetize the animal until a surgical plane is achieved. Clip the fur of the animals on both sides of the spinal column. Remove loose hair by means of vacuum. Clean the clipped area with povidone-iodine solution. Using aseptic technique, make two midline incisions (approximately 1.0 cm long) through the skin at the cranial and caudal regions on the dorsal surface. Using blunt dissection, separate the fascia connecting skin to muscle to form a pocket underneath the skin lateral to each side of the incision (base of pocket approximately 20 mm from the line of implant). Insert a sterile sample into each pocket, and close the incision with wound clips or sutures. Implant two test samples and two control samples in each of five rats. Keep the animals for a period of at least seven days, and sacrifice them at the end of the observation period by CO₂ induced hypoxia or administering an overdose of an anesthetic agent. Allow sufficient time to elapse for the tissue to be cut without bleeding. Cut the skin (dorsal surface) longitudinally and lay back. Carefully examine macroscopically the area of the tissue surrounding the implant. Cut the sample in half and remove for close examination of the tissue in direct contact with the sample. Use a magnifying lens and auxiliary light source, if appropriate. Observe the *Sample* and *Control* implant sites for hemorrhage, necrosis, discolorations, and infections, and record the observations. Measure encapsulation, if present, by recording the width of the capsule (from the periphery of the space occupied by the implant *Control* or *Sample* to the periphery of the capsule) rounded to the nearest 0.1 mm. Score encapsulation according to *Table 6*. Calculate the differences between average scores for the *Sample* and *Control* sites. The requirements of the test are met if the difference does not exceed 1.0.

SAFETY TESTS—BIOLOGICALS

The safety test set forth here is intended to detect in an article any unexpected, unacceptable biological reactivity. This *in vivo* test is provided for the safety assessment of biologics (see *Biologics* (1041)) and biotechnology-derived products.

Safety Test

Select five healthy mice not previously used for testing, weighing between 17 and 23 g, unless otherwise directed in the individual monograph or elsewhere in this chapter, and maintained on an adequate balanced diet. Prepare a test solution as directed in the individual monograph. Unless otherwise directed in the individual monograph or elsewhere in this chapter, inject intravenously a dose of 0.5 mL of the test solution into each of the mice, using a 26-gauge needle of suitable length, or of the length specified below as applicable. Observe the animals over the 48 hours following the injection. If, at the end of 48 hours, all of the animals survive and not more than one of the animals shows outward symptoms of a reaction not normally expected of the level of toxicity related to the article, the requirements of this test are met. If one or more animals die or if more than one of the animals shows signs of abnormal or unto-

ward toxicity of the article under test, repeat the test using at least another 10 mice similar to those used in the initial test, but weighing 20 ± 1 g. In either case, if all of the animals survive for 48 hours and show no symptoms of a reaction indicative of an abnormal or undue level of toxicity of the article, the requirements of the test are met.

For biologics, perform the test according to the procedures prescribed in the *Federal Regulations* (see *Biologics* (1041)), Section 610.11, using not less than two mice similar to those described above but weighing less than 22 g and not less than two healthy guinea pigs weighing less than 400 g. Unless otherwise directed in the individual monograph, for a liquid product or a freeze-dried product that has been constituted as directed in the labeling, inject a volume of 0.5 mL intraperitoneally into each mouse, and inject a volume of 5.0 mL intraperitoneally into each guinea pig. For freeze-dried products for which the volume of constitution is not indicated in the label, or for nonliquid products other than freeze-dried products, perform the test using the route of administration, test dose, and diluent approved by the Center for Biologics Evaluation and Research (FDA), on the basis of substantial evidence demonstrating that the test variation will assure sensitivity equal to or greater than that of the test described above. Observe the animals for a minimum observation period of 7 days. If all of the animals survive the test period, do not exhibit any response that is not specific for or expected from the product and that may indicate a difference in such product quality, and weigh no less at the end of the test period than at the time of injection, the requirements of the test are met. If the article fails to meet the requirements, the test may be repeated as in the initial test, in the one or both species in which the requirements were not met. If the animals fulfill the criteria specified for the initial test, the article meets the requirements of the test. If the article fails to meet the requirements after the first repeat test, and not less than 50% of the total number of animals of the species in which the requirements of the test were not met in the combined initial and first retests have survived, a second retest may be performed. Use twice the number of animals of the relevant species used in the initial test. If the animals fulfill the criteria specified for the initial test, the requirements of the test are met.

(90) FETAL BOVINE SERUM— QUALITY ATTRIBUTES AND FUNCTIONALITY TESTS

PROCESSING

Fetal bovine serum (FBS) is the light-brown liquid fraction of clotted fetal bovine blood. It is depleted of cells, fibrin, and clotting factors. Although the complete composition of FBS is undefined, FBS contains high levels of growth factors and low levels of immunoglobulins. In addition, it contains other key ingredients that are essential in supporting proliferation of cells in culture. This product is used both in life science basic research and industrial manufacturing. FBS is a by-product of the meat industry and is collected from bovine fetuses removed from cattle found to be pregnant at slaughter. FBS is harvested from abattoirs that are inspected by the competent authority in the country of origin. Trained personnel following written and approved procedures should perform collection and processing. Blood is collected

in a closed system in a dedicated area within the facility, and processed quickly to prevent hemolysis. The blood is allowed to clot and then typically is centrifuged in a refrigerated centrifuge to separate the serum from the other components. Serum typically is removed from the clot, transferred to labeled containers, and frozen. All manufacturers employ sterile filtration before final packaging. Additionally, gamma irradiation provides the highest assurance of the absence of viral activity. Gamma irradiation doses of 25–40 kGy provide significant log reduction of viral and other adventitious agents while preserving cellular growth performance.

The screening of FBS for viral contamination is accomplished by using all applicable testing described in the Code of Federal Regulations 9 CFR 113.53 (known as full 9 CFR testing). Mycoplasma assays are performed as described in *Mycoplasma Tests* (63).

FETAL BOVINE SERUM QUALITY ATTRIBUTES

Packaging and Storage: Store in sealed containers at a temperature of -10° or below.

Labeling: Label it to indicate that contents are Fetal Bovine Serum, and indicate lot number, expiration date, and storage conditions. Also, indicate country of origin on product labeling.

USP Reference Standards (11)

USP Endotoxin RS

USP Fetal Bovine Serum RS

pH (791): 7.00–8.00, in undiluted serum samples

Osmolality (785): 280–360 mOsmol/kg

Bacterial Endotoxins (85): It contains not more than 10 USP Endotoxin Units/mL of serum.

Total Protein Content (1057): 30–45 mg/mL

Sterility Tests (71): Meets the requirements

Identification—Radial Immunodiffusion

Reagents

- FBS test samples
- Horse serum, negative control samples
- Bovine IgG calibrator (500 mg/L)
- Sheep albumin diluent (1% Sheep albumin, 0.18% EDTA, 1.75% NaCl, and 1.21% Tris/HCl pH 7.4).

Materials/Apparatus: Ring measuring device is calibrated in 0.1-mm increments. Radial immunodiffusion (RID) plates are commercially available and contain anti-bovine IgG antiserum in a 1.5% agarose gel, 0.1 M phosphate buffer, pH 7.0, 0.1% sodium azide as bacteriostatic agent, and 1 μ g/mL amphotericin B as an antifungal agent. Store at 2° – 8° . Use RID plates that can measure bovine IgG in the range of 50–500 mg/L.

Standard curve: Use the bovine IgG calibrators for system suitability and for generation of a calibration curve. Prepare two dilutions from a 500 mg/L bovine IgG stock solution. Dilute 120 μ L of the 500 mg/L stock with 80 μ L of diluent (medium dilution) and 25 μ L of the 500 mg/L stock with 225 μ L diluent (low dilution). Label each dilution respectively as 300 mg/L and 50 mg/L calibrators. Use the 500 mg/L, 300 mg/L, and 50 mg/L solutions to generate the standard curve. [NOTE—Prepare and analyse the calibrator bovine IgG solutions in duplicate.] Load 5 μ L of each sample into the 2.5-mm wells of the plate. At 72 h of incubation, measure ring diameters to the nearest 0.1 mm using an appropriate ring measuring device. Record the results and proceed to the generation of a standard curve.

The ring diameter should develop to completion at room temperature for 72 h. Using the result from each data point of the standard curve, generate a single linearity plot where y is the squared diameter (mm^2) of precipitin ring around the well and x is the Bovine IgG concentration (mg/L). Calculate the linear least-squares-fit regression line of the form $y = m(x) + b$ with the help of suitable software and deter-

mine the values for slope (m), y-intercept (b), and coefficient of determination (R^2). The standard curve for the method is linear if R^2 is ≥ 0.98 .

Analysis: Frozen undiluted samples of FBS are thawed and tested within 24 h if stored at 4°. Testing of FBS test and USP Fetal Bovine Serum RS samples is performed in triplicate. Prepare RID plates containing anti-bovine IgG to be tested for the various types of sera. Allow plates and reagents to equilibrate to room temperature before use by leaving the plates open for 10–15 min at room temperature to allow any condensation in the wells or on the gel surface to evaporate. Samples should not be applied to wells where moisture is visible. Prepare serial dilutions, if necessary, of FBS test and USP Fetal Bovine Serum RS samples in diluent. Dilute the negative control horse serum in diluent. Load 5 μ L of each sample into the 2.5-mm wells of the plate, and incubate at room temperature for 72 h. [NOTE—The test samples and the negative control are loaded on the same plate.]

Calculation: After 72 h, measure the diameters of the rings using the ring measuring device, and record the results. Using the regression equation developed under standard curve deviation, calculate the concentration of bovine IgG in FBS samples. Concentration is expressed as mg/L.

Acceptance criteria: Horse serum is negative (should not give a precipitation ring). FBS test and USP Fetal Bovine Serum RS samples are positive and contain NMT 500 mg/L of IgG.

Hemoglobin content:

(See *Spectrophotometry and Light Scattering* (851).)

Sample preparation: FBS samples are thawed, are stored at 4°, and are tested within the same day.

Analysis: Determine the absorbance of the serum sample using a spectrophotometric cell of 1-cm path length at the wavelengths of absorbance at 576, 623, and 700 nm and using water as a blank. Calculate the concentration of hemoglobin in mg/dL:

$$(\text{Abs}_{576} \times 115) - (\text{Abs}_{623} \times 102) - (\text{Abs}_{700} \times 39.1)$$

Acceptance criteria: NMT 30 mg/dL

FBS FUNCTIONALITY TESTS

In the absence of a user-defined functionality assay, the following tests are suitable to determine the functionality of specific lots of FBS and to aid in the optimization of the growth conditions of mammalian cell cultures in the presence of FBS. For valid functionality confirmation independent of user-specific applications, tests are performed on the specified cell lines. For in-house validation of specialized cell culture applications, cell line(s) specific to those applications should be used and characterized. Use appropriate tissue culture vessels. Two tests described in this chapter are the *Growth-Promotion Curve* and the *Clonal Assay*. The decision about which type of test or the number of tests to be performed to assess suitability of a specific lot of FBS depends on the type of cell line used. For adherent cell lines, the number of colonies at the end of the culturing period represents a good assessment of the capacity of these cells, at low concentration, to grow in the presence of a specific lot of FBS. For cell lines growing in suspension cultures, the optimum growth kinetics is measured by counting viable cells after 7 days of culture.

Cell lines: Five cell lines are recommended for use:

- (1) HFL1 (ATCC CCL-153) normal lung, fibroblast
- (2) Mv1 Lu (ATCC CCL-64) mink lung, epithelial
- (3) HL-60 (ATCC CCL-240) peripheral blood promyeloblast, suspension
- (4) VERO (ATCC CCL-81) monkey kidney fibroblast
- (5) CHO (CCL-61) Chinese hamster ovary

The functionality tests described are to be performed on three cell lines, two of which are drawn from the five recommended cell lines and the third of which is the cell line relevant to the user's application. Cell lines are cultured with specific media as recommended by ATCC.

Materials

- Suitable growth vessel/container
- Biological Safety Cabinet Class II, Type A
- Cell counter/hemocytometer
- Inverted microscope with digital camera accessory
- Tissue culture vessels: T25 cm²

Preparation of cells for assays: Quick-thaw a vial in a 37° water bath, and determine cell count and viability. Prepare multiple cultures from each cell line in serum-supplemented growth medium. Incubate the cultures at 37° following instructions provided by ATCC for each of the cell lines used for the test. Examine the prepared cultures under a microscope to ensure uniform, near-confluent monolayers or suspensions. Expand cells until there are enough for assay (about 1×10^7 total cells; >90% viability).

Harvesting of cultures

1. Remove and discard the growth medium, and then rinse each culture with media lacking FBS.
2. For adherent cells, add 1 mL of Trypsin/EDTA for a few minutes for cells to disperse. Incubate at 37°, if necessary. Neutralize with 1 mL culture medium containing at least 10% FBS.
3. Spin down the cells in a centrifuge. Aspirate off wash media, and resuspend cells in an appropriate volume for seeding.

Seeding of cells

1. On day 0: For the three cell lines to be tested prepare multiple cultures using seeding densities that range between 2×10^3 and 2×10^4 viable cells/mL. (Different inocula are chosen initially to determine optimum growth conditions. Once the appropriate inoculum is chosen, that condition is used to propagate the cells.) Following are the recommended seeding densities:
 Low seeding density: 2×10^3 viable cells/mL
 Mid seeding density: 6×10^3 viable cells/mL
 High seeding density: 2×10^4 viable cells/mL
2. Prepare cultures in triplicate for at least five time points (in days or hours according to the cell line), to determine the seeding density that will yield the optimal growth conditions for each cell line used.
3. Incubate the cultures at 37° in a humidified incubator saturated with 5% CO₂.
4. For each time point of measurement (days 0, 1, 2, 3, 4, and 7), take a photograph of each culture, in triplicate, for both the FBS test material and the USP Fetal Bovine Serum RS at each of the three concentrations for each cell line, and record the percentage of confluency for each of the conditions. [NOTE—Perform this step before trypsinization and cell counting.]
5. Harvest the cells from the three different seeding density cultures for each specific time point. For adherent cultures, harvest cells as described above.
6. Perform and record total cell count and viability for each of the nine cultures for the FBS test and the USP Fetal Bovine Serum RS for each cell line using an appropriate cell counter or hemacytometer. [NOTES—The schedule for counting may have to be changed for fast-growing cell lines or large cells that would become confluent before day 7 and/or for slow-growing lines that need to be in culture 8–10 days before reaching a plateau. Some adherent cell lines will never reach confluency.]

Growth-Promotion Curve

Measurements of cell proliferation rates often are used to determine the response of cells to exogenous stimuli. Quantitative assessment of cell growth conditions is an important

factor in monitoring consistency of culture conditions. The optimal cell concentration range for subculturing, optimum inoculum, and doubling time are parameters that can be quantified and trended. Information about the growth kinetics of a culture is critical in the design of cell-based experiments. Cultures vary significantly in their growth properties from lag phase, log phase, and stationary phase. Document the growth characteristics of the culture during the three growth stages to determine population doubling time and cell cycle time. Cells that have entered the stationary phase may demonstrate reduced growth potential and change in morphology. Cells may become polarized and may secrete more extracellular matrix, making them difficult to remove from the substratum. Cells at the end of the log phase give the highest yield and greatest reproducibility.

Reagents

- Growth media without FBS
- FBS test samples
- Growth medium + 10% FBS
- Trypsin/EDTA solution (0.25%/0.53 mM) in Hank's Balanced Salt Solution (HBSS)

Analysis: Once the cells have reached the end of the log phase, subculture the cells for the test. Follow the procedure described under *Seeding Cells* and prepare multiple cultures for the USP Fetal Bovine Serum RS, and test FBS for different cell lines at three seeding densities for which at least one growth curve displays a lag phase, log phase, and stationary phase and for which the log phase is linear at three or more time points.

Viable cell counts are determined on days 0, 1, 2, 3, 4, and 7.

Calculation and Data analysis: Calculate the mean viable count [cells/cm² (adherent) or cells/mL (suspension)] and the mean percent viability for each data point. Plot the data on a semi-log scale graph with the viable count on the log scale on the y-axis and days (or hours) in culture on an arithmetic scale on the x-axis. Estimate the doubling time using a growth curve that is linear over three or more time points.

Acceptance criteria: The R^2 value of the line should be equal to or greater than 0.98 in order to support calculation of a valid doubling time. The doubling time of the test sample should be no less than 90% of the doubling time of USP Fetal Bovine Serum RS.

Clonal Assay

This assay is designed to assess the optimal growth for adherent cell lines. Plating efficiency or colony formation at low cell density is a preferred method for analyzing the proliferative capacity and survival of single cells under optimal growth conditions. This is a very sensitive test and is often used for assessing the quality of serum lots. This technique reveals differences in the growth rate within the cell population and is capable of distinguishing between changes in growth rate (colony size) and cell survival (colony number). Because of the heterogeneous cell population of some cell cultures, remember that cells grow differently as isolated colonies at low densities. Consequently, few cells survive even under ideal conditions because all cell interaction is lost. Cloning is a survival assay that is also used for optimizing growth conditions (selection of medium and serum). If it can be confirmed that a single colony arose from a single cell, then cloning efficiency can be determined.

Reagents

- Growth medium + 10% FBS (test serum)—Eagle minimum essential medium (EMEM) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium carbonate, 0.1 mM nonessential amino acids, and sodium pyruvate containing 100 U/mL penicillin and 100 g/mL streptomycin plus 10% FBS.
- Trypsin/EDTA solution (0.25%/0.53 mM) in HBSS.

- Dulbecco's Phosphate Buffer Saline without calcium or magnesium.
- Carbol Fuchsin–Methylene Blue Solution—Mix 20 g carbol fuchsin stock in 2 L methanol and stir for 10 min (1% carbol fuchsin). Mix 50 g methylene blue in 5 L methanol, and stir for 10 min (1% methylene blue). Prepare Carbol Fuchsin–Methylene Blue working solution by mixing methylene blue, methanol, and carbol fuchsin in a ratio of 3:2:1. Mix for 20 min and filter through four folds of cheesecloth in a funnel. Aliquot and store in brown glass bottles at 15° to 25°.

Sample: Multiple lots of FBS are used for this assay. For each lot of serum to be tested, add 20 mL of FBS to 180 mL of EMEM, and use the same sample for the entire test. Sterilize using 0.22-μm low protein binding filter units. Store growth medium at 4° until ready to use.

Cell preparation: This test is only for adherent cultures and is performed with the adherent cell lines described under *Cell Lines* (HFL1 and Mv 1 Lu). One week before testing serum, expand the cell lines as described under *Seeding of Cells*, change the medium every 2–3 days, and subculture the cells when they are about 90% confluent. Determine the cell count and viability (viability should be >90%) before performing the assay. Harvest cells as described under *Harvesting of Cultures*, wash twice, and resuspend cells in basal EMEM.

Analysis: The procedure involves plating single-cell suspension at low densities (2–50 cells/cm²) from which discrete colonies will form. At the end of the assay, fix, stain, and count the number of colonies as directed below.

1. For each cell line label ten 60-mm × 15-mm tissue culture dishes for each serum lot that will be tested. Label the side of the lower half of each dish, including controls.
2. Transfer 5 mL of medium containing 10% of the appropriate test serum (10 replicates). Add 400 cells per culture dish (aim for a cell concentration of about 800 cells/mL).
3. Incubate for 10–14 days at 37° in a humidified incubator saturated with 5% CO₂.
4. Remove the supernatant and add enough Carbol Fuchsin–Methylene Blue Solution to cover each of the culture dishes for 10 min.
5. Remove the stain; rinse the culture dishes with several changes of distilled water; invert the dishes on paper towels; and allow to dry.
6. Count and record (1) the number of colonies and (2) the total surface of stained colonies (mm²). Calculate means and standard deviations.

Acceptance criteria: Percent plating efficiency is expressed by counting the number of colonies in a defined area divided by the number of cells seeded multiplied by 100. Compare results between lots of FBS, and select a serum lot that is good for various types of cells and optimal for a specific cell culture application.

(91) CALCIUM PANTOTHENATE ASSAY

USP Reference Standards (11)—*USP Calcium Pantothenate RS*

Standard Stock Solution of Calcium Pantothenate—Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and accurately weighed while protected from absorption of

moisture during the weighing, in about 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), then dilute with water to volume. Each mL represents 50 µg of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard Preparation—On the day of the assay, dilute a measured volume of *Standard Stock Solution of Calcium Pantothenate* with sufficient water so that it contains, in each mL, between 0.01 µg and 0.04 µg of calcium pantothenate, the exact concentration being such that the responses obtained as directed for *Procedure*, 2.0 and 4.0 mL of the *Standard Preparation* being used, are within the linear portion of the log-concentration response curve.

Assay Preparation—Proceed as directed in the individual monograph for preparing a solution expected to contain approximately the equivalent of the calcium pantothenate concentration in the *Standard Preparation*.

Basal Medium Stock Solution—

Acid-hydrolyzed Casein Solution	25 mL
Cystine-Tryptophane Solution	25 mL
Polysorbate 80 Solution	0.25 mL
Dextrose, Anhydrous	10 g
Sodium Acetate, Anhydrous	5 g
Adenine-Guanine-Uracil Solution	5 mL
Riboflavin-Thiamine Hydrochloride-Biotin Solution	5 mL
Para-aminobenzoic Acid-Niacin-Pyridoxine Hydrochloride Solution	5 mL
Salt Solution A	5 mL
Salt Solution B	5 mL

Dissolve the anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL, and mix.

Acid-Hydrolyzed Casein Solution—Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8 to 12 hours. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-Tryptophane Solution—Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophane (or 2.0 g of D,L-tryptophane) in 700 to 800 mL of water, heat to 70° to 80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a refrigerator at a temperature not below 10°.

Adenine-Guanine-Uracil Solution—Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid, cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 Solution—Dissolve 25 g of polysorbate 80 in alcohol to make 250 mL.

Riboflavin-Thiamine Hydrochloride-Biotin Solution—Prepare a solution containing, in each mL, 20 µg of riboflavin, 10 µg of thiamine hydrochloride, and 0.04 µg of biotin, by dissolving riboflavin, thiamine hydrochloride, and biotin in 0.02 N acetic acid. Store, protected from light, under toluene in a refrigerator.

Para-aminobenzoic Acid-Niacin-Pyridoxine Hydrochloride Solution—Prepare a solution in neutral 25 percent alcohol to contain 10 µg of para-aminobenzoic

acid, 50 µg of niacin, and 40 µg of pyridoxine hydrochloride in each mL. Store in a refrigerator.

Salt Solution A—Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, and store under toluene.

Salt Solution B—Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and store under toluene.

Stock Culture of *Lactobacillus plantarum*—Dissolve 2.0 g of water-soluble yeast extract in 100 mL of water, add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture, with stirring, on a steam bath, until the agar dissolves. Add approximately 10-mL portions of the hot solution to test tubes, suitably close or cover the tubes, sterilize at 121°, and allow the tubes to cool in an upright position. Prepare stab cultures in 3 or more of the tubes, using a pure culture of *Lactobacillus plantarum*,* incubating for 16 to 24 hours at any selected temperature between 30° and 37° but held constant to within $\pm 0.5^\circ$, and finally store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for inoculum if the culture is more than 1 week old.

Culture Medium—To each of a series of test tubes containing 5.0 mL of *Basal Medium Stock Solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121°, and cool.

Inoculum—Make a transfer of cells from the stock culture of *Lactobacillus plantarum* to a sterile tube containing 10 mL of culture medium. Incubate this culture for 16 to 24 hours at any selected temperature between 30° and 37° but held constant to within $\pm 0.5^\circ$. The cell suspension so obtained is the inoculum.

Procedure—To similar test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL, respectively, of the *Standard Preparation*. To each tube and to 4 similar tubes containing no *Standard Preparation* add 5.0 mL of *Basal Medium Stock Solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Assay Preparation* corresponding to 3 or more of the levels listed above for the *Standard Preparation*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal Medium Stock Solution* and sufficient water to make 10 mL. Place one complete set of Standard and Assay tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series suitably to prevent contamination, and heat in an autoclave at 121° for 5 minutes. Cool, add 1 drop of inoculum to each tube, except 2 of the 4 tubes containing no *Standard Preparation* (to serve as the uninoculated blanks), and mix. Incubate the tubes at a temperature between 30° and 37°, held constant to within $\pm 0.5^\circ$ until, following 16 to 24 hours of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of standard during a 2-hour period.

Determine the transmittance of the tubes in the following manner: Mix the contents of each tube, and transfer to an optical container if necessary. Place the container in a spectrophotometer that has been set at a specific wavelength between 540 nm and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 seconds or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is

* American Type Culture Collection No. 8014 is suitable. This strain formerly was known as *Lactobacillus arabinosus* 17-5.

evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation—Prepare a standard concentration-response curve as follows. For each level of the standard, calculate the response from the sum of the duplicate values of the transmittance as the difference, $y = 2.00 - \Sigma$ (of transmittance). Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard Preparation* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y , adding together the two transmittances for each level of the *Assay Preparation*. Read from the standard curve the logarithm of the volume of the *Standard Preparation* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Assay Preparation* to obtain the difference, x , for each dosage level. Average the values of x for each of three or more

dosage levels to obtain $\bar{x} = M'$, the log-relative potency of the *Assay Preparation*. Determine the quantity, in mg, of USP Calcium Pantothenate RS corresponding to the calcium pantothenate in the portion of material taken for assay as antilog:

$$M = \text{antilog } (M' + \log R)$$

in which R is the number of mg of calcium pantothenate that was assumed to be present in each mg (or capsule or tablet) of the material taken for assay.

Replication—Repeat the entire determination at least once, using separately prepared *Assay Preparations*. If the difference between the two log-potencies M is not greater than 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *The Confidence Interval and Limits of Potency* (111)). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

<92> GROWTH FACTORS AND CYTOKINES USED IN CELL THERAPY MANUFACTURING

INTRODUCTION

Qualification of reagents, source materials, and control of the manufacturing process are key elements that ensure the quality and safety of cell therapies. Growth factors and cytokines are important for the maintenance, growth, selection, and purification of cultures of cell therapy products. This chapter describes the accepted tests, procedures, and acceptance criteria for growth factors and cytokines that may be involved in the manufacturing of cell therapy products.

RECOMBINANT HUMAN INTERLEUKIN 4 (rhIL-4)

MHKCDITLQE	IIKTLNSLTE	QKTLCTELTV	TDIFAASKNT
TEKETFCRAA	TVLRQFYSHH	EKDTRCLGAT	AQQFHRHKQL
IRFLKRLDRN	LWGLAGLNSC	PVKEANQSTL	ENFLERLKT
MREKYSKCSS			

C₆₅₈H₁₀₇₁N₁₉₃O₁₉₇S₈

15,096 Da

rhIL-4 is a single-chain polypeptide of 130 amino acid residues expressed in *Escherichia coli*. It is produced as a lyophilized powder and contains NLT 0.5×10^7 USP Units of IL-4/mg of total protein. Process specific host-cell DNA impurities in IL-4 with limits of less than 1 ng/mg are determined as described in *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* <1130>. Neither manufacturing license nor market approval is required for IL-4 intended for use as an ancillary material during manufacturing. Following are typical IL-4 quality attributes.

IDENTIFICATION

- A.** Amino-terminal sequence analysis of at least eight amino acids is performed with an automated sequencer, as described in *Biotechnology-Derived Articles* <1045>. Stepwise-released phenylthiohydantoin amino acids are identified with on-line reversed-phase high-performance liquid chromatography, on the basis of their elution times.
- B.** Use the electrophoresis method followed by western blotting analysis to visualize the IL-4 protein. The method is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), described in the test for *Purity*.
Phosphate buffered saline; Laemmli sample buffer, reducing; and Laemmli sample buffer, nonreducing: Proceed as directed in the test for *Purity* in the *Assay*.
Standard stock solution: 50 µg/mL of reconstituted USP rHuman Interleukin 4 RS in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]
Standard solution: 20 µg/mL of IL-4, from *Standard stock solution*, in *Phosphate buffered saline*.
Standard solution, reducing: Combine 20 µL of *Standard solution* and 5 µL of *Laemmli sample buffer, reducing*.
Standard solution, nonreducing: Combine 20 µL of *Standard solution* and 5 µL of *Laemmli sample buffer, nonreducing*.
Sample stock solution: 50 µg/mL of reconstituted IL-4 in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]
Sample solution: 20 µg/mL of IL-4, from *Sample stock solution*, in *Phosphate buffered saline*.
Sample solution, reducing: Combine 20 µL of *Sample solution* and 5 µL of *Laemmli sample buffer, reducing*.
Sample solution, nonreducing: Combine 20 µL of *Sample solution* and 5 µL of *Laemmli sample buffer, nonreducing*.
Analysis
Samples: *Standard solution, reducing; Standard solution, nonreducing; Sample solution, reducing; and Sample solution, nonreducing*

Western blotting: After electrophoresis, the proteins are transferred onto a polyvinylidene fluoride (PVDF) membrane using standard procedures. Incubate the membrane for 1 h at room temperature with *Phosphate buffered saline* containing 0.1% Tween 20 and 5% skim milk powder. The membrane is then incubated with an anti-IL-4 antibody¹ (diluted appropriately in *Phosphate buffered saline*), followed by incubation with a secondary antibody at room temperature under gentle agitation for 1 h for each of the antibodies. The IL-4 protein band is identified by developing the membrane using a suitable detection system.²

Acceptance criteria: The developed Western blot should give a positive signal equivalent to the USP rHuman Interleukin 4 RS.

ASSAY

- PURITY:** [NOTE—Purity is determined on the bulk material.] SDS-PAGE is performed as described under *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* <1056> under reducing and nonreducing conditions.
Molecular weight marker: Use a suitable molecular weight marker containing protein bands between 10 and 200 kDa.
Phosphate buffered saline: 2.67 mM of potassium chloride, 1.47 mM of potassium phosphate (KH₂PO₄), 137.93 mM of sodium chloride, and 8.06 mM of dibasic sodium phosphate in water. Adjust to a pH of 7.0–7.3.
Laemmli sample buffer, nonreducing: 100 mM TRIS-HCl, pH 6.8, 50% glycerol, 0.25% bromophenol blue indicator, and 10% sodium lauryl sulfate in water
Laemmli sample buffer, reducing: Add 2.5 µL mercaptoethanol to 50 µL of *Laemmli sample buffer, nonreducing*.
Sample stock solution: 400 µg/mL of bulk IL-4 in *Phosphate buffered saline*
Sample solution 1: Combine 20 µL of *Sample stock solution* and 5 µL of *Laemmli sample buffer, nonreducing*.
Sample solution 2: Combine 20 µL of *Sample stock solution* and 5 µL of *Laemmli sample buffer, reducing*.
Control A stock solution: 4 µg/mL of IL-4, from *Sample stock solution*, in *Phosphate buffered saline*. [NOTE—Control A solutions are run in triplicates in both reducing and nonreducing conditions.]
Control A solution 1: Combine 20 µL of *Control A stock solution* and 5 µL of *Laemmli sample buffer, nonreducing*.
Control A solution 2: Combine 20 µL of *Control A stock solution* and 5 µL of *Laemmli sample buffer, reducing*.
Control B stock solution: 12 µg/mL of IL-4, from *Sample stock solution*, in *Phosphate buffered saline*. [NOTE—Control B solutions are run in duplicates in both reducing and nonreducing conditions.]
Control B solution 1: Combine 20 µL of *Control B stock solution* and 5 µL of *Laemmli sample buffer, nonreducing*.
Control B solution 2: Combine 20 µL of *Control B stock solution* and 5 µL of *Laemmli sample buffer, reducing*.
Electrophoretic conditions
 (See *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* <1056>.)
Mode: Discontinuous PAGE gel
Stacking gel: 4% acrylamide
Resolving gel: 12% acrylamide
Run conditions: 10 min at 100 V; then 30 min at 200 V
Protein detection: Silver stain
Analysis
Samples: *Sample solution 1, Sample solution 2, Control A solution 1, Control A solution 2, Control B solution 1, and Control B solution 2*
 Incubate 25 µL of each *Sample solution* and *Control solution* under nonreducing conditions for 5 min at 60°, and load onto the gel. Incubate 20 µL of each *Sample solution* and

¹A suitable anti-IL-4 antibody can be obtained from commercial sources (e.g., Dianova Inc.).

²A suitable detection system can be obtained from commercial sources (e.g., Pierce/Perbio Science).

Control solution under reducing conditions for 5 min at 60°, and load onto the gel. After silver staining and scanning the whole gel, determine the intensity of all detectable protein bands by densitometry, and calculate the percentage of each detectable protein band, in the *Sample solution*, twice by comparing the pixel intensity of each contaminating band with the mean value of *Control solutions A* and *B*, respectively, by the formulas:

$$\text{Result} = (A_{100}) \times 1/(A_1) \text{ and}$$

$$\text{Result} = (A_{100}) \times 3/(A_3)$$

A_{100} = intensity of one contaminating band of the *Sample solution*

A_1 = mean intensity of all detectable bands of *Control A solution*

A_3 = mean intensity of all detectable bands of *Control B solution*

IL-4 control solutions analysis should yield one detectable band with an apparent molecular weight of approximately 15 kDa. If values calculated by means of *Control A solution* are different from those revealed by comparison with *Control B solution*, the value corresponding to the highest amount of impurity should be taken. If the intensity of one of the contaminating bands is lower than the value of *Control A solution* (corresponding to 1%), the value of this contamination is set to 1%. The purity of the sample solution is then calculated:

$$\text{Result} = 100 - \sum C_n$$

C = percentage of each contamination given in rounded whole numbers

n = number of contaminants of the IL-4 *Sample solution*

Acceptance criteria: The purity of IL-4 is NLT 97%, as determined by SDS-PAGE.

- **PROTEIN CONTENT:** [NOTE—Protein content is determined on the basis of the packaged product.]

Phosphate buffered saline: Proceed as directed in the test for *Purity*.

Sample solution: 50 µg/mL of IL-4 in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]

Blank: *Phosphate buffered saline*

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV

Pathlength: 1 cm

Analytical wavelength: 280 nm

Analysis

Samples: *Sample solution* and *Blank*

Calculate the protein concentration:

$$C = A_{280}/0.63$$

C = IL-4 concentration of the *Sample solution* (mg/mL)

A_{280} = absorbance at 280 nm

SPECIFIC TESTS

- **BIOIDENTITY:** [NOTE—The biological activity measurement is determined on the basis of the packaged product.]

RPMI 1640 medium with L-glutamine: Prepare a mixture of the ingredients in the quantities shown in sufficient water to obtain 1 L of medium, and sterilize by filtration:

Material	Quantity
Calcium nitrate (Ca(NO ₃)-4H ₂ O)	100 mg
Magnesium sulfate (MgSO ₄ -7H ₂ O)	100 mg
Potassium chloride	400 mg
Sodium chloride	6000 mg
Sodium phosphate, dibasic anhydrous	800 mg

Material	Quantity
Sodium bicarbonate	2000 mg
Glycine	10 mg
L-Arginine	200 mg
L-Asparagine	50 mg
L-Aspartic acid	20 mg
L-Polyvinylidene fluoride L-cystine dihydrochloride	20 mg
L-Glutamic acid	20 mg
L-Glutamine	300 mg
L-Histidine	15 mg
L-Hydroxyproline	20 mg
L-Isoleucine	50 mg
L-Leucine	50 mg
L-Lysine hydrochloride	40 mg
L-Methionine	15 mg
L-Phenylalanine	15 mg
L-Proline	20 mg
L-Serine	30 mg
L-Threonine	20 mg
L-Tryptophan	5 mg
L-Tyrosine disodium salt dihydrate	20 mg
L-Valine	20 mg
Biotin	0.2 mg
Choline chloride	3 mg
D-Calcium pantothenate	0.25 mg
Folic acid	1 mg
<i>D</i> -Inositol	35 mg
Niacinamide	1 mg
<i>para</i> -Aminobenzoic acid	1 mg
Pyridoxine hydrochloride	1 mg
Riboflavin	0.2 mg
Thiamine hydrochloride	1 mg
Vitamin B ₁₂	0.005 mg
D-Glucose (dextrose)	2000 mg
Glutathione (reduced)	1 mg
Phenol red	5 mg

Growth medium: Using aseptic procedures, prepare the following tissue culture medium:

RPMI-1640 with L-glutamine	500 mL
Sodium pyruvate 100 mM	5 mL
Fetal bovine serum	50 mL
Human rGM-CSF ^a	3 × 10 ⁴ International Units

^a Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) is added extemporaneously.

Sterilize by filtration, and store at between 2° to 8°. Use within 1 month. Add GM-CSF immediately before use.

Assay medium: Use *Growth medium* containing no GM-CSF.

Phosphate buffered saline: Proceed as directed in the test for *Purity* in the *Assay*.

Resazurin solution: 11 mg of resazurin in 100 mL of *Phosphate buffered saline*. [NOTE—Sterile filter and store solution protected from light at 4°. *Resazurin solution* is stable for at least 6 months if treated under sterile conditions.]

[NOTE—For all *Standard* and *Sample solutions*, IL-4 concentration is determined by photometry at 280 nm using an extinction coefficient (ϵ) of 0.63 mg⁻¹cm⁻¹.]

Standard stock solution: 50 µg/mL of USP rHuman Interleukin 4 RS in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]

Standard solutions: 36, 12, 4, 1.33, 0.44, 0.15, 0.05, 0.016, 0.006 ng/mL of IL-4, from *Standard stock solution* in *Assay medium*

Sample stock solution: 50 µg/mL of IL-4 in *Phosphate buffered saline*. [NOTE—Do not agitate while mixing; swirl gently.]

Sample solutions: 36, 12, 4, 1.33, 0.44, 0.15, 0.05, 0.016, 0.006 ng/mL of IL-4, from *Sample stock solution* in *Assay medium*

Control solution: Use the *Assay medium*.

Cell culture preparation: Prepare cell cultures of the human factor-dependent TF-1 cell line (ATCC No. CRL-2003), following the protocol described in the ATCC information sheet. Passage the cultures every 2–3 days, using 1:3 subcultures of the cells for up to 1 month. Seed density should be 0.5×10^6 cells/mL, and maximal density should be 3×10^6 cells/mL. Viability of the cells should be >90%. Maximal passage number is 24, and maximal cultivation time from thawing is 28 days. After 28 days, initiate a new culture. Cells are propagated using *Growth medium* at 37°, supplemented with air and 5% carbon dioxide.

Analysis

Samples: *Standard solutions*, *Sample solutions*, and *Control solution*

The activity of the *Sample solution* is determined in duplicate. Wash the cells three times in *Phosphate buffered saline*. Plate 2×10^4 TF-1 cells resuspended in 100 µL of *Assay medium* per well in 96-well, flat-bottom microplates. Incubate for 72 h at 37° and 5% CO₂ atmosphere in a humidified incubator in the presence or absence of various concentrations of *Standard solution*, *Sample solution*, or *Control solution* by adding 100 µL of the corresponding

solution to each well. Add 30 µL of *Resazurin solution* to each well and incubate for another 24 h. Determine the fluorescence intensity per well by reading the plate with a microplate reader using 544 nm (excitation) and 590 nm (emission). Convert the fluorescence intensity in each well to a percentage of the maximum fluorescence intensity. For the *Sample solution* and *Standard solution*, plot the percentage of fluorescence intensity versus the concentration of the respective solution. By using the least squares method of regression analysis, compute the ED₅₀ in ng/mL of the *Sample solution* and the *Standard solution*. The coefficient of determination for curve regression should be ≥ 0.98. Calculate the potency in USP Interleukin 4 Units/mg:

$$\text{Result} = A \times E_s/E_u$$

A = activity of USP rHuman Interleukin 4 RS (USP units/mg)

E_s = determined ED₅₀ of *Standard solution* (ng/mL)

E_u = determined ED₅₀ of *Sample solution* (ng/mL)

Acceptance criteria: NLT 0.5×10^7 USP IL-4 Units/mg

- **STERILITY TESTS (71):** Meets the requirements
- **BACTERIAL ENDOTOXINS TEST (85):** It contains NMT 50 USP Endotoxin Units/mg.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at –80°.
- **LABELING:** Material is of recombinant DNA origin.
- **USP REFERENCE STANDARDS (11)**
 - USP Endotoxin RS
 - USP rHuman Interleukin 4 RS

(111) DESIGN AND ANALYSIS OF BIOLOGICAL ASSAYS

General

The potency of several Pharmacopeial drugs must be determined by bioassay. A controlling factor in assay design and analysis is the variability of the biological test system, which may vary in its mean response from one laboratory to another, and from time to time in the same laboratory. To control this type of variation, the response to a Pharmacopeial drug is compared with a USP Reference Standard or other suitable standard. For convenience, each such preparation will be called the "Standard" and each preparation under assay, or Sample, the "Unknown," and these will be designated respectively by the symbols S and U. (The Sample is sometimes referred to as the "test preparation.")

After elimination of extraneous variables from the comparison of the Standard and the Unknown, an error variance is computed from the remaining variation, which, while uncontrolled, can nevertheless be measured. The error variance is required in calculating the confidence interval of the assayed potency. The confidence interval, known also as the fiducial interval, is so computed that its upper and lower limits are expected to enclose the true potency of the Unknown in 19 out of 20 assays. Many assay procedures fix the acceptable width of the confidence interval, and two or more independent assays may be needed to meet the specified limit. The confidence limits of the individual component assays usually overlap.

The aim of this chapter is to present a concise account of biometrical procedures for the USP bioassays. Its various sections are interrelated. Although the procedures are planned primarily for the assay of a single Unknown, equations for the joint assay of several Unknowns are given in context throughout the chapter and are summarized in the last section. Proof that an assayed potency meets its required confidence limits may be based also upon other recognized biometric methods that have a precision equivalent to that of the methods outlined herein.

A glossary of the terms used in the equations is provided at the end of this chapter.

Steps Preceding the Calculation of Potency

Designs for Minimizing the Error Variance—Variation in response is reduced as much as is practicable by the limitations imposed on body weight, age, previous handling, environment, and similar factors. In a number of assays, the test animals or their equivalent are then assigned at random but in equal numbers to the different doses of the Standard and Unknown. This implies an objective random process, such as throwing dice, shuffling cards, or using a table of random numbers. Assigning the same number of individuals to each treatment simplifies the subsequent calculations materially, and usually leads to the shortest confidence interval for a given number of observations.

In some assays, the potential responses can be assembled into homogeneous sets in advance of treatment. The differences between sets are later segregated, so that they do not affect adversely either the computed potency or its confidence interval. One unit within each set, picked at random, receives each treatment. Examples of randomized sets are

the cleared areas on a single plate in the plate assay of an antibiotic, and four successive paired readings in the same rat in the Vasopressin Injection assay. Sets of two occur where each test animal is used twice, as in the assays of Tubocurarine Chloride Injection and Insulin Injection. In these cases, neither the average differences between individuals nor the order of treatment can bias the potency or precision. In the microbial assays for vitamin B₁₂ activity and for calcium pantothenate, replicate tubes are assigned to two or more separate, complete sets, preferably with the tubes arranged at random within each set. This restricts the variation due to position or order within a set to the differences within each complete replicate.

Rejection of Outlying or Aberrant Observations—A response that is questionable because of failure to comply with the procedure during the course of an assay is rejected. Other aberrant values may be discovered only after the responses have been tabulated, but can then be traced to assay irregularities, which justify their omission. The arbitrary rejection or retention of an apparently aberrant response can be a serious source of bias. In general, the rejection of observations solely on the basis of their relative magnitudes is a procedure to be used sparingly. When this is unavoidable, each suspected aberrant response or outlier may be tested against one of two criteria:

1. The first criterion is based upon the variation within a single group of supposedly equivalent responses. On the average, it will reject a valid observation once in 25 or once in 50 trials, provided that relatively few, if any, responses within the group are identical. Beginning with the supposedly erratic value or outlier, designate the responses in order of magnitude from y_1 to y_N , where N is the number of observations in the group. Compute the relative gap $G_1 = (y_2 - y_1)/(y_N - y_1)$ when $N = 3$ to 7, $G_2 = (y_3 - y_1)/(y_{N-1} - y_1)$ when $N = 8$ to 13, or $G_3 = (y_3 - y_1)/(y_{N-2} - y_1)$ when $N = 14$ to 24. If G_1 , G_2 , or G_3 exceeds the critical value in *Table 1* for the observed N , there is a statistical basis for omitting the outlier.

This criterion is applicable also in a microbial assay where each treatment is represented by a transmittance in each of two separate complete sets. Subtract each transmittance in the first set from its paired value in the second set, and record each difference with its sign, either plus or minus. Beginning with the most divergent difference, designate the N differences in order of magnitude from y_1 to y_N and compute the relative gap G_1 , G_2 , or G_3 . If this exceeds its critical value in *Table 1*, one of the two transmittances giving the aberrant difference is suspect and may be identified on inspection or by comparison with its expectation (see next column). Repeat the process with the remaining differences if an outlier is suspected in a second pair.

2. The second criterion compares the ranges from a series of $k = 2$ or more groups. Different groups may receive different treatments, but all f responses within each group represent the same treatment. Compute the range from each group by subtracting the smallest response from the largest within each of the k groups. Divide the largest of the k ranges by the sum of all the ranges in the series. Refer this ratio R^* to *Table 2*. If k is not larger than 10, use the tabular values in the upper part of *Table 2*; if k is larger than 10, multiply R^* by $(k + 2)$ and interpolate, if necessary, between the tabular values in the lower part of *Table 2*. If R^* exceeds the tabular or interpolated value, the group with the largest range is suspect and inspection of its components will usually identify the observation, which is then assumed to be aberrant or an outlier. The process may be repeated with the remaining ranges if an outlier is suspected in a second group.

Table 1

Test for outliers. In samples from a normal population, gaps equal to or larger than the following values of G_1 , G_2 , and G_3 occur with a probability $P = 0.02$ where outliers can occur only at one end, or with $P = 0.04$ where they may occur at either end.										
N	3	4	5	6	7					
G_1	.976	.846	.729	.644	.586					
N	8	9	10	11	12	13				
G_2	.780	.725	.678	.638	.605	.578				
N	14	15	16	17	18	19	20	21	22	23
G_3	.602	.579	.559	.542	.527	.514	.502	.491	.481	.472
									.464	

Table 2

Test for groups containing outliers. Compute the range from the f observations in each of k groups, where all groups in the series are equal in size. The observed ratio R^* of the largest range to the sum of the k ranges will equal or exceed the following critical values at a probability of $P = 0.05$.									
No. of Ranges k	Critical R^* for Ranges Each from f Observations								
	2	3	4	5	6	7	8	9	10
2	0.962	0.862	0.803	0.764	0.736	0.717	0.702	0.691	0.682
3	.813	.667	.601	.563	.539	.521	.507	.498	.489
4	.681	.538	.479	.446	.425	.410	.398	.389	.382
5	.581	.451	.398	.369	.351	.338	.328	.320	.314
6	0.508	0.389	0.342	0.316	0.300	0.288	0.280	0.273	0.267
7	.451	.342	.300	.278	.263	.253	.245	.239	.234
8	.407	.305	.267	.248	.234	.225	.218	.213	.208
9	.369	.276	.241	.224	.211	.203	.197	.192	.188
10	.339	.253	.220	.204	.193	.185	.179	.174	.172

No. of Ranges k	Critical $(k + 2)R^*$ for Ranges Each from f Observations								
	2	3	4	5	6	7	8	9	10
10	4.06	3.04	2.65	2.44	2.30	2.21	2.14	2.09	2.05
12	4.06	3.03	2.63	2.42	2.29	2.20	2.13	2.07	2.04
15	4.06	3.02	2.62	2.41	2.28	2.18	2.12	2.06	2.02
20	4.13	3.03	2.62	2.41	2.28	2.18	2.11	2.05	2.01
50	4.26	3.11	2.67	2.44	2.29	2.19	2.11	2.06	2.01

Replacement of Missing Values—As directed in the monographs and in this section, the calculation of potency and its confidence interval from the total response for each dose of each preparation requires the same number of observations in each total. When observations are lost or additional responses have been obtained with the Standard, the balance may be restored by one of the following procedures, so that the usual equations apply.

1. Reduce the number of observations in the larger groups until the number of responses is the same for each treatment. If animals have been assigned at random to each treatment group, either omit one or more responses, selected at random, from each larger group, or subtract the mean of each larger group from its initial total as often as may be necessary. The latter technique is preferred when extra animals have been assigned deliberately to the Standard. When the assay consists of randomized sets, retain only the complete sets.

2. Alternatively, an occasional smaller group may be brought up to size when the number of missing responses is not more than one in any one treatment or 10% in the entire assay. Estimate a replacement for each missing value by either *Method a* or *Method b*. One degree of freedom (n) is lost from the error variance s^2 for each replacement by either method, except in a microbial assay where each response is based on the sum of two or more transmittances and only one transmittance is replaced.

(a) If animals have been assigned to treatments at random, add the mean of the remaining responses in the incomplete group to their total. In a microbial assay, when one of two transmittances is missing for a given treatment, add the mean difference between sets, computed from all complete pairs, to the remaining transmittance to obtain the replacement.

(b) If the assay consists of randomized sets, replace the missing value by

$$y' = \frac{fT_r' + kT_t' - T'}{(f-1)(k-1)}, \quad (1)$$

where f is the number of sets, k is the number of treatments or doses, and T_r' , T_t' , and T' are the incomplete totals for the randomized set, treatment, and assay from which an observation is missing.

If the assay consists of n' Latin squares with k rows in common, replace a missing value by

$$y' = \frac{k(n'T_c' + T_r' + T_t') - 2T'}{(k-1)(n'k-2)} \quad (1a)$$

where n' is the number of Latin squares with k rows in common, k is the number of treatments or doses, and T_c' , T_r' , T_t' , and T' are respectively the incomplete totals for the column, row, treatment, and assay from which an observation is missing.

If more than one value is missing, substitute the treatment mean temporarily in all but one of the empty places, and compute y' for the other by *Equation 1*. Replace each of the initial substitutions in turn by *Equation 1*, and repeat the process in successive approximations until a stable y' is obtained for each missing observation.

Calculation of Potency from a Single Assay

Directions for calculating potency from the data of a single assay are given in the individual monographs. In those

assays that specify graphical interpolation from dosage-response curves but that meet the conditions for assay validity set forth herein, potency may be computed alternatively by the appropriate method in this section.

Planning the assay involves assigning to the Unknown an assumed potency, to permit administering it in dosages equivalent to those of the Standard. The closer the agreement between this original assumption and the result of the assay, the more precise is the calculated potency. The ratio of a given dose of the Standard, in μg or in USP Units, to the corresponding dose of the Unknown, measured as specified in the monograph, is designated uniformly by R . The log-relative potency in quantities assumed initially to equal those of the Standard is designated as M' .

Ideally, M' should not differ significantly from zero. The log-potency is equation 2

$$M = M' + \log R \quad (2)$$

or

$$\text{Potency} = P_{\star} = \text{antilog } M = (\text{antilog } M')R$$

Assay from Direct Determinations of the Threshold Dose—Tubocurarine Chloride Injection and Metocurarine Iodide are assayed from the threshold dose that just produces a characteristic biological response. The ratio of the mean threshold dose for the Standard to that for the Unknown gives the potency directly. The threshold dose is determined twice in each animal, once with the Standard and once with the Unknown. Each dose is converted to its logarithm, the difference (x) between the two log-doses is determined for each animal, and potency is calculated from the average of these differences.

In the *Bacterial Endotoxins Test* (85), the geometric mean dilution endpoint for the Unknown corresponding to the geometric mean dilution endpoint for the Standard (multiplied by a dilution factor, where applicable) gives the concentration of endotoxin in the test material.

In these assays, the confidence interval depends upon the variability in the threshold dose.

Indirect Assays from the Relationship between the Log-Dose and the Response—Generally, the threshold dose cannot be measured directly; therefore, potency is determined indirectly by comparing the responses following known doses of the Standard with the responses following one or more similar doses of the Unknown. Within a restricted dosage range, a suitable measure of the response usually can be plotted as a straight line against the log-dose, a condition that simplifies the calculation of potency and its confidence interval. Both the slope and position of the log-dose response relationship are determined in each assay by the use of two or more levels of the Standard, or, preferably, of both the Standard and the Unknown.

In the assay of Heparin Sodium, the interval between the dose at which clotting occurs and that which produces no clotting is so small that the dosage-response curve is not determined explicitly. Moving averages are used instead to interpolate the log-dose corresponding to 50% clotting for both the Standard and the Unknown, leading to the log-potency (see *Calculation under Heparin Sodium*). The precision of the potency is estimated from the agreement between independent assays of the same Unknown.

For a drug that is assayed biologically, the response should plot as a straight line against the log-dose over an adequate range of doses. Where a preliminary test is required or the assay depends upon interpolation from a multi-dose Standard curve, plot on coordinate paper the mean response of the Standard at each dosage level on the ordinate against the log-dose x on the abscissa. If the trend is basically linear over the required dosage range, the initial response unit may be used directly as y ; if, instead, the trend is clearly curvilinear, a suitable transformation of each initial reading may bring linearity.

One possible transformation is to logarithms; another, in microbial tube assays, where $y = (100 - \% \text{ transmittance})$ does not plot linearly against the log-dose x , is to probits. In this case, if absorbance cannot be read directly, the percent transmittance for each tube or test solution is first converted to absorbance, $A = 2 - \log(\% \text{ transmittance})$. Each absorbance value, in turn, is converted to % reduction in bacterial growth as

$$\% \text{ reduction} = 100(\bar{A}_c - A)/\bar{A}_c$$

where \bar{A}_c is the mean density for the control tubes (without antibiotic or with excess of vitamin) in the same set or tube rack. Percent reduction is then transformed to a probit (see *Table 3*) to obtain a new y for all later calculation. The probit transformation offers the advantage of extending the working range of linearity even where a portion of the dosage-response relationship is nonlinear in the original units of percent transmittance, provided that the incubation period does not extend beyond the logarithmic phase of growth of the control tubes.

The LD_{50} in the *Safety test for Iron Dextran Injection* is calculated with log-doses and probits. The four doses of the Injection, in mg of iron per kg of body weight, are transformed to $x_1 = 2.574$, $x_2 = 2.699$, $x_3 = 2.875$, and $x_4 = 3.000$. The probits corresponding to the number of deaths observed in each group of 10 mice are designated y_1 , y_2 , y_3 , and y_4 , respectively, and are given in *Table 3* for mortalities from 10 to 90 percent. For observed deaths of 0 and 10 adjacent to doses giving an intermediate mortality, use the approximate probits 3.02 and 6.98, respectively; omit the end value (at x_1 or x_4) if not adjacent to an intermediate mortality. Since the information in a probit varies with its expectation, assign each probit an approximate relative

Table 3

Probits (normal deviates + 5) corresponding to percentages in the margins.										
	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

weight w for computing the LD_{50} of the Injection, as shown in the accompanying table.

No. of Deaths	0 or 10	1 or 9	2 or 8	3 or 7	4 to 6
Weight, w	0.3	0.7	1.0	1.2	1.3

Calculate the weighted means

$$\bar{x} = \Sigma(wx)/\Sigma w$$

and (2a)

$$\bar{y} = \Sigma(wy)/\Sigma w$$

from the sum of the weights, Σw , of the four (or three) acceptable responses and the corresponding weighted sums of the log-doses, $\Sigma(wx)$, and of the probits, $\Sigma(wy)$. From the sums of the weighted products, $\Sigma(wxy)$, and of the weighted squares, $\Sigma(wx^2)$, compute the slope b of the log-dose-probit line as

$$b = [\Sigma(wxy) - \bar{x}\Sigma(wy)]/[\Sigma(wx^2) - \bar{x}\Sigma(wx)] \quad (2b)$$

The LD_{50} for this safety test, in mg of iron per kg of body weight, is calculated as

$$LD_{50} = \text{antilog}[\bar{x} + (5 - \bar{y})/b] \quad (2c)$$

In quantal assays not included in this Pharmacopeia, such as the mouse assay for insulin, the calculation with probits involves other adjustments that are omitted here.

When the mean response \bar{y}_i for each dose of Standard plots linearly against the log-dose, and the k doses are spaced at equal intervals on the logarithmic scale, the predicted responses (Y_L and Y_H) at the extreme ends of the line of best fit can be computed directly with the coefficients x_* in Table 4, which correspond to the k successive log-doses, as

$$Y_L = \Sigma(x_*\bar{y}_i)/\text{divisor}$$

and (3)

$$Y_H = \Sigma(x_*\bar{y}_i)/\text{divisor}$$

where Σ stands uniformly for "the sum of" the values that follow it. When Y_L and Y_H are plotted against the low and

high log-doses, X_L and X_H , respectively, they may be connected by a straight line with the slope

$$b = (Y_H - Y_L)/(X_H - X_L) \quad (4)$$

At any selected log-dose x of *Standard*, the predicted response is

$$Y = \bar{y} + b(x - \bar{x}) \quad (5)$$

where $\bar{x} = \Sigma x/k$, and $\bar{y} = (Y_L + Y_H)/2$, or, for predictions within a set, \bar{y} is the mean response for the *Standard* within the set.

When the log-dose response relationship is linear, but the k doses (expressed in mL) are spaced substantially in an arithmetic sequence as in Table 5 (which refers to the microbial assays set forth under *Antibiotics—Microbial Assays* (81)), the slope b of the straight line of best fit may be computed with the terms in Table 5 and the mean response at each dose \bar{y}_i , or $T_i = f\bar{y}_i$ where the number of y 's(f) is constant at each dose, as

$$b = \Sigma(x_i\bar{y}_i)/e_b i = \Sigma(x_i T_i)/f e_b i \quad (6)$$

The coefficients x_i are convenient multiples of the differences $(x - \bar{x})$ about the mean log-dose \bar{x} , and $e_b i$ is the corresponding multiple of $\Sigma(x - \bar{x})^2$. The predicted response Y at a given log-dose x may be computed by substitution of the assay slope b in Equation 5 and of the mean \bar{y} either of all the responses on the *Standard* in the entire assay or of those for each set separately.

POTENCIES INTERPOLATED FROM A STANDARD CURVE—Where the log-dose response curve of the *Standard* in a given assay is curvilinear and is fitted graphically to the plotted points, the amount of *Standard* that would be expected to produce each observed response y of an *Unknown* is estimated by interpolation from the curve and then adjusted for the known concentration of its test solution.

When the response to the *Standard* can be plotted linearly against the log-dose, it is fitted numerically by a straight line, as described in the preceding section. For assays in randomized sets, a standard curve is computed with b for the assay and \bar{y} for each set and the response y_U in each tube of a given *Unknown* in that set is converted to an estimated log-relative potency,

$$X = (y_U - Y_S)/b \quad (7)$$

where Y_S is the response predicted by the standard curve at the assumed log-dose x of the *Unknown*. The average of the separate estimates from each of f sets, $M' = \Sigma X/f$, is the assayed log-relative potency of the *Unknown*.

Factorial Assays from the Response to Each

Treatment—When some function of the response can be plotted linearly against the log-dose, the assayed potency is computed from the total response for each treatment, and

Table 4

Coefficients x_* for computing the responses y_L and y_H predicted by least squares at the lowest and highest of k log-doses when these are spaced at equal intervals.								
No. of Doses	Predicted End y	Coefficient x_* for Mean Response \bar{y}_i at Log-Dose						Divisor
		1	2	3	4	5	6	
3	y_L	5	2	-1				6
	y_H	-1	2	5				6
4	y_L	7	4	1	-2			10
	y_H	-2	1	4	7			10
5	y_L	3	2	1	0	-1		5
	y_H	-1	0	1	2	3		5
6	y_L	11	8	5	2	-1	-4	21
	y_H	-4	-1	2	5	8	11	21

its precision is measured in terms of confidence intervals. This requires that (1) in suitable units the response (y) depends linearly upon the log-dose within the dosage range of the assay, and (2) the number (f) of responses be the same at each dosage level of both Standard and Unknown. The y 's are totaled at each dosage level of each preparation. In different combinations, these totals, T_i , lead directly to the log-relative potency and to tests of assay validity. The factorial coefficients in Tables 6, 7, and 8 determine how they are combined. In a given row, each T_i is multiplied by the corresponding coefficient and the products summed to obtain T_i . The T_i 's in the successive rows carry the same meaning in all assays.

T_a in the first row measures the difference in the average response to the Standard and to the Unknown. T_b in the second row leads directly to the combined slope of the dosage-response curves for both Standard and Unknown. The third to the fifth rows (ab , q , and aq) provide tests for the validity of an assay, as described in a later section. From the totals T_a and T_b , compute the log-relative potency of the Unknown, before adjustment for its assumed potency, as

$$M' = ciT_a/T_b \quad (8)$$

where i is the interval in logarithms between successive log-doses of both the Standard and the Unknown, and the constant c is given separately at the bottom of each table. Each M' is corrected to its log-potency M by Equation 2.

When doses are spaced unequally on a log scale, as in Table 8, use instead the constant ci at the bottom of the table.

In a fully balanced assay, such as the assay for corticotropin, compute M' with the coefficients in Table 6. If one preparation has one less dose than the other but the successive log-doses of both Standard and Unknown differ by a constant interval i , use the factorial coefficients in Table 7, correcting for the actual difference between the observed mean log-doses, \bar{x}_s and \bar{x}_u , by computing

$$M = \bar{x}_s - \bar{x}_u + M' \quad (9)$$

In assays where the successive doses are not spaced at equal log-intervals, the log-relative potency of a single Unknown may be computed by Equation 8 with the factorial coefficients and ci in Table 8.

In an assay of two or more Unknowns against a common Standard, all with dosage-response lines that are parallel within the experimental error, each log-relative potency may be computed with the same assay slope as follows. For each preparation, determine the slope factor $T_b' = \Sigma(x_1 T_i)$ or $\Sigma(x_1 y)$, where the values of x_1 are the factorial coefficients for the Standard in the appropriate row b of Table 6 or 8. The log-relative potency of each Unknown is

$$M' = cih'T_a/2\Sigma T_b' \quad (10)$$

where h' is the number of values of T_b' summed in the denominator.

Table 5

Coefficients x_1 for computing the slope b of a log-dose response curve when the doses are spaced on an arithmetic scale as shown.								
Coefficients x_1 for Computing b from the Responses y at Doses, in mL, of								
No. of Doses	1	1.5	2	3	4	5	Divisor $e_b i$	Mean Log-Dose \bar{x}
4	—	-29	-12	12	29	—	14.4663	0.38908
5	-34	—	-9	5	15	23	24.7827	0.41584
5	—	-20	-11	2	11	18	13.3249	0.45105
6	-15	-8	-3	4	9	13	14.1017	0.37588

Table 6

Factorial coefficients x_1 for analyzing a balanced bioassay, in which successive log-doses of Standard (S) and of Unknown (U) are spaced equally, each with the same number (f) of responses totaling T_i .											
Factorial Coefficients x_1 for Each Dose											
Design	Row	S_1	S_2	S_3	S_4	U_1	U_2	U_3	U_4	e_i	T_i
2,2	a	-1	-1			1	1			4	T_a
	b	-1	1			-1	1			4	T_b
	ab	1	-1			-1	1			4	T_{ab}
3,3	a	-1	-1	-1		1	1	1		6	T_a
	b	-1	0	1		-1	0	1		4	T_b
	ab	1	0	-1		-1	0	1		4	T_{ab}
	q	1	-2	1		1	-2	1		12	T_q
	aq	-1	2	-1		1	-2	1		12	T_{aq}
4,4	a	-1	-1	-1	-1	1	1	1	1	8	T_a
	b	-3	-1	1	3	-3	-1	1	3	40	T_b
	ab	3	1	-1	-3	-3	-1	1	3	40	T_{ab}
	q	1	-1	-1	1	1	-1	-1	1	8	T_q
	aq	-1	1	1	-1	1	-1	-1	1	8	T_{aq}

		Value of Constant for Design			
For Computing	Equation No.	Constant	2,2	3,3	4,4
M'	8, 10	c	1	4/3	5
L	26, 29	c'	1	8/3	5

Assays from Differences in Response—When doses of the Standard and Unknown are paired and the difference in response is computed for each pair, these differences are not affected by variations in the average sensitivity of the paired readings. The paired 2-dose insulin assay corresponds to the first design in *Table 6*, and requires four equal groups of rabbits each injected twice (see *Insulin Assays* <121>). The difference (y) in the blood sugar response of each rabbit to the two treatments leads to the log-relative potency M' (see the first two paragraphs of the section, *Calculation of Potency from a Single Assay*). The Vasopressin Injection assay

follows a similar design, substituting two or more randomized sets of four successive pairs of injections into rats for the four treatment groups of rabbits in the insulin assay.

Oxytocin Injection is assayed from blood pressure changes in a single test animal following alternating injections of a single dose of Standard and of one of two doses of the Unknown. The calculation of potency from the differences in the response of the Unknown and to the average of the two adjacent responses to the Standard is equivalent to the first design in *Table 7* with S and U reversed, where i is the log-interval between the two dosage levels of the Unknown.

Table 7

Factorial coefficients x_i for analyzing a partially balanced assay, in which successive log-doses of Standard (S_i) and of Unknown (U_i) are spaced equally, each with the same number (f) of responses totaling T_i. If the number of successive doses of the Unknown exceeds by one the number on the Standard, interchange S_i and U_i in the heading and reverse all signs in rows a, ab, and aq.										
Factorial Coefficients x_i for Each Dose										
Design	Row	S_1	S_2	S_3	S_4	U_1	U_2	U_3	e_i	T_i
2,1	a	-1	-1			2			6	T_a
	b	-1	1			0			2	T_b
3,2	a	-2	-2	-2		3	3		30	T_a
	b	-2	0	2		-1	1		10	T_b
	ab	1	0	-1		-2	2		10	T_{ab}
	q	1	-2	1		0	0		6	T_q
4,3	a	-3	-3	-3	-3	4	4	4	84	T_a
	b	-3	-1	1	3	-2	0	2	28	T_b
	ab	3	1	-1	-3	-5	0	5	70	T_{ab}
	q	3	-3	-3	3	2	-4	2	60	T_q
	aq	-1	1	1	-1	1	-2	1	10	T_{aq}

For Computing	Equation No.	Constant	Value of Constant for Design		
			2,1	3,2	4,3
M'	8, 10	c	1/2	5/6	7/6
L	26, 29	c'	3/4	25/12	49/12

Table 8

Factorial coefficients x_i for analyzing assays with a 3- or 4-dose sequence of 1.5, 2.0, 3.0, and 4.0, each dose having the same number (f) of responses.											
Design	Row	Dose of Standard				Dose of Unknown				e_i	T_i
		1.5	2.0	3.0	4.0	1.5	2.0	3.0	4.0		
4,4	a	-1	-1	-1	-1	1	1	1	1	8	T_a
	b	-29	-12	12	29	-29	-12	12	29	3940	T_b
	ab	29	12	-12	-29	-29	-12	12	29	3940	T_{ab}
	q	1	-1	-1	1	1	-1	-1	1	8	T_q
3,3	aq	-1	1	1	-1	1	-1	-1	1	8	T_{aq}
	a	-1	-1	-1		1	1	1		6	T_a
	b	-25	-3	28		-25	-3	28		2836	T_b
	ab	25	3	-28		-25	-3	28		2836	T_{ab}
	q	31	-53	22		31	-53	22		8508	T_q
3,3	aq	-31	53	-22		31	-53	22		8508	T_{aq}
	a		-1	-1	-1		1	1	1	6	T_a
	b		-28	3	25		-28	3	25	2836	T_b
	ab		28	-3	-25		-28	3	25	2836	T_{ab}
	q		22	-53	31		22	-53	31	8508	T_q
3,3	aq		-22	53	-31		22	-53	31	8508	T_{aq}

For Computing	Equation No.	Constant	Value of Constant for Design	
			4,4	3,3
M'	8, 10	c_i	7.2332	5.3695
L	26, 29	$c'i2$	0.10623	0.06100

Experimental Error and Tests of Assay Validity

As the term is used here, "experimental error" refers to the residual variation in the response of biological indicators, not to a mistake in procedure or to an outlier that needs replacement. It is measured in terms of the error variance of a single response or other unit, which is designated uniformly as s^2 , despite differences in the definition of the unit. It is required in tests of assay validity and in computing the confidence interval.

Error Variance of a Threshold Dose—The individual threshold dose is measured directly in some assays. In a Digitalis assay, designate each individual threshold dose by the symbol z , the number or frequency of z 's by f , and the total of the z 's for each preparation by T , with subscripts S and U for Standard and Unknown, respectively. Compute the error variance of z as

$$s^2 = [\Sigma z^2 - T_S^2/f_S - T_U^2/f_U]/n \quad (11)$$

with $n = f_S + f_U - 2$ degrees of freedom. In the assay of Tubocurarine Chloride Injection, each log-threshold dose of the Unknown is subtracted from the corresponding log-dose of the Standard in the same rabbit to obtain an individual difference x . Since each x may be either positive or negative (+ or -), it is essential to carry the correct sign in all sums. Designate the total of the x 's for the animals injected with the Standard on the first day as T_1 , and for those injected with the Standard on the second day as T_2 . Compute the error variance of x with $n = N - 2$ degrees of freedom as

$$s^2 = \{\Sigma x^2 - (T_1^2 + T_2^2)/f\}/n \quad (12)$$

where N is the total number of rabbits that complete the assay, excluding any replacement for a missing value to equalize the size of the two groups.

Error Variance of an Individual Response—In the Pharmacopeial assays, differences in dose that modify the mean response are assumed not to affect the variability in the response. The calculation of the error variance depends upon the design of the assay and the form of the adjustment for any missing values. Each response is first converted to the unit y used in computing the potency. Determine a single error variance from the combined deviations of the y 's around their respective means for each dosage level, summed over all levels. Doubtful values of y may be tested as described under *Rejection of Outlying or Aberrant Observations*, and proved outliers may be replaced as missing values (see *Replacement of Missing Values*).

In the simplest design, the units of response are assigned at random to each dosage level, as in the assay for corticotropin. If a missing value is replaced by adding the mean of the remaining y 's at any given dosage level to their total, the degrees of freedom (n) in the error variance are reduced by one for each replacement but no other change is needed in the calculation. Assuming that f is then the same for all doses or groups, compute the error variance from the variation within doses of all the y 's as

$$s^2 = \{\Sigma y^2 - \Sigma T_i^2/f\}/n \quad (13)$$

where T_i is the total at each dose of the f values of y , there are k totals T_i and the degrees of freedom $n = \Sigma f - k$, with Σf diminished by 1 for each replacement.

If variations in f are adjusted by subtracting a group mean from its group total, compute the error variance from the observed y 's and the *unadjusted* totals T_i as

$$s^2 = \{\Sigma y^2 - \Sigma (T_i^2/f)\}/n \quad (14)$$

where $n = \Sigma f - k$.

In the calculation of the result of an assay using the coefficients of Table 6 or 8, s^2 may be computed from the re-

sponse y for each of the h' preparations, including the h Unknowns and the corresponding dosage levels of the Standard. For each preparation, compute $T' = \Sigma y$ and the slope factor $T_b' = \Sigma (x_1 y)$ where the values of x_1 are the factorial coefficients for the Standard in the appropriate row b of Table 6 or 8. The error variance for the assay is

$$s^2 = \{\Sigma y^2 - \Sigma T'^2/k - 2(\Sigma T_b')^2/h'e_b f\}/n \quad (15)$$

where the degrees of freedom $n = h'(k - 1) - 1$, and e_b is the e_i from the same table and row as the coefficients x_1 .

The Error Variance in Restricted Designs—In some assays, the individual responses occur in randomized sets of three or more. Examples of sets are litter mates in the assay of vitamin D, the cleared areas within each plate in an antibiotic assay, and the responses following four successive pairs of injections in the vasopressin assay. Arrange the individual y 's from these assays in a 2-way table, in which each column represents a different treatment or dose and each row a randomized set. Losses may be replaced as described under *Replacement of Missing Values*. The k column totals are the T_i 's required for the analysis of balanced designs. The f row totals (T_r) represent a source of variation that does not affect the estimated potency and hence is excluded from the assay error. Compute the approximate error variance from the squares of the individual y 's and of the marginal totals as

$$s^2 = \{\Sigma y^2 - \Sigma T_r^2/k - \Sigma T_i^2/f + T^2/N\}/n \quad (16)$$

where $T = \Sigma T_r = \Sigma T_i$, and the $n = (k - 1)(f - 1)$ degrees of freedom must be diminished by one for any gap in the original table that has been filled by computation.

When the order of treatment is an additional potential source of variation, its effect can be corrected by the dose regimen for a series of n' Latin squares with k rows in common, such as that for the two Latin squares in the dose regimens 1 to 4 and 5 to 8 in the assay of Glucagon for Injection. List the observed responses y of each test animal in a separate column in the order of dosing. The responses to each of the k doses then occur equally often in each of the k rows and of the $n'k$ columns, where n' is the number of Latin squares. Total the responses y in each row (T_r) in each column (T_c), and, in a separate listing, for each dose or treatment (T_i). An occasional lost reading may be replaced by Equation 1a as described under *Replacement of Missing Values*. Compute the error variance from the squares of the individual y 's and of the marginal and treatment totals as

$$s^2 = \{\Sigma y^2 - \Sigma T_r^2/n'k - \Sigma T_c^2/k - \Sigma T_i^2/n'k + 2T^2/N\}/n \quad (16a)$$

where $T = \Sigma y = \Sigma T_r = \Sigma T_c = \Sigma T_i$, $N = n'k^2$, and the $n = (k - 1)(n'k - 2)$ degrees of freedom must be diminished by one for any gap in the original table that has been filled by computation.

In assays where the reactions occur in pairs, the differences between test animals or paired reactions are segregated automatically by calculating the assay with the difference within a pair as the response. With insulin, the response is the difference y in the blood sugar of a single rabbit following two injections (see *Insulin Assay* (121)). After adjustment for rabbits lost during the assay, compute the error variance of y from the responses in all four groups and from the group totals $T_i = T_1$ to T_4 as

$$s^2 = \{\Sigma y^2 - \Sigma T_i^2/f\}/n \quad (17)$$

where the number of rabbits f is the same in each group and the degrees of freedom, $n = 4(f - 1)$, are reduced by one for each replacement of a rabbit lost during the assay. In the Oxytocin Injection assay, each y represents the difference between the blood pressure response to a dose of the

Unknown and the average for the two adjacent doses of Standard. Compute the error variance of y as

$$s^2 = \{\Sigma y^2 - (T_1^2 + T_2^2)/f\}/n \quad (18)$$

with $n = 2(f - 1)$ degrees of freedom, where T_1 is the total of the y 's for the low dose of the Unknown and T_2 that for the high dose.

In a microbial assay calculated by interpolation from a standard curve, convert each difference between two paired responses to units of log-dose, X , by the use of Equation 7. With each difference X as the unit, a composite s^2 is computed from the variation in the f values of X for each Unknown, totaled over the h Unknowns in the assay, as

$$s^2 = \{\Sigma X^2 - \Sigma(T_x^2/f)\}/n \quad (19)$$

where $T_x = \Sigma X$ for a single Unknown and the degrees of freedom $n = \Sigma f - h$.

Tests of Assay Validity— In addition to the specific requirements in each monograph and a combined log-dose response curve with a significant slope (see the statistic C in the next section), two conditions determine the validity of an individual factorial assay: (1) the log-dose response curve for the Unknown must parallel that for the Standard within the experimental error, and (2) neither curve may depart significantly from a straight line. When the assay has been completely randomized or consists of randomized sets, the necessary tests are computed with the factorial coefficients for ab , q , and aq from Tables 6 to 8 and the treatment totals T_i . Sum the products of the coefficients in each row by the corresponding T_i 's to obtain the product total T_i , where the subscript i stands in turn for ab , q , and aq , respectively. Each of the three ratios, T_i^2/ef , is computed with the corresponding value of e_i from the table and with f equal to the number of y 's in each T_i . That in row ab tests whether the dosage-response lines are parallel, and is the only test available in a 2-dose assay. With three or more doses of both preparations, that in row q is a test of com-

bined curvature in the same direction, and in row aq of separate curvatures in opposite directions. If any ratio in a 3- or 4-dose assay exceeds s^2 as much as three-fold, compute

$$F_3 = \Sigma(T_i^2/ef)/3s^2 \quad (20)$$

For a 2-dose assay, compute instead

$$F_1 = T_{ab}^2/e_{ab}s^2 \quad (21)$$

and for a 3,2 assay (Table 7) determine

$$F_2 = \Sigma(T_i^2/ef)/2s^2 \quad (22)$$

For a valid assay, F_1 , F_2 , or F_3 does not exceed the value given in Table 9 (at odds of 1 in 20) for the degrees of freedom n in s^2 .

An assay may fail the test for validity and still provide a contributory estimate of potency that can be combined profitably with the result of a second assay of the same Unknown, as described in a later section. An end dosage level for either the Standard or the Unknown, or both, may fall outside the linear zone. With three or more dosage levels and relatively large values of T_{ab} , T_{ab} , and T_{aq} , the total response T_i at an end dose of one preparation may approach an upper or lower limit and be responsible for the large values of T_{ab} and T_{aq} . This T_i may be omitted and the assay recomputed with the appropriate design in Table 7. If the assay then meets the test in Equation 20, or 22, the resulting potency, M , may be combined with that of a second assay in computing the log-potency of the Unknown (see under *Combination of Independent Assays*). If T_a is not significant but T_q shows significant combined curvature, the largest (or smallest) dose of both preparations may be too large (or too small). Their omission may lead to a valid assay with the factorial coefficients for the next smaller design in Table 6 or 8. A statistically significant T_q or ΣT_q may be neglected and all dosage levels retained without biasing the

Table 9

Values of t , t^2 , F , and χ^2 for different degrees of freedom n that will be exceeded with a probability $P = 0.05$ (or 0.95 for confidence intervals). [†]											
n	t	$t^2 = F_1$	F_2	F_3	χ^2	n	t	$t^2 = F_1$	F_2	F_3	χ^2
1	12.706	161.45	—	—	3.84	19	2.093	4.381	3.52	3.13	30.1
2	4.303	18.51	19.00	19.16	5.99	20	2.086	4.351	3.49	3.10	31.4
3	3.182	10.128	9.55	9.28	7.82	21	2.080	4.325	3.47	3.07	32.7
4	2.776	7.709	6.94	6.59	9.49	22	2.074	4.301	3.44	3.05	33.9
5	2.571	6.608	5.79	5.41	11.07	23	2.069	4.279	3.42	3.03	35.2
6	2.447	5.987	5.14	4.76	12.59	24	2.064	4.260	3.40	3.01	36.4
7	2.365	5.591	4.74	4.35	14.07	25	2.060	4.242	3.38	2.99	37.7
8	2.306	5.318	4.46	4.07	15.51	26	2.056	4.225	3.37	2.98	38.9
9	2.262	5.117	4.26	3.86	16.92	27	2.052	4.210	3.35	2.96	40.1
10	2.228	4.965	4.10	3.71	18.31	28	2.048	4.196	3.34	2.95	41.3
11	2.201	4.844	3.98	3.59	19.68	29	2.045	4.183	3.33	2.93	42.6
12	2.179	4.747	3.89	3.49	21.03	30	2.042	4.171	3.32	2.92	43.8
13	2.160	4.667	3.81	3.41	22.36	40	2.021	4.085	3.23	2.84	55.8
14	2.145	4.600	3.74	3.34	23.68	60	2.000	4.001	3.15	2.76	79.1
15	2.131	4.543	3.68	3.29	25.00	120	1.980	3.920	3.07	2.68	146.6
16	2.120	4.494	3.63	3.24	26.30	∞	1.960	3.841	3.00	2.60	
17	2.110	4.451	3.59	3.20	27.59						
18	2.101	4.414	3.55	3.16	28.87						

[†]Adapted from portions of Tables III to V of "Statistical Tables for Biological, Agricultural and Medical Research," by R. A. Fisher and F. Yates, published by Oliver and Boyd, Ltd., Edinburgh.

computed log-potency M' and its confidence interval by more than 5% when the following inequality is true:

$$T_b^2/e_b > 100T_q^2/e_q$$

or

$$(\Sigma T_b')^2/e_b > 100(\Sigma T_q')^2/e_q \quad (23)$$

where each T_b' and T_q' is computed with the T_i 's (or y 's) for a single preparation multiplied by the coefficients for the Standard in rows b and q , respectively. If both T_a and T_{ab} are significant in a 2-dose assay, one T_i may be outside the linear zone. Sometimes a preliminary or contributory estimate of potency can be computed from the remaining three values of T_i and the first design in *Table 7*. In assays of insulin and of other drugs in which the responses are paired, the test for parallelism is so insensitive that it is omitted. If the tubes in each set are arranged systematically instead of at random in a microbial assay, the tests for validity may be subject to bias from positional effects.

The Confidence Interval and Limits of Potency

A bioassay provides an estimate of the true potency of an Unknown. This estimate falls within a confidence interval, which is computed so that the odds are not more than 1 in 20 ($P = 0.05$) that the true potency either exceeds the upper limit of the confidence interval or is less than its lower limit. Since this interval is determined by a number of factors that may influence the estimate of potency, the required precision for most bioassays is given in the monograph in terms of the confidence interval, related either to the potency directly or to its logarithm.

General Calculation—Despite their many forms, bioassays fall into two general categories: (1) those where the log-potency is computed directly from a mean or a mean difference, and (2) those where it is computed from the ratio of two statistics.

(1) When the log-potency of an assay is computed as the mean of several estimated log-potencies that are approximately equal in precision, the log-confidence interval is

$$L = 2st / \sqrt{k} \quad (24)$$

where s is the standard deviation of a single estimated log-potency, t is read from *Table 9* with the n degrees of freedom in s , and k is the number of estimates that have been averaged. The same equation holds where the log-potency is computed as the mean \bar{x} of k differences x , with s the standard deviation of a single x . In either case, the estimated log-potency M is in the center of its confidence interval, so that its confidence limits are

$$X_M = M + 1/2L \text{ and } M - 1/2L, \text{ or } X_M = M \pm 1/2L \quad (25)$$

The upper and lower limits are converted to their antilogarithms to obtain the limits as explicit potencies.

(2) More often, the log-potency or potency is computed from a ratio, and in these cases the length of the confidence interval is typified by the log-interval in the equation

$$L = 2\sqrt{(C-1)(CM'^2 + c'i^2)} \quad (26)$$

where M' is the log-relative potency as defined (see *Calculation of Potency from a Single Assay*), i is the log-interval between successive doses, and c' is a constant characteristic of the assay procedure. The remaining term C depends upon the precision with which the slope of the dosage-response

curve has been determined. (This is sometimes expressed in terms of $g = (C - 1)/C$.) In factorial assays, it is computed as

$$C = T_b^2/(T_b^2 - e_bfs^2t^2) \quad (27)$$

where s^2 is the error variance of a single observation, t^2 is read from *Table 9* with the degrees of freedom in s^2 , f is the number of responses in each T_i used in calculating T_b , and T_b and e_b are computed with the factorial coefficients for row b in *Tables 6* to *8*. The s^2 in *Equation 26* depends upon the design of the assay, as indicated for each drug in the next section. In a valid assay, C is a positive number.

In an assay of two or more Unknowns against a common Standard, all with dosage-response curves that are parallel within the experimental error, C may be computed with the error variance s^2 for the assay and with the assay slope as

$$C = (\Sigma T_b')^2/(\Sigma T_b')^2 - e_bfh's^2t^2/2 \quad (28)$$

The slope factor $T_b' = \Sigma(x_iT_i)$ or $\Sigma(x_iy)$ for each of the h' preparations, including the Standard, is computed with the factorial coefficients x_i for the Standard in the appropriate row b of *Table 6* or *8*. If a treatment total T_i includes one or more replacements for a missing response, replace e_bf in *Equation 27*, or $e_bfh'/2$ in *Equation 28*, by $f^2\Sigma(x_i^2/f')$, where each x_i is a factorial coefficient in row b of *Tables 6* to *8*, in this chapter, and f' is the number of responses in the corresponding T_i before adding the replacement. With this C , compute the confidence interval as

$$L = 2\sqrt{(C-1)(CM'^2 + c'i^2h'/2)} \quad (29)$$

In assays computed from a ratio, the most probable log-potency M is not in the exact center of the confidence interval. The upper and lower confidence limits in logarithms are

$$X_M = \log R + CM' + 1/2L \text{ and } \log R + CM' - 1/2L \quad (30)$$

C is often very little larger than unity, and the more precise the assay, the more nearly C approaches 1 exactly. $R = z_s/z_u$ is the ratio of corresponding doses of the Standard and of the Unknown or the assumed potency of the Unknown. The upper and lower confidence limits in log-potencies are converted separately to their antilogarithms to obtain the corresponding potencies.

Confidence Intervals for Individual Assays—Since the confidence interval may vary in detail from the above general patterns, compute it for each assay by the special directions given under the name of the substance in the paragraphs following.

Antibiotic Assays—The confidence interval may be computed by *Equations 24* and *25*.

Calcium Pantothenate—For log-potencies obtained by interpolation from the Standard curve, the confidence interval may be computed with *Equations 19* and *24*. For log-potencies calculated with *Equation 8* or *10*, s^2 may be computed with *Equation 15*, C with *Equation 27* or *28*, and the confidence interval L with *Equation 26* or *29*.

Corticotropin Injection—Compute the log confidence interval by *Equations 26* and *27*, with the coefficients and constants in *Table 6* for a 3-dose assay, and s^2 as determined by *Equation 13* or *14*.

Digitalis—Compute the confidence interval as

$$L = 2\sqrt{(C-1)\{C(\bar{z}_s/\bar{z}_u)^2 + f_u/f_s\}} \quad (31)$$

where f_U and f_S are the number of observations on the Unknown and on the Standard, and

$$C = \bar{Z}_U^2 / (\bar{Z}_U^2 - s^2 t^2 / f_U) \quad (32)$$

is determined with s^2 from Equation 11. The confidence limits for the potency in USP Units are then

$$X_{P^*} = R\{C(\bar{Z}_S/\bar{Z}_U) \pm 1/2L\} \quad (33)$$

in which R is as defined in the *Glossary of Symbols*.

Glucagon for Injection—Compute the error variance s^2 by Equation 15a, C by Equation 27 with $e_b f = 16n'$, and the log confidence interval L by Equation 26 with $c'i^2 = 0.09062$.

Chorionic Gonadotropin—Proceed as directed under *Coricotropin Injection*.

Heparin Sodium—If two independent determinations of the log-potency M differ by more than 0.05, carry out additional assays and compute the error variance among the N values of M as

$$s^2 = \{\Sigma M^2 - (\Sigma M)^2/N\}/n \quad (34)$$

with $n = N - 1$ degrees of freedom. Given this value, determine the confidence interval in logarithms (L) by Equation 24.

Insulin Injection—Compute the error variance (s^2) of y by Equation 16 and C as

$$C = T_b^2 / (T_b^2 - s^2 t^2 N) \quad (35)$$

where t^2 from Table 9 depends upon $n = 4(f - 1)$ degrees of freedom in s^2 and $N = 4f$ is the total number of differences in the four groups. By Equation 26, compute the confidence interval L in logarithms, where $c'i^2 = 0.09062$. The upper and lower confidence limits in USP Units of insulin are given by the antilogarithms of X_M from Equation 30.

Oxytocin Injection—Compute the approximate log confidence interval by Equation 26, in which

$$C = (T_2 - T_1)^2 / \{(T_2 - T_1)^2 - 4(f + 1)s^2 t^2 / 3\} \quad (36)$$

where s^2 is defined by Equation 18, and

$$c' = (4f - 1)/8(f + 1) \quad (37)$$

Tubocurarine Chloride Injection—Compute the error variance by Equation 12, and the confidence interval by Equation 24.

Vasopressin Injection—Compute the error variance s^2 by Equation 16, C by Equation 35, and the log confidence interval by Equation 26, where $c' = 1$ and i is the log-interval separating the two dosage levels.

Vitamin B₁₂ Activity—Proceed as directed under *Calcium Pantothenate*.

Combination of Independent Assays

When the method permits, additional animals can be added to an insufficiently precise assay until the combined results reduce the confidence interval within the limits specified in the monograph. Where two or more independent assays are required, each leading to a log-potency M , the M 's are combined in determining the *weighted mean potency* of the Unknown. Except in the Heparin Sodium assay, where the log-potencies are weighted equally, the relative precisions of the two or more independent M 's determine the weight assigned to each value in computing their mean and its confidence interval.

Before combining two or more separate estimates of M , test their mutual consistency. If the M 's are consistent, their

respective confidence intervals will overlap. Where the intervals do not overlap or where the overlap is small, compute an approximate χ_M^2 . Assign each of the h individual assays a weight w , defined as

$$w = 4t^2/L^2 \quad (38)$$

where the length of the confidence interval L is computed with the appropriate equation from the preceding section, and t^2 is read from Table 9 for the degrees of freedom n in the error variance of the assay. Sum the individual weights to obtain Σw . Then an approximate χ^2 with $h - 1$ degrees of freedom is determined as

$$\text{Approx. } \chi_M^2 = \Sigma(wM^2) - \{\Sigma(wM)\}^2/\Sigma w \quad (39)$$

For two assays with log-potencies M_1 and M_2 and weights w_1 and w_2 , Equation 35 reduces to

$$\text{Approx. } \chi_M^2 = w_1 w_2 (M_1 - M_2)^2 / (w_1 + w_2) \quad (40)$$

with one degree of freedom. If the approximate χ_M^2 is well under the critical value for χ^2 in Table 9, use the weights w in computing the mean log-potency \bar{M} and its confidence interval, L . If χ_M^2 approaches or exceeds this critical value, use instead the semi-weights w' (Equation 47) when computing \bar{M} .

Compute the mean log-potency \bar{M} of two or more mutually consistent assays as

$$\bar{M} = \Sigma(wM)/\Sigma w \quad (41)$$

This is the most probable single value within a combined confidence interval of length L_c , defined as the square root of

$$L_c^2 = 4t_L^2/\Sigma w \{1 + (4/\Sigma^2 w)\Sigma(w(\Sigma w - w)/n')\} \quad (42)$$

where each $n' = n - 4(h - 2)/(h - 1)$ and t_L^2 is interpolated from Table 9 with the degrees of freedom

$$n_L = \Sigma^2 w / \Sigma(w^2/n)$$

For two assays ($h = 2$) with log-potencies M_1 and M_2 and weights w_1 and w_2 , respectively, the above equation may be rewritten as

$$L_c^2 = 4t_L^2/\Sigma w \{1 + [1/n_1 + 1/n_2]4w_1 w_2/\Sigma^2 w\} \quad (43)$$

where $\Sigma w = w_1 + w_2$. Where L_c , the confidence interval for a combined estimate, does not exceed the requirement in a monograph, upper and lower confidence limits are taken $1/2 L_c$ above and below \bar{M} , to obtain approximately a 95% confidence interval.

Where the variation in the assayed potency between the h independent determinations, as tested by χ_M^2 , approaches or exceeds $P = 0.05$, the several estimates are assigned semi-weights w' . From the weight w , compute the variance of each M as

$$V = 1/w = L^2/4t^2 \quad (44)$$

Calculate the variance of the heterogeneity between assays as

$$v = \Sigma M^2 - (\Sigma M^2/h)/(h - 1) - \Sigma v/h \quad (45)$$

or if $h = 2$,

$$v = (M_1 - M_2)^2/2 - (V_1 + V_2)/2 \quad (46)$$

Where V varies so markedly that v calculated as above is a negative number, compute instead an approximate v by

omitting the term following the minus sign in *Equations 45* and *46*. A semi-weight is defined as

$$w' = 1/(V + v) \quad (47)$$

Substitute w' and $\Sigma w'$ for w and Σw in *Equation 41* to obtain the semi-weighted mean \bar{M} . This falls near the middle of a confidence interval of approximate length L_c' , where

$$L_c'^2 = 4t^2/\Sigma w' \quad (48)$$

and t^2 from *Table 9* has ΣN degrees of freedom.

Where χ_{M2} in *Equation 39*, from $h = 4$ or more estimates of M , exceeds the critical level in *Table 9* by more than 50%, and the weights w differ by less than 30%, the h estimates of M may be checked for a suspected outlier with *Table 1*. Where significant, the outlying M may be omitted in computing \bar{M} with w' .

Where the potency of a drug is determined repeatedly in a given laboratory by the same bioassay method, successive determinations of both the slope b and the error variance s^2 may scatter randomly within the sampling error about a common value for each parameter. Plotting estimates from successive assays on a quality control chart for each statistic and computing the midvalue and control limits defining the allowable random variation make it possible to check continuously the consistency of an assay technique. Where estimates of b and s^2 from a single assay fall within the control limits, they may be replaced by their laboratory means. Reject any assay in which these statistics fall outside the control limits, or accept it only after close scrutiny with respect to its validity.

Joint Assay of Several Preparations

Each monograph describes the assay of a single Unknown against the Standard. Although not provided explicitly, several different Unknowns are often included in the same assay and each is compared separately with the same responses to the Standard. This fact may warrant increasing the number of observations with the Standard. Given f observations at each dosage level of each of h different Unknowns, the number of observations at each dosage level of the Standard may be increased advantageously, if h is large, to

$$f/h$$

This rule can be applied only approximately where litter differences or their equivalent must be segregated, and in any case is merely suggestive.

If all of several assays conducted concurrently meet the requirements for validity, and have linear log-dose response curves with the same slope b and the same error variance s^2 about these lines, these two statistics may be considered as characteristic of the assay. Combining all of the evidence from the same assay into a single value of the assay slope results in a more stable and reliable estimate of b than if each Unknown were analyzed independently. The degrees of freedom and reliability of the error variance s^2 can be increased similarly. Confidence intervals computed with these composite values for b and s^2 are smaller on the average than if based upon only part of the relevant data. For the calculation or application of such assay estimates, see *Equations 10, 15, 16, 19, 28, and 29*. The potency estimated with a slope computed from a single Unknown and the Standard agrees within a fraction of the confidence interval with that computed from the combined slope for the entire assay. Since it is based upon more evidence, the latter is considered the better estimate.

GLOSSARY

Glossary of Symbols

A	absorbance for computing % reduction in bacterial growth from turbidimetric readings.
b	slope of the straight line relating response (y) to log-dose (x) [<i>Equations 2b, 4, 5, 6</i>].
c	constant for computing M' with <i>Equations 8</i> and <i>10</i> .
c'	constant for computing L with <i>Equations 26</i> and <i>29</i> .
ci	constant for computing M' when doses are spaced as in <i>Table 8</i> .
c'i ²	constant for computing L when doses are spaced as in <i>Table 8</i> .
C	term measuring precision of the slope in a confidence interval [<i>Equations 27, 28, 35, 36</i>].
χ^2	statistical constant for testing significance of a discrepancy [<i>Table 9</i>].
χ_{M2}	χ^2 testing the disagreement between different estimates of log-potency [<i>Equations 39, 40</i>].
e_b	e_i from row b in <i>Tables 6</i> to <i>8</i> .
$e_b'i$	multiple of $\Sigma(x - \bar{x})^2$ [<i>Table 5; Equation 6</i>].
e_i	sum of squares of the factorial coefficients in each row of <i>Tables 6</i> to <i>8</i> .
e_q	e_i from row q in <i>Tables 6</i> to <i>8</i> .
f	number of responses at each dosage level of a preparation; number of replicates or sets.
f_s	number of observations on the Standard.
f_u	number of observations on the Unknown.
F_1 to F_3	observed variance ratio with 1 to 3 degrees of freedom in numerator [<i>Table 9</i>].
G_1, G_2 , and G_3	relative gap in test for outlier [<i>Table 1</i>].
h	number of Unknowns in a multiple assay.
h'	number of preparations in a multiple assay, including the Standard and h Unknowns; i.e., $h' = h + 1$.
i	interval in logarithms between successive log-doses, the same for both <i>Standard</i> and <i>Unknown</i> .
k	number of estimated log-potencies in an average [<i>Equation 24</i>]; number of treatments or doses [<i>Table 4; Equations 1, 13, 15, 16</i>]; number of ranges or groups in a series [<i>Table 2</i>]; number of rows, columns, and doses in a single Latin square [<i>Equations 1a, 16a</i>].
L	length of the confidence interval in logarithms [<i>Equations 24, 26, 29, 38</i>], or in terms of a proportion of the relative potency of the dilutions compared [<i>Equations 31, 33</i>].
L_c	length of a combined confidence interval [<i>Equations 42, 43</i>].
L_c'	length of confidence interval for a semi-weighted mean \bar{M} [<i>Equation 48</i>].
LD ₅₀	lethal dose killing an expected 50% of the animals under test [<i>Equation 2c</i>].
M	log-potency [<i>Equation 2</i>].
M'	log-potency of an Unknown, relative to its assumed potency.
\bar{M}	mean log-potency.
n	degrees of freedom in an estimated variance s^2 or in the statistic t or χ^2 .
n'	number of Latin squares with rows in common [<i>Equations 1a, 16a</i>].
N	number; e.g., of observations in a gap test [<i>Table 1</i>], or of responses y in an assay [<i>Equation 16</i>].

Glossary of Symbols (Continued)

P	probability of observing a given result, or of the tabular value of a statistic, usually $P = 0.05$ or 0.95 for confidence intervals [Tables 1, 2, 9].
P_*	potency, P_* = antilog M or computed directly.
R	ratio of a given dose of the Standard to the corresponding dose of the Unknown, or assumed potency of the Unknown [Equations 2, 30, 33].
R_*	ratio of largest of k ranges in a series to their sum [Table 2].
$s = \sqrt{s^2}$	standard deviation of a response unit, also of a single estimated log-potency in a direct assay [Equation 24].
s^2	error variance of a response unit.
S_i	a log-dose of Standard [Tables 6, 7].
Σ	"the sum of."
t	Student's t for n degrees of freedom and probability $P = 0.05$ [Table 9].
T	total of the responses y in an assay [Equation 16].
T'	incomplete total for an assay in randomized sets with one missing observation [Equation 1].
T_1	$\Sigma(y)$ for the animals injected with the Standard on the first day [Equations 18, 36].
T_2	$\Sigma(y)$ for the animals injected with the Standard on the second day [Equations 18, 36].
T_a	T_i for the difference in the responses to the Standard and to the Unknown [Tables 6 to 8].
T_{ab}	T_i for testing the difference in slope between Standard and Unknown [Tables 6 to 8].
T_{aq}	T_i for testing opposed curvature in the curves for Standard and Unknown [Tables 6 to 8].
T_b	T_i for the combined slope of the dosage-response curves for Standard and Unknown [Tables 6 to 8].
T_b'	$\Sigma(x_i T_i)$ or $\Sigma(x_i y)$ for computing the slope of the log-dose response curve [Equations 10, 23, 28].
T_i	sum of products of T_i multiplied by the corresponding factorial coefficients in each row of Tables 6 to 8.
T_q	T_i for testing similar curvature in the curves for Standard and Unknown [Tables 6 to 8].
T_r	row or set total in an assay in randomized sets [Equation 16].
T_r'	incomplete total for the randomized set with a missing observation in Equation 1.
T_t	total of f responses y for a given dose of a preparation [Tables 6 to 8; Equations 6, 13, 14, 16].
T_t'	incomplete total for the treatment with a missing observation in Equation 1.
U_i	a log-dose of Unknown [Tables 6 to 8].
v	variance for heterogeneity between assays [Equation 45].
$V = 1/w$	variance of an individual M [Equations 44 to 47].
w	weight assigned to the M for an individual assay [Equation 38], or to a probit for computing an LD_{50} [Equations 2a, 2b].
w'	semi-weight of each M in a series of assays [Equations 47, 48].
x	a log-dose of drug in a bioassay [Equation 5]; also the difference between two log-threshold doses in the same animal [Equation 12].
x_*	coefficients for computing the lowest and highest expected responses y_L and y_H in a log-dose response curve [Table 4; Equation 3].
x_1	a factorial coefficient that is a multiple of $(x - \bar{x})$ for computing the slope of a straight line [Table 5; Equation 6].
\bar{x}	mean log-dose [Equation 5].

Glossary of Symbols (Continued)

\bar{x}_s	mean log-dose for Standard [Equation 9].
\bar{x}_u	mean log-dose for Unknown [Equation 9].
X	log-potency from a unit response, as interpolated from a standard curve [Equations 7a, 7b, 19].
X_M	confidence limits for an estimated log-potency M [Equations 25, 30].
X_p	confidence limits for a directly estimated potency P. (see <i>Digitalis</i> assay) [Equation 33].
y	an observed individual response to a dose of drug in the units used in computing potency and the error variance [Equations 13 to 16]; a unit difference between paired responses in 2-dose assays [Equations 17, 18].
$y_1 \dots y_N$	observed responses listed in order of magnitude, for computing G_1 , G_2 , or G_3 in Table 1.
y'	replacement for a missing value [Equation 1].
\bar{y}	mean response in a set or assay [Equation 5].
\bar{y}_t	mean response to a given treatment [Equations 3, 6].
Y	a response predicted from a dosage-response relationship, often with qualifying subscripts [Equations 3 to 5].
z	threshold dose determined directly by titration (see <i>Digitalis</i> assay) [Equation 11].
\bar{z}	mean threshold dose in a set (see <i>Digitalis</i> assay) [Equations 31, 32, 33].

(115) DEXPANTHENOL ASSAY

The following procedure is provided for the determination of dexpanthenol as an ingredient of multiple-vitamin preparations. It is applicable also to the determination of the dextrorotatory component of racemic panthenol and of other mixtures containing dextrorotatory panthenol.

Media may be prepared as described hereinafter, or dehydrated mixtures yielding similar formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they have growth-promoting properties equal to or superior to those obtained from the formulas given herein.

USP Reference Standards (11)—*USP Dexpanthenol RS*.

Standard Stock Solution of Dexpanthenol—Dissolve an accurately weighed quantity of *USP Dexpanthenol RS* in water, dilute with water to obtain a solution having a known concentration of about 800 μg per mL, and mix. Store in a refrigerator, protected from light, and use within 30 days.

Standard Preparation—On the day of the assay, prepare a water dilution of the *Standard Stock Solution of Dexpanthenol* to contain 1.2 μg of dexpanthenol per mL.

Assay Preparation—Proceed as directed in the individual monograph for preparing a solution expected to contain approximately the equivalent of the dexpanthenol concentration in the *Standard Preparation*.

Modified Pantothenate Medium—

Acid-Hydrolyzed Casein Solution	25 mL
Cystine-Tryptophane Solution	25 mL
Polysorbate 80 Solution	0.25 mL
Dextrose, Anhydrous	10 g
Sodium Acetate, Anhydrous	5 g

Adenine–Guanine–Uracil Solution	5 mL
Riboflavin–Thiamine Hydrochloride–Biotin Solution	5 mL
Para-aminobenzoic Acid–Niacin–Pyridoxine Hydrochloride Solution	5 mL
Salt Solution A	5 mL
Salt Solution B	5 mL
Pyridoxal–Calcium Pantothenate Solution	5 mL
Polysorbate 40–Oleic Acid Solution	5 mL

Dissolve the anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL, and mix.

Double-Strength Modified Pantothenate Medium—Prepare as directed under *Modified Pantothenate Medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Acid-Hydrolyzed Casein Solution—Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8 to 12 hours. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–Tryptophane Solution—Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophane (or 2.0 g of D,L-tryptophane) in 700 mL to 800 mL of water, heat to $75 \pm 5^\circ$, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, add water to make 1000 mL, and mix. Store under toluene in a refrigerator at a temperature not below 10°.

Adenine–Guanine–Uracil Solution—Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid, cool, add water to make 200 mL, and mix. Store under toluene in a refrigerator.

Polysorbate 80 Solution—Dissolve 25 g of polysorbate 80 in alcohol to make 250 mL, and mix.

Riboflavin–Thiamine Hydrochloride–Biotin Solution—Prepare a solution containing, in each mL, 20 µg of riboflavin, 10 µg of thiamine hydrochloride, and 0.04 µg of biotin, by dissolving riboflavin, thiamine hydrochloride, and biotin in 0.02 N acetic acid. Store, protected from light, under toluene in a refrigerator.

Para-aminobenzoic Acid–Niacin–Pyridoxine Hydrochloride Solution—Prepare a solution in neutral 25 percent alcohol to contain 10 µg of para-aminobenzoic acid, 50 µg of niacin, and 40 µg of pyridoxine hydrochloride in each mL. Store in a refrigerator.

Salt Solution A—Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, mix, and store under toluene.

Salt Solution B—Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, mix, and store under toluene.

Pyridoxal–Calcium Pantothenate Solution—Dissolve 40 mg of pyridoxal hydrochloride and 375 µg of calcium pantothenate in 10 percent alcohol to make 2000 mL, and mix. Store in a refrigerator, and use within 30 days.

Polysorbate 40–Oleic Acid Solution—Dissolve 25 g of polysorbate 40 and 0.25 g of oleic acid in 20 percent alco-

hol to make 500 mL, and mix. Store in a refrigerator, and use within 30 days.

Stock Culture of *Pediococcus acidilactici*—Dissolve in about 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH between 6.5 and 6.6, adjust the volume with water to 1000 mL, and mix. Add approximately 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize at 121° for 15 minutes. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici** on a slant of this medium. Incubate at 35° for 20 to 24 hours, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum—Inoculate three 250-mL portions of *Modified Pantothenate Medium* from a stock culture slant, and incubate at 35° for 20 to 24 hours. Centrifuge the suspension from the combined portions, and wash the cells with *Modified Pantothenate Medium*. Resuspend the cells in sufficient *Modified Pantothenate Medium* so that a 1:50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.]

Procedure—Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0 mL, 4.5 mL, 4.0 mL, 3.5 mL, 3.0 mL, 2.0 mL, 1.0 mL, and 0.0 mL. To these same tubes, and in the same order, add 0.0 mL, 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, 4.0 mL, and 5.0 mL of the *Standard Preparation*.

Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0 mL, 3.5 mL, 3.0 mL, 2.0 mL, and 1.0 mL. To these same tubes, and in the same order, add 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, and 4.0 mL of the *Assay Preparation*.

Add 5.0 mL of *Double-Strength Modified Pantothenate Medium* to each tube, and mix. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 minutes. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 hours. Terminate growth by heating to a temperature not below 80°, such as by steaming at atmospheric pressure in a suitable sterilizer, for 5 to 10 minutes. Cool, and concomitantly determine the percentage transmittance of the suspensions, in cells of equal pathlength, on a suitable spectrophotometer, at 530 nm.

Calculation—Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percent transmittance, for each set of tubes of the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency, in terms of dexpanthenol, of each tube containing portions of the *Assay Preparation*. Divide the potency of each tube by the amount of *Assay Preparation* added to it, to obtain the individual responses. Calculate the mean response by averaging the individual responses that vary from their mean by not more than 15%, using not less than half the total number of tubes. Calculate the potency of the portion of the material taken for assay, in terms of

* American Type Culture Collection No. 8042 is suitable.

dexpanthenol, by multiplying the mean response by the appropriate dilution factor.

(121) INSULIN ASSAYS

The most prominent manifestation of insulin activity, an abrupt decrease in blood glucose, was the basis for biologic assay from the time of its first clinical use. The procedure, although relatively cumbersome, has the great merit of accurately reflecting the effect on the diabetic patient. The advent of practical yet sophisticated physicochemical methods (e.g., liquid chromatography) to measure insulin potency quantitatively has resulted in a more accurate and precise compendial test for insulin and insulin products. However, the bioidentity of insulin and insulin products cannot be assessed by these methods. Thus, a qualitative test in rabbits is included in this chapter, and its use is called for in the appropriate monographs.

The *Rabbit Blood Sugar Method—Quantitative* is used to determine the potency of Insulin Reference Standards, for the validation of the stability of new insulin preparations, and to determine the specific activities of insulin analogs.

RABBIT BLOOD SUGAR METHOD—QUANTITATIVE

USP Reference Standards (11)—USP Dextrose RS. USP Insulin RS. USP Insulin (Beef) RS. USP Insulin Human RS. USP Insulin (Pork) RS.

Diluent—Prepare an aqueous solution containing 0.1% to 0.25% (w/v) of either cresol or phenol, 1.4% to 1.8% (w/v) of glycerin, and sufficient hydrochloric acid to produce a pH between 2.5 and 3.5, unless otherwise directed in the individual monograph.

Standard Stock Solution—Dissolve either a suitable quantity of accurately weighed USP Insulin RS or a vial of lyophilized USP Insulin RS of the appropriate species in *Diluent* to make a *Standard Stock Solution* containing 40 USP Insulin Units per mL and having a pH between 2.5 and 3.5, unless otherwise directed in the individual monograph. Store in a cold place, protected from freezing, and use within 6 months.

Standard Solutions—Dilute portions of the *Standard Stock Solution* with *Diluent* to make two solutions, one to contain 1.0 USP Insulin Unit per mL (*Standard Solution 1*), and the other to contain 2.0 USP Insulin Units per mL (*Standard Solution 2*).

Assay Stock Solution—Proceed as directed under *Standard Stock Solution*, except to use a suitable quantity of the preparation under test in place of USP Insulin RS. The *Assay Stock Solution* contains about 40 USP Insulin Units per mL.

Assay Solutions—Dilute portions of the *Assay Stock Solution* with *Diluent* to make two dilutions of the preparation under test, one of which may be expected, on the basis of the assumed potency, to contain 1.0 USP Insulin Unit per mL (*Assay Solution 1*), and the other to contain 2.0 USP Insulin Units per mL (*Assay Solution 2*). In the case of neutral insulin injection, adjust to a pH of 2.5 to 3.5 prior to making the dilutions.

Doses of the Solutions To Be Injected—Select on the basis of trial or experience the dose of the dilutions to be injected, the volume of which usually will be between 0.30 mL and 0.50 mL. For each animal the volume of the *Standard Solution* is the same as that of the *Assay Solution*.

Preparation of Animal—Select suitable, healthy rabbits each weighing not less than 1.8 kg. Keep the rabbits in the laboratory for not less than 1 week before use in the assay, maintaining them on an adequate uniform diet, with water available at all times.

Procedure—Divide the rabbits into four equal groups of preferably not less than six rabbits each. On the preceding day, approximately 20 hours before the assay, provide each rabbit with an amount of food that will be consumed within 6 hours. Follow the same feeding schedule before each test day. During the assay, withhold all food until after the final blood specimen is taken. Handle the rabbits with care in order to avoid undue excitement, and inject subcutaneously the doses indicated in the following design (see *Table 1*), the second injection being made on the day after the first injection, or not more than 1 week later. The time between the first and second injection is the same for all rabbits.

Table 1

Group	First Injection	Second Injection
1	<i>Standard Solution 2</i>	<i>Assay Solution 1</i>
2	<i>Standard Solution 1</i>	<i>Assay Solution 2</i>
3	<i>Assay Solution 2</i>	<i>Standard Solution 1</i>
4	<i>Assay Solution 1</i>	<i>Standard Solution 2</i>

Blood Samples—At 1 hour \pm 5 minutes and 2½ hours \pm 5 minutes after the time of injection, obtain from each rabbit a suitable blood specimen from a marginal ear vein. Blood can also be collected effectively from the central auricular artery.

Dextrose Determination—Determine the dextrose content of the blood specimens by a suitable procedure that is adapted to automated analysis. The following procedure may be used.

Anticoagulant Solution—Dissolve 1 g of edetate sodium and 200 mg of sodium fluoride in 1 L of water, and mix.

Dextrose Standard Preparations—Transfer known concentrations of USP Dextrose RS to suitable vessels, and dilute quantitatively and stepwise with *Anticoagulant Solution* (1:9) to obtain a range of *Dextrose Standard Preparations* containing between 20 and 100 mg per 100 mL, having known concentrations similar to the concentrations in the rabbit blood samples.

Test Preparations—Pipet into separate, suitable vessels 0.1 mL of each *Blood Sample* and 0.9 mL of *Anticoagulant Solution*.

Procedure—Subject the *Test Preparations* to dialysis across a semipermeable membrane for a sufficient time so that the dextrose passes through the membrane into a saline TS solution containing glucose oxidase, horseradish peroxidase, 3-methyl-2-benzothiazolinone hydrazone hydrochloride TS, and *N,N*-dimethylaniline. The absorbances of the *Test Preparations* are determined at 600 nm in a recording colorimeter. The absorbances of the *Dextrose Standard Preparations* are similarly determined at the start and the end of each run.

Calculation—Calculate the response of each rabbit to each injection from the sum of the two blood-sugar values, and subtract its response, disregarding the chronological order in which the responses were observed, to obtain the individual differences, *y*, as shown in *Table 2*.

When the data for one or more rabbits are missing in an assay, do not use the confidence interval formulas given here, but seek statistical help. The data can still be analyzed with proper analysis of variance.

When the number of rabbits, *f*, carried through the assay is the same in each group, total the *y*'s in each group and compute $T_a = -T_1 + T_2 + T_3 - T_4$ and $T_b = T_1 + T_2 + T_3 + T_4$. The logarithm of the relative potency of the test dilutions is $M' = 0.301T_a/T_b$. The potency of the injection in USP Units per mg equals the antilog ($\log R + M'$), where $R = v_s/v_u$, in

which v_s is the number of USP Units per mL of the Standard solution and v_u is the number of mg of insulin per mL of the corresponding Assay solution.

Determine the 95% confidence interval for the log-relative potency using Fieller's Theorem (see *Appendix* and *Design and Analysis of Biological Assays* (111)). If the confidence interval is more than 0.082, which corresponds at $P = 0.95$ to confidence limits of about $\pm 10\%$ of the computed potency, repeat the assay until the combined data of the two or more assays, redetermined as described in *Combination of Independent Assays* under *Design and Analysis of Biological Assays* (111), meet this acceptable limit.

Table 2

Group	Differences	Individual Response (y)	Total Response (T)	Standard Deviations of Differences (S)
1	Standard Solution 2 – Assay Solution 1	y_1	T_1	S_1
2	Assay Solution 2 – Standard Solution 1	y_2	T_2	S_2
3	Assay Solution 2 – Standard Solution 1	y_3	T_3	S_3
4	Standard Solution 2 – Assay Solution 1	y_4	T_4	S_4

BIOIDENTITY TEST

Proceed as directed for *Rabbit Blood Sugar Method—Quantitative* with the following modifications:

Procedure—Divide the rabbits into four equal groups of two rabbits each.

Calculation—Proceed as directed for *Calculation* under *Rabbit Blood Sugar Method—Quantitative*, but do not determine the confidence interval of the log-relative potency, M' .

Interpretation—If the potency value obtained is not less than 15 USP Units per mg, the *Bioidentity Test* requirement is met. If the potency value is less than 15 USP Units per mg, repeat the test using eight more rabbits. If the average potency of the two sets of tests is not less than 15 USP Units per mg, the requirement of the test is met.

Appendix—Fieller's Theorem for Determining the Confidence Interval for a Ratio

This version of Fieller's Theorem is for the case where the numerator and denominator are uncorrelated. The equation assumes the numerator and denominator are normally distributed and the groups of rabbits are equal-sized.

Then, the 95% confidence interval for the ratio is:

$$(L,U) = \frac{M' \pm \frac{t}{T_b} \sqrt{(1-g)S_N^2 + (M')^2 S_D^2}}{1-g}$$

where f (degrees of freedom in the standard errors) = $4(k - 1)$, where k is the number of rabbits in a group, t is the upper 97.5 percentile of the t -distribution with f degrees of freedom, and

$$g = \frac{t^2 S_D^2}{T_b^2}$$

If $g \geq 1$, the denominator is not significantly different from 0 and the formula does not work.

$$S_N = 0.301 \sqrt{k} \sqrt{S_1^2 + S_2^2 + S_3^2 + S_4^2}$$

$$S_D = \sqrt{k} \sqrt{S_1^2 + S_2^2 + S_3^2 + S_4^2}$$

<123> GLUCAGON BIOIDENTITY TESTS

DEFINITION

Glucagon is a polypeptide hormone that increases blood glucose levels via release of liver glycogen stores and is clinically used to treat hypoglycemia. Human, porcine, and bovine glucagon share an identical 29 amino acid sequence. Commercially available Glucagon was previously purified from bovine and porcine pancreas glands. Today, human glucagon is recombinantly produced (rGlucagon) with various microbial fermentation systems using the human amino acid sequence. The *USP–NF Glucagon for Injection* monograph defines glucagon identification tests. Glucagon bioidentity must be determined using a validated bioassay method approved by a competent authority. The bioassay must demonstrate that the manufacturing process produces Glucagon that has a biologic activity of NLT 0.80 USP Unit/mg of glucagon. This chapter describes a validated glucagon bioidentity test that measures glucose released from freshly prepared rat liver cells (hepatocytes) stimulated with Glucagon in vitro.

ASSAY

• PRIMARY LIVER CELL ASSAY

[NOTE—All buffers are oxygenated, prepared with either *Sterile Water for Injection* or *Sterile Water for Irrigation*, warmed to 37°, and adjusted to a final pH of 7.4 unless otherwise indicated. At least two independent assays (replicates) must be performed utilizing two rat livers for each lot of Glucagon. *Figure 1* demonstrates the process used to generate one replicate value. A minimum of two replicates are combined according to the *Calculations* section. The concentration range of the *Standard preparations* and the *Assay preparations* may be modified to fall within the linear range of the *Assay*, and the calculations can be adjusted accordingly. Alternatively, full curve analysis using validated non-linear statistical methods can be used, provided that similarity is demonstrated when analysts compare the responses of the *Standard preparations* and the *Assay preparations*.]

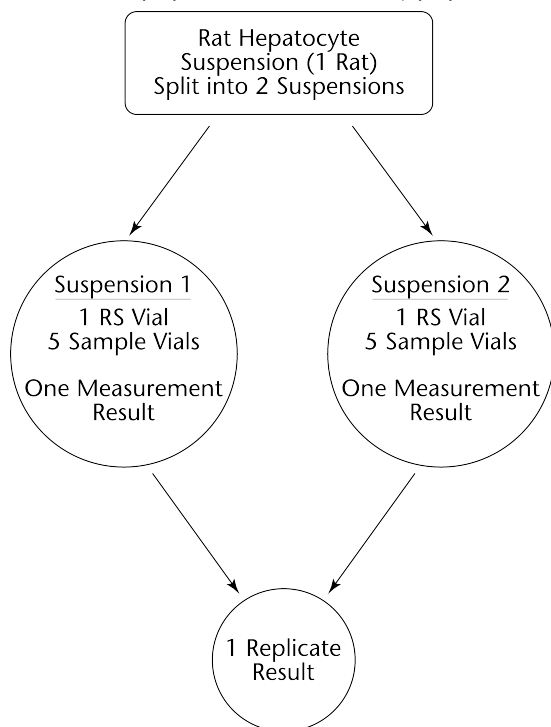


Figure 1. Rat hepatocyte assay method flow diagram (RS = Reference Standard).

Hepatocyte Preparation

Calcium-free perfusion buffer with dextrose: Prepare a solution containing 7.92 g/L of sodium chloride, 0.35 g/L of potassium chloride, 1.80 g/L of dextrose, 0.19 g/L of edetic acid (EDTA), and 2.38 g/L of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Oxygenate before use.

Collagenase buffer: Prepare a solution containing 3.62 g/L of sodium chloride, 23.83 g/L of HEPES, 0.35 g/L of potassium chloride, 0.52 g/L of calcium chloride, and 1.8 g/L of dextrose. Adjust to a pH of 7.6. Immediately before perfusion, dissolve a quantity of collagenase in this solution to obtain a concentration of 0.02%–0.05%. The exact concentration of collagenase is determined empirically for each new lot of enzyme and is the amount that can consistently dissociate the tissue within 10 min of buffer entry and produce a viable cell concentration of NLT 3×10^6 cells/mL.

Wash buffer: Prepare a solution containing 7.92 g/L of sodium chloride, 0.35 g/L of potassium chloride, 0.19 g/L of EDTA, 2.38 g/L of HEPES, 0.11 g/L of calcium chloride, and 0.06 g/L of magnesium sulfate.

Incubation buffer: Prepare a solution containing 6.19 g/L of sodium chloride, 0.35 g/L of potassium chloride, 0.22 g/L of calcium chloride, 0.12 g/L of magnesium sulfate, 0.16 g/L of monobasic potassium phosphate, 11.915 g/L of HEPES, and 10 g/L of bovine serum albumin (1% BSA). Adjust to a pH of 7.5.

Test animals: Male Sprague-Dawley rats are maintained on a standard rat chow diet, given water ad libitum, and allowed to adjust to their new housing before testing. On the morning of the test, select a healthy rat weighing approximately 300 g, and administer 100 Units of Heparin Sodium subcutaneously.

Procedure: [NOTE—Conduct this procedure in the morning to ensure that the rat has optimal glycogen in its liver and so that the procedure can be completed in one day.] Anesthetize the rat with an appropriate anesthetic. Open the abdominal cavity, and isolate the portal vein. Insert an angiocatheter and tie into the portal vein at the general location of the lienal branch and then connect to a perfusion pump. Start the perfusion (25 mL/min) in situ with the previously warmed, oxygenated, *Calcium-free perfusion buffer with dextrose*. As the liver enlarges, cut the inferior vena cava to allow pressure equilibrium. [NOTE—About 300 mL of the perfusate is needed to clear the liver of red blood cells at a flow rate of 25–60 mL/min.] Then circulate *Collagenase buffer* at an appropriate flow rate so that the liver leaks perfusate out of the lobes in approximately 10 min (typically 25–60 mL/min). When the liver significantly increases in size, changes color and consistency, and starts to leak perfusate out of the lobes, change the system to the oxygenated prewarmed *Wash buffer*. About 100 mL of *Wash buffer* is needed to wash the liver of collagenase at a flow rate of 25 mL/min. Surgically remove the liver from the animal, and place in a prewarmed Petri dish containing a small amount of oxygenated *Wash buffer* (37°). Gently comb the liver with a stainless steel, fine-toothed comb to free the hepatocytes. Filter and wash the hepatocytes with *Wash buffer*, through prewetted cheesecloth (3 layers thick, or through a 150-μm mesh polyethylene net) into a beaker. Transfer the cells to two centrifuge tubes, and spin for about 1 min at 600 rpm. Discard the supernatant fractions, and resuspend the two pellets in *Incubation buffer*. Combine the two pellets in a suitable container, and add sufficient *Incubation buffer* to make 150 mL.

System suitability of cell preparation: The cell yield may vary because of the collagenase activity and the viability of the hepatocytes. To check cell viability and to determine viable cell concentration, dilute a 100-μL aliquot of

the cell suspension with 400 μ L of *Wash buffer* and 500 μ L of isotonic 0.4% trypan blue solution. Load aliquots of the cell suspension into both chambers of a hemocytometer, and count all 8 quadrants. To meet system suitability of the cell preparation method, a viable cell concentration of 3×10^6 cells/mL (acceptable range of 2.5×10^6 to 3.4×10^6 cells/mL) must be obtained to proceed with the bioassay. If the viable cell concentration exceeds the upper limit, additional *Incubation buffer* may be added to the cells to adjust the concentration to 3×10^6 cells/mL. In this case, the cells are counted again in a hemocytometer, as described above to verify the concentration. [NOTE—Viable cells are those cells that exclude the trypan blue.]

Glucose Determination

Negative control solution: Prepare a solution containing 0.5% BSA using *Sterile Water for Injection* or *Sterile Water for Irrigation*.

Incubation flasks: Use specially prepared 25-mL conical flasks, the bottoms of which have been heated and pushed inward to form a conically raised center, or similar flasks that allow sufficient mixing when swirling. Place the *Incubation flasks* in an orbital shaker water bath at 35°.

Standard preparations: On the day of the assay, dissolve two vials of USP rGlucagon RS, accurately measured, in 0.01 N hydrochloric acid or other suitable diluent (volume based on the potency of the Reference Standard lot) to obtain two solutions each containing 1 USP rGlucagon Unit/mL. All dilutions thereafter are made using *Negative control solution*. Accurately dilute measured volumes of each solution with *Negative control solution* to obtain an intermediate concentration of 400 μ U/mL, and then dilute the intermediate to produce five concentrations: 200, 100, 50, 25, and 12.5 μ U/mL (*Standard preparations*). Pipet 0.1 mL of each *Standard preparation* into separate *Incubation flasks*. Pipet 0.1 mL of *Negative control solution* into each of two flasks (*Negative control solutions 1 and 2*).

Assay preparations: Using accurately weighed quantities of Glucagon samples, proceed as directed for *Standard preparations* or, if testing *Glucagon for Injection*, reconstitute 10 vials by slowly adding the contents of the accompanying prefilled syringes containing an appropriate glucagon diluent. Gently mix each vial until the glucagon is dissolved. Using the same syringes, withdraw the contents of 5 vials and place the solutions in a 25-mL volumetric flask. Repeat for the second 5 vials, transferring the contents to a second 25-mL volumetric flask. Dilute each flask with 0.01 N hydrochloric acid to volume. Dilute an accurate amount of each solution with 0.5% BSA to yield a concentration of 400 μ U/mL, and dilute the intermediate to produce five *Assay preparation* concentrations: 200, 100, 50, 25, and 12.5 μ U/mL. Then proceed as directed for the *Standard preparations*.

Reference stock solution: Dry USP Dextrose RS, and then transfer 1.0 g, accurately weighed, to a 100-mL volumetric flask. Dissolve in and dilute with saturated benzoic acid solution to volume.

Reference solutions: Transfer suitable quantities of *Reference stock solution* to 4 flasks, and dilute with saturated benzoic acid solution to obtain *Reference solutions* having concentrations of 100, 500, 1000, and 1500 mg/L.

Potassium ferrocyanide solution: Dissolve 1.25 g of trihydrate potassium ferrocyanide in 125 mL of *Sterile Water for Injection*, or use an appropriate commercial source.

System suitability: Analyze the *Potassium ferrocyanide solution*, the *Reference solutions*, and an additional five replicates of either the 500- or 1000-mg/L *Reference solution* in an appropriate glucose analyzer. Prepare a standard curve using the *Reference solutions* as directed for the *Standard preparations*. The square root of the residual error mean square from the regression divided by the average of the response multiplied by 100% (line %RSD) must be NMT 2.0%. In addition, the response of the *Potassium ferrocyanide solution* must be NMT 30 mg/L, and the relative standard deviation must be NMT 2.0% for the replicate analyses of the middle *Reference solution*.

Procedure: Dispense 5 mL of *Hepatocyte Preparation* into the *Incubation flasks* in sequence from high glucagon concentration to low glucagon concentration, alternating the *Standard preparations* with the *Assay preparations*. Swirl the flasks in an orbiting water bath at 125 rpm at 35° for approximately 30 min. Following incubation, remove 1.0-mL aliquots from each *Incubation flask*, transfer to labeled microcentrifuge tubes, and centrifuge at 13,000 rpm for 15 s. Place each supernatant fraction in a labeled sampling tube for a glucose analyzer, and determine the glucose concentration (mg/L) of each *Standard preparation* and *Assay preparation*. Measure the background reading of the *Negative control solutions 1 and 2*, and calculate the average of the two responses.

To conform to the linear range of the instrument being used, analysts may find it necessary to adjust by dilution each of the *Standard preparations* and *Assay preparations*. Use a glucose analyzer that has demonstrated appropriate specificity, accuracy, precision, and linear response over the range of concentrations being determined. Determine the increase in glucose concentration for each *Standard preparation* and *Assay preparation* compared to the average value of the *Negative control solution*.

Calculations

Calculate the relative potency of the Glucagon samples in USP Units/mL using statistical methods for parallel-line assays, comparing the Reference Standard curve (from the *Standard preparations*) to the Glucagon sample curve (from the *Assay preparations*). No dose-response reversals may occur within a run for the 25-, 50-, or 100- μ U/mL *Standard preparations* and *Assay preparations*. [NOTE—Either the low- or high-dose level, but not both, may be excluded from the calculation in order to meet linearity requirements.] Because a minimum of two valid assays (rats) are required, the estimated potencies are combined using the procedures in *Design and Analysis of Biological Assays* (11), *Combination of Independent Assays*, and the width, L , of a 95% confidence interval for the estimated logarithm of the relative potency is calculated. If L is NMT 0.1938, the results are valid. If L is >0.1938, additional assays may be performed and combined until a valid L term results, and the relative potency is then calculated from all valid independent runs. It meets the requirement of bioidentity if the relative potency is NLT 0.80 USP rGlucagon Unit/mg.

ADDITIONAL REQUIREMENTS

• USP REFERENCE STANDARDS (11)

USP Dextrose RS
USP rGlucagon RS

(130) PROTEIN A QUALITY ATTRIBUTES

Introduction

Protein A is coupled to a resin support in order to create protein A affinity chromatography media commonly used in the manufacturing of recombinant therapeutic monoclonal antibodies. Natural protein A is derived from *Staphylococcus aureus* and contains five homologous antibody binding regions and a C-terminal region for cell wall attachment. In addition to naturally derived protein A, recombinant material manufactured in *Escherichia coli*, as well as several engineered versions of the protein, also manufactured recombinantly, have entered the market place. When immobilized on a column, protein A provides a highly efficient and robust purification method for purifying antibodies at various scales. However, protein A ligand from the column can co-elute with the antibody during purification, an effect which is often referred to as protein A leaching. This tendency increases as the chromatography medium ages. Engineered versions of protein A may improve the pH tolerance of the medium, but do not eliminate leaching. It is the current regulatory expectation that leached protein A should be cleared during the purification of antibodies for human use, and manufacturing processes should be validated accordingly. Enzyme-Linked Immunosorbent Assay (ELISA)-based residuals testing is generally employed during process development and validation to assure the efficient removal of residual protein A during process steps following protein A affinity chromatography. In addition, the manufacturer should have a clear understanding and documentation of resin and ligand quality through raw materials qualification and column lifetime studies.

General Chapter (130) describes quality attributes of protein A ligands that are used in chromatography media for the manufacture of therapeutic monoclonal antibodies: Protein A; rProtein A; rProtein A, C-Cys; rProtein A, B4, C-Cys.

Protein A

C₁₉₉₅H₃₁₆₃N₅₉₇O₆₉₇S₃
46,760

N-terminal Sequence AQHDEA

C-terminal Sequence IAADNK

Protein A is derived from *Staphylococcus aureus*. The structure is composed of a single polypeptide chain containing four IgG binding domains. With the exception of IgG₃, all other human IgGs bind to protein A. Each molecule of Protein A is capable of binding two IgG molecules. It is manufactured as a bulk solution at a concentration of greater than 20 mg protein A per mL with an IgG-binding potency of greater than 95%. Because Protein A is used as an ancillary material in the manufacture of recombinant therapeutic drugs, regulatory requirements differ from those for therapeutic drug products.

Packaging and storage—Store in closed containers at the temperature indicated on the label.

Labeling—Preserve in sealed containers, and store at a temperature of –20° or below.

USP Reference standards (11)—USP Endotoxin RS. USP Protein A RS.

Identification—

A: SDS-PAGE—It meets the requirements of Identification test A under rProtein A using USP Protein A RS.

B: IgG Binding—It meets the requirements of Identification test B under rProtein A using USP Protein A RS.

Microbial enumeration tests (61) and **Tests for specified microorganisms** (62)—The total aerobic microbial count does not exceed 100 cfu per mL, and the total yeasts and molds count does not exceed 10 cfu per mL.

Bacterial endotoxins (85)—It contains not more than 1 USP Endotoxin Unit per mg of total protein. [NOTE—The Bacterial endotoxins test for Protein A is used to describe the quality of this ancillary material. This test does not define the acceptable level of bacterial endotoxin in the preparation of injectable dosage forms in which Protein A is used.]

Total protein (see *Spectrophotometry and Light-Scattering* (851))—Prepare triplicate samples for analysis by diluting Protein A to 3.0 mg per mL in *Water for Injection*. Measure the absorbance of each sample at 275 nm after correcting for the absorbance using *Water for Injection* as the blank. Determine the protein concentration using the equation:

$$\text{Protein concentration (mg per mL)} = (A_{275}/0.149)$$

in which A is the absorbance of Protein A at the wavelength of 275 nm and 0.149 is the molar absorptivity. Average the triplicate results, and determine a coefficient of variance (CV): the CV is ≤5%.

Limit of common contaminant protein and corresponding assay—

Enterotoxin B—Enterotoxin B is determined using a commercially available microstrip enzyme-immunoassay kit.¹ Wells of the microstrips are coated with sheep antibodies to enterotoxin B. Standard curves are made using the ELISA kit control. The negative controls are wells coated with serum from nonimmunized sheep. The level of enterotoxin is determined from the standard curve. The specification for the enterotoxin B level is ≤1 ng per mg of total protein.

Chromatographic purity—[NOTE—The size-exclusion chromatographic purity test resolves Protein A from high molecular weight contaminants.]

Mobile phase—Prepare a solution of 50 mM sodium dihydrogen phosphate, pH 6.5 in the following manner. Add 6.9 ± 0.1 g of sodium dihydrogen phosphate into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 5 M sodium hydroxide to a pH of 6.50 ± 0.05. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.22-μm membrane filter.

Column regeneration solution—Prepare a solution of 0.1 M sodium dihydrogen phosphate, pH 3.0 in the following manner. Add 13.8 ± 0.1 g of sodium dihydrogen phosphate into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with hydrochloric acid to a pH of 3.0 ± 0.1. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.22-μm membrane filter.

Column storage solution—Mix 100 mL of methanol with 900 mL of water.

Test solution—Dilute Protein A to approximately 1 mg per mL with Mobile phase.

Calibration standards—Using Mobile phase, prepare separate 1 mg per mL solutions of each of the following: thyroglobulin (670 kD), IgG (150 kD), beta lactoglobulin (36 kD), and lysozyme (14 kD).

Standard solution—Prepare a solution containing 1 mg per mL of USP Protein A RS in Mobile phase.

Chromatographic system (see *Chromatography* (621))—The liquid chromatograph is equipped with a 280-nm detector and a 7.8-mm × 30-cm column that contains packing L33. Equilibrate the column for approximately 30 minutes at 0.5 mL of Mobile phase per minute or until a stable baseline is achieved.

Procedure—Separately inject 100 μL of each sample, and run the samples in the following sequence: Calibration stan-

¹A suitable enzyme immunoassay kit is available from TECRA International Pty Ltd., Australia (No. SETVIA96).

dards, thyroglobulin, IgG, beta lactoglobulin, and lysozyme; the *Standard solution*; and the *Test solution*. Run the sequence three times isocratically using *Mobile phase* at 0.5 mL per minute for 30 minutes. Absorbance is detected at 280 nm. Analyze the 280-nm peak data, and pick the retention time (RT) with the largest peak area. Using the data from the *Calibration standards*, plot the mean RT versus the log molecular weight to produce the standard curve. The purity should be $\geq 95\%$ in the main peak. Use the formula from the standard curve to give the log molecular weights of the *Test solutions*. Convert the log molecular weights of the *Test solutions* and the *Standard solutions* to actual molecular weights. The apparent molecular weight of protein A from the *Standard solution* is between 156 and 205 kDa; and the Protein A from the *Test solution* is within the same range.

Column cleaning and storage—Rinse the column with 100 mL of *Column regeneration solution*, and store by flushing with 100 mL of *Column storage solution*.

rPROTEIN A, C-CYS

C₁₄₇₈H₂₃₂₀N₄₃₂O₅₀₃S₄
34317.5 Da

N-terminal Sequence AQHDEAQQNA

rProtein A, C-Cys is a recombinant Protein A lacking the C-terminal membrane binding part; instead, a C-terminal cysteine has been introduced for directed immobilization purposes. It has five homologous IgG binding domains identical to the native Protein A and is produced using *Escherichia coli* as the host cell followed by purification with conventional chromatography. rProtein A, C-Cys is manufactured as a bulk solution with an IgG-binding potency of greater than 95%. Because rProtein A, C-Cys is used as an ancillary material in the manufacture of recombinant therapeutic drugs, regulatory requirements differ from those for therapeutic drug products.

Packaging and storage—Store in closed containers at the temperature indicated on the label.

Labeling—Preserve in sealed containers, and store at a temperature of -20° or below.

USP Reference standards (11)—*USP Endotoxin RS. USP rProtein A, C-Cys RS.*

Identification—

A: SDS-PAGE—It meets the requirements of *Identification test A* under *rProtein A* using USP rProtein A, C-Cys RS.

B: IgG Binding—It meets the requirements of *Identification test B* under *rProtein A* using USP rProtein A, C-Cys RS.

Microbial enumeration tests (61) and **Tests for specified microorganisms** (62)—The total aerobic microbial count does not exceed 100 cfu per mL, and the total yeasts and molds count does not exceed 10 cfu per mL.

Bacterial endotoxins (85)—It contains not more than 1 USP Endotoxin Unit per mg of total protein. [NOTE—The *Bacterial endotoxins* test for rProtein A, C-Cys is used to describe the quality of this ancillary material. This test does not define the acceptable level of bacterial endotoxin in the preparation of injectable dosage forms in which rProtein A, C-Cys is used.]

Total protein (see *Spectrophotometry and Light-Scattering* (851))—Prepare triplicate samples for analysis by diluting the rProtein A, C-Cys to 3.0 mg per mL in *Water for Injection*. Measure the absorbance of each sample at 275 nm after correcting for the absorbance using *Water for Injection* as the blank. Determine the protein concentration using the equation:

$$\text{Protein concentration (mg per mL)} = (A_{275}/0.22)$$

in which A is the absorbance of rProtein A, C-Cys, at the wavelength of 275 nm and 0.22 is the molar absorptivity.

Average the triplicate results, and determine a coefficient of variance (CV): the CV is $\leq 2.5\%$.

Chromatographic purity—[NOTE—The size-exclusion chromatographic purity test resolves rProtein A, C-Cys from high molecular weight contaminants and low molecular weight contaminants.]

Mobile phase—Prepare a solution of 0.02 M sodium phosphate, pH 7.2 containing 0.15 M sodium chloride in the following manner. Add 0.96 ± 0.02 g of monobasic sodium phosphate hydrate, 2.32 ± 0.02 g of dibasic sodium phosphate dihydrate, and 8.76 ± 0.02 g of sodium chloride into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 1 M sodium hydroxide to a pH of 7.2 ± 0.05 . Transfer this solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45- μ m membrane filter.

EDTA solution—Prepare a 20 mM ethylenediaminetetraacetic acid (EDTA) solution by dissolving 0.74 ± 0.02 g of EDTA in 100 mL of *Mobile phase*.

DTT solution—Prepare a 100 mM DL-dithiothreitol (DTT) solution by dissolving 1.54 ± 0.02 g of DTT in 100 mL of *Mobile phase*.

Pretreatment solution—Prepare a solution containing a mixture of *EDTA solution* and *DTT solution* (1:1, v/v). [NOTE—Prepare fresh just before use.]

Test solution—Dilute rProtein A, C-Cys 1 to 5 in *Pretreatment solution*, and mix gently. Incubate the sample at 40° for 60 minutes.

Chromatographic system (see *Chromatography* (621))—The liquid chromatograph is equipped with a 214-nm detector and a 10-mm \times 30-cm column that contains packing L54. Equilibrate the column with at least two column volumes of *Mobile phase* at a flow rate of 0.4 mL per minute.

Procedure—Inject 100 μ L of *Pretreatment solution*, and allow the chromatography to continue for at least two column volumes. Repeat this twice before injecting 100 μ L of the *Test solution*. Absorbance is detected at 214 nm. Integrate the main peak from the *Test solution* run and all other peaks not present in the *Pretreatment solution* runs. Calculate the percentage of impurities in the portion of the rProtein A, C-Cys taken by the formula:

$$100(r_i/r_s)$$

in which r_i is the peak response for each impurity; and r_s is the sum of the responses of all the peaks: the sum of all impurities is not more than 5%; and the *Test solution* shows a major peak at approximately 35 minutes.

rPROTEIN A

C₁₉₁₇H₃₀₃₉N₅₆₅O₆₅₈S₃
44,618

N-terminal Sequence FLRPVE

Protein A is a component of the cell wall of *Staphylococcus aureus*. Recombinant Protein A (rProtein A) consists of five homologous immunoglobulin (IgG) binding domains (E, D, A, B, C) followed by a partial X domain sequence. It is expressed in *Escherichia coli* and purified via a column chromatography process. IgG columns are not used in the purification process. It is manufactured as a bulk solution with an IgG-binding potency greater than 95%. Release testing methods and specifications are described below. Because rProtein A is used as an ancillary material in the manufacture of recombinant therapeutic drugs, regulatory requirements differ from those of therapeutic drug products.

Packaging and storage—Store in closed containers at the temperature indicated on the label.

Labeling—The labeling states that the material is of recombinant DNA origin along with the lot number, storage conditions, and the statement "Formulated in *Water for Injection*."

USP Reference standards (11)—USP Endotoxin RS. USP rProtein A RS.

Identification—

A: SDS-PAGE—

Molecular weight marker—Use a suitable molecular weight marker (MWM) containing protein bands between 20 and 200 kD.

PBS solution—Prepare a solution that contains 8065.0 mg and 200.0 mg of sodium chloride and potassium chloride, respectively, per L of 0.01 M sodium phosphate buffer, pH 7.4.

4X Sample buffer²—Dissolve 0.666 g of tris-hydrochloride, 0.682 g of tris base, 0.800 g of lithium dodecyl sulfate (LDS), 0.006 g of ethylenedinitrilotetraacetic acid (EDTA), and 4 g of glycerol in 8 mL of water; add 0.75 mL of 1% Coomassie brilliant blue G-250 (see Coomassie Blue G-250 in *Reagents under Reagents, Indicators, and Solutions*) solution and 0.25 mL of 1% phenol red solution. Mix well, and adjust the volume with water to 10 mL.

2X Sample buffer—Prepare a mixture of 4X Sample buffer and water (1:1).

1X Sample buffer—Prepare a mixture of 2X Sample buffer and water (1:1).

1 M Dithiothreitol solution—Dissolve 0.154 g of DL-dithiothreitol (DTT) in 1 mL of water.

2X Reducing sample buffer—Mix 180 μ L of 2X Sample buffer and 20 μ L of 1 M Dithiothreitol solution.

20X Running buffer³—Dissolve 104.6 g of 3-(N-morpholino)propanesulfonic acid (MOPS), 60.6 g of tris base, 10 g of sodium dodecyl sulfate (SDS), and 3.0 g of EDTA in 400 mL of water. Mix well, and adjust with water to 500 mL.

1X Running buffer—Prepare a solution of water and 20X Running buffer (19:1).

Gel staining solution—Prepare a solution of Coomassie brilliant blue R-250 (see Coomassie Brilliant Blue R-250 in *Reagents under Reagents, Indicators, and Solutions*) having a concentration of 0.5 g per L in a mixture of water, isopropanol, and acetic acid (6.5:2.5:1.0). Filter, and store at room temperature. Silver staining is not recommended.

Destaining solution—Mix 100 mL of acetic acid with 900 mL of water.

Standard preparation—Dilute USP rProtein A RS to 0.4 mg per mL with PBS solution. Further dilute this solution 1:1 with 2X Reducing sample buffer, and incubate in a closed tube for 5 minutes at 90°. Mix, and quick spin prior to loading.

Test preparation—Dilute rProtein A with PBS solution to 0.4 mg per mL. Proceed as directed under *Standard preparation* beginning with "Further dilute."

Comix solution—Dilute rProtein A and USP rProtein A RS with PBS solution to 0.8 mg per mL. This solution contains 0.4 mg per mL of each protein. Proceed as directed under *Standard preparation* beginning with "Further dilute."

SDS-PAGE gel and apparatus set-up—Assemble gel apparatus following the manufacturer's instructions. Lock the gel tension wedge in place, and fill approximately 200 mL of 1X Running buffer into the inside chamber. If there are no leaks, pour 600 mL of 1X Running buffer into the outer chamber. Gently pull the comb out of the cassette to immerse the wells in 1X Running buffer. Load 10 μ L of each preparation as directed below under *Gel loading* onto a 10% Bis-Tris SDS-PAGE gel.⁴

Gel loading—Use the following gel loading scheme when running one *Test preparation* (see Table 1). Each *Test preparation* is run by itself and as part of the *Comix solution* that contains the rProtein A and USP rProtein A RS.

Table 1

Lane	Sample	Load Volume (μ L)	Load Amount (μ g)
1	1X Sample buffer	10	N/A
2	MWM	20	N/A
3	Test preparation #1	10	2
4	Comix solution #1	10	4 (total)
5	Test preparation #1	10	2
6	1X Sample buffer	10	N/A
7	Standard preparation	10	2
8	MWM	20	N/A
9	—	—	—
10	—	—	—

Running the gel—Set the voltage to 125 volts, and run at a constant voltage. Run the gels until the bromophenol blue band is approximately 5 mm from the bottom of the gel (approximately 120 to 140 minutes).

Gel staining—Pour approximately 100 mL of *Gel staining solution* into the staining container. Place the gel into the staining container, and allow the stain to completely cover the gel. Cook the gel and container in a microwave for 30 seconds. Place the staining container on an orbital shaker, and stain the gel for 1 hour with gentle shaking.

Destaining—Drain the *Gel staining solution*, and add enough *Destaining solution* to the container to cover the gel. Place the container on an orbital shaker, and shake at low speed. Change the *Destaining solution* as necessary until a clear background is obtained. After destaining, rinse the gel thoroughly with water, and leave the gel in water for 10 minutes before scanning.

Gel scanning—Apply some water to the glass plate of the scanner, and place the gels on a wetted glass plate. Eliminate any bubbles. Using appropriate settings, scan the gels.

Data analysis—Choose a band between the 20 kD and 30 kD bands of the MWM to calculate the percentage of the retention factor. Draw a line in one lane (lane containing 1X Sample buffer) from the well to the apex (region of greatest intensity) of the chosen band.

The length of this line is denoted as the total distance (D_T). For the lanes containing samples draw a line from the well to the apex of each band. For each band the length of this distance is the migration distance (D_M) in mm. Record the D_T and D_M on the report sheet for each peak or band. The total distance should be the same for each lane on a gel. Calculate the percentage of the retention factor (R_F) of each major peak or band, and document on the report sheet using the following equation:

$$\%R_F = D_M/D_T \times 100$$

Also for each gel, record the number of bands and approximate molecular weight of each band in each sample.

System suitability—All bands between 20 kD and 70 kD are present. The lane containing 1X Sample buffer does not contain any bands.

Specificity—The rProtein A has one major band and a similar molecular weight that corresponds to those of the USP rProtein A RS. The *Comix solution* also shows a single major band.

B: IgG Binding—[NOTE—The IgG binding assay is a functional method for determining the percentage of rProtein A capable of binding to immobilized human polyclonal immunoglobulin. Since the percent of functional rProtein A in each lot is not less than 95%, the assay measures unbound protein versus total protein injected. This is done by comparing the absorbance in the flow-through to absorbance from an injection bypassing the column.]

²4X NuPAGE LDS sample buffer is available from Invitrogen (No. NP0007).

³20X NuPAGE MOPS SDS Running Buffer is available from Invitrogen (No. NP0001).

⁴10% Bis-Tris SDS-PAGE gel is available from Invitrogen (No. NP0301).

Sample pretreatment (desalting)—In order to remove any buffer components that may contribute to absorbance in the “unbound” IgG column fraction, samples are desalted with *Solution A*. Desalting may be performed using a suitable desalting column⁵ depending on the volumes required.

IgG column—A 1-mL Sepharose column⁶ with immobilized human polyclonal IgG (hIgG) is required to perform this assay. [NOTE—The IgG column requires washing when it is new, when it has performed several analysis cycles, or after system suitability failure. Column washing procedure is not required for each sample injection.]

Column washing solution A—Prepare a solution of 0.5 M acetic acid, pH 3.4 by adding 28.6 mL of acetic acid into a 1000-mL beaker, diluting to 900 mL with water, and adjusting with ammonium acetate to a pH of 3.4. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45-μm membrane filter.

Column washing solution B—Prepare a solution of 50 mM Tris, pH 7.6, 150 mM sodium chloride, and 0.05% Tween 20 by the following procedure. Add 6.06 ± 0.01 g of Tris and 8.77 ± 0.01 g of sodium chloride into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 0.5 M sodium hydroxide to a pH of 7.60 ± 0.05. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45-μm membrane filter (buffer solution). Add 0.5 mL of Tween 20 into 1 L of the buffer solution and mix thoroughly.

Solution A—Prepare a solution of 20 mM monobasic sodium phosphate and 150 mM sodium chloride, pH 7.6 by the following procedure. Add 2.76 ± 0.01 g monobasic sodium phosphate hydrate and 8.77 ± 0.01 g sodium chloride into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 5 M sodium hydroxide to a pH of 7.60 ± 0.05. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45-μm membrane filter.

Solution B—Prepare a solution of 100 mM phosphoric acid pH 2.8 by the following procedure. Add 6.8 mL of phosphoric acid into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 2 M potassium hydroxide to a pH of 2.80 ± 0.05. Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume.

Mobile phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic system*. Make adjustments if necessary (see *System Suitability* under *Chromatography* (621)).

Standard preparation—Thaw USP rProtein A RS, and use directly.

⁵Zeba columns are available from Pierce; Nap-10 columns are available from GE Healthcare.

⁶HiTrap IgG Sepharose 6 FF column is available from GE Healthcare (No. 90-1003-97).

Test preparation—Prepare a 4.0 to 6.0 mg per mL rProtein A solution in *Solution A*.

Chromatographic system—The liquid chromatograph is equipped with a 280-nm detector and a 1-mL column with immobilized hIgG. The chromatograph is equipped with a bypass valve to allow flow to be diverted from the column. Each analysis consists of a series of two injections, one where the sample is injected onto the column and one where the sample bypasses the column and flows directly into the detector. Perform three replicate analyses. The chromatograph is programmed as follows (see *Table 2*). Chromatograph the *Standard preparation*, record the peak responses, and calculate the percentage of hIgG binding as directed for *Procedure*: the percentage of hIgG binding ≥ 95% and the relative standard deviation for replicate analysis is not more than 1%.

Procedure—Inject a volume (about 100 μL) of the *Test preparation*. Record the chromatogram, and measure the peak responses. Calculate the percentage of hIgG binding activity by the following formula:

$$100 - 100(r_C/r_B)$$

in which r_C is the unbound material peak response from the column injection and r_B is the bypass peak response from the bypass injection. Each replicate analysis of the *Test preparation* is not less than 95% of hIgG binding. Report the average value from three replicate analyses.

Microbial enumeration tests (61) and Tests for specified microorganisms (62)—The total aerobic microbial count does not exceed 100 cfu per mL, and the total yeasts and molds count does not exceed 10 cfu per mL.

Bacterial endotoxins (85)—It contains not more than 0.5 USP Endotoxin Unit per mg of total protein. [NOTE—The *Bacterial endotoxins* test for rProtein A is used to describe the quality of this ancillary material. This test does not define the acceptable level of bacterial endotoxin in the preparation of injectable dosage forms in which rProtein A is used.]

Total protein (see *Spectrophotometry and Light-Scattering* (851))—Prepare triplicate samples for analysis by diluting the rProtein A to 3.0 mg per mL in *Water for Injection*. Measure the absorbance of each sample at 275 nm after correcting for the absorbance using *Water for Injection* as the blank. Determine the protein concentration using the equation:

$$\text{Protein concentration (mg per mL)} = (A_{275}/0.165)$$

in which A is the absorbance of rProtein A at the wavelength of 275 nm and 0.165 is the molar absorptivity.

Table 2

Flow Rate (mL per min- ute)	Time (minutes)	Solution A (%)	Solution B (%)	Valve Position	Elution
1.0	0–6	100	0	Column	Re-equilibration
1.0	6–12	100→0	0→100	Column	Re-equilibration
1.0	12–22	100	0	Column	Equilibration
0.4	22–25	100	0	Column	Equilibration
0.4 (sample injected)	25–35	100	0	Column	Isocratic
1.0	35–49	100→0	0→100	Column	Regeneration
1.0	49–63	0→100	100→0	Column	Re-equilibration
1	63–65	100	0	Bypass	Equilibration
0.4	65–68	100	0	Bypass	Equilibration
0.4 (sample injected)	68–75	100	0	Bypass	Isocratic

Average the triplicate results, and determine a coefficient of variance (CV): the CV is $\leq 5\%$.

UV spectral analysis—Dilute rProtein A to 1 mg per mL in *Water for Injection*. Using a scanning UV spectrophotometer and *Water for Injection* as the blank, obtain spectral scans over the range of 240 to 360 nm. From the resulting data, calculate the absorbance value at 270 nm and the ratio of absorbance at 270 to 250 nm (i.e., E270/E250): the absorbance at 270 nm of a 1 mg per mL solution of rProtein A in *Water for Injection* is within the range 0.14–0.20.

Chromatographic purity—[NOTE—The size-exclusion chromatographic purity test resolves rProtein A from high molecular weight contaminants.]

Mobile phase—A solution of 0.3 M sodium phosphate, pH 7 is prepared by mixing monobasic and dibasic phosphate solutions in the following manner. Weigh 21.3 ± 0.1 g of dibasic anhydrous sodium phosphate, and dissolve in 500 mL of water to obtain a 0.3 M dibasic sodium phosphate solution (*Solution 1*). Into a separate container, weigh 18.0 ± 0.1 g monobasic anhydrous sodium phosphate, and dissolve in 500 mL of water to obtain a 0.3 M monobasic sodium phosphate solution (*Solution 2*). Calibrate a pH meter using pH calibrators at a pH of 7 and 10. Add 400 mL of *Solution 1* to a 1-L beaker. Transfer the pH probe to the beaker. Slowly add *Solution 2* to the solution until the pH is 7.0 ± 0.1 . Pass the solution through a 0.45- μ m membrane filter.

Standard solution—Dilute USP rProtein A RS to 1 mg per mL in *Mobile phase*.

Test solution—Dilute rProtein A to 1 mg per mL in *Mobile phase*.

Chromatographic system (see *Chromatography* (621))—The liquid chromatograph is equipped with a 214-nm and 280-nm detector and a 9.4-mm \times 25-cm column that contains packing L35. The flow rate is 1 mL per minute. Chromatograph the *Standard solution* as directed for *Procedure*: rProtein A shows a single major peak at approximately 9 minutes and the area percentage is $\geq 98\%$ at 214 nm and $\geq 95\%$ at 280 nm.

Procedure—Inject 100 μ L of the *Test solution* into the chromatograph, run isocratically for 15 minutes, and record the chromatogram. The values for the rProtein A from the *Test solution* correspond to the specifications of the USP rProtein A RS from the *Standard solution*.

Isoforms—

Standard solution—Thaw USP rProtein A RS, and use directly.

Test solution—Dilute rProtein A to 4 mg per mL in *Water for Injection*.

pI Markers—Use a suitable marker set containing markers between 3 and 10.⁷

IEF gel—Use a suitable gel with the range of between 3 and 10 and a size of 100 \times 125 mm.⁸

Procedure—Apply 5- μ L aliquots of the *pI Markers*, *Test solution*, and the *Standard solution* to the IEF gel, and run under 1W of power for approximately 10 minutes. Remove the sample mask, and apply power with concurrent cooling between 5° to 10° of the focusing chamber for 40 minutes at a setting of 1000V, 20 mA, 25W. Fix the IEF gel for 1 hour in 20% trichloroacetic acid, then stain using a suitable stain for IEF gels.⁹ Finally, wash and dry the gel: the correlation coefficient of the best fit line for the *pI Markers* versus their migration in cm is ≥ 0.990 , and the rProtein A from the *Standard solution* shows a single major band within the *pI* range of 4.6 to 5.2. A single band is seen in the *Test solution* that corresponds to the *pI* range of the *Standard solution*.

⁷pI markers in the 3–10 range are available from BioRad (No. 161-0310).

⁸IEF gels in the 3–10 range are available from Cambrex (No. 56015).

⁹ISS Pro-Blue is available from Integrated Separation Systems.

Limit of Triton X-100—

Mobile phase—Prepare a filtered and degassed mixture of water and acetonitrile (60:40).

Test solution—Dilute rProtein A to 5 mg per mL in *Water for Injection*.

Triton X-100 spike solution—Combine 5 mg per mL of USP rProtein A RS and 0.15% Triton X-100 (9:1) to obtain a solution having known concentrations of rProtein A and 0.015% Triton X-100.

Chromatographic system (see *Chromatography* (621))—The liquid chromatograph is equipped with a 214-nm and a 280-nm detector and a 4.6-mm \times 25-cm column that contains 5- μ m packing L11. The flow rate is 1 mL per minute. Chromatograph the *Triton X-100 spike solution* as directed for *Procedure*: Triton X-100 has a single major peak at approximately 9 minutes, and the rProtein A shows a smaller or undetectable peak at the same retention time.

Procedure—Inject about 100 μ L of the *Test solution* into the chromatograph, run isocratically for 35 minutes, and record the chromatogram. The absorbance is detected at 223 nm: the Triton X-100 peak is not more than 0.015% (equivalent to the *Triton X-100 spike solution*).

rPROTEIN A, B₄, C-CYS

C₁₁₇₇H₁₈₅₄N₃₂₆O₃₈₄S₁

26747.6 Da

N-terminal Sequence AQGTVDKFD

rProtein A, B₄, C-Cys is a recombinant protein derived from the B-domain of Protein A. The Protein A domain has been alkali-stabilized by site-specific mutagenesis and multimerized to a tetramer with a C-terminal cysteine for directed immobilization purposes. rProtein A, B₄, C-Cys is produced using *Escherichia coli* as the host cell followed by purification with conventional chromatography. rProtein A, B₄, C-Cys is manufactured as a bulk solution with an IgG-binding potency of greater than 95%. Because rProtein A, B₄, C-Cys is used as an ancillary material in the manufacture of recombinant therapeutic drugs, regulatory requirements differ from those for therapeutic drug products.

Packaging and storage—Store in closed containers at the temperature indicated on the label.

Labeling—Preserve in sealed containers, and store at a temperature of -20° or below.

USP Reference standards (11)—USP Endotoxin RS. USP rProtein A, B₄, C-Cys RS.

Identification—

A: SDS-PAGE—It meets the requirements of *Identification test A* under rProtein A using USP rProtein A, B₄, C-Cys RS.

B: IgG Binding—It meets the requirements of *Identification test B* under rProtein A using USP rProtein A, B₄, C-Cys RS.

Microbial enumeration tests (61) and **Tests for specified microorganisms** (62)—The total aerobic microbial count does not exceed 100 cfu per mL, and the total yeasts and molds count does not exceed 10 cfu per mL.

Bacterial endotoxins (85)—It contains not more than 1 USP Endotoxin Unit per mg of total protein. [NOTE—The *Bacterial endotoxins* test for rProtein A, B₄, C-Cys is used to describe the quality of this ancillary material. This test does not define the acceptable level of bacterial endotoxin in the preparation of injectable dosage forms in which rProtein A, B₄, C-Cys is used.]

Total protein (see *Spectrophotometry and Light-Scattering* (851))—

Formulation buffer solution—Prepare a solution of 0.02 M potassium phosphate, pH 7.0, containing 0.15 M potassium chloride and 2 mM of ethylenediaminetetraacetic acid (EDTA) in the following manner. Add 2.72 ± 0.01 g of monobasic potassium phosphate anhydrous, 11.18 ± 0.22 g of potassium chloride, and 0.744 ± 0.02 g of EDTA into a 1000-mL beaker. Dilute with water to 900 mL, and adjust

with 1 M sodium hydroxide to a pH of 7.00 ± 0.05 . Transfer the solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45- μ m membrane filter.

Test preparation—Dilute the rProtein A, B₄, C-Cys to 3.0 mg per mL with *Formulation buffer solution*.

Procedure—Prepare triplicate samples for analysis. Measure the absorbance of each *Test preparation* at 275 nm after correcting for the absorbance using the *Formulation buffer solution* as the blank. Determine the protein concentration using the equation:

$$\text{Protein concentration (mg per mL)} = (A_{275}/0.22)$$

in which A is the absorbance of rProtein A, B₄, C-Cys, at the wavelength of 275 nm and 0.22 is the molar absorptivity. Average the triplicate results, and determine a coefficient of variance (CV): the CV $\leq 2.5\%$.

Chromatographic purity—[NOTE—The size-exclusion chromatographic purity test resolves rProtein A, B₄, C-Cys from high molecular weight contaminants and low molecular weight contaminants.]

Mobile phase—Prepare a solution of 0.02 M sodium phosphate, pH 7.2 containing 0.15 M sodium chloride in the following manner. Add 0.96 ± 0.02 g of monobasic sodium phosphate hydrate, 2.32 ± 0.02 g of dibasic sodium phosphate dihydrate, and 8.76 ± 0.02 g of sodium chloride into a 1000-mL beaker. Dilute with water to 900 mL, and adjust with 1 M sodium hydroxide to a pH of 7.2 ± 0.05 . Transfer this solution into a 1000-mL volumetric flask, and dilute with water to volume. Pass the solution through a 0.45- μ m membrane filter.

EDTA solution—Prepare a 20 mM EDTA solution by dissolving 0.74 ± 0.02 g of EDTA in 100 mL of *Mobile phase*.

DTT solution—Prepare a 100 mM DL-dithiothreitol (DTT) solution by dissolving 1.54 ± 0.02 g of DTT in 100 mL of *Mobile phase*.

Pretreatment solution—Prepare a solution containing a mixture of *EDTA solution* and *DTT solution* (1:1, v/v). [NOTE—Prepare fresh just before use.]

Test solution—Dilute rProtein A, B₄, C-Cys 1 to 5 in *Pretreatment solution*, and mix gently. Incubate the sample at 40° for 60 minutes.

Chromatographic system (see *Chromatography* (621))—The liquid chromatograph is equipped with a 214-nm detector and a 10-mm \times 30-cm column that contains packing L54. Equilibrate the column with at least two column volumes of *Mobile phase* at a flow rate of 0.4 mL per minute.

Procedure—Inject 100 μ L of *Pretreatment solution*, and allow the chromatography to continue for at least two column volumes. Repeat this twice before injecting 100 μ L of the *Test solution*. Absorbance is detected at 214 nm. Integrate the main peak from the *Test solution* run and all other peaks not present in the *Pretreatment solution* runs. Calculate the percentage of impurities in the portion of rProtein A, B₄, C-Cys taken by the formula:

$$100(r_i/r_s)$$

in which r_i is the peak response for each impurity; and r_s is the sum of all the responses of all the peaks: the sum of all impurities is not more than 5%; and the *Test solution* shows a major peak at approximately 37 minutes.

(151) PYROGEN TEST

The pyrogen test is designed to limit to an acceptable level the risks of febrile reaction in the patient to the administration, by injection, of the product concerned. The test involves measuring the rise in temperature of rabbits following the intravenous injection of a test solution and is designed for products that can be tolerated by the test rabbit in a dose not to exceed 10 mL per kg injected intravenously within a period of not more than 10 minutes. For products that require preliminary preparation or are subject to special conditions of administration, follow the additional directions given in the individual monograph or, in the case of antibiotics or biologics, the additional directions given in the federal regulations (see *Biologics* (1041)).

APPARATUS AND DILUENTS

Render the syringes, needles, and glassware free from pyrogens by heating at 250° for not less than 30 minutes or by any other suitable method. Treat all diluents and solutions for washing and rinsing of devices or parenteral injection assemblies in a manner that will assure that they are sterile and pyrogen-free. Periodically perform control pyrogen tests on representative portions of the diluents and solutions for washing or rinsing of the apparatus. Where Sodium Chloride Injection is specified as a diluent, use Injection containing 0.9 percent of NaCl.

TEMPERATURE RECORDING

Use an accurate temperature-sensing device such as a clinical thermometer, or thermistor probes or similar probes that have been calibrated to assure an accuracy of $\pm 0.1^\circ$ and have been tested to determine that a maximum reading is reached in less than 5 minutes. Insert the temperature-sensing probe into the rectum of the test rabbit to a depth of not less than 7.5 cm, and, after a period of time not less than that previously determined as sufficient, record the rabbit's body temperature.

TEST ANIMALS

Use healthy, mature rabbits. House the rabbits individually in an area of uniform temperature between 20° and 23° and free from disturbances likely to excite them. The temperature varies not more than $\pm 3^\circ$ from the selected temperature. Before using a rabbit for the first time in a pyrogen test, condition it not more than seven days before use by a sham test that includes all of the steps as directed for *Procedure* except injection. Do not use a rabbit for pyrogen testing more frequently than once every 48 hours, nor prior to 2 weeks following a maximum rise of its temperature of 0.6° or more while being subjected to the pyrogen test, or following its having been given a test specimen that was adjudged pyrogenic.

PROCEDURE

Perform the test in a separate area designated solely for pyrogen testing and under environmental conditions similar to those under which the animals are housed and free from disturbances likely to excite them. Withhold all food from the rabbits used during the period of the test. Access to water is allowed at all times, but may be restricted during the test. If rectal temperature-measuring probes remain inserted throughout the testing period, restrain the rabbits with light-fitting neck stocks that allow the rabbits to assume a natural resting posture. Not more than 30 minutes prior to the injection of the test dose, determine the "control temperature" of each rabbit: this is the base for the determination of any temperature increase resulting from the injection of a test solution. In any one group of test rabbits, use only those rabbits whose control temperatures do not vary by more than 1° from each other, and do not use any rabbit having a temperature exceeding 39.8°.

Unless otherwise specified in the individual monograph, inject into an ear vein of each of three rabbits 10 mL of the test solution per kg of body weight, completing each injection within 10 minutes after start of administration. The test solution is *either* the product, constituted if necessary as directed in the labeling, *or* the material under test treated as directed in the individual monograph and injected in the dose specified therein. For pyrogen testing of devices or injection assemblies, use washings or rinsings of the surfaces that come in contact with the parenterally administered material or with the injection site or internal tissues of the patient. Assure that all test solutions are protected from contamination. Perform the injection after warming the test solution to a temperature of $37 \pm 2^\circ$. Record the temperature at 30-minute intervals between 1 and 3 hours subsequent to the injection.

TEST INTERPRETATION AND CONTINUATION

Consider any temperature decreases as zero rise. If no rabbit shows an individual rise in temperature of 0.5° or more above its respective control temperature, the product meets the requirements for the absence of pyrogens. If any rabbit shows an individual temperature rise of 0.5° or more, continue the test using five other rabbits. If not more than three of the eight rabbits show individual rises in temperature of 0.5° or more and if the sum of the eight individual maximum temperature rises does not exceed 3.3°, the material under examination meets the requirements for the absence of pyrogens.

RADIOACTIVE PHARMACEUTICALS

Test Dose for Preformulated, Ready-to-Use Products Labeled with Radioactivity

AGGREGATED ALBUMIN and OTHER PARTICLE-CONTAINING PRODUCTS

For the rabbit pyrogen test, dilute the product with Sodium Chloride Injection to not less than 100 μCi per mL, and inject a dose of 3 mL per kg of body weight into each rabbit.

OTHER PRODUCTS

Where Physical Half-life of Radionuclide Is Greater Than 1 Day—Calculate the maximum volume of the product that might be injected into a human subject. This calculation

takes into account the maximum recommended radioactive dose of the product, in μCi , and the radioactive assay, in μCi per mL, of the product at its expiration date or time. Using this information, calculate the maximum volume dose per kg to a 70-kg human subject.

For the rabbit pyrogen test, inject a minimum of 10 times this dose per kg of body weight into each rabbit. If necessary, dilute with Sodium Chloride Injection. The total injected volume per rabbit is not less than 1 mL and not more than 10 mL of solution.

Where Physical Half-life of Radionuclide is Less Than 1 Day—For products labeled with radionuclides having a half-life of less than 1 day, the dosage calculations are identical to those described in the first paragraph under *Other Products*. These products may be released for distribution prior to completion of the rabbit pyrogen test, but such test shall be initiated at not more than 36 hours after release.

Test Dose for Pharmaceutical Constituents or Reagents to Be Labeled

The following test dose requirements pertain to reagents that are to be labeled or constituted prior to use by the direct addition of radioactive solutions such as Sodium Pertechnetate Tc 99m Injection, i.e., "cold kits".

Assume that the entire contents of the vial of nonradioactive reagent will be injected into a 70-kg human subject, or that $1/70$ of the total contents per kg will be injected. If the contents are dry, constitute with a measured volume of Sodium Chloride Injection.

For the rabbit pyrogen test, inject ($1/7$) of the vial contents per kg of body weight into each rabbit. The maximum dose per rabbit is the entire contents of a single vial. The total injected volume per rabbit is not less than 1 mL and not more than 10 mL of solution.

<161> TRANSFUSION AND INFUSION ASSEMBLIES AND SIMILAR MEDICAL DEVICES

The requirements apply to sterile and nonpyrogenic assemblies or devices in contact directly or indirectly with the cardiovascular system, the lymphatic system, or cerebrospinal fluid. This includes, but is not limited to, solution administration sets, extension sets, transfer sets, blood administration sets, intravenous catheters, implants extracorporeal oxygenator tubings and accessories, dialysers and dialysis tubing and accessories, heart valves, vascular grafts, intramuscular drug delivery catheters, and transfusion and infusion assemblies. These requirements do not apply to orthopedic products, latex gloves, or wound dressings.

Sterility—Proceed as directed for *Sterilized Devices* under *Sterility Tests* <71>.

Bacterial Endotoxins—Proceed as directed under *Bacterial Endotoxins Test* <85>.

For medical devices, the endotoxin limit is not more than 20.0 USP Endotoxin Units per device except that for those medical devices in contact with the cerebrospinal fluid the limit is not more than 2.15 USP Endotoxin Units per device.

A device that fails this test can be retested once by another *Bacterial Endotoxins* test. For devices that cannot be tested by the *Bacterial Endotoxins Test* <85> because of non-removable inhibition or enhancement, the *Pyrogen Test* <151> is applied.

Preparation of Devices—Select not less than 3 and not more than 10 devices. Rinse or soak the devices with LAL Reagent Water. The volume of rinsing or extracting solution may be adjusted for the size and configuration of the device.

For devices labeled “nonpyrogenic fluid pathway,” flush the fluid pathway with extracting fluid that has been heated to $37 \pm 1.0^\circ$, keeping the extracting fluid in contact with the relevant pathway for not less than 1 hour at controlled room temperature. Extracts may be combined, where appropriate. The endotoxin limit for the rinsing or extracting solution is calculated by the formula:

$$(K \times N)/(V)$$

where K is equal to the amount of endotoxin allowed per device, N is equal to the number of devices tested, and V is equal to the total volume of the extract or rinse. If the undiluted rinsing or extracting solution is unsuitable for the *Bacterial Endotoxins Test* (85), repeat the inhibition or enhancement test after neutralization and removal of the interfering substances or after the solution has been diluted by a factor not exceeding the Maximum Valid Dilution. The Maximum Valid Dilution for devices is calculated by dividing the endotoxin limit by the labeled sensitivity λ of the LAL reagent used.

Pyrogen—For samples that cannot be tested by the *Bacterial Endotoxins Test* because of nonremovable inhibition or enhancement of the test, the *Pyrogen Test* (151) is applied. Select 10 devices, and obtain a pooled effluent, utilizing preparation methods appropriate to the device as directed for *Bacterial Endotoxins*, but with volumes of rinse or extraction fluid not to exceed 40 mL of sterile saline TS per device. The requirements of the *Pyrogen Test* (151) are met.

Other Requirements—The portions of medical devices that are made of plastics or other polymers meet the requirements specified for *Biological Tests—Plastics and Other Polymers under Containers—Plastics* (661); those made of elastomers meet the requirements under *Elastomeric Closures for Injections* (381). If a class designation for elastomers, plastics, or other polymers is needed, perform the appropriate in vivo tests indicated in the general test chapter *Biological Reactivity Tests, In Vivo* (88).

(171) VITAMIN B₁₂ ACTIVITY ASSAY

USP Reference Standards (11)—*USP Cyanocobalamin RS*.

Assay Preparation—Place a suitable quantity of the material to be assayed, previously reduced to a fine powder if necessary and accurately measured or weighed, in an appropriate vessel containing, for each g or mL of material taken, 25 mL of an aqueous extracting solution prepared just prior to use to contain, in each 100 mL, 1.29 g of disodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 minutes. Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of the clear solution with water so that the final test solution contains vitamin B₁₂ activity approximately equivalent to that of the *Standard Cyanocobalamin Solution* which is added to the assay tubes.

Standard Cyanocobalamin Stock Solution—To a suitable quantity of USP Cyanocobalamin RS, accurately weighed, add sufficient 25 percent alcohol to make a solu-

tion having a known concentration of 1.0 μ g of cyanocobalamin per mL. Store in a refrigerator.

Standard Cyanocobalamin Solution—Dilute a suitable volume of *Standard Cyanocobalamin Stock Solution* with water to a measured volume such that after the incubation period as described for *Procedure*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard Cyanocobalamin Solution* is not less than that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 ng and 0.04 ng per mL of *Standard Cyanocobalamin Solution*. Prepare a fresh standard solution for each assay.

Basal Medium Stock Solution—Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed, carefully dissolving the cystine and tryptophane in the hydrochloric acid before adding the next eight solutions in the resulting solution. Add 100 mL of water, mix, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary, add the polysorbate 80 solution, adjust the solution to a pH between 5.5 and 6.0 with 1 N sodium hydroxide, and add purified water to make 250 mL.

L-Cystine	0.1 g
L-Tryptophane	0.05 g
1 N Hydrochloric Acid	10 mL
Adenine–Guanine–Uracil Solution	5 mL
Xanthine Solution	5 mL
Vitamin Solution I	10 mL
Vitamin Solution II	10 mL
Salt Solution A	5 mL
Salt Solution B	5 mL
Asparagine Solution	5 mL
Acid-hydrolyzed Casein Solution	25 mL
Dextrose, Anhydrous	10 g
Sodium Acetate, Anhydrous	5 g
Ascorbic Acid	1 g
Polysorbate 80 Solution	5 mL

Acid-Hydrolyzed Casein Solution—Prepare as directed under *Calcium Pantothenate Assay* (91).

Asparagine Solution—Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–Guanine–Uracil Solution—Prepare as directed under *Calcium Pantothenate Assay* (91).

Xanthine Solution—Suspend 0.20 g of xanthine in 30 mL to 40 mL of water, heat to about 70° , add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt Solution A—Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL. Add 2 drops of hydrochloric acid, and store under toluene.

Salt Solution B—Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid, and store under toluene.

Polysorbate 80 Solution—Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin Solution I—Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 μ g of biotin, and 20 mg of niacin in 0.02 N glacial acetic acid to make 400 mL. Store, protected from light, under toluene in a refrigerator.

Vitamin Solution II—Dissolve 20 mg of para-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in dilute neutralized alcohol (1 in 4) to make 400 mL. Store, protected from light, in a refrigerator.

Tomato Juice Preparation—Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend about 5 g per L of analytical filter-aid in the supernatant, and filter, with the aid of reduced pressure, through a layer of the filter-aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture Medium—[NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of water-soluble yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of potassium biphosphate in 60 mL to 70 mL of water. Add 10 mL of *Tomato Juice Preparation* and 1 mL of *Polysorbate 80 Solution*. Adjust the solution with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 minutes. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension Medium—Dilute a measured volume of *Basal Medium Stock Solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed above for the *Culture Medium*.

Stock Culture of *Lactobacillus leichmannii*—To 100 mL of *Culture Medium* add 1.0 g to 1.5 g of agar, and heat the mixture, with stirring, on a steam bath, until the agar dissolves. Place approximately 10-mL portions of the hot solution in test tubes, cover the tubes suitably, sterilize at 121° for 15 minutes in an autoclave (exhaust line temperature), and allow the tubes to cool in an upright position. Inoculate three or more of the tubes, by stab transfer of a pure culture of *Lactobacillus leichmannii*.^{*} (Before first using a fresh culture in this assay, make not fewer than 10 successive transfers of the culture in a 2-week period.) Incubate 16 to 24 hours at any selected temperature between 30° and 40° but held constant to within $\pm 0.5^\circ$, and finally store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the inoculum if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid inoculum can be observed 2 to 4 hours after inoculation. A slow-growing culture seldom gives a suitable response curve, and may lead to erratic results.

Inoculum—[NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an inoculum comparable to a fresh culture.] Make a transfer of cells from the *Stock Culture of Lactobacillus leichmannii* to 2 sterile tubes containing 10 mL of the *Culture Medium* each. Incubate these cultures for 16 to 24 hours at any selected temperature between 30° and 40° but held constant to within $\pm 0.5^\circ$. Under aseptic conditions, centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension Medium*, and combine. Using sterile *Suspension Medium*, adjust the volume so that a 1 in 20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1 in 400 dilution of the adjusted suspension using *Basal Medium Stock Solution*, and use it for

the test inoculum. (This dilution may be altered, when necessary, to obtain the desired test response.)

Calibration of Spectrophotometer—Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Procedure—Cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 hours, hard-glass test tubes, about 20 mm \times 150 mm in size, and other necessary glassware because of the high sensitivity of the test organism to minute amounts of vitamin B₁₂ activity and to traces of many cleansing agents.

To test tubes add, in duplicate, 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, 4.0 mL, and 5.0 mL, respectively, of the *Standard Cyanocobalamin Solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal Medium Stock Solution* and water to make 10 mL.

To similar test tubes add, in duplicate, respectively, 1.0 mL, 1.5 mL, 2.0 mL, 3.0 mL, and 4.0 mL of the *Assay Preparation*. To each tube add 5.0 mL of *Basal Medium Stock Solution* and water to make 10 mL. Place one complete set of standard and assay tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes suitably to prevent bacterial contamination, and sterilize the tubes and contents in an autoclave at 121° for 5 minutes, arranging to reach this temperature in not more than 10 minutes by preheating the autoclave, if necessary. Cool as rapidly as practicable to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, since packing tubes too closely in the autoclave, or overloading it, may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard Cyanocobalamin Solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$, for 16 to 24 hours.

Terminate growth by heating to a temperature not lower than 80° for 5 minutes. Cool to room temperature. After agitating its contents, place the container in a spectrophotometer that has been set at a wavelength of 530 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 seconds or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation—Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the standard, calculate the response from the sum of the duplicate values of the transmittances (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard Cyanocobalamin Solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y , adding together the two transmittances for each level of the *Assay Preparation*. Read from the standard curve the logarithm of the volume of the *Stan-*

^{*} Pure cultures of *Lactobacillus leichmannii* may be obtained as No. 7830 from the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110.

Standard Preparation corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Assay Preparation* to obtain the difference, x , for each dosage level. Average the values of x for each of three or more dosage levels to obtain $\bar{x} = M'$, the log-relative potency of the *Assay Preparation*. Determine the quantity, in μg , of USP Cyanocobalamin RS corresponding to the cyanocobalamin in the portion of material taken for assay by the equation $\text{antilog } M = \text{antilog } (M' + \log R)$, in which R is the number of μg of cyanocobalamin that was assumed to be present in each mg (or capsule or tablet) of the material taken for assay.

Replication—Repeat the entire determination at least once, using separately prepared *Assay Preparations*. If the difference between the two log potencies M is not greater than 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Vitamin B₁₂ Activity Assay* under *Design and Analysis of Biological Assays* (111)). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Chemical Tests and Assays

IDENTIFICATION TESTS

〈181〉 IDENTIFICATION—ORGANIC NITROGENOUS BASES

INTRODUCTION

The purpose of this test is the identification of tertiary amine compounds. This spectroscopic test has a limited degree of specificity and, therefore, the conformance with all additional identification tests listed in a particular monograph is necessary to ensure the identity of the specimen under examination.

ASSAY

• PROCEDURE

Standard solution: In a separator dissolve 50 mg of the corresponding USP Reference Standard in 25 mL of 0.01 N hydrochloric acid.

Sample solution: Depending upon the nature of the sample, dissolve 50 mg of the bulk substance under test in 25 mL of 0.01 N hydrochloric acid, or shake a quantity of powdered tablets or the contents of capsules, equivalent to 50 mg of the substance, with 25 mL of 0.01 N hydrochloric acid for 10 min. Transfer the liquid to a separator, filtering if necessary, and washing the filter and the residue with several small portions of water.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* 〈851〉.)

Mode: IR

Wavelength range: 7–15 μm (1430 cm^{-1} to 650 cm^{-1})

Cell: 1-mm

Blank: Carbon disulfide

Analysis

Samples: *Standard solution* and *Sample solution*

Treat each solution as follows: Add 2 mL of 1 N sodium hydroxide and 4 mL of carbon disulfide, and shake for 2 min. Centrifuge if necessary to clarify the lower phase, and pass it through a dry filter, collecting the filtrate in a small flask provided with a glass stopper. Determine the absorption spectra of the filtered *Standard solution* and *Sample solution* without delay.

Acceptance criteria: The spectrum of the *Sample solution* must show all of the significant absorption bands present in the spectrum of the *Standard solution*.

(191) IDENTIFICATION TESTS— GENERAL

Under this heading are placed tests that are frequently referred to in the Pharmacopeia for the identification of official articles. Before using any acid or base to modify the pH of the sample solution, make sure that the added substance will not interfere with the results of the test. [NOTE—The tests are not intended to be applicable to mixtures of substances unless so specified.]

Acetate—Dissolve about 30 mg of the substance to be examined in 3 mL of water, or use 3 mL of the prescribed solution. Adjust the pH of the solution with sodium hydroxide to slightly alkaline. Add 0.25 mL of lanthanum nitrate TS. If a white precipitate is formed, filter the solution. Add successively 0.1 mL of iodine and potassium iodide TS 3 and 0.1 mL of ammonia TS 2 to the solution. If no blue color is observed, heat carefully to boiling. In the presence of acetates, a dark color develops or a blue precipitate is formed. With neutral solutions of acetates, ferric chloride TS produces a red color that is destroyed by the addition of mineral acids.

Aluminum—With 6 N ammonium hydroxide, solutions of aluminum salts yield a gelatinous, white precipitate that is insoluble in an excess of 6 N ammonium hydroxide. 1 N sodium hydroxide or sodium sulfide TS produces the same precipitate, which dissolves in an excess of either of these reagents.

Ammonium—Add 0.2 g of magnesium oxide to the solution under test. Pass a current of air through the mixture, and direct the gas that escapes to just beneath the surface of an indicator solution, prepared by mixing 1 mL of 0.1 M hydrochloric acid and 0.05 mL of methyl red TS 2. In the presence of ammonium, the color of the indicator solution is changed to yellow. After directing the gas into the indicator solution for a sufficient period of time, add 1 mL of freshly prepared sodium cobaltinitrite TS to the indicator solution. Upon the addition of the sodium cobaltinitrite TS, a yellow precipitate is formed when ammonium is present.

Antimony—With hydrogen sulfide, solutions of antimony (III) compounds, strongly acidified with hydrochloric acid, yield an orange precipitate of antimony sulfide that is insoluble in 6 N ammonium hydroxide, but is soluble in ammonium sulfide TS.

Barium—Solutions of barium salts yield a white precipitate with 2 N sulfuric acid. This precipitate is insoluble in hydrochloric acid and in nitric acid. Barium salts impart a yellowish-green color to a nonluminous flame that appears blue when viewed through green glass.

Benzoate—In neutral solutions, benzoates yield a salmon-colored precipitate with ferric chloride TS. In moderately concentrated solutions, benzoates yield a precipitate of benzoic acid upon acidification with 2 N sulfuric acid. This precipitate is readily soluble in ethyl ether.

Bicarbonate—See *Carbonate*.

Bismuth—When dissolved in a slight excess of nitric acid or hydrochloric acid, bismuth salts yield a white precipitate upon dilution with water. This precipitate is colored brown by hydrogen sulfide, and the resulting compound dissolves in a warm mixture of equal parts of nitric acid and water.

Bisulfite—See *Sulfite*.

Borate—To 1 mL of a borate solution, acidified with hydrochloric acid to litmus, add 3 or 4 drops of iodine TS and 3 or 4 drops of polyvinyl alcohol solution (1 in 50): an intense blue color is produced. When a borate is treated with sulfuric acid, methanol is added, and the mixture is ignited, it burns with a green-bordered flame.

Bromide—Solutions of bromides, upon the addition of chlorine TS, dropwise, liberate bromine, which is dissolved

by shaking with chloroform, coloring the chloroform red to reddish brown. Silver nitrate TS produces in solutions of bromides a yellowish-white precipitate that is insoluble in nitric acid and is slightly soluble in 6 N ammonium hydroxide.

Calcium—Solutions of calcium salts form insoluble oxalates when treated as follows. To a solution of the calcium salt (1 in 20) add 2 drops of methyl red TS, and neutralize with 6 N ammonium hydroxide. Add 3 N hydrochloric acid, dropwise, until the solution is acid to the indicator. Upon the addition of ammonium oxalate TS, a white precipitate is formed. This precipitate is insoluble in 6 N acetic acid but dissolves in hydrochloric acid. Calcium salts moistened with hydrochloric acid impart a transient yellowish-red color to a nonluminous flame.

Carbonate—Carbonates and bicarbonates effervesce with acids, evolving a colorless gas that, when passed into calcium hydroxide TS, produces a white precipitate immediately. A cold solution (1 in 20) of a soluble carbonate is colored red by phenolphthalein TS, while a similar solution of a bicarbonate remains unchanged or is only slightly colored.

Chlorate—Solutions of chlorates yield no precipitate with silver nitrate TS. The addition of sulfurous acid to this mixture produces a white precipitate that is insoluble in nitric acid, but is soluble in 6 N ammonium hydroxide. Upon ignition, chlorates yield chlorides, recognizable by appropriate tests. When sulfuric acid is added to a dry chlorate, decrepitation occurs, and a greenish yellow-gas is evolved. [Caution—Use only a small amount of chlorate for this test, and exercise extreme caution in performing it.]

Chloride—With silver nitrate TS, solutions of chlorides yield a white, curdy precipitate that is insoluble in nitric acid but is soluble in a slight excess of 6 N ammonium hydroxide. When testing amine (including alkaloidal) hydrochlorides that do not respond to the above test, add one drop of diluted nitric acid and 0.5 mL of silver nitrate TS to a solution of the substance being examined containing, unless otherwise directed in the monograph, about 2 mg of chloride ion in 2 mL: a white, curdy precipitate is formed. Centrifuge the mixture without delay, and decant the supernatant layer. Wash the precipitate with three 1-mL portions of nitric acid solution (1 in 100), and discard the washings. Add ammonia TS dropwise to this precipitate. It dissolves readily. When a monograph specifies that an article responds to the test for dry chlorides, mix the solid to be tested with an equal weight of manganese dioxide, moisten with sulfuric acid, and gently heat the mixture: chlorine, which is recognizable by the production of a blue color with moistened starch iodide paper, is evolved.

Citrate—To 15 mL of pyridine add a few mg of a citrate salt, dissolved or suspended in 1 mL of water, and shake. To this mixture add 5 mL of acetic anhydride, and shake: a light red color is produced.

Cobalt—Solutions of cobalt salts (1 in 20) in 3 N hydrochloric acid yield a red precipitate when heated on a steam bath with an equal volume of a hot, freshly prepared solution of 1-nitroso-2-naphthol (1 in 10) in 9 N acetic acid. Solutions of cobalt salts, when saturated with potassium chloride and treated with potassium nitrite and acetic acid, yield a yellow precipitate.

Copper—Solutions of cupric compounds, acidified with hydrochloric acid, deposit a red film of metallic copper upon a bright, untarnished surface of metallic iron. An excess of 6 N ammonium hydroxide, added to a solution of a cupric salt, produces first a bluish precipitate and then a deep blue-colored solution. With potassium ferrocyanide TS, solutions of cupric salts yield a reddish-brown precipitate, insoluble in diluted acids.

Hypophosphite—When strongly heated, hypophosphites evolve spontaneously flammable phosphine. Hypophosphites in solution yield a white precipitate with mercuric chloride TS. This precipitate becomes gray when an excess of hypo-

phosphite is present. Solutions of hypophosphites, acidified with sulfuric acid, and warmed with cupric sulfate TS yield a red precipitate.

Iodide—Solutions of iodides, upon the addition of chlorine TS, dropwise, liberate iodine, which colors the solution yellow to red. When the solution is shaken with chloroform, the latter is colored violet. The iodine thus liberated gives a blue color with starch TS. Silver nitrate TS produces, in solutions of iodides, a yellow, curdy precipitate that is insoluble in nitric acid and in 6 N ammonium hydroxide.

Iron—Ferrous and ferric compounds in solution yield a black precipitate with ammonium sulfide TS. This precipitate is dissolved by cold 3 N hydrochloric acid with the evolution of hydrogen sulfide.

Ferric Salts—Acid solutions of ferric salts yield a dark blue precipitate with potassium ferrocyanide TS. With an excess of 1 N sodium hydroxide, a reddish-brown precipitate is formed. With ammonium thiocyanate TS, solutions of ferric salts produce a deep red color that is not destroyed by dilute mineral acids.

Ferrous Salts—Solutions of ferrous salts yield a dark blue precipitate with potassium ferricyanide TS. This precipitate is insoluble in 3 N hydrochloric acid but is decomposed by 1 N sodium hydroxide. With 1 N sodium hydroxide, solutions of ferrous salts yield a greenish-white precipitate, the color rapidly changing to green and then to brown when shaken.

Lactate—When solutions of lactates are acidified with sulfuric acid, potassium permanganate TS is added, and the mixture is heated, acetaldehyde is evolved. This can be detected by allowing the vapor to come into contact with a filter paper that has been moistened with a freshly prepared mixture of equal volumes of 20% aqueous morpholine and sodium nitroferricyanide TS: a blue color is produced.

Lead—With 2 N sulfuric acid, solutions of lead salts yield a white precipitate that is insoluble in 3 N hydrochloric or 2 N nitric acid, but is soluble in warm 1 N sodium hydroxide and in ammonium acetate TS. With potassium chromate TS, solutions of lead salts, free or nearly free from mineral acids, yield a yellow precipitate that is insoluble in 6 N acetic acid but is soluble in 1 N sodium hydroxide.

Lithium—With sodium carbonate TS, moderately concentrated solutions of lithium salts, made alkaline with sodium hydroxide, yield a white precipitate on boiling. The precipitate is soluble in ammonium chloride TS. Lithium salts moistened with hydrochloric acid impart an intense crimson color to a nonluminous flame. Solutions of lithium salts are not precipitated by 2 N sulfuric acid or soluble sulfates (*distinction from strontium*).

Magnesium—Solutions of magnesium salts in the presence of ammonium chloride yield no more than a slightly hazy precipitate when neutralized with ammonium carbonate TS, but on the subsequent addition of dibasic sodium phosphate TS, a white, crystalline precipitate, which is insoluble in 6 N ammonium hydroxide, is formed.

Manganese—With ammonium sulfide TS, solutions of manganous salts yield a salmon-colored precipitate that dissolves in acetic acid.

Mercury—When applied to bright copper foil, solutions of mercury salts, free from an excess of nitric acid, yield a deposit that upon rubbing, becomes bright and silvery in appearance. With hydrogen sulfide, solutions of mercury compounds yield a black precipitate that is insoluble in ammonium sulfide TS and in boiling 2 N nitric acid.

Mercuric Salts—Solutions of mercuric salts yield a yellow precipitate with 1 N sodium hydroxide. They yield also, in neutral solutions with potassium iodide TS, a scarlet precipitate that is very soluble in an excess of the reagent.

Mercurous Salts—Mercurous compounds are decomposed by 1 N sodium hydroxide, producing a black color. With hydrochloric acid, solutions of mercurous salts yield a white precipitate that is blackened by 6 N ammonium hydroxide.

With potassium iodide TS, a yellow precipitate, that may become green upon standing, is formed.

Nitrate—When a solution of a nitrate is mixed with an equal volume of sulfuric acid, the mixture is cooled, and a solution of ferrous sulfate is superimposed, a brown color is produced at the junction of the two liquids. When a nitrate is heated with sulfuric acid and metallic copper, brownish-red fumes are evolved. Nitrates do not decolorize acidified potassium permanganate TS (*distinction from nitrites*).

Nitrite—When treated with dilute mineral acids or with 6 N acetic acid, nitrites evolve brownish-red fumes. The solution colors starch-iodide paper blue.

Oxalate—Neutral and alkaline solutions of oxalates yield a white precipitate with calcium chloride TS. This precipitate is insoluble in 6 N acetic acid but is dissolved by hydrochloric acid. Hot acidified solutions of oxalates decolorize potassium permanganate TS.

Permanganate—Solutions of permanganates acidified with sulfuric acid are decolorized by hydrogen peroxide TS and by sodium bisulfite TS, in the cold, and by oxalic acid TS, in hot solution.

Peroxide—Solutions of peroxides slightly acidified with sulfuric acid yield a deep blue color upon the addition of potassium dichromate TS. On shaking the mixture with an equal volume of ethyl ether and allowing the liquids to separate, the blue color is found in the ethyl ether layer.

Phosphate—[NOTE—Where the monograph specifies the identification test for *Phosphate*, use the tests for orthophosphates, unless the instructions specify the use of the pyrophosphate tests or indicate that the product is to be ignited before performing the test.] With silver nitrate TS, neutral solutions of orthophosphates yield a yellow precipitate that is soluble in 2 N nitric acid and in 6 N ammonium hydroxide. With ammonium molybdate TS, acidified solutions of orthophosphates yield a yellow precipitate that is soluble in 6 N ammonium hydroxide. This precipitate may be slow to form. With silver nitrate TS, pyrophosphates obtained by ignition yield a white precipitate that is soluble in 2 N nitric acid and in 6 N ammonium hydroxide. With ammonium molybdate TS, a yellow precipitate that is soluble in 6 N ammonium hydroxide is formed.

Potassium—Potassium compounds impart a violet color to a nonluminous flame, but the presence of small quantities of sodium masks the color unless the yellow color produced by sodium is screened out by viewing through a blue filter that blocks emission at 589 nm (sodium) but is transparent to emission at 404 nm (potassium). Traditionally, cobalt glass has been used, but other suitable filters are commercially available. In neutral, concentrated or moderately concentrated solutions of potassium salts (depending upon the solubility and the potassium content), sodium bitartrate TS produces a white crystalline precipitate that is soluble in 6 N ammonium hydroxide and in solutions of alkali hydroxides and carbonates. The formation of the precipitate, which is usually slow, is accelerated by stirring or rubbing the inside of the test tube with a glass rod. The addition of a small amount of glacial acetic acid or alcohol also promotes the precipitation.

Salicylate—In moderately dilute solutions of salicylates, ferric chloride TS produces a violet color. The addition of acids to moderately concentrated solutions of salicylates produces a white, crystalline precipitate of salicylic acid that melts between 158° and 161°.

Silver—With hydrochloric acid, solutions of silver salts yield a white, curdy precipitate that is insoluble in nitric acid, but is readily soluble in 6 N ammonium hydroxide. A solution of a silver salt to which 6 N ammonium hydroxide and a small quantity of formaldehyde TS are added deposits, upon warming, a mirror of metallic silver upon the sides of the container.

Sodium—Unless otherwise specified in an individual monograph, prepare a solution to contain 0.1 g of the so-

dium compound in 2 mL of water. Add 2 mL of 15% potassium carbonate, and heat to boiling. No precipitate is formed. Add 4 mL of potassium pyroantimonate TS, and heat to boiling. Allow to cool in ice water and, if necessary, rub the inside of the test tube with a glass rod. A dense precipitate is formed. Sodium compounds impart an intense yellow color to a nonluminous flame.

Sulfate—With barium chloride TS, solutions of sulfates yield a white precipitate that is insoluble in hydrochloric acid and in nitric acid. With lead acetate TS, neutral solutions of sulfates yield a white precipitate that is soluble in ammonium acetate TS. Hydrochloric acid produces no precipitate when added to solutions of sulfates (*distinction from thiosulfates*).

Sulfite—When treated with 3 N hydrochloric acid, sulfites and bisulfites yield sulfur dioxide, which blackens filter paper moistened with mercurous nitrate TS.

Tartrate—Dissolve a few mg of a tartrate salt in 2 drops of sodium metaperiodate solution (1 in 20). Add a drop of 1 N sulfuric acid, and after 5 minutes add a few drops of sulfurous acid followed by a few drops of fuchsin-sulfurous acid TS: a reddish-pink color is produced within 15 minutes.

Thiocyanate—With ferric chloride TS, solutions of thiocyanates yield a red color that is not destroyed by moderately concentrated mineral acids.

Thiosulfate—With hydrochloric acid, solutions of thiosulfates yield a white precipitate that soon turns yellow, and sulfur dioxide, which blackens filter paper moistened with mercurous nitrate TS. The addition of ferric chloride TS to solutions of thiosulfates produces a dark violet color that quickly disappears.

Zinc—In the presence of sodium acetate, solutions of zinc salts yield a white precipitate with hydrogen sulfide. This precipitate is insoluble in acetic acid, but is dissolved by 3 N hydrochloric acid. Ammonium sulfide TS produces a similar precipitate in neutral and in alkaline solutions. With potassium ferrocyanide TS, zinc salts in solution yield a white precipitate that is insoluble in 3 N hydrochloric acid.

(193) IDENTIFICATION— TETRACYCLINES

The following chromatographic procedures are provided to confirm the identity of Pharmacopeial drug substances that are of the tetracycline type, such as doxycycline, oxytetracycline, and tetracycline, and to confirm the identity of such compounds in their respective Pharmacopeial dosage forms. Two procedures are provided, one based on paper chromatography (*Method I*) and the other on thin-layer chromatography (*Method II*). *Method I* is to be used unless otherwise directed in the individual monograph.

Standard Solution—Unless otherwise directed in the individual monograph, dissolve the USP Reference Standard for the drug substance being identified in the same solvent and at the same concentration as for the *Test Solution*.

Test Solution—Prepare as directed in the individual monograph.

METHOD I

pH 3.5 Buffer—Dissolve 13.4 g of anhydrous citric acid and 16.3 g of dibasic sodium phosphate in 1000 mL of water, and mix.

Developing Solvent—On the day of use, mix 10 volumes of chloroform, 20 volumes of nitromethane, and 3 volumes of pyridine.

Mixed Test Solution—Mix equal volumes of the *Standard Solution* and the *Test Solution*.

Chromatographic Sheet—Draw a spotting line 2.5 cm from one edge of a 20-cm × 20-cm sheet of filter paper (Whatman No. 1, or equivalent). Impregnate the sheet with pH 3.5 Buffer by passing it through a trough filled with pH 3.5 Buffer, and remove the excess solvent by firmly pressing the sheet between nonfluorescent blotting papers.

Procedure—To a suitable chromatographic chamber, prepared for ascending chromatography (see *Chromatography* (621)) add *Developing Solvent* to a depth of 0.6 cm. Apply at 1.5-cm intervals 2 µL each of the *Standard Solution*, the *Test Solution*, and the *Mixed Test Solution* to the spotting line of the *Chromatographic Sheet*. Allow the sheet to dry partially, and while still damp place it in the chromatographic chamber with the bottom edge touching the *Developing Solvent*. When the solvent front has risen about 10 cm, remove the sheet from the chamber, and expose the sheet to ammonia vapor. Examine the chromatogram under long-wavelength UV light. Record the positions of the major yellow fluorescent spots: the R_f value of the principal spot obtained from the *Test Solution* and from the *Mixed Test Solution* corresponds to that obtained from the *Standard Solution*.

METHOD II

Resolution Solution—Unless otherwise directed in the individual monograph, prepare a solution in methanol containing 0.5 mg each of USP Chlortetracycline Hydrochloride RS, USP Doxycycline Hyclate RS, USP Oxytetracycline RS, and USP Tetracycline Hydrochloride RS per mL.

Developing Solvent—Prepare a mixture of 0.5 M oxalic acid, previously adjusted with ammonium hydroxide to a pH of 2.0, acetonitrile, and methanol (80:20:20).

Chromatographic Plate—Use a suitable thin-layer chromatographic plate (see *Thin-layer Chromatography* under *Chromatography* (621)) coated with a 0.25-mm layer of octylsilylated chromatographic silica gel mixture. Activate the plate by heating it at 130° for 20 minutes, allow to cool, and use while still warm.

Procedure—Separately apply 1 µL each of the *Standard Solution*, the *Test Solution*, and the *Resolution Solution* to the *Chromatographic Plate*. Allow the spots to dry, and develop the chromatogram in the *Developing Solvent* until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow to air-dry. Expose the plate to ammonia vapors for 5 minutes, and promptly locate the spots on the plate by viewing under long-wavelength UV light: the chromatogram of the *Resolution Solution* shows clearly separated spots, and the principal spot obtained from the *Test Solution* corresponds in R_f value, intensity, and appearance to that obtained from the *Standard Solution*.

(197) SPECTROPHOTOMETRIC IDENTIFICATION TESTS

Spectrophotometric tests contribute meaningfully toward the identification of many compendial chemical substances. The test procedures that follow are applicable to substances that absorb IR and/or UV radiation (see *Spectrophotometry and Light-Scattering* (851)).

The IR absorption spectrum of a substance, compared with that obtained concomitantly for the corresponding USP Reference Standard, provides perhaps the most conclusive evidence of the identity of the substance that can be realized from any single test. The UV absorption spectrum, on the other hand, does not exhibit a high degree of specificity. Conformance with both IR absorption and UV absorption test specifications, as called for in a large proportion of compendial monographs, leaves little doubt, if any, regarding the identity of the specimen under examination.

INFRARED ABSORPTION

Seven methods are indicated for the preparation of previously dried test specimens and Reference Standards for analysis. The reference (197K) in a monograph signifies that the substance under examination is mixed intimately with potassium bromide. The reference (197M) in a monograph signifies that the substance under examination is finely ground and dispersed in mineral oil. The reference (197F) in a monograph signifies that the substance under examination is suspended neat between suitable (for example, sodium chloride or potassium bromide) plates. The reference (197S) signifies that a solution of designated concentration is prepared in the solvent specified in the individual monograph, and the solution is examined in 0.1-mm cells unless a different cell path length is specified in the individual monograph. The reference (197A) signifies that the substance under examination is intimately in contact with an internal reflection element for attenuated total reflectance (ATR) analysis. The reference (197E) signifies that the substance under examination is pressed as a thin sample against a suitable plate for IR microscopic analysis. The reference (197D) in a monograph signifies that the substance under examination is mixed intimately with an IR-transparent material and transferred to a sample container for diffuse reflection (DR) analysis. The ATR (197A) and the (197E) techniques can be used as alternative methods for (197K), (197M), (197F), and (197S) where testing is performed qualitatively and the Reference Standard spectra are similarly obtained.

Record the spectra of the test specimen and the corresponding USP Reference Standard over the range from about 2.6 μm to 15 μm (3800 cm^{-1} to 650 cm^{-1}) unless otherwise specified in the individual monograph. The IR absorption spectrum of the preparation of the test specimen, previously dried under conditions specified for the corresponding Reference Standard unless otherwise specified, or unless the Reference Standard is to be used without drying, exhibits maxima only at the same wavelengths as that of a similar preparation of the corresponding USP Reference Standard.

Differences that may be observed in the spectra so obtained sometimes are attributed to the presence of polymorphs, which are not always acceptable (see *Procedure under Spectrophotometry and Light-Scattering* (851)). Unless otherwise directed in the individual monograph, therefore, continue as follows. If a difference appears in the IR spectra of the analyte and the standard, dissolve equal portions of the test specimen and the Reference Standard in equal volumes of a suitable solvent, evaporate the solution to dry-

ness in similar containers under identical conditions, and repeat the test on the residues.

ULTRAVIOLET ABSORPTION

The reference (197U) in a monograph signifies that a test solution and a Standard solution are examined spectrophotometrically, in 1-cm cells, over the spectral range from 200 to 400 nm unless otherwise specified in the individual monograph.

Dissolve a portion of the substance under examination in the designated *Medium* to obtain a test solution having the concentration specified in the monograph for *Solution*. Similarly prepare a Standard solution containing the corresponding USP Reference Standard.

Record and compare the spectra concomitantly obtained for the test solution and the Standard solution. Calculate absorptivities and/or absorbance ratios where these criteria are included in an individual monograph. Unless otherwise specified, absorbances indicated for these calculations are those measured at the maximum absorbance at about the wavelength specified in the individual monograph. Where the absorbance is to be measured at about the specified wavelength other than that of maximum absorbance, the abbreviations (min) and (sh) are used to indicate a minimum and shoulder, respectively, in an absorption spectrum. The requirements are met if the UV absorption spectra of the test solution and the Standard solution exhibit maxima and minima at the same wavelengths and absorptivities and/or absorbance ratios are within specified limits.

(201) THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

GENERAL PROCEDURE

The following procedure is applicable as an aid in verifying the identities of many compendial drug substances as such and in their respective dosage forms.

Prepare a test solution as directed in the individual monograph. On a line parallel to and about 2 cm from the edge of a suitable thin-layer chromatographic plate, coated with a 0.25-mm layer of chromatographic silica gel mixture (see *Chromatography* (621)) apply 10 μL of this solution and 10 μL of a Standard solution prepared from the USP Reference Standard for the drug substance being identified, in the same solvent and at the same concentration as the test solution, unless otherwise directed in the individual monograph. Allow the spots to dry, and develop the chromatogram in a solvent system consisting of a mixture of chloroform, methanol, and water (180:15:1), unless otherwise directed in the individual monograph, until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Unless otherwise directed in the individual monograph, locate the spots on the plate by examination under short-wavelength UV light. The R_f value of the principal spot obtained from the test solution corresponds to that obtained from the Standard solution.

PROCEDURE FOR BACITRACIN, NEOMYCIN, AND POLYMYXIN B

The following thin-layer chromatographic procedure is applicable as an aid in verifying the identities of bacitracin, neomycin, and polymyxin B active ingredients and in dosage forms when present singly and in two- and three-component mixtures. The reference (201BNP) in a monograph signifies that this procedure is intended.

Prepare a *Test Solution* as follows, unless otherwise directed in the individual monograph.

Test Solution—

FOR DRUG SUBSTANCES—Dissolve a portion of Bacitracin, Bacitracin Zinc, Neomycin Sulfate, or Polymyxin B Sulfate in 0.1 N hydrochloric acid to obtain a solution containing about 500 USP Bacitracin Units per mL, 3.5 mg of neomycin (base) per mL, or 10,000 USP Polymyxin B Units per mL.

FOR SOLUTIONS—Where the Solution contains neomycin and polymyxin B, dilute a portion of it with 0.1 N hydrochloric acid to obtain a solution containing the equivalent of about 3.5 mg of neomycin (base) per mL. Where the Solution contains polymyxin B but not neomycin, dilute a portion of it with 0.1 N hydrochloric acid to obtain a solution containing about 10,000 USP Polymyxin B Units per mL.

FOR CREAMS, LOTIONS, AND OINTMENTS—Where the Cream, Lotion, or Ointment contains Bacitracin or Bacitracin Zinc, transfer a portion of it equivalent to about 500 USP Bacitracin Units, to a 15-mL centrifuge tube. Where the Cream, Lotion, or Ointment contains neomycin, but not Bacitracin or Bacitracin Zinc, transfer a portion of it equivalent to about 3.5 mg of neomycin (base) per mL to a 15-mL centrifuge tube. Add 4 mL of chloroform to the centrifuge tube, and shake well to disperse the Cream, Lotion, or Ointment. Add 1 mL of 0.1 N hydrochloric acid, vortex for 4 minutes, centrifuge, and use the clear supernatant.

NOTE—The *Modified Test Solution* as described below in the *Modified Procedure* may be used in lieu of the *Test Solution*.

Standard Bacitracin Solution—Dissolve a portion of USP Bacitracin Zinc RS in 0.1 N hydrochloric acid to obtain a solution containing 500 USP Bacitracin Units per mL.

Standard Neomycin Solution—Dissolve a portion of USP Neomycin Sulfate RS in 0.1 N hydrochloric acid to obtain a solution containing the equivalent of 3.5 mg of neomycin (base) per mL.

Standard Polymyxin B Solution—Dissolve a portion of USP Polymyxin B Sulfate RS in 0.1 N hydrochloric acid to obtain a solution containing 10,000 USP Polymyxin B Units per mL. Where the article under test also contains Bacitracin or Bacitracin Zinc, dissolve a portion of USP Polymyxin B Sulfate RS in 0.1 N hydrochloric acid to obtain a solution containing 500 USP Polymyxin B Units per mL, *J* being the ratio of the labeled amount of USP Polymyxin B Units to the labeled amount of USP Bacitracin Units in each g of Cream, Lotion, or Ointment.

Developing Solvent Solution—Prepare a mixture of methanol, isopropyl alcohol, methylene chloride, ammonium hydroxide, and water (4:2:2:2:1.5).

Procedure—Apply 10 μ L of the *Test Solution* and each of the relevant *Standard Solutions* to a suitable thin-layer chromatographic plate (see *Chromatography* (621)) coated with a 0.25-mm layer of chromatographic silica gel. Place the plate in a presaturated chromatographic chamber, and develop the chromatogram with the *Developing Solvent System* until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and dry at 105° for 10 minutes. Spray the plate with a 0.2% solution of ninhydrin in butyl alcohol, and heat at 105° for 5 minutes. The R_f value of each principal spot in the chromatogram of the *Test Solution* corresponds to that of the principal spot in the chromatogram obtained from each relevant *Standard Solution* as appropriate for the labeled active

ingredient or ingredients specified on the label. If the chromatogram of the *Test Solution* yields excessive streaking, proceed as directed for *Modified Procedure*.

Modified Procedure—Transfer the *Test Solution* to a 15-mL centrifuge tube, add 10 mL of saturated aqueous picric acid solution (1.2%, w/v), vortex for 1 minute, centrifuge for 10 minutes, and discard the supernatant. Wash the residue with 1-mL portions of water until no yellow color is observed in the washing. Discard the washings, and dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 1 mL of acetone, add 1 mL of a freshly prepared solution of sulfuric acid in acetone (1 in 100), shake, centrifuge for 5 minutes, and discard the supernatant. Rinse the residue with 1 mL of acetone, centrifuge briefly, and discard the washing. Repeat the washing until no yellow color is observed. Dry the residue under a stream of nitrogen at 50°. Dissolve the residue in 0.5 mL of 0.1 N hydrochloric acid (*Modified Test Solution*). Repeat the *Procedure* using this *Modified Test Solution* instead of the *Test Solution*. The R_f value of each principal spot in the chromatogram of the *Modified Test Solution* corresponds to that of the principal spot in the chromatogram obtained from each relevant *Standard Solution* as appropriate for the active ingredient or ingredients specified on the label.

LIMIT TESTS

(206) ALUMINUM

This procedure is provided to demonstrate that the content of aluminum (Al) does not exceed the limit given in the individual monograph of a substance labeled as intended for use in hemodialysis. [NOTE—The *Standard Preparations* and the *Test Preparation* may be modified, if necessary, to obtain solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

Nitric Acid Diluent—Transfer 40 mL of nitric acid to a 100-mL volumetric flask, and dilute with water to volume.

Standard Preparations—Treat some aluminum wire with 6 N hydrochloric acid at 80° for a few minutes. Dissolve about 100 mg of the treated wire, accurately weighed, in a mixture of 10 mL of hydrochloric acid and 2 mL of nitric acid by heating at about 80° for approximately 30 minutes. Continue heating until the volume is reduced to about 4 mL. Cool to room temperature, and add 4 mL of water. Evaporate to about 2 mL by heating. Cool, and transfer this solution, with the aid of water, to a 100-mL volumetric flask, dilute with water to volume, and mix. Transfer 10.0 mL of this solution to a second 100-mL volumetric flask, dilute with water to volume, and mix. Transfer 1.0 mL of this solution to a third 100-mL volumetric flask, dilute with water to volume, and mix. The concentration of aluminum in this *Standard Preparation* is about 1.0 μ g per mL. If a more diluted *Standard Preparation* is required, transfer 1.0-, 2.0-, and 4.0-mL portions of this solution to separate 100-mL volumetric flasks, dilute with *Nitric Acid Diluent* to volume, and mix. These solutions contain 0.01, 0.02, and 0.04 μ g of Al per mL, respectively.

Test Preparation—Unless otherwise directed in the monograph, transfer an accurately weighed amount (in g) of the test substance, as specified in the monograph, to a 100-mL plastic volumetric flask, add 50 mL of water, and sonicate for 30 minutes. Add 4 mL of nitric acid, dilute with water to volume, and mix.

Procedure—Determine the absorbances of the *Standard Preparations* and the *Test Preparation* at the aluminum emission line at 309.3 nm with a suitable atomic absorption spectrophotometer (see *Spectrophotometry and Light-Scattering* (851)) equipped with an aluminum hollow-cathode lamp and a flameless electrically heated furnace, using the *Nitric Acid Diluent* as the blank. Plot the absorbances of the *Standard Preparations* versus the content of Al, in μg per mL, drawing a straight line best fitting the three points. From the graph so obtained, determine the quantity, in μg , of Al in each mL of the *Test Preparation*. Calculate the amount of Al in the specimen taken, in μg per g, by multiplying this value by 100/W, where W is the weight, in g, of the substance taken to prepare the *Test Preparation*.

(207) TEST FOR 1,6-ANHYDRO DERIVATIVE FOR ENOXAPARIN SODIUM

The following procedure is used to determine the levels of 1, 6-anhydro forms in enoxaparin sodium. [NOTE—The test for the 1,6-anhydro derivative is conducted only where specified in the individual monograph.]

INTRODUCTION

The disaccharides specified in this general chapter are listed by name and structure in *Appendix 1*; the oligosaccharides are listed in *Appendix 2*.

Depolymerization of heparin into enoxaparin sodium produces a partial but characteristic conversion of glucosamines at the reducing termini of oligosaccharide chains with terminal glucosamine 6-O sulfate, yielding 1,6-anhydro derivatives (see *Figure 1*).

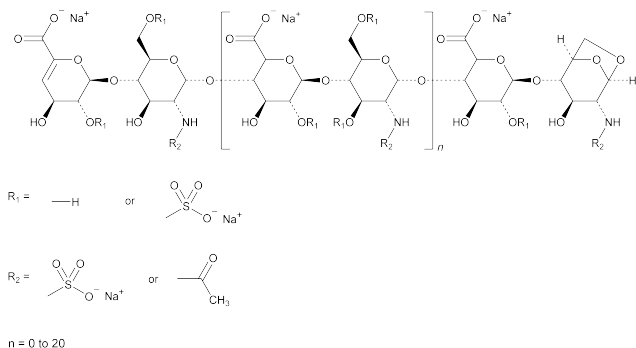


Figure 1. Structure of enoxaparin sodium containing a 1,6-anhydro derivative on the reducing end of the chain.

The percentage of oligosaccharide chains that are cyclized in a 1,6-anhydro ring is a characteristic of enoxaparin sodium.

DEPOLYMERIZATION OF ENOXAPARIN SODIUM BY HEPARINASES AND RESULTING OLIGOSACCHARIDES

The assay involves HPLC analysis of a depolymerized enoxaparin sodium solution by a mixture of heparinases. After enzymatic depolymerization, the main 1,6-anhydro resi-

dues of enoxaparin sodium observed are 1,6-anhydro ΔIIS and 1,6-anhydro $\Delta\text{IIS}^{\text{epi}}$, and 1,6-anhydro ΔIS and 1,6-anhydro $\Delta\text{IS-IS}^{\text{epi}}$ (see *Appendix 2*).

The 1,6-anhydro $\Delta\text{IS-IS}^{\text{epi}}$ tetrasaccharide (2-O-sulfated mannosamine form) is not completely cleaved by the heparinases. The two disaccharides (1,6-anhydro ΔIIS and 1,6-anhydro $\Delta\text{IIS}^{\text{epi}}$), which generally co-elute, are poorly resolved with respect to ΔIIA (see *Appendix 1*), especially because the latter occurs as two anomers: α and β . To allow quantitation of 1,6-anhydro ΔIIS and 1,6-anhydro $\Delta\text{IIS}^{\text{epi}}$, the enoxaparin sodium sample already depolymerized by heparinases is then reduced by sodium borohydride (see *Figure 2*).

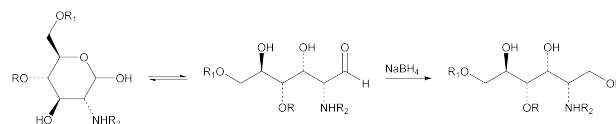


Figure 2. Reduction of oligosaccharides by sodium borohydride

The sodium borohydride reduction eliminates the $\alpha \leftrightarrow \beta$ anomeric effect by opening the terminal oligosaccharide ring. The four 1,6-anhydro derivatives (see *Appendix 2*) are not reduced by sodium borohydride because the ring opening is blocked by the 1,6-anhydro bridge. The reduction of the oligosaccharides decreases their retention time, whereas the retention time of the 1,6-anhydro derivatives remains unchanged. Thus, it is possible to separate the two compounds—1,6-anhydro ΔIIS and 1,6-anhydro $\Delta\text{IIS}^{\text{epi}}$ —from the reduced ΔIIA disaccharide peak. [NOTE—1,6-Anhydro ΔIIS and 1,6-anhydro $\Delta\text{IIS}^{\text{epi}}$ are eluted as two non-resolved peaks and are quantitated together as a single compound, 1,6-anhydro ΔIIS . Therefore, for the purpose of simplification, the epimeric form is not referred to in the remaining text.]

PROCEDURE

USP Reference Standards (11)—*USP Enoxaparin Sodium RS*.

Solutions—

Solution A—Dissolve 0.280 g of monobasic sodium phosphate in 950 mL of water, adjust with phosphoric acid to a pH of 3.0, and dilute with water to 1000 mL.

Solution B—Dissolve 140 g of sodium perchlorate in 950 mL of *Solution A*, adjust with phosphoric acid to a pH of 3.0, and dilute with *Solution A* to 1000 mL.

Mobile Phase—Use variable mixtures of filtered and degassed *Solution A* and *Solution B* as directed in *Chromatographic System*.

Sodium/Calcium Acetate pH 7.0 Solution—Dissolve 10 mg of bovine serum albumin and 32 mg of calcium acetate in 60 mL of water. Add 580 μL of glacial acetic acid, and adjust with 2 M sodium hydroxide to a pH of 7.0. Transfer to a 100-mL volumetric flask, and dilute with water to volume. Pass the solution through a filter having a porosity of 0.45 or 0.22 μm .

Potassium Phosphate pH 7.0 Buffer—Dissolve 68 mg of monobasic potassium phosphate and 10 mg of bovine serum albumin in 30 mL of water in a 50-mL volumetric flask. Adjust with potassium hydroxide, if necessary, to a pH of 7.0, and dilute with water to volume. Pass the solution through a filter having a porosity of 0.45 or 0.22 μm .

Sodium Borohydride Solution—Dissolve 12 mg of sodium borohydride in 400 μL of water, and mix on a vortex mixer. [NOTE—Prepare fresh immediately before use.]

Heparinase 1 Solution—Dissolve heparinase 1 (see *Reagent Specifications* under *Reagents, Indicators, and Solutions*) [reference: heparin lyase I, EC 4.2.2.7] in *Potassium Phosphate*

pH 7.0 Buffer to obtain a solution having an activity of 0.4 IU per mL. Store the solution at -20° until ready to use. [NOTE—Heparinase solutions can be stored for 3 months at -20° .]

Heparinase 2 Solution—Dissolve heparinase 2 (see *Reagent Specifications* under *Reagents, Indicators, and Solutions* [no EC number] in *Potassium Phosphate pH 7.0 Buffer* to obtain a solution having an activity of 0.4 IU per mL. Store the solution at -20° until ready to use.

Heparinase 3 Solution—Dissolve heparinase 3 (see *Reagent Specifications* under *Reagents, Indicators, and Solutions* [reference: heparitinase I, EC 4.2.2.8] in *Potassium Phosphate pH 7.0 Buffer* to obtain a solution having an activity of 0.4 IU per mL. Store the solution at -20° until ready to use.

Heparinases 1, 2, 3, Solution—Prepare a 1:1:1 (v:v:v) mixture of *Heparinase 1 Solution*, *Heparinase 2 Solution*, and *Heparinase 3 Solution*.

Peak Identification Solutions—[NOTE—The depolymerized test solutions and Standard solutions must be prepared at the same time. Depolymerized test solutions are stable for 1 month at -20° . Also, the reduced test solutions and Standard solutions must be prepared at the same time. Reduced solutions are also stable for 1 month at -20° .]

Disaccharide Solutions—Separately prepare a 0.25 mg per mL solution of each disaccharide¹ Δ IA, Δ IIA, Δ IIIA, Δ IVA, Δ IS, Δ IIS, Δ IIIS, Δ IVS (see *Appendix 1*). Chromatograph each disaccharide solution, and record the peak responses.

Reduced Disaccharide Solutions—To 60 μ L of each *Disaccharide Solution*, add 10 μ L of freshly prepared *Sodium Borohydride Solution*. Mix on a vortex mixer, and allow to stand at room temperature for at least 4 hours. Chromatograph each solution, and record the peak response.

Blank Solution—Prepare a mixture of 20 μ L of water, 70 μ L of *Sodium/Calcium Acetate pH 7.0 Solution*, and 100 μ L of the *Heparinases 1, 2, 3 Solution*. Mix gently by inversion, and allow to stand for at least 48 hours in a 25° water bath. Prepare a mixture of 60 μ L of this depolymerized solution with 10 μ L of freshly prepared *Sodium Borohydride Solution*. Homogenize, and allow to stand at room temperature for at least 4 hours. Chromatograph the resulting solution, and record the peak responses.

Test Solution 1—Prepare two solutions, each containing 20 mg of enoxaparin sodium in 1 mL of water.

Standard Solution 1—Prepare one solution containing 20 mg of USP Enoxaparin Sodium RS in 1 mL of water.

Test Solution 2—For each solution, prepare a mixture of 20 μ L of *Test Solution 1*, 70 μ L of *Sodium/Calcium Acetate pH 7 Solution*, and 100 μ L of *Heparinases 1, 2, 3 Solution*. Mix gently by inversion, and allow to stand for at least 48 hours in a 25° water bath. After 48 hours of depolymerization, chromatograph the solution, and record the peak responses.

Standard Solution 2—Prepare a mixture of 20 μ L of *Standard Solution 1*, 70 μ L of *Sodium/Calcium Acetate pH 7 Solution*, and 100 μ L of *Heparinases 1, 2, 3 Solution*. Mix gently, and allow to stand for at least 48 hours in a 25° water bath. After 48 hours of depolymerization, chromatograph the solution, and record the peak responses.

Test Solution 3—For each depolymerized test solution, prepare a mixture of 60 μ L of *Test Solution 2* and 10 μ L of freshly prepared *Sodium Borohydride Solution*. Homogenize, and allow to stand loosely capped at room temperature for at least 4 hours before injecting into the chromatograph. *Test Solution 3* is stable for 48 hours at room temperature.

Standard Solution 3—Prepare a mixture of 60 μ L of *Standard Solution 2* and 10 μ L of freshly prepared *Sodium Borohydride Solution*. Homogenize and mix on a vortex mixer, and allow to stand loosely capped at room temperature for at

least 4 hours before injecting into the chromatograph. *Standard Solution 3* is stable for 48 hours at room temperature.

Chromatographic System (see *Chromatography* (621))—The liquid chromatograph is equipped with a 234-nm detector and a 3-mm \times 25-cm column that contains 5- μ m packing L14. A guard column packed with the same material should also be used. The flow rate is 0.45 mL per minute, the column temperature is maintained at 50° , and the injection volume is 10 μ L. The chromatograph is programmed as follows.

Time (minutes)	Solution A (%)	Solution B (%)	Elution
0–20	97→65	3→35	Linear gradient
20–50	65→0	35→100	Linear gradient
50–60	0	100	Isocratic
60–61	0→97	100→3	Linear gradient for re-equilibration
61–79	97	3	Isocratic for re-equilibration

Chromatograph the reduced *Test Solution 3* and the reduced *Standard Solution 3*, and record the peak responses as directed for *Procedure*.

Depolymerization Suitability Test—The ratio of the peak area of 1,6-anhydro- Δ IS-IS to that of 1,6-anhydro Δ IS is not more than 1.15 for the depolymerized *Standard Solution 2*.

Column Performance Suitability Test—Identify the peaks corresponding to reduced Δ IA and 1,6-anhydro- Δ IS for the *Standard Solution 3*: the retention time of reduced Δ IS is between 27 and 33 minutes for the depolymerized and reduced *Standard Solution 3*; and the resolution, R , between reduced Δ IA and 1,6-anhydro- Δ IS is not less than 1.5.

Reduction Suitability Test—The ratio of the peak area of Δ IS disaccharide to that of reduced Δ IS in the depolymerized and reduced *Standard Solution 3* and *Test Solution 3* is not more than 0.02%.

Procedure/Calculation—Separately inject equal volumes of the reduced *Test Solutions 3* and the reduced *Standard Solution 3* into the chromatograph. Use the normalized area percentage method for calculation. Each peak is integrated from the dwell volume peak to the last detected peak. Measure the area of each analyte peak after excluding solvent peaks at the beginning of the chromatogram and in the *Blank Solution*. Using the previously obtained chromatograms of the *Reduced Disaccharide Solutions*, identify peaks belonging to the eight reduced disaccharides in the chromatograms for *Test Solution 3* and *Standard Solution 3*. The peaks belonging to 1,6-Anhydro Δ IS, 1,6-Anhydro Δ IIS, and 1,6-Anhydro Δ IS-IS^{epi} are identified from the relative retention times provided in *Table 1* and the Reference chromatogram provided with USP Enoxaparin Sodium RS. Once the peaks have been identified, use the values in *Table 1* to calculate the (w/w) percentage of the three main 1,6-anhydro derivatives obtained after depolymerization of enoxaparin sodium using the following formula:

$$\% \text{ 1,6-anhydro } i \text{ (w/w)} = (100 \times \text{MW}_i \times A_i) / \sum (\text{MW}_x \times A_x)$$

in which MW_i and A_i are the molecular weight and the area of the 1,6-anhydro peak i , respectively; and MW_x and A_x are the molecular weight and the area, respectively, of either the peak X or the zone X specified by its retention time. [NOTE—Once the method is established, the peaks belonging to the different di- and tetrasaccharides can be easily identified using the USP Enoxaparin Sodium RS chromatogram. Thus, the use of the disaccharide Standards is only needed during the method-implementation stage.]

Calculate the molar percentage of components containing a 1,6-anhydro structure at the reducing end of their chain

¹Suitable disaccharides are available from Grampian Enzymes (GE-H1001, GE-G1002, GE-H1003, GE-H1004, GE-H1005, GE-H1006, GE-H1007, GE-H1008), Nisithouse, Harray, Orkney, KW17 2LQ, United Kingdom, Tel: 01856 771771, Scottish Local Authority: Orkney Islands.

in the enoxaparin sodium test sample according to the following formula:

$$\%1.6anhydro = 100 \times \frac{MW}{\sum MW_x \times Area_x} \times$$

(AreaΔ1s1.6anhydro + AreaΔ1s1.6anhydro + AreaΔ1s - 1s1.6anhydro)

in which MW is the mass-average molecular mass (see Identification test D under Enoxaparin Sodium); MW_x and Area_x are the molecular weight and the area, respectively, of either the peak X or the range X specified by its retention time. The molar percentage of components having a 1,6-anhydro structure at the reducing end of their chain is between 15% and 25%. Typical retention times and molecular masses attributed to different oligosaccharide structures are provided in Table 1.

Table 1. Typical Relative Retention Times (t_{RR}) and Molecular Masses Attributed to Different Compounds*

Compound	t _{RR}	Molecular Mass (Daltons)
—	< 0.25	741
Reduced ΔIVA	0.25	401
—	0.25 < t _{RR} < 0.51	741
Reduced ΔIVS	0.51	461
—	0.51 < t _{RR} < 0.55	483
Reduced ΔIIA	0.55	503
—	0.55 < t _{RR} < 0.59	503
1,6-Anhydro ΔIIS	0.59	443
—	0.59 < t _{RR} < 0.64	503

* Relative retention times were obtained with a depolymerized and reduced batch of enoxaparin sodium. They are expressed relative to the retention time of the main peak corresponding to reduced ΔIS. Note that according to the quality of the column, relative retention times can change slightly.

Table 1. Typical Relative Retention Times (t_{RR}) and Molecular Masses Attributed to Different Compounds* (Continued)

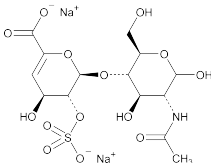
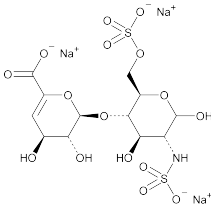
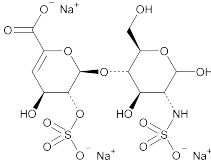
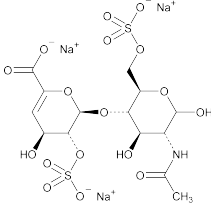
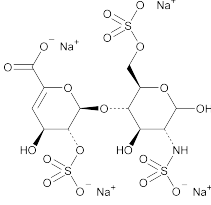
Compound	t _{RR}	Molecular Mass (Daltons)
Reduced ΔIIIA	0.64	503
—	0.64 < t _{RR} < 0.72	533
Reduced ΔIIS	0.72	563
—	0.72 < t _{RR} < 0.80	563
Reduced ΔIIIS	0.80	563
—	0.80 < t _{RR} < 0.88	583
Reduced ΔIA	0.88	605
—	0.88 < t _{RR} < 0.90	635
1,6-Anhydro ΔIS	0.90	545
—	0.90 < t _{RR} < 0.98	635
Reduced ΔIIA-IVSglu	0.98	1066
—	0.98 < t _{RR} < 1.00	635
Reduced ΔIS	1.00	665
—	1.00 < t _{RR} < 1.04	665
ΔIS	1.04	665
—	1.04 < t _{RR} < 1.10	1228
Reduced ΔIIA-IIS-glu	1.10	1168
—	1.10 < t _{RR} < 1.27	1228
1,6-Anhydro ΔIS-IS	1.27	1210
—	t _{RR} > 1.27	1228

* Relative retention times were obtained with a depolymerized and reduced batch of enoxaparin sodium. They are expressed relative to the retention time of the main peak corresponding to reduced ΔIS. Note that according to the quality of the column, relative retention times can change slightly.

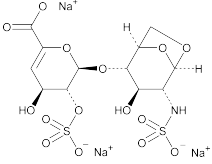
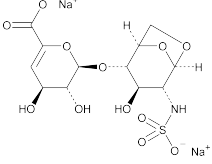
APPENDIX 1: STANDARD DISACCHARIDE STRUCTURES

ΔIVA		ΔUA-(1→4)α-GlcNAc
ΔIVS		ΔUA-(1→4)α-GlcN(NS)
ΔIIA		ΔUA-(1→4)α-GlcNAc(6S)

APPENDIX 1: STANDARD DISACCHARIDE STRUCTURES (Continued)

ΔIIIA		$\Delta\text{UA-2S-(1}\rightarrow\text{4)}\alpha\text{-GlcNAc}$
ΔIIS		$\Delta\text{UA-(1}\rightarrow\text{4)}\alpha\text{-GlcN (NS,6S)}$
ΔIIIS		$\Delta\text{UA-2S-(1}\rightarrow\text{4)}\alpha\text{-GlcN (NS)}$
ΔIA		$\Delta\text{UA-2S-(1}\rightarrow\text{4)}\alpha\text{-GlcNAc(6S)}$
ΔIS		$\text{UA-2S-(1}\rightarrow\text{4)}\alpha\text{-GlcN (NS,6S)}$

APPENDIX 2: OLIGOSACCHARIDE STRUCTURES

1,6-Anhydro ΔIS or 1,6-Anhydro ΔIS glucose	
1,6-Anhydro ΔIIS or 1,6-Anhydro ΔIIS glucose	

APPENDIX 2: OLIGOSACCHARIDE STRUCTURES (Continued)

1,6-Anhydro Δ IS epi or 1,6-Anhydro Δ IS mannose	
Δ IIA-IVSglu	
Δ IIA-IISglu	
1,6-Anhydro Δ IS-IS epi or 1,6-Anhydro Δ IS-IS mannose	

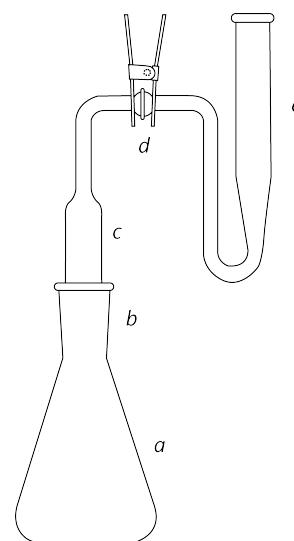
(211) ARSENIC

This procedure is designed to determine the presence of trace amounts of arsenic (As) by converting the arsenic in a substance under test to arsine, which is then passed through a solution of silver diethyldithiocarbamate to form a red complex. The red color so produced is compared, either visually or spectrophotometrically, to the color produced similarly in a control containing an amount of arsenic equivalent to the limit given in the individual monograph. Limits are stated in terms of arsenic (As). The content of arsenic does not exceed the limit given in the individual monograph.

Two methods are provided, the methods differing only in the preliminary treatment of the test substance and the standard. Generally, *Method I* is used for inorganic materials, while *Method II* is used for organic materials.

Apparatus—

The apparatus (see *illustration*) consists of an arsine generator (*a*) fitted with a scrubber unit (*c*) and an absorber tube (*e*) with standard-taper or ground glass ball-and-socket joints (*b* and *d*) between the units. However, any other suitable apparatus, embodying the principle of the assembly described and illustrated, may be used.



Arsenic Test Apparatus

Arsenic Trioxide Stock Solution—Dissolve 132.0 mg of arsenic trioxide, previously dried at 105° for 1 hour and accurately weighed, in 5 mL of sodium hydroxide solution (1 in 5) in a 1000-mL volumetric flask. Neutralize the solution with 2 N sulfuric acid, add 10 mL more of 2 N sulfuric acid, then add recently boiled and cooled water to volume, and mix.

Standard Arsenic Solution—Transfer 10.0 mL of *Arsenic Trioxide Stock Solution* to a 1000-mL volumetric flask, add 10 mL of 2 N sulfuric acid, then add recently boiled and cooled water to volume, and mix. Each mL of *Standard Arsenic Solution* contains the equivalent of 1 µg of arsenic (As). Keep this solution in an all-glass container, and use within 3 days.

METHOD I

Standard Preparation—Pipet 3.0 mL of *Standard Arsenic Solution* into a generator flask, and dilute with water to 35 mL.

Test Preparation—Unless otherwise directed in the individual monograph, transfer to the generator flask the quantity, in g, of the test substance calculated by the formula:

$$3.0/L$$

in which L is the arsenic limit in ppm, dissolve in water, and dilute with water to 35 mL.

Procedure—Treat the *Standard Preparation* and the *Test Preparation* similarly as follows. Add 20 mL of 7 N sulfuric acid, 2 mL of potassium iodide TS, 0.5 mL of stronger acid stannous chloride TS, and 1 mL of isopropyl alcohol, and mix. Allow to stand at room temperature for 30 minutes. Pack the scrubber tube (c) with two pledgets of cotton that have been soaked in saturated lead acetate solution, freed from excess solution by expression, and dried in vacuum at room temperature, leaving a 2-mm space between the two pledgets. Lubricate the joints (b and d) with a suitable stopcock grease designed for use with organic solvents, and connect the scrubber unit to the absorber tube (e). Transfer 3.0 mL of silver diethyldithiocarbamate TS to the absorber tube. Add 3.0 g of granular zinc (No. 20 mesh) to the mixture in the flask, immediately connect the assembled scrubber unit, and allow the evolution of hydrogen and the color development to proceed at room temperature for 45 minutes, swirling the flask gently at 10-minute intervals. Disconnect the absorber tube from the generator and scrubber units, and transfer the absorbing solution to a 1-cm absorption cell. Any red color produced by the *Test Preparation* does not exceed that produced by the *Standard Preparation*. If necessary or desirable, determine the absorbance at the wavelength of maximum absorbance between 535 and 540 nm, with a suitable spectrophotometer or colorimeter, using silver diethyldithiocarbamate TS as the blank.

Interfering Chemicals—Metals or salts of metals, such as chromium, cobalt, copper, mercury, molybdenum, nickel, palladium, and silver, may interfere with the evolution of arsine. Antimony, which forms stibine, produces a positive interference in the color development with silver diethyldithiocarbamate TS; when the presence of antimony is suspected, the red colors produced in the two silver diethyldithiocarbamate solutions may be compared at the wavelength of maximum absorbance between 535 and 540 nm, with a suitable colorimeter, since at this wavelength the interference due to stibine is negligible.

METHOD II

NOTES—

(1) *Caution*—Some substances may react with explosive violence when digested with hydrogen peroxide. Exercise safety precautions at all times.

(2) If halogen-containing compounds are present, use a lower temperature while heating the test specimen with sulfuric acid, avoid boiling the mixture, and add the hydrogen peroxide with caution, before charring begins, to prevent loss of trivalent arsenic.

(3) If the test substance reacts too rapidly and begins charring with 5 mL of sulfuric acid before heating, use in-

stead 10 mL of cooled dilute sulfuric acid (1 in 2), and add a few drops of the hydrogen peroxide before heating.

Standard Preparation—Pipet 3.0 mL of *Standard Arsenic Solution* into a generator flask, add 2 mL of sulfuric acid, mix, and add the total amount of 30 percent hydrogen peroxide used in preparing the *Test Preparation*. Heat the mixture to strong fuming, cool, add cautiously 10 mL of water, and again heat to strong fumes. Repeat this procedure with another 10 mL of water to remove any traces of hydrogen peroxide. Cool, and dilute with water to 35 mL.

Test Preparation—Unless otherwise directed in the individual monograph, transfer to a generator flask the quantity, in g, of the test substance calculated by the formula:

$$3.0/L$$

in which L is the arsenic limit in ppm. Add 5 mL of sulfuric acid and a few glass beads, and digest in a fume hood, preferably on a hot plate and at a temperature not exceeding 120°, until charring begins. (Additional sulfuric acid may be necessary to wet some specimens completely, but the total volume added should not exceed 10 mL.) Cautiously add, dropwise, 30 percent hydrogen peroxide, allowing the reaction to subside and again heating between drops. Add the first few drops very slowly with sufficient mixing, in order to prevent a rapid reaction. Discontinue heating if foaming becomes excessive. When the reaction has abated, heat cautiously, rotating the flask occasionally to prevent the specimen from caking on glass exposed to the heating unit. *Maintain oxidizing conditions at all times during the digestion by adding small quantities of the hydrogen peroxide solution whenever the mixture turns brown or darkens.* Continue the digestion until the organic matter is destroyed, gradually raising the temperature of the hot plate until fumes of sulfur trioxide are copiously evolved, and the solution becomes colorless or retains only a light straw color. Cool, add cautiously 10 mL of water, mix, and again evaporate to strong fuming, repeating this procedure to remove any trace of hydrogen peroxide. Cool, add cautiously 10 mL of water, wash the sides of the flask with a few mL of water, and dilute with water to 35 mL.

Procedure—Proceed as directed for *Procedure* under *Method I*.

Interfering Chemicals—See *Interfering Chemicals* under *Method I*.

(221) CHLORIDE AND SULFATE

The following limit tests are provided as general procedures for use where limits for chloride and sulfate are specified in the individual monographs.

Perform the tests and the controls in glass cylinders of the same diameter and matched as closely as practicable in other respects (see *Visual Comparison* under *Spectrophotometry and Light-Scattering* (851)). Use the same quantities of the same reagents for both the solution under test and the control solution containing the specified volume of chloride or sulfate. If, after acidification, the solution is not perfectly clear, pass it through a filter paper that gives negative tests for chloride and sulfate. Add the precipitant, silver nitrate TS or barium chloride TS as required, to both the test solution and the control solution in immediate sequence.

Where the individual monograph calls for applying the test to a specific volume of a solution of the substance, and the limit for chloride or sulfate corresponds to 0.20 mL or less of 0.020 N hydrochloric acid or sulfuric acid, respectively, apply the test to the solution without further dilution.

In such cases maintain the same volume relationships for the control solution as specified for the solution under test. In applying the test to the salts of heavy metals, which normally show an acid reaction, omit the acidification and do not neutralize the solution. Dissolve bismuth salts in a few mL of water and 2 mL of nitric acid before treating with the precipitant.

Chloride—Dissolve the specified quantity of the substance under test in 30 to 40 mL of water, or, where the substance is already in solution, add water to make a total volume of 30 to 40 mL, and, if necessary, neutralize the solution with nitric acid to litmus. Add 1 mL each of nitric acid and of silver nitrate TS and sufficient water to make 50 mL. Mix, and allow to stand for 5 minutes protected from direct sunlight. Unless otherwise specified in the monograph, compare the turbidity, if any, with that produced in a solution containing the volume of 0.020 N hydrochloric acid specified in the monograph.

Sulfate—Dissolve the specified quantity of the substance under test in 30 to 40 mL of water, or, where the substance is already in solution, add water to make a total volume of 30 to 40 mL, and, if necessary, neutralize the solution with hydrochloric acid to litmus. Add 1 mL of 3 N hydrochloric acid, 3 mL of barium chloride TS, and sufficient water to make 50 mL. Mix, and allow to stand for 10 minutes. Unless otherwise specified in the monograph, compare the turbidity, if any, with that produced in a solution containing the volume of 0.020 N sulfuric acid specified in the monograph.

(223) DIMETHYLANILINE

The following limit test is provided as a general procedure, when specified in the individual monographs for the gas chromatographic determination in compendial articles of traces of dimethylaniline, a hydrochloric acid scavenger that may have been carried over during processing.

Internal Standard Solution—Unless otherwise specified in the individual monograph, prepare a solution of naphthalene in cyclohexane containing about 50 µg per mL.

Standard Preparation—Unless otherwise specified in the individual monograph, transfer 50.0 mg of *N,N*-dimethylaniline to a 50-mL volumetric flask, add 25 mL of 1 N hydrochloric acid, swirl to dissolve, dilute with water to volume, and mix. Transfer 5.0 mL of the resulting solution to a 250-mL volumetric flask, dilute with water to volume, and mix. To a suitable centrifuge tube add 1.0 mL of this solution, 5.0 mL of 1 N sodium hydroxide, and 1.0 mL of *Internal Standard Solution*, shake vigorously for 1 minute, and centrifuge. Use the clear supernatant as the *Standard Preparation*.

Test Preparation—Unless otherwise specified in the individual monograph, transfer 1.0 g of the substance to be tested to a suitable centrifuge tube, add 5 mL of 1 N sodium hydroxide, swirl to dissolve the specimen, add 1.0 mL of *Internal Standard Solution*, shake vigorously for 1 minute, and centrifuge. Use the clear supernatant as the *Test Preparation*.

Chromatographic System (see *Chromatography* (621))—The gas chromatograph is equipped with a flame-ionization detector, maintained at about 250°, and a 0.53-mm × 30-m fused silica capillary column bonded with a 1.0-µm film of phase G42. The carrier gas is helium, with a linear velocity of about 30 cm per second and a split ratio of 10 : 1. The column temperature is maintained at 110° for the first 4 minutes after an injection is made, then increased from 110° to 200° at 8° per minute, and then held at 200° for 5 minutes. The injection port temperature is maintained at

250°. Chromatograph the *Standard Preparation*, and record the responses as directed for *Procedure*: identify the dimethylaniline and naphthalene peaks by their relative retention times, which are 1.0 and 1.3, respectively. The signal-to-noise ratio for the dimethylaniline peak is not less than 10.

Procedure—Inject equal volumes (about 1 µL) of the *Standard Preparation* and the *Test Preparation* into the chromatograph, record the chromatograms, and measure the areas for the major peaks. The ratio of the response of any dimethylaniline peak to the response of the naphthalene peak obtained from the *Test Preparation* is not greater than that obtained from the *Standard Preparation* (0.002%).

(226) 4-EPIANHYDRO-TETRACYCLINE

This chromatographic procedure is provided to demonstrate that the content of 4-epianhydrotetracycline, a degradation product of tetracycline, does not exceed the limit given in the individual monograph.

EDTA Buffer—Dissolve 37.2 g of edetate disodium in 800 mL of water, adjust with ammonium hydroxide to a pH of 7.8, dilute with water to 1000 mL, and mix.

Support Phase—Add 5 mL of *EDTA Buffer* to 10 g of acid-washed chromatographic siliceous earth for column chromatography, and mix until the siliceous earth is uniformly moistened.

Test Solution—Prepare as directed in the individual monograph.

Procedure—Prepare a 15-mm × 170-mm chromatographic tube with a 4-mm × 50-mm outlet by packing it, in increments, with *Support Phase*, firmly tamping down each increment, until the tube is filled to a height of about 10 cm. In a beaker, prepare a mixture of 1 g of acid-washed chromatographic siliceous earth for column chromatography and 1 mL of *Test Solution*. Transfer the mixture to the top of the column. Dry-wash the beaker with *Support Phase*, and transfer to the column to provide an additional 1-cm layer on top of the mixture containing the *Test Solution*. Within 30 minutes, pass chloroform through the column, and collect successive fractions of 5.0 mL, 5.0 mL, 10.0 mL, 10.0 mL, and 5.0 mL. Observe the column during elution, and note the appearance of two separate yellow bands. The fraction or fractions containing the first yellow band contain the anhydrotetracyclines. Discard these fractions. The fractions after the first yellow band contain the 4-epianhydrotetracycline. Determine the absorbance of each 4-epianhydrotetracycline fraction at the wavelength of maximum absorbance at about 438 nm, with a suitable spectrophotometer, diluting each fraction, if necessary, with chloroform, and using chloroform as the blank. Calculate the quantity, in mg, of 4-epianhydrotetracycline in each fraction by the formula:

$$AVD/20.08$$

in which A is the absorbance, V is the volume, in mL, of the fraction taken, D is the dilution factor, if the fraction was diluted, and 20.08 is the absorptivity of 4-epianhydrotetracycline at 438 nm. From the sum of the quantities of 4-epianhydrotetracycline found in the fractions, calculate the percentage of 4-epianhydrotetracycline in relation to the tetracycline hydrochloride equivalent contained in the *Test Solution*.

(228) ETHYLENE OXIDE AND DIOXANE

The following procedure is used to determine the contents of residual ethylene oxide and dioxane in the products prepared from ethylene oxide. Unless otherwise directed in the individual monograph, use *Method I*.

Method I

[CAUTION]—Ethylene oxide is toxic and flammable. Prepare these solutions in a well-ventilated fume hood, using great care. Protect both hands and face by wearing polyethylene protective gloves and an appropriate face mask. Store all solutions in hermetic containers, and refrigerate between 4° and 8°.

[NOTE]—Before using the polyethylene glycol 200 in this test, remove any volatile components from it by placing 500 mL of polyethylene glycol 200 in a 1000-mL round-bottom flask and attaching the flask to a rotary evaporator maintained at 60° and under a vacuum of 10–20 mm Hg for 6 h.]

Acetaldehyde solution: 10 µg/mL of acetaldehyde. **[NOTE]**—Prepare immediately before use.]

Ethylene oxide stock solution: 2.5 mg/g of ethylene oxide. Prepare as follows: Tare a glass-stoppered conical flask, add 50 mL of polyethylene glycol 200, and reweigh the flask. Transfer 5 mL of the liquid ethylene oxide to a 100-mL beaker chilled in a mixture of sodium chloride and ice (1:3). Transfer 300 µL (corresponding to 250 mg) of liquid ethylene oxide to the polyethylene glycol 200, and swirl gently to mix. Replace the stopper, reweigh the flask, and determine the amount of ethylene oxide absorbed by weight difference. Adjust the weight of the mixture with polyethylene glycol 200 to 100.0 g, replace the stopper, and swirl gently to mix. **[NOTE]**—Fill a chilled pressure bottle with liquid ethylene oxide, and store in a freezer when not in use. Use a small piece of polyethylene film to protect the liquid from contact with the rubber gasket. Use an adequately chilled apparatus where appropriate. Prepare this stock solution immediately prior to use, and store in a refrigerator after preparation.]

Ethylene oxide solution: Tare a glass-stoppered conical flask, and chill it in a refrigerator. Add 35 mL of polyethylene glycol 200, and reweigh the flask. Transfer 1 g of chilled *Ethylene oxide stock solution* to the tared conical flask. Adjust the weight of the solution with polyethylene glycol 200 to 50.0 g, replace the stopper, and swirl gently to mix. Transfer 10 g of this solution to a 50-mL volumetric flask. Add 30 mL of water, and mix. Dilute with water to volume, and mix to obtain a solution containing 10 µg/mL of ethylene oxide. **[NOTE]**—Use an adequately chilled apparatus where appropriate. Prepare immediately before use.]

Dioxane solution: 500 µg/mL of dioxane

Standard solution A: Transfer 0.1 mL of *Ethylene oxide solution* to a 10-mL pressure headspace vial. **[NOTE]**—Other sizes such as a 22-mL pressure headspace vial may be used, depending on operating conditions; however, the same size must be used for *Standard solution A*, *Standard solution B*, and the *Sample solution*.] Add 0.1 mL of *Acetaldehyde solution* and 0.1 mL of *Dioxane solution*, seal the vial, and mix.

Standard solution B: Transfer 1.0 g of the test substance to a 10-mL pressure headspace vial, and add 0.1 mL of *Ethylene oxide solution*, 0.1 mL of *Dioxane solution*, and 1.0 mL of *N,N*-dimethylacetamide. Seal the vial, and mix.

Sample solution: Transfer 1.0 g of the test substance to a 10-mL pressure headspace vial, and add 1.0 mL of *N,N*-dimethylacetamide and 0.2 mL of water. Seal the vial, and mix.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: Headspace GC

Detector: Flame ionization

Column: 0.32-mm × 30-m glass or quartz capillary; 1.0-µm layer of phase G1

Temperature

Injector port: 150°

Detector: 250°

Column: See the column temperature table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	—
180	30	230	5

Carrier gas: Helium

Linear velocity: 20 cm/s

Injection volume: 1 mL (gaseous headspace)

Injection type: Split ratio 20:1

Headspace sampler

Temperature equilibration time: 45 min

Equilibration temperature

70° for *Standard solution A*

90° for *Standard solution B*

90° for *Sample solution*

Transfer line temperature: 150°

Pressurization time: 1 min

Injection time: 12 s

System suitability

Sample: *Standard solution A*

[NOTE]—The relative retention times for acetaldehyde and ethylene oxide are 0.94 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between acetaldehyde and ethylene oxide

Signal-to-noise ratio: NLT 5, determined from the dioxane peak

Relative standard deviation: NMT 15%

Analysis

Samples: *Standard solution B* and *Sample solution*

[NOTE]—The relative retention times for ethylene oxide and dioxane are 1.0 and 2.5, respectively.]

Calculate the content of ethylene oxide, in ppm, in the portion of the test substance taken:

$$\text{Result} = A_E \times r_U / [(r_S \times W_U) - (r_U \times W_S)]$$

A_E = quantity of ethylene oxide added to *Standard solution B* (µg)

r_U = ethylene oxide peak responses from the *Sample solution*

r_S = ethylene oxide peak responses from *Standard solution B*

W_U = weight of the test substance taken to prepare the *Sample solution* (g)

W_S = weight of the test substance taken to prepare *Standard solution B* (g)

Calculate the content of dioxane, in ppm, in the portion of the test substance taken:

$$\text{Result} = A_D \times r_U / [(r_S \times W_U) - (r_U \times W_S)]$$

A_D = quantity of dioxane added to *Standard solution B* (µg)

r_U = dioxane peak responses from the *Sample solution*

r_S = dioxane peak responses from *Standard solution B*

W_U = weight of the test substance taken to prepare the *Sample solution* (g)

W_S = weight of the test substance taken to prepare *Standard solution B* (g)

Method II

Ethylene oxide standard solution: Dilute 0.5 mL of ethylene oxide in methylene chloride (50 mg/mL)¹ with water to 50.0 mL. [NOTE—The solution is stable for 3 months if stored in vials with polytetrafluoroethylene (polytef)-coated silicon membrane crimped caps at –20°.] Allow to reach room temperature. Dilute 1.0 mL with water to 250.0 mL to obtain a solution having a concentration of 2 µg/mL of ethylene oxide. [NOTE—Use this solution immediately after preparation.]

Dioxane standard solution: 0.05 µL/mL of dioxane

Acetaldehyde standard solution: 10 µg/mL of acetaldehyde. [NOTE—Prepare immediately before use.]

Resolution solution: Add 2.0 mL of *Acetaldehyde standard solution* and 2.0 mL of *Ethylene oxide standard solution* to a 10-mL headspace vial. Seal the vial immediately with a polytef-coated silicon membrane and an aluminum cap, and mix carefully.

Standard solution A: 0.48 µg/mL of ethylene oxide, from *Ethylene oxide standard solution*, and 0.005 µL/mL of dioxane, from *Dioxane standard solution*, in water

Standard solution B: Transfer 1.0 g of the test substance into a 10-mL headspace vial. Add 2.0 mL of *Standard solution A*, seal the vial immediately with a polytef-coated silicon membrane and an aluminum cap, and mix carefully.

Sample solution: Transfer 1.0 g of the test substance into a 10-mL headspace vial. Add 2.0 mL of water, seal the vial immediately with a polytef-coated silicon membrane and an aluminum cap, and mix carefully.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: Headspace GC

Detector: Flame ionization

Column: 0.53-mm × 50-m fused-silica capillary column; 5.0-µm layer of phase G27

Temperature

Injector port: 85°

Detector: 250°

Column: See the column temperature table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	10	250	5

¹This is a commercially available solution.

Carrier gas: Helium

Flow rate: 4 mL/min

Injection volume: 1 mL (gaseous headspace)

Injection type: Split ratio 3.5 : 1

Headspace sampler

Temperature equilibration time: 30 min

Equilibration temperature: 80°

System suitability

Sample: *Resolution solution*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are 0.9 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between acetaldehyde and ethylene oxide

Analysis

Samples: *Standard solution B* and *Sample solution*

[NOTE—The relative retention times for ethylene oxide and dioxane are 1.0 and 1.9, respectively.]

Calculate the content of ethylene oxide, in ppm, in the portion of the test substance taken:

$$\text{Result} = C_E \times V \times r_U / [(r_S \times W_U) - (r_U \times W_S)]$$

C_E = concentration of ethylene oxide in the *Standard solution A* (µg/mL)

V = volume of *Standard solution A* added to *Standard solution B* (2.0 mL)

r_U = ethylene oxide peak responses from the *Sample solution*

r_S = ethylene oxide peak responses from *Standard solution B*

W_U = weight of the test substance taken to prepare the *Sample solution* (g)

W_S = weight of the test substance taken to prepare *Standard solution B* (g)

Calculate the content of dioxane, in ppm, in the portion of the test substance taken:

$$\text{Result} = C_D \times V \times \rho \times F \times r_U / [(r_S \times W_U) - (r_U \times W_S)]$$

C_D = concentration of dioxane in *Standard solution A* (µL/mL)

V = volume of *Standard solution A* added to *Standard solution B* (2.0 mL)

ρ = density of dioxane (1.03 g/mL = 1.03 mg/µL)

F = conversion factor (1000 µg/mg)

r_U = dioxane peak responses from the *Sample solution*

r_S = ethylene oxide peak responses from *Standard solution B*

W_U = weight of the test substance taken to prepare the *Sample solution* (g)

W_S = weight of the test substance taken to prepare *Standard solution B* (g)

(231) HEAVY METALS

This test is provided to demonstrate that the content of metallic impurities that are colored by sulfide ion, under the specified test conditions, does not exceed the *Heavy metals* limit specified in the individual monograph in percentage (by weight) of lead in the test substance, as determined by concomitant visual comparison (see *Visual Comparison* in the section *Procedure* under *Spectrophotometry and Light-Scattering* (851)) with a control prepared from a *Standard Lead Solution*. [NOTE—Substances that typically will respond to this test are lead, mercury, bismuth, arsenic, antimony, tin, cadmium, silver, copper, and molybdenum.]

Determine the amount of heavy metals by *Method I*, unless otherwise specified in the individual monograph. *Method I* is used for substances that yield clear, colorless preparations under the specified test conditions. *Method II* is used for substances that do not yield clear, colorless preparations under the test conditions specified for *Method I*, or for substances that, by virtue of their complex nature, interfere with the precipitation of metals by sulfide ion, or for fixed and volatile oils. *Method III*, a wet-digestion method, is used only in those cases where neither *Method I* nor *Method II* can be used.

Special Reagents

Lead Nitrate Stock Solution—Dissolve 159.8 mg of lead nitrate in 100 mL of water to which has been added 1 mL of nitric acid, then dilute with water to 1000 mL. Prepare and store this solution in glass containers free from soluble lead salts.

Standard Lead Solution—On the day of use, dilute 10.0 mL of *Lead Nitrate Stock Solution* with water to 100.0 mL. Each mL of *Standard Lead Solution* contains the equivalent of 10 µg of lead. A comparison solution prepared on the basis of 100 µL of *Standard Lead Solution* per g of substance being tested contains the equivalent of 1 part of lead per million parts of substance being tested.

Method I

pH 3.5 Acetate Buffer—Dissolve 25.0 g of ammonium acetate in 25 mL of water, and add 38.0 mL of 6 N hydrochloric acid. Adjust, if necessary, with 6 N ammonium hydroxide or 6 N hydrochloric acid to a pH of 3.5, dilute with water to 100 mL, and mix.

Standard Preparation—Into a 50-mL color-comparison tube pipet 2 mL of *Standard Lead Solution* (20 µg of Pb), and dilute with water to 25 mL. Using a pH meter or short-range pH indicator paper as external indicator, adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, dilute with water to 40 mL, and mix.

Test Preparation—Into a 50-mL color-comparison tube place 25 mL of the solution prepared for the test as directed in the individual monograph; or, using the designated volume of acid where specified in the individual monograph, dissolve in and dilute with water to 25 mL the quantity, in g, of the substance to be tested, as calculated by the formula:

$$2.0/(1000L)$$

in which L is the *Heavy metals* limit, as a percentage. Using a pH meter or short-range pH indicator paper as external indicator, adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, dilute with water to 40 mL, and mix.

Monitor Preparation—Into a third 50-mL color-comparison tube place 25 mL of a solution prepared as directed for

Test Preparation, and add 2.0 mL of *Standard Lead Solution*. Using a pH meter or short-range pH indicator paper as external indicator, adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, dilute with water to 40 mL, and mix.

Procedure—To each of the three tubes containing the *Standard Preparation*, the *Test Preparation*, and the *Monitor Preparation*, add 2 mL of pH 3.5 Acetate Buffer, then add 1.2 mL of thioacetamide–glycerin base TS, dilute with water to 50 mL, mix, allow to stand for 2 minutes, and view downward over a white surface*: the color of the solution from the *Test Preparation* is not darker than that of the solution from the *Standard Preparation*, and the color of the solution from the *Monitor Preparation* is equal to or darker than that of the solution from the *Standard Preparation*.

[NOTE—If the color of the *Monitor Preparation* is lighter than that of the *Standard Preparation*, use *Method II* instead of *Method I* for the substance being tested.]

Method II

NOTE—This method does not recover mercury.

pH 3.5 Acetate Buffer—Prepare as directed under *Method I*.

Standard Preparation—Prepare as directed under *Method I*.

Test Preparation—Use a quantity, in g, of the substance to be tested as calculated by the formula:

$$2.0/(1000L)$$

in which L is the *Heavy metals* limit, in percentage. Transfer the weighed quantity of the substance to a suitable crucible, add sufficient sulfuric acid to wet the substance, and carefully ignite at a low temperature until thoroughly charred. (The crucible may be loosely covered with a suitable lid during the charring.) Add to the carbonized mass 2 mL of nitric acid and 5 drops of sulfuric acid, and heat cautiously until white fumes no longer are evolved. Ignite, preferably in a muffle furnace, at 500° to 600°, until the carbon is completely burned off. Cool, add 4 mL of 6 N hydrochloric acid, cover, digest on a steam bath for 15 minutes, uncover, and slowly evaporate on a steam bath to dryness. Moisten the residue with 1 drop of hydrochloric acid, add 10 mL of hot water, and digest for 2 minutes. Add 6 N ammonium hydroxide dropwise until the solution is just alkaline to litmus paper, dilute with water to 25 mL, and adjust with 1 N acetic acid to a pH between 3.0 and 4.0, using short-range pH indicator paper as an external indicator. Filter if necessary, rinse the crucible and the filter with 10 mL of water, combine the filtrate and rinsing in a 50-mL color-comparison tube, dilute with water to 40 mL, and mix.

Procedure—To each of the tubes containing the *Standard Preparation* and the *Test Preparation*, add 2 mL of pH 3.5 Acetate Buffer, then add 1.2 mL of thioacetamide–glycerin base TS, dilute with water to 50 mL, mix, allow to stand for 2 minutes, and view downward over a white surface*: the color of the solution from the *Test Preparation* is not darker than that of the solution from the *Standard Preparation*.

Method III

pH 3.5 Acetate Buffer—Prepare as directed under *Method I*.

Standard Preparation—Transfer a mixture of 8 mL of sulfuric acid and 10 mL of nitric acid to a clean, dry, 100-mL Kjeldahl flask, and add a further volume of nitric acid equal to the incremental volume of nitric acid added to

* In those countries or jurisdictions where thioacetamide cannot be used, add 10 mL of freshly prepared hydrogen sulfide TS to each of the tubes, mix, allow to stand for 5 minutes, and view downward over a white surface.

the *Test Preparation*. Heat the solution to the production of dense, white fumes; cool; cautiously add 10 mL of water; and, if hydrogen peroxide was used in treating the *Test Preparation*, add a volume of 30 percent hydrogen peroxide equal to that used for the substance being tested. Boil gently to the production of dense, white fumes. Again cool, cautiously add 5 mL of water, mix, and boil gently to the production of dense, white fumes and to a volume of 2 to 3 mL. Cool, dilute cautiously with a few mL of water, add 2.0 mL of *Standard Lead Solution* (20 µg of Pb), and mix. Transfer to a 50-mL color-comparison tube, rinse the flask with water, adding the rinsing to the tube until the volume is 25 mL, and mix.

Test Preparation—Unless otherwise indicated in the individual monograph, use a quantity, in g, of the substance to be tested as calculated by the formula:

$$2.0/(1000L)$$

in which L is the *Heavy metals* limit, as a percentage.

If the substance is a solid—Transfer the weighed quantity of the test substance to a clean, dry, 100-mL Kjeldahl flask. [NOTE—A 300-mL flask may be used if the reaction foams excessively.] Clamp the flask at an angle of 45°, and add a sufficient quantity of a mixture of 8 mL of sulfuric acid and 10 mL of nitric acid to moisten the substance thoroughly. Warm gently until the reaction commences, allow the reaction to subside, and add portions of the same acid mixture, heating after each addition, until a total of 18 mL of the acid mixture has been added. Increase the amount of heat, and boil gently until the solution darkens. Cool, add 2 mL of nitric acid, and heat again until the solution darkens. Continue the heating, followed by addition of nitric acid until no further darkening occurs, then heat strongly to the production of dense, white fumes. Cool, cautiously add 5 mL of water, boil gently to the production of dense, white fumes, and continue heating until the volume is reduced to a few mL. Cool, cautiously add 5 mL of water, and examine the color of the solution. If the color is yellow, cautiously add 1 mL of 30 percent hydrogen peroxide, and again evaporate to the production of dense, white fumes and a volume of 2 to 3 mL. If the solution is still yellow, repeat the addition of 5 mL of water and the peroxide treatment. Cool, dilute cautiously with a few mL of water, and rinse into a 50-mL color-comparison tube, taking care that the combined volume does not exceed 25 mL.

If the substance is a liquid—Transfer the weighed quantity of the test substance to a clean, dry, 100-mL Kjeldahl flask. [NOTE—A 300-mL flask may be used if the reaction foams excessively.] Clamp the flask at an angle of 45°, and cautiously add a few mL of a mixture of 8 mL of sulfuric acid and 10 mL of nitric acid. Warm gently until the reaction commences, allow the reaction to subside, and proceed as directed for *If the substance is a solid*, beginning with “add portions of the same acid mixture.”

Monitor Preparation—Proceed with the digestion, using the same amount of sample and the same procedure as directed in the subsection *If the substance is a solid* in the section *Test Preparation*, until the step “Cool, dilute cautiously with a few mL of water.” Add 2.0 mL of *Lead Standard Solution* (20 µg of lead), and mix. Transfer to a 50-mL color comparison tube, rinse the flask with water, adding the rinsing to the tube until the volume is 25 mL, and mix.

Procedure—Treat the *Test Preparation*, the *Standard Preparation*, and the *Monitor Preparation* as follows. Using a pH meter or short-range pH indicator paper as external indicator, adjust the solution to a pH between 3.0 and 4.0 with ammonium hydroxide (a dilute ammonia solution may be used, if desired, as the specified range is approached), dilute with water to 40 mL, and mix.

To each tube add 2 mL of pH 3.5 *Acetate Buffer*, then add 1.2 mL of thioacetamide–glycerin base TS, dilute with water

to 50 mL, mix, allow to stand for 2 minutes, and view downward over a white surface*: the color of the *Test Preparation* is not darker than that of the *Standard Preparation*, and the color of the *Monitor Preparation* is equal to or darker than that of the *Standard Preparation*.

(232) ELEMENTAL IMPURITIES—LIMITS

INTRODUCTION

This general chapter specifies limits for the amounts of elemental impurities in drug products. Elemental impurities include catalysts and environmental contaminants that may be present in drug substances, excipients, or drug products. These impurities may occur naturally, be added intentionally, or be introduced inadvertently (e.g., by interactions with processing equipment). When elemental impurities are known to be present, have been added, or have the potential for introduction, assurance of compliance to the specified levels is required. A risk-based control strategy may be appropriate when analysts determine how to assure compliance with this standard. Due to the ubiquitous nature of As, Cd, Pb, and Hg, they (at the minimum) must be considered in the risk-based control strategy. Regardless of the approach used, compliance with the limits specified is required for all drug products.

The limits presented in this chapter do not apply to excipients and drug substances, except where specified in this chapter or in the individual monographs. However, elemental impurity levels present in drug substances and excipients must be known and reported.

The limits indicated in this chapter are not required for articles intended only for veterinary use and conventional vaccines. Dietary supplements and their ingredients are addressed in *Elemental Contaminants in Dietary Supplements* (2322).¹

SPECIATION

The determination of the oxidation state, organic complex, or combination is termed speciation. Each of the elemental impurities has the potential to be present in differing oxidation or complexation states. However, arsenic and mercury are of particular concern because of the differing toxicities of their inorganic and complexed organic forms.

The arsenic limits are based on the inorganic (most toxic) form. Arsenic can be measured using a total-arsenic procedure under the assumption that all arsenic contained in the material under test is in the inorganic form. Where the limit is exceeded using a total arsenic procedure, it may be possible to show via a procedure that quantifies the different forms that the inorganic form meets the specification.

The mercury limits are based upon the inorganic (2+) oxidation state. The methyl mercury form (most toxic) is rarely an issue for pharmaceuticals. Thus, the limit was established assuming the most common (mercuric) inorganic form. Limits for articles that have the potential to contain methyl mercury (e.g., materials derived from fish) are to be provided in the monograph.

¹ This dietary supplement chapter is still under revision and appears online in PF 38(3) [May–June 2012].

ROUTES OF EXPOSURE

The toxicity of an elemental impurity is related to its extent of exposure (bioavailability). The extent of exposure has been determined for each of the elemental impurities of interest for three routes of administration: oral, parenteral, and inhalational. These limits are based on chronic exposure. The other two routes of administration, mucosal and topical, are considered to be the same as oral for the purpose of this standard, and the PDEs described in *Table 1* would apply to these products. [NOTE—The routes of administration of drug products are defined in general chapter *Pharmaceutical Dosage Forms* (1151).]

DRUG PRODUCTS

The limits described in the second through fourth columns of *Table 1* are the base daily dose PDEs of the elemental impurities of interest for a drug product taken by the patient according to indicated routes of administration. Parenterals with an intended maximum dose of greater than 10 mL and not more than 100 mL must use the *Summation Option* described below.

Large Volume Parenterals

When the daily dose of an injection is greater than 100 mL (large volume parenteral (LVP)), the amount of elemental impurities present in the drug product must be controlled through the individual components used to produce the product. The amounts of elemental impurities present in each component used in an LVP are less than the values included in the fifth column of *Table 1*.

Table 1. Elemental Impurities for Drug Products

Element	Oral Daily Dose PDE ^a (μg/day)	Parenteral Daily Dose PDE (μg/day)	Inhalational Daily Dose PDE (μg/day)	LVP Component Limit (μg/g)
Cadmium	25	2.5	1.5	0.25
Lead	5	5	5	0.5
Inorganic arsenic ^b	1.5	1.5	1.5	0.15
Inorganic mercury ^b	15	1.5	1.5	0.15
Iridium	100	10	1.5	1.0
Osmium	100	10	1.5	1.0
Palladium	100	10	1.5	1.0
Platinum	100	10	1.5	1.0
Rhodium	100	10	1.5	1.0
Ruthenium	100	10	1.5	1.0
Chromium	*	*	25	*
Molybdenum	100	10	250	1.0
Nickel	500	50	1.5	5.0

^a PDE = Permissible daily exposure based on a 50-kg person.

^b See *Speciation* section.

* Not a safety concern.

Table 1. Elemental Impurities for Drug Products (Continued)

Element	Oral Daily Dose PDE ^a (μg/day)	Parenteral Daily Dose PDE (μg/day)	Inhalational Daily Dose PDE (μg/day)	LVP Component Limit (μg/g)
Vanadium	100	10	30	1.0
Copper	1000	100	70	25

^a PDE = Permissible daily exposure based on a 50-kg person.

^b See *Speciation* section.

* Not a safety concern.

Options for Demonstrating Compliance

DRUG PRODUCT ANALYSIS OPTION

The results obtained from the analysis of a typical dosage unit, scaled to a maximum daily dose, are compared to the *Daily Dose PDE*.

$$\text{Daily Dose PDE} \geq \text{measured value } (\mu\text{g/g}) \times \text{maximum daily dose (g/day)}$$

The measured amount of each impurity is NMT the *Daily Dose PDE*, unless otherwise stated in the individual monograph.

SUMMATION OPTION

Separately add the amounts of each elemental impurity (in μg/g) present in each of the components of the drug product using the following equation:

$$\text{Daily Dose PDE} \geq [\sum M_i(C_M \times W_M)] \times D_D$$

where

M = each ingredient used to manufacture a dosage unit

C_M = element concentration in component (drug substance or excipient) (μg/g)

W_M = weight of component in a dosage unit (g/dosage unit)

D_D = number of units in the maximum daily dose (unit/day)

The result of the summation of each impurity is NMT the *Daily Dose PDE*, unless otherwise stated in the individual monograph. Before products can be evaluated using this option, the manufacturer must validate that additional elemental impurities cannot be inadvertently added through the manufacturing process.

DRUG SUBSTANCE AND EXCIPIENTS

The presence of elemental impurities in drug substances and excipients must be controlled and, where present, reported. The acceptable levels for these impurities depend on the material's ultimate use. Therefore, drug product manufacturers must determine the acceptable level of elemental impurities in the drug substances and excipients used to produce their products.

The values provided in *Table 2* represent concentration limits for components (drug substances and excipients) of drug products dosed at a maximum daily dose of ≤ 10 g/day. These values serve as default concentration limits to aid discussions between drug product manufacturers and the suppliers of the components of their drug products. [NOTE—Individual components may need to be limited at levels different from those in the table depending on monograph-specific mitigating factors.]

Table 2. Default Concentration Limits for Drug Substances and Excipients

Element	Concentration Limits (µg/g) for Oral Drug Products with a Maximum Daily Dose of ≤10 g/day	Concentration Limits (µg/g) for Parenteral Drug Products with a Maximum Daily Dose of ≤10 g/day	Concentration Limits (µg/g) for Inhalational Drug Products with a Maximum Daily Dose of ≤10 g/day
Cadmium	2.5	0.25	0.15
Lead	0.5	0.5	0.5
Inorganic arsenic	0.15	0.15	0.15
Inorganic mercury	1.5	0.15	0.15
Iridium	10	1.0	0.15
Osmium	10	1.0	0.15
Palladium	10	1.0	0.15
Platinum	10	1.0	0.15
Rhodium	10	1.0	0.15
Ruthenium	100	10	1.5
Chromium	*	*	2.5
Molybdenum	10	1.0	25
Nickel	50	5.0	0.15
Vanadium	100	10	30
Copper	100	10	7

* Not a safety concern.

ANALYTICAL TESTING

If, by validated processes and supply-chain control, manufacturers can demonstrate the absence of impurities, then further testing is not needed. When testing is done to demonstrate compliance, proceed as directed in general chapter *Elemental Impurities—Procedures* <233>, and minimally include As, Cd, Pd, and Hg in the *Target Element* evaluation.

<233> ELEMENTAL IMPURITIES—PROCEDURES

INTRODUCTION

This chapter describes two analytical procedures (*Procedures 1* and *2*) for the evaluation of the levels of the elemental impurities. The chapter also describes criteria for acceptable alternative procedures. Alternative procedures that meet the validation requirements described herein may be considered equivalent to *Procedures 1* and *2* for the purposes of this test. In addition, system standardization and suitability evaluation using applicable reference materials should be performed on the day of analysis. The requirement for an elemental impurity test is specified in *General Notices and Requirements* or in the individual monograph. By means of verification studies, analysts will confirm that the analytical procedures described herein, as well as alternative analytical procedures, are suitable for use on specified material.

Speciation

The determination of the oxidation state, organic complex or combination is termed *speciation*. Analytical procedures for speciation are not included in this chapter but examples may be found elsewhere in the *USP–NF* and in the literature.

Definitions

Concentrated Acid: Concentrated ultra-pure nitric, sulfuric, hydrochloric, or hydrofluoric acids or *Aqua Regia*.

Aqua Regia: Aqua regia is a mixture of concentrated hydrochloric and nitric acids, typically at ratios of 3:1 or 4:1, respectively.

Matched Matrix: Solutions having the same solvent composition as the *Sample solution*. In the case of an aqueous solution, *Matched Matrix* would indicate that the same acids, acid concentrations, and mercury stabilizer are used in both preparations.

Target Elements: Elements with the potential of being present in the material under test. Include As, Cd, Pd, and Hg in the target element evaluation when testing is done to demonstrate compliance. Target elements should also include any elements that may be added through material processing or storage, and any elements whose presence may interfere with the operation of the analytical procedures.

Target Limit or Target Concentration: The acceptance value for the elemental impurity being evaluated. Exceeding the target limit indicates that a material under test exceeds the acceptable value. The determination of compliance is addressed in other chapters. [NOTE—When applying this chapter to *Elemental Impurities—Limits* <232> and *Elemental Contaminants in Dietary Supplements* <232>,¹ *Target Limits* can be approximated by dividing the *Daily Dose PDEs* by the maximum daily dose for the *Drug Product Analysis Option* in <232> or the *Daily Serving PDE* divided by the maximum daily serving size in <232>]

J: The concentration (w/w) of the element(s) of interest at the *Target Limit*, appropriately diluted to the working range of the instrument. For example, if the target elements are Pb and As for an analysis of an oral solid drug product with a daily dose of 10 g/day using an inductively coupled plasma–mass spectrometry (ICP–MS). The target limit for these elements would be 0.5 µg/g and 0.15 µg/g (see *Table 2* in chapter <232>). However, in this case, the linear dynamic range of the ICP–MS is known to extend from 0.01 ng/mL to 0.1 µg/mL for these elements. Therefore, a dilution factor of at least 1:10 is required to ensure that the analysis occurs in the linear dynamic range of the instrument. *J* would thus equal 0.05 µg/mL and 0.015 µg/mL for Pb and As, respectively, when the dilution factor is added.

Appropriate Reference Materials: Where *Appropriate Reference Materials* are specified in the chapter, certified reference materials (CRM) from a national metrology institute (NMI), or reference materials that are traceable to the CRM of a NMI should be used. An example of a NMI in the United States is the National Institute of Standards and Technology.

COMPENDIAL PROCEDURES 1 AND 2

Procedure and Detection Technique

Procedure 1 can be used for elemental impurities generally amenable to detection by inductively coupled plasma–atomic (optical) emission spectroscopy (ICP–AES or

¹ This dietary supplement chapter is still under revision and appears online in *PF* 38(3) [May–June 2012].

ICP-OES). *Procedure 2* can be used for elemental impurities generally amenable to detection by ICP-MS. Before initial use, the analyst should verify that the procedure is appropriate for the instrument and sample used (procedural verification) by meeting the *Alternative Procedure Validation* requirements below.

Sample Preparation

Forms of sample preparation include *Neat*, *Direct Aqueous Solution*, *Direct Organic Solution*, and *Indirect Solution*. The selection of the appropriate sample preparation depends on the material under test and is the responsibility of the analyst. When a sample preparation is not indicated in the monograph, an analyst may use any of the following appropriately verified preparation procedures. In cases where spiking of a material under test is necessary to provide an acceptable signal intensity, the blank should be spiked with the same *Target Elements*, and where possible, using the same spiking solution. Standard solutions may contain multiple *Target Elements*. [NOTE—All liquid samples should be weighed.]

Neat: Used for liquids or alternative procedures that allows the examination of unsolvated samples.

Direct Aqueous Solution: Used when the sample is soluble in an aqueous solvent.

Direct Organic Solution: Used where the sample is soluble in an organic solvent.

Indirect Solution: Used when a material is not directly soluble in aqueous or organic solvents. Digest the sample using a closed-vessel digestion procedure, similar to the procedure provided below. The sample preparation scheme should yield sufficient sample to allow quantification of each element at the limit specified in the corresponding monograph or chapter.

Closed Vessel Digestion: This sample-preparation procedure is designed for samples that must be digested in a *Concentrated Acid* using a closed-vessel digestion apparatus. Closed-vessel digestion minimizes the loss of volatile impurities. The choice of a *Concentrated Acid* depends on the sample matrix. The use of any of the *Concentrated Acids* may be appropriate, but each introduces inherent safety risks. Therefore, appropriate safety precautions should be used at all times. [NOTE—Weights and volumes provided may be adjusted to meet the requirements of the digestion apparatus used.]

An example procedure that has been shown to have broad applicability is the following. Dehydrate and predigest 0.5 g of primary sample in 5 mL of freshly prepared *Concentrated Acid*. Allow to sit loosely covered for 30 minutes in a fume hood. Add an additional 10 mL of *Concentrated Acid*, and digest, using a closed vessel technique, until digestion or extraction is complete. Repeat if necessary by adding an additional 5 mL of *Concentrated Acid*. [NOTE—Where closed vessel digestion is necessary, follow the manufacturer's recommended procedures to ensure safe use.]

Reagents: All reagents used for the preparation of sample and standard solutions should be free of elemental impurities, in accordance with *Plasma Spectrochemistry* (730).

Procedure 1: ICP-AES

Standardization solution 1: 2J of the *Target Element(s)* in a *Matched Matrix*

Standardization solution 2: 0.5J of the *Target Element(s)* in a *Matched Matrix*

Sample stock solution: Proceed as directed in *Sample Preparation* above. Allow the sample to cool, if necessary. For mercury determination, add an appropriate stabilizer.

Sample solution: Dilute the *Sample Stock Solution* with an appropriate solvent to obtain a final concentration of the *Target Elements* at NMT 2J.

Blank: *Matched Matrix*

Elemental spectrometric system

(See *Plasma Spectrochemistry* (730).)

Mode: ICP

Detector: Optical detection system

Rinse: Diluent used

Standardization: *Standardization solution 1*, *Standardization solution 2*, and *Blank*

System suitability

Sample: *Standardization solution 1*

Suitability requirements

Drift: Compare results obtained from *Standardization solution 1* before and after the analysis of the *Sample solutions*.

Suitability criteria: NMT 20% for each *Target Element*. [NOTE—If samples are high in mineral content, rinse system well (60 seconds) before introducing the *Sample* in order to minimize carryover.]

Analysis: Analyze according to the manufacturer's suggestions for program and wavelength. Calculate and report results on the basis of the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences (e.g., Wavelength overlaps).]

Procedure 2: ICP-MS

Standardization solution 1: 2J of the *Target Element(s)* in a *Matched Matrix*

Standardization solution 2: 0.5J of the *Target Element(s)* in a *Matched Matrix*

Sample stock solution: Proceed as directed for *Sample Preparation* above. Allow the sample to cool, if necessary. For mercury determination, add an appropriate stabilizer.

Sample solution: Dilute the *Sample stock solution* with an appropriate solvent to obtain a final concentration of the *Target Elements* at NMT 2J.

Blank: *Matched Matrix*

Elemental spectrometric system

(See *Plasma Spectrochemistry* (730).)

Mode: ICP. [NOTE—An instrument with a cooled spray chamber is recommended. (A collision cell or reaction cell may also be beneficial.)]

Detector: Mass spectrometer

Rinse: Diluent used

Standardization: *Standardization solution 1*, *Standardization solution 2*, and *Blank*

System suitability

Sample: *Standardization solution 1*

Suitability requirements

Drift: Compare results obtained from *Standardization solution 1* before and after the analysis of the *Sample solutions*.

Suitability criteria: *Drift* NMT 20% for each *Target Element*. [NOTE—If samples are high in mineral content, rinse system well (60 seconds) before introducing the *Sample* in order to minimize carryover.]

Analysis: Analyze according to the manufacturer's suggestions for program and *m/z*. Calculate and report results based on the original sample size. [NOTE—Appropriate measures must be taken to correct for matrix-induced interferences (e.g., argon chloride interference with arsenic determinations).]

ALTERNATE PROCEDURE VALIDATION

If a specified compendial procedure does not meet the needs of a specific application, an alternative procedure may be used (see *General Notices* 6.30). Alternative procedures must be validated and must be acceptable and therefore equivalent to the compendial procedures for the purposes of the test. The principles of validation are provided in general chapter *Validation of Compendial Procedures* (1225). The level of validation necessary to ensure that an alternative procedure is acceptable depends on whether a limit test or a quantitative determination is necessary. The requirements for validation of an elemental impurities procedure for either type of determination are described below. Where this information differs from that presented in *Validation of Compendial Procedures* (1225), the parameters and acceptance criteria presented in this chapter take precedence. Any alternative procedure that has been validated and meets the acceptance criteria that follow is considered to be equivalent to the compendial procedures for the purposes of this test.

LIMIT PROCEDURES

The following section defines the validation parameters for the acceptability of alternative limit procedures. Meeting these requirements must be demonstrated experimentally using an appropriate system suitability procedure and reference material. Meeting these requirements demonstrates that the procedure is equivalent to the compendial procedure as a limit procedure for the *Target Element*.

The suitability of the method must be determined by conducting studies with material or mixture under test supplemented with known concentrations of each *Target Element* of interest at the appropriate acceptance limit concentration. The material or mixture under test must be spiked before any sample preparation steps are performed.

Detectability

Standard solution: A preparation of reference materials for the *Target Element(s)* at the *Target Concentrations*.

Spiked sample solution 1: Prepare a solution of sample under test, spiked with appropriate reference materials for the *Target Elements* at the *Target Concentration*, solubilized or digested as described in *Sample Preparation*.

Spiked sample solution 2: Prepare a solution of the sample under test, spiked with appropriate reference materials at 80% of the *Target Concentration* for the *Target Elements*, solubilized or digested as described in *Sample Preparation*.

Unspiked sample solution: A sample of material under test, solubilized or digested in the same manner as the *Sample solutions*.

Acceptance criteria

Non-instrumental procedures: *Spiked sample solution 1* provides a signal or intensity equivalent to or greater than that of the *Standard Solution*. *Spiked sample solution 2* must provide a signal or intensity less than that of the *Spiked sample solution 1*. [NOTE—The signal from each *Spiked sample solution* is NLT the *Unspiked sample solution* determination.]

Instrumental procedures: The average value of the three replicate measurements of *Spiked sample solution 1* is within ($\pm 15\%$) of the average value obtained for the replicate measurements of the *Standard solution*. The average value of the replicate measurements of *Spiked sample solution 2* must provide a signal intensity or value less than that

of the *Standard solution*. [NOTE—Correct the values obtained for each of the spiked solutions using the *Unspiked sample solution*.]

Precision for Instrumental Methods (Repeatability)

[NOTE—Non-instrumental precision is demonstrated by meeting the *Detectability* requirement above.]

Sample solutions: Six independent samples of the material under test, spiked with appropriate reference materials for the *Target Elements* at the *Target Concentration*.

Acceptance criteria

Relative standard deviation: NMT 20% for each *Target Element*.

Specificity

The procedure must be able to unequivocally assess (see *Validation of Compendial Procedures* (1225)) each *Target Element* in the presence of components that may be expected to be present, including other *Target Elements*, and matrix components.

QUANTITATIVE PROCEDURES

The following section defines the validation parameters for the acceptability of alternative quantitative procedures. Meeting these requirements must be demonstrated experimentally, using an appropriate system suitability procedure and reference materials. Meeting these requirements demonstrates that the procedure is equivalent to the compendial procedure for the purpose of quantifying the *Target Elements*.

Accuracy

Standard solutions: Prepare solutions containing the *Target Elements* at concentrations ranging from 50% to 150% of *J*, using appropriate reference materials.

Test samples: Prepare samples of the material under test spiked with appropriate reference materials before any sample preparation steps (digestion or solubilization) at concentrations ranging from 50% to 150% of *J* for each *Target Element*.

Acceptance criteria

Spike recovery: 70%–150% for the mean of three replicate preparations at each concentration

Precision

REPEATABILITY

Test samples: Six independent samples of material under test (taken from the same lot) spiked with appropriate reference materials for the *Target Element(s)* at the indicated level.

Acceptance criteria

Relative standard deviation: NMT 20% for each *Target Element*

RUGGEDNESS

Perform the *Repeatability* analysis over three independent events using the following events or combinations thereof:

1. on different days, or
2. with different instrumentation, or
3. with different analysts.

Acceptance criteria

Relative standard deviation: NMT 25% for each *Target Element*

Specificity

The procedure must be able to unequivocally assess (see *Validation of Compendial Procedures* (1225)) each *Target Element* in the presence of components that may be expected to be present, including other *Target Elements*, and matrix components.

Limit of Quantitation, Range, and Linearity

Demonstrated by meeting the *Accuracy* requirement.

<241> IRON

This limit test is provided to demonstrate that the content of iron, in either the ferric or the ferrous form, does not exceed the limit for iron specified in the individual monograph. The determination is made by concomitant visual comparison with a control prepared from a standard iron solution.

Special Reagents—

STANDARD IRON SOLUTION—Dissolve 863.4 mg of ferric ammonium sulfate [$\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$] in water, add 10 mL of 2 N sulfuric acid, and dilute with water to 100.0 mL. Pipet 10 mL of this solution into a 1000-mL volumetric flask, add 10 mL of 2 N sulfuric acid, dilute with water to volume, and mix. This solution contains the equivalent of 0.01 mg (10 μg) of iron per mL.

AMMONIUM THIOCYANATE SOLUTION—Dissolve 30 g of ammonium thiocyanate in water to make 100 mL.

Standard Preparation—Into a 50-mL color-comparison tube pipet 1 mL of *Standard Iron Solution* (10 μg of Fe), dilute with water to 45 mL, add 2 mL of hydrochloric acid, and mix.

Test Preparation—Into a 50-mL color comparison tube place the solution prepared for the test as directed in the individual monograph and if necessary dilute with water to 45 mL; or, dissolve in water, and dilute with water to 45 mL the quantity, in g, of the substance to be tested, as calculated by the formula:

$$1.0/(1000L)$$

in which L is the *Iron* limit in percentage. Add 2 mL of hydrochloric acid, and mix.

Procedure—To each of the tubes containing the *Standard Preparation* and the *Test Preparation* add 50 mg of ammonium peroxydisulfate crystals and 3 mL of *Ammonium Thiocyanate Solution*, and mix: the color of the solution from

the *Test Preparation* is not darker than that of the solution from the *Standard Preparation*.

<251> LEAD

The imposition of stringent limits on the amounts of lead that may be present in pharmaceutical products has resulted in the use of two methods, of which the one set forth following depends upon extraction of lead by solutions of dithizone. For determination of the content of heavy metals generally, expressed as a lead equivalent, see *Heavy Metals* (231).

Select all reagents for this test to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Rinse thoroughly all glassware with warm dilute nitric acid (1 in 2), followed by water.

Special Reagents—

AMMONIA-CYANIDE SOLUTION—Dissolve 2 g of potassium cyanide in 15 mL of ammonium hydroxide, and dilute with water to 100 mL.

AMMONIUM CITRATE SOLUTION—Dissolve 40 g of citric acid in 90 mL of water. Add 2 or 3 drops of phenol red TS, then cautiously add ammonium hydroxide until the solution acquires a reddish color. Remove any lead that may be present by extracting the solution with 20-mL portions of *Dithizone Extraction Solution* (see below), until the dithizone solution retains its orange-green color.

DILUTED STANDARD LEAD SOLUTION—Dilute an accurately measured volume of *Standard Lead Solution* (see *Heavy Metals* (231)) [containing 10 μg of lead per mL], with 9 volumes of dilute nitric acid (1 in 100) to obtain a solution that contains 1 μg of lead per mL.

DITHIZONE EXTRACTION SOLUTION—Dissolve 30 mg of dithizone in 1000 mL of chloroform, and add 5 mL of alcohol. Store the solution in a refrigerator.

Before use, shake a suitable volume of the dithizone extraction solution with about half its volume of dilute nitric acid (1 in 100), discarding the nitric acid.

HYDROXYLAMINE HYDROCHLORIDE SOLUTION—Dissolve 20 g of hydroxylamine hydrochloride in sufficient water to make approximately 65 mL. Transfer to a separator, add 5 drops of thymol blue TS, then add ammonium hydroxide until the solution assumes a yellow color. Add 10 mL of sodium diethyldithiocarbamate solution (1 in 25), mix, and allow to stand for 5 minutes. Extract this solution with successive 10- to 15-mL portions of chloroform until a 5-mL portion of the chloroform extract does not assume a yellow color when shaken with cupric sulfate TS. Add 3 N hydrochloric acid until the solution is pink (if necessary, add 1 or 2 drops more of thymol blue TS), and then dilute with water to 100 mL.

POTASSIUM CYANIDE SOLUTION—Dissolve 50 g of potassium cyanide in sufficient water to make 100 mL. Remove the lead from this solution by extraction with successive portions of *Dithizone Extraction Solution*, as described under *Ammonium Citrate Solution* above, then extract any dithizone remaining in the cyanide solution by shaking with chloroform. Finally dilute the cyanide solution with sufficient water so that each 100 mL contains 10 g of potassium cyanide.

STANDARD DITHIZONE SOLUTION—Dissolve 10 mg of dithizone in 1000 mL of chloroform. Keep the solution in a glass-stoppered, lead-free bottle, suitably wrapped to protect it from light, and store in a refrigerator.

Test Preparation—[NOTE—If, in the following preparation, the substance under test reacts too rapidly and begins charring with 5 mL of sulfuric acid before heating, use in-

stead 10 mL of cooled dilute sulfuric acid (1 in 2), and add a few drops of the hydrogen peroxide before heating.] Where the monograph does not specify preparation of a solution, prepare a *Test Preparation* as follows. [Caution—Exercise safety precautions in this procedure, as some substances may react with explosive violence when digested with hydrogen peroxide.] Transfer 1.0 g of the substance under test to a suitable flask, add 5 mL of sulfuric acid and a few glass beads, and digest on a hot plate in a hood until charring begins. Other suitable means of heating may be substituted. (Add additional sulfuric acid, if necessary, to wet the substance completely, but do not add more than a total of 10 mL.) Add, dropwise and with caution, 30 percent hydrogen peroxide, allowing the reaction to subside and again heating between drops. Add the first few drops very slowly, mix carefully to prevent a rapid reaction, and discontinue heating if foaming becomes excessive. Swirl the solution in the flask to prevent unreacted substance from caking on the walls of the flask. [NOTE—Add peroxide whenever the mixture turns brown or darkens.] Continue the digestion until the substance is completely destroyed, copious fumes of sulfur trioxide are evolved, and the solution is colorless. Cool, cautiously add 10 mL of water, evaporate until sulfur trioxide again is evolved, and cool. Repeat this procedure with another 10 mL of water to remove any traces of hydrogen peroxide. Cautiously dilute with 10 mL of water, and cool.

Procedure—Transfer the *Test Preparation*, rinsing with 10 mL of water, or the volume of the prepared sample specified in the monograph to a separator, and, unless otherwise directed in the monograph, add 6 mL of *Ammonium Citrate Solution* and 2 mL of *Hydroxylamine Hydrochloride Solution*. (For the determination of lead in iron salts use 10 mL of *Ammonium Citrate Solution*.) Add 2 drops of phenol red TS, and make the solution just alkaline (red in color) by the addition of ammonium hydroxide. Cool the solution if necessary, and add 2 mL of *Potassium Cyanide Solution*. Immediately extract the solution with 5-mL portions of *Dithizone Extraction Solution*, draining off each extract into another separator, until the dithizone solution retains its green color. Shake the combined dithizone solutions for 30 seconds with 20 mL of dilute nitric acid (1 in 100), and discard the chloroform layer. Add to the acid solution 5.0 mL of *Standard Dithizone Solution* and 4 mL of *Ammonia-Cyanide Solution*, and shake for 30 seconds: the color of the chloroform layer is of no deeper shade of violet than that of a control made with a volume of *Diluted Standard Lead Solution* equivalent to the amount of lead permitted in the sample under examination, and the same quantities of the same reagents and in the same manner as in the test with the sample.

(261) MERCURY

Method I

NOTE—Mercuric dithizonate is light-sensitive. Perform this test in subdued light.

Reagents—

DITHIZONE STOCK SOLUTION—Dissolve 40 mg of dithizone in 1000 mL of chloroform.

DITHIZONE TITRANT—Dilute 30.0 mL of *Dithizone Stock Solution* with chloroform to 100.0 mL. This solution contains approximately 12 mg of dithizone per L.

MERCURY STOCK SOLUTION—Transfer 135.4 mg of mercuric chloride to a 100-mL volumetric flask, and dilute with 1 N

sulfuric acid to volume. This solution contains the equivalent of 100 mg of Hg in 100 mL.

MERCURY SOLUTION FOR STANDARDIZING DITHIZONE TITRANT—Transfer 2.0 mL of *Mercury Stock Solution* to a 100-mL volumetric flask, and dilute with 1 N sulfuric acid to volume. Each mL of this solution contains the equivalent of 20 µg of Hg.

The following solutions are called for in the limit test for mercury that is specified in the monographs on Ferrous Fumarate, Ferrous Sulfate, and Dried Ferrous Sulfate.

HYDROXYLAMINE HYDROCHLORIDE SOLUTION—Prepare as directed in the test for *Lead* (251).

STANDARD MERCURY SOLUTION—On the day of use, quantitatively dilute 1.0 mL of *Mercury Stock Solution* with 1 N sulfuric acid to 1000 mL. Each mL of the resulting solution contains the equivalent of 1 µg of mercury.

DITHIZONE EXTRACTION SOLUTION—Prepare as directed in the test for *Lead* (251).

DILUTED DITHIZONE EXTRACTION SOLUTION—Just prior to use, dilute 5 mL of *Dithizone Extraction Solution* with 25 mL of chloroform.

Standardization of Dithizone Titrant—Transfer 1.0 mL of *Mercury Solution for Standardizing Dithizone Titrant* to a 250-mL separator, and add 100 mL of 1 N sulfuric acid, 90 mL of water, 1 mL of glacial acetic acid, and 10 mL of hydroxylamine hydrochloride solution (1 in 5). Titrate the solution with *Dithizone Titrant* from a 10-mL microburet, shaking the mixture 20 times after each addition and allowing the chloroform layer to separate, then discarding the chloroform layer. Continue until a final addition of *Dithizone Titrant* is green in color after shaking. Calculate the quantity, in µg, of Hg equivalent to each mL of *Dithizone Titrant* by the formula:

$$20/V$$

in which V is the volume, in mL, of *Dithizone Titrant* added.

Test Preparation—Transfer about 2 g of the substance under test, accurately weighed, to a glass-stoppered, 250-mL conical flask, add 20 mL of a mixture of equal volumes of nitric acid and sulfuric acid, attach a suitable condenser, reflux the mixture for 1 hour, cool, cautiously dilute with water, and boil until fumes of nitrous acid no longer are noticeable. Cool the solution, cautiously dilute with water, transfer to a 200-mL volumetric flask, dilute with water to volume, mix, and filter.

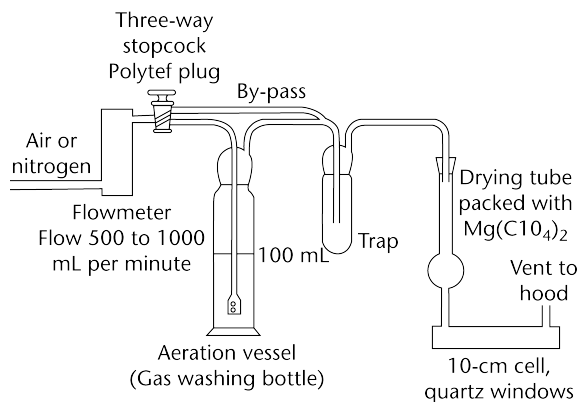
Procedure—Transfer 50.0 mL of *Test Preparation* to a 250-mL separator, and extract with successive small portions of chloroform until the last chloroform extract remains colorless. Discard the chloroform extract, and add to the extracted *Test Preparation* 50 mL of 1 N sulfuric acid, 90 mL of water, 1 mL of glacial acetic acid, and 10 mL of hydroxylamine hydrochloride solution (1 in 5). Proceed as directed under *Standardization of Dithizone Titrant*, beginning with "Titrate the solution." Calculate the amount of mercury.

Method IIa and Method IIb

Mercury Detection Instrument—Use any suitable atomic absorption spectrophotometer equipped with a fast-response recorder and capable of measuring the radiation absorbed by mercury vapors at the mercury resonance line of 253.6 nm. [NOTE—Wash all glassware associated with the test with nitric acid, and rinse thoroughly with water before use.]

Aeration Apparatus—The apparatus (see *accompanying diagram*) consists of a flowmeter capable of measuring flow rates from 500 to 1000 mL per minute, connected via a three-way stopcock fitted with a polytef plug to an aeration vessel (250-mL gas washing bottle), followed by a trap, a drying tube packed with magnesium perchlorate, a 10-cm ×

25-mm flow-through cell with quartz windows, and terminating with a vent to a fume hood.



Connections are glass or polyvinyl chloride

Mercury Aeration Apparatus

Reagents—

Potassium Permanganate Solution—Dissolve 5 g of potassium permanganate in 100 mL of water.

Hydroxylamine Hydrochloride Solution—Dissolve 10 g of hydroxylamine hydrochloride in 100 mL of water.

Stannous Chloride Solution—Dissolve 10 g of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in 20 mL of warm hydrochloric acid, and add 80 mL of water. Prepare fresh each week.

Standard Mercury Solution—Prepare from *Mercury Stock Solution* as directed under *Method I*. Each mL of the *Standard Mercury Solution* contains the equivalent of 1 μg of mercury.

Test Preparation—Unless otherwise directed in the individual monograph, use the quantity, in g, of the test substance calculated by the formula:

$$2.0/L$$

in which L is the mercury limit, in ppm.

Method IIa

Standard Preparation—Pipet 2.0 mL of *Standard Mercury Solution* into a 100-mL beaker, and add 35 mL of water, 3 mL of sulfuric acid, and 1 mL of potassium permanganate solution. Cover the beaker with a watch glass, boil for a few seconds, and cool.

Test Preparation—Transfer the calculated amount of the test substance to a 100-mL beaker, and add 35 mL of water. Stir, and warm to assist solution, if necessary. Add 2 drops of phenolphthalein TS, and, as necessary, slowly neutralize with constant stirring, using 1 N sodium hydroxide or 1 N sulfuric acid. Add 3 mL of sulfuric acid and 1 mL of *Potassium Permanganate Solution*. Cover the beaker with a watch glass, boil for a few seconds, and cool.

Procedure—Assemble the *Aeration Apparatus* as shown in the accompanying diagram, with the aeration vessel and the trap empty, and the stopcock in the bypass position. Connect the apparatus to the absorption cell, and adjust the air or nitrogen flow rate so that, in the following procedure, maximum absorption and reproducibility are obtained without excessive foaming in the test solution. Obtain a smooth baseline reading at 253.6 nm, following the manufacturer's instructions for operating the instrument.

Treat the *Standard Preparation* and the *Test Preparation* similarly, as follows. Destroy the excess permanganate by

adding *Hydroxylamine Hydrochloride Solution*, dropwise, until the solution is colorless. Immediately wash the solution into the aeration vessel with water, and dilute with water to 100 mL. Add 2 mL of *Stannous Chloride Solution*, and immediately reconnect the aeration vessel to the aeration apparatus. Turn the stopcock from the bypass position to the aerating position, and continue the aeration until the absorption peak has been passed and the recorder pen returns to the baseline. Disconnect the aeration vessel from the apparatus, and wash with water after each use. After correcting for any reagent blank, any absorbance produced by the *Test Preparation* does not exceed that produced by the *Standard Preparation*.

Method IIb

Caution—Some substances may react with explosive violence when digested with hydrogen peroxide. Exercise safety precautions at all times.

Standard Preparation—Pipet 2.0 mL of *Standard Mercury Solution* into a 125-mL conical flask, add 3 mL each of nitric acid and sulfuric acid, mix, and add an amount of 30 percent hydrogen peroxide equal to the total amount used in preparing the *Test Preparation*. Attach a suitable water-cooled condenser with a standard-taper joint to fit the flask, and reflux the mixture in a fume hood for 1 hour. Turn off the water circulating through the condenser, and heat until white fumes appear in the flask. Cool, and cautiously add 10 mL of water through the condenser, while swirling the flask. Again heat until white fumes appear, cool, and add an additional 15 mL of water. Remove the condenser, and rinse the sides of the flask to obtain a volume of 35 mL. Add 1 mL of *Potassium Permanganate Solution*, boil for a few seconds, and cool.

Test Preparation—Transfer the calculated amount of the test substance to a 125-mL conical flask. Add 5 mL each of nitric acid and sulfuric acid and a few glass beads. Attach a suitable water-cooled condenser with a standard-taper joint to fit the flask, and digest in a fume hood, preferably on a hot plate, and at a temperature not exceeding 120°, until charring begins. (If additional sulfuric acid is necessary to wet the specimen completely, add it carefully through the condenser, but do not allow the total volume added to exceed 10 mL.) After the test substance has been decomposed by the acid, cautiously add, dropwise through the condenser, 30 percent hydrogen peroxide, allowing the reaction to subside and again heating between drops (add the first few drops very slowly with sufficient mixing, in order to prevent a rapid reaction; discontinue heating if foaming becomes excessive). When the reaction has abated, heat cautiously, rotating the flask occasionally to prevent the specimen from caking on glass exposed to the heating unit. Maintain oxidizing conditions at all times during the digestion by adding small quantities of the hydrogen peroxide solution whenever the mixture turns brown or darkens. Continue the digestion until the organic matter is destroyed, and then reflux the mixture for 1 hour. Turn off the water circulating through the condenser, and heat until fumes of sulfur trioxide are copiously evolved and the solution becomes colorless or retains only a light straw color. Cool, and cautiously add 10 mL of water through the condenser, while swirling the flask. Again heat until white fumes appear. Cool, and cautiously add 15 mL of water. Remove the condenser, and rinse the sides of the flask with a few mL of water to obtain a volume of 35 mL. Add 1 mL of *Potassium Permanganate Solution*, boil for a few seconds, and cool.

Procedure—Proceed as directed for *Procedure* under *Method IIa*.

(267) POROSIMETRY BY MERCURY INTRUSION

In general, different types of pores may be pictured as apertures, channels, or cavities within a solid body or as space (i.e., interstices or voids) between solid particles in a bed, compact, or aggregate. *Porosity* is a term that is often used to indicate the porous nature of solid material and is more precisely defined as the ratio of the volume of accessible pores and voids to the total volume occupied by a given amount of the solid. In addition to the accessible pores, a solid may contain closed pores, which are isolated from the external surface and into which fluids are not able to penetrate. The characterization of closed pores, i.e., cavities with no access to an external surface, is not covered in this general chapter.

Porous materials may take the form of fine or coarse powders, compacts, extrudates, sheets, or monoliths. Their characterization usually involves the determination of the total pore volume or porosity as well as the pore size distribution.

It is well established that the performance of a porous solid (e.g., its strength, reactivity, permeability, or adsorbent power) is dependent on its pore structure. Many different methods have been developed for the characterization of pore structure. In view of the complexity of most porous solids, it is not surprising to find that the results obtained are not always in agreement and that no single technique can be relied upon to provide a complete picture of the pore structure. The choice of the most appropriate method depends on the application of the porous solid, its chemical and physical nature, and the range of pore size.

This chapter provides guidance for measurement of porosity and pore size distribution by mercury porosimetry. It is a comparative test, usually destructive, in which the volume of mercury penetrating a pore or void is determined as a function of an applied hydrostatic pressure that can be related to a pore diameter. Other information such as pore shape and interconnectivity, the internal and external surface area, powder granulometry, and bulk and tapped density can also be inferred from volume-pressure curves; however, these aspects of the technique do not fall under the scope of this chapter.

Practical considerations presently limit the maximum applied absolute pressure reached by some equipment to about 400 MPa, corresponding to a minimum equivalent pore diameter of approximately 0.003 μm . The maximum diameter will be limited for samples having a significant depth because of the difference in the hydrostatic head of mercury from the top to the bottom of the sample. For most purposes this limit may be regarded as 400 μm .

Interparticle and intraparticle porosity can be determined, but the method does not distinguish between these porosities where they coexist.

The method is suitable for the study of most porous materials. Samples that amalgamate with mercury, such as certain metals, may be unsuitable for this technique or may require a preliminary passivation. Other materials may deform or compact under the applied pressure. In some cases, it may be possible to apply sample compressibility corrections, and useful comparative data may still be obtained.

The mercury porosimetry technique should be considered to be comparative, because for most porous media, a theory is not available to allow an absolute calculation of results of pore size distribution. Therefore, this technique is mainly recommended for development studies.

Mercury is toxic. Appropriate precautions must be observed to safeguard the health of the operator and others working in the area. Waste material must also be disposed of in a suitable manner, according to local regulations.

PRINCIPLE

The technique is based on the measurement of the mercury volume intruded into a porous solid as a function of the applied pressure. The measurement includes only those pores into which mercury can penetrate at the pressure applied.

A nonwetting liquid penetrates into a porous system only under pressure. The pressure applied is in inverse proportion to the inner width of the pore aperture. In the case of cylindrical pores, the correlation between pore diameter and pressure is given by the Washburn equation:

$$d_p = -\frac{4 \cdot \sigma}{p} \cos \theta \quad (1)$$

- p = applied pressure, in pascals
- d_p = pore diameter, in meters
- σ = surface tension of mercury, in newtons per meter
- θ = contact angle of mercury on the sample, in degrees

APPARATUS

The sample holder, referred to as a penetrometer or dilatometer, has a calibrated capillary tube, through which the sample can be evacuated and through which mercury can enter. The capillary tube is attached to a wider tube in which the test sample is placed. The change in the volume of mercury intruded is usually measured by the change in capacitance between the mercury column in the capillary tube and a metal sleeve around the outside of the capillary tube. If precise measurements are required, the internal volume of the capillary tube should be between 20% and 90% of the expected pore and void volume of the sample. Because different materials exhibit a wide range of open porosities, a number of penetrometers with different diameter capillary tubes and sample volumes may be required. A typical setup for a mercury porosimeter instrument is given in *Figure 1*. The porosimeter may have separate ports for high- and low-pressure operation, or the low-pressure measurement may be carried out on a separate unit.

The pressure range is typically 4 kPa to 300 kPa for low-pressure operation and above 300 kPa for high-pressure operation, depending on the design of the particular apparatus and on the intended use.

METHOD

Sample Preparation—The sample is pretreated to remove adsorbed material that can obscure its accessible porosity by either heating and/or evacuation or by flowing inert gas. It may be possible to passivate the surface of wettable or amalgam-forming solids by producing a thin layer of oxide, or by coating with stearate. The sample of the pretreated solid is weighed and transferred to the penetrometer. The pore system of the sample is then degassed in a vacuum to a maximum residual pressure of 7 Pa.

Filling the Penetrometer with Mercury—Use mercury of analytical quality. The sample is overlaid with mercury under vacuum. The vacuum is required to ensure the transfer of mercury from the reservoir to the penetrometer. In a filled penetrometer, the filling pressure is comprised of the applied pressure plus the pressure contribution created by the head of mercury contacting the sample. A typical filling pressure would be about 4 kPa. The hydrostatic pressure of the mercury over the sample can be minimized by filling the penetrometer in the horizontal positions.

Low-Pressure Measurement—Admit air or nitrogen in a controlled manner to increase the pressure either in stages corresponding to the particular pore sizes of interest, or

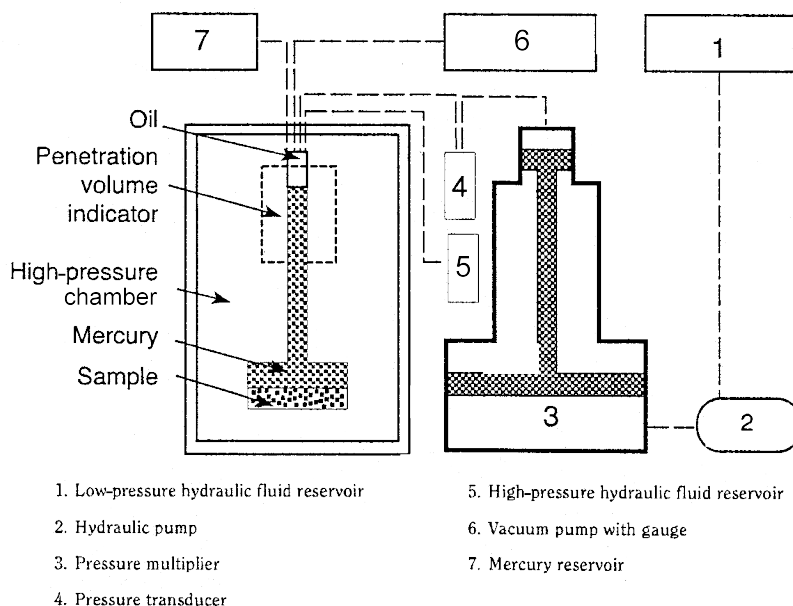


Figure 1. Example of the setup of a mercury porosimeter instrument.

continuously at a slow rate. The concomitant change in the length of the mercury column in the capillary tube is recorded. When the maximum required pressure has been reached, reduce the pressure to ambient.

High-Pressure Measurement—After measurement at low-pressure conditions, the penetrometer filled with mercury is transferred to the high-pressure port or unit of the instrument and overlaid with hydraulic fluid. Mercury is intruded into the pore system via the hydraulic fluid. Increase the pressure in the system to the maximum pressure reached in the low-pressure measurement, and record the intrusion volume at this pressure, because subsequent intrusion volumes are calculated from this initial volume. Increase the pressure either in stages corresponding to the particular pore sizes of interest, or continuously at a slow rate. The fall in the mercury column is measured up to the maximum required pressure. If required, the pressure may be decreased either in stages or continuously at a slow rate to determine the mercury extrusion curve. Make corrections to take account of changes in the volume of the mercury, the penetrometer, and other components of the volume detector system under elevated pressure. The extent of the corrections may be determined by means of blank measurements under the same conditions. An experimentally determined volume–pressure curve is shown in Figure 2.

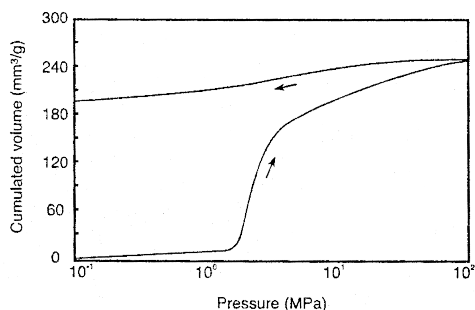


Figure 2. Volume–pressure curve as semilogarithmic plot.

REPORTING OF RESULTS

The pressure readings are converted to pore diameter by means of the Washburn equation or by another model.

The surface tension of mercury, σ , depends not only on the temperature and the material, but also—in the case of markedly curved surface areas—on the radius of curvature. In general, values between $0.41 \text{ N} \cdot \text{m}^{-1}$ and $0.52 \text{ N} \cdot \text{m}^{-1}$ are measured at room temperature. If the value is not known, $\sigma = 0.48 \text{ N} \cdot \text{m}^{-1}$ can be used.

The contact angle of mercury θ in most cases is more than 90° . It may be determined using a contact angle instrument. If the θ value is not known, 130° can be used. Report the values of contact angle, surface tension, and the model used in the calculation. Visualization of the data can be done with several types of graphs. Frequently, in a graphical representation, the pore diameter is plotted on the abscissa and the dependent intruded specific volume on the ordinate to give the pore size distribution. It is appropriate here to choose a logarithmic scale for the abscissa (see Figure 3). The spaces between the particles of the solid sample are included as pores in the calculation. If the pores differ in size from the voids, the latter can be separated by choosing the relevant pore size range.

Extrusion curves may not be used for calculating the pore size distribution (for hysteresis, see Figure 2), because an intruded part of the mercury always remains in the pore system. The retention ratio may be useful for the qualitative characterization of pores that are only accessible via narrow openings (“ink-bottle pores”).

The most common characteristic values, such as the total intruded specific volume, the mean, and the median pore diameter are calculated from the pore size distribution. Moreover, sufficient information should be documented about the sample, the sample preparation, the evacuation conditions, and the instrument used.

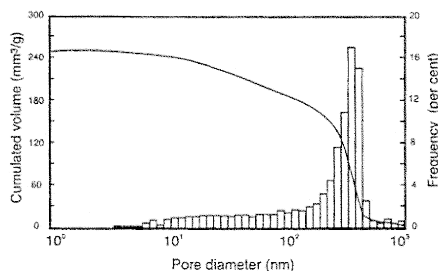


Figure 3. Pore volume distribution as semilogarithmic plot.

CONTROL OF THE INSTRUMENT'S PERFORMANCE

As the mercury porosimetry technique is considered as a comparative test, no details are given in this chapter. However, it is recommended that a stable comparison material should be tested on a regular basis to monitor instrument calibration and performance.

(271) READILY CARBONIZABLE SUBSTANCES TEST

In tests for readily carbonizable substances, unless otherwise directed, add the specified quantity of the substance, finely powdered if in solid form, in small portions to the comparison container, which is made of colorless glass resistant to the action of sulfuric acid and contains the specified volume of sulfuric acid (see under *Reagent Specifications* in the section *Reagents, Indicators, and Solutions*).

Stir the mixture with a glass rod until solution is complete, allow the solution to stand for 15 minutes, unless otherwise directed, and compare the color of the solution with that of the specified Matching Fluid (see *Color and Achromicity* (631)) in a comparison container, which also is of colorless glass and has the same internal and cross-section dimensions, viewing the fluids transversely against a background of white porcelain or white glass.

When heat is directed in order to effect solution of the substance in the sulfuric acid, mix the sample and the acid in a test tube, heat as directed, and transfer the solution to the comparison container for matching with the designated Matching Fluid (see *Color and Achromicity* (631)).

(281) RESIDUE ON IGNITION

Portions of this general chapter have been harmonized with the corresponding texts of the *European Pharmacopoeia* and the *Japanese Pharmacopoeia*. The portions that are not harmonized are marked with symbols (♦). The harmonized texts of these pharmacopoeias are therefore interchangeable, and the methods of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia* may be used for demonstration of compliance instead of the present *United States Pharmacopoeia* general chapter. These pharmacopoeias have undertaken

not to make any unilateral change to this harmonized chapter.

The *Residue on Ignition/Sulfated Ash* test uses a procedure to measure the amount of residual substance not volatilized from a sample when the sample is ignited in the presence of sulfuric acid according to the procedure described below. This test is usually used for determining the content of inorganic impurities in an organic substance.

Procedure—Ignite a suitable crucible (for example, silica, platinum, quartz, or porcelain) at $600 \pm 50^\circ$ for 30 minutes, cool the crucible in a desiccator (silica gel or other suitable desiccant), and weigh it accurately. Weigh accurately ♦1 to 2 g of the substance, or ♦ the amount specified in the individual monograph, in the crucible.

Moisten the sample with a small amount (usually 1 mL) of sulfuric acid, then heat gently at a temperature as low as practicable until the sample is thoroughly charred. Cool; then, ♦ unless otherwise directed in the individual monograph, ♦ moisten the residue with a small amount (usually 1 mL) of sulfuric acid; heat gently until white fumes are no longer evolved; and ignite at $600 \pm 50^\circ$, ♦ unless another temperature is specified in the individual monograph, ♦ until the residue is completely incinerated. Ensure that flames are not produced at any time during the procedure. Cool the crucible in a desiccator (silica gel or other suitable desiccant), weigh accurately, and calculate the percentage of residue.

Unless otherwise specified, if the amount of the residue so obtained exceeds the limit specified in the individual monograph, repeat the moistening with sulfuric acid, heating and igniting as before, using a 30-minute ignition period, until two consecutive weighings of the residue do not differ by more than 0.5 mg or until the percentage of residue complies with the limit in the individual monograph.

♦ Conduct the ignition in a well-ventilated hood, but protected from air currents, and at as low a temperature as is possible to effect the complete combustion of the carbon. A muffle furnace may be used, if desired, and its use is recommended for the final ignition at $600 \pm 50^\circ$.

Calibration of the muffle furnace may be carried out using an appropriate digital temperature meter and a working thermocouple probe calibrated against a standard thermocouple traceable to the National Institute of Standards and Technology.

Verify the accuracy of the measuring and controlling circuitry of the muffle furnace by checking the positions in the furnace at the control set point temperature of intended use. Select positions that reflect the eventual method of use with respect to location of the specimen under test. The tolerance is $\pm 25^\circ$ at each position measured. ♦

(291) SELENIUM

Stock Solution—Dissolve 40.0 mg of metallic selenium in 100 mL of dilute nitric acid (1 in 2) in a 1000-mL volumetric flask, warming gently on a steam bath if necessary to effect solution, add water to volume, and mix. Pipet 5 mL of this solution into a 200-mL volumetric flask, add water to volume, and mix. Each mL of the resulting solution contains the equivalent of 1 μ g of selenium (Se).

Diaminonaphthalene Solution—Dissolve 100 mg of 2,3-diaminonaphthalene and 500 mg of hydroxylamine hydrochloride in 0.1 N hydrochloric acid to make 100 mL. Prepare this solution fresh on the day of use.

Standard Solution—Pipet 6 mL of *Stock Solution* into a 150-mL beaker, and add 25 mL of dilute nitric acid (1 in 30) and 25 mL of water.

Test Solution—Clean combustion of the test material is an important factor in conducting the test. For compounds that burn poorly and produce soot, the addition of magnesium oxide usually results in more thorough combustion and reduces soot formation. Where the need to add magnesium oxide has been identified, it is specified in the individual monograph. Using a 1000-mL combustion flask and using 25 mL of dilute nitric acid (1 in 30) as the absorbing liquid, proceed as directed under *Oxygen Flask Combustion* (471), using a test specimen weighing 100 to 200 mg, unless directed otherwise in the individual monograph. Upon completion of the combustion, place a few mL of water in the cup, loosen the stopper, and rinse the stopper, the specimen holder, and the sides of the flask with about 10 mL of water. Transfer the solution with the aid of about 20 mL of water to a 150-mL beaker, and heat gently to the boiling temperature. Boil for 10 minutes, and allow the solution to cool to room temperature.

Procedure—Treat the *Standard Solution*, the *Test Solution*, and the reagent blank consisting of 25 mL of dilute nitric acid (1 in 30) and 25 mL of water, concomitantly and in parallel, as follows. Add ammonium hydroxide solution (1 in 2) to adjust to a pH of 2.0 ± 0.2 . Dilute with water to 60 mL, and transfer to a low-actinic separator with the aid of 10 mL of water, adding the 10 mL of rinsings to the separator. Add 200 mg of hydroxylamine hydrochloride, swirl to dissolve, immediately add 5.0 mL of *Diaminonaphthalene Solution*, insert the stopper, and swirl to mix. Allow the solution to stand at room temperature for 100 minutes. Add 5.0 mL of cyclohexane, shake vigorously for 2 minutes, and allow the layers to separate. Discard the aqueous layer, and centrifuge the cyclohexane extract to remove any dispersed water. Determine the absorbances of the cyclohexane extracts of the *Test Solution* and the *Standard Solution* in a 1-cm cell at the wavelength of maximum absorbance at about 380 nm, with a suitable spectrophotometer, using the cyclohexane extract of the reagent blank as the blank, and compare the absorbances: the absorbance of the *Test Solution* is not greater than that of the *Standard Solution* where a 200-mg test specimen has been taken, or is not greater than one-half that of the *Standard Solution* where a 100-mg test specimen has been taken.

OTHER TESTS AND ASSAYS

(301) ACID-NEUTRALIZING CAPACITY

NOTE—All tests shall be conducted at a temperature of $37 \pm 3^\circ$.

Standardization of pH Meter—Standardize a pH meter using the 0.05 M potassium biphthalate and 0.05 M potassium tetroxalate standardizing buffers as described under pH (791).

Magnetic Stirrer—Transfer 100 mL of water to a 250-mL beaker containing a 40- × 10-mm (or other suitable size) magnetic stirring bar that is coated with solid perfluorocarbon and has a spin ring at its center. Adjust the power setting of the magnetic stirrer to produce a stirring rate of 300 ± 30 rpm when the stirring bar is centered in the beaker, as determined by a suitable optical tachometer.

Test Preparation—

Powders—Transfer the accurately weighed portion of the substance specified in the individual monograph to a

250-mL beaker, add 70 mL of water, and mix on the *Magnetic Stirrer* for 1 minute.

Effervescent Solids—Transfer an accurately weighed quantity, equivalent to the minimum labeled dosage, to a 250-mL beaker, add 10 mL of water, and swirl the beaker gently while allowing the reaction to subside. Add another 10 mL of water, and swirl gently. Wash the walls of the beaker with 50 mL of water, and mix on the *Magnetic Stirrer* for 1 minute.

Suspensions and Other Liquids—Shake the container until the contents are uniform, and determine the density. Transfer an accurately weighed quantity of the uniform mixture, equivalent to the minimum labeled dosage, to a 250-mL beaker, add water to make a total volume of about 70 mL, and mix on the *Magnetic Stirrer* for 1 minute.

Lozenges—Accurately weigh not fewer than 20 lozenges, and determine the average weight. Select and weigh 2 lozenges, and transfer them to a 250-mL beaker containing 70 mL of water.

Nonchewable Tablets—Weigh not fewer than 20 tablets, and determine the average tablet weight. Grind the tablets to a fine powder, mix to obtain a uniform mixture, and transfer an accurately weighed quantity of it, equivalent to the minimum labeled dosage, to a 250-mL beaker. If wetting is desired, add not more than 5 mL of alcohol (neutralized to an apparent pH of 3.5), and mix to wet the specimen thoroughly. Add 70 mL of water, and mix on the *Magnetic Stirrer* for 1 minute.

Chewable Tablets—Prepare as directed for *Nonchewable Tablets*.

Tablets That Are Required To Be Chewed—Transfer 1 Tablet to a 250-mL beaker, add 50 mL of water, and mix on the *Magnetic Stirrer* for 1 minute.

Capsules—Weigh accurately not fewer than 20 capsules. Remove the capsule contents completely, with the aid of a cotton swab if necessary. Accurately weigh the empty capsules, and determine the average weight of the contents per capsule. Mix the combined capsule contents to obtain a uniform mixture, and proceed as directed for *Nonchewable Tablets*, beginning with "transfer an accurately weighed quantity of it."

Procedure for Powders, Effervescent Solids, Suspensions and Other Liquids, Lozenges, Nonchewable Tablets, Chewable Tablets, and Capsules—Pipet 30.0 mL of 1.0 N hydrochloric acid VS into the *Test Preparation* while continuing to stir with the *Magnetic Stirrer*. [NOTE—Where the acid-neutralizing capacity of the specimen under test is greater than 25 mEq, use 60.0 mL of 1.0 N hydrochloric acid VS, and make the appropriate modifications in the calculation.] Stir for 15 minutes, accurately timed, after the addition of the acid, begin to titrate immediately, and in a period not to exceed an additional 5 minutes, titrate the excess hydrochloric acid with 0.5 N sodium hydroxide VS to attain a stable (for 10 to 15 seconds) pH of 3.5. Calculate the number of mEq of acid consumed by the formula:

$$\text{Total mEq} = (30 \times N_{\text{HCl}}) - (V_{\text{NaOH}} \times N_{\text{NaOH}})$$

in which N_{HCl} and N_{NaOH} are the normalities of the hydrochloric acid VS and the sodium hydroxide VS, respectively; and V_{NaOH} is the volume of sodium hydroxide VS used for titration. Express the result in terms of mEq of acid consumed per g of the substance tested.

Procedure for Tablets That Are Required To Be Chewed—Pipet 30.0 mL of 1.0 N hydrochloric acid VS into the *Test Preparation* while continuing to stir with the *Magnetic Stirrer* for 10 minutes, accurately timed, after the addition of the acid. Discontinue stirring briefly, and without delay remove any gum base from the beaker using a long needle. Promptly rinse the needle with 20 mL of water, collecting the washing in the beaker, and resume stirring for 5 minutes, accurately timed, then begin to titrate immediately, and in a period not to exceed an additional 5 min-

utes, titrate the excess hydrochloric acid with 0.5 N sodium hydroxide VS to attain a stable (for 10 to 15 seconds) pH of 3.5. Calculate the number of mEq of acid consumed by the Tablet tested by the formula:

$$\text{Total mEq} = (30 \times N_{\text{HCl}}) - (V_{\text{NaOH}} \times N_{\text{NaOH}})$$

in which the terms are as defined above.

(311) ALGINATES ASSAY

APPARATUS

The required apparatus (see *Figure 1*) contains a capillary metering valve, A, followed by a flowmeter, B, to control and monitor the flow of nitrogen through the system. Halogenated vinyl plastic tubing* and a rubber fitting, C, are used to connect the flowmeter to a sidearm of a reaction flask, D. Flask D is a 250-mL round-bottom, boiling flask, resting in a suitable heating mantle, E. Flask D is provided with a 225-mm Hopkins coil reflux condenser, F. The condenser terminates in a U-shaped trap, G, which contains two 25-g bands of 20-mesh zinc, the bands being bounded and separated by three 3-inch plugs of glass wool. The trap terminates in an adapter, H, that by means of a halogenated vinyl plastic tubing and a twistcock connector, I, connects with a 250-mL gas washing bottle, J. The inlet (bubbling) tube extends almost to the bottom of the gas washing bottle, and it terminates in a fritted disk having a coarse porosity. The size of all glass joints is $24/40$, except for the $45/50$ joint of the gas washing bottle.

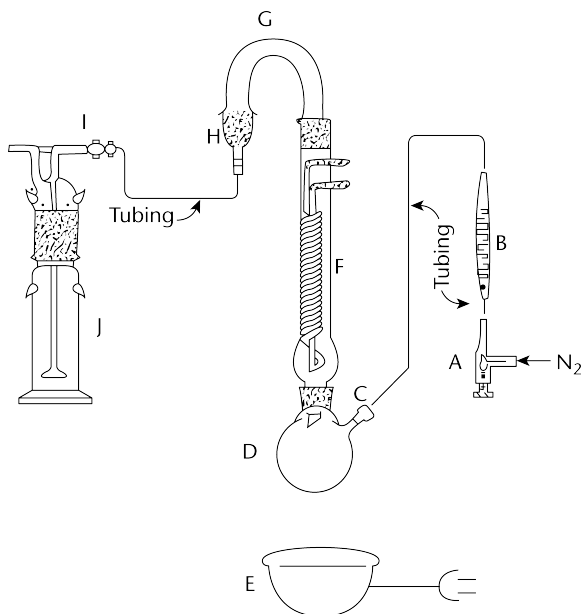


Fig. 1. Apparatus for Alginates Assay

*This type of tubing is commonly referred to as Tygon tubing. This note is added for clarity and it does not constitute USP's endorsement of this product.

SYSTEM SUITABILITY

Using D-glucuronolactone as the standard, proceed as directed for *Procedure*, but do not perform the preboiling steps. The system is suitable if the following criteria are met: (1) a blank determination results in a net titration value, C, between 0.02 and 0.06 mEq, calculated as follows:

$$A_b - B_b$$

in which A_b is the number of mEq of 0.25 N sodium hydroxide in the 25 mL used, and B_b is the number of mEq of 0.1 N hydrochloric acid used in the blank titration; and (2) the percentage of carbon dioxide, CO_2 , obtained from the standard is between 24.2% and 25.7%.

PROCEDURE

Unless otherwise directed in the individual monograph, transfer a specimen of about 250 mg, accurately weighed, into the reaction flask, D, add 50 mL of 0.1 N hydrochloric acid, insert several boiling chips, and connect the flask to the reflux condenser, F, using phosphoric acid as a lubricant. [NOTE—Stopcock grease may be used for the other connections.] Connect the nitrogen line to the sidearm of the flask, and adjust the flow of cooling water to about 2 L per minute.

[NOTE—The following preboiling steps, outlined in this paragraph, are optional and need only be performed when the presence of inorganic carbonates is suspected.] Maintain the flow of nitrogen through the apparatus at 90 to 100 mL per minute. Raise the heating mantle, E, to the flask, heat the specimen to boiling, and boil gently for 2 minutes. Turn the heat off, lower the mantle, E, and allow to cool for about 10 minutes.

Connect the empty gas washing bottle assembly, J, and sweep the system with nitrogen at a rate of 90 to 100 mL per minute for 5 minutes. Reduce the nitrogen flow to 60 to 65 mL per minute, add 10 drops of butyl alcohol, 25.0 mL of 0.25 N sodium hydroxide VS, and 50 mL of distilled water into the bottle, rinsing down the inside of the gas washing bottle, and replace the cap. Detach the rubber fitting, C, from sidearm, and add 46 mL of hydrochloric acid through the sidearm of the boiling flask. Reattach the nitrogen line, raise the heating mantle, and heat the reaction mixture to boiling. After 2 hours of boiling, increase the nitrogen flow to 90 to 100 mL per minute, discontinue the heating, and lower the mantle. Allow to cool for 10 minutes. Disconnect, and disassemble the gas washing bottle. Using a directed stream of distilled water, thoroughly rinse all parts of the bubbling tube and cap, collecting the washings in the gas washing bottle. Use nitrogen to gently force all water out of the bubbling tube. To the bottle immediately add 10 mL of 10% barium chloride solution and a stirring bar. Insert a tight stopper, and stir gently for 1 minute. Allow to stand for at least 5 minutes. Add three drops of phenolphthalein TS, and titrate with 0.1 N hydrochloric acid VS. Perform a blank determination (see *Residual Titrations* under *Titrimetry* (541)). Calculate the percentage of carbon dioxide, CO_2 , by the formula:

$$2200[(A - B) - C]/(1000W)(1 - D)$$

in which A is the number of mEq of 0.25 N sodium hydroxide in the 25 mL used; B is the number of mEq of 0.1 N hydrochloric acid used for the titration of the sample or the standard; C is the net titration value calculated in the blank determination; W is the weight, in g, of the sample or the standard taken; and D is the percentage expressed as a decimal (1 decimal place), obtained in the test for *Loss on drying* for the sample or for the standard.

(341) ANTIMICROBIAL AGENTS— CONTENT

An essential component of Injections preserved in multiple-dose containers is the agent or agents present to reduce the hazard of having introduced, in the course of removing some of the contents, accidental microbial contamination of the contents remaining. It is a Pharmacopeial requirement that the presence and amount added of such agent(s) be declared on the label of the container. The methods provided herein for the most commonly used agents are to be used to demonstrate that the declared agent is present but does not exceed the labeled amount by more than 20% of the labeled amount.

The concentration of an antimicrobial preservative added to a multiple-dose or single-dose parenteral, otic, nasal, and ophthalmic preparation may diminish during the shelf life of the product. Because it is recognized that the antimicrobial preservative concentration in a given preparation may decrease during the product's shelf life, the manufacturer shall determine the lowest level at which the preservative is effective, and the product should be so formulated as to assure that this level is exceeded throughout the product's shelf life. At the time of its manufacture, the product should contain the declared amount of antimicrobial preservative (within $\pm 20\%$ to allow for manufacturing and analytical variations). The quantitative label statement of the preservative content is not intended to mean that the labeled quantity is retained during the shelf life of the product; rather, it is a statement of the amount added, within process limits, and which is not exceeded by more than 20%. An example of such a label statement is "____(unit) added as preservative." [NOTE—"____(unit)" would be a number followed by the unit of measurement, e.g., 0.015 mg per mL or 0.1%.]

The most commonly used agents include the two mercurials, phenylmercuric nitrate and thimerosal and the four homologous esters of p-hydroxybenzoic acid, phenol, benzyl alcohol, and chlorobutanol. The methods for the first two named are polarographic, while quantitative gas chromatography is employed in the determination of the other agents.

GENERAL GAS CHROMATOGRAPHIC METHOD

The general procedures set forth in the following paragraphs are applicable to the quantitative determination of benzyl alcohol, chlorobutanol, phenol, and the methyl, ethyl, propyl, and butyl esters of p-hydroxybenzoic acid, the latter being treated as a group, the individual members of which, if present, are capable of separate determination. Prepare the *Internal Standard Solution* and the *Standard Preparation* for each agent as directed individually below. Unless otherwise directed below, prepare the *Test Preparation* from accurately measured portions of the *Internal Standard Solution* and the sample under test, of such size that the concentration of the agent and the composition of the solvent correspond closely to the concentration and composition of the *Standard Preparation*. Suggested operating parameters of

the gas chromatograph apparatus are given in the accompanying table, the carrier gas being helium or nitrogen, and the detector being the flame-ionization type.

Benzyl Alcohol

Internal Standard Solution—Dissolve about 380 mg of phenol in 10 mL of methanol contained in a 200-mL volumetric flask. Add water to volume, and mix.

Standard Preparation—Dissolve about 180 mg of USP Benzyl Alcohol RS, accurately weighed, in 20.0 mL of methanol contained in a 100-mL volumetric flask. Add *Internal Standard Solution* to volume, and mix.

Procedure—Separately inject equal volumes (about 5 μ L) of the *Standard Preparation* and the *Test Preparation* into the chromatograph, record the chromatograms with the apparatus adjusted to the parameters set forth in the accompanying table, and measure the areas under the peaks for benzyl alcohol and phenol. Calculate the content, in mg per mL, of benzyl alcohol (C_7H_8O) in the specimen taken by the formula:

$$100(C/V)(p_1/p_2)(P_2/P_1)$$

in which C is the concentration, in mg per mL, of benzyl alcohol in the *Standard Preparation*; V is the volume, in mL, of the specimen under test used in preparing each 100 mL of the *Test Preparation*; p_1 and p_2 are the peak areas for benzyl alcohol and phenol, respectively, obtained from the *Test Preparation*; and P_1 and P_2 are the peak areas of benzyl alcohol and phenol, respectively, obtained from the *Standard Preparation*.

Chlorobutanol

Internal Standard Solution—Transfer about 140 mg of benzaldehyde to a 100-mL volumetric flask, add 10 mL of methanol, and swirl to dissolve. Dilute with water to volume, and mix.

Standard Preparation—Transfer about 125 mg of USP Chlorobutanol RS, accurately weighed, to a 25-mL volumetric flask. Add 2 mL of methanol, swirl to dissolve, dilute with water to volume, and mix. Transfer 5.0 mL of this solution and 5.0 mL of *Internal Standard Solution* to a 25-mL flask, and mix to obtain a solution having a known concentration of about 2.5 mg of chlorobutanol per mL.

Test Preparation—Quantitatively dilute, if necessary, an accurately measured volume of the specimen under test with methanol to obtain a solution containing not more than about 5.0 mg of chlorobutanol per mL. Combine 3.0 mL of this solution with 3.0 mL of *Internal Standard Solution*, and mix.

Chromatographic System (see *Chromatography* (621))—[NOTE—See accompanying table for column dimensions, column packing phase and support, flow rate, and column temperature.] The injection port temperature is maintained at 180°, and the detector temperature is maintained at 220°. Chromatograph the *Standard Preparation*, and record the peak responses as directed for *Procedure*: the relative retention times are about 0.8 for benzaldehyde and 1.0 for chlorobutanol; the resolution, R , between benzaldehyde and

Suggested Operating Parameters of Gas Chromatograph Apparatus

Agent	Column Size		Column Packing Phases and Support	Flow Rate, mL per minute	Column Temperature
	Length	ID			
Benzyl Alcohol	1.8 m	3 mm	5% G16/S1A	50	140°
Chlorobutanol	1.8 m	2 mm	5% G16/S1A	20	110°
Phenol	1.2 m	3 mm	5% G16/S1A	50	145°
Parabens	1.8 m	2 mm	5% G2/S1A	20	150°

the chlorobutanol is not less than 2.0; and the relative standard deviation for replicate injections is not more than 2.0%.

Procedure—Separately inject equal volumes (about 1 μ L) of the *Standard Preparation* and the *Test Preparation* into the chromatograph, record the chromatograms, and measure the areas for the major peaks. Calculate the quantity, in mg, of chlorobutanol ($C_4H_7Cl_3O$) in each mL of the specimen under test by the formula:

$$C(L/D)(R_U/R_S)$$

in which C is the concentration, in mg per mL, of chlorobutanol, calculated on the anhydrous basis, in the *Standard Preparation*; L is the labeled quantity, in mg, of chlorobutanol in each mL of the specimen under test; D is the concentration, in mg per mL, of chlorobutanol in the *Test Preparation*, based on the volume of specimen under test taken and the extent of dilution; and R_U and R_S are the ratios of the chlorobutanol peak to the benzaldehyde peak obtained from the *Test Preparation* and the *Standard Preparation*, respectively.

Phenol

Internal Standard Solution—Pipet 1 mL of USP Benzyl Alcohol RS into a 500-mL volumetric flask, add methanol to volume, and mix.

Standard Preparation—Dissolve about 75 mg of USP Phenol RS, accurately weighed, in 7.5 mL of methanol contained in a 100-mL volumetric flask. Add 20.0 mL of *Internal Standard Solution*, then add water to volume, and mix.

Procedure—Separately inject equal volumes (about 3 μ L) of the *Standard Preparation* and the *Test Preparation* into the chromatograph, record the chromatograms with the apparatus adjusted to the parameters set forth in the accompanying table, and measure the areas under the peaks for phenol and benzyl alcohol. Calculate the content, in mg per mL, of phenol (C_6H_6O) in each mL of the specimen taken by the formula:

$$100(C/V)(p_1/p_2)(P_2/P_1)$$

in which C is the concentration, in mg per mL, of phenol in the *Standard Preparation*; V is the volume, in mL, of the specimen under test used in preparing each 100 mL of the *Test Preparation*; p_1 and p_2 are the peak areas for phenol and benzyl alcohol, respectively, obtained from the *Test Preparation*; and P_1 and P_2 are the peak areas of phenol and benzyl alcohol, respectively, obtained from the *Standard Preparation*.

Methylparaben and Propylparaben

Internal Standard Solution—Place about 200 mg of benzophenone in a 250-mL volumetric flask, dilute with ether to volume, and mix.

Standard Preparation—Place 100 mg of USP Methylparaben RS and 10 mg of USP Propylparaben RS, each accurately weighed, in a 200-mL volumetric flask, dilute with *Internal Standard Solution* to volume, and mix. Place 10 mL of this solution in a 25-mL conical flask, and proceed as directed for *Test Preparation*, beginning with "Add 3 mL of pyridine."

Test Preparation—Pipet 10 mL of the specimen under test and 10 mL of the *Internal Standard Solution* into a small separator. Shake vigorously, allow the layers to separate, draw off the aqueous layer into a second separator, and transfer the ether layer into a small flask through a funnel containing anhydrous sodium sulfate. Extract the aqueous layer with two 10-mL portions of ether, also filtering the extracts through the anhydrous sodium sulfate. Evaporate

the combined extracts under a current of dry air until the volume is reduced to about 10 mL, then transfer the residue to a 25-mL conical flask. Add 3 mL of pyridine, complete the evaporation of the ether, and boil on a hot plate until the volume is reduced to about 1 mL. Cool, and add 1 mL of a suitable silylation agent, such as bis(trimethylsilyl)-trifluoroacetamide, bis(trimethylsilyl)acetamide, or a mixture of hexamethyldisilazane and trimethylchlorosilane [2:1 or 3:1 (v/v)]. Mix, and allow to stand for not less than 15 minutes.

Procedure—Separately inject equal volumes (2 μ L) of the silylated solution from the *Standard Preparation* and the *Test Preparation* into the chromatograph, record the chromatograms with the apparatus adjusted to the parameters set forth in the accompanying table, and measure the areas under the peaks for methylparaben, propylparaben, and benzophenone. Calculate the content, in μ g per mL, of methylparaben ($C_8H_8O_3$) in the sample under test by the formula:

$$10(C_M/V)(p_1/p_3)(P_3/P_1)$$

in which C_M is the concentration, in μ g per mL, of methylparaben in the *Standard Preparation*; V is the volume, in mL, of the specimen taken; p_1 and p_3 are the peak areas for methylparaben and benzophenone, respectively, obtained from the *Test Preparation*; and P_1 and P_3 are the peak areas of methylparaben and benzophenone, respectively, obtained from the *Standard Preparation*. Similarly, calculate the content, in μ g per mL, of propylparaben ($C_{10}H_{12}O_3$) in the specimen under test by the formula:

$$10(C_P/V)(p_2/p_3)(P_3/P_2)$$

in which C_P is the concentration, in μ g per mL, of propylparaben in the *Standard Preparation*; V is the volume, in mL, of the specimen taken; p_2 and p_3 are the peak areas for propylparaben and benzophenone, respectively, obtained from the *Test Preparation*; and P_2 and P_3 are the peak areas of propylparaben and benzophenone, respectively, obtained from the *Standard Preparation*.

Ethylparaben and butylparaben may be determined in a similar manner.

POLAROGRAPHIC METHOD

Phenylmercuric Nitrate

Standard Preparation—Dissolve about 100 mg of phenylmercuric nitrate, accurately weighed, in sodium hydroxide solution (1 in 250) contained in a 1000-mL volumetric flask, warming if necessary to effect solution, add the sodium hydroxide solution to volume, and mix. Pipet 10 mL of this solution into a 25-mL volumetric flask, and proceed as directed under *Test Preparation*, beginning with "add 2 mL of potassium nitrate solution (1 in 100)."

Test Preparation—Pipet 10 mL of the specimen under test into a 25-mL volumetric flask, add 2 mL of potassium nitrate solution (1 in 100) and 10 mL of pH 9.2 alkaline borate buffer (see under *Buffer Solutions* in the section *Reagents, Indicators, and Solutions*), and adjust to a pH of 9.2, if necessary, by the addition of 2 N nitric acid. Add 1.5 mL of freshly prepared gelatin solution (1 in 1000), then add the pH 9.2 alkaline borate buffer to volume, and mix.

Procedure—Pipet a portion of the *Test Preparation* into the polarographic cell, and deaerate by bubbling nitrogen through the solution for 15 minutes. Insert the dropping mercury electrode of a suitable polarograph (see *Polarography* (801)), and record the polarogram from -0.6 to -1.5 volts versus the saturated calomel electrode. Determine the diffusion current of the *Test Preparation*, $(i_d)_U$, as the difference between the residual current and the limiting current.

Similarly and concomitantly determine the diffusion current, $(i_d)_s$, of the *Standard Preparation*. Calculate the quantity, in μg , of phenylmercuric nitrate ($\text{C}_6\text{H}_5\text{HgNO}_3$) in each mL of the specimen taken by the formula:

$$2.5C[(i_d)_u/(i_d)_s]$$

in which C is the concentration, in μg per mL, of phenylmercuric nitrate in the *Standard Preparation*.

Thimerosal

Standard Preparation—On the day of use, place about 25 mg of USP Thimerosal RS, accurately weighed, in a 250-mL volumetric flask, add water to volume, and mix. Protect from light. Pipet 15 mL of this solution into a 25-mL volumetric flask, add 1.5 mL of gelatin solution (1 in 1000), then add potassium nitrate solution (1 in 100) to volume, and mix.

Test Preparation—Pipet 15 mL of the test specimen into a 25-mL volumetric flask, add 1.5 mL of gelatin solution (1 in 1000), add potassium nitrate solution (1 in 100) to volume, and mix.

Procedure—Transfer a portion of the *Test Preparation* to a polarographic cell, and deaerate by bubbling nitrogen through the solution for 15 minutes. Insert the dropping mercury electrode of a suitable polarograph (see *Polarography* (801)), and record the polarogram from -0.2 to -1.4 volts versus the saturated calomel electrode. Determine the diffusion current, $(i_d)_u$, as the difference between the residual current and the limiting current. Similarly and concomitantly determine the diffusion current, $(i_d)_s$, of the *Standard Preparation*. Calculate the quantity, in μg , of thimerosal ($\text{C}_6\text{H}_5\text{HgNaO}_2\text{S}$) in each mL of the test specimen taken by the formula:

$$1.667C[(i_d)_u/(i_d)_s]$$

in which C is the concentration, in μg per mL, of thimerosal in the *Standard Preparation*; and the other terms are as defined therein.

(345) ASSAY FOR CITRIC ACID/ CITRATE AND PHOSPHATE

The following ion chromatographic general procedure is provided for the determination of citric acid/citrate and phosphate in compendial articles, when specified in the individual monographs. Identification tests for citrate and phosphate are provided separately under USP general chapter *Identification Tests—General* (191). The procedure for preparation of the *Standard Preparations* used for the assay depends on whether or not citrate and phosphate are being assayed concomitantly, as indicated below.

USP Reference Standards (11)—*USP Citric Acid RS*.

Mobile Phase—Transfer an appropriate volume of water (resistivity not less than 18 megohm-cm) to a suitable container, and degas with helium for not less than 20 minutes. Add an appropriate volume of 50% (w/w) carbonate-free sodium hydroxide or potassium hydroxide to obtain a 20 mM sodium hydroxide or potassium hydroxide solution.

Alternatively, a 20 mM sodium hydroxide or potassium hydroxide eluant can be generated electrolytically using an automatic eluant generator. [NOTE—Protect the *Mobile Phase* from atmospheric carbon dioxide.]

Standard Preparations—Use *Standard Preparation 1* for an assay for citric acid/citrate only. Use *Standard Preparation 2* when a concomitant assay for citrate and phosphate is intended.

Standard Preparation 1—Dissolve USP Citric Acid RS in freshly prepared 1 mM sodium hydroxide to obtain a solution having a known concentration of about 20 μg per mL of citrate ($\text{C}_6\text{H}_5\text{O}_7$).

Standard Preparation 2—Dissolve USP Citric Acid RS and monobasic sodium phosphate in freshly prepared 1 mM sodium hydroxide to obtain a solution having known concentrations of about 20 μg per mL and 12 μg per mL of citrate and phosphate (PO_4), respectively.

Assay Preparation for Citric Acid/Citrate Assay—Unless stated otherwise in the monograph, dissolve an appropriate quantity of a solid dosage form in freshly prepared 1 mM sodium hydroxide to obtain a solution containing about 20 μg per mL of citrate. If the dosage form is a liquid formulation, dilute with water, and add a freshly prepared sodium hydroxide solution to obtain a solution containing about 20 μg per mL of citrate in 1 mM sodium hydroxide.

Assay Preparation for Phosphate Assay—Unless stated otherwise in the monograph, dissolve an appropriate quantity of a solid dosage form in freshly prepared 1 mM sodium hydroxide to obtain a solution containing about 12 μg per mL of phosphate. If the dosage form is a liquid formulation, dilute with water, and add a freshly prepared sodium hydroxide solution to obtain a solution containing about 12 μg per mL of phosphate in 1 mM sodium hydroxide.

Chromatographic System (see *Chromatography* (621))—The liquid chromatograph is equipped with a suitable anion trap column; a 4-mm \times 50-mm guard column and a 4-mm \times 250-mm analytical column, both packed with L61 packing; and an electrochemical detector with suppressed conductivity detection using either a micromembrane anion autosuppressor or a suitable chemical suppression system. All columns are maintained at a temperature of 30° and eluted at a flow rate of 2 mL per minute. [NOTE—An anion trap column designed to remove trace anion contaminants in the *Mobile Phase* should be added to the column assembly before the injector.] Chromatograph *Standard Preparation 1* or *Standard Preparation 2*, as appropriate, and record the peak area responses as directed for *Procedure*: the tailing factor is not more than 2.0; and the relative standard deviation of the peak areas for citrate (and phosphate where appropriate), for six replicate injections of *Standard Preparation 1* or *Standard Preparation 2*, is not more than 1.5%.

Procedure—Separately inject 10 μL each of the appropriate *Standard Preparation* and the *Assay Preparation* into the chromatograph, record the chromatograms, and measure the peak areas for citrate and phosphate, as appropriate. Determine the concentrations of citrate or phosphate in the portion of *Assay Preparation* taken by the formula:

$$C_s(r_u/r_s)$$

in which C_s is the concentration of citrate or phosphate, in μg per mL, in the appropriate *Standard Preparation*; and r_u and r_s are the peak areas of citrate or phosphate obtained from the *Assay Preparation* and the *Standard Preparation*, respectively.

<351> ASSAY FOR STEROIDS

The following procedure is applicable for determination of those Pharmacopeial steroids that possess reducing functional groups such as α -ketols.

Standard Preparation—Dissolve in alcohol a suitable quantity of the USP Reference Standard specified in the individual monograph, previously dried under the conditions specified in the individual monograph and accurately weighed, and dilute quantitatively and stepwise with alcohol to obtain a solution having a concentration of about 10 μ g per mL. Pipet 20 mL of this solution into a glass-stoppered, 50-mL conical flask.

Assay Preparation—Prepare as directed in the individual monograph.

Procedure—To each of the two flasks containing the *Assay Preparation* and the *Standard Preparation*, respectively, and to a similar flask containing 20.0 mL of alcohol to serve as the blank, add 2.0 mL of a solution prepared by dissolving 50 mg of blue tetrazolium in 10 mL of methanol, and mix. Then to each flask add 2.0 mL of a mixture of alcohol and tetramethylammonium hydroxide TS (9:1), mix, and allow to stand in the dark for 90 minutes. Without delay, concomitantly determine the absorbances of the solutions from the *Assay Preparation* and the *Standard Preparation* at about 525 nm, with a suitable spectrophotometer, against the blank. Calculate the result by the formula given in the individual monograph, in which C is the concentration, in μ g per mL, of the Reference Standard in the *Standard Preparation*; and A_U and A_S are the absorbances of the solutions from the *Assay Preparation* and the *Standard Preparation*, respectively.

<361> BARBITURATE ASSAY

Internal Standard, Internal Standard Solution, Standard Preparation, and Assay Preparation—Prepare as directed in the individual monograph.

Chromatographic System—Under typical conditions, the gas chromatograph is equipped with a flame-ionization detector and contains a 4-mm \times 0.9-m glass column packed with 3% liquid phase G10 on support 80- to 100-mesh S1A. The column is maintained at a temperature of $200 \pm 10^\circ$, and the injection port and detector are maintained at about 225° , the column temperature being varied within the designated tolerance, as necessary, to meet *System Suitability* specifications and provide suitable retention times. Use a suitable carrier gas, such as dry nitrogen, at an appropriate flow rate, such as 60 to 80 mL per minute. Use on-column injection. [NOTE—If the instrument is not equipped for on-column injection, use an injection port lined with glass that has been washed successively with chromic acid cleansing solution, water, methanol, chloroform, a 1 in 10 solution of trimethylchlorosilane in chloroform, and chloroform.]

System Suitability (see *Chromatography* <621>)—Chromatograph five replicate injections of the *Standard Preparation*, and record peak responses as directed for *Procedure*: the relative standard deviation for the ratio R_S is not more than 1.5%. In a suitable chromatogram, the resolution, R , between the barbituric acid and the *Internal Standard* is not less than the value given in the individual monograph, and the tailing factor, T , for each of the two peaks is not more than 2.0.

Procedure—Inject a suitable portion (about 5 μ L) of the *Standard Preparation* into a suitable gas chromatograph, and record the chromatogram. Similarly inject a suitable portion of the *Assay Preparation*, and record the chromatogram. Calculate the content of the barbiturate or barbituric acid in the assay specimen by the formula given in the individual monograph, in which R_U is the ratio of the peak response of the barbituric acid to that of the *Internal Standard* obtained for the *Assay Preparation*; Q_S is the ratio of the weight of the barbituric acid to that of the *Internal Standard* in the *Standard Preparation*; C_i is the concentration, in mg per mL, of *Internal Standard* in the *Internal Standard Solution*; and R_S is the ratio of the peak response of the barbituric acid to that of the *Internal Standard* in the *Standard Preparation*.

<371> COBALAMIN RADIOTRACER ASSAY

All radioactive determinations required by this method should be made with a suitable counting assembly over a period of time optimal for the particular counting assembly used. All procedures should be performed in replicate to obtain the greatest accuracy.

USP Reference Standards <11>—*USP Cyanocobalamin RS*.

Cyanocobalamin Tracer Reagent—Dilute an accurately measured volume of a solution of radioactive cyanocobalamin* with water to yield a solution having a radioactivity between 500 and 5000 counts per minute per mL. Add 1 drop of cresol per L of solution prepared, and store in a refrigerator.

Standardization—Prepare a solution of a weighed quantity of USP Cyanocobalamin RS in water to contain 20 to 50 μ g per mL. Perform the entire assay on a 10.0-mL portion of this solution, proceeding as directed under *Assay Preparation*, beginning with "Add water to make a measured volume."

Cresol-Carbon Tetrachloride Solution—Mix equal volumes of carbon tetrachloride and freshly distilled cresol.

Phosphate-Cyanide Solution—Dissolve 100 mg of potassium cyanide in 1000 mL of a saturated solution of dibasic sodium phosphate, and mix.

Butanol-Benzalkonium Chloride Solution—Dilute benzalkonium chloride solution (17 in 100) with water (3:1), and mix with 36 volumes of butyl alcohol.

Alumina-Resin Column—Place a pledget of glass wool in the bottom of a constricted glass tube such as a 50-mL buret. With the tube held in an upright position, add a volume of a slurry of ion-exchange resin (see in the section *Reagents, Indicators, and Solutions*), in water, sufficient to give a column of settled resin 7 cm in height. When the solid has settled somewhat, allow the water to drain so that there is only 1 cm of liquid above the resin column, and tamp the resin lightly. Then add a volume of a slurry of anhydrous alumina (not acid-washed) in water sufficient to increase the height of the settled column to 10 cm, and allow the water to drain to about 1 cm from the top of the alumina. Add a pledget of glass wool, and wash the column, using a total of 50 mL of water, and again drain to within 1 cm of the top of the column. Prepare a fresh column for each determination.

Assay Preparation—Transfer to a beaker a weighed quantity or measured volume of the preparation to be assayed, equivalent in vitamin B₁₂ activity to that of 200 to

* A solution of cyanocobalamin made radioactive by the incorporation of ⁶⁰Co is available from Merck and Co., Inc., Rahway, NJ 07065.

500 µg of cyanocobalamin. Add water to make a measured volume of not less than 25 mL, then add 5.0 mL of *Cyano-cobalamin Tracer Reagent*. Add, while working under a hood, 5 mg of sodium nitrite and 2 mg of potassium cyanide for each mL of the resulting solution. Adjust the solution with diluted hydrochloric acid to a pH of approximately 4, and heat on a steam bath for 15 minutes. Cool, and adjust the solution with 1 N sodium hydroxide to a pH between 7.6 and 8.0. Centrifuge or filter to remove any undissolved solids.

Procedure—Transfer the *Assay Preparation* to a 250-mL centrifuge bottle, add 10 mL of *Cresol–Carbon Tetrachloride Solution*, suitably close the bottle with a glass, polyethylene, or foil-wrapped rubber stopper, shake vigorously for 2 to 5 minutes, and centrifuge. Remove and save the lower, solvent layer. Repeat the extraction using a 5-mL portion of *Cresol–Carbon Tetrachloride Solution*, and combine the lower, solvent-layer extracts in a centrifuge bottle or separator of 50- to 100-mL capacity.

Wash the combined extracts with successive 10-mL portions of 5 N sulfuric acid until the last washing is practically colorless (two washings usually suffice). During each washing, shake for 2 to 5 minutes, allow the layers to separate, centrifuge, if necessary, and discard the acid layer. Wash further with two successive 10-mL portions of *Phosphate–Cyanide Solution*. Finally, wash with 10 mL of water. Discard all of the washings.

To the washed extract add 30 mL of a mixture of *Butanol–Benzalkonium Chloride Solution* and carbon tetrachloride (2:1). Extract with two 5-mL portions of water, each time shaking vigorously for 1 minute, centrifuging, and removing and saving the upper, aqueous layer.

Pass the combined aqueous extracts through the *Alumina–Resin Column* at a rate of about 1 mL per minute, maintaining a 1-cm layer of liquid on the head of the column by adding water as needed. Discard as much of the forerun as is colorless (usually about 5 mL), and collect the colored eluate (usually about 10 mL) in a 50-mL centrifuge tube or separator containing 500 µL of diluted acetic acid. Extract the eluate by shaking for 2 to 5 minutes with 5 mL of *Cresol–Carbon Tetrachloride Solution*, and discard the upper, aqueous layer. To the extract add 5.0 mL of water, 5 mL of carbon tetrachloride, and 10 mL of butyl alcohol. Shake, allow to separate until the upper layer is clear, and remove the upper, aqueous layer.

Determine the absorbances of the aqueous extract, in a 1-cm cell, at 361 nm and 550 nm, with a suitable spectrophotometer, using a tungsten light source. Make the 361-nm reading using a filter capable of reducing stray light. Calculate the ratio A_{361}/A_{550} : the purity of the aqueous extract is acceptable if the ratio is between 3.10 and 3.40. If a ratio outside this range is observed, purify the aqueous extract by repeating the extraction cycle, proceeding as directed in the foregoing paragraph.

If an acceptable absorbance ratio is observed in the aqueous extract, determine the radioactivity, in counts per minute, using a suitable counter over a period optimal for the particular counting assembly used. Average the results, and correct the average for the observed background radioactivity determined over two or more 30-minute periods.

Calculation—Calculate the cobalamin content, expressed in µg of cyanocobalamin, of the portion taken for assay by the formula:

$$R(C_s/C_u)(A_u/A_s)$$

in which R is the quantity, in µg, of cyanocobalamin in the portion of the standard solution taken; C_s and C_u are the corrected average radioactivity values, expressed in counts per minute per mL, of the standard and assay solutions,

respectively; and A_u and A_s are the absorbances determined at 361 nm of the assay and standard solutions, respectively.

(381) ELASTOMERIC CLOSURES FOR INJECTIONS

INTRODUCTION

Elastomeric closures for containers used in the types of preparations defined in the general test chapter *Injections* (1) are made of materials obtained by vulcanization (cross-linking) polymerization, polyaddition, or polycondensation of macromolecular organic substances (elastomers). Closure formulations contain natural or synthetic elastomers and inorganic and organic additives to aid or control vulcanization, impart physical and chemical properties or color, or stabilize the closure formulation.

This chapter applies to closures used for long-term storage of preparations defined in the general test chapter *Injections* (1). Such closures are typically used as part of a vial, bottle, or pre-fill syringe package system.

This chapter applies to closures formulated with natural or synthetic elastomeric substances. This chapter does not apply to closures made from silicone elastomer; however, it does apply to closures treated with silicone (e.g., Dimethicone, NF). When performing the tests in this chapter, it is not required that closures be treated with silicone, although there is no restriction prohibiting the use of siliconized closures.

This chapter also applies to closures coated with other lubricious materials (e.g., materials chemically or mechanically bonded to the closure) that are not intended to, and in fact do not provide, a barrier to the base elastomer. When performing the tests, closures with lubricious nonbarrier coatings are to be tested in their coated state.

The following comments relate solely to closures laminated or coated with materials intended to provide, or in fact function as, a barrier to the base elastomer (e.g., PTFE or lacquer coatings). It is not permissible to use a barrier material in an attempt to change a closure that does not meet compendial requirements to one that does conform. Therefore, all *Physicochemical Tests* apply to the base formula of such closures, as well as to the coated or laminated closure. To obtain *Physicochemical Tests* results, the tests are to be performed on uncoated or nonlaminated closures of the same elastomeric compound, as well as to the laminated or coated closure. The *Functionality Tests* apply to and are to be performed using the laminated or coated elastomeric closure. *Biological Tests* apply to the lamination or coating material, as well as to the base formula. *Biological Tests* may be performed on the laminated or coated closure, or they may be performed on the laminate/coating material and the uncoated or nonlaminated closures of the same elastomeric compound. In the latter case, the results are to be reported separately. The base formula used for physicochemical or biological tests intended to support the compendial compliance of a barrier-coated closure should be similar to the corresponding coated closure in configuration and size.

For all *Elastomeric Closures for Injection* (381) tests performed on any closure type, it is important to document the closure being tested, including a full description of the elastomer, and any lubrication, coating, laminations, or treatments applied.

This chapter states test limits for Type I and Type II elastomeric closures. Type I closures are typically used for aqueous

preparations. Type II closures are typically intended for non-aqueous preparations and are those which, having properties optimized for special uses, may not meet all requirements listed for Type I closures because of physical configuration, material of construction, or both. If a closure fails to meet one or more of the Type I test requirements, but still meets the Type II requirements for the test(s), the closure is assigned a final classification of Type II. All elastomeric closures suitable for use with injectable preparations must comply with either Type I or Type II test limits. However, this specification is not intended to serve as the sole evaluation criteria for the selection of such closures.

It is appropriate to use this chapter when identifying elastomeric closures that might be acceptable for use with injectable preparations on the basis of their biological reactivity, their aqueous extract physicochemical properties, and their functionality.

The following closure evaluation requirements are beyond the scope of this chapter:

- The establishment of closure identification tests and specifications
- The verification of closure–product physicochemical compatibility
- The identification and safety determination of closure leachables found in the packaged product
- The verification of packaged product closure functionality under actual storage and use conditions

The manufacturer of the injectable product (the end user) must obtain from the closure supplier an assurance that the composition of the closure does not vary and that it is the same as that of the closure used during compatibility testing. When the supplier informs the end user of changes in the composition, compatibility testing must be repeated, totally or partly, depending on the nature of the changes. Closures must be properly stored, cleaned for removal of environmental contaminants and endotoxins, and, for aseptic processes, sterilized prior to use in packaging injectable products.

CHARACTERISTICS

Elastomeric closures are translucent or opaque and have no characteristic color, the latter depending on the additives used. They are homogeneous and practically free from flash and adventitious materials (e.g., fibers, foreign particles, and waste rubber.)

IDENTIFICATION

Closures are made of a wide variety of elastomeric materials and optional polymeric coatings. For this reason, it is beyond the scope of this chapter to specify identification tests that encompass all possible closure presentations. However, it is the responsibility of the closure supplier and the injectable product manufacturer (the end user) to verify the closure elastomeric formulation and any coating or laminate

materials used according to suitable identification tests. Examples of some of the analytical test methodologies that may be used include specific gravity, percentage of ash analysis, sulfur content determination, FTIR-ATR test, thin-layer chromatography of an extract, UV absorption spectrophotometry of an extract, or IR absorption spectrophotometry of a pyrolysate.

TEST PROCEDURES

Elastomeric closures shall conform to biological, physicochemical, and functionality requirements both as they are shipped by the closure supplier to the injectable product manufacturer (the end user), and in their final ready-to-use state by the end user.

For those elastomeric closures processed by the supplier prior to distribution to the end user, the supplier shall demonstrate compendial conformance of closures exposed to such processing and/or sterilization steps. Similarly, if elastomeric closures received by the end user are subsequently processed or sterilized, the end user is responsible for demonstrating the continued conformance of closures to compendial requirements subsequent to such processing and/or sterilization conditions (i.e., in their ready-to-use state). This is especially important if closures shall be exposed to processes or conditions that may significantly impact the biological, physicochemical, or functionality characteristics of the closure (e.g., gamma irradiation).

For closures that are normally lubricated with silicone prior to use, it is permissible to perform physicochemical testing on nonlubricated closures, in order to avoid potential method interference and/or difficulties in interpreting test results. For closures supplied with other lubricious nonbarrier coatings, all tests are to be performed using the coated closure.

For closures coated or laminated with coatings intended to provide a barrier function (e.g., PTFE or lacquer coatings), physicochemical compendial tests apply to the uncoated base elastomer, as well as to the coated closure. In this case, suppliers are responsible for demonstrating physicochemical compendial compliance of the coated closure, as well as of the uncoated closure, processed or treated in a manner simulating conditions typically followed by the supplier for such coated closures prior to shipment to the end user. The uncoated closure subject to physicochemical tests should be similar to the corresponding coated closure in size and configuration. End users of coated closures are also responsible for demonstrating the continued physicochemical compendial conformance of the coated closure, processed or treated in a manner simulating conditions typically employed by the end user prior to use.

In all cases, it is appropriate to document all conditions of closure processing, pretreatment, sterilization, or lubrication when reporting test results.

Table 1 summarizes the testing requirements of closures, and the responsibilities of the supplier and the end user.

Table 1

Closure Types (As Supplied or Used)	Test Requirements		
	Physicochemical Tests	Functionality Tests	Biological Tests
Closure with or without Silicone Coating	• Tests are to be performed.	• Tests are to be performed.	• Tests are to be performed.
	• Silicone use is optional.	• Silicone use is optional.	• Silicone use is optional.
	• Responsibility: supplier and end user	• Responsibility: supplier and end user	• Responsibility: supplier and end user
Closures with Lubricious Coating (Nonbarrier Material; Not Silicone)	• Tests are to be performed on coated closures.	• Tests are to be performed on coated closures.	• Tests are to be performed on coated closures.
	• Responsibility: supplier and end user	• Responsibility: supplier and end user	• Responsibility: supplier and end user

Table 1 (Continued)

Closure Types (As Supplied or Used)	Test Requirements		
	Physicochemical Tests	Functionality Tests	Biological Tests
Closures with Barrier Coating	• Tests are to be performed on coated closures.	• Tests are to be performed on coated closures.	• Tests are to be performed on coated closures.
	• Responsibility: supplier and end user	• Responsibility: supplier and end user	OR:
	AND:		• Tests are to be performed on uncoated closures (base formula) and the laminate/coating material (report results separately).
	• Tests are to be performed on uncoated closures (base formula).		• Responsibility: supplier and end user
	• Responsibility: supplier		

Table 2

	Reference Suspension A	Reference Suspension B	Reference Suspension C	Reference Suspension D
Standard of Opalescence	5.0 mL	10.0 mL	30.0 mL	50.0 mL
Water	95.0 mL	90.0 mL	70.0 mL	50.0 mL
Nephelometric Turbidity Units	3 NTU	6 NTU	18 NTU	30 NTU

BIOLOGICAL TESTS

Two stages of testing are indicated. The first stage is the performance of an in vitro test procedure as described in general test chapter *Biological Reactivity Tests, In Vitro* (87). Materials that do not meet the requirements of the in vitro test are subjected to the second stage of testing, which is the performance of the in vivo tests, *Systemic Injection Test and Intracutaneous Test*, according to the procedures set forth in the general test chapter *Biological Reactivity Tests, In Vivo* (88). Materials that meet the requirements of the in vitro test are not required to undergo in vivo testing.

Type I and Type II closures must both conform to the requirements of either the in vitro or the in vivo biological reactivity tests. [NOTE—Also see the general information chapter *The Biocompatibility of Material Used in Drug Containers, Medical Devices, and Implants* (1031).]

PHYSICOCHEMICAL TESTS

Preparation of Solution S

Place whole, uncut closures corresponding to a surface area of 100 ± 10 cm² into a suitable glass container. Cover the closures with 200 mL of Purified Water or Water for Injection. If it is not possible to achieve the prescribed closure surface area (100 ± 10 cm²) using uncut closures, select the number of closures that will most closely approximate 100 cm², and adjust the volume of water used to the equivalent of 2 mL per each 1 cm² of actual closure surface area used. Boil for 5 minutes, and rinse five times with cold Purified Water or Water for Injection.

Place the washed closures into a Type I glass wide-necked flask (see *Containers—Glass* (660)), add the same quantity of Purified Water or Water for Injection initially added to the closures, and weigh. Cover the mouth of the flask with a Type I glass beaker. Heat in an autoclave so that a temperature of $121 \pm 2^\circ$ is reached within 20 to 30 minutes, and maintain this temperature for 30 minutes. Cool to room temperature over a period of about 30 minutes. Add Purified Water or Water for Injection to bring it up to the original mass. Shake, and immediately decant and collect the solution. [NOTE—This solution must be shaken before being used in each of the tests.]

Preparation of Blank

Prepare a blank solution similarly, using 200 mL of Purified Water or Water for Injection omitting the closures.

Appearance of Solution (Turbidity/Opalescence and Color)

Determination of Turbidity (Opalescence)

NOTE—The determination of turbidity may be performed by visual comparison (*Procedure A*), or instrumentally using a suitable ratio turbidimeter (*Procedure B*). For a discussion of turbidimetry, see *Spectrophotometry and Light-Scattering* (851). Instrumental assessment of clarity provides a more discriminatory test that does not depend on the visual acuity of the analyst.

Hydrazine Sulfate Solution—Dissolve 1.0 g of hydrazine sulfate in water and dilute with water to 100.0 mL. Allow to stand for 4 to 6 hours.

Hexamethylenetetramine Solution—Dissolve 2.5 g of hexamethylenetetramine in 25.0 mL of water in a 100-mL glass-stoppered flask.

Opalescence Stock Suspension—Add 25.0 mL of *Hydrazine Sulfate Solution* to the *Hexamethylenetetramine Solution* in the flask. Mix, and allow to stand for 24 hours. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.

Opalescence Standard Suspension—Prepare a suspension by diluting 15.0 mL of the *Opalescence Stock Suspension* with water to 1000.0 mL. *Opalescence Standard Suspension* is stable for about 24 hours after preparation.

Reference Suspensions—Prepare according to Table 2. Mix and shake before use. [NOTE—Stabilized formazin suspensions that can be used to prepare stable, diluted turbidity standards are available commercially and may be used after comparison with the standards prepared as described.]

Procedure A: Visual Comparison—Use identical test tubes made of colorless, transparent, neutral glass with a flat base and an internal diameter of 15 to 25 mm. Fill one tube to a depth of 40 mm with *Solution S*, one tube to the same depth with water, and four others to the same depth with *Reference Suspensions A, B, C, and D*. Compare the solutions in diffuse daylight 5 minutes after preparation of the *Reference Suspensions*, viewing vertically against a black background. The light conditions shall be such that *Reference*

Suspension A can be readily distinguished from water and that *Reference Suspension B* can be readily distinguished from *Reference Suspension A*.

Requirement—*Solution S* is not more opalescent than *Reference Suspension B* for Type I closures, and not more opalescent than *Reference Suspension C* for Type II closures. *Solution S* is considered clear if its clarity is the same as that of water when examined as described above, or if its opalescence is not more pronounced than that of *Reference Suspension A* (refer to *Table 3*).

Procedure B: Instrumental Comparison—Measure the turbidity of the *Reference Suspensions* in a suitable calibrated turbidimeter (see *Spectrophotometry and Light Scattering* (851)). The blank should be run and the results corrected for the blank. *Reference Suspensions A, B, C, and D* represent 3, 6, 18, and 30 Nephelometric Turbidity Units (NTU), respectively. Measure the turbidity of *Solution S* using the calibrated turbidimeter.

Requirement—The turbidity of *Solution S* is not greater than that for *Reference Suspension B* (6 NTU FTU) for Type I closures, and is not greater than that for *Reference Suspension C* (18 NTU FTU) for Type II closures (refer to *Table 3*).

Table 3

Comparison Method		
Opalescence Requirements	Procedure A (Visual)	Procedure B (Instrumental)
Type I closures	No more opalescent than <i>Suspension B</i>	No more than 6 NTU
Type II closures	No more opalescent than <i>Suspension C</i>	No more than 18 NTU

Determination of Color

Color Standard—Prepare a solution by diluting 3.0 mL of *Matching Fluid O* (see *Color and Achromicity* (631)) with 97.0 mL of diluted hydrochloric acid.

Procedure—Use identical tubes made of colorless, transparent, neutral glass with a flat base and an internal diameter of 15 to 25 mm. Fill one tube to a depth of 40 mm with *Solution S*, and the second with the *Color Standard*. Compare the liquids in diffuse daylight, viewing vertically against a white background.

Requirement—*Solution S* is not more intensely colored than the *Color Standard*.

Acidity or Alkalinity

Bromothymol Blue Solution—Dissolve 50 mg of bromothymol blue in a mixture of 4 mL of 0.02 M sodium hydroxide and 20 mL of alcohol. Dilute with water to 100 mL.

Procedure—To 20 mL of *Solution S* add 0.1 mL of *Bromothymol Blue Solution*. If the solution is yellow, titrate with 0.01 N sodium hydroxide until a blue endpoint is reached. If the solution is blue, titrate with 0.01 N hydrochloric acid until a yellow endpoint is reached. If the solution is green, it is neutral and no titration is required.

Blank Correction—Test 20 mL of *Blank* similarly. Correct the results obtained for *Solution S* by subtracting or adding the volume of titrant required for the *Blank*, as appropriate. (*Reference Titrimetry* (541).)

Requirement—Not more than 0.3 mL of 0.01 N sodium hydroxide produces a blue color, or not more than 0.8 mL of 0.01 N hydrochloric acid produces a yellow color, or no titration is required.

Absorbance

Procedure—[NOTE—Perform this test within 5 hours of preparing *Solution S*.] Pass *Solution S* through a 0.45- μ m pore size filter, discarding the first few mL of filtrate. Measure the absorbance of the filtrate at wavelengths between 220 and 360 nm in a 1-cm cell using the blank in a matched cell in the reference beam. If dilution of the filtrate is required before measurement of the absorbance, correct the test results for the dilution.

Requirement—The absorbances at these wavelengths do not exceed 0.2 for Type I closures or 4.0 for Type II closures.

Reducing Substances

Procedure—[NOTE—Perform this test within 4 hours of preparing *Solution S*.] To 20.0 mL of *Solution S* add 1 mL of diluted sulfuric acid and 20.0 mL of 0.002 M potassium permanganate. Boil for 3 minutes. Cool, add 1 g of potassium iodide, and titrate immediately with 0.01 M sodium thiosulfate, using 0.25 mL of starch solution TS as the indicator. Perform a titration using 20.0 mL of blank and note the difference in volume of 0.01 M sodium thiosulfate required.

Requirement—The difference between the titration volumes is not greater than 3.0 mL for Type I closures and not greater than 7.0 mL for Type II closures.

Heavy Metals

Procedure—Proceed as directed for *Method I* under *Heavy Metals* (231). Prepare the *Test Preparation* using 10.0 mL of *Solution S*.

Requirement—*Solution S* contains not more than 2 ppm of heavy metals as lead.

Extractable Zinc

Test Solution—Prepare a *Test Solution* by diluting 10.0 mL of *Solution S* to 100 mL with 0.1 N hydrochloric acid. Prepare a test blank similarly, using the *Blank* for *Solution S*.

Zinc Standard Solution—Prepare a solution (10 ppm Zn) by dissolving zinc sulfate in 0.1 N hydrochloric acid.

Reference Solutions—Prepare not fewer than three *Reference Solutions* by diluting the *Zinc Standard Solution* with 0.1 N hydrochloric acid. The concentrations of zinc in these *Reference Solutions* are to span the expected limit of the *Test Solution*.

Procedure—Use a suitable atomic absorption spectrophotometer (see *Spectrophotometry and Light Scattering* (851)) equipped with a zinc hollow-cathode lamp and an air-acetylene flame. An alternative procedure such as an appropriately validated inductively coupled plasma analysis (ICP) may be used.

Test each of the *Reference Solutions* at the zinc emission line of 213.9 nm at least three times. Record the steady readings. Rinse the apparatus with the test blank solution each time, to ensure that the reading returns to initial blank value. Prepare a calibration curve from the mean of the readings obtained for each *Reference Solution*. Record the absorbance of the *Test Solution*. Determine the ppm zinc concentration of the *Test Solution* using the calibration curve.

Requirement—*Solution S* contains not more than 5 ppm of extractable zinc.

Ammonium

Alkaline Potassium Tetraiodomercurate Solution—Prepare a 100 mL solution containing 11 g of potassium iodide and 15 g of mercuric iodide in water. Immediately before use, mix 1 volume of this solution with an equal volume of a 250 g per L solution of sodium hydroxide.

Test Solution—Dilute 5 mL of *Solution S* to 14 mL with water. Make alkaline if necessary by adding 1 N sodium hydroxide, and dilute with water to 15 mL. Add 0.3 mL of *Alkaline Potassium Tetraiodomercurate Solution*, and close the container.

Ammonium Standard Solution—Prepare a solution of ammonium chloride in water (1 ppm NH_4). Mix 10 mL of the 1 ppm ammonium chloride solution with 5 mL water and 0.3 mL of *Alkaline Potassium Tetraiodomercurate Solution*. Close the container.

Requirement—After 5 minutes, any yellow color in the *Test Solution* is no darker than the *Ammonium Standard Solution* (no more than 2 ppm of NH_4 in *Solution S*).

Volatile Sulfides

Procedure—Place closures, cut if necessary, with a total surface area of $20 \pm 2 \text{ cm}^2$ in a 100-mL flask, and add 50 mL of a 20 g per L citric acid solution. In the same manner and at the same time, prepare a control solution in a separate 100-mL flask by dissolving 0.154 mg of sodium sulfide in 50 mL of a 20 g per L citric acid solution. Place a piece of lead acetate paper over the mouth of each flask, and hold the paper in position by placing over it an inverted weighing bottle. Heat the flasks in an autoclave at $121 \pm 2^\circ$ for 30 minutes.

Requirement—Any black stain on the paper produced by the test solution is not more intense than that produced by the control substance.

FUNCTIONALITY TESTS

NOTE—Samples treated as described for preparation of *Solution S* and air dried should be used for *Functionality Tests* of *Penetrability*, *Fragmentation*, and *Self-Sealing Capacity*. *Functionality Tests* are performed on closures intended to be pierced by a hypodermic needle. The *Self-Sealing Capacity* test is required only for closures intended for multiple-dose containers. The needle specified for each test is a lubricated long bevel (bevel angle $12 \pm 2^\circ$) hypodermic needle¹.

Penetrability

Procedure—Fill 10 suitable vials to the nominal volume with water, fit the closures to be examined, and secure with a cap. Using a new hypodermic needle as described above for each closure, pierce the closure with the needle perpendicular to the surface.

Requirement—The force for piercing is no greater than 10 N (1 kgf) for each closure, determined with an accuracy of $\pm 0.25 \text{ N}$ (25 gf).

Fragmentation

Closures for Liquid Preparations—Fill 12 clean vials with water to 4 mL less than the nominal capacity. Fit the closures to be examined, secure with a cap, and allow to stand for 16 hours.

¹Refer to ISO 7864, Sterile hypodermic needles for single use with an external diameter of 0.8 mm (21 Gauge).

Closures for Dry Preparations—Fit closures to be examined into 12 clean vials, and secure each with a cap.

Procedure—Using a hypodermic needle as described above fitted to a clean syringe, inject into each vial 1 mL of water while removing 1 mL of air. Repeat this procedure four times for each closure, piercing each time at a different site. Use a new needle for each closure, checking that it is not blunted during the test. Filter the total volume of liquid in all the vials through a single filter with a nominal pore size no greater than $0.5 \mu\text{m}$. Count the rubber fragments on the surface of the filter visible to the naked eye.

Requirement—There are no more than five fragments visible. This limit is based on the assumption that fragments with a diameter $>50 \mu\text{m}$ are visible to the naked eye. In case of doubt or dispute, the particles are examined microscopically to verify their nature and size.

Self-Sealing Capacity

Procedure—Fill 10 suitable vials with water to the nominal volume. Fit the closures that are to be examined, and cap. Using a new hypodermic needle as described above for each closure, pierce each closure 10 times, piercing each time at a different site. Immerse the 10 vials in a solution of 0.1% (1 g per L) methylene blue, and reduce the external pressure by 27 kPa for 10 minutes. Restore to atmospheric pressure, and leave the vials immersed for 30 minutes. Rinse the outside of the vials.

Requirement—None of the vials contain any trace of blue solution.

(391) EPINEPHRINE ASSAY

USP Reference Standards (11)—*USP Epinephrine Bitartrate RS*.

Ferro-citrate Solution—On the day needed, dissolve 1.5 g of ferrous sulfate in 200 mL of water to which have been added 1.0 mL of dilute hydrochloric acid (1 in 12) and 1.0 g of sodium bisulfite. Dissolve 500 mg of sodium citrate in 10 mL of this solution, and mix.

Buffer Solution—In a 50-mL volumetric flask mix 4.2 g of sodium bicarbonate, 5.0 g of potassium bicarbonate, and 18 mL of water (not all of the solids will dissolve at this stage). To another 18 mL of water add 3.75 g of aminoacetic acid and 1.7 mL of 6 N ammonium hydroxide, mix to dissolve, and transfer this solution to the 50-mL volumetric flask containing the other mixture. Dilute with water to volume, and mix until solution is complete.

Standard Preparation—Transfer about 18 mg of *USP Epinephrine Bitartrate RS*, accurately weighed, to a 100-mL volumetric flask with the aid of 20 mL of sodium bisulfite solution (1 in 50), dilute with water to volume, and mix. Transfer 5.0 mL of this solution to a 50-mL volumetric flask, dilute with sodium bisulfite solution (1 in 500) to volume, and mix. [NOTE—Make the final dilution when the assay is carried out.] The concentration of *USP Epinephrine Bitartrate RS* in the *Standard Preparation* is about $18 \mu\text{g}$ per mL.

Assay Preparation—Transfer to a 50-mL volumetric flask an accurately measured volume of the Injection under assay, equivalent to about $500 \mu\text{g}$ of epinephrine, dilute with sodium bisulfite solution (1 in 500) to volume, if necessary, and mix. [NOTE—The final concentration of sodium bisulfite is in the range of 1 to 3 mg per mL, any bisulfite present in the Injection under assay being taken into consideration.]

Procedure—Into three 50-mL glass-stoppered conical flasks transfer, separately, 20.0-mL aliquots of the *Standard Preparation*, the *Assay Preparation*, and sodium bisulfite solution (1 in 500) to provide the blank. To each flask add 200 μ L of *Ferro-citrate Solution* and 2.0 mL of *Buffer Solution*, mix, and allow the solutions to stand for 30 minutes. Determine the absorbances of the solutions in 5-cm cells at the wavelength of maximum absorbance at about 530 nm, with a suitable spectrophotometer, using the blank to set the instrument. Calculate the quantity, in mg, of epinephrine ($C_9H_{13}NO_3$) in each mL of the Injection taken by the formula:

$$(183.21/333.30)(0.05C/V)(A_U/A_S)$$

in which 183.21 and 333.30 are the molecular weights of epinephrine and epinephrine bitartrate, respectively; C is the concentration, in μ g per mL, of USP Epinephrine Bitartrate RS in the *Standard Preparation*; and V is the volume, in mL, of Injection taken.

(401) FATS AND FIXED OILS

The following definitions and general procedures apply to fats, fixed oils, waxes, resins, balsams, and similar substances.

PREPARATION OF SPECIMEN

If a specimen of oil shows turbidity owing to separated stearin, warm the container in a water bath at 50° until the oil is clear, or if the oil does not become clear on warming, pass it through dry filter paper in a funnel contained in a hot-water jacket. Mix thoroughly, and weigh at one time as many portions as are needed for the various determinations, using preferably a bottle having a pipet dropper, or a weighing buret. Keep the specimen melted, if solid at room temperature, until the desired portions of specimen are withdrawn.

SPECIFIC GRAVITY

Determine the specific gravity of a fat or oil as directed under *Specific Gravity* (841).

MELTING TEMPERATURE

Determine the melting temperature as directed for substances of *Class II* (see *Melting Range or Temperature* (741)).

ACID VALUE (FREE FATTY ACIDS)

The acidity of fats and fixed oils in this Pharmacopeia may be expressed as the number of mL of 0.1 N alkali required to neutralize the free acids in 10.0 g of substance. Acidity is frequently expressed as the Acid Value, which is the number of mg of potassium hydroxide required to neutralize the free acids in 1.0 g of the substance. Unless otherwise directed in the individual monograph, use *Method I*.

Method I

Procedure—Unless otherwise directed, dissolve about 10.0 g of the substance, accurately weighed, in 50 mL of a

mixture of equal volumes of alcohol and ether (which has been neutralized to phenolphthalein with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide, unless otherwise specified) contained in a flask. If the test specimen does not dissolve in the cold solvent, connect the flask with a suitable condenser and warm slowly, with frequent shaking, until the specimen dissolves. Add 1 mL of phenolphthalein TS, and titrate with 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS until the solution remains faintly pink after shaking for 30 seconds. Calculate either the Acid Value or the volume of 0.1 N alkali required to neutralize 10.0 g of specimen (free fatty acids), whichever is appropriate. Calculate the Acid Value by the formula:

$$56.11V \times N/W$$

in which 56.11 is the molecular weight of potassium hydroxide; V is the volume, in mL; N is the normality of the potassium hydroxide solution or the sodium hydroxide solution; and W is the weight, in g, of the sample taken.

If the volume of 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS required for the titration is less than 2 mL, a more dilute titrant may be used, or the sample size may be adjusted accordingly. The results may be expressed in terms of the volume of titrant used or in terms of the equivalent volume of 0.1 N potassium hydroxide or 0.1 N sodium hydroxide.

If the oil has been saturated with carbon dioxide for the purpose of preservation, gently reflux the alcohol-ether solution for 10 minutes before titration. The oil may be freed from carbon dioxide also by exposing it in a shallow dish in a vacuum desiccator for 24 hours before weighing the test specimens.

Method II

Procedure—Prepare 125 mL of a solvent mixture consisting of equal volumes of isopropyl alcohol and toluene. Before use, add 2 mL of a 1% solution of phenolphthalein in isopropyl alcohol to the 125-mL mixture, and neutralize with alkali to a faint but permanent pink color. Weigh accurately the appropriate amount of well-mixed liquid sample indicated in the table below, and dissolve it in the neutralized solvent mixture. If the test specimen does not dissolve in the cold solvent, connect the flask with a suitable condenser and warm slowly, with frequent shaking, until the specimen dissolves. Shake vigorously while titrating with 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS to the first permanent pink of the same intensity as that of the neutralized solvent before mixing with the sample. Calculate the Acid Value as indicated in *Method I*.

Acid Value	Sample Weight (g)
0–1	20
1–4	10
4–15	2.5
15–74.9	0.5
≥ 75.0	0.1

ESTER VALUE

The Ester Value is the number of mg of potassium hydroxide required to saponify the esters in 1.0 g of the substance. If the Saponification Value and the Acid Value have been determined, the difference between these two represents the Ester Value, i.e., Ester Value = Saponification Value – Acid Value.

Procedure—Place 1.5–2 g of the substance, accurately weighed, in a tared, 250-mL flask, add 20–30 mL of neutralized alcohol, and shake. Add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS

until the free acid is neutralized. Add 25.0 mL of 0.5 N alcoholic potassium hydroxide VS, and proceed as directed under *Saponification Value*, beginning with "Heat the flask" and omitting the further addition of phenolphthalein TS. Calculate the Ester Value by the formula:

$$[56.11(V_B - V_T)N]/W$$

in which 56.11 is the molecular weight of potassium hydroxide; V_B and V_T are the volumes, in mL, of 0.5 N hydrochloric acid consumed in the blank test and in the actual test, respectively; N is the exact normality of the hydrochloric acid; and W is the weight, in g, of the substance taken for the test.

HYDROXYL VALUE

The Hydroxyl Value is the number of mg of potassium hydroxide equivalent to the hydroxyl content of 1.0 g of the substance.

Pyridine–Acetic Anhydride Reagent—Just before use, mix 3 volumes of freshly opened or freshly distilled pyridine with 1 volume of freshly opened or freshly distilled acetic anhydride.

Procedure—Transfer a quantity of the substance, determined by reference to the accompanying table and accurately weighed, to a glass-stoppered, 250-mL conical flask, and add 5.0 mL of *Pyridine–Acetic Anhydride Reagent*. Transfer 5.0 mL of *Pyridine–Acetic Anhydride Reagent* to a second glass-stoppered, 250-mL conical flask to provide the reagent blank. Fit both flasks with suitable glass-jointed reflux condensers, heat on a steam bath for 1 hour, add 10 mL of water through each condenser, and heat on the steam bath for 10 minutes more. Cool, and to each add 25 mL of butyl alcohol, previously neutralized to phenolphthalein TS with 0.5 N alcoholic potassium hydroxide, by pouring 15 mL through each condenser and, after removing the condensers, washing the sides of both flasks with the remaining 10-mL portions. To each flask add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS, recording the volume, in mL, consumed by the residual acid in the test solution as T and that consumed by the blank as B . In a 125-mL conical flask, mix about 10 g of the substance, accurately weighed, with 10 mL of freshly distilled pyridine, previously neutralized to phenolphthalein TS, add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS, recording the volume, in mL, consumed by the free acid in the test specimen as A . Calculate the Hydroxyl Value by the formula:

$$(56.11N/W)[B + (WA/C) - T]$$

in which W and C are the weights, in g, of the substances taken for the acetylation and for the free acid determination, respectively; N is the exact normality of the alcoholic potassium hydroxide; and 56.11 is the molecular weight of potassium hydroxide. If the Acid Value for the test substance is known, calculate the Hydroxyl Value by the formula:

$$(56.11N/W)[B - T] + \text{Acid Value}$$

in which W is the weight, in g, of the substance taken for the acetylation; N is the exact normality of the alcoholic potassium hydroxide; and 56.11 is the molecular weight of potassium hydroxide.

Hydroxyl Value Range	Weight of Test Specimen (g)
0–20	10
20–50	5
50–100	3
100–150	2

Hydroxyl Value Range	Weight of Test Specimen (g)
150–200	1.5
200–250	1.25
250–300	1.0
300–350	0.75

IODINE VALUE

The Iodine Value represents the number of g of iodine absorbed, under the prescribed conditions, by 100 g of the substance. Unless otherwise specified in the individual monograph, determine the Iodine Value by *Method I*.

Method I (Hanus Method)

Procedure—Transfer an accurately weighed quantity of sample, as determined from the accompanying table, into a 250-mL iodine flask, dissolve it in 10 mL of chloroform, add 25.0 mL of iodobromide TS, insert the stopper in the vessel securely, and allow it to stand for 30 minutes protected from light, with occasional shaking. Then add, in the order named, 30 mL of potassium iodide TS and 100 mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, shaking thoroughly after each addition of thiosulfate. When the iodine color becomes quite pale, add 3 mL of starch TS, and continue the titration with 0.1 N sodium thiosulfate VS until the blue color is discharged. Perform a blank test at the same time with the same quantities of the same reagents and in the same manner (see *Residual Titrations* <541>). Calculate the Iodine Value from the formula:

$$[126.90(V_B - V_S)N]/(10W)$$

in which 126.90 is the atomic weight of iodine; V_B and V_S are the volumes, in mL, of 0.1 N sodium thiosulfate VS consumed by the blank test and the actual test, respectively; N is the exact normality of the sodium thiosulfate VS; and W is the weight, in g, of the substance taken for the test. [NOTE—If more than half of the iodobromide TS is absorbed by the portion of the substance taken, repeat the determination, using a smaller portion of the substance under examination.]

Sample Weights

Iodine Value Expected	Weight in g, ±0.1
<5	3.0
5–20	1.0
21–50	0.4
51–100	0.2
101–150	0.13
151–200	0.1

Method II

Potassium Iodide Solution—Dissolve 10.0 g of potassium iodide in water to make 100 mL. Store in light-resistant containers.

Starch Indicator Solution—Mix 1 g of soluble starch with sufficient cold water to make a thin paste. Add, while stirring, to 100 mL of boiling water. Mix, and cool. Use only the clear solution.

Procedure—Melt the sample, if it is not already liquid. [NOTE—The temperature during melting should not exceed the melting point of the sample by more than 10°.] Pass through two pieces of filter paper to remove any solid im-

purities and the last traces of moisture. The filtration may be performed in an air oven at 100° but should be completed within 5 minutes \pm 30 seconds. The sample must be absolutely dry. All glassware must be absolutely clean and completely dry. After filtration, allow the filtered sample to achieve a temperature of 68° to 71 \pm 1° before weighing the sample. Once the sample has achieved a temperature of 68° to 71 \pm 1°, immediately weigh the sample into a 500-mL iodine flask, using the weights and weighing accuracy noted in the accompanying table. [NOTE—The weight of the substance must be such that there will be an excess of iodochloride TS of 50%–60% of the amount added, that is, 100%–150% of the amount absorbed.] Add 15 mL of a fresh mixture of cyclohexane and glacial acetic acid (1:1), and swirl to dissolve the sample. Add 25.0 mL of iodochloride TS, insert the stopper securely in the flask, and swirl to mix. Allow it to stand at 25 \pm 5°, protected from light, with occasional shaking, for 1.0 or 2.0 hours, depending on the Iodine Value (IV) of the sample: IV less than 150, 1.0 hour; IV equal to or greater than 150, 2.0 hours. Then, within 3 minutes after the indicated reaction time, add, in the order named, 20 mL of *Potassium Iodide Solution* and 150 mL of recently boiled and cooled water, and mix. Within 30 minutes, titrate the liberated iodine with 0.1 N sodium thiosulfate VS, while stirring by mechanical means after each addition of thiosulfate. When the yellow iodine color has almost disappeared, add 1–2 mL of *Starch Indicator Solution*, and continue the titration with 0.1 N sodium thiosulfate VS until the blue color is discharged. Perform a blank test at the same time with the same quantities of the same reagents and in the same manner (see *Residual Titrations* (541)). Calculate the Iodine Value as indicated in *Method I*.

PEROXIDE VALUE

The Peroxide Value is the number that expresses, in milliequivalents of active oxygen, the quantity of peroxide contained in 1000 g of the substance. [NOTE—This test must be performed promptly after sampling to avoid oxidation of the test specimen.]

Procedure—Unless otherwise directed, place about 5 g of the substance, accurately weighed, in a 250-mL conical flask fitted with a ground-glass stopper. Add 30 mL of a mixture of glacial acetic acid and chloroform (3:2), shake to dissolve, and add 0.5 mL of saturated potassium iodide solution. Shake for exactly 1 minute, and add 30 mL of water. Titrate with 0.01 N sodium thiosulfate VS, adding the titrant slowly with continuous shaking, until the yellow color is almost discharged. Add 5 mL of starch TS, and continue the titration, shaking vigorously, until the blue color is discharged. Perform a blank determination under the same conditions. [NOTE—The volume of titrant used in the blank determination must not exceed 0.1 mL.] Calculate the Peroxide Value by the formula:

$$[1000 (V_T - V_B)N]/W$$

in which V_T and V_B are the volumes, in mL, of 0.01 N sodium thiosulfate consumed in the actual test and in the blank test, respectively; N is the exact normality of the sodium thiosulfate solution; and W is the weight, in g, of the substance taken for the test.

SAPONIFICATION VALUE

The Saponification Value is the number of mg of potassium hydroxide required to neutralize the free acids and saponify the esters contained in 1.0 g of the substance.

Procedure—Place 1.5–2 g of the substance in a tared, 250-mL flask, weigh accurately, and add to it 25.0 mL of 0.5 N alcoholic potassium hydroxide. Heat the flask on a steam bath, under a suitable condenser to maintain reflux for 30 minutes, frequently rotating the contents. [NOTE—Re-

flux time can be up to 90 minutes to ensure complete saponification, depending on the type of ester to be tested.] Then add 1 mL of phenolphthalein TS, and titrate the excess potassium hydroxide with 0.5 N hydrochloric acid VS. Perform a blank determination under the same conditions (see *Residual Titrations* under *Titrimetry* (541)). The titration also can be carried out potentiometrically. Calculate the Saponification Value by the formula:

$$[56.11(V_B - V_T)N]/W$$

in which 56.11 is the molecular weight of potassium hydroxide; V_B and V_T are the volumes, in mL, of 0.5 N hydrochloric acid consumed in the blank test and in the actual test, respectively; N is the exact normality of the hydrochloric acid; and W is the weight, in g, of the substance taken for the test.

If the oil has been saturated with carbon dioxide for the purpose of preservation, expose it in a shallow dish in a vacuum desiccator for 24 hours before weighing the test specimens.

UNSAAPONIFIABLE MATTER

The term “Unsaponifiable Matter” in oils or fats, refers to those substances that are not saponifiable by alkali hydroxides but are soluble in the ordinary fat solvents, and to products of saponification that are soluble in such solvents.

Procedure—Transfer about 5.0 g of the oil or fat, accurately weighed, to a 250-mL conical flask, add 50 mL of an alcoholic potassium hydroxide solution prepared by dissolving 12 g of potassium hydroxide in 10 mL of water and diluting this solution with alcohol to 100 mL, and heat the flask on a steam bath under a suitable condenser to maintain reflux for 1 hour, swirling frequently. Cool to a temperature below 25°, and transfer the contents of the flask to a separator having a polytetrafluoroethylene stopcock, rinsing the flask with two 50-mL portions of water that are added to the separator (do not use grease on stopcock). Extract with three 100-mL portions of ether, combining the ether extracts in another separator containing 40 mL of water. Gently rotate or shake the separator for a few minutes. [NOTE—Violent agitation may result in the formation of a difficult-to-separate emulsion.] Allow the mixture to separate, and discard the lower aqueous phase. Wash the ether extract with two additional 40-mL portions of water, and discard the lower aqueous phase. Wash the ether extract successively with a 40-mL portion of potassium hydroxide solution (3 in 100) and a 40-mL portion of water. Repeat this potassium hydroxide solution–water wash sequence three times. Wash the ether extract with 40-mL portions of water until the last washing is not reddened by the addition of 2 drops of phenolphthalein TS. Transfer the ether extract to a tared flask, and rinse the separator with 10 mL of ether, adding the rinsings to the flask. Evaporate the ether on a steam bath, and add 6 mL of acetone to the residue. Remove the acetone in a current of air, and dry the residue at 105° until successive weighings differ by not more than 1 mg. Calculate the percentage of unsaponifiable matter in the portion of oil or fat taken by the formula:

$$100(W_R/W_S)$$

in which W_R is the weight, in g, of the residue; and W_S is the weight, in g, of the oil or fat taken for the test.

Dissolve the residue in 20 mL of alcohol, previously neutralized to the phenolphthalein endpoint, add phenolphthalein TS, and titrate with 0.1 N alcoholic sodium hydroxide VS to the first appearance of a faint pink color that persists for not less than 30 seconds. If the volume of 0.1 N alcoholic sodium hydroxide required is greater than 0.2 mL, the separation of the layers was incomplete; the residue weighed cannot be considered as “unsaponifiable matter,” and the test must be repeated.

SOLIDIFICATION TEMPERATURE OF FATTY ACIDS

Preparation of the Fatty Acids—Heat 75 mL of glycerin-potassium hydroxide solution (made by dissolving 25 g of potassium hydroxide in 100 mL of glycerin) in an 800-mL beaker to 150°, and add 50 mL of the clarified fat, melted if necessary. Heat the mixture for 15 minutes with frequent stirring, but do not allow the temperature to rise above 150°. Saponification is complete when the mixture is homogeneous, with no particles clinging to the beaker at the meniscus. Pour the contents of the beaker into 500 mL of nearly boiling water in an 800-mL beaker or casserole, add slowly 50 mL of dilute sulfuric acid (made by adding water and sulfuric acid (3:1)), and heat the solution, with frequent stirring, until the fatty acids separate cleanly as a transparent layer. Wash the acids with boiling water until free from sulfuric acid, collect them in a small beaker, place on a steam bath until the water has settled and the fatty acids are clear, filter into a dry beaker while hot, and dry at 105° for 20 minutes. Place the warm fatty acids in a suitable container, and cool in an ice bath until they congeal.

Test for Complete Saponification—Place 3 mL of the dry acids in a test tube, and add 15 mL of alcohol. Heat the solution to boiling, and add an equal volume of 6 N ammonium hydroxide. A clear solution results.

Procedure—Using an apparatus similar to the “Congealing Temperature Apparatus” specified therein, proceed as directed for *Procedure* under *Congeeing Temperature* (651), reading “solidification temperature” for “congealing point” (the terms are synonymous). The average of not less than four consecutive readings of the highest point to which the temperature rises is the solidification temperature of the fatty acids.

FATTY ACID COMPOSITION

Standard Solution—Prepare an ester mixture of known composition containing the esters required in the individual monograph. This *Standard Solution* may contain other components. [NOTE—Ester mixtures are available commercially from Nu-Chek-Prep, Inc., P.O. Box 295, Elysian, MN 56028. Typical Nu-Chek-Prep ester mixtures useful in this test include Nu-Chek 17A and Nu-Chek 19A.] Nu-Chek mixture 17A has the following composition:

Percentage	Fatty Acid Ester	Carbon-chain Length	No. of Double Bonds
1.0	Methyl myristate	14	0
4.0	Methyl palmitate	16	0
3.0	Methyl stearate	18	0
3.0	Methyl arachidate	20	0
3.0	Methyl behenate	22	0
3.0	Methyl lignocerate	24	0
45.0	Methyl oleate	18	1
15.0	Methyl linoleate	18	2
3.0	Methyl linolenate	18	3
20.0	Methyl erucate	22	1

Nu-Chek mixture 19A has the following composition:

Percentage	Fatty Acid Ester	Carbon-chain Length	No. of Double Bonds
7.0	Methyl caprylate	8	0
5.0	Methyl caprate	10	0
48.0	Methyl laurate	12	0
15.0	Methyl myristate	14	0

Percentage	Fatty Acid Ester	Carbon-chain Length	No. of Double Bonds
7.0	Methyl palmitate	16	0
3.0	Methyl stearate	18	0
12.0	Methyl oleate	18	1
3.0	Methyl linoleate	18	2

0.5 N Methanolic Sodium Hydroxide Solution—Dissolve 2 g of sodium hydroxide in 100 mL of methanol.

Test Solution—[NOTE—If fatty acids containing more than 2 double bonds are present in the test specimen, remove air from the flask by purging it with nitrogen for a few minutes.] Transfer about 100 mg of the test specimen to a 50-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 4 mL of 0.5 N *Methanolic Sodium Hydroxide Solution*, and reflux until fat globules disappear (usually 5–10 minutes). Add 5 mL of a solution prepared by dissolving 14 g of boron trifluoride in methanol to make 100 mL, swirl to mix, and reflux for 2 minutes. Add 4 mL of chromatographic *n*-heptane through the condenser, and reflux for 1 minute. Cool, remove the condenser, add about 15 mL of saturated sodium chloride solution, shake, and allow the layers to separate. Pass the *n*-heptane layer through 0.1 g of anhydrous sodium sulfate (previously washed with chromatographic *n*-heptane) into a suitable flask. Transfer 1.0 mL of this solution to a 10-mL volumetric flask, dilute with chromatographic *n*-heptane to volume, and mix.

System Suitability Solution—Transfer about 20 mg each of stearic acid, palmitic acid, and oleic acid to a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar, and proceed as directed for *Test Solution*, beginning with “Add 5.0 mL of a solution prepared by dissolving.”

Chromatographic System (see *Chromatography* (621))—The gas chromatograph is equipped with a flame-ionization detector, maintained at a temperature of about 260°, a splitless injection system, and a 0.53-mm × 30-m fused-silica capillary column bonded with a 1.0-μm layer of phase G16. The chromatograph is programmed to maintain the column temperature at 70° for about 2 minutes after injection, then to increase the temperature at the rate of 5° per minute to 240°, and finally to maintain this temperature for 5 minutes. The injection port temperature is maintained at about 220°. The carrier gas is helium with a linear velocity of about 50 cm per second.

Chromatograph the *System Suitability Solution*, and record the peak responses as directed for *Procedure*: the relative retention times are about 0.87 for methyl palmitate, 0.99 for methyl stearate, and 1.0 for methyl oleate; the resolution, *R*, between methyl stearate and methyl oleate is not less than 1.5; and the relative standard deviation of the peak area responses for the palmitate and stearate peaks for replicate injections is not more than 6.0%. The relative standard deviation of the peak area response ratio of the palmitate to stearate peaks from these replicate injections is not more than 1.0%.

Procedure—Separately inject equal volumes (about 1 μL) of the *Standard Solution* and the *Test Solution* into the chromatograph, record the chromatograms, identify the fatty acid ester peaks in the chromatogram of the *Test Solution* by comparing the retention times of these peaks with those obtained in the chromatogram of the *Standard Solution*, and measure the peak areas for all of the fatty acid ester peaks in the chromatogram obtained from the *Test Solution*. Calculate the percentage of each fatty acid component in the test specimen by the formula:

$$100(A/B)$$

in which *A* is the area of the peak response obtained for each individual fatty acid ester component; and *B* is the sum

of the peak areas of all of the peaks, excluding the solvent peak, in the chromatogram obtained from the *Test Solution*.

OMEGA-3 FATTY ACIDS DETERMINATION AND PROFILE

The following procedure may be used for the determination of eicosapentaenoic acid (EPA) (C20:5 n-3), docosahexaenoic acid (DHA) (C22:6 n-3) and total omega-3 acids obtained from fish, plant, or microbial sources in bulk oils and encapsulated oil. Protect the solutions from actinic light, oxidizing agents, oxidation catalysts, and air.

Content of EPA and DHA

USP Reference Standards (11)—USP Docosahexaenoic Acid Ethyl Ester RS. USP Eicosapentaenoic Acid Ethyl Ester RS. USP Methyl Tricosanoate RS.

Antioxidant Solution—Dissolve an accurately weighed quantity of butylated hydroxytoluene in 2,2,4-trimethylpentane to obtain a solution having a concentration of 0.05 mg per mL.

Internal Standard Solution—Transfer an accurately weighed quantity of USP Methyl Tricosanoate RS to a volumetric flask. Dissolve in *Antioxidant Solution*, and dilute with the same solvent to obtain a solution having a concentration of about 7.0 mg per mL. [NOTE—Guard the solution against evaporation during usage.]

Approx. Sum EPA + DHA	Amount of Sample to Be Weighed (g)
30%–50%	0.4–0.5
50%–70%	0.3
70%–80%	0.25

Test Solution 1 (for triglycerides)—In a 10-mL volumetric flask, dissolve the mass of sample to be examined, according to the table above, in *Antioxidant Solution*, and dilute with the same solution to volume. Transfer 2.0 mL of this solution to a glass tube, and evaporate the solvent with a gentle stream of nitrogen. Add 1.5 mL of a 2% (w/v) solution of sodium hydroxide in methanol, cap tightly with a polytetrafluoroethylene-lined cap, mix, and heat in a boiling water bath for 7 minutes. Cool, add 2 mL of boron trichloride-methanol solution (120 g in 1000 mL of methanol), cover with nitrogen, cap tightly, mix, and heat in a boiling water bath for 30 minutes. Cool to 40°–50°, add 1 mL of 2,2,4-trimethylpentane, cap, and mix on a vortex mixer or shake vigorously for at least 30 seconds. Immediately add 5 mL of saturated sodium chloride solution containing 1 volume of sodium chloride and 2 volumes of water. [NOTE—Shake from time to time. Before use, decant the solution from any undissolved substance, and filter if necessary.] Cover with nitrogen, cap, and mix on a vortex mixer or shake thoroughly for at least 15 seconds. Allow the upper layer to become clear, and transfer to a separate tube. Shake the methanol layer once more with 1 mL of 2,2,4-trimethylpentane, and combine the 2,2,4-trimethylpentane extracts. Wash the combined extracts with two quantities, 1 mL each, of water, and dry over anhydrous sodium sulfate.

Test Solution 2 (for triglycerides)—Transfer the equivalent amount of sample used to prepare *Test Solution 1* to a 10-mL volumetric flask, and dissolve in and dilute with *Internal Standard Solution* to volume. Gentle heating (up to 60°) may be applied to obtain a clear solution. Then proceed as directed in *Test Solution 1*, starting with “Transfer 2.0 mL”.

Test Solution 3 (for ethyl esters)—In a 10-mL volumetric flask, dissolve the mass of sample to be examined, according to the table above, in the *Internal Standard Solution*, and

dilute with the same solution to volume. Gentle heating (up to 60°) may be applied to obtain a clear solution.

Test Solution 4 (for ethyl esters)—Transfer the equivalent amount of sample used to prepare *Test Solution 3* to a 10-mL volumetric flask, and dissolve in and dilute with *Antioxidant Solution* to volume.

Standard Solution 1—Transfer 0.10 g each of USP Docosahexaenoic Acid Ethyl Esters RS and USP Eicosapentaenoic Acid Ethyl Esters RS, accurately weighed, to a 10-mL volumetric flask, and dissolve in and dilute with *Internal Standard Solution* to volume. Gentle heating (up to 60°) may be applied to obtain a clear solution.

Standard Solution 2—Transfer 2.0 mL of *Standard Solution 1* to a glass tube, and evaporate the solvent with a gentle stream of nitrogen. Then proceed as directed for *Test Solution 1* starting with, “Add 1.5 mL”.

System Suitability Solution 1—Transfer 0.30 g of methyl palmitate, 0.30 g of methyl stearate, 0.30 g of methyl arachidate, and 0.30 g of methyl behenate, accurately weighed, to a 10-mL volumetric flask, and dissolve in and dilute with *Antioxidant Solution* to volume.

System Suitability Solution 2—Transfer 55.0 mg of docosahexaenoic acid methyl ester and about 5.0 mg of tetracos-15-enoic acid (nervonic acid) methyl ester, accurately weighed, to a 10-mL volumetric flask, and dissolve in and dilute with *Antioxidant Solution* to volume.

Chromatographic System (see *Chromatography* (621))—The gas chromatograph is equipped with a flame-ionization detector and a 0.25-mm × 25-m fused silica capillary column coated with a 0.2-μm film of G16. The temperature of the detector is maintained at 270° and that of the injection port at 250°. The column temperature is initially set at 170° for 2 minutes, then increased at a rate of 3° per minute to 240°, and is maintained at this temperature for 2.5 minutes. The carrier gas is helium with a split flow ratio of 1:200 and a flow rate of about 1 mL per minute. [NOTE—If splitless injection mode is used, solutions should be further diluted 1 in 200.] Chromatograph *System Suitability Solution 1* and *System Suitability Solution 2*, and record the peak responses as directed for *Procedure*: using *System Suitability Solution 1*, the area percent increases in the following order; methyl palmitate, methyl stearate, methyl arachidate, methyl behenate; the difference between the percent area of methyl palmitate and that of methyl behenate is less than 2.0 area percent units; using *System Suitability Solution 2*, the resolution, *R*, between docosahexaenoic acid methyl ester and tetracos-15-enoic acid methyl ester is not less than 1.2. [NOTE—In addition to the above system suitability requirements, the following is required for the analysis of triglycerides but not for ethyl esters.] For triglycerides, chromatograph *Standard Solution 1* and *Standard Solution 2*, and record the peak responses as directed for *Procedure*: the derivatization efficiency for the conversion of fatty acid ethyl ester to the fatty acid methyl ester is not less than 90.0% for each (DHA and EPA).

Procedure—Separately inject duplicate equal volumes (about 1 μL) of *Standard Solution 1*, *Standard Solution 2*, *Test Solution 1* (for triglycerides), *Test Solution 2* (for triglycerides), *Test Solution 3* (for ethyl esters), and *Test Solution 4* (for ethyl esters) into the chromatograph, record the chromatograms, and measure the peak responses. Identify the retention time for the internal standard peak by comparing the chromatograms for *Test Solution 1* and *Test Solution 2* (for triglycerides) and by comparing the chromatograms for *Test Solution 3* and *Test Solution 4* (for ethyl esters). Calculate the percentage of EPA or DHA in the triglyceride taken by the formula:

$$100F(C/W)(R_U/R_S)$$

in which *F* is the factor to express the content of DHA (*F* = 0.921) and of EPA (*F* = 0.915) as free fatty acids; *C* is the concentration, in mg per mL, of either DHA or EPA in *Stan-*

Standard Solution 2; W is the weight, in mg, of the sample taken to prepare *Test Solution 1*; R_s is the ratio of peak responses of either EPA or DHA relative to the internal standard in the chromatogram of *Standard Solution 2*; and R_U is the corrected peak response of either EPA or DHA relative to the internal standard in the chromatogram of *Test Solution 1* calculated as follows:

$$1/(r_{U2}/r_{T2} - r_{U1}/r_{T1})$$

in which r_{U2} is the peak response of any peak at the locus of the internal standard in the chromatogram of *Test Solution 2*; r_{U1} is the peak response of any peak at the locus of the internal standard in the chromatogram of *Test Solution 1*; r_{T1} is the peak response of EPA or DHA in the chromatogram of *Test Solution 1*; and r_{T2} is the peak response of EPA or DHA in the chromatogram of *Test Solution 2*. Calculate the percentage of EPA or DHA in the ethyl ester taken by the formula:

$$100F(C/W)(R_U/R_s)$$

in which F is the factor to express the content of DHA ($F = 0.921$) and of EPA ($F = 0.915$) as free fatty acids; C is the concentration, in mg per mL, of either DHA or EPA in *Standard Solution 1*; W is the weight, in mg, of the sample taken to prepare *Test Solution 3*; R_s is the ratio of peak responses of either EPA or DHA relative to the internal standard in the chromatogram of *Standard Solution 1*; and R_U is the corrected peak response of either EPA or DHA relative to the internal standard in the chromatogram of *Test Solution 3*, calculated as follows:

$$1/(r_{U2}/r_{T2} - r_{U1}/r_{T1})$$

in which r_{U2} is the peak response of any peak at the locus of the internal standard in the chromatogram of *Test Solution 3*; r_{U1} is the peak response of any peak at the locus of the internal standard in the chromatogram of *Test Solution 4*; r_{T1} is the peak response of EPA or DHA in the chromatogram of *Test Solution 4*; and r_{T2} is the peak response of EPA or DHA in the chromatogram of *Test Solution 3*.

Content of Total Omega-3 Acids

Calculate the content of the total omega-3 acids by the formula:

$$\text{EPA} + \text{DHA} + ((A_{n-3} \times (\text{EPA} + \text{DHA})) / (A_{\text{EPA}} + A_{\text{DHA}}))$$

in which EPA is the content of EPA, in mg per g, obtained from the test for *Content for EPA and DHA*; DHA is the content of DHA, in mg per g, obtained from test for *Content of EPA and DHA*; A_{n-3} is the sum of the areas of the peaks corresponding to C18:3 n-3, C18:4 n-3, C20:4 n-3, C21:5 n-3, and C22:5 n-3 methyl esters in the chromatogram obtained with *Test Solution 1* for triglycerides or the corresponding ethyl esters in the chromatogram obtained with *Test Solution 4*; A_{EPA} is the area of the peak corresponding to the EPA methyl ester in the chromatogram obtained with *Test Solution 1* for triglycerides or the peak corresponding to the EPA ethyl ester in the chromatogram obtained with *Test Solution 4* for ethyl esters; and A_{DHA} is the area of the peak corresponding to the DHA methyl ester in the chromatogram obtained with *Test Solution 1* for triglycerides or the peak corresponding to the DHA ethyl ester in the chromatogram obtained with *Test Solution 4*.

WATER AND SEDIMENT IN FIXED OILS

Apparatus—The preferred centrifuge has a diameter of swing (d = distance from tip to tip of whirling tubes) of

38–43 cm and is operated at a speed of about 1500 rpm. If a centrifuge of different dimensions is used, calculate the desired rate of revolution by the formula:

$$\text{rpm} = 1500\sqrt{40.6/d}$$

The centrifuge tubes are pear-shaped, and are shaped to accept closures. The total capacity of each tube is about 125 mL. The graduations are clear and distinct, reading upward from the bottom of the tube according to the scale shown in the accompanying table.

Volume (mL)	Scale Division (mL)
0–3	0.1
3–5	0.5
5–10	1.0
10–25	5.0
25–50	25.0
50–100	50.0

Procedure—Place 50.0 mL of benzene in each of two centrifuge tubes, and to each tube add 50.0 mL of the oil, warmed if necessary to re-incorporate separated stearin, and mixed thoroughly at 25°. Insert the stopper tightly into the tubes, and shake them vigorously until the contents are mixed thoroughly, then immerse the tubes in a water bath at 50° for 10 minutes. Centrifuge for 10 minutes. Read the combined volume of water and sediment at the bottom of each tube. Centrifuge repeatedly for 10-minute periods until the combined volume of water and sediment remains constant for three consecutive readings. The sum of the volumes of combined water and sediment in the two tubes represents the percentage, by volume, of water and sediment in the oil.

ANISIDINE VALUE

The Anisidine Value is defined as 100 times the optical density measured in a 1-cm cell of a solution containing 1 g of the substance to be examined in 100 mL of a mixture of solvents and reagents according to the method described below. [NOTE—Carry out the operations as rapidly as possible, avoiding exposure to actinic light.]

Test Solution A—Dissolve 0.500 g of the substance to be examined in isooctane, and dilute with the same solvent to 25.0 mL.

Test Solution B—To 5.0 mL of *Test Solution A* add 1.0 mL of a 2.5 g per L solution of *p*-anisidine in glacial acetic acid, shake, and store protected from light.

Standard Solution—To 5.0 mL of isooctane add 1.0 mL of a 2.5 g per L solution of *p*-anisidine in glacial acetic acid, shake, and store protected from light.

Procedure—Measure the absorbance of *Test Solution A* at 350 nm using isooctane as the blank. Measure the absorbance of *Test Solution B* at 350 nm exactly 10 minutes after its preparation, using the *Standard Solution* as the compensation liquid. Calculate the Anisidine Value from the expression:

$$25(1.2A_s - A_b)/m$$

in which A_s is the absorbance of *Test Solution B* at 350 nm; A_b is the absorbance of *Test Solution A* at 350 nm; and m is the weight, in g, of the substance to be examined in *Test Solution A*.

TOTAL OXIDATION VALUE (TOTOX)

Total Oxidation Value is defined by the formula:

$$2PV + AV$$

in which *PV* is the Peroxide Value, and *AV* is the Anisidine Value.

TRACE METALS

Apparatus

The apparatus typically consists of the following:

Digestion Flasks—Use a polytetrafluoroethylene flask with a volume of about 120 mL, fitted with an airtight closure, a valve to adjust the pressure inside the container, and a polytetrafluoroethylene tube to allow the release of gas.

System—Make the flask airtight, using the same torsional force for each of them.

Microwave Oven—It has a magnetron frequency of 2450 MHz, with a selectable output from 0 to 630 ± 70 W in 1% increments, a programmable digital computer, a polytetrafluoroethylene-coated microwave cavity with a variable speed exhaust fan, a rotating turntable drive system, and exhaust tubing to vent fumes.

Atomic Absorption Spectrometer—It is equipped with a hollow-cathode lamp as the source of radiation and a deuterium lamp as a background corrector; the system is fitted with the following:

1. A graphite furnace as the atomization device for cadmium, copper, iron, lead, nickel, and zinc.
2. An automated continuous-flow hydride vapor generation system for arsenic and mercury.

General Procedure

Caution—When using closed high-pressure digestion vessels and microwave laboratory equipment, the safety precautions and operating instructions given by the manufacturer must be followed.

[NOTE—If an alternative apparatus is used, adjustment of the instrument parameters may be necessary.]

Cleaning—Clean all the glassware and laboratory equipment with a 10 mg per mL solution of nitric acid before use.

Trace Metal-Free Nitric Acid—Nitric acid meets the requirements with the maximum values for arsenic (As), cadmium (Cd), copper (Cu), iron (Fe), mercury (Hg), lead (Pb), nickel (Ni), and zinc (Zn) equal to 0.005, 0.005, 0.001, 0.02, 0.002, 0.001, 0.005, and 0.01 ppm, respectively.

Trace Metal-Free Hydrochloric Acid—Hydrochloric acid meets the requirements with the maximum values for As, Cd, Cu, Fe, Hg, Pb, Ni, and Zn equal to 0.005, 0.003, 0.003, 0.05, 0.005, 0.001, 0.004, and 0.005 ppm, respectively.

Trace Metal-Free Sulfuric Acid—Sulfuric acid meets the requirements with the maximum values for As, Cd, Cu, Fe, Hg, Pb, Ni, and Zn equal to 0.005, 0.002, 0.001, 0.05, 0.005, 0.001, 0.002, and 0.005 ppm, respectively.

Test Stock Solution—In a digestion flask place about 0.5 g of fatty oil, accurately weighed, as indicated in each individual monograph. Add 6 mL of *Trace Metal-Free Nitric Acid* and 4 mL of *Trace Metal-Free Hydrochloric Acid*. Close the flask.

Blank Stock Solution—Mix 6 mL of *Trace Metal-Free Nitric Acid* and 4 mL of *Trace Metal-Free Hydrochloric Acid* in a digestion flask.

Test Solution 1—Place the digestion flask containing the *Test Stock Solution* in the microwave oven. Carry out the digestion in three steps according to the following program: 80% power for 15 minutes, 100% power for 5 minutes, and 80% power for 20 minutes.

At the end of the cycle allow the flask to cool. Add 4 mL of *Trace Metal-Free Sulfuric Acid* to the flask. Repeat the digestion program. After completing the digestion, allow the flask to cool to room temperature. Open the digestion flask, and transfer the clear, colorless solution obtained into a 50-mL volumetric flask. Rinse the digestion flask with 2 quantities, 15 mL each, of water, and collect the rinsings in the volumetric flask. Add 1.0 mL of a 10 mg per mL solution of magnesium nitrate and 1.0 mL of a 100 mg per mL solution of ammonium dihydrogen phosphate to the volumetric flask. Dilute with water to volume, and mix. This solution is *Test Solution 1*.

Blank Solution 1—Place the digestion flask containing *Blank Stock Solution* in the microwave oven. Proceed as directed under *Test Solution 1* beginning with "Carry out the digestion in three steps according to the following program".

Direct Calibration—[NOTE—Concentrations of the standard solutions will depend on the metal contents of the test substance.] For routine measurements, three standard solutions, *Blank Solution 1*, and *Test Solution 1* are prepared and examined.

Use *Test Solution 1* and *Blank Solution 1* as prepared above or as indicated in the monograph. Prepare not fewer than three standard solutions containing all the metal elements to be tested. The expected absorbance value in *Test Solution 1* for each metal element should be within its corresponding calibrated absorbance range, preferably in the middle of the calibrated absorbance range. Any reagents used in the preparation of *Test Solution 1* are added at the same concentration to the standard solutions.

Introduce each of the solutions into the instrument using the same number of replicates for each of the solutions to obtain a steady reading.

Prepare a calibration curve from the mean of the readings obtained with the standard solutions by plotting the means as a function of concentration. Determine the concentration of the element in *Test Solution 1* from the curve obtained.

Standard Additions—Add to at least four identical volumetric flasks equal volumes of *Test Solution 1*, as prepared above or as indicated in the monograph. Add to all but one of the flasks progressively larger volumes of a standard solution containing a known concentration of the test element to produce a series of solutions containing steadily increasing concentrations of that element known to give responses in the linear part of the curve. Dilute the contents of each flask with the solvent specified in the monograph to volume, and mix. The flask without an addition of standard solution is labeled as the test solution.

Introduce each of the solutions into the instrument, using the same number of replicates for each of the solutions, to obtain a steady reading.

Plot the absorbances of the standard solutions and the test solution versus the added quantity of test element. [NOTE—The test solution should be plotted as if it had a content of added test element equivalent to 0 mg or μg .] Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of test element in the test solution.

Table 1

	Cd	Cu	Fe	Pb	Ni	Zn
Wavelength (nm)	228.8	324.8	248.3	283.5	232	213.9
Slit (nm)	0.5	0.5	0.2	0.5	0.2	0.5
Lamp current (mA)	6	7	5	5	10	7
Ignition temperature (°)	800	800	800	800	800	800
Atomization temperature (°)	1800	2300	2300	2200	2500	2000
Background corrector	On	Off	Off	Off	Off	Off
Nitrogen flow (L per minute)	3	3	3	3	3	3

Specific Tests

CADMIUM (Cd), COPPER (Cu), IRON (Fe), LEAD (Pb),
NICKEL (Ni), AND ZINC (Zn)

Standard Stock Solution—Prepare a solution containing known concentrations of 5 µg per mL for each test element.

Standard Solutions—In three identical 10-mL volumetric flasks, introduce 10, 20, and 40 µL of *Standard Stock Solution*, respectively. To each flask, add 5.0 mL of *Test Solution 1*, dilute with water to volume, and mix.

Test Solution 2—In a 10-mL volumetric flask, add 5.0 mL of *Test Solution 1*, dilute with water to volume, and mix.

Blank Solution 2—In a 10-mL volumetric flask, add 5.0 mL of *Blank Solution 1*, dilute with water to volume, and mix.

Procedure—Measure the content of Cd, Cu, Fe, Pb, Ni, and Zn using a suitable graphite furnace atomic absorption spectrophotometer. Concomitantly determine the absorbances of *Blank Solution 2*, the *Standard Solutions*, and *Test Solution 2* at least three times each. The absorbance value of *Blank Solution 2* is subtracted from the value obtained using the *Standard Solutions* and *Test Solution 2*. Proceed as directed in the *Standard Additions* method in *General Procedure* above. See *Table 1* for instrumental parameters that may be used.

ARSENIC AND MERCURY

Measure the content of arsenic and mercury against their standard solutions of arsenic or mercury at a known concentration using the *Direct Calibration* method from the section *General Procedure* above, with an automated continuous-flow hydride vapor generation system.

For 1 ppm arsenic specification limit and 1 ppm mercury specification limit, prepare three working calibration solutions having known concentrations of 5 ng per mL, 10 ng per mL, and 20 ng per mL for each test element, respectively.

The absorbance value of the blank solution is automatically subtracted from the value obtained using the test solution.

Arsenic—

Blank Solution 3—Add 1.0 mL of a 200 mg per mL solution of potassium iodide to 19.0 mL of *Blank Solution 1* prepared above. Allow this solution to stand at room temperature for about 50 minutes or at 70° for about 4 minutes.

Test Solution 3—Add 1.0 mL of a 200 mg per mL solution of potassium iodide to 19.0 mL of *Test Solution 1* prepared above. Allow this solution to stand at room temperature for about 50 minutes or at 70° for about 4 minutes.

Acid Reagent 1: *Trace Metal-Free Hydrochloric Acid*.

Reducing Reagent 1: a 6 mg per mL solution of sodium tetrahydroborate in a 5 mg per mL solution of sodium hydroxide.

The instrumental parameters in *Table 2* may be used.

Mercury—

Blank Solution 4—Proceed as directed for *Blank Solution 3*.

Test Solution 4—Proceed as directed for *Test Solution 3*.

Acid Reagent 2: a 515 mg per mL solution of *Trace Metal-Free Hydrochloric Acid*.

Reducing Reagent 2: a 10 mg per mL solution of stannous chloride in a 200 mg per mL solution of *Trace Metal-Free Hydrochloric Acid*.

The instrumental parameters in *Table 2* may be used.

Table 2

	As	Hg
Wavelength (nm)	193.7	253.7
Slit width (nm)	0.2	0.5
Lamp current (mA)	10	4
Acid reagent flow rate (mL per minute)	1.0	1.0
Reducing reagent flow rate (mL per minute)	1.0	1.0
Flow rate for the blank, standard, test solutions (mL per minute)	7.0	7.0
Absorption cell	Quartz (heated)	Quartz (unheated)
Background corrector	Off	Off
Nitrogen flow rate (L per minute)	0.1	0.1

STEROL COMPOSITION

Separation of the Sterol Fraction

Reference Solution A—Dissolve an accurately weighed quantity of cholesterol in chloroform to obtain a solution of 5% (w/v).

Developing Solvent System: a mixture of toluene and acetone (95:5) or a mixture of hexane and ether (65:35).

Test Solution A—Weigh accurately 5 g of the test substance into a 250-mL flask. Add 50 mL of alcoholic potassium hydroxide TS 2 (2 N alcoholic potassium hydroxide), and heat to gentle boiling with continuous vigorous stirring until saponification takes place (the solution becomes clear). Continue heating for a further 20 minutes, and add 50 mL of water from the top of the condenser. Cool the flask to approximately 30°. Transfer the contents of the flask to a 500-mL separating funnel with several rinses of water, amounting in all to about 50 mL. Add approximately 80 mL of ether, shake vigorously for approximately 30 seconds, and allow to settle. [NOTE—Any emulsion can be destroyed by adding small quantities of ethyl or methyl alcohol by means of a spray.] Separate the lower aqueous phase, and collect it into a second separating funnel. Perform two further extractions on the water-alcohol phase in the same way, using 60–70 mL of ether on each occasion. Pool the ether extracts into a single separating funnel, and wash with water, 50 mL at a time, until the wash water is no longer alkaline to phe-

nolphthalein. Dry the ether phase with anhydrous sodium sulfate, and filter on anhydrous sodium sulfate into a previously weighed 250-mL flask, washing the funnel and filter with small quantities of ether. Distill the ether down to a few mL, and bring to dryness under a slight vacuum or in a stream of nitrogen. Complete the drying at 100° for approximately 15 minutes, and then weigh after cooling in a desiccator. Dissolve the unsaponifiables so obtained in chloroform to obtain a solution having a concentration of approximately 5%.

Test Solution B—Treat 5 g of canola oil in the same way as prescribed for the test substance in *Test Solution A*, beginning with “Add 50 mL of alcoholic potassium hydroxide TS 2 (2 N alcoholic potassium hydroxide)”.

Test Solution C—Treat 5 g of sunflower oil in the same way as prescribed for the test substance in *Test Solution A*, beginning with “Add 50 mL of alcoholic potassium hydroxide TS 2 (2 N alcoholic potassium hydroxide)”.

Procedure—Immerse the thin-layer chromatographic plate (see *Chromatography* (621)), 20-cm × 20-cm silica gel on polyester with a layer thickness of 200 µm and particle size of 5–17 µm,¹ completely in the 0.2 N alcoholic potassium hydroxide for 10 seconds, then allow to dry in a fume cupboard for 2 hours, and finally place at 100° for 1 hour. [NOTE—Remove from the validated heating device, and keep the plate in a desiccator until required for use. The plates must be used within 15 days. Thin-layer chromatographic plates without requiring the preconditioning are also commercially available.] Use a separate plate for each test solution.

Place a mixture of toluene and acetone (95:5) or a mixture of hexane and ether (65:35) in the chamber to a depth of approximately 1 cm. Close the chamber with the appropriate cover, and leave for at least 30 minutes. Strips of filter paper dipping into the eluent may be placed on the internal surfaces of the chamber. [NOTE—The developing mixture should be replaced for every test to ensure reproducible elution conditions.] Apply 0.3 mL of *Test Solution A* approximately 2 cm from the lower edge in a streak which is as thin and as uniform as possible. In line with the streak, place 2–3 µL of *Reference Solution A* at one end of the plate. Develop the chromatograms in an equilibrated chamber with the *Developing Solvent System* until the solvent front reaches approximately 1 cm from the upper edge of the plate. Remove the plate from the developing chamber, and evaporate the solvent under a current of hot air [NOTE—Avoid excessive heat.] or by leaving the plate for a short while under a hood. Spray the plate with a 0.2% alcoholic solution of 2,7-dichlorofluorescein, and examine in UV light at 254 nm. [NOTE—The plates pretreated with UV indicator are also commercially available and used equivalently.] In each of the plates, mark the limits of the sterol band identified through being aligned with the stain obtained from *Reference Solution A* along the edges of the fluorescence, and additionally include the area of the zones 2–3 mm above and below the visible zones corresponding to *Reference Solution A*. Remove the silica gel in the marked area into a filter funnel with a G3 porous septum.² Add 10 mL of hot chloroform, mix carefully with the metal spatula, filter under vacuum, and collect the filtrate in the conical flask attached to the filter funnel. Wash the residue in the funnel three times with ether, about 10 mL each time, and collect the filtrate in the same flask attached to the funnel. Evaporate the filtrate to a volume of 4–5 mL, transfer the residual solution to a previously weighed 10-mL test tube with a tapering bottom and a sealing stopper, and evaporate to dryness by mild heating in a gentle stream of nitrogen. Dissolve the residue in a few drops of acetone, and evaporate again to dryness. Place at 105° for approximately 10 minutes, allow to cool in a desiccator, and weigh.

¹ A commercial TLC plate may be obtained from Sigma-Aldrich, catalog #z122785.

² A commercial product may be obtained from Kimble/Kontes as a filter, buchner with fritted disc, Kimax 28400-152.

Treat *Test Solution B* and *Test Solution C* the same way as directed for *Test Solution A*.

Determination of the Sterols

Test Solution D—To the test tube containing the sterol fraction separated from the test substance by thin-layer chromatography, add a freshly prepared mixture of anhydrous pyridine, hexamethyldisilazane, and chlorotrimethylsilane (9:3:1) [NOTE—This reagent is also commercially available and used equivalently.] in the ratio of 50 µL for every mg of sterols, avoiding any uptake of moisture. Insert the stopper into the test tube, and shake carefully until the sterols are completely dissolved. Allow it to stand for at least 15 minutes at ambient temperature, and centrifuge for a few minutes if necessary. Use the supernatant. [NOTE—The slight opalescence that may form is normal and does not cause an anomaly. However, the formation of a white floc or the appearance of a pink color is indicative of the presence of moisture or deterioration of the reagent. If these occur, the test must be repeated.]

Reference Solution E—To 9 parts of the sterols separated from canola oil by thin-layer chromatography, add 1 part of cholesterol. Treat the mixture in the same way as directed for *Test Solution D*.

Reference Solution F—Treat the sterols separated from sunflower oil by thin-layer chromatography in the same way as directed for *Test Solution D*.

Chromatographic System (see *Chromatography* (621))—The gas chromatograph is equipped with a flame-ionization detector and a glass or fused-silica capillary column of length 20–30 m, internal diameter 0.25–0.32 mm, entirely coated with a 0.10- to 0.30-µm layer of stationary phase G27 or G36. The injection port temperature is maintained at 280°, the detector temperature is maintained at 290°, and the column temperature is maintained at 260 ± 5°. The carrier gas is either helium with a linear velocity of 20–35 cm per second or hydrogen with a linear velocity of 30–50 cm per second. A split ratio of 1:50 to 1:100 is used. Chromatograph *Reference Solution E* and *Reference Solution F*, and record the peak responses as directed for *Procedure*: the retention time should be 20 ± 5 minutes for β -sitosterol, and all the sterols present must be separated. The chromatogram obtained with *Reference Solution E* shows four principal peaks corresponding to cholesterol, brassicasterol, campesterol, and β -sitosterol; and the chromatogram obtained with *Reference Solution F* shows four principal peaks corresponding to campesterol, stigmasterol, β -sitosterol, and Δ^7 -stigmastenol. The retention times of the sterols with reference to β -sitosterol are given in *Table 3*.

Table 3. Relative Retention Times of Sterols for Two Different Columns

Identification	G36 Column	G27 Column
Cholesterol	0.67	0.63
Brassicasterol	0.73	0.71
24-Methylene-cholesterol	0.82	0.80
Campesterol	0.83	0.81
Campestanol	0.85	0.82
Stigmasterol	0.88	0.87
Δ^7 -Campesterol	0.93	0.92
$\Delta^5,23$ -Stigmastadienol	0.95	0.95
Clerosterol	0.96	0.96
β -Sitosterol	1.00	1.00
Sitostanol	1.02	1.02
Δ^5 -Avenasterol	1.03	1.03
$\Delta^5,24$ -Stigmastadienol	1.08	1.08
Δ^7 -Stigmastenol	1.12	1.12
Δ^7 -Avenasterol	1.16	1.16

Procedure—Separately inject equal volumes (about 1 μL) of *Test Solution D*, *Reference Solution E*, and *Reference Solution F* into the chromatograph, record the chromatograms, and measure the peak areas for the sterols. Calculate the percentage of each individual sterol in the sterol fraction of the test substance taken by the formula:

$$100(A/S)$$

in which *A* is the area of the peak due to the sterol component to be determined, and *S* is the sum of the areas of the peaks due to the components indicated in *Table 3*.

(411) FOLIC ACID ASSAY

The following procedure is provided for the estimation of folic acid as an ingredient of Pharmacopeial preparations containing other active constituents.

USP Reference Standards (11)—*USP Folic Acid RS*.

Mobile Phase—Place 2.0 g of monobasic potassium phosphate in a 1-liter volumetric flask, and dissolve in about 650 mL of water. Add 12.0 mL of a 1 in 4 solution of tetrabutylammonium hydroxide in methanol, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with either 3 N phosphoric acid or 6 N ammonium hydroxide to a pH of 7.0, dilute with water to volume, and mix. Pass through a 0.45- μm filter, and recheck the pH before use. [NOTE—The methanol-to-water ratio may be varied by up to 3 percent and the pH may be increased up to 7.15 to achieve better separation.]

Diluting Solvent—Prepare as directed under *Mobile Phase*. Adjust to a pH of 7.0, and bubble nitrogen through the solution for 30 minutes before use.

Internal Standard Solution—Dissolve about 25 mg of methylparaben in 2.0 mL of methanol, dilute with *Diluting Solvent* to 50 mL, and mix.

Standard Folic Acid Solution—Transfer about 12 mg of USP Folic Acid RS, accurately weighed, to a low-actinic, 50-mL volumetric flask, dissolve in 2 mL of ammonium hydroxide, dilute with *Diluting Solvent* to volume, and mix.

Standard Preparation—Transfer 2.0 mL of *Standard Folic Acid Solution* to a low-actinic, 25-mL volumetric flask, add 2.0 mL of *Internal Standard Solution*, add *Diluting Solvent* to volume, and mix.

Assay Preparation—Transfer an accurately weighed or measured portion of the preparation to be assayed, containing about 1 mg of folic acid, to a low-actinic, 50-mL volumetric flask, add 4.0 mL of *Internal Standard Solution*, add *Diluting Solvent* to volume, and mix.

Chromatographic System (see *Chromatography* (621))—The liquid chromatograph is equipped with a 280-nm detector and a 15-cm \times 3.9-mm column that contains packing L1. The flow rate is about 1.0 mL per minute. Chromatograph the *Standard Preparation*, and record the peak responses as directed for *Procedure*: there is baseline separation of folic acid and methylparaben.

Procedure—Separately inject equal volumes (about 10 μL) of *Standard Preparation* and *Assay Preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. The relative retention times are about 0.8 for folic acid and 1.0 for methylpara-

ben. Calculate the quantity, in μg , of $\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$ in the portion of the preparation taken by the formula:

$$50C(R_U/R_S)$$

in which *C* is the concentration, in μg per mL, of USP Folic Acid RS in the *Standard Preparation*; and R_U and R_S are the ratios of the response of the folic acid peak to that of the methylparaben peak obtained from the *Assay Preparation* and the *Standard Preparation*, respectively.

(413) IMPURITIES TESTING IN MEDICAL GASES

INTRODUCTION

This general test chapter defines the safe and proper means to sample high-pressure gas containers of different medical gas compositions using the detector tube manufacturer's suggested total gas volume for the purpose of conducting detector tube analysis to satisfy the USP monographs. See *Reagents* in the section *Reagents, Indicators, and Solutions* for information on each referenced detector tube.

There are two types of detector tubes currently manufactured: those to be used with a manual hand pump of fixed volume (e.g., 100 mL/pump stroke) and those used in a continuous flow system that can be set to pass a volume of gas through the detector tube at approximately 1 atmosphere. It is important to match the appropriate detector tube type to the mode of gas volume exchange.

To ensure that the required gas volume has passed through the detector tube, measure the gas volume at the time of the analysis either by using a hand pump or using a flowmeter that is calibrated to the subject gas or corrected via a calibration chart. Flowmeter manufacturers generally provide a chart of the gas volume flow of common gases for each of the flow tubes identified in this general chapter. [NOTE—See general chapter *Medical Gases Assay* (415) for sampling.]

Continuous Flow System

Identify the gas contained in the container, and select the appropriate gas regulator. Secure the regulator to the gas container. Do not apply lubricant or Teflon tape to the container-to-regulator connections. Purge the regulator with the gas under test. Select float setting to obtain the proper flow rate to achieve total gas volume as recommended by the manufacturer. Attach the detector tube, then adjust the flow rate to the required level as indicated by the charts that accompany the flowmeter and the detector tube. Time the gas flow to achieve the desired total gas volume ± 10 s. At the allotted time close the regulator valve, then the main container valve. Observe the tube while it is still attached to determine the degree of color change, and record the result. Remove the tube, and disconnect the apparatus, vent the regulator gas pressure to atmosphere, and remove the regulator from the container. Dispose of the tube after use.

Hand Pump—Fixed Volume

The alternative approach is to apply the gas detector hand pump. The system draws a consistent volume with

each pump stroke. To ensure the accuracy of the total gas volume, the user must follow the pump manufacturer's suggested recommendation about how to pump.

<415> MEDICAL GASES ASSAY

INTRODUCTION

The evaluation of the purity of a gas used for medical treatment or as a component of a pharmaceutical process is the purpose of a *USP* medical gas monograph. The purity generally is evaluated by an assay for the content of the article and by analyses for trace impurities. The application of gas chromatography, paramagnetic analysis, and detector tubes to medical gases is somewhat different from traditional procedures used for analytes in the liquid phase and therefore warrants a separate description. This general test chapter focuses on the assay for content tests. Sampling for impurities is addressed in general chapter *Impurities Testing in Medical Gases* (413).

This chapter includes sampling and qualification aspects of gas chromatographic and paramagnetic analyses of medical gases. In addition, it includes a description of the initial set-up, validation, and calibration of these instruments. The specific assay procedures are defined in the specific monograph for that gas.

The basic definitions of instrumental qualification and validation are included in general information chapters *Analytical Instrument Qualification* (1058) and *Validation of Compendial Methods* (1225), respectively, and will not be repeated. However, when variations of the materials presented in these chapters are necessary due to the character of the analyte, this chapter will define those variations.

METHODS

Gas Chromatography (GC)

See *Chromatography* (621).

Detectors for Medical Gases Assay

The two most common detectors used in the analyses of medical gases are the thermal conductivity detector (TCD) and flame ionization detector (FID).

The TCD will detect any gas or vapor that has a thermal conductivity (TC) that differs significantly from the high TC of the reference gas, usually helium, therefore it is virtually universal. However, the generally accepted lower detection limit for the TCD is 50 ppm v/v. This represents a limitation for the evaluation of trace impurities in medical gases.

The FID is also used for the evaluation of trace impurities in medical gases, because it is more sensitive to organic compounds but does not produce a signal for most common medical gases.

QUALIFICATION

Installation Qualification (IQ)—The IQ requirements ensure the gas chromatograph hardware and software (or readout device) is installed safely and in accordance with the GC manufacturer's instructions.

Consideration should be given to the following as applicable:

- Suitability of the sample system (including connections);
- Leakage (should be leak free);
- Representative sampling;
- Sample flow rates;
- Response time;
- Correct output signals;
- Power supply (including voltage regulation); and
- Appropriate environmental conditions of the instrument and of the sample itself (e.g., temperature and pressure).

Operational Qualification (OQ)—OQ verifies that the GC performs as intended within its anticipated operating range. For medical gas final product testing, the GC is tested to ensure repeatability (verification that relative standard deviation is consistent with claims) for each analyte of interest. Due to the specific nature of medical gas testing and the limited number of analytes, routine calibration and periodic calibration verification of a GC instrument and the testing procedure may be used in place of initial or periodic OQ. When an instrument is used for a broader range of analytes, the replacement of OQ with calibration and periodic calibration verification is inappropriate.

Performance Qualification (PQ)—For medical gas final product testing, the GC is periodically checked at appropriate intervals during analytical runs with a calibration gas (i.e., verifying that the results are consistent with a named concentration within acceptable accuracy and precision ranges after a specific number of sample injections).

Paramagnetic Oxygen Measurement

Theory—The paramagnetic analyzer measures the displacement of a diamagnetic gas (nitrogen) by a paramagnetic gas (oxygen), in a strong magnetic field. A measuring cell typically employs a glass dumbbell with nitrogen-filled spheres that is suspended on a torsion strip between magnets that concentrate the flux around the dumbbell. When oxygen molecules enter the measuring cell, the dumbbell is deflected by the force exerted by the oxygen molecules that are attracted to the strongest part of the magnetic field. By using optical sensors, a feedback coil, and suitable electronics, analysts measure an output that is directly proportional to the partial pressure of oxygen.

Oxygen is the only paramagnetic gas present above trace levels in the atmosphere. However, paramagnetic analyzers can be affected by the magnetic susceptibility of the background gas. Therefore changes to background gases in *USP* monographs should be avoided.

Design Considerations

The design considerations for the purchase of new instruments may include the following parameters.

Drift—A change of the output of the instrument for a given concentration over a stated period of time under constant conditions and without any adjustments being made to the instrument by external means. Drift is the summation of two components, zero drift and span drift. Drift determines the frequency of instrument calibration.

Zero Drift—A change in the output when zero gas is being measured.

Span Drift—A change in the output at the level of oxygen concentration that is being measured.

Operating Temperature—The ambient temperature range for which the stated performance specification of the instrument will remain valid. A larger temperature coefficient will indicate that a smaller change in ambient temperature is permitted before re-calibration is required.

Operating Pressure—The instrument should operate at the inlet pressures of the samples to be tested.

Qualification Aspects

Installation Qualification (IQ)—The IQ requirements ensure the oxygen analyzer hardware and software (or readout device) is installed safely and in accordance with the oxygen analyzer manufacturer's instructions.

Consideration should be given to the following as applicable:

- Suitability of the sample system (including connections);
- Leakage (should be leak free);
- Representative sampling;
- Sample flow rates;
- Response time;
- Correct output signals;
- Power supply (including voltage regulation); and
- Appropriate environmental conditions of the instrument and of the sample itself (e.g., temperature and pressure).

Operational Qualification (OQ)—The OQ requirements verify that the paramagnetic analyzer performs as intended within its anticipated operating range and is suitable for the actual conditions of use. Instruments and apparatus should be calibrated and used in accordance with the oxygen analyzer manufacturer's instructions. Because of the specific nature of the instrument, routine calibration may be used in place of initial or periodic OQ testing.

Performance Qualification (PQ)—The PQ requirements verify that the paramagnetic analyzer performs as intended in its normal operating environment. For medical gas final product testing, the paramagnetic analyzer is initially calibrated (zeroed and spanned using a certified standard) in accordance with the oxygen analyzer manufacturer's instructions and is periodically recalibrated to ensure continued acceptable performance.

Zeroing the Instrument (establishing the lower limit)—Using the certified standard defined in the monograph, establish a zero setting on the analyzer by passing the zero gas into the analyzer at the oxygen analyzer manufacturer's suggested flow rate. Maintain the flow until a stable reading is observed on the instrument. As necessary, adjust the zero setting to a value of 0.0% according to the oxygen analyzer manufacturer's instructions. Confirm the reading is stable. [NOTE—Depending on the intended use of the instrument, zeroing to a setting other than 0.0% is an acceptable alternative to this procedure if it provides greater measurement precision.]

Spanning the Range of Use—Establish the upper limit (span) with a span gas defined in the monograph and appropriate for the range of use. Pass the span gas through the instrument at the manufacturer's suggested flow rate. Confirm the reading is stable. Adjust the span setting to the certified value of the reference standard according to the oxygen analyzer manufacturer's instructions. Confirm the reading is stable.

VALIDATION

Validation of this instrument is generally completed during the (IQ/OQ) process. Routine verification is performed as described in the OQ/PQ sections of this chapter; and, therefore, specific information on instrument validation is unnecessary.

PROCEDURE

For Off-line Instrument—Before analysis, the instrument is calibrated by zeroing and spanning as described in the PQ section. [NOTE—The calibration need not be run concomitantly with the test samples.] Connect the sample gas to the instrument, and establish a constant flow into the

analyzer at the analyzer manufacturer's suggested flow rate. Maintain the flow until a constant reading is observed on the instrument. The definition of a constant reading is included in the analyzer manufacturer's instructions or in the user's instrument qualification documentation.

For On-line Instrument—The calibration intervals are defined by the analyzer manufacturer, by past history, or by statistical means. Establish a constant flow into the analyzer at the manufacturer's suggested flow rate.

SAMPLING

Sampling from Liquid Phase—Cylinders containing a dip tube allow a liquid sample to be obtained from the valve outlet with the cylinder in the upright position. If a dip tube is not present, the cylinder should be placed in an inverted position with the cylinder and main valve safely supported (so the liquid phase is in contact with the valve).

Sampling of medical gases should always be conducted using the required regulator. Regulators should be purged with the gas that will be sampled. When necessary, the flow to the analyzer should be measured using a calibrated flow-measuring device.

Sampling from Gaseous Phase—Cylinders that do not contain a dip tube allow a gaseous sample to be obtained from the valve outlet with the cylinder in the upright position. If a dip tube is present, the cylinder should be in an inverted position with the cylinder and main valve safely supported (so the gaseous phase is in contact with the end of the dip tube). Sampling of medical gases should always be conducted using the required regulator.

CERTIFIED STANDARDS FOR MEDICAL GAS ANALYSIS

USP monographs for medical gases require tests that use certified standards for instrument calibration and analytical determinations. Such compendial testing may be conducted using reference materials that are traceable to the U.S. National Institute of Standards and Technology or other National standards-setting organizations, e.g., Institute for National Measurement Standards (Canada). The individual monographs and the reagents, indicators, and solutions section refer to the nominal percent of various certified standards required to perform medical gas analysis. The requirements for the actual certified concentrations in terms of variance from the nominal value are indicated in the *Reagents, Indicators, and Solutions* section for each respective certified standard.

(425) IODOMETRIC ASSAY—ANTIBIOTICS

The following method is provided for the assay of most of the Pharmacopeial penicillin antibiotic drugs and their dosage forms, for which iodometric titration is particularly suitable.

Standard Preparation—Dissolve in the solvent specified in the table of *Solvents and Final Concentrations* a suitable quantity of the USP Reference Standard specified in the individual monograph, previously dried under the conditions specified in the individual monograph and accurately weighed, and dilute quantitatively and stepwise with the same solvent to obtain a solution having a known concen-

tration of about that specified in the table. Pipet 2.0 mL of this solution into each of two 125-mL glass-stoppered conical flasks.

Solvents and Final Concentrations

Antibiotic	Solvent*	Final Concentration
Amoxicillin	Water	1.0 mg per mL
Ampicillin	Water	1.25 mg per mL
Ampicillin Sodium	Buffer No. 1	1.25 mg per mL
Cloxacillin Sodium	Water	1.25 mg per mL
Cyclacillin	Water	1.0 mg per mL
Dicloxacillin Sodium	Buffer No. 1	1.25 mg per mL
Methicillin Sodium	Buffer No. 1	1.25 mg per mL
Nafcillin Sodium	Buffer No. 1	1.25 mg per mL
Oxacillin Sodium	Buffer No. 1	1.25 mg per mL
Penicillin G Potassium	Buffer No. 1	2,000 units per mL
Penicillin G Sodium	Buffer No. 1	2,000 units per mL
Penicillin V Potassium	Buffer No. 1	2,000 units per mL
Phenethicillin Potassium	Buffer No. 1	2,000 units per mL

* Unless otherwise noted, the *Buffers* are the potassium phosphate buffers defined in the section *Media and Diluents* under *Antibiotics—Microbial Assays* (81), except that sterilization is not required before use.

Assay Preparation—Unless otherwise specified in the individual monograph, dissolve in the solvent specified in the table of *Solvents and Final Concentrations* a suitable quantity, accurately weighed, of the specimen under test, and dilute quantitatively with the same solvent to obtain a solution having a known final concentration of about that specified in the table. Pipet 2 mL of this solution into each of two 125-mL glass-stoppered conical flasks.

Procedure—

Inactivation and Titration—To 2.0 mL of the *Standard Preparation* and of the *Assay Preparation*, in respective flasks, add 2.0 mL of 1.0 N sodium hydroxide, mix by swirling, and allow to stand for 15 minutes. To each flask add 2.0 mL of 1.2 N hydrochloric acid, add 10.0 mL of 0.01 N iodine VS, immediately insert the stopper, and allow to stand for 15 minutes. Titrate with 0.01 N sodium thiosulfate VS. As the endpoint is approached, add 1 drop of starch iodide paste TS, and continue the titration to the discharge of the blue color.

Blank Determination—To a flask containing 2.0 mL of the *Standard Preparation* add 10.0 mL of 0.01 N iodine VS. If the *Standard Preparation* contains amoxicillin or ampicillin, immediately add 0.1 mL of 1.2 N hydrochloric acid. Immediately titrate with 0.01 N sodium thiosulfate VS. As the endpoint is approached, add 1 drop of starch iodide paste TS, and continue the titration to the discharge of the blue color. Similarly treat a flask containing 2.0 mL of the *Assay Preparation*.

Calculations—Calculate the microgram (or unit) equivalent (F) of each mL of 0.01 N sodium thiosulfate consumed by the *Standard Preparation* by the formula:

$$(2CP)/(B - I)$$

in which C is the concentration, in mg per mL, of Reference Standard in the *Standard Preparation*, P is the potency, in μg (or units) per mg, of the Reference Standard, B is the volume, in mL, of 0.01 N sodium thiosulfate consumed in the *Blank determination*, and I is the volume, in mL, of 0.01 N sodium thiosulfate consumed in the *Inactivation and titra-*

tion. Calculate the potency of the specimen under test by the formula given in the individual monograph.

<429> LIGHT DIFFRACTION MEASUREMENT OF PARTICLE SIZE

INTRODUCTION

The method is based on the ISO standards 13320-1(1999) and 9276-1(1998).

This general chapter has been harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*.

The laser light diffraction technique used for the determination of particle-size distribution is based on the analysis of the diffraction pattern produced when particles are exposed to a beam of monochromatic light. Historically, the early laser diffraction instruments only used scattering at small angles. However, the technique has since been broadened to include laser light scattering in a wider angular range and application of the Mie theory, in addition to the Fraunhofer approximation and anomalous diffraction.

The technique cannot distinguish between scattering by single particles and scattering by clusters of primary particles, i.e., by agglomerates or aggregates. As most particulate samples contain agglomerates or aggregates and as the focus of interest is generally on the size distribution of primary particles, the clusters are usually dispersed into primary particles before measurement.

For nonspherical particles, an equivalent sphere-size distribution is obtained because the technique assumes spherical particles in its optical model. The resulting particle-size distribution may differ from those obtained by methods based on other physical principles (e.g., sedimentation, sieving).

This chapter provides guidance for the measurement of size distributions of particles in different dispersed systems (e.g., powders, sprays, aerosols, suspensions, emulsions, and gas bubbles in liquids), through analysis of their angular light-scattering patterns. It does not address specific requirements of particle-size measurement of specific products.

PRINCIPLE

A representative sample, dispersed at an adequate concentration in a suitable liquid or gas, is passed through a beam of monochromatic light, usually a laser. The light scattered by the particles at various angles is measured by a multi-element detector. Numerical values representing the scattering pattern are then recorded for subsequent analysis. These scattering pattern values are then transformed, using an appropriate optical model and mathematical procedure, to yield the proportion of total volume to a discrete number of size classes, forming a volumetric particle-size distribution.

INSTRUMENT

The instrument is located in an environment where it is not affected by electrical noise, mechanical vibrations, temperature fluctuations, humidity, or direct bright light.

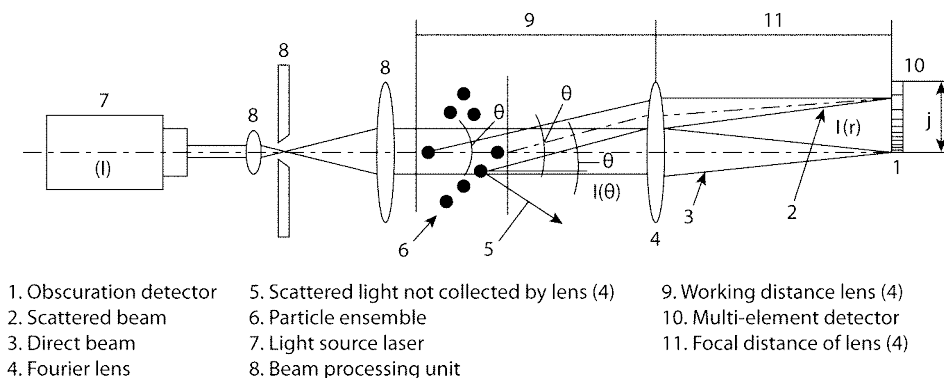


Figure 1. Example of a set-up of a laser light diffraction instrument.

An example of a setup of a laser light diffraction instrument is given in *Figure 1*. Other equipment may be used.

The instrument comprises a laser light source, beam processing optics, a sample measurement region (or cell), a Fourier lens, and a multi-element detector for measuring the scattered light pattern. A data system is also required for deconvolution of the scattering data into a volumetric size distribution and associated data analysis and reporting.

The particles can enter the laser beam in two positions. In the conventional case the particles enter the parallel beam before the collecting lens and within its working distance. In so-called reversed Fourier optics the particles enter behind the collecting lens and thus in a converging beam. The advantage of the conventional setup is that a reasonable path length for the sample is allowed within the working distance of the lens. The second setup allows only small path lengths but enables measurement of scattered light at larger angles, which is useful when submicron particles are present.

The interaction of the incident light beam and the ensemble of dispersed particles results in a scattering pattern with different light intensities at various angles. The total angular intensity distribution, consisting of both direct and scattered light, is then focused onto a multi-element detector by a lens or a series of lenses. These lenses create a scattering pattern that, within limits, does not depend on the location of the particles in the light beam. Hence, the continuous angular intensity distribution is converted into a discrete spatial intensity distribution on a set of detector elements.

It is assumed that the measured scattering pattern of the particle ensemble is identical to the sum of the patterns from all individual single scattering particles presented in random relative positions. Note that only a limited angular range of scattered light is collected by the lens(es) and, therefore, by the detector.

DEVELOPMENT OF THE METHOD

The measurement of particle size by laser diffraction can give reproducible data, even in the submicron region, provided the instrument used and the sample tested are carefully controlled to limit variability of the test conditions (e.g., dispersion medium, method of preparation of the sample dispersion).

Traditionally, the measurement of particle size using laser diffraction has been limited to particles in the range of approximately 0.1 μm to 3 mm. Because of recent advances in lens and equipment design, newer instruments are routinely capable of exceeding this range. With the validation report, the user demonstrates the applicability of the method for its intended use.

Sampling

The sampling technique must be adequate to obtain a representative sample of a suitable volume for the particle-

size measurement. Sample splitting techniques such as rotating riffler or the cone and quartering method may be applied.

Evaluation of the Dispersion Procedure

The sample to be analyzed is inspected, visually or with the aid of a microscope, to estimate its size range and particle shape. The dispersion procedure must be adjusted to the purpose of the measurement. The purpose may be such that it is preferable to deagglomerate clusters into primary particles as far as possible, or it may be desirable to retain clusters as intact as possible. In this sense, the particles of interest may be either primary particles or clusters.

For the development of a method, it is highly advisable to check that comminution of the particles does not occur and, conversely, that dispersion of particles or clusters is satisfactory. This can usually be done by changing the dispersing energy and monitoring the change of the particle-size distribution. The measured size distribution must not change significantly when the sample is well dispersed and the particles are neither fragile nor soluble. Moreover, if the manufacturing process (e.g., crystallization, milling) of the material has changed, the applicability of the method must be verified (e.g., by microscopic comparison).

Sprays, aerosols, and gas bubbles in a liquid should be measured directly, provided that their concentration is adequate, because sampling or dilution generally alters the particle-size distribution.

In other cases (such as emulsions, pastes, and powders), representative samples may be dispersed in suitable liquids. Dispersing aids (wetting agents, stabilizers) and/or mechanical forces (e.g., agitation, sonication) are often applied for deagglomeration or deaggregation of clusters and stabilization of the dispersion. For these liquid dispersions, a recirculating system consisting of an optical measuring cell, a dispersion bath usually equipped with stirrer and ultrasonic elements, a pump, and tubing is most commonly used. Nonrecirculating, stirred cells are useful when only small amounts of a sample are available or when special dispersion liquids are used.

Dry powders can also be converted into aerosols through the use of suitable dry powder dispersers that apply mechanical force for deagglomeration or deaggregation. Generally, the dispersers use the energy of compressed gas or the differential pressure of a vacuum to disperse the particles to an aerosol that is blown through the measuring zone, usually into the inlet of a vacuum unit that collects the particles. However, for free-flowing, coarser particles or granules, the effect of gravity may be sufficient to disperse the particles adequately.

If the maximum particle size of the sample exceeds the measuring range of the instrument, the material that is too coarse can be removed by sieving, and the mass and percentage of removed material are reported. However, after

presieving, note that the sample is no longer representative, unless proven otherwise.

Optimization of the Liquid Dispersion

Liquids, surfactants, and dispersing aids used to disperse powders must

- be transparent at the laser wavelength and practically free from air bubbles or particles;
- have a refractive index that differs from that of the test material;
- be a nonsolvent of the test material (pure liquid or prefiltered, saturated solution);
- not alter the size of the test materials (e.g., by solubility, solubility enhancement, or recrystallization effects);
- favor easy formation and stability of the dispersion;
- be compatible with the materials used in the instrument (such as O-rings, gaskets, tubing, etc.); and
- possess a suitable viscosity to facilitate recirculation, stirring, and filtration.

Surfactants and/or dispersing aids are often used to wet the particles and to stabilize the dispersion. For weak acids and weak bases, buffering of the dispersing medium at low or high pH, respectively, can assist in identifying a suitable dispersant.

A preliminary check of the dispersion quality can be performed by visual or microscopic inspection. It is also possible to take fractional samples out of a well-mixed stock dispersion. Such stock dispersions are formed by adding a liquid to the sample while mixing it with, for example, a glass rod, a spatula or a vortex mixer. Care must be taken to ensure the transfer of a representative sample and that settling of larger particles does not occur. Therefore, a sample paste is prepared or sampling is carried out quickly from a suspension maintained under agitation.

Optimization of the Gas Dispersion

For sprays and dry powder dispersions, a compressed gas free from oil, water, and particles may be used. To remove such materials from the compressed gas, a dryer with a filter can be used. Any vacuum unit should be located away from the measurement zone, so that its output does not disturb the measurement.

Determination of the Concentration Range

In order to produce an acceptable signal-to-noise ratio in the detector, the particle concentration in the dispersion must exceed a minimum level. Likewise, it must be below a maximum level in order to avoid multiple scattering. The concentration range is influenced by the width of the laser beam, the path length of the measurement zone, the optical properties of the particles, and the sensitivity of the detector elements.

In view of the above, measurements must be performed at different particle concentrations to determine the appropriate concentration range for any typical sample of material. [NOTE—In different instruments, particle concentrations are usually represented by differently scaled and differently named numbers, e.g., obscuration, optical concentration, proportional number of total mass.]

Determination of the Measuring Time

The time of measurement, the reading time of the detector, and the acquisition frequency is determined experimentally in accordance with the required precision. Generally, the time for measurement permits a large number of detector scans or sweeps at short time intervals.

Selection of an Appropriate Optical Model

Most instruments use either the Fraunhofer or the Mie theory, though other approximation theories are sometimes applied for calculation of the scattering matrix. The choice of the theoretical model depends on the intended application and the different assumptions (size, absorbance, refractive index, roughness, crystal orientation, mixture, etc.) made for the test material. If the refractive index values (real and imaginary parts for the used wavelength) are not exactly known, then the Fraunhofer approximation or the Mie theory with a realistic estimate of the refractive index can be used. The former has the advantages that it is simple and it does not need refractive index values; the latter usually provides less-biased particle-size distributions for small particles. For instance, if the Fraunhofer model is used for samples containing an appreciable amount of small, transparent particles, a significantly larger amount of small particles may be calculated. In order to obtain traceable results, it is essential to document the refractive index values used, because small differences in the values assumed for the real and imaginary part of the complex refractive index may cause significant differences in the resulting particle-size distributions. Small values of the imaginary part of the refractive index (about 0.01–0.1 *i*) are often applied to allow the correction of the absorbance for the surface roughness of the particles. It should be noted, in general, that the optical properties of the substance to be tested, as well as the structure (e.g., shape, surface roughness, and porosity) bear upon the final result.

Validation

Typically, the validity of a procedure may be assessed by the evaluation of its specificity, linearity, range, accuracy, precision, and robustness. In particle-size analysis by laser light diffraction, specificity as defined by ICH is not applicable as it is not possible to discriminate different components into a sample, nor is it possible to discriminate between agglomerates from dispersed particles unless properly complemented by microscopic techniques. Exploring a linear relationship between concentration and response, or a mathematical model for interpolation, is not applicable to this procedure. Rather than evaluating linearity, this method requires the definition of a concentration range within which the result of the measurements does not vary significantly. Concentrations below that range produce an error due to a poor signal-to-noise ratio, while concentrations above that range produce an error due to multiple scattering. The range depends mostly on the instrument hardware. Accuracy should be confirmed through an appropriate instrument qualification and comparison with microscopy, while precision may be assessed by means of a repeatability determination.

The attainable repeatability of the method mainly depends on the characteristics of the material (milled/not milled, robust/fragile, width of its size distribution, etc.), whereas the required repeatability depends on the purpose of the measurement. Mandatory limits cannot be specified in this chapter, as repeatabilities (different sample preparations) may vary appreciably from one substance to another. However, it is good practice to aim at acceptance criteria for repeatability such as % RSD $\leq 10\%$ [$n = 6$] for any central value of the distribution (e.g., for x_{50}). Values at the sides of the distribution (e.g., x_{10} and x_{90}) are oriented towards less stringent acceptance criteria such as % RSD $\leq 15\%$ [$n = 6$]. Below 10 μm , these values must be doubled. Robustness may be tested during the selection and optimization of the dispersion media and forces. The change of the dispersing energy may be monitored by the change in the particle-size distribution.

MEASUREMENT

Precautions

The instructions given in the instrument manual are followed:

- never look into the direct path of the laser beam or its reflections;
- earth all instrument components to prevent ignition of solvents or dust explosions;
- check the instrument set-up (e.g., warm-up, required measuring range and lens, appropriate working distance, position of the detector, no direct bright daylight); and
- in the case of wet dispersions, avoid air bubbles, evaporation of liquid, schlieren or other inhomogeneities in the dispersion; similarly, avoid improper mass-flow from the disperser or turbulent airflow in the case of dry dispersions; such effects can cause erroneous particle-size distributions.

Measurement of the Light Scattering of Dispersed Sample(s)

After proper alignment of the optical part of the instrument, a blank measurement of the particle-free dispersion medium must be performed using the same method as that used for the measurement of the sample. The background signal must be below an appropriate threshold. The detector data are saved in order to subtract them later from the data obtained with the sample. The sample dispersion is measured according to the developed method.

For each detector element, an average signal is calculated, sometimes together with its standard deviation. The magnitude of the signal from each detector element depends upon the detection area, the light intensity, and the quantum efficiency. The coordinates (size and position) of the detector elements together with the focal distance of the lens determine the range of scattering angles for each element. Most instruments also measure the intensity of the central (unscattered) laser beam. The ratio of the intensity of a dispersed sample to that in its absence (the blank measurement) indicates the proportion of scattered light and hence the particle concentration.

Conversion of Scattering Pattern Into Particle-Size Distribution

This deconvolution step is the inverse of the calculation of a scattering pattern for a given particle-size distribution. The assumption of spherical particle shape is particularly important as most algorithms use the mathematical solution for scattering from spherical particles. Furthermore, the measured data always contain some random and systematic errors, which may vitiate the size distributions. Several mathematical procedures have been developed for use in the available instruments. They contain some weighting of deviations between measured and calculated scattering patterns (e.g., least squares), some constraints (e.g., non-negativity for amounts of particles), and/or some smoothing of the size distribution curve.

The algorithms used are specific to each make and model of equipment, and are proprietary. The differences in the algorithms between different instruments may give rise to differences in the calculated particle-size distributions.

Replicates

The number of replicate measurements (with individual sample preparations) to be performed, depends on the re-

quired measurement precision. It is recommended to set this number in a substance-specific method.

REPORTING RESULTS

The particle-size distribution data are usually reported as cumulative undersize distribution and/or as density distribution by volume. The symbol x is used to denote the particle size, which in turn is defined as the diameter of a volume-equivalent sphere. $Q_3(x)$ denotes the volume fraction undersize at the particle size x . In a graphical representation, x is plotted on the abscissa and the dependent variable Q_3 on the ordinate. Most common characteristic values are calculated from the particle-size distribution by interpolation. The particle sizes at the undersize values of 10%, 50%, and 90% (denoted as x_{10} , x_{50} , and x_{90} , respectively) are frequently used. x_{50} is also known as the median particle size. It is recognized that the symbol d is also widely used to designate the particle size, thus the symbol x may be replaced by d .

Moreover, sufficient information must be documented about the sample, the sample preparation, the dispersion conditions, and the cell type. Because the results depend on the particular instrument, data analysis program, and optical model used, these details must also be documented.

CONTROL OF THE INSTRUMENT PERFORMANCE

Use the instrument according to the manufacturer's instructions and carry out the prescribed qualifications at an appropriate frequency, according to the use of the instrument and substances to be tested.

Calibration

Laser diffraction systems, although assuming idealized properties of the particles, are based on first principles of laser light scattering. Thus, calibration in the strict sense is not required. However, it is still necessary to confirm that the instrument is operating correctly. This can be undertaken using any certified reference material that is acceptable in industrial practice. The entire measurement procedure is examined, including sample collection, sample dispersion, sample transport through the measuring zone, measurement, and the deconvolution procedure. It is essential that the total operational procedure is fully described.

The preferred certified reference materials consist of spherical particles of a known distribution. They must be certified as to the mass-percentage size distribution by an absolute technique, if available, and used in conjunction with an agreed, detailed operation procedure. It is essential that the real and imaginary parts of the complex refractive index of the material are indicated if the Mie theory is applied in data analysis. The representation of the particle-size distribution by volume will equal that of the distribution by mass, provided that the density of the particles is the same for all size fractions.

The response of a laser diffraction instrument meets the requirements if the mean value of x_{50} from at least three independent measurements does not deviate by more than 3% from the certified range of values of the certified reference material. The mean values for x_{10} and x_{90} must not deviate by more than 5% from the certified range of values. Below 10 μm , these values must be doubled.

Although the use of materials consisting of spherical particles is preferable, nonspherical particles may also be employed. Preferably, these particles have certified or typical values from laser diffraction analysis performed according to an agreed, detailed operating procedure. The use of reference values from methods other than laser diffraction may cause a significant bias. The reason for this bias is that the

different principles inherent in the various methods may lead to different sphere-equivalent diameters for the same nonspherical particle.

Although the use of certified reference materials is preferred, other well-defined reference materials may also be employed. They consist of substances of typical composition and particle-size distribution for a specified class of substances. Their particle-size distribution has proven to be stable over time. The results must comply with previously determined data, with the same precision and bias as for the certified reference material.

Qualification of the System

In addition to the calibration, the performance of the instrument must be qualified at regular time intervals or as frequently as appropriate. This can be undertaken using any suitable reference material as mentioned in the previous paragraph.

The qualification of the system is based on the concept that the equipment, electronics, software, and analytical operations constitute an integral system, which can be evaluated as an entity. Thus the entire measurement procedure is examined, including sample collection, sample dispersion, sample transport through the measuring zone, and the measurement and deconvolution procedure. It is essential that the total operational procedure is fully described.

In general, unless otherwise specified in the individual monograph, the response of a laser diffraction instrument is considered to meet the requirements if the x_{50} value does not deviate by more than 10% from the range of values of the reference material. If optionally the values at the sides of the distribution are evaluated (e.g., x_{10} and x_{90}), then these values must not deviate by more than 15% from the certified range of values. Below 10 μm , these values must be doubled.

NOTE—For calibration of the instrument, stricter requirements are laid down in the paragraph on *Calibration*.

(431) METHOXY DETERMINATION

Apparatus—The apparatus for methoxy determination is shown diagrammatically in the *accompanying figure*. The boiling flask, A, is fitted with a capillary side-arm for the introduction of carbon dioxide or nitrogen and is connected to a column, B, which serves to separate aqueous hydriodic acid from the more volatile methyl iodide. The methyl iodide passes through water in a scrubber trap, C, and is finally absorbed in the bromine–acetic acid solution in absorption tube D. The carbon dioxide or nitrogen is introduced through a pressure-regulating device and connected

to the apparatus by a small capillary containing a small cotton pledget. [NOTE—Avoid the use of organic solvents in cleaning this apparatus, since traces remaining may interfere with the determination. This test is used also for ethoxy determination with an 80-minute reaction time and a titrant equivalent of 0.751 mg of $(\text{OC}_2\text{H}_5)_2$.]

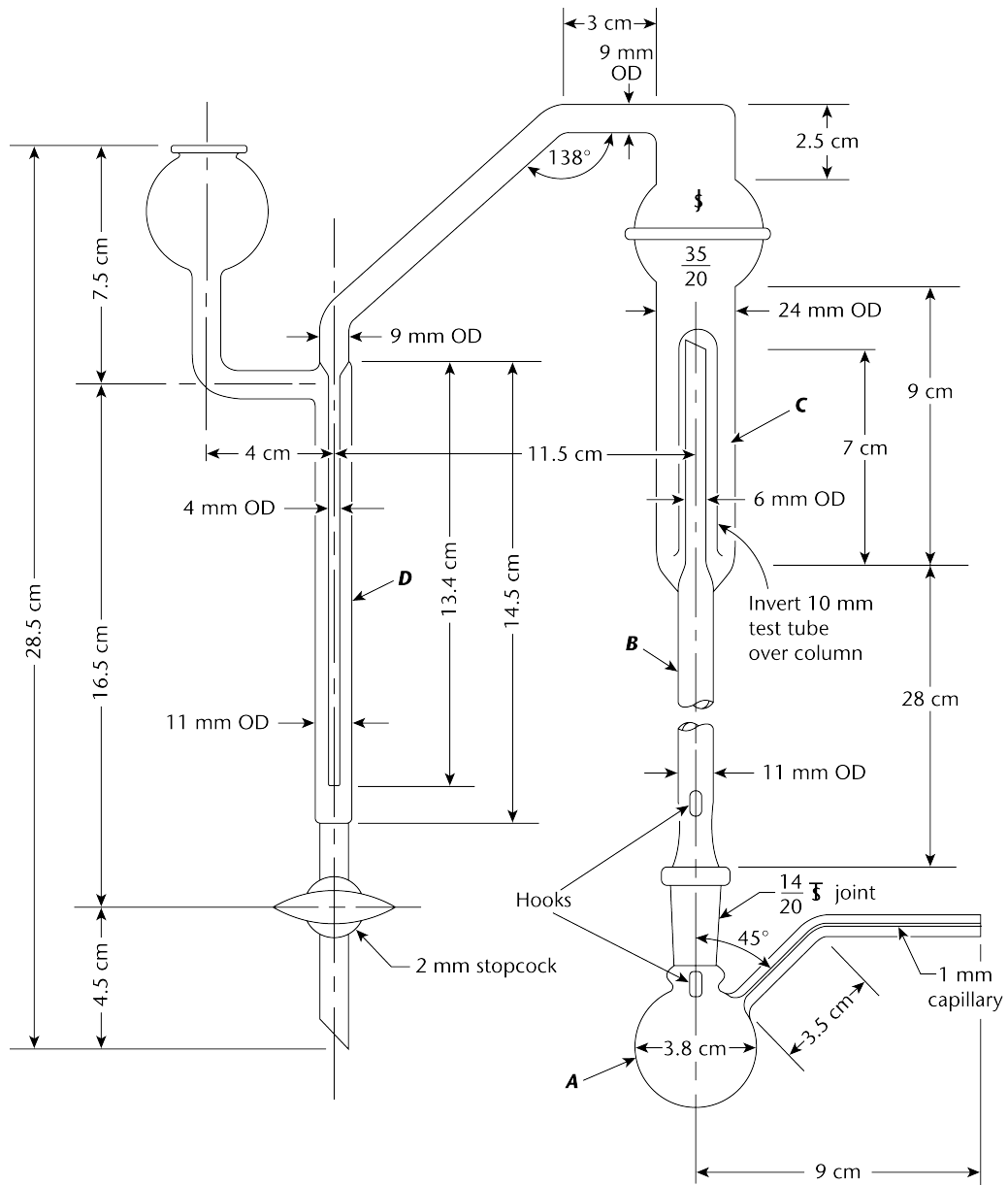
For greater convenience in use and cleaning, a ground-glass ball joint connects the two upright columns of the apparatus. The top of the scrubber C consists of a 35/20 ball joint, the upper half of which is connected to the side-arm leading into tube D. This permits taking the apparatus apart and facilitates adding the water to the trap. Also, it allows access to the loose inverted (10-mm) test tube that serves as the trap over the inner tube of the scrubber C.

Reagents—

BROMINE-ACETIC ACID SOLUTION—Dissolve 100 g of potassium acetate in 1000 mL of a solution consisting of 900 mL of glacial acetic acid and 100 mL of acetic anhydride. On the day of use, to 145 mL of this solution add 5 mL of bromine.

HYDRIODIC ACID—A colorless, or nearly colorless, constant-boiling reagent solution, prepared for this purpose, is available commercially. If not obtained commercially, it may be prepared by distilling hydriodic acid over red phosphorus, passing carbon dioxide or nitrogen through the apparatus during the distillation. Use the constant-boiling mixture (between 55% and 58% of HI) distilling between 126° and 127°, which is colorless or nearly colorless. [Caution—Exercise safety precautions when distilling Hydriodic Acid.] Place the acid in small, amber, glass-stoppered bottles previously flushed with carbon dioxide, or nitrogen, seal with paraffin, and store in a cool, dark place.

Procedure—Prepare the apparatus by disconnecting the ball joint and pouring water into trap C until it is half-full. Connect the two parts, using a minimal amount of a suitable silicone grease to seal the ball joint. Add 7 mL of *Bromine-Acetic Acid Solution* to absorption tube D. Weigh the sample in a tared gelatin capsule, and add it to the boiling flask along with a few boiling chips or pieces of porous plate. Finally add 6 mL of *Hydriodic Acid* and attach the flask to the column, using a minimal amount of a suitable silicone grease to seal the junction. Bubble the carbon dioxide or nitrogen through the apparatus at the rate of 2 bubbles per second, place the boiling flask in an oil bath or heating mantle heated to 150°, and continue the reaction for 40 minutes for methoxy determination, or 80 minutes for ethoxy determination. Drain the contents of the absorption tube into a 500-mL conical flask containing 10 mL of sodium acetate solution (1 in 4). Rinse the tube with water, adding the rinsings to the flask, and finally dilute with water to about 125 mL. Add formic acid, dropwise, with swirling, until the reddish brown color of the bromine is discharged, then add 3 additional drops. A total of 12 to 15 drops usually is required. Allow to stand for 3 minutes, and add 15 mL of diluted sulfuric acid and 3 g of potassium iodide, and titrate immediately with 0.1 N sodium thiosulfate VS, using 3 mL of starch TS as the indicator. Perform a blank determination, including also a gelatin capsule, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 0.517 mg of $(\text{OCH}_3)_2$.



Apparatus for Methoxy Determination

(441) NIACIN OR NIACINAMIDE ASSAY

USP Reference Standards (11)—*USP Niacin RS. USP Niacinamide RS.* [NOTE—The previously dried Reference Standards may be stored in a desiccator over silica gel, protected from light.]

Chemical Method

NOTE—Determine from the labeling if the vitamin in the assay specimen is niacin or niacinamide, and use the corresponding standard preparation (either *Standard Niacin Preparation* or *Standard Niacinamide Preparation*) as directed in the *Procedure*.

Cyanogen Bromide Solution—Dissolve 5 g of cyanogen bromide in water to make 50 mL. [Caution—Prepare this solution under a hood, as cyanogen bromide volatilizes at room temperature, and the vapor is highly irritating and poisonous.]

Sulfanilic Acid Solution—To 2.5 g of sulfanilic acid add 15 mL of water and 3 mL of 6 N ammonium hydroxide. Mix, add, with stirring, more 6 N ammonium hydroxide, if necessary, until the acid dissolves, adjust the solution with 3 N hydrochloric acid to a pH of about 4.5, using bromocresol green TS as an external indicator, and dilute with water to 25 mL.

Standard Niacin Stock Solution—Transfer 25.0 mg of USP Niacin RS to a 500-mL volumetric flask, dissolve in alcohol solution (1 in 4), dilute with alcohol solution (1 in 4) to volume, and mix. Store in a refrigerator. Each mL of this solution contains 50 µg of USP Niacin RS.

Standard Niacin Preparation—Transfer 10.0 mL of *Standard Niacin Stock Solution* to a 100-mL volumetric flask, dilute with water to volume, and mix. Each mL of this solution contains 5 µg of USP Niacin RS.

Standard Niacinamide Stock Solution—Transfer 50.0 mg of USP Niacinamide RS to a 500-mL volumetric flask, dissolve in alcohol solution (1 in 4), dilute with alcohol solution (1 in 4) to volume, and mix. Store in a refrigerator. Each mL of this solution contains 100 µg of USP Niacinamide RS.

Standard Niacinamide Preparation—Transfer 10.0 mL of *Standard Niacinamide Stock Solution* to a 100-mL volumetric flask, dilute with water to volume, and mix. Each mL of this solution contains 10 µg of USP Niacinamide RS.

Assay Preparation—Prepare as directed in the individual monograph.

Procedure—Pipet into four marked tubes the quantities of the appropriate *Standard Preparation*, the *Assay Preparation*, the ammonia dilution, and water indicated in the accompanying table. Then add the other constituents, respectively, as listed in the table, according to the directions given herein.

Reaction Mixtures for Niacin or Niacinamide Assay—Chemical Method

Constituent	Tube 1, mL	Tube 2, mL	Tube 3, mL	Tube 4, mL
Standard Preparation	1.0	1.0	—	—
Assay Preparation	—	—	1.0	1.0
Ammonia Dilution (ammonium hydroxide, diluted to 1 in 50)	0.5	0.5	0.5	0.5
Water	6.5	1.5	6.5	1.5
Cyanogen Bromide Solution	—	5.0	—	5.0

Reaction Mixtures for Niacin or Niacinamide Assay—Chemical Method (Continued)

Constituent	Tube 1, mL	Tube 2, mL	Tube 3, mL	Tube 4, mL
Sulfanilic Acid Solution	2.0	2.0	2.0	2.0
Hydrochloric Acid	1 drop	—	1 drop	—

To Tube 1 add the *Sulfanilic Acid Solution*, shake well, add the hydrochloric acid, mix, place in a suitable spectrophotometer, and adjust to zero absorbance at 450 nm. To Tube 2 add the *Cyanogen Bromide Solution*, mix, and 30 seconds, accurately timed, after completion of the addition of the cyanogen bromide, add the *Sulfanilic Acid Solution*, with swirling. Close the tube, place it in the spectrophotometer, and after 2 minutes measure its absorbance at 450 nm against Tube 1 as a blank, designating the absorbance as A_S . Repeat the procedure with Tubes 3 (as blank) and 4, designating the absorbance of Tube 4 as A_U . Calculate the quantity of niacin or niacinamide in the sample as directed in the individual monograph.

Microbiological Method

Test Solution of Material to be Assayed—Place the prescribed amount of the material to be assayed in a flask of suitable size, and proceed by one of the methods given below. The concentrations of the sulfuric acid and sodium hydroxide solutions used are not stated in each instance because these concentrations may be varied depending upon the amount of material taken for assay, volume of test solution, and buffering effect of material.

(a) *For Dry or Semidry Materials that Contain No Appreciable Amount of Basic Substances*—Add a volume of dilute sulfuric acid (1 in 35) equal, in mL, to not less than 10 times the dry weight of the material, in g, but the resulting solution shall contain not more than 5.0 mg of niacin in each mL. If the material is not readily soluble, comminute it so that it may be evenly dispersed in the liquid, then agitate vigorously, and wash down the sides of the flask with dilute sulfuric acid (1 in 35).

Heat the mixture in an autoclave at 121° to 123° for 30 minutes, and cool. If lumping occurs, agitate the mixture until the particles are evenly dispersed. Adjust the mixture with sodium hydroxide solution to a pH of 6.8, dilute with water to make a final measured volume that has a concentration of niacin equivalent to that of *Standard Niacin Solution*, and filter.

(b) *For Dry or Semidry Materials that Contain Appreciable Amounts of Basic Substances*—Add sufficient sulfuric acid solution to bring the pH of the mixture to between 5.0 and 6.0. Add such an amount of water that the total volume of liquid shall be equal in mL to not less than ten times the dry weight of the assay specimen, in g, but the resulting solution shall contain not more than 5.0 mg of niacin in each mL. Then add the equivalent of 10 mL of dilute sulfuric acid (2 in 7) for each 100 mL of liquid, and proceed as directed under (a), beginning with the second paragraph.

(c) *For Liquid Materials*—Adjust the material with either sulfuric acid solution or sodium hydroxide solution to a pH of 5.0 to 6.0. Add such an amount of water that the total volume of liquid shall be equal, in mL, to not less than 10 times the volume of the specimen, in mL, but the resulting solution shall contain not more than 5.0 mg of niacin in each mL. Then add the equivalent of 10 mL of dilute sulfuric acid (2 in 7) for each mL of liquid, and proceed as directed under (a), beginning with the second paragraph.

Standard Niacin Stock Solution I—Transfer 50.0 mg of USP Niacin RS to a 500-mL volumetric flask, dissolve in alcohol, dilute with alcohol to volume, and mix. Store in a refrigerator. Each mL of this solution contains 100 µg of USP Niacin RS.

Standard Niacin Stock Solution II—To 100.0 mL of *Standard Niacin Stock Solution I* add water to make 1000.0 mL. Store under toluene in a refrigerator. Each mL of this solution contains 10 µg of USP Niacin RS.

Standard Niacin Solution—Dilute a suitable volume of *Standard Niacin Stock Solution II* with water to such a measured volume so that after incubation as described in the *Assay Procedure* the transmittance of the 5.0-mL level of *Standard Niacin Solution* is equivalent to that of a dried cell weight of not less than 1.25 mg, when the inoculated blank is set at 100 percent transmittance. This concentration is usually between 10 ng and 40 ng of niacin per mL. Prepare a fresh *Standard Niacin Solution* for each assay.

Basal Medium Stock Solution—

Acid-hydrolyzed Casein Solution	25 mL
Cystine-Tryptophan Solution	25 mL
Dextrose Anhydrous	10 g
Sodium Acetate Anhydrous	5 g
Adenine-Guanine-Uracil Solution	5 mL
Riboflavin-Thiamine Hydrochloride-Biotin Solution	5 mL
Aminobenzoic Acid-Calcium Pantothenate-Pyridoxine Hydrochloride Solution	5 mL
Salt Solution A	5 mL
Salt Solution B	5 mL

Dissolve the anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, add water to make 250 mL.

Acid-Hydrolyzed Casein Solution—Mix 100 g of vitamin-free casein with 500 mL of constant-boiling hydrochloric acid [approximately 20 percent (w/w) HCl], and reflux the mixture for 24 hours. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 (± 0.1), and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal if the filtrate does not appear straw-colored to colorless. Store under toluene in a refrigerator. Filter the solution if a precipitate forms upon storage.

Cystine-Tryptophan Solution—Suspend 4.0 g of *L*-cystine and 1.0 g of *L*-tryptophan (or 2.0 g of *DL*-tryptophan) in 700 to 800 mL of water, heat to 70° to 80°, and add the 20 percent (w/w) hydrochloric acid, dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a refrigerator at a temperature not below 10°.

Adenine-Guanine-Uracil Solution—Dissolve 100 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 5.0 mL of the 20 percent (w/w) hydrochloric acid, cool, and add water to make 100 mL. Store under toluene in a refrigerator.

Riboflavin-Thiamine Hydrochloride-Biotin Solution—Prepare a solution containing, in each mL, 20 µg of riboflavin, 10 µg of thiamine hydrochloride, and 0.04 µg of biotin by dissolving crystalline riboflavin, crystalline thiamine hydrochloride, and crystalline biotin (free acid) in dilute glacial acetic acid (1 in 850). Store, protected from light, under toluene in a refrigerator.

Aminobenzoic Acid-Calcium Pantothenate-Pyridoxine Hydrochloride Solution—Prepare a solution of neutral 25 percent alcohol having a concentration of 10 µg of aminobenzoic acid, 20 µg of calcium pantothenate, and 40 µg of pyridoxine hydrochloride per mL. Store in a refrigerator.

Salt Solution A—Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in

water to make 500 mL. Add 5 drops of hydrochloric acid, and store under toluene.

Salt Solution B—Dissolve 10 g of magnesium sulfate, 500 mg of sodium chloride, 500 mg of ferrous sulfate, and 500 mg of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and store under toluene.

Stock Culture of *Lactobacillus plantarum*—Dissolve 2.0 g of water-soluble yeast extract in 100 mL of water, add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture with stirring, on a steam bath, until the agar dissolves. Add approximately 10-mL portions of the hot solution to test tubes, plug the tubes with cotton, sterilize for 15 minutes in an autoclave at 121° to 123°, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,* incubating for 16 to 24 hours at any selected temperature between 30° and 37°, but held constant to within $\pm 0.5^\circ$, and finally store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for inoculum if the culture is more than 1 week old.

Culture Medium—To each of a series of test tubes containing 5.0 mL of the *Basal Medium Stock Solution* add 5.0 mL of water containing 1.0 µg of niacin. Plug the tubes with cotton, sterilize for 15 minutes in an autoclave at 121° to 123°, and cool.

Inoculum—Make a transfer of cells from the stock culture of *Lactobacillus plantarum* to a sterile tube containing 10 mL of culture medium. Incubate this culture for 16 to 24 hours at any selected temperature between 30° and 37°, but held constant to within $\pm 0.5^\circ$. The cell suspension so obtained is the inoculum.

Calibration of Spectrophotometer—Add aseptically 1 mL of *Inoculum* to approximately 300 mL of *Culture Medium* containing 1 mL of *Standard Niacin Solution*. Incubate the inoculated medium for the same period and at the same temperature to be employed in the *Assay Procedure*.

Following the incubation period, centrifuge and wash the cells three times with approximately 50-mL portions of saline TS, and then resuspend the cells in about 25 mL of the saline solution.

Dry to constant weight a 10-mL portion, accurately measured, using a steam bath and completing the drying in vacuum at 100°, and calculate the dry weight of the cells, in mg per mL, corrected for the amount of sodium chloride present.

Dilute a second portion, accurately measured, of the saline cell suspension with the saline solution so that each mL contains a known quantity of cells equivalent to 500 µg on a dried basis. To test tubes add, in triplicate, 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 2.5 mL, 3.0 mL, 4.0 mL, and 5.0 mL, respectively, of this diluted cell suspension and 5.0 mL of *Basal Medium Stock Solution*, and make the volume in each tube to 10.0 mL with saline solution. Using as the blanks three similar tubes containing no cell suspension, measure the light transmittance of each tube under the same conditions to be employed in the assay. Plot the observations as the ordinate on cross-section paper against the cell content, expressed as mg of dry weight, as the abscissa.

Repeat this procedure at least twice for the spectrophotometer to be used in the assay. Draw the composite curve best representing the three or more individual curves relating transmittance to cell density for the spectrophotometer under the conditions of the assay.

Assay Procedure—Prepare standard niacin tubes as follows. To test tubes add, in duplicate, 0.0 mL, 0.5 mL, 1.0 mL, 1.5 mL, 2.0 mL, 2.5 mL, 3.0 mL, 3.5 mL, 4.0 mL, 4.5 mL, and 5.0 mL, respectively, of *Standard Niacin Solution*. To each tube add 5.0 mL of *Basal Medium Stock Solution* and water to make 10.0 mL.

* Pure cultures of *Lactobacillus plantarum* may be obtained, as number 8014, from the American Type Culture Collection, P.O. Box 1549, Manassas, VA 20108.

Prepare tubes containing the material to be assayed as follows. To test tubes add, in duplicate, 1.0 mL, 2.0 mL, 3.0 mL, and 4.0 mL, respectively, of the test solution of the material to be assayed. To each tube add 5.0 mL of *Basal Medium Stock Solution* and water to make 10.0 mL. After mixing, plug the tubes with cotton or cover with caps, and sterilize in an autoclave at 121° to 123°. (Overheating the assay tubes may produce unsatisfactory results.) Cool, aseptically inoculate each tube with 1 drop of *Inoculum*, and incubate for 16 to 24 hours at any selected temperature between 30° and 37°, but held constant to within $\pm 0.5^\circ$. Contamination of the assay tubes with any foreign organism invalidates the assay.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, to which 1 drop of a suitable antifoam agent solution may be added, and transfer to an optical container. After agitating its contents, place the container in a spectrophotometer that has been set at a specific wavelength between 540 nm and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 seconds or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. If this transmittance reading corresponds to a dried cell weight greater than 600 μg per tube, or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

Then with the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. Disregard the results of the assay if the difference between the transmittance observed at the highest level of the standard and that of the inoculated blank is less than the difference corresponding to a dried cell weight of 1.25 mg per tube.

Calculation—Prepare a standard curve of the niacin standard transmittances for each level of *Standard Niacin Solution* plotted against μg of niacin contained in the respective tubes. From this standard curve, determine by interpolation the niacin content of the test solution in each tube. Disregard transmittance values equivalent to less than 0.5 mL or more than 4.5 mL of *Standard Niacin Solution*. The niacin content of the test material is calculated from the average values obtained from not less than six tubes that do not vary by more than ± 10 percent from the average. If the transmittance values of less than six tubes containing the test solution are within the range of the 0.5- to 4.5-mL levels of the niacin standard tubes, the data are insufficient to permit calculation of the concentration of niacin in the test material. Transmittance values of inoculated blank exceeding readings corresponding to dried cell weights of more than 600 μg per tube indicate the presence of an excessive amount of niacin in the *Basal Medium Stock Solution* and invalidate the assay.

Multiply the values obtained by 0.992 if the results are to be expressed as niacinamide.

<451> NITRITE TITRATION

The following general method is provided for the determination of most of the Pharmacopeial sulfonamide drugs and their dosage forms, as well as of other Pharmacopeial drugs for which nitrite titration is particularly suitable.

USP Reference Standards (11)—*USP Sulfanilamide RS*.

Procedure—Accurately weigh about 500 mg in the case of a sulfonamide, or otherwise the quantity specified in the individual monograph, and transfer to a suitable open vessel. Add 20 mL of hydrochloric acid and 50 mL of water, stir until dissolved, cool to about 15°, and slowly titrate with 0.1 M sodium nitrite VS that previously has been standardized against USP Sulfanilamide RS.

Determine the endpoint electrometrically, using suitable electrodes (platinum-calomel or platinum-platinum). Place the buret tip below the surface of the solution to eliminate air oxidation of the sodium nitrite, and stir the solution gently, using a magnetic stirrer, without pulling a vortex of air under the surface, maintaining the temperature at about 15°. The titration may be carried out manually, or by means of an automatic titrator. In performing it manually, add the titrant until the titration is within 1 mL of the endpoint, and then add it in 0.1-mL portions, allowing not less than 1 minute between additions. (The instrument needle deflects and then returns to approximately its original position until the endpoint is reached.)

The weight, in mg, of the substance to which each mL of 0.1 M sodium nitrite VS is equivalent is as stated in the individual monograph.

For the assay of Tablets of the sulfonamides or other drugs, reduce not less than 20 tablets to a fine powder, weigh accurately a portion of the powder, equivalent to about 500 mg if a sulfonamide, or the quantity of drug specified in the individual monograph, and proceed as directed in the foregoing, beginning with "transfer to a suitable open vessel."

For the assay of Injections and other liquid forms where the nitrite titration is specified, pipet a portion, equivalent to about 500 mg if a sulfonamide, or the quantity of drug specified in the individual monograph, into a suitable open vessel, and proceed as directed in the foregoing, beginning with "Add 20 mL of hydrochloric acid."

<461> NITROGEN DETERMINATION

Some alkaloids and other nitrogen-containing organic compounds fail to yield all of their nitrogen upon digestion with sulfuric acid; therefore these methods cannot be used for the determination of nitrogen in all organic compounds.

METHOD I

Nitrates and Nitrites Absent—Place about 1 g of the substance, accurately weighed, in a 500-mL Kjeldahl flask of hard borosilicate glass. The material to be tested, if solid or semisolid, may be wrapped in a sheet of nitrogen-free filter paper for convenience in transferring it to the flask. Add 10 g of powdered potassium sulfate or anhydrous sodium sulfate, 500 mg of powdered cupric sulfate, and 20 mL of sulfuric acid. Incline the flask at an angle of about 45°, and gently heat the mixture, keeping the temperature below the boiling point until frothing has ceased. Increase the heat until the acid boils briskly, and continue the heating until the solution has been clear green in color or almost colorless for 30 minutes. Allow to cool, add 150 mL of water, mix the contents of the flask, and again cool. Add cautiously 100 mL of sodium hydroxide solution (2 in 5), in such manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution. Immediately add a few pieces of granulated zinc, and without delay connect the flask to a Kjeldahl connecting bulb (trap), previ-

ously attached to a condenser, the delivery tube from which dips beneath the surface of 100 mL of boric acid solution (1 in 25) contained in a conical flask or a wide-mouth bottle of about 500-mL capacity. Mix the contents of the Kjeldahl flask by gentle rotation, and distill until about four-fifths of the contents of the flask has distilled over. Titrate with 0.5 N sulfuric acid VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.5 N sulfuric acid VS is equivalent to 7.003 mg of nitrogen.

When the nitrogen content of the substance is known to be low, the 0.5 N sulfuric acid VS may be replaced by 0.1 N sulfuric acid VS. Each mL of 0.1 N sulfuric acid VS is equivalent to 1.401 mg of nitrogen.

Nitrates and Nitrites Present—Place a quantity of the substance, accurately weighed, corresponding to about 150 mg of nitrogen, in a 500-mL Kjeldahl flask of hard borosilicate glass, and add 25 mL of sulfuric acid in which 1 g of salicylic acid previously has been dissolved. Mix the contents of the flask, and allow the mixture to stand for 30 minutes with frequent shaking. To the mixture add 5 g of powdered sodium thiosulfate, again mix, then add 500 mg of powdered cupric sulfate, and proceed as directed under *Nitrates and Nitrites Absent*, beginning with "Incline the flask at an angle of about 45°."

When the nitrogen content of the substance is known to exceed 10%, 500 mg to 1 g of benzoic acid may be added, prior to digestion, to facilitate the decomposition of the substance.

METHOD II

Apparatus—Select an appropriate 300-mL Kjeldahl flask, from which the nitrogen is first liberated by acid digestion and then transferred quantitatively to the titration vessel by steam distillation.

Procedure—Place an accurately weighed or measured quantity of the material, equivalent to 2 to 3 mg of nitrogen, in the digestion flask of the apparatus. Add 1 g of a powdered mixture of potassium sulfate and cupric sulfate (10:1), and wash down any adhering material from the neck of the flask with a fine jet of water. Add 7 mL of sulfuric acid, allowing it to rinse down the wall of the flask, then, while swirling the flask, add 1 mL of 30 percent hydrogen peroxide cautiously down the side of the flask. (Do not add hydrogen peroxide during the digestion.)

Heat the flask over a free flame or an electric heater until the solution has a clear blue color and the sides of the flask are free from carbonaceous material. Cautiously add to the digestion mixture 70 mL of water, cool the solution, and arrange for steam distillation. Add through a funnel 30 mL of sodium hydroxide solution (2 in 5) in such manner as to cause the solution to flow down the inner side of the flask to form a layer under the acid solution, rinse the funnel with 10 mL of water, tightly close the apparatus, and begin the distillation with steam immediately. Receive the distillate in 15 mL of boric acid solution (1 in 25), to which has been added 3 drops of methyl red-methylene blue TS and sufficient water to cover the end of the condensing tube. Continue the distillation until the distillate measures 80 to 100 mL. Remove the absorption flask, rinse the end of the condensing tube with a small quantity of water, and titrate the distillate with 0.01 N sulfuric acid VS. Perform a blank determination, and make any necessary correction. Each mL of 0.01 N sulfuric acid VS is equivalent to 140.1 µg of nitrogen.

When a quantity of material containing more than 2 to 3 mg of nitrogen is taken, 0.02 N or 0.1 N sulfuric acid may be employed, provided that at least 15 mL is required for the titration. If the total dry weight of material taken is

greater than 100 mg, increase proportionately the quantities of sulfuric acid and sodium hydroxide.

(466) ORDINARY IMPURITIES

This test, where called for in the individual monograph, is provided to evaluate the presence of ordinary impurities in official articles. Ordinary impurities are defined as those species in drug substances and/or drug products that have no significant, undesirable biological activity in the amounts present. These impurities may arise out of the synthesis, preparation, or degradation of compendial articles. In certain instances, impurities that pose a potential health risk may be detected. Because these impurities would not be individually identified by the strict use of this General Chapter, a separate evaluation may be necessary to ensure that the detected impurities fit the requirements set forth in the definition of Ordinary Impurities. Selections of tests and assays allow for anticipated amounts of impurities that are unobjectionable for the customary use of the article.

Reporting and Specifications—The value of 2.0%, unless otherwise specified in the individual monograph, was selected as the general limit for the total amount of ordinary impurities in monographs where documentation did not support adoption of other values.

Where a monograph sets limits on concomitant components and/or specified impurities/degradation products, these species are not to be included in the estimation of ordinary impurities unless so stated in the individual monograph. Concomitant components are defined as species characteristic of many drug substances that are not considered to be impurities in the Pharmacopeial sense. Examples of concomitant components are geometric and optical isomers (or racemates) and antibiotics that are mixtures. Any component that can be considered a toxic impurity because of significant undesirable biological effect is not considered to be a concomitant component.

Methodology—Unless otherwise specified in an individual monograph, estimation of the amount and number of ordinary impurities is made by relative methods rather than by strict comparison to individual Reference Standards. Non-specific detection of ordinary impurities is also consistent with this classification.

Typical evaluation methods used for ordinary impurities are thin-layer chromatographic (TLC) techniques. See *Chromatography* (621) for a general discussion of the thin-layer chromatographic technique. Tests for related substances or chromatographic purity may also be used to evaluate the presence of ordinary impurities. Other methods (e.g., HPLC, HPTLC, etc.) may also be used with adequate justification as an alternate method. Unless otherwise specified in the individual monograph, use the following method.

Test Solution—Prepare, in the solvent specified in the monograph, a solution of the substance under test having an accurately known final concentration of about 10 mg per mL. [NOTE—Heat or sonication may be used to dissolve the drug substance where use of such does not adversely affect the compound.]

Standard Solutions—Prepare, in the solvent specified in the monograph, solutions of the USP Reference Standard or designated substance having accurately known concentrations of 0.01 mg per mL, 0.05 mg per mL, 0.1 mg per mL, and 0.2 mg per mL. [NOTE—Heat or sonication may be used to dissolve the drug substance where use of such does not adversely affect the compound.]

Procedure—Use a thin-layer chromatographic plate coated with a 0.25-mm layer of chromatographic silica gel

mixture, and the *Eluant* specified in the monograph. Apply equal volumes (20 μ L) of the *Test Solution* and *Standard Solutions* to the plate, using a stream of nitrogen to dry the spots.

Allow the chromatogram to develop in a pre-equilibrated chamber until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the chamber, and air-dry. View the plate using the visualization technique(s) specified. Locate any spots other than the principal spot in the chromatogram of the *Test Solution*, and determine their relative intensities by comparison with the chromatograms of the appropriate *Standard Solutions*. See discussion above with regard to reporting and specifying total ordinary impurities.

KEY FOR VISUALIZATION TECHNIQUES

- (1) Use UV light at 254 nm and at about 366 nm.
- (2) Use Iodoplatinate TS.
- (3) *Solution A*—Mix 850 mg of bismuth subnitrate with 40 mL of water and 10 mL of glacial acetic acid.
Solution B—Dissolve 8 g of potassium iodide in 20 mL of water. Mix A and B together to obtain a Stock Solution which can be stored for several months in a dark bottle. Mix 10 mL of the Stock Solution with 20 mL of glacial acetic acid, and dilute with water to make 100 mL, to prepare the spray reagent.
- (4) *Ninhydrin Spray*—Dissolve 200 mg of ninhydrin in 100 mL of alcohol. Heat the plate after spraying.
- (5) *Acid Spray*—In an ice bath, add slowly and cautiously, with stirring, 10 mL of sulfuric acid to 90 mL of alcohol. Spray the plate, and heat until charred.
- (6) *Acid-Dichromate Spray*—Add sufficient potassium dichromate to 100 mL of sulfuric acid to make a saturated solution. Spray the plate, and heat until charred.
- (7) *Vanillin*—Dissolve 1 g of vanillin in 100 mL of sulfuric acid.
- (8) *Chloramine T-Trichloroacetic Acid*—Mix 10 mL of a 3% aqueous solution of chloramine T with 40 mL of a 25% alcoholic solution of trichloroacetic acid. Prepare immediately before use.
- (9) *Folin-C*—Add 10 g of sodium tungstate and 2.5 g of sodium molybdate to 70 mL of water, add 5 mL of 85% phosphoric acid and 10 mL of 36% hydrochloric acid, and reflux this solution for 10 hours.
- (10) *KMnO₄*—Dissolve 100 mg of Potassium Permanganate in 100 mL of water.
- (11) *DAB*—Mix 1 g of *p*-dimethylaminobenzaldehyde in 100 mL of 0.6 N hydrochloric acid.
- (12) *DAC*—Mix 100 mg of *p*-dimethylaminocinnamaldehyde in 100 mL of 1 N hydrochloric acid.
- (13) *Ferricyanide*—Mix equal volumes of a 1% ferric chloride solution and a 1% potassium ferricyanide solution. Use immediately.
- (14) *Fast Blue B*—Reagent A—Dissolve 500 mg of Fast Blue B Salt in 100 mL of water.
Reagent B—0.1 N sodium hydroxide.
Spray first with A, then with B.
- (15) *Alkaline Ferric Cyanide*—Dilute 1.5 mL of a 1% potassium ferricyanide solution with water to 20 mL, and add 10 mL of 15% sodium hydroxide solution.
- (16) *Iodine Spray*—Prepare a 0.5% solution of iodine in chloroform.
- (17) Expose the plate for 10 minutes to iodine vapors in a pre-equilibrated closed chamber, on the bottom of which there are iodine crystals.
- (18) *Solution A*—Dissolve 0.5 g of potassium iodide in 50 mL of water.
Solution B—Prepare a solution of 0.5 g of soluble starch in 50 mL of hot water.
Just prior to use, mix equal volumes of *Solution A* and *Solution B*.

(19) *PTSS*—Dissolve 20 g of *p*-toluenesulfonic acid in 100 mL of alcohol, spray the plate, dry for 15 minutes at 110°, and view under UV light at 366 nm.

(20) *o-Tolidine Spray*—Dissolve 160 mg of *o*-tolidine in 30 mL of glacial acetic acid, dilute with water to make 500 mL, add 1 g of potassium iodide, and mix until the potassium iodide has dissolved.

(21) Mix 3 mL of chloroplatinic acid solution (1 in 10) with 97 mL of water, followed by the addition of 100 mL of potassium iodide solution (6 in 100) to prepare the spray reagent.

(22) *Iodine-Methanol Spray*—Prepare a mixture of iodine TS and methanol (1:1).

<467> RESIDUAL SOLVENTS

INTRODUCTION

This general chapter applies to existing drug substances, excipients, and products. All substances and products are subject to relevant control of solvents likely to be present in a substance or product.

Where the limits to be applied comply with those given below, tests for residual solvents are not generally mentioned in specific monographs, because the solvents employed may vary from one manufacturer to another.

The objective of this general chapter is to provide acceptable amounts of residual solvents in pharmaceuticals for the safety of the patient. The chapter recommends the use of less toxic solvents and describes levels considered to be toxicologically acceptable for some residual solvents.

For pharmacopeial purposes, residual solvents in pharmaceuticals are defined as organic volatile chemicals that are used or produced in the manufacture of drug substances or excipients, or in the preparation of drug products. The residual solvents are not completely removed by practical manufacturing techniques. Appropriate selection of the solvent for the synthesis of a drug substance or an excipient may enhance the yield, or determine characteristics such as crystal form, purity, and solubility. Therefore, the solvent may sometimes be a critical element in the synthetic process. This general chapter does not address solvents deliberately used as excipients, nor does it address solvents. However, the content of solvents in such products should be evaluated and justified.

Because residual solvents do not provide therapeutic benefit, they should be removed, to the extent possible, to meet ingredient and product specifications, good manufacturing practices, or other quality-based requirements. Drug products should contain no higher levels of residual solvents than can be supported by safety data. Solvents that are known to cause unacceptable toxicities (Class 1, *Table 1*) should be avoided in the production of drug substances, excipients, or drug products unless their use can be strongly justified in a risk-benefit assessment. Solvents associated with less severe toxicity (Class 2, *Table 2*) should be limited in order to protect patients from potential adverse effects. Ideally, less toxic solvents (Class 3, *Table 3*) should be used where practical. The complete list of solvents included in this general chapter is given in *Appendix 1*. These tables and the list are not exhaustive. For the purposes of this Pharmacopeia, when a manufacturer has received approval from a competent regulatory authority for the use of a new solvent not currently listed in this general chapter, it is the responsibility of that manufacturer to notify the USP regarding the identity of this solvent, the approved residual solvent limit in

the article, and the appropriate test procedure for this residual solvent in the article. The USP will then address this topic in the individual monograph. When a new solvent has been approved through the ICH process, this new solvent will be added to the appropriate list in this general chapter. At that time, consideration will be given for removal of the specific solvent test requirement in the individual monograph.

Testing of drug substances, excipients, and drug products for residual solvents should be performed when production or purification processes are known to result in the presence of such residual solvents. It is only necessary to test for residual solvents that are used or produced in the manufacture or purification of drug substances, excipients, or products.

Although manufacturers may choose to test the drug product, a cumulative procedure may be used to calculate the residual solvent levels in the drug product from the levels in the ingredients used to produce the drug product. If the calculation results in a level equal to or below that provided in this general chapter, no testing of the drug product for residual solvents need be considered. If, however, the calculated level is above the recommended level, the drug product should be tested to ascertain whether the formulation process has reduced the relevant solvent level to within the acceptable amount. A drug product should also be tested if a residual solvent is used during its manufacture.

For the purposes of this Pharmacopeia, when a manufacturer has received approval from a competent regulatory authority for a higher level of residual solvent, it is the responsibility of that manufacturer to notify the USP regarding the identity of this solvent and the approved residual solvent limit in the article. The USP will then address this topic in the individual monograph.

See *Appendix 2* for additional background information related to residual solvents.

CLASSIFICATION OF RESIDUAL SOLVENTS BY RISK ASSESSMENT

The term *tolerable daily intake* (TDI) is used by the International Program on Chemical Safety (IPCS) to describe exposure limits of toxic chemicals, and the term *acceptable daily intake* (ADI) is used by the World Health Organization (WHO) and other national and international health authorities and institutes. The term *permitted daily exposure* (PDE) is defined as a pharmaceutically acceptable intake of residual solvents to avoid confusion of differing values for ADIs of the same substance.

Residual solvents assessed in this general chapter are listed in *Appendix 1* by common names and structures. They were evaluated for their possible risk to human health and placed into one of three classes as follows:

Residual Solvent Class	Assessment
Class 1	Solvents to be avoided Known human carcinogens Strongly suspected human carcinogens Environmental hazards
Class 2	Solvents to be limited Nongenotoxic animal carcinogens or possible causative agents of other irreversible toxicity, such as neurotoxicity or teratogenicity Solvents suspected of other significant but reversible toxicities

*For residual solvents with PDEs of more than 50 mg per day, see the discussion in the section *Class 3* under *Limits of Residual Solvents*.

Residual Solvent Class	Assessment
Class 3	Solvents with low toxic potential Solvents with low toxic potential to humans; no health-based exposure limit is needed [NOTE—Class 3 residual solvents have PDEs of 50 mg or more per day.*]

*For residual solvents with PDEs of more than 50 mg per day, see the discussion in the section *Class 3* under *Limits of Residual Solvents*.

METHODS FOR ESTABLISHING EXPOSURE LIMITS

The method used to establish PDEs for residual solvents is presented in *Appendix 3*.

For articles that are designated “for veterinary use only”, higher levels for the PDE and concentration limit may be justified in exceptional cases based upon the actual daily dose, actual target species, and relevant toxicological data and considering consumer safety impact. For the purpose of this Pharmacopeia, when a manufacturer has received approval from a competent regulatory authority for a higher limit, it is the responsibility of that manufacturer to notify the USP regarding the approved residual solvent limit in the article and the justification. The USP will then address this topic in the individual monograph.

OPTIONS FOR DESCRIBING LIMITS OF CLASS 2 RESIDUAL SOLVENTS

Two options are available when setting limits for Class 2 residual solvents.

Option 1

The concentration limits in ppm stated in *Table 2* are used. They were calculated using the equation below by assuming a product weight of 10 g administered daily.

$$\text{Concentration (ppm)} = (1000 \mu\text{g/mg} \times \text{PDE})/\text{dose}$$

Here, PDE is given in terms of mg per day, and dose is given in g per day.

These limits are considered acceptable for all drug substances, excipients, and drug products. Therefore, this option may be applied if the daily dose is not known or fixed. If all drug substances and excipients in a formulation meet the limits given in *Option 1*, these components may be used in any proportion. No further calculation is necessary, provided that the daily dose does not exceed 10 g. Products that are administered in doses greater than 10 g per day are to be considered under *Option 2*.

Option 2

It is not necessary for each component of the drug product to comply with the limits given in *Option 1*. The PDE in terms of mg per day as stated in *Table 2* can be used with the known maximum daily dose and the equation above to determine the concentration of residual solvent allowed in a drug product. Such limits are considered acceptable, provided that it has been demonstrated that the residual solvent has been reduced to the practical minimum. The limits should be realistic in relation to analytical precision, manufacturing capability, and reasonable variation in the manufacturing process. The limits should also reflect contemporary manufacturing standards.

Option 2 may be applied by adding the amounts of a residual solvent present in each of the components of the

drug product. The sum of the amounts of solvent per day should be less than that given by the PDE.

Consider an example of the application of *Option 1* and *Option 2* to acetonitrile concentration in a drug product. The permitted daily exposure to acetonitrile is 4.1 mg per day; thus, the *Option 1* limit is 410 ppm. The maximum administered daily weight of a drug product is 5.0 g, and the drug product contains two excipients. The composition of the drug product and the calculated maximum content of residual acetonitrile are given in the following table.

Component	Amount in Formulation (g)	Acetonitrile Content (ppm)	Daily Exposure (mg)
Drug substance	0.3	800	0.24
Excipient 1	0.9	400	0.36
Excipient 2	3.8	800	3.04
Drug product	5.0	728	3.64

Excipient 1 meets the *Option 1* limit, but the drug substance, excipient 2, and drug product do not meet the *Option 1* limit. Nevertheless, the drug product meets the *Option 2* limit of 4.1 mg per day and thus conforms to the acceptance criteria in this general chapter.

Consider another example, using acetonitrile as the residual solvent. The maximum administered daily weight of a drug product is 5.0 g, and the drug product contains two excipients. The composition of the drug product and the calculated maximum content of residual acetonitrile are given in the following table.

Component	Amount in Formulation (g)	Acetonitrile Content (ppm)	Daily Exposure (mg)
Drug substance	0.3	800	0.24
Excipient 1	0.9	2000	1.80
Excipient 2	3.8	800	3.04
Drug product	5.0	1016	5.08

In this example, the drug product meets neither the *Option 1* nor the *Option 2* limit according to this summation. The manufacturer could test the drug product to determine whether the formulation process reduced the level of acetonitrile. If the level of acetonitrile was not reduced to the allowed limit during formulation, the product fails to meet the solvent limits as described in this chapter, and the manufacturer of the drug product should take other steps to reduce the amount of acetonitrile in the drug product. In some instances the manufacturer may have received approval from a competent regulatory authority for such a higher level of residual solvent. If this is the case, it is the responsibility of that manufacturer to notify the USP regarding the identity of this solvent and the approved residual solvent limit in the article. The USP will then address this topic in the individual monograph.

ANALYTICAL PROCEDURES

Residual solvents are typically determined using chromatographic techniques such as gas chromatography. Compensatory methods for testing for residual solvent content are described under the section *Identification, Control, and Quantification of Residual Solvents* in this general chapter. The *General Notices* discuss the use of other methods in special circumstances (see 6.30. *Alternative and Harmonized Methods and Procedures*). If Class 3 solvents are present, a nonspecific method such as loss on drying may be used.

REPORTING LEVELS OF RESIDUAL SOLVENTS

Manufacturers of pharmaceutical products need certain information about the content of residual solvents in drug substances or excipients in order to meet the criteria of this general chapter. The following statements are given as acceptable examples of the information that could be provided from a supplier of drug substances or excipients to a pharmaceutical manufacturer. The supplier might choose one of the following as appropriate:

- Only Class 3 solvents are likely to be present. Loss on drying is less than 0.5%.
- Only Class 2 solvents X, Y, ... are likely to be present. All are below the *Option 1* limit. (Here the supplier would name the Class 2 solvents represented by X, Y, ...)
- Only Class 2 solvents X, Y, ... and Class 3 solvents are likely to be present. Residual Class 2 solvents are below the *Option 1* limit and residual Class 3 solvents are below 0.5%.

The phrase "likely to be present" as used in the above examples refers to the solvent used or produced in the final manufacturing step and to solvents that are used or produced in earlier manufacturing steps and not removed consistently by a validated process.

If Class 1 solvents are likely to be present, they should be identified and quantified. If solvents of Class 2 or 3 are present at greater than their *Option 1* limits or 0.5%, respectively, they should be identified and quantified.

LIMITS OF RESIDUAL SOLVENTS

Class 1 (solvents to be avoided)

Class 1 residual solvents (*Table 1*) should not be employed in the manufacture of drug substances, excipients, and drug products because of the unacceptable toxicities or deleterious environmental effects of these residual solvents. However, if their use in order to produce a medicinal product with a significant therapeutic advance is unavoidable, their levels should be restricted as shown in *Table 1*, unless otherwise stated in the individual monograph. The solvent 1,1,1-trichloroethane is included in *Table 1* because it is an environmental hazard. The stated limit of 1500 ppm is based on a review of safety data.

When Class 1 residual solvents are used or produced in the manufacture or purification of a drug substance, excipient, or drug product and are not removed by the process, these solvents should be identified and quantified. The procedures described in the section *Identification, Control, and Quantification of Residual Solvents* in this general chapter are to be applied wherever possible. Otherwise an appropriate validated procedure is to be employed.

Table 1. Class 1 Residual Solvents
(solvents that should be avoided)

Solvent	Concentration Limit (ppm)	Concern
Benzene	2	Carcinogen
Carbon tetrachloride	4	Toxic and environmental hazard
1,2-Dichloroethane	5	Toxic
1,1-Dichloroethene	8	Toxic
1,1,1-Trichloroethane	1500	Environmental hazard

Class 2

Class 2 residual solvents (*Table 2*) should be limited in drug substances, excipients, and drug products because of the inherent toxicities of the residual solvents. PDEs are given to the nearest 0.1 mg per day, and concentrations are given to the nearest 10 ppm. The stated values do not reflect the necessary analytical precision of the determination procedure. Precision should be determined as part of the procedure validation.

If Class 2 residual solvents are present at greater than their *Option 1* limits, they should be identified and quantified. The procedures described in the section *Identification, Control, and Quantification of Residual Solvents* in this general chapter are to be applied wherever possible. Otherwise an appropriate validated procedure is to be employed.

[NOTE—The following Class 2 residual solvents are not readily detected by the headspace injection conditions described in the section *Identification, Control, and Quantification of Residual Solvents* in this general chapter: formamide, 2-ethoxyethanol, 2-methoxyethanol, ethylene glycol, *N*-methylpyrrolidone, and sulfolane. Other appropriate validated procedures are to be employed for the quantification of these residual solvents. Such procedures shall be submitted to the USP for review and possible inclusion in the relevant individual monograph. In addition, USP Residual Solvent Class 2—Mixture C RS can be used to develop an alternative procedure.]

Table 2. Class 2 Residual Solvents

Solvent	PDE (mg/day)	Concentration Limit (ppm)
Acetonitrile	4.1	410
Chlorobenzene	3.6	360
Chloroform	0.6	60
Cyclohexane	38.8	3880
1,2-Dichloroethene	18.7	1870
1,2-Dimethoxyethane	1.0	100
<i>N,N</i> -Dimethylacetamide	10.9	1090
<i>N,N</i> -Dimethylformamide	8.8	880
1,4-Dioxane	3.8	380
2-Ethoxyethanol	1.6	160
Ethylene glycol	6.2	620
Formamide	2.2	220
Hexane	2.9	290
Methanol	30.0	3000
2-Methoxyethanol	0.5	50
Methylbutylketone	0.5	50
Methylcyclohexane	11.8	1180
Methylene chloride	6.0	600
<i>N</i> -Methylpyrrolidone	5.3	530
Nitromethane	0.5	50
Pyridine	2.0	200
Sulfolane	1.6	160
Tetrahydrofuran	7.2	720
Tetralin	1.0	100
Toluene	8.9	890
Trichloroethylene	0.8	80
Xylene*	21.7	2170

*Usually 60% *m*-xylene, 14% *p*-xylene, 9% *o*-xylene with 17% ethyl benzene.

Class 3

Class 3 residual solvents (*Table 3*) may be regarded as less toxic and of lower risk to human health than Class 1 and

Class 2 residual solvents. Class 3 includes no solvent known as a human health hazard at levels normally accepted in pharmaceuticals. However, there are no long-term toxicity or carcinogenicity studies for many of the residual solvents in Class 3. Available data indicate that they are less toxic in acute or short-term studies and negative in genotoxicity studies.

It is considered that amounts of these residual solvents of 50 mg per day or less (corresponding to 5000 ppm or 0.5% under *Option 1*) would be acceptable without justification. Higher amounts may also be acceptable, provided that they are realistic in relation to manufacturing capability and good manufacturing practice. For the purposes of this Pharmacopeia, when a manufacturer has received approval from a competent regulatory authority for such a higher level of residual solvent, it is the responsibility of that manufacturer to notify the USP regarding the identity of this solvent and the approved residual solvent limit in the article. The USP will then address this topic in the individual monograph. If a Class 3 solvent limit in an individual monograph is greater than 50 mg per day, that residual solvent should be identified and quantified. The procedures described in the section *Identification, Control, and Quantification of Residual Solvents* in this general chapter, with appropriate modifications to the standard solutions, are to be applied wherever possible. Otherwise an appropriate validated procedure is to be employed.

Table 3. Class 3 Residual Solvents

(limited by GMP or other quality-based requirements in drug substances, excipients, and drug products)

Acetic acid	Heptane
Acetone	Isobutyl acetate
Anisole	Isopropyl acetate
1-Butanol	Methyl acetate
2-Butanol	3-Methyl-1-butanol
Butyl acetate	Methylethylketone
<i>tert</i> -Butylmethyl ether	Methylisobutylketone
Cumene	2-Methyl-1-propanol
Dimethyl sulfoxide	Pentane
Ethanol	1-Pentanol
Ethyl acetate	1-Propanol
Ethyl ether	2-Propanol
Ethyl formate	Propyl acetate
Formic acid	

Other Residual Solvents

The residual solvents listed in *Table 4* may also be of interest to manufacturers of drug substances, excipients, or drug products. However, no adequate toxicological data on which to base a PDE was found.

Table 4. Other Residual Solvents

(for which no adequate toxicological data was found)

1,1-Diethoxypropane	Methyl isopropyl ketone
1,1-Dimethoxymethane	Methyltetrahydrofuran
2,2-Dimethoxypropane	Solvent hexane
Isooctane	Trichloroacetic acid
Isopropyl ether	Trifluoroacetic acid

IDENTIFICATION, CONTROL, AND QUANTIFICATION OF RESIDUAL SOLVENTS

Whenever possible, the substance under test needs to be dissolved to release the residual solvent. Because the USP deals with drug products, as well as active ingredients and excipients, it may be acceptable that in some cases, some of the components of the formulation will not dissolve completely. In those cases, the drug product may first need to be pulverized into a fine powder so that any residual solvent that may be present can be released. This operation should be performed as fast as possible to prevent the loss of volatile solvents during the procedure.

NOTE—The organic-free water specified in the following procedures produces no significantly interfering peaks when chromatographed.

Class 1 and Class 2 Residual Solvents

The following procedures are useful to identify and quantify residual solvents when the information regarding which solvents are likely to be present in the material is not available. When the information about the presence of specific residual solvents is available, only *Procedure C* is needed to quantify the amount of residual solvents present. A flow diagram for the application of the residual solvent limit tests is shown in *Figure 1*.

WATER-SOLUBLE ARTICLES

Procedure A—

Class 1 Standard Stock Solution—[**NOTE**—When transferring solutions, place the tip of the pipet just below the surface of the liquid, and mix.] Transfer 1.0 mL of USP Class 1 Residual Solvents Mixture RS to a 100-mL volumetric flask, previously filled with about 9 mL of dimethyl sulfoxide, dilute with water to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, previously filled with about 50 mL of water, dilute with water to volume, and mix. Transfer 10 mL of this solution to a 100-mL volumetric flask, previously filled with about 50 mL of water, dilute with water to volume, and mix.

Class 1 Standard Solution—Transfer 1.0 mL of *Class 1 Standard Stock Solution* to an appropriate headspace vial containing 5.0 mL of water (place the tip of the pipet just below the surface of the liquid for dispensing), apply the stopper, cap, and mix.

Class 2 Standard Stock Solutions—Transfer 1.0 mL of USP Residual Solvents Class 2—Mixture A RS to a 100-mL volumetric flask, dilute with water to volume, and mix. This is *Class 2 Standard Stock Solution A*. Transfer 1.0 mL of USP Residual Solvents Class 2—Mixture B RS to a 100-mL volumetric flask, dilute with water to volume, and mix. This is *Class 2 Standard Stock Solution B*.

Class 2 Mixture A Standard Solution—Transfer 1.0 mL of *Class 2 Standard Stock Solution A* to an appropriate head-

space vial, add 5.0 mL of water, apply the stopper, cap, and mix.

Class 2 Mixture B Standard Solution—Transfer 5.0 mL of *Class 2 Standard Stock Solution B* to an appropriate headspace vial, add 1.0 mL of water, apply the stopper, cap, and mix.

Test Stock Solution—Transfer about 250 mg of the article under test, accurately weighed, to a 25-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

Test Solution—Transfer 5.0 mL of *Test Stock Solution* to an appropriate headspace vial, add 1.0 mL of water, apply the stopper, cap, and mix.

Class 1 System Suitability Solution—Transfer 1.0 mL of *Class 1 Standard Stock Solution* to an appropriate headspace vial, add 5.0 mL of *Test Stock Solution*, apply the stopper, cap, and mix.

Chromatographic System (see *Chromatography* (621))—The gas chromatograph is equipped with a flame-ionization detector and a 0.32-mm × 30-m fused-silica column coated with a 1.8-μm layer of phase G43 or a 0.53-mm × 30-m wide-bore column coated with a 3.0-μm layer of phase G43. The carrier gas is nitrogen or helium with a linear velocity of about 35 cm per second, and a split ratio of 1:5. [NOTE—The split ratio can be modified in order to optimize sensitivity.] The column temperature is maintained at 40° for 20 minutes, then raised at a rate of 10° per minute to 240°, and maintained at 240° for 20 minutes. The injection port and detector temperatures are maintained at 140° and 250°, respectively. Chromatograph the *Class 1 Standard Solution*, *Class 1 System Suitability Solution*, and *Class 2 Mixture A Standard Solution*, and record the peak responses as directed for *Procedure*: the signal-to-noise ratio of 1,1,1-trichloroethane in the *Class 1 Standard Solution* is not less than 5; the signal-to-noise ratio of each peak in the *Class 1 System Suitability Solution* is not less than 3; and the resolution, R, between acetonitrile and methylene chloride in the *Class 2 Mixture A Standard Solution* is not less than 1.0.

Procedure—[NOTE—It is recommended to increase the temperature of the transfer line between runs to eliminate any potential condensation of solvents.] Separately inject (following one of the headspace operating parameter sets described in *Table 5*) equal volumes of headspace (about 1.0 mL) of the *Class 1 Standard Solution*, *Class 2 Mixture A Standard Solution*, *Class 2 Mixture B Standard Solution*, and *Test Solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. If a peak response of any peak, other than a peak for 1,1,1-trichloroethane, in the *Test Solution* is greater than or equal to a corresponding peak in either the *Class 1 Standard Solution* or either of the two *Class 2 Mixture Standard Solutions*, or a peak response of 1,1,1-trichloroethane is greater than or equal to 150 times the peak response corresponding to 1,1,1-trichloroethane in the *Class 1 Standard Solution*, proceed to *Procedure B* to verify the identity of the peak; otherwise the article meets the requirements of this test.

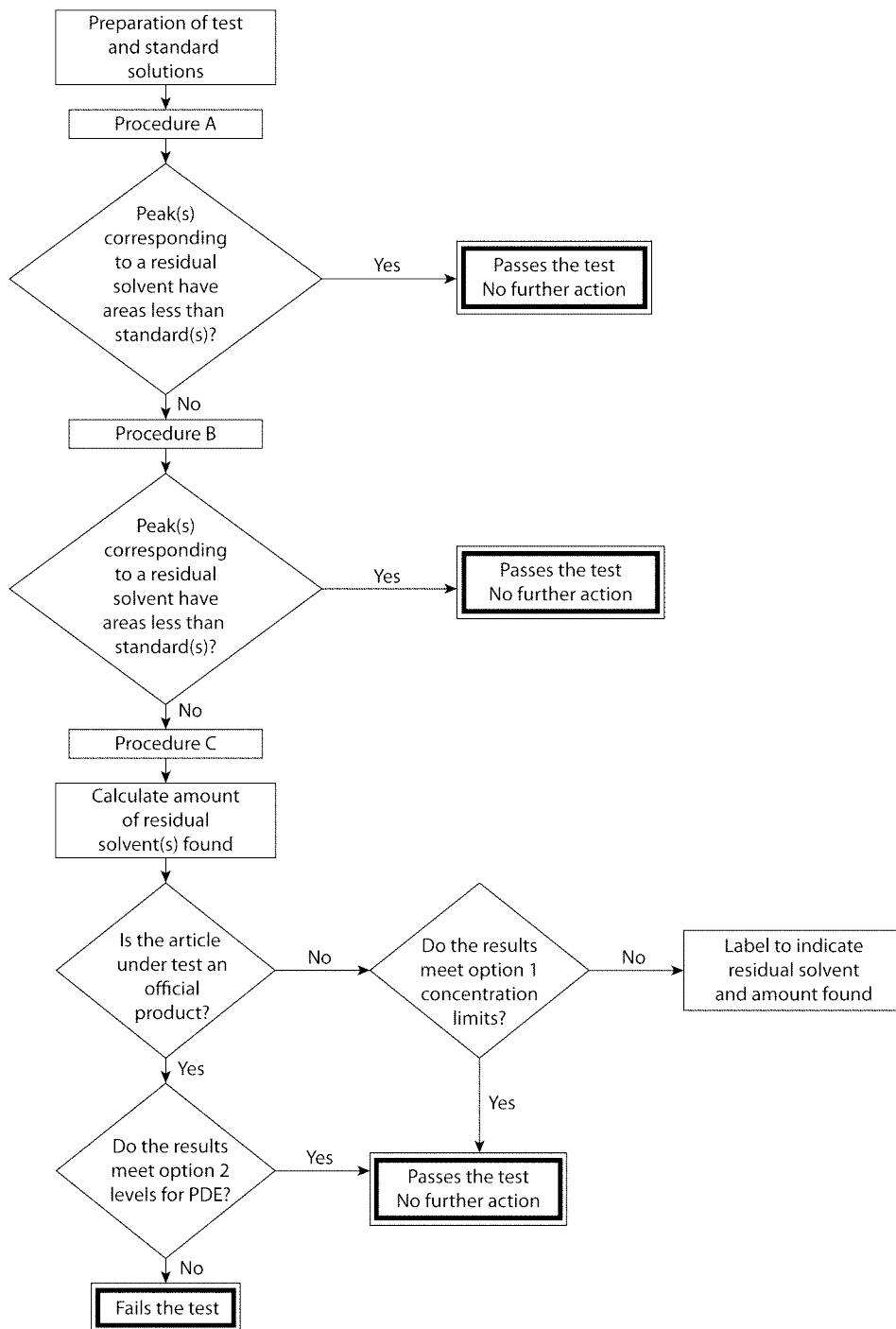


Figure 1. Diagram relating to the identification of residual solvents and the application of limit tests.

Procedure B—

Class 1 Standard Stock Solution, Class 1 Standard Solution, Class 2 Standard Stock Solutions, Class 2 Mixture A Standard Solution, Class 2 Mixture B Standard Solution, Test Stock Solution, Test Solution, and Class 1 System Suitability Solution—Prepare as directed for Procedure A.

Chromatographic System (see *Chromatography* (621))—The gas chromatograph is equipped with a flame-ionization detector and a 0.32-mm × 30-m fused-silica column coated with a 0.25-μm layer of phase G16 or a 0.53-mm × 30-m wide-bore column coated with a 0.25-μm layer of phase G16. The carrier gas is nitrogen or helium with a linear ve-

locity of about 35 cm per second and a split ratio of 1:5. [NOTE—The split ratio can be modified in order to optimize sensitivity.] The column temperature is maintained at 50° for 20 minutes, then raised at a rate of 6° per minute to 165°, and maintained at 165° for 20 minutes. The injection port and detector temperatures are maintained at 140° and 250°, respectively. Chromatograph the *Class 1 Standard Solution* and the *Class 1 System Suitability Solution*, and record the peak responses as directed for Procedure: the signal-to-noise ratio of benzene in the *Class 1 Standard Solution* is not less than 5; the signal-to-noise ratio of each peak in the *Class 1 System Suitability Solution* is not less than 3; and the

resolution, R , between acetonitrile and *cis*-dichloroethene in the *Class 2 Mixture A Standard Solution* is not less than 1.0.

Procedure—[NOTE—It is recommended to increase the temperature of the transfer line between runs to eliminate any potential condensation of solvents.] Separately inject (following one of the headspace operating parameter sets described in *Table 5*) equal volumes of headspace (about 1.0 mL) of the *Class 1 Standard Solution*, the *Class 2 Mixture A Standard Solution*, the *Class 2 Mixture B Standard Solution*, and the *Test Solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. If the peak response(s) in the *Test Solution* of the peak(s) identified in *Procedure A* is/are greater than or equal to a corresponding peak(s) in either the *Class 1 Standard Solution* or either of the two *Class 2 Mixture Standard Solutions*, proceed to *Procedure C* to quantify the peak(s); otherwise the article meets the requirements of this test.

Procedure C—

Class 1 Standard Stock Solution, *Class 1 Standard Solution*, *Class 2 Standard Stock Solution A*, *Class 2 Mixture A Standard Solution*, *Test Stock Solution*, *Test Solution*, and *Class 1 System Suitability Solution*—Prepare as directed for *Procedure A*.

Standard Stock Solution—[NOTE—Prepare a separate *Standard Stock Solution* for each peak identified and verified by *Procedures A* and *B*. For the *Class 1* solvents other than 1,1,1-trichloroethane, prepare the first dilution as directed for the first dilution under *Class 1 Standard Stock Solution* in *Procedure A*.] Transfer an accurately measured volume of each individual USP Reference Standard corresponding to each residual solvent peak identified and verified by *Procedures A* and *B* to a suitable container, and dilute quantitatively, and stepwise if necessary, with water to obtain a solution having a final concentration of 1/20 of the value stated in *Table 1* or *2* (under *Concentration Limit*).

Standard Solution—Transfer 1.0 mL of this solution to an appropriate headspace vial, add 5.0 mL of water, apply the stopper, cap, and mix.

Spiked Test Solution—[NOTE—Prepare a separate *Spiked Test Solution* for each peak identified and verified by *Procedures A* and *B*.] Transfer 5.0 mL of *Test Stock Solution* to an appropriate headspace vial, add 1.0 mL of the *Standard Stock Solution*, apply the stopper, cap, and mix.

Chromatographic System (see *Chromatography* (621))—[NOTE—If the results of the chromatography from *Procedure A* are found to be inferior to those found with *Procedure B*, the *Chromatographic System* from *Procedure B* may be substituted.] The gas chromatograph is equipped with a flame-ionization detector and a 0.32-mm × 30-m fused-silica column coated with a 1.8-μm layer of phase G43 or a 0.53-mm × 30-m wide-bore column coated with a 3.0-μm layer of phase G43. The carrier gas is nitrogen or helium with a linear velocity of about 35 cm per second, and a split ratio of 1:5. [NOTE—The split ratio can be modified in order to optimize sensitivity.] The column temperature is maintained at 40° for 20 minutes, then raised at a rate of 10° per minute to 240°, and maintained at 240° for 20 minutes. The

injection port and detector temperatures are maintained at 140° and 250°, respectively. Chromatograph the *Class 1 Standard Solution*, the *Class 1 System Suitability Solution*, and the *Class 2 Mixture A Standard Solution*, and record the peak responses as directed for *Procedure*: the signal-to-noise ratio of 1,1,1-trichloroethane in the *Class 1 Standard Solution* is not less than 5; the signal-to-noise ratio of each peak in the *Class 1 System Suitability Solution* is not less than 3; and the resolution, R , between acetonitrile and methylene chloride in the *Class 2 Mixture A Standard Solution* is not less than 1.0.

Procedure—[NOTE—It is recommended to increase the temperature of the transfer line between runs to eliminate any potential condensation of solvents.] Separately inject (following one of the headspace operating parameters described in *Table 5*) equal volumes of headspace (about 1.0 mL) of the *Standard Solution*, the *Test Solution*, and the *Spiked Test Solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the amount, in ppm, of each residual solvent found in the article under test by the formula:

$$5(C/W)[r_U / (r_{ST} - r_U)]$$

in which C is the concentration, in μg per mL, of the appropriate USP Reference Standard in the *Standard Stock Solution*; W is the weight, in g, of the article under test taken to prepare the *Test Stock Solution*; and r_U and r_{ST} are the peak responses of each residual solvent obtained from the *Test Solution* and the *Spiked Test Solution*, respectively.

WATER-INSOLUBLE ARTICLES

Procedure A—[NOTE—Dimethyl sulfoxide may be substituted as an alternative solvent to dimethylformamide.]

Class 1 Standard Stock Solution—Transfer 1.0 mL of USP *Class 1 Residual Solvents Mixture RS* to a 100-mL volumetric flask previously filled with about 80 mL of dimethylformamide, dilute with dimethylformamide to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, previously filled with about 80 mL of dimethylformamide, dilute with dimethylformamide to volume, and mix (reserve a portion of this solution for the *Class 1 System Suitability Solution*). Transfer 1.0 mL of this solution to a 10-mL volumetric flask, dilute with dimethylformamide to volume, and mix.

Class 1 Standard Solution—Transfer 1.0 mL of *Class 1 Standard Stock Solution* to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.

Class 2 Standard Stock Solutions—Transfer 1.0 mL of USP *Residual Solvents Class 2—Mixture A RS* to a 100-mL volumetric flask, previously filled with about 80 mL of dimethylformamide, dilute with dimethylformamide to volume, and mix. This is *Class 2 Standard Stock Solution A*. Transfer

Table 5. Headspace Operating Parameters

	Headspace Operating Parameter Sets		
	1	2	3
Equilibration temperature (°)	80	105	80
Equilibration time (min)	60	45	45
Transfer-line temperature (°) (if appropriate)	85	110	105
Syringe temperature (°) (if appropriate)	80–90	105–115	80–90
Carrier gas: nitrogen or helium at an appropriate pressure			
Pressurization time(s) (if appropriate)	≥60	≥60	≥60
Injection volume (mL)*	1	1	1

*Or follow the instrument manufacturer's recommendations, as long as the method criteria are met. Injecting less than this amount is allowed as long as adequate sensitivity is achieved.

0.5 mL of USP Residual Solvents Class 2—Mixture B RS to a 10-mL volumetric flask, dilute with dimethylformamide to volume, and mix. This is *Class 2 Standard Stock Solution B*.

Class 2 Mixture A Standard Solution—Transfer 1.0 mL of *Class 2 Standard Stock Solution A* to an appropriate headspace vial containing 5.0 mL of water, apply the stopper, cap, and mix.

Class 2 Mixture B Standard Solution—Transfer 1.0 mL of *Class 2 Standard Stock Solution B* to an appropriate headspace vial containing 5.0 mL of water, apply the stopper, cap, and mix.

Test Stock Solution—Transfer about 500 mg of the article under test, accurately weighed, to a 10-mL volumetric flask, dissolve in and dilute with dimethylformamide to volume, and mix.

Test Solution—Transfer 1.0 mL of *Test Stock Solution* to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.

Class 1 System Suitability Solution—Mix 5 mL of *Test Stock Solution* with 0.5 mL of the intermediate dilution reserved from *Class 1 Standard Stock Solution*. Transfer 1.0 mL of this solution to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.

Chromatographic System (see *Chromatography* (621))—The gas chromatograph is equipped with a flame-ionization detector and a 0.53-mm × 30-m wide-bore column coated with a 3.0-μm layer of phase G43. The carrier gas is helium with a linear velocity of about 35 cm per second and a split ratio of 1:3. [NOTE—The split ratio can be modified in order to optimize sensitivity.] The column temperature is maintained at 40° for 20 minutes, then raised at a rate of 10° per minute to 240°, and maintained at 240° for 20 minutes. The injection port and detector temperatures are maintained at 140° and 250°, respectively. Chromatograph the *Class 1 Standard Solution*, *Class 1 System Suitability Solution*, and *Class 2 Mixture A Standard Solution*, and record the peak responses as directed for *Procedure*: the signal-to-noise ratio of 1,1,1-trichloroethane in the *Class 1 Standard Solution* is not less than 5; the signal-to-noise ratio of each peak in the *Class 1 System Suitability Solution* is not less than 3; and the resolution, *R*, between acetonitrile and methylene chloride in the *Class 2 Mixture A Standard Solution* is not less than 1.0.

Procedure—[NOTE—It is recommended to increase the temperature of the transfer line between runs to eliminate any potential condensation of solvents.] Separately inject (use headspace operating parameters in column 3 of *Table 5* with a vial pressure of 10 psi) equal volumes of headspace (about 1.0 mL) of the *Class 1 Standard Solution*, *Class 2 Mixture A Standard Solution*, *Class 2 Mixture B Standard Solution*, and *Test Solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. If a peak response of any peak, other than a peak for 1,1,1-trichloroethane, in the *Test Solution* is greater than or equal to a corresponding peak in either the *Class 1 Standard Solution* or either of the two *Class 2 Mixture Standard Solutions*, or a peak response of 1,1,1-trichloroethane is greater than or equal to 150 times the peak response corresponding to 1,1,1-trichloroethane in the *Class 1 Standard Solution*, proceed to *Procedure B* to verify the identity of the peak; otherwise the article meets the requirements of this test.

Procedure B—

Class 1 Standard Stock Solution, *Class 1 Standard Solution*, *Class 1 System Suitability Solution*, *Class 2 Standard Stock Solutions*, *Class 2 Mixture A Standard Solution*, and *Class 2 Mixture B Standard Solution*, *Test Stock Solution*, and *Test Solution*—Proceed as directed for *Procedure A*.

Chromatographic System—Proceed as directed for *Procedure B* under *Water-Soluble Articles* with a split ratio of 1:3. [NOTE—The split ratio can be modified in order to optimize sensitivity.]

Procedure—[NOTE—It is recommended to increase the temperature of the transfer line between runs to eliminate any potential condensation of solvents.] Separately inject (use headspace operating parameters in column 3 of *Table 5* with a vial pressure of 10 psi) equal volumes of headspace (about 1.0 mL) of the *Class 1 Standard Solution*, *Class 2 Mixture A Standard Solution*, *Class 2 Mixture B Standard Solution*, and *Test Solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. If the peak response(s) in *Test Solution* of the peak(s) identified in *Procedure A* is/are greater than or equal to a corresponding peak(s) in either the *Class 1 Standard Solution* or any of the two *Class 2 Mixture Standard Solutions*, proceed to *Procedure C* to quantify the peak(s); otherwise the article meets the requirements of this test.

Procedure C—

Class 1 Standard Stock Solution, *Class 1 Standard Solution*, *Class 1 System Suitability Solution*, *Class 2 Standard Stock Solution A*, and *Class 2 Mixture A Standard Solution*—Proceed as directed for *Procedure A*.

Standard Stock Solution—[NOTE—Prepare a separate *Standard Stock Solution* for each peak identified and verified by *Procedures A* and *B*. For the Class 1 solvents other than 1,1,1-trichloroethane, prepare the first dilution as directed for the first dilution under *Class 1 Standard Stock Solution* in *Procedure A*.] Transfer an accurately measured volume of each individual USP Reference Standard corresponding to each residual solvent peak identified and verified by *Procedures A* and *B* to a suitable container, and dilute quantitatively, and stepwise if necessary, with water to obtain a solution having a final concentration of 1/20 of the value stated in *Table 1* or *Table 2* (under *Concentration Limit*).

Standard Solution—Transfer 1.0 mL of the *Standard Stock Solution* to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.

Test Stock Solution—Proceed as directed for *Procedure A*.

Test Solution—Transfer 1.0 mL of the *Test Stock Solution* to an appropriate headspace vial, containing 5.0 mL of water, apply the stopper, cap, and mix.

Spiked Test Solution—[NOTE—Prepare a separate *Spiked Test Solution* for each peak identified and verified by *Procedures A* and *B*.] Transfer 1.0 mL of *Test Stock Solution* to an appropriate headspace vial, add 1 mL of *Standard Stock Solution* and 4.0 mL of water, apply the stopper, cap, and mix.

Chromatographic System—Proceed as directed for *Procedure C* under *Water-Soluble Articles*.

Procedure—[NOTE—It is recommended to increase the temperature of the transfer line between runs to eliminate any potential condensation of solvents.] Separately inject (use headspace operating parameters in column 3 of *Table 5* with a vial pressure of 10 psi) equal volumes of headspace (about 1.0 mL) of the *Standard Solution*, *Test Solution*, and *Spiked Test Solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the amount, in ppm, of each residual solvent found in the article under test by the formula:

$$10(C/W)[r_U/(r_{ST} - r_U)]$$

in which *C* is the concentration, in μg per mL, of the appropriate USP Reference Standard in the *Standard Stock Solution*; *W* is the weight, in g, of the article under test taken to prepare the *Test Stock Solution*; and *r_U* and *r_{ST}* are the peak responses of each residual solvent obtained from *Test Solution* and *Spiked Test Solution*, respectively.

Class 3 Residual Solvents

If Class 3 solvents are present, the level of residual solvents may be determined as directed under *Loss on Drying* (731) when the monograph for the article under test contains a loss on drying procedure specifying an upper limit of

no more than 0.5% (per *Option 1* in this general chapter), or a specific determination of the solvent may be made. If there is no loss on drying procedure in the monograph for the article under test or if a Class 3 solvent limit in an individual monograph is greater than 50 mg per day (corresponding to 5000 ppm or 0.5% under *Option 1*), the individual Class 3 residual solvent or solvents present in the article under test should be identified and quantified, and the procedures as described above, with appropriate modifications to the standard solutions, are to be applied whenever possible. Otherwise an appropriate validated procedure is to be employed. USP Reference Standards, where available, should be used in these procedures.

GLOSSARY

Acceptable daily intake (ADI): The maximum acceptable intake of toxic chemicals per day. This term is used by the World Health Organization (WHO).

Genotoxic carcinogens: Carcinogens that produce cancer by affecting genes or chromosomes.

Lowest-observed-effect level (LOEL): The lowest dose of a substance in a study or group of studies that produces biologically significant increases in frequency or severity of any effects in exposed humans or animals.

Modifying factor: A factor determined by professional judgment of a toxicologist and applied to bioassay data so that the data can be safely related to humans.

Neurotoxicity: The ability of a substance to cause adverse effects on the nervous system.

No-observed-effect level (NOEL): The highest dose of a substance at which there are no biologically significant increases in frequency or severity of any effects in exposed humans or animals.

Permitted daily exposure (PDE): The maximum acceptable intake per day of a residual solvent in pharmaceutical products.

Reversible toxicity: The occurrence of harmful effects that are caused by a substance and that disappear after exposure to the substance ends.

Strongly suspected human carcinogen: A substance for which there is no epidemiological evidence of carcinogenesis but for which there are positive genotoxicity data and clear evidence of carcinogenesis in rodents.




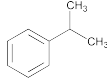

Teratogenicity: The occurrence of structural malformations in a developing fetus when a substance is administered during pregnancy.

Tolerable daily intake (TDI): Tolerable daily exposure to toxic chemicals. Term used by the International Program on Chemical Safety (IPCS).

APPENDIX 1. LIST




See the table *Appendix 1. List of Residual Solvents Included in This General Chapter*.

APPENDIX 1. LIST OF RESIDUAL SOLVENTS INCLUDED IN THIS GENERAL CHAPTER

Solvent	Other Names	Structure	Class
Acetic acid	Ethanoic acid	CH ₃ COOH	Class 3
Acetone	2-Propanone Propan-2-one	CH ₃ COCH ₃	Class 3
Acetonitrile		CH ₃ CN	Class 2
Anisole	Methoxybenzene		Class 3
Benzene	Benzol		Class 1
1-Butanol	<i>n</i> -Butyl alcohol Butan-1-ol	CH ₃ (CH ₂) ₃ OH	Class 3
2-Butanol	<i>sec</i> -Butyl alcohol Butan-2-ol	CH ₃ CH ₂ CH(OH)CH ₃	Class 3
Butyl acetate	Acetic acid butyl ester	CH ₃ COO(CH ₂) ₃ CH ₃	Class 3
<i>tert</i> -Butylmethyl ether	2-Methoxy-2-methylpropane	(CH ₃) ₃ COCH ₃	Class 3
Carbon tetrachloride	Tetrachloromethane	CCl ₄	Class 1
Chlorobenzene			Class 2
Chloroform	Trichloromethane	CHCl ₃	Class 2
Cumene	Isopropylbenzene (1-Methylethyl)benzene		Class 3
Cyclohexane	Hexamethylene		Class 2







*Usually 60% *m*-xylene, 14% *p*-xylene, 9% *o*-xylene with 17% ethyl benzene.

APPENDIX 1. LIST OF RESIDUAL SOLVENTS INCLUDED IN THIS GENERAL CHAPTER (Continued)

Solvent	Other Names	Structure	Class
1,2-Dichloroethane	<i>sym</i> -Dichloroethane Ethylene dichloride Ethylene chloride	$\text{CH}_2\text{ClCH}_2\text{Cl}$	Class 1
1,1-Dichloroethene	1,1-Dichloroethylene Vinylidene chloride	$\text{H}_2\text{C}=\text{CCl}_2$	Class 1
1,2-Dichloroethene	1,2-Dichloroethylene Acetylene dichloride	$\text{ClHC}=\text{CHCl}$	Class 2
1,2-Dimethoxyethane	Ethyleneglycol dimethyl ether Monoglyme Dimethyl cellosolve	$\text{H}_3\text{COCH}_2\text{CH}_2\text{OCH}_3$	Class 2
<i>N,N</i> -Dimethylacetamide	DMA	$\text{CH}_3\text{CON}(\text{CH}_3)_2$	Class 2
<i>N,N</i> -Dimethylformamide	DMF	$\text{HCON}(\text{CH}_3)_2$	Class 2
Dimethyl sulfoxide	Methylsulfinylmethane Methyl sulfoxide DMSO	$(\text{CH}_3)_2\text{SO}$	Class 3
1,4-Dioxane	<i>p</i> -Dioxane [1,4]Dioxane		Class 2
Ethanol	Ethyl alcohol	$\text{CH}_3\text{CH}_2\text{OH}$	Class 3
2-Ethoxyethanol	Cellosolve	$\text{CH}_3\text{CH}_2\text{OCH}_2\text{CH}_2\text{OH}$	Class 2
Ethyl acetate	Acetic acid ethyl ester	$\text{CH}_3\text{COOCH}_2\text{CH}_3$	Class 3
Ethylene glycol	1,2-Dihydroxyethane 1,2-Ethanediol	$\text{HOCH}_2\text{CH}_2\text{OH}$	Class 2
Ethyl ether	Diethyl ether Ethoxyethane 1,1'-Oxybisethane	$\text{CH}_3\text{CH}_2\text{OCH}_2\text{CH}_3$	Class 3
Ethyl formate	Formic acid ethyl ester	$\text{HCOOCH}_2\text{CH}_3$	Class 3
Formamide	Methanamide	HCONH_2	Class 2
Formic acid		HCOOH	Class 3
Heptane	<i>n</i> -Heptane	$\text{CH}_3(\text{CH}_2)_5\text{CH}_3$	Class 3
Hexane	<i>n</i> -Hexane	$\text{CH}_3(\text{CH}_2)_4\text{CH}_3$	Class 2
Isobutyl acetate	Acetic acid isobutyl ester	$\text{CH}_3\text{COOCH}_2\text{CH}(\text{CH}_3)_2$	Class 3
Isopropyl acetate	Acetic acid isopropyl ester	$\text{CH}_3\text{COOCH}(\text{CH}_3)_2$	Class 3
Methanol	Methyl alcohol	CH_3OH	Class 2
2-Methoxyethanol	Methyl cellosolve	$\text{CH}_3\text{OCH}_2\text{CH}_2\text{OH}$	Class 2
Methyl acetate	Acetic acid methyl ester	$\text{CH}_3\text{COOCH}_3$	Class 3
3-Methyl-1-butanol	Isoamyl alcohol Isopentyl alcohol 3-Methylbutan-1-ol	$(\text{CH}_3)_2\text{CHCH}_2\text{CH}_2\text{OH}$	Class 3
Methylbutylketone	2-Hexanone Hexan-2-one	$\text{CH}_3(\text{CH}_2)_3\text{COCH}_3$	Class 2
Methylcyclohexane	Cyclohexylmethane		Class 2
Methylene chloride	Dichloromethane	CH_2Cl_2	Class 2
Methylethylketone	2-Butanone MEK Butan-2-one	$\text{CH}_3\text{CH}_2\text{COCH}_3$	Class 3
Methyl isobutyl ketone	4-Methylpentan-2-one 4-Methyl-2-pentanone MIBK	$\text{CH}_3\text{COCH}_2\text{CH}(\text{CH}_3)_2$	Class 3
2-Methyl-1-propanol	Isobutyl alcohol 2-Methylpropan-1-ol	$(\text{CH}_3)_2\text{CHCH}_2\text{OH}$	Class 3
<i>N</i> -Methylpyrrolidone	1-Methylpyrrolidin-2-one 1-Methyl-2-pyrrolidinone		Class 2
Nitromethane		CH_3NO_2	Class 2
Pentane	<i>n</i> -Pentane	$\text{CH}_3(\text{CH}_2)_3\text{CH}_3$	Class 3

*Usually 60% *m*-xylene, 14% *p*-xylene, 9% *o*-xylene with 17% ethyl benzene.

APPENDIX 1. LIST OF RESIDUAL SOLVENTS INCLUDED IN THIS GENERAL CHAPTER (Continued)

Solvent	Other Names	Structure	Class
1-Pentanol	Amyl alcohol Pentan-1-ol Pentyl alcohol	$\text{CH}_3(\text{CH}_2)_3\text{CH}_2\text{OH}$	Class 3
1-Propanol	Propan-1-ol Propyl alcohol	$\text{CH}_3\text{CH}_2\text{CH}_2\text{OH}$	Class 3
2-Propanol	Propan-2-ol Isopropyl alcohol	$(\text{CH}_3)_2\text{CHOH}$	Class 3
Propyl acetate	Acetic acid propyl ester	$\text{CH}_3\text{COOCH}_2\text{CH}_2\text{CH}_3$	Class 3
Pyridine			Class 2
Sulfolane	Tetrahydrothiophene 1,1-dioxide		Class 2
Tetrahydrofuran	Tetramethylene oxide Oxacyclopentane		Class 2
Tetralin	1,2,3,4-Tetrahydronaphthalene		Class 2
Toluene	Methylbenzene		Class 2
1,1,1-Trichloroethane	Methylchloroform	CH_3CCl_3	Class 1
Trichloroethylene	1,1,2-Trichloroethene	$\text{HC}(\text{Cl})=\text{CCl}_2$	Class 2
Xylene*	Dimethylbenzene Xylol		Class 2

*Usually 60% *m*-xylene, 14% *p*-xylene, 9% *o*-xylene with 17% ethyl benzene.

APPENDIX 2. ADDITIONAL BACKGROUND

A2.1. Environmental Regulation of Organic Volatile Solvents

Several of the residual solvents frequently used in the production of pharmaceuticals are listed as toxic chemicals in *Environmental Health Criteria* (EHC) monographs and in the Integrated Risk Information System (IRIS). The objectives of such groups as the International Programme on Chemical Safety (IPCS), the United States Environmental Protection Agency (EPA), and the United States Food and Drug Administration (FDA) include the determination of acceptable exposure levels. The goal is maintenance of environmental integrity and protection of human health against the possible deleterious effects of chemicals resulting from long-term environmental exposure. The procedures involved in the estimation of maximum safe exposure limits are usually based on long-term studies. When long-term study data are unavailable, shorter-term study data can be used with modification of the approach, such as use of larger safety factors. The approach described therein relates primarily to long-term or lifetime exposure of the general population in the ambient environment (i.e., ambient air, food, drinking water, and other media).

A2.2. Residual Solvents in Pharmaceuticals

Exposure limits in this general chapter are established by referring to methodologies and toxicity data described in EHC and IRIS monographs. However, the following specific assumptions about residual solvents to be used in the synthesis and formulation of pharmaceutical products should be taken into account in establishing exposure limits.

1. Patients (not the general population) use pharmaceuticals to treat their diseases or for prophylaxis to prevent infection or disease.
2. The assumption of lifetime patient exposure is not necessary for most pharmaceutical products but may be appropriate as a working hypothesis to reduce risk to human health.
3. Residual solvents are unavoidable components in pharmaceutical production and will often be a part of medicinal products.
4. Residual solvents should not exceed recommended levels except in exceptional circumstances.
5. Data from toxicological studies that are used to determine acceptable levels for residual solvents should have been generated using appropriate protocols such as those described, for example, by the Organization for Economic Cooperation and Development (OECD), EPA, and the FDA *Red Book*.

APPENDIX 3. PROCEDURES FOR ESTABLISHING EXPOSURE LIMITS

The Gaylor-Kodell method of risk assessment (Gaylor, D. W., and Kodell, R.L. Linear Interpolation Algorithm for Low Dose Assessment of Toxic Substance. *Journal of Environmental Pathology and Toxicology*, 4:305, 1980) is appropriate for Class 1 carcinogenic solvents. Only in cases where reliable carcinogenicity data are available should extrapolation by the use of mathematical models be applied to setting exposure limits. Exposure limits for Class 1 residual solvents could be determined with the use of a large safety factor (i.e., 10,000 to 100,000) with respect to the no-observed-effect level (NOEL). Detection and quantification of these residual solvents should be performed by state-of-the-art analytical techniques.

Acceptable exposure levels in this general chapter for Class 2 residual solvents were established by calculation of PDE values according to the procedures for setting exposure limits in pharmaceuticals (page 5748 of *PF 15(6)* [Nov.–Dec. 1989]), and the method adopted by IPCS for Assessing Human Health Risk of Chemicals (*Environmental Health Criteria 170*, WHO, 1994). These procedures are similar to those used by the U.S. EPA (IRIS) and the U.S. FDA (*Red Book*) and others. The method is outlined here to give a better understanding of the origin of the PDE values. It is not necessary to perform these calculations in order to use the PDE values presented in *Table 2* of this document.

PDE is derived from the no-observed-effect level (NOEL), or the lowest-observed effect level (LOEL), in the most relevant animal study as follows:

$$\text{PDE} = (\text{NOEL} \times \text{Weight Adjustment}) / (F1 \times F2 \times F3 \times F4 \times F5) \quad (1)$$

The PDE is derived preferably from a NOEL. If no NOEL is obtained, the LOEL may be used. Modifying factors proposed here, for relating the data to humans, are the same kind of “uncertainty factors” used in *Environmental Health Criteria (Environmental Health Criteria 170*, WHO, Geneva, 1994) and “modifying factors” or “safety factors” in *Pharmacopeial Forum*. The assumption of 100 percent systemic exposure is used in all calculations regardless of route of administration.

The modifying factors are as follows:

F1 =	A factor to account for extrapolation between species
F1 =	2 for extrapolation from dogs to humans
F1 =	2.5 for extrapolation from rabbits to humans
F1 =	3 for extrapolation from monkeys to humans
F1 =	5 for extrapolation from rats to humans
F1 =	10 for extrapolation from other animals to humans
F1 =	12 for extrapolation from mice to humans

F1 takes into account the comparative surface area to body weight ratios for the species concerned and for man. Surface area (S) is calculated as:

$$S = kM^{0.67} \quad (2)$$

in which M = body weight, and the constant k has been taken to be 10. The body weights used in the equation are those shown below in *Table A3-1*.

F2 =	A factor of 10 to account for variability between individuals. A factor of 10 is generally given for all organic solvents, and 10 is used consistently in this general chapter.
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F3 =	A variable factor to account for toxicity studies of short-term exposure
F3 =	1 for studies that last at least one half-lifetime (1 year for rodents or rabbits; 7 years for cats, dogs, and monkeys)
F3 =	1 for reproductive studies in which the whole period of organogenesis is covered
F3 =	2 for a 6-month study in rodents, or a 3.5-year study in nonrodents
F3 =	5 for a 3-month study in rodents, or a 2-year study in nonrodents
F3 =	10 for studies of a shorter duration

In all cases, the higher factor has been used for study durations between the time points (e.g., a factor of 2 for a 9-month rodent study).

F4 =	A factor that may be applied in cases of severe toxicity, e.g., nongenotoxic carcinogenicity, neurotoxicity, or teratogenicity. In studies of reproductive toxicity, the following factors are used:
F4 =	1 for fetal toxicity associated with maternal toxicity
F4 =	5 for fetal toxicity without maternal toxicity
F4 =	5 for a teratogenic effect with maternal toxicity
F4 =	10 for a teratogenic effect without maternal toxicity

F5 =	A variable factor that may be applied if the no-effect level was not established
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When only a LOEL is available, a factor of up to 10 can be used, depending on the severity of the toxicity. The weight adjustment assumes an arbitrary adult human body weight for either sex of 50 kilograms (kg). This relatively low weight provides an additional safety factor against the standard weights of 60 kg or 70 kg that are often used in this type of calculation. It is recognized that some adult patients weigh less than 50 kg; these patients are considered to be accommodated by the built-in safety factors used to determine a PDE. If the solvent was present in a formulation specifically intended for pediatric use, an adjustment for a lower body weight would be appropriate.

As an example of the application of this equation, consider a toxicity study of acetonitrile in mice that is summarized in *Pharmeuropa*, Vol. 9, No. 1, Supplement, April 1997, page S24. The NOEL is calculated to be 50.7 mg kg⁻¹ day⁻¹. The PDE for acetonitrile in this study is calculated as follows:

$$\text{PDE} = (50.7 \text{ mg kg}^{-1} \text{ day}^{-1} \times 50 \text{ kg}) / (12 \times 10 \times 5 \times 1 \times 1) = 4.22 \text{ mg day}^{-1}$$

In this example,

F1 =	12 to account for the extrapolation from mice to humans
F2 =	10 to account for differences between individual humans
F3 =	5 because the duration of the study was only 13 weeks
F4 =	1 because no severe toxicity was encountered
F5 =	1 because the no-effect level was determined

A3-1. Values Used in the Calculations in This Document

Rat body weight	425 g
Pregnant rat body weight	330 g
Mouse body weight	28 g
Pregnant mouse body weight	30 g
Guinea-pig body weight	500 g
Rhesus monkey body weight	2.5 kg
Rabbit body weight (pregnant or not)	4 kg
Beagle dog body weight	11.5 kg
Rat respiratory volume	290 L/day
Mouse respiratory volume	43 L/day
Rabbit respiratory volume	1440 L/day
Guinea-pig respiratory volume	430 L/day
Human respiratory volume	28,800 L/day
Dog respiratory volume	9000 L/day
Monkey respiratory volume	1150 L/day
Mouse water consumption	5 mL/day
Rat water consumption	30 mL/day
Rat food consumption	30 g/day

The equation for an ideal gas, $PV = nRT$, is used to convert concentrations of gases used in inhalation studies from units of ppm to units of mg/L or mg/m³. Consider as an example the rat reproductive toxicity study by inhalation of carbon tetrachloride (molecular weight 153.84) summarized in *Pharmeuropa*, Vol. 9, No. 1, Supplement, April 1997, page S9.

$$\frac{n}{V} = \frac{P}{RT} = \frac{300 \times 10^{-6} \text{ atm} \times 153.840 \text{ mg mol}^{-1}}{0.082 \text{ L at mK}^{-1} \text{ mol}^{-1} \times 298 \text{ K}} = \frac{46.15 \text{ mg}}{24.45 \text{ L}} = 1.89 \text{ mg/L}$$

The relationship 1000 L = 1 m³ is used to convert to mg/m³.

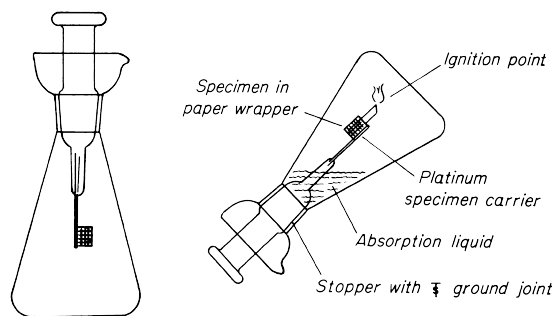
<471> OXYGEN FLASK COMBUSTION

The oxygen flask combustion procedure is provided as the preparatory step in the determination of bromine, chlorine, iodine, selenium, and sulfur in some Pharmacopeial articles. Combustion of the material under test (usually organic) yields water-soluble inorganic products, which are analyzed for specific elements as directed in the individual monograph or general chapter.

The caution statement given for *Procedure* covers minimum safety precautions only, and serves to emphasize the need for exceptional care throughout.

Apparatus—The apparatus¹ consists of a heavy-walled conical, deeply lipped or cupped 500-mL flask (unless a larger flask is specified), fitted with a ground-glass stopper to which is fused a test specimen carrier consisting of heavy-gauge platinum wire and a piece of welded platinum gauze measuring about 1.5 × 2 cm.

¹ A suitable apparatus [Catalog Nos. 6513-C20 (500-ml capacity) and 6513-C30 (1000-ml capacity)] and suitable capsules [Catalog No. 6513-84 (1000 capsules)] are obtainable from Thomas Scientific, 99 High Hill Road, Swedesboro, NJ 08085.



Apparatus for Oxygen Flask Combustion

Procedure—[Caution—Wear safety glasses and use a suitable safety shield between yourself and the apparatus. Exercise care to ensure that the flask is scrupulously clean and free from even traces of organic solvents.] Weigh the substance, if a solid, on a piece of halide-free filter paper measuring about 4 cm square, and fold the paper to enclose it. Liquid substances are weighed in tared capsules, polycarbonate capsules¹ being used for liquids in volumes not exceeding 200 µL, and gelatin capsules being satisfactory for use for larger volumes. [NOTE—Gelatin capsules may contain significant amounts of combined halide or sulfur. If such capsules are used, perform a blank determination, and make any necessary correction.] Place the specimen, together with a filter paper fuse-strip, in the platinum gauze specimen holder. Place the absorbing liquid specified in the individual monograph or general chapter in the flask, moisten the joint of the stopper with water, and flush the air from the flask with a stream of rapidly flowing oxygen, swirling the liquid to favor its taking up oxygen. [NOTE—Saturation of the liquid with oxygen is essential for the successful performance of the combustion procedure.] Ignite the fuse-strip by suitable means. If the strip is ignited outside the flask, immediately plunge the specimen holder into the flask, invert the flask so that the absorption solution makes a seal around the stopper, and hold the stopper firmly in place. If the ignition is carried out in a closed system, the inversion of the flask may be omitted. After combustion is complete, shake the flask vigorously, and allow to stand for not less than 10 minutes with intermittent shaking. Then proceed as directed in the individual monograph or general chapter.

<481> RIBOFLAVIN ASSAY

The following procedure is suitable for preparations in which riboflavin is a constituent of a mixture of several ingredients. In employing it, keep the pH of solutions below 7.0, and protect the solutions from direct sunlight at all stages.

USP Reference Standards <11>—*USP Riboflavin RS*.

Standard Riboflavin Stock Solution—To 50.0 mg of USP Riboflavin RS, previously dried and stored protected from light in a desiccator over phosphorus pentoxide, add about 300 mL of 0.02 N acetic acid, and heat the mixture on a steam bath, with frequent agitation, until the riboflavin has

dissolved. Then cool, add 0.02 N acetic acid to make 500 mL, and mix. Store under toluene in a refrigerator.

Dilute an accurately measured portion of this solution, using 0.02 N acetic acid, to a concentration of 10.0 µg of the dried USP Riboflavin RS per mL, to obtain the *Standard Riboflavin Stock Solution*. Store under toluene in a refrigerator.

Standard Preparation—Dilute 10.0 mL of *Standard Riboflavin Stock Solution* with water in a 100-mL volumetric flask to volume, and mix. Each mL represents 1.0 µg of USP Riboflavin RS. Prepare fresh *Standard Preparation* for each assay.

Assay Preparation—Place an amount of the material to be assayed in a flask of suitable size, and add a volume of 0.1 N hydrochloric acid equal in mL to not less than 10 times the dry weight of the material in g, but the resulting solution shall contain not more than 100 µg of riboflavin per mL. If the material is not readily soluble, comminute it so that it may be evenly dispersed in the liquid. Then agitate vigorously, and wash down the sides of the flask with 0.1 N hydrochloric acid.

Heat the mixture in an autoclave at 121° to 123° for 30 minutes, and cool. If clumping occurs, agitate the mixture until the particles are evenly dispersed. Adjust the mixture, with vigorous agitation, to a pH of 6.0 to 6.5 with sodium hydroxide solution,* then add hydrochloric acid solution* immediately until no further precipitation occurs (usually at a pH of approximately 4.5, the isoelectric point of many of the proteins present). Dilute the mixture with water to make a measured volume that contains about 0.11 µg of riboflavin in each mL, and filter through paper known not to adsorb riboflavin. To an aliquot of the filtrate add, with vigorous agitation, sodium hydroxide solution* to produce a pH of 6.6 to 6.8, dilute the solution with water to make a final measured volume that contains approximately 0.1 µg of riboflavin in each mL, and if cloudiness occurs, filter again.

Procedure—To each of four or more tubes (or reaction vessels) add 10.0 mL of the *Assay Preparation*. To each of two or more of these tubes add 1.0 mL of the *Standard Preparation*, and mix, and to each of two or more of the remaining tubes add 1.0 mL of water, and mix. To each tube add 1.0 mL of glacial acetic acid, mix, then add, with mixing, 0.50 mL of potassium permanganate solution (1 in 25), and allow to stand for 2 minutes. To each tube add, with mixing, 0.50 mL of hydrogen peroxide solution, whereupon the permanganate color is destroyed within 10 seconds. Shake the tubes vigorously until excess oxygen is expelled. Remove any gas bubbles remaining on the sides of the tubes after foaming has ceased, by tipping the tubes so that the solution flows slowly from end to end.

In a suitable fluorophotometer, having an input filter of narrow transmittance range with a maximum at about 440 nm and an output filter of narrow transmittance range with a maximum at about 530 nm, measure the fluorescence of all tubes, designating the average reading from the tubes containing only the *Assay Preparation* as I_u and the average from the tubes containing both the *Assay Preparation* and the *Standard Preparation* as I_s . Then to each of one or more tubes of each kind add, with mixing, 20 mg of sodium hydrosulfite, and within 5 seconds again measure the fluorescence, designating the average reading as I_b .

Calculation—Calculate the quantity, in mg, of $C_{17}H_{20}N_4O_6$ in each mL of the *Assay Preparation* taken by the formula:

$$0.0001(I_u - I_b)/(I_s - I_u).$$

Calculate the quantity, in mg, of $C_{17}H_{20}N_4O_6$ in each capsule or tablet.

* The concentrations of the hydrochloric acid and sodium hydroxide solutions used are not stated in each instance because these concentrations may be varied depending upon the amount of material taken for assay, volume of test solution, and buffering effect of material.

(501) SALTS OF ORGANIC NITROGENOUS BASES

Standard Preparation—Unless otherwise directed, prepare a solution in dilute sulfuric acid (1 in 70) containing, in each mL, about 500 µg of the specified USP Reference Standard, calculated on the anhydrous basis, and accurately weighed.

Assay Preparation—If the dosage form is a tablet, weigh and finely powder not less than 20 tablets, weigh accurately a portion of the powder, equivalent to about 25 mg of the active ingredient, and transfer to a 125-mL separator; or, if the dosage form is a liquid, transfer a volume of it, equivalent to about 25 mg of the active ingredient and accurately measured, to a 125-mL separator. Then to the separator add 20 mL of dilute sulfuric acid (1 in 350), and shake vigorously for 5 minutes. Add 20 mL of ether, shake carefully, and filter the acid phase into a second 125-mL separator. Shake the ether phase with two 10-mL portions of dilute sulfuric acid (1 in 350), filter each portion of acid into the second separator, and discard the ether. To the acid extract add 10 mL of sodium hydroxide TS and 50 mL of ether, shake carefully, and transfer the aqueous phase to a third 125-mL separator containing 50 mL of ether. Shake the third separator carefully, and discard the aqueous phase. Wash the two ether solutions, in succession, with a single 20-mL portion of water, and discard the water. Extract each of the two ether solutions with 20-, 20-, and 5-mL portions of dilute sulfuric acid (1 in 70), in the order listed, but each time extract first the ether solution in the third separator and then that in the second separator. Combine the acid extracts in a 50-mL volumetric flask, dilute with the acid to volume, and mix.

NOTE—Hexane or heptane may be substituted for ether if the distribution ratio of the nitrogenous base between water and hexane, or between water and heptane, favors complete extraction by the organic phase.

Procedure—Unless otherwise directed, dilute 5.0 mL each of the *Standard Preparation* and the *Assay Preparation* with dilute sulfuric acid (1 in 70) to 100.0 mL, and determine the absorbance of each solution at the specified wavelength, using dilute sulfuric acid (1 in 70) as the blank. Designate the absorbance of the solution from the *Standard Preparation* as A_s and that from the *Assay Preparation* as A_u , and calculate the result of the assay as directed in the individual monograph.

(503) ACETIC ACID IN PEPTIDES

The following procedure is to be used to determine the amount of acetate or acetic acid in peptides. Acetate is a common counterion in many peptide preparations.

USP Reference Standards (11)—USP Glacial Acetic Acid RS.

Strong Sodium Hydroxide Solution—Dissolve 42 g of sodium hydroxide in water, and dilute with water to 100 mL.

Solution A—Add 0.7 mL of phosphoric acid to 1000 mL of water, and adjust with *Strong Sodium Hydroxide Solution* to a pH of 3.0.

Solution B—Use methanol.

Diluent—Prepare a mixture of *Solution A* and *Solution B* (95:5).

Standard Solution—[NOTE—The concentration can be adjusted depending on the amount of acetate or acetic acid expected to be present in the test material.] Dissolve an accurately weighed quantity of USP Glacial Acetic Acid RS in *Diluent* to obtain a solution having a known concentration of about 0.1 mg per mL.

Test Solution—Prepare as directed in the individual monograph. The amount of material used can be adapted depending on the amount of acetic acid expected.

Chromatographic System (see *Chromatography* <621>)—The liquid chromatograph is equipped with a 210-nm detector and a 4.6-mm × 25-cm column that contains not greater than 5-μm packing L1. The flow rate is about 1.2 mL per minute. The chromatograph is programmed as follows.

Time (minutes)	Solution A (%)	Solution B (%)	Elution
0	95	5	equilibration
0–5	95	5	isocratic
5–10	95→50	5→50	linear gradient
10–20	50	50	isocratic
20–22	50→95	50→5	linear gradient

Chromatograph the *Standard Solution*, and record the peak responses as directed for *Procedure*: the retention time of acetic acid is between 3 and 4 minutes; and the relative standard deviation for replicate injections is not more than 5%.

Procedure—Separately inject equal volumes (about 10 μL) of the *Standard Solution* and the *Test Solution* into the chromatograph, record the chromatograms, and measure the responses for the acetic acid peaks. Calculate the percentage of acetic acid in the portion of test material taken by the formula:

$$100(C_S/M)(r_U/r_S)$$

in which C_S is the concentration of acetic acid in the *Standard Solution*; M is the concentration, in mg per mL, of the *Test Solution*, based on the weight of test material taken and the extent of dilution; and r_U and r_S are the acetic acid peak responses obtained from the *Test Solution* and the *Standard Solution*, respectively.

<511> SINGLE-STERIOD ASSAY

In the following procedure, the steroid to be assayed is separated from related foreign steroids and excipients by thin-layer chromatography and determined following recovery from the chromatogram.

Preparation of the Plate—Prepare a slurry from 30 g of chromatographic silica gel with a suitable fluorescing substance by the gradual addition, with mixing, of about 65 mL of a mixture of water and alcohol (5:2). Transfer the slurry to a clean, 20- × 20-cm plate, spread to make a uniform layer 250 μm thick, and allow to dry at room temperature for 15 minutes. Heat the plate at 105° for 1 hour, and store in a desiccator.

Solvent A—Mix methylene chloride with methanol (180:16).

Solvent B—Mix chloroform with acetone (4:1).

Standard Preparation—Dissolve in a mixture of equal volumes of chloroform and alcohol a suitable quantity of the

USP Reference Standard specified in the individual monograph, previously dried as directed (see *USP Reference Standards* <11>) and accurately weighed, to obtain a solution having a known concentration of about 2 mg per mL.

Assay Preparation—Prepare as directed in the individual monograph.

Procedure—Divide the area of the chromatographic plate into three equal sections, the left and right sections to be used for the *Assay Preparation* and the *Standard Preparation*, respectively, and the center section for the blank. Apply 200 μL each of the *Assay Preparation* and the *Standard Preparation* as streaks 2.5 cm from the bottom of the appropriate section of the plate. Dry the solution as it is being applied, with the aid of a stream of air. Using the *Solvent* specified in the individual monograph, develop the chromatogram in a suitable chamber, previously equilibrated and lined with absorbent paper, until the solvent front has moved 15 cm above the initial streaks.

Remove the plate, evaporate the solvent, and locate the principal band occupied by the *Standard Preparation* by viewing under UV light. Mark this band, as well as corresponding bands in the *Assay Preparation* and blank sections of the plate. Remove the silica gel from each band separately, either by scraping onto glazed weighing papers or by using a suitable vacuum collecting device, and transfer it to a glass-stoppered, 50-mL centrifuge tube. To each tube add 25.0 mL of alcohol, and shake for not less than 2 minutes. Centrifuge the tubes for 5 minutes, pipet 20 mL of the supernatant from each tube into a glass-stoppered, 50-mL conical flask, add 2.0 mL of a solution prepared by dissolving 50 mg of blue tetrazolium in 10 mL of methanol, and mix. Proceed as directed for *Procedure* under *Assay for Steroids* <351>, beginning with "Then to each flask."

<525> SULFUR DIOXIDE

The following methods are provided for the determination of sulfur dioxide in pharmaceutical excipients.

METHOD I

Procedure

Mix 20 g of the test specimen, accurately weighed, with 200 mL of an appropriate solvent as indicated in each individual monograph, and stir until a smooth suspension is obtained. Allow the test specimen mixture to remain undisturbed until most of the test specimen has settled, and filter the aqueous portion through paper (Whatman No. 1 or equivalent). To 100 mL of the clear filtrate add an additional solvent as indicated in each individual monograph, add 3 mL of starch TS, and titrate with 0.01 N iodine solution VS to the first permanent blue or purple color. Each 1.0 mL of 0.01 N iodine solution VS consumed corresponds to 0.003% of sulfur dioxide found.

METHOD II

Procedure

Transfer about 50 to 100 g of the substance to be tested, accurately weighed, to a 250-mL conical flask, add 100 to 150 mL of water, and mix. Cool to between 5° and 10°.

While stirring with a magnetic stirrer, add 10 mL of cold 1.5 N sodium hydroxide (at a temperature between 5° and 10°). Stir for an additional 20 seconds, and add 10 mL of starch indicator solution, prepared as follows: mix 10 g of soluble starch with 50 mL of cold water, transfer to 1000 mL of boiling water, stir until completely dissolved, cool, and add 1 g of salicylic acid preservative. [NOTE—Discard the solution after 1 month.] Add 10 mL of 2.0 N sulfuric acid (at a temperature between 5° and 10°), and titrate immediately with 0.005 N iodine VS until a light blue color persists for 1 minute (see *Titrimetry* (541)). Perform a blank determination under test, and make any necessary correction. Each mL of 0.005 N iodine is equivalent to 0.16 mg of SO₂.

METHOD III

Procedure

Dissolve 20.0 g of the test specimen in 150 mL of hot water in a flask having a round bottom and a long neck, add 5 mL of phosphoric acid and 1 g of sodium bicarbonate, and at once connect the flask to a condenser. [NOTE—Excessive foaming can be alleviated by the addition of a few drops of a suitable antifoaming agent.] Distill 50 mL, receiving the distillate under the surface of 50 mL of 0.1 N iodine. Acidify the distillate with a few drops of hydrochloric acid, add 2 mL of barium chloride TS, and heat on a steam bath until the liquid is nearly colorless. The precipitate of barium sulfate, if any, when filtered, washed, and ignited, weighs not more than 3 mg, corresponding to not more than 0.004% of sulfur dioxide, correction being made for any sulfate that may be present in 50 mL of the 0.1 N iodine.

METHOD IV

In this test, sulfur dioxide is released from the test specimen in a boiling acid medium and is removed by a stream of carbon dioxide. The separated gas is collected in a dilute hydrogen peroxide solution, in which the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali, using a pH meter to control the pH value and titration. This test is performed under conditions such that the requirements specified in the system suitability test are met.

Special Reagents

Carbon Dioxide—Use carbon dioxide with a flow regulator that will maintain a flow of 100 ± 10 mL per minute.

Hydrogen Peroxide Solution—Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Neutralize the 3% hydrogen peroxide solution with 0.01 N sodium hydroxide to a pH of 4.1 determined potentiometrically.

Potassium Metabisulfite Solution—Transfer 0.87 g of potassium metabisulfite (K₂S₂O₅) and 0.2 g of edetate disodium to a 1000-mL volumetric flask. Dilute with water to volume before mixing. [NOTE—Edetate disodium is used to protect sulfite ion from oxidation.]

Apparatus

A suitable apparatus for sulfur dioxide determination is shown in the accompanying diagram (Figure 1).

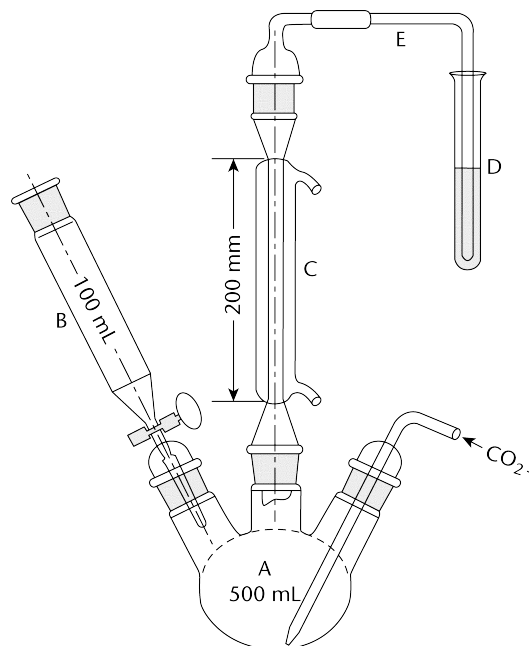


Figure 1. Apparatus for Method IV.

The apparatus consists of a 500-mL three-neck, round-bottom boiling flask, A; a separatory funnel, B, having a capacity of 100 mL or greater; a gas inlet tube of sufficient length to permit introduction of the carbon dioxide within 2.5 cm of the bottom of the boiling flask; a reflux condenser, C, having a jacket length of 200 mm; and a delivery tube, E, connecting the upper end of the reflux condenser to the bottom of a receiving test tube, D. Apply a thin film of stopcock grease to the sealing surfaces of all joints except the joint between the separatory funnel and the boiling flask, and clamp the joints to ensure tightness.

System Suitability Test

Test A—Using the *Potassium Metabisulfite Solution* as the standard, proceed as directed for *Procedure*, except replace the 25.0 g of test substance with 20 mL of *Potassium Metabisulfite Solution*. Calculate the content, in µg per mL, of sulfur dioxide in the *Potassium Metabisulfite Solution* taken by the formula:

$$1000(32.03)V_N/V_P$$

in which the factor 1000 converts mg to µg; 32.03 is the milliequivalent weight of sulfur dioxide; V is the volume, in mL, of titrant consumed; N is the normality of the titrant; and V_P is the volume, in mL, of the *Potassium Metabisulfite Solution* taken for the test.

Test B—In a 100-mL conical flask, add 20 mL of 0.02 N iodine solution and 5 mL of 2 N hydrochloric acid. Add 1 mL of starch TS, and titrate with the *Potassium Metabisulfite Solution* until the first discoloration is observed. Calculate the content, in µg per mL, of sulfur dioxide in the *Potassium Metabisulfite Solution* by the formula:

$$1000(32.03)V_I N_I/V_P$$

in which 1000 and 32.03 are defined above; V_I is the volume, in mL, of the iodine solution used in the test; N_I is the normality of the iodine solution; and V_P is the volume, in mL, of the *Potassium Metabisulfite Solution* consumed.

The difference between the sulfur dioxide contents obtained from *Test A* and *Test B* is not more than 5% of their mean value. *Test B* shall be performed within 15 minutes

after completion of *Test A*. [NOTE—This avoids a potential variation of the sulfur dioxide content in the *Potassium Metabisulfite Solution* when stored at room temperature.]

Procedure

Add 150 mL of water to the boiling flask (A). Close the stopcock of the separatory funnel, and begin the flow of carbon dioxide at a rate of 100 ± 5 mL per minute through the apparatus. Start the condenser coolant flow. Place 10 mL of *Hydrogen Peroxide Solution* in the receiving test tube (D). After 15 minutes, without interrupting the flow of carbon dioxide, remove the separatory funnel (B) from the boiling flask, and transfer 25.0 g of the test specimen to the boiling flask with the aid of 100 mL of water. Apply stopcock grease to the outer joint of the separatory funnel, and replace the separatory funnel in the boiling flask. Close the stopcock of the separatory funnel, and add 80 mL of 2 N hydrochloric acid to the separatory funnel. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the boiling flask, guarding against escape of sulfur dioxide into the separatory funnel by closing the stopcock before the last few mL of hydrochloric acid drain out. Boil the mixture for 1 hour. Open the stopcock of the funnel, stop the flow of carbon dioxide, discontinue heating the flask, and turn off the cooling water in the condenser. Remove the receiving test tube, and transfer its contents to a 200-mL wide-necked, conical flask. Rinse the receiving test tube with a small portion of water, add the rinsing to the 200-mL conical flask, and mix. Heat on a water bath for 15 minutes, and allow to cool. Add 0.1 mL of bromophenol blue TS, and titrate the contents with 0.1 N sodium hydroxide VS until the color changes from yellow to violet-blue, with the color change lasting for at least 20 seconds. Perform a blank determination and make any necessary correction (see *Titrimetry* (541)). Calculate the content, in μg per g, of sulfur dioxide in the test specimen taken by the formula:

1000(32.03)VN/W

in which the factor 1000 converts mg to μg ; 32.03 is the milliequivalent weight of sulfur dioxide; V is the volume, in mL, of titrant consumed; N is the normality of the titrant; and W is the weight, in g, of the test specimen taken.

METHOD V

In this method, similar to *Method IV*, sulfur dioxide is released from the test specimen in a boiling acid medium and is removed by a stream of nitrogen. The separated gas is collected in a dilute hydrogen peroxide solution, in which the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali, using methyl red as an indicator. This test is performed under conditions such that the requirements specified in the system suitability test are met.

Special Reagents

Hydrogen Peroxide Solution—Dilute a portion of 30 percent hydrogen peroxide with water to obtain a 3% solution. Just before use, add 3 drops of methyl red TS, and neutralize to a yellow endpoint with 0.01 N sodium hydroxide. Do not exceed the endpoint.

Nitrogen—Use high-purity nitrogen with a flow regulator that will maintain a flow of 200 ± 10 mL per minute. Guard against the presence of oxygen by passing the nitrogen through a scrubber, such as alkaline pyrogallol, prepared as follows: add 4.5 g of pyrogallol to a gas-washing bottle, add a solution containing 85 mL of water and 65 g of potassium

hydroxide while maintaining an atmosphere of nitrogen in the bottle.

Potassium Metabisulfite Solution—Transfer 0.87 g of potassium metabisulfite ($K_2S_2O_5$) and 0.2 g of edetate disodium to a 1000-mL volumetric flask. Dilute with water to volume before mixing. [NOTE—Edetate disodium is used to protect sulfite ion from oxidation.]

Apparatus

The apparatus (see *Figure 2*) is designed to effect the selective transfer of sulfur dioxide from the specimen in boiling aqueous hydrochloric acid to the *Hydrogen Peroxide Solution* in vessel G. The backpressure is limited to the unavoidable pressure due to the height of the *Hydrogen Peroxide Solution* above the tip of the bubbler, F. Keeping the backpressure as low as possible reduces the likelihood that sulfur dioxide will be lost through leaks. Preboil vinyl and silicone tubing. Apply a thin film of stopcock grease to the sealing surfaces of all joints, except the joint between the separatory funnel and the flask, and clamp the joints to ensure tightness. The separatory funnel, B, has a capacity of 100 mL or greater. The inlet adapter, A, with a hose connector, provides a means of applying headpressure over the solution. [NOTE—A pressure-equalizing dropping funnel is not recommended because condensate, which may contain sulfur dioxide, is deposited in the funnel and the side arm.]

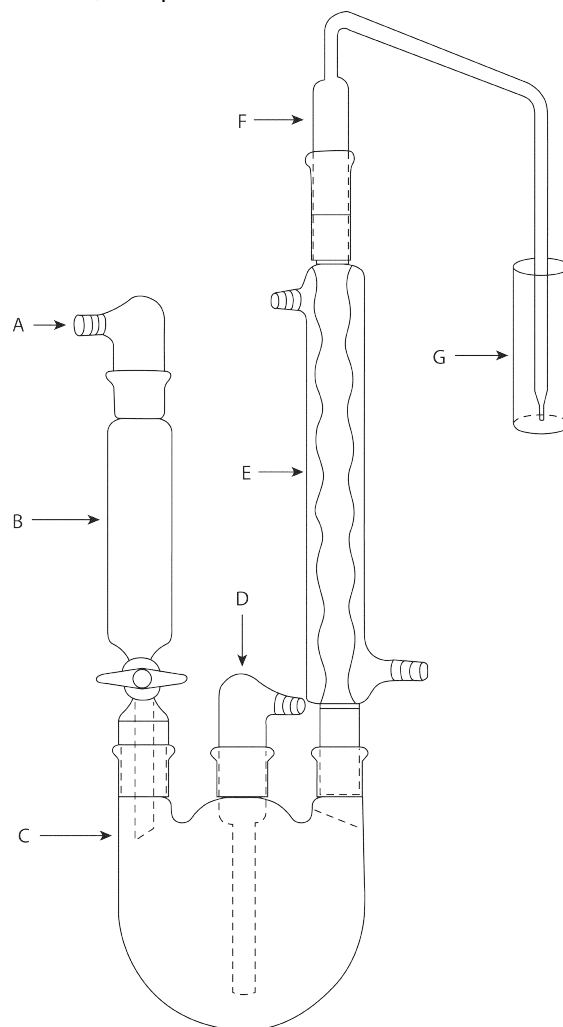


Figure 2. Apparatus for Method V.

The round-bottom flask, C, is a 1000-mL flask with three 24/40 tapered joints. The gas inlet tube, D, is long enough to permit introduction of the nitrogen to within 2.5 cm of the bottom of the flask. The Allihn condenser, E, has a jacket length of 300 mm. The bubbler, F (see Figure 3), is fabricated from glass according to the dimensions given in Figure 3. The Hydrogen Peroxide Solution is contained in the vessel, G, having an inside diameter of about 2.5 cm and a depth of about 18 cm. Circulate coolant, such as a mixture of water and methanol (4:1) maintained at 5°, to chill the condenser.

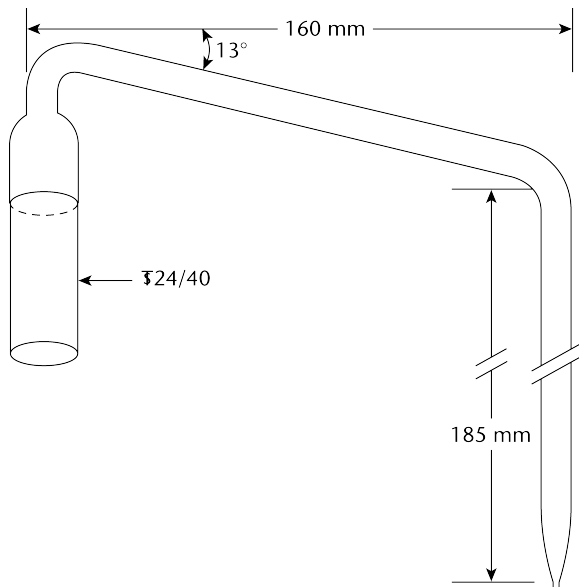


Figure 3. Bubbler (F) for apparatus in Method V.

System Suitability Test

Test A—Using the *Potassium Metabisulfite Solution* as the standard, proceed as directed for *Procedure*, except replace the 50.0 g of test substance with 20 mL of *Potassium Metabisulfite Solution*. Calculate the content, in µg per mL, of sulfur dioxide in the *Potassium Metabisulfite Solution* taken by the formula:

$$1000(32.03)VN/V_P$$

in which the factor 1000 converts mg to µg; 32.03 is the milliequivalent weight of sulfur dioxide; V is the volume, in mL, of titrant consumed; N is the normality of the titrant; and V_P is the volume, in mL, of *Potassium Metabisulfite Solution* taken for the test.

Test B—In a 100-mL conical flask, add 20 mL of 0.02 N iodine solution and 5 mL of 2 N hydrochloric acid. Add 1 mL of starch TS, and titrate with the *Potassium Metabisulfite Solution* until the first discoloration is observed. Calculate the content, in µg per mL, of sulfur dioxide in the *Potassium Metabisulfite Solution* by the formula:

$$1000(32.03)V_iN_i/V_P$$

in which 1000 and 32.03 are defined above; V_i is the volume, in mL, of iodine solution used in the test; N_i is the normality of the iodine solution; and V_P is the volume, in mL, of *Potassium Metabisulfite Solution* consumed.

The difference between the sulfur dioxide contents obtained from *Test A* and *Test B* is not more than 5% of their mean value. *Test B* shall be performed within 15 minutes after completion of *Test A*. [NOTE—This avoids a potential

variation of the sulfur dioxide content in the *Potassium Metabisulfite Solution* when stored at room temperature.]

Procedure

Position the apparatus in a heating mantle controlled by a power-regulating device. Add 400 mL of water to the flask. Close the stopcock of the separatory funnel, and add 90 mL of 4 N hydrochloric acid to the separatory funnel. Begin the flow of nitrogen at a rate of 200 ± 10 mL per minute. Start the condenser coolant flow. Add 30 mL of *Hydrogen Peroxide Solution* to the vessel (G). After 15 minutes, remove the separatory funnel, and transfer a mixture of 50.0 g of the test specimen, accurately weighed, and 100 mL of alcohol solution (5 in 100) to the flask. Apply stopcock grease to the outer joint of the separatory funnel, return the separatory funnel to the tapered joint flask, and concomitantly resume the nitrogen flow. Apply headpressure above the hydrochloric acid solution in the separatory funnel with a rubber bulb equipped with a valve. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the flask. Continue to maintain sufficient pressure above the hydrochloric acid solution to force it into the flask. [NOTE—The stopcock may be temporarily closed, if necessary, to increase the pressure.] To guard against escape of sulfur dioxide into the separatory funnel, close the stopcock before the last few mL of hydrochloric acid drain out. Apply power to the heating mantle sufficient to cause about 85 drops of reflux per minute. After refluxing for 1.75 hours, remove the vessel (G), add 3 drops of methyl red TS, and titrate the contents with 0.01 N sodium hydroxide VS, using a 10-mL buret with an overflow tube and a hose connection to a carbon dioxide-absorbing tube, to a yellow endpoint that persists for at least 20 seconds. Perform a blank determination, and make any necessary correction (see *Titrimetry* (541)). Calculate the quantity, in µg, of SO_2 in each g of the test specimen taken by the formula:

$$1000(32.03)VN/W$$

in which the factor 1000 converts mg to µg; 32.03 is the milliequivalent weight of sulfur dioxide; V is the volume, in mL, of titrant consumed; N is the normality of the titrant; and W is the weight, in g, of the test specimen taken.

(531) THIAMINE ASSAY

USP Reference Standards (11)—*USP Thiamine Hydrochloride RS*.

The following procedure is provided for the determination of thiamine as an ingredient of Pharmacopeial preparations containing other active constituents.

Special Solutions and Solvents—

POTASSIUM FERRICYANIDE SOLUTION—Dissolve 1.0 g of potassium ferricyanide in water to make 100 mL. Prepare fresh on the day of use.

OXIDIZING REAGENT—Mix 4.0 mL of *Potassium Ferricyanide Solution* with sufficient 3.5 N sodium hydroxide to make 100 mL. Use this solution within 4 hours.

QUININE SULFATE STOCK SOLUTION—Dissolve 10 mg of quinine sulfate in 0.1 N sulfuric acid to make 1000 mL. Preserve this solution, protected from light, in a refrigerator.

QUININE SULFATE STANDARD SOLUTION—Dilute 0.1 N sulfuric acid with *Quinine Sulfate Stock Solution* (39:1). This solution fluoresces to approximately the same degree as the thiochrome obtained from 1 µg of thiamine hydrochloride and

is used to correct the fluorometer at frequent intervals for variation in sensitivity from reading to reading within an assay. Prepare this solution fresh on the day of use.

Standard Thiamine Hydrochloride Stock Solution—

Transfer about 25 mg of USP Thiamine Hydrochloride RS, accurately weighed, to a 1000-mL volumetric flask. Dissolve the weighed Standard in about 300 mL of dilute alcohol solution (1 in 5) adjusted with 3 N hydrochloric acid to a pH of 4.0, and add the acidified, dilute alcohol to volume. Store in a light-resistant container, in a refrigerator. Prepare this stock solution fresh each month.

Standard Preparation—Dilute a portion of *Standard Thiamine Hydrochloride Stock Solution* quantitatively and stepwise with 0.2 N hydrochloric acid to obtain the *Standard Preparation*, each mL of which represents 0.2 µg of USP Thiamine Hydrochloride RS.

Assay Preparation—Place in a suitable volumetric flask sufficient amount of the material to be assayed, accurately weighed or measured by volume as directed, such that when diluted to volume with 0.2 N hydrochloric acid, the resulting solution will contain about 100 µg of thiamine hydrochloride (or mononitrate) per mL. If the sample is difficultly soluble, the solution may be heated on a steam bath, and then cooled and diluted with the acid to volume. Dilute 5 mL of this solution, quantitatively and stepwise, using 0.2 N hydrochloric acid, to an estimated concentration of 0.2 µg of thiamine hydrochloride (or mononitrate) per mL.

Procedure—Into each of three or more tubes (or other suitable vessels), of about 40-mL capacity, pipet 5 mL of *Standard Preparation*. To each of two of these tubes add rapidly (within 1 to 2 seconds), with mixing, 3.0 mL of *Oxidizing Reagent*, and within 30 seconds add 20.0 mL of isobutyl alcohol, then mix vigorously for 90 seconds by shaking the capped tubes manually, or by bubbling a stream of air through the mixture. Prepare a blank in the remaining tube of the standard by substituting for the *Oxidizing Reagent* an equal volume of 3.5 N sodium hydroxide and proceeding in the same manner.

Into each of three or more similar tubes pipet 5 mL of the *Assay Preparation*. Treat these tubes in the same manner as directed for the tubes containing the *Standard Preparation*.

Into each of the six tubes pipet 2 mL of dehydrated alcohol, swirl for a few seconds, allow the phases to separate, and decant or draw off about 10 mL of the clear, supernatant isobutyl alcohol solution into standardized cells, then measure the fluorescence in a suitable fluorometer, having an input filter of narrow transmittance range with a maximum at about 365 nm and an output filter of narrow transmittance range with a maximum at about 435 nm.

Calculation—The number of µg of $C_{12}H_{17}ClN_4OS \cdot HCl$ in each 5 mL of the *Assay Preparation* is given by the formula:

$$(A - b)/(S - d)$$

in which A and S are the average fluorometer readings of the portions of the *Assay Preparation* and the *Standard Preparation* treated with *Oxidizing Reagent*, respectively, and b and d are the readings for the blanks of the *Assay Preparation* and the *Standard Preparation*, respectively.

Calculate the quantity, in mg, of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the assay material on the basis of the aliquots taken. Where indicated, the quantity, in mg, of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) may be calculated by multiplying the quantity of $C_{12}H_{17}ClN_4OS \cdot HCl$ found by 0.9706.

(541) TITRIMETRY

Direct Titrations—Direct titration is the treatment of a soluble substance, contained in solution in a suitable vessel (the titrate), with an appropriate standardized solution (the titrant), the endpoint being determined instrumentally or visually with the aid of a suitable indicator.

The titrant is added from a suitable buret and is so chosen, with respect to its strength (normality), that the volume added is between 30% and 100% of the rated capacity of the buret. [NOTE—Where less than 10 mL of titrant is required, a suitable microburet is to be used.] The endpoint is approached directly but cautiously, and finally the titrant is added dropwise from the buret in order that the final drop added will not overrun the endpoint. The quantity of the substance being titrated may be calculated from the volume and the normality or molarity factor of the titrant and the equivalence factor for the substance given in the individual monograph.

Residual Titrations—Some Pharmacopeial assays require the addition of a measured volume of a volumetric solution, in excess of the amount actually needed to react with the substance being assayed, the excess of this solution then being titrated with a second volumetric solution. This constitutes a residual titration and is known also as a "back titration." The quantity of the substance being titrated may be calculated from the difference between the volume of the volumetric solution originally added, corrected by means of a blank titration, and that consumed by the titrant in the back titration, due allowance being made for the respective normality or molarity factors of the two solutions, and the equivalence factor for the substance given in the individual monograph.

Complexometric Titrations—Successful complexometric titrations depend on several factors. The equilibrium constant for formation of the titrant-analyte complex must be sufficiently large that, at the endpoint, very close to 100% of the analyte has been complexed. The final complex must be formed rapidly enough that the analysis time is practical. When the analytical reaction is not rapid, a residual titration may sometimes be successful.

In general, complexometric indicators are themselves complexing agents. The reaction between metal ion and indicator must be rapid and reversible. The equilibrium constant for formation of the metal-indicator complex should be large enough to produce a sharp color change but must be less than that for the metal-titrant complex. Indicator choice is also restricted by the pH range within which the complexation reaction must be carried out and by interference of other ions arising from the sample or the buffer. Interfering ions may often be masked or "screened" via addition of another complexing agent. (The masking technique is also applicable to redox titrations.)

Oxidation-Reduction (Redox) Titrations—Determinations may often be carried out conveniently by the use of a reagent that brings about oxidation or reduction of the analyte. Many redox titration curves are not symmetric about the equivalence point, and thus graphical determination of the endpoint is not possible; but indicators are available for many determinations, and a redox reagent can often serve as its own indicator. As in any type of titration, the ideal indicator changes color at an endpoint that is as close as possible to the equivalence point. Accordingly, when the titrant serves as its own indicator, the difference between the endpoint and the equivalence point is determined only by the analyst's ability to detect the color change. A common example is the use of permanganate ion as an oxidizing titrant since a slight excess can easily be detected by its pink color. Other titrants that may serve as their own indicators are iodine, cerium (IV) salts, and potassium dichromate.

In most cases, however, the use of an appropriate redox indicator will yield a much sharper endpoint.

It may be necessary to adjust the oxidation state of the analyte prior to titration through use of an appropriate oxidizing or reducing agent; the excess reagent must then be removed, e.g., through precipitation. This is nearly always the practice in the determination of oxidizing agents since most volumetric solutions of reducing agents are slowly oxidized by atmospheric oxygen.

Titration in Nonaqueous Solvents—Acids and bases have long been defined as substances that furnish, when dissolved in water, hydrogen and hydroxyl ions, respectively. This definition, introduced by Arrhenius, fails to recognize the fact that properties characteristic of acids or bases may be developed also in other solvents. A more generalized definition is that of Brönsted, who defined an acid as a substance that furnishes protons, and a base as a substance that combines with protons. Even broader is the definition of Lewis, who defined an acid as any material that will accept an electron pair, a base as any material that will donate an electron pair, and neutralization as the formation of a coordination bond between an acid and a base.

The apparent strength of an acid or a base is determined by the extent of its reaction with a solvent. In water solution all strong acids appear equally strong because they react with the solvent to undergo almost complete conversion to oxonium ion and the acid anion (leveling effect). In a weakly protophilic solvent such as acetic acid the extent of formation of the acetate acidium ion shows that the order of decreasing strength for acids is perchloric, hydrobromic, sulfuric, hydrochloric, and nitric (differentiating effect).

Acetic acid reacts incompletely with water to form oxonium ion and is, therefore, a weak acid. In contrast, it dissolves in a base such as ethylenediamine, and reacts so completely with the solvent that it behaves as a strong acid. The same holds for perchloric acid.

This leveling effect is observed also for bases. In sulfuric acid almost all bases appear to be of the same strength. As the acid properties of the solvent decrease in the series sulfuric acid, acetic acid, phenol, water, pyridine, and butylamine, the bases become progressively weaker until all but the strongest have lost their basic properties. In order of decreasing strength, the strong bases are sodium 2-aminoethoxide, potassium methoxide, sodium methoxide, and lithium methoxide.

Many water-insoluble compounds acquire enhanced acidic or basic properties when dissolved in organic solvents. Thus the choice of the appropriate solvent permits the determination of a variety of such materials by nonaqueous titration. Furthermore, depending upon which part of a compound is the physiologically active moiety, it is often possible to titrate that part by proper selection of solvent and titrant. Pure compounds can be titrated directly, but it is often necessary to isolate the active ingredient in pharmaceutical preparations from interfering excipients and carriers.

The types of compounds that may be titrated as acids include acid halides, acid anhydrides, carboxylic acids, amino acids, enols such as barbiturates and xanthines, imides, phenols, pyrroles, and sulfonamides. The types of compounds that may be titrated as bases include amines, nitrogen-containing heterocyclic compounds, oxazolines, quaternary ammonium compounds, alkali salts of organic acids, alkali salts of weak inorganic acids, and some salts of amines. Many salts of halogen acids may be titrated in acetic acid or acetic anhydride after the addition of mercuric acetate, which removes halide ion as the unionized mercuric halide complex and introduces the acetate ion.

For the titration of a basic compound, a volumetric solution of perchloric acid in glacial acetic acid is preferred, although perchloric acid in dioxane is used in special cases. The calomel-glass electrode system is useful in this case. In acetic acid solvent, this electrode system functions as predicted by theory.

For the titration of an acidic compound, two classes of titrant are available: the alkali metal alkoxides and the tetraalkylammonium hydroxides. A volumetric solution of sodium methoxide in a mixture of methanol and toluene is used frequently, although lithium methoxide in methanol-benzene solvent is used for those compounds yielding a gelatinous precipitate on titration with sodium methoxide.

The alkali error limits the use of the glass electrode as an indicating electrode in conjunction with alkali metal alkoxide titrants, particularly in basic solvents. Thus, the antimony-indicating electrode, though somewhat erratic, is used in such titrations. The use of quaternary ammonium hydroxide compounds, e.g., tetra-*n*-butylammonium hydroxide and trimethylhexadecylammonium hydroxide (in benzene-methanol or isopropyl alcohol), has two advantages over the other titrants in that (a) the tetraalkylammonium salt of the titrated acid is soluble in the titration medium, and (b) the convenient and well-behaved calomel-glass electrode pair may be used to conduct potentiometric titrations.

Because of interference by carbon dioxide, solvents for acidic compounds need to be protected from excessive exposure to the atmosphere by a suitable cover or by an inert atmosphere during the titration. Absorption of carbon dioxide may be determined by performing a blank titration. The blank should not exceed 0.01 mL of 0.1 N sodium methoxide VS per mL of solvent.

The endpoint may be determined visually by color change, or potentiometrically, as indicated in the individual monograph. If the calomel reference electrode is used, it is advantageous to replace the aqueous potassium chloride salt bridge with 0.1 N lithium perchlorate in glacial acetic acid for titrations in acidic solvents or potassium chloride in methanol for titrations in basic solvents.

Where these or other mixtures are specified in individual monographs, the calomel reference electrode is modified by first removing the aqueous potassium chloride solution and residual potassium chloride, if any, by rinsing with water, then eliminating residual water by rinsing with the required nonaqueous solvent, and finally filling the electrode with the designated nonaqueous mixture.

In nearly all cases, except those where silver ion might interfere, a silver-silver chloride reference electrode may be substituted for the calomel electrode. The silver-silver chloride electrode is more rugged, and its use helps to eliminate toxic mercury salts from the laboratory. Generally, a salt bridge may be used to circumvent interference by silver ion.

The more useful systems for titration in nonaqueous solvents are listed in *Table 1*.

Indicator and Potentiometric Endpoint Detection—The simplest and most convenient method by which the equivalence point, i.e., the point at which the stoichiometric analytical reaction is complete, may be determined is with the use of indicators. These chemical substances, usually colored, respond to changes in solution conditions before and after the equivalence point by exhibiting color changes that may be taken visually as the endpoint, a reliable estimate of the equivalence point.

A useful method of endpoint determination results from the use of electrochemical measurements. If an indicator electrode, sensitive to the concentration of the species undergoing titrimetric reaction, and a reference electrode, whose potential is insensitive to any dissolved species, are immersed in the titrate to form a galvanic cell, the potential difference between the electrodes may be sensed by a pH meter and used to follow the course of the reaction. Where such a series of measurements is plotted correctly (i.e., for an acid-base titration, pH versus mL of titrant added; for a precipitometric, complexometric, or oxidation-reduction titration, mV versus mL of titrant added), a sigmoid curve results with a rapidly changing portion (the "break") in the vicinity of the equivalence point. The midpoint of this linear vertical portion or the inflection point may be taken as the

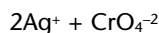
Table 1. Systems for Nonaqueous Titrations

Type of Solvent	Acidic (for titration of bases and their salts)	Relatively Neutral (for differential titration of bases)	Basic (for titration of acids)	Relatively Neutral (for differential titration of acids)
Solvent ¹	Glacial Acetic Acid	Acetonitrile	Dimethylformamide	Acetone
	Acetic Anhydride	Alcohols	<i>n</i> -Butylamine	Acetonitrile
	Formic Acid	Chloroform	Pyridine	Methyl Ethyl Ketone
	Propionic Acid	Benzene	Ethylenediamine	Methyl Isobutyl Ketone
	Sulfuryl Chloride	Toluene	Morpholine	<i>tert</i> -Butyl Alcohol
		Chlorobenzene		
		Ethyl Acetate		
		Dioxane		
Indicator	Crystal Violet	Methyl Red	Thymol Blue	Azo Violet
	Quinaldine Red	Methyl Orange	Thymolphthalein	Bromothymol Blue
	<i>p</i> -Naphtholbenzein	<i>p</i> -Naphtholbenzein	Azo Violet	<i>p</i> -Hydroxyazobenzene
	Alphezurine 2-G		<i>o</i> -Nitroaniline	Thymol Blue
	Malachite Green		<i>p</i> -Hydroxyazobenzene	
	Electrodes		Glass–calomel	Glass–calomel
Glass–silver–silver chloride		Calomel–silver–silver chloride	Antimony–glass	Glass–calomel
Mercury–mercuric acetate			Antimony–antimony ²	Glass–platinum ²
			Platinum–calomel	
			Glass–calomel	

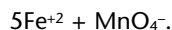
¹Relatively neutral solvents of low dielectric constant such as benzene, toluene, chloroform, or dioxane may be used in conjunction with any acidic or basic solvent in order to increase the sensitivity of the titration end-points.

²In titrant.

endpoint. The equivalence point may also be determined mathematically without plotting a curve. However, it should be noted that in asymmetrical reactions, which are reactions in which the number of anions reacting is not the same as the number of cations reacting, the endpoint as defined by the inflection of the titration curve does not occur exactly at the stoichiometric equivalence point. Thus, potentiometric endpoint detection by this method is not suitable in the case of asymmetric reactions, examples of which are the precipitation reaction,



and the oxidation-reduction reaction,



All acid-base reactions, however, are symmetrical. Thus, potentiometric endpoint detection may be employed in acid-base titrations and in other titrations involving symmetrical reversible reactions where an indicator is specified, unless otherwise directed in the individual monograph.

Two types of automatic electrometric titrators are available. The first is one that carries out titrant addition automat-

ically and records the electrode potential differences during the course of titration as the expected sigmoid curve. In the second type, titrant addition is performed automatically until a preset potential or pH, representing the endpoint, is reached, at which point the titrant addition ceases.

Several acceptable electrode systems for potentiometric titrations are summarized in Table 2.

Blank Corrections—As previously noted, the endpoint determined in a titrimetric assay is an estimate of the reaction equivalence point. The validity of this estimate depends upon, among other factors, the nature of the titrate constituents and the concentration of the titrant. An appropriate *blank correction* is employed in titrimetric assays to enhance the reliability of the endpoint determination. Such a blank correction is usually obtained by means of a *residual blank titration*, wherein the required procedure is repeated in every detail except that the substance being assayed is omitted. In such instances, the actual volume of titrant equivalent to the substance being assayed is the difference between the volume consumed in the residual blank titration and that consumed in the titration with the substance

Table 2. Potentiometric Titration Electrode Systems

Titration	Indicating Electrode	Equation ¹	Reference Electrode	Applicability ²
Acid-base	Glass	$E = k + 0.0591 \text{ pH}$	Calomel or silver–silver chloride	Titration of acids and bases
Precipitometric (silver)	Silver	$E = E^\circ + 0.0591 \log [\text{Ag}^+]$	Calomel (with potassium nitrate salt bridge)	Titration with or of silver involving halides or thiocyanate
Complexometric	Mercury–mercury(II)	$E = E^\circ + 0.0296(\log k' - \text{pM})$	Calomel	Titration of various metals (M), e.g., Mg^{+2} , Ca^{+2} , Al^{+3} , Bi^{+3} , with EDTA
Oxidation–reduction	Platinum	$E = E^\circ + (0.0591/n) \times \log [\text{ox}]/[\text{red}]$	Calomel or silver–silver chloride	Titrations with arsenite, bromine, cerate, dichromate, exacyonoferrate(III), iodate, nitrite, permanganate, thiosulfate

¹Appropriate form of Nernst equation describing the indicating electrode system: k = glass electrode constant; k' = constant derived from Hg–Hg(II)–EDTA equilibrium; M = any metal undergoing EDTA titration; $[\text{ox}]$ and $[\text{red}]$ from the equation, $\text{ox} + n\text{e}^- \rightleftharpoons \text{red}$.

²Listing is representative but not exhaustive.

present. The corrected volume so obtained is used in calculating the quantity of the substance being titrated, in the same manner as prescribed under *Residual Titrations*. Where potentiometric endpoint detection is employed, the blank correction is usually negligible.

(551) ALPHA TOCOPHEROL ASSAY

The following procedure is provided for the determination of tocopherol as an ingredient.

Hydrogenator—A suitable device for low-pressure hydrogenation may be assembled as follows. Arrange in a rack or in clamps two conical centrifuge tubes, connected in series by means of glass and inert plastic tubing and suitable stoppers of glass, polymer, or cork (avoiding all use of rubber). Use one tube for the blank and the other for the assay specimen. Arrange a gas-dispersion tube so that the hydrogen issues as bubbles at the bottom of each tube. Pass the hydrogen first through the blank tube and then through the specimen tube.

Procedure—Pipet into a suitable vessel 25 mL of the final washed ether solution of the unsaponifiable fraction obtained as directed for *When Tocopherol Is Present* under *Procedure* in the *Vitamin A Assay* (571), and evaporate to about 5 mL. *Without applying heat*, remove the remaining ether in a stream of inert gas or by vacuum. Dissolve the residue in sufficient alcohol to give an expected concentration of about 0.15 mg of alpha tocopherol per mL. Pipet 15 mL into a 50-mL centrifuge tube, add about 200 mg of palladium catalyst, stir with a glass rod, and hydrogenate for 10 minutes in the *Hydrogenator*, using hydrogen that has been passed through alcohol in a blank tube. Add about 300 mg of chromatographic siliceous earth, stir with a glass rod, and immediately centrifuge until the solution is clear.

Test a 1-mL aliquot of the solution by removing the solvent by evaporation, dissolving the residue in 1 mL of chloroform, and adding 10 mL of antimony trichloride TS: no detectable blue color appears. [NOTE—If a blue color appears, repeat the hydrogenation for a longer time period, or with a new lot of catalyst.]

Pipet 2 mL of the supernatant into a glass-stoppered, opaque flask, add 1.0 mL of a 1 in 500 solution of ferric chloride in dehydrated alcohol,* and begin timing the reaction, preferably with a stop watch. Add immediately 1.0 mL of a 1 in 200 solution of 2,2'-bipyridine in dehydrated alcohol, mix with swirling, add 21.0 mL of dehydrated alcohol, close the tube, and shake vigorously to ensure complete mixing. When about 9½ minutes have elapsed from the beginning of the reaction, transfer part of the mixture to one of a pair of matched 1-cm spectrophotometer cells. After 10 minutes, accurately timed, following the addition of the ferric chloride-dehydrated alcohol solution, determine the absorbance at 520 nm, with a suitable spectrophotometer, using dehydrated alcohol as the blank. Perform a blank determination with the same quantities of the same reagents and in the same manner, but using 2 mL of dehydrated alcohol in place of the 2 mL of the hydrogenated solution. Subtract the absorbance determined for the blank from that determined for the assay specimen, and designate the difference as A_D .

*NOTE—The absorbance of the blank may be reduced, and the precision of the determination thereby improved, by purification of the dehydrated alcohol that is used throughout the assay. Purification may be accomplished by the addition of a few crystals (about 0.02%) of potassium permanganate and of a few pellets of potassium hydroxide to the dehydrated alcohol, and subsequent redistillation.

Calculate the alpha tocopherol content, in mg, in the assay specimen taken by the formula:

$$30.2 A_D / (LC_D)$$

in which A_D is the corrected absorbance; L is the length, in cm, of the absorption cell; and C_D is the content of the assay specimen in the alcohol solution employed for the measurement of absorbance, expressed as g, capsules, or tablets per 100 mL.

(561) ARTICLES OF BOTANICAL ORIGIN

SAMPLING

In order to reduce the effect of sampling bias in qualitative and quantitative results, it is necessary to ensure that the composition of the sample used be representative of the batch of drugs being examined. The following sampling procedures are the minimum considered applicable to vegetable drugs. Some articles, or some tests, may require more rigorous procedures involving more containers being sampled or more samples per container.

Gross Sample

Where external examination of containers, markings, and labels indicates that the batch can be considered to be homogeneous, take individual samples from the number of randomly selected containers indicated below. Where the batch cannot be considered to be homogeneous, divide it into sub-batches that are as homogeneous as possible, then sample each one as a homogeneous batch. It is recommended to include samples from the first, middle, and last containers where the *No. of Containers in Batch* (N) is 11 or more and each container in the batch is numbered or lettered in order.

No. of Containers in Batch (N)	No. of Containers to be Sampled (n)
1 to 10	all
11 to 19	11
>19	$n = 10 + (N/10)$

(Round calculated "n" to next highest whole number.)

Samples are taken from the upper, middle, and lower sections of each container. If the crude material consists of component parts which are 1 cm or less in any dimension, and in the case of all powdered or ground materials, withdraw the sample by means of a sampling device that removes a core from the top to the bottom of the container, not less than two cores being taken from different angles. For materials with component parts over 1 cm in any dimension, withdraw samples by hand. In the case of large bales or packs, samples should be taken from a depth of 10 cm because the moisture content of the surface layer may be different from that of the inner layers.

Prepare the gross sample by combining and mixing the individual samples taken from each opened container, taking care not to increase the degree of fragmentation or significantly affect the moisture content.

For articles in containers holding less than 1 kg, mix the contents, and withdraw a quantity sufficient for the tests.

For articles in containers holding between 1 and 5 kg, withdraw equal portions from the upper, middle, and lower parts of the container, each of the samples being sufficient to carry out the tests. Thoroughly mix the samples, and withdraw an amount sufficient to carry out the tests. For containers holding more than 5 kg, withdraw three samples, each weighing not less than 250 g, from the upper, middle, and lower parts of the container. Thoroughly mix the samples, and withdraw a portion sufficient to carry out the tests.

Laboratory Sample

Prepare the laboratory sample by repeated quartering of the gross sample.

NOTE—Quartering consists of placing the sample, adequately mixed, as an even and square-shaped heap and dividing it diagonally into four equal parts. The two opposite parts are then taken and carefully mixed. The process is repeated as necessary until the required quantity is obtained.

The laboratory sample should be of a size sufficient for performing all the necessary tests.

Test Sample

Unless otherwise directed in the individual monograph or test procedure below, prepare the test sample as follows:

Decrease the size of the laboratory sample by quartering, taking care that each withdrawn portion remains representative. In the case of unground or unpowdered drugs, grind the withdrawn sample so that it will pass through a No. 20 standard-mesh sieve, and mix the resulting powder well. If the material cannot be ground, reduce it to as fine a state as possible, mix by rolling it on paper or sampling cloth, spread it out in a thin layer, and withdraw the portion for analysis.

METHODS OF ANALYSIS

Foreign Organic Matter

Test Sample—Unless otherwise specified in the individual monograph, weigh the following quantities of the laboratory sample, taking care that the withdrawn portion is representative (quartering if necessary):

Roots, rhizomes, bark, and herbs	500 g
Leaves, flowers, seeds, and fruit	250 g
Cut vegetable drugs (average weight of the pieces is less than 0.5 g)	50 g

Spread the sample out in a thin layer, and separate the foreign organic matter by hand as completely as possible. Weigh it, and determine the percentage of foreign organic matter in the weight of drug taken.

Total Ash

Accurately weigh a quantity of the *Test Sample*, representing 2 to 4 g of the air-dried material, in a tared crucible, and incinerate, gently at first, and gradually increase the temperature to $675 \pm 25^\circ$, until free from carbon, and determine the weight of the ash. If a carbon-free ash cannot be obtained in this way, extract the charred mass with hot water, collect the insoluble residue on an ashless filter paper, incinerate the residue and filter paper until the ash is white or nearly so, then add the filtrate, evaporate it to dryness, and heat the whole to a temperature of $675 \pm 25^\circ$.

If a carbon-free ash cannot be obtained in this way, cool the crucible, add 15 mL of alcohol, break up the ash with a glass rod, burn off the alcohol, and again heat the whole to a temperature of $675 \pm 25^\circ$. Cool in a desiccator, weigh the ash, and calculate the percentage of total ash from the weight of the drug taken.

Acid-Insoluble Ash

Boil the ash obtained as directed under *Total Ash*, above, with 25 mL of 3 N hydrochloric acid for 5 minutes, collect the insoluble matter on a tared filtering crucible or ashless filter, wash with hot water, ignite, and weigh. Determine the percentage of acid-insoluble ash calculated from the weight of drug taken.

Water-Soluble Ash

Boil the ash obtained as directed for *Total Ash* with 25 mL of water for 5 minutes. Collect the insoluble matter in a sintered-glass crucible or on an ashless filter paper. Wash with hot water, and ignite for 15 minutes at a temperature not exceeding 450° . Subtract the weight of this residue, in mg, obtained under *Total Ash*, and calculate the percentage of water-soluble ash with reference to the weight of sample as determined under *Total Ash*.

Alcohol-Soluble Extractives

Method 1 (hot extraction method)—Transfer about 4 g of air-dried, coarsely powdered material, accurately weighed, to a glass-stoppered conical flask. Add 100 mL of alcohol, and weigh the flask. Shake, and allow to stand for 1 hour. Attach a reflux condenser to the flask, and boil gently for 1 hour, cool, and weigh. Readjust to the original weight with alcohol. Shake, and filter rapidly through a dry filter. Transfer 25 mL of the filtrate to a tared flat-bottomed dish, and evaporate on a water bath to dryness. Dry at 105° for 6 hours, cool in a desiccator for 30 minutes, and weigh without delay. Calculate the content, in mg per g, of alcohol-extractable matter in the test specimen.

Method 2 (cold extraction method)—Transfer about 4 g of air-dried, coarsely powdered material, accurately weighed, to a glass-stoppered conical flask. Add 100 mL of alcohol, insert a stopper into the flask, and macerate for 24 hours, shaking frequently during the first 8 hours and then allowing to stand. Filter rapidly, taking precautions against loss of alcohol. Evaporate 25 mL of the filtrate to dryness in a tared, flat-bottomed, shallow dish, and dry at 105° to constant weight. Calculate the content, in mg per g, of alcohol-extractable matter in the test specimen.

Water-Soluble Extractives

Method 1 (hot extraction method)—Proceed as directed for *Method 1* (hot extraction method) under *Alcohol-Soluble Extractives*, except to use water in place of alcohol.

Method 2 (cold extraction method)—Proceed as directed for *Method 2* (cold extraction method) under *Alcohol-Soluble Extractives*, except to use water in place of alcohol.

Crude Fiber

Exhaust a weighed quantity of the *Test Sample*, representing about 2 g of the drug, with ether. Add 200 mL of boiling dilute sulfuric acid (1 in 78) to the ether-exhausted marc, in a 500-mL flask, and connect the flask to a reflux condenser. Reflux the mixture for 30 minutes, accurately timed, then pass through a linen or hardened-paper filter, and wash the residue on the filter with boiling water until the effluent washing is no longer acid. Rinse the residue

back into the flask with 200 mL of boiling sodium hydroxide solution, adjusted to 1.25 percent by titration and free from sodium carbonate. Again reflux the mixture for 30 minutes, accurately timed, then rapidly pass through a tared filter, wash the residue with boiling water until the last washing is neutral, and dry it at 110° to constant weight. Incinerate the dried residue, ignite to constant weight, cool in a desiccator, and weigh the ash: the difference between the weight obtained by drying at 110° and that of the ash represents the weight of the crude fiber.

NOTE—The boiling with acid and alkali should continue for 30 minutes, accurately timed, from the time that the liquid (which is cooled below the boiling point by being added to the cold flask) again boils. After the solution has been brought to boiling, the heat should be turned low enough just to maintain boiling. During the boiling, the flask should be gently rotated from time to time to wash down any particles that may adhere to the walls of the flask. A slow current of air introduced into the flask during the boiling operation aids in preventing excessive frothing.

Starch Content

Method 1—The following is a general procedure for all reducing sugars and may be used to determine the starch content in botanical articles.

Malt Extract—Use clean new barley malt of known efficacy, and grind just before use. Prepare malt extract just prior to use. For every 80 mL of malt extract needed, digest 5 g of ground malt with 100 mL of water at room temperature for 2 hours. [NOTE—If an electric mixer is used, stir the mixture for 20 minutes.] Filter to obtain a clear extract, filtering again, if necessary, and mix the infusion well.

Test Solution—Extract about 5 g of the finely ground test specimen with five 10-mL portions of ether, using a filter that will completely retain the smallest starch granule. Allow the ether to evaporate from the residue, and wash with 250 mL of aqueous alcohol solution (10 in 100). Carefully wash the residue from the paper into a 500-mL beaker with about 100 mL of water. Heat to about 60° (avoiding, if possible, gelatinizing starch), and allow to stand for about 1 hour, stirring frequently to effect complete solution of sugars. Transfer to a wide-mouth bottle, rinse the beaker with a little warm water, and cool. Add an equal volume of alcohol, mix, and allow to stand for not less than 1 hour.

Centrifuge until the precipitate is closely packed on the bottom of the bottle, and decant the supernatant. Wash the precipitate with successive 50-mL portions of alcohol solution (50 in 100) by centrifuging and decanting through a suitable filter until the washings are sugar-free. [NOTE—To test for the presence of sugar, transfer a few drops of the washings to a test tube, add 3 or 4 drops of a 20% solution of 1-naphthol in alcohol, prepared by dissolving 200 mg of 1-naphthol in 1 mL of alcohol and 2 mL of water. Shake the test tube well to allow uniform mixing, allow 2 to 4 mL of sulfuric acid to flow down the sides of the test tube, and hold the test tube upright. If sugar is present, the interface of the two liquids is colored faint to deep violet, and on shaking, the whole solution becomes blue-violet.]

Transfer the residue from the bottle and hardened filter to a beaker with about 50 mL of water. Immerse the beaker in boiling water, and stir constantly for 15 minutes or until all of the starch is gelatinized. Cool the beaker to 55°, add 20 mL of *Malt Extract*, and hold at this temperature for 1 hour. Heat again to boiling for a few minutes, cool to 55°, add 20 mL of *Malt Extract*, and hold at this temperature for 1 hour or until the residue when treated with iodine TS shows no blue tinge upon microscopic examination. Cool, dilute with water to 250 mL, and filter.

General Procedure—Transfer 200 mL of the *Test Solution* to a flask fitted with a reflux condenser, add 20 mL of hydrochloric acid, and heat in a boiling water bath for 2½ hours. Cool, nearly neutralize with sodium hydroxide TS, complete

neutralization with sodium carbonate TS, dilute with water to 500 mL, mix, and filter. The volume of aliquot taken depends on the starch content of the specimen under test (see *Table 1*). The aliquot should contain between 100 and 200 mg of dextrose. Transfer 50 mL of the filtrate to a 400-mL alkali-resistant glass beaker, add 50 mL of alkaline cupric tartrate TS, cover the beaker with a water glass, and heat. Adjust the flame in the burner so that the contents of the flask begins to boil in 4 minutes, and continue boiling for exactly 2 minutes. Filter the hot solution at once through a sintered-glass filter. Wash the precipitate of cuprous oxide thoroughly with water at about 60°, then with 10 mL of alcohol, and finally with 10 mL of ether.

Table 1. Determination of the Optimum Aliquot

% of Expected Starch Content	Aliquot in mL
60	25
50	35
40	50
30	50
20	50

For solutions of reducing sugars of comparatively high purity, proceed as directed under *Method 1A* to determine the amount of reduced copper obtained by weighing the dried cuprous oxide. For solutions of reducing sugars containing large amounts of organic impurities, including sucrose, proceed as directed under *Method 1B* to determine the amount of reduced copper obtained by titration with sodium thiosulfate.

METHOD 1A—Dry the precipitate obtained under *General Procedure* for 30 minutes in an oven at 110 ± 2°, cool to room temperature in a desiccator, and weigh. Refer to *Table 2* to find the quantity of dextrose, in mg, corresponding to the weight of cuprous oxide found. Determine the percentage of dextrose and then the content of starch by the following formula:

$$\text{Percentage of dextrose} = (\text{wt. of dextrose in mg} \times 0.1 \times 500) / (\text{wt. of sample in g} \times \text{aliquot in mL})$$

$$\text{Content of starch} = \% \text{ dextrose} \times 0.9$$

METHOD 1B—

Sodium Thiosulfate Solution—Transfer 3.9 g of sodium thiosulfate, accurately weighed, to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

Potassium Iodide Solution—Dissolve 42 g of potassium iodide in 100 mL of water.

Sodium Acetate Solution—Dissolve 5.74 g of sodium acetate in 10 mL of water.

Copper Solution—Transfer about 0.3 g of pure electrolytic copper, accurately weighed, to a 250-mL flask, add 5 mL of nitric acid to dissolve the copper, add about 25 mL of water, and boil to expel red fumes. Add about 5 mL of bromine TS, and boil until the bromine is completely removed. Cool, add 10 mL of *Sodium Acetate Solution* followed by 10 mL of *Potassium Iodide Solution*, and titrate with *Sodium Thiosulfate Solution* to a light yellow color. Add enough starch TS to produce a marked blue color, and continue the titration. As the endpoint nears, add 2 g of potassium thiocyanate, and stir until completely dissolved. Continue titration until the precipitate is completely white. One mL of sodium thiosulfate solution is equivalent to about 10 mg of copper. [NOTE—It is essential that the concentration of *Potassium Iodide Solution* be carefully regulated. If the solution contains less than 320 mg of copper at the completion of titration, add 4.2 to 5 g of potassium iodide to make a total solution of 100 mL. If greater amounts of Cu are present, add *Potassium Iodide Solution* slowly, with constant agitation, from the buret in amounts proportionately greater.]

Table 2. Calculating Dextrose (Applicable when Cu₂O is weighed directly) (Expressed in mg)

Cu-prous Oxide (Cu ₂ O)	Dex-trose (D-Glu-cose)	Cu-prous Oxide (Cu ₂ O)	Dex-trose (D-Glu-cose)	Cu-prous Oxide (Cu ₂ O)	Dex-trose (D-Glu-cose)	Cu-prous Oxide (Cu ₂ O)	Dex-trose (D-Glu-cose)	Cu-prous Oxide (Cu ₂ O)	Dex-trose (D-Glu-cose)	Cu-prous Oxide (Cu ₂ O)	Dex-trose (D-Glu-cose)
10	4.0	90	38.9	170	75.1	250	112.8	330	152.2	410	193.7
12	4.9	92	39.8	172	76.0	252	113.7	332	153.2	412	194.7
14	5.7	94	40.6	174	76.9	254	114.7	334	154.2	414	195.8
16	6.6	96	41.5	176	77.8	256	115.7	336	155.2	416	196.8
18	7.5	98	42.4	178	78.8	258	116.6	338	156.3	418	197.9
20	8.3	100	43.3	180	79.7	260	117.6	340	157.3	420	199.0
22	9.2	102	44.2	182	80.6	262	118.6	342	158.3	422	200.1
24	10.0	104	45.1	184	81.5	264	119.5	344	159.3	424	201.1
26	10.9	106	46.0	186	82.5	266	120.5	346	160.3	426	202.2
28	11.8	108	46.9	188	83.4	268	121.5	348	161.4	428	203.3
30	12.6	110	47.8	190	84.3	270	122.5	350	162.4	430	204.4
32	13.5	112	48.7	192	85.3	272	123.4	352	163.4	432	205.5
34	14.3	114	49.6	194	86.2	274	124.4	354	164.4	434	206.5
36	15.2	116	50.5	196	87.1	276	125.4	356	165.4	436	207.6
38	16.1	118	51.4	198	88.1	278	126.4	358	166.5	438	208.7
40	16.9	120	52.3	200	89.0	280	127.3	360	167.5	440	209.8
42	17.8	122	53.2	202	89.9	282	128.3	362	168.5	442	210.9
44	18.7	124	54.1	204	90.9	284	129.3	364	169.6	444	212.0
46	19.6	126	55.0	206	91.8	286	130.3	366	170.6	446	213.1
48	20.4	128	55.9	208	92.8	288	131.3	368	171.6	448	214.1
50	21.3	130	56.8	210	93.7	290	132.3	370	172.7	450	215.2
52	22.2	132	57.7	212	94.6	292	133.2	372	173.7	452	216.3
54	23.0	134	58.6	214	95.6	294	134.2	374	174.7	454	217.4
56	23.9	136	59.5	216	96.5	296	135.2	376	175.8	456	218.5
58	24.8	138	60.4	218	97.5	298	136.2	378	176.8	458	219.6
60	25.6	140	61.3	220	98.4	300	137.2	380	177.9	460	220.7
62	26.5	142	62.2	222	99.4	302	138.2	382	178.9	462	221.8
64	27.4	144	63.1	224	100.3	304	139.2	384	180.0	464	222.9
66	28.3	146	64.0	226	101.3	306	140.2	386	181.0	466	224.0
68	29.2	148	65.0	228	102.2	308	141.2	388	182.0	468	225.1
70	30.0	150	65.9	230	103.2	310	142.2	390	183.1	470	226.2
72	30.9	152	66.8	232	104.1	312	143.2	392	184.1	472	227.4
74	31.8	154	67.7	234	105.1	314	144.2	394	185.2	474	228.3
76	32.7	156	68.6	236	106.0	316	145.2	396	186.2	476	229.6
78	33.6	158	69.5	238	107.0	318	146.2	398	187.3	478	230.7
80	34.4	160	70.4	240	108.0	320	147.2	400	188.4	480	231.8
82	35.3	162	71.4	242	108.9	322	148.2	402	189.4	482	232.9
84	36.2	164	72.3	244	109.9	324	149.2	404	190.5	484	234.1
86	37.1	166	73.2	246	110.8	326	150.2	406	191.5	486	235.2
88	38.0	168	74.1	248	111.8	328	151.2	408	192.6	488	236.3

Procedure—Wash the precipitated cuprous oxide obtained under *General Procedure* with water, cover this filter with a watch glass, and dissolve the cuprous oxide with 5 mL of nitric acid directed under the watch glass with a pipet. Collect the filtrate in a 250-mL flask, wash the watch glass and the filter with water. Collect all the washings in the flask. Boil the contents of the flask to expel red fumes. Add about 5 mL of bromine TS, and boil until the bromine is completely removed. Cool, and proceed as directed under *Copper Solution* beginning with "add 10 mL of Sodium Acetate Solution." From the volume of Sodium Thiosulfate Solution consumed, obtain the weight of copper, in mg, and multiply the weight of copper by 1.1259 to obtain the weight, in mg, of cuprous oxide. From Table 2, find the quantity of

dextrose, in mg, corresponding to the weight of cuprous oxide. The content of starch is equivalent to the weight, in mg, of dextrose obtained times 0.9. Conduct a blank determination, using 50 mL of alkaline cupric tartrate TS and 50 mL of *Malt Extract*. If the weight of the cuprous oxide so obtained exceeds 0.5 mg, correct the result of the determination accordingly. [NOTE—The alkaline cupric tartrate TS deteriorates on standing and the quantity of cuprous oxide obtained in the blank determination increases.]

Method 2—The following method is specific for dextrose (glucose), and because of its extreme sensitivity it may account for differences noted between values obtained from

the same specimen. Duplicate determinations do not vary more than 2%.

Glucoamylase Solution—Prepare a solution of glucoamylase in water containing 30 International Units (IU) per mL. Use glucoamylase obtained preferably from *Rhizopus deleamar*. The total glucoamylase activity of the test specimen being used should be not less than 150 IU.

Acetate Buffer Solution—Dissolve 16.4 g of sodium acetate in 100 mL of water, add 12.0 mL of glacial acetic acid, and mix. The pH of this solution is 4.8.

Phosphate Buffer—Dissolve 3.63 g of tris (hydroxymethyl) aminomethane and 5.0 g of monobasic sodium phosphate in 50.0 mL of water. At 37°, adjust with phosphoric acid to a pH of 7.0, dilute with water to 100.0 mL, and mix. [NOTE—The pH of the buffer medium is sensitive to temperature and should be adjusted to the desired pH at the temperature to be used during incubation.]

Enzyme Solution—Dissolve 30 mg of glucose oxidase (Type II from *Aspergillus niger*), 3 mg of peroxidase (Type I from horseradish), and 10 mg of potassium ferrocyanide in 100 mL of Phosphate Buffer. [NOTE—This mixture can be stored in a refrigerator for up to 10 days.]

18 N Sulfuric Acid—Add slowly, while stirring, 54 mL of sulfuric acid to 102 mL of water, allow to cool to 25°, and mix.

Standard Solutions—Dissolve an accurately weighed quantity of USP Dextrose RS in water to obtain a solution containing 1.0 mg of USP Dextrose RS per mL. Quantitatively dilute a known volume of this solution with water to obtain Standard Solutions A, B, C, D, and E, having known concentrations of 10, 20, 25, 40, and 50 µg per mL of USP Dextrose RS, respectively. [NOTE—Allow 4 hours for complete mutarotation before use.]

Test Solutions—Extract about 5 g of finely ground test specimen with five 25-mL portions of 80% alcohol, and filter. Remove all the alcohol from the residue by drying in an air oven at 105° for about 8 hours. [NOTE 1—Any traces of alcohol remaining in the residue will inhibit glucoamylase.] Cool, and transfer the flask containing the dried test specimen to a desiccator. Transfer about 1 g, accurately weighed, of the test specimen to a previously tared flask, add 25 mL of water, and adjust with phosphoric acid to a pH between 5.0 and 7.0, if necessary. Boil the suspension for about 3 minutes, transfer the flask to an autoclave, and heat to 135° for 2 hours. Remove the flask from the autoclave, maintain the temperature near 55°, and add 2.5 mL of Acetate Buffer Solution and sufficient water to adjust the total weight of the solution to 45 ± 1 g. Immerse the flask in a water bath maintained at 55 ± 1°, and add 5 mL of Glucoamylase Solution. Continuously swirl the flask for 2 hours to effect hydrolysis, pass through filter paper into a 250-mL volumetric flask, wash quantitatively with water, and collect all the washings in the flask. Dilute the contents of the flask with water to volume, and mix. Transfer 1 mL of an aliquot containing 20 to 60 µg of D-glucose to each of five test tubes. [NOTE 2—In order to obtain the range of concentration of glucose in the hydrolysate, quantitatively dilute, if necessary, with water to volume.] Add 2 mL of Enzyme Solution to each of the five test tubes, and place the test tubes in the dark at 37 ± 1° for exactly 30 minutes to develop the color. At the end of 30 minutes, add 2 mL of 18 N Sulfuric Acid to each of the test tubes to stop the reaction, and mix.

Control Solution—Transfer an accurately weighed quantity of about 0.4 g of starch to a previously tared flask and proceed as directed under Test Solutions beginning with “add 25 mL of water, and adjust the pH with phosphoric acid.”

Procedure—Concomitantly determine the absorbances of the Standard Solutions and the Test Solutions at the wavelength of maximum absorbance at about 540 nm, with a suitable spectrophotometer, using the Control Solution as the blank to set the instrument. Plot the absorbance values of the Standard Solutions versus concentration, in µg per mL,

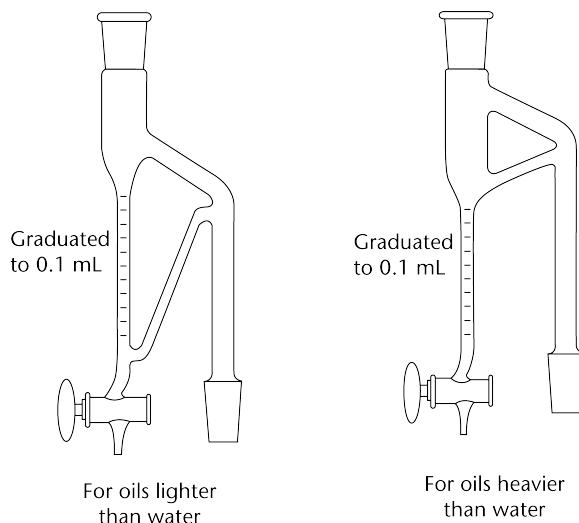
of dextrose, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg per mL, of dextrose in each of the Test Solutions, calculate the average concentration, in µg per mL, of the solution under test. The percentage of starch content in the weight of the test specimen taken is calculated by the formula:

$$(0.9C/10^6)(V_1)(250/V_0)(100/E)(100/W) = 2.25CV_1/V_0EW$$

in which E is the weight, in g, of the test specimen taken; V₀ is the volume, in mL, of the aliquot taken from the 250-mL volumetric flask; W is the percentage of dry weight of the test specimen; and V₁ is the volume, in mL, if extra dilution is done (see Note 2 under Test Solutions). [NOTE—V₀ is 1.0 when no extra dilution is done.]

Volatile Oil Determination

Set up a round-bottom, shortneck, 1-L flask in a heating mantle set over a magnetic stirrer. Insert an egg-shaped stirring bar magnet in the flask, and attach a cold-finger condenser and an appropriate volatile oil trap of the type illustrated.



Traps for Volatile Oil Apparatus

Coarsely comminute a sufficient quantity of the drug to yield from 1 to 3 mL of volatile oil. Small seeds, fruits, or broken leaves of herbs ordinarily do not need comminution. Very fine powders are to be avoided. If this is not possible, it may be necessary to mix them with purified sawdust or purified sand. Place a suitable quantity of the drug, accurately weighed, in the flask, and fill it one-half with water. Attach the condenser and the proper separator. Boil the contents of the flask, using a suitable amount of heat to maintain gentle boiling for 2 hours, or until the volatile oil has been completely separated from the drug and no longer collects in the graduated tube of the separator.

If a proper quantity of the volatile oil has been obtained in the graduated tube of the separator, it can be read to tenths of 1 mL, and the volume of volatile oil from each 100 g of drug can be calculated from the weight of the drug taken. The graduations on the separator “for oils heavier than water” are so placed that oil remains below the aqueous condensate that automatically flows back into the flask.

Water Content

For unground or unpowdered drugs, prepare about 10 g of the Laboratory Sample by cutting, granulating, or shred-

ding, so that the parts are about 3 mm in thickness. Seeds or fruits smaller than 3 mm should be cracked. Avoid the use of high-speed mills in preparing the sample, and exercise care that no appreciable amount of moisture is lost during the preparation and that the portion taken is representative of the *Laboratory Sample*. Determine the water content as directed for *Procedure for Articles of Botanical Origin* under *Water Determination* (921), *Method III* (Gravimetric).

TEST FOR AFLATOXINS

Caution—Aflatoxins are highly dangerous, and extreme care should be exercised in handling aflatoxin materials.

Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are not more than 5 ppb for aflatoxin B₁ (AFB₁) and not more than 20 ppb for the sum of aflatoxins B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁), and G₂ (AFG₂). The extent of testing may be determined using a risk-based approach that considers the likelihood of contamination. The presence of unexpected contamination with aflatoxins is to be considered in determining compliance. The following analytical procedures are provided for determining compliance. Unless otherwise specified in the individual monograph, use *Method I*. If system suitability fails, use either *Method II* or *Method III*.

Method I

This TLC test is provided to detect the possible presence of AFB₁, AFB₂, AFG₁, and AFG₂ in any material of plant origin.

Zinc Acetate–Aluminum Chloride Reagent—Dissolve 20 g of zinc acetate and 5 g of aluminum chloride in sufficient water to make 100 mL.

Sodium Chloride Solution—Dissolve 5 g of sodium chloride in 50 mL of water.

Test Solution 1—Grind about 200 g of plant material to a fine powder. Transfer about 50 g of the powdered material, accurately weighed, to a glass-stoppered flask. Add 200 mL of a mixture of methanol and water (17:3). Shake vigorously by mechanical means for not less than 30 minutes, and filter. [NOTE—If the solution has interfering plant pigments, proceed as directed for *Test Solution 2*.] Discard the first 50 mL of the filtrate, and collect the next 40-mL portion. Transfer the filtrate to a separatory funnel. Add 40 mL of *Sodium Chloride Solution* and 25 mL of solvent hexane, and shake for 1 minute. Allow the layers to separate, and transfer the lower aqueous layer to a second separatory funnel. Extract the aqueous layer in the separatory funnel twice, each time with 25 mL of methylene chloride, by shaking for 1 minute. Allow the layers to separate each time, separate the lower organic layer, and collect the combined organic layers in a 125-mL conical flask. Evaporate the organic solvent on a water bath. Transfer the remaining extract to an appropriate sample tube, and evaporate to dryness on a water bath. Cool the residue. If interferences exist in the residue, proceed as directed for *Cleanup Procedure* under *Test Solution 2*; otherwise, dissolve the residue obtained above in 0.2 mL of a mixture of chloroform and acetonitrile (9.8:0.2), and shake by mechanical means if necessary.

Test Solution 2—Collect 100 mL of the filtrate from the start of the flow, and transfer to a 250-mL beaker. Add 20 mL of *Zinc Acetate–Aluminum Chloride Reagent* and 80 mL of water. Stir, and allow to stand for 5 minutes. Add 5 g of a suitable filtering aid, such as diatomaceous earth, mix, and filter. Discard the first 50 mL of the filtrate, and collect the next 80-mL portion. Proceed as directed for *Test Solution 1*, beginning with "Transfer the filtrate to a separatory funnel."

Cleanup Procedure—Place a medium-porosity sintered-glass disk or a glass wool plug at the bottom of a 10-mm × 300-mm chromatographic tube. Prepare a slurry of 2 g of

silica gel with a mixture of ethyl ether and solvent hexane (3:1), pour the slurry into the column, and wash with 5 mL of the same solvent mixture. Allow the absorbent to settle, and add to the top of the column a layer of 1.5 g of anhydrous sodium sulfate. Dissolve the residue obtained above in 3 mL of methylene chloride, and transfer it to the column. Rinse the flask twice with 1-mL portions of methylene chloride, transfer the rinses to the column, and elute at a rate not greater than 1 mL per minute. Add successively to the column 3 mL of solvent hexane, 3 mL of ethyl ether, and 3 mL of methylene chloride; elute at a rate not greater than 3 mL per minute; and discard the eluates. Add to the column 6 mL of a mixture of methylene chloride and acetone (9:1), and elute at a rate not greater than 1 mL per minute, preferably without the aid of vacuum. Collect this eluate in a small vial, add a boiling chip if necessary, and evaporate to dryness on a water bath. Dissolve the residue in 0.2 mL of a mixture of chloroform and acetonitrile (9.8:0.2), and shake by mechanical means if necessary.

Test Solution 3—If interferences still exist in the residue, proceed as directed for *Cleanup Procedure* with IAC under *Test Solution* in *Method II*.

Aflatoxin Solution—[**Caution**—Aflatoxins are highly toxic. Handle with care.] Dissolve accurately weighed quantities of AFB₁, AFB₂, AFG₁, and AFG₂ in a mixture of chloroform and acetonitrile (9.8:0.2) to obtain a solution having concentrations of 0.5 µg/mL each of AFB₁ and AFG₁, and 0.1 µg/mL each of AFB₂ and AFG₂.

Procedure—Separately apply 2.0, 5, 7.5, and 10 µL of the *Aflatoxin Solution* and three 10-µL applications of either *Test Solution 1*, *Test Solution 2*, or *Test Solution 3* to a suitable thin-layer chromatographic plate (see *Chromatography* (621)) coated with a 0.25-mm layer of chromatographic silica gel mixture. Superimpose 5 µL of the *Aflatoxin Solution* on one of the three 10-µL applications of the *Test Solution*. Allow the spots to dry, and develop the chromatogram in an unsaturated chamber containing a solvent system consisting of a mixture of chloroform, acetone, and isopropyl alcohol (85:10:5) until the solvent front has moved not less than 15 cm from the origin. Remove the plate from the developing chamber, mark the solvent front, and allow the plate to air-dry. Locate the spots on the plate by examination under UV light at 365 nm.

System Suitability—The four applications of the *Aflatoxin Solution* appear as four clearly separated blue fluorescent spots. Observe any spot obtained from the *Test Solution* that coincides in hue and position with those of the *Aflatoxin Solution*. Any spot obtained from the *Test Solution* with the superimposed *Aflatoxin Solution* is not less intense than that of the corresponding *Aflatoxin Solution*.

Acceptance Criteria—No spot from any of the other applications of the *Test Solution* corresponds to any of the spots obtained from the applications of the *Aflatoxin Solution*. If any spot of aflatoxins is obtained in the *Test Solution*, match the position of each fluorescent spot of the *Test Solution* with those of the *Aflatoxin Solution* to identify the type of aflatoxin present. The intensity of the aflatoxin spot, if present in the *Test Solution*, when compared with that of the corresponding aflatoxin in the *Aflatoxin Solution* will give an approximate concentration of aflatoxin in the *Test Solution*. Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are not more than 5 ppb for AFB₁ and not more than 20 ppb for the sum of AFB₁, AFB₂, AFG₁, and AFG₂, except when otherwise indicated.

Method II

Sodium Chloride Solution—See *Method I*.

Phosphate Buffered Saline Solution—Prepare 10 mM phosphate buffer solution containing 0.138 M sodium chlo-

ride and 0.0027 M potassium chloride in water, and adjust with 2 M sodium hydroxide to a pH of 7.4.¹

Immunoaffinity Column (IAC)—Prior to conditioning, adjust the IAC to room temperature. For conditioning, apply 10 mL of *Phosphate Buffered Saline Solution* onto the column and let it flow through the column by gravity force at a rate of 2 to 3 mL/min. Leave 0.5 mL of the *Phosphate Buffered Saline Solution* on top of the column until the *Test Solution* is applied.

Test Solution—

Sample Extraction—Transfer about 5 g of a representative powdered sample, accurately weighed, to a glass-stoppered flask. Add 20 mL of a mixture of methanol and water (17:3). Shake vigorously by mechanical means for not less than 30 minutes, and filter. Discard the first 5 mL of the filtrate, and collect the next 4-mL portion. Transfer the filtrate to a separatory funnel. Add 4 mL of *Sodium Chloride Solution* and 2.5 mL of hexane, and shake for 1 minute. Allow the layers to separate, and transfer the lower aqueous layer to a second separatory funnel. Extract the aqueous layer in the separatory funnel twice, each time with 2.5 mL of methylene chloride, by shaking for 1 minute. Allow the layers to separate each time, separate the lower organic layer, and collect the combined organic layers in a 50-mL conical flask. Evaporate the organic solvent on a water bath. Transfer the remaining extract to an appropriate sample tube, and evaporate to dryness on a water bath. Cool the residue. If interferences exist in the residue, proceed as directed for *Cleanup Procedure with IAC*; otherwise, dissolve the residue obtained above in 200 µL of acetonitrile, and shake by mechanical means if necessary.

Cleanup Procedure with IAC—The residue is dissolved in 5 mL of a mixture of methanol and water (60:40) and then diluted with 5 mL of water. This extract is applied onto a conditioned IAC. The IAC is rinsed twice with 10 mL of *Phosphate Buffered Saline Solution*, and the elution is performed slowly with 2 mL of methanol. Evaporate the eluate with nitrogen, and dissolve the residue in 200 µL of acetonitrile.

Aflatoxin Solution—[Caution—Aflatoxins are highly toxic. Handle with care.] Prepare as directed in *Method I*, and then dilute 10-fold using a volumetric flask. The final solution contains 0.05 µg/mL of AFB₁ and AFG₁ and 0.01 µg/mL of AFB₂ and AFG₂.

Analysis—Separately apply 2.0, 5, 7.5, and 10 µL of *Aflatoxin Solution* and three 10-µL applications of the *Test Solution* to a suitable HPTLC plate (see *Chromatography* (621)) coated with a 200-µm layer of chromatographic silica gel mixture. Superimpose 5 µL of *Aflatoxin Solution* on one of the three 10-µL applications of the *Test Solution*. Allow the spots to dry, and develop the chromatogram in a saturated chamber containing a solvent system consisting of a mixture of chloroform, acetone, and water (140:20:0.3) until the solvent front has moved not less than 72 mm from the origin (80 mm from the lower edge of the plate). Remove the plate from the developing chamber, mark the solvent front, and allow the plate to air-dry for 5 minutes. Locate the spots on the plate by scanning fluorescence density (> 400 nm) under UV light at 366 nm.

System Suitability—The four applications of *Aflatoxin Solution* appear as four clearly separated blue fluorescent spots. Observe any spot obtained from the *Test Solution* that coincides in hue and position with those of *Aflatoxin Solution*. Any spot obtained from the *Test Solution* with the superimposed *Aflatoxin Solution* is not less intense than that of the corresponding *Aflatoxin Solution*. The mean recovery of spiked AFB₁ and AFG₁ is not less than 70%.

¹ A suitable powder mixture is available from Sigma as PBS P-3813.

Acceptance Criteria—Match the position of each fluorescent spot of the *Test Solution* with those of *Aflatoxin Solution* to identify the type of aflatoxin present. The concentration of aflatoxins in the *Test Solution* can be calculated from the calibration curve obtained from the scan data with *Aflatoxin Solution*. Where the individual monograph calls for compliance with the limits for aflatoxins, the limits are not more than 5 ppb for AFB₁ and not more than 20 ppb for the sum of AFB₁, AFB₂, AFG₁, and AFG₂, except when otherwise indicated.

Method III

This test method is provided as an example for the detection of the possible presence of AFB₁ and total aflatoxins (AF: sum of AFB₁, AFB₂, AFG₁, and AFG₂). It has been shown to be suitable for powdered ginseng and ginger. Its suitability to other articles of botanical origin must be demonstrated.

0.1 M Phosphate Buffer Solution—Dissolve 8.69 g of anhydrous disodium phosphate and 4.66 g of anhydrous monosodium phosphate or 5.36 g of monosodium phosphate monohydrate in 800 mL water, adjust with 2 M sodium hydroxide to a pH of 7.4, add 10 mL of polysorbate 20, and dilute to 1 L.

Phosphate Buffered Saline Solution—Prepare as directed in *Method II*.

AFB₁, AFB₂, AFG₁, and AFG₂ Standard Stock Solutions: Weigh 10 mg of each aflatoxin into a separate 100-mL volumetric flask. Add 50 mL of acetonitrile to each flask, mix, dilute with additional acetonitrile to volume, and mix. Then pipet 10 mL of each solution into separate 100-mL volumetric flasks, and dilute with acetonitrile to volume (*Standard Aflatoxin Stock Solutions A, B, C, and D*). Record the UV spectrum of each aflatoxin solution. Determine the concentration of aflatoxin solution by measuring absorbance (A) at a wavelength of maximum absorption close to 360 nm and using the equation:

µg/mL of aflatoxin = (A × MW × 1000)/ε,

where MW is molecular weight and ε is molecular absorptivity (*Table 3*). The concentration is approximately 10 µg/mL.

Table 3. Molecular Weights (MW) and Molar Absorptivities (ε) for Aflatoxins

Aflatoxin	MW	Solvent	ε
AFB ₁	312	Acetonitrile	20700
AFB ₂	314	Acetonitrile	22500
AFG ₁	328	Acetonitrile	17600
AFG ₂	330	Acetonitrile	18900

Aflatoxin Standard Solution: 400 ng/mL (mixture of the AFB₁, AFB₂, AFG₁, and AFG₂ *Standard Stock Solutions* at 200, 50, 100, and 50 ng/mL, respectively). Add an appropriate amount of each aflatoxin standard stock solution to the same volumetric flask, and dilute with acetonitrile to volume. [NOTE—Use the *Aflatoxin Standard Solution* as the spiking solution for the recovery study. Store at –18°. Equilibrate to room temperature before use.]

Working Aflatoxin Standard Solutions—Prepare six solutions in separate 10-mL volumetric flasks according to *Table 4*. Dilute with methanol and water (1:1, v/v) to volume. Store in a refrigerator, and equilibrate to room temperature before use. Prepare the solutions daily.

Table 4. Preparation of Working Aflatoxin Standard Solutions

Working Aflatoxin Standard Solutions	Aflatoxin Standard Solution (μL)	Final Aflatoxin Concentration of Working Aflatoxin Standard Solution (ng/mL)				
		AFB ₁	AFB ₂	AFG ₁	AFG ₂	ΣAF
1	0	0	0	0	0	0
2	12.5	0.25	0.0625	0.125	0.0625	0.5
3	25	0.5	0.125	0.25	0.125	1
4	50	1	0.25	0.5	0.25	2
5	100	2	0.5	1	0.5	4
6	200	4	1	2	1	8

Immunoaffinity Column (IAC)²—Use an immunoaffinity column that contains monoclonal antibodies cross reactive towards AFB₁, AFB₂, AFG₁, and AFG₂. The immunoaffinity columns have a minimum capacity of not less than 100 ng total aflatoxin and give a recovery of not less than 80% for AFB₁, AFB₂, AFG₁, and AFG₂ when 5 ng of each AFB₁, AFB₂, AFG₁, and AFG₂ is applied in 10 mL of 10% methanol in *Phosphate Buffered Saline Solution* (v/v).

Test Solution—

Extraction—Weigh 5 g of a representative test sample in a 50-mL centrifuge tube. Add 1 g of sodium chloride and 25 mL of a mixture of methanol and 0.5% sodium bicarbonate (700:300, v/v). Mix on a vortex mixer until sample particles and extract solvent are well mixed. Shake at 400 rpm for 10 minutes. Centrifuge for 10 minutes at 7000 rpm (g value = 5323 mm/s²) or at a speed that can result in a firm pellet of residues. Immediately pipet 7 mL into a 50-mL centrifuge tube, add 28 mL of 0.1 M *Phosphate Buffer Solution*, mix, and filter through glass microfiber paper. Collect 25 mL of filtrate (equivalent to 1 g of test sample) into a 25-mL graduated cylinder, and proceed immediately with IAC chromatography.

IAC Cleanup—[NOTE—For IAC cleanup, columns must be kept at room temperature for at least 15 minutes before use.] Remove the top cap from the column, and connect it with the reservoir. Remove the end cap from the column and attach it to the column manifold (the fit must be tight). Let the liquid in the column pass through until the liquid is about 2 to 3 mm above the column bed. Pass 25 mL of filtrate into the reservoir. Let the filtrate flow through the column by gravity force. Let the column run dry. In order to start the flow easily again, remove the column from the manifold, add about 2 mL of *Phosphate Buffered Saline Solution* into the column, reattach the column to the reservoir, wash the column with an additional 3 mL of *Phosphate Buffered Saline Solution* and then with 5 mL of water (the 5 mL of *Phosphate Buffered Saline Solution* can be added directly to the column reservoir if other techniques are used to dislodge the air bubble at the end of the column and to start flow easily again). Let the column run dry, then force 3 mL of air through the column with a syringe. Elute with 1 mL of methanol and collect the analytes in a 3-mL volumetric flask, letting the eluate drip freely. Let the column run dry. Let stand for 1 minute, then elute with an additional 1 mL of methanol, and collect in the same volumetric flask. Let the column run dry, and force 10 mL of air through the column. Dilute the eluate with water to volume. Use this as the *Test Solution*, and perform the analysis of aflatoxins immediately.

Chromatographic System—

Flow Rate: 0.8 mL/minute.

Detection: Fluorescence detector set at excitation wavelength (Ex) 362 nm and emission wavelength (Em) 440 nm.

Column: 4.6-mm × 15-cm containing 3-μm packing L1.

Mobile Phase: Isocratic.

FOR POST-COLUMN DERIVATIZATION WITH PHRED CELL: Water, methanol, and acetonitrile (600:250:150, v/v/v).

FOR POST-COLUMN DERIVATIZATION WITH KOBRA CELL: A solution prepared by mixing 1 L of a mixture of water, methanol, and acetonitrile (600:250:150, v/v/v); 350 mL of 4 M nitric acid; and 120 mg of potassium bromide.

Post-Column Derivatization (PCD) Systems—

PHRED CELL: Post-column photochemical derivatization cell.³

KOBRA CELL: Electrochemical cell, post-column bromination derivatization cell.⁴

Analysis—

Post-Column Derivatization for Aflatoxins—Use a UV or Kobra cell. Inject 50 μL of reagent blank (*Working Aflatoxin Standard Solution 1*), the *Working Aflatoxin Standard Solutions 2–6* or the *Test Solution* into the LC column. Identify the aflatoxin peaks in the *Test Solution* by comparing the retention times with those of the working standards. The aflatoxins elute in the order AFG₂, AFG₁, AFB₂, and AFB₁. After passing through the PHRED or Kobra cell, the AFG₁ and AFB₁ have been derivatized to form AFG_{2a} (derivative of AFG₁) and AFB_{2a} (derivative of AFB₁). [NOTE—The chemical structures of the derivatives resulting from electrochemical bromination and photolysis are not the same. The structures of AFB₁ and AFG₁ photolysis products have not been established.] The retention times of AFG₂, AFG_{2a}, AFB₂, and AFB_{2a} are between about 14 and 27 minutes using the PHRED cell; retention times are shorter using the Kobra cell. The peaks should be baseline resolved. Construct standard curves for each aflatoxin. Determine the concentration of each aflatoxin in the *Test Solution* from the calibration curve.

Aflatoxins Calibration Curves—Calibration curves are prepared for each of the aflatoxins using the *Working Aflatoxin Standard Solutions* containing the four aflatoxins described. These solutions cover the range of 0.25 to 4 ng/mL for AFB₁, 0.0625 to 1 ng/mL for AFB₂, 0.125 to 2 ng/mL for AFG₁, and 0.0625 to 1 ng/mL for AFG₂. Make the calibration curves prior to analysis according to *Table 4* and check the plot for linearity. If the test portion area response is outside (higher) the calibration range, then the *Test Solution* should be diluted with a mixture of methanol and water (1:1, v/v) and reinjected into the LC column.

Quantitation of Aflatoxins—Quantitation of aflatoxins is performed by measuring peak areas at each aflatoxin retention time and comparing them with the corresponding calibration curve.

Acceptance Criteria—The mean recovery of spiked AFB₁ (2 μg/kg) and the total of aflatoxins [AFB₁ (2 μg/kg), AFB₂ (0.5 μg/kg), AFG₁ (1 μg/kg) and AFG₂ (0.5 μg/kg)] is not less than 68% and 70%, respectively. The relative standard deviation (RSD) is not more than 10% for AFB₁ and for the total of aflatoxins.

Calculations—Plot the peak area (response, y-axis) of each of the toxin standards against the concentration (ng/mL, x-axis) and determine the slope (S) and y-intercept

³ PHRED™ Photochemical Reactor (AURA Industries, New York, NY, USA) or equivalent. Avoid looking at the UV lamp.

⁴ Kobra Cell™ (R-Biopharm Inc., Marshall, MI, USA) or equivalent. Set at 100 mA. Do not turn on the current until the LC pump is operating to avoid overheating the cell membrane.

² AflaOchraTest column (G1017; Vicam, Watertown, MA, USA) or equivalent. Aflatoxin/OTA immunoaffinity columns are suitable.

(a). Calculate the level of toxin in the sample by the following formula:

Toxin (µg/kg) = [(R - a)/S] × V/W × F

where R is the *Test Solution* peak area; V is the final volume of the injected *Test Solution* (mL); and F is the dilution factor. F = 1 when V = 3 mL. W is 1 g of test sample passed through the immunoaffinity column. The total of aflatoxins is the sum of AFG₂, AFG₁, AFB₂, and AFB₁.

GENERAL METHOD FOR PESTICIDE RESIDUES ANALYSIS

Definition—Where used in this Pharmacopeia, the designation *pesticide* applies to any substance or mixture of substances intended to prevent, destroy, or control any pest, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport, or marketing of pure articles. The designation includes substances intended for use as growth regulators, defoliants, or desiccants, and any substance applied to crops before or after harvest to protect the product from deterioration during storage and transport.

Limits—Within the United States, many botanicals are treated as dietary supplements and are subject to the statutory provisions that govern foods but not drugs in the Federal Food, Drug, and Cosmetic Act. Limits for pesticides for foods are determined by the Environmental Protection Agency (EPA) as indicated in the Code of Federal Regulations (40 CFR Part 180) or the Federal Register (FR). For pesticide chemicals without EPA-established tolerance levels, the limits should be below the detection limit of the specified method. Results less than the EPA detection limits are considered zero values. The limits contained herein, therefore, are not applicable in the United States when articles of botanical origins are labeled for food purposes. The limits, however, may be applicable in other countries where the presence of pesticide residues is permitted. Unless otherwise indicated in the monograph, the article to be examined complies with the limits indicated in *Table 5*. The limits for suspected pesticides that are not listed in *Table 5* must comply with the regulations of the EPA. For instances in which a pesticide is not listed in *Table 5* or in EPA regulations, calculate the limit by the formula:

Limits (mg/kg) = AM/100B

where A is the acceptable daily intake (ADI), as published by FAO-WHO, in mg/kg of body weight; M is body weight, in kg (60 kg); and B is the daily dose of the article, in kg.

If the article is intended for the preparation of extracts, tinctures, or other pharmaceutical forms of which the preparation method modifies the content of pesticides in the finished product, calculate the limits by the formula:

Limits (mg/kg) = AME/100B

where E is the extraction factor of the preparation method, determined experimentally; and A, M, and B are as defined above.

A total or partial exemption from the test may be granted when the complete history (nature and quantity of the pesticides used, date of each treatment during cultivation and after harvest) of the treatment of the batch is known and can be checked precisely according to good agricultural and collection practice (GACP).

Table 5

Substance	Limit (mg/kg)
Acephate	0.1
Alachlor	0.05

Table 5 (Continued)

Substance	Limit (mg/kg)
Aldrin and dieldrin (sum of)	0.05
Azinphos-ethyl	0.1
Azinphos-methyl	1
Bromide, inorganic (calculated as bromide ion)	50
Bromophos-ethyl	0.05
Bromophos-methyl	0.05
Bromopropylate	3
Chlordane (sum of <i>cis</i> -, <i>trans</i> -, and oxychlordane)	0.05
Chlorfenvinphos	0.5
Chlorpyrifos-ethyl	0.2
Chlorpyrifos-methyl	0.1
Chlorthal-dimethyl	0.01
Cyfluthrin (sum of)	0.1
λ-Cyhalothrin	1
Cypermethrin and isomers (sum of)	1
DDT (sum of <i>o,p'</i> -DDE, <i>p,p'</i> -DDE, <i>o,p'</i> -DDT, <i>p,p'</i> -DDT, <i>o,p'</i> -TDE, and <i>p,p'</i> -TDE)	1
Deltamethrin	0.5
Diazinon	0.5
Dichlofluanid	0.1
Dichlorvos	1
Dicofol	0.5
Dimethoate and omethoate (sum of)	0.1
Dithiocarbamates (expressed as CS ₂)	2
Endosulfan (sum of isomers and endosulfan sulphate)	3
Endrin	0.05
Ethion	2
Etrimphos	0.05
Fenchlorophos (sum of fenchlorophos and fenchlorophos-oxon)	0.1
Fenitrothion	0.5
Fenpropathrin	0.03
Fensulfothion (sum of fensulfothion, fensulfothion-oxon, fensulfothion-oxonsulfon, and fensulfothion-sulfon)	0.05
Fenthion (sum of fenthion, fenthion-oxon, fenthion-oxon-sulfon, fenthion-oxon-sulfoxid, fenthion-sulfon, and fenthion-sulfoxid)	0.05
Fenvalerate	1.5
Flucytrinate	0.05
τ-Fluvalinate	0.05
Fonophos	0.05
Heptachlor (sum of heptachlor, <i>cis</i> -heptachlorepoxyde, and <i>trans</i> -heptachlorepoxyde)	0.05
Hexachlorbenzene	0.1
Hexachlorocyclohexane (sum of isomers α-, β-, δ-, and ε-)	0.3
Lindan (γ-hexachlorocyclohexane)	0.6
Malathion and malaoxon (sum of)	1
Mecarbam	0.05
Methacriphos	0.05
Methamidophos	0.05
Methidathion	0.2

Table 5 (Continued)

Substance	Limit (mg/kg)
Methoxychlor	0.05
Mirex	0.01
Monocrotophos	0.1
Parathion-ethyl and Paraoxon-ethyl (sum of)	0.5
Parathion-methyl and Paraoxon-methyl (sum of)	0.2
Pendimethalin	0.1
Pentachloranisole	0.01
Permethrin and isomers (sum of)	1
Phosalone	0.1
Phosmet	0.05
Piperonyl butoxide	3
Pirimiphos-ethyl	0.05
Pirimiphos-methyl (sum of pirimiphos-methyl and N-desethyl-pirimiphos-methyl)	4
Procymidone	0.1
Profenophos	0.1
Prothiophos	0.05
Pyrethrum (sum of cinerin I, cinerin II, jasmolin I, jasmolin II, pyrethrin I, and pyrethrin II)	3
Quinalphos	0.05
Quintozene (sum of quintozene, pentachloroaniline, and methyl pentachlorophenyl sulfide)	1
S-421	0.02
Tecnazene	0.05
Tetradifon	0.3
Vinclozolin	0.4

Reagents—Use reagents and solvents that are free from any contaminants, especially pesticides, that might interfere with the analysis. It is often necessary to use special grade solvents suitable for pesticide residue analysis or solvents that have recently been redistilled in an apparatus made entirely of glass. In any case, suitable blank tests must be performed.

Preparation of Apparatus—Clean all equipment, especially glassware, to ensure that it is free from pesticides. Soak all glassware for a minimum of 16 hours in a solution of phosphate-free detergent, rinse with copious quantities of distilled water, and then wash with acetone, followed by hexane or heptane.

Qualitative and Quantitative Analysis of Pesticide Residues—Use validated analytical procedures (e.g., FDA Pesticide Analytical Manual (PAM) [<http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/PesticideAnalysisManualPAM/default.htm>], or other analytical procedures validated in accordance with EU guideline [NOTE—Document No. SANCO/10232/2006, http://ec.europa.eu/food/plant/resources/qualcontrol_en.pdf] or *Validation of Compendial Procedures* (1225).) that satisfy the following criteria. The method, especially with respect to its purification steps, is suitable for the combination of pesticide residue and substance under test, and is not susceptible to interference from co-extractives. Measure the limits of detection and quantification for each pesticide matrix combination to be analyzed: the method is shown to recover between 70% and 110% of each pesticide; the repeatability and reproducibility of the method are not less than the appropriate values indicated in Table 6; and the concentrations of test and reference solutions and the setting of the apparatus are such that a linear response is obtained from the analytical detector.

Table 6

Concentration Range of the Pesticide (mg/kg)	Repeatability (RSD) (%)	Reproducibility (RSD) (%)
0.001 – 0.01	30	60
> 0.01 – 0.1	20	40
> 0.1 – 1	15	30
> 1	10	20

TEST FOR PESTICIDES

Unless otherwise specified in the individual monograph, the following methods may be used for the analysis of pesticides. Depending on the substance being examined, it may be necessary to modify, sometimes extensively, the procedure described hereafter. Additionally, it may be necessary to perform another method with another column having a different polarity, another detection method (e.g., mass spectrometry), or a different method (e.g., immunochemical method) to confirm the results.

Extraction—[NOTE—Use the following procedure for the analysis of samples of articles having a water content of less than 15%. Samples having a higher water content may be dried, provided that the drying procedure does not significantly affect the pesticide content.] To 10 g of the coarsely powdered substance under test, add 100 mL of acetone, and allow to stand for 20 minutes. Add 1 mL of a solution in toluene containing 1.8 µg of carbophenothion per mL. Mix in a high-speed blender for 3 minutes. Filter this solution, and wash the residue with two 25-mL portions of acetone. Combine the filtrate and the washings, and heat, in a rotary evaporator, maintaining the temperature of the bath below 40° until the solvent has almost completely evaporated. To the residue add a few mL of toluene, and heat again until the acetone is completely removed. Dissolve the residue in 8 mL of toluene. Pass through a membrane filter of 45-µm pore size, rinse the flask and the filter with toluene, dilute with toluene to 10.0 mL (*Solution A*), and mix.

Purification—

Organochlorine, Organophosphorus, and Pyrethroid Insecticides—The size-exclusion chromatograph is equipped with a 7.8-mm × 30-cm stainless steel column containing 5-µm packing L21. Toluene is used as the mobile phase at a flow rate of about 1 mL per minute.

Performance of the Column—Inject 100 µL of a solution in toluene containing, in each mL, 0.5 mg of methyl red and 0.5 mg of oracet blue or equivalent. The column is not suitable unless the color of the eluate changes from orange to blue at an elution volume of about 10.3 mL. If necessary, calibrate the column, using a solution in toluene containing suitable concentrations of the pesticide of interest having the lowest molecular weight (for example, dichlorvos) and that having the highest molecular weight (for example, deltamethrin). Determine which fraction of the eluate contains both pesticides.

Purification of the Test Solution—Inject a suitable volume (100 to 500 µL) of *Solution A* into the chromatograph. Collect the fraction (*Solution B*) as determined above under *Performance of the Column*. Organophosphorus pesticides elute between 8.8 and 10.9 mL. Organochlorine and pyrethroid pesticides elute between 8.5 and 10.3 mL.

Organochlorine and Pyrethroid Insecticides—Into a 5-mm × 10-cm chromatographic column, introduce a piece of fat-free cotton and 0.5 g of silica gel treated as follows. Heat chromatographic silica gel in an oven at 150° for at least 4 hours. Allow to cool, and add dropwise a quantity of water corresponding to 1.5% of the weight of silica gel used. Shake vigorously until agglomerates have disappeared, and continue shaking by mechanical means for 2 hours.

Condition the column with 1.5 mL of hexane. [NOTE—Packed columns containing about 0.50 g of a suitable silica gel may also be used, provided they have been previously validated.] Concentrate *Solution B* almost to dryness, with the aid of a stream of helium or oxygen-free nitrogen, and dilute with toluene to a suitable volume (200 μ L to 1 mL, according to the volume injected in the preparation of *Solution B*). Quantitatively transfer this solution to the column, and proceed with the chromatography, using 1.8 mL of toluene as the mobile phase. Collect the eluate (*Solution C*).

Quantitative Analysis of Organophosphorus Insecticides—

Test Solution—Concentrate *Solution B* almost to dryness, with the aid of a stream of helium, dilute with toluene to 100 μ L, and mix.

Standard Solution—Prepare at least three solutions in toluene containing each of the pesticides of interest and carbophenothion at concentrations suitable for plotting a calibration curve.

Chromatographic System—The gas chromatograph is equipped with an alkali flame-ionization detector or a flame-photometric detector and a 0.32-mm \times 30-m fused silica column coated with a 0.25- μ m layer of phase G1. Hydrogen is used as the carrier gas. Other gases, such as helium or nitrogen, may also be used. The injection port temperature is maintained at 250°, and the detector is maintained at 275°. The column temperature is maintained at 80° for 1 minute, then increased to 150° at a rate of 30° per minute, maintained at 150° for 3 minutes, then increased to 280° at a rate of 4° per minute, and maintained at this temperature for 1 minute. Use carbophenothion as the internal standard. [NOTE—If necessary, use a second internal standard to identify any possible interference with the peak corresponding to carbophenothion.] Inject the chosen volume of each solution, record the chromatograms, and measure the peak responses. Calculate the content of each pesticide from the peak areas and the concentrations of the solution.

Quantitative Analysis of Organochlorine and Pyrethroid Insecticides—

Test Solution—Concentrate *Solution C* almost to dryness, with the aid of a stream of helium or oxygen-free nitrogen, dilute with toluene to 500 μ L, and mix.

Standard Solution—Prepare at least three solutions in toluene containing each of the pesticides of interest and carbophenothion at concentrations suitable for plotting a calibration curve.

Chromatographic System—The gas chromatograph is equipped with an electron-capture detector, a device allowing direct on-column cold injection, and a 0.32-mm \times 30-m fused silica column coated with a 0.25- μ m layer of phase G1. Hydrogen is used as the carrier gas. Other gases, such as helium or nitrogen, may also be used. The injection port temperature is maintained at 275°, and the detector is maintained at 300°. The column temperature is maintained at 80° for 1 minute, then increased to 150° at a rate of 30° per minute, maintained at 150° for 3 minutes, then increased to 280° at a rate of 4° per minute, and maintained at this temperature for 1 minute. Use carbophenothion as the internal standard. [NOTE—If necessary, use a second internal standard to identify any possible interference with the peak corresponding to carbophenothion.] Inject the chosen volume of each solution, record the chromatograms, and measure the peak responses. Calculate the content of each pesticide from the peak areas and the concentrations of the solutions.

(563) IDENTIFICATION OF ARTICLES OF BOTANICAL ORIGIN

Identification of raw plant material intended for use in the manufacture of pharmaceuticals, excipients, or dietary supplements is carried out by examining the morphological and histological features of the article under test and by performing diagnostic chemical tests on the article. The botanical and chemical characteristics of the test article are then compared to the known botanical and chemical characteristics of the plant species. Reference articles may be specified to assist in the proper botanical and chemical identification of the plant and plant part. A reference article may be either a USP Authenticated Reference Material, which may be used for both botanical and chemical identification, or a USP Reference Standard, which is used for chemical identification only.

USP AUTHENTICATED REFERENCE MATERIALS

USP Authenticated Reference Materials are plant organs or tissues certified to have come from a plant that has been properly identified as belonging to the species listed on the label. The authentication is performed by botanical taxonomists, plant anatomists, phytochemists, or other plant scientists contracted by the USP. A USP Authenticated Reference Material is typically a dried, pulverized plant organ or tissue, and it may be obtained from USP. Herbarium samples that may include roots, stems, leaves, flowers, fruits, and seeds of the authenticated plants are archived and made available for examination upon request. Standard herbarium samples usually consist of the entire mature plant. USP Authenticated Reference Materials undergo the same botanical and chemical diagnostic tests as those applied to the test raw materials. A test article must have all botanical and chemical characteristics specified and found in the USP Authenticated Reference Material. To serve its intended purpose, each USP Authenticated Reference Material is properly stored, handled, and used. Generally, USP Authenticated Reference Materials are stored in their original containers under cool and dry conditions and protected from light and insect infestation. Where special storage conditions are necessary, directions are given on the label. Active principles and marker compounds typically degrade with time; therefore, expiration dates are assigned to USP Authenticated Reference Materials for their use in chemical identification. USP Authenticated Reference Materials are not intended for use in the manufacture of pharmaceuticals, excipients, or dietary supplements.

BOTANICAL IDENTIFICATION

The botanical identification of raw plant materials used in the manufacture of pharmaceuticals, excipients, or dietary supplements consists of ascertaining the macroscopic characteristics of the plant part, such as root, stem, leaf, flower, fruit, or seed, used in the manufacture of the article, as well as ascertaining its histological (microscopic) features. It may also include the inspection of organoleptic features of the botanical tissue, such as the presence or absence of a characteristic odor. Individual compendial monographs may include botanical information on possible adulterant species to help ensure their absence in the raw material. For a proper identification of the plant, plant organ, or plant tissue, it is necessary to have a basic knowledge of plant anatomy.

Diagnostic Plant Morphology and Anatomy

This section exclusively addresses the diagnostic morphological and anatomical features of vascular plants and the various plant parts, such as roots, stems, leaves, flowers, fruits, and seeds, from which pharmaceuticals, excipients, or dietary supplements are derived. Vascular plants include pteridophytes (ferns and fern allies; for example, genera *Aspidium*, *Equisetum*, and *Lycopodium*), gymnosperms (seed plants, in which the seed is not enclosed within a fruit; for example, genera *Ephedra*, *Ginkgo*, and *Pinus*), and angiosperms (seed plants, in which the seed is enclosed within a fruit; for example, genera *Allium*, *Digitalis*, *Panax*, *Matricaria*, and *Rauwolfia*). Anatomical diagnostic features that are specified in an individual monograph (see *Botanic characteristics* in individual monographs) may include, but are not limited to, the presence of a particular tissue within an organ; the arrangement and type of cells within a tissue; the presence and type of secretory canal, oil, or resin duct or laticifers within an organ; the number of epithelial cells surrounding a secretory canal; and the presence and type of ergastic substances such as starch, inulin, fat globules, essential oils, calcium oxalate crystals, cystoliths, polyphenols, fluids, or other materials occurring in the cytoplasm, organelles, vacuoles, cavities, or cell wall.

ROOTS

The tissues present in young roots, starting with the most external tissue, include an epidermis with root hairs, cortex, endodermis, pericycle, phloem, xylem, and, in some species, pith. In some species, the outermost layer or layers of cortex are distinct from the inner layers, in which case they are referred to as a hypodermis. In species that undergo secondary growth in the roots, it is typical for all tissues external to the pericycle to be sloughed off. Roots that exhibit secondary growth have a periderm or bark, composed of a phellum (cork), phellogen (cork cambium), and phelloderm as the outermost tissue. Underneath the periderm, remnants of primary phloem, secondary phloem, vascular cambium, primary xylem, and secondary xylem can be found. Secondary vascular tissues have medullary rays separating clusters of the principal conducting cells of phloem (sieve elements or sieve cells) and the principal conducting cells of xylem (vessels and tracheids). Most species of plants that undergo secondary growth lack pith in the root. The type and arrangement of the principal conducting cells of the vascular tissues may be diagnostic of the species. Roots of many species develop into food storage organs. Abundant parenchyma and large amounts of starch or other polysaccharides characterize these types of roots. The presence, type, and arrangement of fibers, sclereids, and other tissues, and the presence and location of ergastic material may also be diagnostic features. Morphologically, roots may be distinguished from rhizomes (the underground stems) primarily by the absence of nodes and internodes, which are present in rhizomes.

STEMS

Several external macroscopic features of stems that may be diagnostic of the species include the attributes of the nodes, internodes, leaf scars, vascular bundle scars, lenticles, and buds; the growth pattern of the buds; position and arrangement of the leaves along the stem; and the presence of tendrils, spines, thorns, or prickles. Starting with the outermost tissue, the internal arrangement of tissues in the young stems of most species is epidermis, cortex, a concentric ring of vascular bundles separated from each other by parenchymatous medullary rays, and pith. Depending on the species, stomata or trichomes or both structures may be present in the epidermis. The cortex of some species may

include a hypodermis or an endodermis or both. In most monocotyledons, the vascular bundles are not arranged concentrically; instead they are scattered throughout a mass of parenchyma tissue internal to the epidermis. Because of this arrangement, neither cortex, medullary rays, nor pith can be discerned. In woody plant stems that undergo secondary growth, it is typical for the epidermis to be sloughed off and replaced by a periderm composed of a phellum, phellogen, and phelloderm. Some species are characterized by having multiple periderms (rhytidome). Lenticles may be present in the periderm and their attributes may serve as diagnostic features. Underneath the periderm are the remnants of the cortex, primary phloem, secondary phloem, vascular cambium, and secondary xylem, and the remnants of primary xylem and pith. Medullary rays are also present. As in the root, the type and arrangement of the principal conducting cells of the vascular tissues; the presence, type, and arrangement of fibers, sclereids, and other tissues; and the presence and location of ergastic material may also be diagnostic features. Rhizomes may have some morphological characteristics similar to those of roots and therefore they may be mistaken for roots. However, rhizomes can be correctly identified as stems because they have distinct nodes and internodes.

LEAVES

Several macroscopic features of leaves that may be diagnostic of the species include the attributes of the leaf blade, petiole, and stipules and the phyllotaxy. The outermost tissue of a leaf blade is the epidermis, followed by mesophyll and vascular tissues. Microscopic diagnostic features of epidermal cells include the cuticle thickness and markings, the shape and arrangement of stomata and guard cells, the arrangement and size of subsidiary cells, stomatal number (number of stomata per unit area), and stomatal index (number of stomata per unit number of epidermal cells). Additional features useful in the identification of leaf material include types and arrangement of trichomes (plant hairs) present; type and arrangement of mesophyll and vascular tissues; palisade mesophyll ratio; presence and appearance of accessory tissues such as parenchymatous or sclerenchymatous bundle sheaths, paraveinal mesophyll, endodermis, and transfusion tissue; type and arrangement of the principal conducting cells of the vascular tissues; presence, type, and arrangement of fibers, sclereids, and other tissues; and presence, location, and physical appearance of ergastic material.

FLOWERS

Flowers are the best diagnostic morphological features of any flowering plant and the floral structure is the principal criterion used in plant taxonomy. The diagnostic features of flowers include type of inflorescence; presence, number, and appearance of the primary floral parts (sepals, petals, stamens, and carpels); type of symmetry displayed by the floral parts; relative position of the ovaries in regards to the other parts of the flower; the number of ovules per ovary; type of placentation of the ovary; physical appearance of the pollen grains; presence of nectaries; presence of covering or glandular trichomes; and physical features of accessory structures, such as the receptacle and bracts. The histological features and the presence of ergastic materials in the tissues of floral parts are also diagnostic of the species.

FRUITS

The identification of the species of plant from which a fruit was derived may be determined by observing several macroscopic criteria. These criteria include the number of pistils found in the fruit, the number of carpels within each

pistil, the number of seeds within each carpel, the placentation of the fruit, and the determination whether the fruit is dehiscent, indehiscent, or fleshy. Additional diagnostic features include the number of sutures in a dehiscent fruit, the determination whether the seeds are fused to or free from the pericarp wall, physical features of the three layers of the pericarp of fleshy fruits (epicarp, mesocarp, and endocarp), and presence and physical appearance of accessory tissues such as the receptacle and bracts. Histological features of fruit tissues may aid in identification. The characteristics of the seeds within the fruit are also diagnostic features of the species.

SEEDS

The macroscopic features of seeds used in identification include the shape and size of the seed; appearance of the seed-coat surface; placement of the hilum and micropyle; and presence of accessory structures of the seed coat such as arils, caruncle, or oil bodies. Physical features of the embryo such as its size, shape, position, and the number and appearance of the cotyledons, as well as the presence and appearance of accessory nutritive tissues such as the remnants of a megagametophyte (in gymnosperms), perisperm (nucellus), or endosperm are also diagnostic of the species. Histological features of the seed coat and other structures and tissues of the seed may also be used for species identification.

Microtechnique

Histological analysis of botanical specimens can be performed on whole plant material or plant powder. The use of cytological stain or other reagents may be necessary to visualize certain histological features. Crossed polarizers can be used to detect structures that rotate plane-polarized light. These structures include starch grains, calcium oxalate crystals, some fibers, and grains of sand (present as a contaminant) that can be observed as bright objects against a dark background. One polarizer is commonly placed in the condenser or the light source, and the second polarizer is placed in the ocular. Light entering the slide from below is plane polarized, permitting only some light waves in a specific plane to pass through. When the two polarizers are aligned, the field becomes bright; and when the two polarizers are crossed, the field becomes dark.

PROCEDURE FOR TEMPORARY MOUNTS AND POWDERED MATERIAL

General Procedure—Plant samples are observed under the microscope by employing different mounting media, stains, or other solutions to assist in the correct identification of the test article. If a USP Authenticated Reference Material is available, prepare it with the same mounting media or reagent solutions used for the test article. Place one or two drops of water, *Glycerin-Alcohol Solution*, *Chloral Hydrate Solution*, or another reagent solution (see *Preparation and Use of Reagent Solutions, Optical Devices, and Mountants*) in the center of a clean slide. Transfer a small plant tissue section or a portion of plant powder into the mountant or reagent solution, and cover with a clean coverslip. (For specific preparation techniques, see *Preparation of Temporary Mounts and Hand Sections, Maceration, or Preparation of Powdered Material*, as appropriate.) To prevent the formation of air bubbles, the coverslip may be carefully placed at an appropriate angle with its edge making the first contact with the slide and then pressed until it covers the specimen. Using a piece of filter paper, remove excess fluid from the margin of the coverslip. Air bubbles can be removed by placing the slide in a vacuum desiccator. When using chloral

hydrate, air bubbles can be removed by gently boiling the sample over a small flame such as that from an alcohol lamp. To replace the mountant or reagent solution, place drops of the new mountant or reagent solution on one edge of the coverslip. Place a strip of filter paper at the opposite edge of the coverslip to remove the old mountant or reagent solution and to cause the new mountant or reagent solution to be drawn over the powdered material or tissue. Plant oils can be also washed away from the tissue in this manner, when solvent hexane or acetone is washed through the slide followed by water and, if necessary, *Chloral Hydrate Solution*. Do not use *Chloral Hydrate Solution* immediately after treating the plant tissue with flammable solvents without thoroughly washing the tissue with water. This is to avoid setting fire to residual solvent when the microscope slide is later placed over a small flame to boil the tissue. Care must be taken when using reagent solutions that are volatile or corrosive to the microscope. To prevent drying of aqueous or chloral hydrate solutions during observation, add a small drop of glycerin to the slide. Observe the mounted sample under an optical microscope (see *Optical Microscopy* (776)), and examine for histological features.

Preparation and Use of Reagent Solutions, Optical Devices, and Mountants—The following reagents, optical devices, and mounting media are used to assist in the identification of cells, tissues, structural features, and ergastic substances in the tissue or powdered material (see *Tables 1 and 2*).

Table 1. The Use of Reagent Solutions and Optical Devices

Detection	Reagent Solutions and Optical Devices
Calcium carbonate concretion	Diluted Acetic Acid
Calcium oxalate crystals	Crossed Polarizers
Cellulose	Carmine Alum–Methyl Green Solution Hydriodic Acid Zinc Chloride–Iodine Solution
Cytoplasm	Alcoholic Picric Acid Solution
1,8-Dihydroxyanthraquinones	1 M Potassium Hydroxide Solution
Essential oils	Osmium Tetroxide Solution Sudan III Solution
Inulin	Naphthol–Sulfuric Acid Solution
Lignin	Carmine Alum–Methyl Green Solution Phloroglucinol–Hydrochloric Acid Solution Universal Reagent
Lipids (cutin, waxes, and suberin included)	Carmine Alum–Methyl Green Solution Osmium Tetroxide Solution Sudan III Solution Universal Reagent
Pectin and mucilage	Ruthenium Red Solution Thionine Solution Toluidine Blue Solution
Phytoglycogen	Ruthenium Red Solution
Protein bodies	Alcoholic Picric Acid Solution Osmium Tetroxide Solution
Saponin	Blood–Gelatin Mixture Iodine–Glycerin Solution (confirm by testing with Blood–Gelatin Mixture)
Starch	Crossed Polarizers Iodine Solution Universal Reagent
Tannins and other polyphenols	Ferric Chloride Solution Osmium Tetroxide Solution

Table 2. Bleaching and Clarifying Agents and Mountants

Use	Mountants and Agents
Bleaching Agents	<i>Sodium Hypochlorite Solution</i>
Clarifying Agents	<i>Chloral Hydrate Solution</i> <i>Lactochloral Solution</i> <i>Lactophenol Solution</i>
Mountants	<i>Glycerin</i> <i>Glycerin-Alcohol Solution</i> <i>Glycerin-Gelatin Mixture</i> <i>Water</i>

Alcoholic Picric Acid Solution—Prepare a 1% solution of picric acid in alcohol. Picric acid is useful to stain cells having dense cytoplasm, such as aleurone cells in seeds. Place a small amount of powdered plant material in a test tube, and shake with about 1 mL of solvent hexane to remove plant oils, which would interfere with the reaction. Centrifuge, and discard solvent hexane. Soak the plant powder in *Alcoholic Picric Acid Solution* for about 30 minutes. Transfer a portion of the powder to a microscope slide, and observe under a microscope: cytoplasm and protein bodies turn bright yellow. [Caution—Picric acid is explosive when dry. Handle appropriately.]

Blood-Gelatin Mixture—Add 4.5 g of gelatin powder to 100 mL of a 0.9% sodium chloride solution, and allow to swell for 30 minutes. Heat the gel, while stirring, to about 80° in a water bath. Cool to 40°, and add 6 mL of defibrinated bovine blood. Heat to 45° to 50°, and pour onto a microscope slide in a thin layer of about 1 mm while the slide is in a horizontal position. To prevent loss of blood-gelatin mixture from the sides, seal the microscope slide edge with a 1-cm wide adhesive tape to form a tray. After cooling and solidification, it is ready for use. [NOTE—Store in a humid chamber for not more than 1 to 2 days at 3° to 4°.] To test for saponins, place small clusters of the powdered plant material on the blood-gelatin layer, spacing them a few millimeters apart from each other, transfer to a humidifier for a few hours, and observe: saponin-containing particles will cause light-transparent zones to appear in the blood-gelatin.

Carmine Alum-Methyl Green Solution—Boil 1.5 g of carmine for 30 minutes in a 15% solution of aluminum potassium sulfate. Cool, filter, and add 10 mL of a 0.75% methyl green solution while stirring. Add 1 to 2 drops to plant material: lignin and suberin turn green and cellulose turns red-violet.

Chloral Hydrate Solution—Use chloral hydrate TS. When using the solution as a clarifying agent, add a few drops to the plant material, and boil briefly over a small flame. Chloral hydrate dissolves cellular contents and intercellular substances and allows cell walls and shapes to be easily observed. It can be used to assist in the identification of cork, fibers, vessels, calcium oxalate crystals (with the aid of crossed polarizers), trichomes, stomata, and pollen.

Crossed Polarizers—This optical device is used to detect calcium oxalate crystals and starch grains (amyloplasts). In polarized light, calcium oxalate crystals and starch grains appear as bright, birefringent objects on a dark background. Starch grains observed under polarized light will also have a Maltese-cross effect with the arms of the cross intersecting at the hilum. Calcium oxalate crystals are usually best viewed after the sample has been clarified with *Chloral Hydrate Solution* or another clarifying agent.

Diluted Acetic Acid—Add 1 to 2 drops to the plant material, and immediately observe under a microscope: calcium carbonate deposits dissolve with effervescence.

Ferric Chloride Solution—Dilute 1 mL of ferric chloride TS with 9 mL of water. For the detection of phenol hydroxyl groups, such as tannins and flavonoids, from the side of the coverslip add the solution to the aqueous sample: tannins and other polyphenols become blue-black to green.

Glycerin—Use as a mountant to prevent the drying of aqueous and chloral hydrate solutions.

Glycerin-Alcohol Solution—Mix equal volumes of glycerin and alcohol. Use as a mounting medium.

Glycerin-Gelatin Mixture—Add 10.0 g of powdered gelatin to 60 mL of water. Allow to stand for 2 hours, and add 70 mL of glycerin containing 1.5 g of dissolved phenol. Heat in a water bath, and filter through a preheated funnel containing glass wool. The filtered mixture is liquefied before use, and it serves as a mounting medium. Add a few drops to the cut or powdered plant material, and cover with a heated coverslip. This preparation is used for long-term storage of specimen mounts. The margins of the coverslip may be sealed with Canada balsam after a few months of drying.

Hydriodic Acid—Add 1 to 2 drops to plant material: cellulosic cell walls become blue to blue-violet.

Iodine Solution—Add 1 to 2 drops of 0.1 N iodine VS to the plant material: starch particles become dark-blue to blue-violet; this reaction is reversible on heating. [NOTE—Proteins, lipids, and cellulose turn yellow to brown; and guaiac powder particles become green to blue, but this reagent is not used for diagnostic identification of these features.]

Iodine-Glycerin Solution—Dissolve 0.3 g of iodine and 1.0 g of potassium iodide in a small quantity of water, and add 10 mL of a mixture of glycerin and water (1:1). Add 1 to 2 drops to the powdered plant material: samples containing saponins form yellow lumps or aggregates. If a sample tests positive for saponin, the result has to be confirmed by testing the sample with *Blood-Gelatin Mixture* as well.

Lactochloral Solution—Dissolve 50.0 g of chloral hydrate in 50 mL of lactic acid with gentle heating. Add a few drops to the plant material. Place the microscope slide in a small vacuum desiccator if it is necessary to eliminate air bubbles. *Chloral Hydrate Solution* and *Lactochloral Solution* are used for the same type of identification, except that *Lactochloral Solution* is a stronger clarifying agent and it is used for plant material that is more difficult to clarify.

Lactophenol Solution—Mix 20 g of lactic acid, 40 g of glycerin, and 20 mL of water. Add 20 g of phenol, and mix. This is a strong clarifying agent suitable for the examination of pollen grains.

Naphthol-Sulfuric Acid Solution—Prepare a 20% solution of 1-naphthol in alcohol. To plant material add 1 drop of 1-naphthol solution and 1 drop of sulfuric acid: inulin crystals turn brownish red and then dissolve.

Osmium Tetroxide Solution—Dissolve 0.1 g of osmium tetroxide in 5 mL of distilled water. Add 1 to 2 drops of the solution so obtained to plant material: essential oils, fatty oils and other lipids, tannins, and protein bodies become brown to black.

Phloroglucinol-Hydrochloric Acid Solution—This solution is used for the identification of lignin and other hydroxyphenylpropane derivatives, lignified tissues such as sclereids, vessels, fibers, and stone cells, and lignified parenchyma. Moisten the powder or the cut sample with phloroglucinol TS, and allow to dry for 2 to 3 minutes before placing the coverslip. Add a few drops of a 25% hydrochloric acid solution, and cover with the coverslip. Lignified cell walls turn carmine red. [NOTE—This stain is not stable.] Cells with hydroxyphenylpropane derivatives, such as vanillin and ferulic acid, also turn red. Alternatively, hydroxyphenylpropane derivatives can be extracted from the plant material and the plant material then examined. To extract hydroxyphenylpropane derivatives repeatedly immerse the untreated material in alcohol, mix on a vortex mixer, centrifuge, and discard the alcohol between washings. Then treat the plant material as specified above, beginning with the addition of phloroglucinol TS.

1 M Potassium Hydroxide Solution—Add 1 drop to plant material: cells containing 1,8-dihydroxyanthraquinones will stain red.

Ruthenium Red Solution—Add a few drops of ammonium hydroxide to ruthenium red TS. [NOTE—Store the solution protected from light.] Add 1 to 2 drops to plant material: pectin-containing cell membranes, acidic mucilage, and phyloglycogen turn red.

Sodium Hypochlorite Solution—This solution is used to bleach deeply colored sections. Immerse the plant material in the solution for a few minutes until sufficiently bleached. Wash the tissue with water, and mount with a suitable mounting agent. [NOTE—Sodium hypochlorite will extract lignin; plant tissue so treated will test negative for lignin.]

Sudan III Solution—Dissolve 0.5 g of Sudan III in 50 mL of alcohol or isopropyl alcohol with reflux boiling. Cool, filter, and add 50 mL of glycerin. Add 1 to 2 drops of this solution to plant powder: essential oils, waxes, cutin, suberin, and fatty oils and other lipids combine with this lipophilic colorant and become orange-red to red after a short time.

Thionine Solution—Prepare a 0.2% thionine acetate solution in 25 percent alcohol. Immerse the dry sample in this solution. After about 15 minutes, wash out the excess of stain with 25 percent alcohol: mucilage will have swollen into spherical globules and turned red-violet, while cellulose, pectin, and lignified septa will turn blue or blue-violet.

Toluidine Blue Solution—Using toluidine blue, proceed as directed for *Thionine Solution*.

Universal Reagent—

SOLUTION A—Dilute 20 mL of a lactic acid-saturated solution of Sudan III with 30 mL of lactic acid.

SOLUTION B—Dissolve 0.55 g of aniline sulfate in 35 mL of water.

SOLUTION C—Dissolve 0.55 g of potassium iodide and 0.05 g of iodine in 5 mL of water, and add 5 mL of alcohol.

PROCEDURE—Combine *Solution A*, *Solution B*, and *Solution C*, and add 2.5 mL of hydrochloric acid while stirring. [NOTE—The solution is used without filtering.] For identification, add 2 to 3 drops to the sample, and gently boil over a small flame. If necessary, small amounts of *Universal Reagent* may be added during boiling. Cover with the coverslip: lignified elements turn yellow; suberin turns red-brown; lipids turn red; and starch turns blue-violet.

Water—Use as a mounting medium. [NOTE—All grades of water are acceptable for this purpose.]

Zinc Chloride-Iodine Solution—Dissolve 20.0 g of zinc chloride and 6.5 g of potassium iodide in 10.5 mL of water. Add 0.5 g of 0.1 N iodine VS, and shake for 15 minutes. Filter if necessary. Store in low-actinic glassware. Add 1 to 2 drops to plant material, and allow to stand for a few minutes: cellulosic cell walls are stained blue to blue-violet.

Preparation of Temporary Mounts and Hand Sections—When using the dry plant tissue, soak or gently boil in water until soft. Do not soften too much. Material can then be treated like fresh plant material. When appropriate, use the mountants or reagent solutions listed for use with plant powder to help visualize features of the tissue (see *Preparation and Use of Reagent Solutions, Optical Devices, and Mountants*).

To make an epidermal peel of the leaf, petal, sepal, bract, and other leaf-like appendages, roll the tissue into a cylinder, and nick with a sharp, polytef-coated razor blade that has been wetted with water. Grasp nicked piece of tissue with forceps, and strip back removing a clear section of the epidermis. Mount in water on a microscope slide, place a coverslip over the tissue, and examine under a microscope. If it is difficult to obtain an epidermal peel using the above procedure, proceed as follows. Soak the tissue in a 40% to 60% nitric acid solution at 60° for 3 to 4 minutes or until the epidermis can be easily peeled. The peel is then washed three to five times in water to remove the excess of nitric acid. Neutralize the tissue in a 1% potassium hydroxide solution or a 1% sodium hydroxide solution. Wash the tissue again with water, mount in water on a microscope slide,

place a coverslip over the tissue, and examine under a microscope.

An alternative method of preparing leaf tissue for the examination of the epidermis is to heat a leaf fragment (about 5 mm × 5 mm) for 15 minutes in *Chloral Hydrate Solution* on a water bath. Transfer the tissue to a microscope slide, add a drop of water, and cover with a coverslip. These procedures can be used to determine the stomatal type, distribution, number, and index.

Stomatal number is determined by counting the number of stomata per unit area of a microscopic field. Determine the stomatal number on at least 10 different sites of the specimen, and calculate a mean value. Keep track of which leaf surface is being observed, abaxial or adaxial, as the stomatal number for different surfaces is frequently significantly different.

To calculate the stomatal index, the specimen is observed under a microscope at a low magnification. The size of the surface is determined with a calibrated micrometer ocular, and the number of stomata and the number of epidermal cells for that area are determined. The stomatal index is calculated by the formula:

$$100S/(E + S)$$

in which *S* is the number of stomata for a given area; and *E* is the number of epidermal cells of the same area. Determine the stomatal index on at least 10 different sites of the specimen, and calculate a mean value. Again, keep track of which leaf surface is being observed, abaxial or adaxial, as the stomatal indices for different surfaces is frequently significantly different.

To make a cross section of a leaf or thin roots, stems, or other thin appendages, lay the appendage to be sectioned on a microscope slide. Place another microscope slide over the appendage with a portion of the tissue exposed. Using a sharp, polytef-coated razor blade that has been wetted, cut straight down along the edge of upper slide. Without moving the upper slide, cut down again with the razor blade at an angle. Some practice may be necessary for one to be able to get sections thin enough so that when they are mounted and covered with a coverslip, these sections can be used to determine tissue arrangements (for instance, the number of palisade layers in leaf, thickness of cuticle, types of trichomes, types of vascular bundles, and the like). Because razor blades dull quickly, they have to be replaced frequently.

Use the cross section of leaf tissue so obtained to determine the palisade mesophyll ratio. Alternatively, boil leaf fragments of about 2 mm² in *Chloral Hydrate Solution*, mount, cover with a coverslip, and observe under a microscope. Identify groups of four adaxial epidermal cells, and count the palisade mesophyll cells that are lying below and are at least 50% covered by the epidermal cells. This value divided by 4 is the palisade mesophyll ratio. Determine the palisade mesophyll ratio of at least 10 groups of epidermal cells, and calculate a mean value. Palisade mesophyll ratio can also be determined on powdered leaf material.

To make a cross section of thick stems, roots, or other plant parts, including woody tissues, hold the tissue in one hand and using a sharp, polytef-coated razor blade that has been wetted with water, shave a cross section from the appendage. Mount in water, another medium, or reagent solution, place a coverslip over the material, and examine under a microscope. Sections thin enough to determine vascular tissue arrangement, ray type, parenchyma distribution, presence of crystals, and the like can usually be made with a little practice.

Maceration—It is sometimes necessary, for the proper identification of a plant material, to macerate the tissue into its individual cells before microscopic examination. This can be an especially useful technique for woody or other hard tissues. The material is cut into small pieces of about 2-mm thickness and 5-mm length or sliced into pieces of about 1-mm thickness. Depending on the nature of the cell wall,

one of the following methods is used. For hard or highly lignified tissues, use *Method I*. For tissues that are not extensively lignified, use *Method II*.

Method I—

SOLUTION A—Use 4 N nitric acid solution.

SOLUTION B—Prepare a mixture of 1.2 M chromium trioxide solution and sulfuric acid (7:4).

PROCEDURE—Place the plant material in a test tube containing about 5 mL of a mixture of *Solution A* and *Solution B* (1:1). Heat in a water bath for 20 minutes. Wash the tissue repeatedly with water, and transfer to a microscope slide. Tease tissue apart with dissecting needle, add 1 to 2 drops of mountant, cover with a coverslip, and examine under a microscope. If necessary, cells can be further separated from each other by pressing down on the coverslip with a gentle, sliding motion. The macerated tissue will test negative for lignin.

Method II—

PROCEDURE—Place the plant material in a test tube containing about 5 mL of 2 M potassium hydroxide solution. Heat in a water bath for 30 minutes. Wash the tissue repeatedly with water, and transfer to a microscope slide. Add 1 to 2 drops of mountant. Place a coverslip over the tissue, press down, squashing the tissue, and examine under a microscope. The macerated tissue will test negative for lignin.

Preparation of Powdered Materials—Place one or two drops of water, another mountant, or a reagent solution in the center of a clean slide. Moisten the tip of a dissecting needle with water, and dip into the powder under test. Transfer a small amount of material that adheres to the needle into the fluid on the slide, and stir thoroughly and carefully. Cover with a clean coverslip. Because the arrangement of the tissue structures within the plant tissue has been destroyed, the important features for observation of the powdered plant material are the chemical and physical features of tissues and cell types, as well as the presence and chemical and physical features of ergastic substances. The specific tissues, cells, and ergastic substances to be examined are specified in the individual monograph.

PROCEDURE FOR THIN, PERMANENT MOUNTS

When it is necessary to reveal detailed histological features of a plant specimen, thin tissue sections have to be obtained. The sections need to be thin enough to transmit light and they have to be cut in such a plane that the desired features are exposed. The plant material is properly killed, fixed, dehydrated, and embedded in paraffin or other embedding media. The embedding medium is used as a solid-support matrix during tissue sectioning. After sectioning and mounting, staining of the specimen is frequently performed to aid in the differentiation of certain structures. [NOTE—The process of fixing, dehydration, embedding, and staining can be significantly expedited by utilizing a microwave oven specifically designed for histological work.]

Killing and Fixation—As a first step in preparing plant material for sectioning, living cells are killed, and the tissue is preserved. This is most frequently done by employing a chemical fixative. A good general purpose fixative for plant material is a mixture of formaldehyde, acetic acid, and alcohol (FAA).

FAA Solution—Mix 50 mL of alcohol, 5 mL of glacial acetic acid, 10 mL of formaldehyde solution, and 35 mL of water. [NOTE—Periodically prepare fresh solution, as it loses effectiveness with storage.]

Procedure—Completely immerse the plant material in *FAA Solution*. Allow the material to remain immersed for 18 to 24 hours at room temperature. Plant material can be kept indefinitely in *FAA Solution*, as long as it remains completely immersed and is not allowed to dry out. Certain plant tissues may require vacuum infiltration to facilitate the penetration of the fixative. Vacuum infiltration is required if the

tissue has abundant air spaces or epidermal hairs or if it floats on top of the fixative solution. Place the tissue in a small vial containing the fixative. Place the uncapped vial into a bell jar or desiccator that is connected to a vacuum source, preferably an oil-sealed vacuum pump. The vacuum is vented into a fume hood to prevent fixative vapors from filling the room. Slowly turn on the vacuum. Do not use a strong vacuum because the fixative may start to boil and damage the tissue. As residual air is pulled from the tissue, it will rise to the surface. Turn the vacuum on and off through several cycles until the tissue stays at the bottom of the container during an "on" cycle.

Tissue Dehydration—Paraffin and other embedding media are hydrophobic; thus, water must be removed from the plant tissue after fixation. This is accomplished by immersing the fixed tissue in dehydration solutions, which are a series of mixtures of alcohol and water with increasing alcohol concentration. The final solution in the series is dehydrated alcohol. Begin by washing the fixed tissue once or twice with fresh 50 percent alcohol to remove traces of FAA. Remove this solution, and subsequently remove any other dehydration solution, by decanting the solution or removing it with the aid of a glass pipet. Add the first dehydration solution (70 percent alcohol) to the vial, completely immersing the tissue. The graded alcohol-water series and the suggested times for tissue immersion are as follows.

Dehydration Solution	Time (hours)
50 percent alcohol	1–2
70 percent alcohol	1–2
90 percent alcohol	1–2
95 percent alcohol	1–2
Dehydrated alcohol containing 0.1% of safranin O	2–4
Dehydrated alcohol	1

Safranin O is added to the penultimate dehydration solution in the series to visualize the tissue when it is has become embedded in paraffin. If the tissue to be sectioned is hard or woody, the time for each step in the series may need to be increased to up to 24 hours. If necessary, the tissue can be stored for several days in 70 percent alcohol or in solutions of even higher alcohol concentrations.

Embedding—

Preparation for Embedding—

ALCOHOL REMOVAL—Paraffin is the most common embedding medium, although other embedding media are available. After dehydration, alcohol is removed from the tissue by using a graded series of dehydrated alcohol-xylene solutions, because paraffin is not soluble in alcohol. The graded dehydrated alcohol-xylene series and the suggested times for tissue immersion are as follows.

Alcohol Removal Solution	Time (hours)
A mixture of dehydrated alcohol and xylene (3:1)	1
A mixture of dehydrated alcohol and xylene (1:1)	1
A mixture of dehydrated alcohol and xylene (1:3)	1
Xylene	1
Xylene	1

XYLENE REMOVAL—Once xylene has completely replaced alcohol, paraffin is added slowly to infiltrate the tissue and remove xylene. Proceed as follows:

- For each mL of xylene add about 1 paraffin chip to the tissue vial, cap, and allow to stand at room temperature for 4 hours. Add additional paraffin chips until no more chips dissolve.

2. Place tissue in an oven maintained at 42° to 45°. Add 2 to 3 paraffin chips every hour until no more chips dissolve at that temperature.
3. Pour off one-third of the volume, and replace with an equal volume of melted paraffin. Do not cap, and transfer vial to an oven maintained at 58° to 60°.
4. After the paraffin remelts (about 4 hours later) pour off one-half of the volume, and replace with equal volume of melted paraffin. Transfer vial to the oven maintained at 58° to 60° if paraffin begins to solidify.
5. Repeat step 4 twice more, then pour off the entire volume of paraffin-xylene. Replace with pure melted paraffin. About 4 hours later, pour off paraffin, and replace with fresh pure melted paraffin. Repeat the pouring off and replacement 4 hours later, and allow to stand overnight. [NOTE—Transfer vial to the oven maintained at 58° to 60° if paraffin begins to solidify at any point.]

Embedding Procedure—Pour the tissue with the paraffin to an embedding boat. Paraffin has to completely cover the tissue by about 3 to 5 mm. Place the embedding boat on top of a preheated warming platform that is designed for histology work. Adjust the tissue in the boat to its proper orientation for sectioning. Slowly cool the paraffin by sliding the boat down to the cool side of the platform until the paraffin has solidified. Immerse the paraffin block in ice water to rapidly cool the block and to prevent paraffin crystals from forming. Store the paraffin block at 4°.

Sectioning and Mounting—Cut the paraffin block into pieces, each containing one tissue sample. Trim the paraffin block, as close to the tissue mass as possible, to form a rectangle or a slight trapezoid. Such trimming will prevent sectioning problems due to excess paraffin around the tissue. To make transverse sections, orient the tissue at a right angle to a wooden tissue block of which the face has been soaked in melted paraffin. Affix the paraffin block to the face of the tissue block. Add a small amount of melted paraffin to the base of the paraffin block to help form a tighter seal. Cool the block to 4°.

Properly mount and adjust the tissue and paraffin block in a microtome. Use a sharp stainless-steel microtome knife that has been properly honed. Set the microtome to cut sections 8 to 15 µm thick (10-µm thickness is optimal for most tissues). Cut individual or serial sections. Prepare a microscope slide as follows. An adhesive may be prepared as a solution containing 1% of gelatin and 0.5% of sodium benzoate that is heated to 30° to 35° to dissolve the gelatin. Smear a thin film of the adhesive so obtained onto the slide, allow to dry, rinse with a 4% solution of formaldehyde TS, and add a small amount of water. Plate the cut sections upside down on the slide, so that they float on water, and flood with a 4% solution of formaldehyde TS. The sections will immediately spread out and wrinkles will disappear.

Place the slide on a warming platform, maintained at 42°, to relax the sections. Pipet, and blot the excess water and formaldehyde solution. Dry overnight in an oven at 42° to ensure adherence of the tissue section to the slide.

Staining—

Preparation for Staining—Immerse the microscope slide with the affixed tissue twice into xylene, each time for 10 to 15 minutes, to remove paraffin. Then immerse the slide into several solutions, leaving it in each solution for 5 minutes and taking care not to dislodge the tissue, the following sequence of solutions being used: a mixture of dehydrated alcohol and xylene (1:1), dehydrated alcohol, alcohol, and a 70 percent alcohol solution. The tissue is bleached prior to staining if it is opaque because of the presence of tannins or other ergastic materials. To bleach, dip the slide into a 1% potassium permanganate solution for 1 minute, rinse with water, dip into a 5% oxalic acid solution for 1 minute, and rinse thoroughly with water. The material is ready for staining. One of the following two staining procedures is recommended for most botanical identification work. The first staining procedure uses safranin O counterstained with fast

green. An alternative staining procedure uses safranin O counterstained with orange G.

Safranin O—Fast Green Staining—

SAFRANIN O STAINING SOLUTION—Prepare a mixture of methoxy-ethanol, dehydrated alcohol, water, and formaldehyde solution (50:25:25:2). Add a sufficient quantity of sodium acetate to obtain a solution containing 1% of sodium acetate, and mix. Add a sufficient quantity of safranin O to obtain a solution containing 1% of safranin O, and mix.

FAST GREEN STAINING SOLUTION—Prepare a mixture of methoxy-ethanol, dehydrated alcohol, and methyl salicylate (1:1:1) containing 0.05% of fast green FCF.

PROCEDURE—Once the tissue has been rehydrated to 70 percent alcohol as described under *Preparation for Staining*, immerse for 2 to 24 hours, depending on the tissue, in *Safranin O Staining Solution*. Remove excess stain by immersing the slide in water several times. Transfer slide to an alcohol solution containing 0.5% of picric acid for 2 to 10 seconds to further remove excess stain from the section and to assist in differentiation of the tissue structures. To stop the action of the picric acid, transfer slide for 10 seconds to 1 minute to an alcohol solution containing 4 drops of ammonium hydroxide in each 100 mL of alcohol. Transfer slide to dehydrated alcohol for 10 seconds. Visually inspect the stained tissue under a microscope to see if further destaining with picric acid is necessary. Counterstain for 10 to 15 seconds in *Fast Green Staining Solution*. Transfer slide through two changes of a mixture of methyl salicylate, dehydrated alcohol, and xylene (2:1:1), each change lasting for 5 to 10 seconds. Then transfer slide to a mixture of xylene and dehydrated alcohol (95:5) for 1 minute. Transfer through two changes of xylene. Store in xylene until ready to mount the coverslip. Chromosomes, nuclei, and lignified, cutinized, or suberized cell walls will be stained red. Cytoplasm and cellulosic cell walls will be stained green to blue, depending on the pH of the tissue.

Safranin O—Orange G Staining—

SAFRANIN O STAINING SOLUTION—Prepare a 0.004% solution of safranin O.

ORANGE G STAINING SOLUTION—Dissolve 2 g of orange G, 5 g of tannic acid, and 4 drops of hydrochloric acid in water, and dilute with water to 100 mL.

PROCEDURE—Once the tissue has been rehydrated to 70 percent alcohol as described under *Preparation for Staining*, sequentially transfer slide through the following series of solutions.

Solution	Time
35 Percent alcohol	5 minutes
A filtered 2% zinc chloride solution	1 minute
Water	5 seconds
<i>Safranin O Staining Solution</i>	5 minutes
Water	5 seconds
<i>Orange G Staining Solution</i>	1 minute
Water	5 seconds
A filtered 5% tannic acid solution	5 minutes
Water	3 seconds
A 1% ferric ammonium sulfate solution	2 minutes
Water	15 seconds
45 Percent alcohol	10 seconds
90 Percent alcohol	10 seconds
Dehydrated alcohol	10 seconds
A mixture of dehydrated alcohol and xylene (1:1)	1 to 2 minutes

Finally, store in xylene until ready to mount the coverslip. Cellulosic cell walls will stain blue-black, nuclei will stain yellow, starch grains will stain black, and lignified cell walls will stain red.

Mounting the Coverslip—The mounting of a coverslip over the tissue completes the preparation of the slide. Canada balsam, diluted with a small portion of xylene, can be used as an adhesive. Other mountants are also commercially available. Upon drying of the mountant, the slide can then be examined under a microscope. The entire process of making permanent microscope slides can be expected to take 5 or more days.

Scanning Electron Microscopy—Botanicals in commerce are often encountered in the form of powder or in pieces, thus making authentication by routine method of cross-sectioning of the article difficult and often impossible. Structures such as xylem vessels and trachids may be broken into smaller bits making detection of pitting and lignifications on the walls difficult if not impossible using an optical microscope. Structures which are resistant to these processes are most useful in identification. Scanning electron microscopy (SEM) is useful for characterizing the size and morphology of microscopic specimens. The more detailed differential characteristics in the structure of trichomes, peculiar elements in the epidermis, along with superficial granular material containing specific compounds, can be observed and identified with SEM, which assists in the identification of particular species. SEM has been used extensively to investigate surface topology of a wide variety of plant materials. It can play a vital role in authentication of an entire botanical, those in powder form, distinguishing between closely related species, and can be used to examine a mixture of powders.

Introduction and general information about SEM as applied to pharmacopeial articles can be found in USP general chapter *Scanning Electron Microscopy* (1181).

SEM produces a higher resolution compared to that possible using an optical microscope, and the images obtained are three-dimensional. SEM has the advantage of providing images with large depth of field, which allows a substantial thickness of the sample to be in focus at one time. It allows the analysis of specimens as large as 50 mm, making it possible to produce detailed topographical electron micrographs of an object clearly visible to the naked eye. The maximum resolution for SEM (minimum distance by which the two objects can be separated and observed as distinct objects) is 10 to 20 nm compared to 200 to 300 nm for optical microscopy. Typical SEM magnification ranges from $\times 10$ to $\times 300,000$. Commercial SEM instruments also are available with magnifications as low as $\times 5$ and as high as $\times 2,000,000$. In comparison, typical modern optical microscopes have a magnification range of $\times 10$ to $\times 2000$. At low magnification, images obtained with SEM provide more information than those of optical microscopy. SEM can produce images for which contrast is based on compositional variations of specimens.

CHEMICAL IDENTIFICATION

To help ensure the authenticity of the article, chemical identification is performed in conjunction with botanical identification outlined above. Chemical identification typically employs chromatographic procedures to detect the presence of marker compounds specified in the individual monograph. Spectroscopic or chromatographic profiles can be used to achieve chemical identification by fingerprint comparison against that of a reference sample or standard. Examples of spectroscopic method include UV, IR, and Fourier transformed IR (see *Spectrophotometric Identification Tests* (197)). Examples of chromatographic methods include high-pressure liquid chromatography (HPLC), thin-layer chromatography (TLC), 2-dimensional-TLC, and gas chromatography (see *Chromatography* (621)). Analytical methods used for fingerprinting should be capable of detecting as many chemical constituents as possible. Multiple fingerprints, using a combination of analytical methods with different separation principles and test conditions, may be useful. In addition to the spectroscopic chromatographic

methods, qualitative wet-chemistry methods may also be specified in the individual monograph.

Chemotaxonomy

Chemotaxonomy is the classification of the plants based on their chemical constituents and it may be useful in botanical articles identification. Metabolic compounds found within plant tissues can be divided into two broad categories based on their functions. The first category comprises primary metabolites—metabolites involved in the physiological plant processes that are absolutely necessary for life and ubiquitous throughout the plant kingdom. These processes include photosynthesis, respiration, and nucleic acid, protein, carbohydrate, and lipid metabolism. The second category comprises secondary metabolites—compounds that are thought not to be absolutely necessary for plant processes, although they may have important functions in the plant's interactions with other organisms, such as allelopathic interactions; in chemical defense against herbivores and plant pathogens; and in signaling to attract pollinating and seed-dispersing animals. Many secondary metabolites are known to have pharmacological activity. They are also the basis for the chemotaxonomy of plants. Secondary metabolites fall into several different chemical classes such as nonprotein amino acids, flavonoids, xanthenes, coumarins, polyacetylenes, cyclic polyketides, monoterpenes, sesquiterpenes, iridoids, triterpenes, sterols, nitrogen-containing terpenes, and alkaloids. These chemical classes are not ubiquitous throughout the plant kingdom, but tend to be specific to certain botanical classes, orders, and families. Moreover, many chemical subclasses and individual secondary compounds are specific to certain subfamilies, genera, or species. It is these chemical subclasses and individual compounds that can be used as marker compounds to aid in the proper identification of plant material.

Active Principles and Marker Compounds

For chemical identification of botanical articles, extracts are prepared. Such extracts are usually complex mixtures of several chemical constituents. For a large majority of botanical extracts it is not known with certainty which of the various components is responsible for the reported pharmacological effect. It is generally believed that several constituents act synergistically to provide the reported effect. For articles for which compendial monographs are provided, certain chemical constituents of the article are chosen and quantitative test procedures for determining their content are provided. The choice of such constituents, known generally as marker compounds, is based on certain considerations. Currently, the following types of marker compounds are specified in compendial monographs and may be identified in raw materials:

Active Principles—These are constituents that have proven clinical activity. A minimum content or range for the active principles is usually specified in the individual monograph. A quantitative determination of active principles during stability studies of botanical dosage forms provides necessary information for arriving at suitable expiration dates.

Active Markers—These are constituents that have known pharmacological activity contributing in some extent to efficacy. However, the clinical efficacy for these constituents may not be proven. A minimum content or range for active markers is usually specified in individual monographs. A quantitative determination of active markers during stability studies of botanical dosage forms provides necessary information for arriving at suitable expiration dates.

Analytical Markers—Where neither defined active principles nor active markers are known, other constituents of the botanical extract amenable to quantitative determination are chosen. These markers aid in the positive identification of the article under test. In addition, maintaining a minimum

content or a specified range of the analytical markers helps to achieve standardization of the plant extract and to arrive at a suitable expiration date during stability studies.

Negative Markers—These are constituents that may have allergenic or toxic properties, rendering their presence in the botanical extract undesirable. For example ginkgolic acids from ginkgo belong to this category. A stringent limit for these negative markers may be specified in individual monographs.

Use of USP Reference Articles

Reference articles are used to assist in the identification of marker compounds within the test article. Reference articles are either USP Authenticated Reference Materials or USP Reference Standards (see *USP Reference Standards* (11)), whichever is specified in the individual monograph. USP Reference Standards used to identify marker compounds in the test articles may be a single purified chemical entity, a mixture of purified chemical entities, or a standardized extract prepared from the authenticated plant article. USP Reference Standards may also be used to quantitate marker compounds, as specified in the individual monograph.

A pulverized test article undergoes a specified extraction procedure (see *Methods of Extraction* under *Botanical Extracts* (565)) and is prepared for chromatographic or wet-chemistry analysis. If a USP Authenticated Reference Material is available, then it undergoes the same extraction procedure as the test article. The test preparation and reference articles then undergo the same chromatographic or wet-chemistry procedure specified in the individual monograph. The response of the test preparation is compared to the response of reference articles to determine the presence of the marker compounds in the test article.

(565) BOTANICAL EXTRACTS

In the extraction practice for articles of botanical origin, the constituents of interest are completely or partially separated from other components with the aid of water, alcohol, alcohol-water mixtures, or other suitable solvents. This extraction process involves the removal of the desired constituents from the plant matter with suitable menstrua, the evaporation of all or nearly all of the solvent, and the adjustment of the residual fluids, masses, or powders to the prescribed standards. Suitable inert substances may be added as carriers or diluents to improve physical characteristics. Suitable antimicrobials and other preservatives may be added to preserve the integrity. Extracts may be subjected to processes that increase the content of characterized constituents, decrease the content of unwanted constituents, or both. Extracts with no added inert substances and no processing beyond the extraction are called native extracts. In some preparations, the plant matter may be pretreated by inactivation of enzymes and microbial contaminants, grinding, defatting, or a similar procedure.

Extracts may be defined as preparations with liquid, solid, or semisolid consistency. The products obtained by extraction are fluidextracts, powdered extracts, semisolid extracts, and tinctures.

METHODS OF EXTRACTION

Percolation

In the manufacture of extracts, percolation is a commonly used method. The crude material being extracted is reduced to pieces of suitable size, if necessary, then mixed thoroughly with a portion of the specified solvent, and allowed to stand for about 15 minutes. The mixture is transferred to a percolator, sufficient amount of the specified solvent is added to cover the entire solid mass, and the mixture is allowed to percolate slowly (at a rate of not more than 1 mL per minute for 1000 g of material), the matter to be extracted being always covered with a layer of solvent. The residue may be pressed, and the obtained fluid is combined with the percolate. The entire percolates are concentrated, generally by distillation under reduced pressure, so as to subject the constituents of interest in the article under extraction to as little heat as possible.

Maceration

Unless otherwise specified, the crude material being extracted is reduced to pieces of suitable size, mixed thoroughly with the specified extracting solvent, and allowed to stand at room temperature in a closed container for an appropriate time, with frequent agitation until soluble matter is dissolved. The mixture is filtered, the insoluble material is washed with the same solvent used for maceration, and the filtrates are combined and concentrated, usually under reduced pressure, to the desired consistency.

PREPARATIONS

Fluidextracts

FLUIDEXTRACTS, also known as liquid extracts, are preparations of plant matter, containing alcohol as a solvent or as a preservative, or both, and are so made that each mL contains the extracted constituents of 1 g of the crude material that it represents, unless otherwise specified in the individual monograph. They may be prepared from suitable extracts and may contain suitable antimicrobial or other preservatives.

Pharmacopeial fluidextracts are made by percolation, often following a period of maceration. The required solvent is specified in the individual monograph. The common manufacturing procedure includes concentration of the more diluted portion of percolate by evaporation or distillation under vacuum at temperatures below 60°. The time of maceration and the rate of flow during percolation may be varied to adjust for the quantity and nature of the crude material under extraction, provided that the composition of the extracted constituents of interest is not adversely affected.

The rate of flow of the percolate can be slow, moderate, or rapid. With reference to the extraction of 1000 g of the starting material, at a slow rate, not more than 1 mL of percolate is produced per minute; at a moderate rate, between 1 and 3 mL per minute is produced; and at a rapid rate, between 3 and 5 mL per minute is produced. A fluidextract that tends to deposit sediment may be aged and filtered, or the clear portion may be decanted, provided that the resulting clarified liquid conforms to the Pharmacopeial standards.

Powdered Extracts

POWDERED EXTRACTS are solid preparations having a powdery consistency obtained by evaporation of the solvent

used for extraction. They may contain suitable added substances such as excipients, stabilizers, and preservatives. Standardized powdered extracts are adjusted to the defined content of constituents, using suitable inert materials or a powdered extract of the plant matter used for preparation. Where applicable, a limit for the solvent used for extraction is specified in the individual monograph.

Semisolid Extracts

SEMISOLID EXTRACTS, also known as soft extracts or pillular extracts, are preparations having consistencies between those of fluidextracts and those of powdered extracts, and are obtained by partial evaporation of the solvent, water, alcohol, or hydroalcoholic mixtures being used as extracting solvents. They may contain suitable antimicrobial or other preservatives. A semisolid extract and a powdered extract obtained from the same material are interchangeable as drugs or as supplements, but each has its own advantages.

General Pharmacopeial Requirements

Unless otherwise specified in the individual monographs, Pharmacopeial requirements for the fluidextracts, powdered extracts, and semisolid extracts are as follows.

Packaging and Storage—Store in tight, light-resistant containers. [NOTE—See *Preservation, Packaging, Storage, and Labeling* under *General Notices and Requirements*.]

Labeling—Label it to indicate the name of the plant part used; the names of solvents, other than the hydroalcoholic solvents, used in preparation; the content, in percentage, of active principles or marker compounds identified in the individual monograph; and the name and concentration of any added antimicrobial or other preservative. Where active principles are unknown, the ratio of starting material to final product is stated. For semisolid extracts and powdered extracts, the identity and quantity of any added excipient is also indicated. In such cases the percentage of native extract may also be stated.

Residue on Evaporation—Transfer promptly about 2 mL, accurately measured, of Fluidextract, about 0.5 g of Powdered Extract, or about 2 g of Semisolid Extract to a suitable tared, round-bottom flask. Evaporate to dryness on a water bath, and dry the residue at 100° to 105° for 3 hours. Allow to cool in a desiccator over phosphorus pentoxide, and determine the weight of the residue obtained: not less than 95% of Powdered Extract specimen remains as residue; or not less than 70% of Semisolid Extract specimen remains as residue. [NOTE—Limits for Fluidextracts are specified in the individual monographs.]

Residual Solvents—If prepared with solvents other than alcohol, water, or alcohol-water mixtures, it meets the requirements for *Residual Solvents* (467). [NOTE—See ICH document *Impurities: Residual Solvents* for related information.]

Pesticide Residues—Proceed as directed under *Articles of Botanical Origin* (561): meets the requirements.

Heavy Metals, Method II (231): 20 µg per g.

Alcohol Content, Method II (611) (if present): between 90% and 110% of the labeled amount of C₂H₅OH is found in Fluidextract and Semisolid Extract.

Tinctures

TINCTURES are liquid preparations usually prepared by extracting plant materials with alcohol or hydroalcoholic mixtures. Traditionally, tinctures of potent articles of botanical

origin represent the activity of 10 g of the drug in each 100 mL of tincture, the strength being adjusted following the test for content of active principles or marker compounds. Most other plant tinctures represent 20 g of the respective plant material in each 100 mL of tincture.

Different tinctures are not always diluted to obtain the same ratio of starting plant material to final tincture. This ratio will depend on the requirements prescribed in the specific tests for content of active principles or marker compounds included in the individual monographs. As tinctures are being prepared, they are assayed in accordance with these content tests. Using the values obtained from such assays, the final concentration of a tincture is adjusted by adding more solvent or by evaporating part of the solvent.

Unless otherwise specified, tinctures are usually prepared from coarse powder or fine cuttings of plant materials either by a percolation process or a maceration process.

PERCOLATION PROCESS

Carefully mix the ground mixture of ingredients with a sufficient quantity of the prescribed extracting solvent to render it evenly and distinctly damp, allow it to stand for 15 minutes, transfer it to a suitable percolator, and pack the mass firmly. Pour on enough of the specified extracting solvent to saturate the drug, and cover the top of the percolator. When the liquid is about to drip from the percolator, close the lower orifice, and allow the drug to macerate for 24 hours or for the time specified in the monograph. If the test for content of active principles or marker compounds is not required in the individual monograph, allow the percolation to proceed slowly or at the specified rate (for definitions of flow rates, see under *Fluidextracts*), gradually adding sufficient quantity of extracting solvent to produce 1000 mL of tincture, and mix. If a test for content of active principles or marker compounds is required, collect only 950 mL of percolate, mix, and test a portion of it as directed in the individual monograph. Dilute the remainder of the percolate with as much of the prescribed extracting solvent as calculation from the content test indicates is necessary to produce a tincture that conforms to the requirements, and mix.

MACERATION PROCESS

Macerate the drug with 750 mL of the prescribed extracting solvent in a closed container, and put in a warm place. Agitate it frequently during 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter. When most of the liquid has drained, wash the residue on the filter with a sufficient quantity of the prescribed extracting solvent, combining the filtrates, to produce 1000 mL of tincture, and mix.

GENERAL PHARMACOPEIAL REQUIREMENTS

Unless otherwise specified in the individual monographs, Pharmacopeial requirements for the tinctures are as follows.

Packaging and Storage—Store in tight, light-resistant containers, and avoid exposure to direct sunlight and excessive heat. [NOTE—See *Preservation, Packaging, Storage, and Labeling* under *General Notices and Requirements*.]

Labeling—Label it to indicate the name of the plant part used for preparation; the name of the solvent or solvent mixture used for extraction; and the content of the constituents of interest and the ratio of starting material to final product.

(571) VITAMIN A ASSAY

CHEMICAL METHOD

The following procedure is provided for the determination of vitamin A as an ingredient of Pharmacopeial preparations. It conforms to that which was adopted in 1956 for international use by the International Union of Pure and Applied Chemistry.

Complete the assay promptly, and exercise care throughout the procedure to keep to a minimum the exposure to actinic light and to atmospheric oxygen and other oxidizing agents, preferably by the use of low-actinic glassware and an atmosphere of an inert gas.

Special Reagents—

ETHER—Use ethyl ether, and use it within 24 hours after opening the container.

ISOPROPYL ALCOHOL—Use spectrophotometric-grade isopropyl alcohol (see *Isopropyl Alcohol* under *Reagent Specifications* in the section *Reagents, Indicators, and Solutions*).

Procedure—Accurately weigh, count, or measure a portion of the test specimen expected to contain the equivalent of not less than 0.15 mg of retinol but containing not more than 1 g of fat. If in the form of capsules, tablets, or other solid, so that it cannot be saponified efficiently by the ensuing instructions, reflux the portion taken in 10 mL of water on a steam bath for about 10 minutes, crush the remaining solid with a blunt glass rod, and warm for about 5 minutes longer.

Transfer to a suitable borosilicate glass flask, and add 30 mL of alcohol, followed by 3 mL of potassium hydroxide solution (9 in 10). Reflux in an all-borosilicate glass apparatus for 30 minutes. Cool the solution, add 30 mL of water, and transfer to a conical separator. Add 4 g of finely powdered sodium sulfate decahydrate. Extract by shaking with one 150-mL portion of ether for 2 minutes, and then, if an emulsion forms, with three 25-mL portions of ether. Combine the ether extracts, if necessary, and wash by swirling gently with 50 mL of water. Repeat the washing more vigorously with three additional 50-mL portions of water. Transfer the washed ether extract to a 250-mL volumetric flask, add ether to volume, and mix.

Evaporate a 25.0-mL portion of the ether extract to about 5 mL. *Without applying heat and with the aid of a stream of inert gas or vacuum*, continue the evaporation to about 3 mL. Dissolve the residue in sufficient isopropyl alcohol to give an expected concentration of the equivalent of 3 µg to 5 µg of vitamin A per mL or to give an absorbance in the range 0.5 to 0.8 at 325 nm. Determine the absorbances of the resulting solution at the wavelengths 310 nm, 325 nm, and 334 nm, with a suitable spectrophotometer fitted with matched quartz cells, using isopropyl alcohol as the blank.

WHEN TOCOPHEROL IS PRESENT—Transfer to a suitable borosilicate glass flask a test specimen, accurately measured, or not less than 5 previously crushed capsules or tablets. Reflux in an all-borosilicate glass apparatus with 30 mL of alcohol and 3 mL of potassium hydroxide solution (9 in 10) for 30 minutes. Add through the condenser 2.0 g of citric acid monohydrate, washing the walls of the condenser with 10 mL of water. Cool, and transfer the solution to a conical separator with the aid of 20 mL of water. Add 4 g of finely powdered sodium sulfate decahydrate. Extract with one 150-mL portion of ether and then, if an emulsion forms, with three 25-mL portions of ether. Combine the ether extracts, if necessary, and wash by swirling gently with 50 mL of water. Repeat the washing more vigorously with three additional 50-mL portions of water. Transfer the washed ether extract to a 250-mL volumetric flask, and add ether to volume. Transfer a 100.0-mL aliquot of the resulting ether solution to

a conical separator, and wash once with 50 mL of potassium hydroxide solution (1 in 33), using alcohol, if necessary, to break any emulsion that forms. Wash by swirling gently with 50 mL of water. Repeat the washing more vigorously with three additional 50-mL portions of water. Transfer the washed ether extract to a 100-mL volumetric flask, add ether to volume, and mix.

Evaporate a 50.0-mL aliquot of the ether solution of the unsaponifiable extract to about 5 mL. *Without applying heat and with the aid of a stream of inert gas or vacuum*, remove the residual ether. Dissolve the residue in 50.0 mL of isopropyl alcohol.

Hydrogenated Portion—Pipet 15.0 mL of the isopropyl alcohol solution into a 50-mL centrifuge tube, add approximately 200 mg of palladium catalyst, stir with a glass rod, and hydrogenate for 10 minutes in a *Hydrogenator* such as is described in the *Alpha Tocopherol Assay* (551), using isopropyl alcohol in the blank tube. Add about 300 mg of chromatographic siliceous earth, stir with a glass rod, and immediately centrifuge until the solution is clear.

Test a 1-mL aliquot of the solution by removing the solvent by evaporation, dissolving the residue in 1 mL of chloroform, and adding 10 mL of phosphomolybdic acid TS: no detectable blue-green color appears. [NOTE—If a blue-green color appears, repeat the hydrogenation for a longer time period, or with a new lot of catalyst.]

Into two separate flasks pipet equal volumes of the *Hydrogenated Portion* and the untreated isopropyl alcohol solution, respectively, and add sufficient isopropyl alcohol to give an expected concentration of vitamin A equivalent to 3 µg to 5 µg per mL. Determine the absorbances of the untreated solution against the solution from the *Hydrogenated Portion* as a blank, at the wavelengths 310 nm, 325 nm, and 334 nm, with a suitable spectrophotometer fitted with matched quartz cells.

Calculation—Calculate the vitamin A content as follows:

$$\text{Content (in mg)} = 0.549A_{325}/LC$$

in which A_{325} is the observed absorbance at 325 nm; L is the length, in cm, of the absorption cell; and C is the amount of test specimen expressed as g, capsule, or tablet in each 100 mL of the final isopropyl alcohol solution, provided that A_{325} has a value not less than $[A_{325}]/1.030$ and not more than $[A_{325}]/0.970$, where $[A_{325}]$ is the corrected absorbance at 325 nm and is given by the equation:

$$[A_{325}] = 6.815A_{325} - 2.555A_{310} - 4.260A_{334}$$

in which A designates the absorbance at the wavelength indicated by the subscript.

Where $[A_{325}]$ has a value less than $A_{325}/1.030$, apply the following equation:

$$\text{Content (in mg)} = 0.549[A_{325}]/LC$$

in which the values are as defined herein. Each mg of vitamin A (alcohol) represents 3333 USP Units of vitamin A.

Confidence Interval—The range of the limits of error, indicating the extent of discrepancy to be expected in the results of different laboratories at $P = 0.05$, is approximately $\pm 8\%$.

CHROMATOGRAPHIC METHOD

The following pressurized liquid chromatographic procedure is provided for the determination of Vitamin A. Where the use of vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the raw material. Use low-actinic glassware throughout this procedure.

USP Reference Standards (11)—[NOTE—Use USP Vitamin A RS, all-*trans* retinyl acetate, for assaying pharmaceutical

dosage forms that are labeled to contain retinol or vitamin A ester (retinyl acetate or retinyl palmitate).]

Mobile Phase—Use *n*-hexane.

System Suitability Preparation—Dissolve an accurately weighed quantity of retinyl palmitate and USP Vitamin A RS in *n*-hexane to obtain a solution containing about 7.5 µg per mL of each.

Standard Preparation—Dissolve an accurately weighed quantity of USP Vitamin A RS in *n*-hexane, and dilute quantitatively, and stepwise if necessary, to obtain a solution having a known concentration of about 15 µg of retinyl acetate per mL.

Assay Preparation—Transfer about 15 mg of vitamin A ester (retinyl acetate or retinyl palmitate), accurately weighed, to a 100-mL volumetric flask, dissolve in and dilute with *n*-hexane to volume, and mix. Pipet 5.0 mL of this solution into a 50-mL volumetric flask, dilute with *n*-hexane to volume, and mix.

Chromatographic System (see *Chromatography* (621))—The liquid chromatograph is equipped with a 325-nm detector and a 4.6-mm × 15-cm column that contains packing L8. The flow rate is about 1 mL per minute. Chromatograph the *System Suitability Preparation*, and record the peak responses as directed for *Procedure*: the resolution, *R*, between retinyl acetate and retinyl palmitate is not less than 10; and the relative standard deviation for replicate injections is not more than 3.0%.

Procedure—Separately inject equal volumes (about 40 µL) of the *Standard Preparation* and the *Assay Preparation* into the chromatograph, record the chromatograms, and measure the responses for retinyl acetate obtained from the *Standard Preparation* and the peak area for retinyl acetate or retinyl palmitate in the chromatogram of the *Assay Preparation*. Calculate the quantity, in mg, of vitamin A as the retinol equivalent (C₂₀H₃₀O) in the portion of vitamin A taken by the formula:

$$0.872CD(r_U/r_S)$$

in which 0.872 is the factor used to convert retinyl acetate, obtained from USP Vitamin A RS to its retinol equivalent; *C* is the concentration, in mg per mL, of USP Vitamin A RS in the *Standard Preparation*; *D* is the dilution factor, in mL, for the *Assay Preparation*; and *r_U* and *r_S* are the peak responses of the retinyl ester obtained from the *Assay Preparation* and the *Standard Preparation*, respectively. [NOTE—The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

(581) VITAMIN D ASSAY

Chromatographic Method

The following pressurized liquid chromatographic procedure is provided for the determination of vitamin D, as cholecalciferol or as ergocalciferol, as an ingredient of Pharmaceutical multiple-vitamin preparations.

Throughout this assay, protect solutions containing, and derived from, the test specimen and the Reference Standard from the atmosphere and light, preferably by the use of a blanket of inert gas and low-actinic glassware.

USP Reference Standards (11)—[NOTE—Use *USP Ergocalciferol RS*, or *USP Cholecalciferol RS*, for assaying pharmaceutical dosage forms that are labeled to contain vitamin D as ergocalciferol, or as cholecalciferol, respectively.] *USP Chole-*

calciferol RS. *USP Δ^{4,6}-Cholestadienol RS*. *USP Ergocalciferol RS*. *USP Vitamin D Assay System Suitability RS*.

Special Reagents and Solutions—

Ether—Use ethyl ether. Use within 24 hours after opening container.

Dehydrated Hexane—Prepare a chromatographic column by packing a chromatographic tube, 60 cm × 8 cm in diameter, with 500 g of 50- to 250-µm chromatographic siliceous earth, activated by drying at 150° for 4 hours (see *Column Adsorption Chromatography* under *Chromatography* (621)). Pass 500 mL of hexanes through the column, and collect the eluate in a glass-stoppered flask.

Butylated Hydroxytoluene Solution—Dissolve a quantity of butylated hydroxytoluene in chromatographic hexane to obtain a solution containing 10 mg per mL.

Aqueous Potassium Hydroxide Solution—Dissolve 500 g of potassium hydroxide in 500 mL of freshly boiled water, mix, and cool. Prepare this solution fresh daily.

Alcoholic Potassium Hydroxide Solution—Dissolve 3 g of potassium hydroxide in 50 mL of freshly boiled water, add 10 mL of alcohol, dilute with freshly boiled water to 100 mL, and mix. Prepare this solution fresh daily.

Sodium Ascorbate Solution—Dissolve 3.5 g of ascorbic acid in 20 mL of 1 N sodium hydroxide. Prepare this solution fresh daily.

Sodium Sulfide Solution—Dissolve 12 g of sodium sulfide in 20 mL of water, dilute with glycerin to 100 mL, and mix.

Mobile Phase A—Prepare a mixture of acetonitrile, methanol, and water (25:25:1). The amount of water and the flow rate may be varied to meet system suitability requirements.

Mobile Phase B—Prepare a 3 in 1000 mixture of *n*-amyl alcohol in *Dehydrated Hexane*. The ratio of components and the flow rate may be varied to meet system suitability requirements.

Internal Standard Solution—Transfer 15 mg of USP Δ^{4,6}-Cholestadienol RS, accurately weighed, to a 200-mL volumetric flask, add a 1 in 10 mixture of toluene and *Mobile Phase B* to volume, and mix.

Standard Preparation—Transfer about 25 mg of USP Ergocalciferol RS or Cholecalciferol RS, accurately weighed, to a 50-mL volumetric flask, dissolve without heat in toluene, add toluene to volume, and mix. Pipet 10 mL of this stock solution into a 100-mL volumetric flask, dilute with toluene to volume, and mix. Prepare stock solution fresh daily.

Assay Preparation—

For oily solutions—Accurately weigh a portion of the specimen to be assayed, preferably more than 0.5 g and equivalent to about 125 µg of cholecalciferol or ergocalciferol (5000 USP Units). Add 1 mL of *Sodium Ascorbate Solution*, 25 mL of alcohol, and 2 mL of *Aqueous Potassium Hydroxide Solution*, and mix.

For capsules or tablets—Reflux not less than 10 capsules or tablets with a mixture of 10 mL of *Sodium Ascorbate Solution* and 2 drops of *Sodium Sulfide Solution* on a steam bath for 10 minutes, crush any remaining solids with a blunt glass rod, and continue heating for 5 minutes. Cool, add 25 mL of alcohol and 3 mL of *Aqueous Potassium Hydroxide Solution*, and mix.

For dry preparations and aqueous dispersions—Accurately weigh a portion of the specimen to be assayed, preferably more than 0.5 g and equivalent to about 125 µg of cholecalciferol or ergocalciferol (5000 USP Units). Add, in small quantities and with gentle swirling, 25 mL of alcohol, 5 mL of *Sodium Ascorbate Solution*, and 3 mL of *Aqueous Potassium Hydroxide Solution*.

SAPONIFICATION AND EXTRACTION—Reflux the mixture prepared from the specimen to be assayed on a steam bath for 30 minutes. Cool rapidly under running water, and transfer the saponified mixture to a conical separator, rinsing the saponification flask with two 15-mL portions of water,

10 mL of alcohol, and two 50-mL portions of ether. Shake the combined saponified mixture and rinsings vigorously for 30 seconds, and allow to stand until both layers are clear. Transfer the aqueous phase to a second conical separator, add a mixture of 10 mL of alcohol and 50 mL of solvent hexane, and shake vigorously. Allow to separate, transfer the aqueous phase to a third conical separator, and transfer the hexane phase to the first separator, rinsing the second separator with two 10-mL portions of solvent hexane, adding the rinsings to the first separator. Shake the aqueous phase in the third separator with 50 mL of solvent hexane, and add the hexane phase to the first separator. Wash the combined ether-hexane extracts by shaking vigorously with three 50-mL portions of *Alcoholic Potassium Hydroxide Solution*, and wash with 50-mL portions of water vigorously until the last washing is neutral to phenolphthalein. Drain any remaining drops of water from the combined ether-hexane extracts, add 2 sheets of 9-cm filter paper, in strips, to the separator, and shake. Transfer the washed ether-hexane extracts to a round-bottom flask, rinsing the separator and paper with solvent hexane. Combine the hexane rinsings with the ether-hexane extracts, add 5.0 mL of *Internal Standard Solution* and 100 μ L of *Butylated Hydroxytoluene Solution*, and mix. Evaporate to dryness in vacuum by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve the residue in 5.0 mL of a mixture of equal volumes of acetonitrile and methanol, or in a measured portion of the acetonitrile-methanol mixture until the concentration of vitamin D is about 25 μ g per mL, to obtain the *Assay Preparation*.

Chromatographic System—Use a chromatograph, operated at room temperature, fitted with an UV detector that monitors absorption at 254 nm, a 30-cm \times 4.6-mm stainless steel cleanup column packed with column packing L7 and using *Mobile Phase A*, and a 25-cm \times 4.6-mm stainless steel analytical column packed with column packing L3 and using *Mobile Phase B*.

Cleanup Column System Suitability Test—Pipet 5 mL of the *Standard Preparation* into a round-bottom flask fitted with a reflux condenser, and add 2 or 3 crystals of butylated hydroxytoluene. Displace the air with nitrogen, and heat in a water bath maintained at a temperature of 90° in subdued light under an atmosphere of nitrogen for 45 minutes, to obtain a solution containing vitamin D and pre-vitamin D. Cool, add 10.0 mL of *Internal Standard Solution*, mix, and evaporate in vacuum to dryness by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve the residue in 10.0 mL of a mixture of equal volumes of acetonitrile and methanol, and mix. Inject 500 μ L of this solution into the cleanup column, and record the chromatogram as directed under *Procedure*. The chromatogram exhibits a peak exhibiting a retention time between 5 and 9 minutes, corresponding to the separation under a single peak of the mixture of vitamin D, pre-vitamin D, and $\Delta^{4,6}$ -cholestadienol from other substances. Adjust the water content or other operating parameters, if necessary (see *Mobile Phase A*).

Analytical Column System Suitability Test—Transfer about 100 mg of USP Vitamin D Assay System Suitability RS to a 100-mL volumetric flask, add a 1 in 20 mixture of toluene and *Mobile Phase B* to volume, and mix. Heat a portion of this solution, under reflux, at 90° for 45 minutes, and cool. Chromatograph five injections of the resulting solution, and measure the peak responses as directed for *Procedure*: the resolution, *R* between *trans*-cholecalciferol and pre-cholecalciferol is not less than 1.0, and the relative standard deviation for the cholecalciferol peak response does not exceed 2.0%. [NOTE—Chromatograms obtained as directed for this

test exhibit relative retention times of approximately 0.4 for pre-cholecalciferol, 0.5 for *trans*-cholecalciferol, and 1.0 for cholecalciferol.]

Calibration—

Vitamin D Response Factor—Transfer 4.0 mL of the *Standard Preparation* and 10.0 mL of *Internal Standard Solution* to a 100-mL volumetric flask, dilute with *Mobile Phase B* to volume, and mix to obtain the *Working Standard Preparation*. Store this *Working Standard Preparation* at a temperature not above 0°, retaining the unused portion for the *Procedure*. Inject 200 μ L of the *Working Standard Preparation* into the analytical column, and measure the peak responses for vitamin D and for $\Delta^{4,6}$ -cholestadienol. The relative retention time of $\Delta^{4,6}$ -cholestadienol is about 1.3. Calculate the response factor, F_D , by the formula:

$$C_S / (R_S C_R)$$

in which C_S and C_R are the concentrations, in μ g per mL, of vitamin D and $\Delta^{4,6}$ -cholestadienol, respectively, in the *Working Standard Preparation*, and R_S is the ratio of the peak response of vitamin D to that of $\Delta^{4,6}$ -cholestadienol.

Pre-Vitamin D Response Factor—Pipet 4 mL of the *Standard Preparation* into a round-bottom flask fitted with a reflux condenser, and add 2 or 3 crystals of butylated hydroxytoluene. Displace the air with nitrogen, and heat in a water bath maintained at a temperature of 90° in subdued light under a nitrogen atmosphere for 45 minutes, to obtain a solution containing vitamin D and pre-vitamin D. Cool, transfer with the aid of several portions of *Mobile Phase B* to a 100-mL volumetric flask containing 10.0 mL of *Internal Standard Solution*, dilute with *Mobile Phase B* to volume, and mix to obtain the *Working Mixture*. Inject 200 μ L of this *Working Mixture* into the analytical column, and measure the peak responses for vitamin D, pre-vitamin D, and $\Delta^{4,6}$ -cholestadienol. Calculate the concentration, C'_S , in μ g per mL, of vitamin D in the (heated) *Working Mixture* by the formula:

$$F_D C_R R'_S$$

in which C_R is the concentration, in μ g per mL, of $\Delta^{4,6}$ -cholestadienol, and R'_S is the ratio of the peak response for vitamin D to that for $\Delta^{4,6}$ -cholestadienol. Calculate the concentration, C'_{PRE} , in μ g per mL, of pre-vitamin D, in the *Working Mixture* by the formula:

$$C'_{PRE} = C_S - C'_S$$

Calculate the response factor, F_{PRE} , for pre-vitamin D by the formula:

$$(F_D R'_S C'_{PRE}) / (R'_{PRE} C'_S)$$

in which R'_{PRE} is the ratio of the peak response of pre-vitamin D to that of $\Delta^{4,6}$ -cholestadienol. [NOTE—Value of F_{PRE} determined in duplicate, on different days, can be used during the whole procedure.]

Procedure—Inject 500 μ L of the *Assay Preparation* into the cleanup column, and collect the fraction representing 0.7 to 1.3 relative to the retention time of the mixed vitamin D peak (see *Cleanup Column System Suitability Test*) in a round-bottom flask. Add 50 μ L of *Butylated Hydroxytoluene Solution*, mix, and evaporate in vacuum to dryness by swirling in a water bath maintained at a temperature not higher than 40°. Cool under running water, and introduce nitrogen sufficient to restore atmospheric pressure. Without delay, dissolve the residue in 5.0 mL of a 1 in 20 mixture of toluene and *Mobile Phase B*, and mix. Inject 200 μ L of this solution into the analytical column, and measure the peak responses for vitamin D, pre-vitamin D, and $\Delta^{4,6}$ -cholestadienol. Calculate the concentration, in μ g per mL, of cholecalciferol

ciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the Assay Preparation by the formula:

$$(R''_D F_D + R''_{PRE} F_{PRE}) C''_R$$

in which R''_D is the ratio of the peak response of vitamin D to that of $\Delta^{4,6}$ -cholestadienol; R''_{PRE} is the ratio of the peak response of pre-vitamin D to that of $\Delta^{4,6}$ -cholestadienol; and C''_R is the concentration, in μg per mL, of $\Delta^{4,6}$ -cholestadienol in the Assay Preparation.

Chemical Method

The following procedure is provided for the determination of vitamin D as an ingredient of Pharmacopeial preparations.

Complete the assay promptly, and exercise care throughout the procedure to keep to a minimum the exposure to air and to actinic light, preferably by the use of a blanket of inert gas and low-actinic glassware.

USP Reference Standards (11)—[NOTE—Use *USP Ergocalciferol RS*, or *USP Cholecalciferol RS*, for assaying pharmaceutical dosage forms that are labeled to contain vitamin D as ergocalciferol, or as cholecalciferol, respectively.] *USP Cholecalciferol RS*. *USP Ergocalciferol RS*.

Special Reagents and Solutions—

Chromatographic Fuller's Earth—Use chromatographic Fuller's earth having a water content corresponding to between 8.5% and 9.0% of loss on drying.

Solvent Hexane—Use solvent hexane (see under *Reagents, Indicators, and Solutions*), redistilling if necessary so that it meets the following additional specification:

SPECTRAL PURITY—Measure in a 1-cm cell at 300 nm, with a suitable spectrophotometer, against air as the blank: the absorbance is not more than 0.070.

Ethylene Dichloride—Purify by passage through a column of granular (20 to 200 mesh) silica gel.

Potassium Hydroxide Solution—Dissolve 500 g of potassium hydroxide in water to make 1000 mL.

Butylated Hydroxytoluene Solution—Dissolve 10 mg of butylated hydroxytoluene in 100 mL of alcohol. Prepare this solution fresh daily.

Ether—Use freshly distilled ether, discarding the first and last 10% portions of the distillate.

Color Reagent—Prepare two stock solutions as follows.

SOLUTION A—Empty, without weighing, the entire contents of a previously unopened 113-g bottle of dry, crystalline antimony trichloride into a flask containing about 400 mL of *Ethylene Dichloride*. Add about 2 g of anhydrous alumina, mix, and pass through filter paper into a clear-glass, glass-stoppered container calibrated at 500 mL. Dilute with *Ethylene Dichloride* to 500 mL, and mix: the absorbance of the solution, measured in a 20-mm cell at 500 nm, with a suitable spectrophotometer, against *Ethylene Dichloride*, does not exceed 0.070.

SOLUTION B—Mix, under a hood, 100 mL of acetyl chloride and 400 mL of *Ethylene Dichloride*.

Mix 45 mL of *Solution A* and 5 mL of *Solution B* to obtain the *Color Reagent*. Store in a tight container, and use within 7 days, but discard any reagent in which a color develops.

Chromatographic Tubes—

First Column—Arrange for descending column chromatography a tube of 2.5-cm (inside) diameter, about 25 cm long, and constricted to 8-mm diameter for a distance of 5 cm at the lower end, by inserting at the point of constriction a coarse-porosity, sintered-glass disk or a small plug of glass wool. The constricted portion may be fitted with an inert, plastic stopcock.

Second Column—Select a tube that is made up of three sections: (1) a flared top section, 18 mm in (inside) diameter and approximately 14 cm long, (2) a middle section, 6 mm in (inside) diameter and approximately 25 cm long, and (3)

a tapered, constricted lower exit tube approximately 5 cm long. Insert a small plug of glass wool in the upper 1-cm portion of the constricted section.

Chromatographic Columns—

First Column—To about 125 mL of isooctane contained in a screw-capped, wide-mouth bottle add 25 g of chromatographic siliceous earth, and shake until a slurry is formed. Add, dropwise and with vigorous mixing, 10 mL of polyethylene glycol 600. Replace the bottle cover, and shake vigorously for 2 minutes. Pour about half of the resulting slurry into the chromatographic tube, and allow it to settle by gravity. Then apply gentle suction, and add the remainder of the slurry in small portions, packing each portion with a 20-mm disk plunger. When a solid surface has formed, remove the vacuum, and add about 2 mL of isooctane.

Second Column—Pack the midsection of the tube with 3 g of moderately coarse *Chromatographic Fuller's Earth* with the aid of gentle suction (about 125 mm of mercury).

Standard Preparation—Dissolve about 25 mg of Reference Standard, accurately weighed, in isooctane to give a known concentration of about 250 μg per mL. Store in a refrigerator.

On the day of assay, pipet 1 mL of this solution into a 50-mL volumetric flask, remove the solvent with a stream of nitrogen, and dissolve the residue in and dilute with *Ethylene Dichloride* to volume, and mix.

Sample Preparation—Accurately weigh or measure a portion of the sample to be assayed, equivalent to not less than 125 μg but preferably about 250 μg of ergocalciferol (10,000 USP Units). If little or no vitamin A is present in the sample, add about 1.5 mg (the equivalent of 3000 USP Units) of vitamin A acetate to provide the needed pilot bands in the subsequent chromatography.

For capsules or tablets, reflux not fewer than 10 of them in 10 mL of water on a steam bath for about 10 minutes, crush the remaining solid with a blunt glass rod, and warm for 5 minutes longer.

Add a volume of *Potassium Hydroxide Solution* representing 2.5 mL for each g of the total weight of the sample, but not less than a total of 3.0 mL. Add 10 mL of *Butylated Hydroxytoluene Solution* and 20 mL of alcohol. Reflux vigorously on a steam bath for 30 minutes. Cool, and transfer the saponified mixture to a conical separator, rinsing the saponification flask with three 10-mL portions of water and three 50-mL portions of *Ether*, adding each rinse to the separator. Add about 4 g of sodium sulfate decahydrate to the separator, and extract by shaking for 2 minutes. If an emulsion forms, extract with three 25-mL portions of *Ether*. Combine the ether extracts, if necessary, and wash by swirling gently with 50 mL of water. Repeat the washing more vigorously with additional 50-mL portions of water until the last portion shows no pink color on the addition of phenolphthalein TS. Transfer the washed ether extract to a 250-mL volumetric flask, dilute with *Ether* to volume, and mix.

Transfer the entire sample or an accurately measured aliquot containing about 250 μg to a tall-form, 400-mL beaker containing about 5 g of anhydrous sodium sulfate. Stir for 2 minutes, then decant the solution into a second 400-mL beaker. Rinse the sodium sulfate with three 25-mL portions of *Ether*, adding each rinse to the main portion. Reduce the total volume to about 30 mL by evaporation on a steam bath, and transfer the concentrate to a small, round-bottom evaporation flask. Rinse the beaker with three 10-mL portions of *Ether*, adding the rinsings to the flask. With the aid of vacuum in a water bath at a temperature not exceeding 40°, or with a stream of nitrogen at room temperature, remove the remaining solvent completely. Dissolve the residue in a small amount of *Solvent Hexane*, transfer to a 10-mL volumetric flask, dilute with *Solvent Hexane* to volume, and mix to obtain the *Sample Preparation*.

Procedure—

First Column Chromatography—Just as the 2 mL of isooctane disappears into the surface of the prepared *First Col-*

umn, pipet 2 mL of the *Sample Preparation* onto the column. As the meniscus of the *Sample Preparation* reaches the column surface, add the first of three 2-mL portions of *Solvent Hexane*, adding each succeeding portion as the preceding portion disappears into the column. Continue adding *Solvent Hexane* in portions of 5 to 10 mL until 100 mL has been added. If necessary, adjust the flow rate to between 3 and 6 mL per minute, by application of gentle pressure at the top of the chromatographic tube.

Discard the first 20 mL of effluent, and collect the remainder. Examine the column under UV light at intervals during the chromatography, and stop the flow when the front of the fluorescent band representing vitamin A is about 5 mm from the bottom of the column. (The UV lamp should provide weak radiation in the 300-nm region. It is frequently necessary to use a narrow aperture or screen with commercial lamps to reduce the amount of radiation to the minimum required to visualize the vitamin A on the column.)

Transfer the eluate to a suitable evaporation flask, and remove the *Solvent Hexane* completely under vacuum at a temperature not higher than 40° or with a stream of nitrogen at room temperature. Dissolve the residue in about 10 mL of *Solvent Hexane*.

Second Column Chromatography—Add the solvent hexane solution obtained as directed under *First Column Chromatography* onto the *Second Column*. Rinse the evaporation flask with a total of 10 mL of *Solvent Hexane* in small portions, adding each portion to the *Second Column* and allowing it to flow through the column, and discard the effluent. When about 1 mL of the hexane remains above the surface of the column, add 75 mL of toluene, and elute with the aid of gentle suction (about 125 mm of mercury), collecting the eluate. Evaporate the toluene under vacuum at a temperature not higher than 40°, or with a stream of nitrogen at room temperature.

Assay Preparation—Dissolve the residue obtained as directed under *Second Column Chromatography* in a small amount of *Ethylene Dichloride*, transfer to a 10-mL volumetric flask, dilute with *Ethylene Dichloride* to volume, and mix to obtain the *Assay Preparation*.

Color Development—Into each of three suitable, matched colorimeter tubes of about 20-mm (inside) diameter, and designated tubes 1, 2, and 3, respectively, pipet 1 mL of the *Assay Preparation*. Into tube 1, pipet 1 mL of the *Standard Preparation*; into tube 2, 1 mL of *Ethylene Dichloride*; and into tube 3, 1 mL of a mixture of equal volumes of acetic anhydride and *Ethylene Dichloride*. To each tube add quickly, and preferably from an automatic pipet, 5.0 mL of *Color Reagent*, and mix. After 45 seconds, accurately timed, following the addition of the *Color Reagent*, determine the absorbances of the three solutions at 500 nm, with a suitable spectrophotometer, using *Ethylene Dichloride* as the blank. Similarly, 45 seconds after making the first reading on each solution, determine the absorbances of the solutions in tubes 2 and 3 at 550 nm, in a similar manner. Designate the absorbances as A_{1500}^1 , A_{2500}^2 , A_{3500}^3 , A_{2550}^2 , and A_{3550}^3 , respectively, in which the superscript indicates the number of the tube and the subscript, the wavelength.

Calculation—Calculate the quantity, in μg , of vitamin D in the portion of the sample taken by the formula:

$$(C_s/C)(A_u/A_s)$$

in which C_s is the concentration of vitamin D, in μg per mL, of the *Standard Preparation*; C is the concentration of the sample (as g, capsules, tablets, etc.) in each mL of the final solution; A_u has the value of $(A_{2500}^2 - A_{3500}^3) - 0.67(A_{2550}^2 - A_{3550}^3)$ determined from the absorbances observed on the solution from the *Assay Preparation*; and A_s has the value of $A_{1500}^1 - A_{2500}^2$ determined on the solutions from the *Standard Preparation*.

Biological Method

The biological assay of vitamin D comprises the recording and interpretation of observations on groups of rats maintained on specified dietary regimens throughout specified periods of their lives whereby the biological response to the preparation under assay is compared with the response to USP Vitamin D Capsules RS.

USP Reference Standards (11)—USP *Cholecalciferol RS*.

Preliminary Period—Throughout the preliminary period in the life of a rat, which is not longer than 30 days and extends from birth to the first day of the depletion period, maintain litters of rats under the immediate supervision of, or according to the directions of, the individual responsible for the assay. During the preliminary period, use a dietary regimen that provides for normal development but is limited in its content of vitamin D, so that when placed upon the *Rachitogenic Diet* in the depletion period the rats develop rickets. At the end of the preliminary period, reject any rat that weighs less than 44 g or more than 60 g, or that shows evidence of injury, disease, or anatomical abnormality.

Depletion Period—Through the depletion period, which extends from the end of the preliminary period to the first day of the assay period, provide each rat ad libitum with the *Rachitogenic Diet* and water, and allow access to no other food or dietary supplement.

Rachitogenic Diet—The *Rachitogenic Diet* consists of a uniform mixture of the following ingredients in the proportions shown in the accompanying table.

Rachitogenic Diet

Ingredient	Parts by weight
Whole yellow corn, ground	76
Wheat gluten, ground	20
Calcium carbonate	3
Sodium chloride	1

When a chemical analysis of the entire ration shows a Ca:P ratio of less than 4:1 or more than 5:1, the proportion of calcium carbonate may be varied to bring the adjusted ratio to a uniform level within this range.

Assigning Rats to Groups for Assay Period—Consider a litter suitable for the assay period when individual rats in the litter show evidence of rickets such as enlarged joints and a distinctive wobbly, rachitic gait, provided that the depletion period is not less than 19 or more than 25 days. The presence of rickets may be established also from the width of the rachitic metaphysis upon X-ray examination or by applying the *Line Test* (described below) to a leg bone of one member of each litter.

Record the weight of each rat, and assign it to a group, in which each rat will be fed a specified dose of the Reference Standard or of an assay sample that is under examination for its vitamin D potency. For each assay sample provide one or more assay groups and not less than two standard groups. The two standard groups may be used for the concurrent assay of more than one assay sample. Within an interval not exceeding 30 days, complete the assignment of rats to groups according to a design that divides litters among the groups, to achieve a complete balance.

For complete balance, whereby each litter is represented equally in every group, use 7 or more litters containing at least as many depleted rats as there are groups. From a given litter, assign one rat, selected at random, to each group on the same day. If a litter contains twice as many rats as there are groups, assign a second series of rats similarly. The last one or two litters to be assigned may be allotted to groups so that at the start of the assay period the average body weight of any completed groups will not differ by more than 8 g from that of any other group.

Assay Doses—Select two dosage levels of the USP *Cholecalciferol RS*, spaced so that the ratio of the larger to the

smaller dose is not less than 1.5 or more than 2.5. Select one or two dosage levels based upon a single assumed potency for each sample. The dosage levels of the sample are equivalent to those of the standard or to a mid-level equal to the square root of the product of the two dosage levels of the standard.

Select dosage levels such that, when fed to rachitic rats, they are expected to produce degrees of calcification within the range specified under the test of data acceptability. Before feeding, the Reference Standard and/or sample may be diluted with cottonseed oil, provided that not more than 0.2 mL is fed on any one day. Store the oil solutions in well-closed bottles, protected from light, at a temperature not exceeding 10°, and use within 5 weeks.

Assign one group of rats to each dosage level of the standard and of the one or more samples.

Assay Period—During the assay period, which extends from the end of the depletion period for a fixed interval of 7 to 10 days, cage each rat individually and provide it ad libitum with the *Rachitogenic Diet* and water. Supply a *Rachitogenic Diet* prepared from the same lots of ingredients to all rats. On the first and on the third (or fourth) day of the assay period, feed each rat one-half of its total assigned dose.

Throughout the assay period, maintain as uniform environmental conditions as possible for all rats, and exclude exposure to antirachitic radiations. At the end of a fixed period of 7 to 10 days, weigh and kill each rat. From those rats that do not weigh less at the end than at the start of the assay period and that have consumed each assigned dose within 24 hours of the time it was fed, dissect out one or more leg bones for examination by the *Line Test*.

Line Test—Remove the proximal end of a tibia or the distal end of a radius, and clean adhering tissue from it, in any one assay using the same bone from all animals. With a clean, sharp blade cut a median, longitudinal section through the juncture of the epiphysis and diaphysis at the same place on each bone. Rinse both sections in purified water, immerse immediately in silver nitrate solution (1 in 50) for 1 minute, and rinse again in purified water. Expose the cut surface of bone, in water, to daylight or another source of actinic light until the calcified areas develop a clearly defined stain without marked discoloration of the uncalcified areas. The staining procedure may be modified to differentiate more clearly between calcified and uncalcified areas.

Score the degree of calcification of the rachitic metaphysis in each rat, according to a scale that allows the average response to be plotted as a straight line against the logarithm of the dose.

Acceptability—Observations are acceptable for use in calculation of the potency only from those groups in which two-thirds or more but not less than 7 rats show calcification at least as great as the lowest level and not greater than the highest level. If the average score of the standard group on the high dosage level is not greater than the average score of the standard group on the low dosage level, discard the results, and repeat the assay. If an assay sample is represented solely by assay groups that are not acceptable for measuring vitamin D potency and in each of which the average score is less than the average score of the standard group on the low dosage level or more than the average score of the standard group on the high dosage level, its assayed content of vitamin D is respectively less than that represented by the low dose or more than that represented by the high dose of the Reference Standard.

Calculation—Tabulate the scores (y), listing each litter in a separate row with treatment groups in columns. Omit any groups that do not meet the test for *Acceptability*. Equalize the number of observations in the acceptable groups by disregarding the results on all litters not equally represented in the groups or by other suitable means (see *Design and Analysis of Biological Assays* (111)). Total the f scores for each of the treatment groups, where f is the number of litters, and

designate each total as T with subscripts 1 and 2 for the low and high dosage levels, respectively. Compute the slope b from the sums of T_1 , i.e., ΣT_1 , and of T_2 , i.e., ΣT_2 , for the standard and sample, provided the latter is represented at both dosage levels, from the equation:

$$b = (\Sigma T_2 - \Sigma T_1) / i h'$$

in which i is the logarithm of the ratio of the high dose to the low dose and is the same for each preparation, and h' is the number of preparations represented by two dosage levels and included in the calculation of the value of b .

Compute the logarithm of the relative potency of each specimen under assay from the equation:

$$\begin{aligned} \log (\text{relative potency}) &= M' \\ &= (\bar{y}_u - \bar{y}_s) / b \\ &= i h' T_a / 2 \Sigma T_b \end{aligned}$$

in which each mean score, \bar{y}_u for the assay sample and \bar{y}_s for the Reference Standard, is the average of the individual scores for an intermediate dosage level or of the two means for the high and the low dosage levels and where $T_b = \Sigma T_2 - \Sigma T_1$ and T_a is as defined (see *Design and Analysis of Biological Assays* (111)). Convert each observed M' to its antilogarithm to obtain the relative potency of the sample. Multiply the relative potency by the assumed potency of the assay oil in Units per g, adopted at the start of the assay, to obtain its assayed content of vitamin D in USP Units per g.

(591) ZINC DETERMINATION

The need for a quantitative determination of zinc in the Pharmacopeial insulin preparations reflects the fact that the element is an essential component of zinc-insulin crystals. In common with lead, zinc may be determined either by the dithizone method or by atomic absorption.

Dithizone Method

Select all reagents for this test to have as low a content of heavy metals as practicable. If necessary, distill water and other solvents into hard or borosilicate glass apparatus. Rinse thoroughly all glassware with warm dilute nitric acid (1 in 2) followed by water. Avoid using on the separator any lubricants that dissolve in chloroform.

Special Solutions and Solvents—

ALKALINE AMMONIUM CITRATE SOLUTION—Dissolve 50 g of dibasic ammonium citrate in water to make 100 mL. Add 100 mL of ammonium hydroxide. Remove any heavy metals that may be present by extracting the solution with 20-mL portions of *Dithizone Extraction Solution* (see *Lead* (251)) until the dithizone solution retains a clear green color, then extract any dithizone remaining in the citrate solution by shaking with chloroform.

CHLOROFORM—Distill chloroform in hard or borosilicate glass apparatus, receiving the distillate in sufficient dehydrated alcohol to make the final concentration 1 mL of alcohol for each 100 mL of distillate.

DITHIZONE SOLUTION—Use *Standard Dithizone Solution* (see *Lead* (251)), prepared with the distilled *Chloroform*.

STANDARD ZINC SOLUTION—Dissolve 625 mg of zinc oxide, accurately weighed, and previously gently ignited to constant weight, in 10 mL of nitric acid, and add water to make 500.0 mL. This solution contains 1.0 mg of zinc per mL.

DILUTED STANDARD ZINC SOLUTION—Dilute 1 mL of *Standard Zinc Solution*, accurately measured, with 2 drops of nitric acid and sufficient water to make 100.0 mL. This solution contains 10 µg of zinc per mL. Use this solution within 2 weeks.

TRICHLOROACETIC ACID SOLUTION—Dissolve 100 g of trichloroacetic acid in water to make 1000 mL.

Procedure—Transfer 1 to 5 mL of the preparation to be tested, accurately measured, to a centrifuge tube graduated at 40 mL. If necessary, add 0.25 *N* hydrochloric acid, dropwise, to obtain a clear solution. Add 5 mL of *Trichloroacetic Acid Solution* and sufficient water to make 40.0 mL. Mix, and centrifuge.

Transfer to a hard-glass separator an accurately measured volume of the supernatant believed to contain from 5 to 20 µg of zinc, and add water to make about 20 mL. Add 1.5 mL of *Alkaline Ammonium Citrate Solution* and 35 mL of *Dithizone Solution*. Shake vigorously 100 times. Allow the chloroform phase to separate. Insert a cotton plug in the stem of the separator to remove any water emulsified with the chloroform. Collect the chloroform extract (discarding the first portion that comes through) in a test tube, and determine the absorbance at 530 nm, with a suitable spectrophotometer.

Calculate the amount of zinc present by reference to a standard absorbance-concentration curve obtained by using 0.5 mL, 1.0 mL, 1.5 mL, and, if the zinc content of the sample extracted exceeds 15 µg, 2.0 mL of the *Diluted Standard Zinc Solution*, corrected as indicated by a blank determination run concomitantly, using all of the reagents but no added zinc.

Physical Tests and Determinations

(601) AEROSOLS, NASAL SPRAYS, METERED-DOSE INHALERS, AND DRY POWDER INHALERS

This general chapter contains test methods for propellants, pressurized topical aerosols, nasal sprays, metered-dose inhalers, and propellant-free dry powder inhalers used to aerosolize, or to aerosolize and meter, doses of powders for inhalation. Apply these methods, where indicated, in the testing of the appropriate dosage forms.

PROPELLANTS

Caution—*Hydrocarbon propellants are highly flammable and explosive. Observe precautions and perform sampling and analytical operations in a well-ventilated fume hood.*

General Sampling Procedure

This procedure is used to obtain test specimens for those propellants that occur as gases at about 25° and that are stored in pressurized cylinders. Use a stainless steel sample cylinder equipped with a stainless steel valve and having a capacity of not less than 200 mL and a pressure rating of 240 psi or more. Dry the cylinder with the valve open at 110° for 2 hours, and evacuate the hot cylinder to less than 1 mm of mercury. Close the valve, cool, and weigh. Connect one end of a charging line tightly to the propellant

container and the other end loosely to the sample cylinder. Carefully open the propellant container, and allow the propellant to flush out the charging line through the loose connection. Avoid excessive flushing, which causes moisture to freeze in the charging line and connections. Tighten the fitting on the sample cylinder, and open the sample cylinder valve, allowing the propellant to flow into the evacuated cylinder. Continue sampling until the desired amount of specimen is obtained, then close the propellant container valve, and finally close the sample cylinder valve. [**Caution**—*Do not overload the sample cylinder; hydraulic expansion due to temperature change can cause overloaded cylinders to explode.*] Again weigh the charged sample cylinder, and calculate the weight of the specimen.

Approximate Boiling Temperature

Transfer a 100-mL specimen to a tared, pear-shaped, 100-mL centrifuge tube containing a few boiling stones, and weigh. Suspend a thermometer in the liquid, and place the tube in a medium maintained at a temperature of 32° above the expected boiling temperature. When the thermometer reading becomes constant, record as the boiling temperature the thermometer reading after at least 5% of the specimen has distilled. Retain the remainder of the specimen for the determination of *High-Boiling Residues*.

High-Boiling Residues, Method I

Allow 85 mL of the specimen to distill as directed in the test for *Approximate Boiling Temperature*, and transfer the centrifuge tube containing the remaining 15 mL of specimen to a medium maintained at a temperature 10° above the boiling temperature. After 30 minutes, remove the tube from the water bath, blot dry, and weigh. Calculate the weight of the residue.

High-Boiling Residues, Method II

Prepare a cooling coil from copper tubing (about 6 mm outside diameter × about 6.1 m long) to fit into a vacuum-jacketed flask. Immerse the cooling coil in a mixture of dry ice and acetone in a vacuum-jacketed flask, and connect one end of the tubing to the propellant sample cylinder. Carefully open the sample cylinder valve, flush the cooling coil with about 50 mL of the propellant, and discard this portion of liquefied propellant. Continue delivering liquefied propellant from the cooling coil, and collect it in a previously chilled 1000-mL sedimentation cone until the cone is filled to the 1000-mL mark. Allow the propellant to evaporate, using a warm water bath maintained at about 40° to reduce evaporating time. When all of the liquid has evaporated, rinse the sedimentation cone with two 50-mL portions of pentane, and combine the rinsings in a tared 150-mL evaporating dish. Transfer 100 mL of the pentane solvent to a second tared 150-mL evaporating dish, place both evaporating dishes on a water bath, evaporate to dryness, and heat the dishes in an oven at 100° for 60 minutes. Cool the dishes in a desiccator, and weigh. Repeat the heating for 15-minute periods until successive weighings are within 0.1 mg, and calculate the weight of the residue obtained from the propellant as the difference between the weights of the residues in the two evaporating dishes.

Water Content

Proceed as directed under *Water Determination* (921), with the following modifications: (a) Provide the closed-system titrating vessel with an opening through which passes a coarse-porosity gas dispersion tube connected to a sampling cylinder. (b) Dilute the *Reagent* with anhydrous methanol to give a water equivalence factor of between 0.2 and 1.0 mg

per mL; age this diluted solution for not less than 16 hours before standardization. (c) Obtain a 100-g specimen as directed under *General Sampling Procedure*, and introduce the specimen into the titration vessel through the gas dispersion tube at a rate of about 100 mL of gas per minute; if necessary, heat the sample cylinder gently to maintain this flow rate.

Other Determinations

For those aerosols that use propellants, perform the tests specified in the individual *NF* propellant monographs.

AEROSOLS

Because leaching of extractable substances into pressurized formulations should be minimized, valve materials and other components that are in contact with the product meet the requirements under *Elastomeric Closures for Injections* (381) (Note that under *Physicochemical Test Procedures* in (381) the directions for preparing a sample require pre-extraction, which may cause an underestimate of the amount of extractables from a given component.) See also *Aerosols* under *Pharmaceutical Dosage Forms* (1151).

TOPICAL AEROSOLS

The following tests are applicable to topical aerosols containing drug, in suspension or solution, packaged under pressure, and released upon activation of an appropriate valve system.

Delivery Rate and Delivered Amount

Perform these tests only on containers fitted with continuous valves.

Delivery Rate—Select not fewer than four aerosol containers, shake, if the label includes this directive, remove the caps and covers, and actuate each valve for 2 to 3 seconds. Weigh each container accurately, and immerse in a constant-temperature bath until the internal pressure is equilibrated at a temperature of 25° as determined by constancy of internal pressure, as directed under the *Pressure Test* below. Remove the containers from the bath, remove excess moisture by blotting with a paper towel, shake, if the label includes this directive, actuate each valve for 5.0 seconds (accurately timed by use of a stopwatch), and weigh each container again. Return the containers to the constant-temperature bath, and repeat the foregoing procedure three times for each container. Calculate the average *Delivery Rate*, in g per second, for each container.

Delivered Amount—Return the containers to the constant-temperature bath, continuing to deliver 5 second actuations to waste, until each container is exhausted. [NOTE—Ensure that sufficient time is allowed between each actuation to avoid significant canister cooling.] Calculate the total weight loss from each container. This is the *Delivered Amount*.

Pressure Test

Perform this test only on topical aerosols fitted with continuous valves.

Select not fewer than four aerosol containers, remove the caps and covers, and immerse in a constant-temperature bath until the internal pressure is constant at a temperature of 25°. Remove the containers from the bath, shake, and remove the actuator and water, if any, from the valve stem. Place each container in an upright position, and determine the pressure in each container by placing a calibrated pressure gauge on the valve stem, holding firmly, and actuating

the valve so that it is fully open. The gauge is of a calibration approximating the expected pressure and is fitted with an adapter appropriate for the particular valve stem dimensions. Read the pressure directly from the gauge.

Minimum Fill

Topical aerosols meet the requirements for aerosols under *Minimum Fill* (755).

Leakage Test

Perform this test only on topical aerosols fitted with continuous valves.

Select 12 aerosol containers, and record the date and time to the nearest half hour. Weigh each container to the nearest mg, and record the weight, in mg, of each as W_1 . Allow the containers to stand in an upright position at a temperature of $25.0 \pm 2.0^\circ$ for not less than 3 days, and again weigh each container, recording the weight, in mg, of each as W_2 , and recording the date and time to the nearest half hour. Determine the time, T , in hours, during which the containers were under test. Calculate the leakage rate, in mg per year, of each container taken by the formula:

$$(365)(24/T)(W_1 - W_2).$$

Where plastic-coated glass aerosol containers are tested, dry the containers in a desiccator for 12 to 18 hours, and allow them to stand in a constant-humidity environment for 24 hours prior to determining the initial weight as indicated above. Conduct the test under the same constant-humidity conditions. Empty the contents of each container tested by employing any safe technique (e.g., chill to reduce the internal pressure, remove the valve, and pour). Remove any residual contents by rinsing with suitable solvents, then rinse with a few portions of methanol. Retain as a unit the container, the valve, and all associated parts, and heat them at 100° for 5 minutes. Cool, weigh, record the weight as W_3 , and determine the net fill weight ($W_1 - W_3$) for each container tested. [NOTE—If the average net fill weight has been determined previously, that value may be used in place of the value ($W_1 - W_3$) above.] The requirements are met if the average leakage rate per year for the 12 containers is not more than 3.5% of the net fill weight, and none of the containers leaks more than 5.0% of the net fill weight per year. If 1 container leaks more than 5.0% per year, and if none of the containers leaks more than 7.0% per year, determine the leakage rate of an additional 24 containers as directed herein. Not more than 2 of the 36 containers leak more than 5.0% of the net fill weight per year, and none of the 36 containers leaks more than 7.0% of the net fill weight per year. Where the net fill weight is less than 15 g and the label bears an expiration date, the requirements are met if the average leakage rate of the 12 containers is not more than 525 mg per year and none of the containers leaks more than 750 mg per year. If 1 container leaks more than 750 mg per year, but not more than 1.1 g per year, determine the leakage rate of an additional 24 containers as directed herein. Not more than 2 of the 36 containers leak more than 750 mg per year, and none of the 36 containers leaks more than 1.1 g per year. This test is in addition to the customary in-line leak testing of each container.

Number of Discharges per Container

Perform this test only on topical aerosols fitted with dosing valves, at the same time as, and on the same containers used for, the test for *Delivered-Dose Uniformity*. Determine the number of discharges or deliveries by counting the number of priming discharges plus those used in determining the spray contents, and continue to fire until the label claim number of discharges. The requirements are met if all

the containers or inhalers tested contain not less than the number of discharges stated on the label.

Delivered-Dose Uniformity

The test for *Delivered-Dose Uniformity* is required for topical aerosols fitted with dose-metering valves. For collection of the minimum dose, proceed as directed in the test for *Delivered-Dose Uniformity* under *Metered-Dose Inhalers and Dry Powder Inhalers*, as described below, except to modify the dose sampling apparatus so that it is capable of quantitatively capturing the delivered dose from the preparation being tested. Unless otherwise stated in the individual monograph, apply the acceptance criteria for *Metered-Dose Inhalers and Dry Powder Inhalers* as described below.

NASAL SPRAYS

The following test is applicable to nasal sprays, formulated as aqueous suspensions or solutions of drug, presented in multi-dose containers and fitted with dose-metering valves. In all cases, and for all tests, prepare and test the nasal spray as directed on the label and the instructions for use.

Delivered-Dose Uniformity

Unless otherwise directed in the individual monograph, the drug content of the minimum delivered doses (minimum number of sprays per nostril as described on the label, or instructions for use) collected at the beginning of unit life (after priming as described on the label, or instructions for use) and at the label claim number of metered sprays, from each of 10 separate containers, must meet the following acceptance criteria: not more than 2 of the 20 doses are outside the range of 80% to 120% of label claim, and none are outside the range of 75% to 125% of label claim, while the mean for each of the beginning and end doses falls within the range of 85% to 115% of label claim. If 3–6 doses of the 20 doses collected are outside of 80% to 120% of the label claim, but none are outside of 75% to 125% of label claim, and the means for each of the beginning and end doses fall within 85% to 115% of label claim, select 20 additional containers for second-tier testing. For second-tier testing, the requirements are met if not more than 6 of the 60 doses collected are outside the range of 80% to 120% of label claim, none are outside the range of 75% to 125% of label claim, and the means for each of the beginning and end doses fall within the range of 85% to 115% of label claim.

SAMPLING FOR DELIVERED-DOSE UNIFORMITY OF METERED-DOSE NASAL SPRAYS

General Sampling Procedure—To ensure reproducible in-vitro dose collection, it is recommended that a mechanical means of actuating the pump assembly be employed to deliver doses for collection. The mechanical actuation procedure should have adequate controls for the critical mechanical actuation parameters (e.g., actuation force, actuation speed, stroke length, rest periods, etc.). The test must be performed on units that have been primed according to the patient-use instructions. The test unit should be actuated in a vertical or near vertical, valve-up, position. The two doses collected at the beginning and end of the container life should be the dose immediately following priming and the dose corresponding to the last label claim number of doses from the container.

For suspension products, the delivered dose should be delivered into a suitable container (e.g., scintillation vial) in which quantitative transfer from the container under test

can be accomplished. A validated analytical method is employed to determine the amount of drug in each delivered dose, and data are reported as a percent of label claim. For solution products, the delivered dose can be determined gravimetrically from the weight of the delivered dose, and the concentration and density of the fill solution of the product under test.

METERED-DOSE INHALERS AND DRY POWDER INHALERS

The following tests are applicable to metered-dose inhalers that are formulated as suspensions or solutions of active drug in propellants and dry powder inhalers presented as single or multidose units. The following test methods are specific to the aforementioned inhalers and may require modification when testing alternative inhalation technologies (for example, breath-actuated metered-dose inhalers, or dose-metering nebulizers). However, Pharmacopeial requirements for all dose-metering inhalation dosage forms require determination of the delivered dose and *Aerodynamic Size Distribution*. In all cases, and for all tests, prepare and test the inhaler as directed on the label and the instructions for use. When these directions are not provided by the product manufacturer, follow the precise dose discharge directions included in the tests below.

Delivered-Dose Uniformity

The test for *Delivered-Dose Uniformity* is required for inhalers (e.g., metered-dose inhalers or dry powder inhalers) containing drug formulation (e.g., solution, suspension, or powder) either in reservoirs or in premetered dosage units, and for drug formulations packaged in reservoirs or in premetered dosage units where these containers are labeled for use with a named inhalation device. (For inhalations packaged in premetered dosage units, see also *Uniformity of Dosage Units* (905).) Note that the target-delivered dose is the expected mean drug content for a large number of delivered doses collected from many inhalers of the chosen product. In many cases, its value may depend upon the manner in which the test for delivered dose is performed. For metered-dose inhalers, the target-delivered dose is specified by the label claim, unless otherwise specified in the individual monograph. For dry powder inhalers, where the label claim is usually the packaged or metered-dose of drug, the target-delivered dose is specified in the individual monograph and is usually less than the label claim. Its value reflects the expected mean drug content for a large number of delivered doses collected from the product, using the method specified in the monograph.

Unless otherwise directed in the individual monograph, the drug content of the minimum delivered dose from each of 10 separate containers is determined in accordance with the procedure described below.

Unless otherwise specified in the individual monograph, the requirements for dosage uniformity are met if not less than 9 of the 10 doses are between 75% and 125% of the specified target-delivered dose and none is outside the range of 65% to 135% of the specified target-delivered dose. If the contents of not more than 3 doses are outside the range of 75% to 125% of the specified target-delivered dose, but within the range of 65% to 135% of the specified target-delivered dose, select 20 additional containers, and follow the prescribed procedure for analyzing 1 minimum dose from each. The requirements are met if not more than 3 results, out of the 30 values, lie outside the range of 75% to 125% of the specified target-delivered dose, and none is outside the range of 65% to 135% of the specified target-delivered dose.

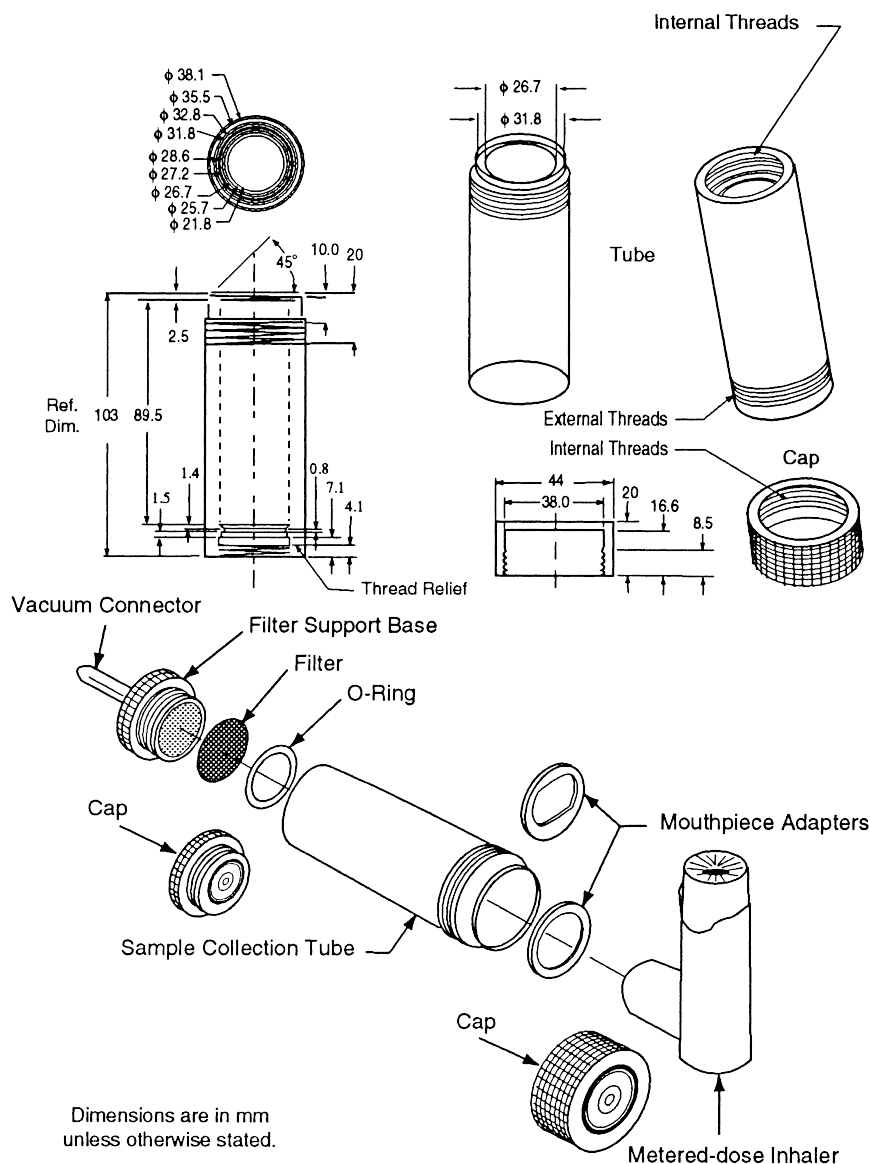


Fig. 1. Sampling apparatus for pressurized metered-dose inhalers.

SAMPLING THE DELIVERED DOSE FROM METERED-DOSE INHALERS

To determine the content of active ingredient in the discharged spray from a metered-dose inhaler, use the sampling apparatus described below, using a flow rate of 28.3 L of air per minute ($\pm 5\%$), unless otherwise stated in the individual monograph.

Apparatus A—The apparatus (see Figure 1) consists of a filter support base with an open-mesh filter support, such as a stainless steel screen, a collection tube that is clamped or screwed to the filter support base, and a mouthpiece adapter to ensure an airtight seal between the collection tube and the mouthpiece. Use a mouthpiece adapter that ensures that the opening of the inhaler mouthpiece is flush with the front face or 2.5-mm indented shoulder in the sample collection tube, as appropriate. The vacuum connector is connected to a system comprising a vacuum source, flow regulator, and flowmeter. The source should be capable of pulling air through the complete assembly, including

the filter and the inhaler to be tested, at the desired flow rate. When testing metered-dose inhalers, air should be drawn continuously through the system to avoid loss of drug into the atmosphere. The filter support base is designed to accommodate 25-mm diameter filter disks. At the airflow being used, the sample collection tube and the filter disk must be capable of quantitatively collecting the *Delivered Dose*. The filter disk and other materials used in the construction of the apparatus must be compatible with the drug and the solvents that are used to extract the drug from the filter. One end of the collection tube is designed to hold the filter disk tightly against the filter support base. When assembled, the joints between the components of the apparatus are airtight so that when a vacuum is applied to the base of the filter, all of the air drawn through the collection device passes through the inhaler.

Procedure—Prepare the inhaler for use according to the label instructions. Unless otherwise specified in the individual monograph, with the vacuum pump running, ensuring an airflow rate through the inhaler of 28.3 L of air per min-

ute ($\pm 5\%$), discharge the minimum recommended dose into the apparatus through the mouthpiece adapter by depressing the valve for a duration sufficient to ensure that the dose has been completely discharged. Detach the inhaler from *Apparatus A*, and disconnect the vacuum. Assay the contents of the apparatus for drug after rinsing the filter and the interior of the apparatus with a suitable solvent.

SAMPLING THE DELIVERED DOSE FROM DRY POWDER INHALERS

To determine the content of active ingredient emitted from the mouthpiece of a dry powder inhaler, use *Apparatus B* (see *Figure 2*).

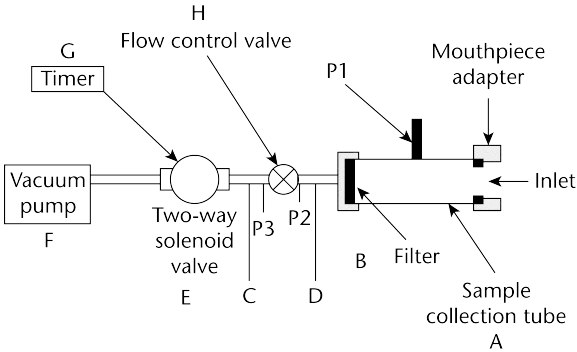


Fig. 2. Apparatus B: Sampling apparatus for dry powder inhalers. (See *Table 1* for component specifications.)

This apparatus is capable of sampling the emitted doses at a variety of airflow rates.

Apparatus B—The apparatus is similar to that described in *Figure 1* for testing metered-dose inhalers. In this case, however, the filter and collection tube have a larger internal diameter to accommodate 47-mm diameter filter disks. This feature enables dosage collection at higher airflow rates—up to 100 L of air per minute—when necessary. A mouthpiece

Table 1. Component Specifications for Apparatus B (see Fig. 2)

Code	Item	Description	Dimensions
A	Sample collection tube ^a	See Fig. 2	34.85-mm ID \times 12-cm length
B	Filter ^b	See Fig. 2	47-mm glass fiber filter
C	Connector	(e.g., short metal coupling with low diameter branch to P3)	≥ 8 -mm ID
D	Vacuum tubing	(e.g., silicon tubing with an outside diameter of 14 mm and an internal diameter of 8 mm)	A length of suitable tubing ≥ 8 mm ID with an internal volume of 25 ± 5 mL.
E	Two-way solenoid valve ^c	See Fig. 2	2-way, 2-port solenoid valve having an ID ≥ 8 mm and an opening response time of ≤ 100 milliseconds.
F	Vacuum pump ^d	See Fig. 2	Pump must be capable of drawing the required flow rate through the assembled apparatus with the dry powder inhaler in the mouthpiece adapter. Connect the pump to the solenoid valve using short and wide (≥ 10 -mm ID) vacuum tubing and connectors to minimize pump capacity requirements.
G	Timer ^e	See Fig. 2	The timer switches current directly to the solenoid valve for the required duration.
P1	Pressure tap	See Fig. 2	2.2-mm ID, 3.1-mm OD flush with the internal surface of the sample collection tube, centered and burr free, 59 mm from its inlet. The pressure taps P1, P2, and P3 must not be open to the atmosphere during dose collection.
P1, P2, P3	Pressure measurements ^f		
H	Flow-control valve ^g	See Fig. 2	Adjustable regulating valve with maximum $C_v \geq 1$ ^h .

^a An example being a Millipore product number XX40 047 00 (Millipore Corporation, 80, Ashby Road, Bedford, MA 01732), modified so that the exit tube has an ID ≥ 8 -mm, fitted with Gelman product number 61631.

^b A/E (Gelman Sciences Inc., 600 South Wagner Road, Ann Arbor, MI 48106) or equivalent.

^c ASCO product number 8030G13, Automatic Switch Company, 60 Hanover Road, Florham Park, NJ 07932.

^d Gast product type 1023, 1423, or 2565 (Gast Manufacturing Inc., PO Box 97, Benton Harbor, MI 49022) or equivalent.

^e Eaton Product number 45610-400 (Eaton Corporation, Automotive Products Division, 901, South 12th Street, Watertown, WI 53094) or equivalent.

^f An example being a PDM 210 pressure meter (Air-Neotronics Ltd., Neotronics Technology plc, Parsonage Road, Takeley, Bishop's Stortford, CM22 6PU, UK), or equivalent.

^g Parker Hannifin type 8FV12LNSS (Parker Hannifin plc., Riverside Road, Barnstable, Devon EX31 1NP, UK) or equivalent.

^h Flow Coefficient, as defined by ISA S75.02 "Control valve capacity test procedure" in *Standards and Recommended Practices for Instrumentation and Control*, 10th ed., Vol. 2, 1989. Published by Instrument Society of America, 67 Alexander Drive, P.O. Box 1227, Research Triangle Park, NC 27709, U.S.A.

adapter ensures an airtight seal between the collection tube and the mouthpiece of the dry powder inhaler being tested. The mouthpiece adapter must ensure that the tip of the inhaler mouthpiece is flush with the open end of the sample collection tube. Tubing connectors, if they are used, should have an internal diameter greater than or equal to 8 mm to preclude their own internal diameters from creating significant airflow resistance. A vacuum pump with excess capacity must be selected in order to draw air, at the designated volumetric flow rate, through both the sampling apparatus and the inhaler simultaneously. A timer-controlled, low resistance, solenoid-operated, two-way valve is interposed between the vacuum pump and the flow-control valve to control the duration of flow. This type of valve enables 4.0 L of air ($\pm 5\%$) to be withdrawn from the mouthpiece of the inhaler at the designated flow rate. Flow control is achieved by ensuring that critical (sonic) flow occurs in the flow-control valve (absolute pressure ratio $P_3/P_2 \leq 0.5$ under conditions of steady-state flow).

Procedure—Operate the apparatus at an airflow rate that produces a pressure drop of 4 kPa (40.8 cm H₂O) over the inhaler to be tested and at a duration consistent with the withdrawal of 4 L of air from the mouthpiece of the inhaler. [NOTE—If the flow rate and duration are defined otherwise in the monograph, adjust the system to within 5% of those values.] Determine the test flow rate using *Apparatus B* as follows. Insert an inhaler into the mouthpiece adapter to ensure an airtight seal. In cases where the drug packaging modifies the inhaler's resistance to airflow, use a loaded, drug-free inhaler (with previously emptied packaging). In other cases, use an unloaded (drug free) inhaler. Connect one port of a differential pressure transducer to the pressure tap, P₁, and leave the other open to the atmosphere. Switch on the pump, and open the two-way solenoid valve. Adjust the flow-control valve until the pressure drop across the inhaler is 4.0 kPa (40.8 cm H₂O). Ensure that critical (sonic) flow occurs in the flow-control valve by determining the individual values for absolute pressure, P₂ and P₃, so that their ratio P₃/P₂ is less than or equal to 0.5. If this criterion cannot be achieved, it is likely that the vacuum pump is worn or of insufficient capacity. Critical (sonic) flow conditions in the flow-control valve are required in order to ensure that the volumetric airflow drawn from the mouthpiece is unaffected by pump fluctuations and changes in airflow resistance of the inhaler. Remove the inhaler from the mouthpiece adapter and without disturbing the flow-control valve, measure the airflow rate drawn from the mouthpiece, Q_{out} , by connecting a flowmeter to the mouthpiece adaptor in an airtight fashion. Use a flowmeter calibrated for the volumetric flow leaving the meter in an airtight fashion to directly determine Q_{out} or, if such a meter is unobtainable, calculate the volumetric flow leaving the meter (Q_{out}) using the ideal gas law. For example, for a meter calibrated for the entering volumetric flow (Q_{in}), use the formula:

$$Q_{out} = Q_{in}P_0 / (P_0 - \Delta P)$$

where P_0 is the atmospheric pressure and ΔP is the pressure drop over the meter. If the flow rate is greater than 100 L of air per minute, adjust the flow-control valve until Q_{out} equals 100 L per minute; otherwise, record the value of Q_{out} , and leave the flow-control valve undisturbed. Define the test flow duration, $T = 240/Q_{out}$, in seconds, so that a volume of 4.0 L of air ($\pm 5\%$) is withdrawn from the inhaler at the test flow rate Q_{out} , and adjust the timer controlling the operation of the two-way solenoid valve accordingly. Prime or load the inhaler with powder for inhalation according to the labeled instructions. With the vacuum pump running and the solenoid valve closed, insert the inhaler mouthpiece horizontally into the mouthpiece adapter. Discharge the powder into the sampling apparatus by activating the timer controlling the solenoid valve and withdrawing 4.0 L of air from the inhaler at the previously defined airflow rate. If the labeled instructions so direct, repeat the operation so as to

simulate the use of the inhaler by the patient (e.g., inhale two or three times, if necessary, to empty the capsule). Repeat the whole operation $n - 1$ times beginning with the text, "Prime or load the inhaler with powder," where n is the number of times defined in the labeling as the minimum recommended dose. Detach the dry powder inhaler from the sampling apparatus, and disconnect the vacuum tubing, D. Assay the contents of the apparatus for drug after rinsing the filter and the interior of the apparatus with a suitable solvent. Where specified in individual monographs, perform this test under conditions of controlled temperature and humidity.

Delivered-Dose Uniformity over the Entire Contents

The test for *Delivered-Dose Uniformity over the Entire Contents* is required for inhalers (e.g., metered-dose inhalers or dry powder inhalers) containing multiple doses of drug formulation (e.g., solution, suspension, or dry powder) either in reservoirs or in premeasured dosage units (e.g., blisters), and for drug formulations packaged in reservoirs or in multiple-dose assemblies of premeasured dosage units that have a predetermined dose sequence, where these multiple-dose assemblies are labeled for use with a named inhalation device. The test for *Delivered-Dose Uniformity over the Entire Contents* also ensures that multidose products supply the total number of discharges stated on the label. Unless otherwise directed in the individual monograph, the drug content of at least 9 of the 10 doses collected from one inhaler, in accordance with the procedure below, are between 75% and 125% of the target-delivered dose, and none is outside the range of 65% to 135% of the target-delivered dose. If the contents of not more than 3 doses are outside the range of 75% to 125%, but within the range of 65% to 135% of the target-delivered dose, select 2 additional inhalers, and follow the prescribed procedure for analyzing 10 doses from each. The requirements are met if not more than 3 results, out of the 30 values, lie outside the range of 75% to 125% of the target-delivered dose, and none is outside the range of 65% to 135% of the target-delivered dose.

METERED-DOSE INHALERS

Apparatus—Use *Apparatus A* as directed in *Sampling the Delivered-Dose from Metered-Dose Inhalers* under *Delivered-Dose Uniformity* at a flow rate of 28.3 L of air per minute ($\pm 5\%$).

Procedure—A single dose is defined as the number of sprays specified in the product labeling as the minimum recommended dose. Select a single metered-dose inhaler, and follow the labeled instructions for priming, shaking, cleaning, and firing the inhaler throughout. Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 seconds, and fire one minimum recommended dose to waste. Wait for 5 seconds, and collect the next dose. Detach the inhaler from *Apparatus A*, and disconnect the vacuum. Assay the contents of the apparatus for drug after rinsing the filter and the interior of the apparatus with a suitable solvent. Collect two more doses, allowing at least 5 seconds between doses. Discharge the device to waste, waiting for not less than 5 seconds between actuations (unless otherwise specified in the individual monograph), until $(n/2) + 1$ minimum recommended doses remain, in which n is the number of minimum recommended doses on the label. Collect four more doses, allowing at least 5 seconds between doses, unless otherwise specified in the individual monograph. Discharge the device to waste, as before, until three doses remain. Collect the final three doses, allowing at least 5 seconds between doses. Note that the rate of discharges to waste should not be such to cause excessive canister cooling.

DRY POWDER INHALERS

Apparatus—Use *Apparatus B* as directed in *Sampling the Delivered Dose from Dry Powder Inhalers under Delivered-Dose Uniformity* at the appropriate airflow rate for testing.

Procedure—Proceed as directed for *Procedure in Sampling the Delivered Dose from Dry Powder Inhalers under Delivered-Dose Uniformity*. A single dose is defined as the number of actuations stated in the product labeling as the minimum recommended dose. Select a single inhaler and follow the labeled instructions for loading with powder, discharging and cleaning throughout. Collect a total of 10 doses—three doses at the beginning, four in the middle $[(n/2) - 1 \text{ to } (n/2) + 2]$, where n is the number of minimum recommended doses on the label], and three at the end—of the labeled contents following the labeled instructions. Prior to collecting each of the doses to be analyzed, clean the inhaler as directed in the labeling.

Particle Size

The particle or droplet size distribution in the spray discharged from metered-dose inhalers, and the particle size distribution in the cloud discharged from dry powder inhalers, are important characteristics used in judging inhaler performance. While particle size measurement by microscopy can be used to evaluate the number of large particles, agglomerates, and foreign particulates in the emissions of metered-dose inhalers (e.g., *Epinephrine Bitartrate Inhalation Aerosol*), whenever possible this test should be replaced with a method to determine the aerodynamic size distribution of the drug aerosol leaving the inhaler. The aerodynamic size distribution defines the manner in which an aerosol deposits during inhalation. When there is a log-normal distribution, the aerodynamic size distribution may be characterized by the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD). The aerodynamic size distribution of the drug leaving metered-dose and dry powder inhalers is determined using *Apparatus 1, 2, 3, 4, 5, or 6* as specified in this chapter. A fine particle dose or fine particle fraction can also be determined as that portion of the inhaler output having an aerodynamic diameter less than the size defined in the individual monograph. This may be expected to correlate with the drug dose or that fraction of the drug dose that penetrates the lung during inhalation. Individual monographs may also define the emitted fractions of the delivered dose in more than one aerodynamic size range.

AERODYNAMIC SIZE DISTRIBUTION

Cascade impaction devices classify aerosol particles and droplets on the basis of those particles' aerodynamic diameters. The principle of their operation, whereby they separate aerosol particles and droplets from a moving airstream on the basis of particle or droplet inertia, is shown in *Figure 3*.

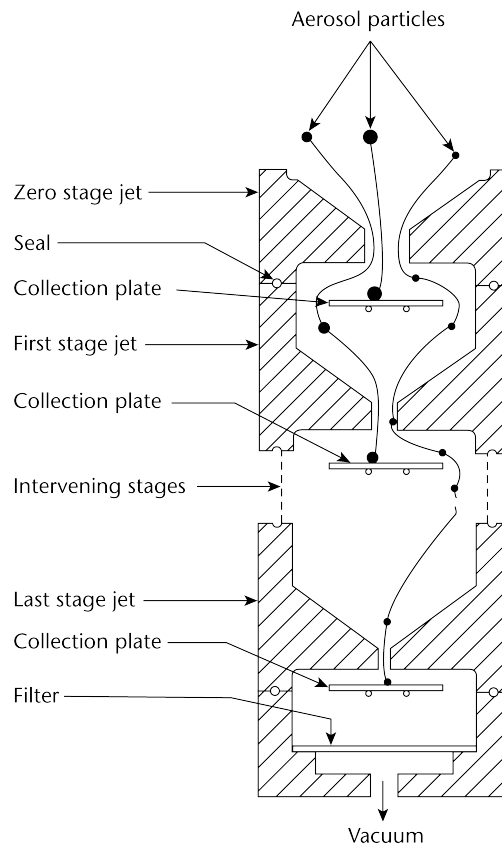


Fig. 3. Schematic representation of the principle of operation of cascade impactors. (A single jet per impactor stage is shown. Impactors with multiple jets in each stage function in the same manner.)

Because the dimensions of the induction port used to connect inhalers to the cascade impactors and impingers (shown in *Apparatus 1, 2, 3, 4, 5, and 6*) also define the mass of drug that enters the aerodynamic sizing device, these are carefully defined and, where possible, are held constant between each apparatus (see *Figures 4, 6, 7, 8, and 9*).

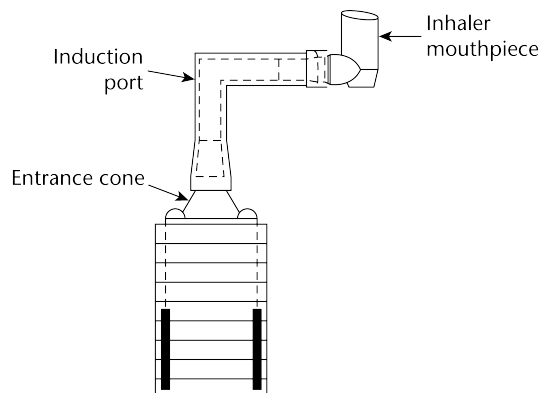


Fig. 4. Apparatus 1: Assembly of induction port and entrance cone mounted on cascade impactor.

Because the size distributions produced by different impactors are often a function of impactor design and the airflow rate through them, there is a need to standardize the instruments that are used to test inhalers (i.e., *Apparatus 1 or 6* for metered-dose inhalers) or to provide guidelines

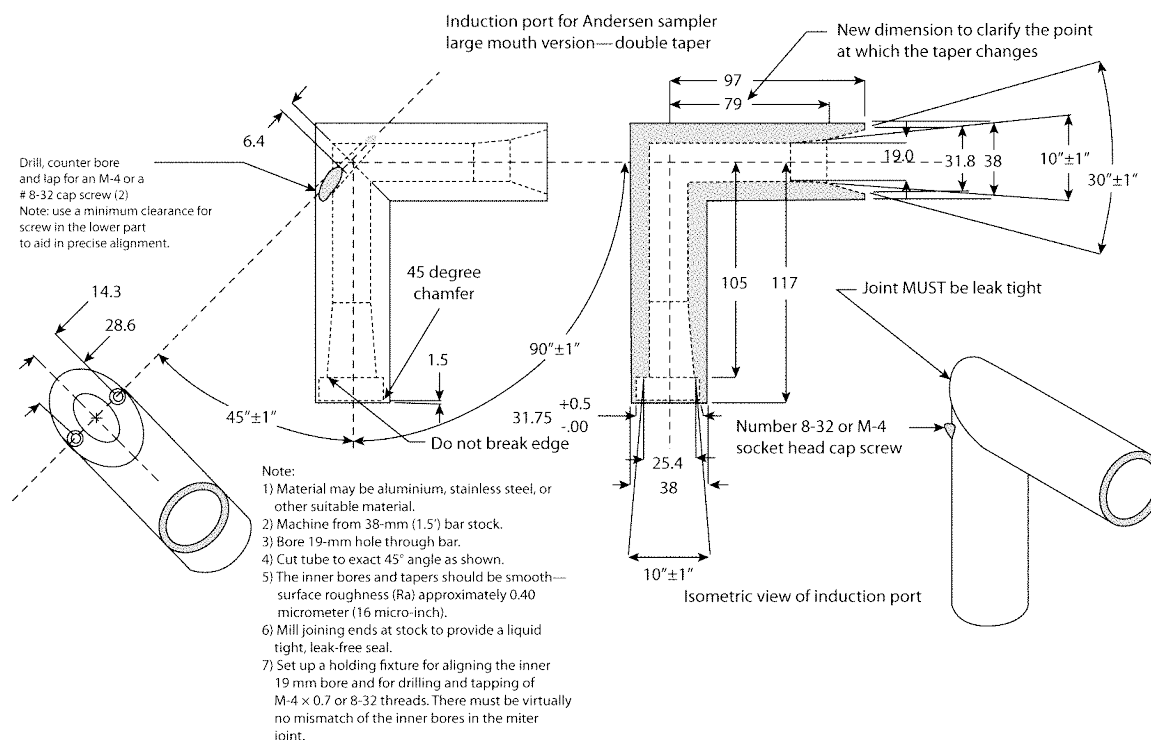


Fig. 4a. Apparatus 1: Expanded view of induction port for use with metered-dose and dry powder inhalers.

on system suitability where different apparatuses may be used (i.e., Apparatus 2, 3, 4, or 5 for dry powder inhalers).

Because of the varied nature of the formulations and devices being tested, the cascade impaction system and technique selected for testing an inhaler should fulfill a number of criteria.

Stage Mensuration—Manufacturers of cascade impaction devices provide a definitive calibration for the separation characteristics of each impaction stage in terms of the relationship between the stage collection efficiency and the aerodynamic diameter of particles and droplets passing through it as an aerosol. Calibration is a property of the jet dimensions, the spatial arrangement of the jet and its collection surface, and the airflow rate passing through it. Because jets can corrode and wear over time, the critical dimensions of each stage, which define that impaction stage's calibration, must be measured on a regular basis. This process, known as stage mensuration, replaces the need for repetitive calibration (using standard aerosols) and ensures that only devices that conform to specifications are used for testing inhaler output. The process involves the measurement and adjustment of the critical dimensions of the instrument.

Interstage Drug Loss (wall losses)—Where method variations are possible and there is no apparatus specified in the monograph, the selected technique should ensure that not more than 5% of the inhaler's total delivered drug mass (into the impactor) is subject to loss between the impaction device's sample collection surfaces. In the event that interstage drug losses are known to be greater than 5%, either the procedure should be performed in such a way that wall losses are included along with the associated collection plate, or an alternative apparatus should be used. As an example, the following procedures described for Apparatus 1 and 3 have been written to include wall losses along with the associated collection plate. Provided, however, that such losses are known to be less than or equal to 5% of the total delivered drug mass into the impactor and that there are no instructions to the contrary in an individual monograph, the

technique may be simplified by only assaying drug on the collection plates.

Re-Entrainment—Where method variations are possible, the selected technique should seek to minimize particle re-entrainment (from an upper to a lower impaction stage) on stages that contribute to size fractions defined in the individual monograph, especially where this may affect the amounts of drug collected. Minimizing the number of sampled doses, the use of coated particle collection surfaces, and proving that multiple-dose techniques produce statistically similar results to those from smaller numbers of doses, are all methods that can be used for this purpose. In the event that re-entrainment cannot be avoided, the number of doses collected, the time interval between doses, and the total duration of airflow through the cascade impaction device should be standardized. Under these circumstances, the presentation of impaction data should not presume the validity of the impactor's calibration (i.e., aerodynamic diameter ranges should not be assigned to drug masses collected on specific stages).

By using appropriate assay methods and a suitable mensurated impaction device, aerodynamic particle size distributions can be determined for drugs leaving the mouthpieces of metered-dose or dry powder inhalers. If temperature or humidity limits for use of the inhaler are stated on the label, it may be necessary to control the temperature and humidity of the air surrounding and passing through the device to conform to those limits. Ambient conditions are presumed, unless otherwise specified in individual monographs.

Mass Balance—In addition to the size distribution, good analytical practice dictates that a mass-balance be performed in order to confirm that the amount of the drug discharged from the inhaler, which is captured and measured in the induction port-cascade impactor apparatus, is within an acceptable range around the expected value. The total mass of drug collected in all of the components (material balance) divided by the total number of minimum recommended doses discharged is not less than 75% and not more than 125% of the average minimum recommended

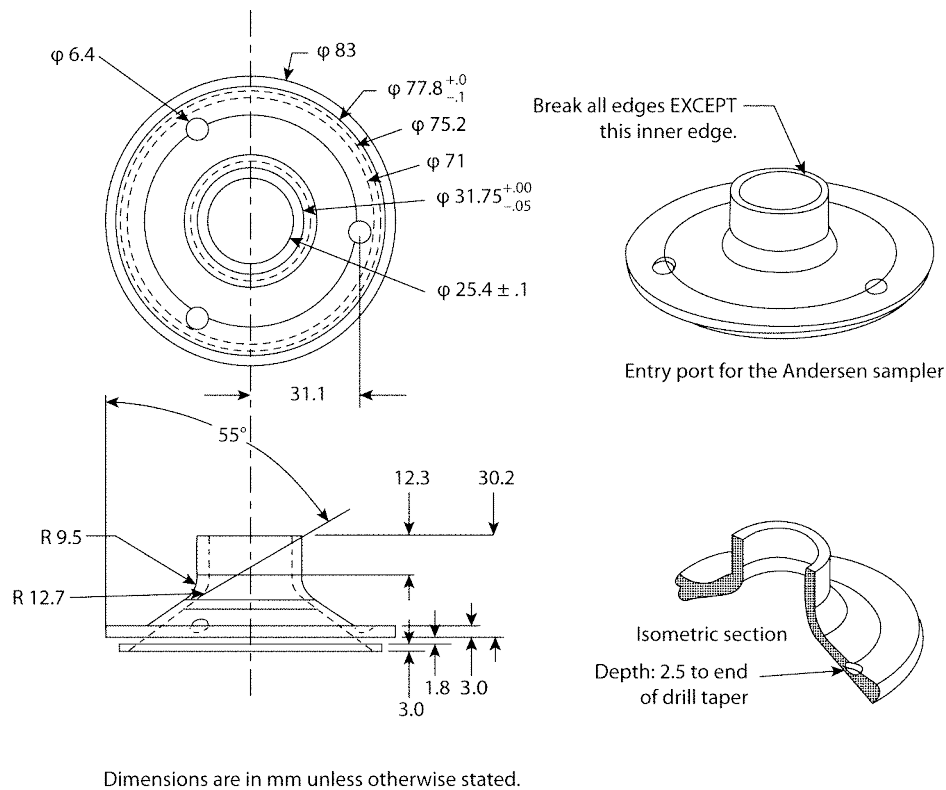


Fig. 4b. Apparatus 1: Expanded view of the entrance cone for mounting induction port on the Andersen cascade impactor without preseparator. Material may be aluminum, stainless steel, or other suitable material. Surface roughness (Ra) should be approximately 0.4 μm .

dose determined during testing for *Delivered-Dose Uniformity*. This is not a test of the inhaler but serves to ensure that the test results are valid.

Use one of the multistage impaction devices shown below, or an equivalent, to determine aerodynamic particle size distributions of drugs leaving the mouthpieces of metered-dose or dry powder inhalers. *Apparatus 1* and 6 [Figures 4 and 9 (without preseparator), respectively] are intended for use with metered-dose inhalers at a single airflow rate. *Apparatus 2, 3, 4, and 5* (Figures 6, 7, 8, and 9, respectively) are intended for use with dry powder inhalers at the appropriate airflow rate, Q_{out} , determined earlier, provided that the value of Q_{out} falls in the range 30–100 L per minute.

NOTE—If Q_{out} is greater than 100 L per minute, testing should be performed with Q_{out} set at 100 L per minute; if Q_{out} is less than 30 L per minute, testing is performed with Q_{out} at 30 L per minute.

Apparatus 1 for Metered-Dose Inhalers—Use this apparatus, or an equivalent, at a flow rate of 28.3 L per minute ($\pm 5\%$), as specified by the manufacturer of the cascade impactor.

Design—The design and assembly of this apparatus and the induction port to connect the device to an inhaler are shown in Figures 4, 4a, and 4b¹.

Critical engineering dimensions applied by manufacturers to the stages of *Apparatus 1* are provided in Table 2. During use, some occlusion and blockage of jet nozzles may occur

and therefore, “in use” mensuration tolerances need to be justified.

Table 2. Critical Dimensions for the Jet Nozzles of Apparatus 1

Stage #	Number of Jets	Nozzle Diameter (mm)
0	96	2.55 ± 0.025
1	96	1.89 ± 0.025
2	400	0.914 ± 0.0127
3	400	0.711 ± 0.0127
4	400	0.533 ± 0.0127
5	400	0.343 ± 0.0127
6	400	0.254 ± 0.0127
7	201	0.254 ± 0.0127

Procedure—Set up the multistage cascade impactor as described in the manufacturer’s literature with an after filter below the final stage to capture any fine particles that otherwise would escape from the device. To ensure efficient particle capture, coat the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid typically deposited from a volatile solvent, unless it has been demonstrated to be unnecessary. Attach the induction port and mouthpiece adapter to produce an airtight seal between the inhaler mouthpiece and the induction port as shown in Figure 4. Use a mouthpiece adapter that ensures that the tip of the inhaler mouthpiece is flush with the open end of the induction port. Ensure that the various stages of the cascade impactor are connected with airtight seals to prevent leaks. Turn on the vacuum pump to draw air through the cascade impactor, and calibrate the airflow through the system with an appropriate flowmeter attached to the open end of the induction port. Adjust the flow-control valve on the vacuum pump to achieve steady flow through the system at the required rate, and ensure that

¹ A suitable cascade impactor is available as Model Mk II from Thermo-Electron, 27 Forge Parkway, Franklin, MA 02038. The impactor is used without the preseparator. The inhaler is connected to the impactor via the induction port, atop the entrance cone shown in Figure 4. If an equivalent impactor is employed, the induction port in Figure 4a should be used, although the entrance cone (Fig. 4b) should be replaced with one to fit the impactor in question. Note that the internal surfaces of the induction port (Fig. 4a) are designed to fit flush with their counterparts in the entrance cone (Fig. 4b). This design avoids aerosol capture at the junction of the two pipes.

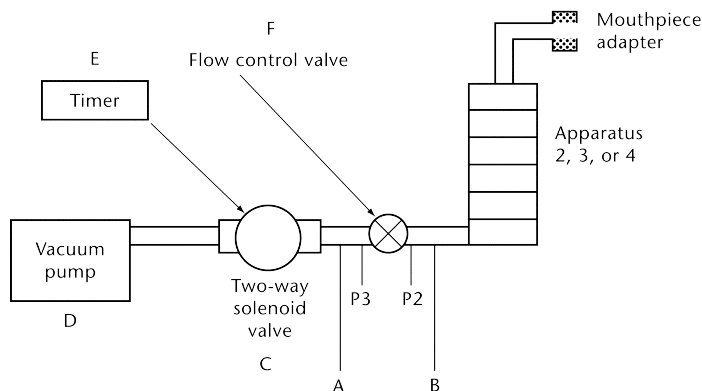


Fig. 5. Apparatus 2, 3, 4, or 5: General control equipment. (See Table 3 for component specifications.)

the airflow through the system is within $\pm 5\%$ of the flow rate specified by the manufacturer. Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 seconds and discharge one delivery to waste. With the vacuum pump running, insert the mouthpiece into the mouthpiece adapter and immediately fire the minimum recommended dose into the cascade impactor. Keep the valve depressed for a duration sufficient to ensure that the dose has been completely discharged. If additional sprays are required for the sample, wait for 5 seconds before removing the inhaler from the mouthpiece adapter, shake the inhaler, reinsert it into the mouthpiece adapter, and immediately fire the next minimum recommended dose. Repeat until the required number of doses have been discharged. The number of minimum recommended doses discharged must be sufficient to ensure an accurate and precise determination of *Aerodynamic Size Distribution*. [NOTE—The number of minimum recommended doses is typically not greater than 10.] After the last dose has been discharged, remove the inhaler from the mouthpiece adapter. Rinse the mouthpiece adapter and induction port with a suitable solvent, and dilute quantitatively to an appropriate volume. Disassemble the cascade impactor, place each stage and its associated collection plate or filter in a separate container, and rinse the drug from each of them. [NOTE—If it has been determined that wall losses in the impactor are less than or equal to 5%, then the collection plates only may be used.]

Dilute each quantitatively to an appropriate volume. Using the method of analysis specified in the individual monograph, determine the mass of drug collected in each of the components. To analyze the data, proceed as directed under *Data Analysis*.

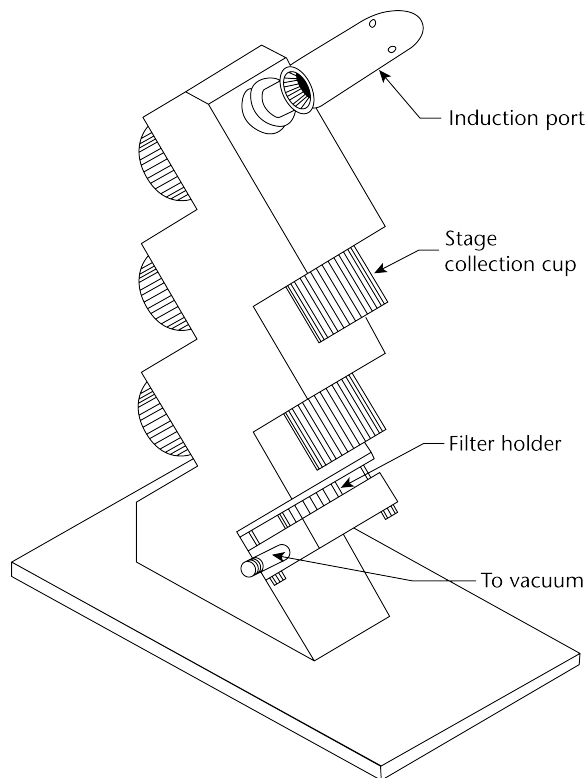


Fig. 6. Apparatus 2: Assembly of induction port, stage collector, and filter holder. (Marple-Miller impactor, Model 160 with USP induction port.)

Apparatus 2 for Dry Powder Inhalers—

Design—The design and assembly of Apparatus 2, and the induction port to connect the device to an inhaler, are shown in Figure 6.² [NOTE—The induction port is shown in detail in Figure 4a.] The impactor has five impaction stages and an after filter. At a volumetric airflow rate of 60 L per minute (the nominal flow rate, Q_n), the cutoff aerodynamic diameters D_{50,Q_n} of Stages 1 to 5 are 10, 5, 2.5, 1.25, and 0.625 μm , respectively. The after filter effectively retains aerosolized drug in the particle size range up to 0.625 μm . Set up the multistage cascade impactor with the control

² The cascade impactor is available as the Model 160 Marple-Miller Impactor from MSP Corporation, Minneapolis, MN. The inhaler should be connected to the impactor via the induction port, shown in Figure 4a.

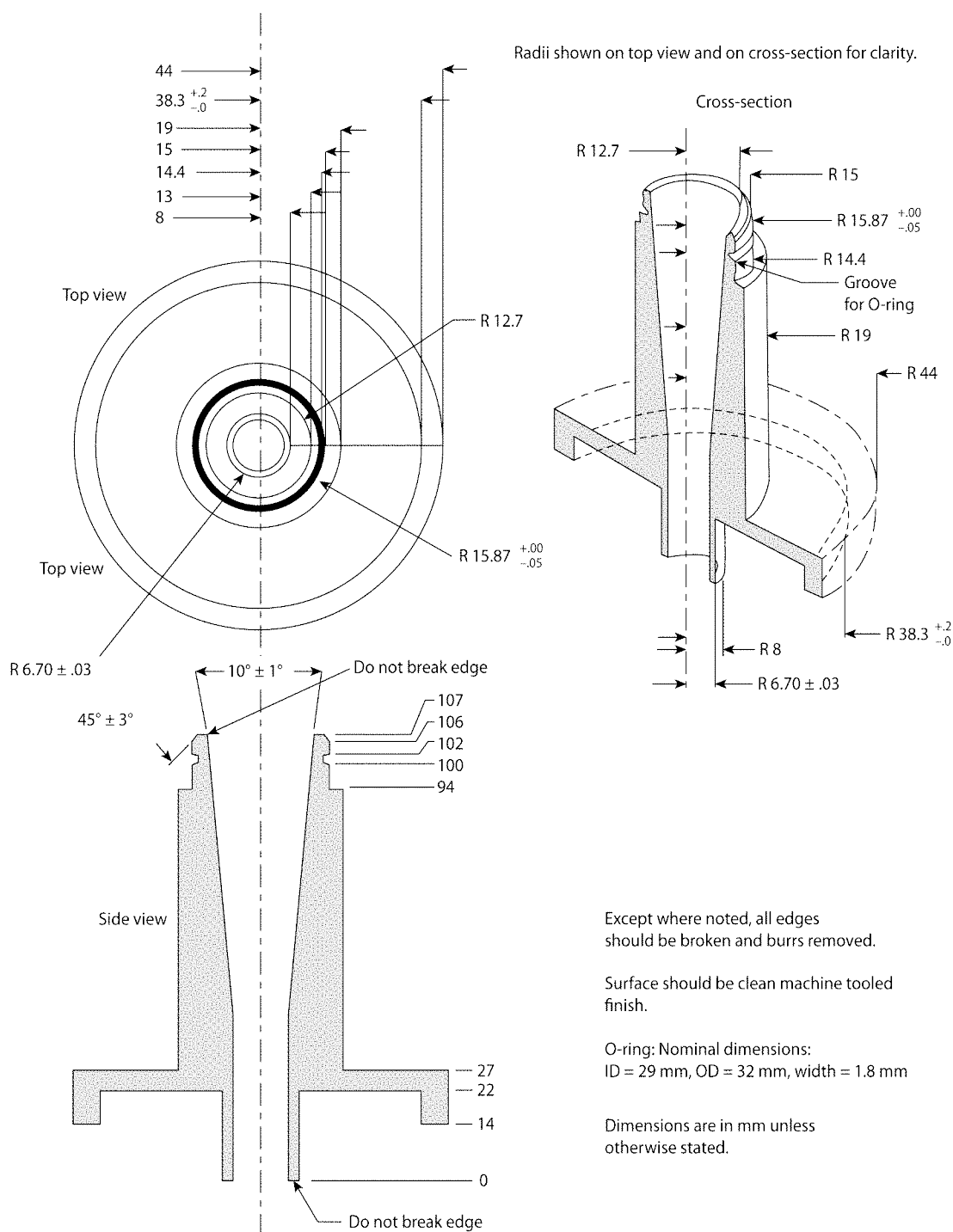


Fig. 7. Apparatus 3: Expanded views of top for the Andersen preseparator adapted to the USP induction port. Material may be aluminum, stainless steel, or other suitable material; interior bore should be polished to surface roughness (Ra) approximately 0.4 μm .

system as specified in Figure 5. To ensure efficient particle capture, coat the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid typically deposited from a volatile solvent, unless it has been demonstrated to be unnecessary. Assemble the impactor as described in the manufacturer's literature with an after filter below the final stage to capture any fine particles that otherwise would escape from the device. Attach the induction port and mouthpiece adapter to produce an airtight seal between the inhaler mouthpiece and the induction port.

Use a mouthpiece adapter that ensures that the tip of the inhaler mouthpiece is flush with the open end of the induction port. Ensure that the various stages of the cascade impactor are connected with airtight seals to prevent leaks.

Turn on the vacuum pump, open the solenoid valve, and calibrate the airflow through the system as follows. Connect a flowmeter to the induction port. Use a flowmeter calibrated for the volumetric flow leaving the meter to directly determine Q_{out} , or, if such a meter is unobtainable, calculate the volumetric flow leaving the meter (Q_{out}) using the ideal

gas law. For example, for a meter calibrated for the entering volumetric flow (Q_{in}), use the formula:

$$Q_{out} = Q_{in}P_0/(P_0 - \Delta P)$$

where P_0 is the atmospheric pressure and ΔP is the pressure drop over the meter. Adjust the flow-control valve to achieve a steady flow through the system at the required rate, Q_{out} , so that Q_{out} is within $\pm 5\%$ of the value determined during testing for *Delivered-Dose Uniformity*. Ensure that critical flow occurs in the flow-control valve, at the air-flow rate to be used during testing, by using the following procedure. With the inhaler in place, and the intended flow running, measure the absolute pressure on both sides of the flow-control valve (P2 and P3 in Figure 5). A ratio of $P3/P2 \leq 0.5$ indicates critical flow. Switch to a more powerful pump, and remeasure the test flow rate if $P3/P2 > 0.5$. Adjust the timer controlling the operation of the two-way solenoid valve so that it opens this valve for a duration of T seconds as determined during testing for *Delivered-Dose Uniformity*. Prime or load the dry powder inhaler with powder for inhalation according to the labeled instructions. With the vacuum pump running and the two-way solenoid valve closed, insert the inhaler mouthpiece, held horizontally, into the induction port mouthpiece adapter. Discharge the powder into the apparatus by opening the two-way solenoid valve for a duration of T seconds. After the two-way solenoid valve has closed, remove the inhaler from the mouthpiece adapter. If additional doses are required for the sample, reload the inhaler according to the labeled instructions, reinsert the mouthpiece into the mouthpiece adapter, and repeat the operation until the required number of doses

have been discharged. After discharge of the last dose, switch off the vacuum pump.

Rinse the mouthpiece adapter and induction port with a suitable solvent, and quantitatively dilute to an appropriate volume. Disassemble the cascade impactor, and place the after filter in a separate container. Rinse the drug from each of the stages and the filter, and quantitatively dilute each to an appropriate volume. Using the method of analysis specified in the individual monograph, determine the mass of drug collected in each of the components. Determine the cutoff diameters of each of the individual stages of the impactor, at the value of $Q = Q_{out}$ employed in the test by the formula:

$$D_{50,Q} = D_{50,Qn}(Q_n/Q)^{1/2}, \quad (\text{Eq. 1})$$

where $D_{50,Q}$ is the cutoff diameter at the flow rate, Q, employed in the test, and the subscript, n, refers to the nominal values determined when Q_n equals 60 L per minute. Thus, when Q equals 40 L per minute, the cutoff diameter of Stage 2 is given by the formula:

$$D_{50,40\text{LPM}} = 5 \mu\text{m} \times [60/40]^{1/2} = 6.1 \mu\text{m}.$$

General Procedure—Perform the test using *Apparatus 2* at the airflow rate, Q_{out} determined earlier, during testing for *Delivered-Dose Uniformity*, provided Q_{out} is less than or equal to 100 L per minute. [NOTE—If Q_{out} is greater than 100 L per minute, use an airflow rate of 100 L per minute.] Connect the apparatus to a flow control system that is based upon critical (sonic) flow as specified in Figure 5 (see also Table 3).

Table 3. Component Specifications for Figure 5

Code	Item	Description	Dimensions
A	Connector	(e.g., short metal coupling with low diameter branch to P3)	≥ 8 -mm ID
B	Vacuum tubing	(e.g., silicon tubing with an outside diameter of 14 mm and an internal diameter of 8 mm)	A length of suitable tubing ≥ 8 mm ID with an internal volume of 25 ± 5 mL.
C	Two-way solenoid valve ^a	See Fig. 5	2-way, 2-port solenoid valve having an ID ≥ 8 mm and an opening response time of ≤ 100 milliseconds.
D	Vacuum pump ^b	See Fig. 5	Pump must be capable of drawing the required flow rate through the assembled apparatus with the dry powder inhaler in the mouthpiece adapter. Connect the pump to the solenoid valve using short and wide (≥ 10 -mm ID) vacuum tubing and connectors to minimize pump capacity requirements.
E	Timer ^c	See Fig. 5	The timer switches current directly to the solenoid valve for the required duration.
P2, P3	Pressure measurements		Determine under steady-state flow conditions with an absolute pressure transducer.
F	Flow control valve ^d	See Fig. 5	Adjustable regulating valve with maximum $C_v \geq 1$.

^aAn example being ASCO product number 8030G13 (Automatic Switch Company, 60 Hanover Road, Florham Park, NJ 07932) or equivalent. See also Footnote *h* in Table 1.

^bGast product type 1023, 1423, or 2565 (Gast Manufacturing Inc., PO Box 97, Benton Harbor, MI 49022) or equivalent.

^cAn example being Eaton Product number 45610-400 (Eaton Corporation, Automotive Products Division, 901 South 12th Street, Watertown, WI 53094) or equivalent.

^dParker Hannifin type 8FV12LNSS, or equivalent (Parker Hannifin plc, Riverside Road, Barnstable, Devon EX31 1NP, UK). See also Footnote *h* in Table 1.

Table 4. Component Units of Multistage Liquid Impinger (see Fig. 8)

Code ¹	Item	Description	Dimensions ²
A,H	Jet tube	Metal tube screwed onto partition wall sealed by gasket (C), polished inner surface	see Figure 8a
B,G	Partition wall	Circular metal plate, diameter	120
		Thickness	see Figure 8a
C	Gasket	e.g., PTFE	to fit jet tube
D	Impaction plate	Porosity O sintered-glass disk,	
		Diameter	see Figure 8a
E	Glass cylinder	Plane polished cut glass tube	
		Height, including gaskets	46
		Outer diameter	100
		Wall thickness	3.5
		Sampling port (F) diameter	18
		Stopper in sampling port	ISO 24/25
I	Metal frame	L-profiled circular frame with slit	
		Inner diameter	to fit impaction plate
		Height	4
		Thickness of horizontal section	0.5
		Thickness of vertical section	2
K	Wire	Steel wire interconnecting metal frame and sleeve (two for each frame)	
		Diameter	1
L	Sleeve	Metal sleeve secured on jet tube by screw	
		Inner diameter	to fit jet tube
		Height	6
		Thickness	5
M	Gasket	e.g., silicone	to fit glass cylinder
N	Bolt	Metal bolt with nut (six pairs), length	205
		Diameter	4
P	O-ring	Rubber O-ring, diameter × thickness	66.34 × 2.62
Q	O-ring	Rubber O-ring, diameter × thickness	29.1 × 1.6
R	Filter holder	Metal housing with stand and outlet	see Figure 8b
S	Filter support	Perforated sheet metal, diameter	65
		Hole diameter	3
		Distance between holes (center-points)	4
T	Snap-locks		
U	Multi-jet tube	Jet tube (H) ending in multijet arrangement	see inserts Figure 8a
V	Outlet	Outlet and nozzle for connection to vacuum	Internal diameter ≥ 10 (Figure 8b)

¹See Fig. 8.²Measurements in mm unless otherwise stated.

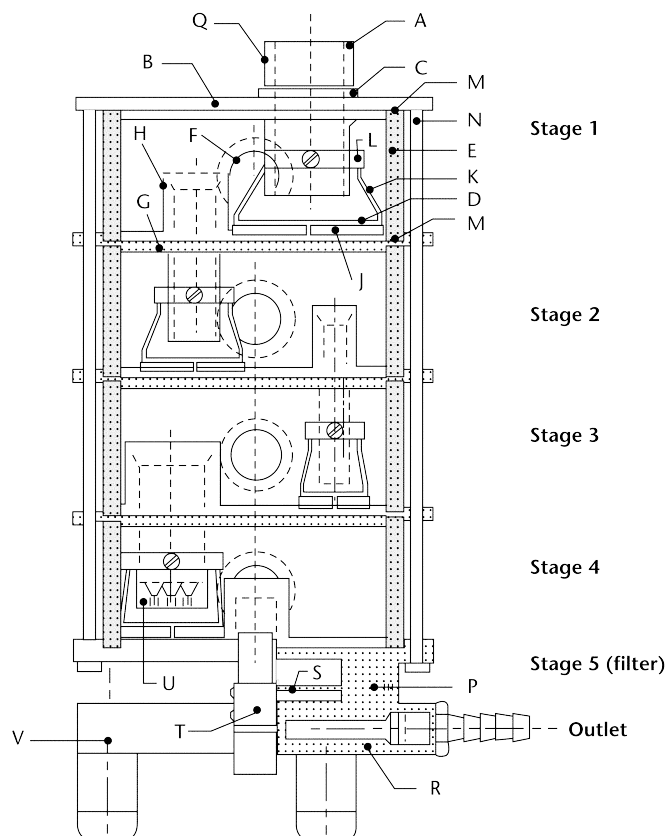


Fig. 8. Apparatus 4: Schematic of multistage liquid impinger. (See Table 4 for component specifications.)

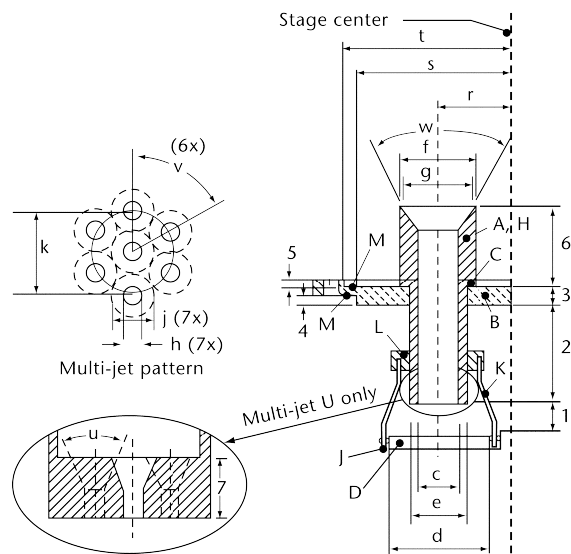


Fig. 8a. Apparatus 4: Details of jet tube and impaction plate. Inserts show end of multi-jet tube U leading to Stage 4. (See Table 5 for dimension specifications.)

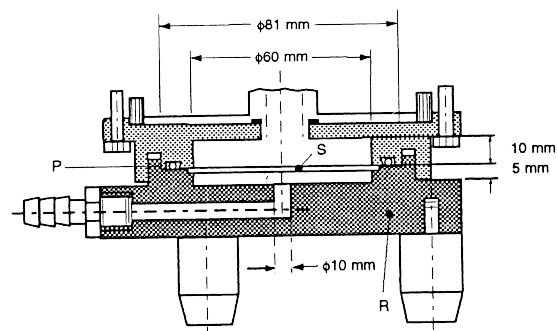


Fig. 8b. Apparatus 4: Expanded view of Stage 5. (See Table 4 for component specifications.)

Table 5. Apparatus 4: Dimensions¹ of Jet Tube with Impaction Plate (see Fig. 8a).

Type	Code ²	Stage 1	Stage 2	Stage 3	Stage 4	Filter (Stage 5)
Distance	1	9.5 (-0, +5)	5.5 (-0, +5)	4.0 (-0, +5)	6.0 (-0, +5)	n.a.
Distance	2	26	31	33	30.5	0
Distance	3	8	5	5	5	5
Distance	4	3	3	3	3	n.a.
Distance	5	0	3	3	3	3
Distance	6 ³	20	25	25	25	25
Distance	7	n.a.	n.a.	n.a.	8.5	n.a.
Diameter	c	25	14	8.0(±0.1)	21	14
Diameter	d	50	30	20	30	n.a.
Diameter	e	27.9	16.5	10.5	23.9	n.a.
Diameter	f	31.75 (-0.05, +0.00)	22	14	31	22

¹Measurements in mm with tolerances according to ISO 2768-m, unless otherwise stated.

²See Fig. 8a.

³Including gasket.

⁴Relative centerline of stage compartment.

n.a.: not applicable.

Table 5. Apparatus 4: Dimensions¹ of Jet Tube with Impaction Plate (see Fig. 8a). (Continued)

Type	Code ²	Stage 1	Stage 2	Stage 3	Stage 4	Filter (Stage 5)
Diameter	g	25.4	21	13	30	21
Diameter	h	n.a.	n.a.	n.a.	2.70 (±.05)	n.a.
Diameter	j	n.a.	n.a.	n.a.	6.3	n.a.
Diameter	k	n.a.	n.a.	n.a.	12.6	n.a.
Radius ⁴	r	16	22	27	28.5	0
Radius ⁴	s	46	46	46	46	n.a.
Radius ⁴	t	n.a.	50	50	50	50
Angle	w	10°	53°	53°	53°	53°
Angle	u	n.a.	n.a.	n.a.	45°	n.a.
Angle	v	n.a.	n.a.	n.a.	60°	n.a.

¹Measurements in mm with tolerances according to ISO 2768-m, unless otherwise stated.

²See Fig. 8a.

³Including gasket.

⁴Relative centerline of stage compartment.

n.a.: not applicable.

Under steady flow conditions, at the appropriate volumetric airflow rate through the entire apparatus, ensure that critical (sonic) flow occurs in the flow control valve by determining the individual values for absolute pressure, P₂ and P₃, so that their ratio P₃/P₂ is less than or equal to 0.5. Coat the particle collection surface of each of the stages of the cascade impactor to ensure that particles that have impacted on a given stage are not re-entrained in the flowing airstream, unless this has been shown to be unnecessary. Analyze the data as directed under *Data Analysis*.

Apparatus 3 for Dry Powder Inhalers—

Design—Apparatus 3 is identical to Apparatus 1 (Figure 4), except that the manufacturer's preseparator is added atop Stage 0 to collect large masses of noninhalable powder prior to their entry into the impactor, and the outlet nipple, used to connect to vacuum tubing B (Figure 5), is replaced with one having an internal diameter ≥ 8 mm. To connect the preseparator of the impactor to the induction port (Figure 4a), a specially designed top for the preseparator must be used. This is shown in Figure 7.³ The impactor, therefore, has eight stages, a preseparator (to collect large particulates), and an after filter. At a volumetric airflow rate of 28.3 L per minute (the nominal flow rate, Q_n), the cutoff aerodynamic diameters D_{50,Qn} of Stages 0 to 7 are 9.0, 5.8, 4.7, 3.3, 2.1, 1.1, 0.7, and 0.4 μm, respectively. The after filter effectively retains aerosolized drug in the particle size range up to 0.4 μm. Connect the cascade impactor into the control system specified in Figure 5. Omit Stage 6 and Stage 7 from the impactor if the test flow rate, Q_{out}, used during testing for *Delivered-Dose Uniformity* was greater than or equal to 60 L per minute. To ensure efficient particle capture, coat the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid typically deposited from a volatile solvent, unless it has been demonstrated to be unnecessary. Assemble the impactor as described in the manufacturer's literature with an after filter below the final stage to capture any fine particles that otherwise would escape from the device. Place an appropriate volume (up to 10 mL) of an appropriate solvent into the preseparator, or coat the particle collection surfaces of the preseparator to prevent re-entrainment of impacted particles. [Caution—Some solvents form flammable vapor-air mixtures that may be ignited during passage through a vacuum pump. Take appropriate precautions (alternative solvents, use of vapor traps, minimal pump operating times, etc.) to ensure operator safety during testing.] Attach a molded mouthpiece adapter to the end of the induction port to produce an airtight seal between the inhaler mouthpiece and the induction port. Use a mouthpiece adapter that ensures that the tip of the inhaler mouthpiece is flush with the open end of the induction

port. Ensure that the various stages of the cascade impactor are connected with airtight seals to prevent leaks.

Turn on the vacuum pump, open the two-way solenoid valve, and calibrate the airflow through the system as follows. Prime or load the dry powder inhaler with powder for inhalation according to the labeled instructions. With the vacuum pump running and the two-way solenoid valve closed, insert the inhaler mouthpiece, held horizontally, into the induction port mouthpiece adapter. Once the inhaler is positioned, discharge the powder into the apparatus by activating the timer and opening the two-way solenoid valve for the required duration, T ± 5%, as determined during testing for *Delivered-Dose Uniformity*. After the two-way solenoid valve has closed, remove the inhaler from the mouthpiece adapter. If additional doses are required for the sample, reload the inhaler according to the labeled instructions, reinsert the mouthpiece into the mouthpiece adapter, and repeat the operation until the required number of doses have been discharged. After discharge of the last dose, remove the inhaler from the mouthpiece adapter, and switch off the vacuum pump.

Carefully disassemble the apparatus. Using a suitable solvent, rinse the drug from the mouthpiece adapter, induction port, and preseparator, and quantitatively dilute to an appropriate volume. Rinse the drug from each stage, and the impaction plate immediately below, into appropriately sized flasks. Quantitatively dilute each flask to an appropriate volume. Using the method of analysis specified in the individual monograph, determine the mass of drug collected in each of the samples. The aerodynamic cutoff diameters of the individual stages of this device, in the airflow range between 30 and 100 L per minute, are currently not well established. Do not use the formula in Equation 1 to calculate cutoff diameters.

Procedure—Proceed as directed in the *General Procedure* under Apparatus 2, except to use Apparatus 3.

Apparatus 4 for Dry Powder Inhalers—

NOTE—Apparatus 4, the multistage liquid impinger, has a small number of stages and is used extensively outside the USA. It is provided here for the benefit of users in countries other than the USA.

Design—The design and assembly of Apparatus 4 are shown in Figs. 8, 8a, and 8b.⁴ The induction port, used to connect the device to an inhaler, is shown in Fig. 4a. The device is a multi-stage liquid impinger consisting of impaction Stages 1, 2, 3, and 4 and an integral after filter (Stage 5). The collection stages of the liquid impinger (see Fig. 8 and Table 4) are kept moist, unlike those of traditional impactors, such as Apparatus 1, 2, 3, 5, and 6; wetting may

³The cascade impactor is available as the Andersen 1ACFM Non-Viable Cascade Impactor (Mark II) from Thermo-Electron, 27 Forge Parkway, Franklin, MA 02038. The impactor is used with the preseparator.

⁴The five-stage impinger is available from Copley Instruments, plc, Nottingham, UK. The inhaler should be connected to the impactor via the induction port, shown in Fig. 4 and Fig. 4a.

produce an effect similar to coating the stages of *Apparatus 2*, *3*, *5*, and *6* at certain flow rates, although this should be confirmed by demonstrating control over re-entrainment as described earlier. An impaction stage comprises an upper horizontal metal partition wall (B) through which a metal inlet jet tube (A) with its impaction plate (D) is protruding; a glass cylinder (E) with sampling port (F), forming the vertical wall of the stage; and a lower horizontal metal partition wall (G) through which a jet tube (H) connects to the lower stage. The tube into Stage 4 (U) ends in a multi-jet arrangement. The impaction plate (D) is secured in a metal frame (J), which is fastened by two wires (K) to a sleeve (L) secured on the jet tube (C). For more detail of the jet tube and impaction plate, see *Fig. 8a*. The horizontal plane of the collection plate is perpendicular to the axis of the jet tube and centrally aligned. The upper surface of the impaction plate is slightly raised above the edge of the metal frame. A recess around the perimeter of the horizontal partition wall guides the position of the glass cylinder. The glass cylinders are sealed against the horizontal partition walls with gaskets (M) and clamped together by six bolts (N). The sampling ports are sealed by stoppers. The bottom side of the lower partition wall of Stage 4 has a concentric protrusion fitted with a rubber O-ring (P) that seals against the edge of a filter placed in the filter holder. The filter holder (R) is a basin with a concentric recess in which a perforated filter support (S) is flush-fitted. The filter holder is designed for 76-mm diameter filters. The whole impaction stage assembly is clamped onto the filter holder by two snap locks (T). The impinger is equipped with an induction port (*Fig. 4a*) that fits onto the Stage 1 inlet jet tube. A rubber O-ring on the jet tube provides an airtight connection to the induction port. An elastomeric mouthpiece adapter to fit the inhaler being tested provides an airtight seal between the inhaler and the induction port.

At a volumetric airflow rate of 60 L per minute (the nominal flow rate, Q_n), the cutoff aerodynamic diameters D_{50,Q_n} of Stages 1 to 4 are 13.0, 6.8, 3.1, and 1.7 μm , respectively. The after filter effectively retains aerosolized drug in the particle size range up to 1.7 μm . Ensure that *Apparatus 4* is clean and free of drug solution from any previous tests. Place a 76-mm diameter filter in the filter stage, and assemble the apparatus. Use a low pressure filter capable of quantitatively collecting the passing drug aerosol, which also allows a quantitative recovery of the collected drug. Set up *Apparatus 4* using the control system as specified in *Figure 5*. Attach the induction port (*Figure 4a*) and mouthpiece adapter to produce an airtight seal between the inhaler mouthpiece and the induction port. Use a mouthpiece adapter that ensures that the tip of the inhaler mouthpiece is flush with the open end of the induction port. Ensure that the various stages of the apparatus are connected with airtight seals to prevent leaks. Turn on the vacuum pump, open the two-way solenoid valve, and calibrate the airflow through the system as follows. Connect a flowmeter, calibrated for the volumetric flow rate leaving the meter, to the induction port. Adjust the flow-control valve to achieve a steady flow through the system at the required rate, Q_{out} , so that Q_{out} is within $\pm 5\%$ of the value determined during testing for *Delivered-Dose Uniformity*. Ensure that critical flow occurs in the flow-control valve, at the value of Q_{out} to be used during testing, using the following procedure. With the inhaler in place, and the intended flow running, measure the absolute pressure on both sides of the flow-control valve (P2 and P3 in *Figure 5*). A ratio of $P3/P2 \leq 0.5$ indicates critical flow. Switch to a more powerful pump, and remeasure the test flow rate if $P3/P2 > 0.5$. Adjust the timer controlling the operation of the two-way solenoid valve so that it opens that valve for the same duration, T , as used during testing for *Delivered-Dose Uniformity*. Dispense 20 mL of a solvent, capable of dissolving the drug, into each of the four upper stages of *Apparatus 4*, and replace the stoppers. [Caution—Some solvents form flammable vapor-air mixtures that may be ignited during passage through a vacuum pump. Take appropriate precautions (alternative solvents, use of vapor

traps, minimal pump operating times, etc.) to ensure operator safety during testing.] Tilt the apparatus to wet the stoppers, thereby neutralizing their electrostatic charge. Adjust the timer controlling the operation of the two-way solenoid valve so that it opens the valve for the same duration, T , as used during testing for *Delivered-Dose Uniformity*. Prime or load the dry powder inhaler with powder for inhalation according to the labeled instructions. With the vacuum pump running and the two-way solenoid valve closed, insert the inhaler mouthpiece, held horizontally, into the induction port mouthpiece adapter. Discharge the powder into the apparatus by activating the timer and opening the two-way solenoid valve for the required duration, $T \pm 5\%$. After the two-way solenoid valve has closed, remove the inhaler from the mouthpiece adapter. If additional doses are required for the sample, reload the inhaler according to the labeled instructions, reinsert the mouthpiece into the mouthpiece adapter, and repeat the operation until the required number of doses have been discharged. After discharge of the last dose, switch off the vacuum pump.

Dismantle the filter stage of *Apparatus 4*. Carefully remove the filter, and extract the drug with solvent. Rinse the mouthpiece adapter and induction port with a suitable solvent, and quantitatively dilute to an appropriate volume. Rinse the inside of the inlet jet tube to Stage 1 (*Figure 8*), allowing the solvent to flow into the stage. Rinse the drug from the inner walls and the collection plate of each of the four upper stages of the apparatus, into the solution in the respective stage, by tilting and rotating the apparatus, while ensuring that no liquid transfer occurs between the stages. Using the method of analysis specified in the individual monograph, determine the mass of drug collected in each of the six volumes of solvent. Ensure that the method corrects for possible evaporation of the solvent during the test. This may involve the use of an internal standard (of known original concentration in the solvent and assayed at the same time as the drug) or the quantitative transfer of the liquid contents from each of the stages, followed by dilution to a known volume. Determine the cutoff diameters of each of the individual stages of the impactor, at the value of $Q = Q_{out}$ employed in the test by the formula:

$$D_{50,Q} = D_{50,Q_n} (Q_n/Q)^{1/2}$$

where $D_{50,Q}$ is the cutoff diameter at the flow rate, Q , employed in the test, and the subscript, n , refers to the nominal values determined when Q_n equals 60 L of air per minute. Thus, when Q equals 40 L of air per minute, the cutoff diameter of Stage 2 is given by the formula:

$$D_{50,40\text{LPM}} = 6.8 \mu\text{m} \times (60/40)^{1/2} = 8.3 \mu\text{m}.$$

Procedure—Proceed as directed in the *General Procedure* under *Apparatus 2*, except to use *Apparatus 4*.

Apparatus 5 for Dry Powder Inhalers—

Design—The design and assembly of *Apparatus 5*⁵ are shown in *Figures 9, 9a, 9b, 9c, and 9d*. The induction port, used to connect the device to an inhaler, is shown in *Figure 4a*. The device is a cascade impactor with seven stages and a micro-orifice collector (MOC). Over the design flow-rate range of 30 to 100 L per minute, the 50% efficiency cut-off diameters of the stages (D_{50} values) range between 0.24 μm to 11.7 μm , evenly spaced on a logarithmic scale. In the design flow-rate range, there are always at least five stages with D_{50} values between 0.5 μm and 6.5 μm . The collection efficiency curves for each stage are sharp and minimize overlap between stages. Material may be aluminum, stainless steel, or other suitable material.

The impactor layout has removable impaction cups with all the cups in one plane (*Figures 9–9c*). There are three main sections to the impactor: the bottom frame that holds the impaction cups, the seal body that holds the jets, and

⁵The cascade impactor is available as the Next Generation Pharmaceutical Impactor from MSP Corporation, Minneapolis, MN.

Table 6. Critical Dimensions for Apparatus 5 and 6

Description	Dimension (mm)
Preseparator (dimension a—see Figure 9d)	12.80 ± 0.05
Stage 1 ¹ Nozzle diameter	14.30 ± 0.05
Stage 2 ¹ Nozzle diameter	4.88 ± 0.04
Stage 3 ¹ Nozzle diameter	2.185 ± 0.02
Stage 4 ¹ Nozzle diameter	1.207 ± 0.01
Stage 5 ¹ Nozzle diameter	0.608 ± 0.01
Stage 6 ¹ Nozzle diameter	0.323 ± 0.01
Stage 7 ¹ Nozzle diameter	0.206 ± 0.01
MOC ¹	approximately 0.070
Cup Depth (Dimension b—see Figure 9b)	14.625 ± 0.10
Collection cup surface roughness	0.5 to 2 μm
Stage 1 Nozzle to seal body distance ² —dimension c	0 ± 1.18
Stage 2 Nozzle to seal body distance ² —dimension c	5.236 ± 0.736
Stage 3 Nozzle to seal body distance ² —dimension c	8.445 ± 0.410
Stage 4 Nozzle to seal body distance ² —dimension c	11.379 ± 0.237
Stage 5 Nozzle to seal body distance ² —dimension c	13.176 ± 0.341
Stage 6 Nozzle to seal body distance ² —dimension c	13.999 ± 0.071
Stage 7 Nozzle to seal body distance ² —dimension c	14.000 ± 0.071
MOC Nozzle to seal body distance ² —dimension c	14.429 – 14.571

¹See Figure 9c.²See Figure 9b.

the lid that contains the interstage passageways (shown in Figures 9–9b). Multiple nozzles are used at all but the first stage (Figure 9c). The flow passes through the impactor in a saw-tooth pattern.

Stage mensuration is performed periodically together with confirmation of other dimensions critical to the effective operation of the impactor. Critical dimensions are provided below in Table 6.

In routine operation, the seal body and lid are held together as a single assembly. The impaction cups are accessible when this assembly is opened at the end of an inhaler test. The cups are held in a support tray, so that all cups can be removed from the impactor simultaneously by lifting out the tray.

An induction port with internal dimensions identical to those defined in Figure 4a is connected to the impactor inlet. When necessary, with dry powder inhalers, a preseparator can be added to avoid overloading the first stage. This preseparator connects between the induction port and the impactor. A suitable mouthpiece adapter is used to provide an airtight seal between the inhaler and the induction port.

At a volumetric airflow rate of 60 L per minute (the assigned reference flow rate for cutoff-diameter calculations, Q_{in}), the cutoff-aerodynamic diameters $D_{50,Q_{in}}$ of Stages 1 to 7 are 8.06, 4.46, 2.82, 1.66, 0.94, 0.55 and 0.34 μm, respectively. The apparatus contains a terminal micro-orifice collector (MOC) that for most formulations may eliminate the need for a final filter as determined by method validation. The MOC is an impactor nozzle plate and collection cup. The nozzle plate contains, nominally, 4032 jets, each approximately 70 μm in diameter. Most particles not captured on Stage 7 of the impactor will be captured on the cup surface below the MOC. (For impactors operated at 60 L per minute, the MOC is capable of collecting 80% of 0.14-μm particles). For formulations with a significant fraction of particles not captured by the MOC, there is an optional filter holder that can replace the MOC or be placed downstream of the MOC containing a suitable after-filter (glass fiber is often suitable).

Procedure—Assemble the apparatus with the preseparator (Figure 9d), unless experiments have shown that its omission does not result in increased interstage drug losses (>5%) or particle re-entrainment, in which case the preseparator may be omitted.

Place cups into the apertures in the cup tray. To ensure efficient particle capture, coat the particle collection surface

of each stage with glycerol, silicone oil, or other suitable liquid typically deposited from a volatile solvent, unless it has been demonstrated to be unnecessary. Insert the cup tray into the bottom frame, and lower into place. Close the impactor lid with the seal body attached, and operate the handle to lock the impactor together so that the system is airtight.

The preseparator may be assembled as follows: assemble the preseparator insert into the preseparator base; fit the preseparator base to the impactor inlet; add 15 mL of the solvent used for sample recovery to the central cup of the preseparator insert; place the preseparator body on top of this assembly; and close the two catches. [Caution—Some solvents form flammable vapor-air mixtures that may be ignited during passage through a vacuum pump. Take appropriate precautions (e.g., alternative solvents, use of vapor traps, minimal pump operating times, etc.) to ensure operator safety during testing.]

Connect an induction port with internal dimensions as defined in Figure 4a either to the impactor inlet or to the preseparator inlet atop the cascade impactor (Figure 9d). Place a suitable mouthpiece adapter in position at the end of the induction port so that the mouthpiece end of the inhaler, when inserted, lines up along the horizontal axis of the induction port. The front face of the inhaler mouthpiece is flush with the front face of the induction port, producing an airtight seal. When attached to the mouthpiece adapter, the inhaler should be positioned in the same orientation as intended for use. Connect the apparatus to a flow system according to the scheme specified in Figure 5.

Unless otherwise prescribed, conduct the test at the flow rate used in the test for *Delivered-Dose Uniformity* drawing 4 L of air from the mouthpiece of the inhaler and through the apparatus. Connect a flowmeter to the induction port. Use a flowmeter calibrated for the volumetric flow leaving the meter, or calculate the volumetric flow leaving the meter (Q_{out}) using the ideal gas law. For a meter calibrated for the entering volumetric flow (Q_{in}), use the formula:

$$Q_{out} = Q_{in}P_0/(P_0 - \Delta P)$$

where P_0 is the atmospheric pressure and ΔP is the pressure drop over the meter. Adjust the flow control valve to achieve steady flow through the system at the required rate, Q_{out} (±5%). Ensure that critical flow occurs in the flow-control valve by the procedure described for *Apparatus 2*. Adjust the timer controlling the operation of the two-way solenoid

valve so that it opens the valve for the same duration, *T*, as used during testing for *Delivered-Dose Uniformity*.

Prime or load the dry powder inhaler with powder for inhalation according to the labeled instructions. With the vacuum pump running and the two-way solenoid valve closed, insert the inhaler mouthpiece, held horizontally, into the induction port mouthpiece adapter. Discharge the powder into the apparatus by activating the timer and opening the two-way solenoid valve for the required duration, *T*(±5%). After the two-way solenoid valve has closed, remove the inhaler from the mouthpiece adapter. If additional doses are required for the sample, reload the inhaler according to the labeled instructions, reinsert the mouthpiece into the mouthpiece adapter, and repeat the operation until the required number of doses have been discharged. After discharge of the last dose, switch off the vacuum pump.

Dismantle the apparatus, and recover drug for analysis as follows: remove the induction port and mouthpiece adapter from the preseparator and extract the drug into an aliquot of solvent; if used, remove the preseparator from the impactor, without spilling the solvent into the impactor; and recover the active ingredient from all inner surfaces.

Open the impactor by releasing the handle and lifting the lid. Remove the cup tray, with the collection cups, and recover the active ingredient from each cup into an aliquot of solvent. Using the method of analysis specified in the individual monograph, determine the mass of drug contained in each of the aliquots of solvent.

Determine the cutoff diameters of each of the individual stages of the impactor, at the value of *Q* = *Q*_{out} employed in the test by the formula:

$$D_{50,Q} = D_{50,Q_n} (Q_n/Q)^x, \text{ (Eq. 2)}$$

where *D*_{50,*Q*} is the cutoff diameter at the flow rate, *Q* employed in the test, and the subscript, *n*, refers to the nominal or reference value for *Q*_{*n*} = 60 L of air per minute (see Table 7). The values for the exponent, *x*, are listed in Table 7. Thus, when *Q* = 40 L of air per minute, the cutoff diameter of Stage 2 is given by the formula:

$$D_{50,40LPM} = 4.46 \mu\text{m} \times (60/40)^{0.52} = 5.51 \mu\text{m}.$$

Analyze the data as directed under *Data Analysis*.

Table 7. Cutoff Aerodynamic Diameter for Stages of Apparatus 5 and 6

Use Eq. 2 to calculate *D*_{50,*Q*} for flow rates, *Q*, in the range 30 to 100 L per minute with *Q*_{*n*} = 60 L per minute.

Stage	<i>D</i> _{50,<i>Q</i>_{<i>n</i>}}	<i>x</i>
1	8.06	0.54
2	4.46	0.52
3	2.82	0.50
4	1.66	0.47
5	0.94	0.53
6	0.55	0.60
7	0.34	0.67

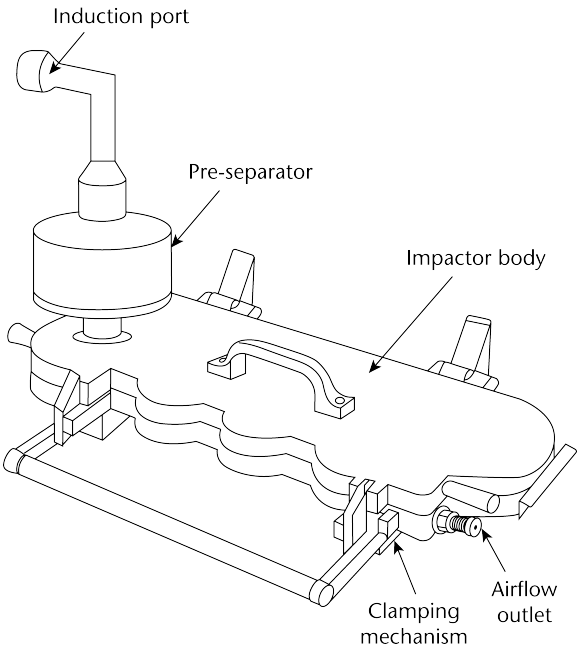


Fig. 9. Apparatus 5 (shown with the preseparator in place).

Apparatus 6 for Metered-Dose Inhalers—

Design—Apparatus 6 is identical to Apparatus 5 (Figures 9-9d), except that the preseparator is not to be used. Use this apparatus at a flow rate of 30 L per minute (±5%), unless otherwise prescribed in the individual monograph.

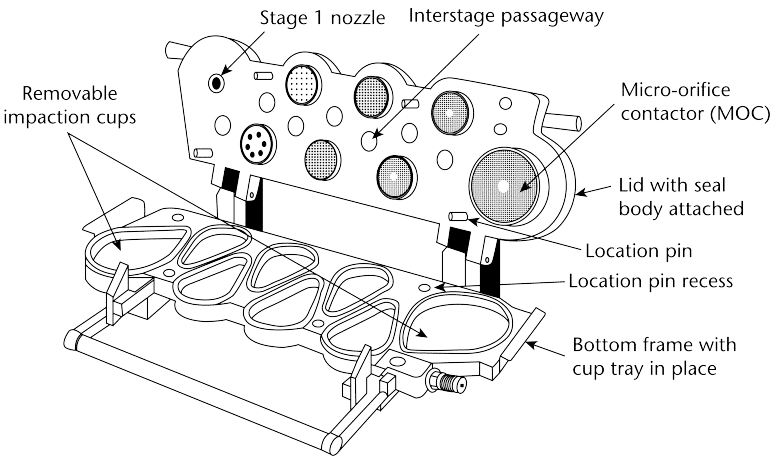


Fig. 9a. Components of Apparatus 5.

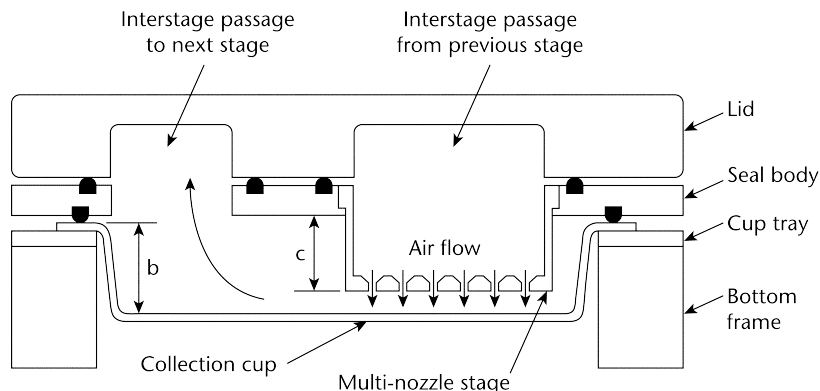


Fig. 9b. Layout of interstage passageways of Apparatus 5.

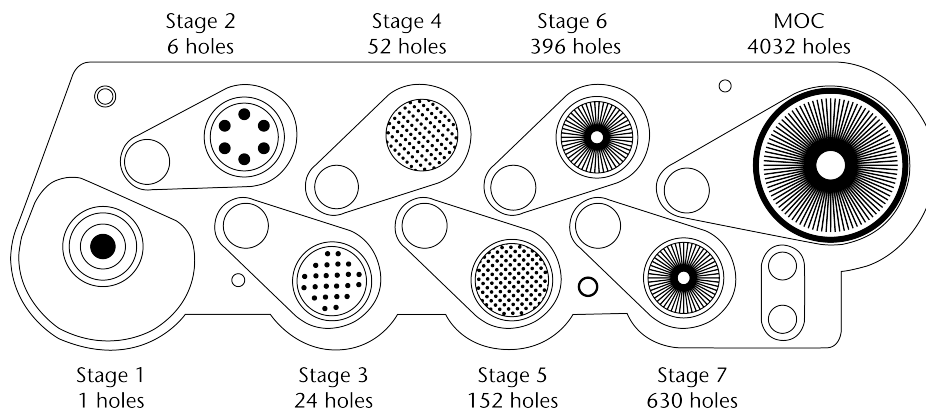


Fig. 9c. Nozzle configuration of Apparatus 5.

Procedure—Assemble the apparatus without the preseparator. Place cups into the apertures in the cup tray. To ensure efficient particle capture, coat the particle collection surface of each stage with glycerol, silicone oil, or other suitable liquid typically deposited from a volatile solvent, unless it has been demonstrated to be unnecessary. Insert the cup tray into the bottom frame, and lower into place. Close the impactor lid with seal body attached, and operate the handle to lock the impactor together so that the system is airtight. Connect an induction port with internal dimensions as defined in Figure 4a to the impactor inlet. Use a mouthpiece adapter that ensures that the tip of the inhaler mouthpiece is flush with the open end of the induction port. Turn on the vacuum pump to draw air through the cascade impactor, and calibrate the airflow through the system with an appropriate flowmeter attached to the open end of the induction port. Adjust the flow-control valve on the vacuum pump to achieve steady flow through the system at the required rate, and ensure that the airflow through the system is within $\pm 5\%$ of this flow rate. Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 seconds, and discharge one delivery to waste. With the vacuum pump running, insert the mouthpiece into the mouthpiece adapter, and immediately fire the minimum recommended dose into the cascade impactor. Keep the valve depressed for a duration sufficient to ensure that the dose has been completely discharged. If additional sprays are required for the sample, shake the inhaler, reinsert it into the mouthpiece adapter, and immediately fire the next minimum recommended dose.

Repeat until the required number of doses have been discharged. The number of minimum recommended doses discharged must be sufficient to ensure an accurate and precise determination of *Aerodynamic Size Distribution*. [NOTE—The number of minimum recommended doses is typically not greater than 10.] After the last dose has been discharged, remove the inhaler from the mouthpiece adapter. Rinse the mouthpiece adapter and induction port with a suitable solvent, and dilute quantitatively to an appropriate volume.

Dismantle the apparatus, and recover the drug for analysis as follows: remove the induction port and mouthpiece adapter from the apparatus, and recover the deposited drug into an aliquot of solvent; open the impactor by releasing the handle and lifting the lid; remove the cup tray, with the collection cups; and extract the active ingredient in each cup into an aliquot of solvent. Using the method of analysis specified in the individual monograph, determine the quantity of active ingredient contained in each of the aliquots of solvent.

Determine the cutoff diameters of each of the individual stages of the impactor, at the value of Q employed in the test by using Eq. 2 with values obtained from Table 7. Thus, when $Q = 30$ L of air per minute, the cutoff diameter of Stage 2 is given by the formula:

$$D_{50,30\text{LPM}} = 4.46 \mu\text{m} \times (60/30)^{0.52} = 6.40 \mu\text{m}.$$

To analyze the data, proceed as directed under *Data Analysis*.

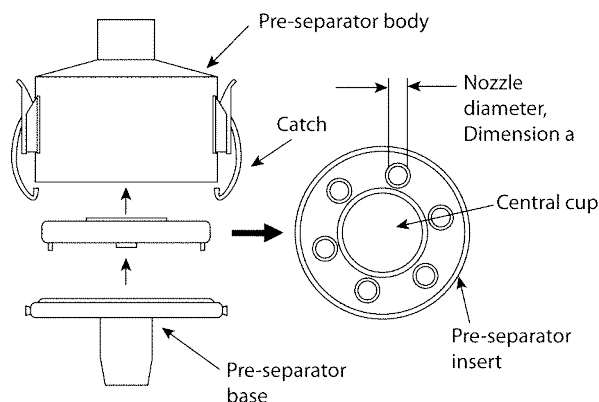


Fig. 9d. Pre-separator layout for Apparatus 5.

Data Analysis

This section describes the data analysis required to define the *Aerodynamic Size Distribution* of the drug output from the test inhaler, after the use of Apparatus 1, 2, 3, 4, 5, or 6. Enter the data collected from Apparatus 1, 2, 3, 4, 5, or 6 in the table of mass summaries as shown in Table 8. Perform only those calculations specified in the individual monograph.

CALCULATIONS

Fine Particle Dose and Fine Particle Fraction—Calculate the total mass, ΣA , of drug delivered from the mouthpiece of the inhaler into the apparatus. Then calculate the total mass, R , of drug found on the stages of the apparatus and the filter that captured the drug in the fine particle size range appropriate for the particular drug being tested. The *Fine Particle Dose* is calculated by the formula:

$$R/n$$

where R is as stated above, and n is the number of doses discharged during the test. The *Fine Particle Fraction* that would be delivered from the inhaler is then calculated by the formula:

$$R/\Sigma A.$$

Cumulative Percentage (Cum%) of Drug Mass Less Than Stated Aerodynamic Diameter—Construct Table 9 by dividing the mass of drug on the filter stage by ΣB (see Table 8). Multiply the quotient by 100, and enter this number as a percentage opposite the effective cutoff diameter of the stage immediately above it in the impactor or impinger stack. For Apparatus 2 or 4, use Equation 1 to calculate the stage cutoff diameters, $D_{50,Q}$, at the airflow rate, Q , employed during the test. For Apparatus 5 and 6, use Equation 2 with Table 7. For Apparatus 1, use the cutoff diameters quoted by the manufacturer. For Apparatus 3, present the data as cumulative percentages of mass on and below the stated stage, and avoid assigning values to stage cutoff diameters.

Table 8. Table of Mass Summaries for Analyses of Metered-Dose Inhalers and Dry Powder Inhalers

Mass	Apparatus 1		Apparatus 2		Apparatus 3 ^a		Apparatus 4 ^b		Apparatus 5 ^d		Apparatus 6 ^d	
Mouthpiece adapter	A _i	—	A _i A _i	—	A _i	—	A _i	—	A _i	—	A _i	—
Preseparator	—	—	—	—	A _p	—	—	—	A _p	—	—	—
Stage 0 of impactor	A ₀	B ₀	—	—	A ₀	B ₀	—	—	—	—	—	—
Stage 1 of impactor/impinger	A ₁	B ₁	A ₁	—	A ₁	B ₁	A ₁	—	A ₁	B ₁	A ₁	B ₁
Stage 2 of impactor/impinger	A ₂	B ₂	A ₂	B ₂	A ₂	B ₂	A ₂	B ₂	A ₂	B ₂	A ₂	B ₂
Stage 3 of impactor/impinger	A ₃	B ₃	A ₃	B ₃	A ₃	B ₃	A ₃	B ₃	A ₃	B ₃	A ₃	B ₃
Stage 4 of impactor/impinger	A ₄	B ₄	A ₄	B ₄	A ₄	B ₄	A ₄	B ₄	A ₄	B ₄	A ₄	B ₄
Stage 5 of impactor/impinger	A ₅	B ₅	A ₅	B ₅	A ₅	B ₅	—	—	A ₅	B ₅	A ₅	B ₅
Stage 6 of impactor/impinger	A ₆	B ₆	—	—	A ₆	B ₆	—	—	A ₆	B ₆	A ₆	B ₆
Stage 7 of impactor/impinger	A ₇	B ₇	—	—	A ₇	B ₇	—	—	A ₇	B ₇	A ₇	B ₇
Filter	A _f	B _f	A _f	B _f	A _f	B _f	A _f	B _f	A _f	B _f	A _f	B _f
Sums of Masses	ΣA^c	ΣB^c	ΣA^c	ΣB^c	ΣA^c	ΣB^c	ΣA^c	ΣB^c	ΣA^c	ΣB^c	ΣA^c	ΣB^c

^aStages 6 and 7 are omitted from Apparatus 3 at airflow rates >60 L per minute.

^bStage 5 of Apparatus 4 is the filter stage (see Figure 8).

^c ΣA is the total drug mass recovered from the apparatus; ΣB is the mass of drug recovered from the impactor (Apparatus 1, 3, 5 and 6) or from the impactor stages beneath the uppermost stage (Apparatus 2 and 4).

^dFor Apparatus 5 and 6, values for the drug masses A_f and B_f refer to collections from the MOC, and/or the after-filter if used.

Repeat the calculation for each of the stages in the impactor or impinger stack, in reverse numerical order (largest to smallest stage number). For each stage, calculate the cumulative percentage of mass less than the stated aerodynamic diameter by adding the percentage of the mass on that stage to the total percentage from the stages below and entering the value opposite the effective cutoff diameter of the stage above it in the stack. Thus, the percentage of drug on the filter can be seen to have aerodynamic diameters less than the cutoff diameter of the stage above the filter, and the percentage on the filter plus the percentage on the stage above have diameters less than the cutoff diameter of the stage above that, and so on. Repeat the calculation for each of the remaining stages in reverse numerical order (see *Table 9*).

If necessary, and where appropriate, plot the percentage of mass less than the stated aerodynamic diameters, versus the aerodynamic diameter, $D_{50,Q}$, on log probability paper. Calculate the GSD by the equation:

$$GSD = \sqrt{\frac{\text{Size X}}{\text{Size Y}}}$$

Use these data and/or plot to determine values for MMAD and GSD etc., as appropriate and when necessary (see *Figure 10*).

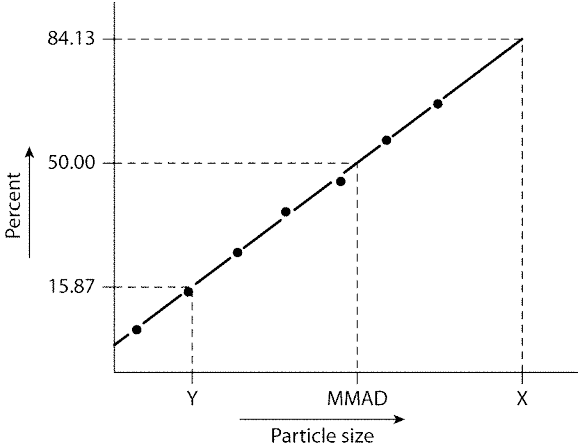


Fig. 10. Plot of cumulative percentage of mass less than stated aerodynamic diameter (probability scale) versus aerodynamic diameter (log scale).

Table 9. Cumulative Percentage (Cum%) of Mass Less than the Stated Aerodynamic Diameter

	Apparatus 1		Apparatus 2		Apparatus 3 ^a		Apparatus 4 ^b		Apparatus 5		Apparatus 6	
Mass	Cum% ^c	D _{50,Q} ^d	Cum% ^c	D _{50,Q} ^d	Cum% ^c	D _{50,Q} ^e	Cum% ^c	D _{50,Q} ^d	Cum% ^c	D _{50,Q} ^d	Cum% ^c	D _{50,Q} ^d
Filter		0.4		0.625		0.4		1.7		0.34		0.34
Stage 7	b	0.7	—	—	b	0.7	—	—	b	0.55	b	0.55
Stage 6	c	1.1	—	—	c	1.1	—	—	c	0.94	c	0.94
Stage 5	d	2.1	b	1.25	d	2.1	—	—	d	1.66	d	1.66
Stage 4	e	3.3	c	2.5	e	3.3	b	3.1	e	2.82	e	2.82
Stage 3	f	4.7	d	5.0	f	4.7	c	6.8	f	4.46	f	4.46
Stage 2	q	5.8	100	10.0	q	5.8	100	13.0	q	8.06	q	8.06
Stage 1	h	9.0	—	—	h	9.0	—	—	—	—	—	—
Stage 0	100	—	—	—	100	—	—	—	100	—	100	—

^aStages 6 and 7 are omitted from *Apparatus 3* at flow rates >60 L per minute; thus, values for b and c should be omitted for *Apparatus 3*, where necessary.

^bThe filter stage in *Apparatus 4* is Stage 5 (see *Figure 8*).

^c [(mass on stage / ΣB)×100] % + (total% of ΣB from stages below).

^dThe 50% cutoff diameter of the stage immediately above that indicated (e.g., for Stage 4, enter the cutoff diameter for Stage 3; for *Apparatus 2* or 4, calculate as $D_{50,Q}$ from Eq. 1; for *Apparatus 5* or 6, calculate as $D_{50,Q}$ from Eq. 2 using *Table 7*). Values entered in the Table are correct for *Apparatus 1, 2, 4, 5, and 6* only when used at 28.3, 60.0, 60.0, 60.0, and 60.0 L per minute, respectively.

^eThe D_{50} values are only valid at a flow rate of 28.3 L per minute.

<610> ALTERNATIVE MICROBIOLOGICAL SAMPLING METHODS FOR NONSTERILE INHALED AND NASAL PRODUCTS

INTRODUCTION

Proper microbiological sampling of microbiologically susceptible nonsterile products can be difficult because these products are often filled into unique primary containers that are designed to protect the product from inadvertent contamination during storage and use. These unique designs may increase the difficulty of taking an aseptic sample of sufficient size or volume for microbiological testing. Unless special approaches are used, products such as inhaled, nasal liquid, or powder dosage forms can be difficult to sample without potential exposure to extraneous microbial contamination. This general test chapter provides these special approaches for sampling either low- or high-content inhaled or nasal dosage forms. Alternative sampling approaches may provide better ways to sample containers in an aseptic manner. Any alternative methodology should employ aseptic techniques and should be conducted under environmental and other conditions that are appropriate for aseptic sampling.

INHALED OR NASAL DOSAGE FORMS

Low-content inhaled and nasal drug products (low-content INDP) are products that have a target fill of less than 100 mg of powder or 1 mL of liquid formulation per unit (primary container). Examples are pre-metered inhalation powders, more commonly known as dry powder inhalers (DPIs), and single-dose nasal sprays.

High-content INDP are multidose drug products that have a target fill of more than 100 mg of powder or more than 1 mL of liquid formulation per unit. Examples are aerosols for inhalation and nasal delivery, known as metered-dose inhalers (MDIs); device-metered inhalation powders; and multidose nasal sprays.

The appropriate sample quantity or volume should be based on the test methodology, including any relevant general test chapters, such as <61> *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* and <62> *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms*. Testing may be performed on the unpackaged bulk dry powder or liquid formulation or the finished product. If testing is performed on the bulk material alone, then the process leading from the bulk to the finished product should be validated for its ability to prevent microbial contamination. Testing should be performed on the finished product if this process is not validated.

SAMPLE SIZE DETERMINATION

For each microbiological test, sample 10 drug product containers or units or a number of units that can provide a minimum of 1 gram of product that are representative of the batch. For batch sizes smaller than 200 units (e.g., batches used in clinical trials), sample size may be reduced to 1% of the units or 1 unit, whichever is greater. The contents of individual containers may be pooled for testing.

BULK TESTING FOR LOW-CONTENT INDP

Bulk lot testing may be preferable for low-content INDP in lieu of finished product testing to allow larger sample sizes that are representative of the batch, without unduly increasing the risk of inadvertent microbial contamination. Bulk testing can be performed on the bulk powder or liquid formulation just before filling. If bulk testing is performed in lieu of finished product testing, then manufacturing processes following bulk sampling (e.g., filling and packaging) must be validated in accordance with current good manufacturing practice (CGMP) for their ability to prevent microbial contamination. For microbial enumeration tests, at least 10 g or 10 mL of bulk material, or, for specified microorganisms tests, 1 g or 1 mL of bulk material may be sampled. For small batch sizes (i.e., less than 1000 g or 1000 mL), the recommended sample size is 1% of the batch for both microbial enumeration and specified microorganisms tests.

SAMPLING METHODS FOR HIGH-CONTENT INDP

Dry Powder Inhalers

DPIs have an internal reservoir that contains a sufficient quantity of formulation for multiple doses that are metered by the device itself during activation by the patient. For DPIs, appropriate validated procedures should be used to sample a nonsterile drug product container.

Inhalation Aerosols

Consider safety issues related to both inhalation of the drug substance and the potential of a flammability hazard. Avoid contamination of samples by employing aseptic techniques whenever necessary.

AUTOMATIC ACTUATION METHOD

The contents of the inhalation aerosol containers may be collected by automatically actuating each aerosol container and collecting the delivered formulation on a suitable sterile filter.

ROOM TEMPERATURE METHOD

Disinfect the outside of the test containers with an appropriate disinfectant, and allow the containers to dry in a controlled environment. Empty the contents of the aerosol container into a sterile vessel using a needle apparatus or similar device (e.g., icemaker water line tap). If it has been demonstrated that the propellant does not inhibit the growth of microorganisms, the contents of the sterile vessel may be added directly to the liquid media or buffer for the test. Otherwise, allow the propellant to evaporate from the vessel by leaving the vessel at room temperature for several minutes. Remove any residual gaseous propellant by tilting the vessel slightly or by allowing a slow stream of microbiologically inert sterile gas to pass over the surface. For some less volatile propellants such as chlorofluorocarbon (CFC) 11/12 combinations, the vessel may be heated slightly (to temperatures $\leq 45^{\circ}$) to assist with evaporation. After the propellant has evaporated, add the liquid media or buffer, and mix the contents to prepare for testing.

Direct expulsion into the broth media or buffer may be feasible if a needle apparatus that is thin and strong enough to puncture the container and to allow slow removal of the contents is available. In this case, the contents may be expelled into and mixed with the aqueous medium. Layering

of the propellant and aqueous medium may occur, in which case a longer time period and slight heating (not to exceed 45°) may be required for propellant evaporation.

CHILLING METHOD

Place the disinfected aerosol containers in dry ice, or a dry ice slurry (ensure the microbial quality of the dry ice and slurry-forming liquid), or a cryofreezer for the period required to liquefy the contents. Disinfect the outside of the test containers with an appropriate disinfectant, and allow the containers to dry in an aseptic environment. Aseptically open the aerosol containers using an appropriate tool. Be aware that freezing can affect the viability of microorganisms. For CFC-based products, pour the contents of the containers into sterile vessels. Allow the propellant to escape, and combine residues with a diluent appropriate for the drug product. Other drug-specific procedures may also be employed. For hydrofluoroalkane-based products, pour the contents of the containers into sterile vessels partially immersed in larger vessels containing dry ice. Drive off the propellant, for example by allowing a slow stream of sterile, filtered, oil-free compressed air to evaporate the material to dryness. Combine residues with a diluent appropriate for the drug product. An alternative procedure when testing the entire contents of a previously chilled container is to pour the contents of the opened container onto a sterile membrane filtration unit, allow the propellant to escape, and then rinse with an appropriate amount of sterile diluent.

Multidose Nasal Sprays

Multidose nasal spray containers usually have a cap that is screwed on, crimped on, or forms a snap-fit. The container can be opened by unscrewing the cap, cutting the seal, or using a decrimping tool, taking care to avoid microbial contamination during the process. Following cap removal, traditional sampling methods as described in general test chapters such as (61) *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* and (62) *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* are typically appropriate for use.

(611) ALCOHOL DETERMINATION

METHOD I—DISTILLATION METHOD

Method I is to be used for the determination of alcohol, unless otherwise specified in the individual monograph. It is suitable for examining most fluid extracts and tinctures, provided the capacity of the distilling flask is sufficient (commonly two to four times the volume of the liquid to be heated) and the rate of distillation is such that clear distillates are produced. Cloudy distillates may be clarified by agitation with talc, or with calcium carbonate, and filtered, after which the temperature of the filtrate is adjusted and the alcohol content determined from the specific gravity. During all manipulations, take precautions to minimize the loss of alcohol by evaporation.

Treat liquids that froth to a troublesome extent during distillation by rendering them strongly acidic with phosphoric, sulfuric, or tannic acid, or treat with a slight excess

of calcium chloride solution or with a small amount of paraffin or silicone oil before starting the distillation.

Prevent bumping during distillation by adding porous chips of insoluble material such as silicon carbide, or beads.

For Liquids Presumed to Contain 30% of Alcohol or Less—By means of a pipet, transfer to a suitable distilling apparatus not less than 25 mL of the liquid in which the alcohol is to be determined, and note the temperature at which the volume was measured. Add an equal volume of water, distill, and collect a volume of distillate about 2 mL less than the volume taken of the original test liquid, adjust to the temperature at which the original test liquid was measured, add sufficient water to measure exactly the original volume of the test liquid, and mix. The distillate is clear or not more than slightly cloudy, and does not contain more than traces of volatile substances other than alcohol and water. Determine the specific gravity of the liquid at 25°, as directed under *Specific Gravity* (841), using this result to ascertain the percentage, by volume, of C₂H₅OH contained in the liquid examined by reference to the *Alcoholometric Table* in the section *Reference Tables*.

For Liquids Presumed to Contain More Than 30% of Alcohol—Proceed as directed in the foregoing paragraph, except to do the following: dilute the specimen with about twice its volume of water, collect a volume of distillate about 2 mL less than twice the volume of the original test liquid, bring to the temperature at which the original liquid was measured, add sufficient water to measure exactly twice the original volume of the test liquid, mix, and determine its specific gravity. The proportion of C₂H₅OH, by volume, in this distillate, as ascertained from its specific gravity, equals one-half that in the liquid examined.

Special Treatment—

VOLATILE ACIDS AND BASES—Render preparations containing volatile bases slightly acidic with diluted sulfuric acid before distilling. If volatile acids are present, render the preparation slightly alkaline with sodium hydroxide TS.

GLYCERIN—To liquids that contain glycerin add sufficient water so that the residue, after distillation, contains not less than 50% of water.

IODINE—Treat all solutions containing free iodine with powdered zinc before the distillation, or decolorize with just sufficient sodium thiosulfate solution (1 in 10), followed by a few drops of sodium hydroxide TS.

OTHER VOLATILE SUBSTANCES—Spirits, elixirs, tinctures, and similar preparations that contain appreciable proportions of volatile materials other than alcohol and water, such as volatile oils, chloroform, ether, camphor, etc., require special treatment, as follows:

For Liquids Presumed to Contain 50% of Alcohol or Less—Mix 25 mL of the specimen under examination, accurately measured, with about an equal volume of water in a separator. Saturate this mixture with sodium chloride, then add 25 mL of solvent hexane, and shake the mixture to extract the interfering volatile ingredients. Draw off the separated, lower layer into a second separator, and repeat the extraction twice with two further 25-mL portions of solvent hexane. Extract the combined solvent hexane solutions with three 10-mL portions of a saturated solution of sodium chloride. Combine the saline solutions, and distill in the usual manner, collecting a volume of distillate having a simple ratio to the volume of the original specimen.

For Liquids Presumed to Contain More Than 50% of Alcohol—Adjust the specimen under examination to a concentration of approximately 25% of alcohol by diluting it with water, then proceed as directed in *For Liquids Presumed to Contain 50% of Alcohol or Less*, beginning with "Saturate this mixture with sodium chloride."

In preparing *Collodion* or *Flexible Collodion* for distillation, use water in place of the saturated solution of sodium chloride directed above.

If volatile oils are present in small proportions only, and a cloudy distillate is obtained, the solvent hexane treatment

not having been employed, the distillate may be clarified and rendered suitable for the specific gravity determination by shaking it with about one-fifth its volume of solvent hexane, or by filtering it through a thin layer of talc.

Method II—Gas Chromatographic Method

Use *Method IIa* when *Method II* is specified in the individual monograph. For a discussion of the principles upon which it is based, see *Gas Chromatography* under *Chromatography* (621).

USP Reference Standards—USP Alcohol Determination—Acetonitrile RS. USP Alcohol Determination—Alcohol RS.

Method IIa

Apparatus—Under typical conditions, use a gas chromatograph equipped with a flame-ionization detector and a 4-mm × 1.8-m glass column packed with 100- to 120-mesh chromatographic column packing support S3, using nitrogen or helium as the carrier. Prior to use, condition the column overnight at 235° with a slow flow of carrier gas. The column temperature is maintained at 120°, and the injection port and detector temperatures are maintained at 210°. Adjust the carrier flow and temperature so that acetonitrile, the internal standard, elutes in 5 to 10 minutes.

Solutions—

Test Stock Preparation—Dilute the specimen under examination stepwise with water to obtain a solution containing approximately 2% (v/v) of alcohol.

Test Preparation—Pipet 5 mL each of the *Test Stock Preparation* and the USP Alcohol Determination—Acetonitrile RS [NOTE—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 50-mL volumetric flask, dilute with water to volume, and mix.

Standard Preparation—Pipet 5 mL each of the USP Alcohol Determination—Alcohol RS and the USP Alcohol Determination—Acetonitrile RS [NOTE—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 50-mL volumetric flask, dilute with water to volume, and mix.

Procedure—Inject about 5 µL each of the *Test Preparation* and the *Standard Preparation*, in duplicate, into the gas chromatograph, record the chromatograms, and determine the peak response ratios. Calculate the percentage of alcohol (v/v) in the specimen under test according to the formula:

$$CD(R_U/R_S)$$

in which C is the labeled concentration of USP Alcohol Determination—Alcohol RS; D is the dilution factor (the ratio of the volume of the *Test Stock Preparation* to the volume of the specimen taken); and R_U and R_S are the peak response ratios obtained from the *Test Preparation* and the *Standard Preparation*, respectively.

System Suitability Test—In a suitable chromatogram, the resolution factor, R , is not less than 2; the tailing factor of the alcohol peak is not greater than 2.0; and six replicate injections of the *Standard Preparation* show a relative standard deviation of not more than 2.0% in the ratio of the peak of alcohol to the peak of the internal standard.

Method IIb

Apparatus—The gas chromatograph is equipped with a split injection port with a split ratio of 5:1, a flame-ionization detector, and a 0.53-mm × 30-m capillary column coated with a 3.0-µm film of phase G43. Helium is used as the carrier gas at a linear velocity of 34.0 cm per second.

The chromatograph is programmed to maintain the column temperature at 50° for 5 minutes, then to increase the temperature at a rate of 10° per minute to 200°, and maintain at this temperature for 4 minutes. The injection port temperature is maintained at 210° and the detector temperature at 280°.

Solutions—

Test Stock Preparation—Dilute the specimen under examination stepwise with water to obtain a solution containing approximately 2% (v/v) of alcohol.

Test Preparation—Pipet 5 mL each of the *Test Stock Preparation* and the USP Alcohol Determination—Acetonitrile RS [NOTE—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 25-mL volumetric flask, dilute with water to volume, and mix.

Standard Preparation—Pipet 5 mL each of the USP Alcohol Determination—Alcohol RS and the USP Alcohol Determination—Acetonitrile RS [NOTE—Alternatively, a 2% aqueous solution of acetonitrile of suitable quality may be used as the internal standard solution] into a 25-mL volumetric flask, dilute with water to volume, and mix.

Procedure—Inject about 0.2 to 0.5 µL each of the *Test Preparation* and the *Standard Preparation*, in duplicate, into the gas chromatograph, record the chromatograms, and determine the peak response ratios. Calculate the percentage of alcohol (v/v) in the specimen under test according to the formula:

$$CD(R_U/R_S)$$

in which C is the labeled concentration of USP Alcohol Determination—Alcohol RS; D is the dilution factor (the ratio of the volume of the *Test Stock Preparation* to the volume of the specimen taken); and R_U and R_S are the peak response ratios obtained from the *Test Preparation* and the *Standard Preparation*, respectively.

System Suitability Test—In a suitable chromatogram, the resolution factor, R , between alcohol and the internal standard is not less than 4; the tailing factor of the alcohol peak is not greater than 2.0; and six replicate injections of the *Standard Preparation* show a relative standard deviation of not more than 4.0% in the ratio of the peak of alcohol to the peak of the internal standard.

<616> BULK DENSITY AND TAPPED DENSITY OF POWDERS

BULK DENSITY

This general chapter has been harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. ♦The portion that is not harmonized is marked with symbols (♦♦) to specify this fact.♦

The bulk density of a powder is the ratio of the mass of an untapped powder sample and its volume including the contribution of the interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the spatial arrangement of particles in the powder bed. The bulk density is expressed in grams per mL (g/mL) although the international unit is kilograms per cubic meter (1 g/mL = 1000 kg/m³) because the measurements are made using cylinders. It may also be expressed in grams per cubic centimeter (g/cm³). The bulking properties of a pow-

der are dependent upon the preparation, treatment, and storage of the sample, i.e., how it was handled. The particles can be packed to have a range of bulk densities; however, the slightest disturbance of the powder bed may result in a changed bulk density. Thus, the bulk density of a powder is often very difficult to measure with good reproducibility and, in reporting the results, it is essential to specify how the determination was made. The bulk density of a powder is determined by measuring the volume of a known weight of powder sample, that may have been passed through a sieve, into a graduated cylinder (*Method I*), or by measuring the mass of a known volume of powder that has been passed through a volumeter into a cup (*Method II*) or a measuring vessel (*Method III*).

Method I and *Method III* are favored.

Method I—Measurement in a Graduated Cylinder

Procedure—Pass a quantity of material sufficient to complete the test through a sieve with apertures greater than or equal to 1.0 mm, if necessary, to break up agglomerates that may have formed during storage; this must be done gently to avoid changing the nature of the material. Into a dry graduated 250-mL cylinder (readable to 2 mL) introduce, without compacting, approximately 100 g of test sample, M , weighed with 0.1% accuracy. Carefully level the powder without compacting, if necessary, and read the unsettled apparent volume (V_0) to the nearest graduated unit. Calculate the bulk density in g/mL by the formula m/V_0 . Generally, replicate determinations are desirable for the determination of this property. If the powder density is too low or too high, such that the test sample has an untapped apparent volume of either more than 250 mL or less than 150 mL, it is not possible to use 100 g of powder sample. Therefore, a different amount of powder has to be selected as the test sample, such that its untapped apparent volume is 150–250 mL (apparent volume greater than or equal to 60% of the total volume of the cylinder); the weight of the test sample is specified in the expression of results. For test samples having an apparent volume between 50 mL and 100 mL, a 100-mL cylinder readable to 1 mL can be used; the volume of the cylinder is specified in the expression of results.

Method II—Measurement in a Volumeter

Apparatus—The apparatus (*Figure 1*) consists of a top funnel fitted with a 1.0-mm sieve.¹ The funnel is mounted over a baffle box containing four glass baffle plates over which the powder slides and bounces as it passes. At the bottom of the baffle box is a funnel that collects the powder and allows it to pour into a cup of specified capacity mounted directly below it. The cup may be cylindrical (25.00 ± 0.05 mL volume with an inside diameter of 30.00 ± 2.00 mm) or cubical (16.39 ± 0.2 mL volume with inside dimensions of 25.400 ± 0.076 mm).

¹ The apparatus (the Scott Volumeter) conforms to the dimensions in ASTM 329 90.

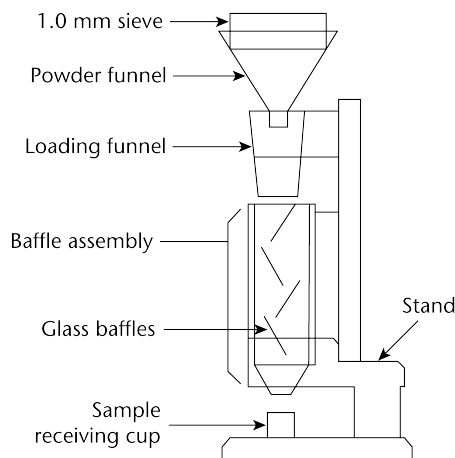


Figure 1.

Procedure—Allow an excess of powder to flow through the apparatus into the sample receiving cup until it overflows, using a minimum of 25 cm³ of powder with the square cup and 35 cm³ of powder with the cylindrical cup. Carefully scrape excess powder from the top of the cup by smoothly moving the edge of the blade of a spatula perpendicular to and in contact with the top surface of the cup, taking care to keep the spatula perpendicular to prevent packing or removal of powder from the cup. Remove any material from the sides of the cup, and determine the weight, M , of the powder to the nearest 0.1%. Calculate the bulk density, in g/mL, by the formula:

$$(M)/(V_0)$$

in which V_0 is the volume, in mL, of the cup. Record the average of three determinations using three different powder samples.

Method III—Measurement in a Vessel

Apparatus—The apparatus consists of a 100-mL cylindrical vessel of stainless steel with dimensions as specified in *Figure 2*.

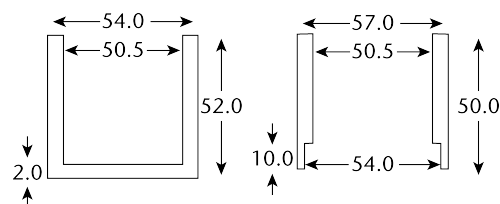


Figure 2.

Procedure—Pass a quantity of powder sufficient to complete the test through a 1.0-mm sieve, if necessary, to break up agglomerates that may have formed during storage, and allow the obtained sample to flow freely into the measuring vessel until it overflows. Carefully scrape the excess powder from the top of the vessel as described for *Method II*. Determine the weight (M_0) of the powder to the nearest 0.1% by subtraction of the previously determined mass of the empty measuring vessel. Calculate the bulk density (g/mL) by the formula $M_0/100$, and record the average of three determinations using three different powder samples.

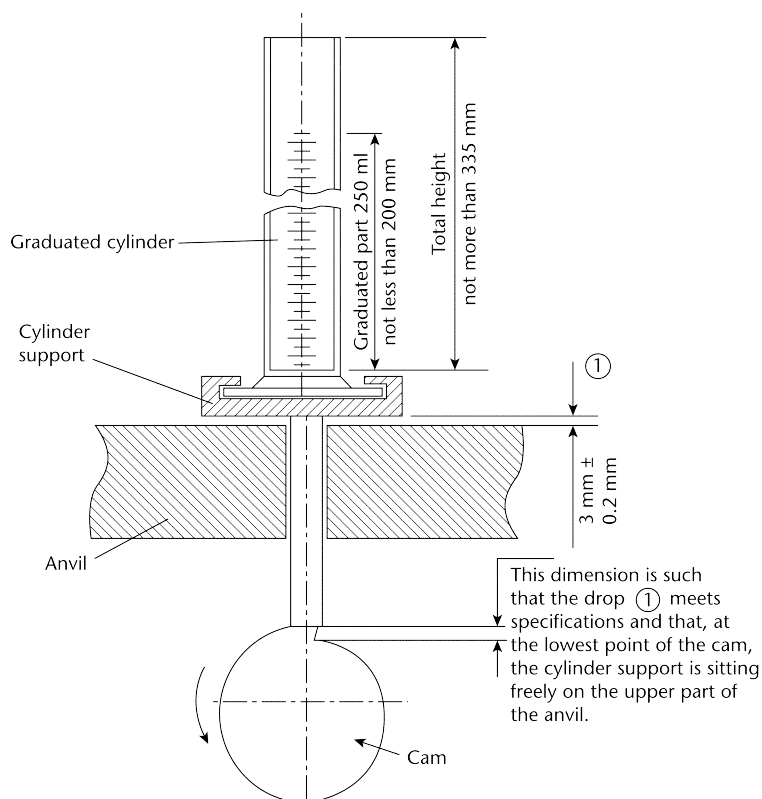


Figure 3.

TAPPED DENSITY

The tapped density is an increased bulk density attained after mechanically tapping a container containing the powder sample. Tapped density is obtained by mechanically tapping a graduated measuring cylinder or vessel containing a powder sample. After observing the initial powder volume or weight, the measuring cylinder or vessel is mechanically tapped, and volume or weight readings are taken until little further volume or weight change is observed. The mechanical tapping is achieved by raising the cylinder or vessel and allowing it to drop under its own weight a specified distance by either of three methods as described below. Devices that rotate the cylinder or vessel during tapping may be preferred to minimize any possible separation of the mass during tapping down.

Method I

Apparatus—The apparatus (Figure 3) consists of the following:

- A 250-mL graduated cylinder (readable to 2 mL with a mass of 220 ± 44 g)
- A settling apparatus capable of producing, in 1 min, either nominally 250 ± 15 taps from a height of 3 ± 0.2 mm, or nominally 300 ± 15 taps from a height of 14 ± 2 mm. The support for the graduated cylinder, with its holder, has a mass of 450 ± 10 g.

Procedure—Proceed as described above for the determination of the bulk volume (V_0). Secure the cylinder in the holder. Carry out 10, 500, and 1250 taps on the same powder sample and read the corresponding volumes V_{10} , V_{500} , and V_{1250} to the nearest graduated unit. If the difference between V_{500} and V_{1250} is less than or equal to 2 mL, V_{1250} is the tapped volume. If the difference between V_{500} and V_{1250} exceeds 2 mL, repeat in increments such as 1250 taps, until the difference between succeeding measurements is less

than or equal to 2 mL. Fewer taps may be appropriate for some powders, when validated. Calculate the tapped density (g/mL) using the formula m/V_f , in which V_f is the final tapped volume. Generally, replicate determinations are desirable for the determination of this property. Specify the drop height with the results. If it is not possible to use a 100-g test sample, use a reduced amount and a suitable 100-mL graduated cylinder (readable to 1 mL) weighing 130 ± 16 g and mounted on a holder weighing 240 ± 12 g. The modified test conditions are specified in the expression of the results.

Method II

Apparatus and Procedure—Proceed as directed under Method I except that the mechanical tester provides a fixed drop of 3 ± 0.2 mm at a nominal rate of 250 taps per min.

Method III

Apparatus and Procedure—Proceed as directed in Method III—Measurement in a Vessel for measuring bulk density using the measuring vessel equipped with the cap shown in Figure 2. The measuring vessel with the cap is lifted 50–60 times per min by the use of a suitable tapped density tester. Carry out 200 taps, remove the cap, and carefully scrape excess powder from the top of the measuring vessel as described in Method III—Measurement in a Vessel for measuring the bulk density. Repeat the procedure using 400 taps. If the difference between the two masses obtained after 200 and 400 taps exceeds 2%, carry out a test using 200 additional taps until the difference between succeeding measurements is less than 2%. Calculate the tapped density (g/mL) using the formula $M_f/100$, where M_f is the mass of powder in the measuring vessel. Record the average of three determinations using three different pow-

der samples. The test conditions including tapping height are specified in the expression of the results.

MEASURES OF POWDER COMPRESSIBILITY

Because the interparticulate interactions influencing the bulking properties of a powder are also the interactions that interfere with powder flow, a comparison of the bulk and tapped densities can give a measure of the relative importance of these interactions in a given powder. Such a comparison is often used as an index of the ability of the powder to flow, for example the *Compressibility Index* or the *Hausner Ratio* as described below.

The *Compressibility Index* and *Hausner Ratio* are measures of the propensity of a powder to be compressed as described above. As such, they are measures of the powder's ability to settle, and they permit an assessment of the relative importance of interparticulate interactions. In a free-flowing powder, such interactions are less significant, and the bulk and tapped densities will be closer in value. For poorer flowing materials, there are frequently greater interparticle interactions, and a greater difference between the bulk and tapped densities will be observed. These differences are reflected in the *Compressibility Index* and the *Hausner Ratio*.

Compressibility Index—Calculate by the formula:

$$100(V_0 - V_F)/V_0$$

V_0 = unsettled apparent volume

V_F = final tapped volume

Hausner Ratio—

$$V_0/V_F$$

Depending on the material, the compressibility index can be determined using V_{10} instead of V_0 . [NOTE—If V_{10} is used, it will be clearly stated in the results.]

(621) CHROMATOGRAPHY

INTRODUCTION

Chromatographic separation techniques are multistage separation methods in which the components of a sample are distributed between two phases, of which one is stationary and the other mobile. The stationary phase may be a solid or a liquid supported on a solid or a gel. The stationary phase may be packed in a column, spread as a layer, distributed as a film, or applied by other techniques. The mobile phase may be gaseous or liquid or supercritical fluid. The separation may be based on adsorption, mass distribution (partition), or ion exchange; or it may be based on differences among the physicochemical properties of the molecules, such as size, mass, and volume. This chapter contains general procedures, definitions, and calculations of common parameters and describes general requirements for system suitability. The types of chromatography useful in qualitative and quantitative analysis employed in *USP* procedures are column, gas, paper, thin-layer (including high-performance thin-layer chromatography), and pressurized liquid chromatography (commonly called high-pressure or high-performance liquid chromatography).

GENERAL PROCEDURES

This section describes the basic procedures used when a chromatographic method is described in a monograph. The following procedures are followed unless otherwise indicated in the individual monograph.

Paper Chromatography

Stationary Phase: The stationary phase is a sheet of paper of suitable texture and thickness. Development may be ascending, in which the solvent is carried up the paper by capillary forces, or descending, in which the solvent flow is also assisted by gravitational force. The orientation of paper grain with respect to solvent flow is to be kept constant in a series of chromatograms. (The machine direction is usually designated by the manufacturer.)

Apparatus: The essential equipment for paper chromatography consists of a vapor-tight chamber with inlets for addition of solvent and a rack of corrosion-resistant material about 5 cm shorter than the inside height of the chamber. The rack serves as a support for solvent troughs and for antisiphon rods that, in turn, hold up the chromatographic sheets. The bottom of the chamber is covered with the prescribed solvent system or mobile phase. Saturation of the chamber with solvent vapor is facilitated by lining the inside walls with paper wetted with the prescribed solvent system.

Spotting: The substance or substances analyzed are dissolved in a suitable solvent. Convenient volumes, delivered from suitable micropipets, of the resulting solution, normally containing 1–20 µg of the compound, are placed in 6- to 10-mm spots not less than 3 cm apart.

Descending Paper Chromatography Procedure

- (1) A spotted chromatographic sheet is suspended in the apparatus, using the antisiphon rod to hold the upper end of the sheet in the solvent trough. [NOTE—Ensure that the portion of the sheet hanging below the rods is freely suspended in the chamber without touching the rack, the chamber walls, or the fluid in the chamber.]
- (2) The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapor. Any excess pressure is released as necessary.
- (3) After equilibration of the chamber, the prepared mobile phase is introduced into the trough through the inlet.
- (4) The inlet is closed, and the mobile solvent phase is allowed to travel the desired distance down the paper.
- (5) The sheet is removed from the chamber.
- (6) The location of the solvent front is quickly marked, and the sheet is dried.
- (7) The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated drug or drugs.

Ascending Paper Chromatography Procedure

- (1) The mobile phase is added to the bottom of the chamber.
- (2) The chamber is sealed to allow equilibration (saturation) of the chamber and the paper with the solvent vapor. Any excess pressure is released as necessary.
- (3) The lower edge of the stationary phase is dipped into the mobile phase to permit the mobile phase to rise on the chromatographic sheet by capillary action.
- (4) When the solvent front has reached the desired height, the chamber is opened, the sheet is removed, the location of the solvent front is quickly marked, and the sheet is dried.
- (5) The chromatogram is observed and measured directly or after suitable development to reveal the location of the spots of the isolated drug or drugs.

Thin-Layer Chromatography

Stationary Phase: The stationary phase is a relatively thin, uniform layer of dry, finely powdered material applied to a glass, plastic, or metal sheet or plate (typically called the plate). The stationary phase of TLC plates has an average particle size of 10–15 μm , and that of high-performance TLC (HPTLC) plates has an average particle size of 5 μm . Commercial plates with a preadsorbent zone can be used if they are specified in a monograph. Sample applied to the preadsorbent region develops into sharp, narrow bands at the preadsorbent-sorbent interface. The separations achieved may be based on adsorption, partition, or a combination of both effects, depending on the particular type of stationary phase.

Apparatus: A chromatographic chamber made of inert, transparent material and having the following specifications is used: a flat-bottom or twin trough, a tightly fitted lid, and a size suitable for the plates. The chamber is lined on at least one wall with filter paper. Sufficient mobile phase or developing solvent is added to the chamber that, after impregnation of the filter paper, a depth appropriate to the dimensions of the plate used is available. The chromatographic chamber is closed and allowed to equilibrate. [NOTE—Unless otherwise indicated, the chromatographic separations are performed in a saturated chamber.]

Detection/Visualization: An ultraviolet (UV) light source suitable for observations under short- (254 nm) and long- (365 nm) wavelength UV light and a variety of other spray reagents used to make spots visible are often used.

Spotting: Solutions are spotted on the surface of the stationary phase (plate) at the prescribed volume in sufficiently small portions to obtain circular spots of 2–5 mm in diameter (1–2 mm on HPTLC plates) or bands of 10–20 mm \times 1–2 mm (5–10 mm \times 0.5–1 mm on HPTLC plates) at an appropriate distance from the lower edge of and sides of the plate. [NOTE—During development, the application position must be at least 5 mm (TLC) or 3 mm (HPTLC) above the level of the mobile phase.] The solutions are applied on a line parallel to the lower edge of the plate with an interval of at least 10 mm (5 mm on HPTLC plates) between the centers of spots, or 4 mm (2 mm on HPTLC plates) between the edges of bands, then allowed to dry.

Procedure

- (1) Place the plate in the chamber, ensuring that the spots or bands are above the surface of the mobile phase.
- (2) Close the chamber.
- (3) Allow the mobile phase to ascend the plate until the solvent front has traveled three-quarters of the length of the plate, or the distance prescribed in the monograph.
- (4) Remove the plate, mark the solvent front with a pencil, and allow to dry.
- (5) Visualize the chromatograms as prescribed.
- (6) Determine the chromatographic retardation factor (R_f) values for the principal spots or zones.
- (7) Presumptive identification can be made by observation of spots or zones of identical R_f value and about equal magnitude obtained, respectively, with an unknown and a standard chromatographed on the same plate. A visual comparison of the size or intensity of the spots or zones may serve for semiquantitative estimation. Quantitative measurements are possible by means of densitometry (absorbance or fluorescence measurements).

Column Chromatography

Solid Support: Purified siliceous earth is used for normal-phase separation. Silanized chromatographic siliceous earth is used for reverse-phase partition chromatography.

Stationary Phase: The solid support is modified by the addition of a stationary phase specified in the individual monograph. If a mixture of liquids is used as the stationary phase, mix the liquids before the introduction of the solid support.

Mobile Phase: The mobile phase is specified in the individual monograph. If the stationary phase is an aqueous solution, equilibrate with water. If the stationary phase is a polar organic fluid, equilibrate with that fluid.

Apparatus: Unless otherwise specified in the individual monograph, the chromatographic tube is about 22 mm in inside diameter and 200–300 mm long. Attached to it is a delivery tube, without stopcock, about 4 mm in inside diameter and about 50 mm long.

APPARATUS PREPARATION: Pack a pledget of fine glass wool in the base of the tube. Combine the specified volume of stationary phase and the specified amount of solid support to produce a homogeneous, fluffy mixture. Transfer this mixture to the chromatographic tube, and tamp, using gentle pressure, to obtain a uniform mass. If the specified amount of solid support is more than 3 g, transfer the mixture to the column in portions of approximately 2 g, and tamp each portion. If the assay or test requires a segmented column with a different stationary phase specified for each segment, tamp after the addition of each segment, and add each succeeding segment directly to the previous one. Pack a pledget of fine glass wool above the completed column packing. [NOTE—The mobile phase should flow through a properly packed column as a moderate stream or, if reverse-phase chromatography is applied, as a slow trickle.]

If a solution of the analyte is incorporated into the stationary phase, complete the quantitative transfer to the chromatographic tube by scrubbing the beaker used for the preparation of the test mixture with a mixture of about 1 g of *Solid Support* and several drops of the solvent used to prepare the sample solution before adding the final portion of glass wool.

Procedure

- (1) Transfer the mobile phase to the column space above the column packing, and allow it to flow through the column under the influence of gravity.
- (2) Rinse the tip of the chromatographic column with about 1 mL of mobile phase before each change in composition of mobile phase and after completion of the elution.
- (3) If the analyte is introduced into the column as a solution in the mobile phase, allow it to pass completely into the column packing, then add mobile phase in several small portions, allowing each to drain completely, before adding the bulk of the mobile phase.
- (4) Where the procedure indicates the use of multiple chromatographic columns mounted in series and the addition of mobile phase in divided portions is specified, allow each portion to drain completely through each column, and rinse the tip of each with mobile phase before the addition of each succeeding portion.

Gas Chromatography (GC)

Liquid Stationary Phase: This type of phase is available in packed or capillary columns.

Packed Column GC: The liquid stationary phase is deposited on a finely divided, inert solid support, such as diatomaceous earth, porous polymer, or graphitized carbon,

which is packed into a column that is typically 2–4 mm in internal diameter and 1–3 m in length.

Capillary Column GC: In capillary columns, which contain no packed solid support, the liquid stationary phase is deposited on the inner surface of the column and may be chemically bonded to it.

Solid Stationary Phase: This type of phase is available only in packed columns. In these columns the solid phase is an active adsorbent, such as alumina, silica, or carbon, packed into a column. Polyaromatic porous resins, which are sometimes used in packed columns, are not coated with a liquid phase. [NOTE—Packed and capillary columns must be conditioned before use until the baseline and other characteristics are stable. The column or packing material supplier provides instructions for the recommended conditioning procedure.]

Apparatus: A gas chromatograph consists of a carrier gas source, injection port, column, detector, and recording device. The injection port, column, and detector are temperature controlled and may be varied as part of the analysis. The typical carrier gas is helium, nitrogen, or hydrogen, depending on the column and detector in use. The type of detector used depends on the nature of the compounds analyzed and is specified in the individual monograph. Detector output is recorded as a function of time, and the instrument response, measured as peak area or peak height, is a function of the amount present.

Temperature Program: The length and quality of a GC separation can be controlled by altering the temperature of the chromatographic column. When a temperature program is necessary, the individual monograph indicates the conditions in table format. The table indicates the initial temperature, rate of temperature change (ramp), final temperature, and hold time at the final temperature.

Procedure

- (1) Equilibrate the column, injector, and detector with flowing carrier gas until a constant signal is received.
- (2) Inject a sample through the injector septum, or use an autosampler.
- (3) Begin the temperature program.
- (4) Record the chromatogram.
- (5) Analyze as indicated in the monograph.

Liquid Chromatography (LC)

The term *liquid chromatography*, as used in the compendia, is synonymous with high-pressure liquid chromatography and high-performance liquid chromatography. LC is a separation technique based on a solid stationary phase and a liquid mobile phase.

Stationary Phase: Separations are achieved by partition, adsorption, or ion-exchange processes, depending on the type of stationary phase used. The most commonly used stationary phases are modified silica or polymeric beads. The beads are modified by the addition of long-chain hydrocarbons. The specific type of packing needed to complete an analysis is indicated by the "L" designation in the individual monograph (see also the section *Chromatographic Columns*, below). The size of the beads is often described in the monograph as well. Changes in the packing type and size are covered in the *System Suitability* section of this chapter.

Chromatographic Column: The term *column* includes stainless steel, lined stainless steel, and polymeric columns, packed with a stationary phase. The length and inner diameter of the column affects the separation, and therefore typical column dimensions are included in the individual monograph. Changes to column dimensions are discussed in the *System Suitability* section of this chapter. Compendial monographs do not include the name of appropriate columns; this omission avoids the appearance of endorsement of a vendor's product and natural changes in the marketplace.

See the section *Chromatographic Columns* for more information.

Mobile Phase: The mobile phase is a solvent or a mixture of solvents, as defined in the individual monograph.

Apparatus: A liquid chromatograph consists of a reservoir containing the mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector, and a data collection device.

Gradient Elution: The technique of continuously changing the solvent composition during the chromatographic run is called gradient elution or solvent programming. The gradient elution profile is presented in the individual monograph as a gradient table, which lists the time and proportional composition of the mobile phase at the stated time.

Procedure

- (1) Equilibrate the column and detector with mobile phase at the specified flow rate until a constant signal is received.
- (2) Inject a sample through the injector, or use an autosampler.
- (3) Begin the gradient program.
- (4) Record the chromatogram.
- (5) Analyze as directed in the monograph.

CHROMATOGRAPHIC COLUMNS

A complete list of packings (L), phases (G), and supports (S) used in *USP–NF* tests and assays is located in *USP–NF* and *PF, Reagents, Indicators, and Solutions—Chromatographic Columns*. This list is intended to be a convenient reference for the chromatographer in identifying the pertinent chromatographic column specified in the individual monograph.

DEFINITIONS AND INTERPRETATION OF CHROMATOGRAMS

Chromatogram: A chromatogram is a graphical representation of the detector response, concentration of analyte in the effluent, or other quantity used as a measure of effluent concentration versus effluent volume or time. In planar chromatography, *chromatogram* may refer to the paper or layer with the separated zones.

Figure 1 represents a typical chromatographic separation of two substances, 1 and 2. t_{R1} and t_{R2} are the respective retention times; and h is the height, $h/2$ the half-height, and $W_{h/2}$ the width at half-height, for peak 1. W_1 and W_2 are the respective widths of peaks 1 and 2 at the baseline. Air peaks are a feature of gas chromatograms and correspond to the solvent front in LC. The retention time of these air peaks, or unretained components, is designated as t_M .

Dwell Volume (D): The dwell volume (also known as gradient delay volume) is the volume between the point at which the eluents meet and the top of the column.

Hold-Up Time (t_M): The hold-up time is the time required for elution of an unretained component (see *Figure 1*, shown as an air or unretained solvent peak, with the baseline scale in min).

Hold-Up Volume (V_M): The hold-up volume is the volume of mobile phase required for elution of an unretained component. It may be calculated from the hold-up time and the flow rate F , in mL/min:

$$V_M = t_M \times F$$

In size exclusion chromatography, the symbol V_0 is used.

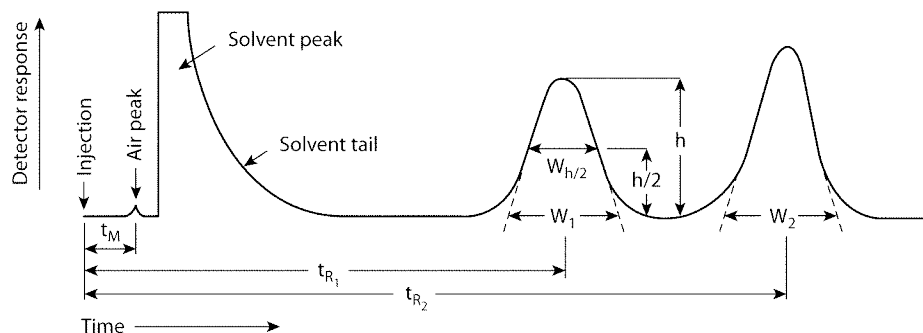


Figure 1. Chromatographic separation of two substances.

Number of Theoretical Plates (N)¹: N is a measure of column efficiency. For Gaussian peaks, it is calculated by:

$$N = 16(t_R/W)^2$$

where t_R is the retention time of the substance, and W is the peak width at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline. The value of N depends upon the substance being chromatographed as well as the operating conditions, such as the flow rate and temperature of the mobile phase or carrier gas, the quality of the packing, the uniformity of the packing within the column, and, for capillary columns, the thickness of the stationary phase film and the internal diameter and length of the column.

Where electronic integrators are used, it may be convenient to determine the number of theoretical plates, by the equation:

$$N = 5.54 \left(\frac{t_R}{W_{h/2}} \right)^2$$

where $W_{h/2}$ is the peak width at half-height. However, in the event of dispute, only equations based on peak width at baseline are to be used.

Peak: The peak is the portion of the chromatographic recording of the detector response when a single component is eluted from the column. If separation is incomplete, two or more components may be eluted as one unresolved peak.

Peak-to-Valley Ratio (p/v): The p/v may be employed as a system suitability criterion in a test for related substances when baseline separation between two peaks is not achieved. Figure 2 represents a partial separation of two substances, where H_p is the height above the extrapolated baseline of the minor peak and H_v is the height above the

extrapolated baseline at the lowest point of the curve separating the minor and major peaks:

$$p/v = H_p/H_v$$

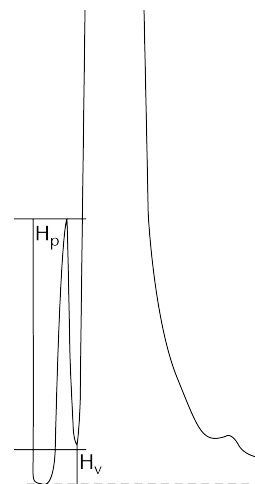


Figure 2. Peak-to-valley ratio determination.

Relative Retardation (R_{ret}): The relative retardation is the ratio of the distance traveled by the analyte to the distance simultaneously traveled by a reference compound (see Figure 3) and is used in planar chromatography.

$$R_{ret} = b / c$$

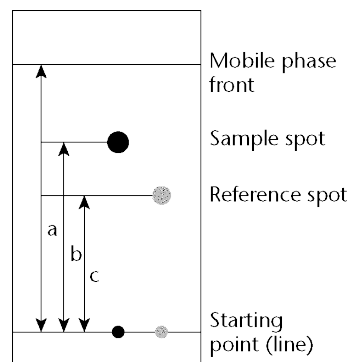


Figure 3. Typical planar chromatography.

¹The parameters k , N , r , and r_c were developed for isothermal GC separations and isocratic HPLC separations. Because these terms are thermodynamic parameters, they are valid only for separations made at constant temperature, mobile phase composition, and flow rate. However, for separations made with a temperature program or solvent gradient, these parameters may be used simply as comparative means to ensure that adequate chromatographic conditions exist to perform the methods as intended in the monographs.

Relative Retention (r)¹: Is the ratio of the adjusted retention time of a component relative to that of another used as a reference obtained under identical conditions:

$$r = t_{R2} - t_M / t_{R1} - t_M$$

where t_{R2} is the retention time measured from the point of injection of the compound of interest; t_{R1} is the retention time measured from the point of injection of the compound used as reference; and t_M is the retention time of a nonretained marker defined in the procedure, all determined under identical experimental conditions on the same column.

Relative Retention Time (RRT): Also known as unadjusted relative retention. Comparisons in USP are normally made in terms of unadjusted relative retention, unless otherwise indicated.

$$RRT = t_{R2}/t_{R1}$$

The symbol r_G is also used to designate unadjusted relative retention values.

Relative Standard Deviation in Percentage

$$\%RSD = \frac{100}{x} \left(\frac{\sum_{i=1}^N (x_i - \bar{x})^2}{N - 1} \right)^{1/2}$$

Retardation Factor (R_F): The retardation factor is the ratio of the distance traveled by the center of the spot to the distance simultaneously traveled by the mobile phase and is used in planar chromatography. Using the symbols in Figure 3:

$$R_F = b/a$$

Retention Factor (k)¹: The retention factor is also known as the capacity factor (k'). Defined as:

$$k = \frac{\text{amount of substance in stationary phase}}{\text{amount of substance in mobile phase}}$$

or

$$k = \frac{\text{time spent by substance in stationary phase}}{\text{time spent by substance in mobile phase}}$$

The retention factor of a component may be determined from the chromatogram:

$$k = (t_R - t_M)/t_M$$

Retention Time (t_R): In liquid chromatography and gas chromatography, the retention time, t_R , is defined as the time elapsed between the injection of the sample and the appearance of the maximum peak response of the eluted sample zone. t_R may be used as a parameter for identification. Chromatographic retention times are characteristic of the compounds they represent but are not unique. Coincidence of retention times of a sample and a reference substance can be used as a partial criterion in construction of an identity profile but may not be sufficient on its own to establish identity. Absolute retention times of a given compound may vary from one chromatogram to the next.

Retention Volume (V_R): The retention volume is the volume of mobile phase required for elution of a compo-

nent. It may be calculated from the retention time and the flow rate in mL/min:

$$V_R = t_R \times F$$

Resolution (R_S): The resolution is the separation of two components in a mixture, calculated by:

$$R_S = 2(t_{R2} - t_{R1})/(W_1 + W_2)$$

where t_{R2} and t_{R1} are the retention times of the two components; and W_2 and W_1 are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Where electronic integrators are used, it may be convenient to determine the resolution, by the equation:

$$R_S = 1.18(t_{R2} - t_{R1})/(W_{1,h/2} + W_{2,h/2})$$

Separation Factor (α): The separation factor is the relative retention calculated for two adjacent peaks (by convention, the value of the separation factor is always >1):

$$\alpha = k_2/k_1$$

Symmetry Factor (A_s)²: The symmetry factor (also known as the tailing factor) of a peak (see Figure 4) is calculated by:

$$A_s = W_{0.05}/2f$$

where $W_{0.05}$ is the width of the peak at 5% height and f is the distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

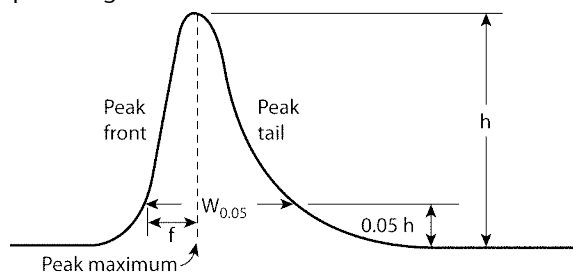


Figure 4. Asymmetrical chromatographic peak.

Tailing Factor (T): See *Symmetry Factor*.

SYSTEM SUITABILITY

System suitability tests are an integral part of gas and liquid chromatographic methods. These tests are used to verify that the chromatographic system is adequate for the intended analysis.

The tests are based on the concept that the equipment, electronics, analytical operations, and samples analyzed constitute an integral system that can be evaluated as such.

Factors that may affect chromatographic behavior include the following:

- Composition, ionic strength, temperature, and apparent pH of the mobile phase
- Flow rate, column dimensions, column temperature, and pressure
- Stationary phase characteristics, including type of chromatographic support (particle-based or monolithic),

²It is also a common practice to measure the Asymmetry Factor as the ratio of the distance between the vertical line connecting the peak apex with the interpolated baseline and the peak front, and the distance between that line and the peak back measured at 10% of the peak height (see Figure 4), would be $(W_{0.10} - f_{0.10})/f_{0.10}$. However, for the purposes of USP, only the formula (A_s) as presented here is valid.

particle or macropore size, porosity, and specific surface area

- Reverse-phase and other surface modification of the stationary phases, the extent of chemical modification (as expressed by end-capping, carbon loading, etc.)

The resolution, R_s , is a function of the number of theoretical plates, N (also referred to as efficiency), the separation factor, α , and the capacity factor, k . [NOTE—All terms and symbols are defined in the preceding section *Definitions and Interpretation of Chromatograms*.] For a given stationary phase and mobile phase, N may be specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug. This is a less reliable means to ensure resolution than is direct measurement. Column efficiency is, in part, a reflection of peak sharpness, which is important for the detection of trace components.

Replicate injections of a standard preparation or other standard solutions are compared to ascertain whether requirements for precision are met. Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation, %RSD, if the requirement is 2.0% or less; data from six replicate injections are used if the relative standard deviation requirement is more than 2.0%.

For the Assay in a drug substance monograph, where the value is 100% for the pure substance, and no maximum relative standard deviation is stated, the maximum permitted %RSD is calculated for a series of injections of the reference solution:

$$\%RSD = KB\sqrt{n}/t_{90\%, n-1}$$

where K is a constant (0.349), obtained from the expression $K = (0.6/\sqrt{2}) \times (t_{90\%, 5}/\sqrt{6})$, in which $0.6/\sqrt{2}$ represents the required percentage relative standard deviation after six injections for $B = 1.0$; B is the upper limit given in the definition of the individual monograph minus 100%; n is the number of replicate injections of the reference solution ($3 \leq n \leq 6$); and $t_{90\%, n-1}$ is the Student's t at the 90% probability level (double sided) with $n - 1$ degrees of freedom.

Unless otherwise prescribed, the maximum permitted relative standard deviation does not exceed the appropriate value given in the table of repeatability requirements. This requirement does not apply to tests for related substances.

Relative Standard Deviation Requirements

	Number of Individual Injections			
	3	4	5	6
B (%)	Maximum Permitted RSD			
2.0	0.41	0.59	0.73	0.85
2.5	0.52	0.74	0.92	1.06
3.0	0.62	0.89	1.10	1.27

The symmetry factor, A_s , a measure of peak symmetry, is unity for perfectly symmetrical peaks; and its value increases as tailing becomes more pronounced (see Figure 4). In some cases, values less than unity may be observed. As peak symmetry moves away from values of 1, integration, and hence precision, become less reliable.

The signal-to-noise ratio (S/N) is a useful system suitability parameter. The S/N is calculated as follows:

$$S/N = 2H/h$$

where H is the height of the peak measured from the peak apex to a baseline extrapolated over a distance ≥ 5 times the peak width at its half-height; and h is the difference between the largest and smallest noise values observed over a distance ≥ 5 times the width at the half-height of the peak

and, if possible, situated equally around the peak of interest (see Figure 5).

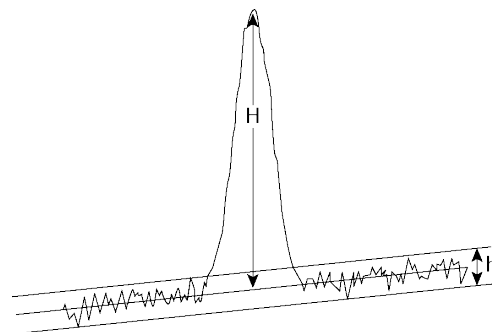


Figure 5. Noise and chromatographic peak, components of the S/N ratio.

These system suitability tests are performed by collecting data from replicate injections of standard or other solutions as specified in the individual monograph.

The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions. Adjustments are permitted only when

- Suitable standards (including Reference Standards) are available for all compounds used in the suitability test; and
- Those standards show that the adjustments improved the quality of the chromatography with respect to the system suitability requirements.

Adjustments to chromatographic systems performed in order to comply with system suitability requirements are not to be made in order to compensate for column failure or system malfunction.

If adjustments of operating conditions are necessary in order to meet system suitability requirements, each of the items in the following list is the maximum variation that can be considered, unless otherwise directed in the monograph; these changes may require additional validation data. To verify the suitability of the method under the new conditions, assess the relevant analytical performance characteristics potentially affected by the change. Multiple adjustments can have a cumulative effect on the performance of the system and are to be considered carefully before implementation. Adjustments to the composition of the mobile phase in gradient elution are not recommended. If adjustments are necessary, only column changes (same packing material) or dwell volume adjustments are recommended.

pH of Mobile Phase (HPLC): The pH of the aqueous buffer used in the preparation of the mobile phase can be adjusted to within ± 0.2 units of the value or range specified.

Concentration of Salts in Buffer (HPLC): The concentration of the salts used in the preparation of the aqueous buffer employed in the mobile phase can be adjusted to within $\pm 10\%$ if the permitted pH variation (see above) is met.

Ratio of Components in Mobile Phase (HPLC): The following adjustment limits apply to minor components of the mobile phase (specified at 50% or less). The amounts of these components can be adjusted by $\pm 30\%$ relative. However, the change in any component cannot exceed $\pm 10\%$ absolute (i.e., in relation to the total mobile phase). Adjustment can be made to one minor component in a ternary mixture. Examples of adjustments for binary and ternary mixtures are given below.

Binary Mixtures

SPECIFIED RATIO OF 50:50: 30% of 50 is 15% absolute, but this exceeds the maximum permitted change of $\pm 10\%$ absolute in either component. Therefore, the mobile phase ratio may be adjusted only within the range of 40:60 to 60:40.

SPECIFIED RATIO OF 2:98: 30% of 2 is 0.6% absolute. Therefore the maximum allowed adjustment is within the range of 1.4:98.6 to 2.6:97.4.

Ternary Mixtures

SPECIFIED RATIO OF 60:35:5: For the second component, 30% of 35 is 10.5% absolute, which exceeds the maximum permitted change of $\pm 10\%$ absolute in any component. Therefore the second component may be adjusted only within the range of 25% to 45% absolute. For the third component, 30% of 5 is 1.5% absolute. In all cases, a sufficient quantity of the first component is used to give a total of 100%. Therefore, mixture ranges of 50:45:5 to 70:25:5 or 58.5:35:6.5 to 61.5:35:3.5 would meet the requirement.

Wavelength of UV-Visible Detector (HPLC): Deviations from the wavelengths specified in the procedure are not permitted. The procedure specified by the detector manufacturer, or another validated procedure, is used to verify that error in the detector wavelength is, at most, ± 3 nm.

Stationary Phase

COLUMN LENGTH (GC, HPLC): Can be adjusted by as much as $\pm 70\%$.

COLUMN INNER DIAMETER (HPLC): Can be adjusted if the linear velocity is kept constant. See *Flow Rate (HPLC)* below.

COLUMN INNER DIAMETER (GC): Can be adjusted by as much as $\pm 50\%$ for GC.

FILM THICKNESS (CAPILLARY GC): Can be adjusted by as much as -50% to 100% .

Particle Size (HPLC): The particle size can be reduced by as much as 50% , but cannot be increased.

Particle Size (GC): Changing from a larger to a smaller or from a smaller to a larger particle size GC mesh support is acceptable if the chromatography meets the requirements of system suitability and the same particle size range ratio is maintained. The particle size range ratio is defined as the diameter of the largest particle divided by the diameter of the smallest particle.

Flow Rate (GC): The flow rate can be adjusted by as much as $\pm 50\%$.

Flow Rate (HPLC): When column dimensions have been modified, the flow rate can be adjusted using:

$$F_2 = F_1 \frac{l_2 d_2^2}{l_1 d_1^2}$$

in which F_1 is the flow rate indicated in the monograph, in mL/min; F_2 is the adjusted flow rate, in mL/min; l_1 is the length of the column indicated in the monograph; l_2 is the length of the column used; d_1 is the column inner diameter indicated in the monograph; and d_2 is the internal diameter of the column used. Additionally, the flow rate can be adjusted by $\pm 50\%$.

Injection Volume (HPLC): The injection volume can be reduced as far as is consistent with accepted precision and detection limits; no increase is permitted.

Injection Volume and Split Volume (GC): The injection volume and split volume may be adjusted if detection and repeatability are satisfactory.

Column Temperature (HPLC): The column temperature can be adjusted by as much as $\pm 10^\circ$. Column thermostating is recommended to improve control and reproducibility of retention time.

Oven Temperature (GC): The oven temperature can be adjusted by as much as $\pm 10\%$.

Oven Temperature Program (GC): Adjustment of temperatures is permitted as stated above. When the specified temperature must be maintained or when the temperature must be changed from one value to another, an adjustment of up to $\pm 20\%$ is permitted.

Unless otherwise directed in the monograph, system suitability parameters are determined from the analyte peak.

Measured values of R_f or R_F or t_R for the sample substance do not deviate from the values obtained for the reference compound and mixture by more than the statistically determined reliability estimates from replicate assays of the reference compound. Relative retention times may be provided in monographs for informational purposes only to aid in peak identification. There are no acceptance criteria applied to relative retention times.

Suitability testing is used to ascertain the effectiveness of the final operating system, which should be subjected to this testing. Make injections of the appropriate preparation(s) as required in order to demonstrate adequate system suitability (as described in the *Chromatographic system* section of the method in a monograph) throughout the run.

The preparation can be a standard preparation or a solution containing a known amount of analyte and any additional materials (e.g., excipients or impurities) useful in controlling the analytical system. Whenever there is a significant change in the chromatographic system (equipment, mobile phase component, or other components) or in a critical reagent, system suitability is to be reestablished. No sample analysis is acceptable unless the suitability of the system has been demonstrated.

QUANTITATION

During quantitation, disregard peaks caused by solvents and reagents or arising from the mobile phase or the sample matrix.

In the linear range, peak areas and peak heights are usually proportional to the quantity of compound eluting. The peak areas and peak heights are commonly measured by electronic integrators but may be determined by more classical approaches. Peak areas are generally used but may be less accurate if peak interference occurs. The components measured are separated from any interfering components. Peak tailing and fronting is minimized, and the measurement of peaks on tails of other peaks are avoided when possible.

Although comparison of impurity peaks with those in the chromatogram of a standard at a similar concentration is preferred, impurity tests may be based on the measurement of the peak response due to impurities and expressed as a percentage of the area of the drug peak. The standard may be the drug itself at a level corresponding to, for example, 0.5% impurity, assuming similar peak responses. When impurities must be determined with greater certainty, use a standard of the impurity itself or apply a correction factor based on the response of the impurity relative to that of the main component.

External Standard Method: The concentration of the component(s) quantified is determined by comparing the response(s) obtained with the sample solution to the response(s) obtained with a standard solution.

Internal Standard Method: Equal amounts of the internal standard are introduced into the sample solution and a standard solution. The internal standard is chosen so that it does not react with the test material, is stable, is resolved from the component(s) quantified (analytes), and does not contain impurities with the same retention time as that of the analytes. The concentrations of the analytes are determined by comparing the ratios of their peak areas or peak heights and the internal standard in the sample solution with the ratios of their peak areas or peak heights and the internal standard in the standard solution.

Normalization Procedure: The percentage content of a component of the test material is calculated by determining the area of the corresponding peak as a percentage of the total area of all the peaks, excluding those due to solvents or reagents or arising from the mobile phase or the sample

matrix and those at or below the limit at which they can be disregarded.

Calibration Procedure: The relationship between the measured or evaluated signal y and the quantity (e.g., concentration, mass) of substance x is determined, and the calibration function is calculated. The analytical results are calculated from the measured signal or evaluated signal of the analyte and its position on the calibration curve.

In tests for impurities for both the *External Standard Method*, when a dilution of the sample solution is used for comparison, and the *Normalization Procedure*, any correction factors indicated in the monograph are applied (e.g., when the response factor is outside the range 0.8–1.2).

When the impurity test prescribes the total of impurities or there is a quantitative determination of an impurity, choice of an appropriate threshold setting and appropriate conditions for the integration of the peak areas is important. In such tests the limit at or below which a peak is disregarded is generally 0.05%. Thus, the threshold setting of the data collection system corresponds to at least half of this limit. Integrate the peak area of any impurity that is not completely separated from the principal peak, preferably by valley-to-valley extrapolation (tangential skim).

<631> COLOR AND ACHROMICITY

Definition—For the purposes of this chapter, color may be defined as the perception or subjective response by an observer to the objective stimulus of radiant energy in the visible spectrum extending over the range 400 nm to 700 nm in wavelength. Perceived color is a function of three variables: spectral properties of the object, both absorptive and reflective; spectral properties of the source of illumination; and visual characteristics of the observer.

Two objects are said to have a color match for a particular source of illumination when an observer cannot detect a color difference. Where a pair of objects exhibit a color match for one source of illumination and not another, they constitute a metameric pair. Color matches of two objects occur for all sources of illumination if the absorption and reflectance spectra of the two objects are identical.

Achromicity or colorlessness is one extreme of any color scale for transmission of light. It implies the complete absence of color, and therefore the visible spectrum of the object lacks absorbances. For practical purposes, the observer in this case perceives little if any absorption taking place in the visible spectrum.

Color Attributes—Because the sensation of color has both a subjective and an objective part, color cannot be described solely in spectrophotometric terms. The common attributes of color therefore cannot be given a one-to-one correspondence with spectral terminology.

Three attributes are commonly used to identify a color: (1) hue, or the quality by which one color family is distinguished from another, such as red, yellow, blue, green, and intermediate terms; (2) value, or the quality that distinguishes a light color from a dark one; and (3) chroma, or the quality that distinguishes a strong color from a weak one, or the extent to which a color differs from a gray of the same value.

The three attributes of color may be used to define a three-dimensional color space in which any color is located by its coordinates. The color space chosen is a visually uni-

form one if the geometric distance between two colors in the color space is directly a measure of the color distance between them. Cylindrical coordinates are often conveniently chosen. Points along the long axis represent value from dark to light or black to white and have indeterminate hue and no chroma. Focusing on a cross-section perpendicular to the value axis, hue is determined by the angle about the long axis and chroma is determined by the distance from the long axis. Red, yellow, green, blue, purple, and intermediate hues are given by different angles. Colors along a radius of a cross-section have the same hue, which become more intense farther out. For example, colorless or achromatic water has indeterminate hue, high value, and no chroma. If a colored solute is added, the water takes on a particular hue. As more is added, the color becomes darker, more intense, or deeper; i.e., the chroma generally increases and value decreases. If, however, the solute is a neutral color, i.e., gray, the value decreases, no increase in chroma is observed, and the hue remains indeterminate.

Laboratory spectroscopic measurements can be converted to measurements of the three color attributes. Spectroscopic results for three chosen lights or stimuli are weighted by three distribution functions to yield the tristimulus values, X , Y , Z (see *Color—Instrumental Measurement* <1061>). The distribution functions were determined in color matching experiments with human subjects.

The tristimulus values are not coordinates in a visually uniform color space; however, several transformations have been proposed that are close to being uniform, one of which is given in the chapter cited <1061> *Color—Instrumental Measurement*. The value is often a function of only the Y value. Obtaining uniformity in the chroma-hue subspace has been less satisfactory. In a practical sense, this means in visual color comparison that if two objects differ significantly in hue, deciding which has a higher chroma becomes difficult. This points out the importance of matching standard to sample color as closely as possible, especially for the attributes of hue and chroma.

Color Determination and Standards—The perception of color and color matches is dependent on conditions of viewing and illumination. Determinations should be made using diffuse, uniform illumination under conditions that reduce shadows and nonspectral reflectance to a minimum. The surface of powders should be smoothed with gentle pressure so that a planar surface free from irregularities is presented. Liquids should be compared in matched color-comparison tubes, against a white background. If results are found to vary with illumination, those obtained in natural or artificial daylight are to be considered correct. Instead of visual determination, a suitable instrumental method may be used.

Colors of standards should be as close as possible to those of test specimens for quantifying color differences. Standards for opaque materials are available as sets of color chips that are arranged in a visually uniform space.* Standards identified by a letter for matching the colors of fluids can be prepared according to the accompanying table. To prepare the matching fluid required, pipet the prescribed volumes of the colorimetric test solutions [see under *Colorimetric Solutions* (CS) in the section *Reagents, Indicators, and Solutions*] and water into one of the matching containers, and mix the solution in the container. Make the comparison as directed in the individual monograph, under the viewing conditions previously described. The matching fluids, or other combinations of the colorimetric solutions, may be used in very low concentrations to measure deviation from achromicity.

*Collections of color chips, arranged according to hue, value, and chroma in a visually uniform space and suitable for use in color designation of specimens by visual matching are available from GretagMacbeth LLC, 617 Little Britain Road, New Windsor, NY 12553-6148.

Matching Fluids

Matching Fluid	Parts of Cobaltous Chloride CS	Parts of Ferric Chloride CS	Parts of Cupric Sulfate CS	Parts of Water
A	0.1	0.4	0.1	4.4
B	0.3	0.9	0.3	3.5
C	0.1	0.6	0.1	4.2
D	0.3	0.6	0.4	3.7
E	0.4	1.2	0.3	3.1
F	0.3	1.2	0.0	3.5
G	0.5	1.2	0.2	3.1
H	0.2	1.5	0.0	3.3
I	0.4	2.2	0.1	2.3
J	0.4	3.5	0.1	1.0
K	0.5	4.5	0.0	0.0
L	0.8	3.8	0.1	0.3
M	0.1	2.0	0.1	2.8
N	0.0	4.9	0.1	0.0
O	0.1	4.8	0.1	0.0
P	0.2	0.4	0.1	4.3
Q	0.2	0.3	0.1	4.4
R	0.3	0.4	0.2	4.1
S	0.2	0.1	0.0	4.7
T	0.5	0.5	0.4	3.6

(641) COMPLETENESS OF SOLUTION

Place the quantity of the substance specified in the individual monograph in a meticulously cleansed, glass-stoppered, 10-mL glass cylinder approximately 13 mm × 125 mm in size. Using the solvent that is specified in the monograph or on the label of the product, fill the cylinder almost to the constriction at the neck. Shake gently to effect solution: the solution is not less clear than an equal volume of the same solvent contained in a similar vessel and examined similarly.

(643) TOTAL ORGANIC CARBON

Total organic carbon (TOC) is an indirect measure of organic molecules present in pharmaceutical waters measured as carbon. Organic molecules are introduced into the water from the source water, from purification and distribution system materials, and from biofilm growing in the system. TOC can also be used as a process control attribute to monitor the performance of unit operations comprising the purification and distribution system. A TOC measurement is not a replacement test for endotoxin or microbiological control. While there can be a qualitative relationship between a food source (TOC) and microbiological activity, there is no direct numerical correlation.

A number of acceptable methods exist for analyzing TOC. This chapter does not endorse, limit, or prevent any tech-

nologies from being used, but this chapter provides guidance on how to qualify these analytical technologies for use as well as guidance on how to interpret instrument results for use as a limit test.

Apparatus commonly used to determine TOC in water for pharmaceutical use have in common the objective of oxidizing the organic molecules in the water to produce carbon dioxide followed by the measurement of the amount of carbon dioxide produced. Then the amount of CO₂ produced is determined and used to calculate the organic carbon concentration in the water.

All technologies must discriminate between the inorganic carbon, which may be present in the water from sources such as dissolved CO₂ and bicarbonate, and the CO₂ generated from the oxidation of organic molecules in the sample. The discrimination may be accomplished either by determining the inorganic carbon and subtracting it from the total carbon (total carbon is the sum of organic carbon and inorganic carbon), or by purging inorganic carbon from the sample before oxidation. While purging may entrain organic molecules, such purgeable organic carbon is present in negligible quantities in water for pharmaceutical use.

Apparatus Requirements—This test method is performed either as an on-line test or as an off-line laboratory test using a calibrated instrument. The suitability of the apparatus must be periodically demonstrated as described below. In addition, it must have a manufacturer's specified limit of detection of 0.05 mg of carbon per L (0.05 ppm of carbon) or lower.

When testing water for quality control purposes, ensure that the instrument and its data are under appropriate control and that the sampling approaches and locations of both on-line and off-line measurements are representative of the quality of the water used. The nature of the water production, distribution, and use should be considered when selecting either on-line or off-line measurement.

USP Reference Standards (11)—USP 1,4-Benzoquinone RS. USP Sucrose RS.

Reagent Water—Use water having a TOC level of not more than 0.10 mg per L. [NOTE—A conductivity requirement may be necessary to ensure method reliability.]

Container Preparation—Organic contamination of containers results in higher TOC values. Therefore, use labware and containers that have been scrupulously cleaned of organic residues. Any method that is effective in removing organic matter can be used (see *Cleaning Glass Apparatus* (1051)). Use *Reagent Water* for the final rinse.

Standard Solution—Unless otherwise directed in the individual monograph, dissolve in the *Reagent Water* an accurately weighed quantity of USP Sucrose RS, to obtain a solution having a concentration of 1.19 mg of sucrose per L (0.50 mg of carbon per L).

System Suitability Solution—Dissolve in *Reagent Water* an accurately weighed quantity of USP 1,4-Benzoquinone RS to obtain a solution having a concentration of 0.75 mg per L (0.50 mg of carbon per liter).

Reagent Water Control—Use a suitable quantity of *Reagent Water* obtained at the same time as that used in the preparation of the *Standard Solution* and the *System Suitability Solution*.

Water Sample—Obtain an on-line or off-line sample that suitably reflects the quality of water used.

Other Control Solutions—Prepare appropriate reagent blank solutions or other specified solutions needed for establishing the apparatus baseline or for calibration adjustments following the manufacturer's instructions, and run the appropriate blanks to zero the instrument, if necessary.

System Suitability—Test the *Reagent Water Control* in the apparatus, and record the response, r_w . Repeat the test using the *Standard Solution*, and record the response, r_s . Calculate the corrected *Standard Solution* response, which is also the limit response, by subtracting the *Reagent Water*

Control response from the response of the *Standard Solution*. The theoretical limit of 0.50 mg of carbon per L is equal to the corrected *Standard Solution* response, $r_s - r_w$. Test the *System Suitability Solution* in the apparatus, and record the response, r_{ss} . Calculate the corrected *System Suitability Solution* response by subtracting the *Reagent Water Control* response from the response of the *System Suitability Solution*, $r_{ss} - r_w$. Calculate the percent Response Efficiency for the *System Suitability Solution* by the formula:

$$\% \text{ Response Efficiency} = 100[(r_{ss} - r_w)/(r_s - r_w)]$$

where r_s is the instrument response to the *Standard Solution*; r_{ss} is the instrument response to the *System Suitability Solution*; and r_w is the instrument response to the *Reagent Water Control*. The system is suitable if the percent Response Efficiency is not less than 85% and not more than 115%.

Procedure—Perform the test on the *Water Sample*, and record the response, r_u . The *Water Sample* meets the requirements if r_u is not more than the limit response, $r_s - r_w$. This method can be performed using on-line or off-line instrumentation that meets the *Apparatus Requirements*.

(645) WATER CONDUCTIVITY

Electrical conductivity in water is a measure of the ion-facilitated electron flow through it. Water molecules dissociate into ions as a function of pH and temperature and result in a very predictable conductivity. Some gases, most notably carbon dioxide, readily dissolve in water and interact to form ions, which predictably affect conductivity as well as pH. For the purpose of this discussion, these ions and their resulting conductivity can be considered intrinsic to the water.

Water conductivity is also affected by the presence of extraneous ions. The extraneous ions used in modeling the conductivity specifications described below are the chloride and sodium ions. The conductivity of the ubiquitous chloride ion (at the theoretical endpoint concentration of 0.47 ppm when it was a required attribute test in *USP XXII* and earlier revisions) and the ammonium ion (at the limit of 0.3 ppm) represent a major portion of the allowed water impurity level. A balancing quantity of cations, such as sodium ions, is included in this allowed impurity level to maintain electroneutrality. Extraneous ions such as these may have significant impact on the water's chemical purity and suitability for use in pharmaceutical applications. The procedure in the section *Bulk Water* is specified for measuring the conductivity of waters such as Purified Water, Water for Injection, Water for Hemodialysis, and the condensate of Pure Steam. The procedure in the section *Sterile Water* is specified for measuring the conductivity of waters such as Sterile Purified Water, Sterile Water for Injection, Sterile Water for Inhalation, and Sterile Water for Irrigation.

Online conductivity testing provides real-time measurements and opportunities for real-time process control, decision, and intervention. Precaution should be taken while collecting water samples for off-line conductivity measurements. The sample may be affected by the sampling method, the sampling container, and environmental factors such as ambient carbon dioxide concentration and organic vapors.

INSTRUMENT SPECIFICATIONS AND OPERATING PARAMETERS

Water conductivity must be measured accurately using calibrated instrumentation. The conductivity cell constant, a factor that represents the geometrical properties of the conductivity sensor, must be known within $\pm 2\%$. The cell constant can be verified directly by using a solution of known or traceable conductivity, or indirectly by comparing the instrument reading taken with the conductivity sensor in question to readings from a conductivity sensor of known or traceable cell constant.

Meter calibration is accomplished by replacing the conductivity sensor with NIST (or equivalent local national authority) -traceable precision resistors (accurate to $\pm 0.1\%$ of the stated value) or an equivalently accurate adjustable resistance device, such as a Wheatstone Bridge, to give a predicted instrument response. Each scale on the meter may require separate calibration prior to use. The frequency of recalibration is a function of instrument design, degree of use, etc. However, because some multiple-scale instruments have a single calibration adjustment, recalibration may be required between each use of a different scale. Excluding the conductivity sensor cell constant accuracy, the instrument accuracy must be $\pm 0.1 \mu\text{S/cm}$.

In order to increase the measurement accuracy on the conductivity ranges used, which can be large, and to ensure a complete equipment calibration, it is suggested that periodic verification of the entire equipment be performed. This could be done by comparing the conductivity/resistivity values displayed by the measuring equipment with those of an external calibrated conductivity-measuring device. The two nontemperature-compensated conductivity or resistivity values must be equivalent to within $\pm 20\%$ of each other, or at a difference that is acceptable on the basis of product water criticality and/or the water conductivity ranges in which the measurements are taken. The two conductivity sensors should be positioned close enough together to measure the same water sample in the same environmental conditions.

In addition to the verification method performed in nontemperature-compensated mode, a similar verification performed in temperature-compensated mode could be performed to ensure an appropriate accuracy of the equipment when such a mode is used for trending or other purposes.

Because temperature has a substantial impact on conductivity readings of specimens at high and low temperatures, many instruments automatically correct the actual reading to display the value that theoretically would be observed at the nominal temperature of 25° . This is typically done using a temperature sensor embedded in the conductivity sensor and an algorithm in the instrument's circuitry. This temperature compensation algorithm may not be accurate. Conductivity values used in this method are nontemperature-compensated measurements. Temperature measurement is required for the performance of the *Stage 1* test. It may be made using the temperature sensor embedded in the conductivity cell sensor. An external temperature sensor positioned near the conductivity sensor is also acceptable. Accuracy of the temperature measurement must be $\pm 2^\circ$.

The procedures below shall be performed using instrumentation that has been calibrated, has conductivity sensor cell constants that have been accurately determined, and has temperature compensation function that has been disabled. For both online and offline measurements, the suitability of instrumentation for quality control testing is also dependent on the sampling location(s) in the water system. The selected sampling instrument location(s) must reflect the quality of the water used.

BULK WATER

The procedure and test limits in this section are intended for Purified Water, Water for Injection, Water for Hemodial-

ysis, the condensate of Pure Steam, and any other monographs which specify this section.

The combined conductivities of the intrinsic and extraneous ions vary as a function of pH and are the basis for the conductivity specifications described in the *Stage 3—pH and Conductivity Requirements* table and used when performing *Stage 3* of the test method. Two preliminary stages are included in the test method. If the test conditions and conductivity limits are met at either of these preliminary stages, the water meets the requirements of this test. Proceeding to the third stage of the test in these circumstances is unnecessary. Only in the event of failure at the final test stage is the sample judged noncompliant with the requirements of the test.

Procedure

STAGE 1

- Stage 1* is intended for online measurement or may be performed offline in a suitable container.
1. Determine the temperature of the water and the conductivity of the water using a nontemperature-compensated conductivity reading.
 2. Using the *Stage 1—Temperature and Conductivity Requirements* table, find the temperature value that is not greater than the measured temperature, i.e., the next lower temperature. The corresponding conductivity value on this table is the limit. [NOTE—Do not interpolate.]
 3. If the measured conductivity is not greater than the table value, the water meets the requirements of the test for conductivity. If the conductivity is higher than the table value, proceed with *Stage 2*.

Stage 1—Temperature and Conductivity Requirements
(for nontemperature-compensated conductivity measurements only)

Temperature	Conductivity Requirement (µS/cm)
0	0.6
5	0.8
10	0.9
15	1.0
20	1.1
25	1.3
30	1.4
35	1.5
40	1.7
45	1.8
50	1.9
55	2.1
60	2.2
65	2.4
70	2.5
75	2.7
80	2.7
85	2.7
90	2.7
95	2.9
100	3.1

STAGE 2

4. Transfer a sufficient amount of water (100 mL or more) to a suitable container, and stir the test specimen. Adjust the temperature, if necessary, and, while maintaining it at $25 \pm 1^\circ$, begin vigorously agitating the test specimen while periodically observing the conductivity. When the change in conductivity (due to uptake of atmospheric carbon dioxide) is less than a net of 0.1 µS/cm per 5 minutes, note the conductivity.
5. If the conductivity is not greater than 2.1 µS/cm, the water meets the requirements of the test for conductivity. If the conductivity is greater than 2.1 µS/cm, proceed with *Stage 3*.

STAGE 3

6. Perform this test within approximately 5 minutes of the conductivity determination in Step 5, while maintaining the sample temperature at $25 \pm 1^\circ$. Add a saturated potassium chloride solution to the same water sample (0.3 mL per 100 mL of the test specimen), and determine the pH to the nearest 0.1 pH unit, as directed under *pH* (791).
7. Referring to the *Stage 3—pH and Conductivity Requirements* table, determine the conductivity limit at the measured pH value. If the measured conductivity in Step 4 is not greater than the conductivity requirements for the pH determined in Step 6, the water meets the requirements of the test for conductivity. If either the measured conductivity is greater than this value or the pH is outside the range of 5.0 to 7.0, the water does not meet the requirements of the test for conductivity.

Stage 3—pH and Conductivity Requirements
(for atmosphere- and temperature-equilibrated samples only)

pH	Conductivity Requirement (µS/cm)
5.0	4.7
5.1	4.1
5.2	3.6
5.3	3.3
5.4	3.0
5.5	2.8
5.6	2.6
5.7	2.5
5.8	2.4
5.9	2.4
6.0	2.4
6.1	2.4
6.2	2.5
6.3	2.4
6.4	2.3
6.5	2.2
6.6	2.1
6.7	2.6
6.8	3.1
6.9	3.8
7.0	4.6

ter occurs, repeat the test, introducing small particles of the material under test in solid form at 1° intervals as the temperature approaches the expected congealing point.

Record the reading of the test tube thermometer every 30 seconds. Continue stirring only so long as the temperature is gradually falling, stopping when the temperature becomes constant or starts to rise slightly. Continue recording the temperature in the test tube every 30 seconds for at least 3 minutes after the temperature again begins to fall after remaining constant.

The average of not less than four consecutive readings that lie within a range of 0.2° constitutes the congealing temperature. These readings lie about a point of inflection or a maximum, in the temperature-time curve, that occurs after the temperature becomes constant or starts to rise and before it again begins to fall. The average to the nearest 0.1° is the congealing temperature.

(659) PACKAGING AND STORAGE REQUIREMENTS

Every monograph in the *USP* and *NF* shall have packaging and storage requirements. For the packaging portion of the statement, the choice of containers is given in this chapter. For drug product packaging requirements, definitions are provided to guide selection and adaptation. For active pharmaceutical ingredients (APIs), the choice would be tight, well-closed, or, where needed, a light-resistant container. For excipients, given their typical presentation as large-volume commodity items (containers ranging from drums to tank cars), a well-closed container is an appropriate default.

Where no specific directions or limitations are provided in the article's labeling, articles shall be protected from moisture, freezing, and excessive heat, and where necessary, from light during shipping and distribution. Drug substances are exempt from this standard.

PACKAGING

Packaging must not interact physically or chemically with official articles in any way that causes their safety, identity, strength, quality, or purity to fail to conform to requirements. This chapter provides definitions of both packaging and storage.

GENERAL DEFINITIONS

Packaging System (also referred to as a container–closure system): The sum of packaging components that together contain and protect the article. This includes primary packaging components and secondary packaging components, if the latter is intended to provide additional protection.

Container: A receptacle that holds an intermediate compound, active pharmaceutical ingredient, excipient, or dosage form and is in direct contact with the articles.

Packaging Component: Any single part of the package or container–closure system including the container (e.g., ampules, prefilled syringes, vials, bottles); container liners (e.g., tube cartridge liners); closures (e.g., screw caps, stoppers); ferrules and overseals; closure liners; inner seals; administration ports; overwraps; administration accessories; and labels.

Primary Packaging Component: Packaging components that are in direct contact or may become in direct contact with the article.

Secondary Packaging Component: Packaging components that are not and will not be in direct contact with the article but may provide additional protection.

Tertiary Packaging: Packaging components that are not in direct contact with the article but facilitate the handling and transport in order to prevent damage from physical handling and storage conditions to which the article is subjected.

Materials of Construction: Refers to the materials (e.g., glass, plastic, elastomers, metal) used to manufacture a packaging component.

Multiple-Dose (also referred to as multi-dose): A packaging system that permits withdrawal of successive portions of an article for parenteral administration without changing the safety, strength, quality, or purity of the remaining portion. See *Multi-Dose Containers in Injections* (1), *Determination of Volume of Injection in Containers*.

Multiple-Unit: A packaging system that permits withdrawal of successive portions of an article without changing the safety, strength, quality, or purity of the remaining portion.

Single-Unit: A packaging system that holds a quantity of an article intended for administration as a single dose or a single finished device intended for single use.

Single-Dose (see also *Injections* (1), *Containers for Injections*): A single-unit package for an article intended for parenteral administration. Examples of single-dose containers include prefilled syringes, cartridges, fusion-sealed containers, and closure-sealed containers when so labeled.

Unit-Dose: A single-unit packaging system for an article intended for administration by other than the parenteral route as a single dose.

Unit-of-Use: A packaging system that contains a specific quantity of an article that is intended to be dispensed as such without further modification except for the addition of appropriate labeling. *Unit-of-Use* packaging may not be repackaged for sale.

Pharmacy Bulk Package: A container of a sterile preparation for parenteral use that contains many single doses. The contents are intended for use in a pharmacy admixture program and are restricted to the preparation of admixtures for infusion or, through a sterile transfer device, for the filling of empty sterile syringes.

After constitution, the closure shall be penetrated only one time with a suitable sterile transfer device or dispensing set that allows measured dispensing of the contents. The *Pharmacy Bulk Package* is to be used only in a suitable work area such as a laminar flow hood (or an equivalent clean air compounding area).

Designation as a *Pharmacy Bulk Package* is limited to *Injections*, for *Injection*, or *Injectable Emulsion* as defined under *Injections* (1), *Nomenclature*.

Pharmacy Bulk Packages, although containing more than one single dose, are exempt from the multiple-dose container volume limit of 30 mL and the requirement that they contain a substance or suitable mixture of substances to prevent the growth of microorganisms. Where a container is offered as a *Pharmacy Bulk Package*, the label shall (a) state prominently "Pharmacy Bulk Packages—Not for direct infusion," (b) contain or refer to information on proper techniques to help assure safe use of the product, and (c) bear a statement limiting the time frame in which the container may be used once it has been entered, provided it is held under the labeled storage conditions.

Small-Volume Injections: A single-dose injection that is intended for intravenous use and is packaged in containers labeled as containing 100 mL or less.

Large-Volume Injections: A single-dose injection that is intended for intravenous use and is packaged in containers labeled as containing more than 100 mL.

Child-Resistant: A packaging system designed or constructed to meet Consumer Product Safety Commission standards pertaining to opening by children (16 CFR § 1700.20).

Senior-Friendly: A packaging system designed or constructed to meet Consumer Product Safety Commission standards pertaining to opening by senior adults (16 CFR § 1700.20).

Tamper-Evident: A packaging system that may not be accessed without obvious destruction of the seal or some portion of the packaging system.

Tight: A packaging system that protects the articles from contamination by extraneous liquids, solids, or vapors; from loss of the article; and from efflorescence, deliquescence, or evaporation under the ordinary or customary conditions of handling, shipment, storage, and distribution. See *Containers—Performance Testing* (671).

Well-Closed: A packaging system that protects the articles from contamination by extraneous solids and liquids and from loss of the article under the ordinary or customary conditions of handling, shipment, storage, and distribution. See *Containers—Performance Testing* (671).

Light-Resistant: A packaging system that protects from the effects of light by virtue of the specific properties of the material of which it is composed, including any coating applied to it. A clear and colorless or a translucent container may be made light-resistant by means of an opaque covering or by use of secondary packaging, in which case the label of the container bears a statement that the opaque covering or secondary packaging is needed until the articles are to be used or administered. See *Containers—Performance Testing* (671), *Light Transmission Test*.

MEDICAL GAS PACKAGING

Gas Cylinder: A gas cylinder is a metallic packaging system constructed of steel or aluminum designed to hold medical gases under pressure. Medical gases include Carbon Dioxide USP, Helium USP, Medical Air USP, Nitric Oxide, Nitrous Oxide USP, Nitrogen NF, and Oxygen USP. As a safety measure, for carbon dioxide, cyclopropane, helium, medical air, nitrous oxide, and oxygen, the Pin-Index Safety System of matched fittings is recommended for cylinders of Size E or smaller.

ASSOCIATED COMPONENTS

Many associated components are graduated for dose administration. It is the responsibility of the manufacturer to ensure the appropriate dosing component is provided or a general purpose component, such as those described in this section, is specified for delivering the appropriate dose with the intended accuracy. The graduations should be legible and indelible.

Graduated associated components described in this section are for general use. Graduated markings should be legible, indelible, and on an extraoral nonproduct contact surface. Under ideal conditions of use, the volume error incurred in measuring liquids for individual dose administration by means of such graduated components should be not greater than 10% of the indicated amount of the liquid preparation with which the graduated component will be used. Few liquid preparations have the same surface and flow characteristics. Therefore, the volume delivered varies materially from one preparation to another.

Polymers and ingredients added to polymers that are used in the fabrication of associated components must conform to the requirements in the applicable sections of the Code of Federal Regulations, Title 21, *Indirect Food Additives*.

Dosing Cups: A measuring device consisting of a small cup that is packaged with oral liquid articles or that may be sold and purchased separately.

Dosing Spoon: A measuring device consisting of a bowl and a handle that is packaged with oral liquid articles or that may be sold and purchased separately. The handle may be a graduated tube.

Medicine Dropper: A measuring device consisting of a transparent or translucent barrel or tube that is generally fitted with a collapsible bulb. It is packaged with oral liquid articles or may be sold and purchased separately.

Droppers typically vary in capacity; however, the delivery end should be a round opening having an external diameter of about 3 mm. The barrel may be graduated. [NOTE—Few medicinal liquids have the same surface and flow characteristics as water, and therefore the size of drops varies materially from one preparation to another.]

Oral Syringe: A measuring device consisting of a plunger and barrel made of suitable rigid transparent or translucent plastic material and a seal on the end. It is packaged with oral liquid articles or may be sold and purchased separately. The syringe should expel a measured amount of a liquid article directly into the patient's mouth. Finger grips located at the open end of the barrel should be the appropriate size, shape, and strength and should allow the syringe to be held securely during use. The barrel may be graduated.

Teaspoon: A measuring device consisting of a shallow bowl, oval or round, at the end of a handle. A teaspoon has been established as containing 4.93 ± 0.24 mL. For the practice of administering articles, the teaspoon may be regarded as representing 5 mL.

Articles intended for administration by teaspoon should be formulated on the basis of dosage in 5-mL units, such that any component used to administer liquid articles should deliver 5 mL wherever a teaspoon calibration is indicated. A household spoon is not an acceptable alternative to the graduated teaspoon described herein.

POISON PREVENTION PACKAGING ACT (PPPA)

This act requires special packaging of most human oral prescription drugs, oral controlled drugs, certain non-oral prescription drugs, certain dietary supplements, and many over-the-counter (OTC) drug preparations in order to protect the public from personal injury or illness from misuse of these preparations (16 CFR §1700.14).

The immediate packaging of substances regulated under the PPPA must comply with the special packaging standards (16 CFR §1700.15 and 16 CFR §1700.16) and applies to all packaging types including reclosable, nonclosable, and unit-dose types.

Special packaging is not required either for drugs dispensed within a hospital setting for inpatient administration or by manufacturers and packagers of bulk-packaged prescription drugs repackaged by the pharmacist. PPPA-regulated prescription drugs may be dispensed in nonchild-resistant packaging upon the request of the purchaser or when directed in a legitimate prescription (15 U.S.C. §1473).

Manufacturers or packagers of PPPA-regulated OTC preparations are allowed to package one size in nonchild-resistant packaging as long as popular-size, special packages are also supplied. The nonchild-resistant packaging requires special labeling (16 CFR §1700.5).

STORAGE CONDITIONS

Specific directions are stated in some monographs with respect to storage conditions, e.g., the temperature or humidity at which an article must be stored and shipped. Such directions apply, except where the label on the article has different storage conditions that are based on stability stud-

ies. Where no specific storage conditions are provided in the individual monograph, but the label of an article states storage conditions based on stability studies, such labeled storage directions apply. Current storage conditions for articles are defined by the following terms.

Freezer: A place in which the temperature is maintained between –25° and –10° (–13° and 14 °F).
Refrigerator: A place in which the temperature is maintained between 2° and 8° (36° and 46 °F).

Cold: Any temperature not exceeding 8° (46 °F).
Cool: Any temperature between 8° and 15° (46° and 59 °F). [NOTE—An article for which storage in a cool place is directed may, alternatively, be stored and shipped as refrigerated, unless otherwise specified by the individual monograph.]

Room Temperature: The temperature prevailing in a work area.

Controlled Room Temperature: The temperature maintained at the usual and customary working environment of 20° to 25° (68° to 77 °F). The following conditions also apply.

The mean kinetic temperature shall not exceed 25°. The mean kinetic temperature is a calculated value that may be used as an isothermal storage temperature that simulates the nonisothermal effects of storage temperature variations. Excursions between 15° and 30° (59° and 86 °F) that are experienced in pharmacies, hospitals, and warehouses, and during shipping are allowed, provided the mean kinetic temperature does not exceed 25°.

Transient spikes up to 40° are permitted as long as they do not last for more than 24 hours. Spikes above 40° may be permitted only if the manufacturer so instructs.

Articles may be labeled for storage at “controlled room temperature” or at “up to 25°”, or other wording based on the same mean kinetic temperature.

An article for which storage at *Controlled Room Temperature* is directed may, alternatively, be stored and shipped in a cool place or refrigerated, unless otherwise specified in the individual monograph or on the label.

Warm: Any temperature between 30° and 40° (86° and 104 °F).

Excessive Heat: Any temperature above 40° (104 °F).

Dry Place: The term “dry place” denotes a place that does not exceed 40% average relative humidity at 20° (68 °F) or the equivalent water vapor pressure at other temperatures. The determination may be made by direct measurement at the place or may be based on reported climatic conditions. Determination is based on not less than 12 equally spaced measurements that encompass either a season, a year, or, where recorded data demonstrate, the storage period of the article. There may be values of up to 45% relative humidity provided that the average value does not exceed 40% relative humidity. Storage in a container validated to protect the article from moisture vapor, including storage in bulk, is considered a dry place.

Protection from Freezing: Where, in addition to the risk of breakage of the container, freezing subjects an article to loss of strength or potency, or to destructive alteration of its characteristics, the container label bears an appropriate instruction to protect the article from freezing.

Protection from Light: Where light subjects an article to loss of strength or potency, or to destructive alteration of its characteristics, the container label bears an appropriate instruction to protect the article from light.

(660) CONTAINERS—GLASS

DESCRIPTION

Glass containers for pharmaceutical use are intended to come into direct contact with pharmaceutical products. Glass used for pharmaceutical containers is either borosilicate (neutral) glass or soda-lime-silica glass. Borosilicate glass contains significant amounts of boric oxide, aluminum oxide, and alkali and/or alkaline earth oxides. Borosilicate glass has a high hydrolytic resistance and a high thermal shock resistance due to the chemical composition of the glass itself; it is classified as Type I glass. Soda-lime-silica glass is a silica glass containing alkaline metal oxides, mainly sodium oxide; and alkaline earth oxides, mainly calcium oxide. Soda-lime-silica glass has a moderate hydrolytic resistance due to the chemical composition of the glass itself; it is classified as Type III glass. Suitable treatment of the inner surface of Type III soda-lime-silica glass containers will raise the hydrolytic resistance from a moderate to a high level, changing the classification of the glass to Type II.

The following recommendations can be made as to the suitability of the glass type for containers for pharmaceutical products, based on the tests for hydrolytic resistance. Type I glass containers are suitable for most products for parenteral and nonparenteral use. Type II glass containers are suitable for most acidic and neutral aqueous products for parenteral and non-parenteral uses. Type II glass containers may be used for alkaline parenteral products where stability data demonstrate their suitability. Type III glass containers usually are not used for parenteral products or for powders for parenteral use, except where suitable stability test data indicate that Type III glass is satisfactory.

The inner surface of glass containers may be treated to improve hydrolytic resistance. The outer surface of glass containers may be treated to reduce friction or for protection against abrasion or breakage. The outer surface treatment is such that it does not contaminate the inner surface of the container.

Glass may be colored to provide protection from light by the addition of small amounts of metal oxides and is tested as described in *Spectral Transmission for Colored Glass Containers*. A clear and colorless container that is made light resistant by means of an opaque enclosure (see *Light-Resistant Container* in (659) *Packaging and Storage Requirements*) is exempt from the requirements for spectral transmission. Containers for aqueous parenteral products are tested for arsenic release.

SPECIFIC TESTS

The *Glass Grains Test* combined with the *Surface Glass Test* for hydrolytic resistance determines the glass type. The hydrolytic resistance is determined by the quantity of alkali released from the glass under the conditions specified. This quantity of alkali is extremely small in the case of the more resistant glasses, thus calling for particular attention to all details of the tests and the use of apparatus of high quality and precision. The tests should be conducted in an area relatively free from fumes and excessive dust. Test selection is shown in *Table 1* and *Table 2*.

Table 1. Determination of Glass Types

Container Type	Test	Reason
I, II, III	Glass Grains Test	Distinguishes Type I borosilicate glass from Type II and III soda-lime-silica glass

The inner surface of glass containers is the contact surface for pharmaceutical preparations, and the quality of this surface is determined by the *Surface Glass Test* for hydrolytic resistance. The *Surface Etching Test* may be used to determine whether high hydrolytic resistance is due to chemical composition or to surface treatment. Alternatively, the comparison of data from the *Glass Grains Test* and the *Surface Glass Test* may be used in Table 2.

Table 2. Determination of Inner Surface Hydrolytic Resistance

Container Type	Test	Reason
I, II, III	<i>Surface Glass Test</i>	Determines hydrolytic resistance of inner surface. Distinguishes between <i>Type I</i> and <i>Type II</i> containers with high hydrolytic resistance and <i>Type III</i> containers with moderate hydrolytic resistance
I, II	<i>Surface Etching Test</i> or comparison of <i>Glass Grains Test</i> and <i>Surface Glass Test</i> data	Where it is necessary to determine whether high hydrolytic resistance is due to inner surface treatment or to the chemical composition of the glass containers

Glass containers must comply with their respective specifications for identity and surface hydrolytic resistance to be classified as Type I, II, or III glass. Type I or Type II containers for aqueous parenteral products are tested for extractable arsenic.

Hydrolytic Resistance

Apparatus

Autoclave—For these tests, use an autoclave capable of maintaining a temperature of $121 \pm 1^\circ$, equipped with a thermometer, a pressure gauge, a vent cock, and a tray of sufficient capacity to accommodate the number of containers needed to carry out the test above the water level. Clean the autoclave and other apparatus thoroughly with Purified Water before use.

Mortar and Pestle—Use a hardened-steel mortar and pestle, made according to the specifications in Figure 1.

Other Apparatus—Also required are a set of three square-mesh stainless steel sieves mounted on frames consisting of US Sieve Nos. 25, 40, and 50 (see *Particle Size Distribution Estimation by Analytical Sieving* (786), Table 1. *Sizes of Standard Sieve Series in Range of Interest*); a tempered, magnetic steel hammer; a permanent magnet; weighing bottles; stoppers; metal foil (e.g. aluminum, stainless steel); a hot air oven, capable of maintaining $140 \pm 5^\circ$; a balance, capable of weighing up to 500 g with an accuracy of 0.005 g; a desiccator; and an ultrasonic bath.

Reagents

Carbon Dioxide-Free Water—This is Purified Water that has been boiled vigorously for 5 minutes or more and allowed to cool while protected from absorption of carbon dioxide from the atmosphere, or Purified Water that has a resistivity of not less than 18 Mohm-cm.

Methyl Red Solution—Dissolve 50 mg of methyl red in 1.86 mL of 0.1 M sodium hydroxide and 50 mL of ethanol (96%), and dilute with Purified Water to 100 mL. To test for sensitivity, add 100 mL of carbon dioxide-free water and 0.05 mL of 0.02 M hydrochloric acid to 0.1 mL of the methyl red solution. The resulting solution should be red.

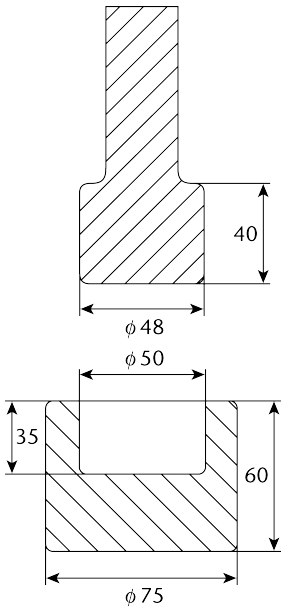


Figure 1. Mortar and pestle for pulverizing glass.

Not more than 0.1 mL of 0.02 M sodium hydroxide is required to change the color to yellow. A color change from red to yellow corresponds to a change in pH from pH 4.4 (red) to pH 6.0 (yellow).

GLASS GRAINS TEST

The *Glass Grains Test* may be performed either on the canes used for the manufacture of tubing glass containers or on the containers.

Sample Preparation: Rinse the containers to be tested with Purified Water, and dry in the oven. Wrap at least three of the glass articles in clean paper, and crush to produce two samples of about 100 g each in pieces not more than 30 mm across. Place in the mortar 30–40 g of the pieces between 10 and 30 mm across taken from one of the samples, insert the pestle, and strike it heavily with the hammer once only. Alternatively, transfer samples into a ball mill-breaker, add the balls, and crush the glass. Transfer the contents of the mortar or ball mill to the coarsest sieve (No. 25) of the set. Repeat the operation until all fragments have been transferred to the sieve. Shake the set of sieves for a short time by hand, and remove the glass that remains on sieves No. 25 and No. 40. Submit these portions to further fracture, repeating the operation until about 10 g of glass remains on sieve No. 25. Reject this portion and the portion that passes through sieve No. 50. Reassemble the set of sieves, and shake for 5 minutes. Transfer to a weighing bottle the glass grains that passed through sieve No. 40 and are retained on sieve No. 50. Repeat the crushing and sieving procedure with the second glass sample until two samples of grains are obtained, each of which weigh more than 10 g.

Spread each sample on a piece of clean glazed paper, and remove any iron particles by passing the magnet over them. Transfer each sample into a beaker for cleaning. Add 30 mL of acetone to the grains in each beaker, and scour the grains, using suitable means such as a rubber-tipped or plastic-coated glass rod. After scouring the grains, allow to settle, and decant as much acetone as possible. Add another 30 mL of acetone, swirl, decant, and add a new portion of acetone. Fill the bath of the ultrasonic vessel with water at room temperature, then place the beaker in the rack, and immerse it until the level of the acetone is at the level of the water; apply the ultrasound for 1 minute. Swirl the beaker, allow to settle, and decant the acetone as completely as

possible; then repeat the ultrasonic cleaning operation. If a fine turbidity persists, repeat the ultrasonic cleaning and acetone washing until the solution remains clear. Swirl, and decant the acetone. Dry the grains, first by putting the beaker on a warm plate and then by heating at 140° for 20 minutes in a drying oven. Transfer the dried grains from each beaker into separate weighing bottles, insert the stoppers, and cool in a desiccator.

Method

Filling and Heating—Weigh 10.00 g of the cleaned and dried grains into two separate conical flasks. Pipet 50 mL of carbon dioxide-free Purified Water into each of the conical flasks (test solutions). Pipet 50 mL of carbon dioxide-free Purified Water into a third conical flask that will serve as a blank. Distribute the grains evenly over the flat bases of the flasks by shaking gently. Close the flasks with neutral glass dishes or aluminum foil rinsed with Purified Water or with inverted beakers so that the inner surfaces of the beakers fit snugly down onto the top rims of the flasks. Place all three flasks in the autoclave containing the water at ambient temperature, and ensure that they are held above the level of the water in the vessel. Carry out the following operations:

- 1. Heat the autoclave to 100°, and allow the steam to issue from the vent cock for 10 minutes.
- 2. Close the vent cock, and raise the temperature from 100° to 121° at a rate of 1° per minute.
- 3. Maintain the temperature at 121 ± 1° for 30 ± 1 minutes.
- 4. Lower the temperature from 121° to 100° at a rate of 0.5° per minute, venting to prevent a vacuum.
- 5. Do not open the autoclave before it has cooled to 95°. Remove the containers from the autoclave, using normal precautions, and cool the flasks in running tap water.

Titration—To each of the 3 flasks add 0.05 mL of *Methyl Red Solution*. Titrate the blank solution immediately with 0.02 M hydrochloric acid, then titrate the test solutions until the color matches that obtained with the blank solution. Subtract the titration volume for the blank solution from that for the test solutions. Calculate the mean value of the results in mL of 0.02 M hydrochloric acid per gram of the sample. Repeat the test if the highest and lowest observed values differ by more than 20%.

NOTE—Where necessary to obtain a sharp endpoint, decant the clear solution into a separate 250-mL flask. Rinse the grains by swirling with three 15-mL portions of Purified Water, and add the washings to the main solution. Add 0.05 mL of the *Methyl Red Solution*. Titrate, and calculate as before. In this case also add 45 mL of Purified Water and 0.05 mL of *Methyl Red Solution* to the blank solution.

Limits: The volume does not exceed the values indicated in *Table 3*.

Table 3. Test Limits for Glass Grains Test

Filling Volume (mL)	Maximum Volume of 0.02 M HCl per g of Test Glass (mL)	
	Type I	Types II and III
All	0.1	0.85

SURFACE GLASS TEST

Determination of the Filling Volume: The filling volume is the volume of Purified Water to be added to the container for the purpose of the test. For vials, bottles, cartridges, and syringes, the filling volume is 90% of the brimful capacity. For ampuls, it is the volume up to the height of the shoulder.

Vials and Bottles—Select six dry vials or bottles from the sample lot, or three if their capacity exceeds 100 mL, and remove any dirt or debris. Weigh the empty containers with an accuracy of 0.1 g. Place the containers on a horizontal surface, and fill them with Purified Water to about the rim edge, avoiding overflow and the introduction of air bubbles. Adjust the liquid levels to the brimful line. Weigh the filled containers to obtain the mass of the water expressed to 2 decimal places, for containers having a nominal volume less than or equal to 30 mL; and expressed to 1 decimal place, for containers having a nominal volume greater than 30 mL. Calculate the mean value of the brimful capacity in mL, and multiply it by 0.9. This volume, expressed to 1 decimal place, is the filling volume for the particular container lot.

Cartridges and Syringes—Select six dry syringes or cartridges, and seal the small opening (mouth of cartridges; Luer cone or staked needle of syringes), using an inert material. Determine the mean brimful capacity and filling volume according to *Vials and Bottles*.

Ampuls—Place at least six dry ampuls on a flat, horizontal surface, and fill them with Purified Water from a buret until the water reaches point A, where the body of the ampul starts to decrease to the shoulder of the ampul (see *Figure 2*). Read the capacities, expressed to 2 decimal places, and calculate the mean value. This volume, expressed to 1 decimal place, is the filling volume for the particular ampul lot. The filling volume may also be determined by weighing.

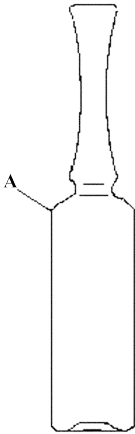


Figure 2. Filling volumes of ampuls up to point A.

Test: The determination is carried out on unused containers. The volumes of the test liquid necessary for the final determination are shown in *Table 4*.

Table 4. Volume of Test Liquid and Number of Titrations

Filling Volume (mL)	Volume of Test Liquid for One Titration (mL)	Number of Titrations
Up to 3	25.0	1
Above 3 and up to 30	50.0	2
Above 30 and up to 100	100.0	2
Above 100	100.0	3

Method

Cleaning—Remove any debris or dust. Shortly before the test, rinse each container carefully at least twice with Puri-

fied Water, and allow to stand. Immediately before testing, empty the containers; rinse once with Purified Water, then with carbon dioxide-free water; and allow to drain. Complete the cleaning procedure from the first rinsing in not less than 20 minutes and not more than 25 minutes. Heat closed ampuls in a water bath or in an air oven at about 50° for approximately 2 minutes before opening. Do not rinse before testing.

Filling and Heating—The containers are filled with carbon dioxide-free water up to the filling volume. Containers in the form of cartridges or prefillable syringes are closed in a suitable manner with material that does not interfere with the test. Each container, including ampuls, shall be loosely capped with an inert material such as a dish of neutral glass or aluminum foil previously rinsed with Purified Water. Place the containers on the tray of the autoclave. Place the tray in an autoclave containing a quantity of water such that the tray remains clear of the water. Close the autoclave, and carry out the autoclaving procedure as described for the *Glass Grains Test*, except that the temperature is maintained at $121 \pm 1^\circ$ for 60 ± 1 minutes. Remove the containers from the autoclave using normal precautions, place them in a water bath at 80°, and run cold tap water into the water bath. To avoid contamination of the extraction solution, take care that the water does not contact the loose foil caps. The cooling time does not exceed 30 minutes. The extraction solutions are analyzed by titration according to the method described below.

Titration—Carry out the titration within 1 hour of removal of the containers from the autoclave. Combine the liquids obtained from the containers, and mix. Introduce the prescribed volume (see *Table 4*) into a conical flask. Transfer the same volume of carbon dioxide-free water, to be used as a blank, into a second similar flask. Add to each flask 0.05 mL of *Methyl Red Solution* for each 25 mL of liquid. Titrate the blank with 0.01 M hydrochloric acid. Titrate the test liquid with the same acid until the color of the resulting solution is the same as that obtained for the blank. Subtract the value found for the blank titration from that found for the test liquid, and express the results in mL of 0.01 M hydrochloric acid per 100 mL of test liquid. Express titration values of less than 1.0 mL to two decimal places; express titration values of more than or equal to 1.0 mL to one decimal place.

Limits: The results, or the average of the results if more than one titration is performed, are not greater than the values stated in *Table 5*.

Table 5. Limit Values for the Surface Glass Test

Filling Volume (mL)	Maximum Volume of 0.01 M HCl per 100 mL of Test Liquid (mL)	
	Types I and II	Type III
Up to 1	2.0	20.0
Above 1 and up to 2	1.8	17.6
Above 2 and up to 5	1.3	13.2
Above 5 and up to 10	1.0	10.2
Above 10 and up to 20	0.80	8.1
Above 20 and up to 50	0.60	6.1
Above 50 and up to 100	0.50	4.8
Above 100 and up to 200	0.40	3.8
Above 200 and up to 500	0.30	2.9
Above 500	0.20	2.2

SURFACE ETCHING TEST

The *Surface Etching Test* is used in addition to the *Surface Glass Test* when it is necessary to determine whether a con-

tainer has been surface treated and/or to distinguish between Type I and Type II glass containers. Alternatively, the *Glass Grains Test* and *Surface Glass Test* may be used. The *Surface Etching Test* may be carried out either on unused samples or on samples used in the *Surface Glass Test*.

Method

Vials and Bottles—The volumes of test liquid required are shown in *Table 4*. Rinse the containers twice with Purified Water, fill to the brimful point with a mixture of 1 volume of hydrofluoric acid and 9 volumes of hydrochloric acid, and allow to stand for 10 minutes. Empty the containers, and rinse carefully five times with Purified Water. Immediately before the test, rinse once again with Purified Water. Submit these containers to the same autoclaving and determination procedure as described for the *Surface Glass Test*. If the results are considerably higher than those obtained from the original surfaces (by a factor of about 5 to 10), the samples have been surface treated. [Caution—Hydrofluoric acid is extremely aggressive. Even small quantities can cause life threatening injuries.]

Ampuls, Cartridges, and Syringes—Apply the test method as described for *Vials and Bottles*. If the ampuls, cartridges, and syringes are not surface treated, the values obtained are slightly lower than those obtained in the previous tests. [NOTE—Ampuls, cartridges, and syringes made from Type I glass tubing are not normally subjected to internal surface treatment.]

Distinction Between Type I and Type II Glass Containers:

The results obtained from the *Surface Etching Test* are compared to those obtained from the *Surface Glass Test*. For Type I glass containers, the values obtained are close to those found in the *Surface Glass Test*. For Type II glass containers, the values obtained greatly exceed those found in the *Surface Glass Test*; and they are similar to, but not larger than, those obtained for Type III glass containers of the same filling volume.

IMPURITIES

Arsenic (211)

Use as the *Test Preparation* 35 mL of the water from one Type I or one Type II glass container, or, in the case of smaller containers, 35 mL of the combined contents of several Type I or Type II glass containers, prepared as directed for the *Surface Glass Test*. The limit does not exceed 0.1 µg per g.

FUNCTIONALITY

Spectral Transmission for Colored Glass Containers

Apparatus: A UV-Vis spectrophotometer, equipped with either a photodiode detector or a photomultiplier tube coupled with an integrating sphere.

Preparation of Sample: Break the glass container or cut it with a circular saw fitted with a wet abrasive wheel, such as a carborundum or a bonded diamond wheel. Select sections representative of the wall thickness, and trim them as suitable for mounting in a spectrophotometer. After cutting, wash and dry each specimen, taking care to avoid scratching the surfaces. If the specimen is too small to cover the opening in the specimen holder, mask the uncovered portion of the opening with opaque paper or tape, provided that the length of the specimen is greater than that of the slit. Before placing in the holder, wash, dry, and wipe the specimen with lens tissue. Mount the specimen with the aid

of wax, or by other convenient means, taking care to avoid leaving fingerprints or other marks.

Method: Place the specimen in the spectrophotometer with its cylindrical axis parallel to the slit and in such a way that the light beam is perpendicular to the surface of the section and the losses due to reflection are at a minimum. Measure the transmission of the specimen with reference to air in the spectral region of 290–450 nm, continuously or at intervals of 20 nm.

Limits: The observed spectral transmission for colored glass containers for products for nonparenteral use does not exceed 10% at any wavelength in the range of 290–450 nm, irrespective of the type and capacity of the glass container. The observed spectral transmission in colored glass containers for parenteral products does not exceed the limits given in Table 6.

Table 6. Limits of Spectral Transmission for Colored Glass Containers for Parenteral Products

Nominal Volume (mL)	Maximum Percentage of Spectral Transmission at Any Wavelength between 290 nm and 450 nm	
	Flame-Sealed Containers	Containers with Closures
Up to 1	50	25
Above 1 and up to 2	45	20
Above 2 and up to 5	40	15
Above 5 and up to 10	35	13
Above 10 and up to 20	30	12
Above 20	25	10

(661) CONTAINERS—PLASTICS

INTRODUCTION

It is the purpose of this chapter to provide standards for plastic materials and components used to package medical articles (pharmaceuticals, biologics, dietary supplements, and devices). Definitions that apply to this chapter are provided in (659) *Packaging and Storage Requirements*. Standards and tests for the functional properties of containers and their components are provided in general chapter *Containers—Performance Testing* (671).

In addition to the standards provided herein, the ingredients added to the polymers, and those used in the fabrication of the containers, must conform to the requirements in the applicable sections of the *Code of Federal Regulations*, Title 21, *Indirect Food Additives*, or have been evaluated by the FDA and determined to be acceptable substances for the listed use.

Plastic articles are identified and characterized by IR spectroscopy and differential scanning calorimetry. Standards are provided in this chapter for the identification and characterization of the different types of plastic, and the test procedures are provided at the end of the chapter. The degree of testing is based on whether or not the container has direct contact with the drug product, and the risk is based on the route of administration.

Plastics are composed of a mixture of homologous polymers, having a range of molecular weights. Plastics may contain other substances such as residues from the polymerization process, plasticizers, stabilizers, antioxidants, pigments, and lubricants. These materials meet the requirements for food contact as provided in the *Code of Federal Regulations*, Title 21. Factors such as plastic composition, processing and cleaning procedures, surface treatment, contacting media, inks, adhesives, absorption and permeability of preservatives, and conditions of storage may also affect the suitability of a plastic for a specific use. Extraction tests are designed to characterize the extracted components and identify possible migrants. The degree or extent of testing for extractables of the component is dependent on the intended use and the degree of risk to adversely impact the efficacy of the compendial article (drug, biologic, dietary supplement, or device). Resin-specific extraction tests are provided in this chapter for polyethylene, polypropylene, polyethylene terephthalate, and polyethylene terephthalate G. Test all other plastics as directed for *Physicochemical Tests* in the section *Test Methods*. Conduct the *Buffering Capacity* test only when the containers are intended to hold a liquid product.

Plastic components used for products of high risk, such as those intended for inhalation, parenteral preparation, and ophthalmics are tested using the *Biological Tests* in the section *Test Methods*.

Plastic containers intended for packaging products prepared for parenteral use meet the requirements for *Biological Tests* and *Physicochemical Tests* in the section *Test Methods*. Standards are also provided for polyethylene containers used to package dry oral dosage forms that are not meant for constitution into solution.

POLYETHYLENE CONTAINERS

Scope

The standards and tests provided in this section characterize containers and components, produced from either low-density polyethylene or high-density polyethylene of either homopolymer or copolymer resins that are interchangeably suitable for packaging dry oral dosage forms not meant for constitution into solution. All polyethylene components are subject to testing by IR spectroscopy and differential scanning calorimetry. Where stability studies have been performed to establish the expiration date of a particular dosage form in the appropriate polyethylene container, then any other polyethylene container meeting these requirements may be similarly used to package such a dosage form, provided that the appropriate stability programs are expanded to include the alternative container, in order to ensure that the identity, strength, quality, and purity of the dosage form are maintained throughout the expiration period.

Background

High-density and low-density polyethylene are long-chain polymers synthesized under controlled conditions of heat and pressure, with the aid of catalysts from not less than 85.0% ethylene and not less than 95.0% total olefins. Other olefin ingredients that are most frequently used are butene, hexene, and propylene. High-density polyethylene and low-density polyethylene both have an IR absorption spectrum that is distinctive for polyethylene, and each possesses characteristic thermal properties. High-density polyethylene has a density between 0.941 and 0.965 g per cm³. Low-density polyethylene has a density between 0.850 and 0.940 g per cm³. Other properties that may affect the suitability of polyethylene include modulus of elasticity, melt index, environ-

mental stress crack resistance, and degree of crystallinity after molding.

High-Density Polyethylene

Infrared Spectroscopy—Proceed as directed for *Multiple Internal Reflectance* in the section *Test Methods*. The corrected spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of USP High-Density Polyethylene RS.

Differential Scanning Calorimetry—Proceed as directed for *Thermal Analysis* in the section *Test Methods*. The thermogram of the specimen is similar to the thermogram of USP High-Density Polyethylene RS, similarly determined, and the temperature of the endotherm (*melt*) in the thermogram of the specimen does not differ from that of the USP Reference Standard by more than 6.0°.

Heavy Metals and Nonvolatile Residue—Prepare extracts of specimens for these tests as directed for *Physicochemical Tests* under *Test Methods*, except that for each 20.0 mL of *Extracting Medium* the portion shall be 60 cm², regardless of thickness.

HEAVY METALS—Containers meet the requirements for *Heavy Metals* in the section *Physicochemical Tests* under *Test Methods*.

NONVOLATILE RESIDUE—Proceed as directed for *Nonvolatile Residue* under *Physicochemical Tests*, except that the *Blank* shall be the same solvent used in each of the following test conditions: the difference between the amounts obtained from the *Sample Preparation* and the *Blank* does not exceed 12.0 mg when water maintained at a temperature of 70° is used as the *Extracting Medium*; does not exceed 75.0 mg when alcohol maintained at a temperature of 70° is used as the *Extracting Medium*; and does not exceed 100.0 mg when hexanes maintained at a temperature of 50° is used as the *Extracting Medium*.

Components Used in Contact with Oral Liquids—Proceed as directed for *Buffering Capacity* in the section *Physicochemical Tests* under *Test Methods*.

Low-Density Polyethylene

Infrared Spectroscopy—Proceed as directed for *Multiple Internal Reflectance* under *Test Methods*. The corrected spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of USP Low-Density Polyethylene RS.

Differential Scanning Calorimetry—Proceed as directed for *Thermal Analysis* under *Test Methods*. The thermogram of the specimen is similar to the thermogram of USP Low-Density Polyethylene RS, similarly determined, and the temperature of the endotherm (*melt*) in the thermogram of the specimen does not differ from that of the USP Reference Standard by more than 8.0°.

Heavy Metals and Nonvolatile Residue—Prepare extracts of specimens for these tests as directed for *Sample Preparation* in the section *Physicochemical Tests* under *Test Methods*, except that for each 20.0 mL of *Extracting Medium* the portion shall be 60 cm², regardless of thickness.

HEAVY METALS—Containers meet the requirements for *Heavy Metals* in the section *Physicochemical Tests* under *Test Methods*.

NONVOLATILE RESIDUE—Proceed as directed for *Nonvolatile Residue* in the section *Physicochemical Tests* under *Test Methods*, except that the *Blank* shall be the same solvent used in each of the following test conditions: the difference between the amounts obtained from the *Sample Preparation* and the *Blank* does not exceed 12.0 mg when water maintained at a temperature of 70° is used as the *Extracting Medium*; does not exceed 75.0 mg when alcohol maintained at a temperature of 70° is used as the *Extracting Medium*; and

does not exceed 350.0 mg when hexanes maintained at a temperature of 50° is used as the *Extracting Medium*.

Components Used in Contact with Oral Liquids—Proceed as directed for *Buffering Capacity* in the section *Physicochemical Tests* under *Test Methods*.

POLYPROPYLENE CONTAINERS

Scope

The standards and tests provided in this section characterize polypropylene containers, produced from either homopolymers or copolymers, that are interchangeably suitable for packaging dry solid and liquid oral dosage forms. Where suitable stability studies have been performed to establish the expiration date of a particular dosage form in the appropriate polypropylene container, then any other polypropylene container meeting these requirements may be similarly used to package such a dosage form, provided that the appropriate stability programs are expanded to include the alternative container, in order to ensure that the identity, strength, quality, and purity of the dosage form are maintained throughout the expiration period.

Background

Propylene polymers are long-chain polymers synthesized from propylene or propylene and other olefins under controlled conditions of heat and pressure, with the aid of catalysts. Examples of other olefins most commonly used include ethylene and butene. The propylene polymers, the ingredients used to manufacture the propylene polymers, and the ingredients used in the fabrication of the containers conform to the applicable sections of the *Code of Federal Regulations*, Title 21.

Factors such as plastic composition, processing and cleaning procedures, contacting media, inks, adhesives, absorption, adsorption and permeability of preservatives, and conditions of storage may also affect the suitability of a plastic for a specific use. The suitability of a specific polypropylene must be established by appropriate testing.

Polypropylene has a distinctive IR spectrum and possesses characteristic thermal properties. It has a density between 0.880 and 0.913 g per cm³. The permeation properties of molded polypropylene containers may be altered when reground polymer is incorporated, depending on the proportion of reground material in the final product. Other properties that may affect the suitability of polypropylene used in containers for packaging drugs are the following: oxygen and moisture permeability, modulus of elasticity, melt flow index, environmental stress crack resistance, and degree of crystallinity after molding. The requirements in this section are to be met when dry solid and liquid oral dosage forms are to be packaged in a container defined by this section.

Infrared Spectroscopy—Proceed as directed for *Multiple Internal Reflectance* under *Test Methods*. The corrected spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of the respective USP Homopolymer Polypropylene RS or copolymer polypropylene standard, similarly determined.

Differential Scanning Calorimetry—Proceed as directed for *Thermal Analysis* under *Test Methods*. The temperature of the endotherm (*melt*) in the thermogram does not differ from that of the USP Reference Standard for homopolymers by more than 6.0°. The temperature of the endotherm obtained from the thermogram of the copolymer polypropylene specimen does not differ from that of the copolymer polypropylene standard by more than 12.0°.

Heavy Metals and Nonvolatile Residue—Prepare extracts of specimens for these tests as directed for *Sample*

Preparation in the section *Physicochemical Tests* under *Test Methods*, except that for each 20 mL of *Extracting Medium* the portion shall be 60 cm², regardless of thickness.

HEAVY METALS—Containers meet the requirements for *Heavy Metals* in the section *Physicochemical Tests* under *Test Methods*.

NONVOLATILE RESIDUE—Proceed as directed for *Nonvolatile Residue* in the section *Physicochemical Tests* under *Test Methods*, except that the *Blank* shall be the same solvent used in each of the following test conditions: the difference between the amounts obtained from the *Sample Preparation* and the *Blank* does not exceed 10.0 mg when water maintained at a temperature of 70° is used as the *Extracting Medium*; does not exceed 60.0 mg when alcohol maintained at a temperature of 70° is used as the *Extracting Medium*; and does not exceed 225.0 mg when hexanes maintained at a temperature of 50° is used as the *Extracting Medium*. Containers meet these requirements for *Nonvolatile Residue* for all of the above extracting media. [NOTE—Hexanes and alcohol are flammable. When evaporating these solvents, use a current of air with the water bath; when drying the residue, use an explosion-proof oven.]

Components Used in Contact with Oral Liquids—Proceed as directed for *Buffering Capacity* in the section *Physicochemical Tests* under *Test Methods*.

POLYETHYLENE TEREPHTHALATE BOTTLES AND POLYETHYLENE TEREPHTHALATE G CONTAINERS

Scope

The standards and tests provided in this section characterize polyethylene terephthalate (PET) and polyethylene terephthalate G (PETG) bottles that are interchangeably suitable for packaging liquid oral dosage forms. Where stability studies have been performed to establish the expiration date of a particular liquid oral dosage form in a bottle meeting the requirements set forth herein for either PET or PETG bottles, any other PET or PETG bottle meeting these requirements may be similarly used to package such a dosage form, provided that the appropriate stability programs are expanded to include the alternative bottle in order to ensure that the identity, strength, quality, and purity of the dosage form are maintained throughout the expiration period. The suitability of a specific PET or PETG bottle for use in the dispensing of a particular pharmaceutical liquid oral dosage form must be established by appropriate testing.

Background

PET resins are long-chain crystalline polymers prepared by the condensation of ethylene glycol with dimethyl terephthalate or terephthalic acid. PET copolymer resins are prepared in a similar way, except that they may also contain a small amount of either isophthalic acid (not more than 3 mole percent) or 1,4-cyclohexanedimethanol (not more than 5 mole percent). Polymerization is conducted under controlled conditions of heat and vacuum, with the aid of catalysts and stabilizers.

PET copolymer resins have physical and spectral properties similar to PET and for practical purposes are treated as PET. The tests and specifications provided in this section to characterize PET resins and bottles apply also to PET copolymer resins and to bottles fabricated from them.

PET and PET copolymer resins generally exhibit a large degree of order in their molecular structure. As a result, they exhibit characteristic composition-dependent thermal behavior,

including a glass transition temperature of about 76° and a melting temperature of about 250°. These resins have a distinctive IR absorption spectrum that allows them to be distinguished from other plastic materials (e.g., polycarbonate, polystyrene, polyethylene, and PETG resins). PET and PET copolymer resins have a density between 1.3 and 1.4 g per cm³ and a minimum intrinsic viscosity of 0.7 dL per g, which corresponds to a number average molecular weight of about 23,000 Da.

PETG resins are high molecular weight polymers prepared by the condensation of ethylene glycol with dimethyl terephthalate or terephthalic acid and 15 to 34 mole percent of 1,4-cyclohexanedimethanol. PETG resins are clear, amorphous polymers, having a glass transition temperature of about 81° and no crystalline melting point, as determined by differential scanning calorimetry. PETG resins have a distinctive IR absorption spectrum that allows them to be distinguished from other plastic materials, including PET. PETG resins have a density of approximately 1.27 g per cm³ and a minimum intrinsic viscosity of 0.65 dL per g, which corresponds to a number average molecular weight of about 16,000 Da.

PET and PETG resins, and other ingredients used in the fabrication of these bottles, conform to the requirements in the applicable sections of the *Code of Federal Regulations*, Title 21, regarding use in contact with food and alcoholic beverages. PET and PETG resins do not contain any plasticizers, processing aids, or antioxidants. Colorants, if used in the manufacture of PET and PETG bottles, do not migrate into the contained liquid.

Infrared Spectroscopy—Proceed as directed under *Multiple Internal Reflectance* in the section *Test Methods*. The corrected spectrum of the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of USP Polyethylene Terephthalate RS, or USP Polyethylene Terephthalate G RS, similarly determined.

Differential Scanning Calorimetry—Proceed as directed under *Thermal Analysis* in the section *Test Methods*. For polyethylene terephthalate, the thermogram of the specimen is similar to the thermogram of USP Polyethylene Terephthalate RS, similarly determined: the melting point (T_m) of the specimen does not differ from that of the USP Reference Standard by more than 9.0°, and the glass transition temperature (T_g) of the specimen does not differ from that of the USP Reference Standard by more than 4.0°. For polyethylene terephthalate G, the thermogram of the specimen is similar to the thermogram of USP Polyethylene Terephthalate G RS, similarly determined: the glass transition temperature (T_g) of the specimen does not differ from that of the USP Reference Standard by more than 6.0°.

Colorant Extraction—Select three test bottles. Cut a relatively flat portion from the side wall of one bottle, and trim it as necessary to fit the sample holder of the spectrophotometer. Obtain the visible spectrum of the side wall by scanning the portion of the visible spectrum from 350 to 700 nm. Determine, to the nearest 2 nm, the wavelength of maximum absorbance. Fill the remaining two test bottles, using 50% alcohol for PET bottles and 25% alcohol for PETG bottles. Fit the bottles with impervious seals, such as aluminum foil, and apply closures. Fill a glass bottle having the same capacity as that of the test bottles with the corresponding solvent, fit the bottle with an impervious seal, such as aluminum foil, and apply a closure. Incubate the test bottles and the glass bottle at 49° for 10 days. Remove the bottles, and allow them to equilibrate to room temperature. Concomitantly determine the absorbances of the test solutions in 5-cm cells at the wavelength of maximum absorbance (see *Spectrophotometry and Light-Scattering* (851)), using the corresponding solvent from the glass bottle as the blank. The absorbance values so obtained are less than 0.01 for both test solutions.

Heavy Metals, Total Terephthaloyl Moieties, and Ethylene Glycol—**EXTRACTING MEDIA—**

Purified Water—(see monograph).

50 Percent Alcohol—Dilute 125 mL of alcohol with water to 238 mL, and mix.

25 Percent Alcohol—Dilute 125 mL of *50 Percent Alcohol* with water to 250 mL, and mix.

n-Heptane.

GENERAL PROCEDURE—[NOTE—Use an *Extracting Medium* of *50 Percent Alcohol* for PET bottles and *25 Percent Alcohol* for PETG bottles.] For each *Extracting Medium*, fill a sufficient number of test bottles to 90% of their nominal capacity to obtain not less than 30 mL. Fill a corresponding number of glass bottles with *Purified Water*, a corresponding number of glass bottles with *50 Percent Alcohol* or *25 Percent Alcohol*, and a corresponding number of glass bottles with *n-Heptane* for use as *Extracting Media* blanks. Fit the bottles with impervious seals, such as aluminum foil, and apply closures. Incubate the test bottles and the glass bottles at 49° for 10 days. Remove the test bottles with the *Extracting Media* samples and the glass bottles with the *Extracting Media* blanks, and store them at room temperature. Do not transfer the *Extracting Media* samples to alternative storage vessels.

HEAVY METALS—Pipet 20 mL of the *Purified Water* extract of the test bottles, filtered if necessary, into one of two matched 50-mL color-comparison tubes, and retain the remaining *Purified Water* extract in the test bottles for use in the test for *Ethylene Glycol*. Adjust the extract with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator. Dilute with water to about 35 mL, and mix.

Into the second color-comparison tube, pipet 2 mL of freshly prepared (on day of use) *Standard Lead Solution* (see *Heavy Metals* (231)), and add 20 mL of *Purified Water*. Adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator. Dilute with water to about 35 mL, and mix.

To each tube add 1.2 mL of thioacetamide–glycerin base TS and 2 mL of pH 3.5 *Acetate Buffer* (see *Heavy Metals* (231)), dilute with water to 50 mL, and mix: any color produced within 10 minutes in the tube containing the *Purified Water* extract of the test bottles does not exceed that in the tube containing the *Standard Lead Solution*, both tubes being viewed downward over a white surface (1 ppm in extract).

TOTAL TEREPHTHALOYL MOITIES—Determine the absorbance of the *50 Percent Alcohol* or *25 Percent Alcohol* extract in a 1-cm cell at the wavelength of maximum absorbance at about 244 nm (see *Spectrophotometry and Light-Scattering* (851)), using as the blank the corresponding *Extracting Medium* blank: the absorbance of the extract does not exceed 0.150, corresponding to not more than 1 ppm of total terephthaloyl moieties.

Determine the absorbance of the *n-Heptane* extract in a 1-cm cell at the wavelength of maximum absorbance at about 240 nm (see *Spectrophotometry and Light-Scattering* (851)), using as the blank the *n-Heptane Extracting Medium*: the absorbance of the extract does not exceed 0.150, corresponding to not more than 1 ppm of total terephthaloyl moieties.

ETHYLENE GLYCOL—

Periodic Acid Solution—Dissolve 125 mg of periodic acid in 10 mL of water.

Dilute Sulfuric Acid—To 50 mL of water add slowly and with constant stirring 50 mL of sulfuric acid, and allow to cool to room temperature.

Sodium Bisulfite Solution—Dissolve 0.1 g of sodium bisulfite in 10 mL of water. Use this solution within 7 days.

Disodium Chromotropate Solution—Dissolve 100 mg of disodium chromotropate in 100 mL of sulfuric acid. Protect this solution from light, and use within 7 days.

Standard Solution—Dissolve an accurately weighed quantity of ethylene glycol in water, and dilute quantitatively, and stepwise if necessary, to obtain a solution having a known concentration of about 1 µg per mL.

Test Solution—Use the *Purified Water* extract.

Procedure—Transfer 1.0 mL of the *Standard Solution* to a 10-mL volumetric flask. Transfer 1.0 mL of the *Test Solution* to a second 10-mL volumetric flask. Transfer 1.0 mL of the *Purified Water Extracting Medium* to a third 10-mL volumetric flask. To each of the three flasks, add 100 µL of *Periodic Acid Solution*, swirl to mix, and allow to stand for 60 minutes. Add 1.0 mL of *Sodium Bisulfite Solution* to each flask, and mix. Add 100 µL of *Disodium Chromotropate Solution* to each flask, and mix. [NOTE—All solutions should be analyzed within 1 hour after addition of the *Disodium Chromotropate Solution*.] Cautiously add 6 mL of sulfuric acid to each flask, mix, and allow the solutions to cool to room temperature. [Caution—Dilution of sulfuric acid produces substantial heat and can cause the solution to boil. Perform this addition carefully. Sulfur dioxide gas will be evolved. Use of a fume hood is recommended.] Dilute each solution with *Dilute Sulfuric Acid* to volume, and mix. Concomitantly determine the absorbances of the solutions from the *Standard Solution* and the *Test Solution* in 1-cm cells at the wavelength of maximum absorbance at about 575 nm (see *Spectrophotometry and Light-Scattering* (851)), using as the blank the solution from the *Purified Water Extracting Medium*: the absorbance of the solution from the *Test Solution* does not exceed that of the solution from the *Standard Solution*, corresponding to not more than 1 ppm of ethylene glycol.

TEST METHODS**Multiple Internal Reflectance**

Apparatus—Use an IR spectrophotometer capable of correcting for the blank spectrum and equipped with a multiple internal reflectance accessory and a KRS-5 internal reflection plate.¹ A KRS-5 crystal 2-mm thick having an angle of incidence of 45° provides a sufficient number of reflections.

Specimen Preparation—Cut two flat sections representative of the average wall thickness of the container, and trim them as necessary to obtain segments that are convenient for mounting in the multiple internal reflectance accessory. Taking care to avoid scratching the surfaces, wipe the specimens with dry paper or, if necessary, clean them with a soft cloth dampened with methanol, and permit them to dry. Securely mount the specimens on both sides of the KRS-5 internal reflection plate, ensuring adequate surface contact. Prior to mounting the specimens on the plate, they may be compressed to thin uniform films by exposing them to temperatures of about 177° under high pressures (15,000 psi or more).

General Procedure—Place the mounted specimen sections within the multiple internal reflectance accessory, and place the assembly in the specimen beam of the IR spectrophotometer. Adjust the specimen position and mirrors within the accessory to permit maximum light transmission of the unattenuated reference beam. (For a double-beam instrument, attenuate the reference beam to permit full-scale deflection during the scanning of the specimen.) Determine the IR spectrum from 3500 to 600 cm⁻¹ for polyethylene

¹The multiple internal reflectance accessory and KRS-5 plate are available from several sources, including Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, CA 92634, and from Perkin Elmer Corp., Main Ave., Norwalk, CT 06856.

and polypropylene and from 4000 to 400 cm^{-1} for PET and PETG.

Thermal Analysis

General Procedure—Cut a section weighing about 12 mg, and place it in the test specimen pan. [NOTE—Intimate contact between the pan and the thermocouple is essential for reproducible results.] Determine the thermogram under nitrogen, using the heating and cooling conditions as specified for the resin type and using equipment capable of performing the determinations as specified under *Thermal Analysis* (891).

For Polyethylene—Determine the thermogram under nitrogen at temperatures between 40° and 200° at a heating rate between 2° and 10° per minute followed by cooling at a rate between 2° and 10° per minute to 40°.

For Polypropylene—Determine the thermogram under nitrogen at temperatures ranging from ambient to 30° above the melting point. Maintain the temperature for 10 minutes, then cool to 50° below the peak crystallization temperature at a rate of 10° to 20° per minute.

For Polyethylene Terephthalate—Heat the specimen from room temperature to 280° at a heating rate of about 20° per minute. Hold the specimen at 280° for 1 minute. Quickly cool the specimen to room temperature, and reheat it to 280° at a heating rate of about 5° per minute.

For Polyethylene Terephthalate G—Heat the specimen from room temperature to 120° at a heating rate of about 20° per minute. Hold the specimen at 120° for 1 minute. Quickly cool the specimen to room temperature, and reheat it to 120° at a heating rate of about 10° per minute.

Biological Tests

The in vitro biological tests are performed according to the procedures set forth under *Biological Reactivity Test, In Vitro* (87). Components that meet the requirements of the in vitro tests are not required to undergo further testing. No plastic class designation is assigned to these materials. Materials that do not meet the requirements of the in vitro tests are not suitable for containers for drug products.

If a plastic class designation is needed for plastics and other polymers that meet the requirements under *Biological Reactivity Test, In Vitro* (87), perform the appropriate in vivo test specified for *Classification of Plastics* under *Biological Reactivity Test, In Vivo* (88).

Physicochemical Tests

The following tests, designed to determine physical and chemical properties of plastics and their extracts, are based on the extraction of the plastic material, and it is essential that the designated amount of the plastic be used. Also, the specified surface area must be available for extraction at the designated temperature.

Testing Parameters—

Extracting Medium—Unless otherwise directed in a specific test below, use *Purified Water* (see monograph) as the *Extracting Medium*, maintained at a temperature of 70° during the extraction of the *Sample Preparation*.

Blank—Use *Purified Water* where a blank is specified in the tests that follow.

Apparatus—Use a water bath and the *Extraction Containers* as described under *Biological Reactivity Tests, In Vivo* (88). Proceed as directed in the first paragraph of *Preparation of Apparatus* under *Biological Reactivity Tests, In Vivo* (88). [NOTE—The containers and equipment need not be sterile.]

Sample Preparation—From a homogeneous plastic specimen, use a portion, for each 20.0 mL of *Extracting Medium*, equivalent to 120 cm^2 total surface area (both sides combined), and subdivide into strips approximately 3 mm in width and as near to 5 cm in length as is practical. Transfer the subdivided sample to a glass-stoppered, 250-mL graduated cylinder of Type I glass, and add about 150 mL of *Purified Water*. Agitate for about 30 seconds, drain off and discard the liquid, and repeat with a second washing.

Sample Preparation Extract—Transfer the prepared *Sample Preparation* to a suitable extraction flask, and add the required amount of *Extracting Medium*. Extract by heating in a water bath at the temperature specified for the *Extracting Medium* for 24 hours. Cool, but not below 20°. Pipet 20 mL of the prepared extract into a suitable container. [NOTE—Use this portion in the test for *Buffering Capacity*.] Immediately decant the remaining extract into a suitably cleansed container, and seal.

Nonvolatile Residue—Transfer, in suitable portions, 50.0 mL of the *Sample Preparation Extract* to a suitable, tared crucible (preferably a fused-silica crucible that has been acid-cleaned), and evaporate the volatile matter on a steam bath. Similarly evaporate 50.0 mL of the *Blank* in a second crucible. [NOTE—If an oily residue is expected, inspect the crucible repeatedly during the evaporation and drying period, and reduce the amount of heat if the oil tends to creep along the walls of the crucible.] Dry at 105° for 1 hour: the difference between the amounts obtained from the *Sample Preparation Extract* and the *Blank* does not exceed 15 mg.

Residue on Ignition (281)—[NOTE—It is not necessary to perform this test when the *Nonvolatile Residue* test result does not exceed 5 mg.] Proceed with the residues obtained from the *Sample Preparation Extract* and from the *Blank* in the test for *Nonvolatile Residue* above, using, if necessary, additional sulfuric acid but adding the same amount of sulfuric acid to each crucible: the difference between the amounts of residue on ignition obtained from the *Sample Preparation Extract* and the *Blank* does not exceed 5 mg.

Heavy Metals—Pipet 20 mL of the *Sample Preparation Extract*, filtered if necessary, into one of two matched 50-mL color-comparison tubes. Adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator, dilute with water to about 35 mL, and mix.

Into the second color-comparison tube pipet 2 mL of *Standard Lead Solution* (see *Heavy Metals* (231)), and add 20 mL of the *Blank*. Adjust with 1 N acetic acid or 6 N ammonium hydroxide to a pH between 3.0 and 4.0, using short-range pH paper as an external indicator, dilute with water to about 35 mL, and mix. To each tube add 1.2 mL of thioacetamide–glycerin base TS and 2 mL of pH 3.5 *Acetate Buffer* (see *Heavy Metals* (231)), dilute with water to 50 mL, and mix: any brown color produced within 10 minutes in the tube containing the *Sample Preparation Extract* does not exceed that in the tube containing the *Standard Lead Solution*, both tubes being viewed downward over a white surface (1 ppm in extract).

Buffering Capacity—Titrate the previously collected 20-mL portion of the *Sample Preparation Extract* potentiometrically to a pH of 7.0, using either 0.010 N hydrochloric acid or 0.010 N sodium hydroxide, as required. Treat a 20.0-mL portion of the *Blank* similarly: if the same titrant was required for both the *Sample Preparation Extract* and the *Blank*, the difference between the two volumes is not greater than 10.0 mL; and if acid was required for either the *Sample Preparation Extract* or the *Blank* and alkali for the

other, the total of the two volumes required is not greater than 10.0 mL.

(670) AUXILIARY PACKAGING COMPONENTS

Auxiliary packaging components are articles that are used to support or enhance container-closure systems. These articles include, but are not limited to, pharmaceutical coil for containers. The components covered in this chapter must meet the applicable requirements provided and the additional applicable requirements provided in other specified chapters.

PHARMACEUTICAL COIL

Pharmaceutical coil is used as a filling material in multiple-unit containers for solid oral dosage forms to prevent breakage of tablets or capsules during shipment. The filling material should be discarded once the bottle is opened.

Solutions

Iodinated Zinc Chloride Solution—Dissolve 20 g of zinc chloride and 6.5 g of potassium iodide in 10.5 mL of Purified Water. Add 0.5 g of iodine, and shake for 15 minutes. Filter if necessary. Protect from light.

Zinc Chloride-Formic Acid Solution—Dissolve 20 g of zinc chloride in 80 g of an 850 g/L solution of anhydrous formic acid.

1% DuPont Fiber Identification Stain No. 4¹

Solution—Dissolve 3.8 g of powdered stain in 378.5 mL of deionized water.

Cotton Pharmaceutical Coil

Purified cotton is the hair of the seed of cultivated varieties of *Gossypium hirsutum* Linné, or of other species of *Gossypium* (Fam. Malvaceae). It is deprived of fatty matter and bleached, and does not contain more than traces of leaf residue, pericarp, seed coat, or other impurities. Cotton pharmaceutical coil is used in bottles of solid oral dosage forms to prevent breakage.

Identification—

A: When examined under a microscope, each fiber is seen to consist of a single cell, up to about 4-cm long and 40- μ m wide, in the form of a flattened tube with thick and rounded walls that are often twisted.

B: When treated with *Iodinated Zinc Chloride Solution*, the fibers become violet.

C: To 0.1 g of fibers add 10 mL of *Zinc Chloride-Formic Acid Solution*, heat to 40°, and allow to stand for 2 hours, shaking occasionally: the fibers do not dissolve.

D: Weigh about 5 g of fibers, wet with water, and squeeze out the excess. Add fibers to 100 mL of a boiling solution of a 1% *DuPont Fiber Identification Stain No. 4 Solution*, and gently boil for at least 1 minute. Remove the fibers, rinse well in cold water, and squeeze out the excess moisture: the fibers become green.

Acidity or Alkalinity—Immerse about 10 g of fibers in 100 mL of recently boiled and cooled Purified Water, and

allow to macerate for 2 hours. Decant 25-mL portions of the water, with the aid of a glass rod, into each of two dishes. To one portion add 3 drops of phenolphthalein TS, and to the other portion add 1 drop of methyl orange TS. Neither portion appears pink when viewed against a white background.

Fluorescence—Examine a layer about 5 mm in thickness under UV light at 365 nm. It displays only a slight brownish-violet fluorescence and a few yellow particles. It shows no intense blue fluorescence, apart from that which may be shown by a few isolated fibers.

Residual Hydrogen Peroxide Concentration—Place 1 g of fibers in a beaker containing 30 mL of Purified Water, and stir for 3 minutes with a stirring rod. Pour contents into another clean container (do not squeeze sample), or alternatively, remove the fibers from the solution with clean tweezers. Remove a peroxide analytical test strip² from its container, and immerse the test end into the sample liquid for 2 seconds. Shake to remove the excess liquid, immediately insert the test strip into a suitable reflectometry instrument, and record the reading in mg/kg (ppm), and calculate residual hydrogen peroxide concentration in ppm.

For an alternate method, place 20 g in a beaker, add 400 mL of Purified Water, stir, add 20 mL of 20% sulfuric acid, and stir contents. Titrate with 0.100 N potassium permanganate solution to a faint pink color that remains for 30 seconds. Record the amount of titer, and calculate the concentration in ppm.

NMT 50 ppm is found using either method.

Loss on Drying (731)—Dry 5.00 g of fibers in an oven at 105° to constant weight: it loses NMT 8.0% of its weight.

Residue on Ignition (281)—Place 5 g of fibers in a porcelain or platinum dish, and moisten with 2 N sulfuric acid. Gently heat the cotton until it is charred, then ignite more strongly until the carbon is completely consumed: NMT 0.20% of residue remains.

Water-Soluble Substances—Place 10.00 g of fibers in a beaker containing 1000 mL of Purified Water, and boil gently for 30 minutes, adding water as required to maintain the volume. Pour the water through a funnel into another vessel, and press out the excess water from the cotton with a glass rod. Wash the cotton in the funnel with two 250-mL portions of boiling water, pressing the cotton after each washing. Filter the combined extract and washings, and wash the filter thoroughly with hot water. Evaporate the combined extract and washings to a small volume, transfer to a tared porcelain or platinum dish, evaporate to dryness, and dry the residue at 105° to constant weight. The residue weighs NMT 0.35%.

Fatty Matter—Pack 10.00 g of fibers in a Soxhlet extractor provided with a tared receiver, and extract with ethyl ether for 4 hours at a rate such that the ether siphons over not less than four times per hour. The ethyl ether solution in the flask shows no trace of blue, green, or brownish color. Evaporate the extract to dryness, and dry at 105° for 1 hour. The weight of the residue does not exceed 0.7%.

Dyes—Pack about 10 g in a narrow percolator, and extract slowly with alcohol until the percolate measures 50 mL. When observed downward through a column 20 cm in depth, the percolate may show a yellowish color, but not a blue or a green tint.

Other Foreign Matter—Pinches contain no oil stains or metallic particles by visual inspection.

Rayon Pharmaceutical Coil

Rayon pharmaceutical coil is a fibrous form of bleached, regenerated cellulose, to be used as a filler in bottles of solid oral dosage forms to prevent breakage. It consists exclu-

¹DuPont Fiber Identification Stain No. 4 is available from Pylam Products Co., 2175 East Cedar Street, Tempe, AZ 85281: www.pylamdyes.com.

²A suitable analysis system consisting of Reflectoquant® peroxide test strips and a RQflex® reflectometry instrument may be obtained from EMD Chemicals Inc., 480 S. Democrat Road, Gibbstown, NJ 08027: www.emdchemicals.com.

sively of rayon fibers except a few isolated foreign fibers may be present.

[NOTE—Rayon pharmaceutical coil has been found to be a potential source of dissolution problems for gelatin capsules or gelatin-coated tablets resulting from gelatin cross-linking.]

Identification—

A: When treated with *Iodinated Zinc Chloride Solution*, the fibers become violet.

B: Add 10 mL of *Zinc Chloride-Formic Acid Solution* to 0.1 g of fibers, heat to 40°, and allow to stand for 2 hours, shaking occasionally: the fibers dissolve completely, except for matt rayon fibers where titanium particles remain.

C: Weigh about 5 g of fibers, wet with water, and squeeze out the excess. Add fibers to 100 mL of a boiling solution of a 1% *DuPont Fiber Identification Stain No. 4 Solution*, and gently boil for at least 1 minute. Remove the fibers, rinse well in cold water, and squeeze out the excess moisture: the fibers become blue-green.

Acidity or Alkalinity, Fluorescence, Fatty Matter, Dyes, and Other Foreign Matter—Proceed as directed under *Cotton Pharmaceutical Coil*, except to use rayon pharmaceutical coil. Sample weight for fatty matter is 5 g and weight of residue does not exceed 0.5%.

Loss on Drying (731)—Dry 5.00 g of fibers in an oven at 105° to constant weight: it loses NMT 11.0% of its weight.

Residue on Ignition (281): NMT 1.50%, determined on a 5.00-g test specimen.

Acid-Insoluble Ash—To the residue obtained in the test for *Residue on Ignition*, add 25 mL of 3 N hydrochloric acid, and boil for 5 minutes. Collect the insoluble matter on a tared filtering crucible, wash with hot water, ignite, and weigh: the residue weighs NMT 1.25%.

Water-Soluble Substances—Proceed as directed under *Cotton Pharmaceutical Coil*, except to use rayon pharmaceutical coil. The residue weighs NMT 1.0%.

Polyester Pharmaceutical Coil

Polyester pharmaceutical coil is a white odorless material, to be used as a filler in bottles of solid oral dosage forms to prevent breakage.

Identification—

A: Proceed as directed under *Infrared Spectroscopy* in the *Test Methods* section. Determine the IR spectrum from 4000 to 650 cm⁻¹ (2.5 to 15 μm). The spectrum obtained from the specimen exhibits major absorption bands only at the same wavelengths as the spectrum of USP Polyethylene Terephthalate RS.

B: Weigh about 5 g of fibers, wet with water, and squeeze out excess. Add fibers to 100 mL of a boiling solution of a 1% *DuPont Fiber Identification Stain No. 4 Solution*, and gently boil for at least 1 minute. Remove the fibers, rinse well in cold water, and squeeze out the excess moisture: the fibers become pale orange.

Acidity or Alkalinity—Proceed as directed under *Cotton Pharmaceutical Coil*, except to use polyester pharmaceutical coil.

Loss on Drying (731)—Dry 5.00 g of fibers in an oven at 105° to constant weight: it loses NMT 1.0% of its weight.

Residue on Ignition (281): NMT 0.5%, determined on a 5.00-g test specimen.

Finish on Fibers—The finish on fibers used for processing should comply with FDA food contact regulations.

Test Methods

INFRARED SPECTROSCOPY³

Apparatus: FTIR or a double-beam spectrometer capable of scanning from 4000 to 650 cm⁻¹ (2.5 to 15 μm).

Specimen Preparation—

Method 1 (Potassium Bromide Disk)—Use scissors to cut polyester fibers (1 to 3 mg) into short lengths (less than 1 mm long), mix with 200 mg of powdered potassium bromide, and grind in a ball mill for 1 to 2 minutes. Transfer to potassium bromide-disc die, and form a disc.

Method 2 (Melt Film)—Produce film by pressing polyester fibers between TFE-fluorocarbon sheets and place between heated plates.

(671) CONTAINERS— PERFORMANCE TESTING

It is the purpose of this chapter to provide standards for the functional properties of containers and their components used to package regulated articles (pharmaceuticals, biologics, dietary supplements, and devices). Definitions that apply to this chapter are provided in (659) *Packaging and Storage Requirements*. The tests that follow are provided to determine the moisture permeability and light transmission of containers utilized for regulated articles. The section *Multiple-Unit Containers for Capsules and Tablets* applies to multiple-unit containers. The section *Single-Unit Containers and Unit-Dose Containers for Capsules and Tablets* applies to single-unit and unit-dose containers. The section *Multiple-Unit Containers for Capsules and Tablets (Without Closure)* applies to polyethylene and polypropylene containers that have no closures. The section *Multiple-Unit and Single-Unit Containers for Liquids* applies to multiple-unit and single-unit containers.

A container intended to provide protection from light or offered as a *light-resistant* container meets the requirements for *Light Transmission*, where such protection or resistance is by virtue of the specific properties of the material of which the container is composed, including any coating applied thereto. A clear and colorless or a translucent container that is made *light-resistant* by means of an opaque enclosure (see *General Notices and Requirements*) is exempt from the requirements for *Light Transmission*. As used herein, the term “container” refers to the entire system comprising, usually, the container itself, the liner (if used), the closure in the case of multiple-unit containers, and the lidding and blister in the case of unit-dose containers.

³Additional information on fiber identification methods may be found in “Standard Test Methods for Identification of Fibers in Textiles”. Current version of ASTM Method D276, published by ASTM International, 100 Barr Harbor Drive, P.O. Box C700, West Conshohocken, PA 19428-2959. www.astm.org.

MOISTURE PERMEATION

Multiple-Unit Containers for Capsules and Tablets

Desiccant—Place a quantity of 4- to 8-mesh, anhydrous calcium chloride¹ in a shallow container, taking care to exclude any fine powder, then dry at 110° for 1 hour, and cool in a desiccator.

Procedure—Select 12 containers of a uniform size and type, clean the sealing surfaces with a lint-free cloth, and close and open each container 30 times. Apply the closure firmly and uniformly each time the container is closed. Close screw-capped containers with a torque that is within the range of tightness specified in the accompanying table. Add *Desiccant* to 10 of the containers, designated *test containers*, filling each to within 13 mm of the closure if the container volume is 20 mL or more, or filling each to two-thirds of capacity if the container volume is less than 20 mL. If the interior of the container is more than 63 mm in depth, an inert filler or spacer may be placed in the bottom to minimize the total weight of the container and *Desiccant*; the layer of *Desiccant* in such a container shall be not less than 5 cm in depth. Close each immediately after adding *Desiccant*, applying the torque designated in the accompanying table when closing screw-capped containers. To each of the remaining 2 containers, designated *controls*, add a sufficient number of glass beads to attain a weight approximately equal to that of each of the *test containers*, and close, applying the torque designated in the accompanying table when closing screw-capped containers. Record the weight of the individual containers so prepared to the nearest 0.1 mg if the container volume is less than 20 mL; to the nearest mg if the container volume is 20 mL or more but less than 200 mL; or to the nearest centigram (10 mg) if the container volume is 200 mL or more; and store at $75 \pm 3\%$ relative humidity and a temperature of $23 \pm 2^\circ$. [NOTE—A saturated system of 35 g of sodium chloride with each 100 mL of water placed in the bottom of a desiccator maintains the specified humidity. Other methods may be employed to maintain these conditions.] After 336 ± 1 hours (14 days), record the weight of the individual containers in the same manner. Completely fill 5 empty containers of the same size and type as the containers under test with water or a noncompressible, free-flowing solid such as well-tamped fine glass beads, to the level indicated by the closure surface when in place. Transfer the contents of each to a graduated cylinder, and determine the average container volume, in mL. Calculate the rate of moisture permeability, in mg per day per L, by the formula:

$$(1000/14V)[(T_F - T_i) - (C_F - C_i)]$$

in which V is the volume, in mL, of the container; $(T_F - T_i)$ is the difference, in mg, between the final and initial weights of each *test container*; and $(C_F - C_i)$ is the difference, in mg, between the average final and average initial weights of the 2 *controls*. For containers used for drugs being dispensed on prescription, the containers so tested are *tight containers* if not more than 1 of the 10 *test containers* exceeds 100 mg per day per L in moisture permeability, and none exceeds 200 mg per day per L.

For containers used for drugs being dispensed on prescription, the containers are *well-closed containers* if not more than 1 of the 10 *test containers* exceeds 2000 mg per day per L in moisture permeability, and none exceeds 3000 mg per day per L.

¹Suitable 4- to 8-mesh, anhydrous calcium chloride is available commercially as Item JT1313-1 from VWR International. Consult the VWR International catalog for ordering information or call 1-800-234-9300.

Table 1. Torque Applicable to Screw-Type Container

Closure Diameter ^a (mm)	Suggested Tightness Range with Manually Applied Torque ^b (inch-pounds)
8	5
10	6
13	8
15	5–9
18	7–10
20	8–12
22	9–14
24	10–18
28	12–21
30	13–23
33	15–25
38	17–26
43	17–27
48	19–30
53	21–36
58	23–40
63	25–43
66	26–45
70	28–50
83	32–65
86	40–65
89	40–70
100	45–70
110	45–70
120	55–95
132	60–95

^aThe torque designated for the next larger closure diameter is to be applied in testing containers having a closure diameter intermediate to the diameters listed.

^bA suitable apparatus is available from SecurePak, PO Box 1210, Maumee, Ohio 43537-8210. MRA Model with indicators on both the removal and application sides available in the following ranges: 1) 0–25 inch lbs., read in 1-inch lb. increments, 2) 0–50 inch lbs., read in 2-inch lb. increments, and 3) 0–100 inch lbs., read in 5-inch lb. increments. For further detail regarding instructions, reference may be made to "Standard Test Method for Application and Removal Torque of Threaded or Lug-Style Closures" ASTM Method D3198-02, published by the American Society for Testing and Materials, 100 Barr Harbor Drive, P.O. Box C700, West Conshohocken, PA 19428-2959.

Multiple-Unit Containers for Capsules and Tablets (Without Closure)

Polyethylene Container—Fit the containers with impermeable seals obtained by heat-sealing the bottles with an aluminum foil–polyethylene laminate or other suitable seal.² Test the containers as specified under *Multiple-Unit Containers for Capsules and Tablets*: the high-density polyethylene containers so tested meet the requirements if the moisture permeability exceeds 10 mg per day per L in not more than 1 of the 10 test containers and exceeds 25 mg per day per L in none of them. The low-density polyethylene containers so tested meet the requirements if the moisture permeability exceeds 20 mg per day per L in not more than 1 of the 10 test containers and exceeds 30 mg per day per L in none of them.

²A suitable laminate for sealing has, as the container layer, polyethylene of not less than 0.025 mm (0.001 inch) and a second layer of aluminum foil of not less than 0.018 mm (0.0007 inch), with additional layers of suitable backing materials. A suitable seal can be obtained also by using glass plates and a sealing wax consisting of 60% of refined amorphous wax and 40% of refined crystalline paraffin wax.

Polypropylene Containers—Fit the containers with impervious seals obtained by heat-sealing the bottles with an aluminum foil-polyethylene laminate or other suitable seal. Test the containers as described under *Multiple-Unit Containers for Capsules and Tablets*. The containers meet the requirements if the moisture permeability exceeds 15 mg per day per L in not more than 1 of the 10 test containers and exceeds 25 mg per day per L in none of them.

Single-Unit Containers and Unit-Dose Containers for Capsules and Tablets

To permit an informed judgment regarding the suitability of the packaging for a particular type of product, the following procedure and classification scheme are provided for evaluating the moisture-permeation characteristics of single-unit and unit-dose containers. Inasmuch as equipment and operator performance may affect the moisture permeation of a container formed or closed, the moisture-permeation characteristics of the packaging system being utilized shall be determined.

Desiccant—Dry suitable desiccant pellets³ at 110° for 1 hour prior to use. Use pellets weighing approximately 400 mg each and having a diameter of approximately 8 mm. [NOTE—If necessary due to limited unit-dose container size, pellets weighing less than 400 mg each and having a diameter of less than 8 mm may be used.]

Procedure—

Method I—Seal not fewer than 10 unit-dose containers with 1 pellet in each, and seal 10 additional, empty unit-dose containers to provide the controls, using finger cots or padded forceps to handle the sealed containers. Number the containers, and record the individual weights⁴ to the nearest mg. Weigh the controls as a unit, and divide the total weight by the number of controls to obtain the average. Store all of the containers at 75 ± 3% relative humidity and at a temperature of 23 ± 2°. [NOTE—A saturated system of 35 g of sodium chloride with each 100 mL of water placed in the bottom of a desiccator maintains the specified humidity. Other methods may be employed to maintain these conditions.] After a 24-hour interval, and at each multiple thereof (see *Results*), remove the containers from the chamber, and allow them to equilibrate for 15 to 60 minutes in the weighing area. Again record the weight of the individual containers and the combined controls in the same manner. [NOTE—If any indicating pellets turn pink during this procedure, or if the pellet weight increase exceeds 10%, terminate the test, and regard only earlier determinations as valid.] Return the containers to the humidity chamber. Calculate the rate of moisture permeation, in mg per day, of each container taken by the formula:

$$(1/N)[(W_F - W_i) - (C_F - C_i)]$$

in which N is the number of days expired in the test period (beginning after the initial 24-hour equilibration period); $(W_F - W_i)$ is the difference, in mg, between the final and initial weights of each test container; and $(C_F - C_i)$ is the difference, in mg, between the average final and average initial weights of the controls, the data being calculated to two significant figures. [NOTE—Where the permeations measured are less than 5 mg per day, and where the controls are observed to reach equilibrium within 7 days, the individual permeations may be determined more accurately by using the 7-day test container and control container

weights as W_i and C_i , respectively, in the calculation. In this case, a suitable test interval for *Class A* (see *Results*) would be not less than 28 days following the initial 7-day equilibration period (a total of 35 days).]

Method II—Use this procedure for packs (e.g., punch-out cards) that incorporate a number of separately sealed unit-dose containers or blisters. Seal a sufficient number of packs, such that not fewer than 4 packs and a total of not fewer than 10 unit-dose containers or blisters filled with 1 pellet in each unit are tested. Seal a corresponding number of empty packs, each pack containing the same number of unit-dose containers or blisters as used in the test packs, to provide the controls. Store all of the containers at 75 ± 3% relative humidity and at a temperature of 23 ± 2°. [NOTE—A saturated system of 35 g of sodium chloride with each 100 mL of water placed in the bottom of a desiccator maintains the specified humidity. Other methods may be employed to maintain these conditions.] After 24 hours, and at each multiple thereof (see *Results*), remove the packs from the chamber, and allow them to equilibrate for about 45 minutes. Record the weights of the individual packs, and return them to the chamber. Weigh the control packs as a unit, and divide the total weight by the number of control packs to obtain the average empty pack weight. [NOTE—If any indicating pellets turn pink during the procedure, or if the average pellet weight increase in any pack exceeds 10%, terminate the test, and regard only earlier determinations as valid.] Calculate the average rate of moisture permeation, in mg per day, for each unit-dose container or blister in each pack taken by the formula:

$$(1/NX)[(W_F - W_i) - (C_F - C_i)]$$

in which N is the number of days expired in the test period (beginning after the initial 24-hour equilibration period); X is the number of separately sealed units per pack; $(W_F - W_i)$ is the difference, in mg, between the final and initial weights of each test pack; and $(C_F - C_i)$ is the difference, in mg, between the average final and average initial weights of the control packs, the rates being calculated to two significant figures.

Results—The individual unit-dose containers as tested in *Method I* are designated *Class A* if not more than 1 of 10 containers tested exceeds 0.5 mg per day in moisture permeation rate and none exceeds 1 mg per day; they are designated *Class B* if not more than 1 of 10 containers tested exceeds 5 mg per day and none exceeds 10 mg per day; they are designated *Class C* if not more than 1 of 10 containers tested exceeds 20 mg per day and none exceeds 40 mg per day; and they are designated *Class D* if the containers tested meet none of the moisture permeation rate requirements.

The packs as tested in *Method II* are designated *Class A* if no pack tested exceeds 0.5 mg per day in average blister moisture permeation rate; they are designated *Class B* if no pack tested exceeds 5 mg per day in average blister moisture permeation rate; they are designated *Class C* if no pack tested exceeds 20 mg per day in average blister moisture permeation rate; and they are designated *Class D* if the packs tested meet none of the above average blister moisture permeation rate requirements.

With the use of the *Desiccant* described herein, as stated for *Method I* and *Method II*, after every 24 hours, the test and control containers or packs are weighed; and suitable test intervals for the final weighings, W_F and C_F , are as follows: 24 hours for *Class D*; 48 hours for *Class C*; 7 days for *Class B*; and not less than 28 days for *Class A*.

Multiple-Unit Containers and Unit-Dose Containers for Liquids

The standards and tests provided in this section measure the functional and performance characteristics of bottles used to package aqueous products by measuring the liquid

³Suitable moisture-indicating desiccant pellets are available commercially from sources such as Medical Packaging, Inc., 470 Route 31, Ringoes, NJ 08551-1409 [Telephone 800-257-5282; in NJ, 609-466-8991; FAX 609-466-3775], as Indicating Desiccant Pellets, Item No. TK-1002.

⁴Accurate comparisons of *Class A* containers may require test periods in excess of 28 days if weighings are performed on a *Class A* prescription balance (see *Prescription Balances and Volumetric Apparatus* (1176)). The use of an analytical balance on which weights can be recorded to 4 or 5 decimal places may permit more precise characterization between containers and/or shorter test periods.

water weight loss as a percent of the contents. This test can also be used to demonstrate performance or functional comparability. [NOTE—Throughout the following procedure, determine the weights of individual container–closure systems (bottle, innerseal if used, and closure) both as tare weights and fill weights, to the nearest 0.1 mg if the bottle capacity is less than 200 mL; to the nearest mg if the bottle capacity is 200 mL or more but less than 1000 mL; or to the nearest centigram (10 mg) if the bottle capacity is 1000 mL or more.]

Procedure—Select 12 bottles of a uniform size and type, and clean the sealing surfaces with a lint-free cloth. Fit each bottle with a seal, closure liner (if applicable), and closure. Number each container–closure system, and record the tare weight.

Remove the closures and, using a pipet, fill 10 bottles with water to the fill capacity. Fill 2 containers with glass beads to the same approximate weight of the filled test containers. If using screw closures, apply a torque that is within the range specified in *Table 1*, and store the sealed containers at a temperature of $25 \pm 2^\circ$ and a relative humidity of $40 \pm 2\%$. After 336 ± 1 hours (14 days), record the weight of the individual containers, and calculate the water weight loss rate, in percent per year, for each bottle taken by the formula:

$$\frac{(W_{1i} - W_T) - (W_{14i} - W_T) - (WC_1 - WC_{14})}{(W_{1i} - W_T)14} = \text{Percent per year}$$

in which W_{1i} is the initial weight of each individual bottle (i); W_T is the tare weight; W_{14i} is the weight of each individual bottle (i) at 14 days; and $(WC_1 - WC_{14})$ is the average weight change of the controls from initial to 14 days.

The containers so tested meet the requirements and are tight containers if the percentage of water weight loss does not exceed 2.5% per year in not more than 1 of the 10 test containers and does not exceeds 5.0% per year in none of them.

LIGHT TRANSMISSION TEST

Apparatus⁵—Use a spectrophotometer of suitable sensitivity and accuracy, adapted for measuring the amount of light transmitted by either transparent or translucent glass or plastic materials used for pharmaceutical containers. In addition, the spectrophotometer is capable of measuring and recording light transmitted in diffused as well as parallel rays.

Procedure—Select sections to represent the average wall thickness. Cut circular sections from two or more areas of the container and trim them as necessary to give segments of a size convenient for mounting in the spectrophotometer. After cutting, wash and dry each specimen, taking care to avoid scratching the surfaces. If the specimen is too small to cover the opening in the specimen holder, mask the uncovered portion of the opening with opaque paper or masking tape, provided that the length of the specimen is greater than that of the slit in the spectrophotometer. Immediately before mounting in the specimen holder, wipe the specimen with lens tissue. Mount the specimen with the aid of a tacky wax, or by other convenient means, taking care to avoid leaving fingerprints or other marks on the surfaces through which light must pass. Place the section in the spectrophotometer with its cylindrical axis parallel to the plane of the slit and approximately centered with respect to the slit. When properly placed, the light beam is normal to

⁵For further detail regarding apparatus and procedures, reference may be made to the following publications of the American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, PA 19428-2959: “Standard Method of Test for Haze and Luminous Transmittance of Transparent Plastics,” ASTM Method D1003-07; “Tentative Method of Test for Luminous Reflectance, Transmittance and Color of System” ASTM Method E308-06.

the surface of the section and reflection losses are at a minimum.

Continuously measure the transmittance of the section with reference to air in the spectral region of interest with a recording instrument or at intervals of about 20 nm with a manual instrument, in the region of 290 to 450 nm.

Limits—The observed light transmission does not exceed the limits given in *Table 2* for containers intended for parenteral use.

Table 2. Limits for Plastic Classes I–VI and Glass Types I, II, and III

Nominal Size (in mL)	Maximum Percentage of Light Transmission at Any Wavelength between 290 and 450 nm	
	Flame-sealed Containers	Closure-sealed Containers
1	50	25
2	45	20
5	40	15
10	35	13
20	30	12
50	15	10

[NOTE—Any container of a size intermediate to those listed above exhibits a transmission not greater than that of the next larger size container listed in the table. For containers larger than 50 mL, the limits for 50 mL apply.]

The observed light transmission for plastic containers for products intended for oral or topical administration does not exceed 10% at any wavelength in the range from 290 to 450 nm.

Delete the following:

▲ <681> REPACKAGING INTO SINGLE-UNIT CONTAINERS AND UNIT-DOSE CONTAINERS FOR NONSTERILE SOLID AND LIQUID DOSAGE FORMS

This chapter is intended to provide guidance to those engaged in pharmaceutical dispensing, not commercial repackaging. An official dosage form is required to bear on its label an expiration date assigned for the particular formulation and package of the article. This date limits the time during which the product may be dispensed or used. Because the expiration date stated on the original manufacturer’s container–closure system has been determined for the drug in that particular system and is not intended to be applicable to the product where it has been repackaged in a different container, repackaged drugs dispensed pursuant to a prescription are exempt from using the expiration date from the original manufacturer’s package. However, under no circumstance should the repackaged pharmaceutical preparation’s expiration date exceed the original manufacturer’s expiration date. It is necessary, therefore, that other precautions be taken by the dispenser to preserve the strength, quality, and purity of drugs that are repackaged for ultimate distribution or sale to patients.

The following guidelines and requirements are applicable where official dosage forms are repackaged into single-unit or unit-dose containers or mnemonic packs for dispensing pursuant to prescription.

Labeling—It is the responsibility of the dispenser, taking into account the nature of the drug repackaged, any packaging and expiration dating information in the manufacturer's product labeling, the characteristics of the containers, and the storage conditions to which the article may be subjected, to place a suitable expiration date on the label. Repackaged dosage forms must bear on their labels expiration dates as determined from information in the product labeling (see (659) *Packaging and Storage Requirements*). Each single-unit or unit-dose container bears a separate label, unless the device holding the unit-dose form does not allow for the removal or separation of the intact single-unit or unit-dose container therefrom.

Storage—Store the repackaged article in a humidity-controlled environment and at the temperature specified in the individual monograph or in the product labeling. Where no temperature or humidity is specified in the monograph or in the labeling of the product, controlled room temperature and a relative humidity corresponding to 60% are not to be exceeded during repackaging or storage.

A refrigerator or freezer shall not be considered to be a humidity-controlled environment. Drugs that are to be stored at a cold temperature in a refrigerator or freezer must be protected during storage in the refrigerator or freezer. An outer container may be necessary for such protection; it is recommended that the drug monograph be referenced for storage.

Reprocessing—Reprocessing of repackaged unit-dose containers (i.e., removing dosage unit from one unit-dose container and placing dosage unit into another unit-dose container) shall not be done. However, reprocessing of the secondary package (e.g., removing the blister card from the cardboard carrier and placing the blister card into another cardboard carrier) is allowed provided that the original expiration date is maintained.

CUSTOMIZED PATIENT MEDICATION PACKAGES

In lieu of dispensing two or more prescribed drug products in separate containers, a pharmacist may, with the consent of the patient, the patient's caregiver, or a prescriber, provide a customized patient medication package (patient med pak).¹

A patient med pak is a package prepared by a pharmacist for a specific patient comprising a series of containers and containing two or more prescribed solid oral dosage forms. The patient med pak is so designed or each container is so labeled as to indicate the day and time, or period of time, that the contents within each container are to be taken.

It is the responsibility of the dispenser to instruct the patient or caregiver on the use of the patient med pak.

Label—The patient med pak shall bear a label stating:

- (1) the name of the patient;
- (2) a serial number for the patient med pak itself and a separate identifying serial number for each of the prescription orders for each of the drug products contained therein;
- (3) the name, strength, physical description or identification, and total quantity of each drug product contained therein;

¹ It should be noted that there is no special exemption for patient med paks from the requirements of the Poison Prevention Packaging Act. Thus the patient med pak, if it does not meet child-resistant standards, shall be placed in an outer package that does comply, or the necessary consent of the purchaser or physician, to dispense in a container not intended to be child-resistant, shall be obtained.

- (4) the directions for use and cautionary statements, if any, contained in the prescription order for each drug product therein;
- (5) any storage instructions or cautionary statements required by the official compendia;
- (6) the name of the prescriber of each drug product;
- (7) the date of preparation of the patient med pak and the beyond-use date or period of time assigned to the patient med pak (such beyond-use date or period of time shall be not longer than the shortest recommended beyond-use date for any dosage form included therein or not longer than 60 days from the date of preparation of the patient med pak and shall not exceed the shortest expiration date on the original manufacturer's bulk containers for the dosage forms included therein); alternatively, the package label shall state the date of the prescription(s) or the date of preparation of the patient med pak, provided the package is accompanied by a record indicating the start date and the beyond-use date;
- (8) the name, address, and telephone number of the dispenser (and the dispenser's registration number where necessary); and
- (9) any other information, statements, or warnings required for any of the drug products contained therein.

If the patient med pak allows for the removal or separation of the intact containers therefrom, each individual container shall bear a label identifying each of the drug products contained therein.

Labeling—The patient med pak shall be accompanied by a patient package insert, in the event that any medication therein is required to be dispensed with such insert as accompanying labeling. Alternatively, such required information may be incorporated into a single, overall educational insert provided by the pharmacist for the total patient med pak.

Packaging—In the absence of more stringent packaging requirements for any of the drug products contained therein, each container of the patient med pak shall comply with the moisture permeation requirements for a Class B single-unit or unit-dose container (see *Containers—Performance Testing* (671)). Each container shall be either not reclosable or so designed as to show evidence of having been opened.

Guidelines—It is the responsibility of the dispenser, when preparing a patient med pak, to take into account any applicable compendial requirements or guidelines and the physical and chemical compatibility of the dosage forms placed within each container, as well as any therapeutic incompatibilities that may attend the simultaneous administration of the medications. In this regard, pharmacists are encouraged to report to USP headquarters any observed or reported incompatibilities. Once a medication has been placed in a patient med pak with another solid dosage form, it may not be returned to stock, redistributed, or resold if unused.

Recordkeeping—In addition to any individual prescription filing requirements, a record of each patient med pak shall be made and filed. Each record shall contain, as a minimum:

- (1) the name and address of the patient;
- (2) the serial number of the prescription order for each drug product contained therein;
- (3) the name of the manufacturer or labeler and lot number for each drug product contained therein;
- (4) information identifying or describing the design, characteristics, or specifications of the patient med pak sufficient to allow subsequent preparation of an identical patient med pak for the patient;

- (5) the date of preparation of the patient med pak and the beyond-use date that was assigned;
- (6) any special labeling instructions; and
- (7) the name or initials of the pharmacist who prepared the patient med pak.

▲ USP36

〈691〉 COTTON

Preparatory to the determination of absorbency and of fiber length, remove the Cotton from its wrappings, and condition it for not less than 4 hours in a standard atmosphere of $65 \pm 2\%$ relative humidity at $21 \pm 1.1^\circ$ ($70 \pm 2^\circ\text{F}$).

Absorbency Test

Procedure—Prepare a test basket, weighing not more than 3 g, from copper wire approximately 0.4 mm in diameter (No. 26 B. & S.) in the form of a cylinder approximately 5 cm in diameter and 8 cm deep, with spaces of about 2 cm between the wires. Take portions of purified cotton weighing 1 ± 0.05 g from five different parts of the package by pulling, not cutting, the specimens, place the combined portions in the basket, and weigh. Hold the basket on its side approximately 12 mm above the surface of water at $25 \pm 1^\circ$, and drop it into the water. Determine, preferably by use of a stop watch, the time in seconds required for complete submersion.

Remove the basket from the water, allow it to drain for 10 seconds in the same horizontal position, then place it immediately in a tared, covered vessel, and weigh, deducting the weight of the test basket and of the purified cotton to find the weight of water absorbed.

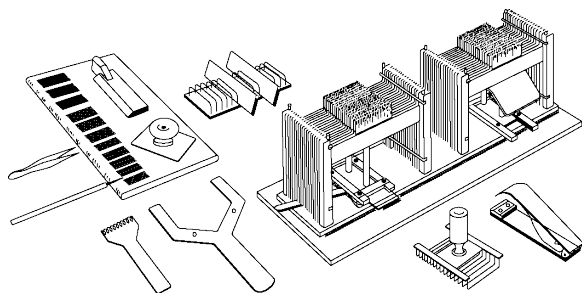
Fiber Length

For the determination of the length and of the length distribution of cotton fibers in purified cotton use the following method:

Carry out all operations associated with the determination of fiber length of purified cotton in an atmosphere maintained at $65 \pm 2\%$ relative humidity at $21 \pm 1.1^\circ$ ($70 \pm 2^\circ\text{F}$).

These directions describe the mode of procedure that is well adapted to the sorter* most extensively used in the United States at the present time.

Apparatus—The sorter (see illustration)



Duplex Cotton Fiber Sorter

* NOTE—The method here described is especially adapted to the Suter-Webb Duplex Cotton Fiber sorting apparatus, but with more or less obvious alteration in procedure, may be carried out with two Baer sorters in tandem arrangement, or with a Johanssen or other similar apparatus.

consists of two banks of combs rigidly mounted side by side on a common base. Each bank of combs consists of at least 12 individual combs spaced 3.2 mm apart, one behind the other, and mounted in grooves so that as they are approached during the fractionating process and no longer needed, they may be dropped below the working plane. Each individual comb has a single row of accurately aligned and sharply pointed teeth, 12 mm long, consisting of needles 0.38 mm in diameter. The teeth are spaced 62 to 25 mm over an extent of approximately 50 mm.

Accessory equipment consists of fiber-sorter forceps, fiber-depressing grid, fiber-depressing smooth plate, and velvet-covered plates. The sorter forceps consist of two brass pieces approximately 75 mm long, hinged on one end and slightly curved to present a beaked aspect at the gripping end for gripping the protruding fibers close to the surfaces of the combs. Usually, one of the gripping edges has a leather or other fibrous padding. The gripping edge is approximately 19 mm wide.

The fiber-depressing grid consists of a series of brass rods spaced 3.2 mm apart so that they may be placed between the combs to press the fibers down between the teeth. The fiber-depressing smooth plate consists of a polished brass plate approximately 25×50 mm, with a knob or handle on the upper surface whereby the plate may be smoothed over the fibers as they are laid on the velvet surface of the array plates. The velvet-covered plates, upon which the fibers may be arrayed, are aluminum sheets approximately $100 \text{ mm} \times 225 \text{ mm} \times 2.4 \text{ mm}$ thick, covered on both sides with high-grade velvet, preferably black.

Selection of Cotton—After unrolling the cotton, prepare a representative laboratory test specimen by taking from a package containing from 8 to 16 ounces, 32 pinches (about 75 mg each) well distributed throughout the bulk of the lap, 16 representative pinches being taken from each longitudinal half of the lap. Avoid the cut ends of the lap, and take particular care to secure portions throughout the thickness of the lap. To avoid biased selection of long or short fibers, remove all fibers of the group pinched and do not allow them to slip from between the fingers.

From packages of not more than 4 ounces in weight, take 8 pinches, and from packages weighing more than 4 ounces and not more than 8 ounces, take 16 pinches, all well distributed.

Mix the pinches in pairs promiscuously, and combine each pair by gently drawing and lapping them in the fingers. Then divide each combined pair by splitting longitudinally into two approximately equal parts and utilize one part in the further mixing. (The other part may be discarded or reserved for any further tests or checks.)

Repeat the process described in the preceding paragraph with the successive halves of the bifurcated series until only 1 pinch, the final composite test portion, results. Gently parallel and straighten the fibers of the final composite test portion by drawing and lapping them in the fingers. Take care to retain all of the fibers, including as far as possible those of the neps (specks of entangled fibers) and naps (matted masses of fibers), discarding only motes (immature seed fragments with fibers) and nonfiber foreign material such as stem, leaf, and fragments of seedcoats.

From the final composite portion described in the preceding paragraph, separate longitudinally a test portion of 75 ± 2 mg, accurately weighed. Retain the residue for any check test necessary.

Procedure—With the fiber-depressing grid carefully insert the weighed test portion into one bank of combs of the cotton sorter, so that it extends across the combs at approximately right angles.

With the sorter forceps, grip by the free ends a small portion of the fibers extending through the teeth of the comb nearest to the operator; gently and smoothly draw them forward out of the combs, and transfer them to the tips of the teeth in the second bank of combs, laying them parallel to themselves, straight, and approximately at right angles to

the faces of the combs, releasing the gripped ends as near to the face of the front comb as possible. With the depressor grid carefully press the transferred fibers down into the teeth of the combs. Continue the operation until all of the fibers are transferred to the second bank of combs. During this transfer of the fibers, drop the combs of the first bank in succession when and as all of the protruding fibers have been removed.

Turn the machine through 180°, and transfer the cotton fibers back to the *first bank* of combs in the manner described in the preceding paragraph.

Take great care in evening up the ends of the fibers during both of the above transfers, arranging them as closely as possible to the front surface of the proximal comb. Such evening out of the ends of the protruding fibers may involve drawing out straggling fibers from both the front and rear aspects of the banks of combs, and re-depositing them into and over the main bundle in the combs.

Turn the machine again through 180°. Drop successive combs if necessary to expose the ends of the longest fibers. It may be necessary to re-deposit some straggling fibers. With the forceps withdraw the few most protuberant fibers. In this way continue to withdraw successively the remaining protuberant fibers back to the front face of the proximal comb. Drop this comb and repeat the series of operations in the same manner until all of the fibers have been drawn out. In order not to disturb seriously the portion being tested, and thereby vitiate the length fractionation into length groups, make several pulls (as many as 8 to 10) between each pair of combs.

Lay the pulls on the velvet-covered plates alongside each other, as straight as possible, with the ends as clearly defined as possible, and with the distal ends arranged in a straight line, pressing them down gently and smoothly with the fiber-depressing smooth plate before releasing the pull from the forceps. Employ not fewer than 50 and not more than 100 pulls to fractionate the test portion.

Group together all of the fibers measuring 12.5 mm (about 1/2 inch) or more in length, and weigh the group to the nearest 0.3 mg. In the same manner, group together all fibers 6.25 mm (about 1/4 inch) or less in length, and weigh in the same manner. Finally, group the remaining fibers of intermediate lengths together and weigh. The sum of the three weights does not differ from the initial weight of the test portion by more than 3 mg. Divide the weight of each of the first two groups by the weight of the test portion to obtain the percentage by weight of fiber in the two ranges of length.

(695) CRYSTALLINITY

This test is provided to determine compliance with the crystallinity requirement where stated in the individual monograph for a drug substance.

Procedure—A detailed test procedure is described under *Optical Microscopy* (776).

(696) CHARACTERIZATION OF CRYSTALLINE SOLIDS BY MICROCALORIMETRY AND SOLUTION CALORIMETRY

For the purpose of this chapter, crystalline material, partially crystalline material, and amorphous material are considered as solids.

INTRODUCTION—THE CONCEPT OF CRYSTALLINITY

The perfectly ordered crystal lattice with every molecule in its expected lattice position is an ideal that is seldom, if ever, achieved. The other extreme is the amorphous state, in which a solid contains the maximum possible density of imperfections (defects of various dimensionalities), such that all long-range order is lost while only the short-range order, imposed by its nearest neighbors, remains. Real crystals lie somewhere between these two extremes. A crystal's position on a scale bounded by these two extremes is termed *crystallinity*.

All real crystals, even in the pure state, possess some lattice imperfections or defects, which increase both the energy (enthalpy under conditions of constant atmospheric pressure) and the disorder (expressed as the entropy) of the crystal lattice. A crystal with a relatively low density of imperfections is said to be highly crystalline and to possess a high crystallinity. By contrast, a particle with a relatively high density of imperfections is said to be partially amorphous and to possess a low crystallinity. In ideal terms, a totally amorphous particle corresponds to zero crystallinity. Amorphous particles may contain somewhat ordered domains that can act as nuclei for crystallization; such so-called amorphous particles are said to possess a low-level, but finite, crystallinity.

The ability to detect and quantify the amount of amorphous material within a highly crystalline substance is of great importance during the development and subsequent manufacture of a pharmaceutical preparation.

In reality, a powder probably contains particles with different degrees of crystallinity, just as it may contain particles with varying sizes and shapes. The lower the crystallinity of a solid, the greater its enthalpy and entropy. The increase in enthalpy is never totally compensated by the increase in entropy; therefore, the Gibbs free energy, which reflects the balance between them, actually increases. Hence, the lower the crystallinity of a material (powder), and consequently the greater its amorphous character, the greater its apparent intrinsic solubility and dissolution rate, but the lower its thermodynamic stability. Because of the great relevance of these properties, crystallinity is also an important property and requires measurement by a suitable method.

In the following chapter, the crystallinity or the content of amorphous parts of a powder are measured by calorimetric methods such as microcalorimetry or solution calorimetry, although other methods could be used (e.g., see general chapter *Characterization of Crystalline and Partially Crystalline Solids by X-ray Powder Diffraction (XRPD)* (941)).

Many substances are capable of crystallizing in more than one type of crystal lattice, which is known as polymorphism.

If water or a solvent is incorporated in the crystal lattice, the crystals are termed hydrates or solvates. Because of the different crystal packing, and/or molecular conformation and lattice energy, they usually exhibit different physical properties. For simplicity, calorimetry measurements for degree of crystallinity determination discussed here assume only one solid crystalline form present in the material of interest. The theory and experimental technique can be easily expanded to polymorphic systems with proper consideration of the enthalpy differences among the polymorphs.

METHOD 1—MICROCALORIMETRY (DETERMINATION OF AMORPHOUS CONTENT)

Most chemical, physical, and biological processes are associated with the exchange of heat. Microcalorimetry is a highly sensitive technique to monitor and quantify both exothermic (heat producing) and endothermic (heat absorbing) changes associated with those processes. The technique allows the determination of the rate and extent of chemical reactions, changes of phase, or changes of structure.

Thermal events producing only a fraction of a microwatt can be observed using microcalorimetry. This means that temperature differences less than 10^{-6} K must be detectable. Microcalorimetry typically uses the heat flow (heat leakage) principle, where, in a thermally defined vessel, the heat produced (or absorbed) flows away from (or into) the vessel in an effort to re-establish thermal equilibrium with its surroundings. Exceptional thermal stability with its surrounding has to be achieved either by a heat sink or an electronically regulated surrounding.

Heat energy from an active sample in the reaction vessel is channeled typically through Peltier elements; they act as thermoelectric generators using the Seebeck effect. The heat energy is converted into a voltage signal proportional to the heat flow.

Results are typically presented as a measure of the thermal energy produced per unit of time (Watt) as a function of time.

Apparatus

Microcalorimeters are typically designed as twin systems with a measuring vessel and a reference vessel. Vessels are typically made of glass or stainless steel. For certain applications, specially designed vessels that allow the addition of a gas, a liquid, or a solid material may be used.

Calibration

The microcalorimeter is calibrated for heat flow (energy per time unit) using either calibrated external or internal electrical heat sources or a suitable standard reaction.

Sensitivity

The sensitivity of the microcalorimetric method can be assessed on the basis of an appropriate standard sample analyzed according to the corresponding method in conjunction with the determination of the instrument baseline noise.

Procedure

Weigh an appropriate quantity of the material in a suitable vessel. Close the vessel carefully, to avoid any evaporation of solvents, and place the vessel in the sample holder. If appropriate, allow the vessel to equilibrate at the temperature of the measurement before placing it in the measuring position.

Begin the analysis, and record the heat flow with the time on the abscissa and the heat flow on the ordinate (specify the direction of exothermic or endothermic heat flow).

Detection and Quantification of Amorphous Content in Powders—The amorphous state is metastable with respect to the crystalline state; recrystallization may therefore occur. The measurement of the heat of recrystallization enables the amorphous content to be determined by the area of the recrystallization peak. By relating the output from the microcalorimeter for a sample to that obtained from an amorphous standard, it is possible to quantify the amorphous content of the sample. The range of amorphous content covered by this method depends on the individual substance to be tested. In favorable cases, limits of detection below 1% can be reached.

Recrystallization can be initiated by subjecting the sample to higher relative humidity or an atmosphere containing organic vapor. The sample is typically placed in an ampul that also contains a small test tube containing an aqueous saturated salt solution, an organic solvent, or a solvent mixture.

The heat of recrystallization is typically measured using a fixed sample mass placed in a glass or steel vessel. The test tube containing a saturated salt solution or an organic solvent is chosen large enough to allow a full saturation of the atmosphere above the sample. The mass of the sample and the nature of the vapor atmosphere above the sample is chosen so that recrystallization occurs in such a way that a distinct peak is observed, clearly separated from initial thermal events caused by introduction of the sample.

The conditions under which the transition of the amorphous phase to a thermodynamically more stable crystalline state occurs will have a significant impact on the time of recrystallization. In particular, physical mixtures of purely amorphous and crystalline material will behave differently from a partially crystalline material. These effects should be considered when developing a method.

A typical response for the recrystallization of a mainly amorphous material is shown in Figure 1. The first part of the curve represents several concurrent processes taking place simultaneously, such as the absorption of water vapor into the amorphous parts of the powder and by the generation of water vapor from the test tube. After this initial response, there is a large exothermic response caused by the recrystallization of the amorphous material. Also included, but not seen, is the expulsion of excess water from the recrystallized parts and its condensation. Thus, the area under this exothermic recrystallization response is proportional to the heat of recrystallization.

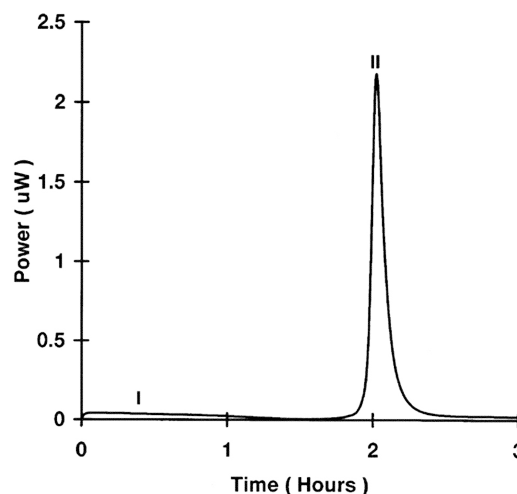


Figure 1. Typical microcalorimetric output of power (in μW) as a function of time (in h): amorphous collapse peak (I) and crystallization peak (II) for mainly amorphous lactose at 25° and 75% relative humidity.

METHOD 2—SOLUTION CALORIMETRY (DETERMINATION OF CRYSTALLINITY)

Solution calorimetry provides a means of determining enthalpy of solution (i.e., heat of solution under constant atmospheric pressure) of a substance. Enthalpy of solution is defined as the enthalpy of the substance dissolved in the solution to a defined concentration minus the enthalpy of the original substance. The solvent for the dissolution process must be such that the mass of solid dissolves within a time frame that matches the response time of the calorimeter, as discussed below. The enthalpy of solution is proportional to the amount of solid being dissolved. This amount may be defined as one mole for molar enthalpy or as one gram for specific enthalpy. If the substance possesses adequate purity (as determined by the degree of accuracy required) and if its molecular mass is known, the molar enthalpy is preferred; otherwise the specific enthalpy must be used. The enthalpy of solution is weakly dependent on both the temperature, which is usually 25.0°, and the final concentration of the dissolved solute.

It is usually preferred to express the crystallinity, P_c , of a substance on a percentage scale. This procedure requires two reference standards, namely a highly crystalline sample assuming 100% crystallinity and having a measured enthalpy of solution of ΔH^s_c , and an amorphous sample assuming 0% crystallinity and having a measured enthalpy of solution of ΔH^s_a . From these values and from the measured enthalpy, ΔH^s_s , of solution of the solid under study, the percentage crystallinity of the solid, P_c , may be calculated as follows:

$$P_c (\%) = 100(\Delta H^s_s - \Delta H^s_a)/(\Delta H^s_c - \Delta H^s_a)$$

Clearly, crystallinity expressed on a percentage scale depends on three measured values, and the enthalpies of solution may be replaced by other corresponding physical quantities that depend on crystallinity. The value of the percentage crystallinity of a sample, however, depends not only on the nature and method of preparation of the two reference standards, but also on the choice of the physical quantity that is measured.

The enthalpy of solution is measured either by an isoperibol (constant perimeter, i.e., jacket) solution calorimeter or by an isothermal (constant temperature) solution calorimeter. Typically, at least three measurements are made with each sample. The mean of these values is then calculated. The exact requirements will depend on the equipment capability and degree of accuracy needed.

Isoperibol Solution Calorimetry

In the isoperibol solution calorimeter, the heat change during the solution process causes a corresponding change in temperature of the solvent-solute system (i.e., solution). This temperature change is measured by a temperature sensor, which is wired to an electrical circuit that records an electrical signal corresponding to the temperature change. Typically, this temperature change in an electronic form is measured at precisely defined time intervals to produce temperature-time data that are collected, analyzed by a computer, and then plotted. A blank run without addition of the solid solute to the solvent normally shows no discernible change in the slope of the temperature-time plot.

For isoperibol solution calorimeters, response is fairly rapid, but corrections must be made for any heat losses to or heat gains from the bath. Therefore, isoperibol solution calorimeters are more advantageous than isothermal solution calorimeters when the solution process is relatively fast. For all measurements of enthalpy of solution using isoperibol solution calorimeters, the choice of solvent is critical. The nature and mass of the solvent and the mass of sample allow the total heat change, corresponding to total dissolution of the solid, to proceed to completion within five min

under vigorous stirring at a constant rotational speed within the range of 400–600 revolutions/min.

The effective heat capacity of the calorimeter cell and its contents is determined for every calorimeter run. This determination is accomplished by electrical heating of the contents of the calorimeter cell. The effective heat capacity is determined according to one of two protocols—either by making one determination after ampul breakage or by making one determination before and a second determination after ampul breakage, and then averaging the two results. The accuracy and reliability of the electrical heating are established by the accuracy and reliability of the aforementioned chemical calibrations.

Isothermal Solution Calorimetry

In the isothermal (constant temperature) solution calorimeter, the heat change during the solution process is compensated for by an equal but opposite energy change, such that the temperature of the solvent-solute system (i.e., solution) remains essentially constant. This equal but opposite energy change is measured and, when its sign is reversed, provides the enthalpy of solution. For isothermal calorimeters, response is relatively slow, but the compensation process eliminates the effects of heat losses to or heat gains from the bath. Therefore, isothermal calorimeters are more advantageous than isoperibol calorimetry when the solution process is relatively slow.

Solution Calorimeter Calibration

To ensure the accuracy of the calorimeter, chemical calibrations must be performed on a regular basis. For an endothermic solution process, the calibration of the calorimeter is checked by measuring the heat absorbed during the dissolution of potassium chloride in distilled water at 298.15 K (25.0°). The established enthalpy change in this endothermic process is 235.5 J/g (17.56 kJ/mol). For an exothermic solution process, the calorimeter is checked by measuring the heat evolved during the dissolution of 5 g/L of trimethamine [tris(hydroxymethyl)aminomethane, THAM] in a 0.1 mol/L aqueous hydrochloric acid solution at 298.15 K (25.0°). The established heat for the aforementioned process is –246.0 J/g (–29.80 kJ/mol).

Sample Handling

The chemical and physical stability of solids may decrease with decreasing crystallinity. In particular, solids of low crystallinity, especially amorphous solids, tend to sorb water vapor from the atmosphere, leading to crystallization and a corresponding gain in crystallinity. For these reasons, anhydrous samples whose crystallinity is to be determined must be stored at zero humidity or below critical humidity levels in sealed chambers containing a desiccant, preferably containing an indicator of effectiveness. If crystallinity-humidity studies are to be carried out, the sample is stored in a sealed chamber containing a saturated salt solution to provide a defined relative humidity.

(698) DELIVERABLE VOLUME

The following tests are designed to provide assurance that oral liquids will, when transferred from the original container, deliver the volume of dosage form that is declared on the label of the article. These tests are applicable to

products labeled to contain not more than 250 mL, whether supplied as liquid preparations or liquid preparations that are constituted from solids upon the addition of a designated volume of a specific diluent. They are not required for an article packaged in single-unit containers when the monograph includes the *Uniformity of Dosage Units* (905) test.

DENSITY DETERMINATION

Because of the tendency of oral liquids to entrain air when transferred, a more accurate method for determining the delivered volume is to first determine the delivered mass, and then, using the density of the material, to convert the mass to delivered volume. In order to do that, a determination of the true density of the material is required. The following is one method to determine true density:

1. Tare a 100-mL volumetric flask containing 50.0 mL of water.
2. Add approximately 25 g of well-shaken product, and gently swirl the contents to mix.
3. Reweigh the flask.
4. From a buret, add an accurately measured amount of water to bring the flask contents to volume, while gently swirling the contents of the flask. Record the volume taken from the buret.
5. Calculate the density of the sample by the formula:

$$W/V$$

in which *W* is the weight, in g, of the material taken, and *V* is 50.0 mL minus the volume, in mL, of water necessary to adjust the contents of the flask to volume. Other methods to determine the true density may be employed depending on the formulation (e.g., substantially nonaqueous formulations).

TEST PREPARATIONS

For the determination of deliverable volume, select not fewer than 30 containers, and proceed as follows for the dosage form designated.

Oral Solutions, Oral Suspensions, and Other Oral Liquid Dosage Forms—Shake the contents of 10 containers individually.

Powders that are Labeled to State the Volume of Oral Liquid that Results when the Powder is Constituted with the Volume of Diluent Stated in the Labeling—Constitute 10 containers with the volume of diluent stated in the labeling, accurately measured, and shake individually.

PROCEDURE

The deliverable volume can be determined as follows:

1. Discharge the container contents into a suitable tared container (allowing drainage for not more than

5 seconds for single dose containers and not more than 30 minutes for multiple unit containers).

2. Determine the mass of the contents.

3. Calculate the volume using the true density.

Alternatively, the following procedure may be used:

1. Being careful to avoid the formation of air bubbles, gently pour the contents of each container into separate dry graduated cylinders of a rated capacity not exceeding two and a half times the volume to be measured, and calibrated "to contain" (see *Volumetric Apparatus* (31)).
2. Allow each container to drain for a period not to exceed 30 minutes for multiple-unit containers and 5 seconds for single-unit containers, unless otherwise specified in the monograph.
3. When free from bubbles, measure the volume of each mixture.

ACCEPTANCE CRITERIA

Use the following criteria to determine compliance with this test.

For Multiple-Unit Containers (see *Figure 1*)—The average volume of liquid obtained from the 10 containers is not less than 100%, and the volume of no container is less than 95% of the volume declared in the labeling. If A, the average volume is less than 100% of that declared in the labeling, but the volume of no container is less than 95% of the labeled amount, or if B, the average volume is not less than 100% and the volume of not more than 1 container is less than 95%, but is not less than 90% of the labeled volume, perform the test on 20 additional containers. The average volume of liquid obtained from the 30 containers is not less than 100% of the volume declared in the labeling; and the volume of liquid obtained from not more than 1 of the 30 containers is less than 95%, but not less than 90% of that declared in the labeling.

For Single-Unit Containers (see *Figure 2*)—The average volume of liquid obtained from the 10 containers is not less than 100%, and the volume of each of the 10 containers lies within the range of 95% to 110% of the volume declared in the labeling. If A, the average volume is less than 100% of that declared in the labeling, but the volume of no container is outside the range of 95% to 110%, or if B, the average volume is not less than 100% and the volume of not more than 1 container is outside the range of 95% to 110%, but within the range of 90% to 115%, perform the test on 20 additional containers. The average volume of liquid obtained from the 30 containers is not less than 100% of the volume declared in the labeling; and the volume obtained from not more than 1 of the 30 containers is outside the range of 95% to 110%, but within the range of 90% to 115% of the volume declared on the labeling.

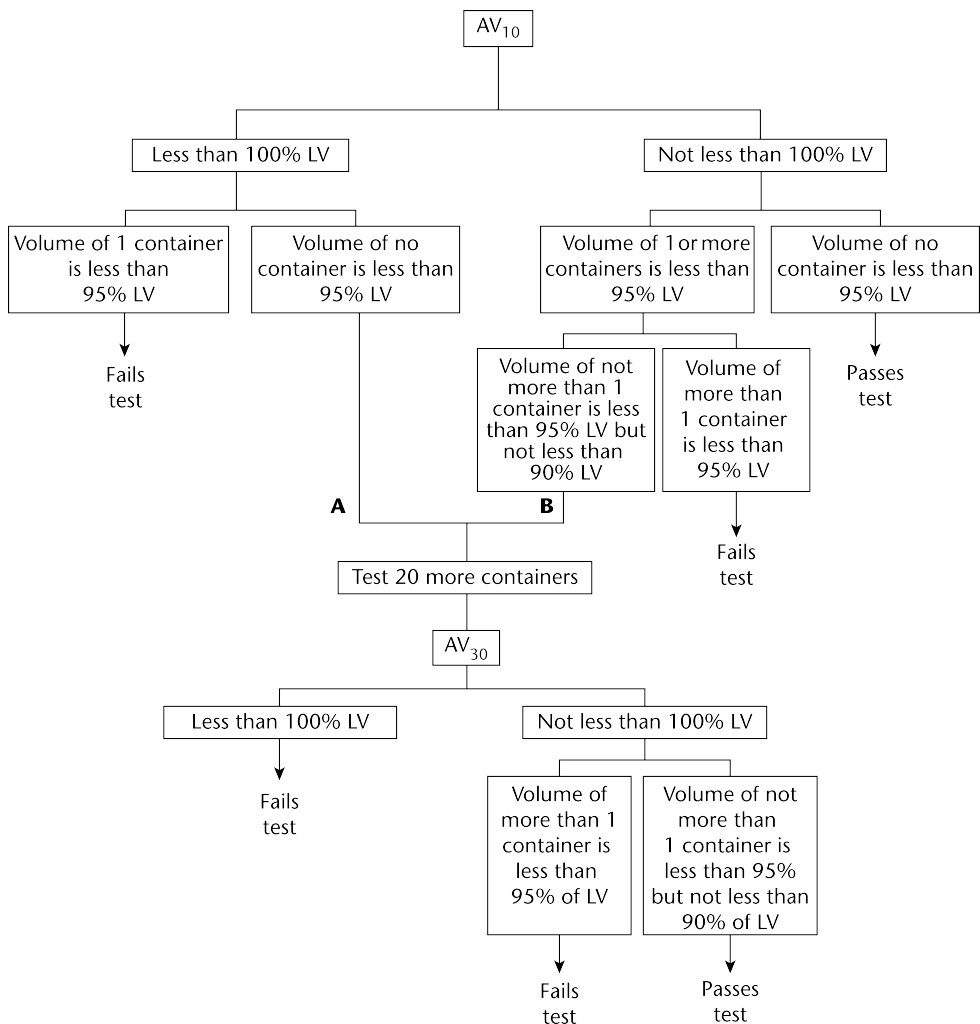


Figure 1. Decision scheme for multiple-unit containers. (AV = Average volume. LV = Labeled volume)

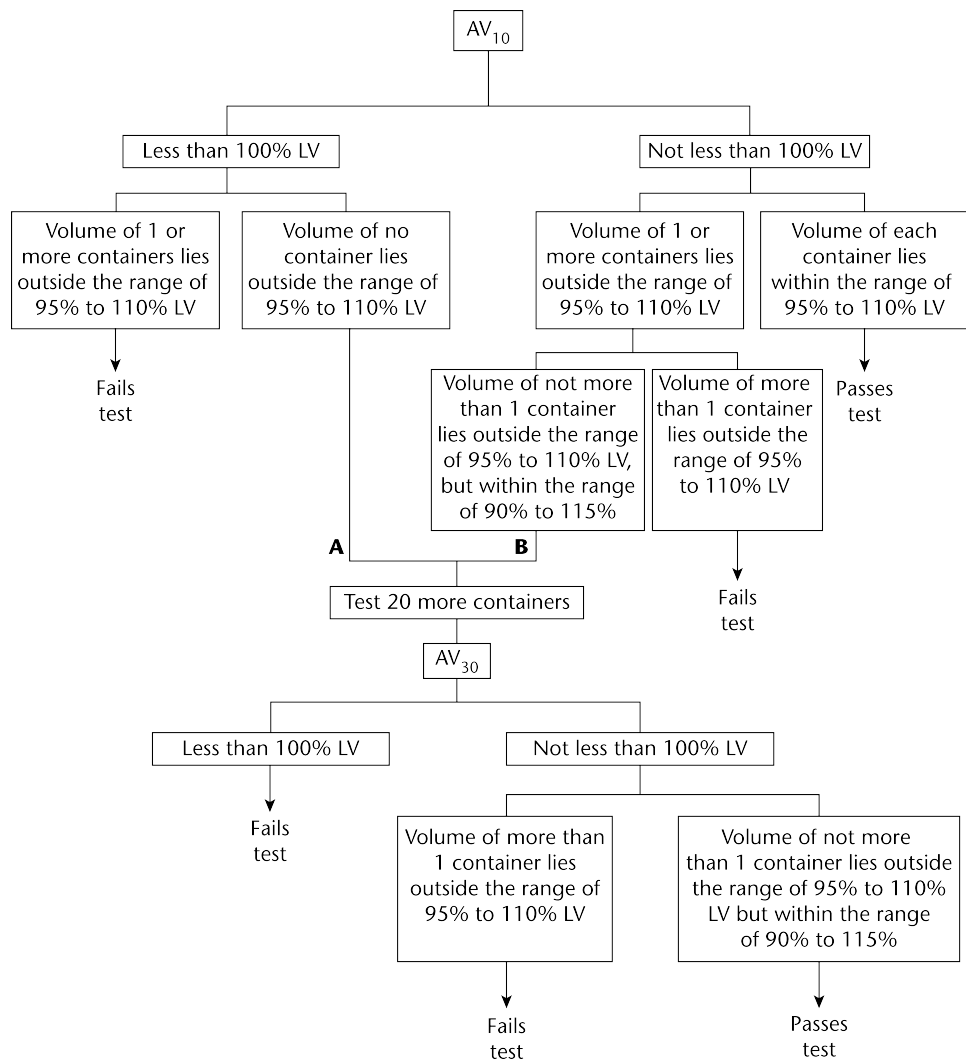


Figure 2. Decision scheme for single-unit containers. (AV = Average volume. LV = Labeled volume)

(699) DENSITY OF SOLIDS

TERMS AND DEFINITIONS

Density refers to the average spatial distribution of mass in a material. The density of solids typically is expressed in g per cm³, in contrast to fluids, where the density is commonly expressed in g per mL at a stated reference temperature.

The density of a solid particle can assume different values depending on the method used to measure the volume of the particle. It is useful to distinguish among three different possibilities.

The *true density* of a substance is the average mass per unit volume, exclusive of all voids that are not a fundamental part of the molecular packing arrangement. It is a property of a particular material, and hence should be independent of the method of determination. The true density of a perfect crystal can be determined from the size and composition of the unit cell.

The *pycnometric density*, as measured by gas pycnometry, is a convenient density measurement for pharmaceutical powders. In a gas pycnometer, the volume occupied by a known mass of powder is determined by measuring the volume of gas displaced by the powder. The quotient of the mass and volume is the pycnometric density. The pycnometric density equals the true density unless the material contains impenetrable voids, or sealed pores, that are inaccessible to the gas used in the pycnometer.

The *granular density* includes contributions to particle volume from open pores smaller than some limiting size. The size limit depends on the method of measurement. A common measurement technique is mercury porosimetry, where the limiting pore size depends upon the maximum intrusion pressure. Because of the additional contribution from pore volume, the granular density will never be greater than the true density. A related concept is the *aerodynamic density*, which is the density of the particle with a volume defined by the aerodynamic envelope of the particle in a flowing stream. Both the closed and open pores contribute to this volume, but the open pores fill with the permeating fluid. The aerodynamic density, therefore, depends on the density of the test fluid if the particle is porous.

For brevity, the pycnometric density and the true density are both referred to as density. If needed, these quantities may be distinguished based on the method of measurement.

The density of a material depends on the molecular packing. For gases and liquids, the density will depend only on temperature and pressure. For solids, the density will also vary with the crystal structure and degree of crystallinity. If the solids are amorphous, the density may further depend upon the history of preparation and treatment. Therefore, unlike fluids, the densities of two chemically equivalent solids may be different, and this difference reflects a difference in solid-state structure. The density of constituent particles is an important physical characteristic of pharmaceutical powders.

Beyond these definitions of particle density, the *bulk density* of a powder includes the contribution of interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the packing of powder particles.

GAS PYCNOMETRY FOR THE MEASUREMENT OF DENSITY

Gas pycnometry is a convenient and suitable method for the measurement of the density of powder particles. A sim-

ple schematic of one type of gas pycnometer is shown in Figure 1.

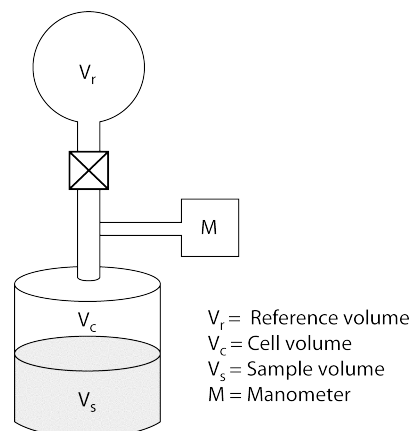


Figure 1. Schematic of gas pycnometer.

The sample, with mass w and volume V_s , is placed inside a sealed test cell with an empty cell volume of V_c . The system reference pressure, P_r , is determined at the manometer while the valve that connects the reference volume with the test cell is open. The valve is closed to separate the reference volume, V_r , from the test cell. The test cell is pressurized with the measurement gas to an initial pressure, P_i . Then the valve is opened to connect the reference volume, V_r , with the test cell, and the pressure drops to the final pressure, P_f . If the measurement gas behaves ideally under the conditions of measurement, the sample volume, V_s , is given by the following expression:

$$V_s = V_c - \frac{V_r}{\left[\frac{P_i - P_r}{P_f - P_r} \right] - 1} \quad (1)$$

The density, ρ , is given by the equation:

$$\rho = \frac{w}{V_s} \quad (2)$$

Details of the instrumental design may differ, but all gas pycnometers rely on the measurement of pressure changes as a reference volume is added to, or deleted from, the test cell.

The measured density is a volume-weighted average of the densities of individual powder particles. The density will be in error if the test gas sorbs onto the powder or if volatile contaminants are evolved from the powder during the measurement. Sorption is prevented by an appropriate choice of test gas. Helium is the common choice. Volatile contaminants in the powder are removed by degassing the powder under a constant purge of helium prior to the measurement. Occasionally, powders may have to be degassed under vacuum. Two consecutive readings should yield sample volumes that are equal within 0.2% if volatile contaminants are not interfering with the measurements. Because volatiles may be evolved during the measurement, the weight of the sample should be taken after the pycnometric measurement of volume.

Method

Ensure that the reference volume and the calibration volume have been determined for the gas pycnometer by an appropriate calibration procedure. The test gas is helium, unless another gas is specified in the individual monograph.

The temperature of the gas pycnometer should be between 15° and 30° and should not vary by more than 2° during the course of the measurement. Load the test cell with the substance under examination that has been prepared according to the individual monograph. Where <699D> is indicated, dry the substance under examination as directed for *Loss on drying* in the monograph unless other drying conditions are specified in the monograph *Density of solids* test. Where <699U> is indicated, the substance under examination is used without drying. Use a quantity of powder recommended in the operating manual for the pycnometer. Seal the test cell in the pycnometer, and purge the pycnometer system with the test gas according to the procedure given in the manufacturer's operating instructions. If the sample must be degassed under vacuum, follow the recommendations in the individual monographs and the instructions in the operating manual for the pycnometer.

The measurement sequence above describes the procedure for the gas pycnometer shown in *Figure 1*. If the pycnometer differs in operation or in construction from the one shown in *Figure 1*, follow the operating procedure given in the manual for the pycnometer.

Repeat the measurement sequence for the same powder sample until consecutive measurements of the sample volume, V_s , agree to within 0.2%. Unload the test cell and measure the final powder weight, w . Calculate the pycnometric density, ρ , of the sample according to *Equation 2*.

<701> DISINTEGRATION

This general chapter is harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. The texts of these pharmacopoeias are therefore interchangeable, and the methods of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia* may be used for demonstration of compliance instead of the present general chapter. These pharmacopoeias have undertaken not to make any unilateral change to this harmonized chapter.

Portions of the present general chapter text that are national USP text, and therefore not part of the harmonized text, are marked with symbols (♦) to specify this fact.

This test is provided to determine whether tablets or capsules disintegrate within the prescribed time when placed in a liquid medium at the experimental conditions presented below. ♦Compliance with the limits on *Disintegration* stated in the individual monographs is required except where the label states that the tablets or capsules are intended for use as troches, or are to be chewed, or are designed as extended-release dosage forms or delayed-release dosage forms. Determine the type of units under test from the labeling and from observation, and apply the appropriate procedure to 6 or more dosage units.♦

For the purposes of this test, disintegration does not imply complete solution of the unit or even of its active constituent. Complete disintegration is defined as that state in which any residue of the unit, except fragments of insoluble coating or capsule shell, remaining on the screen of the test apparatus or adhering to the lower surface of the disk, if used, is a soft mass having no palpably firm core.

APPARATUS

The apparatus consists of a basket-rack assembly, a 1000-mL, low-form beaker, 138 to 160 mm in height and having an inside diameter of 97 to 115 mm for the immersion fluid, a thermostatic arrangement for heating the fluid

between 35° and 39°, and a device for raising and lowering the basket in the immersion fluid at a constant frequency rate between 29 and 32 cycles per minute through a distance of not less than 53 mm and not more than 57 mm. The volume of the fluid in the vessel is such that at the highest point of the upward stroke the wire mesh remains at least 15 mm below the surface of the fluid and descends to not less than 25 mm from the bottom of the vessel on the downward stroke. At no time should the top of the basket-rack assembly become submerged. The time required for the upward stroke is equal to the time required for the downward stroke, and the change in stroke direction is a smooth transition, rather than an abrupt reversal of motion. The basket-rack assembly moves vertically along its axis. There is no appreciable horizontal motion or movement of the axis from the vertical.

Basket-Rack Assembly—The basket-rack assembly consists of six open-ended transparent tubes, each 77.5 ± 2.5 mm long and having an inside diameter of 20.7 to 23 mm and a wall 1.0 to 2.8 mm thick; the tubes are held in a vertical position by two plates, each 88 to 92 mm in diameter and 5 to 8.5 mm in thickness, with six holes, each 22 to 26 mm in diameter, equidistant from the center of the plate and equally spaced from one another. Attached to the under surface of the lower plate is a woven stainless steel wire cloth, which has a plain square weave with 1.8- to 2.2-mm apertures and with a wire diameter of 0.57 to 0.66 mm. The parts of the apparatus are assembled and rigidly held by means of three bolts passing through the two plates. A suitable means is provided to suspend the basket-rack assembly from the raising and lowering device using a point on its axis.

The design of the basket-rack assembly may be varied somewhat, provided the specifications for the glass tubes and the screen mesh size are maintained. The basket-rack assembly conforms to the dimensions found in *Figure 1*.

Disks—The use of disks is permitted only where specified or allowed ♦in the monograph. If specified in the individual monograph,♦ each tube is provided with a cylindrical disk 9.5 ± 0.15 mm thick and 20.7 ± 0.15 mm in diameter. The disk is made of a suitable transparent plastic material having a specific gravity of between 1.18 and 1.20. Five parallel 2 ± 0.1 -mm holes extend between the ends of the cylinder. One of the holes is centered on the cylindrical axis. The other holes are centered 6 ± 0.2 mm from the axis on imaginary lines perpendicular to the axis and parallel to each other. Four identical trapezoidal-shaped planes are cut into the wall of the cylinder, nearly perpendicular to the ends of the cylinder. The trapezoidal shape is symmetrical; its parallel sides coincide with the ends of the cylinder and are parallel to an imaginary line connecting the centers of two adjacent holes 6 mm from the cylindrical axis. The parallel side of the trapezoid on the bottom of the cylinder has a length of 1.6 ± 0.1 mm, and its bottom edges lie at a depth of 1.5 to 1.8 mm from the cylinder's circumference. The parallel side of the trapezoid on the top of the cylinder has a length of 9.4 ± 0.2 mm, and its center lies at a depth of 2.6 ± 0.1 mm from the cylinder's circumference. All surfaces of the disk are smooth. If the use of disks is specified ♦in the individual monograph♦, add a disk to each tube, and operate the apparatus as directed under *Procedure*. The disks conform to dimensions found in *Figure 1*¹.

PROCEDURE

♦Uncoated Tablets—♦Place 1 dosage unit in each of the six tubes of the basket and, if prescribed, add a disk. Oper-

¹The use of automatic detection employing modified disks is permitted where the use of disks is specified or allowed. Such disks must comply with the requirements for density and dimension given in this chapter.

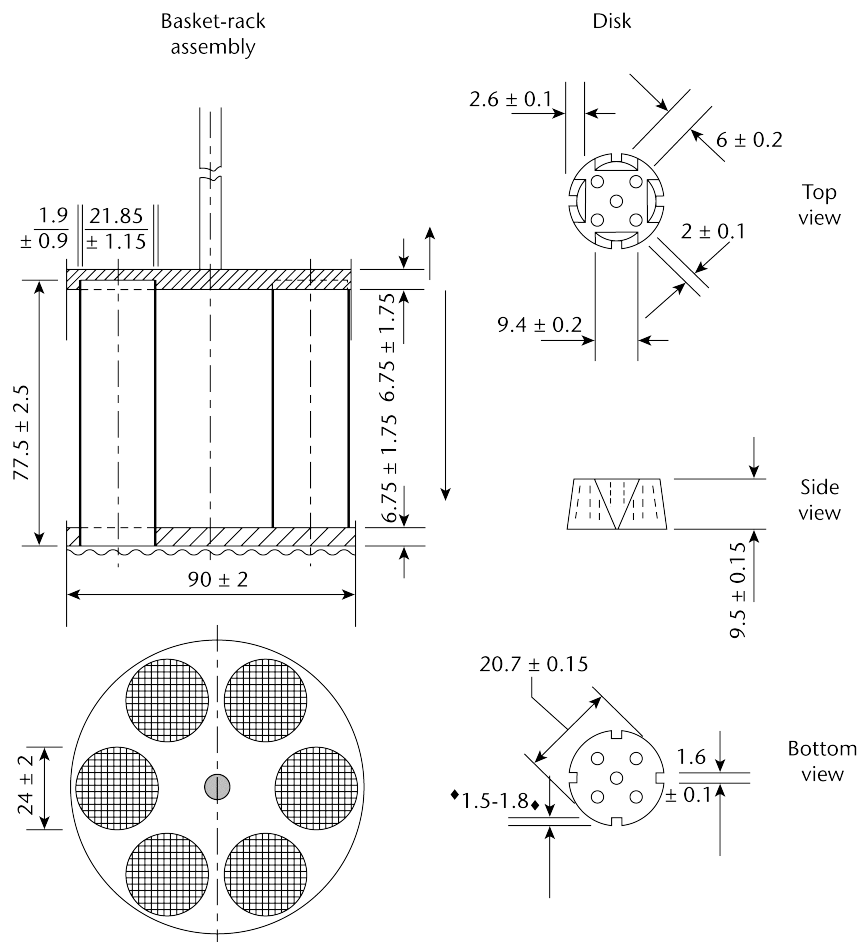


Figure 1. Disintegration apparatus. (All dimensions are expressed in mm.)

ate the apparatus, using ♦water or♦ the specified medium as the immersion fluid, maintained at $37 \pm 2^\circ$. At the end of the time limit specified ♦in the monograph,♦ lift the basket from the fluid, and observe the tablets: all of the tablets have disintegrated completely. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets. The requirement is met if not fewer than 16 of the total of 18 tablets tested are disintegrated.

♦**Plain-Coated Tablets**—Apply the test for *Uncoated Tablets*, operating the apparatus for the time specified in the individual monograph.

Delayed-Release (Enteric-Coated) Tablets—Place 1 tablet in each of the six tubes of the basket and, if the tablet has a soluble external sugar coating, immerse the basket in water at room temperature for 5 minutes. Then operate the apparatus using simulated gastric fluid TS maintained at $37 \pm 2^\circ$ as the immersion fluid. After 1 hour of operation in simulated gastric fluid TS, lift the basket from the fluid, and observe the tablets: the tablets show no evidence of disintegration, cracking, or softening. Operate the apparatus, using simulated intestinal fluid TS maintained at $37 \pm 2^\circ$ as the immersion fluid, for the time specified in the monograph. Lift the basket from the fluid, and observe the tablets: all of the tablets disintegrate completely. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not fewer than 16 of the total of 18 tablets tested disintegrate completely.

Buccal Tablets—Apply the test for *Uncoated Tablets*. After 4 hours, lift the basket from the fluid, and observe the tablets: all of the tablets have disintegrated. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not fewer than 16 of the total of 18 tablets tested disintegrate completely.

Sublingual Tablets—Apply the test for *Uncoated Tablets*. At the end of the time limit specified in the individual monograph: all of the tablets have disintegrated. If 1 or 2 tablets fail to disintegrate completely, repeat the test on 12 additional tablets: not fewer than 16 of the total of 18 tablets tested disintegrate completely.

Hard Gelatin Capsules—Apply the test for *Uncoated Tablets*. Attach a removable wire cloth, which has a plain square weave with 1.8- to 2.2-mm mesh apertures and with a wire diameter of 0.60 to 0.655 mm, as described under *Basket-Rack Assembly*, to the surface of the upper plate of the basket-rack assembly. Observe the capsules within the time limit specified in the individual monograph: all of the capsules have disintegrated except for fragments from the capsule shell. If 1 or 2 capsules fail to disintegrate completely, repeat the test on 12 additional capsules: not fewer than 16 of the total of 18 capsules tested disintegrate completely.

Soft Gelatin Capsules—Proceed as directed under *Hard Gelatin Capsules*.♦

<711> DISSOLUTION

This general chapter is harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. These pharmacopoeias have undertaken not to make any unilateral change to this harmonized chapter.

Portions of the present general chapter text that are national USP text, and therefore not part of the harmonized text, are marked with symbols (♦) to specify this fact.

This test is provided to determine compliance with the dissolution requirements ♦where stated in the individual monograph♦ for dosage forms administered orally. In this general chapter, a dosage unit is defined as 1 tablet or 1 capsule or the amount specified. ♦Of the types of apparatus described herein, use the one specified in the individual monograph. Where the label states that an article is enteric-coated, and where a dissolution or disintegration test that does not specifically state that it is to be applied to delayed-release articles is included in the individual monograph, the procedure and interpretation given for *Delayed-Release Dosage Forms* is applied unless otherwise specified in the indi-

vidual monograph. For hard or soft gelatin capsules and gelatin-coated tablets that do not conform to the *Dissolution* specification, repeat the test as follows. Where water or a medium with a pH of less than 6.8 is specified as the *Medium* in the individual monograph, the same *Medium* specified may be used with the addition of purified pepsin that results in an activity of 750,000 Units or less per 1000 mL. For media with a pH of 6.8 or greater, pancreatin can be added to produce not more than 1750 USP Units of protease activity per 1000 mL.

USP Reference Standards (11)—USP Prednisone Tablets RS.♦

APPARATUS

Apparatus 1 (Basket Apparatus)

The assembly consists of the following: a vessel, which may be covered, made of glass or other inert, transparent

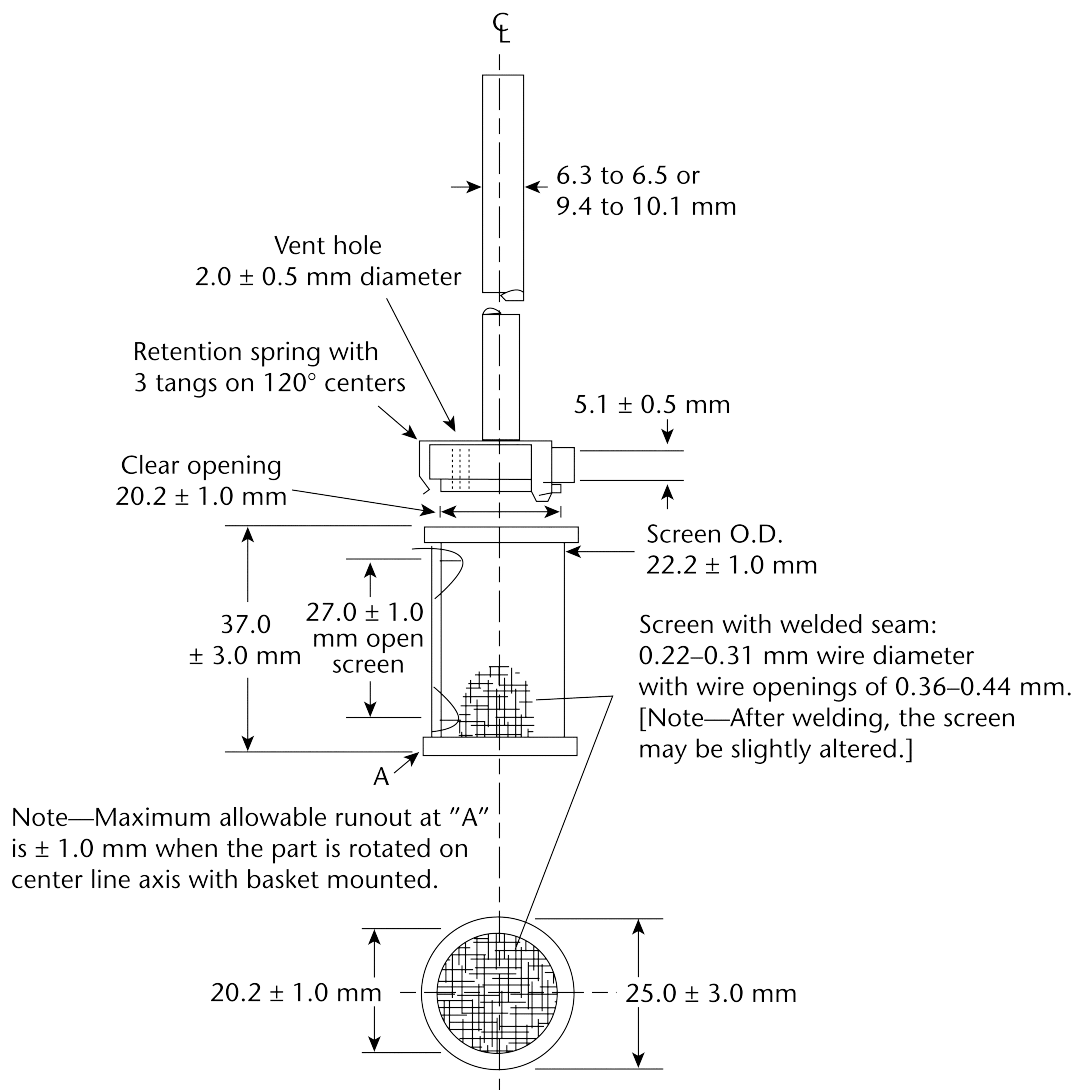


Figure 1. Basket stirring element.

material¹; a motor; a metallic drive shaft; and a cylindrical basket. The vessel is partially immersed in a suitable water bath of any convenient size or heated by a suitable device such as a heating jacket. The water bath or heating device permits holding the temperature inside the vessel at $37 \pm 0.5^\circ$ during the test and keeping the bath fluid in constant, smooth motion. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smoothly rotating stirring element. An apparatus that permits observation of the specimen and stirring element during the test is preferable. The vessel is cylindrical, with a hemispherical bottom and with one of the following dimensions and capacities: for a nominal capacity of 1 L, the height is 160 to 210 mm and its inside diameter is 98 to 106 mm; for a nominal capacity of 2 L, the height is 280 to 300 mm and its inside diameter is 98 to 106 mm; and for a nominal capacity of 4 L, the height is 280 to 300 mm and its inside diameter is 145 to 155 mm. Its sides are flanged at the top. A fitted cover may be used to retard evaporation.² The shaft is positioned so that its axis is not more than 2 mm at any point from the vertical axis of the vessel and rotates smoothly and without significant wobble that could affect the results. A speed-regulating device is used that allows the shaft rotation speed to be selected and maintained at the specified rate given in the individual monograph, within $\pm 4\%$.

Shaft and basket components of the stirring element are fabricated of stainless steel, type 316, or other inert material, to the specifications shown in Figure 1. A basket having a gold coating of about 0.0001 inch (2.5 μm) thick may be used. A dosage unit is placed in a dry basket at the beginning of each test. The distance between the inside bottom of the vessel and the bottom of the basket is maintained at 25 ± 2 mm during the test.

Apparatus 2 (Paddle Apparatus)

Use the assembly from Apparatus 1, except that a paddle formed from a blade and a shaft is used as the stirring element. The shaft is positioned so that its axis is not more than 2 mm from the vertical axis of the vessel at any point and rotates smoothly without significant wobble that could affect the results. The vertical center line of the blade passes through the axis of the shaft so that the bottom of the blade is flush with the bottom of the shaft. The paddle conforms to the specifications shown in Figure 2. The distance of 25 ± 2 mm between the bottom of the blade and the inside bottom of the vessel is maintained during the test. The metallic or suitably inert, rigid blade and shaft comprise a single entity. A suitable two-part detachable design may be used provided the assembly remains firmly engaged during the test. The paddle blade and shaft may be coated with a suitable coating so as to make them inert. The dosage unit is allowed to sink to the bottom of the vessel before rotation of the blade is started. A small, loose piece of nonreactive material, such as not more than a few turns of wire helix, may be attached to dosage units that would otherwise float. An alternative sinker device is shown in Figure 2a. Other validated sinker devices may be used.

¹ The materials should not sorb, react, or interfere with the specimen being tested.

² If a cover is used, it provides sufficient openings to allow ready insertion of the thermometer and withdrawal of specimens.

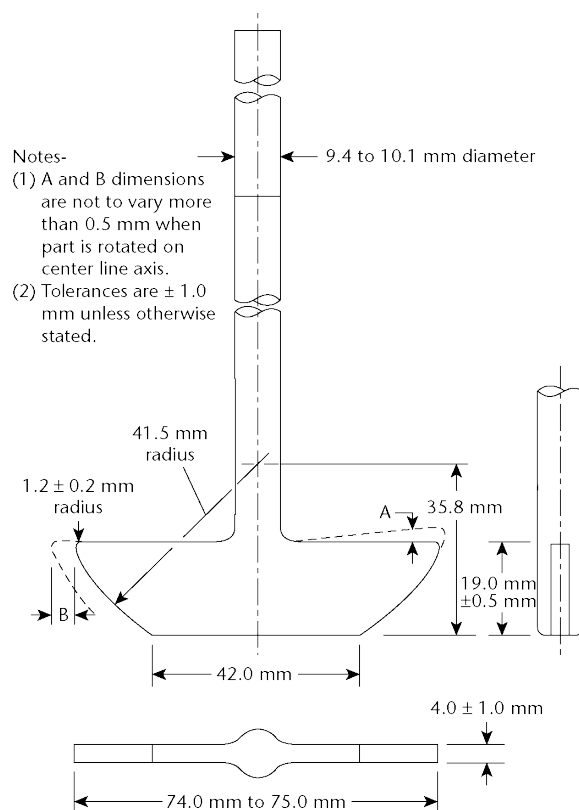
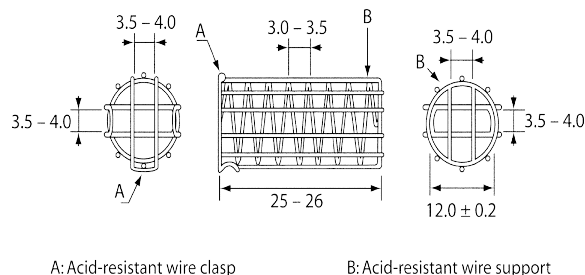


Figure 2. Paddle stirring element.



A: Acid-resistant wire clasp

B: Acid-resistant wire support

Figure 2a. Alternative sinker. All dimensions are expressed in mm.

Apparatus 3 (Reciprocating Cylinder)

NOT ACCEPTED BY THE JAPANESE PHARMACOPOEIA

The assembly consists of a set of cylindrical, flat-bottomed glass vessels; a set of glass reciprocating cylinders; inert fittings (stainless steel type 316 or other suitable material), and screens that are made of suitable nonsorbing and nonreactive material and that are designed to fit the tops and bottoms of the reciprocating cylinders; and a motor and drive assembly to reciprocate the cylinders vertically inside the vessels and, if desired, index the reciprocating cylinders horizontally to a different row of vessels. The vessels are partially immersed in a suitable water bath of any convenient size that permits holding the temperature at $37 \pm 0.5^\circ$ during the test. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smooth, vertically reciprocating cylinder. A device is used

that allows the reciprocation rate to be selected and maintained at the specified dip rate ♦given in the individual monograph♦ within $\pm 5\%$. An apparatus that permits observation of the specimens and reciprocating cylinders is preferable. The vessels are provided with an evaporation cap that remains in place for the duration of the test. The components conform to the dimensions shown in Figure 3 unless otherwise specified ♦in the individual monograph♦.

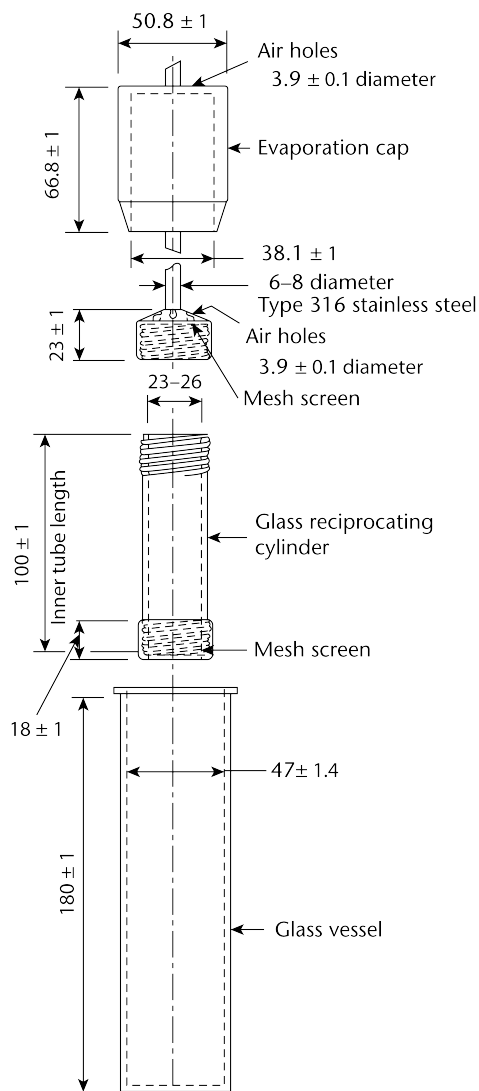


Figure 3. Apparatus 3 (reciprocating cylinder).

Apparatus 4 (Flow-Through Cell)

The assembly consists of a reservoir and a pump for the *Dissolution Medium*; a flow-through cell; and a water bath that maintains the *Dissolution Medium* at $37 \pm 0.5^\circ$. Use the specified cell size ♦as given in the individual monograph♦.

The pump forces the *Dissolution Medium* upwards through the flow-through cell. The pump has a delivery range be-

tween 240 and 960 mL per hour, with standard flow rates of 4, 8, and 16 mL per minute. It must deliver a constant flow ($\pm 5\%$ of the nominal flow rate); the flow profile is sinusoidal with a pulsation of 120 ± 10 pulses per minute. A pump without pulsation may also be used. Dissolution test procedures using a flow-through cell must be characterized with respect to rate and any pulsation.

The flow-through cell (see Figures 4 and 5), of transparent and inert material, is mounted vertically with a filter system (specified in the individual monograph) that prevents escape of undissolved particles from the top of the cell; standard cell diameters are 12 and 22.6 mm; the bottom cone is usually filled with small glass beads of about 1-mm diameter with one bead of about 5 mm positioned at the apex to protect the fluid entry tube; and a tablet holder (see Figures 4 and 5) is available for positioning of special dosage forms, for example, inlay tablets. The cell is immersed in a water bath, and the temperature is maintained at $37 \pm 0.5^\circ$.

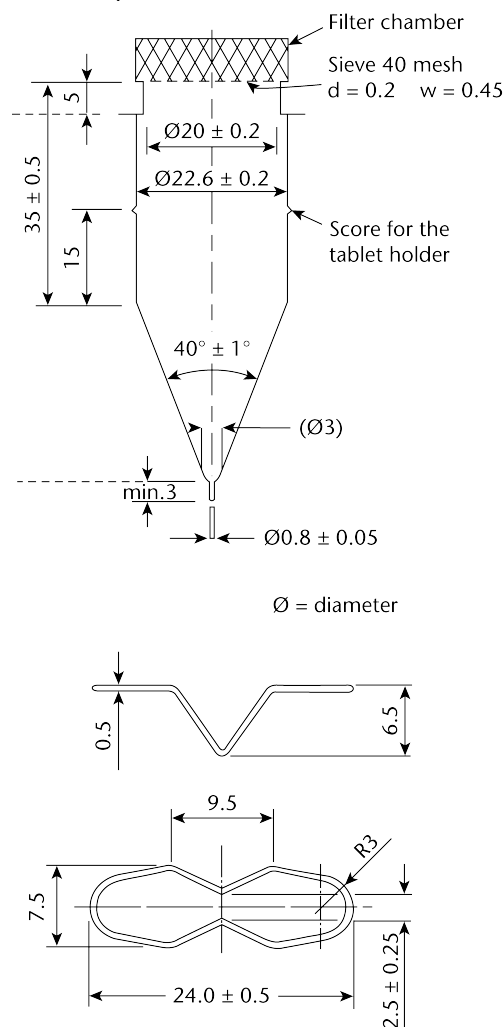


Figure 4. Apparatus 4, large cell for tablets and capsules (top), tablet holder for the large cell (bottom). (All measurements are expressed in mm unless noted otherwise.)

section. Combine equal volumes of the filtered solutions of the six or twelve individual specimens withdrawn, and use the pooled sample as the test specimen. Determine the average amount of the active ingredient dissolved in the pooled sample.♦

EXTENDED-RELEASE DOSAGE FORMS

Proceed as directed for *Immediate-Release Dosage Forms*.

Dissolution Medium—Proceed as directed for *Immediate-Release Dosage Forms*.

Time—The test-time points, generally three, are expressed in hours.

DELAYED-RELEASE DOSAGE FORMS NOT ACCEPTED BY THE JAPANESE PHARMACOPOEIA

Use *Method A* or *Method B* and the apparatus specified ♦in the individual monograph. All test times stated are to be observed within a tolerance of $\pm 2\%$, unless otherwise specified.

Method A—

Procedure ♦(unless otherwise directed in the individual monograph)♦—

ACID STAGE—Place 750 mL of 0.1 N hydrochloric acid in the vessel, and assemble the apparatus. Allow the medium to equilibrate to a temperature of $37 \pm 0.5^\circ$. Place 1 dosage unit in the apparatus, cover the vessel, and operate the apparatus at the specified rate ♦given in the monograph.♦

After 2 hours of operation in 0.1 N hydrochloric acid, withdraw an aliquot of the fluid, and proceed immediately as directed under *Buffer Stage*.

Perform an analysis of the aliquot using a suitable assay method. ♦The procedure is specified in the individual monograph.♦

BUFFER STAGE—[NOTE—Complete the operations of adding the buffer and adjusting the pH within 5 minutes.]

With the apparatus operating at the rate specified ♦in the monograph♦, add to the fluid in the vessel 250 mL of 0.20 M tribasic sodium phosphate that has been equilibrated to $37 \pm 0.5^\circ$. Adjust, if necessary, with 2 N hydrochloric acid or 2 N sodium hydroxide to a pH of 6.8 ± 0.05 . Continue to operate the apparatus for 45 minutes, or for the specified time ♦given in the individual monograph♦. At the end of the time period, withdraw an aliquot of the fluid, and perform the analysis using a suitable assay method. ♦The procedure is specified in the individual monograph. The test may be concluded in a shorter time period than that specified for the *Buffer Stage* if the requirement for the minimum amount dissolved is met at an earlier time.♦

Method B—

Procedure ♦(unless otherwise directed in the individual monograph)♦—

ACID STAGE—Place 1000 mL of 0.1 N hydrochloric acid in the vessel, and assemble the apparatus. Allow the medium to equilibrate to a temperature of $37 \pm 0.5^\circ$. Place 1 dosage unit in the apparatus, cover the vessel, and operate the apparatus at the rate specified ♦in the monograph♦. After 2 hours of operation in 0.1 N hydrochloric acid, withdraw an aliquot of the fluid, and proceed immediately as directed under *Buffer Stage*.

Perform an analysis of the aliquot using a suitable assay method. ♦The procedure is specified in the individual monograph.♦

BUFFER STAGE—[NOTE—For this stage of the procedure, use buffer that previously has been equilibrated to a temperature of $37 \pm 0.5^\circ$.] Drain the acid from the vessel, and add to the vessel 1000 mL of pH 6.8 phosphate buffer, prepared by mixing 0.1 N hydrochloric acid with 0.20 M tribasic sodium phosphate (3:1) and adjusting, if necessary, with 2 N

hydrochloric acid or 2 N sodium hydroxide to a pH of 6.8 ± 0.05 . [NOTE—This may also be accomplished by removing from the apparatus the vessel containing the acid and replacing it with another vessel containing the buffer and transferring the dosage unit to the vessel containing the buffer.]

Continue to operate the apparatus for 45 minutes, or for the specified time ♦given in the individual monograph♦. At the end of the time period, withdraw an aliquot of the fluid, and perform the analysis using a suitable assay method. ♦The procedure is specified in the individual monograph. The test may be concluded in a shorter time period than that specified for the *Buffer Stage* if the requirement for minimum amount dissolved is met at an earlier time.♦

Apparatus 3 (Reciprocating Cylinder)

NOT ACCEPTED BY THE JAPANESE PHARMACOPOEIA IMMEDIATE-RELEASE DOSAGE FORMS

Place the stated volume of the *Dissolution Medium* in each vessel of the apparatus, assemble the apparatus, equilibrate the *Dissolution Medium* to $37 \pm 0.5^\circ$, and remove the thermometer. Place 1 dosage-form unit in each of the six reciprocating cylinders, taking care to exclude air bubbles from the surface of each dosage unit, and immediately operate the apparatus as specified ♦in the individual monograph♦. During the upward and downward stroke, the reciprocating cylinder moves through a total distance of 9.9 to 10.1 cm. Within the time interval specified, or at each of the times stated, raise the reciprocating cylinders and withdraw a portion of the solution under test from a zone midway between the surface of the *Dissolution Medium* and the bottom of each vessel. Perform the analysis as directed ♦in the individual monograph♦. If necessary, repeat the test with additional dosage-form units.

Dissolution Medium—Proceed as directed for *Immediate-Release Dosage Forms* under *Apparatus 1* and *Apparatus 2*.

Time—Proceed as directed for *Immediate-Release Dosage Forms* under *Apparatus 1* and *Apparatus 2*.

EXTENDED-RELEASE DOSAGE FORMS

Proceed as directed for *Immediate-Release Dosage Forms* under *Apparatus 3*.

Dissolution Medium—Proceed as directed for *Extended-Release Dosage Forms* under *Apparatus 1* and *Apparatus 2*.

Time—Proceed as directed for *Extended-Release Dosage Forms* under *Apparatus 1* and *Apparatus 2*.

DELAYED-RELEASE DOSAGE FORMS

Proceed as directed for *Delayed-Release Dosage Forms*, *Method B* under *Apparatus 1* and *Apparatus 2* using one row of vessels for the acid stage media and the following row of vessels for the buffer stage media and using the volume of medium specified (usually 300 mL).

Time—Proceed as directed for *Immediate-Release Dosage Forms* under *Apparatus 1* and *Apparatus 2*.

Apparatus 4 (Flow-Through Cell)

IMMEDIATE-RELEASE DOSAGE FORMS

Place the glass beads into the cell specified ♦in the monograph♦. Place 1 dosage unit on top of the beads or, if specified ♦in the monograph♦, on a wire carrier. Assemble

the filter head, and fix the parts together by means of a suitable clamping device. Introduce by the pump the *Dissolution Medium* warmed to $37 \pm 0.5^\circ$ through the bottom of the cell to obtain the flow rate specified in the individual monograph, and measured with an accuracy of 5%. Collect the eluate by fractions at each of the times stated. Perform the analysis as directed in the individual monograph. Repeat the test with additional dosage-form units.

Dissolution Medium—Proceed as directed for *Immediate-Release Dosage Forms* under *Apparatus 1* and *Apparatus 2*.

Time—Proceed as directed for *Immediate-Release Dosage Forms* under *Apparatus 1* and *Apparatus 2*.

EXTENDED-RELEASE DOSAGE FORMS

Proceed as directed for *Immediate-Release Dosage Forms* under *Apparatus 4*.

Dissolution Medium—Proceed as directed for *Immediate-Release Dosage Forms* under *Apparatus 4*.

Time—Proceed as directed for *Immediate-Release Dosage Forms* under *Apparatus 4*.

DELAYED-RELEASE DOSAGE FORMS

Proceed as directed for *Delayed-Release Dosage Forms* under *Apparatus 1* and *Apparatus 2*, using the specified media.

Time—Proceed as directed for *Delayed-Release Dosage Forms* under *Apparatus 1* and *Apparatus 2*.

INTERPRETATION

Immediate-Release Dosage Forms

Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the dosage units tested conform to *Acceptance Table 1*. Continue testing through the three stages unless the results conform at either S_1 or S_2 . The quantity, Q , is the amount of dissolved active ingredient specified in the individual monograph, expressed as a percentage of the labeled content of the dosage unit; the 5%, 15%, and 25% values in *Acceptance Table 1* are percentages of the labeled content so that these values and Q are in the same terms.

Acceptance Table 1

Stage	Number Tested	Acceptance Criteria
S_1	6	Each unit is not less than $Q + 5\%$.
S_2	6	Average of 12 units ($S_1 + S_2$) is equal to or greater than Q , and no unit is less than $Q - 15\%$.
S_3	12	Average of 24 units ($S_1 + S_2 + S_3$) is equal to or greater than Q , not more than 2 units are less than $Q - 15\%$, and no unit is less than $Q - 25\%$.

Immediate-Release Dosage Forms Pooled Sample—Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the pooled sample conform to the accompanying *Acceptance Table for a Pooled Sample*. Continue testing through the three stages unless the results conform at either S_1 or S_2 . The quantity, Q , is the amount of dissolved active ingredient specified in the individual monograph, expressed as a percentage of the labeled content.

Acceptance Table for a Pooled Sample

Stage	Number Tested	Acceptance Criteria
S_1	6	Average amount dissolved is not less than $Q + 10\%$.
S_2	6	Average amount dissolved ($S_1 + S_2$) is equal to or greater than $Q + 5\%$.
S_3	12	Average amount dissolved ($S_1 + S_2 + S_3$) is equal to or greater than Q .

♦

Extended-Release Dosage Forms

Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient dissolved from the dosage units tested conform to *Acceptance Table 2*. Continue testing through the three levels unless the results conform at either L_1 or L_2 . Limits on the amounts of active ingredient dissolved are expressed in terms of the percentage of labeled content. The limits embrace each value of Q , the amount dissolved at each specified fractional dosing interval. Where more than one range is specified in the individual monograph, the acceptance criteria apply individually to each range.

Acceptance Table 2

Level	Number Tested	Criteria
L_1	6	No individual value lies outside each of the stated ranges and no individual value is less than the stated amount at the final test time.
L_2	6	The average value of the 12 units ($L_1 + L_2$) lies within each of the stated ranges and is not less than the stated amount at the final test time; none is more than 10% of labeled content outside each of the stated ranges; and none is more than 10% of labeled content below the stated amount at the final test time.
L_3	12	The average value of the 24 units ($L_1 + L_2 + L_3$) lies within each of the stated ranges, and is not less than the stated amount at the final test time; not more than 2 of the 24 units are more than 10% of labeled content outside each of the stated ranges; not more than 2 of the 24 units are more than 10% of labeled content below the stated amount at the final test time; and none of the units is more than 20% of labeled content outside each of the stated ranges or more than 20% of labeled content below the stated amount at the final test time.

Delayed-Release Dosage Forms

NOT ACCEPTED BY THE JAPANESE PHARMACOPOEIA

Acid Stage—Unless otherwise specified in the individual monograph, the requirements of this portion of the test are met if the quantities, based on the percentage of the labeled content, of active ingredient dissolved from the units tested conform to *Acceptance Table 3*. Continue testing through all levels unless the results of both acid and buffer stages conform at an earlier level.

Acceptance Table 3

Level	Number Tested	Criteria
A ₁	6	No individual value exceeds 10% dissolved.
A ₂	6	Average of the 12 units (A ₁ + A ₂) is not more than 10% dissolved, and no individual unit is greater than 25% dissolved.
A ₃	12	Average of the 24 units (A ₁ + A ₂ + A ₃) is not more than 10% dissolved, and no individual unit is greater than 25% dissolved.

Buffer Stage—Unless otherwise specified ♦in the individual monograph♦, the requirements are met if the quantities of active ingredient dissolved from the units tested conform to *Acceptance Table 4*. Continue testing through the three levels unless the results of both stages conform at an earlier level. The value of *Q* in *Acceptance Table 4* is 75% dissolved unless otherwise specified ♦in the individual monograph♦. The quantity, *Q*, ♦specified in the individual monograph♦ is the total amount of active ingredient dissolved in both the *Acid* and *Buffer Stages*, expressed as a percentage of the labeled content. The 5%, 15%, and 25% values in *Acceptance Table 4* are percentages of the labeled content so that these values and *Q* are in the same terms.

Acceptance Table 4

Level	Number Tested	Criteria
B ₁	6	Each unit is not less than <i>Q</i> + 5%.
B ₂	6	Average of 12 units (B ₁ + B ₂) is equal to or greater than <i>Q</i> , and no unit is less than <i>Q</i> – 15%.
B ₃	12	Average of 24 units (B ₁ + B ₂ + B ₃) is equal to or greater than <i>Q</i> , not more than 2 units are less than <i>Q</i> – 15%, and no unit is less than <i>Q</i> – 25%.

<721> DISTILLING RANGE

To determine the range of temperatures within which an official liquid distills, or the percentage of the material that distills between two specified temperatures, use Method I or Method II as directed in the individual monograph. The *lower limit* of the range is the temperature indicated by the thermometer when the first drop of condensate leaves the tip of the condenser, and the *upper limit* is the Dry Point, i.e., the temperature at which the last drop of liquid evaporates from the lowest point in the distillation flask, without regard to any liquid remaining on the side of the flask, or the temperature observed when the proportion specified in the individual monograph has been collected.

NOTE—Cool all liquids that distill below 80° to between 10° and 15° before measuring the sample to be distilled.

Method I

Apparatus—Use apparatus similar to that specified for *Method II*, except that the distilling flask is of 50- to 60-mL capacity, and the neck of the flask is 10 to 12 cm long and

14 to 16 mm in internal diameter. The perforation in the upper insulating board, if one is used, should be such that when the flask is set into it, the portion of the flask below the upper surface of the insulating material has a capacity of 3 to 4 mL.

Procedure—Proceed as directed for *Method II*, but place in the flask only 25 mL of the liquid to be tested.

Method II

Apparatus—Use an apparatus consisting of the following parts:

Distilling Flask—A round-bottom distilling flask, of heat-resistant glass, of 200-mL capacity, and having a total length of 17 to 19 cm, and an inside neck diameter of 20 to 22 mm. Attached about midway on the neck, approximately 12 cm from the bottom of the flask, is a side-arm 10 to 12 cm long and 5 mm in internal diameter, which forms an angle of 70° to 75° with the lower portion of the neck.

Condenser—A straight glass condenser 55 to 60 cm in length with a water jacket about 40 cm in length, or a condenser of other design having equivalent condensing capacity. The lower end of the condenser may be bent to provide a delivery tube, or it may be connected to a bent adapter that serves as a delivery tube.

Insulating Boards—Two pieces of insulating board, 5 to 7 mm thick and 14 to 16 cm square, suitable for confining the heat to the lower part of the flask. Each board has a hole in its center, and the two boards differ only with respect to the diameter of the hole, i.e., the diameters are 4 cm and 10 cm, respectively. In use, the boards are placed one upon the other, and rest on a tripod or other suitable support, with the board having the larger hole on top.

Receiver—A 100-mL cylinder graduated in 1-mL subdivisions.

Thermometer—In order to avoid the necessity for an emergent stem correction, an accurately standardized, partial-immersion thermometer having the smallest practical subdivisions (not greater than 0.2°) is recommended. Suitable thermometers are available as the ASTM E-1 series 37C through 41C, and 102C through 107C (see *Thermometers* <21>). When placed in position, the stem is located in the center of the neck, and the top of the contraction chamber (or bulb, if 37C or 38C is used) is level with the bottom of the outlet to the side-arm.

Heat Source—A small Bunsen burner or an electric heater or mantle capable of adjustment comparable to that possible with a Bunsen burner.

Procedure—Assemble the apparatus, and place in the flask 100 mL of the liquid to be tested, taking care not to allow any of the liquid to enter the side-arm. Insert the thermometer, shield the entire burner and flask assembly from external air currents, and apply heat, regulating it so that between 5 and 10 minutes elapse before the first drop of distillate falls from the condenser. Continue the distillation at a rate of 4 to 5 mL of distillate per minute, collecting the distillate in the receiver. Note the temperature when the first drop of distillate falls from the condenser, and again when the last drop of liquid evaporates from the bottom of the flask or when the specified percentage has distilled over. Unless otherwise specified in the individual monograph, apply when necessary the emergent stem correction and report the temperatures adjusting the barometric pressure by the following formula:

$$t = t_0 + [(t_0 10^{-4} + 0.033)(760 - p)]$$

in which *t* is the corrected boiling temperature, in Celsius scale; *t*₀ is the measured boiling temperature, in Celsius

scale; and p is the barometric pressure at the time of measurement, in mm Hg.

<724> DRUG RELEASE

This test is provided to determine compliance with drug-release requirements where specified in individual monographs. Use the apparatus specified in the individual monograph. Replace the aliquots withdrawn for analysis with equal volumes of fresh *Dissolution Medium* at the temperature specified in the monograph or, where it can be shown that replacement of the medium is not necessary, correct for the volume change in the calculation. Keep the vessel covered for the duration of the test, and verify the temperature of the mixture under test at suitable times.

TRANSDERMAL DELIVERY SYSTEMS—
GENERAL DRUG RELEASE STANDARDS

Apparatus 5 (Paddle over Disk)

Apparatus—Use the paddle and vessel assembly from *Apparatus 2* as described under *Dissolution* <711>, with the addition of a stainless steel disk assembly¹ designed for holding the transdermal system at the bottom of the vessel. Other appropriate devices may be used, provided they do not sorb, react with, or interfere with the specimen being tested². The temperature is maintained at $32 \pm 0.5^\circ$. A distance of 25 ± 2 mm between the paddle blade and the surface of the disk assembly is maintained during the test. The vessel may be covered during the test to minimize evaporation. The disk assembly for holding the transdermal system is designed to minimize any “dead” volume between the disk assembly and the bottom of the vessel. The disk assembly holds the system flat and is positioned such that the release surface is parallel with the bottom of the paddle blade (see *Figure 1*).

Apparatus Suitability Test and Dissolution Medium—Proceed as directed for *Apparatus 2* under *Dissolution* <711>.

Procedure—Place the stated volume of the *Dissolution Medium* in the vessel, assemble the apparatus without the disk assembly, and equilibrate the medium to $32 \pm 0.5^\circ$. Apply the transdermal system to the disk assembly, assuring that the release surface of the system is as flat as possible. The system may be attached to the disk by applying a suitable adhesive³ to the disk assembly. Dry for 1 minute. Press the system, release surface side up, onto the adhesive-coated side of the disk assembly. If a membrane⁴ is used to support the system, it is applied so that no air bubbles occur between the membrane and the release surface. Place the disk assembly flat at the bottom of the vessel with the release surface facing up and parallel to the edge of the paddle blade and surface of the *Dissolution Medium*. The bottom edge of the paddle is 25 ± 2 mm from the surface of the disk assembly. Immediately operate the apparatus at the rate specified in the monograph. At each sampling time

¹ Disk assembly (stainless support disk) may be obtained from Millipore Corp., Ashley Rd., Bedford, MA 01730.
² A suitable device is the watchglass-patch-polytetrafluoroethylene mesh sandwich assembly available as the Transdermal Sandwich™ from Hanson Research Corp., 9810 Varrel Ave., Chatsworth, CA 91311.
³ Use Dow Corning, MD7-4502 Silicone Adhesive 65% in ethyl acetate, or the equivalent.
⁴ Use Cuprophane, Type 150 pm, 11 ± 0.5 -μm thick, an inert, porous cellulosic material, which is available from Medicell International Ltd., 239 Liverpool Road, London NI 1LX, England.

interval, withdraw a specimen from a zone midway between the surface of the *Dissolution Medium* and the top of the

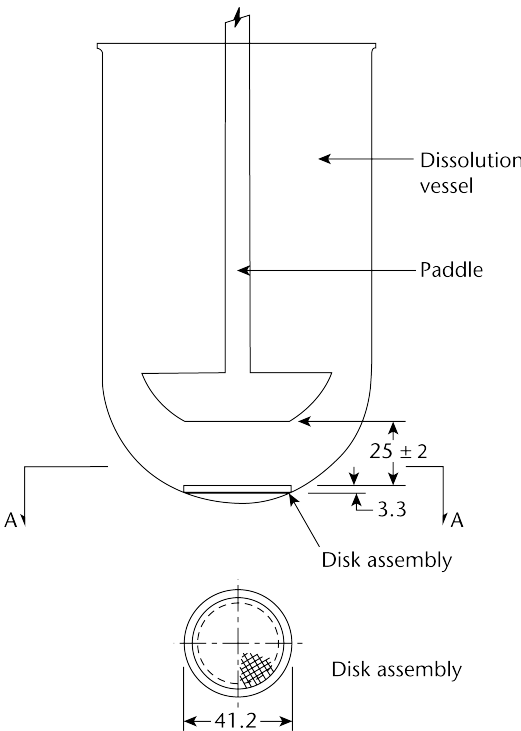


Figure 1. Paddle over disk.
(All measurements are expressed in mm unless noted otherwise.)

blade, not less than 1 cm from the vessel wall. Perform the analysis on each sampled aliquot as directed in the individual monograph, correcting for any volume losses, as necessary. Repeat the test with additional transdermal systems.

Time—The test time points, generally three, are expressed in hours. Specimens are to be withdrawn within a tolerance of ± 15 minutes or $\pm 2\%$ of the stated time, the tolerance that results in the narrowest time interval being selected.

Interpretation—Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient released from the system conform to *Acceptance Table 1* for transdermal drug delivery systems. Continue testing through the three levels unless the results conform at either L₁ or L₂.

Acceptance Table 1

Level	Number Tested	Criteria
L ₁	6	No individual value lies outside the stated range.
L ₂	6	The average value of the 12 units (L ₁ + L ₂) lies within the stated range. No individual value is outside the stated range by more than 10% of the average of the stated range.
L ₃	12	The average value of the 24 units (L ₁ + L ₂ + L ₃) lies within the stated range. Not more than 2 of the 24 units are outside the stated range by more than 10% of the average of the stated range; and none of the units is outside the stated range by more than 20% of the average of the stated range.

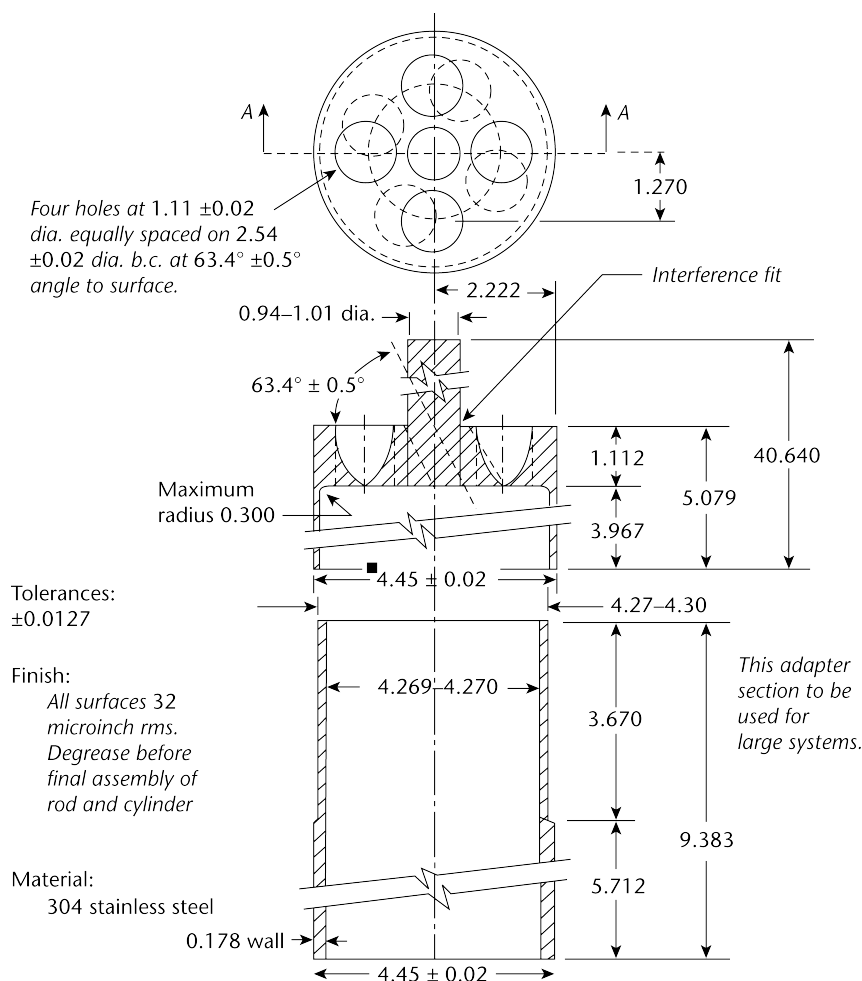


Figure 2. Cylinder stirring element.⁵
(All measurements are expressed in cm unless noted otherwise.)

Apparatus 6 (Cylinder)

Apparatus—Use the vessel assembly from *Apparatus 1* as described under *Dissolution* <711>, except to replace the basket and shaft with a stainless steel cylinder stirring element and to maintain the temperature at $32 \pm 0.5^\circ$ during the test. The shaft and cylinder components of the stirring element are fabricated of stainless steel to the specifications shown in *Figure 2*. The dosage unit is placed on the cylinder at the beginning of each test. The distance between the inside bottom of the vessel and the cylinder is maintained at 25 ± 2 mm during the test.

Dissolution Medium—Use the medium specified in the individual monograph (see *Dissolution* <711>).

Procedure—Place the stated volume of the *Dissolution Medium* in the vessel of the apparatus specified in the individual monograph, assemble the apparatus, and equilibrate the *Dissolution Medium* to $32 \pm 0.5^\circ$. Unless otherwise directed in the individual monograph, prepare the test system prior to test as follows. Remove the protective liner from the system, and place the adhesive side on a piece of Cuprophane⁴ that is not less than 1 cm larger on all sides than the system. Place the system, Cuprophane covered side down, on a clean surface, and apply a suitable adhesive³ to

the exposed Cuprophane borders. If necessary, apply additional adhesive to the back of the system. Dry for 1 minute. Carefully apply the adhesive-coated side of the system to the exterior of the cylinder such that the long axis of the system fits around the circumference of the cylinder. Press the Cuprophane covering to remove trapped air bubbles. Place the cylinder in the apparatus, and immediately rotate at the rate specified in the individual monograph. Within the time interval specified, or at each of the times stated, withdraw a quantity of *Dissolution Medium* for analysis from a zone midway between the surface of the *Dissolution Medium* and the top of the rotating cylinder, not less than 1 cm from the vessel wall. Perform the analysis as directed in the individual monograph, correcting for any volume losses as necessary. Repeat the test with additional transdermal drug delivery systems.

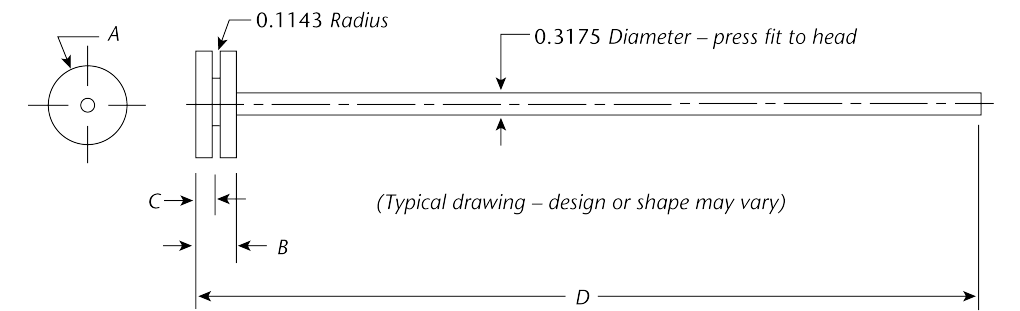
Time—Proceed as directed under *Apparatus 5*.

Interpretation—Unless otherwise specified in the individual monograph, the requirements are met if the quantities of active ingredient released from the system conform to *Acceptance Table 1* for transdermal drug delivery systems. Continue testing through the three levels unless the results conform at either L_1 or L_2 .

Apparatus 7 (Reciprocating Holder)

NOTE—This apparatus may also be specified for use with a variety of dosage forms.

⁵ The cylinder stirring element is available from Accurate Tool, Inc., 25 Diaz St., Stamford, CT 06907, or from VanKel Technology Group, 13000 Weston Parkway, Cary, NC 27513.



Dimensions are in centimeters

System ^a	HEAD			Material ^b	ROD		O-RING
	A (Diameter)	B	C		D	Material ^c	(not shown)
1.6 cm ²	1.428	0.9525	0.4750	SS/VT	30.48	SS/P	Parker 2-113-V884-75
2.5 cm ²	1.778	0.9525	0.4750	SS/VT	30.48	SS/P	Parker 2-016-V884-75
5 cm ²	2.6924	0.7620	0.3810	SS/VT	8.890	SS/P	Parker 2-022-V884-75
7 cm ²	3.1750	0.7620	0.3810	SS/VT	30.48	SS/P	Parker 2-124-V884-75
10 cm ²	5.0292	0.6350	0.3505	SS/VT	31.01	SS/P	Parker 2-225-V884-75

^a Typical system sizes.
^b SS/VT=Either stainless steel or virgin Teflon.
^c SS/P=Either stainless steel or Plexiglas.

Figure 3. Reciprocating disk sample holder.⁷

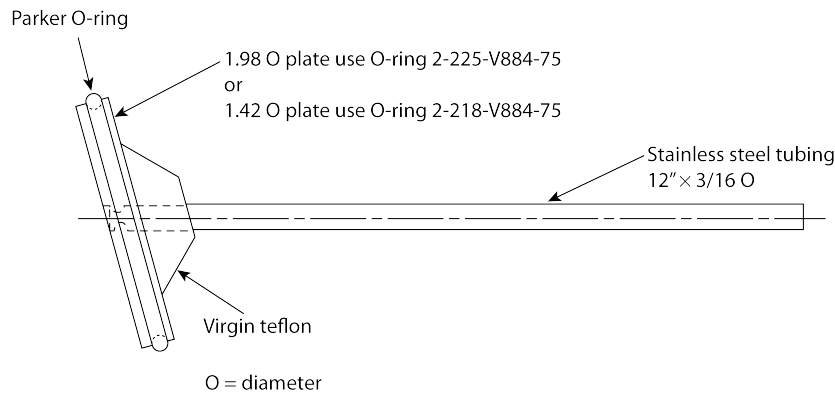


Figure 4a. Transdermal system holder—angled disk.

Apparatus—The assembly consists of a set of volumetrically calibrated or tared solution containers made of glass or other suitable inert material,⁶ a motor and drive assembly to reciprocate the system vertically and to index the system horizontally to a different row of vessels automatically if desired, and a set of suitable sample holders (see Figure 3⁷ and Figures 4a–4d). The solution containers are partially immersed in a suitable water bath of any convenient size that permits maintaining the temperature, T, inside the contain-

⁶ The materials should not sorb, react with, or interfere with the specimen being tested.
⁷ The reciprocating disk sample holder may be purchased from ALZA Corp., 1900 Charleston Road, P.O. Box 7210, Mt. View, CA 94039-7210 or VanKel Technology Group.

ers at 32 ± 0.5° or within the allowable range, as specified in the individual monograph, during the test. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smooth, vertically reciprocating sample holder. Apparatus that permits observation of the system and holder during the test is preferable. Use the size container and sample holder as specified in the individual monograph.

Dissolution Medium—Use the Dissolution Medium specified in the individual monograph (see Dissolution (711)).

Sample Preparation A (Coated tablet drug delivery system)—Attach each system to be tested to a suitable sam-

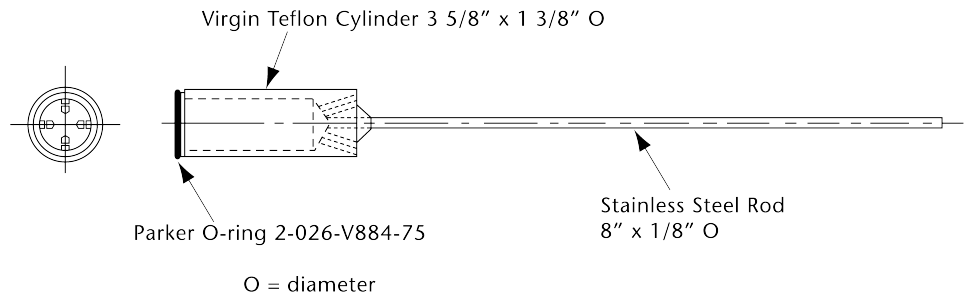


Figure 4b. Transdermal system holder—cylinder.

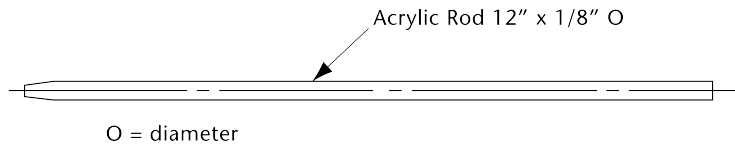


Figure 4c. Oral extended-release tablet holder—rod, pointed for gluing.

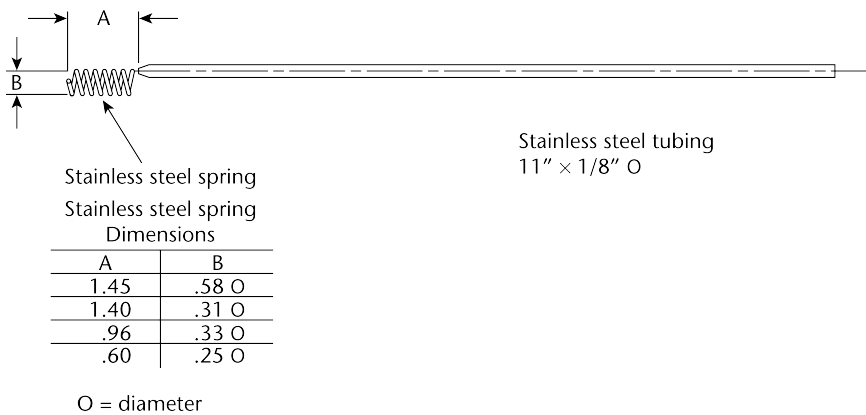


Figure 4d. Oral extended-release tablet holder—spring holder.

ple holder (e.g., by gluing system edge with 2-cyano acrylate glue onto the end of a plastic rod or by placing the system into a small nylon net bag at the end of a plastic rod or within a metal coil attached to a metal rod).

Sample Preparation B (Transdermal drug delivery system)—Press the system onto a dry, unused piece of Cuprophane⁴, nylon netting, or equivalent with the adhesive side against the selected substrate, taking care to eliminate air bubbles between the substrate and the release surface. Attach the system to a suitable sized sample holder with a suitable O-ring such that the back of the system is adjacent to and centered on the bottom of the disk-shaped sample holder or centered around the circumference of the cylindrical-shaped sample holder. Trim the excess substrate with a sharp blade.

Sample Preparation C (Other drug delivery systems)—Attach each system to be tested to a suitable holder as described in the individual monograph.

Procedure—Suspend each sample holder from a vertically reciprocating shaker such that each system is continu-

ously immersed in an accurately measured volume of *Dissolution Medium* within a calibrated container pre-equilibrated to temperature, T. Reciprocate at a frequency of about 30 cycles per minute with an amplitude of about 2 cm, or as specified in the individual monograph, for the specified time in the medium specified for each time point. Remove the solution containers from the bath, cool to room temperature, and add sufficient solution (i.e., water in most cases) to correct for evaporative losses. Perform the analysis as directed in the individual monograph. Repeat the test with additional drug delivery systems as required in the individual monograph.

Interpretation—Unless otherwise specified in the individual monograph, the requirements are met if the quantities of the active ingredients released from the system conform to *Acceptance Table 2* under *Dissolution* <711> for coated tablet drug delivery systems, to *Acceptance Table 1* for transdermal drug delivery systems, or as specified in the individual monograph. Continue testing through the three levels unless the results conform at either L₁ or L₂.

(726) ELECTROPHORESIS

Electrophoresis refers to the migration of electrically charged proteins, colloids, molecules, or other particles when dissolved or suspended in an electrolyte through which an electric current is passed.

Based upon the type of apparatus used, electrophoretic methods may be divided into two categories, one called *free solution* or moving boundary electrophoresis and the other called *zone electrophoresis*.

In the *free solution* method, a buffered solution of proteins in a U-shaped cell is subjected to an electric current which causes the proteins to form a series of layers in order of decreasing mobility, which are separated by boundaries. Only a part of the fastest moving protein is physically separated from the other proteins, but examination of the moving boundaries using a schlieren optical system provides data for calculation of mobilities and information on the qualitative and quantitative composition of the protein mixture.

In *zone electrophoresis*, the sample is introduced as a narrow zone or spot in a column, slab, or film of buffer. Migration of the components as narrow zones permits their complete separation. Remixing of the separated zones by thermal convection is prevented by stabilizing the electrolyte in a porous matrix such as a powdered solid, or a fibrous material such as paper, or a gel such as starch, agar, or polyacrylamide.

Various methods of zone electrophoresis are widely employed. *Gel electrophoresis*, particularly the variant called *disk electrophoresis*, is especially useful for protein separation because of its high resolving power.

Gel electrophoresis, which is employed by the compendium, is discussed in more detail following the presentation of some theoretical principles and methodological practices, which are shared in varying degrees by all electrophoretic methods.

The electrophoretic migration observed for particles of a particular substance depends on characteristics of the particle, primarily its electrical charge, its size or molecular weight, and its shape, as well as characteristics and operating parameters of the system. These latter include the pH, ionic strength, viscosity and temperature of the electrolyte, density or cross-linking of any stabilizing matrix such as gel, and the voltage gradient employed.

Effect of Charge, Particle Size, Electrolyte Viscosity, and Voltage Gradient—Electrically charged particles migrate toward the electrode of opposite charge, and molecules with both positive and negative charges move in a direction dependent on the net charge. The rate of migration is directly related to the magnitude of the net charge on the particle and is inversely related to the size of the particle, which in turn is directly related to its molecular weight.

Very large spherical particles, for which Stokes' law is valid, exhibit an electrophoretic mobility, u_0 , which is inversely related to the first power of the radius as depicted in the equation:

$$u_0 = v/E = Q/6\pi r\eta$$

where v is the velocity of the particle, E is the voltage gradient imposed on the electrolyte, Q is the charge on the particle, r is the particle radius, and η is the viscosity of the electrolyte. This idealized expression is strictly valid only at infinite dilution and in the absence of a stabilizing matrix such as paper or a gel.

Ions, and peptides up to molecular weights of at least 5000, particularly in the presence of stabilizing media, do

not obey Stokes' law, and their electrophoretic behavior is best described by an equation of the type:

$$u_0 = Q/A\pi r^2\eta$$

where A is a shape factor generally in the range of 4 to 6, which shows an inverse dependence of the mobility on the square of the radius. In terms of molecular weight, this implies an inverse dependence of mobility on the $^{2/3}$ power of the molecular weight.

Effect of pH—The direction and rate of migration of molecules containing a variety of ionizable functional groups, such as amino acids and proteins, depends upon the pH of the electrolyte. For instance, the mobility of a simple amino acid such as glycine varies with pH approximately as shown in Figure 1.

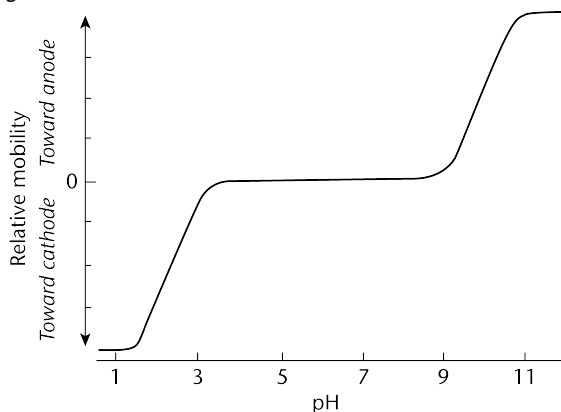


Figure 1

The pK_a values of 2.2 and 9.9 coincide with the inflection points of the sigmoid portions of the plot. Since the respective functional groups are 50% ionized at the pH values where $pH = pK_a$, the electrophoretic mobilities at these points are half of the value observed for the fully ionized cation and anion obtained at very low and very high pH, respectively. The zwitterion that exists at the intermediate pH range is electrically neutral and has zero mobility.

Effect of Ionic Strength and Temperature—Electrophoretic mobility decreases with increasing ionic strength of the supporting electrolyte. Ionic strength, μ , is defined as:

$$\mu = 0.5\sum C_i Z_i^2$$

where C_i is the concentration of an ion in moles per L and Z_i is its valence, and the sum is calculated for all ions in the solution. For buffers in which both the anion and cation are univalent, ionic strength is identical with molarity.

Ionic strengths of electrolytes employed in electrophoresis commonly range from about 0.01 to 0.10. A suitable strength is somewhat dependent on the sample composition, since the buffer capacity must be great enough to maintain a constant pH over the area of the component zones. Zones become sharper or more compact as ionic strength is increased.

Temperature affects mobility indirectly, since the viscosity, η , of the supporting electrolyte is temperature-dependent. The viscosity of water decreases at a rate of about 3% per °C in the range of 0° to 5° and at a slightly lower rate in the vicinity of room temperature. Mobility, therefore, increases with increasing electrolyte temperature.

Considerable heat is evolved as a result of current passing through the supporting electrolyte. This heat increases with the applied voltage and with increasing ionic strength. Particularly in larger apparatus, despite the circulation of a coolant, this heat produces a temperature gradient across the bed which may lead to distortion of the separated zones. Therefore, practical considerations and the design of

the particular apparatus dictate the choice of ionic strength and operating voltage.

Effect of a Stabilizing Medium, Electroosmosis—When an electrical current is passed through an electrolyte contained in a glass tube or contained between plates of glass or plastic, a bulk flow of the electrolyte toward one of the electrodes is observed. This flow is called electroosmosis. It results from the surface charge on the walls of the apparatus, which arises either from ionizable functional groups inherent in the structural material or from ions adsorbed on the cell walls from the electrolyte contacting them. The effect is usually increased when the cell is filled with a bed of porous substance, such as a gel, used to stabilize the supporting electrolyte and prevent remixing of separated zones by thermal convection or diffusion. The solution immediately adjacent to the surface builds up an electrical charge, equal but opposite to the surface charge, and the electrical field traversing the cell produces a movement of solution toward the electrode of opposite charge.

The substances commonly used as stabilizing media in zone electrophoresis develop a negative surface charge, and therefore electroosmotic flow of the electrolyte is toward the cathode. As a result, all zones, including neutral substances, are carried toward the cathode during the electrophoretic run.

The degree of electroosmosis observed varies with the stabilizing substance. It is appreciable with agar gel, while it is negligibly small with polyacrylamide gel.

Molecular Sieving—In the absence of a stabilizing medium or in cases where the medium is very porous, electrophoretic separation of molecules results from differences in the ratio of their electrical charge to their size. In the presence of a stabilizing medium, differences in adsorptive or other affinity of molecules for the medium introduces a chromatographic effect that may enhance the separation.

If the stabilizing medium is a highly cross-linked gel such that the size of the resultant pores is of the order of the dimensions of the molecules being separated, a molecular sieving effect is obtained. This effect is analogous to that obtained in separations based on gel permeation or molecular exclusion chromatography, but in gel electrophoresis the effect is superimposed on the electrophoretic separation. Molecular sieving may be visualized to result from a steric barrier to the passage of larger molecules. Small molecules pass through pores of a wide size range, and therefore their electrophoretic passage through the gel will not be impeded. As size increases, fewer pores will permit passage of the molecules, causing a retardation of the migration of substances of large molecular weight.

Gel Electrophoresis

Processes employing a gel such as agar, starch, or polyacrylamide as a stabilizing medium are broadly termed gel electrophoresis. The method is particularly advantageous for protein separations. The separation obtained depends upon the electrical charge to size ratio coupled with a molecular sieving effect dependent primarily on the molecular weight.

Polyacrylamide gel has several advantages that account for its extensive use. It has minimal adsorptive properties and produces a negligible electroosmotic effect. Gels of a wide range of pore size can be reproducibly prepared by varying the total gel concentration (based on monomer plus cross-linking agent) and the percentage of cross-linking agent used to form the gel. These quantities are conveniently expressed as

$$T(\%) = [(a + b)/V] \times 100$$

$$C(\%) = [b/(a + b)] \times 100$$

where T is the total gel concentration in %; C is the percentage of cross-linking agent used to prepare the gel; V is

the volume, in mL, of buffer used in preparing the gel; and a and b are the weights, in g, of monomer (acrylamide) and cross-linking agent (usually *N,N'*-methylenebisacrylamide) used to prepare the gel. Satisfactory gels ranging in concentration (T) from about 3% to 30% have been prepared. The amount of cross-linking agent is usually about one-tenth to one-twentieth of the quantity of monomer (C = 10% to 5%), a smaller percentage being used for higher values of T.

In the preparation of the gel, the bed of the electrophoresis apparatus is filled with an aqueous solution of monomer and cross-linking agent, usually buffered to the pH desired in the later run, and polymerized in place by a free radical process. Polymerization may be initiated by a chemical process, frequently using ammonium persulfate plus *N,N,N',N'*-tetramethylethylenediamine or photochemically using a mixture of riboflavin and *N,N,N',N'*-tetramethylethylenediamine. Polymerization is inhibited by molecular oxygen and by acidic conditions. The gel composition and polymerization conditions chosen must be adhered to rigorously to ensure reproducible qualities of the gel.

Apparatus for Gel Electrophoresis—In general, the bed or medium in which electrophoresis is carried out may be supported horizontally or vertically, depending upon the design of the apparatus. A series of separations to be compared may also be carried out in several individual tubes or by placing different samples in adjacent wells, cast or cut into a single slab of gel. A vertical slab assembly such as that depicted schematically in Figure 2.

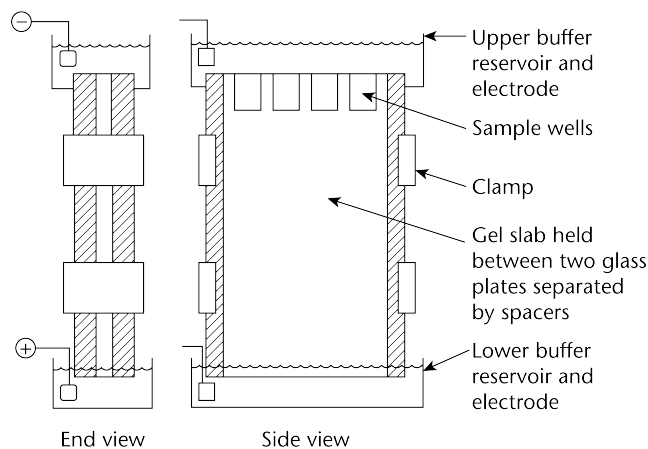


Figure 2. Vertical slab gel electrophoresis apparatus.

is convenient for direct comparison of several samples. A particular advantage derives from the comparison of the samples in a single bed of gel which is likely to be more uniform in composition than gels cast in a series of chambers.

A feature of many types of apparatus, not illustrated in the schematic view, seals the lower buffer chamber to the base of the bed and allows the level of the buffer in the lower chamber to be made equal to that in the upper chamber, thereby eliminating hydrostatic pressure on the gel. In addition, some units provide for the circulation of coolant on one or both sides of the gel bed.

In the preparation of the gel, the base of the gel chamber is closed with a suitable device and the unit is filled with the solution of monomer, cross-linking agent, and catalyst. A comb, having teeth of an appropriate size, is inserted in the top, and polymerization is allowed to proceed to completion. Removal of the comb leaves a series of sample wells in the polymerized gel.

In simple gel electrophoresis, an identical buffer is used to fill the upper and lower buffer chambers as well as in the solution used to prepare the gel. After filling the chambers, the samples, dissolved in sucrose or other dense and somewhat viscous solution to prevent diffusion, are introduced

with a syringe or micropipet into the bottoms of the sample wells, and the electrophoresis is begun immediately thereafter.

DISK ELECTROPHORESIS

An important variant of polyacrylamide gel electrophoresis, which employs a discontinuous series of buffers and often also a discontinuous series of gel layers, is called disk electrophoresis. The name is derived from the discoid shape of the very narrow zones that result from the technique. As a result of the narrow zones produced, this technique exhibits an extremely high resolving power and is to be recommended for the characterization of protein mixtures and for the detection of contaminants that may have mobilities close to that of the major component.

The basis of disk electrophoresis is outlined in the following paragraphs with reference to an anionic system suitable for separating proteins bearing a net negative charge. To understand disk electrophoresis, it is essential to have a knowledge of the general aspects of electrophoresis and the apparatus already described.

Basis of Disk Electrophoresis—The high resolution obtained in disk electrophoresis depends on the use of a buffer system that is discontinuous with respect to both pH and composition. This is usually combined with a discontinuous series of two or three gels that differ in density.

A typical system is illustrated schematically in Figure 3.

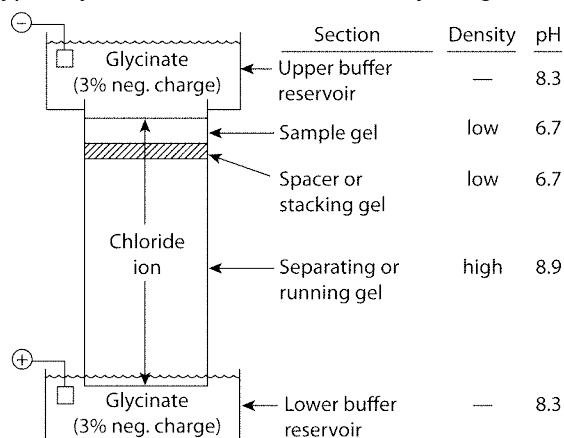


Figure 3. Terminology, buffer pH, and buffer composition for acrylamide gel disk electrophoresis.

A high density ($T = 10\%$ to 30%) separating gel several centimeters high is polymerized in a tris-chloride buffer in the bed of the apparatus. During polymerization the buffer is overlaid with a thin layer of water to prevent fixation of a meniscus in the top of the gel. The overlayer of water is then removed and a thin layer, 3 mm to 10 mm thick, of low density ($T = 3\%$) gel, called the spacer or stacking gel, is polymerized in a tris-chloride buffer on top of the separating gel. An overlayer of water is again used to ensure a flat surface. The sample is mixed with a small amount of the spacer gel monomer solution which is applied on top of the spacer gel and allowed to polymerize. The pH of the separating gel is typically 8.9, while that of the spacer and sample gels is 6.7. All three gels are prepared using chloride as the anion.

The upper and lower buffer reservoirs are filled with a pH 8.3 buffer prepared from tris and glycine. At this pH about 3% of the glycine molecules bear a net negative charge.

When a voltage is applied across the system, the glycinate-chloride interface moves downward toward the anode. It was initially positioned at the junction of the buffer in the upper reservoir and the top of the sample gel layer. The chloride anion, by virtue of its small size, migrates

faster than any of the proteins present in the sample. The pH of the sample and spacer layers was chosen to be about 3 units below the higher pK_a of glycine. Therefore, in traversing these layers, only about 0.1% of the glycine molecules bear a net negative charge. Consequently, glycine migrates more slowly than chloride. The tendency for the faster-moving chloride to move away from glycinate lowers the concentration at the interface, producing a greater voltage drop at the interface, which in turn causes the glycinate to catch up to the chloride. Under these conditions, a very sharp interface is maintained, and as it moves through the sample and spacer layers, the proteins in the sample tend to stack themselves at the interface in very thin layers in order of mobility. The process is called stacking and is the source of the disks which are separated.

When the stacked proteins reach the high-density separating gel, they are slowed down by a molecular sieving process. The higher pH encountered in the running gel also causes the glycinate to migrate faster, so that the discontinuous buffer interface overtakes the proteins and eventually reaches the bottom of the separating gel. During this period, the disks of protein continue to separate by electrophoresis and molecular sieving in the separating gel. At the end of the run, the pH of the separating gel will have risen above its original value of 8.9 to a value of about pH 9.5.

Relative Mobility—Bromophenol blue is often used as a standard for calculating the relative mobility of separated zones and to judge visually the progress of a run. It may be added to one of the sample wells, or mixed with the sample itself, or simply added to the buffer in the upper sample reservoir.

Relative mobility, M_B , is calculated as:

$$M_B = \text{distance from origin to sample zone} / \text{distance from origin to bromophenol blue zone}$$

Visualization of Zones—Since polyacrylamide is transparent, protein bands may be located by scanning in a densitometer with UV light. The zones may be fixed by immersing in protein precipitants such as phosphotungstic acid or 10% trichloroacetic acid. A variety of staining reagents including naphthalene black (amido black) and Coomassie brilliant blue R250 may be used. The fixed or stained zones may be conveniently viewed and photographed with transmitted light from an X-ray film illuminator.

SAFETY PRECAUTIONS

Voltages used in electrophoresis can readily deliver a lethal shock. The hazard is increased by the use of aqueous buffer solutions and the possibility of working in damp environments.

The equipment, with the possible exception of the power supply, should be enclosed in either a grounded metal case or a case made of insulating material. The case should have an interlock that deenergizes the power supply when the case is opened, after which reactivation should be prevented until activation of a reset switch is carried out.

High-voltage cables from the power supply to the apparatus should preferably be a type in which a braided metal shield completely encloses the insulated central conductor, and the shield should be grounded. The base of the apparatus should be grounded metal or contain a grounded metal rim which is constructed in such a way that any leakage of electrolyte will produce a short which will deenergize the power supply before the electrolyte can flow beyond the protective enclosure.

If the power supply contains capacitors as part of a filter circuit, it should also contain a bleeder resistor to ensure discharge of the capacitors before the protective case is opened. A shorting bar that is activated by opening the case may be considered as an added precaution.

Because of the potential hazard associated with electrophoresis, laboratory personnel should be completely familiar with electrophoresis equipment before using it.

〈729〉 GLOBULE SIZE DISTRIBUTION IN LIPID INJECTABLE EMULSIONS

INTRODUCTION

Lipid injectable emulsions for intravenous administration are sterile oil-in-water emulsions of soybean oil, used to provide an ample supply of essential fatty acids, linoleic and linolenic, dispersed with the aid of an emulsifying agent in *Water for Injection*. Alternatively, soybean oil can be mixed with other suitable oils (neutral triglycerides), such as safflower oil, medium-chain triglycerides (MCT) derived from coconut or palm kernel oils, olive oil, or a marine oil, such as menhaden oil. The size of the lipid droplets is critical: because of mechanical filtration, larger-size fat globules ($>5\ \mu\text{m}$) can be trapped in the lungs. The essential size characteristics of a lipid injectable emulsion for intravenous use include the mean diameter of the lipid droplets and the range of the various droplet diameters distributed around the mean diameter, expressed as the standard deviation. In particular, the amounts of fat globules comprising the large-diameter tail of the globule size distribution are especially important with respect to infusion safety. These two regions of the globule size distribution (mean droplet size and large-diameter tail) must be controlled within specified limits.

The two methods described below are used for determination of the mean lipid droplet diameter and the distribution of large-diameter globule sizes in lipid injectable emulsions. *Method I* and *Method II* must be validated. The methods described below to assess the quality of lipid injectable emulsions are to be performed in two stages.

METHOD I—LIGHT-SCATTERING METHOD

For the determination of the mean droplet size of lipid injectable emulsions, either of two common light-scattering techniques may be employed: (1) dynamic light scattering (DLS), also known as photon correlation spectroscopy (PCS), or (2) classical light scattering, based on Mie scattering theory. The DLS, or PCS, technique is based on analyzing the rapid temporal fluctuations in the scattered light intensity that occur due to the random Brownian motion, or diffusion, of any particles, including lipid droplets, suspended in liquid. The intensity is measured at a given angle (usually 90°) by a suitable detector (e.g., photomultiplier tube) able to measure the rapidly fluctuating scattered light intensity produced by the suspended, diffusing droplets. These scattered intensity data are typically used to calculate the intensity autocorrelation function, which is a simple decaying exponential function in time for droplets of uniform size. A distribution of droplet sizes expresses itself by exponential functions of different decay times. The autocorrelation function generated by the scattered intensity data obtained from a given emulsion can be “inverted” by means of an appropriate deconvolution algorithm in order to obtain the approximate distribution of intensity-weighted diffusion coefficients. From the latter, the distribution of small-diameter

droplets is calculated, using the Stokes-Einstein equation and the rules of classical (Mie) light scattering.

By contrast, classical light scattering based on Mie theory analyzes the spatial, rather than temporal, variation of the scattered light intensity by measuring the latter as a function of the scattering angle, typically over a large range of detected angles. The temporal fluctuations in the scattering intensity due to Brownian motion are averaged out in time for each angular measurement. This angular variation occurs as a consequence of the mutual interference of individual scattered waves arriving at the detector with different phases from different points within a given lipid droplet, as well as from different particles. The extent of the angular variation is significant whenever the droplet diameter is not small compared with the wavelength of the laser light (typically $635\ \text{nm}$). Droplets of a given size and refractive index yield a unique curve of scattering intensity vs. angle. A distribution of droplet sizes gives rise to a final angular dependence that represents the superposition, or summation, of individual (different) intensity vs. angle curves. The measured angular dependence of the scattering intensity obtained from a given emulsion sample can be inverted by means of an appropriate deconvolution algorithm and Mie scattering theory in order to obtain the approximate droplet size distribution.

Thus, light scattering, using either dynamic light scattering (i.e., temporal fluctuations due to droplet diffusion) or classical light scattering/Mie theory (i.e., average intensity vs. angle), can provide acceptable results for both the mean diameter and standard deviation of the droplet size distribution. For purposes of illustrating the method used in *Method I*, a dynamic light-scattering technique is described. For guidance regarding instruments employing classical Mie-theory light scattering, see *Light Diffraction Measurement of Particle Size* 〈429〉.

Apparatus—A suitable DLS/PCS instrument with or without the capability of automatic sample dilution is controlled by validated software and is used to perform the measurement, with the scattering angle typically set at 90° . The intensity-weighted results (mean diameter and standard deviation) are reported, provided it is clearly stated which values are given and that the necessary parameter values required for all requisite calculations are also given.

Water—Pass distilled water through a filter of $0.2\text{-}\mu\text{m}$ pore size, and degas by sonication, or use *Sterile Water for Injection* stored in a glass container.

Standard Preparation—To a pre-established volume of *Water* add an appropriate amount of concentrated suspension, containing NIST-traceable polystyrene latex standard particles or other suitable nanospheres. Gently mix the fluids to achieve a homogeneous suspension. The diluted suspension will be slightly turbid in appearance. If the DLS/PCS instrument is equipped with an automatic dilution system, the starting concentrated sample can be analyzed by injection directly into the instrument via a syringe, with further dilution occurring automatically to optimize the droplet concentration for analysis. Alternatively, the sample would require greater manual dilution with *Water* (typically by at least a factor of 10 over the first dilution), and then this sample would be instilled into a “drop-in” cuvette. The optimum dilution scheme that achieves the proper scattering intensity for the cuvette-based analysis will be determined by the instrument specifications. Thus, the concentration of latex in the final sample must be optimized for the DLS/PCS instrument used. This should be performed separately for three different size standards of approximately 100, 250, and $400\ \text{nm}$ (triplicate analyses per size), and the corresponding results of intensity-weighted mean diameter and standard deviation should coincide with the expected values within acceptable errors.

Test Preparation—To a pre-established volume of water add an appropriate volume of sample from the lipid injectable emulsion. Gently mix the fluids to achieve a homogeneous suspension. The diluted suspension will be slightly

turbid in appearance. If the DLS/PCS instrument is equipped with an automatic dilution system, the starting concentrated sample can be analyzed by injection directly into the instrument via a syringe. Further dilution of the sample then occurs automatically to optimize the droplet concentration for analysis, ensuring that it is not so high as to cause artifacts due to multiple scattering or interdroplet interactions. Alternatively, the sample would require greater manual dilution with *Water* (typically by at least a factor of 10 over the first dilution), and then this sample would be instilled into a "drop-in" cuvette. The optimum dilution scheme that achieves the proper scattering intensity for the cuvette-based analysis will be determined by the instrument specifications. Thus, the concentration of lipid injectable emulsion in the final sample must be optimized for the DLS/PCS instrument used.

System Suitability—Using the *Standard Preparation*, measure the intensity-weighted mean particle diameter and the corresponding standard deviation. The system is suitable once the sample temperature has reached equilibration and the results have stabilized and triplicate mean droplet diameter measurements are obtained. The coefficient of variation (CV) should not exceed 10% of the NIST-traceable mean droplet diameter. A larger CV value indicates that the latex microspheres are not suitable as a standard because they either inherently lack uniformity or have become agglomerated to an unacceptable extent. In this case, another standard latex suspension must be selected and tested.

Procedure and Interpretation—If the DLS/PCS instrument is equipped with an automatic dilution system, use a disposable syringe to load the *Standard Preparation* or *Test Preparation*. If no automatic dilution system is used, transfer the appropriately diluted preparation to a cuvette, and place the cuvette in the spectrometer. Allow the sample to equilibrate to a preset controlled temperature close to ambient (between 20° and 25°, as in the USP definition found in (659) *Packaging and Storage Requirements*). Set the instrument scattering angle to 90°, and carry out the measurements. As long as the chi-square (χ^2) goodness-of-fit parameter remains acceptably low (per instrument specifications), the results for the *Test Preparation* are acceptable. Excessive values of the χ^2 parameter suggest that the droplet distribution is not normal and may indicate an unstable emulsion. The intensity-weighted mean droplet diameter (MDD) for lipid injectable emulsions must be less than 500 nm or 0.5 μm , irrespective of the concentration of the dispersed lipid phase.

METHOD II—MEASUREMENT OF LARGE GLOBULE CONTENT BY LIGHT OBSCURATION OR EXTINCTION METHOD

For determination of the extent of the large-diameter droplet tail ($>5 \mu\text{m}$) of lipid injectable emulsions, a light obscuration (LO) or light extinction (LE) method that employs a single-particle (globule) optical sizing (SPOS) technique is used. During application of the LE/SPOS technique, passage of a droplet through a thin optical sensing zone results in blockage of a portion of the incident light beam, causing a momentary decrease in the light intensity reaching the "extinction" detector. The magnitude of this decrease in the signal is ideally proportional to the cross-sectional area of the droplet (assumed smaller than the sensing zone thickness), i.e., to the square of the droplet diameter. During optimization of the LE/SPOS instrument for a given emulsion sample, a series of dilutions should be tested to achieve consistency between samples. The goal is to identify a standard range of dilutions that yield consistent data and are most applicable to the formulation tested. Ideally, when comparing different emulsions, the same approximate number of globules are sized each time, and once a standard is achieved, it should be incorporated into the routine sampling plan for validation testing. As long as the fat globule

concentration is below the "coincidence limit" of the sensor (determined by the flow cell and optical design), only one globule at most will pass through the sensing zone at any given time, allowing it to be counted and accurately sized (with less than 1% coincidence events). Both the coincidence limit and the optimal flow rate must be known for the LE/SPOS sensor used. Furthermore, it is prudent to perform the large-diameter measurements at a reduced emulsion concentration such that the measurable droplet concentration at threshold of detection (e.g., $>1.8 \mu\text{m}$) to an upper limit (e.g., $50 \mu\text{m}$) is only approximately one-third of the nominal coincidence limit for the sensor used. The resulting single pulse heights are converted to droplet diameters using a standard calibration curve previously constructed from NIST-traceable monosized polystyrene microspheres of known diameters. For additional guidance in the use of the light obscuration methodology, see the general chapter *Particulate Matter in Injections* (788).

Apparatus—A suitable light obscuration instrument with or without the capability of automatic sample dilution and controlled by a personal computer (PC) is used for the measurement. The number- and volume-weighted particle size distribution data are reported, provided that it is clearly stated which values are given and that the necessary parameter values required for all necessary calculations are also given.

Water—Pass distilled water through a filter of 0.2- μm pore size, or use *Sterile Water for Injection* stored in a glass container.

Standard Preparation—To a pre-established volume of *Water* add an appropriate amount of concentrated suspension, containing NIST-traceable polystyrene latex standard particles or other suitable microspheres. Gently mix the fluids to achieve a homogeneous suspension. If the light obscuration instrument is equipped with an automatic dilution system, the starting concentrated sample can be analyzed by injection directly into the instrument via a syringe or Teflon sample line. Further dilution of the sample then occurs automatically to optimize the particle concentration for analysis. Alternatively, the sample would require greater manual dilution with water (typically by at least a factor of 10 over the first dilution). The resulting diluted sample is then instilled in an appropriate, clean container, such as a sterile Type I glass container, before being passed through the sensor. In either case the final particle concentration is caused to lie below the coincidence limit of the sensor. The sizing and counting accuracy of the light obscuration instrument should be obtained using two different size standards of approximately 5 and 10 μm (triplicate analyses per size). For the standards after system calibration, set the instrument threshold of detection at 1.8 μm , extended to an upper limit of 50 μm . The corresponding results for the mean diameter should coincide with the expected values, within 10% of the relative standard deviation and 90%–110% size accuracy. In addition, the number of particle counts obtained per mL should also agree within $\pm 10\%$ with the concentration values certified in the documentation provided with each NIST-traceable size standard.

Test Preparation—To a pre-established volume of water add an appropriate volume of sample from the lipid injectable emulsion (triplicate analyses per sample). Gently mix the fluids to achieve a homogeneous suspension. The diluted emulsion will be slightly turbid in appearance. If the light obscuration instrument is equipped with an automatic dilution system, the starting concentrated sample can be analyzed by injection directly into the instrument via a syringe or nonreactive* Teflon sample line. Further dilution then occurs automatically to optimize the droplet/globule concentration for analysis. Alternatively, the sample would require greater manual dilution with water (typically by at least a factor of 10 over the first dilution). The resulting diluted

*Polyvinyl chloride (PVC) with diethylhexylphthalate (DEHP) has been shown to induce breakdown of lipid injectable emulsions (Drug Product Problem Reporting System. USP File Access No. 11173, May 15, 1991).

sample is then instilled in an appropriate, clean container such as a sterile Type I glass container. In either case the final droplet/globule concentration is caused to lie below the coincidence limit of the sensor.

System Suitability—Perform prior to the test procedure, using the *Standard Preparation* of a 5- and 10- μ m NIST-traceable particle. Measure in triplicate the number-weighted particle diameter and the counts/mL of the standard. The system is suitable when the triplicate mean number-weighted particle diameter measurements are within 10% of the target value, both in terms of repeatability (CV) and closeness to the certified size on the label of the NIST-traceable standard.

Procedure and Interpretation—If the light obscuration instrument is equipped with an automatic dilution system, use a disposable syringe or Teflon sample line to load the *Standard Preparation* or *Test Preparation*. If no automatic dilution system is used, transfer the sample to an appropriate large-volume, clean container such as a sterile Type I glass vessel containing an appropriate volume of water. Allow the sample and water to mix thoroughly to achieve a homogeneous suspension. Set the instrument threshold of detection at 1.8 μ m, extended to an upper limit of 50 μ m, and vary the concentration and/or data collection times such that there is at least a factor of two in the difference of the total number of globules that measure >5 μ m between at least two sample runs. In any case, the number of globules that measure >5 μ m should be large enough so that it represents an adequate number of globules that are statistically representative of the large-diameter tail population of the native emulsion. The volume-weighted, large-diameter fat globule limits of the dispersed phase, expressed as the percentage of fat residing in globules larger than 5 μ m (PFAT5) for a given lipid injectable emulsion, must not exceed 0.05%.

(730) PLASMA SPECTROCHEMISTRY

Plasma-based instrumental techniques that are useful for pharmaceutical analyses fall into two major categories: those based on the inductively coupled plasma, and those where a plasma is generated at or near the surface of the sample. An inductively coupled plasma (ICP) is a high-temperature excitation source that desolvates, vaporizes, and atomizes aerosol samples and ionizes the resulting atoms. The excited analyte ions and atoms can then subsequently be detected by observing their emission lines, a method termed inductively coupled plasma-atomic emission spectroscopy (ICP-AES), also known as inductively coupled plasma-optical emission spectroscopy (ICP-OES); or the excited or ground state ions can be determined by a technique known as inductively coupled plasma-mass spectrometry (ICP-MS). ICP-AES and ICP-MS may be used for either single- or multi-element analysis, and they provide good general-purpose procedures for either sequential or simultaneous analyses over an extended linear range with good sensitivity.

An emerging technique in plasma spectrochemistry is laser-induced breakdown spectroscopy (LIBS). In LIBS, a solid, liquid, or gaseous sample is heated directly by a pulsed laser, or indirectly by a plasma generated by the laser. As a result, the sample is volatilized at the laser beam contact point, and the volatilized constituents are reduced to atoms, molecular fragments, and larger clusters in the plasma that forms at or just above the surface of the sample. Emission from the atoms and ions in the sample is collected, typically using fiber optics or a remote viewing system, and meas-

ured using an array detector such as a charge-coupled device (CCD). LIBS can be used for qualitative analysis or against a working standard curve for quantitative analysis. Although LIBS is not currently in wide use by the pharmaceutical industry, it might be suited for at-line or on-line measurements in a production setting as well as in the laboratory. Because of its potential, it should be considered a viable technique for plasma spectrochemistry in the pharmaceutical laboratory. However, because LIBS is still an emerging technique, details will not be further discussed in this general chapter.¹

SAMPLE PREPARATION

Sample preparation is critical to the success of plasma-based analysis and is the first step in performing any analysis via ICP-AES or ICP-MS. Plasma-based techniques are heavily dependent on sample transport into the plasma, and because ICP-AES and ICP-MS share the same sample introduction system, the means by which samples are prepared may be applicable to either technique. The most conventional means by which samples are introduced into the plasma is via solution nebulization. If solution nebulization is employed, solid samples must be dissolved in order to be presented into the plasma for analysis. Samples may be dissolved in any appropriate solvent. There is a strong preference for the use of aqueous or dilute nitric acid solutions, because there are minimal interferences with these solvents compared to other solvent choices. Hydrogen peroxide, hydrochloric acid, sulfuric acid, perchloric acid, combinations of acids, or various concentrations of acids can all be used to dissolve the sample for analysis. Dilute hydrofluoric acid may also be used, but great care must be taken to ensure the safety of the analyst, as well as to protect the quartz sample introduction equipment when using this acid; specifically, the nebulizer, spray chamber, and inner torch tube should be manufactured from hydrofluoric acid-tolerant materials. Additionally, alternative means of dissolving the sample can be employed. These include, but are not limited to, the use of dilute bases, straight or diluted organic solvents, combinations of acids or bases, and combinations of organic solvents.

When samples are introduced into the plasma via solution nebulization, it is important to consider the potential matrix effects and interferences that might arise from the solvent. The use of an appropriate internal standard and/or matching the standard matrix with samples should be applied for ICP-AES and ICP-MS analyses in cases where accuracy and precision are not adequate. In either event, the selection of an appropriate internal standard should consider the analyte in question, ionization energy, wavelengths or masses, and the nature of the sample matrix.

Where a sample is found not to be soluble in any acceptable solvent, a variety of digestion techniques can be employed. These include hot-plate digestion and microwave-assisted digestions, including open-vessel and closed-vessel approaches. The decision regarding the type of digestion technique to use depends on the nature of the sample being digested, as well as on the analytes of interest.

Open-vessel digestion is generally not recommended for the analysis of volatile metals, e.g., selenium and mercury. The suitability of a digestion technique, whether open-vessel or closed-vessel, should be supported by spike recovery experiments in order to verify that, within an acceptable tolerance, volatile metals have not been lost during sample preparation. Use acids, bases, and hydrogen peroxide of ultra-high purity, especially when ICP-MS is employed. Deionized water must be at least 18 megaohm. Check diluents for interferences before they are used in an analysis. Because it is not always possible to obtain organic solvents that are

¹ Yueh F-Y, Singh JP, Zhang H. Laser-induced breakdown spectroscopy, elemental analysis. In: *Encyclopedia of Analytical Chemistry: Instrumentation and Applications*. New York: Wiley; 2000:2066–2087.

free of metals, use organic solvents of the highest quality possible with regard to metal contaminants.

It is important to consider the selection of the type, material of construction, pretreatment, and cleaning of analytical labware used in ICP-AES and ICP-MS analyses. The material must be inert and, depending on the specific application, resistant to caustics, acids, and/or organic solvents. For some analyses, diligence must be exercised to prevent the adsorption of analytes onto the surface of a vessel, particularly in ultra-trace analyses. Contamination of the sample solutions from metal and ions present in the container can also lead to inaccurate results.

The use of labware that is not certified to meet Class A tolerances for volumetric flasks is acceptable if the linearity, accuracy, and precision of the method have been experimentally demonstrated to be suitable for the purpose at hand.

SAMPLE INTRODUCTION

There are two ways to introduce the sample into the nebulizer: by a peristaltic pump and by self-aspiration. The peristaltic pump is preferred and serves to ensure that the flow rate of sample and standard solution to the nebulizer is the same irrespective of sample viscosity. In some cases, where a peristaltic pump is not required, self-aspiration can be used.

A wide variety of nebulizer types is available, including pneumatic (concentric and cross-flow), grid, and ultrasonic nebulizers. Micronebulizers, high-efficiency nebulizers, direct-injection high-efficiency nebulizers, and flow-injection nebulizers are also available. The selection of the nebulizer for a given analysis should consider the sample matrix, analyte, and desired sensitivity. Some nebulizers are better suited for use with viscous solutions or those containing a high concentration of dissolved solids, whereas others are better suited for use with organic solutions.

Note that the self-aspiration of a fluid is due to the Bernoulli, or Venturi, effect. Not all types of nebulizers will support self-aspiration. The use of a concentric nebulizer, for example, is required for self-aspiration of a solution.

Once a sample leaves the nebulizer as an aerosol, it enters the spray chamber, which is designed to permit only the smallest droplets of sample solution into the plasma; as a result, typically only 1% to 2% of the sample aerosol reaches the ICP, although some special-purpose nebulizers have been designed that permit virtually all of the sample aerosol to enter the ICP. As with nebulizers, there is more than one type of spray chamber available for use with ICP-AES or ICP-MS. Examples include the Scott double-pass spray chamber, as well as cyclonic spray chambers of various configurations. The spray chamber must be compatible with the sample and solvent and must equilibrate and wash out in as short a time as possible. When a spray chamber is selected, the nature of the sample matrix, the nebulizer, the desired sensitivity, and the analyte should all be considered.

Gas and liquid chromatography systems can be interfaced with ICP-AES and ICP-MS for molecular speciation, ionic speciation, or other modes of separation chemistry, based on elemental emission or mass spectrometry.

Ultimately, the selection of sample introduction hardware should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

In addition to solution nebulization, it is possible to analyze solid samples directly via laser ablation (LA). In such instances, the sample enters the torch as a solid aerosol. LA-ICP-AES and LA-ICP-MS are better suited for qualitative analyses of pharmaceutical compounds because of the difficulty in obtaining appropriate standards. Nonetheless, quantitative analyses can be performed if it can be demonstrated

through appropriate method validation that the available standards are adequate.²

STANDARD PREPARATION

Single- or multi-element standard solutions, whose concentrations are traceable to primary reference standards, such as those of the National Institute of Standards and Technology (NIST), can be purchased for use in the preparation of working standard solutions. Alternatively, standard solutions of elements can be accurately prepared from standard materials and their concentrations, determined independently, as appropriate. Working standard solutions, especially those used for ultra-trace analyses, may have limited shelf life. As a general rule, working standard solutions should be retained for no more than 24 hours unless stability is demonstrated experimentally. The selection of the standard matrix is of fundamental importance in the preparation of element standard solutions. Spike recovery experiments should be conducted with specific sample matrices in order to determine the accuracy of the method. If sample matrix effects cause excessive inaccuracies, standards, blanks, and sample solutions should be matrix matched, if possible, in order to minimize matrix interferences.

In cases where matrix matching is not possible, an appropriate internal standard or the method of standard additions should be used for ICP-AES or ICP-MS. Internal standards can also be introduced through a T connector into the sample uptake tubing. In any event, the selection of an appropriate internal standard should consider the analytes in question, their ionization and excitation energies, their chemical behavior, their wavelengths or masses, and the nature of the sample matrix. Ultimately, the selection of an internal standard should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

The method of standard additions involves adding a known concentration of the analyte element to the sample at no fewer than two concentration levels plus an unspiked sample preparation. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the x-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

The presence of dissolved carbon at concentrations of a small percentage in aqueous solutions enhances ionization of selenium and arsenic in an inductively coupled argon plasma. This phenomenon frequently results in a positive bias for ICP-AES and ICP-MS selenium and arsenic quantification measurements, which can be remedied by using the method of standard additions or by adding a small percentage of carbon, such as analytically pure glacial acetic acid, to the linearity standards.

ICP

The components that make up the ICP excitation source include the argon gas supply, torch, radio frequency (RF) induction coil, impedance-matching unit, and RF generator. Argon gas is almost universally used in an ICP. The plasma torch consists of three concentric tubes designated as the inner, the intermediate, and the outer tube. The intermediate and outer tubes are almost universally made of quartz. The inner tube can be made of quartz or alumina if the analysis is conducted with solutions containing hydrofluoric acid. The nebulizer gas flow carries the aerosol of the sample solution into and through the inner tube of the torch and into the plasma. The intermediate tube carries the intermediate (sometimes referred to as the auxiliary) gas. The

² For additional information on laser ablation, see Russo R, Mao X, Borisov O, Liu H. Laser ablation in atomic spectrometry. In: *Encyclopedia of Analytical Chemistry: Instrumentation and Applications*. New York: Wiley; 2000.

intermediate gas flow helps to lift the plasma off the inner and intermediate tubes to prevent their melting and the deposition of carbon and salts on the inner tube. The outer tube carries the outer (sometimes referred to as the plasma or coolant) gas, which is used to form and sustain the toroidal plasma. The tangential flow of the coolant gas through the torch constricts the plasma and prevents the ICP from expanding to fill the outer tube, keeping the torch from melting. An RF induction coil, also called the load coil, surrounds the torch and produces an oscillating magnetic field, which in turn sets up an oscillating current in the ions and electrons produced from the argon. The impedance-matching unit serves to efficiently couple the RF energy from the generator to the load coil. The unit can be of either the active or the passive type. An active matching unit adjusts the impedance of the RF power by means of a capacitive network, whereas the passive type adjusts the impedance directly through the generator circuitry. Within the load coil of the RF generator, the energy transfer between the coil and the argon creates a self-sustaining plasma. Collisions of the ions and electrons liberated from the argon ionize and excite the analyte atoms in the high-temperature plasma. The plasma operates at temperatures of 6,000 to 10,000 K, so most covalent bonds and analyte-to-analyte interactions have been eliminated.

ICP-AES

An inductively coupled plasma can use either an optical or a mass spectral detection system. In the former case, ICP-AES, analyte detection is achieved at an emission wavelength of the analyte in question. Because of differences in technology, a wide variety of ICP-AES systems are available, each with different capabilities, as well as different advantages and disadvantages. Simultaneous-detection systems are capable of analyzing multiple elements at the same time, thereby shortening analysis time and improving background detection and correction. Sequential systems move from one wavelength to the next to perform analyses, and often provide a larger number of analytical lines from which to choose. Array detectors, including charge-coupled devices and charge-injection devices, with detectors on a chip, make it possible to combine the advantages of both simultaneous and sequential systems. These types of detection devices are used in the most powerful spectrometers, providing rapid analysis and a wide selection of analytical lines.

The ICP can be viewed in either axial or radial (also called lateral) mode. The torch is usually positioned horizontally in axially viewed plasmas and is viewed end on, whereas it is positioned vertically in radially viewed plasmas and is viewed from the side. Axial viewing of the plasma can provide higher signal-to-noise ratios (better detection limits and precision); however, it also incurs greater matrix and spectral interferences. Methods validated on an instrument with a radial configuration will probably not be completely transferable to an instrument with an axial configuration, and vice versa.

Additionally, dual-view instrument systems are available, making it possible for the analyst to take advantage of either torch configuration. The selection of the optimal torch configuration will depend on the sample matrix, analyte in question, analytical wavelength(s) used, cost of instrumentation, required sensitivity, and type of instrumentation available in a given laboratory.

Regardless of torch configuration or detector technology, ICP-AES is a technique that provides a qualitative and/or quantitative measurement of the optical emission from excited atoms or ions at specific wavelengths. These measurements are then used to determine the analyte concentration in a given sample. Upon excitation, an atom or atomic ion emits an array of different frequencies of light that are characteristic of the distinct energy transition allowed for that element. The intensity of the light is generally proportional to the analyte concentration. It is necessary to correct for

the background emission from the plasma. Sample concentration measurements are usually determined from a working curve of known standards over the concentration range of interest. It is, however, also possible to perform a single-point calibration under certain circumstances, such as with limit tests, if the methodology has been validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness.

Because there are distinct transitions between atomic energy levels, and because the atoms in an ICP are rather dilute, emission lines have narrow bandwidths. However, because the emission spectra from the ICP contain many lines, and because "wings" of these lines overlap to produce a nearly continuous background on top of the continuum that arises from the recombination of argon ions with electrons, a high-resolution spectrometer is required in ICP-AES. The decision regarding which spectral line to measure should include an evaluation of potential spectral interferences. All atoms in a sample are excited simultaneously; however, the presence of multiple elements in some samples can lead to spectral overlap. Spectral interference can also be caused by background emission from the sample or plasma. Modern ICPs usually have background correction available, and a number of background correction techniques can be applied. Simple background correction typically involves measuring the background emission intensity at some point away from the main peak and subtracting this value from the total signal being measured. Mathematical modeling to subtract the interfering signal as a background correction can also be performed with certain types of ICP-AES spectrometers.

The selection of the analytical spectral line is critical to the success of an ICP-AES analysis, regardless of torch configuration or detector type. Though some wavelengths are preferred, the final choice must be made in the context of the sample matrix, the type of instrument being used, and the sensitivity required. Analysts might choose to start with the wavelengths recommended by the manufacturer of their particular instrument and select alternative wavelengths based on manufacturer recommendations or published wavelength tables.^{3,4,5,6,7} Ultimately, the selection of analytical wavelengths should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision of the analysis at hand.

Forward power, gas flow rates, viewing height, and torch position can all be optimized to provide the best signal. However, it must also be kept in mind that these same variables can influence matrix and spectral interferences.

In general, it is desirable to operate the ICP under robust conditions, which can be gauged on the basis of the MgII/MgI line pair at (280.270 nm/285.213 nm). If that ratio of intensities is above 6.0 in an aqueous solution, the ICP is said to be *robust*, and is less susceptible to matrix interferences. A ratio of about 10.0 is generally what is sought. Note that the term *robust conditions* is unrelated to *robustness* as applied to analytical method validation. Operation of an instrument with an MgII/MgI ratio greater than 6.0 is not mandated, but is being suggested as a means of optimizing instrument parameters in many circumstances.

The analysis of the Group I elements can be an exception to this strategy. When atomic ions are formed from elements in this group, they assume a noble gas electron configuration, with correspondingly high excitation energy. Because the first excited state of these ions is extremely high, few are excited, so emission intensity is correspondingly low. This situation can be improved by reducing the frac-

³ Payling R, Larkins P. *Optical Emission Lines of the Elements*. New York: Wiley; 2000.

⁴ Harrison GR. *Massachusetts Institute of Technology Wavelength Tables* [also referred to as *MIT Wavelength Tables*]. Cambridge, MA: MIT Press; 1969.

⁵ Winge RK, Fassel VA, Peterson VJ, Floyd MA. *Inductively Coupled Plasma Atomic Emission Spectroscopy: An Atlas of Spectral Information*. New York: Elsevier; 1985.

⁶ Boumans PWJM. *Spectrochim Acta A*. 1981;36B:169.

⁷ Boumans PWJM. *Line Coincidence Tables for Inductively Coupled Plasma Atomic Emission Spectrometry*. 2nd ed.; Oxford, UK: Pergamon; 1984.

tional ionization, which can in turn be achieved by using lower forward power settings in combination with adjusted viewing height or nebulizer gas flow, or by adding an ionization suppression agent to the samples and standards.

When organic solvents are used, it is often necessary to use a higher forward power setting, higher intermediate and outer gas flows, and a lower nebulizer gas flow than would be employed for aqueous solutions, as well as a reduction in the nebulizer gas flow. When using organic solvents, it may also be necessary to bleed small amounts of oxygen into the torch to prevent carbon buildup in the torch.

Calibration

The wavelength accuracy for ICP–AES detection must comply with the manufacturer's applicable operating procedures. Because of the inherent differences among the types of instruments available, there is no general system suitability procedure that can be employed. Calibration routines recommended by the instrument manufacturer for a given ICP–AES instrument should be followed. These might include, but are not limited to, use of a multi-element wavelength calibration with a reference solution, internal mercury (Hg) wavelength calibration, and peak search. The analyst should perform system checks in accordance with the manufacturer's recommendations.

Standardization

The instrument must be standardized for quantification at time of use. However, because ICP–AES is a technique generally considered to be linear over a range of 6 to 8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a standard curve composed of multiple standards. Once a method has been developed and is in routine use, it is possible to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products if the methodology has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. The use of a single-point standardization is also acceptable for qualitative ICP–AES analyses, where the purpose of the experiment is to confirm the presence or absence of elements without the requirement of an accurate quantification.

An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration, as in the case where the concentration of a known component is being determined within a specified tolerance. However, it is not always possible to employ a bracketing standard when an analysis is performed at or near the detection limit. This lack of use of a bracketing standard is acceptable for analyses conducted to demonstrate the absence or removal of elements below a specified limit. The number and concentrations of standard solutions used should be based on the purpose of the quantification, the analyte in question, the desired sensitivity, and the sample matrix. Regression analysis of the standard plot should be employed to evaluate the linearity of detector response, and individual monographs may set criteria for the residual error of the regression line. Optimally, a correlation coefficient of not less than 0.99, or as indicated in the individual monograph, should be demonstrated for the working curve. Here, too, however, the nature of the sample matrix, the analyte(s), the desired sensitivity, and the type of instrumentation available may dictate a correlation coefficient lower than 0.99. The analyst should use caution when proceeding with such an analysis, and should employ additional working standards.

To demonstrate the stability of the system's initial standardization, a solution used in the initial standard curve must be reassayed as a check standard at appropriate intervals throughout the analysis of the sample set. The reas-

sayed standard should agree with its expected value to within $\pm 10\%$, or as specified in an individual monograph, for single-element analyses when analytical wavelengths are between 200 and 500 nm, or concentrations are $>1 \mu\text{g per mL}$. The reassayed standard should agree with its theoretical value to within $\pm 20\%$, or as specified in an individual monograph, for multi-element analyses, when analytical wavelengths are $<200 \text{ nm}$ or $>500 \text{ nm}$, or at concentrations of $<1 \mu\text{g per mL}$. In cases where an individual monograph provides different guidance regarding the reassayed check standard, the requirements of the monograph take precedence.

Procedure

Follow the procedure as directed in the individual monograph for the instrumental parameters. Because of differences in manufacturers' equipment configurations, the manufacturer's suggested default conditions may be used and modified as needed. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Data collected from a single sample introduction are treated as a single result. This result might be the average of data collected from replicate sequential readings from a single solution introduction of the appropriate standard or sample solution. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions. This calculation is often performed directly by the instrument.

ICP–MS

When an inductively coupled plasma uses a mass spectral detection system, the technique is referred to as inductively coupled plasma–mass spectrometry (ICP–MS). In this technique, analytes are detected directly at their atomic masses. Because these masses must be charged to be detected in ICP–MS, the method relies on the ability of the plasma source to both atomize and ionize sample constituents. As is the case with ICP–AES, a wide variety of ICP–MS instrumentation systems are available.

The systems most commonly in use are quadrupole-based systems. Gaining in interest is time-of-flight ICP–MS. Although still not in widespread use, this approach may see greater use in the future. Additionally, high-resolution sector field instruments are available.

Regardless of instrument design or configuration, ICP–MS provides both a qualitative and a quantitative measurement of the components of the sample. Ions are generated from the analyte atoms by the plasma. The analyte ions are then extracted from the atmospheric-pressure plasma through a sampling cone into a lower-pressure zone, ordinarily held at a pressure near 1 Torr. In this extraction process, the sampled plasma gases, including the analyte species, form a supersonic beam, which dictates many of the properties of the resulting analyte ions. A skimmer cone, located behind the sampling cone, "skims" the supersonic beam of ions as they emerge from the sampling cone. Behind the skimmer cone is a lower-pressure zone, often held near a milliTorr. Lastly, the skimmed ions pass a third-stage orifice to enter a zone held near a microTorr, where they encounter ion optics and are passed into the mass spectrometer. The mass spectrometer separates the ions according to their mass-to-charge (m/z) ratios. The ICP–MS has a mass range up to 240 atomic mass units (amu). Depending on the equipment configuration, analyte adducts can form with diluents, with argon, or with their decomposition products. Also formed are oxides and multiply-charged analyte ions, which can increase the complexity of the resulting mass spectra. Interfer-

ences can be minimized by appropriate optimization of operational parameters, including gas flows (central, intermediate, and outer gas flow rates), sample-solution flow, RF power, extraction-lens voltage, etc., or by the use of collision or reaction cells, or cool plasma operation, if available on a given instrument. Unless a laboratory is generating or examining isotopes that do not naturally occur, a list of naturally occurring isotopes will provide the analyst with acceptable isotopes for analytical purposes. Isotopic patterns also serve as an aid to element identification and confirmation. Additionally, tables of commonly found interferences and polyatomic isobaric interferences and correction factors can be used.

ICP-MS generally offers considerably lower (better) detection limits than ICP-AES, largely because of the extremely low background that it generates. This ability is a major advantage of ICP-MS for determination of very low analyte concentrations or when elimination of matrix interferences is required. In the latter case, some interferences can be avoided simply by additional dilution of the sample solution. In some applications, analytes can be detected below the parts per trillion (ppt) level using ICP-MS. As a general rule, ICP-MS as a technique requires that samples contain significantly less total dissolved solids than does ICP-AES.

The selection of the analytical mass to use is critical to the success of an ICP-MS analysis, regardless of instrument design. Though some masses are often considered to be the primary ones, because of their high natural abundance, an alternative mass for a given element is often used to avoid spectral overlaps (isobaric interferences). Selection of an analytical mass must always be considered in the context of the sample matrix, the type of instrument being used, and the concentrations to be measured. Analysts might choose to start with masses recommended by the manufacturer of their particular instrument and select alternate masses based on manufacturer's recommendations or published tables of naturally occurring isotopes.⁸

Optimization of an ICP-MS method is also highly dependent on the plasma parameters and means of sample introduction. Forward power, gas flow rates, and torch position may all be optimized to provide the best signal. When organic solvents are used, it is often necessary to use a higher forward power setting and a lower nebulizer flow rate than would be used for aqueous solutions. Additionally, when organic solvents are used, it might be necessary to introduce small amounts of oxygen into the central or intermediate gas to prevent carbon buildup in the torch or on the sampler cone orifice. The use of a platinum-tipped sampling or skimmer cone may also be required in order to reduce cone degradation with some organic solvents.

Calibration

The mass spectral accuracy for ICP-MS detection must be in accordance with the applicable operating procedures. Because of the inherent differences between the types of instruments available, there is no general system suitability procedure that can be employed. Analysts should refer to the tests recommended by the instrument manufacturer for a given ICP-MS instrument. These may include, but are not limited to, tuning on a reference mass or masses, peak search, and mass calibration. The analyst should perform system checks recommended by the instrument manufacturer.

Standardization

The instrument must be standardized for quantification at the time of use. Because the response (signal vs. concentration) of ICP-MS is generally considered to be linear over a

range of 6 to 8 orders of magnitude, it is not always necessary to continually demonstrate linearity by the use of a working curve. Once a method has been developed and is in routine use, it is common practice to calibrate with a blank and a single standard. One-point standardizations are suitable for conducting limit tests on production materials and final products, provided that the methodology has been rigorously validated for sufficient specificity, sensitivity, linearity, accuracy, precision, ruggedness, and robustness. An appropriate blank solution and standards that bracket the expected range of the sample concentrations should be assayed and the detector response plotted as a function of analyte concentration. The number and concentration of standard solutions used should be based on the analyte in question, the expected concentrations, and the sample matrix, and should be left to the discretion of the analyst. Optimally, a correlation coefficient of not less than 0.99, or as indicated in the individual monograph, should be demonstrated for the working standard curve. Here, too, however, the nature of the sample matrix, the analyte, the desired sensitivity, and the type of instrumentation available might dictate a correlation coefficient lower than 0.99. The analyst should use caution when proceeding with such an analysis and should employ additional working standards.

To demonstrate the stability of the system since initial standardization, a solution used in the initial standard curve must be reassayed as a check standard at appropriate intervals throughout the analysis of the sample set. Appropriate intervals may be established as occurring after every fifth or tenth sample, or as deemed adequate by the analyst, on the basis of the analysis being performed. The reassayed standard should agree with its expected value to within $\pm 10\%$ for single-element analyses when analytical masses are free of interferences and when concentrations are >1 ng per mL. The reassayed standard should agree with its expected value to within $\pm 20\%$ for multi-element analyses, or when concentrations are <1 ng per mL. In cases where an individual monograph provides different guidance regarding the reassayed check standard, the requirements of the monograph take precedence.

The method of standard additions should be employed in situations where matrix interferences are expected or suspected. This method involves adding a known concentration of the analyte element to the sample solution at no fewer than two concentration levels. The instrument response is plotted against the concentration of the added analyte element, and a linear regression line is drawn through the data points. The absolute value of the x-intercept multiplied by any dilution factor is the concentration of the analyte in the sample.

Procedure

Follow the procedure as directed in the individual monograph for the detection mode and instrument parameters. The specification of definitive parameters in a monograph does not preclude the use of other suitable operating conditions, and adjustments of operating conditions may be necessary. Alternative conditions must be supported by suitable validation data, and the conditions in the monograph will take precedence for official purposes. Because of differences in manufacturers' equipment configurations, the analyst may wish to begin with the manufacturer's suggested default conditions and modify them as needed. Data collected from a single sample introduction are treated as a single result. Data collected from replicate sequential readings from a single introduction of the appropriate standard or sample solutions are averaged as a single result. Sample concentrations are calculated versus the working curve generated by plotting the detector response versus the concentration of the analyte in the standard solutions. With modern instruments, this calculation is often performed by the instrument.

⁸ Horlick G, Montaser A. Analytical characteristics of ICPMS. In: Montaser A, Editor. *Inductively Coupled Plasma Mass Spectrometry*. New York: Wiley-VCH; 1998:516-518.

GLOSSARY

AUXILIARY GAS: See *Intermediate (or Auxiliary) Gas*.

AXIAL VIEWING: A configuration of the plasma for AES in which the plasma is directed toward the spectrometer optical path, also called "end-on viewing."

CENTRAL (OR NEBULIZER) GAS: One of three argon gas flows in an ICP torch. The central gas is used to help create a fine mist of the sample solution when solution nebulization is employed. This fine mist is then directed through the central tube of the torch and into the plasma.

COLLISION CELL: A design feature of some ICP-MS instruments. Collision cells are used to reduce interferences from argon species or polyatomic ions and facilitate the analysis of elements that might be affected by those interferences.

COOL PLASMA: Plasma conditions used for ICP-MS that result in a plasma that is cooler than that normally used for an analysis. This condition is achieved by using a lower forward power setting and higher central-gas flow rate, and is used to help reduce isotopic interferences caused by argon and some polyatomic ions.

COOLANT GAS: See *Outer (or Coolant or Plasma) Gas*.

FORWARD POWER: The number of watts used to ignite and sustain the plasma during an analysis. Forward power requirements may vary, depending on sample matrix and analyte.

INTERMEDIATE (OR AUXILIARY) GAS: Gas used to "lift" the plasma off the surface of the torch, thereby preventing melting of the intermediate tube and the formation of carbon and salt deposits on the inner tube.

INTERNAL STANDARD: An element added to or present in the same concentration in blanks, standards, and samples to act as an intensity reference for the analysis. An internal standard should be used for ICP-AES work and must always be used for quantitative ICP-MS analyses.

LATERAL VIEWING: See *Radial Viewing*.

m: The ion mass of interest.

MULTIPLY-CHARGED IONS: Atoms that, when subjected to the high-ionization temperature of the ICP, can form doubly or triply charged ions (X^{++} , X^{+++} , etc.). When detected by MS, the apparent mass of these ions will be $1/2$ or $1/3$ that of the atomic mass.

NEBULIZER: Used to form a consistent sample aerosol that mixes with the argon gas, which is subsequently sent into the ICP.

OUTER (OR COOLANT OR PLASMA) GAS: The main gas supply for the plasma.

PLASMA GAS: See *Outer (or Coolant or Plasma) Gas*.

RADIAL VIEWING: A configuration of the plasma for AES in which the plasma is viewed orthogonal to the spectrometer optic path. Also called "side-on viewing." See also *Lateral Viewing*.

REACTION CELL: Similar to *Collision Cell*, but operating on a different principle. Designed to reduce or eliminate spectral interferences.

SAMPLING CONE: A metal cone (usually nickel-, aluminum-, or platinum-tipped) with a small opening, through which ionized sample material flows after leaving the plasma.

SEQUENTIAL: A type of detector configuration for AES or MS in which discrete emission lines or isotopic peaks are observed by scanning or hopping across the spectral range by means of a monochromator or scanning mass spectrometer.

SIMULTANEOUS: A type of detector configuration for AES or MS in which all selected emission lines or isotopic peaks are observed at the same time by using a polychromator or simultaneous mass spectrometer, offering increased analysis speed for analyses of multi-element samples.

SKIMMER CONE: A metal cone through which ionized sample flows after leaving the sampling cone and before entering the high-vacuum region of an ICP-MS.

STANDARD ADDITIONS: A method used to determine the actual analyte concentration in a sample when viscosity or matrix effects might cause erroneous results.

TORCH: A series of three concentric tubes, usually manufactured from quartz, in which the ICP is formed.

(731) LOSS ON DRYING

The procedure set forth in this chapter determines the amount of volatile matter of any kind that is driven off under the conditions specified. For substances appearing to contain water as the only volatile constituent, the procedure given in the chapter, *Water Determination* (921), is appropriate, and is specified in the individual monograph.

Unless otherwise directed in the individual monograph, conduct the determination on a 1- to 2-g test specimen. Mix the substance to be tested and, if it is in the form of large particles, reduce the particle size to about 2 mm by quickly crushing before weighing out the test specimen. Tare an appropriate glass-stoppered, shallow weighing bottle that has been dried for about 30 minutes under the same conditions to be employed in the determination and cooled to room temperature in a desiccator. Put the test specimen in the bottle, replace the cover, and accurately weigh the bottle and the contents. By gentle, sidewise shaking, distribute the test specimen as evenly as practicable to a depth of about 5 mm generally, and not more than 10 mm in the case of bulky materials. Place the loaded bottle in the drying chamber, removing the stopper and leaving it also in the chamber. Dry the test specimen at the temperature and for the time specified in the monograph. [NOTE—The temperature specified in the monograph is to be regarded as being within the range of $\pm 2^\circ$ of the stated figure.] When "dry to constant weight" is specified in a monograph, drying shall be continued until two consecutive weighings do not differ by more than 0.50 mg per g of substance taken, the second weighing following an additional hour of drying. Upon opening the chamber, close the bottle promptly, and allow it to come to room temperature in a desiccator before weighing.

If the substance melts at a lower temperature than that specified for the determination of *Loss on Drying*, maintain the bottle with its contents for 1 to 2 hours at a temperature 5° to 10° below the melting temperature, then dry at the specified temperature.

Where capsules are to be tested, use a portion of the mixed contents of not fewer than 4 capsules.

Where tablets are to be tested, use powder from not fewer than 4 tablets.

Where the individual monograph directs that loss on drying be determined by thermogravimetric analysis, a sensitive electrobalance is to be used.

Where drying in vacuum over a desiccant is directed in the individual monograph, a vacuum desiccator or a vacuum drying pistol, or other suitable vacuum drying apparatus, is to be used.

Where drying in a desiccator is specified, exercise particular care to ensure that the desiccant is kept fully effective by frequent replacement.

Where drying in a capillary-stoppered bottle¹ in vacuum is directed in the individual monograph, use a bottle or tube fitted with a stopper having a $225 \pm 25\text{-}\mu\text{m}$ diameter capillary, and maintain the heating chamber at a pressure of 5 mm or less of mercury. At the end of the heating period, admit dry air to the heating chamber, remove the bottle,

¹Available as an "antibiotic moisture content flask" from Kimble-Kontes, 1022 Spruce St., Vineland, NJ 08362-1502.

and with the capillary stopper still in place allow it to cool to room temperature in a desiccator before weighing.

(733) LOSS ON IGNITION

This procedure is provided for the purpose of determining the percentage of test material that is volatilized and driven off under the conditions specified. The procedure, as generally applied, is nondestructive to the substance under test; however, the substance may be converted to another form such as an anhydride.

Perform the test on finely powdered material, and break up lumps, if necessary, with the aid of a mortar and pestle before weighing the specimen. Weigh the specimen to be tested without further treatment, unless a preliminary drying at a lower temperature, or other special pretreatment, is specified in the individual monograph. Unless other equipment is designated in the individual monograph, conduct the ignition in a suitable muffle furnace or oven that is capable of maintaining a temperature within 25° of that required for the test, and use a suitable crucible, complete with cover, previously ignited for 1 hour at the temperature specified for the test, cooled in a desiccator, and accurately weighed.

Unless otherwise directed in the individual monograph, transfer to the tared crucible an accurately weighed quantity, in g, of the substance to be tested, about equal to that calculated by the formula:

$$10/L$$

in which L is the limit (or the mean value of the limits) for *Loss on ignition*, in percentage. Ignite the loaded uncovered crucible, and cover at the temperature ($\pm 25^\circ$) and for the period of time designated in the individual monograph. Ignite for successive 1-hour periods where ignition to constant weight is indicated. Upon completion of each ignition, cover the crucible, and allow it to cool in a desiccator to room temperature before weighing.

(736) MASS SPECTROMETRY

A mass spectrometer produces ions from the substance under investigation, separates them according to their mass-to-charge ratio (m/z), and records the relative abundance of each ionic species present. The instrument consists of three major components (see Figure 1):

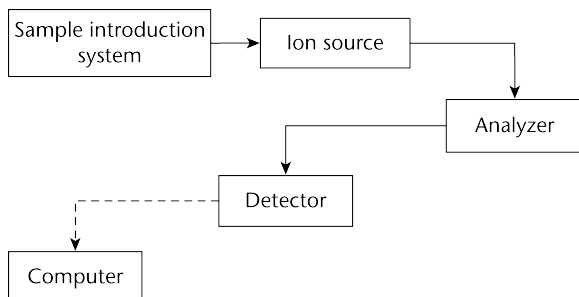


Figure 1. Major components of a mass spectrometer.

an ion source for producing gaseous ions from the substance being studied, an analyzer for resolving the ions into their characteristic mass components according to their mass-to-charge ratios, and a detector system for detecting the ions and recording the relative abundance of each of the resolved ionic species. In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements ($\sim 10^{-6}$ to 10^{-8} mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries.

This chapter gives an overview of the theory, construction, and use of mass spectrometers. The discussion is limited to those instruments and measurements with actual or potential application to compendial and other pharmaceutical requirements: generally, the identification and quantitation of specific compounds.

SAMPLE INTRODUCTION

Samples are introduced either as a gas to be ionized in the ion source, or by ejection of charged molecular species from a solid surface or solution. In some cases sample introduction and ionization take place in a single process, making a distinction between them somewhat artificial.

Substances that are gases or liquids at room temperature and atmospheric pressure can be admitted to the source as a neutral beam via a controllable leak system. Volatilizable compounds dissolved or adsorbed in solids or liquids can be removed and concentrated with a headspace analyzer. Vapors are flushed from the solid or liquid matrix with a stream of carrier gas and trapped on an adsorbing column. The trapped vapors are subsequently desorbed by programmed heating of the trap and introduced into the mass spectrometer by a capillary connection.

For volatilizable solids, the most frequently used method of sample introduction is the direct insertion probe. Here, the sample is placed in a small crucible at the tip of the probe, which is heated under high vacuum in close proximity to the ion source. A variation of this technique involves desorption of samples inside the ionization chamber from a rapidly heated wire or with the aid of a laser beam. Such desorption techniques, in combination with electron, chemical, or field ionization, are preferred for the analysis of heat sensitive or poorly volatile samples.

Sample introduction techniques that involve the ejection of charged molecules from the surface of solid samples include the field desorption method and various sputtering techniques, where the samples are bombarded by high energy photons, by a primary ion beam, or by a neutral particle beam. Similarly, ions can be ejected from solutions either by bombardment with a primary beam, or by one of the various spray techniques described below.

Gas and liquid chromatographs are widely used as sample inlet devices for mass spectrometers. These chromatographs provide for an initial sample purification, since only that portion of the chromatographic effluent containing the compound of interest need be admitted to the mass spectrometer. Gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) combinations are valuable tools for the identification of unknown impurities in drugs. These combination methods have the capacity to separate complex mixtures with the opportunity to obtain structural information on the individual components.

Gas Chromatography/Mass Spectrometry

Gas chromatographic effluents are already in the vapor state and can be admitted directly into the mass spectrometer. Bridging the several orders of magnitude difference in the operating pressures of the two systems was initially ac-

complished with the use of various carrier gas separators. However, with the advent of capillary gas chromatographic columns and high capacity vacuum pumps for mass spectrometers, the gas chromatographic effluents are now fed directly into the ion source.

Liquid Chromatography/Mass Spectrometry

This technique is particularly useful for analyzing materials that cannot be analyzed by GC/MS, either because of thermal instability, high polarity, or high molecular weight. Compounds of biological interest such as drugs and their metabolites, polar endogenous substances, and macromolecules—including peptides, proteins, nucleic acids, and oligosaccharides—often fall into one of these categories.

Currently available LC/MS interfaces encompass a number of approaches to separating the compound of interest from the liquid chromatographic mobile phase and transforming it into an ionized species suitable for mass spectrometry. These include transport devices such as the particle beam; various spray techniques including thermospray, electrospray, and ionspray; and particle-induced desorption such as continuous-flow fast atom bombardment (CF-FAB).

PARTICLE BEAM INTERFACE

The solvent is removed from an aerosol of the liquid chromatographic effluent, and the resulting neutral analyte molecules are introduced into the ion source of the mass spectrometer where they are ionized by electron ionization (EI) or chemical ionization (CI). The resulting spectra are thus classical EI or CI spectra, the former with a wealth of structural information. There are limitations with respect to polarity, thermal lability, and molecular weight, so this technique is best suited for small organic molecules with molecular weights of less than 1000 daltons.

THERMOSPRAY

The compound of interest in a volatile buffer mobile phase, such as ammonium acetate, is passed through heated, narrow bore tubing directly into the ion source of a mass spectrometer. The solution is vaporized in the tubing, and analyte ions desorb into the gas phase and pass into the mass analyzer. Neutral analyte molecules in the gas phase may undergo chemical ionization by reaction with gas phase buffer ions such as NH_4^+ . Thermospray is compatible with relatively high flow rates of 1 to 2 mL per minute, solvents containing a high percentage of water, and many types of polar analytes. Thermal degradation may occur, since the analytes are exposed to relatively high temperatures during the volatilization process.

ELECTROSPRAY

The mobile phase is sprayed through a small opening (needle tip) held at a potential of several kilovolts. The charged droplets so produced are desolvated by passing through a drying gas, and the resulting ions are injected directly into the high vacuum of the analyzer through an orifice or glass capillary. Classical electrospray is limited to flow rates of 1 to 5 μL per minute, and is therefore compatible with either microbore HPLC or post-column stream splitting techniques.

The ions may carry multiple charges, so that the m/z value of high molecular weight substances will fall into the usable range for most quadrupole or magnetic sector mass analyzers ($m/z < 4000$). Analytes of up to 150,000 daltons can thus be successfully analyzed.

IONSpray

A variant of electrospray in which nebulization with a gas flow is used to assist the formation of microdroplets of mobile phase. The technique can extend the upper limit of usable flow rates to 0.1 mL per minute. Volatile buffers must be used with both techniques.

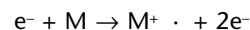
DESORPTION TECHNIQUES

Microflow liquid chromatography can also be interfaced to particle induced desorption techniques such as fast atom bombardment (FAB) and liquid secondary ion mass spectroscopy (LSIMS), described in the following section on ionization techniques. Typically, column effluent flowing at a rate of 1 to 10 μL per minute is mixed with a small percentage of nonvolatile liquid such as glycerol. The mixture is introduced via a capillary inlet onto a target within the ion source where it is bombarded with high energy (5 to 20 keV) atoms or ions. The resulting spectra are similar to FAB or LSIMS spectra but with the background from the sample matrix greatly reduced. Frit-FAB is a variant of continuous flow FAB where the sample is introduced through a porous frit target.

IONIZATION TECHNIQUES

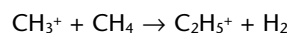
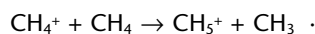
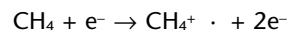
Electron Impact

Molecules of the sample under analysis enter the ionization chamber in the vapor state. Positive ions are produced by passing a beam of electrons, obtained from tungsten or rhenium filaments, through the vapor, which is maintained at a pressure of 10^{-4} to 10^{-6} mm of mercury. Provided the energy of the electron beam is greater than the ionization potential of the sample, the sample is ionized and/or fragmented, as represented by the following equation:

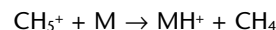


Chemical Ionization (CI)

In this process, a reagent gas at a pressure between 0.1 to 10 mm of mercury is admitted to the source and ionized by a high energy electron beam or discharge. At these pressures, ion-molecule reactions occur and the primary reagent gas ions react further. The most commonly used reagent gases are methane, isobutane, and ammonia. Typical reactions for methane are shown in the following equations:



The CH_5^+ species is a strong Bronsted acid and readily transfers a proton to most organic compounds

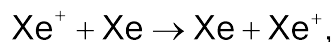
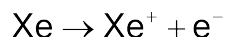


In the case of methane, the protonated ion (MH^+) initially formed may be sufficiently energetic to dissociate further.

Fast Atom Bombardment (FAB)

The sample is ionized by bombardment with a beam of high speed xenon atoms, produced by exchange with

highly accelerated xenon ions in a collision cell. The process is summarized as follows:



where the subscript arrows indicate the fast-moving particles.

FAB is a surface analysis technique, and care must be taken during sample preparation to optimize the condition of the surface. When the sample is coated on a probe by evaporation of a solution, the sample ion beam obtained is often transitory. Molecular adducts with alkali metals, such as (M + Na) and (M + K), favor ion formation. This phenomenon is used to assist in the ionization of biological molecules. Thus, treatment of the sample surface with sodium chloride solution may enhance the yield of adduct ions. Heating the sample during analysis may also increase the ion yield.

The declining yield of sample ions during analysis is probably due to destruction of the sample surface. The surface can, in effect, be continuously replaced by dissolving the sample in a suitable nonvolatile liquid and by coating the mixture onto the top of the probe. Using this approach, the lifetime of samples in the source has been extended to more than 1 hour and the range of compounds amenable to FAB analysis expanded dramatically. The long sample lifetimes and higher sensitivities so achieved make FAB an important mass spectral technique for biochemical analysis, providing the elemental formula of the sample through accurate mass determination. A further advantage of FAB, unlike CI, is the presence of fragment ions within the spectra, which aid in structural elucidation.

Recently, neutral atom bombardment has been replaced by cesium ion bombardment. Although this technique is still referred to as FAB, it is more correctly described as liquid secondary ion mass spectrometry (LSIMS).

Negative and positive ions are formed in the various ionization processes described above, and both are readily analyzed by modern mass spectrometers. Samples with a high electron capture cross section, notably those containing halide atoms, yield an abundance of negative ions. For this reason, halide derivatives of compounds to be studied are often prepared. Negative ion mass spectrometry has been successfully applied to the analysis of pesticide residues, since the structures of these compounds are well suited to the technique.

ANALYZERS

Mass analyzers separate the charged species in the ionized sample according to their m/z ratios, thus permitting the mass and abundance of each species to be determined. Four commonly used analyzers are the magnetic sector, the quadrupole, the time-of-flight, and the Fourier transform analyzers.

Magnetic Sector Analyzers

Ions generated in the ion source are collimated into a beam through the focusing action of a magnetic field and a slit assembly. After exiting the source, ions are subjected to a magnetic field perpendicular to the direction of the beam. Each ion experiences a force at right angles to both its direction of travel and the direction of the magnetic field,

thereby deflecting the beam. The motion of each ion is given by

$$m/z = H^2 r^2 / 2V$$

where m is the mass in atomic mass units, z is the number of electronic charges, H is the magnetic field strength in gauss, r is the radius of the ion trajectory in centimeters, and V is the accelerating voltage. The mass spectrum is scanned by varying the strength of the magnetic field and detecting those ions passing through an exit slit as they come into "focus." The magnetic sector mass spectrometer affords spatial resolution of ions, giving a unique trajectory at a given field strength for each value of m/z .

Quadrupole Analyzers

The instrument is based on four parallel rods in a square array. The ion beam is focused down the axis of the array and an electrical potential of fixed (DC) and radio frequency (RF) components is applied to diagonally opposed rods. For a given combination of DC and RF components, ions of one specific m/z ratio have a stable path down the axis. All others are deflected to the sides and lost. Mass scanning is achieved by changing the DC and RF components of the voltage, while maintaining a fixed ratio. The quadrupole analyzer is a mass filter because it separates ions on the basis of their m/z ratio.

Ion-Trap Analyzer

This quadrupole-type device is composed of a ring electrode placed between two end cap electrodes. Depending upon the commercial version employed, the end caps are either held at ground potential or have an RF voltage applied to them, while an RF voltage is placed on the ring electrode. As a result of these potentials, the hyperbolic surfaces of the three elements form a three-dimensional quadrupole analyzer.

Both ionization and mass analysis take place within the three-dimensional quadrupole field. In the ionization step, the RF voltage on the ring electrode is set low enough so that the ions within the mass range of interest are trapped within the device. Following ionization, mass analysis is accomplished through use of the "mass selective instability" mode of operation. That is, by raising the RF voltage on the ring electrode, ions of successively higher mass are ejected from the ion trap into an electron multiplier detector. In its most common application, the ion-trap analyzer is used in conjunction with a gas chromatograph and covers the mass range of 10 to 560 daltons. However, recent advances in ion-trap technology have extended the workable mass range to many thousands of daltons.

Time-of-Flight Analyzers

Ion separation is based on the principle that ions of different masses, possessing equal kinetic energy, have different velocities. If there is a fixed distance for the ions to travel, the time of travel will vary with their mass, the lighter ions traveling faster and reaching the detector in a shorter period of time. The time-of-flight is given by

$$t_f = k \sqrt{m/z}$$

where t_f is the time-of-flight in seconds. Thus, the time-of-flight of the various ions is simply proportional to the square root of the mass-to-charge ratio of the ions. To measure the time-of-flight, ions are introduced into the mass spectrometer in discrete packets so that a starting point for the timing process can be established. Ion packets are generated either through a pulsed ionization process or through a gating sys-

tem in which ions are produced continuously, but are introduced only at given times into the flight tube.

Fourier Transform Analyzers

In a magnetic field of flux density B , ions move in circular orbits. The angular frequency, ω , of the orbital motion is given by

$$\omega = (z/m)B$$

In this type of mass spectrometer, the orbits are varied by subjecting the ions to a resonant alternating electric field. When the frequency of the alternating field matches the orbital frequency, the ions are steadily accelerated to larger and larger orbits in coherent motion, developing a high level of kinetic energy. After the alternating electric field is turned off, the orbiting ions give rise to an alternating image current on the electrodes. A frequency analysis of this signal yields the mass of the ions involved. Thus, the Fourier transform of the time domain transient signal yields the corresponding frequency spectrum from which the mass spectrum is computed. This is a high resolution technique, yielding m/z ratios accurate to about one thousandth of a dalton.

TANDEM MASS SPECTROMETRY

Two mass spectrometers connected in series (MS/MS), tandem mass spectrometry, refers to the use of two or more sequential mass analysis steps. In its simplest form MS/MS (Figure 2)

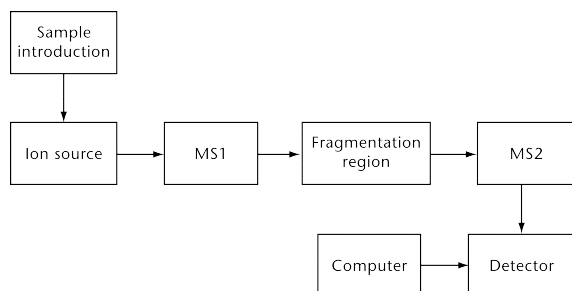


Figure 2. Tandem mass spectrometry.

consists of two mass spectrometers linked in such a way that ions preselected by the first mass analyzer (MS1) are chemically or energetically modified and the results analyzed by the second mass analyzer (MS2).

The basic concept of MS/MS involves the ability to determine the mass relationship between a precursor ion in MS1 and a product ion in MS2. Different mass relationships can be probed depending on how MS1 and MS2 are scanned. These include fragmentation of a precursor and measurement of all its fragments (a product scan), selection of multiple precursors and testing for a common fragment (a precursor scan), or scanning to see if a number of precursors all lose the same neutral species (a constant neutral loss scan).

Fragmentation of the precursor ion can be induced by momentum transfer through collision with gas molecules and/or solid surfaces or by electronic excitation using lasers. These techniques are known as collision-induced dissociation, surface-induced dissociation, or laser-induced dissociation, respectively. Allowing the ion to fragment without additional activation is known as metastable decomposition.

There are many applications of MS/MS to pharmaceutical problems. Product scans can be used to obtain qualitative information from precursor ions of drug substances, impurities, and contaminants. This can aid in the identification of unknowns. The method can also be used to determine the amino acid sequence of peptides and protein fragments.

MS/MS has advantages for mixture analysis. Even when the mass spectrometer is coupled to a separation device such as a liquid or gas chromatograph, the resulting signals may be a result of overlapping or unresolved components. MS/MS can be employed to select the precursor ion from one component and obtain structural information without interference from the others.

Selected reaction monitoring is used to reduce the interference encountered during quantitative analysis for low levels of drugs in biological matrices, as in pharmacokinetic studies. If analysis is for a drug specific ion, interfering signals from other compounds in the matrix can mask the desired signal. Interference is reduced if a drug-specific fragment is selected with MS1 and a structure-specific fragment with MS2. The odds of another molecule producing the same mass relationship are diminishingly small.

MS/MS can also be used in metabolism studies to search for molecules with common structural features such as metabolites related to the drug substance. All of the metabolites might contain the same functional group that is lost as a neutral fragment. In this case a constant-neutral-loss scan will show all of these species. For instance, carboxylic acids will all lose neutral carbon dioxide. If the common functionality is lost as an ionic fragment, then a precursor scan will show all of the molecules that produce that fragment ion.

DATA ANALYSIS AND INTERPRETATION

The mass spectral experiment yields information on the molecular weight of ions derived from the sample and the relative abundance of each of these ions. Spectra are often complex, and not all of the ions may be separated by the mass spectrometer. The ability of the instrument to separate ions is called the resolving power, commonly described by the "10% valley" definition. This states that the resolving power is the highest mass number at which two peaks differing by one molecular weight unit and of equal height have a valley between them that is equal to 10% of the peak height. For low, medium, and high resolution mass spectrometers, this value is between 100 and 2000, 2000 and 10,000, and greater than 10,000, respectively.

If one electron is removed or added to a neutral molecule, a molecular ion of essentially the same molecular weight as the parent molecule results. It is often possible to determine the mass of this ion with sufficient precision to enable the empirical formula of the compound to be calculated. Molecular masses may be determined accurately by using high resolution instruments or by peak-matching measurements using reference compounds.

Fragment ions are those produced from the molecular ion by various bond cleavage processes. Numerous papers in the literature relate bond cleavage patterns (fragmentation patterns) to molecular structure.

In addition to measurement of the mass of a molecular ion and its associated fragment ions, mass spectrometers are also used to quantitate compounds with a high degree of selectivity, precision, and accuracy. Compounds are introduced into the mass spectrometer either via direct insertion probe, gas inlet, or, as is more common, via gas or liquid chromatographic interfaces, which provide sample purification. Ionization may be by EI, CI, FAB, thermospray, or electrospray and mass separation by magnetic sector, quadrupole, or quadrupole ion-trap mass spectrometers. Quantitative mass spectrometry involves measuring the abundance of a specific ion, or set of ions, and relating the response to a known standard. External or internal standards may be used, but the latter are preferred for greater accuracy.

For mass spectrometry, internal standards may be either structural or stable isotope analogs. The former have the advantage of lower cost and availability while precision and accuracy are typically achieved by use of a stable isotope (^2H , ^{13}C , ^{15}N) labeled analog of the analyte. The only requirements for labeling the analyte are that the ion moni-

tored for the internal standard must retain an isotopic label after ionization and the label must not be exchangeable under the sampling, separation, or ionization conditions. Stable isotope internal standards are often required for acceptable quantitation, particularly with FAB and LC/MS techniques such as thermospray and electrospray.

Relative abundances of the analyte and internal standard ions are typically determined by selected ion monitoring, by which only specific ions due to the analyte and the internal standard are monitored. This technique has the advantage over scanning the full mass range in that more time is spent integrating the ion current at a selected mass-to-charge ratio, thereby increasing sensitivity. Chromatographic peak area or amount of analyte in a sample is calculated from the ratio of analyte to internal standard peak area (or height) and the regression parameters as determined by a calibration curve, using standard techniques.

(741) MELTING RANGE OR TEMPERATURE

For Pharmacopeial purposes, the melting range, melting temperature, or melting point is defined as those points of temperature within which, or the point at which, the first detectable liquid phase is detected to the temperature at which no solid phase is apparent, except as defined otherwise for *Classes II* and *III* below. A melting transition may be instantaneous for a highly pure material, but usually a range is observed from the beginning to the end of the process. Factors influencing this transition include the sample size, the particle size, the efficiency of heat diffusion, and the heating rate, among other variables, that are controlled by procedure instructions. In some articles, the melting process is accompanied by simultaneous decomposition, which is visually evidenced as a side event like darkening of the material, charring, bubbling, or other incident. The visual impact of this side reaction frequently obscures the end of the melting process, which it may be impossible to accurately determine. In those circumstances, only the beginning of the melting can be accurately established; and it is to be reported as the melting temperature. The accuracy of the apparatus to be used as described below should be checked at suitable intervals by the use of one or more of the available USP Melting Point Reference Standards, preferably those that melt nearest the melting temperatures of the compounds being tested (see *USP Reference Standards* (11)).

Eight procedures for the determination of melting range or temperature are given herein, varying in accordance with the nature of the substance. When no class is designated in the monograph, use the procedure for *Class Ia* for crystalline or amorphous substances and the procedure for *Class II* for waxy substances.

The procedure known as the mixed-melting point determination, whereby the melting range or temperature of a solid under test is compared with that of an intimate mixture of equal parts of the solid and an authentic specimen of it, e.g., the corresponding USP Reference Standard, if available, may be used as a confirmatory identification test. Agreement of the observations on the original and the mixture constitutes reliable evidence of chemical identity.

Apparatus I—An example of a suitable melting range *Apparatus I* consists of a glass container for a bath of transparent fluid, a suitable stirring device, an accurate thermometer (see *Thermometers* (21)),* and a controlled source of heat.

* ASTM Method E77 deals with "Verification and Calibration of Liquid-in-glass Thermometers."

The bath fluid is selected with a view to the temperature required, but light paraffin is used generally and certain liquid silicones are well adapted to the higher temperature ranges. The fluid is deep enough to permit immersion of the thermometer to its specified immersion depth so that the bulb is still about 2 cm above the bottom of the bath. The heat may be supplied by an open flame or electrically. The capillary tube is about 10 cm long and 0.8 to 1.2 mm in internal diameter with walls 0.2 to 0.3 mm in thickness.

Apparatus II—An instrument may be used in the procedures for *Classes I, Ia*, and *Ib*. An example of a suitable melting range *Apparatus II* consists of a block of metal that may be heated at a controlled rate, its temperature being monitored by a sensor. The block accommodates the capillary tube containing the test substance and permits monitoring of the melting process, typically by means of a beam of light and a detector. The detector signal may be processed by a microcomputer to determine and display the melting point or range, or the detector signal may be plotted to allow visual estimation of the melting point or range.

Procedure for Class I, Apparatus I—Reduce the substance under test to a very fine powder, and, unless otherwise directed, render it anhydrous when it contains water of hydration by drying it at the temperature specified in the monograph, or, when the substance contains no water of hydration, dry it over a suitable desiccant for not less than 16 hours.

Charge a capillary glass tube, one end of which is sealed, with a sufficient amount of the dry powder to form a column in the bottom of the tube 2.5 to 3.5 mm high when packed down as closely as possible by moderate tapping on a solid surface.

Heat the bath until the temperature is about 30° below the expected melting point. Remove the thermometer, and quickly attach the capillary tube to the thermometer by wetting both with a drop of the liquid of the bath or otherwise, and adjust its height so that the material in the capillary is level with the thermometer bulb. Replace the thermometer, and continue the heating, with constant stirring, sufficiently to cause the temperature to rise at a rate of about 3° per minute. When the temperature is about 3° below the lower limit of the expected melting range, reduce the heating so that the temperature rises at a rate of about 1° to 2° per minute. Continue heating until melting is complete.

The temperature at which the column of the substance under test is observed to collapse definitely against the side of the tube at any point indicates the beginning of melting, and the temperature at which the test substance becomes liquid throughout corresponds to the end of melting or the melting point. The two temperatures fall within the limits of the melting range. If melting occurs with decomposition, the melting temperature corresponding to the beginning of the melting is within the range specified.

Procedure for Class Ia, Apparatus I—Prepare the test substance and charge the capillary as directed for *Class I, Apparatus I*. Heat the bath until the temperature is about 10° below the expected melting point and is rising at a rate of 1 ± 0.5° per minute. Insert the capillary as directed under *Class I, Apparatus I* when the temperature is about 5° below the lower limit of the expected melting range, and continue heating until melting is complete. Record the melting range as directed for *Class I, Apparatus I*.

Procedure for Class Ib, Apparatus I—Place the test substance in a closed container and cool to 10°, or lower, for at least 2 hours. Without previous powdering, charge the cooled material into the capillary tube as directed for *Class I, Apparatus I*, then immediately place the charged tube in a vacuum desiccator and dry at a pressure not exceeding 20 mm of mercury for 3 hours. Immediately upon removal from the desiccator, fire-seal the open end of the tube, and as soon as practicable proceed with the determination of the melting range as follows: Heat the bath until a temperature 10 ± 1° below the expected melting range is reached,

then introduce the charged tube, and heat at a rate of rise of $3 \pm 0.5^\circ$ per minute until melting is complete. Record the melting range as directed for *Class I, Apparatus I*.

If the particle size of the material is too large for the capillary, precool the test substance as directed above, then with as little pressure as possible gently crush the particles to fit the capillary, and immediately charge the tube.

Procedure for Class I, Apparatus II—Prepare the substance under test and charge the capillary tube as directed for *Class I, Apparatus I*. Operate the apparatus according to the manufacturer's instructions. Heat the block until the temperature is about 30° below the expected melting point. Insert the capillary tube into the heating block, and continue heating at a rate of temperature increase of about 1° to 2° per minute until melting is complete.

The temperature at which the detector signal first leaves its initial value indicates the beginning of melting, and the temperature at which the detector signal reaches its final value corresponds to the end of melting, or the melting point. The two temperatures fall within the limits of the melting range. If melting occurs with decomposition, the melting temperature corresponding to the beginning of the melting is within the range specified. In the event of dispute, only the melting range or temperature obtained as directed for *Class I, Apparatus I*, is definitive.

Procedure for Class Ia, Apparatus II—Prepare the test substance and charge the capillary as directed for *Class I, Apparatus I*. Operate the apparatus according to the manufacturer's instructions. Heat the block until the temperature is about 10° below the expected melting point and is rising at a rate of $1 \pm 0.5^\circ$ per minute. Insert the capillary as directed under *Class I, Apparatus I* when the temperature is about 5° below the lower limit of the expected melting range, and continue heating until melting is complete. Record the melting range as directed for *Class I, Apparatus I*. If melting occurs with decomposition, the melting temperature corresponding to the beginning of the melting is within the range specified. In the event of dispute, only the melting range or temperature obtained as directed for *Class Ia, Apparatus I*, is definitive.

Procedure for Class Ib, Apparatus II—Place the test substance in a closed container and cool to 10° , or lower, for at least 2 hours. Without previous powdering, charge the cooled material into the capillary tube as directed for *Class I, Apparatus I*, then immediately place the charged tube in a vacuum desiccator, and dry at a pressure not exceeding 20 mm of mercury for 3 hours. Immediately upon removal from the desiccator, fire-seal the open end of the tube, and as soon as practicable proceed with the determination of the melting range as follows: operate the apparatus according to the manufacturer's instructions. Heat the block until the temperature is about $10 \pm 1^\circ$ below the expected melting range, then introduce the charged tube, and heat at a rate of rise of $3 \pm 0.5^\circ$ per minute until melting is complete. Record the melting range as directed for *Class I, Apparatus I*.

If the particle size of the material is too large for the capillary, precool the test substance as directed above, then with as little pressure as possible gently crush the particles to fit the capillary, and immediately charge the tube. In the event of dispute, only the melting range or temperature obtained as directed for *Class Ib, Apparatus I*, is definitive.

Procedure for Class II—Carefully melt the material to be tested at as low a temperature as possible, and draw it into a capillary tube, which is left open at both ends, to a depth of about 10 mm. Cool the charged tube at 10° , or lower, for 24 hours, or in contact with ice for at least 2 hours. Then attach the tube to the thermometer by suitable means, adjust it in a water bath so that the upper edge of the material is 10 mm below the water level, and heat as directed for *Class I, Apparatus I* except, within 5° of the expected melt-

ing temperature, to regulate the rate of rise of temperature to 0.5° to 1.0° per minute. The temperature at which the material is observed to rise in the capillary tube is the melting temperature.

Procedure for Class III—Melt a quantity of the test substance slowly, while stirring, until it reaches a temperature of 90° to 92° . Remove the source of the heat, and allow the molten substance to cool to a temperature of 8° to 10° above the expected melting point. Chill the bulb of a suitable thermometer (see *Thermometers* (21)) to 5° , wipe it dry, and while it is still cold dip it into the molten substance so that approximately the lower half of the bulb is submerged. Withdraw it immediately, and hold it vertically away from the heat until the wax surface dulls, then dip it for 5 minutes into a water bath having a temperature not higher than 16° .

Fix the thermometer securely in a test tube so that the lower point is 15 mm above the bottom of the test tube. Suspend the test tube in a water bath adjusted to about 16° , and raise the temperature of the bath at the rate of 2° per minute to 30° , then change to a rate of 1° per minute, and note the temperature at which the first drop of melted substance leaves the thermometer. Repeat the determination twice on a freshly melted portion of the test substance. If the variation of three determinations is less than 1° , take the average of the three as the melting point. If the variation of three determinations is 1° or greater than 1° , make two additional determinations and take the average of the five.

(751) METAL PARTICLES IN OPHTHALMIC OINTMENTS

The following test is designed to limit to a level considered to be unobjectionable the number and size of discrete metal particles that may occur in ophthalmic ointments.

Procedure—Extrude, as completely as practicable, the contents of 10 tubes individually into separate, clear, flat-bottom, 60-mm Petri dishes that are free from scratches. Cover the dishes, and heat at 85° for 2 hours, increasing the temperature slightly if necessary to ensure that a fully fluid state is obtained. Taking precautions against disturbing the melted sample, allow each to cool to room temperature and to solidify.

Remove the covers, and invert each Petri dish on the stage of a suitable microscope adjusted to furnish 30 times magnification and equipped with an eye-piece micrometer disk that has been calibrated at the magnification being used. In addition to the usual source of light, direct an illuminator from above the ointment at a 45° angle. Examine the entire bottom of the Petri dish for metal particles. Varying the intensity of the illuminator from above allows such metal particles to be recognized by their characteristic reflection of light.

Count the number of metal particles that are $50 \mu\text{m}$ or larger in any dimension: the requirements are met if the total number of such particles in all 10 tubes does not exceed 50, and if not more than 1 tube is found to contain more than 8 such particles. If these results are not obtained, repeat the test on 20 additional tubes: the requirements are met if the total number of metal particles that are $50 \mu\text{m}$ or larger in any dimension does not exceed 150 in all 30 tubes.

tested, and if not more than 3 of the tubes are found to contain more than 8 such particles each.

<755> MINIMUM FILL

The following tests and specifications apply to articles such as creams, gels, jellies, lotions, ointments, pastes, powders, and aerosols, including pressurized and nonpressurized topical sprays that are packaged in containers in which the labeled content is not more than 150 g or 150 mL.

PROCEDURE FOR DOSAGE FORMS OTHER THAN AEROSOLS—For containers labeled by weight, select a sample of 10 filled containers, and remove any labeling that might be altered in weight during the removal of the container contents. Thoroughly cleanse and dry the outside of the containers by a suitable means, and weigh individually. Quantitatively remove the contents from each container, cutting the latter open and washing with a suitable solvent, if necessary, taking care to retain the closure and other parts of each container. Dry, and again weigh each empty container together with its corresponding parts. The difference between the two weights is the net weight of the contents of the container. For containers labeled by volume, pour the contents of 10 containers into 10 suitable graduated cylinders, and allow to drain completely. Record the volume of the contents of each of the 10 containers. The average net content of the 10 containers is not less than the labeled amount, and the net content of any single container is not less than 90% of the labeled amount where the labeled amount is 60 g or 60 mL or less, or not less than 95% of the labeled amount where the labeled amount is more than 60 g or 60 mL but not more than 150 g or 150 mL. If this requirement is not met, determine the content of 20 additional containers. The average content of the 30 containers is not less than the labeled amount, and the net content of not more than 1 of the 30 containers is less than 90% of the labeled amount where the labeled amount is 60 g or 60 mL or less, or less than 95% of the labeled amount where the labeled amount is more than 60 g or 60 mL but not more than 150 g or 150 mL.

PROCEDURE FOR AEROSOLS—Select a sample of 10 filled containers, and remove any labeling that might be altered in weight during the removal of the container contents. Thoroughly cleanse and dry the outsides of the containers by suitable means, and weigh individually. Remove the contents from each container by employing any safe technique (e.g., chill to reduce the internal pressure, remove the valve, and pour). Remove any residual contents with suitable solvents, then rinse with a few portions of methanol. Retain as a unit the container, the valve, and all associated parts, and heat them at 100° for 5 minutes. Cool, and again weigh each of the containers together with its corresponding parts. The difference between the original weight and the weight of the empty aerosol container is the net fill weight. Determine the net fill weight for each container tested. The requirements are met if the net weight of the contents of each of the 10 containers is not less than the labeled amount.

Change to read:

<761> ▲ NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is an analytical method based on the magnetic properties of certain atomic nuclei. As is the case with other types of spectroscopy, absorption or emission of electromagnetic energy at characteristic frequencies provides structural information. NMR differs from other types of spectroscopy because the discrete energy levels between which the transitions take place are present only when the nuclei are placed in a magnetic field.

Although widely recognized as one of the most powerful structure-elucidation tools available, with proper experimental design, it can also be used for accurate qualitative and quantitative measurements. See general information chapter *Applications of Nuclear Magnetic Resonance Spectroscopy* <1761>. [NOTE—Above 1000 chapters are for informational purposes only.]

QUALIFICATION OF NMR INSTRUMENTS

Qualification of an NMR instrument can be divided into three elements: Installation Qualification (IQ), Operational Qualification (OQ), and Performance Qualification (PQ). For further discussion, see general information chapter *Analytical Instrument Qualification* <1058>.

Installation Qualification

The IQ requirements provide evidence that the hardware and software are installed to accommodate safe and effective use of the instrument at the desired location.

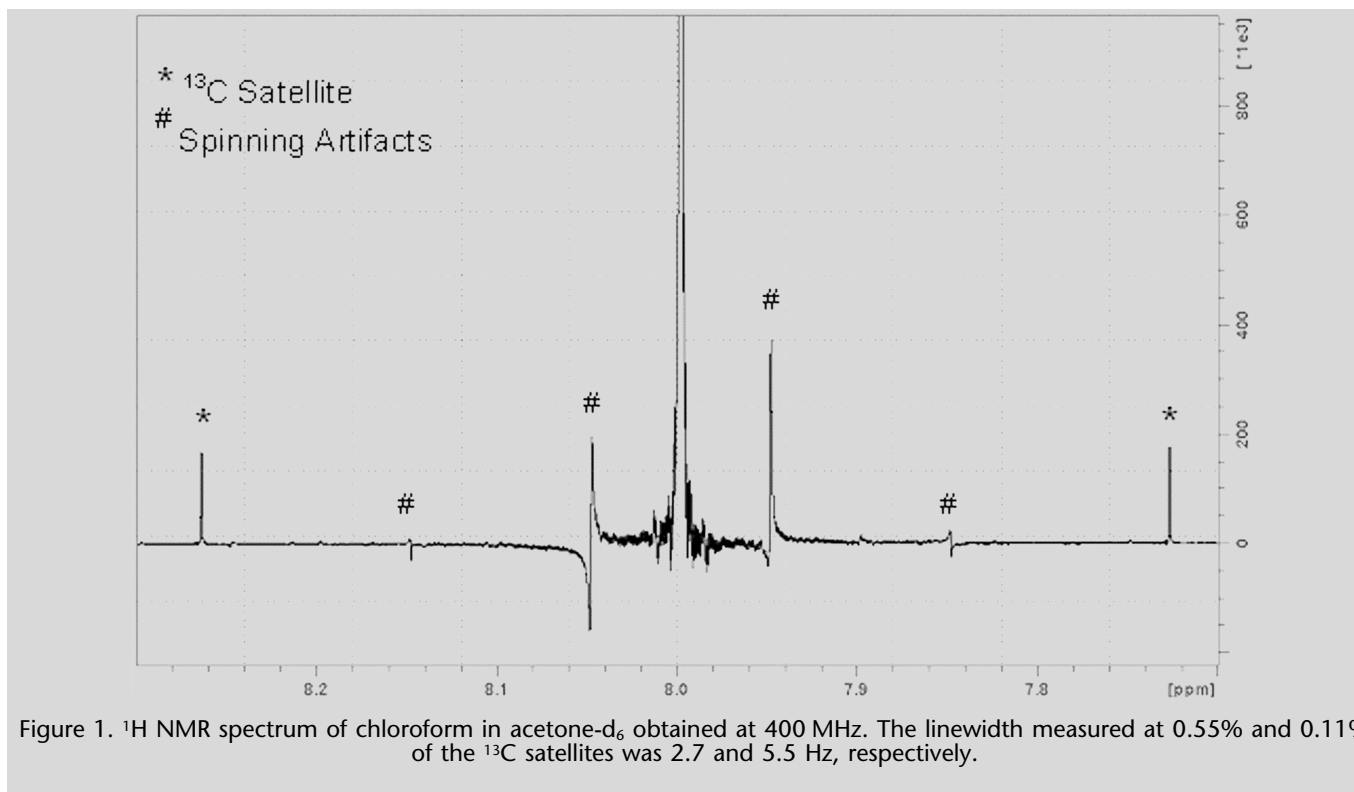
Operational Qualification

In OQ, an instrument's performance is characterized using standards to verify that the system operates within target specifications. The purpose of OQ is to demonstrate that instrument performance is suitable for a given application. Because so many different approaches are available for measuring NMR spectra, OQ using standards with known spectral properties is recommended. Generally, sealed NMR tubes are available as reference standards for measuring signal-to-noise (S/N) and lineshape.

Performance Qualification

PQ helps to determine that the instrument is capable of meeting the user's requirements for all critical-to-quality (CTQ) measures. PQ documentation should describe the following:

1. The definition of the specific performance criteria and detailed test procedures including test samples and instrument parameters.
2. The elements that will be measured to evaluate the criteria and the predefined specifications.
3. The test interval, which may be time-of-use.
4. The use of bracketing samples or groups of samples.
5. The defined corrective actions that will be implemented if the spectrometer does not pass the specifications.



Periodic PQ should include a subset of the OQ tests to ensure that those aspects of the instrument that are being supplied are performing at a level that produces data that are suitable for its intended use. Depending on typical use, the specifications for PQ may be higher or lower than the manufacturer's installation specifications. Typical CTQs include S/N ratio and resolution tests for all nuclei of interest. Method-specific PQ tests, also known as system suitability tests, may be used in lieu of PQ requirements for validated procedures.

The PQ samples and tests in the following subsections are typical examples only. Other tests and samples may be used to establish specifications for specific purposes. Instrument vendors often provide samples and test parameters that can be used as part of the PQ package.

RESOLUTION AND LINESHAPE MEASUREMENT— ^1H NMR (see Figure 1)

Sample: 1% chloroform in acetone- d_6 (≥ 500 MHz), 3% chloroform in acetone- d_6 , degassed and sealed

Spectral width: < 1 KHz

Data acquisition time: NLT 10 s

Tip angle: 90°

Relaxation time: 60 s

Spinning rate: Static or 20 Hz

Pulse sequence: Delay-pulse-acquire with no decoupling

Processing: No line broadening, zero-filling to 128 k
Shim the magnet with special attention to the off-axis shims, acquire a single acquisition, phase to pure absorption, and measure the linewidth at 50%, 0.55%, and 0.11% maximum intensity. The linewidth should pass specifications at these positions, and, in addition, the lineshape should be Lorentzian. On modern NMR spectrometers, the lineshape is frequently obtained on a nonspinning sample because the off-axis shims can be set so well that there is essentially no difference between spectra obtained spinning and nonspinning.

In addition, two-dimensional spectra should be obtained on a static sample.

S/N MEASUREMENTS— ^1H NMR (see Figure 2)

Sample: 0.1% ethylbenzene in chloroform- d , 1% ethylbenzene in chloroform- d (< 200 MHz) degassed and sealed

Spectral width: 10 ppm

Data acquisition time: 400 ms

Tip angle: 90°

Relaxation delay: 60 s

Spinning rate: 0 or approximately 20 Hz

Pulse sequence: Delay-pulse-acquire with no decoupling

Processing: Exponential with 1-Hz line broadening

Referencing: Tetramethylsilane (TMS) = 0.0 ppm or the center of the quartet = 2.65 ppm

The concentration of ethylbenzene should be chosen to achieve S/N ratio specifications in the range of 20–1000. Concentrations that typically result in measurements outside that range are of limited utility in assessing the performance of the instrument. Nevertheless, established standard solutions are conventionally used. The magnet should be shimmed as well as possible. Ideally, this test should be run immediately after the lineshape test because most of the shims will be nearly maximized. Acquire a single acquisition, phase the spectrum in pure absorption mode, and measure the S/N of the ethylbenzene quartet. This experiment can be run with or without sample spinning. With a spinning sample, the S/N value that is measured should be only about 10% higher than that obtained with a nonspinning sample if the off-axis shims are well adjusted. A higher ratio would indicate that the determination would benefit from further shimming with the off-axis shims.

Most modern spectrometers have software that perform the S/N measurement after the operator has identified the signal and noise regions. Manual calculations can also be

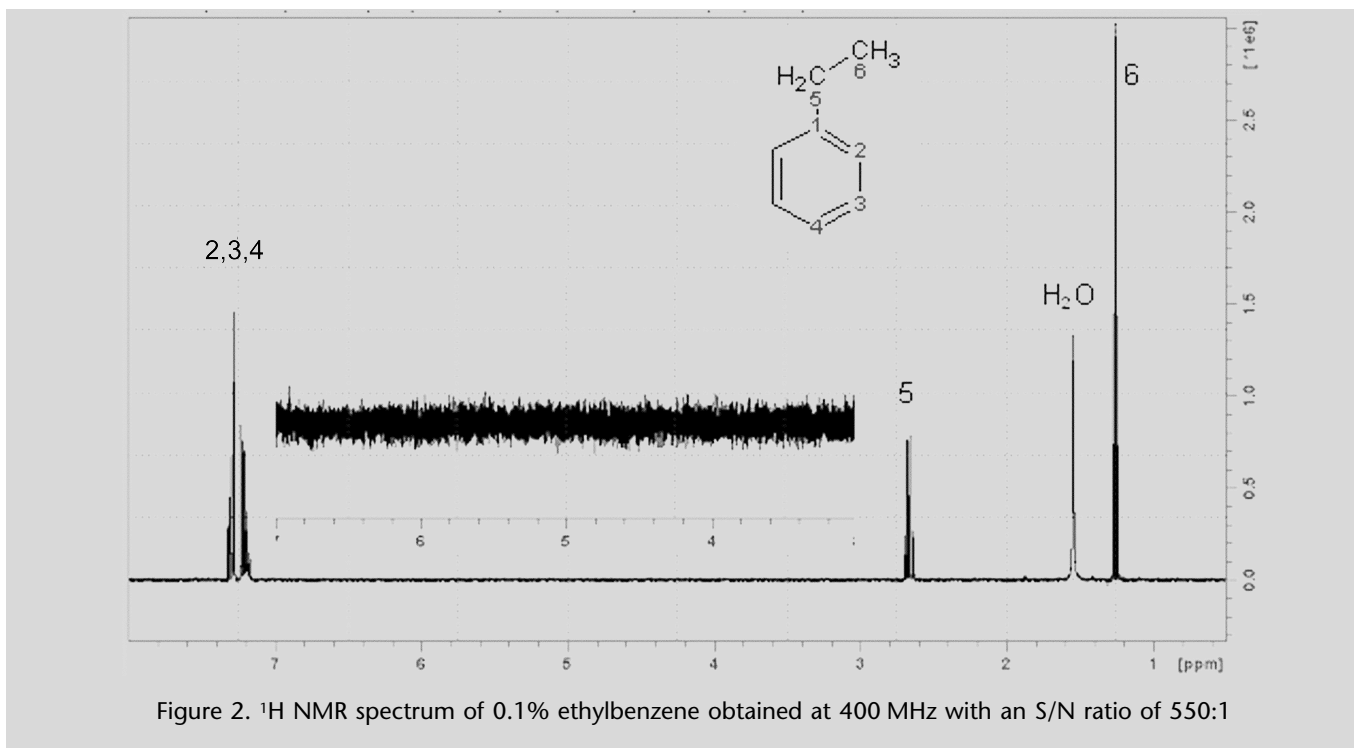


Figure 2. ^1H NMR spectrum of 0.1% ethylbenzene obtained at 400 MHz with an S/N ratio of 550:1

made. Measure the amplitude (A) from the center of the baseline to the peak of the highest of the central two lines in the quartet. Measure the peak-to-peak noise height (H) from the lowest noise peak to the highest noise peak in the 3–5 ppm region. The noise may be vertically multiplied by a factor for accurate measurement of high S/N spectra. Calculate the S/N as follows:

$$\text{S/N} = k \times 2.5 \times A/H \quad [1]$$

where k is the vertical expansion factor of the noise region used. The factor of 2.5 converts the peak-to-peak S/N to root-mean-squared (rms) noise, which is the standard convention for reporting S/N in NMR spectroscopy. Computerized S/N calculations can be used provided the specifications are set and tested by the same procedure. At the discretion of the spectroscopist, an S/N value lower than that specified by the manufacturer may be used if it is judged to be sufficient for the current application.

S/N MEASUREMENTS ^{13}C NMR (see Figure 3)

Sample: 40% *p*-dioxane in benzene- d_6 (v/v) (degassed and sealed)

Spectral width: Approximately 200 ppm

Tip angle: 90°

Relaxation delay: 300 s

Spinning rate: Approximately 20 Hz

Pulse sequence: Delay-pulse-acquire with no decoupling

Processing: Exponential with 3.5-Hz line broadening, zero-filling to 32k

Referencing: TMS = 0.0 ppm or the center of the benzene triplet = 128.4 ppm

With a well-shimmed magnet, acquire a single acquisition following a minimum delay of 300 s, phase the spectrum in pure absorption mode, and measure the height of the benzene triplet at approximately 128.4 ppm from the center of the baseline. The peak-to-peak noise can be measured as above with appropriate vertical expansion of 80–120 ppm.

S/N calculations can be made as in Equation 1 or by computer calculation.

The benzene- d_6 triplet has no nuclear Overhauser enhancement (NOE). Consequently, this test verifies the performance of only the ^{13}C channel.

PERFORMANCE OF BOTH OF THE ^{13}C AND ^1H CHANNELS (see Figure 4)

Sample: up to 10% ethylbenzene in chloroform- d (degassed and sealed)

Spectral width: 200 ppm

Data acquisition length: 64k points

Tip angle: 90°

Relaxation delay: 300 s

Spinning rate: Approximately 20 Hz

Pulse sequence: Delay-pulse-acquire with composite pulse decoupling set to the center of the ^1H spectrum

Processing: Exponential with 0.3-Hz line-broadening

Referencing: TMS = 0.0 ppm or the center of the chloroform- d , triplet = 77.23 ppm

The shimming should be sufficient to pass the resolution and lineshape tests described above. The measurement of S/N is done from the peak height of the larger resonance of the two near 128 ppm. The noise is measured as above in the region of 80–120 ppm, with appropriate vertical expansion. S/N is calculated by the computer or as in Equation 1.

RELAXOMETRY MEASUREMENTS—LOW FIELD-NMR (LF-NMR)

The PQ should be performed before the collection of experimental data.

Dissolve an accurately weighed quantity of manganese (II) chloride tetrahydrate (MW 197.91) in water, and quantitatively dilute with water to obtain check solutions that have known concentrations of 0.9, 2.7, and 4.5 mM.

Place a portion of each of the solutions into sample holders suitable for the configuration of the specific model of the LF-NMR spectrometer. Warm to 40° for NLT 10 min,

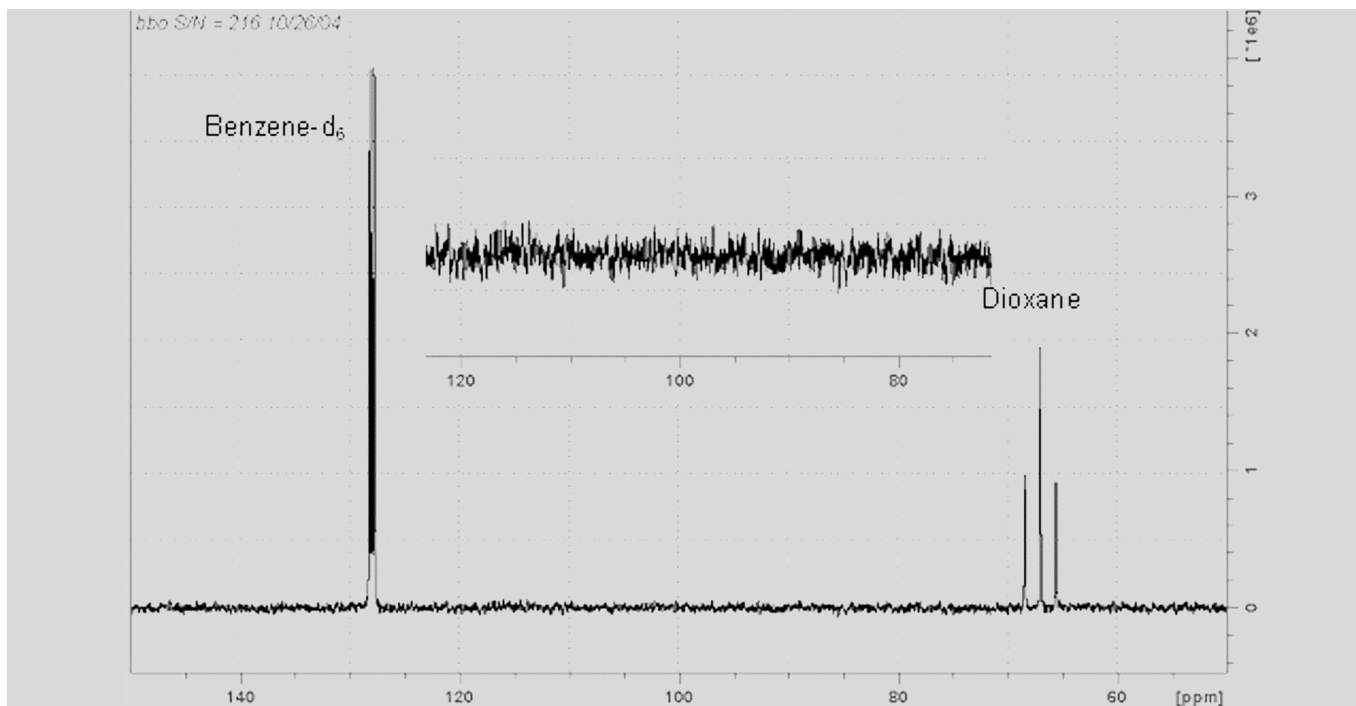


Figure 3. ^{13}C NMR spectrum of the ASTM standard 40% *p*-dioxane in benzene- d_6 (v/v) obtained at 100.6 MHz, with an S/N ratio of 140:1

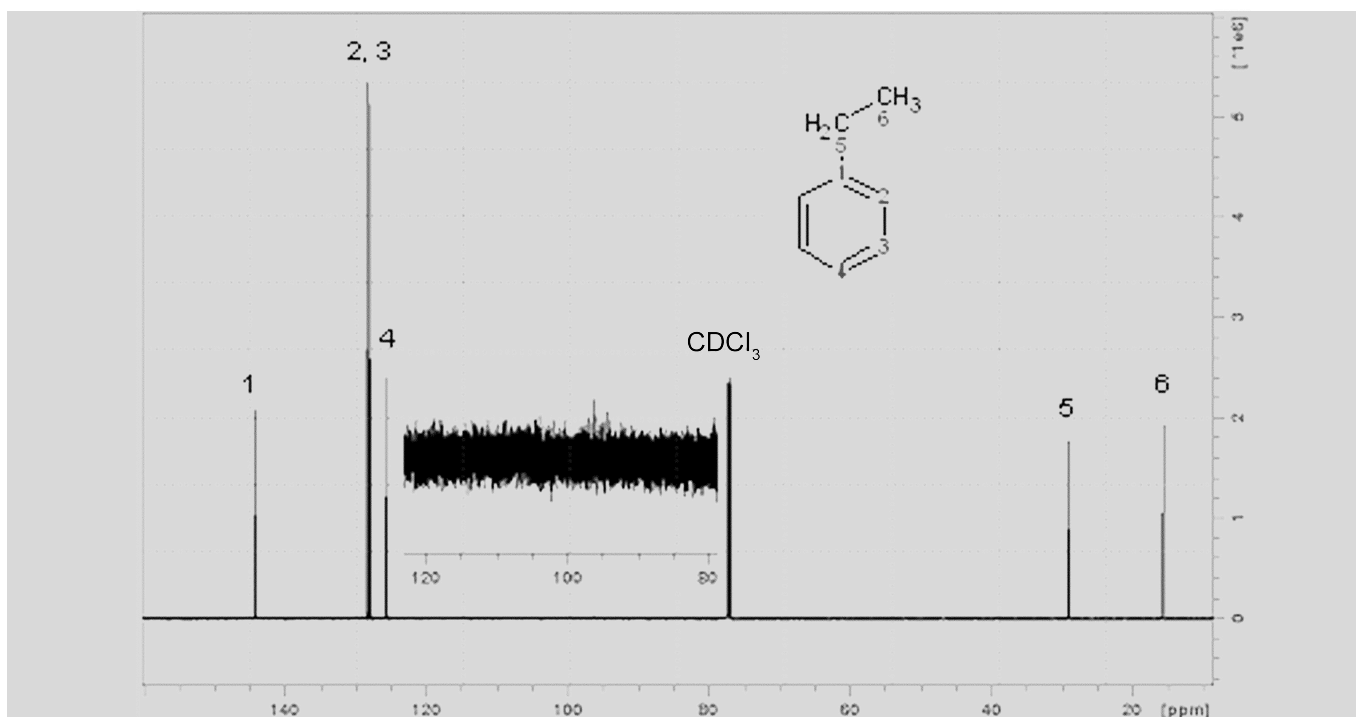


Figure 4. ^{13}C NMR spectrum of 10% ethylbenzene obtained using a cryogenically cooled dual $^1\text{H}/^{13}\text{C}$ probe at 150.9 MHz, with an S/N ratio of 640:1

and measure the spin-lattice relaxation time (T_1) of water. The average T_1 for replicate measurements must be within 5% of 156, 52, and 32 ms for the 0.9, 2.7, and 4.5 mM solutions, respectively.

Characterizing Instrument Performance

Specific procedures, acceptance criteria, and time intervals for characterizing NMR spectrometer performance depend

on the instrument and its intended application. Many NMR applications use previously validated experiments that relate NMR spectra to a physical or chemical property of interest. Stable instrument performance over extended periods of time should be demonstrated. This practice provides some assurance that reliable measurements can be taken from sample spectra using previously validated NMR experiments.

QUALITATIVE AND QUANTITATIVE NMR ANALYSIS

NMR spectroscopy has been used for a wide range of applications such as structure elucidation; thermodynamic, kinetic, and mechanistic studies; and quantitative analysis. Some of these applications are beyond the scope of compendial methods.

All characteristics of the signal—chemical shift, multiplicity, linewidth, coupling constants, relative intensity, and relaxation time—contribute analytical information.

Qualitative Applications

Comparison of a spectrum from the literature or from an authentic standard with that of a test sample may be used to confirm the identity of a compound and to detect the presence of impurities that generate extraneous signals. The NMR spectra of simple structures can be adequately described by the value of the chemical shifts and coupling constants, and by the relative number of nuclei represented by the integral of each signal. (The software of modern instruments have available programs that generate simulated spectra using these data.) Experimental details, such as the solvent used, and the chemical shift reference, must also be provided.

For unknown samples, NMR analysis, usually coupled with other analytical techniques, is a powerful tool for structure elucidation. Chemical shifts provide information on the chemical environment of the nuclei. Extensive literature is available with correlation charts and rules for predicting chemical shifts. The multiplicity of the signals provides important structural information. The magnitude of the scalar coupling constant, J , between residual protons on substituted aromatic, olefinic, or cycloalkyl structures is used to identify the relative position of the substituents. Routine ^{13}C spectra are obtained under proton decoupling conditions that remove all heteronuclear ^{13}C - ^1H couplings. As a result of this decoupling, the carbon signals appear as singlets, unless other nuclei that are not decoupled are present (e.g., ^{19}F , ^{31}P).

Chemical exchange is an example of the effect of intermolecular and intramolecular rate processes on NMR spectra. If a proton can experience different environments by virtue of such a process (tautomerism, rotation about a bond, exchange equilibria, ring inversion, etc.), the appearance of the spectrum will be a function of the rate of the process. Slow processes (on an NMR time scale) result in more than one signal from the interconverting species; fast processes average these signals to one line; and intermediate processes produce broad signals, which sometimes cannot be easily found in the spectra.

The software of modern FT-NMR spectrometers allows for sequences of pulses much more complex than the repetitive accumulation of transients described above. Such experiments include homonuclear or heteronuclear *multidimensional analysis*, which determines the correlation of couplings and may simplify the interpretation of otherwise complex spectra.

See chapter <1761> for detailed descriptions of common two-dimensional experiments.

Quantitative Applications

I. General considerations of quantitative NMR: see <1761>.

II. The scope of this section is limited to quantitation by one-dimensional NMR. Although any of the NMR active nuclei can be used to obtain quantitative data, the discussion here will be limited to ^1H . There are two kinds of quantitation by NMR: relative and absolute.

(A) **Relative quantitation** involves measurement of relative amounts of species in a sample based on integration of peaks due to each of the components measured. The integrals are normalized by factor N , that is, the integral is divided by the number of equivalent nuclei represented by that peak to give the relative molar concentration of each component.

(B) **Absolute quantitation** is the direct measurement of the actual amount of analyte independent of other components contained in that sample. There are two basic methods for absolute quantitation based on the kind of reference standard that is used to calibrate the NMR signal.

(1) **Internal reference standard**

(a) **Definition:** The reference standard is co-dissolved in the analyte test solution.

(b) **Procedure**

Typical NMR solution preparation: An NMR solution is prepared with exact weights of both the analyte and reference standard. The largest source of error in this quantitative NMR method is from weighing, so the use of larger weights is recommended to minimize this error. This quantitative method is based on a comparison of the reference standard and analyte NMR peaks and their respective concentrations. Since the analyte and reference standard are in the same solution, the analyte and reference standard are contained in the same volume, and only their masses are compared. Therefore, the exact volume is not required. Typically, at least three replicates are prepared.

Data acquisition: Data is acquired under quantitative conditions, see <1761>. For example, the pulse repetition time should be at least 5 times the longest T_1 when a 90° pulse is used.

Data processing: Process the data, using zero-filling if necessary, such that a sufficient number of points define a peak. For example, experience has shown that at least 16 points gives a good quantitative representation of a peak.

Analysis: Integrate appropriate peaks. For example, avoid using peaks that are overlapped, or due to hydrogens capable of exchanging. The determination of the amount of analyte derives from the basic proportionality between the peak intensity and the concentration of the solute.

$$\frac{[A]_{1H}}{I_A/N_A} = \frac{[RS]_{1H}}{I_{RS}/N_{RS}} \quad [2]$$

where I = integral; N = normalization factor; and $[]_{1H}$ = ^1H relative molar concentration, and the subscripts A and RS represent the analyte and reference standard, respectively.

The mass of the analyte is thus calculated according to the following equation.

$$M_A = \frac{I_A}{I_{RS}} \times \frac{N_{RS}}{N_A} \times \frac{MM_A}{MM_{RS}} \times M_{RS} \times P \quad [3]$$

where M_A = mass of the analyte, MM = molar mass, and P = purity of the reference standard.

- (c) A common application of absolute quantitation is the determination of the purity of a sample. The weight % purity is given by

$$\text{weight \% purity} = \frac{M_A}{M_S} \times 100\% \quad [4]$$

where M_S is the total mass of the sample with contributions from the analyte plus any contaminants that may be present in the sample such as water and salts. Combining Equations 3 and 4, the weight % purity is given by

$$\text{weight \% purity} = \frac{I_A}{I_{RS}} \times \frac{N_{RS}}{N_A} \times \frac{MM_A}{MM_{RS}} \times \frac{M_{RS}}{M_S} \times P \times 100 \quad [5]$$

(2) External reference standard

- (a) **Definition:** The classical external reference standard method consists of solutions of a reference standard and analyte that are each in separate NMR tubes. One variation of an external reference standard is the standard test solution contained in a coaxial tube and is inserted into an analyte test solution contained in an NMR tube. Another variation is the introduction of a computer-generated signal into the spectrum of a reference standard solution of known concentration to calibrate the signal's response (intensity per ^1H molar concentration, in the case of ^1H NMR), followed by insertion of that calibrated computer-generated signal into the spectrum of an analyte test solution. This section will address the use of an external reference in the classical sense.

(b) Procedure

NMR solution preparation: NMR solutions of known concentrations of each of the analyte and reference standard are prepared using exact weights and volumes. Again, the use of larger weights is recommended to minimize the weighing error. Typically replicates of the analyte solutions and reference standard solutions are prepared. The analyte and reference must be prepared in the same solvent to minimize probe tuning differences.

Data acquisition and processing: Same as in II.B.1.b, internal reference standard. Apply the same acquisition and processing parameters to the analyte and reference standard spectra.

Analysis: Integrate appropriate peaks in the spectra of the analyte and reference standard. The amount of analyte is calculated according to the following equation:

$$M_A = \frac{I_A}{I_{RS}} \times \frac{N_{RS}}{N_A} \times \frac{V_A}{V_{RS}} \times \frac{MM_A}{MM_{RS}} \times M_{RS} \times P \quad [6]$$

where V = volume.

Application to weight % purity: Weight % purity values may be similarly calculated as in Equation 5.

- (C) The internal and external reference standard methods each have their own set of advantages and disadvantages.

(1) **Chemical interactions:** Preparation of the reference standard and the test material in separate solutions avoids chemical interactions between the test sample and reference standard that may otherwise occur with an internal reference standard.

(2) **Spectral overlap:** The use of an external reference standard also avoids potential overlap between peaks of the reference standard and test sample that can occur with an internal standard.

(3) **Calibration:** Once an NMR response has been calibrated with external reference standard solutions, this calibration may be applied to any other sample in the same solvent given that i) the instrument has been demonstrated to be stable over the time between when the calibration is done and when data is acquired on the test material, ii) system suitability has been established on the day that the measurement on the test material is made, and iii) absolute integrals are compared. In the case of internal reference standards, the measurement on the reference standard and test sample is made under absolutely identical conditions.

(4) **Accuracy and precision:** Multiple external reference standard solutions may be prepared to average the errors in the mass and volume measurements during sample preparation, thereby improving the accuracy of the calibrated NMR response. In the case of internal reference standards, single measurements of the reference standard and analyte are made for each replicate test solution. The combined errors from the mass measurements of the reference standard and test sample as well as instrumental electronic variations determine the standard deviation of the average M_A or weight % purity values.

VALIDATION AND VERIFICATION OF NMR ANALYTICAL PROCEDURES

If an NMR procedure is provided in a monograph, verification of suitability (see (1226)) under actual conditions of use is required. Validation is required only when an NMR method is an alternative to the official procedure for testing an official article. The objective of validation of a procedure relying on the NMR method is to demonstrate that the measurement is suitable for its intended purpose, including the following: quantitative determination of the main component in a drug substance or a drug product (Category I assays), quantitative determination of impurities (Category II), and identification tests (Category IV). [NOTE—For a definition of the different categories, see *Validation of Compendial Procedures* (1225).] Depending on the category of the test, analytical procedure validation requires the testing of specificity, linearity, range, accuracy, precision, quantitation limit, and robustness. These analytical performance characteristics apply to externally standardized methods and to the method of standard additions.

Chapter (1225) provides definitions and general guidance on analytical procedures validation without indicating specific validation criteria for each characteristic. The intention of the following sections is to provide the user with specific validation criteria that represent the minimum expectations

for this technology. For each particular application, tighter criteria may be needed in order to demonstrate suitability for the intended use.

Analytical Procedure Validation

The objective of an analytical procedure validation is to demonstrate that the analytical procedure is suitable for its intended purpose by conducting experiments and obtaining results that meet predefined acceptance criteria. NMR analytical procedures can include the following: quantitative tests for major component and impurities content, limit tests for the presence of impurities, quantification of component in a product or formulation, and/or identification tests.

Performance characteristics that demonstrate the suitability of an analytical procedure are similar to those required for any analytical procedure. A discussion of the applicable general principles is found in chapter 〈1225〉. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the analytical procedure.

The performance characteristics that are required as part of a validation for each of the analytical procedure categories is given below.

SPECIFICITY

The purpose of a specificity test is to demonstrate that measurements of the intended analyte signals are free of interference from components and impurities in the test material. Specificity may be applied to all categories and is a requirement for Category IV. Specificity tests can be conducted to compare NMR spectra of other components and impurities that are known from synthetic processes and formulations and test preparations. For an identification NMR analytical procedure (Category IV), validation experiments may include multidimensional NMR experiments to validate correct assignments of chemical shifts and to confirm the structure of the analyte.

Validation criteria: Specificity is ensured by use of a reference standard wherever possible and demonstrable lack of interference from other components.

LINEARITY

A linear relationship is exhibited between the analyte concentration and instrument response; this should be demonstrated by measuring responses of analyte from NLT five standard solutions at concentrations encompassing the anticipated concentration range of analyte(s) of the test solution. For Category I, standard solutions can be prepared from reference materials in an appropriate NMR solvent. For Category II, NMR analytical procedures that are used to quantitate impurities, linearity samples can be prepared by spiking suitable test samples that contain low amounts of analyte or by spiking matrix samples at concentrations of the expected range. The standard curve should then be constructed using appropriate statistical analytical procedures such as a least squares regression. The correlation coefficient (R), y -intercept, and slope of the regression line should be determined. Absolute values determined for these factors should be appropriate for the procedure being validated.

Validation criteria: The correlation coefficient (R) must be NLT 0.995 for Category I assays and NLT 0.99 for Category II quantitative tests.

RANGE

The range between the low and high concentrations of analyte is given by the quantitative NMR analytical procedure. This is normally based on test article specifications in

the USP monograph. It is the range within which the analytical procedure can demonstrate an acceptable degree of linearity, accuracy, and precision, and may be obtained from an evaluation of that analytical procedure. Recommended ranges for various NMR analytical procedures are given below.

Validation criteria: For Category I tests, the validation range for 100.0% centered acceptance criteria is 80.0%–120.0%. For noncentered acceptance criteria, the validation range is 10.0% below the lower limit to 10.0% above the upper limit. For content uniformity, it is 70.0%–130.0%. For Category II quantitative tests, the validation range covers 50.0%–120.0% of the acceptance criteria.

ACCURACY

The accuracy of a quantitative NMR analytical procedure should be determined across the required analytical range. Typically, three levels of concentrations are evaluated using triplicate preparations at each level.

For drug substance assays (Category I), accuracy can be determined by analyzing a reference standard of known purity. For drug product (Category I), a composite sample of reference standard and other components in a pharmaceutical finished product should be used for analytical procedure validation. The assay results are compared to the theoretical value of the reference standard to estimate errors or percent recovery. For the quantitation of impurities (Category II), the accuracy of the analytical procedure can be determined by conducting studies with drug substances or products spiked with known concentrations of the analyte under test. It is also acceptable to compare assay results from the analytical procedure being validated to those of an established, alternative analytical procedure.

Validation criteria: 98.0%–102.0% recovery for drug substances, 95.0%–105.0% recovery for compounded pharmaceutical finished products assay, and 80.0%–120.0% recovery for the quantitative impurity analysis. These criteria should be met throughout the intended range.

PRECISION

Repeatability: The analytical procedure should be assessed by measuring the concentrations of six separate standard solutions at 100% of the test concentration. The relative standard deviation from the replicate measurements should be evaluated to meet acceptance criteria. Alternatively they can measure the concentrations of three replicates of three separate sample solutions at different concentrations. The three concentrations should be close enough so that the repeatability is constant across the concentration range. If this is done, the repeatability at the three concentrations is pooled for comparison to the acceptance criteria.

Validation criteria: The relative standard deviation is NMT 1.0% for drug substances, NMT 2.0% for compounded pharmaceutical finished products, and NMT 20.0% for the quantitative impurity analysis.

Intermediate precision: The effect of random events on the analytical precision of the analytical procedure should be established. Typical variables include performing the analysis on different days, using different instrumentation that are suitable as specified in the analytical procedure, and/or having the analytical procedure performed by two or more analysts. As a minimum, any combination of at least two of these factors totaling six experiments will provide an estimation of intermediate precision.

Validation criteria: The relative standard deviation is NMT 1.0% for drug substances, NMT 3.0% for compounded pharmaceutical finished products, and NMT 25.0% for quantitative impurity analysis.

QUANTITATION LIMIT (QL)

The QL can be validated by measuring six replicates of test samples spiked with analyte at 50% of specification.

From these replicates, accuracy and precision can be determined. Examples of specifications for Category II quantitative determinations are that the measured concentration is within 70.0%–130.0% of the spike concentration and the relative standard deviation is NMT 15%.

ROBUSTNESS

The reliability of an analytical measurement should be demonstrated with deliberate changes to critical experimental parameters. This can include measuring the stability of the analyte under specified storage conditions, slightly varied inter-pulse delay, probe temperature, and possible interfering species, to list a few examples. Robustness is required for Category I and Category II, quantitative methods.

Analytical Procedure Verification

U.S. Current Good Manufacturing Practices regulations [21 CFR 211.194(a)(2)] indicate that users of analytical procedures described in *USP–NF* do not need to validate these procedures if provided in a monograph. Instead, they must simply verify their suitability under actual conditions of use.

The objective of an NMR procedure verification is to demonstrate that the procedure as prescribed in a specific monograph can be executed by the user with suitable accuracy, specificity, and precision using the instruments, analysts, and sample matrices available. According to general information chapter *Verification of Compendial Procedures* (1226), if the verification of the compendial procedure by following the monograph is not successful, the procedure may not be suitable for use with the article under test. It may be necessary to develop and validate an alternative procedure as allowed in *General Notices and Requirements* 6.30.

Verification of a compendial NMR procedure should at minimum include the execution of the validation parameters for specificity, accuracy, precision, and limit of quantitation, when appropriate, as indicated in this section.

GLOSSARY

Internal standard: An internal standard (IS) is a substance added to a sample solution at a known concentration. One should select an IS with at least a single NMR resonance that does not overlap with those of the analyte. The ratio of a specific internal standard peak area and that of an analyte peak area is used to determine the concentration of the analyte. The number of nuclei corresponding to the integrated peaks in the IS and analyte spectra must be known.

NMR reference: An NMR reference, also known as an NMR shift reference, is a substance added to a sample and from which the chemical shift for the δ scale is established. Common examples for proton and carbon NMR analyses are tetramethylsilane (TMS) for use in organic solvents and the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) or sodium-3-trimethylsilylpropionate (TMSP) for use in aqueous media. In both cases, the chemical shift of the methyl peaks is defined as 0.0 ppm.

Reference standard: A reference standard is a substance authenticated by appropriate experimental means to be

of a specific chemical structure. In NMR spectroscopy, a reference standard is typically used for the qualitative analysis of a test material. Structure can be confirmed if one directly compares the chemical shifts and multiplicities of the peaks in the NMR spectrum of the test material against the spectrum of the reference standard acquired under comparable experimental conditions.▲ *USP36*

(771) OPHTHALMIC OINTMENTS

Added Substances—Suitable substances may be added to ophthalmic ointments to increase stability or usefulness, unless proscribed in the individual monograph, provided they are harmless in the amounts administered and do not interfere with the therapeutic efficacy or with the responses to the specified assays and tests. No coloring agent may be added, solely for the purpose of coloring the finished preparation, to an article intended for ophthalmic use (see also *Added Substances* under *General Notices* and under *Antimicrobial Effectiveness Testing* (51)).

A suitable substance or mixture of substances to prevent the growth of microorganisms must be added to ophthalmic ointments that are packaged in multiple-use containers, regardless of the method of sterilization employed, unless otherwise directed in the individual monograph, or unless the formula itself is bacteriostatic. Such substances are used in concentrations that will prevent the growth of or kill microorganisms in the ophthalmic ointments (see also *Antimicrobial Effectiveness Testing* (51) and *Antimicrobial Agents—Content* (341)). Sterilization processes are employed for the finished ointment or for all ingredients, if the ointment is manufactured under rigidly aseptic conditions, even though such substances are used (see also *Parenteral and Topical Preparations* in the section *Added Substances*, under *General Notices*, and *Sterilization and Sterility Assurance of Compendial Articles* (1211)). Ophthalmic ointments that are packaged in single-use containers are not required to contain antibacterial agents; however, they meet the requirements for *Sterility Tests* (71).

Containers—Containers, including the closures, for ophthalmic ointments do not interact physically or chemically with the preparation in any manner to alter the strength, quality, or purity beyond the official requirements under the ordinary or customary conditions of handling, shipment, storage, sale, and use.

Metal Particles—Follow the *Procedure* set forth under *Metal Particles in Ophthalmic Ointments* (751).

Leakage—Select 10 tubes of the Ointment, with seals applied when specified. Thoroughly clean and dry the exterior surfaces of each tube with an absorbent cloth. Place the tubes in a horizontal position on a sheet of absorbent blotting paper in an oven maintained at a temperature of $60 \pm 3^\circ$ for 8 hours. No significant leakage occurs during or at the completion of the test (disregard traces of ointment presumed to originate externally from within the crimp of the tube or from the thread of the cap). If leakage is observed from one, but not more than one, of the tubes, repeat the test with 20 additional tubes of the Ointment. The requirement is met if no leakage is observed from the first

10 tubes tested, or if leakage is observed from not more than one of 30 tubes tested.

〈776〉 OPTICAL MICROSCOPY

Optical microscopy for particle characterization can generally be applied to particles 1 μm and greater. The lower limit is imposed by the resolving power of the microscope. The upper limit is less definite and is determined by the increased difficulty associated with the characterization of larger particles. Various alternative techniques are available for particle characterization outside the applicable range of optical microscopy. Optical microscopy is particularly useful for characterizing particles that are not spherical. This method may also serve as a base for the calibration of faster and more routine methods that may be developed.

Apparatus—Use a microscope that is stable and protected from vibration. The microscope magnification (product of the objective magnification, ocular magnification, and additional magnifying components) must be sufficient to allow adequate characterization of the smallest particles to be classified in the test specimen. The greatest numerical aperture of the objective should be sought for each magnification range. Polarizing filters may be used in conjunction with suitable analyzers and retardation plates. Color filters of relatively narrow spectral transmission should be used with achromatic objectives, are preferable with apochromats, and are required for appropriate color rendition in photomicrography. Condensers corrected at least for spherical aberration should be used in the microscope substage and with the lamp. The numerical aperture of the substage condenser should match that of the objective under the conditions of use and is affected by the actual aperture of the condenser diaphragm and by the presence of immersion oils.

Adjustment—The precise alignment of all elements of the optical system and proper focusing are essential. The focusing of the elements should be done in accordance with the recommendations of the microscope manufacturer. Critical axial alignment is recommended.

Illumination—A requirement for good illumination is a uniform and adjustable intensity of light over the entire field of view; Kohler illumination is preferred. With colored particles, choose the color of the filters used so as to control the contrast and detail of the image.

Visual Characterization—The magnification and numerical aperture should be sufficiently high to allow adequate resolution of the images of the particles to be characterized. Determine the actual magnification using a calibrated stage micrometer to calibrate an ocular micrometer. Errors can be minimized if the magnification is sufficient that the image of the particle is at least 10 ocular divisions. Each objective must be calibrated separately. To calibrate the ocular scale, the stage micrometer scale and the ocular scale should be aligned. In this way, a precise determination of the distance between ocular stage divisions can be made. Several different magnifications may be necessary to characterize materials having a wide particle size distribution.

Photographic Characterization—If particle size is to be determined by photographic methods, take care to ensure that the object is sharply focused at the plane of the photographic emulsion. Determine the actual magnification by photographing a calibrated stage micrometer, using photographic film of sufficient speed, resolving power, and contrast. Exposure and processing should be identical for photographs of both the test specimen and the determination of magnification. The apparent size of a photographic im-

age is influenced by the exposure, development, and printing processes as well as by the resolving power of the microscope.

Preparation of the Mount—The mounting medium will vary according to the physical properties of the test specimen. Sufficient but not excessive contrast between the specimen and the mounting medium is required to ensure adequate detail of the specimen edge. The particles should rest in one plane and be adequately dispersed to distinguish individual particles of interest. Furthermore, the particles must be representative of the distribution of sizes in the material and must not be altered during preparation of the mount. Care should be taken to ensure that this important requirement is met. Selection of the mounting medium must include a consideration of the analyte solubility.

Crystallinity Characterization—The crystallinity of a material may be characterized to determine compliance with the crystallinity requirement where stated in the individual monograph of a drug substance. Unless otherwise specified in the individual monograph, mount a few particles of the specimen in mineral oil on a clean glass slide. Examine the mixture using a polarizing microscope: the particles show birefringence (interference colors) and extinction positions when the microscope stage is revolved.

Limit Test of Particle Size by Microscopy—Weigh a suitable quantity of the powder to be examined (for example, 10 to 100 mg), and suspend it in 10 mL of a suitable medium in which the powder does not dissolve, adding, if necessary, a wetting agent. A homogeneous suspension of particles can be maintained by suspending the particles in a medium of similar or matching density and by providing adequate agitation. Introduce a portion of the homogeneous suspension into a suitable counting cell, and scan under a microscope an area corresponding to not less than 10 μg of the powder to be examined. Count all the particles having a maximum dimension greater than the prescribed size limit. The size limit and the permitted number of particles exceeding the limit are defined for each substance.

Particle Size Characterization—The measurement of particle size varies in complexity depending on the shape of the particle, and the number of particles characterized must be sufficient to ensure an acceptable level of uncertainty in the measured parameters. Additional information on particle size measurement, sample size, and data analysis is available, for example, in ISO 9276. For spherical particles, size is defined by the diameter. For irregular particles, a variety of definitions of particle size exist. In general, for irregularly shaped particles, characterization of particle size must also include information on the type of diameter measured as well as information on particle shape. Several commonly used measurements of particle size are defined below (see Figure 1):

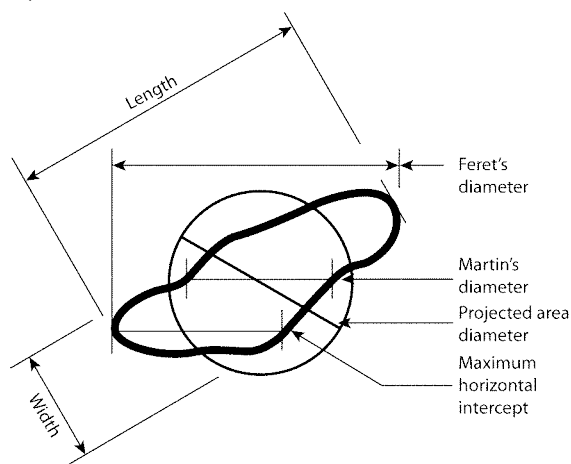


Figure 1. Commonly used measurements of particle size.

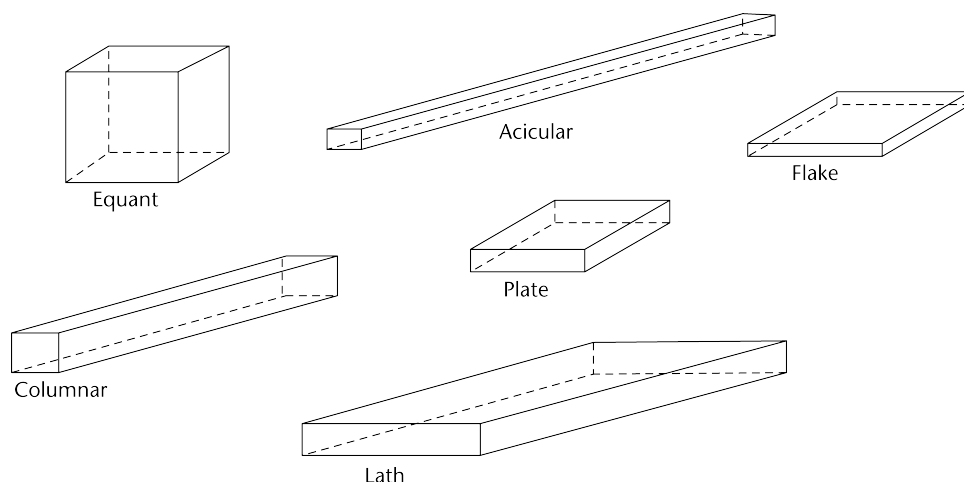


Figure 2. Commonly used descriptions of particle shape.

Feret's Diameter—The distance between imaginary parallel lines tangent to a randomly oriented particle and perpendicular to the ocular scale.

Martin's Diameter—The diameter of the particle at the point that divides a randomly oriented particle into two equal projected areas.

Projected Area Diameter—The diameter of a circle that has the same projected area as the particle.

Length—The longest dimension from edge to edge of a particle oriented parallel to the ocular scale.

Width—The longest dimension of the particle measured at right angles to the length.

Particle Shape Characterization—For irregularly shaped particles, characterization of particle size must also include information on particle shape. The homogeneity of the powder should be checked using appropriate magnification. The following defines some commonly used descriptors of particle shape (see Figure 2):

Acicular—Slender, needle-like particle of similar width and thickness.

Columnar—Long, thin particle with a width and thickness that are greater than those of an acicular particle.

Flake—Thin, flat particle of similar length and width.

Plate—Flat particles of similar length and width but with greater thickness than flakes.

Lath—Long, thin, and blade-like particle.

Equant—Particles of similar length, width, and thickness; both cubical and spherical particles are included.

General Observations—A particle is generally considered to be the smallest discrete unit. A particle may be a liquid or semisolid droplet; a single crystal or polycrystalline; amorphous or an agglomerate. Particles may be associated. This degree of association may be described by the following terms:

Lamellar—Stacked plates.

Aggregate—Mass of adhered particles.

Agglomerate—Fused or cemented particles.

Conglomerate—Mixture of two or more types of particles.

Spherulite—Radial cluster.

Drusy—Particle covered with tiny particles.

Particle condition may be described by the following terms:

Edges—Angular, rounded, smooth, sharp, fractured.

Optical—Color (using proper color-balancing filters), transparent, translucent, opaque.

Defects—Occlusions, inclusions.

Surface characteristics may be described as:

Cracked—Partial split, break, or fissure.

Smooth—Free of irregularities, roughness, or projections.

Porous—Having openings or passageways.

Rough—Bumpy, uneven, not smooth.

Pitted—Small indentations.

(781) OPTICAL ROTATION

Many pharmaceutical substances are optically active in the sense that they rotate an incident plane of polarized light so that the transmitted light emerges at a measurable angle to the plane of the incident light. This property is characteristic of some crystals and of many pharmaceutical liquids or solutions of solids. Where the property is possessed by a liquid or by a solute in solution, it is generally the result of the presence of one or more asymmetric centers, usually a carbon atom with four different substituents. The number of optical isomers is 2^n , where n is the number of asymmetric centers. Polarimetry, the measurement of optical rotation, of a pharmaceutical article may be the only convenient means for distinguishing optically active isomers from each other and thus is an important criterion of identity and purity.

Substances that may show optical rotatory power are *chiral*. Those that rotate light in a clockwise direction as viewed towards the light source are *dextrorotatory*, or (+) *optical isomers*. Those that rotate light in the opposite direction are called *levorotatory* or (–) *optical isomers*. (The symbols *d*- and *l*-, formerly used to indicate dextro- and levorotatory isomers, are no longer sanctioned owing to confusion with *D*- and *L*-, which refer to configuration relative to *D*-glyceraldehyde. The symbols *R* and *S* and α and β are also used to indicate configuration, the arrangement of atoms or groups of atoms in space.)

The physicochemical properties of nonsuperimposable chiral substances rotating plane polarized light in opposite directions to the same extent, *enantiomers*, are identical, except for this property and in their reactions with other chiral substances. Enantiomers often exhibit profound differences in pharmacology and toxicology, owing to the fact that biological receptors and enzymes themselves are chiral. Many articles from natural sources, such as amino acids, proteins, alkaloids, antibiotics, glycosides, and sugars, exist as chiral compounds. Synthesis of such compounds from nonchiral

materials results in equal numbers of the enantiomers, *racemates*. Racemates have a net null optical rotation, and their physical properties may differ from those of the component enantiomers. Use of stereoselective or stereospecific synthetic methods or separation of racemic mixtures can be used to obtain individual optical isomers.

Measurement of optical rotation is performed using a polarimeter.* The general equation used in polarimetry is:

$$[\alpha]_t^{\lambda} = \frac{100a}{lc}$$

where $[\alpha]$ is the specific rotation at wavelength λ , t is the temperature, a is the observed rotation in degrees ($^{\circ}$), l is the pathlength in decimeters, and c is the concentration of the analyte in g per 100 mL. Thus, $[\alpha]$ is 100 times the measured value, in degrees ($^{\circ}$), for a solution containing 1 g in 100 mL, measured in a cell having a pathlength of 1.0 dm under defined conditions of incident wavelength of light and temperature. For some Pharmacopeial articles, especially liquids such as essential oils, the optical rotation requirement is expressed in terms of the observed rotation, a , measured under conditions defined in the monograph.

Historically, polarimetry was performed using an instrument where the extent of optical rotation is estimated by visual matching of the intensity of split fields. For this reason, the D-line of the sodium lamp at the visible wavelength of 589 nm was most often employed. Specific rotation determined at the D-line is expressed by the symbol:

$$[\alpha]_D^{25} \text{ or } [\alpha]_D^{20}$$

and much of the data available are expressed in this form. Use of lower wavelengths, such as those available with the mercury lamp lines isolated by means of filters of maximum transmittance at approximately 578, 546, 436, 405, and 365 nm in a photoelectric polarimeter, has been found to provide advantages in sensitivity with a consequent reduction in the concentration of the test compound. In general, the observed optical rotation at 436 nm is about double and at 365 nm about three times that at 589 nm. Reduction in the concentration of the solute required for measurement may sometimes be accomplished by conversion of the substance under test to one that has a significantly higher optical rotation. Optical rotation is also affected by the solvent used for the measurement, and this is always specified.

It is now common practice to use other light sources, such as xenon or tungsten halogen, with appropriate filters, because these may offer advantages of cost, long life, and broad wavelength emission range, over traditional light sources.

Specific Rotation—The reference *Specific Rotation* <781S> in a monograph signifies that specific rotation is to be calculated from observed optical rotations in the *Test solution* or *Sample solution* obtained as directed therein. Unless otherwise directed, measurements of optical rotation are made at 589 nm at 25°. Where a photoelectric polarimeter is used, a single measurement, corrected for the solvent blank, is made. Where a visual polarimeter is employed, the average of no fewer than five determinations, corrected for the reading of the same tube with a solvent blank, is used. Temperature, which applies to the solution or the liquid under test, should be maintained within 0.5° of the stated value. Use the same cell for sample and blank. Maintain the same angular orientation of the cell in each reading. Place the cell so that the light passes through it in the same direction

*Suitable calibrators are available from the Office of Standard Reference Materials, National Institute of Standards and Technology (NIST), Gaithersburg, MD 20899, as current lots of Standard Reference Materials, Dextrose and Sucrose. Alternatively, calibration may be checked using a Polarization Reference Standard, which consists of a plate of quartz mounted in a holder perpendicular to the light path. These standards are available, traceable to NIST, from Rudolph Research Analytical, 354 Route 206, Flanders, NJ 07836, or from Rudolph Instruments, Inc., 40 Pier Lane, Fairfield, NJ 07004-2113.

each time. Unless otherwise specified, specific rotation is calculated on the dried basis where *Loss on Drying* is specified in the monograph, or on the anhydrous basis where *Water* is specified.

Optical rotation of solutions should be determined within 30 minutes of preparation. In the case of substances known to undergo racemization or mutarotation, care should be taken to standardize the time between adding the solute to the solvent and introduction of the solution into the polarimeter tube.

Angular Rotation—The reference *Angular Rotation* <781A> in a monograph signifies, unless otherwise directed, that the optical rotation of the neat liquid is measured in a 1.0-dm tube at 589 nm at 25°, corrected for the reading of the dry empty tube.

<785> OSMOLALITY AND OSMOLARITY

INTRODUCTION

Osmotic pressure plays a critical role in all biological processes that involve diffusion of solutes or transfer of fluids through membranes. Osmosis occurs when solvent but not solute molecules cross a semipermeable membrane from regions of lower to higher concentrations to produce equilibrium. The knowledge of osmotic pressures is important for practitioners in determining whether a parenteral solution is hypo-osmotic, iso-osmotic, or hyperosmotic. A quantitative measure of osmotic pressure facilitates the dilution required to render a solution iso-osmotic relative to whole blood.

OSMOTIC PRESSURE

The osmotic pressure of a solution depends on the number of particles in solution, and is therefore referred to as a colligative property. A particle can be a molecule or an ion or an aggregated species (e.g., a dimer) that can exist discretely in solution. A solution exhibits ideal behavior when no interaction occurs between solutes and solvent, except where solvent molecules are bound to solutes by hydrogen bonding or coordinate covalency. For such a solution containing a nondissociating solute, the osmotic pressure (π) is directly proportional to its molality (number of moles of solute per kilogram of the solvent):

$$\pi = (\rho RT/1000)m,$$

where ρ is the density of the solvent at the temperature T (in the absolute scale); R is the universal gas constant; and m is the molality of the solution. For a real solution containing more than one solute, the osmotic pressure is given by the formula:

$$\pi = (\rho RT/1000)\sum v_i m_i \Phi_{m,i}$$

where v_i is the number of particles formed by the dissociation of one molecule of the i^{th} solute; $v_i = 1$ for nonionic (nondissociating) solutes; m_i is the molality of the i^{th} solute; and $\Phi_{m,i}$ is the molal osmotic coefficient of the i^{th} solute. The molal osmotic coefficient takes into account the deviation of a solution from ideal behavior. Its value depends upon the concentration of the solute(s) in solution, its chemical properties, and ionic characteristics. The value of the molal osmotic coefficient of a solute can be determined

experimentally by measuring the freezing point depression at different molal concentrations. At concentrations of pharmaceutical interest, the value of the molal osmotic coefficient is less than one. The molal osmotic coefficient decreases with the increase in concentration of the solute (Table 1).

OSMOLALITY

The osmolality of a solution ξ_m is given by

$$\xi_m = \sum v_i m_i \Phi_{m,i}$$

The osmolality of a real solution corresponds to the molality of an ideal solution containing nondissociating solutes and is expressed in osmoles or milliosmoles per kilogram of solvent (Osmol per kg or mOsmol per kg, respectively), a unit that is similar to the molality of the solution. Thus, osmolality is a measure of the osmotic pressure exerted by a real solution across a semipermeable membrane. Like osmotic pressure, other colligative properties of a solution, such as vapor pressure lowering, boiling point elevation, and freezing point depression, are also directly related to the osmolality of the solution. Indeed, the osmolality of a solution is typically determined most accurately and conveniently by measuring freezing point depression (ΔT_f):

$$\Delta T_f = k_f \xi_m$$

where k_f is the molal cryoscopic constant, which is a property of the solvent. For water, the value of k_f is 1.860° per Osmol. That is, 1 Osmol of a solute added to 1 kg of water lowers the freezing point by 1.860°.

OSMOLARITY

Osmolarity of a solution is a theoretical quantity expressed in osmoles per L (Osmol per L) of a solution and is widely used in clinical practice because it expresses osmoles as a function of volume. Osmolarity cannot be measured but is calculated theoretically from the experimentally measured value of osmolality.

Sometimes, osmolarity (ξ_c) is calculated theoretically from the molar concentrations:

$$\xi_c = \sum v_i c_i$$

where v_i is as defined above, and c_i is the molar concentration of the i^{th} solute in solution. For example, the osmolarity of a solution prepared by dissolving 1 g of vancomycin in

100 mL of 0.9% sodium chloride solution can be calculated as follows:

$$[3 \times 10 \text{ g/L} / 1449.25 (\text{mol. wt. of vancomycin}) + 2 \times 9 \text{ g/L} / 58.44 (\text{mol. wt. of sodium chloride})] \times 1000 = 329 \text{ mOsmol/L}$$

The results suggest that the solution is slightly hyperosmotic because the osmolality of blood ranges between 285 and 310 mOsmol per kg. However, the solution is found to be hypo-osmotic and has an experimentally determined osmolality of 255 mOsmol per kg.¹ The example illustrates that osmolarity values calculated theoretically from the concentration of a solution should be interpreted cautiously and may not represent the osmotic properties of infusion solutions.

The discrepancy between theoretical (osmolarity) and experimental (osmolality) results is, in part, due to the fact that osmotic pressure is related to osmolality and not osmolarity. More significantly, the discrepancy between experimental results and the theoretical calculation is due to the fact that the osmotic pressure of a real solution is less than that of an ideal solution because of interactions between solute molecules or between solute and solvent molecules in a solution. Such interactions reduce the pressure exerted by solute molecules on a semipermeable membrane, reducing experimental values of osmolality compared to theoretical values. This difference is related to the molal osmotic coefficient ($\Phi_{m,i}$). The example also illustrates the importance of determining the osmolality of a solution experimentally, rather than calculating the value theoretically.

MEASUREMENT OF OSMOLALITY

The osmolality of a solution is commonly determined by the measurement of the freezing point depression of the solution.

Apparatus—The apparatus, an osmometer for freezing point depression measurement, consists of the following: a means of cooling the container used for the measurement; a resistor sensitive to temperature (thermistor), with an appropriate current- or potential-difference measurement device that may be graduated in temperature change or in osmolality; and a means of mixing the sample.

Osmometers that measure the vapor pressures of solutions are less frequently employed. They require a smaller volume of specimen (generally about 5 μ L), but the accuracy and precision of the resulting osmolality determination are comparable to those obtained by the use of osmometers that depend upon the observed freezing points of solutions.

Standard Solutions—Prepare *Standard Solutions* as specified in Table 1, as necessary.²

¹Kastango, E.S. and Hadaway, L. *International Journal of Pharmaceutical Compounding* 5, (2001) 465-469.

²Commercially available solutions for osmometer calibration, with osmolalities equal to or different from those listed in Table 1 and standardized by methods traceable to NIST, may be used.

Table 1. Standard Solutions for Osmometer Calibration*

Standard Solutions (Weight in g of sodium chloride per kg of water)	Osmolality (mOsmol/kg) (ξ_m)	Molal Osmotic Coefficient ($\Phi_{m,\text{NaCl}}$)	Freezing Point Depression (°) ΔT_f
3.087	100	0.9463	0.186
6.260	200	0.9337	0.372
9.463	300	0.9264	0.558
12.684	400	0.9215	0.744
15.916	500	0.9180	0.930
19.147	600	0.9157	1.116
22.380	700	0.9140	1.302

*Adapted from the *European Pharmacopoeia*, 4th Edition, 2002, p. 50.

Test Solution—For a solid for injection, constitute with the appropriate diluent as specified in the instructions on the labeling. For solutions, use the sample as is. [NOTE—A solution can be diluted to bring it within the range of measurement of the osmometer, if necessary, but the results must be expressed as that of the diluted solution and must NOT be multiplied by a dilution factor to calculate the osmolality of the original solution, unless otherwise indicated in the monograph. The molal osmotic coefficient is a function of concentration. Therefore, it changes with dilution.]

Procedure—First, calibrate the instrument by the manufacturer's instructions. Confirm the instrument calibration with at least one solution from *Table 1* such that the osmolality of the *Standard Solution* lies within 50 mOsmol/kg of the expected value of the *Test Solution* or the center of the expected range of osmolality of the *Test Solution*. The instrument reading should be within ± 4 mOsmol per kg from the *Standard Solution*. Introduce an appropriate volume of each *Standard Solution* into the measurement cell as in the manufacturer's instructions, and start the cooling system. Usually, the mixing device is programmed to operate at a temperature below the lowest temperature expected from the freezing point depression. The apparatus indicates when the equilibrium is attained. If necessary, calibrate the osmometer, using an appropriate adjustment device such that the reading corresponds to either the osmolality or freezing point depression value of the *Standard Solution* shown in *Table 1*. [NOTE—If the instrument reading indicates the freezing point depression, the osmolality can be derived by using the appropriate formula under *Osmolality*.] Repeat the procedure with each *Test Solution*. Read the osmolality of the *Test Solution* directly, or calculate it from the measured freezing point depression.

Assuming that the value of the osmotic coefficient is essentially the same whether the concentration is expressed in molality or molarity, the experimentally determined osmolality of a solution can be converted to osmolality in the same manner in which the concentration of a solution is converted from molality to molarity. Unless a solution is very concentrated, the osmolality of a solution (ξ_c) can be calculated from its experimentally determined osmolality (ξ_m):

$$\xi_c = 1000\xi_m / (1000 / \rho + \sum w_i v_i)$$

where w_i is the weight in g; and v_i is the partial specific volume, in mL per g, of the i^{th} solute. The partial specific volume of a solute is the change in volume of a solution when an additional 1 g of solute is dissolved in the solution. This volume can be determined by the measurement of densities of the solution before and after the addition of the solute. The partial specific volumes of salts are generally very small, around 0.1 mL per g. However, those of other solutes are generally higher. For example, the partial specific volumes of amino acids are in the range of 0.6–0.9 mL per g. It can be shown from the above equation correlating osmolality with osmolality that,

$$\xi_c = \xi_m (\rho - c)$$

where ρ is the density of the solution, and c is the total solute concentration, both expressed in g per mL. Thus, alternatively, the osmolality can also be calculated from experimentally determined osmolality from the measurement of density of the solution by a suitable method and the total

weight of the solute, after correction for water content, dissolved per mL of the solution.

〈786〉 PARTICLE SIZE DISTRIBUTION ESTIMATION BY ANALYTICAL SIEVING

Sieving is one of the oldest methods of classifying powders and granules by particle size distribution. When using a woven sieve cloth, the sieving will essentially sort the particles by their intermediate size dimension (i.e., breadth or width). Mechanical sieving is most suitable where the majority of the particles are larger than about 75 μm . For smaller particles, the light weight provides insufficient force during sieving to overcome the surface forces of cohesion and adhesion that cause the particles to stick to each other and to the sieve, and thus cause particles that would be expected to pass through the sieve to be retained. For such materials, other means of agitation such as air-jet sieving or sonic sifting may be more appropriate. Nevertheless, sieving can sometimes be used for some powders or granules having median particle sizes smaller than 75 μm where the method can be validated. In pharmaceutical terms, sieving is usually the method of choice for classification of the coarser grades of single powders or granules. It is a particularly attractive method in that powders and granules are classified only on the basis of particle size, and in most cases the analysis can be carried out in the dry state.

Among the limitations of the sieving method are the need for an appreciable amount of sample (normally at least 25 g, depending on the density of the powder or granule, and the diameter of test sieves) and difficulty in sieving oily or other cohesive powders or granules that tend to clog the sieve openings. The method is essentially a two-dimensional estimate of size because passage through the sieve aperture is frequently more dependent on maximum width and thickness than on length.

This method is intended for estimation of the total particle size distribution of a single material. It is not intended for determination of the proportion of particles passing or retained on one or two sieves.

Estimate the particle size distribution as described under *Dry Sieving Method*, unless otherwise specified in the individual monograph. Where difficulty is experienced in reaching the endpoint (i.e., material does not readily pass through the sieves) or when it is necessary to use the finer end of the sieving range (below 75 μm), serious consideration should be given to the use of an alternative particle-sizing method.

Sieving should be carried out under conditions that do not cause the test sample to gain or lose moisture. The relative humidity of the environment in which the sieving is carried out should be controlled to prevent moisture uptake or loss by the sample. In the absence of evidence to the contrary, analytical test sieving is normally carried out at ambient humidity. Any special conditions that apply to a particular material should be detailed in the individual monograph.

Principles of Analytical Sieving—Analytical test sieves are constructed from a woven-wire mesh, which is of simple weave that is assumed to give nearly square apertures and is sealed into the base of an open cylindrical container. The basic analytical method involves stacking the sieves on top of one another in ascending degrees of coarseness, and then placing the test powder on the top sieve.

The nest of sieves is subjected to a standardized period of agitation, and then the weight of material retained on each sieve is accurately determined. The test gives the weight percentage of powder in each sieve size range.

This sieving process for estimating the particle size distribution of a single pharmaceutical powder is generally intended for use where at least 80% of the particles are larger than 75 µm. The size parameter involved in determining particle size distribution by analytical sieving is the length of the side of the minimum square aperture through which the particle will pass.

TEST SIEVES

Test sieves suitable for pharmacopeial tests conform to the most current edition of International Organization for Standardization Specification ISO 3310-1: Test Sieves—Technical Requirements and Testing (see *Table 1*). Unless otherwise specified in the monograph, use those ISO sieves listed as principal sizes in *Table 1*. Unless otherwise specified in the monograph, use those ISO sieves listed in *Table 1* as recommended in the particular region.

Table 1. Sizes of Standard Sieve Series in Range of Interest

ISO Nominal Aperture			US Sieve No.	Recommended USP Sieves (microns)	European Sieve No.	Japan Sieve No.
Principal Sizes	Supplementary Sizes					
R 20/3	R 20	R 40/3				
11.20 mm	11.20 mm	11.20 mm			11200	
	10.00 mm					
8.00 mm		9.50 mm				
	9.00 mm					
	8.00 mm	8.00 mm				
	7.10 mm					
5.60 mm		6.70 mm				
	6.30 mm					
	5.60 mm	5.60 mm			5600	3.5
	5.00 mm					4
4.00 mm		4.75 mm				
	4.50 mm					
	4.00 mm	4.00 mm	5	4000	4000	4.7
	3.55 mm					
2.80 mm		3.35 mm	6			5.5
	3.15 mm					
	2.80 mm	2.80 mm	7	2800	2800	6.5
	2.50 mm					
2.00 mm		2.36 mm	8			7.5
	2.24 mm					
	2.00 mm	2.00 mm	10	2000	2000	8.6
	1.80 mm					
1.40 mm		1.70 mm	12			10
	1.60 mm					
	1.40 mm	1.40 mm	14	1400	1400	12
	1.25 mm					
1.00 mm		1.18 mm	16			14
	1.12 mm					
	1.00 mm	1.00 mm	18	1000	1000	16
	900 μm					
710 μm		850 μm	20			18
	800 μm					
	710 μm	710 μm	25	710	710	22
	630 μm					
500 μm		600 μm	30			26
	560 μm					
	500 μm	500 μm	35	500	500	30
	450 μm					
355 μm		425 μm	40			36
	400 μm					
	355 μm	355 μm	45	355	355	42
	315 μm					
250 μm		300 μm	50			50
	280 μm					
	250 μm	250 μm	60	250	250	60
	224 μm					
180 μm		212 μm	70			70
	200 μm					
	180 μm	180 μm	80	180	180	83

Table 1. Sizes of Standard Sieve Series in Range of Interest (Continued)

ISO Nominal Aperture			US Sieve No.	Recommended USP Sieves (microns)	European Sieve No.	Japan Sieve No.
Principal Sizes	Supplementary Sizes					
R 20/3	R 20	R 40/3				
125 μm	160 μm					
		150 μm	100			100
	140 μm					
	125 μm	125 μm	120	125	125	119
90 μm	112 μm					
		106 μm	140			140
	100 μm					
	90 μm	90 μm	170	90	90	166
63 μm	80 μm					
		75 μm	200			200
	71 μm					
	63 μm	63 μm	230	63	63	235
45 μm	56 μm					
		53 μm	270			282
	50 μm					
	45 μm	45 μm	325	45	45	330
	40 μm					
		38 μm			38	391

Sieves are selected to cover the entire range of particle sizes present in the test specimen. A nest of sieves having a $\sqrt{2}$ progression of the area of the sieve openings is recommended. The nest of sieves is assembled with the coarsest screen at the top and the finest at the bottom. Use micrometers or millimeters in denoting test sieve openings. [NOTE—Mesh numbers are provided in the table for conversion purposes only.] Test sieves are made from stainless steel or, less preferably, from brass or other suitable nonreactive wire.

Calibration and recalibration of test sieves is in accordance with the most current edition of ISO 3310-1. Sieves should be carefully examined for gross distortions and fractures, especially at their screen frame joints, before use. Sieves may be calibrated optically to estimate the average opening size, and opening variability, of the sieve mesh. Alternatively, for the evaluation of the effective opening of test sieves in the size range of 212 to 850 µm, Standard Glass Spheres are available. Unless otherwise specified in the individual monograph, perform the sieve analysis at controlled room temperature and at ambient relative humidity.

Cleaning Test Sieves—Ideally, test sieves should be cleaned using only an air jet or a liquid stream. If some apertures remain blocked by test particles, careful gentle brushing may be used as a last resort.

Test Specimen—If the test specimen weight is not given in the monograph for a particular material, use a test specimen having a weight between 25 and 100 g, depending on the bulk density of the material, and test sieves having a 200-mm diameter. For 76-mm sieves, the amount of material that can be accommodated is approximately 1/7th that which can be accommodated on a 200-mm sieve. Determine the most appropriate weight for a given material by test sieving accurately weighed specimens of different weights, such as 25, 50, and 100 g, for the same time period on a mechanical shaker. [NOTE—If the test results are similar for the 25-g and 50-g specimens, but the 100-g specimen shows a lower percentage through the finest sieve, the 100-g specimen size is too large.] Where only a specimen of 10 to 25 g is available, smaller diameter test sieves conforming to the same mesh specifications may be substituted, but the endpoint must be redetermined. The use of test samples having a smaller mass (e.g., down to 5 g) may be needed. For materials with low apparent particle density, or for materials mainly comprising particles with a highly isodiametrical shape, specimen weights below 5 g for a 200-mm screen may be necessary to avoid excessive

blocking of the sieve. During validation of a particular sieve analysis method, it is expected that the problem of sieve blocking will have been addressed.

If the test material is prone to picking up or losing significant amounts of water with varying humidity, the test must be carried out in an appropriately controlled environment. Similarly, if the test material is known to develop an electrostatic charge, careful observation must be made to ensure that such charging is not influencing the analysis. An anti-static agent, such as colloidal silicon dioxide and/or aluminum oxide, may be added at a 0.5 percent (m/m) level to minimize this effect. If both of the above effects cannot be eliminated, an alternative particle-sizing technique must be selected.

Agitation Methods—Several different sieve and powder agitation devices are commercially available, all of which may be used to perform sieve analyses. However, the different methods of agitation may give different results for sieve analyses and endpoint determinations because of the different types and magnitude of the forces acting on the individual particles under test. Methods using mechanical agitation or electromagnetic agitation, and that can induce either a vertical oscillation or a horizontal circular motion, or tapping or a combination of both tapping and horizontal circular motion are available. Entrainment of the particles in an air stream may also be used. The results must indicate which agitation method was used and the agitation parameters used (if they can be varied), because changes in the agitation conditions will give different results for the sieve analysis and endpoint determinations, and may be sufficiently different to give a failing result under some circumstances.

Endpoint Determination—The test sieving analysis is complete when the weight on any of the test sieves does not change by more than 5% or 0.1 g (10% in the case of 76-mm sieves) of the previous weight on that sieve. If less than 5% of the total specimen weight is present on a given sieve, the endpoint for that sieve is increased to a weight change of not more than 20% of the previous weight on that sieve.

If more than 50% of the total specimen weight is found on any one sieve, unless this is indicated in the monograph, the test should be repeated, but with the addition to the sieve nest of a more coarse sieve, intermediate between that carrying the excessive weight and the next coarsest sieve in the original nest, i.e., addition of the ISO series sieve omitted from the nest of sieves.

SIEVING METHODS

Mechanical Agitation

Dry Sieving Method—Tare each test sieve to the nearest 0.1 g. Place an accurately weighed quantity of test specimen on the top (coarsest) sieve, and replace the lid. Agitate the nest of sieves for 5 minutes. Then carefully remove each from the nest without loss of material. Reweigh each sieve, and determine the weight of material on each sieve. Determine the weight of material in the collecting pan in a similar manner. Reassemble the nest of sieves, and agitate for 5 minutes. Remove and weigh each sieve as previously described. Repeat these steps until the endpoint criteria are met (see *Endpoint Determination* under *Test Sieves*). Upon completion of the analysis, reconcile the weights of material. Total losses must not exceed 5% of the weight of the original test specimen.

Repeat the analysis with a fresh specimen, but using a single sieving time equal to that of the combined times used above. Confirm that this sieving time conforms to the requirements for endpoint determination. When this endpoint has been validated for a specific material, then a single fixed time of sieving may be used for future analyses, providing the particle size distribution falls within normal variation.

If there is evidence that the particles retained on any sieve are aggregates rather than single particles, the use of mechanical dry sieving is unlikely to give good reproducibility, and a different particle size analysis method should be used.

Air Entrainment Methods

Air Jet and Sonic Sifter Sieving—Different types of commercial equipment that use a moving air current are available for sieving. A system that uses a single sieve at a time is referred to as air jet sieving. It uses the same general sieving methodology as that described under the *Dry Sieving Method*, but with a standardized air jet replacing the normal agitation mechanism. It requires sequential analyses on individual sieves starting with the finest sieve to obtain a particle size distribution. Air jet sieving often includes the use of finer test sieves than those used in ordinary dry sieving. This technique is more suitable where only oversize or undersize fractions are needed.

In the sonic sifting method, a nest of sieves is used, and the test specimen is carried in a vertically oscillating column of air that lifts the specimen and then carries it back against the mesh openings at a given number of pulses per minute. It may be necessary to lower the sample amount to 5 g, when sonic sifting is employed.

The air jet sieving and sonic sieving methods may be useful for powders or granules when mechanical sieving techniques are incapable of giving a meaningful analysis.

These methods are highly dependent upon proper dispersion of the powder in the air current. This requirement may be hard to achieve if the method is used at the lower end of the sieving range (i.e., below 75 μm), when the particles tend to be more cohesive, and especially if there is any tendency for the material to develop an electrostatic charge. For the above reasons endpoint determination is particularly critical, and it is very important to confirm that the oversize material comprises single particles and is not composed of aggregates.

INTERPRETATION

The raw data must include the weight of test specimen, the total sieving time, and the precise sieving methodology and the set values for any variable parameters, in addition to the weights retained on the individual sieves and in the

pan. It may be convenient to convert the raw data into a cumulative weight distribution, and if it is desired to express the distribution in terms of a cumulative weight undersize, the range of sieves used should include a sieve through which all the material passes. If there is evidence on any of the test sieves that the material remaining on it is composed of aggregates formed during the sieving process, the analysis is invalid.

(788) PARTICULATE MATTER IN INJECTIONS

Change to read:

This general chapter is harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. These pharmacopoeias have undertaken not to make any unilateral change to this harmonized chapter. Portions of the present general chapter text that are national USP text, and therefore not part of the harmonized text, are marked with symbols (♦) to specify this fact.

Particulate matter in injections and parenteral infusions consists of extraneous mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions.

♦As stated in *Injections* (1), solutions for injection administered by the intramuscular or subcutaneous route must meet the requirements of *Particulate Matter in Injections* (788). This requirement has been indefinitely postponed for products for veterinary use. Parenterals packaged and labeled exclusively for use as irrigating solutions are exempt from the requirements of *Particulate Matter in Injections* (788). Radiopharmaceutical preparations are exempt from the requirements of *Particulate Matter in Injections* (788). Parenteral products for which the labeling specifies use of a final filter prior to administration are exempt from the requirements of *Particulate Matter in Injections* (788), provided that scientific data are available to justify this exemption.♦

For the determination of particulate matter, two procedures, *Method 1 (Light Obscuration Particle Count Test)* and *Method 2 (Microscopic Particle Count Test)*, are specified hereinafter. When examining injections and parenteral infusions for subvisible particles, *Method 1* is preferably applied. However, it may be necessary to test some preparations by the *Light Obscuration Particle Count Test* followed by the *Microscopic Particle Count Test* to reach a conclusion on conformance to the requirements.

Not all parenteral preparations can be examined for subvisible particles by one or both of these methods. When *Method 1* is not applicable, e.g., in the case of preparations having reduced clarity or increased viscosity, the test should be carried out according to *Method 2*. Emulsions, colloids, and liposomal preparations are examples. Similarly, products that produce air or gas bubbles when drawn into the syringe may also require microscopic particle count testing. If the viscosity of the preparation to be tested is sufficiently high so as to preclude its examination by either test method, a quantitative dilution with an appropriate diluent may be made to decrease viscosity, as necessary, to allow the analysis to be performed.

The results obtained in examining a discrete unit or group of units for particulate matter cannot be extrapolated with certainty to other units that remain untested. Thus, statistically sound sampling plans must be developed if valid inferences are to be drawn from observed data to characterize the level of particulate matter in a large group of units.

•For the purpose of this chapter, small-volume parenteral is synonymous with small-volume injection, and large-volume parenteral is synonymous with large-volume injection. ♦♦ (RB 1-Jul-2012)

Change to read:

METHOD 1 LIGHT OBSCURATION PARTICLE COUNT TEST

Use a suitable apparatus based on the principle of light blockage that allows for an automatic determination of the size of particles and the number of particles according to size. The definition for *particle-free water* is provided in *Reagents, Indicators, and Solutions—Reagent Specifications*.

The apparatus is calibrated using dispersions of spherical particles of known sizes between 10 μm and 25 μm . These standard particles are dispersed in *particle-free water*. Care must be taken to avoid aggregation of particles during dispersion. ♦System suitability can be verified by using the USP Particle Count RS.♦

General Precautions

The test is carried out under conditions limiting particulate matter, preferably in a laminar flow cabinet.

Very carefully wash the glassware and filtration equipment used, except for the membrane filters, with a warm detergent solution, and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse the equipment from top to bottom, outside and then inside, with *particle-free water*.

Take care not to introduce air bubbles into the preparation to be examined, especially when fractions of the preparation are being transferred to the container in which the determination is to be carried out.

In order to check that the environment is suitable for the test, that the glassware is properly cleaned, and that the water to be used is particle-free, the following test is carried out. Determine the particulate matter in five samples of *particle-free water*, each of 5 mL, according to the method described below. If the number of particles of 10 μm or greater size exceeds 25 for the combined 25 mL, the precautions taken for the test are not sufficient. The preparatory steps must be repeated until the environment, glassware, and water are suitable for the test.

Method

Mix the contents of the sample by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of *particle-free water*, and remove the closure, avoiding any contamination of the contents. Eliminate gas bubbles by appropriate measures such as allowing to stand for 2 min or sonicating.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 mL in volume, the contents of 10 or more units are combined in a cleaned container to obtain a volume of NLT 25 mL; the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 mL with *particle-free water* or with an appropriate particle-free solvent when *particle-free water* is not suitable. Small-volume parenterals having a volume of 25 mL or more may be tested individually.

Powders for parenteral use are reconstituted with *particle-free water* or with an appropriate particle-free solvent when *particle-free water* is not suitable.

▲♦For pharmacy bulk packages for parenteral use labeled "Not for Direct Infusion", proceed as directed for small-volume parenterals when the volume is 25 mL or more. Calcula-

late the test result on a portion that is equivalent to the maximum dose given in the labeling. For example, if the total bulk package volume is 100 mL and the maximum dose volume is 10 mL, then the average particle count per mL would be multiplied by 10 to obtain the test result based on the 10-mL maximum dose. [NOTE—For the calculation of test results, consider this maximum dose portion to be equivalent to the contents of one full container.]

Products packaged with dual compartments meant to hold a drug product and a solvent should be prepared and tested as directed for large-volume parenterals or small-volume parenterals, depending on container volume. Mix each unit as directed in the labeling, activating and agitating to ensure thorough mixing of the separate components and drug dissolution. ♦♦ USP36

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 mL or more, fewer than 10 units may be tested, using an appropriate sampling plan.

Remove four portions, NLT 5 mL each, and count the number of particles equal to or greater than 10 μm and 25 μm . Disregard the result obtained for the first portion, and calculate the mean number of particles for the preparation to be examined.

Evaluation

For preparations supplied in containers with a nominal volume of more than 100 mL, apply the criteria of *Test 1.A*.

For preparations supplied in containers with a nominal volume of less than 100 mL, apply the criteria of *Test 1.B*.

For preparations supplied in containers with a nominal volume of 100 mL, apply the criteria of *Test 1.B*. [NOTE—*Test 1.A* is used in the *Japanese Pharmacopoeia*.]

If the average number of particles exceeds the limits, test the preparation by the *Microscopic Particle Count Test*.

Test 1.A (*Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL*)—The preparation complies with the test if the average number of particles present in the units tested does not exceed 25 per mL equal to or greater than 10 μm and does not exceed 3 per mL equal to or greater than 25 μm .

Test 1.B (*Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL*)—The preparation complies with the test if the average number of particles present in the units tested does not exceed 6000 per container equal to or greater than 10 μm and does not exceed 600 per container equal to or greater than 25 μm .

METHOD 2 MICROSCOPIC PARTICLE COUNT TEST

Use a suitable binocular microscope, a filter assembly for retaining particulate matter, and a membrane filter for examination.

The microscope is adjusted to 100 ± 10 magnifications and is equipped with an ocular micrometer calibrated with an objective micrometer, a mechanical stage capable of holding and traversing the entire filtration area of the membrane filter, and two suitable illuminators to provide episcopic illumination in addition to oblique illumination.

The ocular micrometer is a circular diameter graticule (see *Figure 1*) and consists of a large circle divided by crosshairs into quadrants, transparent and black reference circles 10 μm and 25 μm in diameter at 100 magnifications, and a linear scale graduated in 10- μm increments. It is calibrated using a stage micrometer that is certified by either a domestic or international standard institution. A relative error of the linear scale of the graticule within $\pm 2\%$ is acceptable. The large circle is designated the graticule field of view (GFOV).

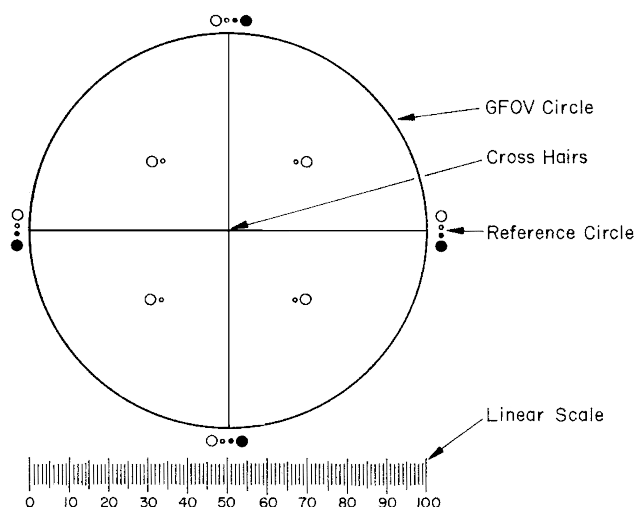


Figure 1. Circular diameter graticule. The large circle divided by crosshairs into quadrants is designated the graticule field of view (GFOV). Transparent and black circles having 10- μ m and 25- μ m diameters at 100 \times are provided as comparison scales for particle sizing.

Two illuminators are required. One is an episcopic brightfield illuminator internal to the microscope, the other is an external, focusable auxiliary illuminator that can be adjusted to give reflected oblique illumination at an angle of 10°–20°.

The filter assembly for retaining particulate matter consists of a filter holder made of glass or other suitable material, and is equipped with a vacuum source and a suitable membrane filter.

The membrane filter is of suitable size, black or dark gray in color, nongridded or gridded, and 1.0 μ m or finer in nominal pore size.

General Precautions

The test is carried out under conditions limiting particulate matter, preferably in a laminar flow cabinet.

Very carefully wash the glassware and filter assembly used, except for the membrane filter, with a warm detergent solution, and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse both sides of the membrane filter and the equipment from top to bottom, outside and then inside, with *particle-free water*.

In order to check that the environment is suitable for the test, that the glassware and the membrane filter are properly cleaned, and that the water to be used is particle-free, the following test is carried out. Determine the particulate matter of a 50-mL volume of *particle-free water* according to the method described below. If more than 20 particles 10 μ m or larger in size or if more than five particles 25 μ m or larger in size are present within the filtration area, the precautions taken for the test are not sufficient. The preparatory steps must be repeated until the environment, glassware, membrane filter, and water are suitable for the test.

Method

Mix the contents of the samples by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of *particle-free water*, and remove the closure, avoiding any contamination of the contents.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 mL in volume, the

contents of 10 or more units are combined in a cleaned container; the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 mL with *particle-free water* or with an appropriate particle-free solvent when *particle-free water* is not suitable. Small-volume parenterals having a volume of 25 mL or more may be tested individually.

Powders for parenteral use are constituted with *particle-free water* or with an appropriate particle-free solvent when *particle-free water* is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 mL or more, fewer than 10 units may be tested, using an appropriate sampling plan.

Wet the inside of the filter holder fitted with the membrane filter with several mL of *particle-free water*. Transfer to the filtration funnel the total volume of a solution pool or of a single unit, and apply a vacuum. If needed, add stepwise a portion of the solution until the entire volume is filtered. After the last addition of solution, begin rinsing the inner walls of the filter holder by using a jet of *particle-free water*. Maintain the vacuum until the surface of the membrane filter is free from liquid. Place the membrane filter in a Petri dish, and allow the membrane filter to air-dry with the cover slightly ajar. After the membrane filter has been dried, place the Petri dish on the stage of the microscope, scan the entire membrane filter under the reflected light from the illuminating device, and count the number of particles that are equal to or greater than 10 μ m and the number of particles that are equal to or greater than 25 μ m. Alternatively, partial membrane filter count and determination of the total filter count by calculation is allowed. Calculate the mean number of particles for the preparation to be examined.

The particle sizing process with the use of the circular diameter graticule is carried out by estimating the equivalent diameter of the particle in comparison with the 10 μ m and 25 μ m reference circles on the graticule. Thereby the particles are not moved from their initial locations within the graticule field of view and are not superimposed on the reference circles for comparison. The inner diameter of the transparent graticule reference circles is used to size white and transparent particles, while dark particles are sized by using the outer diameter of the black opaque graticule reference circles.

In performing the *Microscopic Particle Count Test*, do not attempt to size or enumerate amorphous, semiliquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discoloration on the membrane filter. These materials show little or no surface relief and present a gelatinous or film-like appearance. In such cases, the interpretation of enumeration may be aided by testing a sample of the solution by the *Light Obscuration Particle Count Test*.

Evaluation

For preparations supplied in containers with a nominal volume of more than 100 mL, apply the criteria of *Test 2.A*.

For preparations supplied in containers with a nominal volume of less than 100 mL, apply the criteria of *Test 2.B*.

For preparations supplied in containers with a nominal volume of 100 mL, apply the criteria of *Test 2.B*. [NOTE—*Test 2.A* is used in the *Japanese Pharmacopoeia*.]

Test 2.A (*Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL*)—The preparation complies with the test if the average number of particles present in the units tested does not exceed 12 per mL equal to or greater than 10 μ m and does not exceed 2 per mL equal to or greater than 25 μ m.

Test 2.B (*Solutions for parenteral infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL*)—The preparation complies with the test if the

average number of particles present in the units tested does not exceed 3000 per container equal to or greater than 10 μm and does not exceed 300 per container equal to or greater than 25 μm .

<789> PARTICULATE MATTER IN OPHTHALMIC SOLUTIONS

Particulate matter consists of mobile, randomly sourced, extraneous substances, other than gas bubbles, that cannot be quantitated by chemical analysis because of the small amount of material they represent and because of their heterogeneous composition. Ophthalmic solutions should be essentially free from particles that can be observed on visual inspection. The tests described herein are physical tests performed for the purpose of enumerating extraneous particles within specific size ranges.

Every ophthalmic solution for which the monograph includes a test for *Particulate matter* is subject to the particulate matter limits set forth for the test being applied, unless otherwise specified in the individual monograph. When higher limits are appropriate, they will be specified in the individual monograph. Ophthalmic preparations that are suspensions, emulsions, or gels are exempt from these requirements, as are medical devices. Refer to the specific monograph when a question of test applicability occurs.

Light obscuration and microscopic procedures for the determination of particulate matter in ophthalmic solutions are identical to those for injections; therefore, where appropriate, *Particulate Matter in Injections* (788) is cross-referenced. This chapter provides a test approach in two stages. The ophthalmic solution is first tested by the light obscuration procedure (stage 1). If it fails to meet the prescribed limits, it must pass the microscopic procedure (stage 2) with its own set of test limits. Where for technical reasons the ophthalmic solution cannot be tested by light obscuration, microscopic testing may be used exclusively. Documentation is required, demonstrating that the light obscuration procedure is incapable of testing the ophthalmic solution or that it produces invalid results.

It is expected that most articles will meet the requirements on the basis of the light obscuration test alone; however, it may be necessary to test some articles by the light obscuration test followed by the microscopic test to reach a conclusion on conformance to requirements. Any product that is not a pure solution having a clarity and a viscosity approximating those of water may provide erroneous data when analyzed by the light obscuration counting method. Such materials may be analyzed by the microscopic counting method. In some instances, the viscosity of a material to be tested may be sufficiently high so as to preclude its analysis by either test method. In this event, a quantitative dilution with an appropriate diluent may be made to decrease viscosity, as necessary, to allow the analysis to be performed.

In the tests described below, the results obtained by examining a discrete unit or group of units for particulate matter cannot be extrapolated with certainty to other units that remain untested. Thus, sampling plans based on known operational factors must be developed if valid inferences are to be drawn from observed data to characterize the level of particulate matter in a large group of units. Sampling plans need to be based on consideration of product volume, particle numbers historically found to be present in comparison

to limits, particle size distribution of particles present, and variability of particle counts between units.

LIGHT OBSCURATION PARTICLE COUNT TEST

This test applies to ophthalmic solutions, including solutions constituted from sterile solids, for which a test for *Particulate matter* is specified in the individual monograph. The test counts suspended particles that are solid or liquid.

Test Apparatus, Instrument Standardization, Test Environment, Test Procedure, and Calculations—Proceed as directed for *Light Obscuration Particle Count Test* under *Particulate Matter in Injections* (788).

Interpretation—The ophthalmic solution meets the requirements of the test if the average number of particles present in the units tested does not exceed the appropriate value listed in *Table 1*. If the average number of particles exceeds the limit, test the article by the *Microscopic Particle Count Test*.

Table 1. Light Obscuration Test Particle Count

	Diameter	
	$\geq 10 \mu\text{m}$	$\geq 25 \mu\text{m}$
Number of particles	50 per mL	5 per mL

MICROSCOPIC PARTICLE COUNT TEST

Some articles cannot be tested meaningfully by light obscuration. In such cases, individual monographs clearly specify that only a microscopic particle count is to be performed. The microscopic particle count test enumerates subvisible, essentially solid, particulate matter in ophthalmic solutions, after collection on a microporous membrane filter. Some ophthalmic solutions, such as solutions that do not filter readily because of their high viscosity, may be exempted from analysis using the microscopic test.

When performing the microscopic test, do not attempt to size or enumerate amorphous, semiliquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discoloration on the membrane surface. These materials show little or no surface relief and present a gelatinous or film-like appearance. Because in solution this material consists of units on the order of 1 μm or less, which may be counted only after aggregation or deformation on an analytical membrane, interpretation of enumeration may be aided by testing a sample of the solution by the light obscuration particle count method.

Test Apparatus, Test Environment, Test Procedure, and Enumeration of Particles—Proceed as directed for *Microscopic Particle Count Test* under *Particulate Matter in Injections* (788).

Interpretation—The ophthalmic solution meets the requirements of the test if the average number of particles present in the units tested does not exceed the appropriate value listed in *Table 2*.

Table 2. Microscopic Method Particle Count

	Diameter		
	$\geq 10 \mu\text{m}$	$\geq 25 \mu\text{m}$	$\geq 50 \mu\text{m}$
Number of particles	50 per mL	5 per mL	2 per mL

(791) pH

For compendial purposes, pH is defined as the value given by a suitable, properly standardized, potentiometric instrument (pH meter) capable of reproducing pH values to 0.02 pH unit using an indicator electrode sensitive to hydrogen-ion activity, the glass electrode, and a suitable reference electrode. The instrument should be capable of sensing the potential across the electrode pair and, for pH standardization purposes, applying an adjustable potential to the circuit by manipulation of "standardization," "zero," "asymmetry," or "calibration" control, and should be able to control the change in millivolts per unit change in pH reading through a "temperature" and/or "slope" control. Measurements are made at $25 \pm 2^\circ$, unless otherwise specified in the individual monograph or herein.

The pH scale is defined by the equation:

$$\text{pH} = \text{pH}_s + (E - E_s)/k$$

in which E and E_s are the measured potentials where the galvanic cell contains the solution under test, represented by pH, and the appropriate *Buffer Solution for Standardization*, represented by pH_s, respectively. The value of k is the change in potential per unit change in pH and is theoretically $[0.05916 + 0.000198(t - 25^\circ)]$ volts at any temperature t .

It should be emphasized that the definitions of pH, the pH scale, and the values assigned to the *Buffer Solutions for Standardization* are for the purpose of establishing a practical, operational system so that results may be compared between laboratories. The pH values thus measured do not correspond exactly to those obtained by the definition, $\text{pH} = -\log a_{\text{H}^+}$. So long as the solution being measured is sufficiently similar in composition to the buffer used for standardization, the operational pH corresponds fairly closely to the theoretical pH. Although no claim is made with respect to the suitability of the system for measuring hydrogen-ion activity or concentration, the values obtained are closely related to the activity of the hydrogen-ion in aqueous solutions.

Where a pH meter is standardized by use of an aqueous buffer and then used to measure the "pH" of a nonaqueous solution or suspension, the ionization constant of the acid or base, the dielectric constant of the medium, the liquid-junction potential (which may give rise to errors of approximately 1 pH unit), and the hydrogen-ion response of the glass electrode are all changed. For these reasons, the values so obtained with solutions that are only partially aqueous in character can be regarded only as apparent pH values.

BUFFER SOLUTIONS FOR STANDARDIZATION OF THE pH METER

Buffer Solutions for Standardization are to be prepared as directed in the accompanying table.* Buffer salts of requisite purity can be obtained from the National Institute of Science and Technology. Solutions may be stored in hard glass or polyethylene bottles fitted with a tight closure or carbon dioxide-absorbing tube (soda lime). Fresh solutions should be prepared at intervals not to exceed 3 months using carbon dioxide-free water. The table indicates the pH of the buffer solutions as a function of temperature. The instructions presented here are for the preparation of solutions having the designated molal (m) concentrations. For convenience, and to facilitate their preparation, however, instructions are given in terms of dilution to a 1000-mL volume rather than specifying the use of 1000 g of solvent, which is the basis of the molality system of solution concentration. The indicated quantities cannot be computed simply without additional information.

Potassium Tetraoxalate, 0.05 m—Dissolve 12.61 g of $\text{KH}_3(\text{C}_2\text{O}_4)_2 \cdot 2\text{H}_2\text{O}$ in water to make 1000 mL.

Potassium Biphthalate, 0.05 m—Dissolve 10.12 g of $\text{KHC}_8\text{H}_4\text{O}_4$, previously dried at 110° for 1 hour, in water to make 1000 mL.

Equimolal Phosphate, 0.05 m—Dissolve 3.53 g of Na_2HPO_4 and 3.39 g of KH_2PO_4 , each previously dried at 120° for 2 hours, in water to make 1000 mL.

Sodium Tetraborate, 0.01 m—Dissolve 3.80 g of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ in water to make 1000 mL. Protect from absorption of carbon dioxide.

Calcium Hydroxide, saturated at 25° —Shake an excess of calcium hydroxide with water, and decant at 25° before use. Protect from absorption of carbon dioxide.

Because of variations in the nature and operation of the available pH meters, it is not practicable to give universally applicable directions for the potentiometric determinations of pH. The general principles to be followed in carrying out the instructions provided for each instrument by its manufacturer are set forth in the following paragraphs. Examine the electrodes and, if present, the salt bridge prior to use. If necessary, replenish the salt bridge solution, and observe other precautions indicated by the instrument or electrode manufacturer.

* Commercially available buffer solutions for pH meter standardization, standardized by methods traceable to the National Institute of Standards and Technology (NIST), labeled with a pH value accurate to 0.01 pH unit may be used. For standardization solutions having a pH lower than 4, a labeled accuracy of 0.02 is acceptable. Solutions prepared from ACS reagent grade materials or other suitable materials, in the stated quantities, may be used provided the pH of the resultant solution is the same as that of the solution prepared from the NIST certified material.

pH Values of Buffer Solutions for Standardization

Temperature, $^\circ\text{C}$	Potassium Tetraoxalate, 0.05 m	Potassium Biphthalate, 0.05 m	Equimolal Phosphate, 0.05 m	Sodium Tetraborate, 0.01 m	Calcium Hydroxide, Saturated at 25°
10	1.67	4.00	6.92	9.33	13.00
15	1.67	4.00	6.90	9.28	12.81
20	1.68	4.00	6.88	9.23	12.63
25	1.68	4.01	6.86	9.18	12.45
30	1.68	4.02	6.85	9.14	12.29
35	1.69	4.02	6.84	9.10	12.13
40	1.69	4.04	6.84	9.07	11.98
45	1.70	4.05	6.83	9.04	11.84
50	1.71	4.06	6.83	9.01	11.71
55	1.72	4.08	6.83	8.99	11.57
60	1.72	4.09	6.84	8.96	11.45

To standardize the pH meter, select two *Buffer Solutions for Standardization* whose difference in pH does not exceed 4 units and such that the expected pH of the material under test falls between them. Fill the cell with one of the *Buffer Solutions for Standardization* at the temperature at which the test material is to be measured. Set the “temperature” control at the temperature of the solution, and adjust the calibration control to make the observed pH value identical with that tabulated. Rinse the electrodes and cell with several portions of the second *Buffer Solution for Standardization*, then fill the cell with it, at the same temperature as the material to be measured. The pH of the second buffer solution is within ± 0.07 pH unit of the tabulated value. If a larger deviation is noted, examine the electrodes and, if they are faulty, replace them. Adjust the “slope” or “temperature” control to make the observed pH value identical with that tabulated. Repeat the standardization until both *Buffer Solutions for Standardization* give observed pH values within 0.02 pH unit of the tabulated value without further adjustment of the controls. When the system is functioning satisfactorily, rinse the electrodes and cell several times with a few portions of the test material, fill the cell with the test material, and read the pH value. Use carbon dioxide-free water (see *Water* in the section *Reagents, Indicators, and Solutions*) for solution or dilution of test material in pH determinations. In all pH measurements, allow a sufficient time for stabilization.

Where approximate pH values suffice, indicators and test papers (see *Indicators and Indicator Test Papers*, in the section *Reagents, Indicators, and Solutions*) may be suitable.

For a discussion of buffers, and for the composition of standard buffer solutions called for in compendial tests and assays, see *Buffer Solutions* in the section *Reagents, Indicators, and Solutions*.

<795> PHARMACEUTICAL COMPOUNDING—NONSTERILE PREPARATIONS

INTRODUCTION

The purpose of this chapter is to provide compounders with guidance on applying good compounding practices for the preparation of nonsterile compounded formulations for dispensing and/or administration to humans or animals. Compounding is an integral part of pharmacy practice and is essential to the provision of healthcare. This chapter and applicable monographs on formulation help define good compounding practices. Furthermore, this chapter provides general information to enhance the compounder's ability in the compounding facility to extemporaneously compound preparations that are of acceptable strength, quality, and purity. Pharmacists, other healthcare professionals, and others engaged in the compounding of drug preparations should comply with applicable state and federal compounding laws, regulations, and guidelines.

DEFINITIONS

ACTIVE PHARMACEUTICAL INGREDIENT (API)—Any substance or mixture of substances intended to be used in the compounding of a drug preparation, thereby becoming the active ingredient in that preparation and furnishing pharmaco-

logical activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease in humans and animals or affecting the structure and function of the body.

ADDED SUBSTANCES—Ingredients that are necessary to compound a preparation but are not intended or expected to cause a pharmacologic response if administered alone in the amount or concentration contained in a single dose of the compounded preparation. The term is used synonymously with the terms *inactive ingredients*, *excipients*, and *pharmaceutical ingredients*.

BEYOND-USE DATE (BUD)—The date after which a compounded preparation should not to be used; determined from the date the preparation is compounded.

COMPONENT—Any ingredient used in the compounding of a drug preparation, including any active ingredient or added substance that is used in its preparation.

COMPOUNDER—A professional authorized by the appropriate jurisdiction to perform compounding pursuant to a prescription or medication order by a licensed prescriber.

COMPOUNDING—The preparation, mixing, assembling, altering, packaging, and labeling of a drug, drug-delivery device, or device in accordance with a licensed practitioner's prescription, medication order, or initiative based on the practitioner/patient/pharmacist/compounder relationship in the course of professional practice. Compounding includes the following:

- Preparation of drug dosage forms for both human and animal patients
- Preparation of drugs or devices in anticipation of prescription drug orders based on routine, regularly observed prescribing patterns
- Reconstitution or manipulation of commercial products that may require the addition of one or more ingredients
- Preparation of drugs or devices for the purposes of, or as an incident to, research (clinical or academic), teaching, or chemical analysis
- Preparation of drugs and devices for prescriber's office use where permitted by federal and state law

HAZARDOUS DRUG—Any drug identified by at least one of the following six criteria:

- Carcinogenicity
- Teratogenicity or developmental toxicity
- Reproductive toxicity in humans
- Organ toxicity at low doses in humans or animals
- Genotoxicity
- New drugs that mimic existing hazardous drugs in structure or toxicity [for examples see current National Institute for Occupational Safety and Health (NIOSH) publications]

MANUFACTURING—The production, propagation, conversion, or processing of a drug or device, either directly or indirectly, by extraction of the drug from substances of natural origin or by means of chemical or biological synthesis. Manufacturing may also include any packaging or repackaging of the substance(s) or labeling or relabeling of containers for resale by pharmacies, practitioners, or other persons.

PREPARATION—For the purposes of this chapter, a compounded drug dosage form or dietary supplement or a device to which a compounder has introduced a drug. This term will be used to describe compounded formulations; the term *product* will be used to describe manufactured pharmaceutical dosage forms. (For the definitions of *official substance* and *official products*, see *General Notices and Requirements*.)

STABILITY—The extent to which a preparation retains, within specified limits and throughout its period of storage and use, the same properties and characteristics that it possessed at the time of compounding (see *Stability Considerations in Dispensing Practice* <1191>, the table *Criteria for Acceptable Levels of Stability*)

VEHICLE—A component for internal or external use that is used as a carrier or diluent in which liquids, semisolids, or solids are dissolved or suspended. Examples include, but are not limited to, water, syrups, elixirs, oleaginous liquids, solid and semisolid carriers, and proprietary products.

CATEGORIES OF COMPOUNDING

In the three general categories of nonsterile compounding described in this section, different levels of experience, training, and physical facilities are associated with each category.

Criteria used to determine overall classification include:

- degree of difficulty or complexity of the compounding process
- stability information and warnings
- packaging and storage requirements
- dosage forms
- complexity of calculations
- local versus systemic biological disposition
- level of risk to the compounder
- potential for risk of harm to the patient

See *Pharmaceutical Compounding—Sterile Preparations* (797) for risk levels associated with sterile preparations. Specialty areas such as radiopharmaceuticals require special training and are beyond the scope of this chapter. Compounders shall acquire and maintain knowledge and skills in all areas (e.g., dosage form, patient population, and medical specialty) for which they compound.

Description of Categories

Simple—Making a preparation that has a *United States Pharmacopeia* (USP) compounding monograph or that appears in a peer-reviewed journal article that contains specific quantities of all components, compounding procedure and equipment, and stability data for that formulation with appropriate BUDs; or reconstituting or manipulating commercial products that may require the addition of one or more ingredients as directed by the manufacturer. Examples include *Captopril Oral Solution*, *Indomethacin Topical Gel*, and *Potassium Bromide Oral Solution, Veterinary*.

Moderate—Making a preparation that requires special calculations or procedures (such as calibration of dosage unit mold cavities) to determine quantities of components per preparation or per individualized dosage units; or making a preparation for which stability data for that specific formulation are not available. Examples include *Morphine Sulfate Suppositories*, diphenhydramine hydrochloride troches, and mixing two or more manufactured cream products when the stability of the mixture is not known.

Complex—Making a preparation that requires special training, environment, facilities, equipment, and procedures to ensure appropriate therapeutic outcomes. Examples of possible complex preparation types include transdermal dosage forms, modified-release preparations, and some inserts and suppositories for systemic effects.

RESPONSIBILITIES OF THE COMPOUNDER

The compounder is responsible for compounding preparations of acceptable strength, quality, and purity and in accordance with the prescription or medication order. The compounder is also responsible for dispensing the finished preparation, with appropriate packaging and labeling, and in compliance with the requirements established by the applicable state agencies, state boards of pharmacy, federal law, and other regulatory agencies where appropriate. Individuals who are engaged in drug or dietary supplement compounding shall be proficient in compounding and should continually expand their compounding knowledge by participating in seminars and/or studying appropriate literature. They shall be knowledgeable about the contents

of this chapter and should be familiar with *Pharmaceutical Compounding—Sterile Preparations* (797), *Pharmaceutical Dosage Forms* (1151), *Pharmaceutical Calculations in Prescription Compounding* (1160), *Quality Assurance in Pharmaceutical Compounding* (1163), *Prescription Balances and Volumetric Apparatus* (1176), *Stability Considerations in a Dispensing Practice* (1191), *Written Prescription Drug Information—Guidelines* (1265), and all applicable compounding laws, guidelines, and standards.

To ensure the quality of compounded preparations, compounders shall adhere to the following general principles (additional information on these general principles is provided in the sections that follow).

General Principles of Compounding

1. Personnel are appropriately trained and are capable of performing and qualified to perform their assigned duties. Such training should be documented.
2. Compounding ingredients of the appropriate identity, purity, and quality are purchased from reliable sources and are properly stored according to manufacturer specifications or USP standards.
3. Bulk component containers are labeled with appropriate Occupational Safety and Health Administration (OSHA) hazard communication labels (see OSHA.gov), and Material Safety Data Sheets (MSDSs) are available to compounding personnel for all drugs and chemicals used in compounding.
4. All equipment used in compounding is clean, properly maintained, and used appropriately.
5. The compounding environment is suitable for its intended purpose; and procedures are implemented to prevent cross-contamination, especially when compounding with drugs (e.g., hazardous drugs and known allergens like penicillin) that require special precautions.
6. Only authorized personnel are allowed in the immediate vicinity of the drug compounding operations.
7. There is assurance that processes are always carried out as intended or specified and are reproducible.
8. Compounding conditions and procedures are adequate for preventing errors.
9. All aspects of compounding are appropriately documented.
10. Adequate procedures and records exist for investigating and correcting failures or problems in compounding, testing, or the preparation itself.

COMPOUNDING PROCESS

The compounder is responsible for ensuring that each individual incidence of compounding meets the criteria given in this section (additional information on these criteria is provided in the sections that follow).

Criteria When Compounding Each Drug Preparation

1. The dose, safety, and intended use of the preparation or device has been evaluated for suitability in terms of:
 - the chemical and physical properties of the components
 - dosage form
 - therapeutic appropriateness and route of administration, including local and systemic biological disposition
 - legal limitations, if any
2. A Master Formulation Record should be created before compounding a preparation for the first time. This record shall be followed each time that preparation is made. In addition, a Compounding Record

should be completed each time a preparation is compounded.

3. Ingredients used in the formulation have their expected identity, quality, and purity. If the formulation is for humans, ingredients are not on a list of federally recognized drugs or specific drug products that have been withdrawn or removed from the market for safety or efficacy reasons (see www.FDA.gov). If the formulation is for food-producing animals, ingredients are not on a list of components prohibited for use in food-producing animals. Certificates of Analysis, when applicable, and MSDSs have been consulted for all ingredients used.
4. Compounding is done in an appropriately clean and sanitized area dedicated to this activity (see the section *Compounding Facilities*).
5. Only one preparation is compounded at one time in a specific workspace.
6. Appropriate compounding equipment has been selected and inspected for cleanliness and correct functioning and is properly used.
7. A reliable BUD is established to ensure that the finished preparation has its accepted potency, purity, quality, and characteristics, at least until the labeled BUD.
8. Personnel engaged in compounding maintain good hand hygiene and wear clean clothing appropriate to the type of compounding performed (e.g., hair bonnets, coats, gowns, gloves, facemasks, shoes, aprons, or other items) as needed for protection of personnel from chemical exposures and for prevention of drug contamination.
9. The preparation is made in accordance with this chapter, other official standards referenced in this chapter, and relevant scientific data and information.
10. Critical processes (including but not limited to weighing, measuring, and mixing) are verified by the compounder to ensure that procedures, when used, will consistently result in the expected qualities in the finished preparation.
11. The final preparation is assessed using factors such as weight, adequacy of mixing, clarity, odor, color, consistency, pH, and analytical testing as appropriate; and this information is recorded on the Compounding Record (see Chapter (1163)).
12. The preparation is packaged as recommended in the *Packaging and Drug Preparation Containers* section of this chapter.
13. The preparation container is labeled according to all applicable state and federal laws. The labeling shall include the BUD and storage and handling information. The labeling should indicate that "this is a compounded preparation."
14. The Master Formulation Record and the Compounding Record have been reviewed by the compounder to ensure that errors have not occurred in the compounding process and that the preparation is suitable for use.
15. The preparation is delivered to the patient or caregiver with the appropriate consultation.

COMPOUNDING FACILITIES

Compounding facilities shall have an adequate space that is specifically designated for compounding of prescriptions. This space shall provide for the orderly placement of equipment and materials to prevent mixups among ingredients, containers, labels, in-process materials, and finished preparations and is designed, arranged, and used to prevent adventitious cross-contamination. Areas used for sterile preparations shall be separated and distinct from the nonsterile compounding area (see Chapter (797), *Environmental Quality and Control*).

Potable water shall be supplied for hand and equipment washing. This water meets the standards prescribed in the Environmental Protection Agency's National Primary Drinking Water Regulations (40 CFR Part 141). *Purified Water* (see *Purified Water* monograph) shall be used for compounding nonsterile drug preparations when formulations indicate the inclusion of water. *Purified Water* should be used for rinsing equipment and utensils. In those cases when a water is used to prepare a sterile preparation, follow the appropriate monographs and general chapters (see *Water for Pharmaceutical Purposes* (1231)).

The plumbing system shall be free of defects that could contribute to contamination of any compounded preparation. Adequate hand and equipment washing facilities shall be easily accessible to the compounding areas. Such facilities shall include, but are not limited to, hot and cold water, soap or detergent, and an air-drier or single-use towels. The areas used for compounding shall be maintained in clean, orderly, and sanitary conditions and shall be maintained in a good state of repair. Waste shall be held and disposed of in a sanitary and timely manner and in accordance with local, state, and federal guidelines.

The entire compounding and storage area should be well lighted. Heating, ventilation, and air conditioning systems shall be controlled to avoid decomposition and contamination of chemicals (see the *General Notices and Requirements, Preservation, Packaging, Storage, and Labeling, Storage Temperature and Humidity*; and the manufacturers' labeled storage conditions). Appropriate temperature and humidity monitoring should be maintained as required for certain components and compounded dosage forms. All components, equipment, and containers shall be stored off the floor and in a manner to prevent contamination and permit inspection and cleaning of the compounding and storage area.

Hazardous drugs shall be stored, prepared, and handled by appropriately trained personnel under conditions that protect the healthcare workers and other personnel. The following are references for the safe handling of antineoplastic and hazardous drugs in healthcare settings:

- OSHA Technical Manual—Section VI: Chapter 2, *Controlling Occupational Exposure to Hazardous Drugs*
- NIOSH Alert: *Preventing Occupational Exposure to Antineoplastic and Other Hazardous Drugs in Health Care Settings* (DHHS (NIOSH) Publication No. 2004-165) and updates.

Disposal of all hazardous drug wastes shall comply with all applicable federal and state regulations. All personnel who perform routine custodial waste removal and cleaning activities in storage and preparation areas for hazardous drugs shall be trained in appropriate procedures to protect themselves and prevent contamination.

COMPOUNDING EQUIPMENT

The equipment and utensils used for compounding of a drug preparation shall be of appropriate design and capacity. The equipment shall be of suitable composition that the surfaces that contact components are neither reactive, additive, nor sorptive and therefore will not affect or alter the purity of the compounded preparations. The types and sizes of equipment depend on the dosage forms and the quantities compounded (see Chapter (1176) and equipment manufacturers' instruction manuals).

Equipment shall be stored to protect it from contamination and shall be located to facilitate its use, maintenance, and cleaning. Automated, mechanical, electronic, and other types of equipment used in compounding or testing of compounded preparations shall be routinely inspected, calibrated as necessary, and checked to ensure proper performance. Immediately before compounding operations, the equipment shall be inspected by the compounder to determine its suitability for use. After use, the equipment shall be appropriately cleaned.

Extra care should be used when cleaning equipment used in compounding preparations that require special precaution (e.g., antibiotics and cytotoxic and other hazardous materials). When possible, special equipment should be dedicated for such use, or when the same equipment is being used for all drug products, appropriate procedures shall be in place to allow meticulous cleaning of equipment before use with other drugs. If possible, disposable equipment should be used to reduce chances of bioburden and cross-contamination.

COMPONENT SELECTION, HANDLING, AND STORAGE

The following guidelines shall be followed when selecting, handling, and storing components for compounded preparations.

1. A *United States Pharmacopeia (USP)*, *National Formulary (NF)*, or *Food Chemicals Codex (FCC)* substance is the recommended source of ingredients for compounding all preparations.
2. Compounders shall first attempt to use components manufactured in an FDA-registered facility. When components cannot be obtained from an FDA-registered facility, compounders shall use their professional judgment in selecting an acceptable and reliable source and shall establish purity and safety by reasonable means, which should include Certificate of Analysis, manufacturer reputation, and reliability of source.
3. Official compounded preparations are prepared from ingredients that meet requirements of the compendial monograph for those individual ingredients for which monographs are provided. These preparations may be labeled *USP* or *NF* as appropriate.
4. When components of compendial quality are not obtainable, components of high quality such as those that are chemically pure, analytical reagent grade, or American Chemical Society-certified may be used. However, these components should be used cautiously because the standards for analytical reagents or American Chemical Society-grade materials do not consider whether any impurity present raises human or animal safety concerns.
5. For components in containers that have an expiration date from the manufacturer or distributor, the material may be used in compounding before that expiration date (a) when the material is stored in its original container under conditions to avoid decomposition of the chemicals (see Chapter (1191) and (659) *Packaging and Storage Requirements*, unless other conditions are noted on the label), (b) when there is minimal exposure of the remaining material each time material is withdrawn from the container, and (c) when any withdrawals from the container are performed by those trained in the proper handling of the material. If the component has been transferred to a different container, that container shall be identified with the component name, original supplier, lot or control number, transfer date, and expiration date and shall provide integrity that is equivalent to or better than that of the original container.
6. For components that do not have expiration dates assigned by the manufacturer or supplier, the compounder shall label the container with the date of receipt and assign a conservative expiration date, not to exceed three years after receipt, to the component (see the *General Notices and Requirements, Preservation, Packaging, Storage, and Labeling, Labeling, Expiration Date and Beyond-Use Date*) based on the nature of the component and its degradation mechanism, the container in which it is packaged, and the storage conditions.
7. If a manufactured drug product is used as the source of active ingredient, the drug product shall be manufactured in an FDA-registered facility, and the manufacturer's product container shall be labeled with a batch control number and expiration date. When compounding with manufactured drug products, the compounder shall consider all ingredients, including excipients, present in the drug product relative to the intended use of the compounded preparation and the effect of manipulating the drug product on the therapeutic appropriateness and stability of the components.
8. If the preparation is intended for use as a dietary or nutritional supplement, then the compounder must adhere to this chapter and must also comply with any federal and state requirements. Generally, dietary supplements are prepared from ingredients that meet *USP*, *FCC*, or *NF* standards. Where such standards do not exist, substances may be used in dietary supplements if they have been shown to have acceptable food-grade quality using other suitable procedures.
9. When a component is derived from ruminant animals (e.g., bovine, caprine, ovine), the supplier shall provide written assurance that the component is in compliance with all federal laws governing processing, use, and importation requirements for these materials.
10. When compounding for humans, the compounder should consult the list of components that have been withdrawn or removed from the market for safety or efficacy reasons by FDA (see www.FDA.gov). When compounding for food-producing animals, the compounder should consult the list of components prohibited for use in food-producing animals.
11. All components used in the compounding of preparations must be stored as directed by the manufacturer, or according to *USP*, *NF*, or *FCC* monograph requirements, in a clean area, and under appropriate temperature and humidity conditions (controlled room temperature, refrigerator, or freezer). All components shall be stored off the floor, handled and stored to prevent contamination, and rotated so that the oldest stock is used first. All containers shall be properly labeled.

STABILITY CRITERIA AND BEYOND-USE DATING

The BUD is the date after which a compounded preparation shall not be used and is determined from the date when the preparation is compounded. Because compounded preparations are intended for administration immediately or following short-term storage, their BUDs are assigned on the basis of criteria different from those applied to assigning expiration dates to manufactured drug products.

BUDs should be assigned conservatively. When assigning a BUD, compounders shall consult and apply drug-specific and general stability documentation and literature when available and should consider:

- the nature of the drug and its degradation mechanism
- the dosage form and its components
- the potential for microbial proliferation in the preparation
- the container in which it is packaged
- the expected storage conditions
- the intended duration of therapy (see the *General Notices and Requirements, Preservation, Packaging, Storage, and Labeling, Labeling, Expiration Date and Beyond-Use Date*).

When a manufactured product is used as the source of the API for a nonsterile compounded preparation, the product expiration date cannot be used solely to assign a BUD for the compounded preparation. Instead, the compounder

shall refer to the manufacturer for stability information and to the literature for applicable information on stability, compatibility, and degradation of ingredients; shall consider stability factors in Chapter (1191); and shall use his or her compounding education and experience. All stability data shall be carefully interpreted in relation to the actual compounded formulation.

At all steps in the compounding, dispensing, and storage process, the compounder shall observe the compounded drug preparation for signs of instability. For more specific details of some of the common physical signs of deterioration (see Chapter (1191), *Observing Products for Evidence of Instability*). However, excessive chemical degradation and other drug concentration loss due to reactions may be invisible more often than visible.

General Guidelines for Assigning Beyond-Use Dates

In the absence of stability information that is applicable to a specific drug and preparation, the following table presents maximum BUDs recommended for (1) nonsterile compounded drug preparations that are packaged in tight, light-resistant containers and stored at controlled room temperature, unless otherwise indicated; and for (2) sterile preparations for which a program of sterility testing is in place (see the *General Notices and Requirements, Preservation, Packaging, Storage, and Labeling*). Drugs or chemicals known to be labile to decomposition will require shorter BUDs.

BUD by Type of Formulation ^a
For Nonaqueous Formulations —The BUD is not later than the time remaining until the earliest expiration date of any API or 6 months, whichever is earlier.
For Water-Containing Oral Formulations —The BUD is not later than 14 days when stored at controlled cold temperatures.
For Water-Containing Topical/Dermal and Mucosal Liquid and Semisolid Formulations —The BUD is not later than 30 days.

^a These maximum BUDs are recommended for nonsterile compounded drug preparations in the absence of stability information that is applicable to a specific drug or preparation. The BUD shall not be later than the expiration date on the container of any component.

Susceptible preparations should contain suitable antimicrobial agents to protect against bacteria, yeast, and mold contamination inadvertently introduced during or after the compounding process. When antimicrobial preservatives are contraindicated in such compounded preparations, storage of the preparation at controlled cold temperature is necessary; to ensure proper storage and handling of such compounded preparations by the patient or caregiver, appropriate patient instruction and consultation is essential. Antimicrobial preservatives should not be used as a substitute for good compounding practices.

For information on assigning BUDs when repackaging drug products for dispensing or administration, see the *General Notices and Requirements, Preservation, Packaging, Storage, and Labeling, Labeling, Expiration Date and Beyond-Use Date, and Packaging and Repackaging—Single-Unit Containers* (1136).

Assurance of sterility in a compounded sterile preparation is mandatory. Compounding and packaging of sterile drugs (including ophthalmic preparations) requires strict adherence to guidelines presented in Chapter (797) and in the manufacturers' labeling instructions.

PACKAGING AND DRUG PREPARATION CONTAINERS

The compounder shall ensure that the containers and container closures used in packaging compounded preparations meet USP requirements (see (659) *Packaging and Storage Requirements; Containers—Glass* (660); *Containers—Plastics* (661); *Containers—Performance Testing* (671); Chapter (1136)); and when available, compounding monographs. Compounders are not expected to perform the tests described in these chapters but should be knowledgeable about the standards described in them. Container suppliers shall supply, upon request, verification of USP container compliance. Containers and container closures intended for the compounding of sterile preparations must be handled as described in Chapter (797).

The containers and closures shall be made of suitable clean material in order not to alter the quality, strength, or purity of the compounded drug preparation. The container used depends on the physical and chemical properties of the compounded preparation. Container–drug interaction should be considered for substances that have sorptive or leaching properties.

The containers and closures shall be stored off the floor, handled and stored to prevent contamination, and rotated so that the oldest stock is used first. The containers and container closures shall be stored in such a way as to permit inspection and cleaning of the storage area.

COMPOUNDING DOCUMENTATION

Documentation, written or electronic, enables a compounder, whenever necessary, to systematically trace, evaluate, and replicate the steps included throughout the preparation process of a compounded preparation. All compounders who dispense prescriptions must comply with the record-keeping requirements of their state boards of pharmacy. When the compounder compounds a preparation according to the manufacturer's labeling instructions, then further documentation is not required. All other compounded preparations require further documentation as described in this section.

These records should be retained for the same period of time that is required for any prescription under state law. The record may be a copy of the prescription in written or machine-readable form and should include a Master Formulation Record and a Compounding Record.

Master Formulation Record

This record shall include:

- official or assigned name, strength, and dosage form of the preparation
- calculations needed to determine and verify quantities of components and doses of active pharmaceutical ingredients
- description of all ingredients and their quantities
- compatibility and stability information, including references when available
- equipment needed to prepare the preparation, when appropriate
- mixing instructions that should include:
 1. order of mixing
 2. mixing temperatures or other environmental controls
 3. duration of mixing
 4. other factors pertinent to the replication of the preparation as compounded
- sample labeling information, which shall contain, in addition to legally required information:
 1. generic name and quantity or concentration of each active ingredient
 2. assigned BUD

3. storage conditions
 4. prescription or control number, whichever is applicable
- container used in dispensing
 - packaging and storage requirements
 - description of final preparation
 - quality control procedures and expected results

Compounding Record

The Compounding Record shall contain:

- official or assigned name, strength, and dosage of the preparation
- Master Formulation Record reference for the preparation
- names and quantities of all components
- sources, lot numbers, and expiration dates of components
- total quantity compounded
- name of the person who prepared the preparation, name of the person who performed the quality control procedures, and name of the compounding who approved the preparation
- date of preparation
- assigned control or prescription number
- assigned BUD
- duplicate label as described in the Master Formulation Record
- description of final preparation
- results of quality control procedures (e.g., weight range of filled capsules, pH of aqueous liquids)
- documentation of any quality control issues and any adverse reactions or preparation problems reported by the patient or caregiver

Standard Operating Procedures

All significant procedures performed in the compounding area should be covered by written standard operating procedures (SOPs). Procedures should be developed for the facility, equipment, personnel, preparation, packaging, and storage of compounded preparations to ensure accountability, accuracy, quality, safety, and uniformity in compounding. Implementing SOPs establishes procedural consistency and also provides a reference for orientation and training of personnel.

Material Safety Data Sheets File

MSDSs shall be readily accessible to all employees working with drug substances or bulk chemicals located on the compounding facility premises. Employees should be instructed on how to retrieve and interpret needed information.

QUALITY CONTROL

The safety, quality, and performance of compounded preparations depend on correct ingredients and calculations, accurate and precise measurements, appropriate formulation conditions and procedures, and prudent pharmaceutical judgment. As a final check, the compounding shall review each procedure in the compounding process. To ensure accuracy and completeness, the compounding shall observe the finished preparation to ensure that it appears as expected and shall investigate any discrepancies and take appropriate corrective action before the prescription is dispensed to the patient.

Compounding Controls

1. The Master Formulation Record, the Compounding Record, and associated written procedures shall be followed in execution of the compounding process. Any deviation in procedures shall be documented.
2. The compounding shall check and recheck each procedure at each stage of the process. If possible, a trained second person should verify each critical step in the compounding process.
3. The compounding shall have established written procedures that describe the tests or examinations conducted on the compounded preparation (e.g., the degree of weight variation among capsules) to ensure their uniformity and integrity.
4. Appropriate control procedures shall be established to monitor the output and to verify the performance of compounding processes and equipment that may be responsible for causing variability in the final compounded preparations.
5. For further guidance on recommended quality control procedures, see Chapter (1163).

PATIENT COUNSELING

At the time of dispensing the prescription, the patient or the patient's agent shall be counseled about proper use, storage, handling, and disposal of the compounded preparation. The patient or the patient's agent shall also be instructed to report any adverse event and to observe and report to the compounding any changes in the physical characteristics of the compounded preparation (see Chapter (1191), *Responsibility of the Pharmacist*). The compounding shall investigate and document any reported problem with a compounded preparation and shall take corrective action.

TRAINING

All personnel involved in the compounding, evaluation, packaging, and dispensing of compounded preparations shall be properly trained for the type of compounding conducted. It is the responsibility of the compounding to ensure that a training program has been implemented and that it is ongoing. Compounding personnel should be evaluated at least annually. Steps in the training procedure include the following:

- All employees involved in pharmaceutical compounding shall read and become familiar with this chapter. They should also be familiar with the contents of the *USP Pharmacists' Pharmacopeia* and other relevant publications, including how to read and interpret MSDSs.
- All employees shall read and become familiar with each of the procedures related to compounding, including those involving the facility, equipment, personnel, actual compounding, evaluation, packaging, storage, and dispensing.
- All personnel who compound hazardous drugs shall be fully trained in the storage, handling, and disposal of these drugs. This training shall occur before preparing or handling hazardous drugs. For information on training for personnel who compound hazardous drugs, see the references in *Compounding Facilities* earlier in this chapter.
- All training activities shall be documented. The compounding shall meet with employees to review their work and answer any questions the employees may have concerning compounding procedures.
- The compounding shall demonstrate the procedures for the employee and shall observe and guide the employee throughout the training process. The employee will then repeat the procedure without any

assistance from, but under the direct supervision of, the compounder.

- When the employee has demonstrated to the compounder a verbal and functional knowledge of the procedure, then and only then will the employee be permitted to perform the procedure without direct supervision. However, the compounder should be physically present and shall approve all ingredients and their quantities and the final preparation.
- When the compounder is satisfied with the employee's knowledge and proficiency, the compounder will sign the documentation records to show that the employee was appropriately trained.
- The compounder shall continually monitor the work of the employee and ensure that the employee's calculations and work are accurate and adequately performed.
- The compounder is solely responsible for the finished preparation.

COMPOUNDING FOR ANIMAL PATIENTS

A compounder's responsibility for providing patients with high-quality compounded preparations extends beyond the human species. All portions of this chapter apply to compounded preparations formulated for animal patients. Intended use of any animal patient (e.g., companion, performance, food) shall be determined before compounding for that patient.

Because humans can consume animal patients as food, care must be taken to prevent drug residues from entering the human food chain when compounded preparations are used in animal patients. For this reason, all compounders preparing formulations for animals shall possess a functional knowledge of drug regulation and disposition in animal patients. Veterinarians are required by law to provide food-producing animal caregivers with an accurate length of time to withhold treated animal tissues (e.g., meat, milk, eggs) from the human food supply. This length of time is referred to as a withdrawal time (WDT) and must also, by law, be included on the dispensing label of every prescription prepared for a food-producing species.

Drug use in any performance animal is strictly regulated by federal and state governments, in addition to the governing bodies of each of the specific disciplines. Penalties for violation of these rules may be severe for all contributing to the violation, including the veterinarian, pharmacist, and caregiver.

The pharmacist shall be knowledgeable about the individual species' limitations in physiology and metabolic capacity that can result in toxicity when certain drugs or excipients are used in compounded preparations. For this reason, compounders making preparations for animals should use, when possible, formulations specifically developed for animal patients. If such formulations are not available, the compounder shall conduct a literature review to determine whether a specific component of the formula is toxic to the target species. Extrapolating compounding formulations intended for use in humans may not be appropriate for animal species and may contribute to negative outcomes.

Veterinarians and pharmacists making preparations for animal patients should be familiar with all state and federal regulations regarding drug use in animals, including but not limited to the Food, Drug, and Cosmetic Act; the Animal Drug Amendment; the Animal Medicinal Drug Use Clarifica-

tion Act; and FDA's Compliance Policy Guideline for Compounding of Drugs for Use in Animal Patients.

〈797〉 PHARMACEUTICAL COMPOUNDING—STERILE PREPARATIONS

INTRODUCTION

The objective of this chapter is to describe conditions and practices to prevent harm, including death, to patients that could result from (1) microbial contamination (nonsterility), (2) excessive bacterial endotoxins, (3) variability in the intended strength of correct ingredients that exceeds either monograph limits for official articles (see "official" and "article" in the *General Notices and Requirements*) or 10% for nonofficial articles, (4) unintended chemical and physical contaminants, and (5) ingredients of inappropriate quality in compounded sterile preparations (CSPs). Contaminated CSPs are potentially most hazardous to patients when administered into body cavities, central nervous and vascular systems, eyes, and joints, and when used as baths for live organs and tissues. When CSPs contain excessive bacterial endotoxins (see *Bacterial Endotoxins Test* 〈85〉), they are potentially most hazardous to patients when administered into the central nervous system.

Despite the extensive attention in this chapter to the provision, maintenance, and evaluation of air quality, the avoidance of direct or physical contact contamination is paramount. It is generally acknowledged that direct or physical contact of critical sites of CSPs with contaminants, especially microbial sources, poses the greatest probability of risk to patients. Therefore, compounding personnel must be meticulously conscientious in precluding contact contamination of CSPs both within and outside ISO Class 5 (see *Table 1*) areas.

To achieve the above five conditions and practices, this chapter provides minimum practice and quality standards for CSPs of drugs and nutrients based on current scientific information and best sterile compounding practices. The use of technologies, techniques, materials, and procedures other than those described in this chapter is not prohibited so long as they have been proven to be equivalent or superior with statistical significance to those described herein. The standards in this chapter do not pertain to the *clinical administration* of CSPs to patients via application, implantation, infusion, inhalation, injection, insertion, instillation, and irrigation, which are the routes of administration. Four specific categories of CSPs are described in this chapter: low-risk level, medium-risk level, and high-risk level, and immediate use. Sterile compounding differs from nonsterile compounding (see *Pharmaceutical Compounding—Nonsterile Preparations* 〈795〉 and *Good Compounding Practices* 〈1075〉) primarily by requiring the maintenance of sterility when compounding exclusively with sterile ingredients and components (i.e., with immediate-use CSPs, low-risk level CSPs, and medium-risk level CSPs) and the achievement of sterility when compounding with nonsterile ingredients and components (i.e., with high-risk level CSPs). Some differences between standards for sterile compounding in this chapter and those for nonsterile compounding in *Pharmaceutical Compounding—Nonsterile Preparations* 〈795〉 include, but are not limited to, ISO-classified air environments (see *Table 1*); personnel garbing and gloving; personnel training and testing

in principles and practices of aseptic manipulations and sterilization; environmental quality specifications and monitoring; and disinfection of gloves and surfaces of ISO Class 5 (see *Table 1*) sources.

Table 1. ISO Classification of Particulate Matter in Room Air
(limits are in particles of 0.5 μm and larger per cubic meter [current ISO] and cubic feet [former Federal Standard No. 209E, FS 209E])*

Class Name		Particle Count	
ISO Class	U.S. FS 209E	ISO, m^3	FS 209E, ft^3
3	Class 1	35.2	1
4	Class 10	352	10
5	Class 100	3,520	100
6	Class 1,000	35,200	1,000
7	Class 10,000	352,000	10,000
8	Class 100,000	3,520,000	100,000

*Adapted from former Federal Standard No. 209E, General Services Administration, Washington, DC, 20407 (September 11, 1992) and ISO 14644-1:1999, Cleanrooms and associated controlled environments—Part 1: Classification of air cleanliness. For example, 3,520 particles of 0.5 μm per m^3 or larger (ISO Class 5) is equivalent to 100 particles per ft^3 (Class 100) ($1 \text{ m}^3 = 35.2 \text{ ft}^3$).

The standards in this chapter are intended to apply to all persons who prepare CSPs and all places where CSPs are prepared (e.g., hospitals and other healthcare institutions, patient treatment clinics, pharmacies, physicians' practice facilities, and other locations and facilities in which CSPs are prepared, stored, and transported). Persons who perform sterile compounding include pharmacists, nurses, pharmacy technicians, and physicians. These terms recognize that most sterile compounding is performed by or under the supervision of pharmacists in pharmacies and also that this chapter applies to all healthcare personnel who prepare, store, and transport CSPs. For the purposes of this chapter, CSPs include any of the following:

- (1) Compounded biologics, diagnostics, drugs, nutrients, and radiopharmaceuticals, including but not limited to the following dosage forms that must be sterile when they are administered to patients: aqueous bronchial and nasal inhalations, baths and soaks for live organs and tissues, injections (e.g., colloidal dispersions, emulsions, solutions, suspensions), irrigations for wounds and body cavities, ophthalmic drops and ointments, and tissue implants.
- (2) Manufactured sterile products that are either prepared strictly according to the instructions appearing in manufacturers' approved labeling (product package inserts) or prepared differently than published in such labeling. [NOTE—The FDA states that "Compounding does not include mixing, reconstituting, or similar acts that are performed in accordance with the directions contained in approved labeling provided by the product's manufacturer and other manufacturer directions consistent with that labeling" [21 USC 321 (k) and (m)]. However, the FDA-approved labeling (product package insert) rarely describes environmental quality (e.g., ISO Class air designation, exposure durations to non-ISO classified air, personnel garbing and gloving, and other aseptic precautions by which sterile products are to be prepared for administration). Beyond-use exposure and storage dates or times (see *General Notices and Requirements and Pharmaceutical Compounding—Nonsterile Preparations* (795)) for sterile products that have been either opened or prepared for administration are not specified in all package inserts for all sterile products. Furthermore, when such durations are specified, they may refer to chemical stability and not necessarily to microbiological purity or safety.]

ORGANIZATION OF THIS CHAPTER

The sections in this chapter are organized to facilitate the practitioner's understanding of the fundamental accuracy and quality practices for preparing CSPs. They provide a foundation for the development and implementation of essential procedures for the safe preparation of low-risk, medium-risk, and high-risk level CSPs and immediate-use CSPs, which are classified according to the potential for microbial, chemical, and physical contamination. The chapter is divided into the following main sections:

- Definitions
- Responsibility of Compounding Personnel
- CSP Microbial Contamination Risk Levels
- Personnel Training and Evaluation in Aseptic Manipulation Skills
- Immediate-Use CSPs
- Single-Dose and Multiple-Dose Containers
- Hazardous Drugs as CSPs
- Radiopharmaceuticals as CSPs
- Allergen Extracts as CSPs
- Verification of Compounding Accuracy and Sterility
- Environmental Quality and Control
- Suggested Standard Operating Procedures (SOPs)
- Elements of Quality Control
- Verification of Automated Compounding Devices (ACDs) for Parenteral Nutrition Compounding
- Finished Preparation Release Checks and Tests
- Storage and Beyond-Use Dating
- Maintaining Sterility, Purity, and Stability of Dispensed and Distributed CSPs
- Patient or Caregiver Training
- Patient Monitoring and Adverse Events Reporting
- Quality Assurance (QA) Program
- Abbreviations and Acronyms
- Appendices I–V

The requirements and recommendations in this chapter are summarized in *Appendix I*. A list of abbreviations and acronyms is included at the end of the main text, before the *Appendices*.

All personnel who prepare CSPs shall be responsible for understanding these fundamental practices and precautions, for developing and implementing appropriate procedures, and for continually evaluating these procedures and the quality of final CSPs to prevent harm.

DEFINITIONS

Ante-Area—An ISO Class 8 (see *Table 1*) or better area where personnel hand hygiene and garbing procedures, staging of components, order entry, CSP labeling, and other high-particulate-generating activities are performed. It is also a transition area that (1) provides assurance that pressure relationships are constantly maintained so that air flows from clean to dirty areas and (2) reduces the need for the heating, ventilating, and air-conditioning (HVAC) control system to respond to large disturbances.¹

Aseptic Processing (see *Microbiological Control and Monitoring of Aseptic Processing Environments* (1116))—A mode of processing pharmaceutical and medical products that involves the separate sterilization of the product and of the package (containers—closures or packaging material for medical devices) and the transfer of the product into the container and its closure under at least ISO Class 5 (see *Table 1*) conditions.

Beyond-Use Date (BUD) (see *General Notices and Requirements and Pharmaceutical Compounding—Nonsterile Preparations* (795))—For the purpose of this chapter, the date or time after which a CSP shall not be stored or transported. The date is determined from the date or time the preparation is compounded.

¹ See *American Society of Heating, Refrigerating and Air-Conditioning Engineers, Inc. (ASHRAE), Laboratory Design Guide*.

Biological Safety Cabinet (BSC)—A ventilated cabinet for CSPs, personnel, product, and environmental protection having an open front with inward airflow for personnel protection, downward high-efficiency particulate air (HEPA)-filtered laminar airflow for product protection, and HEPA-filtered exhausted air for environmental protection.

Buffer Area—An area where the primary engineering control (PEC) is physically located. Activities that occur in this area include the preparation and staging of components and supplies used when compounding CSPs.

Clean Room (see *Microbiological Control and Monitoring of Aseptic Processing Environments* 〈1116〉 and also the definition of *Buffer Area*)—A room in which the concentration of airborne particles is controlled to meet a specified airborne particulate cleanliness class. Microorganisms in the environment are monitored so that a microbial level for air, surface, and personnel gear are not exceeded for a specified cleanliness class.

Compounding Aseptic Containment Isolator (CACI)—A compounding aseptic isolator (CAI) designed to provide worker protection from exposure to undesirable levels of airborne drug throughout the compounding and material transfer processes and to provide an aseptic environment for compounding sterile preparations. Air exchange with the surrounding environment should not occur unless the air is first passed through a microbial retentive filter (HEPA minimum) system capable of containing airborne concentrations of the physical size and state of the drug being compounded. Where volatile hazardous drugs are prepared, the exhaust air from the isolator should be appropriately removed by properly designed building ventilation.

Compounding Aseptic Isolator (CAI)—A form of isolator specifically designed for compounding pharmaceutical ingredients or preparations. It is designed to maintain an aseptic compounding environment within the isolator throughout the compounding and material transfer processes. Air exchange into the isolator from the surrounding environment should not occur unless the air has first passed through a microbially retentive filter (HEPA minimum).²

Critical Area—An ISO Class 5 (see *Table 1*) environment.

Critical Site—A location that includes any component or fluid pathway surfaces (e.g., vial septa, injection ports, beakers) or openings (e.g., opened ampuls, needle hubs) exposed and at risk of direct contact with air (e.g., ambient room or HEPA filtered), moisture (e.g., oral and mucosal secretions), or touch contamination. Risk of microbial particulate contamination of the critical site increases with the size of the openings and exposure time.

Direct Compounding Area (DCA)—A critical area within the ISO Class 5 (see *Table 1*) primary engineering control (PEC) where critical sites are exposed to unidirectional HEPA-filtered air, also known as first air.

Disinfectant—An agent that frees from infection, usually a chemical agent but sometimes a physical one, and that destroys disease-causing pathogens or other harmful microorganisms but may not kill bacterial and fungal spores. It refers to substances applied to inanimate objects.

First Air—The air exiting the HEPA filter in a unidirectional air stream that is essentially particle free.

Hazardous Drugs—Drugs are classified as hazardous if studies in animals or humans indicate that exposures to them have a potential for causing cancer, development or reproductive toxicity, or harm to organs. (See current NIOSH publication.)

Labeling [see *General Notices and Requirements* and 21 USC 321 (k) and (m)]—A term that designates all labels and other written, printed, or graphic matter on an immediate container of an article or preparation or on, or in, any pack-

age or wrapper in which it is enclosed, except any outer shipping container. The term “label” designates that part of the labeling on the immediate container.

Media-Fill Test (see *Microbiological Control and Monitoring of Aseptic Processing Environments* 〈1116〉)—A test used to qualify aseptic technique of compounding personnel or processes and to ensure that the processes used are able to produce sterile product without microbial contamination. During this test, a microbiological growth medium such as Soybean-Casein Digest Medium is substituted for the actual drug product to simulate admixture compounding.³ The issues to consider in the development of a media-fill test are media-fill procedures, media selection, fill volume, incubation, time and temperature, inspection of filled units, documentation, interpretation of results, and possible corrective actions required.

Multiple-Dose Container (see *General Notices and Requirements and Containers for Injections under Injections* 〈1〉)—A multiple-unit container for articles or preparations intended for parenteral administration only and usually containing antimicrobial preservatives. The beyond-use date (BUD) for an opened or entered (e.g., needle-punctured) multiple-dose container with antimicrobial preservatives is 28 days (see *Antimicrobial Effectiveness Testing* 〈51〉), unless otherwise specified by the manufacturer.

Negative Pressure Room—A room that is at a lower pressure than the adjacent spaces and, therefore, the net flow of air is *into* the room.¹

Pharmacy Bulk Package (see *Containers for Injections under Injections* 〈1〉)—A container of a sterile preparation for parenteral use that contains many single doses. The contents are intended for use in a pharmacy admixture program and are restricted to the preparation of admixtures for infusion or, through a sterile transfer device, for the filling of empty sterile syringes. The closure shall be penetrated only one time after constitution with a suitable sterile transfer device or dispensing set, which allows measured dispensing of the contents. The pharmacy bulk package is to be used only in a suitable work area such as a laminar flow hood (or an equivalent clean air compounding area).

Where a container is offered as a pharmacy bulk package, the label shall (a) state prominently “Pharmacy Bulk Package—Not for Direct Infusion,” (b) contain or refer to information on proper techniques to help ensure safe use of the product, and (c) bear a statement limiting the time frame in which the container may be used once it has been entered, provided it is held under the labeled storage conditions.

Primary Engineering Control (PEC)—A device or room that provides an ISO Class 5 (see *Table 1*) environment for the exposure of critical sites when compounding CSPs. Such devices include, but may not be limited to, laminar airflow workbenches (LAFWs), biological safety cabinets (BSCs), compounding aseptic isolators (CAIs), and compounding aseptic containment isolators (CACIs).

Preparation—A preparation, or a CSP, that is a sterile drug or nutrient compounded in a licensed pharmacy or other healthcare-related facility pursuant to the order of a licensed prescriber; the article may or may not contain sterile products.

Product—A commercially manufactured sterile drug or nutrient that has been evaluated for safety and efficacy by the FDA. Products are accompanied by full prescribing information, which is commonly known as the FDA-approved manufacturer’s labeling or product package insert.

Positive Pressure Room—A room that is at a higher pressure than the adjacent spaces and, therefore, the net airflow is *out* of the room.¹

Single-Dose Container (see *General Notices and Requirements and Containers for Injections under Injections* 〈1〉)—A single-dose container is a single-unit container for articles

² CETA Applications Guide for the Use of Compounding Isolators in Compounding Sterile Preparations in Healthcare Facilities, CAG-001-2005, Controlled Environment Testing Association (CETA), November 8, 2005.

³ U.S. Food and Drug Administration, Guidance for Industry, *Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice*, September 2004.

(see *General Notices and Requirements*) or preparations intended for parenteral administration only. It is intended for a single use. A single-dose container is labeled as such. Examples of single-dose containers include prefilled syringes, cartridges, fusion-sealed containers, and closure-sealed containers when so labeled.

Segregated Compounding Area—A designated space, either a demarcated area or room, that is restricted to preparing low-risk level CSPs with 12-hour or less BUD. Such area shall contain a device that provides unidirectional airflow of ISO Class 5 (see *Table 1*) air quality for preparation of CSPs and shall be void of activities and materials that are extraneous to sterile compounding.

Sterilizing Grade Membranes—Membranes that are documented to retain 100% of a culture of 10^7 microorganisms of a strain of *Brevundimonas* (*Pseudomonas*) *diminuta* per square centimeter of membrane surface under a pressure of not less than 30 psi (2.0 bar). Such filter membranes are nominally at 0.22- μ m or 0.2- μ m nominal pore size, depending on the manufacturer's practice.

Sterilization by Filtration—Passage of a fluid or solution through a sterilizing grade membrane to produce a sterile effluent.

Terminal Sterilization—The application of a lethal process (e.g., steam under pressure or autoclaving) to sealed containers for the purpose of achieving a predetermined sterility assurance level of usually less than 10^{-6} , or a probability of less than one in one million of a nonsterile unit.³

Unidirectional Flow (see footnote 3)—An airflow moving in a single direction in a robust and uniform manner and at sufficient speed to reproducibly sweep particles away from the critical processing or testing area.

RESPONSIBILITY OF COMPOUNDING PERSONNEL

Compounding personnel are responsible for ensuring that CSPs are accurately identified, measured, diluted, and mixed and are correctly purified, sterilized, packaged, sealed, labeled, stored, dispensed, and distributed. These performance responsibilities include maintaining appropriate cleanliness conditions and providing labeling and supplementary instructions for the proper clinical administration of CSPs.

Compounding supervisors shall ensure, through either direct measurement or appropriate information sources, that specific CSPs maintain their labeled strength within monograph limits for *USP* articles, or within 10% if not specified, until their BUDs. All CSPs are prepared in a manner that maintains sterility and minimizes the introduction of particulate matter.

A written quality assurance procedure includes the following in-process checks that are applied, as appropriate, to specific CSPs: accuracy and precision of measuring and weighing; the requirement for sterility; methods of sterilization and purification; safe limits and ranges for strength of ingredients, bacterial endotoxins, and particulate matter; pH; labeling accuracy and completeness; BUD assignment; and packaging and storage requirements. The dispenser shall, when appropriate and practicable, obtain and evaluate results of testing for identity, strength, purity, and sterility before a CSP is dispensed. Qualified licensed healthcare professionals who supervise compounding and dispensing of CSPs shall ensure that the following objectives are achieved:

1. Compounding personnel are adequately skilled, educated, instructed, and trained to correctly perform and document the following activities in their sterile compounding duties:
 - a. perform antiseptic hand cleansing and disinfection of nonsterile compounding surfaces;
 - b. select and appropriately don protective garb;
 - c. maintain or achieve sterility of CSPs in ISO Class 5 (see *Table 1*) PEC devices and protect personnel

and compounding environments from contamination by radioactive, cytotoxic, and chemotoxic drugs (see *Hazardous Drugs as CSPs* and *Radiopharmaceuticals as CSPs*);

- d. identify, weigh, and measure ingredients; and
 - e. manipulate sterile products aseptically, sterilize high-risk level CSPs, and label and quality inspect CSPs.
2. Ingredients have their correct identity, quality, and purity.
 3. Opened or partially used packages of ingredients for subsequent use in CSPs are properly stored under restricted access conditions in the compounding facility. Such packages cannot be used when visual inspection detects unauthorized breaks in the container, closure, and seal; when the contents do not possess the expected appearance, aroma, and texture; when the contents do not pass identification tests specified by the compounding facility; and when either the BUD or expiration date has been exceeded.
 4. Water-containing CSPs that are nonsterile during any phase of the compounding procedure are sterilized within 6 hours after completing the preparation in order to minimize the generation of bacterial endotoxins.
 5. Sterilization methods achieve sterility of CSPs while maintaining the labeled strength of active ingredients and the physical integrity of packaging.
 6. Measuring, mixing, sterilizing, and purifying devices are clean, appropriately accurate, and effective for their intended use.
 7. Potential harm from added substances and differences in rate and extent of bioavailability of active ingredients for other than oral route of administration are carefully evaluated before such CSPs are dispensed and administered.
 8. Packaging selected for CSPs is appropriate to preserve the sterility and strength until the BUD.
 9. While being used, the compounding environment maintains the sterility or the presterilization purity, whichever is appropriate, of the CSP.
 10. Labels on CSPs list the names and amounts or concentrations of active ingredients, and the labels or labeling of injections (see *Preservation, Packaging, Storage, and Labeling* in the *General Notices and Requirements*) list the names and amounts or concentrations of all ingredients (see *Injections* (1)). Before being dispensed or administered, the clarity of solutions is visually confirmed; also, the identity and amounts of ingredients, procedures to prepare and sterilize CSPs, and specific release criteria are reviewed to ensure their accuracy and completeness.
 11. BUDs are assigned on the basis of direct testing or extrapolation from reliable literature sources and other documentation (see *Stability Criteria and Beyond-Use Dating* under *Pharmaceutical Compounding—Nonsterile Preparations* (795)).
 12. Procedures for measuring, mixing, dilution, purification, sterilization, packaging, and labeling conform to the correct sequence and quality established for the specified CSP.
 13. Deficiencies in compounding, labeling, packaging, and quality testing and inspection can be rapidly identified and corrected.
 14. When time and personnel availability so permit, compounding manipulations and procedures are separated from postcompounding quality inspection and review before CSPs are dispensed.

This chapter emphasizes the need to maintain high standards for the quality and control of processes, components, and environments and for the skill and knowledge of personnel who prepare CSPs. The rigor of in-process quality-control checks and of postcompounding quality inspection and testing increases with the potential hazard of the route

of administration. For example, nonsterility, excessive bacterial endotoxin contamination, large errors in strength of correct ingredients, and incorrect ingredients in CSPs are potentially more dangerous to patients when the CSPs are administered into the vascular and central nervous systems than when administered by most other routes.

CSP MICROBIAL CONTAMINATION RISK LEVELS

The three contamination categories for CSPs described in this section are assigned primarily according to the potential for microbial contamination during the compounding of low-risk level CSPs and medium-risk level CSPs or the potential for not sterilizing high-risk level CSPs, any of which would subject patients to risk of harm, including death. High-risk level CSPs must be sterilized before being administered to patients. The appropriate risk level—low, medium, or high—is assigned according to the corresponding probability of contaminating a CSP with (1) microbial contamination (e.g., microbial organisms, spores, endotoxins) and (2) chemical and physical contamination (e.g., foreign chemicals, physical matter). Potential sources of contamination include, but are not limited to, solid and liquid matter from compounding personnel and objects; nonsterile components employed and incorporated before terminal sterilization; inappropriate conditions within the restricted compounding environment; prolonged presterilization procedures with aqueous preparations; and nonsterile dosage forms used to compound CSPs.

The characteristics described below for low-, medium-, and high-risk level CSPs are intended as a guide to the breadth and depth of care necessary in compounding, but they are neither exhaustive nor prescriptive. The licensed healthcare professionals who supervise compounding are responsible for determining the procedural and environmental quality practices and attributes that are necessary for the risk level they assign to specific CSPs.

These risk levels apply to the quality of CSPs immediately after the final aseptic mixing or filling or immediately after the final sterilization, unless precluded by the specific characteristics of the preparation. Upon subsequent storage and shipping of freshly finished CSPs, an increase in the risks of chemical degradation of ingredients, contamination from physical damage to packaging, and permeability of plastic and elastomeric packaging is expected. In such cases, compounding personnel are responsible for considering the potential additional risks to the integrity of CSPs when assigning BUDs. The pre-administration storage duration and temperature limits specified in the following subsections apply in the absence of direct sterility testing results that justify different limits for specific CSPs.

Low-Risk Level CSPs

CSPs compounded under all the following conditions are at a low risk of contamination.

Low-Risk Conditions—

1. The CSPs are compounded with aseptic manipulations entirely within ISO Class 5 (see *Table 1*) or better air quality using only sterile ingredients, products, components, and devices.
2. The compounding involves only transfer, measuring, and mixing manipulations using not more than three commercially manufactured packages of sterile products and not more than two entries into any one sterile container or package (e.g., bag, vial) of sterile product or administration container/device to prepare the CSP.
3. Manipulations are limited to aseptically opening ampuls, penetrating disinfected stoppers on vials with sterile needles and syringes, and transferring sterile liquids in sterile syringes to sterile administration de-

vices, package containers of other sterile products, and containers for storage and dispensing.

4. For a low-risk level preparation, in the absence of passing a sterility test (see *Sterility Tests* <71>), the storage periods cannot exceed the following time periods: before administration, the CSPs are properly stored and are exposed for not more than 48 hours at controlled room temperature (see *General Notices and Requirements*), for not more than 14 days at a cold temperature (see *General Notices and Requirements*), and for 45 days in solid frozen state between -25° and -10° .

Examples of Low-Risk Compounding—

1. Single-volume transfers of sterile dosage forms from ampuls, bottles, bags, and vials using sterile syringes with sterile needles, other administration devices, and other sterile containers. The solution content of ampuls should be passed through a sterile filter to remove any particles.
2. Simple aseptic measuring and transferring with not more than three packages of manufactured sterile products, including an infusion or diluent solution to compound drug admixtures and nutritional solutions.

Low-Risk Level CSPs with 12-Hour or Less BUD—If the PEC is a CAI or CACI that does not meet the requirements described in *Placement of Primary Engineering Controls* or is a laminar airflow workbench (LAFW) or a biological safety cabinet (BSC) that cannot be located within an ISO Class 7 (see *Table 1*) buffer area, then only low-risk level nonhazardous and radiopharmaceutical CSPs pursuant to a physician's order for a specific patient may be prepared, and administration of such CSPs shall commence within 12 hours of preparation or as recommended in the manufacturers' package insert, whichever is less. Low-risk level CSPs with a 12-hour or less BUD shall meet all of the following four criteria:

1. PECs (LAFWs, BSCs, CAIs, CACIs,) shall be certified and maintain ISO Class 5 (see *Table 1*) as described in *Facility Design and Environmental Controls* for exposure of critical sites and shall be in a segregated compounding area restricted to sterile compounding activities that minimize the risk of CSP contamination.
2. The segregated compounding area shall not be in a location that has unsealed windows or doors that connect to the outdoors or high traffic flow, or that is adjacent to construction sites, warehouses, or food preparation. Note that this list is not intended to be all inclusive.
3. Personnel shall follow the procedures described in *Personnel Cleansing and Garbing and Additional Personnel Requirements* prior to compounding. Sinks should not be located adjacent to the ISO Class 5 (see *Table 1*) PEC. Sinks should be separated from the immediate area of the ISO Class 5 (see *Table 1*) PEC device.
4. The specifications in *Cleaning and Disinfecting the Sterile Compounding Areas, Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures*, and *Viable and Non-viable Environmental Sampling (ES) Testing* shall be followed as described in the chapter.

Compounding personnel must recognize that the absence of an ISO Class 7 (see *Table 1*) buffer area environment in a general uncontrolled environment increases the potential of microbial contamination, and administration durations of microbially contaminated CSPs exceeding a few hours increase the potential for clinically significant microbial colonization, and thus for patient harm, especially in critically ill or immunocompromised patients.

Quality Assurance—Quality assurance practices include, but are not limited to the following:

1. Routine disinfection and air quality testing of the direct compounding environment to minimize micro-

bial surface contamination and maintain ISO Class 5 (see *Table 1*) air quality.

2. Visual confirmation that compounding personnel are properly donning and wearing appropriate items and types of protective garments, including eye protection and face masks.
3. Review of all orders and packages of ingredients to ensure that the correct identity and amounts of ingredients were compounded.
4. Visual inspection of CSPs to ensure the absence of particulate matter in solutions, the absence of leakage from vials and bags, and the accuracy and thoroughness of labeling.

Media-Fill Test Procedure—This test or an equivalent test is performed at least annually by each person authorized to compound in a low-risk level environment under conditions that closely simulate the most challenging or stressful conditions encountered during compounding of low-risk level CSPs. Once begun, this test is completed without interruption. *Example of test procedure:* within an ISO Class 5 (see *Table 1*) air quality environment, three sets of four 5-mL aliquots of sterile Soybean–Casein Digest Medium (also known as trypticase soy broth or trypticase soy agar [TSA]) are transferred with the same sterile 10-mL syringe and vented needle combination into separate sealed, empty, sterile 30-mL clear vials (i.e., four 5-mL aliquots into each of three 30-mL vials). Sterile adhesive seals are aseptically affixed to the rubber closures on the three filled vials, then the vials are incubated at 20° to 25° or at 30° to 35° for a minimum of 14 days. If two temperatures are used for incubation of media-filled samples, then these filled containers should be incubated for at least 7 days at each temperature (see *Microbiological Control and Monitoring of Aseptic Processing Environments* (1116)). Inspect for microbial growth over 14 days as described in *Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures*.

Medium-Risk Level CSPs

When CSPs are compounded aseptically under *Low-Risk Conditions* and one or more of the following conditions exists, such CSPs are at a medium risk of contamination.

Medium-Risk Conditions—

1. Multiple individual or small doses of sterile products are combined or pooled to prepare a CSP that will be administered either to multiple patients or to one patient on multiple occasions.
2. The compounding process includes complex aseptic manipulations other than the single-volume transfer.
3. The compounding process requires unusually long duration, such as that required to complete dissolution or homogeneous mixing.
4. For a medium-risk preparation, in the absence of passing a sterility test (see *Sterility Tests* (71)), the storage periods cannot exceed the following time periods: before administration, the CSPs are properly stored and are exposed for not more than 30 hours at controlled room temperature (see *General Notices and Requirements*), for not more than 9 days at a cold temperature (see *General Notices and Requirements*), and for 45 days in solid frozen state between –25° and –10°.

Examples of Medium-Risk Compounding—

1. Compounding of total parenteral nutrition fluids using manual or automated devices during which there are multiple injections, detachments, and attachments of nutrient source products to the device or machine to deliver all nutritional components to a final sterile container.
2. Filling of reservoirs of injection and infusion devices with more than three sterile drug products and evac-

uation of air from those reservoirs before the filled device is dispensed.

3. Transfer of volumes from multiple ampuls or vials into one or more final sterile containers.

Quality Assurance—Quality assurance procedures for medium-risk level CSPs include all those for low-risk level CSPs, as well as a more challenging media-fill test passed annually or more frequently.

Media-Fill Test Procedure—This test or an equivalent test is performed at least annually under conditions that closely simulate the most challenging or stressful conditions encountered during compounding. Once begun, this test is completed without interruption. *Example of test procedure:* within an ISO Class 5 (see *Table 1*) air quality environment, six 100-mL aliquots of sterile Soybean–Casein Digest Medium are aseptically transferred by gravity through separate tubing sets into separate evacuated sterile containers. The six containers are then arranged as three pairs, and a sterile 10-mL syringe and 18-gauge needle combination is used to exchange two 5-mL aliquots of medium from one container to the other container in the pair. For example, after a 5-mL aliquot from the first container is added to the second container in the pair, the second container is agitated for 10 seconds, then a 5-mL aliquot is removed and returned to the first container in the pair. The first container is then agitated for 10 seconds, and the next 5-mL aliquot is transferred from it back to the second container in the pair. Following the two 5-mL aliquot exchanges in each pair of containers, a 5-mL aliquot of medium from each container is aseptically injected into a sealed, empty, sterile 10-mL clear vial, using a sterile 10-mL syringe and vented needle. Sterile adhesive seals are aseptically affixed to the rubber closures on the three filled vials, then the vials are incubated at 20° to 25° or at 30° to 35° for a minimum of 14 days. If two temperatures are used for incubation of media-filled samples, then these filled containers should be incubated for at least 7 days at each temperature (see *Microbiological Control and Monitoring of Aseptic Processing Environments* (1116)). Inspect for microbial growth over 14 days as described in *Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures*.

High-Risk Level CSPs

CSPs compounded under any of the following conditions are either contaminated or at a high risk to become contaminated.

High-Risk Conditions—

1. Nonsterile ingredients, including manufactured products not intended for sterile routes of administration (e.g., oral), are incorporated or a nonsterile device is employed before terminal sterilization.
2. Any of the following are exposed to air quality worse than ISO Class 5 (see *Table 1*) for more than 1 hour (see *Immediate-Use CSPs*):
 - sterile contents of commercially manufactured products,
 - CSPs that lack effective antimicrobial preservatives, and
 - sterile surfaces of devices and containers for the preparation, transfer, sterilization, and packaging of CSPs.
3. Compounding personnel are improperly garbed and gloved (see *Personnel Cleansing and Use of Barrier Protective Equipment*).
4. Nonsterile water-containing preparations are stored for more than 6 hours before being sterilized.
5. It is assumed, and not verified by examination of labeling and documentation from suppliers or by direct determination, that the chemical purity and content strength of ingredients meet their original or compendial specifications in unopened or in opened packages of bulk ingredients (see *Ingredient Selection*

under *Pharmaceutical Compounding—Nonsterile Preparations* (795)).

For a sterilized high-risk level preparation, in the absence of passing a sterility test, the storage periods cannot exceed the following time periods: before administration, the CSPs are properly stored and are exposed for not more than 24 hours at controlled room temperature (see *General Notices and Requirements*), for not more than 3 days at a cold temperature (see *General Notices and Requirements*), and for 45 days in solid frozen state between -25° and -10° . [NOTE—Sterility tests for autoclaved CSPs are not required unless they are prepared in batches of more than 25 units.]

All nonsterile measuring, mixing, and purifying devices are rinsed thoroughly with sterile, pyrogen-free water, and then thoroughly drained or dried immediately before use for high-risk compounding. All high-risk level CSP solutions subjected to terminal sterilization are prefiltered by passing through a filter with a nominal pore size not larger than $1.2\ \mu\text{m}$ preceding or during filling into their final containers to remove particulate matter. Sterilization of high-risk level CSPs by filtration shall be performed with a sterile $0.2\text{-}\mu\text{m}$ or $0.22\text{-}\mu\text{m}$ nominal pore size filter entirely within an ISO Class 5 (see *Table 1*) or superior air quality environment.

Examples of High-Risk Conditions—

1. Dissolving nonsterile bulk drug and nutrient powders to make solutions that will be terminally sterilized.
2. Exposing the sterile ingredients and components used to prepare and package CSPs to room air quality worse than ISO Class 5 (see *Table 1*) for more than 1 hour (see *Immediate-Use CSPs*).
3. Measuring and mixing sterile ingredients in nonsterile devices before sterilization is performed.
4. Assuming, without appropriate evidence or direct determination, that packages of bulk ingredients contain at least 95% by weight of their active chemical moiety and have not been contaminated or adulterated between uses.

Quality Assurance—Quality assurance procedures for high-risk level CSPs include all those for low-risk level CSPs. In addition, a media-fill test that represents high-risk level compounding is performed semiannually by each person authorized to compound high-risk level CSPs.

Media-Fill Test Procedure for CSPs Sterilized by Filtration—This test or an equivalent test is performed under conditions that closely simulate the most challenging or stressful conditions encountered when compounding high-risk level CSPs. Once begun, this test is completed without interruption. *Example of test procedure* (in the following sequence):

1. Dissolve 3 g of nonsterile commercially available Soybean–Casein Digest Medium in 100 mL of nonbacteriostatic water to make a 3% nonsterile solution.
2. Draw 25 mL of the medium into each of three 30-mL sterile syringes. Transfer 5 mL from each syringe into separate sterile 10-mL vials. These vials are the positive controls to generate exponential microbial growth, which is indicated by visible turbidity upon incubation.
3. Under aseptic conditions and using aseptic techniques, affix a sterile $0.2\text{-}\mu\text{m}$ or $0.22\text{-}\mu\text{m}$ nominal pore size filter unit and a 20-gauge needle to each syringe. Inject the next 10 mL from each syringe into three separate 10-mL sterile vials. Repeat the process for three more vials. Label all vials, affix sterile adhesive seals to the closure of the nine vials, and incubate them at 20° to 25° or at 30° to 35° for a minimum of 14 days. If two temperatures are used for incubation of media-filled samples, then these filled containers should be incubated for at least 7 days at each temperature (see *Microbiological Control and Monitoring of Aseptic Processing Environments* (1116)). Inspect for microbial growth over 14 days as described in *Personnel Training and Competency Evalua-*

tion of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures.

PERSONNEL TRAINING AND EVALUATION IN ASEPTIC MANIPULATION SKILLS

Personnel who prepare CSPs shall be trained conscientiously and skillfully by expert personnel and through audio-video instructional sources and professional publications in the theoretical principles and practical skills of aseptic manipulations and in achieving and maintaining ISO Class 5 (see *Table 1*) environmental conditions before they begin to prepare CSPs. Compounding personnel shall perform didactic review and pass written and media-fill testing of aseptic manipulative skills initially, at least annually thereafter for low- and medium-risk level compounding, and semiannually for high-risk level compounding. Compounding personnel who fail written tests or whose media-fill test vials result in gross microbial colonization shall be immediately re-instructed and re-evaluated by expert compounding personnel to ensure correction of all aseptic practice deficiencies.

Media-Fill Challenge Testing—The skill of personnel to aseptically prepare CSPs may be evaluated using sterile fluid bacterial culture media-fill verification³ (i.e., sterile bacterial culture medium transfer via a sterile syringe and needle). Media-fill testing is used to assess the quality of the aseptic skill of compounding personnel. Media-fill tests represent the most challenging or stressful conditions actually encountered by the personnel being evaluated when they prepare particular risk level CSPs and when sterilizing high-risk level CSPs. Media-fill challenge tests that simulate high-risk level compounding are also used to verify the capability of the compounding environment and process to produce a sterile preparation.

Commercially available sterile fluid culture media, such as Soybean–Casein Digest Medium (see *Sterility Tests* (71)), shall be able to promote exponential colonization of bacteria that are most likely to be transmitted to CSPs from the compounding personnel and environment. Media-filled vials are generally incubated at 20° to 25° or at 30° to 35° for a minimum of 14 days. If two temperatures are used for incubation of media-filled samples, then these filled containers should be incubated for at least 7 days at each temperature (see *Microbiological Control and Monitoring of Aseptic Processing Environments* (1116)). Failure is indicated by visible turbidity in the medium on or before 14 days.

IMMEDIATE-USE CSPs

The immediate-use provision is intended only for those situations where there is a need for emergency or immediate patient administration of a CSP. Such situations may include cardiopulmonary resuscitation, emergency room treatment, preparation of diagnostic agents, or critical therapy where the preparation of the CSP under conditions described for *Low-Risk Level CSPs* subjects the patient to additional risk due to delays in therapy. Immediate-use CSPs are not intended for storage for anticipated needs or batch compounding. Preparations that are medium-risk level and high-risk level CSPs shall not be prepared as immediate-use CSPs.

Immediate-use CSPs are exempt from the requirements described for *Low-Risk Level CSPs* only when all of the following criteria are met:

1. The compounding process involves simple transfer of not more than three commercially manufactured packages of sterile nonhazardous products or diagnostic radiopharmaceutical products from the manufacturers' original containers and not more than two entries into any one container or package (e.g., bag, vial) of sterile infusion solution or administration container/device. For example, anti-neoplastics shall not

be prepared as immediate-use CSPs because they are hazardous drugs.

2. Unless required for the preparation, the compounding procedure is a continuous process not to exceed 1 hour.
3. During preparation, aseptic technique is followed and, if not immediately administered, the finished CSP is under continuous supervision to minimize the potential for contact with nonsterile surfaces, introduction of particulate matter or biological fluids, mix-ups with other CSPs, and direct contact of outside surfaces.
4. Administration begins not later than 1 hour following the start of the preparation of the CSP.
5. Unless immediately and completely administered by the person who prepared it or immediate and complete administration is witnessed by the preparer, the CSP shall bear a label listing patient identification information, the names and amounts of all ingredients, the name or initials of the person who prepared the CSP, and the exact 1-hour BUD and time.
6. If administration has not begun within 1 hour following the start of preparing the CSP, the CSP shall be promptly, properly, and safely discarded.

Compounding in worse than ISO Class 5 (see *Table 1*) conditions increases the likelihood of microbial contamination, and administration durations of microbially contaminated CSPs exceeding a few hours increase the potential for clinically significant microbial colonization and thus for patient harm, especially in critically ill or immunocompromised patients.

SINGLE-DOSE AND MULTIPLE-DOSE CONTAINERS

Opened or needle-punctured single-dose containers, such as bags, bottles, syringes, and vials of sterile products and CSPs shall be used within 1 hour if opened in worse than ISO Class 5 (see *Table 1*) air quality (see *Immediate-Use CSPs*), and any remaining contents must be discarded. Single-dose vials exposed to ISO Class 5 (see *Table 1*) or cleaner air may be used up to 6 hours after initial needle puncture. Opened single-dose ampuls shall not be stored for any time period. Multiple-dose containers (e.g., vials) are formulated for removal of portions on multiple occasions because they usually contain antimicrobial preservatives. The BUD after initially entering or opening (e.g., needle-punctured) multiple-dose containers is 28 days (see *Antimicrobial Effectiveness Testing* (51)) unless otherwise specified by the manufacturer.

HAZARDOUS DRUGS AS CSPs

Although the potential therapeutic benefits of compounded sterile hazardous drug preparations generally outweigh the risks of their adverse effects in ill patients, exposed healthcare workers risk similar adverse effects with no therapeutic benefit. Occupational exposure to hazardous drugs can result in (1) acute effects, such as skin rashes; (2) chronic effects, including adverse reproductive events; and (3) possibly cancer (see Appendix A of NIOSH Publication no. 2004-165).

Hazardous drugs shall be prepared for administration only under conditions that protect the healthcare workers and other personnel in the preparation and storage areas. Hazardous drugs shall be stored separately from other inventory in a manner to prevent contamination and personnel exposure. Many hazardous drugs have sufficient vapor pressures that allow volatilization at room temperature; thus storage is preferably within a containment area such as a negative pressure room. The storage area should have sufficient gen-

eral exhaust ventilation, at least 12 air changes per hour (ACPH)⁴ to dilute and remove any airborne contaminants.

Hazardous drugs shall be handled with caution at all times using appropriate chemotherapy gloves during receiving, distribution, stocking, inventorying, preparation for administration, and disposal. Hazardous drugs shall be prepared in an ISO Class 5 (see *Table 1*) environment with protective engineering controls in place and following aseptic practices specified for the appropriate contamination risk levels defined in this chapter. Access shall be limited to areas where drugs are stored and prepared to protect persons not involved in drug preparation.

All hazardous drugs shall be prepared in a BSC⁵ or a CACI that meets or exceeds the standards for CACI in this chapter. The ISO Class 5 (see *Table 1*) BSC or CACI shall be placed in an ISO Class 7 (see *Table 1*) area that is physically separated (i.e., a different area from other preparation areas) and optimally has not less than 0.01-inch water column negative pressure to adjacent positive pressure ISO Class 7 (see *Table 1*) or better ante-areas, thus providing inward airflow to contain any airborne drug. A pressure indicator shall be installed that can be readily monitored for correct room pressurization. The BSC and CACI optimally should be 100% vented to the outside air through HEPA filtration.

If a CACI that meets the requirements of this chapter is used outside of a buffer area, the compounding area shall maintain a minimum negative pressure of 0.01-inch water column and have a minimum of 12 ACPHs.

When closed-system vial-transfer devices (CSTDs) (i.e., vial-transfer systems that allow no venting or exposure of hazardous substance to the environment) are used, they shall be used within the ISO Class 5 (see *Table 1*) environment of a BSC or CACI. The use of a CSTD is preferred because of their inherent closed system process. In facilities that prepare a low volume of hazardous drugs, the use of two tiers of containment (e.g., CSTD within a BSC or CACI that is located in a non-negative pressure room) is acceptable.

Appropriate personnel protective equipment (PPE) shall be worn when compounding in a BSC or CACI and when using CSTD devices. PPE should include gowns, face masks, eye protection, hair covers, shoe covers or dedicated shoes, double gloving with sterile chemo-type gloves, and compliance with manufacturers' recommendations when using a CACI.

All personnel who compound hazardous drugs shall be fully trained in the storage, handling, and disposal of these drugs. This training shall occur prior to preparing or handling hazardous CSPs, and its effectiveness shall be verified by testing specific hazardous drugs preparation techniques. Such verification shall be documented for each person at least annually. This training shall include didactic overview of hazardous drugs, including mutagenic, teratogenic, and carcinogenic properties, and it shall include ongoing training for each new hazardous drug that enters the marketplace. Compounding personnel of reproductive capability shall confirm in writing that they understand the risks of handling hazardous drugs. The training shall include at least the following: (1) safe aseptic manipulation practices; (2) negative pressure techniques when utilizing a BSC or CACI; (3) correct use of CSTD devices; (4) containment, cleanup, and disposal procedures for breakages and spills; and (5) treatment of personnel contact and inhalation exposure.

NOTE—Because standards of assay and unacceptable quantities of contamination of each drug have not been established in the literature, the following paragraph is a recommendation only. Future standards will be adopted as these assay methods are developed and proven.

In order to ensure containment, especially in operations preparing large volumes of hazardous drugs, environmental

⁴ Guidelines for Environmental Infection Control in Health-Care Facilities, Recommendations of CDC and the Healthcare Infection Control Practices Advisory Committee (HICPAC), MMWR, vol. 52, no. RR-10, June 6, 2003, figure 3, pg. 12.

⁵ NSF/ANSI 49.

sampling to detect uncontained hazardous drugs should be performed routinely (e.g., initially as a benchmark and at least every 6 months or more often as needed to verify containment). This sampling should include surface wipe sampling of the working area of BSCs and CACIs; counter tops where finished preparations are placed; areas adjacent to BSCs and CACIs, including the floor directly under the working area; and patient administration areas. Common marker hazardous drugs that can be assayed include cyclophosphamide, ifosfamide, methotrexate, and fluorouracil. If any measurable contamination (cyclophosphamide levels greater than 1.00 ng per cm² have been found to cause human uptake) is found by any of these quality assurance procedures, practitioners shall make the decision to identify, document, and contain the cause of contamination. Such action may include retraining, thorough cleaning (utilizing high-pH soap and water), and improving engineering controls. Examples of improving engineering controls are (1) venting BSCs or CACIs 100% to the outside, (2) implementing a CSTD, or (3) re-assessing types of BSCs or CACIs.

Disposal of all hazardous drug wastes shall comply with all applicable federal and state regulations. All personnel who perform routine custodial waste removal and cleaning activities in storage and preparation areas for hazardous drugs shall be trained in appropriate procedures to protect themselves and prevent contamination.

RADIOPHARMACEUTICALS AS CSPs

In the case of production of radiopharmaceuticals for positron emission tomography (PET), general test chapter *Positron Emission Tomography Drugs for Compounding, Investigational, and Research Uses* (823) supersedes this chapter. Upon release of a PET radiopharmaceutical as a finished drug product from a production facility, the further handling, manipulation, or use of the product will be considered compounding, and the content of this section and chapter is applicable.

For the purposes of this chapter, radiopharmaceuticals compounded from sterile components in closed sterile containers and with a volume of 100 mL or less for a single-dose injection or not more than 30 mL taken from a multiple-dose container (see *Injections* (1)) shall be designated as, and conform to, the standards for *Low-Risk Level CSPs*.

These radiopharmaceuticals shall be compounded using appropriately shielded vials and syringes in a properly functioning and certified ISO Class 5 (see *Table 1*) PEC located in an ISO Class 8 (see *Table 1*) or cleaner air environment to permit compliance with special handling, shielding, and negative air flow requirements.

Radiopharmaceutical vials designed for multi-use, compounded with technetium-99m, exposed to ISO Class 5 (see *Table 1*) environment, and punctured by needles with no direct contact contamination may be used up to the time indicated by manufacturers' recommendations. Storage and transport of properly shielded vials of radiopharmaceutical CSPs may occur in a limited access ambient environment without a specific ISO class designation.

Technetium-99m/molybdenum-99 generator systems shall be stored and eluted (operated) under conditions recommended by manufacturers and applicable state and federal regulations. Such generator systems shall be eluted in an ISO Class 8 (see *Table 1*) or cleaner air environment to permit special handling, shielding, and air flow requirements. To limit acute and chronic radiation exposure of inspecting personnel to a level that is as low as reasonably achievable (ALARA), direct visual inspection of radiopharmaceutical CSPs containing high concentrations of doses of radioactivity shall be conducted in accordance with ALARA.

Radiopharmaceuticals prepared as *Low-Risk Level CSPs with 12-Hour or Less BUD* shall be prepared in a segregated compounding area. A line of demarcation defining the segre-

gated compounding area shall be established. Materials and garb exposed in a patient care and treatment area shall not cross a line of demarcation into the segregated compounding area.

ALLERGEN EXTRACTS AS CSPs

Allergen extracts as CSPs are single-dose and multiple-dose *intradermal* or *subcutaneous* injections that are prepared by specially trained physicians and personnel under their direct supervision. Allergen extracts as CSPs are not subject to the personnel, environmental, and storage requirements for all *CSP Microbial Contamination Risk Levels* in this chapter only when all of the following criteria are met:

1. The compounding process involves simple transfer via sterile needles and syringes of commercial sterile allergen products and appropriate sterile added substances (e.g., glycerin, phenol in sodium chloride injection).
2. All allergen extracts as CSPs shall contain appropriate substances in effective concentrations to prevent the growth of microorganisms. Nonpreserved allergen extracts shall comply with the appropriate CSP risk level requirements in the chapter.
3. Before beginning compounding activities, personnel perform a thorough hand-cleansing procedure by removing debris from under fingernails using a nail cleaner under running warm water followed by vigorous hand and arm washing to the elbows for at least 30 seconds with either nonantimicrobial or antimicrobial soap and water.
4. Compounding personnel don hair covers, facial hair covers, gowns, and face masks.
5. Compounding personnel perform antiseptic hand cleansing with an alcohol-based surgical hand scrub with persistent activity.
6. Compounding personnel don powder-free sterile gloves that are compatible with sterile 70% isopropyl alcohol (IPA) before beginning compounding manipulations.
7. Compounding personnel disinfect their gloves intermittently with sterile 70% IPA when preparing multiple allergen extracts as CSPs.
8. Ampul necks and vial stoppers on packages of manufactured sterile ingredients are disinfected by careful wiping with sterile 70% IPA swabs to ensure that the critical sites are wet for at least 10 seconds and allowed to dry before they are used to compound allergen extracts as CSPs.
9. The aseptic compounding manipulations minimize direct contact contamination (e.g., from glove fingertips, blood, nasal and oral secretions, shed skin and cosmetics, other nonsterile materials) of critical sites (e.g., needles, opened ampuls, vial stoppers).
10. The label of each multiple-dose vial (MDV) of allergen extracts as CSPs lists the name of one specific patient and a BUD and storage temperature range that is assigned based on manufacturers' recommendations or peer-reviewed publications.
11. Single-dose allergen extracts as CSPs shall not be stored for subsequent additional use.

Personnel who compound allergen extracts as CSPs must be aware of greater potential risk of microbial and foreign material contamination when allergen extracts as CSPs are compounded in compliance with the foregoing criteria instead of the more rigorous standards in this chapter for *CSP Microbial Contamination Risk Levels*. Although contaminated allergen extracts as CSPs can pose health risks to patients when they are injected *intradermally* or *subcutaneously*, these risks are substantially greater if the extract is inadvertently injected *intravenously*.

VERIFICATION OF COMPOUNDING ACCURACY AND STERILITY

The compounding procedures and sterilization methods for CSPs correspond to correctly designed and verified written documentation in the compounding facility. Verification requires planned testing, monitoring, and documentation to demonstrate adherence to environmental quality requirements, personnel practices, and procedures critical to achieving and maintaining sterility, accuracy, and purity of finished CSPs. For example, sterility testing (see *Test for Sterility of the Product To Be Examined* under *Sterility Tests* (71)) may be applied to specimens of low- and medium-risk level CSPs, and standard self-contained biological indicators (BI) shall be added to nondispensable specimens of high-risk level CSPs before terminal sterilization for subsequent evaluation to determine whether the sterilization cycle was adequate (see *Biological Indicators for Sterilization* (1035)). Packaged and labeled CSPs shall be visually inspected for physical integrity and expected appearance, including final fill amount. The accuracy of identities, concentrations, amounts, and purities of ingredients in CSPs shall be confirmed by reviewing labels on packages, observing and documenting correct measurements with approved and correctly standardized devices, and reviewing information in labeling and certificates of analysis provided by suppliers. When the correct identity, purity, strength, and sterility of ingredients and components of CSPs cannot be confirmed (in cases of, for example, unlabeled syringes, opened ampuls, punctured stoppers of vials and bags, containers of ingredients with incomplete labeling), such ingredients and components shall be discarded immediately.

Some individual ingredients, such as bulk drug substances, are not labeled with expiration dates when they are stable indefinitely in their commercial packages under their labeled storage conditions. However, despite retaining full chemical stability, such ingredients may gain or lose moisture during storage and use. Changes in moisture content may require testing (see *Loss on Drying* (731)) to determine the correct amount to weigh for accurate content of active chemical moieties in CSPs (see *Pharmaceutical Calculations in Prescription Compounding* (1160)).

Although not required, a quantitative stability-indicating chemical assay is recommended to ensure compounding accuracy of CSPs, especially those that contain drug ingredients with a narrow therapeutic plasma concentration range.

Sterilization Methods

The licensed healthcare professionals who supervise compounding shall be responsible for determining that the selected sterilization method (see *Methods of Sterilization* under *Sterilization and Sterility Assurance of Compensal Articles* (1211)) both sterilizes and maintains the strength, purity, quality, and packaging integrity of CSPs. The selected sterilization process is obtained from experience and appropriate information sources (e.g., see *Sterilization and Sterility Assurance of Compensal Articles* (1211))—and, preferably, verified wherever possible—to achieve sterility in the particular CSPs. General guidelines for matching CSPs and components to appropriate sterilization methods include the following:

1. CSPs have been ascertained to remain physically and chemically stable when subjected to the selected sterilization method.
2. Glass and metal devices may be covered tightly with aluminum foil, then exposed to dry heat in an oven at a mean temperature of 250° for 30 minutes to achieve sterility and depyrogenation (see *Dry-Heat Sterilization* under *Sterilization and Sterility Assurance of Compensal Articles* (1211) and *Bacterial Endotoxins Test* (85)). Such items are either used immediately or stored until use in an environment suitable for com-

pounding *Low-Risk Level CSPs* and *Medium-Risk Level CSPs*.

3. Personnel ascertain from appropriate information sources that the sterile microporous membrane filter used to sterilize CSP solutions, during either compounding or administration, is chemically and physically compatible with the CSP.

STERILIZATION OF HIGH-RISK LEVEL CSPs BY FILTRATION

Commercially available sterile filters shall be approved for human-use applications in sterilizing pharmaceutical fluids. Sterile filters used to sterilize CSPs shall be pyrogen free and have a nominal pore size of 0.2 or 0.22 µm. They shall be certified by the manufacturer to retain at least 10⁷ microorganisms of a strain of *Brevundimonas* (*Pseudomonas*) *diminuta* on each square centimeter of upstream filter surface area under conditions similar to those in which the CSPs will be sterilized (see *High-Risk Conditions* in *High-Risk Level CSPs*).

The compounding supervisor shall ensure, directly or from appropriate documentation, that the filters are chemically and physically stable at the pressure and temperature conditions to be used, that they have enough capacity to filter the required volumes, and that they will achieve sterility and maintain prefiltration pharmaceutical quality, including strength of ingredients of the specific CSP. The filter dimensions and liquid material to be sterile-filtered shall permit the sterilization process to be completed rapidly, without the replacement of the filter during the process. When CSPs are known to contain excessive particulate matter, a prefilter of larger nominal pore size membrane is placed upstream from the sterilizing filter to remove gross particulate contaminants in order to maximize the efficiency of the sterilizing filter.

Filter units used to sterilize CSPs shall also be subjected to manufacturers' recommended integrity test, such as the bubble point test.

Compounding personnel shall ascertain that selected filters will achieve sterilization of the particular CSPs being sterilized. Large deviations from usual or expected chemical and physical properties of CSPs (e.g., water-miscible alcohols) may cause undetectable damage to filter integrity and shrinkage of microorganisms to sizes smaller than filter nominal pore size.

STERILIZATION OF HIGH-RISK LEVEL CSPs BY STEAM

The process of thermal sterilization employing saturated steam under pressure, or autoclaving, is the preferred method to terminally sterilize aqueous preparations that have been verified to maintain their full chemical and physical stability under the conditions employed (see *Steam Sterilization* under *Sterilization and Sterility Assurance of Compensal Articles* (1211)). To achieve sterility, all materials are to be exposed to steam at 121° under a pressure of about 1 atmosphere or 15 psi for the duration verified by testing to achieve sterility of the items, which is usually 20 to 60 minutes for CSPs. An allowance shall be made for the time required for the material to reach 121° before the sterilization exposure duration is timed.

Not directly exposing items to pressurized steam may result in survival of microbial organisms and spores. Before their sterilization, plastic, glass, and metal devices are tightly wrapped in low-particle-shedding paper or fabrics or sealed in envelopes that prevent poststerilization microbial penetration. Immediately before filling ampuls and vials that will be steam sterilized, solutions are passed through a filter having a nominal pore size not larger than 1.2 µm for removal of particulate matter. Sealed containers shall be able to generate steam internally; thus, stoppered and crimped empty vials shall contain a small amount of moisture to generate steam.

The description of steam sterilization conditions and duration for specific CSPs shall be included in written documentation in the compounding facility. The effectiveness of steam sterilization shall be verified using appropriate BIs of *Bacillus stearothermophilus* (see *Biological Indicators* <1035>) and other confirmation methods such as temperature-sensing devices (see *Sterilization and Sterility Assurance of Compendial Articles* <1211> and *Sterility Tests* <71>).

STERILIZATION OF HIGH-RISK LEVEL CSPS BY DRY HEAT

Dry heat sterilization is usually done as a batch process in an oven designed for sterilization. Heated filtered air shall be evenly distributed throughout the chamber by a blower device. The oven should be equipped with a system for controlling temperature and exposure period. Sterilization by dry heat requires higher temperatures and longer exposure times than does sterilization by steam. Dry heat shall be used only for those materials that cannot be sterilized by steam, when either the moisture would damage the material or the material is impermeable. During sterilization, sufficient space shall be left between materials to allow for good circulation of the hot air. The description of dry heat sterilization conditions and duration for specific CSPs shall be included in written documentation in the compounding facility. The effectiveness of dry heat sterilization shall be verified using appropriate BIs of *Bacillus subtilis* (see *Biological Indicators* <1035>) and other confirmation methods such as temperature-sensing devices (see *Sterilization and Sterility Assurance of Compendial Articles* <1211> and *Sterility Tests* <71>). [NOTE—Dry heat sterilization may be performed at a lower temperature than may be effective for depyrogenation].

Depyrogenation by Dry Heat

Dry heat depyrogenation shall be used to render glassware or containers such as vials free from pyrogens as well as viable microbes. A typical cycle would be 30 minutes at 250°. The description of the dry heat depyrogenation cycle and duration for specific load items shall be included in written documentation in the compounding facility. The effectiveness of the dry heat depyrogenation cycle shall be verified using endotoxin challenge vials (ECVs). The bacterial endotoxin test should be performed on the ECVs to verify that the cycle is capable of achieving a 3-log reduction in endotoxin (see *Sterilization and Sterility Assurance of Compendial Articles* <1211> and *Bacterial Endotoxins Test* <85>).

ENVIRONMENTAL QUALITY AND CONTROL

Achieving and maintaining sterility and overall freedom from contamination of a CSP is dependent on the quality status of the components incorporated, the process utilized, personnel performance, and the environmental conditions under which the process is performed. The standards required for the environmental conditions depend on the amount of exposure of the CSP to the immediate environment anticipated during processing. The quality and control of environmental conditions for each risk level of operation are explained in this section. In addition, operations using nonsterile components require the use of a method of preparation designed to produce a sterile preparation.

Exposure of Critical Sites

Maintaining the sterility and cleanliness (i.e., freedom from sterile foreign materials) of critical sites is a primary

safeguard for CSPs. Critical sites are locations that include any component or fluid pathway surfaces (e.g., vial septa, injection ports, beakers) or openings (e.g., opened ampuls, needle hubs) exposed and at risk of direct contact with air (e.g., ambient room or HEPA filtered), moisture (e.g., oral and mucosal secretions), or touch contamination. The risk of, or potential for, critical sites to be contaminated with microorganisms and foreign matter increases with increasing exposed area of the critical sites, the density or concentration of contaminants, and exposure duration to worse than ISO Class 5 (see *Table 1*) air. Examples include an opened ampul or vial stopper on a 10-mL or larger vial or an injection port on a package of intravenous solution having an area larger than the point of a needle or the tip of a syringe.

The nature of a critical site also affects the risk of contamination. The relatively rough, permeable surface of an elastomeric closure retains microorganisms and other contaminants after swabbing with a sterile 70% IPA pad more readily than does the smoother glass surface of the neck of an ampul. Therefore, the surface disinfection can be expected to be more effective for an ampul.

Protection of critical sites by precluding physical contact and airborne contamination shall be given the highest priority in sterile compounding practice. Airborne contaminants, especially those generated by sterile compounding personnel, are much more likely to reach critical sites than are contaminants that are adhering to the floor or other surfaces below the work level. Furthermore, large and high-density particles that are generated and introduced by compounding manipulations and personnel have the potential to settle on critical sites even when those critical sites are exposed within ISO Class 5 (see *Table 1*) air.

ISO Class 5 Air Sources, Buffer Areas, and Ante-Areas

The most common sources of ISO Class 5 (see *Table 1*) air quality for exposure of critical sites are horizontal and vertical LAFWs, CAIs, and CACIs. A clean room (see *Microbiological Control and Monitoring of Aseptic Processing Environments* <1116>) is a compounding environment that is supplied with HEPA or HEPA-filtered air that meets ISO Class 7 (see *Table 1*), the access to which is limited to personnel trained and authorized to perform sterile compounding and facility cleaning. A buffer area is an area that provides at least ISO Class 7 (see *Table 1*) air quality.

Figure 1 is a conceptual representation of the placement of an ISO Class 5 (see *Table 1*) PEC in a segregated compounding area used for low-risk level CSPs with 12-hour or less BUD. This plan depicts the most critical operation area located within the PEC in a designated area (see definition of *Segregated Compounding Area*) separated from activities not essential to the preparation of CSPs. Placement of devices (e.g., computers, printers) and objects (e.g., carts, cabinets) that are not essential to compounding in the segregated area should be restricted or limited, depending on their effect on air quality in the ISO Class 5 (see *Table 1*) PEC.

Conceptual representation of USP
Chapter <797> facility requirements

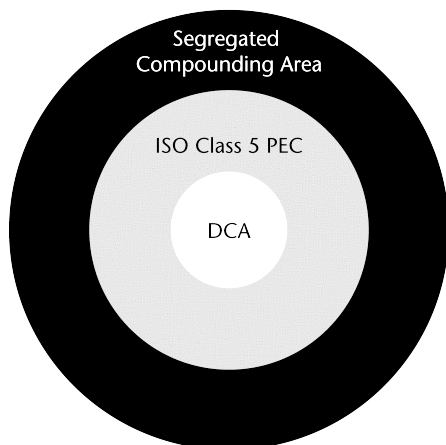


Figure 1. Conceptual representation of the placement of an ISO Class 5 PEC in a segregated compounding area used for low-risk level CSPs with 12-hour or less BUD.

Figure 2 is a conceptual representation of the arrangement of a facility for preparation of CSPs categorized as low-, medium-, and high-risk level. The quality of the environmental air increases with movement from the outer boundary to the direct compounding area (DCA). Placement of devices in ante-areas and buffer areas is dictated by their effect on the designated environmental quality of atmospheres and surfaces, which shall be verified by monitoring (see *Viable and Nonviable Environmental Sampling (ES) Testing*). It is the responsibility of each compounding facility to ensure that each source of ISO Class 5 (see Table 1) environment for exposure of critical sites and sterilization by filtration is properly located, operated, maintained, monitored, and verified.

Conceptual representation of USP
Chapter <797> facility requirements

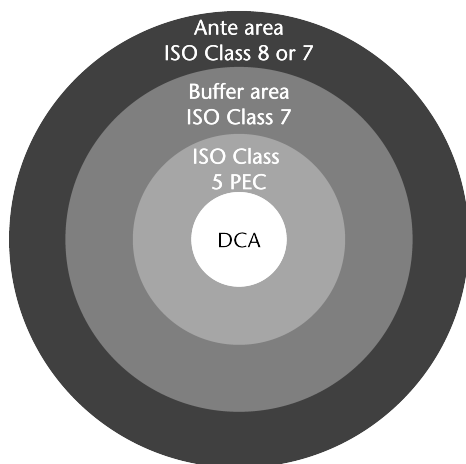


Figure 2. Conceptual representation of the arrangement of a facility for preparation of CSPs categorized as low-, medium-, and high-risk level.

Placement of devices (e.g., computers, printers) and objects (e.g., carts, cabinets) that are not essential to compounding in buffer areas is dictated by their effect on the required environmental quality of air atmospheres and surfaces, which shall be verified by monitoring (see *Viable and Nonviable Environmental Sampling (ES) Testing*). It is the responsibility of each compounding facility to ensure that

each source of ISO Class 5 (see Table 1) environment for exposure of critical sites and sterilization by filtration is properly located, operated, maintained, monitored, and verified.

Facility Design and Environmental Controls

Compounding facilities are physically designed and environmentally controlled to minimize airborne contamination from contacting critical sites. These facilities shall also provide a comfortable and well-lighted working environment, which typically includes a temperature of 20° or cooler, to maintain comfortable conditions for compounding personnel to perform flawlessly when attired in the required aseptic compounding garb. PECs typically include, but are not limited to, LAFWs, BSCs, CAs, and CACIs, which provide an ISO Class 5 (see Table 1) environment for the exposure of critical sites. PECs shall maintain ISO Class 5 (see Table 1) or better conditions for 0.5-μm particles (dynamic operating conditions) while compounding CSPs. Secondary engineering controls such as buffer areas and ante-areas generally serve as a core for the location of the PEC. Buffer areas are designed to maintain at least ISO Class 7 (see Table 1) conditions for 0.5-μm particles under dynamic conditions and ISO Class 8 (see Table 1) conditions for 0.5-μm and larger particles under dynamic conditions for the ante-areas. Airborne contamination control is achieved in the PEC through the use of HEPA filters. The airflow in the PEC shall be unidirectional (laminar flow), and because of the particle collection efficiency of the filter, the “first air” at the face of the filter is, for the purposes of aseptic compounding, free from airborne particulate contamination. HEPA-filtered air shall be supplied in critical areas (ISO Class 5, see Table 1) at a velocity sufficient to sweep particles away from the compounding area and maintain unidirectional airflow during operations. Proper design and control prevents turbulence and stagnant air in the critical area. In situ air pattern analysis via smoke studies shall be conducted at the critical area to demonstrate unidirectional airflow and sweeping action over and away from the product under dynamic conditions.

The principles of HEPA-filtered unidirectional airflow in the work environment shall be understood and practiced in the compounding process in order to achieve the desired environmental conditions. Policies and procedures for maintaining and working within the PEC area shall be written and followed. The policies and procedures will be determined by the scope and risk levels of the aseptic compounding activities utilized during the preparation of the CSPs. The CSP work environment is designed to have the cleanest work surfaces (PEC) located in a buffer area. The buffer area shall maintain at least ISO Class 7 (see Table 1) conditions for 0.5-μm and larger particles under dynamic operating conditions. The room shall be segregated from surrounding, unclassified spaces to reduce the risk of contaminants being blown, dragged, or otherwise introduced into the filtered unidirectional airflow environment, and this segregation shall be continuously monitored. For rooms providing a physical separation through the use of walls, doors, and pass-throughs, a minimum differential positive pressure of 0.02- to 0.05-inch water column is required. For buffer areas not physically separated from the ante-areas, the principle of displacement airflow shall be employed. This concept utilizes a low pressure differential, high airflow principle. Using displacement airflow typically requires an air velocity of 40 ft per minute or more from the buffer area across the line of demarcation into the ante-area.

The displacement concept shall not be used for high-risk compounding.⁶ The PEC shall be placed within a buffer area in such a manner as to avoid conditions that could adversely affect their operation. For example, strong air currents from opened doors, personnel traffic, or air streams from the HVAC systems can disrupt the unidirectional air-

⁶ ISO 14644-4:2001 Cleanrooms and associated controlled environments—Design, construction, and start-up, *Case Postale 56*, CH-1211 Geneva 20, Switzerland, tel. +41 22 749 01 11.

flow in open-faced workbenches. The operators may also create disruptions in airflow by their own movements and by the placement of objects onto the work surface. The PEC shall be placed out of the traffic flow and in a manner to avoid disruption from the HVAC system and room cross-drafts. Room air exchanges are typically expressed as ACPHs. Adequate HEPA-filtered airflow supplied to the buffer area and ante-area is required to maintain cleanliness classification during operational activity through the number of ACPHs. Factors that should be considered when determining air-change requirements include number of personnel working in the room and compounding processes that generate particulates, as well as temperature effects. An ISO Class 7 (see *Table 1*) buffer area and ante-area supplied with HEPA-filtered air shall receive an ACPH of not less than 30. The PEC is a good augmentation to generating air changes in the air supply of an area but cannot be the sole source of HEPA-filtered air. If the area has an ISO Class 5 (see *Table 1*) recirculating device, a minimum of 15 ACPHs through the area supply HEPA filters is adequate, providing the combined ACPH is not less than 30. More air changes may be required, depending on the number of personnel and processes. HEPA-filtered supply air shall be introduced at the ceiling, and returns should be mounted low on the wall, creating a general top-down dilution of area air with HEPA-filtered make-up air. Ceiling-mounted returns are not recommended. All HEPA filters should be efficiency tested using the most penetrating particle size and should be leak tested at the factory and then leak tested again in situ after installation.⁷

Activities and tasks carried out within the buffer area shall be limited to only those necessary when working within a controlled environment. Only the furniture, equipment, supplies, and other material required for the compounding activities to be performed shall be brought into the area, and they shall be nonpermeable, nonshedding, cleanable, and resistant to disinfectants. Whenever such items are brought into the area, they shall first be cleaned and disinfected. Whenever possible, equipment and other items used in the buffer area shall not be taken out of the area except for calibration, servicing, or other activities associated with the proper maintenance of the item.

The surfaces of ceilings, walls, floors, fixtures, shelving, counters, and cabinets in the buffer area shall be smooth, impervious, free from cracks and crevices, and nonshedding, thereby promoting cleanability and minimizing spaces in which microorganisms and other contaminants may accumulate. The surfaces shall be resistant to damage by disinfectant agents. Junctures of ceilings to walls shall be coved or caulked to avoid cracks and crevices where dirt can accumulate. If ceilings consist of inlaid panels, the panels shall be impregnated with a polymer to render them impervious and hydrophobic, and they shall be caulked around each perimeter to seal them to the support frame. Walls may be constructed of flexible material (e.g., heavy gauge polymer), panels locked together and sealed, or of epoxy-coated gypsum board. Preferably, floors are overlaid with wide sheet vinyl flooring with heat-welded seams and coving to the sidewall. Dust-collecting overhangs, such as ceiling utility pipes, and ledges, such as windowsills, should be avoided. The exterior lens surface of ceiling lighting fixtures should be smooth, mounted flush, and sealed. Any other penetrations through the ceiling or walls shall be sealed. The buffer area shall not contain sources of water (sinks) or floor drains. Work surfaces shall be constructed of smooth, impervious materials, such as stainless steel or molded plastic, so that they are easily cleaned and disinfected. Carts should be of stainless steel wire, nonporous plastic, or sheet metal construction with good quality, cleanable casters to promote mobility. Storage shelving, counters, and cabinets shall be smooth, impervious, free from cracks and crevices, nonshed-

ding, cleanable, and disinfectable; their number, design, and manner of installation shall promote effective cleaning and disinfection.

Placement of Primary Engineering Controls

PECs (LAFWs, BSCs, CAIs, and CACIs) shall be located within a restricted access ISO Class 7 (see *Table 1*) buffer area (see *Figure 1*), with the following CAI/CACI exceptions below:

- Only authorized personnel and materials required for compounding and cleaning shall be permitted in the buffer area.
- Presterilization procedures for high-risk level CSPs, such as weighing and mixing, shall be completed in no worse than an ISO Class 8 (see *Table 1*) environment.
- PECs shall be located out of traffic patterns and away from room air currents that could disrupt the intended airflow patterns.

CAIs and CACIs shall be placed in an ISO Class 7 (see *Table 1*) buffer area *unless* they meet all of the following conditions:

- The isolator shall provide isolation from the room and maintain ISO Class 5 (see *Table 1*) during dynamic operating conditions, including transferring ingredients, components, and devices into and out of the isolator and during preparation of CSPs.
- Particle counts sampled approximately 6 to 12 inches upstream of the critical exposure site shall maintain ISO Class 5 (see *Table 1*) levels during compounding operations.
- Not more than 3520 particles (0.5 μm and larger) per m^3 shall be counted during material transfer, with the particle counter probe located as near to the transfer door as possible without obstructing the transfer.⁸

It is incumbent on the compounding personnel to obtain documentation from the manufacturer that the CAI/CACI will meet this standard when located in environments where the background particle counts exceed ISO Class 8 (see *Table 1*) for 0.5- μm and larger particles. When isolators are used for sterile compounding, the recovery time to achieve ISO Class 5 (see *Table 1*) air quality shall be documented and internal procedures developed to ensure that adequate recovery time is allowed after material transfer before and during compounding operations.

If the PEC is a CAI or CACI that does not meet the requirements above or is a LAFW or BSC that cannot be located within an ISO Class 7 (see *Table 1*) buffer area, then only low-risk level nonhazardous and radiopharmaceutical CSPs pursuant to a physician order for a specific patient may be prepared, and administration of the CSP shall commence within 12 hours of preparation or as recommended in the manufacturer's package insert, whichever is less.

Viable and Nonviable Environmental Sampling (ES) Testing

The ES program should provide information to staff and leadership to demonstrate that the PEC is maintaining an environment within the compounding area that consistently ensures acceptably low viable and nonviable particle levels. The compounding area includes the ISO Class 5 (see *Table 1*) PEC (LAFWs, BSCs, CAIs, and CACIs), buffer areas, ante-areas, and segregated compounding areas.

Environmental sampling shall occur as part a comprehensive quality management program and shall occur minimally under any of the following conditions:

- as part of the commissioning and certification of new facilities and equipment;
- following any servicing of facilities and equipment;

⁷ By definition (IEST RP CC 001.4), HEPA filters are a minimum of 99.97% efficient when tested using 0.3- μm thermally generated particles and a photometer or rated at their most penetrating particle size using a particle counter.

⁸ Sample procedures are detailed in CETA Applications Guide CAG-002-2006—section 2.09.

- as part of the re-certification of facilities and equipment (i.e., every 6 months);
- in response to identified problems with end products or staff technique; or
- in response to issues with CSPs, observed compounding personnel work practices, or patient-related infections (where the CSP is being considered as a potential source of the infection).

ENVIRONMENTAL NONVIABLE PARTICLE TESTING PROGRAM

A program to sample nonviable airborne particles differs from that for viable particles in that it is intended to directly measure the performance of the engineering controls used to create the various levels of air cleanliness, for example, ISO Class 5, 7, or 8 (see *Table 1*).

Engineering Control Performance Verification—PECs (LAFWs, BSCs, CAIs, and CACIs) and secondary engineering controls (buffer and ante-areas) are essential components of the overall contamination control strategy for aseptic compounding. As such, it is imperative that they perform as designed and that the resulting levels of contamination be within acceptable limits. Certification procedures such as those outlined in *Certification Guide for Sterile Compounding Facilities* (CAG-003-2006)⁹ shall be performed by a qualified individual no less than every 6 months and whenever the device or room is relocated or altered or major service to the facility is performed.

Total Particle Counts—Certification that each ISO classified area, for example, ISO Class 5, 7, and 8 (see *Table 1*), is within established guidelines shall be performed no less than every 6 months and whenever the LAFW, BSC, CAI, or CACI is relocated or the physical structure of the buffer area or ante-area has been altered. Testing shall be performed by qualified operators using current, state-of-the-art electronic equipment with results of the following:

- ISO Class 5: not more than 3520 particles 0.5 μm and larger size per cubic meter of air for any LAFW, BSC, CAI, and CACI;
- ISO Class 7: not more than 352,000 particles of 0.5 μm size and larger per cubic meter of air for any buffer area;
- ISO Class 8: not more than 3,520,000 particles or 0.5 μm size and larger per cubic meter of air for any ante-area.

All certification records shall be maintained and reviewed by supervising personnel or other designated employees to ensure that the controlled environments comply with the proper air cleanliness, room pressures, and ACPHs.

PRESSURE DIFFERENTIAL MONITORING

A pressure gauge or velocity meter shall be installed to monitor the pressure differential or airflow between the buffer area and the ante-area and between the ante-area and the general environment outside the compounding area. The results shall be reviewed and documented on a log at least every work shift (minimum frequency shall be at least daily) or by a continuous recording device. The pressure between the ISO Class 7 (see *Table 1*) and the general pharmacy area shall not be less than 5 Pa (0.02 inch water column). In facilities where low- and medium-risk level CSPs are prepared, differential airflow shall maintain a minimum velocity of 0.2 meters per second (40 feet per minute) between buffer area and ante-area.

⁹ Controlled Environment Testing Association, 1500 Sunday Drive, Ste. 102, Raleigh, NC 27607; www.CETAIternational.org.

ENVIRONMENTAL VIABLE AIRBORNE PARTICLE TESTING PROGRAM

The risk of contaminating a CSP prepared under low-risk level and medium-risk level conditions is highly dependent on proper hand hygiene and garbing practices, compounding personnel aseptic technique, and the presence of surface contamination, assuming that all work is performed in a certified and properly functioning ISO Class 5 (see *Table 1*) PEC and secondary engineering controls, ISO Class 7 (see *Table 1*) buffer area, and ISO Class 8 (see *Table 1*) ante-area. High-risk level CSPs pose the greatest threat to patients because compounding personnel are tasked with the requirement of processing nonsterile components and devices in order to achieve sterility.

A sampling program in conjunction with an observational audit is designed to evaluate the competency of compounding personnel work practices, allowing for the implementation of corrective actions on an ongoing basis (see *Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures*).

Sampling Plan—An appropriate environmental sampling plan shall be developed for airborne viable particles based on a risk assessment of compounding activities performed.

Selected sampling sites shall include locations within each ISO Class 5 (see *Table 1*) environment and in the ISO Class 7 and 8 (see *Table 1*) areas and in the segregated compounding areas at greatest risk of contamination (e.g., work areas near the ISO Class 5 [see *Table 1*] environment, counters near doors, pass-through boxes). The plan shall include sample location, method of collection, frequency of sampling, volume of air sampled, and time of day as related to activity in the compounding area and action levels.

Review of the data generated during a sampling event may detect elevated amounts of airborne microbial bioburden; such changes may be indicative of adverse changes within the environment. It is recommended that compounding personnel refer to *Microbiological Control and Monitoring of Aseptic Processing Environments* (1116) and the CDC's "Guidelines for Environmental Infection Control in Healthcare Facilities, 2003" for more information.

Growth Medium—A general microbiological growth medium such as Soybean-Casein Digest Medium shall be used to support the growth of bacteria. Malt extract agar or some other media that supports the growth of fungi shall be used in high-risk level compounding environments. Media used for surface sampling must be supplemented with additives to neutralize the effects of disinfecting agents (e.g., TSA with lecithin and polysorbate 80).

Viable Air Sampling—Evaluation of airborne microorganisms using volumetric collection methods in the controlled air environments (LAFWs, CAIs, clean room or buffer areas, and ante-areas) shall be performed by properly trained individuals for all compounding risk levels.

Impaction shall be the preferred method of volumetric air sampling. Use of settling plates for qualitative air sampling may not be able to determine adequately the quality of air in the controlled environment. The settling of particles by gravity onto culture plates depends on the particle size and may be influenced by air movement. Consequently, the number of colony-forming units (cfu) on a settling plate may not always relate to the concentrations of viable particles in the sampled environment.

For low-, medium-, and high-risk level compounding, air sampling shall be performed at locations that are prone to contamination during compounding activities and during other activities such as staging, labeling, gowning, and cleaning. Locations shall include zones of air backwash turbulence within LAFW and other areas where air backwash turbulence may enter the compounding area (doorways, in and around ISO Class 5 [see *Table 1*] PEC and environments). Consideration should be given to the overall effect the chosen sampling method will have on the unidirectional airflow within a compounding environment.

For low-risk level CSPs with 12-hour or less BUD prepared in a PEC (LAFWs, BSCs, CAIs) that maintains an ISO Class 5 (see *Table 1*), air sampling shall be performed at locations inside the ISO Class 5 (see *Table 1*) environment and other areas that are in close proximity to the ISO Class 5 (see *Table 1*) environment during the certification of the PEC.

Air Sampling Devices—There are a number of manufacturers of electronic air sampling equipment. It is important that personnel refer to the manufacturer's recommended procedures when using the equipment to perform volumetric air sampling procedures. The instructions in the manufacturer's user's manual for verification and use of electric air samplers that actively collect volumes of air for evaluation must be followed. A sufficient volume of air (400 to 1000 liters) shall be tested at each location in order to maximize sensitivity. The volumetric air sampling devices need to be serviced and calibrated as recommended by the manufacturer.

It is recommended that compounding personnel also refer to *Methodology and Instrumentation for Quantitation of Viable Airborne Microorganisms under Microbiological Control and Monitoring of Aseptic Processing Environments* (1116), which provides more information on the use of volumetric air samplers and volume of air that should be sampled to detect environmental bioburden excursions.

Air Sampling Frequency and Process—Air sampling shall be performed at least semiannually (i.e., every 6 months) as part of the re-certification of facilities and equipment. If compounding occurs in multiple locations within an institution (e.g., main pharmacy, satellites), environmental sampling is required for each individual compounding area. A sufficient volume of air shall be sampled and the manufacturer's guidelines for use of the electronic air sampling equipment followed. Any facility construction or equipment servicing may require that air sampling be performed during these events.

Incubation Period—At the end of the designated sampling or exposure period for air sampling activities, the microbial growth media plates are recovered and their covers secured (e.g., taped), and they are inverted and incubated at a temperature and for a time period conducive to multiplication of microorganisms. TSA should be incubated at 30° to 35° for 48 to 72 hours. Malt extract agar or other suitable fungal media should be incubated at 26° to 30° for 5 to 7 days. The number of discrete colonies of microorganisms are counted and reported as cfu and documented on an environmental sampling form. Counts from air sampling need to be transformed into cfu per cubic meter of air and evaluated for adverse trends.

Action Levels, Documentation, and Data Evaluation—The value of viable microbial sampling of the air in the compounding environment is realized when the data are used to identify and correct an unacceptable situation. Sampling data shall be collected and reviewed on a periodic basis as a means of evaluating the overall control of the compounding environment. If an activity consistently shows elevated levels of microbial growth, competent microbiology personnel shall be consulted.

Any cfu count that exceeds its respective action level (see *Table 2*) should prompt a re-evaluation of the adequacy of personnel work practices, cleaning procedures, operational procedures, and air filtration efficiency within the aseptic compounding location. An investigation into the source of the contamination shall be conducted. Sources could include HVAC systems, damaged HEPA filters, and changes in personnel garbing or work practices. The source of the problem shall be eliminated, the affected area cleaned, and resampling performed.

Counts of cfu are to be used as an approximate measure of the environmental microbial bioburden. Action levels are determined on the basis of cfu data gathered at each sampling location and trended over time. The numbers in *Table 2* should be used only as guidelines. Regardless of the number of cfu identified in the pharmacy, further corrective ac-

tions will be dictated by the identification of microorganisms recovered (at least the genus level) by an appropriate credentialed laboratory of any microbial bioburden captured as a cfu using an impactation air sampler. Highly pathogenic microorganisms (e.g., Gram-negative rods, coagulase positive staphylococcus, molds and yeasts) can be potentially fatal to patients receiving CSPs and must be immediately remedied, regardless of cfu count, with the assistance of a competent microbiologist, infection control professional, or industrial hygienist.

Table 2. Recommended Action Levels for Microbial Contamination*

†(cfu per cubic meter [1000 liters] of air per plate)

Classification	Air Sample†
ISO Class 5	> 1
ISO Class 7	> 10
ISO Class 8 or worse	> 100

* Guidance for Industry—Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice—US HHS, FDA September 2004.

Additional Personnel Requirements

Food, drinks, and materials exposed in patient care and treatment areas shall not enter ante-areas, buffer areas, or segregated compounding areas where components and ingredients of CSPs are present. When compounding activities require the manipulation of a patient's blood-derived or other biological material (e.g., radiolabeling a patient's or donor's white blood cells), the manipulations shall be clearly separated from routine material-handling procedures and equipment used in CSP preparation activities, and they shall be controlled by specific SOPs in order to avoid any cross-contamination. Packaged compounding supplies and components, such as needles, syringes, tubing sets, and small- and large-volume parenterals, should be uncartoned and wiped down with a disinfectant that does not leave a residue (e.g., sterile 70% IPA), when possible in an ante-area of ISO Class 8 (see *Table 1*) air quality, before being passed into the buffer areas. Personnel hand hygiene and garbing procedures are also performed in the ante-area, which may contain a sink that enables hands-free use with a closed system of soap dispensing to minimize the risk of extrinsic contamination. There shall be some demarcation designation that separates the ante-area from the buffer area. Adequate provision for performing antiseptic hand cleansing using an alcohol-based surgical hand scrub with persistent activity followed by the donning of sterile gloves should be provided after entry into the buffer area.

Cleaning and Disinfecting the Compounding Area

Environmental contact is a major source of microbial contamination of CSPs. Consequently, scrupulous attention to cleaning and disinfecting the sterile compounding areas is required to minimize this as a source of CSP contamination.

The cleaning and disinfecting practices and frequencies in this section apply to ISO Class 5 (see *Table 1*) compounding areas for exposure of critical sites as well as buffer areas, ante-areas, and segregated compounding areas. Compounding personnel are responsible for ensuring that the frequency of cleaning is in accordance with the requirements stated in *Table 3* and determining the cleaning and disinfecting products to be used (see *Appendix II*). Any organizational or institutional policies regarding disinfectant selection should be considered by compounding personnel. All cleaning and disinfecting practices and policies for the compounding of CSPs shall be included in written SOPs and shall be followed by all compounding personnel.

The selection and use of disinfectants in healthcare facilities is guided by several properties, such as microbicidal activity, inactivation by organic matter, residue, and shelf life (see *Appendix II*). In general, highly toxic disinfectants, such as glutaraldehyde, are not used on housekeeping surfaces (e.g., floors, countertops). Many disinfectants registered by the EPA are one-step disinfectants. This means that the disinfectant has been formulated to be effective in the presence of light to moderate soiling without a pre-cleaning step.

Surfaces in LAFWs, BSCs, CAIs, and CACIs, which are intimate to the exposure of critical sites, require disinfecting more frequently than do housekeeping surfaces such as walls and ceilings. Disinfecting sterile compounding areas shall occur on a regular basis at the intervals noted in *Table 3* when spills occur, when the surfaces are visibly soiled, and when microbial contamination is known to have been or is suspected of having been introduced into the compounding areas.

When the surface to be disinfected has heavy soiling, a cleaning step is recommended prior to the application of the disinfectant. Trained compounding personnel are responsible for developing, implementing, and practicing the procedures for cleaning and disinfecting the DCAs written in the SOPs. Cleaning and disinfecting shall occur before compounding is performed. Items shall be removed from all areas to be cleaned, and surfaces shall be cleaned by removing loose material and residue from spills; for example, water-soluble solid residues are removed with sterile water (for injection or irrigation) and low-shedding wipes. This shall be followed by wiping with a residue-free disinfecting agent such as sterile 70% IPA, which is allowed to dry before compounding begins.

Cleaning and disinfecting surfaces in the LAFWs, BSCs, CAIs, and CACIs are the most critical practices before the preparation of CSPs. Consequently, such surfaces shall be cleaned and disinfected frequently, including at the beginning of each work shift, before each batch preparation is started, every 30 minutes during continuous compounding periods of individual CSPs, when there are spills, and when surface contamination is known or suspected from procedural breaches.

Work surfaces in the ISO Class 7 (see *Table 1*) buffer areas and ISO Class 8 (see *Table 1*) ante-areas as well as segregated compounding areas shall be cleaned and disinfected at least daily, and dust and debris shall be removed when necessary from storage sites for compounding ingredients and supplies using a method that does not degrade the ISO Class 7 or 8 (see *Table 1*) air quality (see *Disinfectants and Antiseptics* <1072>).

Table 3. Minimum Frequency of Cleaning and Disinfecting Compounding Areas

Site	Minimum Frequency
ISO Class 5 (see <i>Table 1</i>) Primary Engineering Control (e.g., LAFW, BSC, CAI, CACI)	At the beginning of each shift, before each batch, not longer than 30 minutes following the previous surface disinfection when ongoing compounding activities are occurring, after spills, and when surface contamination is known or suspected
Counters and easily cleanable work surfaces	Daily
Floors	Daily
Walls	Monthly
Ceilings	Monthly
Storage shelving	Monthly

Floors in the buffer or clean area, ante-area, and segregated compounding area are cleaned by mopping with a cleaning and disinfecting agent once daily at a time when no aseptic operations are in progress. Mopping shall be performed by trained personnel using approved agents and

procedures described in the written SOPs. It is incumbent on compounding personnel to ensure that such cleaning is performed properly. In the buffer or clean area, ante-area, and segregated compounding area, walls, ceilings, and shelving shall be cleaned and disinfected monthly. Cleaning and disinfecting agents are to be used with careful consideration of compatibilities, effectiveness, and inappropriate or toxic residues (see *Appendix II*). Their schedules of use and methods of application shall be in accordance with written SOPs and followed by custodial or compounding personnel.

All cleaning materials, such as wipers, sponges, and mops, shall be nonshedding, preferably composed of synthetic micro fibers, and dedicated to use in the buffer or clean area, ante-area, and segregated compounding areas and shall not be removed from these areas except for disposal. Floor mops may be used in both the buffer or clean area and ante-area, but only in that order. Ideally, all cleaning tools are discarded after one use by collection in suitable plastic bags and removed with minimal agitation. If cleaning materials (e.g., mops) are reused, procedures shall be developed (based on manufacturers' recommendations) that ensure that the effectiveness of the cleaning device is maintained and that repeated use does not add to the bioburden of the area being cleaned.

Supplies and equipment removed from shipping cartons shall be wiped with a suitable disinfecting agent (e.g., sterile 70% IPA) delivered from a spray bottle or other suitable delivery method. After the disinfectant is sprayed or wiped on a surface to be disinfected, the disinfectant shall be allowed to dry, during which time the item shall not be used for compounding purposes.

Wiping with small sterile 70% IPA swabs that are commercially available in individual foil-sealed packages (or a comparable method) is preferred for disinfecting entry points on bags and vials, allowing the IPA to dry before piercing stoppers with sterile needles and breaking necks of ampuls. The surface of the sterile 70% IPA swabs used for disinfecting entry points of sterile packages and devices shall not contact any other object before contacting the surface of the entry point. Sterile 70% IPA wetted gauze pads or other particle-generating material shall not be used to disinfect the sterile entry points of packages and devices.

When sterile supplies are received in sealed pouches designed to keep them sterile until opening, the sterile supplies may be removed from the covering pouches as the supplies are introduced into the ISO Class 5 (see *Table 1*) PEC (LAFW, BSC, CAI, CACI) without the need to disinfect the individual sterile supply items. No shipping or other external cartons may be taken into the buffer or clean area or segregated compounding area.

Personnel Cleansing and Garbing

The careful cleansing of hands and arms and the correct donning of PPE by compounding personnel constitute the first major step in preventing microbial contamination in CSPs. Personnel shall also be thoroughly competent and highly motivated to perform flawless aseptic manipulations with ingredients, devices, and components of CSPs. Squamous cells are normally shed from the human body at a rate of 10⁶ or more per hour, and those skin particles are laden with microorganisms.^{10, 11} When individuals are experiencing rashes, sunburn, weeping sores, conjunctivitis, active respiratory infection, as well as when they wear cosmetics, they shed these particles at even higher rates. Particles shed from compounding personnel pose an increased risk of microbial contamination of critical sites of CSPs. Therefore, compounding personnel with such conditions as mentioned above shall be excluded from working in ISO Class 5 (see

¹⁰ Agalloco J, Akers JE. Aseptic Processing: A Vision of the Future. *Pharmaceutical Technology*. 2005. Aseptic Processing supplement, s16.
¹¹ Eaton T. Microbial Risk Assessment for Aseptically Prepared Products. *Am Pharm Rev*. 2005; 8 (5, Sep/Oct): 46–51.

Table 1) and ISO Class 7 (see Table 1) compounding areas until their conditions are remedied.

Before entering the buffer area or segregated compounding area (see *Low-Risk Level CSPs with 12-Hour or Less BUD*), compounding personnel shall remove personal outer garments (e.g., bandannas, coats, hats, jackets, scarves, sweaters, vests); all cosmetics, because they shed flakes and particles; and all hand, wrist, and other visible jewelry or piercings (e.g., earrings, lip or eyebrow piercings) that can interfere with the effectiveness of PPE (e.g., fit of gloves and cuffs of sleeves). The wearing of artificial nails or extenders is prohibited while working in the sterile compounding environment. Natural nails shall be kept neat and trimmed.

Personnel shall don the following PPE in an order that proceeds from those activities considered the dirtiest to those considered the cleanest. Garbing activities considered the dirtiest include donning of dedicated shoes or shoe covers, head and facial hair covers (e.g., beard covers in addition to face masks), and face masks/eye shields. Eye shields are optional unless working with irritants such as germicidal disinfecting agents or when preparing hazardous drugs.

After donning dedicated shoes or shoe covers, head and facial hair covers, and face masks, a hand cleansing procedure shall be performed by removing debris from underneath fingernails using a nail cleaner under running warm water followed by vigorous hand washing. Hands and forearms shall be washed to the elbows for at least 30 seconds with soap (either nonantimicrobial or antimicrobial) and water while in the ante-area. The use of antimicrobial scrub brushes is not recommended because they can cause skin irritation and skin damage. Hands and forearms to the elbows will be completely dried using either lint-free disposable towels or an electronic hand dryer. After completion of hand washing, a nonshedding gown with sleeves that fit snugly around the wrists and enclosed at the neck is donned. Gowns designated for buffer area use shall be worn, and preferably they should be disposable. If reusable gowns are worn, they should be laundered appropriately for buffer area use.

Once inside the buffer area or segregated compounding area (see *Low-Risk Level CSPs with 12-Hour or Less BUD*), and prior to donning sterile powder-free gloves, antiseptic hand cleansing shall be performed using a waterless alcohol-based surgical hand scrub with persistent activity¹² following manufacturers' recommendations. Hands are allowed to dry thoroughly before donning sterile gloves.

Sterile gloves shall be the last item donned before compounding begins. Gloves become contaminated when they contact nonsterile surfaces during compounding activities. Disinfection of contaminated gloved hands may be accomplished by wiping or rubbing sterile 70% IPA to all contact surface areas of the gloves and letting the gloved hands dry thoroughly. Only use gloves that have been tested for compatibility with alcohol disinfection by the manufacturer. Routine application of sterile 70% IPA shall occur throughout the compounding process and whenever nonsterile surfaces (e.g., vials, counter tops, chairs, carts) are touched. Gloves on hands shall also be routinely inspected for holes, punctures, or tears and replaced immediately if such are detected. Antiseptic hand cleansing shall be performed as indicated above. Compounding personnel shall be trained and evaluated in the avoidance of touching critical sites.

When compounding personnel exit the compounding area during a work shift, the exterior gown may be removed and retained in the compounding area if not visibly soiled, to be re-donned during that same work shift only. However, shoe covers, hair and facial hair covers, face masks/eye shields, and gloves shall be replaced with new ones before re-entering the compounding area, and proper hand hygiene shall be performed.

During high-risk compounding activities that precede terminal sterilization, such as weighing and mixing of nonster-

ile ingredients, compounding personnel shall be garbed and gloved the same as when performing compounding in an ISO Class 5 (see Table 1) environment. Properly garbed and gloved compounding personnel who are exposed to air quality that is either known or suspected to be worse than ISO Class 7 (see Table 1) shall re-garb PPE along with washing their hands properly, performing antiseptic hand cleansing with a waterless alcohol-based surgical hand scrub, and donning sterile gloves upon re-entering the ISO Class 7 (see Table 1) buffer area. When CAls and CACIs are the source of the ISO Class 5 (see Table 1) environment, the garbing and gloving requirements for compounding personnel should be as described above, unless the isolator manufacturer can provide written documentation based on validated environmental testing that any component(s) of PPE or personnel cleansing are not required.

Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices, and Cleaning/Disinfection Procedures

Personnel who prepare CSPs shall be trained conscientiously and skillfully by expert personnel and through multimedia instructional sources and professional publications in the theoretical principles and practical skills of garbing procedures, aseptic work practices, achieving and maintaining ISO Class 5 (see Table 1) environmental conditions, and cleaning and disinfection procedures. This training shall be completed and documented before any compounding personnel begin to prepare CSPs. Compounding personnel shall complete didactic training, pass written competence assessments, undergo skill assessment using observational audit tools, and media-fill testing (see *Appendices III–V*).

Media-fill testing of aseptic work skills shall be performed initially before beginning to prepare CSPs and at least annually thereafter for low- and medium-risk level compounding and semiannually for high-risk level compounding.

Compounding personnel who fail written tests or observational audits or whose media-fill test vials have one or more units showing visible microbial contamination shall be re-instructed and re-evaluated by expert compounding personnel to ensure correction of all aseptic work practice deficiencies. Compounding personnel shall pass all evaluations prior to resuming compounding of sterile preparations. In addition to didactic evaluation and aseptic media fill, compounding personnel must demonstrate proficiency of proper hand hygiene, garbing, and consistent cleaning procedures.

In the event that cleaning and disinfecting procedures are also performed by other support personnel (e.g., institutional environmental services, housekeeping), thorough training of proper hand hygiene, garbing, and cleaning and disinfection procedures shall be done by a qualified aseptic compounding expert. After completion of training, support personnel shall routinely undergo performance evaluation of proper hand hygiene, garbing, and all applicable cleaning and disinfecting procedures conducted by a qualified aseptic compounding expert.

COMPETENCY EVALUATION OF GARBING AND ASEPTIC WORK PRACTICE

The risk of contaminating a CSP prepared under low-risk level and medium-risk level conditions is highly dependent on proper hand hygiene and garbing practices, compounding personnel aseptic technique, and the presence of surface contamination, assuming that all work is performed in a certified and properly functioning ISO Class 5 (see Table 1) PEC and secondary engineering controls, ISO Class 7 (see Table 1) buffer area, and ISO Class 8 (see Table 1) ante-area. High-risk level CSPs pose the greatest threat to patients because compounding personnel are tasked with the requirement of processing nonsterile components and devices in order to achieve sterility. Compounding personnel shall be

¹² *Guideline for Hand Hygiene in Health care Settings*, MMWR, October 25, 2002, vol. 51, No. RR-16 available on the Internet at <http://www.cdc.gov/handhygiene/>.

evaluated initially prior to beginning compounding CSPs and whenever an aseptic media fill is performed using a form such as the *Sample Form for Assessing Hand Hygiene and Garbing Related Practices of Compounding Personnel* (see Appendix III) and the personnel glove fingertip sampling procedures indicated below.

Aseptic Work Practice Assessment and Evaluation via Personnel Glove Fingertip Sampling—Sampling of compounding personnel glove fingertips shall be performed for all CSP risk level compounding because direct touch contamination is the most likely source of introducing microorganisms into CSPs prepared by humans. Glove fingertip sampling shall be used to evaluate the competency of personnel in performing hand hygiene and garbing procedures in addition to educating compounding personnel on proper work practices, which include frequent and repeated glove disinfection using sterile 70% IPA during actual compounding of CSPs. All personnel shall demonstrate competency in proper hand hygiene and garbing procedures and in aseptic work practices (e.g., disinfection of component surfaces, routine disinfection of gloved hands).

Sterile contact agar plates shall be used to sample the gloved fingertips of compounding personnel after garbing in order to assess garbing competency and after completing the media-fill preparation (without applying sterile 70% IPA) in order to assess the adequacy of aseptic work practices prior to being initially allowed to prepare CSPs for human use and for more experienced personnel to maintain their qualifications to prepare CSPs for human use.

Garbing And Gloving Competency Evaluation—Compounding personnel shall be visually observed during the process of performing hand hygiene and garbing procedures (see *Personnel Cleansing and Garbing* under *Personnel Training and Evaluation in Aseptic Manipulation Skills* above). The visual observation shall be documented on a form such as the *Sample Form for Assessing Hand Hygiene and Garbing Related Practices of Compounding Personnel* (see Appendix III) and maintained to provide a permanent record and long-term assessment of personnel competency.

Gloved Fingertip Sampling—All compounding personnel shall successfully complete an initial competency evaluation and gloved fingertip/thumb sampling procedure (zero cfu) no less than three times before initially being allowed to compound CSPs for human use. Immediately after the compounding employee completes the hand hygiene and garbing procedure (e.g., donning of sterile gloves prior to any disinfection with sterile 70% IPA), the evaluator will collect a gloved fingertip and thumb sample from both hands of the compounding employee onto appropriate agar plates by lightly pressing each fingertip into the agar. The plates will be incubated for the appropriate incubation period and at the appropriate temperature (see *Incubation Period*). After completing the initial gowning and gloving competency evaluation, re-evaluation of all compounding personnel for this competency shall occur at least annually for personnel who compound low- and medium-risk level CSPs and semi-annually for personnel who compound high-risk level CSPs using one or more sample collections during any media-fill test procedure before they are allowed to continue compounding CSPs for human use.

Immediately prior to sampling, gloves shall not be disinfected with sterile 70% IPA. Disinfecting gloves immediately before sampling will provide false negative results. Plates filled with nutrient agar with neutralizing agents such as lecithin and polysorbate 80 added shall be used when sampling personnel fingertips. Personnel shall “touch” the agar with the fingertips of both hands in separate plates in a manner to create a slight impression in the agar. The sampled gloves shall be immediately discarded and proper hand hygiene performed after sampling. The nutrient agar plates shall be incubated as stated below (see *Incubation Period*). Results should be reported separately as number of cfu per employee per hand (left hand, right hand). The cfu action

level for gloved hands will be based on the total number of cfu on both gloves, not per hand.

Incubation Period—At the end of the designated sampling period for compounding personnel competency assessment activities (surface or personnel), the agar plates are recovered and covers secured and they are inverted and incubated at a temperature and for a time period conducive to multiplication of microorganisms. TSA with lecithin and polysorbate 80 shall be incubated at 30° to 35° for 48 to 72 hours.

Aseptic Manipulation Competency Evaluation—After successful completion of an initial Hand Hygiene and Garbing Competency Evaluation, all compounding personnel shall have their aseptic technique and related practice competency evaluated initially during the *Media-Fill Test Procedure* and subsequent annual or semi-annual *Media-Fill Test Procedures*. Records of these evaluations will be maintained using a form such as the *Sample Form for Assessing Aseptic Technique and Related Practices of Compounding Personnel* (see Appendix IV) and maintained to provide a permanent record of and long-term assessment of personnel competency.

Media-Fill Test Procedure—The skill of personnel to aseptically prepare CSPs shall be evaluated using sterile fluid bacterial culture media-fill verification, (i.e., sterile bacterial culture medium transfer via a sterile syringe and needle). Media-fill testing is used to assess the quality of the aseptic skill of compounding personnel. Media-fill tests shall represent the most challenging or stressful conditions actually encountered by the personnel being evaluated when they prepare low- and medium-risk level CSPs and when sterilizing high-risk level CSPs. Media-fill challenge tests are also used to verify the capability of the compounding environment and processes to produce sterile preparations.

A commercially available sterile fluid culture media, such as Soybean–Casein Digest Medium (see *Sterility Tests* (71)), that is able to promote exponential colonization of bacteria that are most likely to be transmitted to CSPs from the compounding personnel and environment is commonly used. For high-risk level CSPs nonsterile commercially available Soybean–Casein Digest Medium may be used to make a 3% solution. Normal processing steps, including filter sterilization, shall be mimicked. Media-filled vials shall be incubated at 20° to 25° or at 30° to 35° for a minimum of 14 days. If two temperatures are used for incubation of media-filled samples, then these filled containers should be incubated for at least 7 days at each temperature (see *Microbiological Control and Monitoring of Aseptic Processing Environments* (1116)). Failure is indicated by visible turbidity in any one of the media-fill units on or before 14 days. Other methodologies recommended by a competent microbiologist to enhance recovery time and sensitivity to detect microbial contamination may be considered (see *CSP Microbial Contamination Risk Levels* for examples of media-fill procedures).

SURFACE CLEANING AND DISINFECTION SAMPLING AND ASSESSMENT

Surface sampling is an important component of the maintenance of a suitable microbially controlled environment for compounding CSPs, especially since transfer of microbial contamination from improperly disinfected work surfaces via inadvertent touch contact by compounding personnel can be a potential source of contamination into CSPs. It is useful for evaluating facility and work surface cleaning and disinfecting procedures and employee competency in work practices such as disinfection of component/vial surface cleaning. Surface sampling shall be performed in all ISO classified areas on a periodic basis. Sampling can be accomplished using contact plates or swabs, and it shall be done at the conclusion of compounding. Locations to be sampled shall be defined in a sample plan or on a form. The size of the

plate to be used for each sampled location usually ranges from 24 to 30 cm². Contact plates are filled with general solid agar growth medium and neutralizing agents above the rim of the plate, and they are used for sampling regular or flat surfaces. Swabs may be used for sampling irregular surfaces, especially for equipment (see *Microbiological Control and Monitoring of Aseptic Processing Environments* (1116)).

Cleaning and Disinfecting Competency Evaluation—Compounding personnel and other personnel responsible for cleaning shall be visually observed during the process of performing cleaning and disinfecting procedures, during initial personnel training on cleaning procedures, during changes in cleaning staff, and at the completion of any media-fill test procedure (see *Cleaning and Disinfecting of Compounding Areas*).

The visual observation shall be documented using a form such as the *Sample Form for Assessing Cleaning and Disinfection Procedures* (see Appendix V) and maintained to provide a permanent record and long-term assessment of personnel competency.

Surface Collection Methods—To sample surfaces using a contact plate, gently touch the sample area with the agar surface and roll the plate across the surface to be sampled. The contact plate will leave a growth media residue behind; therefore, immediately after sampling with the contact plate, the sampled area shall be thoroughly wiped with a nonshedding wipe soaked in sterile 70% IPA.

If an area is sampled via the swab method, collection of the sample is processed by using appropriate procedures that will result in the surface location equivalent to that of a contact plate. After swabbing the surface to be sampled, swabs are placed in an appropriate diluent; an aliquot is planted on or in the specified nutrient agar. Results should be reported as cfu per unit of surface area.

Action Levels, Documentation, and Data Evaluation

The value of viable microbial monitoring of gloved fingertips and surfaces of components and the compounding environment are realized when the data are used to identify and correct an unacceptable work practice. Sampling data shall be collected and reviewed on a routine basis as a means of evaluating the overall control of the compounding environment. If an activity consistently shows elevated levels of microbial growth, competent microbiology personnel shall be consulted.

Any cfu count that exceeds its respective action level (see Table 4) should prompt a re-evaluation of the adequacy of personnel work practices, cleaning procedures, operational procedures, and air filtration efficiency within the aseptic compounding location. An investigation into the source of the contamination shall be conducted. Sources could include HVAC systems, damaged HEPA filters, and changes in personnel garbing or working practices. The source of the problem shall be eliminated, the affected area cleaned, and resampling performed.

When gloved fingertip sample results exceed action levels after proper incubation, a review of hand hygiene and garbing procedures as well as glove and surface disinfection procedures and work practices shall be performed and documented. Employee training may be required to correct the source of the problem.

Counts of cfu are to be used as an approximate measure of the environmental microbial bioburden. Action levels are determined on the basis of cfu data gathered at each sampling location and trended over time. The numbers in Table 4 should be used only as guidelines. Regardless of the number of cfu identified in the compounding facility, further corrective actions will be dictated by the identification of microorganisms recovered (at least the genus level) by an appropriate credentialed laboratory of any microbial bi-

oburden captured as a cfu using an impaction air sampler. Highly pathogenic microorganisms (e.g., Gram-negative rods, coagulase positive staphylococcus, molds and yeasts) can be potentially fatal to patients receiving CSPs and shall be immediately remedied, regardless of cfu count, with the assistance of a competent microbiologist, infection control professional, or industrial hygienist.

Table 4. Recommended Action Levels for Microbial Contamination*

Classification	Fingertip Sample	Surface Sample (Contact Plate) (cfu per plate)
ISO Class 5	> 3	> 3
ISO Class 7	N/A	> 5
ISO Class 8 or worse	N/A	> 100

* Pharmaceutical Inspection Co-operation Scheme (PIC/S) Guide to Good Manufacturing Practice for Medicinal Products Annexes PE 009-6, 5 April 2007.

SUGGESTED STANDARD OPERATING PROCEDURES (SOPs)

The compounding facility shall have written, properly approved SOPs designed to ensure the quality of the environment in which a CSP is prepared. The following procedures are recommended:

1. Access to the buffer area is restricted to qualified personnel with specific responsibilities or assigned tasks in the compounding area.
2. All cartoned supplies are decontaminated in the area by removing them from shipping cartons and wiping or spraying them with a nonresidue-generating disinfecting agent while they are being transferred to a clean and properly disinfected cart or other conveyance for introduction into the buffer area. Manufacturers' directions or published data for minimum contact time will be followed. Individual pouched sterile supplies need not be wiped because the pouches can be removed as these sterile supplies are introduced into the buffer area.
3. Supplies that are required frequently or otherwise needed close at hand but not necessarily needed for the scheduled operations of the shift are decontaminated and stored on shelving in the ante-area.
4. Carts used to bring supplies from the storeroom cannot be rolled beyond the demarcation line in the ante-area, and carts used in the buffer area cannot be rolled outward beyond the demarcation line unless cleaned and disinfected before returning.
5. Generally, supplies required for the scheduled operations of the shift are wiped down with an appropriate disinfecting agent and brought into the buffer area, preferably on one or more movable carts. Supplies that are required for back-up or general support of operations may be stored on the designated shelving in the buffer area, but excessive amounts of supplies are to be avoided.
6. Nonessential objects that shed particles shall not be brought into the buffer area, including pencils, cardboard cartons, paper towels, and cotton items (e.g., gauze pads).
7. Essential paper-related products (e.g., paper syringe overwraps, work records contained in a protective sleeve) shall be wiped down with an appropriate disinfecting agent prior to being brought into the buffer area.
8. Traffic flow in and out of the buffer area shall be minimized.

9. Personnel preparing to enter the buffer area shall remove all personal outer garments, cosmetics (because they shed flakes and particles), and all hand, wrist, and other visible jewelry or piercings that can interfere with the effectiveness of PPE.
10. Personnel entering the ante-area shall don attire as described in *Personnel Cleansing and Garbing* and *Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures*.
11. Personnel shall then thoroughly wash hands and forearms to the elbow with soap and water for at least 30 seconds. An air dryer or disposable nonshedding towels are used to dry hands and forearms after washing.
12. Personnel entering the buffer area shall perform anti-septic hand cleansing prior to donning sterile gloves using a waterless alcohol-based surgical hand scrub with persistent activity.
13. Chewing gum, drinks, candy, or food items shall not be brought into the buffer area or ante-area. Materials exposed in patient care and treatment areas shall never be introduced into areas where components and ingredients for CSPs are present.
14. At the beginning of each compounding activity session, and whenever liquids are spilled, the surfaces of the direct compounding environment are first cleaned with USP Purified Water to remove water-soluble residues. Immediately thereafter, the same surfaces are disinfected with a nonresidue-generating agent using a nonlinting wipe.
15. Primary engineering controls shall be operated continuously during compounding activity. When the blower is turned off and before other personnel enter to perform compounding activities, only one person shall enter the buffer area for the purposes of turning on the blower (for at least 30 minutes) and disinfecting the work surfaces.
16. Traffic in the area of the DCA is minimized and controlled.
17. Supplies used in the DCA for the planned procedures are accumulated and then decontaminated by wiping or spraying the outer surface with sterile 70% IPA or removing the outer wrap at the edge of the DCA as the item is introduced into the aseptic work area.
18. All supply items are arranged in the DCA so as to reduce clutter and provide maximum efficiency and order for the flow of work.
19. After proper introduction into the DCA of supply items required for and limited to the assigned operations, they are so arranged that a clear, uninterrupted path of HEPA-filtered air will bathe all critical sites at all times during the planned procedures. That is, no objects may be placed between the first air from HEPA filters and an exposed critical site.
20. All procedures are performed in a manner designed to minimize the risk of touch contamination. Gloves are disinfected with adequate frequency with an approved disinfectant such as sterile 70% IPA.
21. All rubber stoppers of vials and bottles and the necks of ampuls are disinfected by wiping with sterile 70% IPA and waiting for at least 10 seconds before they are used to prepare CSPs.
22. After the preparation of every CSP, the contents of the container are thoroughly mixed and then inspected for the presence of particulate matter, evidence of incompatibility, or other defects.
23. After procedures are completed, used syringes, bottles, vials, and other supplies are removed, but with a minimum of exit and re-entry into the DCA so as to minimize the risk of introducing contamination into the aseptic workspace.

ELEMENTS OF QUALITY CONTROL

A written description of specific training and performance evaluation program for individuals involved in the use of aseptic techniques for the preparation of sterile products shall be developed for each site. This program equips personnel with the appropriate knowledge and trains them in the required skills necessary to perform the assigned tasks. Each person assigned to the aseptic area in the preparation of sterile products shall successfully complete specialized training in aseptic techniques and aseptic area practices prior to preparing CSPs (see *Personnel Training and Evaluation in Aseptic Manipulation Skills* and *Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures*).

Ingredients and Devices

Compounding personnel ascertain that ingredients for CSPs are of the correct identity and appropriate quality using the following information: vendor labels, labeling, certificates of analysis, direct chemical analysis, and knowledge of compounding facility storage conditions.

STERILE INGREDIENTS AND DEVICES

Commercially available sterile drug products, sterile ready-to-use containers, and devices are examples of sterile components. A written procedure for unit-by-unit physical inspection preparatory to use is followed to ensure that these components are sterile, free from defects, and otherwise suitable for their intended use.

NONSTERILE INGREDIENTS AND DEVICES

If any nonsterile components, including containers and ingredients, are used to make a CSP, such CSPs must be high risk. Nonsterile active ingredients and added substances or excipients for CSPs should preferably be official *USP* or *NF* articles. When nonofficial ingredients are used, they shall be accompanied by certificates of analysis from their suppliers to aid compounding personnel in judging the identity, quality, and purity in relation to the intended use in a particular CSP. Physical inspection of a package of ingredients is necessary in order to detect breaks in the container, looseness in the cap or closure, and deviation from the expected appearance, aroma, and texture of the contents.

Bulk or unformulated drug substances and added substances or excipients shall be stored in tightly closed containers under temperature, humidity, and lighting conditions that are either indicated in official monographs or approved by suppliers. The date of receipt by the compounding facility shall be clearly and indelibly marked on each package of ingredient. After receipt by the compounding facility, packages of ingredients that lack a supplier's expiration date cannot be used after 1 year unless either appropriate inspection or testing indicates that the ingredient has retained its purity and quality for use in CSPs.

Careful consideration and evaluation of nonsterile ingredient sources is especially warranted when the CSP will be administered into the vascular system, central nervous system, or eyes.

Upon receipt of each lot of the bulk drug substance or excipient used for CSPs, the individual compounding the preparation performs a visual inspection of the lot for evidence of deterioration, other types of unacceptable quality, and wrong identification. For bulk drug substances or excipients, visual inspection is performed on a routine basis as described in the written protocol.

Equipment

It is necessary that equipment, apparatus, and devices used to compound a CSP be consistently capable of operating properly and within acceptable tolerance limits. Written procedures outlining required equipment calibration, annual maintenance, monitoring for proper function, and controlled procedures for use of the equipment and specified time frames for these activities are established and followed. Routine maintenance and frequencies shall be outlined in these SOPs. Results from the equipment calibration, annual maintenance reports, and routine maintenance are kept on file for the lifetime of the equipment. Personnel are prepared through an appropriate combination of specific training and experience to operate or manipulate any piece of equipment, apparatus, or device they may use when preparing CSPs. Training includes gaining the ability to determine whether any item of equipment is operating properly or is malfunctioning.

VERIFICATION OF AUTOMATED COMPOUNDING DEVICES (ACDs) FOR PARENTERAL NUTRITION COMPOUNDING

ACDs for the preparation of parenteral nutrition admixtures are widely used by pharmacists in hospitals and other healthcare settings. They are designed to streamline the labor-intensive processes involved in the compounding of these multiple-component formulations by automatically delivering the individual nutritional components in a predetermined sequence under computerized control. Parenteral nutrition admixtures often contain 20 or more individual additives representing as many as 50 or more individual components (e.g., 15 to 20 crystalline amino acids, dextrose monohydrate, and lipids; 10 to 12 electrolyte salts; 5 to 7 trace minerals; and 12 vitamins). Thus, ACDs can provide improved accuracy and precision of the compounding process over the traditional manual compounding methods.

Accuracy

The accuracy of an ACD can be determined in various ways to ensure that the correct quantities of nutrients, electrolytes, or other nutritional components are delivered to the final infusion container. Initially, the ACD is tested for its volume and weight accuracy. For volume accuracy, a suitable volume of Sterile Water for Injection, USP, which represents a typical additive volume (e.g., 40 mL for small-volume range of 1 to 100 mL, 300 mL for large-volume range of 100 to 1000 mL), is programmed into the ACD and delivered to the appropriate volumetric container. The compounding personnel should then consult *Volumetric Apparatus* (31) for appropriate parameters to assess the volumetric performance of the ACD. For gravimetric accuracy, the balance used in conjunction with the ACD is tested using various weight sizes that represent the amounts typically used to deliver the various additives. Compounding personnel should consult *Weights and Balances* (41) for acceptable tolerances of the weights used. In addition, the same volume of *Sterile Water for Injection* used to assess volumetric accuracy is then weighed on the balance used in conjunction with the ACD. For example, if 40 mL of water was used in the volumetric assessment, its corresponding weight should be about 40 g (assuming the relative density of water is 1.0). In addition, during the use of the ACD, certain additives, such as potassium chloride (corrected for density differences), can also be tested in the same manner as with an in-process test.

Finally, additional tests of accuracy may be employed that determine the content of certain ingredients in the final volume of the parenteral nutrition admixture. Generally, pharmacy departments do not have the capability to routinely

perform chemical analyses such as analyses of dextrose or electrolyte concentrations. Consequently, hospital or institutional laboratories may be called upon to perform these quality assurance tests. However, the methods in such laboratories are often designed for biological, not pharmaceutical, systems. Thus, their testing procedures shall be verified to meet the USP requirements stated in the individual monograph for the component being tested. For example, under *Dextrose Injection*, the following is stated: It contains not less than 95.0% and not more than 105.0% of the labeled amount of $C_6H_{12}O_6 \cdot H_2O$. The hospital or institutional chemistry laboratories must validate their methods to apply to this range and correct for their typical measurement of anhydrous dextrose versus dextrose monohydrate. Similar ranges and issues exist, for example, for injections of calcium gluconate, magnesium sulfate, and potassium chloride. The critical point is the use of USP references and possible laboratory procedural differences.

Precision

The intermediate precision of the ACD can be determined on the basis of the day-to-day variations in performance of the accuracy measures. Thus, compounding personnel shall keep a daily record of the above-described accuracy assessments and review the results over time. This review shall occur at least at weekly intervals to avoid potentially clinically significant cumulative errors over time. This is especially true for additives with a narrow therapeutic index, such as potassium chloride.

FINISHED PREPARATION RELEASE CHECKS AND TESTS

The following quality metrics shall be performed for all CSPs before they are dispensed or administered.

Inspection of Solution Dosage Forms and Review of Compounding Procedures

All CSPs that are intended to be solutions shall be visually examined for the presence of particulate matter and not administered or dispensed when such matter is observed. The prescription orders, written compounding procedure, preparation records, and expended materials used to make CSPs at all contamination risk levels are inspected for accuracy of correct identities and amounts of ingredients, aseptic mixing and sterilization, packaging, labeling, and expected physical appearance before they are administered or dispensed.

PHYSICAL INSPECTION

Finished CSPs are individually inspected in accordance with written procedures after compounding. If not distributed promptly, these CSPs are individually inspected just prior to leaving the storage area. Those CSPs that are not immediately distributed are stored in an appropriate location as described in the written procedures. Immediately after compounding, and as a condition of release, each CSP unit, where possible, should be inspected against lighted white or black background or both for evidence of visible particulates or other foreign matter. Prerelease inspection also includes container-closure integrity and any other apparent visual defect. CSPs with observed defects should be immediately discarded or marked and segregated from acceptable products in a manner that prevents their administration. When CSPs are not distributed promptly after preparation, a predistribution inspection is conducted to ensure that a CSP with defects, such as precipitation, cloudiness, and leakage, which may develop between the time of release and the time of distribution, is not released.

Compounding Accuracy Checks

Written procedures for double-checking compounding accuracy shall be followed for every CSP during preparation and immediately prior to release. The double-check system should meet state regulations and include label accuracy and accuracy of the addition of all drug products or ingredients used to prepare the finished product and their volumes or quantities. The used additive containers and, for those additives for which the entire container was not expended, the syringes used to measure the additive should be quarantined with the final products until the final product check is completed. Compounding personnel shall visually confirm that ingredients measured in syringes match the written order being compounded. Preferably, a person other than the compounder can verify that correct volumes of correct ingredients were measured to make each CSP. For example, compounding personnel would pull the syringe plunger back to the volume measured.

When practical, the accuracy of measurements is confirmed by weighing a volume of the measured fluid, then calculating that volume by dividing the weight by the accurate value of the density, or specific gravity, of the measured fluid. Correct density or specific gravity values programmed in ACDs, which measure by weight using the quotient of the programmed volume divided by the density or specific gravity, shall be confirmed to be accurate before and after delivering volumes of the liquids assigned to each channel or port. These volume accuracy checks and the following additional safety and accuracy checks in this section shall be included in the SOP manual of the CSP facility.

Sterility Testing

All high-risk level CSPs that are prepared in groups of more than 25 identical individual single-dose packages (e.g., ampuls, bags, syringes, vials) or in multiple-dose vials (MDVs) for administration to multiple patients or that are exposed longer than 12 hours at 2° to 8° and longer than 6 hours at warmer than 8° before they are sterilized shall meet the sterility test (see *Sterility Tests* (71)) before they are dispensed or administered. The *Membrane Filtration* method is the method of choice where feasible (e.g., components are compatible with the membrane). A method not described in the *USP* may be used if verification results demonstrate that the alternative is at least as effective and reliable as the *USP Membrane Filtration* method or the *USP Direct Inoculation of the Culture Medium* method where the *Membrane Filtration* method is not feasible.

When high-risk level CSPs are dispensed before receiving the results of their sterility tests, there shall be a written procedure requiring daily observation of the incubating test specimens and immediate recall of the dispensed CSPs when there is any evidence of microbial growth in the test specimens. In addition, the patient and the physician of the patient to whom a potentially contaminated CSP was administered are notified of the potential risk. Positive sterility test results should prompt a rapid and systematic investigation of aseptic technique, environmental control, and other sterility assurance controls to identify sources of contamination and correct problems in the methods or processes.

Bacterial Endotoxin (Pyrogen) Testing

All high-risk level CSPs, except those for inhalation and ophthalmic administration, that are prepared in groups of more than 25 identical individual single-dose packages (e.g., ampuls, bags, syringes, vials) or in MDVs for administration to multiple patients or that are exposed longer than 12 hours at 2° to 8° and longer than 6 hours at warmer than 8° before they are sterilized shall be tested to ensure that they do not contain excessive bacterial endotoxins (see

Bacterial Endotoxins Test (85) and *Pyrogen Test* (151)). In the absence of a bacterial endotoxins limit in the official monograph or other CSP formula source, the CSP shall not exceed the amount of USP Endotoxin Units (per hour per kilogram of body weight or square meters of body surface area) specified in *Bacterial Endotoxins Test* (85) referenced above for the appropriate route of administration.

Identity and Strength Verification of Ingredients

Compounding facilities shall have at least the following written procedures for verifying the correct identity and quality of CSPs before they are dispensed and administered:

1. That labels of CSPs bear correct names and amounts or concentrations of ingredients, the total volume, the BUD, the appropriate route(s) of administration, the storage conditions, and other information for safe use.
2. That there are correct identities, purities, and amounts of ingredients by comparing the original written order with the written compounding record for the CSP.
3. That correct fill volumes in CSPs and correct quantities of filled units of the CSPs were obtained. When the strength of finished CSPs cannot be confirmed to be accurate, based on the above three inspections, the CSPs shall be assayed by methods that are specific for the active ingredients.

STORAGE AND BEYOND-USE DATING

BUDs for compounded preparations are usually assigned on the basis of professional experience, which should include careful interpretation of appropriate information sources for the same or similar formulations (see *Stability Criteria and Beyond-Use Dating* under *Pharmaceutical Compounding—Nonsterile Preparations* (795)). BUDs for CSPs are rarely based on preparation-specific chemical assay results, which are used with the Arrhenius equation to determine expiration dates (see *General Notices and Requirements*) for manufactured products. The majority of CSPs are aqueous solutions in which hydrolysis of dissolved ingredients is the most common chemical degradation reaction. The extent of hydrolysis and other heat-catalyzed degradation reactions at any particular time point in the life of a CSP represents the thermodynamic sum of exposure temperatures and durations. Such lifetime stability exposure is represented in the mean kinetic temperature calculation (see *Pharmaceutical Calculations in Prescription Compounding* (1160)). Drug hydrolysis rates increase exponentially with arithmetic temperature increase; thus, exposure of a beta-lactam antibiotic solution for 1 day at controlled room temperature (see *General Notices and Requirements*) will have an equivalent effect on the extent of hydrolysis of approximately 3 to 5 days in cold temperatures (see *General Notices and Requirements*).

Personnel who prepare, dispense, and administer CSPs shall store them strictly in accordance with the conditions stated on the label of ingredient products and finished CSPs. When CSPs are known to have been exposed to temperatures warmer than the warmest labeled limit or to temperatures exceeding 40° (see *General Notices and Requirements*) for more than 4 hours, such CSPs should be discarded unless direct assay data or appropriate documentation confirms their continued stability.

Determining Beyond-Use Dates

BUDs and expiration dates are not the same (see *General Notices and Requirements*). Expiration dates for the chemical and physical stability of manufactured sterile products are determined from results of rigorous analytical and performance testing, and they are specific for a particular formula-

tion in its container and at stated exposure conditions of illumination and temperature. When CSPs deviate from conditions in the approved labeling of manufactured products contained in CSPs, compounding personnel may consult the manufacturer of particular products for advice on assigning BUDs based on chemical and physical stability parameters. BUDs for CSPs that are prepared strictly in accordance with manufacturers' product labeling shall be those specified in that labeling or from appropriate literature sources or direct testing. BUDs for CSPs that lack justification from either appropriate literature sources or by direct testing evidence shall be assigned as described in *Stability Criteria and Beyond-Use Dating under Pharmaceutical Compounding—Nonsterile Preparations* <795>.

In addition, compounding personnel may refer to applicable publications to obtain relevant stability, compatibility, and degradation information regarding the drug or its congeners. When assigning a beyond-use date, compounding personnel should consult and apply drug-specific and general stability documentation and literature where available, and they should consider the nature of the drug and its degradation mechanism, the container in which it is packaged, the expected storage conditions, and the intended duration of therapy (see *Expiration Date and Beyond-Use Date under Labeling in the General Notices and Requirements*). Stability information must be carefully interpreted in relation to the actual compounded formulation and conditions for storage and use. Predictions based on other evidence, such as publications, charts, and tables, would result in theoretical BUDs. Theoretically predicted beyond-use dating introduces varying degrees of assumptions and, hence, a likelihood of error or at least inaccuracy. The degree of error or inaccuracy would be dependent on the extent of differences between the CSPs' characteristics (e.g., composition, concentration of ingredients, fill volume, container type and material) and the characteristics of the products from which stability data or information is to be extrapolated. The greater the doubt of the accuracy of theoretically predicted beyond-use dating, the greater the need to determine dating periods experimentally. Theoretically predicted beyond-use dating periods should be carefully considered for CSPs prepared from nonsterile bulk active ingredients having therapeutic activity, especially where these CSPs are expected to be compounded routinely. When CSPs will be distributed to and administered in residential locations other than healthcare facilities, the effect of potentially uncontrolled and unmonitored temperature conditions shall be considered when assigning BUDs. It must be ascertained that CSPs will not be exposed to warm temperatures (see *General Notices and Requirements*) unless the compounding facility has evidence to justify stability of CSPs during such exposure.

It should be recognized that the truly valid evidence of stability for predicting beyond-use dating can be obtained only through product-specific experimental studies. Semi-quantitative procedures such as thin-layer chromatography (TLC) may be acceptable for many CSPs. However, quantitative stability-indicating assays such as high-performance liquid chromatographic (HPLC) assays would be more appropriate for certain CSPs. Examples include CSPs with a narrow therapeutic index, where close monitoring or dose titration is required to ensure therapeutic effectiveness and to avoid toxicity; where a theoretically established beyond-use dating period is supported by only marginal evidence; or where a significant margin of safety cannot be verified for the proposed beyond-use dating period. In short, because beyond-use dating periods established from product-specific data acquired from the appropriate instrumental analyses are clearly more reliable than those predicted theoretically, the former approach is strongly urged to support dating periods exceeding 30 days.

To ensure consistent practices in determining and assigning BUDs, the compounding facility should have written policies and procedures governing the determination of the BUDs for all compounded products. When attempting to

predict a theoretical BUD, a compounded or an admixed preparation should be considered as a unique system that has physical and chemical properties and stability characteristics that differ from its components. For example, antioxidant, buffering, or antimicrobial properties of a sterile vial for injection (SVI) might be lost upon its dilution, with the potential of seriously compromising the chemical stability of the SVI's active ingredient or the physical or microbiological stability of the SVI formulation in general. Thus, the properties stabilized in the SVI formulation usually cannot be expected to be carried over to the compounded or admixed preparation. Preparation-specific, experimentally determined stability data evaluation protocols are preferable to published stability information.

Compounding personnel who assign BUDs to CSPs when lacking direct chemical assay results must critically interpret and evaluate the most appropriate available information sources to determine a conservative and safe BUD. The SOP manual of the compounding facility and each specific CSP formula record shall describe the general basis used to assign the BUD and storage conditions.

When manufactured MDVs (see *Multiple-Dose Container under Preservation, Packaging, Storage, and Labeling in the General Notices and Requirements*) of sterile ingredients are used in CSPs, the stoppers of the MDVs are inspected for physical integrity and disinfected by wiping with a sterile 70% IPA swab before each penetration with a sterile withdrawal device. When contaminants or abnormal properties are suspected or observed in MDVs, such MDVs shall be discarded. The BUD after initially entering or opening (e.g., needle puncturing) multiple-dose containers is 28 days (see *Antimicrobial Effectiveness Testing* <51>) unless otherwise specified by the manufacturer.

Proprietary Bag and Vial Systems

The sterility storage and stability beyond-use times for attached and activated (where activated is defined as allowing contact of the previously separate diluent and drug contents) container pairs of drug products for intravascular administration (e.g., ADD-Vantage®, Mini Bag Plus®) shall be applied as indicated by the manufacturer. In other words, follow manufacturers' instructions for handling and storing ADD-Vantage®, Mini Bag Plus®, Add A Vial®, Add-Ease® products, and any others.

Monitoring Controlled Storage Areas

To ensure that product potency is retained through the manufacturer's labeled expiration date, compounding personnel shall monitor the drug storage areas within the compounding facility. Controlled temperature areas in compounding facilities include controlled room temperature, 20° to 25° with mean kinetic temperature 25°; controlled cold temperature, 2° to 8° with mean kinetic temperature 8°; cold temperature, 2° to 8°; freezing temperature, –25° and –10° (see *General Notices and Requirements*) if needed to achieve freezing, and the media-specific temperature range for microbial culture media. A controlled temperature area shall be monitored at least once daily and the results documented on a temperature log. Additionally, compounding personnel shall note the storage temperature when placing the product into or removing the product from the storage unit in order to monitor any temperature aberrations. Suitable temperature recording devices may include a calibrated continuous recording device or a National Institute of Standards and Technology (NIST) calibrated thermometer that has adequate accuracy and sensitivity for the intended purpose, and it shall be properly calibrated at suitable intervals. If the compounding facility uses a continuous temperature recording device, compounding personnel shall verify at least once daily that the recording device itself is functioning properly.

The temperature-sensing mechanisms shall be suitably placed in the controlled temperature storage space to reflect accurately its true temperature. In addition, the compounding facility shall adhere to appropriate procedures of all controlled storage spaces to ensure that such spaces are not subject to significantly prolonged temperature fluctuations as may occur, for example, by leaving a refrigerator door open too long.

MAINTAINING STERILITY, PURITY, AND STABILITY OF DISPENSED AND DISTRIBUTED CSPs

This section summarizes the responsibilities of compounding facilities for maintaining quality and control of CSPs that are dispensed and administered within their parent health-care organizations.

Compounding personnel shall ensure proper storage and security of CSPs prepared by or dispensed from the compounding facility until either their BUDs are reached or they are administered to patients. In fulfilling this general responsibility, the compounding facility is responsible for the proper packaging, handling, transport, and storage of CSPs prepared by or dispensed from it, including the appropriate education, training, and supervision of compounding personnel assigned to these functions. The compounding facility should assist in the education and training of noncompounding personnel responsible for carrying out any aspect of these functions.

Establishing, maintaining, and ensuring compliance with comprehensive written policies and procedures encompassing these responsibilities is a further responsibility of the compounding facility. Where noncompounding personnel are assigned tasks involving any of these responsibilities, the policies and procedures encompassing those tasks should be developed by compounding supervisors. The quality and control activities related to distribution of CSPs are summarized in the following five subsections. Activities or concerns that should be addressed as the compounding facility fulfills these responsibilities are as follows.

Packaging, Handling, and Transport

Inappropriate processes or techniques involved with packaging, handling, and transport can adversely affect quality and package integrity of CSPs. Although compounding personnel routinely perform many of the tasks associated with these functions, some tasks, such as transport, handling, and placement into storage, may be fulfilled by noncompounding personnel who are not under the direct administrative control of the compounding facility. Under these circumstances, appropriate SOPs shall be established by the compounding facility with the involvement of other departments or services whose personnel are responsible for carrying out those CSP-related functions for which the compounding facility has a direct interest. The performance of the noncompounding personnel is monitored for compliance to established policies and procedures.

The critical requirements that are unique to CSPs and that are necessary to ensure CSP quality and packaging integrity shall be addressed in SOPs. For example, techniques should be specified to prevent the depression of syringe plungers or dislodging of syringe tips during handling and transport. Additionally, disconnection of system components (e.g., where CSPs are dispensed with administration sets attached to them) shall be prevented through the BUD of the CSP. Foam padding or inserts are particularly useful where CSPs are transported by pneumatic tube systems. Regardless of the methods used, the compounding facility must evaluate their effectiveness and the reliability of the intended protection. Evaluation should be continuous—for example, through a surveillance system, including a system of problem reporting to the compounding facility.

Inappropriate transport and handling can adversely affect the quality of certain CSPs having unique stability concerns. For example, the physical shaking that might occur during pneumatic tube transport or undue exposure to heat or light must be addressed on a preparation-specific basis. Alternative transport modes or special packaging measures might be needed for the proper assurance of quality of these CSPs. The use of tamper-evident closures and seals on CSP ports can add an additional measure of security to ensure product integrity regardless of the transport method used.

Chemotoxic and other hazardous CSPs require safeguards to maintain the integrity of the CSP and to minimize the exposure potential of these products to the environment and to personnel who may come in contact with them. Transportation by pneumatic tube should be discouraged because of potential breakage and contamination. Special requirements associated with the packaging, transport, and handling of these agents include the prevention of accidental exposures or spills and the training of personnel in the event of an exposure or spill. Examples of special requirements of these agents also include exposure-reducing strategies such as the use of Luer lock syringes and connections, syringe caps, the capping of container ports, sealed plastic bags, impact-resistant containers, and cautionary labeling.

Use and Storage

The compounding facility is responsible for ensuring that CSPs in the patient-care setting maintain their quality until administered. The immediate labeling of the CSP container will display prominently and understandably the requirements for proper storage and expiration dating. Delivery and patient-care-setting personnel shall be properly trained to deliver the CSP to the appropriate storage location. Outdated and unused CSPs shall be returned to the compounding facility for disposition.

SOPs must exist to ensure that storage conditions in the patient-care setting are suitable for the CSP-specific storage requirements. Procedures include daily monitoring and documentation of drug storage refrigerators to ensure temperatures between 2° and 8° and the monthly inspection of all drug storage locations by compounding personnel. Inspections shall confirm compliance with appropriate storage conditions, separation of drugs and food, proper use of MDVs, and the avoidance of using single-dose products as MDVs. CSPs, as well as all other drug products, shall be stored in the patient-care area in such a way as to secure them from unauthorized personnel, visitors, and patients.

Readying for Administration

Procedures essential for generally ensuring quality, especially sterility assurance, when readying a CSP for its subsequent administration include proper hand washing, aseptic technique, site care, and change of administration sets. Additional procedures may also be essential for certain CSPs, devices, or techniques. Examples where such special procedures are needed include in-line filtration, the operation of automated infusion control devices, and the replenishment of CSPs into the reservoirs of implantable or portable infusion pumps. When CSPs are likely to be exposed to warmer than 30° for more than 1 hour during their administration to patients, the maintenance of their sterility and stability should be confirmed from either relevant and reliable sources or direct testing.

Redispensed CSPs

The compounding facility shall have the sole authority to determine when unopened, returned CSPs may be redispensed. Returned CSPs may be redispensed only when personnel responsible for sterile compounding can ensure that

such CSPs are sterile, pure, and stable (contain labeled strength of ingredients). The following may provide such assurance: the CSPs were maintained under continuous refrigeration and protected from light, if required, and no evidence of tampering or any readying for use outside the compounding facility exists. Assignment of new storage times and BUDs that exceed the original dates for returned CSPs is permitted only when there is supporting evidence from sterility testing and quantitative assay of ingredients. Thus, initial preparation and thaw times should be documented and reliable measures should have been taken to prevent and detect tampering. Compliance with all procedures associated with maintaining product quality is essential. The CSPs shall not be redispensed if there is not adequate assurance that preparation quality and packaging integrity (including the connections of devices, where applicable) were continuously maintained between the time the CSPs left and the time they were returned. Additionally, CSPs shall not be redispensed if redispensing cannot be supported by the originally assigned BUD.

Education and Training

The assurance of CSPs' quality and packaging integrity is highly dependent on the proper adherence of all personnel to the pertinent SOPs. Compounding personnel shall design, implement, and maintain a formal education, training, and competency assessment program that encompasses all the functions and tasks addressed in the foregoing sections and all personnel to whom such functions and tasks are assigned. This program includes the assessment and documentation of procedural breaches, administration mishaps, side effects, allergic reactions, and complications associated with dosage or administration, such as extravasation. This program should be coordinated with the institution's adverse-events and incident reporting programs.

Packing and Transporting CSPs

The following sections describe how to maintain sterility and stability of CSPs until they are delivered to patient care locations for administration.

PACKING CSPs FOR TRANSIT

When CSPs are distributed to locations outside the premises in which they are compounded, compounding personnel select packing containers and materials that are expected to maintain physical integrity, sterility, and stability of CSPs during transit. Packing is selected that simultaneously protects CSPs from damage, leakage, contamination, and degradation, and protects personnel who transport packed CSPs from harm. The SOP manual of the compounding facility specifically describes appropriate packing containers and insulating and stuffing materials, based on information from product specifications, vendors, and experience of compounding personnel. Written instructions that clearly explain how to safely open containers of packed CSPs are provided to patients and other recipients.

TRANSIT OF CSPs

Compounding facilities that ship CSPs to locations outside their own premises shall select modes of transport that are expected to deliver properly packed CSPs in undamaged, sterile, and stable condition to recipients.

Compounding personnel should ascertain that temperatures of CSPs during transit by the selected mode will not exceed the warmest temperature specified on the storage temperature range on CSP labels. It is recommended that compounding personnel communicate directly with the

couriers to learn shipping durations and exposure conditions that CSPs may encounter.

Compounding personnel shall include specific handling and exposure instructions on the exteriors of containers packed with CSPs to be transported and obtain reasonable assurance of compliance therewith from transporters. Compounding personnel shall periodically review the delivery performance of couriers to ascertain that CSPs are being efficiently and properly transported.

Storage in Locations Outside Compounding Facilities

Compounding facilities that ship CSPs to patients and other recipients outside their own premises shall ascertain or provide, whichever is appropriate, the following assurances:

1. Labels and accessory labeling for CSPs include clearly readable BUDs, storage instructions, and disposal instructions for out-of-date units.
2. Each patient or other recipient is able to store the CSPs properly, including the use of a properly functioning refrigerator and freezer if CSPs are labeled for such storage.

PATIENT OR CAREGIVER TRAINING

A formal training program is provided as a means to ensure understanding and compliance with the many special and complex responsibilities placed on the patient or caregiver for the storage, handling, and administration of CSPs. The instructional objectives for the training program include all home care responsibilities expected of the patient or caregiver and is specified in terms of patient or caregiver competencies.

Upon the conclusion of the training program, the patient or caregiver should, correctly and consistently, be able to do the following:

1. Describe the therapy involved, including the disease or condition for which the CSPs are prescribed, goals of therapy, expected therapeutic outcome, and potential side effects of the CSPs.
2. Inspect all drug products, CSPs, devices, equipment, and supplies on receipt to ensure that proper temperatures were maintained during transport and that goods received show no evidence of deterioration or defects.
3. Handle, store, and monitor all drug products, CSPs, and related supplies and equipment in the home, including all special requirements related to same.
4. Visually inspect all drug products, CSPs, devices, and other items the patient or caregiver is required to use immediately prior to administration in a manner to ensure that all items are acceptable for use. For example, CSPs must be free from leakage, container cracks, particulates, precipitate, haziness, discoloration, or other deviations from the normal expected appearance, and the immediate packages of sterile devices must be completely sealed, with no evidence of loss of package integrity.
5. Check labels immediately prior to administration to ensure the right drug, dose, patient, and time of administration.
6. Clean the in-home preparation area, scrub hands, use proper aseptic technique, and manipulate all containers, equipment, apparatus, devices, and supplies used in conjunction with administration.
7. Employ all techniques and precautions associated with CSP administration; for example, preparing supplies and equipment, handling of devices, priming the tubing, and discontinuing an infusion.
8. Care for catheters, change dressings, and maintain site patency as indicated.

9. Monitor for and detect occurrences of therapeutic complications such as infection, phlebitis, electrolyte imbalance, and catheter misplacement.
10. Respond immediately to emergency or critical situations such as catheter breakage or displacement, tubing disconnection, clot formation, flow blockage, and equipment malfunction.
11. Know when to seek and how to obtain professional emergency services or professional advice.
12. Handle, contain, and dispose of wastes, such as needles, syringes, devices, biohazardous spills or residuals, and infectious substances.

Training programs include a hands-on demonstration and practice with actual items that the patient or caregiver is expected to use, such as CSP containers, devices, and equipment. The patient or caregiver practices aseptic and injection technique under the direct observation of a health professional.

The compounding facility, in conjunction with nursing or medical personnel, is responsible for ensuring initially and on an ongoing basis that the patient or caregiver understands, has mastered, and is capable of and willing to comply with all of these home care responsibilities. This is achieved through a formal, written assessment program. All specified competencies in the patient or caregiver training program are formally assessed. The patient or caregiver is expected to demonstrate to appropriate healthcare personnel mastery of assigned activities before being allowed to administer CSPs unsupervised by a health professional.

Printed material such as checklists or instructions provided during training may serve as continuing post-training reinforcement of learning or as reminders of specific patient or caregiver responsibilities. Post-training verbal counseling can also be used periodically, as appropriate, to reinforce training and to ensure continuing correct and complete fulfillment of responsibilities.

PATIENT MONITORING AND ADVERSE EVENTS REPORTING

Compounding facilities shall clinically monitor patients treated with CSPs according to the regulations and guidelines of their respective state healthcare practitioner licensure boards or of accepted standards of practice. Compounding facilities shall provide patients and other recipients of CSPs with a way to address their questions and report any concerns that they may have with CSPs and their administration devices.

The SOP manuals of compounding facilities shall describe specific instructions for receiving, acknowledging, and dating receipts, and for recording, or filing, and evaluating reports of adverse events and of the quality of preparation claimed to be associated with CSPs. Reports of adverse events with CSPs shall be reviewed promptly and thoroughly by compounding supervisors to correct and prevent future occurrences. Compounding personnel are encouraged to participate in adverse event reporting and product defects programs of the FDA and USP.

QUALITY ASSURANCE (QA) PROGRAM

A provider of CSPs shall have in place a formal QA program intended to provide a mechanism for monitoring, evaluating, correcting, and improving the activities and processes described in this chapter. Emphasis in the QA program is placed on maintaining and improving the quality of systems and the provision of patient care. In addition, the QA program ensures that any plan aimed at correcting identified problems also includes appropriate follow-up to make certain that effective corrective actions were performed.¹³

Characteristics of a QA program include the following:

1. Formalization in writing;
2. Consideration of all aspects of the preparations and dispensing of products as described in this chapter, including environmental testing and verification results;
3. Description of specific monitoring and evaluation activities;
4. Specification of how results are to be reported and evaluated;
5. Identification of appropriate follow-up mechanisms when action limits or thresholds are exceeded; and
6. Delineation of the individuals responsible for each aspect of the QA program.

In developing a specific plan, focus is on establishing objective, measurable indicators for monitoring activities and processes that are deemed high risk, high volume, or problem prone. In general, the selection of indicators and the effectiveness of the overall QA program is reassessed on an annual basis.

ABBREVIATIONS AND ACRONYMS

ACD	automated compounding device
ACPH	air changes per hour
ALARA	as low as reasonably achievable
ASHRAE	American Society of Heating, Refrigerating and Air-Conditioning Engineers
BI	biological indicator
BSC	biological safety cabinet
BUD	beyond-use date
CACI	compounding aseptic containment isolator
CAI	compounding aseptic isolator
CDC	Centers for Disease Control and Prevention
CETA	Controlled Environment Testing Association
cfu	colony-forming unit(s)
CSP	compounded sterile preparation
CSTD	closed-system vial-transfer device
DCA	direct compounding area
ECV	endotoxin challenge vial
EU	Endotoxin Unit
FDA	Food and Drug Administration
HEPA	high efficiency particulate air
HICPAC	Healthcare Infection Control Practices Advisory Committee
HVAC	heating, ventilation, and air conditioning
IPA	isopropyl alcohol
ISO	International Organization for Standardization
LAFW	laminar airflow workbench
MDVs	multiple-dose vials
MMWR	Morbidity and Mortality Weekly Report
NIOSH	National Institute for Occupational Safety and Health
NIST	National Institute of Standards and Technology
PEC	primary engineering control
PET	positron emission tomography
PPE	personnel protective equipment
psi	pounds per square inch
QA	quality assurance
SOP	standard operating procedure
SVI	sterile vial for injection
TSA	trypticase soy agar
USP	United States Pharmacopeia

¹³ The use of additional resources, such as the Accreditation Manual for Home Care from the Joint Commission on Accreditation of Healthcare Organizations, may prove helpful in the development of a QA plan.

APPENDICES**Appendix I. Principal Competencies, Conditions, Practices, and Quality Assurances That Are Required († “shall”) and Recommended (‡ “should”) in USP Chapter <797>**

NOTE—This tabular appendix selectively abstracts and condenses the full text of <797> for rapid reference only. Compounding personnel are responsible for reading, understanding and complying with the full text and all official USP terminology, content, and conditions therein.

INTRODUCTION

- ‡ Chapter purpose is to prevent harm and death to patients treated with CSPs.
- † Chapter pertains to preparation, storage, and transportation, but not administration, of CSPs.
- † Personnel and facilities to which <797> applies; therefore, for whom and which it may be enforced by regulatory and accreditation authorities.
- † Types of preparations designated to be CSPs according to their physical forms, and their sites and routes of administration to patients.
- † Compounding personnel must be meticulously conscientious to preclude contact contamination of CSPs both within and outside ISO Class 5 areas.

ORGANIZATION

- † All compounding personnel shall be responsible for understanding fundamental practices and precautions within USP <797>, for developing and implementing appropriate procedures, and for continually evaluating these procedures and the quality of final CSPs to prevent harm.

DEFINITIONS

- † Twenty-eight terms are defined and integral to complying with USP <797>.

RESPONSIBILITY OF COMPOUNDING PERSONNEL

- † Practices and quality assurances required to prepare, store, and transport CSPs that are sterile, and acceptably accurate, pure, and stable.

CSP MICROBIAL CONTAMINATION RISK LEVELS

- † Proper training and evaluation of personnel, proper cleansing and garbing of personnel, proper cleaning and disinfecting of compounding work environments, and proper maintenance and monitoring of controlled environmental locations (all of which are detailed in their respective sections).

Low-Risk Level CSPs

- † Aseptic manipulations within an ISO Class 5 environment using three or fewer sterile products and entries into any container.
- † In absence of passing sterility test, store not more than 48 hours at controlled room temperature, 14 days at cold temperature, and 45 days in solid frozen state at –25° to –10° or colder.
- † Media-fill test at least annually by compounding personnel.

Low-Risk Level CSPs with 12-Hour or Less BUD

- † Fully comply with all four specific criteria.
- ‡ Sinks should not be located adjacent to the ISO Class 5 primary engineering control.
- ‡ Sinks should be separated from the immediate area of the ISO Class 5 primary engineering control device.

Medium-Risk Level CSPs

- † Aseptic manipulations within an ISO Class 5 environment using prolonged and complex mixing and transfer, more than three sterile products and entries into any container, and pooling ingredients from multiple sterile products to prepare multiple CSPs.
- † In absence of passing sterility test, store not more than 30 hours at controlled room temperature, 9 days at cold temperature, and 45 days in solid frozen state at –25° to –10° or colder.
- † Media-fill test at least annually by compounding personnel.

High-Risk Level CSPs

- † Confirmed presence of nonsterile ingredients and devices, or confirmed or suspected exposure of sterile ingredients for more than one hour to air quality inferior to ISO Class 5 before final sterilization.
- † Sterilization method verified to achieve sterility for the quantity and type of containers.
- † Meet allowable limits for bacterial endotoxins.
- † Maintain acceptable strength and purity of ingredients and integrity of containers after sterilization.
- † In absence of passing sterility test, store not more than 24 hours at controlled room temperature, 3 days at cold temperature, and 45 days in solid frozen state at –25° to –10° or colder.
- † Media-fill test at least semiannually by compounding personnel.

PERSONNEL TRAINING AND EVALUATION IN ASEPTIC MANIPULATIONS SKILLS

- † Pass didactic, practical skill assessment and media-fill testing initially, followed by an annual assessment for a low- and medium-risk level compounding and semi-annual assessment for high-risk level compounding.
- † Compounding personnel who fail written tests, or whose media-fill test vials result in gross microbial colonization, shall be immediately reinstructed and re-evaluated by expert compounding personnel to ensure correction of all aseptic practice deficiencies.

IMMEDIATE-USE CSPs

- † Fully comply with all six specified criteria.

APPENDICES**Appendix I. Principal Competencies, Conditions, Practices, and Quality Assurances That Are Required († “shall”) and Recommended (§ “should”) in USP Chapter <797> (Continued)**

SINGLE-DOSE AND MULTIPLE-DOSE CONTAINERS

- † Beyond-use date 28 days, unless specified otherwise by the manufacturer, for closure sealed multiple-dose containers after initial opening or entry.
- † Beyond-use time of 6 hours, unless specified otherwise by the manufacturer, for closure sealed single-dose containers in ISO Class 5 or cleaner air after initial opening or entry.
- † Beyond-use time of 1 hour for closure sealed single-dose containers after being opened or entered in worse than ISO Class 5 air.
- † Storage of opened single-dose ampuls is not permitted.

HAZARDOUS DRUGS AS CSPs

- † Appropriate personnel protective equipment.
- † Appropriate primary engineering controls (BSCs and CACIs) are used for concurrent personnel protection and exposure of critical sites.
- † Hazardous drugs shall be stored separately from other inventory in a manner to prevent contamination and personnel exposure.
- † At least 0.01 inch water column negative pressure and 12 air changes per hour in non-cleanrooms in which CACIs are located.
- † Hazardous drugs shall be handled with caution at all times using appropriate chemotherapy gloves during receiving, distribution, stocking, inventorying, preparing for administration, and disposal.
- † Hazardous drugs shall be prepared in an ISO Class 5 environment with protective engineering controls in place, and following aseptic practices specified for the appropriate contamination risk levels.
- † Access to drug preparation areas shall be limited to authorized personnel.
- † A pressure indicator shall be installed that can readily monitor room pressurization, which is documented daily.
- † Annual documentation of full training of personnel regarding storage, handling, and disposal of hazardous drugs.
- † When used, a CSTD shall be used in an ISO Class 5 primary engineering control device.
- † At least 0.01 inch water column negative pressure is required for compounding of hazardous drugs.
- ‡ Negative-pressure buffer area is not required for low-volume compounding operations when CSTD is used in BSC or CACI.
- † Compounding personnel of reproductive capability shall confirm in writing that they understand the risks of handling hazardous drugs.
- † Disposal of all hazardous drug wastes shall comply with all applicable federal and state regulations.
- ‡ Total external exhaust of primary engineering controls.
- ‡ Assay of surface wipe samples every 6 months.

RADIOPHARMACEUTICALS AS CSPs

- † Positron Emission Tomography is according to USP chapter <823>.
- † Appropriate primary engineering controls and radioactivity containment and shielding.
- † Radiopharmaceuticals compounded from sterile components, in closed sterile containers, with volume of 100 mL or less for a single-dose injection or not more than 30 mL taken from a multiple-dose container shall be designated as and conform to the standards for low-risk level CSPs.
- † Radiopharmaceutical vials, designed for multi-use, compounded with technetium-99m, exposed to ISO Class 5 environment and punctured by needles with no direct contact contamination may be used up to the time indicated by manufacturers' recommendations.
- † Location of primary engineering controls permitted in ISO Class 8 controlled environment.
- † Technetium-99m/Molybdenum-99 generators used according to manufacturer, state, and federal requirements.
- † Radiopharmaceuticals prepared as low-risk level CSPs with 12-hour or less BUD shall be prepared in a segregated compounding area.
- † Materials and garb exposed in patient-care and treatment area shall not cross a line of demarcation into the segregated compounding area.
- † Technetium-99m/Molybdenum-99 generators must be eluted in ISO Class 8 conditions.
- † Segregated compounding area will be designated with a line of demarcation.
- ‡ Storage and transport of properly shielded vials of radiopharmaceutical CSPs may occur in a limited access ambient environment without a specific ISO class designation.

ALLERGEN EXTRACTS AS CSPs

- † Allergen extracts as CSPs are not subject to the personnel, environmental, and storage requirements for all CSP Microbial Contamination Risk Levels when certain criteria are met.

VERIFICATION OF COMPOUNDING ACCURACY AND STERILITY

- † Review labels and document correct measurements, aseptic manipulations, and sterilization procedures to confirm correct identity, purity, and strength of ingredients in, and sterility of, CSPs.
- ‡ Assay finished CSPs to confirm correct identity and, or, strength of ingredients.
- ‡ Sterility test finished CSPs.

Sterilization Methods

- † Verify that methods achieve sterility while maintaining appropriate strength, purity, quality, and packaging integrity.
- ‡ Prove effectiveness by USP chapter <71>, equivalent, or superior sterility testing.

Sterilization of High-Risk Level CSPs by Filtration

- † Nominal 0.2-µm pore size sterile membranes that are chemically and physically compatible with the CSP.
- † Complete rapidly without filter replacement.
- † Subject filter to manufacturer's recommended integrity test (e.g., bubble point test) after filtering CSPs.

Sterilization of High-Risk Level CSPs by Steam

- † Test to verify the mass of containers to be sterilized will be sterile after the selected exposure duration in the particular autoclave.

APPENDICES

Appendix I. Principal Competencies, Conditions, Practices, and Quality Assurances That Are Required († “shall”) and Recommended (§ “should”) in USP Chapter <797> (Continued)

- † Ensure live steam contacts all ingredients and surfaces to be sterilized.
- † Pass solutions through a 1.2-µm or smaller nominal pore size filter into final containers to remove particulates before sterilization.
- † Heated filtered air shall be evenly distributed throughout the chamber by a blower device.
- † Dry heat shall only be used for those materials that cannot be sterilized by steam, when the moisture would either damage or be impermeable to the materials.
- † Sufficient space shall be left between materials to allow for good circulation of the hot air.
- † The description of dry heat sterilization conditions and duration for specific CSPs shall be included in written documentation in the compounding facility. The effectiveness of dry heat sterilization shall be verified using appropriate biological indicators and other confirmation.
- ‡ The oven should be equipped with a system for controlling temperature and exposure period.

Depyrogenation by Dry Heat

- † Dry heat depyrogenation shall be used to render glassware or containers, such as vials free from pyrogens as well as viable microbes.
- † The description of the dry heat depyrogenation cycle and duration for specific load items shall be included in written documentation in the compounding facility.
- † The effectiveness of the dry heat depyrogenation cycle shall be verified using endotoxin challenge vials (ECVs).
- ‡ The bacterial endotoxin test should be performed on the ECVs to verify the cycle is capable of achieving a 3 log reduction in endotoxin.

ENVIRONMENTAL QUALITY AND CONTROL

Exposure of Critical Sites

- † ISO Class 5 or better air.
- † Preclude direct contact (e.g., touch and secretions) contamination.

ISO Class 5 Air Sources, Buffer Areas, and Ante-Areas

- † A buffer area is an area that provides at least ISO Class 7 air quality.
- † New representations of facility layouts.
- † Each compounding facility shall ensure that each source of ISO Class 5 environment for exposure of critical sites and sterilization by filtration is properly located, operated, maintained, monitored, and verified.
- † Devices (e.g., computers and printers) and objects (e.g., carts and cabinets) can be placed in buffer areas and shall be verified by testing or monitoring.

Viable and Nonviable Environmental Sampling (ES) Testing

- † Environmental sampling shall occur as part a comprehensive quality management program and shall occur minimally when several conditions exist.
- ‡ The ES program should provide information to staff and leadership to demonstrate that the engineering controls are maintaining an environment within the compounding area that consistently maintains acceptably low viable and nonviable particle levels.

Environmental Nonviable Particle Testing Program

- † Certification and testing of primary (LAFWs, BSCs, CAIs and CACIs) and secondary engineering controls (buffer and ante areas) shall be performed by a qualified individual no less than every six months and whenever the device or room is relocated, altered, or major service to the facility is performed. Certification procedures such as those outlined in the CETA Certification Guide for Sterile Compounding Facilities (CAG-003-2006) shall be used.

Total Particle Counts

- † Certification that each ISO classified area (e.g., ISO Class 5, 7 and 8) is within established guidelines shall be performed no less than every 6 months and whenever the LAFW, BSC, CAI, or CACI is relocated or the physical structure of the buffer room or ante-area has been altered.
- † Testing shall be performed by qualified operators using current, state-of-the-art electronic equipment with results meeting ISO Class 5, 7, or 8 depending on the requirements of the area.
- † All certification records shall be maintained and reviewed by supervising personnel or other designated employee to ensure that the controlled environments comply with the proper air cleanliness, room pressures, and air changes per hour.

Pressure Differential Monitoring

- † A pressure gauge or velocity meter shall be installed to monitor the pressure differential or airflow between the buffer area and ante-area, and the ante-area and the general environment outside the compounding area.
- † The results shall be reviewed and documented on a log at least every work shift (minimum frequency shall be at least daily) or by a continuous recording device.
- † The pressure between the ISO Class 7 and general pharmacy area shall not be less than 5 Pa (0.02 inch water column (w.c.)).
- † In facilities where low- and medium-risk level CSPs are prepared, differential airflow shall maintain a minimum velocity of 0.2 meter/second (40 fpm) between buffer area and ante-area.

Environmental Viable Airborne Particle Testing Program—Sampling Plan

- † An appropriate environmental sampling plan shall be developed for airborne viable particles based on a risk assessment of compounding activities performed.
- † Selected sampling sites shall include locations within each ISO Class 5 environment and in the ISO Class 7 and 8 areas, and the segregated compounding areas at greatest risk of contamination (e.g., work areas near the ISO Class 5 environment, counters near doors, pass-through boxes).
- † The plan shall include sample location, method of collection, frequency of sampling, volume of air sampled, and time of day as related to activity in the compounding area and action levels.
- ‡ It is recommended that compounding personnel refer to USP Chapter *Microbiological Control and Monitoring of Aseptic Processing Environments* <1116> and the CDC Guidelines for Environmental Infection Control in Healthcare Facilities-2003 for more information.

Growth Media

- † A general microbiological growth medium such as Soybean–Casein Digest Medium (also known as trypticase soy broth (TSB) or agar (TSA)) shall be used to support the growth of bacteria.

APPENDICES**Appendix I. Principal Competencies, Conditions, Practices, and Quality Assurances That Are Required († “shall”) and Recommended (§ “should”) in USP Chapter <797> (Continued)**

† Malt extract agar (MEA) or some other media that supports the growth of fungi shall be used in high-risk level compounding environments.

† Media used for surface sampling shall be supplemented with additives to neutralize the effects of disinfecting agents (e.g., TSA with lecithin and polysorbate 80).

Viable Air Sampling

† Evaluation of airborne microorganisms using volumetric collection methods in the controlled air environments shall be performed by properly trained individuals for all compounding risk levels.

† Impaction shall be the preferred method of volumetric air sampling.

† For low-, medium-, and high-risk level compounding, air sampling shall be performed at locations that are prone to contamination during compounding activities and during other activities like staging, labeling, gowning, and cleaning.

† Locations shall include zones of air backwash turbulence within laminar airflow workbench and other areas where air backwash turbulence may enter the compounding area.

† For low-risk level CSPs with 12-hour or less BUD, air sampling shall be performed at locations inside the ISO Class 5 environment and other areas that are in close proximity to the ISO class 5 environment, during the certification of the primary engineering control.

‡ Consideration should be given to the overall effect the chosen sampling method will have on the unidirectional airflow within a compounding environment.

Air Sampling Devices

† The instructions in the manufacturer’s user manual for verification and use of electric air samplers that actively collect volumes of air for evaluation shall be followed.

† A sufficient volume of air (400–1000 liters) shall be tested at each location in order to maximize sensitivity.

‡ It is recommended that compounding personnel also refer to USP Chapter (1116), which can provide more information on the use of volumetric air samplers and volume of air that should be sampled to detect environmental bioburden excursions.

Air Sampling Frequency and Process

† Air sampling shall be performed at least semiannually (i.e. every 6 months), as part of the re-certification of facilities and equipment for area where primary engineering controls are located.

† A sufficient volume of air shall be sampled and the manufacturer’s guidelines for use of the electronic air sampling equipment followed.

‡ Any facility construction or equipment servicing may require the need to perform air sampling during these events.

Incubation Period

† The microbial growth media plates used to collect environmental sampling are recovered, covers secured (e.g., taped), inverted, and incubated at a temperature and for a time period conducive to multiplication of microorganisms.

† The number of discrete colonies of microorganisms shall be counted and reported as colony-forming units (cfu) and documented on an environmental monitoring form. Counts from air monitoring need to be transformed into cfu/cubic meter of air and evaluated for adverse trends.

‡ TSA should be incubated at $35^{\circ} \pm 2^{\circ}$ for 2–3 days.

‡ MEA or other suitable fungal media should be incubated at $28^{\circ} \pm 2^{\circ}$ for 5–7 days.

Action Levels, Documentation and Data Evaluation

† Sampling data shall be collected and reviewed on a periodic basis as a means of evaluating the overall control of the compounding environment.

† Competent microbiology personnel shall be consulted if an environmental sampling consistently shows elevated levels of microbial growth.

† An investigation into the source of the environmental contamination shall be conducted.

‡ Any cfu count that exceeds its respective action level should prompt a re-evaluation of the adequacy of personnel work practices, cleaning procedures, operational procedures, and air filtration efficiency within the aseptic compounding location.

‡ Table titled, Recommended Action Levels for Microbial Contamination should only be used as a guideline

Facility Design and Environmental Controls

† Compounding facilities are physically designed and environmentally controlled to minimize airborne contamination from contacting critical sites.

† Compounding facilities shall provide a comfortable and well-lighted working environment, which typically includes a temperature of 20° or cooler to maintain comfortable conditions for compounding personnel when attired in the required aseptic compounding garb.

† Primary engineering controls provide unidirectional (i.e., laminar) HEPA air at a velocity sufficient to prevent airborne particles from contacting critical sites.

† In situ air pattern analysis via smoke studies shall be conducted at the critical area to demonstrate unidirectional airflow and sweeping action over and away from the product under dynamic conditions.

† Policies and procedures for maintaining and working within the primary engineering control area shall be written and followed. The policies and procedures will be determined by the scope and risk levels of the aseptic compounding activities used during the preparation of the CSPs.

† The principles of HEPA-filtered unidirectional airflow in the work environment shall be understood and practiced in the compounding process in order to achieve the desired environmental conditions.

† Clean rooms for nonhazardous and nonradioactive CSPs are supplied with HEPA that enters from ceilings with return vents low on walls, and that provides not less than 30 air changes per hour.

† Buffer areas maintain 0.02- to 0.05-inch water column positive pressure, and do not contain sinks or drains.

† Air velocity from buffer rooms or zones to ante-areas is at least 40 feet/minute.

† The primary engineering controls shall be placed within a buffer area in such a manner as to avoid conditions that could adversely affect their operation.

† The primary engineering controls shall be placed out of the traffic flow and in a manner to avoid disruption from the HVAC system and room cross-drafts.

† HEPA-filtered supply air shall be introduced at the ceiling.

† All HEPA filters shall be efficiency tested using the most penetrating particle size and shall be leak tested at the factory and then leak tested again in situ after installation.

APPENDICES

Appendix I. Principal Competencies, Conditions, Practices, and Quality Assurances That Are Required († “shall”) and Recommended (§ “should”) in USP Chapter <797> (Continued)

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- † Activities and tasks carried out within the buffer area shall be limited to only those necessary when working within a controlled environment.
- † Only the furniture, equipment, supplies, and other material required for the compounding activities to be performed shall be brought into the room.
- † Surfaces and essential furniture in buffer rooms or zones and clean rooms shall be nonporous, smooth, nonshedding, impermeable, cleanable, and resistant to disinfectants.
- † The surfaces of ceilings, walls, floors, fixtures, shelving, counters, and cabinets in the buffer area shall be smooth, impervious, free from cracks and crevices, and nonshedding, thereby promoting cleanability, and minimizing spaces in which microorganisms and other contaminants may accumulate.
- † The surfaces shall be resistant to damage by disinfectant agents.
- † Junctures of ceilings to walls shall be coved or caulked to avoid cracks and crevices where dirt can accumulate.
- † Ceiling tiles shall be caulked around each perimeter to seal them to the support frame.
- † The exterior lens surface of ceiling lighting fixtures shall be smooth, mounted flush, and sealed.
- † Any other penetrations through the ceiling or walls shall be sealed.
- † The buffer area shall not contain sources of water (sinks) or floor drains. Work surfaces shall be constructed of smooth, impervious materials, such as stainless steel or molded plastic, so that they are easily cleaned and disinfected.
- † Carts shall be of stainless steel wire, nonporous plastic, or sheet metal construction with good quality, cleanable casters to promote mobility.
- † Storage shelving, counters, and cabinets shall be smooth, impervious, free from cracks and crevices, nonshedding, cleanable, and disinfectable.
- † Their number, design, and manner of installation the itmes above shall promote effective cleaning and disinfection.
- ‡ If ceilings consist of inlaid panels, the panels should be impregnated with a polymer to render them impervious and hydrophobic.
- ‡ Dust-collecting overhangs, such as ceiling utility pipes, or ledges, such as windowsills, should be avoided.
- ‡ Air returns should be mounted low on the wall creating a general top-down dilution of room air with HEPA-filtered make-up air.
- Placement of Primary Engineering Controls Within ISO Class 7 Buffer Areas**
- † Primary engineering controls for nonhazardous and nonradioactive CSPs are located in buffer areas, except for CAIs that are proven to maintain ISO Class 5 air when particle counts are sampled 6 to 12 inches upstream of critical site exposure areas during performance of normal inward and outward transfer of materials, and compounding manipulations when such CAIs are located in air quality worse than ISO Class 7.
- † Presterilization procedures for high-risk level CSPs, such as weighing and mixing, shall be completed in no worse than an ISO Class 8 environment.
- † Primary engineering controls shall be located out of traffic patterns and away from room air currents that could disrupt the intended airflow patterns.
- † When isolators are used for sterile compounding, the recovery time to achieve ISO Class 5 air quality shall be documented and internal procedures developed to ensure that adequate recovery time is allowed after material transfer before and during compounding operations.
- † When compounding activities require the manipulation of a patient’s blood-derived or other biological material (e.g., radiolabeling a patient’s or a donor’s white blood cells), the manipulations shall be clearly separated from routine material-handling procedures and equipment used in CSP preparation activities, and they shall be controlled by specific standard operating procedures in order to avoid any cross-contamination.
- † Food, drinks, and items exposed in patient care areas, and unpacking of bulk supplies and personnel cleansing and garbing are prohibited from buffer areas or rooms.
- † Demarcation designation between buffer areas or rooms and ante-areas.
- † Antiseptic hand cleansing and sterile gloves in buffer areas or rooms.
- ‡ Packaged compounding supplies and components, such as needles, syringes, tubing sets, and small- and large-volume parenterals, should be uncanted and wiped down with a disinfectant that does not leave a residue (e.g., sterile 70% IPA) when possible in an ante-area, of ISO Class 8 air quality, before being passed into the buffer areas.
- Cleaning and Disinfecting the Sterile Compounding Areas**
- † Trained personnel write detailed procedures including cleansers, disinfectants, and non-shedding wipe and mop materials.
- † Cleaning and disinfecting surfaces in the LAFWs, BSCs, CAIs, and CACIs shall be cleaned and disinfected frequently, including at the beginning of each work shift, before each batch preparation is started, every 30 minutes during continuous compounding periods of individual CSPs, when there are spills, and when surface contamination is known or suspected from procedural breaches.
- † Trained compounding personnel are responsible for developing, implementing, and practicing the procedures for cleaning and disinfecting the DCAs written in the SOPs.
- † Cleaning and disinfecting shall occur before compounding is performed. Items shall be removed from all areas to be cleaned, and surfaces shall be cleaned by removing loose material and residue from spills, e.g., water-soluble solid residues are removed with Sterile Water (for Injection or Irrigation) and low-shedding wipes. This shall be followed by wiping with a residue-free disinfecting agent, such as sterile 70% IPA, which is allowed to dry before compounding begins.
- † Work surfaces in ISO Class 7 and 8 areas and segregated compounding areas are cleaned at least daily.
- † Dust and debris shall be removed when necessary from storage sites for compounding ingredients and supplies, using a method that does not degrade the ISO Class 7 or 8 air quality.
- † Floors in ISO Class 7 and 8 areas are cleaned daily when no compounding occurs.
- † IPA (70% isopropyl alcohol) remains on surfaces to be disinfected for at least 30 seconds before such surfaces are used to prepare CSPs.
- † Emptied shelving, walls, and ceilings in ante-areas are cleaned and disinfected at least monthly.
- † Mopping shall be performed by trained personnel using approved agents and procedures described in the written SOPs.
- † Cleaning and disinfecting agents, their schedules of use and methods of application shall be in accordance with written SOPs and followed by custodial and/or compounding personnel.
- † All cleaning materials, such as wipers, sponges, and mops, shall be nonshedding, preferably composed of synthetic micro fibers, and dedicated to use in the buffer area, or ante-area, and segregated compounding areas and shall not be removed from these areas except for disposal.

APPENDICES**Appendix I. Principal Competencies, Conditions, Practices, and Quality Assurances That Are Required († “shall”) and Recommended (§ “should”) in USP Chapter (797) (Continued)**

- † If cleaning materials are reused (e.g., mops), procedures shall be developed (based on manufacturer recommendations) that ensure that the effectiveness of the cleaning device is maintained and repeated use does not add to the bioburden of the area being cleaned.
- † Supplies and equipment removed from shipping cartons shall be wiped with a suitable disinfecting agent (e.g., sterile 70% IPA) delivered from a spray bottle or other suitable delivery method.
- † After the disinfectant is sprayed or wiped on a surface to be disinfected, the disinfectant shall be allowed to dry, and during this time the item shall not be used for compounding purposes.
- † Sterile 70% IPA wetted gauze pads or other particle-generating material shall not be used to disinfect the sterile entry points of packages and devices.

Personnel Cleansing and Garbing

- † Personnel shall also be thoroughly competent and highly motivated to perform flawless aseptic manipulations with ingredients, devices, and components of CSPs.
- † Personnel with rashes, sunburn, weeping sores, conjunctivitis, active respiratory infection, and cosmetics are prohibited from preparing CSPs.
- † Compounding personnel shall remove personal outer garments; cosmetics; artificial nails; hand, wrist, and body jewelry that can interfere with the fit of gowns and gloves; and visible body piercing above the neck.
- † Order of compounding garb and cleansing in ante-area: shoes or shoe covers, head and facial hair covers, face mask, fingernail cleansing, hand and forearm washing and drying; non-shedding gown.
- † Order of cleansing and gloving in buffer room or area: hand cleansing with a persistently active alcohol-based product with persistent activity; allow hands to dry; don sterile gloves.
- † Routinely disinfect gloves with sterile 70% IPA after contacting nonsterile objects.
- † Inspect gloves for holes and replace when breaches are detected.
- † Personnel repeat proper procedures after they are exposed to direct contact contamination or worse than ISO Class 8 air.
- † These requirements are exempted only for immediate-use CSPs and CAls for which manufacturers provide written documentation based on validated testing that such personnel practices are not required to maintain sterility in CSPs.

Personnel Training and Competency Evaluation of Garbing, Aseptic Work Practices and Cleaning/Disinfection Procedures

- † Personnel who prepare CSPs shall be trained conscientiously and skillfully by expert personnel, multi-media instructional sources, and professional publications in the theoretical principles and practical skills of garbing procedures, aseptic work practices, achieving and maintaining ISO Class 5 environmental conditions, and cleaning and disinfection procedures.
- † This training shall be completed and documented before any compounding personnel begin to prepare CSPs.
- † Compounding personnel shall complete didactic training, pass written competence assessments, undergo skill assessment using observational audit tools, and media-fill testing.
- † Media-fill testing of aseptic work skills shall be performed initially before beginning to prepare CSPs and at least annually thereafter for low- and medium-risk level compounding; and semiannually for high-risk level compounding.
- † Compounding personnel who fail written tests, observational audits, or whose media-fill test vials have one or more units showing visible microbial contamination, shall be re-instructed and re-evaluated by expert compounding personnel to ensure correction of all aseptic work practice deficiencies.
- † Compounding personnel shall pass all evaluations prior to resuming compounding of sterile preparations.
- † Compounding personnel must demonstrate proficiency of proper hand hygiene, garbing, and consistent cleaning procedures in addition to didactic evaluation and aseptic media fill.
- † Cleaning and disinfecting procedures performed by other support personnel shall be thoroughly trained in proper hand hygiene, and garbing, cleaning, and disinfection procedures by a qualified aseptic compounding expert.
- † Support personnel shall routinely undergo performance evaluation of proper hand hygiene, garbing, and all applicable cleaning and disinfecting procedures conducted by a qualified aseptic compounding expert.

Competency Evaluation of Garbing and Aseptic Work Practices

- † Compounding personnel shall be evaluated initially prior to beginning compounding CSPs and whenever an aseptic media fill is performed using a Sample Form for Assessing Hand Hygiene and Garbing Related Practices of Compounding Personnel and the personnel glove fingertip sampling procedures.

Aseptic Work Practice Assessment and Evaluation via Personnel Glove Fingertip Sampling

- † Monitoring of compounding personnel glove fingertips shall be performed for all CSP risk level compounding.
- † Glove fingertip sampling shall be used to evaluate the competency of personnel in performing hand hygiene and garbing procedures in addition to educating compounding personnel on proper work practices.
- † All personnel shall demonstrate competency in proper hand hygiene and garbing procedures in addition to aseptic work practices.
- † Sterile contact agar plates shall be used to sample the gloved fingertips of compounding personnel after garbing to assess garbing competency and after completing the media-fill preparation.
- † Gloves shall not be disinfected with sterile 70% IPA immediately prior to sampling.

Garbing and Gloving Competency Evaluation

- † Compounding personnel shall be visually observed during the process of performing hand hygiene and garbing procedures.
- † The visual observation shall be documented on a Sample Form for Assessing Hand Hygiene and Garbing Related Practices of Compounding Personnel and maintained to provide a permanent record of and long-term assessment of personnel competency.

Gloved Fingertip Sampling

- † Immediately after the compounder completes the hand hygiene and garbing procedure, the evaluator shall collect a gloved fingertip and thumb sample from both hands of the compounder onto appropriate agar plates by lightly pressing each finger tip into the agar.
- † The plates shall be incubated for the appropriate incubation period and at the appropriate temperature.
- † All employees shall successfully complete an initial competency evaluation and gloved fingertip/thumb sampling procedure (0 cfu) no less than three times before initially being allowed to compound CSPs for human use.

APPENDICES**Appendix I. Principal Competencies, Conditions, Practices, and Quality Assurances That Are Required († “shall”) and Recommended (§ “should”) in USP Chapter <797> (Continued)**

† After completing the initial gowning and gloving competency evaluation, re-evaluation of all compounding personnel shall occur at least annually for low- and medium-risk level CSPs and semiannually for high-risk level CSPs before being allowed to continue compounding CSPs.

† Gloves shall not be disinfected with sterile 70% IPA prior to testing.

† The sampled gloves shall be immediately discarded and proper hand hygiene performed after sampling. The nutrient agar plates shall be incubated as stated below.

† The cfu action level for gloved hands shall be based on the total number of cfu on both gloves and not per hand.

‡ Results should be reported separately as number of cfu per employee per hand (left hand, right hand).

Incubation Period

† At the end of the designated sampling period, the agar plates are recovered, covers secured, inverted and incubated at a temperature and for a time period conducive to multiplication of microorganisms. Trypticase soy agar (TSA) with lecithin and polysorbate 80 shall be incubated at $35^{\circ} \pm 2^{\circ}$ for 2–3 days.

Aseptic Manipulation Competency Evaluation

† All compounding personnel shall have their aseptic technique and related practice competency evaluated initially during the media-fill test procedure and subsequent annual or semiannual media-fill test procedures on the Sample Form for Assessing Aseptic Technique and Related Practices of Compounding Personnel.

Media-Fill Test Procedure

† The skill of personnel to aseptically prepare CSPs shall be evaluated using sterile fluid bacterial culture media-fill verification.

† Media-filled vials shall be incubated within a range of $35^{\circ} \pm 2^{\circ}$ for 14 days.

Surface Cleaning and Disinfection Sampling and Assessment

† Surface sampling shall be performed in all ISO classified areas on a periodic basis and can be accomplished using contact plates and/or swabs and shall be done at the conclusion of compounding.

† Locations to be sampled shall be defined in a sample plan or on a form.

Cleaning and Disinfecting Competency Evaluation

† Compounding personnel and other personnel responsible for cleaning shall be visually observed during the process of performing cleaning and disinfecting procedures during initial personnel training on cleaning procedures, changes in cleaning staff and at the completion of any Media-Fill Test Procedure.

† Visual observation shall be documented on a Sample Form for Assessing Cleaning and Disinfection Procedures and maintained to provide a permanent record of, and long-term assessment of, personnel competency.

Surface Collection Methods

† Immediately after sampling a surface with the contact plate, the sampled area shall be thoroughly wiped with a non-shedding wipe soaked in sterile 70% IPA.

‡ Results should be reported as cfu per unit of surface area.

Action Levels, Documentation, and Data Evaluation

† Environmental sampling data shall be collected and reviewed on a routine basis as a means of evaluating the overall control of the compounding environment.

† If an activity consistently shows elevated levels of microbial growth, competent microbiology personnel shall be consulted.

† An investigation into the source of the contamination shall be conducted.

† When gloved fingertip sample results exceeds action levels after proper incubation, a review of hand hygiene and garbing procedures as well as glove and surface disinfection procedures and work practices shall be performed and documented.

‡ Any cfu count that exceeds its respective action level should prompt a re-evaluation of the adequacy of personnel work practices, cleaning procedures, operational procedures, and air filtration efficiency within the aseptic compounding location.

SUGGESTED STANDARD OPERATING PROCEDURES

† All facilities are required to have these, and they must include at least the items enumerated in this section.

FINISHED PREPARATION RELEASE CHECKS AND TESTS**Inspection of Solution Dosage Forms and Review of Compounding Procedures**

† Review procedures and documents to ensure sterility, purity, correct identities and amounts of ingredients, and stability.

† Visually inspect for abnormal particulate matter and color, and intact containers and seals.

Sterility Testing

† High-risk level CSPs prepared in batches of more than 25 identical containers, or exposed longer than 12 hours at 2° to 8° , and 6 hours at warmer than 8° before being sterilized.

Bacterial Endotoxin (Pyrogen) Testing

† High-risk level CSPs, excluding those for inhalation and ophthalmic administration, prepared in batches of more than 25 identical containers, or exposed longer than 12 hours at 2° to 8° , and 6 hours at warmer than 8° , before being sterilized.

Identity and Strength Verification of Ingredients

† Written procedures to verify correct identity, quality, amounts, and purities of ingredients used in CSPs.

† Written procedures to ensure labels of CSPs contain correct names and amounts or concentrations of ingredients, total volumes, beyond-use dates, storage conditions, and route(s) of administration.

APPENDICES**Appendix I. Principal Competencies, Conditions, Practices, and Quality Assurances That Are Required († “shall”) and Recommended (§ “should”) in USP Chapter <797> (Continued)**

STORAGE AND BEYOND-USE DATING**Determining Beyond-Use Dates**

† Use the general criteria in USP <795> in the absence of direct stability-indicating assays or authoritative literature that supports longer durations.

MAINTAINING STERILITY, PURITY, AND STABILITY OF DISPENSED AND DISTRIBUTED CSPs

† Written procedures for proper packaging, storage, and transportation conditions to maintain sterility, quality, purity, and strength of CSPs.

Redispensed CSPs

† When sterility, and acceptable purity, strength, and quality can be ensured.

† Assignment of sterility storage times and stability beyond-use dates that occur later than those of originally dispensed CSPs must be based on results of sterility testing and quantitative assay of ingredients.

Packaging and Transporting CSPs

† Packaging maintains physical integrity, sterility, stability, and purity of CSPs.

† Modes of transport that maintain appropriate temperatures and prevent damage to CSPs.

PATIENT OR CAREGIVER TRAINING

† Multiple component formal training program to ensure patients and caregivers understand the proper storage, handling, use, and disposal of CSPs.

PATIENT MONITORING AND ADVERSE EVENTS REPORTING

† Written standard procedures describe means for patients to ask questions and report concerns and adverse events with CSPs, and for compounding supervisors to correct and prevent future problems.

‡ Adverse events and defects with CSPs reported to FDA’s MedWatch and USP’s MEDMARX programs.

Appendix II. Common Disinfectants Used in Health Care for Inanimate Surfaces and Noncritical Devices, and Their Microbial Activity and Properties¹

Chemical Category of Disinfectant							
		Isopropyl alcohol	Accelerated hydrogen peroxide	Quaternary Ammonium (e.g., dodecyl dimethyl ammonium chloride)	Phenolics	Chlorine (e.g., sodium hypochlorite)	Iodophors (e.g., povidone-iodine)
Concentration Used		60–95%	0.5%³	0.4–1.6% aq	0.4–1.6% aq	100–5000 ppm	30–50 ppm
Microbial Inactivation ²	Bacteria	+	+	+	+	+	+
	Lipophilic viruses	+	+	+	+	+	+
	Hydrophilic viruses	±	+	±	±	+	±
	M.tuberculosis	+	+	±	+	+	±
	Mycotic agents (fungi)	+	+	+	+	+	±
	Bacterial Spores	–	–	–	–	+	–
Important Chemical & Physical Properties	Shelf life >1 week	+	+	+	+	+	+
	Corrosive or deleterious effects	±	–	–	–	±	±
	Non-evaporable residue	–	–	+	+	–	+
	Inactivated by organic matter	+	±	+	±	+	+
	Skin irritant	±	–	+	+	+	±
	Eye irritant	+	–	+	+	+	+
	Respiratory irritant	–	–	–	–	+	–
	Systemic toxicity	+	–	+	+	+	+

Key to abbreviation and symbols: aq = diluted with water; ppm = parts per million; + = yes; – = no; ± = variable results.

¹ Modified from World Health Organization, Laboratory Bio Safety Manual 1983 and Rutala WA, "Antisepsis, disinfection and sterilization in the hospital and related institutions," *Manual of Clinical Microbiology*, American Society for Microbiology, Washington, DC, 1995, pages 227-245.

² Inactivation of the most common microorganisms (i.e., bacteria) occurs with a contact time of ≤1 minute; inactivation of spores requires longer contact times (e.g., 5-10 minutes for 5,000 ppm chlorine solution against *C. difficile* spores). Reference: Perez J, Springthorpe VS, Sattar SA, "Activity of selected oxidizing microbicides against the spores of *Clostridium difficile*: Relevance to environmental control," *American Journal of Infection Control*, August 2005, pages 320-325.

³ Accelerated hydrogen peroxide is a new generation of hydrogen peroxide-based germicides in which the potency and performance of the active ingredient have been enhanced and accelerated through the use of appropriate acids and detergents.

Appendix III. Sample Form for Assessing Hand Hygiene and Garbing Related Practices of Compounding Personnel

Printed name and position/title of person assessed: _____
Name of facility or location: _____

Hand Hygiene and Garbing Practices: The qualified evaluator will check each space for which the person being assessed has acceptably completed the described activity, prints N/A if the activity is not applicable to the assessment session or N/O if the activity was not observed.*

- _____ Presents in a clean appropriate attire and manner.
- _____ Wears no cosmetics or jewelry (watches, rings, earrings, etc. piercing jewelry included) upon entry into ante-areas.
- _____ Brings no food or drinks into or stored in the ante-areas or buffer areas.
- _____ Is aware of the line of demarcation separating clean and dirty sides and observes required activities.
- _____ Dons shoe covers or designated clean-area shoes one at a time, placing the covered or designated shoe on clean side of the line of demarcation, as appropriate.
- _____ Dons beard cover if necessary.
- _____ Dons head cover assuring that all hair is covered.
- _____ Dons face mask to cover bridge of nose down to include chin.
- _____ Performs hand hygiene procedure by wetting hands and forearms and washing using soap and warm water for at least 30 seconds.
- _____ Dries hands and forearms using lint-free towel or hand dryer.
- _____ Selects the appropriate sized gown examining for any holes, tears, or other defects.
- _____ Dons gown and ensures full closure.
- _____ Disinfects hands again using a waterless alcohol-based surgical hand scrub with persistent activity and allows hands to dry thoroughly before donning sterile gloves.
- _____ Dons appropriate sized sterile gloves ensuring that there is a tight fit with no excess glove material at the fingertips.
- _____ Examines gloves ensuring that there are no defects, holes, or tears.
- _____ While engaging in sterile compounding activities, routinely disinfects gloves with sterile 70% IPA prior to work in the direct compounding area (DCA) and after touching items or surfaces that may contaminate gloves.
- _____ Removes PPE on the clean side of the ante-area.
- _____ Removes gloves and performs hand hygiene.
- _____ Removes gown and discards it, or hangs it on hook if it is to be reused within the same work day.
- _____ Removes and discards mask, head cover, and beard cover (if used).
- _____ Removes shoe covers or shoes one at a time, ensuring that uncovered foot is placed on the dirty side of the line of demarcation and performs hand hygiene again. (Removes and discards shoe covers every time the compounding area is exited).

***The person assessed is immediately informed of all unacceptable activities (i.e., spaces lacking check marks, N/A, or N/O) and shown and informed of specific corrections.**

Signature of Person Assessed	Printed Name	Date
Signature of Qualified Evaluator	Printed Name	Date

Appendix IV. Sample Form for Assessing Aseptic Technique and Related Practices of Compounding Personnel

Printed name and position/title of person assessed: _____
 Name of facility or location: _____

Aseptic Technique, Safety, and Quality Assurance Practices: The qualified evaluator checks each space for which the person being assessed has acceptably completed the described activity, prints N/A if the activity is not applicable to the assessment session or N/O if the activity was not observed.*

- _____ Completes the Hand Hygiene and Garbing Competency Assessment Form.
- _____ Performs proper hand hygiene, garbing, and gloving procedures according to SOPs.
- _____ Disinfects ISO Class 5 device surfaces with an appropriate agent.
- _____ Disinfects components/vials with an appropriate agent prior to placing into ISO Class 5 work area.
- _____ Introduces only essential materials in a proper arrangement in the ISO Class 5 work area.
- _____ Does not interrupt, impede, or divert flow of first-air to critical sites.
- _____ Ensures syringes, needles, and tubing remain in their individual packaging and are only opened in ISO Class 5 work area.
- _____ Performs manipulations only in the appropriate DCA of the ISO Class 5 device.
- _____ Does not expose critical sites to contact contamination or worse than ISO Class 5 air.
- _____ Disinfects stoppers, injection ports, and ampul necks by wiping with sterile 70% IPA and allows sufficient time to dry.
- _____ Affixes needles to syringes without contact contamination.
- _____ Punctures vial stoppers and spikes infusion ports without contact contamination.
- _____ Labels preparation(s) correctly.
- _____ Disinfects sterile gloves routinely by wiping with sterile 70% IPA during prolonged compounding manipulations.
- _____ Cleans, sets up, and calibrates automated compounding device (e.g., "TPN compounder") according to manufacturer's instructions.
- _____ Disposes of sharps and waste according to institutional policy or recognized guidelines.

***The person assessed is immediately informed of all unacceptable activities (i.e., spaces lacking check marks, N/A, or N/O) and shown and informed of specific corrections.**

Signature of Person Assessed	Printed Name	Date
Signature of Qualified Evaluator	Printed Name	Date

Appendix V. Sample Form for Assessing Cleaning and Disinfection Procedures

Printed name and position/title of person assessed: _____
Name of facility or location: _____

Cleaning and Disinfection Practices: The qualified evaluator will check each space for which the person being assessed has acceptably completed the described activity, prints N/A if the activity is not applicable to the assessment session or N/O if the activity was not observed.*

Daily Tasks:

- _____ Prepares correct concentration of disinfectant solution according to manufacturer’s instructions.
- _____ Uses appropriately labeled container for the type of surface to be cleaned (floor, wall, production bins, etc.).
- _____ Documents disinfectant solution preparation.
- _____ Follows garbing procedures when performing any cleaning activities.
- _____ At the beginning of each shift, cleans all ISO Class 5 devices prior to compounding in the following order: walls, IV bar, automated compounders, and work surface.
- _____ Uses a lint free wipe soaked with sterile 70% IPA or other approved disinfectant solution and allows to dry completely.
- _____ Removes all compounder components and cleans all ISO Class 5 areas as stated above at the end of each shift.
- _____ Cleans all counters and easily cleanable work surfaces.
- _____ Mops floors, using the mop labeled “floors,” starting at the wall opposite the room entry door; mops floor surface in even strokes toward the operator. Moves carts as needed to clean entire floor surface. Use of a microfiber cleaning system is an acceptable alternative to mops.
- _____ In the ante-area, cleans sink and all contact surfaces; cleans floor with a disinfectant solution or uses microfiber cleaning system.

Monthly Tasks:

- _____ Performs monthly cleaning on a designated day. Prepares a disinfectant solution as stated in daily tasks that is appropriate for the surfaces to be cleaned.
- _____ Cleans buffer area and ante-area ceiling, walls, and storage shelving with a disinfectant solution and a mop or uses a microfiber cleaning system.
- _____ Once ISO Class 5 area is clean, cleans compounding room ceiling, followed by walls and ending with the floor. Uses appropriate labeled mops or microfiber cleaning system.
- _____ Cleans all buffer area totes and storage shelves by removing contents and using a germicidal detergent soaked lint free wipe, cleans the inside surfaces of the tote and then the entire exterior surfaces of the tote. Allows totes to dry. Prior to replacing contents into tote, wipes tote with sterile 70% IPA to remove disinfectant residue. Uses new wipe as needed.
- _____ Cleans all buffer area carts by removing contents and using germicidal detergent soaked lint free wipe, cleans all carts starting with the top shelf and top of post, working down to wheels. Cleans the under side of shelves in a similar manner. Uses a new wipe for each cart. Allows to dry. Wipes carts with sterile 70% IPA wetted lint-free wipe to remove any disinfectant residue. Uses new wipe as needed.
- _____ Cleans buffer area chairs, the interior and exterior of trash bins, and storage bins using disinfectant solution soaked lint free wipe.
- _____ Documents all cleaning activities as to who performed such activities with date and time noted.

*The person assessed is immediately informed of all unacceptable activities (i.e., spaces lacking check marks, N/A, or N/O) and shown and informed of specific corrections.

Signature of Person Assessed	Printed Name	Date
Signature of Qualified Evaluator	Printed Name	Date

<801> POLAROGRAPHY

Polarography is an electrochemical method of analysis based on the measurement of the current flow resulting from the electrolysis of a solution at a polarizable microelec-trode, as a function of an applied voltage. The polarogram (see Figure 1) obtained by this measurement provides quali-tative and quantitative information on electro-reducible and electro-oxidizable substances. The normal concentration range for substances being analyzed is from 10⁻² molar to 10⁻⁵ molar.

In direct current (dc) polarography, the microelectrode is a dropping mercury electrode (DME) consisting of small re-producible drops of mercury flowing from the orifice of a capillary tube connected to a mercury reservoir. A saturated calomel electrode (SCE) with a large surface area is the most commonly employed reference electrode. As the voltage ap-plied to the cell increases, only a very small residual current flows until the substance under assay undergoes reduction or oxidation. Then the current increases, at first gradually, then almost linearly with voltage, and it gradually reaches a limiting value as is shown in Figure 1.

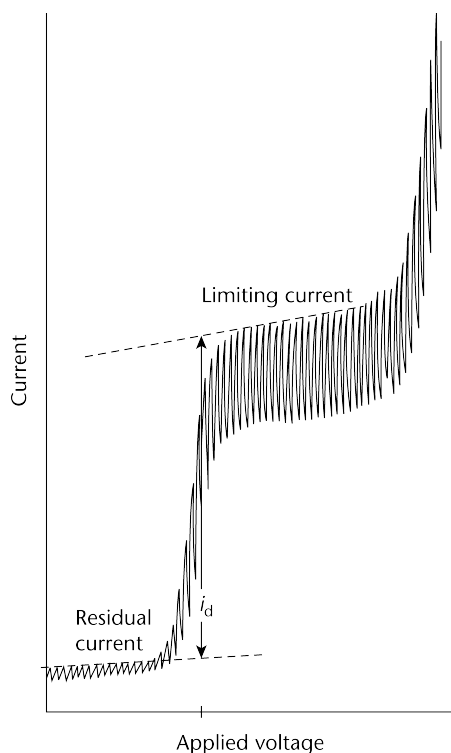


Fig. 1. Typical Polarogram Showing Change in Current Flow with Increasing Potential Applied to the Dropping Mercury Electrode.

On the initial rising portion of the polarographic wave, the increased flow of current results in a decrease in the concentration of the electro-active species at the electrode surface. As the voltage and current increase, the concentration of the reactive species decreases further to a minimal value at the electrode surface. The current is then limited by the rate at which the reacting species can diffuse from the bulk of the solution to the surface of the microelectrode. The final current rise is caused by the reaction of the supporting electrolyte. This large concentration of electrolyte is inert within the potential range used in the analysis, and it prevents the reactive species from reaching the electrode by electrical migration, thus assuring that the limiting current is diffusion-controlled.

Since, in the case of the DME, the electrode surface is being constantly renewed in a cyclic fashion, the current increases from a small value as the drop begins to form to a maximum value as the drop falls. By the use of a suitable recorder to measure the current, the characteristic saw-toothed record is obtained. The limiting current is the sum of the residual and the diffusion currents. The residual current is subtracted from the limiting current to give the wave height.

Ilkovic Equation—The linear relationship between the diffusion current (i_d) and the concentration of electro-active species is shown by the Ilkovic equation:

$$i_d = 708nD^{1/2}Cm^{2/3}t^{1/6}$$

in which i_d is the maximum current in microamperes; n is the number of electrons required per molecule of electro-active substance; D is its diffusion coefficient, in square cm per second; C is the concentration, in millimoles per L; m is the rate of mercury flow from the DME, in mg per second; and t is the drop time, in seconds.

Modern polarographs are equipped with recorders capable of following the current during the latter portion of the drop life; consequently, the maximum of the oscillations is the measure of the current. When the current is measured

only at the end of the drop life, the technique is termed sampled dc polarography. In this case, only the maximum currents are recorded and oscillations due to drop growth are not observed.

For instruments equipped with galvanometers to measure the current or recorders operated in a damped mode, the saw-toothed waves correspond to oscillations about the average current. In the latter case, the average of the oscillations is the measure of the current. For polarograms obtained in this manner, the i_d given by the Ilkovic equation is the average current in microamperes observed during the life of the drop, when the coefficient 708 is replaced by 607.

Control of the Diffusion Current—The Ilkovic equation identifies the variables that must be controlled to ensure that the diffusion current is directly proportional to the concentration of electro-active material. At 25° the diffusion coefficients for aqueous solutions of many ions and organic molecules increase 1% to 2% per degree rise in temperature. Thus the temperature of the polarographic cell must be controlled to within $\pm 0.5^\circ$. The quantities m and t depend upon the dimensions of the capillary and the height of the mercury column above the electrode. Although results obtained with different capillaries can be compared if the product $m^{2/3}t^{1/6}$ is known, it is advisable to use the same capillary with a constant head of mercury during a series of analyses. The diffusion current is proportional to the square root of the height of the mercury column. A mercury reservoir with a diameter greater than 4 cm prevents any significant drop in the mercury level during a series of runs.

The capillary for the DME has a bore of approximately 0.04 mm and a length of 6 cm to 15 cm. The height of the mercury column, measured from the tip of the capillary to the top of the mercury pool, ranges from 40 cm to 80 cm. The exact length of the capillary and the height of the mercury column are adjusted to give a drop-time of between 3 and 5 seconds at open circuit with the capillary immersed in the test solution.

Equipment is available that allows controlled drop-times of fractions of a second to several seconds. As detail within a polarogram is related to the number of drops delivered during a given potential change, such short drop-times allow more rapid recording of the polarogram.

The current flowing through the test solution during the recording of a polarogram is in the microampere range. Thus, the current flow produces negligible changes in the test solution and several polarograms can be run on the same test solution without significant differences.

Half-wave Potential—The half-wave potential ($E_{1/2}$) occurs at the point on the polarogram one-half the distance between the residual current and the limiting current plateau. This potential is characteristic of the electro-active species and is largely independent of its concentration or the capillary used to obtain the wave. It is dependent upon the solution composition and may change with variations in the pH or in the solvent system or with the addition of complexing agents. The half-wave potential thus serves as a criterion for the qualitative identification of a substance.

The potential of the DME is equal to the applied voltage versus the reference electrode after correction for the iR drop (that potential need to pass the current, i , through the solution with a resistance R). It is especially important to make this correction for nonaqueous solutions, which ordinarily possess high resistance, if an accurate potential for the DME is needed. Correction of the half-wave potential is not required for quantitative analysis. Unless otherwise indicated, it is to be understood that potentials represent measurements made against the SCE.

Removal of Dissolved Oxygen—Inasmuch as oxygen is reduced at the DME in two steps, first to hydrogen peroxide and then to water, it interferes where polarograms are to be made at potentials more negative than about 0 volt versus SCE, and must be removed. This may be accomplished by bubbling oxygen-free nitrogen through the solution for 10

to 15 minutes immediately before recording the wave, the nitrogen first having been "conditioned" to minimize changes due to evaporation, by being passed through a separate portion of the solution.

It is necessary that the solution be quiet and vibration-free during the time the wave is recorded, to ensure that the current is diffusion-controlled. Therefore, the nitrogen aeration should be stopped and the gas be directed to flow over the surface of the solution before a polarogram is recorded.

In alkaline media, sodium bisulfite may be added to remove oxygen, provided the reagent does not react with other components of the system.

Measurement of Wave Height—To use a polarogram quantitatively, it is necessary to measure the height of the wave. Since this is a measure of the magnitude of the diffusion current, it is measured vertically. To compensate for the residual current, the segment of the curve preceding the wave is extrapolated beyond the rise in the wave. For a well-formed wave where this extrapolation parallels the limiting current plateau, the measurement is unambiguous. For less well-defined waves, the following procedure may be used unless otherwise directed in the individual monograph. Both the residual current and the limiting current are extrapolated with straight lines, as shown by the graph (Figure 7). The wave height is taken as the vertical distance between these lines measured at the half-wave potential.

Procedure—[Caution—Mercury vapor is poisonous, and metallic mercury has a significant vapor pressure at room temperature. The work area in which mercury is used should be constructed in such a way that any spilled or spattered droplets of mercury can be completely recovered with relative ease. Scrupulously clean up mercury after each use of the instrument. Work in a well-ventilated laboratory, taking care to clean up any spilled mercury.] Transfer a portion of the final dilution of the substance being assayed to a suitable polarographic cell immersed in a water bath regulated to $25 \pm 0.5^\circ$. Pass a stream of nitrogen through the solution for 10 to 15 minutes to remove dissolved oxygen. Start the mercury dropping from the capillary, insert the capillary into the test solution, and adjust the height of the mercury reservoir. Switch the flow of nitrogen to pass over the surface of the solution, and record the polarogram over the potential range indicated in the individual monograph, using the appropriate recorder or galvanometer sensitivity to give a suitable wave. Measure the height of the wave, and unless otherwise directed in the monograph, compare this with the wave height obtained with the appropriate USP Reference Standard, measured under the same conditions.

Pulse Polarography—In conventional dc polarography, the current is measured continuously as potential is applied as a linear ramp (see Figure 2).

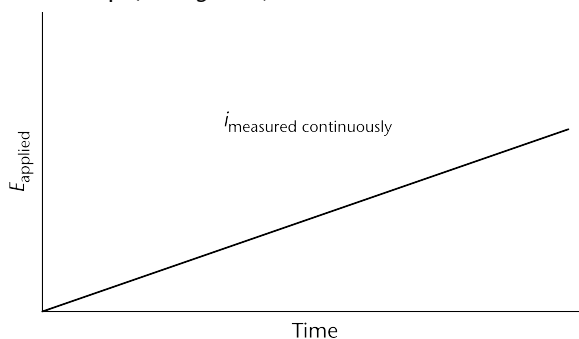


Fig. 2. Direct Current (dc) Polarography.

This current is composed of two elements. The first, the diffusion (faradaic) current, is produced by the substance undergoing reduction or oxidation at the working electrode, and is directly proportional to the concentration of this substance. The second element is the capacitive current

(charging of the electrochemical double layer). The changes in these currents as the mercury drop varies in size produce the oscillations present in typical dc polarograms.

In normal pulse polarography, a potential pulse is applied to the mercury electrode near the end of the drop life, with the drop being held at the initial potential during growth period (see Figure 3).

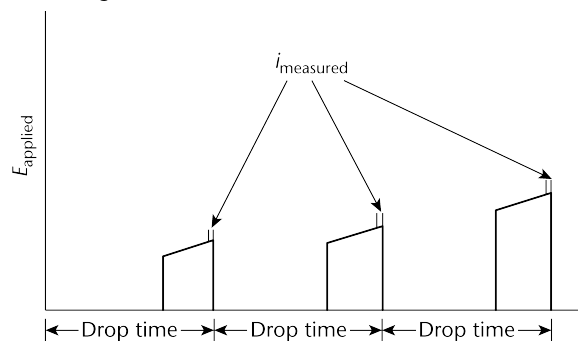


Fig. 3. Pulse Polarography.

Each succeeding drop has a slightly higher pulse applied to it, with the rate of increase being determined by the selected scan rate. The current is measured at the end of the pulse where the capacitive current is nearly zero, and thus primarily faradaic current is measured (see Figure 4).

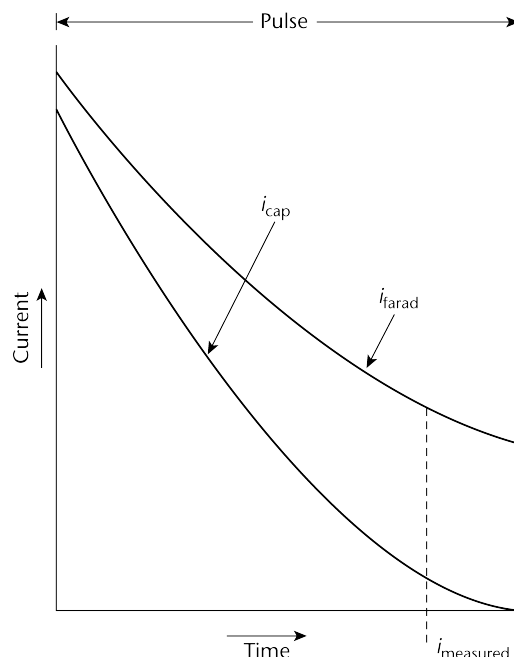


Fig. 4. Plot of Current Versus Time in Pulse Polarography.

In addition, since the pulse is applied for only a short duration, the diffusion layer is not depleted as extensively as in dc polarography and larger current levels are obtained for equivalent concentrations. Concentrations as low as 10^{-6} M can be measured, providing approximately a ten-fold increase in sensitivity over that with dc polarography. Limiting current values are more easily measured, since the waves are free from oscillations.

Differential pulse polarography is a technique whereby a fixed-height pulse applied at the end of the life of each drop is superimposed on a linear increasing dc ramp (see Figure 5).

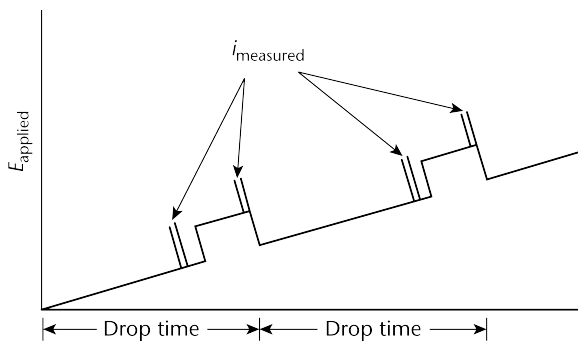


Fig. 5. Differential Pulse Polarography.

Current flow is measured just before application of the pulse and again at the end of the pulse. The difference between these two currents is measured and presented to the recorder. Such a differential signal provides a curve approximating the derivative of the polarographic wave, and gives a peak presentation. The peak potential is equivalent to:

$$E_{1/2} - \Delta E/2$$

where ΔE is the pulse height. The peak height is directly proportional to concentration at constant scan rates and constant pulse heights. This technique is especially sensitive (levels of $10^{-7}M$ may be determined) and affords improved resolution between closely spaced waves.

Anodic Stripping Voltammetry—Anodic stripping voltammetry is an electrochemical technique whereby trace amounts of substances in solution are concentrated (reduced) onto an electrode and then stripped (oxidized) back into solution by scanning the applied voltage anodically. The measurement of the current flow as a function of this voltage and scanning rate provides qualitative and quantitative information on such substances. The concentration step permits analyses at $10^{-7}M$ to $10^{-9}M$ levels.

Basic instrumentation includes a voltage ramp generator; current-measuring circuitry; a cell with working, reference, and counter electrodes; and a recorder or other read-out device. Instruments having dc or pulse-polarographic capabilities are generally quite adequate for stripping application. The working electrode commonly used is the hanging mercury drop electrode (HMDE), although the mercury thin-film electrode (MTFE) has acquired acceptance. For analysis of metals such as silver, platinum, and gold, whose oxidation potentials are more positive than mercury, and mercury itself, the use of solid electrodes such as platinum, gold, or carbon is required. A saturated calomel electrode or a silver-silver chloride electrode serves as the reference except for the analysis of mercury or silver. A platinum wire is commonly employed as the counter electrode.

Test specimens containing suitable electrolyte are pipeted into the cell. Dissolved oxygen is removed by bubbling nitrogen through the cell for 5 to 10 minutes.

Generally, an electrolysis potential equivalent to 200 to 300 mV more negative than the half-wave potential of the material to be analyzed is applied (although this potential is to be determined experimentally), with stirring for 1 to 10 minutes. For reproducible results, maintain constant conditions (i.e., deposition time, stirring rate, temperature, specimen volume, and drop size if HMDE is used).

After deposition, the stirring is discontinued and the solution and electrode are allowed to equilibrate for a short period. The potential is then rapidly scanned anodically (10 mV/second or greater in dc polarography and 5 mV/second in differential pulse polarography). As in polarography, the limiting current is proportional to concentration of the species (wave height in dc and pulse; peak height in differential pulse), while the half-wave potential (dc, pulse) or peak potential (differential pulse) identifies the

species. It is imperative that the choice of supporting electrolyte be made carefully in order to obtain satisfactory behavior. Quantitation is usually achieved by a standard addition or calibration method.

This technique is appropriate for trace-metal analysis, but has limited use in organic determinations, since many of these reactions are irreversible. In analyzing substances such as chloride, cathodic stripping voltammetry may be used. The technique is the same as anodic stripping voltammetry, except that the substance is deposited anodically and then stripped by a cathodic voltage scan.

(811) POWDER FINENESS

The particle size distribution should be estimated by *Particle Size Distribution Estimation by Analytical Sieving* (786) or by application of other methods where practical. A simple descriptive classification of powder fineness is provided in this chapter. For practical reasons, sieves are commonly used to measure powder fineness. Sieving is most suitable where a majority of the particles are larger than about 75 μm , although it can be used for some powders having smaller particle sizes where the method can be validated. Light diffraction is also a widely used technique for measuring the size of a wide range of particles.

Classification of Powder Fineness—Where the cumulative distribution has been determined by analytical sieving or by application of other methods, powder fineness may be classified in the following manner:

X_{90} = particle dimension corresponding to 90% of the cumulative undersize distribution

X_{50} = median particle dimension (i.e., 50% of the particles are smaller and 50% of the particles are larger)

X_{10} = particle dimension corresponding to 10% of the cumulative undersize distribution

It is recognized that the symbol d is also widely used to designate these values. Therefore, the symbols d_{90} , d_{50} , and d_{10} may be used.

The following parameters may be defined based on the cumulative distribution. $Q_R(x)$ = cumulative distribution of particles with a dimension less than or equal to x where the subscript R reflects the distribution type.

R	Distribution Type
0	Number
1	Length
2	Area
3	Volume

Therefore, by definition:

1. $Q_R(x) = 0.90$ when $x = X_{90}$

2. $Q_R(x) = 0.50$ when $x = X_{50}$

3. $Q_R(x) = 0.10$ when $x = X_{10}$

An alternative but less informative method of classifying powder fineness is by use of the terms in the following table.

Classification of Powders by Fineness

Descriptive Term	X_{50} (μm)	Cumulative Distribution by Volume Basis, $Q_3(x)$
Coarse	>355	$Q_3(355) < 0.50$
Moderately Fine	180–355	$Q_3(180) < 0.50$ and $Q_3(355) \geq 0.50$

Classification of Powders by Fineness (Continued)

Descriptive Term	X_{50} (μm)	Cumulative Distribution by Volume Basis, $Q_3(x)$
Fine	125–180	$Q_3(125) < 0.50$ and $Q_3(180) \geq 0.50$
Very Fine	≤ 125	$Q_3(125) \geq 0.50$

(821) RADIOACTIVITY

Radioactive pharmaceuticals require specialized techniques in their handling and testing in order that correct results may be obtained and hazards to personnel be minimized. All operations should be carried out or supervised by personnel having had expert training in handling radioactive materials.

The facilities for the production, use, and storage of radioactive pharmaceuticals are generally subject to licensing by the federal Nuclear Regulatory Commission, although in certain cases this authority has been delegated to state agencies. The federal Department of Transportation regulates the conditions of shipment of radioactive materials. State and local agencies often have additional special regulations. Each producer or user must be thoroughly cognizant of the applicable regulations of the federal Food, Drug, and Cosmetic Act, and any additional requirements of the U. S. Public Health Service and of state and local agencies pertaining to the articles concerned.

Definitions, special considerations, and procedures with respect to the Pharmacopeial monographs on radioactive drugs are set forth in this chapter.

GENERAL CONSIDERATIONS

Fundamental Decay Law

The decay of a radioactive source is described by the equation:

$$N_t = N_0 e^{-\lambda t}$$

in which N_t is the number of atoms of a radioactive substance at elapsed time t , N_0 is the number of those atoms when $t = 0$, and λ is the transformation or decay constant, which has a characteristic value for each radionuclide. The *half-life*, $T_{1/2}$, is the time interval required for a given activity of a radionuclide to decay to one-half of its initial value, and is related to the decay constant by the equation:

$$T_{1/2} = 0.69315/\lambda$$

The activity of a radioactive source (A) is related to the number of radioactive atoms present by the equation:

$$A = \lambda N$$

from which the number of radioactive atoms at time t can be computed, and hence the mass of the radioactive material can be determined.

The activity of a pure radioactive substance as a function of time can be obtained from the exponential equation or from decay tables, or by graphical means based on the half-life (see *Normalized Decay Chart*, Figure 1).

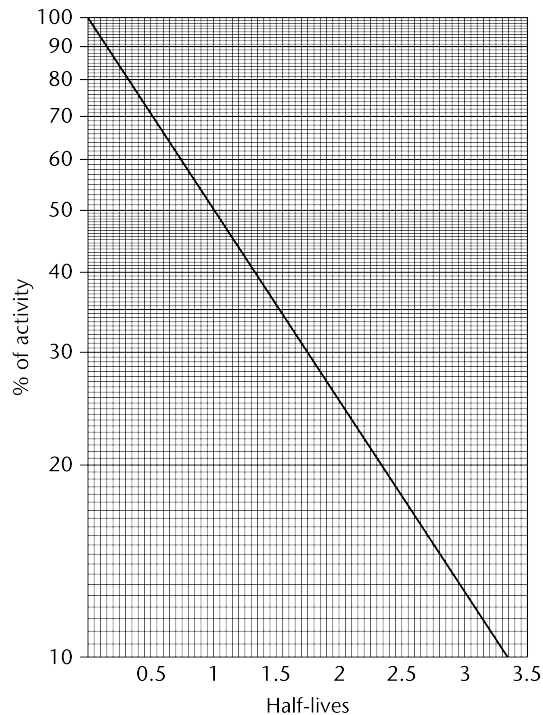


Fig. 1. Normalized Decay Chart.

The activity of a radioactive material is expressed as the number of nuclear transformations per unit time. The fundamental unit of radioactivity, the *curie* (Ci), is defined as 3.700×10^{10} nuclear transformations per second. The *millicurie* (mCi) and *microcurie* (μCi) are commonly used subunits. The “number of nuclear transformations per unit time” is the sum of rates of decay from all competing modes of disintegration of the parent nuclide. Before the activity of any given radionuclide in a measured specimen can be expressed in curies, it is often necessary to know the abundance(s) of the emitted radiation(s) measured.

Geometry

The validity of relative calibration and measurement of radionuclides is dependent upon the reproducibility of the relationship of the source to the detector and its surroundings. Appropriate allowance must be made for source configuration.

Background

Cosmic rays, radioactivity present in the detector and shielding materials, and radiation from nearby radioactive sources not properly shielded from the measuring equipment, all contribute to the background count rate. All radioactivity measurements must be corrected by subtracting the background count rate from the gross count rate in the test specimen.

Statistics of Counting

Since the process of radioactive decay is a random phenomenon, the events being counted form a random sequence in time. Therefore, counting for any finite time can yield only an estimate of the true counting rate. The precision of this estimate, being subject to statistical fluctuations, is dependent upon the number of counts accumulated in a

given measurement and can be expressed in terms of the standard deviation σ . An *estimate* for σ is

$$\sqrt{n}$$

where n is the number of counts accumulated in a given measurement. The probability of a single measurement falling within

$$\pm 100/\sqrt{n}\%$$

of the mean of a great many measurements is 0.68. That is, if many measurements of n counts each were to be made, approximately two-thirds of the observations would lie within

$$\pm 100/\sqrt{n}\%$$

of the mean, and the remainder outside.

Because of the statistical nature of radioactive decay, repeated counting of an undisturbed source in a counting assembly will yield count-rate values in accordance with the frequency of a normal distribution. Deviations in these values from the normal distribution conform to the χ^2 test. For this reason, the χ^2 test is frequently applied to determine the performance and correct operation of a counting assembly. In the selection of instruments and conditions for assay of radioactive sources, the figure of merit ε^2/B should be maximized (where ε = counter efficiency = observed count rate/sample disintegration rate, and B = background count rate).

Counting Losses

The minimum time interval that is required for the counter to resolve two consecutive signal pulses is known as the dead time. The dead time varies typically from the order of microseconds for proportional and scintillation counters, to hundreds of microseconds for Geiger-Müller counters. Nuclear events occurring within the dead time of the counter will not be registered. To obtain the corrected count rate, R , from the observed count rate, r , it is necessary to use the formula:

$$R = r/(1 - r\tau)$$

in which τ is the dead time. The foregoing correction formula assumes a nonextendable dead time. Thus, for general validity, the value of $r\tau$ should not exceed 0.1. The observed count rate, r , refers to the gross specimen count rate and is not to be corrected for background before use in the foregoing equation.

Calibration Standards

Perform all radioactivity assays using measurement systems calibrated with appropriately certified radioactivity standards. Such calibration standards may be purchased either direct from the National Institute of Standards and Technology or from other sources that have established traceability to the National Institute of Standards and Technology through participation in a program of inter-comparative measurements. Where such calibration standards are unavailable, the Pharmacopeia provides the nuclear decay data required for calibration. These data, as well as half-life values, are obtained from the Evaluated Nuclear Structure Data File of the Oak Ridge Nuclear Data Project, and reflect the most recent values at the time of publication.

Carrier

The total mass of radioactive atoms or molecules in any given radioactive source is directly proportional to the activ-

ity of the radionuclide for a given half-life, and the amount present in radiopharmaceuticals is usually too small to be measured by ordinary chemical or physical methods. For example, the mass of ^{131}I having an activity of 100 mCi is 8×10^{-7} g. Since such small amounts of material behave chemically in an anomalous manner, carriers in the form of nonradioactive isotopes of the same radionuclide may be added during processing to permit ready handling. In many cases, adsorption can be prevented merely by increasing the hydrogen-ion concentration of the solution. Amounts of such material, however, must be sufficiently small that undesirable physiological effects are not produced. The term "carrier-free" refers only to radioactive preparations in which nonradioactive isotopes of the radionuclide are absent. This implies that radioactive pharmaceuticals produced by means of (n, γ) reactions cannot be considered carrier-free.

The activity per unit volume or weight of a medium or vehicle containing a radionuclide either in the carrier-free state or in the presence of carrier is referred to as the radioactive concentration, whereas the term specific activity is used to express the activity of a radionuclide per gram of its element.

Radiochemical Purity

Radiochemical purity of a radiopharmaceutical preparation refers to the fraction of the stated radionuclide present in the stated chemical form. Radiochemical impurities in radiopharmaceuticals may result from decomposition and from improper preparative procedures. Radiation causes decomposition of water, a main ingredient of most radiopharmaceuticals, leading to the production of reactive hydrogen atoms and hydroxyl radicals, hydrated electrons, hydrogen, hydrogen ions, and hydrogen peroxide. The last-mentioned is formed in the presence of oxygen radicals, originating from the radiolytic decomposition of dissolved oxygen. Many radiopharmaceuticals show improved stability if oxygen is excluded. Radiation may also affect the radiopharmaceutical itself, giving rise to ions, radicals, and excited states. These species may combine with one another and/or with the active species formed from water. Radiation decomposition may be minimized by the use of chemical agents that act as electron or radical scavengers. Electrons trapped in solids cause discoloration due to formation of F-centers and the darkening of glass containers for radiopharmaceuticals, a situation that typifies the case. The radiochemical purity of radiopharmaceuticals is determined by column, paper, and thin-layer chromatography or other suitable analytical separation techniques as specified in the individual monograph.

Radionuclidic Purity

Radionuclidic purity of a radiopharmaceutical preparation refers to the proportion of radioactivity due to the desired radionuclide in the total radioactivity measured. Radionuclidic purity is important in the estimation of the radiation dose received by the patient when the preparation is administered. Radionuclidic impurities may arise from impurities in the target materials, differences in the values of various competing production cross-sections, and excitation functions at the energy or energies of the bombarding particles during production.

Terms and Definitions

The *date of manufacture* is the date on which the manufacturing cycle for the finished product is completed.

The *date of assay* is the date (and time, if appropriate) when the actual assay for radioactivity is performed.

The *date of calibration* is an arbitrary assigned date and time to which the radioactivity of the product is calculated for the convenience of the user.

The *expiration date* is the date that establishes a limit for the use of the product. The expiration period (i.e., the period of time between the date of manufacture and the expiration date) is based on a knowledge of the radioactive properties of the product and the results of stability studies on the finished dosage form.

Labeling

Individual radiopharmaceutical monographs indicate the expiration date, the calibration date, and the statement, "Caution—Radioactive Material." The labeling indicates that in making dosage calculations, correction is to be made for radioactive decay, and also indicates the radioactive half-life of the radionuclide. Articles that are Injections comply with the requirements for *Labeling* under *Injections* (1), and those that are Biologics comply with the requirements for *Labeling* under *Biologics* (1041).

IDENTIFICATION AND ASSAY OF RADIONUCLIDES

Instrumentation

IONIZATION CHAMBERS

An ionization chamber is an instrument in which an electric field is applied across a volume of gas for the purpose of collecting ions produced by a radiation field. The positive ions and negative electrons drift along the lines of force of the electric field, and are collected on electrodes, producing an ionization current. In a properly designed well-type ionization chamber, the ionization current should not be too dependent on the position of the radioactive specimen, and the value of the current per unit activity, known as the calibration factor, is characteristic of each gamma-ray-emitting radionuclide.

The ionization current produced in an ionization chamber is related to the mean energy of the emitted radiation and is proportional to the intensity of the radiation. If standard sources of known disintegration rates are used for efficiency calibration, the ionization chamber may then be used for activity determinations between several microcuries and several hundred millicuries or more. The upper limit of activity that may be measured in an ionization chamber usually is not sharply defined and may be limited by saturation considerations, range of the amplifier, and design of the chamber itself. The data supplied with or obtained from a particular instrument should be reviewed to ascertain the useful ranges of energies and intensities of the device.

Reproducibility within approximately 5% or less can be readily obtained in about 10 seconds, with a deep re-entrant well-type chamber. The most commonly used form of ionization chamber for measurement of the activities of radiopharmaceuticals is known as a dose calibrator.

Although the calibration factor for a radionuclide may be interpolated from an ionization chamber energy-response curve, there are a number of sources of error possible in such a procedure. It is therefore recommended that all ionization chamber calibrations be performed with the use of authentic reference sources of the individual radionuclides, as described hereinafter.

The calibration of a dose calibrator should be maintained by relating the measured response of a standard to that of a long-lived performance standard, such as radium 226 in equilibrium with its daughters. The instrument must be checked daily with the ^{226}Ra or other source to ascertain the stability over a long period of time. This check should include performance standard readings at all radionuclide set-

tings employed. To obtain the activity (A_x) of the radionuclide being measured, use the relationship:

$$A_x = R_x R / R_n$$

in which R_n is the new reading for the radium or other source, R_c is the reading for the same source obtained during the initial calibration procedure, and R is the observed reading for the radionuclide specimen. Obviously, any necessary corrections for radioactive decay of the reference source must first be applied. Use of this procedure should minimize any effects due to drift in the response of the instrument. The recommended activity of the ^{226}Ra or other monitor used in the procedure described above is 75 to 150 μCi . It is recommended also that the reproducibility and/or stability of multirange instruments be checked for all ranges with the use of appropriate standards.

The size and shape of a radioactive source may affect the response of a dose calibrator, and it is often necessary to apply a small correction when measuring a bulky specimen.

SCINTILLATION and SEMICONDUCTOR DETECTORS

When all or part of the energy of beta or gamma radiation is dissipated within scintillators, photons of intensity proportional to the amount of dissipated energy are produced. These pulses are detected by an electron multiplier phototube and converted to electrical pulses, which are subsequently analyzed with a pulse-height analyzer to yield a pulse-height spectrum related to the energy spectrum of the radiation emitted by the source. In general, a beta-particle scintillation pulse-height spectrum approximates the true beta-energy spectrum, provided that the beta-particle source is prepared in such a manner that self-absorption is minimized. Beta-ray spectra may be obtained by using calcium fluoride or anthracene as the scintillator, whereas gamma-ray spectra are usually obtained with a thallium-activated sodium iodide crystal or a large-volume lithium-drifted germanium semiconductor detector. The spectra of charged particles also may be obtained using silicon semiconductor detectors and/or gas proportional counters. Semiconductor detectors are in essence solid-state ionization chambers, but the energy required to create an electron-hole pair or to promote an electron from the valence band to the conduction band in the semiconductor is about one-tenth the energy required for creation of an ion-pair in a gas-filled ionization chamber or proportional counter and is far less than the energy needed to produce a photon in a NaI(Tl) scintillation crystal. In gamma-ray spectrometry, a Ge(Li) detector can yield an energy resolution of 0.33% for 1.33 MeV gamma-rays from ^{60}Co , while a 3×3 -inch NaI(Tl) crystal can give a value of 5.9% for the same gamma-ray energy. The energy resolution is a measure of the ability to distinguish the presence of two gamma rays closely spaced in energy and is defined by convention as the full width of the photopeak at its half maximum (FWHM), expressed in percentage of the photopeak energy.

Gamma-ray spectra exhibit one or more sharp, characteristic photopeaks, or full-energy peaks, as a result of total absorption in the detector of the full energy of gamma radiations from the source; these photopeaks are useful for identification purposes. Other secondary peaks are observed as a consequence of backscatter, annihilation radiation, coincidence summing, fluorescent X-rays, etc., accompanied by a broad band known as the Compton continuum arising from scattering of the photons in the detector and from surrounding materials. Since the photopeak response varies with gamma-ray energy, calibration of a gamma-ray spectrometer should be achieved with radionuclide standards having well-known gamma-ray energies and emission rates. The shape of the gamma-ray spectrum is dependent upon the shape and size of the detector and the types of shielding materials used.

When confirming the identity of a radionuclide by gamma-ray spectrometry, it is necessary to make a comparison of the specimen spectrum with that of a specimen of known purity of the same radionuclide obtained under *identical instrument parameters and specimen geometry*. Where the radionuclides emit coincident X- or gamma-radiations, the character of the pulse-height distribution often changes quite dramatically because of the summing effect of these coincident radiations in the detector as the efficiency of detection is increased (e.g., by bringing the source closer to the detector). Such an effect is particularly evident in the case of iodine 125. Among the more useful applications of gamma-ray spectrometry are those for the identification of radionuclides and the determination of radionuclidic impurities.

Where confirmation of the identity of a given radionuclide by means of a direct comparison with the spectrum of a specimen of the same radionuclide of known purity is not possible, the identity of the radionuclide in question must then be established by the following method. Two or more of the following nuclear decay scheme parameters of the radionuclide specimen to be identified shall be measured, and agreement shall be within $\pm 10\%$: (1) half-life, (2) energy of each gamma- or X-ray emitted, (3) the abundance of each emission, and (4) E_{\max} for those radionuclides that decay with beta-particle emissions. Such measurements are to be performed as directed in the *Identification and Assay* sections of this chapter. Agreement of two or more of the measured parameters with the corresponding published nuclear decay scheme data constitutes confirmation of the identity of the radionuclide.

LIQUID-SCINTILLATION COUNTERS

Alpha- and beta-emitting radionuclides may be assayed with the use of a liquid-scintillation detector system. In the liquid scintillator, the radiation energy is ultimately converted into light quanta that are usually detected by two multiplier phototubes so arranged as to count only coincidence radiation. The liquid scintillator is a solution consisting of a solvent, primary and secondary solutes, and additives. The charged particle dissipates its energy in the solvent, and a fraction of this energy is converted into fluorescence in the primary solute. The function of the secondary solute is to shift the fluorescence radiation to longer wavelengths that are more efficiently detected by the multiplier phototubes. Frequently used solvents are toluene and *p*-xylene; primary solutes are 2,5-diphenyloxazole (PPO) and 2-(4'-*tert*-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole (butyl-PBD); and secondary solutes are 2,2'-*p*-phenylenebis[4-methyl-5-phenyloxazole] (dimethyl-POPOP) and *p*-bis(*o*-methylstyryl)benzene (bis-MSB). As a means of attaining compatibility and miscibility with aqueous specimens to be assayed, many additives, such as surfactants and solubilizing agents, are also incorporated into the scintillator. For an accurate determination of radioactivity of the specimen, care must be exercised to prepare a specimen that is truly homogeneous. The presence of impurities or color in solution causes a decrease in photon output of the scintillator; such a decrease is known as quenching. Accurate radioactivity measurement requires correcting for count-rate loss due to quenching.

The disintegration rate of a beta-particle source may be determined by a procedure in which the integral count rate of the specimen is measured as a function of the pulse-height discriminator bias, and the emission rate is then obtained by extrapolation to zero bias. Energetic alpha-particle emitters may be similarly measured by this method.

Identification

A radionuclide can be identified by its mode of decay, its half-life, and the energies of its nuclear emissions.

The radioactive half-life is readily determined by successive counting of a given source of the radionuclide over a period of time that is long compared to its half-life. The response of the counting assembly when employed for the decay measurement of long-lived radionuclides should be monitored with an even longer-lived reference source to assess and compensate for errors arising from electronic drift. In the case of short-lived radionuclides, when the counting period constitutes a significant fraction of the half-life of the radionuclide, the recorded count rate must be corrected to the time when the count is initiated, as follows:

$$R_t = r\lambda t / (1 - e^{-\lambda t})$$

in which R_t is the count rate at the beginning of a counting period, r is the count rate observed over the entire counting period, t is the duration of the counting period, λ is the decay constant of the radionuclide, and e is the base of the natural logarithm. When t is small compared to the half-life of the radionuclide under study so that $\lambda t < 0.05$, then $(1 - e^{-\lambda t})$ approaches λt , and no such correction is necessary.

The energy of nuclear emissions is often determined by the maximum range of penetration of the radiation in matter (in the case of alpha- and beta-particles) and by the full-energy peak or photopeak in the gamma-ray spectrum (in the case of X- and gamma-rays). Since beta-particles are emitted with a continuous energy spectrum, the maximum beta-energy, E_{\max} , is a unique index for each beta-emitting radionuclide. In addition to the maximum range and energy spectrum of the beta-particles, the absorption coefficient, when obtained under reproducible counting conditions, can serve as a reliable index for identification of a beta-emitter. Fortuitously, beta-particles are absorbed in matter in an approximately exponential manner, and a plot of the logarithm of the beta-particle count rate as a function of the absorber thickness is known as the absorption curve. The initial portion of the absorption curve shows linearity from which the absorption coefficient can be obtained. The maximum range is determined by the use of absorbers of varying thickness, and the energy spectrum is measured by beta-ray scintillation spectrometry.

The absorption of gamma-rays in matter is strictly exponential, but the half-value layers of attenuation have not been very useful for the purpose of radionuclide characterization. Gamma-rays from each isomeric transition are monoenergetic; their energy can be directly measured by gamma-ray spectrometry. Because of their high energy resolution, solid-state detectors [Ge(Li)] are vastly superior to scintillation detectors [NaI(Tl)] in gamma-ray spectrometry.

The activities of radiopharmaceutical solutions are frequently in the range of millicuries per mL. Such solutions usually must be extensively diluted before they can be accurately assayed. The diluent should be compatible with the radiopharmaceutical with respect to factors such as pH and redox potentials, so that no hydrolysis or change in oxidation state occurs upon dilution, which could lead to adsorption and separation of the radionuclide from solution.

BETA-EMITTING RADIONUCLIDES

Mass Absorption Coefficient Procedure—Deposit and dry an aliquot of the radioactive phosphorus 32 solution on a thin plastic film to minimize backscattering, and place it under a suitable counter. Determine the counting rates successively, using not less than six different "thicknesses" of aluminum each between 20 and 50 mg/cm² and a single absorber thicker than 800 mg/cm², which is used to measure the background. (The absorbers are inserted between the test specimen and the counter but are placed nearer the counter window to minimize scattering.) Net beta-particle count rates are obtained after subtraction of the count rate found with the absorber having a thickness of 800 mg/cm² or greater. Plot the logarithm of the net beta-particle count

rate as a function of the total absorber "thickness." The total absorber "thickness" is the "thickness" of the aluminum absorbers plus the "thickness" of the counter window (as stated by the manufacturer) plus the air-equivalent "thickness" (the distance in centimeters of the specimen from the counter window multiplied by 1.205 mg/cm³ at 20° and 76 cm of mercury), all expressed in mg/cm². An approximately straight line results.

Choose two total absorber "thicknesses" that differ by 20 mg/cm² or more and that fall on the linear plot, and calculate the mass absorption coefficient, μ , by the equation:

$$\mu = 1/(t_2 - t_1) \cdot \ln(N_{t1}/N_{t2}) = (2.303/(t_2 - t_1)) \times (\log N_{t1} - \log N_{t2})$$

in which t_1 and t_2 represent the total absorber "thicknesses," in mg/cm², t_2 being the thicker absorber, and N_{t1} and N_{t2} being the net beta-particle rates with the t_1 and t_2 absorbers, respectively.

For characterization of the radionuclide, the mass absorption coefficient should be within $\pm 5\%$ of the value found for a pure specimen of the same radionuclide when determined under identical counting conditions and geometry.

Other Methods of Identification—Other methods for determining the identity of a beta emitter also rely upon the determination of E_{\max} . This may be accomplished in several ways. For example, (1) utilization of the range energy relationships of beta particles in an absorber, or (2) determination of E_{\max} from a beta-particle spectrum obtained on an energy-calibrated beta-spectrometer using a thin source of the radionuclide (see *Scintillation and Semiconductor Detectors* in this chapter).

GAMMA-EMITTING RADIONUCLIDES

The gamma-ray spectrum of a radionuclide is a valuable tool for the qualitative identification of gamma-ray emitting radionuclides. The full-energy peak, or the photopeak, is identified with the gamma-ray transition energy that is given in the decay scheme of the radionuclide.

In determining radionuclidic identity and purity, the gamma-ray spectrum of a radioactive substance is obtained with either a NaI(Tl) crystal or a semiconductor Ge(Li) detector. The latter has an energy resolution more than an order of magnitude better than the former and is highly preferred for analytical purposes. The spectrum obtained shall be identical in shape to that of a specimen of the pure radionuclide, measured with the same detection system and in the same geometry. The gamma-ray spectrum of the radiopharmaceutical shall contain only photopeaks identifiable with the gamma-ray transition energies found in the decay scheme of the same radionuclide. For low geometrical efficiencies, the areas under the photopeaks, after correction for the measured detector efficiency, shall be proportional to the abundances or emission rates of the respective gamma-rays in the radionuclide.

RADIONUCLIDIC IMPURITIES

Because they are extremely toxic, alpha-emitting nuclides must be strictly limited in radiopharmaceutical preparations. Procedures for identifying beta- and gamma-active radionuclides as given in the foregoing text are applicable to the detection of gamma and usually beta contaminants.

The gross alpha-particle activity in radiopharmaceutical preparations can be measured by the use of a windowless proportional counter or a scintillation detector employing a silver-activated zinc-sulfide phosphor or by the techniques of liquid-scintillation counting.

The heavy ionization caused by alpha particles allows the measurement of alpha-emitting radionuclides in the presence of large quantities of beta- and gamma-active nuclides

by the use of appropriate techniques for discriminating the amplitudes of signal pulses. In proportional counting, the operating voltage region for counting alpha particles, referred to as the "alpha plateau," is considerably lower than the "beta plateau" for counting beta and gamma radiations. Typical "alpha plateau" and "beta plateau" voltage settings with P-10 counting gas are 900 to 1300 and 1600 to 2000 volts, respectively.

When silver-activated zinc-sulfide phosphor is employed for alpha-particle detection, the alpha particles can be distinguished from other interfering radiation by pulse-height discrimination. Care must be exercised to minimize self-absorption at the source whenever specimens are prepared for alpha-particle counting.

Assay

BETA-EMITTING RADIONUCLIDES

Procedure—The disintegration rate (A) of a beta-particle-emitting specimen is obtained by counting a quantitatively deposited aliquot in a fixed geometry according to the formula:

$$A = R/(\epsilon \times f_r \times f_b \times f_s)$$

in which ϵ is the counting efficiency of the counter; f_r is the correction factor for counter dead time; f_b is the correction factor for backscatter; and f_s is the correction factor for self-absorption. The count rate for zero absorber is obtained by extrapolation of the initial linear portion of the absorption curve to zero absorber "thickness," taking into consideration the mg/cm² "thickness" of specimen coverings, counter window, and the intervening air space between specimen and the counter window. The counter efficiency, ϵ , is determined by use of a long-lived secondary standard with similar spectral characteristics. RaD + E has frequently been used for efficiency calibration of counters for phosphorus 32. By the use of identical measurement conditions for the specimen and the standard (and extrapolation to zero absorber), the ratio of the values of f_r , f_b , and f_s for the standard and the specimen approaches unity.

The previous relationship is valid also when the counter has been calibrated with a standard of the radionuclide to be assayed. In this case, however, the extrapolations to zero absorber "thickness" for the specimen and standard are not required, as the two absorption corrections cancel for a given geometry.

Another useful and frequently employed method for the determination of the disintegration rate of beta-emitting radionuclides is liquid-scintillation counting, which also utilizes an extrapolation of the specimen count rate to zero pulse-height discriminator bias.

GAMMA-EMITTING RADIONUCLIDES

For the assay of gamma-emitting radionuclides, three methods are provided. The selection of the preferred method is dictated by the availability of a calibration standard of the radionuclide to be assayed and the radionuclidic purity of the article itself.

Direct comparison with a calibration standard is required if a calibration standard of the radionuclide to be assayed is available and if the upper limit of conceivable error in the activity determination arising from the presence of radionuclidic impurities has been determined to be less than 3%. If the required calibration standard is not routinely available, as would probably be the case for a short-lived radionuclide, but was available at some time prior to the performance of the assay for determination of efficiency of the counting system for the radionuclide to be assayed, use a calibrated

counting system, provided the radionuclidic impurity content of the specimen meets the requirements stated for the direct comparison method. If the requirements for either of the first two methods cannot be met, use the method for determination of activity from a calibration curve.

With the exception of the first method, the counting systems used are monitored for stability. This requirement is met by daily checks with a long-lived performance check source and weekly checks with at least three sources covering a broad range of gamma-ray emission energies (e.g., ^{57}Co , ^{137}Cs , and ^{60}Co). If a discrepancy for any of the aforementioned measurements is found, either completely recalibrate or repair and recalibrate the system prior to further use.

Assay by Direct Comparison with a Calibration

Standard—An energy selective measurement system (e.g., pulse-height analyzer) is not required for this procedure. Use either an ionization chamber or an integral counting system with a NaI(Tl) detector. A consistently reproducible geometrical factor from specimen to specimen is essential for accurate results. With proper precautions, the accuracy of this method approaches the accuracy with which the disintegration rate of the calibration standard is known.

Determine the counting rate of the detector system for a calibration standard of the radionuclide to be assayed (e.g., active enough to give good measurement statistics in a reasonable time, but not so active as to cause serious dead-time problems), selecting such a standard as to provide optimum accuracy with the particular assembly used. Place an accurately measured aliquot of the unknown assay specimen (diluted, if necessary) in a container identical to that used for the standard, and measure this specimen at approximately the same time and under the same geometrical conditions as for the standard. If the elapsed time between the measurements of the calibration standard and the specimen exceeds 12 hours, check the stability of the measurement system within 8 hours of the specimen measurement time with a long-lived performance check source. Record the system response with respect to the same check source at the time of calibration, and if subsequent checks exceed the original recorded response by more than $\pm 3\%$, recalibration is required. Correct both activity determinations for background, and calculate the activity, in μCi per mL, by the formula:

$$SD(g/b)$$

in which S is the μCi strength of the standard, D is the dilution factor, and g and b are the measured values of counting rate for the specimen and the standard, respectively.

Assay with a Calibrated Integral Counting System—

The procedure and precautions given for the preceding direct-comparison method apply, except that the efficiency of the detector system is determined and recorded for each radionuclide to be assayed, rather than simply recording the counting rate of the standard. Thus, the efficiency for a given radionuclide, x , is determined by $\epsilon_x = b_x/s_x$, in which b_x is the counting rate, corrected for background and dead-time, for the calibration standard of the radionuclide, x , and s_x is the corresponding activity of the certified calibration standard in nuclear transformations per second. For subsequent specimen assays, the activity is given by the formula:

$$A_x = Dg_x/\epsilon_x$$

in which D is the dilution factor, g_x is the specimen counting rate (corrected for background and dead-time), and ϵ_x is the corresponding efficiency for the radionuclide.

Determination of Activity from a Calibration Curve—

Versatility in absolute gamma-ray intensity measurements can be achieved by employing multi-channel pulse-height analysis. The photopeak efficiency of a detector system can be determined as a function of gamma-ray energy by

means of a series of gamma-ray emission rate standard specimens, and the gamma-ray emission rate of any radionuclide for which no standard is available can be determined by interpolation from this efficiency curve. However, exercise care to ensure that the efficiency curve for the detector system is adequately defined over the entire region of interest by using a sufficient number of calibration points along the photopeak-energy axis.

Selection of a Counting Assembly—A gamma-ray spectrometer is used for the identification of radionuclides that emit X-rays or gamma rays in their decay. Requirements for an assembly suitable for identification and assay of the radionuclides used in radiopharmaceuticals are that (a) the resolution of the detector based on the 662-keV photopeak of ^{137}Cs - ^{137m}Ba must be 8.0% or better, (b) the detector must be equipped with a specimen holder designed to facilitate exact duplication of counting geometry, and (c) the pulse-height analyzer must have enough channels to delineate clearly the photopeak being observed.

Procedure—Minimal requirements for the maintenance of instrument calibrations shall consist of weekly performance checks with a suitable reference source and a complete recalibration semi-annually. Should the weekly performance check deviate from the value determined at the time of calibration by more than 4.0%, a complete recalibration of the instrument is required at that time.

This method involves three basic steps, namely photopeak integration, determination of the photopeak efficiency curve, and calculation of the activity of the specimen.

PHOTOPEAK INTEGRATION—The method for the determination of the required photopeak area utilizes a Gaussian approximation for fitting the photopeak. A fixed fraction of the total number of photopeak counts can be obtained by taking the peak width, a , at some fraction of the maximum, where the shape has been experimentally found to be very close to Gaussian, and multiplying by the counting rate of the peak channel, P , after correction for any Compton and background contributions to the peak channel count rate. This background usually can be adequately determined by linear interpolation. This is illustrated in Figure 2.

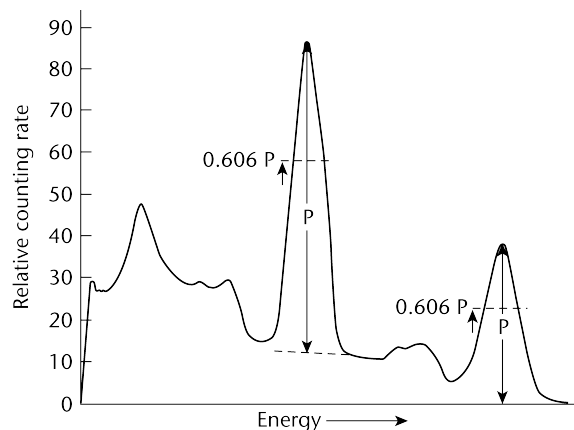


Fig. 2. Typical Gamma-ray Spectrum Showing the Selection of the Peak Channel Counting Rate, P , after the Correction for Compton and Background Contributions.

The photopeak-curve shape is closest to a straight line at $0.606P$, and the contribution of the fractional channels to a can be accurately estimated by interpolation. Calculate a by the equation:

$$a = D' - D + [(d - 0.606P)/(d - c)] + [(d' - 0.606P)/(d' - c)]$$

in which c and d and also c' and d' are the single channel counting rates on either side of $0.606P$, and D and D' are the channel numbers (locations) of d and d' , respectively.

The location of the required variables on the photopeak is illustrated in Figure 3.

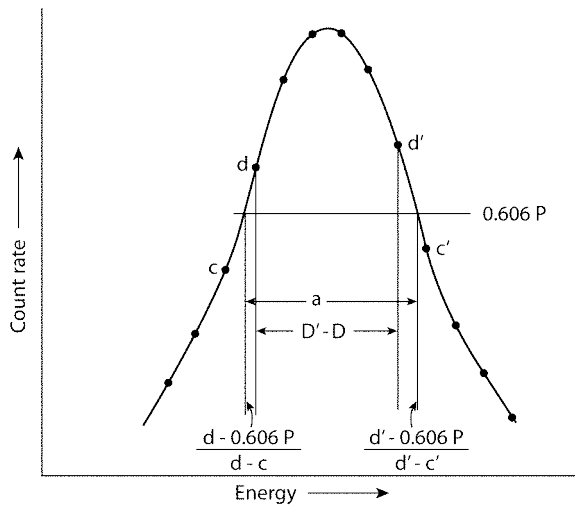


Fig. 3. Location of the Variables Required for the Determination of the Peak Width, a , at $0.606P$.

From the known values for the counting rate in the peak channel of the photopeak, P , and the width of the peak at $0.606P$, a , a calibrated fraction of the photopeak area is then obtained from the product, (aP) .

To summarize the procedures involved in obtaining a calibrated fraction of a photopeak area using this method, the necessary steps or calculations are presented below in a stepwise manner:

(1) Subtract any Compton and background contributions from the photopeak to be measured.

(2) Determine the counting rate of the peak channel (maximum channel counting rate after subtracting Compton and background), P .

(3) Multiply P by 0.606 , and locate the horizontal line corresponding to the peak width, a .

(4) Obtain the peak width, a , by inserting the values of variables (obtained as shown in the preceding figure) into the equation defining a .

(5) The desired calibrated fraction of the peak area is then equal to the product of a times P or $F = aP$, where F is a fractional area of the peak proportional to the emission rate of the source.

This method provides a quick and accurate means of determining the gamma-ray emission rate of sources while avoiding, to a large extent, subjective estimates of the detailed shape of the tails of the peaks. The error due to using the maximum channel counting rate, rather than the theoretical maximum or peak channel rate, is of the order of 1.0% if a is 6 or greater.

PHOTOPEAK EFFICIENCY CALIBRATION—Radionuclides such as those listed in the accompanying table together with some of their nuclear decay data are available as certified reference standards.* A sufficient number of radioactive standard reference sources should be selected in order to obtain the calibration curve over the desired range. Where possible, standard sources of those radionuclides that are to be assayed should be included.

* These certified reference standards are obtainable from the National Institute of Standards and Technology, Washington, DC 20234.

Nuclear Properties of Selected Calibration Standards^(1,2)

Principal Photon Emissions	Energy (ke V)	Photons per 100 Disintegrations
¹³³ Ba ($T_{1/2} = 10.5$ years)		
$K_{\alpha 1}$	30.97	63.4
$K_{\alpha 2}$	30.62	34.2
K_{β}	35.0	22.8
γ_1	53.15	2.14
γ_2	79.62	2.55
γ_3	80.99	33.0
γ_6	276.39	6.9
γ_7	302.83	17.8
γ_8	356.0	60.0
γ_9	383.85	8.7
¹³⁷ Cs— ^{137m} Ba ($T_{1/2} = 30.17$ years)		
$K_{\alpha 1}$	32.19	3.82
$K_{\alpha 2}$	31.82	2.07
K_{β}	36.4	1.39
Weighted Mean ⁽⁴⁾	(32.9)	(7.28)
γ_1	661.6	89.98
²² Na ($T_{1/2} = 2.60$ years)		
h ν	511	179.80 ⁽⁵⁾
γ_1	1274.54	99.94
⁶⁰ Co ($T_{1/2} = 5.27$ years)		
γ_1	1173.2 ⁽⁶⁾	100.0
γ_2	1332.5 ⁽⁶⁾	100.0
⁵⁷ Co ($T_{1/2} = 270.9$ days)		
ΣX_K	7.0	56.0
γ_1	14.4	9.5
γ_2	122.06	85.51
γ_3	136.47	10.60
Weighted Mean	(125.0)	(96.11)
$(\gamma_2 + \gamma_3)^{(4)}$		
⁵⁴ Mn ($T_{1/2} = 312.7$ days)		
ΣX_K	6.0	25.0
γ_1	834.83	99.98
¹⁰⁹ Cd— ^{109m} Ag ($T_{1/2} = 464$ days)		
$K_{\alpha 1}$	22.16	35.3
$K_{\alpha 2}$	21.99	18.6
K_{β}	24.9	11.4
Weighted Mean ⁽⁴⁾		63.5
γ_1	88.0	3.72
¹²⁹ I ($T_{1/2} = 1.57 \times 10^7$ years)		
$K_{\alpha 1}^{(3)}$	29.78	37.0
$K_{\alpha 2}$	29.46	20.0
K_{β}	13.2	37.0

(1) In measurements for gamma- (or X-)ray assay purposes, fluorescent radiation from lead shielding (specifically, lead K X-rays ~76 ke V) may interfere with quantitative results. Allowance must be made for these effects, or the radiation suppressed; a satisfactory means of absorbing this radiation is covering the exposed lead with cadmium sheet 0.06 to 0.08 inch thick, and then covering the cadmium with copper 0.02 to 0.04 inch thick.

(2) Only those photon emissions having an abundance $\geq 1\%$ are normally included.

(3) The K notation refers to X-ray emissions.

(4) The weighted mean energies and total intensities are given for groups of photons that would not be resolved by a NaI(Tl) detector.

(5) For this photon intensity to be usable, all emitted positrons must be annihilated in the source material.

(6) Cascade.

Nuclear Properties of Selected Calibration Standards ^(1,2) (Continued)		
Principal Photon Emissions	Energy (ke V)	Photons per 100 Disintegrations
γ_1	39.58	7.52
Weighted Mean ⁽⁴⁾	(31.3)	(77.80)

- (1) In measurements for gamma- (or X-)ray assay purposes, fluorescent radiation from lead shielding (specifically, lead K X-rays ~76 ke V) may interfere with quantitative results. Allowance must be made for these effects, or the radiation suppressed; a satisfactory means of absorbing this radiation is covering the exposed lead with cadmium sheet 0.06 to 0.08 inch thick, and then covering the cadmium with copper 0.02 to 0.04 inch thick.
- (2) Only those photon emissions having an abundance $\geq 1\%$ are normally included.
- (3) The K notation refers to X-ray emissions.
- (4) The weighted mean energies and total intensities are given for groups of photons that would not be resolved by a NaI(Tl) detector.
- (5) For this photon intensity to be usable, all emitted positrons must be annihilated in the source material.
- (6) Cascade.

Calculate the gamma-ray emission rate from the equation:

$$\Gamma = A_s b$$

in which A_s is the activity, in disintegrations per second, of the standard used, and b is the number of gamma rays per disintegration at that energy. Accurately measure quantities of standard solutions of each radionuclide into identical containers, and determine the fractional photopeak area (F) for each of the standards.

Using the equation $\epsilon_p = F/\Gamma$, calculate the photopeak efficiency, ϵ_p , and construct a log-log plot of ϵ_p versus the gamma-ray energy as shown in Figure 4.

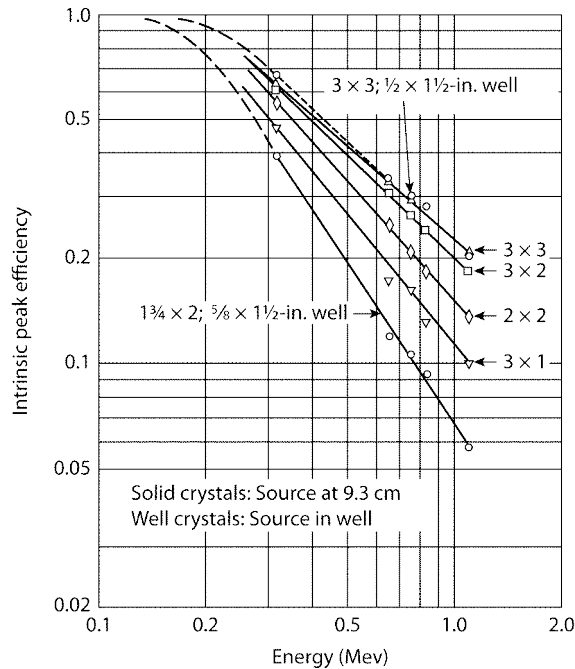


Fig. 4. Typical Photopeak Efficiency Calibration Curves for Various NaI(Tl) Detectors.

DETERMINATION OF SPECIMEN ACTIVITY—In the same manner as in the preparation of the calibration curve, determine the fractional area (F) of the principal photopeak of the specimen under assay or an accurately measured aliquot adjusted

to the same volume in an identical container as used for the standards. From the calibration curve, find the value of ϵ_p for this radionuclide. Using the equation $\Gamma = F/\epsilon_p$, calculate the gamma-ray emission rate (Γ). Calculate the activity (A), in disintegrations per second, of the specimen using the equation $A = (\Gamma/b)(D)$, in which b is the number of gamma rays per disintegration and D is the dilution factor. To obtain the activity, in μCi or mCi , divide A by 3.7×10^4 or 3.7×10^7 , respectively. The above relationship is equally valid for obtaining the activity of an undiluted specimen or capsule; in this case, the dilution factor, D , is unity.

<823> POSITRON EMISSION TOMOGRAPHY DRUGS FOR COMPOUNDING, INVESTIGATIONAL, AND RESEARCH USES

INTRODUCTION

Radionuclides used in positron emission tomography (PET) typically possess short physical half-lives, $T_{1/2}$ (e.g., $T_{1/2}$ of $^{15}\text{O} = 2.03$ min, $^{62}\text{Cu} = 9.67$ min, $^{13}\text{N} = 9.96$ min, $^{11}\text{C} = 20.4$ min, $^{68}\text{Ga} = 67.7$ min, $^{18}\text{F} = 109.8$ min, $^{64}\text{Cu} = 12.7$ h). As a result, these radionuclides usually are produced using particle acceleration techniques (e.g., cyclotrons) or from generators, and then are processed into the final PET drug product in close proximity to the site where the PET procedure will be conducted.

The short half-lives of PET radionuclides create unique constraints for the preparation and testing of PET drug products. This chapter describes guidelines for making and testing PET drug products based on the following constraints:

- It is not possible to complete all testing before the use of PET drug products.
- An entire batch or sub-batch of a PET drug product may be contained in a single vial. Samples withdrawn for quality control (QC) testing are representative of the entire batch or sub-batch.
- An entire batch or sub-batch may be administered to a single patient.
- The mass of the PET drug in a PET drug product usually ranges from nanogram to microgram quantities.
- PET drug products do not enter a traditional drug distribution chain. Instead, PET drug products are used in-house or are delivered to the point of use by dedicated couriers.
- Small-scale facilities for the preparation of PET drug products have limited personnel and resources, which require the following:
 - Allowance for multiple operations in one area with adequate controls;
 - Allowance for the making and testing of multiple PET drug products using shared equipment;
 - Appropriate requirements for aseptic operations;
 - Appropriate requirements for system suitability and other day-of-use activities;
 - QC requirements for components, materials, and supplies;

- Self-verification of significant steps in radionuclide production, PET drug production, or compounding and testing; and
- Single-person oversight of production and compounding, review of batch records, and release authorization.

The scope of this chapter includes the production and compounding of PET drug products for human administration as used (a) according to state-regulated practice of medicine and pharmacy, (b) according to an approved investigational new drug (IND) application (see 21 CFR 312), and (c) according to research uses under the supervision of a Radioactive Drug Research Committee (RDRC; see 21 CFR 361). The scope of this chapter does not include dispensing activities as defined in other USP general chapters.

DEFINITIONS

The following definitions apply to words and phrases as they are used in this chapter.

Batch: A quantity of PET drug product that is intended to have uniform character and quality, within specified limits, and that is made in a single operational cycle produced according to one or more production order(s).

Conditional Final Release: A final release for patient administration before completion of required tests because of a malfunction of analytical equipment.

Lot: A quantity of materials (e.g., reagents, solvents, gases, purification columns, and other auxiliary materials) that have uniform character and quality within specified limits and are used to make a PET drug product.

PET Drug: A radioactive substance (active pharmaceutical ingredient) that exhibits spontaneous disintegration of unstable nuclei by the emission of positrons and is incorporated into a PET drug product to furnish direct effect in the diagnosis or monitoring of a disease or a manifestation of a disease in humans, or monitoring treatment of disease or therapeutic procedures (e.g., tumor therapy).

PET Drug Product: A finished dosage form that contains a PET drug, whether or not in association with one or more other ingredients.

Compounding: The practice as described in the Food, Drug and Cosmetic Act (1997) Chapter II, Section 121 (a) (ii) (1) (B) of synthesizing or formulating a PET drug product, by or on the order of a practitioner who is licensed by a State to compound or order compounding for a PET drug product, and is compounded in accordance with that State's law, for a patient or for research, teaching, or quality control.

Line Clearance: The segregation and cleaning of different processing and work areas to avoid cross-contamination and mix-ups between the production and/or compounding of different PET drug products.

Manufacturer's Certification: Documentation, including, but not limited to, certificates of analysis, certificates of conformance, or certificates of quality obtained from the manufacturer, supplier, or vendor of a material or component that describes critical quality characteristics used to determine acceptability of use.

Out of Specification (OOS): A quality control test result for a PET drug product that does not conform to established acceptance criteria.

Production: The process of synthesis or formulation of a PET drug product including processing, packaging, labeling, reprocessing, and testing for investigational or research use.

Quality Assurance (QA): A planned system for ensuring that a PET drug product possesses defined identity, strength, quality, and purity required for its intended purpose by procedures, tests, and analytical methods.

Quality Control (QC): A system for testing the quality of components, materials, supplies, and PET drug products by procedures, tests, analytical methods, and acceptance criteria.

Specific Activity: The radioactivity of a radionuclide per unit mass of the element or compound. The unit of specific activity is radioactivity per mass expressed on a gram or mole basis (e.g., mCi/μg [MBq/μg], Ci/mmol [GBq/mmol]).

Strength: The radioactivity concentration of the PET drug in the PET drug product on a volume basis at the time of calibration. The unit of strength is the amount of radioactivity per volume at the time of calibration (e.g., mCi/mL [MBq/mL]).

Sub-batch: A quantity of PET drug product having uniform character and quality, within specified limits, that is produced during one succession of multiple irradiations using a given synthesis or purification operation. A group of sub-batches collectively form a batch that is intended to have uniform character and quality, within specified limits. Sub-batches may be required for PET drug products with very short-lived radionuclides (e.g., ¹³N and ¹⁵O) because QC tests cannot be completed before use.

Validation: Establishment of documented evidence that a method, process, or system meets its intended requirements.

Verification: Confirmation that an established method, process, or system meets predetermined acceptance criteria.

PERSONNEL

Sufficient numbers of personnel with the appropriate education, training, and experience are needed for the preparation and testing of PET drug products. The number depends on the size and complexity of the operations executed at each facility.

Training Requirements: Personnel should be trained before they begin to make and test PET drug products. Training can be performed by various methods, including live instruction, audio-video instruction, and study of publications. Training should address but is not limited to radionuclide production techniques, synthetic and purification methods, materials, components, reagents, stock solutions, automated and manual apparatus used to make PET drug products, and QC methods, including equipment, software, and documentation. Training must be documented.

Aseptic Operations Training: Training should address aseptic manipulations as well as the techniques and equipment used to achieve and maintain International Organization for Standardization (ISO) Class 5 environmental conditions. Training also should address all aseptic operations, including the assembly of sterile components, compounding, and filtration. Manipulations of sterile solutions should be performed by operators who are qualified to use aseptic techniques (see *Facilities and Equipment* below).

Personnel involved in aseptic operations should be evaluated periodically by aseptic simulations in which a microbiological growth medium is used to assess the quality of the aseptic operation. Aseptic simulations should provide the following:

- Include all manipulations required for the aseptic assembly of the PET drug product vial assembly (e.g., vial, filter, and syringe assembly, etc.).
- Represent worst-case scenarios for aseptic operations.
- Be performed in triplicate to qualify a new operator. Each operator should be requalified annually by conducting at least one media fill.
- Be performed any time procedures are changed significantly.

After the simulation process, the media should show the absence of contamination after incubation at a suitable tem-

perature for 14 days. An operator who fails written assessments or whose aseptic simulations result in microbial growth should be immediately re-instructed and re-evaluated to ensure correction of aseptic practice deficiencies.

QUALITY ASSURANCE

QA is a broad concept that covers all matters that influence identity, strength, quality, and purity of a PET drug product. QC is a subset of QA that deals with testing of materials and PET drug products to determine if they meet acceptance criteria. The QA function typically consists of oversight activities, and the QC function consists of execution activities.

QC functions include the following.

- Evaluate each lot of incoming material to ensure that it meets its established specifications before use in the preparation or testing of PET drug products.
- Evaluate each batch of a PET drug product to ensure the batch meets its established specifications before authorizing the final release of the batch.

The oversight functions associated with QA include the following:

- Review completed batch records for accuracy and completeness.
- Approve procedures, specifications, processes, and methods.
- Ensure that personnel are properly trained and qualified, as appropriate.
- Ensure that PET drug products have adequately defined identity, strength, quality, and purity.
- Ensure that changes to component quality, suppliers, changes to production procedures, and changes to testing procedures and specifications are appropriate and implemented properly.
- Investigate errors and ensure that appropriate corrective and preventive actions are taken to prevent their recurrence.
- Handle complaints.
- Ensure that the PET drug products are produced, tested, labeled, released, and distributed according to the facility's established procedures and practices for PET drug products.
- Conduct periodic audits to monitor compliance with established procedures and practices for PET drug products.

Personnel at the facility may perform both QA and QC functions.

FACILITIES AND EQUIPMENT

Facilities should be adequate for the production, compounding, and testing of PET drug products. Work areas should be organized to prevent cross-contamination, mix-ups, and errors, especially in areas used for making multiple PET drug products. Work areas should be periodically cleaned to prevent the contamination of equipment, materials, components, or PET drug products by personnel or environmental conditions that could reasonably be expected to adversely affect PET drug product quality. These requirements should be described in written procedures, and their routine execution should be documented.

Environmental Controls for Parenteral PET Drug Products: Because the sterility test results for parenteral PET drug products are obtained after release, facilities and equipment should ensure a sterile PET drug product.

Aseptic Workstation—The primary environmental control for aseptic operations is a high-efficiency particulate air (HEPA) filter that is capable of producing air with a cleanliness rating of ISO Class 5. This can be achieved with a laminar airflow workstation, aseptic isolator, biological safety cabi-

net, or other suitable device (generically, aseptic workstations). The aseptic workstation should be protected from sources of microbial contamination and should be located in an area where personnel traffic is limited. The area around the aseptic workstation should not be used for storage of materials that shed large quantities of particulate matter (e.g., corrugated boxes).

The proper operation of the aseptic workstation must be certified by measurement of airborne particles, HEPA filter integrity testing, pressure differential testing, or other means. The specific tests depend on the type of aseptic workstation. Certification should be performed at the inception of operation and at least annually thereafter or after repair or replacement of the HEPA filter. These requirements supersede those in other USP general information chapters (e.g., *Microbiological Control and Monitoring of Aseptic Processing Environments* <1116>).

The work area inside the aseptic workstation should be clean. The internal surfaces should allow easy cleaning and disinfection. The internal surfaces should be cleaned and disinfected with appropriate disinfectants that are sterile filtered or certified sterile with a manufacturer's certification.

Microbiological Testing—Microbiological testing of the environment should be performed to assess air quality and surface disinfection of the aseptic areas. This can be achieved by either settling plates or active air-sampling plates. Surface disinfection of critical surfaces (e.g., the work surface of the aseptic workstation or operators' fingers) should be assessed with swab or contact plates. For microbiological testing of the aseptic workstation, the air should be tested as part of the workstation qualification (e.g., every six months) and the surface (using swab or contact plates) should be assessed after use, each day of use. Nonviable particle counts may be determined less frequently following certification of the *Aseptic workstation* (see above).

Alert and action limits should be established for samples obtained during microbiological testing. Typical alert levels are set at less than three colony-forming units (cfu) per plate. More than three cfu require corrective actions that may include operator retraining, recertification of the aseptic workstation, or other actions. The results of microbiological testing also should be used in the investigation of positive sterility tests.

Equipment: Equipment used to make and test PET drug products should be appropriate for its intended purpose and should be installed, cleaned, and maintained in an appropriate manner. Equipment should be capable of producing consistent results.

The following requirements should be described in written procedures, and performance of these procedures should be documented.

1. **Installation of New Equipment**—Newly installed equipment should be qualified before it is used to make or test PET drug products at an appropriate level of detail based on complexity. All qualification activities should be properly documented, including the date and the name of the person who performed the qualification. For more complex equipment, qualification consists of three phases:
 - **Installation Qualification (IQ)**—IQ is a check of items required for proper installation of the equipment, including physical location, required utilities and supplies, communications, and environmental conditions. IQ should describe the installation procedure for the equipment.
 - **Operational Qualification (OQ)**—OQ is a check of operational specifications for the equipment, including equipment set-up, functional testing of subsystems, and proper overall operation. OQ should describe operational procedures for the equipment.

- **Performance Qualification (PQ)**—PQ demonstrates that the equipment is capable of performing tasks required to make and test PET drug products in the operating environment and that the equipment provides the intended results. PQ should describe the required performance tasks for the equipment.
- 2. **Calibration of Equipment**—Analytical equipment calibration should be performed before use, as appropriate. A schedule should be developed for recalibration and should have a sufficient frequency to ensure accurate results. Calibration activities should be properly documented, including the date and the name of the person who performed the calibration.
- 3. **Preventive Maintenance of Equipment**—A preventive maintenance schedule should be developed for major production and testing equipment, including automated chemistry modules, gas chromatographs, high-performance liquid chromatographs, and others. The schedule should have a sufficient frequency to minimize equipment downtime. Major repairs may require recalibration and requalification. Preventative maintenance activities should be properly documented, including the date of such performance and the name of the person who performed them.

Cleaning Equipment and Components: Equipment used in production or compounding of PET drug products includes automated, computer-controlled devices, as well as manually operated apparatus. Before it is used in making PET drug products, equipment should be properly cleaned to ensure that the resulting PET drug product meets established specifications for identity, strength, quality, and purity (see *Controls and Acceptance Criteria for Finished PET Drug Products* below). Once cleaned, equipment should be maintained in a state of cleanliness before use.

Equipment may be used to make multiple batches of one or more PET drug products. Documented studies should demonstrate the effectiveness of the cleaning process between batches. All impurities should be controlled at levels that conform to established specifications for identity, strength, quality, and purity. Written procedures for line clearance between batches of different PET drug products should describe routine execution of cleaning processes.

Day-of-Use Checks: Day-of-use checks are necessary for processing equipment to ensure proper function. Written procedures for the day-of-use checks should be established and followed. These procedures should be designed to check key parameters at the beginning of each operational cycle (e.g., temperature, pressure integrity, gas supply, vacuum supply, proper delivery line selection, reagent delivery volumes, gas flow rates, radiation monitors, and other process sensors). Some parameters may be periodically checked as part of the calibration and preventive maintenance schedules as described above.

System Suitability for QC Equipment: System suitability tests are necessary for QC equipment to ensure that the equipment, components, and personnel (i.e., the system) function as a whole to execute the desired analytical method. System suitability tests should be performed prior to using the equipment according to established procedures. Written procedures should be established and followed for system suitability tests, and the test results should be documented.

The system suitability tests required for chromatographic methods include tailing factor, replicate injections, and resolution. When the test chromatogram used for system suitability contains only a single peak, then tailing factor, replicate injections, and column efficiency (theoretical plates) are adequate. The use of internal or external standards with a known concentration is necessary for these determinations. Standards should be prepared from well-characterized materials or from materials that have a manufacturer's certification. Two acceptable approaches that may be used for chromatographic methods are the following:

1. Create a calibration curve from a range of standards with known concentrations. The concentrations of the standards should bracket the conditions of use for the chromatographic method. The calibration curve should be used over a suitable specified period of time (e.g., six months), after which time a new one should be created. A new calibration curve should be created each time an alteration is made to the chromatographic system. Routine system suitability for replicate injections consists of a single injection of a known standard and a measurement of the concentration based on the calibration curve. If the measured concentration agrees with the known concentration within a predefined range (e.g., 10% for manual injections and 5% for automated injections), this demonstrates the suitability of the system for replicate injections and ensures that the calibration curve is appropriate for use in subsequent sample injections. The tailing factor and resolution (or column efficiency, as appropriate) should be determined from the same chromatogram.
 2. At the beginning of each testing cycle, create a single-point calibration from two injections of a known standard. The measured area of the peaks for these injections should agree within a predefined range (e.g., 10% for manual injections and 5% for automated injections). Then the results are averaged and used with the standard concentration to provide a calibration factor that is used in subsequent sample injections for that day. The tailing factor and resolution (or column efficiency as appropriate) should be determined from one of the two chromatograms.
- Other chromatographic parameters such as signal-to-noise ratio, limit of detection, and limit of quantitation can be determined as part of routine system suitability testing.
- System suitability tests also may be appropriate for other QC equipment, including dose calibrators, scanners for radio-thin layer chromatography (radio-TLC), and multichannel analyzers. When used, these tests should be performed at installation, relocation, and appropriate intervals thereafter. These tests should use known standards to demonstrate the proper function of the equipment, for example:
1. **Dose Calibrator**—Accuracy, geometry, and linearity should be assessed at installation and at appropriate intervals thereafter. The instrument should be calibrated in accordance with nationally recognized standards or the manufacturer's instructions. Routine system suitability testing should include a constancy check with a suitable high-energy radionuclide source.
 2. **Radio-TLC Scanner**—Uniformity, positional accuracy, detector linearity, and resolution should be assessed with a suitable radionuclide source. Routine system suitability testing should include checks for these parameters.
 3. **Multichannel Analyzer**—Sensitivity and resolution should be assessed at installation and at appropriate intervals thereafter. Routine system suitability testing should include a constancy check with a suitable high-energy radionuclide source.

CONTROL OF COMPONENTS, MATERIALS, AND SUPPLIES

Components, materials, and supplies that are used in the preparation of PET drug products should be controlled to avoid contamination, mix-ups, and errors. A designated person should be responsible for ensuring that these activities are carried out and completed properly. Records of completed examinations and tests for components, materials, and supplies should be maintained for one year after their expiration or for one year after batch release, whichever is longer. The following activities should be established and performed:

1. Establish written specifications for the identity, strength, quality, and purity of ingredients, reagents, target materials, and gases.
 2. Establish written specifications for the identity and quality of sterile empty vials, transfer lines, sterile stopcocks, sterile needles, sterile membrane filters, and other components used in the PET drug product vial assembly.
 3. Establish written specifications for the identity, strength, quality, and purity of analytical supplies (e.g., solvents, chromatography columns, and authentic standards), sterility test media, and endotoxin test reagents used in the testing of PET drug products.
 4. Establish appropriate storage conditions (based on heat, light, humidity, and other factors) for components, materials, and supplies used to make and test PET drug products.
 5. Store components, materials, and supplies in a controlled-access area according to established storage conditions. Segregate components, materials, and supplies as appropriate to avoid mix-ups and errors.
 6. Log each lot of shipment of components, materials, and supplies, and record the date of receipt, quantity received, manufacturer, manufacturer's lot number, and expiration date. If no expiration date is designated by the manufacturer, assign one based on knowledge of its physical and chemical properties and previous experience with its use. For organic substrates and reagents that are potentially susceptible to degradation or to a change in composition, the expiration date should be based on the material's stability.
 7. Determine that each lot of components, materials, and supplies complies with established written specifications. Compliance with specifications can be demonstrated by inspection of the labeling or inspection of the manufacturer's certification. The identity of each lot of components, materials, and supplies should be verified by defined procedures, tests, or documented manufacturer's certification, as appropriate. Perform an identity test for precursors (e.g., melting point determination or other appropriate tests). Alternatively, the manufacturer's certification can be used as the only acceptance criterion for a precursor if final testing of the PET drug product ensures that the correct precursor has been used. Reference standards used in chromatographic procedures should have suitable documentation of identity and purity. Other components can be accepted on the basis of a manufacturer's certification only.
 8. Membrane filters used with parenteral PET drug products should have a manufacturer's certification. Examine the manufacturer's certification for each lot to ensure compliance with written specifications.
 9. Media used in the sterility testing of PET drug products may be obtained from commercial sources. If the media is obtained from commercial sources, then growth-promotion testing that uses a suitable single species of organism should be performed on initial qualification of the supplier and periodically (e.g., quarterly) thereafter.
- include sterility and bacterial endotoxins. If a *USP* monograph exists or if there are specifications that have been previously accepted by the appropriate regulatory agency (e.g., FDA), then these standards, if applicable, may be applied as the minimum acceptance criteria.
2. Written procedures for the preparation of each PET drug product should provide the following:
 - Incorporate, for each PET drug product intended for parenteral administration, sterile membrane filtration (0.22 μm) or steam sterilization;
 - Incorporate, for each PET drug product intended for inhalation, particulate filtration (0.45 μm);
 - Describe routine cleaning procedures for equipment and facilities;
 - Describe components, materials, and supplies used to make PET drug products, including precursors, standards, reagents, stock solutions, and related items;
 - Describe the process and the steps used to make the PET drug product;
 - Describe the formulation process, including the use of stabilizers, buffers, and other agents;
 - Describe calculations performed for quantitative parameters associated with making and QC testing the PET drug product (e.g., including radiochemical yield, radiochemical purity, specific activity, solvent amounts, etc.);
 - Describe QC tests for the final PET drug product (see *Controls and Acceptance Criteria for Finished PET Drug Products* below), including a schedule that defines whether or not each test should be performed on each batch and that states if the test results should be complete at the time of release.
 3. The quality of each batch of a PET drug product should be verified by full finished product testing prior to use to ensure the product meets all specifications.
 4. In cases where testing as described in the previous paragraph is not possible or impractical, the quality of a PET drug product may also be ensured by documented validation studies in lieu of prerelease tests. Such studies should provide the following:
 - Demonstrate a consistent process that is suitable for the intended preparation of the PET drug product;
 - Be completed on three batches made according to the master formula, and all three batches should meet all acceptance criteria;
 - Include evaluation of radiochemical identity and purity, radionuclidic identity and purity, specific activity, sterility (for parenteral PET drug products), bacterial endotoxins (for parenteral PET drug products), pH, appearance, stereochemical purity (for applicable compounds), residual solvents, other toxic chemicals that may have been used during the synthesis or purification procedure, effective concentration of a stabilizer (if any), chemical purity of the PET drug product, and equivalence of initial and final sub-batches (see *Definitions* above);
 - Be repeated if the process and steps described in the master formula have been altered in a way that could change the identity, strength, quality, or purity of the PET drug product;
 5. The processes and steps described in the master formula should be updated as needed and should be reviewed annually to ensure they are current. Prior to the implementation of updates, appropriate validation and/or verification should be approved and performed.

Appropriate controls of computer-controlled equipment should ensure that process changes are instituted only by authorized personnel and that such changes are docu-

PROCESS AND OPERATIONAL CONTROLS

Process Controls: The following process controls should be established and summarized in a master formula for the PET drug product. A designated person should be responsible for ensuring that these activities are carried out and completed properly.

1. Written acceptance criteria for the identity, strength, quality, and purity of each PET drug product should be established. For PET drug products intended for parenteral administration, specifications should in-

mented and verified. Production, compounding, and test methods should be backed up and controlled to avoid accidental use of outdated methods. In the case of processes or test methods from a vendor that are used without alteration, it is acceptable to rely on vendor certification for software verification and proper operation.

Operational Controls: The following operational controls should be established and summarized in a batch record that is a subset of the master formula for the PET drug product. The batch record should adequately document the routine process for making the PET drug product. A designated person should be responsible for ensuring that these activities are carried out and completed properly. Completed batch records and associated documentation should be maintained for one year after batch release.

1. Execute suitable line clearance procedures to avoid mix-ups and cross-contamination, including the inspection of areas used to make and test PET drug products, and the inspection of all equipment for cleanliness and suitability before use. Remove extraneous materials and labels from these areas and equipment.
2. Ensure the correct identity, strength, quality, and purity of components, materials, and supplies used in the preparation of the PET drug product. Label components as appropriate for identity and traceability purposes.
3. Execute routine cleaning procedures for equipment and facilities.
4. Prepare the PET drug product according to the current master formula, and for each batch maintain a batch record. Batch records may consist of paper documents, electronic records, or combinations thereof. Spreadsheets and other electronic record-keeping tools should be verified to ensure traceability, data integrity, accuracy of results for calculations, and so on. The batch record should include the following:
 - Lot numbers or other unique identifiers for all components, materials, and supplies used to make the PET drug product;
 - A description of the individual procedures that were followed;
 - The initials, signature, or other identifier of the responsible individual indicating that critical steps and processes used to make and test the PET drug product were completed;
 - The percent yield calculated on the basis of the known or expected amount of the starting radionuclide that is synthetically incorporated into the PET drug product;
 - Raw analytical data on each batch of the PET drug product;
 - Labeling for the PET drug product (see *Labeling* below);
 - Calculations for key parameters defined in the master formula;
 - Results obtained from QC tests of the PET drug product, including chromatograms, print-outs, and other test data;
 - The initials of the analyst who performed each QC test;
 - A notation of the result for each QC test and whether or not the result meets the acceptance criteria;
 - The date and time of release and the signature of the individual who assumes overall responsibility for, and adherence to, the procedures used to make the batch and authorizes the release of the batch for human administration; and
 - Documentation on the batch record of process deviations, when applicable.

Entries in batch records should be made immediately after the activity is performed and should include the initials, signature, or other identifier for the person making the entry.

Corrections to paper entries should be dated and initialed, signed, or noted with an identifier of the person making the corrections but leaving the original entry still readable.

Aseptic Operations for Parenteral PET Drug Products:

Because the sterility test results for parenteral PET drug products are obtained after release for human administration, aseptic operations and procedures should adequately ensure a sterile PET drug product. All aseptically prepared PET drug products for parenteral administration should be filtered through a sterile membrane filter of 0.22- μ m or finer pore size into a closed sterile vial or container or sterilized by steam sterilization. Although the chemical synthesis of a parenteral PET drug product may take place in an open or closed apparatus, the membrane filtration of the PET drug product should be a closed system downstream of the membrane filter. This system should be aseptically assembled from presterilized, commercially available components.

Components—The sterile components used in the aseptically assembled apparatus typically consist of an empty vial, needles, membrane filters, vent needles, syringes, tubing, stopcocks, and perhaps others. All components should be single-use, commercially available, presterilized items. If components in the aseptically assembled apparatus are sterilized by the PET facility, the sterilization processes should be verified. The exact configuration of the PET drug product vial assembly is process dependent. A typical example is a sterile, empty vial with a membrane filter of 0.22- μ m pore size attached to a needle that is inserted through the vial septum for filtration, a membrane filter of 0.22- μ m pore size attached to a needle that is inserted through the vial septum for venting the vial during filtration, and a syringe with needle inserted through the vial septum for removal of the QC sample after filtration is complete.

PET Drug Product Vial Assembly—Aseptic techniques should be used in the preparation of the PET drug product vial assembly, especially the assembly of all components downstream from the membrane sterilizing filter. These operations should be performed in an ISO Class 5 environment (see *Facilities and Equipment* above).

Following the creation of the PET drug product vial assembly in the ISO Class 5 environment, the assembly can be removed to another location for filtration. The location can be a noncontrolled environment as long as the integrity of the PET drug product vial assembly is not compromised during the process. Any PET drug product vial assembly that is compromised during this process should be discarded.

Aseptic Techniques—Any sterile component downstream from the membrane filter that contacts the PET drug product should be handled using suitable aseptic techniques inside the aseptic workstation. During aseptic operations, operators should wear proper attire, including a clean laboratory jacket, forearm sleeves, hair cover, sanitized gloves that cover the wrist, and beard/moustache covers (as appropriate). Multiple PET drug product vial assemblies can be prepared in a single aseptic operational cycle. The storage conditions and time for assembled vials should be based on data from aseptic simulations.

Sterility Test Inoculations—Sterility tests should be performed to assess the quality of PET drug products intended for parenteral administration. The inoculation of sterility test media should be performed in a manner that is consistent with personnel radiation exposure requirements but that also minimizes the risk of false positives caused by adventitious contamination during the inoculation process. For media tubes with a screw-cap opening, the inoculation should be performed in the aseptic workstation. Media tubes with a septum cap can be inoculated in a shielded area that does not contain a HEPA filter.

STABILITY

Written specifications for the expiration time and storage conditions should be established for each PET drug product.

The expiration time should be based on the results of stability testing (and specific activity requirements, as appropriate). Stability testing of the PET drug product should be performed at the highest strength of the PET drug product and in the intended final vial or container. At least three batches of the PET drug product should be stored according to proposed conditions and should be examined after a time period equal to the proposed shelf life. In addition, the PET drug product should meet acceptance criteria for radiochemical purity, appearance (color and clarity), pH, and stabilizer effectiveness (as appropriate) and chemical purity at expiry. Analytical methods should be reliable, meaningful, and specific. Stability studies should be repeated if there is a change in strength, stabilizer (or preservative) content that has the potential to affect the stability, the final vial or container, storage conditions, or expiration time. The results of stability testing should be documented.

CONTROLS AND ACCEPTANCE CRITERIA FOR FINISHED PET DRUG PRODUCTS

Written specifications for identity, strength, quality, and purity should be established for each PET drug product. For PET drug products intended for parenteral administration, specifications should be included for sterility and bacterial endotoxins.

Written procedures should be developed for QC tests. QC and documentation requirements should be established for each batch or sub-batch of a PET drug product (see *Process and Operational Controls* above). All QC tests should be executed by qualified and trained personnel according to written procedures.

The short half-life of PET radionuclides frequently precludes the completion of all QC tests before shipment of the PET drug product. This effectively creates two levels of release, one for distribution and the other for human administration. This is acceptable as long as the QC tests required for release of the PET drug product for human administration (see below) are completed before administration. The controls used in the release for distribution should be previously established in writing and should be documented in routine practice. It is not necessary to retain reserve samples of PET drug products.

If a *USP* compendial test procedure is used, the procedure should be verified to demonstrate that the test works under the conditions of actual use. Noncompendial test procedures used in the testing of a PET drug product should be reliable and specific. Supporting data for use of all analytical methods should be documented. Data derived from process studies or from in-process controls can be used as a basis for the omission of some QC tests. An example of this approach is the chlorodeoxyglucose determination in the testing of [¹⁸F]fluorodeoxyglucose. Supporting data from process studies or in-process controls should be documented.

Quality Control Tests: The following QC tests should be performed on each batch before release for administration:

1. Appearance by visual inspection for color and clarity (absence of particulate matter) for parenteral dosage forms.
2. Measurement of the pH for parenteral dosage forms.
3. Determination of the radiochemical purity and identity of all dosage forms.
4. Determination of the radionuclidic identity of all dosage forms by half-life measurement.
5. Determination of the strength.
6. Determination of the specific activity of PET drug products that have mass-dependent localization or toxicity concerns.
7. Determination of residual solvents used in the synthesis or purification processes.
8. Determination of the chemical purity and residual compounds used in the synthesis or purification processes (e.g., cryptand [2.2.2]).

9. Determination of preservative or stabilizer, if present.

For PET drug products with very short-lived radionuclides, prepare an initial QC sub-batch that is representative of successive sub-batches prepared in a defined operational cycle. The QC tests described in the previous paragraph should be considered for the QC sub-batch before release of subsequent sub-batches for human administration. For subsequent sub-batches of parenteral and inhaled dosage forms, visual inspection should be performed before human administration. In certain cases, limited testing of each sub-batch before administration may be appropriate (e.g., for pH determination of [¹³N]ammonia produced by Devarda's alloy).

Periodic Quality Indicating Tests: For all PET drug products, periodically measure the radionuclidic purity of decayed samples of the PET drug product to assess the presence of long-lived radionuclides that are produced in targetry associated with the particle accelerator. For PET drug products labeled with certain radionuclides (e.g., ^{94m}Tc, ¹²⁴I, ⁶⁴Cu, ⁷⁶Br, and others), consider the measurement of radionuclidic purity by gamma spectrometry. Periodic quality indicating tests for PET drug products also include low-level nontoxic impurities (e.g., Class 3 residual solvents). The periodic testing should be performed at predetermined intervals rather than on a batch-to-batch basis.

Microbiological Tests for Sterile PET Drug Products: For PET drug products intended for parenteral administration, perform the following QC tests in addition to those described previously:

1. Determine the integrity of the membrane filter. Filter units used to sterilize PET drug products should be subjected to manufacturers' recommended integrity tests such as the bubble point test. Perform the filter integrity test after completion of filtration and before release of the PET drug product for human administration. In the case of PET drug products with $T_{1/2} < 10$ min, the PET drug product can be released for human administration before completion of the filter integrity test. In this case, the test should be completed as soon as possible after release.
2. Perform a test for bacterial endotoxins on each batch or QC sub-batch of a PET drug product. The test can be performed using recognized procedures in *USP* (see *Bacterial Endotoxins Test* (85)). Regardless of which test is used, it should be initiated before release of each batch for human administration. For PET drug products with very short-lived radionuclides, complete the test on the QC sub-batch before the release of subsequent sub-batches for human administration. After a record of successful bacterial endotoxin tests is established for a particular PET drug product, it is necessary only to test the first batch prepared each day for that PET drug product.
3. Perform a test for sterility on each batch or QC sub-batch. The sterility test consists of the inoculation and incubation of a sample into each of two media: tryptic soy broth and fluid thioglycollate. The inoculated volume may be adjusted to avoid excessive losses because of sterility testing (e.g., 0.1 mL inoculated into 10 mL of media). The incubation period for sterility tests should begin within 30 hours of the membrane filtration. The samples can be inoculated immediately after completion of the membrane filtration, or they can be allowed to decay in a shielded area for as long as 30 hours before inoculation. It is acceptable to exceed the 30-hour period because of weekends or holidays provided it is shown that the extended period does not significantly reduce the viability of a suitable indicator organism in the sample. The sterility test may be performed using other recognized procedures in *USP* (see *Sterility Tests* (71)). Samples should be tested individually and may not be pooled. After a record of successful sterility tests is established for a particular PET drug product, it is only necessary to

test the first batch prepared each day for that PET drug product.

Conditional Final Release Tests: When a required QC test for a PET drug product cannot be completed because of a malfunction of testing equipment, it may be appropriate to conditionally release the batch. PET drug products may not be released without determination of radiochemical identity and purity. The batch may be released if the following conditions are met:

1. Review historical QC data to assess the frequency of out-of-specification (OOS) results or failures associated with the QC test. A conditional release is appropriate only if the historical data reveal a record of successful completion of the QC test.
2. Confirm that the acceptance criteria are met for all other QC tests for the batch.
3. Retain a sample of the conditionally released batch.
4. Promptly correct the malfunction of the testing equipment.
5. Complete the omitted QC test on the sample as soon as possible after the malfunction has been corrected. This is not necessary if the omitted QC result is meaningless after decay of the PET drug product.
6. If the sample fails the omitted QC test, immediately notify the physician or receiving facility that ordered the PET drug product.
7. Document all actions regarding the conditional release of the PET drug product, including the justification for the release, results of completed testing, and any notifications and corrective and preventive actions resulting from the incident.

In addition to the finished QC testing, other appropriate laboratory determinations could involve in-process testing of an attribute that is equivalent to finished-product testing of that attribute; continuous statistical process monitoring; or some combination of these approaches with finished testing of each PET drug product.

IF A PET DRUG PRODUCT DOES NOT CONFORM TO SPECIFICATIONS

When the result of a QC test for a PET drug product does not meet established acceptance criteria, the result is OOS. An OOS result does not necessarily mean that the final PET drug product is a failure and should be rejected. Instead, an OOS investigation should be performed to determine if the OOS result indicates a true failure or an analytical error.

If an OOS investigation concludes that the OOS result was caused by an analytical error, invalidate the original test. If a printout is associated with this test, mark the printout *invalid*, retain it for the batch record, and repeat the test.

If an OOS investigation concludes that the OOS result was a true failure, the batch should be rejected and cannot be released for human administration. Segregate the batch to avoid its potential use. Investigate all failures and document the results according to written procedures. The investigation should include, but is not limited to, the examination of processes, operations, and records from previous batches, as well as complaints and other relevant sources of information. If possible, assign an actual or probable cause to the failure, and document corrective actions undertaken as a result of the investigation. Depending on the nature of the failure, the PET drug product may be reprocessed according to pre-established written procedures (see *Reprocessing* below).

When a sterility test for a PET drug product shows signs of microbial growth, the test result is OOS and should be investigated. Upon completion of the investigation, immediately notify all receiving facilities if the product fails to meet the criterion for sterility, including the microbiological findings from the investigation.

REPROCESSING

If a PET drug product is rejected as a true failure, the batch may be reprocessed according to established procedures. It is not possible to describe all possible reprocessing operations, but some examples could include the following:

- pH adjustment;
- A second passage through a membrane filter in the event of a failed filter integrity test; and
- A second passage through a purification column to remove an impurity.

If a PET drug product is reprocessed, the reprocessed batch should be tested to ensure it meets the established acceptance criteria for the PET drug product before release for human administration.

LABELING

The following information should appear on the label attached to the final PET drug container:

- The name of the PET drug product, including the dosage form;
- The assigned batch number; and
- Any required warning statements or symbols (e.g., investigational use, radioactive).

The following information should appear on the shielding for the PET drug product:

- The name of the PET drug product, including the dosage form;
- The assigned batch number;
- The date and time of calibration;
- Any required warning statements or symbols (e.g., investigational use, radioactive);
- As appropriate, the total radioactivity in MBq (or mCi) or the strength in MBq/mL (or mCi/mL) at time of calibration;
- Expiration time and date;
- Added substance(s) (e.g., stabilizer inactive ingredients);
- The name of the producer where the PET drug product was made or the name of the distributor;
- Other applicable warning statement(s) (e.g., "Do not use if cloudy or if it contains particulate matter" or investigational use labeling); and
- Other pertinent information (if required), such as storage condition(s), half-life.

(831) REFRACTIVE INDEX

The refractive index (n) of a substance is the ratio of the velocity of light in air to the velocity of light in the substance. It is valuable in the identification of substances and the detection of impurities.

Although the standard temperature for Pharmacopeial measurements is 25°, many of the refractive index specifications in the individual monographs call for determining this value at 20°. The temperature should be carefully adjusted and maintained, since the refractive index varies significantly with temperature.

The values for refractive index given in this Pharmacopeia are for the D line of sodium (doublet at 589.0 nm and 589.6 nm). Most instruments available are designed for use with white light but are calibrated to give the refractive index in terms of the D line of sodium light.

The Abbé refractometer measures the range of refractive index for those Pharmacopeial materials for which such values are given. Other refractometers of equal or greater accuracy may be employed.

To achieve the theoretical accuracy of ± 0.0001 , it is necessary to calibrate the instrument against a standard provided by the manufacturer and to check frequently the temperature control and cleanliness of the instrument by determining the refractive index of distilled water, which is 1.3330 at 20° and 1.3325 at 25°.

<841> SPECIFIC GRAVITY

Unless otherwise stated in the individual monograph, the specific gravity determination is applicable only to liquids, and, unless otherwise stated, is based on the ratio of the weight of a liquid in air at 25° to that of an equal volume of water at the same temperature. Where a temperature is specified in the individual monograph, the specific gravity is the ratio of the weight of the liquid in air at the specified temperature to that of an equal volume of water at the same temperature. When the substance is a solid at 25°, determine the specific gravity of the melted material at the temperature directed in the individual monograph, and refer to water at 25°.

Unless otherwise stated in the individual monograph, the density is defined as the mass of a unit volume of the substance at 25°, expressed in kilograms per cubic meter or grams per cubic centimeter ($1 \text{ kg/m}^3 = 10^{-3} \text{ g/cm}^3$).

Unless otherwise directed in the individual monograph, use *Method I*.

METHOD I

Procedure—Select a scrupulously clean, dry pycnometer that previously has been calibrated by determining its weight and the weight of recently boiled water contained in it at 25°. Adjust the temperature of the liquid to about 20°, and fill the pycnometer with it. Adjust the temperature of the filled pycnometer to 25°, remove any excess liquid, and weigh. When the monograph specifies a temperature different from 25°, filled pycnometers must be brought to the temperature of the balance before they are weighed. Subtract the tare weight of the pycnometer from the filled weight.

The specific gravity of the liquid is the quotient obtained by dividing the weight of the liquid contained in the pycnometer by the weight of water contained in it, both determined at 25°, unless otherwise directed in the individual monograph.

METHOD II

The procedure includes the use of the *Oscillating transducer density meter*. The apparatus consists of the following:

- a U-shaped tube, usually of borosilicate glass, which contains the liquid to be examined;
- a magneto-electrical or piezo-electrical excitation system that causes the tube to oscillate as a cantilever oscillator at a characteristic frequency depending on the density of the liquid to be examined;
- a means of measuring the oscillation period (T), which may be converted by the apparatus to give a direct reading of density or used to calculate density by using the constants A and B described below; and
- a means to measure and/or control the temperature of the oscillating transducer containing the liquid to be tested.

The oscillation period is a function of the spring constant (c) and the mass of the system:

$$T^2 = ((M/c) + ((\rho \times V)/c)) \times 4\pi^2$$

where ρ is the density of the liquid to be tested, M is the mass of the tube, and V is the volume of the filled tube.

Introduction of two constants $A = c/(4\pi^2 \times V)$ and $B = (M/V)$, leads to the classical equation for the oscillating transducer:

$$\rho = A \times T^2 - B$$

The specific gravity of the liquid is given by the formula:

$$\rho_{(L)}/\rho_{(W)}$$

where $\rho_{(L)}$ and $\rho_{(W)}$ are the densities of the liquid and water, respectively, both determined at 25°, unless otherwise directed in the individual monograph.

Calibration—The constants A and B are determined by operating the instrument with the U-tube filled with two different samples of known density (e.g., degassed water and air). Perform the control measurements daily, using degassed water: the results displayed for the control measurement using degassed water do not deviate from the reference value ($\rho_{25} = 0.997043 \text{ g/cm}^3$) by more than its specified error. Precision is a function of the repeatability and stability of the oscillator frequency. Density meters are able to achieve measurements with an error on the order of $1 \times 10^{-3} \text{ g/cm}^3$ to $1 \times 10^{-5} \text{ g/cm}^3$ and a repeatability of $1 \times 10^{-4} \text{ g/cm}^3$ to $1 \times 10^{-6} \text{ g/cm}^3$. For example, an instrument specified to $\pm 1 \times 10^{-4} \text{ g/cm}^3$ must display $0.9970 \pm 0.0001 \text{ g/cm}^3$ in order to be suitable for further measurement, otherwise a readjustment is necessary. Calibration with certified reference materials should be carried out regularly.

Procedure—Using the manufacturer's instructions, perform the measurements using the same procedure as for *Calibration*. If necessary, equilibrate the liquid to be examined at 25° before introduction into the tube to avoid the formation of bubbles and to reduce the time required for measurement. Factors affecting accuracy include the following:

- temperature uniformity throughout the tube,
- nonlinearity over a range of density,
- parasitic resonant effects, and
- viscosity, if the oscillating transducer density meters used do not provide automatic compensation of sample viscosity influence.

<846> SPECIFIC SURFACE AREA

INTRODUCTION

The specific surface area of a powder is determined by physical adsorption of a gas on the surface of the solid and by calculating the amount of adsorbate gas corresponding to a monomolecular layer on the surface. Physical adsorption results from relatively weak forces (van der Waals forces) between the adsorbate gas molecules and the adsorbent surface of the test powder. The determination is usually carried out at the temperature of liquid nitrogen. The amount of gas adsorbed can be measured by a volumetric or continuous flow procedure.

BRUNAUER, EMMETT AND TELLER (BET) THEORY AND SPECIFIC SURFACE AREA DETERMINATION

Multipoint Measurement

The data are treated according to the Brunauer, Emmett and Teller (BET) adsorption isotherm equation:

$$\frac{1/[V_a((P_o/P) - 1)]}{[(C - 1)/(V_m C)] \times (P/P_o) + (1/V_m C)} \quad (1)$$

P	=	partial vapor pressure of adsorbate gas in equilibrium with the surface at 77.4 K (b.p. of liquid nitrogen), in Pa,
P _o	=	saturated pressure of adsorbate gas, in Pa,
V _a	=	volume of gas adsorbed at standard temperature and pressure (STP) [273.15 K and atmospheric pressure (1.013 × 10 ⁵ Pa)], in mL,
V _m	=	volume of gas adsorbed at STP to produce an apparent monolayer on the sample surface, in mL,
C	=	dimensionless constant that is related to the enthalpy of adsorption of the adsorbate gas on the powder sample.

A value of V_a is measured at each of not less than three values of P/P_o.

Then the BET value

$$1/[V_a((P_o/P) - 1)]$$

is plotted against P/P_o, according to equation (1). This plot should yield a straight line usually in the approximate relative pressure range 0.05 to 0.3. The data are considered acceptable if the correlation coefficient, *r*, of the linear regression is not less than 0.9975; that is, *r*² is not less than 0.995. From the resulting linear plot, the slope, which is equal to (C - 1)/V_mC, and the intercept, which is equal to 1/V_mC, are evaluated by linear regression analysis. From these values, V_m is calculated as 1/(slope + intercept), while C is calculated as (slope/intercept) + 1. From the value of V_m so determined, the specific surface area, *S*, in m² · g⁻¹, is calculated by the equation:

$$S = (V_m N_a)/(m \times 22400) \quad (2)$$

N	=	Avogadro constant (6.022 × 10 ²³ mol ⁻¹),
a	=	effective cross-sectional area of one adsorbate molecule, in square meters (0.162 nm ² for nitrogen and 0.195 nm ² for krypton),
m	=	mass of test powder, in g,
22,400	=	volume, in mL, occupied by one mole of the adsorbate gas at STP allowing for minor departures from the ideal.

A minimum of three data points is required. Additional measurements may be carried out especially when nonlinearity is obtained at a P/P_o value close to 0.3. Because nonlinearity is often obtained at a P/P_o value below 0.05, values in this region are not recommended. The test for linearity, the treatment of the data, and the calculation of the specific surface area of the sample are described above.

Single-Point Measurement

Normally, at least three measurements of V_a, each at different values of P/P_o, are required for the determination of specific surface area by the dynamic flow gas adsorption technique (*Method I*) or by volumetric gas adsorption (*Method II*). However, under certain circumstances described below, it may be acceptable to determine the specific surface area of a powder from a single value of V_a measured at a single value of P/P_o such as 0.300 (corresponding to 0.300 mole of nitrogen or 0.001038 mole fraction of krypton), using the following equation for calculating V_m:

$$V_m = V_a[1 - (P/P_o)] \quad (3)$$

The specific surface area is then calculated from the value of V_m by equation (2) given above.

The single-point method may be employed directly for a series of powder samples of a given material for which the material constant C is much greater than unity. These circumstances may be verified by comparing values of specific surface area determined by the single-point method with that determined by the multipoint method for the series of powder samples. Close similarity between the single-point values and multipoint values suggests that 1/C approaches zero.

The single-point method may be employed indirectly for a series of very similar powder samples of a given material for which the material constant C is not infinite but may be assumed to be invariant. Under these circumstances, the error associated with the single-point method can be reduced or eliminated by using the multipoint method to evaluate C for one of the samples of the series from the BET plot, from which C is calculated as (1 + slope/intercept). Then V_m is calculated from the single value of V_a measured at a single value of P/P_o, by the equation:

$$V_m = V_a[(P_o/P) - 1] [(1/C) + ((C - 1)/C) \times (P/P_o)] \quad (4)$$

The specific surface area is calculated from V_m by equation (2) given above.

EXPERIMENTAL TECHNIQUES

This section describes the methods to be used for the sample preparation, the dynamic flow gas adsorption technique (*Method I*) and the volumetric gas adsorption technique (*Method II*).

Sample Preparation

OUTGASSING

Before the specific surface area of the sample can be determined, it is necessary to remove gases and vapors that may have become physically adsorbed onto the surface after manufacture and during treatment, handling, and storage. If outgassing is not achieved, the specific surface area may be reduced or may be variable because an intermediate area of the surface is covered with molecules of the previously adsorbed gases or vapors. The outgassing conditions are critical for obtaining the required precision and accuracy of specific surface area measurements on pharmaceuticals because of the sensitivity of the surface of the materials.

The outgassing conditions must be demonstrated to yield reproducible BET plots, a constant weight of test powder, and no detectable physical or chemical changes in the test powder.

The outgassing conditions defined by the temperature, pressure, and time are chosen so that the original surface of

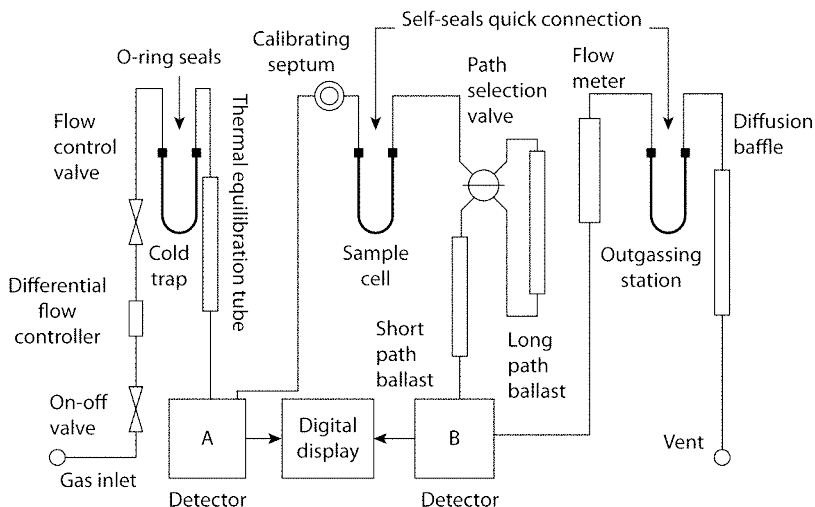


Fig. 1. Schematic diagram of the dynamic flow method apparatus.

the solid is reproduced as closely as possible. Outgassing of many substances is often achieved by applying a vacuum by purging the sample in a flowing stream of a nonreactive, dry gas or by applying a desorption-adsorption cycling method. In either case, elevated temperatures are sometimes applied to increase the rate at which the contaminants leave the surface. Caution should be exercised when outgassing powder samples using elevated temperatures to avoid affecting the nature of the surface and the integrity of the sample.

If heating is employed, the recommended temperature and time of outgassing are as low as possible to achieve reproducible measurement of specific surface area in an acceptable time. For outgassing sensitive samples, other outgassing methods such as the desorption-adsorption cycling method may be employed.

ADSORBATE

The standard technique is the adsorption of nitrogen of analytical quality at liquid nitrogen temperature.

For powders of low specific surface area ($< 0.2 \text{ m}^2\text{g}^{-1}$), the proportion adsorbed is low. In such cases, the use of krypton at the liquid nitrogen temperature is preferred because the low vapor pressure exerted by this gas greatly reduces error. The use of larger sample quantities, where feasible (equivalent to 1 m^2 or greater total surface area using nitrogen), may compensate for the errors in determining low surface areas.

All gases used must be free from moisture.

QUANTITY OF SAMPLE

A quantity of the test powder is accurately weighed such that the total surface of the sample is at least 1 m^2 when the adsorbate is nitrogen and 0.5 m^2 when the adsorbate is krypton.

Lower quantities of sample may be used after appropriate validation.

Measurements

Because the amount of gas adsorbed under a given pressure tends to increase when the temperature is decreased, adsorption measurements are usually made at a low temper-

ature. Measurement is performed at 77.4 K , the boiling point of liquid nitrogen.

Method I: The Dynamic Flow Method

PRINCIPLE

In the dynamic flow method (see *Figure 1*), the recommended adsorbate gas is dry nitrogen or krypton, while helium is employed as a diluent gas, which is not adsorbed under the recommended conditions.

A minimum of three mixtures of the appropriate adsorbate gas with helium are required within the P/P_0 range 0.05 to 0.30.

The gas detector-integrator should provide a signal that is approximately proportional to the volume of the gas passing through it under defined conditions of temperature and pressure. For this purpose, a thermal conductivity detector with an electronic integrator is one among various suitable types. A minimum of three data points within the recommended range of 0.05 to 0.30 for P/P_0 is determined.

PROCEDURE

A known mixture of the gases, usually nitrogen and helium, is passed through a thermal conductivity cell, through the sample again, through the thermal conductivity cell, and then to a recording potentiometer.

The sample cell is immersed in liquid nitrogen, and the sample adsorbs nitrogen from the mobile phase. This unbalances the thermal conductivity cell, and a pulse is generated on a recorder chart.

The sample is removed from the coolant; this gives a desorption peak equal in area and in the opposite direction to the adsorption peak. Because this is better defined than the adsorption peak, it is the one used for the determination.

To effect the calibration, a known quantity of adsorbate, sufficient to give a peak of similar magnitude to the desorption peak, is injected into the system, and the proportion of gas volume per unit peak area is obtained.

A mixture of nitrogen and helium is used for a single-point determination; and several such mixtures or premixing two streams of gas are used for a multipoint determination.

The calculation is the same as the volumetric method.

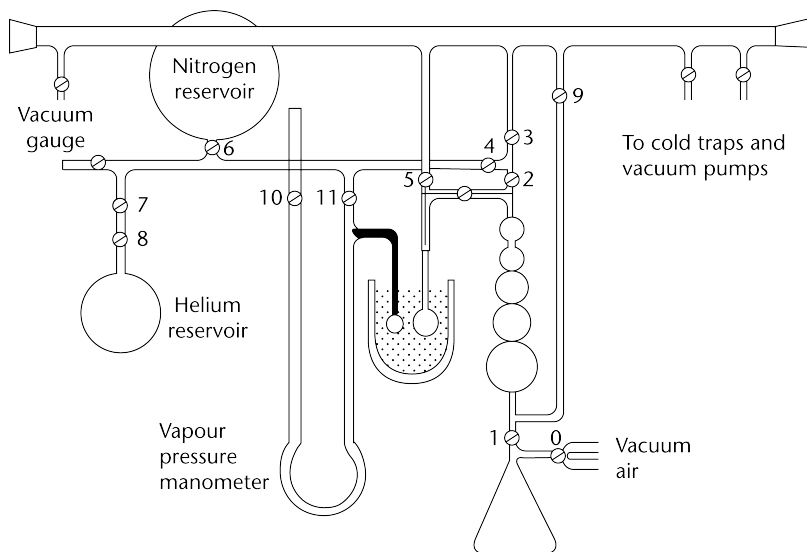


Fig. 2. Schematic diagram of the volumetric method apparatus.

Method II: The Volumetric Method

PRINCIPLE

In the volumetric method (see *Figure 2*), the recommended adsorbate gas is nitrogen, which is admitted into the evacuated space above the previously outgassed powder sample to give a defined equilibrium pressure, P , of the gas. The use of a diluent gas, such as helium, is therefore unnecessary, although helium may be employed for other purposes, such as to measure the dead volume.

Because only pure adsorbate gas, instead of a gas mixture, is employed, interfering effects of thermal diffusion are avoided in this method.

PROCEDURE

A small amount of dry nitrogen is admitted into the sample tube to prevent contamination of the clean surface, the sample tube is removed, a stopper is inserted, the tube is weighed, and the weight of the sample is calculated. Then the sample tube is attached to the volumetric apparatus. The sample is cautiously evacuated down to the specified pressure (e.g., between 2 Pa and 10 Pa). Alternately, some instruments are operated by evacuating to a defined rate of pressure change (e.g., less than 13 Pa/30 s) and by holding for a defined period of time before commencing the next step.

If the principle of operation of the instrument requires the determination of the dead volume in the sample tube, for example, by the admission of a nonadsorbed gas, such as helium, this procedure is carried out at this point, followed by evacuation of the sample. The determination of dead volume may be avoided using difference measurements: that is, by means of reference and sample tubes connected by a differential transducer. The adsorption of nitrogen gas is then measured as described below.

Raise a Dewar vessel containing liquid nitrogen at 77.4 K up to a defined point on the sample cell. Admit a sufficient volume of adsorbate gas to give the lowest desired relative pressure. Measure the volume adsorbed, V_a . For multipoint measurements, repeat the measurement of V_a at successively higher P/P_0 values. When nitrogen is used as the adsorbate gas, P/P_0 values of 0.10, 0.20, and 0.30 are often suitable.

Reference Materials

Periodically verify the functioning of the apparatus using appropriate reference materials of known surface area that have a specific surface area similar to that of the sample to be examined.

⟨851⟩ SPECTROPHOTOMETRY AND LIGHT-SCATTERING

ULTRAVIOLET, VISIBLE, INFRARED, ATOMIC ABSORPTION, FLUORESCENCE, TURBIDIMETRY, NEPHELOMETRY, AND RAMAN MEASUREMENT

Absorption spectrophotometry is the measurement of an interaction between electromagnetic radiation and the molecules, or atoms, of a chemical substance. Techniques frequently employed in pharmaceutical analysis include UV, visible, IR, and atomic absorption spectroscopy. Spectrophotometric measurement in the visible region was formerly referred to as *colorimetry*; however, it is more precise to use the term "colorimetry" only when considering human perception of color.

Fluorescence spectrophotometry is the measurement of the emission of light from a chemical substance while it is being exposed to UV, visible, or other electromagnetic radiation. In general, the light emitted by a fluorescent solution is of maximum intensity at a wavelength longer than that of the exciting radiation, usually by some 20 to 30 nm.

Light-Scattering involves measurement of the light scattered because of submicroscopic optical density inhomogeneities of solutions and is useful in the determination of weight-average molecular weights of polydisperse systems in the molecular weight range from 1000 to several hundred million. Two such techniques utilized in pharmaceutical analysis are *turbidimetry* and *nephelometry*.

Raman spectroscopy (inelastic light-scattering) is a light-scattering process in which the specimen under examination is irradiated with intense monochromatic light (usually laser light) and the light scattered from the specimen is analyzed for frequency shifts.

The wavelength range available for these measurements extends from the short wavelengths of the UV through the IR. For convenience of reference, this spectral range is roughly divided into the UV (190 to 380 nm), the visible (380 to 780 nm), the near-IR (780 to 3000 nm), and the IR (2.5 to 40 μm or 4000 to 250 cm^{-1}).

COMPARATIVE UTILITY OF SPECTRAL RANGES

For many pharmaceutical substances, measurements can be made in the UV and visible regions of the spectrum with greater accuracy and sensitivity than in the near-IR and IR. When solutions are observed in 1-cm cells, concentrations of about 10 μg of the specimen per mL often will produce absorbances of 0.2 to 0.8 in the UV or the visible region. In the IR and near-IR, concentrations of 1 to 10 mg per mL and up to 100 mg per mL, respectively, may be needed to produce sufficient absorption; for these spectral ranges, cell lengths of from 0.01 mm to upwards of 3 mm are commonly used.

The UV and visible spectra of substances generally do not have a high degree of specificity. Nevertheless, they are highly suitable for quantitative assays, and for many substances they are useful as additional means of identification.

There has been increasing interest in the use of near-IR spectroscopy in pharmaceutical analysis, especially for rapid identification of large numbers of samples, and also for water determination.

The near-IR region is especially suitable for the determination of $-\text{OH}$ and $-\text{NH}$ groups, such as water in alcohol, $-\text{OH}$ in the presence of amines, alcohols in hydrocarbons, and primary and secondary amines in the presence of tertiary amines.

The IR spectrum is unique for any given chemical compound with the exception of optical isomers, which have identical spectra. However, polymorphism may occasionally be responsible for a difference in the IR spectrum of a given compound in the solid state. Frequently, small differences in structure result in significant differences in the spectra. Because of the large number of maxima in an IR absorption spectrum, it is sometimes possible to quantitatively measure the individual components of a mixture of known qualitative composition without prior separation.

The Raman spectrum and the IR spectrum provide similar data, although the intensities of the spectra are governed by different molecular properties. Raman and IR spectroscopy exhibit different relative sensitivities for different functional groups, e.g., Raman spectroscopy is particularly sensitive to $\text{C}-\text{S}$ and $\text{C}-\text{C}$ multiple bonds, and some aromatic compounds are more easily identified by means of their Raman spectra. Water has a highly intense IR absorption spectrum, but a particularly weak Raman spectrum. Therefore, water has only limited IR "windows" that can be used to examine aqueous solutes, while its Raman spectrum is almost completely transparent and useful for solute identification. The two major limitations of Raman spectroscopy are that the minimum detectable concentration of specimen is typically 10^{-1} M to 10^{-2} M and that the impurities in many substances fluoresce and interfere with the detection of the Raman scattered signal.

Optical reflectance measurements provide spectral information similar to that obtained by transmission measurements. Since reflectance measurements probe only the surface composition of the specimen, difficulties associated with the optical thickness and the light-scattering properties of the substance are eliminated. Thus, reflectance measurements are frequently more simple to perform on intensely absorbing materials. A particularly common technique used

for IR reflectance measurements is termed attenuated total reflectance (ATR), also known as multiple internal reflectance (MIR). In the ATR technique, the beam of the IR spectrometer is passed through an appropriate IR window material (e.g., KRS-5, a TlBr-TlI eutectic mixture), which is cut at such an angle that the IR beam enters the first (front) surface of the window, but is totally reflected when it impinges on the second (back) surface (i.e., the angle of incidence of the radiation upon the second surface of the window exceeds the critical angle for that material). By appropriate window construction, it is possible to have many internal reflections of the IR beam before it is transmitted out of the window. If a specimen is placed in close contact with the window along the sides that totally reflect the IR beam, the intensity of reflected radiation is reduced at each wavelength (frequency) that the specimen absorbs. Thus, the ATR technique provides a reflectance spectrum that has been increased in intensity, when compared to a simple reflectance measurement, by the number of times that the IR beam is reflected within the window. The ATR technique provides excellent sensitivity, but it yields poor reproducibility, and is not a reliable quantitative technique unless an internal standard is intimately mixed with each test specimen.

Fluorescence spectrophotometry is often more sensitive than absorption spectrophotometry. In absorption measurements, the specimen transmittance is compared to that of a blank; and at low concentrations, both solutions give high signals. Conversely, in fluorescence spectrophotometry, the solvent blank has low rather than high output, so that the background radiation that may interfere with determinations at low concentrations is much less. Whereas few compounds can be determined conveniently at concentrations below 10^{-5} M by light absorption, it is not unusual to employ concentrations of 10^{-7} M to 10^{-8} M in fluorescence spectrophotometry.

THEORY AND TERMS

The power of a radiant beam decreases in relation to the distance that it travels through an absorbing medium. It also decreases in relation to the concentration of absorbing molecules or ions encountered in that medium. These two factors determine the proportion of the total incident energy that emerge. The decrease in power of monochromatic radiation passing through a homogeneous absorbing medium is stated quantitatively by Beer's law, $\log_{10}(1/T) = A = abc$, in which the terms are as defined below.

Absorbance [Symbol: A]—The logarithm, to the base 10, of the reciprocal of the transmittance (T). [NOTE—Descriptive terms used formerly include optical density, absorbancy, and extinction.]

Absorptivity [Symbol: a]—The quotient of the absorbance (A) divided by the product of the concentration of the substance (c), expressed in g per L, and the absorption path length (b) in cm. [NOTE—It is not to be confused with absorbancy index; specific extinction; or extinction coefficient.]

Molar Absorptivity [Symbol: ϵ]—The quotient of the absorbance (A) divided by the product of the concentration, expressed in moles per L, of the substance and the absorption path length in cm. It is also the product of the absorptivity (a) and the molecular weight of the substance. [NOTE—Terms formerly used include molar absorbancy index; molar extinction coefficient; and molar absorption coefficient.]

For most systems used in absorption spectrophotometry, the absorptivity of a substance is a constant independent of the intensity of the incident radiation, the internal cell length, and the concentration, with the result that concentration may be determined photometrically.

Beer's law gives no indication of the effect of temperature, wavelength, or the type of solvent. For most analytical work the effects of normal variation in temperature are negligible.

Deviations from Beer's law may be caused by either chemical or instrumental variables. Apparent failure of Beer's law may result from a concentration change in solute molecules because of association between solute molecules or between solute and solvent molecules, or dissociation or ionization. Other deviations might be caused by instrumental effects such as polychromatic radiation, slit-width effects, or stray light.

Even at a fixed temperature in a given solvent, the absorptivity may not be truly constant. However, in the case of specimens having only one absorbing component, it is not necessary that the absorbing system conform to Beer's law for use in quantitative analysis. The concentration of an unknown may be found by comparison with an experimentally determined standard curve.

Although, in the strictest sense, Beer's law does not hold in atomic absorption spectrophotometry because of the lack of quantitative properties of the cell length and the concentration, the absorption processes taking place in the flame under conditions of reproducible aspiration do follow the Beer relationship in principle. Specifically, the negative log of the transmittance, or the absorbance, is directly proportional to the absorption coefficient, and, consequently, is proportional to the number of absorbing atoms. On this basis, calibration curves may be constructed to permit evaluation of unknown absorption values in terms of concentration of the element in solution.

Absorption Spectrum—A graphic representation of absorbance, or any function of absorbance, plotted against wavelength or function of wavelength.

Transmittance [Symbol: T]—The quotient of the radiant power transmitted by a specimen divided by the radiant power incident upon the specimen. [NOTE—Terms formerly used include transmittancy and transmission.]

Fluorescence Intensity [Symbol: I]—An empirical expression of fluorescence activity, commonly given in terms of arbitrary units proportional to detector response. The *fluorescence emission spectrum* is a graphical presentation of the spectral distribution of radiation emitted by an activated substance, showing intensity of emitted radiation as ordinate, and wavelength as abscissa. The *fluorescence excitation spectrum* is a graphical presentation of the activation spectrum, showing intensity of radiation emitted by an activated substance as ordinate, and wavelength of the incident (activating) radiation as abscissa. As in absorption spectrophotometry, the important regions of the electromagnetic spectrum encompassed by the fluorescence of organic compounds are the UV, visible, and near-IR, i.e., the region from 250 to 800 nm. After a molecule has absorbed radiation, the energy can be lost as heat or released in the form of radiation of the same or longer wavelength as the absorbed radiation. Both absorption and emission of radiation are due to the transitions of electrons between different energy levels, or orbitals, of the molecule. There is a time delay between the absorption and emission of light; this interval, the duration of the excited state, has been measured to be about 10^{-9} second to 10^{-8} second for most organic fluorescent solutions. The short lifetime of fluorescence distinguishes this type of luminescence from phosphorescence, which is a long-lived afterglow having a lifetime of 10^{-3} second up to several minutes.

Turbidity [Symbol: S]—The light-scattering effect of suspended particles. The amount of suspended matter may be measured by observation of either the transmitted light (turbidimetry) or the scattered light (nephelometry).

Turbidity [Symbol: τ]—In light-scattering measurements, the turbidity is the measure of the decrease in incident beam intensity per unit length of a given suspension.

Raman Scattering Activity—The molecular property (in units of cm^4 per g) governing the intensity of an observed Raman band for a randomly oriented specimen. The scattering activity is determined from the derivative of the molecular polarizability with respect to the molecular motion giving

rise to the Raman shifted band. In general, the Raman band intensity is linearly proportional to the concentration of the analyte.

USE OF REFERENCE STANDARDS

With few exceptions, the Pharmacopeial spectrophotometric tests and assays call for comparison against a USP Reference Standard. This is to ensure measurement under conditions identical for the test specimen and the reference substance. These conditions include wavelength setting, slit-width adjustment, cell placement and correction, and transmittance levels. It should be noted that cells exhibiting identical transmittance at a given wavelength may differ considerably in transmittance at other wavelengths. Appropriate cell corrections should be established and used where required.

The expressions, "similar preparation" and "similar solution," as used in tests and assays involving spectrophotometry, indicate that the reference specimen, generally a USP Reference Standard, is to be prepared and observed in a manner identical for all practical purposes to that used for the test specimen. Usually in making up the solution of the specified Reference Standard, a solution of about (i.e., within 10%) the desired concentration is prepared and the absorptivity is calculated on the basis of the exact amount weighed out; if a previously dried specimen of the Reference Standard has not been used, the absorptivity is calculated on the anhydrous basis.

The expressions, "concomitantly determine" and "concomitantly measured," as used in tests and assays involving spectrophotometry, indicate that the absorbances of both the solution containing the test specimen and the solution containing the reference specimen, relative to the specified test blank, are to be measured in immediate succession.

APPARATUS

Many types of spectrophotometers are available. Fundamentally, most types, except those used for IR spectrophotometry, provide for passing essentially monochromatic radiant energy through a specimen in suitable form, and measuring the intensity of the fraction that is transmitted. Fourier transform IR spectrophotometers use an interferometric technique whereby polychromatic radiation passes through the analyte and onto a detector on an intensity and time basis. UV, visible, and dispersive IR spectrophotometers comprise an energy source, a dispersing device (e.g., a prism or grating), slits for selecting the wavelength band, a cell or holder for the test specimen, a detector of radiant energy, and associated amplifiers and measuring devices. In *diode array* spectrophotometers, the energy from the source is passed through the test specimen and then dispersed via a grating onto several hundred light-sensitive diodes, each of which in turn develops a signal proportional to the number of photons at its small wavelength interval; these signals then may be computed at rapid chosen intervals to represent a complete spectrum. Fourier transform IR systems utilize an interferometer instead of a dispersing device and a digital computer to process the spectral data. Some instruments are manually operated, whereas others are equipped for automatic and continuous recording. Instruments that are interfaced to a digital computer have the capabilities also of co-adding and storing spectra, performing spectral comparisons, and performing difference spectroscopy (accomplished with the use of a digital absorbance subtraction method).

Instruments are available for use in the visible; in the visible and UV; in the visible, UV, and near-IR; and in the IR regions of the spectrum. Choice of the type of spectrophotometric analysis and of the instrument to be used depends upon factors such as the composition and amount of available test specimen, the degree of accuracy, sensitivity, and

selectivity desired, and the manner in which the specimen is handled.

The apparatus used in atomic absorption spectrophotometry has several unique features. For each element to be determined, a specific source that emits the spectral line to be absorbed should be selected. The source is usually a hollow-cathode lamp, the cathode of which is designed to emit the desired radiation when excited. Since the radiation to be absorbed by the test specimen element is usually of the same wavelength as that of its emission line, the element in the hollow-cathode lamp is the same as the element to be determined. The apparatus is equipped with an aspirator for introducing the test specimen into a flame, which is usually provided by air-acetylene, air-hydrogen, or, for refractory cases, nitrous oxide-acetylene. The flame, in effect, is a heated specimen chamber. A detector is used to read the signal from the chamber. Interfering radiation produced by the flame during combustion may be negated by the use of a chopped source lamp signal of a definite frequency. The detector should be tuned to this alternating current frequency so that the direct current signal arising from the flame is ignored. The detecting system, therefore, reads only the change in signal from the hollow-cathode source, which is directly proportional to the number of atoms to be determined in the test specimen. For Pharmacopeial purposes, apparatus that provides the readings directly in absorbance units is usually required. However, instruments providing readings in percent transmission, percent absorption, or concentration may be used if the calculation formulas provided in the individual monographs are revised as necessary to yield the required quantitative results. Percent absorption or percent transmittance may be converted to absorbance, A , by the following two equations:

$$A = 2 - \log_{10} (100 - \% \text{ absorption})$$

or:

$$A = 2 - \log_{10} (\% \text{ transmittance})$$

Depending upon the type of apparatus used, the readout device may be a meter, digital counter, recorder, or printer. Both single-beam and double-beam instruments are commercially available, and either type is suitable.

Measurement of fluorescence intensity can be made with a simple *filter fluorometer*. Such an instrument consists of a radiation source, a primary filter, a specimen chamber, a secondary filter, and a fluorescence detection system. In most such fluorometers, the detector is placed on an axis at 90° from that of the exciting beam. This right-angle geometry permits the exciting radiation to pass through the test specimen and not contaminate the output signal received by the fluorescence detector. However, the detector unavoidably receives some of the exciting radiation as a result of the inherent scattering properties of the solutions themselves, or if dust or other solids are present. Filters are used to eliminate this residual scatter. The primary filter selects short-wavelength radiation capable of exciting the test specimen, while the secondary filter is normally a sharp cut-off filter that allows the longer-wavelength fluorescence to be transmitted but blocks the scattered excitation.

Most fluorometers use photomultiplier tubes as detectors, many types of which are available, each having special characteristics with respect to spectral region of maximum sensitivity, gain, and electrical noise. The photocurrent is amplified and read out on a meter or recorder.

A *spectrofluorometer* differs from a filter fluorometer in that filters are replaced by monochromators, of either the prism or the grating type. For analytical purposes, the spectrofluorometer is superior to the filter fluorometer in wavelength selectivity, flexibility, and convenience, in the same way in which a spectrophotometer is superior to a filter photometer.

Many radiation sources are available. Mercury lamps are relatively stable and emit energy mainly at discrete wave-

lengths. Tungsten lamps provide an energy continuum in the visible region. The high-pressure xenon arc lamp is often used in spectrofluorometers because it is a high-intensity source that emits an energy continuum extending from the UV into the IR.

In spectrofluorometers, the monochromators are equipped with slits. A narrow slit provides high resolution and spectral purity, while a large slit sacrifices these for high sensitivity. Choice of slit size is determined by the separation between exciting and emitting wavelengths as well as the degree of sensitivity needed.

Specimen cells used in fluorescence measurements may be round tubes or rectangular cells similar to those used in absorption spectrophotometry, except that they are polished on all four vertical sides. A convenient test specimen size is 2 to 3 mL, but some instruments can be fitted with small cells holding 100 to 300 μ L, or with a capillary holder requiring an even smaller amount of specimen.

Light-scattering instruments are available and consist in general of a mercury lamp, with filters for the strong green or blue lines, a shutter, a set of neutral filters with known transmittance, and a sensitive photomultiplier to be mounted on an arm that can be rotated around the solution cell and set at any angle from -135° to 0° to +135° by a dial outside the light-tight housing. Solution cells are of various shapes, such as square for measuring 90° scattering; semioctagonal for 45°, 90°, and 135° scattering; and cylindrical for scattering at all angles. Since the determination of molecular weight requires a precise measure of the difference in refractive index between the solution and solvent, $[(n - n_0)/c]$, a second instrument, a differential refractometer, is needed to measure this small difference.

Raman spectrometers include the following major components: a source of intense monochromatic radiation (invariably a laser); optics to collect the light scattered by the test specimen; a (double) monochromator to disperse the scattered light and reject the intense incident frequency; and a suitable light-detection and amplification system. Raman measurement is simple in that most specimens are examined directly in melting-point capillaries. Because the laser source can be focused sharply, only a few microliters of the specimen is required.

PROCEDURE

Absorption Spectrophotometry

Detailed instructions for operating spectrophotometers are supplied by the manufacturers. To achieve significant and valid results, the operator of a spectrophotometer should be aware of its limitations and of potential sources of error and variation. The instruction manual should be followed closely on such matters as care, cleaning, and calibration of the instrument, and techniques of handling absorption cells, as well as instructions for operation. The following points require special emphasis.

Check the instrument for accuracy of calibration. Where a continuous source of radiant energy is used, attention should be paid to both the wavelength and photometric scales; where a spectral line source is used, only the photometric scale need be checked. A number of sources of radiant energy have spectral lines of suitable intensity, adequately spaced throughout the spectral range selected. The best single source of UV and visible calibration spectra is the quartz-mercury arc, of which the lines at 253.7, 302.25, 313.16, 334.15, 365.48, 404.66, and 435.83 nm may be used. The glass-mercury arc is equally useful above 300 nm. The 486.13-nm and 656.28-nm lines of a hydrogen discharge lamp may be used also. The wavelength scale may be calibrated also by means of suitable glass filters, which have useful absorption bands through the visible and UV regions. Standard glasses containing didymium (a mixture

of praseodymium and neodymium) have been used widely, although glasses containing holmium were found to be superior. Standard holmium oxide solution has superseded the use of holmium glass.¹ The wavelength scales of near-IR and IR spectrophotometers are readily checked by the use of absorption bands provided by polystyrene films, carbon dioxide, water vapor, or ammonia gas.

For checking the photometric scale, a number of standard inorganic glass filters as well as standard solutions of known transmittances such as potassium dichromate are available.²

Quantitative absorbance measurements usually are made on solutions of the substance in liquid-holding cells. Since both the solvent and the cell window absorb light, compensation must be made for their contribution to the measured absorbance. Matched cells are available commercially for UV and visible spectrophotometry for which no cell correction is necessary. In IR spectrophotometry, however, corrections for cell differences usually must be made. In such cases, pairs of cells are filled with the selected solvent and the difference in their absorbances at the chosen wavelength is determined. The cell exhibiting the greater absorbance is used for the solution of the test specimen and the measured absorbance is corrected by subtraction of the cell difference.

With the use of a computerized Fourier transform IR system, this correction need not be made, since the same cell can be used for both the solvent blank and the test solution. However, it must be ascertained that the transmission properties of the cell are constant.

Comparisons of a test specimen with a Reference Standard are best made at a peak of spectral absorption for the compound concerned. Assays prescribing spectrophotometry give the commonly accepted wavelength for peak spectral absorption of the substance in question. It is known that different spectrophotometers may show minor variation in the apparent wavelength of this peak. Good practice demands that comparisons be made at the wavelength at which peak absorption occurs. Should this differ by more than ± 1 nm from the wavelength specified in the individual monograph, recalibration of the instrument may be indicated.

TEST PREPARATION

For determinations utilizing UV or visible spectrophotometry, the specimen generally is dissolved in a solvent. Unless otherwise directed in the monograph, determinations are made at room temperature using a path length of 1 cm. Many solvents are suitable for these ranges, including water, alcohols, chloroform, lower hydrocarbons, ethers, and dilute solutions of strong acids and alkalis. Precautions should be taken to utilize solvents free from contaminants absorbing in the spectral region being used. It is usually advisable to use water-free methanol or alcohol, or alcohol denatured by the addition of methanol but not containing benzene or other interfering impurities, as the solvent. Solvents of special spectrophotometric quality, guaranteed to be free from contaminants, are available commercially from several sources. Some other analytical reagent-grade organic solvents may contain traces of impurities that absorb strongly in the UV region. New lots of these solvents should be checked for their transparency, and care should be taken to use the same lot of solvent for preparation of the test solution and the standard solution and for the blank.

No solvent in appreciable thickness is completely transparent throughout the near-IR and IR spectrum. Carbon tetra-

chloride (up to 5 mm in thickness) is practically transparent to $6\text{ }\mu\text{m}$ (1666 cm^{-1}). Carbon disulfide (1 mm in thickness) is suitable as a solvent to $40\text{ }\mu\text{m}$ (250 cm^{-1}) with the exception of the $4.2\text{-}\mu\text{m}$ to $5.0\text{-}\mu\text{m}$ (2381-cm^{-1} to 2000-cm^{-1}) and the $5.5\text{-}\mu\text{m}$ to $7.5\text{-}\mu\text{m}$ (1819-cm^{-1} to 1333-cm^{-1}) regions, where it has strong absorption. Other solvents have relatively narrow regions of transparency. For IR spectrophotometry, an additional qualification for a suitable solvent is that it must not affect the material, usually sodium chloride, of which the cell is made. The test specimen may also be prepared by dispersing the finely ground solid specimen in mineral oil or by mixing it intimately with previously dried alkali halide salt (usually potassium bromide). Mixtures with alkali halide salts may be examined directly or as transparent disks or pellets obtained by pressing the mixture in a die. Typical drying conditions for potassium bromide are 105° in vacuum for 12 hours, although grades are commercially available that require no drying. Infrared microscopy or a mineral oil dispersion is preferable where disproportionation between the alkali halide and the test specimen is encountered. For suitable materials the test specimen may be prepared neat as a thin sample for IR microscopy or suspended neat as a thin film for mineral oil dispersion. For Raman spectrometry, most common solvents are suitable, and ordinary (nonfluorescing) glass specimen cells can be used. The IR region of the electromagnetic spectrum extends from 0.8 to $400\text{ }\mu\text{m}$. From 800 to 2500 nm (0.8 to $2.5\text{ }\mu\text{m}$) is generally considered to be the near-IR (NIR) region; from 2.5 to $25\text{ }\mu\text{m}$ (4000 to 400 cm^{-1}) is generally considered to be the mid-range (mid-IR) region; and from 25 to $400\text{ }\mu\text{m}$ is generally considered to be the far-IR (FIR) region. Unless otherwise specified in the individual monograph, the region from 3800 to 650 cm^{-1} (2.6 to $15\text{ }\mu\text{m}$) should be used to ascertain compliance with monograph specifications for IR absorption.

Where values for IR line spectra are given in an individual monograph, the letters s, m, and w signify strong, medium, and weak absorption, respectively; sh signifies a shoulder, bd signifies a band, and v means very. The values may vary as much as $0.1\text{ }\mu\text{m}$ or 10 cm^{-1} , depending upon the particular instrument used. Polymorphism gives rise to variations in the IR spectra of many compounds in the solid state. Therefore, when conducting IR absorption tests, if a difference appears in the IR spectra of the analyte and the standard, dissolve equal portions of the test substance and the standard in equal volumes of a suitable solvent, evaporate the solutions to dryness in similar containers under identical conditions, and repeat the test on the residues.

In NIR spectroscopy much of the current interest centers around the ease of analysis. Samples can be analyzed in powder form or by means of reflectance techniques, with little or no preparation. Compliance with in-house specifications can be determined by computerized comparison of spectra with spectra previously obtained from reference materials. Many pharmaceutical materials exhibit low absorptivity in this spectral region, which allows incident near-IR radiation to penetrate samples more deeply than UV, visible, or IR radiation. NIR spectrophotometry may be used to observe matrix modifications and, with proper calibration, may be used in quantitative analysis.

In atomic absorption spectrophotometry, the nature of the solvent and the concentration of solids must be given special consideration. An ideal solvent is one that interferes to a minimal extent in the absorption or emission processes and one that produces neutral atoms in the flame. If there is a significant difference between the surface tension or viscosity of the test solution and standard solution, the solutions are aspirated or atomized at a different rate, causing significant differences in the signals generated. The acid concentration of the solutions also affects the absorption processes. Thus, the solvents used in preparing the test specimen and the standard should be the same or as much alike in these respects as possible, and should yield solutions that are easily aspirated via the specimen tube of the burner-aspirator. Since undissolved solids present in the so-

¹ National Institute of Standards and Technology (NIST), Gaithersburg, MD 20899: "Spectral Transmittance Characteristics of Holmium Oxide in Perchloric Acid," *J. Res. Natl. Bur. Stds.* **90**, No. 2, 115 (1985). The performance of an uncertified filter should be checked against a certified standard.

² For further detail regarding checks on photometric scale of a spectrophotometer, reference may be made to the following NIST publications: *J. Res. Natl. Bur. Stds.* **76A**, 469 (1972) [re: SRM 931, "Liquid Absorbance Standards for Ultraviolet and Visible Spectrophotometry"]; as well as potassium chromate and potassium dichromate; *NIST Spec. Publ.* 260-116 (1994) [re: SRM 930 and SRM 1930, "Glass Filters for Spectrophotometry."]

lutions may give rise to matrix or bulk interferences, the total undissolved solids content in all solutions should be kept below 2% wherever possible.

CALCULATIONS

The application of absorption spectrophotometry in an assay or a test generally requires the use of a Reference Standard. Where such a measurement is specified in an assay, a formula is provided in order to permit calculation of the desired result. A numerical constant is frequently included in the formula. The following derivation is provided to introduce a logical approach to the deduction of the constants appearing in formulas in the assays in many monographs.

The Beer's law relationship is valid for the solutions of both the Reference Standard (S) and the test specimen (U):

$$A_S = abC_S \quad (1)$$

$$A_U = abC_U \quad (2)$$

in which A_S is the absorbance of the Standard solution of concentration C_S ; and A_U is the absorbance of the test specimen solution of concentration C_U . If C_S and C_U are expressed in the same units and the absorbances of both solutions are measured in matching cells having the same dimensions, the absorptivity, a , and the cell thickness, b , are the same; consequently, the two equations may be combined and rewritten to solve for C_U :

$$C_U = C_S(A_U/A_S) \quad (3)$$

Quantities of solid test specimens to be taken for analysis are generally specified in mg. Instructions for dilution are given in the assay and, since dilute solutions are used for absorbance measurements, concentrations are usually expressed for convenience in units of μg per mL. Taking a quantity, in mg, of a test specimen of a drug substance or solid dosage form for analysis, it therefore follows that a volume (V_U), in L, of solution of concentration C_U may be prepared from the amount of test specimen that contains a quantity W_U , in mg, of the drug substance [NOTE— C_U is numerically the same whether expressed as μg per mL or mg per L], such that:

$$W_U = V_U C_U \quad (4)$$

The form in which the formula appears in the assay in a monograph for a solid article may be derived by substituting C_U of equation (3) into equation (4). In summary, the use of equation (4), with due consideration for any unit conversions necessary to achieve equality in equation (5), permits the calculation of the constant factor (V_U) occurring in the final formula:

$$W_U = V_U C_S(A_U/A_S) \quad (5)$$

The same derivation is applicable to formulas that appear in monographs for liquid articles that are assayed by absorption spectrophotometry. For liquid dosage forms, results of calculations are generally expressed in terms of the quantity, in mg, of drug substance in each mL of the article. Thus it is necessary to include in the denominator an additional term, the volume (V), in mL, of the test preparation taken.

Assays in the visible region usually call for comparing concomitantly the absorbance produced by the *Assay preparation* with that produced by a *Standard preparation* containing approximately an equal quantity of a USP Reference Standard. In some situations, it is permissible to omit the use of a Reference Standard. This is true where spectrophotometric assays are made with routine frequency, and where a suitable standard curve is available, prepared with the respective USP Reference Standard, and where the substance

assayed conforms to Beer's law within the range of about 75% to 125% of the final concentration used in the assay. Under these circumstances, the absorbance found in the assay may be interpolated on the standard curve, and the assay result calculated therefrom.

Such standard curves should be confirmed frequently, and always when a new spectrophotometer or new lots of reagents are put into use.

In spectrophotometric assays that direct the preparation and use of a standard curve, it is permissible and preferable, when the assay is employed infrequently, not to use the standard curve but to make the comparison directly against a quantity of the Reference Standard approximately equal to that taken of the specimen, and similarly treated.

Fluorescence Spectrophotometry

The measurement of fluorescence is a useful analytical technique. *Fluorescence* is light emitted from a substance in an excited state that has been reached by the absorption of radiant energy. A substance is said to be *fluorescent* if it can be made to fluoresce. Many compounds can be assayed by procedures utilizing either their inherent fluorescence or the fluorescence of suitable derivatives.

Test specimens prepared for fluorescence spectrophotometry are usually one-tenth to one-hundredth as concentrated as those used in absorption spectrophotometry, for the following reason. In analytical applications, it is preferable that the fluorescence signal be linearly related to the concentration; but if a test specimen is too concentrated, a significant part of the incoming light is absorbed by the specimen near the cell surface, and the light reaching the center is reduced. That is, the specimen itself acts as an "inner filter." However, fluorescence spectrophotometry is inherently a highly sensitive technique, and concentrations of 10^{-5} M to 10^{-7} M frequently are used. It is necessary in any analytical procedure to make a working curve of fluorescence intensity versus concentration in order to establish a linear relationship. All readings should be corrected for a solvent blank.

Fluorescence measurements are sensitive to the presence of dust and other solid particles in the test specimen. Such impurities may reduce the intensity of the exciting beam or give misleading high readings because of multiple reflections in the specimen cell. It is, therefore, wise to eliminate solid particles by centrifugation; filtration also may be used, but some filter papers contain fluorescent impurities.

Temperature regulation is often important in fluorescence spectrophotometry. For some substances, fluorescence efficiency may be reduced by as much as 1% to 2% per degree of temperature rise. In such cases, if maximum precision is desired, temperature-controlled specimen cells are useful. For routine analysis, it may be sufficient to make measurements rapidly enough so that the specimen does not heat up appreciably from exposure to the intense light source. Many fluorescent compounds are light-sensitive. Exposed in a fluorometer, they may be photo-degraded into more or less fluorescent products. Such effects may be detected by observing the detector response in relationship to time, and may be reduced by attenuating the light source with filters or screens.

Change of solvent may markedly affect the intensity and spectral distribution of fluorescence. It is inadvisable, therefore, to alter the solvent specified in established methods without careful preliminary investigation. Many compounds are fluorescent in organic solvents but virtually non-fluorescent in water; thus, a number of solvents should be tried before it is decided whether or not a compound is fluorescent. In many organic solvents, the intensity of fluorescence is increased by elimination of dissolved oxygen, which has a strong quenching effect. Oxygen may be removed by bubbling an inert gas such as nitrogen or helium through the test specimen.

A semiquantitative measure of the strength of fluorescence is given by the ratio of the fluorescence intensity of a

test specimen and that of a standard obtained with the same instrumental settings. Frequently, a solution of stated concentration of quinine in 0.1 N sulfuric acid or fluorescein in 0.1 N sodium hydroxide is used as a reference standard.

Light-Scattering

Turbidity can be measured with a standard photoelectric filter photometer or spectrophotometer, preferably with illumination in the blue portion of the spectrum. Nephelometric measurements require an instrument with a photocell placed so as to receive scattered rather than transmitted light; this geometry applies also to fluorometers, so that, in general, fluorometers can be used as nephelometers, by proper selection of filters. A ratio turbidimeter combines the technology of 90° nephelometry and turbidimetry: it contains photocells that receive and measure scattered light at a 90° angle from the sample as well as receiving and measuring the forward scatter in front of the sample; it also measures light transmitted directly through the sample. Linearity is attained by calculating the ratio of the 90° angle scattered light measurement to the sum of the forward scattered light measurement and the transmitted light measurement. The benefit of using a ratio turbidimetry system is that the measurement of stray light becomes negligible.

In practice, it is advisable to ensure that settling of the particles being measured is negligible. This is usually accomplished by including a protective colloid in the liquid suspending medium. It is important that results be interpreted by comparison of readings with those representing known concentrations of suspended matter, produced under precisely the same conditions.

Turbidimetry or nephelometry may be useful for the measurement of precipitates formed by the interaction of highly dilute solutions of reagents, or other particulate matter, such as suspensions of bacterial cells. In order that consistent results may be achieved, all variables must be carefully controlled. Where such control is possible, extremely dilute suspensions may be measured.

The specimen solute is dissolved in the solvent at several different accurately known concentrations, the choice of concentrations being dependent on the molecular weight of the solute and ranging from 1% for $M_w = 10,000$ to 0.01% for $M_w = 1,000,000$. Each solution must be very carefully cleaned before measurement by repeated filtration through fine filters. A dust particle in the solution vitiates the intensity of the scattered light measured. A criterion for a clear solution is that the dissymmetry, 45°/135° scattered intensity ratio, has attained a minimum.

The turbidity and refractive index of the solutions are measured. From the general 90° light-scattering equation, a plot of HC/τ versus C is made and extrapolated to infinite dilution, and the weight-average molecular weight, M , is calculated from the intercept, $1/M$.

Visual Comparison

Where a color or a turbidity comparison is directed, color-comparison tubes that are matched as closely as possible in internal diameter and in all other respects should be used. For color comparison, the tubes should be viewed downward, against a white background, with the aid of a light source directed from beneath the bottoms of the tubes, while for turbidity comparison the tubes should be viewed horizontally, against a dark background, with the aid of a light source directed from the sides of the tubes.

In conducting limit tests that involve a comparison of colors in two like containers (e.g., matched color-comparison

tubes), a suitable instrument, rather than the unaided eye, may be used.

(861) SUTURES—DIAMETER

The gauge for determining the diameter of sutures is of the dead-weight type, mechanical or electrical, and equipped with a direct-reading dial, a digital readout, or a printed readout. Use a gauge graduated to 0.002 mm or smaller. The anvil of the gauge is about 50 mm in diameter, and the presser foot is 12.70 ± 0.02 mm in diameter. The presser foot and moving parts connected therewith are weighted so as to apply a total load of 210 ± 3 g to the specimen. The presser foot and anvil surfaces are plane to within 0.005 mm and parallel to each other to within 0.005 mm. For measuring the diameter of sutures of metric size 0.4 and smaller, remove the additional weight from the presser foot so that the total load on the suture does not exceed 60 g.

Collagen Absorbable Surgical Suture—Determine the diameter immediately after removal from the immediate container and without stretching. Lay the strand across the center of the anvil and presser foot, and gently lower the foot until its entire weight rests upon the suture. Measure the diameter of each strand at three points corresponding roughly to one-fourth, one-half, and three-fourths of its length.

Synthetic Absorbable Surgical Suture—Proceed as directed for *Nonabsorbable Surgical Suture*.

Nonabsorbable Surgical Suture—Lay the strand across the center of the anvil and presser foot, and gently lower the foot until its entire weight rests upon the suture. Measure nonabsorbable sutures, whether packaged in dry form or in fluid, immediately after removal from the container, without prior drying or conditioning.

Measure the diameter of the suture at three points corresponding roughly to one-fourth, one-half, and three-fourths of its length. In the case of braided suture of sizes larger than 3-0 (metric size 2), make two measurements at each point at right angles to each other, and use the average as the observed diameter at that point.

In measuring multifilament sutures, attach a portion of the designated section of the strand in a fixed clamp in such a way that the strand lies across the center of the anvil. While holding the strand in the same plane as the surface of the anvil, place the strand under tension by suitable means, such as by passing the free end of the strand around a cylinder or a pulley and attaching to the free end a weight of about one-half of the knot-pull limit for the non-sterilized Class I suture of the size concerned, taking care not to permit the strand, if twisted, to untwist. Measure the diameter at the designated points on the strand, and calculate the average diameter likewise as directed.

(871) SUTURES—NEEDLE ATTACHMENT

Absorbable (collagen) surgical sutures and nonabsorbable surgical sutures with *Standard Needle Attachment* are such

that the needles are firmly attached and are not intended to be separated. Sutures supplied with eyeless needles attached fall into either the category of *Standard Needle Attachment* or the category of *Removable Needle Attachment*. *Removable Needle Attachment* of both absorbable and nonabsorbable surgical sutures is such that the needle may be deliberately separated from the suture by means of a quick tug. Both types of attachments are tested on equipment as specified under *Tensile Strength* <881>.

Procedure—Clamp each of 5 sutures in the tensilometer so that the needle is in the fixed clamp with all of the swaged portion exposed and in line with the direction of force applied to the suture by the moving clamp. Determine the force required to detach the suture from the needle. In

the case of *Standard Needle Attachment*, the suture may break without needle detachment.

Standard Needle Attachment—The requirements are met if neither the average of the 5 values nor any individual value is less than the limit given for the designated size in *Table 1*.

Removable Needle Attachment—The requirements are met if the individual values of the 5 sutures are within the limits shown in *Table 2*. [NOTE—For either type of attachment, if not more than 1 of the individual values falls outside the prescribed limits, repeat the test on an additional 10 sutures: the requirements of the test are met if none of the 10 additional values falls outside the individual limit requirements.]

Table 1. Standard Needle Attachment for Absorbable and Nonabsorbable Sutures

Metric Size (Gauge No.)		Limits on Needle Attachment				
Absorbable Collagen Suture	Nonabsorbable and Synthetic Absorbable Sutures	USP Size	Average (in kgf) (Min.)	Individual (in kgf) (Min.)	Average (in N) (Min.)	Individual (in N) (Min.)
	0.1	11-0	0.007	0.005	0.069	0.049
	0.2	10-0	0.014	0.010	0.137	0.098
0.4	0.3	9-0	0.021	0.015	0.206	0.147
0.5	0.4	8-0	0.050	0.025	0.490	0.245
0.7	0.5	7-0	0.080	0.040	0.784	0.392
1	0.7	6-0	0.17	0.08	1.67	0.784
1.5	1	5-0	0.23	0.11	2.25	1.08
2	1.5	4-0	0.45	0.23	4.41	2.25
3	2	3-0	0.68	0.34	6.67	3.33
3.5	3	2-0	1.10	0.45	10.8	4.41
4	3.5	0	1.50	0.45	14.7	4.41
5	4	1	1.80	0.60	17.6	5.88
6 and larger	5 and larger	2 and larger	1.80	0.70	17.6	6.86

Table 2. Removable Needle Attachment for Absorbable and Nonabsorbable Sutures

Metric Size (Gauge No.)		Limits on Needle Attachment				
Absorbable Collagen Suture	Nonabsorbable and Synthetic Absorbable Sutures	USP Size	Minimum (in kgf)	Maximum (in kgf)	Minimum (in N)	Maximum (in N)
1.5	1	5-0	0.028	1.59	0.274	15.6
2	1.5	4-0	0.028	1.59	0.274	15.6
3	2	3-0	0.028	1.59	0.274	15.6
3.5	3	2-0	0.028	1.59	0.274	15.6
4	3.5	0	0.028	1.59	0.274	15.6
5	4	1	0.028	1.59	0.274	15.6
6	5	2	0.028	1.59	0.274	15.6

(881) TENSILE STRENGTH

Devices for measurement of tensile strength used in the United States may be calibrated in the English units of measure. The following directions are given in metric units with the understanding that the corresponding English equivalents may be used.

Surgical Suture

Determine the tensile strength of surgical suture on a motor-driven tensile strength testing machine having suitable clamps for holding the specimen firmly and using either the principle of constant rate of load on specimen or the principle of constant rate of elongation of specimen, as described below. The apparatus has two clamps for holding the strand. One of these clamps is mobile. The clamps are designed so that the strand being tested can be attached without any possibility of slipping. Gauge length is defined as the interior distance between the two clamps. For gauge lengths of 125 to 200 mm, the mobile clamp is driven at a constant rate of elongation of 30 ± 5 cm per minute. For gauge lengths of less than 125 mm, the rate of elongation per minute is adjusted to equal 2 times the gauge length per minute. For example, a 5-cm gauge length has a rate of elongation of 10 cm per minute.

Determine the tensile strength of the suture, whether packaged in dry form or in fluid, promptly after removal from the container, without prior drying or conditioning. Attach one end of the suture to the clamp at the load end of the machine, pass the other end through the opposite clamp, applying sufficient tension so that the specimen is taut between the clamps, and engage the second clamp. Perform as many breaks as are specified in the individual monograph. If the break occurs at the clamp, discard the reading on the specimen.

Procedure for a machine operating on the principle of constant rate of load on specimen—This description applies to the machine known as the Incline Plane Tester.

The carriage used in any test is of a weight such that when the break occurs, the position of the recording pen on the chart is between 20% and 80% of the capacity that may be recorded on the chart. The friction in the carriage is low enough to permit the recording pen to depart from the zero line of the chart at a point not exceeding 2.5% of the capacity of the chart when no specimen is held in the clamps.

For surgical sutures of intermediate and larger sizes, the clamp for holding the specimen is of the roll type, with a flat gripping surface. The roll has a diameter of 19 mm and the flat gripping surface is not less than 25 mm in length. The length of the specimen, when inserted in the clamps, is at least 127 mm from nip to nip. The speed of inclination of the plane of the tester is such that it reaches its full inclination of 30° from the horizontal in 20 ± 1 seconds from the start of the test.

For surgical sutures of small sizes, the suitable clamp has a flat gripping surface of not less than 13 mm in length. The speed of inclination of the plane is such that it reaches its full inclination of 30° from the horizontal in 60 ± 5 seconds from the start of the test.

Except where straight pull (no knot required) is indicated in the suture monograph, tie the test suture into a surgeon's knot with one turn of suture around flexible rubber tubing of 6.5-mm inside diameter and 1.6-mm wall thickness. The surgeon's knot is a square knot in which the free end is first passed twice, instead of once, through the loop, and pulled taut, then passed once through a second loop, and the ends are drawn taut so that a single throw is superimposed upon a double throw. Start the first knot with the left end over the right end, exerting sufficient tension to tie

the knot securely. Where the test specimen includes a knot, place the specimen in the testing device with the knot approximately midway between the clamps. Leave the flexible rubber tubing in place for the duration of the test.

Procedure for a machine operating on the principle of constant rate of elongation of specimen—This description applies to any suitable tensile testing machine that operates on the principle of constant rate of elongation of specimen.

Except where straight pull (no knot required) is indicated in the suture monographs, tie the test suture into a simple knot formed by placing one end of a strand held in the right hand over the other end held in the left hand, passing one end over the strand and through the loop so formed, and pulling the knot tight. The specimen is placed in the testing device with the knot approximately midway between the clamps.

Textile Fabrics and Films

Determine the tensile strength of textile fabrics, including adhesive tape, on a constant-speed or pendulum type of testing machine of the following general description.

The clamps for holding the specimen are smooth, flat, parallel jaws that are not less than 25 mm in length in the dimension parallel to the direction of application of the load. When the width of the strip being tested does not exceed 19 mm, the jaws of the clamp should be at least 25 mm wide. If the width of the strip is greater than 19 mm and not greater than 44 mm, the width of the jaws of the clamp should be at least 50 mm. If the width of the specimen is greater than 44 mm, cut a 25-mm strip, and use a clamp with jaws not less than 50 mm wide. Round all edges that might have a cutting action on the specimen to a radius of 0.4 mm. The jaws are 76.2 mm apart at the beginning of the test, and they separate at the rate of $30.5 \text{ cm} \pm 13 \text{ mm}$ per minute. The machine is of such capacity that when the break occurs, the deviation of the pendulum from the vertical is between 9° and 45° .

(891) THERMAL ANALYSIS

INTRODUCTION

Precisely determined thermodynamic events, such as a change of state, can indicate the identity and purity of drugs. Compendial standards have long been established for the melting or boiling temperatures of substances. These transitions occur at characteristic temperatures, and the compendial standards therefore contribute to the identification of the substances. Because impurities affect these changes in predictable ways, the same compendial standards contribute to the control of the purity of the substances.

Thermal analysis in the broadest sense is the measurement of physical-chemical properties of materials as a function of temperature. Instrumental methods have largely supplanted older methods dependent on visual inspection and measurements under fixed or arbitrary conditions, because they are objective, provide more information, afford permanent records, and are generally more sensitive, precise, and accurate. Furthermore, they may provide information on desolvation, dehydration, decomposition, crystal perfection, polymorphism, melting temperature, sublimation, glass transitions, evaporation, pyrolysis, solid-solid interactions, and purity. Such data are useful in the characterization of sub-

stances with respect to compatibility, stability, packaging, and quality control. The measurements used most often in thermal analysis, i.e., transition and melting point temperatures by differential scanning calorimetry (DSC), thermogravimetric analysis, hot-stage microscopy, and eutectic impurity analysis, are described here.

TRANSITION AND MELTING POINT TEMPERATURES

As a specimen is heated, transitions can be observed using differential scanning calorimetry (DSC), differential thermal analysis (DTA), or hot-stage microscopy. In heat-flux DSC, the heat differential between the sample and reference material is determined. In power compensation DSC, the sample and reference materials are maintained at the same temperature, using individual heating elements, and the difference in power input to the two heaters is recorded. DTA monitors the difference in temperatures between the sample and the reference. The transitions that may be observed include those shown in *Table 1* below. In the case of melting, both an "onset" and a "peak" temperature can be determined objectively and reproducibly, often to within a few tenths of a degree. Although these temperatures are useful for characterizing substances, and the difference between the two temperatures is indicative of purity, the values cannot be directly compared to visual "melting-range" or "melting-point" values or with constants such as the triple point of the pure material.

Furthermore, caution should be used when comparing results obtained by different methods of analysis. Optical methods may measure the melting point as the temperature where the last trace of solid coalesces. In contrast, melting points measured by DSC may refer to the onset temperature or the temperature where the maximum melting rate (vertex) was observed. However, the vertex is sensitive to sample weight, heating rate, and other factors, whereas the onset temperature is less affected by these factors. With thermal techniques, it is necessary to consider the limitations of solid solution formation, insolubility in the melt, polymorphism, and decomposition during the analysis.

Table 1

Solid to liquid	Melting	Endothermic
Liquid to gas	Vaporization	Endothermic
Liquid to solid	Freezing	Exothermic
	Crystallization	Exothermic
Solid to gas	Sublimation	Endothermic
Solid to solid	Glass transition	Second order event
	Desolvation	Endothermic
	Amorphous to crystalline	Exothermic
	Polymorphic	Endothermic or Exothermic

Reporting Results of Instrumental Methods—A complete description of the conditions employed should accompany each thermogram, including make and model of instrument; record of last calibration; specimen size and identification (including previous thermal history); container; identity, flow rate, and pressure of gaseous atmosphere; direction and rate of temperature change; and instrument and recorder sensitivity.

DETERMINATION OF TRANSITION TEMPERATURE (MELT ONSET TEMPERATURE) AND MELTING POINT TEMPERATURE

Apparatus—Use DTA or DSC instrumentation equipped with a temperature-programming device, thermal detector(s), and a recording system that can be connected to a computer, unless otherwise prescribed by the specific monograph for which this chapter is being used.

Calibration—Calibrate instrumentation for temperature and enthalpy changes, using indium or another suitable certified material. Temperature calibration is performed by heating a standard through the melting transition and comparing the extrapolated onset of melting point of the standard to the certified onset of melting point. The temperature calibration should be conducted at the same heating rate as the experiment. Enthalpy calibration is performed by heating a standard through the melting transition and comparing the calculated heat of fusion to the theoretical value.

Procedure—Accurately weigh an appropriate quantity of the substance to be examined in the sample pan, as described in the specific monograph. Set initial temperature, heating rate, direction of temperature change, and final temperature as specified in the monograph. If not specified in the monograph, these parameters are determined as follows: make a preliminary examination over a wide range of temperatures (typically, room temperature to decomposition temperature, or about 10° to 20° above the melting point) and over a wide range of heating rates (1° to 20° per minute) to reveal any unexpected effects. Then determine a lower heating rate such that decomposition is minimized and the transition temperature is not compromised. Determine a temperature range bracketing the transition of interest such that the baseline can be extended to intersect with the tangent of the melt (see *Figure 1*).

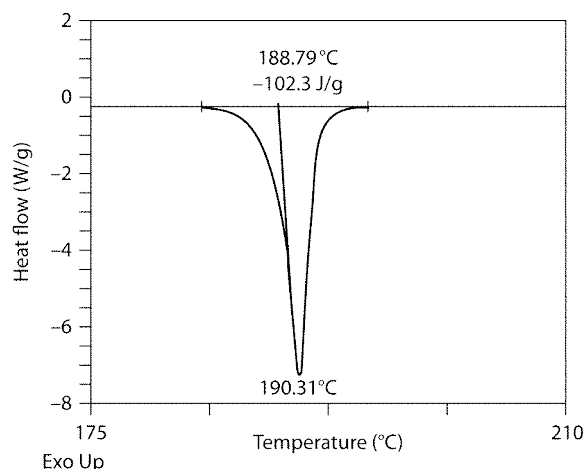


Figure 1. Thermogram.

In examining pure crystalline materials, rates as low as 1° per minute may be appropriate, whereas rates of up to 20° per minute may be more appropriate for polymeric and other semicrystalline materials. Begin the analysis, and record the differential thermal analysis curve with the temperature on the x-axis and the energy change on the y-axis. The melting temperature (melt onset temperature) is the intersection (188.79°) of the extension of the baseline with the tangent at the point of greatest slope (inflection point) of the curve (see *Figure 1*). The vertex is the temperature at the peak of the curve (190.31°). The enthalpy of the event is proportional to the area under the curve after application of a baseline correction.

THERMOGRAVIMETRIC ANALYSIS

Thermogravimetric analysis involves the determination of the mass of a specimen as a function of temperature, or time of heating, or both. It is often used to investigate dehydration/desolvation processes and compound decomposition. When thermogravimetry is properly applied, it provides more useful information than loss on drying at fixed temperature, often for a fixed time and in what is usually an ill-defined atmosphere. Usually, loss of surface-absorbed solvent can be distinguished from solvent in the crystal lattice and from degradation losses. The measurements can be carried out in atmospheres having controlled humidity and oxygen concentration to reveal interactions with the drug substance, between drug substances, and between active substances and excipients or packaging materials.

Apparatus—While the details depend on the manufacturer, the essential features of the equipment are a recording balance and a programmable heat source. Equipment differs in the ability to handle specimens of various sizes, the means of sensing specimen temperature, and the range of atmosphere control.

Calibration—Calibration is required with all systems: that is, the mass scale is calibrated by the use of standard weights, and calibration of the temperature scale involves the use of standard materials, because it is assumed that the specimen temperature is the furnace temperature. Weight calibration is conducted by measuring the mass of a certified or standard weight and comparing the measured mass with the certified value. Temperature calibration is performed by analyzing a high-purity magnetic standard such as nickel for its curie temperature and comparing the measured value to the theoretical value.

Procedure—Apply the method to the sample, using the conditions described in the monograph, and calculate the mass gain or loss, expressing the change in mass as percentage. Alternatively, place a suitable quantity of material in the sample holder, and record the mass. Because the test atmosphere is critical, the pressure or flow rate and the composition of the gas are specified. Set the initial temperature, heating rate, and final temperature according to the manufacturer's instructions, and initiate the temperature increase. Alternatively, conduct an examination of the thermogram over a wide range of temperatures (typically, from room temperature to the decomposition temperature, or 10° to 20° above the melting point at a heating rate of 1° to 20° per minute). Calculate the mass gain or loss, expressing the change in mass as percentage.

HOT-STAGE MICROSCOPY

Hot-stage microscopy is an analytical technique that involves monitoring the optical properties of the sample using a microscope as a function of temperature. Hot-stage microscopy may be used as a complementary technique to other thermal analysis techniques such as DSC, DTA, and variable temperature X-ray powder diffraction for the solid-state characterization of pharmaceutical compounds. It is useful to confirm transitions such as melts, recrystallizations, and solid-state transformations using a visual technique. The hot-stage microscope must be calibrated for temperature.

EUTECTIC IMPURITY ANALYSIS

The basis of any calorimetric purity method is the relationship between the melting and freezing point depression and the level of impurity. The melting of a compound is characterized by the absorption of latent heat of fusion, ΔH_f , at a specific temperature, T_o . In theory, a melting transition for an absolutely pure crystalline compound should occur within an infinitely narrow range. A broadening of the melting range, due to impurities, provides a sensitive criterion of

purity. The effect is apparent visually by examination of thermograms of specimens differing by a few tenths percent in impurity content. A material that is 99% pure is about 20% molten at 3° below the melting point of the pure material (see Figure 2).

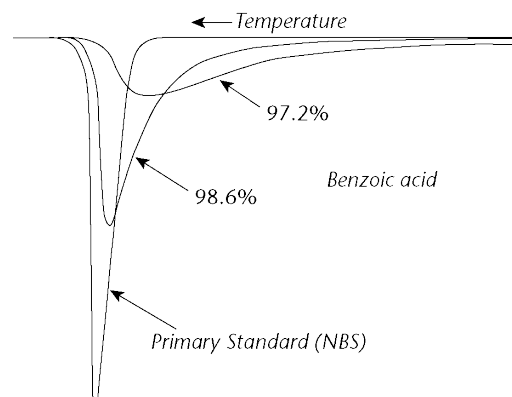


Figure 2. Superimposed thermograms illustrating the effect of impurities on DSC melting peak shape.

The parameters of melting (melting range, ΔH_f , and calculated eutectic purity) are readily obtained from the thermogram of a single melting event using a small test specimen, and the method does not require multiple, precise actual temperature measurements. Thermogram units are directly convertible to heat transfer, millicalories per second.

The lowering of the freezing point in *dilute solutions* by molecules of nearly equal size is expressed by a modified van't Hoff equation:

$$\frac{dT}{dX_2} = \frac{RT^2}{\Delta H_f} (K_D - 1) \quad (1)$$

in which T = absolute temperature in kelvins; X_2 = mole fraction of minor component (solute, impurity); ΔH_f = molar heat of fusion of the major component in Joules per mol; R = gas constant in Joules per mol \times kelvins; and K_D = distribution ratio of solute between the solid and liquid phases.

Assuming that the temperature range is small and that no solid solutions are formed ($K_D = 0$).

Integration of the van't Hoff equation yields the following relationship between the mole fraction of impurity and the melting-point depression:

$$X_2 = \frac{(T_o - T_m)\Delta H_f}{RT_o^2} \quad (2)$$

in which T_o = melting point of the pure compound, in kelvins, and T_m = melting point of the test specimen, in kelvins.

With no solid solution formation, the concentration of impurity in the liquid phase at any temperature during the melting is inversely proportional to the fraction melted at that temperature, and the melting-point depression is directly proportional to the mole fraction of impurity. A plot of the observed test specimen temperature, T_s , versus the reciprocal of the fraction melted, $1/F$, at temperature T_s , should yield a straight line with the slope equal to the melting-point depression ($T_o - T_m$). The theoretical melting point of the pure compound is obtained by extrapolation to $1/F = 0$:

$$T_s = T_o - \frac{RT_o^2 X_2 (1/F)}{\Delta H_f} \quad (3)$$

Substituting the experimentally obtained values for $T_o - T_m$, ΔH_f , and T_o in equation (2) yields the mole fraction of the total eutectic impurity, which, when multiplied by 100, gives the mole percentage of total eutectic impurities.

Deviations from the theoretical linear plot also may be due to solid solution formation ($K_D \neq 0$), so that care must be taken in interpreting the data.

To observe the linear effect of the impurity concentration on the melting-point depression, the impurity must be *soluble* in the liquid phase or melt of the compound, but *insoluble* in the solid phase, i.e., no solid solutions are formed. Some chemical similarities are necessary for solubility in the melt. For example, the presence of ionic compounds in neutral organic compounds and the occurrence of thermal decomposition may not be reflected in purity estimates. The extent of these theoretical limitations has been only partially explored.

Impurities present from the synthetic route often are similar to the end product, hence there usually is no problem of solubility in the melt. Impurities consisting of molecules of the same shape, size, and character as those of the major component can fit into the matrix of the major component without disruption of the lattice, forming solid solutions or inclusions; such impurities are not detectable by DSC. Purity estimates are too high in such cases. This is more common with less-ordered crystals as indicated by low heats of fusion.

In addition, the method is reliable when the purity of the major component is greater than 98.5 mol% and the materials are not decomposed during the melting phase.

Impurity levels calculated from thermograms are reproducible and generally reliable within 0.1% for ideal compounds.

Compounds that exist in polymorphic form cannot be used in purity determination unless the compound is completely converted to one form. On the other hand, DSC and DTA are inherently useful for detecting, and therefore monitoring, polymorphism.

Procedure—The actual procedure and the calculations to be employed for eutectic impurity analysis are dependent on the particular instrument used. Consult the manufacturer's literature and/or the thermal analysis literature for the most appropriate technique for a given instrument. In any event, it is imperative to keep in mind the limitations of solid solution formation, insolubility in the melt, polymorphism, and decomposition during the analysis.

(905) UNIFORMITY OF DOSAGE UNITS

This general chapter is harmonized with the corresponding texts of the *European Pharmacopoeia* and the *Japanese Pharmacopoeia*. Portions of the general chapter text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (♦) to specify this fact.

♦NOTE—In this chapter, *unit* and *dosage unit* are synonymous.♦

To ensure the consistency of dosage units, each unit in a batch should have a drug substance content within a narrow range around the label claim. Dosage units are defined as dosage forms containing a single dose or a part of a dose of drug substance in each unit. The uniformity of dosage units specification is not intended to apply to suspensions, emulsions, or gels in unit-dose containers intended for external, cutaneous administration.

The term “uniformity of dosage unit” is defined as the degree of uniformity in the amount of the drug substance

among dosage units. Therefore, the requirements of this chapter apply to each drug substance being comprised in dosage units containing one or more drug substances, unless otherwise specified elsewhere in this *Pharmacopoeia*.

The uniformity of dosage units can be demonstrated by either of two methods, *Content Uniformity* or ♦*Weight♦ Variation* (see *Table 1*). The test for *Content Uniformity* of preparations presented in dosage units is based on the assay of the individual content of drug substance(s) in a number of dosage units to determine whether the individual content is within the limits set. The *Content Uniformity* method may be applied in all cases.

The test for ♦*Weight♦ Variation* is applicable for the following dosage forms:

(W1)	Solutions enclosed in unit-dose containers and into soft capsules;
(W2)	Solids (including powders, granules, and sterile solids) that are packaged in single-unit containers and contain no active or inactive added substances;
(W3)	Solids (including sterile solids) that are packaged in single-unit containers, with or without active or inactive added substances, that have been prepared from true solutions and freeze-dried in the final containers and are labeled to indicate this method of preparation; and
(W4)	Hard capsules, uncoated tablets, or film-coated tablets, containing 25 mg or more of a drug substance comprising 25% or more, by weight, of the dosage unit or, in the case of hard capsules, the capsule contents, except that uniformity of other drug substances present in lesser proportions is demonstrated by meeting the requirements for <i>Content Uniformity</i> .

The test for *Content Uniformity* is required for all dosage forms not meeting the above conditions for the ♦*Weight♦ Variation* test.¹

Table 1. Application of Content Uniformity (CU) and Weight Variation (WV) Tests for Dosage Forms

Dosage Form	Type	Subtype	Dose & Ratio of Drug Substance	
			≥25 mg and ≥25%	<25 mg or <25%
Tablets	Uncoated		WV	CU
	Coated	Film	WV	CU
		Others	CU	CU
Capsules	Hard		WV	CU
	Soft	Suspension, emulsion, or gel	CU	CU
		Solutions	WV	WV
Solids in single-unit containers	Single component		WV	WV
	Multiple components	Solution freeze-dried in final container	WV	WV
		Others	CU	CU

¹ ♦European Pharmacopoeia and Japanese Pharmacopoeia text not accepted by the United States Pharmacopoeia: Alternatively, products listed in item (4) above that do not meet the 25 mg/25% threshold limit may be tested for uniformity of dosage units by Mass Variation instead of the Content Uniformity test if the concentration relative standard deviation (RSD) of the drug substance in the final dosage units is not more than 2%, based on process validation data and development data, and if there has been regulatory approval of such a change. The concentration RSD is the RSD of the concentration per dosage unit (w/w or w/v), where concentration per dosage unit equals the assay result per dosage unit divided by the individual dosage unit weight. See the RSD formula in *Table 2*.♦

Table 1. Application of Content Uniformity (CU) and Weight Variation (WV) Tests for Dosage Forms (Continued)

Dosage Form	Type	Subtype	Dose & Ratio of Drug Substance	
			≥25 mg and ≥25%	<25 mg or <25%
Solutions in unit-dose containers and into soft capsules			WV	WV
Others			CU	CU

CONTENT UNIFORMITY

Select not fewer than 30 units, and proceed as follows for the dosage form designated.

Where different procedures are used for assay of the preparation and for the *Content Uniformity* test, it may be necessary to establish a correction factor to be applied to the results of the latter.

Solid Dosage Forms

Assay 10 units individually using an appropriate analytical method. Calculate the acceptance value (see *Table 2*).

Liquid or Semi-Solid Dosage Forms

Assay 10 units individually using an appropriate analytical method. Carry out the assay on the amount of well-mixed material that is removed from an individual container in conditions of normal use, and express the results as delivered dose. Calculate the acceptance value (see *Table 2*).

Calculation of Acceptance Value

Calculate the acceptance value by the formula:

$$|M - \bar{X}| + ks$$

in which the terms are as defined in *Table 2*.

•WEIGHT• VARIATION

Carry out an assay for the drug substance(s) on a representative sample of the batch using an appropriate analytical method. This value is result A, expressed as percentage of label claim (see *Calculation of Acceptance Value*). Assume that the concentration (weight of drug substance per weight of dosage unit) is uniform. Select not fewer than 30 dosage units, and proceed as follows for the dosage form designated.

Uncoated or Film-Coated Tablets

Accurately weigh 10 tablets individually. Calculate the content, expressed as percentage of label claim, of each tablet from the •weight• of the individual tablet and the result of the *Assay*. Calculate the acceptance value.

Hard Capsules

Accurately weigh 10 capsules individually, taking care to preserve the identity of each capsule. Remove the contents of each capsule by a suitable means. Accurately weigh the emptied shells individually, and calculate for each capsule the net •weight• of its contents by subtracting the •weight• of the shell from the respective gross •weight•. Calculate the drug substance content of each capsule from the •net weight• of the individual capsule •content• and the result of the *Assay*. Calculate the acceptance value.

Soft Capsules

Accurately weigh 10 intact capsules individually to obtain their gross •weights•, taking care to preserve the identity of each capsule. Then cut open the capsules by means of a suitable clean, dry cutting instrument such as scissors or a sharp open blade, and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature over a period of about 30 minutes, taking precautions to avoid uptake or loss of moisture. Weigh the individual shells, and calculate the net contents. Calculate the drug substance content in each capsule from the •weight• of product removed from the individual capsules and the result of the *Assay*. Calculate the acceptance value.

Solid Dosage Forms Other Than Tablets and Capsules

Proceed as directed for *Hard Capsules*, treating each unit as described therein. Calculate the acceptance value.

Liquid Dosage Forms

Accurately weigh the amount of liquid that is removed from each of 10 individual containers in conditions of normal use. If necessary, compute the equivalent volume after determining the density. Calculate the drug substance content in each container from the mass of product removed from the individual containers and the result of the *Assay*. Calculate the acceptance value.

Calculation of Acceptance Value

Calculate the acceptance value as shown in *Content Uniformity*, except that the individual contents of the units are replaced with the individual estimated contents defined below.

$\chi_1, \chi_2, \dots, \chi_n$	=	individual estimated contents of the units tested, where $\chi_i = w_i \times A/\bar{W}$
w_1, w_2, \dots, w_n	=	individual •weights• of the units tested
A	=	content of drug substance (% of label claim) obtained using an appropriate analytical method
\bar{W}	=	mean of individual •weights• (w_1, w_2, \dots, w_n)

CRITERIA

Apply the following criteria, unless otherwise specified.

Solid, Semi-Solid, and Liquid Dosage Forms

The requirements for dosage uniformity are met if the acceptance value of the first 10 dosage units is less than or

equal to L1%. If the acceptance value is > L1%, test the next 20 units, and calculate the acceptance value. The requirements are met if the final acceptance value of the 30 dosage units is ≤ L1%, and no individual content of any dosage unit is less than $[1 - (0.01)(L2)]M$ nor more than $[1 + (0.01)(L2)]M$ as specified in the *Calculation of Acceptance Value* under *Content Uniformity* or under *Weight Variation*. Unless otherwise specified, L1 is 15.0 and L2 is 25.0.

Table 2

Variable	Definition	Conditions	Value
\bar{X}	Mean of individual contents ($\chi_1, \chi_2, \dots, \chi_n$), expressed as a percentage of the label claim		
$\chi_1, \chi_2, \dots, \chi_n$	Individual contents of the units tested, expressed as a percentage of the label claim		
n	Sample size (number of units in a sample)		
k	Acceptability constant	If n = 10, then k =	2.4
		If n = 30, then k =	2.0
s	Sample standard deviation		$\left[\frac{\sum_{i=1}^n (\chi_i - \bar{X})^2}{n-1} \right]^{1/2}$
RSD	Relative standard deviation (the sample standard deviation expressed as a percentage of the mean)		$100s/\bar{X}$
M (case 1) to be applied when $T \leq 101.5$	Reference value	If $98.5\% \leq \bar{X} \leq 101.5\%$, then	$M = \bar{X}$ (AV = ks)
		If $\bar{X} < 98.5\%$, then	$M = 98.5\%$ (AV = $98.5 - \bar{X} + ks$)
		If $\bar{X} > 101.5\%$, then	$M = 101.5\%$ (AV = $\bar{X} - 101.5 + ks$)
M (case 2) to be applied when $T > 101.5$	Reference value	If $98.5 \leq \bar{X} \leq T$, then	$M = \bar{X}$ (AV = ks)
		If $\bar{X} < 98.5\%$, then	$M = 98.5\%$ (AV = $98.5 - \bar{X} + ks$)
		If $\bar{X} > T$, then	$M = T\%$ (AV = $\bar{X} - T + ks$)
Acceptance value (AV)			General formula: $ M - \bar{X} + ks$ (Calculations are specified above for the different cases.)
L1	Maximum allowed acceptance value		L1 = 15.0 unless otherwise specified
L2	Maximum allowed range for deviation of each dosage unit tested from the calculated value of M	On the low side, no dosage unit result can be less than $[1 - (0.01)(L2)]M$, while on the high side, no dosage unit result can be greater than $[1 + (0.01)(L2)]M$. (This is based on an L2 value of 25.0.)	L2 = 25.0 unless otherwise specified
T	Target content per dosage unit at the time of manufacture, expressed as a percentage of the label claim. Unless otherwise stated, T is 100.0%, or T is the manufacturer's approved target content per dosage unit.		

<911> VISCOSITY—CAPILLARY VISCOMETER METHODS

The following procedures are used to determine the viscosity of a Newtonian fluid, i.e. a fluid having a viscosity that is independent of the shearing stress rate or rate of shear. Unless otherwise directed in the individual monograph, use *Method I*.

• METHOD I. UBBELOHDE-TYPE CAPILLARY VISCOMETER

Apparatus: The determination may be carried out with an Ubbelohde-type capillary viscometer (*Figure 1*) that has the specifications described in *Table 1* or *Table 2*.

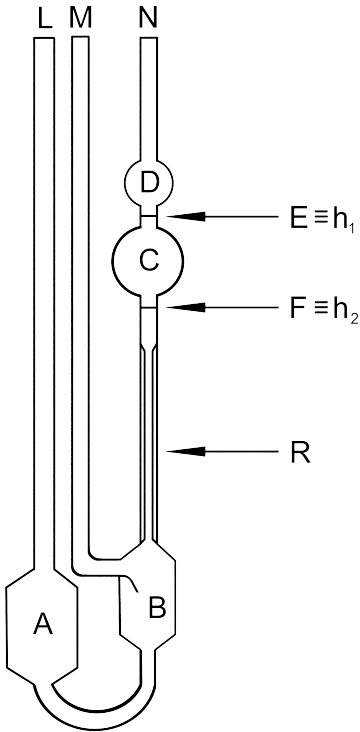


Figure 1. Ubbelohde-Type Capillary Viscometer

Procedure: Fill the viscometer through tube (L) with a sufficient quantity of the sample liquid that is appropriate for the viscometer being used or by following the manufacturer's instructions. Carry out the experiment with the tube in a vertical position. Fill bulb (A) with the liquid, and also ensure that the level of liquid in bulb (B) is below the exit to the ventilation tube (M). Immerse the viscometer in a

water or oil bath stabilized at the temperature specified in the individual monograph, and control the temperature to $\pm 0.1^\circ$, unless otherwise specified in the individual monograph. Maintain the viscometer in a vertical position for a time period of NLT 30 min to allow the sample temperature to reach equilibrium. Close tube (M), and raise the level of the liquid in tube (N) to a level about 8 mm above mark (E $\equiv h_1$). Keep the liquid at this level by closing tube (N) and opening tube (M). Open tube (N), and measure the time required for the level of the liquid to drop from mark (E $\equiv h_1$) to (F $\equiv h_2$), using an appropriate accurate timing device. [NOTE—In *Table 1*, the minimum flow time should be 350 s for size no. 1, and 200 s for all other sizes. In *Table 2*, the minimum flow time should be 300 s for size no. 0, and 200 s for all other sizes.]

Calibration: Calibrate each viscometer at the test temperature by using fluids of known viscosities of appropriate viscosity standards to determine the viscometer constant, k . The viscosity values of the calibration standards should bracket the expected viscosity value of the sample liquid. Determine the viscometer constant at the same temperature as the sample liquid under test.

Calculate the viscometer constant, k , in mm^2/s^2 , from the equation:

$$k = \eta / (\rho \times t)$$

η = known viscosity of the liquid ($\text{mPa} \cdot \text{s}$)
 ρ = density of the liquid (g/mL)
 t = flow time for the liquid to pass from the upper mark to the lower mark (s)

Calculation of kinematic and Newtonian viscosities of sample fluid: A capillary viscometer is chosen so that the flow time, t , ranges between 200 and 1000 s, and the kinematic energy correction is typically less than 1%. If the viscosity constant, k , is known, use the following equation to calculate the kinematic viscosity, ν , in mm^2/s , from the flow time, t , in s.

$$\nu = k \times t$$

If the density of the fluid is known at the temperature of the viscosity measurement, then the Newtonian viscosity, η , in $\text{mPa} \cdot \text{s}$, is calculated by the following equation:

$$\eta = \nu \times \rho$$

ρ = density of the fluid (g/mL)
The flow time of the fluid under examination is the mean of NLT three consecutive determinations. The result is valid if the percentage of the relative standard deviation (%RSD) for the three readings is NMT 2.0%.

• METHOD II. OSTWALD-TYPE CAPILLARY VISCOMETER

Apparatus: The determination may be carried out with an Ostwald-type capillary viscometer (*Figure 2*).

Table 1

Size Number	Nominal Constant of Viscometer (mm^2/s^2)	Measurable Kinematic Viscosity Range (mm^2/s)	Internal Diameter of Tube, R (mm) ($\pm 2\%$)	Volume of Bulb, C (mL) ($\pm 5\%$)	Internal Diameter of Tube, N (mm)
1	0.01	3.5–10	0.64	5.6	2.8–3.2
1A	0.03	6–30	0.84	5.6	2.8–3.2
2	0.1	20–100	1.15	5.6	2.8–3.2
2A	0.3	60–300	1.51	5.6	2.8–3.2
3	1.0	200–1,000	2.06	5.6	3.7–4.3
3A	3.0	600–3,000	2.74	5.6	4.6–5.4
4	10	2,000–10,000	3.70	5.6	4.6–5.4
4A	30	6,000–30,000	4.07	5.6	5.6–6.4
5	100	20,000–100,000	6.76	5.6	6.8–7.5

Table 2

Size Number	Nominal Constant of Viscometer (mm ² /s ²)	Measurable Kinematic Viscosity Range (mm ² /s)	Internal Diameter of Tube, R (mm) (±2%)	Volume of Bulb, C (mL) (±5%)	Internal Diameter of Tube, N (mm)
0	0.001	0.3–1	0.24	1.0	6.0
0C	0.003	0.6–3	0.36	2.0	6.0
0B	0.005	1–5	0.46	3.0	6.0
1	0.01	2–10	0.58	4.0	6.0
1C	0.03	6–30	0.78	4.0	6.0
1B	0.05	10–50	0.88	4.0	6.0
2	0.1	20–100	1.03	4.0	6.0
2C	0.3	60–300	1.36	4.0	6.0
2B	0.5	100–500	1.55	4.0	6.0
3	1.0	200–1,000	1.83	4.0	6.0
3C	3.0	600–3,000	2.43	4.0	6.0
3B	5.0	1,000–5,000	2.75	4.0	6.5
4	10	2,000–10,000	3.27	4.0	7.0
4C	30	6,000–30,000	4.32	4.0	8.0
4B	50	10,000–50,000	5.20	5.0	8.5
5	100	20,000–100,000	6.25	5.0	10.0

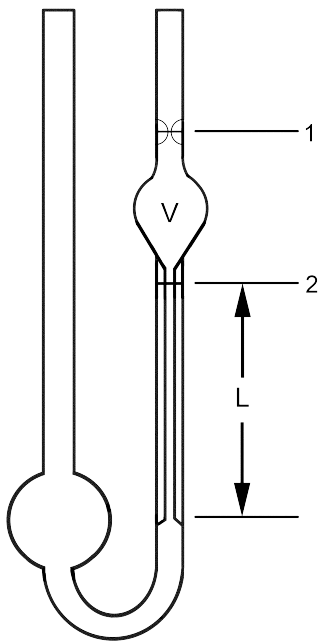


Figure 2. Ostwald-Type Capillary Viscometer

Procedure: Fill the tube with an amount of the sample that is appropriate for the viscometer being used or by following the manufacturer's instructions. The volume of fluid used should be such that the lower bulb is not entirely emptied when the fluid is drawn up through the capillary tube to the uppermost graduation mark. Carry out the experiment with the tube in a vertical position. Immerse the viscometer in a water or oil bath stabilized at the temperature specified in the individual monograph, and control the temperature to $\pm 0.1^\circ$, unless otherwise

specified in the individual monograph. Maintain the viscometer in a vertical position for a time period of NLT 30 min to allow the sample temperature to reach equilibrium. Using suction, draw the fluid up through the capillary tube until the meniscus is at the level of the uppermost graduation. With both the filling and capillary tubes open to atmospheric pressure, record the time, in s, required for the liquid to flow from the upper mark to the lower mark in the capillary tube. [NOTE—The minimum flow time should be 200 s.]

Calibration and Calculation of kinematic and Newtonian viscosities of sample fluid: Proceed as directed in *Method I*.

<912> ROTATIONAL RHEOMETER METHODS

The principle of the method is to measure the force (torque) acting on a rotor when it rotates at a constant angular velocity (rotational speed) in a liquid. Rotational rheometers/viscometers are used for measuring the viscosity of Newtonian fluids, i.e., a fluid having a viscosity that is independent of the shearing stress or rate of shear, or the apparent viscosity of non-Newtonian fluids, which may exhibit different rheological behavior, depending on shear rate, shear stress, and temperature. The following procedures are used to determine the viscosity of Newtonian fluids or the apparent viscosity of non-Newtonian fluids. The calculated viscosity of Newtonian fluids should be the same (within experimental error), regardless of the rate of shear (or rotational speed). Given the dependence of viscosity on temperature, the temperature of the substance being measured should be controlled to within $\pm 0.1^\circ$, unless otherwise specified in the individual monograph. Unless otherwise directed in the individual monograph, use *Method I*.

- **METHOD I. SPINDLE RHEOMETERS (RELATIVE RHEOMETERS—SPINDLE VISCOMETERS)**
Apparatus: In the spindle rheometer, the apparent viscosity is determined by rotating a cylinder- or disc-shaped spindle, as shown in *Figures 1* and *2*, respectively, immersed in a large volume of liquid.

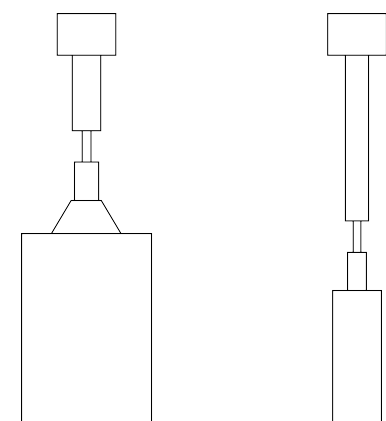


Figure 1. Cylinder-shaped spindles

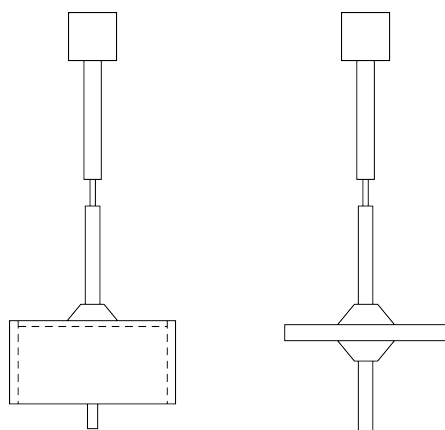


Figure 2. Disc-shaped spindles

Procedure: Under these test conditions the shear rate varies between the outer surface of the spindle and the inner surface of the beaker or cup containing the test substance. As a result, the following additional information must be described along with the measured viscosity:

1. Size and geometry of spindle
2. Angular velocity of the spindle
3. Inner dimensions of the test substance container
4. Temperature of the test substance
5. Use of instrument accessories, such as a spindle guard

The preparation of the test specimen, including its temperature equilibration, is specified in each individual monograph. Follow the instrument manufacturer's recommendations regarding sample loading, spindle selection, and rheometer operation.

Calibration: Select at least two calibration standards whose viscosities differ by an appropriate value within the viscosity range of the test substance under measurement for a particular rheometer configuration. Measure the apparent viscosities of each standard, as described above, at multiple rotational speeds.

A rheometer is deemed to be calibrated if the measured apparent viscosities are within $\pm 5\%$ of the stated values. Generally, calibration, operation, and cleaning of rheometers should be performed according to the recommendations of the instrument manufacturer.

• METHOD II. CONCENTRIC CYLINDER RHEOMETERS

Apparatus: In the concentric cylinder rheometer, the apparent viscosity is determined by placing the liquid in the gap between the inner cylinder and the outer cylinder. Both controlled-stress and controlled-rate rotational rheometers are available commercially in configurations with absolute geometries (e.g., very small annular gaps between concentric cylinders) that can provide consistent meaningful rheological data for non-Newtonian fluids. Controlled-stress rheometers provide controlled-stress input and measurement of the resulting shear rate. Controlled-rate rheometers provide controlled-shear rate input and determination of the resultant shear stress, measured as torque, on the rotor axis. Concentric cylinder rotational rheometers are sometimes referred to as cup-and-bob rheometers. These rheometers involve an additional design consideration depending on whether the outer cylinder (the cup) or the inner cylinder (the bob) rotates. Rotating-cup rheometers are called Couette systems, while rotating-bob rheometers are called Searle systems, as shown in Figures 3 and 4, respectively.

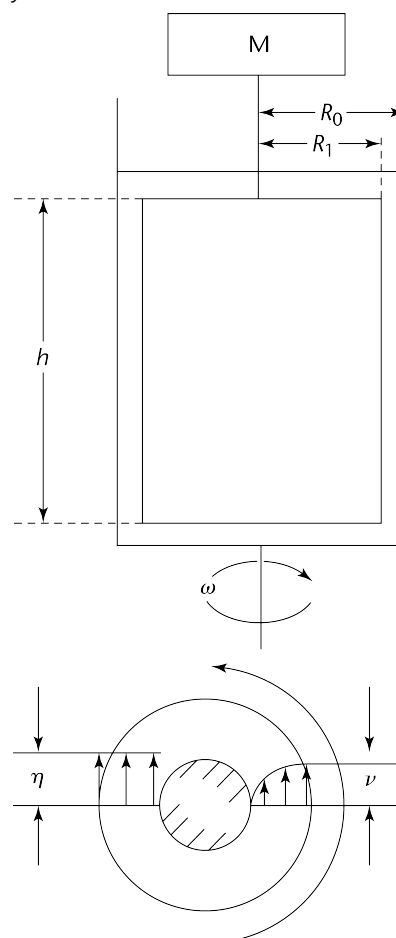


Figure 3. Couette concentric cylinder system for rotational rheometry

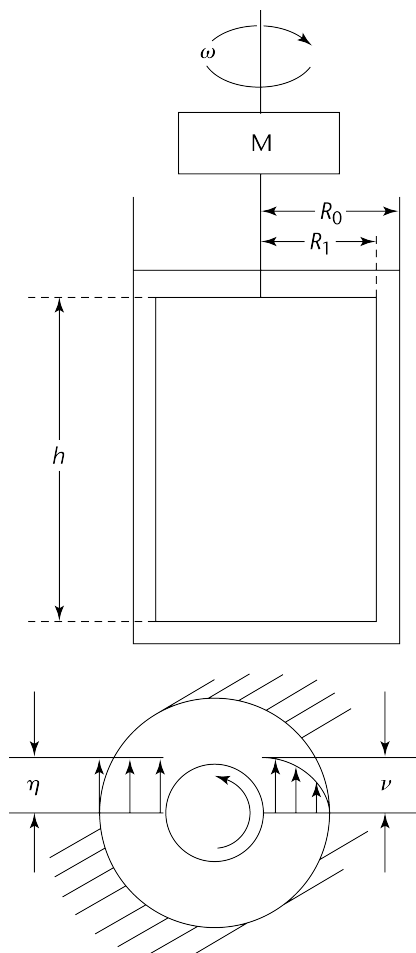


Figure 4. Searle concentric cylinder system for rotational rheometry

Procedure: Place a sufficient quantity of a test solution or fluid in the rheometer, and allow the sample to reach thermal equilibrium, as indicated in the individual monograph. Operate the rheometer following the procedure recommended by the instrument manufacturer. For non-Newtonian systems, the monograph indicates the type of rheometer that should be used and the shear rate(s) at which the measurements should be made. [NOTE—If there is evidence of time-dependent (e.g., thixotropic or rheopectic) rheological behavior, this should be noted as well.] As noted above, apparent viscosity should be determined preferably over a range of shear rates appropriate to the material under test. The procedure employed to measure the apparent viscosity of the liquid is repeated, using a series of different rotational speeds or torques. From a series of such viscosity measurements, the relationship between the shear rate and the shear stress of a non-Newtonian

liquid—that is, the flow characteristics of the non-Newtonian liquid—can be obtained.

Calculation of shear rate, shear stress, and viscosity: For non-Newtonian liquids, it is essential to specify the shear stress, σ , or the shear rate $\dot{\gamma}$, at which the viscosity is measured. Under narrow gap conditions (conditions satisfied in absolute rheometers), the shear rate $\dot{\gamma}$ in s^{-1} , and the shear stress σ , in Pa ($N \cdot m^{-2}$ or $kg \cdot m^{-1} \cdot s^{-2}$), are calculated using Equations (1) and (2) below:

$$\dot{\gamma} = \left(\frac{R_o^2 + R_i^2}{R_o^2 - R_i^2} \right) \omega \quad (1)$$

$$\sigma = \left(\frac{1}{4\pi h R_o^2} + \frac{1}{4\pi h R_i^2} \right) M \quad (2)$$

R_o = radius of the outer cylinder (m)

R_i = radius of the inner cylinder (m)

ω = angular velocity (radians/s)

M = torque acting on the cylinder surface ($N \cdot m$)

h = height of immersion of the inner cylinder in the liquid medium (m)

Generally, the angular velocity can be calculated using Equation (3):

$$\omega = \left(\frac{2\pi}{60} \right) n \quad (3)$$

n = rotational speed, in revolutions/min (rpm)

For laminar flow, the viscosity η (or apparent viscosity η_{app}), in $Pa \cdot s$, is given by the following equation: [NOTE—1 $Pa \cdot s$ = 1000 $mPa \cdot s$.]

$$\eta \text{ or } \eta_{app} = \frac{1}{4\pi h} \left(\frac{1}{R_i^2} - \frac{1}{R_o^2} \right) \frac{M}{\omega} = k \frac{M}{\omega} \quad (4)$$

k = the constant of the apparatus (radians/ m^3)

Calibration: Rotational rheometers require calibration with rheological standards appropriate for the shear rate or shear stress ranges and the nature of the fluid or material under evaluation. To determine or confirm the apparatus constant, perform the necessary tests beforehand, using fluids of known viscosities of appropriate viscosity standards at the required temperature.

• METHOD III. CONE-AND-PLATE RHEOMETERS

Apparatus: In the cone-and-plate rheometer, the liquid is introduced into the gap between a flat disc or plate and a cone forming a defined angle. Viscosity measurement can be performed by rotating the cone or the plate, as shown in Figures 5 and 6, respectively. [NOTE—Because the volume of sample is small, even a small absolute loss of solvents can cause a large percentage change in viscosity. Such a loss is particularly relevant for volatile solvents but could be significant even for nonvolatile solvents such as water.]

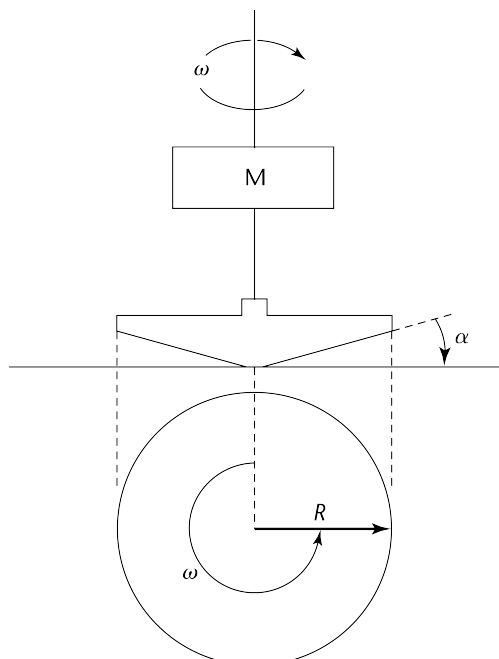


Figure 5. Cone-and-plate rotational rheometer with rotating cone

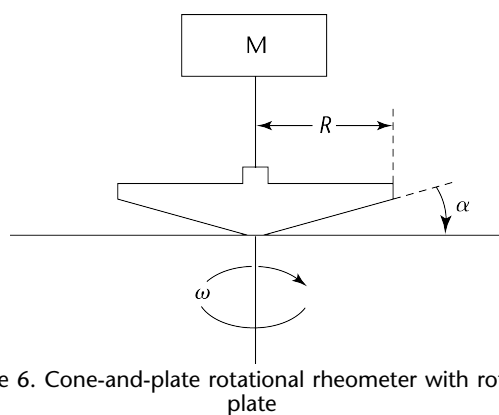


Figure 6. Cone-and-plate rotational rheometer with rotating plate

Procedure: Proceed as directed for *Method II. Concentric Cylinder Rheometers*.

Calculation of shear rate, shear stress, and viscosity: The shear rate $\dot{\gamma}$ in s^{-1} , and the shear stress σ , in Pa, are calculated by Equations (5) and (6).

$$\dot{\gamma} = \left(\frac{1}{\alpha} \right) \omega \quad (5)$$

$$\sigma = \left(\frac{1}{\frac{2}{3} \pi R^3} \right) M \quad (6)$$

α = angle between the flat plate and the cone (radians)

R = radius of the cone (m)

ω = angular velocity (radians/s)

M = torque acting on the flat plate or cone surface (N · m)

For laminar flow, the viscosity η (or apparent viscosity η_{App}), in Pa · s, is given by the following equation:

$$\eta \text{ or } \eta_{App} = \left(\frac{3\alpha}{2\pi R^3} \right) \left(\frac{M}{\omega} \right) = k \frac{M}{\omega} \quad (7)$$

k = constant of the apparatus (radians/m³)

Calibration: Proceed as directed for *Calibration in Method II. Concentric Cylinder Rheometers*.

<913> ROLLING BALL VISCOMETER METHOD

The following procedure is used to determine the viscosity of a Newtonian fluid, i.e., a fluid having a viscosity that is independent of the shearing stress or rate of shear.

Apparatus: See Figure 1.

The basic design of a rolling ball viscometer consists of a tube (or capillary) that contains the sample liquid under test and a ball chosen so that it will require a minimum rolling time of 20 s at the measuring angle in the sample liquid.

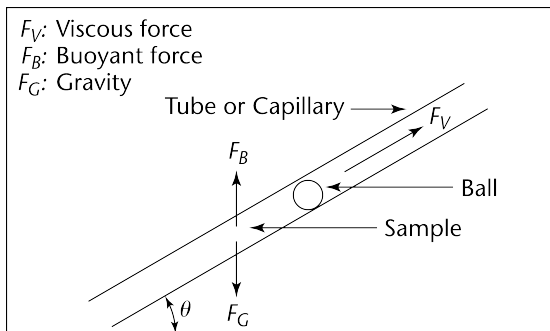


Figure 1. Basic design for rolling ball viscometer.

Measuring Principle: The rolling ball measurement is based on Stokes's Law, as influenced by the angle of inclination of the tube (or capillary). The Newtonian viscosity, η , in $\text{mPa} \cdot \text{s}$, is calculated using the following equation:

$$\eta = [(\rho_1 - \rho_2) \times g \times r^2 \times \sin\theta] / v_{\infty}$$

ρ_1 = density of the ball used (g/mL)

ρ_2 = density of the sample liquid (g/mL)

g = gravitational constant (mm/s^2)

r = radius of the ball (mm)

θ = angle of inclination of the tube (or capillary)

v_{∞} = terminal velocity of the ball (mm/s)

Determine the viscosity of a liquid by observing the rolling time of a solid sphere (ball) under the influence of gravity in an inclined cylindrical tube filled with the sample liquid. Measure the time taken by the ball to travel the fixed distance between two ring marks or measuring sensors. For each single measured rolling time, the resulting viscosity can be expressed as dynamic viscosity ($\text{mPa} \cdot \text{s}$) as well as kinematic viscosity (mm^2/s) for a sample of known density.

Procedure: Select a measuring system [tube (or capillary) and ball combination] within the anticipated range of viscosity of the sample liquid. As necessary, heat the clean and dry tube and ball of the viscometer to the temperature specified in the individual monograph, and control the temperature to $\pm 0.1^\circ$, unless otherwise specified in the individual monograph. Select a measuring angle to obtain a minimum rolling time of 20 s. Fill the tube with the sample liquid, being careful to avoid bubble formation. Close the tube, and insert it in the instrument. Allow to equilibrate at the specified temperature for NLT 15 min for a rolling ball viscometer with a tube of large diameter. For a micro rolling ball viscometer, follow the instrument manufacturer's instructions regarding temperature equilibration. Release the ball, and record the time required for the ball to roll from the upper to the lower ring mark (or measuring sensor). Repeat the test run at least four times.

The rolling time in the fluid under examination is the mean of NLT four consecutive determinations. The result is valid if the percent relative standard deviation (%RSD) for the four readings is NMT 2.0%.

Calculation and Calibration: Calculate the Newtonian viscosity, η , in $\text{mPa} \cdot \text{s}$, using the formula:

$$\eta = k \times (\rho_1 - \rho_2) \times t$$

k = calibration constant of the instrument (mm^2/s^2) at a specified measuring angle and temperature

ρ_1 = density of the ball used (g/mL)

ρ_2 = density of the sample liquid (g/mL)

t = rolling time of the ball (s).

Calibrate each tube (or capillary) and ball combination at the test temperature and test angle using fluids of known viscosities and densities (viscosity standards) to determine the measuring system constant, k . [NOTE—Some automated viscometers use a polynomial function to determine the calibration for different angles and temperatures.] The viscosity values of the calibration standards should bracket the expected viscosity value of the sample liquid.

Calibrations are specific to the ball radius, ball density, temperature, and test angle. Recalibration is necessary when any of these parameters is changed.

Where no reference values at the required test temperature are available, follow the manufacturer's instructions for mathematical corrections to the calibration function. When the materials of the ball and tube are dissimilar, apply corrections calculated using the linear thermal expansion coefficients of the materials.

<921> WATER DETERMINATION

Many Pharmacopeial articles either are hydrates or contain water in adsorbed form. As a result, the determination of the water content is important in demonstrating compliance with the Pharmacopeial standards. Generally one of the methods given below is called for in the individual monograph, depending upon the nature of the article. In rare cases, a choice is allowed between two methods. When the article contains water of hydration, *Method I (Titrimetric)*, *Method II (Azeotropic)*, or *Method III (Gravimetric)* is employed, as directed in the individual monograph, and the requirement is given under the heading *Water*.

The heading *Loss on Drying* (see *Loss on Drying* <731>) is used in those cases where the loss sustained on heating may be not entirely water.

Change to read:

METHOD I (TITRIMETRIC)

Determine the water by *Method Ia*, unless otherwise specified in the individual monograph.

Method Ia (Direct Titration)

Principle—The titrimetric determination of water is based upon the quantitative reaction of water with an anhydrous solution of sulfur dioxide and iodine in the presence of a buffer that reacts with hydrogen ions.

In the original titrimetric solution, known as Karl Fischer Reagent, the sulfur dioxide and iodine are dissolved in pyridine and methanol. The test specimen may be titrated with the *Reagent* directly, or the analysis may be carried out by a residual titration procedure. The stoichiometry of the reaction is not exact, and the reproducibility of a determination depends upon such factors as the relative concentrations of

the *Reagent* ingredients, the nature of the inert solvent used to dissolve the test specimen, and the technique used in the particular determination. Therefore, an empirically standardized technique is used in order to achieve the desired accuracy. Precision in the method is governed largely by the extent to which atmospheric moisture is excluded from the system. The titration of water is usually carried out with the use of anhydrous methanol as the solvent for the test specimen. ▲ In some cases, other suitable solvents may be used for special or unusual test specimens. In these cases, the addition of at least 20% of methanol or other primary alcohol is recommended.▲ *USP36*

Apparatus—Any apparatus may be used that provides for adequate exclusion of atmospheric moisture and determination of the endpoint. In the case of a colorless solution that is titrated directly, the endpoint may be observed visually as a change in color from canary yellow to amber. The reverse is observed in the case of a test specimen that is titrated residually. More commonly, however, the endpoint is determined electrometrically with an apparatus employing a simple electrical circuit that serves to impress about 200 mV of applied potential between a pair of platinum electrodes immersed in the solution to be titrated. At the endpoint of the titration a slight excess of the reagent increases the flow of current to between 50 and 150 microamperes for 30 s to 30 min, depending upon the solution being titrated. The time is shortest for substances that dissolve in the reagent. With some automatic titrators, the abrupt change in current or potential at the endpoint serves to close a solenoid-operated valve that controls the buret delivering the titrant. Commercially available apparatus generally comprises a closed system consisting of one or two automatic burets and a tightly covered titration vessel fitted with the necessary electrodes and a magnetic stirrer. The air in the system is kept dry with a suitable desiccant, and the titration vessel may be purged by means of a stream of dry nitrogen or current of dry air.

Reagent—Prepare the Karl Fischer Reagent as follows. Add 125 g of iodine to a solution containing 670 mL of methanol and 170 mL of pyridine, and cool. Place 100 mL of pyridine in a 250-mL graduated cylinder, and, keeping the pyridine cold in an ice bath, pass in dry sulfur dioxide until the volume reaches 200 mL. Slowly add this solution, with shaking, to the cooled iodine mixture. Shake to dissolve the iodine, transfer the solution to the apparatus, and allow the solution to stand overnight before standardizing. One mL of this solution when freshly prepared is equivalent to approximately 5 mg of water, but it deteriorates gradually; therefore, standardize it within 1 h before use, or daily if in continuous use. Protect from light while in use. Store any bulk stock of the reagent in a suitably sealed, glass-stoppered container, fully protected from light, and under refrigeration. ▲ For determination of trace amounts of water (less than 1%), it is preferable to use a *Reagent* with a water equivalency factor of not more than 2.0, which will lead to the consumption of a more significant volume of titrant.▲ *USP36*

A commercially available, stabilized solution of Karl Fischer type reagent may be used. Commercially available reagents containing solvents or bases other than pyridine or alcohols other than methanol may be used also. These may be single solutions or reagents formed in situ by combining the components of the reagents present in two discrete solutions. The diluted *Reagent* called for in some monographs should be diluted as directed by the manufacturer. Either methanol or other suitable solvent, such as ethylene glycol monomethyl ether, may be used as the diluent.

Test Preparation—Unless otherwise specified in the individual monograph, use an accurately weighed or measured amount of the specimen under test estimated to contain 2–250 mg of water. The amount of water depends on the water equivalency factor of the *Reagent* and on the method

of endpoint determination. In most cases, the minimum amount of specimen, in mg, can be estimated using the formula:

$$FCV/KF$$

in which *F* is the water equivalency factor of the *Reagent*, in mg per mL; *C* is the used volume, in percent, of the capacity of the buret; *V* is the buret volume, in mL; and *KF* is the limit or reasonable expected water content in the sample, in percent. *C* is generally between 30% and 100% for manual titration, and between 10% and 100% for the instrumental method endpoint determination. [NOTE—It is recommended that the product of *FCV* be greater than or equal to 200 for the calculation to ensure that the minimum amount of water titrated is greater than or equal to 2 mg.]

Where the specimen under test is an aerosol with propellant, store it in a freezer for not less than 2 h, open the container, and test 10.0 mL of the well-mixed specimen. In titrating the specimen, determine the endpoint at a temperature of 10° or higher.

Where the specimen under test is capsules, use a portion of the mixed contents of not fewer than four capsules.

Where the specimen under test is tablets, use powder from not fewer than four tablets ground to a fine powder in an atmosphere of temperature and relative humidity known not to influence the results.

Where the monograph specifies that the specimen under test is hygroscopic, ▲ take an accurately weighed portion of the solid into the titration vessel, proceeding as soon as possible and taking care to avoid moisture uptake from the atmosphere. If the sample is constituted by a finite amount of solid as a lyophilized product or a powder inside a vial,▲ *USP36* use a dry syringe to inject an appropriate volume of methanol, or other suitable solvent, accurately measured, into a tared container, and shake to dissolve the specimen. Using the same syringe, remove the solution from the container and transfer it to a titration vessel prepared as directed for *Procedure*. Repeat the procedure with a second portion of methanol, or other suitable solvent, accurately measured, add this washing to the titration vessel, and immediately titrate. Determine the water content, in mg, of a portion of solvent of the same total volume as that used to dissolve the specimen and to wash the container and syringe, as directed for *Standardization of Water Solution for Residual Titration*, and subtract this value from the water content, in mg, obtained in the titration of the specimen under test. Dry the container and its closure at 100° for 3 h, allow to cool in a desiccator, and weigh. Determine the weight of specimen tested from the difference in weight from the initial weight of the container.

▲ When appropriate, the water may be desorbed or released from the sample by heat in an external oven connected with the vessel, to where it is transferred with the aid of an inert and dried gas such as pure nitrogen. Any drift due to the transport gas should be considered and corrected. Care should be taken in the selection of the heating conditions to avoid the formation of water coming from dehydration due to decomposition of the sample constituents, which may invalidate this approach.▲ *USP36*

Standardization of the Reagent—Place enough methanol or other suitable solvent in the titration vessel to cover the electrodes, and add sufficient *Reagent* to give the characteristic endpoint color, or 100 ± 50 microamperes of direct current at about 200 mV of applied potential.

▲ *USP36* **Purified Water**, sodium tartrate dihydrate, a USP Reference Standard, or commercial standards with a certificate of analysis traceable to a national standard may be used to standardize the *Reagent*. The reagent equivalency factor, the recommended titration volume, buret size, and amount of standard to measure are factors to consider

when deciding which standard and how much to use.¹ For *Purified Water* or water standards, quickly add the equivalent of between 2 and 250 mg of water. Calculate the water equivalency factor, F , in mg of water per mL of reagent:

$$W/V$$

in which W is the weight, in mg, of the water contained in the aliquot of standard used; and V is the volume, in mL, of the *Reagent* used in the titration. For sodium tartrate dihydrate, quickly add 20–125 mg of sodium tartrate dihydrate ($C_4H_4Na_2O_6 \cdot 2H_2O$), accurately weighed by difference, and titrate to the endpoint. The water equivalence factor F , in mg of water per mL of reagent, is given by the formula:

$$W/V (36.04/230.08)$$

in which 36.04 is two times the molecular weight of water and 230.08 is the molecular weight of sodium tartrate dihydrate; W is the weight, in mg, of sodium tartrate dihydrate; and V is the volume, in mL, of the *Reagent* consumed in the second titration. Note that the solubility of sodium tartrate dihydrate in methanol is such that fresh methanol may be needed for additional titrations of the sodium tartrate dihydrate standard.

Procedure—Unless otherwise specified, transfer enough methanol or other suitable solvent to the titration vessel, ensuring that the volume is sufficient to cover the electrodes (approximately 30–40 mL), and titrate with the *Reagent* to the electrometric or visual endpoint to consume any moisture that may be present. (Disregard the volume consumed, because it does not enter into the calculations.) Quickly add the *Test Preparation*, mix, and again titrate with the *Reagent* to the electrometric or visual endpoint. Calculate the water content of the specimen taken, in mg:

$$SF$$

in which S is the volume, in mL, of the *Reagent* consumed in the second titration; and F is the water equivalence factor of the *Reagent*.

Method Ib (Residual Titration)

Principle—See the information given in the section *Principle* under *Method Ia*. In the residual titration, excess *Reagent* is added to the test specimen, sufficient time is allowed for the reaction to reach completion, and the unconsumed *Reagent* is titrated with a standard solution of water in a solvent such as methanol. The residual titration procedure is applicable generally and avoids the difficulties that may be encountered in the direct titration of substances from which the bound water is released slowly.

Apparatus, Reagent, and Test Preparation—Use *Method Ia*.

Standardization of Water Solution for Residual Titration—Prepare a *Water Solution* by diluting 2 mL of water with methanol or other suitable solvent to 1000 mL. Standardize this solution by titrating 25.0 mL with the *Reagent*, previously standardized as directed under *Standardization of the Reagent*. Calculate the water content, in mg per mL, of the *Water Solution* taken:

$$V'F/25$$

in which V' is the volume of the *Reagent* consumed, and F is the water equivalence factor of the *Reagent*. Determine the

¹ Consider a setup in which the reagent equivalency factor is 5 mg/mL, and the buret volume is 5 mL and an instrumental endpoint. Standard amounts equivalent to between 2.5 mg and 22.5 mg of water (10%–90% of buret capacity) could be used based on the buret and the reagent equivalency factor. The upper end of this range would involve an excessive amount of sodium tartrate dihydrate. If *Purified Water* or a standard is weighed, an analytical balance appropriate to the amount weighed is required.

water content of the *Water Solution* weekly, and standardize the *Reagent* against it periodically as needed.

Procedure—Where the individual monograph specifies that the water content is to be determined by *Method Ib*, transfer enough methanol or other suitable solvent to the titration vessel, ensuring that the volume is sufficient to cover the electrodes (approximately 30–40 mL), and titrate with the *Reagent* to the electrometric or visual endpoint. Quickly add the *Test Preparation*, mix, and add an accurately measured excess of the *Reagent*. Allow sufficient time for the reaction to reach completion, and titrate the unconsumed *Reagent* with standardized *Water Solution* to the electrometric or visual endpoint. Calculate the water content of the specimen, in mg, taken:

$$F(X' - XR)$$

in which F is the water equivalence factor of the *Reagent*; X' is the volume, in mL, of the *Reagent* added after introduction of the specimen; X is the volume, in mL, of standardized *Water Solution* required to neutralize the unconsumed *Reagent*; and R is the ratio, $V/25$ (mL *Reagent*/mL *Water Solution*), determined from the *Standardization of Water Solution for Residual Titration*.

Method Ic (Coulometric Titration)

Principle—The Karl Fischer reaction is used in the coulometric determination of water. Iodine, however, is not added in the form of a volumetric solution but is produced in an iodide-containing solution by anodic oxidation. The reaction cell usually consists of a large anode compartment and a small cathode compartment that are separated by a diaphragm. Other suitable types of reaction cells (e.g., without diaphragms) may also be used. Each compartment has a platinum electrode that conducts current through the cell. Iodine, which is produced at the anode electrode, immediately reacts with water present in the compartment. When all the water has been consumed, an excess of iodine occurs, which usually is detected electrometrically, thus indicating the endpoint. Moisture is eliminated from the system by pre-electrolysis. Changing the Karl Fischer solution after each determination is not necessary because individual determinations can be carried out in succession in the same reagent solution. A requirement for this method is that each component of the test specimen is compatible with the other components, and no side reactions take place. Samples are usually transferred into the vessel as solutions by means of injection through a septum. Gases can be introduced into the cell by means of a suitable gas inlet tube. Precision in the method is predominantly governed by the extent to which atmospheric moisture is excluded from the system; thus, the introduction of solids into the cell may require precautions, such as working in a glove-box in an atmosphere of dry inert gas. Control of the system may be monitored by measuring the amount of baseline drift, ▲ which does not preclude the need of any blank correction when used as a vehicle for sample introduction.▲^{USP36} This method is particularly suited to chemically inert substances like hydrocarbons, alcohols, and ethers. In comparison with the volumetric Karl Fischer titration, coulometry is a micro-method.

▲ When appropriate, the water may be desorbed or released from the sample by heat in an external oven connected with the vessel, to where it is transferred with the aid of an inert and dried gas such as pure nitrogen. Any drift due to the transport gas should be considered and corrected. Care should be taken in the selection of the heating conditions to avoid the formation of water coming from dehydration due to decomposition of the sample constituents, which may invalidate this approach.▲^{USP36}

Apparatus—Any commercially available apparatus consisting of an absolutely tight system fitted with the necessary electrodes and a magnetic stirrer is appropriate. The

instrument's microprocessor controls the analytical procedure and displays the results. Calibration of the instrument is not necessary, as the current consumed can be measured absolutely.

Reagent—See the manufacturer's recommendations.

Test Preparation—Where the specimen is a soluble solid, an appropriate quantity, accurately weighed, may be dissolved in anhydrous methanol or other suitable solvents.

Where the specimen is an insoluble solid, an appropriate quantity, accurately weighed, may be extracted using a suitable anhydrous solvent, and may be injected into the anolyte solution. Alternatively, an evaporation technique may be used in which water is released and evaporated by heating the specimen in a tube in a stream of dry inert gas. The gas is then passed into the cell.

Where the specimen is to be used directly without dissolving in a suitable anhydrous solvent, an appropriate quantity, accurately weighed, may be introduced into the chamber directly.

Where the specimen is a liquid, and is miscible with anhydrous methanol or other suitable solvents, an appropriate quantity, accurately weighed, may be added to anhydrous methanol or other suitable solvents.

Procedure—Using a dry device, inject or add directly an accurately measured amount of the sample or sample preparation estimated to contain between 0.5 and 5 mg of water, or an amount recommended by the instrument manufacturer into the anolyte, mix, and perform the coulometric titration to the electrometric endpoint. Read the water content of the liquid *Test Preparation* directly from the instrument's display, and calculate the percentage that is present in the substance. Perform a blank determination, as needed, and make any necessary corrections.

Change to read:

METHOD II (AZEOTROPIC—TOLUENE DISTILLATION)

Apparatus—Use a 500-mL glass flask A connected by means of a trap B to a reflux condenser C by ground glass joints (see Figure 1).

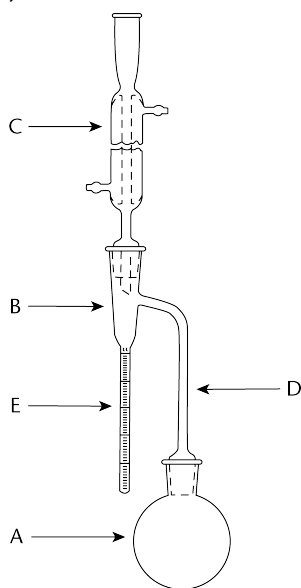


Figure 1. Toluene moisture apparatus.

The critical dimensions of the parts of the apparatus are as follows. The connecting tube D is 9–11 mm in internal diameter. The trap is 235–240 mm in length. The condenser, if of the straight-tube type, is approximately 400 mm in length and not less than 8 mm in bore diameter. The receiving tube E has a 5-mL capacity, and its cylindrical portion, 146–156 mm in length, is graduated in 0.1-mL subdivisions, so that the error of reading is not greater than 0.05 mL for any indicated volume. The source of heat is preferably an electric heater with rheostat control or an oil bath. The upper portion of the flask and the connecting tube may be insulated.

Clean the receiving tube and the condenser with ▲ a suitable cleanser, ▲ *USP36* thoroughly rinse with water, and dry in an oven. Prepare the toluene to be used by first shaking with a small quantity of water, separating the excess water, and distilling the toluene.

Procedure—Place in the dry flask a quantity of the substance, weighed accurately to the nearest centigram, which is expected to yield 2–4 mL of water. If the substance is of a pasty character, weigh it in a boat of metal foil of a size that will just pass through the neck of the flask. If the substance is likely to cause bumping, add enough dry, washed sand to cover the bottom of the flask, or a number of capillary melting-point tubes, about 100 mm in length, sealed at the upper end. Place about 200 mL of toluene in the flask, connect the apparatus, and fill the receiving tube E with toluene poured through the top of the condenser. Heat the flask gently for 15 min and, when the toluene begins to boil, distill at the rate of about two drops per s until most of the water has passed over, then increase the rate of distillation to about four drops per s. When the water has apparently all distilled over, rinse the inside of the condenser tube with toluene while brushing down the tube with a tube brush attached to a copper wire and saturated with toluene. Continue the distillation for five min, then remove the heat, and allow the receiving tube to cool to room temperature. If any droplets of water adhere to the walls of the receiving tube, scrub them down with a brush consisting of a rubber band wrapped around a copper wire and wetted with toluene. When the water and toluene have separated completely, read the volume of water, and calculate the percentage that was present in the substance.

METHOD III (GRAVIMETRIC)

Procedure for Chemicals—Proceed as directed in the individual monograph preparing the chemical as directed under *Loss on Drying* (731).

Procedure for Biologics—Proceed as directed in the individual monograph.

Procedure for Articles of Botanical Origin—Place about 10 g of the drug, prepared as directed (see *Methods of Analysis* under *Articles of Botanical Origin* (561)) and accurately weighed, in a tared evaporating dish. Dry at 105° for 5 h, and weigh. Continue the drying and weighing at 1-h inter-

vals until the difference between two successive weighings corresponds to not more than 0.25%.

$\langle 941 \rangle$ CHARACTERIZATION OF CRYSTALLINE AND PARTIALLY CRYSTALLINE SOLIDS BY X-RAY POWDER DIFFRACTION (XRPD)

INTRODUCTION

Every crystalline phase of a given substance produces a characteristic X-ray diffraction pattern. Diffraction patterns can be obtained from a randomly oriented crystalline powder composed of crystallites or crystal fragments of finite size. Essentially three types of information can be derived from a powder diffraction pattern: the angular position of diffraction lines (depending on geometry and size of the unit cell), the intensities of diffraction lines (depending mainly on atom type and arrangement, and particle orientation within the sample), and diffraction line profiles (depending on instrumental resolution, crystallite size, strain, and specimen thickness).

Experiments giving angular positions and intensities of lines can be used for applications such as qualitative phase analysis (e.g., identification of crystalline phases) and quantitative phase analysis of crystalline materials. An estimate of the amorphous and crystalline fractions¹ can also be made.

The X-ray powder diffraction (XRPD) method provides an advantage over other means of analysis in that it is usually nondestructive in nature (to ensure a randomly oriented sample, specimen preparation is usually limited to grinding). XRPD investigations can also be carried out under *in situ* conditions on specimens exposed to nonambient conditions such as low or high temperature and humidity.

PRINCIPLES

X-ray diffraction results from the interaction between X-rays and electron clouds of atoms. Depending on atomic arrangement, interferences arise from the scattered X-rays. These interferences are constructive when the path difference between two diffracted X-ray waves differs by an integral number of wavelengths. This selective condition is described by the Bragg equation, also called Bragg's law (see Figure 1).

$$2d_{hkl} \sin \theta_{hkl} = n\lambda$$

The wavelength, λ , of the X-rays is of the same order of magnitude as the distance between successive crystal lattice planes, or d_{hkl} (also called d-spacings). θ_{hkl} is the angle between the incident ray and the family of lattice planes, and $\sin \theta_{hkl}$ is inversely proportional to the distance between successive crystal planes or d-spacings.

The direction and spacing of the planes with reference to the unit cell axes are defined by the Miller indices $\{hkl\}$. These indices are the reciprocals, reduced to the next-lower integer, of the intercepts that a plane makes with the unit

cell axes. The unit cell dimensions are given by the spacings a , b , and c , and the angles between them α , β , and γ .

The interplanar spacing for a specified set of parallel hkl planes is denoted by d_{hkl} . Each such family of planes may show higher orders of diffraction where the d values for the related families of planes nh , nk , nl are diminished by the factor $1/n$ (n being an integer: 2, 3, 4, etc.).

Every set of planes throughout a crystal has a corresponding Bragg diffraction angle, θ_{hkl} , associated with it (for a specific λ).

A powder specimen is assumed to be polycrystalline so that at any angle θ_{hkl} there are always crystallites in an orientation allowing diffraction according to Bragg's law.² For a given X-ray wavelength, the positions of the diffraction peaks (also referred to as "lines", "reflections", or "Bragg reflections") are characteristic of the crystal lattice (d-spacings), their theoretical intensities depend on the crystallographic unit cell content (nature and positions of atoms), and the line profiles depend on the perfection and extent of the crystal lattice. Under these conditions, the diffraction peak has a finite intensity arising from atomic arrangement, type of atoms, thermal motion, and structural imperfections, as well as from instrument characteristics.

The intensity is dependent upon many factors such as structure factor, temperature factor, crystallinity, polarization factor, multiplicity, and Lorentz factor.

The main characteristics of diffraction line profiles are 2θ position, peak height, peak area, and shape (characterized by, e.g., peak width, or asymmetry, analytical function, and empirical representation). An example of the type of powder patterns obtained for five different solid phases of a substance are shown in Figure 2.

In addition to the diffraction peaks, an X-ray diffraction experiment also generates a more or less uniform background, upon which the peaks are superimposed. Besides specimen preparation, other factors contribute to the background—for example, sample holder, diffuse scattering from air and equipment, and other instrumental parameters such as detector noise and general radiation from the X-ray tube. The peak-to-background ratio can be increased by minimizing background and by choosing prolonged exposure times.

INSTRUMENT

Instrument Setup

X-ray diffraction experiments are usually performed using powder diffractometers or powder cameras.

A powder diffractometer generally comprises five main parts: an X-ray source; the incident beam optics, which may perform monochromatization, filtering, collimation, and/or focusing of the beam; a goniometer; the diffraction beam optics, which may include monochromatization, filtering, collimation, and focusing or parallelizing of beam; and a detector. Data collection and data processing systems are also required and are generally included in current diffraction measurement equipment.

Depending on the type of analysis to be performed (phase identification, quantitative analysis, lattice parameters determination, etc.), different XRPD instrument configurations and performance levels are required. The simplest instruments used to measure powder patterns are powder cameras. Replacement of photographic film as the detection method by photon detectors has led to the design of diffractometers in which the geometric arrangement of the optics is not truly focusing, but parafofocusing, such as in Bragg-Brentano geometry. The Bragg-Brentano parafofocusing con-

¹ There are many other applications of the X-ray powder diffraction technique that can be applied to crystalline pharmaceutical substances, such as determination of crystal structures, refinement of crystal structures, determination of the crystallographic purity of crystalline phases, and characterization of crystallographic texture. These applications are not described in this chapter.

² An ideal powder for diffraction experiments consists of a large number of small, randomly oriented spherical crystallites (coherently diffracting crystalline domains). If this number is sufficiently large, there are always enough crystallites in any diffracting orientation to give reproducible diffraction patterns.

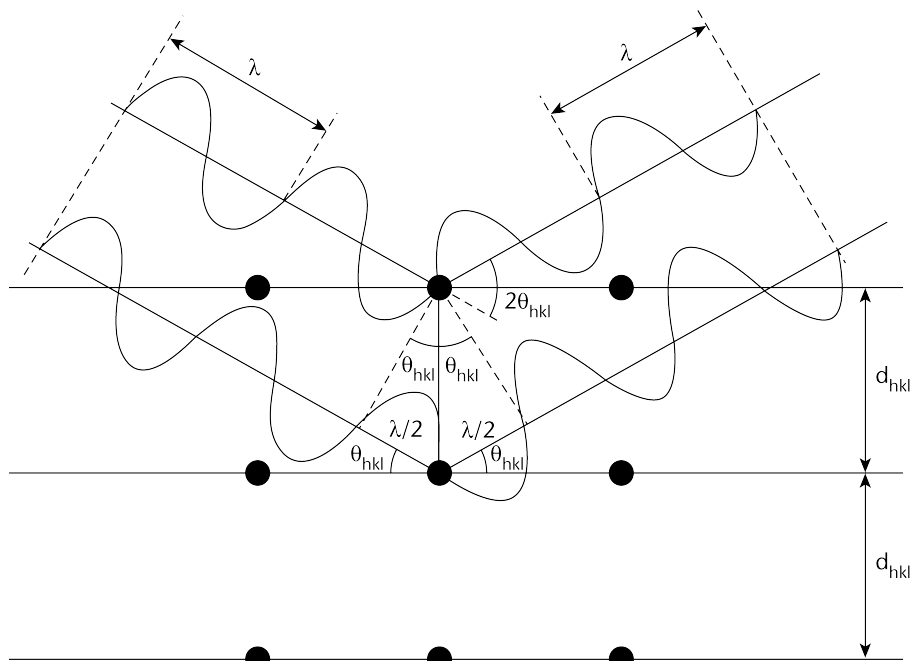


Figure 1. Diffraction of X-rays by a crystal according to Bragg's Law.

figuration is currently the most widely used and is therefore briefly described here.

A given instrument may provide a horizontal or vertical $\theta/2\theta$ geometry or a vertical θ/θ geometry. For both geometries, the incident X-ray beam forms an angle θ with the specimen surface plane, and the diffracted X-ray beam forms an angle 2θ with the direction of the incident X-ray beam (an angle θ with the specimen surface plane). The basic geometric arrangement is represented in Figure 3. The divergent beam of radiation from the X-ray tube (the so-called primary beam) passes through the parallel plate collimators and a divergence slit assembly and illuminates the flat surface of the specimen. All the rays diffracted by suitably oriented crystallites in the specimen at an angle 2θ converge to a line at the receiving slit. A second set of parallel plate collimators and a scatter slit may be placed either behind or before the receiving slit. The axes of the line focus and of the receiving slit are at equal distances from the axis of the goniometer. The X-ray quanta are counted by a radiation detector, usually a scintillation counter, a sealed-gas proportional counter, or a position-sensitive solid-state detector such as an imaging plate or CCD detector. The receiving slit assembly and the detector are coupled together and move tangentially to the focusing circle. For $\theta/2\theta$ scans, the goniometer rotates the specimen around the same axis as that of the detector, but at half the rotational speed, in a $\theta/2\theta$ motion. The surface of the specimen thus remains tangential to the focusing circle. The parallel plate collimator limits the axial divergence of the beam and hence partially controls the shape of the diffracted line profile.

A diffractometer may also be used in transmission mode. The advantage with this technology is to lessen the effects due to preferred orientation. A capillary of about 0.5- to 2-mm thickness can also be used for small sample amounts.

X-Ray Radiation

In the laboratory, X-rays are obtained by bombarding a metal anode with electrons emitted by the thermionic effect and accelerated in a strong electric field (using a high-volt-

age generator). Most of the kinetic energy of the electrons is converted to heat, which limits the power of the tubes and requires efficient anode cooling. A 20- to 30-fold increase in brilliance can be obtained by using rotating anodes and by using X-ray optics. Alternatively, X-ray photons may be produced in a large-scale facility (synchrotron).

The spectrum emitted by an X-ray tube operating at sufficient voltage consists of a continuous background of polychromatic radiation and additional characteristic radiation that depends on the type of anode. Only this characteristic radiation is used in X-ray diffraction experiments. The principal radiation sources used for X-ray diffraction are vacuum tubes using copper, molybdenum, iron, cobalt, or chromium as anodes; copper, molybdenum, or cobalt X-rays are employed most commonly for organic substances (the use of a cobalt anode can especially be preferred to separate distinct X-ray lines). The choice of radiation to be used depends on the absorption characteristics of the specimen and possible fluorescence by atoms present in the specimen. The wavelengths used in powder diffraction generally correspond to the K_α radiation from the anode. Consequently, it is advantageous to make the X-ray beam "monochromatic" by eliminating all the other components of the emission spectrum. This can be partly achieved using K_β filters—that is, metal filters selected as having an absorption edge between the K_α and K_β wavelengths emitted by the tube. Such a filter is usually inserted between the X-ray tube and the specimen. Another more commonly used way to obtain a monochromatic X-ray beam is via a large monochromator crystal (usually referred to as a "monochromator"). This crystal is placed before or behind the specimen and diffracts the different characteristic peaks of the X-ray beam (i.e., K_α and K_β) at different angles so that only one of them may be selected to enter into the detector. It is even possible to separate $K_{\alpha 1}$ and $K_{\alpha 2}$ radiations by using a specialized monochromator. Unfortunately, the gain in getting a monochromatic beam by using a filter or a monochromator is counteracted by a loss in intensity. Another way of separating K_α and K_β wavelengths is by using curved X-ray mirrors that can simultaneously monochromate and focus or parallelize the X-ray beam.

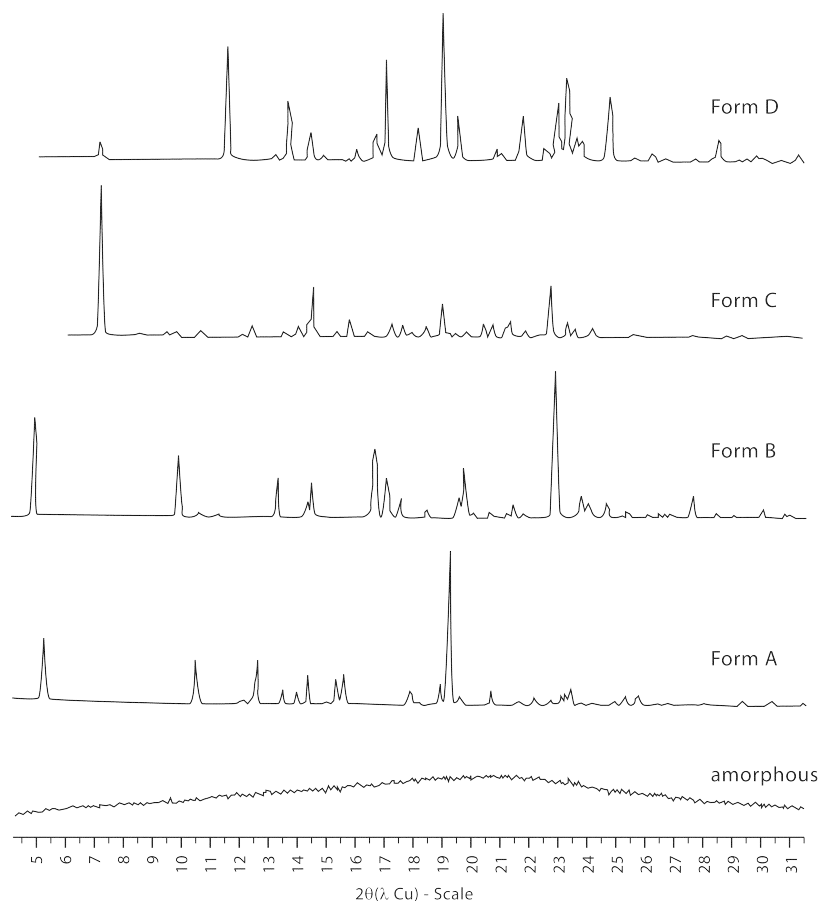


Figure 2. X-ray powder diffraction patterns collected for five different solid phases of a substance (the intensities are normalized).

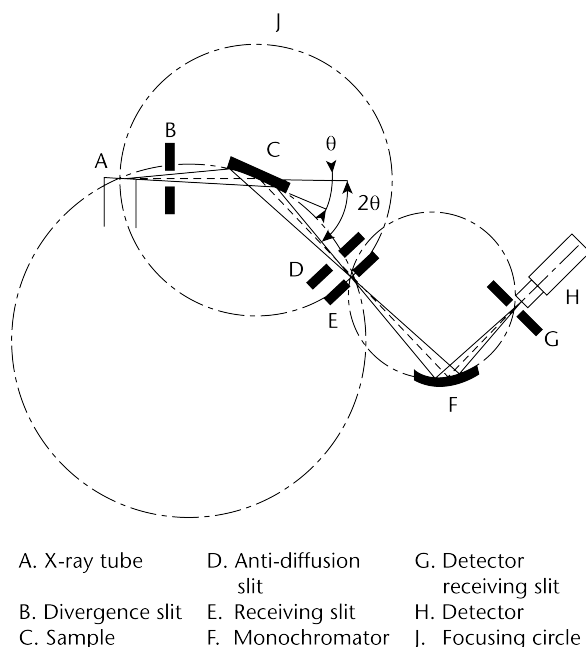


Figure 3. Geometric arrangement of the Bragg-Brentano parafocusing geometry.

RADIATION PROTECTION

Exposure of any part of the human body to X-rays can be injurious to health. It is therefore essential that whenever X-ray equipment is used, adequate precautions be taken to protect the operator and any other person in the vicinity. Recommended practice for radiation protection as well as limits for the levels of X-radiation exposure are those established by national legislation in each country. If there are no official regulations or recommendations in a country, the latest recommendations of the International Commission on Radiological Protection should be applied.

SPECIMEN PREPARATION AND MOUNTING

The preparation of the powdered material and the mounting of the specimen in a suitable holder are critical steps in many analytical methods, particularly for X-ray powder diffraction analysis, since they can greatly affect the quality of the data to be collected.³ The main sources of errors due to specimen preparation and mounting are briefly discussed in the following section for instruments in Bragg-Brentano parafocusing geometry.

Specimen Preparation

In general, the morphology of many crystalline particles tends to give a specimen that exhibits some degree of preferred orientation in the specimen holder. This is particularly evident for needle-like or platelike crystals when size reduc-

³ Similarly, changes in the specimen can occur during data collection in the case of a nonequilibrium specimen (temperature, humidity).

tion yields finer needles or platelets. Preferred orientation in the specimen influences the intensities of various reflections so that some are more intense and others less intense, compared to what would be expected from a completely random specimen. Several techniques can be employed to improve randomness in the orientation of crystallites (and therefore to minimize preferred orientation), but further reduction of particle size is often the best and simplest approach. The optimum number of crystallites depends on the diffractometer geometry, the required resolution, and the specimen attenuation of the X-ray beam. In some cases, particle sizes as large as 50 μm will provide satisfactory results in phase identification. However, excessive milling (crystallite sizes less than approximately 0.5 μm) may cause line broadening and significant changes to the sample itself, such as

- specimen contamination by particles abraded from the milling instruments (mortar, pestle, balls, etc.),
- reduced degree of crystallinity,
- solid-state transition to another polymorph,
- chemical decomposition,
- introduction of internal stress, and
- solid-state reactions.

Therefore, it is advisable to compare the diffraction pattern of the nonground specimen with that corresponding to a specimen of smaller particle size (e.g., a milled specimen). If the X-ray powder diffraction pattern obtained is of adequate quality considering its intended use, then grinding may not be required.

It should be noted that if a sample contains more than one phase and if sieving is used to isolate particles to a specific size, the initial composition may be altered.

Specimen Mounting

EFFECT OF SPECIMEN DISPLACEMENT

A specimen surface that is offset by D with reference to the diffractometer rotation axis causes systematic errors that are very difficult to avoid entirely; for the reflection mode, this results in absolute $D \cdot \cos\theta$ shifts⁴ in 2θ positions (typically of the order of 0.01° in 2θ at low angles

$$[\cos\theta \approx 1]$$

for a displacement $D = 15 \mu\text{m}$) and asymmetric broadening of the profile toward low 2θ values. Use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen transparency. This effect is by far the largest source of errors in data collected on well-aligned diffractometers.

EFFECT OF SPECIMEN THICKNESS AND TRANSPARENCY

When the XRPD method in reflection mode is applied, it is often preferable to work with specimens of "infinite thickness". To minimize the transparency effect, it is advisable to use a nondiffracting substrate (zero background holder)—for example, a plate of single crystalline silicon cut parallel to the 510 lattice planes.⁵ One advantage of the transmission mode is that problems with sample height and specimen transparency are less important.

⁴Note that a goniometer zero alignment shift would result in a constant shift on all observed 2θ -line positions; in other words, the whole diffraction pattern is, in this case, translated by an offset of Z° in 2θ .

⁵In the case of a thin specimen with low attenuation, accurate measurements of line positions can be made with focusing diffractometer configurations in either transmission or reflection geometry. Accurate measurements of line positions on specimens with low attenuation are preferably made using diffractometers with parallel beam optics. This helps to reduce the effects of specimen thickness.

The use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen displacement.

CONTROL OF THE INSTRUMENT PERFORMANCE

The goniometer and the corresponding incident and diffracted X-ray beam optics have many mechanical parts that need adjustment. The degree of alignment or misalignment directly influences the quality of the results of an XRPD investigation. Therefore, the different components of the diffractometer must be carefully adjusted (optical and mechanical systems, etc.) to adequately minimize systematic errors, while optimizing the intensities received by the detector. The search for maximum intensity and maximum resolution is always antagonistic when aligning a diffractometer. Hence, the best compromise must be sought while performing the alignment procedure. There are many different configurations, and each supplier's equipment requires specific alignment procedures. The overall diffractometer performance must be tested and monitored periodically, using suitable certified reference materials. Depending on the type of analysis, other well-defined reference materials may also be employed, although the use of certified reference materials is preferred.

QUALITATIVE PHASE ANALYSIS (IDENTIFICATION OF PHASES)

The identification of the phase composition of an unknown sample by XRPD is usually based on the visual or computer-assisted comparison of a portion of its X-ray powder pattern to the experimental or calculated pattern of a reference material. Ideally, these reference patterns are collected on well-characterized single-phase specimens. This approach makes it possible in most cases to identify a crystalline substance by its 2θ -diffraction angles or d -spacings and by its relative intensities. The computer-aided comparison of the diffraction pattern of the unknown sample to the comparison data can be based on either a more or less extended 2θ range of the whole diffraction pattern or on a set of reduced data derived from the pattern. For example, the list of d -spacings and normalized intensities, I_{norm} , a so-called (d, I_{norm}) list extracted from the pattern, is the crystallographic fingerprint of the material and can be compared to (d, I_{norm}) lists of single-phase samples compiled in databases.

For most organic crystals, when using $\text{Cu K}\alpha$ radiation, it is appropriate to record the diffraction pattern in a 2θ -range from as near 0° as possible to at least 40° . The agreement in the 2θ -diffraction angles between specimen and reference is within 0.2° for the same crystal form, while relative intensities between specimen and reference may vary considerably due to preferred orientation effects. By their very nature, variable hydrates and solvates are recognized to have varying unit cell dimensions, and as such, shifting occurs in peak positions of the measured XRPD patterns for these materials. In these unique materials, variance in 2θ positions of greater than 0.2° is not unexpected. As such, peak position variances such as 0.2° are not applicable to these materials. For other types of samples (e.g., inorganic salts), it may be necessary to extend the 2θ region scanned to well beyond 40° . It is generally sufficient to scan past the 10 strongest reflections identified in single-phase X-ray powder diffraction database files.

It is sometimes difficult or even impossible to identify phases in the following cases:

- noncrystallized or amorphous substances,
- the components to be identified are present in low mass fractions of the analyte amounts (generally less than 10% m/m),
- pronounced preferred orientation effects,
- the phase has not been filed in the database used,

- the formation of solid solutions,
- the presence of disordered structures that alter the unit cell,
- the specimen comprises too many phases,
- the presence of lattice deformations,
- the structural similarity of different phases.

QUANTITATIVE PHASE ANALYSIS

If the sample under investigation is a mixture of two or more known phases, of which not more than one is amorphous, the percentage (by volume or by mass) of each crystalline phase and of the amorphous phase can in many cases be determined. Quantitative phase analysis can be based on the integrated intensities, on the peak heights of several individual diffraction lines,⁶ or on the full pattern. These integrated intensities, peak heights, or full-pattern data points are compared to the corresponding values of reference materials. These reference materials must be single phase or a mixture of known phases. The difficulties encountered during quantitative analysis are due to specimen preparation (the accuracy and precision of the results require, in particular, homogeneity of all phases and a suitable particle size distribution in each phase) and to matrix effects.

In favorable cases, amounts of crystalline phases as small as 10% may be determined in solid matrices.

Polymorphic Samples

For a sample composed of two polymorphic phases *a* and *b*, the following expression may be used to quantify the fraction F_α of phase *a*:

$$F_\alpha = 1/[1 + K(I_b/I_\alpha)]$$

The fraction is derived by measuring the intensity ratio between the two phases, knowing the value of the constant *K*. *K* is the ratio of the absolute intensities of the two pure polymorphic phases I_{0a}/I_{0b} . Its value can be determined by measuring standard samples.

Methods Using a Standard

The most commonly used methods for quantitative analysis are

- the external standard method,
- the internal standard method, and
- the spiking method (also often called the standard addition method).

The external standard method is the most general method and consists of comparing the X-ray diffraction pattern of the mixture, or the respective line intensities, with those measured in a reference mixture or with the theoretical intensities of a structural model, if it is fully known.

To limit errors due to matrix effects, an internal reference material can be used that has a crystallite size and X-ray absorption coefficient comparable to those of the compo-

⁶If the crystal structures of all components are known, the Rietveld method can be used to quantify them with good accuracy. If the crystal structures of the components are not known, the Pawley method or the partial least-squares (PLS) method can be used.

nents of the sample and with a diffraction pattern that does not overlap at all that of the sample to be analyzed. A known quantity of this reference material is added to the sample to be analyzed and to each of the reference mixtures. Under these conditions, a linear relationship between line intensity and concentration exists. This application, called the internal standard method, requires precise measurement of diffraction intensities.

In the spiking method (or standard addition method), some of the pure phase *a* is added to the mixture containing the unknown concentration of *a*. Multiple additions are made to prepare an intensity-versus-concentration plot in which the negative x-intercept is the concentration of the phase *a* in the original sample.

ESTIMATE OF THE AMORPHOUS AND CRYSTALLINE FRACTIONS

In a mixture of crystalline and amorphous phases, the crystalline and amorphous fractions can be estimated in several ways. The choice of the method used depends on the nature of the sample:

- If the sample consists of crystalline fractions and an amorphous fraction of different chemical compositions, the amounts of each of the individual crystalline phases may be estimated using appropriate standard substances, as described above. The amorphous fraction is then deduced indirectly by subtraction.
- If the sample consists of one amorphous and one crystalline fraction, either as a 1-phase or a 2-phase mixture, with the same elemental composition, the amount of the crystalline phase (the "degree of crystallinity") can be estimated by measuring three areas of the diffractogram:

A = total area of the peaks arising from diffraction from the crystalline fraction of the sample,

B = total area below area *A*,

C = background area (due to air scattering, fluorescence, equipment, etc).

When these areas have been measured, the degree of crystallinity can be roughly estimated as:

$$\% \text{ crystallinity} = 100A/(A + B - C)$$

It is noteworthy that this method does not yield an absolute degree of crystallinity values and hence is generally used for comparative purposes only. More sophisticated methods are also available, such as the Ruland method.

SINGLE CRYSTAL STRUCTURE

In general, the determination of crystal structures is performed from X-ray diffraction data obtained using single crystals. However, crystal structure analysis of organic crystals is a challenging task, since the lattice parameters are comparatively large, the symmetry is low, and the scattering properties are normally very low. For any given crystalline form of a substance, the knowledge of the crystal structure allows for calculating the corresponding XRPD pattern, thereby providing a preferred orientation-free reference XRPD pattern, which may be used for phase identification.

General Chapters

General Information

General Information

The chapters in this section are information, and aside from excerpts given herein from Federal Acts and regulations that may be applicable, they contain no standards, tests, assays, nor other mandatory specifications, with respect to any Pharmacopeial articles. The excerpts from pertinent Federal Acts and regulations included in this section are placed here inasmuch as they are not of Pharmacopeial authorship. Revisions of the federal requirements that affect these excerpts will be included in *USP Supplements* as promptly as practical. The official requirements for Pharmacopeial articles are set forth in the *General Notices*, the individual monographs, and the *General Tests and Assays* chapters of this *Pharmacopeia*.

<1005> ACOUSTIC EMISSION

INTRODUCTION

Ultrasound techniques can be categorized into two distinct types: acoustic emission (passive mode) and ultrasound spectroscopy (active mode). Both of these techniques have many applications.

The technique of acoustic emission is based on the detection and analysis of sound produced by a process or system. This is essentially equivalent to listening to the process or system, although these sounds are often well above the frequencies that can be detected by the human ear. Generally, frequencies up to about 15 kHz are audible.

In the case of ultrasound spectroscopy, the instrument is designed to generate ultrasound waves across a defined frequency range. These waves travel through the sample and are measured using a receiver. An analogy can be drawn with UV-visible or IR spectroscopy in that the detected ultrasound spectrum reflects changes in velocity or sound attenuation due to the interaction with a sample across a range of frequencies. However, as the scope of this chapter is limited to acoustic emission, ultrasound spectroscopy will not be discussed further.

Acoustic emission is well-known in the study of fracture mechanics and therefore is used extensively by material scientists. It is also widely used as a nondestructive testing technique and is applied routinely for the inspection of aircraft wings, pressure vessels, load-bearing structures, and components. Acoustic emission is also used in the engineering industry for the monitoring of machine tool wear.

In terms of pharmaceutical applications, the dependence of the acoustic emission measurement on physical properties such as particle size, mechanical strength, and cohesivity of solid materials allows the technique to be used for the control and endpoint detection of processes such as high shear granulation, fluid bed drying, milling, and micronization.

General Principles

Acoustic emissions can propagate by a number of modes. In solids, compressional and shear or transverse modes are important. Compressional modes have the highest velocity and thus reach the acoustic detector (or acoustic emission transducer) first. However, in most process applications of acoustic emission, there are many sources—each producing short bursts of energy—and, consequently, the different modes cannot easily be resolved. The detected signal, for example on the wall of a vessel, is a complex mixture of many overlapping waveforms resulting from many sources and many propagation modes.

At interfaces, depending on the relative acoustic impedance of the two materials, much of the energy is reflected back towards the source. In a fluidized bed, for example, acoustic emissions will only be detected from particles directly impacting the walls of the bed close to the transducer.

A convenient method of studying acoustic emission from processes is to use the “average signal level”. A root mean square-to-direct current (RMS-to-DC) converter may be used to convert the amplitude-modulated (AM) carrier into a more slowly varying DC signal. This is referred to as the average signal level (ASL). The ASL can then be digitally sampled (typically at a sampling frequency of about 50 Hz) and stored electronically for further signal processing.

The simplest way of studying the acoustic data is to examine changes in the ASL. However, other information can be derived from examining the power spectrum of the ASL. The power spectrum is calculated by taking the complex square of the amplitude spectrum and can be obtained by performing a Fast Fourier Transform (FFT) on the digitized raw data record. Power spectra may be averaged to produce a reliable estimate of power spectral density or to give a “fingerprint” of a particular process regime. Interpretation of the power spectrum is complicated by the fact that the acoustic signal originating in the system is distorted by several factors including transmission, reflection, and signal transfer characteristics.

The shape of the power spectrum of the ASL record is a function of the process dynamics. Periodic processes (e.g., mechanical stirring or periodic bubbling of a fluidized bed) show high power at certain discrete frequencies. Random processes show either flicker type properties, where power is inversely proportional to frequency, or white noise type properties in which power is independent of frequency. The amplitude of the power spectrum is also affected by the energy of the acoustic emissions produced by the process. For example, if hard material is being processed, the acous-

tic emission produced by particle impact will be greater than that produced by soft material.

INSTRUMENTATION

Generally, piezoelectric sensors are used to detect and quantify the acoustic signals produced by a process. Piezoelectric transducers are constructed from piezoelectric crystalline solids connected to transducer control circuitry by electrical leads. When configured as a detector, an acoustic wave that impinges on the piezoelectric element is transformed into an electrical signal in the transducer control circuitry. When configured as an acoustic generator, an electrical signal applied to the piezoelectric element by the control circuitry creates an acoustic wave that can propagate into the medium to which the transducer is attached. Typically, this means that acoustic emission detectors can also be operated as acoustic wave generators and this feature is used to ensure good sensor performance as described later (see *Qualification and Verification of Acoustic Emission Instruments*).

In general acoustic emission applications, sensors with different resonance frequencies are often used (e.g., 70 and 190 kHz, although higher frequencies may be more appropriate at smaller scales of operation), incorporating various band-passes. As sound (ultrasound) of the appropriate frequency range reaches these sensors, an electrical signal is generated, the amplitude of which is directly proportional to the energy (amplitude) of the incident sound waves.

These signals are processed through the following:

- (1) a pre-amplifier (which incorporates signal filtering),
- (2) an RMS-to-DC converter,
- (3) a variable gain amplifier, and
- (4) a PC-based data acquisition board.

The controlling software is also incorporated into the PC.

Acoustic emission equipment generally allows several sensors to be used simultaneously by incorporating multiple electronic channels into a single instrument.

Signal Processing

The signal from a resonant transducer resembles an AM radio signal. At the resonance frequency of the transducer, the signal consists of a carrier wave that is modulated in amplitude by the process. An RMS-to-DC converter is used to demodulate the signal. The output of this device is the modulation signal or envelope.

The envelope is digitally resampled at a frequency appropriate for the process. For example, 50 Hz is a typical digital sampling rate for a fluid bed drier or high shear granulator.

FACTORS AFFECTING MEASUREMENT

The following factors can affect the acoustic data obtained and should be considered when installing an acoustic emission system.

1. *Failure or Physical Damage*—As with any other type of sensor, acoustic emission sensors can fail with time or as a result of physical damage. It is important to check the sensor function as part of routine maintenance of the instrument. If multiple sensors are installed on the same vessel, an active signal can be generated from one sensor and this can be used to check the detection on another sensor. This exercise would ensure that the sensors are detecting the acoustic signals generated by the process. A statistically valid "minimum acceptable acoustic signal" for the sensor(s) should also be determined and monitored at the start, middle, and end of a process to ensure the performance of the sensor(s) during a process run. This may be established from the routine maintenance signal experiments or on the basis of historical data for the sensors.

2. *Issues of Sensor Interfacing*—Sensors are typically installed on the outer wall of the process vessel. Several types of adhesives (temporary or permanent) can be used to attach the sensor to the vessel wall. Through repeated cleaning and vessel movement, it is possible for the bonding between the sensor and vessel to be compromised. Checking the integrity of the installation should be part of routine maintenance. Similar to item 1 above, an active signal can be used to ensure proper bonding between sensor and vessel and helps to confirm the matching of acoustic impedance.
3. *Influence of Mechanical Noise*—The use of high frequencies significantly reduces the contribution of mechanical noise to the acoustic signal detected, especially at smaller scales of operation, although it does not eliminate it completely. Testing the effect of various motor settings, for example, can determine if the acoustic signal detected is a function of mechanical noise. If the effect is significant, using higher frequencies may be necessary. Awareness of the contribution of the mechanical noise, no matter how small, is important to consider as the motors age or are replaced.
4. *Influence of Vessel Wall Characteristics*—Because the sensors are often placed on the outer vessel wall, wall thickness can affect the quality of the signal detected. If the vessel is jacketed, the amplitude of the acoustic signal may be reduced. Adding more sensors on the vessel can improve signal quality. Alternatively, an increase in signal may be obtained by positioning sensor(s) at a location where contact exists between the inner and outer walls, essentially providing a waveguide between the sensor and sound source. Waveguides may also be incorporated into the design of manufacturing equipment to enable utilization of acoustic emission monitoring. Appropriate validation is required to ensure that this does not adversely affect the performance of equipment.
5. *Effect of Material Properties*—During operation, the acoustic signal collected is a summation of various events occurring within the process. For example, the acoustic signal generated as particles hit the wall in a granulator is a function of the material properties of the granules (i.e., density, size, porosity). Therefore, significant changes to any of these parameters can affect the acoustic signal and the quality of the ensuing prediction.
6. *Influence of Process-Related Factors*—Similar to item 5 above, the process-related properties (i.e., force of impact, frequency of impact, amount of material) can also affect the acoustic signal and the quality of the ensuing prediction.
7. *Impact of Environmental Conditions*—Finally, the influence of environmental factors (i.e., temperature, humidity) must also be considered.

The acoustic emission data collected is vessel/equipment specific. It is not advisable to apply a model generated on one piece of equipment to another because the acoustic information can differ as a result of the issues discussed in items 3, 4, and 5 above.

Qualification and Verification of Acoustic Emission Instruments

A system suitability approach should be taken around instrument performance, establishing optimum measurement configuration, then comparing the instrument performance to the values obtained during routine use to those obtained during installation qualification (IQ).

This approach effectively answers the issues related to sampling because, unlike other on-line analytical systems, the transducers can be optimally positioned and attached to receive the maximum signal without vessel modification.

Sample rates need to comply with the Nyquist sampling theorem, which states that a signal must be sampled at a rate that is twice the highest frequency component in the signal. A low-pass filter should be used to remove the frequency components greater than half the sampling frequency (Nyquist frequency). Failure to comply with this criterion will result in aliasing.

Owing to the nature of the piezoelectric transducers and because resonance frequencies are natural properties of the crystals, it is not necessary to test the variation (reproducibility) or drift in the frequency domain. If other types of transducers are used, this may be necessary. Any gross change in the frequency domain will be recorded as a drop in the power intensity at the resonance frequency, and therefore is covered by the power intensity tests.

The two main areas for instrument performance verification are power intensity and timings. Any change in the signal intensity will affect the raw signal and the ASL and, therefore, will also affect the power spectrum. Changes in power intensity can occur as a result of changes in the process (e.g., variation in hardness or moisture in the particles impacting the vessel wall) or changes in the acoustic conduit from the process to transducer.

Reproducibility of the acoustic conduit should be tested using a second transducer to input a pulse or "ping" at the resonance frequency of the receiving sensor. This reproducibility value represents the noise of the signal and can be used in calculations of limit of detection (LOD) and limit of quantitation (LOQ), where LOD is defined as three times the noise of the signal and LOQ is ten times the noise of the signal. The noise on the background signal level (in acoustic emission this background signal is mainly due to amplifier noise) should be calculated from twenty sequential ASL values acquired at the sampling frequency used for normal operation. This test should be repeated in reverse in order to establish that statistically similar intensity values can be obtained on both channels.

Short term reproducibility allows the calculation of noise. However, it does not give a measure of integrity of acoustic conduit over time or, more specifically, of changes caused by the process (e.g., variations in adhesive properties with process changes such as heating/cooling). The noise test should be repeated while executing the normal processing parameters (using an empty vessel) and the drift in the ASL should be calculated. Care should be taken to make sure that signal drift (due to normal variation in processing parameters) does not impact chemometric models used for endpoint determination. For trend plots, it should be shown that drift is not statistically significant; otherwise, drift correction will need to be applied. Values for noise, drift, and absolute ASL should be recorded and logged, and the tests re-executed if changes are made to the processing equipment or to the acoustic emission system. If no changes are made, then the tests should be re-executed every month. In this way the quality of the acoustic conduit can be shown to be intact and any changes to the signal intensity isolated and attributed to the process itself.

During routine use, it is recommended that the noise test be executed (as above) before each process run, and that power intensity and noise be calculated. These values should be logged and compared to those generated both during previous use and during installation. Impact of the deviation from previous values will be a function of the prediction model and should be addressed by method validation.

The noise data (from above) can also be used to calculate the time of flight of the pulse. If the pulse activation and signal reception are synchronized, the time taken for the pulse to transmit across the vessel can be measured. This is a good indication of the measurement electronics as well as the overall condition of the acoustic conduit. However, this test should be regarded as a measure of the "system" condition and needs to be executed only if changes have been made to the process equipment or the acoustic emission system, or every 6 months. Correlation of the measured tim-

ings with the historical ones should be statistically valid. If not, it is an indicator that the acoustic emission system may need requalification by the instrument manufacturer or supplier, or that there are changes in the acoustic conduit.

All of these tests require the use of an acoustic pulse generated electrically. Failure in any of the above tests could be attributed to the signal generation itself. It is recommended that the electrical pulse generation system be requalified and certified against National Institute of Standards and Technology (NIST) traceable standards every 12 months.

DATA ANALYSIS

Acoustic emission from granulators and fluid bed driers is known as continuous acoustic emission. Continuous acoustic emission is aphasical (i.e., there are no starts or stops to the signal). This means that it is unnecessary to use signal processing techniques that preserve phase. Power spectral analysis is a useful technique in processing acoustic emission signals. The information in the power spectra, unlike the raw acoustic emission signals, is coherent in the short term, allowing signal averaging to be performed. This provides a better estimate of power spectral density than that provided by a single power spectrum.

To detect endpoints in batch processes (e.g., granulation or drying endpoint), a qualitative multivariate model is appropriate (e.g., PCA or SIMCA). The following sequence of operations is performed:

- (1) *Training/Calibration*—Acoustic emission spectra that are representative of the endpoint condition are obtained.
- (2) *Modeling*—A multivariate model describing the distribution of acoustic emission signals at the endpoint condition is created.
- (3) *Prediction*—Acoustic emission spectra are compared against the model. The fit to the model (usually expressed in terms of a number of standard deviations) is monitored. As the system approaches the endpoint, the fit improves and completion of the process is established once the model fits predefined criteria. The prediction model is generated from acoustic emission spectra obtained from the process operating under normal conditions. Upsets (e.g., unwanted agglomeration in coaters) are detected by observing statistically valid deviations from the model.

Adaptive modeling has also been proposed for upset detection. This involves generating multivariate models continuously as the acoustic emission signals are acquired. Unusual deviation of the acoustic emission signal indicates the occurrence of a process upset. The advantage of adaptive modeling is that it is not necessary to perform a separate calibration step.

GLOSSARY

Acoustic Emission Transducer—A solid state device usually incorporating a piezoelectric element to convert the acoustic emission wave to an electrical signal.

Acoustic Impedance—Acoustic impedance (Z) is defined as $Z = \rho v$ (where ρ is density and v is the sound velocity). It is an important quantity and gives the proportion of sound energy transmitted from one medium to another and the amount of energy reflected at the interface.

Adaptive Modeling—A method that predicts the state of a process without the use of a previously generated model (i.e., there is no prior training or calibration step).

Aliasing—Spurious low frequency components, appearing in the signal, that are really frequencies above the Nyquist frequency.

Amplitude—The magnitude or strength of a varying waveform.

Average Signal Level (ASL)—A measure of the average power in an acoustic emission signal.

Band-Pass—The range of frequencies within which a component operates.

Compressional Mode—A longitudinal mode of acoustic transmission encountered in solids, liquids, and gases.

Continuous Acoustic Emission—Acoustic emission signals that cannot be separated in time and are typical of pharmaceutical processes such as granulation and fluid bed drying.

Flicker Type Properties—A type of signal associated with many natural processes. The characteristics of flicker noise are that the power of the noise is directly proportional to the signal and has approximately a $1/f$ (f = frequency) spectral density distribution.

Gain—The amplification factor for a component usually expressed in terms of decibels (dB).

Gain in dB = $20 \log_{10} (\text{Voltage}_{\text{out}} / \text{Voltage}_{\text{in}})$.

Nyquist Frequency—The Nyquist frequency is defined as half the digital sampling rate and is the highest frequency that can be reproduced faithfully.

Piezoelectric—A material which generates an electric field when compressed. Piezoelectric materials are used in the construction of acoustic emission sensors. A common material is PZT (lead zirconium titanate).

Power Spectrum—A power spectrum of a signal is a representation of the signal power as a function of frequency. A power spectrum is calculated from the time domain signal by means of the Fast Fourier Transform (FFT) algorithm. It is useful to study acoustic emission signals in the frequency or spectral domain, as the spectrum is often characteristic of the mechanism. Improvements in signal-to-noise ratio can be obtained by averaging a number of power spectra, as they are coherent.

Power Spectral Density—The measure of acoustic emission power in each resolution element of the power spectrum.

Resonance Frequency—The frequency at which an acoustic emission sensor is most sensitive. Resonant acoustic emission sensors have a clearly defined resonance frequency, but are usually sensitive to other frequencies.

RMS-to-DC Converter—An electronic device that converts an alternating signal to a voltage level proportional to the average power in the signal.

Shear Mode—A transverse mode of acoustic transmission, encountered only in solids.

Signal Filtering—Filtering a signal means attenuating frequencies outside a prescribed range. In acoustic emission work, band-pass filtering is used to improve the signal-to-noise ratio by attenuating noise outside the bandwidth of the sensor. Low-pass filtering is used to remove frequencies higher than the Nyquist frequency in order to prevent aliasing.

Transverse Mode—A mode of wave propagation where the displacement of the material is perpendicular to the direction of propagation. These modes are only encountered in solid materials.

White Noise—The characteristic of white noise is a power spectrum of uniform spectral density and is associated with purely random processes.

<1010> ANALYTICAL DATA— INTERPRETATION AND TREATMENT

INTRODUCTION

This chapter provides information regarding acceptable practices for the analysis and consistent interpretation of data obtained from chemical and other analyses. Basic statistical approaches for evaluating data are described, and the treatment of outliers and comparison of analytical methods are discussed in some detail.

NOTE—It should not be inferred that the analysis tools mentioned in this chapter form an exhaustive list. Other, equally valid, statistical methods may be used at the discretion of the manufacturer and other users of this chapter.

Assurance of the quality of pharmaceuticals is accomplished by combining a number of practices, including robust formulation design, validation, testing of starting materials, in-process testing, and final-product testing. Each of these practices is dependent on reliable test methods. In the development process, test procedures are developed and validated to ensure that the manufactured products are thoroughly characterized. Final-product testing provides further assurance that the products are consistently safe, efficacious, and in compliance with their specifications.

Measurements are inherently variable. The variability of biological tests has long been recognized by the USP. For example, the need to consider this variability when analyzing biological test data is addressed under *Design and Analysis of Biological Assays* <111>. The chemical analysis measurements commonly used to analyze pharmaceuticals are also inherently variable, although less so than those of the biological tests. However, in many instances the acceptance criteria are proportionally tighter, and thus, this smaller allowable variability has to be considered when analyzing data generated using analytical procedures. If the variability of a measurement is not characterized and stated along with the result of the measurement, then the data can only be interpreted in the most limited sense. For example, stating that the difference between the averages from two laboratories when testing a common set of samples is 10% has limited interpretation, in terms of how important such a difference is, without knowledge of the intralaboratory variability.

This chapter provides direction for scientifically acceptable treatment and interpretation of data. Statistical tools that may be helpful in the interpretation of analytical data are described. Many descriptive statistics, such as the mean and standard deviation, are in common use. Other statistical tools, such as outlier tests, can be performed using several different, scientifically valid approaches, and examples of these tools and their applications are also included. The framework within which the results from a compendial test are interpreted is clearly outlined in *Test Results, Statistics, and Standards* under *General Notices and Requirements*. Selected references that might be helpful in obtaining additional information on the statistical tools discussed in this chapter are listed in *Appendix F* at the end of the chapter. USP does not endorse these citations, and they do not rep-

resent an exhaustive list. Further information about many of the methods cited in this chapter may also be found in most statistical textbooks.

PREREQUISITE LABORATORY PRACTICES AND PRINCIPLES

The sound application of statistical principles to laboratory data requires the assumption that such data have been collected in a traceable (i.e., documented) and unbiased manner. To ensure this, the following practices are beneficial.

Sound Record Keeping

Laboratory records are maintained with sufficient detail, so that other equally qualified analysts can reconstruct the experimental conditions and review the results obtained. When collecting data, the data should generally be obtained with more decimal places than the specification requires and rounded only after final calculations are completed as per the *General Notices and Requirements*.

Sampling Considerations

Effective sampling is an important step in the assessment of a quality attribute of a population. The purpose of sampling is to provide representative data (the sample) for estimating the properties of the population. How to attain such a sample depends entirely on the question that is to be answered by the sample data. In general, use of a random process is considered the most appropriate way of selecting a sample. Indeed, a random and independent sample is necessary to ensure that the resulting data produce valid estimates of the properties of the population. Generating a nonrandom or "convenience" sample risks the possibility that the estimates will be biased. The most straightforward type of random sampling is called *simple random sampling*, a process in which every unit of the population has an equal chance of appearing in the sample. However, sometimes this method of selecting a random sample is not optimal because it cannot guarantee equal representation among factors (i.e., time, location, machine) that may influence the critical properties of the population. For example, if it requires 12 hours to manufacture all of the units in a lot and it is vital that the sample be representative of the entire production process, then taking a simple random sample after the production has been completed may not be appropriate because there can be no guarantee that such a sample will contain a similar number of units made from every time period within the 12-hour process. Instead, it is better to take a *systematic random sample* whereby a unit is randomly selected from the production process at systematically selected times or locations (e.g., sampling every 30 minutes from the units produced at that time) to ensure that units taken throughout the entire manufacturing process are included in the sample. Another type of random sampling procedure is needed if, for example, a product is filled into vials using four different filling machines. In this case it would be important to capture a random sample of vials from each of the filling machines. A *stratified random sample*, which randomly samples an equal number of vials from each of the four filling machines, would satisfy this requirement. Regardless of the reason for taking a sample (e.g., batch-release testing), a sampling plan should be established to provide details on how the sample is to be obtained to ensure that the sample is representative of the entirety of the population and that the resulting data have the required sensitivity. The optimal sampling strategy will depend on knowledge of the manufacturing and analytical measurement processes. Once the sampling scheme has been defined, it is likely that the sampling will include some

element of random selection. Finally, there must be sufficient sample collected for the original analysis, subsequent verification analyses, and other analyses. Consulting a statistician to identify the optimal sampling strategy is recommended.

Tests discussed in the remainder of this chapter assume that simple random sampling has been performed.

Use of Reference Standards

Where the use of the USP Reference Standard is specified, the USP Reference Standard, or a secondary standard traceable to the USP Reference Standard, is used. Because the assignment of a value to a standard is one of the most important factors that influences the accuracy of an analysis, it is critical that this be done correctly.

System Performance Verification

Verifying an acceptable level of performance for an analytical system in routine or continuous use can be a valuable practice. This may be accomplished by analyzing a control sample at appropriate intervals, or using other means, such as, variation among the standards, background signal-to-noise ratios, etc. Attention to the measured parameter, such as charting the results obtained by analysis of a control sample, can signal a change in performance that requires adjustment of the analytical system. An example of a controlled chart is provided in *Appendix A*.

Method Validation

All methods are appropriately validated as specified under *Validation of Compendial Procedures* <1225>. Methods published in the *USP–NF* have been validated and meet the Current Good Manufacturing Practices regulatory requirement for validation as established in the Code of Federal Regulations. A validated method may be used to test a new formulation (such as a new product, dosage form, or process intermediate) only after confirming that the new formulation does not interfere with the accuracy, linearity, or precision of the method. It may not be assumed that a validated method could correctly measure the active ingredient in a formulation that is different from that used in establishing the original validity of the method. [NOTE on terminology—The definition of *accuracy* in *Validation of Compendial Procedures* <1225> and in ICH Q2 corresponds to unbiasedness only. In the International Vocabulary of Metrology (VIM) and documents of the International Organization for Standardization (ISO), *accuracy* has a different meaning. In ISO, *accuracy* combines the concepts of unbiasedness (termed *trueness*) and precision. This chapter follows the definition in chapter <1225>, which corresponds only to trueness.]

MEASUREMENT PRINCIPLES AND VARIATION

All measurements are, at best, estimates of the actual ("true" or "accepted") value for they contain random variability (also referred to as random error) and may also contain systematic variation (bias). Thus, the measured value differs from the actual value because of variability inherent in the measurement. If an array of measurements consists of individual results that are representative of the whole, statistical methods can be used to estimate informative properties of the entirety, and statistical tests are available to investigate whether it is likely that these properties comply with given requirements. The resulting statistical analyses should address the variability associated with the measurement process as well as that of the entity being measured. Statistical measures used to assess the direction and magnitude of

these errors include the mean, standard deviation, and expressions derived therefrom, such as the percent coefficient of variation (%CV, also called the percent relative standard deviation, %RSD). The estimated variability can be used to calculate confidence intervals for the mean, or measures of variability, and tolerance intervals capturing a specified proportion of the individual measurements.

The use of statistical measures must be tempered with good judgment, especially with regard to representative sampling. Data should be consistent with the statistical assumptions used for the analysis. If one or more of these assumptions appear to be violated, alternative methods may be required in the evaluation of the data. In particular, most of the statistical measures and tests cited in this chapter rely on the assumptions that the distribution of the entire population is represented by a normal distribution and that the analyzed sample is a representative subset of this population. The normal (or Gaussian) distribution is bell-shaped and symmetric about its center and has certain characteristics that are required for these tests to be valid. The data may not always be expected to be normally distributed and may require a transformation to better fit a normal distribution. For example, there exist variables that have distributions with longer right tails than left. Such distributions can often be made approximately normal through a log transformation. An alternative approach would be to use "distribution-free" or "nonparametric" statistical procedures that do not require that the shape of the population be that of a normal distribution. When the objective is to construct a confidence interval for the mean or for the difference between two means, for example, then the normality assumption is not as important because of the central limit theorem. However, one must verify normality of data to construct valid confidence intervals for standard deviations and ratios of standard deviations, perform some outlier tests, and construct valid statistical tolerance limits. In the latter case, normality is a critical assumption. Simple graphical methods, such as dot plots, histograms, and normal probability plots, are useful aids for investigating this assumption.

A single analytical measurement may be useful in quality assessment if the sample is from a whole that has been prepared using a well-validated, documented process and if the analytical errors are well known. The obtained analytical result may be qualified by including an estimate of the associated errors. There may be instances when one might consider the use of averaging because the variability associated with an average value is always reduced as compared to the variability in the individual measurements. The choice of whether to use individual measurements or averages will depend upon the use of the measure and its variability. For example, when multiple measurements are obtained on the same sample aliquot, such as from multiple injections of the sample in an HPLC method, it is generally advisable to average the resulting data for the reason discussed above.

Variability is associated with the dispersion of observations around the center of a distribution. The most commonly used statistic to measure the center is the sample mean (\bar{x}):

$$\bar{x} = \frac{\sum_{i=1}^n x_i}{n} = \frac{x_1 + x_2 + \dots + x_n}{n}$$

Method variability can be estimated in various ways. The most common and useful assessment of a method's variability is the determination of the standard deviation based on

repeated independent¹ measurements of a sample. The sample standard deviation, s , is calculated by the formula:

$$s = \sqrt{\sum_{i=1}^n (x_i - \bar{x})^2 / (n-1)}$$

in which x_i is the individual measurement in a set of n measurements; and \bar{x} is the mean of all the measurements. The percent relative standard deviation (%RSD) is then calculated as:

$$\%RSD = \frac{s}{\bar{x}} \cdot 100\%$$

and expressed as a percentage. If the data requires log transformation to achieve normality (e.g., for biological assays), then alternative methods are available.²

A precision study should be conducted to provide a better estimate of method variability. The precision study may be designed to determine intermediate precision (which includes the components of both "between run" and "within-run" variability) and repeatability ("within-run" variability). The intermediate precision studies should allow for changes in the experimental conditions that might be expected, such as different analysts, different preparations of reagents, different days, and different instruments. To perform a precision study, the test is repeated several times. Each run must be completely independent of the others to provide accurate estimates of the various components of variability. In addition, within each run, replicates are made in order to estimate repeatability. See an example of a precision study under *Appendix B*.

A confidence interval for the mean may be considered in the interpretation of data. Such intervals are calculated from several data points using the sample mean (\bar{x}) and sample standard deviation(s) according to the formula:

$$\left(\bar{x} - t_{\alpha/2, n-1} \frac{s}{\sqrt{n}}, \bar{x} + t_{\alpha/2, n-1} \frac{s}{\sqrt{n}} \right)$$

in which $t_{\alpha/2, n-1}$ is a statistical number dependent upon the sample size (n), the number of degrees of freedom ($n - 1$), and the desired confidence level ($1 - \alpha$). Its values are obtained from published tables of the Student t -distribution. The confidence interval provides an estimate of the range within which the "true" population mean (μ) falls, and it also evaluates the reliability of the sample mean as an estimate of the true mean. If the same experimental set-up were to be replicated over and over and a 95% (for example) confidence interval for the true mean is calculated each time, then 95% of such intervals would be expected to contain the true mean, μ . One cannot say with certainty whether or not the confidence interval derived from a specific set of data actually collected contains μ . However, assuming the data represent mutually independent measurements randomly generated from a normally distributed

¹ Multiple measurements (or, equivalently, the experimental errors associated with the multiple measurements) are independent from one another when they can be assumed to represent a random sample from the population. In such a sample, the magnitude of one measurement is not influenced by, nor does it influence the magnitude of, any other measurement. Lack of independence implies the measurements are correlated over time or space. Consider the example of a 96-well microtiter plate. Suppose that whenever the unknown causes that produce experimental error lead to a low result (negative error) when a sample is placed in the first column and these same causes would also lead to a low result for a sample placed in the second column, then the two resulting measurements would not be statistically independent. One way to avoid such possibilities would be to randomize the placement of the samples on the plate.

² When data have been log (base e) transformed to achieve normality, the %RSD is:

$$\%RSD = 100\% \cdot \sqrt{e^{s^2} - 1}$$

This can be reasonably approximated by:

$$\%RSD = 100\% \cdot (e^{s^2} - 1)$$

where s is the standard deviation of the log (base e) transformed data.

population, the procedure used to construct the confidence interval guarantees that 95% of such confidence intervals contain μ . Note that it is important to define the population appropriately so that all relevant sources of variation are captured. [NOTE on terminology—In the documents of the International Organization for Standardization (ISO), different terminology is used for some of the concepts described here. The term s/\sqrt{n} , which is commonly called the standard error of the mean, is called the standard uncertainty in ISO documents. The term $t_{\alpha/2, n-1} S/\sqrt{n}$ is called the expanded uncertainty, and $t_{\alpha/2, n-1}$ is called the coverage factor, by ISO. If the standard deviation is found by combining estimates of variability from multiple sources, it is called the combined standard uncertainty. Some of these sources could have nonstatistical estimates of uncertainty, called Type B uncertainties, such as uncertainty in calibration of a balance.]

OUTLYING RESULTS

Occasionally, observed analytical results are very different from those expected. Aberrant, anomalous, contaminated, discordant, spurious, suspicious or wild observations; and flyers, rogues, and mavericks are properly called outlying results. Like all laboratory results, these outliers must be documented, interpreted, and managed. Such results may be accurate measurements of the entity being measured, but are very different from what is expected. Alternatively, due to an error in the analytical system, the results may not be typical, even though the entity being measured is typical. When an outlying result is obtained, systematic laboratory and process investigations of the result are conducted to determine if an assignable cause for the result can be established. Factors to be considered when investigating an outlying result include—but are not limited to—human error, instrumentation error, calculation error, and product or component deficiency. If an assignable cause that is not related to a product or component deficiency can be identified, then retesting may be performed on the same sample, if possible, or on a new sample. The precision and accuracy of the method, the Reference Standard, process trends, and the specification limits should all be examined. Data may be invalidated, based on this documented investigation, and eliminated from subsequent calculations.

If no documentable, assignable cause for the outlying laboratory result is found, the result may be tested, as part of the overall investigation, to determine whether it is an outlier.

However, careful consideration is warranted when using these tests. Two types of errors may occur with outlier tests: (a) labeling observations as outliers when they really are not; and (b) failing to identify outliers when they truly exist. Any judgment about the acceptability of data in which outliers are observed requires careful interpretation.

“Outlier labeling” is informal recognition of suspicious laboratory values that should be further investigated with more formal methods. The selection of the correct outlier identification technique often depends on the initial recognition of the number and location of the values. Outlier labeling is most often done visually with graphical techniques. “Outlier identification” is the use of statistical significance tests to confirm that the values are inconsistent with the known or assumed statistical model.

When used appropriately, outlier tests are valuable tools for pharmaceutical laboratories. Several tests exist for detecting outliers. Examples illustrating three of these procedures, the Extreme Studentized Deviate (ESD) Test, Dixon’s Test, and Hampel’s Rule, are presented in *Appendix C*.

Choosing the appropriate outlier test will depend on the sample size and distributional assumptions. Many of these tests (e.g., the ESD Test) require the assumption that the data generated by the laboratory on the test results can be thought of as a random sample from a population that is normally distributed, possibly after transformation. If a transformation is made to the data, the outlier test is applied to

the transformed data. Common transformations include taking the logarithm or square root of the data. Other approaches to handling single and multiple outliers are available and can also be used. These include tests that use robust measures of central tendency and spread, such as the median and median absolute deviation and exploratory data analysis (EDA) methods. “Outlier accommodation” is the use of robust techniques, such as tests based on the order or rank of each data value in the data set instead of the actual data value, to produce results that are not adversely influenced by the presence of outliers. The use of such methods reduces the risks associated with both types of error in the identification of outliers.

“Outlier rejection” is the actual removal of the identified outlier from the data set. However, an outlier test cannot be the sole means for removing an outlying result from the laboratory data. An outlier test may be useful as part of the evaluation of the significance of that result, along with other data. Outlier tests have no applicability in cases where the variability in the product is what is being assessed, such as content uniformity, dissolution, or release-rate determination. In these applications, a value determined to be an outlier may in fact be an accurate result of a nonuniform product. All data, especially outliers, should be kept for future review. Unusual data, when seen in the context of other historical data, are often not unusual after all but reflect the influences of additional sources of variation.

In summary, the rejection or retention of an apparent outlier can be a serious source of bias. The nature of the testing as well as scientific understanding of the manufacturing process and analytical method have to be considered to determine the source of the apparent outlier. An outlier test can never take the place of a thorough laboratory investigation. Rather, it is performed only when the investigation is inconclusive and no deviations in the manufacture or testing of the product were noted. Even if such statistical tests indicate that one or more values are outliers, they should still be retained in the record. Including or excluding outliers in calculations to assess conformance to acceptance criteria should be based on scientific judgment and the internal policies of the manufacturer. It is often useful to perform the calculations with and without the outliers to evaluate their impact.

Outliers that are attributed to measurement process mistakes should be reported (i.e., footnoted), but not included in further statistical calculations. When assessing conformance to a particular acceptance criterion, it is important to define whether the reportable result (the result that is compared to the limits) is an average value, an individual measurement, or something else. If, for example, the acceptance criterion was derived for an average, then it would not be statistically appropriate to require individual measurements to also satisfy the criterion because the variability associated with the average of a series of measurements is smaller than that of any individual measurement.

COMPARISON OF ANALYTICAL METHODS

It is often necessary to compare two methods to determine if their average results or their variabilities differ by an amount that is deemed important. The goal of a method comparison experiment is to generate adequate data to evaluate the equivalency of the two methods over a range of concentrations. Some of the considerations to be made when performing such comparisons are discussed in this section.

Precision

Precision is the degree of agreement among individual test results when the analytical method is applied repeatedly to a homogeneous sample. For an alternative method to be considered to have “comparable” precision to that of a cur-

rent method, its precision (see *Analytical Performance Characteristics* under *Validation of Compendial Procedures* (1225)) must not be worse than that of the current method by an amount deemed important. A decrease in precision (or increase in variability) can lead to an increase in the number of results expected to fail required specifications. On the other hand, an alternative method providing improved precision is acceptable.

One way of comparing the precision of two methods is by estimating the variance for each method (the sample variance, s^2 , is the square of the sample standard deviation) and calculating a one-sided upper confidence interval for the ratio of (true) variances, where the ratio is defined as the variance of the alternative method to that of the current method. An example, with this assumption, is outlined under *Appendix D*. The one-sided upper confidence limit should be compared to an upper limit deemed acceptable, *a priori*, by the analytical laboratory. If the one-sided upper confidence limit is less than this upper acceptable limit, then the precision of the alternative method is considered acceptable in the sense that the use of the alternative method will not lead to an important loss in precision. Note that if the one-sided upper confidence limit is less than one, then the alternative method has been shown to have improved precision relative to the current method.

The confidence interval method just described is preferred to applying the two-sample F-test to test the statistical significance of the ratio of variances. To perform the two-sample F-test, the calculated ratio of sample variances would be compared to a critical value based on tabulated values of the F distribution for the desired level of confidence and the number of degrees of freedom for each variance. Tables providing F-values are available in most standard statistical textbooks. If the calculated ratio exceeds this critical value, a statistically significant difference in precision is said to exist between the two methods. However, if the calculated ratio is less than the critical value, this does not prove that the methods have the same or equivalent level of precision; but rather that there was not enough evidence to prove that a statistically significant difference did, in fact, exist.

Accuracy

Comparison of the accuracy (see *Analytical Performance Characteristics* under *Validation of Compendial Procedures* (1225)) of methods provides information useful in determining if the new method is equivalent, on the average, to the current method. A simple method for making this comparison is by calculating a confidence interval for the difference in true means, where the difference is estimated by the sample mean of the alternative method minus that of the current method.

The confidence interval should be compared to a lower and upper range deemed acceptable, *a priori*, by the laboratory. If the confidence interval falls entirely within this acceptable range, then the two methods can be considered equivalent, in the sense that the average difference between them is not of practical concern. The lower and upper limits of the confidence interval only show how large the true difference between the two methods may be, not whether this difference is considered tolerable. Such an assessment can be made only within the appropriate scientific context.

The confidence interval method just described is preferred to the practice of applying a *t*-test to test the statistical significance of the difference in averages. One way to perform the *t*-test is to calculate the confidence interval and to examine whether or not it contains the value zero. The two methods have a statistically significant difference in averages if the confidence interval excludes zero. A statistically significant difference may not be large enough to have practical importance to the laboratory because it may have arisen as a result of highly precise data or a larger sample size. On the other hand, it is possible that no statistically significant

difference is found, which happens when the confidence interval includes zero, and yet an important practical difference cannot be ruled out. This might occur, for example, if the data are highly variable or the sample size is too small. Thus, while the outcome of the *t*-test indicates whether or not a statistically significant difference has been observed, it is not informative with regard to the presence or absence of a difference of practical importance.

Determination of Sample Size

Sample size determination is based on the comparison of the accuracy and precision of the two methods³ and is similar to that for testing hypotheses about average differences in the former case and variance ratios in the latter case, but the meaning of some of the input is different. The first component to be specified is δ , the largest acceptable difference between the two methods that, if achieved, still leads to the conclusion of equivalence. That is, if the two methods differ by no more than δ , on the average, they are considered acceptably similar. The comparison can be two-sided as just expressed, considering a difference of δ in either direction, as would be used when comparing means. Alternatively, it can be one-sided as in the case of comparing variances where a decrease in variability is acceptable and equivalency is concluded if the ratio of the variances (new/current, as a proportion) is not more than $1.0 + \delta$. A researcher will need to state δ based on knowledge of the current method and/or its use, or it may be calculated. One consideration, when there are specifications to satisfy, is that the new method should not differ by so much from the current method as to risk generating out-of-specification results. One then chooses δ to have a low likelihood of this happening by, for example, comparing the distribution of data for the current method to the specification limits. This could be done graphically or by using a tolerance interval, an example of which is given in *Appendix E*. In general, the choice for δ must depend on the scientific requirements of the laboratory.

The next two components relate to the probability of error. The data could lead to a conclusion of similarity when the methods are unacceptably different (as defined by δ). This is called a false positive or Type I error. The error could also be in the other direction; that is, the methods could be similar, but the data do not permit that conclusion. This is a false negative or Type II error. With statistical methods, it is not possible to completely eliminate the possibility of either error. However, by choosing the sample size appropriately, the probability of each of these errors can be made acceptably small. The acceptable maximum probability of a Type I error is commonly denoted as α and is commonly taken as 5%, but may be chosen differently. The desired maximum probability of a Type II error is commonly denoted by β . Often, β is specified indirectly by choosing a desired level of $1 - \beta$, which is called the "power" of the test. In the context of equivalency testing, power is the probability of correctly concluding that two methods are equivalent. Power is commonly taken to be 80% or 90% (corresponding to a β of 20% or 10%), though other values may be chosen. The protocol for the experiment should specify δ , α , and power. The sample size will depend on all of these components. An example is given in *Appendix E*. Although *Appendix E* determines only a single value, it is often useful to determine a table of sample sizes corresponding to different choices of δ , α , and power. Such a table often allows for a more informed choice of sample size to better balance the competing priorities of resources and risks (false negative and false positive conclusions).

³ In general, the sample size required to compare the precision of two methods will be greater than that required to compare the accuracy of the methods.

APPENDIX A: CONTROL CHARTS

Figure 1 illustrates a control chart for individual values. There are several different methods for calculating the upper control limit (UCL) and lower control limit (LCL). One method involves the moving range, which is defined as the absolute difference between two consecutive measurements ($|x_i - x_{i-1}|$). These moving ranges are averaged (\overline{MR}) and used in the following formulas:

$$UCL = \bar{x} + 3 \frac{\overline{MR}}{d_2}$$

$$LCL = \bar{x} - 3 \frac{\overline{MR}}{d_2}$$

where \bar{x} is the sample mean, and d_2 is a constant commonly used for this type of chart and is based on the number of observations associated with the moving range calculation. Where $n = 2$ (two consecutive measurements), as here, $d_2 = 1.128$. For the example in Figure 1, the \overline{MR} was 1.7:

$$UCL = 102.0 + 3 \frac{1.7}{1.128} = 106.5$$

$$LCL = 102.0 - 3 \frac{1.7}{1.128} = 97.5$$

Other methods exist that are better able to detect small shifts in the process mean, such as the cumulative sum (also known as "CUSUM") and exponentially weighted moving average ("EWMA").

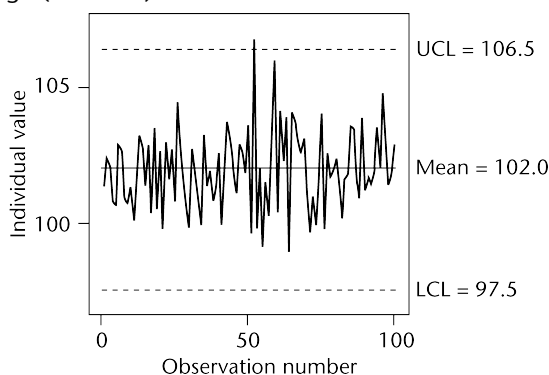


Figure 1. Individual X or individual measurements control chart for control samples. In this particular example, the mean for all the samples (\bar{x}) is 102.0, the UCL is 106.5, and the LCL is 97.5.

APPENDIX B: PRECISION STUDY

Table 1 displays data collected from a precision study. This study consisted of five independent runs and, within each run, results from three replicates were collected.

Performing an analysis of variance (ANOVA) on the data in Table 1 leads to the ANOVA table (Table 1A). Because there were an equal number of replicates per run in the precision study, values for $\text{Variance}_{\text{Run}}$ and $\text{Variance}_{\text{Rep}}$ can be derived from the ANOVA table in a straightforward manner. The equations below calculate the variability associated with both the runs and the replicates where the $\text{MS}_{\text{within}}$ repre-

sents the "error" or "within-run" mean square, and $\text{MS}_{\text{between}}$ represents the "between-run" mean square.

$$\text{Variance}_{\text{Rep}} = \text{MS}_{\text{within}} = 0.102$$

$$\text{Variance}_{\text{Run}} = \frac{\text{MS}_{\text{between}} - \text{MS}_{\text{within}}}{\# \text{ of reps per run}} = \frac{3.550 - 0.102}{3} = 1.149$$

[NOTE—It is common practice to use a value of 0 for $\text{Variance}_{\text{Run}}$ when the calculated value is negative.] Estimates can still be obtained with unequal replication, but the formulas are more complex. Many statistical software packages can easily handle unequal replication. Studying the relative magnitude of the two variance components is important when designing and interpreting a precision study. The insight gained can be used to focus any ongoing method improvement effort and, more important, it can be used to ensure that methods are capable of supporting their intended uses. By carefully defining what constitutes a result (i.e., reportable value), one harnesses the power of averaging to achieve virtually any desired precision. That is, by basing the reportable value on an average across replicates and/or runs, rather than on any single result, one can reduce the %RSD, and reduce it in a predictable fashion.

Table 2 shows the computed variance and %RSD of the mean (i.e., of the reportable value) for different combinations of number of runs and number of replicates per run using the following formulas:

Variance of the mean =

$$\frac{\text{Variance}_{\text{Run}}}{(\# \text{ of runs})} + \frac{\text{Variance}_{\text{rep}}}{(\# \text{ of runs})(\# \text{ of reps per run})}$$

Standard deviation of the mean = $\sqrt{\text{Variance of the mean}}$

$$\text{RSD} = \frac{\text{Standard deviation of the mean}}{\text{Average of all results}} \times 100\%$$

For example, the *Variance of the mean*, *Standard deviation of the mean*, and %RSD of a test involving two runs and three replicates per each run are 0.592, 0.769, and 0.76% respectively, as shown below.

$$\text{Variance of the mean} = \frac{1.149}{2} + \frac{0.102}{(2 \cdot 3)} = 0.592$$

$$\text{Standard deviation of the mean} = \sqrt{0.592} = 0.769$$

$$\text{RSD} = (0.769/100.96) \times 100\% = 0.76\%$$

where 100.96 is the mean for all the data points in Table 1. As illustrated in Table 2, increasing the number of runs from one to two provides a more dramatic reduction in the variability of the reportable value than does increasing the number of replicates per run.

No distributional assumptions were made on the data in Table 1, as the purpose of this Appendix is to illustrate the calculations involved in a precision study.

APPENDIX C: EXAMPLES OF OUTLIER TESTS FOR ANALYTICAL DATA

Given the following set of 10 measurements: 100.0, 100.1, 100.3, 100.0, 99.7, 99.9, 100.2, 99.5, 100.0, and

95.7 (mean = 99.5, standard deviation = 1.369), are there any outliers?

Generalized Extreme Studentized Deviate (ESD) Test

This is a modified version of the ESD Test that allows for testing up to a previously specified number, r , of outliers from a normally distributed population. For the detection of a single outlier ($r = 1$), the generalized ESD procedure is also known as Grubb's test. Grubb's test is not recommended for the detection of multiple outliers. Let r equal 2, and n equal 10.

Stage 1 ($n = 10$)—Normalize each result by subtracting the mean from each value and dividing this difference by the standard deviation (see Table 3).⁴

Take the absolute value of these results, select the maximum value ($|R_1| = 2.805$), and compare it to a previously specified tabled critical value λ_1 (2.290) based on the selected significance level (for example, 5%). The maximum value is larger than the tabled value and is identified as being inconsistent with the remaining data. Sources for λ -values are included in many statistical textbooks. Caution should be exercised when using any statistical table to ensure that the correct notations (i.e., level of acceptable error) are used when extracting table values.

Stage 2 ($n = 9$)—Remove the observation corresponding to the maximum absolute normalized result from the original data set, so that n is now 9. Again, find the mean and standard deviation (Table 3, right two columns), normalize each value, and take the absolute value of these results. Find the maximum of the absolute values of the 9 normalized results ($|R_2| = 1.905$), and compare it to λ_2 (2.215). The maximum value is not larger than the tabled value.

Conclusion—The result from the first stage, 95.7, is declared to be an outlier, but the result from the second stage, 99.5, is not an outlier.

Dixon-Type Tests

Dixon's Test can be one-sided or two-sided, depending on an a priori decision as to whether outliers will be considered on one side only. As with the ESD Test, Dixon's Test assumes that the data, in the absence of outliers, come from a single normal population. Following the strategy used for the ESD Test, we proceed as if there were no a priori decision as to side, and so use a two-sided Dixon's Test. From examination of the example data, we see that it is the two smallest that are to be tested as outliers. Dixon provides for testing for two outliers simultaneously; however, these procedures are beyond the scope of this Appendix. The stepwise procedure discussed below is not an exact procedure for testing for the second outlier, because the result of the second test is conditional upon the first. And because the sample size is also reduced in the second stage, the end result is a procedure that usually lacks the sensitivity of Dixon's exact procedures.

Stage 1 ($n = 10$)—The results are ordered on the basis of their magnitude (i.e., X_n is the largest observation, X_{n-1} is the second largest, etc., and X_1 is the smallest observation). Dixon's Test has different ratios based on the sample size (in this example, with $n = 10$), and to declare X_1 an outlier, the following ratio, r_{11} , is calculated by the formula:

$$r_{11} = \frac{X_2 - X_1}{X_{n-1} - X_1}$$

⁴ The difference between each value and the mean is termed the residual. Other Studentized residual outlier tests exist where the residual, instead of being divided by the standard deviation, can be divided by the standard deviation times the square root of $n - 1$ divided by n .

A different ratio would be employed if the largest data point was tested as an outlier. The r_{11} result is compared to an $r_{11, 0.05}$ value in a table of critical values. If r_{11} is greater than $r_{11, 0.05}$, then it is declared an outlier. For the above set of data, $r_{11} = (99.5 - 95.7)/(100.2 - 95.7) = 0.84$. This ratio is greater than $r_{11, 0.05}$, which is 0.52979 at the 5% significance level for a two-sided Dixon's Test. Sources for $r_{11, 0.05}$ values are included in many statistical textbooks.⁵

Stage 2—Remove the smallest observation from the original data set, so that n is now 9. The same r_{11} equation is used, but a new critical $r_{11, 0.05}$ value for $n = 9$ is needed ($r_{11, 0.05} = 0.56420$). Now $r_{11} = (99.7 - 99.5)/(100.2 - 99.5) = 0.29$, which is less than $r_{11, 0.05}$ and not significant at the 5% level.

Conclusion—Therefore, 95.7 is declared to be an outlier but 99.5 is not an outlier.

Hampel's Rule

Step 1—The first step in applying Hampel's Rule is to normalize the data. However, instead of subtracting the mean from each data point and dividing the difference by the standard deviation, the median is subtracted from each data value and the resulting differences are divided by MAD (see below). The calculation of MAD is done in three stages. First, the median is subtracted from each data point. Next, the absolute values of the differences are obtained. These are called the *absolute deviations*. Finally, the median of the absolute deviations is calculated and multiplied by the constant 1.483 to obtain MAD.⁶

Step 2—The second step is to take the absolute value of the normalized data. Any such result that is greater than 3.5 is declared to be an outlier. Table 4 summarizes the calculations.

The value of 95.7 is again identified as an outlier. This value can then be removed from the data set and Hampel's Rule re-applied to the remaining data. The resulting table is displayed as Table 5. Similar to the previous examples, 99.5 is not considered an outlier.

APPENDIX D: COMPARISON OF METHODS—PRECISION

The following example illustrates the calculation of a 90% confidence interval for the ratio of (true) variances for the purpose of comparing the precision of two methods. It is assumed that the underlying distribution of the sample measurements are well-characterized by normal distributions. For this example, assume the laboratory will accept the alternative method if its precision (as measured by the variance) is no more than four-fold greater than that of the current method.

To determine the appropriate sample size for precision, one possible method involves a trial and error approach using the following formula:

$$\text{Power} = \Pr \left[F > \frac{1}{4} F_{\alpha, n-1, n-1} \right]$$

where n is the smallest sample size required to give the desired power, which is the likelihood of correctly claiming the alternative method has acceptable precision when in fact the two methods have equal precision; α is the risk of wrongly claiming the alternative method has acceptable precision; and the 4 is the allowed upper limit for an increase in variance. F-values are found in commonly available

⁵ The critical values for r in this example are taken from Reference 2 under Appendix F, Outlier Tests.

⁶ Assuming an underlying normal distribution, 1.483 is a constant used so that the resulting MAD is a consistent estimator of the population standard deviation. This means that as the sample size gets larger, MAD gets closer to the population standard deviation.

tables of critical values of the F-distribution. $F_{\alpha, n-1, n-1}$ is the upper α percentile of an F-distribution with $n - 1$ numerator and $n - 1$ denominator degrees of freedom; that is, the value exceeded with probability α . Suppose initially the laboratory guessed a sample size of 11 per method was necessary (10 numerator and denominator degrees of freedom); the power calculation would be as follows:⁷

$$\begin{aligned}\Pr [F > 1/4 F_{\alpha, n-1, n-1}] &= \Pr [F > 1/4 F_{.05, 10, 10}] \\ &= \Pr [F > (2.978/4)] = 0.6751\end{aligned}$$

In this case the power was only 68%; that is, even if the two methods had exactly equal variances, with only 11 samples per method, there is only a 68% chance that the experiment will lead to data that permit a conclusion of no more than a four-fold increase in variance. Most commonly, sample size is chosen to have at least 80% power, with choices of 90% power or higher also used. To determine the appropriate sample size, various numbers can be tested until a probability is found that exceeds the acceptable limit (e.g., power > 0.90). For example, the power determination for sample sizes of 12–20 are displayed in Table 6. In this case, the initial guess at a sample size of 11 was not adequate for comparing precision, but 15 samples per method would provide a large enough sample size if 80% power were desired, or 20 per method for 90% power.

Typically the sample size for precision comparisons will be larger than for accuracy comparisons. If the sample size for precision is so large as to be impractical for the laboratory to conduct the study, there are some options. The first is to reconsider the choice of an allowable increase in variance. For larger allowable increases in variance, the required sample size for a fixed power will be smaller. Another alternative is to plan an interim analysis at a smaller sample size, with the possibility of proceeding to a larger sample size if needed. In this case, it is strongly advisable to seek professional help from a statistician.

Now, suppose the laboratory opts for 90% power and obtains the results presented in Table 7 based on the data generated from 20 independent runs per method.

$$\text{Ratio} = \text{alternative method variance/current method variance} = 45.0/25.0 = 1.8$$

$$\text{Lower limit of confidence interval} = \text{ratio}/F_{.05} = 1.8/2.168 = 0.83$$

$$\text{Upper limit of confidence interval} = \text{ratio}/F_{.95} = 1.8/0.461 = 3.90$$

For this application, a 90% (two-sided) confidence interval is used when a 5% one-sided test is sought. The test is one-sided, because only an increase in standard deviation of the alternative method is of concern. Some care must be exercised in using two-sided intervals in this way, as they must have the property of equal tails—most common intervals have this property. Because the one-side upper confidence limit, 3.90, is less than the allowed limit, 4.0, the study has demonstrated that the alternative method has acceptable precision. If the same results had been obtained from a study with a sample size of 15—as if 80% power had been chosen—the laboratory would not be able to conclude that the alternative method had acceptable precision (upper confidence limit of 4.47).

⁷ This could be calculated using a computer spreadsheet. For example, in Microsoft® Excel the formula would be: $\text{FDIST}((R/A)*\text{FINV}(\alpha, n-1, n-1), n-1, n-1)$, where R is the ratio of variances at which to determine power (e.g., $R = 1$, which was the value chosen in the power calculations provided in Table 6) and A is the maximum ratio for acceptance (e.g., $A = 4$). Alpha is the significance level, typically 0.05.

APPENDIX E: COMPARISON OF METHODS— DETERMINING THE LARGEST ACCEPTABLE DIFFERENCE, δ , BETWEEN TWO METHODS

This Appendix describes one approach to determining the difference, δ , between two methods (alternative-current), a difference that, if achieved, still leads to the conclusion of equivalence between the two methods. Without any other prior information to guide the laboratory in the choice of δ , it is a reasonable way to proceed. Sample size calculations under various scenarios are discussed in this Appendix.

Tolerance Interval Determination

Suppose the process mean and the standard deviation are both unknown, but a sample of size 50 produced a mean and standard deviation of 99.5 and 2.0, respectively. These values were calculated using the last 50 results generated by this specific method for a particular (control) sample. Given this information, the tolerance limits can be calculated by the following formula:

$$\bar{x} \pm Ks$$

in which \bar{x} is the mean; s is the standard deviation; and K is based on the level of confidence, the proportion of results to be captured in the interval, and the sample size, n . Tables providing K values are available. In this example, the value of K required to enclose 95% of the population with 95% confidence for 50 samples is 2.382.⁸ The tolerance limits are calculated as follows:

$$99.5 \pm 2.382 \times 2.0$$

hence, the tolerance interval is (94.7, 104.3).

Comparison of the Tolerance Limits to the Specification Limits

Assume the specification interval for this method is (90.0, 110.0) and the process mean and standard deviation have not changed since this interval was established. The following quantities can be defined: the lower specification limit (LSL) is 90.0, the upper specification limit (USL) is 110.0, the lower tolerance limit (LTL) is 94.7, and the upper tolerance limit (UTL) is 104.3. Calculate the acceptable difference, (δ), in the following manner:

$$A = \text{LTL} - \text{LSL for } \text{LTL} \geq \text{LSL}$$

$$(A = 94.7 - 90.0 = 4.7);$$

$$B = \text{USL} - \text{UTL for } \text{USL} \geq \text{UTL}$$

$$(B = 110.0 - 104.3 = 5.7); \text{ and}$$

$$\delta = \text{minimum } (A, B) = 4.7$$

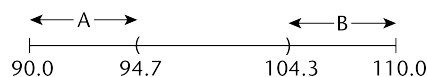


Figure 2. A graph of the quantities calculated above.

With this choice of δ , and assuming the two methods have comparable precision, the confidence interval for the difference in means between the two methods (alternative-

⁸ There are existing tables of tolerance factors that give approximate values and thus differ slightly from the values reported here.

current) should fall within -4.7 and $+4.7$ to claim that no important difference exists between the two methods.

Quality control analytical laboratories sometimes deal with 99% tolerance limits, in which cases the interval will widen. Using the previous example, the value of K required to enclose 99% of the population with 99% confidence for 50 samples is 3.390. The tolerance limits are calculated as follows:

$$99.5 \pm 3.390 \times 2.0$$

The resultant wider tolerance interval is (92.7, 106.3). Similarly, the new LTL of 92.7 and UTL of 106.3 would produce a smaller δ :

$$A = \text{LTL} - \text{LSL for } \text{LTL} \geq \text{LSL}$$

$$(A = 92.7 - 90.0 = 2.7);$$

$$B = \text{USL} - \text{UTL for } \text{USL} \geq \text{UTL}$$

$$(B = 110.0 - 106.3 = 3.7); \text{ and}$$

$$\delta = \text{minimum } (A, B) = 2.7$$

Though a manufacturer may choose any δ that serves adequately in the determination of equivalence, the choice of a larger δ , while yielding a smaller n , may risk a loss of capacity for discriminating between methods.

Sample Size

Formulas are available that can be used for a specified δ , under the assumption that the population variances are known and equal, to calculate the number of samples required to be tested per method, n . The level of confidence and power must also be specified. [NOTE—Power refers to the probability of correctly concluding that two identical methods are equivalent.] For example, if $\delta = 4.7$, and the two population variances are assumed to equal 4.0, then, for a 5% level test⁹ and 80% power (with associated z -values of 1.645 and 1.282, respectively), the sample size is approximated by the following formula:

$$n \geq \frac{2\sigma^2}{\delta^2} (z_\alpha + z_{\beta/2})^2$$

$$n \geq \frac{2(4)}{(4.7)^2} (1.645 + 1.282)^2 = 3.10$$

Thus, assuming each method has a population variance, σ^2 , of 4.0, the number of samples, n , required to conclude with 80% probability that the two methods are equivalent (90% confidence interval for the difference in the true means falls between -4.7 and $+4.7$) when in fact they are identical (the true mean difference is zero) is 4. Because the normal distribution was used in the above formula, 4 is actually a lower bound on the needed sample size. If feasible, one might want to use a larger sample size. Values for z for common confidence levels are presented in Table 8. The formula above makes three assumptions: 1) the variance used in the sample size calculation is based on a sufficiently large amount of prior data to be treated as known; 2) the prior known variance will be used in the analysis of the new experiment, or the sample size for the new experiment is sufficiently

⁹ When testing equivalence, a 5% level test corresponds to a 90% confidence interval.

ciently large so that the normal distribution is a good approximation to the t distribution; and 3) the laboratory is confident that there is no actual difference in the means, the most optimistic case. It is not common for all three of these assumptions to hold. The formula above should be treated most often as an initial approximation. Deviations from the three assumptions will lead to a larger required sample size. In general, we recommend seeking assistance from someone familiar with the necessary methods.

When a log transformation is required to achieve normality, the sample size formula needs to be slightly adjusted as shown below. Instead of formulating the problem in terms of the population variance and the largest acceptable difference, δ , between the two methods, it now is formulated in terms of the population %RSD and the largest acceptable proportional difference between the two methods.

$$n \geq \frac{2\sigma_L^2}{\delta_L^2} (z_\alpha + z_{\beta/2})^2$$

where

$$\sigma_L^2 = \log((\% \text{RSD}/100)^2 + 1)$$

$$\delta_L^2 = (\log(\rho + 1))^2$$

and ρ represents the largest acceptable proportional difference between the two methods ((alternative-current)/current), and the population %RSDs are assumed known and equal.

APPENDIX F: ADDITIONAL SOURCES OF INFORMATION

There may be a variety of statistical tests that can be used to evaluate any given set of data. This chapter presents several tests for interpreting and managing analytical data, but many other similar tests could also be employed. The chapter simply illustrates the analysis of data using statistically acceptable methods. As mentioned in the *Introduction*, specific tests are presented for illustrative purposes, and USP does not endorse any of these tests as the sole approach for handling analytical data. Additional information and alternative tests can be found in the references listed below or in many statistical textbooks.

Control Charts:

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Detectable Differences and Sample Size

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2. Kateman, G., Buydens, L., *Quality Control in Analytical Chemistry*, 2nd ed., John Wiley and Sons, New York, 1993.
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4. Mandel, J., *Evaluation and Control of Measurements*, Marcel Dekker, New York, 1991.
5. Melveger, A.J., "Statistics in the pharmaceutical analysis laboratory," *Analytical Chemistry in a GMP Environment*, Miller J.M., Crowther J.B., eds., John Wiley and Sons, New York, 2000.
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Table 1. Data from a Precision Study

Replicate Number	Run Number				
	1	2	3	4	5
1	100.70	99.46	99.96	101.80	101.91
2	101.05	99.37	100.17	102.16	102.00
3	101.15	99.59	101.01	102.44	101.67
Mean	100.97	99.47	100.38	102.13	101.86
Standard Deviation	0.236	0.111	0.556	0.321	0.171
% RSD ¹	0.234%	0.111%	0.554%	0.314%	0.167%

¹ %RSD (percent relative standard deviation) = $100\% \times (\text{standard deviation}/\text{mean})$

Table 1A. Analysis of Variance Table for Data Presented in Table 1

Source of Variation	Degrees of Freedom (df)	Sum of Squares (SS)	Mean Squares ¹ (MS)	F = MS _B /MS _W
Between Runs	4	14.200	3.550	34.886
Within Runs	10	1.018	0.102	
Total	14	15.217		

¹ The Mean Squares Between (MS_B) = SS_{Between}/df_{Between} and the Mean Squares Within (MS_W) = SS_{Within}/df_{Within}

Table 2. The Predicted Impact of the Test Plan (No. of Runs and No. of Replicates per Run) on the Precision of the Mean

No. of Runs	No. of Replicates per Run	Variance of the Mean	SD of the Mean	% RSD ¹
1	1	1.251	1.118	1.11
1	2	1.200	1.095	1.09
1	3	1.183	1.088	1.08
2	1	0.625	0.791	0.78
2	2	0.600	0.775	0.77
2	3	0.592	0.769	0.76

¹ A mean value of 100.96, based on the 15 data points presented in Table 1, was used (as the divisor) to compute the %RSD.

Table 3. Generalized ESD Test Results

	n = 10		n = 9	
	Data	Normalized	Data	Normalized
	100.3	+0.555	100.3	+1.361
	100.2	+0.482	100.2	+0.953
	100.1	+0.409	100.1	+0.544
	100.0	+0.336	100.0	+0.136
	100.0	+0.336	100.0	+0.136
	100.0	+0.336	100.0	+0.136
	99.9	+0.263	99.9	-0.272
	99.7	+0.117	99.7	-1.089
	99.5	-0.029	99.5	-1.905
	95.7	-2.805		
Mean =	99.54		99.95	
SD =	1.369		0.245	

Table 4. Test Results Using Hampel's Rule

	n = 10			
	Data	Deviations from the Median	Absolute Deviations	Absolute Normalized
	100.3	0.3	0.3	1.35
	100.2	0.2	0.2	0.90
	100.1	0.1	0.1	0.45
	100	0	0	0
	100	0	0	0
	100	0	0	0
	99.9	-0.1	0.1	0.45

Table 4. Test Results Using Hampel's Rule (Continued)

n = 10				
	Data	Deviations from the Median	Absolute Deviations	Absolute Normalized
	99.7	-0.3	0.3	1.35
	99.5	-0.5	0.5	2.25
	95.7	-4.3	4.3	19.33
Median =	100		0.15	
MAD =			0.22	

Table 5. Test Results of Re-Applied Hampel's Rule

n = 9				
	Data	Deviations from the Median	Absolute Deviations	Absolute Normalized
	100.3	0.3	0.3	2.02
	100.2	0.2	0.2	1.35
	100.1	0.1	0.1	0.67
	100	0	0	0
	100	0	0	0
	100	0	0	0
	99.9	-0.1	0.1	0.67
	99.7	-0.3	0.3	2.02
	99.5	-0.5	0.5	3.37
Median =	100		0.1	
MAD =			0.14	

Table 6. Power Determinations for Various Sample Sizes (Specific to the Example in Appendix D)

Sample Size	$\Pr[F > 1/4 F_{0.05, n-1, n-1}]$
12	0.7145
13	0.7495
14	0.7807
15	0.8083
16	0.8327
17	0.8543
18	0.8732
19	0.8899
20	0.9044

Table 7. Example of Measures of Variance for Independent Runs (Specific to the Example in Appendix D)

Method	Variance (standard deviation)	Sample Size	Degrees of Freedom
Alternative	45.0 (6.71)	20	19
Current	25.0 (5.00)	20	19

Table 8. Common Values for a Standard Normal Distribution

Confidence level	z-values	
	One-sided (α)	Two-sided ($\alpha/2$)
99%	2.326	2.576
95%	1.645	1.960
90%	1.282	1.645
80%	0.842	1.282

(1015) AUTOMATED RADIOCHEMICAL SYNTHESIS APPARATUS

The preparation and quality control of diagnostic radiopharmaceuticals labeled with the very short-lived positron-emitting nuclides (e.g., ^{15}O , ^{13}N , ^{11}C and ^{18}F having half-lives of 2, 10, 20, and 110 minutes, respectively), are subject to constraints different from those applicable to therapeutic drugs: (1) Synthesis must be rapid, yet must be arranged to protect the chemist or pharmacist from excessive radiation exposure. (2) Except to a limited extent for ^{18}F , synthesis must occur at the time of use. (3) With the exception of ^{18}F , *each batch of radiopharmaceutical generally leads to only a single administration.*

These factors raise the importance of quality control of the final drug product relative to validation of the synthesis process. Since with few exceptions every dose is individually manufactured, ideally every dose should be subjected to quality control tests for radiochemical purity and other key aspects of quality before administration. Because quality testing of every batch is not possible, batches are selected at regular intervals for examination to establish and completely characterize their radiopharmaceutical purity. This routine and thorough quality testing of selected batches forms the basis of process validation, which is absolutely essential for prospective assessment of batch quality and purity when dealing with such extremely short-lived radiopharmaceuticals. Since radiopharmaceuticals used in positron emission tomography (PET) are administered intravenously or (for radioactive gases) by inhalation, batch-to-batch variability in bioavailability is not an issue. Furthermore, the very small scale of radiopharmaceutical syntheses (almost always less than 1 milligram and often in the microgram range) and the fact that patients generally receive only a single dose of radioactive drug minimize the likelihood of administering harmful amounts of chemical impurities. These statements are not intended to contest the need for quality control in the operation of automated synthesis equipment, but to place the manufacture of positron-emitting radiopharmaceuticals in an appropriate perspective and to reemphasize the overwhelming importance of prospective process validation and finished product quality control.

The routine synthesis of radiopharmaceuticals can result in unnecessarily high radiation doses to personnel. Automated radiochemical synthesis devices have been developed, partly to comply with the concept of reducing personnel radiation exposures to "as low as reasonably achievable" (ALARA). These automated synthesis devices can be more efficient and precise than existing manual methods. Such automated methods are especially useful where a radiochemical synthesis requires repetitive, uniform manipulations on a daily basis.

The products from these automated radiosynthesis devices must meet the same quality assurance criteria as the products obtained by conventional manual syntheses. In the case of positron-emitting radiopharmaceuticals, these criteria will include many of the same determinations used for conventional nuclear medicine radiopharmaceuticals, for example, tests for sterility and bacterial endotoxins. Many of the same limitations apply. Typical analytical procedures such as spectroscopy are not generally applicable because the small amount of product is below the minimum detection level of the method. In all cases, the applicable Pharmacopeial method is the conclusive arbiter (see *Procedures under Tests and Assays in the General Notices*).

Preparation of Fludeoxyglucose F 18 Injection and other positron-emitting radiopharmaceuticals can be adapted readily to automated synthesis. In general, the equipment required for the manual methods is simpler and less expen-

sive than that used in automated methods but is more labor-intensive. Of special concern are the methods involved in validating the correct performance of an automated apparatus. For a manual procedure, human intervention and correction by inspection can nullify many procedural errors. In an automated system, effective feedback also can begin during the synthesis. For example, radiation detectors can monitor activity at various stages of radiosynthesis. Failure to obtain the appropriate activity could activate an alarm system that would lead to human intervention.

Radiochemicals versus Radiopharmaceuticals—It is appropriate to draw a distinction between a radiochemical and a corresponding radiopharmaceutical. In research PET centers, automated equipment is used to prepare labeled compounds for animal experiments. These radiochemicals are not regarded as radiopharmaceuticals if (1) they are not prepared according to a validated process that provides a high degree of assurance that the preparation meets all established requirements for quality and purity; and (2) have not been certified by qualified personnel (licensed pharmacists and approved physicians) in accordance with published Pharmacopeial methods for individual radiopharmaceuticals.

Automated Equipment—The considerations in this chapter apply to synthesis conducted by general purpose robots and by special purpose apparatus. Both are automated devices used in the synthesis of radiochemicals. The exact method of synthesis device control is variable. Both hard-wired and software-controlled synthesis devices fall under the general designation, and there is a spectrum ranging from traditional manual equipment through semi-automated devices to completely automatic devices.

Common Elements of Automated Synthesis Equipment—To manipulate a chemical apparatus to effect the synthesis of a radiochemical, control of parameters such as time, temperature, pressure, volume, and sequencing are needed. These parameters can be monitored and constrained to fall within certain bounds.

Equipment Quality Assurance—The goal of quality assurance is to help ensure that the subsequent radiopharmaceutical meets Pharmacopeial standards. Although the medical device good manufacturing practice regulations (21 CFR 820) are not applicable, they may be helpful in developing a quality assurance program. As a practical matter this involves documented measurement and control of all relevant physical parameters controlled by the synthesis apparatus.

Routine Quality Control Testing—Routine quality control testing of automated equipment implies periodic testing of all parameters initially certified during the quality assurance qualification. Depending on the criticality and the stability of the parameter setting, testing may be as often as daily. This process performance assessment must be augmented by regular end product testing. For example, variations in the temperature of an oil bath may be acceptable if the radiochemical (end product) can be shown to meet all relevant testing criteria.

Reagent Audit Trail—Materials and reagents used for the synthesis of radiopharmaceuticals should conform to established quality control specifications and tests. Procedures for the testing, storage, and use of these materials should be established. In this context, a reagent is defined as any chemical used in the procedure leading to the final radiochemical product, whereas materials are defined as ancillary supplies (tubing, glassware, vials, etc.). For example, in some processes compressed nitrogen is used to move liquid reagents. In this case, both the nitrogen and the tubing should meet established specifications.

Documentation of Apparatus Parameters—Key synthesis variables should be identified, monitored, and documented. These characteristics include meaningful physical, chemical, electrical, and performance attributes. A method for specifying, testing, and documenting computer software and hardware is especially important for microprocessor- and computer-controlled devices. This program should in-

clude periodic generalized testing of the computer hardware. In addition, the software program code should be periodically examined to determine that it has not been modified and that it continues to result in the final product's meeting all specifications. In-process feedback is one means of confirming that the synthesis is under control. Changes to the software code should involve a formal authorization procedure, and changes should be documented.

Each type of radiochemical synthesis device requires a set of specific procedures for testing and monitoring the reliability and reproducibility of the various subsystems that make up the total synthesis system.

It is essential that calibration of each of the components be confirmed according to an established maintenance timetable and that measurements or monitoring be made under actual synthesis conditions.

Delivery times, reagent volumes, temperatures, gas pressures, and rates of flow need to be measured and shown to be stable and reproducible within established limits. Delivery of the reagents and solvents needs to be calibrated periodically. Other components to be routinely calibrated include the radiation detection system and process monitoring sensors and system.

For illustration, elements of system validation of several representative components of an automatic synthetic device are as follows:

Reaction vessels may be cleaned and inspected by an established documented method. The vessels themselves may be numbered and their performance tracked.

Heating and cooling systems (such as oil baths) may be monitored by thermometers or thermocouples. The temperatures may be recorded in a batch sheet, or they may be automatically printed out as part of a computerized log. Maintenance involves periodic calibration.

Gases and gas delivery system performance may be tracked by pressure gauges and flowmeters. Gas purity may be established via supplier certificates of analysis or may be verified by independent testing. Maintenance of gauges and flowmeters involves periodic calibration with standards.

Position-dependent motor performance may be verified by limit switches. Maintenance could involve actual measurement of distance traversed and elapsed time.

Solenoid valves may be checked electrically, by flow and pressure tests.

Heater output is evidenced by proper thermocouple readings. Additional tests could involve resistance measurements.

Reagents may be accepted on the basis of suppliers' certificates of analysis. Alternatively, the chemical could be tested in-house or sent to an independent testing laboratory. Periodic retesting may be necessary depending on stability.

Computer programs may be tested by documenting elapsed time of synthesis, with printouts verifying that all appropriate manipulations occurred, including printing of relevant parameters such as times, temperature, pressures, and activities.

Patterns of activity distribution such as absolute amount of product, percentage yield, and individual impurity activity levels afford the experienced user an opportunity to discern systems failures.

Changes in the Synthesis Method—Some changes in the synthesis apparatus can be considered to be trivial. This category would often include changes not affecting any of the monitored parameters. However, it is important that care be taken to ensure that seemingly innocuous changes do not have an unexpected impact. For example, changes in a comment line of a computer program may result in inadvertently changing or deleting a vital instruction. Any changes in monitored parameters have the potential for changing the process output. If the resultant radiochemical does not meet specifications or if the subsequent radiopharmaceutical does not meet Pharmacopeial criteria, the process change is unacceptable; the fault must be corrected and the process revalidated.

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〈1024〉 BOVINE SERUM

INTRODUCTION

Bovine serum is the liquid fraction of clotted blood, obtained from an ox (*Bos taurus*, among others), that has been depleted of cells, fibrin, and clotting factors. Since the advent of modern cell culture, manufacturers of biological products have used bovine serum extensively as a cell culture growth supplement. Its rich nutritional composition of proteins, growth factors, hormones, amino acids, vitamins, sugars, lipids, trace elements, and other components supports a broad range of cell culture applications in research and commercial manufacture of vaccines, natural source and recombinant biologics (hereafter biologics), engineered tissues, and other emerging cell-based therapeutic products intended for human or veterinary use. The predominant type of serum used in research applications is Fetal Bovine Serum (FBS). Calf serum (from newborn and older animals) is used much less frequently, but because of its lower cost it may be widely used in commercial manufacturing.

As is the case with other animal-derived products, bovine serum carries a potential risk of introducing extraneous agents into cell culture. Serum manufacturers and regulators must adopt rigorous sourcing and testing procedures and strict processing and production guidelines to ensure the quality of bovine serum.

The objective of increasing the quality and safety of biologics produced with bovine serum, coupled with attempts to mitigate regulatory burden, have caused developers to investigate alternatives to serum supplementation, resulting in application-specific serum-free medium formulations. Although it is recognized that bovine serum should be avoided when there is an option to use serum-free medium, there are cases where this is technically impossible or impractical.

This chapter describes issues related to sourcing, production, and characterization of bovine serum to ensure its safe use. A list of relevant regulatory and guidance documents is presented in *Appendix 1*. Serum manufacturers and serum end users (manufacturers of biological products) should consider and apply as needed the controls and procedures outlined in this chapter to ensure the safe use of bovine serum components in research and pharmaceutical manufacturing.

Types of Bovine Serum

- FBS is obtained from the fetuses of healthy, prepartum bovine dams that had been deemed fit for human consumption through ante- and postmortem inspection by licensed veterinarians. It is collected in government-inspected and -registered slaughterhouses.
- Newborn calf serum (also known as newborn bovine serum) is obtained in government-inspected and -registered slaughterhouses from animals aged less than 20 days.
- Calf serum is obtained in government-inspected and -registered slaughterhouses from animals aged between 20 days and 12 months.
- Donor bovine serum (also known as donor calf serum) is obtained by the repeated bleeding of donor animals from controlled government-inspected and -reg-

istered donor herds. The animals are 12–36 months old.

- Adult bovine serum is obtained in government inspected and -registered slaughterhouses from cattle older than 12 months that are declared fit for human consumption.

BOVINE SERUM: HISTORY AND TYPES OF USE

History of Bovine Serum Use

Animal serum and other complex biological materials have been employed in the cultivation of mammalian cells for approximately 100 years. Several factors led to the wide adoption of bovine serum as a standard tissue culture supplement. In comparison to serum from other animal species (horse, goat), bovine serum is easily sourced, and thereby more affordable. Many investigators choose to use fetal serum in their experimental systems because of concerns associated with antibodies present in newborn and adult serum that could cross-react with cells in culture and cause cell lysis through complement-mediated pathways. To eliminate that concern, heat was introduced to inactivate complement that was potentially present in the serum. Studies of FBS undertaken in the 1950s on the cultivation of low-density human cells to elucidate mechanisms of cell growth found that (1) the albumin component may serve as a carrier of essential small molecules; (2) fetuin, a glycoprotein present at high levels in the alpha globulin fraction, facilitates cell attachment and stretching; and (3) fetuin markedly inhibits trypsin, and this antiproteolytic activity may play a role in the ability of fetuin to stimulate cell growth.

In the 1960s and 1970s, serum supplementation of tissue culture media became the norm, thus facilitating biomedical research as well as the first large-scale vaccine manufacturing processes. Serum supplementation reduced the requirement for optimizing medium formulations for different cell types. FBS was shown to provide a variety of polypeptide growth factors. Albumin promoted cell growth presumably because of its abilities to function as a carrier protein for small molecules or lipids, to bind metal ions, to serve as a pH buffer, and to protect cells against shear. Similar functions were found for other serum components such as transferrin, hormones, and other serum-derived attachment factors such as fibronectin, vitronectin, and laminin.

Uses of Bovine Serum

Serum is a complex mixture of macromolecules that is required for cell growth and virus production, and its use as a raw material presents a number of challenges. These include its batch-to-batch composition and the risk of contamination by adventitious agents. The development of serum-free media has replaced serum in some new biotechnology manufacturing applications, but many cell lines used in manufacturing have not been adapted to these serum-free media. Regulatory constraints and scientific challenges generally make it impractical to alter existing manufacturing processes in which serum is used as a raw material.

FBS sometimes is required in cell and tissue bioprocessing, which often involves the cultivation of cells from tissue explants and biopsies. Some bioprocesses may also require the maintenance of specific cellular characteristics during cultivation. FBS often appears to facilitate such procedures and may affect the biological behavior of fastidious cell types. FBS has been shown to affect the transcription of developmentally important genes, apoptosis, and apoptosis-related gene expression, and to provide neuroprotective and antioxidative factors, all of which may be beneficial to the survival and development of cells in culture. Therefore, FBS will con-

tinue to play an important role as a cell culture supplement for production of cell- and tissue-based therapies.

In most viral vaccine manufacturing processes the media used for cell culture expansion and virus infection/production are supplemented with different types of serum at different concentrations. In these processes, bovine serum helps generate a mass of cells in an optimal physiological state for efficient viral replication.

BOVINE SERUM HARVESTING AND PRODUCTION

Blood Collection

For all types of bovine sera, blood should be collected in government-inspected and -registered premises (slaughterhouses, abattoirs, and donor farms). Blood should be collected by trained operators following the written procedures approved by the serum manufacturer and using either single-use disposable collection devices or reusable collection equipment for which cleaning procedures have been validated.

DONOR BOVINE SERUM

For each lot of serum from donor animals, serum manufacturers should ensure traceability to the donor herd of origin via production records and animal health and origin certificates. Donor animals are subjected to regular veterinary inspections and are bled multiple times following established procedures. Animals introduced into the herd should be traceable by source, breeding, and rearing history. Collectors should introduce new animals into the herd following specified and approved procedures that include prepurchase animal inspection and testing, proper transportation, a quarantine period, veterinary examination and testing during the quarantine period, and animal release criteria from quarantine to serum production. The collectors should not vaccinate donor animals for bovine viral diarrhea (BVD). Collectors should test animals for any agent and antibody from which the herd is claimed to be free.

NEWBORN CALF SERUM, CALF SERUM, AND ADULT BOVINE SERUM

Certificates of animal health and origin and/or serum production records should ensure that serum manufacturers can trace bovine serum derived from slaughtered animals back to the abattoir. Serum manufacturers should require abattoirs to maintain documentation of the origin of animals for slaughter. Blood should be collected from animals that have been slaughtered, for human consumption, in abattoirs inspected by the competent authority of the country of origin. Inspectors should routinely inspect animals both antemortem and postmortem to check for the clinical appearance of infections and parasitic diseases and other animal health-related problems or conditions. The animals must be free of clinical evidence of infectious diseases at the time of slaughter. Blood collection procedures must be in place to prevent cross-contamination with other tissues and body fluids and the surrounding environment. The standard procedure of slaughter consists of an approved method of animal stunning followed by exsanguination.

FETAL BOVINE SERUM

FBS product specifications and test procedures are presented in the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* (90). Serum manufacturers should collect fetal bovine blood from bovine

fetuses whose dams have been slaughtered. The dams must have been deemed fit for human consumption and must have been slaughtered in abattoirs that were inspected by the competent authority of the country of origin. Inspectors should examine all animals both antemortem and post-mortem to check for the clinical appearance of infections and parasitic diseases and other animal health-related problems or conditions. The animals must be free of clinical evidence of infectious diseases at the time of slaughter. The uterus is removed and transported to a dedicated space for fetal bovine blood harvest, where blood collection personnel evaluate the fetus for signs of fetal death, including bloating, skin discoloration, odor, deformation, and hair sloughing. Collectors also should check the amniotic fluid for color, quantity, and clarity. Serum manufacturers should collect blood from acceptable fetuses by cardiac puncture into a closed collection system under conditions designed to minimize microbial contamination. Manufacturers should have in place procedures that will prevent cross-contamination with other fetal tissues and bodily fluids and the surrounding environment.

Serum Harvesting and Processing

Trained personnel should perform serum separation (harvesting) and further processing activities following written and approved procedures. Serum is first separated and pooled, followed by filtration and filling into clean and disinfected containers. If the serum is subjected to one or more virus inactivation treatments in the production process, serum manufacturers should validate the virus inactivation processes against a range of relevant viruses. It is recommended that bovine viral diarrhea virus (BVDV) be included in any virus validation study because it is ubiquitous.

SERUM SEPARATION AND HARVESTING

Bovine blood should be processed and serum separated (harvested) in such a way as to minimize bacterial and mycoplasmal contamination, which in turn minimizes endotoxin levels in serum product. Gentle, quick blood processing helps to minimize hemolysis, further enhancing the quality of the serum product. After collection, blood is first allowed to clot for a specified period of time and under controlled conditions, then centrifuged in a refrigerated centrifuge. Serum is then removed from the clot, typically by centrifugation; pooled and mixed in a pooling vessel; transferred to labeled containers; and frozen, unless it is filter-sterilized immediately. Serum manufacturers should describe each process step and carry out serum processing activities, including sample collection and in-process quality control testing, following the manufacturer's approved procedures.

POOLING BEFORE FILTRATION

Because limited amounts of blood can be collected from individual animals, serum manufacturers pool the raw serum from many animals in order to create commercial-sized lots. Serum is pooled, after raw serum thawing and before filtration, in a pooling vessel and mixed at a controlled mixing rate and temperature. Pools or lots of donor bovine serum may consist of many separate collections from individual members of the herd. Lots of FBS may consist of pooled serum from thousands of animals. Serum manufacturers should describe each prefiltration pooling process step and should carry out serum thawing, prefiltration pooling, and mixing activities following the manufacturer's approved procedures.

FILTRATION

Pooled serum is mixed and aseptically passed through filters of pore size 0.2 μm or smaller, depending on the intended application. Filtration processes should be validated. Triple filtration using filters of pore size 0.1 μm has been shown to result in a high degree of mycoplasma removal. Although filtration may remove some large viruses and viral aggregates from the serum, generally viruses cannot be completely eliminated in this manner. Furthermore, the filters are not known to eliminate the causative agent of bovine spongiform encephalopathy (BSE). Following filtration, serum manufacturers fill filtered serum into sterile containers by aseptic processing in a suitably controlled environment. Serum manufacturers should describe each filtration process step and should perform serum filtration, filling, and sample collection activities following the manufacturer's approved procedures.

IRRADIATION

Serum treatment by gamma irradiation is very common and one of the most effective methods of virus inactivation. The most frequently used minimum dose is 25 kilograys (kGy). Some countries specify higher dose requirements (>30 kGy) for imported serum. Gamma irradiation may inactivate viruses, mycoplasma, and bacteria, but serum end users should ensure that the gamma irradiation process does not negatively affect their specific applications. Irradiation may have adverse effects on serum quality, and these adverse effects tend to increase with higher doses.

Validation of gamma irradiation has two aspects: (1) dose delivery in a defined load configuration and (2) inactivation capacity. Critical irradiation process parameters include product (serum) temperature, packaging size and configuration, dosimeter distribution, and defined minimum/maximum dose received. Dosimeters should be used to monitor the established high-dose and low-dose positions in each irradiation run. If the serum manufacturer makes inactivation claims, these should be supported by the manufacturer's own well-designed viral inactivation studies.

ULTRAVIOLET (UV) TREATMENT

Serum manufacturers may use UV treatment to inactivate viruses, mycoplasma, and bacteria, but manufacturers must validate the process to demonstrate its efficacy. In addition, manufacturers must be aware that UV treatment may have an adverse effect on serum quality and accordingly should consider the effects of UV treatment for each application, as should serum end users.

HEAT INACTIVATION

Heat inactivation involves elevating the temperature of the serum to >56° for at least 30 minutes to inactivate complement. Heat inactivation may also inactivate viruses, mycoplasma, and bacteria; but it may have an adverse effect on serum quality, and manufacturers must validate the procedure's suitability for specific applications. Heat inactivation provides significantly less assurance of virus inactivation than does irradiation.

VIRAL CLEARANCE STUDIES

Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin (1050) and other regulatory documents give guidance about conducting viral clearance studies that help validate removal/inactivation processes. Serum manufacturers should also perform formal spiking studies with relevant and representative (model) vi-

ruses, and should test and compare inactivated spiked serum samples, negative controls, and positive controls.

CHARCOAL STRIPPING

Some serum manufacturers use charcoal/dextran treatment to reduce the levels of hormones in serum.

DIALYSIS

Some manufacturers use dialysis or diafiltration to remove low molecular weight components from serum.

CLEANING AND STERILITY OF EQUIPMENT

Stainless steel systems and tubing used in the manufacture of bovine serum must be cleaned and sterilized to prevent cross-contamination and growth of adventitious agents. Serum manufacturers must validate their cleaning processes for removing and inactivating agents of concern. Thereafter, manufacturers should implement process controls that routinely verify cleaning cycles. Steam sterilization-in-place is a common and effective sterilization technique. Serum manufacturers that use this technology must validate steam cycles to demonstrate their uniformity and ability to destroy heat-resistant bacterial spores. Alternatively, manufacturers can use sterile disposable systems that do not require cleaning validation.

Quality Control

TRACEABILITY

Abattoir Collection

Materials collected in the U.S. should originate from U.S. Department of Agriculture (USDA)-registered facilities. Serum manufacturers should maintain documentation that traces a given serum sub-lot to the abattoir where it was collected. Slaughterhouses maintain records of animal source. General industry practice is to keep this information as part of the Device Master Record. General record-keeping requirements at USDA-licensed abattoir facilities are outlined in 9 Code of Federal Regulations (CFR) 320.

Materials collected from countries approved by the USDA for importation of bovine products into the U.S. should meet the requirements of the competent authority of the country of origin. In addition, serum manufacturers should keep USDA-required safety testing records of imported materials (if applicable) as part of their Device History Record.

Serum manufacturers should consult 9 CFR 309 and 9 CFR 310 about requirements for inspection of animals for various diseases pre- and post-slaughter. These requirements are recommended for materials collected outside the U.S.

Donor Herd Collection

Serum manufacturers should maintain traceability to the donor animal farm where blood was collected from donor animals. In most cases, manufacturers individually identify farm animals and keep records for bleed and processing dates, making it possible to trace blood collection to an individual animal. A licensed veterinarian or a designee under the guidance of a veterinarian should inspect animals regularly and should certify that the animals are free of disease and fit for human consumption, consistent with 9 CFR 309.

PRODUCT STORAGE AND STABILITY

Serum should be stored in the frozen state at -10° or below. Serum is frozen as quickly as possible to preserve product quality and is stored under controlled storage conditions. Serum manufacturers should establish serum product stability in support of a proposed expiration date. Typical expiration dating for bovine serum is 5 years from the date of filtration and filling. Use of any type of bovine serum beyond the stated expiration date depends on the application, and the serum user must establish the product's continued suitability for use.

Labeling

Finished product labels must contain the following information: product description, lot number, storage conditions, name and address of manufacturer, and a statement indicating the intended use. Materials intended for research purposes are exempt from labeling regulations (21 CFR 801). Typically, serum manufacturers supply a lot-specific Certificate of Analysis (COA) that is classified as part of the product's labeling. See COA requirements in the following section.

Certification/Documentation

CERTIFICATE OF ANALYSIS

The COA should provide information about a specific lot of serum, including tests performed and test results (according to the serum manufacturer's specifications for release), as well as critical labeling identifiers such as lot number, catalog number, description of type of bovine serum, country of origin, and either or both dates of manufacture and expiration. This document is distinct from the certificate of health issued by the competent authority of the country of origin.

CERTIFICATE OF ORIGIN AND CERTIFICATION OF ANIMAL STATE OF HEALTH

The Certificate of Origin establishes the country in which the bovine blood was collected and veterinary certification of the health of the animals pre- and postcollection (9 CFR 327.4).

IMPORT/EXPORT DOCUMENTS

Import/export documents contain formal certification of animal disease status of the country of origin and negotiated/agreed certification statements. These vary from country to country. Each country defines import/export requirements in order to control introduction of exotic animal diseases and their economic impact as well as product safety assessments (risk vs. research, diagnostic, and/or therapeutic benefits).

PRODUCTION REPORTS

Production reports typically are batch records that document the raw materials in identifiable and traceable ways, production methods (centrifugation or filtration) used in manufacturing, equipment and facility cleaning, quality control testing, and personnel performing required activities. Raw material with Certificates of Origin or serum production records facilitates traceability to the source of the blood that was used to create the serum. When serum is used as a raw material for further manufacturing, process documentation

also helps demonstrate controlled manufacture of the bovine serum.

BSE RISK ASSESSMENT

Despite the low risk potential of transmissible spongiform encephalopathies (TSEs) in bovine serum, various U.S. and international regulatory agencies have developed guidance to help manage and further reduce the potential risks of transmission. In the absence of appropriate test methods of detecting the infectious agent in fluids such as blood, the consensus recommendation from various regulatory agencies is to adopt good risk assessment strategies. This section of the chapter provides some background information on the disease and current methods of detection; it also highlights risk assessment and risk reduction strategies to potentially prevent transmission of the disease through the use of serum in the manufacturing of medicinal products.

Description of the Disease

TSEs are transmissible animal and human diseases that are characterized by degeneration of the brain, associated with severe neurological signs and symptoms. Since the outbreaks of TSE in cattle, termed BSE, which were transmitted to other species, public health officials have been concerned about the risk of TSE infection, including the possibility of TSE transmission by the use of therapeutic products manufactured using bovine serum. In cattle infected with BSE, lower titers have been found in the cerebrospinal fluid, lung, lymph tissue, spleen, kidney, liver, and ileum. Studies have shown that transfusion of blood from sheep infected with either BSE or scrapie but without evident disease can infect naive sheep. Although the risk of cross-contamination is always present, to date no studies have shown that blood can transmit disease from cattle with BSE. Embryos from BSE-affected cattle have not transmitted diseases to mice. Calves born of dams that received embryos from BSE-affected cattle have survived for up to 7 years, and examination of the brains of both the unaffected dams and their offspring revealed no spongiform encephalopathy.

Detection Strategies

No currently available procedures have been validated as being sufficiently sensitive for routine antemortem screening of asymptomatic animals, although analytical methods are under development for detection and quantitation from low-infectivity materials such as blood. The classic diagnostic test for TSEs is postmortem histological examination of brain tissue to confirm characteristic vacuolar degeneration. Other testing options include immunohistochemical tests that can confirm the presence of PrP^{Sc}, the abnormal disease-specific conformation of prion-related protein (PrP), in the vacuolated regions of the brain; and immunochemical tests such as Western blots and enzyme-linked immunosorbent assays that can detect PrP^{Sc} in tissues with high or moderately high titers. These tests typically take less time to perform than histological examination (6–8 hours vs. weeks, respectively) and can be partially or fully automated. Although most of these are postmortem tests, studies have demonstrated the feasibility of antemortem testing of lymphoid tissue samples from the tonsils or from the third eyelid of infected animals. Immunochemical tests require extensive sample collection and preparation and can be cost prohibitive for routine testing and monitoring the disease state of large herds. Diagnostic strategies must consider the sensitivity of testing in certain tissues as well as the test's ability to detect infectivity in animals before the development of clinical signs of disease. Negative results do not ensure the absence of infectivity. Detection of infectivity is possible if suspect tissue is inoculated into experimental animals intracranially where the causative agent can amplify. This ap-

proach for detection of low infectivity can take months to years to yield a positive result.

Risk Assessment and Risk Reduction Strategies

Serum manufacturers should employ risk reduction strategies to eliminate the danger of cross-contamination of fetal blood with other tissues, including appropriate sourcing of animal-derived articles and using practices that have been shown to eliminate or minimize the risk of transmitting TSE, via either foods or health care products. Serum end users should perform a risk assessment of their sourcing strategy that takes into account the amount of bovine serum used in their application and should conduct supplier audits to ensure traceability of sourcing, handling, and appropriate quality control systems.

SOURCE AND AGE OF ANIMALS

Serum manufacturers should monitor the traceability of each lot of serum to ensure the qualification of bovine serum, as described previously in the two sections *Serum Harvesting and Processing* and *Quality Control*. In addition to traceability, careful selection of source materials is the most important criterion for the safety of medicinal products. Certification of the origin must be available from the supplier, and manufacturers should keep this information on file. The U.S. Food and Drug Administration (FDA) recommendations prohibit the use in FDA-regulated products (except gelatin) of any bovine-derived materials that originate from countries that report indigenous cases of BSE. The current proposed rule qualifies FBS as an unlikely source of BSE infectious material, because current evidence suggests that cow-to-calf transmission of BSE is unlikely. The proposed rule also states that prohibited cattle materials do not include materials sourced from fetal calves of cows that were inspected and passed, as long as the materials were obtained by procedures that can prevent contamination with specified risk materials. For veterinary biologics, current regulations enforced by the USDA's Center for Veterinary Biologics (CVB) indicate that ingredients of animal origin should be sourced from countries with no or low BSE risk, as defined by the U.S. National Center for Import and Export and 9 CFR 94.18.

The most satisfactory sources of materials are from countries with the following:

- No reported cases of indigenous BSE
- Compulsory notification of positive tests
- Compulsory clinical and laboratory verification of suspected cases
- Prohibition of the use in ruminant feed of meat and bone meal containing any ruminant protein
- No importation of cattle from countries where a high incidence of BSE has occurred
- No importation of progeny of affected females

BSE infectivity may increase with animal age. Although bovine serum is considered a low-risk material for TSE transmission, some end users consider it prudent to source serum from dams below a set maximum age. If manufacturers cannot determine the date of the dam's birth, they should consider both the implementation date of the feed ban in the country of origin and the incubation period of BSE in order to determine the safety of the source. A ruminant feed ban was imposed in the United Kingdom in July of 1988. These considerations are lot specific, so audits of the raw material supplier should include a review of records.

PRODUCTION PROCESS

End user manufacturing systems should be in place for monitoring the production process and for batch delineation (definition of batch, separation of batches, and cleaning between batches). Of primary importance is control of the

potential for cross-contamination with possible infectious material. Because of the documented resistance of TSE agents to most inactivation procedures, controlled sourcing is the most important criterion in achieving acceptable product safety.

Whenever possible, manufacturers should identify steps that theoretically or demonstratively remove or inactivate agents during the manufacture of the material. Manufacturers should continue their investigations into removal and inactivation methods to identify steps/processes that will help ensure the removal or inactivation of TSE agents. Manufacturers should design production processes using available methods that have the greatest likelihood of inactivating or removing TSE agents. For example, prolonged exposure of tissues to high moist heat and high pH inactivates the BSE agent. Such treatments, however, are inappropriate for the extraction of many other types of bovine-derived articles, such as serum, because these treatments lead to the destruction of the material. Conventional chemical and biochemical extraction and isolation procedures may be sufficient to remove the infectious agent. Similar techniques may be effective for other bovine-derived articles. Further research will help to develop an understanding of the most appropriate methodology for validation studies. Issues to consider during validation of a process for removal of TSE agents include the following:

- The nature of the spiked material and its relevance to the natural situation
- Design of the study (including scale-down approaches)
- Method of detecting the agent (in vitro or in vivo assay) after spiking and after the treatment
- Characterization and standardization of reference materials for spiking
- Data treatment and analysis (see *Design and Analysis of Biological Assays* (111))

Because no studies have successfully validated analytical methods for the detection of small amounts of the TSE agent, TSE clearance validation studies typically employ the intracranial injection of in-process material into rodents for amplification and detection of potential residual infectivity.

TESTING AND CONTROL OF ADVENTITIOUS AGENTS

Introduction

Rigorous testing procedures, strict processing and production guidelines, and appropriate risk assessments help ensure the safety of the different types of bovine serum. This section discusses specific tests that can detect and control adventitious agents.

Adventitious Agents Testing

The adventitious agents testing required for the evaluation of master seeds, master cells, and bulk and final products is described in 9 CFR 113.53 and by directives from the European Agency for the Evaluation of Medicinal Products (EMA) (EMA/CVMP/743/00 and EMA/CPMP/BWP/1793/02). The testing methods outlined in these documents can detect a wide range of bovine microbial agents in serum products. These testing methods meet the requirements for most of the world's regulatory agencies. Serum manufacturers should test a representative sample of each batch of serum to determine the presence of adventitious agents. Testing is performed after filtration but before any further processing that is intended to inactivate or remove viruses.

Filtration with 100-nm (0.1- μ m) pore size filters is an accepted method for removing mycoplasmas and gamma irradiation (> 25 kGy while frozen), and chemical treatments (e.g., with betapropiolactone) are accepted methods of inactivating viruses and mycoplasmas; serum manufacturers

routinely use these tools in both production and testing facilities. These treatments do not remove antibodies that may interfere with some applications. Additionally, the treatments do not ensure complete viral removal or inactivation, but can significantly reduce the risk of viral activity. The testing series to screen bovine serum for the absence of adventitious agents typically includes the following:

- Bacterial and fungal sterility testing as described in 9 CFR 113.26
- Mycoplasma testing as described in 9 CFR 113.28
- Viral testing as described in 9 CFR 113.53

The procedures described in *Sterility Tests* (71) confirm the absence of bacterial and fungal infection. For viruses, only cultivation using suitable substrate cells can indicate viral infectivity and replication. Those who use serum for research or production should test the serum for the absence of adventitious agents in a manner that is consistent with the product's intended application, bearing in mind that testing indicates only presence or absence of adventitious agents within the limits of the test procedures used.

Mycoplasma Testing

Mycoplasma contamination in tissue culture can arise from many animal origin sources, including serum, but more commonly it results from cross-contamination of infected cultures. Mycoplasmas are particularly insidious contaminants in cell culture because they

- cannot be visualized by light microscopy even at high density (>10⁷ colony-forming units/mL);
- cause no observable change in turbidity or pH of the culture fluid;
- cannot routinely be removed by single sterilizing filters, although removal can be obtained through a triple series of 0.1- μ m filters;
- are unaffected by traditional antibiotics used in cell culture; and
- exert an extremely wide variety of adverse effects in tissue culture.

Classical mycoplasma detection is described in *Mycoplasma Tests* (63).

In addition to these methods, more recent detection procedures include luminescent and polymerase chain reaction (PCR) assay procedures. *Nucleic Acid-Based Techniques—Amplification* (1127) describes the general principles of PCR assays. The sensitive 20-minute luminescent assay measures a specific enzyme activity of mollicutes that converts adenosine diphosphate to adenosine triphosphate via a luciferase/luciferin reaction. Results are unequivocal and semiquantitative. PCR methods are quick and sensitive and display with good reliability, but occasional false positive results are a source of concern with commercial testing service labs. PCR may detect mycoplasmal DNA fragments that are non-infectious.

Viral Testing

The virus testing procedures for serum products are outlined in 9 CFR 113.52 and 9 CFR 113.53. In addition, there are other documents that may include equivalent or relevant testing such as EMA/CVMP/743/00-Rev.2 from the Committee for Veterinary Medicinal Products (CVMP) *Revised Guideline on Requirements and Controls Applied to Bovine Serum Used in the Production of Immunological Veterinary Medicinal Products* and EMA/CPMP/BWP/1793/02 from the Committee for Proprietary Medicinal Products (CPMP) *Note for Guidance on the Use of Bovine Serum in the Manufacture of Human Biological Medicinal Products*. Serum manufacturers should perform virus testing in compliance with this regulation, using at least two different and sensitive detector cell lines, one of which should be of bovine origin. The tests include cultivation of detector cells in cell culture media supplemented with 15% test serum for at least 21 days. Cells are subcultured at least twice during this period, usu-

ally 7 and 14 days post inoculation. At the conclusion of the last subculture (after a total of at least 21 days of incubation), cells are examined for general signs of virus amplification. The following end points are used for general virus detection: microscopic cell examination for cytopathogenic agents such as infectious bovine rhinotracheitis virus, cell staining and microscopic examination for inclusion bodies, and hemadsorption test to detect hemadsorbing agents such as PI-3. In addition to this series of testing and at the conclusion of the last subculture (after a total of at least 21 days of incubation), cells are stained with specific fluorescent antibodies against the following specific viral agents:

- BVDV
- Bovine parvovirus
- Bovine adenovirus
- Bluetongue virus
- Bovine respiratory syncytial virus
- Reovirus
- Rabies virus

In addition to the viruses listed above, other viruses can be causative agents of disease and may require testing in various bovine serum applications. The serum end user is responsible for determining whether full 9 CFR testing is sufficient, and if other specific viral agents should be tested for. Examples of specific viruses not covered by the current virus testing guide may include akabane, bovine herpesvirus 1 (BHV-1), Parainfluenza-3 virus (PI-3), bovine leukemia, bovine rotavirus, bovine circovirus, bovine polyomavirus, coronavirus, torovirus, bovine enterovirus, bovine astrovirus, foot-and-mouth disease virus (FMDV), and rinderpest. *Appendix 2* provides a general description of some of these viruses as well as the ones for which testing is required. A serum end user's thorough risk analysis should determine the scope of testing and serum treatment options.

Risk Assessment and Detection Strategies

Serum manufacturers and serum end users should carry out a comprehensive, science-based risk assessment (e.g. Failure Modes and Effects Analysis) in order to better understand the safety profile of the serum product. The following risk assessment elements can be taken into consideration, but other elements can be included as appropriate: country of origin, region of the country, animal disease status of the country/region of origin, animal age, blood collection process, animal stunning method and exsanguination method, serum manufacturing process, type of production quality system, production in-process controls, final product testing, virus inactivation, equipment segregation, equipment cleaning procedure, personnel training, serum use/application, pharmaceutical product type, and intended use.

The species barrier provides a degree of protection against infection by some animal etiologic agents. This barrier is not an alternative to proactively ensuring that pharmaceutical products are manufactured only from raw materials of animal origin that have undetectable levels of adventitious agents. Inoculation of viable organisms into a nonhost species carries a risk that the organisms could cross the species barrier. An appropriate test regimen of serum material should therefore include examination for potential contaminants associated with the species of origin and the species of intent. Serum treatments to inactivate viral agents are a factor in establishing the appropriate test regimen for a particular material. Lowest risk of contamination is associated with biological materials that are terminally sterilized.

Zero risk is neither possible nor reasonable. The serum manufacturers should fully describe specific testing regimens in the product specifications, and these will vary depending on the type and source of the serum. Therefore, the guidelines for screening described in this chapter are examples only, and screening for all viruses listed may not be required for a particular material. Some manufacturers may perform certain tests on the finished product or on in-process materials rather than on individual component(s). Manufacturers

must also evaluate the dilution effect in relation to the limit of detection of the test procedure. Interference with growth or neutralization of viral activity by serum may be an indication of a specific antibody or certain nonspecific factors in serum masking the viral agent. It is recommended that serum manufacturers consider this possibility when determining an adequate level of treatment in their viral inactivation studies or in virus testing applications.

Serum manufacturers should confirm that the species of origin is bovine to ensure that no other nonbovine agents may be present. Manufacturers should perform extraneous virus testing in appropriate cell cultures (see *Virology Test Methods* (1237) for appropriate cell line choices dependent on assay and targeted agent). If necessary, seroconversion studies should be conducted in susceptible animal species using a host species immune antibody response as the method of detection. Studies should use this procedure following an inactivation step to detect whether the virus was present before the virus inactivation process.

Serum manufacturing processes should be conducted in a consistent manner, following the established manufacturing procedures, with adequate quality systems built into the production process. Furthermore, equipment segregation (by species of origin), equipment and facility cleaning procedures, and personnel training are important elements in the risk assessment of the process.

Safety Considerations

End users of donor bovine serum may require serum that does not have detectable antibodies against BVDV or other specific agents so that the users can propagate cell cultures used in vaccine production, diagnostic testing, and test kit preparation, especially for the maintenance of master seed and master cell stocks. More than 40 cell types are available for the production of veterinary biologicals, but fewer than 10 media types are available for their propagation. Some researchers have proposed serum-free media as an alternative in propagating certain cells and viruses; but this means adapting culture procedures, which may alter the cells and change production results. If new or different sera are imported into the U.S., serum end users will require confirmation of source, species, and documentation of the origin of the sera in countries that are free of FMD and rinderpest.

CHARACTERIZATION OF BOVINE SERUM

Introduction

In the absence of end product-specific requirements, each lot of FBS should be tested to confirm that the serum meets the requirements of the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* (90). For all other types of bovine sera, this section describes several key procedures for characterization. These procedures are not mandatory but are guidelines that manufacturers may consider for their individual applications. The table in the *Hemoglobin* section shows samples of specifications for the different types of bovine sera.

Species Identification

Both inter- and intraspecies identification assays should be performed on bovine sera to confirm species identity and the integrity of the serum products, and to ensure that nonbovine agents are not present. The most commonly used assay for the identification of bovine species identity is based on the electrophoretic profile of specific serum proteins. With electrophoresis, the serum proteins usually separate into as many as six fractions: albumin, alpha 1, alpha 2, beta 1, beta 2, and gamma globulins.

Other procedures used for bovine speciation include radial immunodiffusion (RID) and the double diffusion Ouchterlony method. These procedures allow either qualitative or quantitative measurements of the immunoglobulin G levels in serum. The RID method is based on the diffusion of an antigen from a circular well into a homogeneous gel that contains specific antiserum for each particular antigen. A circle of precipitated antigen and antibody forms and continues to grow until it reaches equilibrium. The diameters of the rings are a function of antigen concentration. The Ouchterlony method is a double gel diffusion test wherein antigen and antibody diffuse toward each other in a semi-solid medium to a point in the medium where optimum concentration of each is reached, forming a precipitate. The Ouchterlony plates contain cylindrical wells—a central 8-mm diameter antigen well, surrounded by six 3-mm antisera wells—which make possible the simultaneous monitoring of multiple antigen–antibody systems and the identification of particular antigens in a preparation. The proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* (90) describes the accepted procedure.

Hemoglobin

Hemoglobin is a multi-subunit protein that forms an unstable reversible bond with oxygen in the red blood cells. The oxygen-loaded form is called oxyhemoglobin and is bright red. The oxygen-unloaded form is called deoxyhemoglobin and is purple-blue. Oxyhemoglobin is the predominant form in red blood cells.

Low hemoglobin content in sera is widely accepted as a good general indication of rapid and careful processing of blood that will be used for serum. Red blood cells are fragile and rupture easily, releasing hemoglobin into the serum. Rough handling of the harvested blood, poor temperature control, or delayed processing elevates hemoglobin content in serum. Acceptable levels of hemoglobin may vary with intended application. The hemoglobin levels are measured using spectrophotometric procedures (see *Spectrophotometry and Light-Scattering* (851)) as described in the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* (90).

Chemical Profile

The testing of components such as cholesterol, alpha globulin, beta globulin, gamma globulin, albumin, creatinine, bilirubin, glucose, alanine aminotransferase, aspartate aminotransferase, phosphorus, potassium, calcium, and sodium usually is not considered a criterion for bovine serum lot release. Some manufacturers do not perform the tests on a routine basis but only as auxiliary tests. In some instances hospital clinical laboratories may run the tests. The levels of these chemicals in serum are important to end users and may also be used to assess lot-to-lot variability.

Endotoxin Levels

Although high endotoxin levels are not suitable for applications involving injectables, acceptable levels in bovine sera vary depending on the intended application. Some manufacturers may overlook the importance of low endotoxin levels in bovine sera used in cell culture applications. Endotoxin influences more than 30 biological activities. Some of these are macrophage activation, mitogenic stimulation, and induction of interferon and colony-stimulating factor (for some applications, these may be positive activities). Endotoxin can also lead to cytotoxicity by initiating complement activation. The most commonly used methods for endotoxin detection are the semiquantitative gel clot Limulus amoebocyte lysate procedure and the quantitative kinetic chromogenic method described in *Bacterial Endotoxins Test* (85). For both the gel clot and the kinetic chromogenic assays, valid endotoxin assays require appropriate treatment by heat or dilution in order to avoid adverse effects of interfering substances in serum. Researchers should include a positive product control in each assay to confirm that any interference has been overcome by the heat or dilution treatment.

Osmolality

The osmolality test is designed to evaluate the electrolyte concentration in bovine serum. Chemicals that affect serum osmolality include sodium, chloride, bicarbonate, potassium, proteins, and glucose. Serum manufacturers should measure the osmolality of each serum batch to verify compliance with product specifications, using equipment calibrated with standards that are traceable to the National Institute of Standards and Technology. *Osmolality and Osmolarity* (785) describes how osmolality is determined by freezing-point depression of the bovine serum solution. Scientists use at least two standards to calibrate the instrument. The osmolality of each sample is calculated and related to the serum water content and is expressed as mOsmol/kg H₂O.

Total Protein Level

The total protein level in serum is measured to verify animal age and compliance with product specifications. *Biotechnology-Derived Articles—Total Protein Assay* (1057) describes two procedures, the Biuret and Bradford methods, for determining protein concentration. The acceptable level of protein in serum should be assessed by the end user based on the intended application.

Cell Growth Properties

Each lot of serum should be tested for its ability to support in vitro growth of specific cell lines. Bovine sera are highly variable, and different lots may yield different results. Because of this variability, end users should characterize and standardize the cell lines that they will use for this type of testing. End users should design cell growth procedures that

	FBS	Newborn Calf Serum	Calf Serum	Donor Bovine Serum	Adult Bovine Serum
Sterility test	No growth detected	No growth detected	No growth detected	No growth detected	No growth detected
Mycoplasma	Not detected	Not detected	Not detected	Not detected	Not detected
Virus testing	Not detected	Not detected	Not detected	Not detected	Not detected
Hemoglobin (mg/dL)	<30	<30	<30	<30	<30
Total protein (g/dL)	3.0–4.5	3.5–6.0	5.0–8.0	5.0–8.0	6.0–10.0
pH	7.00–8.00	7.00–8.00	7.00–8.00	7.00–8.00	7.00–8.00
Osmolality (mOsmol/Kg)	280–360	240–340	240–340	240–340	240–340

will help them check the efficacy of bovine serum in promoting cell growth. Serum manufacturers will benefit from monitoring growth promotion over several generations of subcultures to detect any evidence of cytotoxicity or changes in cell morphology. Different serum manufacturers use different cell types, and the growth studies and cell lines used by serum manufacturers also may differ from those applied by serum end users. When serum manufacturers evaluate the growth properties of a specific cell line in response to a specific lot of serum, they should take into account plating efficiency and/or growth promotion or some other functionality tests that qualify the serum lot for its intended use.

Plating efficiency at low cell density is a preferred method for analyzing the proliferative capacity and survival of single cells under optimal growth conditions. This procedure can reveal differences in the growth rate within the population and is capable of distinguishing between changes in growth rate (colony size) and cell survival (colony number). The growth kinetic is another important aspect in the design of cell-based experiments. Determining the growth curve of each cell line helps define optimal culture conditions, because variation in serum and other growth additives may influence growth parameters, which may affect the experimental outcome.

In the absence of specific tests designed for their particular products, serum users can refer to the functionality tests described in the proposed general chapter *Fetal Bovine Serum—Quality Attributes and Functionality Tests* (90) to determine whether a lot of serum is suitable for their application. This chapter provides guidance about how to perform growth promotion and plating efficiency tests.

In Vitro Cytotoxicity

Serum manufacturers should use an appropriate cell line for testing each lot of serum, and should perform growth studies through several subcultured generations to ensure that the serum has no cytotoxic effect on the cells. The choice of cell line depends on the intended use of serum. The cell growth and cytotoxicity assays should be performed on the final batch of serum after any viral inactivation step or any further processing.

CONCLUSION

Bovine serum is likely to remain an important component in the manufacture of many biologics, particularly those relying on cell culture. As with similar materials, bovine serum displays inherently variable quality. As a result, serum end users must establish suitable tests, procedures, and acceptance criteria for introduction of materials into a particular application process that uses serum. This may mean screening multiple lots of bovine serum to determine which lots meet the specification (see the section *Characterization of Bovine Serum*).

Manufacturers of therapeutic products using bovine serum are responsible for ensuring and documenting its quality and its impact on the quality, safety, and efficacy of the final product. In addition, it is important to ensure that each lot of serum performs in an equivalent manner during manufacturing. Serum can also interfere with final product purification; therefore it is important to understand the effect of bovine serum on the manufacturing process in order to understand the effect that various processes might have on the final product. Finally, risks can also be mitigated through the design of processes to include steps to adequately remove the bovine material through dilution, separation, or inactivation as well as the development of analytical assays to assess the bovine-derived residual content during processes and in the final therapeutic product. A number of sensitive assays can provide a quantitative means of detecting bovine material at picogram levels.

APPENDIX 1

Bovine sera and serum-related products used in the manufacture of biological products are regulated in the context of *Requirements for Ingredients of Animal Origin Used for the Production of Biologics*, 9 CFR 113.53. Currently, individual serum manufacturers perform detection studies to identify contaminating viruses. Because of the potential international market for serum, serum manufacturers need to be mindful of other regulatory requirements. Manufacturers can use the documents listed here as guidance for screening bovine sera for contamination by adventitious agents. Because of the risk carried by animal-derived serum products, serum manufacturers and end users should ensure that the country of origin of the material complies with applicable regulatory requirements. Although no cell performance assays currently demonstrate lack of BSE in serum, serum manufacturers must comply with the regulatory requirements of countries where the serum is sourced and marketed to ensure a minimal risk of infection with BSE/TSE.

Beyond relevant USP chapters referenced in this chapter, the following list of documents includes regulatory documents as well as best practices in product and process development, manufacturing, quality control, and quality assurance.

CFR

- 9 CFR 94.18 (CVB, 2001)
- 9 CFR 113.46
- 9 CFR 113.47
- 9 CFR 113.52
- 9 CFR 113.53
- 9 CFR 113.55
- 9 CFR 320
- 9 CFR 327.4
- 21 CFR 211 Subpart E
- 21 CFR 801.1
- 21 CFR 809.10

FDA

- FDA. Center for Biologics Evaluation and Research (CBER). 2000. Letter to manufacturers of biological products. Available at: <http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/ucm105877.htm>
- Use of materials derived from cattle in medicinal products intended for use in humans and drugs intended for use in ruminants (Proposed Rule). *Federal Register*. 2007; 72(8): 1582–1619. Available at: <http://www.reginfo.gov/public/>

International Regulations and Guidance Documents

- CPMP/Biotechnology Working Party/EMA (CPMP/BWP/EMA). 1996. *Note for guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses*. Available at <http://www.emea.europa.eu/pdfs/human/bwp/026895en.pdf>.
- CPMP/BWP/EMA. 2003. *Note for guidance on the use of bovine serum in the manufacture of human biological medicinal products*. Available at <http://www.emea.europa.eu/pdfs/human/bwp/179302en.pdf>.
- EMA/CVMP/743/00-Rev.2 from the Committee for Veterinary Medicinal Products (CVMP). *Revised guideline on requirements and controls applied to bovine serum used in the production of immunological veterinary medicinal products*. Available at <http://www.emea.europa.eu/pdfs/vet/iwp/074300en.pdf>.
- EMA/CPMP/BWP/1793/02, from the CPMP. *Note for guidance on the use of bovine serum in the manufacture of human biological medicinal products*. Available at <http://www.emea.europa.eu/pdfs/human/bwp/179302en.pdf>.
- CPMP/CVMP. *Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products*. Available

at <http://www.emea.europa.eu/pdfs/human/bwp/TSE%20NFC%20410-rev2.pdf>.

- World Health Organization (WHO), Office International des Epizooties. *Terrestrial animal health code 2007*. Available at http://www.oie.int/eng/normes/mcode/code2007/anc-en_summry.htm.
- WHO. 2006. *WHO guidelines on tissue infectivity distribution in transmissible spongiform encephalopathies*. <http://www.who.int/bloodproducts/cs/TSEPUBLICSHEDREPORT.pdf>.

APPENDIX 2

Following is a general description of viruses that manufacturers can consider when testing bovine serum for the absence of adventitious agents. The list is intended only to provide general information. The list of required testing is described in this chapter in the section *Viral Testing*.

Akabane—An insect-transmitted virus that causes congenital abnormalities of the central nervous system in ruminants. Disease due to *Akabane* virus has been recognized in Australia, Israel, Japan, and Korea. Antibodies to it have been found in a number of countries in Southeast Asia, the Middle East, and Africa. The disease affects fetuses of cattle, sheep, and goats. Asymptomatic infection has been demonstrated serologically in horses, buffalo, and deer (but not in humans or pigs) in endemic areas.

Bluetongue—An infectious, noncontagious arthropod-borne viral disease primarily of domestic and wild ruminants. Infection with bluetongue virus is common worldwide but is usually subclinical or mild. *Bluetongue* virus is the type-species of the genus *Orbivirus* in the family *Reoviridae*. Worldwide, 24 serotypes have been identified, although not all serotypes exist in any one geographic area: e.g., only 5 serotypes (2, 10, 11, 13, and 17) have been reported in the U.S. Distribution throughout the world parallels the spatial and temporal distribution of vector species of *Culicoides* biting midges, which are the only significant natural transmitters of the virus.

Bovine adenovirus—Associated with a wide spectrum of diseases. *Bovine adenovirus* type 3 is the serotype most often associated with bovine respiratory disease. *Bovine adenoviruses* are DNA viruses that have been separated into two genera: the *Mastadenovirus*, or *mammalian adenoviruses*, and the *Aviadenovirus*, or *avian adenoviruses*. Within the genus *Mastadenovirus* are numerous species-specific serotypes, nine of which have been identified in cattle. *Epitheliotropic adenoviruses* have also been isolated from ruminants, and usually are clinically unapparent. Clinical disease is dictated by various factors, including the strain of virus, concurrent infection, stress, environmental conditions, and management practices.

Bovine herpesvirus 1 (BHV-1)—Associated with several diseases in cattle, including infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, balanoposthitis, conjunctivitis, abortion, encephalomyelitis, and mastitis. BHV-1 infections are widespread in the cattle population. In feedlot cattle the respiratory form is most common.

Bovine leukemia—An exogenous C-type oncovirus in the family *Retroviridae*. Bovine leukemia is a viral disease of adult cattle characterized by neoplasia of lymphocytes and lymph nodes. Infection occurs by iatrogenic transfer of infected lymphocytes and is followed by a permanent antibody response. The prevalence of infection in a herd may be high, but only a few animals develop fatal lymphosarcoma. Infection is spread by contact with contaminated blood from an infected animal.

Bovine reovirus—Double-stranded ribonucleic acid (RNA) (dsRNA) viruses with nonenveloped spherical virions

60–80 nm in diameter. They cause bovine respiratory diseases.

Bovine respiratory syncytial virus (BRSV)—An RNA virus classified as a pneumovirus in the *Paramyxovirus* family. This virus was named for its characteristic cytopathic effect—the formation of syncytial cells. In addition to cattle, sheep and goats can also be infected by respiratory syncytial viruses. Human respiratory syncytial virus (HRSV) is an important respiratory pathogen in infants and young children. HRSV has antigenic subtypes, and preliminary evidence suggests the existence of antigenic subtypes of BRSV. BRSV is distributed worldwide, and the virus is indigenous in the cattle population. BRSV infections associated with respiratory disease occur predominantly in young beef and dairy cattle.

Bovine rotavirus—A dsRNA spherical virion 60–80 nm in diameter without an envelope. It is the most common viral cause of diarrhea in calves and lambs.

Bovine viral diarrhea virus (BVDV)—An RNA virus classified as a *Pestivirus* in the family *Flaviviridae*. BVDV can cross the placenta and appears to be capable of inducing immunosuppression, which allows the development of secondary bacterial pneumonia. BVDV has been reported to be the virus most frequently associated with multiple viral infections of the respiratory tract of calves.

Foot-and-mouth disease (FMD)—A highly infectious viral disease of cattle, pigs, sheep, goats, buffalo, and artiodactyl wildlife species. In a susceptible population, morbidity approaches 100%. The disease is rarely fatal except in young animals. FMD is caused by an *Aphthovirus* of the family *Picornaviridae*. Seven immunologically distinct serotypes are known, and within each serotype exist a large number of strains that exhibit a spectrum of antigenic characteristics.

Parainfluenza-3 virus (PI-3)—An RNA virus classified in the *Paramyxovirus* family. Although PI-3 is capable of causing disease, the virus usually is associated with mild to subclinical infection. The most important role of PI-3 is to serve as an initiator that can lead to the development of secondary bacterial pneumonia. Infections caused by PI-3 are common in cattle.

Parvovirus—A relatively heat-stable single-stranded DNA virus approximately 20 nm in diameter that has been recovered from cattle but under natural conditions is not known to cause disease.

Rabies—An acute viral encephalomyelitis that principally affects carnivores and bats, although it can affect any mammal. Rabies is caused by *Lyssaviruses* in the *Rhabdovirus* family. Although they are usually confined to one major reservoir species in a given geographic area, spillover to other species is common.

Rinderpest—A *Morbillivirus*, closely related to the viruses that cause canine distemper and measles. Strains may vary markedly in host range and virulence. Sera from recovered or vaccinated cattle cross-react with all strains in neutralization tests, but minor antigenic differences have been demonstrated. The virus is fragile and becomes rapidly inactivated by heat and light but remains viable for long periods in chilled or frozen tissues. Rinderpest is endemic in many countries in Asia and Africa. Historically, the virus has been widely distributed throughout Europe and Africa but to date has not established itself in North America, Central America, the Caribbean Islands, South America, Australia, or New Zealand. Rinderpest is included in the WHO's Office International des Epizooties list of communicable diseases that have the potential for very serious and rapid spread, irrespective of national borders; that are of serious socioeconomic or public health consequence; and that are of major impor-

tance in the international trade of livestock and livestock products.

<1027> FLOW CYTOMETRY

INTRODUCTION

Flow cytometry is an analytical method that plays a critical role in the quantitative and qualitative assessment of cell populations in patient and cellular product samples. The power of flow cytometry lies in its ability to rapidly and reliably analyze multiple attributes of individual cells within heterogeneous cell populations. Despite the value of flow cytometry data, method validation is challenging—perhaps more so than for other analytical methods—because of errors and artifacts from multiple sources.

Although flow cytometric methods can also be used to sort and isolate cells as part of the manufacturing process for cell- and tissue-based biological products, the scope of this chapter is limited to the use of flow cytometry as an analytical method. This chapter presents the technical aspects of the method, including instrumentation, sample handling and staining, and data analysis. Sources of error are considered in the context of technical features, as well as in the discussion of quality control, quality assurance, and standardization. Finally, current applications and assay troubleshooting principles are presented. For additional information on the basics and practical aspects of flow cytometry, see the current edition of *Practical Flow Cytometry* (Shapiro, 2003).

Flow cytometry is widely used to characterize cell and tissue-based products, but most assay methods are not yet standardized. In addition to issues related to technical complexity, there are also challenges to standardization of flow cytometric methods for specific product classes or types related to the heterogeneous nature of these products, even among those with similar manufacturing processes and clinical uses. Current and future innovations in instrumentation, analytic reagents, analytic algorithms, and automation are likely to improve the technology's capabilities but are unlikely to eliminate challenges (e.g., bioassay, identification tests, and other applications).

PRINCIPLES OF OPERATION, METHODS, QUALITY, AND STANDARDIZATION

The process of flow cytometry requires that cells move past a fixed light source consisting of one or more lasers so that individual cells can be observed or interrogated for characteristics such as size, granularity, and presence of surface membrane or intracellular antigens or molecules. The cells are suspended in fluid in which movement is controlled by the size and configuration of tubing, chambers, and pumps specific to the flow cytometry instrument. The pattern of light signals produced from the laser light's interaction with the cells is captured by a detection system, also specific to the instrument, and the detected signals are transformed into data elements that can be analyzed and combined with data from other cells in a given sample. Data from a cell suspension can then be expressed and presented in one-, two-, or three-dimensional visual formats, or in numerical formats, to characterize the cellular sample and its subpopulations both qualitatively and quantitatively.

Flow Cytometry Instrumentation

Flow cytometers, which incorporate fluidic, optical, and electronic signal processing elements, are described below.

FLUIDICS

The fluidics system moves a bulk mixture of cells so that a stream of single cells is formed. Within the flow cytometer, the single-cell suspension passes through a confined region where each cell is sequentially illuminated by a uniform light source at the observation point (interrogation point). Most instruments use a flow chamber (flow cell) that, after the cell sample is drawn into the sample injection tip, combines the cells with isotonic sheath fluid, using a conical nozzle assembly that is geometrically designed to produce a laminar flow of fluid (Figure 1). The fluid outlet nozzle typically has an orifice of 50–250 μm in diameter through which fluid exits at a high flow rate. Differential pressures between the sample stream of cells (lower pressure) and the sheath stream (higher pressure) draw the cells/particles out into a confined stream. The resulting coaxial stream within a stream is highly efficient, and the sample stream at the observation point is typically only slightly larger than the cells or particles contained within. At least one manufacturer uses an alternative approach in which the coaxial stream strategy is replaced by the use of microcapillaries to focus and direct the cells. The light signal deflected or emitted by the cell is then measured and analyzed.

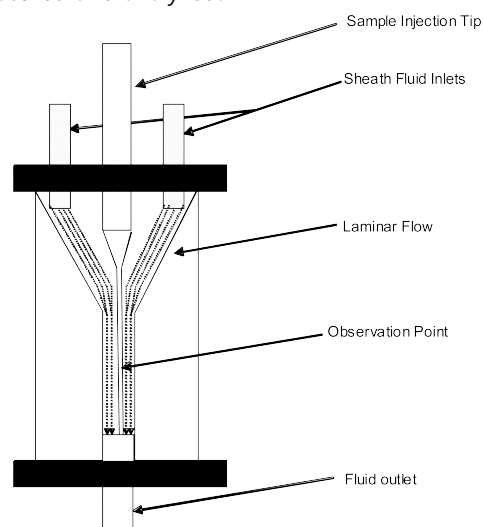


Figure 1. Schematic diagram of a flow cell.

OPTICS

When cells are stained with fluorescent dyes or with fluorescent-labeled antibody reagents, light emitted from the laser interacts with the fluorescent dye to produce a stimulated emission that has coherent (parallel) waves of light of uniform wavelength, phase, and polarization. Fluorescent light signals generated from the interaction of the laser light with the cells are collected by an array of detectors oriented in direct line with, and at 90° to, the incoming laser beam. The most common commercially available flow cytometer lasers (with corresponding wavelengths) are the blue argon laser (488 nm), the red diode laser (635 nm), and the violet laser (405 nm).

ELECTRONIC SIGNAL PROCESSING AND DATA OUTPUT

When a cell passes through the optical system of a flow cytometer, the light-scattering patterns or fluorescence from any fluorochrome on or in the cell are detected by various types of photodetectors or photomultiplier tubes (PMT) that transform the information about the characteristics of the cell into a computerized readout. Each analyzed cell generates an event in each parameter (forward scatter, side scatter, fluorescence) for which it is measured. *Figure 2* shows an example of a typical two-color flow cytometer configuration. Different cell types have distinctive sets of signals in the various parameters. For example, when the cell passes through a beam of light, the light deflected in the forward direction (usually about 20° from the forward direction of the laser) is called forward scatter and is collected by a detector known as the forward scatter channel (FSC). The amount of deflection in the FSC is proportional to cell size. Light deflected at a 90° angle is known as side scatter and is collected by the side scatter channel (SSC). This provides a measure of the cell's structural complexity caused by granules, membrane roughness, or nucleus, all of which are associated with higher SSC. The light deflected by other PMTs using a specific band-pass filter is collected by specific fluorescence channels (FL1 and FL2 in *Figure 2*). The electrical pulses, originating from light detected by the PMTs, are processed by a series of linear and log amplifiers. Logarithmic amplification is often used to measure fluorescence in cells. *Figures 3–7* show histograms for cells stained with antibodies conjugated with specific fluorochromes (see *Table 1*). The antibodies are specific to some of the cluster of differentiation (CD) markers discussed in *Immunophenotyping* (see below).

Table 1. Fluorochromes Commonly Used in Flow Cytometry

Fluorochrome	Typical Excitation Laser (nm)	Emission Peak (nm)
Cascade Blue	375; 401	423
Pacific Blue	403	455
R-Phycoerythrin (R-PE)	480; 565	578
PE-Cy5 conjugates	480; 565; 650	670
PE-Cy7 conjugates	480; 565; 743	767
Red 613 (Texas Red)	480; 565	613
Peridinin Chlorophyll (PerCP)	490	675
Fluorescein (FITC)	495	519
Allophycocyanin (APC)	650	660
APC-Cy7 conjugates	650; 755	767

The versatility of flow cytometry comes from the ability to attach fluorescent tags to the cell's surface, cytoplasm, or nucleus or to products of the cell. Fluorescent markers attached to the cell can be excited by lasers to emit light of specific wavelengths, and this light is then detected and analyzed in the manner described above. The type and amount of fluorescence detected provide both quantitative and qualitative information about the cell.

The photodetectors convert light into an analyzable output by generating a small current of which the voltage has amplitude proportional to the amount of light. The voltage is amplified and converted into electrical signals large enough to be plotted by the computer in several different ways. Thus, the FSC, SSC, and fluorescent detectors collect the light and convert it into electrical signals that can be analyzed by the computer. In this way, the signals coming

from each photodetector can be measured for their intensity (low to high) and sorted into channels. The channels are arranged as a continuum so that a cell population with many large cells will have many events in the higher channels, and one with many small cells will have many events in the lower channels.

DATA ANALYSIS

Data output from the flow cytometer can be represented in several ways, the most basic of which is the single-parameter histogram (*Figure 3*), in which events with similar intensity of light (forward scatter, side scatter, or fluorescence) are collected in channels and then plotted. This plot demonstrates the number of cells with similar optical characteristics. *Figure 4* is an example of graphs that display two measurement parameters, one on the x-axis and one on the y-axis, and the cell count as a density (dot) plot or contour map. The parameters could be SSC, FSC, or fluorescence. These parameters can be collected in one channel.

A dot plot displays a dot for each cell, and density plots and contour plots show a heat map or a topographical linear map, respectively, based on the relative number of cells in each channel. The forward versus side scatter histogram is the most common method of identifying different hematopoietic cell types. *Figure 5* shows a contour plot that is a 3-dimensional representation of the relative number of cells in the various channels.

When cells are stained with antibodies for different epitopes carrying two different fluorochromes, the data are presented as a plot of the two parameters plotted against each other. Cursors can be set on each axis to separate positive populations from negative populations for each of the attributes. This results in a graphic representation of cells that are positive for both markers, negative for both markers, and positive for only one of the two markers (*Figure 6*).

The flow cytometer allows the user to set the limits of positive and negative for each marker. Flow cytometric data are collected in list mode, where each electronic signal from a respective cell is displayed in the sequence in which it was acquired. List mode files can be edited at a later time to include or exclude any event. A basic advantage of flow cytometry is the ability to separate the data about the cells of interest from both the background and dead cells (e.g., noncellular particles or debris) when dealing with forward and side scatter and cells of other populations. The user must decide which signals are the actual light outputs from the cells and must construct an electronic gate to tell the computer to count as positive only the events that fall within the gate. Cell populations can vary widely depending on the tissue or cell source and the characteristics of the flow cytometer used. Gating allows the user to determine which outputs to consider actual events, so this process is of prime importance in standardizing flow cytometry data (*Figure 7*).

A technique known as compensation can be used to separate spectral overlap of fluorochromes that have similar emission wavelengths. For analog-style flow cytometers manufactured before the late 1990s, compensation must be set before data acquisition. In modern digital instruments, compensation can be set either before or after data acquisition. The adjustment of compensation can be more of an art than a science, and considerable literature has been focused on the relative merits of various methods to determine the correct compensation for cell types or experimental conditions. The analyst should have considerable knowledge of the cell type under analysis in order to prevent errors in phenotyping that can result from improper adjustment of compensation.

The number of events counted should be adequate for statistical confidence in the results. The instrument can be set so that data are collected until a certain number of

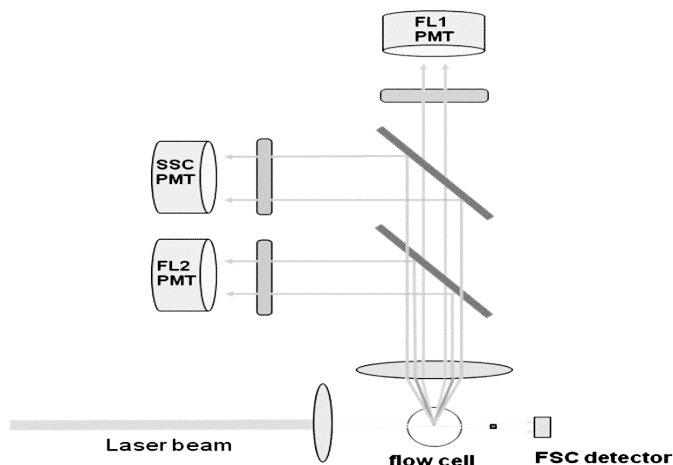


Figure 2. Typical 2-color flow cytometer with detectors for FSC, SCC, and fluorescence.

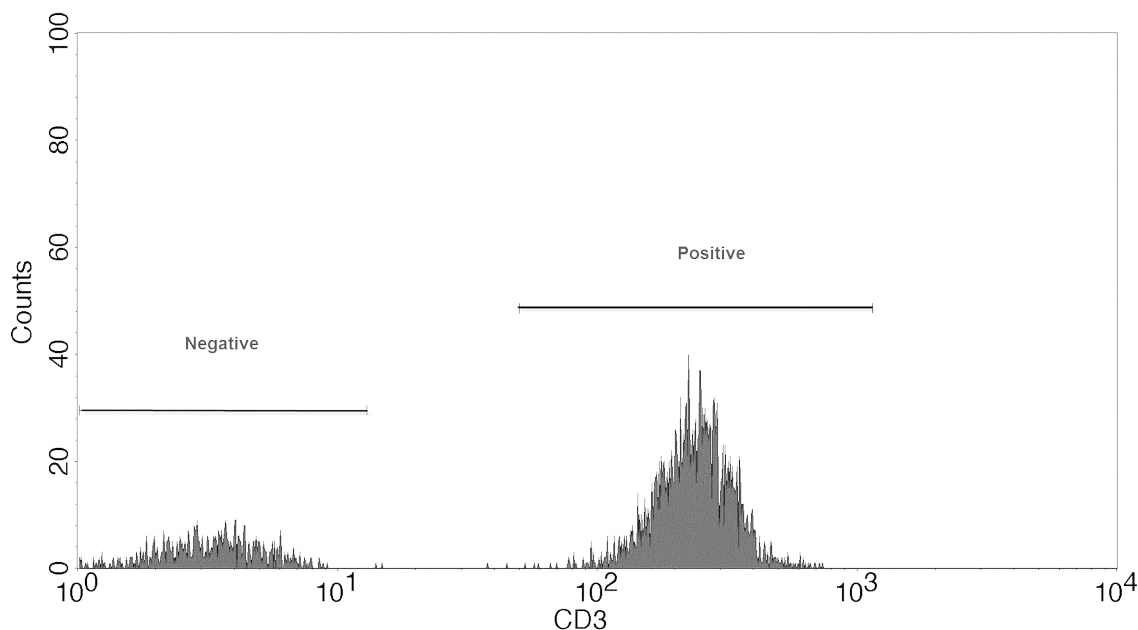


Figure 3. Single-parameter histogram showing expression of the cellular antigen CD3 in a mixture of cells.

events in a channel have been measured. This feature allows the operator to vary the length of time or number of events from the sample so that statistically reliable data are generated. Thus, a sample that measures a rare event will analyze more total cells than one that measures a common event. Use of list mode files, the electronic data files that represent the most uncensored data, provides an advantage because these files can be further analyzed after data acquisition. Investigators need to ensure that all raw data, documentation, protocols, specimens, and final reports are archived at the close of the study. To ensure the integrity and quality of raw data collected, researchers need to abide by U.S. FDA Regulations for Good Laboratory Practices (GLP) as prescribed in 21 CFR Part 58, Good Laboratory Practice for Nonclinical Laboratory Studies; and Part 11, Electronic Records and Electronic Signatures.

Flow Cytometry: Elements of a Procedure

Flow cytometric methods incorporate sample handling, preparation, and staining; instrument setup and operation;

data collection, analysis, and storage; and associated quality control measures.

SAMPLE HANDLING AND STAINING

Sample Collection, Handling, and Anticoagulation

In order to make accurate conclusions about the cell-based drug product, the analyst should ensure that samples from cellular products are as representative as possible of the whole product. Blood, apheresis samples, and cell suspension products should be well mixed before sampling, and care should be taken to obtain adequate sample volumes.

Cellular samples containing human blood/plasma must be anticoagulated. Citrate-based anticoagulants (e.g., Anticoagulant Citrate Dextrose Solution A) or heparin are recommended more highly than EDTA because they will optimally preserve samples being held for more than a few hours. For longer-term samples, specific transport/storage media may be required, and validation studies should be performed to

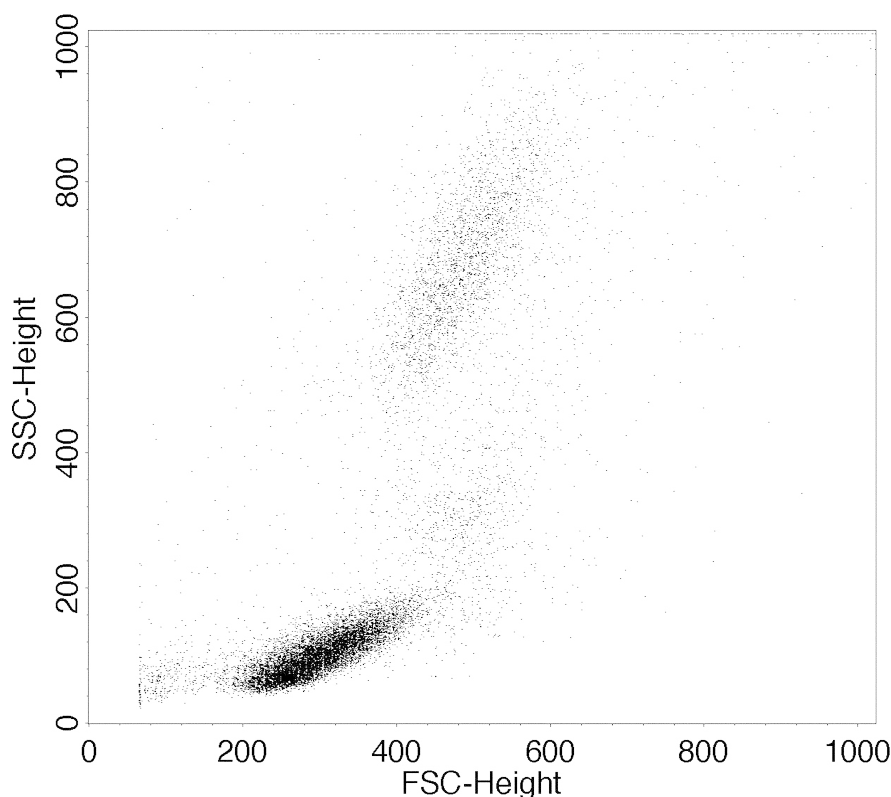


Figure 4. Bivariate dot plot of cells displayed by FSC and SSC.

ensure that those samples are equivalent to fresh samples at the time of flow cytometric analysis.

Samples intended for whole blood lysis and surface antigen staining should be transported and stored, preferably on a slow oscillating mixer, at room temperature. Fixed samples or live cell preparations should be stored at 4°. When the sample may be exposed to extreme temperatures, temperature-control materials (room temperature packs, wet ice/cold packs, and insulation) may be necessary, and validation studies should be performed to ensure sample integrity. For critical or high-value samples, temperature-monitoring devices may be needed during transport.

After acquisition, specimens should be analyzed as soon as possible. Special attention should be given to situations in which cellular proliferation and metabolic depletion of energy sources within the transport/storage media can lead to apoptosis. When accurate counting is not required or if infectious agents are suspected, a commercial lyse/fix solution can increase storage time and reduce the risk of disease transmission. For specimens separated by density-gradient centrifugation, storage in a solution of buffered paraformaldehyde (0.1% to 2.0%) is recommended after cell labeling has occurred.

Sample Processing, Staining, and Fixation

Reagents used in sample processing, staining, and fixation should be qualified for their intended use. Further guidance is available in ICH Q6B, *Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*. When using reagent kits, follow the manufacturer's sample processing instructions.

Sample processing that involves centrifugation, washing, red blood cell (RBC) removal or lysis, or density-gradient separation is commonly done during many flow cytometric applications but can introduce error and artifact. Several techniques and reagents are available for RBC removal and lysis. Clinical grade [in vitro diagnostics (IVD) or analyte-specific reagents (ASR)] reagents are recommended for optimal quality, but artifacts can still occur. Density-gradient

centrifugation can introduce error associated with variable cell losses among subpopulations that are being measured. These sources of error and artifact can be avoided by analyzing live whole blood whenever possible.

Most whole blood lysate instructions recommend staining at room temperature and in the dark. Many methods include a dilute fixative to prevent capping and internalization of fluorochrome. In contrast, cell preparations (density-gradient cell preparations, apheresis specimens, tissue culture) should be stained at 4°, washed with cold buffer, and stored cold until analyzed.

Fixation that also preserves cell surface antigens can be accomplished using commercial leukocyte preservatives or with buffered formaldehyde or paraformaldehyde. Very little validation of storage times, antibody binding, or fluorochrome intensity has been reported. Any laboratory that considers batch analysis of fixed specimens should validate these techniques thoroughly before implementing.

USE AND CHOICE OF FLUOROCHROMES

Fluorochromes

Fluorochromes are used for direct staining of cells or as agents bound to antibody or other reagents to stain cellular antigens or other structures. *Table 1* lists examples of common fluorochromes used for flow cytometry and their excitation and emission wavelengths. Wavelengths (nm) may vary slightly depending on the environment. Synthetic probes from specific manufacturers are also available.¹ Fluorochromes must match the spectral range for the lasers and filter sets specific to the user's flow cytometer.

When choosing fluorochromes for multicolor phenotyping, the operator should refer to established methods for

¹ AlexaFluor series (Invitrogen/Molecular Probes) or the Cy series (GE Healthcare).

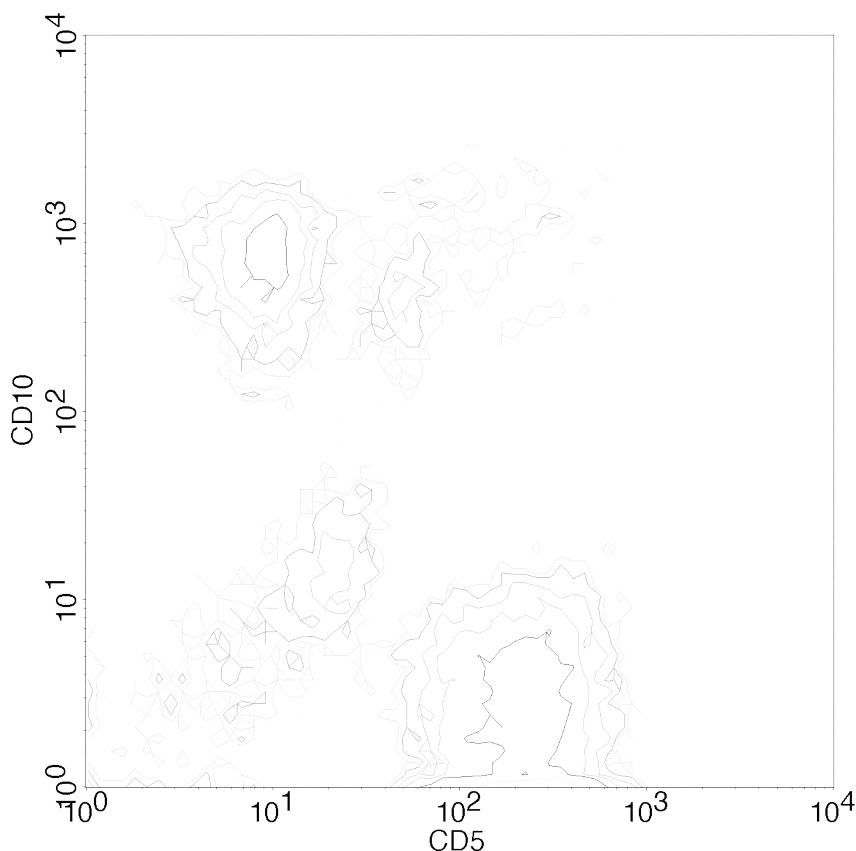


Figure 5. Bivariate contour plot showing relative numbers of cells present in each channel that co-express 2 CD markers.

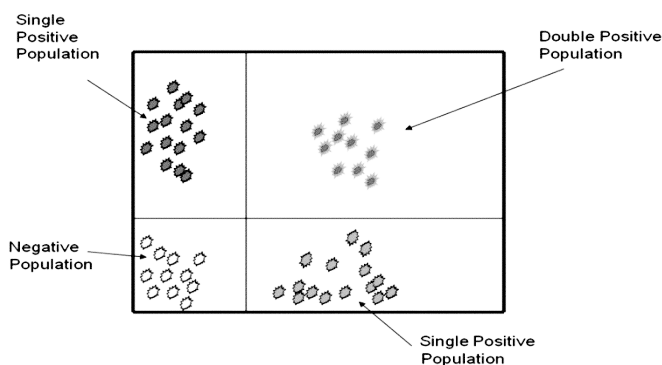


Figure 6. Schematic presentation of a 2-parameter histogram.

the particular instrument. In general, the brightest fluorochromes should be matched with the antigens that are expected to have the lowest expression on the cell surface. The brightness of tandem dyes can also be reduced by the use of certain fixatives, some of which are less problematic than others. When designing a multicolor flow and tandem dye procedure that has not been previously validated, the operator should consult the manufacturer's technical service, compare tandem dye/fixative combinations, and validate the final fluorochrome combination to ensure sample-to-sample consistency.

Tandem fluorescent dyes are dual-conjugated fluorescent molecules. When the two labels are in close proximity, energy produced by the laser exciting the donor fluor is transferred to the acceptor fluor, releasing a photon at the emission wavelength of the acceptor fluor (also known as

fluorescence resonance energy transfer, or FRET). For example, PECy5 will excite at the excitation wavelength for PE (565 nm), transfer energy to Cy5, and emit at the emission wavelength for Cy5 (670 nm).

Fluorescently Labeled Antibodies

Most commercially available antibody reagents are monoclonal, but polyclonal reagents may be available, and desirable, for some applications. The quality and specificity of an antibody can vary widely. Antibodies directed at a given antigen may differ in their binding specificity for different antigenic epitopes or in the strength of binding to the same epitope. If possible, use directly conjugated fluorochrome-antibody combinations that are IVD or ASR grade. Optimization of antibody concentration for the desired cell population is protocol specific but is generally accomplished by using increasing concentrations of antibody with a fixed

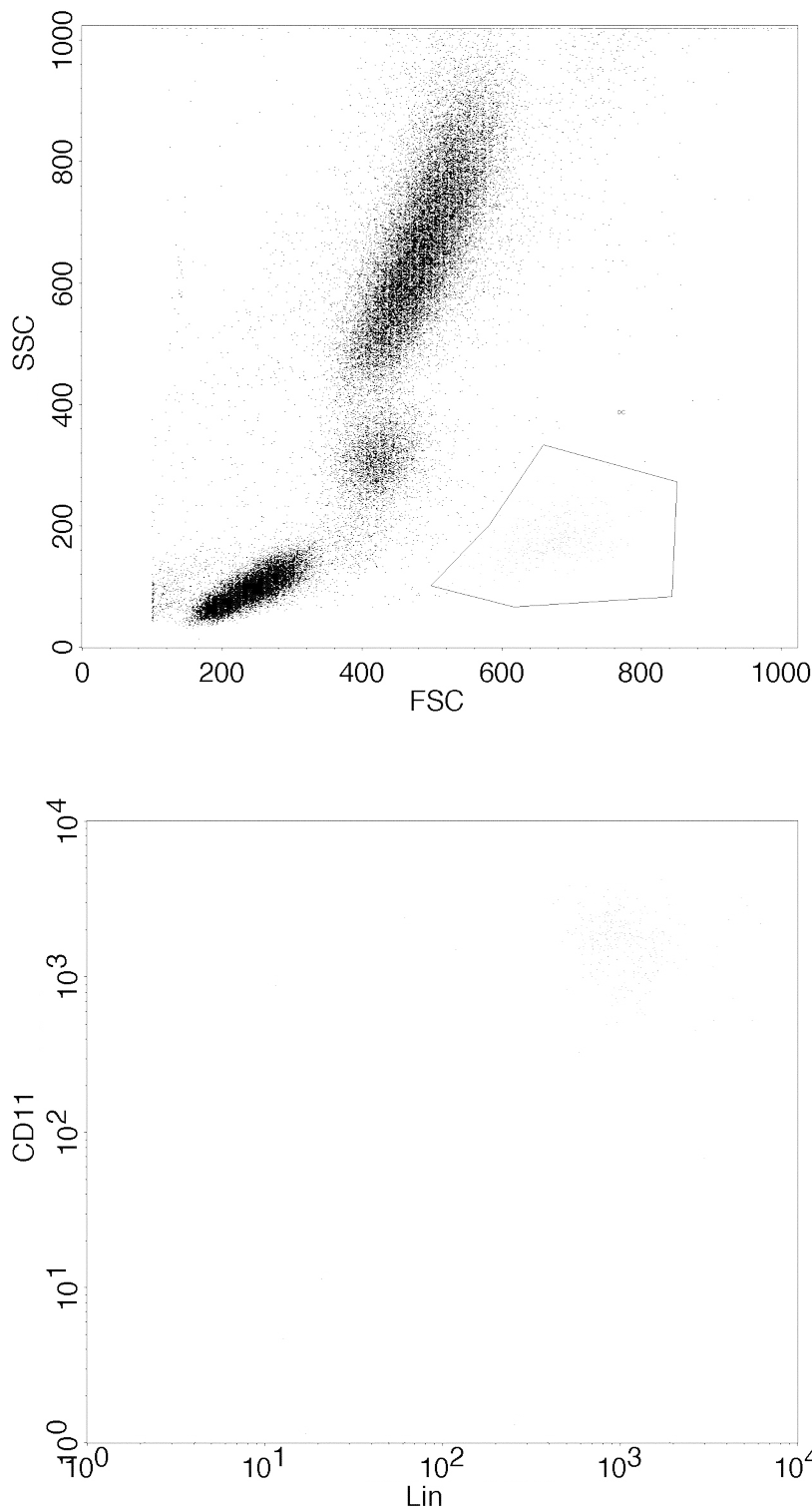


Figure 7. Gating of a cell population with low side scatter and high forward scatter, which is distinct from other cell populations in the sample.

number of cells to bracket the optimal brightness between autofluorescence and quenching. Quenching is caused by the prozone phenomenon, which occurs when excess antibody leads to immunoprecipitation and loss of fluorescence intensity. Further details are available in methods manuals such as *Current Protocols in Immunology* (Coligan et al. 1994).

Cell Surface Antigen Staining

Techniques for surface antigen staining vary with the type of specimen. Whole blood lysis techniques generally require surface labeling at room temperature in darkness for 15–30 min, followed by RBC lysis and, if desired, fixation. Published techniques use ammonium chloride (NH₄Cl) lysis of whole blood or marrow specimens followed by washing

before antibody labeling for leukemia immunophenotyping. Mononuclear cell or cultured cell samples that are stained live should be kept at 4° or in an azide-containing buffer to prevent capping and internalization of the antibodies.

Intracellular Staining

Several standardized procedures also exist for labeling intracellular antigens and cytokines. The operator should consult the manufacturer's protocol and standardized reagents for these procedures. Because permeabilizing reagents vary among procedures and manufacturers, do not mix and match reagents. For cytokine labeling it is often necessary to use an activating step and a Golgi block to allow a sufficient amount of cytokine to accumulate for detection. If standardized reagents or procedures are not available from the manufacturer or if analysis of specialized functions is required, many common procedures and techniques can be found in sources such as *Current Protocols in Immunology* (Coligan et al., 1994).

Quantitation of Antigens

Some applications require quantitation of the average number and density of antigen molecules per cell in order to give a more complete picture of the immunological behavior of cells (e.g., in studies where extracellular antigens are expressed differentially in relation to activating stimuli). The intensity of an antibody/fluorochrome labeled cell preparation is compared to the intensity of a set of microbead fluorescence standards collected at the same PMT voltage settings. The standards are calibrated in molecules of soluble fluorescence (MESF) units, from which one can determine effective fluorescence to antibody (F/P) ratio, the number of antigen molecules per cell, and the density of available sites per cell.

INSTRUMENT SETUP AND OPERATION

Compensation

Most instrument manufacturers supply software and test reagents (usually fluorescent beads) to set PMTs and compensation in order to target values found with the most common clinical tests (e.g., lymphocyte phenotyping, CD34 analysis). The operator should also use a biological control such as preserved blood or mononuclear cells, which are commercially available. Compensation must be set before acquisition on analog instruments. Digital instruments' PMTs must be set correctly because the values for these settings cannot be changed once the list mode file has been generated. When rare events are examined and/or intracellular dyes (e.g., 7-amino actinomycin D [7-AAD], propidium iodide [PI], Syto-16, etc.) are used in conjunction with fluorescently labeled antibodies, the balance of PMT voltage and spectral overlap must be closely monitored.

Autofluorescence (AF) is fluorescence above baseline in the absence of fluorochrome staining. This occurs in some cells, typically myeloid cells (especially alveolar macrophages) and cultured primary cells. If desired or necessary, AF can be measured directly on a fixed PMT voltage or can be calculated from a reference standard of fluorescent reference bead preparations (see *Quantitation of Antigens*, above). Avoid use of the 488 or 532 nm excitation wavelength and subsequent spectral compensation of the AF as an additional fluorochrome.

Data Acquisition and Gating Strategies

When possible, all events should be acquired in list mode, i.e., without selective gating of events. *Live gating*, defined as selective gating of events during acquisition, should be employed only when the desired subset is sufficiently rare that >2 million total events must be analyzed in order to count a significant (100 or greater) number of events of the desired population. List mode data can be acquired uncompensated when digital instrumentation is used, but most operators find that analysis is much less difficult and time-consuming if the data are in the range of proper compensation before acquisition. In addition, it is often desirable to

set thresholds for exclusion of debris. Setting a forward scatter threshold, for instance, excludes events below a predetermined size in order to prevent the large list mode file size that can occur when these events are counted.

Use of Controls

Fluorochrome-conjugated bead preparations are used for standardizing PMTs and compensation and for quantifying the expression of specific markers. The use of biologic controls is also highly recommended. Stain the cell samples with an isotype control and primary and secondary antibodies to assess nonspecific binding unless the laboratory has ascertained by rigorous validation procedures that nonspecific binding does not interfere with assay results.

Antigen-positive and -negative cell populations (prepared and stained in a manner identical to that for the test articles) provide internal system suitability standards. Such control cell populations also allow the laboratory to assess lot-to-lot variations in antibody preparations and staining reagents.

Use of Dyes and Gating for Cell Viability

Cell viability dyes such as 7-AAD, PI, and TO-PRO iodide are commonly used to determine the proportion of dead cells in a cell therapy product. These dyes are typically excluded from live cells but pass through the cell membranes of dead cells, staining their DNA. Cell viability staining can be combined with surface membrane or intracellular staining to evaluate subpopulations and the proportion of live and dead cells stained with a given marker. Viability staining can also be used in conjunction with a membrane dye in flow cytometry-based cytotoxicity assays. These viability dyes should not be confused with the many apoptosis-detection reagents now available. Validation techniques for non-IVD viability dyes involve the preparation of a dead cell population that is added in serial dilution to a live cell product, and the cell mixture is then assessed for fidelity to the known proportion by staining with the dye of interest.

Cell Enumeration

Absolute cell count, expressed as the number of cells in a given sample volume, can be determined by dual- and single-platform methods. The dual-platform method relies on a separate automated cell-counting instrument or manual counting method to first enumerate the cell population. The percentage of a subset(s) of interest is then determined by flow cytometry, that percentage is multiplied by the cell count, and the result is divided by 100. Single-platform methods enumerate the cell population and subset counts directly by counting the cells in the sample simultaneously while counting reference beads that have been added to that sample volume in a known concentration. Reference beads are often provided as a bead suspension that is added to the specimen. Alternatively, a given volume of sample may be added to a known number of reference beads provided as a solid phase matrix in polystyrene tubes. These approaches are subject to pipetting error, so extra care must be taken to ensure accuracy and reproducibility.

Instrument Setup and Quality Assurance

Each laboratory should have a quality plan that defines the standard operating procedures for instrument setup and calibration, as well as regular instrument monitoring, maintenance, and cleaning. Instrument logs should document these activities and operators. In general, the instrument manufacturer's quality program should be followed unless a suitable alternative has been established.

Instrument parameters such as laser current, voltage, output, and PMT voltages during calibration should be monitored and recorded whenever the instrument is in use. Careful monitoring of instrument setup parameters can be helpful in detecting trends and predicting laser or PMT failure. Biological control testing results should also be monitored and recorded to detect and prevent analytical method drift.

The laboratory should also participate in a proficiency-testing program that reflects the test menu. Depending on need, this could range from a formal program such as the

one administered by the College of American Pathologists (CAP) to simple sharing of specimens and analysis with another laboratory. Ensuring that operators are trained, qualified, and periodically evaluated for proficiency to perform specific procedures will also help ensure the consistency of techniques and controls.

DATA MANAGEMENT AND STATISTICAL CONSIDERATIONS

Data Management and Storage

Quality control assays and sample test assays (in list mode) should be stored in a manner that complies with regulatory requirements applicable to the laboratory. This can be accomplished by transfer to fixed drives, removable media, or to a server such as a commercial laboratory information system. Storage of results should always be traceable to the original FCS list mode file, instrument settings, and quality control parameters for that particular specimen. Data should be backed up to avoid loss of files. Storage and backup procedures should also be established for manual (paper) records that may be used for calculations and summary data.

Data Analysis and Statistical Considerations

For most flow cytometry applications, data analysis involves displaying the data from list mode files or live gating in a plot (single-parameter histogram plot, two-parameter dot plot with regions, or three-dimensional plot), and measuring the distribution of events within that plot. Further analysis of data within selected populations can be done by gating on specific cell populations. Description of the data typically includes the percentage of events within the population with a given characteristic (forward scatter, side scatter, fluorescent marker). The numerator is the number of events with the characteristic, and the denominator is either the number of total events counted or the number of gated events counted. For two-dimensional plots, analysis is typically done using computer software that analyzes and reports regional (e.g., quadrant) statistics. Because cell population clusters may shift their positions from one data file to the next, software has been developed for cluster analysis.

Statistical analysis of quantitative flow cytometry applications differs from qualitative applications in which a cell is considered either positive or negative for a given marker. For a typical quantitative application in which the number of molecules on the cell surface is estimated, the mean or median fluorescence intensity of the sample cells labeled with a fluorescent antibody bound to the molecule of interest can be compared to appropriate controls, including standard curves of cells or particles with known quantities of that molecule/antibody.

A common practical consideration for flow cytometric analysis of cell therapy products, especially autologous and related donor allogeneic products, is that sample size for analytical testing is often limited because of the limited cell content of the therapeutic product itself. This creates special challenges if cells containing the flow marker of interest are rare events. In these cases, before making a decision about sample size, the user must consider the expectations for detecting the rare events within a given number of total cells (i.e., prevalence, variability, sampling error) in relationship to the desired precision of the estimate.

Quality and Standardization

Standardization of flow cytometry practices and equipment requires validation, quality assurance (QA), and quality control (QC) practices. Although flow cytometry is used widely in both research and clinical laboratories, testing of cells for the development of clinical diagnostic and therapeutic applications is increasing, leading to more comprehensive regulatory requirements and attention to standardization. As an example, flow cytometry operators have traditionally used fluorescent microspheres (beads) or cells

for instrument setup and QC, frequently based upon manufacturers' recommendations. However, consistent instructions about how these control standards should be applied to instrument setup and QC remain elusive.

Properly applied, validation provides documented evidence that the manufacturing or testing process consistently produces product that meets predetermined specifications. Based on a thorough understanding of critical process parameters, validation helps to define product quality and helps to ensure a consistent and well-controlled manufacturing or testing process. Validation of flow cytometric methods should incorporate instrument qualification, analytical method validation, and operator qualification.

DOCUMENTATION

GLP and GMP processes require appropriate documentation such as standard operating procedures (SOPs) for all lab processes. SOPs must also be periodically updated and approved to reflect current practices. Training and qualification are required so that laboratory staff have the appropriate level of competence for their assigned responsibility. Operator competencies must be continually reviewed and assessed in relationship to SOPs and policies.

Integrating both internal and external quality processes is an important element of quality assurance. These involve equipment validation, manufacturing controls and limits, and product specifications. Process and equipment validation processes generally require installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ).

EQUIPMENT AND ASSAY QUALIFICATION

IQ establishes that the instrument is received as designed and specified, and that it is properly installed in a suitable environment. This generally means checking physical and facility requirements to determine whether the flow cytometer can be suitably installed. Qualification factors verified typically include temperature, humidity, space, and electrical facility capabilities in relationship to the instrument manufacturer's requirements. IQ procedures also ensure that all hardware and software components purchased are installed properly by the instrument manufacturer's representative.

OQ demonstrates that an instrument will function according to the manufacturer's specifications. This generally means component-level testing by the instrument manufacturer's representative or using a manufacturer's validation package that guides the end user to perform this function. Where possible, these tests should have specifications with corresponding quantitative control limits. This testing ensures that the instrument hardware and software are operational by comparison with the manufacturer's specifications.

PQ demonstrates that both the instrument and the assay consistently perform according to specifications. For flow cytometry, these specifications are generally determined by the laboratory performing the flow cytometric testing and usually include daily instrument and assay control test specifications. For specific assays, PQ should incorporate standardized methodology, application-specific setup and compensation, and specifications for linearity, precision, and accuracy of reported assay results. On some digital flow cytometers, a baseline instrument setup may be necessary in order to determine the optimal instrument settings for a given assay.

INSTRUMENT PERFORMANCE

Performance specifications may be identified by the flow cytometer manufacturer and may not address all of the operator's specification requirements. After identifying the manufacturer's specifications, the laboratory must establish

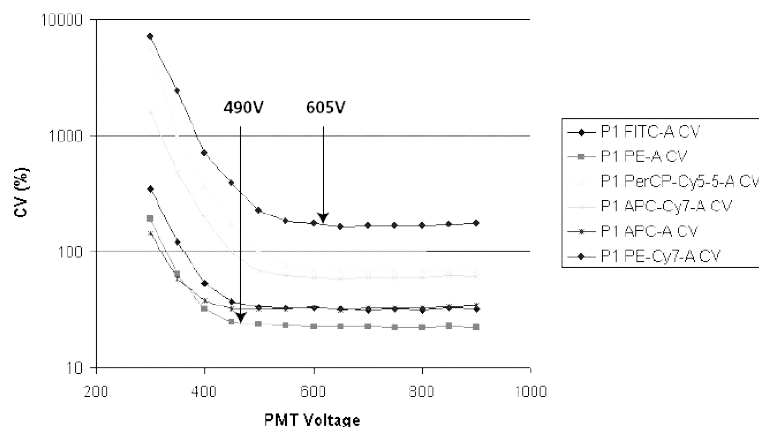


Figure 8. PMT voltages can be increased to a range that provides a lower CV.

specifications that are appropriate for the hardware configuration(s) that will be used, and the specifications must be standardized. As an example, the manufacturer may have a base specification for a four-color dual-laser flow cytometer. If the base specification calls for the standard red diode 635-nm laser but a helium–neon air-cooled 633-nm laser is substituted, the base specifications are no longer valid for the system. Similarly, if the specifications are based on the use of a 660-nm band-pass filter but a 675-nm long-pass filter is substituted, the base specifications are not valid because of differences in the emission filter characteristics.

PERFORMANCE MONITORING

Instrument performance can be monitored on a daily basis, using commercially available fluorescent beads. Light detectors such as PMTs, photodiodes (PD), and avalanche photodiodes (APDs) are used in most systems to detect signals, and their gains can be changed to increase or decrease the sensitivity of the detectors. Therefore, monitoring the settings of these detectors is as important as monitoring the signals, and these settings should be associated with each raw data file. The easiest approach is to maintain the same instrument detector settings from day to day and to measure the intensity of the fluorescence signals. This approach should be implemented for all parameters that must be validated using the appropriate beads. Instrument sensitivity is based on the ability of the detection system to resolve dim cell or bead populations. For this reason, measuring the coefficient of variation (CV) of dim to moderately intense fluorescent bead populations is a means to monitor fluorescence sensitivity on a daily basis. The instrument manufacturer's recommendations should be used to monitor performance.

Ambient high temperature can affect laser and PMT performance and should also be monitored on a daily basis.

Incorrect compensation for spectral overlap can strongly affect data during multicolor analysis. Many approaches have been established for this purpose, and recently mathematical algorithms have been used rather than analog circuitry. Algorithms, such as those that use matrix algebra, enable the operator or investigators to apply objective criteria to compensate for spectral overlap after all data have been collected. On older systems, the standard approach has been to compensate using a subtractive hardware adjustment to the observed preliminary data before all data have been collected. This approach can be subjective and is not as likely to produce accurate results as compensation by newer methods. Antibody-bound capture beads are valuable compensation tools because they can be used with the same antibody and tandem dyes for all fluorophores that will be used on cells. Validate the use of beads in place of cells for compensation purposes.

STANDARDS

Microsphere-based fluorescence standards for flow cytometry have been categorized by their purpose:

- Type I standards are alignment standards that are used to make adjustments to the instrument's optical alignment. These are typically used by field service engineers and by users of operator-adjusted systems to check the optical signal alignment in order to improve instrument sensitivity. These particles are typically small ($\approx 2 \mu\text{m}$) and bright, and they provide the most uniform illumination.
- Type II standards are reference beads and are the most commonly used bead standards. These typically are used on a daily basis, have dim to moderate fluorescence intensity, and can be obtained with various attached fluorophores. These can be used to mimic cells and, with dedicated software, to determine relative instrument sensitivity.
- Type III standards are used for fluorescence calibration. These are used for specialized applications that require calibration of one or more fluorescence detectors for quantitation of molecules of fluorochrome. Determination of the ratio of fluorophores to antibody (F/P ratio) allows subsequent calculation of the number of antibodies bound per cell.

INSTRUMENT SETUP AND QUALITY ASSURANCE

Instrument setup and quality assurance should be activities independent of the biological assay. Many variables related to instrumentation can lead to artifacts in the biological assay results. Two activities can be used for instrument quality assurance: baseline setup and daily setup.

Baseline Setup

On newer digital instruments it is desirable to establish a baseline setup of instrument settings that provide optimal sensitivity. This setup is not a daily procedure but should be performed if the instrument configuration is changed or if the instrument is serviced. Because PMT voltages and instrument configuration can strongly affect instrument sensitivity, this method should be used to provide objectivity as well as improved sensitivity. PMT voltages can be increased to a range that provides a lower CV (Figure 8). These settings can be, but may not always be, used for the biological assay.

Daily Setup

Type II standards, as reference particles, can be used to monitor signal intensity, separation of moderately bright and dim particles, and signal resolution. Fluorophore-matched beads provide a compensation tool as well as a means of knowing that the instrument is able to detect

those wavelengths. Fluorophore-matched beads, however, do not have the fluorescence uniformity required for measuring CVs. Based on the optical performance of the instrument, CVs are best measured using dim and moderately bright hard-dyed beads such as coumarin-dyed microparticles that fluoresce in a broad spectral range.

It is easy to confuse assay controls and instrument controls. Beads provide a fluorescence uniformity and consistency that cannot be obtained with cells, and, accordingly, beads are useful for monitoring instrument performance. For this reason, it is better to use a process control cell preparation to verify compensation and fluorophore acceptability.

Instrument settings for daily setup are generally the same settings used for assays. Not all assays can be used with the same instrument settings, and it is not always necessary to perform these activities for every instrument setting unless it is required by validation.

Daily activities include consistent instrument setting from day to day, use of a broad range of dim to moderate intensity beads, and monitoring key parameters, including bead fluorescence intensity (absolute and % CV), PMT values, temperature, laser power, and laser wavelength.

ASSAY QUALITY CONTROL AND QUALITY ASSURANCE

Assay-specific instrument settings should be established to demonstrate that all cell populations can be identified in bivariate plots for fluorescence and light scatter. Most importantly, positive populations must be on scale and properly compensated. This is critical when exciting cells with red lasers, which do not cause cells to generate significant autofluorescence. For this reason, it is important to verify appropriate PMT settings so that the positive population is in the upper part of its fluorescence scale, because it may be extremely difficult to identify the negative cells.

Fluorescence compensation is a critical adjustment. Digital instruments provide objective offline adjustments during analysis, and detailed instructions for proper compensation settings are available. Using cells or capture beads stained with a single antibody-fluorochrome is generally the best approach, but specialized fluorochrome-labeled bead mixtures can also work well to compensate for multicolor acquisition and analysis.

ISOTYPE CONTROLS

An isotype control is a negative-control antibody that should not react with the antigen of interest and is the same isotype as the test antibody. Myeloma protein or immunoglobulin that has no specificity to the species being tested and has the same Ig chain class and subclass as the test antibody is conjugated to a fluorochrome identical to that on the test antibody. Ideally, very little or no binding occurs when the isotype control is used in parallel with the test. Idiotypic nonspecific binding frequently occurs, however, and is independent of the isotype of the antibody. This is most likely related to other differences in antibody chemistry and can be especially problematic with rare-event detection assays, such as those for hematopoietic stem cell assays in peripheral blood.

FLUORESCENCE MINUS ONE CONTROLS

Fluorescence minus one (FMO) controls are used to control nonspecific staining during a multicolor assay. After compensation has been set, a tube containing all of the fluorochrome-labeled antibodies, except one, is run. If the compensation has been properly set, any positive fluorescence in the parameter corresponding to the missing fluorochrome-labeled antibody is caused by nonspecific staining and can be an indication of antibody excess or degradation of related tandem dyes. Although FMO controls are

very useful for estimating the sensitivity of a particular detector in the context of other reagents, the controls do not take into account nonspecific binding that can occur with the addition of the test antibody. FMO control tubes are most appropriately used for troubleshooting or when establishing a new multicolor reagent cocktail.

PROCESS CONTROLS

Process controls, also known as system suitability standards, account for sample preparation and data acquisition. They can include commercially available preserved control cells, cell lines, or primary cells such as normal peripheral blood. Process controls can also be used to test new lots of antibody reagent against old lots.

BIOLOGICAL CONTROLS

When treated or stimulated cells are compared to untreated or unstimulated cells, the untreated or unstimulated cells may in some cases be the most useful control for setting a positive/negative boundary. However, use of isotype controls may also apply to these situations, because stimulation may lead to Fc receptor upregulation, leading in turn to increased background staining, the presence of which can be elucidated by an isotype control.

FLOW CYTOMETRY APPLICATIONS FOR CELLULAR SAMPLES AND CELL THERAPY PRODUCTS

A wide variety of flow cytometry applications have been developed for research, clinical diagnosis and monitoring, drug development, and cellular product characterization and quality assessment (i.e., control to allow batch release). Traditional clinical applications include monitoring HIV disease and diagnosis and monitoring leukemia and lymphoma. Both pharmaceutical and academic research laboratories have increasingly broadened the application of flow cytometry from immunophenotyping to functional cellular assays, as well as microsphere-based multiplex assays capable of measuring multiple functional parameters on individual cells. Current functional assays include those that allow direct study of cellular activation status by measuring intracellular cytokine production or secretion of chemokines or cytokines, using a ligand-binding sandwich assay on microspheres.

Immunophenotyping

Flow cytometry allows the characterization of leukocyte subtypes by labeling cells with fluorochrome-conjugated monoclonal antibodies. The CD system defines monoclonal antibodies that recognize unique cell-surface antigens. Many clinical applications take advantage of flow cytometry's unique capabilities to measure multiple CD antigens on thousands of individual cells.

CD4 ENUMERATION

In the early 1980s, investigators discovered that HIV infects CD4 T cells and that a patient's peripheral blood CD4 T cell count is a useful indicator of immune status. CD4 enumeration has become the most commonly used diagnostic test in HIV-infected patients to determine the need for anti-retroviral (ARV) therapy and for monitoring the effectiveness of ARV drugs. T cell subset counts are typically expressed in terms of cells per microliter and as a percentage of lymphocytes, using a standardized reagent, software, and instrument system.

LEUKEMIA AND LYMPHOMA

Multidimensional flow cytometric analysis enables identification of aberrant cell populations in bone marrow, lymphatic tissue, and peripheral blood of patients with leukemia or lymphoma. This is accomplished with oncology-relevant and lineage-specific cocktails of monoclonal antibodies. With optimal fluorophores and improved optical/electronic configurations in flow cytometry instrumentation, additional cell markers can be detected to more precisely identify leukemia or lymphoma cell phenotypes and to improve the physician's assessment of patient status. Rare-event detection methods have improved the ability to detect minimal residual disease.

DENDRITIC CELLS

Dendritic cells (DCs) act as antigen-presenting cells that can influence the nature and strength of the immune response to specific antigens. This finding has led to the development of DCs as cell-based therapies for cancer, infectious disease, and autoimmune disease. DCs are morphologically and phenotypically diverse and can be derived from several cell types. Two major DC lineages, known as myeloid and plasmacytoid DCs, can be segregated on the basis of their expression of CD11c and CD123, respectively. Additionally, the expression of the costimulatory molecules CD80 and CD86 can be monitored to determine DC maturation state.

STEM AND PROGENITOR CELLS

CD34 expression is commonly used to characterize hematopoietic stem cells (HSCs) in peripheral blood, cord blood, bone marrow, and purified HSC preparations from these sources. Flow cytometric identification and enumeration of HSCs is possible by using monoclonal antibodies specific to the CD34 class III epitope, along with other well-characterized reagents, analysis software, and protocols. The reagent combination of anti-CD45, anti-CD34, and a viability dye such as 7-AAD is widely used for clinical applications. Increasing interest in developing cell-based therapies from embryonic, fetal, and adult tissue sources has led to the use of a wide variety of conventional and novel phenotypic markers for characterization of source cells and their more differentiated progeny. Flow cytometric assays are being developed as part of assay batteries to assess differentiated cellular products derived from pluripotent stem cell sources. These assays will help define appropriate numbers and types of desired cell populations, as well as help detect undesired cells such as residual pluripotent cells that could prove tumorigenic in the recipient.

LEUKOCYTES

Leukoreduction of blood products is a process used to produce blood products with a residual leukocyte content of less than 5×10^6 per unit. Clinical data suggest that nonhemolytic febrile transfusion reactions can be prevented by leukodepletion. Leukodepletion also prevents alloimmunization to HLA antigens in patients who will repeatedly require transfusion of blood products. Flow cytometry is routinely used to quantitate leukocyte contamination in leukocyte-depleted blood products.

PLATELETS

Flow cytometry is a rapid and useful method for diagnosing many primary thrombocytopathies related to defects in structural or functional glycoproteins (e.g., abnormal expression of gpIIb/IIIa in Glanzmann thrombasthenia or gpIb in

Bernard-Soulier disease). The use of thiazole orange, a fluorescent dye that binds RNA, allows immature platelets (reticulated platelets) to be quantified. The reticulated platelet count can be used to determine the rate of thrombopoiesis. This measurement can separate unexplained thrombocytopenias into those with increased destruction and those with defects in platelet production.

ERYTHROCYTES

Rhesus D-negative women receive prophylactic Rh-immunoglobulin to prevent alloimmunization from Rh(D)⁺ erythrocytes (RBCs). If fetomaternal hemorrhage is suspected, the mother's blood is tested for the presence and quantity of fetal RBCs, using fluorescently labeled antibodies to the Rh(D) antigen or to hemoglobin F.

The reticulocyte count is used to help determine whether the bone marrow is responding adequately to the body's need for RBCs and to help classify different types of anemia. Reticulocyte counts are based on the identification of residual ribosomes and RNA in immature nonnucleated RBCs. Flow cytometric enumeration of reticulocytes and their discrimination from mature RBCs uses fluorescent dyes that bind the residual RNA (e.g., thiazole orange).

Bead-Based Immunoassays

Multiplex microsphere-based flow cytometric assays combine a series of particles of discrete size and/or fluorescence intensity with matched antibody pairs to allow simultaneous detection of multiple soluble analytes on a flow cytometer. The flow cytometer's capacity to discriminate particles on the basis of size and color enables determination of multiple results from a single tube or well. Many investigators use such assays to measure secreted chemokines or cytokines, kinases, and anti-HLA antibodies.

Proliferation Assays

DYE INCORPORATION INTO DNA

Bromodeoxyuridine (BrdU) is a thymidine analog that can be incorporated into the DNA of cells during S phase, and then can be detected using specific, labeled monoclonal antibodies. By pulsing a stimulated cell culture with BrdU, cells can be identified that have proliferated (passed through S phase) during the time of the pulse. This assay has become a useful alternative to ³H-thymidine incorporation as a measure of proliferation because it is nonradioactive and can identify phenotypes of proliferating cells by the use of multiple markers and flow cytometry.

DYE INCORPORATION INTO CELLULAR PROTEINS OR CELL MEMBRANE

Cell-tracking dyes such as carboxyfluorescein succinimidyl ester (CFSE) and PKH26 have proven useful in the assessment of cell proliferation. CFSE binds covalently to cytosol and membrane proteins, and PKH26 binds non-covalently to cell membranes. When cells divide, CFSE/PKH26 labeling is partitioned equally between the daughter cells, which are therefore half as fluorescent as the parents. The fluorescence of each cell is further halved with each succeeding generation. This property makes CFSE/PKH26 assays useful not only for determining the fraction of cells that have proliferated in a stimulated culture but also, under ideal conditions, the number of generations that have elapsed. In this manner, the precursor frequencies of small populations that have proliferated over several days in culture can be calculated.

Functional Assays

INTRACELLULAR CYTOKINE EXPRESSION

Cell surface and intracellular labeling techniques have been applied to the identification of cell subsets with specific functional characteristics. For example, brief stimulation of cells such as PBMC with protein or peptide antigens can result in the expression of activation markers and cytokines that can then be measured along with other phenotypic markers on the surface of the responding cells. The use of a secretion inhibitor such as brefeldin A or monensin allows the intracellular accumulation of cytokines. The cells are then fixed, permeabilized, and detected by a flow cytometric method. Such assays are useful for monitoring T cell subpopulations that respond to vaccines, infectious disease agents, or cancer. Functional properties of other cell types, including monocytes, DCs, and NK cells, can also be monitored using functional assays with appropriate stimuli.

KINASES

Phosphorylation-specific cell activation intermediates can be identified using phospho-specific antibodies and flow cytometry. These reagents are useful for mapping intracellular signaling mechanisms, often in the context of other cell-surface phenotypic markers. Thus, multicolor flow cytometry can provide single-cell assessment of intracellular activation states in complex cell populations. These assays may have utility in detecting altered signaling states in cancer cells or in directing appropriate therapies based on the signaling properties of a patient's tumor cells.

APOPTOSIS

Apoptosis, commonly described as programmed cell death, is the process of cell death caused by regulated, physiologic processes. The apoptotic process manifests itself as a series of morphological, biochemical, and molecular changes to the cell and can be initiated by external or internal stimuli. A central event during apoptosis is the activation of caspases, a family of proteolytic enzymes. Caspases are synthesized as inactive proenzymes and are activated by other caspases or by similar molecules. They form a cascade that can lead to the cleavage of various cytoplasmic or nuclear proteins. One of the caspases that is reported to be crucial for the apoptotic process is caspase-3, which is activated during the early stages of apoptosis.

Flow cytometric methods for detecting apoptotic cells include measuring morphology, changes in membrane structure, DNA cleavage by endonucleases, and mitochondrial membrane potential. Natural or artificial caspase substrates or antibodies against the activated form of the enzyme have also been used for this purpose.

CELL VIABILITY

Flow cytometry is often used to discriminate live cells from dead cells. The principle of nucleic acid dye exclusion is the basis of this application. A nucleic acid dye such as PI or 7-AAD is added to cells in suspension. During flow cytometric analysis, cells that fluoresce above background are considered nonviable because they cannot exclude the dye, which fluoresces when it binds to cellular DNA.

Flow Cytometry Immunoglobulin Assays

FLOW CYTOMETRY CROSS-MATCHING

Before organ transplantation, flow cytometry cross-matching (FCXM) is performed on recipients to screen for anti-HLA antibodies that can cause rejection. Anti-HLA antibodies are detected by incubating HLA-defined leukocytes, B-cell lines, or HLA antigen-coated beads with the serum sample, followed by anti-human immunoglobulin fluorescently labeled antibodies. Leukocytes are immunostained to identify T and B cells in order to distinguish between anti-HLA class I and II activity, respectively. In addition, screening of blood donations for anti-HLA antibodies is also increasingly employed to identify donors whose blood products may have increased risk of causing transfusion-related acute lung injury (TRALI) in recipients.

ANTI-HUMAN NEUTROPHIL ANTIBODIES

Anti-human neutrophil antibodies (HNA) can cause neutropenia and have been implicated in TRALI. Autoimmune neutropenias may develop in patients who have autoimmune disorders such as Felty syndrome, systemic lupus erythematosus, and Hashimoto thyroiditis. The absence of anti-HNA antibodies narrows the differential diagnosis to nonimmune causes such as bone marrow failure, myelodysplasia, or marrow-infiltrative processes. Flow cytometry can detect anti-neutrophil antibodies and can confirm the origin of neutropenia or TRALI.

ANTI-HUMAN PLATELET ANTIBODIES

Anti-human platelet antibodies (HPA) are detected by both indirect and direct flow cytometry-based platelet-associated immunoglobulin assays. In autoimmune thrombocytopenic purpura, free serum antibodies are not found as frequently as are platelet-bound antibodies. In cases of alloantibody formation, serum antibodies may be detected without evidence of platelet-associated antibodies.

FLOW CYTOMETRY ASSAY TROUBLESHOOTING

When developing a flow cytometry method, first determine the ultimate purpose of the assay. For assays intended for research, the cell samples, reagents, and protocols may be difficult to standardize. Assays intended for patient diagnosis or to qualify a cellular product for release before administration to a patient demand more stringent assay and sample standardization. Regulatory guidelines, the type and stage of clinical investigation, and the ultimate purpose of the assay determine the level of assay rigor required.

Flow cytometry assay development should include the establishment and qualification of staining, handling, instrument, and analysis parameters and limits. Assuming that the method has been well developed, the operators are properly trained, the instrument has been properly set up, appropriate assay and instrument controls have been applied, and, if necessary, the instrument and method validations have been performed, operators may encounter and address instances when troubleshooting is necessary.

The most common flow cytometry challenges are high fluorescence or side scatter background, abnormal event rates, high fluorescence intensity, and low fluorescence signal. Approaches to alleviating these issues are described below.

High Particulate Background

Excessive cell handling (e.g., vortexing), improper fixation, and bacterial contamination of the cells can all increase the particulate background. In addition, if the instrument's forward threshold is set too low, cell debris will be detected as events. Gentle cell handling, fresh reagents, and appropriate instrument settings help ensure consistent side scatter profiles.

High Fluorescence Background

High fluorescence intensity can be attributed to excessive antibody concentration, inadequate cell washing, or inadequate Fc receptor blocking. In addition, improperly high instrument PMT gain can also result in a high background. Consistent antibody concentration and cell density, adequate washing and blocking, and appropriate instrument settings will help avoid abnormally high fluorescence background.

High Event Rate

Abnormally high event rates are often attributed to high cell densities during antibody staining or in the final cell sample. Inadequate mixing and settling of the cell sample can result in high cell event rates, as can improper or inconsistent gating.

Low Event Rate

Cell clumping, low final sample cell densities, blockages in the instrument fluidics, or improper gating can often result in abnormally low event detection. Proper cleaning, maintenance, and setup of the instrument, as well as consistent staining protocols, can help achieve consistent results with sufficient sensitivity.

High Fluorescence Intensity

As in the case for high fluorescence background, high mean cell fluorescence can result from too much labeled antibody, inadequate or inconsistent cell washing, or inadequate blocking. Including detergent in the wash buffer, especially during intracellular staining, can help prevent non-specific antibody binding.

Weak Fluorescence Intensity

Many factors can result in weak fluorescence intensity. Instrument parameters such as poor laser alignment, improper compensation, improper setup, inconsistent gain settings, and weak laser output can all negatively affect fluorescence intensity. In addition, cell physiology or reagent preparation issues, such as insufficient antibody concentration, labile or secreted target antigen, poor-quality or improperly stored reagents (resulting in fluorochrome fading), or inaccessible target antigen, can all result in a weak signal. Adequate assay development, proper instrument maintenance, and adherence to qualified protocols can all improve the fluorescence signal intensity.

Flow cytometry enables investigators to analyze cells for many different applications. Types of immunophenotypic and functional assays are increasing in number and in scope. The presence of proteins and cellular processes and detection of rare or abnormal cell populations can be studied. The reader is referred to the technical literature for application and method details.

REFERENCES

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<1031> THE BIOCOMPATIBILITY OF MATERIALS USED IN DRUG CONTAINERS, MEDICAL DEVICES, AND IMPLANTS

This chapter provides guidance on the identification and performance of procedures for evaluating the biocompatibility of drug containers, elastomeric closures, medical devices, and implants. Biocompatibility refers to the tendency of these products to remain biologically inert throughout the duration of their contact with the body. The biocompatibility testing procedures referenced in this chapter are designed to detect the nonspecific, biologically reactive, physical or chemical characteristics of medical products or the materials used in their construction. In combination with chemical assays, these biological procedures can be used to detect and identify the inherent or acquired toxicity of medical products prior to or during their manufacturing and processing.

Preclinical testing procedures to evaluate the safety of the elastomers, plastics, or other polymers used in the construction of medical products are referenced or described in the following general chapters: *Injections* <1>, *Biological Reactivity Tests, In Vitro* <87>, *Biological Reactivity Tests, In Vivo* <88>, *Transfusion and Infusion Assemblies and Similar Medical Devices* <161>, *Elastomeric Closures for Injections* <381>, and *Containers—Plastics* <661>. Specific in vitro and in vivo testing procedures to evaluate the biocompatibility of medical products in patients are described under *Biological Reactivity Tests, In Vitro* <87> and under *Biological Reactivity Tests, In Vivo* <88>.

The procedures used to evaluate the biocompatibility of a medical product or its construction materials have been categorized as a panel of biological effects (toxicity procedures): cytotoxicity, sensitization, irritation or intracutaneous reactivity, acute systemic toxicity, subchronic toxicity (subacute toxicity), genotoxicity, implantation, hemocompatibility, chronic toxicity (extending beyond 10% of the life span of the test animal or beyond 90 days), carcinogenicity, reproductive or developmental toxicity, and biodegradation.¹ The USP general chapters referring to the toxicity procedures for these categories are indicated in *Table 1*. In addition, pyrogenicity, an area of special toxicity, is evaluated under *Pyrogen Test* <151> and under *Bacterial Endotoxins Test* <85>. There are currently no general chapters that detail sensitization, subchronic toxicity, genotoxicity, chronic toxicity, carcinogenicity, hemotoxicity, reproductive toxicity, or biodegradation testing requirements.

¹ ISO document 10993-1:1997 entitled *Biological Evaluation of Medical Devices—Part 1: Evaluation and Testing*.

Table 1. Toxicity Procedures in the USP General Chapters

Biological Effect	USP General Chapter
Cytotoxicity	Biological Reactivity Tests, In Vitro (87)*
Sensitization	[to come]
Irritation or intracutaneous reactivity	Biological Reactivity Tests, In Vivo (88)†
Systemic toxicity (acute toxicity)	Biological Reactivity Tests, In Vivo (88)
Subchronic toxicity (subacute toxicity)	[to come]
Genotoxicity	[to come]
Implantation	Biological Reactivity Tests, In Vivo (88)
Hemocompatibility	Under development in the USP monograph Sterile Single-Use Plastic Large-Volume Containers for Human Blood and Blood Components
Chronic toxicity	[to come]
Carcinogenicity	[to come]
Reproductive or developmental toxicity	[to come]
Biodegradation	[to come]

* Additional general chapters referring to this biological effect include *Transfusion and Infusion Assemblies and Similar Medical Devices* (161), *Elastomeric Closures for Injections* (381), and *Containers—Plastics* (661).
† Additional general chapters referring to this biological effect include *Injections* (1), *Transfusion and Infusion Assemblies and Similar Medical Devices* (161), *Elastomeric Closures for Injections* (381), and *Containers—Plastics* (661).

DRUG CONTAINERS

Biocompatibility of Plastic and Other Polymeric Drug Containers

Pharmaceutical containers consist of a container and a closure. Plastic containers may consist of polymers that upon extraction do not alter the stability of the contained product or do not exhibit toxicity. The biocompatibility testing requirements for drug containers are stated under *Injections* (1) and *Containers—Plastics* (661). As directed in these chapters, the plastic or other polymeric portions of these products are tested according to the procedures set forth under *Biological Reactivity Tests, In Vitro* (87). A plastic or other polymer that does not meet the requirements of *Biological Reactivity Tests, In Vitro* (87) is not a suitable material for a drug container. Materials that meet the in vitro requirements qualify as biocompatible materials without the need for further testing and may be used in the construction of a drug container. If a class designation (classes I–VI) for plastics or other polymers is desired, the appropriate testing procedures are performed as discussed in the section *In Vivo Testing and Class Designation*.

Elastomeric Closures

Elastomeric closures are closures that can be pierced by a syringe and maintain their integrity because of their elastic properties. Elastomeric materials may be composed of several chemical entities including fillers, pigments, plasticizers, stabilizers, accelerators, vulcanizing agents, and a natural or a synthetic polymer. These materials are used for manufacturing a product with the desired elastomeric physical properties, and they frequently demonstrate biological reactivity—cellular degeneration and malformation—when tested with in vitro cell cultures.

The biocompatibility of an elastomeric material is evaluated according to the two-stage testing protocol specified in the *Biological Test Procedures* under *Elastomeric Closures for Injections* (381). Unlike plastics or other polymers, an elastomeric material that does not meet the requirements of the first-stage (in vitro) testing may qualify as a biocompatible material by passing the second-stage (in vivo) testing, which consists of the *Systemic Injection Test* and the *Intracutaneous Test* described under *Biological Reactivity Tests, In Vivo* (88). No class or type distinction is made between elastomeric materials that meet the requirements of the first stage of testing and those that qualify as biocompatible materials by

meeting the second-stage requirements. Elastomeric materials are not assigned class I–VI designation.

MEDICAL DEVICES AND IMPLANTS

Medical devices and implants, labeled nonpyrogenic, in direct or indirect contact with the cardiovascular system or other soft body tissues, meet the requirements described under *Transfusion and Infusion Assemblies and Similar Medical Devices* (161). The products listed in this chapter that meet the criteria are solution administration sets, extension sets, transfer sets, blood administration sets, intravenous catheters, dialyzers and dialysis tubing and accessories, transfusion and infusion assemblies, and intramuscular drug delivery catheters. The outlined criteria do not apply to medical products such as orthopedic products, latex gloves, and wound dressings.

The testing requirements described or referenced under *Transfusion and Infusion Assemblies and Similar Medical Devices* (161) include *Sterility*, *Bacterial endotoxins*, *Pyrogen*, and *Other requirements*. A procedure to evaluate the presence of bacterial endotoxins is set forth under *Bacterial Endotoxins Test* (85), and the limits are set in *Bacterial Endotoxins* under *Transfusion and Infusion Assemblies and Similar Medical Devices* (161). For devices that cannot be tested by the *Bacterial Endotoxins Test* (85) because of nonremovable inhibition or enhancement, the *Pyrogen Test* (151) is applied. The procedures for evaluating medical devices purported to contain sterile pathways are set forth in *Sterile Devices* under *Sterility Tests* (71). A procedure for evaluating the safety of medical devices is set forth in the *Safety Test* under *Biological Reactivity Tests, In Vivo* (88).

The plastic or other polymer components of medical devices meet the requirements specified for plastics and other polymers under *Containers—Plastics* (661); those made of elastomers meet the requirements under *Elastomeric Closures for Injections* (381). As directed in these chapters, the biocompatibility of the plastic, other polymeric, and elastomeric portions of these products are tested according to the procedures described under *Biological Reactivity Tests, In Vitro* (87). If a class designation for a plastic or other polymer is also required, the appropriate testing procedures described under *Biological Reactivity Tests, In Vivo* (88) are performed.

As required for elastomeric closures, elastomeric materials that do not meet the in vitro requirements may qualify as biocompatible materials and may be used in the construction of medical devices if they meet the requirements of the *Systemic Injection Test* and the *Intracutaneous Test* under *Biological Reactivity Tests, In Vivo* (88). As required for drug containers, plastics and other polymers that do not meet the in

vitro testing requirements are not suitable materials for use in medical devices.

IN VITRO TESTING, IN VIVO TESTING, AND CLASS DESIGNATION FOR PLASTICS AND OTHER POLYMERS

The testing requirements specified under *Biological Reactivity Tests, In Vitro* (87) and *Biological Reactivity Tests, In Vivo* (88) are designed to determine the biological reactivity of mammalian cell cultures and the biological response of animals to elastomeric, plastic, and other polymer materials with direct or indirect patient contact. The biological reactivity of these materials may depend on both their surface characteristics and their extractable chemical components. The testing procedures set forth in these chapters can often be performed with the material or an extract of the material under test, unless otherwise specified.

Preparation of Extracts

Evaluation of the biocompatibility of a whole medical product is often not realistic; thus, the use of representative portions or extracts of selected materials may be the only practical alternative for performing the assays. When representative portions of the materials or extracts of the materials under test are used, it is important to consider that raw materials may undergo chemical changes during the manufacturing, processing, and sterilization of a medical product. Although in vitro testing of raw materials can serve as an important screening procedure, a final evaluation of the biocompatibility of a medical product is performed with portions of the finished and sterilized product.

The preparation of extracts is performed according to the procedures set forth under *Biological Reactivity Tests, In Vitro* (87) and under *Biological Reactivity Tests, In Vivo* (88). Extractions may be performed at various temperatures (121°, 70°, 50°, or 37°), for various time intervals (1 hour, 24 hours, or 72 hours), and in different extraction media. The choice of extraction medium for the procedures under *Biological Reactivity Tests, In Vitro* (87) includes *Sodium Chloride Injection* (0.9% NaCl) or tissue culture medium with or without serum. When medium with serum is used, the extraction temperature cannot exceed 37°. In vivo extraction medium includes the choices described under *Biological Reactivity Tests, In Vivo* (88) or the solvent to which the drug or medical device is exposed.

When choosing extraction conditions, select the temperature, solvent, and time variables that best mimic the "in use" conditions of the product. The performance of multiple tests at various conditions can be used to simulate variations in the "in use" conditions. Although careful selection of extraction conditions allows the simulation of manufacturing and processing conditions in the testing of raw materials, an evaluation of the biocompatibility of the product is performed with the finished and sterilized product.

In Vitro Testing

The procedures described under *Biological Reactivity Tests, In Vitro* (87) include an *Agar Diffusion Test* (indirect contact test), a *Direct Contact Test*, and an *Elution Test* (extraction test). The sample is biocompatible if the cell cultures do not exhibit greater than a mild reactivity (Grade 2) to the material under test, as described under *Biological Reactivity Tests, In Vitro* (87). The *Agar Diffusion Test* is designed to evaluate the biocompatibility of elastomeric materials. The material is placed on the agar overlay of the cell monolayer, which cushions the cells from physical damage by the material and allows leachable chemicals or materials to diffuse from the elastomer and contact the cell monolayer. Extracts of elastomeric materials are tested by placing the filter paper satu-

rated with an extract of the elastomer on the solidified surface of the agar. The *Direct Contact Test* is designed for elastomeric or plastic materials that will not physically damage cells with which they are in direct contact. Any leachable chemicals diffuse from the material into the serum-supplemented growth medium and directly contact the cell monolayer. The *Elution Test* is designed to evaluate the extracts of polymeric materials. The material may be applied directly to the tissue culture media.

The performance of either the *Agar Diffusion Test* or the *Direct Contact Test* in combination with the *Elution Test* is the preferred testing protocol. Extraction of the product or materials for the *Agar Diffusion Test* or the *Elution Test* is performed as described in the *Preparation of Extracts*.

In Vivo Testing and Class Designation

According to the injection and implantation requirements specified in *Table 1* under *Biological Reactivity Tests, In Vivo* (88), plastics and other polymers are assigned a class designation between class I and class VI. To obtain a plastic or other polymer class designation, extracts of the test material are produced according to the specified procedures in various media. To evaluate biocompatibility, the extracts are injected systemically and intracutaneously into mice and rabbits. According to the specified injection requirements, a plastic or other polymer may initially be graded as class I, II, III, or V. If in addition to injection testing, implantation testing using the material itself is performed, the plastic or other polymer may be classified as class IV or class VI.

BIOCOMPATIBILITY OF MEDICAL DEVICES AND IMPLANTS

In addition to evaluating medical products for compendial purposes according to the procedures specified under *Injections* (1), *Sterility* (71), *Biological Reactivity Tests, In Vitro* (87), *Biological Reactivity Tests, In Vivo* (88), *Transfusion and Infusion Assemblies and Similar Medical Devices* (161), *Elastomeric Closures for Injections* (381), and *Containers—Plastics* (661), medical devices and implants are evaluated for sensitization, subchronic toxicity, genotoxicity, hemocompatibility, chronic toxicity, carcinogenicity, reproductive or developmental toxicity, and biodegradation as required by the regulatory agencies.

The guidance provided by the regulatory agencies indicates that the extent of testing that is performed for a medical device or an implant depends on the following factors: (1) the similarity and uniqueness of the product relative to previously marketed ("predicate") products as considered in the *Decision Flowchart*; (2) the extent and duration of the contact between the product and the patient as described in the *Categorization of Medical Devices*; and (3) the material composition of the product as considered in the sections *Decision Flowchart* and *In Vivo Testing and Class Designation*.

Decision Flowchart

Guidance on comparing a medical device or an implant to previously marketed products is provided by the Biocompatibility Decision Flowchart (see *Figure 1.2*) as adapted from the FDA's Blue Book Memorandum #G95-1. The purpose of the flowchart is to determine whether the available data from previously marketed devices are sufficient to ensure the safety of the device under consideration. As indicated by the flowchart, the material composition and the manufacturing techniques of a product are compared to those of the previ-

²Adapted from the FDA Blue Book Memorandum #G95-1 ("Use of International Standard ISO-10993: Biological Evaluation of Medical Devices-Part 1: Evaluation and Testing.")

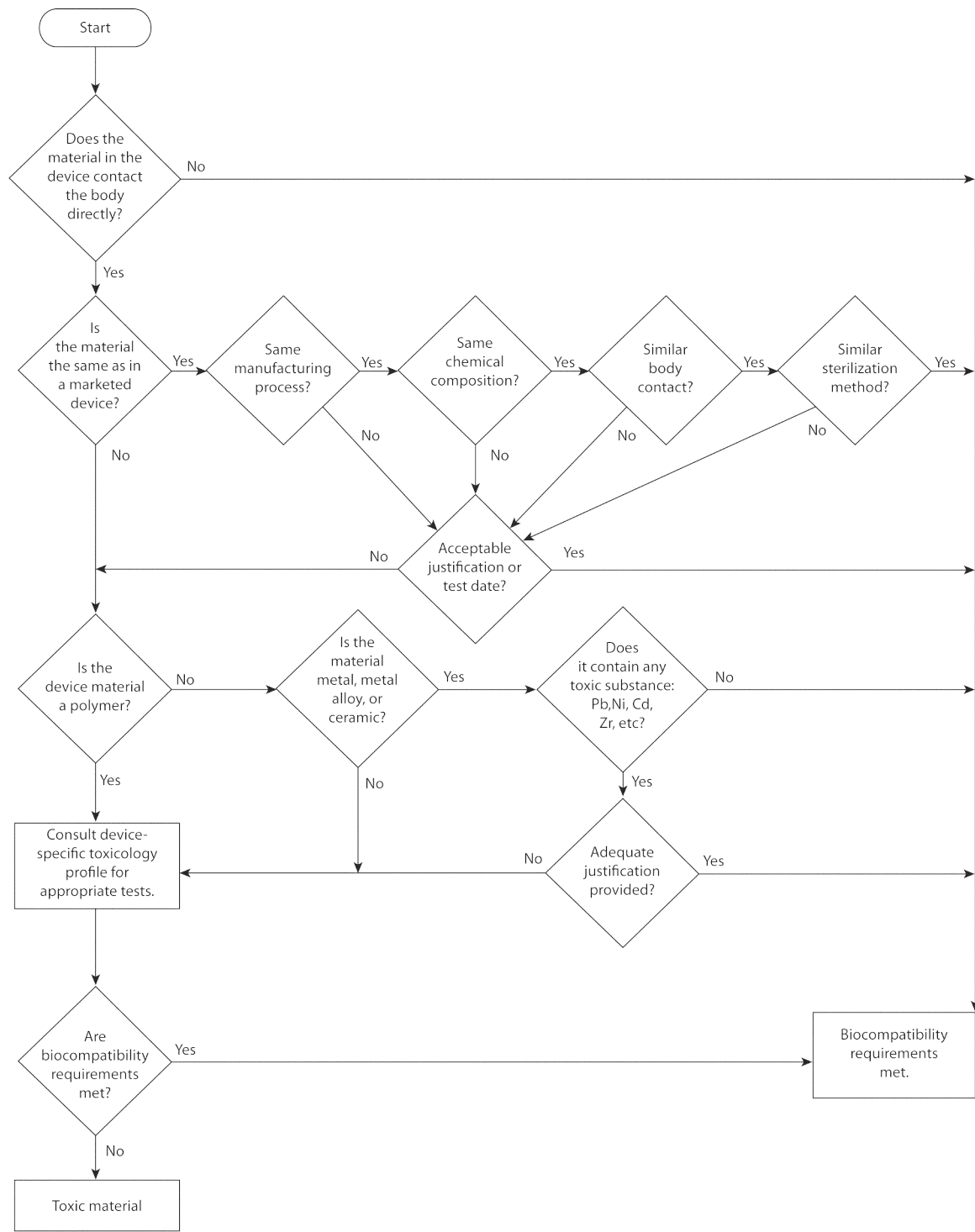


Figure 1. Biocompatibility flowchart.

ously marketed products for the devices that come in direct contact with the body. In addition, the flowchart requires an evaluation of the toxicity of any unique material that has not been used in predicate devices. Responses to the questions posed in the flowchart lead to the conclusion that either the available data are sufficient or additional testing is required to ensure the safety of the product. When additional testing is required, guidance on the identification of appropriate testing procedures is provided in the section *Test Selection Matrix*.

Categorization of Medical Devices

To facilitate the identification of appropriate testing procedures, medical devices are divided and subdivided, as shown in *Table 2*, according to the nature and extent of their contact with the body. Major categories of medical devices are surface devices, external communicating devices, and implant devices. These are then further subcategorized.

Some examples of medical devices and implants belonging to each of the subcategories are also presented in *Table 2*.

Test Selection Matrix

The matrix provides guidance on the identification of appropriate biological testing procedures for the three categories of medical devices: tests for *Surface Devices* (see *Table 3*), tests for *External Communicating Devices* (see *Table 4*), and tests for *Implant Devices* (see *Table 5*). Each category of devices is subcategorized and then even further subdivided according to the duration of the contact between the device and the body. The duration of contact is defined as (A) limited (less than 24 hours); (B) prolonged (24 hours to 30 days); or (C) permanent (more than 30 days). The biological effects that are included in the matrix are cytotoxicity, sensitization, irritation or intracutaneous reactivity, systemic toxicity, subchronic toxicity, genotoxicity, implantation, hemocompatibility, chronic toxicity, carcinogenicity, reproductive or developmental toxicity, and biodegradation. The general chapters that contain toxicity testing procedure for these biological effects are indicated in *Table 1*.

Each subcategory in the matrix has an associated panel of testing requirements. Generally, the number of tests in the panel increases as the duration of the contact between the device and the body is extended and as the device or implant comes in closer contact with the circulatory system. Within several subcategories, the option of performing additional tests beyond those required should be considered on a case-by-case basis. Specific situations such as use of per-

manent implant devices or external communicating devices for pregnant women have to be taken into consideration in the manufacturer's decision to include reproductive or developmental testing. Guidance on the identification of possible additional testing procedures is provided in the matrix for each subcategory of medical devices.

GUIDANCE IN SELECTING THE PLASTIC OR OTHER POLYMER CLASS DESIGNATION FOR A MEDICAL DEVICE

To provide guidance on selecting the appropriate plastic or other polymer class designation for a medical device, each subcategory of *Surface Devices* (see *Figure 2*) and *External Communicating Devices* (see *Figure 3*) is assigned a USP Plastic Class designation (see *Biological Reactivity Tests, In Vivo* (88)). If the tests for each USP class designation are not sufficient for a specific device, the manufacturer or the practitioner must develop an appropriate set of tests. The indicated numerical class number increases relative to the duration (risk) of contact between the device and the body. In the category of *Implant Devices*, the exclusive use of class VI is mandatory. The assignment of USP Plastic Class designation is based on the test selection matrices illustrated in *Tables 3, 4, and 5*.

The assignment of a plastic or other polymer class designation to a subcategory is not intended to restrict the use of higher classes of plastics or other polymers. Although the assigned class defines the lowest numerical class of plastic or

Table 2. Classification and Examples of Medical Devices

Device Category	Device Subcategory	Nature or Extent of Contact	Some Examples
Surface Devices	Skin	Devices that contact intact skin surfaces only	Electrodes, external prostheses, fixation tapes, compression bandages, and monitors of various types
	Mucosal Membrane	Devices communicating with intact mucosal membranes	Contact lenses, urinary catheters, intravaginal and intrainestinal devices (stomach tubes, sigmoidoscopes, colonoscopes, gas troscopes), endotracheal tubes, bronchoscopes, dental prostheses, orthodontic devices, and intrauterine devices
	Breached or Compromised Surfaces	Devices that contact breached or otherwise compromised body surfaces	Ulcer, burn, and granulation tissue dressings or healing devices and occlusive patches
External Communicating Devices	Blood Path, Indirect	Devices that contact the blood path at one point and serve as a conduit for entry into the vascular system	Solution administration sets, extension sets, transfer sets, and blood administration sets
	Tissue, Bone, or Dentin Communicating	Devices and materials communicating with tissue, bone, or pulp and dentin system	Laparoscopes, arthroscopes, draining systems, dental cements, dental filling materials, and skin staples
	Circulating blood	Devices that contact circulating blood	Intravascular catheters, temporary pacemaker electrodes, oxygenators, extracorporeal oxygenator tubing and accessories, dialyzers, dialysis tubing and accessories, hemoadsorbents, and immunoabsorbents
Implant Devices	Tissue or Bone	Devices principally contacting bone or principally contacting tissue and tissue fluid	Examples of the former are orthopedic pins, plates, replacement joints, bone prostheses, cements, and intraosseous devices; examples of the latter are pacemakers, drug supply devices, neuromuscular sensors and simulators, replacement tendons, breast implants, artificial larynxes, subperiosteal implants, and ligation clips
	Blood	Devices principally contacting blood	Pacemaker electrodes, artificial arteriovenous fistulae, heart valves, vascular grafts, internal drug delivery catheters, and ventricular-assist devices

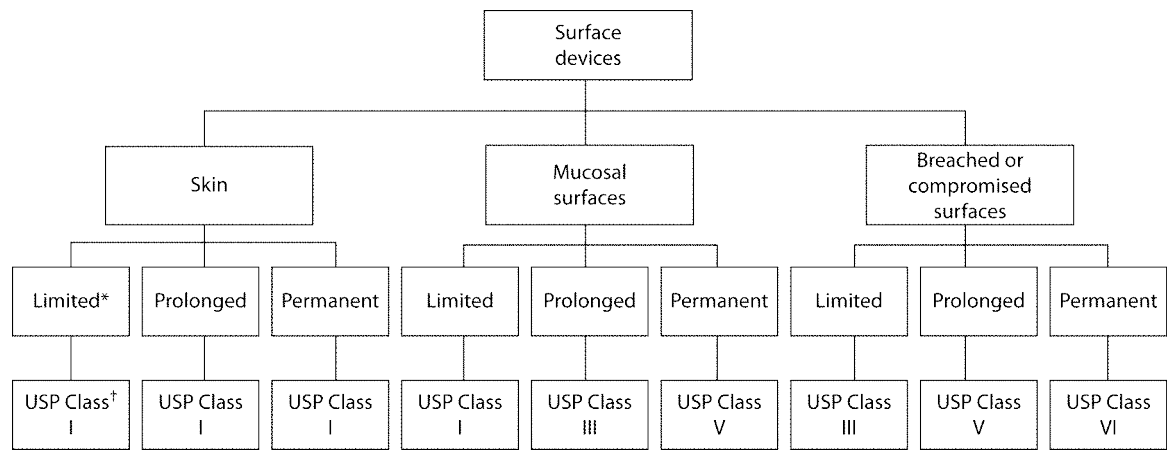


Figure 2. USP plastic and other polymer class requirements for surface devices.

* Categorization based on duration of contact: limited—less than 24 hours; prolonged—24 hours to 30 days; permanent—more than 30 days.
† USP Plastic Class designation.

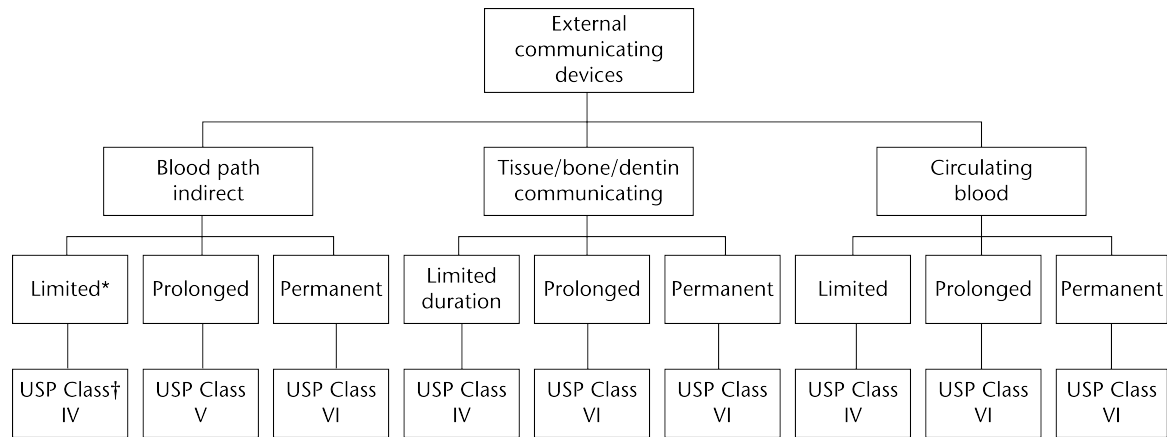


Figure 3. USP plastic and other polymer class requirements for external communicating devices.

* Categorization based on duration of contact: limited—less than 24 hours; prolonged—24 hours to 30 days; permanent—more than 30 days.
† USP Plastic Class designation.

other polymer that may be used in the corresponding device, the use of a numerically higher class of plastic is optional. When a device can be defined as belonging to more than one device category, the plastic or other polymer

should meet the requirements of the highest numerical class.

Table 3. Test Selection Matrix for Surface Devices*

Device Categories			Biological Effect ^b												
		Contact Duration ^a	Cytotoxicity	Sensitization	Irritation or Intracutaneous Reactivity	Systemic Toxicity (Acute)	Sub-chronic Toxicity (Subacute)	Genotoxicity	Implantation	Hemocompatibility	Chronic Toxicity	Carcinogenicity	Reproductive or Development Toxicity	Biodegradation	
Surface Devices	Body Contact	A	X	X	X	—	—	—	—	—	—	—	—	—	
		B	X	X	X	—	—	—	—	—	—	—	—	—	
		C	X	X	X	—	—	—	—	—	—	—	—	—	
	Skin	A	X	X	X	—	—	—	—	—	—	—	—	—	—
		B	X	X	X	O	O	—	—	—	—	—	—	—	—
		C	X	X	X	O	X	X	—	O	—	O	—	—	—
	Mucosal Membrane	A	X	X	X	—	—	—	—	—	—	—	—	—	—
		B	X	X	X	O	O	—	—	—	—	—	—	—	—
		C	X	X	X	O	X	X	—	O	—	O	—	—	—
	Breached or Compromised Surfaces	A	X	X	X	—	—	—	—	—	—	—	—	—	—
		B	X	X	X	O	O	O	—	O	—	—	—	—	—
		C	X	X	X	O	X	X	X	O	—	O	—	—	—

^a Legend A—limited (less than 24 hours); B—prolonged (24 hours to 30 days); C—permanent (more than 30 days).^b Legend X—ISO evaluation tests for consideration; O—additional tests that may be applicable.

* Adapted from the FDA's Blue Book Memorandum #G95-1 (Table 1. Initial Evaluation Tests for Consideration and Table 2. Supplementary Evaluation Tests for Consideration).

Table 4. Test Selection Matrix for External Communicating Devices*

Device Categories		Biological Effect ^b											
Body Contact	Con- tact Dura- tion ^a	Cyto- tox- icity	Sensi- tiza- tion	Irritation or Intracuta- neous Reac- tivity	Sys- temic Toxic- ity (Acute)	Sub- chronic Toxic- ity (Sub- acute)	Geno- toxic- ity	Im- plan- tation	Hemo- com- pat- abili- ty	Chronic Toxic- ity	Cardi- no- genic- ity	Re- pro- ductive or De- velop- ment Toxic- ity	Bio- degra- dation
	A	X	X	X	X	X	—	—	X	—	—	—	—
External Communicating Devices	B	X	X	X	X	O	—	—	X	—	—	—	—
	C	X	X	O	X	X	X	O	X	X	X	—	—
	A	X	X	X	O	—	—	—	—	—	—	—	—
	B	X	X	O	O	O	X	X	—	—	—	—	—
	C	X	X	O	O	O	X	X	—	X	X	—	—
	A	X	X	X	X	X	—	—	X	—	—	—	—
	B	X	X	X	X	X	O	O	X	—	—	—	—
	C	X	X	X	X	X	X	X	X	X	X	—	—
	Circulating Blood	X	X	X	X	X	X	X	X	X	X	X	—

^a Legend A—limited (less than 24 hours); B—prolonged (24 hours to 30 days); C—permanent (more than 30 days).^b Legend X—ISO evaluation tests for consideration; O—additional tests that may be applicable.

* Adapted from the FDA's Blue Book Memorandum #G95-1 (Table 1. Initial Evaluation Tests for Consideration and Table 2. Supplementary Evaluation Tests for Consideration).

Table 5. Test Selection Matrix for Implant Devices*

Device Categories		Biological Effect ^b													
Body Contact	Implant Devices	Contact Duration ^a	Cytotoxicity	Sensitization	Irritation or Intracutaneous Reactivity	Systemic Toxicity (Acute)	Subchronic Toxicity (Subacute)	Genotoxicity	Implantation	Hemocompatibility	Chronic Toxicity	Cardiogenicity	Reproductive or Development Toxicity	Biodegradation	
	Tissue or Bone	A	X	X	X	X	O	—	—	—	—	—	—	—	—
		B	X	X	O	O	O	X	X	X	—	—	—	—	—
		C	X	X	O	O	O	X	X	X	—	X	X	—	—
Blood	A	X	X	X	X	X	—	—	X	X	—	—	—	—	
	B	X	X	X	X	X	O	X	X	X	—	—	—	—	
	C	X	X	X	X	X	X	X	X	X	X	X	—	—	

^a Legend A—limited (less than 24 hours); B—prolonged (24 hours to 30 days); C—permanent (more than 30 days).^b Legend X—ISO evaluation tests for consideration; O—additional tests that may be applicable.

* Adapted from the FDA's Blue Book Memorandum #G95-1 (Table 1. Initial Evaluation Tests for Consideration and Table 2. Supplementary Evaluation Tests for Consideration).

(1032) DESIGN AND DEVELOPMENT OF BIOLOGICAL ASSAYS

1. INTRODUCTION

1.1 Purpose and Scope

General chapter *Design and Development of Biological Assays* (1032) presents methodology for the development of bioassay procedures that have sound experimental design, that provide data that can be analyzed using well-founded statistical principles, and that are fit for their specific use.

General chapter (1032) is one of a group of five general chapters that focus on relative potency assays, in which the activity of a Test material is quantified by comparison to the activity of a Standard material. However, many of the principles can be applied to other assay systems.

This general chapter is intended to guide the design and development of a bioassay for a drug substance or product intended for commercial distribution. Although adoption of this chapter's recommended methods may be resource intensive during assay development, early implementation can yield benefits. Lastly, the perspectives and methods described herein are those recommended from among the many alternatives which contemporary bioassay theory and practice offers.

Focus on Relative Potency—Because of the inherent variability in biological test systems (including that from animals, cells, instruments, reagents, and day-to-day and between-lab), an absolute measure of potency is more variable than a measure of activity relative to a Standard. This has led to the adoption of the relative potency methodology. Assuming that the Standard and Test materials are biologically *similar*, *statistical similarity* (a consequence of the Test and Standard similarity) should be present, and the Test sample can be expected to behave like a concentration or dilution of the Standard. Relative potency is a unitless measure obtained from a comparison of the dose-response relationships of Test and Standard drug preparations. For the purpose of the relative comparison of Test to Standard, the potency of the Standard is usually assigned a value of 1 (or 100%). The Standard can be a material established as such by a national (e.g., USP) or international (e.g., WHO) organization, or it could be an internal Standard.

1.2 Audience

This chapter is intended for both the practicing bioassay analyst and the statistician who are engaged in developing a bioassay. The former will find guidance for implementing bioassay structure and methodology to achieve analytical goals while reliably demonstrating the biological activity of interest, and the latter will gain insights regarding the constraints of biology that can prove challenging to balance with a rigorous practice of statistics.

2. BIOASSAY FITNESS FOR USE

To evaluate whether an assay is fit for use, analysts must specify clearly the purpose(s) for performing the bioassay. Common uses for a bioassay include lot release of drug substance (active pharmaceutical ingredient) and drug product; assessment of stability; qualification of Standard and other critical reagents; characterization of process intermediates and formulations; characterization of contaminants and deg-

radation products; and support of changes in the product production process. The relative accuracy, specificity, precision, and robustness requirements may be different for each of these potential uses. It is a good strategy to develop and validate a bioassay to support multiple intended uses; for example, a bioassay primarily developed for batch release may serve other purposes. Decisions about fitness for use are based on scientific and statistical considerations, as well as practical considerations such as cost, turnaround time, and throughput requirements for the assay.

When assays are used for lot release, a linear-model bioassay may allow sufficient assessment of similarity. For bioassays used to support stability, comparability, to qualify reference materials or critical reagents, or in association with changes in the production or assay processes, it is generally useful to assess similarity using the entire concentration-response curve, including the asymptotes (if present).

2.1 Process Development

Bioassays are generally required in the development and optimization of product manufacturing, including formulation and scale-up processes. Bioassays can be used to evaluate purification strategies, optimize product yield, and measure product stability. Because samples taken throughout the process are often analyzed and compared, sample matrix effects that may affect assay response should be carefully studied to determine an assay's fitness for use. For relative potency measures, the Standard material may require dilution into a suitable matrix for quantitation. The bioassay's precision and accuracy should be sufficient for measuring process performance or for assessing and comparing the stability of candidate formulations.

2.2 Process Characterization

Bioassays may be performed to assess the effect on drug potency associated with different stages of drug manufacture or with changes in the manufacturing process (e.g., to demonstrate product equivalence before and after process changes are made). Bioassays used in this type of application may be qualitative or quantitative.

2.3 Product Release

Bioassays are used to evaluate the potency of the drug before commercial product release. To the extent possible, the assay should reflect or mimic the product's known or intended mechanism of action. If the bioassay does not include the functional biology directly associated with the mechanism of action, it may be necessary to demonstrate a relationship between the bioassay's estimated potency determinations and those of some other assay that better or otherwise reflects putative functional activity.

For product-release testing, product specifications are established to define a minimum or range of potency values that are acceptable for product. The precision of the reportable value from the bioassay must support the number of significant digits listed in the specification (see general chapter *Biological Assay Validation* (1033)), and, in conjunction with relative accuracy, support the specification range. In order to meet these specifications, manufacturing quality control will have sufficiently narrow product release specifications in order to accommodate any loss of activity due to instability and uncertainty in the release assay.

2.4 Process Intermediates

Bioassay assessment of process intermediates can provide information regarding specificity. Formulation and fill strategies may rely on bioassays in order to ensure that drug product, including that in final container, will meet its es-

established specifications. For example, unformulated bulk materials may be held and evaluated for potency. Bulks may be pooled with other bulk lots, diluted, or reworked based on the potency results. For these types of applications, the bioassay must be capable of measuring product activity in different matrices. In some cases, a separate Standard material is made and is used to calculate relative potency for the process intermediate.

2.5 Stability

The potency assay may be used to assess biotechnology and vaccine product stability. Information from stability studies, performed during development under actual and/or accelerated or stressed storage conditions, may be used to establish shelf life duration as well as to identify and estimate degradation products and degradation rates. Post licensure stability studies may be used to monitor product stability. Knowledge of both short-term and long-term variability of the bioassay is important to assure an acceptable level of uncertainty in potency measures obtained.

2.6 Qualification of Reagents

The quantitative characterization of a new Standard requires an accurate and precise measurement of the new Standard's biological activity. This measurement is used either to establish that the new Standard lot is equivalent to the previous lot or to assign it a label potency to which Test samples can be compared. Additional replication (beyond routine testing) may be required to achieve greater precision in the potency measurement of the new Standard material. Additionally, the bioassay may be used to qualify a cell culture reagent such as fetal bovine serum. The fitness for use in such cases is tied to the ability of the assay to screen reagent lots and to ensure that lots that may bias or compromise the relative potency measurements are not accepted.

2.7 Product Integrity

Biotechnology, biological, and vaccine products may contain a population of heterogeneous material, including the intended predominant product material. Some process impurities and degradation products may be active, partially active, inactive in, or antagonistic to, the response measured in the bioassay. For product variants or derivatives for which changes in structure or relative composition may be associated with subtle yet characteristic changes in the bioassay response (e.g., change in slope or asymptote), the bioassay may be useful in the detection and measurement of these variants or derivatives. Studies that identify characteristic changes associated with variants of the intended product help ensure consistent product performance. Whenever practical, the bioassay should be accompanied by orthogonal methods that are sensitive to product variants, process impurities, and/or degradation products.

3. BIOASSAY FUNDAMENTALS

3.1 In Vivo Bioassays

In vivo potency assays are bioassays in which sets of dilutions of the Standard and Test materials are administered to animals and the concentration-response relationships are used to estimate potency. For some animal assays, the endpoint is simple (e.g., rat body weight gain assay for human growth hormone or rat ovarian weight assay for follicle stimulating hormone), but others require further processing of samples collected from treated animals (e.g., reticulocyte count for erythropoietin, steroidogenesis for

gonadotropins, neutrophil count for granulocyte colony stimulating factor, or antibody titer after administration of vaccines). With the advent of cell lines specific for the putative physiological mechanism of action (MOA), the use of animals for the measurement of potency has substantially diminished. Cost, low throughput, ethical, and other practical issues argue against the use of animal bioassays. Regulatory agencies have encouraged the responsible limitation of animal use whenever possible (see The Interagency Coordinating Committee on the Validation of Alternative Methods, Mission, Vision, and Strategic Priorities; February 2004). When in vitro activity is not strongly associated with in vivo activity (e.g., EPO), the combination of an in vitro cell-based assay and a suitable physicochemical method (e.g., IEF, glycan analysis) may substitute for in vivo assays. However, a need for in vivo assays may remain when in vitro assays cannot detect differences that are critical in regard to a drug's intended biological function.

Animals' physiological responses to biological drugs (including vaccines) may predict patients' responses. Selection of animal test subjects by species, strain, gender, and maturity or weight range is guided by the goal of developing a representative and sensitive model with which to assess the activity of Test samples.

Some assay methods lend themselves to the use of colony versus naive animals. For example, pyrogen and insulin testing benefit from using experienced colony rabbits that provide a reliable response capacity. If animals recently introduced to the colony fail to respond as expected after several administrations of a compound, they should be culled from the colony so they do not cause future invalid or indeterminate assay results. In the case of assaying highly antigenic compounds for pyrogens, however, naive animals should be used to avoid generating inaccurate or confounded results. Other colony advantages include common controlled environmental conditions (macro/room, and micro/rack), consistent feeding schedule, provision of water, and husbandry routine.

Historical data including colony records and assay data can be used to identify factors that influence assay performance. The influence of biasing factors can be reduced by applying randomization principles such as distribution of weight ranges across dose groups, group assignments from shipping containers to different cages, or use of computer-generated or deck patterns for injection/dosing. A test animal must be healthy and have time to stabilize in its environment to be suitable for use in a bioassay. Factors that combine to influence an animal's state of health include proper nutrition, hydration, freedom from physical and psychological stressors, adequate housing sanitization, controlled light cycle (diurnal/nocturnal), experienced handling, skillful injections and bleedings, and absence of noise or vibration. Daily observation of test animals is essential for maintenance of health, and veterinary care must be available to evaluate issues that have the potential to compromise the validity of bioassay results.

3.2 Ex Vivo Bioassays

Cells or tissues from human or animal donors can be cultured in the laboratory and used to assess the activity of a Test sample. In the case of cytokines, the majority of assays use cells from the hematopoietic system or subsets of hematopoietic cells from peripheral blood such as peripheral blood mononuclear cells or peripheral blood lymphocytes. For proteins that act on solid tissues, such as growth factors and hormones, specific tissue on which they act can be removed from animals, dissociated, and cultured for a limited period either as adherent or semi-adherent cells. Although an ex vivo assay system has the advantage of similarity to the natural milieu, it may also suffer from substantial donor-to-donor variability, as well as challenging availability of appropriate cells.

Bioassays that involve live tissues or cells from an animal (e.g., rat hepatocyte glucagon method) require process management similar to that of *in vivo* assays to minimize assay variability and bias. The level of effort to manage bias (e.g., via randomization) should be appropriate for the purpose of the assay. Additional factors that may affect assay results include time of day, weight or maturity of animal, anesthetic used, buffer components/reagents, incubation bath temperature and position, and cell viability.

3.3 In Vitro (Cell-Based) Bioassays

Bioassays using cell lines that respond to specific ligands or infectious agents can be used for lot-release assays. These cell lines can be derived from tumors, immortalized as factor-dependent cell lines, or engineered cell lines transfected with appropriate receptors. Additionally, nontransformed cell lines which can be maintained over a sufficient number of passages (e.g., fibroblasts) may also be used. Regardless of cell line, there is an expectation of adequately equivalent potency response through some number of continuous passages. Advances in recombinant DNA technology and the understanding of cellular signaling mechanisms have allowed the generation of engineered cell lines with improved response, stable expression of receptors and signaling mechanisms, and longer stability. The cellular responses to the protein of interest depend on the drug's MOA and the duration of exposure. Such responses include cell proliferation, cell killing, antiviral activity, differentiation, cytokine/mediator secretion, and enzyme activation. Assays involving these responses may require incubation of the cells over several days, during which time contamination, uneven evaporation, or other location effects may arise. Comparatively rapid responses based on an intracellular signaling mechanism—such as second messengers, protein kinase activation, or reporter gene expression—have proven acceptable to regulatory authorities. Lastly, most cell lines used for bioassays express receptors for multiple cytokines and growth factors. This lack of specificity may not be detrimental if the Test sample's specificity is demonstrated.

Cell-based bioassay design should reflect knowledge of the factors that influence the response of the cells to the active analyte. Response variability is often reflected in parameters such as slope, EC_{50} of the concentration–response curve, or the response range (maximum minus minimum response). Even though relative potency methodology minimizes the effects on potency estimates of variation in these parameters among assays, and among blocks within an assay, such response variability can make an assay difficult to manage (i.e., it may be difficult to assess system suitability). Hence, while assay development should be focused primarily on the properties of potency, efforts to identify and control variation in the concentration–response relationship are also appropriate. For blocked assays (e.g., multiple cell culture plates in an assay) with appreciable variation in curve shape among blocks, an analysis that does not properly include blocks will yield inflated estimates of within-assay variation, making similarity assessment particularly difficult. Two strategies are available for addressing variation among blocks: one, a laboratory effort to identify and control sources of variation and two, a statistical effort to build and use a blocked design and analysis. Combining these strategies can be particularly effective.

The development of a cell-based bioassay begins with the selection or generation of a cell line. An important first step when developing a cell-based assay to assess a commercial product is to verify that the cell line of interest is not restricted to research use only. To ensure an adequate and consistent supply of cells for product testing, a cell bank should be generated if possible. To the extent possible, information regarding functional and genetic characteristics of the bioassay's cell line should be documented, including details of the cell line's history from origin to banking. For example, for a recombinant cell line this might include the

identification of the source of the parental cell line (internal cell bank, external repository, etc.), of the DNA sequences used for transfection, and of the subsequent selection and functional testing regimen that resulted in selection of the cell line. Ideally, though not always practical, sufficient information is available to permit recreation of a similar cell line if necessary. Pertinent information may include identity (e.g., isoenzyme, phenotypic markers, genetic analysis); morphology (e.g., archived photographic images); purity (e.g., mycoplasma, bacteria, fungus and virus testing); cryopreservation; thaw and culture conditions (e.g., media components, thaw temperature and method, methods of propagation, seeding densities, harvest conditions); thaw viability (immediately after being frozen and after time in storage); growth characteristics (e.g., cell doubling times); and functional stability (e.g., ploidy).

Cell characterization and vigilance regarding aspects of assay performance that reflect on cell status are necessary to ensure the quality and longevity of cell banks for use in the QC environment. The general health and metabolic state of the cells at the time of bioassay can substantially influence the test results. After a cell line has been characterized and is ready for banking, analysts typically prepare a two-tiered bank (Master and Working). A Master Cell Bank is created as the source for the Working Cell Bank. The Working Cell Bank is derived by expansion of one or more vials of the Master Cell Bank. The size of the banks depends on the growth characteristics of the cells, the number of cells required for each assay, and how often the assay will be performed. Some cells may be sensitive to cryopreservation, thawing, and culture conditions, and the banks must be carefully prepared and characterized before being used for validation studies and for regular use in the QC laboratory.

There follow factors that may affect bioassay response and the assessment of potency, that are common to many cell-based bioassays: cell type (adherent or nonadherent); cell thawing; plating density (at thaw and during seed train maintenance) and confluence (adherent cells); culture vessels; growth, staging, and assay media; serum requirements (source, heat inactivation, gamma irradiation); incubation conditions (temperature, CO_2 , humidity, culture times from thaw); cell harvesting reagents and techniques (for adherent cells, method of dissociation); cell sorting; cell counting; determination of cell health (growth rate, viability, yield); cell passage number and passaging schedule; cell line stability (genetic, receptor, marker, gene expression level); and starvation or stimulation steps. This list is not exhaustive, and analysts with comprehensive understanding and experience with the cell line should be involved during assay development. These experienced individuals should identify factors that might influence assay outcomes and establish strategies for an appropriate level of control whenever possible.

3.4 Standard

The Standard is a critical reagent in bioassays because of the necessity to have a reliable material to which a Test preparation can be quantitatively compared. The Standard may be assigned a unitage or specific activity that represents fully (100%) potent material. Where possible, a Standard should be representative of the samples to be tested in the bioassay. Testing performed to qualify a Standard may be more rigorous than the routine testing used for lot release.

A Standard must be stored under conditions that preserve its full potency for the intended duration of its use. To this end, the Standard may be stored under conditions that are different from the normal storage of the drug substance or drug product. These could include a different temperature (e.g., -70° or -20° , instead of 2° – 8°), a different container (e.g., plastic vials instead of syringes), a different formulation (e.g., lyophilizable formulation or the addition of carrier proteins such as human serum albumin, stabilizers, etc.). The Standard material should be tested for stability at ap-

propriate intervals. System suitability criteria of the bioassay such as maximum or background response, EC_{50} slope, or potency of assay control may be used to detect change in the activity of the Standard. Accelerated stability studies can be performed to estimate degradation rates and establish recognizable characteristics of Standard instability.

At later stages in clinical development, the Standard may be prepared using the manufacturing process employed in pivotal clinical trials. If the Standard formulation is different from that used in the drug product process, it is important to demonstrate that the assay's assessment of similarity and estimate of potency is not sensitive to the differences in formulation. An initial Standard may be referred to as the *Primary Standard*. Subsequent Standards can be prepared using current manufacturing processes and can be designated *Working Standards*. Separate SOPs may be required for establishing these standards for each product. Bias in potency measurements sometimes can arise if the activity of the Standard gradually changes. Also, loss of similarity may be observed if, with time, the Standard undergoes changes in glycosylation. It is prudent to archive aliquots of each Standard lot for assessment of comparability with later Standards and for the investigation of assay drift.

4. STATISTICAL ASPECTS OF BIOASSAY FUNDAMENTALS

The statistical elements of bioassay development include the type of data, the measure of response at varying concentration, the assay design, the statistical model, pre-analysis treatment of the data, methods of data analysis, suitability testing, and outlier analysis. These form the constituents of the bioassay system that will be used to estimate the potency of a Test sample.

4.1 Data

Fundamentally, there are two bioassay data types: quantitative and quantal (categorical). Quantitative data can be either continuous (not limited to discrete observations; e.g., collected from an instrument), count (e.g., plaque-forming units), or discrete (e.g., endpoint dilution titers). Quantal data are often dichotomous; for example, life/death in an animal response model or positivity/negativity in a plate-based infectivity assay that results in destruction of a cell monolayer following administration of an infectious agent. Quantitative data can be transformed to quantal data by selecting a threshold that distinguishes a positive response from a negative response. Such a threshold can be calculated from data acquired from a negative control, as by adding (or subtracting) a measure of uncertainty (such as two or three times the standard deviation of negative control responses) to the negative control average. Analysts should be cautious about transforming quantitative data to quantal data because this results in a loss of information.

4.2 Assumptions

A key assumption for the analysis of most bioassays is that the Standard and Test samples contain the same effective analyte or population of analytes and thus may be expected to behave similarly in the bioassay. This is termed *similarity*. As will be shown in more detail in the general chapter *Analysis of Biological Assays* (1034) for specific statistical models, biological similarity implies that statistical similarity is present (for parallel-line and parallel-curve models, the Standard and Test curves are parallel; for slope-ratio models, the Standard and Test lines have a common intercept). The reverse is not true. Statistical similarity (parallel lines, parallel curves, or common intercept, as appropriate) does not ensure biological similarity. However, failure to satisfy statistical similarity may be taken as evidence against biological similarity. The existence of a Standard-Test sample pair that passes the

assessment of statistical similarity is thus a necessary but not sufficient condition for the satisfaction of the key assumption of biological similarity. Biological similarity thus remains, unavoidably, an assumption. Departures from statistical similarity that are consistent in value across replicate assays may be indicative of matrix effects or of real differences between Test and Standard materials. This is true even if the departure from statistical similarity is sufficiently small to support determination of a relative potency.

In many assays multiple compounds will yield similar concentration-response curves. It may be reasonable to use a biological assay system to describe or even compare response curves from different compounds. But it is not appropriate to report relative potency unless the Standard and Test samples contain only the same active analyte or population of analytes. Biological products typically exhibit lot-to-lot variation in the distribution of analytes (i.e., most biological products contain an intended product and, at acceptably low levels, some process contaminants that may be active in the bioassay). Assessment of similarity is then, at least partially, an assessment of whether the distribution of analytes in the Test sample is close enough to that of the distribution in the Standard sample for relative potency to be meaningful; that is, the assay is a comparison of like to like. When there is evidence (from methods other than the bioassay) that the Standard and Test samples do not contain the same active compound(s), the assumption of biological similarity is not satisfied, and it is not appropriate to report relative potency.

Other common statistical assumptions in the analysis of quantitative bioassays are constant variance of the responses around the fitted model (see section 4.3 *Variance Heterogeneity, Weighting, and Transformation* for further discussion), normally distributed residuals (a residual is the difference between an observed response and the response predicted by the model), and independence of the residuals.

Constant variance, normality, and independence are interrelated in the practice of bioassay. For bioassays with a quantitative response, a well-chosen data transformation may be used to obtain approximately constant variance and a nearly normal distribution of residuals. Once such transformation has been imposed, the remaining assumption of independence then remains to be addressed via reflection of the assay design structure in the analysis model. Independence of residuals is important for assessing system and sample suitability.

4.3 Variance Heterogeneity, Weighting, and Transformation

Simple analysis of quantitative bioassay data requires that the data be approximately normally distributed with near constant variance across the range of the data. For linear and nonlinear regression models, the variance referred to here is the residual variance from the fit of the model. Constant variance is often not observed; *variance heterogeneity* may manifest as an increase in variability with increase in response. If the variances are not equal but the data are analyzed as though they are, the estimate of relative potency may still be reasonable; however, failure to address nonconstant variance around the fitted concentration-response model results in an unreliable estimate of within-assay variance. Further, the assessment of statistical similarity may not be accurate, and standard errors and confidence intervals for all parameters (including a Fieller's Theorem-based interval for the relative potency) should not be used. Confidence intervals for relative potency that combine potency estimates from multiple assays may be erroneous if within-assay error is used for confidence interval calculation.

Constancy of variance may be assessed by means of residual plots, Box-Cox (or power law) analysis, or Levene's test. With Levene's test, rather than relying on the p value, change in the statistic obtained is useful as a basis for judging whether homogeneity is improved or worsened. Vari-

ance is best assessed on a large body of assay data. Using only the variance among replicates from the current assay is not appropriate, because there are too few data to properly determine truly representative variances specific to each concentration. Data on variance is sparse during development; it is prudent to re-assess variance during validation and to monitor it periodically during ongoing use of the assay.

Two methods used to mitigate variance heterogeneity are transformation and weighting. Lack of constant variance can be addressed with a suitable transformation. Additionally, transformation can improve the normality of residuals and the fit of some statistical models to the data. A transformation should be chosen for an assay system during development, checked during validation, used consistently in routine assay practice, and checked periodically. Bioassay data are commonly displayed with log-transformed concentration; slope-ratio assays are displayed with concentration on the original scale.

Transformation may be performed to the response data as well as to the concentration data. Common choices for a transformation of the response include log, square root (for counts), reciprocal, and, for count data with known asymptotes, logit of the percent of maximum response. Log transformations are commonly used, as they may make nearly linear a useful segment of the concentration–response relationship, and because of the ease of transforming back to the original scale for interpretation. A log–log fit may be performed on data exhibiting nonlinear behavior. Other alternatives are available; i.e., data may be transformed by the inverse of the *Power of the Mean* (POM) function. A POM coefficient of $k = 2$ corresponds to a log transformation of the data. For further discussion of relationships between log-transformed and untransformed data, see *Appendix* in the general chapter *Biological Assay Validation* (1033).

Note that transformation of the data requires re-evaluation of the model used to fit the data. From a statistical perspective there is nothing special about the original scale of measurement; any transformation that improves accordance with assumptions is acceptable. Analysts should recognize, however, that transformations, choice of statistical model, and choice of weighting scheme are interrelated. If a transformation is used, that may affect the choice of model. That is, transforming the response by a log or square root, for example, may change the shape of the response curve, and, for a linear model, may change the range of concentrations for which the responses are nearly straight and nearly parallel.

For assays with non-constant variance, a weighted analysis may be a reasonable option. Though weighting cannot address lack of residual normality, it is a valid statistical approach to placing emphasis on more precise data. Ideally, weights may be based on the inverse of the predicted within-assay (or within-block) variance of each response where the predictors of variance are independent of responses observed in a specific assay.

In practice, many bioassays have relatively large variation in log EC_{50} (compared to the variation in log relative potency) among assays (and sometimes among blocks within assay). If not addressed in the variance model, this variation in log EC_{50} induces what appears to be large variation in response near the mean log EC_{50} , often yielding too-low weights for observations near the EC_{50} .

If the assay is fairly stable (low variability in EC_{50}), an alternative is to look at variance as a function of concentration. While not ideal, an approach using concentration-dependent variances may be reasonable when the weights are estimated from a large number of assays, the variances are small, any imbalance in the number of observations across concentrations is addressed in the variance model, and there are no unusual observations (outliers). This possibility can be examined by plotting the response variance at each concentration (preferably pooled across multiple assays) against concentration and then against a function of concentration (e.g., concentration squared). Variance will be

proportional to the function of concentration where this plot approximates a straight line. The apparent slope of this line is informative, in that a horizontal line indicates no weighting is needed. If a function that yields a linear plot can be found, then the weights are taken as proportional to the reciprocal of that function. There may be no such function, particularly if the variation is higher (or lower) at both extremes of the concentration range studied.

Whether a model or historical data are used, the goal is to capture the relative variability at each concentration. It is not necessary to assume that the absolute level of variability of the current assay is identical to that of the data used to determine the weighting, but only that the ratios of variances among concentrations are consistent with the historical data or the data used to determine the variance function.

Appropriate training and experience in statistical methods are essential in determining an appropriate variance-modeling strategy. Sources of variability may be misidentified if the wrong variance model is used. For example, data may have constant variation throughout a four-parameter logistic concentration–response curve but can also have appreciable variation in the EC_{50} parameter from block to block within the assay, or from assay to assay. If the between-block or between-assay variability is not recognized, this assay can appear to have large variation in the response for concentrations near the long-term average value of the EC_{50} . A weighted model with low weights for concentrations near the EC_{50} would misrepresent a major feature of such an assay system.

4.4 Normality

Many statistical methods for the analysis of quantitative responses assume normality of the residuals. If the normality assumption is not met, the estimate of relative potency and its standard error may be reasonable, but suitability tests and a confidence interval for the relative potency estimate may be invalid. Most methods used in this chapter are reasonably robust to departures from normality, so the goal is to detect substantial nonnormality. During assay development, in order to discover substantial departure from normality, graphical tools such as a normal probability plot or a histogram (or something similar like stem-and-leaf or box plots) of the residuals from the model fit may be used. The histogram should appear unimodal and symmetric. The normal probability plot should approximate a straight line; a normal probability plot that is not straight (e.g., curved at one end, both ends, or in the middle) indicates the presence of nonnormality. A pattern about a straight line is an indication of nonnormality. Nonnormal behavior may be due to measurements that are log normal and show greater variability at higher levels of response. This may be seen as a concave pattern in the residuals in a normal plot.

Statistical tests of normality may not be useful. As per the previous discussion of statistical testing of constancy of variance, change of the value of a normality test statistic, rather than reliance on a p value, is useful for judging whether normality is improved or worsened. As for variance assessment, evaluate normality on as large a body of assay data as possible during development, re-assess during validation, and monitor periodically during ongoing use of the assay. Important departures from normality can often be mitigated with a suitable transformation. Failure to assess and mitigate important departure from normality carries the risks of disabling appropriate outlier detection and losing capacity to obtain reliable estimates of variation.

4.5 Linearity of Concentration–Response Data

Some bioassay analyses assume that the shape of the concentration–response curve is a straight line or approximates a straight line over a limited range of concentrations. In those cases, a linear-response model may be assessed to de-

termine if it is justified for the data in hand. Difference testing methods for assessing linearity face the same problems as do difference testing methods applied to parallelism—more data and better precision make it more likely to detect nonlinearity. Because instances in which lack of linearity does not affect the potency estimate are rare, analysts should routinely assess departure from linearity if they wish to use a linear-response model to estimate potency.

If an examination of a data plot clearly reveals departure from linearity, this is sufficient to support a conclusion that linearity is not present. High data variability, however, may mask departure from linearity. Thus a general approach for linearity can conform to that for similarity, developed more elaborately in section 4.7 *Suitability Testing, Implementing Equivalence Testing for Similarity (parallelism)*.

- (1) Specify a measure of departure from linearity which can either combine across samples or be sample specific. Possibilities include the nonlinearity sum of squares or quadratic coefficients.
- (2) Use one of the four approaches in *Step 2 of Implementing Equivalence Testing for Similarity (parallelism)* to determine, during development, a range of acceptable values (acceptance interval) for the measure of nonlinearity.
- (3) Determine a 90% two-sided confidence interval on the measure on nonlinearity, following the Two One-Sided Test (TOST) procedure, and compare the result to the acceptance interval as determined in (2).

Often a subset of the concentrations measured in the assay will be selected in order to establish a linear concentration–response curve. The subset may be identified graphically. The concentrations at the extreme ends of the range should be examined carefully as these often have a large impact on the slope and calculations derived from the slope. If, in the final assay, the intent is to use only concentrations in the linear range, choose a range of concentrations that will yield parallel straight lines for the relative potencies expected during routine use of the assay; otherwise, the assay will fail parallelism tests when the potency produces assay response values outside the linear range of response. When potency is outside the linear range, it may be appropriate to adjust the sample concentration based on this estimated potency and test again in order to obtain a valid potency result. The repeat assays together with the valid assays may generate a biased estimate of potency because of the selective process of repeating assays when the response is in the extremes of the concentration–response curve.

The problem is more complex in assays where there is even modest variation in the shape or location of the concentration–response curve from run to run or from block to block within an assay. In such assays it may be appropriate to choose subsets for each sample in each assay or even in each block within an assay. Note that a fixed-effects model will mask any need for different subsets in different blocks, but a mixed-effects model may reveal and accommodate different subsets in different blocks (see section 4.9 *Fixed and Random Effects in Models of Bioassay Response*).

Additional guidance about selection of data subset(s) for linear model estimation of relative potency includes the following: use at least three, and preferably four, adjacent concentrations; require that the slope of the linear segment is sufficiently steep; require that the lines fit to Standard and Test samples are straight; and require that the fit regression lines are parallel. One way to derive a steepness criterion is to compute a t-statistic on the slope difference from zero. If the slope is not significant the bioassay is likely to have poor performance; this may be observed as increased variation in the potency results. Another aspect that supports requiring adequate steepness of slope is the use of subset selection algorithms. Without a slope steepness criterion, a subset selection algorithm that seeks to identify subsets of three or more contiguous data points that are straight and parallel might select concentrations on an asymptote. Such subsets are obviously inappropriate to use for potency estimation.

How steep or how significant the steepness of the slope should be depends on the assay. This criterion should be set during assay development and possibly refined during assay validation.

4.6 Common Bioassay Models

Most bioassays consist of a series of concentrations or dilutions of both a Test sample and a Standard material. A mathematical model is fit to the concentration–response data, and a relative potency may then be calculated from the parameters of the model. Choice of model may depend on whether quantitative or qualitative data are being analyzed.

For quantitative data, models using parallel response profiles which support comparative evaluation for determining relative potency may provide statistical advantages. If such a model is used, concentrations or dilutions are usually scaled geometrically, e.g., usually in two-fold, log, or half-log increments. If a slope-ratio model is used, concentrations or dilutions can be equally spaced on concentration, rather than log concentration. Several functions may be used for fitting a parallel response model to quantitative data, including a linear function, a higher-order polynomial function, a four-parameter logistic (symmetric sigmoid) function, and a five-parameter logistic function for asymmetric sigmoids. Such functions require a sufficient number of concentrations or dilutions to fit the model. To assess lack of fit of any model it is necessary to have at least one, and preferably several, more concentrations (or dilutions) than the number of parameters that will be estimated in the model. Also, at least one, and better, two, concentrations are commonly used to support each asymptote.

A linear model is sometimes selected because of apparent efficiency and ease of processing. Because bioassay response profiles are usually nonlinear, the laboratory might perform an experiment with a wide range of concentrations in order to identify the approximately linear region of the concentration–response profile. For data that follow a four-parameter logistic model, these are the concentrations near the center of the response region, often from 20% to 80% response when the data are rescaled to the asymptotes. Caution is appropriate in using a linear model because for a variety of reasons the apparently linear region may shift. A stable linear region may be identified after sufficient experience with the assay and with the variety of samples that are expected to be tested in the assay. Data following the four-parameter logistic function may also be linearized by transformation. The lower region of the function is approximately linear when the data are log transformed (log–log fit).

Quantal data are typically fit using more complex mathematical models. A probit or logit model may be used to estimate a percentile of the response curve (usually the 50th percentile) or, more directly, the relative potency of the Test to the Standard. Spearman–Kärber analysis is a non-modeling method that may be employed for determining the 50th percentile of a quantal concentration–response curve.

4.7 Suitability Testing

System suitability and sample suitability assessment should be performed to ensure the quality of bioassay results. System suitability in bioassay, as in other analytical methods, consists of pre-specified criteria by which the validity of an assay (or, perhaps, a run containing several assays) is assessed. Analysts may assess system suitability by determining that some of the parameters of the Standard response are in their usual ranges and that some properties (e.g., residual variation) of the data are in their usual range. To achieve high assay acceptance rates, it is advisable to accept large fractions of these usual ranges (99% or more) and to assess system suitability using only a few uncorrelated Standard response parameters. The choice of system suitability parameters and their ranges may also be informed by empirical or

simulation studies that measure the influence of changes in a parameter on potency estimation.

Sample suitability in bioassay is evaluated using pre-specified criteria for the validity of the potency estimate of an individual Test sample, and usually focuses on similarity assessment. System and sample suitability criteria should be established during bioassay development and before bioassay validation. Where there is limited experience with the bioassay, these criteria may be considered provisional.

System Suitability—System suitability parameters may be selected based on the design and the statistical model. Regardless of the design and model, however, system suitability parameters should be directly related to the quality of the bioassay. These parameters are generally based on standard and control samples. In parallel-line assays, for example, low values of the Standard slope typically yield estimates of potency with low precision. Rather than reject assays with low slope, analysts may find it more effective to use additional replicate assays until the assay system can be improved to consistently yield higher-precision estimates of potency. It may be particularly relevant to monitor the range of response levels and location of asymptotes associated with controls or Standard sample to establish appropriate levels of response. A drift or a trend in some of the criteria may indicate the degradation of a critical reagent or Standard material. Statistical process control (SPC) methods should be implemented to detect trends in system suitability parameters.

Two common measures of system suitability are assessment of the adequacy of the model (goodness of fit) and of precision. With replicates in a completely randomized design, a pure error term may be separated from the assessment of lack of fit. Care should be taken in deriving a criterion for lack of fit; the use of the wrong error term may result in an artificial assessment. The lack of fit sum of squares from the model fit to the Standard may, depending on the concentrations used and the way in which the data differ from the model, be a useful measure of model adequacy. A threshold may be established, based on *sensitivity analysis* (assessment of assay sensitivity to changes in the analyte) and/or historical data, beyond which the lack of fit value indicates that the data are not suitable. Note that the Test data are not used here; adequacy of the model for the Test is part of sample suitability.

For assessment of precision, two alternatives may be considered. One approach uses the mean squared error (residual variance) from the model fit to the Standard alone. Because this approach may have few degrees of freedom for the variance estimate, it may be more useful to use a pooled mean squared error from separate model fits to Standard and Test. Once the measure is selected, use historical data and sensitivity analysis to determine a threshold for acceptance.

Sample Suitability—Sample suitability in bioassay generally consists of the assessment of similarity, which can only be done within the assay range. Relative potency may be reported only from samples that both show similarity to Standard, exhibit requisite quality of model fit, and have been diluted to yield an EC_{50} (and potency) within the range of the assay system.

Similarity—In the context of similarity assessment, classical hypothesis (*difference*) testing evaluates a null hypothesis that a measure (a nonsimilarity parameter measuring the difference between Standard and Test concentration–response curves) is zero, with an implicit alternative hypothesis that the measure is non-zero or the statistical assumptions are not satisfied. The usual (“difference test”) criterion that the p-value must be larger than a certain critical value in order to declare the sample similar to reference controls the probability that samples are falsely declared nonsimilar; this is the producer’s risk of failing good samples. The consumer’s risk (the risk that nonsimilar samples are declared similar) is controlled via the precision in the nonsimilarity

measure and amount of replication in the assay; typically these are poorly assessed, leaving consumer risk uncontrolled.

In contrast to difference testing, equivalence testing for similarity (assessing whether a 90% confidence interval for a measure of nonsimilarity is contained within specified equivalence bounds) allows only a 5% probability that samples with nonsimilarity measures outside the equivalence boundaries will be declared similar (controlling the consumer’s risk). With equivalence testing it is practical to examine and manage the producer’s risk by ensuring that there is enough replication in the assay to have good precision in estimating the nonsimilarity measure(s).

For the comparison of slopes, difference tests have traditionally been used to establish parallelism between a Test sample and the Standard sample. Using this approach the laboratory cannot conclude that the slopes are equal. The data may be too variable, or the assay design may be too weak to establish a difference. The laboratory can, however, conclude that the slopes are sufficiently similar using the equivalence testing approach.

Equivalence testing has practical advantages compared to difference testing, including that increased replication yielding improved assay precision will increase the chances that samples will pass the similarity criteria; that decreased assay replication or precision will decrease the chances that samples will pass the similarity criteria; and that sound approaches to combining data from multiple assays of the same sample to better understand whether a sample is truly similar to Standard or not are obtained.

Because of the advantages associated with the use of equivalence testing in the assessment of similarity, analysts may transition existing assays to equivalence testing or may implement equivalence testing methods when changes are made to existing assays. In this effort, it is informative to examine the risk that the assay will fail good samples. This risk depends on the precision of the assay system, the replication strategy in the assay system, and the critical values of the similarity parameters (this constitutes a *process capability analysis*). One approach to transitioning an established assay from difference testing to equivalence testing (for similarity) is to use the process capability of the assay to set critical values for similarity parameters. This approach is reasonable for an established assay because the risks (of falsely declaring samples similar and falsely declaring samples nonsimilar) are implicitly acceptable, given the assay’s history of successful use.

Similarity measures may be based on the parameters of the concentration–response curve and may include the slope for a straight parallel-line assay; intercept for a slope-ratio assay; the slope and asymptotes for a four-parameter logistic parallel-line assay; or the slope, asymptotes, and nonsymmetry parameter in a five-parameter sigmoid model. In some cases, these similarity measures have interpretable, practical meaning in the assay; certain changes in curve shape, for example, may be associated with specific changes (e.g., the presence of a specific active contaminant) in the product. When possible, discussion of these changes and their likely effects is a valuable part of setting appropriate equivalence boundaries.

Implementing Equivalence Testing for Similarity (parallelism)—As previously stated, many statistical procedures for assessing similarity are based on a null hypothesis stating that similarity is present and the alternative hypothesis of there being a state of nonsimilarity. Failure to find that similarity is statistically improbable is then taken as a conclusion of similarity. In fact, however, this failure to establish a probabilistic basis for nonsimilarity does not prove similarity. Equivalence testing provides a method for the analyst to proceed to a conclusion (if warranted by the data) of *sufficiently similar* while controlling the risk of doing so inappropriately. The following provides a sequence for this process of implementing equivalence testing.

Step 1: Choose a measure of nonsimilarity.

For the parallel-line case, this could be the difference or ratio of slopes. (The ratio of slopes can be less sensitive to the value of the slope. Framing the slope difference as a proportional change from Standard rather than in absolute slope units has an advantage because it is invariant to the units on the concentration and response axes.) For a slope-ratio assay, the measure of nonsimilarity can be the difference in y-intercepts between Test and Standard samples. Again, it can be advantageous to frame this difference as a proportion of the (possibly transformed) response range of Standard to make the measure invariant to the units of the response.

The determination of similarity could be based on the individual parameters, one at a time; for the four-parameter logistic model, similarity between Standard and Test samples can be assessed discretely for the upper asymptote, the slope, and the lower asymptote. If sigmoid curves with additional parameters are used to fit bioassay data, it is also important to consider addressing similarity between Standard and Test preparations of the additional curve parameters (e.g., asymmetry parameter of the five-parameter model). Alternatively, evaluation of similarity can be based on a single composite measure of nonparallelism, such as the *parallelism sum of squares*. This is found as the difference in residual sum of squared errors (RSSE) between the value obtained from fitting the Standard and Test curves separately and the value obtained from imposing parallelism:

$$\text{Parallelism sum of squares} = \text{RSSE}_p - \text{RSSE}_s - \text{RSSE}_t$$

where the subscripts P, S, and T denote Parallel model, Standard model, and Test model, respectively. With any composite measure, the analyst must consider the implicit relative weighting of the importance of the three (or more) curve regions and whether the weighting is appropriate for the problem at hand. For the parallelism sum of squares, for example, with nonlinear models, the weighting given to the comparison of the asymptotes depends on the amount of data in the current assay on and near the asymptotes.

Step 2: Specify a range of acceptable values, typically termed an equivalence interval or "indifference zone," for the measure of nonsimilarity.

The challenge in implementing equivalence testing is in setting appropriate equivalence bounds for the nonsimilarity measures. Ideally, information is available to link variation in similarity measures to meaningful differences in biological function (as measured by the bioassay). Information may be available from evaluation of orthogonal assays. The following four approaches can be used to determine this interval. If pharmacopeial limits have been specified for a defined measure of nonsimilarity, then the assay should satisfy those requirements.

- a. The first approach is to compile historical data that compare the Standard to itself and using these data to determine the equivalence interval as a tolerance interval for the measure of nonparallelism. The advantage of using historical data is that they give the laboratory control of the false failure rate (the rate of failing a sample that is in fact acceptable). The disadvantage is that there is no control of the false pass rate (the rate of passing a sample that may have an unacceptable difference in performance relative to the Standard). The equivalence interval specification developed in this way is based solely on assay capability. Laboratories that use this approach should take caution that an imprecise assay in need of improvement may yield

such a wide equivalence interval that no useful discrimination of nonsimilarity is possible.

- b. Approach (a) is simple to implement in routine use and can be used with assay designs that do not provide reliable estimates of within-assay variation and hence confidence intervals. However, there is a risk that assays with larger than usual amounts of within-assay variation can pass inappropriately. The preferable alternative to (a) is therefore to determine a tolerance interval for the confidence interval for the measure of nonparallelism. The following is particularly appropriate to transition an existing assay with a substantial body of historical data on both Standard and Test samples from a difference testing approach to an equivalence approach:
 - i. For each value of the measure of nonparallelism from the historical data, determine a 95% confidence interval, (m, n) .
 - ii. For each confidence interval, determine its maximum departure from perfect parallelism. This is $\max(|m|, |n|)$ for differences, $\max(1/m, 1/n)$ for ratios, and simply n for quantities that must be positive, such as a sum of squares.
 - iii. Determine a tolerance interval for the maximum departures obtained in (ii). This will be a one-sided tolerance interval for these necessarily positive quantities. A nonparametric tolerance interval approach is preferred.
 - iv. "Sufficiently parallel" is concluded for new data if the confidence interval for the measure of nonparallelism falls completely within the interval determined in (iii).

Approaches (a) and (b), through their reliance on assay capability, control only the false fail rate, and neglect the false pass rate. Incorporating information from sources other than the evaluation of assay capability provides control of the false pass rate. Approaches (c) and (d) are means to this end.

- c. The third approach starts with historical data comparing the Standard to itself and adds data comparing the Standard to known failures, e.g., to degraded samples. Compare values of the measure of nonsimilarity for data for which a conclusion of similarity is appropriate (Standard against itself) and data for which a conclusion of similarity is not appropriate, e.g., degraded samples. Based on this comparison, determine a value of the measure of nonsimilarity that discriminates between the two cases. If this approach is employed, a range of samples for which a conclusion of similarity is not appropriate should be utilized, including samples with the minimal important nonsimilarity. For nonlinear models, this comparison also can be used to determine which parameters should be assessed; some may not be sensitive to the failures that can occur with the specific assay or collection of nonsimilar samples.
- d. The fourth approach is based on combining a sensitivity analysis of the assay curve to nonsimilarity parameters with what is known about the product and the assay. It is particularly helpful if information is available that links a shift in one or more nonsimilarity measures to properties of the product. These measures may be direct (e.g., conformational changes in a protein) or indirect (e.g., changes in efficacy or safety in an animal model). A complementary approach is provided by a limited sensitivity analysis that combines analyst and biologist judgment regarding the magnitude of shifts in a nonsimilarity parameter that are meaningful, with simulation and/or laboratory experiments, to demonstrate thresholds for similarity parameters.

ters that provide protection against important nonsimilarity. Additionally, risk analysis may be informed by the therapeutic index of the drug.

Step 3. *Examine whether the value of the nonsimilarity measure is found within the equivalence interval of acceptable values.*

For approaches (a) and (b), compare the obtained value of the measure of nonparallelism (a) or its confidence interval (b) to the interval obtained at the beginning of Step 2. The value must be within the limits if one uses (a), or the confidence interval must be completely within the limits if one uses (b).

An alternative to the approach described above [for (a)] is to use an average (historical) value for the variance of the ratio or difference in a similarity parameter—obtained from some number of individual assays—to compute an acceptance interval for a point estimate of the similarity parameter. This approach is simpler to implement in routine use and can be used with assay designs that are unable to provide reliable estimates of within-assay variation. However, there is a price. The equivalence testing approach that relies on assay-specific (within-assay) measure(s) of variation (i.e., the confidence intervals) is conservative in the sense that it will fail to pass similarity for samples from assays that have larger than usual amounts of within-assay variation. Using an acceptance region for a similarity parameter—rather than an acceptance region for confidence intervals for the similarity parameter—loses this conservative property and hence is not preferred where alternatives exist.

For approach (c), an approach that essentially treats the parallelism as a discrimination problem may be used. The choice of the cut point in (c) should take into account the rates of false positive and false negative decisions (and the acceptable risks to the laboratory) and should reflect the between-assay variability in precision. Thus it is reasonable to compare the point estimate of the measure of nonparallelism to the cut point and to not use confidence intervals. This approach is simpler to implement in routine use and can be used with assay designs that cannot provide reliable estimates of within-assay variation.

For approach (d), demonstrate that the measure of nonsimilarity is significantly greater than the lower endpoint of the acceptance interval and significantly less than the upper endpoint. (If the acceptance interval is one-sided, then apply only the single applicable test.) This is use of the TOST approach. For most situations, TOST can be most simply implemented by calculating a 90% two-sided confidence interval, which corresponds to a 5% equivalence test. If this confidence interval lies entirely within the equivalence interval specified at the beginning of Step 2, then similarity is sufficiently demonstrated. For parallel-line models, one can use either (1) a confidence interval based on the value of the difference of the slopes $\pm k$ times the standard error of that value, or (2) Fieller's Theorem for the ratio of slopes may be used. For slope ratio models use the confidence interval for the difference of intercepts. For nonlinear models, there is evidence that these simple confidence interval methods do not attain the stated level of confidence, and methods based on likelihood profile or resampling are more appropriate.

Range—The range for a relative potency bioassay is the interval between the upper and lower relative potencies for which the bioassay is shown to have suitable levels of precision, relative accuracy, linearity of log potency, and success rates for system and sample suitability. It is straightforward to determine whether or not a sample that is similar to a Standard has a relative potency within the (validated) range of the assay system. For samples that are not similar according to established criteria, it is more challenging to deter-

mine whether a relative potency estimate for the sample might be obtained. In a nonlinear parallel-line assay a sample that does not have data on one asymptote might be assumed to be out of the potency range of the assay. In a parallel straight-line assay a sample that does not have three or more points on the steep portion of the response curve may be out of the potency range of the assay. For samples that have not been shown to be similar to reference it is not appropriate to report potency or to construct a ratio of EC_{50} s from unrestricted fits. As such samples may be out of the assay range, it may be useful to shift the dilution of the test sample for a subsequent assay on the basis of an estimate of relative activity. This estimated relative activity may be obtained via the ratio of the concentrations of Standard and Test that yields responses that match the reference response at the reference EC_{50} .

4.8 Outliers

Bioassay data should be screened for outliers before relative potency analysis. Outliers may be simple random events or a signal of a systematic problem in the bioassay. Systematic error that generates outliers may be due to a dilution error at one or more concentrations of a Test sample or the Standard or due to a mechanical error (e.g., system malfunction). Several approaches for outlier detection can be considered. Visual inspection is frequently utilized but should be augmented with a more objective approach to avoid potential bias.

An outlier is a datum that appears not to belong among the other data present. An outlier may have a distinct, identifiable cause, such as a mistake in the bench work, equipment malfunction, or a data recording error, or it could just be an unusual value relative to the variability typically seen and may appear without an identifiable cause. The essential question pertaining to an outlier becomes: Is the apparent outlier sampled from the same population as the other, less discordant, data, or is it from another population? If it comes from the same population and the datum is, therefore, an unusual (yet still legitimate) value obtained by chance, then the datum should stand. If it comes from another population and the datum's excursive value is due to human error or instrument malfunction, then the datum should be omitted from calculations. In practice, the answer to this essential question is often unknown, and investigations into causes are often inconclusive. Outlier management relies on procedures and practices to yield the best answer possible to that essential question and to guide response accordingly.

General chapter *Analytical Data—Interpretation and Treatment* (1010) addresses outlier labeling, identification, and rejection; statistical methods are included. General chapter (1010) also lists additional sources of information that can provide a comprehensive review of the relevant statistical methodology. General chapter (1010) makes no explicit remarks regarding outlier analysis in linear or nonlinear regression. Outlier analysis techniques appropriate for data obtained from regression of response on concentration can be used. Some remarks about outliers are provided here in the context of bioassays to emphasize or complement the information in (1010).

Of the procedures employed for analysis of drug compounds and biological drugs, the bioassay may be expected to be the most prone to outlying data. The management of outliers is appropriate with bioassay data on at least two levels: where an individual datum or a group of data (e.g., data at a concentration) can be checked against expected responses for the sample and concentration; and, separately, when estimates of relative potency from an assay can be checked for consistency with other independent estimates of the potency of the same material.

Three important aspects of outlier management are prevention, labeling, and identification.

Outlier prevention is preferred for obvious reasons, and is facilitated by procedures that are less subject to error and by checks that are sensitive to the sorts of errors that, given the experience gained in assay development, may be expected to occur. In effect, the error never becomes an outlier because it is prevented from occurring.

Good practice calls for the examination of data for outliers and labeling (“flagging”) of the apparently outlying observation(s) for investigation. If investigation finds a cause, then the outlying datum may be excluded from analysis. Because of the ordinary occurrence of substantial variability in bioassay response, a laboratory’s investigation into the outlying observation is likely to yield no determinable cause. However, the lack of evidence regarding an outlier’s cause is not a clear indication that statistical outlier testing is warranted. Knowledge of the typical range of assay response variability should be the justification for the use of statistical outlier tests.

Outlier identification is the use of rules to confirm that the values are inconsistent with the known or assumed statistical model. For outliers with no determined cause, it is tempting to use statistical outlier identification procedures to discard unusual values. Discarding data solely because of statistical considerations should be a rare event. Falsely discarding data leads to overly optimistic estimates of variability and can bias potency estimates. The laboratory should monitor the failure rate for its outlier procedure and should take action when this is significantly higher than expected.

Statistical procedures for outlier identification depend on assumptions about the distribution of the data without outliers. Identification of data as outliers may mean only that the assumption about distribution is not correct. If dropping outliers because of statistical considerations is common, particularly if outliers tend to occur more often at high values or at high responses, then this may be an indication that the data require some adjustment, such as log transformation, as part of the assay procedure. Two approaches to statistical assessment of outlying data are replication-based and model-based.

Replication-Based Approaches—When replicates are performed at concentrations of a Test sample and the Standard, an “extra variability” (EV) criterion may be employed to detect outliers. Historical data can be analyzed to determine the range in variability commonly observed among replicates, and this distribution of ranges can be used to establish an extreme in the range that might signal an outlier. Metrics that can be utilized are the simple range (maximum replicate minus minimum replicate), the standard deviation, or the CV or RSD among replicates. However, if the bioassay exhibits heterogeneity of variability, assumptions about uniform scatter of data are unsupported. Analysts can use a variable criterion across levels in the bioassay, or they can perform a transformation of the data to a scale that yields homogeneity of variability. Transformation can be performed with a POM approach as discussed previously. Where heterogeneity exists nonnormality is likely present, and the range rather than standard deviation or RSD should be used.

The actions taken upon detection of a potential outlier depend in part on the number of replicates. If EV is detected within a pair ($n = 2$) at a concentration of a Test sample or the Standard, it will not always be clear which of the replicates is aberrant, and the laboratory should eliminate the concentration from further processing. If more than two replicates are performed at each dilution the laboratory may choose to adopt a strategy that identifies which of the extremes may be the outlier. Alternatively, the laboratory may choose to eliminate the dilution from further processing.

Model-Based Approaches—Model-based approaches may be used to detect outliers within bioassay data. These approaches use the residuals from the fit of an appropriate model. In general, if using model-based methods to identify potential outliers, the models used may make fewer assump-

tions about the data than the models used to assess suitability and estimate potency. For example, a non-parametric regression (smoothing) model may be useful.

Lastly, an alternative to discarding outlying data is to use robust methods that are less sensitive to influence by outlying observations. Use of the median rather than the mean to describe the data’s center exemplifies a robust perspective. Also, regression using the method of least squares, which underlies many of the methods in this chapter, is not robust in the presence of outliers. The use of methods such as robust regression may be appropriate but is not covered in the USP bioassay chapters.

4.9 Fixed and Random Effects in Models of Bioassay Response

The choice of treating factors as fixed or random is important for the bioassay design, the development experiments, the statistical analysis of data, and the bioassay validation. Fixed effects are factors for which all levels, or all levels of interest, are discretely present, like sample, concentration, temperature and duration of thaw, and incubation time. Data for a response at some level, or combination of levels, of a fixed factor, can predict future responses. Fixed effects are expected to cause a consistent shift in responses. Analysts study fixed effects by controlling them in the design and examining changes in means across levels of the factor.

Random effects are factors of which the levels in a particular run of an assay are considered representative of levels that could be present. That is, there is no expectation that any specific value of the random factor will influence response. Rather, that value may vary subject to some expected distribution of values and thus may be a source of variability. For example, there is no desire to predict assay response for a specific day, but there is interest in predicting the variation in response associated with the factor “day”. Examples of random effects include reagent lot, operator, or day if there is no interest in *specific* reagent lots, operators, or day as sources of variability. Analysts may study random effects by measuring the variance components corresponding to each random effect. Variance components can be estimated well only if there are an appreciable number of levels of each random effect. If there are, for example, only two or three reagent lots or analysts present, the variation associated with these factors will be poorly estimated.

Making a correct choice regarding treating a factor as fixed or random is important to the design of the assay and to proper reporting of its precision. Treating all factors as fixed, for example, leads to an understatement of assay variability because it ignores all sources of variability other than replication. The goal is to identify specific sources of variability that can be controlled, to properly include those factors in the design, and then to include other factors as random.

If the factor may switch from random to fixed effect or vice versa, the factor should normally be modeled as a random effect. For example, reagent lots cannot be controlled, so different lots are typically considered to cause variability, and reagent lot would be considered a random effect. However, if a large shift in response values has been traced to a particular lot, a comparison among a set of lots could be performed using reagent lot as a fixed effect. Similarly, within-assay location (e.g., block, plate, plate row, plate column, or well) or sequence may be considered a source of random variation or a source of a consistent (fixed) effect.

Assay designs that consist of multiple factors are efficient, but require corresponding statistical techniques that incorporate the factors as fixed or random effects in the analysis. If all factors are fixed, the statistical model is termed a fixed-effects model. If all are random, it is termed a random-effects model. If some factors are fixed and some random, the model is a mixed-effects model. Note that the concepts of fixed and random effects apply to models for quantitative, qualitative and integer responses. For assay designs that in-

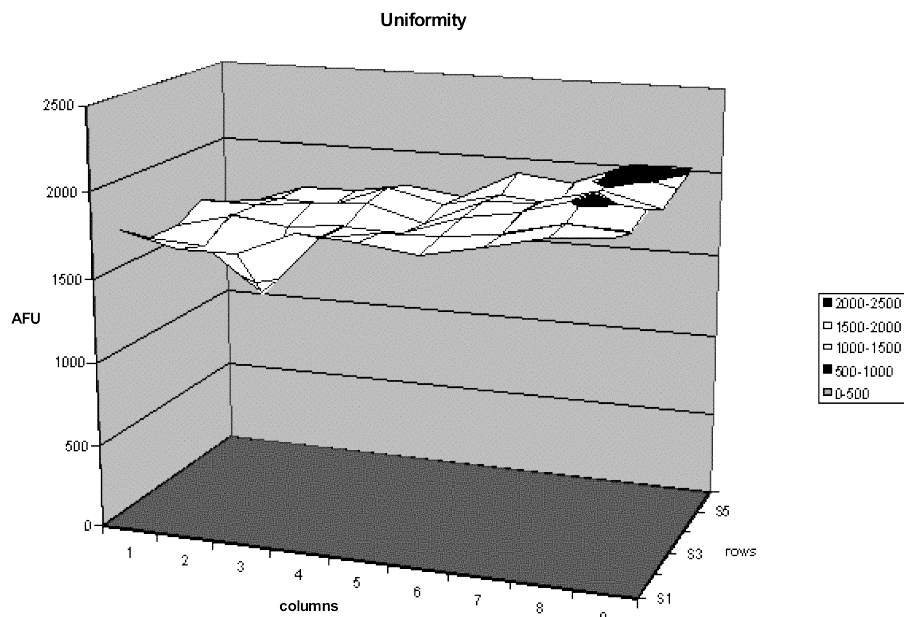


Figure 1. Plot of change in assay response across a plate.

clude multiple experimental units (e.g., samples assigned to sets of tubes and concentrations assigned to pre-plate tubes) a mixed-effects model in which the experimental units are treated as random effects is particularly effective. Additional complexity is added by the presence of designs with crossed random effects (e.g., each operator used material from one or more reagent batches, but many reagent batches were used by multiple operators). This can cause methodological and computational challenges for model fitting, especially when the designs are unbalanced.

5. STAGES IN THE BIOASSAY DEVELOPMENT PROCESS

Given the ubiquity of cell-based assays and the motivation to use one bioassay system to provide context for discussion, the development of a cell-based bioassay will be used to illustrate the stages in the bioassay development continuum.

5.1 Design: Assay Layout, Blocking, and Randomization

Most cell-based assays are performed using a cell culture plate (6-, 12-, 96-, or 384-well micro titer plate). Ideally, a plate is able to provide a uniform substrate for experimental treatments in all wells, including after wash steps and incubations. However, regardless of assay conditions intended to minimize the potential for bias (e.g., good analyst technique, careful calibration of pipets, controlled incubation time, and temperature), systematic gradients on the plate, independent of experimental treatments, may be observed. These gradients may occur across rows, across columns, or from the edge to the center of the plate and are often called *plate effects*. Even moderate or inconsistent plate effects should be addressed during assay development, by means of plate layout strategies, blocking, randomization, and replication.

Plate effects can be evaluated in a *uniformity trial* in which a single experimental treatment, such as an assay concentration chosen from the middle section of the concentration-response curve, is used in all wells of the plate. Figure 1 provides an example of what may be observed; a trend of

decreasing signal is evident from right to left. In this case, it was discovered that the plate washer was washing more briskly on the left side of the plate, and required adjustment to provide uniform washing intensity and eliminate the gradient. Another common plate effect is a differential cell-growth pattern in which the outer wells of the plate grow cells in such a way that the assay signal is attenuated. This is such a persistent problem that the choice is often made to not use the outer wells of the assay plate. Because location effects are so common, designs that place replicates (e.g., of sample by concentration combinations) in adjacent wells should be avoided.

Blocking is the grouping of related experimental units in experimental designs. Blocks may consist of individual 96-well plates, sections of 96-well plates, or 96-well plates grouped by analyst, day, or batch of cells. The goal is to isolate any systematic effects so that they do not obscure the effects of interest. A *complete block design* occurs when all levels of a treatment factor (in a bioassay, the primary treatment factors are sample and concentration) are applied to experimental units for that factor within a single block. An *incomplete block design* occurs when the number of levels of a treatment factor exceeds the number of experimental units for that factor within the block.

Randomization is a process of assignment of treatment to experimental units based on chance so that all such experimental units have an equal chance of receiving a given treatment. Although challenging in practice, randomization of experimental treatments has been advocated as the best approach to minimizing assay bias or, more accurately, to protecting the assay results from known and unknown sources of bias by converting bias into variance. While randomization of samples and concentrations to individual plate wells may not be practical, a plate layout can be designed to minimize plate effects by alternating sample positions across plates and the pattern of dilutions within and across plates. Where multiple plates are required in an assay, the plate layout design should, at a minimum, alternate sample positions across plates within an assay run to accommodate possible bias introduced by the analyst or equipment on a given day. It is prudent to use a balanced rotation of layouts on plates so that the collection of replicates (each of which uses a different layout) provides some protection against likely sources of bias.

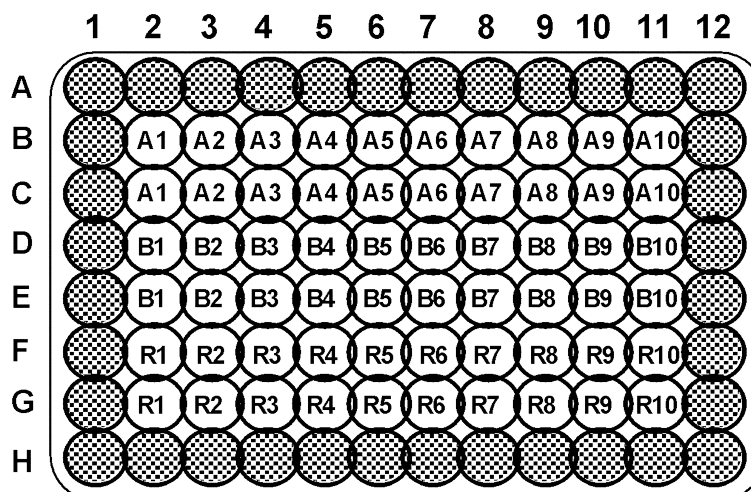


Figure 2. A highly patterned plate.

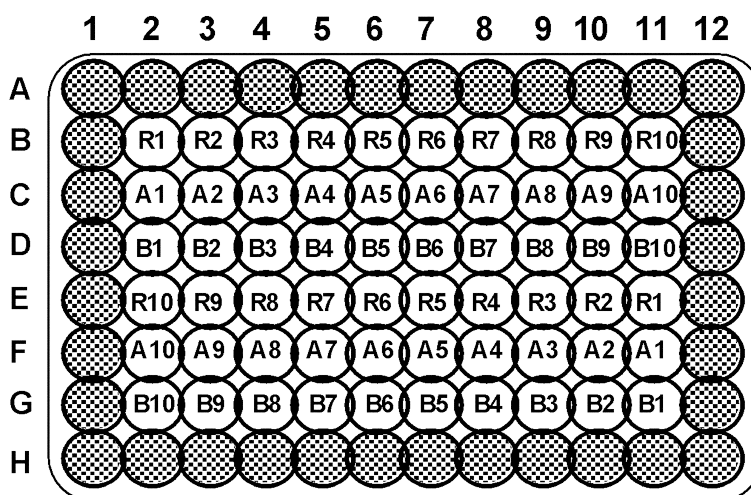


Figure 3. A strip-plot design.

Figure 2 illustrates a patterned assay design that lacks randomization and is susceptible to bias. Dilutions and replicates of the Test preparations (A and B) and the Standard (R) are placed together sequentially on the plate. Bias due to a plate or incubator effect can influence some or all of the concentrations of one of the samples. Note that in Figures 2 through 5 all outer plate wells are left as blanks to protect against edge effect.

A layout that provides some protection from plate effects and can be performed manually is a *strip-plot design*, shown in Figure 3. Here samples are randomized to rows of a plate and dilution series are performed in different directions in different sections (blocks) on the plate to mitigate bias across columns of the plate. An added advantage of the strip-plot design is the ability to detect location effects by the interaction of sample and dilution direction (left-to-right or right-to-left).

Figure 4 illustrates an alternation of Test (Test sample 1 = "1"; Test sample 2 = "2") and Standard ("R") positions on multiple plates, within a single assay run; this protects against plate row effects. Combining the two methods illustrated in Figures 3 and 4 can effectively help convert plate bias into assay variance. Assay variance may then be ad-

ressed, as necessary, by increased assay replication (increased number of plates in an assay).

A *split-plot design*, an alternative that assigns samples to plate rows randomly and randomizes dilutions (concentrations) within each row, is seen in Figure 5. Such a strategy may be difficult to implement even with the use of robotics.

Dilution Strategy—Assay concentrations of a Test sample and the Standard can be obtained in different ways. Laboratories often perform serial dilutions, in which each dilution is prepared from the previous one, in succession. Alternatively, the laboratory may prepare wholly independent dilutions from the Test sample and Standard to obtain independent concentration series. These two strategies result in the same nominal concentrations, but they have different properties related to error. Serial dilutions are subject to propagation of error across the dilution series, and a dilution error made at an early dilution will result in correlated, non-independent observations. Correlations may also be introduced by use of multichannel pipets. Independent dilutions help mitigate the bias resulting from dilution errors.

It is noteworthy that when working to improve precision, the biggest reductions in variance come when replicating at the highest possible levels of nested random effects. This is particularly effective when these highest levels are sources of variability. To illustrate: replicating extensively within a day

Plate Row	Plate 1	Plate 2	Plate 3
B	R	2	1
C	1	R	2
D	2	1	R
E	R	2	1
F	1	R	2
G	2	1	R

Figure 4. A multi-plate assay with varied Test and Reference positions.

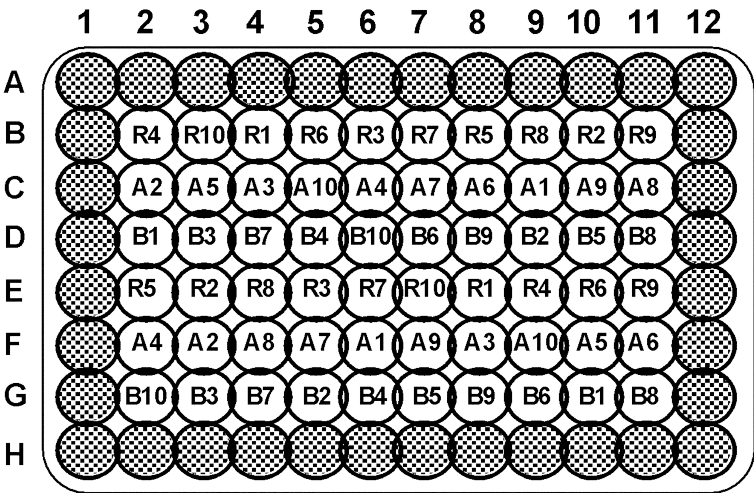


Figure 5. A split-plot design.

for an assay known to have great day-to-day variation is not effective in improving precision of reportable values.

5.2 Development

A goal of bioassay development is to achieve optimal bioassay relative accuracy and precision of the potency estimate. An endpoint of assay development is the completed development of the assay procedure, a protocol for the performance of the bioassay. The procedure should include enough detail so that a qualified laboratory with a trained analyst can perform the procedure in a routine manner. A strategic part of development is a look forward toward performance maintenance. Standard operating procedures for reagent and technician qualification, as well as for calibration of the working Standard, help complete the bioassay development package.

One Factor at a Time versus Design of Experiments—Bioassay development proceeds through a series of experiments in which conditions and levels of assay factors are varied to identify those that support a reliable and robust bioassay qualified for routine use. Those experiments may be conducted one factor at a time (OFAT), studying each parameter separately to identify ideal conditions, or through the use of multi-factor design of experiments (DOE). DOE is an efficient and effective strategy for developing a bioassay and improving bioassay performance, thus helping to obtain a measurement system that meets its requirements. In comparison to OFAT, DOE generally requires fewer experiments and also provides insight into interactions of factors that

affect bioassay performance. Assay development using DOE may proceed through a series of steps: process mapping and risk analysis; screening; response optimization; and confirmation.

Process Mapping and Risk Analysis—Bioassay optimization may begin with a systematic examination and risk assessment to identify those factors that may influence bioassay response. It is useful to visualize bioassay factors using a bioassay process map such as a cause-and-effect or fishbone diagram. Using the process map as a guide, the laboratory can examine assay factors that might affect assay performance, such as buffer pH, incubation temperature, and incubation time. Historical experience with one or several of the bioassay steps, along with sound scientific judgment, can identify key factors that require further evaluation. One tool that may be used to prioritize factors is a failure mode and effects analysis. Factors are typically scored by the combination of their potential to influence assay response and the likelihood that they will occur. The laboratory must be careful to recognize potential interactions between assay factors.

Screening—Once potential key factors have been identified from process mapping and risk analysis, the laboratory may conduct an initial screening experiment to probe for effects that may require control. Screening designs such as factorial and fractional factorial designs are commonly used for this purpose. Software is available to assist the practitioner in the selection of the design and in subsequent analysis. Analysts should take care, however, to understand their assumptions

about design selection and analysis to ensure accurate identification of experimental factors.

Response Optimization—A screening design will usually detect a few important factors from among those studied. Such factors can be further studied in a response-optimization design. Response-optimization designs such as central composite designs are performed to determine optimal settings for combinations of bioassay factors for achieving desired response. The information obtained from response optimization may be depicted as a response surface and can be used to establish ranges that yield acceptable assay performance and will be incorporated into the bioassay procedure.

In the parlance of Quality by Design (QbD), the “region” where the combined levels of input variables and process parameters have been demonstrated to provide acceptable assay performance is described as the *design space* for the bioassay. Establishing a true design space for a bioassay is challenging; some but not all factors and levels of random factors will be included in the development DOE, and there is no assurance that the design space is not sensitive to unstudied random factors. Similarly, there is little assurance that the assay (design space) is robust to random factors that are studied using small samples (or non-random samples of levels). Elements of DOE that may be considered include the use of blocks; deliberate confounding among interactions that are of lower interest, or known to be unimportant; robust design (response surface designs with random effects); and use of split-plot, strip-plot, or split-lot designs.

Confirmation—The mathematical model depicting assay performance as a function of changes in key assay factors is an approximation; thus, it is customary to confirm performance at the ideal settings of the bioassay. Confirmation can take the form of a qualification trial in which the assay is performed, preferably multiple independent times using optimal values for factors. Alternatively, the laboratory may determine that the bioassay has been adequately developed and may move to validation. Qualification is a good practice, not a regulatory requirement. The decision to perform confirmatory qualifying runs or to proceed to validation depends upon the strength of the accumulated information obtained throughout development.

5.3 Data Analysis during Assay Development

Analysis of bioassay data during assay development enables analysts to make decisions regarding the statistical model that will be used for routine analysis, including transformation and/or weighting of data, and the development of system and sample suitability criteria. The analysis also provides information regarding which elements of design structure should be used during outlier detection and the fitting of a full model. This may also include a plan for choosing subsets of data, such as a linear portion, for analysis or, for nonlinear bioassays, a model reduction strategy for samples similar to Standard. Once these decisions are made and proven sound during validation, they don't need to be reassessed with each performance of the assay. A process approach to enabling these decisions follows.

Step 1: Choose an appropriate statistical model (also see section 4.6 Common Bioassay Models).

Given the complexity of bioassays and the motivation to use an approach proven reliable, fairly standardized analytical models are common in the field of bioassay analysis. Nonetheless, many considerations are involved in choosing the most appropriate statistical model. First, the model should be appropriate for the type of assay endpoint—continuous, count, or dichotomous. Second, the model should incorporate the structure of the assay design. For any design other than completely randomized, there will be terms in the model for the structural elements. These could be, for example, within-plate blocking, location of

cage in the animal facility, day, etc. A third consideration, applicable to continuous endpoints, involves whether to use a regression model or a means model (an analysis of variance model that fits a separate mean at each dilution level of each sample tested), with appropriate error terms. A means model can be appropriate at this stage because it makes no assumptions about the shape of the concentration–response curve.

Step 2: Fit the chosen statistical model to the data without the assumption of parallelism, and then assess the distribution of the residuals, specifically examining them for departures from normality and constant variance.

Transform the data as necessary or, if needed, choose a weighting scheme (see section 4.3 *Variance Heterogeneity, Weighting, and Transformation*). Use as large a body of assay data, from independent assays, as possible. The primary goal is to address any departure from normality and from constant variance of responses across the range of concentrations in the assay. Step 2 will likely alternate between imposing a transformation and assessing the distribution of the residuals.

Step 3: Screen for outliers, and remove as is appropriate.

This step normally follows the initial choice of a suitable transformation and/or weighting method. Ideally the model used for outlier detection contains the important elements of the assay design structure, allows nonsimilar curves, and makes fewer assumptions about the functional shape of the concentration–response curve than did the model used to assess similarity. See section 4.8 *Outliers* and general chapter (1010) for discussion of outlier detection and removal. In some cases, outliers may be so severe that a reasonable model cannot be fit, and thus residuals will not be available. In such cases, it is necessary to screen the raw data for outliers before attempting to fit the model.

During assay development, a strategy should be developed for the investigation and treatment of an outlier observation, including any limits on how many outliers are acceptable. Include these instructions in the assay SOP. Good practice includes recording the process of an investigation, outlier test(s) applied, and results therefrom. Note that outlier procedures must be considered apart from the investigation and treatment of an out-of-specification (OOS) result (reportable value). Decisions to remove an outlier from data analysis should not be made on the basis of how the reportable value will be affected (e.g., a potential OOS result). Removing data as outliers should be rare. If many values from a run are removed as outliers, that run should be considered suspect.

Step 4: Refit the model with the transformation and/or weighting previously imposed (Step 2) without the observations identified as outliers (Step 3) and re-assess the appropriateness of the model.

Step 5: If necessary or desired, choose a scheme for identifying subsets of data to use for potency estimation, whether the model is linear or nonlinear (see section 4.5 Linearity of Concentration–Response Data).

Step 6: Calculate a relative potency estimate by analyzing the Test and Standard data together using a model constrained to have parallel lines or curves, or equal intercepts.

5.4 Bioassay Validation

The bioassay validation is a protocol-driven study that demonstrates that the procedure is fit for use. A stage-wise approach to validation may be considered, as in a “suitable for intended use” validation to support release of clinical trial material, and a final, comprehensive validation prior to BLA or MAA filing. Preliminary system and sample suitability

controls should be established and clearly described in the assay procedure; these may be finalized based on additional experience gained in the validation exercise. Chapter (1033) provides validation comprehensive discussion of bioassay validation.

5.5 Bioassay Maintenance

The development and validation of a bioassay, though discrete operations, lead to ongoing activities. Assay improvements may be implemented as technologies change, as the laboratory becomes more skilled with the procedure, and as changes to bioassay methodology require re-evaluation of bioassay performance. Some of these changes may be responses to unexpected performance during routine processing. Corrective action should be monitored using routine control procedures. Substantial changes may require a study verifying that the bioassay remains fit for use. An equivalence testing approach can be used to show that the change has resulted in acceptable performance. A statistically-oriented study can be performed to demonstrate that the change does not compromise the previously acceptable performance characteristics of the assay.

Assay Transfer—Assay transfer assumes both a known intended use of the bioassay in the recipient lab and the associated required capability for the assay system. These implicitly, though perhaps not precisely, demarcate the limits on the amount of bias and loss of precision allowed between labs. Using two laboratories interchangeably to support one product will require considering the variation between labs in addition to intermediate precision for sample size requirements to determine process capability. For a discussion and example pertaining to the interrelationship of bias, process capability, and validation, see *A Bioassay Validation Example* in (1033).

Improving or Updating a Bioassay System—A new version of a bioassay may improve the quality of bias, precision, range, robustness, specificity, lower the operating costs or offer other compelling advantages. When improving or updating a bioassay system a bridging study may be used to compare the performance of the new to the established assay. A wide variety of samples (e.g., lot release, stability, stressed, critical isoforms) can be used for demonstrating equivalence of estimated potencies. Even though the assay systems may be quite different (e.g., an animal bioassay versus a cell-based bioassay), if the assays use the same Standard and mechanism of action, comparable potencies may reasonably be expected. If the new assay uses a different Standard, the minimum requirement for an acceptable comparison is a unit slope of the log linear relationship between the estimated potencies. An important implication of this recommendation is that poor precision or biased assays used early can have lasting impact on the replication requirements, even if the assay is later replaced by an improved assay.

and marketing of many biological and some non-biological drug products. Bioassays commonly used for drug potency estimation can be distinguished from chemical tests by their reliance on a biological substrate (e.g., animals, living cells, or functional complexes of target receptors). Because of multiple operational and biological factors arising from this reliance on biology, they typically exhibit a greater variability than do chemically-based tests.

Bioassays are one of several physicochemical and biologic tests with procedures and acceptance criteria that control critical quality attributes of a biological drug product. As described in the ICH Guideline entitled Specifications: Test Procedures And Acceptance Criteria For Biotechnological/Biological Products (Q6B), section 2.1.2, bioassay techniques may measure an organism's biological response to the product; a biochemical or physiological response at the cellular level; enzymatic reaction rates or biological responses induced by immunological interactions; or ligand- and receptor-binding. As new biological drug products and new technologies emerge, the scope of bioassay approaches is likely to expand. Therefore, general chapter *Biological Assay Validation* (1033) emphasizes validation approaches that provide flexibility to adopt new bioassay methods, new biological drug products, or both in conjunction for the assessment of drug potency.

Good manufacturing practice requires that test methods used for assessing compliance of pharmaceutical products with quality requirements should meet appropriate standards for accuracy and reliability. Assay validation is the process of demonstrating and documenting that the performance characteristics of the procedure and its underlying method meet the requirements for the intended application and that the assay is thereby suitable for its intended use. USP general chapter *Validation of Compendial Procedures* (1225) and ICH Q2(R1) describe the assay performance characteristics (parameters) that should be evaluated for procedures supporting small-molecule pharmaceuticals. Although evaluation of these validation parameters is straightforward for many types of analytical procedures for well-characterized, chemically-based drug products, their interpretation and applicability for some types of bioassays has not been clearly delineated. This chapter addresses bioassay validation from the point of view of the measurement of activity rather than mass or other physicochemical measurements, with the purpose of aligning bioassay performance characteristics with uses of bioassays in practice.

Assessment of bioassay performance is a continuous process, but bioassay validation should be performed when development has been completed. Bioassay validation is guided by a validation protocol describing the goals and design of the validation study. General chapter (1033) provides validation goals pertaining to *relative potency* bioassays. Relative potency bioassays are based on a comparison of bioassay responses for a Test sample to those of a designated Standard that provides a quantitative measure of the Test bioactivity relative to that of the Standard.

Validation parameters discussed include *relative accuracy*, *specificity*, *intermediate precision*, and *range*. Laboratories may use *dilutional linearity* to verify the *relative accuracy* and *range* of the method. Although robustness is not a requirement for validation, general chapter (1033) recommends that a bioassay's robustness be assessed prior to validation. In addition, (1033) describes approaches for validation design (sample selection and replication strategy), validation acceptance criteria, data analysis and interpretation, and finally bioassay performance monitoring through quality control. Documentation of bioassay validation results is also discussed, with reference to pre-validation experiments performed to optimize bioassay performance. In the remainder of general chapter (1033) the term "bioassay" should be interpreted as meaning "relative potency bioassay".

(1033) BIOLOGICAL ASSAY VALIDATION

1. INTRODUCTION

Biological assays (also called bioassays) are an integral part of the quality assessment required for the manufacturing

2. FUNDAMENTALS OF BIOASSAY VALIDATION

The goal of bioassay validation is to confirm that the operating characteristics of the procedure are such that the procedure is suitable for its intended use. The issues involved in developing a bioassay are described in greater detail in general chapter <1032> and are assumed resolved by the time the bioassay is in validation. Included in those decisions will be identification of what constitutes an assay and a run for the bioassay. Multiple dilutions (concentrations) of the Standard and one or more Test samples constitute a *replicate set* (also known as a minimal set), which contain a test substrate (e.g., group of animals or vessel of cells) at each dilution for each sample [Test(s) and Standard]. A *run* is defined as work performed during a period when the accuracy (trueness) and precision in the assay system can reasonably be expected to be stable. In practice, a run frequently consists of the work performed by a single analyst in one lab, with one set of equipment, in a short period of time (typically a day). An assay is the body of data used to assess similarity and estimate potency relative to a Standard for each Test sample in the assay. A run may contain multiple assays, a single assay, or part of an assay. Multiple assays may be combined to yield a reportable value for a sample. The reportable value is the value that is compared to a product specification.

In assays that involve groups at each dilution (e.g., 6 samples, each at 10 dilutions, in the non-edge wells of each of several 96-well cell culture plates) the groups (plates) constitute statistical *blocks* that should be elements in the assay and validation analyses (blocks are discussed in <1032>). Within-block replicates for Test samples are rarely cost-effective. Blocks will not be further discussed in this chapter; more detailed discussion is found in <1032>.

The amount of activity (potency) of the Standard is initially assigned a value of 1.0 or 100%, and the potency of the Test sample is calculated by comparing the concentration-response curves for the Test and Standard pair. This results in a unitless measure, which is the relative potency of the Test sample in reference to the potency of the Standard. In some cases the Standard is assigned a value according to another property such as protein concentration. In that case the potency of the Test sample is the relative potency times the assigned value of the Standard. An assumption of parallel-line or parallel-curve (e.g., four-parameter logistic) bioassays is that the dose-response curves that are generated using a Standard and a Test sample have similar (parallel) curve shape distinguished only by a horizontal shift in the log dose. For slope-ratio bioassays, curves generated for Standard and Test samples should be linear, pass through a common intercept, and differ only by their slopes. Information about how to assess parallelism is provided in general chapters <1032> and <1034>.

In order to establish the *relative accuracy* and *range* of the bioassay, validation Test samples may be constructed using a dilution series of the Standard to assess dilutional linearity (linearity of the relationship between known and measured relative potency). In addition, the validation study should yield a representative estimate of the variability of the relative potency determination. Although robustness studies are usually performed during bioassay development, key factors in these studies such as incubation time and temperature and, for cell-based bioassays, cell passage number and cell number may be included in the validation, particularly if they interact with another factor that is introduced during the validation (e.g., a temperature sensitive reagent that varies in its sensitivity from lot-to-lot). Because of potential influences on the bioassay from inter-run factors such as multiple analysts, instruments, or reagent sources, the design of the bioassay validation should include consideration of these factors. The variability of potency from these combined elements defines the *intermediate precision* (IP) of the bioassay. An appropriate study of the variability of the po-

tency values obtained, including the impact of intra-assay and inter-run factors, can help the laboratory confirm an adequate testing strategy and forecast the inherent variability of the *reportable value* (which may be the average of multiple potency determinations). Variability estimates can also be utilized to establish the sizes of differences (fold difference) that can be distinguished between samples tested in the bioassay. (See section 3.4 *Use of Validation Results for Bioassay Characterization*.)

Demonstrating specificity (also known as selectivity) requires evidence of lack of influence from matrix components such as manufacturing process components or degradation products so that measurements quantify the target molecule only. Other analytical methods may complement a bioassay in measuring or identifying other components in a sample.

2.1 Bioassay Validation Protocol

A bioassay validation protocol should include the number and types of samples that will be studied in the validation; the study design, including inter-run and intra-run factors; the replication strategy; the intended validation parameters and justified target acceptance criteria for each parameter; and a proposed data-analysis plan. Note that in regard to satisfying acceptance criteria, failure to find a statistically significant effect is not an appropriate basis for defining acceptable performance in a bioassay; conformance to acceptance criteria may be better evaluated using an equivalence approach.

In addition, assay, run, and sample acceptance criteria such as system suitability and similarity should be specified before performing the validation. Depending on the extent of development of the bioassay, these may be proposed as tentative and can be updated with data from the validation. Assay, run, or sample failures may be reassessed according to criteria which have been defined in the validation protocol and, with sound justification, included in the overall validation assessment. Additional validation trials may be required in order to support changes to the method.

The bioassay validation protocol should include target acceptance criteria for the proposed validation parameters. Steps to be taken upon failure to meet a target acceptance criterion should be specified in the validation protocol, and may result in a limit on the range of potencies that can be measured in the bioassay or a modification to the replication strategy in the bioassay procedure.

2.2 Documentation of Bioassay Validation Results

Bioassay validation results should be documented in a bioassay validation report. The validation report should support the conclusion that the method is fit for use or should indicate corrective action (such as an increase in the replication strategy) that will be undertaken to generate sufficiently reliable results to achieve fitness for use. The report could include the raw data and intermediate results (e.g., variance component estimates should be provided in addition to overall intermediate precision) which would facilitate reproduction of the bioassay validation analysis by an independent reviewer. Estimates of validation parameters should be reported at each level and overall as appropriate. Deviations from the validation protocol should be documented with justification. The conclusions from the study should be clearly described with references to follow-up action as necessary. Follow-up action can include amendment of system or sample suitability criteria or modification of the bioassay replication strategy. Reference to prevalidation experiments may be included as part of the validation study report. Prevalidation experiments may include robustness experiments, where bioassay parameters have been identified and ranges have been established for significant parameters, and also may include qualification experiments, where the final

procedure has been performed to confirm satisfactory performance in routine operation. Conclusions from prevalidation and qualification experiments performed during development contribute to the description of the operating characteristics of the bioassay procedure.

2.3 Bioassay Validation Design

The biological assay validation should include samples that are representative of materials that will be tested in the bioassay and should effectively establish the performance characteristics of the procedure. For *relative accuracy*, sample relative potency levels that bracket the range of potencies that may be tested in the bioassay should be used. Thus samples that span a wide range of potencies might be studied for a drug or biological with a wide specification range or for a product that is inherently unstable, but a narrower range can be used for a more durable product. A minimum of three potency levels is required, and five are recommended for a reliable assessment. If the validation criteria for relative accuracy and IP are satisfied, the potency levels chosen will constitute the *range* of the bioassay. A limited range will result from levels that fail to meet their target acceptance criteria. Samples may also be generated for the bioassay validation by stressing a sample to a level that might be observed in routine practice (i.e., stability investigations). Additionally, the influences of the sample matrix (excipients, process constituents, or combination components) can be studied strategically by intentionally varying these together with the target *analyte*, using a multifactorial approach. Often this will have been done during development, prior to generating release and stability data.

The bioassay validation design should consider all facets of the measurement process. Sources of bioassay measurement variability include sample preparation, intra-run factors, and inter-run factors. Representative estimation of bioassay variability necessitates consideration of these factors. Test sample and Standard preparation should be performed independently during each validation run.

The replication strategy used in the validation should reflect knowledge of the factors that might influence the measurement of potency. Intra-run variability may be affected by bioassay operating factors that are usually set during development (temperature, pH, incubation times, etc.); by the bioassay design (number of animals, number of dilutions, replicates per dilution, dilution spacing, etc.); by the assay acceptance and sample acceptance criteria; and by the statistical analysis (where the primary endpoints are the similarity assessment for each sample and potency estimates for the reference samples). Operating restrictions and bioassay design (intra- and inter-run formulae that result in a *reportable value* for a test material) are usually specified during development and may become a part of the bioassay operating procedure. IP is studied by independent *runs* of the procedure, perhaps using an experimental design that alters those factors that may have an impact on the performance of the procedure. Experiments (including those that implement formalized design of experiments [DOE]) with nested or crossed design structure can reveal important sources of variability in the procedure, as well as ensure a representative estimate of long-term variability. During the validation it is not necessary to employ the format required to achieve the reportable value for a Test sample. A well-designed validation experiment that combines both intra-run and inter-run sources of variability provides estimates of independent components of the bioassay variability. These components can be used to verify or forecast the variability of the bioassay format.

A thorough analysis of the validation data should include graphical and statistical summaries of the validation parameters' results and their conformance to target acceptance criteria. The analysis should follow the specifics of the data-analysis plan outlined in the validation protocol. In most cases, log relative potency should be analyzed in order to

satisfy the assumptions of the statistical methods (see section 2.7 *Statistical Considerations, Scale of Analysis*). Those assumptions include *normality* of the distribution from which the data were sampled and *homogeneity of variability* across the range of results observed in the validation. These assumptions can be explored using graphical techniques such as box plots and probability plots. The assumption of normality can be investigated using statistical tests of normality across a suitably sized collection of historical results. Alternative methods of analysis should be sought when the assumptions can be challenged. *Confidence intervals* should be calculated for the validation parameters, using methods described here and in general chapter *Analytical Data—Interpretation and Treatment* (1010).

2.4 Validation Strategies for Bioassay Performance Characteristics

Parameters that should be verified in a bioassay are *relative accuracy*, *specificity*, IP (which incorporates repeatability), and *range*. Other parameters discussed in general chapter (1225) and ICH Q2(R1) such as detection limit and quantitation limit have not been included because they are usually not relevant to a bioassay that reports relative potency. These may be relevant, however, to the validation of an ancillary assay such as one used to score responders or measure response in conjunction with an *in vivo* potency assay. Likewise linearity is not part of bioassay validation, except as it relates to relative accuracy (dilutional linearity). There follow strategies for addressing bioassay validation parameters.

Relative Accuracy—The *relative accuracy* of a relative potency bioassay is the relationship between measured relative potency and known relative potency. Relative accuracy in bioassay refers to a unit slope (slope = 1) between log measured relative potency and log known relative potency. The most common approach to demonstrating relative accuracy for relative potency bioassays is by construction of target potencies by dilution of the standard material or a Test sample with known potency. This type of study is often referred to as a *dilutional linearity study*. The results from a dilutional linearity study should be assessed using the estimated relative bias at individual levels and via a trend in *relative bias* across levels. The *relative bias* at individual levels is calculated as follows:

$$\text{Relative Bias} = 100 \cdot \left(\frac{\text{Measured Potency}}{\text{Target Potency}} - 1 \right) \%$$

The trend in bias is measured by the estimated slope of log measured potency versus log target potency, which should be held to a target acceptance criterion. If there is no trend in *relative bias* across levels, the estimated *relative bias* at each level can be held to a prespecified target acceptance criterion that has been defined in the validation protocol (see section 3 *A Bioassay Validation Example*).

Specificity—For products or intermediates associated with complex matrices, specificity involves demonstrating lack of interference from matrix components or product-related components that can be expected to be present. This can be assessed via parallel dilution of the Standard with and without a spike addition of the potentially interfering compound. If the curves are similar and the potency conforms to expectations of a Standard-to-Standard comparison, the bioassay is specific against the compound. For these assessments both similarity and potency may be assessed using appropriate equivalence tests.

Specificity may also refer to the capacity of the bioassay to distinguish between different but related biopharmaceutical molecules. An understanding should be sought of the molecule and any related forms, and of opportunities for related molecules to be introduced into the bioassay.

Intermediate Precision—Because of potential influences on the bioassay by factors such as analysts, instruments, or reagent lots, the design of the bioassay validation should include evaluation of these factors. The overall variability from measurements taken under a variety of normal test conditions within one laboratory defines the IP of the bioassay. IP is the ICH and USP term for what is also commonly referred to as inter-run variability. IP measures the influence of factors that will vary over time after the bioassay is implemented. These influences are generally unavoidable and include factors like change in personnel (new analysts), receipt of new reagent lots, etc.

When the validation has been planned using multifactor DOE, the impact of each factor can first be explored graphically to establish important contributions to potency variability. The identification of important factors should lead to procedures that seek to control their effects, such as further restrictions on intra-assay operating conditions or strategic qualification procedures on inter-run factors such as analysts, instruments, and reagent lots.

Contributions of validation study factors to the overall IP of the bioassay can be determined by performing a *variance component analysis* on the validation results. *Variance component analysis* is best carried out using a statistical software package that is capable of performing a mixed-model analysis with restricted maximum likelihood estimation (REML).

A variance component analysis yields variance component estimates such as

$$\hat{\sigma}_{\text{Intra}}^2$$

and

$$\hat{\sigma}_{\text{Inter}}^2$$

corresponding to intra-run and inter-run variation. These can be used to estimate the IP of the bioassay, as well as the variability of the *reportable value* for different bioassay formats (format variability). IP expressed as percent *geometric coefficient of variation* (%GCV) is given by the following formula, in this case using the natural log of the relative potency in the analysis (see section 2.7 *Statistical Considerations, Scale of Analysis*):

$$\text{Intermediate Precision} = 100 \cdot \left(e^{\sqrt{\hat{\sigma}_{\text{Inter}}^2 + \hat{\sigma}_{\text{Intra}}^2}} - 1 \right) \%$$

The variability of the *reportable value* from testing performed with *n* replicate sets in each of *k* runs (*format variability*) is equal to:

$$\text{Format Variability} = 100 \cdot \left(e^{\sqrt{\hat{\sigma}_{\text{Inter}}^2/k + \hat{\sigma}_{\text{Intra}}^2/(nk)}} - 1 \right) \%$$

This formula can be used to determine a testing format suitable for various uses of the bioassay (e.g., release testing and stability evaluation).

Range—The *range* of the bioassay is defined as the true or known potencies for which it has been demonstrated that the analytical procedure has a suitable level of relative accuracy and IP. The range is normally derived from the dilutional linearity study and minimally should cover the product specification range for potency. For stability testing and to minimize having to dilute or concentrate hyper- or hypopotent Test samples into the bioassay range, there is value in validating the bioassay over a broader range.

2.5 Validation Target Acceptance Criteria

The validation target acceptance criteria should be chosen to minimize the risks inherent in making decisions from bioassay measurements and to be reasonable in terms of the

capability of the art. When there is an existing product specification, acceptance criteria can be justified on the basis of the risk that measurements may fall outside of the product specification. Considerations from a process capability (Cp) index can be used to inform bounds on the *relative bias* (RB) and the IP of the bioassay. This chapter uses the following Cpm index:

$$\text{Cpm} = \frac{\text{USL} - \text{LSL}}{6 \cdot \sqrt{\sigma_{\text{Product}}^2 + \text{RB}^2 + \sigma_{\text{RA}}^2}}$$

where USL and LSL are the upper and lower release specification, RB is a bound on the degree of relative bias in the bioassay, and

$$\sigma_{\text{Product}}^2$$

and

$$\sigma_{\text{RA}}^2$$

are target product variance (i.e., lot-to-lot variability) and release assay variance (with associated format) respectively. (See section 3 *A Bioassay Validation Example* for an example of determination of

$$\sigma_{\text{RA}}^2$$

and Cpm.) This formulation requires prior knowledge regarding target product variability, or the inclusion of a random selection of lots to estimate this characteristic as part of the validation. Given limited understanding of assay performance, manufacturing history, and final specifications during development, this approach may be used simply as a guide for defining validation acceptance criteria.

The choice of a bound on Cpm is a business decision. The proportion of lots that are predicted to be outside their specification limits is a function of Cpm. Some laboratories require process capability corresponding to Cpm greater than or equal to 1.3. This corresponds to approximately a 1 in 10,000 chance that a lot with potency at the center of the specification range will be outside the specification limits.

When specifications have yet to be established for a product, a restriction on *relative bias* or IP can be formulated on the basis of the capability of the art of the bioassay methodology. For example, although chemical assays and immunoassays are often capable of achieving near single digit *percent coefficient of variation* (%CV, or percent relative standard deviation, %RSD), a more liberal restriction might be placed on bioassays, such as animal potency bioassays, that operate with much larger variability (measured as %GCV which can be compared to %CV; see *Appendix*). In this case the validation goal might be to *characterize* the method, using the validation results to establish an assay format that is predicted to yield reliable product measurements. A sound justification for target acceptance criteria or use of *characterization* should be included in the validation protocol.

2.6 Assay Maintenance

Once a bioassay has been validated it can be implemented. However, it is important to monitor its behavior over time. This is most easily accomplished by maintaining *statistical process control* (SPC) charts for suitable parameters of the Standard response curve and potency of assay QC samples. The purpose of these charts is to identify at an early stage any shift or drift in the bioassay. If a trend is observed in any SPC chart, the reason for the trend should be identified. If the resolution requires a modification to the

bioassay or if a serious modification of the bioassay has occurred for other reasons (for example, a major technology change), the modified bioassay should be revalidated or linked to the original bioassay by an adequately designed bridging study with acceptance criteria that use equivalence testing.

2.7 Statistical Considerations

Several statistical considerations are associated with designing a bioassay validation and analyzing the data. These relate to the properties of bioassay measurements as well as the statistical tools that can be used to summarize and interpret bioassay validation results.

Scale of Analysis—The scale of analysis of bioassay validation, where data are the relative potencies of samples in the validation study, must be considered in order to obtain reliable conclusions from the study. This chapter assumes that appropriate methods are already in place to reduce the raw bioassay response data to relative potency (as described in general chapter (1034)). Relative potency measurements are typically nearly *log normally distributed*. *Log normally distributed* measurements are skewed and are characterized by *heterogeneity of variability*, where the standard deviation is proportional to the level of response. The statistical methods outlined in this chapter require that the data be symmetric, approximating a normal distribution, but some of the procedures require *homogeneity of variability* in measurements across the potency range. Typically, analysis of potency after *log transformation* generates data that more closely fulfill both of these requirements. The base of the log transformation does not matter as long as a consistent base is maintained throughout the analysis. Thus, for example, if the natural log (log to the base e) is used to transform relative potency measurements, summary results are converted back to the bioassay scale utilizing base e.

The distribution of potency measurements should be assessed as part of bioassay development (as described in (1032)). If it is determined that potency measurements are normally distributed, the validation can be carried out using methods described in the general chapter *Validation of Compensatory Procedures* (1225).

As a consequence of the usual (for parallel-line assays) log transformation of relative potency measurements, there are advantages if the levels selected for the validation study are evenly spaced on the log scale. An example with five levels would be 0.50, 0.71, 1.00, 1.41, and 2.00. Intermediate levels are obtained as the *geometric mean* of two adjacent levels. Thus for example, the mid-level between 0.50 and 1.0 is derived as follows:

$$GM = \sqrt{0.50 \cdot 1.0} = 0.71$$

Likewise, summary measures of the validation are influenced by the log normal scale. Predicted response should be reported as the *geometric mean* of individual relative potency measurements, and variability expressed as %GCV. GCV is calculated as the anti-log of the standard deviation, S_{\log} , of log transformed relative potency measurements. The formula is given by:

$$GCV = \text{antilog}(S_{\log}) - 1$$

Variability is expressed as GCV rather than RSD of the log normal distribution in order to preserve continuity using the log transformation (see additional discussion in the *Appendix* to this chapter). Intervals that might be calculated from GCV will be consistent with intervals calculated from mean and standard deviation of log transformed data. *Table 1* presents an example of the calculation of geometric mean (GM) and associated RB, with %GCV for a series of relative potency measurements performed on samples tested at the 1.00 level. The log base e is used in the illustration.

Table 1. Illustration of calculations of GM and %GCV

RP ¹	ln RP	
1.1299	0.1221	
0.9261	-0.0768	
1.1299	0.1221	
1.0143	0.0142	
1.0027	0.0027	
1.0316	0.0311	
1.1321	0.1241	
1.0499	0.0487	
Average	0.0485	GM = 1.0497
		RB = 4.97%
SD	0.0715	%GCV = 7.4%

¹ Relative potency (RP) is the geometric mean of duplicate potencies measured in the eight runs of the example given in *Table 4*.

Here the GM of the relative potency measurements is calculated as the anti-log of the average log relative potency measurements and then expressed as relative bias, the percent deviation from the target potency:

$$GM = e^{\text{Average}} = e^{0.0485} = 1.0497$$

$$RB = 100 \cdot \left(\frac{GM}{\text{Target}} - 1 \right) \% = 100 \cdot \left(\frac{1.0497}{1.00} - 1 \right) \% = 4.97\%$$

and the percent *geometric coefficient of variation* (%GCV) is calculated as:

$$\%GCV = 100 \cdot (e^{SD} - 1) \% = 100 \cdot (e^{0.0715} - 1) \% = 7.4\%$$

Note that the %GCV calculated for this illustration is not equal to the IP determined in the bioassay validation example for the 1.00 level (8.5%); see *Table 6*. This illustration utilizes the average of within-run replicates, while the IP in the validation example represents the variability of individual replicates.

Reporting Validation Results Using Confidence Intervals—Estimates of bioassay validation parameters should be presented as a *point estimate* together with a *confidence interval*. A *point estimate* is the numerical value obtained for the parameter, such as the GM or %GCV. A *confidence interval's* most common interpretation is as the likely range of the true value of the parameter. The previous example determines a 90% *confidence interval* for average log relative potency, Cl_{ln} , as follows:

$$Cl_{ln} = \text{Average} \pm t_{df} \cdot SD / \sqrt{n} \\ = 0.0485 \pm 1.89 \cdot 0.0715 / \sqrt{8} = (0.0007, 0.0963)$$

For percent relative bias this is:

$$Cl_{RB} = 100 \cdot \left(\frac{e^{0.0007}}{1.00} - 1 \right) \%, 100 \cdot \left(\frac{e^{0.0963}}{1.00} - 1 \right) \% = (0.07\%, 10.1\%)$$

The statistical constant (1.89) is from a t-table, with degrees of freedom (df) equal to the number of measurements minus one ($df = 8 - 1 = 7$). A confidence interval for IP or format variability can be formulated using methods for variance components; these methods are not covered in this general chapter.

Assessing Conformance to Acceptance Criteria—Bioassay validation results are compared to target acceptance criteria in order to demonstrate that the bioassay is fit for use. The process of establishing conformance of validation parameters to validation acceptance criteria should not be confused with establishing conformance of relative potency measure-

ments to product specifications. Product specifications should inform the process of setting validation acceptance criteria.

A common practice is to apply acceptance criteria to the estimated validation parameter. This does not account, however, for the uncertainty in the estimated validation parameter. A solution is to hold the *confidence interval* on the validation parameter to the acceptance criterion. This is a standard statistical approach used to demonstrate conformance to expectation and is called an *equivalence test*. It should not be confused with the practice of performing a significance test, such as a t-test, which seeks to establish a difference from some target value (e.g., 0% relative bias). A significance test associated with a P-value > 0.05 (equivalent to a confidence interval that includes the target value for the parameter) indicates that there is insufficient evidence to conclude that the parameter is different from the target value. This is not the same as concluding that the parameter conforms to its target value. The study design may have too few *replicates*, or the validation data may be too variable to discover a meaningful difference from target. Additionally, a significance test may detect a small deviation from target that is of negligible importance. These scenarios are illustrated in Figure 1.

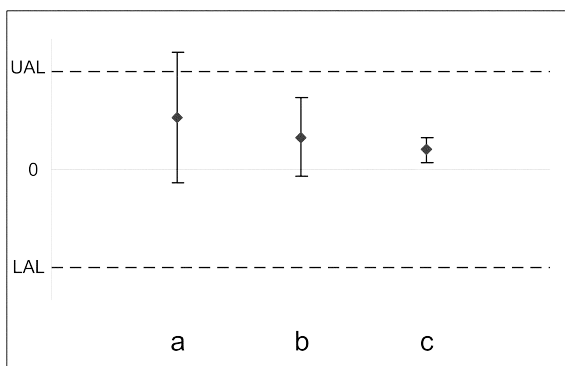


Figure 1. Use of confidence intervals to establish that validation results conform to an acceptance criterion.

The solid horizontal line represents the target value (perhaps 0% relative bias), and the dashed lines form the lower (LAL) and upper (UAL) acceptance limits. In scenario a, the confidence bound includes the target, and thus one could conclude there is insufficient evidence to conclude a difference from target (the significance test approach). However, although the *point estimate* (the solid diamond) falls within the acceptance range, the interval extends outside the range, which signifies that the true relative bias may be outside the acceptable range. In scenario b, the interval falls within the acceptance range, signifying conformance to the acceptance criterion. The interval in scenario c also falls within the acceptance range but excludes the target. Thus, for scenario c, although the difference of the point estimate from the target is statistically significant, c is acceptable because the confidence interval falls within the target acceptance limits.

Using the 90% confidence interval calculated previously, we can establish whether the bioassay has acceptable relative bias at the 1.00 level compared to a target acceptance criterion of no greater than +12%, for example. Because the 90% confidence interval for percent relative bias (0.07%, 10.1%) falls within the interval $(100 \cdot [(1/1.12) - 1]\%, 100 \cdot [(1.12/1) - 1]\%) = (-11\%, 12\%)$, we conclude that there is acceptable relative bias at the 1.00 level. Note that a 90% confidence interval is used in an equivalence test rather than a conventional 95% confidence interval. This is common practice and is the same as the *two one-sided tests* (TOST) approach used in pharmaceutical bioequivalence testing.

Risks in Decision-Making and Number of Validation Runs

The application of statistical tests, including the assessment of conformance of a validation parameter to its acceptance criteria, involves risks. One risk is that the parameter does not meet its acceptance criterion although the property associated with that parameter is satisfactory; another, the converse, is that the parameter meets its acceptance criterion although the parameter is truly unsatisfactory. A consideration related to these risks is sample size.

The two types of risk can be simultaneously controlled via strategic design, including choice of the number of *runs* that will be conducted in the validation. Specifically, the minimum number of *runs* needed to establish conformance to an acceptance criterion for relative bias is given by:

$$n \geq \frac{(t_{\alpha, df} + t_{\beta/2, df})^2 \hat{\sigma}_{IP}^2}{\theta^2}$$

where $t_{\alpha, df}$ and $t_{\beta/2, df}$ are distributional points from a Student's t-distribution; α and β are the one-sided type I and type II errors, and represent the risks associated with drawing the wrong conclusion in the validation; df is the degrees of freedom associated with the study design (usually $n - 1$);

$$\hat{\sigma}_{IP}^2$$

is a preliminary estimate of IP ; and θ is the acceptable deviation (target acceptance criterion).

For example, if the acceptance criterion for relative bias is $\pm 0.11 \log$ (i.e., $\theta = 0.11$), the bioassay variability is

$$\hat{\sigma}_{IP} = 0.076 \log$$

and $\alpha = \beta = 0.05$,

$$n \geq \frac{(1.89 + 2.36)^2 0.076^2}{0.11^2} \approx 8 \text{ runs}$$

Note that this formulation of sample size assumes no intrinsic bias in the bioassay. A more conservative solution includes some nonzero bias in the determination of a sample size. This results in a greater sample size to offset the impact of the bias on the conclusions of the validation. In the current example the sample size increases to 10 runs if one assumes an intrinsic bias equal to 2%. Note also that this calculation represents a recursive solution (because the degrees of freedom depend on n) requiring statistical software or an algorithm that employs iterative methodology.

Note further that the selection of α and β should be justified on the basis of the corresponding risks of drawing the wrong conclusion from the validation.

Modeling Validation Results Using Mixed Effects

Models—Many analyses associated with bioassay validation must account for multiple design factors such as *fixed effects* (e.g., potency level), as well as *random effects* (e.g., analyst, run, and replicate). Statistical models composed of both *fixed* and *random effects* are called *mixed effects models* and usually require sophisticated statistical software for analysis. The results of the analysis may be summarized in an *analysis of variance* (ANOVA) table or a table of variance component estimates. The primary goal of the analysis is to estimate critical parameters rather than establish the significance of an effect. The modeling output provides parameter estimates together with their *standard errors* of estimates that can be utilized to establish conformance of a validation parameter to its acceptance criterion. Thus the average *relative bias* at each level is obtained as a portion of the analysis together with its associated variability. These compose a *confidence interval* that is compared to the acceptance criterion as described above. If variances across levels can be

pooled, statistical modeling can also determine the overall *relative bias* and IP by combining information across levels performed in the validation. Similarly, mixed effects models can be used to obtain variance components for validation study factors and to combine results across validation study samples and levels.

Statistical Design—Statistical designs, such as multifactor DOE or *nesting*, can be used to organize assay and runs in a bioassay validation. It is useful to incorporate factors that are believed to influence the bioassay response and that vary during long-term use of the procedure into these designs. Using these methods of design, the sources of variability may be characterized and a strategic test plan to manage the variability of the bioassay may be developed.

Table 2 shows an example of a multifactor DOE that incorporates multiple analysts, multiple cell culture preparations, and multiple reagent lots into the validation plan.

Table 2. Example of a Multifactor DOE with 3 Factors

Run	Analyst	Cell Prep	Reagent Lot
1	1	1	1
2	1	1	2
3	1	2	1
4	1	2	2
5	2	1	1
6	2	1	2
7	2	2	1
8	2	2	2

In this design each analyst performs the bioassay with both cell preparations and both reagent lots. This is an example of a *full factorial* design because all combinations of the factors are performed in the validation study. To reduce the number of runs in the study, *fractional factorial* designs may be employed when more than three factors have been identified. For example, if it is practical for an analyst to perform four assays in a run, a split-unit design could be used with analysts as the whole-plot factor and cell preparation and reagent lot as sub-plot factors. Unlike screening experiments, the validation design should incorporate as many factors at as many levels as possible in order to obtain a representative estimate of IP. More than two levels of a factor should be employed in the design whenever possible. This may be accomplished in a less structured manner, without regard to strict factorial layout. Validation runs should be randomized whenever possible to mitigate the potential influences of run order or time.

Figure 2 illustrates an example of a validation using *nesting* (replicates nested within plate, plate nested within analyst).

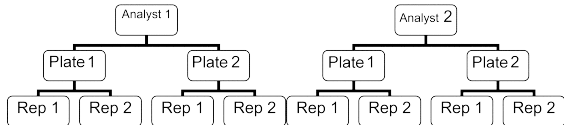


Figure 2. Example of a nested design using two analysts.

For both of these types of design as well as combinations of the two, components of variability can be estimated from the validation results. These components of variability can be used to identify significant sources of variability as well as to derive a bioassay *format* that meets the procedure’s requirements for precision. It should be noted that significant sources of variability may have been identified during bioassay development. In this case the validation should confirm both the impact of these factors and the assay format that meets the requirement for precision.

Significant Figures—The number of significant figures in a reported result from a bioassay is related to the latter’s precision. In general, a bioassay with %GCV between 2% and 20% will support two significant figures. The number of sig-

nificant figures should not be confused with the number of decimal places—reported values equal to 1.2 and 0.12 have the same number (two) of significant figures. This standard of rounding is appropriate for log scaled measurements that have constant variation on the log scale and proportional rather than additive variability on the original scale (or the scale commonly used for interpretation). Note that rounding occurs at the end of a series of calculations when the final measurement is reported and used for decision making such as conformance to specifications. Thus if the final measurement is a reportable value from multiple assays, rounding should not occur prior to determination of the reportable value. Likewise, specifications should be stated with the appropriate number of significant figures.

3. A BIOASSAY VALIDATION EXAMPLE

An example illustrates the principles described in this chapter. The bioassay will be used to support a specification range of 0.71 to 1.41 for the product. Using the Cpm described in section 2.5 *Validation Target Acceptance Criteria*, a table is derived showing the projected rate of OOS results for various restrictions on RB and IP. Cpm is calculated on the basis of the variability of a reportable value using three independent runs of the bioassay (see discussion of format variability, above). Product variability is assumed to be equal to 0 in the calculations. The laboratory may wish to include target product variability. An estimate of target product variability can be obtained from data from a product, for example, manufactured by a similar process.

Table 3. Cpm and Probability of OOS for Various Restrictions on RB and IP

LSL-USL	IP (%)	RB (%)	Cpm	Prob(OOS) (%)
0.71–1.41	20	20	0.54	10.5
0.71–1.41	8	12	0.94	0.48
0.71–1.41	10	5	1.55	0.0003

The calculation is illustrated for IP equal to 8% and relative bias equal to 12% (n = 3 runs):

Cpm = (ln(1.41) – ln(0.71)) / (6 * sqrt([ln(1.08)]^2 / 3 + [ln(1.12)]^2)) = 0.94

Prob(OOS) = 2 * Φ(–3 * 0.94) = 0.0048 (0.48%),

where Φ represents the standard normal cumulative distribution function.

From Table 3, acceptable performance (less than 1% chance of obtaining an OOS result due to bias and variability of the bioassay) can be expected if the IP is ≤8% and relative bias is ≤12%. The sample size formula given in section 2.7 *Statistical Considerations, Risks in Decision-Making and Number of Validation Runs* can be used to derive the number of runs required to establish conformance to an acceptance criterion for relative bias equal to 12% (using %GCV_{IP} = 8%; α = β = 0.05):

n ≥ ((1.89 + 2.36)^2 * [ln(1.08)]^2) / [ln(1.12)]^2 ≈ 8 runs

Thus eight runs would be needed in order to have a 95% chance of passing the target acceptance criterion for relative bias if the true relative bias is zero. Note that the calculation of sample size assumes that a singlet of the validation samples will be performed in each validation run. The use of

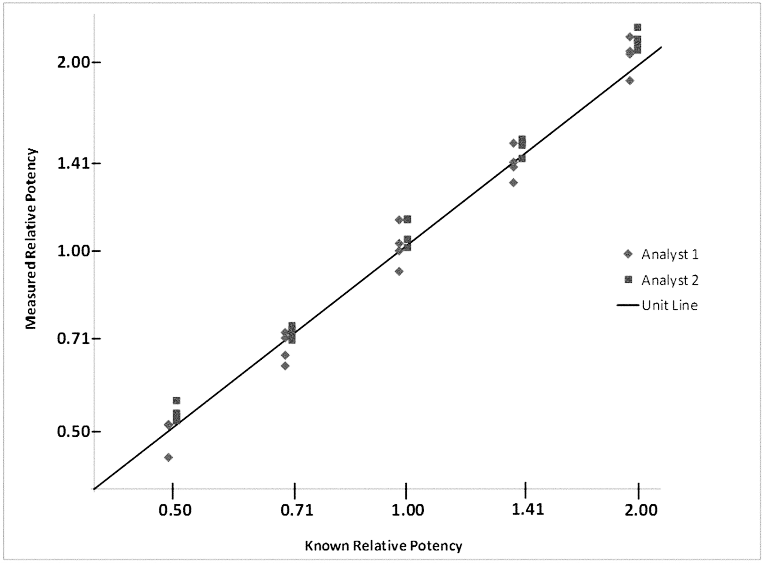


Figure 3. A plot of the validation results versus the sample levels.

multiple replication sets and/or multiple assays will provide valuable information that allows separate estimates for intra-run and inter-run variability, and will decrease the risk of failing to meet the validation target acceptance criteria.

Five levels of the target analyte are studied in the validation: 0.50, 0.71, 1.00, 1.41, and 2.00. Two runs at each level are generated by two trained analysts using two media lots. Other factors may be considered and incorporated into the design using a fractional factorial layout. The laboratory should strive to design the validation with as many levels of each factor as possible in order to best model the long-term performance of the bioassay. In this example each analyst performs two runs at each level using each media lot. A run consists of a full dilution series of the Standard as described in the bioassay’s operating procedure, together with two independent dilution series of the Test sample. This yields duplicate measurements of relative potency in each run; see Table 4 for all relative potency observations. Note that the two potency estimates at each level of potency in a run are not independent due to common analysts and media lots.

A plot is used to reveal irregularities in the experimental results. In particular, a properly prepared plot can reveal a failure in agreement of validation results with validation levels, as well as *heterogeneity of variability* across levels (see discussion of the log transformation in section 2.7 *Statistical Considerations*). The example plot in Figure 3 includes the unit line (line with slope equal to 1, passing through the origin). The analyst 1 and analyst 2 data are deliberately

offset with respect to the expected potency to allow clear visualization and comparison of the data sets from each analyst.

A formal analysis of the validation data might be undertaken in the following steps: (1) an assessment of variability (IP) should precede an assessment of relative accuracy or specificity in order to establish conformance to the assumption that variances across sample levels can be pooled; and (2) *relative accuracy* is assessed either at separate levels or by a combined analysis, depending on how well the data across levels can be pooled. These steps are demonstrated using the example validation data, along with some details of the calculations for illustrative purposes. Note that the calculations illustrated in the following sections are appropriate only with a balanced dataset. Imbalanced designs or datasets with missing relative potency measurements should be analyzed using a mixed model analysis with restricted maximum likelihood estimation (REML).

3.1 Intermediate Precision

Data at each level can be analyzed using *variance component analysis*. With balanced data, as in this example, variance components can be determined from a standard one-way ANOVA. An example of the calculation performed at a single level (0.50) is presented in Table 5.

Table 4. Example of Bioassay Validation with Two Analysts, Two Media Lots, and Runs per Level for Each Combination of Analyst and Lot

Media Lot/Analyst	1/1		1/2		2/1		2/2	
	1	2	1	2	1	2	1	2
0.50	0.5215	0.4532	0.5667	0.5054	0.5222	0.5179	0.5314	0.5112
0.50	0.5026	0.4497	0.5581	0.5350	0.5017	0.5077	0.5411	0.5488
0.71	0.7558	0.6689	0.6843	0.7050	0.6991	0.7463	0.6928	0.7400
0.71	0.7082	0.6182	0.8217	0.7143	0.6421	0.6877	0.7688	0.7399
1.00	1.1052	0.9774	1.1527	0.9901	1.0890	1.0314	1.1459	1.0273
1.00	1.1551	0.8774	1.1074	1.0391	0.9233	1.0318	1.1184	1.0730
1.41	1.5220	1.2811	1.5262	1.4476	1.4199	1.3471	1.4662	1.5035
1.41	1.5164	1.3285	1.5584	1.4184	1.4025	1.4255	1.5495	1.5422
2.00	2.3529	1.8883	2.3501	2.2906	2.2402	2.1364	2.3711	2.0420
2.00	2.2307	1.9813	2.4013	2.1725	2.0966	2.1497	2.1708	2.3126

Table 5. Variance Component Analysis Performed on Log Relative Potency Measurements at the 0.5 Level

Source	df	Sum of Squares	Mean Square	Expected Mean Square
Run	7	0.055317	0.007902	Var(Error) + 2 Var(Run)
Error	8	0.006130	0.000766	Var(Error)
Corrected total	15	0.061447		
Variance Component Estimates				
Var(Run) = 0.003568				
Var(Error) = 0.000766				

The top of the table represents a standard ANOVA analysis. Analyst and media lot have not been included because of the small number of levels (2 levels) for each factor. The factor "Run" in this analysis represents the combined runs across the analyst by media lot combinations. The Expected Mean Square is the linear combination of variance components that generates the measured *mean square* for each source. The variance component estimates are derived by solving the equation "Expected Mean Square = Mean Square" for each component. To start, the *mean square* for Error estimates Var(Error), the within-run component of variability, is

$$\text{Var(Error)} = \text{MS(Error)} = 0.000766$$

The between-run component of variability, Var(Run), is subsequently calculated by setting the mean square for Run to the mathematical expression for the expected mean square, then solving the equation for Var(Run) as follows:

$$\begin{aligned} \text{MS(Run)} &= \text{Var(Error)} + 2 \cdot \text{Var(Run)} \\ \text{Var(Run)} &= \frac{\text{MS(Run)} - \text{MS(Error)}}{2} \\ &= \frac{0.007902 - 0.000766}{2} = 0.003568 \end{aligned}$$

These *variance component estimates* are combined to establish the overall IP of the bioassay at 0.50:

$$\begin{aligned} \text{IP} &= 100 \cdot \left(e^{\sqrt{\text{Var(Run)} + \text{Var(Error)}}} - 1 \right) \% \\ &= 100 \cdot \left(e^{\sqrt{0.003568 + 0.000766}} - 1 \right) \% = 6.8\% \end{aligned}$$

The same analysis was performed at each level of the validation, and is presented in *Table 6*.

A combined analysis can be performed if the variance components are similar across levels. Typically a heuristic method is used for this assessment. One might hold the ratio of the maximum variance to the minimum variance to no greater than 10 (10 is used because of the limited number of runs performed in the validation). Here the ratios associated with the between-run variance component, $0.003639/0.000648 = 5.6$, and the within-run component, $0.004303/0.000577 = 7.5$, meet the 10-fold criterion. Had the ratio exceeded 10 and if this was due to excess variability in one or the other of the extremes in the levels tested,

that extreme would be eliminated from further analysis and the range would be limited to exclude that level.

The analysis might proceed using statistical software that is capable of applying a *mixed effects model* to the validation results. That analysis should account for any imbalance in the design, random effects such as analyst and media lot, and fixed effects such as level (see section 2.7 *Statistical Considerations, Modeling Validation Results Using Mixed Effects Models*). Variance components can be determined for analyst and media lot separately in order to characterize their contributions to the overall variability of the bioassay.

In the example, variance components can be averaged across levels to report the IP of the bioassay. This method of combining estimates is exact only if a balanced design has been employed in the validation (i.e., the same replication strategy at each level). A balanced design was employed for the example validation, so the IP can be reported as 7.2% GCV.

Because of the recommendation to report validation results with some measure of uncertainty, a one-sided 95% upper confidence bound can be calculated for the IP of the bioassay. The literature contains methods for calculating confidence bounds for variance components. The upper bound on IP for the bioassay example is 11.8% GCV. The upper confidence bound was not calculated at each level separately because of the limited data at an individual level relative to the overall study design.

3.2 Relative Accuracy

The analysis might proceed with an assessment of relative accuracy at each level. *Table 7* shows the average and 90% confidence interval of validation results in the log scale, as well as corresponding potency and relative bias.

The analysis has been performed on the average of the duplicates from each run ($n = 8$ runs) because duplicate measurements are correlated within a run by shared IP factors (analyst, media lot, and run in this case). A plot of relative bias versus level can be used to examine patterns in the experimental results and to establish conformance to the target acceptance criterion for relative bias (12%).

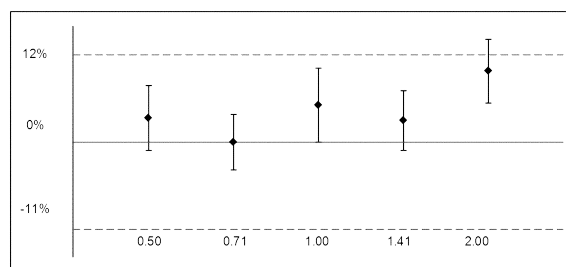


Figure 4. Plot of 90% confidence intervals for relative bias versus the acceptance criterion. Note lower acceptance criterion is equal to $100 \cdot [(1/1.12) - 1] = -11\%$.

Figure 4 shows an average positive bias across sample levels (i.e., the average relative bias is positive at all levels). This consistency is due in part to the lack of independence of bioassay results across levels. In addition there does not appear to be a trend in relative bias across levels. The latter would indicate that a comparison of samples with different

Table 6. Variance Component Estimates and Overall Variability for Each Validation Level and the Average

Component	Level					
	0.50	0.71	1.00	1.41	2.00	Average
Var(Run)	0.003568	0.000648	0.003639	0.003135	0.002623	0.002723
Var(Error)	0.000766	0.004303	0.002954	0.000577	0.002258	0.002172
Overall	6.8%	7.3%	8.5%	6.3%	7.2%	7.2%

Table 7. Average Potency and Relative Bias at Individual Levels

Level	n ^a	Log Potency		Potency		Relative Bias	
		Average	(90% CI)	Average	(90% CI)	Average	(90% CI)
0.50	8	-0.6613	(-0.7034, -0.6192)	0.52	(0.49, 0.54)	3.23%	(-1.02, 7.67)
0.71	8	-0.3419	(-0.3773, -0.3064)	0.71	(0.69, 0.74)	0.06%	(-3.42, 3.67)
1.00 ^b	8	0.0485	(0.0006, 0.0964)	1.05	(1.00, 1.10)	4.97%	(0.06, 10.12)
1.41	8	0.3723	(0.3331, 0.4115)	1.45	(1.40, 1.51)	2.91%	(-1.04, 7.03)
2.00	8	0.7859	(0.7449, 0.8269)	2.19	(2.11, 2.29)	9.72%	(5.31, 14.32)

^aAnalysis performed on averages of duplicates from each run.

^bCalculation illustrated in section 2.7 *Statistical Considerations, Scale of Analysis*.

measured relative potency (such as stability samples) is biased, resulting perhaps in an erroneous conclusion. Trend analysis can be performed using a regression of log relative potency versus log level. Introduction during the development of the bioassay validation protocol of an acceptance criterion on a trend in relative accuracy across the range can be considered.

After establishing that there is no meaningful trend across levels, the analysis proceeds with an assessment of the relative accuracy at each level. The bioassay has acceptable relative bias at levels from 0.50 to 1.41, yielding 90% confidence bounds (equivalent to a *two one-sided t-test*) that fall within the acceptance region of -11% to 12% relative bias. The 90% confidence interval at 2.0 falls outside the acceptance region, indicating that the relative bias may exceed 12%.

A combined analysis can be performed utilizing statistical software that is capable of applying a *mixed effects model* to the validation results. That analysis accurately accounts for the validation study design. The analysis also accommodates *random effects* such as analyst, media lot, and run (see section 2.7 *Statistical Considerations, Modeling Validation Results Using Mixed Effects Models*).

3.3 Range

The conclusions derived from the assessment of IP and *relative accuracy* can be used to establish the bioassay's range that demonstrates satisfactory performance. Based on the acceptance criterion for IP equal to 8% GCV (see Table 6) and for relative bias equal to 12% (see Table 7), the range of the bioassay is 0.50 to 1.41. In this range, level 1.0 has a slightly higher than acceptable estimate of IP (8.5% versus the target acceptance criterion ≤8.0%), which may be due to the variability of the estimate that results from a small dataset. Because of this and other results in Table 6, one may conclude that satisfactory IP was demonstrated across the range.

3.4 Use of Validation Results for Bioassay Characterization

When the study has been performed to estimate the characteristics of the bioassay (characterization), the *variance component estimates* can also be used to predict the variability for different bioassay *formats* and thereby can determine a format that has a desired level of precision. The predicted variability for *k* independent *runs*, with *n* individual dilution series of the test preparation within a run, is given by the following formula for *format variability*:

$$\text{Format Variability} = 100 \cdot (e^{\sqrt{\text{Var}(\text{Run})/k + \text{Var}(\text{Error})/(nk)}} - 1)$$

Using estimates of intra-run and inter-run variance components from Table 6 [Var(Run) = 0.002723 and Var(Error) = 0.002172], if the bioassay is performed in three indepen-

dent *runs*, the predicted variability of the *reportable value* (geometric mean of the relative potency results) is equal to:

$$\text{Format Variability} = 100 \cdot (e^{\sqrt{0.002723/3 + 0.002172/(1 \cdot 3)}} - 1) = 4.1\%$$

This calculation can be expanded to include various combinations of *runs* and *minimal* sets (assuming that the numbers of samples, dilutions, and replicates in the minimal sets are held constant) within *runs* as shown in Table 8.

Table 8. Format Variability for Different Combinations of Number of Runs (k) and Number of Minimal Sets within Run (n)

Reps (n)	Number of Runs (k)			
	1	2	3	6
1	7.2%	5.1%	4.1%	2.9%
2	6.4%	4.5%	3.6%	2.6%
3	6.0%	4.2%	3.4%	2.4%
6	5.7%	4.0%	3.3%	2.3%

Clearly the most effective means of reducing the variability of the *reportable value* (the geometric mean potency across runs and minimal sets) is by independent runs of the bioassay procedure. In addition, confidence bounds on the variance components used to derive IP can be utilized to establish the bioassay's format variability.

Significant sources of variability must be incorporated into runs in order to effect variance reduction. A more thorough analysis of the bioassay validation example would include analyst and media lot as factors in the statistical model. Variance component estimates obtained from such an analysis are presented in Table 9.

Table 9. REML Estimates of Variance Components Associated with Analyst, Media Lot, and Run

Variance	Component Estimate
Var(Media Lot)	0.0000
Var(Analyst)	0.0014
Var(Analyst*Media Lot)	0.0000
Var(Run (Analyst*Media Lot))	0.0019
Var(Error)	0.0022

Identification of analyst as a significant bioassay factor should ideally be addressed during bioassay development. Nonetheless the laboratory may choose to address the apparent contribution of analyst-to-analyst variability through improved training or by using multiple analysts in formatting the assay for routine performance of the bioassay.

Estimates of intra-run and inter-run variability can also be used to determine the sizes of differences (fold difference) that can be distinguished between samples tested in the bioassay. For *k* runs, with *n* minimal sets within each run, using an approximate two-sided critical value from the standard normal distribution with *z* = 2, the critical fold differ-

ence between reportable values for two samples that are tested in the same runs of the bioassay is given by:

$$\text{Critical Fold Difference} = e^2 \cdot \sqrt{\text{Var}(\text{Run})/k + \text{Var}(\text{Error})/(nk)}$$

When samples have been tested in different runs of the bioassay (such as long-term stability samples), the critical fold difference is given by (assuming the same format is used to test the two series of samples):

$$\text{Critical Fold Difference} = e^2 \cdot \sqrt{2 \cdot [\text{Var}(\text{Run})/k + \text{Var}(\text{Error})/(nk)]}$$

For comparison of samples the laboratory can choose a design (bioassay format) that has suitable precision to detect a practically meaningful fold difference between samples.

3.5 Confirmation of Intermediate Precision and Revalidation

The estimate of IP from the validation is highly uncertain because of the small number of runs performed. After the laboratory gains suitable experience with the bioassay, the estimate can be confirmed or updated by analysis of control sample measurements such as the variability of a positive control. This analysis can be done with the control prepared and tested like a Test sample (i.e., same or similar dilution series and replication strategy). This assessment should be made after sufficient assays have been performed to obtain an alternative estimate of the bioassay's intermediate precision, including implementation of changes (e.g., different analysts, different key reagent lots, and different cell preparations) associated with the standardized assay protocol. The reported IP of the bioassay should be modified as an amendment to the validation report if the assessment reveals a substantial disparity of results.

The bioassay should be revalidated whenever a substantial change is made to the method. This includes but is not limited to a change in technology or a change in readout. The revalidation may consist of a complete re-enactment of the bioassay validation or a bridging study that compares the current and the modified methods.

4. ADDITIONAL SOURCES OF INFORMATION

Additional information and alternative methods can be found in the references listed below.

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3. Burdick R, Graybill F. *Confidence Intervals on Variance Components*. New York: Marcel Dekker; 1992:28–39.
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APPENDIX—MEASURES OF LOCATION AND SPREAD FOR LOG NORMALLY DISTRIBUTED VARIABLES

Two assumptions of common statistical procedures, such as ANOVA or confidence interval estimation, are (1) the vari-

ation in the bioassay response about its mean is normally distributed and (2) the standard deviation of the observed response values is constant over the range of responses that are of interest. Such responses are said to have a “normal distribution” and an “additive error structure”. When these two conditions are not met, it may be useful to consider a transformation before using common statistical procedures.

The variation in bioassay responses is often found to be non-normal (skewed toward higher values) with a standard deviation approximately proportional (or nearly so) to the mean response. Such responses often have a “multiplicative error structure” and follow a “log normal distribution” with a percent coefficient of variation (%CV) that is constant across the response range of interest. In such cases, a log transformation of the bioassay response will be found to be approximately normal with a nearly constant standard deviation over the response range. After log transformation, then, the two assumptions are met, and common statistical procedures can be performed on the log transformed response. The following discussion presumes a log normal distribution for the bioassay response.

We refer to an observed bioassay response value, X , as being on the “original scale of measurement” and to the log transformed response, $Y = \log(X)$, as being on the “log transformed scale”. Although common statistical procedures may be appropriate only on the log transformed scale, we can summarize bioassay response results by estimating measures of location (e.g., mean or median), measures of spread (e.g., standard deviation), or confidence intervals on either scale of measurement, as long as the scale being used is indicated. The %CV is useful on the original scale where it is constant over the response range. For the same reason, the standard deviation (SD) is relevant on the log transformed scale. There may be advantages to reporting statistical summaries on the basis of the log transformed (Y) scale. However, it is often informative to back transform the reported measures to the original scale of measurement (X).

For any given value of X , there is only one unique value of $Y = \log(X)$, and vice versa. Similarly for measures of location and spread, there is a unique one-to-one correspondence between measures of location and spread obtained on the original and log transformed scales. Further, just as there is a simple relationship between X and $Y = \log(X)$, there are relatively simple relationships that allow conversion between the corresponding measures on each scale, as indicated in Table A-1 below. In the table, “Average” and “SD”, wherever they appear, refer to measures calculated on the log transformed (Y) scale.

The geometric mean (GM) should not be misinterpreted as an estimate of the mean of the original scale (X) variable, but is instead an estimate of the median of X . The median is a more appropriate measure of location for variables with skewed error distributions such as the log normal, as well as symmetric error distributions where the median is equal to the mean.

Similarly, the geometric standard deviation (GSD) should not be misinterpreted as the standard deviation of the original scale (X) variable. GSD is, however, a useful multiplicative factor for obtaining confidence intervals on the original (X) scale that correspond to those on the log transformed (Y) scale, as shown in the above table. A GSD of 1 corresponds to no variation (SD of $Y = 0$). The ratio of the Upper to the Lower confidence bounds, on the untransformed (X) scale, will be equal to $\text{GSD}^{2k/\sqrt{n}}$, as can be seen from Table A-1.

The geometric coefficient of variation (%GCV) approximates the %CV on the original (X) scale when the %CV is below 20%. It is important not to confuse these different measures of spread. The %GCV is a measure relevant to the log transformed (Y) scale, and the %CV is a measure relevant to the original (X) scale. Depending on the preferred frame of reference, either or both measures may be useful.

Table A-1. Comparison of Measures of Location and Spread

Measure		Scale of Measurement	
		Log Transformed (V)	Original (X)
Location		Mean (average)	Geometric mean (GM) $= \sqrt[n]{\prod_{i=1}^n x_i} = e^{\text{Average}}$
Spread		Standard deviation (SD)	Geometric standard deviation (GSD) = e^{SD}
Confidence intervals (k is an appropriate constant based on the t-distribution or large sample z approximation)	Lower	Average – $k \cdot \text{SD}/\sqrt{n}$	$\text{GM}/\text{GSD}^{k/\sqrt{n}}$
	Upper	Average + $k \cdot \text{SD}/\sqrt{n}$	$\text{GM} \cdot \text{GSD}^{k/\sqrt{n}}$
	Size	Width (upper – lower) = $2 \cdot k \cdot \text{SD}/\sqrt{n}$	Ratio(upper/lower) = $\text{GSD}^{2k/\sqrt{n}}$
Percent coefficient of variation (%CV)		%GCV = $100 \cdot (\text{GSD} - 1)$	%CV = $100\sqrt{e^{\text{SD}^2} - 1} \approx \% \text{GCV}$

APPENDIX INFORMATION SOURCES

1. Limpert E, Stahel WA, Abbt M. (2001) Log-normal distributions across the sciences: keys and clues. *Bio-Science* 51(5): 341–252.
2. Kirkwood TBL. (1979) Geometric means and measures of dispersion. *Biometrics* 35: 908–909.
3. Bohidar NR. (1991) Determination of geometric standard deviation for dissolution. *Drug Development and Industrial Pharmacy* 17(10): 1381–1387.
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6. <1010> Analytical data: interpretation and treatment. USP 34. In: USP34–NF 29. Vol. 1. Rockville (MD): United States Pharmacopeial Convention; c2011. p. 419.
7. Tan CY. (2005) RSD and other variability measures of the lognormal distribution. *Pharmacopeial Forum* 31(2): 653–655.

Topics addressed in <1034> include statistical concepts and methods of analysis for the calculation of potency and confidence intervals for a variety of relative potency bioassays, including those referenced in *USP*. Chapter <1034> is intended for use primarily by those who do not have extensive training or experience in statistics and by statisticians who are not experienced in the analysis of bioassays. Sections that are primarily conceptual require only minimal statistics background. Most of the chapter and all the methods sections require that the nonstatistician be comfortable with statistics at least at the level of *USP* general chapter *Analytical Data—Interpretation and Treatment* <1010> and with linear regression. Most of sections 3.4 *Nonlinear Models for Quantitative Response* and 3.6 *Dichotomous (Quantal) Assays* require more extensive statistics background and thus are intended primarily for statisticians. In addition, <1034> introduces selected complex methods, the implementation of which requires the guidance of an experienced statistician. Approaches in <1034> are recommended, recognizing the possibility that alternative procedures may be employed. Additionally, the information in <1034> is presented assuming that computers and suitable software will be used for data analysis. This view does not relieve the analyst of responsibility for the consequences of choices pertaining to bioassay design and analysis.

<1034> ANALYSIS OF BIOLOGICAL ASSAYS

1. INTRODUCTION

Although advances in chemical characterization have reduced the reliance on bioassays for many products, bioassays are still essential for the determination of potency and the assurance of activity of many proteins, vaccines, complex mixtures, and products for cell and gene therapy, as well as for their role in monitoring the stability of biological products. The intended scope of general chapter *Analysis of Biological Assays* <1034> includes guidance for the analysis of results both of bioassays described in the *United States Pharmacopeia* (*USP*), and of non-USP bioassays that seek to conform to the qualities of bioassay analysis recommended by USP. Note the emphasis on analysis—design and validation are addressed in complementary chapters (*Development and Design of Bioassays* <1032> and *Biological Assay Validation* <1033>, respectively).

2. OVERVIEW OF ANALYSIS OF BIOASSAY DATA

Following is a set of steps that will help guide the analysis of a bioassay. This section presumes that decisions were made following a similar set of steps during development, checked during validation, and then not required routinely. Those steps and decisions are covered in general information chapter *Design and Development of Biological Assays* <1032>. Section 3 *Analysis Models* provides details for the various models considered.

1. As a part of the chosen analysis, select the subset of data to be used in the determination of the relative potency using the prespecified scheme. Exclude only data known to result from technical problems such as contaminated wells, non-monotonic concentration–response curves, etc.
2. Fit the statistical model for detection of potential outliers, as chosen during development, including any weighting and transformation. This is done first without assuming similarity of the Test and Standard curves but should include important elements of the design structure, ideally using a model that makes fewer assumptions about the functional form of the response than the model used to assess similarity.

3. Determine which potential outliers are to be removed and fit the model to be used for suitability assessment. Usually, an investigation of outlier cause takes place before outlier removal. Some assay systems can make use of a statistical (noninvestigative) outlier removal rule, but removal on this basis should be rare. One approach to "rare" is to choose the outlier rule so that the expected number of false positive outlier identifications is no more than one; e.g., use a 1% test if the sample size is about 100. If a large number of outliers are found above that expected from the rule used, that calls into question the assay.
4. Assess system suitability. System suitability assesses whether the assay Standard preparation and any controls behaved in a manner consistent with past performance of the assay. If an assay (or a run) fails system suitability, the entire assay (or run) is discarded and no results are reported other than that the assay (or run) failed. Assessment of system suitability usually includes adequacy of the fit of the model used to assess similarity. For linear models, adequacy of the model may include assessment of the linearity of the Standard curve. If the suitability criterion for linearity of the Standard is not met, the exclusion of one or more extreme concentrations may result in the criterion being met. Examples of other possible system suitability criteria include background, positive controls, max/min, max/background, slope, IC_{50} (or EC_{50}), and variation around the fitted model.
5. Assess sample suitability for each Test sample. This is done to confirm that the data for each Test sample satisfy necessary assumptions. If a Test sample fails sample suitability, results for that sample are reported as "Fails Sample Suitability." Relative potencies for other Test samples in the assay may still be reported. Most prominent of sample suitability criteria is similarity, whether parallelism for parallel models or equivalence of intercepts for slope-ratio models. For nonlinear models, similarity assessment involves all curve parameters other than EC_{50} (or IC_{50}).
6. For those Test samples in the assay that meet the criterion for similarity to the Standard (i.e., sufficiently similar concentration–response curves or similar straight-line subsets of concentrations), calculate relative potency estimates assuming similarity between Test and Standard, i.e., by analyzing the Test and Standard data together using a model constrained to have exactly parallel lines or curves, or equal intercepts.
7. A single assay is often not sufficient to achieve a reportable value, and potency results from multiple assays can be combined into a single potency estimate. Repeat steps 1–6 multiple times, as specified in the assay protocol or monograph, before determining a final estimate of potency and a confidence interval.
8. Construct a variance estimate and a measure of uncertainty of the potency estimate (e.g., confidence interval). See section 4 *Confidence Intervals*.

A step not shown concerns replacement of missing data. Most modern statistical methodology and software do not require equal numbers at each combination of concentration and sample. Thus, unless otherwise directed by a specific monograph, analysts generally do not need to replace missing values.

3. ANALYSIS MODELS

A number of mathematical functions can be successfully used to describe a concentration–response relationship. The first consideration in choosing a model is the form of the assay response. Is it a number, a count, or a category such as Dead/Alive? The form will identify the possible models that can be considered.

Other considerations in choosing a model include the need to incorporate design elements in the model and the possible benefits of means models compared to regression models. For purposes of presenting the essentials of the model choices, section 3 *Analysis Models* assumes a completely randomized design so that there are no design elements to consider and presents the models in their regression form.

3.1 Quantitative and Qualitative Assay Responses

The terms *quantitative* and *qualitative* refer to the nature of the response of the assay used in constructing the concentration–response model. Assays with either quantitative or qualitative responses can be used to quantify product potency. Note that the *responses* of the assay at the concentrations measured are not the relative potency of the bioassay. Analysts should understand the differences among responses, concentration–response functions, and relative potency.

A quantitative response results in a number on a continuous scale. Common examples include spectrophotometric and luminescence responses, body weights and measurements, and data calculated relative to a standard curve (e.g., cytokine concentration). Models for quantitative responses can be linear or nonlinear (see sections 3.2–3.5).

A qualitative measurement results in a categorical response. For bioassay, qualitative responses are most often quantal, meaning they entail two possible categories such as Positive/Negative, 0/1, or Dead/Alive. Quantal responses may be reported as proportions (e.g., the proportion of animals in a group displaying a property). Quantal models are presented in section 3.6. Qualitative responses can have more than two possible categories, such as end-point titer assays. Models for more than two categories are not considered in this general chapter.

Assay responses can also be counts, such as number of plaques or colonies. Count responses are sometimes treated as quantitative, sometimes as qualitative, and sometimes models specific to integers are used. The choice is often based on the range of counts. If the count is mostly 0 and rarely greater than 1, the assay may be analyzed as quantal and the response is Any/None. If the counts are large and cover a wide range, such as 500 to 2500, then the assay may be analyzed as quantitative, possibly after transformation of the counts. A square root transformation of the count is often helpful in such analyses to better satisfy homogeneity of variances. If the range of counts includes or is near 0 but 0 is not the preponderant value, it may be preferable to use a model specific for integer responses. Poisson regression and negative binomial regression models are often good options. Models specific to integers will not be discussed further in this general chapter.

Assays with quantitative responses may be converted to quantal responses. For example, what may matter is whether some defined threshold is exceeded. The model could then be quantal—threshold exceeded or not. In general, assay systems have more precise estimates of potency if the model uses all the information in the response. Using above or below a threshold, rather than the measured quantitative responses, is likely to degrade the performance of an assay.

3.2 Overview of Models for Quantitative Responses

In quantitative assays, the measurement is a number on a continuous scale. Optical density values from plate-based assays are such measurements. Models for quantitative assays can be linear or nonlinear. Although the two display an apparent difference in levels of complexity, parallel-line (linear) and parallel-curve (nonlinear) models share many common-

alities. Because of the different form of the equations, slope-ratio assays are considered separately (section 3.5 *Slope-Ratio Concentration–Response Models*).

Assumptions—The basic parallel-line, parallel-curve, and slope-ratio models share some assumptions. All include a residual term, e , that represents error (variability) which is assumed to be independent from measurement to measurement and to have constant variance from concentration to concentration and sample to sample. Often the residual term is assumed to have a normal distribution as well. The assumptions of independence and equal variances are commonly violated, so the goal in analysis is to incorporate the lack of independence and the unequal variances into the statistical model or the method of estimation.

Lack of independence often arises because of the design or conduct of the assay. For example, if the assay consists of responses from multiple plates, observations from the same plate are likely to share some common influence that is not shared with observations from other plates. This is an example of intraplate correlation. A simple approach for dealing with this lack of independence is to include a block term in the statistical model for plate. With three or more plates this should be a random effects term so that we obtain an estimate of plate-to-plate variability.

In general, the model needs to closely reflect the design. The basic model equations given in sections 3.3–3.5 apply only to completely randomized designs. Any other design will mean additional terms in the statistical model. For example, if plates or portions of plates are used as blocks, one will need terms for blocks.

Calculation of Potency—A primary assumption underlying methods used for the calculation of relative potency is that of similarity. Two preparations are similar if they contain the same effective constituent or same effective constituents in the same proportions. If this condition holds, the Test preparation behaves as a dilution (or concentration) of the Standard preparation. Similarity can be represented mathematically as follows. Let F_T be the concentration–response function for the Test, and let F_S be the concentration–response function for the Standard. The underlying mathematical model for similarity is:

$$F_T(z) = F_S(\rho z), \quad [3.1]$$

where z represents the concentration and ρ represents the relative potency of the Test sample relative to the Standard sample.

Methods for estimating ρ in some common concentration–response models are discussed below. For linear models, the distinction between parallel-line models (section 3.3 *Parallel-Line Models for Quantitative Response*) and slope-ratio models (section 3.5 *Slope-Ratio Concentration–Response Models*) is based on whether a straight-line fit to log concentration or concentration yields better agreement between the model and the data over the range of concentrations of interest.

3.3 Parallel-Line Models for Quantitative Responses

In this section, a linear model refers to a concentration–response relationship, which is a straight-line (linear) function between the logarithm of concentration, x , and the response, y . y may be the response in the scale as measured or a transformation of the response. The functional form of this relationship is $y = a + bx$. Straight-line fits may be used for portions of nonlinear concentration–response curves, although doing so requires a method for selecting the concentrations to use for each of the Standard and Test samples (see <1032>).

Means Model versus Regression—A linear concentration–response model is most often analyzed with least

squares regression. Such an analysis results in estimates of the unknown coefficients (intercepts and slope) and their standard errors, as well as measures of the goodness of fit [e.g., R^2 and root-mean-square error (RMSE)].

Linear regression works best where all concentrations can be used and there is negligible curvature in the concentration–response data. Another statistical method for analyzing linear concentration–response curves is the *means model*. This is an analysis of variance (ANOVA) method that offers some advantages, particularly when one or more concentrations from one or more samples are not used to estimate potency. Because a means model includes a separate mean for each unique combination of sample and dose (as well as block or other effects associated with the design structure) it is equivalent to a saturated polynomial regression model. Hence, a means model provides an estimate of error that is independent of regression lack of fit. In contrast, a regression residual based estimate of error is a mixture of the assay error, as estimated by the means model, combined with lack of fit of the regression model. At least in this sense, the means model error is a better estimate of the residual error variation in an assay system.

Parallel-Line Concentration–Response Models—If the general concentration–response model (3.1 *Quantitative and Qualitative Assay Responses*) can be made linear in $x = \log(z)$, the resulting equation is then:

$$y = \alpha + \beta \log(z) + e = \alpha + \beta x + e,$$

where e is the residual or error term, and the intercept, α , and slope, β , will differ between Test and Standard. With the parallelism (equal slopes) assumption, the model becomes

$$y_S = \alpha + \beta \log(z) + e = \alpha_S + \beta x + e \quad [3.2]$$

$$y_T = \alpha + \beta \log(\rho z) + e = [\alpha + \beta \log(\rho)] + \beta x + e = \alpha_T + \beta x + e,$$

where S denotes Standard, T denotes Test, $\alpha_S = \alpha$ is the y -intercept for the Standard, and $\alpha_T = \alpha + \beta \log(\rho)$ is the y -intercept for the Test (see Figure 3.1).

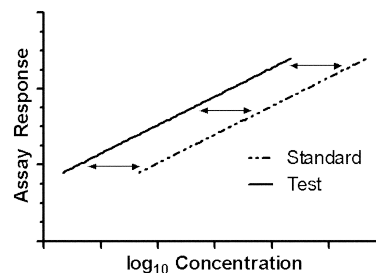


Figure 3.1. Example of parallel-line model.

Where concentration–response lines are parallel, as shown in Figure 3.1, a separation or horizontal shift indicates a difference in the level of biological activity being assayed. This horizontal difference is numerically $\log(\rho)$, the logarithm of the relative potency, and is found as the vertical distance between the lines α_T and α_S divided by the slope, β . The relative potency is then

$$\rho = \text{antilog} \left(\frac{\alpha_T - \alpha_S}{\beta} \right)$$

Estimation of Parallel-line Models—Parallel-line models are fit by the method of least squares. If the equal variance

assumption holds, the parameters of equation [3.2] are chosen to minimize

$$\sum (y - \hat{\alpha}_s - \hat{\delta}T - \hat{\beta}x)^2 \quad [3.3]$$

where the carets denote estimates. This is a linear regression with two independent variables, T and x , where T is a variable that equals 1 for observations from the Test and 0 for observations from the Standard. The summation in equation [3.3] is over all observations of the Test and Standard. If the equal variance assumption does not hold but the variance is known to be inversely proportional to a value, w , that does not depend on the current responses, the y 's, and can be determined for each observation, then the method is weighted least squares

$$\sum w(y - \hat{\alpha}_s - \hat{\delta}T - \hat{\beta}x)^2 \quad [3.4]$$

Equation 3.4 is appropriate only if the weights are determined without using the response, the y 's, from the current data (see (1032) for guidance in determining weights). In equations [3.3] and [3.4] β is the same as the β in equation [3.2] and $\delta = \alpha_T - \alpha_s = \beta \log \rho$. So, the estimate of the relative potency, ρ , is

$$\hat{\rho} = \text{antilog} \left(\frac{\hat{\delta}}{\hat{\beta}} \right)$$

Commonly available statistical software and spreadsheets provide routines for least squares. Not all software can provide weighted analyses.

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to $\hat{\delta}/\hat{\beta}$.

Measurement of Nonparallelism—Parallelism for linear models is assessed by considering the difference or ratio of the two slopes. For the difference, this can be done by fitting the regression model,

$$y = \alpha_s + \delta T + \beta_s x + \gamma x T + e$$

where $\delta = \alpha_T - \alpha_s$, $\gamma = \beta_T - \beta_s$, and $T = 1$ for Test data and $T = 0$ for Standard data. Then use the standard t-distribution confidence interval for γ . For the ratio of slopes, fit

$$y = \alpha_s + \delta T + \beta_s x(1 - T) + \beta_T x T + e$$

and use Fieller's Theorem, equation [4.3], to obtain a confidence interval for β_T/β_s .

3.4 Nonlinear Models for Quantitative Responses

Nonlinear concentration-response models are typically S-shaped functions. They occur when the range of concentrations is wide enough so that responses are constrained by upper and lower asymptotes. The most common of these models is the four-parameter logistic function as given below.

Let y denote the observed response and z the concentration. One form of the four-parameter logistic model is

$$y = D + \frac{A - D}{1 + \left(\frac{z}{C}\right)^B} + e \quad [3.5]$$

One alternative, but equivalent, form is

$$y = a_0 + \frac{d}{1 + \text{antilog} \left[M(\log z - b) \right]} + e$$

The two forms correspond as follows:

Lower asymptote: $D = a_0$

Upper asymptote: $A = a_0 + d$

Steepness: $B = M$ (related to the slope of the curve at the EC_{50})

Effective concentration 50% (EC_{50}): $C = \text{antilog}(b)$ (may also be termed ED_{50}).

Any convenient base for logarithms is suitable; it is often convenient to work in log base 2, particularly when concentrations are twofold apart.

The four-parameter logistic curve is symmetric around the EC_{50} when plotted against log concentration because the rates of approach to the upper and lower asymptotes are the same (see Figure 3.2). For assays where this symmetry does not hold, asymmetrical model functions may be applied. These models are not considered further in this general chapter.

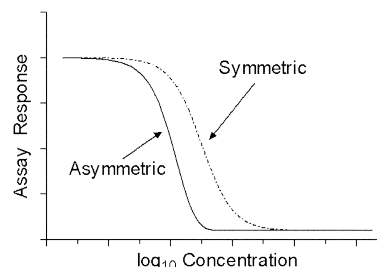


Figure 3.2. Examples of symmetric (four-parameter logistic) and asymmetric sigmoids.

In many assays the analyst has a number of strategic choices to make during assay development (see *Development and Design of Biological Assays* (1032)). For example, the responses could be modeled using a transformed response to a four-parameter logistic curve, or the responses could be weighted and fit to an asymmetric sigmoid curve. Also, it is often important to include terms in the model (often random effects) to address variation in the responses (or parameters of the response) associated with blocks or experimental units in the design of the assay. For simple assays where observations are independent, these strategic choices are fairly straightforward. For assays performed with grouped dilutions (as with multichannel pipets), assays with serial dilutions, or assay designs that include blocks (as with multiple plates per assay), it is usually a serious violation of the statistical assumptions to ignore the design structure. For such assays, a good approach involves a transformation that approximates a solution to non-constant variance, non-normality, and asymmetry combined with a model that captures the important parts of the design structure.

Parallel-Curve Concentration-Response Models—The concept of parallelism is not restricted to linear models. For nonlinear curves, parallel or similar means the concentration-response curves can be superimposed following a horizontal displacement of one of the curves, as shown in Figure 3.3 for four-parameter logistic curves. In terms of the parameters of equation [3.5], this means the values of A , D , and B for the Test are the same as for the Standard.

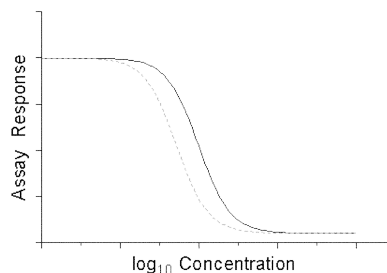


Figure 3.3. Example of parallel curves from a nonlinear model.

The equations corresponding to the figure (with error term, e , added) are

$$y_s = D + \frac{A - D}{1 + \left(\frac{z}{C}\right)^B} + e$$

$$y_T = D + \frac{A - D}{1 + \left(\frac{\rho z}{C}\right)^B} + e$$

or

$$y_s = D + \frac{A - D}{1 + \text{antilog} \left[\frac{M(\log z - b)}{1 + \log p} \right]} + e$$

$$y_T = D + \frac{A - D}{1 + \text{antilog} \left[\frac{M(\log z - b + \log p)}{1 + \log p} \right]} + e$$

Log p is the log of the relative potency and the horizontal distance between the two curves, just as for the parallel-line model. Because the EC_{50} of the standard is $\text{antilog}(b)$ and that of the Test is $\text{antilog}(b - \log p) = \text{antilog}(b)/p$, the relative potency is the ratio of EC_{50} 's (standard over Test) when the parallel-curve model holds.

Estimation of Parallel-Curve Models—Estimation of nonlinear, parallel-curve models is similar to that for parallel-line models, possibly after transformation of the response and possibly with weighting. For the four-parameter logistic model, the parameter estimates are found by minimizing:

$$\sum \left(y - \hat{D} - \frac{\hat{A} - \hat{D}}{1 + \text{antilog} \left[\frac{\hat{M}(\log z - \hat{b} + \hat{r}T)}{1 + \log p} \right]} \right)^2$$

without weighting, or

$$\sum w \left(y - \hat{D} - \frac{\hat{A} - \hat{D}}{1 + \text{antilog} \left[\frac{\hat{M}(\log z - \hat{b} + \hat{r}T)}{1 + \log p} \right]} \right)^2 \quad [3.6]$$

with weighting. (As for equation [3.4], equation [3.6] is appropriate only if the weights are determined without using the responses, y 's, from the current data.) In either case, the estimate of r is the estimate of the log of the relative potency. For some software, it may be easier to work with $d = A - D$.

The parameters of the four-parameter logistic function and those of the asymmetric sigmoid models cannot be found with ordinary (linear) least squares regression routines. Computer programs with nonlinear estimation techniques must be used.

Analysts should not use the nonlinear regression fit to assess parallelism or estimate potency if any of the following are present: a) inadequate asymptote information is available; or b) a comparison of pooled error(s) from nonlinear regression to pooled error(s) from a means model shows that the nonlinear model does not fit well; or c) other ap-

propriate measures of goodness of fit show that the nonlinear model is not appropriate (e.g., residual plots show evidence of a "hook").

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, advanced techniques, such as likelihood profiles or bootstrapping are needed to obtain a confidence interval for the log relative potency, r .

Measurement of Nonparallelism—Assessment of parallelism for a four-parameter logistic model means assessing the slope parameter and the two asymptotes. During development (see <1032>), a decision should be made regarding which parameters are important and how to measure nonparallelism. As discussed in <1032>, the measure of non-similarity may be a composite measure that considers all parameters together in a single measure, such as the parallelism sum of squares (see <1032>), or may consider each parameter separately. In the latter case, the measure may be functions of the parameters, such as an asymptote divided by the difference of asymptotes or the ratio of the asymptotes. For each parameter (or function of parameters), confidence intervals can be computed by bootstrap or likelihood profile methods. These methods are not presented in this general chapter.

3.5 Slope-Ratio Concentration-Response Models

If a straight-line regression fits the nontransformed concentration-response data well, a slope-ratio model may be used. The equations for the slope-ratio model assuming similarity are then:

$$y_s = \alpha + \beta z + e = \alpha + \beta_s z + e \quad [3.7]$$

$$y_T = \alpha + \beta(\rho z) + e = \alpha + \beta_T \rho z + e = \alpha + \beta_T z + e$$

An identifying characteristic of a slope-ratio concentration-response model that can be seen in the results of a ranging study is that the lines for different potencies from a ranging study have the same intercept and different slopes. Thus, a graph of the ranging study resembles a fan. Figure 3.4 shows an example of a slope-ratio concentration-response model. Note that the common intercept need not be at the origin.

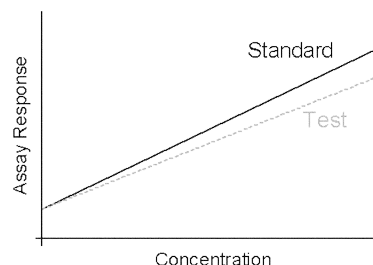


Figure 3.4. Example of slope-ratio model.

An assay with a slope-ratio concentration-response model for measuring relative potency consists, at a minimum, of one Standard sample and one Test sample, each measured at one or more concentrations and, usually, a measured response with no sample (zero concentration). Because the concentrations are not log transformed, they are typically equally spaced on the original, rather than log, scale. The model consists of one common intercept, a slope for the Test sample results, and a slope for the Standard sample

results as in equation [3.7]. The relative potency is then found from the ratio of the slopes:

$$\text{Relative Potency} = \frac{\text{Test sample slope/Standard sample slope}}{\beta_T/\beta_S = \rho}$$

Assumptions for and Estimation of Slope-Ratio Models—The assumptions for the slope-ratio model are the same as for parallel-line models: The residual terms are independent, have constant variance, and may need to have a normal distribution. The method of estimation is also least squares. This may be implemented either with or without weighting, as demonstrated in equations [3.8] and [3.9], respectively.

$$\sum (y - \hat{\alpha} - \hat{\beta}_S z(1-T) - \hat{\beta}_T zT)^2 \quad [3.8]$$

$$\sum w(y - \hat{\alpha} - \hat{\beta}_S z(1-T) - \hat{\beta}_T zT)^2 \quad [3.9]$$

Equation [3.9] is appropriate only if the weights are determined without using the response, the y 's, from the current data. This is a linear regression with two independent variables, $z(1-T)$ and zT , where $T = 1$ for Test data and $T = 0$ for Standard data. $\hat{\beta}_T$ is the estimated slope for the Test, $\hat{\beta}_S$ the estimated slope for the Standard, and then the estimate of relative potency is

$$R = \frac{\hat{\beta}_T}{\hat{\beta}_S}$$

Because the slope-ratio model is a linear regression model, most statistical packages and spreadsheets can be used to obtain the relative potency estimate. In some assay systems, it is sometimes appropriate to omit the zero concentration (e.g., if the no-dose controls are handled differently in the assay) and at times one or more of the high concentrations (e.g., if there is a hook effect where the highest concentrations do not have the highest responses). The discussion about using a means model and selecting subsets of concentrations for straight parallel-line bioassays applies to slope-ratio assays as well.

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to

$$\hat{\beta}_T / \hat{\beta}_S$$

Measurement of Nonsimilarity—For slope-ratio models, statistical similarity corresponds to equal intercepts for the Standard and Test. To assess the similarity assumption it is necessary to have at least two nonzero concentrations for each sample. If the intercepts are not equal, equation [3.7] becomes

$$\begin{aligned} y_S &= \alpha_S + \beta_S z + e \\ y_T &= \alpha_T + \beta_T z + e \end{aligned}$$

Departure from similarity is typically measured by the difference of intercepts, $\alpha_T - \alpha_S$. An easy way to obtain a confidence interval is to fit the model,

$$y = \alpha_S + \delta T + \beta_S z(1-T) + \beta_T zT + e,$$

where $\delta = \alpha_T - \alpha_S$ and use the standard t-distribution-based confidence interval for δ .

3.6 Dichotomous (Quantal) Assays

For quantal assays the assay measurement has a dichotomous or binary outcome, e.g., in animal assays the animal is dead or alive or a certain physiologic response is or is not observed. For cellular assays, the quantal response may be whether there is or is not a response beyond some threshold in the cell. In cell-based viral titer or colony-forming assays, the quantal response may be a limit of integer response such as an integer number of particles or colonies. When one can readily determine if any particles are present—but not their actual number—then the assay can be analyzed as quantal. Note that if the reaction can be quantitated on a continuous scale, as with an optical density, then the assay is not quantal.

Models for Quantal Analyses—The key to models for quantal responses is to work with the probability of a response (e.g., probability of death), in contrast to quantitative responses for which the model is for the response itself. For each concentration, z , a treated animal, as an example, has a probability of responding to that concentration, $P(z)$. Often the curve $P(z)$ can be approximated by a sigmoid when plotted against the logarithm of concentration, as shown in Figure 3.5. This curve shows that the probability of responding increases with concentration. The concentration that corresponds to a probability of 0.5 is the EC_{50} .

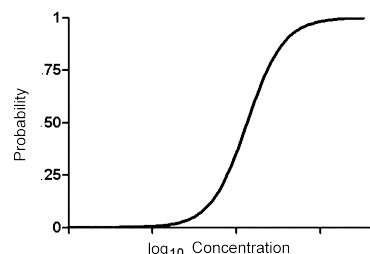


Figure 3.5. Example of sigmoid for $P(z)$.

The sigmoid curve is usually modeled based on the normal or logistic distribution. If the normal distribution is used, the resulting analysis is termed probit analysis, and if the logistic is used the analysis is termed logit or logistic analysis. The probit and logit models are practically indistinguishable, and either is an acceptable choice. The choice may be based on the availability of software that meets the laboratory's analysis and reporting needs. Because software is more commonly available for logistic models (often under the term logistic regression) this discussion will focus on the use and interpretation of logit analysis. The considerations discussed in this section for logit analysis (using a logit transformation) apply as well to probit analysis (using a probit transformation).

Logit Model—The logit model for the probability of response, $P(z)$, can be expressed in two equivalent forms. For the sigmoid,

$$\begin{aligned} P(z) &= \frac{1}{1 + \text{antilog}[-\beta_0 - \beta_1 \log(z)]} \\ &= \frac{1}{1 + (z/ED_{50})^{-\beta_1}} \end{aligned}$$

where $\log(ED_{50}) = -\beta_0/\beta_1$. An alternative form shows the relationship to linear models:

$$\text{logit transform of } P = \log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 \log(z) \quad [3.10]$$

The linear form is usually shown using natural logs and is a useful reminder that many of the considerations, in particular linearity and parallelism, discussed for parallel-line models in section 3.3 *Parallel-Line Models for Quantitative Responses* apply to quantal models as well.

For a logit analysis with Standard and Test preparations, let T be a variable that takes the value 1 for animals receiving the Test preparation and 0 for animals receiving the Standard. Assuming parallelism of the Test and Standard curves, the logit model for estimating relative potency is then:

$$\log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 \log(z) + \beta_2 T$$

The log of the relative potency of the Test compared to the Standard preparation is then β_2/β_1 . The two curves in Figure 3.6 show parallel Standard and Test sigmoids. (If the corresponding linear forms equation [3.10] were shown, they would be two parallel straight lines.) The log of the relative potency is the horizontal distance between the two curves, in the same way as for the linear and four-parameter logistic models given for quantitative responses (sections 3.3 *Parallel-Line Models for Quantitative Responses* and 3.4 *Non-linear Models for Quantitative Responses*).

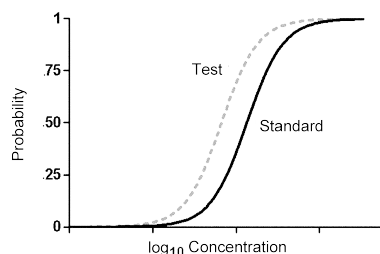


Figure 3.6. Example of Parallel Sigmoid Curves.

Estimating the Model Parameters and Relative Potency—Two methods are available for estimating the parameters of logit and probit models: maximum likelihood and weighted least squares. The difference is not practically important, and the laboratory can accept the choice made by its software. The following assumes a general logistic regression software program. Specialized software should be similar.

Considering the form of equation [3.10], one observes a resemblance to linear regression. There are two independent variables, $x = \log(z)$ and T . For each animal, there is a yes/no dependent variable, often coded as 1 for yes or response and 0 for no or no response. Although bioassays are often designed with equal numbers of animals per concentration, that is not a requirement of analysis. Utilizing the parameters estimated by software, which include β_0 , β_1 , and β_2 and their standard errors, one obtains the estimate of the natural log of the relative potency:

$$\text{Estimate of log of relative potency} = \frac{\hat{\beta}_2}{\hat{\beta}_1}$$

See section 4 for methods to obtain a confidence interval for the estimated relative potency. For a confidence interval based on combining relative potency estimates from multiple assays, use the methods of section 4.2. For a confidence interval from a single assay, use Fieller's Theorem (section 4.3) applied to $\hat{\beta}_2/\hat{\beta}_1$. The confidence interval for the rela-

tive potency is then $[\text{antilog}(L), \text{antilog}(U)]$, where $[L, U]$ is the confidence interval for the log relative potency.

Assumptions—Assumptions for quantal models have two parts. The first concerns underlying assumptions related to the probability of response of each animal or unit in the bioassay. These are difficult to verify assumptions that depend on the design of the assay. The second part concerns assumptions for the statistical model for $P(z)$. Most important of these are parallelism and linearity. These assumptions can be checked much as for parallel-line analyses for quantitative responses.

In most cases, quantal analyses assume a standard binomial probability model, a common choice of distribution for dichotomous data. The key assumptions of the binomial are that at a given concentration each animal treated at that concentration has the same probability of responding and the results for any animal are independent from those of all other animals. This basic set of assumptions can be violated in many ways. Foremost among them is the presence of litter effects, where animals from the same litter tend to respond more alike than do animals from different litters. Cage effects, in which the environmental conditions or care rendered to any specific cage makes the animals from that cage more or less likely to respond to experimental treatment, violates the equal-probability and independence assumptions. These assumption violations and others like them (that could be a deliberate design choice) do not preclude the use of logit or probit models. Still, they are indications that a more complex approach to analysis than that presented here may be required (see <1032>).

Checking Assumptions—The statistical model for $P(z)$ assumes linearity and parallelism. To assess parallelism, equation [3.10] may be modified as follows:

$$\log\left(\frac{P}{1-P}\right) = \beta_0 + \beta_1 \log(z) + \beta_2 T + \beta_3 T * \log(z)$$

Here, β_3 is the difference of slopes between Test and Standard and should be sufficiently small. [The $T * \log(z)$ term is known as an *interaction term* in statistical terminology.] The measure of nonparallelism may also be expressed in terms of the ratio of slopes, $(\beta_1 + \beta_3)/\beta_1$. For model-based confidence intervals for these measures of nonparallelism, bootstrap or profile likelihood methods are recommended. These methods are not covered in this general chapter.

To assess linearity, it is good practice to start with a graphical examination. In accordance with equation [3.10], this would be a plot of $\log[(y + 0.5)/(n - y + 0.5)]$ against $\log(\text{concentration})$, where y is the total number of responses at the concentration and n is the number of animals at that concentration. (The 0.5 corrections improve the properties of this calculation as an estimate of $\log[P/(1 - P)]$.) The lines for Test and Standard should be parallel straight lines as for the linear model in quantitative assays. If the relationship is monotonic but does not appear to be linear, then the model in [3.10] can be extended with other terms. For example, a quadratic term in $\log(\text{concentration})$ could be added: $[\log(\text{concentration})]^2$. If concentration needs to be transformed to something other than log concentration, then the quantal model analogue of slope-ratio assays is an option. The latter is possible but sufficiently unusual that it will not be discussed further in this general chapter.

Outliers—Assessment of outliers is more difficult for quantal assays than for quantitative assays. Because the assay response can be only yes or no, no individual response can be unusual. What may appear to fall into the outlier category is a single response at a low concentration or a single no-response at a high concentration. Assuming that there has been no cause found (e.g., failure to properly administer the drug to the animal), there is no statistical basis for distinguishing an outlier from a rare event.

Alternative Methods—Alternatives to the simple quantal analyses outlined here may be acceptable, depending on

the nature of the analytical challenge. One such challenge is a lack of independence among experimental units, as may be seen in litter effects in animal assays. Some of the possible approaches that may be employed are Generalized Estimating Equations (GEE), generalized linear models, and generalized linear mixed-effects models. A GEE analysis will yield standard errors and confidence intervals whose validity does not depend on the satisfaction of the independence assumption.

There are also methods that make no particular choice of the model equation for the sigmoid. A commonly seen example is the Spearman-Kärber method.

4. CONFIDENCE INTERVALS

A report of an assay result should include a measure of the uncertainty of that result. This is often a standard error or a confidence interval. An interval (c, d), where c is the lower confidence limit and d is the upper confidence limit, is a 95% confidence interval for a parameter (e.g., relative potency) if 95% of such intervals upon repetition of the experiment would include the actual value of the parameter.

A confidence interval may be interpreted as indicating values of the parameter that are consistent with the data. This interpretation of a confidence interval requires that various assumptions be satisfied. Assumptions also need to be satisfied when the width or half width $[(d-c)/2]$ are used in a monograph as a measure of whether there is adequate precision to report a potency. The interval width is sometimes used as a suitability criterion without the confidence interpretation. In such cases the assumptions need not be satisfied.

Confidence intervals can either be *model-based* or *sample-based*. A model-based interval is based on the standard errors for each of the one or more estimates of log relative potency that come from the analysis of a particular statistical model. Model-based intervals should be avoided if sample-based intervals are possible. Model-based intervals require that the statistical model correctly incorporate all the effects and correlations that influence the model's estimate of precision. These include but are not limited to serial dilution and plate effects. Section 4.3 *Model-Based Methods* describes Fieller's Theorem, a commonly used model-based interval.

Sample-based methods combine independent estimates of log relative potency. Multiple assays may arise because this was determined to be required during development and validation or because the assay procedure fixes a maximum acceptable width of the confidence interval and two or more independent assays may be needed to meet the specified width requirement. Some sample-based methods do not require that the statistical model correctly incorporate all effects and correlations. However, this should not be interpreted as dismissing the value of addressing correlations and other factors that influence within-assay precision. The within-assay precision is used in similarity assessment and is a portion of the variability that is the basis for the sample-based intervals. Thus minimizing within-assay variability to the extent practical is important. Sample-based intervals are covered in section 4.2 *Combining Independent Assays (Sample-Based Confidence Interval Methods)*.

4.1 Combining Results from Multiple Assays

In order to mitigate the effects of variability, it is appropriate to replicate independent bioassays and combine their results to obtain a single reportable value. That single reportable value (and not the individual assay results) is then compared to any applicable acceptance criteria. During assay development and validation, analysts should evaluate whether it is useful to combine the results of such assays and, if so, in what way to proceed.

There are two primary questions to address when considering how to combine results from multiple assays:

Are the assays mutually independent?

A set of assays may be regarded as mutually independent when the responses of one do not in any way depend on the distribution of responses of any of the others. This implies that the random errors in all essential factors influencing the result (for example, dilutions of the standard and of the preparation to be examined or the sensitivity of the biological indicator) in one assay must be independent of the corresponding random errors in the other assays. Assays on successive days using the original and retained dilutions of the Standard, therefore, are not independent assays. Similarly, if the responses, particularly the potency, depend on other reagents that are shared by assays (e.g., cell preparations), the assays may not be independent.

Assays need not be independent in order for analysts to combine results. However, methods for independent assays are much simpler. Also, combining dependent assay results may require assumptions about the form of the correlation between assay results that may be, at best, difficult to verify. Statistical methods are available for dependent assays, but they are not presented in this general chapter.

Are the results of the assays homogeneous?

Homogeneous results differ only because of random within-assay errors. Any contribution from factors associated with intermediate precision precludes homogeneity of results. Intermediate precision factors are those that vary between assays within a laboratory and can include analyst, equipment, and environmental conditions. There are statistical tests for heterogeneity, but lack of statistically significant heterogeneity is not properly taken as assurance of homogeneity and so no test is recommended. If analysts use a method that assumes homogeneity, homogeneity should be assessed during development, documented during validation, and monitored during ongoing use of the assay.

Additionally, before results from assays can be combined, analysts should consider the scale on which that combination is to be made. In general, the combination should be done on the scale for which the parameter estimates are approximately normally distributed. Thus, for relative potencies based on a parallel-line, parallel-curve, or quantal method, the relative potencies are combined in the logarithm scale.

4.2 Combining Independent Assays (Sample-Based Confidence Interval Methods)

Analysts can use several methods for combining the results of independent assays. A simple method described below (Method 1) assumes a common distribution of relative potencies across the assays and is recommended. A second procedure is provided and may be useful if homogeneity of relative potency across assays can be documented. A third alternative is useful if the assumptions for Methods 1 and 2 are not satisfied. Another alternative, analyzing all assays together using a linear or nonlinear mixed-effects model, is not discussed in this general chapter.

Method 1—Independent Assay Results From a Common Assay Distribution—The following is a simple method that assumes independence of assays. It is assumed that the individual assay results (logarithms of relative potencies) are from a common normal distribution with some nonzero variance. This common distribution assumption requires that all assays to be combined used the same design and laboratory procedures. Implicit is that the relative potencies may differ between the assays. This method thus captures inter-assay variability in relative potency. Note that the individual

relative potencies should not be rounded before combining results.

Let R_i denote the logarithm of the relative potency of the i^{th} assay of N assay results to be combined. To combine the N results, the mean, standard deviation, and standard error of the R_i are calculated in the usual way:

$$\text{Mean } \bar{R} = \sum_{i=1}^N R_i / N$$

$$\text{Standard Deviation } S = \sqrt{\frac{1}{N-1} \sum_{i=1}^N (R_i - \bar{R})^2}$$

$$\text{Standard Error } SE = S / \sqrt{N}$$

A $100(1 - \alpha)\%$ confidence interval is then found as

$$\bar{R} \pm t_{N-1, \alpha/2} SE,$$

where $t_{N-1, \alpha/2}$ is the upper $\alpha/2$ percentage point of a t -distribution with $N - 1$ degrees of freedom. The quantity $t_{N-1, \alpha/2} SE$ is the expanded uncertainty of \bar{R} . The number, N , of assays to be combined is usually small, and hence the value of t is usually large.

Because the results are combined in the logarithm scale, the combined result can be reported in the untransformed scale as a confidence interval for the geometric mean potency, estimated by $\text{antilog}(\bar{R})$,

$$\text{antilog}(\bar{R} - t_{N-1, \alpha/2} SE), \text{ antilog}(\bar{R} + t_{N-1, \alpha/2} SE)$$

Method 2—Independent Assay Results, Homogeneity Assumed—This method can be used provided the following conditions are fulfilled:

- (1) The individual potency estimates form a homogeneous set with regard to the potency being estimated. Note that this means documenting (usually during development and validation) that there are no contributions to between-assay variability from intermediate precision factors. The individual results should appear to be consistent with homogeneity. In particular, differences between them should be consistent with their standard errors.
- (2) The potency estimates are derived from independent assays.
- (3) The number of degrees of freedom of the individual residual errors is not small. This is required so that the weights are well determined.

When these conditions are not fulfilled, this method cannot be applied and Method 1, Method 3, or some other method should be used. Further note that Method 2 (because it assumes no inter-assay variability) often results in narrower confidence intervals than Method 1, but this is not sufficient justification for using Method 2 absent satisfaction of the conditions listed above.

Calculation of Weighting Coefficients—It is assumed that the results of each of the N assays have been analyzed to give N estimates of log potency with associated confidence limits. For each assay, i , the logarithmic confidence interval for the log potency or log relative potency and a value L_i are obtained by subtracting the lower confidence limit from the upper. (This formula, using the L_i , accommodates asymmetric confidence intervals such as from Fieller's Theorem, section 4.3 *Model-Based Methods*). A weight W_i for each value of the log relative potency, R_i , is calculated as follows, where t_i has the same value as that used in the calculation of confidence limits in the i^{th} assay:

$$W_i = \frac{4t_i^2}{L_i^2} \quad [4.1]$$

Calculation of the Weighted Mean and Confidence Limits—The products $W_i R_i$ are formed for each assay, and their sum is divided by the total weight for all assays to give the weighted mean log relative potency and its standard error as follows:

$$\text{Mean } \bar{R} = \sum_{i=1}^N W_i R_i / \sum_{i=1}^N W_i$$

$$\text{Standard Error } SE = 1 / \sqrt{\sum_{i=1}^N W_i}$$

A $100(1 - \alpha)\%$ confidence interval in the log scale is then found as

$$\bar{R} \pm t_{k, \alpha/2} SE \quad [4.2]$$

where $t_{k, \alpha/2}$ is the upper $\alpha/2$ percentage point of a t -distribution with degrees of freedom, k , equal to the sum of the number of degrees of freedom for the error mean squares in the individual assays. This confidence interval can then be transformed back to the original scale as for Method 1.

Method 3—Independent Assay Results, Common Assay Distribution Not Assumed—Method 3 is an approximate method that may be considered if the conditions for Method 1 (common assay distribution) or Method 2 (homogeneity) are not met.

The observed variation then has two components:

- the intra-assay variation for assay i :

$$S_i^2 = 1 / W_i$$

- the inter-assay variation:

$$S_B^2 = \frac{1}{N-1} \sum_{i=1}^N (R_i - \bar{R})^2 - \frac{1}{N} \sum_{i=1}^N S_i^2$$

For each assay, a weighting coefficient is then calculated as

$$W'_i = \frac{1}{S_i^2 + S_B^2}$$

which replaces W_i in equation [4.1] and where t in equation [4.2] is often approximated by the value 2.

4.3 Model-Based Methods

Many confidence intervals are of the form:

Confidence interval = value $\pm k$ times the standard error of that value.

For such cases, as long as the multiplier k can be easily determined (e.g., from a table of the t -distribution), reporting the standard error and the confidence interval are largely equivalent because the confidence interval is then easily determined from the standard error. However, the logarithms of relative potencies for parallel-line models and some parameterizations of nonlinear models and the relative potencies from slope-ratio models are ratios. In such cases, the confidence intervals are not symmetric around the estimated log relative potency or potency, and Fieller's Theorem is needed. For these asymmetric cases the confidence interval should be reported because the standard error by itself does not capture the asymmetry.

Fieller's Theorem is the formula for the confidence interval for a ratio. Let $R = a/b$ be the ratio for which we need a confidence interval. For the estimates of a and b , we have their respective standard errors, SE_a and SE_b , and a covari-

ance between them, denoted Cov. (The covariance is a measure of the degree to which the estimates of a and b are related and is proportional to the correlation between the estimates of a and b.) The covariance may be 0, as for some parameterizations of standard parallel-line analyses, but it need not be. The confidence interval for R then is as follows:

$$(R_L, R_U) = \frac{\left\{ \hat{R} - \frac{g \text{Cov}}{SE_b^2} \pm \frac{t}{b} \sqrt{(1-g)SE_a^2 + \hat{R}^2 SE_b^2 - 2\hat{R} \text{Cov} + \frac{g \text{Cov}^2}{SE_b^2}} \right\}}{1-g}$$

where

$$g = \frac{t^2 SE_b^2}{b^2}$$

and t is the appropriate t deviate value that will depend on the sample size and confidence level chosen (usually 95%). If $g > 1$, it means that the denominator, b , is not statistically significantly different from 0 and the use of the ratio is not sensible for those data.

For those cases where the estimates of a and b are statistically uncorrelated (Cov = 0), the confidence interval formula simplifies to

$$(R_L, R_U) = \frac{\left\{ \hat{R} \pm \frac{t}{b} \sqrt{(1-g)SE_a^2 + \hat{R}^2 SE_b^2} \right\}}{1-g} \quad [4.3]$$

5. ADDITIONAL SOURCES OF INFORMATION

A variety of statistical methods can be used to analyze bioassay data. This chapter presents several methods, but many other similar methods could also be employed. Additional information and alternative procedures can be found in the references listed below and other sources.

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APPENDIX—GLOSSARY

[NOTE—This glossary is applicable to (111), (1032), (1033), and (1034).]

GLOSSARY

The following is a glossary pertinent to biological assays. For some of this document's terms, the derivation may be clear. Rather than claiming originality, the authors seek to associate with this work a compendial perspective that will broadly provide clarity going forward; consistency with previous authoritative usage; and a useful focus on the bioassay context. In many cases the terms cited here have common usages or are defined in USP general chapter *Validation of Compendial Procedures* (1225), and the International Conference on Harmonization (ICH) Guideline Q2(R1), *Text on Validation of Analytical Procedures* (1). In such cases, the authors seek to be consistent, and they have made notes where a difference arose due to the bioassay context. Definitions from (1225) and ICH Q2 are identified as "1225" if taken without modification or "adopted from 1225" if taken with minor modification for application to bioassay. (Q2 and (1225) agree on definitions.) Most definitions are accompanied by notes that elaborate on the bioassay context.

I. General Terms Related to Bioassays

Analytical procedure (adopted from Q2A)—Detailed description of the steps necessary to perform the assay.

Notes: 1. The description may include but is not limited to the sample, the reference standard and the reagents, use of the apparatus, generation of the standard curve, use of the formulas for the calculation, etc. 2. An FDA Guidance provides a list of information that typically should be included in the description of an analytical procedure (2).

Assay—Analysis (as of a drug) to determine the quantity of one or more components or the presence or absence of one or more components.

Notes: 1. *Assay* often is used as a verb synonymous with *to determine*, as in, "I will assay the material for impurities." In this glossary, *assay* is a noun and is synonymous with the *analytic procedure (protocol)*. 2. The phrase "to run the assay" means to perform the analytical procedure(s) as specified.

Assay data set—The set of data used to determine a single potency or relative potency for all samples included in the bioassay.

Notes: 1. The definition of an assay data set can be subject to interpretation as necessarily a *minimal* set. It is important to understand that it may be possible to determine a potency or relative potency from a set of data but not to do this well. It is *not* the intent of this definition to mean that an assay data set is the *minimal* set of data that can be used to determine a relative potency. In practice, an assay data set should include, at least, sufficient data to assess similarity

(q.v.). It also may include sufficient data to assess other assumptions. 2. It is also not an implication of this definition that assay data sets used together in determining a reportable value (q.v.) are necessarily independent from one another, although it may be desirable that they be so. When a run (q.v.) consists of multiple assay data sets, independence of assay sets within the run must be evaluated.

Bioassay, biological assay (these terms are interchangeable)—Analysis (as of a drug) to quantify the biological activity/activities of one or more components by determining its capacity for producing an expected biological activity, expressed in terms of units.

Notes: 1. Typically a bioassay involves controlled administration of the drug substance to living matter, in vivo or in vitro, followed by observation and assessment of the extent to which the expected biological activity has been manifested. 2. The description of a bioassay includes the analytic procedure, which should include the statistical design for collecting data and the method of statistical analysis that eventually yields the estimated potency or relative potency. 3. Bioassays can be either direct or indirect.

Direct bioassays—Measure the concentration of a substance that is required to elicit a specific response. For example, the potency of digitalis can be directly estimated from the concentration required to stop a cat's heart. In a direct assay, the response must be distinct and unambiguous. The substance must be administered in such a manner that the exact amount (threshold concentration) needed to elicit a response can be readily measured and recorded.

Indirect bioassays—Compare the magnitude of responses for nominally equal concentrations of reference and test preparations rather than test and reference concentrations that are required to achieve a specified response. Most biological assays in *USP* are indirect assays that are based on either quantitative or quantal (yes/no) responses.

Potency—[21 CFR 600.3(s)] The specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result.

Notes: 1. A wholly impotent sample has no capacity to produce the expected specific response, as a potent sample would. Equipotent samples produce equal responses at equal dosages. Potency is typically measured relative to a reference standard or preparation that has been assigned a single unique value (e.g., 100%) for the assay; see *relative potency*. At times, additional qualifiers are used to indicate the physical standard employed (e.g., "international units"). 2. Some biological products have multiple uses and multiple assays. For such products there may be different reference lots that do not have consistently ordered responses across a collection of different relevant assays. 3. [21 CFR 600.10] Tests for potency shall consist of either in vitro or in vivo tests, or both, which have been specifically designed for each product so as to indicate its potency in a manner adequate to satisfy the interpretation of potency given by the definition in 21 CFR 600.3(s).

Relative potency—A measure obtained from the comparison of a Test to a Standard drug substance on the basis of capacity to produce the expected biological activity.

Notes: 1. A frequently invoked perspective is that relative potency is the degree to which the test preparation is diluted or concentrated relative to the standard. 2. Relative potency is unitless and is given definition, for any test material, solely in relation to the reference material and the assay.

Reportable value—The potency or relative potency estimate of record that is intended to achieve such measurement accuracy and precision as are required for use.

Notes: 1. The reportable value is the value that will be compared to a product specification. The specification may be in the *USP* monograph, or it may be set by the company, e.g., for product release. 2. The term *reportable value*

is inextricably linked to the "intended use" of an analytical procedure. Tests are performed on samples in order to yield results that can be used to evaluate some parameter of the sample in some manner. One type of test may be configured in two different ways because the resulting data will be used for two different purposes (e.g., lot release versus stability). The reportable value would likely be different even if the mechanics of the test itself were identical. Validation is required to support the properties of each type of reportable value. In practice there may be one physical document that is the analytical procedure used for more than one application, but each application must be detailed separately within that document. Alternatively, there may be two separate documents for the two applications. 3. When the inherent variability of a biological response, or that of the log potency, precludes a single assay data set's attaining a value sufficiently accurate and precise to meet an assay specification, the assay may consist of multiple blocks or complete replicates, as necessary. The number of blocks or complete replicates needed depends on the assay's inherent accuracy and precision and on the intended use of the reported value. It is practical to improve the precision of a reported value by reporting the geometric mean potency from multiple assays. The number of assays used is determined by the relationship between the precision required for the intended use and the inherent precision of the assay system.

Run—That performance of the analytical procedure that can be expected to have consistent precision and trueness; usually, the assay work that can be accomplished by a laboratory team in a set time with a given unique set of assay factors (e.g., standard preparations).

Notes: 1. There is no necessary relationship of *run* to *assay data set* (q.v.). The term *run* is laboratory specific and relates to the physical capability of a team and its physical environment. An example of a run is given by one analyst's simultaneous assay of several samples in one day's bench work. During the course of a single run, it may be possible to determine multiple reportable values. Conversely, a single assay or reportable value may include data from multiple runs. 2. From a statistical viewpoint, a run is one realization of the factors associated with intermediate precision (q.v.). It is good practice to associate runs with factors that are significant sources of variation in the assay. For example, if cell passage number is an important source of variation in the assay response obtained, then each change in cell passage number initiates a new run. If the variance associated with all factors that could be assigned to runs is negligible, then the influence of runs can be ignored in the analysis and the analysis can focus on combining independent analysis data sets. 3. When a run contains multiple assays, caution is required regarding the independence of the assay results. Factors that are typically associated with runs and that cause lack of independence include cell preparations, groups of animals, analyst, day, a common preparation of reference material, and analysis with other data from the same run. Even though a strict sense of independence may be violated because some elements are shared among the assay sets within a run, the degree to which independence is compromised may have negligible influence on the reportable values obtained. This should be verified and monitored.

Similar preparations (similarity)—The property that the Test and Standard contain the same effective constituent, or the same effective constituents in fixed proportions, and all other constituents are without effect.

Notes: 1. Similarity is often summarized as the property that the Test behaves as a dilution (or concentration) of the Standard. 2. Similarity is fundamental to methods for determination of relative potency. Bioassay similarity requires that the reference and test samples should be sufficiently similar for legitimate calculation of relative potency. Given demonstration of similarity, a relative potency can be calculated, reported, and interpreted. Relative potency is valuable in assessing consistency and also intra- and intermanufacturer comparability in the presence of change. In the absence of

similarity, a meaningful relative potency cannot be reported or interpreted. 3. The practical *consequence* of similarity is a comparable form of dose and/or concentration–response behavior. 4. Failure to statistically demonstrate dissimilarity between a reference and a test sample does not amount to demonstration of similarity. To assess similarity it is not sufficient to fail to find evidence that a reference and a test sample are not similar.

II. Terms Related to Performing a Bioassay

Configuration, assay (also known as assay format)—The arrangement of experimental units (q.v.) by number, position, location, temporal treatment, etc. and the corresponding test, control, or reference sample dilution that will be applied to each.

Notes: 1. The assay configuration must be specified in the formalized assay protocol. 2. Assay configuration can include nested dimensions like plate design, multiple plates per day, single plates on multiple days, etc. The configuration will depend on what the variance analysis (performed during assay development) reveals regarding sources of variability on assay response.

Out of specification—The property of a measurement in which it falls outside its acceptable range.

Sample suitability—A sample is suitable (may be described as having a potency) if its response curve satisfies certain properties defined in the protocol.

Note: Most significant of these properties is that of similarity to the standard response curve. If this property of similarity is satisfied, then the sample is suitable for the assay and can be described via a relative potency estimate.

System suitability—The provision of assurance that the laboratory control procedure is capable of providing legitimate measurements as defined in the validation report.

Notes: 1. System suitability may be thought of as an assessment of current validity achieved at the time of assay performance. An example is provided by positive and negative controls giving values within their normal ranges, ensuring that the assay system is working properly. 2. As described in USP general chapter *Validation of Compendial Procedures* (1225) and ICH Q2, system suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. USP–NF is a source of many system suitability tests.

III. Terms Related to Precision and Accuracy

Accuracy (1225)—An expression of the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found.

Notes: 1. ICH and ISO give the same definition of accuracy. However, ISO specifically regards accuracy as having two components, bias and precision (3). That is, to be accurate as used by ISO, a measurement must be both “on target” (have low bias) and precise. In contrast, ICH Q2 says that accuracy is sometimes termed “trueness” but does not define trueness. ISO defines trueness as the “closeness of agreement between the average value obtained from a large series of test results and an accepted reference value” and indicates that “trueness is usually expressed in terms of bias.” The 2001 FDA guidance on Bioanalytical Method Validation defines accuracy in terms of “closeness of *mean* test results” (emphasis added) and is thus consistent with the ICH usage. This glossary adopts the USP/ICH approach. That is, it uses the phrase “accurate and precise” to indicate low bias (accurate) and low variability (precise). 2. Considerable

caution is needed when using or reading the term *accuracy*. In addition to the inconsistency between USP/ICH and ISO, common usage is not consistent.

Error, types of—Two sources of uncertainty that affect the results of a biological assay are systematic and random error.

A **systematic error** is one that happens with similar magnitude and consistent direction repeatedly. This introduces a *bias* in the determination. Effective experimental design, including randomization and/or blocking, can reduce systematic error.

A **random error** is one whose magnitude and direction vary without pattern. Random error is an inherent variability or uncertainty of the determination. Transformation of systematic into random error will increase the robustness of a biological assay and allow a comparatively simple analysis of assay data.

Format (configuration) variability—Predicted variability for a particular assay format.

Geometric standard deviation (%GSD)—The variability of the log-transformed values of a log normal response expressed as a percent in the untransformed scale.

Note: For example, if the standard deviation of log potency is σ using log base 2, the %GSD of potency is $100 \cdot 2^{\sigma}$.

Intermediate precision (adopted from 1225)—Expresses within-laboratory precision associated with changes in operating conditions.

Notes: 1. Factors contributing to intermediate precision involve anything that can change within a given laboratory and that may affect the assay, including different days, different analysts, different equipment, etc. Intermediate precision is thus “intermediate” in scope between the extremes of repeatability and reproducibility. 2. Any statement of intermediate precision should include clarification about which factors varied. For example, “The intermediate precision associated with changing equipment and operators is ...” 3. There can also be value in separately identifying the precision associated with each source, e.g., interanalyst precision. This may be part of assay development and validation when there is value in identifying which are the important contributors to intermediate precision. 4. When reporting intermediate precision, particularly for individual sources, analysts should take care to distinguish between intermediate precision variance and components of that variance. The variance includes repeatability and thus must be necessarily at least as large as the repeatability variance. A variance component, e.g., for analyst, is also a part of the intermediate precision variance for analyst, but it could be negligible and need not be larger in magnitude than the repeatability variance.

Precision (1225)—The closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Notes: 1. Precision may be considered at three levels: repeatability (q.v.), intermediate precision (q.v.), and reproducibility (q.v.). 2. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample, precision may be investigated using artificially prepared samples or a sample solution. 3. Precision is usually expressed as the variance, standard deviation, coefficient of variation, or geometric standard deviation.

Relative bias—Degree of difference from the true value expressed as a percent.

Repeatability (1225)—The expression of the precision under the same operating conditions over a short interval of time.

Notes: 1. ICH Q2A says that repeatability is also termed “intra-assay” precision. In the bioassay context, the better term is “intra-run,” and a “short interval of time” is meant to connote “within-run.” 2. The idea of a “short interval of time” can be problematic with bioassay. If a run takes multi-

ple weeks and consists of a single assay set, then intra-run precision cannot be determined. Alternatively, if a run consists of two assay data sets and a run can be done in a single day, repeatability of the relative potency determination can be assessed. 3. Operating conditions include, but not limited to, equipment and analyst.

Reproducibility (1225)—Expresses the precision between laboratories.

Notes: 1. Reproducibility includes contributions from repeatability and all factors contributing to intermediate precision, as well as any additional contributions from interlaboratory differences. 2. Reproducibility applies to collaborative studies such as those for standardization or portability of methodology. Depending on the design of the collaborative study, it may be possible to separately describe variance components associated with intra- and interlaboratory sources of variability.

Specificity (1225)—The ability to assess unequivocally the analyte in the presence of components that may be expected to be present.

Note: Typically these components may include impurities, degradants, matrix, etc.

IV. Terms Related to Validation

Detection limit (adopted from 1225)—The lowest amount of analyte in a sample that can be detected but not necessarily quantified or quantified to any given level of precision and accuracy.

Linearity, dilutional (adopted from 1225)—The ability (within a given range) of a bioassay to obtain log relative potencies that are directly proportional to the log relative potency of the sample.

Notes: 1. Dilutional linearity, sometimes called bioassay linearity, is demonstrated across a range of known relative potency values by considering a plot of true log potency versus observed log potency. If that plot yields an essentially straight line with a y-intercept of 0 and a slope of 1, the assay has direct proportionality. If that plot yields an essentially straight line but either the y-intercept is not 0 or the slope is not 1 (or both), the assay has a proportional linear response. 2. To assess whether the slope is (near) 1.0 requires an a priori equivalence or indifference interval. It is not proper statistical practice to test the null hypothesis that the slope is 1.0 against the alternative that it is not 1.0 and conclude a slope of 1.0 if this is not rejected. Assay linearity is separate from consideration of the shape of the concentration–response curve. Linearity of concentration–response is not a requirement of assay linearity.

Quantitation limits (adopted from 1225)—The limits of true relative potencies between which the assay has suitable precision and accuracy.

Note: This applies to assay results (log potency) rather than the reportable value.

Range (adopted from 1225)—The interval between the upper and lower true relative potencies for which the bioassay is demonstrated to have a suitable level of precision, accuracy, and assay linearity.

Note: This applies to assay results (log potency) rather than the reportable value.

Robustness (1225)—A measure of an analytical procedure's capacity to remain unaffected by small but deliberate variations in method parameters.

Notes: 1. Robustness is an indication of a bioassay's reliability during normal usage. For example, a cell culture assay system that is robust to the passage number of the cells would provide potency values with equivalent accuracy and precision across a consistent range of passage numbers. 2. ICH Q2 states:

the evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in

method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled, or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability [q.v.] parameters is established to ensure that the validity of the analytical procedure is maintained whenever used.

Validation, assay—A formal, archived demonstration of the analytical capacity of an assay that provides justification for use of the assay for an intended purpose and a range of acceptable potency values.

Note: Formal validations are conducted prospectively according to a written, approved plan.

V. Terms Related to Statistical Design and Analysis

Analysis of variance (ANOVA)—A statistical tool used to assess contributions of variability from experimental factors.

Blocking—The grouping of related experimental units in experimental designs.

Notes: 1. Blocking is often used to reduce the variability of a measure of interest. 2. Blocks may consist of groups of animals (a cage, a litter, or a shipment), individual 96-well plates, sections of 96-well plates, or whole 96-well plates grouped by analyst, day, or batch of cells. 3. The goal is to isolate a systemic effect, such as cage, so that it does not obscure the effects of interest.

A **complete block design** occurs when all levels of a treatment factor (in a bioassay, the primary treatment factors are sample and concentration) can be applied to experimental units for that factor within a single block. Note that the two treatment factors, sample and concentration, may have different experimental units. For example, if the animals within a cage are all assigned the same concentration but are assigned unique samples, then the experimental unit for concentration is cage and the experimental unit for sample is animal; cage is a blocking factor for sample.

An **incomplete block design** occurs when the number of levels of a treatment factor exceeds the number of experimental units for that factor within the block.

Confidence interval—A statistical interval expressing the likely value of a parameter.

Confounded design—Two factors are confounded if their levels vary together (they are not crossed).

Notes: 1. For example, in a bioassay validation experiment in which one analyst performs assays on a set of samples for three days using cells from one passage number, then another analyst performs assays on the same set of samples for another three days using cells from a different passage number, the passage number of the cells and the analysts are confounded. [Also note that days are nested (q.v.) within analyst and cell passage number.] When factors are confounded one cannot tell which of the factors has caused an observed experimental difference. 2. Fractional factorial designs (q.v.), in which factors are only partially crossed, also are partially confounded. A full factorial design also can be confounded if the number of treatment combinations (sample and concentration) is greater than the block size.

Crossed (and partially crossed)—Two factors are crossed (or fully crossed) if each level of each factor appears with each level of the other factor. Two factors are partially crossed when they are not fully crossed but multiple levels of one factor appear with a common level of the other factor.

Notes: 1. For example, in a bioassay in which all samples appear at all dilutions, samples and dilutions are (fully) crossed. In a bioassay validation experiment in which two of four analysts each perform assays on the same set of samples on each of six days and a different pair of analysts is used on each day the analysts are partially crossed with days. 2. Each factor may be applied to different experimen-

tal units, and the factors may be both fully crossed and nested (q.v.), creating a split-unit or split-plot design (q.v.). 3. Experiments with factors that are partially crossed require particular care for proper analysis. 4. A randomized complete block design (RCBD) (q.v.) is a design in which the block factor (which often is treated as a random effect) is crossed with the treatment factor (which is usually treated as a fixed effect).

Design of experiments (DOE)—A systematic approach for studying multiple factors.

Note: DOE is used in bioassay development and validation; see (1032) and (1033).

Equivalence test—A test of conformance to interval-based target acceptance criteria.

Notes: 1. An equivalence test differs from most common statistical tests in the nature of the statistical hypotheses. For most common tests, the statistical null hypothesis is no difference and the alternative is that there is some difference, without regard to the magnitude or importance of the difference. In equivalence testing the alternative hypothesis is that the difference is sufficiently small so there is no important difference. 2. A common statistical procedure used for equivalence tests is the two one-sided test (TOST) procedure.

Expected mean square—A mathematical expression of variances estimated by an ANOVA mean square.

Experimental design—The structure of assigning treatments to experimental units.

Notes: 1. Blocking (q.v.), randomization (q.v.), replication (q.v.), and specific choice of design (cf. general chapter *Design and Development of Biological Assays* (1032)) are some aspects of experimental design. 2. Important components of experimental design include the number of samples, the number of concentrations, and how samples and concentrations are assigned to experimental units and are grouped into blocks. 3. The experimental design influences which statistical methodology should be used to achieve the analytical objective.

Experimental unit—The smallest unit to which a distinct level of a treatment is randomly allocated.

Notes: 1. Randomization of treatment factors to experimental units is essential in bioassays. 2. Different treatment factors can be applied to different experimental units. For example, samples may be assigned to rows on a 96 well plate while dilutions are assigned to columns on the plate. In this case, rows are the experimental units for samples, columns are the experimental units for concentrations, and wells are the experimental units for the interaction of sample and concentration. 3. An experimental unit needs to be distinguished from a sampling unit, the smallest unit on which a distinct measurement is recorded (e.g., a well). Because the sampling unit is often smaller than the experimental unit, it is an easy mistake to treat sampling units as if they are experimental units. This mistake is called pseudoreplication (q.v.).

Factor—An assay design element that may affect assay response and that varies in an experiment.

Note: In a bioassay there will be at least two treatment factors—sample and concentration.

Fixed factor (fixed effect) is a factor that is deliberately set at specific levels in an experiment. Inference is made only to the levels used in the experiment. In a bioassay, sample and concentration are both fixed factors.

Random factor (random effect) is one for which its levels represent a sample of ways in which that factor might vary. In a bioassay, the test organisms, plate, and day often are considered random factors.

Factorial design—One in which there are multiple factors and the factors are partially or fully crossed.

In a **full factorial design**, each level of a factor appears with each combination of levels of all other factors. For example, if factors are sample (test and reference), concentra-

tion, and analyst, for a full factorial design each analyst must analyze all combinations of sample and concentration.

A **fractional factorial design** is one in which some factors are deliberately partially confounded with interactions associated with other combinations of factors.

General linear model—A statistical linear model that relates study factors, which can be continuous or discrete, to experimental responses.

Independence—For two measurements or observations A and B (raw data, assay sets, or relative potencies) to be independent, values for A must be unaffected by B's responses and vice versa.

Note: A consequence of nonrecognition of lack of independence is poor characterization of variance. In practice this means that if two potency or relative potency measurements share a common factor that might influence assay outcome such as analyst, cell preparation, incubator, group of animals, or aliquot of Standard samples, then the correct initial assumption is that these relative potency measurements are not independent. As assay experience is gained, an empirical basis may be established so that it is reasonable to treat potency measures as independent even if they share a common level of a factor. The same concern for lack of independence holds if the two potency or relative potency measurements are estimated together from the same model or are in any way associated without including in the model some term that captures that there are two or more potency measurements.

Interaction—Two factors are said to interact if the effect of one factor depends on the level of the other factor.

Level—A location on the scale of measurement of a factor.

Notes: 1. Factors have two or more distinct levels. For example, if a bioassay contains two samples, test and reference, then there are two levels for the factor sample. 2. Levels of a factor in a bioassay may be quantitative, such as concentration, or categorical, such as sample (i.e., test and reference).

Log normal distribution—A skewed distribution characterized by increased variability with increased level of response.

Note: A normal distribution is generated by taking the log of the response.

Mean square—A calculation in ANOVA representing the variability associated with an experimental factor.

Mixed-effects model—A statistical model including both fixed and random effects.

Modeling, statistical—The mathematical specification of the concentration–response relationship and important sources of variation in the bioassay.

Notes: 1. Modeling includes methods to capture the dependence of the response on the samples, concentration, and groups or blocking factors in the assay configuration. 2. Modeling of bioassay data includes making many choices, some of which are driven by data. For continuous data there is a choice between linear and nonlinear models. For discrete data there is a choice among logit/log models within a larger family of generalized linear models. In limiting dilution assays there is published literature advocating Poisson models and Markov chain binomial models. One can use either fixed-effects models or mixed-effects models for bioassay data. The fixed-effects models are more widely available in software and are somewhat less demanding for statisticians to set up. On the other hand, mixed models have advantages over fixed ones. The former are more accommodating of missing data and, more importantly, can allow each block to have different slopes, asymptotes, median effective concentrations required to induce a 50% effect (EC_{50}), or relative potencies. Particularly when the analyst is using straight-line models fit to nonlinear responses or in assay systems in which the concentration–response curve varies from block to block, the mixed model captures the behavior of the assay system in a much more realistic and interpretable way. 3. It is essential that any modeling approach for bioassay data use all available data simultane-

ously to estimate the variation (or, in a mixed model, each of several sources of variation). It may be necessary to transform the observations before this modeling; to include a variance model; or to fit a "means" model (in which there is a predicted effect for each combination of sample and concentration) to get pooled estimate(s) of variation.

Multiplicity—The property of compound risk with multiple independent events, all with fixed risk.

Nested—A factor A is nested within another factor B if the levels of A are different for every level of B.

Notes: 1. For example, in a bioassay validation experiment two analysts may perform assays on the same set of samples on each of six days when no analyst performs the assay on more than one day (this requires 12 analysts who are qualified to perform the assay); these analysts are nested within days. 2. Nested factors have a hierarchical relationship. 3. For two factors to be nested they must satisfy the following: a) be applied to different-sized experimental units; b) the larger experimental unit contains more than one of the smaller experimental units; and c) the factor applied to the smaller experimental unit is not fully crossed with the factor applied to the larger experimental unit. When conditions (a) and (b) are satisfied and the factors are partially crossed, then the experiment is partially crossed and partially nested. Experiments with this structure require particular care for proper analysis.

Parallelism (of concentration–response curves)—The concentration–response curves of the test and standard are identical in shape and differ only in a constant horizontal difference.

Notes: 1. When test and reference preparations are similar (q.v.) and assay responses are plotted against log concentrations, the resulting curve for the test preparation will be the same as that for the standard but shifted horizontally by an amount that is the logarithm of the relative potency. Because of this relationship, similarity (q.v.) is generally referred to as *parallelism*. Note that similarity is the primary concept and that parallelism is not necessary for similarity. See slope-ratio models in general chapter *Analysis of Biological Assays* <1034> in which samples with similar concentration–response relationships have a common (or nearly common) y-intercept but may differ in their slopes. 2. In practice, it is not possible to demonstrate that the shapes of two curves are exactly the same. Instead, the two curves are shown to be sufficiently similar (equivalent) in shape. Note that *similar* should be interpreted as "we have evidence that the two values are close enough" rather than "we don't have evidence that the two values are different." 3. The assessment of parallelism depends on the type of function used to fit the response curve. Parallelism for a nonlinear assay using a four-parameter logistic fit means that: a) the slopes of the rapidly changing parts of the sample and reference standard curves (that is, slope at tangent to the curve, where the first derivative is at a maximum) should be similar; and b) the upper and lower asymptotes of the response curves (plateaus) should be similar. For straight-line analysis, the slopes of the lines should be similar.

Point estimate—A single-value estimate obtained from statistical calculations.

Note: Examples are the average, standard deviation, and relative potency.

P-value (significance probability)—A statistical calculation representing the probability associated with observing an experimental outcome that is different from expectation.

Notes: 1. The P-value is the probability of observing what was seen or something more extreme under the assumption that the statistical null hypothesis is true. "More extreme" means further from the null hypothesis. 2. Commonly, $P < 0.05$ is taken as indicating statistical significance, though any value may be used.

Randomization—A process of assignment of treatment to experimental units based on chance so that all equal-sized

groups of units have an equal chance of receiving a given treatment.

Notes: 1. The chance mechanism may be an unbiased physical process (rolling unbiased dice, flipping coins, drawing from a well-mixed urn), random-number tables, or computer-generated randomized numbers. Care must be taken in the choice and use of method. Good practice is to use a validated computerized random-number generator. 2. The use of randomization results in systematic error becoming random error not associated with particular samples or a dilution pattern but distributed throughout the assay. In 96-well bioassays, plate effects can be substantial and cause bias or trending, particularly in assays involving long-term cell culturing or multiple addition and wash steps. In animal studies, a variety of factors associated with individual animals can influence responses. If extraneous factors that influence either plate assays or animal assays are not routinely demonstrated to have been eliminated or minimized to be negligible, randomization is essential to obtain unbiased data required for the calculation of true potency. Randomization is central to the experimental design and analysis of data obtained from most biological assays.

Replication—A process in which multiple independent experimental units receive the same level of a treatment factor.

Notes: 1. The purpose of replication is to minimize the effects of uncontrollable sources of random variability. 2. Replication can occur either completely at random or across blocks. Generally, replication within blocks is pseudoreplication (see below).

True replicates—Samples based on independent experimental units.

Pseudoreplication—Is the identification of samples from experimental units as independent and thus true replicates when they are actually not independent.

Notes: 1. Pseudoreplication results in wrong inferences and the appearance of more replicates than are actually present. 2. Pseudoreplication is dangerous because it is an easy mistake to make, it is easy to overlook, and the consequences can be serious. For example, pseudoreplicates commonly arise when analysts are making a dilution series for each sample in tubes (the dilution series can be made with serial dilutions, by single-point dilutions, or with any convenient dilution scheme). The analyst then transfers each dilution of each sample to several wells on one or more assay plates. The wells are then pseudoreplicates because they are simply aliquots of a single dilution process. 3. In general, pseudoreplication should be avoided because, unless it is properly addressed in the analysis, it leads to underestimation of replicate variance. 4. The simple way to analyze data from pseudoreplicates is to average over the pseudoreplicates (if a transformation of the observed data is used, the transformation should be applied before averaging over pseudoreplicates) before fitting any sort of concentration–response model. In many assay systems averaging over pseudoreplicates will leave the assay without any replication. A more complex way to use data containing pseudoreplicates is to use a mixed model that treats the pseudoreplicates as a separate random effect. The only case in which pseudoreplication is useful is when the pseudoreplicate (i.e., well-to-well) variation is very large compared to the variation associated with replicates and the cost of pseudoreplicates is much lower than the cost of replicates.

Standard error of estimate—The variability associated with an estimate of a reportable value or other parameter.

Note: The standard error is also known as the standard uncertainty.

Statistical process control (SPC)—A set of statistical tools used to monitor for shifts and trends in a process.

Type I error (α)—The error made in judging data analysis, wherein the alternative hypothesis is accepted when it is false.

Type II error (β)—The error made in judging data analysis, wherein the alternative hypothesis is rejected when it is true.

Variance component analysis—A statistical analysis that divides total variability into its component parts.

GLOSSARY REFERENCES

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(1035) BIOLOGICAL INDICATORS FOR STERILIZATION

A biological indicator is broadly defined as a characterized preparation of a specific microorganism that provides a defined and stable resistance to a specific sterilization process. Microorganisms widely recognized as suitable for biological indicators are spore-forming bacteria, because, with the exception of ionizing radiation processes, these microorganisms are significantly more resistant than normal microflora. A biological indicator can be used to assist in the performance qualification of the sterilization equipment and in the development and establishment of a validated sterilization process for a particular article. Biological indicators are used in processes that render a product sterile in its final package or container, as well as for the sterilization of equipment, materials, and packaging components used in aseptic processing. Biological indicators may also be used to monitor established sterilization cycles and in periodic revalidation of sterilization processes. Biological indicators may also be used to evaluate the capability of processes used to decontaminate isolators or aseptic clean-room environments.

The principles and requirements for these applications are described under *Sterilization and Sterility Assurance of Compensial Articles* (1211).

TYPES OF BIOLOGICAL INDICATORS

There are at least three types of biological indicators. Each type of indicator incorporates a known species of a microorganism of known sterilization resistance to the sterilization mode. Some biological indicators may also contain two different species and concentrations of microorganisms.

One form of biological indicator includes spores that are added to a carrier (a disk or strip of filter paper, glass, plastic, or other materials) and packaged to maintain the integrity and viability of the inoculated carrier.

Carriers and primary packaging shall not contain any contamination (physical, chemical, or microbial) that would adversely affect the performance or the stability characteristics of the biological indicator. The carrier and primary packaging shall not be degraded by the specific sterilization process,

which is used in a manner that will affect the performance of the biological indicator. The carrier should withstand transport in the primary and secondary packaging and handling at the point of use. The design of the carrier and primary packaging should minimize the loss of the original inoculum during transport, handling, and shelf life storage.

Another form of biological indicator is a spore suspension that is inoculated on or into representative units of the product to be sterilized. This represents an inoculated product; however, a simulated inoculated product may be used if it is not practical to inoculate the actual product. A simulated product is a preparation that differs in one or more ways from the actual product, but performs as the actual product using test conditions or during actual production sterilization processing. Spore suspensions with a known D value should be used to inoculate the actual or simulated product. If a simulated inoculated product is used, it must be demonstrated that it will not degrade the sterilization resistance of the bioindicator. The physical design of actual or simulated product can affect the resistance of spore suspensions that are inoculated on or into the products. In the case of liquid inoculated products, it is often advisable to determine both the D value and z value of the specific biological indicator microorganism in the specific liquid product. The population, D value, z value where applicable, and endpoint kill time of the inoculated actual or simulated product should be determined.

A third form of biological indicator is a self-contained indicator. A self-contained biological indicator is designed so that the primary package, intended for incubation following sterilization processing, contains the growth medium for recovery of the process-exposed microorganisms. This form of biological indicator together with the self-contained growth medium can be considered a system. In the case of self-contained biological indicators, the entire system provides resistance to the sterilization process.

If the biological indicator is a paper strip or disk in a self-contained package that includes an available culture medium, the package design should be readily penetrable by the sterilizing agent. To allow for the time lag that may occur while the sterilizing agent reaches the contained microorganisms in the system, the D value, process endpoint kill time, and the survival time should be characterized for the system and not solely for the paper strip in the self-contained unit. Following the sterilizing treatment, the spore strip or disk is immersed in the self-contained medium by manipulation, which allows contact with the culture medium.

Self-contained biological indicators may also consist of a spore suspension in its own medium, and they often also contain a dye, which indicates positive or negative growth following incubation. Resistance of the self-contained system is dependent upon penetration of the sterilant into the package. Penetration may be controlled by the manufacturer through varying designs and composition of the self-contained biological indicator package, ampul, or container. Self-contained ampul biological indicators may be incubated directly following exposure to the sterilization process. The entire system is then incubated under the specified conditions. Growth or no growth of the treated spores is determined visually (either by observing a specified color change of an indicator incorporated in the medium or by turbidity) or by microscopic examination of the inoculated medium.

The self-contained system resistance characteristics must also comply with the labeling of the self-contained system and the relevant biological indicator monograph. The self-contained biological indicator system should withstand transport in the secondary packaging and handling at the point of use without breakage. The design of the self-contained system should be such to minimize the loss of the original inoculum of microorganisms during transport and handling. During or after the sterilization process, the materials used in the self-contained system shall not retain or release any substance that can inhibit the growth of low

numbers of surviving indicator microorganism under culture conditions. Adequate steps must be taken to demonstrate that the recovery medium has retained its growth support characteristics after exposure to the sterilization process.

Preparation

All operations associated with the preparation of biological indicators are controlled by a documented quality system. Traceability is maintained for all materials and components incorporated in or coming into direct contact with the microorganism suspension, the inoculated carrier, or the biological indicator.

The preparation of stock spore suspensions of selected microorganisms used as biological indicators requires the development of appropriate procedures, including mass culturing, harvesting, purification, and maintenance of the spore suspensions. The stock suspension should contain predominantly dormant (nongerminating) spores that are held in a nonnutritive liquid.

The finished product (microbial suspension, inoculated carriers, or biological indicators) supplied by commercial manufacturers shall have no microorganisms, other than the test microorganism, present in sufficient numbers to adversely affect the product. The system to minimize the presence of microorganisms other than the biological indicator microorganism in the product will be validated, monitored, and recorded.

Selection for Specific Sterilization Processes

The selection of a biological indicator requires a knowledge of the resistance of the biological indicator system to the specific sterilization process. It must be established that the biological indicator system provides a challenge to the sterilization process that exceeds the challenge of the natural microbial burden in or on the product.

The effective use of biological indicators for the cycle development, process, and product validation, and routine production monitoring of a sterilization process requires a thorough knowledge of the product being sterilized, along with its component parts (materials and packaging). Only the widely recognized biological indicators specified in the particular biological indicator monograph should be used in the development or validation of a sterilization process. This will ensure that the biological indicator selected provides a greater challenge to the sterilization process than the bioburden in or on the product. Some users may require biological indicators with characteristics that differ from those widely available commercially. In such cases, users may grow their own spore cultures for the express purpose of preparing in-house biological indicators for their specific use. In such a case, the user is well advised to use organisms already described in the scientific literature as indicator organisms, and the user must have the capability of determining D and z values for in-house biological indicators. When biological indicators are prepared in-house, users must confirm the population, purity, and shelf life of the biological indicator to ensure the validity of any test conducted using the in-house biological indicator. When a bioburden-based sterilization process design is used, data comparing the resistance of the biological indicator to that of bioburden are essential. Enumeration of the bioburden content of the articles being sterilized is also required. The process must result in a biologically verified lethality sufficient to achieve a probability of obtaining a nonsterile unit that is less than one in a million.

Alternatively, the overkill method may be used in the design of a sterilization process. In this case, specific assumptions are made regarding the resistance assumption used in establishing sterilization process lethality requirements. In general, all overkill processes are built upon the assumption that the bioburden is equal to one million organisms and that the organisms are highly resistant. Thus, to achieve the

required probability of a nonsterile unit that is less than one in a million, a minimum 12 D process is required. A 12 D process is defined as a process that provides a lethality sufficient to result in a 12 log reduction, which is equivalent to 12 times a D value for organisms with sufficiently higher resistance than the mean resistance of bioburden. Because the bioburden is assumed to be one million, an overkill process will result in a probability of nonsterility at much less than 10^{-6} in actual practice. Overkill process design and evaluation may differ depending upon the sterilization process under test. The use of an overkill design and validation approach may minimize or obviate the need for bioburden enumeration and identification.

Moist Heat—For moist heat sterilization process, spores of suitable strains of *Bacillus stearothermophilus* are commercially available as biological indicators and frequently employed. Other heat-resistant spore-forming microorganisms such as *Clostridium sporogenes*, *Bacillus subtilis*, and *Bacillus coagulans* have also been used in the development and validation of moist heat sterilization processes.

Dry Heat—For dry heat sterilization, spores of *Bacillus subtilis* spp. are sometimes used to validate the process. During the validation of dry heat sterilization processes, endotoxin depyrogenation studies are frequently conducted in lieu of microbial inactivation studies during the establishment of sterilization cycles because the inactivation rate of endotoxin is slower than the inactivation rate of *Bacillus subtilis* spores. In practice the reduction of endotoxin titer by three or more logs will result in a process that also achieves a probability of nonsterility substantially lower than 10^{-6} .

Ionizing Radiation—Spores of *Bacillus pumilus* have been used to monitor sterilization processes using ionizing radiation; however, this is a declining practice. Radiation dose-setting methods that do not use biological indicators have been widely used to establish radiation processes. Furthermore, certain bioburden microorganisms can exhibit greater resistance to radiation than *Bacillus pumilus*.

Ethylene Oxide—For ethylene oxide sterilization, spores of a subspecies of *Bacillus subtilis* (*Bacillus subtilis* var. *niger*) are commonly used. The same biological indicator systems are generally used when 100% ethylene oxide or different ethylene oxide and carrier gas systems are used as sterilants.

Vapor-Phase Hydrogen Peroxide (VPHP)—This process has been shown to be an effective surface sterilant or decontaminant. VPHP is capable of achieving sterilization (probability of nonsterility of less than one in a million) when process conditions so dictate and if the target of sterilization is suitably configured. However, VPHP is also commonly used as a surface decontaminating agent in the treatment of sterility testing, biological and chemical containment, manufacturing isolators, and clean rooms.

Surface decontamination is a process that is distinct from sterilization of product contact materials, container-closure systems, or product. It is a process designed to render an environment free of detectable or recoverable microorganisms. Biological indicators are widely used to verify the efficacy of the decontamination process. However, in the case of decontamination, a spore log reduction value of three to four is adequate because the goal is decontamination rather than sterilization.

Bacillus stearothermophilus is the most prevalently used biological indicator for validating VPHP. Other microorganisms that may be useful as biological indicators in VPHP processes are spores of *Bacillus subtilis* and *Clostridium sporogenes*. Other microorganisms may be considered if their performance responses to VPHP are similar to those of the microorganisms cited above.

These spores may be inoculated on the surface of various gas-impermeable carrier systems having glass, metal, or plastic surfaces. Highly absorbent surfaces, such as fibrous substrates, or any other substrate that readily absorbs VPHP or moisture may adversely influence the VPHP concentration available for inactivation of inoculated microorganisms.

Table 1. Typical Characteristics for Commercially Supplied Biological Indicator Systems

Sterilization Mode	Example of a Typical D value (minutes)	Range of D values for Selecting a Suitable Biological Indicator (minutes)	Limits for a Suitable Resistance (depending on the particular D value [minutes])	
			Survival Time	Kill Time
Dry heat ^a	1.9	Min. 1.0	Min. 4.0	10.0
160°		Max. 3.0	Max. 14.0	32.0
Ethylene oxide ^b				
600 mg per L	3.5	Min. 2.5	Min. 10.0	25.0
54°		Max. 5.8	Max. 27.0	68.0
60% relative humidity				
Moist heat ^c	1.9	Min. 1.5	Min. 4.5	13.5
121°		Max. 3.0	Max. 14.0	32.0

^a For 1.0×10^6 to 5.0×10^6 spores per carrier.

^b For 1.0×10^6 to 5.0×10^7 spores per carrier.

^c For 1.0×10^5 to 5.0×10^6 spores per carrier.

Paper substrates are not used because VPHP will degrade cellulose-based materials.

For representative characteristics of commercially supplied biological indicators, see *Table 1*.

The biological indicator may also be individually packaged in a suitable primary overwrap package that does not adversely affect the performance of the indicator, and is penetrable by VPHP. Spunbound polyolefin materials have proven to be well suited as an overwrap of biological indicators intended for use in evaluation of VPHP processes. The overwrap material may facilitate laboratory handling of the biological indicators following exposure to VPHP. Also, the use of an overwrap material to package VPHP biological indicators must be carefully assessed to ensure that, following VPHP exposure, residual hydrogen peroxide is not retained by the packaging material, possibly inducing bacteriostasis during the recovery steps. Microbial D values will be influenced by the presence of a biological indicator overwrap material relative to the rate of inactivation and the potential presence of residual VPHP. In cases where biological indicators (inoculated carriers) are being used without the primary package, stringent adherence to aseptic techniques is required.

PERFORMANCE EVALUATION

Manufacturer's Responsibility

The initial responsibility for determining and providing to the users the performance characteristics of a biological indicator¹ lot resides with the manufacturer of biological indicators. The manufacturer should provide with each lot of biological indicators a certificate of analysis that attests to the validity of biological indicator performance claims cited on the biological indicator package label or in the package insert of the label package. The manufacturer should define the sterilization process that the biological indicator will be used to evaluate. The characterization of each type of biological indicator, which provides the basis for label claims, should be performed initially by the manufacturer of the biological indicator using specialized and standardized apparatus under precisely defined conditions.¹ The manufacturer should also provide information concerning the D value, the method by which the D value was determined, and microbial count and resistance stability of the biological indicator throughout the labeled shelf life of the indicator. Optimum storage conditions should be provided by the manufacturer, including temperature, relative humidity, and any other requirements for controlled storage. The data obtained from the various required performance assays should be cited in a

¹ See *Apparatus under Biological Indicators—Resistance Performance Tests (55)*. These apparatuses have been designed to provide consistent physical conditions applicable to the characterization of biological indicators. The required performance characteristics are also indicated.

package insert or on the label of the biological indicator package. The manufacturer should provide directions for use, including the medium and conditions to be used for the recovery of microorganisms after exposure to the sterilization process. Disposal instructions should also be provided by the manufacturer of the biological indicator.

User's Responsibility

Commercial Product—When biological indicators are purchased from a commercial source, their suitability for use in a specific sterilization process should be established through developmental sterilization studies unless existing data are available to support their use in the process. The user should establish in-house acceptance standards for biological indicator lots and consider rejection in the event the biological indicator lot does not meet the established in-house performance standards. A Certificate of Performance should be obtained for each lot of indicators, and the user should routinely perform audits of the manufacturer's facilities and procedures. If certificates are not obtained and audits have not been performed, or if the biological indicators are to be used outside of the manufacturer's label claims, verification and documentation of performance under conditions of use must exist.

Upon initial receipt of the biological indicator from a commercial supplier, the user should verify the purity and morphology of the purchased biological indicator microorganisms. Verification of at least the proper genus is desirable. Also, a microbial count to determine the mean count per biological indicator unit should be conducted. The manufacturer's comments relative to D value range, storage conditions, expiration dating, and stability of the biological indicator should be observed and noted. The user may consider conducting a D value assessment before acceptance of the lot. Laboratories that have the capability of performing D value assays could conduct a D value determination using one of the three methods cited in the general test chapter *Biological Indicators—Resistance Performance Tests (55)* and in the appropriate USP monographs for specific biological indicators. Particularly important is the verification of the D value and count stability of the biological indicator system if long-term storage is employed.

In the event the spore crop is maintained for longer than 12 months under documented storage conditions, both spore count and resistance analysis must be conducted, unless performance of an original parent crop has been validated for a longer storage period. The result of spore count and resistance assays should be within the range of acceptability established during initial acceptance of the spore crop lot.

Noncommercial Product—A user of biological indicator systems may elect to propagate microorganisms for developing in-house biological indicators to develop or validate sterilization processes. In the event a user becomes a "man-

ufacturer" of biological indicators, biological indicator performance requirements must be met. If the biological indicator system is used for the development of new sterilization processes or validation of existing processes, the same performance criteria described for commercial manufacturers of biological indicators must be followed.

Spore Crop Preparation

Because most biological indicators use microbial spores, accurate records of spore crop identification must be maintained by commercial and noncommercial biological indicator manufacturers. These records should include records pertaining to the source of the initial culture, identification, traceability to the parent spore crop, subculture frequency, media used for sporulation, changes in media preparation, any observation of crop contamination, and pre- and post-heat shock data. Records of usage of the spore crop and resistance to sterilization (namely, D values and z values where applicable) should also be maintained.

Instrumentation

The instrumentation used to evaluate the sterilization resistance of spore crops must be consistent with existing standards² related to the performance evaluation of biological indicator systems.

Equipment for the determination of D values of microorganisms exposed to VPHP should be able to closely control equipment operating parameters as described for other biological indicator systems under *Biological Indicators—Resistance Performance Tests* <55>. Particularly important is the assurance of a consistently reproducible VPHP concentration, delivered within a finite time, and maintained within a specified concentration range or VPHP pressure range for a defined increment of time. Introduction of biological indicators into a stabilized concentration of VPHP conditions should be via a system that permits rapid entry and removal of the test units from the chamber. Also, the design of the test chamber should allow for the attainment of steady-state VPHP concentrations and pressure, or the use of a defined amount of cubic feet of free flowing VPHP at a standardized pressure and temperature. Currently, VPHP concentration measurement devices may not be widely used. Therefore, exposure conditions may need to be based on the maintenance of steady-state VPHP pressures or flow rates resulting from a known initial weight of hydrogen peroxide, admitted to the chamber in a defined unit of time. Using this information, together with the known fixed volume of the chamber environment, a calculation of the approximate VPHP concentration can be made. If conditions are maintained constant throughout each D value assessment run, comparisons of relative resistance among different biological indicator lots may be readily determined.

USE FOR IN-PROCESS VALIDATION

Regardless of the mode of sterilization, the amount of the initial population of the microorganisms, its resistance to sterilization, and the site of inoculation on or in the product can all influence the rate of biological indicator inactivation.

During product microbial challenges, various areas of the product should be inoculated with biological indicators. If, for example, a container with a closure system is sterilized, both the product solution and the closure should be challenged to ensure that sterilization equivalent to a 10^{-6} (one in a million probability of a nonsterile unit) sterilization assurance level (SAL) will be obtained in the solution as well as at the closure site.

One may need to determine through laboratory studies whether product components are more difficult to sterilize

than, for example, a solution or drug within the product. Depending on the locations of the product components most difficult to sterilize, different process parameters may be involved in assuring microbial inactivation to an SAL of 10^{-6} . The product performance qualification phase should identify the most important process parameters for inactivation of microorganisms at the sites most difficult to sterilize. Once these critical processing parameters are determined, during sterilization in-process validation of the product, they should be operated at conditions less than the conditions stated in the sterilization process specifications. Biological indicator survival is predicated upon both resistance and population. Therefore, a 10^6 biological indicator population is not always required to demonstrate a 10^{-6} SAL. The appropriate use for biological indicators is to employ them to confirm that the developed process parameters result in the desired SAL. In moist heat sterilization, the biological indicator is used to establish that physically measured lethality can be verified biologically. Biological indicators with substantive D values and populations substantially less than 10^6 are adequate to validate many sterilization and decontamination processes. It is important that the users be able to scientifically justify their selection of a biological indicator.

<1041> BIOLOGICS

Products such as antitoxins, antivenins, blood, blood derivatives, immune serums, immunologic diagnostic aids, toxoids, vaccines, and related articles that are produced under license in accordance with the terms of the federal Public Health Service Act (58 Stat. 682) approved July 1, 1944, as amended, have long been known as "biologics." However, in Table III, Part F, of the Act, the term "biological products" is applied to the group of licensed products as a whole. For Pharmacopeial purposes, the term "biologics" refers to those products that must be licensed under the Act and comply with Food and Drug Regulations—Code of Federal Regulations, Title 21 Parts 600-680, pertaining to federal control of these products (other than certain diagnostic aids), as administered by the Center for Biologics Evaluation and Research or, in the case of the relevant diagnostic aids, by the Center for Devices and Radiological Health of the federal Food and Drug Administration.

Each lot of a licensed biologic is approved for distribution when it has been determined that the lot meets the specific control requirements for that product as set forth by the Office. Licensing includes approval of a specific series of production steps and in-process control tests as well as end-product specifications that must be met on a lot-by-lot basis. These can be altered only upon approval by the Center for Biologics Evaluation and Research and with the support of appropriate data demonstrating that the change will yield a final product having equal or superior safety, purity, potency, and efficacy. No lot of any licensed biological product is to be distributed by the manufacturer prior to the completion of the specified tests. Provisions generally applicable to biologic products include tests for potency, general safety, sterility, purity, water (residual moisture), pyrogens, identity, and constituent materials (Sections 610.10 to 610.15 and see *Safety Tests—Biologicals* under *Biological Reactivity Tests*, *In Vivo* <88>, *Sterility Tests* <71>, *Water Determination* <921>, and *Pyrogen Test* <151>), as well as *Bacterial Endotoxins Test* <85>). Constituent materials include ingredients, preservatives, diluents and adjuvants (which generally should meet compendial standards), extraneous protein in cell-culture produced vaccines (which, if other than serum-originating, is excluded) and antibiotics other than penicillin added to the production substrate of viral vaccines (for

² BIER/Steam Vessels, American National Standards, ANSI/AAMI ST45:1992.

which compendial monographs on antibiotics and antibiotic substances are available). Additional specific safety tests are also required to be performed on live vaccines and certain other items. Where standard preparations are made available by the Center for Biologics Evaluation and Research (Section 610.20), such preparations are specified for comparison in potency or virulence testing. The U.S. Opacity Standard is used in estimating the bacterial concentration of certain bacterial vaccines and/or evaluating challenge cultures used in tests of them. (See also *Units of Potency* in the *General Notices*.)

The Pharmacopeial monographs conform to the Food and Drug Regulations in covering those aspects of identity, quality, purity, potency, and packaging and storage that are of particular interest to pharmacists and physicians responsible for the purchase, storage, and use of biologics. Revisions of the federal requirements affecting the USP monographs will be made the subjects of *USP Supplements* as promptly as practicable.

Vehicles and Added Substances—Vehicles and added substances suitable for biologics are those named in the Food and Drug Regulations.

Containers for Injections—Containers for biologics intended to be administered by injection meet the requirements for *Containers for Injections* under *Injections* <1>.

Container Content—The volumes in containers of biologics intended to be administered by injection meet the requirements for *Container Content* under *Injections* <1>.

Labeling—Biologics intended to be administered by injection comply with the requirements for *Labeling* under *Injections* <1>. In addition, the label on the final container for each biologic states the following: the title or proper name (the name under which the product is licensed under the Public Health Service Act); the name, address, and license number of the manufacturer; the lot number; the expiration date; and the recommended individual dose for multiple-dose containers. The package label includes all of the above, with the addition of the following: the preservative used and its amount; the number of containers, if more than one; the amount of product in the container; the recommended storage temperature; a statement, if necessary, that freezing is to be avoided; and such other information as the Food and Drug regulations may require.

Packaging and Storage—The labeling gives the recommended storage temperature (see *General Notices*). Precautions should be taken where products labeled to be stored at a temperature between 2° and 8° are stored in a refrigerator, in order to assure that they will not be frozen. Diluents packaged with biologics should not be frozen. Some products (as defined in Section 600.15) are to be maintained during shipment at specified temperatures.

Expiration Date—For compendial articles the expiration date identifies the time during which the article may be expected to meet the requirements of the Pharmacopeial monograph, provided it is kept under the prescribed storage conditions. This date limits the time during which the product may be dispensed or used (see *General Notices*, page 1). However, for biological products, the stated date on each lot determines the dating period, which begins on the date of manufacture (Section 610.50) and beyond which the product cannot be expected beyond reasonable doubt to yield its specific results and to retain the required safety, purity, and potency (Section 300.3 (1) and (m)). Such a dating period may comprise an in-house storage period during which it is permitted to be held under prescribed conditions in the manufacturer's storage, followed by a period after issue therefrom. The individual monographs usually indicate both the latter period and (in parentheses) the permissible in-house storage period. If the product is held in the manufacturer's storage for a longer period than that indicated (in parentheses), the expiration date is set so as to reduce the dating period after issue from the manufacturer's storage by a corresponding amount.

<1043> ANCILLARY MATERIALS FOR CELL, GENE, AND TISSUE-ENGINEERED PRODUCTS

INTRODUCTION

A wide variety of reagents and materials, many of which are unique or complex, are required for the manufacture of cell, gene, and tissue-engineered products. These materials include plasma- or serum-derived products, biological extracts, antibiotics, cytokines, culture media, antibodies, polymeric matrices, separation devices, density gradient media, toxins, conditioned media supplied by "feeder cell layers," fine chemicals, enzymes, and processing buffers. Many of these items are used to ensure the survival and promote the growth of certain cell populations, although their mechanism of action may not be entirely understood. Examples include fetal bovine serum (FBS) and various media supplements. Other items, such as highly purified cholera toxin, are introduced into the processing stream during manufacturing to exert a specific biochemical effect and are immediately washed out in subsequent processing steps to avoid unwanted toxicity at a later point. The finished biological products produced in such processes are often complex mixtures that, in some cases, cannot be completely characterized. Careful scrutiny of the materials used in manufacturing is necessary to prevent the introduction of adventitious agents or toxic impurities, as well as to ensure the ultimate safety, effectiveness, and consistency of the final product.

In cell, gene, and tissue-engineered product manufacturing, these reagents and materials are collectively called ancillary materials (AMs). AMs have also been referred to as ancillary products, ancillary reagents, processing aids, and process reagents. AMs were first discussed under the synonym ancillary products in the U.S. Food and Drug Administration Notice, "Application of Current Statutory Authorities to Human Somatic Cell Therapy Products and Gene Therapy Products" (Federal Register 58(197), October 14, 1993, pp. 53248–53251). This document established the FDA's authority to regulate human somatic cell therapy products and gene therapy products. AMs are also synonymous with "processing materials" that were defined in 21 CFR Part 1271, "Current Good Tissue Practice for Manufacturers of Human Cellular and Tissue-Based Products; Inspection and Enforcement; Proposed Rule" (Federal Register 66(5), January 8, 2001, pp. 1508–1559). AMs can be analogous to "components," and in some cases, "containers" as described in the current good manufacturing practice (cGMP) regulations for finished pharmaceuticals as outlined in 21 CFR 211.80 through 211.94 and 211.101(b) and (c).

The defining property of AMs is that they are not intended to be present in the final product. They are materials used as processing and purification aids or agents that exert their effect on the therapeutic substance. Materials or components that are intended to be in the final product dosage form (e.g., genetic materials, biopolymeric supports, physiological buffers) are not AMs. Cell banks and virus banks are also not considered AMs; there are a number of guidances that describe requirements for their certification. However "helper" viruses and "helper" plasmids may be considered AMs when they are not intended to be part of the final product.

The quality of an AM can affect the stability, safety, potency, and purity of a cell, gene, or tissue-engineered product. For example, the mechanism by which an AM exerts its effect may not be known, and the impact of normal variation of the AM on the quality and safety of the therapeutic product may not be understood. Alternatively, AMs of

human or animal origin may present an infectious disease transmission risk. Other AMs, if administered to humans, may cause an immune reaction. Finally, an AM with toxic properties that is introduced into a manufacturing process and is not adequately removed in subsequent processing steps will expose the patient to a toxic substance and may impair the effectiveness of the therapeutic entity. These risks to the quality and safety of the therapeutic product are often heightened with cell, gene, and tissue-engineered products, due to the limited ability to conduct extensive in-process and release tests. For example, lack of in-process holding steps or limited shelf life may create the need to administer the cell, gene, or tissue-engineered products before in-process or final-release testing results are available. In other cases, the scarcity of suitable donor tissue or the complex logistics in the transport of biological materials may limit the amount of material available for testing. To minimize these risks, whenever possible, it is necessary to implement rigorous material qualification and prudent application of manufacturing process controls.

Frequently, these novel therapeutic products are created using complicated biological processes. The AMs employed in these procedures may be selected primarily for their unique functional contributions or biological effects. Whenever possible, it is preferable to source AMs that are approved or licensed therapeutic products because they are well characterized, have an established toxicological profile, and are manufactured according to controlled and documented procedures. Conversely, the AM may be intended "for research use" and may, therefore, lack the level of qualification necessary for use in the production of a therapeutic product. In either case, the manufacturer of the cell, gene, or tissue-engineered product should develop comprehensive and scientifically sound qualification plans to ensure the traceability, consistency, suitability, purity, and safety of the AM. In cases where AMs are products approved for use for therapeutic purposes, the level of qualification will probably be less extensive than that for a material intended for research purposes. However, their suitability in the manufacturing process will still need to be established when the AM is being used beyond the scope of its intended use or labeling. The purpose of this chapter is to provide guidance in developing appropriate qualification programs for AMs employed in cell, gene, and tissue-engineered product manufacturing.

QUALIFICATION OF ANCILLARY MATERIALS

Qualification is the process of acquiring and evaluating data to establish the source, identity, purity, biological safety, and overall suitability of a specific AM. The responsibility for AM qualification resides with the developer or manufacturer of the cell, gene, or tissue-engineered product. This section outlines the basis by which a manufacturer can establish rational and scientifically sound programs for qualifying AMs, although the broad nature of the cell, gene, and tissue-engineered products and of the AM used to produce these products make it difficult to recommend specific tests or protocols for a qualification program. Thorough documentation is the cornerstone of any qualification program.

A well-designed qualification program becomes more comprehensive as product development progresses. In the early stages of product development, safety is the primary focus. In the later stages, AM production and qualification activities should be comprehensively developed to support eventual licensure of the cell, gene, and tissue-engineered product. On some occasions, complex or unique substances that have been shown to be essential for process control or production may not be available from suppliers that produce them in compliance with cGMP. In these situations, the manufacturer will have to develop a scientifically sound strategy for qualification. A qualification program for AMs used in cell, gene, and tissue-engineered product manufac-

turing should address each of the following areas: (1) identification, (2) selection and suitability for use in manufacturing, (3) characterization, (4) vendor qualification, and (5) quality assurance and control.

Identification

The first step in any qualification program is the listing of all of the AMs used in a given product manufacturing and where in the manufacturing process they are to be employed. The source and intended use for each material should be established, and the necessary quantity or concentration of each material should be determined. Also, alternate sources for each material should be identified.

Selection and Suitability for Use

Developers of cell, gene, and tissue-engineered products should establish and document selection criteria for AMs and qualification criteria for each vendor early in the design phase of product development. Selection criteria should include assessments of microbiological and chemical purity, identity, and biological activity pertinent to the specific manufacturing process. It is important to address these issues early in product development because certain AMs that are initially considered necessary may be impossible or prohibitively expensive to qualify, thereby justifying the investigation of alternatives or replacements. Examples include some animal- or human-derived materials that in some cases have alternate (i.e., plant or chemically synthesized) sources.

AMs of animal or human origin should be selected cautiously due to the potential infectious or zoonotic disease risks associated with these materials. Vendors should be selected that can supply documentation regarding the country of origin for animal-derived AMs to address concerns regarding transmissible spongiform encephalopathies and other diseases of agricultural concern, like tuberculosis and brucellosis. In many cases, the chain of custody for animal-derived AMs (i.e., abattoir → intermediate processing center → final processing center) will need to be documented. Vendors of human-derived AMs should be able to supply documentation regarding material traceability. For instance, human plasma-derived AMs should be sourced from licensed facilities that control the donor pool and appropriately screen the individual donors for relevant human infectious diseases. In some cases, vendors of animal- and human-derived AMs supply different grades of materials, some of which will be more suitable for use in cell, gene, and tissue-engineered product manufacturing than other grades. For example, FBS can be obtained that has been processed to reduce the risk of bovine viral contamination by subjecting it to validated irradiation and nanofiltration processes. Also, many animal and human plasma-derived components are subjected to chemical (detergent or solvent treatment) or physical (heat exposure for extended periods of time) treatments that have been shown through validation studies to significantly reduce the risk of adventitious microbial or viral contamination associated with starting AMs. Such AMs are preferred for use in cell, gene, and tissue-engineered product manufacturing processes because they significantly reduce the risks associated with the original material.

The complexity of risk assessment can be reduced by employing one of a number of quantitative or semiquantitative approaches, such as failure mode effects analysis (FMEA), quality function deployment (QFD), or hazard analysis and critical control point (HACCP). These programs typically assign a point value to each risk parameter for an AM that results in cumulative scores that make it easier to prioritize effort and resources for decreasing the risks associated with AMs. For example, an AM that has a strong safety profile and is used in minimal amounts in upstream steps of the manufacturing process and is thoroughly washed from the system would accumulate a low point score. Conversely, an

AM that is known to be toxic and is employed in downstream processing would, therefore, possess a higher potential for appearing as a residual in the final product and would be assigned a higher point value. One can also assign points based on the risk classification (see *Risk Classification*).

Characterization

Specific quality control characterization tests need to be developed or adopted and implemented for each AM. The set of tests for each AM should assess a variety of quality attributes, including identity, purity, functionality, and freedom from microbial or viral contamination. The appropriate level of testing for each AM is derived from its risk assessment profile and the knowledge gained during development. Test specifications should be developed for each AM to ensure consistency and performance of the manufacturing process. Acceptance criteria should be established and justified on the basis of the data obtained from lots used in preclinical and early clinical studies, lots used for demonstration of manufacturing consistency, and relevant development data, such as those arising from analytical procedure development and stability studies.

Some AMs that are biological in nature may be difficult to fully characterize. Because these materials exert their effects through complex biological activities, and biochemical testing may not be predictive of the AM's process performance, functional or performance testing may be needed. Performance variability of such materials may have a detrimental impact on the potency and consistency of the final therapeutic product. Examples of complex functionality testing for AMs include growth promotion testing of individual lots of FBS on the cell line used in manufacturing, performance testing of digestive enzyme preparations, and *in vitro* tissue culture cytotoxicity assays. (see aspects of *Performance Testing*).

Vendor Qualification

Vendors supplying AMs should be qualified at the earliest opportunity. An early audit of the vendor's manufacturing facility, including their GMP and AM testing program, are basic elements of a vendor qualification program. A review of the vendor's processing procedures and documentation program is essential in establishing confidence in the vendor as a reliable supplier. Additionally, vendors that have been certified through an ISO inspection program or audited by other governmental agencies tend to have robust quality systems in place. Reports of past audits of U.S. suppliers obtained through the Freedom of Information (FOI) Act may augment the qualification process.

It is important to develop a good working relationship with a vendor. In some cases, the vendor may provide higher manufacturing standards, custom formulation services, or replacement of substandard components upon request, with or without additional costs. A good rapport is essential if further investigation into AM suppliers is warranted. It is also critical to ensure that the vendor takes appropriate steps to prevent cross contamination between its products during manufacture. Vendors should be familiar with the principles of validation, especially cleaning validation, as well as viral inactivation and sterilization validation. Finally, systems should be established where vendors supply written certification of processing or sourcing changes to customers, well in advance of the implementation of the changes so that customers can evaluate the potential impact of such changes.

Quality Control and Quality Assurance

Because the components of the qualification program are multifaceted and need to be in compliance with cGMP, they should be monitored by a quality assurance/quality control

unit (QAU). Typical QAU activities include the following systems or programs: (1) incoming receipt, segregation, inspection, and release of materials prior to use in manufacturing, (2) vendor auditing and certification, (3) certificate of analysis verification testing, (4) formal procedures and policies for out-of-specification materials, (5) stability testing, and (6) archival sample storage.

RISK CLASSIFICATION

A scientifically sound and rational qualification program should be designed for each AM and should take into account the source and processes employed in its manufacture. Whenever available, AMs that are approved or licensed therapeutic products are preferable because they are well-characterized with an established toxicological profile and are manufactured according to controlled and documented procedures. Licensed biologics, approved drugs, and approved or cleared medical devices or implantable materials that have been incorporated into cell, gene, or tissue-engineered product manufacturing processes present a known or more favorable safety profile for the patient than nonapproved or nonlicensed versions. Qualification programs for these AMs should reflect the extensive scrutiny that these items were subjected to in their development and manufacture. Consequently, greater emphasis should be placed on the investigation of the impact of inherent variability of these AMs on final product function. For instance, a manufacturer may utilize human serum albumin, intended for human administration, as a supplement to a cell cultivation medium for a cell-based product. Because the cell-based product is marketed as a licensed biological, one need not repeat all the testing already performed by the supplier as part of material qualification. In contrast, the impact of lot-to-lot variability on cell growth rate or maintenance of an important differentiated cellular property may be a prudent area of investigation. Alternatively, the stability of this material at the concentration employed in processing or its potential for interaction with other processing components may also be areas worthy of investigation. Such approaches to AM qualification therefore focus on the AM as a potential source of variability that may influence final product potency and safety. Qualification programs for these AMs should be comprehensive to minimize consumer risk and ensure that unacceptable lots or adulteration will be detected.

The qualification program must also take into account the quantity of the AM employed in manufacturing as well as its point of introduction in the manufacturing process. A relevant example is the use of FBS as a supplement to a tissue culture medium used to expand a stem cell population from a specific tissue for eventual administration to a patient (see *Manufacturing Overview* under *Cell and Gene Therapy Products* (1046)). A qualification program for such an AM would include (a) assurance that the serum was sourced from a country or region known to be free of bovine spongiform encephalopathy (BSE); (b) assurance that the source herds are monitored and test negative for specific diseases relevant in agricultural settings (e.g., tuberculosis, brucellosis, foot and mouth disease); (c) testing of the serum for sterility, mycoplasma, endotoxin content, and adventitious bovine viruses known to be associated with the material;¹ (d) the review and archiving of the supplier's certificate of analysis; (e) lot-to-lot assessment of the ability of the serum to consistently expand a representative cell population using a standardized cell culture quality control assay; and (f) on-site audit of the supplier to ensure that the material is sourced and processed in a manner deemed acceptable by a responsible QA unit.

¹Most suppliers test for adventitious agents according to 9 CFR 113, which was developed by the Center for Veterinary Biologics, Animal and Plant Health Inspection Service, United States Department of Agriculture. These tests may differ from those used to test products developed for human use (e.g., mycoplasma).

To aid manufacturers and developers in the design of their qualification programs for a variety of AMs, tiers of sample risk categories are presented in *Tables 1–4* and are provided as a guide. Risk is also dependent on the amount and the stage at which the AM is used in the manufacturing process. *Tables 1–4* do not address the impact of quantity or stage of use.

Tier 1—These AMs are low-risk, highly qualified materials that are well-suited for use in manufacturing. The AM is either a licensed biologic, an approved drug, an approved or cleared medical device, or it is intended for use as an implantable biomaterial. Generally these components or materials are obtained as a sterile packaging system or dosage form intended for their label use, but are instead utilized “off label” in the manufacturing process for the cell, gene, or tissue-engineered product.

Tier 2—These AMs are low-risk, well-characterized material that are well-suited for use in manufacturing. Their intended use is for drug, biologic, or medical device manufacture, including cell, gene, and tissue-engineered products as AMs, and they are produced under relevant cGMPs. Most animal-derived materials are excluded from this category.

Tier 3—These AMs are a moderate risk material that will require a higher level of qualification than previous tier materials. Frequently, these materials are produced for in vitro diagnostic use and are not intended for use in the production of cell, gene, or tissue-engineered products. In some cases, upgrade of AM manufacturing processes may be necessary in order to employ the AM in manufacturing of these products (e.g., modification of the production process for a diagnostic grade monoclonal antibody to include robust viral removal steps in purification).

Tier 4—This is the highest risk level for AMs. Extensive qualification is necessary prior to use in manufacturing. The

material is not produced in compliance with cGMPs. AMs are not intended for use in the production of cell, gene, or tissue-engineered products. This risk level includes highly toxic substances with known biological mechanisms of action, and also includes most complex, animal-derived fluid materials not subjected to adventitious viral removal or inactivation procedures. These materials may require (a) an upgrade of AM manufacturing processes; (b) treatment of AMs to inactivate or remove adventitious agents, disease-causing substances, or specific contaminants (e.g., animal viruses, prions); (c) testing of each lot of material to ensure that it is free of adventitious agents, disease-causing substances, or specific contaminants; (d) validation of the manufacturing process of the cell, gene, or tissue-engineered product to assess consistency of removal of a known toxic substance or lot-release testing to demonstrate reduction levels considered to be safe; or (e) validation of the manufacturing process of the cell, gene, or tissue-engineered product to assess consistency of removal or inactivation of adventitious agents, disease-causing substances, or specific contaminants associated with the material. Developers in the early stages of development should evaluate the necessity of these materials and explore alternative substances or sources.

PERFORMANCE TESTING

In cases where AMs are chosen for their ability to provide a particular biological function in producing the therapeutic product, performance testing becomes an essential component of their overall qualification. This is especially true when the AM plays a critical role in modulating a complex biochemical effect and has a large impact on product manufacturing yield, purity, or final product potency. These AMs tend to be complex substances or mixtures, are frequently

Table 1. AM Risk Tier 1
Low-Risk, Highly Qualified Materials with Intended Use as Therapeutic Drug or Biologic, Medical Device, or Implantable Material

Example	Typical Use in Cell, Gene, or Tissue-Engineered Product Manufacturing	Qualification or Risk Reduction Activities
Recombinant insulin for injection	Cell culture medium additive	DMF cross reference (when possible or practical) Certificate of analysis
Organ preservation fluid	Process biological fluid employed in tissue transport or processing	
Human serum albumin for injection	Cell culture medium	Assess lot-to-lot effect on process performance ¹
Sterile fluids for injection	Process biological fluid employed in tissue transport, cell processing, purification	Assess removal from final product
Implantable biomaterials (formed collagen, silicone, polyurethane constructs intended for surgical implantation)	Scaffolds, matrices for immobilized cellular cultivation	Stability assessment on AM as stored for use in manufacturing ²
Recombinant deoxyribonuclease for inhalation or injection	Process enzyme employed in viral vector manufacturing, stem cell processing	
Antibiotics for injection ³	Cell culture medium and biopsy transport fluid additive to reduce risk of bacterial contamination	
Injectable monoclonal antibodies	Immunologically targeting specific cell populations for selection or removal	
Injectable cytokines	Cell culture medium	
Vitamins for injection; defined nutrients, chemicals, or excipients intended for injection	Cell culture medium additive employed in cell expansion, controlled cellular differentiation/activation step, or manufacture of a viral vector	
IV bags, transfer sets and tubing, cryopreservation bags, syringes, needles	Storage vessels or container closure systems, closed aseptic transfer systems	

¹ See *Performance Testing*.

² Often AMs are aliquoted or stored at different concentrations, in different buffers, or under conditions that are different from those stated on the label or previously validated. Data should be generated that demonstrate the stability and preservation of activity of the AM under the conditions that are specific to the manufacturing application.

³ Beta lactam antibiotics should not be used as AMs due to the risk of patient hypersensitivity.

biologically sourced, and can exhibit significant lot-to-lot variability. As a result, these AMs usually have no simple identity test, nor can they be easily characterized by physical or chemical tests. The development of well-defined performance assays for complex AMs will not only ensure process reproducibility and final product quality, but in many cases

will satisfy the identity testing criteria in accordance with 21 CFR 211.84(d).

In some cases, the initial qualification of an AM for use in manufacturing should be the investigation of the effect of the amount of the AM on the desired response (increased yield, purity, or potency of the therapeutic product). The

Table 2. AM Risk Tier 2
Low-Risk, Well Characterized Materials with Intended Use as AMs, Produced in Compliance with GMPs

Example	Typical Use in Cell, Gene, or Tissue-Engineered Product Manufacturing	Qualification or Risk Reduction Activities
Recombinant growth factors, cytokines ¹	Cell culture medium additive	DMF cross reference (when possible or practical)
Immunomagnetic beads	Immunomagnetic separation of cells	Certificate of analysis
Human AB serum	Cell culture medium additive	Assess lot-to-lot effect on process performance ²
Progesterone, estrogen, vitamins, purified chemicals (USP-grade)	Cell culture medium additives, induction agents, buffer components	Assess removal from final product
Sterile process buffers	Process biological fluid employed in tissue transport, cell processing, purification	Stability assessment on AM as stored for use in manufacturing ³
Biocompatible polymers, scaffolds, hydrogels	Scaffolds, matrices for immobilized cellular cultivation	When relevant, confirm certificate of analysis test results critical to product (could include functional assay)
Proteolytic enzymes	Process enzyme	Vendor audit
Tissue culture media	Cell culture medium additive	
Monoclonal antibodies	Immunologically targeting specific cell populations for selection or removal	
Density gradient media	Cell separation via centrifugation	

¹ These AMs should be produced from nonmammalian, recombinant sources (i.e., microbially grown in the absence of animal-derived growth medium components).

² See *Performance Testing*.

³ Often AMs are aliquoted or stored at different concentrations, in different buffers, or under conditions that are different from those stated on the label or previously validated. Data should be generated that demonstrates the stability and preservation or activity of the AMs under the conditions that are specific to the manufacturing application.

Table 3. AM Risk Tier 3
Moderate-Risk Materials Not Intended for Use as AMs
(frequently produced for in vitro diagnostic use or reagent grade materials)

Example	Typical Use in Cell, Gene, or Tissue-Engineered Product Manufacturing	Qualification or Risk Reduction Activities
Recombinant growth factors, cytokines	Cell culture medium additive	DMF cross reference (when possible or practical) Certificate of analysis Assess lot-to-lot effect on process performance ¹ Assess removal from final product Stability assessment on AM as stored for use in manufacturing ² When relevant, confirm certificate of analysis test results critical to product (could include functional assay)
Tissue culture media	Cell culture medium additive	Vendor audit
Monoclonal antibodies (diagnostic-grade produced in cell culture)	Immunologically targeting specific cell populations for selection or removal	Upgrade manufacturing process for material to GMP
Process buffers	Process biological fluid employed in tissue transport, cell processing, purification	Develop stringent internal specifications
Novel polymers, scaffolds, hydrogels	Scaffolds, matrices for immobilized cellular cultivation	Determine if lot-to-lot biocompatibility, cytotoxicity, or adventitious agent testing are needed
Proteolytic enzymes	Process enzyme	
Purified chemicals (reagent-grade)	Culture medium additives, induction agents, buffer components	

¹ See *Performance Testing*.

² Often AMs are aliquoted or stored at different concentrations, in different buffers, or under conditions that are different from those stated on the label or previously validated. Data should be generated that demonstrate the stability and preservation or activity of the AM under the conditions that are specific to the manufacturing application.

amount of the AM used in manufacturing should be chosen to consistently yield the desired effect while minimizing issues by removing the AM in subsequent processing steps. Such testing frequently assesses the important functional attribute expected of the AM in a scaled-down or simulated manufacturing process. Some examples follow:

- If an AM is added to the culture media because it promotes cellular proliferation or the secretion of a critical therapeutic agent, the assay could demonstrate that each lot of AMs produces the expected rate and amount of cellular proliferation or the expected level of secreted therapeutic agent.
- If a monoclonal antibody is used to purify a particular cell type, the new lot of monoclonal antibody could be shown to purify the cell population with the expected recovery and purity for the desired cell type.
- If a deoxyribonuclease is used to degrade cellular DNA, new lots could be tested for the ability of the deoxyribonuclease to degrade DNA.
- If a particular type of density gradient material is used to purify a vector or cell, new lots of the material used to make the gradient could be shown to purify the vector or cell to an acceptable level.
- If a plasmid or viral vector is used in the production of a gene therapy vector (e.g., helper function), new lots of the helper vector could be shown to produce the expected amounts of the gene therapy vector.
- If a cell therapy is produced in a hollow-fiber bioreactor, new lots of the bioreactor could be shown to produce the anticipated amount of cell product.

The actual assay used may well evolve as the manufacturing process is developed further and the critical relationships of the AM and the final product are better understood.

Because most performance testing yields relative results, it is often helpful to assay a new lot of AMs side by side with an approved lot of AMs or an official reference standard, if available. This simultaneous comparison helps to reduce the variability due to different lots of cells or vectors and will help discern variability associated with the different lots of AMs. If performance testing involves assays to demonstrate that the new lot of AMs does not affect the impurity profile of the final therapeutic product, either by generating new impurities or by increasing the level of existing impurities, it is helpful to assay both for the total level of impurities, as well as look for the presence of new impurities. An immunologically-based binding assay can typically assess only the total level of impurities. For example, a Western blot of the gene therapy product that is probed both with antibodies to the product and antibodies to host cell proteins is useful for detecting new protein species and significant increases in the levels of host cell impurities. This initial qualification is enhanced by a performance assay that has a quantitative readout with a clear change in the signal when a significant

change in the amount of AMs is introduced into the assay (e.g., dose response). A threshold-type response (i.e., there are two levels of response to the AM and neither large changes in an AM below a certain dose nor above a certain dose change the response) can make it more difficult to select a concentration of AM that consistently results in the desired effect and minimizes the residual levels of the AM in the final therapeutic product.

ANCILLARY MATERIALS RESIDUAL LEVEL ASSESSMENT AND REMOVAL

AMs are not intended to be present in the final dosage form in cell, gene, and tissue-engineered products. Their presence in the final product could lead to undesired effects in the recipient or have a detrimental effect on product potency. Undesired effects in humans include direct toxicity of the AM or an unwanted immunogenic response. Some examples include the following:

- In the generation of a tumor vaccine using a patient's tumor biopsy as the starting material, a chemical entity is introduced to denature the cell surface proteins and tumor antigens to enhance their antigenicity. The chemical entity is known to be highly toxic.
- Antibiotics may be added to a transport solution for human cells to address microbial contamination issues associated with the procurement procedure. Residual levels of the antibiotic may affect the proliferative capacity of the final engineered cellular product. Residual antibiotics could also cause an anaphylactic response in some individuals.
- FBS, employed in the cultivation of an engineered human skin graft, may cause the development of a humoral antibody response directed against bovine proteins.
- Aggregated mouse immunoglobulin, a trace impurity in a purified preparation of mouse monoclonal antibody used to target a cell population for immunoselection, may be immunogenic.
- A cytokine, employed as an immunomodulator in the generation of a gene-modified autologous tumor vaccine product, may elicit a severe reaction in the recipient.
- Cholera toxin, employed as part of a cell culture medium for a cell therapy product intended for intravenous administration, will be highly toxic to the recipient if it is not removed during processing.

These risks can be mitigated through the design of processes to include steps to adequately remove the AM through dilution, separation, or inactivation, as well as the development of analytical detection assays to assess the AM levels during processing and in the final therapeutic prod-

**Table 4. AM Risk Tier 4
High-Risk Materials**

Example	Typical Use in Cell, Gene, or Tissue-Engineered Product	Qualification or Risk Reduction Activities
FBS	Cell culture medium additive	Same as in Table 3, plus
Animal-derived (including human) extracts	Cell culture medium additive	
Animal-derived polymers, scaffolds, hydrogels	Scaffolds, matrices for immobilized cellular cultivation	
Purified enzymes	Process enzyme	Verify traceability to country of origin
Ascites-derived antibodies or proteins	Immunologically targeting specific cell populations for selection or removal	Assure country of origin is qualified as safe with respect to source-relevant animal diseases, including TSE
Animal or human cells used as feeder layers	Cell culture substratum or source of medium components	
Chemical entities with known toxicities (i.e. methotrexate, cholera toxin, <i>Staphylococcus aureus</i> pore-forming hemolysin, <i>Staphylococcus enterotoxins A and B</i> , toxic shock syndrome toxin)	Selection agents used in cell culture to improve or maintain transgene expression, enhance cellular proliferation, improve cell survival upon cryopreservation, superantigens for the activation of T cells	Adventitious agent testing for animal source-relevant viruses

uct. Assessment and removal strategies for residual AMs should be considered in the early phases of process development. There are two different approaches for assessing residual AM levels in the final therapeutic product: (1) Validation studies can demonstrate that the process is capable of removing more of the AM than would be present in a worst-case scenario. (2) The residual levels of an AM can be measured for each lot at an appropriate step in the manufacturing process.

Validation of an AM removal is often best performed by spiking the impure product with "worst case" or higher levels of the AM and showing the purification process is capable of removing the AM to "undetectable levels." Clearance factors can then be generated for each purification step in a manner analogous to that done in viral clearance studies. When designing the validation studies, the following three considerations should be included: (1) The assay should be able to accurately quantitate the AM in each sample matrix. (2) If the validation is conducted at a scale smaller than that used for routine lot production, the comparability of this smaller scale process to the full scale process needs to be demonstrated. This usually means that the smaller scale process is operated using the same critical parameters as the full scale process with the product generated at each step having a similar purity and yield. (3) As with any spiking study, one has to demonstrate that the additional, higher level of AM has not affected the purification process. If the second approach of measuring residual levels of the AM in each lot is used, the specification for the maximum amount of AM in the final therapeutic product is based on the amount of the AM in the lots used in toxicological or clinical studies or known toxicological data.

The development of sensitive and reproducible analytical assays for AMs is another important component of a risk reduction approach. Two types of assays are useful in assessing the levels of residual AM impurity: a limit test and a quantitative test. Either test should be accurate, precise, robust, and have a low limit of detection. Assays for residual AMs may be performed on the product before it is formulated (e.g., on the drug substance) to avoid any interference of the components used in the formulation with the assay for residual AMs or in the final drug product. Spike-recovery controls are often included in such assays to demonstrate that the sample matrix does not inhibit the detection of the AM. Preferably, assays should be designed to detect all forms of AMs including aggregates, fragments, or conjugates. Aggregated protein has been shown to be particularly immunogenic.

Immunoassays such as ELISA are most commonly used to assess residual levels of AMs. An ELISA for bovine serum albumin (BSA) has been used to assess residual levels of FBS. Polymerase chain reaction (PCR) technology has been employed to assess residual levels of host cell DNA. Labeling cells with ^3H thymidine or performing PCR for a feeder cell-specific gene sequence are two ways to assess for residual levels of feeder cells. If "wash out" of the AM is achieved by exhaustive dilution associated with further processing activities, it may be useful to calculate the dilution factor for the AM during this processing. In some cases, this is sufficient to ensure that the AM has been reduced to safe levels for early clinical development. Data should be obtained later in clinical development to confirm the wash out of the AM at the expected step(s). This approach is particularly useful when there is pre-existing knowledge of the therapeutic levels and toxicity of the AM. In other cases, information regarding the safety and tolerability of the AM should be collected (in preclinical toxicology studies or later with human clinical studies) in order to determine the safe or nontoxic levels that must be achieved. These data may be needed even for an AM that is approved for use for therapeutic purposes if it is being used in a manner inconsistent with its intended use or labeling or if the route of administration or dosage level of the AM may present risks not previously encountered or considered.

CONCLUSION

While many types of AMs are used during the manufacture of cell, gene, and tissue-engineered products, they have received less emphasis than the final products. However, the importance of AM quality to the quality of the final product cannot be overstated. Good quality AMs should perform as intended in a consistent manner, batch-to-batch, if they are carefully selected and appropriately used. AMs of insufficient quality will affect the quality and the effectiveness of the final product and endanger the health of patients. Thus, implementing an AM qualification program that addresses the risks associated with the AM, the stage of manufacture at which it is used, and the amount of the AM used during manufacture will ensure the safety and effectiveness of the final product.

APPENDIX

AMs used in cell, gene, and tissue-engineered products will be regulated in the context of the manufacturing process of the cell, gene, and tissue-engineered products. Certain AMs may already be approved for uses other than for cell, gene, and tissue-engineered product manufacture. It is preferable to source AMs that are approved therapeutic products when they are available because they are well-characterized with an established toxicological profile and are manufactured according to controlled and documented procedures. The following list of documents should provide relevant regulatory guidance and a description of best practices in product and process development, manufacturing, quality control, and quality assurance:

- *Biological Reactivity Tests, In Vitro* (87)
- *Biological Reactivity Tests, In Vivo* (88)
- *Biotechnology-Derived Articles* (1045)
- *Cell and Gene Therapy Products* (1046)
- *Biotechnology-Derived Articles—Amino Acid Analysis* (1052)
- *Biotechnology-Derived Articles—Capillary Electrophoresis* (1053)
- *Biotechnology-Derived Articles—Isoelectric Focusing* (1054)
- *Biotechnology-Derived Articles—Peptide Mapping* (1055)
- *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* (1056)
- *Biotechnology-Derived Articles—Total Protein Assay* (1057)
- 21 CFR 211 Subpart E, 211.80 through 211.94 and 211.101
- 21 CFR 312
- 21 CFR 314
- 21 CFR 801.109 (b) (1)
- 21 CFR 807.81 through 21 CFR 807.97
- 21 CFR 812
- 21 CFR 814
- FDA Center for Biologics Evaluation (CBER) "Draft Guidance for Monoclonal Antibodies Used as Reagents in Drug Manufacturing" (1999)
- FDA Center for Biologics Evaluation (CBER) "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals" (1993)
- FDA Center for Devices and Radiological Health (CDRH) "Class II Special Controls Guidance Document: Tissue Culture Media for Human ex vivo Tissue and Cell Culture Processing Applications; Final Guidance for Industry and FDA Reviewers" (May 16, 2001)
- CDRH Blue Book Memorandum C95-1
- ISO 10993-1: 1997 "Biological Evaluation of Medical Devices—Part 1: Evaluation and Testing"
- International Conference on Harmonization (ICH) Q5A "Guidance for Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human and Animal Origin"
- International Conference on Harmonization (ICH) Q5D "Guidance on Quality of Biotechnological/Biological Products: Derivation and Characterisation of Cell Sub-

strates Used for Production of Biotechnological/Biological Products"

- Public Health Service Guideline on Infectious Diseases Issues in Xenotransplantation (October 18, 2000)

<1045> BIOTECHNOLOGY-DERIVED ARTICLES

Macromolecular substances can be obtained by a number of methods including extraction from natural sources, modification of naturally occurring protein, mammalian cell culture in vitro, mammalian cell culture in vivo, production by microorganisms, and chemical syntheses. From a compendial perspective, macromolecular articles derived from biotechnology processes—or more specifically from recombinant-DNA (rDNA) technology, hybridoma technology, and transformed continuous cell lines—are those articles for which official names have been established. These articles have official public standards for identity, strength (potency), quality, and purity. Advances in genetics and the applications of genetic engineering have made the production of new and existing macromolecular articles technologically and economically feasible.

The technologies involved in producing a protein by biotechnological processes have been widely documented and general guidelines have been established by the federal government. The products of biotechnology may be regulated as drugs, biologics, or diagnostics, depending on their source, composition, and intended use. The novel approaches permitted by biotechnology can make it difficult to apply classic definitions of these categories and FDA has advised manufacturers to seek clarification in the early stages of development for how a product will be regulated when classification is not obvious.¹ The overall regulatory scheme for biotechnology-derived products is the same as for products in the same category produced by traditional manufacturing methods, with the addition of specific requirements suited to the biotechnology-derived product. The general requirements are described primarily in the applicable parts of the Code of Federal Regulations, Title 21. NIH has published a guideline for rDNA research that is mandatory for both public and private NIH-supported research. This guideline has wide acceptance and voluntary compliance is common by institutions and corporations not specifically governed by it.² Laboratory safety practices, particularly protection from potentially infectious materials, are a concern.³ Producing macromolecular articles by biotechnological processes involves initially the cloning of a specific gene in the laboratory, or the construction of a synthetic gene, with subsequent insertion into a host cell and subcloning in a microorganism or cell culture; then a process development on a pilot scale to optimize yield and quality; and finally large-scale fermentation or cell culture processes. The next step, which is the most relevant to the development of compendial monographs, is the purification of the macromolecular proteins. This is followed by animal testing, clinical testing, regulatory approval, and marketing.

¹ A series of documents entitled *Points to Consider* are available from the Director, FDA Center for Biologics Evaluation and Research, HFB-1, 8800 Rockville Pike, Bethesda, MD 20892.

² This guideline was originally published in the Federal Register, *Guidelines for Research Involving Recombinant DNA Molecules* 1986; 51 (88): 16957-16985. Copies may be obtained from the Office of Recombinant DNA Activities, 12441 Parklawn Drive, Suite 58, Rockville, MD 20852.

³ A comprehensive guideline, *Biosafety in Microbiological and Biomedical Laboratories*, is available from the Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402, stock #107-040-000508-3.

Development of relevant public standards for these macromolecular articles is generally closely linked to the processing technology used and the physicochemical and biological characteristics of a specific drug. Characterizations of these articles to ensure safety, purity, and activity should incorporate classical techniques as well as methods specific to the technology. There is always the possibility that these articles may cause some untoward effects in patients using them due to immunological sensitization as a result of a single (or multiple) molecular modification. Such a possibility requires precise characterization of these substances. Although it is theoretically possible to develop public standards for a macromolecular article, it is not possible to develop specific standards that incorporate all prospective methods of production. The compendial perspective is to develop public standards that can be applied to a final product without comprehensive knowledge of production details but which can ensure maintenance of safety, identity, strength, quality, and purity.

Testing for identity, purity, and activity generally requires the use of USP Reference Standards. It will be necessary to consider what USP Reference Standards might be required and how relevant they might be to the method of production as it relates to a final product's characteristics. Such decisions will be made on a product-by-product basis. Favorable consideration will be given to the use of USP Reference Standards that are representative of the specific products that have undergone clinical testing and are fully characterized.

Although early adoption in USP of general methods of analysis of macromolecular drugs could be conducive to early standardization of methods, the technology and analytical procedures are evolving very rapidly. Analytical procedures—chemical, physical, microbiological, and immunological—will be included in the specific product monographs.

SCOPE OF BIOTECHNOLOGY IN THE DEVELOPMENT OF PHARMACOPEIAL ARTICLES

Definition of Biotechnology—Historical Perspective

In its broadest definition, biotechnology refers to the use of living organisms, including isolated mammalian cells, in the production of products having beneficial use. This definition would place alcohol, antibiotic production, and dairy processing, for example, within the scope of biotechnology. However, the current interest in biotechnology is primarily a result of two major advances. The first advance was the development of rDNA technology, which allowed the genes of one species to be transplanted into another species. Thus, gene coding for the expression of a desired protein (usually human) could be inserted into a host prokaryotic or eukaryotic cell in such a manner that the host cell would then express usable quantities of the desired protein. The second major advance was the development of techniques for producing large quantities of monoclonal antibodies (i.e., antibodies arising from a single lymphocyte).

Biotechnology within the pharmaceutical industry generally refers either to the production of protein products using rDNA techniques or to the production of monoclonal antibodies. Other technologies, such as transgenic animals and plants, gene therapy, and antisense DNA, may have potential implications for the pharmaceutical industry in the future but are not within the scope of this chapter.

rDNA Technology

The major steps in the application of rDNA technology for production of a desired protein are outlined in this section.

The critical first step is identification of the protein that is to be produced, followed by the isolation of the gene of interest (i.e., the DNA sequence coding for the desired protein). Once this gene is isolated and fully characterized, it is inserted into a suitable vector such as a plasmid, which is an extrachromosomal segment of DNA usually found in certain bacteria. The plasmid is then inserted into the host cell. Clones of the transformed host cell line are isolated, and those that produce the protein of interest in the desired quantities are preserved under suitable conditions as a cell bank. As manufacturing needs arise, the cloned cells can be scaled up in a fermentation or cell culture process to produce the protein product.

Although the rDNA process is more fully described elsewhere in this chapter, the following important points should be recognized. The vector (plasmid) generally contains a selectable marker that can be used to identify cells that contain this gene. This is in addition to the gene coding for the protein of interest and the regulatory nucleotide sequences necessary for plasmid replication and messenger RNA (mRNA) transcription (the first step in protein synthesis). Selection of the desired cells is simplified because only properly transformed cells containing the selectable marker gene will survive under the growth conditions used to identify and propagate the transformed cells. Typically, the bacterial and eukaryotic selectable markers may include both antibiotic resistance or genes that complement an auxotrophic host mutation. There are numerous examples of both types of markers in each system.

Significant differences exist in the rDNA production process between prokaryotic and eukaryotic cells. In general, bacterial cells express greater concentrations of protein product and require relatively simple media components. However, prokaryotic cells do not perform many important post-translational modifications such as glycosylation and, historically, it was not possible to express large proteins in *E. coli*. These limitations necessitate the use of eukaryotic cells in many cases. The production differences between eukaryotic and prokaryotic host cells have significant impacts that are reflected in the requirements for process validation, purification, and analytical methodology. These requirements are addressed later in this chapter.

Monoclonal Antibodies

Antibodies are proteins produced by differentiated B lymphocytes. Each lymphocyte produces an antibody of defined specificity (i.e., the antibody molecule recognizes a specific site or epitope on the antigen). Antibodies that are produced in immunized animals are formed from many different clones of B lymphocytes; hence, the name polyclonal antibodies. Because the harvest of blood from these animals, by definition, results in polyclonal antibody mixtures, the antisera have multiple epitope recognition sites with a wide variety of binding constants (avidity) and therefore vary from lot to lot. Antibodies that are produced by immortalized cell lines (hybridomas) derived from single B cells are referred to as monoclonal antibodies. The harvest of these cultures leads to an antibody of specific epitope recognition with a homogeneous binding constant.

B lymphocytes have a finite life span in culture and have to be immortalized to enable continuous monoclonal antibody production. At present, the most common procedure is through chemically-induced fusion of a mouse spleen cell with a mouse myeloma cell. The resultant mouse-mouse hybridoma cell inherits from the myeloma cell the ability to replicate continuously in culture and inherits from the spleen cell the ability to produce the desired monoclonal antibody. Cell banks of the hybridoma cell line can be used to produce a continuous supply of the monoclonal antibody, either *in vivo* (i.e., by injection into mice and subsequent collection of the ascites fluid), or *in vitro* (i.e., by conventional cell culture techniques). It should be mentioned that recent advances in molecular genetics have led

to the development of transfectomas and *E. coli*- and bacteriophage-based production schemes that may offer advantages for future production of monoclonal antibodies.

Process validation, purification, and analytical considerations for monoclonal antibodies are conceptually similar to those for rDNA products. This is because both types of products are proteins and therefore require similar handling and assay procedures. Because monoclonal antibodies are the products of immortalized cell lines, there is concern that potential viral nucleic acid contaminants be effectively excluded or inactivated by the manufacturing processes, just as for recombinant products of continuous cell lines.

Commercial applications of monoclonal antibodies include both diagnostic and therapeutic uses. In some cases, the monoclonal antibody is coupled to another substance (e.g., an oncolytic agent, radionuclide, toxin), with the resultant antibody conjugate being the final product of interest. In this case, both the antibody intermediate and the final product require extensive process development and analytical characterization.

For the purposes of this chapter, the scope of biotechnology will be confined to rDNA and monoclonal antibody pharmaceutical products.

CHARACTERISTIC PRODUCTION PROCESSES

The major difference between biotechnology-derived products and other pharmaceutical products is the means of production used to generate the product. Biotechnology makes use of genetically modified living organisms to produce protein or peptidyl products. This statement is true for both rDNA-derived products as well as monoclonal antibody products. Biotechnology-derived products are therefore readily differentiated from proteins or peptides that have been obtained by isolation from natural source materials such as plasma, serum, or tissue, or by chemical synthesis.

Biotechnology-derived products are not significantly different from other protein pharmaceuticals after the protein purification process. Thus, the basic requirements for process validation, environmental control, aseptic manufacturing, and quality control/quality assurance systems are fundamentally the same for all pharmaceutical products. However, the complexity of these systems is often greater for biotechnology-derived products because the production of such biomolecules generally requires highly developed cell propagation processes, complicated purification methods, and analytical control to ensure their homogeneity, lot-to-lot consistency, and safety.

This section describes in some detail only those significant factors that are unique to the processing of biotechnology-derived products. This includes descriptions of the various biological production systems now in use, and a discussion of purification issues.

rDNA Production

rDNA products are presently produced in prokaryotic (bacteria) or eukaryotic systems (e.g., yeast, mammalian cell culture). The choice of the production organism is generally a direct function of the molecular complexity of the protein that is to be produced as well as the economics and efficiency of the fermentation or cell culture process. The earliest biotechnology-derived products were produced in *E. coli* based on the high degree of understanding of its molecular biology. Within the last few years, however, the use of large-scale eukaryotic cell culture has become relatively commonplace.

PROKARYOTIC (BACTERIAL) PRODUCTION

Bacterial production of biotechnology-derived products offers a number of distinct advantages as well as certain disadvantages. As previously stated, the biology of bacteria

is quite well understood and the safe and effective use of *E. coli* as the host organism for production has been well documented. Thus, the expression of a new protein in *E. coli*, if possible, is often easier to accomplish than in other, more theoretically suitable, expression systems. This may be offset, however, by the fact that *E. coli* produces proteins usually in a chemically reduced state. For proper folding, such proteins require the production of intramolecular disulfide bonds by oxidation. A second disadvantage is that all *E. coli* proteins begin their sequence with an N-formyl methionine residue that may not always be removed by *E. coli* proteolytic systems, thus possibly yielding a methionyl derivative of the desired natural protein. A third disadvantage of expression in *E. coli* is the potential for product degradation because of trace protease impurities. A fourth disadvantage is the requirement for endotoxin removal during purification. These limitations aside, the ease of use of *E. coli* and their generally high-expression yields for most proteins often have resulted in the continued preferential use of these bacteria, where feasible.

As previously described, the key element in rDNA technology is the recombinant plasmid, which contains the gene that codes for the protein of interest. Plasmids are simple and small circular extrachromosomal segments of bacterial DNA that are isolated from a bacterium and are self-replicating. The basic technology involves the specific enzymatic cleavage of a plasmid using endonucleases followed by the insertion of a new piece of DNA that contains the gene of interest. The resultant recombinant plasmid is considered the key raw material of rDNA technology. The recombinant plasmid is introduced into the host organism through a process called transformation, where it passes on its new genetic information and results in the production of the protein product. The large-scale growth of recombinant organisms can be conducted in commercial fermenters at scales in excess of 100,000 L, making these types of production systems extremely economical. There are, however, a number of issues that complicate *E. coli* fermentation systems. In some cases, the expressed protein product may cause cellular toxicity, and/or be extremely difficult to recover or purify because it may be sequestered into bacterial inclusion bodies as large semisoluble aggregates. Recent advances in *E. coli* molecular biology have led to the ability to express proteins into the periplasmic space, allowing for the removal of unwanted N-terminal methionine groups and leading to more readily purified proteins.

EUKARYOTIC (MAMMALIAN CELL AND YEAST) PRODUCTION

The development of eukaryotic cell culture for the production of vaccines has long been established in the pharmaceutical industry and an extensive database has been developed to ensure the suitability of such protein products in humans. The extension of this technology to rDNA products was primarily a response to the limitations in the use of *E. coli*. Particularly with respect to large proteins or glycoproteins, eukaryotic cell expression is an attractive alternative to a bacterial system because eukaryotic cells can secrete proteins that are properly folded and identical in primary, secondary, and tertiary structure to the natural human protein. Concerns about the economics of this production system originally hindered its development. Recent advances, however, in improved expression levels, in large-scale cell culture using Chinese Hamster Ovary (CHO) cells, and in the formulation of more highly defined growth media have combined to dramatically improve the economic feasibility of eukaryotic cell substrates. The number of cell passages required for cloning, selection, amplification, and cell banking prior to production generally necessitates the use of immortal cell lines because nonimmortalized strains (i.e., diploid cultures) cannot be propagated long enough to provide an economically useful time in the production stage. Initial questions regarding the safety of such immortal cell lines were based on concerns over potential oncogenes and potential viral

and retroviral contamination. These concerns have been minimized by the exhaustive analysis and characterization of master cell banks for adventitious (accidentally introduced) agents, by effective process validation studies, and by the safety data gathered to date for products produced by this method. The resultant thoroughly characterized master cell bank is used for full-scale production. Other eukaryotic cell lines, such as those derived from insect cells, may be useful in achieving many of the conformational and post-translational advantages that have been described for mammalian cell culture.

The use of yeast strains such as *Saccharomyces cerevisiae* for production has been extensively explored. The production of proteins in yeast offers many theoretical advantages over *E. coli* while raising certain new concerns. Like *E. coli*, yeast can maintain stable plasmids extrachromosomally; however, unlike *E. coli*, yeast possesses the ability to produce glycoproteins.

MONOCLONAL ANTIBODY PRODUCTION

Monoclonal antibodies can be produced in two major ways, depending on whether they are of human or murine (mouse) origin. For antibodies of murine origin, appropriate lymphocytes are selected from the spleens of previously inoculated mice or rats. The cell is then fused with a transformed cell line such as a myeloma cell line, producing a hybridoma cell. The hybridoma cells are then clonally selected and used to produce the monoclonal antibody products. For antibodies of human origin, human B lymphocytes can be clonally selected for the hapten binding specificity of their product antibodies; these selected cells can then be immortalized by infection with a virus. The resultant fused or transformed cell can proliferate indefinitely in a bioreactor/cell culture environment or can be injected into mice from whose ascites fluid the protein can be obtained. Antibody is produced as directed by the chromosomal information that resides in the cell or was acquired during fusion and is secreted into the medium from which it can be readily purified. The hybridoma cells must be thoroughly analyzed and characterized in the same general way as an rDNA cell bank. The resultant cell bank is used for production of product either by large-scale cell culture or by harvesting ascites fluid from mice inoculated with transformed cells.

Control of Fermentation and Cell Culture Processes

Because the production process using a living system is the fundamental cornerstone of biotechnology, the issues that relate directly to the control of biotechnology processes need to be examined. Concerns over the production of proteins in bacteria, for example, primarily involve systems for ensuring genetic stability, consistent product yield, and evidence of the lack of contamination by adventitious organisms. These same concerns apply to large-scale eukaryotic cell culture, where, as stated above, there are also significant issues relating to the use of immortalized cell lines such as the putative presence of oncogenic DNA/RNA and impurities from media proteins.

FERMENTATIONS (BACTERIA AND YEAST)

A considerable amount of knowledge has been obtained for the production of recombinant proteins in bacteria and yeast; therefore, the major fermentation issues typically are resolved by the demonstration of consistency in fermentation conditions. Fermentations with bacteria and yeast usually are performed over short, well-defined time periods to monitor and control growth rate and product expression conditions. The presence of contaminating foreign organ-

isms may be detected by effects on growth rate, culture purity, fatty acid profile, etc., and is cause for termination of the fermentation. The genetic stability of the production plasmid for bacteria may be addressed by isolation and nucleotide sequence analysis or by DNA restriction mapping. These results may be confirmed by peptide mapping of the expressed protein for each product lot manufactured. It is very important to optimize the fermentation conditions so that the amount of proteolytic processing of the target protein that may occur can be either limited or avoided completely. Proteolytic processing is often a problem in *E. coli* fermentations and may lead to recovery difficulties and low product yields. Finally, the conformation of the protein and its effects on potency must be addressed by the fermentation process.

EUKARYOTIC CELL CULTURES

The origin of large-scale cell culture techniques for the production of biotechnology-derived products can be traced back to the vaccine industry. Developments such as large-scale cell suspension cultures using recombinant organisms that secrete the desired protein into the media have had a significant impact on biotechnology. Large glycosylated proteins in quantities sufficient for the marketplace can now be produced. The use of eukaryotic cell cultures, however, is complicated by issues such as genetic stability, protein folding, and culture conditions, including cell viability and growth rates. For example, the genetic stability of cell cultures cannot be addressed as readily as *E. coli* fermentations by techniques such as plasmid sequence analysis because the gene that codes for the product is incorporated into the cell genome and is not easily recovered. One alternative is peptide mapping of the expressed protein, which requires a resolution and sensitivity adequate to detect subtle mutations.

The absence of adventitious organisms in cell cultures is critical. In addition to demonstrating that bacteria, yeast, and molds are not present in cell cultures, the manufacturer must provide for each culture evidence that mycoplasmas and adventitious viruses are not present. It is important to recognize that certain hybridomas used for monoclonal antibody production may contain endogenous retroviruses. However, it must be demonstrated that any viruses present in the culture are removed from the final product. This requires the development of suitable analytical techniques to ensure the absence of contamination by mycoplasmas or human and animal adventitious viruses.

The degree and type of glycosylation may be important in the design of cell culture conditions for the production of glycosylated proteins. The degree of glycosylation present may affect the half-life of the product in vivo as well as its potency and antigenicity. Although the glycosylation status of a cell culture product is difficult to determine, it can be verified to be consistent if the culture conditions are highly reproducible.

Process for Recovery and Purification

The recovery of protein products obtained from either fermentation or cell culture is generally based on efficient protein separation techniques such as those listed in Table 1. The recovery process begins with isolation of the desired protein from the fermentation or cell culture medium, often in a very impure form. The advantage of cell culture and yeast-derived products is that many of these proteins are secreted directly into the medium, thus requiring only cell separation to obtain a significant purification. For *E. coli*-derived products, lysis of the bacteria is often necessary to recover the desired protein. It is important in each case to achieve rapid purification of the desired protein because proteases released by the lysed organisms may cleave the desired product. Such trace proteases are a major concern

in the purification of biotechnology-derived products because they can be very difficult to remove, may complicate the recovery process, and can significantly affect final product stability.

Table 1. Chromatographic Purification Methods Used for Biotechnology-derived Products

Chromatofocusing
Reversed-phase chromatography
Hydrophobic interaction chromatography
Charge-transfer chromatography
Size-exclusion chromatography (molecular sizing)
Ion-exchange chromatography
Anion
Cation
Affinity chromatography
Chemical
Monoclonal antibodies
Cellular receptors
Dye/Ligand
Metal chelate

The recovery process is usually designed to purify the final product to a high level. The purity requirement for a product depends on many factors, although chronic use products may be required to have much higher purity than those intended for single-use purposes. Biotechnology products contain certain impurities that the recovery processes are specifically designed to eliminate or minimize. These impurities include trace amounts of DNA, growth factors, residual host proteins, endotoxins, and residual cellular proteins from the media. The most common impurities of concern and suitable assay methods to detect them are presented in Table 2.

Chromatofocusing and reversed-phase chromatography are purification methods that use chemicals, either in the stationary (bonded) phase or in the mobile phase, that may become impurities in the final product. As in any new technology, the burden of validation (i.e., demonstrating removal of potentially harmful chemicals) is incumbent on the manufacturer. Validation is necessary when isolating end product monoclonal antibodies or using a technique that contains a monoclonal antibody purification step. The process must demonstrate removal of leaching antibody or antibody fragments. It is necessary to ensure the absence of adventitious agents such as viruses and mycoplasmas in the cell line that is the source of the monoclonal antibodies. The main concern is the possibility of contamination of the product with an antigenic substance whose administration could be detrimental to patients. Continuous monitoring of the process is necessary to avoid or limit such contamination. The problem of antigenicity related to the active as well as host proteins is one that is unique to biotechnology-derived products in contrast to traditional pharmaceuticals. Manufacturing methods that use certain solvents should be monitored if these solvents are able to cause chemical rearrangements that could alter the antigenic profile of the drug substance. The manufacturer is also obligated to produce evidence regarding performance consistency of novel chromatographic columns. Considerations for single-use products such as vaccines may differ because they are not administered continuously and, in this case, antigenicity is desirable. On the other hand, validating the removal of ligand or extraneous protein contamination is necessary. Unlike drugs derived from natural sources, manufacturers of biotechnology-derived products have been required to provide validation of the removal of nucleic acids during purification. Vaccines may again be different in this regard because of the accumulated clinical history on these products.

QUALITY CONTROL

In general, quality control systems for biotechnology-derived products are very similar to those quality control systems routinely employed for traditional pharmaceutical products in such areas as raw material testing and release, manufacturing and process control documentation, and aseptic processing. Quality control systems of biotechnology-derived products incorporate some of the same philosophies applied to the analysis of low molecular weight pharmaceutical products. These include the use of chemical reference standards and validated methods to evaluate a broad spectrum of known and/or potential product impurities and potential breakdown products. The quality control systems for biotechnology-derived products are generally analogous to those established for traditional biologicals with respect to determining product sterility, product safety in experimental animals, and product potency. For example, refer to *Injections* <1>, *pH* <791>, *Particulate Matter in Injections* <788>, *Bacterial Endotoxins Test* <85>, and *Impurities in Official Articles* <1086>.

The fundamental difference between quality control systems for biotechnology-derived products and traditional pharmaceuticals is in the types of methods that are used to determine product identity, consistency, purity, and impurity profiling. Furthermore, in biotechnology quality control, it is frequently necessary to use a combination of final product and validated in-process testing and process validation to ensure the removal of undesired real or potential impurities to the levels suggested by regulatory agencies. Biotechnology-derived products generally require a detailed characterization of the production organism (cell), a complete assessment of the means of cell growth/propagation, and explicit analysis of the final product recovery process.

The complexity of the quality control systems for biotechnology-derived products is related to both the size and structural characteristics of the product and manufacturing process. In general, the quality control systems required for

products produced in prokaryotic cells are less complex than the systems required for products produced in eukaryotic cells. The quality control systems for prokaryotic production organisms usually entail documentation of the origin of the producer strain and encompass traditional testing for adventitious organisms, karyology, phenotyping, and antibiotic resistance. In addition, newer techniques such as DNA restriction mapping, DNA sequence analysis, and routine monitoring that may include measurement of mRNA and/or plasmid DNA levels may be useful. The quality control of the master cell bank and working cell bank for eukaryotic production organisms generally includes testing for adventitious organisms, karyology, identity, and stability monitoring. All eukaryotic cell lines (except yeast) are generally tested for the presence of retroviruses, retroviral activity markers, and tumorigenicity, although many of these tests may be of limited value.

PRODUCT FORMULATION

The products of biotechnology are proteins and peptides that are relatively unstable molecules compared to most organic pharmaceuticals. Most biotechnology processes involve the transfer of proteins from one stabilizing or solubilizing buffer to another during the purification process. Ultimately, the protein is exchanged into its final solution dosage form where long-term stability is achieved. In addition, these products often require lyophilization to achieve long-term stability because of the potential for degradation by a variety of mechanisms, including deamidation, aggregation, oxidation, and possible proteolysis by trace levels of host cell proteases. The final dosage form of the protein usually contains stabilizing compounds that result in the optimal pH and solution conditions necessary for long-term product stability and/or the desired properties for administration of the product (tonicity). These compounds include proteins, polyhydric alcohols, amino acids, carbohydrates, bulking agents, inorganic salts, and nonionic surfactants. In

Table 2. Potential Impurities and Contaminants in Biotechnology-derived Products

Impurities or Contaminants	Detection Method
Impurities	
Endotoxin	<i>Bacterial Endotoxins Test</i> <85>, <i>Pyrogen Test</i> <151>
Host cell proteins	SDS-PAGE ^a , Immunoassays
Other protein impurities (media)	SDS-PAGE, HPLC ^b , Immunoassays
DNA	DNA hybridization, UV spectrophotometry, Protein binding
Protein mutants	Peptide mapping, HPLC, IEF ^c , MS ^d
Formyl methionine	Peptide mapping, HPLC, MS
Oxidized methionines	Peptide mapping, amino acid analysis, HPLC, Edman degradation analysis, MS
Proteolytic Cleavage	IEF, SDS-PAGE (reduced), HPLC, Edman degradation analysis
Aggregated proteins	SDS-PAGE, HPSEC ^e
Deamidation	IEF, HPLC, MS, Edman degradation analysis
Monoclonal antibodies	SDS-PAGE, immunoassays
Amino acid substitutions	Amino acid analysis, peptide mapping, MS, Edman degradation analysis
Contaminants	
Microbial (bacteria, yeast, fungi)	<i>Microbial Enumeration Tests</i> <61>, <i>Tests for Specified Microorganisms</i> <62>, <i>Sterility Tests</i> <71>, microbiological testing
Mycoplasma	Modified 21 CFR Method ^f , DNAF ^g
Viruses (endogenous and adventitious)	CPE ^h and HAd ⁱ (exogenous virus only), reverse transcriptase activity, MAP ^j

^a Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

^b High-performance liquid chromatography.

^c Isoelectric focusing.

^d Mass spectrometry.

^e High-performance size-exclusion chromatography.

^f Draft guidelines relating to Code of Federal Regulations, Title 21.

^g DNA-binding fluorochrome.

^h Cytopathic effect.

ⁱ Hemadsorption.

^j Murine antibody production.

addition, these excipients may be required for stable lyophilized cake formation. There are special requirements for lyophilized products, such as the control of moisture levels, that generally are defined in the individual USP monograph and that may be important to product stability. Significantly, the assessment of protein stability usually requires the use of multiple analytical methods, each of which may be used to assess a specific mode of protein degradation. Many of these assays are described in the following section. The use of accelerated stability studies to predict the shelf life of protein formulations is often complicated by the effects of temperature on protein conformation, resulting in non-Arrhenius behavior. Thus, reliance on real-time, recommended storage condition stability studies is often required for establishing the expiration dating of biotechnology-derived products.

ANALYTICAL METHODOLOGY

The analysis of biotechnology-derived products relies heavily on the use of sophisticated analytical methods for demonstrating the structural identity and homogeneity of proteins and for evaluating the shelf life or stability of these products. This section discusses accuracy, precision, informational content, and general applicability of the most commonly used methods. Some methods, such as host cell impurity assays and residual DNA procedures, may be both highly process- and product-specific and thus should be included in the individual monographs.

Reference Standard Considerations

The use of suitable reference standards and/or reference materials is extremely important in the analysis of biotechnology-derived products. These standards may be either natural materials or proteins produced by genetic engineering. Many biotechnology-derived products require the availability of accurately characterized reference standards from internationally recognized sources such as the USP (see *USP Reference Standards* (11)), WHO, NIH, and FDA. Currently, reference standards with defined activity units are available from these sources for some biologicals. These standards are used by the manufacturers in testing or to calibrate secondary standards using many of the assays described in this section. The potency value of the reference standard is obtained through collaborative studies that, when statistically evaluated, are used to determine the ultimate potency value assigned to the reference standard. The secondary standard can be used to determine the labeled amount of drug substance or potency defined on a product label. Thus, reference standards/reference materials for biotechnology-derived products that are used for the analytical purposes described in specific USP monographs will be approved and made available from USP. Ideally, these reference standards should be in use worldwide and should always be calibrated against the U.S. standard that is deposited by the manufacturer at FDA for those products licensed by FDA. This ensures the accurate and consistent determination of the activity, strength, and purity of these products. Because of a number of issues unique to biotechnology-derived products, such as process and product specificity, separate reference standards for similar products may be required. In addition, thorough development and recalibration of reference standards to replace depleted or expired standards will be conducted by USP to ensure that the label claims of the drug products do not change. One caveat in the assignment of the potency of the primary standard through collaborative studies is that units of activity so defined are only meaningful when compared in a single assay that is both suitably accurate and well described. Attempts to compare activity values from even subtly different assays can be expected to yield widely varying results.

Typical Methodology

There are a number of specific analytical methods that pertain to biotechnology-derived products. Many of the assays and tests described may be performed in different ways and, because some of these may be product specific as well, there is a need for clear guidelines on the application of specific methods to particular situations. See the chapters *Design and Analysis of Biological Assays* (111) and *Validation of Compendial Procedures* (1225) for some general information on methodology.

PROTEIN CONTENT

Protein content assays are used to quantitatively determine the amount of protein in a given biotechnology-derived product. The determination of protein content is often one of the most difficult measurements that needs to be made and often requires independent confirmation by alternate methods. Where applicable, methods such as UV spectrophotometry with a valid absorptivity and Kjeldahl nitrogen analysis can be used to determine absolute amounts of protein independent of reference standards. However, methods such as Lowry protein, biuret, and quantitative amino acid analysis, which require reference standards, also yield accurate values. Protein content assays are among the most important of all the methods used for these products because the results of other types of assays, such as potency, are also dependent on them.

There are several assays for the determination of protein content that are commonly used. These assays may be used at different points in the production process of a given biotechnology-derived product. For highly pure proteins, the simplest protein content method is based on the determination of the UV absorbance of a protein solution by spectrophotometry. The absorbance at the absorption maximum is determined and the protein concentration is calculated with the use of an empirically determined absorptivity. This technique is applicable to proteins containing the aromatic amino acid residues tryptophan, tyrosine, and/or phenylalanine. The absorption wavelength often used is 280 nm. The extinction coefficient, or molar absorptivity, should be determined in the same solvent that is used for the sample to be measured. If necessary, the product may be diluted prior to analysis to obtain solutions with absorbance values in the linear range of detection. Higher molecular weight aggregates and particulates may give rise to light-scattering effects, which provide artificially high absorbance values. Excipient components that have significant absorbance at 280 nm will also interfere with this test. UV spectrophotometry is unique among the protein content methods in that it is an absolute measure of concentration of a specific protein requiring no calibration with standards.

A commonly used general protein content method is the Lowry assay. This is based on the biuret reaction of proteins with copper (II) in a basic solution and the Folin-Ciocalteu phosphomolybdic-phosphotungstic acid reduction to heteropolymolybdenum blue by the copper-catalyzed oxidation of the aromatic amino acids tyrosine, tryptophan, and phenylalanine in the protein. The reaction products are blue and are quantitated spectrophotometrically in the visible region between 540 and 560 nm. This reaction is linear at microgram protein levels. The assay, however, is prone to interferences from a number of substances such as alcohols, sugars, and detergents. In some cases, interfering substances or product may be removed prior to analysis, e.g., by precipitation. Also, the preparation of controls containing interfering substances that are in the drug product may correct for their presence. Although bovine serum albumin historically has been used to prepare the standard curve, different proteins are known to react with differing intensity, so that a reference material of the same product should be used for calibration. The bicinchoninic acid (BCA) assay is a useful

alternative to the Lowry assay because it is less sensitive to interfering substances. The working reagent is a BCA-copper (II) solution. The copper (II) complex is reduced to copper (I) in the presence of protein, and the purple color may be quantitated spectrophotometrically at approximately 560 nm.

Other colorimetric assays can also be used. The Bradford method, for example, employs the binding of the dye Coomassie Brilliant Blue to the protein in an acidic environment. The concentration of the protein in solution is then determined by comparing the absorbance at 595 nm with a standard curve of a reference material.

Fluorescent methods used are normally based on either fluorescamine or o-phthalaldehyde (OPA). The main advantage of these assays is increased sensitivity. Another advantage is their use with hydrophobic proteins. Fluorescamine and OPA react with primary amines both at the N-terminus of the polypeptide and with amino acid side chains, such as lysine.

The Kjeldahl nitrogen method, *Nitrogen Determination* <461>, provides an accurate and precise determination of protein concentration and is often used in the determination of UV protein absorptivities. The assay is performed in two stages. The sample is first decomposed with sulfuric acid to produce ammonium sulfate, carbon dioxide, and water. The decomposition is performed at the boiling point of sulfuric acid in long-necked, pear-shaped flasks. These flasks serve to condense water vapor and prevent the loss of material. Depending on the efficiency of decomposition, various salts such as potassium sulfate may be added to increase the boiling point of the sulfuric acid solution. Oxidizing agents such as perchloric acid or potassium permanganate have also been used to improve the decomposition. The second stage of the assay involves the direct determination of ammonia. In most macrodeterminations, ammonia is steam distilled from the mixture after basification with sodium hydroxide. The ammonia can typically be quantitatively distilled out of the mixture in 5 to 20 minutes and absorbed quantitatively into a standardized acidic solution of known volume and normality. The excess acid is then back-titrated with standardized base. For crude determinations of protein, the ammonia value (and therefore the nitrogen content), is multiplied by a factor of 6.25 mg of protein per mg of nitrogen, which corresponds to a nitrogen content of 16%. The protein value so obtained is generally valid for most proteins. If a more accurate value is required, as for an absorptivity determination, then the conversion factor must be calculated for the nitrogen content of the individual pure protein from the known amino acid composition. For glycoproteins that contain amino sugars, the calculated value is biased high unless a correction is applied.

Amino acid analysis is used in the determination of the appropriate absorptivity of the protein and may also be used quantitatively for the determination of protein content. This procedure, although more complicated than those described above, can also yield accurate results.

AMINO ACID ANALYSIS

Amino acid analysis is a classical protein chemistry method for the determination of the amino acid composition of proteins and peptides. The method consists of the complete hydrolysis of a protein or peptide to its component amino acids, which are then chromatographically separated and quantitated. Amino acid analysis, therefore, can be used to determine both the amino acid composition of a product (i.e., identity) and the total amount of protein present. The method has some inherent difficulties, such as complete or partial destruction of some amino acids, that can be circumvented by appropriate analytical methodology. The amino acid tryptophan is destroyed by 6 N hydrochloric acid hydrolysis and thus requires the use of alternate hydrolysis conditions. The amino acids serine and threonine may be partially destroyed, whereas peptide bonds between

bulky hydrophobic residues such as valine and isoleucine may be more resistant to hydrolysis, in both cases yielding values lower than actual. Accordingly, analysis of time-course hydrolysis samples may be used to compensate for these factors. Cysteine and methionine may require preoxidation to cysteic acid and methionine sulfone, respectively, for accurate quantitation. Each specific protein may require a procedure of optimized hydrolysis conditions for its amino acid analysis to obtain the optimal results.

Amino acid analysis is performed in two stages. The first stage involves the hydrolysis of the protein into its component amino acids. This hydrolysis is normally performed with 6 N hydrochloric acid at about 110° for 24 hours. Some proteins may require longer or more stringent hydrolysis conditions. The second stage is the separation and quantitation of the individual amino acids by some form of chromatography that can be performed with either precolumn or postcolumn derivatization. A number of precolumn derivatization procedures are available, such as with OPA, phenylisothiocyanate (PITC), and fluorenylmethoxycarbonyl (FMOC). These derivatives are then separated by reversed-phase (RP) high-performance liquid chromatography (HPLC) and quantitated following UV or fluorescence detection. Postcolumn derivative methods involve separation of the component amino acids by high-performance ion-exchange chromatography (HPIEC) followed by postcolumn reaction with a chromophore, such as ninhydrin, and quantitation following UV/visible detection. All of these methods are suitable for performing amino acid analyses and each has its inherent advantages and disadvantages. OPA derivatives are very simple to prepare and are sensitive, requiring only a small amount of sample, but they are unstable and have to be chromatographed immediately upon preparation. Phenylthiocarbamyl (PTC) derivatives, on the other hand, are relatively more stable. Postcolumn derivatization with ninhydrin is often performed in the low-pressure mode and has the advantage of stability of the amino acid hydrolysate. Its disadvantage is the need for dual detection at 440 and 570 nm and for post-column apparatus.

PROTEIN SEQUENCING

Protein sequencing is useful in the control of quality of protein biologicals because it can provide primary structure information, i.e., amino terminal and/or carboxy terminal structure. For rDNA-derived biologicals, this methodology has the additional purpose of confirming the complementary DNA (cDNA)-predicted amino acid sequence, protein homogeneity, and the potential extent of proteolytic clips. For monoclonal antibodies, this technique is used for determining protein homogeneity. Protein sequencing is divided into amino-terminal and carboxy-terminal sequencing applications and procedures.

Amino-Terminal Sequencing—Amino-terminal sequence analysis is a classical protein chemistry technique that yields significant information about primary structure (sequence), homogeneity, and the presence of known or unknown cleavages in the polypeptide. N-terminal sequence analysis is performed with a number of commercially available automatic peptide sequencers. The method is based on the coupling reaction of the amino terminal residue of a protein or peptide with PITC. The resulting PTC-amino acid derivative is cleaved from the protein by a perfluorinated organic acid (generally trifluoroacetic or heptafluorobutyric acid), which exposes the adjacent amino acid. This next amino acid serves as a new N-terminus and is derivatized in the subsequent coupling and cleavage cycle. This process is repeated until an appropriate number, normally 8 to 10, of the amino acids are removed. The modified amino acid residue resulting from the cleavage cycle (anilinothiazolinone [ATZ]) is generally converted in the presence of acid and heat to a phenylthiohydantoin-amino acid (PTH-AA). The PTH-AA may then be determined following RP-HPLC analysis. Any intrachain cleavages as well as heterogeneity of the

N-termini (e.g., N-terminal methionine) on the polypeptide will also be sequenced at the same time. These result in smaller peaks in the chromatogram and may enable both the relative quantitation of the amount of the N-termini and the identification of the location of the cleavage site on the polypeptide. This procedure for protein sequence analysis may also be performed manually. The limitations of the PITC sequencing method are that the method is only semi-quantitative (i.e., the amount of the N-termini can only be estimated) and the PTH derivatives of serine and threonine may be severely degraded, making their determination difficult. Cysteine residues in order to be determined, must first be modified, for example by alkylation. In addition, the amino acids glycine and proline are slow to rearrange, resulting in minor difficulty in their determination.

Carboxy-Terminal Sequencing—Sequencing of the protein from the carboxy terminus also yields valuable primary structure information as well as possible C-terminal cleavages. The sequential degradation of a protein from the C-terminus can be performed by either chemical or enzymatic methods. The reaction of hydrazine, ammonium thiocyanate, or cyanogen bromide with a protein can be used to sequentially degrade the protein at or near the C-terminus. The ammonium thiocyanate reaction has been extended for use on proteins coupled to solid supports. The C-terminal amino acids can be sequentially cleaved enzymatically with exopeptidases such as carboxypeptidases. Limitations of the carboxypeptidase approach are the potential contamination with endopeptidase and the inherent difficulty and unpredictable nature of the sequencing. Mass spectrometry can be used either directly on protein digests or in conjunction with HPLC peptide mapping to identify the C-terminus of the protein. However, these methods are only semiquantitative.

PEPTIDE MAPPING BY HPLC

For pharmaceutical proteins, peptide mapping has two primary purposes: it is a highly specific identity method and, in the case of biotechnology-derived products, may serve as a confirmation of genetic stability. Peptide mapping is used to compare the protein structure of a specific lot of material to that of a suitable reference material/reference standard or to those structures of previous lots to confirm correctness of the primary structure and to confirm lot-to-lot consistency of primary structure (within the limits of this technique). The amino- and carboxy-terminal peptides and carbohydrate-containing peptides often can be separated and identified. The latter are valuable in the peptide maps of glycosylated proteins such as monoclonal antibodies. Peptide mapping may be used to determine the presence of single or multiple incorrect amino acids that may result from such events as a single point mutation or mistranslation of the cDNA sequence.

The procedure involves the selective fragmentation of the protein into discrete peptides that are resolved by some chromatographic technique. The fragmentation is accomplished with endoproteases, such as trypsin, chymotrypsin, thermolysin, or V8 protease, or by selective chemical degradation with cyanogen bromide, which cleaves at specific sites on the molecule. Selection of the appropriate endoprotease to be used is directed by the primary sequence of the protein. Trypsin cleaves on the C-terminal side of the basic residues lysine and arginine; chymotrypsin cleaves after the aromatic residues phenylalanine, tyrosine, and tryptophan; thermolysin cleaves after the hydrophobic residues leucine, isoleucine, and valine; V8 protease cleaves after the acidic residues glutamic acid and aspartic acid; and cyanogen bromide cleaves at methionyl residues. Other enzymes, such as clostripain (arginine) and endoproteinase lys-C (lysine), and chemical methods, such as 2-nitro-5-thiocyanobenzoic acid (cysteine), may also be used. Each of these methods has its own set of advantages and disadvantages. One common disadvantage to all these techniques is that

nonspecific cleavages occur to some degree. It is important that the peptides generated from the digestion are large enough to provide structural information about the protein, and yet small enough to allow their analysis and separation by a technique such as RP-HPLC. For this reason and the fact that cleavage with this enzyme is almost quantitative, trypsin is the enzyme with the most general applicability for most proteins. For large proteins of greater than 60,000 daltons (about 520 amino acids), cleavage with trypsin may result in too many fragments, so another endoprotease may be chosen. L-TPCK (tosyl-L-phenylalanine chloromethyl ketone)-treated trypsin normally is used because TPCK inhibits the action of chymotrypsin, a contaminant present in many trypsin preparations. Although reaction with cyanogen bromide cleaves at methionyl residues, proteins do not contain many of these residues. As a result, relatively few peptides are obtained and these may be too large or hydrophobic for HPLC separation.

Once the digestion is complete, the peptides are generally separated by either RP-HPLC and/or HPIEC. Selection of the appropriate column is empirically based and will vary for different proteins. For RP-HPLC, both 100-Å and 300-Å pore size supports work well, and the selection of the silica support may be an important criterion for optimal separation. For the smaller peptides generated by these digestions, C8 and C18 stationary phases generally have been found to be more efficient than C4 supports. The most common solvents used for reversed-phase separations are water and acetonitrile containing a constant (~0.1%) amount of trifluoroacetic acid. Buffered mobile phases containing phosphate also offer excellent selectivity depending on the pH. Screening the effect of pH in the 3.0 to 5.0 range causes a shift of peptides containing the acidic residues, glutamic acid and aspartic acid. For ion-exchange separations, less information is available, but both silica and polymeric supports with both weak and strong ion-exchange stationary support can be used successfully. Because many of the peptides are somewhat hydrophobic, the addition of small amounts of organic solvents in the mobile phase, such as 5% to 10% methanol or acetonitrile, may be necessary. A potential disadvantage of HPIEC analysis of peptide mixtures is that sometimes neutral peptides or peptides that have the same charge as the support may not be retained on the column and thus may not be separated or identified by this method.

IMMUNOASSAYS

Immunoassays are used either as active drug substance methods to identify and quantitate the protein of interest or as impurity profile methods to detect and quantitate known host cell protein impurities. Because these protein impurities may represent a large number of potential impurities at trace levels rather than a single impurity, the immunoassays must be sensitive and selective to detect as many of these impurities as possible. Immunoassays that can measure these impurities to very low levels have been developed for *E. coli* proteins (ECPs) and CHO proteins. Immunoassays additionally may serve as potency assays for monoclonal antibodies using an appropriate antigen.

Immunoassays consist of a large group of assays that depend on specific high-affinity antibody:antigen interactions. These assays include the radioimmunoassays (RIAs) and the enzyme-linked immunosorbent assays (ELISAs). RIAs are performed in a liquid or solid phase using an unlabeled antibody directed against the radiolabeled protein of interest. The principle of the RIA is that the inhibition of binding of labeled antigen to unlabeled antibody by samples is compared to the inhibition by known standards, thus allowing quantitation of the protein of interest.

Immunoradiometric assays (IRMAs) or sandwich RIAs employ two antibody preparations that are used to sandwich the protein of interest. The first antibody is unlabeled and is directed against the protein, and the second antibody is

radiolabeled and may be directed against the protein or the first antibody. The entire antibody:antigen complex is isolated and the amount of radioactivity, and, therefore, the protein of interest, is determined. The development of an RIA or IRMA for a biotechnology-derived product requires careful attention to production of the antisera, preparation of the labeled tracer, preparation of a suitable reference standard, and methods for the separation of free antigen from bound antigen.

The most commonly used ELISA format of trace impurity analysis is the sandwich ELISA that utilizes two antibody preparations like the IRMA, but without radiolabeling. The first antibody is unlabeled and the second antibody has an enzyme such as horseradish peroxidase (HRP) or alkaline phosphatase attached. Basically, the ELISA method consists of applying a layer of purified antibodies to the host cell proteins onto microtiter plates, followed by the protein product. The enzyme antibody conjugate is added and allowed to bind to the antibody-bound host cell proteins. An appropriate substrate is added for color development, which is analyzed with a spectrophotometric plate reader. Such a multiantigen ELISA requires a representative reference standard preparation of appropriate host cell protein impurities to serve as the immunogen for preparation of the antibodies used for the assay. This reference standard preparation is usually prepared from a manufacturing production run yielding all of the expected host cell proteins except the product protein. The total absence of the product protein is necessary in this preparation to avoid the production of antibodies to the product itself when the reference standard is used as an immunogen. Because of varying affinities of polyclonal antibodies to multiantigen preparations, the absolute accuracy of the multiantigen methods and the ability to detect every potential antigen cannot be guaranteed.

ELECTROPHORESIS

Electrophoretic assays are among the most common and powerful of the assays used to evaluate protein purity and homogeneity. They are valuable not only for the initial evaluation and release of biotechnology-derived products but also as stability-indicating methods for detecting molecular or chemical changes in the molecule as a result of denaturation, aggregation, oxidation, deamidation, etc. The use of these methods is facilitated by their simplicity and their requirement of only microgram quantities of sample. The two types of electrophoretic assays most often used for biotechnology-derived products are sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF).

The SDS-PAGE method separates proteins primarily by their molecular weight because, in the presence of the anionic detergent SDS, a net negatively charged protein SDS complex is formed. The sample is first denatured in the detergent, which disrupts the noncovalent intramolecular and intermolecular bonds that hold proteins together, and then it is electrophoresed through a polyacrylamide gel support. Protein migration through the gel is proportional to size so that smaller proteins migrate faster through the gel than larger ones. Samples are often electrophoresed under both reduced and nonreduced conditions to determine if impurities of the same molecular weight or if intramolecular proteolytic cleavages of the protein of interest are present. Although nonreducing SDS-PAGE is commonly used to estimate the state of aggregation and/or oligomerization of the protein of interest, this method will only permit observation of aggregates or oligomers that are stable in the presence of SDS and the conditions used for sample preparation and electrophoresis. Proteins consisting of multiple chains held together by disulfide bonds are broken down and separated into their individual polypeptide chains. Sample detection following electrophoresis can be quantitative with densitometric analysis of Coomassie Brilliant Blue stain or

qualitative, but with increased sensitivity in the nanogram range, with silver stain. Silver stain SDS-PAGE may also be performed quantitatively under suitable conditions. With proper validation, Coomassie Brilliant Blue staining and densitometry may also be used to give quantitative determination of polypeptides in the nanogram range. SDS-PAGE coupled with Coomassie Brilliant Blue stain is used to quantitatively determine the purity of the sample with regard to dimer and larger covalent aggregates and fragments. When the method is combined with the silver stain technique, an assessment of low/trace levels of a new impurity can be made by directly comparing the electrophoresed sample to the electrophoresed reference material or standard under reduced and nonreduced conditions. Generally, silver staining is used qualitatively because of potential major variations in binding of silver from protein to protein, and relatively inconsistent background on a routine basis. An estimation of the quantity of an impurity can be obtained by electrophoresis of a known amount of an internal standard such as bovine serum albumin or lesser dilutions of the protein of interest on other lanes of the same gel. The SDS-PAGE separation of a protein can be combined with an immunological method such as immunoblotting. The resulting Western blot is used to determine the identity of an electrophoretic band (i.e., product related or host cell protein impurity). After electrophoresis, the separated proteins are transferred onto a nitrocellulose or polyvinylidene difluoride (PVDF) membrane and reacted with the antibody of interest. Visualization of the complex is done with an enzymatically or radiolabeled antibody.

The IEF method separates proteins on the basis of their charge in an electrical field. The charges on a protein originate from various sources within its amino acid composition, such as protonated amino groups, unprotonated carboxyl groups, deprotonated sulfhydryl groups or tyrosine residues, oxidized cysteine residues, and deamidated residues. However, for each protein there is a pH at which the protein is isoelectric and these charges cancel each other, with the net charge being effectively zero. IEF is performed in the native state in a support of loose-pore polyacrylamide or agarose gel containing ampholytes (amphoteric low molecular weight ions) that set up a pH gradient because of their migration within the support matrix when an electrical field is applied. Simultaneously, in the presence of the electrical field, positively charged proteins migrate towards the cathode and negatively charged proteins migrate towards the anode. Migration stops when each protein reaches the pH value in the support gradient where its net charge is zero. This is the apparent pI or isoelectric point of the protein. Because the migration of a protein is dependent on its amino acid composition, altered forms of the protein and other proteins will migrate to different points on the support. IEF gels may be stained for protein visualization with either Coomassie Brilliant Blue or silver stains. IEF is employed as an identity tool or to ensure the homogeneity of a protein (e.g., monoclonal antibodies) as demonstrated by a banding pattern with the correct pI range. The method can also be used to evaluate the stability of a biological product. Protein deamidation (i.e., glutamine or asparagine residue deamidation) over time leading to the production of a new carboxylic acid group results in molecules with a more acidic pI. IEF can provide information on the state of glycosylation of glycoproteins such as monoclonal antibodies, which may appear as many bands because of changes in the apparent charge on the protein molecule as a result of the sialic acid residues. IEF gel patterns are usually more complicated to interpret than those of SDS-PAGE and interpretation may require many assumptions or subjective judgment.

High-performance capillary electrophoresis (HPCE), which offers the potential advantages of very high protein resolution, is being thoroughly investigated because of recent advances in the technology.

CHROMATOGRAPHIC METHODS

Chromatographic methods have long been used in the determination of the purity of small organic molecules and proteins such as insulin (see *Chromatography* (621)) and in the determination of the active ingredient and/or excipient concentration of pharmaceutical products. Chromatographic methods are also very effective in the determination of the purity of recombinant pharmaceuticals. However, the chromatography of proteins is far more difficult because of multiple modes of interaction with the chromatographic support as a result of the size and/or shape, charge, and hydrophobicity of the proteins. The most common chromatographic methods used to profile recombinant proteins are RP-HPLC, HPIEC, size-exclusion chromatography (HPSEC), and hydrophobic interaction chromatography (HIC). These methods involve the separation of proteins and are used to determine the purity of drug substances as well as the levels of known impurities or degradation products. A complication with all column chromatographic methods is determining the mass balance between column load and column eluate. Nevertheless, HPLC techniques are valuable for determining the purity and strength of protein pharmaceuticals.

The most common RP-HPLC analyses are performed on columns containing a C4 or C8 stationary phase on a silica-based or polymeric support. C18 stationary phases are also used but more often with smaller peptides in an application such as peptide mapping. Supports with pore sizes of at least 300 Å are preferred for proteins of molecular weight greater than about 10,000. For most RP-HPLC analyses, the proteins are eluted with aqueous acetonitrile gradients and the trifluoroacetic acid is kept constant at 0.1%. Other buffers such as phosphate or tris(hydroxymethyl)aminomethane (Tris) are also used, where the pH may be adjusted for added selectivity to achieve optimal separations.

HPIEC is an important method for purity determination. These separations are based on changes in the charge of the molecule and are useful for identifying and quantitating in protein pharmaceuticals common impurities such as oxidized (primarily oxidized methionine) and deamidated forms (glutamine and asparagine) and clipped or truncated forms. Both strong and weak ion-exchange stationary phases on either silica or polymeric supports can be used. Cation-exchange chromatography may be performed on sulfopropyl-type resins and are effective in distinguishing oxidation and deamidation products. Proteins are typically loaded onto a column equilibrated with water or a weak buffer, and eluted with a salt gradient, such as 0 to 1 M sodium chloride.

HPSEC is a technique that may provide information on the levels of aggregation and fragmentation in a protein pharmaceutical. Depending on the information needed, the mobile phase may be native, containing an aqueous buffer such as 100 mM phosphate, pH 7, or it may be denaturing, containing a low level of a chaotrope or detergent such as 0.1% SDS. The analyses are performed isocratically, with detection typically between 210 and 220 nm depending on the buffer used. Detection at 280 nm may also be used but is less sensitive. Classical size-exclusion chromatography was performed on soft polymeric supports such as cross-linked dextrans, polyacrylamide, or agarose. These, however, are better suited for low-pressure applications. As a result, a number of supports with increased mechanical strength have been developed. Commercially available silica-based and cross-linked agarose supports are now commonly used. HPSEC is also useful for the determination of clipped forms of proteins. Clipped chains often remain attached through the disulfide bonds of cysteine residues. Treatment of the sample with a reducing agent such as dithiothreitol or mercaptoethanol will cleave the disulfide bond and separate the chains. The clipped chains may then be resolved from unclipped forms by HPSEC.

HIC provides separation of proteins based on differences in their hydrophobicity under mild adsorption and elution

conditions that generally prevent denaturation and subsequent loss of biological activity. A stationary phase that is weakly hydrophobic is used with a buffered aqueous mobile phase and an initial high-salt concentration to adsorb the protein, which is then selectively eluted using a decreasing salt gradient. Interactions occur between nonpolar amino acid residues that are exposed on the surface of the protein and hydrophobic groups that are present on the chromatographic matrix. A number of silica-based and polymeric supports combined with weakly hydrophobic ligands, such as polyethers, phenyl ethers, or short alkyl chains, have been developed for use in HIC. This technique can be used in the analysis, purification, and characterization of more labile hydrophobic proteins. Protein retention and selectivity can be modified by control of variables such as salt type and concentration, pH and selective-ion effects, temperature and gradient design, as well as by careful selection of the stationary phase.

QUANTITATIVE ASSAYS

Biomimetic assays (assays that mimic the biological effect of the product) are of major significance in the discussion of assays for biotechnology-derived products. These assays measure the activity of the product and ensure that it is efficacious. Essentially, there are three major types of quantitative assays: animal model assays, cell culture-based assays and in-vitro (physicochemical) assays. Each of these assays has application in the control of biological products. Regardless of the type of quantitative assay employed, it is desirable and, in some cases, necessary, to use a biomimetic assay.

Animal Model Assays—Biomimetic assays in animal models have been developed for routine use. Although these assays have a relatively long history of use, they have several major disadvantages such as the large number of animals and appropriate animal facilities and handlers required, the high cost of analysis, the long analysis time (i.e., several days to weeks), and poor reproducibility of results. They are, however, in use mainly because a cell culture-based or in-vitro assay has not been developed and demonstrated to be of equal or greater value. An example of such an assay is that used for the determination of the activity of human growth hormone (somatrem and somatropin). The potency of human growth hormone is determined with a rat weight gain bioassay. Hypophysectomized female rats are monitored for weight gain over an 11-day period after daily injections with human growth hormone. The relative potency of the test sample is obtained by statistical comparison of the activity of the sample to that of a reference material/reference standard. Animal models can be used as bioidentity tests if and when appropriate in-vitro biological and/or physicochemical assays are developed for the measurement of potency of products.

Cell Culture-Based Bioassays—This group of assays is comparatively easier to perform, gives results faster (1 to 3 days), and is considerably less expensive and less wasteful of resources than the animal model assays. Cell culture-based bioassays provide information on the effect of the biological product in a living system, but they are imprecise as a consequence of the variances of living cells but not as imprecise as an animal model assay. However, they can be automated and therefore can be repeated sufficiently to provide relatively reproducible and accurate results. An example of this type of assay is the measurement of antiviral activity of human α -interferon in a human diploid foreskin cell line or in a human lung carcinoma cell line (A549). This assay is performed in microtitration plates by incubation of cells with α -interferon and subsequent challenge with encephalomyocarditis virus. The cells that survive are detected by dye binding and the dilution of α -interferon where 50% protection of the monolayer occurs is calculated.

In Vitro (Physicochemical) Assays—This group of assays does not rely on a living model, but is usually based on the chemical action of a biological product. These methods are comparatively simple, fast, precise, and accurate. The activity of tissue-type plasminogen activator (alteplase), for example, can be determined with an in vitro clot lysis assay that can be automated and can provide the required results within hours. A synthetic fibrin clot is formed in the presence of plasminogen as a result of the action of the enzyme thrombin on fibrinogen. When alteplase is added, the plasminogen is converted to the active enzyme plasmin, which then lyses the synthetic clot. The assay endpoint is followed spectrophotometrically or visually by noting the release of entrapped air bubbles. Another advantage of this type of assay, because of its precision and accuracy, is that it can be used to provide reliable estimates of the stability of the product. Examples of antibody:antigen and protein:ligand (receptor)-based in-vitro bioassays have also been developed for specific applications. These types of assays offer many advantages in their application to determine the potency of monoclonal antibodies or other highly ligand-specific proteins whose reactivity includes a binding step.

DNA DETERMINATION

Residual host cell DNA is a potential process-specific impurity in a biotechnology-derived product. The residual DNA is unique for each product because it is dependent on the host organism and the process recovery procedure used to manufacture the product. Although adverse health effects have not been reported from biologicals because of their DNA content, regulatory agencies have requested manufacturers to ensure that the DNA level in biotechnology-derived products is reduced to low levels.

The technique of DNA hybridization (dot blot analysis) is the most sensitive, routine DNA assay available to determine the DNA content of products. It is valuable as a purification process assay to demonstrate that a low level of DNA has been attained early in the manufacturing process. The method relies on the hybridization of cellular DNA from the sample with either specific ^{32}P -labeled or chemically modified DNA probes obtained from the DNA of the host cell. The analysis is performed by first isolating any residual DNA in the sample by a procedure that may include hydrolysis of the protein, chromatography, organic extractions, and alcohol precipitation. The isolated DNA is denatured and then applied to a nitrocellulose or nylon membrane along with a set of serially-diluted host DNA standards. Positive and negative DNA controls are also applied and the membrane may be baked at approximately 80° or placed under UV light to complete binding of the DNA to the membrane. A DNA probe is then prepared either by nick translation, random primer synthesis, or chemical modification of a DNA extract of the host cell. The DNA probe is purified and thermally denatured at 95° . It is then added to the baked or UV-treated membrane and allowed to hybridize with the DNA of the samples at approximately 42° in the presence of formamide or at higher temperatures without formamide for 24 to 48 hours. The membrane is subsequently placed between two X-ray films and exposed to produce an autoradiograph or is developed by immunochemical means using an enzyme conjugate/substrate system similar to ELISA and/or Western blot. The DNA of the sample is estimated by visual comparison of the dot intensity of the sample to those of the diluted DNA standards. The autoradiogram can also be scanned by optical densitometry. The sensitivity of the assay, i.e., 10 to 250 pg, is determined by the limit of visual detection above background of the serially-diluted DNA standards.

Other methods for DNA determination have been developed using biosensor technology. This methodology currently determines total DNA/nucleic acid impurities rather than specific host cell DNA. This technology may become quite valuable in the future, especially when more specific

DNA binding methods are developed. Finally, the recently developed polymerase chain reaction (PCR) technology, which involves DNA amplification, may prove useful in detection and identification of contaminant DNA. Quantitative use of this technology, however, will require further development.

CARBOHYDRATE DETERMINATION

One of the possible post-translational modifications that occurs on proteins is the covalent attachment of oligosaccharide chains. Glycosylation is a characteristic of recombinant proteins that are expressed from eukaryotic cell lines. Although the polypeptide chain of a glycoprotein is synthesized under the direct control of the genetic code, oligosaccharides are not primary gene products, but are synthesized by enzymes known as glycosyltransferases. This synthesis results in microheterogeneity of the carbohydrate chains. Also, glycosylation is cell-line dependent, so glycoproteins with identical polypeptide chains made in different cell lines may have considerably different carbohydrate structures. The sugars commonly found in glycoproteins include neutral sugars (D-galactose, D-mannose, and L-fucose), amino sugars (N-acetylglucosamine and N-acetylgalactosamine), and the acidic sugar, sialic acid.

Two main approaches can be taken to determine the sugars covalently attached to the glycoprotein. Both are based on the understanding that microheterogeneity is a common phenomenon among glycoproteins, and that the information represents either average composition or representative structures.

The first approach is the determination of the composition of sugars in a glycoprotein, which can be performed by several methods. Neutral sugars and sialic acid may be determined by simple colorimetric tests. Total neutral sugars can be determined following reaction with phenol and sulfuric acid and measuring the absorbance of the solution at about 490 nm compared to a standard curve. Following mild acid hydrolysis and periodate oxidation, free sialic acid content can be determined with thiobarbituric acid and the absorbance of the solution at about 550 nm compared to a standard curve. Individual neutral sugars can be determined following acid hydrolysis by several methods. Underivatized, they can be separated by HPLC at high pH and quantitated by pulsed amperometric detection. They may also be converted to the alditol peracetates with acetic anhydride or to the aldonitrile acetates with hydroxylamine hydrochloride and pyridine prior to peracetylation, and the derivatives separated by gas chromatography.

The second approach in determining the carbohydrate composition is to release and separate individual oligosaccharide structures covalently attached to the glycoprotein. This requires an understanding of the types of structures attached. The attachment of sugars to proteins can occur in two major ways: through an O-glycosidic bond involving the hydroxyl group of serine, threonine, or modified amino acids such as hydroxylysine or hydroxyproline, or through the N-glycosidic bond of asparagine. O-linked oligosaccharides can be released from the protein following beta-elimination under alkaline conditions and reduction of the reducing end sugar with sodium borohydride. N-linked oligosaccharides can be released chemically by hydrazinolysis or enzymatically by one of a variety of specific glycosidases, such as endo H, endo F, or peptido-N-glycanase. The oligosaccharides can then be separated by HPLC at high pH and quantitated with pulsed amperometric detection. This results in an oligosaccharide or carbohydrate map analogous to the peptide map for the protein.

ADVENTITIOUS AND ENDOGENOUS AGENT DETECTION

Specific assays pertaining to biotechnology focus on the detection of bacteria, fungi, mycoplasma, and viruses. These

reflect the possible contaminants that may occur in both bacterial fermentation and mammalian cell culture. Control is exerted in a variety of ways including characterization of the master seed bank and the working cell banks to ensure freedom from these contaminants, evaluation of raw materials, the design and operation of closed manufacturing systems, testing of production lots, and validation of specific manufacturing processes to ensure that contaminants would be inactivated or removed if present.

Freedom of final sterile dosage forms from bacteria and fungi is usually evaluated by tests for sterility as described in *Sterility Tests* (71). Mycoplasma assays are performed by standard cultivation methods employing aerobic and anaerobic incubation of solid medium in plates and semisolid broth in tubes and must comply with the code of federal regulations (21 CFR 610.12). In addition, noncultivable mycoplasma are detected microscopically by using the Hoechst bisbenzimidazole staining method.

Various methods that are used for the detection of adventitious virus contamination in cell lines include inoculation of indicator cell lines selected for their ability to support the replication of a broad range of viruses and monitoring these for markers of virus infection such as cytopathology, hemadsorption, hemagglutination, and immunofluorescence; inoculation of intact animals and monitoring for illness and death; inoculation of animals and, after four weeks, collection and evaluation of serum for antibodies to specific viruses of concern; and specific immunologic assays or genetic probes for some viruses of concern that cannot be detected by the other methods listed.

The expression of endogenous retrovirus genes is highly variable among different mammalian cells and cell lines. The unpredictable nature of their expression and the diversity of their biochemical and biological properties preclude the use of a single test and instead require an integrated testing strategy. Test methods generally used include transmission electron microscopy of cells from the master seed bank and ultracentrifuged pellets of cell-free, cell culture harvests; various assays for infectious retroviruses that use retrovirus-susceptible indicator cell lines; reverse transcriptase activity; and induction of retroviruses in cells of the master cell bank with chemicals known to induce retroviruses. In addition to classical virological methods, newer techniques such as molecu-

lar probe hybridization are also beginning to be used for these evaluations.

(1046) CELLULAR AND TISSUE-BASED PRODUCTS

INTRODUCTION

This general chapter provides a comprehensive overview of considerations for the development of cellular and tissue-based products. A collection of terms commonly used in this field is provided under *Glossary and Definition of Terms*. Cell and tissue-based therapies are medical products that contain human or animal cells that will be administered to humans to repair, replace, regenerate, or augment a recipient's cells, tissue, or organs that are diseased, dysfunctional, or injured. The source cells or tissue can be harvested for use without manipulation or may be propagated, expanded, pharmacologically treated, or otherwise altered in biological characteristics *ex vivo* before administration. The diversity of clinical indications and types of cell and tissue-based products are shown in *Table 1*.

Cell therapy products can be modified by treatment with integrating or nonintegrating genetic materials (DNA, RNA, siRNA, etc.) so that the pattern of gene expression is changed. Typically, cells are taken from the patient and are modified outside of the body before they are returned to the patient. Regulatory bodies consider the *ex vivo* gene-modified cellular product to be a gene therapy product. A great deal of information in this general chapter is relevant to processing, characterization, manufacturing, and administration of genetically modified cells. However detailed information about the use of various gene transfer systems, patient monitoring considerations, genetic analysis, and other

Table 1. Examples of Cell-Based Therapy Products

Indication	Product
Hematopoietic stem cell transplantation following ablative therapy	Hematopoietic stem and progenitor cells that have been harvested, propagated, selected, and/or treated for removal of contaminating cells by means of devices and/or reagents
Cancer	T cells, NK cells, dendritic cells, or macrophages exposed to cancer-specific peptides to elicit an anticancer response; autologous or allogeneic cancer cells, genetically or biochemically modified and irradiated to elicit an anticancer response
Diabetes	Encapsulated β -islet cells
Myocardial infarction	Autologous or allogeneic stem/progenitor cells; skeletal myocytes; cardiac-derived stem cells
Graft-versus-host disease	Allogeneic mesenchymal stem cells
Wound healing	Autologous keratinocytes or allogeneic dermal fibroblasts on a biocompatible scaffold
Focal defects in knee cartilage	Autologous or allogeneic chondrocytes with or without a biocompatible scaffold
Bone repair	Mesenchymal stem cells in a biocompatible scaffold
Neurodegenerative diseases	Neuronal progenitor cells derived from embryonic, fetal, or adult source tissues; cells genetically modified to secrete neurotrophic factors, with or without encapsulation
Infectious disease	Activated T-cells
Autoimmune disease	Regulatory T-cells (T _{reg})
Spinal cord injury	Neuronal progenitor cells
Organ repair or regeneration	Autologous or allogeneic cells on biocompatible biomaterials (gels) or 3-dimensional scaffold structure

issues pertinent to gene therapy products are addressed in *Gene Therapy Products* (1047).

This general chapter describes issues related to the manufacturing, sourcing of components, and characterization of cellular or tissue-based products to ensure their safety and efficacy. A list of relevant regulatory and guidance documents is presented in the *Appendix*. Manufacturers of cellular or tissue-based products should consider and apply the controls and procedures outlined in this chapter to ensure the products' safe use in humans. New methodologies are continually being developed and validated and will be included in the United States Pharmacopeia (USP) as they become available. USP monographs for specific tissue and tissue-based products outline test specifications that should be met throughout a product's time in the market place. The term cellular product refers to living human or animal cells or tissues that have been manipulated or are used in ways that result in their regulation as somatic cellular therapies, as defined by the US Food and Drug Administration (FDA). A *tissue-based product* refers to human tissues subject to regulation under good tissue practices (GTPs). *Combination products* refer to cells combined with medical devices, such as a natural or synthetic scaffold.

Considerations for Incorporating Quality System Concepts Early in Cellular and Tissue-Based Product Development

Current and future regulatory requirements will continue to challenge developers of cellular and tissue-based products to incorporate robust quality attributes early in the design phase to ensure a focus on patient safety by means of a high degree of process understanding. Modern quality systems that harmonize current Good Manufacturing Practices (cGMPs) with other non-US pharmaceutical regulatory systems [such as the International Conference on Harmonization (ICH) and the International Organization for Standardization (ISO)] and the FDA medical device quality system are being recognized as the new global standards. These new standards include product development concepts such as Quality by Design (QbD) and Process Analytical Technology (PAT). Moreover, these quality systems integrate approaches to continual improvement and risk management that promote adoption of the latest scientific advances and innovative manufacturing technologies.

Employing the principles of Quality Risk Management (QRM) early in product development may identify areas of risk that can be mitigated before they are incorporated into the manufacturing process and affect the safety and efficacy of the product. Developers of cell and tissue-based products should employ risk management and assessment techniques as a key component of their quality systems. *Risk management* is defined as a systematic process for the identification, assessment, and control of risks to the quality of the cell or tissue-based product across the product lifecycle. Using QRM techniques can help achieve safe and efficacious products by assessing patient risks, determining design space boundaries, or ranking quality attributes. QRM can also establish and maintain a state of control by using risk management to drive process control. Finally, QRM can be used to facilitate continual improvement by prioritizing opportunities for improvement. The level of effort, formality, and documentation of the risk management process should be commensurate with the level of risk, should be based on scientific knowledge, and ultimately should be linked to patient protection.

The elements of risk management have become an accepted paradigm and can be readily accessed in FDA and international regulatory guidance documents, especially ICH Q9. A number of tools have been developed to facilitate this assessment. These tools provide a quantifiable means of prioritizing risk so that higher-risk elements of a process can be identified and corrected.

Depending on the objective of the risk management program, risk analysis can be more or less formalized. Preliminary, less formal risk analysis comes into play when a risk assessment needs to be expedited, as in the resolution of a manufacturing nonconformance. A more formalized risk assessment system is necessary for process or product development. This is especially important when limited resources must be prioritized. Formalized systems are predicated on well-established tools that can quantify risk in every phase or step of manufacturing. These systems can also be used in evaluating raw material choices, validation prioritization, facility alterations, equipment changes, and utility deliberations.

Formal risk analysis tools include process mapping, preliminary hazard analysis, Hazard Analysis of Critical Control Points (HACCP), Hazard Operability Analysis (HAZOP), Fault Tree Analysis (FTA), Failure Mode Effects Analysis (FMEA), and Failure Mode Effects and Criticality Analysis (FMECA).

For cell and tissue-based products, FMEA has been commonly used to identify, quantify, and prioritize risk. FMEA can assign a numerical rating in one of three categories:

- *Severity*, which is the consequence of a failure;
- *Occurrence*, which is the likelihood of the failure happening based on past experience or nonconformance; and
- *Detection*, based on the ability to detect the failure.

Each category is assigned a numerical rating (typically 1 to 5 or 1 to 10) consistent with the severity of the excursion from the operating parameter range, the probability of an excursion, and the likelihood of detecting an excursion before it has an effect on the product. Lower numbers refer to an unlikely probability of detection whereas higher numbers refer to the likelihood of a failure or hazardous effect. The product of the severity, occurrence, and detection values is a Risk Priority Number (RPN). In the risk-evaluation process RPNs are prioritized, and the most immediate remediation can be directed to areas of highest risk.

COMPONENTS USED IN CELL AND TISSUE-BASED PRODUCT MANUFACTURING

Introduction

Manufacturers of cellular or tissue-based products must ensure that all components used in manufacturing are appropriately qualified. Examples of components used in the production of cellular or tissue-based therapies include the source cells and tissues; natural or synthetic biomaterials; ancillary materials required during manufacturing but not intended to be present in the final therapeutic product; and excipients used in the formulation of cellular or tissue-based therapies.

Qualification is the process of acquiring and evaluating data to establish the source, identity, purity, biological safety, and overall suitability of a specific component to ensure quality. The diversity of cellular and tissue-therapy products and of the materials used to produce them makes it difficult to recommend specific tests or protocols for a qualification program. Therefore, rational and scientifically sound programs must be developed for each component.

Material qualification activities will change as products move from clinical trials to licensure and commercialization. A well-designed qualification program becomes more comprehensive as product development progresses. In the early stages of product development, safety concerns should be the primary focus of a material qualification plan. In the later stages, material qualification activities should be completely developed and should comply with cGMP.

Qualification of Source Cells and Tissues

Various human- and animal-derived cells and tissues serve as source material for cell and tissue-based products. Three sources of donor cells for cell-therapy products include:

1. The patient's own cells (autologous cell products)
2. Cells from another human being (allogeneic cell products)
3. Cells derived from animals (xenogeneic cell products)

The source of cells used for a particular cell or tissue-based therapy largely depends on compatibility, purity, and availability. Use of autologous cells has the advantage of minimal concerns regarding immune rejection. However, an autologous source is not always available and appropriate if the cell type is dysfunctional, malignant, not readily obtainable, or contaminated.

The alternative is a compatible allogeneic cell source that may be more readily available. Of primary concern with the use of allogeneic cell sources is immune incompatibility, which could lead to rejection of the administered cell or tissue-based therapy. In immunocompromised recipients, the donor cells may react to the patient's cells, leading to graft-versus-host disease, which can be life threatening. Despite the potential complications of using allogeneic donor cells or tissues, in the absence of other alternatives the risk-to-benefit ratio is often favorable. A number of approaches successfully circumvent immune barriers for the use of allogeneic sources. Immunosuppressive drugs developed for solid organ transplantation and advances in inducing immune tolerance are increasingly applied to cell transplantation. Certain allogeneic cells elicit minimal immune reactions, even in HLA-mismatched recipients. Examples include mesenchymal stem cells, certain dermal and epidermal cells, and fibroblasts.

Despite advances in the derivation of new types of therapeutic cells, particularly stem cells (adult, fetal, embryonic and induced pluripotent cells), the ability to generate certain types of cells or tissues remains elusive. As a result, ongoing efforts use xenogeneic cells and tissues to treat a number of human diseases or conditions. Use of xenogeneic cells must address concerns about both immune rejection and transmission of animal viruses to humans (see *Animal Sources of Cells and Tissues*, below).

Some general principles in the sourcing of tissues include: (1) systems must allow the material to be traced back to the donor, while adhering to privacy legislation; (2) steps must be taken to prevent the transmission of infectious diseases from the donor to the recipient; and (3) aseptic procurement and processing must ensure the safety of the final product because terminal sterilization of products containing living cells and tissues is not possible. FDA has promulgated a specific set of regulations, referred to as GTPs, that specifically address the need to procure and process tissues in a manner that avoids transmission of a communicable disease. GTPs and/or GMPs must be followed for cell or tissue-based therapy products, depending on cell source and place in the product life cycle.

DONOR ELIGIBILITY

FDA has enacted a comprehensive set of regulations governing human tissues and human cells that are intended for implantation, transplantation, infusion, or transfer into a human recipient. These materials are referred to as human cells, tissues, or cellular or tissue-based products (HCT/Ps). Paramount for procurement of HCT/Ps for medical use is adherence to donor eligibility requirements. These dictate that a donor's relevant medical records must be reviewed to evaluate risk factors and clinical evidence of communicable disease agents. This includes obtaining a health history and performing a physical examination on a donor to screen for communicable diseases. In addition, donors must also undergo appropriate laboratory testing using FDA-cleared or

-approved test kits for specific relevant communicable disease agents and diseases (RCDADs). Required disease testing will expand as new RCDADs are identified and FDA-approved or -cleared test kits become available. Two sources for information about communicable disease testing are FDA's *Guidance on Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products* and AABB's *Circular of Information* (http://www.aabb.org/Content/About_Blood/Circulars_of_Information/aabb_coi.htm). Donor eligibility determination is not required for autologous HCT/Ps.

HUMAN CELLS, TISSUES, OR CELLULAR OR TISSUE-BASED PRODUCTS

HCT/Ps may be sourced from normal healthy donors, cadaveric donors, or patients with diseases such as cancer. The suitability of tissue sourced from patients with cancer and other diseases should be assessed before collection to ensure adequate safety and function of the final cell therapy product. Additionally, the regulations in 45 CFR Part 46 apply to all federally supported human subject research. These regulations require that an Institutional Review Board review and approve the use of any tissue taken from a human donor. The regulations also include special considerations for research on prisoners, children, pregnant women, or gestational tissue. In all cases appropriate written consent must be obtained from the donor or the donor's next of kin describing the tissue that is being procured and its intended use.

The risk of disease transmission to the manufacturing operator should be minimized by appropriate training for handling potentially infectious materials and by the use of protective equipment and clothing. Tissues should be obtained under environmental conditions and controls that provide a high degree of assurance for aseptic recovery.

Hematopoietic progenitor cells (HPCs) are one of the most extensively used cell sources for human transplantation. These cells can be collected from the bone marrow, peripheral blood, or umbilical cord blood. The source of cells depends on the patient, the disease, and the clinical protocol. Regardless of the cell source, methods for processing the cells are similar. HPCs can be sourced from healthy donors or patients with hematological disorders. In addition to FDA's HCT/Ps regulations, applicable guidelines and standards for the collection and processing of these materials have been published by the American Association of Blood Banks (AABB), the Foundation for the Accreditation of Hematopoietic Cell Therapy, and the National Marrow Donor Program (NMDP).

For cell or tissue sources obtained from surgical specimens or cadaveric donors, standard hospital operating room practices are applicable. The air quality in a typical limited-access operating room is adequate for such procedures. Procurement personnel must be appropriately trained in all aspects of tissue recovery, such as surgical scrubbing, gowning, operating room behavior, anatomy, surgical site preparation, and aseptic technique. Special care is required when tissue or organ procurement requires extensive manipulation of the bowel, which may result in the inadvertent puncture of the bowel. Tissue that contains microbial flora (e.g., skin) at the time of procurement can be adequately disinfected with antimicrobial or bactericidal agents and extensive scrubbing.

ANIMAL SOURCES OF CELLS AND TISSUE

Ideally, cellular therapy products would consist of human cells manufactured with minimal exposure to animal-based materials. However, at present important unmet medical needs may potentially be addressed by cellular therapy products from animal cells or tissues. One example is pancreatic islets intended to treat diabetes. Human sources of pancreatic islets are available only from pancreas donated at

the time of death. The quality of donor organ islets is variable, and the available supply is inadequate to meet potential demand. One approach is procurement of pancreatic islets from appropriately qualified animal sources for subsequent use in humans (xenotransplantation).

Developers who intend to use animal cells or tissues in a cellular therapy product must adequately address public health concerns and must develop approaches to mitigate the potential risk of introduction and propagation of zoonotic infectious agents into the general human population. The *PHS Guideline on Infectious Disease Issues in Xenotransplantation* (January 2001) describes potential risks. The FDA guidance *Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans* (April 2003) reflects updated approaches and expectations to minimize risks of xenogeneic cellular products.

The use of animal tissue in the manufacture of cell therapy products requires that the tissue be sourced in a controlled and documented manner from designated pathogen-free animals bred and raised in captivity in countries or geographic regions that have appropriate disease prevention and control systems. In addition, the care and use of animals should be approved by a certified institutional animal care and use committee. Donor animals must have documented lineage, be obtained from closed herds or colonies, and be under health maintenance and monitoring programs. The animal housing facility should be USDA certified (large vertebrate animals) or Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) certified (small vertebrate animals) and should meet the recommendations stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996), which can be obtained from AAALAC (www.aaalac.org). Such facilities should be staffed with veterinarians and other trained personnel who ensure animal health and disease prevention. The facility's procedures should be documented, and records should be kept. Health maintenance and monitoring programs should be based on standard veterinary care for the species, including physical examinations, monitoring, laboratory diagnostic tests, and vaccinations. A stepwise *batch* or *all-in-all-out* method of source animal movement through the facility can minimize the potential for transmission of infectious agents.

Feed components should be documented and should exclude recycled or rendered materials in order to reduce the risk of prion-associated diseases.

To provide a high degree of assurance of product safety, animal donors and tissues should be screened at several stages throughout the process to rule out the presence of microbial agents. These control tests should utilize assays that are sufficiently sensitive and specific to detect bacteria, mycoplasma, fungi, or viruses of interest. Donor animals should be screened for relevant diseases before tissue procurement. Post-tissue-retrieval necropsies, sentinel animal programs, and archival storage of donor organs, tissues, blood, and other specimens also ensure the safety of animal tissue for use in cellular therapeutic applications.

In general, similar aseptic procurement issues apply to animal and human tissues. The tissue should be obtained under environmental conditions and controls that provide a high degree of assurance of aseptic recovery. Specifically designed procurement facilities, usually closely associated with the animal holding facility, should be employed. Recommended design features and attributes of the animal tissue procurement facility should include the following: (1) staging of events such as shaving, sedation, and operating room preparation in separate rooms with appropriate environmental controls; (2) high-efficiency particulate air (HEPA) filtration; (3) adjacent but separate facilities for further tissue processing; and (4) dedicated areas for carcass removal. Issues relating to personnel training, bowel manipulation and puncture, and disinfection apply to the surgical procurement of both human and animal tissues (see *Human Cells, Tissues, or Cellular or Tissue-Based Products*, above). When researchers establish animal cell lines for use as feeder layer

cells, cell banks should be created, tested, and characterized as described in the next section.

CELL BANK SYSTEM

A cell bank is a collection of cells obtained from pooled cells or derived from a single cell clone or donor tissue that is stored in bags or vials under defined conditions that maintain genotypic and phenotypic stability. The cell bank system usually consists of a master cell bank (MCB) and a working cell bank (WCB), although alternative approaches are possible. The MCB is produced in accordance with cGMP and preferably is obtained from a qualified source (one that is free from adventitious agents) with known and documented history. Human cells and tissues should be obtained by means of a licensed tissue acquisition vendor with a donor qualification program in accordance to 21 CFR 1271. The WCB is produced or derived by expanding one or more vials of the MCB. The WCB, or MCB in early trials, becomes the source of cells for every batch produced for human use. Cell bank systems contribute greatly to production batch consistency because the starting cell material is always the same. However, it may not be possible or feasible to create a cell bank, so appropriately tested and qualified primary cells may be used in lieu of creation of cell banks. The MCB and WCB should be minimally tested for identity, sterility, purity, viability, and the presence of viruses and mycoplasma.

CELL BANK QUALIFICATION

Cell bank safety testing and characterization are important steps toward obtaining a uniform final product with lot-to-lot consistency and freedom from adventitious agents. ICH Q5A, *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin*, gives specific recommendations for testing cell banks for viral agents. While this guideline is not specifically intended to cover cell or tissue-based products, the same tests are generally applicable. Additional virus testing may be needed depending on the prevalence of viral diseases endemic in the donor population. Testing to qualify the MCB is performed once and can be done on an aliquot of the banked material or on cell cultures derived from the cell bank. Specifications for qualification of the MCB should be prospectively established. It is important to document the MCB history, the methods and reagents used to produce the bank, and the storage conditions. All the ancillary materials required for production of the banks, such as media, sera, cytokines, growth factors, and enzymes, should also be qualified, documented, and appropriately tested.

SAFETY TESTING OF MCB AND WCB

Master Cell Bank—Safety testing to qualify the MCB includes testing to demonstrate freedom from adventitious agents and endogenous viruses. The testing for adventitious agents should include tests for bacteria, fungi, mycoplasma, and viruses. Freedom from adventitious viruses should be demonstrated using both *in vitro* and *in vivo* test systems and appropriate species-specific tests.

Working Cell Bank—Safety testing of the WCB is less extensive and generally focuses on the potential for introduction of adventitious viruses or activation of latent virus during the additional culture required to create the WCB. End-of-production (EOP) safety testing should also be performed to ensure that the cells can be expanded a known maximum number of generations while still producing an acceptable product. For information about which types of adventitious virus testing should be performed on the MCB, WCB, and EOP cells, consult *Viral Safety Evaluation of Bio-*

technology Products Derived from Cell Lines of Human or Animal Origin (1050).

CHARACTERIZATION OF MCB AND WCB

Characterization of the MCB and WCB includes identity testing to establish species origin, e.g., isoenzyme analyses to confirm the human origin of the cells. However, cell bank characterization should encompass additional assessments such as the following:

- Growth kinetics and population doubling time
- Morphological assessment
- Percent confluence at passage
- Cell counts
- Viability (pre- and postcryopreservation)
- Phenotypic expression of desired and undesired cell types (pre- and postcryopreservation)
- Monitoring of unique biochemical markers (pre- and postcryopreservation)
- Assessments of functional activity (pre- and postcryopreservation)
- Gene and protein expression analysis (pre- and postcryopreservation)
- Expression of immune histocompatibility antigens (HLA/M HC)
- Molecular fingerprinting
- Chromosomal stability

Biocompatible Scaffold Materials

Most natural or synthetic scaffold materials are regulated as medical devices, although scaffolds derived from human tissues such as dermis are regulated as HCT/Ps. When possible, use scaffolds that have previously been approved for other clinical uses because these materials should have already undergone extensive safety and quality testing. For applications in cell or tissue-based products, the scaffold material should allow cells to attach, proliferate, and migrate, and high porosity is often desired to facilitate cell seeding within the material. The scaffold must provide adequate diffusion of nutrients for cell health and release of cell-excreted products. The material must have adequate mechanical strength and must be amenable to manipulation, chemical modification, and manufacture. The scaffold material should be biocompatible, relatively inert, and immunologically benign.

Scaffolds can generally be classified as hard or soft. Hard scaffolds are used in applications where a specific shape is required, such as forming a blood vessel or a bladder. Soft scaffolds are used in applications where the product needs to conform flexibly to an existing shape in the body.

Scaffold materials can be synthetic or natural polymers, biodegradable or permanent. Biodegradation allows the scaffold to be resorbed or removed from the body without manipulation. The scaffold degradation rate must coincide with the rate of formation or regeneration of the tissue. The natural scaffold structure must replace the degrading scaffold in such a way that it maintains the structural integrity of the tissue or organ being regenerated. For example, a newly formed blood vessel must withstand both the internal blood pressure as well as external mechanical forces.

The most commonly used synthetic biodegradable polymer is polyglycolic acid (PGA). Polylactic acid (PLA) is also widely used, sometimes in combination with PGA. These polymers degrade within the body, are readily removed before degradation, and have a long history of use in suture materials. Polycaprolactum (PCL), which exhibits a slower rate of degradation than PLA or PGA, is used in applications that require a long presence in the body.

Extracellular matrix (ECM) and its derivatives are natural materials used for scaffolds in the manufacture of cell-bio-material combination products. Proteins such as collagen or fibrin and polysaccharides such as chitosan or glycosami-

noglycans (GAGs) have also been used in growing cells to make combination products. Collagen is by far the most popular substrate for cells and has been molded into scaffolds for a variety of products, mainly in tissue-engineered skin applications. Cross-linking agents such as glutaraldehyde and water-soluble carbodiimides have been used to enhance the strength of natural scaffolds. Depending on the source of the material, natural scaffolds can be immunogenic.

When cells must proliferate after seeding, the scaffold and the supporting culture system must allow the exchange of nutrients and waste products. A thick, impermeable matrix will lead to regions of necrotic tissue. Many tissue devices are designed so that they can eventually be removed from the patient.

The safety and biocompatibility of the scaffold and product-contact materials must be established. A full battery of tests recommended by *Biological Reactivity Tests, In Vitro* (87), *Biological Reactivity Tests, In Vivo* (88), ISO 10993, or FDA Blue Book G95-1 should be performed. Process residuals and degradation products from the preparation of the scaffold should be quantified and limits should be established. The stability and storage conditions of scaffold materials should be established.

Qualification of Ancillary Materials

Ancillary products include a wide variety of raw materials and components used in manufacturing. They may include relatively simple materials or complex substances such as culture media, buffers, growth factors, cytokines, cultivation and processing components, monoclonal antibodies, and cell-separation devices.

Ancillary materials are not intended to be present in the final therapeutic product. Defined media formulations typically include components such as albumin and transferrin that are purified from animal or human sources. The purification, processing, and extensive testing of such components further minimize—but do not eliminate—the risk of viral or microbial contamination. Residual amounts of ancillary materials used in the manufacturing process, including recombinant proteins or other defined media components, may be potentially antigenic so their removal from the final product should be assessed, and appropriate limits should be established when necessary.

Known risks are associated with the use of ancillary materials in the production of cell-therapy products. The quality of ancillary materials used in the production of a cellular therapy product can affect the safety, potency, and purity of the product. Ideally, each ancillary material employed in the manufacture of a cellular or tissue-therapy product should be produced under conditions that are in compliance with cGMP. However, complex or unique substances essential for process control or production may not be available from suppliers that produce them in compliance with cGMP. In these situations, the cellular or tissue-therapy product manufacturer should develop a scientifically sound strategy for qualifying the raw material. A qualification program for ancillary materials used in cell and tissue therapy manufacturing should address each of the following areas: (1) identification and selection, (2) suitability for use in manufacturing, (3) characterization and acceptance criteria, (4) vendor qualification, and (5) quality assurance. Lot history files should be constructed for each ancillary material.

Conformance to established specifications should be compared to the data supplied on the Certificate of Analysis. Traceability is essential, and lot numbers for each ancillary material used should be noted in the production records of the cell-based product. USP general information chapter *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* (1043) should be consulted for specific information about implementing an appropriate qualification program for these materials. Other USP chapters provide considerations about the qualification of specific ancillary materials (e.g.,

Bovine Serum <1024>, *Fetal Bovine Serum—Quality Attributes and Functionality Tests* <90>, and *Growth Factors and Cytokines Used in Cell Manufacturing* <92>).

Qualification of Excipients

During the final steps in the manufacturing process, excipients or substances that increase the stability of the therapeutic cells may be included. Examples of excipients include culture media, USP saline or other electrolyte solutions approved for injection, exogenous proteins such as human serum albumin, or cryoprotectants such as dimethyl sulfoxide (DMSO). Excipients are not intended to exert a direct therapeutic effect upon the patient; rather they are intended to contribute to maintenance of the quality attributes of the final cellular product. Because excipients will be administered to the patient along with the cells, particular attention must be paid to their qualification. In general, excipients that are already FDA approved for human use should be used whenever possible. If nonapproved excipients are used, a complete safety assessment should be done. For novel excipients such as cryopreservation solutions, appropriately designed preclinical safety studies may be needed.

MANUFACTURING OF CELL OR TISSUE-BASED PRODUCTS

Introduction

The manufacturing of cell or tissue-based products requires a number of operations and manipulations by individuals who are well trained in aseptic processing techniques. The technical competence of the personnel is particularly crucial to product safety and efficacy. Autologous products present more challenges for cell and tissue processing because lot segregation, line clearance, and operational processes must be developed to decrease the chance of mix-up of patient-specific lots (see *Facility Design and Operation Considerations*, below).

Cell Isolation and Selection

The general principles for processing human or animal tissues following aseptic procurement are independent of the cell or tissue source. The manufacture of cell products may occur at a cell manufacturing facility located in close proximity to the clinical site or at a distant central cell-manufacturing facility. The source cellular or tissue material should be packaged in sterile, leak-proof containers and transported from the procurement area to the processing area under controlled conditions that maintain cell viability. The fluid medium in which the specimens are bathed during transportation should be optimized to maintain cell and tissue viability. This transport medium can be supplemented with antibiotics. The antibiotic levels in process buffers should be decreased and eventually eliminated during subsequent processing steps so that antibiotics are not present in the final cellular product. In the case of blood products or tissues containing substantial amounts of blood, the transport media or buffered electrolyte solution should contain an anticoagulant.

ISOLATION

Solid organs or tissues are usually dissected to expose a desired region. This material may be used as is for transplantation, or it may be further processed. If multicellular organoids (for instance, islets of Langerhans) or single-cell suspensions are desired, the tissue may be subjected to mechanical or enzymatic disaggregation. Physical disaggregation

may be accomplished by the use of instruments that homogenize the material by imparting high shear forces or breaking the tissue into smaller pieces. Alternatively, the material can be pressed or passed through screens of defined mesh sizes.

Enzymatic digestion of the extracellular connective tissue is another common method for dissociating solid tissue. Various enzymes are used to accomplish this, including collagenase, dispase, trypsin, elastase, hyaluronidase, papain, and chymotrypsin. Enzymes with nuclease activity, such as deoxyribonuclease, may be added to digest nucleic acids released from damaged cells, preventing excessive cell clumping. At the end of the incubation process, the cell suspension may be subjected to a mild pumping action to further break up multicellular clusters into those of desired size or composition. Enzymatic and physical disaggregation methods are often combined to achieve the desired result.

Because blood and bone marrow cells are inherently suspensions, mechanical manipulation is usually limited to plasma and aggregate removal, which is accomplished by centrifugation and filtration.

Cell and tissue isolation activities involving open manipulation steps should be carried out in an ISO 5 (class 100) biological safety cabinet. The environment surrounding the biological safety cabinet should be suitable to maintain aseptic processing operations. For minimally manipulated HCT/PS in closed systems, these environments may be controlled but unclassified. However, for cell and tissue-based therapies that are manipulated and manufactured under cGMPs, the environment surrounding the biological safety cabinet should be controlled and classified, usually as an ISO 7 (class 10,000) clean room. Precautions should be taken to segregate patient-specific tissue and cell isolates.

SELECTION

Cell suspensions often consist of a mixture of cell types that may require further processing in order to isolate a cell population of interest or to decrease the level of an undesirable cell type such as potentially contaminating tumor cells. Various cell isolation and separation techniques provide high yields of pure cell populations.

Cell populations can be selectively enriched by varying the force and duration of centrifugation. Separation can also be achieved by isopycnic centrifugation in which the cell suspension is centrifuged in a gradient medium that encompasses all of the densities of cells in the sample. Specifically designed continuous-flow elutriation centrifuges separate cell populations by subjecting a cell suspension to opposite centrifugal and fluid stream forces in a special chamber within the centrifuge rotor mechanism. Cell populations separate within the rotor on the basis of their various sizes and densities, and they are selectively eluted out of the rotor chamber by increasing the fluid stream force. Finally, other methods involve the addition of high-density agents such as hydroxyethyl starch to the cell suspension. Concentration and separation procedures such as these frequently result in cell loss because of clumping and aggregation.

Cell separation can also be achieved by applying techniques that take advantage of unique cytological or biochemical characteristics of different cell populations. Soybean agglutinin aggregates cells that bear a particular carbohydrate moiety expressed on mature blood cells, but not stem cells, allowing enrichment of the stem cells. Lymphocytes possess the CD2 antigen that acts as a receptor for sheep red blood cells. When sheep red blood cells are added to the cell mixture, the lymphocytes form rosettes around the sheep red blood cells and are then separated via differential centrifugation. Some applications take advantage of the ability of certain cell populations to adhere to the surface of specific solid substrates such as tissue-culture plastic, collagen-coated materials, and natural and synthetic polymeric scaffolds. The specifically bound cell type is selec-

tively recovered onto the surface and removed from the initial cell suspension.

Monoclonal antibodies directed against specific cell-surface proteins can be used for both positive and negative cell selection. For example, a monoclonal antibody-bound cell population can be removed from the cell suspension after exposure to magnetic particles coated with anti-monoclonal antibody. The magnetic particles and their bound cells are removed from the cell suspension magnetically. Unlabeled cell suspensions can be poured over or incubated on surfaces such as plastic flasks or microspheres coated with monoclonal antibodies as a means of isolating particular cell populations. In addition, a fluorescence-activated cell sorter (FACS) can be used to separate different cell types by binding antibodies tagged with fluorescent markers to a particular cell type.

Other techniques enrich cell populations by destroying unwanted cells. For example, certain cell-bound monoclonal antibodies are able to fix and activate exogenously added complement, resulting in cell lysis. Some procedures use cytotoxic agents or mitotic inhibitors to selectively kill unwanted cells. These methods typically target cell subpopulations with high growth rates, such as tumor cells. Finally, an antibody can be conjugated to a toxic moiety, such as ricin, allowing delivery of the cytotoxic agent to the targeted cell population. Most of these procedures require several washing steps to ensure the removal of the dead cells, cell fragments, and cytotoxic agents from the final cell product.

Cell Ex Vivo Expansion and Differentiation

EX VIVO EXPANSION

A key issue for manufacturers of cell and tissue-based products is the ability to produce and deliver a therapeutically relevant dose of the required cell population to the patient. Depending on the application, the product may be a pure, homogeneous cell type, or it may be a mixture of different functional cell types. Many target cell populations are present at a low level or low purity in complex primary source tissues. In such cases, production of a therapeutic dose may be achieved only by specific enrichment and ex vivo expansion of the required cells.

Ex vivo expansion of cells may occur in suspension culture (e.g., T cells or hematopoietic stem and progenitor cells), adherent culture (e.g., mesenchymal stem cells, embryonic stem cells, induced pluripotent stem cells, neuronal stem cells, or dermal fibroblasts), or a mixture of both (e.g., bone marrow stroma expansion). Numerous technologies exist for cell culture. Cells can be propagated in tissue-culture flasks (T flasks), in roller bottles, on polymeric scaffolds, or in nonrigid, gas-permeable bags, usually inside incubator units controlled for temperature, humidity, and gas composition. Multilayered, high-capacity cell culture systems composed of tissue culture plastic, multibag systems, and bioreactors using microcarriers enable expansion, harvesting, and formulation to be carried out in a closed system. Traditional small-scale fermenter units can be used for expansion of cells in suspension culture. It is also possible to expand adherent cells in such units either by providing a surface for attachment (microcarriers, coated beads, or disks) or by adapting the cells to propagate in suspension culture. Some culture systems are specifically designed for the propagation of cells for therapeutic applications. These tend to be closed systems that use disposable bioreactor cartridges in automated processing units with direct control of temperature, gas composition, and media perfusion rate. In some cases automated software allows patient-donor tracking and documentation of culture conditions and manipulations. These features are useful in the design and implementation of QC product release testing programs and for the QA documentation of processing runs.

In adherent culture, the cells are usually harvested from the surface upon which they have expanded. Methods of release include physical agitation, enzymatic cleavage, and chelation of metal ions and competitive inhibition of adhesion or matrix molecules. As described above, consideration must be given to the source, safety, toxicology, and residual testing for any reagent used to release adherent cells during manufacturing. Some product-specific systems do not require the release of adherent cells. Cells are expanded on a biocompatible synthetic or natural scaffold that is then applied topically (for example, engineered skin substitutes), or the cells are grown inside or outside of fibers for ex vivo perfusion (for example, hepatocytes in hollow-fiber devices to treat liver disease).

In all cases standard cell culture parameters should be optimized for maximum process efficiency. Such parameters include composition of cellular source material, initial seeding density, media composition, rate of media exchange, temperature, gas composition, pH, and rate of delivery. Depending on the nature of the product, the potential effect of process parameters on the potency and function of the target cells should be defined.

Bioreactors—Specialized bioreactors and devices are required for manufacturing three-dimensional combination products. These bioreactors hold the biocompatible scaffold/matrices for the manufacture of the construct. Although the bioreactor can provide a closed system for construct manufacturing, it creates a challenge in providing access to the scaffold for seeding cells and sampling for product release testing while maintaining sterility. Bioreactors are often single-use devices that ensure that no cross-contamination occurs between products. Preferably the product will not be repackaged for transport and delivery. For example, bioreactors may also serve as the final container for product shipment.

Container-closure testing must be performed for all final container-closure systems. Compatibility for sterilization of the bioreactor and the scaffold should be verified, and the sterilization process must be validated for each product configuration. Leachables and extractables from product-contact materials such as bioreactors and packaging components should be quantified, and limits should be established.

In closed bioreactor systems it can be difficult to observe or sample cells. Measurement of metabolic parameters can provide a surrogate method that is amenable to validation with which to evaluate the rate of proliferation and predict when to harvest the cell product. The relationship of such parameters to the viability, potency, and function of the cell product should be well defined. Postexpansion purification and enrichment of target cells by using methods such as those described above may be required.

DIFFERENTIATION

Some cell therapies require lineage or functional differentiation of the source cells. For example, hematopoietic stem cell expansion processes normally result in products that contain a mixture of multipotent stem cells, lineage-committed progenitor cells, and lineage-differentiated cells. The composition of these products can be manipulated by different combinations of growth factors and cytokines during the expansion process. The inverse is true for processes in which mature cells are *de-differentiated* to enable them to then be recommitted to a lineage pathway (for example, chondrocytes in cartilage repair). Specific examples of ex vivo manipulation are the production of antigen-specific T cells to target various specific disease indications or derivation of therapeutic cell types from embryonic stem cells. Before release for clinical use, the resulting differentiated target cells must be fully characterized. Assessing the potential for de-differentiation of multipotent cells that have undergone differentiation may be necessary to ensure the safety of the product. Where the cells have been expanded and subsequently differentiated, karyotype analysis or in vitro

transformation assays may be performed to demonstrate the cells are acceptable for clinical use.

EX VIVO GENETIC MANIPULATION

Genetic modification of cells *ex vivo* is a common processing procedure that is used to alter the pattern of gene expression in a defined population. The introduction of integrating or nonintegrating genetic materials (DNA, RNA, siRNA) is performed in order to induce the expression of new genes and products or to inhibit endogenous gene expression. *Ex vivo* genetic modification in autologous transplantation settings involves the manipulation of a harvested or expanded cell population from a patient and subsequent readministration of the cells to the donor. In a typical allogeneic transplant setting, a stable, genetically modified cell population that has been characterized and banked is administered to a broad patient population. In order to control graft-versus-host disease in allogeneic bone marrow transplants, selected donor T cells have been treated with lethal genes such as thymidine kinase that make the cells susceptible to gancyclovir treatment after transplant. Examples of autologous genetically modified cell therapy products include the transduction of tumor cells with cytokine or other immunomodulatory genes, lymphocytes transduced with receptors for tumor antigens, and the introduction into harvested lymphocytes of an antiviral ribozyme vector as a strategy to treat human immunodeficiency virus infection. Allogeneic cell therapy product examples include genetically modified and irradiated tumor cell lines used as tumor vaccines and encapsulated cells transfected with a gene to express a neurotrophic factor for localized therapeutic protein delivery in the central nervous system.

Ex vivo genetically modified cells are considered gene therapy. Issues associated with gene therapy products are addressed in detail in *Gene Therapy Products* (1047), especially the production of the vector or genetic material used to accomplish gene transfer, analytical testing strategies, patient safety, and monitoring. The manufacturing, cell processing, and process control methodologies addressed above are applicable in the procedures used for genetic manipulation. Frequently cell populations that are genetically modified are isolated and expanded or selected before the introduction of the genetic material. Specialized equipment and processes for introduction of genetic material exist and must be validated and monitored. Issues associated with cell banking and stability apply to cell lines used in allogeneic cell therapy products that are established and cryopreserved in MCBs and WCBs. Finally, issues associated with analysis and administration of the genetically modified cell population are discussed later in this chapter.

Formulation of Cell and Tissue-Based Products

Approaches for formulating cell and tissue-based products depend largely on the planned storage time for the cells before delivery to the patient. For some cell-based products, the time between completion of manufacturing and delivery to the intended recipient can be measured on the order of hours to days. Other cell-based products may be cryopreserved in order to extend their shelf life. A different approach for formulating cell and tissue-based products may involve the addition of a natural or synthetic scaffold that can facilitate handling, protecting the cells from immunological responses, and creating a specific shape that contributes to the therapeutic effect. Considerations for formulating each of these types of cell and tissue-based products are discussed below.

NONCRYOPRESERVED CELL AND TISSUE-BASED PRODUCTS

Products consisting of suspensions of cells for delivery to patients within hours after the completion of manufacturing

frequently are formulated in sterile, buffered solutions suitable for direct administration. For other noncryopreserved cell or tissue-based products extension of shelf life from hours to days may be possible by use of solutions that contain appropriate nutrients and antioxidants. In most cases these excipients are not intended for direct administration into patients. Consequently, the excipients may require removal before delivery to the patient (see *Clinical Site Preparation and Administration*). If an unapproved formulation buffer will be administered to patients, preclinical toxicology testing should be performed.

CRYOPRESERVED CELL AND TISSUE-BASED PRODUCTS

Most cell cryopreservation medium formulations are supplemented with 5% to 10% DMSO with or without hydroxyethyl starch (generally 6%) and a plasma protein such as 4% to 10% human serum albumin in a balanced salt solution. DMSO prevents dehydration by altering the increased concentration of nonpenetrating extracellular solutions during ice formation. The high molecular weight polymeric hydroxyethyl solution protects the cells from dehydration as water is incorporated into extracellular ice crystals. The use of protein often results in maximum recovery and viability of cells after thawing. Serum (5% to 90%) is sometimes used in place of specific proteins. Some cryopreservation formulations are completely free of protein.

The optimal concentration of cells for cryopreservation depends on the cell type and should be determined empirically, but it generally ranges from 10^6 to 10^7 cells per mL. The homogeneity and viability of the cell population being cryopreserved can also differ after thawing and should be carefully assessed. In situations where the final cell or tissue-based product is intended to be thawed and administered immediately, the presence of DMSO in the formulation buffer does subject the patient to an increased level of infusion-related toxicity, although this is related to the volume administered and the final concentration of the cryopreservative. Refer to section *Clinical Site Preparation and Administration* for additional considerations.

CELLS COMBINED WITH BIOCOMPATIBLE SCAFFOLDS

Many cell and tissue-therapy products are administered in combination with a biocompatible scaffold. For instance, wound healing or skin substitute products contain cells seeded on a scaffold. The biochemical and physical structure of the scaffold and the method for combining cells with the scaffold are specific to the product.

Cells can be loaded into a semipermeable membrane device for delivery. Usually the pore size of the membrane is large enough to allow the cell-secreted therapeutic factors to pass, but it is small enough to stop immunoglobulins and host cells from making contact with, destroying, or mounting an immune response to the therapeutic cells. The device can be a single hollow fiber or a semipermeable capsule with cells inside that secrete therapeutic compounds, or it can be part of a larger system of pumps and filters such as hollow-fiber modules with hepatocytes for the treatment of liver disease.

Cells can be seeded onto a three-dimensional scaffold and allowed to propagate and form a tissue-like structure. In the resulting product, the cells are oriented in a unique manner that is important for the intended use of the product (e.g., skin substitutes).

Cells can be encapsulated in a gel or cross-linkable polymer solution, and the resulting implantable structure can serve as a culture vessel, as a means to shield the cells from the host's immune system, or as a way to mold cells into a defined shape. Some of the polymers used include alginate, hyaluronic acid, collagen, chitin, or synthetic polymers. Encapsulated pancreatic β -islet cells have been implanted in patients to treat diabetes. To treat urinary incontinence,

chondrocytes have been mixed with alginate to form a structure upon injection.

Cells can be adhered to scaffolds of defined shape that are then implanted. Some examples include osteogenic precursor cells on scaffolds of demineralized cadaveric human bone, ceramic hydroxyapatite, ceramic hydroxyapatite-tricalcium phosphate, or biodegradable glass, which can be used in the repair of bone defects.

ANALYTICAL METHODS

General Considerations

The complexity and scope of cell-based therapies are reflected in the wide range of analytical methods that are used to establish in-process controls and final product release criteria. Quality specifications for cell and tissue products should be chosen to confirm the product's quality, safety, and potency. Selected tests should be product specific and should have appropriate acceptance criteria to ensure that the product exhibits consistent quality parameters within acceptable levels of biological variation, loss of activity, physicochemical changes, or degradation throughout the product's shelf life. The development and setting of specifications for cell and tissue products should follow the principles outlined in ICH Q6B *Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*.

Specifications are established on the basis of thorough characterization of the product during the development phase and an understanding of the process and its capability. Characterization should include measurements of the physicochemical properties, safety, purity, process and product-related impurities, potency, viability, sterility, and quantity. Manufacturers should develop specifications for each product developed from this information by application of appropriate statistical methods. The data should include lots used in preclinical and clinical studies and should also include assay and process validation data that can be correlated to stability, safety, and efficacy assessments.

In-process controls and specifications for the product should be anchored by use of an appropriate reference standard. An autologous product may rely on a reference standard generated from processing cells or tissue from a healthy donor or from a source that supplies cells and tissues to research institutions. The reference standard ensures that the process, as measured by the release assays, does not change significantly over time, and it verifies that a test produces acceptable results, i.e. system suitability requirements are met. The reference standard is made from a lot that is produced under controlled conditions and passes all in-process and final release testing. In addition, this reference standard is subjected to an additional level of characterization that includes tests not normally performed for product release. The reference standard need not be stored at the same dose, formulation, or temperature as the final product. However, the stability of this reference standard must be determined.

Alternatively, a working standard can be used. If so, in the test it should behave like the reference standard. Changing to a new reference standard should include many tests, all of which are run side by side with the existing reference standard. The impact of any change in the properties of the new reference standard should be carefully evaluated before it is adopted. One option for a reference standard for a cell product with a short shelf life or for an autologous or patient-specific application can be a bank of normal donor cells of the appropriate cell type. This cell bank can be used to ensure that the manufacturing process is capable of making a consistent product.

In-Process Controls

Manufacturing processes should have well-defined go-no go decision criteria that are established for key in-process manufacturing steps. In-process controls are the assays or tests that are performed to ensure that the in-process material is of sufficient quality and quantity to ensure manufacture of an acceptable final product. Examples of in-process controls include:

- Enumeration and viability
- Microbiological (sterility, endotoxin, mycoplasma)
- Expression of phenotypic or genotypic markers
- Verification of morphology against visual reference standards
- Production of a desired bioactive substance
- Determination of population doublings, passage number, age of culture
- Assays of potential process impurities
- Monitoring of culture system parameters (% CO₂, % relative humidity, pH, glucose, lactate, etc.)
- Functional tests such as colony forming units (CFU) and expression of cell-specific proteins.

A primary reason for establishing in-process control tests is to ensure that the correct product with specified quality and yield is obtained. A secondary reason for performing in-process tests is to gather process and product characterization data that can be useful in assessing the impact of process changes or excursions. Intermediate in-process material that fails to satisfy in-process control criteria should not be used for further manufacturing. This material can be reprocessed if there are procedures in place for such activities. The reprocessed material must satisfy the original in-process specifications, and the effect of reprocessing on other quality attributes such as stability must be defined before the material can undergo further manufacturing. If several sublots (e.g., cells harvested from different culture vessels) will be pooled for further processing, sublots that fail to satisfy specified criteria should not be included in the pool even if the pool containing these failed sublots would pass the in-process assay criteria.

During clinical process development, assays for product quality and yield should be performed after most processing steps to determine which steps are critical and which assays are most sensitive to deviations in the process. Statistical process controls and critical parameters should be used to establish limits for process validations and manufacturing investigations. Statistical sampling tools should be used to ensure a valid sample size. In-process controls should be performed for fully validated processes to ensure that the process continues to be under control. The results of these assays should be trended, and actions should be taken to correct problems as they arise.

Final Product Release Specifications

Cell-based therapies regulated as biological products must comply with applicable sections of 21 CFR 211 and 21 CFR 610 to ensure their identity, purity, potency, microbiological safety, and other essential attributes, such as viability, are met.

Because terminal sterilization is not possible for a living cell-based product, essentially all cell-based products are required to meet acceptance criteria for product tests such as sterility, mycoplasma, and endotoxin—typically, negative or no growth demonstrates sterility and the absence of mycoplasma, and products must demonstrate <5 endotoxin units (EU) per kilogram of patient body weight. In the case of intrathecal injection, the specified endotoxin limit is more stringent ≤0.2 EU per kilogram of patient body weight. Adventitious virus testing is rarely performed on the final cell-based therapy product because the source cells or cell banks and ancillary materials of biological origin have been

screened and tested for viral agents of concern before manufacturing.

For almost all other final product release criteria, such as those for identity, purity, and potency, the analytical procedures with methods and acceptance criteria are specific to the individual cell-based product. Table 2 provides an overview of the expected final product release tests for cell-based therapies and lists examples of approaches that are used to satisfy the testing requirements.

STERILITY

Cell-based products are required to comply with final product release testing requirements, including sterility. Sterility testing is also frequently performed in-process to establish microbial purity for cells that require extended culturing. Suitable sterility tests include the test described in 21 CFR 610.12 and USP general test chapter *Sterility Tests* <71>. These culture-based test methods require 14 days and thus are suitable only for cell-based therapy products that have extended shelf lives (e.g., following cryopreservation). Many cell-based therapies have short shelf lives and must be delivered to patients before the 14-day test results are available. In such situations, FDA has identified an approach that will allow the administration of the cell-based product to patients in this setting [see *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)*]:

- In-process sterility testing on a sample taken 48 to 72 hours before final harvest or after the last refeeding of the cultures
- A rapid microbial detection test such as a Gram stain or other procedure on the final formulated product
- Sterility testing compliant with 21 CFR 610.12 on the final formulated product

Under this alternative approach, the release criteria for sterility would be based on a negative result of the Gram stain and a no-growth result from the 48- to 72-hour in-process sterility test. In the event that the 14-day sterility

test is determined to be positive after the product is administered to the subject, the manufacturer is required to report the sterility failure, results of investigation of the cause, and any corrective actions as an amendment to the IND within 30 calendar days after initial receipt of the positive culture test result.

Because of concerns regarding the sensitivity of a Gram stain and the inability to obtain full sterility results for 14 days after administration to the patient, there is widespread interest in the use of rapid microbiological methods as an alternative to the 14-day culture method. This is discussed under *Alternative Test Methodologies*.

MYCOPLASMA

Mycoplasma and ureaplasma are the smallest free-living microorganisms. Mycoplasma lacks a rigid cell wall and ranges in size from 0.2 to 0.3 µm. Mycoplasma can be observed as round or filamentous in cell culture using dark-field or phase-contrast microscopy. On solid agar, colonies of mycoplasma can range in diameter from approximately 15 to 300 µm, and the larger colonies are distinguished by a typical "fried egg" appearance.

Mycoplasma is ubiquitous and can be isolated from practically all mammals. Historically it has been one of the main problems in the contamination of tissue cultures. Mycoplasma tends to be fastidious and requires preformed nucleic acids supplied by media components. These components may be readily available in the cell culturing materials that are employed during manufacturing. Mycoplasma can arise from bovine or other animal-derived culture components, cell or tissue materials from asymptomatic patients, or possibly from operators who shed it during manufacturing.

Testing for mycoplasma is recommended for all raw materials derived from a human or animal source and is required as a lot-release assay for cell-based products. FDA has published a document (*Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals*) describing in detail the accepted methods for the cultivation and isolation

Table 2. Overview of Final Product Release Testing

Release Test	Examples	Criteria
Sterility	USP <71>	Negative
Mycoplasma	Direct and indirect culture method (FDA Points to Consider)	Negative; not detected
Endotoxin	USP <85>	<5 EU/kg (<0.2 EU/kg intrathecal)
Identity	<ul style="list-style-type: none"> • Surface marker determination • Isoenzyme analysis • Genetic fingerprint • Morphology • Bioassay • Biochemical marker 	Product specific
Purity	<ul style="list-style-type: none"> • Percentage of viable cells • Percentage of cells expressing specific marker(s) • Limits on undesired cell types • Limits on process contaminants (e.g., serum) 	Product specific
Potency	<ul style="list-style-type: none"> • Viable cell number • Colony-formation assay • Change in expression of specific genes • Secretion of desired macromolecule • Induction of secondary effect (e.g., human leukocyte antigen (HLA)) • Evidence of metabolic activity • Evidence of cell function 	Product specific
Dose	<ul style="list-style-type: none"> • Viable cell number • Enumeration of specific cell population • Total DNA • Total protein 	Product specific
Others	<ul style="list-style-type: none"> • Appearance • Morphology • Size 	Product specific

tion of mycoplasma. Methods for mycoplasma detection are also described in USP general test chapter *Mycoplasma Tests* (63). Because the classical assay takes one month of testing to complete, alternative methods are being developed and validated for the rapid detection of mycoplasma. This is discussed under *Alternative Test Methodologies*.

ENDOTOXINS

Endotoxins exert a number of biological effects on the mammalian cell membrane and can affect secretion and cytokine production, can induce fever in recipients, or can serve as powerful mitogens. Because of the possibility of wide-ranging biologic effects of endotoxins on cell and tissue culture, raw materials and components used for the manufacture of cell-based products must be assessed for the presence of endotoxin as part of the raw materials qualification process. Control of endotoxin in the manufacture of cell therapy products is an essential element of any quality control program.

The presence of endotoxins in products administered to patients is a significant safety concern. USP general test chapter *Bacterial Endotoxins Test* (85) describes a number of different methods for measurement of endotoxins, all based on the *Limulus* amoebocyte lysate assay. This assay can be validated for a wide range of biopharmaceutical products. An important feature of the assay with respect to cell therapy products is the ability to conduct the assay before release of products that have short shelf lives.

IDENTITY

Lot-release testing for cell-based products must include an identity test. This test serves unequivocally to identify the product. The complexity of the identity test depends on the nature of the specific product and the array of products being manufactured. For example, more extensive and rigorous testing may be performed for an autologous cell therapy product at a manufacturing facility where multiple patient products are manufactured by comparison with an allogeneic cell therapy that is the only product manufactured in a facility.

Identity tests for cell-based products must be relevant to the cell type and manipulations applied during processing. Differential surface markers are frequently used to ascertain product identity. Flow-cytometric immunoassay methods are the most common means of detecting and quantifying these markers. Identification and quantitation of particular cell subsets is accomplished by multiparameter analysis, usually of size and granularity and of one or more identity markers. Other examples of identity tests include isoenzyme analyses to confirm species of origin, which would be desirable if the product consists of xenogeneic cells. Cell morphology may be used to distinguish specific cell types. There is also an increasing trend to use genetic fingerprint technologies such as short tandem repeats to establish the identity of cell lines (e.g., human embryonic stem cells used to derive therapeutic cell types).

PURITY

Purity methods specifically quantify the intended active product components. Impurities are either product- or process-related residual contaminants that can be detected in the final product. The requirement to test for a particular impurity for product lot release will depend on the following: (1) the demonstrated capability of the manufacture and purification process to remove or inactivate the impurity by process validation and (2) the toxicity potential or functional product impact associated with the impurity.

Examples of process-related impurities associated with cell therapy products include residual production-medium com-

ponents (e.g., serum, antibiotics, or exogenous cytokines), ancillary materials used in downstream processing (e.g., nucleases or proteases), and leachables (e.g., plasticizers from tubing or culture plastic). Impurities may be bioactive (e.g., cytokines or hormones) or immunogenic (e.g., aggregates, degradation products, or animal-derived proteins). Impurities may have other deleterious effects, depending on the dose of the product.

Product-related impurities are specific to each product type. Examples include cell debris, presence of undifferentiated cells in a cell-based product that should contain specific types of differentiated therapeutic cells; unacceptable levels of nonviable cells; replication-competent cells in a cell product that should contain mitotically inactivated cells; or changes in the composition of functional cells following cryopreservation and thaw. Analytical methodologies to assess purity require quantitation or analytical separation of the intended product from its impurities. Emphasis should be placed on demonstrating the consistency of the product-impurity profile. It may be possible to validate the manufacturing process to the extent that specific lot-release testing for impurities can be limited.

Testing for impurities is often extensive during product characterization and process validation when the consistency of the manufacturing and purification process is being demonstrated. Testing for impurities as part of lot-release testing should reflect the safety risks associated with the impurity and the ability of the process to consistently remove that impurity.

POTENCY

Potency is defined under 21 CFR 600.3(s) as "the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result." Together with dose, potency defines the biological activity of each lot (see *Dose-Defining Assays*, below). The relationship between product potency measurements during development and manufacturing to clinical safety and efficacy is key to their use in batch release. Potency may be assessed by in vitro or in vivo bioassays or a combination of the two. It is not uncommon for these assays to have coefficients of variation between 30% and 50%. These assays require a well-defined, representative reference material that can be used as a positive control for the assay. The positive control serves to qualify the performance of an individual assay. Potency assay development should focus on characterizing and controlling variability. High-precision assays are effective tools in monitoring product quality. Information about potency-assay variability should be incorporated in the stability study design and the proposed statistical approach to assignment of expiration date (see *Stability*, below).

The types of assays that can be used to establish the potency of a cell-based product vary widely and depend on its unique characteristics and its shelf life. For some cell-based products such as hematopoietic progenitor cells, assays for product potency have been correlated with clinical efficacy. In this case, a traditional colony-forming assay that quantifies committed progenitor cells such as colony-forming unit-granulocyte-macrophage (CFU-GM) has been correlated with clinical engraftment outcomes in some studies. For other cell-based products, in vivo animal models of disease have been used to establish product potency. If the cell-based product releases a bioactive macromolecule, a potency assay could be based on units of activity released. For example, the production of insulin in response to changes in glucose levels could be the basis of a potency assay for cells that are intended to treat diabetes.

Patient-specific products such as autologous immunotherapies present a challenge in demonstrating therapeutic activity in an in vitro or in vivo assay system. Novel approaches to measuring potency, such as the correlation of

clinical outcome to other characterization tests such as identity tests, may be appropriate and should be discussed with regulatory authorities early in development. For example, the ability to determine specific cell-surface identity markers by employing flow cytometry techniques or vital stains may be an acceptable measurement of potency if properly validated and correlated with clinical outcome. FDA has issued guidance that discusses the possibility of using a matrix of biological and nonbiological assays, including both qualitative and quantitative assays, to establish product potency. Information in this guidance is particularly relevant for cell-based products that have a short shelf life or complex mechanisms of action or multiple biological activities (see *Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products*).

A validated potency assay is typically required before regulatory approval. During investigational clinical studies, regulators typically require that a well-defined assay or assays intended to establish product potency should be in place before the initiation of pivotal clinical trials. Early implementation of one or more candidate assays intended to establish product potency is strongly encouraged. Data from these candidate potency or functional assays can be particularly important when assessing proposed manufacturing changes, during technology transfer, and in determining product stability.

DOSE-DEFINING ASSAYS

An assay that precisely measures the amount of the product is referred to as a dose-defining assay, and it is selected on the basis of its accuracy and precision. An assay that measures therapeutic activity of the product is referred to as a potency assay and it is designed to measure product function. This type of assay is different from a dose-defining assay. The design of the assay depends on the type of product. In the case of drugs, assays that measure the amount of active ingredient (dose) are referred to as strength assays. For these medicines, product dose can be defined as the concentration or amount of the final product administered to the patient, and it is typically measured as product mass. For cell-based products, attributes such as viable number of therapeutic cells are often used to define the dose of the product.

Cell therapy products may be dosed on the basis of enumeration of one or more cell populations. For products in the form of a homogeneous, single-cell suspension, viable cell number is the most frequently used assay. Such assays may include enumeration of all cells, total nucleated cells, or another subset of cells. Viability assays are usually based on a cell's ability to exclude a supravital dye, such as trypan blue. Results are expressed as the number of cells that exclude the dye and are therefore considered viable. Fluorescent compounds that bind to nuclear proteins and are excluded by viable cells may be incorporated into flow-cytometric methods for simultaneous determination of viability and cell-identity markers.

Cell counting may be performed rapidly by manual or automated methods. Manual cell counting by visual enumeration of cells in a hemacytometer chamber is a readily available technique with acceptable accuracy but a lower degree of precision than most automated methods. Typical instruments for automated cell counting provide reproducible enumeration of nonnucleated cells (e.g., erythrocytes and platelets) and nucleated cells and differential counting of the nucleated cells into mononuclear and polymorphonuclear leukocyte populations. Further discrimination of specific cell populations usually requires cell-surface phenotype analysis by flow cytometric or other methods (see *Identity* above). The proportion of a specific subpopulation of cells may be determined by FACS analysis or by flow cytometry. An example of a cell enumeration assay is the enumeration of CD34-positive (CD34+) hematopoietic progenitor cells.

For products that contain cells in a nonhomogeneous suspension, such as cells that are combined with a biomaterial (e.g., a scaffold), alternative measures have been used for cell enumeration, including total area of a cell sheet, wet weight, total protein, and total DNA. If such measures are used to determine product dose, then supplemental tests should be performed to establish relevance.

Considerations for Release Testing of Cell-Biomaterial Constructs

For some cell-based products such as cells combined with biomaterials to form combination products, it may not be feasible to directly test the cell-biomaterial construct. This is frequently the case when autologous cells are involved and the cell-biomaterial construct consists of a single unit and sampling of the construct is not feasible. In such cases, the individual components are tested before they are combined, and the final construct is not subjected to direct testing. Indirect measures such as sampling of the culture media can be employed to address regulatory requirements. The quality and stability of the formulated cell-biomaterial construct and relevance of indirect measures must be established by validation studies during product development.

Sampling Issues

As required by GMPs, product samples must be retained after release testing is completed. If rapid-release strategies are employed, manufacturers may need to retain additional samples so that product quality can be reassessed by alternative or traditional test methodologies if necessary.

Sampling for lot-release testing should be based on the potential distribution for the parameter tested. See *Stability Protocol Development* (below) for additional considerations. Samples from each lot should be retained in case there is a safety or quality issue with the lot. Even if the product has a very short shelf life, these retained samples can be used to detect impurities and other substances. The need for proper design of the sampling plan deserves special consideration. In such cases, process validation will assist in determining the appropriate statistically based sampling design.

Alternative Test Methodologies

As described under *Final Product Release Specifications* (above), cell-based therapies must undergo testing for sterility, mycoplasma, and endotoxin. Additional acceptance criteria for tests relating to identity, purity, potency, dose, and other relevant characteristics must be met before clinical use. With the exception of sterility, mycoplasma, and endotoxin, most of the test procedures and their underlying methods used to ensure that the final product meets release acceptance criteria are unique to the product and can be adapted to meet the specific characteristics and applications of the cell-based product. In general, test methods should be developed based on the best available science and should be suitable for use in a GMP manufacturing environment. The assays should be robust, reliable, and capable of being validated and should provide results before release for clinical use. *Validation of Compendial Procedures* (1225) provides basic considerations for methods validation.

For some cell-based therapies, the sample size and volume of material required for testing or the length of time necessary to obtain test results can consume significant amounts of the final product, or the time required for obtaining results may exceed the product's shelf life—or both. This creates problems with the available supply of product to treat patients and in other situations precludes the possibility of obtaining results before administration to patients. This is a particular problem for the compendial sterility test as well as the FDA-recommended broth-agar culture method for mycoplasma. Consequently, both industry and

regulatory authorities have shown considerable interest in facilitating the development of alternative test methods for both sterility and mycoplasma.

FDA regulations for biological products specifically address the use of equivalent methods provided they ensure that the safety, purity, potency, and effectiveness of the biological product is equal to or greater than the assurances provided by the specified method (21 CFR 610.9). Some of the available alternative test methods for sterility and mycoplasma are describe below.

The range of available technologies is broad and continues to be developed by assay designers for use in the cell therapy industry. Attributes that should be included in any review of proposed technology include accuracy for the intended purpose, speed in productivity, cost, acceptability by the scientific community and regulatory agencies, simplicity of operation, training requirements and reagents, the reputation of the vendor, technical services provided by the vendor, and, finally, utility and space requirements.

Validation of these test methods and demonstration of equivalence as described in 21 CFR 610.9 are required at the time of biologics license application (BLA) or a premarket approval (PMA) submission.

STERILITY

Detection platforms for alternative microbiological methods have been generally based on growth, viability, artifacts, or nucleic acids. Growth-based technologies use either biochemical or physiological measures that reflect the growth of microorganisms. Test samples are transferred to traditional or enhanced media formulations that encourage microbial proliferation, and microbial growth is detected chemically or spectrophotometrically. The primary advantage of these systems is the automated nature of the test results and recovery of microorganisms for failure investigations and other microbial characterization methodologies. FDA has published guidance for validation of growth-based rapid microbiological methods (*Guidance for Industry: Validation of Growth-Based Rapid Microbiological Methods for Sterility Testing of Cellular and Gene Therapy Products*, CBER, 2008). Principles of validation of alternative microbiological methods are also described in USP chapter *Validation of Alternative Microbiological Methods* (1223).

Viability-based technologies do not require cells to grow. These technologies are based on detecting the presence of individual living contaminants by vital dyes, stains, or cell-surface markers. Cells labeled by a specific fluorochrome metabolic substrate are collected on a membrane for detection.

Artifact-based technologies analyze cellular components or molecular probes that are designed specifically for a particular microbial species. For example, individual species can be characterized by unique patterns of fatty acid composition after samples of whole cells have been saponified to induce the formation of methyl esters. Other technologies use time-of-flight mass spectrometry.

Nucleic acid technologies (NAT) are based on polymerase chain reaction (PCR) DNA amplification, 16s or 23s rRNA typing, and gene sequencing. Some technologies identify microorganisms by sequencing a portion of the chromosome of an unknown organism and comparing the sequence of 16s rRNA to a database. This technology is capable of identifying fungi, mycoplasma, and bacteria, including slow growers and nonfermenters. For more details on PCR-based methods, see the USP chapter *Nucleic Acid-Based Techniques—Amplification* (1127).

MYCOPLASMA

Compendial testing methodologies for mycoplasma are growth based in agar and broth cultures and require at least 28 days to monitor appropriately the presence of myco-

plasma contamination. Because of this limitation, a number of rapid mycoplasma testing technologies have been developed based on nucleic acid amplification techniques such as PCR, as well as nonamplified nucleic acid hybridization assays, ELISA, and enzyme-based assays.

QUALITY SYSTEMS

Quality systems weave together the various aspects of manufacturing. Quality control (QC) and quality assurance (QA) programs should exert control over the manufacturing facilities, the manufacturing process, the validation efforts, and all testing of the raw materials, in-process material, bulk product, and final formulated product. Training and certification programs are central to maintaining a technically competent manufacturing staff. A documentation program should be implemented to support all manufacturing, training, validation, and quality operations. Changes to processes and procedures should follow a formal program based on well-established change control principles.

When allogeneic human cells or tissues are used as the source material for the manufacturing of cell or tissue-based product, cell or tissue donors should undergo appropriate screening and testing (see *Donor Eligibility*, above). In all cases the source human cells and tissue must be handled in accordance with GTP as described in 21 CFR 1271.

In addition to GTPs, cGMP as outlined by FDA in 21 CFR 210, 211, 600s (especially 21 CFR 610), and 820 apply to the manufacturing of cell and tissue-based products that are subject to premarket approval. The manufacturing facility, equipment and process, raw materials, quality systems, and trained personnel are some of the key elements of cGMP. GMPs apply throughout the clinical development to both the manufacturing process and facility. The extent of control increases as clinical development progresses, and full cGMP compliance is expected by the time Phase III clinical trial(s) begin.

Data obtained from in-process and final product release testing should be monitored. Results that are out of specification (OOS), or even those that are out of trend, must be investigated before disposition of the material. FDA's *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production* (October 2006) provides a systematic approach for conducting an investigation. An assay result can be rejected if it can be confirmed that an error, such as an analyst error, calculation error, or equipment failure, has taken place. If the investigation concludes that results of tests of the product do not fall with specified acceptance criteria, the lot should be rejected. In some situations, especially with autologous or allogeneic patient-specific product, a product that does not meet all specifications or that has only incomplete test results may have to be administered to a patient as a life-saving measure. However, procedures must be in place to govern the communication of the OOS results to the physician or to the person responsible for making the decision to use the product and to provide instruction for any follow-up testing, patient monitoring, and communication of those results to all parties, including regulatory authorities.

As discussed earlier an effective risk-management approach at the earliest stages of product development can ensure the highest quality of a cell-based product by providing a proactive measure to identify and mitigate potential quality issues. The probability for failure in cell therapy products can arise from a number of sources including personnel errors, aseptic processing failures, equipment failures, facilities and utilities failures, cleaning, disinfection, and component and raw material failures. A proactive understanding of risk can lead to improved decision making if a quality problem arises. Effective risk management can facilitate better and more informed decisions and may provide regulators with greater assurance of a developer's ability to deal with potential risks. Such assurance can affect the extent and level of direct regulatory oversight. Quality risk

management can be integrated into key parts of the quality system such as change management, Corrective and Preventive Action (CAPA), GMPs, validation, etc., and can be used to establish meaningful specifications and Critical Process Parameters (CPPs) to ensure that the quality attributes are met.

Risk analysis is qualitative in nature. It can be achieved by using experience and process knowledge to define risk categories that can form the basis of a system of risk assessment and mitigation after the identification of manufacturing errors. For example, it is common practice to develop nonconformance or deviation risk assessment categories that can be incorporated into a nonconformance or failure investigation procedure. The categories are particularly useful if the risk assessment must be expedited to facilitate a CAPA. As an example, Risk Levels 1 through 4 are defined below and can be adopted as one means of conducting a preliminary risk assessment:

Risk Level 1: Technicality—Poses no risk to the patient and does not impact the safety and effectiveness of the product. Example: A missing signature on a batch record.

Risk Level 2: Alert—May pose a safety risk to the patient or may have a potential impact on the safety and efficacy of the product. Compliance must be re-established with appropriate justification to proceed after submission to QA for review and approval. Example: Digestion time for biopsy processing falls outside a defined range.

Risk Level 3: Do not ship/reject lot—May pose a safety risk to the patient or impair the efficacy of the product even after corrective action. Shipment is not permitted. Example: Cultures fail to demonstrate adequate cell growth.

Risk Level 4: Post-distribution Event—May pose a safety risk to the patient or a potential impact on the safety, potency, or purity of the product. The safety signal is identified after product distribution. Example: Failed sterility test occurred after distribution of product.

FACILITY DESIGN AND OPERATION CONSIDERATIONS

Manufacturing facilities for cell and tissue therapy products must be carefully designed to maintain GMP aseptic processing operations while also accommodating any unique aspects of the product. Incoming cells or tissue can have bioburden and other contaminants and may need to be received and processed in a segregated area under quarantine to avoid compromising the main facility. Also, tissue processing to obtain cells of interest may require specialized equipment and processes that should be considered during the facility design and subsequent operations. For manufacture of combination products involving biocompatible scaffolds, the facility may need to be capable of handling operations that involve chemical processing, handling, and disposal. This may place constraints on the design of the facility, especially air-handling systems in clean room environments.

Although the primary emphasis in manufacturing a cell or tissue-based product is protection of the product from inadvertent contamination, risk to the manufacturing operator must be assessed and minimized by appropriate training for handling blood-borne pathogens and the use of equipment/protective clothing. Protection of the operator and aseptic processing are complementary and include the use of certified biological safety cabinets and aseptic protective clothing consisting of gowns, gloves, sleeves, surgical masks, eye protection, and head coverings. Human tissue should be obtained under environmental conditions and controls that provide a high degree of assurance for aseptic recovery.

The degree of control required for cell and tissue processing operations depends on a number of factors, including the complexity of the aseptic manufacturing process, the primary site of manufacturing, and the final product shelf

life. Manufacturing processes that involve open manipulation of cells, even in a biological safety cabinet, are at greater risk of contamination than are processes done in closed bioreactors or bag systems that use sterile connections and tube-sealing devices. Typically, ISO 7 (class 10,000) clean rooms and ISO 5 (class 100) biological safety cabinets are essential components for cell therapy manufacturing processes, especially those that involve open manipulations.

The controlled environment of a carefully designed, constructed, validated, and maintained clean room can minimize the risks of environmental contamination during aseptic processing and decrease the possibility of cross-contamination of patient-specific products. The differential pressures between classified manufacturing should comply with the September 2004 guidance document, *Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice*. The facility and processing areas should be monitored for air quality in a manner that provides a high level of process asepsis. For guidance in this area, see *Microbiological Control and Monitoring of Aseptic Processing Environments* (1116).

Facility cleaning, component and product segregation, and sanitization procedures must be in place to avoid microbial contamination and cross-contamination between lots produced in the facility.

Cell or tissue products based on autologous cells add another level of complexity to the manufacturing facility design and operation. For autologous products one product lot is made for each person, and complete segregation during manufacturing is needed. Unlike facility designs for allogeneic products, which are based on volume scale-up to achieve maximum manufacturing efficiency, facilities for autologous products require unit scale-up (scale out), which must be considered in the design and operation of the facility. Automation can be used effectively to manage repetitive manual manipulation of cells. The initial scale of operation may not justify an upfront capital investment in automation but should be considered as manufacturing operations increase in size.

Product segregation in the facility is another key consideration for design and operation. Labeling and QA oversight are traditionally used for tracking and segregation. Techniques such as bar-coding and radio-frequency (RF) tags can be used for product tracking and segregation. For guidance in this area see 21 CFR 211.42, 211.113, 1271, and FDA's September, 2004 *Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing—Current Good Manufacturing Practice*.

Manufacturing equipment should be robust, should provide consistent product, and should allow periodic calibration and preventive maintenance. Qualification is necessary for equipment from which critical process parameters or measurements are derived. In most cases, this includes the software that controls the equipment or system's operation. Critical equipment such as incubators and freezers need to be fitted with alarm systems that can remotely signal failure. Additionally, critical equipment should be connected to an emergency back-up generator, and the generator should be tested periodically to verify its operational state.

CONSIDERATIONS FOR VALIDATION AND QUALIFICATION

The principles of validation recommended by ICH and FDA guidance documents and USP chapters <1225> and *Validation of Microbial Recovery from Pharmacopeial Articles* (<1227>) apply to cell or tissue-based products. Biological variation in the source cells and tissues used to create most cell or tissue-based therapies may affect validation efforts.

Validation activities should include risk assessments, training and personnel qualification, equipment and facilities qualifications, analytical methods validation, aseptic processing, the manufacturing process, and cleaning.

Process validation should take into account safety, consistency (process and step yields, clearance of impurities), robustness (operator-to-operator, day-to-day), and final product quality (identity, purity, potency). Analytical methods used to assess the process should be validated or well qualified.

Process validation for patient-specific products such as autologous cell and tissue products presents some unique issues. First, the starting materials for patient-specific products typically arise from patient or matched donor materials such as biopsy material, apheresis products, scrap tissues from surgical procedures, and cadaver organs that do not qualify for transplantation. The process may require manufacturers to accept a range in the quality and quantity of starting material and yet still produce a final product that satisfies release testing. Second, manual processing of cells and tissues exhibit a degree of inherent variability. Processing steps should be developed that successfully and consistently result in appropriate process components and final product, even if the process relies on nonstandard or variable tissue materials. Process validation should take this variability into consideration and should ensure that critical manufacturing and testing endpoints consistently meet specifications.

Aseptic process validations should be performed using microbiological media to show that the manufacturing staff can execute the procedures and produce a product free of microbial contamination. Procedures intended to maintain segregation during manufacturing should be challenged to verify that there is minimal opportunity for cross-contamination or mix ups among different patient product lots.

Depending on the variability in the source cells or tissues and the complexity of the manufacturing process, it may be necessary to manufacture more than three qualification lots to verify the consistency and the robustness of the manufacturing process. Not every manufacturing effort will be successful for autologous and patient-specific therapies. However, the success rate should be established and tracked to enable manufacturers to discover any decrease in that rate and to take actions to correct the problem. Well-characterized banked primary cells may be used in the validation of the process if the donors have a range of profiles expected for the patient population. Trending of a number of statistically acceptable product administrations can also be appropriate.

Equipment and facility cleaning validations should be performed to demonstrate the efficacy of cleaning agents on standard microbial and fungal contaminants as well as environmental contaminants isolated from the manufacturing facility. Measurement of residual cleaning agents should be addressed in equipment cleaning validations. Equipment, facilities, and electronic monitoring and control systems such as building monitoring and inventory control systems should be validated.

Analytical and manufacturing equipment and methods should be validated following the principles described in (1225) in addition to guidance documents issued by ICH (see Q2 R1). Training plans should be established, and personnel qualifications should be performed. Tissue transport and product shipping validations should be performed.

CLINICAL SITE PREPARATION AND ADMINISTRATION

General Considerations

Before administration of some cell or tissue-based products, one or more product modifications or preparative steps may be required. These modifications or steps are frequently performed close to the time of administration, and, therefore, they are not under the control of the original manufacturer. The nature of these modifications is largely dictated by characteristics of the product.

Preparative steps may include thawing, washing, or filtration to remove unwanted cells or substances accumulated during storage, transfer to an infusible solution, or formulation with a vehicle or structural material such as a scaffold. In addition, patient considerations, such as the need to dose or modify the product according to the patient's anatomical structure, weight, or blood volume may influence these steps.

At the clinical site, additional procedures and process controls must be established for all product storage intervals, transport steps, and modifications, starting with a clear definition of critical control points. Operational requirements include designation of a physical space suitable for aseptic handling, such as an ISO 5 (class 100) biological safety cabinet, trained personnel, detailed standard operating procedures, and quality oversight. The unique and irreplaceable nature of many cell or tissue-based products heightens the need for well-established procedures for clinical site preparation and administration.

Product Manipulations

Before administration of medical products that contain cells, on-site preparation may involve one or more manipulations. Typical manipulations include the following:

Change in Final Container—The manufactured product may have been stored or transported in one container and may require transfer to a different container for administration.

Change in Physical State or Temperature—A product may require thawing or warming.

Change in Solution or Suspension—A product may have to be dissolved, diluted, or suspended in a liquid.

Combination with a Biomaterial—Therapeutic cells may require combination with a scaffold material such as decellularized extracellular matrix sheets, gels, plugs, capsules, sponges, particles, or granules. In other cases, cells can be added to an existing medical device such as a hollow-fiber filtration unit before use.

Admixture or Compounding—For some cell products, mixing or compounding at the clinical site may be necessary.

Filtration or Washing—The presence of unwanted materials in the manufactured product, such as particulates, cellular debris, metabolites, or compounds remaining from previous manipulations, may require washing or filtration steps.

Sampling—Sampling of the final product before administration may be required to test the final formulation.

Clinical Site Facility Considerations

Facility requirements for performing on-site preparative steps or administration of cell therapy products depend on the products and the manipulations required. The most important determinant of facility features is the level of risk for microbial contamination associated with each step. See *Pharmaceutical Compounding—Sterile Preparations* (797) for guidance that relates the type of manipulation and levels of environmental control needed to ensure aseptic handling.

Thawing Cell-Based Products

Thawing is performed rapidly. If a small number of cells will be reinfused or transplanted, DMSO does not need to be removed from the suspension because most cell preparations can be concentrated adequately to keep the DMSO concentration within tolerable limits. DMSO use has two effects on cells after thawing: Cells may clump if damaged, and DMSO reduces cell viability in minutes. If the DMSO must be removed or cells must be concentrated for administration, the thawed cell suspension is generally serially diluted (to avoid osmotic shock) and resuspended in a pro-

tein-containing medium. Cell viability and potency may be monitored after thawing, but the information is frequently intended only to gather information rather than as a specification that must be met for clinical use of the cellular product.

Some cell therapy products require that the product be shipped fresh (i.e., not frozen). In certain situations cellular components are stored frozen but are thawed and applied to scaffolds at the manufacturing site just before shipment at ambient or refrigerator temperatures. Combination products composed of cells on a scaffold may require shipping at higher than refrigerated temperatures (i.e., room temperature) to avoid dislodging cells from the scaffold. Because the fresh product is metabolically active, the shipping container must be designed and validated to maintain the metabolic activity of the product in addition to the standard shipping validation testing. Metabolism can be slowed down by lowering the shipping temperature, but this requires reliable temperature control during shipment. Because shipping containers depend somewhat on the outside temperature, the shipping container must be validated to maintain strict temperature control in all weather conditions.

Additional Release Testing of Clinical Site-Manipulated Cell Products

Cell therapy products that undergo preparative steps or manipulations at clinical sites must be subjected to appropriate checks or tests to ensure that all quality specifications are met before release for patient administration. The nature and extent of manipulations determines whether additional release requirements or critical specifications must be added to those required immediately after initial manufacture.

Prerelease steps usually include the following:

- Physical inspection of the product, including product appearance (color, turbidity, particulates, or foreign matter), container integrity, temperature, and accuracy and convenience of labeling
- Review of process records and/or certificate of analysis
- For patient-specific products, verification of product labeling and records related to identity of the intended recipient

High-risk products (defined in <797>) should undergo additional testing. For all high-risk products, assess the need for and, as appropriate, perform additional quality assays for the identity, potency, and purity of the active ingredients. For high-risk products in Category II, perform sterility and endotoxin testing.

Administration to Patients

Depending on the specific cell or tissue therapy application, patient-care staff may be required to take certain steps to prepare the patient. These steps help ensure that the product will provide the intended therapeutic outcome and help minimize the risk of adverse effects.

Determination of patient suitability for the therapy, including histocompatibility evaluation, typically occurs before the product is prepared. A patient's clinical status can change after tissue collection (because of fever, infection, recurrence or spread of tumors, or organ dysfunction), so the patient's general condition and suitability for therapy should be reviewed before product administration. This evaluation may include a patient history, physical examination, and laboratory studies such as blood counts and chemistries. In addition, relevant baseline physical or functional measurements, laboratory tests, or imaging studies may be obtained.

Depending on the route of administration, the patient may require preparation before treatment. For cellular therapies that require intravenous administration, patients with impaired peripheral circulation may require placement of a central venous catheter. When cells or tissues combined

with structural materials are implanted into the patient, the site requires preparation. This may involve establishing surgical access to the site, removing degenerated or damaged tissue, trimming adjacent tissue to accommodate the implant, and excising tissue from a second site for anchorage or support for the implant. For instance, in the case of cell products for wound healing, the site for grafting must be free from infection and must have a well-prepared wound bed. For cells to repair cartilage defects, the site of damage needs to be prepared. Before direct therapeutic administration into an organ system (e.g., the bronchioalveolar system) or vascular network (e.g., coronary arteries), the patient may require surgical, endoscopic, or radiographically directed catheter access.

In all cases, adequate anesthesia and premedication must be carefully evaluated. For example, if DMSO will remain in a thawed, cryopreserved cellular product, the patient may be given an antihistamine before administration. Pre-administration evaluation must also include assessment of concurrent therapies that may interact with the cell or tissue-therapy product to modify its effects. Some therapies may be adjunctive to the cell or tissue therapy, such as cytokines that promote proliferation or differentiation of the infused or implanted tissue. Other commonly used drugs such as antibiotics, antineoplastics, anticoagulants, and anti-inflammatory agents must be evaluated for possible effects.

DELIVERY OF CELL-BASED THERAPY TO PATIENTS

Some cell or tissue therapy products are patient specific because they are manufactured from a selected autologous or allogeneic tissue source, cells, or tissue. Certain patient-specific products have a defined potential for benefit or adverse immunoreactivity. Systems must be in place to prevent administration of such a product to the wrong patient. Recommended systems include procedures similar to those used for administration of human blood products, and at least two people should verify the identity of the patient and patient-specific product immediately before administration.

Cell and tissue therapy products can be administered by a variety of routes, including the common parenteral routes (intravenous, subcutaneous, intramuscular, and intra-arterial) and the respiratory or gastrointestinal tract. Other possibilities include direct application into regional vasculature, organs, tissues, or body cavities by means of needles or catheters or following surgical exposure of the tissue. Although parenteral administration can be accomplished in routine outpatient or inpatient facilities, the other means of administration may require specialized facilities such as an aseptic operating theater or endoscopic suite. A variety of delivery systems such as catheters, syringes, and IV lines are frequently used to administer cells to patients. Before clinical use manufacturers should ensure that these medical device components are compatible with the cells and formulation solutions. In all cases, standard operating procedures and a quality program must be in place to ensure that the product is administered in the intended manner.

POST-ADMINISTRATION MONITORING

Written policies and procedures for monitoring patient outcomes and for reporting and managing adverse events should be in place. Patient outcome assessments should include indicators that are likely to detect errors or problems related to the entire manufacturing process, and special attention should be given to manipulations, storage, or transportation after manufacturing. Management of adverse reactions should include procedures for ensuring prompt medical evaluation and treatment of patients and a system for reporting and evaluating adverse effects that may identify potential product defects. Reporting includes informa-

tion required for federal or state adverse-event monitoring programs.

STABILITY

General Considerations

The stability of cell or tissue products and the components used to create them will vary depending on the nature of the product, its intended clinical use, its specific attributes, and storage, packaging, and shipping conditions. For this reason, comprehensive guidelines covering a broad array of products are usually not possible. In all cases stability studies should be based of scientifically sound principles and a comprehensive understanding of the final therapeutic product and its intended use. Manufacturers also should assess the stability of in-process hold steps, cell banks, critical raw materials, and reference standards. A well-designed and executed stability program provides a high degree of assurance that the product is stable during its specified shelf life.

Where feasible, stability testing should be carried out in accordance with the principles described in ICH guideline Q5C, presented in *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* (1049). Stability data should also be collected for bulk and other in-process materials that are stored before final processing and filling.

Depending on the formulation used and storage conditions, shelf life can vary from hours to years. If a product has a short shelf life or if a product stabilizer must be removed, the final formulation may require preparation at the clinic just before administration. Instability is frequently observed as aggregation in cell products and as structural nonuniformity in tissue products. The stability of the final cell or tissue product must be established by validation studies during development.

For some cell and tissue products such as autologous or patient-specific cell products, final lots tend to have small volumes and perhaps shelf lives of only a few hours or days. In such cases, stability protocols should be based on materials from multiple donors. Because it is frequently difficult to obtain sufficient cells or tissues from autologous or patient-specific products, cells or tissues from several sources such as normal donors, research tissue repositories, cadaveric sources, or well-characterized banked primary cells can be used in stability studies to validate storage, shipping, and expiration dating. However, the results obtained with such "surrogate" cells or tissues must be interpreted cautiously and conservatively until data confirm that the actual autologous or patient-specific cells exhibit stability profiles that are similar to those of the surrogate cells or tissue.

For combination products that include cells and biomaterials, the stability of both components must be considered. When biodegradable scaffold materials are present, scaffold degradation should be considered in determining the stability and shelf life of the combination product.

Stability Protocol Development

Formal stability studies to support licensure and early-phase product stability information gathering should be detailed in a written plan that describes how stability data will be collected and analyzed to support the expiration date. Protocols should follow the format recommended in existing regulatory guidelines and should include the scope, storage conditions, and number of lots to be tested, test schedule, assays, data analysis, and product specifications. Any assay used in a formal stability study for licensure must be validated before the study begins. The specific study design should take into account the reasonably expected challenges the product may encounter. For instance, if the final

product formulation is performed at the clinical site, stability studies on this final formulation should establish the time frame and conditions under which the product can be held. For cryopreserved cells or tissues, stability should be established following cryopreservation of the final product, in-process intermediates, or any hold step.

Stability studies must verify that the storage conditions maintain the quality attributes of the product so that the latter complies with stability specifications. These specifications may differ from release specifications, but they must address product potency. Measuring and calculating the decay of product activity by employing statistical methodologies may require frequent sampling during an extended period and may require analysis of multiple production lots to compensate for the variability of the assays or products.

Initial studies to establish a provisional expiration date must be conducted before administration to the first patient. Initial studies are also useful for determining which assays are stability indicating, that is, the best indicators of product degradation. Because existing compendial methods do not address the unique characteristics of all cell and tissue products, manufacturers should develop assays that address these unique characteristics.

Shipping validations are a special type of stability study with predetermined protocols, testing requirements, and acceptance criteria. Typically, the product is packaged and shipped under normal and extreme conditions, and the material is tested before and after shipping to ensure that it still meets the product release requirements. As described in *Storage and Shipping* (below), special attention must be given to the specific thermal, mechanical, and radiological stresses products will likely encounter.

Stability Challenge Conditions

The stability-indicating profile of a cell or tissue product may vary with time under the influence of a wide variety of environmental conditions, including temperature, mechanical stress, and light. Multifactorial degradation pathways must be considered in the development of a program to investigate the effects of these parameters on product stability. Based on risk assessment, studies should include conditions that are outside of the specified storage ranges, that is, challenge conditions such as those encountered during periods of abnormal storage, shipping, or handling. Examples include brief incubator malfunctions, incubator or cold storage failure, periods of extreme temperature fluctuation caused by shipping to hot or cold climates, hypobaric conditions in the cargo hold of a commercial airliner, or temperatures likely to be encountered in the surgical suite.

A short exposure to an environmental condition well outside of an established limit and a long exposure to one just outside of an established limit may be equally detrimental. The slow and constant rate of product degradation at a specified temperature may increase if a different set of storage conditions is applied. The effect of light on the stability-indicating profile should be investigated if it is scientifically warranted. Special attention should be given to products stored in fluids containing light-sensitive or -reactive components that may give rise to cytotoxic by-products.

Studies analogous to accelerated aging studies typically used in pharmaceutical stability-monitoring programs are also useful to characterize how the product degrades and which assays are stability indicating. For example, if a cell-based product will be held under refrigeration (4°–6°) until use, then studies performed while holding the product for extended times at room temperature (25°) may provide useful information about product integrity as well as the analytical methods used. Such studies should be performed before formal stability studies begin so that the formal studies incorporate the validated stability-indicating assays into the protocol.

STORAGE AND SHIPPING

General Considerations

Storage conditions are chosen to preserve the purity and potency of the product so that the specifications for the product are maintained throughout storage, shipping, and handling at the clinic. Before clinical trial use, initial studies must be conducted to determine acceptable storage, shipping, and handling conditions. The storage conditions and expiration date for the product must be specified. The initial storage and shipping conditions need not be exactly the same as those envisioned for the commercial product, but they should ensure that the product specifications are maintained beyond the initially proposed expiration dating. Once stability-indicating methods are developed and the final container-closure, storage, and shipping conditions are chosen, these conditions must be validated, as discussed under *Stability* (above).

For products with short shelf lives, storage and shipping conditions—even within a single medical center—should be considered together because shipping constitutes the bulk of storage time after manufacturing. The product should be placed in a lightproof, leakproof container with adequate physical support to ensure stability and prevention of leakage during typical conditions of shipment. Special consideration should be given to the ability of gas to permeate the shipping container, especially if the cell-therapy product is stored or shipped on dry ice or liquid nitrogen.

Storage

For each type of cellular therapy product, the manufacturer should establish product storage specifications and acceptable storage conditions, including temperature range or liquid nitrogen level. Storage units require a system that continuously monitors and records temperature and/or liquid nitrogen levels. This includes an alarm system to immediately notify lab personnel of unacceptable storage conditions. The stability of the product during routine storage should be monitored by a stability program (see *Stability*, above).

Cryopreservation is the main mode for the long-term storage of cells. Cellular products are cryopreserved using controlled-rate freezing procedures or equivalent procedures that are known to maintain viability. The temperature of products during freezing and storage should be monitored and documented according to the facility's policies. The stability of the product under the holding conditions at the manufacturing facility and clinical site should also be validated.

The cooling rate for cell solutions during cryopreservation is important because of the mechanical and dehydration injuries resulting from the formation and growth of ice crystals. The ideal temperatures depend on the type of cells and the concentration of the cryopreservative. The optimal cooling rate for most cells is between 1° and 3° per minute. Controlled-rate freezers that can reproducibly duplicate this optimal cooling rate are critical when large numbers of vials or large volumes of cells in bags are being frozen. Once cooled to below freezing, cells should be stored at temperatures below –130°. This can be achieved with electric freezers or with liquid nitrogen.

Storage of cells in the vapor phase of a liquid nitrogen freezer reduces the risk of cross-contamination with other material in the freezer. Freezer equipment should be validated and temperature mapped so that cells are not subjected to temperatures above –130° as the liquid nitrogen evaporates or during freezer opening. Some cells can be stored at –80° if the cells will be used within a few weeks. Cross-contamination can also be prevented by the use of sealed overwraps covering the cryobags.

Many cell-based products cannot be cryopreserved. Because cells continue to metabolize during storage, their expiration period is short—on the order of hours or days. The expiration date can be extended by increasing the volume of storage medium, by adjusting the storage temperature, or by attaching a series of bags or compartments that allow the medium to be exchanged without breaching system sterility.

Storage conditions at the clinical site must also be defined and monitored. Cell-processing centers or clinics involved with bone marrow transplantation generally have liquid nitrogen freezers, but most clinical pharmacies do not. Storage temperatures and characteristics must be defined for each product. The clinical site may need to hold the product in the shipping container until the product can be administered to the patient. If thawing and administering of the cell-based product are performed at the clinic, the laboratory storing or shipping the cells must closely collaborate with the clinic because cells in a concentrated suspension may survive for only a few hours.

Shipping

Shipping containers and shipping procedures need to ensure temperatures are maintained within acceptable ranges for the duration of transportation and under conditions of actual use. These conditions include temperatures within the shipping container, extremes of temperature outside the shipping container (such as those encountered on a hot airport tarmac or in the chilly cargo hold of an airplane), and other shipping challenges (such as x-rays or mechanical vibration). Shipping studies should be conducted during product development in order to identify stresses to which the products may be subjected. Bracing and insulating materials should then be chosen and validated to provide a packaging system that will protect against extreme temperatures and mechanical stresses.

Most products are shipped by commercial shippers or courier systems. In some cases, critical products are hand-carried onto commercial aircraft. Commercial carriers must obtain special permission in order to bypass scanning by airport x-ray equipment. Special attention should be paid to shipping container labels because both biohazard and patient-specific information may be required in specific areas of the packaging. Shipping validations must be conducted under predefined protocols with predetermined acceptance criteria to ensure that the product meets quality specifications (including potency) once it reaches its final destination.

Cryopreserved cell-based products are typically shipped to medical centers on dry ice or in liquid nitrogen dry shippers. Dry shippers may be preferable because temperature is more readily maintained and monitored. Dry shippers also allow continuous monitoring of the shipper's temperature, which can be collected and logged for up to 14 days. Dry ice and liquid nitrogen are both considered hazardous materials during shipping and must be labeled accordingly.

LABELING

Labeling of cell therapy products is regulated by FDA under 21 CFR 201, 601, 610, and 1271. For biologics, 21 CFR 610 Subpart G outlines the requirements for container and package labeling. When possible, a full label should be affixed to the product container. This includes the proper name of the product obtained from the US Adopted Names (USAN) Council; the name, address, and license number of the manufacturer; the lot number; the expiration date; for multiple-dose containers, the recommended individual dose; the statement "Rx Only"; instructions to the dispenser to provide a Medication Guide, if one is required, to each patient; and if the container is not enclosed in a package, all items required for a package label. If the label is

too small to accommodate all this information, a partial label can be used, but the following information must appear on the partial label: the name expressed either as the proper or USAN name; the lot number and the name of the manufacturer; and for multiple-dose containers, the recommended individual dose. When partial labels are used, the container must be placed in a package that contains a label bearing all the items required for the package label. For containers that cannot accommodate any label, the container must be placed in a package that bears all the information required for a package label. In addition, when affixed to the container the label should not impede inspection of the contents. For products with very short shelf lives, expiration dating requires adjustment and correction for time zones to provide the user an accurate assessment of shelf life.

The package label must contain the proper or USAN name of the product, the name, address, and license number of manufacturer, the lot number, the expiration date, the preservative used and its concentration, or the words "No Preservative" if appropriate, the number of containers if more than one, the amount of product in the container, the recommended storage temperature, the words "Shake Well," "Do Not Freeze," or other instructions as indicated, the recommended individual dose for multi-dose containers, the route of administration, known sensitizing substances, the type and amount of antibiotics added during manufacture, inactive ingredients if they are a safety factor, the adjuvant, the source of the product when this is a factor for safe administration, minimum potency expressed in terms of official standard for potency or the statement "No U.S. Standard of Potency," and finally the statement "Rx Only." Regulations in 21 CFR 610.62 regard the position and prominence of the proper or USAN name in relation to a trade name.

Additional labeling requirements apply because cellular therapy products are also considered HCT/PS in 21 CFR 1271.90. For autologous cell therapies, the manufacturer is exempt from the requirements of determining donor eligibility. However, if the recommended testing for pathogenic or microbial contaminants is not performed before release, the label must contain the statement "FOR AUTOLOGOUS USE ONLY" or "NOT EVALUATED FOR INFECTIOUS SUBSTANCES." The label must also contain the Biohazard legend shown in 21 CFR 1271.3(h) with the statement "WARNING: Advise patient of communicable disease risks." For patient-specific products, the patient's full name, initials, or a combination of these must appear on the labeling to ensure that the product will be administered to the appropriate patient.

In addition, regulations govern the content and format of labeling for human prescription drug products (including biological products), otherwise known as the package insert. These regulations, which apply to approved therapeutics, went into effect 30 June 2006. Details about this content and format can be found in 21 CFR 201.56 and 201.57. These changes were designed to enhance the ability of the health care practitioners to access, read, and use prescription drug labeling. The main change is the addition of a half-page highlights section. Otherwise most of the changes involve rearrangement of sections to move to the front the most critical information for prescribing.

In addition to the specific FDA regulatory requirements, several groups have designed ISBT 128, a standard for uniform labeling of cellular therapy products, where ISBT stands for International Society of Blood Transfusion and the number 128 reflects the choice of barcode symbology known as Code 128. ISBT 128 defines the data structures and the placement of bar codes and their corresponding eye-readable text that appears beneath the bar code. In addition, this standard provides class names for different types of cellular products, modifier text for cell processing, manipulation text for how the cells are manufactured, cryoprotectant text for frozen cell products, and various other texts that must go on the labels. Although this voluntary standard

meets different organizations' requirements for labeling cellular products, it does not currently meet FDA regulatory requirements. Consequently, labels that comply with ISBT 128 must be supplemented with additional information required by FDA.

CONSIDERATIONS FOR TECHNOLOGY TRANSFER

Transfer of the skills, knowledge, technologies, and methods of manufacturing necessary to create a cell or tissue-based product is essential to ensure that scientific and technological developments are accessible to users who can then further develop and advance the technology into new products, processes, applications, materials, or services. Some general considerations for technology transfer activities are summarized below.

The process of developing a cellular or tissue-based therapy is complex and often involves several rounds of technology transfer throughout the product's life cycle. Some examples of technology transfer activities include: from bench research to translational research; transfer from research and development to GMP-compliant manufacturing; and change in manufacturing facility (for example, from in-house manufacturing to a contract manufacturer).

Manufacturers should anticipate the need for technology transfer during the research and development stage of a cell or tissue-therapy process. This should result in good documentation practices for product research and development, including testing procedures. Data and results should be retained in the format of development reports or technical reports to provide historical information that can be referenced and used in regulatory filings. Critical raw materials, procedures, and equipment should also be identified during technology transfer. Product and process development progress should be monitored against milestones established as part of risk assessment and gap identification in the technology transfer plan. *Table 3* provides an overview of the steps involved in technology transfer.

The ultimate goal of technology transfer is for the recipient to consistently reproduce a process to make comparable product in compliance with regulations. It is not atypical for manufacturers to develop and implement process improvements during early stages of technology transfer to support scale-up and manufacturing for Phase I/II clinical trials. However, during technology transfer for Phase III studies, pivotal trials, or commercial manufacturing, changes to the process or product should be avoided because they could require additional clinical studies and adversely affect time to market.

REGULATIONS AND STANDARDS

The Federal Food, Drug, and Cosmetic Act (FD&C Act) and the Public Health Service Act (PHS Act) provide the legal framework for FDA regulation of biological products, including cell-based therapy products. A list of frequently used terms in regulation of cellular-therapy products is presented in *Table 4*. In 1993 FDA provided notice that it intended to regulate cellular and gene-therapy products as biological products (Federal Register 1993;58:53248–53251). FDA defined somatic cell therapy products as autologous (i.e., self), allogeneic (i.e., intraspecies), or xenogeneic (i.e., interspecies) cells that have been propagated, expanded, selected, pharmacologically treated, or otherwise altered *ex vivo* for administration to humans for the prevention, treatment, cure, diagnosis, or mitigation of disease or injuries. For other biological products and drugs, clinical trials involving somatic cellular therapy products must be initiated under an investigational new drug (IND) application. After a sponsor submits sufficient evidence of product safety and clinical effectiveness, FDA approval can be obtained for marketing in the form of a biologics license application (BLA) or PMA.

Table 3. Technology Transfer—Fundamental Steps

Preparation	<ul style="list-style-type: none"> • Define the scope, strategy, and risks associated with the project that will be transferred • Identify overall gaps and process transferability • Assess availability of documentation such as manufacturing and testing procedures, sampling plans, in-process and final product data and specifications, material specifications (including source, testing requirements, and quantities required for a manufacturing procedure or test procedure), equipment specifications, specialized training requirements, facility requirements, and infrastructure requirements • Establish a governance body consisting of leads, experts, and mentors from both the sending and receiving sides; determine responsibilities for each group and individual • Define communication and reporting channels • Identify performance measurements, milestones, and timelines
Development and Implementation	<ul style="list-style-type: none"> • Establish a risk management plan • Establish a technology transfer master plan • Develop a training plan • Establish documents at the receiving site (specifications, SOPs, batch records, and standard test methods) • Train operations, quality, and support personnel for sustainability • Qualify materials and vendors • Establish and execute equipment comparability/suitability protocols • Calibrate equipment at receiving site • Qualify personnel, equipment, and facility at receiving site (includes execution of aseptic process validations, sterile media fills, and cleaning validations) • Establish and execute methods/assay qualifications • Establish a product stability program • Perform engineering and consistency/qualification runs • Assess need to establish comparability and prepare regulatory filings • Establish and execute shipping qualifications
Maintenance	<ul style="list-style-type: none"> • Collect and trend process/product data • Monitor product stability • Manage change control • Train and requalify personnel • Recalibrate and requalify equipment • Update regulatory filings

Table 4. Frequently Used Terms in Regulation of Cellular-Therapy Products

TERM	DEFINITION
351 products	Regulated under Section 351 of the PHS Act
361 products	Regulated under 21 CFR 1271, Human Cells, Tissues, and Cellular and Tissue-Based Products
BLA	Biologics License Application
CBER	Center for Biologics Evaluation and Research
CDRH	Center for Devices and Radiologic Health
GMPs	Good Manufacturing Practices
GTP	Good Tissue Practices, 21 CFR 1271, Human Cells, Tissues, and Cellular and Tissue-Based Products
IDE	Investigational Device Exemption. An investigational device exemption (IDE) allows the investigational device to be used in a clinical study in order to collect safety and effectiveness data required to support a Premarket Approval (PA) application or a Premarket Notification [510(k)] submission to FDA.
IND	Investigational New Drug. An IND is a request for FDA authorization to administer an investigational drug to humans. IND regulations are contained in 21 CFR 312.
PMA	Premarket approval

As defined by FDA, cellular therapy products are considered to be drugs, biological products but also HCT/Ps that are regulated under Section 351 and/or Section 361 of the PHS Act. This means that cell-based therapies are subject to cGMP (21 CFR 210 and 211), Biologics Product regulations (21 CFR 610), and HCT/P regulations (21 CFR 1271) including cGTP.

In recent years FDA has issued a number of regulations and guidance documents for human cell and tissue products (see *Appendix* and www.fda.gov/cber/). Of particular importance are the regulations at 21 CFR 1271 that establish a tiered, risk-based approach for HCT/Ps. In this regulatory framework, many conventional human cells or tissues are not subject to premarket approval and have only to comply with GTPs, including donor eligibility. This lower tier of regulatory oversight is intended to prevent the introduction, transmission, or spread of communicable disease. When

human cells or tissue are the starting material for the creation of a novel cell-based product, additional regulatory requirements are applicable. This higher tier of regulatory oversight includes compliance with GMPs, biological product standards, and premarket approval (see 21 CFR 1271.10). In almost all cases, the cell-based products described in this general chapter should comply with the higher tier of regulatory oversight.

In addition to cellular therapy-specific regulations and guidance, many general guidelines such as those related to aseptic processing, GMP expectations during development, process validation, and others are relevant and applicable (see www.fda.gov). Additionally, ICH has issued guidance documents for qualifying cell and tissue-based products (see *Appendix* and www.ich.org). Some of the guidelines and concepts in these documents are reproduced in *USP–NF*.

Table 5. Cellular Therapy Product Standards-Setting Organizations

AABB	AABB, formerly known as the American Association of Blood Banks, is an international association representing individuals and institutions involved in activities related to transfusion and cellular therapies, including transplantation medicine.	http://www.aabb.org/
AATB	The American Association of Tissue Banks is an educational and scientific, tax-exempt organization that facilitates the provision of transplantable tissues of uniformly high quality to meet national needs. AATB publishes standards to ensure that the conduct of tissue banking meets acceptable norms of technical and ethical performance. AATB conducts an accreditation program for establishments that retrieve, process, store, or distribute human tissue for transplant. A certification program is administered for tissue-bank personnel to ensure that tissue-banking activities are performed in a professional manner consistent with the standards of the association.	http://www.aatb.org/
ASTM	ASTM International (ASTM), originally known as the American Society for Testing and Materials, is one of the largest voluntary standards-development organizations in the world and provides technical standards for materials, products, systems, and services. ASTM International standards are used in the information infrastructure that guides design, manufacturing, and trade in the global economy.	http://www.astm.org/
FACT	The Foundation for the Accreditation of Cellular Therapy is a nonprofit corporation co-founded by the International Society for Cellular Therapy (ISCT) and the American Society of Blood and Marrow Transplantation (ASBMT) for voluntary inspection and accreditation in the field of cellular therapy.	http://www.factwebsite.org/
NMDP	The National Marrow Donor Program is a nonprofit organization that operates the federally funded registry of volunteer hematopoietic cell donors and umbilical cord blood units in the United States.	http://www.nmdp.org/
ICCBBA	The International Council for Commonality in Blood Banking Automation was established and given the responsibility for implementation and management of the ISBT 128 standard, a system for identification, labeling, and processing of human blood, tissue, and cellular-therapy products using an internationally standardized system.	http://www.iccbba.org/

The regulatory pathway for cellular-therapy products parallels that of pharmaceuticals, and as the product moves from early research through pivotal trials and finally marketing approval, the degree of manufacturing control becomes increasingly stringent. This has implications for the manufacturing unit and may dictate that the site be moved. Standards-setting organizations encourage the use of a fully functional quality unit to oversee manufacturing progress. Information is available on the FDA Web site, along with references to groups charged with guiding the medical community and the manufacturing unit during development.

In addition to USP general chapters and monographs for cell and tissue-based therapies, a number of professional standards-setting organizations (see *Table 5* and *Appendix*) have worked closely with regulatory authorities to develop standards and practices. These organizations ensure that standards are current and comply with governmental regulations. Such standards are a supplemental source of knowledge in identification of donors, donor screening and testing, product collections, processing of cellular products, administration, adverse event reporting, and follow-up after treatment. AATB has developed guidelines for sourcing allogeneic tissue. Over the years various organizations have tried to harmonize standards, including the development of common information circulars that can be compared with package inserts. At present, however, compliance with one organization's standards does not ensure compliance with those of any other organization.

Many benefits accrue to manufacturing facilities that participate in voluntary standards programs. Professional standards-setting organizations participate in educational workshops and disseminate information about operational issues. They also maintain close surveillance of FDA activity and training of inspectors. Further, FDA relies on accreditation by voluntary standards program, and FDA's unannounced

inspections have led to an increasingly high level of compliance in laboratory and clinical settings and has also undoubtedly increased patient safety. Third-party payors and hospital-ranking services have begun to use accreditation reports in their evaluation of quality programs.

APPENDIX

Cellular therapies and cell-therapy components are regulated by FDA as biological products. The general requirements are listed in national laws and international guidance. In the United States, national requirements are codified in different sections of 21 CFR, and additional recommendations are available in FDA guidance documents. International guidance documents are available from ICH, the European Agency of Medicines (EMA), and the World Health Organization (WHO). Although guidance documents from ICH are well referenced in this general chapter, those from WHO and EMEA are not, and manufacturers of cellular or tissue-based products intended for markets outside the United States are advised to refer to relevant guidances from relevant nations. Beyond USP chapters referenced in this chapter, the following list includes regulatory documents as well as best practices in product and process development, manufacturing, quality control, and quality assurance:

Code of Federal Regulations (CFR)

- 21 CFR 201.56–57
- 21 CFR 210
- 21 CFR 211
- 21 CFR 600.3
- 21 CFR 601
- 21 CFR 610
- 21 CFR 820
- 21 CFR 1271
- 21 CFR 46

FDA Guidance Documents

- *Draft Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)*
- *Draft Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products*
- *Draft Guidance for Industry: Current Good Tissue Practice (cGTP) and Additional Requirements for Manufacturers of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)*
- *Guidance for Industry: Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)*, February 2007. <http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/ucm073964.htm>
- *Guidance for Industry: Source Animal, Product, Preclinical, and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans*, April 2003. <http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Xenotransplantation/ucm074354.htm>
- *PHS Guideline on Infectious Disease Issues in Xenotransplantation*, January 2001. <http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Xenotransplantation/ucm074727.htm>
- *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production*, October 2006. www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070287.pdf
- *Guidance for Industry: Validation of Growth-Based Rapid Microbiological Methods for Sterility Testing of Cellular and Gene-Therapy Products*, CBER, 2008. <http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/ucm072612.htm>
- FDA Blue Book G95-1, <http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm080735.htm>

National and International Regulatory Documents

- The United States Consensus Standard for the Uniform Labeling of Cellular Therapy Products using ISBT 128, available at: http://www.iccbba.org/usconsensus_standard_cellulartherapy.pdf
- ISO 10993-1:2003, Biological evaluation of medical devices—Part 1: Evaluation and testing, available at: <http://www.iso.org>
- ICH Q2(R1): Validation of Analytical Procedures: Text and Methodology, available at: <http://www.ich.org>
- ICH Q5C: Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products, available at: <http://www.ich.org>
- ICH Q6B: Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products, available at: <http://www.ich.org>
- ICH Q9: Quality Risk Management, available at: <http://www.ich.org>
- Naming Scheme for Cell Therapies by the United States Adopted Names (USAN) Council, available at: <http://www.ama-assn.org/>
- Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), available at: <http://www.nap.edu/>

GLOSSARY AND DEFINITION OF TERMS

Adventitious Agent—A foreign material that is introduced inadvertently; not natural or hereditary (as in microbial, chemical, or biochemical contamination of a purified substance).

Allogeneic—From an unrelated member of the same species but with a different genotype.

Ancillary Materials—Components used during manufacturing that should not be present in the final product. Examples: growth factors, cytokines, monoclonal antibodies, cell-separation devices, and media components.

Apheresis—Procedure of withdrawing blood from a donor, removing select components (e.g., platelets or leukocytes), and transfusing the remainder into the donor.

Autologous—From one's own body.

Bioassay—Measurement of the effectiveness of a compound by its effect on animals or cells in comparison with a standard preparation. (See also *Potency*.)

Biological Product—Any virus, therapeutic serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment, or cure of diseases or injuries in humans. (The term analogous product has been interpreted to include essentially all biotechnology-derived products and procedures including gene therapy, transgenics, and somatic cell therapy.)

Biotechnology—Any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses. The newer definition refers to the industrial and pharmaceutical use of rDNA, cell fusion, novel bioprocessing techniques, and gene therapy.

B Lymphocytes (B Cells)—A class of lymphocytes that produce antibodies and are derived from bone marrow.

Bone Marrow Cells—A variety of undifferentiated cells (stem cells) and differentiated cells (lymphocytes, granulocytes, erythrocytes, and platelets) found in the internal cavities of bones or bone marrow.

Bone Marrow Transplantation—Transplantation of bone marrow cells that are capable of maintaining the hematological functions indefinitely. Technique used in the treatment of immunological disorders (severe combined immune deficiencies such as ADA deficiency), hematological disorders (anemia), metabolic disorders (Gaucher disease), and malignant diseases (leukemia, lymphoma, or solid tumor).

CD34—Cluster of differentiation cell-surface marker 34. CD34 is a protein that distinguishes stem and progenitor cells from more mature blood cells.

Cell Lines—Cells that are derived from primary culture embryos, tissue, or organs. Such cell lines may have a finite life span or be immortalized (made to replicate indefinitely).

Cellular Therapy—Therapy that uses whole cells to treat a disease, condition, or injury.

cGMP—Current good manufacturing practice.

Chondrocytes—Cells that produce the components of cartilage.

Clonal—Genes, cells, or entire organisms derived from and genetically identical to a single common ancestor gene, cell, or organism.

Clonogenic Assay—Procedure based on the ability to give rise to a clone of cells.

Combination Products—Therapeutic products that combine drugs, devices, and/or biological products.

Cytokine—Any factor that acts on cells; usually a protein that promotes growth.

Cytoplasm—Cellular material that is within the cell membrane and surrounds the nucleus.

Cytotoxic—Able to cause cell death.

Culture Medium—The liquid that covers cells in a culture vessel and contains ingredients to nourish and support the cells. Culture medium may also include growth factors added to produce desired changes in the cells.

Dendritic Cells—Cells that sensitize T cells to antigens.

Differentiation—A process of biochemical and structural changes by which cells become specialized in form and function.

ELISA—Enzyme-linked immunosorbent assay. An immunoassay that uses an enzyme-labeled antigen or antibody to detect the binding of a molecule to a solid matrix.

Embryonic Stem Cell, Human (hESC)—Stem cell derived from the inner cell mass of the blastocyst.

Endothelial Cells—Epithelial cells of mesodermal origin that line the internal cavities of the body, such as heart and blood and lymph vessels.

Engraftment—Process whereby cells, tissues, or organs are implanted or transplanted into another organism. Refers both to the mechanical and the biological processes necessary to have a fully functional graft.

Epidermal—Pertaining to the outermost and nonvascular layer of the skin derived from embryonic ectoderm.

Epithelial Cells—Cells from the linings of various organs, e.g., respiratory, intestinal, or vascular epithelial cells.

Ex Vivo—Outside of the living body. Refers to a medical procedure in which an organ, cells, or tissue are taken from a living body for a treatment or procedure, and then returned to the living body.

Feeder Cells—Cells used in co-culture to maintain pluripotent stem cells. For hESC, typical feeder layers include mouse embryonic fibroblasts or human embryonic fibroblasts that have been treated to prevent them from dividing.

Fibroblasts—Connective tissue cells that have the capacity to produce collagen.

Fluorescence-Activated Cell Sorter (FACS)—A machine that sorts cells based on fluorescent markers attached to them.

Formulated—Prepared in accordance with a prescribed method or conditions.

Graft-versus-Host Disease—Rejection of the transplanted tissue by the host. It is the leading cause of patient death when mismatched allogeneic tissue is used.

Granulocyte—One of three types of white blood cells. These cells digest bacteria and parasites.

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)—A natural hormone that stimulates white blood cell production, particularly that of granulocytes and monocytes.

Growth Factors—Factors responsible for regulatory cell proliferation, function, and differentiation.

Hemocytometer—A device used to manually count cells.

Hematopoietic—Pertaining to or affecting the formation of blood cells.

Hematopoietic Stem Cells—Stem cells that give rise to all red and white blood cells and platelets.

Hepatocytes—The predominant cell type in the liver that has an important role in metabolism and is a source of serum proteins. These cells are generally not dividing, but when injured they can divide and regenerate until the injured cells are replaced.

Human Leukocyte Antigen (HLA)—Proteins controlled by the major histocompatibility complex. These proteins play a key role in determining transplant compatibility.

Immunoassay—Technique for identifying substances based on the use of antibodies.

Immunofluorescence—Technique that combines an antibody detection strategy with a fluorescent label for visualization often used in combination with microscopy or fluorescence activated cell sorting.

Immunogenic—Substance capable of inducing an immune response; a form of antigen that induces an immune response, as opposed to a tolerogen that induces tolerance.

In Vivo—Procedure performed in the living organism.

In Vitro—In the laboratory (outside the body). The opposite of in vivo (in the body).

Islet Cells— β -islet cells of the pancreas that secrete insulin.

Keratinocytes—Differentiated epidermal cells that constitute the top layer of cells in the skin.

Lineage (Committed Progenitor Cells, Differentiated Cells)—Specific path of cell differentiation that can be traced to a single cell of origin.

Macrophage—Any of many forms of mononuclear phagocytes that are found in tissues and arise from hematopoietic stem cells in the bone marrow.

Mesenchymal Stem Cells—Multipotent stem cells that can differentiate into a variety of cell types.

Monoclonal Antibodies—Antibodies that are derived from a single cell clone.

Myocytes—Fundamental cell units in the muscle. Target cells for insertion of genes that encode secretory proteins.

Natural Killer Cells (or NK Cells)—Cytotoxic lymphocytes that constitute a major component of the innate immune system.

Neuronal Stem Cells—Stem cells found in neural tissue that can give rise to neurons and glial cells.

Osteogenic Cells—Derived from or involved in the growth or repair of bone.

Passage—The process in which cells are disassociated, washed, and seeded into new cultures after a round of cell growth and proliferation. The number of passages is a good indication of the age of the cultures and expected stability.

Process Validation—Means for providing documentation that the manufacturing process is controlled, reproducible, and capable of consistently producing a product that meets predetermined specifications.

Polymerase Chain Reaction (PCR)—Technique to amplify a target DNA or RNA sequence of nucleotides by cycles of polymerase-based copying, resulting in geometric increases in copy number.

Potency—A quantitative measure of biological activity based on the attribute of the product linked to the relevant biological properties.

Progenitor Cell—Parent or ancestral cell, usually one that is already committed to differentiate into a specific type or lineage of cells.

Regenerative Medicine—An emerging interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function using a combination of approaches including, but not limited to, the use of soluble molecules, gene therapy, stem cell transplantation, tissue engineering, and the reprogramming of cell and tissue types.

Serum-Free—Refers to cell growth medium that lacks a serum component.

Somatic Cells—Cells other than the germ cells.

Stem Cell—Immortal cell that is capable of proliferating and differentiating into different types of specialized cells. Each major tissue system is thought to have its own putative stem cell.

Supravital Dye—A dye that stains only living cells.

Suspension Culture—Growth, in suspension, of cells not requiring attachment to substrate in order to undergo cell division.

T Cells—Lymphocytes that acquire functional repertoires and the concept of self in the thymus and are responsible for cell-mediated immunity. There are several subsets of T cells (helper T cells, suppressor T cells, and cytotoxic T cells).

Umbilical Cord Blood Stem Cells—Stem cells derived from the blood that remains in the placenta and in the attached umbilical cord after childbirth.

Undifferentiated Cells—Cells that have not yet developed into a specialized cell type or tissue.

Xenogeneic—From a different species.

Xenotransplantation—Transplantation of organs from one species to another (e.g., from pigs to humans).

<1047> GENE THERAPY PRODUCTS

INTRODUCTION

Gene therapy products allow administration of nucleic acids to modify the genetic material of cells. Gene therapy products can be broadly classified based on the approach to delivery and include the following: (1) viral vectors [viruses that harbor the gene(s) of interest but usually without the mechanism to self-replicate in vivo]; (2) nucleic acids in a simple formulation (naked DNA); and (3) nucleic acids formulated with agents such as liposomes that enhance their ability to penetrate the cell. Where introduction of nucleic acid to cells takes place ex vivo, the cell population that is administered becomes the gene therapy product. Guidance specific to the manufacturing, processing, characterization, and administration of cell-based products is provided in *Cellular and Tissue-Based Products* <1046>.

Decisions regarding the choice of a gene vector can be complex (see *Design Considerations for Gene Vectors* under *Manufacturing of Gene Therapy Products*). The viruses most commonly used include murine retroviruses, human adenoviruses, and human adeno-associated viruses (AAVs). It is inherent in the definition of gene therapy in this chapter that the administration of nucleic acid through transduction is expressed as RNA and then as protein. Examples of gene therapy products are shown in *Table 1*.

Chapter Purpose and Organization

Clinical uses for gene therapy products, their manufacturing processes, and analytical schemes for determining identity, dose, potency, purity, and safety are rapidly evolving, and are as diverse as the products themselves. This chapter summarizes the issues and best current practices in the manufacturing, testing, and administration of gene therapy products. Usually *USP* chapters focus on materials that are commercially available. This chapter, however, not only discusses products for commercial applications, but it also addresses the production of clinical trial materials. When different approaches are options for clinical trial material compared to those used for commercial product, this is discussed.

Where appropriate, reference is made to applicable guidance including International Conference on Harmonization (ICH) quality guidelines because the principles apply even though gene therapy products may be outside the official scope. A list of regulatory and guidance documents applicable to gene therapy is presented in the *Appendix*, together with a list of terms commonly used in the gene therapy field. The traditional compendial perspective is to develop public standards that can be applied to a particular final product without providing production details. This chapter attempts to specify when traditional methodologies or standards can be adapted.

This chapter is extensive because of the diverse nature of the products and the special considerations that they require. Manufacturing has been divided into two sections: the first discusses general aspects of manufacturing and process development, and the second discusses vector design and class-specific topics. *On-Site Preparation and Administration* follows the manufacturing sections because the handling of these products at the clinic often requires facilities and expertise not found in a typical hospital. Other manufacturing-related sections include: *Analytical Methods*; *Stability*; *Storage and Shipping*; and *Labeling*. The section *Regulations and Standards* summarizes existing guidelines and highlights the need for the development and validation of new methodologies to assess product quality. The *Glossary of Terms* lists and defines the terms and abbreviations used in this chapter and those commonly employed in this field.

Table 1. Examples of Gene Therapy Products

Categories or Strategies	Indication: Administered Product
Gene replacement Short-term Long-term	Cardiovascular disease: growth factor vector on a biocompatible scaffold ^a Cystic fibrosis: transmembrane conductance regulatory vector Hemophilia: factor VIII or IX vector
Direct cell killing	Cancer: recombinant oncolytic viruses
Immunotherapy	Cancer: autologous tumor cells transduced with cytokine or other immunomodulatory genes; lymphocytes transduced with receptors for tumor antigens Arthritis: gene-modified autologous lymphocytes
Conditionally lethal genes ^b	Cancer (solid tumor): thymidine kinase (TK) or cytosine deaminase (CD) vector into tumor cells Graft-versus-host disease (GVHD): TK or CD vector transduced into donor T cells
Gene disruption via antisense RNAs, ribozymes, and inhibitory RNAs expressed via a vector	Cancer: anti-oncogene vector Cytomegalovirus retinitis: anti-viral vector Human immunodeficiency virus (HIV): autologous lymphocytes transduced with antiviral ribozyme vector
Intrabodies	Cancer or HIV: vector encoding single-chain antibody to a tumor protein or a viral protein, respectively

^a This product promotes formation of new blood vessels.

^b Cells with conditionally lethal genes as well as their neighboring cells are killed after the administration of a second drug in vivo. For TK, the drug is gancyclovir. For CD, the drug is 5-fluorocytosine.

MANUFACTURING OVERVIEW

Introduction

The manufacturing of gene therapy products has been divided into two sections. This section, *Manufacturing Overview*, discusses five topics that apply to manufacturing of all gene therapy products: (1) raw materials, (2) characterization of banked materials, (3) in-process controls, (4) specifications, and (5) validation considerations. The second section, *Manufacturing of Gene Therapy Products*, addresses manufacturing of gene therapy vectors, both viral and nonviral, and discusses the design of gene vectors in detail.

All the general principles of current good manufacturing practice (cGMP) outlined by FDA in 21 CFR 210, 211, 600s (especially 21 CFR 610), and 820, as well as other USP chapters apply to the manufacturing of gene therapy products. The manufacturing facility, equipment and process, raw materials, quality systems, and trained personnel are some of the key elements of cGMP. cGMPs are applied throughout clinical development. Typically, the extent of control increases as clinical development progresses, and full cGMP compliance is expected by initiation of manufacturing in support of Phase III clinical trial(s). The facility and equipment should be carefully designed, built, and validated to support the manufacturing process and to maintain the required product/facility segregation. Preventive maintenance and calibration should be performed routinely on critical equipment. Incubators, bioreactors, and freezers should be fitted with alarm systems that can remotely signal failure. Quality systems should be established to ensure manufacturing is consistent and in control. Systems include but are not limited to the following: change control, document control, environmental monitoring, training, validation master plans, raw material testing and release, vendor approval, product testing and release, stability testing, and corrective/preventive action (CAPA).

Ancillary Materials

A wide variety of raw materials, including ancillary materials, may be used in manufacturing. Raw materials may include complex substances such as cells, tissues, biological fluids, growth factors, and monoclonal antibodies. Some of these materials may remain in the final therapeutic product as active substances, cryoprotectants, or excipients. An ancillary material exerts an effect on a therapeutic material (for example, a cytokine may activate a population of cells) but is not intended to be present in the final therapeutic product. The quality of raw materials used in the production of a gene therapy product can affect the safety, potency, and purity of the product. Therefore, qualification of this type of materials is necessary to ensure the consistency and quality of all gene therapy products. Activities involved with raw material qualification will change as products move through various stages of clinical development and on to licensure and commercialization. A well-designed qualification program becomes more comprehensive as product development progresses. A qualification program for raw materials used in the manufacturing of gene therapy products should address each of the following areas: (1) identification and selection, (2) suitability for use, (3) characterization, (4) animal-derived components, and (5) quality assurance. For all raw materials, it must be considered when and where each is used in the manufacturing process because this can help define selection criteria. USP chapter *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* (1043) should be consulted for specific information about implementing an appropriate qualification program for these materials. Other USP chapters provide information about the qualification and standards of specific ancillary materials (e.g., *Bovine Serum* (1024), *Fetal Bovine Serum—Quality Attributes and*

Functionality Tests (90), and *Growth Factors and Cytokines Used in Cell Therapy Manufacturing* (92)).

Characterization of Cell and Virus Banks

CELL BANKS

A cell bank is a collection of vials containing cells stored under defined conditions, with uniform composition, and obtained from pooled cells derived from a single cell clone. The cell bank system usually consists of a master cell bank (MCB) and a working cell bank (WCB), although more tiers are possible. The MCB is manufactured in accordance with cGMP and preferably is obtained from a qualified repository source (source free from adventitious agents) with known and documented history. The WCB is produced or derived by expanding one or more vials of the MCB. The WCB, or MCB in early trials, becomes the source of cells for every batch produced for human use. Cell bank systems contribute greatly to consistency of production of clinical or licensed product batches because the starting cell material is always the same. Cell banks used for the preparation of virus banks or clinical product should be suitably characterized before use. Aspects of cell banking and validation are addressed in *Cellular and Tissue-Based Products* (1046), *Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of rDNA-Derived Protein Products* (1048), and *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* (1050).

VIRUS BANKS

The master virus bank (MVB) is similar in concept to the MCB because it is derived from a single production run and is uniform in composition. The working virus bank (WVB) is derived directly from the MVB. As with the cell banks, the purpose of a virus bank is to have a consistent source of virus that is shown to be free of adventitious agents for use in production of clinical or product batches. In keeping with cGMP regulations, testing of the cell bank that will be used for production of the virus banks, including quality assurance testing, should be completed before the use of this cell bank for production of virus banks.

QUALIFICATION

Cell and viral bank characterization is an important step toward obtaining a uniform final product with lot-to-lot consistency and freedom from adventitious agents. Testing to qualify the MCB or MVB is performed once, and can be done on an aliquot of the banked material or on cell cultures derived from the cell bank. Specifications for qualification of the MCB or MVB should be established. It is important to document the MCB and MVB history, the methods and reagents used to produce the bank, and the storage conditions. All the raw materials required for production of the banks—media, sera, trypsin, and similar substances—must also be tested for adventitious agents.

QUALIFYING THE MASTER CELL BANK

The FDA *Guidance for Industry: Human Somatic Cell Therapy and Gene Therapy* (March 1998) provides specific recommendations for qualifying MCBs. Additional guidance is provided in ICH Q5D. A description and history of the cell line is required, along with a description of the freezing process, storage conditions, and the number of vials prepared. The identity of the cells should be analyzed by genotypic and/or phenotypic markers. For MCB containing vector se-

quences, the presence and integrity of the vector should be demonstrated using molecular assays (restriction endonuclease mapping and/or nucleic acid sequencing) and/or measurement of vector gene expression. Purity must be analyzed to exclude bacterial, mycoplasma, fungal, and viral contamination (other than vector sequences). Freedom from adventitious viruses should be demonstrated using both in vitro and in vivo virus tests and appropriate species-specific tests such as the mouse antibody production (MAP) test. Special attention should be given to the detection of replication-competent virus (RCV) arising from recombination of the vector and viral sequences. The MCB is further qualified by tests conducted on cells (from the MCB or WCB) expanded to the limit of in vitro cell age for production.

QUALIFYING THE MASTER VIRUS BANK

Testing of the MVB is similar to that of the MCB and should include testing for freedom from adventitious agents in general (such as bacteria, fungi, mycoplasma, or viruses) and for organisms specific to the production cell line, including RCV. Identity testing of the MVB should establish the properties of the virus and the stability of these properties during manufacture.

QUALIFYING THE WORKING CELL OR VIRUS BANK

Characterization of the WCB or WVB is generally less extensive and requires the following: (1) testing for freedom from adventitious agents that may have been introduced during generation of the WCB, (2) testing for RCV, if relevant, (3) routine identity tests to check for cell line cross-contamination, and (4) demonstration that aliquots can consistently be used for final product production. This assumes that the WCB and WVB were prepared in a controlled environment using media and equipment that were screened appropriately for adventitious agents. If not, additional release testing is required.

In-Process Controls

Manufacturing processes should have well-defined go-no go decision criteria that are applied to key in-process intermediates and are used to pool material that has been processed through a step in several sublots. Quality must be built into the product as well as tested during batch release. In-process controls are the assays or tests that are performed to ensure that the in-process intermediate is of sufficient quality and quantity to ensure manufacture of a quality final product. Examples of in-process controls are listed in Table 2. The main reason for performing the in-process control is to ensure that the correct product with anticipated quality and yield is obtained. Intermediate in-process material that fails to satisfy the in-process control criteria should not be used for further manufacturing. This material may be reprocessed if there are procedures in place for such activities. The reprocessed material must satisfy the original in-process specifications before it can undergo further manufacturing. If several sublots will be pooled for further processing, sublots that fail to satisfy the criteria should not be

included in the pool, even if the pool containing these failed sublots would pass the in-process assay criteria. During clinical development, assays for product quality and yield should be performed after most processing steps to determine which steps are critical and which assays are most sensitive to deviations in the process. The information from these runs is also used to set the criteria for the selected assays. In-process controls are performed for fully validated processes to ensure that the process continues to be under control. The results of these assays should be trended, and actions should be taken to correct problems as they arise.

Specifications

The specification for a gene therapy product should be chosen to ensure the safety and efficacy of the product before use. Selected tests should be product specific and should have appropriate acceptance criteria to ensure that the product exhibits consistent quality parameters within acceptable levels of biological variation, loss of activity, physicochemical changes, or degradation throughout the product's shelf life. The development and setting of specifications for cell and gene products should follow the principles outlined in ICH Q6B and should reflect the FDA *Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)*.

Establishing specifications for a drug substance and drug product is part of an overall manufacturing control strategy that includes control of raw materials, excipients, and cell and virus banks; in-process testing; process evaluation and validation; stability testing; and testing for consistency of lots. When combined, these elements provide assurance that the process is in control and that the key quality attributes of the product are maintained. Appropriate specifications are established on the basis of thorough characterization of the product during the development phase and an understanding of the process and its capability. Characterization should include measurements of the physicochemical properties, safety, purity, process and product-related impurities, potency, viability, sterility, and quantity. Specifications for each product and its ingredients should be developed from this information by application of appropriate statistical methods. The data should include lots used in preclinical and clinical studies and should also include assay and process validation data that can be correlated to safety and efficacy assessments. Specifications should accommodate the inherent variabilities exhibited by the production process and by the assay. Some lot-release specifications typically applied to biologics may require re-examination for these product types.

The procedures in a specification for the product are anchored by appropriate reference standards. The reference standard for the product ensures that the product, as measured by the release assays, does not change significantly over time. The reference standard is manufactured using the same process as used for clinical production and is subject to all in-process and final release testing. In addition, the reference standard may be subjected to additional characterization not typically performed as part of lot release. The reference standard need not be stored at the same dose,

Table 2. Examples of In-Process Control Applications

Type of Product	Attribute to Control
Viral gene therapy	Quantity of virus after virus culture
	Specific activity of virus in fractions after column chromatography
	Quantity of host-cell DNA in fractions after column chromatography
Nonviral gene therapy	Optical density or change in oxygen consumption during culture
	Amount and form of plasmid before culture harvesting
	Amount and form of plasmid after extraction steps
	Amount of pyrogen or endotoxin after extraction steps in plasmid pool

formulation, or temperature as the product, but the stability of this reference standard should be determined. The reference standard verifies that a test produces acceptable results (passes its system suitability tests). A specific assay standard (working standard) can be used in the test, but it should be calibrated against and behave like the reference standard. Changing to a new reference standard (lot) should include many tests, all of which are run side by side with the existing reference standard. The impact of any change in the properties of the new reference standard should be carefully evaluated before it is adopted.

Additional specifications may be needed to produce a safe and effective gene therapy product. These might relate to some of the controls and action limits used to maintain standards and consistency for raw materials, excipients, and the manufacturing process (see *Ancillary Materials and In-Process Controls*). Specifications should be established to allow acceptance of raw materials and excipients used in the final formulation of the product. In addition, tests should be performed at critical decision steps during manufacture or at points where data serve to confirm consistency of the process. In-process release specifications should be established for each control step. Heterogeneity can result from the manufacturing process or storage of the product. Therefore, the manufacturer should define the pattern of heterogeneity within the product and should establish limits that will maintain the therapeutic efficacy and safety of the product.

In some cases, specifications may be established for lot release as well as for shelf life. As discussed in ICH Q5C and *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* (1049), the use of different specifications should be supported by sufficient data to demonstrate that the clinical performance is not affected. Acceptance criteria should be established and justified on the basis of data obtained from lots used in preclinical and clinical studies and lots used for demonstration of manufacturing consistency and on the basis of relevant development data such as those arising from validated analytical procedures and stability studies. Acceptance criteria should also be correlated with safety and efficacy assessments.

Once specifications have been established, test results should be trended. Results that are out of specification (OOS)—or even those that are out of trend—should be investigated before the material is considered for further processing. The purpose of an investigation is to determine the cause of the discordant result. FDA's *Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production* provides a systematic approach for conducting an investigation. An assay result can be rejected if it can be confirmed that an error, such as analyst error, calculation error, or equipment failure, has taken place. If the investigation concludes that the product is not within the specification, the lot should be rejected. In unique situations, a product that does not meet all specifications may have to be administered to a patient. However, procedures must be in place to govern the communication of the OOS results to the physician or to the person responsible for making the decision to use the product and to provide instruction for any follow-up testing, patient monitoring, and communication of those results.

Considerations for Validation

The potential for wide biological variation in gene therapy products, particularly for patient-specific treatments, affects the validation effort. Nevertheless, the basic principles of process validation for any biological product, including recommendations by ICH, FDA guidance documents, *Validation of Compendial Procedures* (1225), and *Validation of Microbial Recovery from Pharmacopeial Articles* (1227), apply to the validation of most gene therapy products. Guidelines for validating viral vaccines can be relevant to gene therapy processes that produce viral vectors. The hold steps in a manufacturing process should be validated to ensure that

in-process intermediates are within specification and that the quality attributes of the final product are maintained. Product-release assays should be validated before production of the materials for Phase III pivotal clinical trials.

Process validation demonstrates that the unit operations of the manufacturing process perform consistently and can generate a quality product that meets specifications. Because biological processes are prone to variability, the consistency and robustness of the manufacturing process should be determined by validating the process on at least three lots. Process validation issues pertinent to cell-based products are addressed in (1046).

If possible, the process should be validated for virus clearance according to principles discussed in ICH Q5A. If this is difficult because of the nature of the gene therapy vector (e.g., enveloped virus), additional characterization of cells and animal-derived components used in the production process should be considered. If the gene therapy product is manufactured in a multiproduct facility, validate cleaning of multiproduct equipment and rooms to demonstrate the effectiveness of cleaning agents to inactivate or remove virus.

MANUFACTURING OF GENE THERAPY PRODUCTS

Introduction

Principles for the production of pharmaceutical or biological products are also relevant to the production of gene therapy vectors for use in humans. The same cGMP requirements are applied to ensure that a high-quality product is delivered to the patient. Because of the nature of gene therapy manufacturing systems, most manufacturers face development issues such as scalability, yield, cost efficiency, and product stability.

Most gene therapy vectors have been produced only in relatively small batches necessary to meet the needs of early clinical trials conducted in small numbers of patients. However, the promise of gene therapy in larger patient populations has led to progress in large-scale production and purification technology. This section focuses on designing vectors for gene therapy and choosing a suitable production technology.

Design Considerations for Gene Vectors

TYPES OF VECTORS

A typical gene therapy vector is composed of the following: (1) the vector backbone; (2) a promoter; (3) the therapeutic gene, either as cDNA or genomic sequence; and (4) a polyadenylation signal. A wide array of viruses—including murine and human retroviruses, adenoviruses, parvoviruses such as AAV, herpes viruses, poxviruses, toga viruses, and nonviral plasmid therapy systems—have been developed for gene therapy applications. These vectors (see *Table 3*) differ greatly in terms of their capacity to deliver genetic material and the duration of expression. Some viral vectors preferentially target dividing cells, but others are capable of transducing both dividing and nondividing cells. There are significant variations in transgene capacity (i.e., there are limitations on the size of the foreign DNA fragment that can be incorporated into the vector genome). The level, timing, and duration of gene expression required for a gene therapy product depends on the clinical indication. Low-level, long-term gene expression may be required for some diseases, including adenosine deaminase (ADA) deficiency or type A and type B hemophilia. High-level, short-term expression may be more appropriate for cancer when genes that induce apoptosis are used or for cardiovascular disease when

preventing hyperproliferation of smooth-muscle cells that may impede restenosis of saphenous vein grafts.

VECTOR DESIGN CRITERIA

Many types of gene therapy vectors are being developed, and the vector selected for a particular clinical application depends on the disease state, the target cell, and the intended route of administration. As shown in *Table 3*, capacity depends on vector type, so clinical applications that require a large amount of genetic material will limit the choice of vector system. The payload of a vector system becomes increasingly important when one designs vectors with genomic DNA or a vector that contains extensive regulatory sequences.

Vectors are also selected based on the intended duration of expression and the target cell. For example, retroviral vectors integrate stably into target cells and are therefore well suited for stem cells or lymphocytes that are expected to undergo extensive cell division. In contrast, adenoviral and plasmid vectors are episomal and may be lost during cell division. However, adenoviral vectors are attractive for vaccine development and cancer applications where tumor cell elimination is the goal. Other vectors, such as AAV, do not integrate at high efficiency but can be expressed long-term in nondividing cells such as neurons or hepatocytes.

Target cell type can also play into the selection of an appropriate vector system (see *Targeting Transduction*). For example, the adenoviral Coxsackie virus B and adenovirus receptor (CAR) is expressed poorly on hematopoietic tissues, which limits the usefulness of the vector system for blood-derived cells. Vectors based on murine retroviruses require cell cycling and are not well suited to the transduction of nondividing cells such as neurons.

The immune system can target both the viral components of the vector and the expressed transgene. Pre-existing antibodies or cellular immunity to certain vector systems can exist and may limit their usefulness. Vectors can elicit an innate immune response that can decrease the efficiency of gene transfer and may also induce a severe adverse event. A

large number of current gene therapy approaches seek to limit toxicity and immune response by administration of vector to cells *ex vivo*. Nevertheless, the majority of diseases suitable for gene therapy will require *in vivo* administration, and ongoing research seeks vectors with limited immune recognition.

The route of administration and manipulation of the total dose of vector are strategies that can be used to compensate for some limitations of specific vector systems. Additionally, there are advantages and disadvantages for the manufacture of each of the different vector systems that should be considered when planning a clinical application. Production consistency favors systems with well-defined fermentation or culture systems, such as plasmid, retroviral, or adenoviral vectors. For viral vector systems that require helper functions (see below), a rationally engineered cell line can overcome the scalability and consistency limitations of co-transfections. Use of a cell line that is adapted to suspension culture can affect scalability and cost efficiency.

TARGETING TRANSDUCTION

To be effective, a vector must first find and transduce its target cell. Viruses have a natural host range that is strongly influenced by the expression of specific cell-surface receptors, the current phase of the cell cycle, and the route of administration. Integrins are a class of cell-adhesion receptors that interact with either the penton base or the fiber protein of adenoviruses. The fiber and penton base proteins of adenoviruses mediate binding to the CAR, CD46, and integrins. Adeno-associated viruses primarily interact with heparan sulfate proteoglycan and sialic acid receptors on the cell surface. However, interaction with secondary receptors such as integrins, laminin, and growth factor receptors is required for efficient cell entry and trafficking of virus particles to the nucleus. An amphotropic variant of the murine leukemia virus (MLV), commonly used for gene therapy applications, utilizes the sodium-dependent phosphate transporter RAM-1 to enter cellular targets. Expression levels of

Table 3. Types of Gene Vectors

	Viral							Nonviral
Family	Retroviridae		Adenoviridae	Parvoviridae	Herpesviridae	Togaviridae	Poxviridae	
Examples species	Murine Leukemia Virus	HIV	Adenovirus	AAV	Herpes Simplex Virus	Sindbis	Poxvirus (Vaccinia)	Plasmid derived
Vector Characteristics								
Insert size limit	8 kb	8 kb	4.3–34 kb	4–5 kb	40–150 kb	5 kb	25–50 kb	12 kb
Chromosome integration	Yes	Yes	No, episomal	Can be integrated or episomal	Can be integrated or episomal	No	No	Yes, but at very low frequency
Therapeutic expression	Stable	Stable	Stable or transient	Stable	Stable or transient	Transient	Transient	Stable or transient
Vector localization	Nucleus	Nucleus	Nucleus	Nucleus	Nucleus	Cytoplasm	Cytoplasm	Nucleus
Types of cells transduced	Dividing only	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent	Dividing and quiescent
Efficiency of gene transfer	High	High	High	High	High	High	High	Low
Expression of viral proteins	No	No	Yes, unless viral genes deleted	No	Yes	Yes	Yes	No
Other	Tropism can be altered by pseudotyping					Can be used as a plasmid therapy system		

each of these receptors vary according to tissue type, which dictates the transduction efficiency of the vector.

The host and tissue range can be modified or targeted by biochemical and genetic manipulation of the vector. Alterations in the tissue and cell specificity of retroviruses—and lentiviruses in particular—occur largely through genetic pseudotyping. During this process, the envelope proteins that dictate virus binding and entry via a specific cellular receptor of one virus are replaced with the envelope protein of another retrovirus or with a protein from an entirely different virus such as the vesicular stomatitis virus glycoprotein. The relative complexity of adenovirus and adeno-associated virus capsids allows them to be genetically modified in several ways. Substitution of a single virus protein (e.g., adenovirus fiber or AAV VP1) with that of another serotype within the same family is very much like the pseudotyping process for retroviruses. However, mosaic virus particles created by interspersing individual capsid proteins from several different virus serotypes in one virion, and chimeric particles created by capsid proteins of two distinctly different serotypes on a particle of yet another serotype (e.g., adenovirus 35 knob on adenovirus 41 fiber on an adenovirus 5 particle) can also effectively change the types of cells and tissues that a vector can transduce. Although this approach may seem straightforward, certain modifications of virus capsid proteins at the genetic level do not facilitate virus particle formation. Although most modifications improve virus uptake in one specific cellular target, they may also increase uptake in several other tissues in which gene transfer would not be desirable. Thus, selection of proteins and ligands must be carefully considered and tested in preclinical models of disease before these vectors can be used extensively.

Viral coat proteins and nonviral vectors can be chemically modified for ligand-mediated receptor targeting. They can be conjugated to cell-targeting ligands by antibody–virus interactions with bi-specific antibodies. Molecular bridges like biotin–avidin complexes and chemical crosslinkers such as bifunctional polyethylene glycol (PEG) tethered to cell specific receptor-binding proteins are easily conjugated to virus particles and often are incorporated in targeting strategies. These approaches can easily be combined and/or exchanged with genetic modifications and with each other to create vectors that effectively target several receptors. Although the biochemical approach avoids the functional complications of introducing foreign domains into viral proteins, each lot of vector must be modified because progeny virus will default to their original genetically encoded tropism. Biochemical processes also require the use of multiple reagents, which may complicate transfer to the clinic.

Another common and effective strategy to target viral vectors to tumor cells takes advantage of the virus replication cycle. Deletion of genes critical for taking over functional cellular checkpoints to support normal virus replication allows the virus to replicate only in cancerous cells where those checkpoints are either defective or inactive. This effect can be further enhanced creating small mutations in virus replication genes driven by tumor- and tissue-specific promoters. With respect to cell cycling, adenoviruses and adeno-associated viruses easily infect both quiescent and dividing cells, but MLV-based retroviral vectors are efficient only when transducing rapidly dividing cells. Lentiviral vectors can infect quiescent cells, including cells of neuronal origin. In general, nonviral vectors can enter both dividing and nondividing cells but have lower transduction efficiencies than viral vectors. Transduction efficiencies of nonviral vectors can be enhanced by the formulation and direct injection in the tissue of interest.

INFLUENCE OF HUMORAL IMMUNE SYSTEM AND COMPLEMENT

One of the most significant barriers to effective gene transfer is the humoral immune response to the vector. Re-

gardless of the route of administration, the intended target cell, and the dose, the vector is likely to encounter some component of the immune system. For viral vectors, the humoral immune system cannot readily distinguish between wild-type viral infections and recombinant viral vectors because the humoral response is directed against proteins in the viral envelope or capsid. Protein-containing formulations of nonviral vectors can also elicit a humoral immune response. Specific and cross-reacting humoral responses may pre-exist because of natural exposure to wild-type versions of viral vectors, or they may be elicited during dosing, and the antibody response may vary in its capacity to diminish gene transduction in individual patients. Because neutralizing capacity is frequently enhanced upon multiple dosing, repeated administration can also be problematic. Some of these issues may be remedied by the use of nonviral systems. However, the level of transduction efficiency of these vectors is not currently sufficient for many gene-transfer applications. Transduction efficiency of all vectors is also importantly compromised by the complement system. Many complement proteins have a natural affinity for virus capsid proteins. This interaction initiates release of cytokines and chemokines that facilitate rapid removal of vector from the systemic circulation. This affinity is often heightened (especially in the case of retroviruses) when nonhuman cellular proteins and culture components (e.g., fetal bovine serum) are incorporated into and/or coat the virus particle during large-scale production. Use of producer cell lines of human origin and serum-free culture conditions has decreased inactivation of vectors by complement.

Several strategies for mitigating and avoiding the humoral immune response and complement inactivation have been developed. Increasing the vector dose to compensate for the neutralizing activity of the antibodies and altering dosing regimens to coincide with periods of low antibody titer are logical choices, but possible toxicity associated with high doses of virus and the individual variability of the immune response are potential negative consequences of this approach. Alternatively, viral vectors can be engineered to evade the immune system. One approach involves increasing expression of specific viral genes that allow the virus to evade the host's humoral response. Recombinant viruses constructed from serotypes with limited exposure rates such as simian adenovirus 7 can avoid neutralization in those previously exposed to more common serotypes. Mosaic and chimeric viruses can also avoid neutralization. Both approaches effectively address the issue of efficient gene transfer in those with pre-existing immunity, but both approaches require further investigation in response to concerns regarding safety and large-scale production as well as induction of immune responses in naive patient populations. Covalent attachment of polyethylene glycol (PEGylation) to the virus capsid can both protect the virus from neutralization and blunt the immune response. Pharmaceutical methods such as embedding viral vectors in polymer matrices and administration of vectors to the mucosa (oral and nasal) can also protect viral vectors from the humoral immune response.

INFLUENCE OF CELLULAR IMMUNE RESPONSES

Once transgene expression is initiated, cellular immune responses rapidly remove cells transduced by both viral and nonviral vectors. This decreases the overall therapeutic effectiveness of gene transfer of low-to-moderate vector doses and can be highly toxic when higher doses are administered. Active protein synthesis is not required for cellular immune responses to viral capsid proteins. For example, the capsid proteins of recombinant AAV vectors have been shown to be long-lived, leading to a delayed immune response and elimination of vector-transduced cells. De novo synthesis of viral gene products can also exacerbate host-cellular responses. Viral vectors have been designed with specific backbone deletions to eliminate the expression of

viral structural genes and reduce this effect. Examples of such vectors include gutless adenoviruses, herpes viruses, and adeno-associated viruses in which all viral genes have been deleted, making them dependent on another helper virus for subsequent replication. Certain plasmid sequences, especially those with unmethylated CpG dinucleotides, can elicit a strong cellular immune response and have been used as adjuvants in some DNA-based vaccines. Amplifying plasmids in bacteria that express the CpG Methylase (M.SssI), removal of CpG sequences by site-directed mutagenesis, and removal of unnecessary prokaryotic sequences to create minimal plasmids have reduced the incidence of unwanted cellular responses. One nonmolecular approach to minimize the cellular response against the vector involves the use of immunosuppressants (cyclosporine, sirolimus, daclizumab) at the time of initial vector administration as well as methods described for reducing the humoral response (use of chimeric vectors, PEGylation) outlined above.

ANTIGENICITY OF THE GENE THERAPY PRODUCT

In many cases, the gene therapy product and associated promoter and enhancer elements are antigenic in certain cellular targets. When proteins that are retained in the target cell are used, cellular responses may eliminate the target cell. If sustained protein expression is required, the cellular immune response may decrease the effectiveness of the therapy or eliminate it entirely. In terms of treating genetic diseases, patients with a null mutation who have never seen the transgene product may be at a higher risk for immune response than patients who produce a defective protein. Also, truncation of a gene such as the cystic fibrosis transmembrane conductance regulator (CFTR) so that it fits within a chosen vector may result in creation of a distinct antigen.

In other applications, the transgene product is a foreign protein, e.g., thymidine kinase derived from the herpes simplex virus (HSV), and thus may elicit an immune response. In some cases this is the desired therapeutic effect, particularly in antigen-based immunotherapy for cancer or a viral disease. Efforts to minimize the immune response against elements associated with the transgene cassette include designing vectors with the ability to carry full-length humanized sequences for the transgene of interest and administration of immunosuppressants at initial dosing and other intervals throughout the treatment protocol.

VECTOR LOCALIZATION WITHIN THE TARGET CELL

Once the vector reaches the target cell, several factors can affect the level and duration of therapeutic gene expression, and these factors dictate the choice of an appropriate vector system for a specific clinical indication. The localization of the vector genome within the cell, the strength of the gene expression control elements, the stability of the message, and the stability of the translated protein all affect therapeutic impact. Alphavirus-based vectors, such as those derived from Sindbis or Semliki Forest virus, reside in the cytoplasm and typically exhibit a very high level of gene expression. Retroviral, adenoviral, and other viral vectors have advantages in gene delivery with their natural mechanisms for nuclear delivery of the therapeutic gene and reasonable levels of gene expression from viral or other promoters. Nonviral plasmid vectors are episomal and are often susceptible to DNA degradation when they are shunted into cell endosomes. However, some nonviral systems incorporate nuclear targeting signals as a means of increasing therapeutic gene transcription efficiency.

TISSUE-SPECIFIC EXPRESSION

Another means of controlling gene expression is the incorporation of tissue-specific promoters to stimulate or to restrict expression of the therapeutic gene. Unfortunately, many tissue-specific promoters do not provide high levels of gene expression, and incorporating these sequences into viral vectors may result in loss of specificity or low-level expression in cells that do not normally express the promoter. Tagging vector with sequences recognized by the microRNA system has also permitted tissue-specific expression and may offer tighter control than typically seen with tissue-specific promoter systems.

Drug-responsive promoters are being used to control gene expression. Rapamycin, mifepristone, and tetracycline (tet-on) systems have been used to repress gene expression. This type of regulation is particularly useful when constitutive expression of the vector transgene is toxic.

IMPACT OF REPLICATION STATUS OF VECTOR

Replication status is another important consideration for vector design and selection. Viral vectors are most frequently constructed to be incompetent or replication-defective in order to limit uncontrolled vector spread and pathogenicity. However, the ability to replicate and spread within a specific cell population, e.g., within a tumor or to metastatic sites, may provide a significant therapeutic advantage over cell-type-specific targeting of replication-incompetent vectors. Replication can be engineered to be conditional when, for example, specific viral gene interactions are matched with intracellular pathway targets by means of targeted deletions and/or changes in transcriptional or translational control. When these targets are defective or missing, as in cancer cells, the virus can replicate, but when the target cell is functioning normally, viral replication is repressed. Viruses that have been genetically engineered for selective oncolytic replication include: adenovirus, HSV, vaccinia, measles virus, picornaviruses, influenza virus, Coxsackie virus, and Sendai virus. Some nongenetically-modified viruses are inherently oncolytic in human cells, e.g., reovirus and Newcastle disease virus.

One of the risks inherent in the use of conditionally replicating viral vectors is that such systems are leaky, i.e., the growth of the virus is not absolutely restricted to a single cell type. Also, subsequent rounds of viremia become considerations in the evaluation of tissue distribution/exposure and shedding. The therapeutic promise of these approaches depends on the reliability with which conditionality of replication can be selected or engineered. This therapeutic potential will be realized only if balanced with steps to control the potential risks to patients (associated with replication competence of the viruses/viral vectors) and to address associated shedding-related issues of third party exposure and environmental concerns.

As a proactive contribution to the safety profile and to take advantage of scientific and clinical information already available, virus strains that have been used for human vaccination are often used as the vector backbone. Nevertheless, because the product is replication competent, it presents specific technical challenges for adventitious agent testing and product characterization.

Nonviral vectors are normally designed as nonreplicating systems, but some groups are experimenting with replicating nonviral plasmids to increase gene expression levels (because of the low transduction efficiency of most nonviral systems) and to increase the duration of gene expression. Additional preclinical studies are needed to establish the safety of these systems. Artificial chromosomes have also been designed to take advantage of normal mechanisms for retaining gene expression in rapidly dividing target cells.

VECTOR INTEGRATION

The duration of gene expression is also a function of the persistence of the vector genome in target cells. Retroviral vectors can stably integrate into the host-cell genome, providing long-term expression. Adenoviruses and nonviral plasmid vectors, e.g., those not administered using electroporation, do not integrate, and expression generally decreases over time. Recombinant AAV vectors generally do not integrate, and when they do, it is not site specific. However, stable episomes have been observed in certain cell types such as muscle cells.

Site-specific integration can be a desirable feature for vectors that are intended to correct genetic disorders. Although it is not currently efficient enough to be useful, the control of the site of integration is desirable in order to prevent insertional mutagenesis. Insertional mutagenesis has the potential to kill a cell if a critically functioning gene is inactivated or to predispose a cell to malignant transformation if a tumor-suppressor gene is inactivated. Of clinical relevance, promoter or enhancer elements within vectors can lead to activation of cellular oncogenes and have been associated with malignant transformation in children undergoing retroviral gene transfer for X-linked severe combined immunodeficiency.

The success of any gene therapy product depends on the relationship between the vector-delivery system and the requirements of the disease in terms of the site, level, and duration of therapeutic gene expression. A universal vector now appears unlikely, and the challenge lies in fitting one of several possible vectors to the disease and to the gene to be delivered.

Manufacturing and Purification Strategies

VECTOR CONSTRUCTION

Viral and nonviral gene-transfer vectors are constructed by using standard molecular biology protocols. For viral vectors, the vector backbone consists of viral RNA or DNA sequences from which the regions encoding viral structural genes or the regions required for replication have been deleted. The deleted region of the vector is usually modified with specific restriction endonuclease sites used to allow insertion of the gene of interest. For nonviral vectors, the plasmid DNA backbone contains multiple restriction sites for cloning and the bacterial elements necessary for plasmid production. Vector backbones can accommodate single or multiple gene insertions depending on the maximum amount of sequence they can carry. The promoter that facilitates transcription of the gene insert can be a related viral promoter, such as the murine leukemia virus long terminal repeat (MuLV LTR), or a heterologous promoter that is either tissue specific, such as the alpha crystalline promoter (of the eye), or constitutive, such as the cytomegalovirus (CMV) late gene promoter. For example, in a retroviral vector construct containing two gene inserts, transcription of one is regulated from the 5'-LTR-promoter sequence, and a second gene insert can be linked to an internal heterologous promoter from Simian virus 40 (SV40).

The complementary DNA (cDNA) containing the therapeutic gene of interest, including its introns, is excised from its source using restriction enzymes and is inserted at the multiple cloning site of the gene-transfer vector. The polyadenylation signal can be derived from multiple sources such as the SV40 virus or human growth hormone gene. Characterization and testing of gene therapy vectors are described under *Analytical Methods*.

HELPER FUNCTION SYSTEMS

Recombinant viral vectors are most often modified to be replication defective, a condition created by deletion or modification of the viral genes needed for replication and production of infectious virus. Because the vectors are stripped of some or all of the viral genes, a system must be developed to supply viral proteins and to encapsulate the vector into a viral particle. Generally, this is accomplished by two methods: transient transfection or stable packaging cell lines.

In the transient transfection method, a series of different plasmids are generated, including a plasmid containing the vector and another vector containing the viral genes. For example, retroviral vectors can be generated using a three-plasmid system: (1) the transgene-containing vector plasmid; (2) a plasmid containing the *gag/pol* viral gene region; and (3) a plasmid containing the viral envelope. All three plasmids are transfected into cells, e.g., HEK293 or HT1080, and vector-containing virions are harvested after two to three days. The separation of vector and viral genes on different plasmids, along with vector designs that minimize the homology between vector and viral sequences, decrease the chance for recombination and generation of replication-competent virus. Similar approaches can be taken with most viral vector systems.

The transient transfection method has the advantage of a rapid production time and flexibility when changing components of the vector or viral constructs. Nevertheless, it can be cumbersome when scaling up for manufacturing, and special care must be taken to provide consistent production yields. An alternative method has been the use of vector packaging cell lines. In this scenario, the viral genes are introduced stably into an immortalized cell line that yields persistent expression of viral genes. As with transient transfection, the viral genes generally are expressed from different plasmids to decrease the risk of recombination. Since plasmids integrate infrequently, considerable time and effort are required to isolate a high-titer packaging cell line and to generate an MCB. Vector constructs can be introduced into cells from the MCB, and researchers, by screening for a high-titer clone may allow isolation of a stable cell line that generates the vector of interest. These cell lines generally can be expanded to great numbers and often produce vector for up to a week at a time, facilitating vector scale-up and product consistency.

Typical helper function systems are as follows:

Retroviral Vector Systems—Initial packaging cells were based on the murine fibroblast cell line NIH 3T3. The PG13 cell line (expressing the Gibbon Ape Leukemia Virus envelope) has been used extensively with a low incidence of recombination events leading to RCV. More recently, the human HEK293 and HT1080 cell lines have been modified to serve as packaging cell lines for retroviruses. The use of a human cell line decreases elimination of vector particles by the human complement system (although this is generally not a concern for vectors used in ex vivo protocols).

Adenoviral Vector Systems—HEK293 cells are widely used to supply the E1 function necessary for efficient adenoviral replication that is deleted from first-generation adenoviral vectors. Other complementing cell lines, such as E1-modified A549 cells (human lung carcinoma) and the PER.C6 cell line (human embryonic retinoblast), have also been created to supply E1 or other missing functions. PER.C6 contains the E1 region under the control of a phosphoglycerate kinase (PGK) promoter and has no flanking adenoviral sequences in order to eliminate production of replication-competent adenovirus (RCA).

AAV Vector Systems—These systems classically use adenovirus-infected HEK293 cell lines transiently transfected with AAV helper plasmid containing the *rep* and *cap* genes, which are required for AAV replication and capsid formation, respectively, and are deleted from the AAV vector. In some AAV production systems, wild-type adenovirus has been re-

moved from the process by using triple transfection of plasmids expressing *Ad* early genes, *rep* and *cap*, and the vector transgene. The HeLa cell line (from human uterine cervical carcinoma) has also been used as a transient production system. More recently, both of these cell lines have been used to establish stably transfected packaging cell lines that express *rep* and *cap* genes and in some cases express the adenoviral functions needed for AAV replication when *rep* and *cap* are present (E1a, E1b, E2a, E4, and VA RNA). AAV production systems using recombinant HSV and Baculovirus have also been developed.

Gutless Adenoviruses—Early manufacturing systems for the adenovirus vector known as gutless adenovirus were similar to classical AAV vector manufacturing systems because HEK293 cells were transiently transfected with helper plasmid containing required adenoviral functions. Development of helper viruses housing a packaging signal flanked by loxP sites and complementing HEK293 cell lines that express the bacteriophage P1 site-specific Cre recombinase has greatly improved the yield of the gutless virus. This technology notably reduces the amount of helper virus contamination by preventing packaging of the helper virus genome while permitting it to replicate and support replication and encapsidation of the gutless vector.

VIRAL GENE THERAPY VECTORS

Retrovirus and adenovirus vectors typically have been produced at laboratory, non-GMP scale by use of traditional cultivation methods for anchorage- and serum-dependent cell lines employing flasks, trays, and roller bottles. Initially, gene therapy vectors were produced by these methods because large volumes of product were not required for early clinical studies. Cell-bank systems are used as the source of cells, and virus banks are the source of virus for clinical production. In many cases, supernatant is collected, clarified, and stored frozen in bags at -70° . In many early clinical trials, unpurified supernatant has been used for ex vivo gene transfer.

Larger-scale upstream production methods have been reported and are commonly used. They include suspension, bioreactor, and fixed-bed or microcarrier culture methods. Some groups have reported adapting their process cells to serum-free culture conditions. Cells are harvested and lysed or supernatant is collected. The harvest is clarified and purified to remove host-cell debris, host-cell DNA, and other process-derived contaminants.

Traditionally, viruses are purified by gradient ultracentrifugation, but this is time-consuming and unsuitable for larger-scale production purposes. The selection of downstream process steps and their sequence is determined by the nature of the virus itself and the upstream process used for manufacturing the virus. As processes are being developed for the manufacture of gene therapy vectors, many different purification steps have been reported. These include ion-exchange and sulfonated-cellulose chromatography, zinc ion affinity chromatography, and size-exclusion chromatography. Typically, DNase or other nuclease treatments are used in the process in order to reduce host-cell or plasmid DNA. AAV production and lentiviral production are complicated by a need for transient transfection or co-transfection of plasmid or helper virus. These processes have generally required anchorage-dependent cell lines that are difficult to scale up. The development of stably producing cell lines would allow large-scale production.

PURIFICATION METHODS: VIRAL VECTORS

Retroviruses—To date, purification of retrovirus preparations for phase I clinical trials has often been minimal at best, i.e., the simple concentration of culture supernatants is insufficient to meet the stringent quality standards required for in vivo therapy. Centrifugation and microfiltration tech-

niques are very useful for clarification of culture supernatants and removal of cellular debris. Ion-exchange, size-exclusion, and affinity chromatography techniques have also been employed to remove excess salt, serum, and low molecular weight contaminants also concentrated with the virus.

Adenoviruses—Recombinant adenoviral vectors were often purified by cesium chloride density gradient ultracentrifugation. This is still used for research-scale preparations, but the procedure is neither scalable nor efficient for large quantities of clinical-grade virus. The most recent scalable purification methods use anion-exchange chromatography because of the strong affinity of intact virus particles for the resin with respect to that of cellular and individual capsid proteins. Published loading estimates for anion-exchange resins range from 0.5×10^{12} to 5×10^{12} virus particles/mL of resin or 0.14 mg of virus to 1.4 mg of virus/mL resin. Gel filtration and immobilized metal affinity chromatography are often used in polishing steps following anion-exchange purification of recombinant adenovirus-based products.

Adeno-Associated Viruses—The toxicity of cesium chloride, the aggregation of AAV particles, and the fact that adenovirus is not completely removed after extensive centrifugation complicate AAV purification by cesium chloride density gradient ultracentrifugation. Another density separation medium, iodixanol, which is less toxic than cesium chloride and prevents AAV aggregation, has been employed in a single centrifugation step. Passage of the AAV fraction over an affinity column consisting of either a heparinized support matrix or monoclonal antibodies produced against AAV2 strongly increased purity and infectivity of final preparations. These methods are appropriate only for specific AAV serotypes. Ion-exchange chromatography is the most powerful and versatile method for AAV purification, although buffer pH, detergent concentration, and column medium must be tailored for each AAV serotype. Infectivity and purity of preparations obtained from these purification strategies are comparable to those obtained from affinity chromatographic methods and are complete within three hours.

PLASMID OR NONVIRAL VECTORS

Plasmids are double-stranded, circular DNA molecules that exist in bacteria as extrachromosomal, self-replicating molecules. They have been modified to serve as cloning systems, to contain multiple restriction endonuclease recognition sites for insertion of the cloned transgene, and to contain selectable genetic markers for identification of cells that carry the recombinant vector. Plasmid-based, nonviral vectors are frequently used as gene delivery systems for both in vivo and ex vivo gene therapies. These vectors are in the form of naked DNA or are complexed with lipids or other agents that facilitate transfer across the cell membrane and delivery to the cell nucleus without degradation. An advantage of a plasmid-vector system is the efficient production of large quantities of the vector that is easily characterized and avoids the risk of RCV associated with many viral systems.

Nonviral vectors are typically produced by using an *Escherichia coli* bacterial system. Plasmids are transfected into *E. coli*, and an appropriate single bacterial colony is selected and expanded to create an MCB. After reselection of a colony from a bacterial plate inoculated from the MCB, plasmid DNA is isolated from cultures that can range in size from 1 L on a laboratory scale to hundreds of L in bacterial fermenters. Plasmid DNA can be purified by several methods including affinity or ion-exchange chromatography and cesium chloride-ethidium bromide density gradients. Cesium chloride-ethidium bromide density gradients are not recommended for production of clinical-grade material.

PRODUCTION AND PROCESSING OF NONVIRAL VECTORS

One benefit of nonviral vectors for gene transfer is that the production process is rather generic and can be applied

to any plasmid preparation regardless of composition or application. Because the current average human dose of plasmid DNA for gene transfer and vaccination is approximately 1 mg, the primary challenge associated with large-scale production of plasmids is to develop a process that is both scalable and economical. Thus, process development for plasmid-based vectors remains an active area of research and development. A standard process for large-scale production of recombinant DNA plasmids consists of the following five unit operations.

Fermentation—Fermentation processes must support growth of transformed bacteria and maximize the amount of plasmid produced by each cell. *E. coli* is the most common strain used for plasmid production. Amino acids, nucleosides, and the ratio of nitrogen to carbon-containing compounds present in a rich media formulation greatly improve plasmid yield.

Harvest—Bacterial cells are harvested either by centrifugation or microfiltration. Centrifugation under GMP conditions can be costly, which makes microfiltration the accepted method of cell harvest. This also allows spent media, metabolic byproducts, extracellular debris, and impurities to be washed away before purification.

Lysis—Bacterial cells must be lysed to release the recombinant plasmids. This is one of the most critical steps in the production process because it can significantly affect the amount of usable [covalently closed circular (ccc)] and unusable [sheared, partially denatured, and open circular (oc)] forms of DNA in a preparation. The most widely used method of lysis for clinical-scale manufacturing is treatment with alkaline detergent and precipitation of cellular debris with acetate. This removes a large fraction of cellular impurities from the lysate, but it also increases the sensitivity of plasmids to mixing and localized concentrations of detergent, which are hard to manipulate on a large scale. Lysis of cells by heat exposure addresses this issue and effectively denatures cellular proteins and bacterial DNA.

Isolation/Purification—Some processes include additional steps for removal of cellular debris and other contaminants from crude bacterial lysates by precipitation with detergents, polyethylene glycol, or salt. These reagents affect plasmid stability and are removed by column chromatography. Size-exclusion chromatography can effectively separate plasmid DNA from RNA, proteins, and other small molecules present in the cleared lysate. The degree of separation of plasmid DNA from contaminants is highly dependent on the

type and concentration of salt in the running buffer. Resins used in anion-exchange chromatography have a high affinity for plasmid DNA and provide maximal sample concentration. Hydrophobic interaction and thiophilic aromatic chromatography are the methods of choice for selective separation of the different plasmid DNA isoforms and endotoxin reduction.

Bulk Preparation—After purification, the bulk plasmid is placed in a suitable buffer and formulation by ultrafiltration using a membrane with a pore size of 50–100 kDa.

Plasmids for clinical use must be highly characterized. Impurities from production and processing steps are well known. Tests necessary to confirm the identity, purity, and potency of a plasmid-based product are well established and routine. These tests and the current specifications set by FDA and the World Health Organization are summarized in Table 4.

Introduction of Genetic Material into Cells—Gene-Modified Cells

A common extension of cell therapy involves the introduction of genetic material, usually DNA, into cells to alter their pattern of gene expression. While discussion focuses on DNA, similar scenarios can be applied to RNA or a derivative of DNA, except that the stability and solubility of the particular nucleic acid may dictate modifications of certain steps. The general process is often referred to as ex vivo gene therapy because the cells are removed from the patient or donor and the genetic material is introduced while the cells are outside of the body. The genetically modified cells are then administered to the patient. The genetic material introduced can either cause expression of new genes and products or inhibit the expression of already expressed genes and products. The latter represents a type of antisense therapy. The genetic material can be introduced by the same range of reagents that are involved with gene therapy: viral vectors, nucleic acids in a simple formulation (naked DNA), or nucleic acids formulated with agents such as liposomes that enhance their ability to penetrate the cell. Most of the steps and considerations discussed above also apply to the ex vivo introduction of genetic material into cells. The main goal of ex vivo therapy is to develop robust processes that will work with the majority of the patient's or donor's cells. This takes considerably more effort than processes for cell lines.

Table 4

Assay Type	Issue	Determined By	Acceptable Level in Final Product
Identity	Cross-contamination with other products	Restriction digest/gel electrophoresis	N/A
Purity	Residual bacterial chromosomal DNA	Real-time PCR	<2 µg/mg DNA
	Residual RNA	Analytical HPLC	<0.2 µg/mg DNA
	Residual bacterial protein	BCA protein assay	<3 µg/mg DNA
	Endotoxin	LAL assay	<10 EU/mg DNA
	Sterility (bacterial and fungal)	Method outlined in 21 CFR 610.12	No growth
	Appearance	Visual inspection	Clear solution free of particulates
	pH	pH meter	Physiologic (7.0–7.4) but may be product specific
Potency	Plasmid confirmation (ccc vs oc)	HPLC or CGE	>97% ccc
	Labeled dose	In vitro ELISA FACS RT-PCR Light absorbance (A ₂₆₀)	Transgene/plasmid specific

The method for introducing new genetic material into cells depends on the biology of the system and the desired stability of gene expression. If a simple retroviral vector such as Molony murine leukemia virus is used for transduction, the cells must be actively dividing because vector DNA is integrated into the cellular DNA only during replication. This usually leads to long-lasting expression of the desired gene product. Adenoviral vectors, naked DNA, or formulated DNA can be introduced into nondividing cells. However, gene expression will be transient because the introduced DNA will usually be extrachromosomal.

The main challenge in *ex vivo* gene therapy is to achieve efficient transduction or transfection, introducing sufficient DNA into the cell before the DNA degrades. In the case of transduction by simple retroviral vectors, cells are stimulated with reagents that cycle them into the S phase (replication) at the time that the vector is applied. Most retroviral vectors are stable in cell culture for a period up to a few hours. Because diffusion is minimal, only a small fraction of viral particles will come into contact with cells during this period. The following techniques can be used to increase the number of viral particles that contact the cell in a given time period:

1. Maximization of viral particle concentration and minimization of the media volume during the transduction step
2. Multiple applications of the virus
3. Centrifugation of virus particles onto the cells
4. Placing cells on a filter and slow pulling viral media through the filter
5. Addition of binding-enhancing polymers to the media. [NOTE—Co-culturing the target cells with the viral producer cells is not recommended. This technique increases the chance of a recombinant event and production of RCV. Furthermore, any product for which co-culturing is used to transduce the human cells would be considered a xenotransplant if the producer cells were not human. The second cell type, whether human or not, may cause inflammation.]

Each of these techniques has its own set of issues that must be addressed in order to develop a robust process. In technique 1, reduction of the volume during transduction results in rapid exhaustion of the medium, so supplemental medium should be added within a few hours. In technique 2, the cells may no longer be in the correct cell cycle phase during later applications, or cells may have become refractory because of unproductive transformation during the previous application. Techniques 3 and 4 can work well on a very small scale, but the number of cells that can be transduced may be insufficient to obtain an efficacious dose. In technique 5, polymers may fail to provide a benefit because virus binding may involve specific receptors for which surface density may prove to be the limiting factor.

Similar issues and techniques can apply with other viruses or DNA preparations. The issue of slow diffusion is even more marked for the use of DNA preparations. Factors such as the cell type in which the viral vector was produced, the media used for vector production, and the purity of the vector can have a pronounced effect on the efficiency of transduction. Although certain methods may not require cells to be actively cycling, in practice, most processes require that cells be capable of replication because of the following considerations:

1. Safety considerations may dictate that only cells that express the new DNA are returned to the patient, which requires that these cells be selected. As described below, the most common selection method uses an antibiotic-resistant gene that is co-introduced with the new genetic material.
2. Further propagation may be required to achieve the therapeutic dose of cells.
3. Economic, biological, or technical reasons may dictate that the DNA introduction step be carried out at a low cell number and that the desired cell population then be expanded to the required dose.

Therefore, conditions that enable the cell or maintain its ability to proliferate must be developed in almost all cases. The biology of the cells, the available technology, and process economics will determine whether cells are propagated before, after, or during the introduction of new genetic material. Most processes do in fact expand the population after the introduction of the new gene.

Whether cells that do not productively express the gene can be administered to patients depends on the biology of the application, the dose required versus the handling capability of the manufacturing system, and, most importantly, the toxicity of the nonproductive cell population. Selection of the genetically modified cell population is commonly carried out using an antibiotic-resistance marker gene, such as neomycin, which is co-introduced into the cell with the new genetic material. For neomycin selection, cells in culture are treated with the antibiotic G418 at a concentration and for a period that has been shown to kill cells with nonproductive expression while allowing the productively expressing cells to proliferate. In this manner cells that are resistant to the antibiotic are presumed also to express the DNA of interest. The expression should be tested as a lot-release requirement or verified in a series of mock runs. Because most antibiotics decrease cellular proliferation, optimization of the culture media composition may be necessary for efficient selection and propagation of the gene-modified cells.

Following the antibiotic selection step, a second phase of antibiotic-free cell propagation may be required in order to achieve the desired dose and to rinse residual G418 out of the system. The selected medium and the total time that the cells are in culture can be critical to maintaining the desired expression of the original differentiated functions. An additional issue associated with the use of selection markers is that they generally are nonhuman genes. The expression of these genes usually elicits an immune response. Process development is often carried out with cells from healthy donors. Consideration should be given to the fact that for very sick patients, healthy cells that can be stimulated to undergo efficient, sustained replication are difficult to obtain.

Manufacturing, cell processing, and analytical testing issues pertinent to cell-based products are addressed in chapter (1046).

Formulation of Gene Therapy Products

Final formulations for gene therapy products are still in early development, and currently most gene transfer vectors are stored in solution at ultra-low temperatures. Successful formulation of candidates for gene transfer relies on a thorough understanding of the physicochemical and biological characteristics unique to each vector system. Factors like solution pH, ionic strength, and osmolality influence the thermal stability of viral and nonviral vectors. Organic carbohydrates such as mannitol, sorbitol, sucrose, and trehalose have been incorporated into preparations to prevent disruption of the native conformation of the vector in solution, during the freeze-thaw process, and during lyophilization. Amino acids such as arginine and leucine have been incorporated into formulations for their buffering effects and to prevent aggregation. Surfactants such as the Tweens, Spans, and Pluronics have been effective at preventing aggregation, but this effect is somewhat vector specific because some vector products are easily disrupted by these reagents. Lipids, polymer and extraneous proteins (human albumin and gelatin) have also been incorporated in many vector preparations because of their ability to prevent loss of vector from direct interaction with pharmaceutical surfaces and during freeze-thaw cycles.

Before initiating a program for formal screening of formulations for a vector, the following factors should be considered. The required dose and/or storage concentration of the final product, as well as the specifics of the container-closure and/or delivery system should be established. Analytical

methods to assess potency and identify degradants should be in place. The expectations of the formulation must also be defined. Some pragmatic criteria for the design and selection of vaccine formulations for use worldwide are: (a) the final product should be in a formulation that affords an 18- to 36-month shelf life when stored at 2°–8° or above, (b) the formulation should have an acceptable stability profile at ambient temperatures to cover short-term storage and transportation in the field, (c) it should adequately protect the vector from damage during freeze-thaw cycling, and (d) it should consist of reagents that are pharmaceutically acceptable and within physiologically acceptable concentrations. Formulation changes during clinical development must be supported with preclinical studies and stability data. Sufficient time should be built into plans to account for this.

To date, little work in the area of formulation development of retroviral vectors with additives approved for human use has been described. The most significant effort to develop stable formulations for gene transfer has been with recombinant adenoviruses. Recently, identification of the mechanisms by which recombinant adenoviruses degrade in solution led to the development of several liquid formulations that stabilize the virus for up to 18 months at 4°. Adeno-associated viruses are regarded as one of the most stable viral vectors. This virus has been documented to be stable for approximately 4 months in phosphate buffered saline at 4°. Addition of cryoprotectants and surfactants prevents aggregation of virus particles and extends the shelf life to one year. Lyophilized formulations of both adenovirus and AAV with shelf lives of several years at room temperature have also been described. Although nonviral vectors have been found to be generally robust in standard buffers at 4°, their stability may be extensively influenced by extraneous components included to promote gene targeting. Consider the nature of those components when developing stability protocols and strategies (see below).

ON-SITE PREPARATION AND ADMINISTRATION

One or more product modifications or preparative steps may be required before administration of the gene therapy product to the patient. These modifications or steps are frequently performed close to the time of administration, and, therefore, they are performed under conditions not under control of the original manufacturing facility. The nature of these modifications is dictated largely by characteristics of the product in relationship to the particular application. These include thawing, washing, or filtration to remove unwanted product manufacture-related materials and also include defined physical space with appropriate environmental controls, trained personnel, detailed standard operating procedures, and a comprehensive quality program.

The unique and irreplaceable nature of many gene therapy products, e.g., gene-modified cells, many of which have originated from an autologous or a selected allogeneic tissue source, creates special considerations for product manufacture, release, and administration. Issues pertinent to the administration of cell-based products are addressed in detail in chapter (1046).

On-Site Preparation

PRODUCT MANIPULATIONS

Before administration, on-site preparation of the gene therapy product may involve one or more manipulations, including the following:

- **Change in final container**—The manufactured product may have been stored or transported in one container but may require transfer to a different container for administration.
- **Change in physical state or temperature**—A product may require thawing from the frozen state or warming from the refrigerated state.
- **Change in solution or suspension**—A product may have to be dissolved, diluted, or suspended in a liquid.
- **Addition to biocompatible structural material**—A gene therapy product may need to be combined with living, natural, or synthetic structural tissue or matrix. Examples of matrix material include hollow fibers, fibrous sheets, gels, plugs, capsules, sponges, or granules.
- **Admixture or compounding with other nonstructural materials**—A product may require mixing or compounding with drugs, cytokines, biologics, or other nonstructural materials.
- **Filtration or washing**—Unwanted materials in the manufactured product, such as particulates, cellular debris, metabolites, or compounds remaining from previous manipulations may require washing or filtration steps.
- **Sampling**—Sampling of the final product immediately before administration may be required for certain clinical protocols.

FACILITY REQUIREMENTS

Facility requirements for performing on-site preparative steps or administration of gene therapy products depend on the nature of the products, their applications, and the manipulations required. The most important determinant of facility features is the level of risk for microbial contamination associated with each step. Definition of low-risk and high-risk conditions can be made according to a framework similar to that defined for Low-Risk-Level Compounded Sterile Preparations (CSPs) and High-Risk-Level CSPs in *Pharmaceutical Compounding—Sterile Preparations (797)*, *CSP Microbial Contamination Risk Levels*.

RELEASE OF FINAL PRODUCT

Gene therapy products that undergo on-site preparative steps or manipulations must be subjected to appropriate checks or tests to ensure that all quality specifications are met before release for patient administration. The nature and extent of manipulations will determine whether release requirements or critical specifications must be added to those required immediately after initial manufacture. Pre-release requirements usually include the following:

1. Physical inspection of the product, which typically includes measures to ensure appropriate product appearance with regard to color, turbidity, particulates or foreign matter, container integrity; product temperature; and accuracy and convenience of labeling
2. Review of process records
3. For patient-specific products, clerical checking of product labeling or records related to the identity of the intended recipient.

In addition, products considered to be high-risk products according to the description in chapter (797) should undergo additional product testing. For all high-risk products, quality assays for the identity, potency, and purity of the active ingredients should be defined and performed. For high-risk products in Category II, sterility and endotoxin testing should be performed.

Administration to Patients

PRE-ADMINISTRATION REQUIREMENTS

Depending on the specific gene therapy application, trained patient-care staff must take steps to prepare the patient for product administration. These steps are aimed at ensuring that the product will provide the intended therapeutic outcome and at minimizing the risk of adverse effects. Issues pertinent to administration of cell-based products are addressed in chapter <1046>. Generally, a thorough re-evaluation of the patient's general condition and suitability for therapy must be performed close to the time of product administration. This evaluation usually includes a patient history, physical examination, and laboratory studies such as blood counts and chemistries. In addition, staff may obtain baseline physical or functional measurements, laboratory tests, or imaging studies relevant to the specific application. Examples include pulmonary function tests for a therapy aimed at improving lung function, measurement of blood levels of an enzyme that is the gene product in a gene therapy application, and nuclear imaging of organs before anticancer therapies.

A variety of patient interventions related to route of administration may be required before product administration. For therapies that require intravenous administration, patients with poor peripheral venous access may require placement of a central venous catheter. In applications where gene-modified cells or matrices combined with cells are implanted into the patient, the site of implantation may require preparation in the operating room. This may involve surgically opening the site, removing the degenerated or damaged tissue, trimming of the adjacent tissue to accommodate the implant, and excising the tissue from a second site to be used as an anchor or support for the implant. For instance, in the case of products for wound healing, it is critical that the site for grafting be free from infection and that it demonstrates a well-prepared wound bed. Where gene-modified cells are intended to repair cartilage defects, the site of damage needs to be prepared so that the cells can be applied to a water-tight compartment. For applications involving direct administration of the product into an organ system (for example, bronchioalveolar system) or vascular network (for example, coronary arteries), the patient may require endoscopic or surgical access to these sites.

In all cases, the need for adequate anesthesia and premedication must be carefully evaluated in conjunction with these steps before product administration. Pre-administration patient evaluation must also include assessment of concurrent therapies that may interact with the gene therapy product to modify its effects. Some therapies may be considered adjunctive to the gene therapy, such as cytokines that promote proliferation or differentiation of the infused or implanted tissue. Other commonly used drugs such as antibiotics, antineoplastics, anticoagulants, and anti-inflammatory agents must be evaluated for possible effects on the efficacy of the gene therapy product.

PATIENT TREATMENT

Some gene therapy products are patient-specific because they are manufactured from a selected tissue source, such as autologous, selected allogeneic, or xenogeneic tissue. Certain patient-specific products have a defined potential for benefit or adverse immunoreactivity. Systems must be in place to prevent administration of such a product to the wrong patient. Recommended systems include procedures similar to those used for administration of human blood products, including special attention to the correct identification of the patient and patient-specific product by at least two people immediately before administration. These issues

are addressed in greater detail in <1046>. Gene therapy products can be administered by a variety of routes. These include parenteral injection, inhalation, and gastrointestinal routes. Other possibilities include direct application of gene therapy products into regional vasculature, organs, tissues, or body cavities by means of needles or catheters or following surgical exposure of the tissue. Although parenteral administration can be accomplished in routine outpatient or inpatient facilities, the other means of administration may require specialized facilities such as an aseptic operating theater or endoscopic suite. In all cases, standard operating procedures and a quality program must be in place to ensure that the product is administered in the intended manner.

POST-ADMINISTRATION MONITORING OF PATIENT

There should be written policies and procedures for monitoring patient outcomes and managing reports of adverse events. Patient outcome assessment should include indicators that are likely to detect errors or problems related to the entire manufacturing process, with special attention to manipulations, storage, or transportation after the initial manufacture of the product. Management of adverse reactions should include procedures for ensuring prompt medical evaluation and treatment of patients with suspected adverse effects and a system for reporting and evaluating adverse effects that may point to a potential defect in the administered product. Reporting procedures include providing details required for federal, state, or USP adverse-event reporting programs.

Follow-up and monitoring procedures should be implemented for patients who have received gene therapy vectors or ex vivo gene therapies. To the extent that it is relevant and that it can be assessed, vector or gene-modified cell biodistribution and persistence in vivo should be monitored. With direct administration of vectors, localization to the germ line may be an issue. Although preclinical studies can address this issue, useful information may be gained by patient monitoring. When a retroviral vector has been administered, patients should be monitored for replication-competent retrovirus (RCR) according to FDA's *Guidance for Industry: Supplemental Guidance on Testing for Replication Competent Retrovirus in Retroviral Vector-Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors* (October 2000). This involves active monitoring during the first year and archiving of patient samples thereafter if RCR is not detected initially.

Database systems to collate and track patient-monitoring results are essential to management of this information. National registries or publication of data should be considered for establishing the collective safety of gene therapy.

ANALYTICAL METHODS

The complexity and scope of gene therapy products are reflected in the wide range of analytical procedures and their methods that are used to assess product quality. Approved gene therapy products must comply with applicable sections of 21 CFR 211 and 610 to ensure their identity, dose, potency, purity, and safety. Specific guidance for the identification, development, and validation of analytical methodologies to support cell and virus bank characterization, final-product release, and stability studies is currently provided in FDA guidelines for gene therapy manufacturing and testing (see *Appendix*); in *Validation of Compendial Procedures* <1225>; and in the ICH guidelines Q2(R1) and Q6B. Most product-specific analytical methods for gene therapy products have not been standardized. Even well-defined tests such as those described under *Sterility Tests* <71> may not be directly applicable to certain gene therapy products. For some gene therapy products, large quantities of clinical material may not be available during early clinical develop-

ment. Some required tests (e.g., sterility) may require modification. Consultation with regulatory authorities is advised.

Table 5 provides an overview of product-specific testing parameters for the biological component and general methods or approaches used to satisfy the testing requirements for nonviral, viral, and gene-modified cellular gene therapy products. The analysis of gene therapy products relies heavily on biological assays, but it also uses methodologies developed for biotechnology-derived products. The intent of this section is to outline the types of methods and their specific applications with regard to product characterization, stability, and release testing. Process validation may alleviate the need for certain specific lot-release tests. Development of appropriate reference materials and standards for viral, nonviral, and gene-modified cellular gene therapy products should be a part of product development. Reference materials should be fully characterized in order to provide continuity between standards over time. In the case of gene-modified cellular gene therapy products, the reference material may be a surrogate tissue or simulated product. Reference materials are briefly addressed in the *FDA Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products*.

Gene-modified cellular gene therapy products may require a rapid-release approach if they have a limited shelf life (see *Cellular and Tissue-Based Products* (1046)). The rapid-release approach is not usually applied to viral and nonviral gene therapy products because these products are sufficiently stable for completion of testing before release. Some formulated nonviral gene therapy products also have limited shelf lives. In such cases, the individual components are tested before release and the formulated complex is not

tested. The formation and stability of the formulated nonviral gene therapy complex is established via validation studies during product development.

As specified in CFR, product samples must be retained after product-release testing is completed. Retain additional samples if rapid-release strategies are employed so that product quality can be reassessed by alternative or traditional test methodologies if necessary.

Sampling Issues

Sampling for lot-release testing should be based on the potential distribution for the parameter tested. See *Stability Protocol Development* under *Stability* (below) for additional considerations. Samples from each lot should be retained in case of a safety or quality issue with the lot. Even if the product has a very short shelf life, these retained samples can be used to detect impurities and other substances. The need for proper design of the sampling scheme is highlighted in safety testing for adventitious agents or in assessment of RCV for gene-modified cell or viral gene therapy products. In such cases, process validation assists in determining the appropriate statistically based sampling design.

Safety

Safety testing for gene therapy products focuses on three issues: (1) detecting contamination from adventitious sources during product processing, (2) preventing the use of packaging cell lines and plasmids that potentially permit ge-

Table 5. Analytical Tests for Cell and Gene Therapy Biological Products

Test	Gene-Modified Cellular Gene Therapy Product	Gene Therapy Products	
		Viral	Nonviral
Identity of Biological Substance	<ul style="list-style-type: none"> • Surface marker determination • Species • Morphology • Bioassay • Biochemical Marker 	<ul style="list-style-type: none"> —Restriction enzyme map —PCR —Immunoassay for expressed gene —Sequencing 	<ul style="list-style-type: none"> —Restriction enzyme map —PCR —Immunoassay for expressed gene —Sequencing
Dose	<ul style="list-style-type: none"> • Viable cell number • Enumeration of specific cell population • Total DNA • Total protein 	<ul style="list-style-type: none"> —Particle number —Transducing units (DNA hybridization assay) —Total protein —HPLC assay using authenticated reference standard 	<ul style="list-style-type: none"> —Plasmid-DNA weight —Formulated-complex weight HPLC or capillary electrophoresis assay using authenticated reference standard
Potency	<ul style="list-style-type: none"> • Viable cell number (cells intended for structural repair) • Bioassays <ul style="list-style-type: none"> — Colony-formation assay — Function of expressed gene — Induction of secondary effect (e.g., human leukocyte antigen (HLA) induction, secretion of cytokines, and up-regulation of surface marker) 	<ul style="list-style-type: none"> —Function of expressed gene (induction of secondary effect and other bioassays) 	<ul style="list-style-type: none"> —Function of expressed gene (induction of secondary effect and other bioassays) other bioassays)
Purity	<ul style="list-style-type: none"> • Percentage of viable cells • Percentage of transduced cells • Percentage of cells with specific surface marker • Process contaminants (e.g., serum) 	<ul style="list-style-type: none"> —Residual host-cell DNA —Process contaminants (e.g., serum and cesium chloride) —Residual helper virus —Optical density ratio —Residual host-cell proteins —Viral protein profile (HPLC assay for defective or immature particles) —Residual RNA 	<ul style="list-style-type: none"> —Percentage of specific physical form (e.g., percentage supercoiled) —Residual host-cell DNA —Residual RNA —Residual host-cell proteins —Residual solvents —Optical density ratio —Process contaminants (e.g., cesium chloride)
Safety	<ul style="list-style-type: none"> • Mycoplasma • Sterility • Pyrogen and endotoxins • Adventitious viruses • Residual virus • Replication-competent vector 	<ul style="list-style-type: none"> —General safety —Sterility —Pyrogen and endotoxins —Adventitious viruses —RCV 	<ul style="list-style-type: none"> —Mycoplasma —Sterility —Pyrogen and endotoxins

netic recombination between vectors and the packaging cell lines or plasmids—or among the vectors themselves, and (3) testing the final product to ensure a safe level of undesired genetic and/or structural variants or other viruses used in processing.

The primary means of assessing safety are the performance of biological assays to measure adventitious agents directly. Molecular biology-based assays that measure adventitious agent DNA or RNA or detect undesired genetic variants are also used. Although live genetically engineered vectors officially fall outside its scope, the detailed information available in the ICH Q5A guideline, presented in chapter (1050), should be consulted because the principles apply.

VIRAL GENE THERAPY PRODUCTS

One of the primary safety concerns associated with viral vectors used for gene therapy is the occurrence of undesired genetic variants. Among them the most critical type, and probably the best studied, is RCV. RCV is more clearly defined for replication-incompetent viral vectors, but for conditionally replication-competent viruses it refers to undesired genetic variants that have lost selectivity toward the target cells and thus might raise safety concerns. Regardless of the virus, these concerns are based on the potential lack of predictability for the pathogenicity of a contaminating virus for a specific route of administration, particularly if it is not the normal route of infection or if humans are not a natural host for the virus. The pathogenesis of a wild-type adenovirus infection is known but may not be predictive for the routes of administration employed with recombinant adenoviral vectors. For replication-incompetent adenoviral vectors, a limit of one RCA per 3×10^{10} viral particles is currently considered acceptable (see the *FDA Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)*).

Typically, RCA levels are determined by a cell-based assay that allows amplification of the RCA while preventing replication of the product. The cell line most often used for amplification and detection of RCA is the A549 cell line. However, some recombinant adenoviral vectors express therapeutic genes that interfere with analysis on A549 cells. In such cases, a bioassay-based two cell lines is used. The first cell line is chosen on the basis of resistance to the effects of expression of the transgene and with subsequent passage of cell lysate or supernatant onto A549 cells for amplification and detection of the RCA. RCA is most often detected by visual observation of the cytopathic effect, but it can also be detected in the A549 cell culture by immunological or PCR-based methods.

Quantitation of the RCA level is based on the quantity of sample tested and the detection limit of the assay. Typically, RCA bioassays are validated as being able to detect 1 plaque-forming unit or infectious unit of RCA in the test sample over a wide range of test-sample sizes. Test-sample sizes can range, but they are typically based on the FDA RCA acceptance limit. To verify detection limits, include spike controls as part of the test, even with validated assays. For recombinant adenoviruses produced using HEK293 cells, RCA detection by PCR on the final products or the progeny virus amplified in HEK293 cells can be confounded by detection of residual HEK293 host-cell DNA (detection of the E1 region). PCR assays, however, can be designed to specifically quantitate host cell DNA contamination and can be made specific to particular forms of slow-growing RCA. Quantitative PCR assays can be used in conjunction with a cell-based method for precise quantitation of RCA levels. When a tested sample is found to be positive, the identity of the RCA is usually confirmed by conducting PCR analysis. This rules out the possibility that contamination of the assay by exogenous wild-type adenovirus or other adventitious agents is responsible for the positive result.

For conditionally replication-competent adenoviruses or other replication-competent viral vectors, testing for RCV or undesired genetic variants is usually more complicated and vector specific. Usually one or two nonpermissive cell lines that are not target cells are infected with the replication-competent virus in attempts to produce progeny virus. In order to generate a sufficient quantity of progeny population for analysis, analysts subject the infection to multiple passages and extended culture time. Two normal fibroblast cell lines that are easy to culture, WI-38 and MRC-5, have been used as the model nonpermissive cell lines for detecting RCA in replication-competent adenovirus products. Even after multiple passages on the nonpermissive cell lines, it may be necessary to amplify the progeny (which tend to appear only in minute quantities) in permissive or packaging cell lines to a sufficient quantity for subsequent testing. The resulting progeny should be tested for changes in biological selectivity and genetic composition. Usually the genetic characterization of the progeny population includes restriction enzyme mapping followed by Southern blotting, PCR, or nucleotide sequencing. After the genetic elements unique to RCV or undesired genetic variants are identified, quantitative PCR assays can be designed to monitor the level of RCV after amplification in nonpermissive cell lines or sometimes, if the sensitivity is adequate, directly in the final product without biological amplification. Using a spike control in the biological assay for detecting RCV is encouraged but may not be applicable to all cases. Currently there is no specified acceptable limit of RCA for conditionally replication-competent adenovirus, although clinical safety has been reported for an oncology application with several thousands of RCA per dose.

For retroviral vectors, testing for RCR is required for cell banks, viral vector production lots, and any resulting ex vivo product lots (see *FDA's Guidance for Industry: Supplemental Guidance on Testing for Replication-Competent Retrovirus in Retroviral Vector-Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors*). Standard assays have been designed to detect replication-competent MLV. The pathogenesis and potential long-term toxicity of low-level amphotropic MLV in human beings is not known. Methods commonly used to detect RCR include an amplification of virus titer by application of product to a replication-permissive cell line such as Mus dunni. Because infection is limited by the ability of a virus to reach the cells by means of Brownian motion, procedures (e.g., centrifugation and filtration) that physically bring the virus into contact with the cells can be used to enhance detection. However, high-titer recombinant vector can interfere with the detection of low-level RCR, and this interference may be enhanced by such methods. Infected cells are passaged several times to allow viral replication. Culture medium is harvested at the end of the culture period, and RCR is detected by using an indicator cell line. If the product is an amphotropic MLV, RCR can be detected by using a feline cell-based PG4 S+L- assay, a mink cell-based MiCl S+L- assay, or a marker rescue assay. In S+L- assays, the RCR expresses proteins that lead to transformation and subsequent plaque formation on the monolayer. In a marker rescue assay, RCR infects a cell line that expresses a retroviral vector encoding a marker gene such as β -galactosidase, drug resistance, or a fluorescent protein. The vector is packaged by the proteins supplied to it in trans by the RCR. The potentially vector-laden supernatant is transferred to naive target cells that are then screened for expression of the marker vector.

Testing for RCR is performed by co-cultivation of the cell line or amplification of vector supernatant with an RCR replication-permissive cell line, typically *M. dunni*, for several passages. Culture medium is harvested at the end of this co-cultivation process and applied to an appropriate indicator cell line as described above. Note that artifacts may be generated during the co-cultivation assay by expression of an endogenous virus in the permissive cell line or by fusion if the vector-producing cell line is cultured directly with a marker rescue cell line. In addition, co-cultivation may not

be possible for ex vivo cell products that have specific culture requirements or limited culture life spans.

Methodologies for testing the presence of RCR in crude, purified bulk or final vector products are not specified. FDA has deposited a reference standard of an amphotropic hybrid MLV with the American Type Culture Collection (ATCC). This viral stock has been assigned a label titer and should be used in assay validation. Method validation should demonstrate the ability to reproducibly detect a single RCR particle in individual product types because the product and its related impurities can interfere with the detection of RCR. Currently, there are no acceptable limits for RCR contamination in products. Any product lot found to contain RCR cannot be used for human use. Reference standards for assessing RCV in other viral vectors including ecotropic, xenotropic, or pseudotyped MLV, adenovirus, and lentivirus have not been developed. The adenovirus reference material, which consists of wild type human adenovirus type 5, has been used as a spike control and during validation of RCA assays, but this practice may not be applicable to all RCA assays. Amplification and detection of replication-competent HIV, especially its pseudotyped variants, may warrant special containment and handling procedures.

Additional safety testing usually focuses on methods similar to those described in *Biological Reactivity Tests, In Vivo* (88), *Safety Tests—Biologicals*, and chapter (71). For viral gene therapy vectors produced using a human cell line, performance of the in vitro adventitious agent bioassays using three cell lines is recommended. For adenoviral vectors, specific tests for adeno-associated virus are also recommended. For adeno-associated virus, specific tests for adenovirus and herpes virus are recommended. Material for testing should be derived from the stage of manufacture that provides the greatest chance of detection, which could be pre-bulk (e.g., late-stage fermentation), the bulk, or the final product.

NONVIRAL GENE THERAPY PRODUCTS

Safety testing usually focuses on methods similar to those described in chapters (88) and (71). Safety testing should be performed on nonviral formulated material. If the shelf life of the formulated nonviral product is very short, then the components should be tested individually.

Safety testing for undesired genetic variants that might emerge during the manufacturing process in nonviral gene therapy products is similar to that for RCV testing for viral vectors but with more vector-specific considerations. Typically, molecular biology-based methods are applied to the final product to test for variants. When genetic stability is established by process validation, the assays for monitoring the levels of undesired genetic variants may be limited to restriction enzyme mapping followed by further confirmation of critical genetic elements (such as transgenes or regulatory elements) by PCR or Southern blotting.

Dose-Defining Assays

An assay that precisely measures the amount of the product is referred to as a dose-defining assay, and it is selected on the basis of its accuracy and precision. An assay that measures therapeutic activity of the product is referred to as a potency assay, and it is designed to measure product function. The design of the assay depends on the type of product. In the case of chemical and protein drugs, the assays measuring the amount of active ingredient (dose) are referred to as strength assays. Product dose can be defined as the concentration or amount of the drug product administered to the patient, and it is typically measured as product mass.

Particle concentration is a commonly used measure for viral vector product dose. Particle concentration may be measured by physical, biophysical, or in vitro cell-based assays. For example, quantitation of purified adenoviral parti-

cles may be determined by using the optical density of a solution of virus in 0.1% (w/v) sodium dodecyl sulfate (SDS) solution at 260 nm, because a relationship between absorption and particle concentration has been published for adenovirus. The particle number concentration is equivalent to the product of the absorbance at 260 nm in a 1-cm cell, the dilution factor, and 1.1×10^{12} particles. A method that has become standard in determining particle concentration is integration of viral peak area of 260 nm and/or 280 nm absorbance against an authenticated reference standard in an anion-exchange resin-based high-performance liquid chromatographic (HPLC) assay. Compared to the optical density method, the HPLC method has the advantage of eliminating the interference of free DNA and/or capsid proteins on quantitation of viral particles. An adenoviral reference material (ARM) from ATCC has an HPLC-determined particle concentration established from a large-scale collaboration that involved many laboratories. Whenever possible, the ARM should be used to calibrate the internal HPLC method and reference material.

Virus concentration can also be assessed by the measurement of selected structural proteins with known molecular masses and known copy numbers within the virion. For this method, the virus must be lysed, and the structural proteins must be separated by using an appropriate, high-recovery chromatographic procedure (e.g., reversed-phase HPLC). The chromatographic separation and the identity and the purity of the selected structural protein must be verified during assay validation by methods such as SDS polyacrylamide gel electrophoresis (SDS-PAGE), peptide sequencing, and mass spectroscopy. The selected structural proteins can be quantified, for example, by integrating chromatographic peaks at 214 nm and comparing the area to that of an authenticated reference standard. The virus concentration can then be calculated based on the molecular mass, the copy number, and the measured mass of the protein. Very importantly, the virus concentration can be estimated simultaneously for multiple structural proteins, which allows the use of this assay in relatively impure virus preparations. This method has been applied to adenovirus and should be applicable to other viral vector types.

Biophysical methods of determining particle number include direct quantitation of vector nucleic acid by radio-labeled-probe hybridization and indirect quantitation by amplification of template nucleic acid (e.g., PCR and RT-PCR) or by signal amplification (e.g., branched-chain DNA using multiple-probe hybridization).

In cases where biophysical methods are not available, bioassays that measure gene-vector titer have been used. These involve infection, transfection, or transduction of a susceptible cell line in vitro, followed by some measure of the product uptake. Methods for quantitation or estimation of the number of infection, transfection, or transduction events include plaque-forming unit assays, tissue culture infectious dose assays based on cytopathic effect of 50% (TCID₅₀) or immunofluorescent detection of an expressed vector protein, or a quantitative DNA-hybridization assay. Examples follow: For replication-competent adenoviral gene therapy products, the ARM available from ATCC has a defined range of TCID₅₀ titer determined via a collaborative effort. Whenever applicable, it should be used in validation of an internal reference standard or assay control of infectious titer assays. However, because of the likelihood of genetic differences between the ARM, which is wild-type human adenovirus type 5, and the replication-competent adenoviral gene therapy product, it may not be reasonable to normalize the titer of the vector of interest to that of the ARM.

For retroviral or lentiviral gene therapy products or AAVs that carry a selectable marker (e.g., that for neomycin resistance) or a reporter gene (e.g., β -galactosidase) in addition to the therapeutic gene, the infectious titer is commonly determined by measuring the number of transduced or infected cells expressing these nontherapeutic proteins. Vector titer is typically reported as the number of colony-forming units (cfu) per mL for cells transduced with viral vectors that

contain drug-resistance markers and are selected for growth in drug-containing medium. Titer based on β -galactosidase can be expressed in terms of blue cfu per mL after staining and counting the cells that convert the β -galactosidase substrate X-Gal into a blue chromophore. For vectors without a marker gene, quantitation of transduction has been measured precisely by using quantitative PCR or has been estimated by hybridization methods.

Most nonviral gene therapy products contain plasmid DNA, and their usual measure of dose is the DNA mass. The DNA mass may be determined in the formulated state, and, if recombinant protein is included in the formulation, the total combined mass of all formulation components based on a specific ratio can be used. DNA concentrations greater than 500 ng/mL are most simply determined by using optical density measurement at 260 nm. This method is not generally applicable to lipid-formulated DNA. Because RNA and proteins also have significant absorbance at 260 nm, other analyses must be performed to demonstrate minimal contamination with RNA, protein, or residual host-cell chromosomal DNA. Dyes that specifically bind to double-stranded DNA allow accurate measurement of DNA concentrations of less than 500 ng/mL when calculated against an authenticated DNA standard curve. PicoGreen is one such fluorescent dye, and it is minimally affected by single-stranded DNA, RNA, proteins, salts, and detergents. The fluorescent dye Hoechst 33258 also binds to both double-stranded and single-stranded DNA and it can be used to determine DNA concentrations as low as 0.3 ng per mL. The Hoechst 33258 does not bind to protein or RNA, and it can accurately determine the DNA concentrations in crude samples.

Methods such as capillary electrophoresis and HPLC employing an authenticated reference material can also be used to determine the strength of nonviral products.

Potency

Potency is defined as the therapeutic activity of the drug product. Together with dose, potency defines the biological activity of each lot (see *Dose-Defining Assays*). Potency can be assessed by in vitro or in vivo bioassays. It is not uncommon for these assays to have coefficients of variation between 30% and 50%, although stringent assay design with good statistical consideration could help reduce assay variation. These assays require a well-defined, representative reference material that can be used as a positive control for the assay and/or in calculation of the relative potency of the test article. The general consideration for bioassays in current USP chapters on design and development of biological assays should be applied to the potency assay design for gene therapy products. The positive control qualifies the performance of an individual assay. Potency assay development should focus on characterizing and controlling variability. High-precision assays are more effective tools in monitoring product quality. Information about potency assay variability should be incorporated into the stability study design and the proposed statistical approach to assignment of expiration date (see *Stability*).

Bioassays employed to measure the potency of viral and nonviral gene therapy products generally involve infection, transfection, or transduction of a susceptible cell line in vitro, followed by some functional measure of the expressed gene of interest. Functional assays for the therapeutic gene (e.g., those measuring enzyme activity and cell growth stimulation or inhibition) should generally be used instead of analytical methods such as enzyme-linked immunosorbent assay (ELISA). When the biological function of the expressed transgene exhibits a broad range of activities or only generates semiquantitative results, the ELISA or other immunological or biochemical readouts can be used as a surrogate potency assay with a tight specification range if extensive characterization data is available to demonstrate that all expressed protein is biologically active. For example, in the

case of a gene therapy product expressing a cytokine, cytokine expression is usually quantified by ELISA first, and the result is used to adjust the sample dilution for the functional assay. The potency of such vectors may be better controlled by the ELISA quantitation results, but the biological activity of expressed cytokine could be used to verify that the measured mass is biologically active without the requirement to meet a narrow specification range for the biological activity itself.

HPLC or flow cytometry, which provide information about the level of expression but only infer function, have also been used in a context like that described for immunoassays. In addition, for viral vectors, infectious titer measurements by themselves are generally not considered an adequate measure of product potency. For example, the TCID₅₀ titer or plaque-forming-unit assays for adenoviral vectors on HEK293 cells can indicate that the infectivity of adenovirus is preserved but do not confirm that the adenoviral product has maintained full biological function(s), especially transgene biological activity. The design and ultimate suitability of an assay system for determining product potency depends on the relationship between the intended human target cell in vivo and the following: (1) the transduction or transfection efficiency of the cell line used in vitro; (2) the protein expression levels; and, (3) the duration of expression required for the therapeutic effect.

In vivo tests can also be used to measure vector-product potency. Readouts can be based on a response per animal (e.g., blood levels of therapeutic protein 24 hours after treatment) or a group response rate (e.g., percentage of animals that elicited an immune response or survived virus challenge). The availability of an appropriate in vivo test system depends on the vector-host range (for viral vectors), the pharmacokinetics and biodistribution of the vector and the resulting gene product relative to its human counterpart, and the time frame required to observe the therapeutic effect or surrogate. Issues of cost, facilities, validation, and ethics determine the practicality of an in vivo potency test.

Purity

Analytical methods that separate, isolate, and specifically quantify the intended active product components determine product purity. Impurities are either product- or process-related components that can be carried through to the final product. The manufacturing and purification process should be optimized to consistently remove impurities while retaining product activity. The requirement to test for a particular impurity for product lot release depends on the following: (1) the demonstrated capability of the manufacture and purification process to remove or inactivate the impurity through process validation, and (2) the potential toxicity associated with the impurity.

Examples of process-related impurities associated with gene therapy products include residual production-medium components (e.g., FBS, antibiotics, cytokines, and *E. coli* chromosomal DNA in a plasmid product), ancillary products used in downstream processing (e.g., nucleases such as DNase I), and residual moisture for lyophilized vector products. Impurities may be bioactive (e.g., cytokines and hormones) or immunogenic (e.g., product aggregates, degradation products, plasmid-selection markers, and nonhuman-derived proteins), or they may have other deleterious effects (e.g., they may compete with the product) if administered at a dose equal to that of the product. Product-related impurities are specific to each product type. Examples include nicked plasmid forms in nonviral products and defective or immature virus particles in retroviral or adenoviral vector products. Analytical methodologies to assess purity require quantitation or physical separation of intended product from its impurities. Common sense should drive the need to quantify specific impurities. Suitable validation of the manufacturing process may limit the need for specific lot-release

testing for impurities. Manufacturers may place an emphasis on demonstrating the consistency of the product-impurity profile.

Testing for impurities is often extensive during product characterization and process validation when the consistency of the manufacturing and purification process is being demonstrated. Testing for impurities as part of lot-release testing should reflect the safety risks associated with the impurity and the ability of the process to consistently remove that impurity.

VIRAL GENE THERAPY PRODUCTS

Product-related impurities for viral vectors include aggregates and defective and immature particles that may be produced during the manufacture or purification of the recombinant vector. Aggregates of vector may form if the product is highly concentrated, stored under certain conditions (e.g., under a certain pH or temperature), or reconstituted after lyophilization. Assays to detect aggregates include particle size analysis by laser light-scattering and the use of nonreducing, nondenaturing PAGE, followed by staining of the gel or transfer and detection of viral proteins by Western blot analysis. Sedimentation rate analysis also allows separation of aggregates from monomers based on size. Optical density analyses of light scattering are also used to assess vector aggregation.

Defective particles are viral particles that do not contain the appropriate recombinant genome—that is, they contain some other nucleic acid or contain no genome at all, or the vector has some missing, defective, or otherwise altered structural component that impairs its ability to transduce a cell. For viral vector systems that have capsomeric symmetry that requires the appropriate nucleic acid incorporation for configuration, empty particles may be readily distinguished from those carrying genomes. For enveloped viruses, empty particles may not be as readily separated from those with encapsidated nucleic acid.

For some viral vector products, active viral particles can be separated from defective particles by using analytical HPLC. Anion-exchange resins have been used to separate active adenovirus from defective virus particles. However, this method might not be useful for an adenoviral vector purified by anion-exchange chromatography unless the resin for the assay is different from that used during manufacture. Depending on the nature of the viral vector and its nonactive or defective forms, other methods of separation, such as equilibrium centrifugation in a cesium chloride density gradient, may need to precede the quantitation of the active particle. Ideally, the method of separation will allow quantitation.

Defective particles that carry a non-cell-derived oncogene or other undesirable genes may pose a special concern. For example, in murine-based retroviral packaging cell lines, small viral elements called VL30 sequences can be packaged in about one-third of all particles. Assays may be needed to quantify specific defective particles if they are known to be present in quantities sufficient to pose a safety concern.

Virus quality and the comparability of preparations can also be assessed by measuring selected structural proteins with known molecular masses and known copy numbers within the virion. For this method, the virus is lysed, and the structural proteins are separated by using reverse-phase HPLC or some other high-recovery chromatographic procedure. The chromatographic separation should be validated, and the identity of the selected structural proteins should be verified by methods such as SDS-PAGE, peptide sequencing, or mass spectroscopy. Fingerprinting of the batch can be conducted based on quantification of the selected structural proteins and comparison to a reference standard. When the method incorporates mass spectroscopy, impurities such as structural variants can also be identified. For adenovirus preparations, some precursor and most mature virion pro-

teins can be detected and distinguished, thus allowing monitoring of the product and of the immature virion forms.

Host cell-derived proteins may be considered impurities for some viral vector products and may be separated and quantified by PAGE or HPLC or detected by amino acid analysis, Western blot, or immunoassay-based methods. However, for enveloped viruses such as retroviruses, host cell-derived membrane proteins are an integral part of the product. In those vector systems, it may be difficult to determine the presence of contaminating exogenous host-derived proteins.

Presence of specific process-related impurities depends on the manufacture and purification process of each vector or product type. However, most products need to be tested for residual endotoxins (see *Bacterial Endotoxins Test* (85)). Acceptable limits of endotoxins have been determined and can be directly applied to viral vector products. Although genomic DNA derived from continuous cell substrates used to manufacture biological product historically has been considered potentially tumorigenic, recent studies suggest that the risks are very low. However, every attempt should be made during process development to reduce levels of contaminating DNA. The need to test for residual DNA as part of product lot release should be evaluated on a case-by-case basis and may depend on the size distribution of the DNA, its association with the product or its formulation components, and the product's route of administration. Quantitative PCR assays can analyze the amount of residual host-cell DNA by using primers designed to amplify evolutionarily conserved and abundant target sequences such as 18S for HEK293 cells.

Quantitation of residual serum components such as bovine serum albumin (BSA) can be achieved by using ELISA and a BSA reference standard. Researchers may need to develop specific functional or immunological methods for other ancillary products, including other culture media or purification process components such as cytokines or enzymes (e.g., deoxyribonuclease 1 or benzon nuclease).

NONVIRAL GENE THERAPY PRODUCTS

A plasmid used as a drug substance is considered a well-characterized biologic, and key impurities from the manufacturing process are well known. Testing is usually performed on each individual component: the plasmid DNA, lipid or lipoplex reagents, and protein components if any are present in the formulation. Plasmid DNA is characterized for a variety of impurities, including residual host-cell DNA, residual RNA, and residual protein. Residual protein testing is frequently included in lot-release testing. Optical density ratios (usually the measurement at 260 nm to that at 280 nm) are frequently used in purity specifications for plasmid DNA.

In addition, the plasmid DNA should also be characterized with regard to its conformation in solution. Plasmid DNA exists as monomeric supercoiled, relaxed monomer, and linear forms. Because all forms can be generated during large-scale fermentation, and data about their relative in vivo potency is scarce, the relative quantity of each form is monitored to verify batch-to-batch consistency in the relative amounts of each conformation. Agarose gel electrophoresis can resolve each of these forms but is not highly quantitative for each individual species. Analytical anion-exchange HPLC serves as a quantitative assay for monomeric supercoil and other forms, including concatamers. Other analytical methods that have been valuable for characterization of plasmid constructs during process development and validation such as capillary gel electrophoresis, linear-flow dichroism, and atomic-force microscopy are also viable methods to assess the purity of a given plasmid preparation. The most appropriate method for lot release depends on how each plasmid conformation affects product potency. Specific details for each of these methods are outlined in *Nucleic Acid-Based Techniques—General* (1125).

Tests should be conducted for process-related impurities such as residual organic solvents (phenol, alcohol), salts, and certain antibiotics such as kanamycin used during the fermentation process. Lipid and lipoplex formulation components must also be tested for their chemical purity. Testing for specific chemical impurities is commonly performed by using gas chromatography–mass spectroscopy (GC–MS), HPLC, or thin-layer chromatography (TLC) methods. If protein is part of the formulated complex, then the protein must also be tested for purity. HPLC is capable of detecting trace amounts of residual antibiotics and can therefore be used during process validation or lot-release testing to confirm that they have been effectively removed. The specifics of these methods are outlined in *Biotechnology-Derived Articles—Peptide Mapping* <1055> or in *Biotechnology-Derived Articles—Total Protein Assay* <1057>.

Bacterial protein, DNA, RNA, and endotoxins are the major types of host-derived process contaminants. Standard protein assays (e.g., Lowry, Bradford, or Coomassie), PAGE followed by silver staining or Western blot analysis, or ELISA can be used to detect residual host protein in the nanogram range. Host chromosomal DNA can be detected by slot blot hybridization (detection in picogram range) or by real-time PCR (detection sensitivity < 1 pg) using highly conserved target sequences (e.g., 18S for *E. coli*). PCR assays for this purpose must use recombinant polymerases that are highly purified to minimize residual bacterial DNA for which the presence can create background signals. PAGE or agarose gel electrophoresis followed by fluorescent dye staining can be used to detect residual RNA. Quantitation may not be required because of the labile nature of RNA and the low-level toxicity associated with it. The *Limulus* amoebocyte lysate (LAL) test is the most sensitive and widely used method for endotoxins determination. Colorimetric assays offer sensitivities of 0.005 EU/mL. Details of the methods described here are outlined in <1057>, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* <1056>, *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids* <1130>, <1125>, and <85>.

LYOPHILIZED VIRAL AND NONVIRAL VECTOR PRODUCTS

Residual moisture can affect the stability of a lyophilized vector product. FDA's *Guideline for the Determination of Residual Moisture in Dry Biological Products* recommends a 1% residual moisture level, although data indicating no adverse effects on product stability at higher levels is considered acceptable. Residual moisture levels can be determined by using a standard method (see *Water Determination* <921>) that is compatible with the formulated product.

Identity

Lot-release testing for gene therapy products must include an identity test. This test clearly identifies the product and confirms that inadvertent substitution with another product has not occurred. The complexity of the identity test depends on the nature of the specific product and the array of products being manufactured. For example, more extensive and rigorous testing may be performed for an autologous gene-modified cell therapy product at a facility where multiple patient products are manufactured than for a viral vector product produced at a site that manufactures a single vector product.

VIRAL GENE THERAPY PRODUCTS

For characterization purposes, restriction enzyme mapping and sequencing of the transcription unit DNA are the most commonly used approaches. PCR-based methods, restriction enzyme mapping, and transgene-expression-based immunoassays are commonly used to confirm the identity during lot-release testing.

NONVIRAL GENE THERAPY PRODUCTS

Restriction enzyme mapping is the most common identity method for plasmid-based products. The number of enzymes used to create the vector fingerprint will vary according to the complexity of the DNA and the degree of similarity between multiple products. If lipids, lipoplex agents, or proteins are used to formulate the DNA, then their identity must also be tested and confirmed. Lipids and lipoplex components may be identified by procedures used for traditional pharmaceuticals, such as GC–MS and TLC. Protein components of the formulation may be identified by peptide mapping or other means outlined under <1057>.

STABILITY

The shelf lives of gene therapy products vary widely depending on the nature of the product, its intended clinical use, its specific attributes, and the recommended storage, packaging, and shipping conditions. Therefore, it is difficult to draft uniform guidelines regarding stability-study duration and testing frequency for all products. In all cases, the study should be designed on the basis of scientifically sound principles and approaches and a comprehensive understanding of the final therapeutic product and its intended use. The product's stability during in-process hold steps, cell and virus banks, critical raw materials, and reference standards also must be assessed. A well-designed and executed stability program will provide a high degree of assurance that the product is stable within the specified shelf life.

For viral and nonviral vector gene therapy products and gene-modified cellular gene therapy products that are not patient specific, the selection of batches to support license application and final-product labeling should be carried out in accordance with the principles of stability testing, such as those described in ICH guideline Q5C and chapter <1049>. Stability data should also be collected for bulk material and at other in-process points if material is stored before final processing and filling. Issues related to the stability of cell-based products are addressed in chapter <1046>.

Nonviral DNA plasmid vectors are often formulated with specific mixtures of lipids, proteins, or lipoconjugates to form liposomes or encapsulated complexes. Depending on the formulation, a shelf life of hours to years can be attained. Where a product has a short shelf life, the final formulation may require preparation at the clinic just before administration. Instability is frequently observed as aggregation and precipitation. Formation and stability of the formulated complex should be characterized and established by validation studies during product development. Stability data should also be collected for major components of the formulated complex, such as the lipids, the liposomes, and the DNA itself.

Stability-Protocol Development

Stability studies verify that the storage conditions maintain the purity and potency of the product for a defined period so that product administered to the patient is still capable of meeting the stability specifications. These specifications may differ from the manufacturing release specifications, but they must be verified with clinical data. Formal stability studies to support licensure as well as plans for gathering early-phase product stability information should be detailed in a written plan that describes how data will be collected and analyzed to support the product's expiration period. Protocols should follow the format recommended in existing guidelines and should include the scope, storage conditions, number of lots that will be tested, test schedule, assays that will be used, data analysis, and product specifications. Any assay used in a formal stability study for licensure must be validated before the study begins. The specific study design should take into account the problems the product may en-

counter during manufacturing, shipping, and processing at the clinical site (see *Accelerated and Most Appropriate Challenge Conditions*, below). The study design should also incorporate the latest knowledge in the biological sciences and should address existing regulatory requirements. For instance, if the product's final formulation is performed at the clinical site, stability studies on this final formulation should be performed to establish the time and conditions under which the product can be held.

Stability assessment should include an evaluation of product functionality (potency). The potency assay often has a high degree of inherent variability. Measuring and calculating the decay of product activity by employing the standard statistical methodologies may require multiple, frequent sampling intervals over an extended period of time and may require analysis of more than three production lots to compensate for assay variability. Initial studies to establish a provisional expiration date must be conducted before administration to the first patient. Initial studies are also useful for determining which assays are stability indicating, that is, the best indicators of product degradation. Because existing compendial methods do not address the unique characteristics of gene therapy products, the development of assays that would address these unique characteristics is encouraged.

Accelerated and Most Appropriate Challenge Conditions

The stability-indicating profile of a gene therapy product varies over time under the influence of a wide variety of environmental conditions, including temperature, extremes in physiological storage conditions, and light. Multifactorial degradation pathways must be considered when researchers investigate the effects of these parameters on the stability of the product. Studies should include conditions that are outside the specified storage ranges, that is, challenge conditions such as those encountered during periods of abnormal storage, shipping, or handling. Examples include brief incubator malfunctions, incubator or cold storage failure, periods of extreme temperature fluctuation due to shipping to hot or cold climates, hypobaric conditions experienced in the cargo hold of a commercial airliner, or temperatures likely to be encountered in the surgical suite. A short exposure to an environmental condition well outside of an established limit and a long exposure to an environmental condition just outside of an established acceptable range may be equally detrimental to the overall stability profile. The slow and constant rate of product degradation at a specified temperature may increase if a different set of storage conditions is applied. The effect of light on the stability-indicating profile should be investigated if it is scientifically warranted. Give special attention to products stored in fluids that contain light-sensitive or reactive components that may give rise to cytotoxic by-products.

Studies analogous to accelerated aging studies typically used in pharmaceutical stability-monitoring programs are also useful to determine how the product degrades and which assays are stability indicating. These studies can be the same as some of those mentioned in the preceding paragraph. Other studies include placing a product at 37°, or at 18° when its normal storage temperature is 25 ± 2°, or placing a lyophilized product in a high-humidity environment. Such studies should be performed before formal stability studies begin so that the latter can incorporate the validated stability-indicating assays.

STORAGE AND SHIPPING

Appropriate conditions are chosen to preserve the purity and potency of the product so that its specification and those of its ingredients are maintained throughout storage, shipping, and handling at the clinic. Initial studies must be

conducted before patient administration to determine acceptable storage, shipping, and handling conditions. The initial storage and shipping conditions need not be those envisioned for the commercial product but should ensure that the product specifications are maintained beyond the initial expiration date. For products with short shelf lives, storage and shipping conditions, even within a medical center, must be considered at the same time because shipping constitutes the bulk of storage time after manufacturing. Give special consideration to the ability of gas to permeate the shipping container, especially if the gene therapy product is stored or shipped on dry ice. Once stability-indicating methods are developed and the final storage and shipping conditions are chosen, these must be validated as discussed under *Stability*.

Most products with limited shelf lives are shipped by reliable overnight courier systems. In some cases, highly fragile products are hand-carried onto commercial aircraft. Special permission must be obtained by commercial carriers if scanning by airport X-ray equipment must be avoided. Cargo shipping studies should be designed during the development of packaging systems to identify stresses to which the product may be subjected. Bracing and insulating materials should then be chosen and validated to provide a packaging system that will tolerate, and protect the product against, the extreme conditions of shipping.

Most gene therapy products can be either lyophilized or formulated by means similar to those employed for many recombinant proteins or cell therapy products. These storage formulations typically have expiration periods longer than one year and no unusual shipping requirements. Nonviral gene therapy products, which may be unstable in their final formulation, can have similar expiration periods if they are stored in a multiple-vial kit with the nucleic acid in one vial and a carrier, such as lipids, in the other. The final formulation is performed at the medical center just before administration.

LABELING

Product labeling is regulated by the FDA, and compliance with existing policies is required. Because gene therapy products are regulated biologics, their labeling is subject to these rules. Biologics and devices must meet labeling requirements specific to the container and the package (21 CFR 610 and 801, respectively). Both the container label and the package label must include the expiration date. If the container is packaged, then the recommended storage conditions should be included on the outer package label. If the container is not packaged, the recommended storage conditions and all other requirements of a package label must appear on the container. Labeling must also comply with relevant national and international requirements.

If a product must be applied to the patient in a particular physical orientation or in a specifically defined area, labeling that indicates the correct orientation and/or area should be apparent even after the package is opened. Unless the product has been screened for pathogenic or microbial contaminants before release, appropriate biohazard labeling may be required. For products with very short shelf lives, expiration dating requires adjustment and correction for time zones to provide the user an accurate assessment of shelf life. Clinical procedures must be scheduled around these crucial time frames. For patient-specific products, the patient's full name, initials, or a combination of these will need to appear on the labeling, in addition to lot designation, to ensure that the product is administered to the appropriate patient.

REGULATIONS AND STANDARDS

The technologies involved in manufacturing gene therapy products have been widely documented in the literature and continue to evolve. These products can be regulated as

drugs or biologics, or uncommonly as devices, depending on how they are manufactured and used. The novel approaches permitted by these technologies may make it difficult to determine which FDA centers will be involved in their regulation, and the FDA has advised manufacturers to seek clarification in the early stages of development. Currently, the Center for Biologics Evaluation and Research (CBER) regulates most human gene therapy products. CBER relies on both the Public Health Service Act and the Federal Food Drug and Cosmetic Act. Regulation is the same as that for biotechnology-derived products. The general requirements are described primarily in 21 CFR. The federal government has issued many guidance documents as *Points to Consider* or *Guidelines* (see www.fda.gov and, in particular, www.fda.gov/cber/publications.htm). ICH guidance documents for many of the quality-related areas are relevant in varying degrees to qualifying gene therapy products (although some products are nominally outside the scope of the guidance documents, the principles still apply; see www.ifpma.org or www.ich.org). Some of these documents are reproduced in *USP-NF* as general chapters. ICH has also held a number of meetings about gene therapy products and has a Gene Therapy Discussion Group (GTDG) that addresses current issues in gene therapy product development and research and has released several ICH Considerations that reflect harmonized principles. The National Institutes of Health (NIH) has published *Guidelines for Research Involving Recombinant DNA Molecules* that require NIH review of research, including clinical research or trials conducted or sponsored by institutions that receive NIH funding. These guidelines apply to many gene therapy products.

Biological and biochemical standards for QA of the production and analysis of gene therapy products are highly desirable. The diversity of gene therapy products, in particular viral vectors, has so far limited the development of standards that have wide applicability. A MuLV RCR preparation (VR-1450) with an assigned infectivity titer is available from ATCC for testing murine retroviral vectors for the presence of RCR. A wild-type adenovirus type 5 reference standard with assigned particle number and infectivity titer for characterization of adenoviral vectors is also available from ATCC. A working group has been set up to oversee the development of an AAV reference standard. However, several obstacles to choosing, developing, establishing, and circulating suitable standards are apparent. These include decisions about which virus serotype will be most commonly and successfully used for gene therapy, availability of GMP prepared materials, safety, long-term stability, transportation, and initiation and completion of collaborative studies to evaluate candidate standards. Thus, development of standards for other viral vectors, including lentiviral-, herpes viral-, and poxviral-vectors, remains challenging.

New methodologies, including proteomics, novel nucleic acid technologies (NATs), protein modification methods, and stem cell isolation and culture, are now available and, in many cases, are applicable to the development, characterization, and analysis of gene therapy products. In addition, the use of synthetic polymers both for the modification of existing viral vectors and for the development of chemically dynamic synthetic vectors provides advantages, e.g., improved systemic circulation, better targeting and delivery, and lower levels of immunostimulation and inflammation. The availability of defined stem cell populations and improved engrafting methods should lead to greater effectiveness of ex vivo transduced cells used in gene therapy protocols. The introduction of new methodologies will require the continual review and regulatory oversight to ensure the quality and safety of gene therapy products of the future.

APPENDIX

Gene therapy products are regulated by the FDA as biologics, and therefore their manufacturing, testing, labeling, and other factors are subject to the requirements codified in

CFR and FDA guidance documents (www.fda.gov). Additional guidance is provided in ICH guidelines (www.ich.org). Manufacturers of gene therapy products that seek markets outside the United States should refer to regulatory documents from relevant countries. Beyond *USP* chapters, the following list includes regulatory documents, as well as best practices for the development, manufacturing, quality control, and quality assurance of gene therapy products:

CFR

- 21 CFR 210
- 21 CFR 211
- 21 CFR 600s
- 21 CFR 610 Subpart G
- 21 CFR 801
- 21 CFR 820

FDA Guidance Documents

Guideline for the Determination of Residual Moisture in Dried Biological Products (January 1990)

Guidance for Industry: Human Somatic Cell Therapy and Gene Therapy (March 1998)

Guidance for Industry: Supplemental Guidance on Testing for Replication-Competent Retrovirus in Retroviral Vector-Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors (October 2000)

FDA Guidance for Industry: Investigating Out-of-Specification (OOS) Test Results for Pharmaceutical Production (October 2006)

Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs) (April 2008)

Draft Guidance for Industry: Potency Tests for Cellular and Gene Therapy Products (October 2008)

National and International Regulatory Documents

Q5A(R1): Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin

ICH Q5C: Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products

ICH Q5D: Quality of Biotechnological Products: Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products

ICH Q6B Specification: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products

ICH Q2 (R1): Validation of Analytical Procedures: Text and Methodology

NIH Guidelines for Research Involving Recombinant DNA Molecules (<http://www4.od.nih.gov/oba/guidelines.html>)

GLOSSARY OF TERMS

Adenovirus—Virus belonging to the family Adenoviridae of DNA viruses having a nonenveloped virion with 252 capsomeres and a diameter between 70 and 90 nm; a single linear molecule of double-stranded DNA (36 to 38 kb); at least 10 structural ether-resistant and acid-stable proteins; virions are released by cell destruction.

Adenovirus-Associated Virus (AAV)—Human parvovirus contains a single-stranded DNA genome and depends on helper viruses (adenovirus, herpes virus, or vaccinia-virus) for replication. Without co-infection, the wild-type virions integrate at a specific site on chromosome 19 and remain latent.

Adventitious Agent—A foreign agent that is introduced accidentally or inadvertently; not natural or hereditary (as in microbial, chemical, or biochemical contamination of a purified substance).

Amphotropic Virus—A virus that infects and replicates in cells from multiple species.

Ancillary Materials—Components used during manufacturing that are not intended to be present in the final product.

Examples: growth factors, monoclonal antibodies, cell-separation devices, and media components.

Autologous—From one's own body.

Base Pair—Two nucleotide bases on different strands of the nucleic acid molecule that bond together.

Bioassay—Measurement of the effectiveness of a compound by its effect on animals or cells in comparison with a standard preparation. (See also *Potency*.)

Biological Product—Any virus, therapeutic serum, toxin, antitoxin, or *analogous product* applicable to the prevention, treatment, or cure of diseases or injuries in humans. (In this US FDA-derived definition, the term *analogous product* has been interpreted to include essentially all biotechnology-derived products and procedures including gene therapy, transgenics, and somatic cell therapy.)

Biotechnology—Any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses. The newer definition refers to the industrial and pharmaceutical use of rDNA, cell fusion, novel bioprocessing techniques, and gene therapy.

Cell Lines—Cells that are derived from primary culture embryos, tissue, or organs. Such cell lines may have a finite life span or may be immortalized (modified to be able to replicate indefinitely).

Cell Therapy—Therapy that uses whole cells to treat a disease, condition, or injury. Distinct from tissue and organ transplantation.

CFTR—Cystic fibrosis transmembrane conductance regulator.

cGMP—Current good manufacturing practices. FDA outlined cGMPs in the 21 CFR, and in the *Federal Register*, and its *Points to Consider*.

Complementary DNA (cDNA)—DNA synthesized from an mRNA rather than a DNA template. It is used for cloning or as a DNA probe for locating specific genes.

Cytokine—Any factor that acts on cells; usually a protein that promotes growth.

Cytoplasm—Cellular material that is within the cell membrane and surrounds the nucleus.

Cytotoxic—Able to cause cell death.

Differentiation—A process of biochemical and structural changes by which cells become specialized in form and function.

Ecotropic Virus—A virus that infects and replicates in cells from only the original host species.

Electroporation—Method for enabling transfer of material into cells that involves use of a brief electrical field to create temporary pores in the cell membrane.

ELISA—Enzyme-linked immunosorbent assay. An immunoassay that utilizes an enzyme-labeled antigen or antibody to detect the binding of a molecule to a solid matrix.

Enveloped Viruses—Viruses containing a lipoprotein bilayer surrounding the capsid and acquired by budding through the cell membrane of the host cells.

Episomal—Pertaining to any accessory extra-chromosomal genetic material.

Ex Vivo—Procedure performed outside of the living organism.

Fluorescence-Activated Cell Sorter (FACS)—A machine that sorts cells based on fluorescent markers attached to them.

Formulated—Prepared in accordance with a prescribed method or conditions.

Fusion—Joining of the membrane of two cells, creating a daughter that contains some of the same properties from each parent cell. It is used in making hybridoma cells in which antibody-producing cells are fused to mouse myeloma cells.

Gene Construct—Expression vector that contains the coding sequence of the protein and the necessary elements for its expression.

Gene Therapy—Therapy that uses nucleic acid that is subsequently expressed as RNA or protein to treat a disease or condition. The US FDA defines gene therapy products as products containing genetic material administered to modify or manipulate the expression of genetic material to alter the biological properties of living cells.

Genome—Total hereditary material of a cell.

Germ Cell—Reproductive cell (sperm or egg), gamete, or sex cell.

Graft-Versus-Host Disease (GVHD)—Rejection of the transplanted tissue by the host. It is the leading cause of patient death when mismatched allogeneic tissue is used.

Growth Factors—Factors responsible for regulatory cell proliferation, function, and differentiation.

Helper Virus—Aids the development of a defective virus by supplying or restoring the activity of a viral gene or by enabling the defective virus to form a functional envelope.

Hematopoietic—Pertaining to or affecting the formation of blood cells.

Hepatocyte—The predominant cell type in the liver that has an important role in metabolism and is a source of serum proteins. These cells generally do not divide, but when injured they can divide and regenerate until the injured cells are replaced.

Herpes Simplex Virus (HSV)—A DNA virus that is a member of the family Herpesviridae. It can infect both warm- and cold-blooded vertebrates by contact between moist mucosal surfaces.

Human Leukocyte Antigen (HLA)—Proteins controlled by the major histocompatibility complex. These proteins play a key role in determining transplant compatibility.

Humoral—Pertaining to elements found in body fluids (for example, humoral immunity and neutralizing antibodies).

Hybridization Dot Blot (DNA or RNA)—A technique for detecting, analyzing, and identifying protein; similar to the Western blot but without electrophoretic separation of proteins.

Immunoassay—Technique for identifying substances based on the use of antibodies.

Insertional Mutagenesis—A type of mutation that is caused by the insertion of nucleic acid into a host-cell chromosome. There are multiple possible negative consequences of such an event, including death of a cell if an essential gene is inactivated or predisposition to cancer if a tumor suppressor gene is inactivated.

Integration—Assimilation (insertion via covalent binding) of genetic material (DNA) into the chromosome of a recipient cell.

Intrabodies—Intracellular antibodies that are not secreted and that are designed to bind and inactivate target molecules inside cells.

In Vivo—Procedure performed in the living organism.

In Vitro—Procedure performed outside of the living organism. It may involve cells or tissues derived from the organisms.

Leukemia—Malignant neoplasm of the blood-forming tissues.

Lipoplex—A formulation of lipids and polymers and/or proteins.

Liposome—A spherical lipid bilayer enclosing an aqueous compartment.

Mock Run—A test run that deliberately omits some critical reagents.

Monoclonal Antibodies—Antibodies that are derived from a single cell clone.

Naked DNA—Isolated, purified, and uncomplexed DNA (no protein or lipid).

Oligonucleotide—A polymer consisting of a small number of nucleotides, usually 5 to 30.

Oncogenes—Genes associated with neoplastic proliferation (cancer) following a mutation or perturbation in their expression.

Oncogenic—Cancer-causing.

Packaging Cell Line—Cell line that produces proteins required for packaging and production of viral vectors in an active form but does not produce replication-competent virus. It complements at the protein level what the vector is lacking genetically.

Parvovirus—DNA viruses of the family Parvoviridae. Host range includes many vertebrate species. Small, linear chain, single-stranded DNA with terminal hairpin loops.

Plasmid—A small circular form of DNA that carries certain genes and is capable of replicating independently in a host cell.

Process Validation—Means for providing documentation that the manufacturing process is controlled, reproducible, and capable of consistently producing a product that meets predetermined specifications.

Producer Cell Line—An established cell line used to produce virus vectors, often at a large scale.

Polymerase Chain Reaction (PCR)—Technique to amplify a target DNA or RNA sequence of nucleotides by repeated cycles of polymerase-based copying, resulting in geometric increases in copy number.

Potency—A quantitative measure of biological activity based on the attribute of the product linked to the relevant biological properties.

Promoter—DNA sequence that is located at the front of a gene and controls gene expression. It is required for binding of RNA polymerase to initiate transcription.

Recombinant DNA—DNA produced by joining fragments of DNA from different sources by in vitro manipulations.

Replication-competent Virus—A virus that can complete an entire replication cycle without a need for a helper virus; an autonomously replicating virus.

Restriction Endonuclease—An endonuclease that recognizes a specific sequence of bases within double-stranded DNA.

Retrovirus—A virus that contains reverse transcriptase, which converts viral RNA into DNA that then integrates into the host cell in a form called a provirus.

Serum-Free—Refers to cell growth medium that lacks a serum component.

S Phase—Part of the cell cycle during which DNA replication occurs (*Synthesis Phase*).

Stem Cell—Immortal cell that is capable of proliferating and differentiating into different types of specialized cells. Each major tissue system is thought to have its own putative stem cell.

Suspension Culture—Cells capable of growth in suspension, not requiring substrate (attachment) on which to grow.

Transduction—Transfer and expression of genetic material into a cell by means of a virus or phage vector.

Transfection—Transfer of DNA into cells by physical means such as by calcium phosphate coprecipitation.

Transgene—Refers to the foreign genetic material delivered as part of a vector construct.

Vector—The agent (plasmid, virus, or liposome-protein or DNA-protein complex) used to introduce nucleic acid into a cell.

Viability—State of being alive and functional.

Virion—An elementary viral particle consisting of genetic material (nucleocapsid) and a protein covering.

Virus—Submicroscopic infectious agent that contains genetic information necessary for reproduction. It is an obligate intracellular parasite.

Western Blot—An electroblotting method in which proteins are transferred from a gel to a thin, rigid support (e.g., nitrocellulose membrane) and detected by binding radioactively labeled antibody or antibody coupled to an enzyme, allowing use of a precipitating chromogenic or chemiluminescent substrate.

Xenogeneic—From a different species.

〈1048〉 QUALITY OF BIOTECHNOLOGICAL PRODUCTS: ANALYSIS OF THE EXPRESSION CONSTRUCT IN CELLS USED FOR PRODUCTION OF r-DNA DERIVED PROTEIN PRODUCTS¹

I. INTRODUCTION

This document presents guidance regarding the characterization of the expression construct for the production of recombinant DNA (r-DNA) protein products in eukaryotic and prokaryotic cells. The document is intended to describe the types of information that are considered valuable in assessing the structure of the expression construct used to produce r-DNA derived proteins. The document is not intended to cover the entire quality aspect of r-DNA derived medicinal products.

The expression construct is defined as the expression vector containing the coding sequence of the recombinant protein. Segments of the expression construct should be analyzed using nucleic acid techniques in conjunction with other tests performed on the purified recombinant protein for assuring the quality and consistency of the final product. Analysis of the expression construct at the nucleic acid level should be considered as part of the overall evaluation of quality, taking into account that this testing only evaluates the coding sequence of a recombinant gene and not the translational fidelity nor other characteristics of the recombinant protein, such as secondary structure, tertiary structure, and posttranslational modifications.

¹This guideline was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at Step 4 of the ICH process, November 29, 1995. At Step 4 of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA. This guideline was published in the Federal Register on February 23, 1996 (61 FR 7006) and is applicable to drug and biological products. Although this guideline does not create or confer any rights for or on any person and does not operate to bind FDA or the industry, it does represent the agency's current thinking on the production of r-DNA derived protein products. For additional copies of this guideline, contact the Drug Information Branch, HFD-210, CDER, FDA, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-827-4573) or the Manufacturers Assistance and Communication Staff (HFM-42), CBER, FDA, 1401 Rockville Pike, Rockville, MD 20852-1448. Send one self-addressed adhesive label to assist the offices in processing your request. An electronic version of this guidance is also available via Internet using the World Wide Web (WWW) (connect to the CDER Home Page at <http://www.fda.gov/cder> and go to the "Regulatory Guidance" section).

II. RATIONALE FOR THE ANALYSIS OF THE EXPRESSION CONSTRUCT

The purpose of analyzing the expression construct is to establish that the correct coding sequence of the product has been incorporated into the host cell and is maintained during culture to the end of production. The genetic sequence of recombinant proteins produced in living cells can undergo mutations that could alter the properties of the protein with potential adverse consequences to patients. No single experimental approach can be expected to detect all possible modifications to a protein. Protein analytical techniques can be used to assess the amino acid sequence of the protein and structural features of the expressed protein due to posttranslational modifications such as proteolytic processing, glycosylation, phosphorylation, and acetylation. Data from nucleic acid analysis may be useful because protein analytical methods may not detect all changes in protein structure resulting from mutations in the sequence coding for the recombinant protein. The relative importance of nucleic acid analysis and protein analysis will vary from product to product.

Nucleic acid analysis can be used to verify the coding sequence and the physical state of the expression construct. The nucleic acid analysis is performed to ensure that the expressed protein will have the correct amino acid sequence, but is not intended to detect low levels of variant sequences. Where the production cells have multiple integrated copies of the expression construct, not all of which may be transcriptionally active, examination of the transcription product itself by analysis of m-RNA or c-DNA may be more appropriate than analysis of genomic DNA. Analytical approaches that examine a bulk population of nucleic acids, such as those performed on pooled clones or material amplified by the polymerase chain reaction, may be considered as an alternative to approaches that depend on selection of individual DNA clones. Other techniques could be considered that allow for rapid and sensitive confirmation of the sequence coding for the recombinant protein in the expression construct.

The following sections describe information that should be supplied regarding the characterization of the expression construct during the development and validation of the production system. Analytical methodologies should be validated for the intended purpose of confirmation of sequence. The validation documentation should, at a minimum, include estimates of the limits of detection for variant sequences. This should be performed for either nucleic acid or protein sequencing methods. The philosophy and recommendations for analysis expressed in this document should be reviewed periodically to take advantage of new advances in technology and scientific information.

III. CHARACTERIZATION OF THE EXPRESSION SYSTEM

A. Expression Construct and Cell Clone Used to Develop the Master Cell Bank (MCB)

The manufacturer should describe the origin of the nucleotide sequence coding for the protein. This should include identification and source of the cell from which the nucleotide sequence was originally obtained. Methods used to prepare the DNA coding for the protein should be described.

The steps in the assembly of the expression construct should be described in detail. This description should include the source and function of the component parts of the expression construct, e.g., origins of replication, antibiotic resistance genes, promoters, enhancers, and whether or not the protein is being synthesized as a fusion protein. A detailed component map and a complete annotated sequence of the plasmid should be given, indicating those regions that have been sequenced during the construction and those taken from the literature. Other expressed proteins encoded by the plasmid should be indicated. The nu-

cleotide sequence of the coding region of the gene of interest and associated flanking regions that are inserted into the vector, up to and including the junctions of insertion, should be determined by DNA sequencing of the construct.

A description of the method of transfer of the expression construct into the host cell should be provided. In addition, methods used to amplify the expression construct and criteria used to select the cell clone for production should be described in detail.

B. Cell Bank System

Production of the recombinant protein should be based on well-defined MCB and Working Cell Banks (WCB). A cell bank is a collection of ampoules of uniform composition stored under defined conditions, each containing an aliquot of a single pool of cells. The MCB is generally derived from the selected cell clone containing the expression construct. The WCB is derived by expansion of one or more ampoules of the MCB. The cell line history and production of the cell banks should be described in detail, including methods and reagents used during culture, in vitro cell age, and storage conditions. All cell banks should be characterized for relevant phenotypic and genotypic markers, which could include the expression of the recombinant protein or presence of the expression construct.

The expression construct in the MCB should be analyzed as described below. If the testing cannot be carried out on the MCB, it should be carried out on each WCB.

Restriction endonuclease mapping or other suitable techniques should be used to analyze the expression construct for copy number, for insertions or deletions, and for the number of integration sites. For extrachromosomal expression systems, the percent of host cells retaining the expression construct should be determined.

The protein coding sequence for the recombinant protein product of the expression construct should be verified. For extrachromosomal expression systems, the expression construct should be isolated and the nucleotide sequence encoding the product should be verified without further cloning. For cells with chromosomal copies of the expression construct, the nucleotide sequence encoding the product could be verified by recloning and sequencing of chromosomal copies. Alternatively, the nucleic acid sequence encoding the product could be verified by techniques such as sequencing of pooled c-DNA clones or material amplified by the polymerase chain reaction. The nucleic acid sequence should be identical, within the limits of detection of the methodology, to that determined for the expression construct as described in section III.A., and should correspond to that expected for the protein sequence.

C. Limit for In Vitro Cell Age for Production

The limit for in vitro cell age for production should be based on data derived from production cells expanded under pilot plant-scale or full-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could be used to prepare the production cells with appropriate justification.

The expression construct of the production cells should be analyzed once for the MCB as described in section III.B., except that the protein coding sequence of the expression construct in the production cells could be verified by either nucleic acid testing or analysis of the final protein product. Increases in the defined limit for in vitro cell age for production should be supported by data from cells that have been expanded to an in vitro cell age that is equal to or greater than the new limit for in vitro cell age.

IV. CONCLUSION

The characterization of the expression construct and the final purified protein are both important to ensure the consistent production of a r-DNA derived product. As described above, analytical data derived from both nucleic acid

analysis and evaluation of the final purified protein should be evaluated to ensure the quality of a recombinant protein product.

GLOSSARY OF TERMS

Expression Construct

The expression vector that contains the coding sequence of the recombinant protein and the elements necessary for its expression.

Flanking Control Regions

Noncoding nucleotide sequences that are adjacent to the 5' and 3' end of the coding sequence of the product that contain important elements that affect the transcription, translation, or stability of the coding sequence. These regions include, e.g., promoter, enhancer, and splicing sequences, and do not include origins of replication and antibiotic resistance genes.

Integration Site

The site where one or more copies of the expression construct is integrated into the host cell genome.

In Vitro Cell Age

Measure of time between thaw of the MCB vial(s) to harvest of the production vessel measured by elapsed chronological time in culture, by population doubling level of the cells, or by passage level of the cells when subcultured by a defined procedure for dilution of the culture.

Master Cell Bank (MCB)

An aliquot of a single pool of cells that generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers, and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB, or WCB) should be the same as for the MCB unless justified.

Pilot Plant Scale

The production of a recombinant protein by a procedure fully representative of and simulating that to be applied on a full commercial manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

Relevant Genotypic and Phenotypic Markers

Those markers permitting the identification of the strain of the cell line that should include the expression of the recombinant protein or presence of the expression construct.

Working Cell Bank (WCB)

The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

(1049) QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS¹

I. INTRODUCTION (1)

The guidance stated in the ICH harmonized tripartite guideline entitled "Stability Testing of New Drug Substances and Products" (issued by ICH on October 27, 1993) applies in general to biotechnological/biological products. However, biotechnological/biological products have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence, of biological activity, is dependent on non-covalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. To ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary.

The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physicochemical, biochemical, and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies.

With these concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions that can affect the product's potency, purity, and quality. Primary data to support a requested storage period for either drug substance or drug product should be based on long-term, real-time, real-condition stability studies.

¹This guideline was developed within the Expert Working Group (Quality) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at Step 4 of the ICH process, November 20, 1995. At Step 4 of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and the USA. This guideline was published in the *Federal Register* on July 10, 1996 (61 FR 36466) and is applicable to drug and biological products. Although this guideline does not create or confer any rights for or on any person and does not operate to bind FDA or the industry, it does represent the agency's current thinking on stability testing of biotechnological/biological products. For additional copies of this guideline, contact the Drug Information Branch, HFD-210, CDER, FDA, 5600 Fishers Lane, Rockville, MD 20857 (Phone: 301-827-4573) or the Manufacturers Assistance and Communication Staff (HFM-42), CDER, FDA, 1401 Rockville Pike, Rockville, MD 20852-1448. Send one self-addressed adhesive label to assist the offices in processing your request. An electronic version of this guidance is also available via Internet using the World Wide Web (WWW) (connect to the CDER Home Page at <http://www.fda.gov/cder> and go to the "Regulatory Guidance" section).

Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product. The purpose of this document is to give guidance to applicants regarding the type of stability studies that should be provided in support of marketing applications. It is understood that during the review and evaluation process, continuing updates of initial stability data may occur.

II. SCOPE OF THE ANNEX (2)

The guidance stated in this annex to "Stability Testing of New Drug Substances and Products" applies to well-characterized proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using recombinant deoxyribonucleic acid (r-DNA) technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony-stimulating factors, tumor necrosis factors), erythropoietins, plasminogen activators, blood plasma factors, growth hormones and growth factors, insulins, monoclonal antibodies, and vaccines consisting of well-characterized proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins, whole blood, or cellular blood components.

III. TERMINOLOGY (3)

For the basic terms used in this annex, the reader is referred to the "Glossary" in "Stability Testing of New Drug Substances and Products." However, because manufacturers of biotechnological/biological products sometimes use traditional terminology, traditional terms are specified in parentheses to assist the reader. A supplemental glossary is also included that explains certain terms used in the production of biotechnological/biological products.

IV. SELECTION OF BATCHES (4)

A. Drug Substance (Bulk Material) (4.1)

Where bulk material is to be stored after manufacture, but before formulation and final manufacturing, stability data should be provided on at least three batches for which manufacture and storage are representative of the manufacturing scale of production. A minimum of 6 months stability data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug substances with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Data from pilot-plant scale batches of drug substance produced at a reduced scale of fermentation and purification may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval.

The quality of the batches of drug substance placed into the stability program should be representative of the quality of the material used in preclinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the drug substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The drug substance entered into the stability program should be stored in containers that properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable

for drug substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.

B. Intermediates (4.2)

During manufacture of biotechnological/biological products, the quality and control of certain intermediates may be critical to the production of the final product. In general, the manufacturer should identify intermediates and generate in-house data and process limits that assure their stability within the bounds of the developed process. Although the use of pilot-plant scale data is permissible, the manufacturer should establish the suitability of such data using the manufacturing scale process.

C. Drug Product (Final Container Product) (4.3)

Stability information should be provided on at least three batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of 6 months data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug products with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Product expiration dating should be based upon the actual data submitted in support of the application. Because dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of drug product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first three manufacturing scale batches into the long-term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that the product produced at manufacturing scale does not meet those long-term stability specifications throughout the dating period or is not representative of the material used in preclinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

D. Sample Selection (4.4)

Where one product is distributed in batches differing in fill volume (e.g., 1 milliliter (mL), 2 mL, or 10 mL), unitage (e.g., 10 units, 20 units, or 50 units), or mass (e.g., 1 milligram (mg), 2 mg, or 5 mg), samples to be entered into the stability program may be selected on the basis of a matrix system and/or by bracketing.

Matrixing, i.e., the statistical design of a stability study in which different fractions of samples are tested at different sampling points, should only be applied when appropriate documentation is provided that confirms that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure, and, possibly, in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

Where the same strength and exact container/closure system is used for three or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability program, i.e., bracketing. The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples are represented by those at the extremes. In certain cases, data may

be needed to demonstrate that all samples are properly represented by data collected for the extremes.

V. STABILITY-INDICATING PROFILE (5)

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity, and potency of the product will be detected.

At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile, and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasized in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to demonstrate product stability adequately.

A. Protocol (5.1)

The dossier accompanying the application for marketing authorization should include a detailed protocol for the assessment of the stability of both drug substance and drug product in support of the proposed storage conditions and expiration dating periods. The protocol should include all necessary information that demonstrates the stability of the biotechnological/biological product throughout the proposed expiration dating period including, for example, well-defined specifications and test intervals. The statistical methods that should be used are described in the tripartite guideline on stability.

B. Potency (5.2)

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable *in vivo* or *in vitro* quantitative method. In general, potencies of biotechnological/biological products tested by different laboratories can be compared in a meaningful way only if expressed in relation to that of an appropriate reference material. For that purpose, a reference material calibrated directly or indirectly against the corresponding national or international reference material should be included in the assay.

Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognized standards. Where no national or international reference standards exist, the assay results may be reported in *in-house* derived units using a characterized reference material.

In some biotechnological/biological products, potency is dependent upon the conjugation of the active ingredient(s) to a second moiety or binding to an adjuvant. Dissociation of the active ingredient(s) from the carrier used in conjugates or adjuvants should be examined in real-time/real-temperature studies (including conditions encountered during shipment). The assessment of the stability of such products may be difficult because, in some cases, *in vitro* tests for biological activity and physicochemical characterization are impractical or provide inaccurate results. Appropriate strategies (e.g., testing the product before conjugation/binding, assessing the release of the active compound from the second moiety, *in vivo* assays) or the use of an appropriate surrogate test should be considered to overcome the inadequacies of *in vitro* testing.

C. Purity and Molecular Characterization (5.3)

For the purpose of stability testing of the products described in this guideline, purity is a relative term. Because of

the effect of glycosylation, deamidation, or other heterogeneities, the absolute purity of a biotechnological/biological product is extremely difficult to determine. Thus, the purity of a biotechnological/biological product should be typically assessed by more than one method and the purity value derived is method-dependent. For the purpose of stability testing, tests for purity should focus on methods for determination of degradation products.

The degree of purity, as well as the individual and total amounts of degradation products of the biotechnological/biological product entered into the stability studies, should be reported and documented whenever possible. Limits of acceptable degradation should be derived from the analytical profiles of batches of the drug substance and drug product used in the preclinical and clinical studies.

The use of relevant physicochemical, biochemical, and immunochemical analytical methodologies should permit a comprehensive characterization of the drug substance and/or drug product (e.g., molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulfoxidation, aggregation, or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS09Page, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g., reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping.

Wherever significant qualitative or quantitative changes indicative of degradation product formation are detected during long-term, accelerated, and/or stress stability studies, consideration should be given to potential hazards and to the need for characterization and quantification of degradation products within the long-term stability program. Acceptable limits should be proposed and justified, taking into account the levels observed in material used in preclinical and clinical studies.

For substances that cannot be properly characterized or products for which an exact analysis of the purity cannot be determined through routine analytical methods, the applicant should propose and justify alternative testing procedures.

D. Other Product Characteristics (5.4)

The following product characteristics, though not specifically relating to biotechnological/biological products, should be monitored and reported for the drug product in its final container:

Visual appearance of the product (color and opacity for solutions/suspensions; color, texture, and dissolution time for powders), visible particulates in solutions or after the reconstitution of powders or lyophilized cakes, pH, and moisture level of powders and lyophilized products.

Sterility testing or alternatives (e.g., container/closure integrity testing) should be performed at a minimum initially and at the end of the proposed shelf life.

Additives (e.g., stabilizers, preservatives) or excipients may degrade during the dating period of the drug product. If there is any indication during preliminary stability studies that reaction or degradation of such materials adversely affect the quality of the drug product, these items may need to be monitored during the stability program.

The container/closure has the potential to affect the product adversely and should be carefully evaluated (see below).

VI. STORAGE CONDITIONS (6)

A. Temperature (6.1)

Because most finished biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real-temperature stability studies may be confined to the proposed storage temperature.

B. Humidity (6.2)

Biotechnological/biological products are generally distributed in containers protecting them against humidity. Therefore, where it can be demonstrated that the proposed containers (and conditions of storage) afford sufficient protection against high and low humidity, stability tests at different relative humidities can usually be omitted. Where humidity-protecting containers are not used, appropriate stability data should be provided.

C. Accelerated and Stress Conditions (6.3)

As previously noted, the expiration dating should be based on real-time/real-temperature data. However, it is strongly suggested that studies be conducted on the drug substance and drug product under accelerated and stress conditions. Studies under accelerated conditions may provide useful support data for establishing the expiration date, provide product stability information or future product development (e.g., preliminary assessment of proposed manufacturing changes such as change in formulation, scale-up), assist in validation of analytical methods for the stability program, or generate information that may help elucidate the degradation profile of the drug substance or drug product. Studies under stress conditions may be useful in determining whether accidental exposures to conditions other than those proposed (e.g., during transportation) are deleterious to the product and also for evaluating which specific test parameters may be the best indicators of product stability. Studies of the exposure of the drug substance or drug product to extreme conditions may help to reveal patterns of degradation; if so, such changes should be monitored under proposed storage conditions. Although the tripartite guideline on stability describes the conditions of the accelerated and stress study, the applicant should note that those conditions may not be appropriate for biotechnological/biological products. Conditions should be carefully selected on a case-by-case basis.

D. Light (6.4)

Applicants should consult the appropriate regulatory authorities on a case-by-case basis to determine guidance for testing.

E. Container/Closure (6.5)

Changes in the quality of the product may occur due to the interactions between the formulated biotechnological/biological product and container/closure. Where the lack of interactions cannot be excluded in liquid products (other than sealed ampules), stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality. Data should be supplied for all different container/closure combinations that will be marketed.

In addition to the standard data necessary for a conventional single-use vial, the applicant should demonstrate that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its full potency, purity, and quality for the maximum period specified in the instructions-for-use on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

F. Stability after Reconstitution of Freeze-Dried Product (6.6)

The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national/regional requirements.

VII. TESTING FREQUENCY (7)

The shelf lives of biotechnological/biological products may vary from days to several years. Thus, it is difficult to draft uniform guidelines regarding the stability study duration and testing frequency that would be applicable to all types

of biotechnological/biological products. With only a few exceptions, however, the shelf lives for existing products and potential future products will be within the range of 0.5 to 5 years. Therefore, the guidance is based upon expected shelf lives in that range. This takes into account the fact that degradation of biotechnological/biological products may not be governed by the same factors during different intervals of a long storage period.

When shelf lives of 1 year or less are proposed, the real-time stability studies should be conducted monthly for the first 3 months and at 3 month intervals thereafter. For products with proposed shelf lives of greater than 1 year, the studies should be conducted every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter.

While the testing intervals listed above may be appropriate in the preapproval or prelicense stage, reduced testing may be appropriate after approval or licensure where data are available that demonstrate adequate stability. Where data exist that indicate the stability of a product is not compromised, the applicant is encouraged to submit a protocol that supports elimination of specific test intervals (e.g., 9-month testing) for postapproval/postlicensure, long-term studies.

VIII. SPECIFICATIONS (8)

Although biotechnological/biological products may be subject to significant losses of activity, physicochemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf life specifications. Recommendations for maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that the clinical performance is not affected, as discussed in the tripartite guideline on stability.

IX. LABELING (9)

For most biotechnological/biological drug substances and drug products, precisely defined storage temperatures are recommended. Specific recommendations should be stated, particularly for drug substances and drug products that cannot tolerate freezing. These conditions, and where appropriate, recommendations for protection against light and/or humidity, should appear on containers, packages, and/or package inserts. Such labeling should be in accordance with relevant national and regional requirements.

X. GLOSSARY (10)

- **Conjugated Product**—A conjugated product is made up of an active ingredient (e.g., peptide, carbohydrate) bound covalently or noncovalently to a carrier (e.g., protein, peptide, inorganic mineral) with the objective of improving the efficacy or stability of the product.
- **Degradation Product**—A molecule resulting from a change in the drug substance (bulk material) brought about over time. For the purpose of stability testing of the products described in this guideline, such changes could occur as a result of processing or storage (e.g., by deamidation, oxidation, aggregation,

- proteolysis). For biotechnological/biological products, some degradation products may be active.
- **Impurity**—Any component of the drug substance (bulk material) or drug product (final container product) that is not the chemical entity defined as the drug substance, an excipient, or other additives to the drug product.
 - **Intermediate**—For biotechnological/biological products, a material produced during a manufacturing process that is not the drug substance or the drug product but for which manufacture is critical to the successful production of the drug substance or the drug product. Generally, an intermediate will be quantifiable and specifications will be established to determine the successful completion of the manufacturing step before continuation of the manufacturing process. This includes material that may undergo further molecular modification or be held for an extended period before further processing.
 - **Manufacturing Scale Production**—Manufacture at the scale typically encountered in a facility intended for product production for marketing.
 - **Pilot-Plant Scale**—The production of the drug substance or drug product by a procedure fully representative of and simulating that to be applied at manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

(1050) VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

I. INTRODUCTION

This document is concerned with testing and evaluation of the viral safety of biotechnology products derived from characterized cell lines of human or animal origin (i.e., mammalian, avian, insect), and outlines data that should be submitted in the marketing application/registration package. For the purposes of this document, the term virus excludes nonconventional transmissible agents like those associated with Bovine Spongiform Encephalopathy (BSE) and scrapie. Applicants are encouraged to discuss issues associated with BSE with the regulatory authorities.

The scope of the document covers products derived from cell cultures initiated from characterized cell banks. It covers products derived from in vitro cell culture, such as interferons, monoclonal antibodies, and recombinant deoxyribonucleic acid (DNA)-derived products including recombinant subunit vaccines, and also includes products derived from hybridoma cells grown in vivo as ascites. In this latter case, special considerations apply and additional information on testing cells propagated in vivo is contained in *Appendix 1*. Inactivated vaccines, all live vaccines containing self-replicating agents, and genetically engineered live vectors are excluded from the scope of this document.

The risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can

arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of virus during production. To date, however, biotechnology products derived from cell lines have not been implicated in the transmission of viruses. Nevertheless, it is expected that the safety of these products with regard to viral contamination can be reasonably assured only by the application of a virus testing program and assessment of virus removal and inactivation achieved by the manufacturing process, as outlined below.

Three principal, complementary approaches have evolved to control the potential viral contamination of biotechnology products:

(1) Selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable viruses which may be infectious and/or pathogenic for humans;

(2) Assessing the capacity of the production processes to clear infectious viruses;

(3) Testing the product at appropriate steps of production for absence of contaminating infectious viruses.

All testing suffers from the inherent limitation of quantitative virus assays, i.e., that the ability to detect low viral concentrations depends for statistical reasons on the size of the sample. Therefore, no single approach will necessarily establish the safety of a product. Confidence that infectious virus is absent from the final product will in many instances not be derived solely from direct testing for their presence, but also from a demonstration that the purification regimen is capable of removing and/or inactivating the viruses.

The type and extent of viral tests and viral clearance studies needed at different steps of production will depend on various factors and should be considered on a case-by-case and step-by-step basis. The factors that should be taken into account include the extent of cell bank characterization and qualification, the nature of any viruses detected, culture medium constituents, culture methods, facility and equipment design, the results of viral tests after cell culture, the ability of the process to clear viruses, and the type of product and its intended clinical use.

The purpose of this document is to describe a general framework for virus testing, experiments for the assessment of viral clearance, and a recommended approach for the design of viral tests and viral clearance studies. Related information is described in the appendices and selected definitions are provided in the glossary.

Manufacturers should adjust the recommendations presented here to their specific product and its production process. The approach used by manufacturers in their overall strategy for ensuring viral safety should be explained and justified. In addition to the detailed data that is provided, an overall summary of the viral safety assessment would be useful in facilitating the review by regulatory authorities. This summary should contain a brief description of all aspects of the viral safety studies and strategies used to prevent virus contamination as they pertain to this document.

II. POTENTIAL SOURCES OF VIRUS CONTAMINATION

Viral contamination of biotechnology products may arise from the original source of the cell lines or from adventitious introduction of virus during production processes.

A. Viruses That Could Occur in the Master Cell Bank (MCB)

Cells may have latent or persistent virus infection (e.g., herpesvirus) or endogenous retrovirus which may be transmitted vertically from one cell generation to the next, since the viral genome persists within the cell. Such viruses may be constitutively expressed or may unexpectedly become expressed as an infectious virus. Viruses can be introduced into the MCB by several routes such as: (1) Derivation of cell lines from infected animals; (2) use of virus to establish the cell line; (3) use of contaminated biological reagents such as animal serum components; (4) contamination during cell handling.

B. Adventitious Viruses That Could Be Introduced During Production

Adventitious viruses can be introduced into the final product by several routes including, but not limited to, the following: (1) Use of contaminated biological reagents such as animal serum components; (2) use of a virus for the induction of expression of specific genes encoding a desired protein; (3) use of a contaminated reagent, such as a monoclonal antibody affinity column; (4) use of a contaminated excipient during formulation; and (5) contamination during cell and medium handling. Monitoring of cell culture parameters can be helpful in the early detection of potential adventitious viral contamination.

III. CELL LINE QUALIFICATION: TESTING FOR VIRUSES

An important part of qualifying a cell line for use in the production of a biotechnology product is the appropriate testing for the presence of virus.

A. Suggested Virus Tests for MCB, Working Cell Bank (WCB) and Cells at the Limit of In Vitro Cell Age Used for Production

Table 1 shows examples of virus tests to be performed once only at various cell levels, including MCB, WCB, and cells at the limit of in vitro cell age used for production.

1. Master Cell Bank

Extensive screening for both endogenous and nonendogenous viral contamination should be performed on the MCB. For heterohybrid cell lines in which one or more partners are human or nonhuman primate in origin, tests should be performed in order to detect viruses of human or nonhuman primate origin because viral contamination arising from these cells may pose a particular hazard.

Testing for nonendogenous viruses should include in vitro and in vivo inoculation tests and any other specific tests, including species-specific tests such as the mouse antibody production (MAP) test, that are appropriate, based on the passage history of the cell line, to detect possible contaminating viruses.

2. Working Cell Bank

Each WCB as a starting cell substrate for drug production should be tested for adventitious virus either by direct testing or by analysis of cells at the limit of in vitro cell age, initiated from the WCB. When appropriate nonendogenous virus tests have been performed on the MCB and cells cultured up to or beyond the limit of in vitro cell age have been derived from the WCB and used for testing for the presence of adventitious viruses, similar tests need not be performed on the initial WCB. Antibody production tests are

usually not necessary for the WCB. An alternative approach in which full tests are carried out on the WCB rather than on the MCB would also be considered acceptable.

3. Cells at the Limit of In Vitro Cell Age Used for Production

The limit of in vitro cell age used for production should be based on data derived from production cells expanded under pilot-plant scale or commercial-scale conditions to the proposed in vitro cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could also be used to prepare the production cells. Cells at the limit of in vitro cell age should be evaluated once for those endogenous viruses that may have been undetected in the MCB and WCB. The performance of suitable tests (e.g., in vitro and in vivo) at least once on cells at the limit of in vitro cell age used for production would provide further assurance that the production process is not prone to contamination by adventitious virus. If any adventitious viruses are detected at this level, the process should be carefully checked in order to determine the cause of the contamination, and should be completely redesigned if necessary.

B. Recommended Viral Detection and Identification Assays

Numerous assays can be used for the detection of endogenous and adventitious viruses. Table 2 outlines examples for these assays. They should be regarded as assay protocols recommended for the present, but the list is not all-inclusive or definitive. Since the most appropriate techniques may change with scientific progress, proposals for alternative techniques, when accompanied by adequate supporting data, may be acceptable. Manufacturers are encouraged to discuss these alternatives with the regulatory authorities. Other tests may be necessary depending on the individual case. Assays should include appropriate controls to ensure adequate sensitivity and specificity. Wherever a relatively high possibility of the presence of a specific virus can be predicted from the species of origin of the cell substrate, specific tests and/or approaches may be necessary. If the cell line used for production is of human or nonhuman primate origin, additional tests for human viruses, such as those causing immunodeficiency diseases and hepatitis, should be performed unless otherwise justified. The polymerase chain reaction (PCR) may be appropriate for detection of sequences of other human viruses as well as for other specific viruses. The following is a brief description of a general framework and philosophical background within which the manufacturer should justify what was done.

Table 1. Examples of Virus Tests to be Performed Once At Various Cell Levels

	MCB	WCB ¹	Cells at the Limit ²
<i>Tests for Retroviruses and Other Endogenous Viruses</i>			
Infectivity	+	—	+
Electron microscopy ³	+ ³	—	+ ³
Reverse transcriptase ⁴	+ ⁴	—	+ ⁴
Other virus-specific tests ⁵	as appropriate ⁵	—	as appropriate ⁵
<i>Tests for Nonendogenous or Adventitious Viruses</i>			
In vitro Assays	+	— ⁶	+
In vivo Assays	+	— ⁶	+
Antibody production tests ⁷	+ ⁷	—	—
Other virus-specific tests ⁸	+ ⁸	—	—

¹ See text—section III.A.2.
² Cells at the limit: Cells at the limit of in vitro cell age used for production (See text—section III.A.3.).
³ May also detect other agents.
⁴ Not necessary if positive by retrovirus infectivity test.
⁵ As appropriate for cell lines which are known to have been infected by such agents.
⁶ For the first WCB, this test should be performed on cells at the limit of in vitro cell age, generated from that WCB; for WCB's subsequent to the first WCB, a single in vitro and in vivo test can be done either directly on the WCB or on cells at the limit of in vitro cell age.
⁷ e.g., MAP, RAP, HAP—usually applicable for rodent cell lines.
⁸ e.g., tests for cell lines derived from human, nonhuman primate, or other cell lines as appropriate.

1. Tests for Retroviruses

For the MCB and for cells cultured up to or beyond the limit of in vitro cell age used for production, tests for retroviruses, including infectivity assays in sensitive cell cultures and electron microscopy (EM) studies, should be carried out. If infectivity is not detected and no retrovirus or retrovirus-like particles have been observed by EM, reverse transcriptase (RT) or other appropriate assays should be performed to detect retroviruses that may be noninfectious. Induction studies have not been found to be useful.

2. In Vitro Assays

In vitro tests are carried out by the inoculation of a test article (see *Table 2*) into various susceptible indicator cell cultures capable of detecting a wide range of human and relevant animal viruses. The choice of cells used in the test is governed by the species of origin of the cell bank to be tested, but should include a human and/or a nonhuman primate cell line susceptible to human viruses. The nature of the assay and the sample to be tested are governed by the type of virus which may possibly be present based on the origin or handling of the cells. Both cytopathic and hemadsorbing viruses should be sought.

3. In Vivo Assays

A test article (see *Table 2*) should be inoculated into animals, including suckling and adult mice, and in embryonated eggs to reveal viruses that cannot grow in cell cultures. Additional animal species may be used, depending on the nature and source of the cell lines being tested. The

health of the animals should be monitored and any abnormality should be investigated to establish the cause of the illness.

4. Antibody Production Tests

Species-specific viruses present in rodent cell lines may be detected by inoculating test article (see *Table 2*) into virus-free animals and examining the serum antibody level or enzyme activity after a specified period. Examples of such tests are the mouse antibody production (MAP) test, rat antibody production (RAP) test, and hamster antibody production (HAP) test. The viruses currently screened for in the antibody production assays are discussed in *Table 3*.

C. Acceptability of Cell Lines

It is recognized that some cell lines used for the manufacture of product will contain endogenous retroviruses, other viruses, or viral sequences. In such circumstances, the action plan recommended for manufacture is described in section V. of this document. The acceptability of cell lines containing viruses other than endogenous retroviruses will be considered on an individual basis by the regulatory authorities, by taking into account a risk/benefit analysis based on the benefit of the product and its intended clinical use, the nature of the contaminating viruses, their potential for infecting humans or for causing disease in humans, the purification process for the product (e.g., viral clearance evaluation data), and the extent of the virus tests conducted on the purified bulk.

Table 2. Examples of the Use and Limitations of Assays Which May be Used to Test for Virus

Test	Test Article	Detection Capability	Detection Limitation
Antibody production	Lysate of cells and their culture medium	Specific viral antigens	Antigens not infectious for animal test system
In vivo virus screen	Lysate of cells and their culture medium	Broad range of viruses pathogenic for humans	Agents failing to replicate or produce diseases in the test system
In vitro virus screen for:		Broad range of viruses pathogenic for humans	Agents failing to replicate or produce diseases in the test system
1. Cell bank characterization	1. Lysate of cells and their culture medium (for co-cultivation, intact cells should be in the test article)		
2. Production screen	2. Unprocessed bulk harvest or lysate of cells and their cell culture medium from the production reactor		
TEM on:		Virus and virus-like particles	Qualitative assay with assessment of identity
1. Cell substrate	1. Viable cells		
2. Cell culture supernatant	2. Cell-free culture supernatant		
Reverse transcriptase (RT)	Cell-free culture supernatant	Retroviruses and expressed retroviral RT	Only detects enzymes with optimal activity under preferred conditions. Interpretation may be difficult due to presence of cellular enzymes; background with some concentrated samples
Retrovirus (RV) infectivity	Cell-free culture supernatant	Infectious retroviruses	RV failing to replicate or form discrete foci or plaques in the chosen test system
Cocultivation	Viable cells	Infectious retroviruses	RV failing to replicate
1. Infectivity endpoint			1. See above under RV infectivity
2. TEM endpoint			2. See above under TEM ¹
3. RT endpoint			3. See above under RT
PCR (Polymerase chain reaction)	Cells, culture fluid and other materials	Specific virus sequences	Primer sequences must be present. Does not indicate whether virus is infectious.

¹ In addition, difficult to distinguish test article from indicator cells.

Table 3. Virus Detected in Antibody Production Tests

MAP	HAP	RAP
Ectromelia Virus ^{2,3}	Lymphocytic Choriomeningitis Virus (LCM) ^{1,3}	Hantaan Virus ^{1,3}
Hantaan Virus ^{1,3}	Pneumonia Virus of Mice (PVM) ^{2,3}	Kilham Rat Virus (KRV) ^{2,3}
K Virus ²	Reovirus Type 3 (Reo3) ^{1,3}	Mouse Encephalomyelitis Virus (Theilers, GDVII) ²
Lactic Dehydrogenase Virus (LDM) ^{1,3}	Sendai Virus ^{1,3}	Pneumonia Virus of Mice (PVM) ^{2,3}
Lymphocytic Choriomeningitis Virus (LCM) ^{1,3}	SV5	Rat Coronavirus (RCV) ²
Minute Virus of Mice ^{2,3}		Reovirus Type 3 (Reo3) ^{1,3}
Mouse Adenovirus (MAV) ^{2,3}		Sendai Virus ^{1,3}
Mouse Cytomegalovirus (MCMV) ^{2,3}		Sialocryoadenitis Virus (SDAV) ²
Mouse Encephalomyelitis Virus (Theilers, GDVII) ²		Toolan Virus (HI) ^{2,3}
Mouse Hepatitis Virus (MHV) ²		
Mouse Rotavirus (EDIM) ^{2,3}		
Pneumonia Virus of Mice (PVM) ^{2,3}		
Polyoma Virus ²		
Reovirus Type 3 (Reo3) ^{1,3}		
Sendai Virus ^{1,3}		
Thymic Virus ²		

¹ Viruses for which there is evidence of capacity for infecting humans or primates.

² Viruses for which there is no evidence of capacity for infecting humans.

³ Virus capable of replicating in vitro in cells of human or primate origin.

IV. TESTING FOR VIRUSES IN UNPROCESSED BULK

The unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media. When cells are not readily accessible (e.g., hollow fiber or similar systems), the unprocessed bulk would constitute fluids harvested from the fermenter. A representative sample of the unprocessed bulk, removed from the production reactor prior to further processing, represents one of the most suitable levels at which the possibility of adventitious virus contamination can be determined with a high probability of detection. Appropriate testing for viruses should be performed at the unprocessed bulk level unless virus testing is made more sensitive by initial partial processing (e.g., unprocessed bulk may be toxic in test cell cultures, whereas partially processed bulk may not be toxic).

In certain instances, it may be more appropriate to test a mixture consisting of both intact and disrupted cells and their cell culture supernatants removed from the production reactor prior to further processing. Data from at least three lots of unprocessed bulk at pilot-plant scale or commercial scale should be submitted as part of the marketing application/registration package.

It is recommended that manufacturers develop programs for the ongoing assessment of adventitious viruses in production batches. The scope, extent, and frequency of virus testing on the unprocessed bulk should be determined by taking several points into consideration, including the nature of the cell lines used to produce the desired products, the results and extent of virus tests performed during the qualification of the cell lines, the cultivation method, raw material sources, and results of viral clearance studies. In vitro screening tests, using one or several cell lines, are generally employed to test unprocessed bulk. If appropriate, a PCR test or other suitable methods may be used.

Generally, harvest material in which adventitious virus has been detected should not be used to manufacture the product. If any adventitious viruses are detected at this level, the process should be carefully checked to determine the cause of the contamination, and appropriate actions taken.

V. RATIONALE AND ACTION PLAN FOR VIRAL CLEARANCE STUDIES AND VIRUS TESTS ON PURIFIED BULK

It is important to design the most relevant and rational protocol for virus tests from the MCB level, through the various steps of drug production, to the final product including evaluation and characterization of viral clearance from unprocessed bulk. The evaluation and characterization of viral clearance plays a critical role in this scheme. The goal should be to obtain the best reasonable assurance that the product is free of virus contamination.

In selecting viruses to use for a clearance study, it is useful to distinguish between the need to evaluate processes for their ability to clear viruses that are known to be present and the desire to estimate the robustness of the process by characterizing the clearance of nonspecific "model" viruses (described later). Definitions of "relevant," specific, and non-specific "model" viruses are given in the glossary. Process evaluation requires knowledge of how much virus may be present in the process, such as the unprocessed bulk, and how much can be cleared in order to assess product safety. Knowledge of the time dependence for inactivation procedures is helpful in assuring the effectiveness of the inactivation process. When evaluating clearance of known contaminants, in-depth, time-dependent inactivation studies, demonstration of reproducibility of inactivation/removal, and evaluation of process parameters should be provided. When a manufacturing process is characterized for robustness of clearance using nonspecific "model" viruses, particular attention should be paid to nonenveloped viruses in the study design. The extent of viral clearance characterization studies may be influenced by the results of tests on cell lines and unprocessed bulk. These studies should be performed as described in section VI. below.

Table 4 presents an example of an action plan in terms of process evaluation and characterization of viral clearance as well as virus tests on purified bulk, in response to the results of virus tests on cells and/or the unprocessed bulk. Various cases are considered. In all cases, characterization of clearance using nonspecific "model" viruses should be performed. The most common situations are Cases A and B. Production systems contaminated with a virus other than a rodent retrovirus are normally not used. Where there are convincing and well justified reasons for drug production using a cell line from Cases C, D, or E, these should be discussed with the regulatory authorities. With Cases C, D,

Table 4. Action Plan for Process Assessment of Viral Clearance and Virus Tests on Purified Bulk

	Case A	Case B	Case C²	Case D²	Case E²
<i>Status</i>					
Presence of virus ¹	—	—	+	+	(+) ³
Virus-like particles ¹	—	—	—	—	(+) ³
Retrovirus-like particles ¹	—	+	—	—	(+) ³
Virus identified	not applicable	+	+	+	—
Virus pathogenic for humans	not applicable	— ⁴	— ⁴	+	unknown
<i>Action</i>					
Process characterization of viral clearance using nonspecific “model” viruses	yes ⁵	yes ⁵	yes ⁵	yes ⁵	yes ⁷
Process evaluation of viral clearance using “relevant” or specific “model” viruses	no	yes ⁶	yes ⁶	yes ⁶	yes ⁷
Test for virus in purified bulk	not applicable	yes ⁸	yes ⁸	yes ⁸	yes ⁸

¹ Results of virus tests for the cell substrate and/or at the unprocessed bulk level. Cell cultures used for production which are contaminated with viruses will generally not be acceptable. Endogenous viruses (such as retroviruses) or viruses that are an integral part of the MCB may be acceptable if appropriate viral clearance evaluation procedures are followed.

² The use of source material which is contaminated with viruses, whether or not they are known to be infectious and/or pathogenic in humans, will only be acceptable under very exceptional circumstances.

³ Virus has been observed by either direct or indirect methods.

⁴ Believed to be nonpathogenic.

⁵ Characterization of clearance using nonspecific “model” viruses should be performed.

⁶ Process evaluation for “relevant” viruses or specific “model” viruses should be performed.

⁷ See text under Case E.

⁸ The absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least 3 lots of purified bulk manufactured at pilot-plant or commercial scale should be provided. However for cell lines such as CHO cells for which the endogenous particles have been extensively characterized and adequate clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk.

and E, it is important to have validated effective steps to inactivate/remove the virus in question from the manufacturing process.

Case A: Where no virus, virus-like particle, or retrovirus-like particle has been demonstrated in the cells or in the unprocessed bulk, virus removal and inactivation studies should be performed with nonspecific “model” viruses as previously stated.

Case B: Where only a rodent retrovirus (or a retrovirus-like particle that is believed to be nonpathogenic, such as rodent A- and R-type particles) is present, process evaluation using a specific “model” virus, such as a murine leukemia virus, should be performed. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For marketing authorization, data from at least three lots of purified bulk at pilot-plant scale or commercial scale should be provided. Cell lines such as Chinese hamster ovary (CHO), C127, baby hamster kidney (BHK), and murine hybridoma cell lines have frequently been used as substrates for drug production with no reported safety problems related to viral contamination of the products. For these cell lines in which the endogenous particles have been extensively characterized and clearance has been demonstrated, it is not usually necessary to assay for the presence of the noninfectious particles in purified bulk. Studies with nonspecific “model” viruses, as in Case A, are appropriate.

Case C: When the cells or unprocessed bulk are known to contain a virus, other than a rodent retrovirus, for which there is no evidence of capacity for infecting humans (such as those identified by footnote 2 in Table 3, except rodent retroviruses (Case B)), virus removal and inactivation evaluation studies should use the identified virus. If it is not possible to use the identified virus, “relevant” or specific “model” viruses should be used to demonstrate acceptable clearance. Time-dependent inactivation for identified (or “relevant” or specific “model”) viruses at the critical inactivation step(s) should be obtained as part of process evaluation for these viruses. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing au-

thorization, data from at least three lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

Case D: Where a known human pathogen, such as those indicated by footnote 1 in Table 3, is identified, the product may be acceptable only under exceptional circumstances. In this instance, it is recommended that the identified virus be used for virus removal and inactivation evaluation studies and specific methods with high specificity and sensitivity for the detection of the virus in question be employed. If it is not possible to use the identified virus, “relevant” and/or specific “model” viruses (described later) should be used. The process should be shown to achieve the removal and inactivation of the selected viruses during the purification and inactivation processes. Time-dependent inactivation data for the critical inactivation step(s) should be obtained as part of process evaluation. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorization, data from at least three lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

Case E: When a virus that cannot be classified by currently available methodologies is detected in the cells or unprocessed bulk, the product is usually considered unacceptable since the virus may prove to be pathogenic. In the very rare case where there are convincing and well justified reasons for drug production using such a cell line, this should be discussed with the regulatory authorities before proceeding further.

VI. EVALUATION AND CHARACTERIZATION OF VIRAL CLEARANCE PROCEDURES

Evaluation and characterization of due virus removal and/or inactivation procedures play an important role in establishing the safety of biotechnology products. Many instances of contamination in the past have occurred with agents whose presence was not known or even suspected, and though this happened to biological products derived from various source materials other than fully characterized cell

lines, assessment of viral clearance will provide a measure of confidence that any unknown, unsuspected, and harmful viruses may be removed. Studies should be carried out in a manner that is well documented and controlled.

The objective of viral clearance studies is to assess process step(s) that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the process. This should be achieved by the deliberate addition ("spiking") of significant amounts of a virus to the crude material and/or to different fractions obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps. It is not considered necessary to evaluate or characterize every step of a manufacturing process if adequate clearance is demonstrated by the use of fewer steps. It should be borne in mind that other steps in the process may have an indirect effect on the viral inactivation/removal achieved. Manufacturers should explain and justify the approach used in studies for evaluating virus clearance.

The reduction of virus infectivity may be achieved by removal of virus particles or by inactivation of viral infectivity. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. For inactivation steps, the study should be planned in such a way that samples are taken at different times and an inactivation curve constructed (see section VI.B.5.).

Viral clearance evaluation studies are performed to demonstrate the clearance of a virus known to be present in the MCB and/or to provide some level of assurance that adventitious viruses which could not be detected, or might gain access to the production process, would be cleared. Reduction factors are normally expressed on a logarithmic scale, which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

In addition to clearance studies for viruses known to be present, studies to characterize the ability to remove and/or inactivate other viruses should be conducted. The purpose of studies with viruses exhibiting a range of biochemical and biophysical properties that are not known or expected to be present is to characterize the robustness of the procedure rather than to achieve a specific inactivation or removal goal. A demonstration of the capacity of the production process to inactivate or remove viruses is desirable (see section VI.C.). Such studies are not performed to evaluate a specific safety risk. Therefore, a specific clearance value need not be achieved.

A. The Choice of Viruses for the Evaluation and Characterization of Viral Clearance

Viruses for clearance evaluation and process characterization studies should be chosen to resemble viruses which may contaminate the product and to represent a wide range of physico-chemical properties in order to test the ability of the system to eliminate viruses in general. The manufacturer should justify the choice of viruses in accordance with the aims of the evaluation and characterization study and the guidance provided in this document.

1. "Relevant" Viruses and "Model" Viruses

A major issue in performing a viral clearance study is to determine which viruses should be used. Such viruses fall into three categories: "Relevant" viruses, specific "model" viruses, and nonspecific "model" viruses.

"Relevant" viruses are viruses used in process evaluation of viral clearance studies which are either the identified viruses, or of the same species as the viruses that are known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. The purification and/or inactivation process should demonstrate the capability to remove and/or inactivate such viruses. When a "relevant" virus is not available or when it is not well adapted to process evaluation of viral clearance studies

(e.g., it cannot be grown in vitro to sufficiently high titers), a specific "model" virus should be used as a substitute. An appropriate specific "model" virus may be a virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to the observed or suspected virus.

Cell lines derived from rodents usually contain endogenous retrovirus particles or retrovirus-like particles, which may be infectious (C-type particles) or noninfectious (cytoplasmic A- and R-type particles). The capacity of the manufacturing process to remove and/or inactivate rodent retroviruses from products obtained from such cells should be determined. This may be accomplished by using a murine leukemia virus, a specific "model" virus in the case of cells of murine origin. When human cell lines secreting monoclonal antibodies have been obtained by the immortalization of B lymphocytes by Epstein-Barr Virus (EBV), the ability of the manufacturing process to remove and/or inactivate a herpes virus should be determined. Pseudorabies virus may also be used as a specific "model" virus.

When the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the clearance process, viral clearance characterization studies should be performed with nonspecific "model" viruses with differing properties. Data obtained from studies with "relevant" and/or specific "model" viruses may also contribute to this assessment. It is not necessary to test all types of viruses. Preference should be given to viruses that display a significant resistance to physical and/or chemical treatments. The results obtained for such viruses provide useful information about the ability of the production process to remove and/or inactivate viruses in general. The choice and number of viruses used will be influenced by the quality and characterization of the cell lines and the production process.

Examples of useful "model" viruses representing a range of physico-chemical structures and examples of viruses which have been used in viral clearance studies are given in *Appendix 2* and *Table A-1*.

2. Other Considerations

Additional points to be considered are as follows:

(a) Viruses which can be grown to high titer are desirable, although this may not always be possible.

(b) There should be an efficient and reliable assay for the detection of each virus used, for every stage of manufacturing that is tested.

(c) Consideration should be given to the health hazard which certain viruses may pose to the personnel performing the clearance studies.

B. Design and Implications of Viral Clearance Evaluation and Characterization Studies

1. Facility and Staff

It is inappropriate to introduce any virus into a production facility because of good manufacturing practice (GMP) constraints. Therefore, viral clearance studies should be conducted in a separate laboratory equipped for virological work and performed by staff with virological expertise in conjunction with production personnel involved in designing and preparing a scaled-down version of the purification process.

2. Scaled-down Production System

The validity of the scaling down should be demonstrated. The level of purification of the scaled-down version should represent as closely as possible the production procedure. For chromatographic equipment, column bed-height, linear flow-rate, flow-rate-to-bed-volume ratio (i.e., contact time), buffer and gel types, pH, temperature, and concentration of protein, salt, and product should all be shown to be representative of commercial-scale manufacturing. A similar elution profile should result. For other procedures, similar considerations apply. Deviations that cannot be avoided should be discussed with regard to their influence on the results.

Table A-1. Examples of Viruses Which Have Been Used in Viral Clearance Studies

Virus	Family	Genus	Natural Host	Genome	Env	Size (nm)	Shape	Resistance¹
Vesicular Stomatitis Virus	Rhabdo	Vesiculovirus	Equine Bovine	RNA	yes	70 × 150	Bullet	Low
Parainfluenza Virus	Paramyxo	Paramyxovirus	Various	RNA	yes	100–200	Pleo/Spher	Low
MuLV	Retro	Type C oncovirus	Mouse	RNA	yes	80–110	Spherical	Low
Sindbis Virus	Toga	Alphavirus	Human	RNA	yes	60–70	Spherical	Low
BVDV	Flavi	Pestivirus	Bovine	RNA	yes	50–70	Pleo/Spher	Low
Pseudo-rabies Virus	Herpes		Swine	DNA	yes	120–200	Spherical	Med
Poliovirus Sabin Type 1	Picorna	Enterovirus	Human	RNA	no	25–30	Icosahedral	Med
Encephalomyocarditis Virus (EMC)	Picorna	Cardiovirus	Mouse	RNA	no	25–30	Icosahedral	Med
Reovirus 3	Reo	Orthoreovirus	Various	DNA	no	60–80	Spherical	Med
SV 40	Papova	Polyomavirus	Monkey	DNA	no	40–50	Icosahedral	Very high
Parvoviruses (canine, porcine)	Parvo	Parvovirus	Canine Porcine	DNA	no	18–24	Icosahedral	Very high

¹ Resistance to physico-chemical treatments based on studies of production processes. Resistance is relative to the specific treatment and it is used in the context of the understanding of the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment. These viruses are examples only and their use is not considered mandatory.

3. Analysis of Step-wise Elimination of Virus

When viral clearance studies are being performed, it is desirable to assess the contribution of more than one production step to virus elimination. Steps which are likely to clear virus should be individually assessed for their ability to remove and inactivate virus and careful consideration should be given to the exact definition of an individual step. Sufficient virus should be present in the material of each step to be tested so that an adequate assessment of the effectiveness of each step is obtained. Generally, virus should be added to in-process material of each step to be tested. In some cases, simply adding high titer virus to unpurified bulk and testing its concentration between steps will be sufficient. Where virus removal results from separation procedures, it is recommended that, if appropriate and if possible, the distribution of the virus load in the different fractions be investigated. When virucidal buffers are used in multiple steps within the manufacturing process, alternative strategies such as parallel spiking in less virucidal buffers may be carried out as part of the overall process assessment. The virus titer before and after each step being tested should be determined. Quantitative infectivity assays should have adequate sensitivity and reproducibility and should be performed with sufficient replicates to ensure adequate statistical validity of the result. Quantitative assays not associated with infectivity may be used if justified. Appropriate virus controls should be included in all infectivity assays to ensure the sensitivity of the method. Also, the statistics of sampling virus when at low concentrations should be considered (*Appendix 3*).

4. Determining Physical Removal Versus Inactivation

Reduction in virus infectivity may be achieved by the removal or inactivation of virus. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. If little clearance of infectivity is achieved by the production process, and the clearance of virus is considered to be a major factor in the safety of the product, specific or additional inactivation/removal steps should be introduced. It may be necessary to distinguish between removal and inactivation for a particular step, for example, when there is a possibility that a buffer used in more than one clearance step may contribute to inactivation during each step, i.e., the contribution to inactivation by a buffer shared by several chromatographic steps and the removal achieved by each of these chromatographic steps should be distinguished.

5. Inactivation Assessment

For assessment of viral inactivation, unprocessed crude material or intermediate material should be spiked with infectious virus and the reduction factor calculated. It should be recognized that virus inactivation is not a simple, first order reaction and is usually more complex, with a fast "phase 1" and a slow "phase 2." The study should, therefore, be planned in such a way that samples are taken at different times and an inactivation curve constructed. It is recommended that studies for inactivation include at least one time point less than the minimum exposure time and greater than zero, in addition to the minimum exposure time. Additional data are particularly important where the virus is a "relevant" virus known to be a human pathogen and an effective inactivation process is being designed. However, for inactivation studies in which nonspecific "model" viruses are used or when specific "model" viruses are used as surrogates for virus particles, such as the CHO intracytoplasmic retrovirus-like particles, reproducible clearance should be demonstrated in at least two independent studies. Whenever possible, the initial virus load should be determined from the virus that can be detected in the spiked starting material. If this is not possible, the initial virus load may be calculated from the titer of the spiking virus preparation. Where inactivation is too rapid to plot an inactivation curve using process conditions, appropriate controls should be performed to demonstrate that infectivity is indeed lost by inactivation.

6. Function and Regeneration of Columns

Over time and after repeated use, the ability of chromatography columns and other devices used in the purification scheme to clear virus may vary. Some estimate of the stability of the viral clearance after several uses may provide support for repeated use of such columns. Assurance should be provided that any virus potentially retained by the production system would be adequately destroyed or removed prior to reuse of the system. For example, such evidence may be provided by demonstrating that the cleaning and regeneration procedures do inactivate or remove virus.

7. Specific Precautions

(a) Care should be taken in preparing the high-titer virus to avoid aggregation which may enhance physical removal and decrease inactivation, thus distorting the correlation with actual production.

(b) Consideration should be given to the minimum quantity of virus which can be reliably assayed.

(c) The study should include parallel control assays to assess the loss of infectivity of the virus due to such reasons as

the dilution, concentration, filtration or storage of samples before titration.

(d) The virus "spike" should be added to the product in a small volume so as not to dilute or change the characteristics of the product. Diluted, test-protein sample is no longer identical to the product obtained at commercial scale.

(e) Small differences in, for example, buffers, media, or reagents can substantially affect viral clearance.

(f) Virus inactivation is time-dependent; therefore, the amount of time a spiked product remains in a particular buffer solution or on a particular chromatography column should reflect the conditions of the commercial-scale process.

(g) Buffers and product should be evaluated independently for toxicity or interference in assays used to determine the virus titer, as these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, or dialysis of the buffer containing spiked virus might be necessary. If the product itself has anti-viral activity, the clearance study may need to be performed without the product in a "mock" run, although omitting the product or substituting a similar protein that does not have anti-viral activity could affect the behavior of the virus in some production steps. Sufficient controls to demonstrate the effect of procedures used solely to prepare the sample for assay (e.g., dialysis, storage) on the removal/inactivation of the spiking virus should be included.

(h) Many purification schemes use the same or similar buffers or columns repetitively. The effects of this approach should be taken into account when analyzing the data. The effectiveness of virus elimination by a particular process may vary with the manufacturing stage at which it is used.

(i) Overall reduction factors may be underestimated where production conditions or buffers are too cytotoxic or virucidal and should be discussed on a case-by-case basis. Overall reduction factors may also be overestimated due to inherent limitations or inadequate design of viral clearance studies.

C. Interpretation of Viral Clearance Studies; Acceptability

The object of assessing virus inactivation/removal is to evaluate and characterize process steps that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process. For virus contaminants, as in Cases B through E, it is important to show that not only is the virus eliminated or inactivated, but that there is excess capacity for viral clearance built into the purification process to assure an appropriate level of safety for the final product. The amount of virus eliminated or inactivated by the production process should be compared to the amount of virus which may be present in unprocessed bulk.

To carry out this comparison, it is important to estimate the amount of virus in the unprocessed bulk. This estimate should be obtained using assays for infectivity or other methods such as transmission electron microscopy (TEM). The entire purification process should be able to eliminate substantially more virus than is estimated to be present in a single-dose-equivalent of unprocessed bulk. See *Appendix 4* for calculation of virus reduction factors and *Appendix 5* for calculation of estimated particles per dose.

Manufacturers should recognize that clearance mechanisms may differ between virus classes. A combination of factors should be considered when judging the data supporting the effectiveness of virus inactivation/removal procedures. These include:

- (i) The appropriateness of the test viruses used;
- (ii) The design of the clearance studies;
- (iii) The log reduction achieved;
- (iv) The time dependence of inactivation;
- (v) The potential effects of variation in process parameters on virus inactivation/removal;
- (vi) The limits of assay sensitivities;

(vii) The possible selectivity of inactivation/removal procedure(s) for certain classes of viruses.

Effective clearance may be achieved by any of the following: Multiple inactivation steps, multiple complementary separation steps, or combinations of inactivation and separation steps. Since separation methods may be dependent on the extremely specific physico-chemical properties of a virus which influence its interaction with gel matrices and precipitation properties, "model" viruses may be separated in a different manner than a target virus. Manufacturing parameters influencing separation should be properly defined and controlled. Differences may originate from changes in surface properties such as glycosylation. However, despite these potential variables, effective removal can be obtained by a combination of complementary separation steps or combinations of inactivation and separation steps. Therefore, well-designed separation steps, such as chromatographic procedures, filtration steps, and extractions, can be effective virus removal steps provided that they are performed under appropriately controlled conditions. An effective virus removal step should give reproducible reduction of virus load shown by at least two independent studies.

An overall reduction factor is generally expressed as the sum of the individual factors. However, reduction in virus titer of the order of $1 \log_{10}$ or less would be considered negligible and would be ignored unless justified.

If little reduction of infectivity is achieved by the production process, and the removal of virus is considered to be a major factor in the safety of the product, a specific, additional inactivation/removal step or steps should be introduced. For all viruses, manufacturers should justify the acceptability of the reduction factors obtained. Results would be evaluated on the basis of the factors listed above.

D. Limitations of Viral Clearance Studies

Viral clearance studies are useful for contributing to the assurance that an acceptable level of safety in the final product is achieved but do not by themselves establish safety. However, a number of factors in the design and execution of viral clearance studies may lead to an incorrect estimate of the ability of the process to remove virus infectivity. These factors include the following:

1. Virus preparations used in clearance studies for a production process are likely to be produced in tissue culture. The behavior of a tissue culture virus in a production step may be different from that of the native virus, for example, if native and cultured viruses differ in purity or degree of aggregation.

2. Inactivation of virus infectivity frequently follows a biphasic curve in which a rapid initial phase is followed by a slower phase. It is possible that virus escaping a first inactivation step may be more resistant to subsequent steps. For example, if the resistant fraction takes the form of virus aggregates, infectivity may be resistant to a range of different chemical treatments and to heating.

3. The ability of the overall process to remove infectivity is expressed as the sum of the logarithm of the reductions at each step. The summation of the reduction factors of multiple steps, particularly of steps with little reduction (e.g., below $1 \log_{10}$), may overestimate the true potential for virus elimination. Furthermore, reduction values achieved by repetition of identical or near identical procedures should not be included unless justified.

4. The expression of reduction factors as logarithmic reductions in titer implies that, while residual virus infectivity may be greatly reduced, it will never be reduced to zero. For example, a reduction in the infectivity of a preparation containing $8 \log_{10}$ infectious units per milliliter (mL) by a factor of $8 \log_{10}$ leaves zero \log_{10} per mL or one infectious unit per mL, taking into consideration the limit of detection of the assay.

5. Pilot-plant scale processing may differ from commercial-scale processing despite care taken to design the scaled-down process.

6. Addition of individual virus reduction factors resulting from similar inactivation mechanisms along the manufacturing process may overestimate overall viral clearance.

E. Statistics

The viral clearance studies should include the use of statistical analysis of the data to evaluate the results. The study results should be statistically valid to support the conclusions reached (see *Appendix 3*).

F. Reevaluation of Viral Clearance

Whenever significant changes in the production or purification process are made, the effect of that change, both direct and indirect, on viral clearance should be considered and the system re-evaluated as needed. For example, changes in production processes may cause significant changes in the amount of virus produced by the cell line; changes in process steps may change the extent of viral clearance.

VII. SUMMARY

This document suggests approaches for the evaluation of the risk of viral contamination and for the removal of virus from product, thus contributing to the production of safe biotechnology products derived from animal or human cell lines, and emphasizes the value of many strategies, including:

- A. Thorough characterization/screening of cell substrate starting material in order to identify which, if any, viral contaminants are present;
- B. Assessment of risk by determination of the human tropism of the contaminants;
- C. Establishment of an appropriate program of testing for adventitious viruses in unprocessed bulk;
- D. Careful design of viral clearance studies using different methods of virus inactivation or removal in the same production process in order to achieve maximum viral clearance; and
- E. Performance of studies which assess virus inactivation and removal.

GLOSSARY

Adventitious Virus. See *Virus*.

Cell Substrate. Cells used to manufacture product.

Endogenous Virus. See *Virus*.

Inactivation. Reduction of virus infectivity caused by chemical or physical modification.

In Vitro Cell Age. A measure of the period between thawing of the MCB vial(s) and harvest of the production vessel measured by elapsed chronological time in culture, population doubling level of the cells, or passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

Master Cell Bank (MCB). An aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers, and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB, or WCB) should be the same as for the original MCB, unless justified.

Minimum Exposure Time. The shortest period for which a treatment step will be maintained.

Nonendogenous Virus. See *Virus*.

Process Characterization of Viral Clearance. Viral clearance studies in which nonspecific "model" viruses are used to assess the robustness of the manufacturing process to remove and/or inactivate viruses.

Process Evaluation Studies of Viral Clearance. Viral clearance studies in which "relevant" and/or specific "model" viruses are used to determine the ability of the manufacturing process to remove and/or inactivate these viruses.

Production Cells. Cell substrate used to manufacture product.

Unprocessed Bulk. One or multiple pooled harvests of cells and culture media. When cells are not readily accessible, the unprocessed bulk would constitute fluid harvested from the fermenter.

Virus. Intracellularly replicating infectious agents that are potentially pathogenic, possess only a single type of nucleic acid (either ribonucleic acid (RNA) or DNA), are unable to grow and undergo binary fission, and multiply in the form of their genetic material.

Adventitious Virus. Unintentionally introduced contaminant virus.

Endogenous Virus. Viral entity whose genome is part of the germ line of the species of origin of the cell line and is covalently integrated into the genome of animal from which the parental cell line was derived. For the purposes of this document, intentionally introduced, nonintegrated viruses such as EBV used to immortalize cell substrates or Bovine Papilloma Virus fit in this category.

Nonendogenous Virus. Virus from external sources present in the MCB.

Nonspecific Model Virus. A virus used for characterization of viral clearance of the process when the purpose is to characterize the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterize the robustness of the purification process.

Relevant Virus. Virus used in process evaluation studies which is either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process.

Specific Model Virus. Virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus.

Viral Clearance. Elimination of target virus by removal of viral particles or inactivation of viral infectivity.

Virus-like Particles. Structures visible by electron microscopy which morphologically appear to be related to known viruses.

Virus Removal. Physical separation of virus particles from the intended product.

Working Cell Bank (WCB). The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

APPENDIX 1

Products Derived from Characterized Cell Banks Which Were Subsequently Grown In Vivo

For products manufactured from fluids harvested from animals inoculated with cells from characterized banks, additional information regarding the animals should be provided.

Whenever possible, animals used in the manufacture of biotechnological/biological products should be obtained from well defined, specific pathogen-free colonies. Adequate testing for appropriate viruses, such as those listed in *Table 3*, should be performed. Quarantine procedures for newly arrived as well as diseased animals should be described, and assurance provided that all containment, cleaning, and decontamination methodologies employed within the facility are adequate to contain the spread of adventitious agents. This may be accomplished through the use of a sentinel program. A listing of agents for which testing is performed should also be included. Veterinary support services should be available on-site or within easy access. The degree to which the vivarium is segregated from other areas of the manufacturing facility should be described. Personnel practices should be adequate to ensure safety.

Procedures for the maintenance of the animals should be fully described. These would include diet, cleaning and feeding schedules, provisions for periodic veterinary care if appli-

cable, and details of special handling that the animals may require once inoculated. A description of the priming regimen(s) for the animals, the preparation of the inoculum, and the site and route of inoculation should also be included.

The primary harvest material from animals may be considered an equivalent stage of manufacture to unprocessed bulk harvest from a bioreactor. Therefore, all testing considerations previously outlined in section IV. of this document should apply. In addition, the manufacturer should assess the bioburden of the unprocessed bulk, determine whether the material is free of mycoplasma, and perform species-specific assay(s) as well as in vivo testing in adult and suckling mice.

APPENDIX 2

The Choice of Viruses for Viral Clearance Studies

A. Examples of Useful "Model" Viruses:

1. Nonspecific "model" viruses representing a range of physico-chemical structures:

- SV40 (Polyomavirus maccacae 1), human polio virus 1 (Sabin), animal parvovirus or some other small, nonenveloped viruses;
- a parainfluenza virus or influenza virus, Sindbis virus or some other medium-to-large, enveloped, RNA viruses;
- a herpes virus (e.g., HSV-1 or a pseudorabies virus), or some other medium-to-large, DNA viruses.

These viruses are examples only and their use is not mandatory.

2. For rodent cell substrates murine retroviruses are commonly used as specific "model" viruses.

B. Examples of Viruses That Have Been Used in Viral Clearance Studies

Several viruses that have been used in viral clearance studies are listed in Table A-1. However, since these are merely examples, the use of any of the viruses in the table is not considered mandatory and manufacturers are invited to consider other viruses, especially those that may be more appropriate for their individual production processes. Generally, the process should be assessed for its ability to clear at least three different viruses with differing characteristics.

APPENDIX 3

A. Statistical Considerations for Assessing Virus Assays

Virus titrations suffer the problems of variation common to all biological assay systems. Assessment of the accuracy of the virus titrations and reduction factors derived from them and the validity of the assays should be performed to define the reliability of a study. The objective of statistical evaluation is to establish that the study has been carried out to an acceptable level of virological competence.

1. Assay methods may be either quantal or quantitative. Quantal methods include infectivity assays in animals or in tissue-culture-infectious-dose (TCID) assays, in which the animal or cell culture is scored as either infected or not. Infectivity titers are then measured by the proportion of animals or culture infected. In quantitative methods, the infectivity measured varies continuously with the virus input. Quantitative methods include plaque assays where each plaque counted corresponds to a single infectious unit. Both quantal and quantitative assays are amenable to statistical evaluation.

2. Variation can arise within an assay as a result of dilution errors, statistical effects, and differences within the assay system which are either unknown or difficult to control. These effects are likely to be greater when different assay runs are compared (between-assay variation) than when results within a single assay run are compared (within-assay variation).

3. The 95 percent confidence limits for results of within-assay variation normally should be on the order of $\pm 0.5 \log_{10}$ of the mean. Within-assay variation can be assessed by standard textbook methods. Between-assay variation can be monitored by the inclusion of a reference preparation, the estimate of whose potency should be within approximately $0.5 \log_{10}$ of the mean estimate established in the laboratory for the assay to be acceptable. Assays with lower precision may be acceptable with appropriate justification.

4. The 95 percent confidence limits for the reduction factor observed should be calculated wherever possible in studies of clearance of "relevant" and specific "model" viruses. If the 95 percent confidence limits for the viral assays of the starting material are $\pm s$, and for the viral assays of the material after the step are $\pm a$, the 95 percent confidence limits for the reduction factor are

$$\pm \sqrt{S^2 + a^2}$$

B. Probability of Detection of Viruses at Low Concentrations

At low virus concentrations (e.g., in the range of 10 to 1,000 infectious particles per L) it is evident that a sample of a few milliliters may or may not contain infectious particles. The probability, p , that this sample does not contain infectious viruses is:

$$p = ((V-v)/V)^n$$

where V (L) is the overall volume of the material to be tested; v (L) is the volume of the sample; and n is the absolute number of infectious particles statistically distributed in V .

If $V \gg v$, this equation can be approximated by the Poisson distribution:

$$p = e^{-cv}$$

where c is the concentration of infectious particles per L.

$$\text{or, } c = \ln p / -v$$

As an example, if a sample volume of 1 mL is tested, the probabilities p at virus concentrations ranging from 10 to 1,000 infectious particles per L are:

c	10	10	1,000
p	0.99	0.90	0.37

This indicates that for a concentration of 1,000 viruses per L, in 37 percent of sampling, 1 mL will not contain a virus particle.

If only a portion of a sample is tested for virus and the test is negative, the amount of virus which would have to be present in the total sample in order to achieve a positive result should be calculated and this value taken into account when calculating a reduction factor. Confidence limits at 95 percent are desirable. However, in some instances, this may not be practical due to material limitations.

APPENDIX 4

Calculation of Reduction Factors in Studies to Determine Viral Clearance

The virus reduction factor of an individual purification or inactivation step is defined as the \log_{10} of the ratio of the virus load in the pre-purification material and the virus load in the post-purification material which is ready for use in the next step of the process. If the following abbreviations are used:

Starting material: vol v' ; titer $10^{a'}$;
virus load: $(v')(10^a)$,

Final material: vol v'' ; titer $10^{a''}$;
virus load: $(v'')(10^{a''})$,
the individual reduction factors R_i are calculated according to

$$10^{R_i} = (v')(10^a) / (v'')(10^{a''})$$

This formula takes into account both the titers and volumes of the materials before and after the purification step.

Because of the inherent imprecision of some virus titrations, an individual reduction factor used for the calculation of an overall reduction factor should be greater than 1.

The overall reduction factor for a complete production process is the sum logarithm of the reduction factors of the individual steps. It represents the logarithm of the ratio of the virus load at the beginning of the first process clearance step and at the end of the last process clearance step. Reduction factors are normally expressed on a logarithmic scale which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

APPENDIX 5

Calculation of Estimated Particles per Dose

This is applicable to those viruses for which an estimate of starting numbers can be made, such as endogenous retroviruses.

Example:

I. Assumptions

Measured or estimated concentration of virus in cell culture harvest = 10^6 /mL

Calculated viral clearance factor = $> 10^{15}$

Volume of culture harvest needed to make a dose of product = 1 L (10^3 mL)

II. Calculation of Estimated Particles/Dose

$$\frac{(10^6 \text{ virus units/mL}) \times (10^3 \text{ mL/dose})}{\text{Clearance factor} > 10^{15}}$$

$$= \frac{10^9 \text{ particles/dose}}{\text{Clearance factor} > 10^{15}}$$

$$= < 10^{-6} \text{ particles/dose}$$

Therefore, less than one particle per million doses would be expected.

<1051> CLEANING GLASS APPARATUS

Change to read:

▲ Success in conducting many Pharmacopeial assays and tests depends upon the cleanliness of the glassware apparatus used. Usage of commercial detergents or inorganic reagents for cleaning should be used when necessary.

In all cases, it is important to verify that the cleaning procedure is appropriate for the particular test or assay being undertaken. This can be accomplished in a number of ways,

including use of experimental controls or verification of cleaning by utilization of residue/residual testing to ensure removal of any potential contaminants. A statement should be included in the cleaning protocol describing how the success of the cleaning procedure will be assessed.

For optical measurements, special care is required for cleaning containers, but the use of chromic acid or highly alkaline solutions should be avoided.

Some particular tests, though not inclusive, wherein the use of clean glassware is critical for success include the following: pyrogen and total organic carbon tests as well as assays of heparin sodium and vitamin B₁₂ activity.

Selected references that might be helpful in obtaining additional information on cleaning glass apparatus are listed in the *Appendix*. USP does not endorse these citations, and they do not represent an exhaustive list. Further information about the cleanliness of the glassware apparatus procedures mentioned in this chapter may also be found in most quantitative chemical analytical textbooks. ▲ USP36

Add the following:

▲ APPENDIX

Additional information and guidance can be found in the references listed below or in many quantitative chemical analytical textbooks:

1. Parenteral Drug Association. *Draft—Points to Consider for Cleaning Validation* (Technical Report Number 29). Bethesda, MD: Parenteral Drug Association; 1998.
2. Anderson NR. Container cleaning and sterilization. In: Olson WP, Groves MJ, eds. *Aseptic Pharmaceutical Manufacturing*. 1st ed. Buffalo Grove, IL: Interpharm Press; 1987:15–22.
3. Green C. Cleaning validation—application in the laboratory; Montalvo M. The cleaning validation policy and the cleaning validation plan; Verghese G, Kaiser N. Cleaning agents and cleaning chemistry; Verghese G, Lopolito P. Cleaning engineering and equipment design. In: Pluta PL, ed. *Cleaning and Cleaning Validation, Volume 1*. Bethesda, MD: Parenteral Drug Association; 2009.
4. Gordon AJ, Ford RA. Standard glassware cleaning solutions. In: Gordon AJ, Ford RA, eds. *The Chemist's Companion*. Hoboken, NJ: Wiley and Sons; 1973.

▲ USP36

<1052> BIOTECHNOLOGY-DERIVED ARTICLES—AMINO ACID ANALYSIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by amino acid analysis. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Portions of the chapter that are not harmonized with the other two pharmacopeias are marked by the symbol ♦. The footnote below is in the *USP* but is not in the *EP* or *JP*. Other characterization tests, also harmonized, are shown in *Biotechnology-Derived Articles—Capillary Electrophoresis* <1053>, *Biotechnology-Derived Articles—Isoelectric Focusing* <1054>, *Biotechnology-Derived Articles—Peptide Mapping* <1055>, *Biotechnology-Derived Arti-*

cles—Polyacrylamide Gel Electrophoresis (1056), and Biotechnology-Derived Articles—Total Protein Assay (1057).

INTRODUCTION

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organized as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify protein and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide. It is necessary to hydrolyze a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations. The amino acid constituents of the test sample are typically derivatized for analysis.

APPARATUS

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have postcolumn derivatization capability, unless the sample is analyzed using precolumn derivatization. The detector is usually a UV-visible or fluorescence detector depending on the derivatization method used. A recording device (e.g., integrator) is used for transforming the analog signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

GENERAL PRECAUTIONS

Background contamination is always a concern for the analyst in performing amino acid analysis. High-purity reagents are necessary (e.g., low-purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with their hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a

routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

REFERENCE STANDARD MATERIAL

Acceptable amino acid standards are commercially available* for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analyzed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

CALIBRATION OF INSTRUMENTATION

Calibration of amino acid analysis instrumentation typically involves analyzing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino acid analysis technique. Then, replicates at each of the different analyte levels can be analyzed. Peak areas obtained for each amino acid are plotted versus the known concentration for each of the amino acids in the standard dilution. These results will allow the analyst to determine the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important that the analyst prepare the samples for amino acid analysis so that they are within the analytical limits (e.g., linear working range) of the technique employed in order to obtain accurate and repeatable results.

Four to six amino acid standard levels are analyzed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nmol of amino acid present in the standard. A calibration file consisting of the response factor for each amino acid is prepared and is used to calculate the concentration of each amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given amino acid by the response factor for that amino acid to give the nmol of the amino acid. For routine analysis, a single-point calibration may be sufficient; however, the calibration file is updated frequently and tested by the analysis of analytical controls to ensure its integrity.

REPEATABILITY

Consistent high quality amino acid analysis results from an analytical laboratory require attention to the repeatability of the assay. During analysis of the chromatographic separation of the amino acids or their derivatives, numerous peaks can be observed on the chromatogram that corresponds to the amino acids. The large number of peaks makes it necessary to have an amino acid analysis system that can repeatedly identify the peaks based on retention time and integrate the peak areas for quantitation. A typical repeatability evaluation involves preparing a standard amino acid solution and analyzing many replicates (i.e., six analyses or more) of the same standard solution. The relative standard deviation (RSD) is determined for the retention time and integrated peak area of each amino acid. An evaluation of the repeatability is expanded to include multiple assays con-

Suitable standards may be obtained from NIST (Gaithersburg, MD), Beckman Instruments (Fullerton, CA), Sigma Chemical (St. Louis, MO), Pierce (Rockford, IL), or Agilent (Palo Alto, CA).

ducted over several days by different analysts. Multiple assays include the preparation of standard dilutions from starting materials to determine the variation due to sample handling. Often, the amino acid composition of a standard protein (e.g., bovine serum albumin) is analyzed as part of the repeatability evaluation. By evaluating the replicate variation (i.e., RSD), the laboratory can establish analytical limits to ensure that the analyses from the laboratory are under control. It is desirable to establish the lowest practical variation limits to ensure the best results. Areas to focus on to lower the variability of the amino acid analysis include sample preparation, high background spectral interference due to the quality of reagents and/or to laboratory practices, instrument performance and maintenance, data analysis and interpretation, and analyst performance and habits. All parameters involved are fully investigated in the scope of the validation work.

SAMPLE PREPARATION

Accurate results from amino acid analysis require purified protein and peptide samples. Buffer components (e.g., salts, urea, detergents) can interfere with the amino acid analysis and are removed from the sample before analysis. Methods that utilize postcolumn derivatization of the amino acids are generally not affected by buffer components to the extent seen with precolumn derivatization methods. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labor. Common techniques used to remove buffer components from protein samples include the following methods: (1) injecting the protein sample onto a reverse-phase HPLC system, removing the protein with a volatile solvent containing a sufficient organic component, and drying the sample in a vacuum centrifuge; (2) dialysis against a volatile buffer or water; (3) centrifugal ultrafiltration for buffer replacement with a volatile buffer or water; (4) precipitating the protein from the buffer using an organic solvent (e.g., acetone); and (5) gel filtration.

INTERNAL STANDARDS

It is recommended that an internal standard be used to monitor physical and chemical losses and variations during amino acid analysis. An accurately known amount of internal standard can be added to a protein solution prior to hydrolysis. The recovery of the internal standard gives the general recovery of the amino acids from the protein solution. Free amino acids, however, do not behave in the same way as protein-bound amino acids during hydrolysis because their rates of release or destruction are variable. Therefore, the use of an internal standard to correct for losses during hydrolysis may give unreliable results. It will be necessary to take this particular point into consideration when interpreting the results. Internal standards can also be added to the mixture of amino acids after hydrolysis to correct for differences in sample application and changes in reagent stability and flow rates. Ideally, an internal standard is an unnaturally occurring primary amino acid that is commercially available and inexpensive. It should also be stable during hydrolysis, its response factor should be linear with concentration, and it needs to elute with a unique retention time without overlapping other amino acids. Commonly used amino acid standards include norleucine, nitrotyrosine, and α -aminobutyric acid.

PROTEIN HYDROLYSIS

Hydrolysis of protein and peptide samples is necessary for amino acid analysis of these molecules. The glassware used for hydrolysis must be very clean to avoid erroneous results.

Glove powders and fingerprints on hydrolysis tubes may cause contamination. To clean glass hydrolysis tubes, boil tubes for 1 hour in 1 N hydrochloric acid or soak tubes in concentrated nitric acid or in a mixture of concentrated hydrochloric acid and concentrated nitric acid (1:1). Clean hydrolysis tubes are rinsed with high-purity water followed by a rinse with HPLC-grade methanol, dried overnight in an oven, and stored covered until use. Alternatively, pyrolysis of clean glassware at 500° for 4 hours may be used to eliminate contamination from hydrolysis tubes. Adequate disposable laboratory material can also be used.

Acid hydrolysis is the most common method for hydrolyzing a protein sample before amino acid analysis. The acid hydrolysis technique can contribute to the variation of the analysis due to complete or partial destruction of several amino acids. Tryptophan is destroyed; serine and threonine are partially destroyed; methionine might undergo oxidation; and cysteine is typically recovered as cystine (but cystine recovery is usually poor because of partial destruction or reduction to cysteine). Application of adequate vacuum (less than 200 μ m of mercury or 26.7 Pa) or introduction of an inert gas (argon) in the headspace of the reaction vessel can reduce the level of oxidative destruction. In peptide bonds involving isoleucine and valine, the amido bonds of Ile-Ile, Val-Val, Ile-Val, and Val-Ile are partially cleaved; and asparagine and glutamine are deamidated, resulting in aspartic acid and glutamic acid, respectively. The loss of tryptophan, asparagine, and glutamine during an acid hydrolysis limits quantitation to 17 amino acids. Some of the hydrolysis techniques described are used to address these concerns. Some of the hydrolysis techniques described (i.e., *Methods 4–11*) may cause modifications to other amino acids. Therefore, the benefits of using a given hydrolysis technique are weighed against the concerns with the technique and are tested adequately before employing a method other than acid hydrolysis.

A time-course study (i.e., amino acid analysis at acid hydrolysis times of 24, 48, and 72 hours) is often employed to analyze the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (i.e., serine and threonine) versus hydrolysis time, the line can be extrapolated to the origin to determine the starting concentration of these amino acids. Time-course hydrolysis studies are also used with amino acids that are slow to cleave (e.g., isoleucine and valine). During the hydrolysis time course, the analyst will observe a plateau in these residues. The level of this plateau is taken as the residue concentration. If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

An acceptable alternative to the time-course study is to subject an amino acid calibration standard to the same hydrolysis conditions as the test sample. The amino acid in free form may not completely represent the rate of destruction of labile amino acids within a peptide or protein during the hydrolysis. This is especially true for peptide bonds that are slow to cleave (e.g., Ile-Val bonds). However, this technique will allow the analyst to account for some residue destruction. Microwave acid hydrolysis has been used and is rapid but it requires special equipment as well as special precautions. The optimal conditions for microwave hydrolysis must be investigated for each individual protein/peptide sample. The microwave hydrolysis technique typically requires only a few minutes, but even a deviation of 1 minute may give inadequate results (e.g., incomplete hydrolysis or destruction of labile amino acids). Complete proteolysis, using a mixture of proteases, has been used but can be complicated, requires the proper controls, and is typically more applicable to peptides than proteins. [NOTE—During initial analyses of an unknown protein, experiments with various hydrolysis time and temperature conditions are conducted to determine the optimal conditions.]

Method 1

Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

Hydrolysis Solution: 6 N hydrochloric acid containing 0.1% to 1.0% of phenol.

Procedure—

Liquid Phase Hydrolysis—Place the protein or peptide sample in a hydrolysis tube, and dry. [NOTE—The sample is dried so that water in the sample will not dilute the acid used for the hydrolysis.] Add 200 μ L of *Hydrolysis Solution* per 500 μ g of lyophilized protein. Freeze the sample tube in a dry ice–acetone bath, and flame seal in vacuum. Samples are typically hydrolyzed at 110° for 24 hours in vacuum or inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 and 72 hours) are investigated if there is a concern that the protein is not completely hydrolyzed.

Vapor Phase Hydrolysis—This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimized by using vapor phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of *Hydrolysis Solution*. The *Hydrolysis Solution* does not come in contact with the test sample. Apply an inert atmosphere or vacuum (less than 200 μ m of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110° for a 24-hour hydrolysis time. Acid vapor hydrolyzes the dried sample. Any condensation of the acid in the sample vials is minimized. After hydrolysis, dry the test sample in vacuum to remove any residual acid.

Method 2

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid (MESA) as the reducing acid.

Hydrolysis Solution: 2.5 M MESA solution.

Vapor Phase Hydrolysis—About 1 to 100 μ g of the protein/peptide under test is dried in a hydrolysis tube. The hydrolysis tube is placed in a larger tube with about 200 μ L of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 μ m of mercury or 6.7 Pa) to vaporize the *Hydrolysis Solution*. The hydrolysis tube is heated to between 170° to 185° for about 12.5 minutes. After hydrolysis, the hydrolysis tube is dried in vacuum for 15 minutes to remove the residual acid.

Method 3

Tryptophan oxidation during hydrolysis is prevented by using thioglycolic acid (TGA) as the reducing acid.

Hydrolysis Solution: a solution containing 7 M hydrochloric acid, 10% of trifluoroacetic acid, 20% of thioglycolic acid, and 1% of phenol.

Vapor Phase Hydrolysis—About 10 to 50 μ g of the protein/peptide under test is dried in a sample tube. The sample tube is placed in a larger tube with about 200 μ L of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 μ m of mercury or 6.7 Pa) to vaporize the TGA. The sample tube is heated to 166° for about 15 to 30 minutes. After hydrolysis, the sample tube is dried in vacuum for 5 minutes to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

Method 4

Cysteine-cystine and methionine oxidation is performed with performic acid before the protein hydrolysis.

Oxidation Solution—The performic acid is prepared fresh by mixing formic acid and 30 percent hydrogen peroxide (9:1), and incubating at room temperature for 1 hour.

Procedure—The protein/peptide sample is dissolved in 20 μ L of formic acid, and heated at 50° for 5 minutes; then 100 μ L of the *Oxidation Solution* is added. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine sulfone. The oxidation is allowed to proceed for 10 to 30 minutes. The excess reagent is removed from the sample in a vacuum centrifuge. This technique may cause modifications to tyrosine residues in the presence of halides. The oxidized protein can then be acid hydrolyzed using *Method 1* or *Method 2*.

Method 5

Cysteine-cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

Hydrolysis Solution: 6 N hydrochloric acid containing 0.2% of phenol, to which sodium azide is added to obtain a final concentration of 0.2% (w/v). The added phenol prevents halogenation of tyrosine.

Liquid Phase Hydrolysis—The protein/peptide hydrolysis is conducted at about 110° for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the sodium azide present in the *Hydrolysis Solution*. This technique allows better tyrosine recovery than *Method 4*, but it is not quantitative for methionine. Methionine is converted to a mixture of the parent methionine and its two oxidative products, methionine sulfoxide and methionine sulfone.

Method 6

Cysteine-cystine oxidation is accomplished with dimethyl sulfoxide (DMSO).

Hydrolysis Solution: 6 N hydrochloric acid containing 0.1% to 1.0% of phenol, to which DMSO is added to obtain a final concentration of 2% (v/v).

Vapor Phase Hydrolysis—The protein/peptide hydrolysis is conducted at about 110° for 24 hours. During the hydrolysis, the cysteine-cystine present in the sample is converted to cysteic acid by the DMSO present in the *Hydrolysis Solution*. As an approach to limit variability and to compensate for partial destruction, it is recommended to evaluate the cysteic acid recovery from oxidative hydrolyses of standard proteins containing 1 to 8 mol of cysteine. The response factors from protein/peptide hydrolysates are typically about 30% lower than those for nonhydrolyzed cysteic acid standards. Because histidine, methionine, tyrosine, and tryptophan are also modified, a complete compositional analysis is not obtained with this technique.

Method 7

Cysteine-cystine reduction and alkylation is accomplished by a vapor phase pyridylethylation reaction.

Reducing Solution—Transfer 83.3 μ L of pyridine, 16.7 μ L of 4-vinylpyridine, 16.7 μ L of tributylphosphine, and 83.3 μ L of water to a suitable container, and mix.

Procedure—Add the protein/peptide (between 1 and 100 μ g) to a hydrolysis tube, and place in a larger tube. Transfer the *Reducing Solution* to the large tube, seal in vacuum (about 50 μ m of mercury or 6.7 Pa), and incubate at about 100° for 5 minutes. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 minutes to

remove residual reagents. The pyridylethylated protein/peptide can then be acid hydrolyzed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1 to 8 mol of cysteine to improve accuracy in the pyridylethyl-cysteine recovery. Longer incubation times for the pyridylethylation reaction can cause modifications to the α -amino terminal group and the ϵ -amino group of lysine in the protein.

Method 8

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

Stock Solutions—Prepare and filter three solutions: 1 M Tris hydrochloride (pH 8.5) containing 4 mM edetate disodium (*Stock Solution 1*), 8 M guanidine hydrochloride (*Stock Solution 2*), and 10% of 2-mercaptoethanol in water (*Stock Solution 3*).

Reducing Solution—Prepare a mixture of *Stock Solution 2* and *Stock Solution 1* (3:1) to obtain a buffered solution of 6 M guanidine hydrochloride in 0.25 M Tris hydrochloride.

Procedure—Dissolve about 10 μ g of the test sample in 50 μ L of the *Reducing Solution*, and add about 2.5 μ L of *Stock Solution 3*. Store under nitrogen or argon for 2 hours at room temperature in the dark. To achieve the pyridylethylation reaction, add about 2 μ L of 4-vinylpyridine to the protein solution, and incubate for an additional 2 hours at room temperature in the dark. The protein/peptide is desalted by collecting the protein/peptide fraction from a reverse-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis.

Method 9

Cysteine-cystine reduction and alkylation is accomplished by a liquid phase carboxymethylation reaction.

Stock Solutions—Prepare as directed for *Method 8*.

Carboxymethylation Solution—Prepare a solution containing 100 mg of iodoacetamide per mL of alcohol.

Buffer Solution—Use the *Reducing Solution*, prepared as directed for *Method 8*.

Procedure—Dissolve the test sample in 50 μ L of the *Buffer Solution*, and add about 2.5 μ L of *Stock Solution 3*. Store under nitrogen or argon for 2 hours at room temperature in the dark. Add the *Carboxymethylation Solution* in a 1.5 fold ratio per total theoretical content of thiols, and incubate for an additional 30 minutes at room temperature in the dark. [NOTE—If the thiol content of the protein is unknown, then add 5 μ L of 100 mM iodoacetamide for every 20 nmol of protein present.] The reaction is stopped by adding excess of 2-mercaptoethanol. The protein/peptide is desalted by collecting the protein/peptide fraction from a reverse-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis. The S-carboxyamidomethylcysteine formed will be converted to S-carboxymethyl-cysteine during acid hydrolysis.

Method 10

Cysteine-cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulfide. [NOTE—The choice of dithiodiglycolic acid or dithiodipropionic acid depends on the required resolution of the amino acid analysis method.]

Reducing Solution: a solution containing 10 mg of dithiodiglycolic acid (or dithiodipropionic acid) per mL of 0.2 M sodium hydroxide.

Procedure—Transfer about 20 μ g of the test sample to a hydrolysis tube, and add 5 μ L of the *Reducing Solution*. Add 10 μ L of isopropyl alcohol, and then remove all of the sam-

ple liquid by vacuum centrifugation. The sample is then hydrolyzed using *Method 1*. This method has the advantage that other amino acid residues are not derivatized by side reactions, and the sample does not need to be desalted prior to hydrolysis.

Method 11

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by Asx, while glutamine and glutamic acid residues are added and represented by Glx. Proteins/peptides can be reacted with bis(1,1-trifluoroacetoxy)iodobenzene (BTI) to convert the asparagine and glutamine residues to diaminopropionic acid and diaminobutyric acid residues, respectively, upon acid hydrolysis. These conversions allow the analyst to determine the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

Reducing Solutions—Prepare and filter three solutions: a solution of 10 mM trifluoroacetic acid (*Solution 1*), a solution of 5 M guanidine hydrochloride and 10 mM trifluoroacetic acid (*Solution 2*), and a freshly prepared solution of dimethylformamide containing 36 mg of BTI per mL (*Solution 3*).

Procedure—In a clean hydrolysis tube, transfer about 200 μ g of the test sample, and add 2 mL of *Solution 1* or *Solution 2* and 2 mL of *Solution 3*. Seal the hydrolysis tube in vacuum. Heat the sample at 60° for 4 hours in the dark. The sample is then dialyzed with water to remove the excess reagents. Extract the dialyzed sample three times with equal volumes of *n*-butyl acetate, and then lyophilize. The protein can then be acid hydrolyzed using previously described procedures. The α -, β -diaminopropionic and α -, γ -diaminobutyric acid residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid assayed contents with underivatized and BTI-derivatized acid hydrolysis. [NOTE—The threonine, methionine, cysteine, tyrosine, and histidine assayed content can be altered by BTI derivatization; a hydrolysis without BTI will have to be performed if the analyst is interested in the composition of these other amino acid residues.]

METHODOLOGIES OF AMINO ACID ANALYSIS GENERAL PRINCIPLES

Many amino acid analysis techniques exist, and the choice of any one technique often depends on the sensitivity required from the assay. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by postcolumn derivatization (e.g., with ninhydrin or o-phthalaldehyde). Postcolumn detection techniques can be used with samples that contain small amounts of buffer components, such as salts and urea, and generally require between 5 and 10 μ g of protein sample per analysis. The remaining amino acid techniques typically involve precolumn derivatization of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-*N*-hydroxysuccinimide carbamate or o-phthalaldehyde; (dimethylamino)azobenzenesulfonyl chloride; 9-fluorenyl-methylchloroformate; and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reverse-phase HPLC. Precolumn derivatization techniques are very sensitive and usually require between 0.5 and 1.0 μ g of protein sample per analysis but may be influenced by buffer salts in the samples. Precolumn derivatization techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpreta-

tion. Postcolumn derivatization techniques are generally influenced less by performance variation of the assay than precolumn derivatization techniques.

The following *Methods* may be used for quantitative amino acid analysis. Instruments and reagents for these procedures are available commercially. Furthermore, many modifications of these methodologies exist with different reagent preparations, reaction procedures, and chromatographic systems. Specific parameters may vary according to the exact equipment and procedure used. Many laboratories will utilize more than one amino acid analysis technique to exploit the advantages offered by each. In each of these *Methods*, the analog signal is visualized by means of a data acquisition system, and the peak areas are integrated for quantification purposes.

Method 1—Postcolumn Ninhydrin Detection General Principle

Ion-exchange chromatography with postcolumn ninhydrin detection is one of the most common methods employed for quantitative amino acid analysis. As a rule, a Li-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster Na-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

When the amino acid reacts with ninhydrin, the reactant has characteristic purple or yellow color. Amino acids, except imino acids, give a purple color, and show maximum absorption at 570 nm. The imino acids, such as proline, give a yellow color, and show maximum absorption at 440 nm. The postcolumn reaction between ninhydrin and amino acid eluted from the column is monitored at 440 nm and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

Detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for proline. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 1 µg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

Method 2—Postcolumn OPA Fluorometric Detection General Principle

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of thiol compound to form highly fluorescent isoindole products. This reaction is utilized for the postcolumn derivatization in analysis of amino acids by ion-exchange chromatography. The rule of the separation is the same as *Method 1*. Instruments and reagents for this form of amino acid analysis are available commercially. Many modifications of this method exist.

Although OPA does not react with secondary amines (imino acids, such as proline) to form fluorescent substances, the oxidation with sodium hypochlorite allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by postcolumn oxidation with sodium hypochlorite and postcolumn derivatization using OPA and thiol compound, such as *N*-acetyl-L-cysteine and 2-mercaptoethanol. The derivatization of primary amino acids are not noticeably affected by the continuous supply of sodium hypochlorite.

Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes of pH and cation strength. After postcolumn derivatization of

eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limit is considered to be a few tens of pmol level for most of the amino acid derivatives. Response linearity is obtained in the range of a few pmol level to a few tens of nmol level. To obtain good compositional data, a sample greater than 500 ng before hydrolysis is best suited for the amino acid analysis of protein/peptide.

Method 3—Precolumn PITC Derivatization General Principle

Phenylisothiocyanate (PITC) reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives, which can be detected with high sensitivity at 254 nm. Therefore, precolumn derivatization of amino acids with PITC followed by a reverse-phase HPLC separation with UV detection is used to analyze the amino acid composition.

After the reagent is removed under vacuum, the derivatized amino acids can be stored dry and frozen for several weeks with no significant degradation. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after three days.

Separation of the PTC-amino acids on a reverse-phase HPLC with ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from the column are monitored at 254 nm.

Detection limit is considered to be 1 pmol for most of the amino acid derivatives. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, a sample larger than 500 ng of protein/peptide before hydrolysis is best suited for this amino acid analysis of proteins/peptides.

Method 4—Precolumn AQC Derivatization General Principle

Precolumn derivatization of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by reverse-phase HPLC separation with fluorometric detection is used.

AQC reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are readily amenable to analysis by reverse-phase HPLC. Therefore, precolumn derivatization of amino acids with AQC followed by reverse-phase HPLC separation is used to analyze the amino acid composition.

Separation of the AQC-amino acids on an ODS column is accomplished through a combination of changes in the concentrations of acetonitrile and salt. Selective fluorescence detection of the derivatives with an excitation wavelength at 250 nm and an emission wavelength at 395 nm allows for the direct injection of the reaction mixture with no significant interference from the only major fluorescent reagent byproduct, 6-aminoquinoline. Excess reagent is rapidly hydrolyzed ($t_{1/2}$ < 15 seconds) to yield 6-aminoquinoline, *N*-hydroxysuccinimide and carbon dioxide, and after 1 minute no further derivatization can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature, and the derivatives have more than sufficient stability to allow for overnight automated chromatographic analysis.

The detection limit is considered to be ranging from about 40 fmol to 320 fmol for each amino acid, except for Cys. The detection limit for Cys is approximately 800 fmol. Response linearity is obtained in the range of 2.5 µM to 200 µM with correlation coefficients exceeding 0.999. Good compositional data can be obtained from the analysis of derivatized protein hydrolysates containing as little as 30 ng of protein/peptide.

Method 5—Precolumn OPA Derivatization General Principle

Precolumn derivatization of amino acids with *o*-phthalaldehyde (OPA) followed by reverse-phase HPLC separation with fluorometric detection is used. This technique does not detect amino acids that exist as secondary amines (e.g., proline).

OPA in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. 2-Mercaptoethanol and 3-mercaptopropionic acid can be used as thiol. OPA itself does not fluoresce and consequently produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with the rapid kinetics for the reactions, make it amenable to automated derivatization and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been a predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in *Method 7* or *Method 8*.

Precolumn derivatization of amino acids with OPA is followed by reverse-phase HPLC separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatized amino acids. Fluorescence intensity of the OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

The detection limits as low as 50 fmol via fluorescence have been reported, although the practical limit of analysis remains at 1 pmol.

Method 6—Postcolumn DABS-Cl Derivatization General Principle

Precolumn derivatization of amino acids with (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) followed by reverse-phase HPLC separation with visible light detection is used.

DABS-Cl is a chromophoric reagent employed for the labeling of amino acids. Amino acids labeled with DABS-Cl (DABS-amino acids) are highly stable and show the maximum absorption at 436 nm.

DABS-amino acids, all 19 naturally occurring amino acid derivatives, can be separated on an ODS column of a reverse-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

This method can analyze the imino acids, such as proline, together with the amino acids, at the same degree of sensitivity. DABS-Cl derivatization method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein/peptide with sulfonic acids, such as mercaptoethanesulfonic acid, *p*-toluenesulfonic acid, or methanesulfonic acid, described for *Method 2* in *Protein Hydrolysis*. The other acid-labile residues, asparagine and glutamine, can also be analyzed by previous conversion into diaminopropionic acid and diaminobutyric acid, respectively, by treatment of protein/peptide with BTI, described for *Method 11* in *Protein Hydrolysis*.

The nonproteinogenic amino acid, norleucine, cannot be used as an internal standard in this method as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard because it is eluted in a clean region.

The detection limit of DABS-amino acid is about 1 pmol. As little as 2 to 5 pmol of an individual DABS-amino acid

can be quantitatively analyzed with reliability, and only 10 ng to 30 ng of the dabsylated protein hydrolysate is required for each analysis.

Method 7—Precolumn FMOC-Cl Derivatization General Principle

Precolumn derivatization of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reverse-phase HPLC separation with fluorometric detection is used.

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction of FMOC-Cl with amino acid proceeds under mild conditions, in aqueous solution, and is completed in 30 seconds. The derivatives are stable, with only the histidine derivative showing any breakdown. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by reverse-phase HPLC using an ODS column. The separation is carried out by gradient elution varied linearly from a mixture of acetic acid buffer, methanol, and acetonitrile (50:40:10) to a mixture of acetonitrile and acetic acid buffer (50:50), and 20 amino acid derivatives that are separated in 20 minutes. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

The detection limit is in the low fmol range. A linearity range of 0.1 μ M to 50 μ M is obtained for most amino acids.

Method 8—Precolumn NBD-F Derivatization General Principle

Precolumn derivatization of amino acids with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) followed by reverse-phase HPLC separation with fluorometric detection is used.

7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatized with NBD-F by heating to 60° for 5 minutes.

NBD-amino acid derivatives are separated on an ODS column of reverse-phase HPLC by employing a gradient elution system consisting of acetonitrile and aqueous buffer mixture, and 17 amino acid derivatives that are separated in 35 minutes. *L*-aminocaproic acid can be used as an internal standard because it is eluted in a clean chromatographic region. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as that for the precolumn OPA derivatization method (*Method 5*), excluding proline to which OPA is not reactive and might be advantageous for NBD-F against OPA.

The detection limit for each amino acid is about 10 fmol. Profile analysis was achieved for about 1.5 mg of protein hydrolysates in the final precolumn labeling reaction mixture for HPLC.

DATA CALCULATION AND ANALYSIS

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine. Serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants. Application of adequate vacuum (less than 200 μ m of mercury or 26.7 Pa) or introduction of inert gas (argon) in the headspace of the reaction vessel during vapor phase hydrolysis can reduce the level of oxidative de-

struction. Therefore, the quantitative results obtained for cysteine, tryptophan, threonine, isoleucine, valine, methionine, glycine, and serine from a protein/peptide hydrolysate may be variable and may warrant further investigation and consideration.

Calculations

Amino Acid Mole Percent—This is the number of specific amino acid residues per 100 residues in a protein. This result may be useful for evaluating amino acid analysis data when the molecular weight of the protein/peptide under investigation is unknown. This information can be used to corroborate the identity of a protein and has other applications. Carefully identify and integrate the peaks obtained as directed for each *Procedure*. Calculate the mole percent for each amino acid present in the test sample by the formula:

$$100r_u/r$$

in which r_u is the peak response, in nmol, of the amino acid under test; and r is the sum of peak responses, in nmol, for all amino acids present in the test sample. Comparison of the mole percent of the amino acids under test to data from known proteins can help establish or corroborate the identity of the sample protein.

Unknown Protein Samples—This data analysis technique can be used to estimate the protein concentration of an unknown protein sample using the amino acid analysis data. Calculate the mass, in μg , of each recovered amino acid by the formula:

$$mM_w/1000$$

in which m is the recovered quantity, in nmol, of the amino acid under test; and M_w is the molecular weight, for that amino acid, corrected for the weight of the water molecule that was eliminated during peptide bond formation. The sum of the masses of the recovered amino acids will give an estimate of the total mass of the protein analyzed after appropriate correction for partially and completely destroyed amino acids. If the molecular weight of the unknown protein is available (i.e., by SDS-PAGE analysis or mass spectroscopy), the amino acid composition of the unknown protein can be predicted. Calculate the number of residues of each amino acid by the formula:

$$m/(1000M/M_w)$$

in which m is the recovered quantity, in nmol, of the amino acid under test; M is the total mass, in μg , of the protein; and M_w is the molecular weight of the unknown protein.

Known Protein Samples—This data analysis technique can be used to investigate the amino acid composition and protein concentration of a protein sample of known molecular weight and amino acid composition using the amino acid analysis data. When the composition of the protein being analyzed is known, one can exploit the fact that some amino acids are recovered well, while other amino acid recoveries may be compromised because of complete or partial destruction (e.g., tryptophan, cysteine, threonine, serine, methionine), incomplete bond cleavage (i.e., for isoleucine and valine), and free amino acid contamination (i.e., by glycine and serine).

Because those amino acids that are recovered best represent the protein, these amino acids are chosen to quantify the amount of protein. Well-recovered amino acids are, typically, aspartate-asparagine, glutamate-glutamine, alanine, leucine, phenylalanine, lysine, and arginine. This list can be modified based on experience with one's own analysis system. Divide the quantity, in nmol, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein con-

tent results calculated. The protein content determined for each of the well-recovered amino acids should be evenly distributed about the mean. Discard protein content values for those amino acids that have an unacceptable deviation from the mean. Typically, a greater than 5% variation from the mean is considered unacceptable. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample. Divide the content of each amino acid by the calculated mean protein content to determine the amino acid composition of the sample by analysis.

Calculate the relative compositional error, in percentage, by the formula:

$$100m/m_s$$

in which m is the experimentally determined quantity, in nmol per amino acid residue, of the amino acid under test; and m_s is the known residue value for that amino acid. The average relative compositional error is the average of the absolute values of the relative compositional errors of the individual amino acids, typically excluding tryptophan and cysteine from this calculation. The average relative compositional error can provide important information on the stability of analysis run over time. The agreement in the amino acid composition between the protein sample and the known composition can be used to corroborate the identity and purity of the protein in the sample.

♦APPENDIX

AMINO ACID ANALYSIS PROCEDURES

The examples of the specific procedures for each *Method* described in *Methodologies of Amino Acid Analysis* are shown.

Method 1—Postcolumn Ninhydrin Detection

One method for postcolumn ninhydrin detection is shown below. Many other methods are also available, with instruments and reagents available commercially.

Mobile Phase Preparation—

Solution A—Transfer about 1.7 g of anhydrous sodium citrate and 1.5 mL of hydrochloric acid to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Adjust, if necessary, with hydrochloric acid to a pH of 3.0.

Solution B—Transfer about 1.7 g of anhydrous sodium citrate and 0.7 mL of hydrochloric acid to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Adjust, if necessary, with hydrochloric acid to a pH of 4.3.

Solution C—Prepare a solution containing 5% of sodium chloride, 1.9% of anhydrous sodium citrate, and 0.1% of phenol in water, and adjust to a pH of 6.

Column Regeneration Solution—Prepare a solution containing 0.8% of sodium hydroxide in water, and adjust to a pH of 13.

Mobile Phase—Use variable mixtures of *Solution A*, *Solution B*, and *Solution C* as directed for *Chromatographic System*.

Postcolumn Reagent—Transfer about 18 g of ninhydrin and 0.7 g of hydrindantin to 900 mL of a solution containing 76.7% of dimethyl sulfoxide, 0.7% of dihydrate lithium acetate, and 0.1% of acetic acid, and mix for at least 3 hours under inert gas, such as nitrogen. [NOTE—This reagent is stable for 30 days if kept between 2° and 8° under inert gas.]

Buffer Solution—Prepare a solution containing 2% of anhydrous sodium citrate, 1% of hydrochloric acid, 0.5% of

thiodiglycol, and 0.1% of benzoic acid in water, and adjust to a pH of 2.

Chromatographic System—The liquid chromatograph is equipped with a detector with appropriate interference filters at 440, 570, or 690 nm and a 4.0-mm \times 120-mm column that contains 7.5- μ m sulfonated styrene-divinylbenzene copolymer packing. The flow rate is about 14 mL per hour. The system is programmed as follows. Initially equilibrate the column with *Solution A*; at 25 minutes, the composition of the *Mobile Phase* is changed to 100% *Solution B*; and at 37 minutes, the composition is changed to 100% *Solution C*. At 75 minutes into the run, the last amino acid has been eluted from the column, and the column is regenerated with *Column Regeneration Solution* for 1 minute. The column is then equilibrated with *Solution A* for 11 minutes before the next injection. The column temperature is programmed as follows. The initial temperature is 48°; after 11.5 minutes, the temperature is increased to 65° at a rate of 3° per minute; at about 35 minutes, the temperature is increased to 77° at a rate of 3° per minute; and finally at about 52 minutes, the temperature is decreased to 48° at a rate of 3° per minute.

Procedure and Postcolumn Reaction—Reconstitute the lyophilized protein/peptide hydrolysate in the *Buffer Solution*, inject an appropriate amount into the chromatograph, and proceed as directed for *Chromatographic System*. As the amino acids are eluted from the column, they are mixed with the *Postcolumn Reagent*, which is delivered at a flow rate of 7 mL per hour, through a tee. After mixing, the column effluent and the *Postcolumn Reagent* pass through a tubular reactor at a temperature of 135°, where a characteristic purple or yellow color is developed. From the reactor, the liquid passes through a colorimeter with a 12-mm flow-through cuvette. The light emerging from the cuvette is split into three beams for analysis by the detector with interference filters at 440, 570, or 690 nm. The 690-nm signal may be electronically subtracted from the other signals for improved signal-to-noise ratios. The 440-nm (imino acids) and the 570-nm (amino acids) signals may be added in order to simplify data handling.

Method 2—Postcolumn OPA Fluorometric Detection

One method of postcolumn OPA fluorometric detection is shown below.

Mobile Phase Preparation—

Solution A—Prepare a solution of sodium hydroxide, citric acid, and alcohol in HPLC-grade water having a 0.2 N sodium concentration and containing 7% of alcohol (w/v), adjusted to a pH of 3.2.

Solution B—Prepare a solution of sodium hydroxide and citric acid in HPLC-grade water having a 0.6 N sodium concentration, adjusted to a pH of 10.0.

Solution C: 0.2 N sodium hydroxide.

Mobile Phase—Use variable mixtures of *Solution A*, *Solution B*, and *Solution C* as directed for *Chromatographic System*.

Postcolumn Reagent Preparation—

Alkaline Buffer—Prepare a solution containing 384 mM sodium carbonate, 216 mM boric acid, and 108 mM potassium sulfate, and adjust to a pH of 10.0.

Hypochlorite Reagent—To 1 L of *Alkaline Buffer*, add 0.4 mL of sodium hypochlorite solution (10% chlorine concentration). [NOTE—The hypochlorite solution is stable for 2 weeks.]

OPA Reagent—Transfer 2 g of *N*-acetyl-L-cysteine and 1.6 g of OPA to a 15-mL volumetric flask, dissolve in and dilute with alcohol to volume, and mix. Transfer this solution and 4 mL of 10% aqueous polyethylene (23) lauryl

ether to a 1-L volumetric flask, dilute with 980 mL of *Alkaline Buffer*, and mix.

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set to an excitation wavelength of 348 nm and an emission wavelength of 450 nm and a 4.0-mm \times 150-mm column that contains 7.5- μ m packing L17. The flow rate is about 0.3 mL per minute, and the column temperature is set at 50°. The system is programmed as follows. The column is equilibrated with *Solution A*; over the next 20 minutes, the composition of the *Mobile Phase* is changed linearly to 85% *Solution A* and 15% *Solution B*; then there is a step change to 40% *Solution A* and 60% *Solution B*; over the next 18 minutes, the composition is changed linearly to 100% *Solution B* and held for 7 minutes; then there is a step change to 100% *Solution C*, and this is held for 6 minutes; then there is a step change to *Solution A*, and this composition is maintained for the next 8 minutes.

Procedure and Postcolumn Reaction—Inject about 1.0 nmol of each amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*. As the effluent leaves the column, it is mixed with the *Hypochlorite Reagent*. The mixture passes through the first postcolumn reactor which consists of stainless steel 0.5-mm \times 2-m tubing. A second postcolumn reactor of similar design is placed immediately downstream from the first postcolumn reactor and is used for the OPA postcolumn reaction. The flow rates for both the *Hypochlorite Reagent* and the *OPA Reagent* are 0.2 mL per minute, resulting in a total flow rate (i.e., *Hypochlorite Reagent*, *OPA Reagent*, and column effluent) of 0.7 mL per minute exiting from the postcolumn reactors. Postcolumn reactions are conducted at 55°. This results in a residence time of about 33 seconds in the OPA postcolumn reactor. After postcolumn derivatization, the column effluent passes through the fluorometric detector.

Method 3—Precolumn PITC Derivatization

One method of precolumn PITC derivatization is described below.

Mobile Phase Preparation—

Solution A: 0.05 M ammonium acetate, adjusted with phosphoric acid to a pH of 6.8.

Solution B—Prepare 0.1 M ammonium acetate, adjust with phosphoric acid to a pH of 6.8, and then prepare a mixture of this solution and acetonitrile (1:1).

Solution C: a mixture of acetonitrile and water (70:30).

Mobile Phase—Use variable mixtures of *Solution A*, *Solution B*, and *Solution C* as directed for *Chromatographic System*.

Derivatization Reagent Preparation—

Coupling Buffer: a mixture of acetonitrile, pyridine, triethylamine, and water (10:5:2:3).

Sample Solvent: a mixture of water and acetonitrile (7:2).

Sample Derivatization Procedure—Dissolve the lyophilized test sample in 100 μ L of the *Coupling Buffer*, and then dry in a vacuum centrifuge to remove any hydrochloride if a protein hydrolysis step was used. Dissolve the test sample in 100 μ L of *Coupling Buffer*, add 5 μ L of PITC, and incubate at room temperature for 5 minutes. The test sample is again dried in a vacuum centrifuge, and is dissolved in 250 μ L of *Sample Solvent*.

Chromatographic System—The liquid chromatograph is equipped with a 254-nm detector and a 4.6-mm \times 250-mm column that contains 5- μ m packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 52°. The system is programmed as follows. The column is equilibrated with *Solution A*; over the next 15 minutes, the composition of the *Mobile Phase* is changed linearly to 85% *Solution A* and 15% *Solution B*; over the next

15 minutes, the composition is changed linearly to 50% *Solution A* and 50% *Solution B*; then there is a step change to 100% *Solution C*, and this is held for 10 minutes; then there is a step change to 100% *Solution A*, and the column is allowed to equilibrate before the next injection.

Procedure—Inject about 1.0 nmol of each PTC-amino acid under test (10- μ L sample in *Sample Solvent*) into the chromatograph, and proceed as directed for *Chromatographic System*.

Method 4—Precolumn AQC Derivatization

One method of precolumn AQC derivatization is shown below.

Mobile Phase Preparation—

Solution A—Prepare a solution having a composition of 140 mM sodium acetate and 17 mM triethylamine, and adjust with phosphoric acid to a pH of 5.02.

Solution B: a mixture of acetonitrile and water (60:40).

Mobile Phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

Sample Derivatization Procedure—Dissolve about 2 μ g of the test sample in 20 μ L of 15 mM hydrochloric acid, and dilute with 0.2 M borate buffer (pH 8.8) to 80 μ L. The derivatization is initiated by the addition of 20 μ L of 10 mM AQC in acetonitrile, and allowed to proceed for 10 minutes at room temperature.

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 250 nm and an emission wavelength of 395 nm and a 3.9-mm \times 150-mm column that contains 4- μ m packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 37°. The system is programmed as follows. The column is equilibrated with *Solution A*; over the next 0.5 minute, the composition of the *Mobile Phase* is changed linearly to 98% *Solution A* and 2% *Solution B*; then over the next 14.5 minutes to 93% *Solution A* and 7% *Solution B*; then over the next 4 minutes to 87% *Solution A* and 13% *Solution B*; over the next 14 minutes to 68% *Solution A* and 32% *Solution B*; then there is a step change to 100% *Solution B* for a 5-minute wash; over the next 10 minutes, there is a step change to 100% *Solution A*; and the column is allowed to equilibrate before the next injection.

Procedure—Inject about 0.05 nmol of each AQC-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*.

Method 5—Precolumn OPA Derivatization

One method of precolumn OPA derivatization is shown below.

Mobile Phase Preparation—

Solution A: a mixture of 100 mM sodium acetate (pH 7.2), methanol, and tetrahydrofuran (900:95:5).

Solution B: methanol.

Mobile Phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

Derivatization Reagent—Dissolve 50 mg of OPA in 1.25 mL of methanol (protein sequencing grade). Add 50 μ L of 2-mercaptoethanol and 11.2 mL of 0.4 M sodium borate (pH 9.5), and mix. [NOTE—This reagent is stable for 1 week.]

Sample Derivatization Procedure—Transfer about 5 μ L of the test sample to an appropriate container, add 5 μ L of the *Derivatization Reagent*, and mix. After 1 minute, add not less than 20 μ L of 0.1 M sodium acetate (pH 7.0). Use 20 μ L of this solution for analysis. [NOTE—Use of an internal standard (e.g., norleucine) is recommended for quantitative

analysis because of potential reagent volume variations in the sample derivatization. The sample derivatization is performed in an automated on-line fashion. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatization.]

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 348 nm and an emission wavelength of 450 nm and a 4.6-mm \times 75-mm column that contains 3- μ m packing L3. The flow rate is about 1.7 mL per minute, and the column temperature is maintained at 37°. The system is programmed as follows. The column is equilibrated with 92% *Solution A* and 8% *Solution B*; over the next 2 minutes, the composition of the *Mobile Phase* is changed to 83% *Solution A* and 17% *Solution B*, and held for an additional 3 minutes; then changed to 54% *Solution A* and 46% *Solution B* over the next 5 minutes, and held for an additional 2 minutes; then changed to 34% *Solution A* and 66% *Solution B* over the next 2 minutes, and held for 1 minute; then over the next 0.3 minute changed to 20% *Solution A* and 80% *Solution B*, and held for an additional 2.6 minutes; and then finally over 0.6 minute changed to 92% *Solution A* and 8% *Solution B*, and held for an additional 0.6 minute.

Procedure—Inject about 0.02 nmol of each OPA-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*.

Method 6—Postcolumn DABS-Cl Derivatization

One method for precolumn DABS-Cl derivatization is shown below.

Mobile Phase Preparation—

Solution A: 25 mM sodium acetate (pH 6.5) containing 4% of dimethylformamide.

Solution B: acetonitrile.

Mobile Phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

Derivatization Reagent Preparation—

Sample Buffer: 50 mM sodium bicarbonate, adjusted to a pH of 8.1.

Derivatization Reagent—Dissolve 1.3 mg of DABS-Cl in 1 mL of acetonitrile. [NOTE—This reagent is prepared fresh shortly before the derivatization step.]

Sample Dilution Buffer—Prepare a mixture of 50 mM sodium phosphate (pH 7.0) and alcohol (1:1).

Sample Derivatization Procedure—Dissolve the test sample in 20 μ L of *Sample Buffer*, add 40 μ L of *Derivatization Reagent*, and mix. The sample container is sealed with a silicon-rubber stopper, and heated to 70° for 10 minutes. During the sample heating, the mixture will become completely soluble. After the derivatization, dilute the test sample with an appropriate quantity of the *Sample Dilution Buffer*.

Chromatographic System—The liquid chromatograph is equipped with a 436-nm detector and a 4.6-mm \times 250-mm column that contains packing L1. The flow rate is about 1 mL per minute, and the column temperature is maintained at 40°. The system is programmed as follows. The column is equilibrated with 85% *Solution A* and 15% *Solution B*; over the next 20 minutes, the composition of the *Mobile Phase* is changed to 60% *Solution A* and 40% *Solution B*; over the next 12 minutes, the composition is changed to 30% *Solution A* and 70% *Solution B*, and held for an additional 2 minutes.

Procedure—Inject about 0.05 nmol of the DABS-amino acids into the chromatograph, and proceed as directed for *Chromatographic System*.

Method 7—Precolumn FMOC-Cl Derivatization

One method for precolumn FMOC-Cl derivatization is shown below.

Mobile Phase Preparation—

Acetic Acid Buffer—Transfer 3 mL of glacial acetic acid and 1 mL of triethylamine to a 1-L volumetric flask, and dilute with HPLC-grade water to volume. Adjust with sodium hydroxide to a pH of 4.20.

Solution A: a mixture of *Acetic Acid Buffer*, methanol, and acetonitrile (50:40:10).

Solution B: a mixture of acetonitrile and *Acetic Acid Buffer* (50:50).

Mobile Phase—Use variable mixtures of *Solution A* and *Solution B* as directed for *Chromatographic System*.

Derivatization Reagent Preparation—

Borate Buffer—Prepare a 1 M boric acid solution, and adjust with sodium hydroxide to a pH of 6.2.

FMOC-Cl Reagent—Dissolve 155 mg of 9-fluorenylmethyl chloroformate in 40 mL of acetone, and mix.

Sample Derivatization Procedure—To 0.4 mL of the test sample add 0.1 mL of *Borate Buffer* and 0.5 mL of *FMOC-Cl Reagent*. After about 40 seconds, extract the mixture with 2 mL of pentane, and then extract again with fresh pentane. The aqueous solution with amino acid derivatives is then ready for injection.

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm and a 4.6-mm × 125-mm column that contains 3-μm packing L1. The flow rate is about 1.3 mL per minute. The system is programmed as follows. The column is equilibrated with *Solution A*, and this composition is maintained for 3 minutes; over the next 9 minutes, it is changed to 100% *Solution B*; then over the next 0.5 minute, the flow rate is increased to 2 mL per minute, and held until the final FMOC-amino acid is eluted from the column. The total run time is about 20 minutes.

Procedure—Inject not less than 0.01 nmol of each FMOC-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*. The FMOC-histidine derivative will generally give a lower response than the other derivatives.

Method 8—Precolumn NBD-F Derivatization

One method for precolumn NBD-F derivatization is shown below.

Mobile Phase Preparation—

Solution A: a solution of 10 mM sodium citrate containing 75 mM sodium perchlorate, adjusted with hydrochloric acid to a pH of 6.2.

Solution B: a mixture of acetonitrile and water (50:50).

Derivatization Reagent Preparation—

Sample Buffer: a 0.1 M boric acid solution, adjusted with sodium hydroxide to a pH of 9.2.

Derivatization Reagent—Dissolve 5 mg of NBD-F in 1.0 mL of alcohol, and mix.

Sample Derivatization Procedure—Dissolve the test sample in 20 μL of *Sample buffer*, add 10 μL of *Derivatization Reagent*, and mix. The sample container is heated at 60° for 5 minutes. After the derivatization, dilute the test sample with 300 μL of *Solution A*.

Chromatographic System—The liquid chromatograph is equipped with a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm and a 4.6-mm × 150-mm column that contains 5-μm particle size ODS silica packing. The flow rate is about 1.0 mL per minute, and the column temperature is main-

tained at 40°. The system is programmed as follows. The column is equilibrated with 94% *Solution A* and 6% *Solution B*; over the next 16 minutes, the composition is changed linearly to 63% *Solution A* and 37% *Solution B*; over the next 5 minutes, the composition is changed linearly to 62% *Solution A* and 38% *Solution B*; over the next 9 minutes, the composition is changed linearly to 100% *Solution B*, and held for an additional 5 minutes; then finally over 2 minutes, the composition is changed linearly to 94% *Solution A* and 6% *Solution B*; and then the column is allowed to equilibrate before the next injection.

Procedure—Inject about 15 pmol of each NBD-amino acid under test into the chromatograph, and proceed as directed for *Chromatographic System*.♦

(1053) CAPILLARY ELECTROPHORESIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by capillary electrophoresis. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Other characterization tests, also harmonized, are shown in *Biotechnology-Derived Articles—Amino Acid Analysis* (1052), *Biotechnology-Derived Articles—Isoelectric Focusing* (1054), *Biotechnology-Derived Articles—Peptide Mapping* (1055), *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* (1056), and *Biotechnology-Derived Articles—Total Protein Assay* (1057).

INTRODUCTION

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution under the influence of a direct-current electric field. In this section we are describing four capillary electrophoresis methods: *Capillary Zone Electrophoresis*, *Capillary Gel Electrophoresis*, *Capillary Isoelectric Focusing*, and *Micellar Electrokinetic Chromatography*.

GENERAL PRINCIPLES

The migration velocity of the analyte under an electric field of intensity E is determined by the electrophoretic mobility of the analyte and the electroosmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute (μ_{ep}) depends on the characteristics of the solute (electrical charge, molecular size, and shape) and the characteristics of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity, and additives). The electrophoretic velocity (v_{ep}) of a solute, assuming a spherical shape, is as follows:

$$v_{ep} = \mu_{ep} E = \left(\frac{q}{6\pi\eta r} \right) \left(\frac{V}{L} \right)$$

in which q is the effective charge of the solute; η is the viscosity of the electrolyte solution; r is the Stoke's radius of the solute; V is the applied voltage; and L is the total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent, called electroosmotic flow, is generated inside the capillary. Its velocity depends on the electroosmotic mobility (μ_{eo}), which in turn depends

on the charge density on the capillary internal wall and the buffer characteristics. The electroosmotic velocity (v_{eo}) is given by the equation:

$$v_{eo} = \mu_{eo} E = \left(\frac{\epsilon \zeta}{\eta} \right) \left(\frac{V}{L} \right)$$

in which ϵ is the dielectric constant of the buffer; ζ is the zeta potential of the capillary surface; and the other terms are as defined above.

The velocity of the solute (v) is given by the equation:

$$v = v_{ep} + v_{eo}$$

The electrophoretic mobility of the analyte and the electroosmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the electroosmotic flow and their velocities will be smaller than the electroosmotic velocity. Cations will migrate in the same direction as the electroosmotic flow and their velocities will be greater than the electroosmotic velocity. Under conditions in which there is a fast electroosmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run. The time (t) taken by the solute to migrate the distance (l) from the injection end of the capillary to the detection point (capillary effective length) is as follows:

$$t = \frac{l}{v_{ep} + v_{eo}} = \frac{l(L)}{V(\mu_{ep} + \mu_{eo})}$$

in which the other terms are as defined above.

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionized silanol groups in the inner wall. Consequently, the electroosmotic flow is from anode to cathode. The electroosmotic flow must remain constant from run to run to obtain good reproducibility in the migration velocity of the solutes. For some applications, it might be necessary to reduce or suppress the electroosmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition, and/or the pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions, the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this ideal case, the efficiency of the zone, expressed as the number of theoretical plates (N), is given by:

$$N = \frac{(\mu_{ep} + \mu_{eo})(Vl)}{2DL}$$

in which D is the molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena, such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size, and unlevelled buffer reservoirs, can also significantly contribute to band dispersion. Separation between two bands (expressed by the resolution, R_s) can be obtained by modification of the electrophoretic mobility of the analytes, by the electroosmotic mobility induced in the

capillary, and by increasing the efficiency for the band of each analyte as follows:

$$R_s = \frac{\sqrt{N}(\mu_{epb} - \mu_{epa})}{4(\bar{\mu}_{ep} + \mu_{eo})}$$

in which μ_{epa} and μ_{epb} are the electrophoretic mobilities of the two analytes to be separated; $\bar{\mu}_{ep}$ is the average electrophoretic mobility of the two analytes calculated as:

$$\bar{\mu}_{ep} = \frac{1}{2}(\mu_{epb} + \mu_{epa})$$

APPARATUS

An apparatus for capillary electrophoresis is composed of a high voltage controllable direct current power supply; two buffer reservoirs held at the same level and containing specified anodic and cathodic solutions; two electrode assemblies (cathode and anode) immersed in the buffer reservoirs and connected to the power supply; a separation capillary usually made of fused-silica, sometimes with an optical viewing window aligned with the detector, depending on the detector type, with the ends of the capillary placed in the buffer reservoirs and the capillary being filled with a solution specified in a given monograph; a suitable injection system; a detector capable of monitoring the amount of substance of interest passing through a segment of the separation capillary at a given time, generally based on absorption spectrophotometry (UV and visible), fluorimetry, conductimetric, amperometric, or mass spectrometric detection, depending on the specific applications, or even indirect detection to detect non-UV-absorbing and nonfluorescent compounds; a thermostatic system capable of maintaining a constant temperature inside the capillary, recommended to obtain good separation reproducibility; a recorder; and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum, or electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

It is expected that the capillary, the buffer solutions, the preconditioning method, the sample solution, and the migration conditions will be specified in the individual monograph. The electrolytic solution employed is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. To achieve reproducible migration time of the solutes, it would be necessary to develop, for each analytical method, a rigorous rinsing routine.

CAPILLARY ZONE ELECTROPHORESIS

Principle

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. In this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electroosmotic flow on the capillary (see *General Principles*). Coated capillaries can be used to increase the

separation capacity of those substances adsorbing on fused-silica surfaces.

This mode of capillary electrophoresis is appropriate for the analysis of small ($MW < 2000$) and large ($2000 < MW < 100,000$) molecules. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

Optimization

Optimization of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of the separations are instrumental and electrolytic solution parameters.

Instrumental Parameters

Voltage—A Joule heating plot is useful in optimizing the applied voltage and column temperature. The separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result, viscosity gradients in the buffer inside the capillary, which causes band broadening and decreases resolution.

Polarity—Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electroosmotic flow will move toward the cathode. If the electrode polarity is reversed, the electroosmotic flow is away from the outlet and only charged analytes with electroosmotic mobilities greater than the electroosmotic flow will pass to the outlet.

Temperature—The main effect of temperature is observed on buffer viscosity and electrical conductivity, thus affecting migration velocity. In some cases, an increase in capillary temperature can cause a conformational change of some proteins, modifying their migration time and the efficiency of the separation.

Capillary—The length and internal diameter of the capillary affects the analysis time, the efficiency of separations, and the load capacity. Increasing both effective length and total length can decrease the electric fields, at a constant voltage, which increases migration time. For a given buffer and electric field, heat dissipation (thus, sample band broadening) depends on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected into the capillary and the detection system used.

The adsorption of sample components on the capillary wall limits efficiency; therefore, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. Other strategies include the coating of the internal wall of the capillary with a polymer covalently bonded to the silica that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic, and anionic polymers are commercially available.

Electrolytic Solution Parameters

Buffer Type and Concentrations—Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimize current generation.

To minimize band distortion, it is important to match buffer-ion mobility to solute mobility whenever possible. The type of sample solvent used is important to achieve on-column sample focusing, which increases separation efficiency and improves detection. Also, an increase in buffer concentration at a given pH decreases electroosmotic flow and solute velocity.

Buffer pH—The pH of the buffer can affect separation by modifying the charge of the analyte or additives and by changing the electroosmotic flow. For protein and peptide separation, a change in the pH of the buffer from above the isoelectric point to below the isoelectric point changes the net charge of the solute from negative to positive. An increase in the buffer pH generally increases the electroosmotic flow.

Organic Solvents—Organic modifiers, such as methanol, acetonitrile, and others, may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the ionization degree of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electroosmotic flow.

Additives for Chiral Separations—To separate optical isomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, although in some cases crown ethers, certain polysaccharides, or even proteins can be used. Because chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. While developing a given separation it may be useful to test cyclodextrins having a different cavity size (α -, β -, or γ -cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionizable (aminomethyl, carboxymethyl, sulfobutylether, etc.) moieties. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account because it will influence the selectivity. The resolution of chiral separations is also controlled by the concentration of the chiral selector, the composition and pH of the buffer, and the separation temperature. Organic additives, such as methanol or urea, can also affect the resolution of separation.

CAPILLARY GEL ELECTROPHORESIS

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size because smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

Characteristics of Gels

Two types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels. Permanently coated gels are prepared inside the capillary by polymerization of monomers. One example of such a gel is a cross-linked polyacrylamide. This type of gel is usually bonded to the fused-silica wall and cannot be removed without destroying the capillary. For protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulfate, and the sample is denatured by heating in a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When nonreducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Optimization of separation in a cross-linked

gel is obtained by modifying the separation buffer (see *Capillary Zone Electrophoresis*) and by controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the ratio of the cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of this type of gel, only electrokinetic injection can be used.

Dynamically coated gels are hydrophilic polymers (i.e., linear polyacrylamide, cellulose derivatives, dextran, etc.) which can be dissolved in aqueous separation buffers, giving rise to a separation medium that also acts as a molecular sieve. These polymeric separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary with no electroosmotic flow. Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the dynamically coated gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A decrease in gel porosity leads to a decrease in the mobility of the solute for the same buffer. Both hydrodynamic and electrokinetic injection techniques can be used because the dissolution of these polymers in the buffer gives low viscosity solutions.

CAPILLARY ISOELECTRIC FOCUSING

Principle

In isoelectric focusing the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (polyaminocarboxylic acids), dissolved in the separation buffer.

The three basic steps in capillary isoelectric focusing are loading, focusing, and mobilization.

LOADING

Two methods may be employed.

Loading in One Step—The sample is mixed with ampholytes and introduced into the capillary by pressure or vacuum.

Sequential Loading—A leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone, and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough so as not to modify the pH gradient.

FOCUSING

When the voltage is applied, ampholytes migrate toward the cathode or the anode according to their net charge, creating the pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point, and the current drops to very low values.

MOBILIZATION

If mobilization is required for detection, use one of the following methods. Three methods are available.

Method 1—Mobilization is accomplished during *Focusing*, under the influence of the electroosmotic flow when this flow is small enough to allow the focusing of the components.

Method 2—Mobilization is accomplished by application of positive pressure after *Focusing*.

Method 3—Mobilization is achieved after *Focusing*, by adding salts to the cathode reservoir or the anode reservoir, depending on the direction chosen for mobilization, in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilized in the direction of the reservoir, which contains added salts, and pass the detector.

The separation achieved is expressed as ΔpI and depends on the pH gradient (dpH/dx), the number of ampholytes having different pI values, the molecular diffusion coefficient (D), the intensity of the electric field (E), and the variation of the electrophoretic mobility of the analyte with the pH ($-d\mu/dpH$):

$$\Delta pI = 3 \sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

Optimization

The major parameters that need to be considered in the development of separations are the following:

Voltage—The use of high fields from 300 V/cm to 1,000 V/cm during *Focusing*.

Capillary—Depending on the *Mobilization* strategy selected (see above), the electroosmotic flow must be reduced or suppressed. Coated capillaries tend to reduce the electroosmotic flow.

Solutions—The anode buffer reservoir is filled with a solution of a lower pH than the pI of the most acidic ampholyte, and the cathode reservoir is filled with a solution with a higher pH than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, like methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electroosmotic flow by increasing the viscosity. Commercial ampholytes covering many pH ranges are available and may also be mixed to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point (pI), whereas narrower ranges are employed to improve accuracy. Calibration can be made by correlating migration time with the isoelectric point of a series of standard protein markers. During *Focusing*, precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea, or zwitterionic buffers. However, depending on the concentration, urea can denature proteins.

MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

Principle

Separation takes place in an electrolytic solution that contains a surfactant at a concentration above the critical micellar concentration (cmc). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed by the micelles according to the solute's partition coefficient. The technique can be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes maintaining the efficiency, speed, and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants in MEKC is the anionic surfactant, sodium dodecyl sulfate, although other

surfactants, such as cationic surfactant cetyl trimethyl ammonium salts, have also been used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong electroosmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is used as surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, because the analyte can partition between the micelle and the aqueous buffer and has no electrophoretic mobility, the analyte migration velocity will depend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the electroosmotic flow marker and that of the micelle; and the time elapsed between these two peaks is called the separation window. For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer and on the electrophoretic mobility of the solute in the absence of micelles.

Because the mechanism in MEKC of neutral and weakly ionized solutes is essentially chromatographic, migration of the solute and resolution can be rationalized in terms of the retention factor of the solute (k'), also referred to as mass distribution ratio (D_m), which is the ratio between the number of moles of solute in the micelle to those in the mobile phase. For a neutral compound, k' is as follows:

$$k' = \frac{t_r - t_0}{t_0(1 - t_r/t_{mc})} = K \left(\frac{V_s}{V_M} \right)$$

in which t_r is the migration time of the solute; t_0 is the analysis time of the unretained solute obtained by injecting an electroosmotic flow marker that does not enter the micelle (e.g., methanol); t_{mc} is the micelle migration time measured by injecting a micelle marker, such as Sudan III, which migrates continuously associated in the micelle; K is the partition coefficient of the solute; V_s is the volume of the micellar phase; and V_M is the volume of the mobile phase.

The resolution between two closely-migrating solutes (R_s) is as follows:

$$R_s = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_b'}{k_b' + 1} \times \frac{1 - \left(\frac{t_0}{t_{mc}} \right)}{1 + k_a' \times \left(\frac{t_0}{t_{mc}} \right)}$$

in which N is the number of theoretical plates for one of the solutes; α is the selectivity; and k_a' and k_b' are retention factors for both solutes, respectively ($k_b' > k_a'$).

Similar, but not identical, equations give k' and R_s values for electrically charged solutes.

Optimization

The main parameters to be considered in the development of separations by MEKC are instrumental and electrolytic solution parameters.

INSTRUMENTAL PARAMETERS

Voltage—Separation time is inversely proportional to applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross section of the capillary. This effect can be significant with high

conductivity buffers, such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

Temperature—Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration, and the viscosity of the buffer. These parameters contribute to the migration time of the solutes. The use of a good cooling system improves the reproducibility of the migration time for the solutes.

Capillary—As in *Capillary Zone Electrophoresis*, length and internal diameter of the capillary contribute to analysis time and efficiency of separations. Increasing both effective length and total length can decrease the electrical fields, working at constant voltage, and will increase migration time and improve the separation efficiency. The internal diameter controls heat dissipation, for a given buffer and electrical field, and consequently broadening of the sample band.

ELECTROLYTIC SOLUTION PARAMETERS

Surfactant Type and Concentration—The type of surfactant, as the stationary phase in chromatography, affects the resolution because it modifies separation selectively. The log k' of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. When k' approaches the value of

$$\sqrt{t_{mc}/t_0}$$

resolution in MEKC reaches a maximum. Modifying the concentration of surfactant in the mobile phase changes the resolution.

Buffer pH—pH does not modify the partition coefficient of nonionized solutes, but it can modify the electroosmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electroosmotic flow and, therefore, increases the resolution of the neutral solutes in MEKC, resulting in a longer analysis time.

Organic Solvents—To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolytic solution. The addition of these modifiers generally decreases migration time and selectivity of the separation. The addition of organic modifiers affects critical micellar concentration, thus, a given surfactant concentration can be used only with a certain percentage of organic modifier before the micellization is inhibited or adversely affected, resulting in the absence of micelles and, therefore, the absence of the partition. The dissociation of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible, because in some cases, the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

Additives for Chiral Separations—For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts, *N*-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions that contain micellized achiral surfactants.

Other Additives—Selectivity can be modified by adding chemicals to the buffer. Addition of several types of cyclodextrins to the buffer is also used to reduce the interaction of hydrophobic solutes with the micelle, increasing the selectivity for this type of compound. The addition of substances able to modify solute-micelle interactions by ad-

sorption on the latter has been used to improve the selectivity of the separations in MEKC. These additives may consist of a second surfactant (ionic or nonionic), which gives rise to mixed micelles or metallic cations that dissolve in the micelle and form coordination complexes with the solutes.

Quantification

Peak areas must be divided by the corresponding migration time to give the corrected area in order to compensate for the shift in migration time from run to run, thus reducing the variation of the response. Dividing the peak areas by migration time will also compensate for the different responses of sample constituents with different migration times. Where an internal standard is used, check that no peak of the substance to be examined is masked by that of the internal standard.

Calculations—From the values obtained, calculate the content of a component or components being determined. When indicated, the percentage of one (or more) components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total of the corrected areas of all the peaks, excluding those due to solvents or any added reagents (normalization procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

SYSTEM SUITABILITY

In order to check the behavior of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. The parameters include the following: retention factor k' used only for *Micellar Electrokinetic Chromatography*, apparent number of theoretical plates (N), the symmetry factor (A_s), and the resolution (R_s). Note that in previous sections, the theoretical expressions for N and R_s have been described, but more practical equations that allow for the determination of these suitability parameters using the electropherograms are described below.

Apparent Number of Theoretical Plates—The apparent number of theoretical plates (N) may be calculated from the formula:

$$N = 5.54 (t_R/w_h)^2$$

in which t_R is the migration time or distance along the baseline between the point of injection and the perpendicular dropped from the maximum of the peak corresponding to the component; and w_h is the peak width at half-height.

Resolution—The resolution (R_s) between peaks of similar heights of two components may be calculated from the formula:

$$R_s = 1.18 (t_{R2} - t_{R1}) / (w_{h1} + w_{h2})$$

$$t_{R2} > t_{R1}$$

in which t_{R1} and t_{R2} are the migration times or distances along the baseline between the point of injection and the perpendiculars dropped from the maxima of two adjacent peaks; and w_{h1} and w_{h2} are the peak widths at half-height.

When appropriate, the resolution (R_s) may also be calculated by measuring the height of the valley (H_v) between two partly resolved peaks in a standard preparation, the

height of the smaller peak (H_p), and calculating the peak-to-valley ratio:

$$p/v = H_p/H_v$$

Symmetry Factor—The symmetry factor of a peak (A_s) may be calculated using the formula:

$$A_s = w_{0.05}/2d$$

in which $w_{0.05}$ is the width of the peak at one-twentieth of the peak height; and d is the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Other suitability parameters include tests for area repeatability (standard deviation of areas or of area/migration time) and tests for migration time repeatability (standard deviation of migration time). Migration time repeatability provides a test for the suitability of the capillary washing procedures. An alternative practice to avoid the lack of repeatability of the migration time is to use a migration time relative to an internal standard.

Signal-To-Noise Ratio—A test for the verification of the signal-to-noise ratio for a standard preparation or the determination of the limit of quantification may also be useful for the determination of related substances. The detection limit and quantification limit correspond to a signal-to-noise ratio of 3 and 10, respectively. The signal-to-noise ratio (S/N) is calculated as follows:

$$S/N = 2H/h$$

in which H is the height of the peak corresponding to the component concerned in the electropherogram obtained with the specified reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to twenty times the width at half-height; and h is the range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to twenty times the width at the half-height of the peak in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

<1054> BIOTECHNOLOGY-DERIVED ARTICLES—ISOELECTRIC FOCUSING

This chapter provides guidance and procedures used for the characterization of biotechnology-derived articles by isoelectric focusing. This chapter is harmonized with the corresponding chapters in *JP* and *EP*. Other characterization tests, also harmonized, are shown in the USP general information chapters *Biotechnology-Derived Articles—Amino Acid Analysis* <1052>, *Biotechnology-Derived Articles—Capillary Electrophoresis* <1053>, *Biotechnology-Derived Articles—Peptide Mapping* <1055>, *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* <1056>, and *Biotechnology-Derived Articles—Total Protein Assay* <1057>.

GENERAL PRINCIPLES

Isoelectric focusing (IEF) is a method of electrophoresis that separates proteins according to their isoelectric points.

Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes). When subjected to an electrical field, the ampholytes migrate in the gel to create a pH gradient. In some cases, gels containing an immobilized pH gradient, prepared by incorporating weak acids and bases to specific regions of the gel network during the preparation of the gel, are used. When the applied proteins reach the gel fraction that has a pH that is the same as their isoelectric point (pI), their charge is neutralized and migration ceases. Gradients can be made over various ranges of pH, according to the mixture of ampholytes chosen.

THEORETICAL ASPECTS

When a protein is at the position of its isoelectric point, it has no net charge and cannot be moved in a gel matrix by the electric field. It may, however, move from that position by diffusion. The pH gradient forces a protein to remain in its isoelectric point position, thus concentrating it; this concentration effect is called "focusing". Increasing the applied voltage or reducing the sample load results in improved separation of bands. The applied voltage is limited by the heat generated because the heat must be dissipated. The use of thin gels and an efficient cooling plate controlled by a thermostatic circulator prevents the burning of the gel while allowing sharp focusing. The separation is estimated by determining the minimum pI difference (ΔpI), which is necessary to separate two neighboring bands, as follows:

$$\Delta pI = 3 \sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

in which D is the diffusion coefficient of the protein; dpH/dx is the pH gradient; E is the intensity of the electric field, in volts per centimeter; and $-d\mu/dpH$ is the variation of the solute mobility with the pH in the region close to the pI. Because D and $-d\mu/dpH$ for a given protein cannot be altered, the separation can be improved by using a narrower pH range and by increasing the intensity of the electric field.

Resolution between protein bands on an IEF gel prepared with carrier ampholytes can be quite good. Better resolution may be achieved by using immobilized pH gradients where the buffering species, which are analogous to carrier ampholytes, are copolymerized within the gel matrix. Proteins exhibiting pI values differing by as little as 0.02 pH units may be resolved using a gel prepared with carrier ampholytes, whereas immobilized pH gradients can resolve protein differing by approximately 0.001 pH units.

PRACTICAL ASPECTS

From an operational point, special attention must be paid to sample characteristics and/or preparation. Salt in a sample can be problematic, and it is best to prepare the sample, if possible, in deionized water or 2% ampholytes using dialysis or gel filtration if necessary. The time required for completion of focusing in thin-layer polyacrylamide gels is determined by placing a colored protein (e.g., hemoglobin) at different positions on the gel surface and by applying the electric field: the steady state is reached when all applications give an identical band pattern. In some procedures the completion of the focusing is indicated by the time elapsed after the sample application.

The IEF gel can be used as an identity test when the migration pattern on the gel is compared to a suitable standard preparation and IEF calibration proteins; the IEF gel can be used as a limit test when the density of a band on IEF is compared subjectively with the density of bands appearing in a standard preparation, or it can be used as a quantitative test when the density is measured using a den-

sitometer or similar instrumentation to determine the relative concentration of protein in the bands subject to validation.

APPARATUS

An apparatus for isoelectric focusing consists of a controllable generator for constant potential, current, and power. Potentials of 2500 V have been used and are considered optimal under a given set of operating conditions. Supply of up to 30 W of constant power is recommended. The apparatus also includes a rigid plastic isoelectric focusing chamber that contains a cooled plate of suitable material to support the gel; and a plastic cover with platinum electrodes that are connected to the gel by means of paper wicks of suitable width, length, and thickness, impregnated with solutions of anodic and cathodic electrolytes.

ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS: DETAILED PROCEDURE

The following method is a detailed description of an IEF procedure in thick polyacrylamide slab gels, which is used unless otherwise stated in the monograph.

Preparation of the Gels

Mold—The mold is composed of a glass plate (A) on which a polyester film (B) is placed to facilitate handling of the gel, one or more spacers (C), a second glass plate (D), and clamps to hold the structure together (see Figure 1).

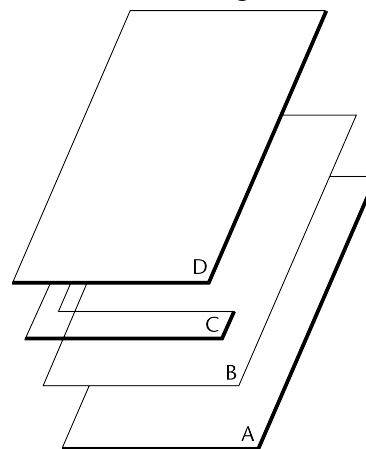


Figure 1. Mold

7.5% Polyacrylamide Gel—Dissolve 29.1 g of acrylamide and 0.9 g of methylenebisacrylamide in 100 mL of water. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the individual monograph, and dilute to 10 volumes with water. Mix carefully, and degas the solution.

Preparation of the Mold—Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate, and fit the clamps. Before use, place the mixture on a magnetic stirrer, and add 0.25 volumes of a 100 g/L solution of ammonium persulfate and 0.25 volumes of tetramethylethylenediamine. Immediately fill the space between the glass plates of the mold with the solution.

Fixing Solution for Isoelectric Focusing Polyacrylamide Gel—Mix 35 g of sulfosalicylic acid and 100 g of trichloroacetic acid in 1000 mL of water.

Coomassie Staining Solution and Destaining Solution—Use the same solutions indicated in general infor-

mation chapter *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* (1056).

Procedure

Dismantle the *Mold*, and using the polyester film, transfer the gel onto the cooled support wetted with a few mL of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the individual monograph. Place strips of paper for sample application, about 10 mm × 5 mm in size, on the gel, and impregnate each with the prescribed amount of the test and reference solutions. Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some procedures, the gel has precast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut two strips of paper to the length of the gel, and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode. The compositions of the anode and cathode solutions are given in the individual monograph. Apply these paper wicks to each side of the gel several mm from the edge. Fit the cover so that the electrodes are in contact with the wicks (with respect to the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the individual monograph. Switch off the current when the migration of the mixture of standard proteins has stabilized. Using forceps, remove the sample application strips and the two electrode wicks. Immerse the gel in *Fixing Solution for Isoelectric Focusing Polyacrylamide Gel*. Incubate with gentle shaking at room temperature for 30 minutes. Drain off the solution, and add 200 mL of *Destaining Solution*. Incubate with shaking for 1 hour. Drain the gel, and add *Coomassie Staining Solution*. Incubate for 30 minutes. Destain the gel by passive diffusion with *Destaining Solution* until the bands are well visualized against a clear background. Locate the position and intensity of the bands in the electropherogram, as prescribed in the individual monograph.

Variations to the Detailed Procedure (Subject to Validation)

Where reference to the general method on isoelectric focusing is made, variations in methodology or procedure may be made subject to validation. These variations include the use of commercially available precast gels and of commercial staining and destaining kits; the use of immobilized pH gradients; the use of rod gels, and the use of cassettes of different dimensions, including ultra-thin (0.2 mm) gels; variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper; the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than subjective interpretation of band stability; the inclusion of a prefocusing step; the use of automated instrumentation; and the use of agarose gels.

Validation of Isoelectric Focusing Procedures

Where alternative methods to the detailed procedure are employed, they must be validated. The following criteria may be used to validate the separation: formation of a stable pH gradient of desired characteristics, assessed for example using colored pH markers of known isoelectric points; comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined; and any other validation criteria as prescribed in the individual monograph.

SPECIFIED VARIATIONS TO THE GENERAL METHOD

Variations to the general method required for the analysis of specific substances may be specified in detail in individual monographs. Variations may include the addition of urea in the gel (a 3 M concentration is often satisfactory to keep the protein in solution, but up to 8 M can be used). Some proteins precipitate at their isoelectric point. In this case, urea is included in the gel formulation to keep the protein in solution. If urea is used, only fresh solutions should be used to prevent carbamylation of the protein. Other variations include the use of alternative staining methods and the use of gel additives such as nonionic detergents (e.g., octylglucoside) or zwitterionic detergents (e.g., CHAPS or CHAPSO) and the addition of ampholyte to the sample to prevent proteins from aggregating or precipitating.

Points To Consider

1. Samples can be applied to any area on the gel, but to protect the proteins from extreme pH environments, samples should not be applied close to either electrode. During method development, the analyst can try applying the protein in three positions on the gel (e.g., middle and both ends); the pattern of a protein applied at opposite ends of the gel may not be identical.
2. A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, electroendosmosis and absorption of carbon dioxide may be factors that lead to cathodic drift. Cathodic drift is observed as focused protein migrating off the cathode end of the gel. Immobilized pH gradients may be used to address this problem.
3. Efficient cooling (approximately 4°) of the bed that the gel lies on during focusing is important. High field strengths used during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

(1055) BIOTECHNOLOGY-DERIVED ARTICLES—PEPTIDE MAPPING

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by peptide mapping. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Portions of the chapter that are not harmonized with the other two pharmacopeias are marked by the symbol ♦. Other characterization tests, also harmonized, are shown in *Biotechnology-Derived Articles—Amino Acid Analysis* (1052), *Biotechnology-Derived Articles—Capillary Electrophoresis* (1053), *Biotechnology-Derived Articles—Isoelectric Focusing* (1054), *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* (1056), and *Biotechnology-Derived Articles—Total Protein Assay* (1057).

INTRODUCTION

Peptide mapping is an identity test for proteins, especially those obtained by rDNA technology. It involves the chemical or enzymatic treatment of a protein, resulting in the

formation of peptide fragments, followed by separation and identification of the resultant fragments in a reproducible manner. It is a powerful test that is capable of identifying single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a Reference Standard or Reference Material similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics that must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterize the desired protein product, to evaluate the stability of the expression construct of cells used for recombinant DNA products, to evaluate the consistency of the overall process, and to assess product stability, as well as to ensure the identity of the protein product or to detect the presence of protein variant. ♦The validation scheme presented differentiates between qualification of the method at an early stage in the regulatory process, the Investigational New Drug (IND) level, and full validation in support of New Drug Application (NDA), Product License Application (PLA), or Marketing Authorization Application (MAA). The validation concepts described are consistent with the general information chapter *Validation of Compendial Procedures* <1225> and with the International Conference on Harmonization (ICH) document *Analytical Methods Validation*.♦

THE PEPTIDE MAP

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analyzed. Four major steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a Reference Standard or Reference Material. Complete cleav-

age of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used instead of chemical cleavage reagents. A map should contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

Isolation and Purification

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form should be validated.

Selective Cleavage of Peptide Bonds

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed—enzymatic or chemical—and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in *Table 1*. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

PRETREATMENT OF SAMPLE

Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. For monoclonal antibodies, the heavy and light chains will need to be separated before mapping. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100,000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

PRETREATMENT OF THE CLEAVAGE AGENT

Pretreatment of cleavage agents, especially enzymatic agents, might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by HPLC or immobilization of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

Table 1. Examples of Cleavage Agents

Type	Agent	Specificity
Enzymatic	Trypsin, EC 3.4.21.4	C-terminal side of Arg and Lys
	Chymotrypsin, EC 3.4.21.1	C-terminal side of hydrophobic residues (e.g., Leu, Met, Ala, aromatics)
	Pepsin, EC 3.4.23.1 and EC 3.4.23.2	Nonspecific digest
	Lysyl endopeptidase (Lys-C endopeptidase), EC 3.4.21.50	C-terminal side of Lys
	Glutamyl endopeptidase; (from <i>S. aureus</i> strain V8), EC 3.4.21.19	C-terminal side of Glu and Asp
	Peptidyl-Asp metalloendopeptidase (Asp-N endoproteinase), EC 3.4.24.33	N-terminal side of Asp
	Clostripain, EC 3.4.22.8	C-terminal side of Arg
	Cyanogen bromide	C-terminal side of Met
Chemical	2-Nitro-5-thiocyanobenzoic acid	N-terminal side of Cys
	o-Iodosobenzoic acid	C-terminal side of Trp and Tyr
	Dilute acid	Asp and Pro
	BNPS-skatole	Trp

PRETREATMENT OF THE PROTEIN

Under certain conditions, it might be necessary to concentrate the sample, or to separate the protein from added substances and stabilizers used in the formulation of the product if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultrafiltration, column chromatography, and lyophilization.

Other pretreatments, such as the addition of chaotropic agents (e.g., urea) can be used to unfold the protein prior to mapping. To allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein, it is often necessary to reduce and alkylate the disulfide bonds prior to digestion.

Digestion with trypsin can introduce ambiguities in the tryptic map as a result of side reactions occurring during the digestion reaction, such as nonspecific cleavage, deamidation, disulfide isomerization, oxidation of methionine residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the *N*-terminal side of a peptide. Furthermore, peaks may be produced by autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g., at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

ESTABLISHMENT OF OPTIMAL DIGESTION CONDITIONS

Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

pH—The digestion mixture pH is empirically determined to ensure the optimal performance of the given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g., pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu should not alter the chemical integrity of the protein during the digestion and should not change during the course of the fragmentation reaction.

Temperature—A temperature between 25° and 37° is adequate for most digestions. The temperature used is intended to minimize chemical side reactions. The type of protein under test will dictate the temperature of the reaction milieu because some proteins are more susceptible to denaturation as the temperature of the reaction increases. For example, digestion of recombinant bovine somatotropin is conducted at 4° because at higher temperatures it will precipitate during digestion.

Time—If a sufficient amount of sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 to 30 hours. The reaction is stopped by the addition of an acid that does not interfere with the tryptic map, or by freezing.

Amount of Cleavage Agent—Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e., 6 to 20 hours), the amount of cleavage agent is minimized to avoid its contribution to the chromatographic map pattern. A protein-to-protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent be added in two or more stages to optimize cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping—the separation step. To sort out digestion artifacts that might be interfering with the subsequent analysis, a blank determination is performed using a digestion control with all the reagents except the test protein.

Chromatographic Separation

Many techniques are used to separate peptides for mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for the separation of peptides are shown in *Table 2*.

Table 2. Techniques Used for the Separation of Peptides

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)
Ion-Exchange Chromatography (IEC)
Hydrophobic Interaction Chromatography (HIC)
Polyacrylamide Gel Electrophoresis (PAGE), nondenaturing
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Capillary Electrophoresis (CE)
Paper Chromatography—High Voltage (PCHV)
High-Voltage Paper Electrophoresis (HVPE)

In this section, a most widely used reverse-phase HPLC (RP-HPLC) method is described as one of the procedures of chromatographic separation.

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC-grade solvents and water that are commercially available are recommended for RP-HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. When solid particles in the solvents are drawn into the HPLC system, they can damage the sealing of pump valves or clog the top of the chromatographic column. Both pre- and post-pump filtration are also recommended.

Chromatographic Column—The selection of a chromatographic column is empirically determined for each protein. Columns with 100 Å or 300 Å pore size and silica support can give optimal separation. For smaller peptides, octylsilane chemically bonded to totally porous silica particles 3 to 10 µm in diameter (L7) and of octadecylsilane chemically bonded to porous silica or ceramic microparticles 3 to 10 µm in diameter (L1) column packings are more efficient than the butyl silane chemically bonded to totally porous silica particles 5 to 10 µm in diameter (L26) packing.

Solvent—The most commonly used solvent is water with acetonitrile as the organic modifier to which less than 0.1% of trifluoroacetic acid is added. If necessary, add isopropyl alcohol or *n*-propyl alcohol to solubilize the digest components, provided that the addition does not unduly increase the viscosity of the components.

Mobile Phase—Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, because shifts of pH in the 3.0 to 5.0 range enhance the separation of peptides containing acidic residues (e.g., glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid, and a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile-containing trifluoroacetic acid is also used quite often.

Gradient Selection—Gradients can be linear, nonlinear, or include step functions. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimized to provide clear resolution of one or two peaks that will become "marker" peaks for the test.

Isocratic Selection—Isocratic HPLC systems using a single mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain clear resolution of each peak is sometimes difficult to establish. Mobile phases for which slight changes in component ratios or in pH significantly affect retention times of peaks in peptide maps should not be used in isocratic HPLC systems.

Other Parameters—Temperature control of the column is usually necessary to achieve good reproducibility. The flow rates for the mobile phases range from 0.1 to 2.0 mL per minute, and the detection of peptides is performed with a UV detector at 200 to 230 nm. Other methods of detection have been used (e.g., postcolumn derivatization), but they are not as robust or as versatile as UV detection.

System Suitability—The section *System Suitability* under *Chromatography* (621) provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria that monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint was achieved is the comparison with a Reference Standard or Reference Material, which is treated exactly as the article under test. The use of a USP Reference Standard in parallel with the protein under test is critical in the development and establishment of system suitability limits. In addition, a specimen chromatogram should be included with the USP Reference Standard or Reference Material for additional comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion-dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection, and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides cover selectivity and precision. In this case, as well as when identification of variant proteins is done, the identification of the primary structure of the peptide fragments in the peptide map provides both a verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the USP Reference Standard or Reference Material for the specified protein. The use of a digested USP Reference Standard or Reference Material for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterized mixture of a variant and a Reference Standard can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters—such as peak-to-peak resolution, maximum peak width, peak area, peak tailing factors, and column efficiency—may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the USP Reference Standard or Reference Material for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The precision is expressed as the relative standard deviation (RSD). Differences in the recovery and precision of the identified peptides are expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the individual monograph.

Visual comparison of the relative retention times, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (v/v) mixture of sample and USP Reference Standard or Reference Material digest. If all peaks in the sample digest and in the USP Reference Standard or Reference Material digest have the same relative retention

times and peak response ratios, then the identity of the sample under test is confirmed.

If peaks that initially eluted with significantly different relative retention times are then observed as single peaks in the 1:1 mixture, the initial difference would be an indication of system variability. However, if separate peaks are observed in the 1:1 mixture, this would be evidence of the nonequivalence of the peptides in each peak. If a peak in the 1:1 mixture is significantly broader than the corresponding peak in the sample and USP Reference Standard or Reference Material digest, it may indicate the presence of different peptides. The use of computer-aided pattern recognition software for the analysis of peptide mapping data has been proposed and applied, but issues related to the validation of the computer software preclude its use in a compendial test in the near future. Other automated approaches have been used that employ mathematical formulas, models, and pattern recognition. Such approaches, for example the automated identification of compounds by IR spectroscopy and the application of diode-array UV spectral analysis for identification of peptides, have been proposed. These methods have limitations due to inadequate resolutions, co-elution of fragments, or absolute peak response differences between USP Reference Standard or Reference Material and sample fragments.

The numerical comparison of the retention times and peak areas or peak heights can be done for a selected group of relevant peaks that have been correctly identified in the peptide maps. Peak areas can be calculated using one peak showing relatively small variation as an internal reference, keeping in mind that peak area integration is sensitive to baseline variation and is likely to introduce error into the analysis. Alternatively, the percentage of each peptide peak height relative to the sum of all peak heights can be calculated for the sample under test. The percentage is then compared to that of the corresponding peak of the USP Reference Standard or Reference Material. The possibility of autohydrolysis of trypsin is monitored by producing a blank peptide map, that is, the peptide map obtained when a blank solution is treated with trypsin.

The minimum requirement for the qualification of peptide mapping is an approved test procedure that includes system suitability as a test control. In general, early in the regulatory process, qualification of peptide mapping for a protein is sufficient. As the regulatory approval process for the protein progresses, additional qualifications of the test can include a partial validation of the analytical procedure to provide assurance that the method will perform as intended in the development of a peptide map for the specified protein.

Analysis and Identification of Peptides

This section gives guidance on the use of peptide mapping during development in support of regulatory applications.

The use of a peptide map as a qualitative tool does not require the complete characterization of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterization of each of the individual peaks in the peptide map. Methods to characterize peaks range from *N*-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterization purposes, when *N*-terminal sequencing and amino acid analysis are used, the analytical separation is scaled up. Because scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to ensure that there is no loss of resolution due to scale-up. Eluates corresponding to specific peptide peaks are collected, vacuum-concentrated, and chromatographed again, if necessary. Amino acid analysis of fragments may be limited by the peptide size. If the *N*-terminus is blocked, it may need to be cleared before sequencing. C-terminal sequencing of proteins in combination with carboxypeptidase diges-

tion and matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS can also be used for characterization purposes.

The use of MS for characterization of peptide fragments is by direct infusion of isolated peptides or by the use of on-line LC-MS for structure analysis. In general, it includes electrospray and MALDI-TOF analyzers as well as fast atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulfide bonds to the various sulfhydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterization of a protein through peptide mapping is to reconcile and account for at least 95% of the theoretical composition of the protein structure.

♦THE USE OF PEPTIDE MAPPING FOR GENETIC STABILITY EVALUATION

A validated peptide map can be used to assess the integrity of the predicted primary sequence of a protein product (i.e., its genetic stability). It can also be used to determine lot-to-lot consistency of the biotechnology-derived product process. Furthermore, the performance of the protein expression of the production system is best assessed by peptide mapping of the expressed protein. Peptide maps of protein produced at various times of the protein expression process, including a point well beyond the normal protein expression time, compared with those of a USP Reference Standard or Reference Material, will evaluate the genetic stability of the expression system as a function of time.

Variant protein sequences can arise from a genetic variation at the DNA level (point mutation) or as an error in the translation process. A validated peptide map is the best approach to the detection of protein variants. However, the limitations of the peptide mapping itself must be taken into consideration. The detection of a structured variant is possible only if the corresponding peptide variant is easily isolated and characterized. To establish genetic stability will require the use of a battery of biochemical methods, provided that the variants have properties different from those of the “normal” protein.♦

♦VALIDATION

Critical Factors

Validation of peptide mapping requires that a protocol be designed, outlining in detail the experiment to be conducted and the criteria for acceptance of the map. Criteria for acceptance of mapping include detection limit, specificity, linearity, range, accuracy, precision, and reagent stability. Reproducibility of the peptide map is a critical element in the utilization of such a map as an identity test and for confirming genetic stability. Those technical aspects of peptide mapping that influence the reproducibility of the map will be discussed.

The setting of limits, with respect to quantification (peak area or height) and identification (retention times) for the selected group of relevant peaks is based on empirical observations. These limits detect significant differences between the sample and USP Reference Standard or Reference Material within a series of analyses.

Another critical issue is the recovery of peptides and its impact on peak area determination and reproducibility and on the establishment of acceptance criteria. The recovery criteria address all aspects of test methodology, from digestion to chromatographic conditions. Determination of pep-

tide recovery includes quantitative amino acid analysis, spike addition, radiolabeling, and UV summation. An overall recovery of about 80% is considered satisfactory. Recovery of individual peptides is more problematic and is handled on a case-by-case basis. The critical factors considered in the validation of a peptide map are as follows.

Written Test Procedures—These procedures include a detailed description of the analytical method in which reagents, equipment, sample preparation, method of analysis, and analysis of the data are defined.

Validation Protocol—A protocol is prepared that contains a procedure for test validation.

Acceptance Criteria—The criteria can be minimal at the early stages, but need to be better defined as validation studies progress.

Reporting of Results—Results from the validation study are documented with respect to the analytical parameters listed in the validation protocol.

Revalidation of the Test Procedure—If the method used requires alteration that could affect the analytical parameter previously assessed in the validation of the procedure, the test procedure must be revalidated. Significant changes in the processing of the article, in laboratories performing the analysis, in formulation of the bulk or the finished products, and in any other significant parameter will require revalidation of the methods.

Requirements

PRECISION

Intratest Precision—This is a measure of the reproducibility of peptide mapping. The two critical steps in peptide mapping are fragmentation (i.e., digestion) and separation of peptides. An acceptable precision occurs where the absolute retention times and the relative peak areas are constant from run to run, and the average variation in retention time is small relative to that of a selected internal reference peak. The reproducibility of the map can be enhanced if a temperature-controlled column oven is used, if an extensive equilibration of the system is performed prior to the start of the test, if a blank (control digest mixture without protein) is run first to minimize “first run effects,” and if a USP Reference Standard or a Reference Material digest is interspersed periodically with test samples to evaluate chromatographic drift.

The criteria for validation of the fragmentation step are similar to those described below for separation of peptides, but they are met for consecutive tests of a series of separately prepared digests of the protein under test.

The criteria for validation of the separation of peptides step include the following:

1. The average standard deviation of the absolute retention times of all major peaks for a set of consecutive tests of the same digest does not exceed a specified acceptance criterion.
2. The average standard deviation of absolute peak area for all fully resolved major peaks does not exceed a specified percentage.

Intertest Precision—This is a measure of the reproducibility of the peptide mapping when the test is performed on different days, by different analysts, in different laboratories, with reagents or enzymes from different suppliers or different lots from the same supplier, with different instruments, on columns of different makes or columns of the same make from different lots, and on individual columns of the same make from the same lot. Although it would be desirable, from a scientific perspective, to validate all of these variables in terms of their impacts on precision, a practical approach is to validate the test using those vari-

ables most likely to be encountered under operational conditions. Additional variables can be included when needed.

The experimental design allows the analyst to make comparisons using peak retention times and areas that are expressed relative to a highly reproducible internal reference peak within the same chromatogram. The relative peak area is expressed as the ratio of the peak area to that of the internal reference peak. The relative retention time can be expressed as the difference between the absolute retention time and that of the reference peak. The use of relative values eliminates the need to make separate corrections for differences due to injector-to-injector volumes, units of measure for peak areas, column dimensions, and instrument dead volumes. The variability in the retention times and peak areas for the *Intertest Precision* experiments is expected to be slightly higher than the variability observed for *Intratest Precision*.

ROBUSTNESS

Factors such as composition of the *Mobile Phase*, protease quality or chemical reagent purity, column variation and age, and digest stability are likely to affect the overall performance of the test and its reproducibility. Tolerances for each of the key parameters are evaluated and baseline limits established in case the test is used for routine lot release purposes.

Mobile Phase—The composition of the *Mobile Phase* is optimized to obtain the maximum resolution of peptides throughout the elution profile. A balance between optimal resolution and overall reproducibility is desired. A lower pH might improve peak separation but might shorten the life of the column, resulting in lack of reproducibility. Peptide maps at a pH above and below the pH of the procedure are compared to the peptide map obtained at the pH of the procedure and checked for significant differences; they are also reviewed with respect to the acceptance criteria established in the validation protocol.

Protease Quality or Chemical Reagent Purity—A sample of the USP Reference Standard or Reference Material for the protein under test is prepared and digested with different lots of cleavage agent. The chromatograms for each digest are compared in terms of peak areas, shape, and number. The same procedure can be applied to other critical chemicals or pretreatment procedures used during sample preparation, such as reducing and carboxymethylation reagents.

Column Considerations—Column-to-column variability, even within a single lot, can affect the performance of the column in the development of peptide maps. Column size may also lead to significant differences. A USP Reference Standard or Reference Material of the protein under test is digested and the digest is chromatographed on different lots of column from a single manufacturer. The maps are then evaluated in terms of the overall elution profile, retention times, selectivity resolution, and recovery. To evaluate the overall lifetime of the column in terms of robustness, perform a peptide mapping test on different columns and vary significantly the number of injections (e.g., from 10 injections to 250 injections). The resulting maps can then be compared for significant differences in peak broadening, peak area, and overall resolution. As a column ages, an increase in back pressure might be observed that might affect the peptide maps.

A sensible precaution in the use of peptide mapping columns is to select alternative columns in case the original columns become unavailable or are discontinued. Perform a peptide mapping test using equivalent columns from different manufacturers, and examine the maps. Differences in particle shape and size, pore size and volume, carbon load, and end-capping can lead to significant differences in retention times, elution profile selectivity, resolution, and recovery. Slight modifications in the gradient profile may be re-

quired to achieve equivalency of mapping when using columns from different manufacturers. [NOTE—The equivalency between instrumentation used for the validation of the test and for routine quality control testing should be considered. It might be preferable to use the same HPLC system for all applications. Otherwise, equivalency of the systems is determined, which may require some changes in the chromatographic test conditions.]

Digest Stability—The length of time a digest can be kept before it is chromatographed, as well as the conditions under which the digest is stored before chromatography, is assessed. Several aliquots from a single digest are stored at different storage conditions and chromatographed. These maps are then evaluated for significant differences.

REPRODUCIBILITY

Determination of various parameters indicated above is repeated using the same USP Reference Standard or Reference Material and test sample in at least two different laboratories by two analysts equipped with similar HPLC systems. The generated peptide maps are evaluated for significant differences.♦

<1056> BIOTECHNOLOGY-DERIVED ARTICLES—POLYACRYLAMIDE GEL ELECTROPHORESIS

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles by polyacrylamide gel electrophoresis. Portions of the chapter that are not harmonized with the other two pharmacopeias are marked by the symbol ♦. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Other characterization tests, also harmonized, are shown in *Biotechnology-Derived Articles—Amino Acid Analysis* (1052), *Biotechnology-Derived Articles—Capillary Electrophoresis* (1053), *Biotechnology-Derived Articles—Isoelectric Focusing* (1054), *Biotechnology-Derived Articles—Peptide Mapping* (1055), and *Biotechnology-Derived Articles—Total Protein Assay* (1057).

INTRODUCTION

Scope

Polyacrylamide gel electrophoresis (PAGE) is used for the qualitative characterization of proteins in biological preparations, for control of purity and for quantitative determinations.

Purpose

Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in drug substances. The method is routinely used for the estimation of protein subunit molecular masses and for the determination of the subunit compositions of purified proteins.

Ready-to-use gels and reagents are widely available on the market and can be used instead of those described in this

chapter, provided that they give equivalent results and that they meet the validity requirements given below under *Validation of the Test*.

♦ General Principle of Electrophoresis

Under the influence of an electrical field, charged particles migrate in the direction of the electrode bearing the opposite polarity. In gel electrophoresis, the movements of the particles are retarded by interactions with the surrounding gel matrix, which acts as a molecular sieve. The opposing interactions of the electrical force and molecular sieving result in differential migration rates according to the sizes, shapes, and charges of particles. Because of their different physicochemical properties, different macromolecules of a mixture migrate at different speeds during electrophoresis and thus are separated into discrete fractions. Electrophoretic separations can be conducted in systems without support phases (e.g., free solution separation in capillary electrophoresis) and in stabilizing media, such as thin-layer plates, films, or gels.♦

CHARACTERISTICS OF POLYACRYLAMIDE GELS

The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibers and pores that is formed as the bifunctional bisacrylamide cross-links adjacent to polyacrylamide chains. Polymerization is catalyzed by a free-radical-generating system composed of ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED).

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties, that is, by the resistance it imparts to the migration of macromolecules. There are limits to the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel, through manipulating the acrylamide concentration, the resolution of the method can be optimized for a given protein product. Thus, a given gel is physically characterized by its respective composition of acrylamide and bisacrylamide.

In addition to the composition of the gel, the state of the protein is an important component of electrophoretic mobility. In the case of proteins, electrophoretic mobility is dependent on the pK value of the charged groups and the size of the molecule. It is influenced by the type, the concentration, and the pH of the buffer; by the temperature and the field strength; and by the nature of the support material.

Denaturing Polyacrylamide Gel Electrophoresis

This method cited is limited to the analysis of monomeric polypeptides with a mass range of 14,000 to 100,000 Da. It is possible to extend the mass range by various techniques (e.g., gradient gels or particular buffer systems), but those techniques are not discussed in this chapter.

Denaturing PAGE using sodium dodecyl sulfate (SDS) (SDS-PAGE) is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and will be the focus of the example method. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strongly anionic detergent SDS is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, be-

come negatively charged, and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent-polypeptide complexes all assume the same functional relationship to their molecular masses. Migration of SDS complexes is toward the anode in a predictable manner, with low molecular mass complexes migrating faster than larger ones. The molecular mass of a protein can therefore be estimated from its relative mobility in calibrated SDS-PAGE and the occurrence of a single band in such a gel is a criterion of purity.

Modifications to the polypeptide backbone, such as *N*- or *O*-linked glycosylation, however, have a significant impact on the apparent molecular mass of a protein, because SDS does not bind to a carbohydrate moiety in a manner similar to the polypeptide. Thus, a consistent charge-to-mass ratio is not maintained. The apparent molecular mass of proteins having undergone post-translational modifications is not a true reflection of the mass of the polypeptide chain.

Reducing Conditions

Polypeptide subunits and three-dimensional structure is often maintained in proteins by the presence of disulfide bonds. A goal of SDS-PAGE analysis under reducing conditions is to disrupt this structure by reducing disulfide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol or dithiothreitol (DTT) will result in the unfolding of the polypeptide backbone and subsequent complexation with SDS. In these conditions, the molecular mass of the polypeptide subunits can be calculated by linear regression in the presence of suitable molecular mass standards.

Nonreducing Conditions

For some analyses, complete dissociation of protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as 2-mercaptoethanol or DTT, disulfide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS-protein complexes migrate more slowly than their SDS-polypeptide subunits. In addition, nonreduced proteins may not be completely saturated with SDS and hence may not bind the detergent in a constant mass ratio. This makes molecular mass determinations of these molecules less straightforward than analyses of fully denatured polypeptides, because it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons. However, the staining of a single band in such a gel is a criterion of purity.

Characteristics of a Discontinuous Buffer System Gel Electrophoresis

The most popular electrophoretic method for the characterization of a complex mixture of proteins involves the use of a discontinuous buffer system consisting of two contiguous, but distinct, gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pHs, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity concentrates large volumes of sample in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution that drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is

rapidly formed with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localized high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS-protein complexes to form into a thin zone (stack) and migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface between the stacking and resolving gels, the proteins experience a sharp retardation due to the restrictive pore size of the resolving gel. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by tris(hydroxymethyl)aminomethane (Tris) and glycine. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular masses.

Preparing Vertical Discontinuous Buffer SDS-Polyacrylamide Gels

GEL STOCK SOLUTIONS

30% Acrylamide-Bisacrylamide Solution—Prepare a solution containing 290 g of acrylamide and 10 g of methylene bisacrylamide per L of warm water, and filter. [NOTE—Acrylamide and methylene bisacrylamide are slowly converted during storage to acrylic acid and bisacrylic acid, respectively. This deamidation reaction is catalyzed by light and alkali. The pH of the solution must be 7.0 or lower. Store the solution in dark bottles at room temperature. Fresh solutions are prepared every month.]

Ammonium Persulfate Solution—Prepare a small quantity of solution having a concentration of 100 g of ammonium persulfate per L, and store at 4°. [NOTE—Ammonium persulfate provides the free radicals that drive polymerization of acrylamide and bisacrylamide. Ammonium persulfate decomposes slowly; therefore, prepare fresh solutions weekly.]

TEMED—Use an electrophoresis-grade reagent. [NOTE—TEMED accelerates the polymerization of acrylamide and bisacrylamide by catalyzing the formation of free radicals from ammonium persulfate. Because TEMED works only as a free base, polymerization is inhibited at low pH.]

SDS Solution—Use an electrophoresis-grade reagent. Prepare a solution having a concentration of about 100 g of SDS per L, and store at room temperature.

1.5 M Buffer Solution—Transfer about 90.8 g of Tris to a 500-mL flask, dissolve in 400 mL of water, adjust with hydrochloric acid to a pH of 8.8, dilute with water to volume, and mix.

1 M Buffer Solution—Transfer about 60.6 g of Tris to a 500-mL flask, add 400 mL of water, adjust with hydrochloric acid to a pH of 6.8, dilute with water to volume, and mix.

PLATE PREPARATION

Clean two glass plates (size e.g., 10 cm × 8 cm), the polytetrafluoroethylene comb, the two spacers, and the silicone rubber tubing (diameter e.g., 0.6 mm × 35 cm) with mild detergent, and rinse thoroughly with water. Dry all items with a paper towel or tissue.

Lubricate the spacers and the tubing with nonsilicone grease. Apply the spacers along each of the two short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel.

Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer, and follow the long side of the glass plate. While holding the tubing with one finger along the long side, twist the tubing again, and lay it on the second short side of the glass plate, using the spacer as a guide.

Place the second glass plate in perfect alignment and hold the gel mold together by hand pressure. Apply two clamps on each of the two short sides of the mold. Carefully apply four clamps on the longer side of the mold, thus forming the bottom of the gel mold. Verify that the tubing is running along the edge of the glass plates and has not been extruded while placing the clamps. The gel mold is now ready for pouring the gel.

PREPARATION OF THE GEL

In a discontinuous buffer SDS-polyacrylamide gel, it is important to pour the resolving gel, let the gel set, and then pour the stacking gel, because the composition of the two gels in the acrylamide-bisacrylamide, the buffer, and the pH are different.

Resolving Gel—In a conical flask, prepare the appropriate volume of solution, containing the desired concentration of acrylamide for the resolving gel using the values given in Table 1. Mix the components in the order shown. Where appropriate, before adding the *Ammonium Persulfate Solution* and the *TEMED*, filter the solution if necessary under vacuum through a cellulose acetate membrane filter (pore diameter 0.45-μm), and keep the solution under vacuum swirling the filtration unit until no more bubbles are formed in the solution. Add appropriate amounts of *Ammonium Persulfate Solution* and *TEMED*, as indicated in Table 1; swirl and pour immediately into the gap between the two glass plates of the mold. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a tapered glass pipet, carefully overlay the solution with water-saturated isobutyl alcohol. Leave the gel in a vertical position at room temperature to allow polymerization.

After polymerization is complete (about 30 minutes later), pour off the isobutyl alcohol and wash the top of the gel several times with water to remove the isobutyl alcohol overlay and any unpolymerized acrylamide. Drain as much fluid as possible from the top of the gel, then remove any remaining water with the edge of a paper towel.

Stacking Gel—In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in Table 2. Mix the components in the order shown. Where appropriate, before adding the *Ammonium Persulfate Solution* and the *TEMED*, filter the solution if necessary under vacuum through a cellulose acetate membrane filter (pore diameter 0.45-μm), and keep the solution under vacuum swirling the filtration unit until no more bubbles are formed in the solution. Add appropriate amounts of *Ammonium Persulfate Solution* and *TEMED* as indicated in Table 2, swirl, and pour immediately into the gap between the two glass plates of the mold directly onto the surface of the polymerized *Resolving Gel*. Immediately insert a clean polytetrafluoroethylene comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Leave the gel in a vertical position, and allow it to polymerize at room temperature.

Electrophoretic Separation

Sample Buffer 1—Dissolve 1.89 g of Tris, 5.0 g of SDS, 50 mg of bromophenol blue, and 25.0 mL of glycerol in 100 mL of water. Adjust with hydrochloric acid to a pH of 6.8, and dilute with water to 125 mL. Before use, dilute with an equal volume of water or sample, and mix.

Sample Buffer 2 (for reducing conditions)—Prepare as directed in *Sample Buffer 1* except to add 12.5 mL of 2-mercaptoethanol before adjusting the pH. Alternatively, prepare as directed for *Sample Buffer 1* except to start with about 1.93 g of Tris and add a suitable quantity of DTT to obtain a final 100 mM DTT concentration.

Running Buffer—Dissolve 151.4 g of Tris, 721.0 g of aminoacetic acid (glycine), and 50.0 g of SDS in water; di-

lute with water to 5000 mL; and mix to obtain a stock solution. Immediately before use, dilute this stock solution with water to 10 times its volume, mix, and adjust to a pH between 8.1 and 8.8.

Procedure—After polymerization is complete (about 30 minutes later), carefully remove the polytetrafluoroethylene comb. Rinse the wells immediately with water or with the *Running Buffer* to remove any unpolymerized acrylamide.

Table 1. Preparation of Resolving Gel

Solution Component	Component Volume (mL) per Gel Mold Volume Below							
	5 mL	10 mL	15 mL	20 mL	25 mL	30 mL	40 mL	50 mL
6% Acrylamide								
Water	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
30% Acrylamide–Bisacrylamide Solution	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8% Acrylamide								
Water	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
30% Acrylamide–Bisacrylamide Solution	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10% Acrylamide								
Water	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
30% Acrylamide–Bisacrylamide Solution	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12% Acrylamide								
Water	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
30% Acrylamide–Bisacrylamide Solution	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
14% Acrylamide								
Water	1.4	2.7	3.9	5.3	6.6	8.0	10.6	13.8
30% Acrylamide–Bisacrylamide Solution	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
1.5 M Buffer Solution	1.2	2.5	3.6	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15% Acrylamide								
Water	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
30% Acrylamide–Bisacrylamide Solution	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Buffer Solution	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
SDS Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
Ammonium Persulfate Solution	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

Table 2. Preparation of Stacking Gel

Solution Component	Component Volume (mL) per Gel Mold Volume Below							
	1 mL	2 mL	3 mL	4 mL	5 mL	6 mL	8 mL	10 mL
Water	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% Acrylamide–Bisacrylamide Solution	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Buffer Solution	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
SDS Solution	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
Ammonium Persulfate Solution	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

If necessary, straighten the teeth of the *Stacking Gel* with a blunt hypodermic needle attached to a syringe. Remove the clamps on one short side, carefully pull out the tubing, and replace the clamps. Proceed similarly on the other short side. Remove the tubing from the bottom part of the gel.

Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never prerun the gel before loading the samples, because that will destroy the discontinuity of the buffer systems. Before loading the sample, carefully rinse the slot with *Running Buffer*.

Prepare the test and standard solutions in the recommended *Sample Buffer*, and treat as directed in the individual monograph. Apply the appropriate volume of each solution to the *Stacking Gel* wells.

Start the electrophoresis under the conditions recommended by the manufacturer of the equipment. Manufacturers of SDS-PAGE equipment may provide gels of different surface area and thickness. Electrophoresis running time and current/voltage may need to vary as described by the manufacturer of the apparatus in order to achieve optimum separation. Check that the dye front is moving into the *Resolving Gel*. When the dye is reaching the bottom of the gel, stop the electrophoresis. Remove the gel assembly from the apparatus, and separate the glass plates. Remove the spacers, cut off and discard the *Stacking Gel*, and immediately proceed with staining.

Detection of Proteins in Gels

Coomassie staining is the most common protein staining method, with a detection level on the order of 1 to 10 µg of protein per band. Silver staining is the most sensitive method for staining proteins in gels and a band containing 10 to 100 ng can be detected.

All of the steps in gel staining are done at room temperature with gentle shaking (e.g., on an orbital shaker platform). Gloves must be worn when staining the gels, because fingertips will stain.

REAGENTS

Coomassie Staining Solution—Prepare a solution of Coomassie brilliant blue R-250 having a concentration of 1.25 g per L in a mixture of water, methanol, and glacial acetic acid (5:4:1). Filter, and store at room temperature.

Destaining Solution—Prepare a mixture of water, methanol, and glacial acetic acid (5:4:1).

Fixing Solution 1—Prepare a mixture of water, methanol, and trichloroacetic acid (5:4:1).

Fixing Solution 2—Transfer 250 mL of methanol to a 500-mL volumetric flask, add 0.27 mL of formaldehyde, dilute with water to volume, and mix.

Silver Nitrate Reagent—To a mixture of 40 mL of 1 M sodium hydroxide and 3 mL of ammonium hydroxide, add,

dropwise and with stirring, 8 mL of a 200 g per L solution of silver nitrate; dilute with water to 200 mL, and mix.

Developing Solution—Transfer 2.5 mL of a citric acid solution (2 in 100) and 0.27 mL of formaldehyde to a 500.0-mL volumetric flask, dilute with water to volume, and mix.

Stopping Solution—Prepare a 10% (v/v) solution of acetic acid.

COOMASSIE STAINING

Immerse the gel in a large excess of *Coomassie Staining Solution*, and allow to stand for at least 1 hour. Remove the *Coomassie Staining Solution*. Destain the gel with a large excess of *Destaining Solution*. Change the *Destaining Solution* several times, until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller the amount of protein that can be detected by the method. Destaining can be speeded up by including a few grams of anion-exchange resin or a small sponge in the *Destaining Solution*. [NOTE—The acid-alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low molecular mass proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by allowing the gel to stand in *Fixing Solution 1* for 1 hour before it is immersed in the *Coomassie Staining Solution*.]

SILVER STAINING

Immerse the gel in a large excess of *Fixing Solution 2*, and allow to stand for 1 hour. Remove *Fixing Solution 2*, add fresh *Fixing Solution 2*, and incubate for at least 1 hour, or overnight if convenient. Discard *Fixing Solution 2*, and wash the gel in a large excess of water for 1 hour. Soak the gel for 15 minutes in a 1% solution of glutaraldehyde (v/v). Wash the gel twice, for 15 minutes each time in a large excess of water. Soak the gel in fresh *Silver Nitrate Reagent* for 15 minutes in darkness. Wash the gel three times, for 5 minutes each time, in a large excess of water. Immerse the gel for about 1 minute in *Developing Solution* until satisfactory staining has been obtained. Stop the development by incubation in the *Stopping Solution* for 15 minutes. Rinse the gel with water.

Drying of Gels

Depending on the method used, the gels are treated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a 100 g per L glycerol solution for at least 2 hours. For silver staining, add to the final rinsing step a 5-minute incubation in a 20 g per L glycerol solution.

Immerse two sheets of porous cellulose film in water, and incubate for 5 to 10 minutes. Place one of the sheets on a drying frame. Carefully lift the gel, and place it on the cellulose film. Remove any trapped air bubbles, and pour a few mL of water around the edges of the gel. Place the second sheet on top, and remove any trapped air bubbles. Com-

plete the assembly of the drying frame. Place in an oven, and leave at room temperature until dry.

Molecular Mass Determination

Molecular masses of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular mass. Mixtures of proteins with precisely known molecular masses blended for uniform staining are available for calibrating gels. They are available in various molecular mass ranges. Concentrated stock solutions of proteins of known molecular mass are diluted in an appropriate sample buffer and loaded on the same gel as the protein sample to be studied.

Immediately after the gel has been run, the position of the bromophenol blue tracking dye is marked to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the *Resolving Gel*. Divide the migration distance of each protein by the distance traveled by the tracking dye. The normalized migration distances so obtained are called the relative mobilities of the proteins (relative to the dye front) and conventionally denoted as R_F . Construct a plot of the logarithm of the molecular masses (M_R) of the protein standards as functions of the R_F values. Note that the graphs are slightly sigmoid. Unknown molecular masses can be estimated by linear regression analysis of interpolation from the curves of $\log M_R$ against R_F as long as the values obtained for the unknown samples are positioned along the linear part of the graph.

VALIDATION OF THE TEST

The test is not valid unless the proteins of the molecular mass marker are distributed along 80% of the length of the gel and over the required separation range (e.g., the range covering the product and its dimer or the products and its related impurities); the separation obtained for the relevant protein bands shows a linear relationship between the logarithm of the molecular mass and the R_F . Additional validation requirements with respect to the solution under test may be specified in individual monographs.

Quantification of Impurities

Where the impurity limit is specified in the individual monograph, a reference solution corresponding to that level of impurity should be prepared by diluting the test solution. For example, where the limit is 5.0%, a reference solution would be a 1:20 dilution of the test solution. No impurity—any band other than the main band—in the electropherogram obtained from the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions impurities may be quantified by normalization to the main band, using an integrating

densitometer. In this case, the responses must be validated for linearity.

(1057) BIOTECHNOLOGY-DERIVED ARTICLES—TOTAL PROTEIN ASSAY

This chapter provides guidance and procedures used for characterization of biotechnology-derived articles. This chapter is harmonized with the corresponding chapter in *JP* and *EP*. Other characterization tests, also harmonized, are provided in *Biotechnology-Derived Articles—Amino Acid Analysis* (1052), *Biotechnology-Derived Articles—Capillary Electrophoresis* (1053), *Biotechnology-Derived Articles—Isoelectric Focusing* (1054), *Biotechnology-Derived Articles—Peptide Mapping* (1055), and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* (1056).

INTRODUCTION

The following procedures are provided as illustrations of the determination of total protein content in pharmacopeial preparations. Other techniques, such as HPLC, are also acceptable if total protein recovery is demonstrated. Many of the total protein assay methods described below can be performed successfully using kits from commercial sources. [NOTE—Where water is required, use distilled water.]

Method 1

Protein in solution absorbs UV light at a wavelength of 280 nm due to the presence of aromatic amino acids, mainly tyrosine and tryptophan. This property is the basis of *Method 1*. Protein determination at 280 nm is mainly a function of the tyrosine and tryptophan content of the protein. If the buffer used to dissolve the protein has a high absorbance relative to that of water, there is an interfering substance in the buffer. This interference can be compensated for when the spectrophotometer is adjusted to zero buffer absorbance. The results may be compromised if the interference results in a large absorbance that challenges the limit of sensitivity of the spectrophotometer. Furthermore, at low concentrations protein can be adsorbed onto the cuvette, thereby reducing the content in solution. This can be prevented by preparing samples at higher concentrations or by using a nonionic detergent in the preparation. [NOTE—Keep the *Test Solution*, the *Standard Solution*, and the buffer at the same temperature during testing.]

Test Solution—Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration of 0.2 to 2 mg per mL.

Standard Solution—Unless otherwise specified in the individual monograph, prepare a solution of USP Reference Standard or reference material for the protein under test in the same buffer and at the same concentration as the *Test Solution*.

Procedure—Concomitantly determine the absorbances of the *Standard Solution* and the *Test Solution* in quartz cells at a wavelength of 280 nm with a suitable spectrophotometer (see *Spectrophotometry and Light-Scattering* (851)), using the buffer as the blank. To obtain accurate results, the response should be linear in the range of protein concentrations to be assayed.

Light-Scattering—The accuracy of the UV spectroscopic determination of protein can be decreased by the scattering of light by the test specimen. If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250 to 300 nm), scattering of the light beam results in an apparent increase in absorbance of the test specimen. To calculate the absorbance at 280 nm due to light-scattering, determine the absorbances of the *Test Solution* at wavelengths of 320, 325, 330, 335, 340, 345, and 350 nm. Using the linear regression method, plot the log of the observed absorbance versus the log of the wavelength, and determine the standard curve best fitting the plotted points. From the graph so obtained, extrapolate the absorbance value due to light-scattering at 280 nm. Subtract the absorbance due to light-scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a filter having a 0.2- μ m porosity or clarification by centrifugation may be performed to reduce the effect of light-scattering, especially if the solution is noticeably turbid.

Calculations—Calculate the concentration, C_u , of protein in the test specimen by the formula:

$$C_s(A_u/A_s)$$

in which C_s is the concentration of the *Standard Solution*; and A_u and A_s are the corrected absorbances of the *Test Solution* and the *Standard Solution*, respectively (see *Spectrophotometry and Light-Scattering* (851)).

Method 2

This method, commonly referred to as the Lowry assay, is based on the reduction by protein of the phosphomolybdic-tungstic mixed acid chromogen in the Folin-Ciocalteu's phenol reagent, resulting in an absorbance maximum at 750 nm. The Folin-Ciocalteu's phenol reagent reacts primarily with tyrosine residues in the protein, which can lead to variation in the response of the assay to different proteins. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test specimen may be used. Where separation of interfering substances from the protein in the test specimen is necessary, proceed as directed below for *Interfering Substances* prior to preparation of the *Test Solution*. The effect of interfering substances can be minimized by dilution, provided the concentration of the protein under test remains sufficient for accurate measurement.

Standard Solutions—Unless otherwise specified in the individual monograph, dissolve the USP Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test Solution*. Dilute portions of this solution with the same buffer to obtain not fewer than five *Standard Solutions* having concentrations between 5 and 100 μ g of protein per mL, the concentrations being evenly spaced.

Test Solution—Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard Solutions*. An appropriate buffer will produce a pH in the range of 10.0 to 10.5.

Blank—Use the buffer used for the *Test Solution* and the *Standard Solutions*.

Reagents and Solutions—

Copper Sulfate Reagent—Dissolve 100 mg of cupric sulfate and 200 mg of sodium tartrate in water, dilute with water to 50 mL, and mix. Dissolve 10 g of sodium carbonate in water to a final volume of 50 mL, and mix. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Prepare this solution fresh daily.

SDS Solution—Dissolve 5 g of sodium dodecyl sulfate in water, and dilute with water to 100 mL.

Sodium Hydroxide Solution—Dissolve 3.2 g of sodium hydroxide in water, dilute with water to 100 mL, and mix.

Alkaline Copper Reagent—Prepare a mixture of *Copper Sulfate Reagent*, *SDS Solution*, and *Sodium Hydroxide Solution* (1:2:1). This reagent may be stored at room temperature for up to 2 weeks.

Diluted Folin-Ciocalteu's Phenol Reagent—Mix 10 mL of Folin-Ciocalteu's phenol TS with 50 mL of water. Store in an amber bottle, at room temperature.

Procedure—To 1 mL of each *Standard Solution*, the *Test Solution*, and the *Blank*, add 1 mL of *Alkaline Copper Reagent*, and mix. Allow to stand at room temperature for 10 minutes. Add 0.5 mL of the *Diluted Folin-Ciocalteu's Phenol Reagent* to each solution, mix each tube immediately, and allow to stand at room temperature for 30 minutes. Determine the absorbances of the solutions from the *Standard Solutions* and the *Test Solution* at the wavelength of maximum absorbance at 750 nm with a suitable spectrophotometer (see *Spectrophotometry and Light-Scattering* (851)), using the solution from the *Blank* to set the instrument to zero.

Calculations—[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the *Standard Solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the *Test Solution*, determine the concentration of protein in the *Test Solution*.

INTERFERING SUBSTANCES

In the following procedure, deoxycholate-trichloroacetic acid is added to a test specimen to remove interfering substances by precipitation of proteins before testing. This technique also can be used to concentrate proteins from a dilute solution.

Sodium Deoxycholate Reagent—Prepare a solution of sodium deoxycholate in water having a concentration of 150 mg in 100 mL.

Trichloroacetic Acid Reagent—Prepare a solution of trichloroacetic acid in water having a concentration of 72 g in 100 mL.

Procedure—Add 0.1 mL of *Sodium Deoxycholate Reagent* to 1 mL of a solution of the protein under test. Mix on a vortex mixer, and allow to stand at room temperature for 10 minutes. Add 0.1 mL of *Trichloroacetic Acid Reagent*, and mix on a vortex mixer. Centrifuge at $3000 \times g$ for 30 minutes, decant the liquid, and remove any residual liquid with a pipet. Redissolve the protein pellet in 1 mL of *Alkaline Copper Reagent*. Proceed as directed for the *Test Solution*.

NOTE—Color development reaches a maximum in 20 to 30 minutes during incubation at room temperature, after which there is a gradual loss of color. Most interfering substances cause a lower color yield; however, some detergents cause a slight increase in color. A high salt concentration may cause a precipitate to form. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.

Method 3

This method, commonly referred to as the Bradford assay, is based on the absorption shift from 470 nm to 595 nm observed when the brilliant blue G dye binds to protein. The brilliant blue G dye binds most readily to arginyl and lysyl residues in the protein, which can lead to variation in the response of the assay to different proteins.

Standard Solutions—Unless otherwise specified in the individual monograph, dissolve the USP Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test Solution*. Dilute portions of this so-

lution with the same buffer to obtain not fewer than five *Standard Solutions* having concentrations between 100 µg and 1 mg of protein per mL, the concentrations being evenly spaced.

Test Solution—Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard Solutions*.

Blank—Use the buffer used to prepare the *Test Solution* and the *Standard Solutions*.

Coomassie Reagent—Dissolve 100 mg of brilliant blue G* in 50 mL of alcohol. [NOTE—Not all dyes have the same brilliant blue G content, and different products may give different results.] Add 100 mL of phosphoric acid, dilute with water to 1 L, and mix. Pass the solution through filter paper (Whatman No. 1 or equivalent), and store the filtered reagent in an amber bottle at room temperature. [NOTE—Slow precipitation of the dye will occur during storage of the reagent. Filter the reagent before use.]

Procedure—Add 5 mL of the *Coomassie Reagent* to 100 µL of each *Standard Solution*, the *Test Solution*, and the *Blank*, and mix by inversion. Avoid foaming, which will lead to poor reproducibility. Determine the absorbances of the solutions from the *Standard Solutions* and the *Test Solution* at 595 nm with a suitable spectrophotometer (see *Spectrophotometry and Light-Scattering* (851)), using the *Blank* to set the instrument to zero. [NOTE—Do not use quartz (silica) spectrophotometer cells: the dye binds to this material. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.]

There are relatively few interfering substances, but detergents and ampholytes in the test specimen should be avoided. Highly alkaline specimens may interfere with the acidic reagent.

Calculations—[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the *Standard Solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the *Test Solution*, determine the concentration of protein in the *Test Solution*.

Method 4

This method, commonly referred to as the bicinchoninic acid or BCA assay, is based on reduction of the cupric (Cu^{2+}) ion to cuprous (Cu^{1+}) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. The method has few interfering substances. When interfering substances are present, their effect may be minimized by dilution, provided that the concentration of the protein under test remains sufficient for accurate measurement.

Standard Solutions—Unless otherwise specified in the individual monograph, dissolve the USP Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test Solution*. Dilute portions of this solution with the same buffer to obtain not fewer than five *Standard Solutions* having concentrations between 10 and 1200 µg of protein per mL, the concentrations being evenly spaced.

Test Solution—Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard Solutions*.

Blank—Use the buffer used to prepare the *Test Solution* and the *Standard Solutions*.

*Dye purity is important in the reagent preparation. Serva Blue G (Crescent Chemical Company, Hauppauge, NY) is an acceptable grade.

Reagents—

BCA Reagent—Dissolve about 10 g of bicinchoninic acid, 20 g of sodium carbonate monohydrate, 1.6 g of sodium tartrate, 4 g of sodium hydroxide, and 9.5 g of sodium bicarbonate in water. Adjust, if necessary, with sodium hydroxide or sodium bicarbonate to a pH of 11.25. Dilute with water to 1 L, and mix.

Copper Sulfate Reagent—Dissolve about 2 g of cupric sulfate in water to a final volume of 50 mL.

Copper-BCA Reagent—Mix 1 mL of *Copper Sulfate Reagent* and 50 mL of *BCA Reagent*.

Procedure—Mix 0.1 mL of each *Standard Solution*, the *Test Solution*, and the *Blank* with 2 mL of the *Copper-BCA Reagent*. Incubate the solutions at 37° for 30 minutes, note the time, and allow to come to room temperature. Within 60 minutes following the incubation time, determine the absorbances of the solutions from the *Standard Solutions* and the *Test Solution* in quartz cells at 562 nm with a suitable spectrophotometer (see *Spectrophotometry and Light-Scattering* (851)), using the *Blank* to set the instrument to zero. The color intensity continues to increase gradually after the solutions are cooled to room temperature. If substances that will cause interference in the test are present, proceed as directed for *Interfering Substances* under *Method 2*. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.

Calculations—[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the *Standard Solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the absorbance of the *Test Solution*, determine the concentration of protein in the *Test Solution*.

Method 5

This method, commonly referred to as the Biuret assay, is based on the interaction of cupric (Cu^{2+}) ion with protein in an alkaline solution and the resultant development of absorbance at 545 nm.

Standard Solutions—Unless otherwise specified in the individual monograph, prepare a solution of Albumin Human for which the protein content has been previously determined by nitrogen analysis (using the nitrogen-to-protein conversion factor of 6.25) or of the USP Reference Standard or reference material for the protein under test in sodium chloride solution (9 in 1000). Dilute portions of this solution with sodium chloride solution (9 in 1000) to obtain not fewer than three *Standard Solutions* having concentrations between 0.5 and 10 mg per mL, the concentrations being evenly spaced. [NOTE—Low responses may be observed if the sample under test has a significantly different level of proline than that of Albumin Human. A different standard protein may be employed in such cases.]

Test Solution—Prepare a solution of the test protein in sodium chloride solution (9 in 1000) having a concentration within the range of the concentrations of the *Standard Solutions*.

Blank—Use sodium chloride solution (9 in 1000).

Biuret Reagent—Dissolve about 3.46 g of cupric sulfate in 10 mL of hot water, and allow to cool (*Solution 1*). Dissolve about 34.6 g of sodium citrate dihydrate and 20.0 g of sodium carbonate in 80 mL of hot water, and allow to cool (*Solution 2*). Mix *Solution 1* and *Solution 2*, and dilute with water to 200 mL. This *Biuret Reagent* is stable at room temperature for 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

Procedure—To one volume of a solution of the *Test Solution* add an equal volume of sodium hydroxide solution (6

in 100), and mix. Immediately add a volume of *Biuret Reagent* equivalent to 0.4 volume of the *Test Solution*, and mix. Allow to stand at a temperature between 15° and 25° for not less than 15 minutes. Within 90 minutes after the addition of the *Biuret Reagent*, determine the absorbances of the *Standard Solutions* and the solution from the *Test Solution* at the wavelength of maximum absorbance at 545 nm with a suitable spectrophotometer (see *Spectrophotometry and Light-Scattering* <851>), using the *Blank* to set the instrument to zero. [NOTE—Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.]

Calculations—Using the least-squares linear regression method, plot the absorbances of the *Standard Solutions* versus the protein concentrations, determine the standard curve best fitting the plotted points, and calculate the correlation coefficient for the line. [NOTE—Within the given range of the standards, the relationship of absorbance to protein concentration is approximately linear.] A suitable system is one that yields a line having a correlation coefficient of not less than 0.99. From the standard curve and the absorbance of the *Test Solution*, determine the concentration of protein in the test specimen, making any necessary correction.

Interfering Substances—To minimize the effect of interfering substances, the protein can be precipitated from the initial test specimen as follows. Add 0.1 volume of 50% trichloroacetic acid to 1 volume of a solution of the test specimen, withdraw the supernatant layer, and dissolve the precipitate in a small volume of 0.5 N sodium hydroxide. Use the solution so obtained to prepare the *Test Solution*.

Comments—This test shows minimal difference between equivalent IgG and albumin samples. Addition of the sodium hydroxide and the *Biuret Reagent* as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the *Biuret Reagent* will give IgG samples a higher response than albumin samples. The trichloroacetic acid method used to minimize the effects of interfering substances can also be used to determine the protein content in test specimens at concentrations below 500 µg per mL.

Method 6

This fluorometric method is based on the derivatization of the protein with o-phthalaldehyde (OPA), which reacts with the primary amines of the protein (i.e., NH₂-terminal amino acid and the ε-amino group of the lysine residues). The sensitivity of the test can be increased by hydrolyzing the protein before testing. Hydrolysis makes the α-amino group of the constituent amino acids of the protein available for reaction with the o-phthalaldehyde reagent. The method requires very small quantities of the protein.

Primary amines, such as tris(hydroxymethyl)amino-methane and amino acid buffers, react with o-phthalaldehyde and must be avoided or removed. Ammonia at high concentrations will react with o-phthalaldehyde as well. The fluorescence obtained when amine reacts with o-phthalaldehyde can be unstable. The use of automated procedures to standardize this procedure may improve the accuracy and precision of the test.

Standard Solutions—Unless otherwise specified in the individual monograph, dissolve the USP Reference Standard or reference material for the protein under test in the buffer used to prepare the *Test Solution*. Dilute portions of this solution with the same buffer to obtain not fewer than five *Standard Solutions* having concentrations between 10 and 200 µg of protein per mL, the concentrations being evenly spaced.

Test Solution—Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the *Standard Solutions*.

Blank—Use the buffer used to prepare the *Test Solution* and the *Standard Solutions*.

Reagents—

Borate Buffer—Dissolve about 61.83 g of boric acid in water, and adjust with potassium hydroxide to a pH of 10.4. Dilute with water to 1 L, and mix.

Stock OPA Reagent—Dissolve about 120 mg of o-phthalaldehyde in 1.5 mL of methanol, add 100 mL of *Borate Buffer*, and mix. Add 0.6 mL of polyoxyethylene (23) lauryl ether, and mix. This solution is stable at room temperature for at least 3 weeks.

OPA Reagent—To 5 mL of *Stock OPA Reagent* add 15 µL of 2-mercaptoethanol. Prepare at least 30 minutes prior to use. This reagent is stable for one day.

Procedure—Adjust each of the *Standard Solutions* and the *Test Solution* to a pH between 8 and 10.5. Mix 10 µL of the *Test Solution* and each of the *Standard Solutions* with 100 µL of *OPA Reagent*, and allow to stand at room temperature for 15 minutes. Add 3 mL of 0.5 N sodium hydroxide, and mix. Using a suitable fluorometer (see *Spectrophotometry and Light-Scattering* <851>), determine the fluorescent intensities of solutions from the *Standard Solutions* and the *Test Solution* at an excitation wavelength of 340 nm and an emission wavelength between 440 and 455 nm. [NOTE—The fluorescence of an individual specimen is read only once because irradiation decreases the fluorescent intensity.]

Calculations—The relationship of fluorescence to protein concentration is linear. Using the linear regression method, plot the fluorescent intensities of the solutions from the *Standard Solutions* versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the fluorescent intensity of the *Test Solution*, determine the concentration of protein in the test specimen.

Method 7

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test specimen can affect the determination of protein by this method. Nitrogen analysis techniques destroy the protein under test but are not limited to protein presentation in an aqueous environment.

Procedure 1—Determine the nitrogen content of the protein under test as directed under *Nitrogen Determination* <461>. Commercial instrumentation is available for the Kjeldahl nitrogen assay.

Procedure 2—Commercial instrumentation is available for nitrogen analysis. Most nitrogen analysis instruments use pyrolysis (i.e., combustion of the sample in oxygen at temperatures approaching 1000°), which produces nitric oxide (NO) and similar oxides of nitrogen (NO_x) from the nitrogen present in the test protein. Some instruments convert the nitric oxides to nitrogen gas, which is quantified with a thermal conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O₃) to produce excited nitrogen dioxide (NO₂), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference material or reference standard that is relatively pure and is similar in composition to the test proteins is used to optimize the injection and pyrolysis parameters and to evaluate consistency in the analysis.

Calculations—The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with the nitro-

gen content of the USP Reference Standard or reference material.

(1058) ANALYTICAL INSTRUMENT QUALIFICATION

INTRODUCTION

A large variety of laboratory equipment, instruments, and computerized analytical systems, ranging from simple nitrogen evaporators to complex multiple-function technologies (see *Instrument Categories*), are used in the pharmaceutical industry to acquire data to help ensure that products are suitable for their intended use. An analyst's objective is to consistently obtain reliable and valid data suitable for the intended purpose. Depending on the applications, users validate their procedures, calibrate their instruments, and perform additional instrument checks, such as system suitability tests and analysis of in-process quality control check samples to help ensure that the acquired data are reliable. With the increasing sophistication and automation of analytical instruments, an increasing demand has been placed on users to qualify their instruments.

Unlike method validation and system suitability activities, analytical instrument qualification (AIQ) currently has no specific guidance or procedures. Competing opinions exist regarding instrument qualification and validation procedures and the roles and responsibilities of those who perform them. Consequently, various approaches have been used for instrument qualification, approaches that require varying amounts of resources and generate widely differing amounts of documentation. This chapter provides a scientific approach to AIQ and considers AIQ as one of the major components required for generating reliable and consistent data. Note that the amount of rigor applied to the qualification process will depend on the complexity and intended use of the instrumentation. This approach emphasizes AIQ's place in the overall process of obtaining reliable data from analytical instruments.

Validation versus Qualification

In this chapter, the term validation is used for manufacturing processes, analytical procedures, and software procedures and the term qualification is used for instruments. Thus, the phrase "analytical instrument qualification" (AIQ) is used for the process of ensuring that an instrument is suitable for its intended application.

COMPONENTS OF DATA QUALITY

There are four critical components involved in the generation of reliable and consistent data (quality data). *Figure 1* shows these components as layered activities within a quality triangle. Each layer adds to the overall quality. Analytical instrument qualification forms the base for generating quality data. The other components essential for generating quality data are analytical method validation, system suitability tests, and quality control check samples. These quality components are described below.

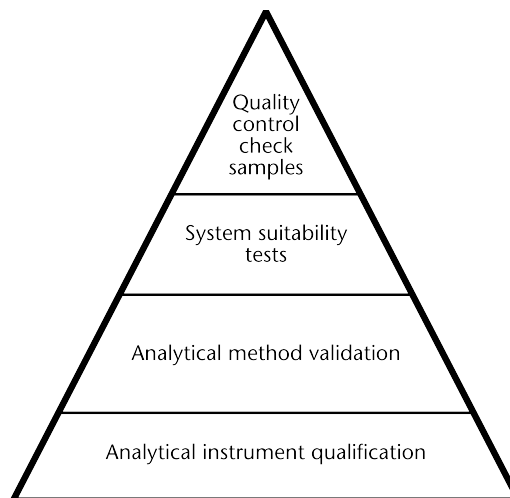


Figure 1. Components of data quality.

Analytical Instrument Qualification

AIQ is the collection of documented evidence that an instrument performs suitably for its intended purpose. Use of a qualified instrument in analyses contributes to confidence in the validity of generated data.

Analytical Method Validation

Analytical method validation is the collection of documented evidence that an analytical procedure is suitable for its intended use. Use of a validated procedure with qualified analytical instruments provides confidence that the procedure will generate test data of acceptable quality. Additional guidance on validation of compendial procedures may be found in the general information chapter *Validation of Compendial Procedures* (1225).

System Suitability Tests

System suitability tests verify that the system will perform in accordance with the criteria set forth in the procedure. These tests are performed along with the sample analyses to ensure that the system's performance is acceptable at the time of the test. USP general chapter *Chromatography* (621) presents a more detailed discussion of system suitability tests as related to chromatographic systems.

Quality Control Check Samples

Many analysts carry out their tests on instruments standardized using reference materials and/or calibration standards. Some analyses also require the inclusion of quality control check samples to provide an in-process or ongoing assurance of the test's suitable performance. In this manner, AIQ and analytical method validation contribute to the quality of analysis *before* analysts conduct the tests. System suitability tests and quality control checks help ensure the quality of analytical results *immediately before* or *during* sample analysis.

ANALYTICAL INSTRUMENT QUALIFICATION PROCESS

The following sections address in detail the AIQ process. The other three components of building quality into analytical data—analytical method validation, system suitability

tests, and quality control check samples—are not within the scope of this chapter.

Qualification Phases

Instrument qualification is not a single continuous process, but instead results from several discrete activities. For convenience, these activities can be grouped into four phases: design qualification (DQ), installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ).

Some AIQ activities cover more than one qualification phase, and analysts potentially could perform them during more than one of the phases (see *Table 1*). However, in many instances there is need for specific order to the AIQ activities; for example, installation qualification must occur first in order to initiate other qualification activities. The AIQ activities will be defined and documented.

DESIGN QUALIFICATION

Design qualification (DQ) is the documented collection of activities that define the functional and operational specifications of the instrument and criteria for selection of the vendor, based on the intended purpose of the instrument. Design qualification (DQ) may be performed not only by the instrument developer or manufacturer but also may be performed by the user. The manufacturer is generally responsible for robust design and maintaining information describing how the analytical instrument is manufactured (design specifications, functional requirements, etc.) and tested before shipment to users. Nonetheless, the user should ensure that commercial off-the-shelf (COTS) instruments are suitable for their intended application and that the manufacturer has adopted a quality system that provides for reliable equipment. Users should also determine the manufacturer's capability for support installation, services, and training. This determination might be aided by the user's previous interaction with the manufacturer.

INSTALLATION QUALIFICATION

Installation qualification (IQ) is the documented collection of activities necessary to establish that an instrument is de-

livered as designed and specified, and is properly installed in the selected environment, and that this environment is suitable for the instrument. IQ applies to an instrument that is new or was pre-owned, or to any instrument that exists on site but has not been previously qualified. Relevant parts of IQ would also apply to a qualified instrument that has been transported to another location or is being reinstalled for other reasons, such as prolonged storage. The activities and documentation typically associated with IQ are as follows.

Description—Provide a description of the instrument or the collection of instrument components, including its manufacturer, model, serial number, software version, and location. Use drawings and flow charts where appropriate.

Instrument Delivery—Ensure that the instrument, software, manuals, supplies, and any other instrument accessories arrive as specified in the purchase order and that they are undamaged. For a pre-owned or existing instrument, manuals and documentation should be obtained.

Utilities/Facility/Environment—Verify that the installation site satisfactorily meets manufacturer-specified environmental requirements.

Assembly and Installation—Assemble and install the instrument, and perform any preliminary diagnostics and testing. Assembly and installation may be done by the manufacturer, vendor, specialized engineers, or qualified in-house personnel. Manufacturer-established installation tests and guides provide a valuable baseline reference for determining instrument acceptance. Any abnormal event observed during assembly and installation merits documenting. Installation packages purchased from the manufacturer or the vendor may, however, need to be supplemented with user-specific criteria.

Network and Data Storage—Some analytical systems require users to provide network connections and data storage capabilities at the installation site. When required, connect the instrument to the network, and check its functionality.

Installation Verification—Perform the initial diagnostics and testing of the instrument after installation.

OPERATIONAL QUALIFICATION

After a successful IQ, the instrument is ready for OQ testing. Operational qualification (OQ) is the documented col-

Table 1. Timing, Applicability, and Activities for Each Phase of Analytical Instrument Qualification*

Design Qualification	Installation Qualification		Operational Qualification		Performance Qualification
Timing and Applicability					
Prior to purchase of a new model of instrument	At installation of each instrument (new, old, or existing unqualified)		After installation or major repair of each instrument		Periodically at specified intervals for each instrument
Activities					
Assurance of manufacturer's DQ	Description	↔	Fixed parameters		Preventive maintenance and repairs
Assurance of adequate support availability from manufacturer	Instrument delivery				Establish practices to address operation, calibration, maintenance, and change control
Instrument's fitness for use in laboratory	Utilities/facility	↔	Environment		
	Assembly and installation				
	Network and data storage	↔	Secure data storage, backup, and archive		
	Installation verification	↔	Instrument function tests	↔	Performance checks

* Activities under each phase are usually performed as given in the table. However, in some cases, it may be more appropriate to perform or combine a given activity with another phase. Such activities spanning more than one qualification phase are shown as connected by double arrows. If an activity listed under a given phase is performed under another phase, it is not necessary to repeat the activity under the phase where the activity is listed. Performing the activity is far more important than the phase under which the activity is performed.

lection of activities necessary to demonstrate that an instrument will function according to its operational specification in the selected environment. Testing activities in the OQ phase may consist of these test parameters.

Fixed Parameters—These tests measure the instrument's nonchanging parameters such as length, height, weight, voltage inputs, acceptable pressures, and loads. If the manufacturer-supplied specifications for these parameters satisfy the user, the test requirements may be waived. However, if the user wants to confirm the parameters, testing can be performed at the user's site. Fixed parameters do not change over the life of the instrument, and therefore never need redetermination. [NOTE—These tests could also be performed during the IQ phase (see *Table 1*); if so, fixed parameters need not be redetermined as part of OQ testing.]

Secure Data Storage, Backup, and Archiving—When applicable, test secure data handling such as storage, backup, audit trails, and archiving at the user's site according to written procedures.

Instrument Function Tests—Instrument functions required by the user should be tested to verify that the instrument operates as intended by the manufacturer. Manufacturer-supplied information is useful in identifying specifications for these parameters and in designing tests to evaluate the identified parameters. Users, or their qualified designees, should perform these tests to verify that the instrument meets manufacturer or user specifications in the user's environment.

The extent of OQ testing that an instrument undergoes depends on its intended applications. Therefore, no specific OQ tests for any instrument or application are offered in this chapter.

Routine analytical tests do not constitute OQ testing. OQ tests are specifically designed to verify the instrument's operation according to specifications in the user's environment, and repeating the testing at regular intervals may not be required. However, when the instrument undergoes major repairs or modifications, relevant OQ and/or PQ tests should be repeated to verify whether the instrument continues to operate satisfactorily. If an instrument is moved to another location, an assessment should be made of what, if any, OQ test should be repeated.

OQ tests can be modular or holistic. Modular testing of individual components of a system may facilitate interchanging of such components without requalification. Holistic tests, which involve the entire system, are also acceptable.

PERFORMANCE QUALIFICATION

Performance qualification (PQ) is the documented collection of activities necessary to demonstrate that an instrument consistently performs according to the specifications defined by the user, and is appropriate for the intended use. After IQ and OQ have been performed, the instrument's continued suitability for its intended use is demonstrated through performance qualification. The PQ phase may include the following parameters.

Performance Checks—Set up a test or series of tests to verify the acceptable performance of the instrument for its intended use. PQ tests are usually based on the instrument's typical on-site applications and may consist of analyzing known components or standards. The tests should be based on good science and reflect the general intended use of the instrument. Some system suitability tests or quality control checks that are performed concurrently with the test samples can be used to demonstrate that the instrument is performing suitably. PQ tests may resemble those performed during OQ, but the specifications for their results may be set differently if required. Nevertheless, user specifications for PQ tests should demonstrate trouble-free instrument operation for the intended applications. As is the case with OQ testing, PQ tests may be modular or holistic.

Testing frequency depends on the ruggedness of the instrument and the criticality of the tests performed. Testing may be unscheduled—for example, each time the instrument is used. It may also be scheduled for regular intervals. Experience with the instrument can influence this decision. It may be useful to repeat the same PQ tests each time the instrument is used so that a history of the instrument's performance can be compiled. Alternatively, the instrument may be incorporated into an integrated support system to assure that it remains continually qualified. Some system suitability tests or quality control checks that are performed concurrently with the test samples also imply that the instrument is performing suitably.

Preventive Maintenance and Repairs—When an instrument fails to meet PQ test specifications, it requires maintenance or repair. A periodic preventive maintenance may also be recommended for many instruments. The relevant PQ test(s) should be repeated after the needed maintenance or repair to ensure that the instrument remains qualified.

Practices for Operation, Calibration, Maintenance, and Change Control—Establish practices to maintain and calibrate the instrument. Each maintenance and calibration activity should be documented.

ROLES AND RESPONSIBILITIES

Users

Users are ultimately responsible for instrument operations and data quality. The user's group encompasses analysts, their supervisors, instrument specialists, and organization management. Users should be adequately trained in the instrument's use, and their training records should be maintained as required by the regulations.

Users should also be responsible for qualifying their instruments because their training and expertise in the use of instruments make them the best-qualified group to design the instrument test(s) and specification(s) necessary for successful AIQ. Consultants, equipment manufacturer or vendors, validation specialists, and quality assurance (QA) personnel can advise and assist as needed, but the final responsibility for qualifying instruments lies with the users. The users must also maintain the instrument in a qualified state by routinely performing PQ.

Quality Unit

The role of the Quality Unit in AIQ remains the same as for any other regulated activity. Quality personnel are responsible for assuring that the AIQ process meets compliance requirements, that processes are being followed, and that the intended use of the equipment is supported by valid and documented data.

Manufacturers

Manufacturers and developers are responsible for DQ when designing the instrument. They are also responsible for validation of relevant processes used in manufacturing and assembly of the instrument. Manufacturers should test the assembled instruments before shipping them to users.

Finally, it is desirable that manufacturers and vendors should notify all known users about hardware defects discovered after a product's release; offer user training, service, repair, and installation support; and invite user audits as necessary.

SOFTWARE VALIDATION

Software used for analytical work can be classified into three categories: firmware; instrument control, data acquisition, and processing software; and stand-alone software. Although software validation is not the primary focus of this chapter, the following sections describe in which cases this activity is under the scope of the analytical instrument qualification.

Firmware

Computerized analytical instruments contain integrated chips with low-level software (firmware). Such instruments will not function without properly operating firmware, and users generally cannot alter firmware design or function. Firmware is therefore considered a component of the instrument itself. Indeed, the qualification of hardware is not possible without operating it via its firmware. Thus, when the hardware (that is, the analytical instrument) is qualified at the user's site, the integrated firmware is also essentially qualified. No separate on-site qualification of the firmware is needed. Whenever possible, the firmware version should be recorded as part of the IQ activities. Any changes made to firmware versions should be tracked through change control of the instrument (see *Change Control*, below).

Instrument Control, Data Acquisition, and Processing Software

Software for instrument control, data acquisition, and processing for many of today's computerized instruments is loaded on a computer connected to the instrument. Operation of the instrument is then controlled via the software, leaving fewer operating controls on the instrument. Also, the software is needed for data acquisition and postacquisition calculations. Thus, both hardware and software, their functions inextricably intertwined, are critical to providing analytical results.

The manufacturer should perform DQ, validate this software, and provide users with a summary of validation. At the user site, holistic qualification, which involves the entire instrument and software system, is more efficient than modular validation of the software alone. Thus, the user qualifies the instrument control, data acquisition, and processing software by qualifying the instrument according to the AIQ process.

Stand-Alone Software

An authoritative guide for validating stand-alone software, such as LIMS, is available.¹ The validation process is administered by the software developer, who also specifies the development model appropriate for the software. Validation takes place in a series of activities planned and executed through various stages of the development cycle.

CHANGE CONTROL

Changes to instruments, including software, become inevitable as manufacturers add new features and correct known defects. However, implementing all such changes may not always benefit users. Users should therefore adopt changes they deem useful or necessary and should also assess the effects of changes to determine what, if any, requalification is required. The change control process enables them to do this.

¹General Principles of Software Validation: Final Guidance for Industry and FDA Staff, U.S. Department of Health and Human Services, Food and Drug Administration, Rockville, MD, January 11, 2002. <http://www.fda.gov/cdrh/comp/guidance/938.html> (accessed September 2004).

Change control may follow the DQ/IQ/OQ/PQ classification process. For DQ, evaluate the changed parameters, and determine whether need for the change warrants implementing it. If implementation of the change is needed, install the changes to the system during IQ. Evaluate which of the existing OQ and PQ tests need revision, deletion, or addition as a result of the installed change. Where the change calls for additions, deletions, or revisions to the OQ or PQ tests, follow the procedure outlined below.

Operational Qualification—Revise OQ tests as necessitated by the change. Perform the relevant tests affected by the change. This ensures the instrument's effective operation after the change is installed.

Performance Qualification—Revise PQ tests as necessitated by the change. Perform the PQ testing after installation of the change if similar testing is not already performed during OQ. In the future, perform the revised PQ testing.

For changes to firmware and to software for instrument control, data acquisition, and processing, change control is performed through DQ/IQ/OQ/PQ of the affected instrument. Change control for stand-alone software requires user-site testing of changed functionality.

AIQ DOCUMENTATION

Documents obtained during instrument qualification should be retained in an accessible manner. Where multiple instruments of one kind exist, documents common to all instruments and documents specific to an instrument may be stored separately. During change control, additional documents may supplement those obtained during the qualification process, and both sets of documents should be retained and maintained in a suitable manner that allows for appropriate protection and access.

INSTRUMENT CATEGORIES

Modern laboratories typically include a suite of instruments and equipment varying from simple nitrogen evaporators to complex automated instruments. Therefore, applying a single set of principles to qualifying such dissimilar instruments would be scientifically inappropriate. Users are most capable of establishing the level of qualification needed for an instrument. On the basis of the level needed, it is convenient to categorize instruments into three groups: A, B, and C, as defined below. Examples of instruments in each group are provided. Note that the list of instruments provided here is for illustration only and is not meant to be exhaustive. It does not provide the exact category for an instrument at a user site. That category should be determined by users for their specific instruments or applications.

The exact grouping of an instrument must be determined by users for their specific requirements. Depending on individual user requirements, the same instrument may appropriately fall into one group for one user and another group for another user. Therefore, a careful selection of groups by users is highly encouraged.

Group A

Group A includes standard equipment with no measurement capability or usual requirement for calibration, where the manufacturer's specification of basic functionality is accepted as user requirements. Conformance of Group A equipment with user requirements may be verified and documented through visual observation of its operation. Examples of equipment in this group are nitrogen evaporators, magnetic stirrers, vortex mixers, and centrifuges.

Group B

Group B includes standard equipment and instruments providing measured values as well as equipment controlling physical parameters (such as temperature, pressure, or flow) that need calibration, where the user requirements are typically the same as the manufacturer's specification of functionality and operational limits. Conformance of Group B instruments or equipment to user requirements is determined according to the standard operating procedures for the instrument or equipment, and documented during IQ and OQ. Examples of instruments in this group are balances, melting point apparatus, light microscopes, pH meters, variable pipets, refractometers, thermometers, titrators, and viscometers. Examples of equipment in this group are muffle furnaces, ovens, refrigerator-freezers, water baths, pumps, and dilutors.

Group C

Group C includes instruments and computerized analytical systems, where user requirements for functionality, operational, and performance limits are specific for the analytical application. Conformance of Group C instruments to user requirements is determined by specific function tests and performance tests. Installing these instruments can be a complicated undertaking and may require the assistance of specialists. A full qualification process, as outlined in this document, should apply to these instruments. Examples of instruments in this group include the following:

- atomic absorption spectrometers
- differential scanning calorimeters
- dissolution apparatus
- electron microscopes
- flame absorption spectrometers
- high-pressure liquid chromatographs
- mass spectrometers
- microplate readers
- thermal gravimetric analyzers
- X-ray fluorescence spectrometers
- X-ray powder diffractometers
- densitometers
- diode-array detectors
- elemental analyzers
- gas chromatographs
- IR spectrometers
- near-IR spectrometers
- Raman spectrometers
- UV/Vis spectrometers
- inductively coupled plasma-emission spectrometers

properties of excipients that may not be provided in *National Formulary (NF)* monographs.

An excipient may have different functional purposes and may possess various required characteristics (e.g., particle size, particle size distribution, or surface area), depending on its use in a formulation or manufacturing process. A listing of excipients grouped by functional category is included in the *NF* and summarizes the most typically identified purposes these excipients serve in drug products. The list of excipients included in each category is not comprehensive and is not intended to limit in any way the choice or use of the excipient. For the complete list, refer to the *USP* and *NF* Excipients, Listed by Category in the *National Formulary*, under *Contents*.

Excipient functional category (sometimes referred to as functionality) is a broad, qualitative, and descriptive term for the purpose or role an excipient serves in a formulation. Of greater importance, however, are the quantitative performance requirements (e.g., critical material attributes) of excipients that must be evaluated and controlled to ensure consistent performance throughout the product life cycle. Not all critical material attributes of an excipient may be identified or evaluated by tests, procedures, and acceptance criteria in *NF* monographs. Excipient suppliers and users therefore at times may wish to identify and control critical excipient attributes that go beyond monograph specifications. This requires a thorough understanding of the formulation, the manufacturing processes, and the physical and chemical properties of each ingredient. Manufacturers should anticipate lot-to-lot and supplier-to-supplier variability in excipient properties and should have in place appropriate controls if needed to ensure consistent excipient performance.

This general chapter provides an overview of the key functional categories of excipients, tests that may assess excipient performance, and test procedures that may not be presented in compendial monographs. The functional categories have been organized by their most typical use in common pharmaceutical dosage forms (Tablets and Capsules; Oral Liquids; Semisolids, Topicals and Suppositories; Parenterals; and Aerosols) to provide a greater level of specificity for each functional category. Several functional categories (e.g., antioxidant) can apply to multiple dosage form types. The association of a functional category with a particular dosage form in this chapter is not absolute and does not limit use of an excipient to a single type of dosage form. Because of the complex nature and interplay of formulation ingredients, processing, and dosage form performance requirements, the information provided in this chapter should not be viewed as either restrictive or completely comprehensive. Each functional category includes a general description; the mechanisms by which the excipients achieve their activity; physical properties common to these excipients; chemical properties; and a list of pharmacopeial general chapters that may be useful in the development of specific tests, procedures, and acceptance criteria, and that help to ensure that the critical material attributes are adequately monitored and controlled.

(1059) EXCIPIENT PERFORMANCE

INTRODUCTION

Excipients are used in virtually all drug products and are essential to product performance. Thus, the successful manufacture of a robust product requires the use of well-defined excipients and processes that together yield a consistent product. Typically, excipients are manufactured and supplied to comply with compendial standards. The development, manufacture, and performance of pharmaceutical dosage forms often depend upon the physical and chemical

TABLETS AND CAPSULES

Functional Category: Diluent

Description: Diluents are components that are incorporated into tablet or capsule dosage forms to increase dosage form volume or weight. Sometimes referred to as fillers, diluents often comprise a significant proportion of the dosage form, and the quantity and type of diluent selected often depend on its physical and chemical properties. Because the diluent may comprise a large portion of the dosage form, successful and robust manufacturing and dosage form per-

formance depend on the measurement and control of the critical material attributes.

Functional Mechanism: Among the most important functional roles diluents play is their ability to impart desirable manufacturing properties (e.g., powder flow, tablet compaction strength, wet or dry granule formation, homogeneity) and performance (e.g., content uniformity, disintegration, dissolution, tablet integrity, friability, physical and chemical stability). Some diluents (e.g., microcrystalline cellulose) are occasionally referred to as dry binders because of the high degree of tablet strength they impart to the final compressed tablet.

Physical Properties: The primary physical properties relevant to tablet/capsule diluents are those that can have a direct effect on diluent and formulation performance. These include: (1) particle size and size distribution, (2) particle shape, (3) bulk/tapped/true density, (4) specific surface area, (5) crystallinity, (6) moisture content, (7) powder flow, (8) solubility, and (9) compaction properties for tablet dosage forms.

Chemical Properties: Tablet diluents comprise a large and diverse group of materials that include inorganics (e.g., dibasic calcium phosphate, calcium carbonate), single-component organic materials (e.g., lactose monohydrate, mannitol), and multicomponent or complex organics (e.g., microcrystalline cellulose, starch). They may be soluble or insoluble in water, and they may be neutral, acidic, or alkaline in nature. These chemical properties may have a positive or negative affect on the drug substance physical or chemical stability and on performance. Appropriate selection of excipients with desirable physical and chemical properties can enhance the physical and chemical stability as well as the performance of the drug substance and dosage form. The detailed composition of an excipient may be important because excipient function could be influenced by the presence of minor concomitant components that are essential for proper performance. Pharmaceutical scientists may need to control the presence of undesirable components (e.g., heavy metals or peroxides) to ensure adequate dosage form stability and performance.

General Chapters: The following general chapters may be useful in ensuring consistency in diluent functions: *Bulk Density and Tapped Density of Powders* (616), *Density of Solids* (699), *Crystallinity* (695), *Crystallinity Determination by Solution Calorimetry* (696), *Loss on Drying* (731), *Water Determination* (921), *Optical Microscopy* (776), *Particle Size Distribution Estimation by Analytical Sieving* (786), *Light Diffraction Measurement of Particle Size* (429), *Powder Fineness* (811), *Specific Surface Area* (846), and *Powder Flow* (1174).

Functional Category: Binder

Description: Tablet/capsule binders are incorporated into formulations to facilitate the agglomeration of powder into granules during mixing with a granulating fluid such as water, hydroalcoholic mixtures, or other solvents. The binder may be either dissolved or dispersed in the granulation liquid or blended in a dry state; other components and the granulation liquid may be added separately during agitation. Following evaporation of the granulation liquid, binders typically produce dry granules that achieve the desired properties such as granule size, size distribution, shape, content, mass, and active content. Wet granulation facilitates the further processing of the granules by improving one or more of the granule properties such as flow, handling, strength, resistance to segregation, dustiness, appearance, solubility, compaction, or drug release.

Functional Mechanism: Binders are soluble or partially soluble in the granulating solvent or, as in the case of native starches, can be made soluble. Concentrated binder solutions also have adhesive properties. Upon addition of liquid, binders typically facilitate the production of moist granules (agglomerates) by altering interparticle adhesion. They may

also modify interfacial properties, viscosity, and/or other properties. During drying they may produce solid bridges that yield significant residual dry granule strength.

Physical Properties: Dispersion or dissolution of a binder in the granulation liquid depends on its physical properties: surface tension, particle size, size distribution, solubility, and viscosity are among the important properties depending on the application. Homogeneous incorporation of binder into a dry blend also depends on its physical properties such as particle size, shape, and size distribution. Viscosity often is an important property to consider for binders and, for polymers, is influenced by the nature of the polymer structure, molecular weight, and molecular weight distribution. Polymeric binders may form gels.

Chemical Properties: Tablet/capsule binders may be categorized as (1) natural polymers, (2) synthetic polymers, or (3) sugars. The chemical nature of polymers, including polymeric structure, monomer properties and sequence, functional groups, degree of substitution, and cross-linking influence the complex interactions that can occur during granulation. Natural polymers in particular may exhibit greater variation in their properties because of variations in their sources and therefore their composition.

General Chapters: The following general chapters may be useful in ensuring consistency in binder functions: *Bulk Density and Tapped Density of Powders* (616), *Crystallinity* (695), *Density of Solids* (699), *Loss on Drying* (731), *Particle Size Distribution Estimation by Analytical Sieving* (786), *Specific Surface Area* (846), *Viscosity* (911), *Powder Flow* (1174), and *Chromatography* (621).

Functional Category: Disintegrant

Description: Disintegrants are functional components that are added to formulations to promote rapid disintegration into smaller units and to allow a drug substance to dissolve more rapidly. Disintegrants are natural, synthetic, or chemically modified natural polymeric substances. When disintegrants come in contact with water or stomach or intestinal fluid they function by absorbing liquid and start to swell, dissolve, or form gels. This causes the tablet structure to rupture and disintegrate, producing increased surfaces for enhanced dissolution of the drug substance.

Functional Mechanism(s): The ability to interact strongly with water is essential to disintegrant function. Four major mechanisms describe the function of the various disintegrants: volume increase by swelling, deformation, capillary action (wicking), and repulsion. In tablet formulations, the function of disintegrants is best described as a combination of two or more of these effects. The onset and degree of the locally achieved actions depend on various parameters of a disintegrant, such as its chemical nature and its particle size distribution and particle shape, as well as some important tablet parameters such as hardness and porosity.

Physical Properties: The primary physical properties relevant to a disintegrant are those that describe the product's particle structure as a dry powder or its structure when in contact with water. These properties include (1) particle size distribution, (2) water absorption rate, (3) swelling ratio or swelling index, and (4) the characterization of the resulting product whether it is still particulate or a gel is formed.

Chemical Properties: Polymers used as disintegrants are either nonionic or anionic with counterions such as sodium, calcium, or potassium. Nonionic polymers are natural or physically modified polysaccharides such as starches, celluloses, pullulan, or cross-linked polyvinylpyrrolidone. The anionic polymers mainly are chemically modified cellulose products or low-crosslinked polyacrylates. These chemical properties should be considered in the case of ionic polymers. Disintegration performance will be affected by pH changes in the gastrointestinal tract or by complex formation with ionic active pharmaceutical ingredients (APIs).

General Chapters: The following general chapters may be useful in ensuring consistency in disintegrant functions: *Light Diffraction Measurement of Particle Size* (429), *Particle Size Distribution Estimation by Analytical Sieving* (786), *Optical Microscopy* (776), and *Powder Flow* (1174).

Functional Category: Lubricant

Description: Lubricants typically are used to reduce the frictional forces between particles, and between particles and metal contact surfaces of manufacturing equipment such as tablet punches and dies used in the manufacture of solid dosage forms. Liquid lubricants may be absorbed into the granule matrix before compaction. Liquid lubricants also may be used to reduce metal-metal friction on manufacturing equipment.

Functional Mechanism: Boundary lubricants function by adhering to solid surfaces (granules and machine parts) and reducing the particle-particle friction or the particle-metal friction. The orientation of the adherent lubricant particles is influenced by the properties of the substrate surface. For optimal performance, the boundary lubricant particles should be composed of small, plate-like crystals or stacks of plate-like crystals. Fluid film lubricants melt under pressure and thereby create a thin fluid film around particles and on the surface of punches and dies in tablet presses, which helps to reduce friction. Fluid film lubricants resolidify after the pressure is removed. Liquid lubricants are released from the granules under pressure and create a fluid film. They do not resolidify when the pressure is removed but are reabsorbed or redistributed through the tablet matrix over the course of use.

Physical Properties: The primary physical properties that may be important for the function of boundary lubricants include particle size, surface area, hydration state, and polymorphic form. Purity (e.g., stearate:palmitate ratio) and moisture content also may be important. The primary physical properties of possible importance for fluid film lubricants are particle size and solid state/thermal behavior. Purity may also be important.

Chemical Properties: Lubricants can be classified as boundary lubricants, fluid film lubricants, or liquid lubricants. Boundary lubricants are salts of long-chain fatty acids (e.g., magnesium stearate) or fatty acid esters (e.g., sodium stearyl fumarate) with a polar head and fatty acid tail. Fluid film lubricants are solid fats (e.g., hydrogenated vegetable oil, type 1), glycerides (glyceryl behenate and distearate), or fatty acids (e.g., stearic acid) that melt when subjected to pressure. Liquid lubricants are liquid materials that are released from granules under pressure.

General Chapters: The following general chapters may be useful in ensuring consistency in lubricant functions: *Light Diffraction Measurement of Particle Size* (429), *Particle Size Distribution Estimation by Analytical Sieving* (786), *Specific Surface Area* (846), *Characterization of Crystalline and Partially Crystalline Solids by X-Ray Powder Diffraction (XRPD)* (941), *Loss on Drying* (731), *Water Determination* (921), *Crystallinity* (695), *Crystallinity Determination by Solution Calorimetry* (696), *Optical Microscopy* (776), and *Thermal Analysis* (891).

Other Information: Certain lubricants, particularly those used in effervescent dosage forms, do not fall into the chemical categories defined above. These materials are used in special situations, and they are not suitable for universal application. Talc is an inorganic material that may have some lubricant properties. It is generally used in combination with fluid film lubricants to reduce sticking to punches and dies.

Functional Category: Glidant and/or Anticaking Agent

Description: Glidants and anticaking agents are used to promote powder flow and to reduce the caking or clumping that can occur when powders are stored in bulk. In addition, glidants and anticaking agents reduce the incidence of bridging during the emptying of powder hoppers and during powder processing.

Functional Mechanism: Glidants are thought to work by a combination of adsorption onto the surface of larger particles and reduction of particle-particle adhesive and cohesive forces, thus allowing particles to move more easily relative to one another. In addition, glidants may be dispersed between larger particles and thus may reduce friction between larger particles. Anticaking agents may absorb free moisture that otherwise would allow the development of particle-particle bridges that are implicated in caking phenomena.

Physical Properties: Primary physical properties of potential importance for glidants and anticaking agents are particle size, particle size distribution, and surface area. They may be slightly hygroscopic.

Chemical Properties: Glidants and anticaking agents typically are finely divided inorganic materials. They are insoluble in water but are not hydrophobic. Some of these materials are complex hydrates.

General Chapters: The following general chapters may be useful in ensuring consistency in glidant or anticaking agent functions: *Light Diffraction Measurement of Particle Size* (429), *Particle Size Distribution Estimation by Analytical Sieving* (786), *Specific Surface Area* (846), *Loss on Drying* (731), and *Water Determination* (921).

Functional Category: Coloring Agent

Description: Coloring agents are incorporated into dosage forms in order to produce a distinctive appearance that may serve to differentiate a particular formulation from others that have a similar physical appearance. These substances are subdivided into dyes (water-soluble substances), lakes (insoluble forms of a dye that result from its irreversible adsorption onto a hydrous metal oxide), inorganic pigments (substances such as titanium dioxide or iron oxides), and natural colorants (colored compounds not considered dyes per se, such as riboflavin). Coloring agents are subject to federal regulations, and consequently the current regulatory status of a given substance must be determined before its use.

The Federal Food, Drug, and Cosmetic Act defines three categories of coloring agents:

- FD&C colors: those certifiable for use in coloring foods, drugs, and cosmetics
- D&C colors: dyes and pigments considered safe in drugs and cosmetics when in contact with mucous membranes or when ingested
- Ext. D&C colors: colorants that, because of their oral toxicity, are not certifiable for use in ingestible products but are considered safe for use in externally applied products.

Functional Mechanism: Water-soluble dyes usually are dissolved in a granulating fluid for use, although they may also be adsorbed onto carriers such as starch, lactose, or sugar from aqueous or alcoholic solutions. These latter products are often dried and used as formulation ingredients. Because of their insoluble character, lakes are almost always blended with other dry excipients during formulation. For this reason, direct-compression tablets are often colored with lakes.

Physical Properties: Particle size and size distribution of dyes and lakes can influence product processing times (blending and dissolution), color intensity, and uniformity of appearance.

Chemical Properties: The most important properties of a coloring agent are its depth of color and resistance to fading over time. Substances can be graded on their efficiency in reflecting desired colors of visible light, as well as on their molar absorptivities at characteristic wavelengths. A coloring agent should be physically and chemically nonreactive with other excipients and the drug substances. The quality of a coloring agent ordinarily is measured by a determination of its strength, performance, or assay. The impurity profile is established by measurements of insoluble matter, inorganic salt content, metal content, and organic impurities.

General Chapters: Two general chapters are useful in ensuring consistency in selected coloring agent functions: *Color—Instrumental Measurement* <1061> and *Light Diffraction Measurement of Particle Size* <429>. Instrumental methods should be used to determine the absolute color of a coloring agent.

Other Information: Coloring agents are subject to federal regulations, and consequently the current regulatory status of a given substance must be determined before it is used. Following is a list of coloring agents and currently applicable sections of the Code of Federal Regulations (CFR).

Color	CFR
Ferric Oxides	21 CFR 73.1200
Titanium Dioxide	21 CFR 73.575 & 21 CFR 73.1575
FD&C Blue #1/Brilliant Blue FCF Aluminum Lake	21 CFR 82.51 & 21 CFR 82.101
FD&C Blue #2/Indigo Carmine Aluminum Lake	21 CFR 82.51 & 21 CFR 82.102
FD&C Red #40/Allura Red AC Aluminum Lake	21 CFR 74.340 & 21 CFR 74.1340
FD&C Yellow #5/Tartrazine Aluminum Lake	21 CFR 82.51 & 21 CFR 82.705
FD&C Yellow #6/Sunset Yellow FCF Aluminum Lake	21 CFR 82.51 & 21 CFR 82.706
D&C Yellow #10 Aluminum Lake	21 CFR 82.1051 & 21 CFR 82.1710
D&C Red #30/Helendons Pink Aluminum Lake	21 CFR 82.1051 & 21 CFR 82.1330
D&C Red #7/Lithol Rubin B Calcium Lake	21 CFR 82.1051 & 21 CFR 82.1307
D&C Red #27/Phloxine Aluminum Lake	21 CFR 82.1051 & 21 CFR 82.1327

Functional Category: Capsule Shell

Description: The word capsule is derived from the Latin *capsula*, which means a small container. Among other benefits, capsules enable pharmaceutical powders and liquids to be formulated for dosing accuracy as well as ease of transportation. The capsule material should be compatible with all other ingredients in the drug product. Hard capsules typically consist of two parts: both are cylindrical, and one part is slightly longer than the other and is called the body. The cap fits closely on the body to enclose the capsule. In contrast, the soft capsule is a one-piece unit that may be seamed along an axis or may be seamless. The capsule material may be derived from hydrolysis of collagen that originates from porcine, bovine, or fish sources, or it can be of non-animal origin, e.g., cellulosic or polysaccharide chemical entities. The capsule shell also contains other additives such as plasticizers, colorants, and preservatives. In some cases, capsule shells are sterilized to prevent microbial growth. The capsule shell is an integral part of the formulation, and therefore robust manufacturing and formulation performance depends on the measurement and control of critical attributes.

Functional Mechanism: Capsules can enclose solid as well as semisolid and liquid formulations. Capsules have a variety of benefits including the following: masking unpleasant taste, facilitating blinding in clinical studies, promoting ease of swallowing, and presenting a unique appearance. Conventional capsule shells should dissolve rapidly at 37° in biological fluids such as gastric and intestinal media. However, the solubility properties of the shell can be modified, e.g., with enteric and controlled-release polymers, to control the release of capsule contents.

Physical Properties: The primary physical properties relevant to the capsule shell are those that can have a direct effect on product performance: (1) moisture content, (2) gas permeability, (3) stability on storage, (4) disintegration, (5) compactness, and (6) brittleness. The moisture content varies with the type of capsule. Hard gelatin capsules typically contain 13%–16% water compared to hypromellose (hydroxypropyl methylcellulose/HPMC) capsules that typically contain 4%–7% water content. Soft gelatin capsules contain 6%–8% water. Moisture content has a significant impact on capsule brittleness. Equilibrium water content also may be crucial to dosage form stability because water migration will take place between the shell and capsule contents. Gas permeability may be important and generally is greater for HPMC capsules than gelatin capsules because of the presence of open structures. Gelatin capsules may undergo cross-linking upon storage at elevated temperature and humidity (e.g. 40°/75% RH), but under these conditions HPMC capsules do not cross-link. The aldehyde content in the powder fill should be considered because it can promote cross-linking of gelatin shell material. Gelatin capsules should disintegrate within 15 minutes when exposed to 0.5% hydrochloric acid at 36°–38° but not below 30°. HPMC capsules also can disintegrate below 30°.

Chemical Properties: Gelatin is a commercial protein derived from native protein collagen. The product is obtained by partial hydrolysis of collagen derived from skin, white connective tissue, and bones of animals. Type A gelatin is derived by acid treatment, and Type B gelatin is derived from base treatment. The common sources of commercial gelatin are pigskin, cattle hide, cattle bone, cod skin, and tilapia skin. The gelatin capsule shell also typically contains coloring agents, plasticizers such as polyhydric alcohols, natural gums and sugars, and preservatives such as sodium metabisulfite and esters of *p*-hydroxybenzoic acid. The more commonly used nongelatin capsules today are made from HPMC. Different capsule types contain different moisture levels and may thus influence drug product stability. The detailed composition of an excipient may be important because the shell function can be influenced by small amounts of impurities in the excipients (e.g., peroxides in oils or aldehydes in lactose and starches) that can cause capsule cross-linking. The presence in capsule shells of undesirable materials such as metals, odorants, water-insoluble substances, and sulfur dioxide should be evaluated to ensure stability and performance.

General Chapters: The following general chapters may be useful in ensuring consistency in selected capsule shell functions: *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* <61>, *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* <62>, *Residue on Ignition* <281>, *Arsenic* <211>, *Heavy Metals* <231>, *Water Determination* <921>, *Color—Instrumental Method* <1061>, *Disintegration* <701>, *Dissolution* <711>, and *Gel Strength of Gelatin* <1081>.

Functional Category: Coating Agent

Description: Reasons for coating pharmaceutical dosage forms include masking unpleasant tastes or odors, improving ingestion and appearance, protecting active ingredients from the environment, and modifying the release of the active ingredient (e.g., controlled-release rates or gastrointesti-

nal targeting). The materials used as coating agents include natural, semisynthetic, and synthetic materials. These may be powders or colloidal dispersions (latexes or pseudo-latexes) that usually are applied as solutions or dispersions in aqueous or nonaqueous systems. Waxes and lipids may be applied as coatings in the molten state without the use of solvents.

Functional Mechanism: Coating agents are composed of film-forming materials that impart desirable pharmaceutical properties such as appearance, patient acceptance, and ease of swallowing. Coating agents also may serve other functional purposes such as providing a barrier against undesirable chemical reactions or untimely release of a drug from its components. After intake, the coating may dissolve by processes such as hydration, solubilization, or disintegration, depending on the nature of the material used. Enteric coatings are insoluble in acidic (low pH) media but dissolve readily in neutral pH conditions. However, most common coatings do not have pH-specific solubility. The coating thickness may vary by application and the nature of the coating agents. In the coating process, the polymer chains spread out on the core surface and coalesce into a continuous film as the solvent evaporates. Plastic polymers, waxes, and lipid-based coatings may be applied without solvents by melting and atomization. Molten fluid droplets, upon impact on the surface of the fluidized drug particles, spread and resolidify to form film layers. Therefore, coating materials generally have the ability to form a complete and stable film around the substrate. The coating preparation typically is applied uniformly and is carefully dried to ensure that a consistent product is produced. Suitable plasticizers may be required to lower the minimum film-forming temperature of the polymer, and their potential effect on drug release should be considered.

Physical Properties: Film coating is a complex process, and the characteristics of a film-forming polymer play an important role: the particle size of colloidal dispersions varies with their origin (latex, pseudolatex, or redispersed powder) and may have an effect on the film-forming mechanism. Polymer solutions or dispersions with a low viscosity and high pigment-binding capacity reduce the coating time and facilitate relatively simple and cost-effective manufacturing. The concentration-viscosity relationship for the film-forming agent should be evaluated for process optimization. The surface tension of coating preparations can influence the spray pattern in the manufacturing process. The applied coating must withstand mechanical stress during coating or packaging operations. Therefore, the film should possess high elasticity and sufficient mechanical strength. It can be useful to analyze tensile properties of isolated films. For coatings that are applied in a molten state without solvents (plastic polymers, waxes, and lipid-based coatings), melting range and melt viscosity are the properties of prime consideration.

Chemical Properties: Film-forming agents are of natural, semisynthetic, or synthetic origin and are available in different chemical grades. NF monographs often describe classes of polymeric materials that allow a considerable range of composition, structure, or molecular weight. These factors should be considered when pharmaceutical scientists identify and quantitate critical material attributes to ensure consistent performance.

General Chapters: The following general chapters may be useful in ensuring consistency in selected excipient functions: *Viscosity* (911), *Tensile Strength* (881), *Light Diffraction Measurement of Particle Size* (429), *Fats and Fixed Oils* (401), *Thermal Analysis* (891), and *Dissolution* (711).

Additional Information: Additives often are included in a coating formulation. Fillers (e.g., sugar alcohols, microcrystalline cellulose) may be added to increase the solids content of the coating agent without increasing viscosity. Stearic acid can be used to improve the protective function/moisture barrier of a coating. Water-soluble or -insoluble ingredients may be added to create pores in the film to adjust the release pattern of sustained-release formulations. Color-

ing agents (e.g., titanium dioxide, kaolin) may be added to modify appearance.

Functional Category: Plasticizer

Description: A plasticizer is a low molecular weight substance that, when added to another material—usually a polymer—makes the latter flexible, resilient, and easier to handle. Modern plasticizers are synthetic organic chemicals, the majority of which are esters such as citrates and phthalates. They are key components that determine the physical properties of polymeric pharmaceutical systems such as tablet film coatings and capsule shells.

Functional Mechanism: Plasticizers function by increasing the intermolecular and intramolecular mobility of the macromolecules that comprise polymeric materials. They achieve this by interfering with the normal intermolecular and intramolecular bonding mechanisms in such systems. The most effective plasticizers exert their effect at low concentrations, typically less than 5% w/w. Plasticizers commonly are added to film coatings (aqueous and nonaqueous systems) and capsule shells (hard and soft varieties) to improve their workability and mechanical ruggedness. Without the addition of plasticizers, such materials can split or fracture prematurely. Plasticizers also are added to semisolid pharmaceutical preparations such as creams and ointments to enhance their rheological properties.

Physical Properties: The most common plasticizers are low molecular weight (< 500 Da) solids or liquids. They typically have low melting points (< 100°) and can be volatile (i.e., exert an appreciable vapor pressure) at ambient temperature. Plasticizers can significantly reduce the glass transition temperature of the system to which they are added.

Chemical Properties: As noted, many modern plasticizers are synthetic esters such as citrates and phthalates. Traditional pharmaceutical plasticizers include oils, sugars, and their derivatives.

General Chapters: The following general chapters may be useful in ensuring consistency in selected excipient functions: *Melting Range or Temperature* (741), *Water Determination* (921), *Residual Solvents* (467), *Specific Gravity* (841), *Refractive Index* (831), and *Thermal Analysis* (891).

Other Information: The choice of an appropriate plasticizer often is guided by reference to its “solubility parameter”, which is related to its cohesive energy density. Solubility parameter values for many common materials are tabulated in standard reference texts. To ensure maximum effectiveness, the solubility parameter of the plasticizer and the polymeric system being plasticized should be matched as closely as possible.

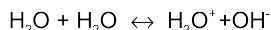
ORAL LIQUIDS

Functional Category: pH Modifier (Acidifying/Alkalizing/Buffering Agent)

Description: The hydrogen ion concentration, $[H^+]$, in an aqueous solution is expressed as $pH = -\log(H^+)$. The pH of pure water is 7 at 25°. An aqueous solution is acidic at $pH < 7$ and alkaline at $pH > 7$. An acid may be added to acidify a solution. Similarly, a base may be used to alkalize a solution. A buffer is a weak acid (or base) and its salt. When a buffer is present in a solution, the addition of small quantities of strong acid or base leads to only a small change in solution pH. Buffer capacity is influenced by salt/acid (or base/salt) ratio and total concentration of acid (or base) and salt. The pH of pharmaceutical solutions typically is controlled using acidifying/alkalizing and buffering agents to (1) maintain a pH close to that of relevant body fluid to avoid irritation; (2) improve drug stability that is pH depen-

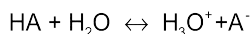
dent; (3) control equilibrium solubility of weak acids or bases; and (4) maintain a consistent ionization state of molecules during chemical analysis, e.g., high-performance liquid chromatography (HPLC).

Functional Mechanism: The ionization equilibria of weak bases, weak acids, and water are the key to the functions of acidifying, alkalizing, and buffering agents. The autoprotolytic reaction of water can be expressed as



The autoprotolysis constant (or ion product) of water is $K_w = 1 \times 10^{-14}$ at 25° and varies significantly with temperature. Because the concentrations of hydrogen and hydroxyl ions in pure water are equal, each has the value of approximately 1×10^{-7} mole/L, leading to the neutral pH of 7 at 25°. When an acid, base, or salt of weak acid (or base) is added, the ionization equilibrium of water is shifted so that $[\text{H}^+][\text{OH}^-]$ remains constant, thus resulting in a solution pH that is different from 7.

Physical Properties: The ionization equilibrium of a weak acid, HA, can be written as



The ionization constant of a weak acid (or conjugate acid of a base) is commonly expressed as $\text{pK}_a = -\log(K_a)$, where $K_a = [\text{H}_3\text{O}^+][\text{A}^-]/[\text{HA}]$. A lower pK_a corresponds to a stronger acid. Similarly, the ionization constant of a weak base (or conjugate base of an acid) is expressed as $\text{pK}_b = -\log(K_b)$. The ionization equilibrium of water ($\text{pK}_a + \text{pK}_b = \text{pK}_w$) equals 14 at 25°. Buffers and pH modifiers influence solution osmolality, osmolarity, and water conductivity.

Chemical Properties: When used in chemical analysis, buffers must be chemically compatible with the reagents and test substance. Buffers, when used in physiological systems, should not interfere with pharmacological activity of the medicament or normal function of the organism.

General Chapters: The following general chapters may be useful in ensuring consistency in selected pH modifier or buffering agent functions: *Water Conductivity* (645), *pH* (791), and *Osmolality and Osmolarity* (785).

Functional Category: Wetting and/or Solubilizing Agent

Description: Solubilizers can be used to dissolve insoluble molecules. They function by facilitating spontaneous phase transfer to yield a thermodynamically stable solution. A number of solubilizers are available commercially. Acceptable solubilizers for pharmaceutical applications have been fully evaluated in animals for safety and toxicology.

Functional Mechanism: Solubilizers comprise a variety of different chemical structures/classes. Some solubilizers may have unique chemical structures. For example, a hydrophilic moiety may be tethered with a hydrophobic moiety to yield distinct micelle shapes and morphologies in water, thus facilitating solubilization. The mechanism of solubilization often is associated with a favorable interaction of the insoluble agent and the interior core of the solubilizer assembly (e.g. micelles). In other cases, unique hydrophobic sites that are capable of forming inclusion complexes are present. Other types of solubilizers utilize a range of polymeric chains that interact with hydrophobic molecules to increase solubility by dissolving the insoluble agent into the polymeric chains.

Physical Properties: Solubilizers are solid, liquid, or waxy materials. Their physical properties depend on their chemical structures. The physical properties and performance of the solubilizers, however, depend on the surface-active properties of the solubilizers and on the hydrophilic-lipophilic balance (HLB). Solubilizers with lower HLB values behave as emulsifiers, and those with higher HLB values be-

have as solubilizers. For example, sodium lauryl sulfate (HLB 40) is hydrophilic and highly water soluble and, upon dispersion in water, spontaneously forms micelles.

The unique hydrophilicity and hydrophobicity properties of solubilizers are characterized by their aggregate numbers or critical micelle concentrations (CMC). The CMC value is unique to an individual solubilizer bearing hydrophilic, lipophilic, and/or hydrophobic chains. CMC is a measure of the concentration at which the surface-active molecule aggregates and solubilizes the solute by incorporating part into the hydrophobic interior and accommodating the rest in the hydrophilic exterior aqueous layer. Such interactions with the insoluble molecule further stabilize the molecules in the entire assemblies without precipitation to yield a continuous solution.

Chemical Properties: The chemical and surface-active properties depend on the structures of the solubilizers. Because of the complex nature of solute-solvent-solubilizer interactions, pharmaceutical scientists must carefully consider, identify, and control the critical material attributes of solubilizers.

General Chapters: The following general chapters may be useful in ensuring consistency in selected solubilizing agent function: *Fats and Fixed Oils* (401), *Specific Gravity* (841), *pH* (791), *Specific Surface Area* (846), *Thermal Analysis* (891), *Spectrophotometry and Light-Scattering* (851), *Scanning Electron Microscopy* (1181), *Viscosity* (911), and *Light Diffraction Measurement of Particle Size* (429).

Functional Category: Antimicrobial Preservative

Description: Antimicrobial preservatives are used to kill or prevent growth of bacteria, yeast, and mold in the dosage form.

Functional Mechanism: Preservatives work by a variety of mechanisms to control microbes. Most of them work at the cell membrane, causing membrane damage and cell leakage. Other modes of action include transport inhibition, protein precipitation, and proton-conducting uncoupling. Some preservatives are -cidal (kill bacteria or yeast and mold); some are -static (inhibit growth of microorganisms); and others are sporicidal (kill spores). Several of the preservatives can act synergistically (e.g., combinations of parabens).

Physical Properties: Antimicrobials generally are soluble in water at concentration ranges at which they are effective. The vapor pressure of these agents is important, especially if the dosage form is intended to be lyophilized or spray dried. Several of these agents are flammable. Understanding of an excipient's partition coefficient is important because partitioning of a preservative into an oil phase will diminish the preservative's concentration in the aqueous phase, which in turn can reduce its value as a preservative.

Chemical Properties: Phenolic preservatives can undergo oxidation and color formation. Incompatibilities of preservatives (cationic and anionic mixtures, adsorption to tubes or filters, binding to surfactants and proteins) should be taken into account during product development.

General Chapters: The following general chapters may be useful in ensuring consistency in selected excipient functions: *Injections* (1), *Antimicrobial Effectiveness Testing* (51), *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* (62), and *Antimicrobial Agents—Content* (341).

Other Information: Be aware of safety and labeling requirements, specifically for benzalkonium chloride (eye and skin irritation), benzoic acid and benzoate salts (risk of jaundice in newborn babies), benzyl alcohol (should not be given to premature babies or neonates and may cause allergic reactions in children aged 3 years or less), bronopol

(may cause skin reaction), chlorocresol (allergic reactions), organic mercury compounds (allergic reaction), parabens (allergic reactions), and sorbic acid and salts (skin reactions). Because of the risk of organic mercury toxicity, thimerosal should not be used. Use of preservative is contraindicated in parenteral products in which the fill volume is greater than 30 mL or comes in contact with cerebrospinal fluid. Antioxidants and chelating agents tend to potentiate antimicrobial efficacy.

Functional Category: Chelating and/or Complexing Agents

Description: Chelating/complexing agents form soluble complex molecules with certain metal ions (e.g., copper, iron, manganese, lead, and calcium) and essentially remove the ions from solution to minimize or eliminate their ability to react with other elements and/or to precipitate. The agents are used in pharmaceuticals (oral, parenteral, and topical formulations), cosmetics, and foods to sequester ions from solution and to form stable complexes. Chelating agents are also referred to as chelants, chelators, or sequestering agents.

Functional Mechanism: Chelating/complexing agents are used to sequester undesirable metal ions from solution. Their chemical structure acts as a "claw" to associate with the metal atom by forming a heterocyclic ring structure. Complexing agents function similarly but mechanistically do not (by definition) require a two-point claw structure because they can associate via one or more binding sites. All chelating agents are complexing agents, but not all complexing agents are chelating agents. As excipients, chelating agents are used as antioxidant synergists, antimicrobial synergists, and water softeners. By "removing" metal ions from solution, chelating agents reduce the propensity for oxidative reactions. Chelating agents also have the ability to enhance antimicrobial effectiveness by forming a metal-ion-deficient environment that otherwise could feed microbial growth.

Physical Properties: Chelating and complexing agents are freely soluble in water. Various salt (disodium and calcium disodium) and hydrated forms (anhydrous, dihydrate, and trihydrate) of edetic acid exist. Edetic acid and its derivatives appear as white to off-white crystalline solids. Oxyquinoline sulfate appears as a pale yellow, crystalline powder. *USP-NF* recognizes that chelating/complexing agents are stable below 100°, but dehydration and/or decomposition can occur at higher temperatures. Chelating agents exhibit different degrees of hygroscopicity. Because low proportions of chelating agents are used in formulations (typically not more than 0.2%), they are not expected to significantly affect the bulk solid mechanical and flow properties of solid formulations. Because these agents are used in very low levels, their particle size distribution is important to enable acceptable dosage form content uniformity.

Chemical Properties: Chelating/complexing agents complex with metal ions via any combination of ionic and covalent bonds. Dilute aqueous solutions may be neutral, acidic, or alkaline. Edetic acid and its salts are incompatible with strong oxidizers, strong bases, and polyvalent metal ions (e.g., copper and nickel). Specific agents are selected for a formulation based on their solubility, affinity for the target metal ion, and stability. Edetate salts are more soluble than the free acid. Unlike other edetate salts and the free acid, edetate calcium disodium does not sequester calcium and therefore is preferred to prevent hypocalcemia. It is also preferred to chelate heavy metals with the release of calcium ions. Alternatively, disodium edetate can be used to treat hypercalcemia. Edetic acid will decarboxylate if heated above 150°.

General Chapters: The following general chapters may be useful in ensuring consistency in selected excipient functions: *Antimicrobial Effectiveness Testing* (51); *Microbiological*

Examination of Nonsterile Products: Microbial Enumeration Tests (61); *Heavy Metals* (231); *Iron* (241); *Lead* (251); *Antimicrobial Agents—Content* (341); *Light Diffraction Measurement of Particle Size* (429); *Loss on Drying* (731); *pH* (791); *Water Determination* (921); *Biotechnology-derived Articles* (1045); and *Cell and Gene Therapy Products* (1046), *Manufacturing of Cell Therapy Products*.

Functional Category: Antioxidant

Description: This category applies to antioxidants used as in vitro stabilizers of pharmaceutical preparations to mitigate oxidative processes. Antioxidants used for their biological activity in vivo may be regarded as active ingredients with therapeutic effects and are not discussed. Antioxidants delay the onset and/or significantly reduce the rate of complex oxidative reactions that could otherwise have a detrimental impact on the drug substance. Antioxidants also can be considered for protecting nonactive components like unsaturated oils, pegylated lipids, flavors, and essential oils. Thus antioxidants preserve the overall integrity of the dosage form against oxidative stress. Antioxidants are most effective when incorporated in the formula to prevent or delay the onset of chain reactions and to inhibit free radicals and hydroperoxides from engaging in the cascading processes described above. Effective application of antioxidants and evaluation of their efficacy necessitate an understanding of oxidative mechanisms and the nature of the byproducts they generate. Autooxidation is initiated when oxygen reacts with a substrate to form highly reactive species known as free radicals ($RH \rightarrow R\cdot$). After "initiation" the free radicals in the presence of oxygen can trigger chain reactions ($R\cdot + O_2 \rightarrow ROO\cdot$ and $ROO\cdot + RH \rightarrow R\cdot + ROOH$) to form peroxy radicals, hydroperoxides, and new alkyl radicals that can initiate and then propagate their own chain reactions. The cascading reactions during the propagation phase can be accelerated by heat, light, and metal catalysts. In the presence of trace amounts of metal catalysts (Cu^+ , Cu^{2+} , Fe^{2+} , and Fe^{3+}), hydroperoxides ($ROOH$) readily decompose to $RO\cdot$ and $ROO\cdot$ and can subsequently trigger reactions with the API and/or the excipients (e.g., hydrocarbons) to form hydroxyl acids, keto acids, and aldehydes that can have further undesirable effects. Note that hydroperoxides are not solely the reaction products of oxidative mechanisms within a formulation. Residual amounts of hydroperoxides can also be found in commonly used excipients like polyethylene glycols (PEG), polyvinylpyrrolidone (PVP), and polysorbates. The initiation phase generally is slow and has limited impact on the quality of the finished product. The propagation phase, in contrast, involves rapid, irreversible degradation of chemical species.

Functional Mechanism: Antioxidants can be grouped by their mode of action. Phenolic antioxidants that block free radical chain reactions are also known as true or primary antioxidants. This group consists of monohydroxy or polyhydroxy phenol compounds with ring substitutions. They have very low activation energy to donate hydrogen atom(s) in exchange for the radical electrons that are rapidly delocalized by free radicals. By accepting the radical electrons they stabilize free radicals. The reaction yields antioxidant free radicals that can also react with lipid free radicals to form other stable compounds. Thus they can block oxidative chain reactions both in the initiation and propagation stages. Because of their solubility behavior, phenolic antioxidants are most effective in protecting oils and oil-soluble actives against oxidative stress. Reducing agents generally are water-soluble antioxidants (e.g., L-ascorbic acid) with lower redox potential than the drug or the excipient they are protecting. They delay the onset and the rate of oxidative reactions by sacrificially reacting with oxygen and other reactive species. The oxygen-scavenging potential of the reducing agents may be sensitive to pH and can also be negatively affected in the presence of trace metals. Chelating agents bind with free metals (Cu^+ , Cu^{2+} , Fe^{2+} , and Fe^{3+}) that

may be present in trace amounts in the formulation. The newly formed complex ions are nonreactive. Chelating agents therefore remove the capacity of the metal catalysts to participate in oxidative reactions that occur during the propagation stage.

The utility of antioxidants can be maximized by synergistic use of one or two primary antioxidants along with reducing and chelating agents. The combined effect is often greater than the sum of the individual effects of each antioxidant (synergistic effect).

Physical Properties: Solubility of the antioxidant should be greatest in the formulation phase (oily, aqueous, or emulsion interface) where the drug substance is most soluble. The temperature at which the antioxidant decomposes is critical for autoclaved preparations where loss of antioxidant activity may occur. Stability of the antioxidant also must be considered and may be a function of pH and processing conditions. Metal ions may react with propyl gallate to form colored complexes. At alkaline pH, certain proteins and sodium salts may bring about discoloration of *tert*-butylhydroquinone (TBHQ).

Chemical Properties: Activation energy, oxidation-reduction potential, stability at different formulation (e.g., pH), and processing (e.g., heat) conditions are important chemical properties. If the dosage form's expected shelf life depends on the antioxidant's function, the concentration must be factored in and periodically assessed to ensure that a sufficient amount of antioxidant remains but does not exceed safety limits.

General Chapters: The following general chapters may be useful for assessing selected excipient antioxidant functions: *Specific Surface Area* <846>, *Crystallinity* <695>, *Chromatography* <621>, *Water Determination* <921>, *Melting Range or Temperature* <741>, and *Iron* <241>.

Functional Category: Sweetening Agent

Description: Sweetening agents are used to sweeten oral dosage forms and to mask unpleasant flavors.

Functional Mechanism: Sweetening agents bind to receptors on the tongue that are responsible for the sensation of sweetness. The longer the sweetener molecule remains attached to the receptor, the sweeter the substance is perceived to be. The standard for sweetness is sucrose.

Physical Properties: The primary physical properties relevant to sweeteners relate to their compatibility with the other ingredients in the formulation (e.g., acidic ingredients), processing conditions (e.g., heating), particle size and distribution, moisture content, isomerism, sweetness, and taste-masking capability. These properties may be formulation dependent.

Chemical Properties: Sweeteners can be divided into three main groups: sugars (which have a ring structure), sugar alcohols (sugars that do not have a ring structure), and artificial sweeteners. All sweeteners are water soluble. The stability of many sweeteners is affected by pH and other ingredients in the formulation. Some sweeteners may catalyze the degradation of some active ingredients, especially in liquids and in cases in which the manufacturing processes involve heating.

General Chapters: The following general chapters may be useful in ensuring consistency in selected excipient functions: *Optical Rotation* <781>, *Specific Rotation*, *Water Determination* <921>, *Loss on Drying* <731>, and *Melting Range or Temperature* <741>.

Other Information: Products that contain aspartame must include a warning on the label stating that the product contains phenylalanine. Sugar alcohols have a glycemic index well below that of glucose. However, sorbitol is slowly metabolized to fructose and glucose, which raises blood sugar levels. Sugar alcohols in quantities generally greater than 20 g/day act as an osmotic laxative, especially when they

are contained in a liquid formulation. Preservative systems should be carefully chosen to avoid incompatibility with the sweetener; some sweeteners are incompatible with certain preservatives.

SEMISOLIDS, TOPICALS, AND SUPPOSITORIES

Functional Category: Suppository Base

Description: Suppository bases are used in the manufacture of suppositories (for rectal administration) and pessaries (for vaginal administration). They can be hydrophobic or hydrophilic.

Functional Mechanism: Suppositories should melt at just below body temperature (37°), thereby allowing the drug to be released either by erosion and partition if the drug is dissolved in the base or by erosion and dissolution if the drug is suspended in the base. Hard fat suppository bases melt at approximately body temperature. Hydrophilic suppository bases also melt at body temperature and typically also dissolve or disperse in aqueous media. Thus release takes place via a combination of erosion and dissolution.

Physical Properties: The important physical characteristic of suppository bases is melting range. In general suppository bases melt between 27° and 45°. However, individual bases usually have a much narrower melting range within these temperature boundaries, typically 2°–3°. The choice of a particular melting range is dictated by the influence of the other formulation components on the melting range of the final product.

Chemical Properties: Hard fat suppository bases are mixtures of semisynthetic triglyceride esters of longer-chain fatty acids. They may contain varying proportions of mono- and di-glycerides and may also contain ethoxylated fatty acids. They are available in many different grades that are differentiated by melting range, hydroxyl number, acid value, iodine value, solidification range, and saponification number.

Hydrophilic suppository bases are mixtures of hydrophilic semisolid materials that in combination are solid at room temperature and yet release the drug by melting, erosion, and dissolution when administered to the patient. Hydrophilic suppository bases have much higher levels of hydroxyl groups or other hydrophilic groups than do hard fat suppository bases. Polyethylene glycols that show appropriate melting behavior are examples of hydrophilic suppository bases.

General Chapters: The following general chapters may be useful in ensuring consistency in selected excipient functions: *Fats and Fixed Oils* <401>, *Congeeing Temperature* <651>, *Melting Range or Temperature* <741>, and *Pharmaceutical Dosage Forms* <1151>.

Other Information: Some materials included in suppositories based on hard fats have much higher melting ranges. These materials typically are microcrystalline waxes that help stabilize molten suspension formulations. Suppositories may also be manufactured from glycerinated gelatin.

Functional Category: Suspending and/or Viscosity-Increasing Agent

Description: Suspending and/or viscosity-increasing agents are used in pharmaceutical formulations to stabilize disperse systems (e.g., suspensions or emulsions), to reduce the rate of solute or particulate transport, or to decrease the fluidity of liquid formulations.

Functional Mechanism(s): A number of mechanisms contribute to the dispersion stabilization or viscosity-increasing effect of these agents. The most common is the increase in viscosity—due to the entrapment of solvent by macromolecu-

lar chains or clay platelets—and the disruption of laminar flow. Other mechanisms include gel formation via a three-dimensional network of excipient molecules or particles throughout the solvent continuum and steric stabilization wherein the macromolecular or mineral component in the dispersion medium adsorbs to the surfaces of particles or droplets of the dispersed phase. The latter two mechanisms increase formulation stability by immobilizing the dispersed phase.

Physical Properties: Each of the mechanisms—increased viscosity, gel formation, or steric stabilization—is a manifestation of the rheological character of the excipient. Because of the molecular weights and sizes of these excipients, the rheological profiles of their dispersions are non-Newtonian. Dispersions of these excipients display viscoelastic properties. The molecular weight distribution and polydispersity of the macromolecular excipients in this category are important criteria for their characterization.

Chemical Properties: The majority of the suspending and/or viscosity-increasing agents are (a) hydrophilic carbohydrate macromolecules (acacia, agar, alginic acid, carboxymethylcellulose, carrageenans, dextrin, gellan gum, guar gum, hydroxyethyl cellulose, hydroxypropyl cellulose, hypromellose, maltodextrin, methylcellulose, pectin, propylene glycol alginate, sodium alginate, starch, tragacanth, and xanthan gum) and (b) noncarbohydrate hydrophilic macromolecules, including gelatin, povidone carbomers, polyethylene oxide, and polyvinyl alcohol. Minerals (e.g., attapulgite, bentonite, magnesium aluminum silicate, and silicon dioxide) comprise the second-largest group of suspending and/or viscosity-increasing agents. Aluminum monostearate is the one non-macromolecular, non-mineral excipient in this functional category. It consists chiefly of variable proportions of aluminum monostearate and aluminum monopalmitate.

General Chapter: The following general chapter may be useful in ensuring consistency in selected excipient functions: *Viscosity* (911).

Functional Category: Ointment Base

Description: An ointment is a viscous semisolid preparation used topically on a variety of body surfaces. An ointment base is the major component of an ointment and controls its physical properties.

Functional Mechanism: Ointment bases serve as vehicles for topical application of medicinal substances and also as emollients and protective agents for skin.

Physical Properties: Ointment bases are liquids with a relatively high viscosity so that solids can be suspended as a stable mixture.

Ointment bases are classified as (a) oleaginous ointment bases that are anhydrous, do not absorb water readily, are insoluble in water, and are not removable by water (e.g., petrolatum); (b) absorption ointment bases that are anhydrous and absorb some water but are insoluble in water and are not water removable (e.g., lanolin); (c) emulsion ointment bases that are water-in-oil or oil-in-water emulsions and are hydrous, absorb water, and are insoluble in water (e.g., creams of water, oils, waxes, and/or paraffins); and (d) water-soluble ointment bases that are anhydrous and absorb water and are soluble in water and are water removable (e.g., polyethylene glycol).

Chemical Properties: Ointment bases are selected to be inert and chemically stable.

General Chapters: The following general chapters may be useful in ensuring consistency in selected excipient functions: *Viscosity* (911) and *Congeeing Temperature* (651).

Functional Category: Stiffening Agent

Description: A stiffening agent is an agent or a mixture of agents that increases the viscosity or hardness of a preparation, especially in ointments and creams.

Functional Mechanism: In general, stiffening agents are high melting point solids that increase the melting point of ointments or increase the consistency or body of creams. Stiffening agents can be either hydrophobic (e.g., hard fat or paraffin) or hydrophilic (e.g., polyethylene glycol, high molecular weight).

Physical Properties: The primary physical property relevant to stiffening agents is their high melting point or melting range. Typical melting ranges for stiffening agents range from 43° to 47° (cetyl esters wax), 53° to 57° (glyceryl distearate), 69° to 74° (glyceryl behenate), and 85° to 88° (castor oil, hydrogenated).

Chemical Properties: Stiffening agents comprise a diverse group of materials that include glycerides of saturated fatty acids, solid aliphatic alcohols, esters of saturated fatty alcohols and saturated fatty acids, saturated hydrocarbons, blends of fatty alcohols and a polyoxyethylene derivative of a fatty acid ester of sorbitan, and higher ethylene glycol polymers.

General Chapters: The following general chapters may be useful in ensuring consistency in selected excipient functions: *Melting Range or Temperature* (741), *Congeeing Temperature* (651), and *Viscosity* (911).

Other Information: Some of the materials included as stiffening agents increase the water-holding capacity of ointments (e.g., petrolatum) or function as co-emulsifiers in creams. Examples include stearyl alcohol and cetyl alcohol.

Functional Category: Emollient

Description: Emollients are excipients used in topical preparations to impart lubrication, spreading ease, texture, and softening of the skin and to counter the potentially drying/irritating impact of surfactants on the skin.

Functional Mechanism: Emollients help form a protective film and maintain the barrier function of the epidermis. Their efficacy may be described by three mechanisms of action: protection against the delipidizing and drying effects of surfactants, humectancy due to occlusion (by providing a layer of oil on the surface of the skin, emollients slow water loss and thus increase the moisture-retention capacity of the stratum corneum), and lubricity, adding slip or glide to the preparation.

Physical Properties: Emollients impart one or more of the following attributes to a pharmaceutical preparation: spreading capacity, pleasant feel to the touch, softness of the skin, and indirect moisturization of the skin by preventing trans-epidermal water loss.

Chemical Properties: Emollients are either oils or are derived from components of oils as esters of fatty acids. Depending on the nature of its fatty acid ester, an emollient may be liquid, semisolid, or solid at room temperature. Generally, the higher the molecular weight of the fatty acid moiety (carbon chain length) the richer the feel and softness of the touch. Fluidity generally is imparted by shorter chain length and higher degree of unsaturation in the fatty acid moiety. The degree of branching of ester bonds also influences the emollient properties.

General Chapter: The following general chapter may be useful in ensuring consistency in selected excipient functions: *Fats and Fixed Oils* (401).

PARENTERALS

Functional Category: Pharmaceutical Water

Description: Water is used as a solvent, vehicle, diluent, or filler for many drug products, especially those supplied in liquid form. These can include injectible drugs, ophthalmic drugs, oral solutions, inhalation solutions, and others. Water is also a vehicle for buffers and antimicrobial agents and is a volume expander for infusion solutions. Its use in dosage form preparation also can include granulation preparation for solid oral dosage forms and applications in the preparation of ointments and gels.

USP includes monographs for eight grades of pharmaceutical waters. One of these types of USP water is always the water of choice when pharmaceutical scientists prepare a pharmaceutical dosage form for human or animal use. However, USP also contains references to other types of water, such as distilled water, deionized water, and others according to specific use as summarized in general information chapter *Water for Pharmaceutical Purposes* <1231>.

Functional Mechanism: A solvent is able to dissolve materials because it is able to disrupt the intermolecular attractive forces and to allow the individual molecules to become dispersed throughout the bulk solvent. Water is a favored solvent and vehicle in the majority of applications because it is easy to handle, safe, and inexpensive.

Physical Properties: Water is liquid at normal temperature and pressure. It forms ice at the freezing temperatures of 0° or lower; and it vaporizes at a normal boiling temperature of 100°, depending upon atmospheric pressure. Vaporized water in the form of steam is used for sterilization purposes because the latent heat of steam is significantly higher than that of boiling water.

Chemical Properties: Water in its pure form is neutral in pH and has very low conductivity and total organic carbon (TOC). However, pH, conductivity, and TOC are affected by storage conditions and exposure of water to gases in the air. Exposure of water to atmospheric carbon dioxide lowers the pH of water. Storage of water in plastic containers may increase the TOC content of water over time. Water stored in glass containers may result in an increase in pH and conductivity of the water over time.

General Chapters: The following general chapters may be useful in ensuring consistency in selected excipient functions: *Injections* <1>, *Water for Pharmaceutical Purposes* <1231>, *Water for Health Applications* <1230>, *Bacterial Endotoxins Test* <85>, *Total Organic Carbon* <643>, and *Water Conductivity* <645>.

Functional Category: Diluent

Description: Diluents or bulking agents used in lyophilized pharmaceuticals include various saccharides, sugar alcohols, amino acids, and polymers. The primary functions of bulking agents are to provide a pharmaceutically elegant lyophilized cake with non-collapsed structural integrity and to prevent drug loss due to blow-out. In addition, bulking agents are selected to facilitate efficient drying and to provide a physically and chemically stable formulation matrix. Frequently, complementary combinations of bulking agents are used to improve performance.

So-called "good cake forming" excipients, such as mannitol, are frequently used because they tend to crystallize during freezing, thereby allowing efficient drying and the formation of a structurally robust cake. For some active ingredients, crystallization during lyophilization helps improve stability. Therefore the use of bulking agents that promote crystallization during lyophilization is important. Amino acids and cosolvents have been used to achieve this effect. Most biopolymer active ingredients remain amor-

phous upon freeze-drying, and bulking agents such as disaccharides may function as lyoprotectants by helping to maintain a stable amorphous phase during freezing and drying to prevent denaturation. Solubility enhancement of an insoluble crystalline active ingredient is sometimes achieved with the use of a biopolymer that enhances solubility or prevents crystallization of the active ingredient during lyophilization or subsequent reconstitution. Bulking agents are also selected on the bases of biocompatibility, buffering capability, and tonicity-modifying properties.

Functional Mechanisms: A bulking agent that readily crystallizes during lyophilization helps maintain the structural integrity of the cake formed during primary drying, thereby preventing macroscopic collapse and pharmaceutical inelegance. Microscopic collapse of amorphous components in the formulation may still occur (with some potentially undesirable results) but will not result in macroscopic collapse if the bulking agent properties and concentration are adequate. The bulking agent also should possess a high eutectic melting temperature with ice to permit relatively high primary drying temperatures with commensurate rapid and efficient drying.

Lyoprotectant properties of lyophilization diluents (i.e., those that protect the drug substance during lyophilization) typically are achieved by the formation of a highly viscous glassy phase that includes the biopolymer drug substance in combination with low molecular weight amorphous saccharides such as sucrose, trehalose, or certain amino acids. A typical approach for protein pharmaceutical formulation is to combine a sugar alcohol that readily crystallizes and an amorphous diluent; this mixture acts as a lyoprotectant.

Physical Properties: Bulk agents are dissolved in aqueous solution before lyophilization. Therefore chemical purity and the absence of bioburden and pyrogenic materials are essential properties of the bulk excipient. However, the physical form and particle properties of the bulk excipient are generally not relevant to the final properties of the lyophilized formulation.

The physical properties that are essential to product performance during and after lyophilization include the glass transition temperature of the amorphous frozen concentrate before drying, the glass transition temperature of the final dried formulation cake, and the eutectic melting temperature of the crystalline bulking agent with ice. The glass transition temperature of the formulation depends on the glass transition temperatures of the individual components, concentrations, and interactions. Although approximations can be made based on reported transition temperatures for individual components, current practice includes the measurement of formulation glass transition temperatures by thermal analysis or freeze-drying microscopy.

The physical states of the bulking agent during and after lyophilization are important physical properties. Both formulation composition and processing parameters play roles in determining whether the bulking agent is amorphous or takes a specific crystalline form. For example, although mannitol is easily crystallized during lyophilization, it can also be amorphous based on formulation composition or can crystallize as a hydrate or metastable polymorph. Rate of freezing, drying temperatures, and annealing are among the important process parameters used to control the physical state of the formulation and its components. Moisture retention and adsorption after lyophilization also may contribute to formulation stability and performance.

Chemical Properties: Reactivity of the bulking agent with respect to other formulation components, especially the active ingredient, may be critical. Reducing sugars are well known to react with aromatic and aliphatic amines. Glycols may contain trace peroxide levels that can initiate oxidative degradation. The ability of saccharides and polyhydric alcohols to form hydrogen bonds to biopolymers may play a role in their lyoprotection effects.

General Chapters: The following general chapters may be useful in ensuring consistency in selecting bulking agent

functions: *Injections* <1>; *Biotechnology-Derived Articles* <1045>; *Product Formulation*; *Crystallinity* <695>; *Crystallinity Determination by Solution Calorimetry* <696>; *Pharmaceutical Dosage Forms* <1151>; and *Water—Solid Interactions in Pharmaceutical Systems* <1241>.

Functional Category: Tonicity Agent

Description: To avoid crenation or hemolysis of red blood cells and to mitigate pain and discomfort if solutions are injected or introduced into the eyes and nose, solutions should be made isotonic. This requires that the effective osmotic pressure of solutions for injection is approximately the same as that in the blood. When drug products are prepared for administration to membranes such as eyes or nasal or vaginal tissues, solutions should be made isotonic with respect to these tissues.

Functional Mechanism: Tonicity is equal to the sum of the concentrations of the solutes that have the capacity to exert an osmotic force across a membrane and thus reflects overall osmolality. Tonicity applies to the impermeant solutes within a solvent—in contrast to osmolality, which takes into account both permeant and impermeant solutes. For example, urea is a permeant solute, meaning that it can pass through the cell membrane freely and is not factored when determining the tonicity of a solution. In contrast, sodium chloride is impermeant and cannot pass through a membrane without the help of a concentration gradient and will therefore contribute to a solution's tonicity.

Physical Properties: Solutions of sodium chloride, dextrose, and Lactated Ringer's are common examples of pharmaceutical preparations that contain tonicity agents. Not all solutes contribute to the tonicity, which in general depends only on the number of solute particles present in a solution, not the kinds of solute particles. For example, mole for mole, sodium chloride solutions display a higher osmotic pressure than do glucose solutions of the same molar concentration. This is because when glucose dissolves it remains one particle, but when sodium chloride dissolves, it becomes two particles: Na^+ and Cl^- .

Chemical Properties: Tonicity agents may be present as ionic and/or nonionic types. Examples of ionic tonicity agents are alkali metal or earth metal halides such as CaCl_2 , KBr , KCl , LiCl , NaI , NaBr or NaCl , Na_2SO_4 , or boric acid. Nonionic tonicity agents include glycerol, sorbitol, mannitol, propylene glycol, or dextrose.

General Chapters: The following general chapters may be useful in ensuring consistency in selected excipient functions: *Injections* <1>; *Biotechnology-Derived Articles* <1045>; *Product Formulation*; *Pharmaceutical Dosage Forms* <1151>; *Ophthalmic Preparations*; and *Pharmaceutical Calculations in Prescription Compounding* <1160>.

AEROSOLS

Functional Category: Propellant

Description: Propellants are compounds that are gaseous under ambient conditions. They are used in pharmaceuticals (nasal sprays and respiratory and topical formulations), cosmetics, and foods to provide force to expel contents from a container.

Functional Mechanism: Propellant substances are low boiling point liquids that are relatively inert toward active ingredients and excipients. They can be characterized by three properties: whether they form a liquid phase at ambient temperatures and useful pressures, their solubility and/or miscibility in the rest of the formulation, and their flammability. Their performance is judged by their ability to provide

adequate and predictable pressure throughout the usage life of the product.

Propellants that have both a liquid and gas phase in the product provide consistent pressures as long as there is liquid phase present—the pressure in the headspace is maintained by the equilibrium between the two phases. In contrast, the pressure provided by propellants that have no liquid phase may change relatively rapidly as the contents of the container are expelled. As the headspace becomes larger, the pressure within the container falls proportionately. Propellants that have no liquid phase but have significant pressure-dependent solubility in the rest of the formulation have performance characteristics between the other two systems. In such cases, as the headspace increases, the propellant comes out of solution to help to maintain the pressure of the system.

In metered-dose inhalers the propellant has a liquid phase that is an integral part of the dispensed pharmaceutical product. Actuating the metering valve dispenses a defined volume of the liquid contents. The propellant spontaneously boils and provides atomizing and propulsive force. A predictable change in active concentration occurs from the beginning to the end of the container life cycle as the liquid phase of the propellant vaporizes to reestablish the equilibrium pressure of the system as the headspace increases.

Physical Properties: Propellants have boiling points well below ambient temperatures. Density and solubility properties are significant considerations when one selects a propellant. Aflurane and norflurane have liquid-phase densities that are greater than that of water. Hydrocarbon propellants (butane, isobutene, and propane) and dimethyl ether have liquid-phase densities that are less than that of water.

Chemical Properties: Propellants typically are stable materials that contribute to long shelf lives of formulations. However, the hydrocarbon propellants (butane, isobutene, and propane) and dimethyl ether are all flammable materials. Aflurane, carbon dioxide, nitrogen, and norflurane are nonflammable. Nitrous oxide is not flammable but supports combustion. Chlorofluorocarbon propellants are considered to be ozone-depleting substances. Their use in foods, drugs, devices, or cosmetics is regulated by 21 CFR 2.125. Albuterol metered-dose inhalers formulated with chlorofluorocarbon propellants have not been available in the United States since January 1, 2009.

General Chapters: The following general chapters may be useful in ensuring consistency in selected excipient functions: *Aerosols*, *Nasal Sprays*, *Metered-dose Inhalers*, and *Dry Powder Inhalers* <601>; *Chromatography* <621>; and *Water Determination* <921>.

<1061> COLOR—INSTRUMENTAL MEASUREMENT

The observed color (see <631> *Color and Achromicity*) of an object depends on the spectral energy of the illumination, the absorbing characteristics of the object, and the visual sensitivity of the observer over the visible range. Similarly, it is essential that any instrumental method that is widely applicable take these same factors into account.

Instrumental methods for measurement of color provide more objective data than the subjective viewing of colors by a small number of individuals. With adequate maintenance and calibration, instrumental methods can provide accurate and precise measurements of color and color differences that do not drift with time. The basis of any instrumental measurement of color is that the human eye has been

shown to detect color via three “receptors.” Hence, all colors can be broken down into a mixture of three radiant stimuli that are suitably chosen to excite all three receptors in the eye. Although no single set of real light sources can be used to match all colors (i.e., for any three lights chosen, some colors require a negative amount of one or more of the lights), three arbitrary stimuli have been defined, with which it is possible to define all real colors. Through extensive color-matching experiments with human subjects having normal color vision, distributing coefficients have been measured for each visible wavelength (400 nm to 700 nm) giving the relative amount of stimulation of each receptor caused by light of that wavelength. These distribution coefficients \bar{x} , \bar{y} , \bar{z} , are shown below. Similarly, for any color the amount of stimulation of each receptor in the eye is defined by the set of *Tristimulus values* (X, Y, and Z) for that color.

The relationships between the distribution coefficient (see accompanying figure) and the tristimulus values are given in the equations

$$X = \int_0^{\infty} f_{\lambda} \bar{x}_{\lambda} P_{\lambda} d\lambda / Y',$$

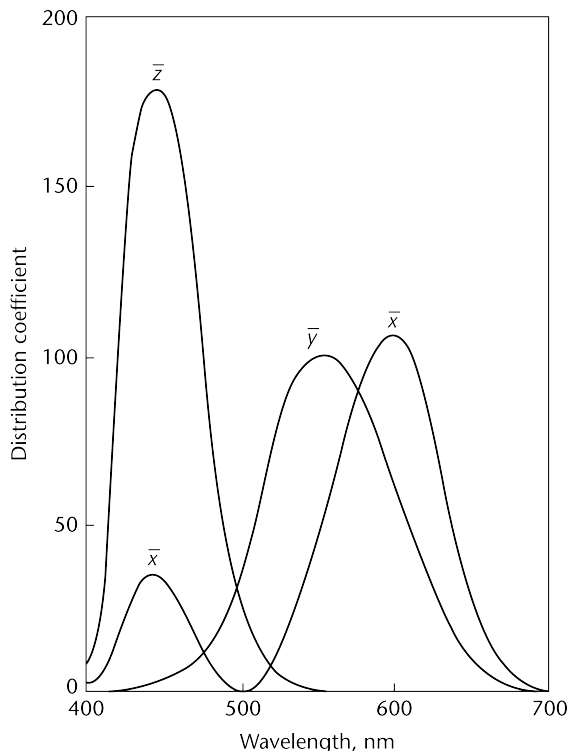
$$Y = \int_0^{\infty} f_{\lambda} \bar{y}_{\lambda} P_{\lambda} d\lambda / Y', \text{ and}$$

$$Z = \int_0^{\infty} f_{\lambda} \bar{z}_{\lambda} P_{\lambda} d\lambda / Y',$$

in which

$$Y' = \int_0^{\infty} \bar{y}_{\lambda} P_{\lambda} d\lambda,$$

is the spectral power of the illuminant, and f_{λ} is either the spectral reflectance (ρ_{λ}) or spectral transmittance (τ_{λ}) of the material.



Distribution Coefficients from 400 to 700 nm

Once the tristimulus values of a color have been determined, they may be used to calculate the coordinates of the color in an idealized three-dimensional color space referred to as a *visually uniform color space*. Many sets of color equa-

tions have been developed in an attempt to define such a space. The equations given in this chapter represent a compromise between simplicity of calculation and conformance with ideality.

The coordinates of a color in a visually uniform color space may be used to calculate the deviation of a color from a chosen reference point. Where the instrumental method is used to determine the result of a test requiring color comparison of a test preparation with that of a standard or matching fluid, the parameter to be compared is the difference, in visually uniform color space, between the color of the blank and the color of the test specimen or standard.

Procedure

The considerations discussed under *Spectrophotometry and Light-Scattering* (851) apply to instrumental color measurement as well. In the spectrophotometric method, reflectance or transmittance values are obtained at discrete wavelengths throughout the visible spectrum, a band width of 10 nm or less being used. These values are then used to calculate the tristimulus values through the use of weighting factors.¹ In the colorimetric method, the weighting is performed through the use of filters.

In the measurement of the spectral reflectance of opaque solids, the angle of viewing is separated from the angle of illumination in such a manner that only rays reflected diffusely from the test specimen enter the receptor. Specular reflection and stray light are excluded.

For the measurement of the spectral transmittance of clear liquids, the specimen is irradiated from within 5 degrees of the normal to its surface, and the transmitted energy measured is that confined within 5 degrees from the normal. The color of solutions changes with the thickness of the layer measured. Unless special considerations dictate otherwise, a layer 1 cm thick should be used.

The methods described here are not applicable to hazy liquids or translucent solids.

CALIBRATION

For purposes of calibration, one of the following reference materials may be used, as required by instrument geometry. For transmittance measurements, purified water may be used as a white standard and assigned a transmittance of 1.000 at all wavelengths. Then the tristimulus values X, Y, and Z for CIE source C are 98.0, 100.0, and 118.1, respectively. For reflectance measurements, opaque porcelain plaques, whose calibration base is the perfect diffuse reflector and whose reflectance characteristics have been determined for the appropriate instrumental geometry, may be used.² If the geometry of sample presentation precludes the use of such plaques, pressed barium sulfate, white reflectance standard grade, may be used.³

After calibration with the above-mentioned materials, it is desirable whenever possible to measure a reference material as close to the color of the sample as possible. If a sample of the material being tested is not suitable for use as a long-term standard, color chips are available⁴ which span the entire visually uniform color space in small increments. The use of such a reference standard is encouraged as a means of monitoring instrument performance even for absolute color determinations.

¹ Typical weighting factors are given by ASTM Z58.7.1-1951 as reported in the Journal of the Optical Society of America, Vol. 41, 1951, pages 431-439.

² Suitable items are available from BYK-Gardner USA, 2431 Linden Lane, Silver Spring, MD 20910, or from Hunter Associates Laboratory, Inc., 11491 Sunset Hills Road, Reston, VA 22090.

³ Suitable material is available from Eastman Kodak Company, Rochester, NY 14650, as "White Reflectance Standard."

⁴ Centroid Color Charts may be obtained from suppliers of instruments for measurement of color.

SPECTROPHOTOMETRIC METHOD

Determine the reflectance or transmittance from 380 to 770 nm at intervals of 10 nm. Express the result as a percentage, the maximum being 100.0. Calculate the tristimulus values X , Y , and Z as follows.

Reflecting Materials—For reflecting materials the quantities X , Y , and Z are

$$X = \sum_{380}^{770} \rho_{\lambda} \bar{x}_{\lambda} P_{\lambda} \Delta\lambda / Y',$$

$$Y = \sum_{380}^{770} \rho_{\lambda} \bar{y}_{\lambda} P_{\lambda} \Delta\lambda / Y', \text{ and}$$

$$Z = \sum_{380}^{770} \rho_{\lambda} \bar{z}_{\lambda} P_{\lambda} \Delta\lambda / Y',$$

in which

$$Y' = \sum_{380}^{770} \bar{y}_{\lambda} P_{\lambda} \Delta\lambda \rho_{\lambda}$$

is the spectral reflectance of the material, $\bar{x}_{\lambda} P_{\lambda}$, $\bar{y}_{\lambda} P_{\lambda}$, and $\bar{z}_{\lambda} P_{\lambda}$ are known values associated with each Standard Source,^{1,2} and $\Delta\lambda$ is expressed in nm.

Transmitting Materials—For transmitting materials, the quantities X , Y , and Z are calculated as above, τ_{λ} (spectral transmittance) being substituted for ρ_{λ} .

COLORIMETRIC METHOD

Operate a suitable colorimeter⁵ to obtain values equivalent to the tristimulus values, X , Y , and Z . The accuracy with which the results obtained from the filter colorimeter match the tristimulus values may be indicated by determining the tristimulus values of plaques of strongly saturated colors and comparing these values with those computed from spectral measurements on a spectrophotometer.

Interpretation

COLOR COORDINATES

The Color Coordinates, L^* , a^* , and b^* are defined by

$$L^* = 116(Y/Y_0)^{1/3} - 16,$$

$$a^* = 500[(X/X_0)^{1/3} - (Y/Y_0)^{1/3}], \text{ and}$$

$$b^* = 200[(Y/Y_0)^{1/3} - (Z/Z_0)^{1/3}]$$

in which X_0 , Y_0 , and Z_0 are the tristimulus values of the nominally white or colorless standard, and $Y/Y_0 > 0.01$. Usually they are equal to the tristimulus values of the standard illuminant, with Y_0 set equal to 100.0. In this case $X_0 = 98.0$ and $Z_0 = 118.1$.

⁵ A suitable tristimulus colorimeter is available from BYK-Gardner USA, 2431 Linden Lane, Silver Spring, MD 20910, or from Hunter Associates Laboratory, Inc., 11491 Sunset Hills Road, Reston, VA 22090.

COLOR DIFFERENCE

The total Color Difference ΔE^* is

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

in which ΔL^* , Δa^* , and Δb^* are the differences in color coordinates of the specimens being compared.

Instrumental variables can influence results. Although reliable comparisons can be made between similar colors measured concomitantly, results obtained on different instruments or under different operating conditions should be compared with caution. If it is necessary to compare data obtained from different instruments or taken at different times, etc., it is very helpful to have concomitant data obtained on a standard reference material such as color chips for opaque materials. Comparison of the readings on the reference material helps to identify variations caused by instrument performance.

<1065> ION CHROMATOGRAPHY

INTRODUCTION

Ion chromatography (IC) is a high-performance liquid chromatography (HPLC) instrumental technique used in USP test procedures such as identification tests and assays to measure inorganic anions and cations, organic acids, carbohydrates, sugar alcohols, aminoglycosides, amino acids, proteins, glycoproteins, and potentially other analytes.

As dictated by the nature of the analyte, IC has been applied to all aspects of the manufacturing and disposition of pharmaceutical products, including characterization of active ingredients, excipients, degradation products, impurities, and process streams. The following sample types are among those that have been analyzed: raw materials, intermediates (including media and culture broths), bulk active ingredients, diluents, formulated products, production equipment cleaning solutions, and waste streams. The technique is especially valuable for ionic or ionizable (in the mobile phase) analytes that have little or no native UV absorbance. The ability to couple the ion-exchange separation with numerous detection strategies, e.g., pulsed amperometric detection (PAD), expands IC applications to instances where analyte-specific detection strategies can provide the required degree of sensitivity or specificity. Utilization of such strategies allows IC applications to be implemented on appropriately configured HPLC systems. Additionally, ion-exclusion separations and pulsed amperometric detection expand the range of application of IC to aliphatic organic acids as well as to nonionic analytes of significant pharmaceutical interest including alcohols, alditols, carbohydrates, and amino acids. The wide dynamic range of the methodology makes it applicable for the quantification of trace contaminants as well as major product components.

Because IC typically uses dilute acids, alkalis, or salt solutions as the mobile phase, and does not use an organic solvent, IC does not require the purchase of costly organic solvents and hazardous disposal of the waste effluent. The effluent can be disposed of after appropriate neutralization (to ~pH 7) and, when necessary, after dilution with water.

IC allows separation using ion exchange, ion exclusion, or ion-pair approaches. IC separations are based on differences in charge density of the analyte species, which in turn depend on the valence and size of the individual ionic species to be measured. Separations are also performed on the basis

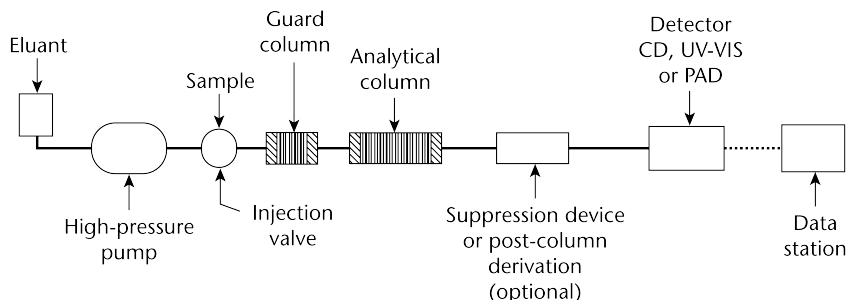


Figure 1. Components of a typical IC system illustrated schematically; CD = conductivity detector and PAD = pulsed amperometric detector.

of differences in the hydrophobic character of the ionic species. IC is typically performed at ambient temperature. As with other forms of HPLC, IC separations are based on varying capacity factors and typically follow the Knox equation. Ion chromatography is a technique complementary to the more commonly used reversed-phase and normal-phase HPLC and to atomic absorption and ion-coupled plasma (plasma spectrochemistry) techniques in pharmaceutical analysis.

APPARATUS

IC instruments closely resemble conventional HPLC instruments. Typical components include an autosampler, a high-pressure pump, an injection valve with a sample loop of suitable size (typically 10 to 250 μL), a guard column, an analytical column, an optional suppressor or other forms of a post-column reaction system, a flow-through detector, and a data system ranging in complexity from an integrator to a computerized data system (Figure 1). Because mobile phases generally consist of dilute acids, alkalis, or salt solutions, the components in contact with the mobile phase and the sample are typically made from inert materials, such as polyetheretherketone. Conventional HPLC systems also may be used provided that their components are compatible with the mobile phase and injected sample solutions. A metal-free system should be used for trace metal analysis. Following suitable preparation, the sample is introduced via the injection valve. After the optional chemical suppression or other post-column reaction on the column effluent, the analyte species are detected using conductivity, amperometry, UV/VIS, or other detection modes. Because IC uses a predominantly ionic mobile phase, a suppressor is often necessary prior to conductometric detection, although non-suppressed conductometric detection has been successfully used in pharmaceutical analysis.

Stationary and Mobile Phases

As IC has developed and matured as an instrumental technique, the number of ion-exchange materials developed for IC has increased, facilitated by the understanding of the processes taking place at the surface of the stationary phase. In contrast to the silica-based column packing prevalent in classical HPLC, organic polymers are predominately used as support materials for IC. Such materials have a higher stability with respect to extremes in pH and in many cases are compatible with organic solvents. Typically, separation of anions requires the use of polymer-based anion exchangers and dilute bases as mobile phases. However, for cation separations, the stability over the entire pH range that is typical of organic polymers is not necessary, because dilute acids serve as mobile phases. Therefore, silica-based cation exchangers that exhibit a significantly higher chromatographic efficiency are commonly used for the separation of cations.

Depending on the separation mode (ion exchange, ion exclusion, or ion-pair), different types of stationary phases

are used. For ion exchange, the stationary phase is either an anion or a cation exchanger. Typically, a strong cation exchanger is used for the ion-exclusion separation of organic acids, and a reversed-phase stationary phase is used when ion-pair is the separation mode. The ion-exchange capacity of a resin is defined as the number of ion-exchange sites per weight equivalent of the column packing and is typically expressed in terms of mEq per g of resin. With ion exchange, the retention times for the analyte ions increase with increasing ion-exchange capacity of the resin. This effect can be partly compensated for by using mobile phases of higher ionic strength. Styrene/divinylbenzene copolymers, polymethacrylate, and polyvinyl resins are the substrate materials used in the manufacturing process of the polymer-based ion exchangers. Organic polymers are functionalized directly at their surface, with the exception of latex-based ion exchangers, where the totally porous latex particle acts as an ion-exchange material. Surface-functionalized, "pellicular" substrates show a much higher chromatographic efficiency compared with the fully functionalized resins.

With ion exchange, a mobile phase consisting of mono- or divalent ionic species, alone or mixed at an optimum ratio, is used to accomplish the separation. In ion-exclusion methods, particularly for organic acids, the mobile phase consists of mineral acids to maintain organic acids in their undissociated forms. Often, the nature of the analyte dictates the mobile phase and the detection mode used. Typical mobile phases used in IC are described below in the section on detectors.

Detectors

Conductivity detection is by far the most commonly employed mode of detection in IC. Although the original IC development work included the use of low-capacity ion-exchange resins for efficient chromatographic separation and conductometric detection of ions in a chemically suppressed mobile phase, the advances in column technologies as well as instrumentation development allow the use of high-capacity ion exchange today.

In suppressed IC, the background conductance of the ionic mobile phase is significantly reduced as it flows through the suppression device. For example, dilute NaOH, about 10 to 50 mM, used as the mobile phase in IC of anions is converted to H_2O (poor conductivity) when the column effluent containing NaOH flows through a suppressor device present in an acidic form. The analyte ionic species in the column effluent are converted from their sodium or other metal salt forms to highly conducting acid forms (due to higher equivalent conductance of hydrogen ions compared to other cations). Analogous reactions occur in the hydroxide form suppressor in IC of cations, wherein the acidic mobile phase is converted to water, and the analyte cations are converted to highly conducting hydroxide forms (due to higher equivalent conductance of hydroxide ions compared to other anions).

The reduced background conductance and the enhanced signal due to the ionic species result in an enhanced signal-

to-noise ratio for the conductometric detection of ions in suppressed IC. This results in reduced background noise and increasing sensitivity and reproducibility of the analysis. The commonly used chemical suppression devices fall into three broad categories. In the first type, the reactions occur across an ion-exchange membrane with the regenerant ions furnished by either a chemical or as products of electrolysis of water. In the second type, the suppression reactions occur in a packed bed of high-exchange capacity resin material, with regeneration either by a chemical or by electrolysis of water. In the third type, although not commonly used, the suppression reactions occur as the eluant stream mixes with the flowing stream of high-capacity resin material.

For pharmaceutical analyses, suppressed conductometric detection may be used for detection of trace ions in high purity waters. The commonly used mobile phases for the separation of anions by suppressed IC include hydroxide ions or a mixture of bicarbonate and carbonate ions. The common mobile phases for separation of cations usually consist of mineral acids or methanesulfonic acid.

Ion-chromatographic analyses also can be performed without chemical suppression, in which case the analytical column effluent flows directly to a conductivity detector. The typical eluants used in nonsuppressed IC are phthalic acid and *p*-hydroxybenzoic acid for the determination of anions and methanesulfonic acid for the determination of cations. The equivalent conductance values of chloride, sulfate, and other common anions are significantly greater than that of the eluant anion, and therefore, a positive peak is detected as the anions are carried through the detector. The equivalent conductance values of sodium, potassium, calcium, magnesium, and other common cations are significantly lower than that of the cation (H^+) in the eluant. In this instance, a negative peak is detected as the cations are carried through the detector.

Nonsuppressed IC is easier to perform, and it is a useful technique for determining ions of weak acids such as cyanide and sulfide, which are nonconductive after chemical suppression but show a higher baseline noise. Pharmaceutical analyses can be performed in the nonsuppressed mode because the quantification limits are usually in the upper mg per L to low percentage levels. While suppressor-based methodologies must often be implemented on the instrument systems specifically designed for this purpose, IC may be performed without the suppressor on an existing HPLC. This is possible because the commonly used eluants in IC include dilute bases or acids that are compatible for use on existing HPLC instruments. When this approach is considered, analysts are encouraged to consult the instrument manufacturer for applicability of the instrument for the IC analysis.

OTHER DETECTORS

Other commonly used detection modes in IC include pulsed amperometry, direct UV detection, or post-column derivatization followed by UV/VIS detection.

Pulsed Amperometric Detection Mode (PAD)—PAD uses a specialized mode of the conventional amperometric technique. This type of detector is commonly used for the detection of electroactive species, e.g., organic compounds such as carbohydrates, sugar alcohols, amino acids, and organic sulfur species. In PAD, analytes are detected by an oxidative desorption process at the surface of an electrode located in the column effluent stream. Following the detection process, a series of potentials are applied for fixed time periods to clean the electrode surface. Unlike conventional amperometry that suffers from electrode surface fouling, a rapidly repeating sequence of different working potentials, referred to as waveform, helps the removal of the products of redox reactions from the electrode surface.

Direct and Indirect UV Detection—Direct UV Detection is used for inorganic and organic ions that possess a UV

chromophore. These include organic acids, bromide, iodide, nitrate, nitrite, thiosulfate, and cyano-metal complexes. Analogous to the inverse conductometric detection of cations, UV detection may also be performed indirectly. This method is called indirect photometric chromatography (IPC).

Photometric Detection—Photometric detection involves chelation of the metal ions in column effluent with a color-forming reagent prior to detection with a visible wavelength. A classic example is the separation of metal ions in which the column effluent is chelated with 4-(2-pyridylazo)-resorcinol followed by detection at 510 to 530 nm.

SAMPLE PREPARATION

Typically sample preparation for IC includes dilution or filtering through a 0.45- μ m filter, or both. Under certain circumstances, samples may require removal of undesirable species through solid-phase extraction (SPE) techniques. For example, a highly alkaline sample can be neutralized by having it pass through an SPE cartridge packed with cation-exchange material in the acidic form.

PROCEDURE

Conductometric detection requires high purity water (generally, resistivity greater than 18 megohm-cm) and high-purity chemicals for the preparation of the mobile phase. For ion-pair separation with UV detection, water and mobile phase components of low UV absorbance should be used.

For ion exchange, the retention time of ions increases with a decrease in the ionic strength and valency (charge) of the mobile phase components. For example, at equimolar concentrations of sodium hydroxide or sodium carbonate mobile phase, capacity factors (k') for anions are smaller with sodium hydroxide as the mobile phase than with sodium carbonate as the mobile phase. Some mobile phases, such as sodium hydroxide, can absorb ambient carbon dioxide, resulting in its composition change and often in baseline artifacts. In this instance, care should be taken to prevent absorption of carbon dioxide by the sodium hydroxide mobile phase.

For ion exclusion, capacity factors of organic acids increase with an increase in ionic strength or concentration of mineral acids but decrease with the increase of the column temperature. Because permeation volume remains constant, these effects are usually small. Addition of a solvent such as acetonitrile shortens the retention of organic acids.

Like other HPLC techniques, IC systems are calibrated by plotting peak responses in comparison with known concentrations of a reference standard, using either an external or internal standardization procedure.

<1066> PHYSICAL ENVIRONMENTS THAT PROMOTE SAFE MEDICATION USE

PURPOSE OF THIS CHAPTER

The work environment has been identified as one of the most commonly reported factors contributing to medication errors reported to the United States Pharmacopeia (USP).

This chapter describes optimal physical environment standards that promote accurate medication use and improve the performance of persons involved in the medication use process (e.g., procurement, prescribing, transcribing, order entry, preparation, dispensing, administration, and monitoring of medications) in any practice setting, including the patient's home. Accuracy and safety of the medication use system is the result of interactions between humans, the physical work environment, equipment employed, and procedures performed. This chapter focuses on one aspect of this system: the characteristics of the physical environment that can promote accurate medication use. Standards are provided when justified by evidence and expert opinion.

DEFINITIONS

Color Rendering Index (CRI)—is an expression of how a light source affects the color appearance of objects or humans compared to how they would appear under a reference light source (29).

Constraint—is a rule stating under what conditions an action is allowed or prohibited. Constraints are used in designing procedures or tools to prevent unsafe practices.

Crowding—occurs when multiple workers utilize the same workspace, adversely affecting the amount of space available for each to organize, and also increasing the negative factors of distractions, interruptions, and noise.

Decibel—a unit used to measure the intensity of a sound by comparing it with a given level on a logarithmic scale, thereby indicating the degree of loudness. The A scale is commonly used when measuring decibels because it most closely represents what the human ear perceives in terms of loudness.

Distractions—occur when there is a continuation of work while responding to anything that diverts or disturbs attention, such as a telephone call or question from a co-worker (19, 38).

Ergonomic Design—refers to a workspace that accommodates each individual's capacities and limitations, allowing them to work safely and efficiently (24). This includes an optimum ambient environment and adjustable furniture.

Forcing Function—is an aspect of a design that prevents a target action from being performed, or that allows its performance only if another specific action is performed first. Forcing functions need not involve device design. One of the first forcing functions identified in healthcare was the removal of concentrated potassium from hospital units. This was designed to eliminate the risk of inadvertent preparation of intravenous solutions with concentrated potassium, an error that has produced a small but stable number of deaths over the years (17).

Human Factors or Ergonomics—the scientific discipline concerned with the understanding of interactions among humans and other elements of a system, and the profession that applies theory, principles, data and methods to design in order to optimize human well-being and overall system performance (30).

Illumination Level—is the rate of light energy emission falling on an area as measured by a photometer with an illuminance sensor in lux or foot-candles (fc) (8) and indicates brightness. A lux is a unit of illuminance, measured in lumens per square meter (34). A foot-candle (fc) is lumens per square foot (28), and is also commonly measured by light meters. The term candela replaced foot-candle as the International System (SI) measure of luminous intensity (29), and represents one lumen per steradian (lm/st).

Interruptions—are the cessation of productive activity before a task is completed and are caused by an externally imposed reason (19).

Lean Production—is the increase of high-quality work output, while eliminating waste and decreasing resources, time, and errors (53).

Medication Safety Zone—is a critical area where medications are prescribed, orders are entered into a computer or transcribed onto paper documents, or where medications are prepared or administered. The characteristics of an optimal physical environment for accurate medication use will apply to medication safety zones.

Noise and Sound—Noise is defined as an auditory stimulus that bears no informational relationship to the task at hand (9, 18). Sound is a change in volume that has some informational relationship to the task at hand (18). A quiet work environment is defined as an area where noise is absent and the worker is free from disturbance.

Override—to neutralize the action of (as an automatic control). See MERRIAM-WEBSTER ONLINE (www.Merriam-Webster.com).

Photometer—an instrument for measuring photometric quantities such as illuminance (28).

Physical Design and Organization of Workspace—accuracy of medication preparation may be influenced by the amount of workspace in which a worker can process one medication order at a time, with only those items involved in the process in the active work area.

Physical Environment—consists of the surroundings that can affect one or more human senses (36).

Workaround—a plan or method to circumvent a problem (as in computer software) without eliminating it. See MERRIAM-WEBSTER ONLINE (www.Merriam-Webster.com).

Working Conditions—include the physical environment, workforce staffing, workflow design, personal/social factors, and organizational factors (25). The focus of this general chapter is on how the physical environment can be designed to improve safe medication use.

FACTORS TO CONSIDER WHEN ASSESSING PHYSICAL ENVIRONMENT NEEDS

There are five work system elements that may affect the importance of meeting the physical environment standards (11, 12, 13, 47):

- (1) Characteristics of the individual performing the work (e.g., visual and hearing acuity, age, experience level, distractibility, and level of attention). Humans vary in their responses to the physical environment. Therefore, the ideal situation is to make it possible to modify the physical environment on an individual basis, so it can be adapted to match the needs of the current user in a way that will optimize the accuracy of his/her performance.
- (2) Tasks performed, and characteristics of these tasks that contribute to unsafe patient care. If the worker is pressured by excessive workload or interruptions, are there opportunities for workarounds or overrides that risk patient safety?
- (3) Tools and technologies used to perform the tasks, and affecting the likelihood of medication errors. Are the tools and technologies readily understandable and available when needed? Is a bar code medication verification system present? Automated medication dispensing devices? Electronic medication administration records (eMAR)? Unit-dose packaging? Ready access to patient- and medication-related clinical information? Are tools and technologies user-friendly? Have they passed usability tests, and failure mode and effects analysis (FMEA)?
- (4) The status of the physical work environment in terms of compliance with the recommendations of this general chapter.
- (5) Support within the organization that promotes or hinders patient safety.

Because of their interrelatedness, work design should consider all these elements. Whenever one work element changes, there will be implications for the other elements (11). This general chapter focuses on recommendations for the physical environment.

PHYSICAL ENVIRONMENT GUIDELINES FOR MEDICATION SAFETY ZONES

Sensory interference resulting from extreme temperatures, noise, poor lighting, glare-producing surfaces, interruptions, or clutter can affect the work of healthcare practitioners and adversely affect working memory (48, 8, 18, 19, and 20). The guidelines described here for the physical environment apply to medication safety zones.

Methods for Assessing the Physical Environment

An illuminance meter (also referred to as a light level meter or photometer) is an instrument that consists of a photodetector and a digital or analog display that measures illuminance in lux or foot-candles (fc) (28). Illuminance meters should be recalibrated annually (29). Lighting levels should be measured in medication safety zones using point illuminance measurements. The photodetector should be placed in the area where the critical medication task is performed (e.g., a work counter medication inspection location), with the worker standing in a normal working position when the measurement is taken (28). Measurements of medication storage areas should include light levels at the top, middle, and bottom shelves, because levels depend on the distance from the lighting source. Photometers are commercially available, or management engineers may be able to provide them.

Sound level meters capable of reading from 30–130 decibels A scale (dBA) should be used to measure sound levels. The A scale is commonly used when measuring decibels because it most closely represents what the human ear hears in terms of loudness. The meters should be calibrated prior to each use. Measurements are taken while standing in a working position, using the instructions provided in the manual for the specific sound meter. Type 1 or Type 2 meters have acceptable levels of accuracy.

Illumination

Proper illumination levels can improve both accuracy and efficiency of performance. Prescription-filling accuracy improved significantly from 96.2% to 97.4% when lighting levels in a busy outpatient pharmacy were increased from 450 to 1460 lux (45 to 146 fc) (8). One study found that pharmacists rating lighting levels as at least adequate detected 38% more errors when filling prescriptions (22). In addition, as visual fatigue increases over a shift, more light is needed. Pharmacists using task lights to increase illumination had a 10.7% reduction in product verification errors (22). A study of luminance in homes, offices, and public places found lower levels than recommended for reading, and age affected performance in different lighting conditions (14). Efforts should be made to prevent medication errors caused directly or indirectly by low lighting. For example, one incident report showed that poor lighting contributed to improperly connected patient controlled analgesia (PCA) administration tubing, causing medication to run onto the floor, resulting in uncontrolled patient pain (27). Low lighting contributed to difficulty in seeing that the tubing was not connected properly. A study of lighting in a retail pharmacy detected an error in strength and dosage form: dicyclomine 10-mg capsules were used to fill a prescription for 20-mg tablets. The light level at the shelf

where the medications were stored was 220 lux (22 fc) (20).

The recommendations described here consider the level of task visibility required, the need for speed and accuracy during medication handling, and worker comfort (33). Architects and lighting engineers can consult the Illuminating Engineering Society of North America (IESNA) reference "Lighting for Hospitals and Healthcare Facilities" for details about lighting medication areas (29). It is important to note that the illuminance levels recommended in the IESNA reference are below those listed in this standard, because of evidence of relationships between higher lighting levels and medication errors. Fluorescent cool white deluxe lamps or compact fluorescent lamps are recommended, because they have a color rendering index of 80 or more (28, 29). Fluorescent lamps also have a high efficacy, and emit more lumens per watt than incandescent lamps (29). The recommended color rendering index can help avoid misidentification of medications.

Task lighting is required in areas where critical visual tasks are performed if illuminance levels are below recommendations. If task lighting is not available, then workers can cast shadows on the workspace, resulting in lower lighting levels (29). Critical tasks include reading small print on labels and handwritten prescriptions, and inspecting medication dosage forms. Because individuals perceive lighting levels differently, adjustable 50-watt high-intensity task lights are recommended when difficult-to-read prescriptions and product labels (e.g., unit-dose package labels) are encountered (22). Key healthcare provider work areas for which lighting levels are important are computer order entry (e.g., physicians or pharmacists), prescription filling, inspection, and patient counseling. Illumination levels for computer order entry areas should be at least 750 lux (75 fc). Higher levels are recommended when handwritten orders are read—1000 lux (100 fc) are recommended in these situations. Lighting should be positioned so there is no glare on the computer monitor that may make it difficult to view the screen accurately (44). Prescription preparation areas, medication inspection stations (double-checking), and counseling areas should have illumination levels between 900 and 1500 lux (90 and 150 fc) (8, 20). These standards are all above the minimum of 200 lux (20 fc) for accurate reading of medication labels set by the American Society for Testing and Materials International (ASTM International). An ASTM International standard prescribes a legibility test requiring that the name and amount of the drug on the label be legible in 20 fc of light at a distance of about 20 inches (500 mm) by a person with 20/20 unaided or corrected vision (3). Lighting levels should be increased where the work force has an average age beyond 45 years to optimize legibility (general recommendation for treatment of presbyopia) (32). Healthcare providers should also have a magnifying glass available to assist in the careful reading of labels with very small script and in situations where low lighting levels are unavoidable. Using a magnification lens along with a task light reduced pharmacist product verification errors by 22% compared to a control group (22).

Key medication-related nursing work areas for which lighting is important include the following: medication order review, medication selection, preparation, and administration. These tasks may take place in one or more locations on the nursing unit, such as the nursing station where patient charts are stored, the medication room, or a patient's room. Transitional lighting is recommended for medication areas on nursing stations and other patient care units to avoid dark and bright spots located next to dimly lit areas. Luminance should enable good color rendering (color rendering index of 80 or more) to assist with proper medication checking (29). Task lighting can help achieve appropriate levels of lighting and should be included on mobile medication carts (including those used with bar code medication verification systems). Glare should be controlled by ensuring that light reflections that can wash out the screen and make it difficult to read are not visible in computer monitors (29).

Illumination levels for medication rooms located on nursing units should be at least 1000 lux (100 fc) based on the complexity of the task, reading small type font on medication packages, and the need for accuracy and speed (28, 44). The higher range of the lighting level should be used when the task requires reading small print. Lighting level recommendations are summarized in *Table 1*. Lighting levels can decrease by 25% over a 2-year period, so it is important that lighting fixtures are cleaned routinely to maintain recommended luminance levels. Lighting levels should be measured on a quarterly basis. Burned out or flickering bulbs should be promptly replaced (29).

Proper lighting is also essential at the point of care. Attempting to be patient- and family-friendly may run contrary to the necessary lighting conditions for safe medication administration. Administration of medication at night under low luminance to avoid disturbing the patient or family is an unsafe practice. Task or spot lighting must be available, so that visual confirmation of the correct patient (reading armband), medication, and administration site is not compromised.

Interruptions and Distractions

Workplace designers need to be keenly aware of the significant impact that interruptions and distractions can have on accurate patient care, so that they design workspaces to counter these effects. Distraction from competing tasks is likely to impair performance in several ways, such as sensory/perceptual interference (you don't hear the alarm because a coworker interrupts with a question), cognitive cost of switching tasks (you respond to an alarm more slowly because it takes time to reorient to the alarmed task after a coworker's question), or prospective memory failure (you forget to perform a step because you forget where you left off when returning to the task after interruption). Countermeasures may address some or all of these problems (e.g., use of checklists). Nurses frequently cite distractions and interruptions as contributing to the incidence of medication errors (27, 51, 52). Interruptions and distractions have been associated with higher prescription dispensing error rates in an ambulatory pharmacy (19). According to the 2008 USP MEDMARX Data Report, distractions continue to rank high (approximately 45%) as contributing to medication errors in hospitals and health systems (27).

Interruptions and distractions can be prevented by providing staff with the ability to control and manage their exposure to these disturbances. Workers can be allowed to adjust features of the medication safety zone to maximize their concentration and attention levels, and to optimize their performance. Adjustable features include provision of a work station that is protected from interruptions and distractions, such as a separate medication room, or a mobile cart with workspace for those that are not adversely affected by distractions. Individuals have different levels of distractibility—workers should be sensitive to their own need for a distraction-free work area (19). Heightened worker awareness of the adverse impact of interruptions and distractions can

help minimize problems. Workers can be trained in how to avoid interrupting coworkers for nonurgent reasons, while their coworkers are performing medication-related tasks. Coworkers asking for assistance were found to be the most frequent source of interruptions in a pharmacy study (19). Techniques to decrease interruptions and distractions include visual cues (such as wearing orange safety vests), physical barriers (e.g., preparing doses in a medication room), and the use of checklists that assist attention focus or refocus (38). Medication safety zones should be located in areas where the potential for distraction and interruption is minimized.

Sound and Noise

The Environmental Protection Agency (EPA) recommends peak sound levels of 45 dB during the day and 35 dB at night in hospitals (10). The World Health Organization (WHO) guidelines state that background sound levels in a patient room should not exceed 35 dB (5). The International Noise Council recommends 45 dB during the day and 20 dB at night for acute care areas (10). Ear protection is required when workers are exposed to sound levels averaging 90 dB.

The standard for sound levels in medication safety zones is set at the level of conversation, 50 dBA. This is intended to ensure that critical verbal information can be heard accurately (7). Healthcare providers should be sensitive to their individual need for quiet, depending on the task being performed, and they should have a quiet area available to promote accurate performance. The total elimination of noise in patient-care settings is not feasible or desirable. Patient counseling areas in pharmacies should include sound-reduction methods to enhance audibility and learning—for example, use of a closed room.

Noise is recognized as a serious health hazard to hospitalized patients, and as an interference with effective work performance. Most studies of the effects of noise in the work environment have been conducted in non-healthcare settings. However, noise levels as a contributing factor of stress for nurses is increasingly being documented. In healthcare facilities, sources of noise can range from overhead paging systems, equipment alarms, heating, ventilation, air-conditioning (HVAC) systems, plumbing, televisions, and radios to ice machines (5). Noise has been cited as one obstacle to the effective performance of nurses (23). An in-depth study developed a noise map of a hospital, and found sound levels of 55 dB, which is 10–20 dB above EPA recommendations, depending on the time of day. Average sound levels in other hospitals have been measured between 45 and 68 dB, with peaks between 85 and 90 dB (50). A study of sound levels during shift changes measured 113 dB (15).

The following sound-related features may affect accuracy when dispensing medication: predictability; controllability (16); type of task (simple vs. complex) (6); multitasking; distraction due to noise (which may mask environmental cues and the worker's internal voice, used to rehearse and recall important tasks) (39, 40). Out of 58 studies, 7 showed that

Table 1. Lighting Level Recommendations for Healthcare Settings

Work Area	Illumination Level	
	Lux	Foot-Candle (fc)
Computer order entry (44, p. 408)	1000	100
Handwritten order processing (44, p. 408)	1000	100
Medication filling and checking (pharmacy) (8, 20)	900–1500	90–150
Patient counseling (pharmacy) (8, 20)	900–1500	90–150
Sterile compounding and preparation (8)	1000–1500	100–150
Pharmacy medication storeroom (29)	500	50
Medication preparation area, e.g., nursing station (2)	1000	100
Medication administration work area (e.g., cart surface, patient room) (2)	1000	100

noise improved performance, while 29 showed that it impaired performance (27). Unpredictable but controllable sounds and noise were found in one study to improve prescription filling accuracy, contrary to previous research (18). This may indicate that some environmental stimuli are needed to maintain proper alertness and attention of workers. Researchers are attempting to identify optimal levels of arousal due to sound and noise for people performing different kinds of tasks (e.g., Yerkes-Dodson law) (54).

Noise and other sensory interference can be reduced by employing activities, tools, and principles developed by human factors and engineering experts—many of these principles are already being used by some healthcare organizations. The effect of these and other design characteristics of nursing workspaces on patient outcomes and facility performance are being studied as part of a research project (http://www.pebbleproject.org/pebble_data.php) sponsored by the Center for Health Design, a nonprofit research and advocacy organization, and a network of 11 healthcare providers. The project reported decreases in medical errors, as well as decreases in patient transfers, nosocomial infections, patient falls, and medication usage (49). When permitted by infection control guidelines, reducing noise by installing materials that absorb sound (e.g., ceiling and wall materials, and carpeting) can be accomplished at modest cost. Acoustical engineers can provide additional methods for noise reduction. Workers who don't have to respond to any audible signals such as telephone calls or alarms may be able to wear noise-canceling headphones and listen to music, provided that performance is not adversely affected.

Physical Design and Organization of Workspace

Ergonomic design of the workplace environment can influence the ability of providers to effectively utilize information and accurately perform tasks (2). Counter height, height of supplies, and lighting changes in lower drawers and cabinets that decrease visibility of products can contribute to errors if improperly adjusted. The provision of adjustable fixtures and counter heights can improve efficiency as well as safety. Work counter clutter is typically an indicator of disorganization and a lack of sufficient space to perform key tasks. One study found that more dispensing errors occurred when medication storage containers were placed on shelves in a cluttered fashion (less than 1 inch between distinct drugs) (20). Older workers have more difficulty discriminating between different items on a cluttered work surface, which is an important consideration given the increasing average age of nurses (35).

Medication Safety Zones

A medication safety zone is defined as a critical area where medications are prescribed, orders are entered into a computer or transcribed onto paper documents, and where medications are prepared, dispensed, or administered. Examples include the work surface of a medication cart on a nursing unit, any location where prescribing decisions are made, the work surface of an automated medication dispensing device, a pharmacy where prescriptions are prepared, inspected, and dispensed, and patient homes where medications are prepared, administered, or consumed. The patient's bedside in a hospital is another important medication safety zone that presents unique challenges. The physical environment of the operating room during surgery is an area that deserves special attention due to increased noise levels and distractions when critical life-and-death medications are in use.

One critically important medication safety zone for nurses is the medication preparation and administration area, which should be analogous to the cockpit of an airplane. Information must be readily available and user-friendly in

order to increase ease of information synthesis. Access to medication-related information should be efficient, with materials and records readily available at the proper sites (i.e., drug information and patient-specific information used to make a decision about drug administration should be near each other to support fact finding) (2). Information and components within the space should be arranged according to specific principles that promote correct choices and decrease distractions when seeking information.

As described in the human factors literature (45), these principles include the following:

Importance Principle—Important components should be placed in convenient locations. This includes information systems near the medication safety zone so that lab results, drug information, vital signs, and pertinent patient information are readily obtained. Information regarding equipment function and trouble-shooting should be located near or on the equipment to provide clarification or a quick answer to questions that arise.

Frequencies of Use Principle—Items that are used frequently are easily found and accessible. This prevents workarounds, in which alternate equipment is used as a substitute.

Function Principle—Items that are related to a function are grouped together. Examples include syringes, needles, and alcohol swabs; and IV tubing and connectors that are used in preparation of infusions. It is important to ensure that appropriate supply levels are maintained and that product expiration dates and applicable storage conditions (e.g., temperature-sensitive products) are routinely checked.

Sequence of Use Principle—Items are placed in an order that supports the sequence needed to perform the task correctly (e.g., sterile gloves are in or with sterile dressing kits; needleless connectors are with the IV administration sets; and epidural medications and epidural supplies are all in one place).

Methods for workplace analysis are available (31). Bedside medication administration areas should follow the same design as the centralized medication safety zone. Distractions are an even greater challenge at the bedside, and measures should be taken to minimize them whenever possible. Information and supplies should follow the same principles and be placed in an uncluttered area with adequate lighting. Sharps containers should be placed within easy reach and out of high-traffic areas. Each bedside work station should be standardized in design, so that information and supplies do not need to be relocated when moving from one patient bed to another.

The incorporation of lean operation techniques to enhance desirable, value-added activities and eliminate the undesirable, often invisible activities that result in waste in the work process is one approach to workspace redesign (7). An efficient and effective workplace is less conducive to errors. Lean operational techniques that eliminate waste and improve timeliness include the following:

- **Visual Controls**—keeping work processes and indicators in view to allow everyone to understand the status of the work system at a glance.
- **Streamlined Layout**—optimizing the sequencing of work processes through facility design.
- **Point-of-Use Storage**—locating supplies, equipment, information, and procedure rules in convenient, easily accessible locations (37).

Simplifying and standardizing the patient-care environment and equipment decrease the cognitive load, making slips and lapses less likely to occur during routine tasks by minimizing decision and manipulation time (37). Standardization can be used for facility and room design, medical equipment (e.g., IV infusion devices), and medication areas (e.g., medication delivery and storage of patient-specific medications). Ensuring ready access to clinical information, both patient-specific and medication-related, is essential for all areas in which steps in the medication-use process occur.

Another medication safety zone design approach is to involve workers in innovating solutions to work station problems. There is a need to incorporate flexibility into medication safety zone design in order to support worker innovation (41).

Medication safety-related tools and technologies, such as automated drug dispensing devices with point-of-care bar code verification and an integrated electronic medication record, can decrease or avert medication errors. Constraint and forcing functions are an effective means of reducing error, particularly for high-risk medications and situations. The simplest of these do not require technology. For example, sealing neuromuscular blockers in an intubation kit lessens the chance of a paralyzing agent being administered to a patient without a means of ventilation support. An enteral product that is physically unable to connect to an intravenous tubing luer lock connector would avert a wrong route error, even if the nurse was working in low-light conditions and initially misidentified the intended route for the tubing (46).

The availability of medication safety technology is never a substitute for safe medication practices within a medication safety zone. Reports have warned of errors as a result of ignoring or overriding safety checks such as smart infusion pump drug libraries and alarms (42).

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(1072) DISINFECTANTS AND ANTISEPTICS

INTRODUCTION

A sound cleaning and sanitization program is needed for controlled environments used in the manufacture of Pharmacopeial articles to prevent the microbial contamination of these articles. Sterile drug products may be contaminated via their pharmaceutical ingredients, process water, packaging components, manufacturing environment, processing equipment, and manufacturing operators. Current Good Manufacturing Practices (cGMPs) emphasize the size, design, construction, and location of buildings and construction materials, and the appropriate material flow to facilitate cleaning, maintenance, and proper operations for the manufacture of drug products. When disinfectants are used in a manufacturing environment, care should be taken to prevent the drug product from becoming contaminated with chemical disinfectants as a result of the inherent toxicity of the disinfectants. The requirements for aseptic processing include readily cleanable floors, walls, and ceilings that have smooth and nonporous surfaces; particulate, temperature, and humidity controls; and cleaning and disinfecting procedures to produce and maintain aseptic conditions. The cleaning and sanitization program should achieve specified cleanliness standards, control microbial contamination of products, and be designed to prevent the chemical contamination of pharmaceutical ingredients, product-contact surfaces and/or equipment, packaging materials, and ultimately the drug products. These principles also apply to nonsterile dosage forms where the microbial contamination is controlled by the selection of appropriate pharmaceutical ingredients, utilities, manufacturing environments, sound equipment cleaning procedures, products especially formulated to control water activity, inclusion of suitable preservatives, and product packaging design.

In addition to disinfectants, antiseptics are used to decontaminate human skin and exposed tissue and may be used by personnel prior to entering the manufacturing area. Chemical sterilants may be used to decontaminate surfaces in manufacturing and sterility testing areas. Furthermore, sterilants may be used for the sterilization of Pharmacopeial articles, and UV irradiation may be used as a surface sanitizer.

This general information chapter will discuss the selection of suitable chemical disinfectants and antiseptics; the demonstration of their bactericidal, fungicidal, and sporicidal efficacy; the application of disinfectants in the sterile pharmaceutical manufacturing area; and regulation and safety considerations. Biofilm formation and its relationship to disinfectants are outside the scope of this chapter. Additional

information not covered in the chapter may be obtained from standard texts on disinfectants and antiseptics.¹

DEFINITIONS

Antiseptic—An agent that inhibits or destroys microorganisms on living tissue including skin, oral cavities, and open wounds.

Chemical Disinfectant—A chemical agent used on inanimate surfaces and objects to destroy infectious fungi, viruses, and bacteria, but not necessarily their spores. Sporicidal and antiviral agents may be considered a special class of disinfectants. Disinfectants are often categorized as high-level, intermediate-level, and low-level by medically oriented groups based upon their efficacy against various microorganisms.

Cleaning Agent—An agent for the removal from facility and equipment surfaces of product residues that may inactivate sanitizing agents or harbor microorganisms.

Decontamination—The removal of microorganisms by disinfection or sterilization.

Disinfectant—A chemical or physical agent that destroys or removes vegetative forms of harmful microorganisms when applied to a surface.

Sanitizing Agent—An agent for reducing, on inanimate surfaces, the number of all forms of microbial life including fungi, viruses, and bacteria.

Sporicidal Agent—An agent that destroys bacterial and fungal spores when used in sufficient concentration for a specified contact time. It is expected to kill all vegetative microorganisms.

Sterilant—An agent that destroys all forms of microbial life including fungi, viruses, and all forms of bacteria and their spores. Sterilants are liquid or vapor-phase agents.

Microorganisms differ greatly in their resistance to disinfection agents. The order of resistance of clinically significant microorganisms to chemical disinfectants from most to least resistant is listed in Table 1.

Table 1. The Resistance of Some Clinically Important Microorganisms to Chemical Disinfectants (Listed in Order of Decreasing Resistance)

Type of Microorganisms	Examples
Bacterial spores	<i>Bacillus subtilis</i> and <i>Clostridium sporogenes</i>
Mycobacteria	<i>Mycobacterium tuberculosis</i>
Nonlipid-coated viruses	Poliovirus and rhinovirus
Fungal spores and vegetative molds and yeast	<i>Trichophyton</i> , <i>Cryptococcus</i> , and <i>Candida</i> spp.
Vegetative bacteria	<i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> , and <i>Salmonella</i> spp.
Lipid-coated viruses	Herpes simplex virus, hepatitis B virus, and human immunodeficiency virus

CLASSIFICATION OF DISINFECTANTS

Chemical disinfectants are classified by their chemical type. This includes aldehydes, alcohols, halogens, peroxides, quaternary ammonium compounds, and phenolic compounds (see Table 2).

¹Ascenzi, J.M., Ed. *Handbook of Disinfectants and Antiseptics*, 5th ed.; Marcel Dekker: New York, 1995; Block, S.S., Ed. *Disinfection, Sterilization, and Preservation*, 5th ed.; Lippincott Williams & Wilkins Publishers: Philadelphia, 2000. Russell, A.D.; Hugo, W.B.; Ayliffe, G.A.J., Eds. *Principles and Practices of Disinfection, Preservation and Sterilization*, 3rd ed.; Blackwell Science Inc.: London, 1999.

Table 2. General Classification of Antiseptics, Disinfectants, and Sporocidal Agents

Chemical Entity	Classification	Example
Aldehydes	Sporicidal agent	2% Glutaraldehyde
Alcohols	General purpose disinfectant, antiseptic, antiviral agent	70% Isopropyl alcohol, 70% alcohol
Chlorine and sodium hypochlorite	Sporicidal agent	0.5% Sodium hypochlorite
Phenolics	General purpose disinfectant	500 µg per g Chlorocresol, 500 µg per g chloroxylenol
Ozone	Sporicidal agent	8% Gas by weight
Hydrogen peroxide	Vapor phase sterilant, liquid sporicidal agent, antiseptic	4 µg per g H ₂ O ₂ vapor, 10%–25% solution, 3% solution
Substituted diguanides	Antiseptic agent	0.5% Chlorhexidine gluconate
Peracetic acid	Liquid sterilant, vapor phase sterilant	0.2% Peracetic acid, 1 µg per g peracetic acid
Ethylene oxide	Vapor-phase sterilant	600 µg per g Ethylene oxide
Quaternary ammonium compounds	General purpose disinfectant, antiseptic	Concentration dependent on application, Benzalkonium chloride
β-Propiolactone	Sporicidal agent	100 µg per g β-Propiolactone

The effectiveness of a disinfectant depends on its intrinsic biocidal activity, the concentration of the disinfectant, the contact time, the nature of the surface disinfected, the hardness of water used to dilute the disinfectant, the amount of organic materials present on the surface, and the type and the number of microorganisms present. Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Environmental Protection Agency (EPA) registers chemical disinfectants marketed in the United States and requires manufacturers to supply product information on the use dilution, type of microorganisms killed, and the necessary contact time. Certain liquid chemical sterilizers intended for use on critical or semicritical medical devices are defined and regulated by the U.S. Food and Drug Administration (FDA).

SELECTION OF AN ANTISEPTIC FOR HAND AND SURGICAL SITE DISINFECTION

Hands and surgical sites are disinfected in a hospital setting to reduce the resident flora and to remove transient flora (e.g., *Streptococcus pyogenes*) and methicillin-resistant *S. aureus* and *P. aeruginosa* that have been implicated in hospital-associated infection. Use of antiseptics to disinfect hands has been shown to be more effective than soap and water in reducing the counts of bacteria on the skin; repeated antiseptic use further reduces these counts. These principles may be applied to clean-room operators in the pharmaceutical industry.

Common antiseptics include 4% chlorhexidine, 10% povidone-iodine, 3% hexachlorophene, 70% isopropyl alcohol, and 0.5% chlorhexidine in 95% alcohol.

SELECTION OF A DISINFECTANT FOR USE IN A PHARMACEUTICAL MANUFACTURING ENVIRONMENT

When selecting a disinfectant for use in a pharmaceutical manufacturing area, the following points should be considered: the number and types of microorganisms to be con-

trolled; the spectrum of activity of commercially available disinfectants; the claims as a sterilant; the disinfectant or sanitizer supported by the EPA registrations; the concentration, application method, and contact time of the disinfectant; the nature of the surface material being disinfected and its compatibility with the disinfectant; the amount of organic compounds on the surface that may inactivate a disinfectant; the possible need to maintain a residual bactericidal activity of the disinfectant on the surface; the corrosiveness of the disinfectant to equipment with repeated application; the safety considerations for operators applying the disinfectant; the compatibility of the disinfectant with cleaning agents and other disinfectants; the planned disinfectant rotation; and the steps that need to be taken to avoid the contamination of pharmaceutical products by a disinfectant.²

THEORETICAL DISCUSSION OF DISINFECTANT ACTIVITY

Plots of the log of the number of microorganisms per mL surviving in a disinfectant solution indicate that first-order kinetics can be applied as a gross approximation to the reduction in microbial count with respect to time. In practice, the plots show a more sigmoid curve with a slower initial reduction in numbers followed by an increasing rate with respect to time.

The rate constant, K , for the disinfection process can be calculated by the formula:

$$(1/t)(\log N_0/N)$$

in which t is the time, in minutes, for the microbial count to be reduced from N_0 to N ; N_0 is the initial number of organisms, in cfu per mL; and N is the final number, in cfu per mL, of organisms.

As with a first-order chemical reaction, the same concentration of disinfectant reduces the number of organisms more rapidly at elevated temperatures. This can be expressed as a temperature, T , coefficient per 10° rise in temperature, Q_{10} , calculated by the formula:

$$\text{Time to decontamination at } T^\circ / \text{Time to decontamination at } T$$

in which T is $T^\circ - 10$.

Further evidence that a first-order reaction is an inadequate description of disinfection is that the Q_{10} values for chemical and enzyme reactions are 2 to 3, while the common disinfectants phenol and alcohol have a Q_{10} of 4 and 45, respectively.

Critical to the successful employment of disinfectants is an understanding of the effect of disinfectant concentration on microbial reduction. A plot of the log of the time to reduce the microbial population in a standard inoculum to zero against the log of the disinfectant concentration is a straight line with the slope of the line termed the concentration exponent, n . The relationship can be expressed as follows:

$$n = (\log \text{ of the kill time at concentration } C_2) - (\log \text{ of the kill time at concentration } C_1) / (\log C_1 - \log C_2)$$

in which C_1 and C_2 are the higher and lower disinfectant concentrations, respectively.

The wide differences in concentration exponents, n , have practical consequences in picking the use dilution of different disinfectants and in using dilution to neutralize a disinfectant in disinfectant-effectiveness testing and routine microbial monitoring of the manufacturing environment. For example, mercuric chloride has a concentration exponent of 1, so a 3-fold dilution will reduce the disinfectant activity by

3^1 (or by one-third), while phenol with a concentration exponent of 6 will have a 3^6 (or a 729-fold) reduction in disinfectant activity. Disinfectants with a larger concentration exponent or dilution coefficient rapidly lose activity when diluted. The concentration exponents for some disinfectants are listed in Table 3.

Table 3. Concentration Exponents of Common Antiseptics, Disinfectants, and Sterilants

Disinfectant	Concentration Exponents
Hydrogen peroxide	0.5
Sodium hypochlorite	0.5
Mercuric chloride	1
Chlorhexidine	2
Formaldehyde	1
Alcohol	9
Phenol	6
Quaternary ammonium compounds	0.8 to 2.5
Aliphatic alcohols	6.0 to 12.7
Phenolic compounds	4 to 9.9

Another important consideration may be the pH of the disinfectant. Many disinfectants are more active in the ionized form, while others are more active in the nonionized form. The degree of ionization will depend on the pK_a of the agent and the pH of the disinfection environment. For example, phenol, with a pK_a of 10, will be more effective at a pH below 7 where it is nonionized.

MECHANISM OF DISINFECTANT ACTIVITY

Table 4 lists the sites and modes of action of some representative disinfectants.

Table 4. Mechanism of Disinfectant Activity Against Microbial Cells

Target	Disinfectant
Cell wall	Formaldehyde, hypochlorite, and glutaraldehyde
Cytoplasmic membrane, action on membrane potential	Anilides and hexachlorophene
Membrane enzymes, action on electron-transport chain	Hexachlorophene
Action on ATP	Chlorhexidine and ethylene oxide
Action on enzymes with -SH groups	Ethylene oxide, glutaraldehyde, hydrogen peroxide, hypochlorite, and iodine
Action on general membrane permeability	Alcohols, chlorhexidine, and quaternary ammonium compounds
Cell contents, general coagulation	Chlorhexidine, aldehydes, and quaternary ammonium compounds
Ribosomes	Hydrogen peroxide
Nucleic acids	Hypochlorites
Thiol groups	Ethylene oxide, glutaraldehyde, hydrogen peroxide, and hypochlorite
Amino groups	Ethylene oxide, glutaraldehyde, and hypochlorite
General oxidation	Hypochlorite

MICROBIAL RESISTANCE TO DISINFECTANTS

The development of microbial resistance to antibiotics is a well-described phenomenon. The development of microbial resistance to disinfectants is less likely to occur at significant levels, as disinfectants are more powerful biocidal agents than antibiotics. In addition, they are normally applied in

²Denny, V.F.; Marsik, F.J. Current Practices in the Use of Disinfectants within the Pharmaceutical Industry. *PDA J. of Pharmaceutical Sci. and Tech.*, 1997, 51, (6), 227-228.

high concentrations against low populations of microorganisms usually not growing actively, so the selective pressure for the development of resistance is less profound. However, the most frequently isolated microorganisms from an environmental monitoring program may be periodically subjected to use-dilution testing with the agents used in the disinfection program to confirm their susceptibility, as there are real differences among different species in resistance to the lethal effects of different sanitizers.

DISINFECTANT CHALLENGE TESTING

Under FIFRA, the EPA requires companies that register public health antimicrobial pesticide products including disinfectants, sanitization agents, sporicidal agents, and sterilants to ensure the safety and effectiveness of their products before they are sold or distributed. Companies registering these products must address the chemical composition of their product, include toxicology data to document that their product is safe if used as directed on the label, include efficacy data to document their claims of effectiveness against specific organisms and to support the directions for use provided in the labeling, and provide labeling that reflects the required elements for safe and effective use. While these directions provide valuable information, they may not be helpful in terms of the products' use as disinfectants in a manufacturing environment.

In the United States, the official disinfectant testing methods are published by AOAC International³ and include the Phenol-Coefficient Test, Use-Dilution Method Test, Hard Surface Carrier Method, and Sporidical Carrier Test. A scientific study submitted for EPA review in support of disinfectant registration must be conducted at a laboratory facility that follows the Good Laboratory Practices (GLP) regulations (21 CFR 58). To demonstrate the efficacy of a disinfectant within a pharmaceutical manufacturing environment, it may be deemed necessary to conduct the following tests: (1) use-dilution tests (screening disinfectants for their efficacy at various concentrations and contact times against a wide range of standard test organisms and environmental isolates); (2) surface challenge tests (using standard test microorganisms and microorganisms that are typical environmental isolates, applying disinfectants to surfaces at the selected use concentration with a specified contact time, and determining the log reduction of the challenge microorganisms); and (3) a statistical comparison of the frequency of isolation and numbers of microorganisms isolated prior to and after the implementation of a new disinfectant. This is considered necessary because critical process steps like disinfection of aseptic processing areas, as required by GMP regulations, need to be validated, and the EPA registration requirements do not address how disinfectants are used in the pharmaceutical, biotechnological, and medical device industries. For the surface challenge tests, the test organisms are enumerated using swabs, surface rinse, or contact plate methods. Neutralizers that inactivate the disinfectants should be included in either the diluent or microbiological media used for microbial enumeration or both. Information on disinfectant neutralization may be found in *Validation of Microbial Recovery from Pharmacopeial Articles* (1227).

The disinfectant efficacy test must have realistic acceptance criteria. In practice, sufficient organisms need to be inoculated on a 2-inch × 2-inch square of the surface being decontaminated, i.e., a coupon, to demonstrate at least a 2 (for bacterial spores) to 3 (for vegetative bacteria) log reduction during a predetermined contact time (i.e., 10 minutes over and above the recovery observed with a control disinfectant application). The efficacy of the neutralizers and their ability to recover inoculated microorganisms from the material should be demonstrated during the use-dilution or surface-challenge studies. Points to remember are that disinfectants are less effective against the higher numbers of mi-

croorganisms used in laboratory challenge tests than they are against the numbers that are found in clean rooms (see *Microbiological Control and Monitoring of Aseptic Processing Environments* (1116)); that inocula from the log growth phase that are typically employed in laboratory tests are more resistant, with the exception of spores formed during the static phase, than those from a static or dying culture or stressed organisms in the environment; and that microorganisms may be physically removed during actual disinfectant application in the manufacturing area.

Although not all inclusive, typical challenge organisms that may be employed are listed in Table 5.

Table 5. Typical Challenge Organisms

AOAC Challenge Organisms	Typical Environmental Isolates
Bactericide: <i>E. coli</i> , ATCC 11229; <i>S. aureus</i> , ATCC 6538; <i>P. aeruginosa</i> , ATCC 15442	Bactericide: <i>M. luteus</i> , <i>S. epidermidis</i> , <i>Corynebacterium jeikeium</i> , <i>P. vesicularis</i>
Fungicide: <i>C. albicans</i> , ATCC 10231 or 2091; <i>Penicillium chrysogenum</i> , ATCC 11709; <i>A. brasiliensis</i> , ATCC 16404	Fungicide: <i>P. chrysogenum</i> , <i>A. brasiliensis</i>
Sporicide: <i>B. subtilis</i> , ATCC 19659	Sporicide: <i>B. spizizenii</i> , <i>B. thuringiensis</i>

Because a wide range of different materials of construction are used in clean rooms and other controlled areas, each material needs to be evaluated separately to validate the efficacy of a given disinfectant. Table 6 contains a list of common materials used in clean room construction.

Table 6. Typical Surfaces to be Decontaminated by Disinfectants in a Pharmaceutical Manufacturing Area

Material	Application
Stainless steel 304L and 316L	Work surfaces, filling equipment, and tanks
Glass	Windows and vessels
Plastic, vinyl	Curtains
Plastic, polycarbonate	Insulation coating
Lexan® (plexiglass)	Shields
Epoxyl-coated gypsum	Walls and ceilings
Fiberglass-reinforced plastic	Wall paneling
Tyvek®	Equipment wraps
Terrazzo tiles	Floors

DISINFECTANTS IN A CLEANING AND SANITIZATION PROGRAM

The selection of suitable disinfectants and the verification of their effectiveness in surface challenge testing is critical in the development of a cleaning and sanitization program.

Issues associated with the successful implementation of such a program are the development of written procedures, staff training, decisions on disinfectant rotation, institution of application methods and contact times, environmental monitoring to demonstrate efficacy, and personnel safety.

The cGMP 21 CFR 211.67, *Equipment Cleaning and Maintenance*, details the requirements for written procedures for cleaning, maintenance, and sanitization of pharmaceutical manufacturing equipment. These procedures should address the assignment of responsibility, establishment of schedules, details of cleaning operations, protection of clean equipment prior to use, inspection for cleanliness immediately prior to use, and maintenance of cleaning and sanitization records.

Staff involved in disinfection require training in microbiology, industry practices for cleaning and sanitization, safe handling of concentrated disinfectants, the preparation and

³AOAC International Official Methods of Analysis, 15th, 16th, and 17th editions. Arlington, VA.

disposal of disinfectants, and appropriate application methods. It should be emphasized that the preparation of the correct dilutions is critical because many disinfectant failures can be attributed to use of disinfectant solutions that are too dilute. Typically disinfectants used in aseptic processing and filling areas are diluted with Sterile Purified Water, and are prepared aseptically. Alternately, the disinfectant may be diluted with Purified Water, and then sterile filtered to eliminate microorganisms that may potentially persist in a disinfectant. Diluted disinfectants must have an assigned expiration dating justified by effectiveness studies.

The rotation of an effective disinfectant with a sporicide is encouraged. It is prudent to augment the daily use of a bactericidal disinfectant with weekly (or monthly) use of a sporicidal agent. The daily application of sporicidal agents is not generally favored because of their tendency to corrode equipment and because of the potential safety issues with chronic operator exposure. Other disinfection rotation schemes may be supported on the basis of a review of the historical environmental monitoring data. Disinfectants applied on potential product contact surfaces are typically removed with 70% alcohol wipes. The removal of residual disinfectants should be monitored for effectiveness as a precaution against the possibility of product contamination.

The greatest safety concerns are in the handling of concentrated disinfectants and the mixing of incompatible disinfectants. For example, concentrated sodium hypochlorite solutions (at a concentration of more than 5%) are strong oxidants and will decompose on heating, on contact with acids, and under the influence of light, producing toxic and corrosive gases including chlorine. In contrast, dilute solutions (at a concentration of less than 0.5%) are not considered as hazardous. Under no circumstances should disinfectants of different concentrations be mixed. Material Safety Data Sheets for all the disinfectants used in a manufacturing area should be available to personnel handling these agents. Appropriate safety equipment such as face shields, safety glasses, gloves, and uniforms must be issued to personnel handling the disinfectant preparation, and personnel must be trained in the proper use of this equipment. Safety showers and eye wash stations must be situated in the work area where disinfectant solutions are prepared.

assessments of excipients and active drug substances will differ. Therefore, it is important to note that the guidelines presented in this informational chapter apply only to the safety assessment of excipients, not to the safety assessment of active drug substances.

These testing guidelines are informational in nature and are intended to be used by professionals having a knowledge of toxicology and associated sciences. It is also intended that the applicable safety test method requirements of the receiving regulatory authority would be used in a proposal for market entry. For example, if a proposal is to be submitted to the U.S. Food and Drug Administration, that agency's safety test requirements would have to be met. These guidelines do not provide specific details regarding test methodology and data interpretation. Test procedures that are generally recognized by experts and by the regulatory agencies should be used. Alternatives to the use of living animals are encouraged wherever these alternative procedures have been validated for the intended purpose and where it is known that the alternative procedure will provide sufficient data upon which to base a safety judgment. It is recommended that the *Guiding Principles on the Use of Animals in Toxicology* of the Society of Toxicology (1996) and, in other countries, the appropriate legal and professional codes, be adhered to in the conduct of all test procedures. All studies must meet the requirements of the appropriate national good laboratory practice guidelines in effect in the country where the studies are being conducted.

In cases of extensive human experience based upon food use, there may be sufficient information to fulfill the requirements of the guidelines for orally-ingested excipients only. In addition, there may be animal-based data, which was developed for other purposes, that may be used to fulfill the testing guidelines requirements. If the data requirements have been met through prior human use experience and pertinent human data have been collected in a scientifically sound manner, there is no need to provide animal data for those endpoints evaluated by prior clinical experience.

Some dosage routes offer unique toxicological challenges, and the guidelines include provisions for these routes (e.g., inhalation). Also, further explanation is provided regarding numbers of species and other basic information (e.g., two species, one rodent and one nonrodent).

The extent of information required to define a set of baseline data, which constitute a toxicological and chemical database, is dependent upon the intended use of, and duration of, dosing of the candidate excipient material. It is critical that a thorough review of background information be conducted before embarking on a testing regimen. In addition to literature database reviews, information should be obtained regarding the physical and chemical properties of the compound; its manufacturing process (or processes); and product specifications including limits of impurities, potential for pharmacological activity, exposure conditions (i.e., dose, duration, frequency of use, dosage formulation, and route of administration), and potential user population. Also, base toxicity information covering the topics is fundamental. Particular attention should be addressed to the absorption/distribution/metabolism/excretion/pharmacokinetics (ADME/PK) studies because much of the later decision process will be dependent upon these data.

These guidelines provide a mechanism for obtaining sets of baseline data for all candidate excipient materials. The background information and baseline toxicity information alone may support the use of the candidate excipient either in a short half-life product that is not administered in a frequency that results in a residual excipient build-up in body tissue or in a product used only once or twice in a lifetime, such as a diagnostic agent. Additional tests, listed under *Step 4* of the *Safety Assessment Guidelines*, are necessary for candidate excipient material that is to be used in a manner that will result in short- or intermediate-term repeated exposure in humans—that is, a pharmaceutical product that will be administered for less than 10 days or for 30 to 90 con-

(1074) EXCIPIENT BIOLOGICAL SAFETY EVALUATION GUIDELINES

INTRODUCTION

This informational chapter presents a scientifically-based approach for the safety assessment of new pharmaceutical excipients (i.e., those excipients that have not been previously used or permitted for use in a pharmaceutical preparation). The guidelines presented herein provide a protocol for developing an adequate database upon which to establish conditions for the safe use of a new excipient intended for use in products administered by various dosage routes.

[NOTE—The final section of this chapter, *Definition of Terms*, lists some terms referred to in this chapter.]

An excipient may perform a variety of functionality roles in a pharmaceutical product; but, unlike pharmacologically active drug entities, the excipient displays either no pharmacological activity or very limited and directed activity. Because of these differences between excipients and active drug substances in terms of risk and benefit relationships and expected biological activities, the approaches for safety

secutive days, respectively. For a candidate excipient material that is intended for use in a pharmaceutical product intended for either intermittent or chronic administration over a long time period, such as a treatment for psoriasis or an insulin preparation, further tests are required. These tests are listed under *Step 7* of the guidelines and in the appropriate section under *Additional Requirements for Specific Exposure Routes*. While providing guidance for consumer safety, some of the required tests are intended to provide information to address occupational safety (e.g., skin and eye irritation).

The guidelines are summarized in *Table 1*. Tests that are required (R) by the guidelines are distinct from those that are recommended conditionally (C). Whether conditional tests are conducted is dependent upon the conditions of use and available biological data. Consideration must also be given to the requirements of the regulatory authorities when making the decision to test.

SAFETY ASSESSMENT GUIDELINES

Background Information

Before proceeding to the steps under *Data Requirements and Checkpoints*, the following points should be reviewed and defined:

- Review literature information using all appropriate databases

- Define chemical and physical properties
 - Define manufacturing process
 - Define product specifications, including impurities and residual solvents (see applicable ICH guidelines)
 - Estimate exposure conditions (dose, duration, frequency route)
 - Define user population
 - Assess potential for pharmacologic activity.
- At this point evaluate what is known, and develop the initial approach to testing.

Data Requirements and Checkpoints

STEP 1: Toxicity Data (see *Baseline Toxicity Data*)

The toxicity data should take into account the following information:

- Effects of acute exposure by oral and intended routes
- Effects of repeated exposures by intended routes
- Effects of in vitro genotoxicity assays
- ADME/PK by oral or appropriate routes; single or multiple doses.

STEP 2: Depending on results of above, evaluate effects of a single dose in humans.

STEP 3: Checkpoint: Evaluate results of above and proposed exposure conditions and exposed population. The above data might allow use in a single product with a short half-life (e.g., a diagnostic agent).

Table 1. Summary of Excipient Guidelines

Tests	Routes of Exposure for Humans					
	Oral	Mucosal	Dermal/Topical/Trans-dermal	Injectable*	Inhalation/Intranasal	Ocular
<i>Baseline Toxicity Data</i>						
Acute Oral Toxicity	R	R	R	R	R	R
Acute Dermal Toxicity	R	R	R	R	R	R
Acute Inhalation Toxicity	C	C	C	C	R	C
Eye Irritation	R	R	R	R	R	R
Skin Irritation	R	R	R	R	R	R
Skin Sensitization	R	R	R	R	R	R
Acute Injectable Toxicity	—	—	—	R	—	—
Application Site Evaluation	—	—	R	R	—	—
Pulmonary Sensitization	—	—	—	—	C	—
Phototoxicity/Photoallergy	R	—	R	R	R	—
Genotoxicity Assays	R	R	R	R	R	R
ADME/PK-Intended Route	R	R	R	R	R	R
28-Day Toxicity (2 Species)-Intended Route	R	R	R	R	R	R
<i>Additional Data: Short- or Intermediate-term Repeated Use</i>						
90-Day Toxicity (Most Appropriate Species)	R	R	R	R	R	R
Embryo-Fetal Toxicol.	R	R	R	R	R	R
Additional Assays	C	C	C	C	C	C
Genotoxicity Assays	R	R	R	R	R	R
Immunosuppression Assays	R	C	C	R	C	C
<i>Additional Data: Intermittent Long-term or Chronic Use</i>						
Chronic Toxicity (Rodent, Nonrodent)	C	C	C	C	C	C
Reproductive Toxicity	R	R	R	R	R	R
Photocarcinogenicity	C	—	C	C	C	—
Carcinogenicity	C	C	C	C	C	C

R = Required

C = Conditional

*Intravenous, intramuscular, subcutaneous, intrathecal, etc.

STEP 4: Gather the following additional data:

- Effects of subchronic exposure in appropriate species and routes
- Embryo-fetal development studies via appropriate route of exposure
- Additional in vitro and in vivo genotoxicity tests.

STEP 5: Depending on results of above, consideration should be given to testing in humans as part of the clinical trials of an active ingredient or as a stand-alone procedure.

STEP 6: Checkpoint: Evaluate all of above information. Data might allow use in a variety of products intended for short-term, repeated intake (e.g., an antibiotic). If the ADME/PK studies for a noninjectable excipient show no absorption, data may permit using a product for 30 to 90 consecutive days.

STEP 7: Additional data should be obtained for use in a product taken chronically, either daily or intermittently, over a long time period depending on:

- Results of subchronic studies and long-term toxicity in appropriate mammalian nonrodents
- Reproductive toxicity studies
- Other test results and human exposure data and long-term toxicity or carcinogenicity in rodents.

Baseline Toxicity Data

The following data should be taken into account:

Appropriate Acute Toxicity by Intended Dose Routes: skin sensitization, approximate lethal dose method, limit test, etc.

Other Appropriate Acute Toxicity Studies: oral toxicity by limit test or approximate lethal dose method, skin irritation, etc.

ADME/PK: single or multiple doses.

Genotoxicity: for example, Ames Test, in vitro chromosome aberration test, mammalian cell mutation assay.

28-Day Repeated Dosing Studies in Two Species by Appropriate Routes (One Rodent, One Mammalian Nonrodent): evaluation of injection site or similar considerations might be necessary depending on route of administration.

NOTES—

1. In those cases where intended route restrictions (e.g., volume, concentration) preclude an adequate assessment of the toxicity of the excipient, development of a toxicity profile by other relevant routes may be needed.
2. The comparison of toxicity and ADME/PK data between oral and intended routes is critical at this point because that knowledge may set the direction for future toxicity testing (e.g., reproductive toxicity testing conducted by oral route rather than intended route). In addition, relevant studies using the intended route and anticipated duration of exposure may preclude performance of additional studies.

Additional Requirements for Specific Exposure Routes

FOR ORAL EXPOSURE: No additional requirements beyond those presented for *Baseline Toxicity Data*.

FOR MUCOSAL EXPOSURE: No additional requirements beyond those presented for *Baseline Toxicity Data*.

FOR DERMAL, TOPICAL, OR TRANSDERMAL EXPOSURE:

Baseline Toxicity Data—

- *Effects of Acute Exposure by Transdermal Dose Route:* dermal sensitization study for repeat applications
- *Effects of Repeated Exposures by Transdermal Route*
 1. Photoallergy/phototoxicity study
 2. Studies in two species (one rodent, one mammalian nonrodent) by transdermal route.

- *Effects of Subchronic Exposure, Reproductive Toxicity Effects*—Initial toxicity studies may be performed by the IV route to adequately profile the toxicity of the excipient. This will provide an assessment of potential target organs if an adequate amount of the compound cannot be delivered via a transdermal dosage form. This is dependent upon the results from the ADME/PK studies.

Reproductive studies may also be conducted via oral or IV route with demonstration of absorption (oral) and pharmacokinetic comparisons of the chosen route versus transdermal.

Photocarcinogenicity studies may be required and should be considered if data and the proposed use indicate when evaluating materials to be placed on the skin for prolonged periods of time and exposure to UV light is a factor (e.g., sun block). This also applies to oral, parenteral, and inhalation products where skin drug concentrations exceed plasma drug concentrations for a substantial period of time, or where the candidate material would appear to have the potential for photo-activity or has demonstrated photo-activity.

FOR INJECTABLE DOSAGE FORMS:

Background Information—

1. Define compatibility of the dosage form with blood, if appropriate, based on route of exposure.
2. Define the pH and tonicity of injectable dose form, if appropriate, based on the route of exposure.

Baseline Toxicity Data—

- *Effects of Acute Exposure by Intended Injectable Dose Routes*
 1. Include evaluation of injection site irritation in rabbit or dog
 2. Include evaluation of rate of administration.

FOR INHALATION OR INTRANASAL EXPOSURE:¹

Baseline Toxicity Data—

- *Acute Inhalation Toxicity*—A limit test that would, for example, use the highest achievable concentration in a 4-hour exposure to vapor, aerosol, or solid particulate. Pulmonary sensitization may be performed along with other appropriate studies. If exposure is to be to an aerosol or solid particulate, particulates of appropriate mass median diameter should be generated.
- *Single and Repeated Dose ADME/PK by Inhalation or Intranasal and Oral Routes*
- *28-Day Repeated Dose Inhalation Study in Two Mammalian Species Using Vapor or Particulates of Appropriate Mass Median Diameter:* compare to similar oral toxicity data.

FOR OPHTHALMIC EXPOSURE:

Background Information: define pH and osmolarity of topical ocular dose form.

Baseline Toxicity Data—

- *Effects of Acute Exposure by Ophthalmic Routes:* cytotoxicity tests (e.g., agar overlay)
- *Effects of Repeated Exposures by Ophthalmic Routes*
 1. Studies in two species (one rodent, one mammalian nonrodent)
 2. Examination of anterior and posterior segments of the eye
 3. Studies on allergenicity potential.

Other Data—Comparison of pharmacokinetic parameters of the route chosen for reproductive studies and the ophthalmic exposure are essential for extrapolation of potential toxicity via the ophthalmic route.

¹ When designing studies to evaluate use in products intended for use by the inhalation or intranasal route, consideration should be given to the dosing regimen that will be used by humans. The appropriate study protocol for a product intended for inhalation therapy that will result in prolonged exposures (e.g., several hours per day) may differ from that used to evaluate a product that would result in exposure to several metered doses per day.

DEFINITION OF TERMS

Acute: exposure to a test agent within a single, 24-hour period. Doses may be single, multiple or continuous during a 24-hour period.

Subacute: repeated dosing of a test agent for up to 29 days. Daily doses may be single, multiple or continuous during a 24-hour period.

Subchronic: repeated dosing of a test agent for 30 days to 10% of the lifespan of the test species (90 days in rodents). Daily doses may be single, multiple or continuous during a 24-hour period.

Chronic: repeated dosing of a test agent for more than 10% of the lifespan of the test species (more than 90 days in rodents). Daily doses may be single, multiple or continuous during a 24-hour period.

〈1078〉 GOOD MANUFACTURING PRACTICES FOR BULK PHARMACEUTICAL EXCIPIENTS

BACKGROUND

This general information chapter provides guidelines for methods, facilities, and manufacturing controls to be used in the production of pharmaceutical excipients in order to ensure that excipients possess the quality, purity, safety, and suitability for use that they purport to possess. The principles and information in this chapter can be applied to the manufacture of all pharmaceutical excipients (referred to throughout this document as *excipient(s)*) intended for use in human drugs, veterinary drugs, and biologics. It covers the quality management system and the extent of good manufacturing practices (GMP) necessary throughout manufacturing for both batch and continuous processes. It is intended to assist manufacturers as well as auditors in establishing whether the facilities and controls used for the manufacture of excipients are adequate and whether the excipients possess the quality and purity that they purport to possess and are suitable for their intended use. The manufacture of certain excipients for specialist applications presents additional challenges that are outside the scope of this chapter. Examples include excipients for parenteral, ocular, inhalation, and open wound use and those that are sterile and/or pyrogen-free. It does not provide information for all national legal requirements nor does it cover in detail the particular characteristics of every excipient. The quality system standard used as a framework for this chapter is ISO 9001, which is appropriate to manufacturing. Because of the diversity of excipients, some principles in this information chapter may not be applicable to certain products and processes.

This chapter combines the concepts of existing GMP principles from the following sources:

- World Health Organization (WHO) GMP Guidelines for Excipients,
- International Pharmaceutical Excipients Council (IPEC) Good Manufacturing Practices Guide for Bulk Pharmaceutical Excipients 2001,
- Institute of Quality Assurance (IQA) Pharmaceutical Quality Group (PQG) PS 9100:2002, Pharmaceutical Excipients,

- International quality management system requirements as developed by the International Organization for Standardization (ISO).

In view of the increasing globalization of the pharmaceutical industry and the harmonization of pharmaceutical registration requirements, deference to all schemes is becoming necessary. Therefore, relevant portions of the manufacturing concepts are employed throughout this chapter.

The *General Guidance* section provides an overview of the appropriate manufacturing practice criteria applicable to excipient manufacture and the points of application of excipient good manufacturing practices and quality systems. The section also recommends measures to limit contamination of an excipient. Finally, it discusses the relationship of excipients to finished dosage forms. No attempt has been made to include details specific to particular excipients.

The information in *Appendix 1. Auditing Considerations* sets forth key criteria to aid in the audit of an excipient manufacturing facility.

For a list of terms used in this chapter and their definitions, see *Appendix 2. Glossary*.

INTRODUCTION

Purpose and Scope

This chapter defines the extent and point of application of appropriate GMP principles for excipient manufacture and is applicable to the manufacture of excipients intended for use in drug products. It covers the quality management system and the extent of GMP necessary throughout manufacturing for both batch and continuous processes. It is intended to aid both auditors and manufacturers in establishing whether the facilities and controls used for the manufacture of excipients are adequate and whether the excipients possess the quality, purity, and safety that they purport to possess and are suitable for their intended use.

The manufacture of certain excipients for specialist applications presents additional challenges that are outside the scope of this chapter. Examples include excipients

- for parenteral, ocular, inhalation, and open wound use; and
- those that are purported to be sterile and/or pyrogen-free.

In these cases, detailed information pertaining to the intended use of an excipient as provided by the end user can be useful in determining appropriate GMP. This chapter does not address the specific GMP relating to good trade and distribution practices (GTDP).

Principles Adopted

The Chapter and Its Use—Pharmaceutical excipients are diverse and often have uses other than pharmaceutical applications. Each manufacturer should consider how the chapter might apply to its products and processes (for example, batch versus continuous processes). Because excipients are so diverse, some principles of this chapter may not be applicable to certain products and manufacturing processes.

Application—The text provides information necessary for the manufacture of excipients but does not provide all the details. It cannot specify national legal requirements or cover particular characteristics of every excipient.

Quality System Standard—The quality management system standard chosen as a framework for this chapter is ISO 9001, which is appropriate for manufacturing facilities. A manufacturer may apply the ISO standard with or without certification; but this possibility, as a business decision, is not discussed in this chapter. However, ISO certification has the benefit of providing assurance to customers that the ex-

excipient manufacturer's quality management system has been independently verified.

The headings in this chapter have been aligned with the ISO 9001 clause numbers, because many excipient manufacturers already use that standard as a basis for their quality management system. Additional headings are included as needed to introduce additional guidance on GMP when not covered by current ISO 9001 clauses.

Document Structure—The chapter combines the concepts of existing GMP principles from the following:

- World Health Organization (WHO), GMP Guidelines for Excipients,
- International Pharmaceutical Excipients Council (IPEC), Good Manufacturing Practices Guide for Bulk Pharmaceutical Excipients 2001,
- Institute of Quality Assurance (IQA) Pharmaceutical Quality Group (PQG) PS 9100:2002, Pharmaceutical Excipients,
- International quality management system requirements as developed by the International Organization for Standardization (ISO).

In view of the increasing globalization of the pharmaceutical industry and the harmonization of pharmaceutical registration requirements, relevant portions of the manufacturing concepts detailed in these schemes are employed throughout this chapter.

The *General Guidance* section provides an overview of the GMP criteria applicable to excipient manufacture and the point of application of excipient GMP.

The remaining sections provide guidance on GMP principles and implementation of a quality management system suitable for excipient manufacture. For example, these sections suggest measures to limit excipient contamination. No attempt has been made to include details specific to particular excipients, and individual manufacturers should address these as they apply to their own products and processes.

The Appendixes provide supporting guidance for excipient GMP. *Appendix 1. Auditing Considerations* describes key criteria to be considered in the audit of an excipient manufacturing facility. *Appendix 2. Glossary* provides definitions of terms used in this chapter.

GENERAL GUIDANCE

Pharmaceutical Excipients—Pharmaceutical excipients are substances other than the active pharmaceutical ingredient (API) that have been appropriately evaluated for safety and are intentionally included in a drug delivery system. For example, excipients can do the following:

- aid in the processing of the drug delivery system during its manufacture,
- protect, support, or enhance stability, bioavailability, or patient acceptability,
- assist in product identification, and
- enhance any other attribute of the overall safety, effectiveness, or delivery of the drug during storage or use.

A more complete classification of excipients according to their functions can be found in *USP and NF Excipients, Listed by Category* in the *USP–NF*.

Excipient GMP Implementation—The application of GMP is relevant once it has been determined that a chemical is intended for use as a component of a drug product. Excipient manufacture should be carried out in accordance with the GMP concepts consistent with this chapter. The objective of excipient GMP is to ensure that the manufacture of an excipient results in a consistent material with the desired quality characteristics. The emphasis of GMP for excipients is to ensure product integrity, avoid product contamination, and ensure that records are maintained.

As the excipient manufacturing process progresses, the degree of assurance concerning the quality of the product

should increase. Manufacturing processes should be controlled and documented. However, at some logical processing step, as determined by the manufacturer, the GMP as described in this chapter should be applied and maintained.

Judgment based on risk analysis and a thorough knowledge of the process is required in order to determine from which processing step GMP should be implemented. This is usually well before the final finishing operation and, for example, may be identified using methods such as hazard analysis and critical control point (HACCP), failure mode and effects analysis (FMEA), or a detailed process flow diagram. Consideration should also be given to other factors such as batch versus continuous processing, dedicated versus multipurpose equipment, and open versus closed processes.

QUALITY MANAGEMENT SYSTEM: EXCIPIENT QUALITY SYSTEMS

General Recommendations

The principles outlined in this chapter provide a comprehensive basis for the quality management system used in the manufacture of pharmaceutical excipients. Excipient manufacturers should identify the quality management processes required to ensure excipient quality. Where manufacturing, testing, or other operations that could affect excipient quality are outsourced, the responsibility for quality remains with the excipient manufacturer, and control measures should be defined (see also the subsection *Purchasing Information* in the *Product Realization* section).

Documentation Recommendations

General—The excipient manufacturer should have a system in place to control documents and data that relate to the requirements of the quality management system.

Quality Manual—The excipient manufacturer should prepare a quality manual describing the quality management system, the quality policy, and the commitment of the excipient manufacturer to applying the appropriate GMP and quality management standards contained in this chapter. This manual should include the scope of the quality management system, reference to supporting procedures, and a description of the interaction between quality management processes.

Control of Documents—The excipient manufacturer should establish and maintain procedures for the identification, collection, indexing, filing, storage, maintenance, and disposition of controlled documents, including documents of external origin that are part of the quality management system.

Procedures used in the manufacture of excipients should be documented, implemented, and maintained. In addition, there should be formal controls relating to procedure approval, revision, and distribution. These controls should provide assurance that the current version of a procedure is being used throughout the operational areas and that previous revisions of documents have been removed.

Documents and subsequent changes to documents should be reviewed and approved by designated qualified personnel before issuance to the appropriate areas, as identified in the documents. Documents that affect product quality should be reviewed and approved by the quality unit (see also *Responsibility and Authority* in the section *Responsibility, Authority, and Communication under Management Responsibility*).

Controlled documents may include a unique identifier, the date of issue, and a revision number to facilitate identification of the most recent document. The department with the responsibility for issuing the documents should be identified.

tified. When it is practical, changes and the reasons for the changes should be documented.

Electronic documentation should meet the requirements for the document control system stated above. If electronic signatures are used on documents, they should be controlled to provide security equivalent to that provided by a handwritten signature. Electronic documents and signatures may also have to satisfy local regulatory requirements.

Control of Records—The excipient manufacturer should establish and maintain procedures for the identification, collection, indexing, filing, storage, maintenance, and disposition of records.

Records should be maintained to demonstrate achievement of the required quality and the effective operation of the quality management system. Records should be legible and identifiable with the product involved. Pertinent subcontractor quality data should be an element of these records.

Entries in records should be clear, indelible, made directly after performing the activity (in the order performed), and signed and dated by the person making the entry. Corrections to entries should be signed and dated, leaving the original entry legible.

Records should be kept for a defined period. This period should be appropriate to the excipient and to its expiry date or reevaluation interval. Records should be stored and maintained in such a manner that they are readily retrievable, in facilities that provide an environment suitable for minimizing deterioration or damage.

Change Control—The excipient manufacturer should establish and maintain procedures to evaluate and approve changes that may affect the quality of the excipient. For example, this may include changes to the following:

- raw materials or packaging and their sources,
- material specifications,
- test methods,
- manufacturing and analytical equipment,
- production processes,
- manufacturing or packaging sites.

A unit with a function that is independent from production (such as regulatory affairs or quality assurance) should have the responsibility and authority for the final approval of changes.

Customers should be notified, and, where applicable, excipient regulatory submissions (for example, for Drug Master Files [DMFs] or Certificates of Suitability to the *European Pharmacopoeia* [CEPs]) should be amended to reflect significant changes from established production and process control procedures that may affect excipient quality (see also *Customer Communication* in the section *Customer-Related Processes* under *Product Realization*).

MANAGEMENT RESPONSIBILITY

Management Commitment

Top management should demonstrate to the organization the importance it places on customer satisfaction and compliance with the appropriate regulations and standards. This should be accomplished through the development of a quality policy and establishment of quality objectives. Progress toward the documented quality objectives should be reviewed at planned intervals.

Customer Focus

It is the responsibility of top management to ensure that customer requirements are determined and met. The excipient manufacturer should permit the customer or its representative to conduct audits of the manufacturer's quality

management system, manufacturing processes, buildings, and facilities.

Quality Policy

Top management should demonstrate its commitment to the corporate quality policy and ensure that it is implemented within the operational unit. The quality policy should support continual improvement of the quality management system. Management should participate in the development of the company's quality policy and provide the resources necessary for its development, maintenance, and deployment.

Planning

Quality Objectives—Top management should set objectives for adherence to GMP to ensure that the excipient manufacturer maintains and improves its performance. Objectives should be deployed throughout the organization and should be measurable and consistent with the quality policy.

Quality Management System Planning—Top management should provide adequate resources to ensure conformity to the provisions of this chapter. There should be a process for the identification of resources needed for adherence to GMP. A gap analysis based on audits by internal personnel, customers, regulatory agencies, or outside contractors, or based on the use of this chapter, could be created to identify resource requirements. Top management should ensure that the integrity of the quality management system is maintained when changes are planned and implemented.

Responsibility, Authority, and Communication

Responsibility and Authority—Responsibility and authority should be clearly defined by top management and communicated within the organization. It should be the responsibility of a unit that is independent of production, such as the quality unit, to do the following:

- ensure that quality-critical activities are undertaken as defined,
- approve suppliers of quality-critical materials and services,
- approve or reject raw materials, packaging components, intermediates, and finished excipients,
- ensure that there is a review of production records to confirm that no errors have occurred or, if errors have occurred, that they are fully investigated,
- participate in reviewing and authorizing changes to processes, specifications, procedures, and test methods that potentially affect quality (also see above, *Change Control* in the section *Documentation Recommendations* under *Quality Management System: Excipient Quality Systems*) and participate also in investigating failures and complaints,
- retain responsibility for approval or rejection of the excipient if it is produced, processed, packaged, or held under contract by another company,
- develop and implement a self-inspection program of the quality management system.

The excipient manufacturer may delegate some of the quality unit's activities to other personnel if appropriate controls (for example, periodic audits, training, and documentation) are in place.

An organization chart by function should show interdepartmental relationships as well as relationships to top management of the company. Personnel whose positions affect excipient quality should have job descriptions.

Management Representative—The excipient manufacturer should appoint a management representative with sufficient authority to ensure that the provisions of this chapter

are properly implemented. The representative should periodically report to top management on conformity to the quality management system, including changing customer and regulatory requirements.

Internal Communication—The excipient manufacturer should ensure that appropriate systems are established to communicate GMP and regulatory requirements, quality policies, quality objectives, and procedures throughout the organization. The communication should also provide information about the effectiveness of the quality management system. Top management should be notified promptly of quality-critical situations, such as product retrievals, in accordance with a documented procedure.

Management Review

General—The top management of the company should hold periodic reviews of the quality management system to confirm the organization's continued conformity to this chapter. The review should be recorded and should include assessing opportunities for improvement and the need for changes to the quality management system.

Review Input—Management review inputs should include, for example, the following:

- results of internal and external audits,
- customer feedback of the company performance,
- product conformity and process performance,
- action items from the previous management review,
- customer complaints,
- status of corrective or preventive actions,
- changes that could affect the quality management system.

Review Output—The management review should identify the resources needed and the opportunities presented for improving the quality management system and improving product conformity to customer and regulatory requirements. A record should be made of actions recommended and taken.

RESOURCE MANAGEMENT

Provision of Resources—There should be sufficient qualified personnel and resources (e.g., equipment, materials, buildings, and facilities) to implement, maintain, and improve the quality management system and to produce, package, test, store, and release each excipient in a manner consistent with this chapter.

Human Resources

General—Personnel performing work affecting the quality of excipients should have the appropriate combination of education, training, and experience for their assigned tasks. Consultants advising on the design, production, packaging, testing, or storage of excipients should have sufficient education, training, and experience or any combination thereof to advise on the subject for which they are retained. Records should be maintained listing the name, address, and qualifications of consultants and the type of service they provide.

Competence, Awareness, and Training—The excipient manufacturer should establish and maintain procedures for identifying training needs and for providing the necessary training to personnel performing activities affecting excipient quality. Appropriate records of training should be maintained. Training should address the particular operations that the employee performs and GMP as they relate to the employee's functions. Qualified individuals should conduct GMP training frequently enough to ensure that employees remain familiar with applicable GMP principles. Management should establish adequate and continued personal-hy-

giene training for personnel who handle materials so that they understand the precautions necessary for preventing contamination of excipients. The training program should ensure that personnel understand that deviations from procedures may affect the customer's product quality.

Personnel Hygiene—To protect excipients from contamination, protective apparel such as head, face, hand, and arm coverings should be worn as appropriate to the duties performed. Jewelry and other loose items, including those in pockets, should be removed or covered. Only authorized personnel should enter the areas of the buildings and facilities designated as limited-access areas.

Personnel should practice good sanitation and health habits. Any person shown by either medical examination or supervisory observation to have an apparent illness or open lesions that may adversely affect the safety or quality of the excipient should be excluded from direct contact with raw materials, packaging components, intermediates, and finished excipients until the condition is corrected or until competent personnel determine that it will not jeopardize the safety or quality of the excipient. Personnel should be instructed to report to supervisory personnel any health conditions that may have an adverse effect on excipients. The storage and use of food, drink, personal medication, tobacco products, or similar items should be restricted to designated locations separate from manufacturing areas.

Infrastructure—The infrastructure should be managed, operated, cleaned, and maintained in accordance with GMP principles to ensure excipient quality and to avoid contamination (including, where critical to excipient quality, control of particulate matter, microbiological control, and control of water quality).

Buildings and Facilities—The prevention of contamination should be considered in the design of the manufacturing processes and facilities, particularly when the excipient is exposed. Buildings and facilities used in the production, processing, packaging, testing, or storage of an excipient should be maintained in a good state of repair and should be of suitable size, construction, and location to facilitate cleaning, maintenance, and correct operation appropriate to the type of processing.

Manufacturing processes associated with the production of highly sensitizing or toxic products (for example, herbicides and pesticides) should be located in dedicated facilities or should use equipment separate from that used for excipient manufacture. If this is not possible, appropriate measures (for example, cleaning, inactivation) should be implemented to avoid cross-contamination. The effectiveness of these measures should be demonstrated. There should be adequate facilities for the testing of raw materials, packaging components, intermediates, and finished excipients.

Equipment—Equipment used in the production, processing, packaging, testing, or storage of an excipient should be maintained in a good state of repair and should be of suitable size, construction, and location to facilitate cleaning, maintenance, and correct operation, depending on the type of processing (for example, batch versus continuous). Equipment should be commissioned before use to ensure that it is functioning as intended. Where equipment is located outdoors, there should be suitable controls to minimize the risk to excipient quality from the environment (for example, processing within a closed system).

Equipment Construction—Process equipment should be constructed so that contact surfaces will not be reactive, additive, or absorptive and thus will not alter the quality of the excipient. Substances required for operation, such as lubricants or coolants, should preferably not come into contact with raw materials, packaging materials, intermediates, or finished excipients. Where contact is possible, substances suitable for use in food applications should be employed.

Equipment should be designed to minimize the possibility of contamination caused by direct operator contact in activities such as the unloading of centrifuge bags, the use of transfer hoses (particularly those used to transfer powders),

and the operation of drying equipment and pumps. The sanitary design of transfer and processing equipment should be evaluated. To control the risk of contamination, equipment with moving parts should be assessed with regard to the integrity of seals and packing materials.

Equipment Maintenance—Documented procedures should be established and followed for maintenance of critical equipment used in the production, processing, packaging, testing, or holding of the excipient. There should be records of the use and maintenance of quality-critical equipment. These records can be in the form of a log, computer database, or other appropriate documentation.

Computer Systems—Computer systems that may affect excipient quality should have sufficient controls for operation and maintenance and for prevention of unauthorized access or changes to computer software, hardware, or data, including the following:

- systems and procedures that show that the equipment and software are performing as intended,
- procedures for checking the equipment at appropriate intervals,
- retention of suitable back-up or archival systems such as copies of the program and files,
- assurance that changes are verified and documented and are made only by authorized personnel.

Utilities—Utilities (for example, nitrogen, compressed air, and steam) used in the production, storage, or transfer of materials that could affect excipient quality should be assessed and appropriate action taken to control the risk of contamination and cross-contamination.

Water—Water used in the manufacture of excipients should be demonstrated to be of appropriate quality in consideration of purity requirements and the intended use of the excipient. Unless otherwise justified, process water should, at a minimum, meet regulatory requirements for drinking (potable) water. If drinking (potable) water is insufficient to ensure quality, or if tighter chemical and/or microbiological water quality specifications are required, appropriate controls and specifications should be set: for example, physical and chemical attributes, total microbial counts, and limits on objectionable organisms and/or endotoxins.

Where water used in the process is treated by the manufacturer to achieve a defined quality, the treatment process should be specified and monitored with appropriate action limits. Water that comes into contact with the excipient should be supplied under continuous positive pressure (or other means of preventing back flow) in a system free of defects to control the risk of contamination to the excipient.

Work Environment—Where the excipient is exposed during manufacture, it should be in an environment appropriate for minimizing contamination. The manufacturer should apply suitable controls to maintain that environment.

Air Handling—Where an air-handling system is installed to provide protection to the excipient, the excipient manufacturer should demonstrate its effectiveness. Excipient production unit air-handling systems should be designed to prevent cross-contamination. For dedicated areas processing the same excipient, it is permissible to recycle a portion of the exhaust air back into the same area. The adequacy of such a system for multiuse areas, especially if several products are processed simultaneously, should be assessed for potential cross-contamination.

Controlled Environment—A controlled environment may be necessary in order to avoid contamination or degradation caused by exposure to heat, air, or light. The degree of protection required may vary depending on the stage of the process. Special environments required by some processes should be monitored to ensure product quality (for example, inert atmosphere or protection from light). Where an inert atmosphere is required, the gas should be treated as a raw material. If interruptions in the special environment occur, adequate evidence and an appropriate rationale should be documented to show that such interrup-

tions have not compromised the quality of the excipient. Such environmental concerns become increasingly important following purification of the excipient.

Cleaning and Sanitary Conditions—Adequate cleanliness is an important consideration in the design of excipient manufacturing facilities. Buildings used in the production, processing, packaging, or holding of an excipient should be maintained in an appropriately clean and sanitary condition according to the type of processing conducted (for example, open/closed systems). Where maintenance of clean and sanitary conditions is critical to excipient quality, documented procedures should assign responsibility for cleaning and sanitation, describing in sufficient detail the cleaning schedules, methods, equipment, and materials to be used in cleaning the buildings and facilities. These procedures should be followed, and cleaning should be documented. Waste should be segregated and disposed of in a timely and appropriate manner. If waste is not disposed of immediately, it should be suitably identified.

Pest Control—Buildings should be free from infestation by rodents, birds, insects, and other vermin. Some raw materials, particularly botanicals, may contain some unavoidable contamination, such as rodent or other animal filth or infestation. The manufacturer should have sufficient control methods to prevent the increase of such contamination or infestation in holding areas and its spread to other areas of the plant.

Lighting—Adequate lighting should be provided to facilitate cleaning, maintenance, and proper operations.

Drainage—In areas where the excipient is open to the environment, drains should be of adequate size and, where connected directly to a sewer, should be provided with an air break or other mechanical device to prevent back-siphoning.

Washing and Toilet Facilities—Adequate personal washing facilities should be provided, including hot and cold water, soap or detergent, air dryers or single-service towels, and clean toilet facilities easily accessible to working areas. Adequate facilities for showering and/or changing clothes should be provided, where appropriate.

PRODUCT REALIZATION

Planning of Product Realization—The excipient manufacturer should plan and develop the processes and controls needed for product manufacture. These plans and controls should be appropriate to the production process, excipient specification, equipment, and facilities used in the manufacture of the product. Key aspects of the planning of a suitable process and its controls should include the following, as appropriate:

- documented testing programs, for quality-critical materials including excipients, that include appropriate specifications, sampling plans, and test and release procedures,
- generation and maintenance of records (also see above, *Control of Records* in the section *Documentation Recommendations* under *Quality Management System: Excipient Quality Systems*) that provide evidence that these plans have been realized as intended and that enable traceability to be demonstrated (also see below in this section, *Traceability* under *Identification and Traceability*),
- provision of resources to implement these plans,
- environmental and hygiene control programs to minimize contamination.

Customer-Related Processes

Determination of Requirements Related to the Product—The excipient manufacturer should determine the excipient quality, labeling, and delivery requirements of the

customer. Additional requirements, whether customer-specific, legal, or regulatory (for example, pharmacopeia material and general monographs), should be agreed on by both parties. Requirements not stated by the customer but necessary for specified or intended use, where known, should be considered.

Review of Requirements Related to the Product—The excipient manufacturer and customer should mutually agree upon the requirements identified in the section above, *Determination of Requirements Related to the Product*, before supply commences. The manufacturer should have the facility and process capability to consistently meet the mutually agreed-upon specifications. Where the requirements determined in the section *Determination of Requirements Related to the Product* are changed, this review should be repeated before supply recommences.

Customer Communication—There should be provision for providing accurate and pertinent communication to the customer. Master copies of documents such as specifications and technical reports should be controlled documents. Provision should be made for replying to customer inquiries, contracts, and order-handling requirements. Customer feedback and complaints should be documented. Customers should be notified of significant changes (also see above, *Change Control* in the section *Documentation Recommendations* under *Quality Management System: Excipient Quality Systems*).

Design and Development—ISO 9001 includes requirements for ensuring control over design and development activities. It is recommended that companies involved in such activities follow the requirements of ISO 9001. Full GMP are not always applicable during the design and development of new excipients and/or manufacturing processes. However, development batches of excipients that are intended for use in drug products should be manufactured in accordance with the applicable provisions of this chapter.

Purchasing

Purchasing Process—Excipient manufacturers should have a system for selecting and approving suppliers of quality-critical materials and services (for example, subcontract manufacturers and laboratories). Supplier approval by the quality unit should require an evaluation of the supplier's quality management system, including adequate evidence that they can consistently meet agreed-upon specifications and maintain traceability. This may require periodic audits of the supplier's manufacturing facility. Records of these activities should be maintained. Materials should be purchased against an agreed specification from approved suppliers.

Purchasing Information—Purchasing agreements should describe the material or service ordered, including, where critical to excipient quality, the following:

- the name, type, class, style, grade, item code number or other precise identification traceable to the raw material and packaging specifications,
- drawings, process requirements, inspection instructions and other relevant technical data, including requirements for approval or qualification of product, procedures, process equipment, and personnel,
- adherence to the appropriate sections of this chapter for relevant contract manufacturers or laboratories, and
- a statement to notify the excipient manufacturer of significant changes in quality-critical raw materials.

Verification of Purchased Product—There should be procedures for the approval and release of quality-critical material. Upon receipt, quality-critical materials should be placed in quarantine and should not be used prior to acceptance. Effective quarantine can be established with suitable identifying labels, signs, and/or other manual documentation systems. When quarantine and stock control are managed with computer systems in lieu of a physical stock con-

trol, system controls should prevent the use of unreleased material. Quarantine may not be feasible for materials supplied via pipelines. In these cases the excipient manufacturer should establish an agreement with the supplier so that the manufacturer is notified of material that does not meet specification. Sampling activities should be conducted under defined conditions, in accordance with a defined sampling method and using procedures designed to prevent contamination and cross-contamination.

Quality-critical materials used in the manufacture of an excipient should be tested or otherwise verified prior to use. Verification should include availability and a check of the supplier certificate of analysis and, wherever feasible, at least an identification test. Testing schedules should be organized to separate routine tests from those that are performed infrequently or only for new suppliers. Bulk deliveries should have additional controls to ensure material purity and freedom from contamination (for example, dedicated tankers, tamper-evident seals, a certificate of cleaning, analytical testing, or audit of the supplier). These procedures, activities, and results should be documented.

Production and Service Provision

Control of Production and Service Provision—Production activities should be carried out under controlled conditions (also see above, *Planning of Product Realization* under *Product Realization*). Specific examples of important controls, some of which may not be applicable to all excipient manufacturers, are illustrated in the following sections.

Production Instructions and Records—Production instructions and records are required but may differ for the type of operation: for example, batch versus continuous processes. There should be a controlled document that describes how the excipient is produced (for example, master production instructions, master production and control records, or process definitions). For batch processes, an accurate reproduction of the appropriate master production instructions should be issued to the production area. For continuous processes, a current processing log should be available. Records should be available for each batch of excipient produced and should include complete information relating to the production and control of each batch. For continuous processes, the batch and its records should be defined (for example, based on time or defined quantity). Records may be in different locations but should be readily retrievable. Records for both batch and continuous processing, where critical to excipient quality, should include the following:

- date and time each step was completed or date and time log of key parameters,
- identification of persons performing and directly supervising or checking each significant step, operation or control parameter,
- identification of major equipment and lines used,
- material inputs to enable traceability: for example, batch number and quantities of raw material/intermediate and time it was added,
- in-process and laboratory control results,
- the quantity produced for the defined batch and a statement of the percentage of theoretical yield, unless not quantifiable (for example, as in some continuous processes),
- inspection of the packaging and labeling area before and after use,
- labeling control records,
- description of excipient product containers and closures,
- description of sampling performed,
- failures, deviations and their investigations,
- results of final product inspection.

Equipment Cleaning—The manufacturer should design and justify cleaning and sanitization procedures and provide

evidence of their effectiveness. In multipurpose plants the use of the *model product* approach (groups of product of similar type) may be used in justifying a suitable procedure. Cleaning and sanitization procedures should be documented. They should contain sufficient detail to allow operators to clean each type of equipment in a reproducible and effective manner. There should be a record confirming that these procedures have been followed. Equipment and utensils should be cleaned and sanitized where critical to excipient quality and at appropriate intervals to prevent contamination and cross-contamination of the excipient. The cleaning status of equipment should be recorded appropriately.

Where multipurpose equipment is in use, it is important to be able to determine previous usage when investigating cross-contamination or the possibility of such contamination (also see below in this section, *Records of Equipment Use*). During a production campaign, incidental carryover frequently occurs, and it is usually acceptable because cleanup between successive batches of the same excipient is not normally required in order to maintain quality levels. Products that leave residues that cannot be effectively removed should be produced in dedicated equipment. For continuous processing, the frequency of equipment cleaning should be determined by the manufacturer and justified.

Recovery of Solvents, Mother Liquors, and Second Crop Crystallizations—Where solvents are recovered and reused in the same process or different processes, they should meet appropriate standards prior to reuse or mixing with other approved material. Mother liquors or filtrates containing recoverable amounts of excipient, reactants, or intermediates are frequently reused. Such processes should be documented in the production records or logs to enable traceability.

In-Process Blending or Mixing—In-process blending or mixing to ensure batch uniformity or to facilitate processing should be controlled and documented. If the intent of the operation is to ensure batch uniformity, it should be performed so as to ensure homogeneous mixing of materials to the extent feasible and should be reproducible from batch to batch.

In-Process Control—In-process inspection and testing, based on monitoring the process or actual sample analysis at defined locations and times, should be performed. Sampling methods should be documented to ensure that the sample is representative and clearly labeled. In-process samples should not be returned to production for incorporation into the final batch.

The results of in-process tests should be recorded and should conform to established process parameters or acceptable tolerances. Work instructions should define the procedure to follow and should indicate how to use the inspection and test data to control the process. There should be defined actions to be taken when the results are outside specified limits. Where approval to continue with the process is issued within the production department, the specified tests should be performed by trained personnel and the results recorded.

Packaging and Labeling—Procedures should be employed to protect the quality and purity of the excipient when it is packaged and to ensure that the correct label is applied to all containers. Packaging and labeling operations should be designed to prevent mix-ups. Procedures should be implemented to ensure that the correct labels are printed and issued and that the labels contain the correct information. The procedure should also specify that excess labels are immediately destroyed or returned to controlled storage. Excess labels bearing batch numbers should be destroyed. Packaging and labeling facilities should be inspected immediately before use to ensure that materials that are not required for the next packaging operation have been removed. When excipients are labeled on the packaging line, packaged in preprinted bags, or bulk-shipped in tank cars,

there should be documentation of the system used to satisfy the intent of the above procedures.

Records of Equipment Use—Records of quality-critical equipment use should be retained. These records should allow the sequence of cleaning, maintenance, and production activities to be determined.

Validation of Processes for Production and Service Provision—An important factor in the assurance of product quality includes the adequate design and control of the manufacturing process, because product testing alone is not sufficient to reveal variations that may have occurred. Each step of the manufacturing process should be controlled to the extent necessary for ensuring that the excipient meets established specifications. The concept of process validation is a key element in ensuring that these quality assurance goals are met. The process reactions, operating parameters, purification steps, impurities, and key tests needed for process control should be documented, thus providing the basis for validation.

The full validation program that is typically performed in the pharmaceutical industry may not always be carried out by the excipient manufacturer. However, the excipient manufacturer should demonstrate the consistent operation of each manufacturing process: for example, through process capability studies, development, and scale-up reports.

Identification and Traceability

Traceability—Quality-critical items (for example, raw materials, packaging materials, intermediates, and finished excipients) should be clearly identified and traceable through records. These records should allow traceability of the excipient both upstream and downstream. Identification of raw materials used in batch production processes should be traceable through the batch numbering system or other appropriate system. Identification of raw materials used in excipients produced by continuous processing should indicate the time frame during which a particular batch of raw material was processed through the plant. Excipient manufacturers should also have adequate knowledge about the origin of any raw materials derived from plant or animal matter.

Raw materials, including solvents, are sometimes stored in bulk tanks or other large containers, making precise separation of batches difficult. Nevertheless, the use of such materials and containers should be documented in production records.

Inspection and Test Status—There should be a system for identifying the inspection status of quality-critical items, including raw materials, packaging materials, intermediates, and finished excipients. Although storing materials in identified locations is preferred, any means that clearly identifies the test status is satisfactory. Continuously fed materials may need special consideration in order to satisfy these requirements.

Labeling—Labeling for excipient packages is subject to national and international regulatory requirements, which may include transportation and safety measures. At a minimum, labels should include the following:

- the name of the excipient and grade, if applicable,
- the excipient manufacturer's and/or distributor's name,
- the batch number from which the complete batch history can be determined,
- special storage conditions, if applicable.

Customer Property—The excipient manufacturer should establish and maintain procedures for verification, storage, and maintenance of customer-supplied materials intended for incorporation into the customer's excipient. Verification by the manufacturer does not relieve the customer of the responsibility of providing an acceptable material. Material that is lost or that is damaged or otherwise unsuitable for use should be recorded and reported to the customer. In this case, procedures should be in place for acceptable dis-

position and replacement of the material. The manufacturer should also make provisions for protecting other real and intellectual property that is provided by the customer (for example, test equipment, test methods, and specifications).

Preservation of Product

Handling, Storage, and Preservation—Excipients, intermediates, and raw materials should be handled and stored under appropriate conditions of temperature, humidity, and light so that their identity, quality, and purity are not affected. Outdoor storage of raw materials (for example, acids, other corrosive substances, explosive materials) or excipients is acceptable, provided that the containers give suitable protection against deterioration or contamination of their contents, identifying labels remain legible, and containers are adequately cleaned prior to opening and use. Records of storage conditions should be maintained if they are critical for the continuing conformity of the material to specifications.

Packaging Systems—An excipient packaging system should include the following features:

- documented specifications and examination or testing methods,
- cleaning procedures, where containers are reused,
- tamper-evident seals,
- containers that provide adequate protection against deterioration or contamination of the excipient during transportation and recommended storage,
- containers that do not interact with or contaminate the excipient,
- storage and handling procedures that protect containers and closures and minimize the risk of contamination, damage, or deterioration and that will avoid mix-ups (for example, between containers that have different specifications but are similar in appearance).

If returnable excipient containers are reused, previous labeling should be removed or defaced. If the containers are reused solely for the same excipient, previous batch numbers or the entire label should be removed or completely obliterated.

Delivery and Distribution—Identification and traceability of quality-critical aspects are required of excipient manufacturers. Distribution records of excipient shipments should be kept. These records should identify, by excipient batch, where and to whom the excipient was shipped, the amount shipped, and the date of shipment so as to facilitate retrieval if necessary. Where excipients are handled by a series of different distributors, it should be possible to trace them back to the original manufacturer, and not only to the previous supplier. The manufacturer should maintain the integrity and the quality of the product after final inspection and test. Where contractually specified, this protection should be extended to include delivery to the final destination. Excipients should be supplied only within their expiry and/or re-test period.

Control of Measuring and Monitoring Devices—Measuring and test equipment, including computerized systems, identified as being quality-critical should be calibrated and maintained. This includes in-process instruments as well as test equipment used in the laboratory. The control program should include the standardization or calibration of instruments and equipment at suitable intervals in accordance with an established, documented program. This program should contain specific directions, schedules, limits for accuracy and precision, and provisions for remedial action in the event that accuracy and/or precision limits are not met. Calibration standards should be traceable to recognized national or compendial standards as appropriate.

Instruments and equipment not meeting established specifications should not be used, and an investigation should be conducted to determine the validity of the previous results since the last successful calibration. The current calibration

status of quality-critical equipment should be known and verifiable to users.

MEASUREMENT, ANALYSIS, AND IMPROVEMENT

The organization should plan and implement the monitoring, measurement, and improvement activities that are required in order to demonstrate conformity of the excipient to customer requirements and to ensure conformity of the quality management system to this chapter. The organization should evaluate opportunities for improvements through the measurement and analysis of product and process trends.

Monitoring and Measurement

Customer Satisfaction—The excipient manufacturer should establish measurement activities to assess customer satisfaction. Such measurements can include customer complaints, return of excipients, and customer feedback. This information should drive activities that strive to continuously improve customer satisfaction.

Internal Audit—The excipient manufacturer should carry out a comprehensive system of planned and documented internal quality audits. These should determine whether quality activities comply with planned arrangements and should also determine the effectiveness of the quality management system. Audits should be scheduled on the basis of the status and importance of the activity. Audits and follow-up actions should be carried out in accordance with documented procedures. Audit results should be documented and discussed with management personnel having responsibility in the area audited. Management personnel responsible for the area audited should take corrective action on the nonconformities found. *Appendix 1. Auditing Considerations* will be of assistance in establishing an internal audit program.

Monitoring and Measurement of Processes—The excipient manufacturer should identify the tests and measurements necessary for adequately controlling manufacturing and quality management system processes. When critical to excipient quality, techniques used to verify that the processes are under control should be established. When deviations from planned results occur, corrective action should be taken to ensure that the excipient meets requirements. Periodic reviews of key indicators such as process quality attributes and process failures should be conducted to assess the need for improvements.

Monitoring and Measurement of Product—The excipient manufacturer should establish the test methods and procedures to ensure that the product consistently meets specifications. Analytical methods should be suited to their purposes. The analytical methods may be those included in the current edition of the appropriate pharmacopeia or another accepted standard. However, the methods may also be noncompendial. If the excipient manufacturer claims that its product is in compliance with a pharmacopeia or an official compendium, then

- noncompendial analytical tests should be demonstrated to be equivalent to those in the compendia;
- the product should comply with applicable USP general chapters and notices.

Laboratory Controls—Laboratory controls should include complete data derived from tests necessary for ensuring conformity with specifications and standards, including the following:

- a description of the sample received for testing, together with the material name, a batch number or other distinctive code, and the date the sample was taken,
- a statement referencing each test method used,

- a record of raw data secured during each test, including graphs, chromatograms, charts, and spectra from laboratory instrumentation, identified to show the specific material and batch tested,
- a record of calculations performed in connection with the test,
- test results and how they compare with established specifications,
- a record of the person who performed each test and the date(s) the tests were performed.

There should be a documented procedure for the preparation of laboratory reagents and solutions. Purchased reagents and solutions should be labeled with the proper name, concentration, and expiry date. Records should be maintained for the preparation of solutions and should include the name of the solution, the date of preparation, and the quantities of material used. Volumetric solutions should be standardized according to an internal method or by using a recognized standard. Records of the standardization should be maintained.

Where used, primary reference reagents and standards should be appropriately stored and need not be tested upon receipt, provided that a certificate of analysis from the supplier is available. Secondary reference standards should be appropriately prepared, identified, tested, approved, and stored. There should be a documented procedure for the qualification of secondary reference standards against primary reference standards. The reevaluation period should be defined for secondary reference standards, and each batch should be periodically requalified in accordance with a documented protocol or procedure.

Finished Excipient Testing and Release—Finished excipient testing should be performed on each batch to ensure that the excipient conforms to documented specifications. There should be a procedure to ensure that appropriate manufacturing documentation, in addition to the test results, is evaluated prior to release of the finished excipient. The quality unit should be responsible for the release of the finished excipient. For excipients produced by continuous processes, assurance that the excipient conforms to documented specifications may be achieved through the results of in-process testing or other process control records.

Out-of-Specification Test Results—Out-of-specification (OOS) test results should be investigated and documented according to a documented procedure. Retest sample results may be used to replace the original test result only if it is demonstrated on the basis of a documented investigation that the original result is erroneous. When statistical analysis is used, both the original and retest data must be included. The OOS procedure should define which statistical techniques are to be used and under what circumstances. These same principles apply when the sample is suspected of not being representative of the material from which it was taken.

Retained Samples—When practical, a representative sample of each batch of the excipient should be retained. The retention period should be appropriate to the expiry or reevaluation date. The retained samples should be stored and maintained in such a manner that they are readily retrievable in facilities that provide a suitable environment. The sample size should be at least twice the amount required to perform complete specification testing.

Certificates of Analysis—The organization should provide certificates of analysis to the required specification for each batch of excipient.

Impurities—When possible, excipient manufacturers should identify and set appropriate limits for impurities. The limits should be based on appropriate safety data, limits as described in official compendia or other requirements, and sound GMP considerations. Manufacturing processes should be adequately controlled so that the impurities do not exceed such established limits. Many excipients are extracted from or purified using organic solvents. These solvents are

normally removed by drying. It is important that excipient specifications include tests and limits for solvent residues.

Stability—Although many excipient products are stable and may not require extensive testing to ensure stability, the stability of excipients is an important factor in the overall quality of the drug product. For excipients that have been on the market for a long time, historical data may be used to indicate stability. Where historical data do not exist, a documented testing and/or evaluation program designed to assess the stability characteristics of the excipient should be undertaken. The results of such stability testing and/or evaluation should be used in determining appropriate storage conditions and retest or expiry dates. The testing program should include the following:

- the number of batches, sample sizes and test intervals,
- storage conditions for samples retained for testing,
- suitable stability-indicating test methods,
- storage of the excipient in containers that simulate the market container, where possible.

The stability of excipients may be affected by undetected changes in raw materials or subtle changes in manufacturing procedures or storage conditions. Excipients may also be shipped in a variety of packaging types that can affect their stability (for example, plastic or glass bottles, metal or plastic drums, bags, tank cars, or other bulk containers).

Some excipients may be available in different grades (for example, various molecular weights of a polymer or different monomer ratios, different particle sizes, bulk densities) or may be mixtures of other excipients. These excipients may be very similar to others within a product group. Minor quantitative differences of some of the components may be the only significant variation from one product to another. For these types of excipients, a model product approach may be appropriate for assessment of the stability of similar excipients. Stability studies of this type should involve selection of several model products that would be expected to simulate the stability of the product group being assessed. This selection should be scientifically sound and documented. Data from stability studies of these model products can be used to determine theoretical stability for similar products.

Expiry/Retest Periods—An expiry or retest period should be assigned to each excipient and communicated to the customer. Common practice is to use a retest period rather than an expiry period.

Control of Nonconforming Product—Raw material, intermediate, or finished excipient found not to meet its specifications should be clearly identified and controlled to prevent inadvertent use or release for sale. A record of nonconforming product should be maintained. Incidences of nonconformity should be investigated to identify the cause. The investigation should be documented and action taken to prevent recurrence. There should be a documented procedure defining how the retrieval of an excipient from distribution should be conducted and recorded. Procedures should exist for the evaluation and subsequent disposition of nonconforming products. Nonconforming product should be reviewed in accordance with documented procedures to determine if it can be

- reprocessed or reworked to meet the specified requirements,
- accepted by the customer with customer agreement,
- regraded for other applications,
- destroyed.

Reprocessing—Repetition of an activity that is a normal part of the manufacturing process (reprocessing) should occur only when it has already been documented that the excipient may be made in that manner. In all other cases, the guidance for reworking should be followed.

Reworking—An activity that is not a normal part of the manufacturing process (reworking) should be conducted only following a documented review of risk to excipient quality and approval by the quality unit. As appropriate,

when performing the risk assessment, consideration should be given to the following:

- new impurities that may be introduced as a result of reworking,
- additional testing to control the reworking,
- records and traceability to the original batches,
- suitable acceptance criteria for the reworked excipient,
- impact on stability or the validity of the reevaluation interval,
- performance of the excipient.

When the need to rework an excipient is identified, an investigation and evaluation of the cause are required. The equivalence of the quality of reworked material to original material should also be evaluated and documented to ensure that the batch will conform to established specifications and characteristics. Batches of excipients that do not conform to specifications individually must not be blended with other batches that do conform in an attempt to hide adulterated or substandard material.

Returned Excipients—Returned excipients should be identified and quarantined until the quality unit has completed an evaluation of their quality. There should be procedures for holding, testing, reprocessing, and reworking of the returned excipient. Records for returned products should be maintained and should include the name and the batch number of the excipient, the reason for the return, the quantity returned, and the ultimate disposition of the returned excipient.

Analysis of Data—The excipient manufacturer should develop methods for evaluating the effectiveness of its quality management system and use those data to identify opportunities for improvement. Such data can be derived from customer complaints, product reviews, process capability studies, internal audits, and customer audits. The analysis of such data may be used as part of the management review (also see above, *Management Review* under the *Management Responsibility* section). A periodic review of key indicators such as product quality attributes, customer complaints, and product nonconformities may be conducted to assess the need for improvements.

Improvement

Continual Improvement—The excipient manufacturer should take proactive measures to continuously improve manufacturing and quality management system processes. To identify opportunities for continual improvement, analysis of the following performance indicators may be considered:

- causes of nonconforming product,
- results of internal and external audits,
- customer returns and complaints,
- process and operational failures.

Corrective Action—The excipient manufacturer should establish, document, and maintain procedures for the following:

- determining the root causes of nonconformities,
- ensuring that corrective actions are implemented and effective,
- implementing and recording changes in procedures resulting from corrective action.

Preventive Action—The excipient manufacturer should establish, document, and maintain procedures for the following:

- initiating preventive actions to deal with problems at a level corresponding to the risks,
- implementing and recording changes in procedures resulting from preventive action.

APPENDIX 1. AUDITING CONSIDERATIONS

Introduction

Many excipients are used in food, cosmetic, and industrial products as well as in pharmaceuticals. Thus, environmental conditions, equipment, and operational techniques employed in excipient manufacture are often those of the chemical industry as opposed to the pharmaceutical industry. Chemical processes can produce impurities from side reactions. Careful process control is therefore essential to minimize levels of impurities and contamination.

Excipients are often manufactured on a large scale, using continuous processing and automated process controls. Production equipment and processes vary depending on the type of excipient being produced, the scale of production, and the type of operation (for example, batch versus continuous process).

This appendix is intended as an aid in preparing for an audit of an excipient manufacturer. Both external and internal auditors (see also *Internal Audit in Monitoring and Measurement* under the *Measurement, Analysis, and Improvement* section) will find this appendix useful in identifying the significant issues with respect to GMP and quality that require examination. This section will assist excipient manufacturers in identifying key deliverables when adopting the GMP standards listed in the other sections of this chapter; in planning an audit, it will also help to verify the quality of the excipient manufacturing process and the manufacturer's quality management system.

GMP Principles

Control of Impurities and Contamination—In general, the pharmaceutical customer does not perform further chemistry or purification steps on the excipient; it is used as purchased. Consequently, impurities present in the excipient are likely to be present in the drug product. Although dosage form manufacturers have some control over excipient quality through specifications, excipient manufacturers have greater control over the physical characteristics, quality, and presence of impurities in the excipients they produce.

External contamination of the excipient can arise from the manufacturing environment. However, chemical processes used to manufacture excipients are often performed in closed systems that afford protection against such contamination, even when the reaction vessels are not located in buildings. The external environment may require suitable controls to avoid potential contamination wherever the excipient or in-process material is exposed.

Excipient Properties and Functionality—Excipients are frequently used in those types of drug products for which physical characteristics, such as particle size, may be important. Although the manufacturer of the finished dosage form is primarily responsible for identifying the particular physical characteristics needed, it is also the responsibility of the excipient manufacturer to control excipient manufacturing processes to ensure consistent conformity to excipient specifications. Wherever possible, consideration should be given to the end use of the excipient. This is particularly important if the excipient is a direct component of a sterile drug product or one that is claimed to be pyrogen-free.

Consistency of Manufacture and Change Control—A thorough understanding of the manufacturing process and effective control of change can best ensure consistency of excipient quality from batch to batch. Implementation of changes may also have consequences for registration filings with regulatory agencies.

Changes in excipient manufacturing processes may result in changed physical or chemical properties of the excipient that are evident only during subsequent processing or in the finished dosage form. This is particularly important in the

context of the pharmaceutical product approval process where bioequivalence comparisons are made between pivotal, clinical trial batch (*bio batch*) production and commercial scale-up batches. Changes made to the excipient supplied for the commercial product from the excipient supplied for the bio batch should not affect the quality and performance of the commercial drug product. Scale-up of excipients to commercial production may involve several stages, and data may be required to demonstrate consistency between batches through the scale-up process.

Traceability—Traceability of batch-related records to facilitate investigations and retrieval of product is also a key requirement of GMP.

Application of GMP Principles

It is the responsibility of the excipient manufacturer to designate and document the rationale for the point in the manufacturing process at which appropriate GMP are to be applied. From this point on, appropriate GMP should be applied. The manufacturer should apply a level of GMP to each manufacturing stage commensurate with the importance of that step in ensuring product integrity. This may be demonstrated by means of the use of a risk assessment procedure (for example, HACCP, FMEA).

The stringency of GMP in excipient production should increase as the process proceeds from early manufacturing to final stages, purification, and packaging. Physical processing (for example, granulation, coating, or physical manipulation of particle size such as milling or micronizing) as well as chemical processing of excipients should be conducted at least to the standards suggested by this chapter.

It should be recognized that not all intermediates may require testing. An excipient manufacturer should, however, be able to identify critical or key points in the manufacturing process where selective intermediate sampling and testing are necessary in order to monitor process performance.

General Auditing Considerations

Audits of an excipient operation will be influenced by the purpose of the audit and the intended use of the excipient. The key stages of a production process should be examined to determine whether the manufacturer controls these steps so that the process performs consistently. Overall, an audit should assess the excipient manufacturer's capability to deliver a product that consistently meets established specifications.

The audit team may consist of engineers, laboratory analysts, purchasing agents, computer experts, maintenance staff, and other personnel as appropriate to the scope and purpose of the audit. External auditors must respect the confidentiality of the manufacturer's processes and other disclosures.

An audit should focus on the quality-critical processing steps that are necessary for producing an excipient that meets established physical and chemical criteria. These steps should be identified and controlled by the excipient manufacturer. Quality-critical processing steps can involve a number of unit operations or unit processes. Quality-critical steps can include, but are not limited to, the following:

- phase changes involving the desired molecule, solvent, inert carrier or vehicle (for example, dissolution, crystallization, evaporation, drying, sublimation, distillation, or absorption),
- phase separation (for example, filtration or centrifugation),
- chemical changes involving the desired molecule (for example, removal or addition of water of hydration, acetylation or formation of a salt),
- adjustments of the solution containing the molecule (for example, pH adjustment),

- precise measurement of added excipient components, in-process solutions, and recycled materials (for example, weighing or volumetric measurements),
- mixing of multiple components,
- changes that occur in surface area, particle size, or batch uniformity (for example, milling, agglomeration, or blending).

Audit Check Points

A good approach for an excipient plant audit is a review of the following areas:

- nonconformities—such as the rejection of a batch that did not meet specifications, customer complaints, return of a product by a customer, or retrieval of a product. The manufacturer should have determined the cause of the nonconformity, prepared a report of the investigation, and initiated and documented subsequent corrective action. Records and documents should be reviewed to ensure that nonconformities are not the result of a poorly developed or inconsistent process;
- customer complaint files—such as reports that some aspect of the product is not entirely suitable for use, because such problems may be caused by impurities or inconsistencies in the excipient manufacturing process;
- change control logs—to ascertain whether the company evaluates its significant changes to decide whether the customer and/or regulatory authority should be notified;
- nonconforming products meeting or Material Review Board documents and/or equivalent records that demonstrate that the disposition of nonconforming product is handled in an appropriate manner by responsible individuals;
- master formula and production records for frequent revisions that may reveal problems in the excipient production process;
- evidence for the presence of unreacted intermediates and solvent residues in the finished excipient;
- materials management systems, to ensure adequate control over nonconforming materials so that they cannot be sold to customers or used in manufacturing without authorization;
- review of a process flow diagram, to aid understanding of the various processing stages. The critical stages and sampling points should be identified as part of the review of the processing records;
- review of contamination control measures.

In evaluating the adequacy of measures taken to prevent contamination and cross-contamination of materials in the process, it is appropriate to consider the following risk factors:

- the type of system (for example, open or closed). Enclosed systems in chemical plants often are not closed when they are being charged and/or when the final product is being emptied. In addition, the same reaction vessels are sometimes used for different reactions;
- the form of the material (for example, wet or dry);
- the stage of processing and use of the equipment and/or area (for example, multipurpose or dedicated);
- continuous versus batch production.

Documentation and Record Review

Documentation required for the early steps in the process need not be as comprehensive as in the latter stages of the process. It is important that a chain of documentation exist and that it be complete when the following is the case:

- the excipient can be identified and quantified for processes where the molecule is produced during the course of the process. For batch production, a theo-

retical mass balance may also be established with appropriate limits, because deviations from tolerance are a good indicator of a loss of control;

- an impurity or other substance likely to adversely affect the impurity profile or form of the molecule is identified, and subsequent attempts are made to remove it.

As chemical processing proceeds, a chain of documentation should be established that includes the following:

- a documented process,
- the identification of critical processing steps,
- appropriate production records,
- records of initial and subsequent batch numbers,
- records of raw materials used,
- comparison of test results against meaningful standards.

If significant deviations from the normal manufacturing process are recorded, there should be evidence of suitable investigations and a review of the quality of the excipient. Complete documentation should be continued throughout the remainder of the process for quality-critical processing steps until the excipient is packaged and delivered to the end user. The batch should be homogeneous within the manufacturer's specifications. This does not necessitate the final blending of continuous-process material if process controls can demonstrate compliance with specifications throughout the batch.

In order to promote uniformity in excipient GMP inspections, the following basic requirements should be established:

- assignment of a unique batch number to the excipient, enabling it to be traced through manufacture to release and certification,
- suitable controls for the preparation of a batch record for batch processing and/or a production record, log sheet, or other appropriate documentation for continuous processing,
- demonstration that the batch has been prepared using GMP guidelines from the processing point at which excipient GMP have been determined to apply,
- confirmation that the batch is not combined with material from other batches for the purpose of either hiding or diluting an adulterated batch,
- records showing that the batch has been sampled in accordance with a sampling plan that ensures a representative sample of the batch,
- records showing that the batch has been analyzed using scientifically established test methods designed to ensure that the product meets established standards, specifications, and characteristics,
- stability data adequate to support the intended period of use of the excipient; these data can be obtained from historical data, from actual studies on the specific excipient, or from applicable model product studies that can reasonably be expected to simulate the performance of the specific excipient.

APPENDIX 2. GLOSSARY

The terms below are defined as used in this chapter. Wherever possible, definitions used by the International Conference on Harmonization have been used as the basis for the glossary.

Acceptance Criteria: numerical limits, ranges, or other suitable measures of acceptance for test results.

Active Pharmaceutical Ingredient (API): any substance or mixture of substances that is intended to be used in the manufacture of a drug product and that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or animals.

Adulterated Material: a material that either has been contaminated with a foreign material or has not been manufactured using GMP. This does not pertain to a material that simply does not meet physical or chemical specifications.

Batch (Lot): a specific quantity of material produced in a process or series of processes so that it can be expected to be homogeneous. In the case of continuous processes, a batch may correspond to a defined fraction of the production. The batch size can be defined either by a fixed quantity or by the amount produced in a fixed time interval.

Batch Number (Lot Number): a unique combination of numbers, letters, and/or symbols that identifies a batch and from which the production and distribution history can be determined.

Batch Process: a process that produces the excipient from a discrete supply of raw materials that are present before the completion of the reaction.

Batch Record: documentation that provides a history of the manufacture of a batch of excipient.

Blending (Mixing): intermingling different conforming grades into a homogeneous lot.

Calibration: the demonstration that a particular instrument or measuring device produces results within specified limits by comparison with those produced by a reference or traceable standard, over an appropriate range of measurements.

CEP (Certificate of Suitability to the European Pharmacopoeia): certification granted to individual manufacturers by the European Directorate for the Quality of Medicines (EDQM) when a specific excipient or active ingredient is judged to be in conformity with a *European Pharmacopoeia* monograph.

Certificate of Analysis: a document listing the test methods, specification, and results of testing a representative sample from the batch to be delivered.

Commissioning: the introduction of equipment for use in a controlled manner.

Contamination: the undesired introduction of impurities of a chemical or microbiological nature or foreign matter into or onto a raw material, intermediate, or excipient during production, sampling, packaging or repackaging, storage, or transport.

Continuous Process: a process that continuously produces material from a continuing supply of raw material.

Critical: a process step, process condition, test requirement, or other relevant parameter or item that must be controlled within predetermined criteria to ensure that the excipient meets its specification.

Cross-Contamination: contamination of a material or product with another material or product.

Customer: the organization receiving the excipient once it has left the control of the excipient manufacturer; includes brokers, agents, and users.

Deviation: departure from an approved instruction or established standard.

Drug Master File (DMF): detailed information about the manufacture of an excipient that is submitted to the U.S. Food and Drug Administration (FDA).

Drug (Medicinal) Product: the dosage form in the final immediate packaging intended for marketing.

Excipient: substances other than the API that have been appropriately evaluated for safety and are intentionally included in a drug delivery system.

Expiry (Expiration) Date: the date designating the time during which the excipient is expected to remain within specifications and after which it should not be used.

Impurity: a component of an excipient that is not intended to be present but arises as a consequence of the manufacturing process.

In-Process Control/Testing: checks performed during production to monitor and, if appropriate, to adjust the process and/or to ensure that the intermediate or excipient conforms to its specification.

Intermediate: material that must undergo further manufacturing steps before it becomes an excipient.

Lot: See *Batch*.

Manufacturer/Manufacturing Process: all operations of receipt of materials, production, packaging, repackaging, labeling, relabeling, quality control, release, and storage of excipients and related controls.

Master Production Instruction (Master Production and Control Record): documentation that describes the manufacture of the excipient from raw material to completion.

Material: a general term used to denote raw materials (starting materials, reagents, and solvents), process aids, intermediates, excipients, packaging, and labeling materials.

Model Product: a product that represents a group of similar products with respect to composition, functionality, or specification.

Mother Liquor: the residual liquid that remains after crystallization or isolation processes.

Packaging Material: a material intended to protect an intermediate or excipient during storage and transport.

Production: operations involved in the preparation of an excipient from receipt of materials through processing and packaging of the excipient.

Quality Assurance: the sum total of the organized arrangements made with the object of ensuring that all excipients are of the quality required for their intended use and that quality systems are maintained.

Quality Control: checking or testing that specifications are met.

Quality-Critical: describes a material, process step or process condition, test requirement, or any other relevant parameter that directly influences the quality attributes of the excipient and that must be controlled within predetermined criteria.

Quarantine: the status of materials isolated physically or by other effective means pending a decision on their subsequent approval or rejection.

Raw Material: a general term used to denote starting materials, reagents, and solvents intended for use in the production of intermediates or excipients.

Record: a document stating results achieved and/or providing evidence of activities performed. The medium may be paper, magnetic, electronic or optical, photographic, or another medium, or a combination thereof.

Reevaluation Date (Retest Date): the date when the material should be reexamined to ensure that it is still in conformity with the specification.

Reprocessing: repetition of an activity that is a normal part of the manufacturing process and that has been documented previously.

Retrieval: process for the removal of an excipient from the distribution chain.

Reworking: subjecting previously processed material that did not conform to standards or specifications to processing steps that differ from the normal process.

Specifications: list of tests, references to analytical procedures, and appropriate acceptance criteria that are numerical limits, ranges, or other criteria for the tests described for a material.

Stability: continued conformity of the excipient to its specifications.

Top Management: person or group of people who direct and control an organization at the highest level. The highest level can be at either the site level or the corporate level and will depend on the way in which the quality management system is organized.

Traceability: ability to determine the history, application, or location that is under consideration: for example, origin of materials and parts, processing history, or distribution of the product after delivery.

Validation: a documented program that provides a high degree of assurance that a specific process, method, or system

will consistently produce a result meeting predetermined acceptance criteria.

<1079> GOOD STORAGE AND DISTRIBUTION PRACTICES FOR DRUG PRODUCTS

INTRODUCTION

This general information chapter describes good storage and distribution practices to ensure that drug products (medicines) reach the end user (practitioners and patient/consumers) with quality intact.

In the context of this chapter, the following definitions are used.

Definitions

Adulteration: FDA FDC Act, SEC. 501 (351), A drug or device shall be deemed to be adulterated, if (2)(A) It has been prepared, packed, or held under insanitary conditions it may have been contaminated with filth, or whereby it may have been rendered injurious to health; or (B) the methods used in, or the facilities or controls used for, its manufacture, processing, packing, or holding do not conform to or are not operated or administered in conformity with current good manufacturing practice to assure that such drug meets the requirements of this Act as to safety and has the identify and strength, and meets the quality and purity characteristics, which it purports or is represented to possess.

Continuous improvement: Recurring activity to increase the ability to fulfill requirements (see *Quality Management Systems—Fundamentals and Vocabulary. ISO Standard 9000:2005*).

Distribution: Refers to elements such as shipping and transportation activities that are associated with the movement and supply of drug products.

Distribution Management System: A program that covers the movement, including storage and transportation, of drug products.

Documentation: Recorded information.

Drug products: Medicines, including marketed human and veterinary prescription finished dosage medications, in-process/intermediate/bulk materials, drug product samples, clinical trial materials, over-the-counter products (OTC).

End user: The patient as well as the healthcare provider administering the drug product to the patient.

Environmental Management System: A management system that allows for the identification of quality critical environmental aspects (such as temperature, humidity, and/or other environmental factors) for the drug product and ensures that adequate processes to maintain that environment are in place.

Hazardous materials and/or dangerous goods: Any item or chemical which, when being transported or moved, is a risk to public safety or the environment, and is regulated as such under any of the following: Hazardous Materials Regulations (49 CFR 100–180); International Maritime Dangerous Goods Code; Dangerous Goods Regulations of the International Air Transport Association; Technical Instructions of the International Civil Aviation Organization; or the U.S. Air Force Joint

Manual, *Preparing Hazardous Materials for Military Air Shipments*.

International Conference on Harmonization (ICH) Guidance for Industry, Q10 Pharmaceutical Quality System; ICH Q9, *Quality Risk Management*; and, ICH Q1A R2, *Stability Testing of New Drug Substances and Products*: Internationally harmonized documents intended to assist the pharmaceutical industry.

Mean Kinetic Temperature (MKT): The single calculated temperature at which the total amount of degradation over a particular period is equal to the sum of the individual degradations that would occur at various temperatures.

Preventive actions: The measures to eliminate the cause of a potential nonconformity or other undesirable potential situation.

Quality: The physical, chemical, microbiological, biological, bioavailability, and stability attributes that a drug product should maintain in order to be deemed suitable for therapeutic or diagnostic use. In this chapter, the term is also understood to convey the properties of safety, identity, strength, quality, and purity.

Quality Management System (QMS): In the context of this chapter, minimally a set of policies, processes, and procedures that enable the identification, measurement, control, and improvement of the distribution and storage of drug product. It is the management system used to direct and control a company with regard to quality (see ICH Q10 model and *Quality System—Fundamentals and Vocabulary, ISO Standard 9000:2005*).

Risk Management System: A systematic process used to assess, control, communicate, and review risks to the quality of a drug product across the product lifecycle. Integral to an effective pharmaceutical quality system, it is a systematic and proactive approach to identifying, scientifically evaluating, and controlling potential risks to quality as described in ICH Q10. It facilitates continual improvement of process performance and product quality throughout the product lifecycle. ICH Q9 Quality Risk Management provides principles and examples of tools that can be applied to different aspects of pharmaceutical quality.

Written Agreement or Contract (commonly referred to as a Quality Agreement, Technical Agreement, Service Level Agreement, or other): A negotiated, documented agreement between the drug product owner and service provider that defines the common understanding about materials or service, quality specifications, responsibilities, guarantees, and communication mechanisms. It can be either legally binding or an information agreement. A Service Level Agreement may also specify the target and minimum level of performance, operation, or other service attributes.

Storage Management System: A program that is used to control the storage of drug products.

Supply chain: The continuum of entities spanning the storage and distribution lifecycle of a product to the end user.

Temperature stabilizer: A material or combination of materials that stores and releases thermal energy used to maintain a specified temperature range within an active or passive packaging container or system (e.g., water-, chemical-, or oil-based phase change material, such as carbon dioxide solid/dry ice and liquid nitrogen).

Transport vehicles: Vehicles used in the supply chain including semitrailer trucks, vans, trains, airplanes, sea vessels, and mail delivery vehicles. Other vehicles, when used to transport drug products are included here, such as emergency medical service vehicles and industry representatives' automobiles.

SCOPE

Good storage and distribution practices apply to all organizations and individuals involved in any aspect of the storage and distribution of all drug products, including but not limited to the following:

- Manufacturers of drug products for human and veterinary use where manufacturing may involve operations at the application holder's facilities (i.e., facilities that belong to the holder of an approved New Drug Application or Abbreviated New Drug Application) or at those of a contractor for the applicant holder
- Packaging operations by the manufacturer or a designated contractor for the applicant holder
- Repackaging operations in which the drug product may be owned by an organization other than the primary manufacturer
- Laboratory operations at the manufacturer's or at the contractor's site
- Physician and veterinary offices
- Pharmacies including but not limited to retail, compounding, specialty, mail order, hospital, and nursing home pharmacies
- Importers and exporters of Record
- Wholesale distributors; distribution companies involved in automobile, rail, sea, and air services
- Third-party logistics providers, freight forwarders, and consolidators
- Health care professional dispensing or administering the drug product to the end user
- Mail distributors including the U.S. Postal Service (USPS) and other shipping services including expedited shipping services

The information is intended to apply to all drug products regardless of environmental storage or distribution requirements.

It is recognized that conceivably there are special cases and many alternative means of fulfilling the intent of this chapter and that these means should be scientifically justified. Although this chapter is not intended to address the storage and distribution of active pharmaceutical ingredients (APIs), excipients, radioactive products, reagents, solvents, medical devices, medical gases, or clinical trial materials for which storage requirements may not yet be defined (e.g., Phase I clinical trial drug products), the general principles outlined here may be useful if applied selectively or comprehensively.

This general information chapter does not supersede or supplant any applicable national, federal, and/or state storage and distribution requirements, or USP monographs. General Chapter (659) *Packaging and Storage Requirements* contains definitions for storage conditions. This chapter is not intended to cover counterfeiting, falsified medicines, drug pedigrees, or other supply chain security, including chain of custody issues.

BACKGROUND INFORMATION

Storage and distribution processes may involve a complex movement of product around the world, differences in documentation and handling requirements, and communication among various entities in the supply chain. The translation of best practices into good storage and distribution meets these challenges and sets forth a state of control.

The good storage and distribution practices described in this chapter should facilitate the movement of drug products throughout a supply chain that is controlled, measured, and analyzed for continuous improvements and should maintain the integrity of the drug product in its packaging during storage and distribution.

RESPONSIBILITIES

The holder of the drug product application, the drug product manufacturer (in the case of many OTCs, where there is no application) and the repackager bear primary responsibility and accountability including but not limited to the following:

- The decision for regulatory submissions, where applicable, relative to the contents of this chapter for the storage and distribution of drug products. If breaches occur in any of the QMS systems and cannot be justified or documented with scientific evidence, the appropriate entity should consider action with the product to ensure the public safety.
- Determining proper storage and handling practices
- Communicating storage and distribution practices through the supply chain
- Drug product stability profiles or the associated stability information from the holder, inclusive of distribution conditions and excursions that may be allowable should they occur. These stability profiles include the approved storage conditions for the shelf life of the drug product and, where appropriate, supporting data for the distribution conditions, if these differ from the storage conditions.
- Appropriate firms, such as an applicant holder, are to convey relevant environmental requirements (e.g., when appropriate, product-specific lifecycle stability data), when needed to support deviations or temperature excursions. If stability data cannot be reviewed or is not shared, an assessment may be needed to consider regulatory review or other appropriate actions (e.g., destruction of product or additional stability testing).
- Recalling the drug product if it is found to be adulterated in any part of the supply chain

However, all organizations along the supply chain bear responsibility for ensuring that they handle drug products within adequate storage and distribution parameters that will not affect the drug product identity, strength, quality, purity, or safety.

Each holder of drug product is responsible and accountable for the receipt from an entity and transfer out of the drug product to the next entity.

LABELING CONSIDERATIONS FOR DRUG PRODUCTS

The environmental requirements for drug product storage conditions should be indicated on the drug product primary container–closure system. If space on the immediate container is too small (e.g., an ampule) or is impractical for the container–closure system (e.g., blister package), this information can be placed on the most immediate container of appropriate size (e.g., carton). Environmental storage conditions and/or environmental warning statements should be evident, securely fixed, and indelible on the outermost container (generally the shipping container).

Products classified as hazardous materials and/or dangerous goods by the U.S. Department of Transportation or other relevant authorities or bodies should be labeled, stored, and handled in accordance with applicable federal/state/local regulations. Drug products classified as controlled substances by the U.S. Drug Enforcement Administration or by individual state requirements should be labeled and handled in accordance with applicable regulations.

Good practices and controls for labeling should provide the receiver with instructions for the correct handling of the drug product upon receipt. When a drug product's storage conditions are not readily available, use the storage conditions described in USP's *General Notices and Requirements* or the applicable USP monograph; or, contact the drug manufacturer for further information.

Product labels with expanded information beyond the single long-term storage temperature ensure ease of transport and use for shippers, distributors, healthcare professionals, and patients. Product labels should clearly define the storage temperature range, and broader distribution or in-use temperature ranges where allowable. Products labeled "Keep in a cold place" or "Do not freeze" are subject to interpretation and are discouraged if used without accompanying temperature ranges. USP storage definitions and temperature ranges are defined in *General Notices and Requirements*.

During international transport, the proper language(s) should be used to ensure that handlers understand the requirements set forth on drug product labeling. The use of symbols that are recognized by international organizations is advisable.

Drug products can be transported at temperatures outside of their labeled storage temperatures if stability data and relevant scientific justification demonstrate that product quality is maintained. The length of the stability studies and the storage conditions for a drug product should be sufficient to cover the shipment, distribution, and subsequent use of the drug product. The data gathered from ICH, Q1A R2, accelerated testing or from testing at an ICH intermediate condition may be used to evaluate the effect of short-term excursions outside of the label storage conditions that might occur during storage and/or distribution.

QUALITY MANAGEMENT SYSTEM

Good storage and distribution practices require that entities involved in the storage and/or distribution of drug products maintain a Quality Management System (QMS) that is based on standard quality concepts, includes good manufacturing practice (GMP) in compliance with the appropriate regulatory agency(s), and is complementary to the ICH quality guidances, including ICH Q10 *Pharmaceutical Quality System* and ICH Q9 *Quality Risk Management*. In the context of this chapter, the QMS includes the following management system programs: (1) *Storage Management System*, (2) *Distribution Management System*, (3) *Environmental Management System*, and (4) *Risk Management System*.

The storage and distribution QMS should, at minimum, cover the following elements: corrective and preventive actions (CAPA), change management, deviation/investigation management, and the management review process.

Written agreements (e.g., Quality Agreement, Technical Agreement, Service Level Agreements) should be in place between applicable organizations involved in the drug product supply chain. This means that the originating manufacturer may not be required to hold a Written Agreement with all parties in the supply chain. The use of written agreements ensures clarity and transparency, and delineates the responsibilities of each organization in the supply chain.

Good Documentation Practices

Good documentation practices should be practiced in the QMS. This documentation includes standard operating procedures and corporate policies and standards, as well as protocols and other written documents that delineate the elements of the QMS. The QMS programs should describe events and actions that must be documented as well as the proper verbiage to be used, the copies required, and any other items that will ensure adequate processing of the drug product and prevent delays. The documentation process should use a standard such as a quality manual or other practice and, should include routine assessment for review and update as needed.

Written procedures should ensure that drug products are held in accordance with their labeling instructions and associated regulatory requirements. Procedures should provide the written steps needed to complete a process and ensure

consistency and standard outcomes. The following elements should be included: (1) how and when a product should be moved from one transport container/vehicle into another, (2) how products are handled when equipment malfunctions or when there are delays in distribution due to Customs hold, and (3) how to communicate with the necessary parties.

The QMS should require monitoring of processes to demonstrate that a state of control is being maintained, where the set of controls consistently provides assurance of continued process performance and product quality (ICH Q10).

If deviations occur, a nonconformance should be documented, and investigation should be performed and documented as appropriate. The investigative process should determine the root cause(s) of the deviation. For example, the following should be determined: whether the drug product experienced stress, damage, delays, or environmental lapses, or whether there were errors in documentation. The associated supply quality management staff should have final responsibility for approving or rejecting the investigation. The investigation process should be linked to the risk management program to ensure that proper mitigation occurs and preventive measures are put in place.

For example, a written investigation should be performed if the receiving and/or transferring processes result in a drug product being subjected to unacceptable temperature conditions or contamination (e.g., pests, microorganisms, or moisture). Any breach of standard operating procedures should be documented with a risk justification as needed. This information should be forwarded to the appropriate organization responsible for the drug product. The drug product should be quarantined, and final disposition should be based on good science with appropriate evidence to justify the decision(s).

Manufacturers should develop written procedures for recording the security process that confirms container-closure integrity for drug products that require special handling, such as security seals for controlled substances. Returned and salvaged goods records should address how the drug product is assessed through a written procedure. In addition, training on such procedures should be part of the QMS.

Records should be retained for purchases and sales of drug products and should show the date of purchase or supply; the name of the drug product and the amount; the name and address of the supplier or consignee; and the associated lot numbers. These records should allow for the traceability of a drug product in the supply chain.

All records and documents should be maintained in accordance with a traceable records-retention program and should be made available upon request to regulatory agencies. These documents should be approved, signed, and dated by the department responsible for the QMS.

Storage Management System

STORAGE LOCATIONS AND PROCESSES

It is important that each entity define their appropriate storage locations to ensure that adequate controls are in place. These locations include buildings and facilities for drug product storage (e.g., warehouse, storage or hold area, the original manufacturer's warehouses, contractor warehouses, wholesale distribution warehouses, mail order or retail pharmacy storage area, hospital or nursing home pharmacy storage areas; and border Customs storage areas).

In these locations, two basic processes can occur. First, receiving for storage is the act of bringing a drug product into a facility, while transferring refers to the moving of a drug product internally within a facility or into or out of a vehicle. Second, storing and holding refers to the act of

maintaining temporary possession of a drug product in the supply chain process, during which no movement of the product will occur.

STORAGE IN BUILDINGS AND FACILITIES

Drug product storage areas are required to maintain the product temperature between the limits as defined on the product label. Buildings and facilities used for the warehousing, storage, and/or holding of drug products should be of adequate size for their intended use. These facilities should be adequate to prevent overcrowding. The building and facility should be designed to control environmental conditions where necessary and should be made of readily or easily cleanable materials. Sanitation and pest control procedures should be written, indicating frequency of cleaning and the materials and methods used. The pest-control program should ensure the prevention of contamination as well as the safe use of pesticides. Records of all cleaning and pest-control activities should be maintained.

Storage should be orderly and should provide for the segregation of approved, quarantined, rejected, returned, or recalled drug product. If computerized systems are used for the control of storage conditions, the software should be appropriately qualified for its intended purposes. Facilities should have controls that mitigate risks such as fire, water, or explosion. Certain drug products may cause these risks and should be stored accordingly. Storage areas, when not computerized, should be appropriately visually labeled.

Storage facilities themselves, unless thermostatically controlled, cannot be validated; however, they can be qualified via a mapping process. The generator back-up power supply should be qualified.

RECEIVING AND TRANSFERRING DRUG PRODUCTS

Storage of a drug product includes not only the period during which the drug product is held in the manufacturer's storage areas but also time spent at the receiving bay area. When drug products arrive at warehouse loading docks and other arrival areas, they should be transferred as quickly as possible to a designated storage or within a time period that is consistent with the risk and exposure of the product in the receiving area to a designated storage environment to ensure minimal time outside specified storage conditions as described in a written procedure.

Relative to the incoming receipt of drug product, it is recognized that the process of product reaction to ambient conditions begins immediately and may occur quickly (e.g., reach temperature equilibrium within minutes to a few hours depending on details such as the product mass, volume, and packaging density taking into account secondary and tertiary packaging)¹. Time spent in a transport vehicle is considered to be part of the distribution process and is not a storage location.

Receiving docks should protect drug product deliveries from inclement weather during unloading. Any storage area, including loading and unloading docks for receipt and distribution of drug products, should be clean, cleanable, and free from pests. The incoming receiving area should limit access to authorized persons. Where appropriate, the delivery vehicle/container should be examined before unloading to ensure that adequate protection from contamination was maintained during transit. Deliveries should be examined at receipt in order to check that containers are not damaged and that the consignment corresponds to the order. The results of this examination should be documented.

Areas should be designated to provide an adequate space in which containers of drug products can be cleaned and opened for sampling. If sampling is performed in the receiving area, it should be done in a manner that prevents con-

¹ JP Edmond, *Study for Temperature Sensitive Product: Preliminary Testing*, October 2009, University of Florida.

tamination and cross-contamination and ensures that environmental requirements for the drug product are not breached.

Adequate precautions should be taken to prevent theft and diversion of drug products. Drug products that have been identified as counterfeit should be quarantined to prevent further distribution. The appropriate regulatory agencies should be contacted according to established procedures.

Appropriate delivery records (e.g., as applicable, transport vehicle movement papers, receiving/delivery records, data logging records, temperature recorders and similar devices, bill of lading, house air waybill, master air waybill, etc.) should be reviewed by each receiving entity in the supply chain to determine if the product has been subjected to any transportation delays or other events that could have exposed the product to undesirable conditions. Each entity should ensure that their respective Service Level Agreement documents and supporting documents such as SOPs cover delivery and receiving responsibilities of the transactional parties.

Smoking, eating, and drinking should not be permitted in any storage/hold areas.

REFRIGERATORS AND FREEZERS

Refrigerators and freezers used to store drug products are required to maintain the product temperature between the limits as defined on the product label. Typically, a refrigeration unit specification would be set to 5° with an allowable range of $\pm 3^\circ$ to store products labeled 2°–8°. Freezer temperatures may vary and typically range from -25° to -10° . Some frozen drug products, however, require lower temperatures, e.g., dry ice or liquid nitrogen temperatures.

Regular operating procedures and maintenance protocols should be in place along with written contractual agreements for all maintenance and evaluation procedures including the following:

1. Items should be stored in the units in a manner that allows adequate air flow to maintain the specified conditions.
2. Units should be positioned in the facility so that they are not subjected to environmental extremes that could affect their performance. If this cannot be prevented, the mapping protocol should include a provision for testing during the anticipated environmental extremes.
3. Large commercial units such as walk-in cold rooms are qualified via a temperature mapping study or other type of qualification process to determine the unit's suitability for storing drug products. A suitable number of temperature-recording devices should be utilized to record temperatures and to provide temperature area maps. Thereafter, the units should be monitored as determined by the results of the mapping study. Refer to the *Temperature Monitoring* section under *Environmental Management System*.
4. Units should utilize recording systems to log and track temperatures. Alarm systems should be an integral part of the monitoring system for both refrigerators and freezers. While automated systems monitor units continuously, manual checks should be performed as appropriate to the validation program. When automated systems are not available, manual systems may be used.

Distribution Management System

Distribution of drug products occurs within a facility or location such as a manufacturer, wholesaler, pharmacy dispensing area, retail site, clinic/hospital/nursing home pharmacy, and the physician's practice. Distribution of drug products occurs as point-to-point movement within the sup-

ply chain between distribution facilities via semitrailer trucks, vans, emergency medical service vehicles, industry representatives' automobiles, trains, aircraft, sea vessels, and mail delivery vehicles.

Communication within the supply chain should be coordinated to determine proper timing for drug products to be transported and received, taking into account holiday schedules, weekends, or other forms of interruption. When international distribution is required, alerts should be made in advance and proper language should be used to ensure understanding of the requirements set forth on drug product labeling.

PACKAGING FOR THE DISTRIBUTION AND TRANSPORTATION PROCESSES

Pharmaceutical manufacturers should consider primary, secondary, and tertiary packaging that best protects the drug product during storage and distribution. Package performance testing should be documented as part of a manufacturer's QMS. Several standard test procedures are available for evaluating package performance for factors such as shock, vibration, pressure, compression, and other transit events. Organizations with standard test methods include the following: the American Society for Testing and Materials (ASTM) *Standard Practice for Performance Testing of Shipping Containers and Systems*, and the International Safe Transit Association (ISTA) specifications for various types of transit modes such as less-than-truckload, small package, rail car, and air freight.

It is important to be aware that removal or modification of the original packaging may subject the product to unacceptable conditions.

The packaging (tertiary or thereafter) for the distribution of the drug product should be selected and tested to ensure that product quality is maintained and to protect the contents from the rigors of distribution including environmental or physical damage.

All drug products have storage requirements that may contain specific controls. The container used for transporting the drug product should be qualified on the basis of the labeled conditions of the product as well as anticipated environmental conditions. Consideration should be made for seasonal temperature differences, transportation between hemispheres, and the routes and modes of transport.

The type, size, location, and amount of the temperature stabilizers required to protect the product should be based on documented studies of specific distribution environments including domestic and international lanes, mode(s) of transport, duration, temperature, and other potential environmental exposures or sensitivities that may impact product quality. Transportation container materials such as warm/cold packs and materials used to control temperature conditions should be properly conditioned before use. Barrier protection may be important in helping to determine the position of materials such as gel packs in order to avoid direct contact with the drug product. It should be determined if studies are required to ensure that the dry ice and its vapors do not adversely affect the drug product, including the drug product labeling.

VALIDATION AND THERMAL PERFORMANCE QUALIFICATION FOR TRANSPORT SYSTEMS

Drug product transport systems should be continuously monitored by calibrated monitoring systems, (continuous verification), or shipping systems should be qualified and based on historical data relative to the process. However, it may be acceptable to use product stability data and supply chain risk assessment to justify shipping without either continuous monitoring or qualification of the shipping system.

Operational and performance shipping studies should on a generic level be part of a formal qualification protocol that

may use controlled environments or actual field testing, depending on the projected transport channel. These studies should reflect actual load configurations, conditions, and expected environmental extremes. Testing should be performed on both active and passive thermal packaging systems.

Environmental Management System

While storage and distribution temperature ranges for drug products are labeled on the packaging, relative humidity effects occur over a much longer time frame. The primary container is designed and tested to protect the product from moisture; therefore, humidity monitoring should be considered when a product will be stored in an uncontrolled facility.

TEMPERATURE MONITORING

Environmental conditions are important parameters to consider in the storage and distribution of all drug products and may require monitoring depending on the requirements. When specific storage conditions are required and transportation qualification has not been performed, and in the absence of active or passive containers, environmental recorders or devices should be used to confirm that an acceptable range has been properly maintained during each stage in the supply chain.

Temperature is one of the most important conditions to control, and requirements for each drug product should be based on stability data. Temperatures should be tracked using a monitoring system, and the monitoring devices used should be included in a calibration and/or preventive maintenance program. Environmental monitoring devices should be calibrated for their range of operation. The monitoring devices used should provide an alert mechanism if the preset ranges are breached. The following practices and controls are examples of appropriate measures that should be put in place to ensure environmental control (see also *Monitoring Devices—Time, Temperature, and Humidity* (1118)):

- Temperature-monitoring equipment, a monitoring device, a temperature data logger, or other such device that is suitable for its intended purpose should be used.
- An appropriate number of temperature monitors or some other form of recordation or proof of temperature control. Temperature monitor(s) should be used with every distribution process unless another process has been put in place to ensure specified temperature ranges.
- Electronic temperature monitors should be calibrated to National Institute of Standards and Technology (NIST) or other suitable standard.
- Chemical temperature indicators may be used as appropriate.
- Predetermined temperature ranges should be set for all applicable areas, as well as a plan of action in the event of an unacceptable excursion.

TEMPERATURE MAPPING

The basis of any temperature mapping in a temperature controlled space (e.g., facility, vehicle, shipping containers, refrigerator, freezer) is the identification and documentation of a sound rationale used for a given mapping procedure. The temperature variability associated with mapped locations and the level of thermal risk to the product should be defined, unless another process has been put in place to ensure environmental control.

A temperature mapping study should be designed to assess temperature uniformity and stability over time and

across a three-dimensional space. Completing a three-dimensional temperature profile should be achieved by measuring points at not less than three dimensional planes in each direction/axis—top-to-bottom, left-to-right, front-to-back, where product will be present.

When temperature mapping is necessary, it should begin with an inspection of the facility, equipment and/or vehicle and should be re-evaluated as appropriate. Environmental mapping also should be performed after any significant modification to the distribution system that could affect drug product temperature.

Facility temperature mapping: The following factors, which may contribute to temperature variability, should be considered during the process of temperature mapping storage locations: (1) size of the space; (2) location of HVAC equipment, space heaters, and air conditioners; (3) sun-facing walls; (4) low ceilings or roofs; (5) geographic location of the area being mapped; (6) airflow inside the storage location; (7) temperature variability outside the storage location; (8) workflow variation and movement of equipment (weekday vs. weekend); (9) loading or storage patterns of product; (10) equipment capabilities (e.g., defrost mode, cycle mode); and (11) SOPs.

The recording of temperatures during the thermal mapping of a warehouse or cold room should be sufficient in time frame to capture workflow variation that may impact air flow and the resulting temperature fluctuation (i.e., a period of one week is recommended for data collection and should capture workflow cycles).

Equipment (container/trailer) temperature mapping: To minimize risk of product exposure to damaging temperatures during transport, dedicated containers/vehicles cargo space should be mapped. When complete fleet mapping (i.e., wholesaler or distributor vehicles) is not realistic or appropriate, minimally at least one container/vehicle from the fleet must be mapped. Thereafter, the following conditions should be considered: (1) SOPs, including loading and unloading procedures; (2) route-specific operation of the temperature control equipment; (3) seasonal effects encountered on expected routes; (4) loading patterns; and (5) transport durations.

When nondedicated (i.e., mail carriers) transport containers/vehicles and equipment are used, they should be designed to minimize the risk of contamination of the product being handled. If environmental mapping of such vehicles is not performed, some other means of control should be in place to ensure that the drug product is adequately protected. Mapping by the shipper may not be necessary if the shipper uses a transport container that is properly insulated and has been previously qualified for the duration of the distribution process by the transport container manufacturer via a mapping study or if drug products are continuously monitored by calibrated monitoring systems (continuous verification).

The vehicle in which drug products are transported should be mapped to determine the appropriate placement of temperature-recording devices and to confirm that the load configuration is not restricting air flow. The following are recommended practices and controls for vehicles that receive and transfer drug products:

1. Transport containers/vehicles and equipment used to store and transport drug products should be suitable for their intended function.
2. Procedures should be established that describe how to operate, clean, and maintain transport containers/vehicles and equipment used in the storage and distribution of drug products.
3. Transport containers/vehicles should be designed to prevent damage to the drug product, and pharmaceutical manufacturers should collaborate with their transporter to determine contingency response plans for how drug products are handled when equipment malfunction.

4. When drug product must be moved from one transport container/vehicle into another, the proper load configuration should be followed.
5. It should be understood how communication is made to the necessary entities when such transfer occurs.
6. Subcontracted vehicles should be considered in contractual agreements and audits, and documentation should be maintained for their use.

Temperature mapping should account for maximum and minimum loads to capture temperature variability resulting from variations in temperature mass of the payload. Performance of equipment under extreme scenarios including door open, door closed, and simulated equipment failure should be taken into account.

Thermal mapping of vehicles should be representative of the fleet with the intention of capturing variability across the range of vehicles (type of vehicle including non-refrigerated equipment, use, heating and/or cooling system). A periodic requalification program should be documented.

Mapping for both facilities and transportation containers/vehicles should be done in a way that confirms their fitness for operation during periods of expected extreme weather (e.g., summer and winter). Facilities should be mapped under varying operating conditions—ideally during periods of greater variability, accounting for and capturing the result of any seasonal fluctuations of inventory movement, equipment movement, or workflow variation.

The temperature-mapping protocol and associated number of temperature data loggers used to map a three-dimensional space should meet the intent of demonstrating three-dimensional uniformity and compliance with product requirements. For both facility and trailer/container temperature mapping, the ambient conditions should be recorded and correlations between ambient conditions and potential thermal risks inside the controlled space should be identified. Drug products should not be stored in areas where a thermal risk has been identified as a result of the temperature mapping. Areas identified as being unsuitable for storage should be clearly labeled as such to ensure that they are not used.

Temperature data loggers should be used for temperature mapping and PQ testing of facilities, equipment, and transportation containers used for storage or transportation of temperature-sensitive medicinal products. Temperature data loggers and any associated software applications should be appropriately validated. Certificates of calibration to an NIST or other international traceable standard should be available for individual monitoring devices.

EXCURSIONS

The mapping process will help determine when excursions could occur and are useful when pharmaceutical manufacturers develop a plan for dealing with them. Alarms should be used to reveal environmental excursions during operations. Temperature excursions for brief periods outside of respective storage label conditions may be acceptable provided stability data and scientific/technical justification exists demonstrating that product quality is not affected (see Health Canada's GUI 0069 entitled, *Guidelines for Temperature Control of Drug Products During Storage and Transportation*, 2011).

MEAN KINETIC TEMPERATURE (MKT) CALCULATION

The MKT is the single calculated temperature at which the total amount of degradation over a particular period is equal to the sum of the individual degradations that would occur at various temperatures. MKT may be considered as an isothermal storage temperature that simulates the non-isothermal effects of storage temperature variation. It is not a simple arithmetic mean.

The temperatures used for calculating MKT can be conveniently collected using electronic devices that measure temperatures at frequent intervals (e.g., every 15 minutes). MKT can be calculated directly or the data can be downloaded to a computer for processing. Software to compute the MKT is available commercially.

For dispensing sites, such as pharmacies and hospitals, where the use of such instruments may not be feasible, devices such as high-low thermometers capable of indicating weekly high and low temperatures may be employed. The arithmetic mean of the weekly high and low temperatures is then used in the calculation of MKT. MKT is calculated by the following equation (derived from the Arrhenius equation):

$$T_k = \frac{\Delta H/R}{-\ln \left(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \dots + e^{-\Delta H/RT_n}}{n} \right)}$$

where T_k is the mean kinetic temperature; ΔH is the heat of activation, 83.144 kJ · mole⁻¹ (unless more accurate information is available from experimental studies); R is the universal gas constant, 8.3144 × 10⁻³ kJ · mole⁻¹ · degree⁻¹; T_1 is the value for the temperature recorded during the first time period, e.g., the first week; T_2 is the value for the temperature recorded during the second time period, e.g., second week; and T_n is the value for the temperature recorded during the n th time period, e.g., n th week, n being the total number of storage temperatures recorded during the observation period. [NOTE—All temperatures, T , are absolute temperatures in degrees Kelvin (K).]

MKT DURING STORAGE AND DISTRIBUTION

The holding of a drug may occur as part of storage and distribution practices. Drug products in the distribution supply chain may be held at temperatures outside their labeled storage requirements as determined by an appropriate stability study. Drug products stored either in warehouse conditions or in transportation modes may experience excursions from their acceptable temperature ranges. Each product excursion must be evaluated to determine the final product effect. The means of evaluation must be scientifically sound with documented technical justification that the integrity of the drug product has not been affected. One method of analysis for drug product stored outside its respective label storage conditions is the use of an MKT calculation.

Because MKT expresses the cumulative thermal stress a drug product experiences, it is considered an acceptable practice for storage, and it follows that it should be considered for transit excursions in the process of distribution. The calculation must be justified for use with distribution excursions by confirming that the stability limiting characteristic of the product follows first order kinetics over the temperature range encountered. The ICH stability-testing guidelines define MKT as a "single" derived temperature, which, if maintained over a defined period, would afford the same thermal challenge to a pharmaceutical product as would have been experienced over a range of both higher and lower temperatures for an equivalent defined period.

The MKT analysis must be based on good science and should take into account the integrity of the product. The calculated MKT is not sensitive to the impact of excursions that may occur if the baseline is a long period of time such as a storage segment or the entire lifetime of the drug product. For shorter baseline periods of time, such as transport segments, an excursion can have a significant impact on the resulting MKT for that segment; however, this would not necessarily have a significant impact on product quality.

The MKT analysis may be used for storage conditions that have exceeded the acceptable parameters for a drug prod-

uct, for a short period of time and is not intended to be a measure for long-term storage.

Knowing the MKT for an excursion is useful for evaluating the potential impact on product quality. However, it is also essential to know the upper and lower temperature limits of any excursion. If these extreme temperatures are outside available stability data, it may not be possible to predict the quality impact of the excursion with any confidence regardless of the MKT. Although higher temperatures are given greater weight in the calculation, the calculation of MKT for nonfrozen product that becomes frozen for any amount of time may not result in an acceptable temperature although the product may not be adulterated. At higher temperatures the kinetics of degradation may change or new degradation reactions may occur; at lower temperatures (near freezing) a phase change may occur that is known to have a negative impact on the quality of some drug products (e.g., some proteins and vaccines). For an example of a calculation, see *Pharmaceutical Calculations in Prescription Compounding* <1160>.

Emergency Medical Service Vehicles, Automobiles, and Van Transportation

Road vehicles used to transport drug products (e.g., ambulances and other emergency response vehicles, vans, or automobiles, including those used by sales representatives to transport physicians' samples) should be suitable for their purpose. Monitoring devices should be placed in different areas of the trunk or cabin where the drug product will be positioned during seasonal extremes (e.g., summer and winter). The monitor should be secured so that it is immobile, and there should be no ambiguity about its exact position within the payload so that the monitor is always placed in the same position. Monitoring devices used on or in packages or on containers may also be used. Suitable measures should be taken to maintain the drug product within the allowable limits of the labeled storage requirements. Storage of physician drug product samples by sales representatives is regulated under 21 CFR Part 203.34(b)(4).

Mail Order Pharmacy Distribution

The mailing party is accountable for the appropriate mailing process. Mail distributors including the U.S. Postal Service (USPS) and other shipping services including expedited shipping services are responsible to provide the service contracted.

In the event that the package cannot be delivered as scheduled, the package should be returned to the mailing pharmacy.

Risk Management System

Risk Management System strategies should ensure that each organization's best interests are served by adhering to proper practices, controls, and procedures, including but not limited to the following: the nature of the drug products; distribution requirements on the readable container labeling; exposure to adverse environmental conditions; number of stages/receipts in the supply chain; manufacturer's written instructions; contractors; and drugs at risk from freezing (vaccines, insulin, and biological products) or elevated temperatures (fatty-based suppositories, vaccines, insulin, and biological products).

Examples of risks include the following: (1) vibration that can cause aggregation of some drug products such as proteins and peptide-based drugs; (2) temperature excursions that may lead to phase changes (melting or freezing); (3) loss of container-closure integrity in transit that could cause glass fractures or loss of sterility in sterile drug product containers; and (4) ingress of water or oxygen that could lead to an increase in degradation products. Appropriate firms

such as applicant holders are recommended to convey relevant environmental requirements when needed to support deviations or excursions. There may be alternate ways of determining acceptable environmental conditions and these should be documented and justified.

Pharmaceutical manufacturers should ensure that suppliers of drug product transportation are monitored. Auditing transportation firms should be carried out routinely to ensure adequate product handling. The manufacturer's change control system should capture and evaluate changes in logistic factors such as warehouse or receiving areas and vehicle changes.

CONCLUSION

The practices and processes set forth in this general information chapter apply to storage and distribution as part of the life-cycle management of drug products. All involved should ensure the product to its point of use, creating a contiguous supply network that is collaborative and emphasizes preventive measures to protect drug product quality. The increase in global processes coupled with products requiring special environmental controls highlights the need for a strong QM program. QM should provide the foundation for maintaining the storage and distribution practices in a continual improvement program and part of an overall management system review by each entity, as appropriate, in the supply chain.

It is equally important to stay current and be ready to change as new solutions evolve. These new technologies should be considered in developing strategies for good distribution practices, controls, and procedures.

<1080> BULK PHARMACEUTICAL EXCIPIENTS—CERTIFICATE OF ANALYSIS

BACKGROUND

This general information chapter is derived from the *Certificate of Analysis Guide for Bulk Pharmaceutical Excipients*, prepared by The International Pharmaceutical Excipients Council of the Americas (IPEC-Americas), an international guidance document on the preparation and appropriate use of a Certificate of Analysis (COA) for these excipients, referenced throughout the chapter as "excipient(s)". The chapter defines the suggested elements of a Certificate of Analysis, provides a template for organizing required and optional data in a logical manner, and assists in establishing a uniform understanding of the roles and responsibilities of excipient manufacturers, distributors, and users.

The principles and information in this chapter can be applied to the manufacture of all bulk pharmaceutical excipients intended for use in human drugs, veterinary drugs, and biologics. As an international guidance document, it cannot specify all national legal requirements nor cover in detail the particular characteristics of every excipient. When considering how to use this chapter, each manufacturer, distributor, or user should consider how it may apply to that specific manufacturer's product and processes. The diversity of excipients means that some principles of the chapter may not be applicable to certain products and processes.

The chapter is divided into several parts. The first part provides background discussion necessary for the design and suggested elements of a COA. A template is provided to show the format and placement of information in the COA. This is followed by a detailed discussion to ensure that the purpose and meaning of the specific information contained in the COA is understood. For a list of terms used in this information chapter and their definitions, see *Appendix 1*.

GENERAL GUIDANCE

International regulations governing drugs require that components of the drugs be manufactured, processed, packed, and held in accordance with good manufacturing practices (GMPs). For a thorough discussion of GMPs that apply to excipient manufacture, see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* <1078>. The excipient is often a natural substance, mixture, or polymer whose chemical and physical properties are difficult to quantify and that is often used with a broad range of active pharmaceutical ingredients and in a diverse range of finished dosage forms. Until now, there were no guidance documents that specifically focused on the content or format of COAs for excipients and that addressed the diversity of both the excipients and their usage.

Preparation and Appropriate Use of a Certificate of Analysis—The Certificate of Analysis for excipients should be prepared and issued by the supplier of the material, following the general guidelines discussed below. Primary responsibility for the preparation of the COA belongs to the excipient manufacturer. It is most important that a complete and accurate COA be provided to the excipient user for specific lots or batches intended for use in the pharmaceutical industry. Additional considerations should be made for the preparation and issuance of a COA by a distributor of excipients.

The user of a bulk pharmaceutical excipient should always receive a COA for material to be used in the manufacture of a drug product. At a minimum, the user should perform adequate identification tests on each lot of excipient received before releasing it for use in the drug product. Specific identity tests should be used whenever possible. It is a regulatory requirement that excipients be assessed for conformity with all appropriate specifications. However, testing of all specification parameters may not be required for lot release if adequate compliance assurances are provided on the supplier's COA. Before using an excipient in a pharmaceutical product based on COA data, the user also should have an understanding of the supplier's control systems and compliance with GMPs, through appropriate auditing or qualification of the supplier.

Nevertheless, it is the responsibility of the user of the excipient to verify any of the analytical data contained in the COA if knowledge of such information is deemed essential to the use of that excipient. Such testing may go beyond the scope of the compendial methods described in the *NF*, or beyond those used to develop the information in the COA.

To use test results from a COA, the user must also establish the reliability of the supplier's COA test results by periodically performing all required tests and comparing the results obtained to the supplier's test results. Occasionally, it may not be possible to perform all the required tests because of special equipment requirements, etc., that may not be available to the user. Performing fewer than all these tests may be acceptable provided that the reliability of the supplier has been adequately determined using other appropriate supplier qualification techniques.

It is important to understand that these results may not always specifically correlate, especially when an excipient is produced as a continuous lot. However, the user's test re-

sults should demonstrate compliance with the specification requirement.

Use of Contract Facilities—Contract facilities are frequently used in the manufacture, testing, and distribution of excipients. When such facilities are used, the supplier of the excipient has the obligation to ensure that the facilities operate under appropriate quality standards (i.e., cGMP, GLP, etc.).

DESIGN AND SUGGESTED ELEMENTS OF A CERTIFICATE OF ANALYSIS

The suggested elements of a COA are listed below and are included in the following *Certificate of Analysis Template* section of this chapter. Excipient suppliers may organize the suggested elements presented in the COA template at their discretion; however, the parts of the template were designed to present the suggested and optional information in a logical manner. For a detailed description of each element and examples of statements, see the appropriate section below in this chapter.

The origin and the identity of the excipient are typically established in a *Header* section. The manufacturer and manufacturing site should be identified if different from the supplier and supplier location, enabling the user to make certain that the excipient comes from a qualified source. Although the manufacturer should be made known to the user, the use of codes for manufacturers and manufacturing sites on the COA to protect confidentiality is acceptable. The identity of the excipient must be definitively established by stating the compendial and trade name, the grade of the material, and applicable compendial designations.

A lot/batch number or other means of uniquely identifying the quantity of material covered by the COA and information relating specifically to it are typically included in a *Body* section. The lot number or other unique identification of the material, its date of manufacture, and product code or number should be stated and traceable to a specified lot. If applicable, the expiration date, recommended re-evaluation date, or other relevant statement regarding the stability of the excipient is typically included in this section. Any information required by the customer would also be included here.

The actual test results applicable to the quantity of material covered by the COA are included in an *Analysis* section. The test name, the result, the acceptance criteria or specifications, and a reference to the test method used should be included for each characteristic listed. Reporting of actual data and observations is recommended rather than nonspecific "passes" or "conforms" statements. If the reported results are derived from a skip-lot or reduced frequency testing program, or an average or in-process test result, this should be noted on the COA.

The *Certification and Compliance Statement* section is used to list various types of statements that may be required depending on the excipient and specific user needs. These statements are usually negotiated between supplier and user based on specific application requirements. Any declaration of the supplier that includes compliance of additional compendial or other regulatory requirements is typically included in this section.

Many excipients have applications other than pharmaceuticals, such as food, cosmetics, or industrial products. Any product listed as being in compliance with specific regulations should meet the specifications and requirements of that regulation and must be manufactured under appropriate GMPs.

The identity of the individual approving the content of the COA should appear on the COA. The page number and total number of pages should also appear on the COA. This information is usually included in a *Footer* section.

CERTIFICATE OF ANALYSIS TEMPLATE

Listed below is a template for the content and format of a COA.

Header

- Titled "Certificate of Analysis"
- Company Name, Address, Phone Number, and Identity of Manufacturer and Manufacturing Site
- Name (compendial/trade) of Excipient
- Grade of Excipient
- Compendial Designation

Body

- Lot/Batch Number
- Date of Manufacture
- Product Code or Number
- Expiration Date (if required)
- Recommended Re-Evaluation Date (if required)
- Stability Statement (if required)
- Customer Required Information

Analysis

- Test Name
- Test Results
- Acceptance Criteria (i.e., specifications)
- Reference to the Test Method
- Reference to Skip-Lot Testing (if appropriate)
- Reference to Average or In-Process Test Results (if appropriate)
- Date Retested (if appropriate)
- Summary of Noncompendial Testing (if any)

Certification and Compliance Statements

- GMP Compliance
- Additional Regulatory References
- Potential to Meet Additional Compendial Standards
- Content Listing and Grade of Ingredients (if a mixture)
- Other Specific Compliance Statements [e.g., organic volatile impurities (OVI), residual solvents, transmissible spongiform encephalopathy (TSE), etc.]

Footer

- Identity of Authorized Individual for Approval
- Date of Approval
- Page Number (i.e., 1 of __)

COMPENDIAL DESIGNATION

For a supplier to claim a compendial grade on the COA for an excipient, two requirements should be met. The first requirement is that the excipient be manufactured according to recognized principles of GMPs (see *General Notices and Requirements*). Adequate conformance to GMPs should also be demonstrated for subsequent steps in the distribution of the excipient. The second requirement is that the excipient meet all the specifications contained in the appropriate compendial monograph, unless its difference is stated on its label, as defined under *General Notices and Requirements*. When an excipient is listed as compendial grade, it is understood that the above requirements have been met for the material, and the user would be able to confirm this through an appropriate audit of the supplier.

Compendial standards define what is considered an acceptable article and also give test procedures that demonstrate that the article is in compliance. These standards ap-

ply at any time in the life of the article from production to consumption. The supplier's release specifications and compliance with GMPs are developed and followed to ensure that the article, when stored according to recommended conditions, will comply with compendial standards until its expiration or recommended re-evaluation date.

Every compendial article shall be so constituted that when examined in accordance with these assay and test procedures, it meets all the requirements in the monograph defining it, as well as meeting any provisions under *General Notices and Requirements* and in the general chapters, as applicable. However, it is not to be inferred that application of every analytical procedure in the monograph to samples from every production batch is necessarily a prerequisite for ensuring compliance with compendial standards before the batch is released for distribution.

Data derived from manufacturing process validation studies and from in-process controls may provide greater assurance that a batch meets a particular monograph requirement than analytical data derived from examination of finished units drawn from the batch. On the basis of such assurances, the analytical procedures in the monograph may be omitted by the supplier when judging compliance of the batch with the compendial standards.

DATES ON A CERTIFICATE OF ANALYSIS

Part of the overall goal to standardize COA for excipients includes a provision for the consistent reporting of appropriate, meaningful, and well-defined dates. The discussion below indicates specific dates that are expected on the COA, along with definitions of the dates, in order to provide suppliers and users of excipients with a mutual understanding of their meaning. Use of the recommended terminology will be helpful in reducing the number of questions on dating information reported for excipients. Use of terminology other than that discussed below is discouraged, because the terms may be ill-defined and have different meanings for the excipient supplier and user. Examples of such terms that should **not** be used include "shelf life", "use-by date", "warranty date", and "expiration period".

In reporting dates on COA for excipients, it is important that a clear and unambiguous format be used to prevent possible misinterpretation. To accomplish this, it is recommended that an alpha designation be used for the month (may be abbreviated), rather than a numerical representation. It is also recommended that the year include all 4 digits (e.g., Jan. 1, 2005, or 1 Jan. 2005).

Date of Manufacture—The date of manufacture should be included on the COA for each excipient lot and should be assigned by the suppliers on the basis of their established policies and procedures. It is recognized that excipients may be manufactured using a variety of processes (e.g., continuous or batch) that may require a period of several days or more to complete. In addition, some excipients may be mixtures or blends of other excipients, and excipient production may include reprocessing steps. Because of this diversity, the date of manufacture should be clearly defined by the supplier and consistently applied for the particular excipient and process. In reporting the date of manufacture, the excipient supplier should indicate the date of completion of the final manufacturing process (as defined by the supplier).

It is important to note that repackaging alone is not considered a processing step to be used in determining the date of manufacture. To provide traceability for a specific excipient lot, other dates may be required in addition to the date of manufacture in order to reflect additional steps such as repackaging.

Expiration Date and Recommended Re-Evaluation Date—The stability of excipients may be an important factor in the stability of the finished pharmaceutical dosage forms that contain them. Many excipients are very stable

and may not require extensive testing to demonstrate continued conformance to appropriate specifications. Other excipients may undergo chemical, physical, and microbiological changes over time that cause the material to fall outside established specifications.

Appropriate expiration and/or recommended re-evaluation dates for excipients should be established from the results of a documented stability-testing program or from historical data. The testing program should include defined and controlled storage conditions (e.g., temperature and humidity), a consideration of different packaging types that may be used as market containers, and meaningful, specific test methods to adequately assess the stability characteristics of the excipient. Stability testing should determine whether possible degradation, moisture gain or loss, viscosity changes, or other possible changes occur to make the excipient unacceptable for use (e.g., unstable or hygroscopic materials). For additional information on excipient stability, see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* <1078>.

The expiration date for an excipient is defined as the date after which the supplier recommends that the material should not be used. Prior to the assigned expiration date, the excipient is expected to remain within established specifications, if stored according to the supplier's recommended conditions.

The recommended re-evaluation date for an excipient is the date suggested by the supplier after which the material should be re-evaluated to ensure continued compliance with specifications. Re-evaluation of the excipient may include physical inspection and appropriate chemical, physical, and microbiological testing. Prior to the re-evaluation date, the excipient is expected to remain within established specifications, provided it has been stored according to the supplier's recommended conditions. But beyond the recommended re-evaluation date, the excipient should not be used without adequate evaluation at appropriate intervals, to determine whether the material continues to be acceptable for use. The recommended re-evaluation date differs from the expiration date in that the excipient may be re-evaluated to extend the length of time the material may be used, if supported by the results of the evaluation and appropriate stability data.

In reporting the expiration and recommended re-evaluation dates, the excipient supplier is providing important information to the user about the stability of the material. As discussed previously, the assignment of an expiration date and a recommended re-evaluation date should be based on appropriate evaluation of potential changes that may occur in the material's properties. It is acceptable to report both an expiration date and a recommended re-evaluation date on the COA for excipients, if applicable, but both dates may not always be required. Expiration and recommended re-evaluation dates should not be reported by a supplier without sufficient stability data or product history to support the assigned dates.

For excipients determined to be very stable (greater than 2 years), either the specific expiration date and/or the recommended re-evaluation date should be reported on the COA for the material, or a general stability statement may be included (e.g., stability greater than 2 years). If available data indicate that an excipient has limited stability (2 years or less) under anticipated storage conditions, a specific expiration date and/or recommended re-evaluation date should be reported on the COA for the material.

If data from formalized stability studies are not available for an excipient, an appropriate statement should be included on the COA to indicate what is known about the stability of the material and whether stability studies are in progress.

Date Retested—If retesting is performed by an excipient supplier and the results are used to extend the length of time that the material may be used, the date retested should also be reported on the COA. The specific tests that

were subject to retesting should be clearly identified, and the results obtained upon retesting should be reported. After retesting, a new recommended re-evaluation date should be reported on the COA.

Additional Dates—Other dates may appear on a COA, if desired by the excipient supplier or requested by the user. Examples include the release date, shipping date, date of testing, and date the COA was printed or approved. Any additional dates that appear on a COA for excipients should include a clear indication of what the date represents or means.

TESTING FREQUENCY

For the excipients listed in the *USP-NF*, the product specifications are set by the supplier to include all parameters listed in the monograph. It is not required that analysis of all specification parameters be made on each lot (see *General Notices and Requirements*). However, sufficient analysis and process validation data should exist to ensure that the lot meets all specifications before it is released. This is an established practice that has been successfully used in industry for many years. Periodic testing of all parameters should be performed to revalidate the control system. The frequency of these periodic tests should be determined by the suppliers on the basis of their understanding of the manufacturing control system. At a minimum, the parameters should be checked once a year.

For excipients that are not included in *USP-NF*, specifications should be set by the supplier to ensure that the quality of the material is maintained on a continuing basis and reflects both the excipient manufacturing process and inherent properties. The analytical methods used to evaluate the characteristics of noncompendial excipients may be the same as those contained in the compendia, or may be unique to the supplier or the material. The methods should be demonstrated to provide accurate, reproducible, and consistent results for the characteristic being tested. It may be appropriate for noncompendial excipients to have some tests performed at reduced frequency.

The excipient user should evaluate the supplier's specifications and methods to ensure that they are appropriate and acceptable for the quality control needed for the manufacturing process of their drug product. The user should determine which of the supplier's specifications and methods are required for release of the excipient for use in their process. If additional tests or alternative methods are required by the user, appropriate specifications and methods, along with responsibility for performing the testing, must be agreed upon by the excipient supplier and user.

Reduced Frequency Testing

When analysis of some parameters is carried out at a reduced frequency (for example, every 10th lot), this should be clearly stated on the COA. Each specific test subject to reduced frequency testing should be indicated. Reduced frequency testing should be used only for excipients manufactured using a stable process. There should be a sound technical basis and sufficient documentation to support testing any parameter at a reduced frequency. This would normally include the following points:

- Appropriate validation of the manufacturing process
- Process control—attribute charting (when appropriate)
- GMP controls

As part of the justification for reduced testing, it is important that there be assurances in place showing that the manufacturer's process complies with appropriate excipient GMP requirements.

Some tests, because of their significance, should always be performed on each lot, whereas others may be candidates for reduced frequency testing. Attribute testing results in qualitative data that provide pass/fail results or results ex-

pressed as less than or greater than a specified value. The result merely establishes compliance with a specification parameter. There are no data to indicate how well the material complies, as would be obtained from variable or quantitative test results.

Reduced frequency testing of an attribute requires that the manufacturer show that the qualitative parameter is in a state of statistical control. This necessitates tabulating the test results for consecutive lots produced.

Skip-Lot Testing—Skip-lot testing may be applied to an excipient that is made by either a batch or a continuous process. Various commonly accepted statistical sampling plans may be used to demonstrate appropriate process control. Examples of each are listed below.

EXAMPLE 1: For an average outgoing quality level (AOQL) of 1% and a test frequency of 1 in 10, the supplier should find 100 consecutive lots in conformance. At a 2% AOQL and a test frequency of 1 in 10, the supplier would test 50 consecutive lots. For a 1% AOQL and a 1 in 5 test frequency, the supplier would test 70 consecutive lots. Nomographs are available to determine the test requirements.

EXAMPLE 2: When the excipient is manufactured by a continuous process, no discrete lot is produced. The sampling plan again is based upon the risk of approving a lot that was nonconforming. By testing 140 consecutive lots before going to a test frequency of 1 in 10, the plan establishes a low risk of approving a lot that is noncompliant.

Once the requirement is met, the supplier can monitor conformance to the specification parameter by testing 1 in 10 lots. Should any lot fail the analysis, the supplier should return to 100% testing until the results once again meet the specification above.

Because excipients vary greatly in chemical and physical properties, the supplier of the excipient should determine which tests should be routinely performed and which tests may be appropriate for reduced frequency testing. This determination must be justified and documented on the basis of the adequacy of the supplier's control system. Documentation should be kept detailing the assumptions and the data supporting the skip-lot testing plan.

Type A and Type B Tests—Only certain types of tests are appropriate for reduced frequency testing. *Type A* is defined as tests that may not be easily controlled through standard process control techniques or that may change with time. These tests should normally be performed on each lot. *Type B* is defined as tests that normally can be controlled using standard process control techniques and that are not expected to change with time. These tests are candidates for reduced frequency testing. Examples of both types of tests are listed below.

TYPE A: EXAMPLES OF TESTS THAT TYPICALLY NEED TO BE PERFORMED ON EVERY LOT

- **Identification**—Required by GMPs for users (candidate for reduced frequency testing by suppliers)
- **Assay**—Critical quality parameter (if specified)
- **Viscosity**—Usually indicates grade
- **Loss on drying** (or moisture determination)—Indication of stability and appropriate process controls
- **Color**—Indication of stability and appropriate process controls
- **pH**—Indication of stability and appropriate process controls

TYPE B: EXAMPLES OF TESTS THAT MAY BE CANDIDATES FOR REDUCED FREQUENCY TESTING

- **Manufacturing impurities**—Based on starting materials and processes (e.g., Chloride, Sulfate, Nitrate, Glyoxal)
- **Heavy metals**
- **Lead**
- **Arsenic**
- **Residue on ignition**
- **Residual solvents**

This is not meant to be an exhaustive list of tests. It simply provides some direction on how a supplier can assess

the importance of each test to the overall control of the process. Tests listed as possible candidates for reduced frequency testing (*Type B*) may need to be routinely tested (*Type A*), depending on the raw materials and process. Determinations can also be made for some *Type A* tests to become *Type B* tests. In a dedicated facility, identification testing by the supplier may not be necessary.

Documentation—The supplier of an excipient should develop and maintain documentation that outlines the process control systems and validation data to justify the use of reduced frequency testing. This documentation should also include procedures for handling the impact of significant changes on the reduced frequency testing program.

The minimum number of lots to be fully tested for all specification parameters after a change has been made depends on the process and the significance of the change and should be based on sound statistical considerations.

Additionally, the documentation should contain procedures for re-evaluating the reduced frequency testing program when a testing failure occurs. Decisions regarding the continuance of reduced frequency testing should be justified on the basis of the reasons for the failure and the supplier's ability to provide assurances that the reduced frequency testing program or other in-process parameters would identify these types of failures in the future.

Justifications for Reduced Frequency Testing—The following are examples of situations where a sound technical basis can be demonstrated and where reduced frequency testing might therefore be justified. [NOTE—There may be other such examples.]

- An impurity, by-product, or unreacted raw material could not be present in the product because the raw materials and chemical reactions used could not contain or generate such substances above the specified limits.
- The process capability index (C_p) on the relevant parameter is high and based on a stable process. Statistical analysis of the reduced frequency data should show that the property remains stable and within specifications. A process is considered stable when the output of the process, regardless of the nature of the processing (batch or continuous), can be demonstrated by appropriate means to show a level of variability that consistently meets all aspects of the stated specification (both Pharmacopeia-specific and customer-specific) and is thus acceptable for its intended use. For continuous processing, it is also important to demonstrate that the material has been produced under conditions in which the process has achieved a form of "steady state", i.e., in which there is minimal operator intervention and in which the in-process parameters have been stabilized (see *Appendix 2* for further definition of this concept and for determining levels of control).
- For a continuous process, the in-process analyses show that the property that is determined at reduced frequency is stable and within specification. Repeating the test on each lot would be redundant.
- An analysis that is determined on every lot has been shown to strongly correlate with an analysis that is run at a reduced frequency. The correlation shows that if a lot is within specification on the first analysis, it will be within specification on the second analysis.

USE OF ELECTRONIC SIGNATURES

Because of the growing dependence on computers and the need to accommodate paperless record systems, an electronic alternative to handwritten records and signatures is suggested. Excipient suppliers have added computer information systems to enhance productivity.

The primary issue with transfer of a COA without a handwritten signature is the validation of data. There are several

considerations that should be met before an electronic signature or name attachment to a COA is considered acceptable.

- Computer systems access must be limited to authorized individuals: access is gained only after inputting a user name and a password. The system should require frequent changes of each individual password.
- A confirmation of the integrity and accuracy of the information stored in the system should be completed.
- The operation of the system must be checked routinely to ensure that the correct information is transferred from the database to the printed record.
- Data entered into a database from which information is extracted for a COA should be accompanied by time- and date-stamped audit trails.

When these criteria are met, the issuance of COAs with electronic signatures or the responsible person's name attached to the document, in lieu of a handwritten signature, is acceptable. [NOTE—Computer systems are currently regulated by 21 CFR 11 of the FDA. Users should monitor the FDA's approach to compliance in this area.]

DISTRIBUTOR INFORMATION

The presentation of a COA issued by a distributor presents some challenges. Because COAs are important documents characterizing the excipients and the state of their quality, the source of that information becomes very important to the end user(s). Because distributors take on different roles in fulfilling the services for which they are contracted, it is necessary to ensure that procedures and methods are appropriate for the functions performed.

Distributors may function in a number of different capacities relating to the movement of excipients and to services associated with their production. Some are simply pass-through locations in which nothing is done to the excipient with the exception of storage and handling. Others serve as extensions of the manufacturer's process by taking bulk quantities and repackaging them for the manufacturer. Still others purchase excipients and repackage them under a different label for sale and distribution. These scenarios should be understood and properly documented with programs that will protect the integrity and safety of the excipients as they move through the distribution process.

Original Manufacturer and Manufacturing Site—The identity of the original manufacturer and the manufacturing site should be included on the COA for excipients. This information is important because it provides traceability for specific excipient lots and assures the excipient users that they are consistently obtaining material from the same manufacturer and site.

Reporting the identity and location of the manufacturer does not represent an issue when the original manufacturer is also the direct supplier of the excipient to the pharmaceutical customers. However, it is recognized that this information may be considered proprietary by an excipient distributor. To adequately address this issue, excipient distributors should either list the specific information identifying the original manufacturer and location or provide the information by reporting an appropriate code, which is assigned in order to unambiguously identify the original manufacturer and manufacturing site. To protect the secrecy of this information, the meaning of the code does not have to be revealed to intermediary distributors.

Certificate of Analysis Data—When a distributor is primarily used as a pass-through of the excipient without any changes to the excipient and packaging, the COA that accompanies the excipient from the manufacturer can be passed on in the original form. If the data are extracted, translated, or rewritten on other letterhead, a system should be in place to check the rewritten information, and justification should be demonstrated upon request. Alternatively,

the source of the data should be indicated on the document.

For a distributor that takes bulk quantities of an excipient from a manufacturer and introduces the bulk quantities into a process (e.g., conveyance and storage system), analysis of the packaged excipient should be performed to demonstrate the same quality as the lot (batch) introduced. Appropriate analytical data should be included on the COA to verify the quality. The distributor should use equivalent methodology and equipment for the analytical evaluation. Some data may be used from the original manufacturer's COA with appropriate justification.

In all scenarios, it is expected that the distributor will have the appropriate level of GMP in place.

APPENDIX 1

DEFINITIONS

Acceptance Criteria—The specifications and acceptance or rejection limits—such as acceptable quality level or unacceptable quality level with an associated sampling plan—that are necessary for making a decision to accept or reject a lot or batch of raw material, intermediate, packaging material, or excipient.

Batch (or Lot)—A defined quantity of excipient processed so that it could be expected to be homogeneous. In a continuous process, a batch corresponds to a defined portion of the production, based on time or quantity (e.g., vessel's volume, 1 day's production, etc.).

Batch Number (or Lot Number)—A unique and distinctive combination of numbers and/or letters from which the complete history of the manufacture, processing, packaging, coding, and distribution of a batch can be determined.

Batch Process—A manufacturing process that produces the excipient from a discrete supply of raw materials that is present before the completion of the reaction.

Certificate of Analysis (COA)—A document relating specifically to the results of testing a representative sample drawn from the batch of material to be delivered.

Chemical Property—A quality parameter that is measured by chemical or physicochemical test methods.

Continuous Process—A manufacturing process that continually produces the excipient from a continuous supply of raw material.

Contract Facility—An internal or external facility that provides services to the manufacturer or distributor of an excipient. These can include, but are not limited to, the following: manufacturing facilities, laboratories, repackaging facilities (including labeling), and warehouses.

Date of Manufacture—A date indicating the completion of the final manufacturing process (as defined by the supplier for the particular excipient and process).

Date Retested—The date when retesting is performed by an excipient supplier to extend the length of time that the material may be used.

Distributor—A party other than the manufacturer who sells the excipient.

Excipient—Any substance, other than the active pharmaceutical ingredient or drug product, that has been appropriately evaluated for safety and is included in a drug delivery system to aid the processing of the drug delivery system during manufacture; to protect, support, or enhance stability, bioavailability, or patient acceptability; to assist in product identification; or to enhance any other attribute of the overall safety and effectiveness of the drug delivery system during storage or use.

Expiration Date—The date after which the supplier recommends that the material should not be used.

Impurity—Any component of an excipient that is not the intended chemical entity but is present as a consequence of either the raw materials used or the manufacturing process.

Lot—See *Batch*.

Lot Number—See *Batch Number*.

Manufacturer—A party who performs the final processing step.

Packaging—The container and its components that hold the excipient for storage and transport to the customer.

Periodic Testing Program—See *Skip-Lot Testing Program*.

Physical Property—A quality parameter that can be measured solely with mechanical equipment.

Process—The set of operating instructions describing how the excipient is to be synthesized, isolated, purified, etc.

Process Capability Index (Cp)—A statistical measurement that can be used to assess whether the process is adequate to meet specifications. A state of statistical control can be said to exist if the random variation in test results for a process parameter is such that the calculated process capability is greater than 1.33 (see *Appendix 2* for further definition).

Process Step—An instruction to the excipient manufacturing personnel directing that an operation be performed.

Recommended Re-Evaluation Date—The date suggested by the supplier when the material should be re-evaluated to ensure continued compliance with specifications. Differs from the *Expiration Date* in that the excipient may be re-evaluated to extend the length of time the material may be used, if supported by the results of the evaluation and appropriate stability data.

Reduced Frequency Testing Program—See *Skip-Lot Testing*.

Repackaging—Transfer of an excipient from one container to another.

Reprocessing—Introducing previously processed material that did not conform to standards or specifications back into the process and repeating steps that are already part of the normal manufacturing process.

Significant Change—Any change that alters an excipient's physical or chemical property from the norm or that is likely to alter the excipient's performance in the dosage form.

Site—A location where the excipient is manufactured. This may be within the facility but in a different operational area, or at a remote facility, including a contract manufacturer.

Skip-Lot Testing Program—Periodic or intermittent testing performed for a particular test parameter that is justified by historical data demonstrating a state of statistical process control.

Specification—The quality parameters to which the excipient, component, or intermediate must conform and that serve as a basis for quality evaluation.

Stable Process—A process whose output, regardless of the nature of the processing (batch or continuous), can be demonstrated by appropriate means to show a level of variability that consistently meets all aspects of the stated specification (both USP-specific and customer-specific) and is thus acceptable for its intended use.

Supplier—A manufacturer or distributor who directly provides the excipient to the user.

User—A party who uses an excipient in the manufacture of a drug product or another excipient.

APPENDIX 2

STATE OF STATISTICAL CONTROL: PROCESS CAPABILITY PARAMETERS FOR DETERMINING LEVELS OF CONTROL

A process is considered to be in a state of statistical control if variations among the observed sampling results from the process can be attributed to a constant system of chance causes. Process capability index (Cp) or capability index adjusted for the process average (Cpk) or performance index (Pp) or performance index adjusted for the process average (Ppk) can be used to assess whether the process is adequate to meet specifications. Values of these parameters exceeding 1.33 show that the process is adequate to meet specifications. Values between 1.00 and 1.33 indicate that the process, although adequate to meet specifications, will require close control. Values below 1.00 indicate that the process is not adequate to meet specifications and that the process and/or specifications should be changed. Pp/Ppk will always be less than or equal to Cp/Cpk, respectively. The essential difference between the capability and the performance indices is the data used. Capability indices require the calculation of σ , the population standard deviation, whereas the performance indices require the calculation of s , the sample standard deviation. Thus for pharmaceutical excipients a state of statistical control can be said to exist if the random variation in test results for a process parameter is such that the calculated process capability index or performance index is greater than 1.33.

(1081) GEL STRENGTH OF GELATIN

Pipet 105 mL of water at 10° to 15° into a standard Bloom bottle, add 7.5 g of Gelatin, and stir. Allow to stand for 1 hour, then bring to a temperature of 62° in 15 minutes by placing in a water bath regulated at 65° (the substance may be swirled several times to aid solution). Finally mix by inversion, allow to stand for 15 minutes, and place in a water bath at 10 ± 0.1°. Chill, without disturbance, for 17 hours. Determine the gel strength in a Bloom Gelometer (a device developed to make this determination under standardized conditions) adjusted for 4-mm depression and to

deliver 200 ± 5 g of shot per 5 seconds, using the 12.7-mm diameter (nonbeveled) plunger.

<1084> GLYCOPROTEIN AND GLYCAN ANALYSIS—GENERAL CONSIDERATIONS

OVERVIEW

A number of glycoprotein drugs have been developed as a result of advances in biotechnology, and many naturally derived protein drugs possess complex glycan structures. Glycosylation, a posttranslational modification of these proteins, can play an important role in determining the function, pharmacokinetics, pharmacodynamics, stability, and immunogenicity of these agents. The two main types of protein glycosylation are *N*-glycosylation and *O*-glycosylation. Unlike transcription and translation, glycosylation is not a template-driven process; therefore variability in the glycosylation pattern of a protein can arise, caused by different sources or different manufacturing processes. Differences in this pattern are known to affect biological activity. Glycosylation patterns may therefore be an important set of attributes that arise in characterizing a candidate glycoprotein intended for therapeutic use and in ensuring its stability and quality.

The first part of this chapter provides a brief introduction to glycobiology and describes the complexity of glycan structures. The subsequent parts provide flow charts and a series of general analytical strategies that can be used to characterize glycoprotein glycans by means of the following:

1. Direct analysis of glycoproteins; and
2. Analysis of released nonderivatized or derivatized glycans by various methods of chromatographic and electrophoretic separation and mass spectrometry (MS).

Different approaches to analyzing monosaccharides are described at the end of the chapter.

For selected analytical methods, this chapter cross-references other USP chapters, particularly those relating to biotechnology-derived articles (see chapters *Biotechnology-Derived Articles—Capillary Electrophoresis* <1053>, *Biotechnology-Derived Articles—Isoelectric Focusing* <1054>, *Biotechnology-Derived Articles—Peptide Mapping* <1055>, and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* <1056>).

PROTEIN GLYCOSYLATION

Most proteins in eukaryotic cells undergo glycosylation and other posttranslational modifications before being trafficked to lysosomes, becoming membrane bound at the cell surface, or being secreted. Glycosylation varies significantly from cell to cell, tissue to tissue, and species to species because of the varying expression of hundreds of glycosyl-

transferases and glycosidases located throughout the Golgi apparatus and endoplasmic reticulum (ER). Four main types of enzymatic glycosylation are found in proteins:

1. *N*-Glycosylation, which involves the initial transfer of oligosaccharides to the nitrogen on the terminal amide group of asparagine and their subsequent processing and modification to a series of glycan chains;
2. *O*-Glycosylation, which in general involves the initial transfer of monosaccharides to the hydroxyl groups of serine and threonine and subsequent elongation and branching of the saccharide chain by the addition of monosaccharides;
3. Glycosylphosphatidylinositol (GPI) anchor, which is a glycolipid linked to the C-terminus of a protein; and
4. C-Glycosylation, which involves the formation of a carbon-carbon bond between the C2 carbon of the indole ring of tryptophan and the C1 carbon of an α -mannopyranosyl residue.

Any given protein may contain multiple *N*-, *O*-, or C-glycosylations, but not more than one GPI anchor. A nonenzymatic addition of saccharides, called glycation, can occur when proteins are mixed with reducing sugars via a complex series of reactions. The two protein glycosylation types that are generally of concern and that are analyzed in glycoprotein drug substances are *N*- and *O*-glycosylation. Each of these is discussed below.

N-Glycosylation

The biosynthesis of *N*-glycans in glycoproteins can be described as a four-step process:

1. Lipid-linked glycan chain initiation and elongation;
2. Transfer of oligosaccharide to the protein or nascent polypeptide chain;
3. Processing of the *N*-glycan chain by removal of specific glucose and mannose residues; and
4. Modification of the *N*-glycan chain by the addition of residues to the nonreducing ends of the glycan chain.

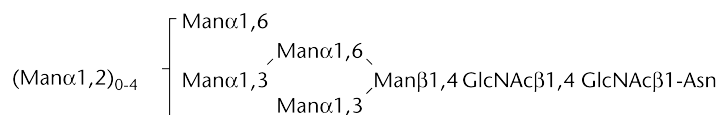
The consensus amino acid sequence for *N*-glycosylation is Asn-Xaa-Thr/Ser (where Xaa is any amino acid other than proline). Overall, only about two-thirds of all potential sequences, termed sequons, are glycosylated, and currently there is no method to predict which sequon will be glycosylated. The role of protein *N*-glycosylation is usually protein trafficking and secretion.

N-glycans can be categorized as high-mannose, hybrid, or complex, depending on the extent of processing (*Figure 1*). High-mannose structures (*Appendix 1*) lack galactose or *N*-acetylglucosamine (GlcNAc) residues in the antennae, branches at the distal end of the chain. In hybrid structures, both substituted GlcNAc residues and terminal mannose residues are present in the antennae, whereas complex structures have both α 1,6- and α 1,3-mannose residues substituted with GlcNAc moieties. Hybrid and complex glycans can exist with two or more branches, frequently termed antennae; such glycans are therefore often termed, for example, biantennary, triantennary, or tetraantennary. Both monoantennary and pentaantennary *N*-glycans are also known to exist. In complex glycans, antennae frequently carry terminal sialic acid (neuraminic acid) residues. Sialylation has been shown to have a great effect on both the pharmacokinetics and the pharmacodynamics of many therapeutic glycoproteins.

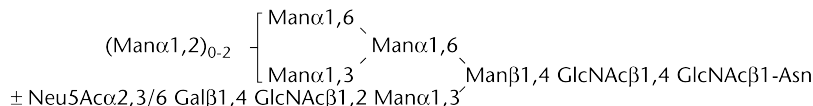
Common type of asparagine (Asn) linked glycans (*N*-glycans).

Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid

High mannose type

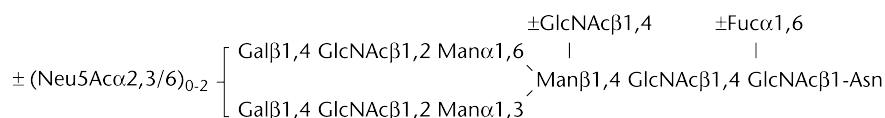


Hybrid type

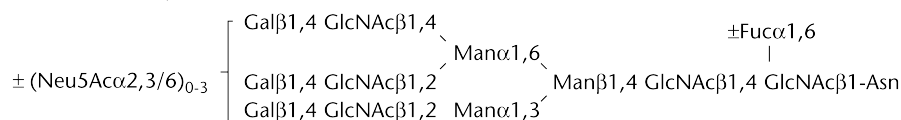


Complex types

Bi-antennary



Tri-antennary



Tetra-antennary

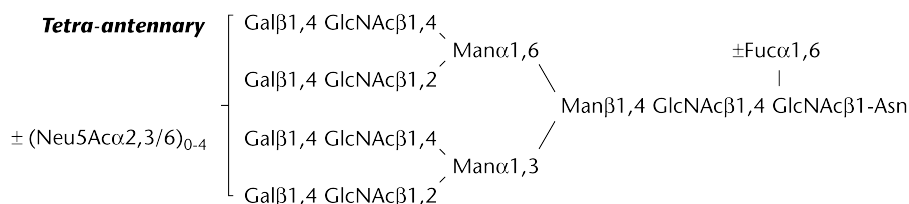


Figure 1. Common types of *N*-glycans. For abbreviations, see Appendix 1.

O-Glycosylation

O-Glycan chains are built up sequentially via an initial GalNAc residue linked to serine, threonine, and tyrosine, as well as to the less common amino acids hydroxyproline and hydroxylysine. Multiple glycan core structures are known. The sequence and isomeric linkage of monosaccharides show greater variety than that in *N*-glycans, and at least eight different types have been identified (Figure 2). Although no consensus amino acid sequence for O-glycosylation has been determined, glycosylation is usually favored by the presence of proline one residue before or three residues after the glycosylation site and the absence of charged amino acid residues proximal to serine or threonine. The disaccharide unit *N*-acetylglucosamine, Gal β 1,4GlcNAc, is the most common chain extension. Additional modifications, including terminal capping of Gal with sialic acid and fucosylation along the chain, are also frequent.

O-Glycosylation can occur in cluster form, the mucin type, which usually forms part of the cell surface extracellular matrix or secreted glycoproteins. Other O-glycosylation, such

as O-GlcNAc, is found on many nucleocytoplasmic proteins; and O-Man-linked glycosylation is found in some muscular and neural glycoproteins and in yeast. O-Fuc- and O-Glc-linked glycosylation types are found on many epidermal growth factor-like proteins that are associated with the Notch signaling pathway.

Glycan Heterogeneity

Not only the type of glycosylation (*N*- or *O*-linked), site occupancy, and the site of glycosylation can vary from glycoprotein to glycoprotein, but also the actual oligosaccharide structures (branching and linkages) can differ, even on the same site. This structural variation arises because glycosylation is a process that is not driven by a template. The glycosylation pattern at a given site depends on many factors, including cell-specific and growth-dependent availability of glycosyltransferases and *exo*-glycosidases found in the Golgi bodies and ER. Heterogeneity leads to different physical and biochemical properties and, therefore, also to functional diversity.



Figure 2. Common core structures of O-glycans (bold and underlined). Sugar abbreviations as in *Appendix 1*.

Host-Cell Expression Systems and Glycosylation

BACTERIA

Although both O- and N-glycosylation have been shown to occur in a variety of prokaryotes, *Escherichia coli*, the bacterium of choice for many therapeutic products, does not produce glycosylated proteins.

YEAST

Yeast produces both N-glycosylated and O-glycosylated proteins. In yeast hypermannosylation with the N-glycan chain that contains more than 100 mannose residues can occur, but sialylation does not occur unless the organism is genetically modified. The development of recombinant strains of *Pichia pastoris* that contain inserted heterologous genes for various glycosylation enzymes has allowed the humanization of N-glycosylation pathways in this yeast. O-Glycosylation in yeast is also significantly different from that in mammalian cells. In contrast to mammalian cells, serine or threonine O-glycosylation is linked via mannose and often consists of linear chains of as many as six mannose residues.

INSECT CELLS

N-glycan chains of insect cells usually are of the high-mannose, trimannose or paucimannose, and truncated complex types (see *Appendix 1* for definitions). Insect cells also produce glycoproteins bearing the Fucα1,3 residue linked to the proximal GlcNAc residue in the core chitobiose. This core fucose residue is a potent immunogen and allergen. O-Glycosylation in insect cells has not been well-studied, and although O-linked GalNAc–Ser(Thr) residues have been found, very few are processed further beyond the Galβ1,3GalNAc–Ser(Thr) sequence. Sialic acid residues have not been found on proteins produced in insect cells.

PLANTS AND PLANT CELLS

Plant N-glycans contain mainly oligosaccharides of the oligomannose type, but also present are hybrid and truncated complex types of structures, with or without Xylβ1,2 attached to the β-linked mannose residue of the trimannosyl core and Fucα1,3 attached to the proximal GlcNAc residue of the core chitobiose. Both the Fuc and Xyl residues are immunogenic and have been shown to be part of the glyco-epitopes of several plant allergens. O-Glycosylation in plants has not been well studied but is known to consist predominantly of the addition of arabinogalactan chains attached to hydroxyproline, threonine, and serine residues that are located in the plant cell wall or on the outer surface of the plasma cell membrane. These glycans are immunogenic.

ANIMAL CELLS

The majority of glycosylated therapeutic proteins are produced in continuous animal cell lines. Chinese hamster ovary (CHO), baby hamster kidney (BHK), human embryonic kidney (HEK), and mouse myeloma (SP2/0 or NS0) cells have all been employed. These animal cells generally produce proteins with humanlike glycosylation. Although there are several differences in glycosylation between rodent and human cells, such as the presence of *N*-glycolylneuraminic acid not found in humans, CHO cells have become a workhorse of the biotechnology industry.

GLYCAN ANALYSIS FOR GLYCOSYLATED BIOLOGICAL DRUGS

Glycosylation of proteins may affect biological activity, either directly or indirectly, and variability in glycosylation arises not only from cellular diversity but also from the manufacturing process. The glycosylation pattern thus may be important as a part of characterization studies in assuring process consistency and may also be important in ensuring the consistent quality of a biological drug product after market access. Appropriately characterized reference materials are needed in order to support biological and physico-chemical testing of production batches to ensure batch-to-batch consistency. Glycosylation analysis may be appropriate for the following:

1. Characterizing the structure and stability of novel products and their stability to processing steps and storage;
2. Batch release testing and in process control testing; and
3. Assessing comparability between products (e.g., when one or more process changes have been made).

An understanding of the relationship between glycan structure and biological function underpins decisions about the information required at each development stage. For biological/biotechnological drug substances, the characterization criteria and specifications for batch release are generally set forth in the guidelines ICH Q6B, *Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*,¹ and ICH Q5E, *Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process*.² Numerous approaches and methodologies are applied for glycan mapping. This variety is a consequence of the diversity and complexity of glycan structures and the available technology and detection systems.

Because of the diversity and complexity of glycan structures and the increasing availability and improvement of various detection systems and technology, analytical methods are wide ranging. Different methods that support step-by-step procedures depend on the glycoproteins, the availability of equipment, the expertise of individual scientists and groups, and the information required. The two most studied types of protein glycosylation that affect bioactivity are *N*- and *O*-glycosylation.

Glycan analysis can serve in different applications; the most important are general product characterization, process validation, comparability evaluation, stability testing, monitoring manufacturing process consistency, and release testing. The selection of the analytical techniques and their applications in product development and routine manufacturing depend on many factors, such as the complexity of the glycoprotein, the understanding of the relationships between glycosylation and safety and efficacy, and the overall design of the strategy for manufacturing process control. For example, even when the biological relevance of glycosylation is not certain, control of glycosylation could be considered as a measure of manufacturing consistency.

The *Figure 3A* flow chart assists in the choice of applications for glycan analysis, and *Figure 3B* provides an overview of available analytical techniques and equipment employed.

CHOICE OF GLYCAN ANALYSIS FOR CHARACTERIZATION AND SPECIFICATION OF GLYCOSYLATED BIOLOGICAL DRUGS

Analysis of Intact Glycoprotein

The most direct mode of analysis is direct study of the intact molecule. This mode provides information about the glycosylation profile of the glycoprotein. However, this approach provides limited information when the molecule is large and contains multiple glycosylation sites. One of the most important glycosylation factors defining biological activity is the degree of sialylation, which often determines the half-life of glycoproteins in circulation. This makes ionic-charge-based electrophoresis and ion-exchange chromatography obvious choices of technique. Nearly all types of gel electrophoresis have been used to probe protein glycosylation, including polyacrylamide gel electrophoresis (PAGE), (see *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* (1056)) and isoelectric focusing (IEF), (see *Biotechnology-Derived Articles—Isoelectric Focusing* (1054)). Similarly, capillary electrophoresis (CE), (see *Biotechnology-Derived Articles—Capillary Electrophoresis* (1053)) has also been found suitable. Strong anion-exchange chromatography has been used for the same purpose, but the resolution is often inferior to that of IEF and CE. Direct mass spectrometry (MS) is another option for the analysis of posttranslational modification. Along with ongoing improvements in the resolution of MS, more and more complex glycoproteins become accessible for direct characterization by this method.

Analysis of Glycopeptides

Analysis of glycopeptides provides information about site-specific glycosylation properties, the degree of occupancy, and oligosaccharide structures. Site-specific glycosylation can be affected by cell culture process conditions. Therefore, if a known glycosylation site is critical, manufacturers must monitor site-specific glycan structures. The typical approach is first to generate glycopeptides by protease digestion and to separate them by, for example, RP (reversed-phase) HPLC (see *Biotechnology-Derived Articles—Peptide Mapping* (1055)). Subsequently the separated glycopeptides can be further characterized individually by, e.g., direct analysis using MS, or deglycosylation and subsequent glycan profiling, as described below in the section *Profiling of Cleaved Oligosaccharides*.

Direct identification of the mixture of glycopeptides and nonglycosylated peptides by MS is limited by masking effects (ion suppression) of peptide signals on glycopeptide signals. One approach to overcoming this effect is to separate peptides and glycopeptides before analysis by MS, e.g., by offline coupling (matrix-assisted laser desorption/ionization [MALDI]) or online coupling (electrospray ionization [ESI]). MS analysis of glycopeptides plays an important role in the characterization of *O*-glycans because these glycans are not always released quantitatively and because, as a result of their smaller size, they are more amenable to characterization by MS as glycopeptides.

The use of CE for high-resolution separation may also be appropriate, especially for the analysis of sialylation.

Profiling of Cleaved Oligosaccharides

Profiling of total glycans cleaved from glycoprotein is the most common approach for the characterization of glycoproteins. It provides a way to obtain information about the

¹<http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html>; accessed 12/15/2011.

²<http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html>; accessed 12/15/2011.

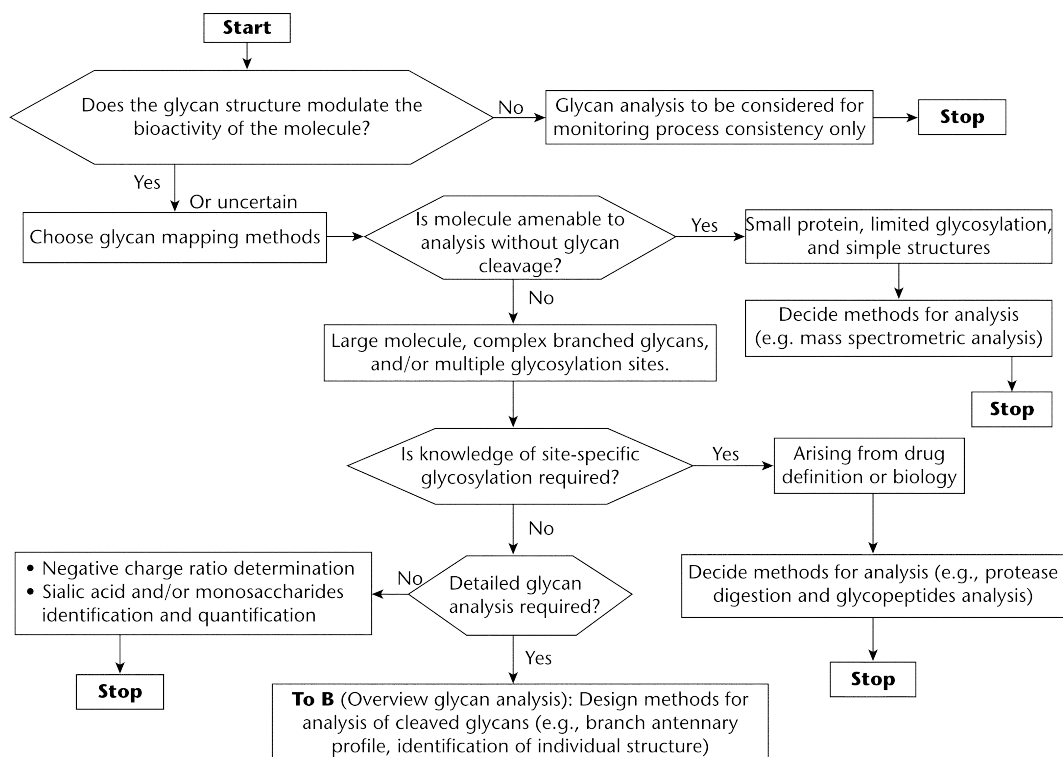


Figure 3A. Flow diagram assisting in the choice of options for glycan analysis.

Overview of glycan analysis methods

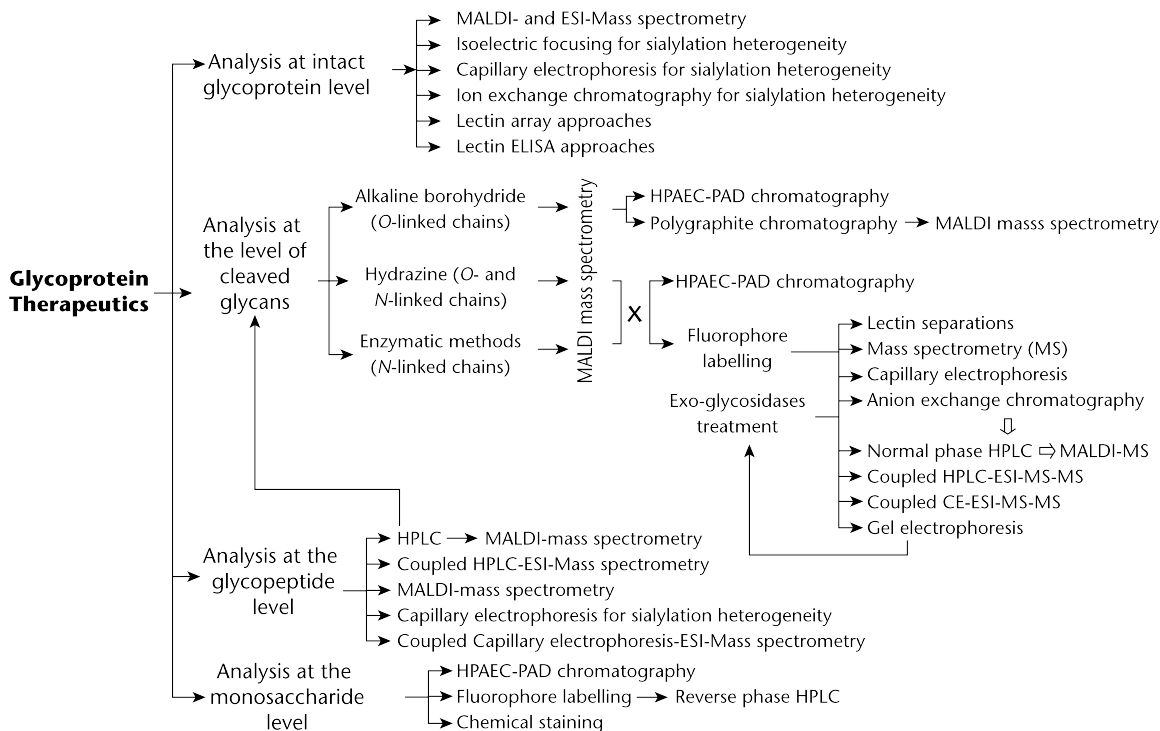


Figure 3B. Overview of glycan analysis techniques and equipment employed.

various populations of glycans present on the protein. The degree of sialylation can also be addressed at this stage. Depending on the chosen method, prior derivatization/labeling may be needed to allow the detection of the glycans.

Many protocols are available, and most of the steps in the analysis are well established. The possible drawback with such flexibility is the lack of consensus about which methods to choose under which circumstances; because of the

variety of analytical techniques, comparison of results obtained by different platforms may not always be possible. So far, the majority of the work has been done on *N*-glycosylation, because of the following factors:

1. *N*-glycans usually are more clinically relevant in biologicals than *O*-glycans; or
2. The release of *N*-glycans, either by chemical means (hydrazine) or by enzymes (endoglycosidases and peptide *N*-glycosidase [PNGase] F), is more straightforward than is the release of *O*-glycans.

DEGLYCOSYLATION

The approach used for the release of glycans depends on the glycoprotein under test. The cleavage agent is chosen according to the type of cleavage needed and the level of information required. Enzymatic or chemical cleavage may be used. Table 1 gives a nonexhaustive list of enzymatic cleavage agents and their specificity. Digestion efficiency generally depends on the accessibility of the glycans on the protein, and hence the protein should be denatured to maximize glycosylation site exposure unless analysts want to distinguish between surface and buried glycans. Chemical cleavage agents can also be used, e.g., hydrazine or alkaline borohydride for β -elimination of *O*-linked glycans.

Chemical or Enzymatic Release of *N*-Glycans—PNGase F (*Flavobacterium meningosepticum*) is the enzyme of choice for the release of *N*-glycans for most glycoproteins except for some insect cell and plant glycoproteins that may contain a Fuc α 1,3 linked to the chitobiosyl core. *N*-Glycan chains having this structure can be cleaved from the glycopeptide only by the almond enzyme, PNGase A. Chemical release by anhydrous hydrazine is much less common, mainly because of the limited availability of the reagent, which is considered a hazardous chemical. In addition, hydrazinolysis produces de-*N*-acetylated *N*-glycans.

Chemical or Enzymatic Release of *O*-Glycans—Currently only one enzyme, *O*-glycanase from *Diplococcus pneumoniae*, is available to release *O*-glycans, and this enzyme has a limited usage because of its high substrate specificity: it cleaves only Gal β 1,3GalNAc α 1-Ser/Thr. In addition, no ideal chemical procedure is available; but Ser- and Thr-linked *O*-linked glycan can usually be released by the reductive alkali-catalyzed β -elimination reaction (alkaline borohydride reaction), in which the released glycans are reduced as soon as they are cleaved in order to prevent formation of degradation products due to peeling. However, this reaction is not specific, and in the reaction, approximately 10%–20% of *N*-glycans are generally known to be released as well. The released glycans lack a reducing group used for the attachment of fluorescent labels by reductive amination. Fortunately, with advances in sensitive MS, direct identification of reduced glycans is possible. Relatively good quality reducing

O-glycans can be obtained by alkali-catalyzed β -elimination using primary amines such as ethylamine and hydrazine. However, both reagents have the potential to produce peeled degradation products. Furthermore, *O*-glycan release by ethylamine is not quantitative. Hydrazine, although it may be better for use than ethylamine, requires strict control of reaction conditions and handling and does not have a commercial source in Europe.

Separation of Cleaved Glycans Without Fluorescent Labeling—*N*-Glycans can also be resolved by HPAEC high-pH anion-exchange chromatography with pulsed amperometric detection (PAD; see *Chromatography* (621)), which shows high sensitivity, can also separate some isomers, and affords the ability to directly detect native glycans without labels or tags. However, LC/MS for this separation approach is challenging because this HPAEC system uses high-pH and high-salt mobile phases that interfere with ionization of glycans. In addition, absolute quantification of the glycan is only possible if the individual PAD response factors for the different glycan structures are known, e.g., if an appropriate oligosaccharide reference library is available. Porous graphitic carbon (PGC) chromatography can also be used to separate glycans, and this method adds an orthogonal selectivity compared to other columns. A PGC-electrospray-ionization-MS approach also can be applied for direct glycan analysis.

MALDI/ESI-MS is a powerful method for the analysis of glycan mixtures either in the native or derivatized form. Permethylation of released glycans is a common method for direct analysis using MALDI/ESI-MS especially for sialylated glycans.

LABELING OF GLYCANS TO INCREASE DETECTION SENSITIVITY AND/OR TO MODIFY THEIR PHYSICOCHEMICAL PROPERTIES

Chemical derivatization is the most commonly used method for labeling glycans at their reducing end by reductive amination. One fluorescent label can be attached to each mono- and oligosaccharide, which facilitates determination of molar quantities. Table 2 illustrates the most common examples of fluorescent labels and their most common uses.

N-GLYCAN PROFILING

Released glycans can be analyzed or profiled by chromatographic, electrophoretic, or MS procedures and, in general, by a combination of these. The choice of method can be grouped according to the nature of the glycans and level of information required. Analysis of glycans provides informa-

Table 1. Examples of Enzymatic Cleavage Agents

Agent	Specificity
<i>N</i>-linked glycan release	
Peptide- <i>N</i> -(<i>N</i> -acetyl- β -glucosaminyl) asparagine amidase (EC 3.5.1.52)	Hydrolysis of peptide- <i>N</i> -(<i>N</i> -acetyl- β -glucosaminyl) asparagine residue in which the glucosamine residue may be further glycosylated, to yield a (substituted) <i>N</i> -acetyl- β -D-glucosaminylamine and a peptide containing an aspartate residue
Peptide <i>N</i> -glycosidase F (PNGase F)	Release of <i>N</i> -glycan chain but no release of <i>N</i> -glyc an chain containing (α 1,3)-linked core fucose
Peptide <i>N</i> -glycosidase A (PNGase A)	Release of <i>N</i> -glycan chain containing (α 1,3)-linked core fucose
Mannosyl-glycoprotein endo- β - <i>N</i> -acetylglucosaminidase (EC 3.2.1.96)	Endohydrolysis of the <i>N,N'</i> -diacetylchitobiosyl unit in high-mannose glycopeptides/ glycoproteins containing the –[Man(GlcNAc) ₂]Asn structure
Endo- β - <i>N</i> -acetylglucosaminidase F (endo F)	Release of high-mannose, hybrid, and complex oligosaccharides
Endo- β - <i>N</i> -acetylglucosaminidase H (endo H)	Release of high-mannose and hybrid oligosaccharides
<i>O</i>-linked glycan release	
Glycopeptide α - <i>N</i> -acetylgalactosaminidase (EC 3.2.1.97)*	Hydrolysis of terminal D-galactosyl- <i>N</i> -acetyl- α -D-galactosaminidic residues

* This enzyme has limited usage because of its high substrate specificity.

tion about the various populations of glycans present on the protein (high-mannose, hybrid, complex).

Profiling of Glycans by HPLC and/or by Electrophoresis and MS—Profiling of fluorescent-tag-labeled glycans by HPLC has become the most common approach. One label can be attached to every single mono- and oligosaccharide by reductive amination at their reducing end, which facilitates determination of molar quantities. With the appropriate label, glycans can be profiled with high sensitivity using reversed-phase, normal-phase, and anion-exchange HPLC (see *Chromatography* <621>). Routinely, analysts use a combination of these methods in order to increase separation resolution and to better differentiate glycan structures. The accuracy of the glycan identification can be validated by means of glycan standards and/or by coupling the HPLC system with MS. Thus, anion-exchange, normal-phase, and reversed-phase HPLC–ESI–MS–MS form powerful combinations; and in-line analysis, if possible, may provide both relative quantitative profiling and information on glycan structure in a single run. Peak identification through retention time is acceptable if their identities have been previously validated by complementary methods and peak homogeneity can be assured.

The degree of sialylation of glycan chains can be a crucial factor for clinical efficacy, because sialylation often defines the half-life of the molecule in vivo. Anion-exchange HPLC is the simplest method for its determination, and glycan structures based on charge can then be identified by MS. De-salting of each fraction is required before MS if the ioniza-

tion interface is designed for low-salt-containing sample flows only.

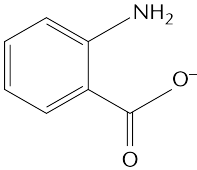
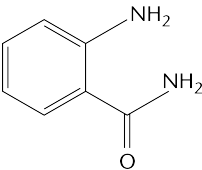
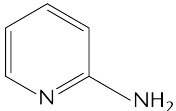
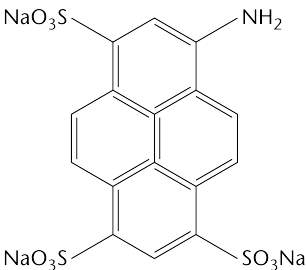
High-resolution separation systems such as CE have been used to identify glycan structures without MS when well characterized standards are used for comparison. The development of an online CE–MS system has further increased the power of glycan analysis using this approach.

Structural Identification by Micro-Enzyme Sequencing and Mass Spectrometry—Traditionally, when detailed structural information is required, the analysis is usually performed using micro-enzyme sequencing. This procedure is highly dependent on the specificity and quality of the enzymes used. Recently, tandem MS has been used more regularly to confirm, determine, and sequence known and novel glycan structures; this method is feasible especially when glycans are released from well-known glycoproteins and production sources.

Monosaccharide Analysis

Different quantitative monosaccharide assays are carried out for a number of purposes. In the glycoprotein field, they provide information about the relative amounts of saccharide in a glycoprotein and about the degree of sialylation of a glycoprotein; and by the measurement of monosaccharide composition, they provide some information about the structure of the glycan chains present.

Table 2. Examples of Fluorescent Labels

Name	Acronym	Structure	Analytical Technique
2-Aminobenzoic acid	2-AA		HPLC
2-Aminobenzamide	2-AB		HPLC MS
2-Aminopyridine	2-AP		HPLC
Trisodium 8-aminopyrene-1,3,6-trisulfonic salt	APTS		CE

The simplest assays used are colorimetric tests to demonstrate that the product is glycosylated and to quantify the total amount of saccharide present in the product. These have poor specificity between different types of sugar residues.

Assays of monosaccharide composition are generally simpler to perform than is oligosaccharide profiling, but they provide less information. The most widely used assay is quantification of sialic acid content, because loss of sialylation and exposure of terminal Gal residues may lead to faster clearance of the glycoprotein from the circulation.

The assays can be divided into two types: (1) those that provide compositional information about the intact sample without prior degradation; and (2) others, principally chromatographic, that require hydrolysis of the saccharide chains before analysis and generate quantitative information about several different monosaccharide species simultaneously. In general, the former are colorimetric and the latter are chromatographic. The hydrolysis step is a significant source of assay variability and may require careful optimization for specific samples.

The presence of certain monosaccharides is diagnostic of specific glycan structures. For example, observation of GalNAc is usually a marker for the presence of O-linked glycan chains, and fucose denotes the presence of specific types of chains. As a consequence of the limited diversity of monosaccharide residues present in glycoprotein glycans, accurate quantification of Man, Gal, or GlcNAc residues is required in order to distinguish between large numbers of structurally diverse glycans. The monosaccharide *N*-glycolylneuraminic acid (Neu5Gc) is not produced in humans and is generally regarded as an unwelcome and potentially immunogenic component of biopharmaceutical products.

SAMPLE PREPARATION

Glycoprotein samples for monosaccharide analysis should be free of salts, excipients, and other carrier materials (low molecular weight sugars are often used as excipients for biopharmaceuticals). This can be achieved by a number of methods, including the following:

1. Dialysis against water or a volatile buffer, using an appropriate membrane, and lyophilization;
2. HPLC on an appropriate gel-permeation column eluted with water or a volatile buffer, monitored by UV absorbance or refractive index, and followed by lyophilization of the sample; or
3. Sample trapping on a conventional RP-SPE cartridge such as a C18 or C8 SPE system, followed by washing away of salts and excipients and elution of the required sample.

QUANTIFICATION

The common method for quantification of neutral sugars in glycoproteins depends on the color generated by heating glycans or glycoproteins in the presence of aqueous phenol in concentrated sulfuric acid. In many cases, the heat required for this reaction is generated by addition of concentrated sulfuric acid to the glycoprotein-phenol mixture in water. Rapid and efficient mixing of the solutions is critical for consistent results. Quantitative results are obtained by the simultaneous analysis of standards to generate a standard curve of absorbance against amount of saccharide and/or against a reference sample of the product under analysis.

HYDROLYSIS PROCEDURES FOR POLYSACCHARIDES AND GLYCOPROTEIN GLYCAN CHAINS

Chromatographic methods for the identification and quantification of monosaccharide components require hydrolysis of the sample before analysis. Appropriate sample preparation is required because excipients or process-related impurities may be saccharides, and residual salts may interfere with the hydrolysis or the subsequent chromatographic separation or with fluorophore labeling. Sialic acid residues can be released either by mild acid hydrolysis or by enzymatic treatment, which leaves other sugar residues attached to the peptide backbone. Quantification of the amount of saccharide present is based on addition of an internal standard before or after hydrolysis. The most commonly used standard for sialic acid analysis by HPAEC is 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid (KDN), and 2-deoxyglucose is widely used for neutral sugars. Both of these sugars are acid labile and should be added after the hydrolysis step. Accurate quantification depends both on stoichiometric hydrolysis and a lack of degradation of the monosaccharide products during hydrolysis.

DETERMINATION OF TOTAL SIALIC ACIDS

Sialic acids occur in bacterial polysaccharides and glycoproteins generally as *N*-acetyl and *N*-glycolyl derivatives of neuraminic acid (Neu5Ac and Neu5Gc). The sialic acids can be determined together with other monosaccharides by a procedure that includes acid hydrolysis to liberate constituent monosaccharides, followed by HPLC using an appropriate standard mixture. Alternatively, total sialic acid content can be determined by colorimetric procedures without the need for hydrolysis. One method, commonly referred to as the Warren method, is based on the reaction of thiobarbituric acid with the product of periodate oxidation of neuraminic acid released *in situ* from the glycoprotein. Alternatively, the color can be generated by the reaction of resorcinol with neuraminic acid. For accurate quantification, include a reference standard sample is included in each measurement.

Selective Release of Sialic Acids—Mild acid hydrolysis or enzymatic digestion can be used to selectively release sialic acid from glycoprotein glycan chains for quantification by chromatographic methods and for quantification of unwelcome forms such as Neu5Gc. More aggressive acid conditions are required in order to release neutral and amino sugars before chromatographic analysis. The protocol must be optimized with respect to yield and saccharide degradation for each protein to be analyzed.

Neuraminidase Digestion for the Release of Sialic Acid from Intact Glycoproteins—Several types of neuraminidases have been isolated and studied; the enzyme derived from *Clostridium perfringens* is the one most commonly used for the enzymatic release of sialic acids from glycoproteins. Recombinant enzyme is available from commercial suppliers. Other enzymes with different specificities are available and can be used to distinguish different types of linkages. Hydrolysis conditions should be optimized for each product, because kinetic parameters for different linkages and for Neu5Ac and Neu5Gc may differ. Selective removal of Neu5Ac α 2 \rightarrow ,3-linked and Neu5Ac α 2 \rightarrow ,6- from cleaved glycans is a convenient means of defining linkages. For quantitative analyses, a known quantity of a suitable internal standard, often 2-deoxyglucose, is added after hydrolysis and removal of the acid.

SEPARATION AND QUANTITATION OF UNLABELED MONOSACCHARIDES

Essentially the only method used for the simultaneous identification and quantification of unlabeled monosaccha-

ride in hydrolysates is HPAEC-PAD. HPAEC-PAD methods are also applicable to oligosaccharide separations, and a single instrumental approach can be used for both applications.

For the general principles and components of chromatography, see *Chromatography* (621). HPAEC-PAD facilitates analysis of monosaccharides and all classes of oligosaccharides without derivatization. Carbohydrates, because they are polyhydric compounds, are weak acids that have pK_a values of 12–14, and at high pH even neutral carbohydrates are ionized and can be separated as oxyanions by ion-exchange chromatography. Although separations can be performed on alkali-stable porous polystyrene–divinylbenzene anion exchangers, carbohydrates tend to exhibit broad peaks as a result of mass transfer problems. In microbead pellicular anion-exchange column packings, small functionalized latex beads (<0.1-μm diameter) are attached to larger (>10-μm diameter) uniform nonporous beads. The carbohydrate analyte interacts with the functional groups at the surface of the latex microbeads, eliminating diffusion into and out of pores and the associated peak broadening.

PAD is the method of choice for the detection of carbohydrates in HPAEC because it relies on the high-pH solutions that HPAEC provides by default. Amperometric detection measures the current, or charge, resulting from the oxidation or reduction of analyte molecules at the surface of a working electrode. Electrons are transferred from the electroactive analyte to the electrode during oxidation reactions and in the opposite direction during reduction reactions. This process allows sensitive and highly selective detection of analytes that can be oxidized or reduced, but interfering species that are not electroactive remain undetected. Carbohydrates are easily oxidized at gold and platinum electrodes at high pH, and the current generated is proportional to the carbohydrate concentration.

A typical amperometric detection system contains a working electrode and a reference electrode. Gold electrodes are most common for carbohydrate analysis, but oxidation products poison the electrode surface and inhibit further oxidation. Maintaining a stable, active electrode surface is accomplished by cyclical pulsing between high positive and negative potentials. This timed series of different potentials is referred to as a waveform, and repeated application of a waveform is the basis of pulsed amperometry. Different waveforms are used for different HPAEC-PAD applications and for different working electrodes: disposable gold electrodes require the use of fast, quadruple waveforms, but other gold electrodes allow a wider range of waveforms to be used without damaging the electrode surface. Disposable electrodes and fast waveforms were introduced to minimize the influence of electrode recess on the sensitivity and precision of quantitative monosaccharide applications.

FLUOROPHORE LABELING OF MONOSACCHARIDES BEFORE SEPARATION AND QUANTIFICATION

An alternative approach to the identification and quantification of monosaccharides present in a hydrolysate is to modify the monosaccharides by reductive amination with an easily detected fluorophore label that allows high-sensitivity detection and improves the chromatographic separation of monosaccharides. Essentially standard HPLC equipment can be used and, because the same labeling approaches are applicable to cleaved oligosaccharides, a consistent analytical approach can be applied. Fluorophore labeling has been much less widely used than HPAEC-PAD for monosaccharide identification and quantification. Labeling of sialic acid derivatives is usually undertaken with 1,2-phenylenediamine (or DMB, the 4,5-methylenedioxy derivatives), and the resulting products are separated on a C-18 column and using fluorescence detection.

CONCLUSION

Because of the complexity of glycoprotein glycan structures and their inherent variation during production processes, manufacturers are generally required by means of characterization studies to develop criteria for the control of the glycosylation pattern of a biological drug substance when glycosylation occurs, as well as to develop the level of information required at each stage of production and at batch release. Then analytical procedures can be derived in a manner that provides information relevant to fulfilling quality requirements. In general, a combination of approaches and techniques is needed, and more detailed glycan structural analysis at early drug development stages is required. Validation considerations are central as method development and product knowledge progress.

APPENDIX 1

Abbreviations

Fuc	L-Fucose
Gal	D-Galactose
GalNAc	N-Acetyl-D-galactosamine
Glc	D-Glucose
GlcNAc	N-Acetyl-D-glucosamine
Man	D-Mannose
Neu5Ac	N-Acetylneuraminic acid
Neu5Gc	N-Glycolylneuraminic acid
Xyl	D-Xylose

Additional Definitions

High mannose—Glycan chains containing two core GlcNAc residues and between five and nine Man residues, and lacking Gal, GlcNAc, or Neu5Ac residues in the antennae. Such chains are typically found in mammalian glycans.

Hypermannosylation—(i) Addition of Man residues to high mannose chains creating chains with large numbers of Man residues, and (ii) O-Man linked glycan chains with multiple Man residues synthesized by yeast.

Paucimannose—Glycan chains containing two core GlcNAc residues between two and four Man residues. Core-linked Fucα1,3 and/or Fucα1,6 residues may be present.

Oligomannose—Used here as a generic term to include high mannose, paucimannose, and N-linked hypermannosylated chains.

(1086) IMPURITIES IN DRUG SUBSTANCES AND DRUG PRODUCTS

INTRODUCTION

This general information chapter is intended to provide common terminology for impurities and degradation products that may be present in compendial drug substances and drug products. Impurities or degradation products in drug substances can arise during the manufacturing process or during storage of the drug substance. The degradation products in drug products can arise from drug substances or

reaction products of the drug substance with the environment, with an excipient, or an immediate container-closure system. Biological and biotechnological products, fermentation products and semisynthetic products derived therefrom, and radiopharmaceutical products are not covered in this chapter.

Communications about impurities and degradation products in compendial articles may be improved by including in this Pharmacopeia the definitions of terms and the contexts in which these terms are used. (See *Definitions* below.) There has been much activity and discussion in recent years about the definition of terms. Certain industry-wide concerns about terminology and context deserve widespread publication and ready retrievability and are included here. See section 5.60, *Impurities and Foreign Substances* in section 5, *Monograph Components under General Notices and Requirements*, as well as the general chapter *Ordinary Impurities* (466). Some other general chapters added over the years have also addressed topics of purity or impurity as these have come into focus or as analytical methodology has become available. Analytical aspects are enlarged upon in the chapter *Validation of Compendial Procedures* (1225).

Purity or impurity measurements for drug products present a challenge to Pharmacopeial standards-setting. Where degradation of a drug product over time is at issue, the same analytical methods that are stability-indicating are also purity-indicating. Resolution of the active ingredient(s) from the excipients necessary to the preparation presents the same qualitative problem. Thus, many monographs for Pharmacopeial preparations feature chromatographic assays. Where more significant impurities are known, some monographs set forth specific limit tests. In general, however, this Pharmacopeia does not repeat impurity tests in subsequent preparations where these appear in the monographs of drug substances and where these impurities are not expected to increase. It is presumed that adequate retention specimens are in storage for the exact batch of drug substances used in any specific lot of a drug product. Whenever analysis of an official article raises a question of the official attributes of any of the drug substances used, subsequent analysis of retention specimens is in order.

DRUG SUBSTANCE

Classification of Impurities—Impurities can be classified into the following categories:

1. Organic impurities (process- and drug-related)
2. Inorganic impurities
3. Residual solvents

Organic impurities can arise during the manufacturing process and/or storage of the drug substance. They can be identified or unidentified, volatile or nonvolatile, and include the following:

1. Starting materials
2. Byproducts
3. Intermediates
4. Degradation products
5. Reagents, ligands, and catalysts
6. Geometric and stereoisomers

Inorganic impurities can result from the manufacturing process. They are normally known and identified and include the following:

1. Reagents, ligands, and catalysts
2. Heavy metals or other residual metals
3. Inorganic salts
4. Other materials (e.g. filter aids, charcoal)

Residual solvents are organic liquids used as vehicles for the preparation of solutions or suspensions in the synthesis of a drug substance. Because these are generally of known toxicity, the selection of appropriate controls is easily accomplished (see *Residual Solvents* (467)).

Concepts for setting impurity or degradation product limits in drug substances are based on chemistry and safety

concerns. As such, limits for organic and inorganic impurities and residual solvents should be established for drug substances. The basic tenet for setting limits is that levels of impurities or degradation products in a drug substance must be controlled throughout its development to ensure its safety and quality for use in a drug product.

Documented evidence that the analytical procedure used to evaluate impurities or degradation products is validated and suitable for the detection and quantification of impurities or degradation products should be established.

DRUG PRODUCT

The specification for a drug product should include a list of degradation products expected to occur during manufacture of the commercial product and under recommended storage conditions. Stability studies, knowledge of degradation pathways, product development studies, and laboratory studies should be used to characterize the degradation profile. The selection of degradation products in the drug product specification should be based on the degradation products found in batches manufactured by the proposed commercial process.

This rationale should include a discussion of the degradation profiles observed in the safety and clinical development batches and in stability studies, together with a consideration of the degradation profile of batches manufactured by the proposed commercial process. For degradation products known to be unusually potent or to produce toxic or unexpected pharmacological effects, the quantitation/detection limit of the analytical procedures should be commensurate with the level at which the degradation products should be controlled.

For drug products the concept for setting degradation product limits is based on sound scientific judgment as applied to available data on the safety and stability of the drug product, data that may include the degradation pathways of the drug substance, the manufacturing process, known excipient interactions, any safety assessment studies, stability studies conducted under the recommended storage conditions, and ancillary studies that may provide additional information on the stability profile of the drug product. Impurities that are not degradation products (e.g., process impurities from the drug substance) are often not controlled in the drug product, as they are typically controlled in the drug substance and these impurities are not expected to increase over time. Additional guidance for setting limits can be found in various ICH and FDA guidance documents, as well as in the USP monograph submission guidelines.

Documented evidence that the analytical procedure used to evaluate impurities or degradation products is validated and suitable for the detection and quantification of impurities or degradation products should be established.

Drug products should contain levels of residual solvents no higher than can be supported by safety data (see *Residual Solvents* (467)).

DEFINITIONS

Concomitant Components—Concomitant components are characteristic of many drug substances and are not considered to be impurities in the Pharmacopeial sense. Limits on contents, or specified ranges, or defined mixtures are set forth for concomitant components in this Pharmacopeia. Examples of concomitant components are geometric and optical isomers (or racemates) and antibiotics that are mixtures. Any component that can be considered a toxic impurity because of significant undesirable biological effect is not considered to be a concomitant component.

Degradation Product—An impurity resulting from a chemical change in the drug substance brought about during manufacture and/or storage of the drug product by the effect of, for example, light, temperature, pH, water, or by

reaction with an excipient and/or the immediate container–closure system.

Foreign Substances (Extraneous Contaminants)—An impurity that arises from any source extraneous to the manufacturing process and that is introduced by contamination or adulteration. These impurities cannot be anticipated when monograph tests and assays are selected. The presence of objectionable foreign substances not revealed by monograph tests and assays constitutes a variance from the official standard. Examples of foreign substances include ephedrine in Ipecac or a pesticide in an oral liquid analgesic. Allowance is made in this Pharmacopeia for the detection of foreign substances by unofficial methods. (See section 5.60, *Impurities and Foreign Substances* in section 5, *Monograph Components* under *General Notices and Requirements*.)

Identified Impurities and Identified Degradation Products—Impurities or degradation products for which structural characterizations have been achieved.

Impurity—Any component of a drug substance that is not the chemical entity defined as the drug substance and in addition, for a drug product, any component that is not a formulation ingredient.

Inorganic Impurities—Inorganic impurities can result from the manufacturing process (e.g., residual metals, inorganic salts, filter aids, etc.). Inorganic impurities are typically controlled by tests such as *Heavy Metals* <231> and *Residue on Ignition* <281>. Information found in *Plasma Spectrochemistry* <730> and *Ion Chromatography* <1065> may also be of value.

Intermediate—A material that is produced during steps of the synthesis of a drug substance and that undergoes further chemical transformation before it becomes a drug substance. The intermediate is often isolated during the process.

Ordinary Impurities—Some monographs make reference to ordinary impurities. For more details see *Ordinary Impurities* <466>.

Other impurities—See section 5. *Monograph Components* under *General Notices and Requirements*.

Polymorphs—Different crystalline forms of the same drug substance. These can include solvation or hydration products (also known as pseudopolymorphs) and amorphous forms. Although polymorphs are not impurities by definition, an understanding of the crystalline forms, hydration or solvation states, or amorphous nature is critical to the overall characterization of the drug substance.

Process Contaminants—Process contaminants are identified or unidentified substances (excluding related substances and water), including reagents, catalysts, other inorganic impurities (e.g., heavy metals, chloride, or sulfate); and may also include foreign substances (extraneous contaminants). These contaminants may be introduced during manufacturing or handling procedures.

Reagent—A substance other than a starting material, intermediate, or solvent that is used in the manufacture of a drug substance.

Related Substances—Related substances are structurally related to a drug substance. These substances may be (a) identified or unidentified impurities arising from the synthesis manufacturing process, such as starting materials, intermediates, or by-products, and do not increase on storage, or (b) identified or unidentified degradation products that result from drug substance or drug product manufacturing processes or arise during storage of a material.

Residual Solvents—An organic liquid used as a vehicle for the preparation of solutions or suspensions in the synthesis of a drug substance (see *Residual Solvents* <467>).

Specified Impurities and Specified Degradation Products—Previously referred to as Signal Impurities, specified impurities or specified degradation products are impurities or degradation products that are individually listed and limited with specific acceptance criteria in individual mono-

graphs as applicable. Specified impurities or specified degradation products can be identified or unidentified.

Starting Material—A material that is used in the synthesis of a drug substance and is incorporated as an element into the structure of an intermediate and/or of the drug substance. Starting materials are often commercially available and have well-defined chemical and physical properties and structure.

Stereomeric Impurity—A compound with the same 2-dimensional chemical structure as the drug substance but differs in the 3-dimensional orientation of substituents at chiral centers within that structure. In those cases where all chiral centers are in the opposite orientation, the impurity is an enantiomer (enantiomeric impurity). Determinations of impurities in this category often require special chiral chromatographic approaches. Diastereomeric or epimeric impurities occur when only some of the chiral centers are present in the opposite orientation.

Toxic Impurities—Toxic impurities have significant undesirable biological activity, even as minor components, and require individual identification and quantification by specific tests. These impurities may arise out of the synthesis, preparation, or degradation of compendial articles. Based on validation data, individualized tests and specifications are selected. These feature comparison to a Reference Standard of the impurity, if available. It is incumbent on the manufacturer to provide data that would support the classification of such impurities as toxic impurities.

Unidentified Impurities and Unidentified Degradation Products—Impurities or degradation products for which structural characterizations have not been achieved and that are identified solely by qualitative analytical properties (e.g., chromatographic retention times).

Unspecified Impurities and Unspecified Degradation Products—Impurities or degradation products that are limited by general acceptance criteria but not individually listed with their own specific acceptance criteria in individual monographs.

<1087> APPARENT INTRINSIC DISSOLUTION—DISSOLUTION TESTING PROCEDURES FOR ROTATING DISK AND STATIONARY DISK

This general information chapter *Apparent Intrinsic Dissolution—Dissolution Testing Procedures for Rotating Disk and Stationary Disk* <1087> discusses the determination of dissolution rates from nondisintegrating compacts exposing a fixed surface area to a given solvent medium. Compact, as used here, is a nondisintegrating mass resulting from compression of the material under test using appropriate pressure conditions. A single surface having specified physical dimensions is presented for dissolution. Determination of the rate of dissolution can be important during the course of the development of new chemical entities because it sometimes permits prediction of potential bioavailability problems and may also be useful to characterize compendial articles such as excipients or drug substances. Intrinsic dissolution studies are characterization studies and are not referenced in individual monographs. Information provided in this general information chapter is intended to be adapted via a specific protocol appropriate to a specified material.

Dissolution rate generally is expressed as the mass of solute appearing in the dissolution medium per unit time (e.g., mass sec⁻¹), but dissolution flux is expressed as the rate per unit area (e.g., mass cm⁻² sec⁻¹). Reporting dissolution flux is preferred because it is normalized for surface area, and for a pure drug substance is commonly called intrinsic dissolution rate. Dissolution rate is influenced by intrinsic solid-state properties such as crystalline state, including polymorphs and solvates, as well as degree of noncrystallinity. Numerous procedures are available for modifying the physicochemical properties of chemical entities so that their solubility and dissolution properties are enhanced. Among these are coprecipitates and the use of racemates and enantiomeric mixtures. The effect of impurities associated with a material can also significantly alter its dissolution properties. Dissolution properties are also influenced by extrinsic factors such as surface area, hydrodynamics, and dissolution medium properties, including solvent (typically water), presence of surfactants, temperature, fluid viscosity, pH, buffer type, and buffer strength.

Rotating disk and stationary disk dissolution procedures are sufficiently versatile to allow the study of characteristics of compounds of pharmaceutical interest under a variety of test conditions. Characteristics common to both apparatuses include the following:

- (1) They are adaptable to use with standard dissolution testing stations, and both use a tablet die to hold the nondisintegrating compact during the dissolution test.
- (2) They rely on compression of the test compound into a compact that does not flake or fall free during the dissolution test.
- (3) A single surface of known geometry and physical dimension is presented for dissolution.
- (4) The die is located at a fixed position in the vessel, which decreases the variation of hydrodynamic conditions.

A difference between the two procedures is the source of fluid flow over the dissolving surface. In the case of the rotating disk procedure, fluid flow is generated by the rotation of the die in a quiescent fluid, but fluid flow is generated by a paddle or other stirring device for the stationary disk procedure.

EXPERIMENTAL PROCEDURE

The procedure for carrying out dissolution studies with the two types of apparatus consists of preparing a nondisintegrating compact of material using a suitable compaction device, placing the compact and surrounding die assembly in a suitable dissolution medium, subjecting the compact to the desired hydrodynamics near the compact surface, and measuring the amount of dissolved solute as a function of time.

Compacts are typically prepared using an apparatus that consists of a die, an upper punch, and a lower surface plate fabricated out of hardened steel or other material that allows the compression of material into a nondisintegrating compact. An alternative compaction apparatus consists of a die and two punches. Other configurations that achieve a nondisintegrating compact of constant surface area also may be used. The nondisintegrating compact typically has a diameter of 0.2 cm to 1.5 cm.

Compact Preparation

Attach the smooth lower surface plate to the underside of the die, or alternatively, insert the lower punch using an appropriate clamping system. Accurately weigh a quantity of material necessary to achieve an acceptable compact and transfer to the die cavity. Place the upper punch into the die cavity, and compress the powder on a hydraulic press at a compression pressure required to form a nondisintegrating

compact that will remain in the die assembly for the length of the test. Compression for 1 minute at 15 MPa usually is sufficient for many organic crystalline compounds, but alternative compression conditions that avoid the formation of capillaries should be evaluated. For a given substance, the compact preparation, once optimized is standardized to facilitate comparison of different samples of the substance.

Changes in crystalline form may occur during compression; therefore, confirmation of solid state form should be performed by powder X-ray diffraction or other similar technique. Remove the surface plate or lower punch. Remove loose powder from the surface of the compact and die by blowing compressed air or nitrogen over the surface.

Dissolution Medium

The choice of dissolution medium is an important consideration. Whenever possible, testing should be performed under sink conditions to avoid artificially retarding the dissolution rate due to approach of solute saturation of the medium. Dissolution measurements are typically made in aqueous media. To approximate in vivo conditions, measurements may be run in the physiological pH range at 37°. The procedure when possible is carried out under the same conditions that are used to determine the intrinsic solubility of the solid state form being tested. Dissolution media should be deaerated immediately prior to use to avoid air bubbles forming on the compact or die surface.¹

The medium temperature and pH must be controlled, especially when dealing with ionizable compounds and salts. In the latter cases, the dissolution rate may depend strongly on the pH, buffer species, and buffer concentration. A simplifying assumption in constant surface area dissolution testing is that the pH at the surface of the dissolving compact is the same as the pH of the bulk dissolution medium. For nonionizable compounds, this is relatively simple because no significant pH dependence on dissolution rate is expected. For acids and bases, the solute can alter the pH at and near the surface of the compact as it dissolves. Under these conditions, the pH at the surface of the compact may be quite different from the bulk pH due to the self-buffering capacity of the solute. To assess intrinsic solubility, experimental conditions should be chosen to eliminate the effect of solute buffering, alteration of solution pH, and precipitation of other solid state forms at the surface of the compact. For weak acids, the pH of the dissolution medium should be one to two pH units below the pKa of the dissolving species. For weak bases, the pH of the dissolution medium should be one to two pH units above the pKa of the dissolving species.

Apparatus

Rotating Disk—A typical apparatus (*Figure 1*) consists of a punch and die fabricated out of hardened steel. The base of the die has three threaded holes for the attachment of a surface plate made of polished steel, providing a mirror-smooth base for the compacted pellet. The die has a cavity into which is placed a measured amount of the material whose intrinsic dissolution rate is to be determined. The punch is then inserted in the die cavity and the test material is compressed with a hydraulic press. [NOTE—A hole through the head of the punch allows insertion of a metal rod to facilitate removal from the die after the test.] A compacted pellet of the material is formed in the cavity with a single face of defined area exposed on the bottom of the die.

The die assembly is then attached to a shaft constructed of an appropriate material (typically steel). The shaft holding

¹One method of deaeration is as follows: Heat the medium, while stirring gently, to about 41°, immediately filter under vacuum using a filter having a porosity of 0.45 µm or less, with vigorous stirring, and continue stirring under vacuum for about 5 minutes. Other deaeration techniques for removal of dissolved gases may be used.

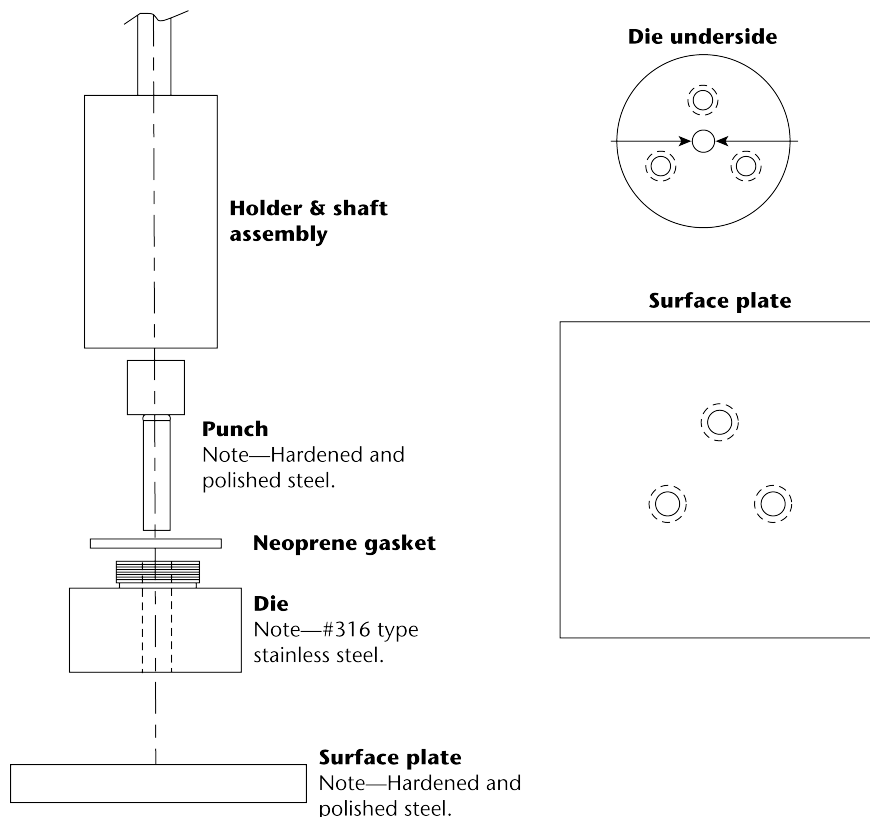


Figure 1

the die assembly is positioned so that when the die assembly is lowered into the dissolution medium (*Figure 2*) the exposed surface of the compact will be not less than 1.0 cm from the bottom of the vessel and nominally in a horizontal position. The die assembly should be aligned to minimize wobble, and air bubbles should not be allowed to form on the compact or die surface.

A rotating disk speed of 300 rpm is recommended. Typical rotation speeds may range from 60 rpm to 500 rpm. The dissolution rate depends on the rotation speed used. This parameter should be selected in order to admit at least five sample points during the test, but excessive stirring speeds may create shear patterns on the surface of the dissolving material that could cause aberrant results (i.e., non-linearity). Typically, the concentration of the test specimen is measured as a function of time, and the amount dissolved is then calculated. The sampling interval will be determined by the speed of the dissolution process. If samples are removed from the dissolution medium, the cumulative amount dissolved at each time point should be corrected for losses due to sampling.

Stationary Disk—The apparatus (*Figure 3*) consists of a steel punch, die, and a base plate. The die base has three holes for the attachment of the base plate. The three fixed screws on the base plate are inserted through the three holes on the die and then fastened with three washers and nuts. The test material is placed into the die cavity. The punch is then inserted into the cavity and compressed, with the aid of a bench top press. The base plate is then disconnected from the die to expose a smooth compact pellet surface. A gasket is placed around the threaded shoulder of the die and a polypropylene cap is then screwed onto the threaded shoulder of the die.

The die assembly is then positioned at the bottom of a specially designed dissolution vessel with a flat bottom (*Figure 4*). The stirring unit (e.g., paddle) is positioned at an appropriate distance (typically 2.54 cm) from the compact surface. The die assembly and stirring unit should be aligned to ensure consistent hydrodynamics, and air bubbles should not be present on the compact surface during testing. Alternative configurations may be utilized if adequate characterization and control of the hydrodynamics can be established.

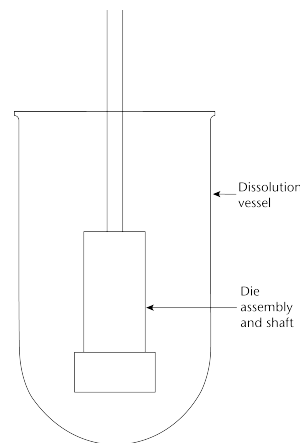


Figure 2

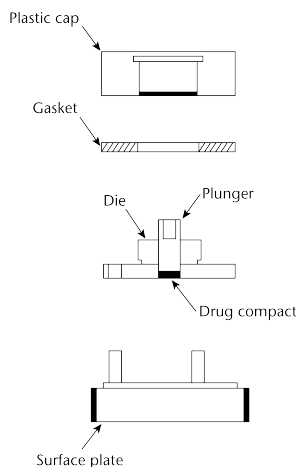


Figure 3

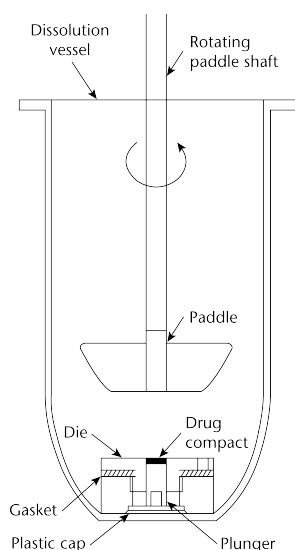


Figure 4

The dissolution rate depends on the rotation speed and precise hydrodynamics that exist. Typically, the concentration of the test specimen is measured as a function of time, and the amount dissolved is then calculated. The sampling interval will be determined by the speed of the dissolution process (see *Rotating Disk*). If samples are removed from the dissolution medium, the cumulative amount dissolved at each time point should be corrected for losses due to sampling.

DATA ANALYSIS AND INTERPRETATION

The dissolution rate is determined by plotting the cumulative amount of solute dissolved against time. Linear regression analysis is performed on data points in the initial linear region of the dissolution curve. The slope corresponds to the dissolution rate (mass sec⁻¹). (More precise estimates of slope can be obtained using a generalized linear model that takes into account correlations among the measurements of the cumulative amounts dissolved at the various sampling times.)

The amount versus time profiles may show curvature. When this occurs, only the initial linear portion of the profile is used to determine the dissolution rate. Upward curvature (positive second derivative) of the concentration versus time

data is typically indicative of a systematic experimental problem. Possible problems include physical degradation of the compact by cracking, delaminating, or disintegration. Downward (negative second derivative) curvature of the dissolution profile is often indicative of a transformation of the solid form of the compact at the surface or when saturation of the dissolution medium is inadvertently being approached. This often occurs when a less thermodynamically stable crystalline form converts to a more stable form. Examples include conversion from an amorphous form to a crystalline form or from an anhydrous form to a hydrate form, or the formation of a salt or a salt converting to the corresponding free acid or free base. If such curvature is observed, the crystalline form of the compact may be assessed by removing it from the medium and examining it by powder X-ray diffraction or another similar technique to determine if the exposed surface area is changing.

The constant surface area dissolution rate is reported in units of mass sec⁻¹, and the dissolution flux is reported in units of mass cm⁻² sec⁻¹. The dissolution flux is calculated by dividing the dissolution rate by the surface area of the compact. Test conditions, typically a description of the apparatus, rotation speed, temperature, buffer species and strength, pH, and ionic strength should also be reported with the analyses.

(1088) IN VITRO AND IN VIVO EVALUATION OF DOSAGE FORMS

PURPOSE

This chapter provides an overview of the methodology for characterizing the physicochemical properties of a drug substance as well as its associated drug product and discusses the relationship of these methods and properties to the pharmacokinetic and pharmacodynamic properties of the drug product. Results of in vitro methods are linked with information from in vivo evaluations through an in vitro-in vivo correlation (IVIVC).

SCOPE

The ultimate goal of these characterization studies is an understanding of the relationship between the physicochemical and pharmacological properties of the drug substance to the pharmacokinetic properties and in vitro performance of the drug product. This chapter outlines the in vitro and in vivo testing that goes into the development of the body of data that informs decision making relating to the formulation, manufacturing, and related regulatory activities necessary for the development, regulatory approval, and marketing of any drug product. The chapter complements the information in general chapters, *Assessment of Drug Product Performance—Bioavailability, Bioequivalence, and Dissolution* (1090) and *The Dissolution Procedure: Development and Validation* (1092) by detailing the essential in vitro and in vivo data elements underlying an understanding of bioequivalence and bioavailability. The chapter text recognizes that regulatory guidances and a wealth of text books are available to elaborate on the content provided, and it is not the purpose to provide an exhaustive disquisition on the subjects presented but rather to provide a guide and listing of the issues of interest.

BACKGROUND INFORMATION

Establishing a meaningful relationship between dissolution behavior and in vivo drug performance (i.e., IVIVC) has long been sought from the perspectives of both bioavailability (BA) and bioequivalence (BE) and quality control considerations. In setting dissolution acceptance criteria for a product monograph, USP's policy has been to give predominant consideration to valid BA or BE studies, when available.

The earliest achievable in vitro characteristic thought to predict an acceptable in vivo performance was tablet and capsule disintegration. A test for disintegration was adopted in *USP XIV* (1950). At that time, no quantitative work was done to attempt to demonstrate such a relationship, especially with regard to in vivo product performance. Advances in instrumental methods and analytical precision ultimately opened up prospects for this work. The USP-NF Joint Panel on Physiologic Availability recognized that the disintegration test was insufficiently sensitive and in 1968 directed the identification of candidate articles for the first 12 official dissolution tests that used *Apparatus 1*.

USP requires drug release testing via the USP performance test in the majority of monographs for non-solution oral, sublingual, and transdermal dosage forms. In the current state of science, in vivo testing is necessary during the development and evaluation of both immediate-release and modified-release dosage forms. In some cases, depending on the Biopharmaceutics Classification System (BCS) classification of the drug, and depending on regulatory policy, in vivo testing may not be necessary. The special sensitivity of the dissolution test to changes in composition or method of manufacturing that do not result in significant changes in performance in vivo is well recognized. An understanding of the full complement of information given by in vitro and in vivo evaluation of the drug substance and product is the starting point in the development of a meaningful in vitro performance test.

IN VITRO EVALUATION

Physicochemical Properties—Drug Substance

Physicochemical information typically includes polymorphism, stability, particle size distribution, solubility, dissolution rate, lipophilicity, permeability, and other release-controlling variables of the drug substance under conditions that may mimic the extremes of the physiologic environment experienced by the dosage form.

Physicochemical Properties—Drug Product

The variables tested to characterize the physicochemical properties of the drug product should be the same as those that are tested to characterize the drug substance. Dissolution profiles over a relevant pH range, usually from pH 1–6.8, should be obtained with particular attention to formulation effects. Characterization of formulations that are insoluble in aqueous systems may require the addition of sodium lauryl sulfate or another surfactant. The BCS classification of the drug substance should be determined, especially for immediate-release dosage forms.

Dissolution Testing

Dissolution testing is required for all non-solution oral, including sublingual, Pharmacopeial dosage forms in which absorption of the drug is necessary for the product to exert the desired therapeutic effect. Exceptions include tablets that meet a requirement for completeness of solution, products that contain radiolabeled drugs, or products that contain a soluble drug and demonstrate rapid (10–15 min) dis-

integration. Dissolution testing should be conducted on equipment that conforms to the requirements in *Dissolution* <711> and on which a performance verification test has been conducted when one is available. On its website, USP provides a guidance for optimizing dissolution instrument performance by mechanical calibration and performance verification testing (<http://www.usp.org/pdf/EN/dissolutionProcedureToolkit2010-03.pdf>).

In vitro dissolution testing generally should attempt to mimic in vivo dissolution, but such in vitro conditions cannot be selected reliably a priori. A range of in vitro dissolution test conditions (e.g., media of varying pH, surfactant, and apparatus rotational speed) should be evaluated. Knowledge of drug substance properties, product formulation, gastrointestinal physiology, in vitro dissolution, and in vivo pharmacokinetics will aid in the selection of in vitro dissolution test conditions and specifications.

For products that contain more than a single active ingredient, dissolution typically should be determined for each active ingredient. When a dissolution test is added to an existing monograph, the disintegration test is deleted, but in the case of sublingual preparations and orally disintegrating tablets, disintegration may be a critical quality attribute in addition to dissolution. In such cases one or both tests can be included in the monograph.

When a single set of specifications cannot be established for multisource products described in monographs, multiple dissolution tests are allowed, and labeling is required to indicate the appropriate dissolution test for the specific product.

Detailed information about method development and validation can be found in *The Dissolution Procedure: Development and Validation* <1092>.

IMMEDIATE-RELEASE DOSAGE FORMS

For immediate-release dosage forms the in vitro dissolution process typically requires no more than 60 min, and in most cases a single time-point specification is adequate for Pharmacopeial purposes. To allow for typical disintegration times, test times of less than 30 min should be based on demonstrated need.

EXTENDED-RELEASE DOSAGE FORMS

For extended-release products in vivo dissolution generally is rate limiting, which results in protracted drug absorption and thus facilitates the identification of in vitro test conditions that may be predictive of in vivo dissolution. Multiple sampling time points, therefore, are necessary to define a dissolution profile for a modified-release dosage form.

The choice of apparatus should be based on knowledge of the formulation and actual dosage form performance in the in vitro test system. *Apparatus 1* (basket) or *Apparatus 2* (paddle) may be more useful at higher rotation rates (e.g., the paddle at 100 rpm). *Apparatus 3* (reciprocating cylinder) has been especially useful for bead-type modified-release dosage forms. *Apparatus 4* (flow cell) may offer advantages for modified-release dosage forms that contain active ingredients that have limited solubility. *Apparatus 7* (reciprocating disk) is applicable to nondisintegrating oral modified-release dosage forms, as well as to transdermal dosage forms. *Apparatus 5* (paddle over disk) and *Apparatus 6* (cylinder) also are useful for evaluating and testing transdermal dosage forms.

At least three timepoints are chosen to characterize the in vitro drug release profile of an extended-release dosage form for Pharmacopeial purposes. Additional sampling times may be required for drug approval purposes. An early time point, usually 1–2 h, is chosen to show that dose dumping is not probable. An intermediate time point is chosen to define the in vitro release profile of the dosage form, and a

final time point is chosen to show essentially complete release of the drug.

IN VIVO EVALUATION OF DOSAGE FORMS

In evaluating a drug product's performance, analysts fundamentally must ask what type of study should be performed to give reasonable assurance of BE of a marketed product to the clinical trial product that demonstrated safety and efficacy. Although they provide important information concerning the release characteristics of the drug from the dosage form, in vitro dissolution studies at present are used primarily for setting or supporting specifications for drug products (e.g., shelf life) and manufacturing process control (e.g., scale-up or postapproval changes). Normally BE is best demonstrated by in vivo evaluation but can sometimes be replaced by in vitro studies.¹ BE assessment of modified-release dosage forms is best achieved by observing in vivo drug pharmacokinetic and/or pharmacodynamic behavior by means of well-designed clinical studies. Multiple guidelines for the conduct of such studies are provided by regulatory agencies. Moreover, when a well-defined, predictive relationship exists between plasma concentrations of a drug or its active metabolites and the clinical response (therapeutic and adverse), it is possible to use plasma drug concentration data alone as a basis for the approval of a modified-release dosage form that is designed to replace an immediate-release dosage form.

Although human pharmacokinetic studies often are used to assess BE of immediate-release solid oral dosage forms, in some cases in vitro studies can be used to assess BE. The principal advantage of in vitro studies is that they reduce development costs. For example, an in vitro test is preferable when one is testing BCS Class I drugs with rapid dissolution. Some regulatory agencies permit this type of testing in lieu of in vivo testing.

The following discussions are intended to provide guidance for drug substance evaluation and the design, conduct, and evaluation of studies involving dosage forms. Although these guidelines focus on oral drug delivery systems, the principles may be applicable to other routes of drug administration (e.g., transdermal, subcutaneous, intramuscular, etc.).

CHARACTERIZATION OF DRUG SUBSTANCE

The Biopharmaceutics Classification System (BCS)

FDA has issued a guidance titled "Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System" (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070246.pdf). A key assumption in the approach is that drug release and dissolution is sufficiently rapid so that an in vitro-in vivo correlation is not possible and/or useful. When applicable, the BCS allows dissolution rate data in lieu of BA or BE studies for product approval.

Pharmacokinetic Properties

Analysts should thoroughly characterize the input absorption profile of the active drug entity from a formulation that shows rapid BA (an intravenous solution, oral solution, or a well-characterized immediate-release drug product). In turn,

¹21 CFR 320.22 Criteria for waiver of evidence of in vivo bioavailability or bioequivalence.

this formulation serves as a reference to evaluate the input profile of the modified-release dosage form. This information, together with the pharmacokinetics of the active drug entity, can characterize drug absorption and can predict changes in drug BA when input is modified as in modified-release dosage forms. For example, if the active drug entity exhibits saturable first-pass hepatic metabolism, a reduction in systemic availability could result after oral administration if the input rate is decreased.

In designing an oral modified-release dosage form, analysts may find it useful to determine the absorption of the active drug entity in various segments of the gastrointestinal tract, particularly in the lower gastrointestinal tract (colon) for delayed-release dosage forms that release drug in this region. Food effects also may be important and should be investigated.

Drug Disposition

The information required to characterize drug disposition may include the following.

1. Disposition parameters—clearance, area under the time—plasma concentration curve (AUC), maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}), volume of distribution, half-life, mean residence time, or model-dependent parameters.
2. Linearity or characterization of nonlinearity over the dose or concentration range that could be encountered.
3. Drug/metabolite accumulation.
4. Metabolic profile and excretory pathway, with special attention to the active metabolites and active enantiomers of racemic mixtures.
5. Enterohepatic circulation.
6. Protein-binding parameters and effect of dialysis.
7. The effects of age, gender, race, and relevant disease states.
8. Plasma: blood ratios.
9. A narrow therapeutic index or a clinical response that varies significantly as a function of the time of day (chronopharmacokinetics).

Pharmacodynamic Properties

Before developing a dosage form, analysts should obtain concentration-response relationships over a dose range sufficiently wide to encompass important therapeutic and adverse responses. In addition, the equilibration-time² characteristics between plasma concentration and effect should be evaluated. For modified-release products that typically have larger drug doses in the dosage form, these concentration-response relationships should be sufficiently characterized so that a reasonable prediction of the safety margin can be made if dose dumping should occur. If there is a well-defined relationship between the plasma concentration of the active drug substance or active metabolites and the clinical response (therapeutic and adverse), the clinical performance of a new modified-release dosage form could be characterized by plasma concentration-time data. If such data are not available, clinical trials of the modified-release dosage form should be carried out with concurrent pharmacokinetic and pharmacodynamic measurements.

² Equilibration time is a measure of the time-dependent discontinuity between measured plasma concentrations and measured effects. The discontinuity is more often characterized by the degree of hysteresis observed when the effect-concentration plot for increasing concentrations is compared with that for decreasing concentrations. Where the equilibration time is very short (i.e., rapid equilibration with no active metabolites generated), there will be little or no hysteresis. That is, the same effect will be observed for a given concentration independent of the interval between the time of dosing and the time that measurements are made.

CHARACTERIZATION OF THE DOSAGE FORM

Pharmacokinetic Properties: Immediate-Release Products

The types of pharmacokinetic studies that should be conducted are based on how much is known about the active drug substance, its clinical pharmacokinetics, and its BCS Class. For example, a new chemical entity requires greater pharmacokinetic characterization than does an FDA-approved formulation that is undergoing scale-up and postapproval changes (SUPAC) evaluation.

The latter is seen when an FDA-approved drug product undergoes changes in the manufacturing of the product after the product has been approved. Such changes are common and can be caused by expansion in the size of the lots manufactured, new manufacturing locations, or the introduction of new technology. Necessary in vitro dissolution tests and/or in vivo BE tests are described in the FDA "Guidance for Industry: Immediate-release Solid Oral Dosage Forms: Scale-up and Postapproval Changes: Chemistry, Manufacturing, and Controls, In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation" (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070636.pdf).

Similar requirements apply to a generic equivalent of an approved immediate-release dosage form that must be BE to the innovator drug, known as the reference listed drug. The two most frequently used methods for meeting bioequivalence requirements are in vivo pharmacokinetic studies and BCS-based in vitro studies.

Pharmacokinetic Properties: Modified-Release Products

Like the approaches for immediate-release products, the types of pharmacokinetic studies that should be conducted for modified-release products are based on how much is known about the drug substance, its pharmacokinetics, biopharmaceutics, and whether pharmacokinetic studies are intended to be the sole basis for product approval. At a minimum, two studies are required to characterize the product when no reference modified-release product exists: (1) a single-dose crossover study for each strength of a modified-release dosage form and (2) a multiple-dose, steady-state study using the highest strength of a modified-release dosage form. A food effects study to evaluate the potential for dose dumping from extended-release dosage forms also is required as a separate study or is included as an arm of a crossover study. In the demonstration of interchangeability, a single-dose, fasting crossover study vs. the reference product usually will suffice. In some cases, a food-effects study is required if the reference product has demonstrated a food effect on BA. Some appropriate single-dose crossover and multiple-dose steady-state studies are described below.

For modified-release products, intravenous solutions, oral solutions, or well-characterized immediate-release drug products are possible reference products to evaluate a modified-release formulation. For example, if the active drug entity exhibits saturable first-pass hepatic metabolism from the small intestine, a reduction in systemic availability could result after oral administration if the input rate is decreased. An increase in systemic availability could be observed if a drug is absorbed from the colon from a delayed-release dosage form that targets the colon, thus avoiding a first-pass effect.

In some modified-release capsule dosage forms, the strengths differ from each other only in the amount of identical beaded material contained in each capsule. In this case, single-dose and multiple-dose steady-state studies at the highest dosage strength are sufficient. Other strengths can

be characterized on the basis of comparative in vitro dissolution data.

The pharmacokinetic studies described below are needed for most modified-release dosage forms. These studies may be the basis for characterization of the dosage form. If regulatory approval is sought without conducting clinical trials, manufacturers should consult with the regulatory authorities to ensure that an adequate database exists for the approval. The types of pharmacokinetic studies generally conducted can be categorized as follows.

CASE A

Case A applies to an original modified-release oral dosage form for a drug already marketed in an immediate-release dosage form and for which extensive pharmacokinetic/pharmacodynamic data exist.

Single-dose crossover study: A single-dose crossover study should include the following treatments: the modified-release dosage form administered under fasting conditions; a dosage form that is rapidly available administered under fasting conditions; and the modified-release dosage form administered immediately after a high-fat standardized meal. The food effects study should control the ambient-temperature fluid intake (e.g., 6–8 oz.) at the time of drug administration. The dosage form should be administered within 5 min after completion of the meal. Ideally all subjects should consume the meal in approximately 15 min. If there are no significant differences in the rate or extent of bioavailability (AUC , C_{max} , and T_{max}) as a function of the meal, then additional food effect studies are not necessary. If significant differences in bioavailability are found, researchers must define how food affects the modified-release dosage form,³ as well as how the food–drug effect relates to time.

Use the following guidelines in evaluating food effects.

1. If no well-controlled studies have previously defined the effects of a concurrent high-fat meal on an immediate-release dosage form, studies should be performed to determine whether a food effect is a result of problems with the dosage form. Does the dosage form show food-related changes in release, or are there food effects that are unrelated to the dosage form, e.g., changes in the drug's absorption from the gastrointestinal tract or changes in the drug's disposition that are independent of absorption? The cause of the food effect should be determined by a single-dose crossover study comparing the solution (or immediate-release dosage form) under fed and fasting conditions. If there is no food effect, then one concludes that there are problems with the dosage form. If there is a food effect, then one concludes that the effect is unrelated to the dosage form.
2. The influence of timing on the food effect should be tested by a four-way crossover study, in which the modified-release dosage form is administered under the following treatment conditions: fasting, taken with a high-fat meal, 1 h before a high-fat meal, and 2 h after a high-fat meal.
3. If the food effect on an immediate-release dosage form is determined to result from changes in the dissolved drug's absorption from the gastrointestinal tract or from changes in drug disposition, studies should define the appropriate relationship between drug dosing and meals.
4. Alternative appropriate studies can be conducted if the applicant labels the drug for administration with a meal that is not fat loaded. In this case, an alternative meal composition should be considered.
5. Analysts should monitor the entire single-dose, modified-release absorption profile. Where appropriate

³ Wagner-Nelson, Loo-Riegelman, and other deconvolution methods are found in textbooks on biopharmaceutics.

(e.g., in a multiple-dose study) for specific drugs and drug delivery systems, blood samples should be taken following breakfast on the second day, before the second dose is administered. This sampling schedule is particularly important for once-a-day products.

6. For delayed-release (enteric-coated) dosage forms, analysts should perform BA studies to characterize food effect and to support the dosing claims stated in the labeling.

The purpose of these studies is twofold: first, to determine whether a need exists for labeling instructions describing special conditions for administration with respect to meals; and second, to provide information concerning the pattern of absorption of the modified-release dosage form compared to that of the immediate-release dosage form. Drug input function should be defined for modified-release dosage forms. This will aid in the development of an appropriate in vitro dissolution test. For dosage forms that exhibit high variability, a replicate study design is recommended.

Multiple-dose, steady-state studies

Study I—When data demonstrating linear pharmacokinetics exist for an immediate-release dosage form, a steady-state study should be conducted with the modified-release dosage form at one dose rate (preferably at the high end of the usual dosage regimen) using a comparable total daily dose of an immediate-release dosage form as a control. At least three trough plasma drug concentration (C_{min}) determinations at the same time of day should be made to demonstrate that steady-state conditions have been achieved.

Plasma drug concentration determinations, over at least one dosing interval of the modified-release dosage form, should be made in each phase of the crossover study. It may be preferable (as in the case of rhythmic variation in absorption or disposition of the drug) to measure concentrations over an entire day in each phase. The presence or absence of circadian variation should be verified. The modified-release dosage form should produce an AUC that is equivalent to that of the immediate-release dosage form if the extent of absorption from the modified-release dosage form is comparable to the immediate-release dose. The degree of fluctuation for the modified-release product should be the same as, or less than, that for the immediate-release dosage form given by the approved regimen. Appropriate concentration measurements should include unchanged drug and major active metabolites. For racemic drug entities, analysts should consider measurement of the active enantiomers.

Study II—When comparisons of the pharmacokinetic properties of an immediate-release dosage form at different doses are not available, or when the data demonstrate non-linearity, steady-state crossover studies comparing effects of the modified-release dosage form and those of the immediate-release dosage form should be conducted at two different dose rates: one at the low end of the recommended dosing range and the second at the high end of the dosing range. In each case, the modified-release dosage form must meet the criteria described in *Study I* with respect to AUC and fluctuations in plasma drug concentrations. If there are significant differences between the modified-release dosage form and the immediate-release dosage form at either the low or the high dosing rate, these data alone are not adequate to characterize the product. Data can be misleading when obtained from subjects with atypical drug disposition or physiologic characteristics relative to the target population. Therefore, subject selection should be from an appropriate target population with randomized assignment to dosage form population. If the modified-release dosage form is for use in a specific subpopulation (e.g., for children), it should be tested in that population. Whether a drug exhibits linear or nonlinear pharmacokinetics, the basis for characterization is equivalence of AUC and of the relative degree of fluctuation of concentrations of the modified-release and immediate-release dosage forms.

Steady-state studies in selected patient populations or drug interaction studies may also be necessary, depending

on the therapeutic use of the drug and the types of individuals for whom the modified-release dosage form will be recommended. For drugs that have narrow therapeutic indices, it may be necessary to perform more extensive plasma concentration measurements to determine the potential for unusual drug-release patterns in certain subpopulations. In such studies, researchers should perform more than one AUC measurement per patient to assess variability with both the modified-release and the immediate-release dosage forms.

CASE B

Case B applies to a non-oral, modified-release dosage form of an already marketed active drug entity for which extensive pharmacokinetic and pharmacodynamic data exist.

Case A studies (omitting the food effects studies) are appropriate for the evaluation of a modified-release dosage form designed for a non-oral route of administration if the pattern of biotransformation to active metabolites is identical for the two routes. If the biotransformation patterns are different, then clinical efficacy studies should be performed with the modified-release dosage form. In addition, special studies may be necessary to assess specific risk factors related to the dosage form (e.g., irritation and/or sensitization at the site of application of a transdermal drug delivery system).

CASE C

Case C applies to a generic equivalent of an approved modified-release dosage form, which should be BE to the reference drug in its rate and extent of drug exposure (i.e., AUC, C_{max} , C_{min} , and degree of fluctuation) in crossover single-dose studies. For an oral modified-release dosage form, the food studies described under Case A also should be performed.

CASE D

Case D applies to an FDA-approved product that has undergone SUPAC. Necessary in vitro dissolution tests and/or in vivo bioequivalence tests are described in the FDA guidance, *SUPAC-MR: Modified Release Solid Oral Dosage Forms; Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation* (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070640.pdf).

Statistical Analysis of In Vivo Bioequivalence

An appropriate statistical method should be selected. (See *Assessment of Drug Product Performance—Bioavailability, Bioequivalence, and Dissolution* (1090)).

IN VITRO-IN VIVO CORRELATIONS

The term IVIVC first appeared in the pharmaceutical literature as a result of the awareness of the importance of bioavailability concepts and in vitro dissolution rate determinations. IVIVC refers to the establishment of a rational relationship between a biological property, or a parameter derived from drug plasma concentrations produced by a dosage form, and a physicochemical property or characteristic of the same dosage form. The biological properties most commonly used are one or more pharmacokinetic parameters such as C_{max} or AUC, obtained following the administration of the dosage form. The physicochemical property most commonly used is a dosage form's in vitro dissolution

behavior (e.g., percent of drug released under a given set of conditions). The quantitative relationship between the two properties, biological and physicochemical, is an IVIVC. The most important use of an IVIVC is for predictability. In many cases the actual drug plasma concentration profile can be predicted from in vitro dissolution data.

Historically, IVIVC analysis has been more successful for extended-release products than for immediate-release products. This difference probably reflects the application of specific data analysis techniques and interpretations that require dissolution rate-limited drug absorption. However some correlations with immediate-release products have been demonstrated using methods that rely on the current, broad availability of computers and nonlinear regression software, along with new correlation methods.

General Considerations

With the proliferation of modified-release products, it becomes necessary to examine IVIVC in greater detail. Unlike immediate-release dosage forms, modified-release products, particularly extended-release dosage forms, cannot be characterized using a single time point dissolution test. These products are designed to deliver drug so that a patient has a specific plasma level profile over a prolonged period, usually 12–24 h. Analysts require an in vitro means of ensuring that each batch of the product will perform identically in vivo. An IVIVC satisfies this requirement. Initially, it was thought that developing a meaningful correlation for immediate-release dosage forms would be an easier task than for extended-release products. However, because of the nature of the principles on which each type is based, analysts now believe that an IVIVC is more readily achieved for modified-release dosage forms.

One expects all extended-release products to be dissolution rate limited. For these products, the formulation significantly contributes to the prolongation of drug release from the dosage form. Because of the impact of formulation on BA from an extended-release product, numerous attempts have been made to correlate one or more pharmacokinetic parameters determined from in vivo studies with the amount released in a given time during an in vitro dissolution test. Single-point correlations can indicate that increasing or decreasing the in vitro dissolution rate of the modified-release dosage form would result in a corresponding directional change in the product's performance. However, such single-point correlations reveal little about the overall plasma level curve, which is a major factor for drug performance in the patient. Rather, correlation methods that utilize all plasma drug concentration data and all in vitro dissolution data are preferred. Three correlation procedures are available that use all dissolution and plasma data, along with statistical moment analysis. Each procedure displays important differences in the quality of the correlation. These methods are discussed in terms of the advantages of each along with its potential utility as a predictive tool for pharmaceutical scientists.

Correlation Levels

Three correlation levels have been defined and categorized in descending order of quality. The concept of correlation level is based on the ability of the correlation to reflect the entire plasma drug concentration–time curve that results from administration of the given dosage form. The relationship of the entire in vitro dissolution curve to the entire plasma concentration–time profile defines the strength of the correlation and, therefore, the predictability.

LEVEL A

This level is the highest category of correlation. It represents a point-to-point relationship between in vitro dissolu-

tion and the in vivo input rate (absorption rate of the drug from the dosage form). For a *Level A* correlation, a product's in vitro dissolution curve is compared to its in vivo input curve, i.e., the curve produced by deconvolution of the plasma profile. Deconvolution can be accomplished using mass balance model-dependent methods, such as the Wagner–Nelson or Loo–Riegelman methods, or by model-independent, mathematical deconvolution. In an ideal correlation, the in vitro dissolution and in vivo absorption rate curves are superimposable or can be made superimposed by the use of a constant offset value of the time scale. The equations describing each curve are the same. This procedure often is found with modified-release dosage systems that demonstrate an in vitro release rate that is essentially independent of the dissolution media and stirring speeds used in a dissolution apparatus. Superimposition is not an absolute requirement for a *Level A* correlation. If the dissolution and absorption curves are different and a mathematical relationship can be developed to relate the two, the plasma level profile still is predictable from the in vitro dissolution data. This relationship must be true not only at that single input rate but also over the entire quality control dissolution range for the product. Furthermore, when the dissolution rate depends on mixing speed, the two curves can be made to superimpose by either increasing or decreasing the in vitro mixing speed or some other alteration of the dissolution method.

The advantages of a *Level A* correlation are as follows.

1. It develops a point-to-point correlation. This is not found with any other correlation level. It is developed using every plasma level and dissolution point collected at different time intervals, so it reflects the complete plasma level curve. As a result, in the case of a *Level A* correlation an in vitro dissolution curve can serve as a surrogate for in vivo performance. A change in manufacturing site, method of manufacture, raw material supplies, minor formulation modifications, and even product strength using the same formulation can be justified without the need for additional BA-BE studies.^{4,5}
2. A truly meaningful quality control procedure that indicates in vivo performance and is predictive of a dosage form's performance is defined for the dosage form.
3. The extremes of the in vitro quality control standards can be justified either by convolution (simulating the plasma level profile from the dissolution curve) or by deconvolution (using the upper and lower confidence interval limits).

LEVEL B

This correlation uses the principles of statistical moment analysis. The mean in vitro dissolution time is compared to either the mean residence time or the mean in vivo dissolution time. As with a *Level A* correlation, *Level B* uses all of the in vitro and in vivo data but is not considered a point-to-point correlation. It does not correlate the actual in vivo plasma profiles but rather a parameter that results from statistical moment analysis of a plasma profile component such as mean residence time. Because a number of different plasma profiles can produce similar mean residence time values, one cannot rely on a *Level B* correlation alone to predict a plasma profile from in vitro dissolution data. In addition, in vitro data from such a correlation cannot be

⁴ FDA Guidance SUPAC-MR: Modified Release Solid Oral Dosage Forms—Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation (1997).

⁵ FDA Guidance Extended-Release Solid Oral Dosage Form—Development, Evaluation, and Application of In Vitro/In Vivo Correlations, "If an IVIVC is developed with the highest strength, waivers for changes made on the highest strength and any lower strengths may be granted if these strengths are compositionally proportional or qualitatively the same, the in vitro dissolution profiles of all the strengths are similar, and all strengths have the same release mechanism."

used to justify values at the extremes of quality control standards.

LEVEL C

This category relates one dissolution time point ($t_{50\%}$, $t_{90\%}$, etc.) to one pharmacokinetic parameter such as AUC, C_{max} , or T_{max} . It represents a single-point correlation and does not reflect the complete shape of the plasma profile, which best defines the performance of modified-release products. Because this type of correlation is not predictive of actual in vivo product performance, generally it is useful only as a guide in formulation development or as a production quality control procedure. Because of its obvious limitations, a *Level C* correlation has limited usefulness in predicting in vivo drug performance and is subject to the same caveats as a *Level B* correlation in its ability to support product and site changes as well as justification of the extreme values in quality control standards. The FDA Guidance "Extended-Release Solid Oral Dosage Forms—Development, Evaluation, and Application of In Vitro/In Vivo Correlations" (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070239.pdf) states that manufacturers can obtain biowaivers based on multiple *Level C* correlations. The guidance shows how manufacturers can achieve this correlation. The FDA also indicates that if such a correlation is achievable, it is likely that the development of a *Level A* correlation is also feasible.

DEVELOPING A CORRELATION

This chapter does not define the only procedures for developing an IVIVC, and any well-designed and scientifically valid approach is acceptable. To assist the pharmaceutical scientist, one possible procedure for developing a *Level A* correlation is described below:

1. In order to perform deconvolution properly, analysts should be familiar with the pharmacokinetics of the drug itself as well as when it is incorporated into a modified-release dosage form. For example, if a drug is known to be fully absorbed but demonstrates saturable first-pass kinetics, it is best to assume 100% bioavailability for purposes of absorption rate calculation. This is based upon the fact that the drug is fully absorbed, but because of liver metabolism, one sees less than if the drug were administered as an immediate-release bolus. If one utilizes the extent of absorption relative to an immediate-release or solution dosage form, the input profiles will not superimpose with that calculated assuming 100% absorption. However, point-to-point correlations most likely will be possible.
2. Different dissolution profiles of a formulation should be obtained as illustrated in *Figure 1*. The formulation should be modified only sufficiently to produce different dissolution profiles so that the formulation has the same excipients in all the lots that will be tested. The formulation modifications used in these batches should be based on factors that would be expected to influence the product's modified-release rate and could occur during normal product manufacture. In vitro drug release is performed on the batches that will be used in the bioavailability study, and the effect of varying the dissolution conditions is investigated. Some of the variables that should be studied are the apparatus (it is preferable to use official dissolution equipment), mixing intensity, and dissolution medium (i.e., pH value, enzymes, surfactants, osmotic pressure, ionic strength, etc.). The dissolution behavior of the dosage form need not be studied under all

of the conditions indicated. The number of conditions investigated depends largely on whether a correlation can be developed with the in vitro results obtained under the more commonly investigated conditions such as apparatus, agitation intensity, or dissolution medium and pH value. Each formulation and every drug represents an individual challenge. The resulting dissolution profiles from the use of different dissolution media are illustrated in *Figures 1* and *2* in which the same formulations were tested in water and an acid buffer.

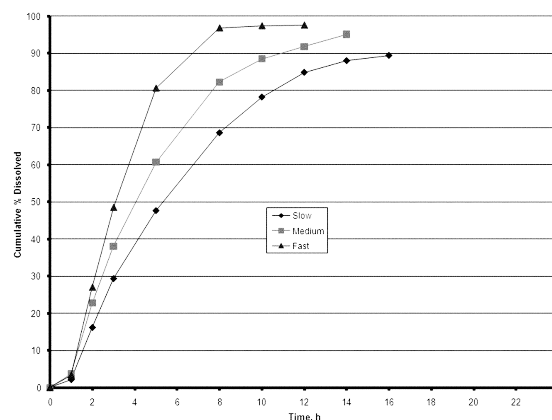


Figure 1. Mean dissolution profiles of three modifications of a new modified-release formulation (USP Apparatus 2, 50 rpm, 0.9 L water, 37°).

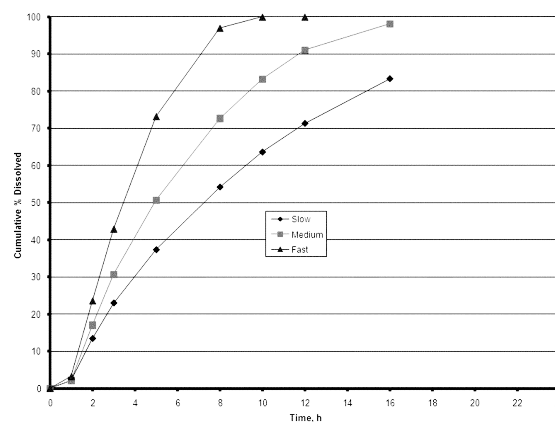


Figure 2. Mean dissolution profiles of a new modified-release formulation (USP Apparatus 2, 50 rpm, 0.9 L, pH 4.5 buffer, 37°).

3. The plasma level or urinary excretion data obtained in the definitive bioavailability study of the modified-release dosage form are treated by a deconvolution procedure. The resulting data may represent the drug input rate of the dosage form. They also represent in vivo dissolution when the rate-controlling step of the dosage form is its dissolution rate (i.e., drug absorption after dissolution is considered to be instantaneous). Any deconvolution procedure (e.g., mass balance or mathematical deconvolution) will produce acceptable results. *Figure 3* illustrates the results of numerical deconvolution of the plasma profiles obtained for the batches in *Figures 1* and *2*.

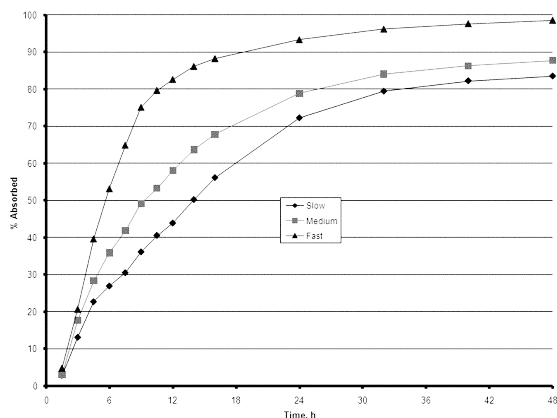


Figure 3. Mean absorption profiles from numerical deconvolution of plasma concentration-time plots.

4. The in vitro dissolution curve is then compared to the drug absorption rate curve. This can be performed by various methods. Simply positioning one curve on the other often can indicate the existence of a correlation. This may then be quantified by defining the equation for each curve and comparing the corresponding constants. The simplest way to demonstrate a correlation is to plot the fraction absorbed in vivo vs. the fraction released in vitro, as illustrated in Figures 4 and 5. With a *Level A* correlation, this relationship is often linear with a slope approaching 1. As illustrated in Figures 4 and 5, a correlation may be curvilinear. The intercept may or may not be zero depending upon whether there is a lag time before the system begins to release drug in vivo, or the absorption rate is not instantaneous, resulting in the presence of some finite quantity of dissolved but unabsorbed drug. In either case, it is a point-to-point or a *Level A* correlation when the least-squares fit of the line approaches a coefficient of determination, R^2 , of 1. For the correlations illustrated in Figures 4 and 5, the IVIVC using the acid buffer dissolution profiles was superior to that obtained from water.

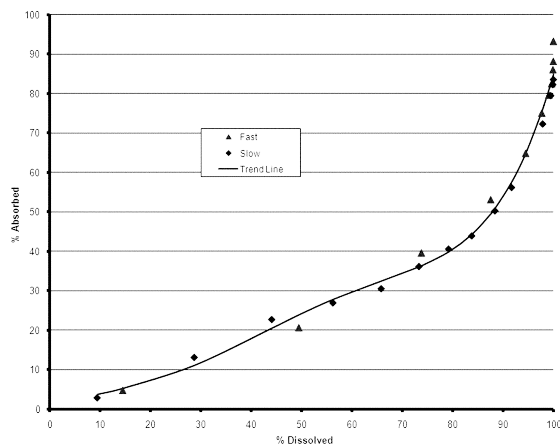


Figure 4. IVIVC attempt: water (using slow and fast formulations).

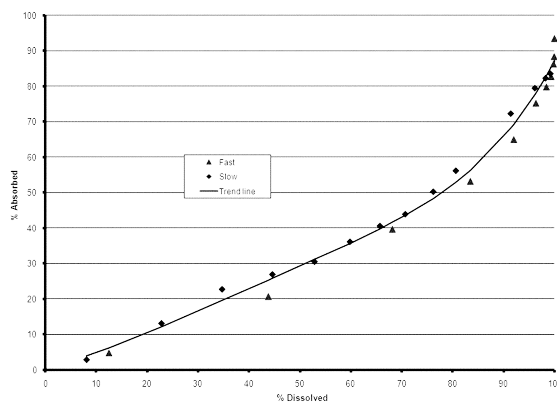


Figure 5. IVIVC attempt: pH 4.5 buffer.

5. If from the studies indicated in the in vitro dissolution evaluation, given above, the modified-release dosage form exhibits dissolution behavior that is independent of the variables studied and a *Level A* correlation is demonstrated when the in vitro dissolution curve is compared to the drug input rate curve, then it is likely that the correlation is general and can be extrapolated within a reasonable range for that formulation of the active drug substance. If the dosage form exhibits dissolution behavior that varies with the in vitro conditions, analysts must determine which set of dissolution conditions best correlates with in vivo performance. One can then establish whether the correlation is real or an artifact. This is achieved by preparing at least two formulations that have significantly different in vitro behavior. One should demonstrate a more rapid release and the other a slower release than the clinical bioavailability lot (biobatch). A pilot BA-BE study should be performed with these formulations, and the previously established correlation should be demonstrated for both. The formulation modifications of these batches should be based upon formulation factors that would be expected to influence the product's modified-release mechanism, and modification of these formulation factors are expected to influence the dosage form's release rate.
6. Alternatively, the in vivo performance of the biobatch formulation can be simulated based on the correlation developed with these formulations that were used in the BA-BE study. Analysts then can compare the predicted and experimentally determined values, the prediction error. The exercise illustrated in Figures 6 and 7 serves as an internal validation of the *Level A* correlation. An external validation would involve simulating data for a formulation batch that was not included in the *Level A* correlation calculations. Such a validation was performed using the in vivo data from the medium lot of the formulation, and the results are illustrated in Figure 8.

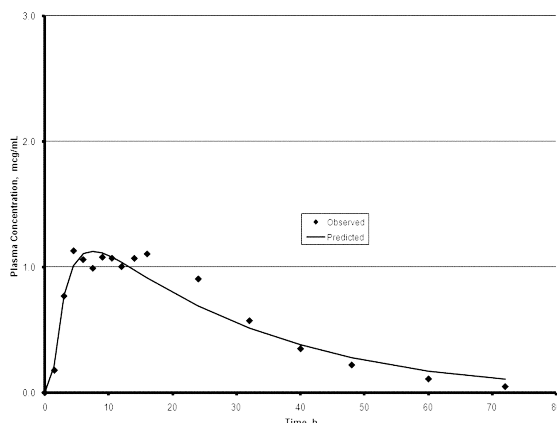


Figure 6. Observed and predicted mean plasma profiles: slow formulation.

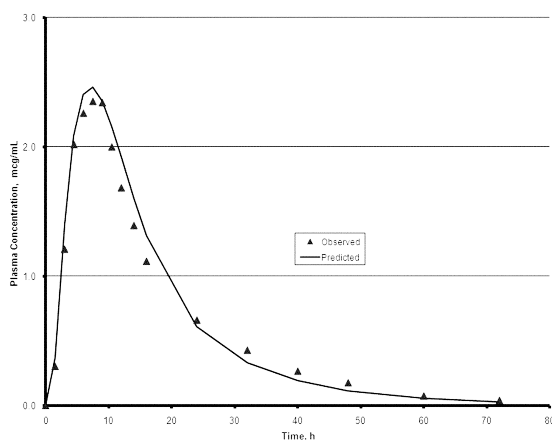


Figure 7. Observed and predicted mean plasma profiles: fast formulation.

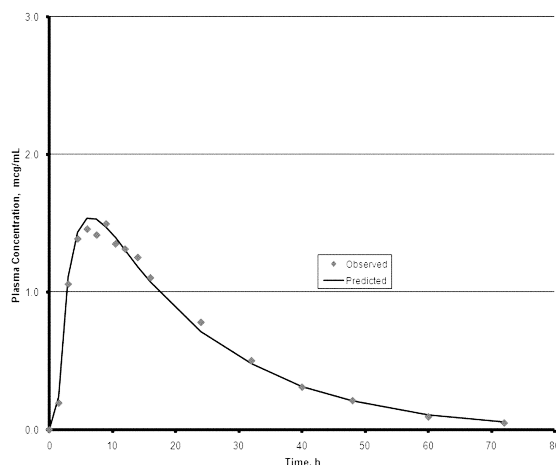


Figure 8. Observed and predicted mean plasma profiles: medium formulation.

- Once a *Level A* correlation is established, in vitro testing can be used to establish dissolution specifications, biowaivers to facilitate SUPAC, and changes in dosage form strength for the same formulation. It is questionable whether such an extrapolation with *Level B* and *C* correlations is possible.

Establishment of Dissolution Specification Ranges

It is relatively easy to establish a multipoint dissolution specification for a modified-release dosage form. The dissolution behavior of the biobatch can be used to define the amount that will be released at each time point. The difficulty arises in the variation that will be allowed around each time point. In the case of a *Level A* correlation, this can be done in two ways, both of which use IVIVC: convolution and deconvolution.

CONVOLUTION

Reasonable upper and lower dissolution values are selected for each time point established from the biobatch. Historically, dissolution specifications have been selected by using the average dissolution of the development batches, with a range of ± 2.5 –3 standard deviations. It is now expected that the average dissolution values be approximately the same as those of the biobatch. The dissolution curves defined by the upper and lower extremes are convoluted to project the anticipated plasma level curves that would result from administration of these formulations to the same patients to whom the biobatch was administered. If the resulting plasma level data fall within the 95% confidence intervals obtained in the definitive BA-BE study, these ranges can be considered acceptable. An alternative acceptance approach that can be used after the therapeutic window for a drug has been defined, is to establish whether the upper and lower limits of the convolution results fall within the therapeutic window, even if they fall outside the confidence interval. If they fall outside the window, a more limited range must be established. This procedure should be continued until the predicted values meet the desired ranges.

DECONVOLUTION

An acceptable set of plasma-level data is established both for a batch of material demonstrating a more rapid release and for one demonstrating a slower release than that of the biobatch. These can be selected by using the extremes of the 95% confidence intervals or ± 1 standard deviation of the mean plasma level. These curves are then deconvoluted, and the resulting input rate curve is used to establish the upper and lower dissolution specifications at each time point. In the case of *Level B* and *C* correlations, batches of product must be made at the proposed upper and lower limits of the dissolution range, and it must be demonstrated that these batches are acceptable by a BA-BE study.

Immediate-Release Dosage Forms

GENERAL CONSIDERATIONS

Because the mechanisms for drug release from modified-release dosage forms are more complex and variable than those associated with immediate-release dosage forms, one would anticipate that an IVIVC would be easier to develop with the latter formulations. Unfortunately, most of the correlation efforts to date with immediate-release dosage forms have been based on the correlation *Level C* approach, although there also have been efforts employing statistical moment theory (*Level B*). Although it is conceivable that the same *Level A* correlation approach can be used with immediate-release dosage forms, until data have been gathered to support this concept, *Level B* and *Level C* are the best

approaches that can be recommended with these dosage forms.

<1090> ASSESSMENT OF DRUG PRODUCT PERFORMANCE—BIOAVAILABILITY, BIOEQUIVALENCE, AND DISSOLUTION

BACKGROUND

This chapter provides recommendations for the in vivo and in vitro assessment of drug product performance. The chapter is intended as a guide to scientists and clinicians seeking to evaluate drug product performance by surrogate procedures correlative and/or antecedent to clinical trials in humans. *USP–NF* provides quality standards for drug substances, excipients, and finished preparations. A *USP–NF* monograph for an official substance or preparation includes the article's definition; packaging, storage, and other requirements; and a specification. The specification consists of a series of universal tests (description, identification, impurities, and assay) and specific tests, one or more analytical procedures for each test, and acceptance criteria. Quality standards are important attributes that must be built into the drug product. Meeting *USP–NF* standards is accepted globally as assurance of high quality and is part of the requirements necessary for approval of a bioequivalent (BE), interchangeable multisource drug product. Multisource drug products must meet certain in vivo and/or in vitro performance standards to be considered therapeutically equivalent and interchangeable. Regulatory approval for interchangeable multisource products may differ somewhat in each country (see the forthcoming chapter *Essentials for Drug Product Selection* <1096> for further discussion). Drug product performance may be defined as the release of the active pharmaceutical ingredient (API) from the drug product dosage form, leading to systemic availability of the API necessary for achieving a desired therapeutic response. This chapter discusses in vivo and in vitro approaches to determining drug product performance. The focus of the chapter is primarily on the performance of solid oral drug products.

The chapter references a Food and Drug Administration (FDA) guidance, *Guidance for Industry—Bioavailability and Bioequivalence Studies for Orally Administered Drug Products—General Considerations* (2003) (<http://www.fda.gov/>; search by document title) and a World Health Organization (WHO) document titled *Annex 7 Multisource (Generic) Pharmaceutical Products: Guidelines on Registration Requirements to Establish Interchangeability* (2006) (<http://who.int/en/>; search by document title). FDA guidances are used in the United States; and WHO, FDA, and national/regional guidelines may be used by national/regional drug regulatory authorities. Following approval, control of the quality of a drug product can be achieved in part by the private and/or public specification, which can include a performance test. USP provides the general chapters *Disintegration* <701>, *Dissolution* <711>, *Drug Release* <724>, *In Vitro and In Vivo Evaluation of Dosage Forms* <1088>, and *The Dissolution Procedure: Development and Validation* <1092>, which describe these tests and procedures.

This chapter provides general information about the conduct of bioequivalence (BE) studies as a surrogate measure of in vivo drug product performance and dissolution profile comparisons as a measure of in vitro drug product performance. The chapter also discusses conditions when an in vivo BE requirement may be waived (biowaiver) for certain drug products and shows how the Biopharmaceutics Classification System (BCS) can be used as a predictor of a drug product's performance. An appendix to this chapter defines key scientific terminology and provides a comparison between FDA and WHO in drug product performance assessment.

BIOAVAILABILITY, BIOEQUIVALENCE, AND DISSOLUTION

Bioavailability (BA) studies focus on determining the process and time frame by which a drug is released from the oral dosage form and moves to the site of action [see FDA *Guidance for Industry—Bioavailability and Bioequivalence Studies for Orally Administered Drug Products—General Considerations* (2003)]. BA is an indirect or surrogate measure of the rate and extent to which the API or active moiety is absorbed from a drug product and becomes available at its target sites of action. BA data provide an estimate of systemic drug exposure, including fraction of drug absorbed. For drug products that are not intended to be absorbed into the bloodstream, availability may be assessed by measurements that reflect the rate and extent to which the active ingredient or active moiety becomes available at the sites of action. Drug products are considered BE if a test drug product does not show a significant difference in rate and extent of absorption by comparison with a designated reference drug when administered at the same molar dose of the same active moiety in the same dosage form under similar experimental conditions in either a single dose or in multiple doses.

BA and BE generally can be obtained by serially measuring drug and/or metabolite concentrations in the systemic circulation over a prescribed period. BE studies can use other approaches when systemic drug concentrations cannot be measured or are not appropriate. For these cases, more indirect approaches to BE determination include acute pharmacodynamic endpoints, clinical endpoints, and in vitro studies that typically involve comparisons of the dissolution profiles of test and reference drug products.

BA and BE information are important in regulatory submissions. BA information broadly addresses the absorption, distribution, metabolism, and excretion of the API. For an innovator product, BE studies establish the performance of the product intended for marketing by comparing the bioavailability of the product as developed for marketing approval to the clinical trial material, the drug product used in safety/efficacy trials. For the development and regulatory approval of a generic drug product, the test drug product must be BE to the reference listed drug (RLD) product (usually the brand or innovator drug product that is designated by the applicable regulatory authority).

The ICH document titled *Guidance on Q6A Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances* (2000) (<http://www.fda.gov/>; search by document title) provides approaches for setting acceptance criteria for drug product performance. This approach relies on dissolution or disintegration based on clinically acceptable batches, as does FDA's. BE studies focus on the performance of the drug product and usually involve comparisons of two drug products: the test (T) and reference (R) or comparator product. The required studies and the determination of BE are the province of regulatory agencies. In the United States, R is termed the *reference listed drug* (RLD) and is so noted in FDA's *Approved Drug Products with Therapeutic Equivalence Ratings* [Orange Book (2008) (<http://www.fda.gov/cder/ob/>)]. To assist countries and regions where the R product may

not always be readily identifiable, WHO has prepared a document titled *Annex 11 Guidance on the Selection of Comparator Pharmaceutical Products for Equivalence Assessment of Interchangeable Multisource (Generic) Products* (2005) (<http://www.who.int/en/search-by-document-title>). In the WHO document, R is termed the *comparator pharmaceutical product* (CPP). When a country or region has a clearly defined set of CPPs, the task becomes one of requiring that a manufacturer demonstrate, to the satisfaction of its regulatory authority, that its multisource product is pharmaceutically equivalent and BE to the corresponding CPP.

BIOEQUIVALENCE

An interchangeable multisource (generic) product must be pharmaceutically equivalent (PE). The WHO document allows pharmaceutical alternatives to be considered therapeutically equivalent and interchangeable if they are BE. Further, generic products must be shown to be BE in order to be considered therapeutically equivalent (TE) to the R product (CPP). For the product to be considered PE, it must have the same active ingredient, same strength, same dosage form, same route of administration, and same labeling as the comparator product. Several methods exist to assess and document BE. These include the following:

1. *Comparative pharmacokinetic studies in humans.* In these studies, the active drug and/or its metabolite(s) are measured as a function of time in accessible biological fluid such as blood, plasma, serum, or urine to obtain pharmacokinetic measures such as area under the plasma drug concentration vs. time curve (AUC) and maximum concentration (C_{max}) that are reflective of systemic exposure. BE studies are designed to compare the in vivo performance of a generic product with an R product. Generally the design is a two-period, two-sequence, single-dose, crossover randomized one carried out in 18 to 36 subjects. The number of subjects should be statistically justified and not less than 12. During the study, blood samples are collected at sufficient intervals for assessing C_{max} , AUC, and other parameters. Blood samples are analyzed using appropriately validated bioanalytical methodology with standard pharmacokinetic measures and statistical approaches. The statistical method for testing pharmacokinetic BE is based on the determination of the 90% confidence interval around the geometric mean ratio of the log-transformed population means (generic/R) for AUC and C_{max} by carrying out two one-sided tests at the 5% level of significance.
2. *Other options.* In addition, comparative pharmacodynamic studies in humans and comparative clinical trials can be used to document or supplement BE assessment. Beyond these clinical studies, in vitro dissolution based on the BCS can ensure BE between T and R products. In vivo documentation of equivalence is especially important for the following: narrow therapeutic range drugs; documented evidence of BE problems; modified-release pharmaceutical products designed to act by systemic absorption; and fixed-dose combination products with systemic action when at least one of the APIs requires an in vivo study.

Immediate-Release Drug Products

Single-dose, crossover BE studies are carried out at the highest dose comparing T and R products under fasting conditions. A parallel study design can be used for drugs that have a very long elimination half-life ($t_{1/2}$). Sampling truncation at 72 hours may be allowable by regulatory agencies. Lower strength(s) of the dosage form can be given a biowaiver based on dosage form proportionality and dissolution profile similarity. Food-effect studies are required

if there is an indication in the labeling that concomitant administration of food may diminish, increase, or not influence the BA of the drug product.

Modified-Release Drug Products

BE studies for extended-release dosage forms are carried out as single-dose, crossover studies under fasting and fed conditions at the highest dose to compare T and R products. A single-dose study is more sensitive than multiple-dose, steady-state studies in assessing in vivo drug product performance, particularly with regard to the phenomenon of dose dumping, i.e., the rapid and unintended premature release of the active ingredient from an extended-release product into the bloodstream. Lower strengths of an extended-release dosage form may not require an in vivo study based on use of the same drug-releasing mechanism, dosage form proportionality, and similar dissolution profile.

Orally Administered Drug Products, Not for Systemic Effect

Some oral drug products are intended for local activity. Mesalamine and cholestyramine are examples of drugs that are intended for local activity. For these types of drugs, systemic absorption from the gastrointestinal tract is minimal; thus a comparative clinical trial is required while a systemic drug exposure profile also may be required. In some cases, in vitro studies may be appropriate; such as including comparison of cholestyramine binding to bile salts.

Bioequivalence Studies

Objective—The objective of a BE study is to measure and compare formulation performance between two or more pharmaceutically equivalent drug products. Drug availability from T and R products should not be statistically different when the drug is administered to patients or subjects at the same molar dose under similar experimental conditions.

Design—The design of a BE study depends on the objectives of the study, the ability to analyze the drug (and metabolites) in biological fluids, the pharmacodynamics of the drug substance, the route of drug administration, and the nature of the drug and drug product. Pharmacokinetic parameters, pharmacodynamic parameters, clinical observations, and/or in vitro studies may be used to determine drug BA from a drug product.

Some possible BE study designs include the following:

1. Single-dose, two-way crossover study under fasted conditions
2. Single-dose, two-way crossover study under fed conditions
3. Single-dose, parallel study under fasted conditions
4. Single-dose, replicate design
5. Single-dose, partial replicate design
6. Multiple-dose, two-way crossover study, fasted conditions
7. Pharmacodynamic or clinical endpoint study
8. In vitro dissolution profile comparisons

The standard BE study is a crossover design (e.g., Latin square crossover design) in which each subject receives the test drug product and the reference product on separate occasions. Studies are usually evaluated by a single-dose, two-period, two-treatment, two-sequence, open-label, randomized crossover design comparing equal doses of the test and reference products in fasted or fed adult healthy subjects. A multiple-dose study may be required for some extended-release drug products. A washout period is scheduled between the two periods to allow the subjects to completely eliminate the drug absorbed from the first dose before administration of the second dose. If the predose concentration is $\leq 5\%$ of the C_{max} value in that subject, the

subject's data without any adjustments can be included in all pharmacokinetic measurements and calculations. Samples of an accessible biologic fluid such as blood characterize the drug concentration vs. time profile. During the fasting study subjects are fasted at least 10 hours. A pre-dose (0 time) blood sample is taken. The drug product is given with 240 mL (8 fluid ounces) of water. No food is allowed for at least 4 hours post-dose. Blood sampling is performed periodically after dose administration according to protocol. A food intervention or food effect study is conducted with standard meal conditions that are expected to provide the greatest effects on gastrointestinal physiology so that systemic drug availability is maximally affected. In addition, the high lipid content of the meal may affect the rate of drug release from the product, *in situ*. A high-fat (approximately 50% of total caloric content of the meal) and high-calorie (approximately 800 to 1000 calories) meal is recommended as a test meal for food-effect BA and fed BE studies. This test meal should derive approximately 150, 250, and 500–600 calories from protein, carbohydrate, and fat, respectively. The drug product is given with 240 mL (8 fluid ounces) of water after ingestion of the standard meal. Subjects should consume identical meals at the same time during a testing period.

Analysis of Samples—Samples, usually plasma, are analyzed for the active drug and, on occasion, active metabolite concentrations by a validated bioanalytical method.

Pharmacokinetic Parameters—Pharmacokinetic parameters are obtained from the resulting concentration-time curves. Two major pharmacokinetic parameters are used to assess the rate and extent of systemic drug absorption. AUC reflects the extent of drug absorption, and the peak drug concentration (C_{max}) reflects the rate of drug absorption. Other pharmacokinetic parameters may include the time to peak drug concentration (T_{max}), the elimination rate constant (k), elimination half-life ($t_{1/2}$), lag time (T_{lag}), and others.

Statistical Analysis

Pharmacokinetic parameters are analyzed statistically to determine whether the T and R products yield comparable values. Because BE studies may use small sample sizes, log transformation of the data allows the frequency distribution of the data to be more normalized so that parametric statistical analyses may be performed (FDA, *Guidance for Industry: Statistical Approaches to Establishing Bioequivalence* (2001) (<http://www.fda.gov/>; search by document title).

Parametric (normal-theory) general linear model procedures are recommended for the analysis of pharmacokinetic data derived from *in vivo* BE studies. An analysis of variance (ANOVA) should be performed on the pharmacokinetic parameters AUC and C_{max} using appropriate statistical programs and models. For example, for a conventional two-treatment, two-period, two-sequence (2×2) randomized crossover study design, the statistical model often includes factors accounting for the following sources of variation:

- Sequence (sometimes called Group or Order)
- Subjects, nested in sequences
- Period (or Phase)
- Treatment (sometimes called Drug or Formulation)

The sequence effect should be tested using the [subject (sequence)] mean square from the ANOVA as an error term. All other main effects should be tested against the residual error (error mean square) from the ANOVA. The least-squares means (LSMEANS) statement should be used to calculate least-squares means for treatments. Estimates should be obtained for the adjusted differences between treatment means and the standard error associated with these differences.

The statistical assumptions underlying the ANOVA are as follows:

- Randomization of samples
- Homogeneity of variances
- Additivity (linearity) of the statistical model

- Independence and normality of residuals
In BE studies, these assumptions can be interpreted as follows:
 - The subjects chosen for the study should be randomly assigned to the sequences of the study.
 - The variances associated with the two treatments, as well as between the sequence groups, should be equal or at least comparable.
 - The main effects of the statistical model, such as subject, sequence, period, and treatment effect for a standard 2×2 crossover study, should be additive. There should be no interactions between these effects.
 - The residuals of the model should be independently and normally distributed.

If these assumptions are not met, additional steps should be taken prior to the ANOVA, including data transformation to improve the fit of the assumptions or use of a nonparametric statistical test in place of ANOVA. However, the normality and constant variance assumptions in the ANOVA model are known to be relatively robust (i.e., a small or moderate departure from each, or both of these assumptions, will not have a significant effect on the final result). The rationale for log transformation is provided in FDA's *Guidance Statistical Approaches to Establishing Bioequivalence*. Justification should be provided if untransformed data is to be used.

The Two One-Sided Tests Procedure—A testing procedure termed the two one-sided tests procedure is used to determine the comparability of geometric mean values for pharmacokinetic parameters measured after administration of the test and reference products.¹ The two one-sided tests procedure decides whether T is not importantly less than R and whether R is not importantly less than T. Most often, 20% defines an important difference. The statistical procedure involves the calculation of a confidence interval for the ratio (or difference) between T and R pharmacokinetic variable averages. The limits of the observed confidence interval must fall within a predetermined range for the ratio (or difference) of the product averages. Point estimate mean ratios (T/R) derived from the log-transformed AUC and C_{max} data must be between 80% and 125%. Because data are log transformed, $T/R = 80/100 = 80\%$ and $R/T = 100/80 = 125\%$. In addition, the 90% confidence intervals for the geometric mean ratios (T/R) for AUC and C_{max} must be between 80% and 125%. The regulatory requirements for the range of 90% confidence intervals for C_{max} may be different in countries outside the United States.

Bio-Inequivalence—The failure to demonstrate BE may be due to a performance failure of the T product or to an inadequate study design. The failure to demonstrate BE due to an inadequate study design can be due to improper sampling in which (1) the sampling time for C_{max} was not properly obtained or (2) the number of samples taken did not adequately describe the plasma drug concentration vs. time profile. Often with highly variable drugs (e.g., %CV >30%), too few subjects were used in the study, and therefore the study was not powered adequately.

Presentation of Data. The drug concentration in biological fluid at each sampling time point should be furnished untransformed for all the subjects who participated in the study. The derived pharmacokinetic parameters also should be furnished untransformed. The mean, the standard deviation, and the coefficient of variation (CV) for each variable should be computed and tabulated in the final report.

To facilitate BE comparisons, pharmacokinetic parameters for each individual should be displayed in parallel for the formulations tested. In particular, for AUC and C_{max} , the difference ($T - R$), the ratio (T/R), and the log of ratio ($\log T/R$ or $\ln T/R$) between the T and R values should be tabulated side by side for all the subjects. For each subject, the sum-

¹Schirmann DJ. A comparison of the two one-sided tests procedure and the power approach for assessing the equivalence of average bioavailability. *J. Pharmacokinetics and Biopharmaceutics*, 1987;15:657–680.

mary tables should indicate in which sequence (T, R or R, T) the subject received the product. Histograms showing the frequency distribution of the difference and ln ratio (or log ratio) for the major pharmacokinetic parameters (AUC and C_{max}) are useful in the submission.

In addition to the arithmetic mean for the T and R products, the geometric means (antilog of the means of the logs), means of the logs, and standard deviations of the logs should be calculated for AUC and C_{max} . All means, including arithmetic mean, geometric mean, and means of the logs, as well as standard deviations and CVs, should be included in the report.

DISSOLUTION AND IN VITRO PRODUCT PERFORMANCE

As noted for an official preparation, *USP* monographs provide a public specification that includes a list of tests, references to analytical procedures, and acceptance criteria. Most solid oral dosage forms, including oral suspensions, require a dissolution or a drug release test. Drug dissolution and drug release testing are described in *USP* general chapters *Dissolution* (711) and *Drug Release* (724). These public specifications are used for quality control tests and for market approval. The *USP* dissolution test in the monograph is related to BA and BE only when closely allied with a sound regulatory determination. Without this link it should be regarded solely as a quality control test for batch release. FDA Guidances are (1) *Guidance for Industry—Dissolution Testing of Immediate Release Solid Oral Dosage Forms* (1977) (<http://www.fda.gov/>; search by document title) and (2) *Guidance for Industry—Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlation* (1977) (<http://www.fda.gov/>; search by document title).

Dissolution and In Vitro Bioavailability

Drug dissolution and release tests are very useful during drug product development in identifying critical manufacturing attributes such as the impact of ingredient properties and the impact of the manufacturing process on drug product performance. During product development, optimum dissolution conditions need to be developed to discriminate drug product formulations and changes in manufacturing processes. After the finished dosage form is approved for marketing, drug dissolution and release tests are useful in predicting possible changes in performance due to scale-up and postapproval changes (SUPAC). See the following FDA guidances:

Guidance for Industry—Immediate Release Solid Oral Dosage Forms, Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, In Vitro Dissolution Testing, and In Vivo Bioequivalence Documentation (1995) (<http://www.fda.gov/>; search by document title) and *Guidance for Industry—SUPAC-MR: Modified-Release Solid Oral Dosage Forms: Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Dissolution Testing and In Vivo Bioequivalence Documentation* (1977) (<http://www.fda.gov/>; search by document title).

For some oral drug products, in vitro drug dissolution can be related to in vivo performance, such as bioavailability and/or systemic drug exposure. *USP* general information chapter *In Vitro and In Vivo Evaluation of Dosage Forms* (1088) describes various approaches to in vitro–in vivo correlation (IVIVC).

Dissolution and In Vitro Equivalence

The dissolution test is a powerful in vitro physiochemical test that measures drug product quality and performance for a variety of dosage forms, such as solid oral dosage

forms, transdermal dosage forms, suspensions, and certain semisolid dosage forms. The *USP* tests for finished dosage forms can be divided into two types: (1) drug product quality tests and (2) drug product performance tests. Product quality tests are intended to assess attributes such as assay and content uniformity; product performance tests are designed to assess product performance and in many cases relate to dissolution. For details regarding the performance of a dissolution test, see *USP* general chapters (711), (724), (1088), and (1092).

The in vitro dissolution test was initially developed as a quality control tool to ensure drug product quality and batch-to-batch consistency. The test procedures for conducting dissolution tests are described in *USP* general chapters (711) and (724). The development of the BCS brings new understanding and power to the dissolution test. The BCS classifies the drug substance according to the solubility and the permeability of the drug through a biomembrane such as the intestinal mucosal cells. The dissolution rate of the drug from the dosage form is important in substantiating biowaivers based on the BCS.

Dissolution Profile Comparisons

In vitro drug dissolution and release testing can be related to in vivo drug performance, such as BA. The comparisons of dissolution profiles are gaining importance as a means of documenting comparative BA studies—that is, BE. A biowaiver is the replacement or waivers of in vivo BE studies by an in vitro test.

A model independent mathematical approach is used to compare the dissolution profile of two products: (1) to compare the dissolution profile between the T (generic, multisource) product and R (comparator) product in biowaiver considerations; (2) to compare the dissolution profile between the two strengths of products from a given manufacturer; and (3) for SUPAC after the product is approved. For comparing the dissolution profile, the similarity factor f_2 should be computed using the equation

$$f_2 = 50 \cdot \log \left\{ \left[1 + \left(\frac{1}{n} \right) \sum_{t=1}^n (R_t - T_t)^2 \right]^{-0.5} \cdot 100 \right\}$$

where R_t and T_t are the cumulative percentage of the drug dissolved at each of the selected n time points of the reference and test product, respectively. An f_2 value of 50 or greater (50 to 100) ensures dissolution profile similarity and the sameness or equivalence of the two curves, and thus the performance of the two products. At a minimum, three points, no more than one point exceeding 85%, should be used for similarity profile comparison. For products that dissolve very rapidly ($\geq 85\%$ dissolution in 15 minutes) a profile comparison is not necessary.

BIOWAIVER

The term *biowaiver* is applied to a regulatory approval process when the application (dossier) is approved on the basis of evidence of equivalence other than an in vivo BE test. For solid oral dosage forms, the evidence of equivalence is determined on the basis of an in vitro dissolution profile comparison between the multisource and the comparator product.

Biowaiver Based on the Pharmaceutical Dosage Form

A drug product's in vivo comparative BA or BE study requirement may be waived if the products compared contain the same API(s) in the same concentration, contain the

same excipients in comparable concentrations, and meet one of the following criteria:

- Aqueous solutions to be administered parenterally
- Solutions for oral use that do not contain an excipient that is known or is suspected to affect gastro-intestinal transit or absorption of the active substance
- Gases
- Powders for reconstitution as a solution
- Otic or ophthalmic products prepared as aqueous solutions
- Topical products prepared as aqueous solutions
- Inhalation products or nasal sprays tested to be administered with essentially the same device. Special in vitro performance testing should be required to document comparable device performance.

Biowaiver Based on Dosage Form Proportionality

When a single-dose fasting BE study is conducted on the designated (usually highest) strength of the drug product, the requirement for the conduct of additional in vivo BE studies on the lower strengths of the same product can be waived, provided that the lower strength (1) is in the same dosage form; (2) is proportionally similar in its active and inactive ingredients; (3) has the same drug release mechanism (for extended-release products); (4) meets an appropriate in vitro dissolution profile comparison criterion ($f_2 \geq 50$); and (5) both lower and higher strengths are within the linear pharmacokinetic range.

Biowaiver Based on the Biopharmaceutics Classification System

The BCS is based on aqueous solubility and intestinal permeability of the API. When the properties of the API are evaluated in conjunction with the dissolution of the pharmaceutical dosage form, the BCS takes into account three major factors that govern the rate and extent of drug absorption from immediate-release dosage forms. On the basis of the solubility and permeability of the dosage form, the drug substance is placed in one of four classes:

- Class 1: high solubility, high permeability
- Class 2: low solubility, high permeability
- Class 3: high solubility, low permeability
- Class 4: low solubility, low permeability

Use of the BCS has become a means of documenting BE without the conduct of an in vivo study; see the FDA Guidance *Guidance for Industry: Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System* (2000) (<http://www.fda.gov/>; search by document title).

The in vitro dissolution studies are generally carried out by basket method at 100 rpm or by paddle method at 50 rpm (FDA Guidance cited immediately above) or 75 rpm [WHO Guidance, *Annex 7 Multisource (Generic) Pharmaceutical Products: Guidelines on Registration Requirements to Establish Interchangeability* (2006)] (<http://www.who.int/en/>; search by document title) in 900 mL of medium at pH 1.2, 4.5, and 6.8. On the basis of dissolution rate, the pharmaceutical dosage forms are classified as (1) very rapidly dissolving, if 85% or greater of the dosage form dissolves in 15 minutes or less; (2) rapidly dissolving, if 85% or greater of the dosage form dissolves in 30 minutes; or (3) slowly dissolving, if the dosage form takes more than 30 minutes for 85% of drug dissolution.

For biowaiver, the dissolution tests should be carried out for both generic and reference product under the same test conditions. For the generic product to be eligible for bi-

owaiver, the reference product should belong to the same BCS class and should meet dissolution profile comparison criteria. Based on BCS classification and dissolution profile comparison, biowaiver can be considered by regulatory authorities provided the dissolution profile similarity criteria provided in the next sections are met.

CLASS 1 DRUG PRODUCTS (ALLOWED IN WHO AND FDA APPROACHES)

Dosage forms of drug substances that are highly soluble, highly permeable, and rapidly dissolving are eligible for biowaivers under the following conditions:

1. 85% or more of the dosage form dissolves in 30 minutes or less and the dissolution profile of the generic product is similar to that of the reference product in pH 1.2, 4.5, and 6.8 buffer, using the basket method at 100 rpm or the paddle method at 50 rpm (FDA) or 75 rpm (WHO), and meets the criterion of dissolution profile similarity, $f_2 \geq 50$.
2. If both the reference and the generic dosage forms are very rapidly dissolving (i.e., 85% dissolution in 15 minutes or less in all three media under the above test conditions), then profile determination is not necessary.

CLASS 2 DRUG PRODUCTS (WHO APPROACH)

Dosage forms of drug substances with high solubility only in pH 6.8 and high permeability (low solubility by definition, BCS Class 2) are eligible for biowaivers, provided that:

1. The dosage form is rapidly dissolving (85% or more in 30 minutes or less) in pH 6.8 buffer.
2. The generic product exhibits dissolution profiles similar to those of the comparator product in buffers at pH 1.2, 4.5, and 6.8.

CLASS 3 DRUG PRODUCTS (WHO APPROACH)

Dosage forms of drug substances that are highly soluble and have low permeability are eligible for biowaivers under the following conditions:

1. Both the reference and the generic dosage forms are very rapidly dissolving (85% dissolution in 15 minutes or less in all three media under the test conditions given above), and they do not contain any excipients and/or inactive substances that are known to alter gastrointestinal motility and/or permeability or influence drug absorption.
2. Firms should show that the quantity of excipients used is consistent with the intended use. When new excipients and/or atypically large amounts of commonly used excipients are included in the dosage form, additional information documenting the absence of any significant impact on bioavailability of the drug is required.

DISSOLUTION AS A QUALITY CONTROL TEST AND A BE TEST

There is a clear difference between dissolution as a quality control test and dissolution as an in vitro equivalence (BE) test. For immediate-release dosage forms, the quality control test involves a single-point dissolution test in only one medium (generally a compendial test). On the other hand, the in vitro equivalence test (BE test) involves dissolution profile comparison in pH 1.2, 4.5, and 6.8 between the T product and the R product.

APPENDIX

Comparison of FDA and WHO Definitions

Term	FDA	WHO
<i>Pharmaceutical Equivalents</i>	Drug products are considered pharmaceutical equivalents if they contain the same active ingredient(s), are of the same dosage form, have the same route of administration, and are identical in strength or concentration. Pharmaceutically equivalent drug products are formulated to contain the same amount of active ingredient in the same dosage form and to meet the same or compendial or other applicable standards (strength, quality, purity, and identity); but they may differ in characteristics such as shape, scoring configuration, release mechanisms, packaging, excipients, expiration time, and, within certain limits, labeling.	Products are pharmaceutical equivalents if they contain the same molar amount of the same API(s) in the same dosage form; if they meet comparable standards; and if they are intended to be administered by the same route. Pharmaceutical equivalence does not necessarily imply therapeutic equivalence, because differences in the excipients and/or the manufacturing process and some other variables can lead to differences in product performance.
<i>Pharmaceutical Alternatives</i>	Drug products are considered pharmaceutical alternatives if they contain the same therapeutic moiety but are different salts, esters, or complexes of that moiety or are different dosage forms or strengths.	Products are pharmaceutical alternative(s) if they contain the same molar amount of the same active pharmaceutical moiety or moieties but differ in dosage form (e.g., tablets vs. capsules) and/or chemical form (e.g., different salts, different esters). Pharmaceutical alternatives deliver the same active moiety by the same route of administration but are otherwise not pharmaceutically equivalent. They may or may not be BE or TE with the comparator product.
<i>Therapeutic Equivalents</i>	Drug products are considered to be therapeutic equivalents only if they are pharmaceutical equivalents and if they can be expected to have the same clinical effect and safety profile when administered to patients under the conditions specified in the labeling.	Two pharmaceutical products are considered to be therapeutically equivalent if they are pharmaceutically equivalent or pharmaceutical alternatives and after administration in the same molar dose, their effects, with respect to both efficacy and safety, are essentially the same when administered to patients by the same route under the conditions specified in the labeling.
<i>Bioavailability (BA)</i>	This term means the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action.	The rate and extent to which the active pharmaceutical ingredient or active moiety is absorbed from a pharmaceutical dosage form and becomes available [at the site(s) of action] in the general circulation.
<i>Bioequivalent Drug Products (BE)</i>	This term describes pharmaceutical equivalent or pharmaceutical alternative products that display comparable BA when studied under similar experimental conditions.	Two pharmaceutical products are BE if they are pharmaceutically equivalent or pharmaceutical alternatives and their BA, in terms of peak concentration (C_{max}), time to peak concentration (T_{max}), and total exposure (AUC) after administration of the same molar dose under the same conditions, are similar to such a degree that their effects can be expected to be essentially the same.
<i>RLD (Reference Product) or Comparator Product</i>	An RLD [21 CFR 314.94(a)(3)] means the listed drug identified by FDA as the drug product upon which an applicant relies in seeking approval of its ANDA.	The comparator product is a pharmaceutical product with which the multisource product is intended to be interchangeable in clinical practice. The comparator product normally will be the innovator product for which efficacy, safety, and quality have been established. The selection of the comparator product usually is made at the national level by the drug regulatory authority.
<i>Generic Product</i>	A generic product is a product that is therapeutically equivalent to the RLD and is intended to be interchangeable with the innovator product.	
<i>Multisource Pharmaceutical Products</i>		Pharmaceutically equivalent or pharmaceutically alternative products that may or may not be therapeutically equivalent. Multisource pharmaceutical products that are therapeutically equivalent are interchangeable.
<i>Interchangeable Pharmaceutical Product</i>		An interchangeable pharmaceutical product is one that is therapeutically equivalent to a comparator product and can be interchanged with the comparator in clinical practice.

〈1091〉 LABELING OF INACTIVE INGREDIENTS

This informational chapter provides guidelines for labeling of inactive ingredients present in dosage forms.

Within the past few years a number of trade associations representing pharmaceutical manufacturers have adopted voluntary guidelines for the disclosure and labeling of inactive ingredients. This is helpful to individuals who are sensitive to particular substances and who wish to identify the presence or confirm the absence of such substances in drug products. Because of the actions of these associations, the labeling of therapeutically inactive ingredients currently is deemed to constitute good pharmaceutical practice.

Although the manufacturers represented by these associations produce most of the products sold in this country, not all manufacturers, repackagers, or labelers here or abroad are members of these associations. Further, there are some differences in association guidelines. The guidelines presented here are designed to help promote consistency in labeling.

In accordance with good pharmaceutical practice, all dosage forms [NOTE—for requirements on parenteral and topical preparations, see the *General Notices*] should be labeled to state the identity of all added substances (therapeutically inactive ingredients) present therein, including colors, except that flavors and fragrances may be listed by the general term “flavor” or “fragrance.” Such listing should be in alphabetical order by name and be distinguished from the identification statement of the active ingredient(s).

The name of an inactive ingredient should be taken from the current edition of one of the following reference works (in the following order of precedence): (1) the *United States Pharmacopeia* or the *National Formulary*; (2) *USAN and the USP Dictionary of Drug Names*; (3) *CTFA Cosmetic Ingredient Dictionary*; (4) *Food Chemicals Codex*. An ingredient not listed in any of the aforementioned reference works should be identified by its common or usual name (the name generally recognized by consumers or health-care professionals) or, if no common or usual name is available, by its chemical or other technical name.

An ingredient that may be, but not always is, present in a product should be qualified by words such as “or” or “may also contain.”

The name of an ingredient whose identity is a trade secret may be omitted from the list if the list states “and other ingredients.” For the purposes of this guideline, an ingredient is considered to be a trade secret only if its presence confers a significant competitive advantage upon its manufacturer and if its identity cannot be ascertained by the use of modern analytical technology.

An incidental trace ingredient having no functional or technical effect on the product need not be listed unless it has been demonstrated to cause sensitivity reactions or allergic responses.

Inactive ingredients should be listed on the label of a container of a product intended for sale without prescription, except that in the case of a container that is too small, such

information may be contained in other labeling on or within the package.

〈1092〉 THE DISSOLUTION PROCEDURE: DEVELOPMENT AND VALIDATION

The USP dissolution procedure is a performance test applicable to many dosage forms. It is one test in a series of tests that constitute the dosage form’s public specification (tests, procedures for the tests, acceptance criteria). To satisfy the performance test, USP provides the general test chapters *Disintegration* 〈701〉, *Dissolution* 〈711〉, and *Drug Release* 〈724〉. These chapters provide information about conditions of the procedure. For dissolution, these include information about (1) medium, (2) apparatus/agitation rate, (3) study design, (4) assay, and (5) acceptance criteria. Overall the dissolution procedure yields data to allow an accept/reject decision relative to the acceptance criteria, which are frequently based on a regulatory decision. This chapter provides recommendations on how to develop and validate a dissolution procedure.

GENERAL COMMENTS

The dissolution procedure requires an apparatus, a dissolution medium, and test conditions that provide a method that is discriminating yet sufficiently rugged and reproducible for day-to-day operation and capable of being transferred between laboratories.

The acceptance criteria should be representative of multiple batches with the same nominal composition and manufacturing process, typically including key batches used in pivotal studies, and representative of performance in stability studies.

The procedure should be appropriately discriminating, capable of distinguishing significant changes in a composition or manufacturing process that might be expected to affect in vivo performance. It is also possible for the procedure to show differences between batches when no significant difference is observed in vivo. This situation requires careful evaluation of whether the procedure is too sensitive or appropriately discriminating. Assessing the results from multiple batches that represent typical variability in composition and manufacturing parameters may assist in this evaluation. It is sometimes valuable to intentionally vary manufacturing parameters, such as lubrication, blend time, compression force, or drying parameters, to further characterize the discriminatory power of the procedure.

With regard to stability, the dissolution test should appropriately reflect relevant changes in the drug product over time that are caused by temperature, humidity, photosensitivity, and other stresses.

A properly designed test should result in data that are not highly variable and should not be associated with significant analytical solution stability problems. High variability in results can make it difficult to identify trends or effects of formulation changes. Dissolution results may be considered highly variable if the relative standard deviation (RSD) is greater than 20% at time points of 10 minutes or less and greater than 10% RSD at later time points.¹ However, most

¹The Biopharmaceutics Classification System is outlined in the *FDA Guidance for Industry: Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System*, August 2000; <http://www.fda.gov/cder/guidance/3618fnl.htm>, accessed 6/22/2005.

dissolution results exhibit less variability than this. The source of the variability should be investigated when practical, and attempts should be made to reduce variability whenever possible. The two most likely causes are the formulation itself (e.g., drug substance, excipients, or manufacturing process) or artifacts associated with the test procedure (e.g., coning, tablets sticking to the vessel wall or basket screen). Visual observations are often helpful for understanding the source of the variability and whether the dissolution test itself is contributing to the variability. Any time the dosage contents do not disperse freely throughout the vessel in a uniform fashion, aberrant results can occur. Depending on the problem, the usual remedies include changing the apparatus type, speed of agitation, or deaeration; consideration and/or examination of sinker type; and changing the composition of the medium. Modifications to the apparatus may also be useful, with proper justification and validation.

Many causes of variability can be found in the formulation and manufacturing process. For example, poor content uniformity, process inconsistencies, a reaction taking place at different rates during dissolution, excipient interactions or interference, film coating, capsule shell aging, and hardening or softening of the dosage form on stability may be sources of variability and interferences. During routine testing of the product, variability outside the expected range should be investigated from analytical, formulation, and processing perspectives.

MEDIUM

Physical and chemical data for the drug substance and dosage unit need to be determined before selecting the dissolution medium. Two key properties of the drug are the solubility and solution state stability of the drug as a function of the pH value. When selecting the composition of the medium, the influence of buffers, pH value, and surfactants on the solubility and stability of the drug need to be evaluated. Key properties of the dosage unit that may affect dissolution include release mechanism (immediate, delayed, or modified) and disintegration rate as affected by hardness, friability, presence of solubility enhancers, and presence of other excipients.

Generally, when developing a dissolution procedure, one goal is to have *sink conditions*, defined as the volume of medium at least three times that required in order to form a saturated solution of drug substance. When sink conditions are present, it is more likely that dissolution results will reflect the properties of the dosage form. A medium that fails to provide sink conditions may be acceptable if it is shown to be more discriminating or otherwise appropriately justified.

Using an aqueous-organic solvent mixture as a dissolution medium is discouraged; however, with proper justification this type of medium may be acceptable.

Purified water is often used as the dissolution medium, but is not ideal for several reasons. First, the quality of the water can vary depending on the source of the water, and the pH value of the water is not controlled. Second, the pH value can vary from day to day and can also change during the run, depending on the active substance and excipients. Despite these limitations, water is inexpensive, readily available, easily disposed of, ecologically acceptable, and suitable for products with a release rate independent of the pH value of the medium.

The dissolution characteristics of an oral formulation should be evaluated in the physiologic pH range of 1.2 to 6.8 (1.2 to 7.5 for modified-release formulations). During method development, it may be useful to measure the pH before and after a run to discover whether the pH changes during the test. Selection of the most appropriate conditions for routine testing is then based on discriminatory capability, ruggedness, stability of the analyte in the test medium, and relevance to in vivo performance, where possible.

Typical media for dissolution may include the following (not listed in order of preference): dilute hydrochloric acid, buffers in the physiologic pH range of 1.2 to 7.5, simulated gastric or intestinal fluid (with or without enzymes), water, and surfactants (with or without acids or buffers) such as polysorbate 80, sodium lauryl sulfate, and bile salts.

The molarity of the buffers and acids used can influence the solubilizing effect, and this factor may be evaluated.

For compounds with high solubility and high permeability (as defined by the Biopharmaceutics Classification System), the choice of medium and apparatus may be influenced by the referenced FDA Guidance¹.

For very poorly soluble compounds, aqueous solutions may contain a percentage of a surfactant (e.g., sodium lauryl sulfate, polysorbate, or lauryldimethylamine oxide) that is used to enhance drug solubility. The need for surfactants and the concentrations used can be justified by showing profiles at several different concentrations. Surfactants can be used either as wetting agents or to solubilize the drug substance.

Volume

Normally, for basket and paddle apparatus, the volume of the dissolution medium is 500 mL to 1000 mL, with 900 mL as the most common volume. The volume can be raised to between 2 and 4 L, using larger vessels and depending on the concentration and sink conditions of the drug; justification for this procedure is expected.

Deaeration

The significance of deaeration of the medium should be determined, because air bubbles can interfere with the test results, acting as a barrier to dissolution if present on the dosage unit or basket mesh. Further, bubbles can cause particles to cling to the apparatus and vessel walls. On the other hand, bubbles on the dosage unit may increase buoyancy, leading to an increase in the dissolution rate, or may decrease the available surface area, leading to a decrease in the dissolution rate. A deaeration method is described as a footnote in the *Procedure* section under *Dissolution* (711). Typical steps include heating the medium, filtering, and drawing a vacuum for a short period of time. Other methods of deaeration are available and in routine use throughout the industry. Media containing surfactants are not usually deaerated because the process results in excessive foaming. To determine whether deaeration of the medium is necessary, results from dissolution samples run in nondeaerated medium and deaerated medium should be compared.

Enzymes

The use of enzymes in the dissolution medium is permitted in accordance with *Dissolution* (711) when dissolution failures occur as a result of cross-linking with gelatin capsules or gelatin-coated products.

In Vitro–In Vivo Correlation (IVIVC)

An in-depth discussion on IVIVC can be found in *In Vitro and In Vivo Evaluation of Dosage Forms* (1088). A brief discussion follows.

Biorelevant medium is a medium that has some relevance to the in vivo performance of the dosage unit. Choice of a biorelevant medium is based on (1) a mechanistic approach that considers the absorption site, if known, and (2) whether the rate-limiting step to absorption is the dissolution or permeability of the compound. In some cases, the biorelevant medium will be different from the test conditions chosen for the regulatory test, and the time points are

also likely to be different. If the compound dissolves quickly in the stomach and is highly permeable, gastric emptying time may be the rate-limiting step to absorption. In this case, the dissolution test should demonstrate that the drug is released quickly under typical gastric (acidic) conditions. On the other hand, if dissolution occurs primarily in the intestinal tract (e.g., for a poorly soluble, weak acid), a higher pH range (e.g., simulated intestinal fluid with a pH of 6.8) may be more appropriate. The fed and fasted states may also have significant effects on the absorption or solubility of a compound. Compositions of media that simulate the fed and fasted states can be found in the literature. These media reflect changes in pH, bile concentrations, and osmolarity after meal intake and therefore have a composition different from that of typical compendial media. They are primarily used to establish in vitro–in vivo correlations during formulation development and to assess potential food effects and are not intended for quality control purposes. For quality control purposes, the substitution of natural surfactants (bile components) with appropriate synthetic surfactants is permitted and encouraged because of the expense of the natural substances and the labor-intensive preparation of the biorelevant media.

APPARATUS/AGITATION

Apparatus

The choice of apparatus is based on knowledge of the formulation design and the practical aspects of dosage form performance in the in vitro test system. For solid oral dosage forms, *Apparatus 1* and *Apparatus 2* are used most frequently.

When *Apparatus 1* or *2* is not appropriate, another official apparatus may be used. *Apparatus 3* (*Reciprocating Cylinder*) has been found to be especially useful for bead-type modified-release dosage forms. *Apparatus 4* (*Flow-Through Cell*) may offer advantages for modified-release dosage forms that contain active ingredients with limited solubility. In addition, *Apparatus 3* or *Apparatus 4* may have utility for soft gelatin capsules, bead products, suppositories, or poorly soluble drugs. *Apparatus 5* (*Paddle over Disk*) and *Apparatus 6* (*Rotating Cylinder*) have been shown to be useful for evaluating and testing transdermal dosage forms. *Apparatus 7* (*Reciprocating Holder*) has been shown to have application to nondisintegrating oral modified-release dosage forms, as well as to transdermal dosage forms.

Some changes can be made to the apparatus; for example, a basket mesh size other than the typical 40-mesh basket (e.g., 10, 20, 80 mesh) may be used when the need is clearly documented by supporting data. In countries where available mesh sizes vary from the USP-specified mesh value, basket material with the nearest metric dimension should be used. Care must be taken that baskets are uniform and meet the dimensional requirements specified under *Dissolution* 〈711〉. If the basket screens become clogged during dissolution of capsule or tablet formulations, it may be advisable to switch to the paddle method. The volume can be increased from the typical 900 to 1000 mL by using 2- and 4-L vessels to assist in meeting sink conditions for poorly soluble drugs.

A noncompendial apparatus may have some utility with proper justification, qualification, and documentation of superiority over the standard equipment. For example, a small-volume apparatus with mini paddles and baskets may be considered for low-dosage strength products. The rotating bottle or static tubes (jacketed stationary tubes enclosed with a water jacket and equipped with a magnetic stirrer) may also have utility for microspheres and implants, peak vessels for eliminating coning, and modified flow-through cells for special dosage forms, including powders and stents.

Sinkers

When sinkers are used, a detailed description of the sinker must be stated in the written procedure. It may be useful to evaluate different sinkers, recognizing that sinkers can significantly influence the dissolution profile of a dosage unit. When transferring the procedure, the sinkers should be duplicated as closely as possible in the next facility. There are several types of commercially available sinkers. A method for making sinkers by hand, sinkers that are similar to “a few turns of wire helix” as described in *Apparatus 2* (*Paddle Apparatus*) under *Dissolution* 〈711〉, is described below.

Materials—Use 316 stainless steel wire or other inert material, typically 0.032 inch/20 gauge; and cylinders of appropriate diameter (e.g., cork borers). Sizes are shown in the accompanying table.

Capsule Shell Type	Length of Wire (cm)	Diameter Size (cm)	Cork Bore Number
#0, elongated	12	0.8	4
#1 and #2	10	0.7	3
#3 and #4	8	0.55	2

Procedure—Cut the specified length of wire, coil around a cylinder of the appropriate size, and use small pliers to curve in the ends. Use caution, because wire ends may be rough and may need to be filed.

If the sinker is handmade, the sinker material and construction procedure instructions should be documented; if a commercial sinker is used, the vendor part number should be reported.

Agitation

For immediate-release capsule or tablet formulations, *Apparatus 1* (baskets) at 100 rpm or *Apparatus 2* (paddles) at 50 or 75 rpm are most commonly used. Other agitation speeds and apparatus are acceptable with appropriate justification.

Rates outside 25 to 150 rpm are usually inappropriate because of the inconsistency of hydrodynamics below 25 rpm and because of turbulence above 150 rpm. Agitation rates between 25 and 50 rpm are generally acceptable for suspensions. For dosage forms that exhibit coning (mounding) under the paddle at 50 rpm, the coning can be reduced by increasing the paddle speed to 75 rpm, thus reducing the artifact and improving the data. If justified, 100 rpm may be used, especially for extended-release products. Decreasing or increasing the apparatus rotation speed may be justified if the profiles better reflect in vivo performance and/or the method results in better discrimination without adversely affecting method reproducibility.

Selection of the agitation and other study design elements for modified-release dosage forms is similar to that for immediate-release products. These elements should conform to the requirements and specifications given in *Dissolution* 〈711〉 when the apparatus has been appropriately calibrated.

STUDY DESIGN

Time Points

For immediate-release dosage forms, the duration of the procedure is typically 30 to 60 minutes; in most cases, a single time point specification is adequate for Pharmacopeial purposes. Industrial and regulatory concepts of product comparability and performance may require additional time points, which may also be required for product registration or approval. A sufficient number of time points should be

selected to adequately characterize the ascending and plateau phases of the dissolution curve. According to the Biopharmaceutics Classification System referred to in several FDA Guidances, highly soluble, highly permeable drugs formulated with rapidly dissolving products need not be subjected to a profile comparison if they can be shown to release 85% or more of the active drug substance within 15 minutes. For these types of products a one-point test will suffice. However, most products do not fall into this category. Dissolution profiles of immediate-release products typically show a gradual increase reaching 85% to 100% at about 30 to 45 minutes. Thus, dissolution time points in the range of 15, 20, 30, 45, and 60 minutes are usual for most immediate-release products. For rapidly dissolving products, including suspensions, useful information may be obtained from earlier points, e.g., 5 to 10 minutes. For slower-dissolving products, time points later than 60 minutes may be useful. Dissolution test times for compendial tests are usually established on the basis of an evaluation of the dissolution profile data.

So-called infinity points can be useful during development studies. To obtain an infinity point, the paddle or basket speed is increased at the end of the run for a sustained period (typically 15 to 60 minutes), after which time an additional sample is taken. Although there is no requirement for 100% dissolution in the profile, the infinity point can provide data that may supplement content uniformity data and may provide useful information about formulation characteristics during initial development or about method bias.

For an extended-release dosage form, at least three test time points are chosen to characterize the *in vitro* drug release profile for Pharmacopeial purposes. Additional sampling times may be required for drug approval purposes. An early time point, usually 1 to 2 hours, is chosen to show that there is little probability of dose dumping. An intermediate time point is chosen to define the *in vitro* release profile of the dosage form, and a final time point is chosen to show the essentially complete release of the drug. Test times and specifications are usually established on the basis of an evaluation of drug release profile data. For products containing more than a single active ingredient, drug release is to be determined for each active ingredient.

Observations

Visual observations and recordings of product dissolution and disintegration behavior are very useful because dissolution and disintegration patterns can be indicative of variables in the formulation or manufacturing process. To accomplish visual observation, proper lighting (with appropriate consideration of photodegradation) of the vessel contents and clear visibility in the bath are essential. Documenting observations by drawing sketches and taking photographs or videos can be instructive and helpful for those who are not able to observe the real time dissolution test. Observations are especially useful during method development and formulation optimization. Examples of typical observations include, but are not limited to, the following:

1. Uneven distribution of particles throughout the vessel. This can occur when particles cling to the sides of the vessel, when there is coning or mounding directly under the apparatus, when particles float at the surface of the medium, when film-coated tablets stick to the vessel, and/or when off-center mounds are formed.
2. Air bubbles on the inside of the vessel or on the apparatus or dosage unit. Sheen on the apparatus is also a sign of air bubbles. This observation would typically be made when assessing the need to deaerate the medium.
3. Dancing or spinning of the dosage unit, or the dosage unit being hit by the paddle.
4. Adhesion of particles to the paddle or the inside of the basket, which may be observed upon removal of the stirring device at the end of the run.
5. Pellicles or analogous formations, such as transparent sacs or rubbery, swollen masses surrounding the capsule contents.
6. Presence of large floating particles or chunks of the dosage unit.
7. Observation of the disintegration rate (e.g., percentage reduction in size of the dosage unit within a certain time frame).
8. Complex disintegration of the coating of modified or enteric-coated products—for example, the partial opening and splitting apart (like a clamshell) or incomplete opening of the shell accompanied by the release of air bubbles and excipients.

Sampling

Manual—Manual sampling uses plastic or glass syringes, a stainless steel cannula that is usually curved to allow for vessel sampling, a filter, and/or a filter holder. The sampling site must conform to specifications under *Dissolution* (711).

Autosampling—Autosampling is a useful alternative to manual sampling, especially if the test includes several time points. However, because regulatory labs may perform the dissolution test using manual sampling, autosampling requires validation with manual sampling.

There are many brands of autosamplers, including semiautomated and fully automated systems. Routine performance checks, cleaning, and maintenance as described in the pertinent standard operating procedures or metrology documents are useful for reliable operation of these devices.

Some instruments are equipped with sampling through the basket or paddle shaft. Proper validation (e.g., demonstrated equivalence to results with the usual sampling procedure) may be required.

The disturbance of the hydrodynamics of the vessel by sampling probes should be considered and adequate validation performed to ensure that the probes are not introducing a significant change in the dissolution rate.

Comparison of manual and automated procedures should be performed to evaluate the interchangeability of the procedures. This can be accomplished by comparing data from separate runs or, in some cases, by sampling both ways from the same vessel. Results should be consistent with the requirements for intermediate precision (described in this chapter in *Validation*) if the procedures are to be considered interchangeable.

Other aspects of automation validation may include carry-over of residual drug, effect of an in-residence probe (simultaneous sampling as mentioned above may not be suitable in this case), adsorption of drug, and cleaning and/or rinse cycles.

Filters

Filtration of the dissolution samples is usually necessary to prevent undissolved drug particles from entering the analytical sample and further dissolving. Also, filtration removes insoluble excipients that may otherwise cause high background or turbidity. Prewetting of the filter with the medium may be necessary.

Filters can be in-line or at the end of the sampling probe or both. The pore size can range from 0.45 to 70 μm . The usual types of filters are depth, disk, and flow-through. However, if the excipient interference is high, if the filtrate has a cloudy appearance, or if the filter becomes clogged, an alternative type of filter or pore size should be evaluated.

Adsorption of the drug(s) onto the filter needs to be evaluated. If drug adsorption occurs, the amount of initial filtrate discarded may need to be increased. If results are still unsuitable, an alternative filter material may be sought.

Filter validation may be accomplished by preparing a suitable standard solution or a completely dissolved sample solution (e.g., prepared as a typical sample in a vessel or a sample put in a beaker and stirred with a magnetic stirrer for 1 hour). For standard solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for the unfiltered solutions. For sample solutions, compare the results for filtered solutions (after discarding the appropriate volume) to those for centrifuged, unfiltered solutions.

Centrifugation

Centrifugation of samples is not preferred, because dissolution can continue to occur and because there may be a concentration gradient in the supernatant. A possible exception might be for compounds that adsorb onto all common filters.

ASSAY

The usual assay for a dissolution sample is either spectrophotometric determination or HPLC. The preferred method of analysis is spectrophotometric determination because results can be obtained faster, the analysis is simpler, and fewer solvents are used. HPLC methods are used when there is significant interference from excipients or among drugs in the formulation to improve analytical sensitivity and/or when the analysis can be automated. It may be useful to obtain data for the drug with a stability-indicating assay (e.g., HPLC chromatograms) in the medium of choice, even if the primary assay is based on a spectrophotometric method.

VALIDATION

The validation topics described in this section are typical but not all-inclusive. The validation elements addressed may vary, depending on the phase of development or the intended use for the data.² The acceptance criteria are presented as guidelines only and may differ for some products. Firms should document the appropriate acceptance criteria for their products in pertinent SOPs. Other considerations may be important for special dosage forms. The extent of validation depends on the phase of the product development. Full validation takes place by the time of Phase III clinical studies. Validation studies should address the variations associated with different profile time points. For products containing more than a single active ingredient, the dissolution method needs to be validated for each active ingredient.

Specificity/Placebo Interference

It is necessary to demonstrate that the results are not unduly affected by placebo constituents, other active drugs, or degradates.

The placebo consists of all the excipients and coatings (inks, sinker, and capsule shell are also included when appropriate) without the active ingredient. Placebo interference may be determined by weighing samples of the placebo blend and dissolving or dispersing them in dissolution medium at concentrations that would be encountered during testing. It may be desirable to perform this experiment

²Boudreau, S.P.; McElvain, J.S.; Martin, L.D.; Dowling, T.; Fields, S.M. Method Validation by Phase of Development, an Acceptable Analytical Practice. *Pharmaceutical Technology* 2004; 28(11):54–66.

at 37° by comparing it to the 100% standard by the formula:

$$100C(A_p/A_s)(V/L)$$

in which C is the concentration, in mg per mL, of the standard; A_p and A_s are the absorbances of the placebo and the standard, respectively; V is the volume, in mL, of the medium; and L is the label claim, in mg. The interference should not exceed 2%.

NOTE—For extended-release products, a placebo version of the finished dosage form may be more appropriate to use than blends, because this placebo formulation will release the various excipients in a manner more nearly reflecting the product than will a simple blend of the excipients. In this case, it may be appropriate to evaluate potential interference at multiple sampling points in the release profile.

If the placebo interference exceeds 2%, then method modification—such as (1) choosing another wavelength, (2) baseline subtraction using a longer wavelength, or (3) using HPLC—may be necessary in order to avoid the interference. When other active drugs or significant levels of degradates are present, it is necessary to demonstrate that these do not significantly affect the results. One procedure for doing this is to measure the matrix in the presence and absence of the other active drug or degradate: any interference should not exceed 2%.

Linearity and Range

Linearity and range are typically established by preparing solutions of the drug, ranging in concentration from below the lowest expected concentration to above the highest concentration during release. This may be done in conjunction with accuracy/recovery determination. The scheme may be altered if different flow-cell sizes or injection volumes are used.

Typically, solutions are made from a common stock if possible. For the highest concentration, the determination may not exceed the linearity limits of the instrument.

Organic solvents may be used to enhance drug solubility for the preparation of the standard solutions; however, no more than 5% (v/v) of organic solvent in the final solution should be used, unless validated.

Linearity is typically calculated by using an appropriate least-squares regression program. Typically, a square of the correlation coefficient ($r^2 \geq 0.98$) demonstrates linearity. In addition, the y-intercept must not be significantly different from zero.

Accuracy/Recovery

Accuracy/recovery are typically established by preparing multiple samples containing the drug and any other constituents present in the dosage form (e.g., excipients, coating materials, capsule shell) ranging in concentration from below the lowest expected concentration to above the highest concentration during release.

In cases of poor drug solubility, it may be appropriate to prepare a stock solution by dissolving the drug substance in a small amount of organic solvent (typically not exceeding 5%) and diluting to the final concentration with dissolution medium. An amount of stock solution equivalent to the targeted label claim may be added to the vessel instead of the drug powder. Similarly, for very low strengths, it may be more appropriate to prepare a stock solution than to attempt to weigh very small amounts. The measured recovery is typically 95% to 105% of the amount added. Bracketing or matrixing of multiple strengths may be useful.

A special case for validation is the *Acid Stage* procedure described in *Delayed-Release Dosage Forms* under *Dissolution* <711>. The limit of not more than 10% needs to be validated. If the compound degrades in acid, the validation experiment must address this fact.

Precision

Repeatability—Repeatability is determined by replicate measurements of standard and/or sample solutions. It can be measured by calculating the RSD of the multiple injections or spectrophotometric readings for each standard solution, or from the accuracy or linearity data.

Intermediate Precision—Intermediate precision may be evaluated to determine the effects of random events on the precision of the analytical procedure. This evaluation is typically done later in the development of the drug product. The precision can be across the range of product strengths. Typical variations to study include days, analysts, and equipment. The use of an experimental matrix design is encouraged for evaluation of intermediate precision. If possible, intermediate precision can be evaluated using a well-characterized lot of drug product of tight content uniformity. In cases where a well-characterized product is not available, placebo and active ingredient may be used to identify intermediate precision.

The dissolution profiles on the same sample may be run by at least two different analysts, each analyst preparing the standard solutions and the medium. Typically, the analysts use different dissolution baths, spectrophotometers or HPLC equipment (including columns), and autosamplers; and they perform the test on different days. This procedure may not need to be performed for each strength; instead, bracketing with high and low strengths may be acceptable.

A typical acceptance criterion is that the difference in the mean value between the dissolution results at any two conditions using the same strength does not exceed an absolute 10% at time points with less than 85% dissolved and does not exceed 5% for time points above 85%. Acceptance criteria may be product-specific, and other statistical tests and limits may be used.

Robustness

The evaluation of robustness, which assesses the effect of making small, deliberate changes to the dissolution conditions, typically is done later in the development of the drug product. The number of replicates (typically 3 or 6) is dependent on the intermediate precision.

Parameters to be varied are dependent on the dissolution procedure and analysis type. They may include medium composition (e.g., buffer or surfactant concentration), pH, volume, agitation rate, and temperature. For HPLC analysis, parameters may include mobile phase composition (percentage organic, buffer concentration, pH), flow rate, wavelength, column temperature, and multiple columns (of the same type). For spectrophotometric analysis, the wavelength may be varied.

Standard and Sample Solution Stability

The standard solution is stored under conditions that ensure stability. The stability of the standard is analyzed over a specified period of time, using a freshly prepared standard solution at each time interval for comparison. The acceptable range for standard solution stability is typically between 98% and 102%.

The sample solution is typically stored at room temperature. The sample is analyzed over a specified period of time using the original sample solution response for comparison. The typical acceptable range for sample solution stability may be between 98% and 102% compared with the initial analysis of the sample solutions. If the solution is not stable, aspects to consider could be temperature (refrigeration may be needed), light protection, and container material (plastic or glass).

The procedure may state that the standards and samples need to be analyzed within a time period demonstrating acceptable standard and sample solution stability.

Spectrophotometric Analysis

Samples may be automatically introduced into the spectrophotometer using autosippers and flow cells. Routine performance checks, cleaning, and maintenance as described in the standard operating procedures or metrology documents are useful for reliable operation of these instruments. Cells with path lengths ranging from 0.02 to 1 cm are typically used. Cell alignment and air bubbles could be sources of error. The smaller path length cells are used to avoid diluting the sample; however, acceptable linearity and standard error need to be demonstrated.

During analysis, standard solutions are typically prepared and analyzed at just one concentration at 100% (or the selected Q value) of the dosage strength. During profile analysis, other concentrations may be useful. A typical blank, standard, and sample may be analyzed in a sequence that brackets the sample with standards and blanks, especially at the beginning and end of the analysis.

In most cases, the mean absorbance of the dissolution medium blank may not exceed 1% of the standard. Values higher than 1% must be evaluated on a case-by-case basis. The typical RSD for UV analysis is usually not more than 2%.

The absorptivity is calculated by dividing the mean standard absorbance by the concentration, in mg per mL, divided by the flow-cell path length in cm. After enough historical data are accumulated, an acceptable absorptivity range for the analyte (using the appropriate flow cell) may be determined. This value may be useful in troubleshooting aberrant data.

Fiber optics as a sampling and determinative method, with proper validation, is an option.

It may be useful to examine the UV spectrum of the drug in solution to select the optimum wavelength.

HPLC

For HPLC analysis, the compatibility of dissolution media and mobile phase may be examined, especially if large injector volumes (over 100 μ L) are needed. Samples are normally analyzed with HPLC using a spectrophotometric detector and an auto-injector. Single injections of each vessel time point with standards throughout the run constitute a typical run design. System suitability tests include, at a minimum, the retention window and injection precision. Typically, the repeatability of an HPLC analysis should be less than or equal to 2% RSD for five or six standard determinations. The standard level is typically at the 100% label claim level, especially for a single-point analysis.

Preparation of the placebo samples for the HPLC analysis is to be performed in the same way as in the spectrophotometric analysis. Examine the chromatogram for peaks eluting at the same retention time as the drug. If there are extraneous peaks, inject the standard solution, and compare retention times. If the retention times are too close, spike the placebo solution with the drug. Chromatograms may also be obtained over an extended run time using the blank (dissolution medium), standard, and sample solution to identify late eluters that may interfere with subsequent analyses.

The validation documentation may include overlaid representative chromatograms or spectra of blank dissolution medium, a filtered placebo solution, a standard solution, and a filtered dissolution sample. Absence of interfering peaks in the placebo chromatogram or lack of absorbance by the placebo at the analytical wavelength demonstrates specificity.

ACCEPTANCE CRITERIA

Typical acceptance criteria for the amount of active ingredient dissolved, expressed as a percentage of the labeled content (Q), are in the range of 75% to 80% dissolved. A Q value in excess of 80% is not generally used, because allowance needs to be made for assay and content uniformity ranges.³ Acceptance criteria including test times are usually established on the basis of an evaluation of the dissolution profile data. Acceptance criteria should be consistent with historical data, and there is an expectation that acceptable batches (e.g., no significant differences in in vivo performance, composition, or manufacturing procedure) will have results that fall within the acceptance criteria.

(1097) BULK POWDER SAMPLING PROCEDURES

INTRODUCTION

The goals of this chapter are to provide guidance on bulk powder sampling procedures, identify important bulk powder sampling concepts, and collect a knowledge base of useful practices and considerations that can lead to the ideal physical sampling of bulk powder materials. The terminology used here is well established in the field of material sampling (see *Appendix 3*, for instance reference 7). Sampling is undertaken as part of an estimation process. The parameter of primary interest here is the mean level of some analyte in the bulk powder as a whole.

The purpose of a sampling plan is to obtain a representative sample of a population so that reliable inferences about the population sampled can be drawn to a certain level or degree of confidence. Acquiring a representative sample from a lot is critical because without a representative sample all further analyses and data interpretations about the lot are in doubt. An ideal sampling process is a process in which every particle or at least every equal-size portion of the population has an equal probability of being chosen in the sample. In addition, sampling procedures should be reproducible, i.e., if the sampling protocol were repeated, a high probability should exist of obtaining similar results. Also, the integrity of the sample should be preserved during and after sampling. The details of how to sample depend on a variety of factors. For example, criteria for sampling to evaluate particle segregation may differ from criteria for evaluating moisture content or identification.

Because of the propensity of a powder to segregate, heterogeneous powder systems can make it difficult to obtain an ideal sample. Thus, to extract representative samples requires careful development of a sampling plan that accounts for and mitigates the segregation tendencies of a particular powder system. Developing a general guidance for bulk powder sampling is challenging because every situation is different, and therefore different approaches must be used to deal with each situation. Thus, the goal of this general information chapter is to outline recommended steps for developing a sampling scheme or plan for a particular system that is consistent with good sampling practices.

The primary difficulty in acquiring a representative sample is that the size of the sample for measurement, typically a few milligrams to grams, must be withdrawn from a large

population on the order of hundreds to thousands of kilograms. The few milligrams analyzed in a laboratory must be taken from a large population of particles in a warehouse in such a manner that the measurement sample is representative of all the particles in the lot. Any bias or error in the sampling process will cause all future inferences to be in error. Over the years methods have been developed and refined to attempt to ensure that the measurement sample is representative of the whole population. A typical strategy is shown in *Figure 1*. The strategy is to sample in stages, starting with the initial gross or primary sample withdrawn directly from the received containers. In the laboratory, the gross sample must be reduced in size until it is the appropriate size for measurement. This should be done in a manner that minimizes the introduction of sampling errors. The key to reducing the sampling error is to ensure that every particle of the population has an equal probability of being included in the sample. However, because of segregation or the nonrandom nature of powders, many obstacles can cause bias and contribute to sampling errors. Following the flow chart in *Figure 1* and the steps outlined in subsequent discussions will help to minimize sampling errors.

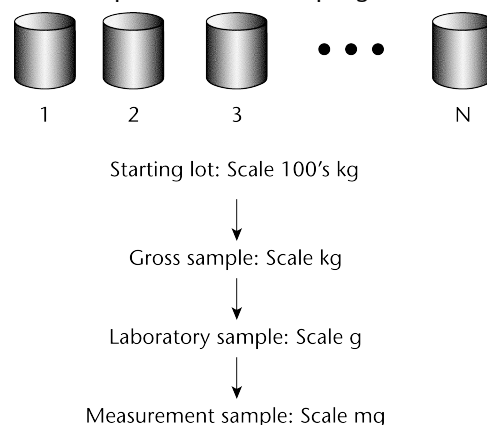


Figure 1. Overall sampling strategy for reducing the sample size from the hundreds of kg scale to the mg scale.

To acquire a representative sample, a suitable sampling plan must be developed and implemented. A good sampling plan includes: (1) population determination and sample size selection, (2) a sample collection procedure and a method for sample size reduction, and (3) summary calculations that demonstrate that the sampling plan will yield samples that accurately characterize the population to within a stated level of acceptance. In addition, an infrastructure is needed to maintain the integrity of the samples and sampled materials.

This chapter begins with a brief introduction to sampling theory and terminology. The technical content of the chapter requires a basic scientific understanding of physical particle characteristics (e.g., mass, density, shape, and size) and statistics (e.g., acceptance sampling and binomial distribution).

SAMPLING THEORY AND TERMINOLOGY

Fundamental Sample Size (Sample Mass)

Sample size is considered from two perspectives: (1) the mass of the sample intended to represent the entire population, sometimes termed the composite sample, and (2) the number of samples taken with a mass sufficient to independently evaluate, compare, or provide confidence to ensure the reproducibility of the composite or the uniformity of the population. The key to obtaining an ideal sample is to un-

³See the *FDA Guidance for Industry: Dissolution Testing of Immediate-Release Solid Oral Dosage Forms*, August 1997; <http://www.fda.gov/cder/guidance/1713bp1.pdf>, accessed 6/22/2005.

derstand and account for the degree of heterogeneity of the characteristic being evaluated in the system under study. For example, heterogeneity of a particle system arises from two sources: the intrinsic, constitutive, or compositional heterogeneity and the spatial distribution heterogeneity. The intrinsic heterogeneity of the powder system reflects the fundamental differences in the individual particles. Statistical heterogeneity (differences between individuals), or variance, is expected to maintain assumed properties. For a normal population the general expression for a statistical sample size suggests that the number of independent samples is proportional to the square of the normal quantile at the desired confidence level (Z) and the population variance (σ^2) and is inversely proportional to the square of the minimum detectable difference required (δ), as shown in equation 1:

$$n \propto \frac{Z^2 \sigma^2}{\delta^2} \quad (1)$$

In order to apply the normal theory sample size equation to sample mass with a discrete number of particles, consideration for material characteristics is needed. For a heterogeneous bulk material, such as a bulk powder, the sample mass required to ensure adequate representation of the intrinsic or fundamental population heterogeneity or variation is determined by the size, shape, and density of the particles. The total sampling error (TSE) measures the difference between the analyte concentration estimated in the sample (a_{sample}) and the mean analyte concentration in the lot (a_{lot}) relative to the mean analyte concentration in the lot (a_{lot}), as shown in equation 2:

$$\text{TSE} = \frac{a_{\text{sample}} - a_{\text{lot}}}{a_{\text{lot}}} \quad (2)$$

When ideal sampling is employed, the TSE is reduced to a fundamental sampling error, limited only by the intrinsic heterogeneity of the material. The relative variance of the fundamental sampling error (S_{fse}^2) has been empirically estimated in particle size applications by characterizing the critical particle mass, heterogeneity, size (diameter), shape, density, and weights of the material. Empirical estimates require a thorough and complete knowledge of the material and process. Established material characterization and methods are critical aspects of avoiding unacceptable estimates. As shown in equation 3:

$$S_{\text{fse}}^2 \propto f_{\text{shape}} g_{\text{CF}} c_{\text{max}} l d_{\text{max}}^3 \left(\frac{1}{m_{\text{sample}}} - \frac{1}{m_{\text{lot}}} \right) \quad (3)$$

where f_{shape} is a measure of cubicity or shape factor of the analyte particles; g_{CF} , the granulometric factor, is an empirical correction factor of differences in particle size; c_{max} is the compositional maximum heterogeneity and is calculated as if the material consists of the analyte particles and everything else; l , the liberation factor, is an empirical factor representing the proportion of critical content particles separated from the non-analyte containing particles of the lot; d_{max} is the particle diameter [e.g., the maximum diameter or the diameter (cm) of the size of the opening of a screen retaining 5% by weight of the lot to be sampled]; m_{sample} is the mass of the sample; and m_{lot} is the mass of the lot being sampled. [NOTE—A liberation factor is needed when the analyte does not appear as separate particles. A high liberation value (1.0) suggests heterogeneity of particles. A low liberation value (0.05) suggests very homogeneous par-

ticles. See *Appendix 1* for examples of potential applications of equation 3 in the estimation of the fundamental sample mass needed to account for constitutional heterogeneity of the powder mixture.] Use of equation 3 requires prior estimates of f_{shape} , g_{CF} , c_{max} , l , and d_{max} .

Segregation Error

Distribution heterogeneity is the difference between samples or groups of particles spatially or temporally. For example, small particles are located preferentially in the lower portion of a powder bed. This type of situation can arise as a result of powder bed segregation and is common in some particle systems with a broad particle size distribution. In other words, smaller particles may not be randomly distributed throughout the lot. This spatial heterogeneity introduces variation in the sample and is a source of variation that contributes to the total variation. Together, fundamental and segregation error give rise to sampling error, which dictates how variable the samples will be, how large the sample size and numbers of samples should be (e.g., 10 containers, sampled at top and bottom, with sample sizes of 50 g each), and how hard it will be to obtain a representative sample.

Minimizing the effects of segregation error during lot material characterization while still ensuring a representative sample mass requires collecting many small samples that average out the variation of the segregation error. This assumes one is interested in estimating the overall average, not characterizing lot heterogeneity. Segregation error is difficult to control because segregation may be the result of changes in particle size, shape, and density, as well as inputs into the determination of sample mass. Minimizing the effects of segregation error when reducing the primary sample size requires adequate physical mixing or randomization of the primary samples before analysis, thus providing equal selection probability.

Total Sampling Method Error

Intrinsic or compositional heterogeneity is a function of the powder system and represents the true characteristics of the material (e.g., equation 3). Thus, intrinsic heterogeneity is often the minimal variance a system can have. The difference between the true state of the system and what is actually measured when ideal sampling is employed is called the fundamental error (equation 2). The relative variance of TSE (S_{Total}^2) is represented in equation 4 as the sum of the relative variances of all error components:

The S_{Total}^2 can be reduced by employing ideal sampling. Ideal sampling will limit or adjust for the effects of error contributed by particle segregation, extraction error created by the sampling device, delimitation error created by not considering the three-dimensional nature of the bulk material, and sample handling errors such as product degradation. The total variation is the sum of these sources of error, illustrated in equation 4 as independent, additive components. To the end of reducing these errors, an important goal of material characterization by sampling is the determination of the relevant errors within the bulk sample. Knowing the source of the error helps determine how to best minimize these errors.

Fundamental error arises from the intrinsic heterogeneity of particles within a sample of the material population. Reducing fundamental error requires changing the intrinsic characteristics of the material, such as reducing the particle size by milling or grinding. Segregation error is the spatial distribution difference of particles across the population. This type of error can be minimized by mixing or randomization of the particles being selected. Segregation

$$S_{\text{Total}}^2 = S_{\text{fundamental}}^2 + S_{\text{segregation}}^2 + S_{\text{extraction}}^2 + S_{\text{delimitation}}^2 + S_{\text{preparation}}^2 + S_{\text{trends, shifts}}^2 + S_{\text{cycles}}^2 + S_{\text{analytical method}}^2 \quad (4)$$

error is affected by the characteristics of fundamental error. Additionally, for the determination of both fundamental and segregation error, it is assumed that mechanical sampling is carried out correctly and is not invasive, i.e., that mechanical sampling does not alter the characteristics being measured and provides a true representation. In instances where sampling of the bulk material does not provide unbiased representation or is so invasive that it alters material characteristics, then, in order to obtain noninvasive, unbiased samples, operators may need to change sampling from a bulk form to a stream form of processing, either upstream or downstream from the sample point (see *Appendix 2*). The mechanical sampler may need to mix the sample sufficiently to facilitate random sampling with equal probability of selection in order to obtain an adequate representation of the entire bulk lot. The process may also require mixing or sampling from a location in the process that will provide a random sample from material that is susceptible to segregation.

Extraction, delimitation, and handling errors occur as a result of the mechanical sampler and sample handling prior to analysis, which also are affected by fundamental error. Trends, shifts, and cycles are temporal sources of error that affect total error. The analytical error of the method of analysis contributes to the overall error of the reported result. In addition to obtaining representative subsamples from the bulk material, the method must also obtain a representative subsample from the particulate laboratory sample before analysis.

Sampling Strategy

A typical sampling strategy consists of two basic steps: (1) the primary or gross sample, followed by (2) the secondary sample, which reduces the primary sample to a size that is suitable for laboratory measurement. In short, the goal is to select from the lot a quantity of material suitable for measurement without significantly changing the attribute for which one is sampling. In parallel with the sample size reduction, sample size calculations must be done in such a way that the sampling strategy has sufficient statistical power to determine whether the attributes of interest lie within the specification ranges with a reasonable degree of certainty. Each step must be done correctly, or the sampling strategy as a whole will not provide a sample that is representative of the original population.

To successfully withdraw a sample from a bulk container that is representative of the population, one needs to have an idea of the population's heterogeneity, i.e., how segregated or stratified the system is. Knowing what factors can accentuate segregation and knowing the patterns of segregation that are likely will help one to account for segregation in a powder bed and to take better samples. Many factors can affect the degree of powder bed segregation. For segregation to occur, sufficient energy needs to be put into the powder bed to induce motion between particles. When a sufficient amount of energy is supplied, segregation can occur via three modes: percolation (in the powder bed), rolling (on the free surfaces of a powder bed), and free

flight (when the powder bed is fluidized). These modes are illustrated in *Figure 2*.

Within the powder bed, segregation can occur by means of percolation, also called sifting segregation, as well as through the movement of coarse particles to the top via vibration. During sifting segregation, smaller particles acting under the influence of gravity can more easily migrate downward into the void spaces between larger particles when the particle bed is perturbed. The net effect of these

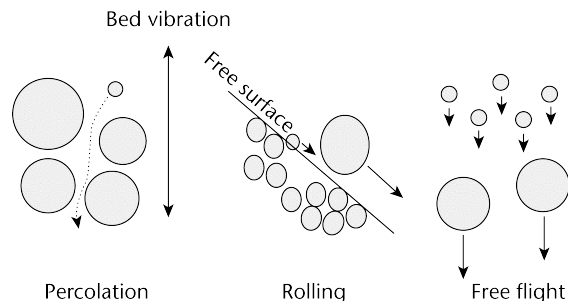


Figure 2. Illustration of the three modes of particle segregation: percolation, rolling, and free flight.

movements is that the smaller particles percolate down into the powder bed, resulting in the top of the powder bed having a higher proportion of larger particles. A common example of sifting segregation is unpopped corn kernels that are found at the bottom of a bag of popped popcorn.

For free surfaces, rolling segregation can occur any time that particles can roll down a free surface. In other words, segregation can occur on any non-level surface that allows the relative movement of particles. When particles roll down these free surfaces, larger particles tend to tumble farther down the surface than the smaller particles (see *Figure 3*). For example, if a conical heap or pile is formed in the middle of a hopper during loading, larger particles are more likely to roll farther down the heap, toward the outer edge of the hopper. This creates a situation in which the smaller particles tend to be in the center of the hopper, and the larger particles accumulate toward the outer wall of the hopper. The formation of these free surfaces can be a major factor in segregation.

When powder beds are fluidized, a large amount of air is incorporated into the powder bed and, when this air is moving, the air velocity may exceed the terminal velocity of the smaller particles. When this happens, the fine particles are suspended in the air stream while the coarse particles settle out. The fine particles eventually settle on top of the powder bed, forming a top layer that has a higher concentration of fine particles. This type of segregation, sometimes called elutriation segregation, can occur when a powder is discharged from a hopper, or is poured into the top of a hopper, and a large volume of air is displaced.

In summary, for a highly segregating system, the powder bed could have a particle distribution similar to that shown

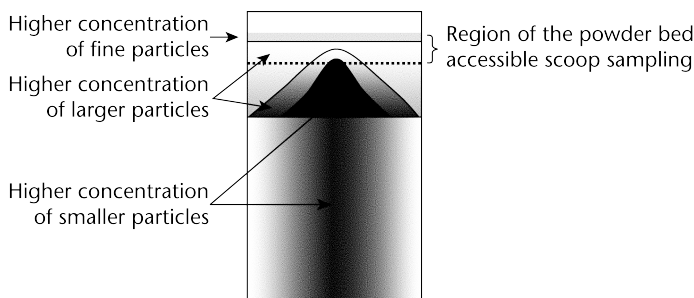


Figure 3. Example of extensive powder segregation within a drum.

in *Figure 3*, where, as a result of elutriation segregation, a layer of fine particles on the top overlies larger particles deposited by percolation segregation, and a radial distribution of larger particles appears toward the outer wall as a result of rolling segregation.

In general, the primary factors that affect segregation are particle size and size distribution, density, and shape and shape distribution. Of secondary importance are surface roughness, surface coefficient of friction, moisture content, and container shape and design. Particle size is the most important single factor, and subtle differences in particle size can cause measurable segregation. If the attribute of interest is associated with particle size, then this attribute will segregate along with the different particle sizes. For example, if a manufacturer makes a granulation in which the larger particles contain more drug than the smaller particles, then drug content can be very prone to segregation—i.e., drug content will show segregation patterns similar to those associated with particle size segregation.

Segregation can notably increase sampling error because it decreases the probability that certain particle types will be in the sample. In addition, the powder bed may already be segregated when material is received, and poor sample handling can also cause segregation. To avoid further segregation during sample handling, the operator should avoid situations that promote segregation, such as the following: pouring where the powder forms a sloping surface, pouring into the core of a hopper, vibrations, shaking, and stirring (unless done to promote mixing). In addition, the use of mass flow hoppers reduces segregation.

Two basic strategies help promote ideal sampling: (1) use of a sampling thief and (2) sampling from a moving powder stream.

A sampling thief is a long spearlike probe that can be inserted into the powder bed and, once inserted, can collect powder samples from points adjacent to the spear. With a sampling thief, particles from almost any point in the powder bed can be included in the sample. The second method relies on fundamental principles of sampling, namely that (1) a powder should always be sampled when in motion, and (2) the whole stream of powder should be sampled for many short periods rather than sampling a part of the stream for a longer period.

For example, if the container to be sampled is emptied onto a conveyor belt, all the material will pass by a single point that can be sampled. Thus, no matter how segregated the system is, the collection of the powder at random time points ensures that every particle has an equal probability of being included in the sample. The second fundamental principle accounts for material segregation on the conveyor belt: by collecting the entire stream, one gets a cross section of all the particles, no matter how much segregation occurs on the conveyor belt.

Many methods are available for obtaining a sample from a powder system. Unfortunately, many of these methods involve setting the powder bed in motion or performing in-process sampling. Because of concerns about cross-contamination and containment of potentially toxic materials, most of these methods are impractical for the bulk sampling required for compliance with current Good Manufacturing Practices (cGMPs). Hence, most of the sampling done in the pharmaceutical industry is static sampling, done by either (1) scoop or grab sampling or (2) stratified sampling, typically employing a sampling thief. The choice of method is dictated by the distribution of the attribute being sampled in the container, as discussed below.

GENERAL SAMPLE COLLECTION: CONSIDERATIONS AND TOOLS

Types of Systems and General Considerations

Homogeneous Systems: For powder systems where the attribute of interest is uniformly distributed throughout the container—so that any sample is an unbiased representation of the entire container, lot, or population of interest—scoop sampling is adequate. Scoop sampling is a straightforward procedure in which the operator, after selecting representative containers for sampling, opens a container, scoops out a sufficient amount of material from the top of the powder bed, and then seals the container. If a thin layer of material on top of the powder bed is suspected of being different from the bulk, samples should be taken from a point below this top layer. For example, in cases of elutriation segregation, a thin layer of fine particles may lie on top of the powder bed, and the operator should dig down into the powder bed to avoid sampling from this layer. The scoop should be large enough that no material is lost during handling, because lost material may result in sample bias. In other words, one should avoid the use of a heaping scoop from which material can roll off the sides. The advantages of scoop sampling are convenience and cost, and, for highly potent materials, low-cost disposable scoops that can be used to minimize cross-contamination.

Heterogeneous Systems: If the attribute of interest is spatially distributed in a heterogeneous manner throughout the sample, then scoop sampling is prone to potentially significant errors. Scoop sampling is a non-probabilistic method because only the most accessible fraction of the container is sampled. Obviously, only the material in the top layer can be reached with a scoop. For example, a sample from the top outer edge of the drum shown in *Figure 3* could be biased because, in this example, the larger particles are preferentially distributed toward the top and outer edges of the drum. Hence the smaller particles have a lower probability of appearing in the sample. As a result, the smaller particles will be underrepresented in the sample, and any analysis of particle size will not reflect the true particle size distribution of the original population.

For heterogeneous systems, the initial primary sample is the most difficult to obtain. Use of a sampling thief, sometimes called a grain probe or sampling spear, is needed. The advantage of a sampling thief is that much more of the powder bed is accessible because the sampling thief can sample from different points in the powder bed, thus helping to reduce sampling bias. Many types of sampling thieves are available, including: (1) the concentric sleeve with slotted compartments, (2) the concentric sleeve with grooves, sometimes called the open-handled probe, (3) the end sampler, and (4) the core sampler. Each type has its own unique operating procedures, as described below.

The concentric sleeve with slotted compartments is probably the most popular type of sampling thief used in the pharmaceutical industry. This type consists of two concentric tubes or cylinders in which the inner tube is divided into compartments. This design makes it possible to detect differences in the attribute of interest across the depth of the container. To collect a sample, the operator closes the compartments and inserts the sampling thief into the powder bed with the collection zone openings facing upward. The handle is turned to open the sample zones, then the handle is moved up and down with two quick short strokes to help fill the compartments. The sampling thief is then closed and removed from the powder bed. The operator should visually inspect the powder bed through its depth before emptying the sampling thief. The powder from the individual compartments can be combined on a clean surface or in a collection container. In certain situations the material from

each compartment may be analyzed separately, that is, without mixing.

In the concentric sleeve with grooves (open-handled probe), the inner tube is not divided into compartments. The probe is first inserted into the powder bed with the groove open, the outer sleeve is rotated to close, and the sampling thief is then withdrawn from the powder bed. The probe's contents are emptied from the handle end by holding the probe upright and letting the sample slide out from the handle, a method more convenient than the one using the thief with slotted compartments. However, this type of thief makes it more difficult to perform visual inspection to examine for material inconsistencies according to depth.

An end sampler probe, often used to sample slurries, has a single entry zone at the bottom of the sampling thief. Frequently the end sampling zone is larger than the rest of the sampling thief. This feature is a disadvantage because the larger the probe, the more it perturbs the powder bed, possibly resulting in the introduction of sampling bias.

Core samplers have a hollow outer cylinder with a tapered outer wall on the open end. This probe is inserted into the powder bed, and the intrinsic cohesion of the particles keeps them from flowing out when the probe is withdrawn. The contents of the cylinder are then emptied into a clear container.

General Considerations: The most reliable and reproducible results in powder size measurements are obtained when the particle size ranges from 2 to 10 μm ; otherwise, the powder is too cohesive and does not flow properly into the sampling thief. In addition, particles larger than about one-third the width of the slot give poor results. Samples should be taken from several sites throughout the container. The probe should be long enough to penetrate at least three-quarters of the depth of the powder bed, ensuring that material from all depths can be captured in the sample. The choice of sites should be dictated by an understanding (often subjective) of the powder bed's degree of heterogeneity, which may have been caused by handling or movement during transport. Sampling plans can call for the insertion of the probe either at random locations and random angles or at predetermined locations and angles. For example, the plan may call for the probe to be inserted at the center and at two locations near the edges. Also, many operators recommend that probes always be inserted at a 10° angle from vertical, which increases the range of locations sampled.

Some of the disadvantages of sampling thieves include the labor-intensive nature of the procedure. The probe must be physically inserted into the powder bed, often multiple times; the contents of the probe must be emptied; and then the probe must be thoroughly cleaned. For settled powder beds, the sampling probe can be difficult to insert. In addition, the sampling probe can introduce errors as a result of the following: fine particles can lodge between the inner and outer tubes; particles can fracture; fine particles can compact and not flow well into the sampling compartments; segregation can occur during flow into the sampling zone; and the act of inserting the probe can disrupt the powder bed by dragging powder from the top layers of the bed down through the bed.

Representative Lot Sampling

Statistically-based sampling plans are based on statistical principles and depend on the population's spatial heterogeneity and intrinsic variability. Statistically-based plans are efficient and allow the collection of a sufficient number of samples to yield the desired degree of certainty without collecting too many or too few samples for the test method, scale, product variation, risk requirements, and tolerance for a stated product's quality level or specification. The commonly used $\sqrt{N} + 1$ sampling plan given in *Table 1* is not a statistically based sampling plan and may result in collection of too few samples for small populations and too many

samples for large populations. The use of statistically-based sampling plans is advantageous because it facilitates risk management. However, in cases where prior knowledge of the population to be sampled is insufficient, a nonstatistical sampling plan such as that given in *Table 1* can be considered.

Figure 4 illustrates the sample size selection scheme paths. The first choice is whether to use a statistical or nonstatistical sampling plan. Statistical plans are preferred when a variable attribute like particle size or drug content is being determined. General sampling approaches are outlined in *USP* general information chapter *Analytical Data—Interpretation and Treatment* (1010). Statistically-based lot acceptance sampling plans require a valid rationale with known quality levels for the determination of product lot characteristics. As noted, the application of statistical sampling plans, including lot acceptance sampling plans, requires specific and thorough knowledge of the material being sampled. Reference statistical sampling plans state the rationale for sampling as part of the sampling scheme. Manufacturers who use a statistically-based lot acceptance sampling method should refer to an appropriate standard such as ANSI/ASQ Z1.9-2003 for bulk materials or ANSI/ASQ Z1.4-2003 for multiunit or discrete populations. These standards are readily available via sources such as the American Society for Quality (<http://www.asq.org/>) or the American National Standards Institute (<http://www.ansi.org/>).

If one is developing a nonstatistical sampling plan for which the quality level is not known, *Table 1* gives suggested sample sizes for the number of containers in the lot that should be sampled.

The *Level 1* sampling plan is relevant to materials when heterogeneity does not affect the analysis and the customer seeks to sample more than one container, when the sampling plan can be proportional to the square root of the number of containers received, and when the material comes from a known and trusted source. In such cases, the sample can be withdrawn from any point in the container. For adequately homogenous systems, scoop sampling from the top of the container is suitable.

The *Level 2* sampling plan involves a 50% increase in sample size when compared with *Level 1* and is used when a larger proportion of the number of containers is needed, for example, when a material's heterogeneity is suspected of being consequential and acceptance sampling quality levels are not defined, or when the material comes from a less trusted source. Depending on the material's degree of heterogeneity, a sampling thief may be used. However, if the degree of heterogeneity will not significantly affect the results for the attribute being sampled, then scoop sampling from the top of the drum may still be suitable.

Table 1 shows the number of containers, n , to be sampled for a lot segregated into N containers. Note that the value of n from the formula is rounded at 0.5 up to the next higher integer. For example, if $N = 6$: for *Level 1*, $n = \sqrt{6} + 1 = 3.45$ rounds to $n = 3$; for *Level 2*, $n = 1.5 \times \sqrt{6} = 3.67$, which rounds to $n = 4$.

Table 1

N (Number of Containers Comprising Lot)	n (Sample Size)	
	Level 1	Level 2
$N \leq 3$	All	All
$N \geq 4$	$\sqrt{N} + 1$	$1.5 \times \sqrt{N}$

These initial decisions, as illustrated in *Figure 4*, are often difficult and sometimes must be made without sufficient information. If there is uncertainty about which method or level is appropriate, sometimes a quick, small-scale informal test of the system may help determine the best way to proceed. In addition, for some systems and attributes, the *Level 1* and *Level 2* sampling plans may result in oversampling. For example, when one is sampling for identification from the same lot, the suggested levels may result in collecting more samples than are statistically needed; in such cases,

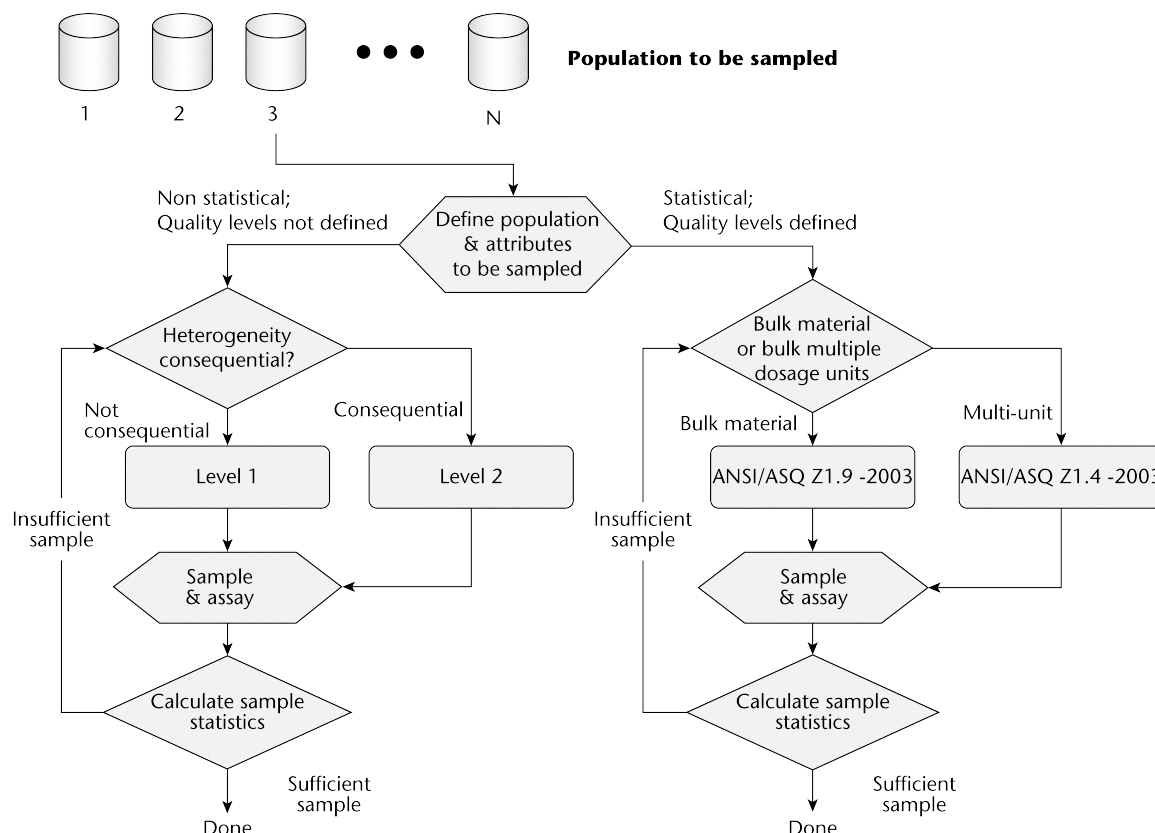


Figure 4. Sample size selection scheme.

the statistically-based sampling plans referenced in *Figure 4* can be used.

Sample Collection

Acquiring a representative sample from a lot of bulk powder is a difficult procedure that requires special consideration, and the basic procedures for acquiring a representative sample are discussed below. Note that every situation requires techniques that are appropriate for the given population to be sampled. The methods presented here are applicable to the sampling of static powders stored in midsize bulk containers such as 1-ton super sacks, 50-kg drums, or 50-lb bags. These methods are not necessarily applicable to the sampling of liquids, large storage containers such as train cars or silos, or in-process systems such as blenders or moving conveyor belts. In addition, the procedures described here are most applicable to particles in the size range from approximately $\sim 1\ \mu\text{m}$ to approximately $\sim 1000\ \mu\text{m}$. Significantly smaller or larger particles require special procedures that are not covered here.

PRIMARY SAMPLE COLLECTION

Lot acceptance samples are generally transferred or delivered in containers. To collect a representative primary or gross sample (see *Figure 1*), the appropriate container or containers must first be selected from the population of N containers; second, a representative sample must be withdrawn from each of the selected containers.

Container Selection

To avoid bias and other sampling errors, the containers to be sampled must be randomly selected. To make a random

selection, first number all containers in the lot, then use a random number table (or computer-generated random numbers) to choose from which container or containers to withdraw the samples.

For systems in which containers are grouped together in such a manner that many of the individual containers are not practically accessible (e.g., 50-lb bags stacked and bound in shrink wrap on a pallet), the sampling plan may need to take into account the larger container, in addition to the smaller container, as a sampling unit, in order to ensure a representative sample.

Withdrawing Sample from a Container

Container Types: The three most popular container types are the bag, drum, and super sack. Because bags are generally closed and not resealable, special sampling thieves, sometimes called bag triers, have been designed to puncture the bag. If the system to be sampled is heterogeneous, the samples should be obtained from the bottom, center, and top of the bag; and, depending on how the bags are stacked on the pallet, they should also be sampled from the front and the back. When sampling from bags, particular attention should be paid to the corners, because they can disproportionately trap fine particles. If no bag trier is available, use a knife to cut open the bag for sampling. When sampling from a bag, be sure to clean the external surface sufficiently that the sample is not contaminated and foreign material is not introduced into the bulk material. Once the sample has been taken, place a compatible material over the hole in the bag, then fix this patch with an appropriate adhesive tape. Depending on the heterogeneity of the drum, a scoop or a sampling thief is used. Super sacks are large sack containers that usually have a fill spout on the top and a discharge spout on the bottom. For adequately homogeneous material, scoop sampling is appropriate.

ate; but if there is any concern about the heterogeneity of the material, a thief should be used. The large size of super sacks makes the use of a thief more important for representative sampling than in the case of a drum or bag, in order to limit potential delimitation error.

Sample Handling

The samples collected can be either assayed individually or combined; then a subset of the gross sample can be assayed, as depicted in *Figure 1* and described below. Sample increments should be combined on a clean, dry surface or in a suitable container or bag. All containers with which the sample comes into contact should be inert and should not chemically or physically react with the sample. In addition, samples should be accurately labeled and good records kept. A portion should be kept for possible future analysis.

PRIMARY SAMPLE SIZE REDUCTION

As mentioned above, the primary sample typically consists of multiple samples taken from containers and mixed together. To obtain an analysis or measurement sample (*Figure 1*), the gross or primary sample must be reduced to a size appropriate for the analytical method. Gross or primary sample size reduction is an often overlooked aspect of a sampling plan, but it is an important step. The factors that cause segregation in a container can also cause segregation in the primary sample, and any bias in the size reduction method for the primary sample will lead to erroneous results. The advantage of secondary samples is that the mass has been reduced to a point at which it is much easier to obtain a representative sample because every element in the powder bed is readily accessible. Such accessibility makes it easier to adhere to sampling best practices. Generally speaking, sample measurement takes place under either wet or dry conditions; the choice is dictated by the requirements of the analytical method. For example, the Coulter counter requires that samples be uniformly suspended in an electrolyte, but other methods, like sieving, are typically performed with dry powders.

Before dividing an agglomerated sample, the agglomerates should be broken apart by a suitable technique such as sieving.

Dry Analysis Methods

Many laboratory devices are available for the reduction of the primary sample to an analytical sample. The three most important methods used in the pharmaceutical industry are: (1) scoop sampling, (2) cone and quartering, and (3) the spinning riffler or rotary sample divider (manual method of fractional shoveling); see *Figure 5*.

Scoop Sampling: Scoop sampling is done as previously described, but generally with a smaller scoop or spatula. Great care must be taken when removing material from the primary sample, because this material could be highly segregated as a result of handling. Scoop sampling is appropriate for homogeneous or cohesive powders. However, if the powder is prone to segregation, scoop sampling can introduce significant errors. Moreover, scoop sampling has several serious disadvantages. First, the method depends on the operator's deciding from which part of the primary sample to scoop the material and what quantity of the sample to extract, which are features that can introduce operator bias. Second, in scoop sampling, operators have a natural tendency to withdraw the sample from the free surface, which is highly prone to segregation and is not representative of the bulk. Third, operators need to avoid creating a heap where rolling segregation can occur, because material could fall off the edges of the spatula or scoop and bias the sample. Ideally, the operator should make some attempt to mix the primary sample before using the scoop, but this too can

exacerbate segregation problems and should be done only with great caution.

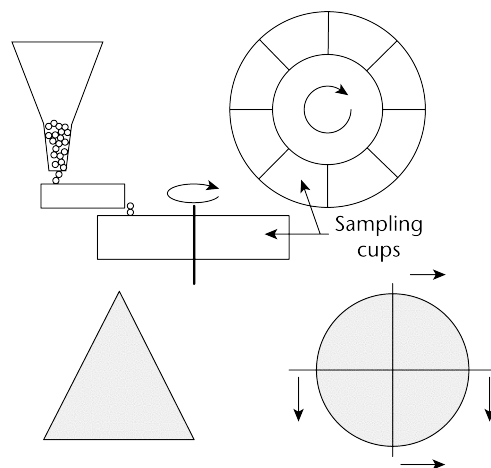


Figure 5. Two procedures for dividing samples. Top: spinning riffler, in which a circular holder rotates at a constant speed, and the sample is loaded at a constant rate into the containers via a vibratory chute, which is fed by a mass-flow hopper. Bottom: cone and quartering. (Cone, left, is flattened and quartered; quarters can be formed into cones and further subdivided.)

Cone and Quartering: Cone and quartering is done by pouring the primary sample into a symmetric cone on a flat surface. The cone is then flattened by a flat surface such as a spatula, and is divided into four identical quarters (*Figure 5*). One quarter is taken as the sample. This procedure can be repeated (e.g., quarter-samples can be subdivided into quarters) until the desired sample size is obtained. The theory of this method is that when a symmetric cone is created, all the segregation processes also occur symmetrically around the cone, and hence symmetry is used to mitigate the effect of segregation. In practice, it is very difficult to actually make a symmetric powder cone, and the method becomes very operator-dependent and often unreliable. Differences in how operators form the heap and subdivide it can lead to a lack of precision and significant errors. In addition, if the method is done more than once, errors can propagate each time the cone and quartering is performed. Some experts do not recommend this method.

Spinning Riffler: A spinning riffler (*Figure 5*) includes a series of containers mounted on a circular holder. The circular holder rotates at a constant speed, and the sample is loaded at a constant rate into the containers via a vibratory chute, which is fed by a mass-flow hopper. Once the material has been divided among the different holders, an individual holder can be removed for testing or further sample division. The angular velocity of the circular holders and the amplitude of the vibratory feeder can be controlled to accommodate powders with different flow properties. The holder velocity and feed rate should be adjusted so that the containers fill uniformly and so that a heap does not form on the vibratory feeder. Spinning riffles are available in different sizes, making possible subdivisions of powders ranging from a few milligrams to hundreds of grams. The only drawbacks of the spinning riffler are the time required to process the sample and clean the device, and the capital expense. Despite these minor disadvantages, the spinning riffler is by far the best method for subdivision of free-flowing powders.

Fractional shoveling is the manual version of the spinning riffler. In this method, scoop samples are taken from the original sample and placed into a sufficient number of aliquots, and then subsequent scoops are taken from the original sample and placed into one of the aliquots in sequential order. This process is repeated until the original samples are

gone. Then one of the aliquots is randomly taken as the reduced sample. As is the case with all manual methods, operator error and variability can be significant factors.

Wet Analysis Methods

Wet analysis methods require dispersing the sample in a liquid suitable for analysis, and then withdrawing an aliquot using a syringe or pipet. Effective secondary sampling requires making a stable homogenous suspension (i.e., the sample must be stable from the time of formation of a suspension to the time when the analysis is complete). Some important factors in wet analysis are sample solubility in the dispersion vehicle, aggregation of sample, the use of suspending agents, and deaggregation of primary particles in the dispersion vehicle. Even though a uniform suspension is created, the sample should be homogenized, typically by shaking, immediately before withdrawing the sample with a syringe or pipet. The diameter of the syringe or pipet should be large enough so that particles are not excluded and clogging does not occur. The diameters of the largest particles should not exceed 40% of the syringe or pipet tip diameter. If for practical reasons the amount of material from the primary sample is too large, the sample size should be reduced before a suspension is made. To reduce the sample size, use the methods described above in the *Dry Analysis Methods* section. As a precaution, collect and retain enough sample to repeat all tests a minimum of five times.

APPENDIX 1: SUBSAMPLING EXAMPLES

The examples provided below describe the importance of material particle characterization during the selection of an appropriate sample mass. Four examples are presented. In the first example, similarity in the fundamental or intrinsic material characteristics is assumed. In the second example, the density of the heavy metal analyte being measured is changed. In the third example, the effect of changing the particle size is evaluated. In the fourth example, the adequacy of the fundamental particle characteristics in a formulation needed for a given unit dose or mass is evaluated.

Example 1. Sample Mass Determination

Assuming the lot size is 1 kg, the maximum particle diameter is 1000 μm , and the concentration of the analyte is expected to be 1%, what sample mass of round, equal-sized and -shaped 1000- μm particles with a density of 1 g/cm^3 would be needed to estimate the average concentration of the analyte with a percent relative standard deviation (%RSD) of 5%?

Rearranging equation 3, one can estimate the sample mass as shown in equation 5:

$$m_{\text{sample}} \approx \frac{1}{\frac{s_{\text{fse}}^2}{f_{\text{shape}} g_{\text{CF}} C_{\text{max}} l d_{\text{max}}^3} + \frac{1}{m_{\text{lot}}}} \quad (5)$$

The compositional maximum heterogeneity (C_{max}) can be estimated by considering the analyte and matrix density (λ_a and λ_m , respectively, and their average λ) and analyte concentration (a_L) (equation 6):

$$C_{\text{max}} = \frac{(1 - a_L)^2 \lambda_a \lambda_m}{a_L \bar{\lambda}} \quad (6)$$

For low analyte concentrations, the compositional maximum heterogeneity is simplified to equation 7:

$$C_{\text{max}} \approx \lambda_a / a_L \quad (7)$$

For high analyte concentrations, the compositional maximum heterogeneity is simplified to equation 8:

$$C_{\text{max}} \approx \lambda_m (1 - a_L) \quad (8)$$

The shape factor is approximated by equation 9:

$$f_{\text{shape}} \approx \text{Volume} / d^3 \quad (9)$$

Where d is the nominal particle diameter for a sphere, and the shape factor is $[(4/3)\pi/8]$, or approximately 0.5.

The granulometric factor can be approximated by the typical minimum diameter noted as the 5th percentile size, divided by the typical maximum diameter noted as the 95th percentile size, as shown in equation 10:

$$g_{\text{CF}} \approx d_{5\%} / d_{95\%} \quad (10)$$

Because all particles are the same size, the granulometric factor, g_{CF} , is 1.0. Because the analyte exists in a state liberated from the matrix particles, the liberation factor is also 1.0. The sample mass for a 5% RSD (using equation 5) is then:

$$m_{\text{sample}} = \frac{1}{\frac{0.05^2}{0.5 \times 1 \times 100 \times 0.1^3} + \frac{1}{1000}} = 19.6 \text{ g}$$

A sample mass of 19.6 g will provide a sampling error of approximately 5% RSD. Note that in this example the particle characteristics are simplified to demonstrate that a lot mass of 1000 g contains 2×10^6 particles of 0.5 mg mass. The sample mass of 19.6 g contains approximately 39,216 particles, yielding a 5% RSD, using the binomial distribution where p is the concentration of the analyte (a_L) and n is the number of particles sampled, as shown in equation 11.

$$\text{Binomial RSD} = \sqrt{(1-p)/np} = \sqrt{(1-0.01)/39,216 \times 0.01} = 0.0498 \approx 0.05 \quad (11)$$

(See Table 2 for a summary of calculations.)

In determining the required sample mass, it is assumed that the sample is representative of the population. Moreover, when using a single representative sample, it is assumed that the uniformity of the sample mass is consistent with the remaining population. Note that the granulometric and liberation factors allow proportional adjustment of the sample size, depending on the nature of the particles. The inclusion of a liberation factor in the equation allows for particles to exist with a proportion of the analyte residing within every particle or a proportion thereof. The granulometric factor permits adjustment of the sample mass by accounting for the relationship in size between the smallest and largest particles represented in the lot.

This approximation also can be applied to liquid suspensions in which each particle is considered discrete and the sample can be characterized with respect to size, density, mass, and volume.

Example 2. Heavy Metal

In this example, it is assumed that the analyte is the heavy metal lead, with a density of 11.34 g/cm³, with a limit of not more than 5 ppm, where the shapes of the particles are cubes ($f_{\text{shape}} = 1.0$), the particles are approximately 50 μm , and a 5-g sample is taken from screened material ($g_{\text{CF}} = 0.55$). On the basis of equation 3, the %RSD is 17.7%. Using equation 5, one finds that a sample mass of approximately 60 g is needed to achieve a 5% RSD, assuming that a_L is equal to the limit allowed and that the analyte cannot be assumed to be liberated from the material ($l = 1.0$). (See Table 3 for a summary of calculations.)

If the sample were assumed to be homogeneous ($l = 0.1$) with respect to presence of the analyte with all particles, then a sample mass of 6.2 g would be required. Moreover, if the shape of the particles were between round and cubic ($f_{\text{shape}} = 0.8$), then a sample mass of 5 g would be required to complete the analysis.

Example 3. Subsampling

Ideal sampling, as noted earlier, is fundamental to understanding the important role of subsampling. In many instances it is desirable to reduce the sample size in a manner that results in a representative sample and lessens the need to test a large sample mass. In some cases the particle size and compositional heterogeneity can result in an unwieldy sample mass. This may occur with larger-sized particles or when a composite sample of many containers is required. Samples with larger-sized particles may need to be physically reduced.

For example, using Example 2 above, if the maximum particle size were 1000 μm or 1 mm, then a 997-g sample would be suggested by equation 3. Reducing the particle size by grinding and subsampling to achieve a predetermined sampling %RSD may require subsampling more than once to achieve the desired particle size. For example, the entire sample may be reduced to 100 μm to reduce the %RSD to approximately 3%; then, with ideal sampling, a subsample could be selected and entirely reduced to 50 μm to achieve a 5% RSD. Finally, a 5-g subsample could be correctly taken and tested. If certain particles have a large size with high concentration of the analyte, then samples should be selected to ensure that at least 1, but preferably at least 5–6, particles would be selected with 95% probability or chance of selection.

Example 4. Minimum Unit Dosage Mass

A formulator would like to know the minimum mass required for a dosage form to ensure with 95% confidence a unit dosage of 1% active drug powder. The drug and the excipient have a similar round shape ($f_{\text{shape}} = 0.5$) and a density of 0.33 g/cm³. The active drug is milled to 1 μm , but the size of excipients can be as large as 200 μm . The value for g_{CF} is taken from equation 10 using the expected range of the excipient that accounts for 95% of the formulation, as 10 $\mu\text{m}/200 \mu\text{m}$, or $g_{\text{CF}} = 0.05$. The quantity C_{max} from equation 7 is taken as 0.33/0.01. The drug particles are completely liberated from the excipient. The batch size is 100 kg.

A minimum sample mass of approximately 3 mg is needed to ensure with 95% confidence (2 RSDs) that the average drug content is 0.9%–1.1%. The proposed dosage form has an active concentration of 100 $\mu\text{m}/10 \text{ mg}$ total unit mass. The unit dosage form mass is adequate, but the formulation requires that the mixing process, unit dosage production, bulk sampling device, and lab sample preparation or subsampling from bulk samples result in equal probability of selection of drug particles. Only if these conditions for mixing, production, sampling, and testing are met can it be reliably demonstrated that the unit dosage and test determination acceptance criteria of 1% (0.01 $\mu\text{g}/\text{mg}$) are met. Acceptable outcomes of such testing also indicate that the particle size, shape, and density must be controlled. For example, an increase in the sizes of particles to 500 μm results in a need for a 42-mg sample mass and dose. Assuming a cubic, as opposed to a rounded, particle increases the sample mass to 5 mg, which for a fixed dosage form mass may result in less room for the variation contributed by other characteristics, or in lesser confidence. If the acceptance criteria were changed to 0.95%–1.05%, requiring a 1% RSD, then the minimum sample mass would increase to approximately 70 mg. (See Table 4 for a summary of calculations.)

APPENDIX 2: MATERIAL CHARACTERIZATION AND SAMPLING

Specific and thorough knowledge of the material's synthesis, composition, and usage is critical to developing a bulk material sampling plan. Material characterization is important because bulk material can exist in many forms throughout the material process flow. As illustrated in Figure 6, the type of sampling can vary by process step and ultimately

Table 2. Summary of Calculations for Example 1, Equal-Sized and -Shaped Particles

$m_{\text{Lot}} \text{ (g)}$	$d \text{ (cm)}$	f_{shape}	g_{CF}	C_{max}	a_L	$\lambda_a \text{ (g/cm}^3\text{)}$	l	$m_s \text{ (g)}$
1000	0.1	0.5	1	100.0	0.01	1	1	19.6
Mass per Particle P_s $d^3 f_{\text{shape}} \lambda_a$		Particles in 19.6 g $m_s / (P_s / g_{\text{CF}})$					Binomial RSD $p = a(L) = 0.01$ $n = 39,216$ (Eq. 11)	
0.005		39,216					0.05	

Table 3. Summary of Calculations for Example 2, Heavy Metal

$m_{\text{Lot}} \text{ (g)}$	$d \text{ (cm)}$	f_{shape}	g_{CF}	C_{max}	a_L	$\lambda_a \text{ (g/cm}^3\text{)}$	l	$m_s \text{ (g)}$
1000	0.005	1.0	0.55	2.3×10^6	5×10^{-6}	11.34	1.0	58.71

Table 4. Summary of Calculations for Example 4, Minimum Unit Dose Mass

$m_{\text{Lot}} \text{ (g)}$	$d \text{ (cm)}$	f_{shape}	g_{CF}	C_{max}	a_L	$\lambda_a \text{ (g/cm}^3\text{)}$	l	$m_s \text{ (g)}$
10^5	0.02	0.5	0.05	33	0.01	0.33	1.0	0.00264

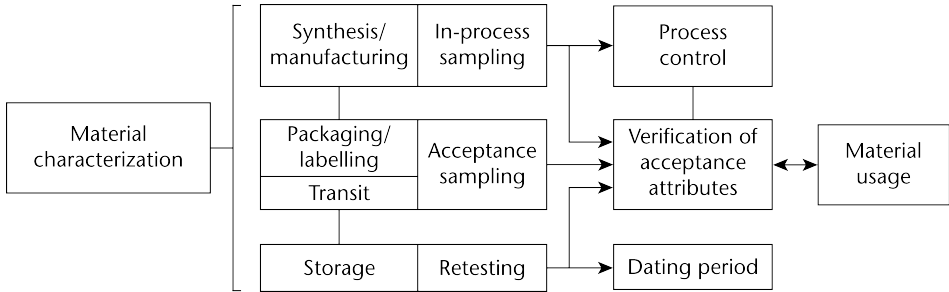


Figure 6. Material process flow.

affects the use of the material in the drug product. Appropriate material characterization considers the material process step, the type of sampling, the objective of the process step, and ultimately the drug product.

For example, material can be synthesized or mixed in a large container where sampling may be limited or ideal for the characteristic needed. If the characteristic is important but the sampling conditions are not ideal, perhaps because of the heterogeneity of a powder mixture, then sampling for that characteristic may be more appropriately performed at a different stage upstream or downstream to assess the heterogeneity of the contents and ensure ideal sampling. This is sometimes performed to reduce the sampling dimension. The sampling dimension is reduced when the 3 dimensional bulk container space is sampled in a 1 or 2 dimensional stream sampled over time. Reducing the spatial sampling dimension may result in conditions that will allow for more accurate measurement of the heterogeneity of material while limiting sampling error through ideal sampling.

Acceptance attributes (see Table 5) depend on material characterization and process. Acceptance attributes may be applicable throughout the life of the bulk material. Both the number and size of samples require an understanding of the material's variation.

Table 5. Examples of Acceptance Attributes

Acceptance Attributes			
Physical	Chemical	Micro-biological	Packaging
Particle size	Purity	Sterility	Label
Viscosity	pH	Pyrogens	accuracy
Density	Identity	Microbial load	Integrity
	Strength		

APPENDIX 3: ADDITIONAL SOURCES OF INFORMATION

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<1102> IMMUNOLOGICAL TEST METHODS—GENERAL CONSIDERATIONS

INTRODUCTION

This general information chapter provides a high-level description of principles for immunological test methods (ITMs) that can be used in specified monograph tests, along with information and approaches to analytical development and validation for ITMs. The scope of this chapter is to provide general information that is applicable to all ITMs. The chapter provides a foundation for specific chapters about different types of ITMs, e.g., *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* <1103>, *Immunological Test Methods—Immunoblot Analysis* <1104> (proposed), and *Immunological Test Methods—Surface Plasmon Resonance* <1105>. This suite of general information chapters is related to the bioassay general information chapters. Use of ITMs for process monitoring, diagnosis, and evaluation of clinical response, assessment of pharmacokinetics/pharmacodynamics/absorption, distribution, metabolism, and excretion (PK/PD/ADME), and other product characterization (nonrelease testing) is outside the scope of this chapter.

The basis of all ITMs used to measure a quality attribute of a biologic drug substance or drug product is the highly specific noncovalent binding interaction between an antibody and antigen. The antigen typically is an analyte of interest (e.g., protein, carbohydrate, virus, or cell), and the binder is usually an antibody (e.g., monoclonal antibody or polyclonal antiserum). ITMs are applicable to molecules that are either directly antigenic (immunogens) or can be rendered indirectly antigenic (haptens). The measurand in ITM is directly related to a quality attribute of the product under test.

ITMs are valuable because they exhibit high sensitivity and specificity for an analyte in complex matrices. They typically are used for qualitative and quantitative assessment of both an antibody and antigen, but their application also extends to the measurement of hapten, complement, antigen–antibody complexes, and other protein–protein interactions. These properties of ITMs allow their use for assessing identity, potency (strength), purity, impurities, stability, and other quality attributes of biological drug substances and drug products.

ITMs are useful for many applications because they can measure molecules over a wide range of sizes and binding types. In general, antibodies are stable during various chemical modifications that do not have a significant adverse influence on interactions with an antigen. Antibody molecules tend to withstand moderate acidic and alkaline pH changes better than other proteins do. Because of this characteristic, a variety of ITMs with high degrees of sensitivity and specificity are possible. The ability to accelerate contact between an antigen and antibody enables ITM formats that provide rapid or real-time results.

Generally, ITMs have higher precision and shorter turnaround time than do traditional biologically-based (i.e., cell-based and animal) assays. Although in some cases these advantages can support the replacement of a biological assay with an immunoassay, such changes should be approached

systematically and with caution. Often it is challenging to prove the equivalence, or comparability, of results from bioassays and immunoassays because the interaction between antigen and antibody may not reflect the functional attributes observed in bioassays.

One major limitation of ITMs compared to physicochemical methods (such as liquid or gas chromatography) is that the latter generally are more precise and can simultaneously identify a set of impurities or unexpected substance(s). Another major limitation is that generally ITMs operate at high molar dilutions at which they are sensitive to disturbances caused by environmental factors in the sample matrix (i.e., matrix effects). Matrix effects can depend on ITM format and are not fully understood. Their specificity, a hallmark of ITMs, is sometimes compromised by structural or sequence similarities between the analyte and a closely related molecular impurity (cross-reactivity).

Most ITMs reflect physical interaction (binding) between an antigen and antibody and not the analyte's functional properties. Therefore, analysts must pay attention in the selection and execution of ITM format. Cell-based ITMs that can provide functional information about the analyte are beyond the scope of this chapter.

GENERAL CHARACTERISTICS OF ITMs

ITMs are based on the principle of specific, noncovalent, and reversible interactions between an antigen and antibody. In general, the primary antigen–antibody reaction is brought about by complementarity, which creates macromolecular specificity. This noncovalent interaction determines the degree of intrinsic affinity. Intrinsic affinity contributes to functional and/or relative affinity that depends on factors like reaction phase and valency, which in turn determines the degree of reversibility of an interaction. A better understanding of factors that affect antigen–antibody interactions provides the rationale for the development of a suitable ITM format (e.g., solid or liquid phase, competitive or noncompetitive binding, etc.).

A defining characteristic of ITMs is that they employ an antigen (or hapten) and antibody. In addition, ITMs may contain companion molecules such as complement components. The components of ITMs are defined as follows:

- **Antigens**—Comprise a wide range of molecules that are capable of binding to the antibody in a specific interaction. Generally, part(s) of an antigen (the immunogenic epitope[s]) is/are capable of eliciting an antibody response.
- **Haptens**—Small molecules that, by themselves, are not capable of eliciting an antibody response but are capable of eliciting an immune response when attached to a large carrier such as a protein. Antibodies produced to a hapten–carrier adduct also may bind to the small-molecule hapten in a specific interaction.
- **Complements**—Companion molecules that, under certain conditions, aid in the functionality of antigen–antibody complexes but are not required for antigen–antibody or hapten–antibody interaction.
- **Antibodies**—Proteins with regions that impart a high degree of specific binding to antigens (and haptens). The structural elements of an immunoglobulin G (IgG) antibody are shown in *Figure 1*.

In addition to these components, ITMs require some means to detect or monitor the binding reaction between the antigen and antibody.

TYPES OF ITMs

Measurement of antigen–antibody binding can be performed in a variety of assay types and formats: solid or liquid phase, manual or automated, labeled or nonlabeled, competitive or noncompetitive, qualitative or quantitative, homogeneous or heterogeneous, or combinations of some of these. The distinguishing characteristic of all these assays

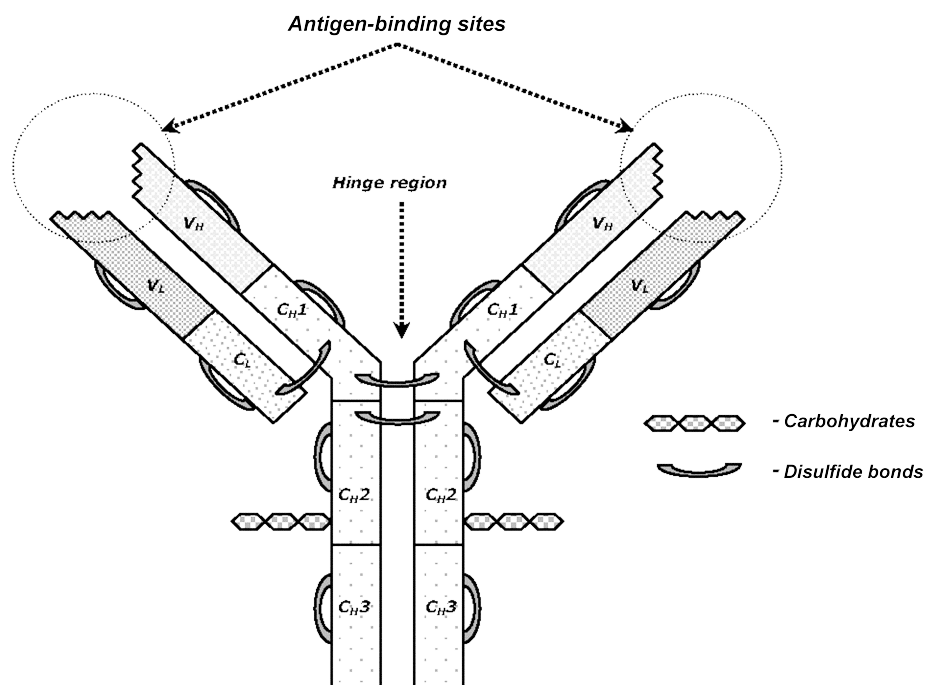


Figure 1. The structure of IgG. The IgG molecule is characterized by a distinctive domain structure of heavy (H) and light (L) chains, both of which are divided into variable and constant regions (V and C, respectively). Light chains consist of V_L and C_L domains, and heavy chains consist of a variable domain (V_H) and three constant domains (C_H1, C_H2, and C_H3). All domains are stabilized by disulfide bonds, and C_H2 domains contain carbohydrates. The flexible hinge region between the C_H1 and C_H2 domains allows the independent behavior of two antigen-binding sites formed by variable domains.

is the binding of an antibody or antigen to the analyte (which can be an antigen or antibody as well), followed by detection of the antigen–antibody complex. Although many different formats can be used for the binding reaction, along with different methods for detection, quantification of the analyte in the test article is always performed by comparison of the measurement to a reference standard. Thus a number of ITM technologies support investigations of product quality. Commonly used assay designs include enzyme-linked immunosorbent assay (ELISA), Western blotting, flow cytometry, competitive enzyme-linked immunosorbent assay, surface plasmon resonance (SPR), rate nephelometry, radioimmunoassay (RIA), radial immunodiffusion, precipitation, and agglutination. These methods are described below.

Enzyme-Linked Immunosorbent Assay

An ELISA is a quantitative, solid-phase immunological method for the measurement of an analyte following binding to an immunosorbent and its subsequent detection using enzymatic hydrolysis of a reporter substrate either directly (analyte has enzymatic properties) or indirectly (e.g., horseradish peroxidase- or alkaline phosphatase-linked antibody subsequently bound to the immunosorbed analyte). The analyte usually is quantitated by interpolation against a standard curve of a reference material. General information chapter *Immunological Test Methods—Enzyme-Linked Immunosorbent Assay (ELISA)* (1103) discusses ELISA in greater detail, including ELISA development for quantitative analysis.

Western Blotting

A Western blot is a semiquantitative or qualitative method for measurement of a protein analyte that has been resolved by polyacrylamide gel electrophoresis and subsequently

transferred to a solid membrane (e.g., nitrocellulose, nylon, or polyvinylidene difluoride). Detection can be achieved directly by reacting with a labeled primary antibody (antibody specific to the analyte of interest) or indirectly by reacting labeled secondary antibody (antibody against the primary antibody) to the primary antibody bound to the membrane-immobilized antigen. The label can be a radioisotope or an enzyme that uses the substrate to produce color, fluorescence, or luminescence. This method is semiquantitative, especially when proteins are present in low concentration and in very complex mixtures. It is commonly used in early process development (e.g., antibody screening, protein expression, protein purification, etc.). Western blotting is a powerful method for analyzing and identifying proteins in complex mixtures, particularly after separation using 2-dimensional gel electrophoresis, which separates proteins based on size and charge (pI).

Flow Cytometry

Flow cytometry is a laser-based semiquantitative technology that permits measurement of fluorophore-conjugated probes as they interact with their respective ligands on cells or particles. More details for flow cytometry can be found in *Flow Cytometry* (1027).

Surface Plasmon Resonance

SPR is a quantitative method for measurement of an analyte in a sample where the antibody–antigen complex formation can be measured in real time at the interface of a liquid and solid (e.g., gold surfaces or particles). The measurement taken is the real-time change in refraction of a polarized light and occurs during the formation of the antibody–antigen complex, resulting in changes to the plasmon resonance minima (i.e., the sensorgram). The

quantity of analyte is determined by comparison to the measurement of a reference standard curve determined in the same assay. More details for SPR can be found in general information chapter *Immunological Test Methods—Surface Plasmon Resonance* <1105>.

Rate Nephelometry

Rate nephelometry is a quantitative method for measurement of an analyte in a sample in solution by measuring the light scatter introduced by small aggregates formed by the antigen–antibody complex. The quantity of analyte is determined by comparison to the measurement of a reference standard curve determined in the same assay.

Radioimmunoassay

RIA, a sensitive ITM first developed in the 1950s, is a quantitative method for measurement of an analyte in a sample. RIA usually uses a competitive antibody–antigen binding reaction, but it also can be used in sandwich immunoassay format, including immunoprecipitation. In competitive RIAs the analyte competes for binding with a radio-labeled (e.g., using ^{125}I or ^3H) reference antigen that is identical to the analyte; therefore, the analyte and the antigen both compete for binding to a fixed and limiting dilution of a specific (often polyclonal) antibody. The radio-labeled antigen is present in excess. The same unlabeled antigen in the test sample competes in binding to the same site on the antibody, which is present in a fixed quantity. Binding of the unlabeled antigen to the antibody leads to the displacement of the labeled antigen, resulting in a decrease in the radioactivity of the antigen–antibody complex fraction. To separate the antigen–antibody complex from the excess unbound antigen, the complex generally is either precipitated with a secondary antibody (or protein G) immobilized on a solid matrix (e.g., glass or resin beads) or with an already immobilized primary antibody. The quantity of analyte usually is determined by interpolation against a standard curve of a reference material, where a fixed amount of antibody and radiolabeled antigen is mixed with an increasing amount of unlabeled antigen. Hence, even a small quantity of unlabeled antigen will result in a relative quantitative decrease in total bound radioactivity.

Single Radial Immunodiffusion

Single radial immunodiffusion (SRID or SRD) is a quantitative method for measurement of an analyte in a sample by

measuring the diameter of the ring of precipitin formed by the antigen–antibody complex. Antigen is applied to a well in a gel infused with a constant level of antibody. Solutions with higher concentrations of antigen diffuse farther before being saturated with antibody and then precipitated. The quantity of analyte is determined by comparison to a reference standard curve measured by the same assay.

Precipitation

The underlying principle for this method is that the interaction of a multivalent antibody and antigen leads to the formation of a complex. In some cases a visible precipitate is formed. Other immunoprecipitation techniques involve the use of Protein A or Protein G beads to capture the antigen–antibody complex and facilitate the separation of the antigen–antibody complexes from the other antigens in the solution. Precipitation is not commonly used for quantitative analytical purposes because of the time required (days to complete), lack of sensitivity, and requirement for large quantities of antigen and antibodies.

Agglutination

Agglutination and inhibition of agglutination, respectively, provide qualitative and quantitative measures of certain antigens and antibodies. Inhibition of agglutination is a modification of the agglutination reaction that provides higher sensitivity to detect small quantities of proteins, chemicals, viruses, and other analytes. The principle of agglutination is similar to that for precipitation except that the interaction takes place between an antibody and a particulate antigen and leads to a visible clump or agglutination. The most common example of this application is for blood typing (i.e., A, B, or O antigen).

CHOICE OF ITM

When choosing an ITM, analysts should consider sensitivity and specificity as well as the complexity of the sample. *Table 1* provides an assay developer with a comparative view of the advantages and disadvantages of a variety of ITM formats. The intended application of the ITM should govern the choice of the most suitable format.

Table 1. ITMs Used in Biopharmaceutical Laboratories

Method	Advantages	Disadvantages	Typical Industry Uses
ELISA	<ul style="list-style-type: none"> • High sensitivity • Often wide dynamic range • High throughput • Low cost 	<ul style="list-style-type: none"> • Multistage process highly dependent on proper execution of each stage • Wash steps add time and often biohazardous waste • Reagent labeling required 	<ul style="list-style-type: none"> • Potency assessment • Specific protein concentration analysis in complex samples • Protein identification • Purity assessment • Immunogenicity assessment
Western blot	<ul style="list-style-type: none"> • Gives information about antigen size and/or charge • Allows separation of various antigens (or degradation/aggregation products) bearing same epitope • Can tolerate complex mixtures 	<ul style="list-style-type: none"> • Typically works only with linear epitopes • Labor intensive • Low throughput, output • Subject to interpretation • Immobilization can alter binding • Limited to proteins 	<ul style="list-style-type: none"> • Protein purity assessment • Protein stability assessment • Protein identity test
Flow cytometry	<ul style="list-style-type: none"> • High throughput • Highly automated 	<ul style="list-style-type: none"> • Use limited to cells, particles, and samples bound to beads • Sensitive to aggregates and sample matrix 	<ul style="list-style-type: none"> • Potency assessment • Cell identity in cell-therapy products

Table 1. ITMs Used in Biopharmaceutical Laboratories (Continued)

Method	Advantages	Disadvantages	Typical Industry Uses
SPR	<ul style="list-style-type: none"> • Direct detection of binding • Can measure affinity precisely, including on and off rates 	<ul style="list-style-type: none"> • Immobilization can alter binding • Regeneration can alter binding • Low throughput, output 	<ul style="list-style-type: none"> • Immunogenicity assessment • Potency assessment • Specific protein concentration analysis in complex samples
Rate nephelometry	<ul style="list-style-type: none"> • Easily automated • Rapid 	<ul style="list-style-type: none"> • Small detection range • High background for turbid samples 	<ul style="list-style-type: none"> • Assay for individual vaccine components for check of stability and purity
RIA	<ul style="list-style-type: none"> • Binding occurs in native conformation • Low-concentration samples can be analyzed • High sensitivity antibody used at limiting dilution that conserves reagent • Can be plate-based for higher throughput (e.g., scintillation proximity assays) 	<ul style="list-style-type: none"> • Requires radioactive labeling for detection • Shorter half-life of some radioisotopes requires periodic preparation of the tracer • Hazardous waste 	<ul style="list-style-type: none"> • Protein identification (e.g., hormones) • Specific protein concentration analysis in complex samples
SRD	<ul style="list-style-type: none"> • Precise • Simple setup 	<ul style="list-style-type: none"> • Semiquantitative • Low precision • Low sensitivity 	<ul style="list-style-type: none"> • Vaccine release test
Precipitation	<ul style="list-style-type: none"> • Low equipment cost 	<ul style="list-style-type: none"> • Subject to interpretation • Slow • Poor sensitivity (μg range) 	<ul style="list-style-type: none"> • Vaccine identification
Agglutination	<ul style="list-style-type: none"> • Rapid • Low equipment cost 	<ul style="list-style-type: none"> • Subject to interpretation • Slow • Low specificity because of interfering substances 	<ul style="list-style-type: none"> • Vaccine identification

KEY CONSIDERATIONS IN ITM DEVELOPMENT

The goal during method development is to produce an accurate assay that is practically feasible and possesses an acceptable degree of intra- and inter-assay precision. To minimize the overall imprecision, the sources of variability should be identified and minimized.

Reagent Selection

Immunoassays are subject to several sources of interference such as cross-reactivity, endogenous interfering substances, buffer matrices, sample components, exposed versus masked epitopes, conformation changes in the antigen of interest, and other factors. Hence, during method development, analysts must identify possible sources of interference both to develop a robust method and to aid future troubleshooting.

Cross-reactivity is a major obstacle during immunoassay development. This arises when the specificity of an antigen–antibody reaction is compromised by the cross-reactivity binding of structurally similar molecules with the reaction binder. Some common examples are protein isoforms, degraded analyte entities, molecules of the same class, precursor proteins, metabolites, etc. Cross-reactivity can be minimized by rigorous reagent characterization and selection.

Reagents used in ITM applications generally fall into one of two categories: critical reagents and noncritical reagents. Critical reagents are specific and unique to the particular ITM or reagents that are intolerant of very small changes in composition or stability. Examples of critical reagents generally include assay-specific antibodies and reference or method calibration standards. Equivalence in the assay format must be established before replacement with a new lot. Noncritical reagents are those that can vary to some degree in composition without adversely affecting ITM performance. Reagents are often assumed to be noncritical (e.g., buffers, water quality, blocking buffer, or substrate) but later may be identified as critical components if assay ruggedness fails and troubleshooting of ITM reagents begins. ITM-spe-

cific reagents, including vendor and catalog number, should be defined in test procedure documents.

Antibody selection is critical for development of a successful immunoassay because it defines the assay's specificity and sensitivity. Furthermore, during antibody generation, analysts should ensure that the immunization protocols support the end use of the antibodies. For some applications a more specific antibody can be generated by the selection of a small and specific immunogen and affinity purification of the antibody, resulting in highly defined epitope coverage. In other applications it may be critical to ensure broad coverage of the different available epitopes on the molecules of interest, and a polyclonal antibody (pAb) pool may be the best choice. Currently, monoclonal antibodies (mAb) are preferred for some applications for the detection of single analytes because of their high specificity, lot-to-lot consistency, and indefinite supply. Compared to polyclonal antibodies, mAb have a higher initial cost to produce, but for these applications, the advantages generally outweigh the initial cost. Other applications may require more comprehensive epitope selection to ensure that subtle changes in the molecule(s) do not prevent recognition of the entire antigen, and thus a pool of monoclonal antibodies, or a pAb pool, would be the preferred choice. The latter are widely used for detection in a complex mixture of analytes (e.g., host-cell proteins). Similarly, immunoassays may use two distinct epitopes on an antigen—one for capture and the other for detection—which greatly reduces cross-reactivity. Another approach to minimize cross-reactivity is to purify the antigen before immunoanalysis. Variations in incubation temperature and time can affect the reaction kinetics of antibody interactions with similar yet different antigens. Thus this property should be optimized to increase the specificity of antigen–antibody interactions.

Development of Immunoassays

Development is an important stage in the establishment of a suitable ITM. During development of an ITM, analysts explore various settings of assay parameters and interactions between parameters to identify conditions under which the assay will consistently produce reliable results using minimal reagents, effort, and time. In Quality by Design terminology,

the “possible operating space” is the collection of settings of assay parameters explored, and the “design space” refers to the conditions under which the assay performs well. The necessary performance properties of the ITM (precision, accuracy, specificity, etc.) required depend on the intended use(s). During ITM development, analysts should consider the following:

- Antigen–antibody ratio;
- In sandwich immunoassays, the ratio of capture antibody to detector antibody;
- Antigen–antibody reaction kinetics in the sample matrix (antigen–antibody binding generally is not linear);
- Selection of the standard (full-length antigen for the standard or just a small portion of the antigen containing the antibody-binding epitope, among other considerations); and
- Matrix effects.

The use of design of experiments (DOE) is strongly recommended, and different DOE methods may be appropriate in each stage of development. Early in development, screening designs are particularly useful (generally two-level geometric fractional factorial designs). After screening (with a modest number of factors to study), full factorials or response surface designs are often appropriate. As development activities shift to qualification (ideally, if not typically, as the focus shifts to robustness), robust response surface designs often are a good choice. During qualification or validation, analysts may find it practical to simultaneously study robustness to assay operating conditions (using a small geometric fractional factorial) and validation parameters such as precision (via nested or crossed designs for random factors associated with repeatability, intermediate precision, and reproducibility).

Experiments that assess dilutional linearity and components of specificity, including matrix effects, usually involve construction of spiked samples. Although spiking often is performed in a dilution matrix, spiking a collection of actual samples or mixing actual samples is an important component of demonstrating robustness of dilutional linearity and components of specificity to the sample and matrix components.

Reagent Considerations

A procedure for qualifying reagent sources and vendors (including audits), ordering, receiving, and disposing of commercial reagents and consumables should be outlined in a standard operating procedure (SOP). The preparation of internal reagents must be documented in a manner that allows reconstruction. Commercial and internally prepared reagents must be labeled with identity, concentration, lot number, expiration, and storage conditions. The stability and assignment of expiration dates for internally prepared reagents often are based on available literature and scientific experience, but analysts may need to confirm these empirically. An SOP for extending expiration dating of critical reagents is recommended. In addition, analysts should implement a mechanism for reagent tracking and linking lot numbers to analytical run numbers. Unacceptable reagent performance is detected by tracking QC samples. Shifts in QC samples should prompt a review of analytical runs and changes in reagent lot numbers or review of possible deterioration of critical reagents. To avoid such shifts, analysts can cross-validate critical reagent lot changes.

The impact of collection and storage containers on analytical performance often is overlooked. When defining the stability and expiration of in-house reagents, analysts should record information about the storage container vendor, catalog, and lot number. The importance of a suitable reference standard and its characterization cannot be overemphasized for ITMs for biological products. Because of their inherent complexity, reference and calibration standards of macromolecular biologics often are less well characterized than are conventional small-molecule drug reference stan-

dards. If the calibration standard represents a mixture of different antigens (e.g., host-cell proteins), it should be shown to be representative of the antigen profile in the samples being tested. Consistency in ITM results depends on the availability of a suitable representative reference standard material.

VALIDATION

Analytical validation involves the systematic execution of a defined protocol and prespecified analysis that includes prespecified acceptance criteria. A validation demonstrates that an analytical method is suitable for one or more intended uses [see *Validation of Compendial Procedures* (1225), *Biological Assay Validation* (1033), and ICH Q2(R1)]. Qualification may involve similar or identical experiments and procedures as validation, but qualification does not require prespecified protocols, analyses, or acceptance criteria. In certain situations (e.g., use of a commercial kit), assay development may not be required before qualification. General information chapter (1225) discusses which assay performance characteristics must be examined during validation for four primary categories of intended uses. For example, analytical procedures that quantitate major bulk drug substances or active ingredients may not require validation of the detection and quantitation limits but do require validation of accuracy, precision, specificity, linearity, and range.

System Suitability or Assay Acceptance Criteria

The purpose of system suitability or assay acceptance criteria is to ensure that the complete system—including the instrumentation, software, reagents, and analyst—is qualified to perform the intended action for the intended purpose. All processes should be controlled by well-defined SOPs that ensure consistency, reduce errors, and promote reproducibility of laboratory processes. Training files for all personnel should be contemporaneous and should include some demonstration that analysts are qualified to perform the method and the specific ITM.

Instrument and software qualification begins with a definition of the design qualifications, including a risk assessment and gap analysis that identify potential threats to the collection, integrity, and permanent capture of ITM data. Qualification also includes installation qualifications (IQ) and operational qualifications (OQ). Purchased commercial instrument validation packages may require modification to meet the intended use at each facility. Instrumentation and software should be continuously monitored for acceptable functionality by performance qualification (PQ) and software validation test script reviews. Routine instrument maintenance is performed according to the manufacturer's recommendations, and additional maintenance may be required based on specific needs in the working environment. A complete history of routine and nonroutine instrument maintenance should be archived for each instrument. Software updates should be handled with change control and typically require additional validation. Adherence to 21 CFR 11 should be maintained.

To ensure robustness, establish a defined process for implementing new ITMs in the laboratory. Control documents should be in place, including method validation plans containing a priori method acceptance criteria and validation reports for the establishment of a new ITM. Well-written analytical test method documents are needed to ensure reconstruction of analytical results and to minimize laboratory errors.

Analytical test methods should include acceptance criteria for critical aspects of the assay, including the performance of the calibration curve, quality controls, agreement between sample replicates, procedures for repeat sample analysis, and identification and treatment of outliers, when ap-

plicable. Furthermore, an SOP should be implemented for unexpected event investigation and resolution.

DATA REPORTING

Units of Measurement

Quantitative ITMs generate test sample data with an estimated concentration based on a calibration curve fit to reference (or standard) samples using an appropriate mathematical model. When determining the amount of analyte in a manufacturing process, analysts often express the unit of measure in terms of mass of analyte per volume of solution (concentration) or mass of analyte per mass of product (e.g., parts per million). Depending on the nature of the measured analyte, the degree of measurement standardization, the geographic region, and the history of the method, analysts may express concentration in terms of weight per volume, mole per volume, or weight of analyte per weight of product. In some circumstances, concentration may be converted to an activity unit of measure in which the analyte mass is assumed to be 100% active. In certain circumstances, qualitative analysis using a predetermined cut-off value may be an acceptable alternative to quantitative methods.

Immunoassay Data Analysis

ITMs employ calibration curves prepared with reference standards of known (nominal) concentrations and are included in every bioanalytical method. This helps control variation associated with repeatability, intermediate precision, and reproducibility and permits the estimation of results for unknown test samples. Common simple statistical analyses assume that the (possibly transformed) data are normally distributed, have constant variance, are independent, and that an appropriate model has been used. For many assays, one or more of these assumptions may be inappropriate. Analysts should assess these assumptions using a substantial body of data (typically tens of assays). When these assumptions are not reasonable, the analysis becomes more complex.

Calibration curves generally are characterized by a nonlinear relationship between the mean response and the analyte concentration and typically are plotted in a log-linear manner with the (possibly transformed and/or weighted) response variable (ordinate) plotted against the nominal calibrator concentration (abscissa) in log scale. The resulting curve that encompasses the assay's validated range is inherently nonlinear and often has a sigmoid shape with horizontal asymptotes at very low and high concentrations of analyte. Competitive ITMs have a negative slope, and noncompetitive ITMs are characterized by a positive slope. The analyte concentration in a test sample is estimated by inverse regression against the calibration curve. The final result often is obtained after multiplication of the estimated concentration in the assay by a dilution factor that is required to yield a response within the ITM's quantification range.

Under the guidance of a qualified biostatistician, analysts can implement outlier tests in controlled documents that permit the exclusion of spurious sample results. A well-defined procedure should be in place regarding how to identify, repeat, and report outliers. Outlier tests and interpretation of results are described in *Analytical Data—Interpretation and Treatment* (1010). Test results that fall outside of their predefined specifications or acceptance criteria should be evaluated by an out-of-specification investigation to identify a root cause.

Trending

A quality system includes monitoring of ITM performance by collection and review of ITM performance characteristics. Trending may detect shifts in assay performance that may be related to events such as assay reagent lot changes, addition of new analysts, shifts in environmental conditions, and others. SOPs, study protocols, analytical test methods, and decision flow charts are recommended to strictly define the handling, use, editing, rejection, acceptability, and interpretation of calibration data and test sample results for ITMs. It is not uncommon to have several raw data reviews, including peer, QC, and quality assurance review. Analysts must be able to distinguish such analytical issues from true changes in the measured analyte caused by changes or errors in the manufacturing process that have affected the product. Two of the most important outcomes of proper trend monitoring are detecting potential problems before they occur and identifying areas for corrective and/or preventive action. General information chapters *Analytical Data—Interpretation and Treatment* (1010) and *Biological Assay Validation* (1033), as well as the statistical literature, contain guidance for various trending methods. Several ITM performance characteristics could be considered for monitoring. The most common trending value is evaluation of QC samples. Ideally, one or more QC sample is available for long-term trending in sufficient quantity and with demonstrated stability so that quality aspects can be assayed in every run and across multiple manufacturing lots. As the long-term QC sample is depleted or expires, crossover comparison and establishment of a new long-term QC sample should be completed. Systematic review of QC data across assays assists in troubleshooting failed ITM runs, providing confidence in the evaluation of spurious results, and controlling the introduction of replenished assay components that may not perform exactly like previous reagents.

Other ITM performance characteristics that may be monitored include calibration curve response variables, curve fit parameters, assay background, and comparison of in-study QC data with validation data.

Tracking

Regulatory agencies have strict requirements about maintaining the identity and integrity of both samples and data. A quality process driven by SOPs must be implemented to ensure the correct identity and integrity of test and reserve samples. Ideally, a bar code system should be used to track the collection, identity, location, chain of custody, number of sample freeze/thaw cycles, storage temperature, and length of time that a sample is stored. This information should be captured and should be auditable from the time of collection to disposal (or sample depletion). The ability to track the sample history permits reconstruction of the events leading to generation of a data result. This information is used by regulatory agencies to ensure that the proper procedures were followed and by internal auditors to ensure that pre-analytical sample handling did not compromise study data. In addition, sample tracking allows a mechanism for ensuring that the analyte measurement occurred within the demonstrated window of stability for that analyte.

The final result generated from a bioanalytical laboratory is a number that represents an analyte measurement in a test sample. The steps necessary to generate that data and preserve it in a report are numerous and are susceptible to error. Therefore, quality systems must be in place to minimize data errors. Errors may be introduced by test sample misplacement or identification, incorrect data reduction, miscalculations, transcription errors, omissions, and other factors. Ideally, validated software and laboratory information management systems are used when possible to generate, transfer, and archive data. Typically, redundancy checks are built into automated processes by visual data review of

at least 10% of the data-transfer processes. In the absence of validated electronic transfer, all data should be reviewed by at least one reviewer. As with sample tracking, data generation, manipulation, and storage should be reconstructible. In addition, all data should be backed up using a format that is stable. Plans should be in place to update archived data so that, as technology changes, archived data can still be retrieved. Regulatory agencies require that raw data be available for various lengths of time after the completion of a study or regulatory filing. Finally, data must be secure from corruption, alteration, or access by unauthorized personnel.

<1103> IMMUNOLOGICAL TEST METHODS—ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

INTRODUCTION

Immunological Test Methods (ITM) utilize binding between an antigen (Ag) and antibody (Ab). (See *Appendix 1* for a complete list of acronyms used in this chapter.) Enzyme-linked immunosorbent assay (ELISA) is one of the most widely used ITM for characterization, release, and stability testing of biotechnology products to help ensure the quality of biological drug substances and drug products. The term ELISA is used here in a broader sense and includes enzyme immunoassays (EIA), as well as alternative detection methods, e.g., chemiluminescence and fluorescence.

This chapter provides analysts with general information about principles, procedures, experimental configurations, assay development, and validation for solid-phase ITM like ELISA and can be used for the other immunoassay variations mentioned above. The chapter also covers reference standard(s) and control(s) used for immunoassays. The information can be adapted to the specific procedures of a monograph. This chapter does not cover immunoassays for the measurement of immune responses to product in animals or humans (e.g., serological or cellular assays), non-immunoassays (e.g., receptor-ligand interactions), or other related approaches.

The chapter is part of a group of general information chapters for immunological test methods [*Immunological Test Methods—General Considerations* <1102>, *Immunological Test Methods—Immunoblot Analysis* <1104> (proposed), and *Immunological Test Methods—Surface Plasmon Resonance* <1105>], and also is related to the general information chapters for bioassays [*Design and Development of Biological Assays* <1032>, *Biological Assay Validation* <1033>, and *Analysis of Biological Assays* <1034>].

Definition

ELISA can be defined as a qualitative or quantitative solid-phase immunological method to measure an analyte following its binding to an immunosorbent surface and its subsequent detection by the use of enzymatic hydrolysis of a reporter substrate, either directly (as with an analyte that has enzymatic properties or is directly labeled with an enzyme) or indirectly (by means of an enzyme-linked antibody that binds to the immunosorbed analyte). Qualitative results provide a simple positive or negative result for a sample. Converting quantitative to qualitative results based on a cutoff

value that separates positive and negative results is common practice. Because the performance properties of the assay depend heavily on the cutoff value, the process used to determine the cutoff should be evidence-based and well documented. Quantitative assays determine the quantity of the analyte based on the interpolation of a standard calibration curve with known analyte concentration, run simultaneously in the same assay. This standard should be an appropriate, preferably homologous, reference or calibration material that is representative of the analyte(s) of interest. The power of immunoassays has been demonstrated by the variety of procedures that have evolved, including alternative solid surfaces such as beads of different sorts, various plastics in plates of different configurations, and alternative detection methods, e.g., chemiluminescence and fluorescence. ELISA assays are widely used in the biopharmaceutical industry for various applications such as identity, purity, potency, detection or quantitation of antibody or antigen, and other purposes.

Basic Principles

The essential steps of an ELISA can be broken down as follows (see *Figure 1*):

1. Binding of the capture reagent (generally an antibody or antigen), which functions as an immunosorbent for capture of the analyte, to a solid surface;
2. Removal of excess, unbound capture reagent followed by blocking of unoccupied binding sites with a blocking protein such as albumin, gelatin, casein, or other suitable material;
- 3a. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and detection of the analyte. Direct detection occurs when the analyte has enzymatic activity or has been linked to a detector molecule (e.g., enzyme); or
- 3b. Incubation of the analyte (in the test sample or reference standard) with the capture reagent to bind the analyte onto the solid surface, followed by the washing away of unbound material in the test sample and subsequent detection of the analyte (*Figure 1*, step 3a). Indirect detection occurs when the analyte is detected by the addition of a secondary enzyme-labeled reagent (*Figure 1*, step 3b); and
4. Quantification of the analyte by addition of a substrate suitable for the detector used (e.g., TMB, 3,3', 5,5'-tetramethylbenzidine), followed by comparison of the test sample to the reference standard.

ASSAY DESIGN

Five general categories of ELISA are described in *Table 1* and in the sections that follow. The assay designs are flexible and, depending on specific needs, can be modified from these procedures. The choice of format depends primarily on the amounts and purity of reagents and equipment available. On some occasions the analyte being characterized actually is an antibody, as in the case of a monoclonal antibody that is being developed as a drug. In this case, anti-idiotypic or other antibodies specific for the antibody are used to develop the assays.

Direct ELISA

Directly Labeled Antibody: In this assay an antigen is coated onto a solid surface and the remaining unbound reactive sites are blocked (*Figure 2A*). Then a solution containing a specific antibody labeled with a detector is added. After incubation, the unbound antibody is washed away,

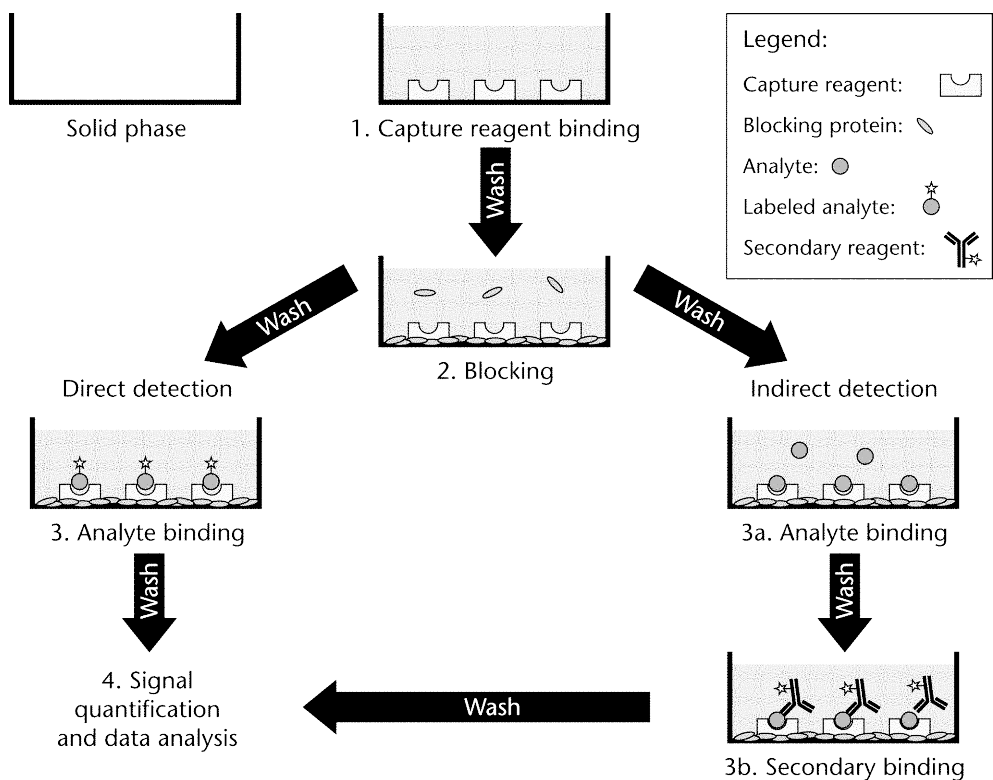


Table 1. Representative ELISA Types

ELISA Type	Required Reagents	Attributes	Disadvantages
Direct detection	<ul style="list-style-type: none">• Capture analyte^a• Labeled primary antibody specific for antigen	<ul style="list-style-type: none">• Rapid because only one antibody is used• Uses less reagent• Analyte is immobilized	<ul style="list-style-type: none">• May modify the conformation of the analyte• Sensitive to matrix and adjuvant components• Not commonly used• Poor sensitivity
Indirect detection	<ul style="list-style-type: none">• Capture analyte^a• Primary antibody specific for antigen• Labeled secondary detector antibody that binds to the primary antibody	<ul style="list-style-type: none">• Versatile because a variety of primary antibodies can be used with the same secondary detector• Improved sensitivity because of signal amplification• Analyte is immobilized	<ul style="list-style-type: none">• Longer because of more incubation and washing steps
Competitive	<ul style="list-style-type: none">• Analyte^a can be used as a capture reagent or can be labeled with a detection label• Antibody specific for analyte can be used for capture or labeled for detection• Labeled secondary antibodies to bind to primary antibody if an indirect format is used	<ul style="list-style-type: none">• Good for assessing antigenic cross-reactivity• Appropriate for smaller proteins with single epitopes• Requires only a single antibody• Analyte in solution competes for binding to primary antibody	<ul style="list-style-type: none">• Format difficult to troubleshoot• Limited dynamic range
Sandwich	<ul style="list-style-type: none">• Primary capture antibody specific for analyte• Sample solution containing analyte^a• A different primary enzyme-antibody conjugate specific for analyte	<ul style="list-style-type: none">• Improved sensitivity• Good for quantitative assays for larger multi-epitope molecules• Analyte measured in solution	<ul style="list-style-type: none">• Requires relatively large amounts of pure or semipure specific antibody• Not suited for smaller proteins that may have only a single epitope or a few closely spaced epitopes

^a This reagent can be either purified or partially purified. The terms “analyte” and “antigen” are used interchangeably when describing ELISAs.

¹ Capture reagent binding, blocking, analyte binding, detector antibody binding, and analysis are the five basic steps in an ELISA. Capture reagent binding, blocking, and analyte binding steps are each followed by a washing step to remove unbound reagents before the addition of the next reagent. Before analysis an appropriate substrate is added, followed by measurement of the substrate by appropriate equipment for detection. Quantitation of unknowns takes place by comparison with a standard curve.

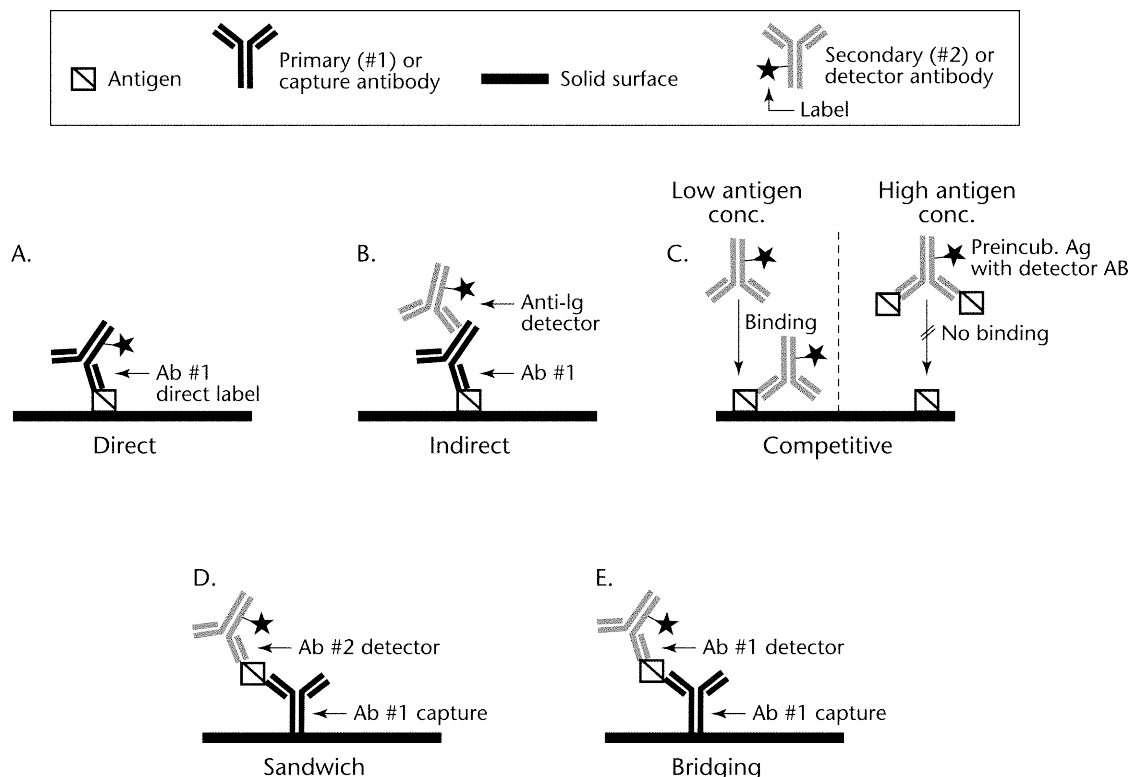


Figure 2. Schematic representations of direct, indirect, competitive, sandwich, and bridging ELISAs.² (Ab = antibody; Ag = antigen (or analyte); Preincub = preincubation)

followed by the addition of an appropriate substrate for the detector used.

Directly Labeled Antigen: This assay is similar to that using a directly labeled antibody except that the antibody is coated onto the solid surface and a labeled antigen is used as the detector.

Indirect ELISA

In this assay an antigen is coated onto a solid surface and then, after blocking, a solution containing a specific antibody is added (Figure 2B). After incubation, the unbound antibody is washed away, followed by the addition of an anti-immunoglobulin (anti-Ig) detector antibody. Anti-Ig detectors are available commercially for specific Ig classes and subclasses from a variety of species, which makes this assay format useful for isotyping of antibodies. In addition, the use of a labeled anti-Ig detector amplifies the signal compared to a *Direct ELISA*, thereby increasing assay sensitivity.

Competitive ELISAs

Direct Antibody Competitive ELISA: This assay is used to detect or quantitate soluble antigens (Figure 2C). It requires an antigen-specific antibody that has been conjugated to an appropriate detector, e.g., horseradish peroxidase, alkaline phosphatase, ruthenium, or fluorescein. It also requires a purified or partially purified antigen for coating. The antigen is coated onto a solid surface, followed by a blocking step. The antibody–conjugate is incubated with the test solution containing soluble antigen. The mixture is then added to the immobilized antigen, incubated, and unbound antigen–

antibody complex is washed away. Substrate is added, and the inhibition of the reaction (e.g., colorimetric, electrochemiluminescence, fluorescence, or chemiluminescence) is measured relative to the reaction when no competitor antigen is added. The amount of inhibition is inversely proportional to the amount of antigen in the test sample. Competitive assays can also measure small molecules by coating an antibody to the plate that is specific to the small molecule. The small molecule is often biotinylated with a long linker that does not interfere with binding between the capture antibody on the plate and the small molecule. Antigen (the small molecule) in the sample then competes with the labeled small molecule for binding to the capture antibody. After washing, a detection reagent (e.g., streptavidin labeled with HRP) is added to detect the binding complex.

Direct Antigen Competitive ELISA: This assay is similar to the *Direct Antibody Competitive ELISA*, except that it is used to detect soluble antibodies. The antigen is conjugated to the detector and the antibody is coated onto the solid surface.

Indirect Antibody Competitive ELISA: This assay is similar to the *Direct Antibody Competitive ELISA*, except that instead of directly labeling the antibody, the test uses a labeled anti-Ig reagent for detection.

Indirect Antigen Competitive ELISA: This assay is similar to the *Direct Antigen Competitive ELISA*, except that instead of directly labeling the antigen, the test uses a labeled secondary antibody for detection.

Sandwich ELISA

Direct Sandwich ELISA: In this assay an antibody is immobilized onto a solid surface and blocked, and then a solution containing a specific antigen is added (Figure 2D). After an incubation step, the unbound material is washed away, and a labeled detector antibody is added. This assay format requires two antibodies, each of which binds to different

² The type of ELISA format depends on the availability of reagents, the intended purpose of the assay, and the physicochemical characteristics of the analyte of interest. For a *Bridging ELISA*, the capture and detector antibodies recognize the same epitope, and therefore the target antigen must have at least two epitopes available for binding.

epitopes on the surface of the large and complex molecule. The two antibodies are specific for the antigen, and the antigen should be sufficiently large and complex to accommodate the binding of two antibodies.

Indirect Sandwich ELISA: Alternatively, instead of directly labeling the detector antibody, an anti-Ig antibody detector can be used. Indirect sandwich immunoassay formats can be considered only if each binding reagent is from a unique species (e.g., a sandwich assay using two mouse monoclonal antibodies for capture and detector could not be detected indirectly because the resulting signal may become independent of the antigen concentration).

Bridging ELISA: This subset of *Sandwich ELISA* assays often uses a single antibody for both capture and detection (*Figure 2E*). If a monoclonal antibody is used, it requires that the target antigen have at least two identical epitopes that are adequately spaced to prevent steric hindrance so that one epitope binds to the capture antibody and the other epitope binds to the detector antibody. Alternatively, a polyclonal antibody can be used but still requires that the target antigen be large enough to accommodate the binding of two antibody molecules. With respect to specificity and sensitivity, bridging assays usually are suitable for most large molecules.

CHOICE OF ASSAY

Deciding which ELISA procedure or format to use often depends on individual choice and availability of reagents, instruments, and other equipment. For example, sometimes a laboratory repeatedly engineers a particular epitope into multiple fusion proteins. In this case, the laboratory can use certain common qualified reagents (e.g., an antibody to a glutathione S-transferase region in multiple fusion proteins), facilitating rapid sandwich immunoassay development. Small antigens with a limited number of epitopes available for antibody binding restrict ELISA format choices. If there is only one binding epitope, then ELISA methods that use the sandwich/two-site binding or other bridging formats cannot be used because they require at least two available epitopes for antibody binding. In addition, small molecules are not usually used as a capture reagent on a plate because the process may interfere with binding to the detection reagent. Examples of such small molecules are some peptides, oligosaccharides, nucleotides, and antibacterials. Analysts usually adopt a competitive assay format for such small analytes.

Different assays and formats may demonstrate different properties and characteristics, e.g., specificity, precision, accuracy, sensitivity, dynamic range, dose-response ratio, sample throughput, sensitivity to interference, and simplicity or efficiency for automation. Ease of validation also may vary between different assay protocols and formats. Assay designs with replicates in adjacent wells could be biased if there are location effects; hence, in this case, replicates should not be in adjacent wells. Assay designs that are convenient to perform on 96-well plates, using relatively few single-channel pipet actions and more multi-channel pipet actions, are usually easier to adapt to automation. Assays with steep dose-response curves are generally better able to deliver high precision estimates; however, some assays with steep dose-response curves are imprecise in the EC_{50} and require a wider dose range.

PROCEDURES

Solid Phase

Solid phases are available in a variety of forms (e.g., membrane, plate, or bead) and chemistries (e.g., nylon, nitrocellulose, polyvinylidene fluoride (PVDF), polyvinyl, polystyrene, or a chemically derivatized surface). The selection of the

solid phase determines the most likely binding mechanism, i.e., hydrophobic, hydrophilic, or covalent interactions. In general, compared to plates, beads offer higher capacity and are more commonly used in clinical assays whereas plates are more commonly used to test biotechnology products. Additional information on plates is provided below.

Coating the Solid Phase—Immobilization of Capture Reagent: Capture reagents are coated onto a solid phase by adding a solution containing the capture reagent to the surface. The most commonly used solid-phase materials for capture reagent immobilization are plastic 96-well microtiter plates. Those with flat-bottom wells are recommended for spectrophotometric readings, and round-bottom well plates are useful for visual assessment of a dye's color development. The degree of coating is influenced by the concentration of capture reagent, temperature during coating, duration of capture reagent adsorption, the surface properties of the solid-phase material, and the nature of the buffer of the capture reagent solution. Although the optimum coating concentration must be determined for each capture reagent, concentrations of 1–10 $\mu\text{g}/\text{well}$ are most commonly used. The volume of capture reagent added to each well usually corresponds to the sample volume that will be analyzed, i.e., 50–100 μL . Coating duration, temperature, and buffers are discussed separately below. During the coating procedure analysts should avoid introducing bubbles. Proteins that bind to plastic can be denatured, which alters antigenicity. In such cases, a capture antibody or an intermediary protein such as Protein A or Protein G can be used. In addition, streptavidin can be used if the reagent is biotinylated. The pH of the coating buffer should be optimized based on the isoelectric point of the capture reagent and the surface properties of the assay plate chosen.

Microtiter Plates: The composition and commercial source of the microtiter plate can influence binding of the capture reagent during coating. Several microtiter plates from different suppliers should be compared using a single coating procedure to select those that provide high specificity for the capture reagent of interest and low nonspecific background. Comparisons of different grades of plates from a single supplier also may be needed. Clear plates typically are used for colorimetric ELISA, and opaque plates often are used for chemiluminescent and fluorometric ELISA. Acidic capture reagents may require a lower pH solution to neutralize repulsive forces between the protein and solid phase. Peptides often require optimization of buffer pH based on their charge for optimal coating conditions during assay development. Polysaccharides, lipopolysaccharides, or glycoproteins may be difficult to coat directly to the plate and may require a capture antibody or a buffer that contains lysine or glutaraldehyde. Coating with an antibody can be enhanced by precoating the microtiter plate with Protein A or Protein G or a combination of the two, which allows binding to the Fc region so that the Fab portion can bind to the analyte of interest. However, care must be taken to ensure that subsequent secondary antibodies do not react with the Protein A- or Protein G-coated wells. In this case, for example, chicken IgY or another appropriate antibody class could be used. Microtiter plate formats other than the 96-well variety, such as half volume 96-well or 384-well plates, can be used to increase throughput and/or conserve reagents.

Coating Time: Coating time depends on binding kinetics, stability, concentration of capture reagent, and incubation temperature. Although different combinations of coating times and temperatures often result in the same coating efficiency, the stability of the capture reagent (which should be determined during method development) influences which conditions to select. Analysts must assess the impact of varying the coating time in order to determine the robustness of the assay procedure.

Coating Temperature: Coating temperature and time are closely related assay parameters. The coating temperature depends on the binding kinetics and stability of the antigen.

Higher temperatures can increase the rate of adsorption and may shorten the coating time, but they are likely to affect interaction sites and to reduce antigen-antibody affinity. Typical combinations of time and temperature are 1–4 h at ambient temperature, 15 min to 2 h at 37°, or overnight at 4°. Analysts should determine the effects of variations in temperature in order to assess the robustness of the assay procedure.

Buffers: Buffers used for diluents, coating, blocking, and washing plates can affect overall assay performance. Buffer components can interact with the test sample and inhibit binding. They also can cause low antigen sensitivity or high nonspecific background activity.

Diluent—Buffers [e.g., phosphate-buffered saline (PBS) or imidazole-buffered saline] with polysorbate 20 (0.01%–0.1%) are used commonly for different ELISA steps as a diluent and washing buffer.

Coating Buffers—Coating buffers should maximize assay consistency and promote binding of the capture reagent to the solid phase. Commonly used coating buffers include 50 mM carbonate, pH 9.6; 20 mM Tris-HCl, pH 8.5; and 10 mM PBS, pH 7.2. The choice of coating buffer depends on the nature of the individual antigens and should be determined empirically.

Blocking Agents and Buffers—A blocking agent is a compound (e.g., protein or detergent) that should saturate the remaining immunosorbent binding sites following capture reagent (antibody or antigen) binding. This reduces nonspecific binding of analyte and nonanalyte components to the immunosorbent matrix and/or the absorbed reagent. Nonspecific binding occurs when protein in the test sample binds to the plastic of the microtiter plate or absorbed reagent instead of specifically binding to the capture reagent of interest. Nonspecific binding can be reduced by adding blocking reagent to the wells and by the addition of another protein such as bovine serum albumin (BSA) to the dilution buffer. The choice of blocking agent should be governed by the nature of the capture reagent, plate, coating buffer, test sample diluent, and related factors. If any of these parameters changes, a change in blocking agent may be needed. Commonly used blocking agents include BSA, nonfat milk, gelatin, casein, normal horse serum, fetal bovine serum, polysorbate 20, and others. Several grades of BSA are available commercially, and the optimal grade should be empirically determined for each assay. In addition, many commercial blocking and assay diluent reagents are available for ITMs.

Adding Samples and Reagents

Samples and reagents generally are pipetted into the ELISA plate wells. Care should be taken to avoid cross-contamination, frothing, or bubbles. Labor-saving equipment such as electronic pipets, automated liquid handlers, plate washers, and robotic pipets also can be used to improve precision, reduce analyst-to-analyst variability, and increase throughput.

Pipets: Single, multichannel, and robotic pipets with set or fixed volumes are available. The type and accuracy of pipets should be evaluated for each application. Regular maintenance and professional calibration of pipets should be performed and documented.

Pipet Tips: A variety of pipet tips are available, some of which are specific to the type of pipet. The type and accuracy of the pipet tip, particularly related to the viscosity and nonspecific binding of the materials, should be evaluated for each application.

Washing

Wash steps are included throughout the ELISA procedure to remove the unbound coating antigen, sample, and de-

tection reagents. Washing is critical for assay performance, can be a source of assay failure, and is important to evaluate during method development. Multiple approaches can be used for washing. Manual procedures include using a squeeze bottle, dipping the microtiter plate in wash buffer, and adding wash buffer with a multichannel pipet or hand-held multi-channel (8- or 12-pin) manifolds. Analysts should wash carefully to avoid cross-well contamination. Automatic microplate washers generally provide more washing consistency. Strip-well and multiwell washers are available. Most automatic washers can be programmed for different dispensing volumes and speeds, number of washes, speed of buffer aspiration, and amount of residual buffer left in the well. Incorrectly programmed or maintained as well as incompletely cleaned automatic washers can cause assay variation and elevated assay background.

Incubation

ELISAs are incubated following the addition of samples and reagents. The optimal time, conditions, and temperature of each incubation step should be determined during method development. Incubation times vary from minutes to overnight. Commonly used incubation temperatures are ambient temperature, 4°, and 37°. ELISA plates commonly are sealed or placed in a secondary container to avoid evaporation or contamination during incubation. Atmospheric conditions such as dry or humidified incubation should be evaluated during method development. Rocking, shaking, or rotating the microtiter plates may be necessary or desirable depending on the kinetics of binding.

Blocking Conditions and Nonspecific Reactions

After immobilization and removal of the unbound antigen or antibody, unoccupied binding sites are blocked to ensure that the measured analyte in the test article or subsequent (detection) reagents does not bind nonspecifically to the solid surface or to the coated antigen or antibody. If nonspecific binding occurs, any reported signal could bias the measurement and may reduce the sensitivity and dynamic range of the assay. Blocking is critical to ensure the sensitivity and/or specificity of the assay. Sources of nonspecific binding fall into two general categories:

1. *Ionic or hydrophobic interactions* occur when binding is mediated by nonspecific ionic or hydrophobic interactions between assay reagents and the solid surface or another assay reagent.
2. *Immunological interactions* occur when binding is mediated by unintended antigen-antibody interactions. This occurs when antibody preparations used in the assay interact with other assay reagents. For example, if an ELISA was designed to test a serum-derived analyte using murine capture and detection antibodies, antibodies in the test article with reactivity to murine Ig (also known as heterophilic antibodies) could be nonspecifically detected in the assay.

The choice of blocking agent (examples are found in the *Blocking Agents and Buffers* section above) is determined empirically, and the balance between the reduction in nonspecific binding and the impact on assay sensitivity should be assessed during method development. Cross-reactivity with other assay reagents should be considered; for example, endogenous biotin is found in milk and serum, and serum may contain antibody to viral or bacterial proteins. Therefore, screening of serum lots may be necessary. The volume of blocking solution added to the well should be greater than the maximum reaction volume used for later steps so that all of the potential surface area that may interfere with the binding reaction is blocked.

In addition, Ig in the test materials can be removed by using buffers that inhibit antibody conformation or aggregate the heterophilic antibodies, by blocking with nonimmune serum, or by removing Fc regions in critical antibody

reagents, thereby reducing or eliminating undesired immunological interactions that cannot be addressed by the blocking reagents described above. Negative control wells can be included to monitor nonspecific reactions. The nature of the negative control wells depends on the assay but can include blocked wells without coating antigen, eliminating the primary or secondary antibody, or using buffer in place of sample. Control wells also can be useful as part of system suitability testing.

Pretreatment of Samples

Although ELISA methods are designed to measure an analyte in complex mixtures, the presence of other materials can prove problematic if they interfere with analyte detection. In order to ensure assay specificity, the specific procedure to treat samples to remove nonspecific interfering substances (e.g., reducing agents or precipitates) can be determined empirically during method development and then can be incorporated into the validated assay. Any sample-processing step should be evaluated against the potential that the treatment will alter the test article's properties and/or introduce further variability that results in biased measurements. Samples, standards, and controls should be prepared and handled in processes as similar to each other as possible. Analysts should verify that sample pretreatments have not damaged the sample so much that it can no longer be measured (e.g., by spiking experiments).

Detector Antibodies

Depending on ELISA format, detector antibodies labeled with enzyme or other labels can be used as primary or secondary reagents to enable detection of the immobilized analyte. In a direct or competitive ELISA (Figure 2A and Figure 2C), after the analyte is bound to the immunosorbent surface, excess analyte is washed away and the immobilized analyte is detected using a detector antibody that is considered to be the primary antibody. In other ELISA formats (Figure 2B, 2D, and 2E), the analyte-specific Ig (nonconjugated primary antibody) is allowed to bind to the immobilized analyte, and any excess antibody is washed away before the addition of a detector antibody, which is termed the secondary antibody.

To facilitate detection, in all ELISA formats that use enzyme-conjugated antibodies, a substrate specific for the con-

jugated enzyme is introduced into the assay system. An enzymatic reaction ensues, converting a substrate into a soluble product that can be measured using appropriate wavelengths and a suitable reader.

ELISA sensitivity depends on the quality of the reagents and the detection system, including the label and substrate. If multiple differently conjugated antibodies are available, analysts should select one appropriate for the assay. During this evaluation, the dilution of each conjugate that yields desirable sensitivity and specificity should be determined using appropriate controls.

The most commonly used labeling enzymes for conjugating to antibodies include alkaline phosphatase (AP), horseradish peroxidase (HRP), and galactosidase. These enzymes are highly specific, sensitive, and stable in catalyzing chromogenic, luminescent, or fluorescent reactions. *para*-Nitrophenyl phosphate (pNPP) is a commonly used substrate for AP. Commonly used substrates for HRP include TMB, OPD (*o*-phenylenediamine dihydrochloride), and ABTS [2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt] (see Table 2). The substrates for AP and HRP are chromogenic and result in the formation of a colorimetric product that can be measured using a spectrophotometer. Chemiluminescent and fluorescent substrates for AP and HRP also are available, and in many cases they are available as commercial kits. Disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan]-4-yl)phenyl phosphate (CSPD) is a known chemiluminescent substrate for AP (see Table 2). Well-known fluorescent substrates for galactosidase include MG (4-methylumbelliferyl galactoside) and NG (nitrophenyl galactoside). If a chemiluminescent substrate is used, then a luminometer is required to quantitate the formed product. A fluorometer is needed if a fluorescent substrate is used in the ELISA.

Table 2 also provides a summary of the advantages and disadvantages of different types of ELISA substrates. Colorimetric substrates have been prevalent since the origin of ELISAs and may yield robust assays that generally are more cost efficient than assays that use chemiluminescent and fluorescent substrates. Nevertheless, chemiluminescent and fluorescent ELISA methods may yield more rapid and sensitive assays with a wider dynamic range than assays that use a colorimetric readout. The final choice of readout should be governed by the assay's purpose and the requirements of the assay.

Table 2. Enzyme Conjugates and Substrates

Readout	Principle of the Enzymatic Reaction	Enzyme	Substrate	Reader	Advantages	Disadvantages
Colorimetric	Produces a colored product that yields absorbance values directly proportional to analyte concentration	AP, HRP	pNPP TMB OPD ABTS	Spectrophotometer	<ul style="list-style-type: none"> Robust Economical Reagent availability 	<ul style="list-style-type: none"> Less sensitive
Chemiluminescent	Produces a light emission that is directly proportional to analyte concentration	AP	CSPD	Luminometer	<ul style="list-style-type: none"> Wide assay dynamic range Lower coating concentrations More sensitive Rapid signal generation 	<ul style="list-style-type: none"> Requires special plates Costly
Fluorescent	Produces excitation-induced light emission that is directly proportional to analyte concentration	Galactosidase	MG NG	Fluorometer	<ul style="list-style-type: none"> Rapid Sensitive 	<ul style="list-style-type: none"> Requires special plates Costly Interference by excipients

ASSAY DEVELOPMENT AND VALIDATION PLAN

Critical Reagent Development

Key considerations for critical reagents are source, purity, specificity, and stability. For quality measurements, ITMs use reference standards along with critical reagents for analyte capture and detection. Any changes of critical biological reagents should be evaluated (see, for example, guidance contained in *Design and Development of Biological Assays* <1032>).

Source: The availability and quality of the starting material should be controlled so that manufacturing of the (purified) reagent can be reproducibly and consistently performed, potentially over several decades. Because critical reagents are biological molecules, sources can range from chemical synthesis (e.g., peptides) to complex biological matrices (e.g., antibodies prepared from serum, monoclonal antibody from ascites/cell culture, or fermentation/cell culture products). When appropriate for the intended use of the assay, a single lot of a critical reagent can be manufactured to establish a substantial supply and to prevent lot-to-lot variability. In other instances it may be appropriate to include in the validation multiple lots or multiple suppliers in order to demonstrate that the assay is sufficiently robust for its intended use.

Purity: In general, the purity of critical reagents should be assessed to ensure the removal of impurities and manufacturing process residuals that can influence reagent performance and/or stability.

Specificity: The specificity of a critical reagent refers to its ability to capture or detect only the analyte of interest. The reagent must be specific to the analyte and should show little nonspecific binding or no cross-binding to off-target molecules in complex test materials.

Stability: The stability of critical reagents should be empirically determined to ensure assay performance over time (issues include accuracy, precision, reproducibility, and assay drift). Long-term (months to years) stability of critical reagents under required storage conditions (e.g., with defined temperatures and containers) should be determined so that appropriate expiry dating can be assigned. Short-term (minutes to days) stability (and freeze/thaw and room temperature stability for frozen critical reagents) also is required to ensure day-to-day assay accuracy, precision, and reproducibility.

Feasibility/Pilot Studies

The steps of the process by which an ELISA method is developed, validated, and used in routine sample analysis are described below:

- Generate or purchase critical reagents to measure the analyte. Determine storage conditions and stability.
- Understand the performance goals for the assay system.
- Develop the assay to the point that there is a detectable concentration response curve.
- Perform method development/robustness testing.
- Prepare the reference/calibration standard and control and assess stability.
- Establish assay procedures, appropriate controls and limits, assay and sample acceptance criteria, and instrumentation.
- Determine method performance, and qualify method for accuracy, specificity, precision, and robustness, including qualification of all applicable sample types to be analyzed.
- Validate the assay.

- Implement the method (technology transfer) in the testing laboratory, including training and qualification of analysts.
- Monitor assay performance.

During assay development, the critical parameters and reagents that are required for the assay should be assessed and set at levels that yield desired assay performance. In many instances several parameters may be evaluated, and well-designed experiments can accelerate assay development, particularly for assessing the potential interaction of several inputs.

Many ELISA procedures are product specific, and external reference/calibration standards may not be available. The preparation and stability of reference/calibration standards should be considered early in assay development.

Assay Validation

Assay validation is executed according to guidances from appropriate regulatory bodies (e.g., ICH Q2) to demonstrate that the particular test used for an analyte is appropriate for its intended use. More information about assay validation can be found in the general information chapter *Validation of Compendial Procedures* <1225> or in general information chapter *Biological Assay Validation* <1033> if the ELISA is used as a surrogate potency assay. See *Appendix 2* for additional information.

DATA ANALYSIS

The analysis of ELISA data can be simple (e.g., a linear calibration with inverse regression) or complex (e.g., a non-linear calibration curve with inverse regression). The type and rigor of data analysis depend largely on the assay system and the intended uses of the assay. For example, data reduction may estimate a concentration (e.g., ng/mL) of an unknown sample using a calibration curve. Other approaches include estimation of the half-maximal inhibitory concentration (IC_{50}) or effective concentration (EC_{50}), estimation of the amount of a sample that yields the same response as the EC_{50} (or IC_{50}) on a standard curve, and an estimate of the relative activity of a test sample compared to a reference/calibration standard. More extensive guidance about statistical methods for potency analysis are given in general information chapters *Design and Development of Biological Assays* <1032> and *Analysis of Biological Assays* <1034>.

In general, ELISA assay curves are characterized by a non-linear relationship between the concentration of the analyte of interest and the calculated mean response. Typically, this response curve is defined by a sigmoidal relationship of response to concentration. A wide range of mathematical models can fit standard/calibration curves, and analysts should take care in the selection of an appropriate curve-fitting algorithm. In other cases, ELISA assays are used for qualitative purposes to determine whether a sample is positive or negative based on a sensitivity threshold.

Basic Statistical Analysis

Basic statistical methods are not detailed here. General information chapter *Analytical Data—Interpretation and Treatment* <1010> addresses important fundamentals, including data handling; computation of means, standard deviations, and standard errors; detection of and methods to address nonconstant or nonnormal variation; detection of and management of outliers; and procedures for and interpretation of statistical tests and confidence intervals. The concepts behind validation, goals, designs, analysis, and practical methods for validation are described in general information chapters *Analytical Data—Interpretation and Treatment* <1010>, *Validation of Compendial Procedures* <1225>, and *Biological Assay Validation* <1033>. General test chapter *Design and Analy-*

sis of Biological Assays <111> contains guidance on combining results from independent assays.

Nonlinear Statistical Analysis

Nonlinear calibration for immunoassays draws on many sources for statistical design and analysis. These include methods for assessing and addressing nonconstant variance, designs and analysis methods for experiments with complex structures, and validation. The concepts behind linear calibration design, analysis, and inverse regression apply in nonlinear calibration, and professional statisticians can help apply these appropriately.

Reporting Results

Reported estimates of concentration should be understood as having an associated confidence interval based on the results of the validation. The reported value or estimate used to describe a sample can be based on a combined result from multiple assays.

APPENDIX 1

Abbreviations

- Ab—Antibody
- Ag—Antigen
- ABTS—2,2'-Azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]diammonium salt
- Anti-Ig—anti-immunoglobulin
- AP—Alkaline phosphatase
- BSA—Bovine serum albumin
- CSPD—Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate
- EIA—Enzyme immunoassay
- ELISA—Enzyme-linked immunosorbent assay
- HRP—Horseradish peroxidase
- Ig—Immunoglobulin
- ITM—Immunological test methods
- MG—4-Methylumbelliferyl galactoside
- NG—Nitrophenyl galactoside
- OPD—o-Phenylenediamine dihydrochloride
- PBS—Phosphate-buffered saline
- PVDF—Polyvinylidene fluoride
- pNPP—para-Nitrophenyl phosphate
- TMB—3,3',5,5'-Tetramethylbenzidine

APPENDIX 2

Additional Sources of Information about Specific Topics in Validation and Data Analysis

	Analytical Data—Interpretation and Treatment<1010>	Design and Analysis of Biological Assays<111>	Validation of Compendial Procedures<1225>	Biological Assay Chapters<1032>, <1033>, and <1034>
Means	X			
Standard deviations	X			
Standard errors	X			
Non-normality	X			X
Nonconstant variance	X			X
Outliers	X			X
Tests	X			
Confidence intervals	X			X
Validation			X	X
Combining results from multiple assays		X		X

<1105> IMMUNOLOGICAL TEST METHODS—SURFACE PLASMON RESONANCE

Introduction

Surface plasmon resonance (SPR) optical detection is a useful method for the label-free assays (procedures) that study biomolecular interactions. Commercially available SPR biosensors that incorporate these assays can collect real-time, information-rich data from binding events. These data can be used widely from basic research to drug discovery and development to manufacturing and quality control (QC). SPR can characterize binding events with samples ranging from proteins, nucleic acids, and small molecules to complex mixtures, lipid vesicles, viruses, bacteria, and eukaryotic cells. Typical quality and safety attributes addressed with SPR analysis include:

- Interaction specificity
- Interaction affinity
- Kinetic binding parameters
- Thermodynamic parameters
- Biologically active concentration of an analyte

This chapter provides an overview of the physics underlying SPR and common instrument configurations, as well as the range of molecules that can be studied and general considerations for experimental design as determined by the assay objective.

Overview

History

The physical principles of SPR were first explained in the early 1900s, starting with a description of the uneven distribution of light in a diffraction grating spectrum caused by the excitation of surface plasmon waves. A landmark series of experiments showed the optical excitation of surface plasmons under conditions of total internal reflection and fostered detailed studies of the application of SPR for chemical and biological sensing. Since then, SPR's potential for characterizing thin films and monitoring interactions at metal interfaces has been recognized, and significant research and development have yielded instruments that can quantitatively evaluate the binding interactions of small and large molecules.

Physics

SPR is an optical phenomenon that occurs when a thin conducting film is placed between two media that have different refractive indices. In many commercially available instruments, the two media are glass and the sample solution, and the conducting film is preferentially a gold layer applied to the glass, although other conducting metals such as silver have been used. The glass-metal component comprises a solid support that is often referred to as a *sensor*.

Light applied to the glass under conditions of total internal reflection produces an electromagnetic component that is called an *evanescent wave*. The evanescent wave penetrates the medium of lower refractive index (typically the sample solution) without losing net energy. The amplitude of the evanescent wave decays exponentially with distance from the surface, roughly one-half of the wavelength of the incident light (e.g., for a light source of 760 nm the evanescent wave penetrates approximately 300 nm).

For a specific combination of wavelength and angle of incident light, electron charge density waves called *plasmons* are excited in the gold film. As energy is absorbed via the evanescent wave, a decrease in the intensity of the reflected light at a specific angle (the SPR angle) is observed. Analysts can conduct an SPR experiment by fixing the wavelength and varying the angle of incident light.

An increase in mass at the sensor surface caused by a binding interaction between two or more molecules causes a

change in the local refractive index (RI) that gives rise to an SPR response, which is observed as a shift in the SPR angle. By monitoring the shift in the SPR angle as a function of time, an analyst can generate a *sensorgram* (Figure 1). The change in RI is very similar for different proteins, so the SPR measurement depends primarily on the mass change at the sensor surface and is relatively independent of the nature of the molecules being measured.

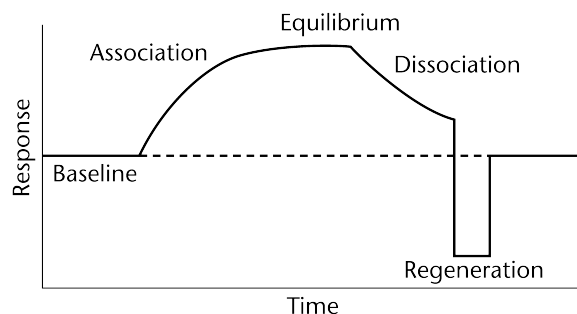


Figure 1. Representative sensorgram.

Instruments

The main components of commercially available SPR instruments are (1) a light source, typically a high-efficiency light-emitting diode, (2) an optical detector such as a diode-array or charge-coupled device camera, (3) a solid support containing the conducting film and some means for attaching molecules, (4) a sample delivery system, frequently a microfluidic device capable of delivering samples using single serial or parallel injections via single or multiple needles, and (5) a computer with appropriate software for instrument control, data collection, and analysis.

Prism-based and diffraction-grating instrument systems are commercially available. Most prism-based systems follow the Kretschmann configuration (Figure 2). The light is focused onto the sensor surface (away from the samples) via a prism with a refractive index matching that of the surface. In this configuration the incident light does not penetrate the sample solution, which permits SPR measurements for heterogeneous, turbid, or opaque samples. In systems that utilize a diffraction grating (Figure 3) the analyte solution is placed over a plastic surface on which a metal has been deposited. The plastic acts as an attenuated total internal reflection prism in which light reflected from the grating is reflected many times back to the grating surface. In this configuration light passes through the analyte sample solution, and thus turbid or opaque samples are not suitable for measurement. The diffraction grating does permit sampling of a larger surface area and is applicable for SPR measurements of arrays.

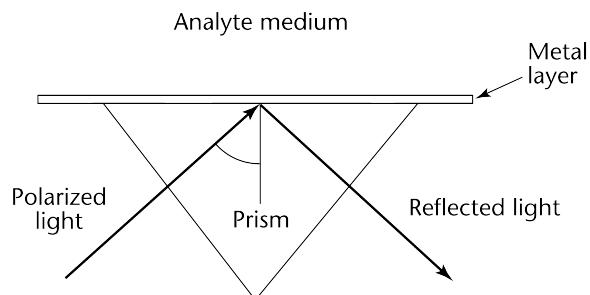


Figure 2. Kretschmann SPR configuration.

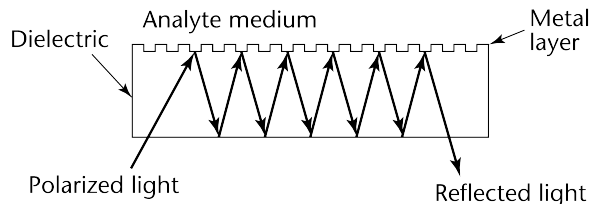


Figure 3. Diffraction grating SPR configuration.

The instruments are compatible with a wide range of biological samples and buffers as well as some organic solvents.

Biomolecular Interactions That Can Be Studied By Assays Using SPR

A diverse range of biological entities can be studied using SPR, including small molecules (<100 Da), proteins, nucleic acids, lipids, bacteria, viruses, and whole cells. Most published SPR research involves protein–protein interactions, of which antibody–antigen interactions represent a dominant subset. Improvements in instrument sensitivity and experimental protocols have helped analysts make studies of small molecules, lipids, and nucleic acids. Protein interactions with larger entities such as whole cells and some bacteria and viruses are limited by the exponential decay of the evanescent wave as described above. In practice these large molecules can be studied effectively, but the information obtained may be limited to qualitative or semiquantitative (e.g., relative ranking) data.

Assay Types

Several types of SPR assays are useful, including binding specificity, concentration analysis, kinetics and affinity analysis, and thermodynamics. Each assay type generates unique information that is helpful for profiling biomolecules.

SPR is also suitable for use in qualitative studies to confirm the specificity of interactions. Analysts can monitor a number of sequential binding events because each individual event yields a mass increase on the sensor chip surface, and all stages in the binding process are monitored. Examples include epitope mapping, antibody isotyping, and immunogenicity measurements.

Most chemical and spectroscopic methods used to quantify proteins (1) measure total protein content, (2) do not distinguish active from inactive molecules, and (3) cannot be used in conjunction with unpurified samples. Because SPR is a noninvasive method (no light penetrates the sample), it can measure small amounts of analyte molecules from complex matrices such as food products, serum or plasma, and cell extracts. Direct or indirect (inhibition or competitive) formats for measuring concentration are possible. SPR biosensors are uniquely suited for measurement of kinetic association and dissociation rate constants from real-time measurement of binding interactions. Affinity can be derived either from interactions that have reached equilibrium or from the ratio of the dissociation and association rate constants. The typical working range for affinity measurements is pM to high μ M concentrations. Association rate constants that can be measured typically range from 10^3 to 10^7 $M^{-1}s^{-1}$ and dissociation rate constants from 10^{-5} to 0.5 s^{-1} . By studying temperature dependence of rate and affinity constants, analysts can determine thermodynamic parameters for a binding interaction. Not only can the equilibrium values for changes in enthalpy (ΔH) and entropy (ΔS) associated with complex formation be determined, but transition state energetics can also be evaluated. Subsequent sections of this chapter address the specific details for these different assay types.

The SPR Assay

The typical SPR assay involves five steps:

1. Sample and buffer preparation
2. Surface preparation
3. Analyte binding

4. Surface regeneration
5. Data analysis and interpretation

Careful attention to experimental design leads to high-quality data and results. In SPR experiments, mass transport is essential for binding interactions to take place in instruments that use thin-layer flow-cell systems. Analyte molecules are transferred from the bulk solution to the binding surface via mass transport. When a limitation for binding occurs as a result of fast binding kinetics combined with high surface density, the binding interaction is considered mass-transport limited. In this case, the binding kinetics and complex formation are influenced by the availability of analyte molecules. The advantages and disadvantages of mass-transport-limited binding are discussed later in the application examples.

Sample and Buffer Preparation

Both purified and crude samples can be analyzed in a variety of matrices including serum, plasma, cell supernatants, and lysates. Crude samples containing particulates (e.g., cell debris or precipitates) may require clarification in order to help minimize unwanted binding. A short spin (30–60 s) in a benchtop centrifuge or filtration (0.22–1.0 μ m) using low-protein-binding filters is recommended. The concentration range for evaluation depends on the experimental objective (yes/no binding, concentration, or kinetic/affinity analysis) as well as the binding affinity of the interacting molecules. In general, sample concentrations an order of magnitude below the equilibrium dissociation constant (K_D), can be detected by SPR, but determination of an exact concentration is influenced by the analyte size (large vs. small molecules), binding specificity, and overall biological activity of the samples.

Most biological buffers and several organic solvents can be used in SPR experiments. The addition of salts and detergents to buffer solutions frequently can stabilize biomolecules. High-quality grade (e.g., molecular biology grade or higher) buffer components should be used. To simplify experiments, analysts should add only components that are absolutely required for biological activity or function. Buffers should be filtered and degassed before use.

Surface Preparation

Surface preparation involves the attachment of one of the binding partners to a solid support (surface). This process is frequently referred to as *immobilization*, and the resulting surface with the attached biomolecule is the sensor for the experiment. The choices of binding partner, solid support, and immobilization method are influenced by (1) the nature and demands of the application or experimental objective; (2) the availability of surfaces with different properties (e.g., charge density, hydrophobicity, or hydrophilicity); (3) the characteristics and supply of biomolecule to be used for immobilization; and, most importantly, that (4) biological activity be maintained and binding sites be available to interacting partners. Depending on the experimental objective, homogeneous or orientation-specific attachment of biomolecules also may be desired. The two main categories of immobilization methods are (1) direct immobilization, in which the molecule is covalently attached to the surface, and (2) indirect or capture immobilization, which takes advantage of tags or native groups on the protein or biomolecules (Table 1).

Direct Immobilization: For direct immobilization, several chemistries are available for attaching proteins or other biomolecules to the surface. The properties of the surface determine the specific sequence of steps and length of time required to prepare the surface. Many commercially available surfaces have a biologically compatible layer (e.g., a hydrogel) that contains functional groups such as carboxyl that can be used for immobilization. To ensure binding specificity, the purity of the biomolecule that is attached to the surface should be 95% or greater and the required concentration should range from 1 to 1000 μ g/mL. Direct immobilization chemistries frequently

result in heterogeneous surfaces because of random orientation of biomolecules on the surface. Immobilization via free primary amine groups such as lysine residues in proteins or the amino terminus of proteins or peptides is one of the most generally applicable covalent chemistries for attaching proteins to a surface. Carboxyl groups on the surface are converted to reactive esters using a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) or sulfo-NHS (sNHS). The protein or biomolecule is applied in high concentrations (mg/mL) to maximize the efficiency of amine coupling. Finally, free esters are blocked with ethanolamine. The contact time with the surface, the protein concentration, or the EDC/NHS concentration can be varied to adjust the immobilization level.

When amine coupling interferes with the binding site, the biomolecule can be attached using alternative coupling chemistries or a high-affinity capture approach. For example, for biomolecules with free thiol groups (typically cysteine residues), a disulfide group is introduced by treating the surface with NHS and EDC to attach 2-(2-pyridinyldithio)ethaneamine (PDEA). Adding the biomolecule to the surface results in thiol–disulfide exchange, and excess PDEA groups are inactivated with cysteine–HCl. If the biomolecule lacks a free thiol group, a reactive disulfide (PDEA) can be linked to carboxyl groups. Subsequently the pyridyldisulfide groups can be attached to thiol groups on the surface that have been derivatized via injection of NHS and EDC, followed by cystamine, then reduction with dithioerythritol (DTE) or dithiothreitol (DTT). Attachment of maleimide groups to the surface makes possible an alternative form of immobilization via thiol groups in which a stable thioether bond is formed. Surfaces prepared using this method have the capacity to withstand basic pH (> 9.5) and reducing agents such as β -mercaptoethanol and dithiothreitol. Several heterobifunctional reagents are available commercially for introduction of reactive maleimido groups to the surface, including sulfo-MBS (*m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester), sulfo-SMCC (sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate), GMBS [*N*-(γ -maleimidobutyroxy)sulfosuccinimide ester], EMCH [*N*-(ϵ -maleimidocaproic acid)-hydrazide] or BMPH [*N*-(β -maleimidopropionic acid)-hydrazide]. For biomolecules containing either native aldehyde groups or *cis*-diols, which may be converted into aldehydes by mild oxidation, surface attachment via a hydrazone bond is an option. Hydrazone groups on the sensor surface react with aldehyde groups on the biomolecule to form a stable bond. Immobilization via aldehyde groups is most useful for glycoconjugates, glycoproteins, and polysaccharides.

Indirect (High-affinity Capture) Immobilization: Indirect or high-affinity capture immobilization approaches use tags commonly used for protein purification. This technique exploits the high-affinity capture of the biomolecule by a capturing molecule that has been immobilized covalently using one of the techniques described above. The requirement for biomolecular purity is less stringent for indirect versus direct immobilization because the capturing step for the biomolecule can also provide purification. Indirect immobilization frequently yields a homogenous surface because all biomolecules are oriented similarly via the tag. The affinity between the biomolecule and its capturing agent should be sufficiently high to ensure little or no dissociation from the surface for the duration of an analysis cycle. Monoclonal antibodies are frequently used as capture molecules. For example anti-GST antibodies can be attached to the sensor chip surface via amine chemistry in order to capture GST-tagged molecules. Protein A, Protein G, and anti-IgG antibodies are useful capturing molecules for use with antibodies.

The high-affinity interaction between streptavidin or related molecules and biotin ($K_D \approx 10^{-15}$ M) makes it a useful system for the capture of biotinylated molecules (e.g., proteins, peptides, nucleic acids, membranes, and liposomes). Frequently, the biotin-binding protein is attached to the surface using primary amine groups. Because of the high affinity of the interaction, biotinylated molecules are considered permanently immobilized, and in contrast to most other capture approaches biotinylated molecules cannot be removed without damaging the surface. Histidine (His)-tagged recombinant proteins can be captured via nickel–NTA chemistry or covalently immobilized anti-His antibodies.

Lipids and membrane-associated proteins can be captured to the surface as either a lipid monolayer or bilayer.

Lipids from micelles or liposomes adsorb to a hydrophobic surface, creating a lipid monolayer with the hydrophobic lipid tails oriented toward the solid support and the hydrophilic heads towards the aqueous sample. This approach provides a stable environment for proteins associated with a membrane surface or partially inserted into the membrane, but it is not ideal for transmembrane proteins because the resulting surface presents only half the membrane structure for binding interactions. Intact membrane structures (lipid bilayers) with associated or incorporated proteins can be captured by preparing liposomes with a specific antigenic component or with biotinylated lipids, allowing capture of the liposomes with immobilized antibody or streptavidin, respectively.

Additional Considerations: Once the biomolecule has been attached to the sensor using either a direct or indi-

Table 1. Surface Preparation Techniques

Chemistry	Immobilization Method	Biomolecules	Comments
Amine	Direct	Proteins, peptides	Amino terminus, Lys residues
Thiol—native	Direct	Proteins, peptides	Native Cys residue
Thiol—added	Direct	Proteins, peptides	Carboxyl groups derivatized
Aldehyde	Direct	Glycoproteins	<i>Cis</i> -diol required
Biotin capture	Indirect	Biotinylated peptides, nucleic acids, proteins	Stable, irreversible capture
Affinity tags	Indirect	Proteins, peptides	His, Glutathione <i>S</i> -transferase (GST), etc.
Protein A, Protein G	Indirect	Antibodies, IgG-tagged molecules	IgG species-dependent
Protein A, Protein G	Indirect	Biomolecules specific to the capturing antibody	Mono- or polyclonal antibodies may be suitable—testing recommended
Hydrophobic adsorption, membrane capture	Indirect	Lipids, membranes, membrane-associated proteins	Monolayer or bilayer attachment possible

rect immobilization approach, analysts should assess the baseline stability of the newly created surface. If the baseline is decreasing (downward drift), the most likely cause is the presence of unattached biomolecules, possibly because of self-association or aggregation. If the baseline is increasing (upward drift) refolding or re-orientation may be causing the change. In either case, the newly created surface should be conditioned before use by one or more of the following: (1) multiple injections of biologically compatible buffer; (2) washing the surface with buffer at a fast flow rate; (3) multiple injections of either high ionic strength (e.g., 1 M NaCl) or detergent (e.g., 20 mM CHAPS or 0.05% Polysorbate 20 (P20)) solutions; or (4) repeated analyte binding and regeneration injections. NOTE: recommendations (3) and (4) should be used only if the activity of the biomolecule in the presence of these reagents has been evaluated previously.

Large baseline drifts caused by low-affinity capture may be overcome by using EDC/NHS as a cross-linking step, but this may compromise biomolecule activity if active sites of the biomolecule are involved in the cross-linkages. The effect of cross-linking on biomolecule activity must be tested empirically for each biomolecule–analyte system. In general, cross-linking should be as brief as possible: 15 s is often sufficient to achieve acceptable baseline stability without compromising biomolecule activity.

How Much to Immobilize: The amount of biomolecule to immobilize depends on the experimental objective. Equations 1 and 2 are useful for calculating the appropriate surface density:

$$R_{max} = (MW_A/MW_L) \times R_L \times S_m \quad [\text{Equation 1}]$$

$$R_L = R_{max} \times (1/S_m) \times (MW_L/MW_A) \quad [\text{Equation 2}]$$

R_{max} = theoretical maximum binding response (assuming a surface that is 100% active and 100% bound with analyte)

R_L = response of the immobilized molecule

MW_A = molecular weight of the analyte

MW_L = molecular weight of the immobilized molecule

S_m = molar binding stoichiometry

For kinetic experiments, a low density of immobilized molecule is preferred in order to avoid steric hindrance, aggregation, and/or mass-transport-limited binding. Low density is defined as R_L that limits R_{max} to 5–50 response units. For other applications, e.g., concentration analysis where mass-transport-limited binding is desired, R_{max} can be 100–200 times higher than for kinetic experiments provided that steric hindrance or aggregation are not induced. Specific recommendations for immobilization density are included in the application examples for this chapter.

Analyte Binding

Samples that will be evaluated for binding using SPR do not require the same purity as biomolecules intended for direct immobilization onto the surface. Because the light source does not penetrate the sample, turbid or opaque samples can be analyzed by SPR. Whenever practical, samples should be clarified according to the recommendations given under *Sample and Buffer Preparation*, and buffer additives should be minimized, including only the amount required for biological activity. Differences between the refractive index of the bulk and sample buffers give rise to a response. The use of control surfaces and samples aids in demonstrating binding specificity for the molecules in SPR. For direct immobilization methods, suitable control surfaces can be (1) the sensor surface without any modification or biomolecule attached, (2) a surface that has been chemically treated in the same manner as the surface containing the biomolecule, or (3) a related but known nonbinding biomolecule. For surfaces prepared using

indirect (capture) immobilization the capturing molecule in the absence of the tagged binding partner should be used as the control surface. The difference in response between the control and active surfaces gives an initial indication of the binding specificity. Concentration-dependent responses and inhibition of binding by incubating the sample with the biomolecule on the surface can further establish the binding specificity.

If nonspecific or unwanted binding is observed, analysts should determine the source. Frequently changes in pH or ionic strength of the buffers used in the experiment will reduce or eliminate the unwanted binding. Additional suggestions for reducing nonspecific binding are summarized in Table 2.

Table 2. Suggested Actions for Reducing Nonspecific Binding

Category	Action
Experimental Design	<ol style="list-style-type: none"> Optimize running buffers: <ol style="list-style-type: none"> increase salt (150 to 500 mM) add detergent (0.001% to 0.05%) match composition of sample and running buffers Change ligand immobilization method Evaluate ligand quality Increase or decrease temperature in detection chamber
Choice of Surface	<ol style="list-style-type: none"> Change properties of sensor surface: <ol style="list-style-type: none"> reduce electrostatic interactions evaluate hydrophobic vs. hydrophilic character of surface consider alternative ligand to use for control surface Pre-immobilize amino—PEG Change blocking molecule (e.g., ethylenediamine)
Additions to Sample	<ol style="list-style-type: none"> Add nonspecific binding reducer to sample: <ol style="list-style-type: none"> increase ionic strength of running and sample buffers (e.g. 150 to 500 mM NaCl) add detergent to running and sample buffers (e.g. 0.001% to 0.05% surfactant P20) add soluble carboxymethyl dextran (1–10 mg/mL, for dextran-based surfaces only) Simplify sample buffer—include only components required for biological activity Evaluate analyte quality

Equations 1 and 2 are also useful for assessing surface activity. The higher the binding response, the more active the surface is unless the observed binding response exceeds the calculated R_{max} value. In this case, the molar binding stoichiometry is incorrect, the analyte molecule is aggregated, or the analyte is binding nonspecifically to the surface. Binding responses that are low (< 10% of R_{max}) suggest that the analyte concentration selected for the experiment is too low or that the surface activity of the immobilized molecule is low. In the former case, increasing the analyte concentration should increase the binding response, and in the latter situation using a different immobilization method may be helpful.

Surface Regeneration

Surface regeneration refers to the process of removing bound analyte from the surface in order to reuse the surface for subsequent binding interactions. In some instances, complex dissociation is fast and bound analyte is simply washed away with buffer, so regeneration is not needed. Alternatively, the instrument configuration may allow multiple samples to be injected either sequentially or in parallel across several immobilized surfaces simultaneously, thereby limiting the need for regeneration. Inadequate surface regeneration may affect

the reproducibility of an assay and negatively affect the overall quality of the resulting data. To identify the correct conditions, analysts should consider the nature of the specific interaction and the experimental objective. For example, a slight baseline drift will not affect the results when a simple yes/no answer is sought, but in concentration determination or kinetic studies, optimization of the regeneration step is critical.

Most biochemical interactions involve non-covalent bonds such as hydrogen, electrostatic, van der Waals, and hydrophobic bonds. Because the combination of physical forces responsible for binding and the regeneration conditions critical for not causing irreversible conformational changes are unknown for most interactions, the final conditions must be evaluated empirically.

The ideal condition for regeneration dissociates all the bound material without affecting the biological properties of the immobilized biomolecule. An incomplete regeneration or too stringent conditions may result in decreased analyte binding capacity in subsequent cycles because of either blocking of binding sites by nondissociated analyte or partial denaturation of the biomolecule. Regeneration buffers and solutions can be divided into different classes by the effect they have on the interaction. Any combination of buffers can be used.

The major classes of regeneration buffers are: acidic, basic, ionic/chaotropic, detergent, hydrophobic/nonpolar, and chelating (see *Table 3* for examples of each class). Analysts should start with mild conditions, moving progressively to more harsh conditions. In many cases, especially when one is working with antibodies, change in pH is the most effective method of regenerating the surface. Contact time with the surface is important for efficient regeneration. When analysts use pH change, the contact times should be short, one-half to 2 min. When analysts use high ionic strength or chaotropes, longer contact times of 2–4 min are usually effective.

The purpose of optimizing the regeneration conditions is to find the mildest possible regeneration solution that completely dissociates the complex. Analysts should maintain a constant level of activity over the binding–regeneration cycles even if the baseline changes a little. Repeated cycles of analyte binding followed by regeneration of the surface will provide insight into the overall performance of the surface. Ideally the surface performance should be evaluated for the same number of cycles that will be used during the SPR experiment.

The surface must be monitored for signs of accumulation and also degradation of the immobilized ligand (*Figure 4*). This can be accomplished by monitoring both the baseline at the beginning of each injection cycle and binding signal (slope or bound response) of a quality control sample. Appropriately defined acceptance criteria

for system suitability such as baseline drift and quality control performance help to monitor the integrity of the immobilized ligand on the surface.

If the binding response is slowly decreasing, there are two possible explanations:

1. If the baseline of the raw data sensorgram remains constant but the binding response still decreases, the regeneration conditions cause an irreversible change to the biomolecule that decreases the binding capacity of the surface, which in turn decreases the amount of analyte that can be bound on the surface. Analysts can decrease the strength of the regeneration solution slightly or can change to another regeneration solution of equal strength within the same class.
2. If the baseline increases, the accumulation of analyte causes a binding-capacity decrease on the surface, which in turn decreases the amount of analyte that can be bound to the surface. Analysts can increase the strength of the regeneration solution slightly or can change to a regeneration solution of equal strength within the same class. There may be a difference between regeneration solutions in their ability to solubilize the analyte.

Once a suitable regeneration solution has been determined, it should be tested in a series of analyte binding and regeneration cycles. Because the binding activity of the surface typically decreases with time and/or use, analysts must empirically determine the binding response threshold and consequent number of cycles for surface use. Examples for determining binding threshold and number of cycles are presented below (see Applications 1–3).

In some cases the baseline will drop or rise and/or the binding capacity will decrease somewhat in the first few injections before it stabilizes. This is caused by either the dissociation of electrostatically bound biomolecules from the surface (depending on surface characteristics) or to binding to a high-affinity non-regenerable fraction of the surface. For this reason, each newly immobilized surface should be conditioned with repeated analyte binding and regeneration cycles before collection of quantitative data. Alternative immobilization methods, such as a different chemistry or indirect capture, should be evaluated when the immobilized biomolecule is difficult to regenerate.

Data Analysis and Interpretation

Analysis and interpretation of the data are specific to the experimental objective. Several data analysis programs exist to aid in the calculation of kinetics and affinity constants from SPR data. The validity and quality of the results are linked directly to experimental design. The fitting process is purely mathematical, without regard to the biological significance of the values obtained.

Table 3. Examples of Regeneration Solutions

Acid	Base	Ionic/ Chaotropic	Detergent	Hydrophobic/ Nonpolar	Chelating
1–100 mM HCl	1–100 mM NaOH	0.5–5 M NaCl	0.02%–0.5% SDS	25%–100% ethylene glycol	10–20 mM EDTA or EGTA
10–100 mM glycine, pH 1.3–3.0	10–100 mM glycine, pH 9.0–10.0	1–4 M MgCl ₂	40 mM octyleneglycol + 20 mM CHAPS	5%–50% DMSO	10–200 mM imidazole
10–100 mM phosphoric acid	1 M ethanolamine HCl, pH 9.0 or above	1 M KSCN	40 mM octylglucoside, 40 mM octylglucoside	1%–10% acetonitrile	
0.1% TFA	100 mM sodium carbonate + 1 M NaCl, pH 9–11	2–6 M guanidine HCl			
100 mM Formic acid	20–100 mM NaOH containing 0.5% surfactant P20 or 0.05% SDS				

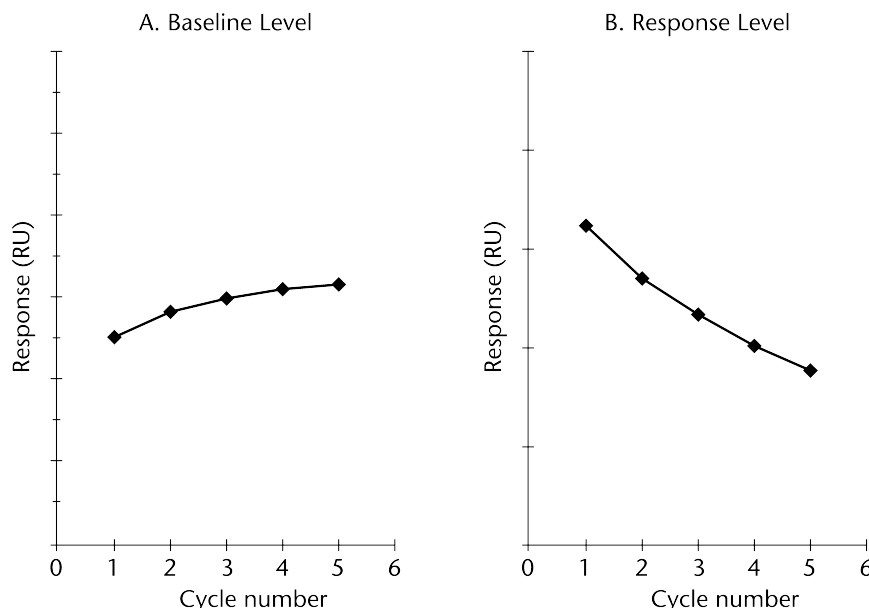


Figure 4. Evaluating surface performance (A) accumulation on surface and (B) degradation of immobilized ligand.

Data Analysis Algorithm: Global analysis seeks a single set of kinetic rate constants for all of the analyte concentrations used in the experiment. Using a data-fitting algorithm such as Marquardt-Levenberg the data analysis software begins an iterative process starting with an initial approximation to find the best set of parameters that produces agreement between the experimental data (sensorgram) and the calculated fit to the data. The iterative process continues until the difference between the experimental and calculated (theoretical) curves is minimized as measured by the sum of the squared residuals.

Preparing the Data for Analysis: Before conducting kinetic analysis, analysts should inspect the experimental data visually for anomalies or artifacts such as baseline disturbances or out-of-range data (often due to air bubbles) lasting for a predefined time period (e.g., 4–8 s). Outliers should be removed from the data set according to pre-established criteria. Nonessential data, such as capture or regeneration injections, should be removed from the sensorgram, and the data at each analyte concentration should be adjusted using the double-referencing procedure described below.

Before analysis the raw data should be processed in the following manner:

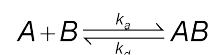
- Align the injection start to zero seconds for all concentrations and buffer injections for both the reference and active surfaces.
- Align the baseline to zero response for all sensorgrams.
- Subtract the reference surface sensorgram from the active surface sensorgram in order to create a corrected data set.
- Subtract the corrected buffer sensorgram from the sensorgrams at different concentrations in order to create a double-referenced data set.

The double-referencing procedure removes systematic errors (e.g., instrument noise) and low levels (less than 5% of total binding response) of nonspecific binding. It should not be used to correct for significant nonspecific binding events because this can lead to erroneous measurements.

When analyzing the data for kinetic information, analysts use the association (injection) and dissociation (buffer flow) phases for all of the concentrations in the series. For steady-state affinity analysis, the response at

equilibrium R_{eq} (data plateau or no change in response vs. time) is measured for each sensorgram to create a binding isotherm with R_{eq} vs. concentration. This isotherm is analyzed using the equations described below.

Kinetics and Steady-state Affinity Models: The Langmuir kinetic model assumes a 1:1 interaction between the binding partners so that



The association and dissociation rate constants are defined below:

$$\frac{d[AB]}{dt} = k_a \times [A] \times [B]$$

$$-\frac{d[AB]}{dt} = k_d \times [AB]$$

Combining these two equations and defining $[B_{free}] = [B_{tot} - AB]$, the net rate expression is

$$d[AB]/dt = k_a \times [A_{free}] [B_{tot} - AB] - k_d \times [AB]$$

which can be translated into terms from the SPR experiment as follows:

$$dR/dt = k_a \times C \times (R_{max} - R) - k_d \times R$$

where R is the binding response at any point along the sensorgram and C is the known analyte concentration. Using global analysis as described above, k_a , k_d , and R_{max} are calculated from the experimental data using the rate equations shown below:

$$\text{Association: } dR/dt = k_a \times C \times (R_{max} - R) - k_d \times R$$

$$\text{Dissociation: } dR/dt = -k_d \times R$$

Because the concentration of analyte is zero during dissociation, the rate equation for dissociation depends only on the response, R , and the dissociation rate constant, k_d .

Application of the equilibrium condition where the complex formation (association) equals complex decay (dissociation)

$$k_a \times [A] \times [B] = k_d \times [AB]$$

yields the following equation for the equilibrium dissociation constant

$$K_D = \frac{[A] \times [B]}{[AB]} = \frac{C(R_{max} - R_{eq})}{R_{eq}}$$

where R_{eq} is the binding response at equilibrium that is measured in the experiment for a given analyte concentration and C , K_D , and R_{max} are calculated using global analysis.

Kinetics binding models can be used to describe non-1:1 interactions, e.g., bivalent interactions that occur if an antibody is used as the analyte, heterogeneity in binding partners, conformational change, or more complex interactions such as cooperative binding. SPR analysts are cautioned against using more complex models to assess data unless experimental design has been confirmed.

Assessing the Fit: The quality and validity of the fit to the kinetic data can be assessed by (1) visual inspection of the agreement between the experimental and calculated curves, (2) the size and the shape of residual plots, (3) the biological relevance of the results, and (4) statistical parameters such as χ^2 (average of squared residuals), and standard error (SE), T -value or U (uniqueness) factor. The best parameter fit to the experimental data should be superimposed on the curve for each concentration in the experiment. The residual plot visualizes the difference between the calculated and experimental data. The shape of the residuals should be random without trending (waviness or curving up or down). The height of the residual plot should reflect the instrument noise. Further, χ^2 should be minimized for a good fit with values that depend on the instrument noise, number of data points, and overall binding response. The parameter values should be considered for biological and experimental relevance. For example, is the calculated k_D value slow when the interaction is known to be fast, or is the calculated R_{max} value higher than the value that was calculated using Equations 1 and 2. Parameter significance is evaluated based on standard error, T -values, and U factor. Parameters that are significant cannot be changed without affecting the quality of the fit. All of the criteria should be within acceptable limits.

A similar set of criteria can be used for assessing the fit to steady-state affinity data, but because there are fewer data points (6–12 total, depending on the number of concentrations used), the statistical parameters and residual plots are less predictive of fit quality. Visual inspection of the agreement between the experimental and calculated binding isotherms and the parameter relevance are good tools to use for assessing the fit. Additionally, according to the relationship between concentration, K_D , and R_{max} , when concentration equals the K_D for the interaction R equals 50% of R_{max} . Confirming that the analyzed data follow this relationship provides another way to check the validity of the calculated result. When it is practical to calculate the K_D using both kinetic and steady-state analysis approaches, the K_D values should agree within experimental error.

Addressing a Lack of Fit: When the data do not fit or the parameter values do not make sense, often the problem can be resolved by a systematic approach that considers potential sources for deviations and tests each hypothesis. Items to consider include reagent purity, immobilization chemistry or surface density, analyte concentration errors, nonspecific binding, loss of ligand activity, or mass-transport-limited binding. Reviewing the raw (uncorrected) data helps determine the source of nonspecific binding or concentration errors. Table 4 lists potential sources for deviations from 1:1 binding and recommended actions.

Table 4. Common Sources for Deviation from 1:1 Binding Kinetics

Source of Deviation	Recommended Action
Nonzero baseline before injection	<ul style="list-style-type: none"> • Normalize response to zero and reanalyze
Incorrect injection start and stop times or poorly defined injection start/stop	<ul style="list-style-type: none"> • Adjust injection start/stop • Remove sensorgram artifacts (e.g., injection or air bubble spikes)
Concentration input errors	<ul style="list-style-type: none"> • Verify concentration values and reanalyze
Bulk refractive index contribution too high	<ul style="list-style-type: none"> • Use double-referencing approach before analysis • Set RI = 0
Mass-transport-limited binding	<ul style="list-style-type: none"> • Vary flow rate (slow to fast) for a single concentration and overlay sensorgrams (should be identical for same association and dissociation time) • Include mass transport term, k_m, in fitting model • Reduce surface density
Nonspecific binding	<ul style="list-style-type: none"> • Change immobilization chemistry • Change sensor surface properties • Buffer additives—add or minimize • Reagent purity—repurify samples
Loss in binding partner activity	<ul style="list-style-type: none"> • Change immobilization chemistry • Change regeneration solution • Re-analyze data using local instead of global parameter fitting for R_{max}
Multi-valent binding interaction	<ul style="list-style-type: none"> • Immobilize multivalent binding partner

Recommendations for data analysis will be introduced in the subsequent sections of this chapter.

Application 1—Immunogenicity Assessment: SPR has emerged as a powerful technique for assessing immunogenicity of protein therapeutics. An advantage of this platform for detecting antibodies in serum (or plasma) samples is that it allows label-free detection based on mass accumulation in real time, which potentially allows detection of low-affinity antibodies of all classes and subclasses. This technology is useful both for screening assays (first-tier immunoassays that are used to detect the presence of antibodies capable of binding to a protein therapeutic) and also characterization assays. Characterization assays are useful for defining generated antibodies that bind to the protein and can include analysis of antibody concentration, isotype(s) represented, relative binding affinity, and binding specificity. A limitation is that SPR it is not appropriate for determination of the neutralizing capability of antibodies, which is best determined using cell-based biological assays. When designing and validating SPR assays for immunogenicity assessment, analysts should consider critical parameters including protein immobilization to ensure immunological reactivity, immobilized protein stability, and surface regeneration conditions.

Protein Immobilization: The first step in the development of immunogenicity assessment assays is to identify the optimum mechanism for immobilization of the target protein. When considering the target density for immobilization, analysts often recommend that a high-density surface be used. The advantage of a high-density surface is that it maximizes the opportunity that anti-therapeutic antibodies will come in contact with an immobilized ligand. A high-density surface also provides excellent assay sensitivity. An important aspect of these assays is that the chemistry chosen for immobilization

should provide random orientation rather than a site-directed orientation so that all potential epitopes on the therapeutic protein are available for binding by the anti-therapeutic antibodies. The effectiveness of immobilization is determined by evaluating the ability of positive control antibodies to bind to the immobilized protein. When evaluating the effectiveness of immobilization, analysts should test multiple antibodies with different epitope specificities. When panels of antibodies that cover a range of affinities and bind to different epitopes on the target protein are all capable of binding, this provides confidence that antibodies contained in clinical specimens also will be detected. If any of the positive control antibodies do not demonstrate binding, this suggests that the immobilization is not optimal and should be modified. Although SPR is demonstrably efficient at detecting low-affinity antibodies, analysts should confirm that the immobilization protocol chosen is effective for detection of low- and high-affinity antibodies.

Protein Stability Upon Immobilization: The positive control antibody must be able to bind to the immobilized protein in order for an assay result to be acceptable. This confirmation of binding provides confidence that if antibodies against a protein are present in a sample, they will bind to the immobilized protein on the surface of the sensor. Because SPR relies on re-using the immobilized protein surface for multiple analyses, a regeneration protocol is required to effectively remove any bound material from the immobilized protein. This regeneration procedure is based on the ability to remove bound material without damaging or removing the immobilized protein. Several regeneration protocols can be used, and most often the regeneration solution is an acidic solution, commonly dilute HCl. The immobilized protein must remain intact and functional after repeated regeneration steps. Because the immobilized protein will be used routinely for multiple analyses involving repeated cycles of serum samples, analysts should verify the stability of the immobilized protein after regeneration cycles. The stability of the immobilized protein can be monitored effectively by tracking the response units after regeneration and also after addition of positive control antibody. If there is a change in baseline or a decrease in the magnitude of binding by the positive control antibody, then the immobilized protein likely is no longer suitable for further analyses. The stability following regeneration should be established during assay development and should be confirmed during assay validation. In order to monitor the performance of the sensor during an assay, analysts should periodically test a positive control sample during an assay run. If the performance of the positive control samples indicates the immobilized protein has been compromised, analysts should re-analyze test samples obtained after the performance of the assay dropped below acceptable limits. Acceptance parameters for immobilization may vary by compound and should be established for each assay.

Availability of Epitopes After Immobilization: Once the protein is immobilized, the availability of multiple epitopes should be confirmed. Ideally this is done by testing for binding of positive control antibodies with different epitope specificity. One method for testing epitope availability is to use a panel of monoclonal antibodies that are known to recognize different regions of the protein. If the protein has been randomly immobilized, all the different positive control antibodies should be able to bind. The reason for evaluating epitope availability is to prevent false-negative results when serum samples are evaluated. If the immobilization is not random, it would be possible to consistently immobilize the protein via a specific epitope, thus making that epitope unavailable for

binding by an antibody. Another possibility is that chemical modification of the protein to facilitate immobilization altered the protein's conformation.

Surface Regeneration and Subsequent Protein

Stability: Using the previous guidelines, analysts should monitor the surface for signs of accumulation and degradation of the immobilized ligand and discontinue use when necessary. For example, when the binding capacity of a positive control antibody (diluted in test serum) drops below 80% of initial capacity the surface should not be used.

Assay Cut-point Determination: When performing assays to determine if a serum sample contains antibodies against a protein, analysts sometimes observe a background level of binding. That background binding can vary depending on the nature of the immobilized protein and also the patient population being tested. In order to determine if a test sample contains antibodies, analysts compare binding to control samples that do not contain antibodies against the protein. A cut-point is established, and when a sample contains antibodies the binding is greater than that cut-point. Analysts determine the assay cut-point by analyzing a series of serum samples that do not contain antibodies against the immobilized protein and then performing statistical analysis to determine the level of binding consistent with a sample that does not contain antibodies. The cut-point should be established using the same conditions that will be used for sample analysis. Although different approaches are used for determining a cut-point, a common approach is to establish the mean from the binding of 50–100 serum samples from healthy volunteers and set the cut-point at 95% (equivalent to the mean plus 1.645 times the standard deviation for a normal distribution). Analysts should remove statistical outliers from the calculations because their inclusion can cause a high bias and raise the cut-point. This higher cut-point will result in identification of fewer samples with antibodies against the immobilized protein. The statistically evaluated cut-point is the response unit value that serum samples must exceed to be considered positive for the presence of antibodies against the therapeutic protein. An important feature of cut-point determination is that it may be different in different patient populations. For example, patients with inflammatory disease, may show a higher level of nonspecific reactivity compared to a normal population. This higher level of nonspecific binding would result in samples being identified as positive when they did not contain any antibodies specific for the protein. When this situation arises, predose serum samples can be used to establish a new patient population-specific mean and assay cut-point.

Analytical Procedure Development and Validation:

Once the stability of the immobilized protein is confirmed, a regeneration procedure has been defined, and the cut-point established, the antibody testing method can be developed and validated. The conditions used for analyzing samples should be identical to those used to establish the assay cut-point. An important parameter to consider is the optimal dilution of the serum sample. Increasing the dilution factor reduces nonspecific binding by serum proteins but also reduces overall sensitivity. Most antibody assessment procedures use between 5% and 50% serum. As the percentage of serum that is tested decreases, the percentage of the binding signal that is due to nonspecific interaction also decreases, and subsequently the percentage of the signal mediated by antibodies binding to the immobilized protein increases. Besides dilution, other means to reduce nonspecific interaction include adding surfactants, increasing salt concentration, adding BSA or HSA, or adding soluble sensor surface support material such as carboxymethyl dextran or alginate to the dilution and running buffer.

Other important variables to optimize include flow rate and sample volume. The combination of flow rate and sample volume defines the contact time, the length of time during which a given sample is in contact with the immobilized protein. The longer a sample is in contact with the immobilized protein, the greater the chances for antibody binding. The next important aspect to consider is verification that initial binding is a result of an antibody and not some other serum component. This can be accomplished by adding an anti-human immunoglobulin reagent and monitoring subsequent binding. If the initial binding observed was due to an anti-protein antibody, this reagent will bind to that antibody (the anti-protein antibody remains bound to the immobilized protein). When a therapeutic monoclonal antibody is the immobilized protein, the confirmatory reagent must be screened and verified not to bind directly to the immobilized therapeutic monoclonal antibody. One option here is the immobilization of the Fab' fragment rather than the intact therapeutic monoclonal antibody. The confirmatory reagent must be verified for specificity. Once all of the parameters are optimized, the assay can be validated. Validation parameters include those typically associated with immunogenicity assays (precision, specificity, sensitivity, and robustness) as well as parameters specific to SPR assays (protein immobilization, stability of immobilized surface, and number of regeneration cycles).

Interference by Serum Components: Depending on the immobilized protein, serum components other than antibodies specifically directed against the immobilized protein possibly could bind to the immobilized surface. It is also possible that serum components that block the ability of antibodies to bind to the immobilized protein could be present. Both of these can be evaluated by testing the binding of serum samples from the target subject population that are known not to contain antibodies against the immobilized protein and then monitoring to determine if any binding does occur. If nonspecific binding is identified, steps can be taken to reduce or eliminate it. These steps can include pretreatment of samples to remove the nonspecific reactant, addition of surfactant, or alteration of salt concentration in sample buffers to reduce nonspecific binding.

Analysts should verify that serum samples do not contain agents that are capable of inhibiting antibody binding to the immobilized protein (these could include soluble forms of the immobilized protein or soluble receptors that could bind to the immobilized protein and block binding of the antibodies to the immobilized protein). Analysts can add the positive control antibody to target serum samples and can evaluate binding. If binding is inhibited by the target serum samples compared with binding to normal human serum samples, steps can be taken to remove the inhibiting agent. Failure to identify target serum interference can result in either false-positive or false-negative results.

Implementation of Multiplex Assays: When a therapeutic protein is a second-generation product that has been modified from an original therapeutic protein (e.g., via pegylation or increased glycosylation), the presence of antibodies against both the original and the second-generation product should be evaluated simultaneously. This can be accomplished by immobilizing each protein on separate channels in the microfluidic device and allowing serum samples to bind in series or in parallel to both immobilized proteins. The rationale for testing for binding to both the original and the modified therapeutic protein is that antibodies generated against the modified protein could have binding specificity to the original protein as well. As part of the characterization of the immune response, analysts must understand the specificity of antibodies

for both first- and second-generation products. When possible, binding to an endogenous counterpart might also be tested by immobilizing the endogenous protein on a separate flow cell or channel.

Characterization of Anti-Therapeutic Protein

Antibodies: Once antibodies against a therapeutic protein have been captured by binding to the immobilized therapeutic protein, those antibodies can be characterized. The important features of anti-therapeutic antibodies that can be studied include the relative binding affinity, the amount of antibodies present in the serum sample, the isotype(s) of antibodies present in the sample, and binding specificity.

By monitoring the rate at which the response units decrease after the conclusion of sample addition to the sensor, analysts can determine the relative affinity of the antibodies. A high rate of dissociation is characteristic of a low-affinity antibody, and a slow rate of dissociation suggests the presence of high-affinity antibodies. It is useful to compare the dissociation rates with both the positive control antibody (typically a high-affinity antibody preparation) as well as a panel of monoclonal antibodies of known binding affinities.

The relative active concentration of antibodies present in a sample can be estimated by comparing the binding signal with the signal produced from a dilution series of the positive control. Analysts can generate a standard curve from the standard and can compare the active concentration of antibodies in the sample with that standard curve. Because the positive control does not exactly mimic the mixture of antibodies contained in the sample—in fact, the positive control is often obtained from hyperimmunized animals such as rabbits—the concentration value obtained is relative to the standard. This value only approximates the actual concentration of human antibodies. Because the same positive control can be used throughout clinical development, analysts can compare the amount of antibodies obtained from different subjects using this strategy. Because the instrument's signal is proportional to the mass that is binding to the sensor, this type of analysis provides value. Analysts should consider that IgM antibodies have five times the mass of IgG antibodies. Another approach for determining the concentration of antibodies is described in the concentration analysis section of this chapter (see Application 2 below).

The isotype of captured antibodies can be readily determined by monitoring binding associated with sequential addition of isotyping reagents. For example, if IgM antibodies are present and have bound to the immobilized protein, the addition of an anti-human IgM reagent will produce an additional signal. Isotyping reagents can be found with specificity towards IgM, IgG, IgE, IgA, IgG1, IgG2, IgG3, and IgG4. Because of steric hindrance, analysts may be required to repeat isotyping analyses in different sequences to be certain the presence of previously bound isotyping reagents has not hindered subsequent analyses. For example, assume a sample contains both IgG1 and IgG4 antibodies against a protein and both species have bound to the immobilized protein. Because the anti-IgG1 isotyping reagent has bound to the IgG1 antibodies, the isotyping reagent bound to them may prevent subsequent additions of an anti IgG4 reagent from binding to the IgG4, and the presence of the IgG4 would be undiscovered. Analysts will conclude that only IgG1 antibodies are present, but if the order of isotyping reagent addition were reversed the IgG4 antibodies would be discovered. This example underscores the importance of careful interpretation of isotyping results. This is a problem for subsequent analysis only if there is observed binding by a previous cycle of isotype reagent addition. The specificity of

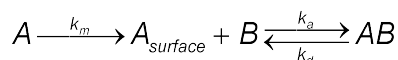
isotyping reagents should be confirmed before use. Analysts should, for example, verify that an anti-human IgG reagent binds only to human IgG and does not cross-react with human IgM.

The region of the therapeutic protein recognized by the antibodies can sometimes be determined by immobilizing versions of the protein that have been truncated, have point mutations, or contain only a fragment of the protein. If the antibodies fail to bind to the changed version of the protein, it suggests that the epitope toward which the antibody is directed was influenced by the change. It should be kept in mind that point mutations and truncations not only influence the primary sequence of a protein, but can also influence the tertiary structure (i.e. folding, conformation) of a protein. Also, a subject is likely to generate a population of antibodies with different specificities for a variety of epitopes. Despite this concern, the strategy just described can prove useful for identifying the region on the protein where the antibodies are binding.

Application 2—Concentration Analysis: SPR can be used to determine the concentration of biologics in defined buffer systems, e.g., eluates from purification columns, formulation buffers, and complex mixtures such as serum, fermentation broths, crude cell extracts, and cell suspensions. The concentration of an analyte is measured by its binding to the specific ligand or other molecules that can interact with any portion of the analyte. The analyte concentration is determined on a surface where the analyte-specific ligand or an analyte-specific capture reagent is immobilized. The binding rate or the mass of analyte bound is determined, and the analyte concentration is calculated using either a standard curve obtained from a concentration series of a purified and well characterized reference material or by a calibration-free analysis that is based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration.

Immobilization of Ligand: To determine concentration the ligand is immobilized covalently or non-covalently on the surface. Analysts select an appropriate coupling mechanism and chemistry to ensure the ligand's functional integrity. In order to provide conditions that favor partial or full independence of kinetic parameters, a high surface density of the ligand is desired.

A high-density surface allows the analyte to bind to the ligand under conditions that limit mass transport. The interaction between the analyte and the ligand can be described by the following two-step process:



where A is the concentration of analyte in the sample, A_{surface} is the concentration of analyte at the sensor surface, k_m is the mass transport coefficient, B is the immobilized ligand, AB is the analyte–ligand complex, and k_a and k_d are the rate constants for the reaction between A and B . The mass transport coefficient k_m depends on the flow rate, the dimensions of the flow cell, and the diffusion coefficient of the analyte. The analyte first must be transported from the bulk to the sensor surface to react with the immobilized ligand molecules. If this mass transport of the analyte is much faster than the association step between ligand and analyte, the overall observed binding will be driven by the kinetic rate constants of A and B , a prerequisite for accurately determining the kinetic parameters. If the mass transport of analyte is much slower than the association step, the binding will be limited by the mass transport process, and kinetic parameters for the specific interaction between A and B will be difficult to obtain. However, these conditions are desired for determining the active concentration of an analyte. A high density of

ligand on the sensor surface and slower flow rates favor limited mass transport. Between these extremes, the overall binding is determined by contributions from both mass transport and interaction kinetics. It may not be possible to achieve limited conditions of mass transport for concentration analyses of interactions with relatively slow association rate constants (e.g., $k_a = 10^4 \text{ M}^{-1}\text{s}^{-1}$).

The ligand and the analyte reference material should be of sufficient purity with special attention to the presence of aggregated material. Aggregates of the analyte can interfere with the regeneration of the ligand surface because they can bind with multiple binding sites.

The reference material must be comparable (e.g., molecular weight and kinetic parameters) to the test samples. Under certain conditions the active concentration in unknown samples can be determined using a calibration-free procedure that is based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration. These two methodologies are described separately below.

Concentration Determination with a Reference

Standard Curve: In typical concentration-determination assays the analyte concentration is calculated from a standard curve that is obtained with a reference material injected at select concentrations. Three different approaches can be used to measure concentration with a reference standard calibration curve:

Direct Binding Assay: Determine the quantity of analyte bound after an arbitrarily fixed sample injection time. A sandwich method can be performed as an extension of the single-step direct binding approach in order to increase assay sensitivity.

Binding Rate Determination: Determine the initial binding rate for a sample rather than the amount bound. Under conditions of mass-transport limited binding, the binding rate is directly proportional to analyte concentration, and is independent of binding kinetics. This allows one to measure the concentration of related molecules that might have different binding characteristics.

Inhibition or Competition Assays: When the mechanism of action for an analyte is binding to a soluble ligand and thereby disrupting a ligand–receptor interaction, an inhibition assay can be used. In an inhibition assay, a receptor is attached to the sensor surface by a covalent linkage. The interaction between the analyte and the soluble ligand is indirectly measured by mixing a fixed concentration of ligand with varying concentrations of the analyte and injecting the ligand–analyte mixture across the immobilized receptor surface. Competitive methods in solution can also be used for large molecules and particles such as viruses, as well as for small analytes that give low direct responses. In parallel systems the assay can be designed so that the standard samples and the unknown sample are injected in parallel. This method can be useful for ligands that are difficult to regenerate.

Analysts can plot the signal (amount bound or rate of binding) of the reference material standards against concentrations and then can generate a standard curve using an appropriate mathematical model such as a linear or a logistic four-parameter curve fit. Samples can be injected at one or more dilutions. Fewer dilutions can be employed if a linear relationship between sample and reference standard has been demonstrated. Concentrations of unknown samples are either obtained by back-calculation from the standard curve or, if they are analyzed at the same target concentrations as those of the reference standard curve, by comparison of curve-fit parameters.

Parameters that can influence assay performance and results include but are not limited to flow rate, ligand

density on the surface, sample purity, sample matrix, and reproducibility of surface regeneration. These parameters must be evaluated during assay qualification or validation. Interference with binding of analyte to the immobilized ligand can be minimized by salts, detergents, or sensor-surface support material. A commonly used sensor surface consists of carboxymethylated dextran, so the addition of dextran to the sample dilution buffer can minimize nonspecific interactions. Injections over a negative control surface can also be used to mathematically subtract the nonspecific binding data from the data obtained on the positive surface. A qualified or validated concentration determination SPR assay should include QC samples that can serve as measure to determine the accuracy of the standard curve that has been prepared to analyze samples with unknown analyte concentration. They can be conveniently prepared in larger batches, qualified for use with a Certificate of Analysis for the target concentration, and stored in small aliquots under appropriate storage conditions.

After each analyte injection the ligand surface is regenerated and all bound analyte is removed. This regeneration must be strong enough to remove all bound analyte, but the conditions also must leave the immobilized ligand intact so that injections can be compared to each other.

Concentration Determination Without Calibration:

Calibration-free concentration assays are based on the relationship between the diffusion properties of the analyte and the absolute analyte concentration. By measuring the initial binding rate analysts can derive the analyte concentration if specific properties of analyte and the analytical environment are known. This approach can be useful when no satisfactory reference standard is available.

To determine the analyte concentration in a sample, analysts use the relationship between initial binding rate and analyte concentration. On a sensor surface with a high immobilization level, the initial binding rate (slope) can be described as a function of the molecular weight, the mass transport coefficient k_m , and the concentration of the analyte. Before a sample is analyzed analysts must determine the mass transport coefficient. It depends on the diffusion coefficient (D), flow rate, and flow cell dimensions and is described by the following formula:

$$k_m = 0.98 \times \sqrt[3]{\frac{D^2 \times f}{0.3 \times h^2 \times w \times l}}$$

where D is the diffusion coefficient, f is the flow rate, and h , w , and l are the flow cell height, width, and length, respectively. Flow rate and flow cell dimensions typically are known for a given instrument, and the diffusion coefficient is determined by the size and shape of the molecule by the use of instrument-specific tools, literature references, or experiments, e.g., by analytical ultracentrifugation or light scattering.

In a typical experimental setup the evaluation requires two flow rates. By using measurements at two widely separated flow rates, analysts can assess the influence of flow rate on binding rate. The robustness of the assay is also improved by fitting the data obtained at two different flow rates, which give correspondingly two different values for k_m (because k_m depends on the flow rate), to a model with a global variable for analyte concentration (so that the model is constrained to find a single concentration value that best fits both curves simultaneously).

Calibration-free concentration analysis is suitable only for proteins with MW \geq 5000 Da. It requires fast analyte–ligand association ($k_a > 5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) and it

cannot handle mixtures of analytes with different diffusion properties. The dynamic range of the method is approximately 0.05–5 $\mu\text{g/mL}$.

Application 3—Kinetic and Affinity Analysis: Because of its ability to detect binding interactions in real time, SPR provides valuable information about the kinetics of complex formation and dissociation. SPR instruments can be used to determine the association rate constant and dissociation rate constant for a particular binding interaction, and these values can be used subsequently to calculate the dissociation equilibrium constant ($K_D = k_d/k_a$). Obtaining K_D from a ratio of k_a and k_d is useful when the binding interaction does not reach equilibrium in a timeframe that is suitable for an SPR binding experiment. For binding interactions that reach equilibrium (rate of complex formation equals the rate of complex decay) in min (vs. h), K_D can be determined directly from a steady-state binding response. The length of time required to reach equilibrium is influenced by the dissociation rate, so quickly dissociating complexes (e.g. $k_d = 10^{-2} \text{ s}^{-1}$) will reach equilibrium faster than those that dissociate slowly (e.g., $k_d = 10^{-5} \text{ s}^{-1}$). Software programs capable of simulating 1:1 binding kinetics are useful for predicting the length of time required to reach equilibrium. The typical working range for affinity measurements with commercially available SPR instruments is from 10^{-12} M (pM) to 10^{-4} M (μM).

Proper experimental design is required to accurately measure k_a , k_d , and K_D . Several questions must be considered when designing kinetic analysis or steady-state affinity experiments, including:

- Which binding partner should be immobilized?
- How will the analyst immobilize one of the binding partners?
- What type of reference surface should be used?
- How much binding partner should be immobilized?
- Does the binding partner maintain activity after immobilization?
- Is binding to the immobilized binding partner specific?
- What regeneration conditions, if necessary, should be used?

When selecting which binding partner to immobilize for most protein–protein interactions, analysts must consider several factors: (1) the purity and availability of the proteins, (2) the presence of a tag or functional group to aid in immobilization, (3) maintaining biological activity, and (4) the binding valency (e.g., monovalent vs. multivalent binding).

A reference surface is required for all detailed kinetic and affinity analysis experiments. If direct immobilization is used, then a reference surface is created using the same immobilization protocol, omitting the protein during the coupling step. Alternatively, a mutant form of the protein with a modified binding site can be used. The reference surface for high-affinity capture typically consists of either the capture molecule and no binding partner, or uses an unrelated molecule for a mock capture surface. For the specific case of antibody–antigen interactions, an unrelated monoclonal antibody often serves as the capture reagent on the reference surface.

After deciding on the immobilization approach, analysts must decide how much binding partner to immobilize. For kinetic analysis, the primary consideration is to minimize the surface density to avoid mass-transport-limited binding of the analyte molecule to the immobilized binding partner. Analysts also must consider the immobilization level when conducting steady-state affinity analysis because high immobilization levels can cause steric hindrance or can induce secondary effects such as nonspecific binding or aggregation.

Before performing a kinetic experiment or steady-state affinity analysis, analysts must assess the activity of the

surface by injecting the analyte molecule at a single concentration. The concentration should be high enough that the equilibrium binding response provides a close approximation of the experimental maximum response (R_{max}). This condition is typically met when the target molecule concentration is at least 10-fold higher than the K_D of the binding interaction. For a protein–protein interaction having a K_D value of 100 pM this means that the target molecule should be injected at a concentration of at least 1 nM. Using the R_{max} equation (Equations 1 and 2), analysts can calculate the theoretical R_{max} based on the amount of binding partner immobilized or captured. If the experimental R_{max} exceeds the theoretical R_{max} , then the analyte molecule is larger than expected or the analyte exists in a higher-order structure than expected (e.g., following aggregation or as a multimer). If the experimental R_{max} is significantly lower (<50%) than the theoretical R_{max} , this suggests that the immobilization procedure has compromised the binding site, and an alternative immobilization procedure should be investigated. An advantage of using high-affinity capture instead of direct coupling is that the surface activity typically remains close to 100%, provided that the specific activity of the immobilized binding partner is 100% before capture.

Injecting the target molecule across the reference surface also provides a quick assessment of the amount of nonspecific binding that exists on the sensor surface. Nonspecific binding that is electrostatic in nature can be eliminated or reduced by addition of salt (e.g., 0.5–1.0 M NaCl) to the sample diluent buffer and the running buffer or by using sensor surfaces with a low charge density. Nonspecific binding that arises from hydrophobic interactions can be minimized or eliminated by the addition of detergents such as 0.05% Polysorbate 20 or 10 mM CHAPS to the sample diluent buffer and the running buffer. Before using buffer additives, analysts should test whether the specific binding interaction or binding activity is affected. Nonspecific binding to a capture molecule can be resolved by switching to a different capture molecule. Reducing surface density also may eliminate nonspecific binding.

Many protein–protein interactions dissociate slowly ($k_d = 10^{-3}$ to 10^{-6} s $^{-1}$), with complex half-lives ($t_{1/2}$) of more than 2 h. A regeneration step is important for these types of binding interactions.

In some protein–protein interactions, surface regeneration may not be possible because of a high-affinity binding interaction between the molecules. If this situation occurs, analysts can consider a titration kinetic experiment or the use of instruments that are capable of performing parallel analyte injections. In a kinetic titration, increasing concentrations of target molecule are injected consecutively across the immobilized surface, and the resulting data are analyzed using a titration kinetics model. In parallel instruments the analyte concentration series is injected in one step, thus eliminating the need for regeneration.

Surface regeneration typically is not performed for steady-state affinity experiments because the binding interactions in this type of experiment have k_d values in the range of 10^{-2} to 0.5 s $^{-1}$, and therefore have $t_{1/2}$ values of <2 min. The bound analyte dissociates from the surface as buffer flows over the surface, and regeneration is not required.

Analysts should have an accurate analyte concentration for kinetic analysis because the calculation of k_d depends on the analyte concentration. Typically an absorbance

reading at a wavelength of 280 nm (A_{280}) is used for this purpose, but analysts should remember that the A_{280} value reflects the total bulk protein in solution and does not reflect the actual concentration of protein that is capable of binding (i.e., the active concentration).

Sample diluent injections should be included in replicate for kinetics and steady-state affinity experiments so that nonspecific responses due to instrumentation or sample diluent can be removed during the data-evaluation process.

The recommended analyte concentration range is 10-fold below and above the K_D for the interaction. By keeping the surface density low ($R_{max} = 5$ –50 RU) and extending the association time, analysts should be able to collect data with enough curvature to accurately define k_d and k_d values. When the affinity of the interaction is high ($K_D =$ low nM to pM), higher analyte concentrations may be required to build a kinetic profile with sufficient curvature to complete the kinetic analysis. Increasing the analyte concentration should not be a substitute for changing other experimental design parameters (e.g., surface density and contact time). Although it is desirable to use analyte concentrations that approach R_{max} , unusually high concentrations may induce aggregation of the analyte in solution or nonspecific binding to the surface.

Within an analyte concentration series, replicate samples should be used to assess surface activity. Additionally, each concentration series should be tested 3–5 times using different surfaces in order to establish confidence intervals for the resulting kinetics and affinity constants. The amount of data that is collected during the kinetic or steady-state affinity experiment affects the accuracy of the results. For kinetic experiments involving binding interactions with slow k_d values, analysts should collect enough dissociation data so that a measurable dissociation response (vs. instrument noise) is acquired. For example, a binding interaction between a therapeutic monoclonal antibody and its target molecule that has a k_d of 10^{-5} s $^{-1}$ will require the collection of at least 4 h of dissociation data. Rather than collecting this much dissociation data for all analyte concentrations, analysts can use a long dissociation time for the highest concentration of analyte that will be injected, and they can use a short dissociation time (2–5 min) for all other analyte concentrations. With parallel-injection instruments one can collect the long dissociation data for all of the concentrations. For data evaluation analysts should collect both long and short dissociation time data for sample diluent injections. For steady-state affinity experiments, the analyte injection time should be long enough to allow for a steady-state binding response to occur at all analyte concentrations that are tested. Dissociation data do not have to be collected for a steady-state affinity experiment because dissociation data are not used in the evaluation of this type of experiment, but the complete dissociation of analyte is required before beginning the next injection.

Use of SPR in a Regulated Environment

When SPR assays are used for lot release and stability testing, the assay must exist within a controlled setting so that decisions can be made about the use of product within the clinic or marketplace. SPR instrumentation, including software, should be 21 CFR Part 11 compliant and should be amenable to validation. These requirements are important because they help ensure the integrity of both data acquisition and data evaluation.

Besides using SPR instrumentation that meets regulatory requirements, analysts should establish system suitability criteria for an SPR assay. Including these criteria in an SPR assay ensures that the results obtained for the test sample are generated by an assay that is performing within its operating parameters. A discussion of assessing system suitability parameters is not within the scope of this chapter, but the reader is referred to USP general chapters *Design and Analysis of Biological Assays* <111> and *Analysis of Biological Assays* <1034> for more detailed discussions. Some examples of system suitability parameters for an SPR assay can include:

- ligand immobilization density
- parameters from a curve fitting model (e.g. four parameter logistic curve fit)
- EC_{50} values for reference standard curve and positive QC curve
- Effective asymptotes (response range) for reference standard curve and positive QC curve
- R^2 values for reference standard curve and positive QC curve
- parallelism between reference standard curve and positive QC curve
 - relative bioactivity for positive QC (EC_{50} ratio of reference standard to positive control)
 - calculated concentration for positive QC (single-point positive QC measurement)
 - binding response for negative QC (nonspecific analyte or diluent)

Multiple lots of ligand, analyte, coupling reagents, and sensor surfaces should be used to establish assay system suitability

criteria that reflect normal assay conditions. Assay results for reference standard and QC samples should be tracked over time. Regular trending analyses should be done on the data to show whether the SPR assay remains in control over its required lifecycle. If trending in the data is observed, remedies can be performed proactively, preventing assay failure.

For SPR assays that are used in lot-release testing, it is also important to establish sample acceptance criteria. These criteria are used to accept or reject sample data and can include:

- relative bioactivity for test sample
- coefficient of variation (CV) for sample replicates
- parallelism between reference standard curve and positive QC curve

Another point to consider for SPR assays in regulated environments is the identification of noncritical and critical reagents. Noncritical reagents typically include coupling buffer, regeneration buffer, and continuous flow (running) buffer. Critical reagents typically include the ligand and analyte for direct binding assays, and ligand, analyte, and competitor molecule in inhibition binding assays. Critical reagents should be qualified/requalified on a routine basis to ensure that they are suitable for use in the SPR assay. Best practices for the characterization of critical reagents are not within the scope of this chapter, so the reader is referred to current regulatory documents for such discussion. Analysts should use current regulatory documents such as ICH Guideline Q2(R1) *Validation of Analytical Procedures* and *USP-NF General Chapter Validation of Compendial Procedures* <1225> when they validate SPR assays in a regulated laboratory.

<1111> MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: ACCEPTANCE CRITERIA FOR PHARMACEUTICAL PREPARATIONS AND SUBSTANCES FOR PHARMACEUTICAL USE

The presence of certain microorganisms in nonsterile preparations may have the potential to reduce or even inactivate the therapeutic activity of the product and has a potential to adversely affect the health of the patient. Manufacturers have therefore to ensure a low bioburden of finished dosage forms by implementing current guidelines on Good Manufacturing Practice during the manufacture, storage, and distribution of pharmaceutical preparations.

Microbial examination of nonsterile products is performed according to the methods given in the texts on *Microbial Enumeration Tests* <61> and *Tests for Specified Microorganisms* <62>. Acceptance criteria for nonsterile pharmaceutical products based upon the total aerobic microbial count (TAMC) and the total combined yeasts and molds count (TYMC) are given in *Tables 1* and *2*. Acceptance criteria are based on individual results or on the average of replicate counts when

replicate counts are performed (e.g., direct plating methods).

When an acceptance criterion for microbiological quality is prescribed, it is interpreted as follows:

- 10^1 cfu: maximum acceptable count = 20;
- 10^2 cfu: maximum acceptable count = 200;
- 10^3 cfu: maximum acceptable count = 2000; and so forth.

Table 1 includes a list of specified microorganisms for which acceptance criteria are set. The list is not necessarily exhaustive, and for a given preparation it may be necessary to test for other microorganisms depending on the nature of the starting materials and the manufacturing process.

If it has been shown that none of the prescribed tests will allow valid enumeration of microorganisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.

In addition to the microorganisms listed in *Table 1*, the significance of other microorganisms recovered should be evaluated in terms of the following:

- The use of the product: hazard varies according to the route of administration (eye, nose, respiratory tract).
- The nature of the product: does the product support growth? does it have adequate antimicrobial preservation?
- The method of application.
- The intended recipient: risk may differ for neonates, infants, the debilitated.
- Use of immunosuppressive agents, corticosteroids.
- The presence of disease, wounds, organ damage.

Table 1. Acceptance Criteria for Microbiological Quality of Nonsterile Dosage Forms

Route of Administration	Total Aerobic Microbial Count (cfu/g or cfu/mL)	Total Combined Yeasts/Molds Count (cfu/g or cfu/mL)	Specified Microorganism(s)
Nonaqueous preparations for oral use	10^3	10^2	Absence of <i>Escherichia coli</i> (1 g or 1 mL)
Aqueous preparations for oral use	10^2	10^1	Absence of <i>Escherichia coli</i> (1 g or 1 mL)
Rectal use	10^3	10^2	—
Oromucosal use	10^2	10^1	Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL) Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL)
Gingival use	10^2	10^1	Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL) Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL)
Cutaneous use	10^2	10^1	Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL) Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL)
Nasal use	10^2	10^1	Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL) Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL)
Auricular use	10^2	10^1	Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL) Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL)
Vaginal use	10^2	10^1	Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL) Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL) Absence of <i>Candida albicans</i> (1 g or 1 mL)
Transdermal patches (limits for one patch including adhesive layer and backing)	10^2	10^1	Absence of <i>Staphylococcus aureus</i> (1 patch) Absence of <i>Pseudomonas aeruginosa</i> (1 patch)
Inhalation use (special requirements apply to liquid preparations for nebulization)	10^2	10^1	Absence of <i>Staphylococcus aureus</i> (1 g or 1 mL) Absence of <i>Pseudomonas aeruginosa</i> (1 g or 1 mL) Absence of bile-tolerant Gram-negative bacteria (1 g or 1 mL)

Table 2. Acceptance Criteria for Microbiological Quality of Nonsterile Substances for Pharmaceutical Use

	Total Aerobic Microbial Count (cfu/g or cfu/mL)	Total Combined Yeasts/Molds Count (cfu/g or cfu/mL)
Substances for pharmaceutical use	10 ³	10 ²

Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialized training in microbiology and in the interpretation of microbiological data. For raw materials, the assessment takes account of the processing to which the product is subjected, the current technology of testing, and the availability of materials of the desired quality.

(1112) APPLICATION OF WATER ACTIVITY DETERMINATION TO NONSTERILE PHARMACEUTICAL PRODUCTS

The determination of the water activity of nonsterile pharmaceutical dosage forms aids in the decisions relating to the following:

- optimizing product formulations to improve antimicrobial effectiveness of preservative systems,
- reducing the degradation of active pharmaceutical ingredients within product formulations susceptible to chemical hydrolysis,
- reducing the susceptibility of formulations (especially liquids, ointments, lotions, and creams) to microbial contamination, and
- providing a tool for the rationale for reducing the frequency of microbial limit testing and screening for objectionable microorganisms for product release and stability testing using methods contained in the general test chapter *Microbial Enumeration Tests* (61) and *Tests for Specified Microorganisms* (62).

Reduced water activity (a_w) will greatly assist in the prevention of microbial proliferation in pharmaceutical products; and the formulation, manufacturing steps, and testing of nonsterile dosage forms should reflect this parameter.

Low water activity has traditionally been used to control microbial deterioration of foodstuffs. Examples where the available moisture is reduced are dried fruit, syrups, and pickled meats and vegetables. Low water activities make these materials self-preserved. Low water activity will also prevent microbial growth within pharmaceutical drug products. Other product attributes, for example, low or high pH, absence of nutrients, presence of surfactants, and addition of antimicrobial agents, as well as low water activity, help to prevent microbial growth. However, it should be noted that more resistant microorganisms, including spore-forming *Clostridium* spp., *Bacillus* spp., *Salmonella* spp. and filamentous fungi, although they may not proliferate in a drug product with a low water activity, may persist within the product.

When formulating an aqueous oral or topical dosage form, candidate formulations should be evaluated for water activity so that the drug product may be self-preserving, if possible. For example, small changes in the concentration of

sodium chloride, sucrose, alcohol, propylene glycol, or glycerin in a formulation may result in the creation of a drug product with a lower water activity that can discourage the proliferation of microorganisms in the product. This is particularly valuable with a multiple-use product that may be contaminated by the user. Packaging studies should be conducted to test product stability and to determine that the container-closure system protects the product from moisture gains that would increase the water activity during storage.

Reduced microbial limits testing may be justified through risk assessment. This reduction in testing, when justified, may entail forgoing full microbial limits testing, implementing skip-lot testing, or eliminating routine testing.

Nonaqueous liquids or dry solid dosage forms will not support spore germination or microbial growth due to their low water activity. The frequency of their microbial monitoring can be determined by a review of the historic testing database of the product and the demonstrated effectiveness of microbial contamination control of the raw materials, ingredient water, manufacturing process, formulation, and packaging system. The testing history would include microbial monitoring during product development, scale-up, process validation, and routine testing of sufficient marketed product lots (e.g., up to 20 lots) to ensure that the product has little or no potential for microbial contamination. Because the water activity requirements for different Gram-reactive bacteria, bacterial spores, yeasts, and molds are well described in the literature,¹ the appropriate microbial limit testing program for products of differing water activities can be established. For example, Gram-negative bacteria including the specific objectionable microorganisms, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella* species will not proliferate or survive in preserved products with water activities below 0.91, while Gram-positive bacteria such as *Staphylococcus aureus* will not proliferate below 0.86, and *Aspergillus niger* will not proliferate below 0.77. Furthermore, even the most osmophilic yeast and xerophilic fungi will not proliferate below 0.60, and they cannot be isolated on compendial microbiological media.¹ The water activity requirements measured at 25° for the growth of a range of representative microorganisms are presented in Table 1.

Pharmaceutical drug products with water activities well below 0.75 (e.g., direct compression tablets, powder and liquid-filled capsules, nonaqueous liquid products, ointments, and rectal suppositories) would be excellent candidates for reduced microbial limit testing for product release and stability evaluation. This is especially true when pharmaceutical products are made from ingredients of good microbial quality, when manufacturing environments do not foster microbial contamination, when there are processes that inherently reduce the microbial content, when the formulation of the drug product has antimicrobial activity, and when manufacturing sites have an established testing history of low bioburden associated with their products. Table 2 contains suggested microbial limit testing strategies for typical pharmaceutical and over-the-counter (OTC) drug products based on water activity. Other considerations, as listed above, would be applied when setting up the microbial limits testing program for individual drug products because water activity measurements cannot solely be used to justify the elimination of microbial content testing for product release.

Similar arguments could be made for the microbial limits testing of pharmaceutical ingredients. However, this would require pharmaceutical manufacturers to have a comprehensive knowledge of the pharmaceutical ingredient manufacturer's manufacturing processes, quality programs, and testing record. This could be obtained through a supplier audit program.

¹ J. A. Troller, D. T. Bernard, and V. W. Scott. Measurement of Water Activity. In: *Compendium of Methods for the Microbiological Examination of Foods*. American Public Health Association, Washington, DC, 1984 pp.124-134.

Table 1. Water Activities (a_w) Required to Support the Growth of Representative Microorganisms

Bacteria	Water Activity (a_w)	Molds and Yeast	Water Activity (a_w)
<i>Pseudomonas aeruginosa</i>	0.97	<i>Rhizopus nigricans</i>	0.93
<i>Bacillus cereus</i>	0.95	<i>Mucor plumbeus</i>	0.92
<i>Clostridium botulinum</i> , Type A	0.95	<i>Rhodotorula mucilaginosa</i>	0.92
<i>Escherichia coli</i>	0.95	<i>Saccharomyces cerevisiae</i>	0.90
<i>Clostridium perfringens</i>	0.95	<i>Paecilomyces variotti</i>	0.84
<i>Lactobacillus viridescens</i>	0.95	<i>Penicillium chrysogenum</i>	0.83
<i>Salmonella</i> spp.	0.95	<i>Aspergillus fumigatus</i>	0.82
<i>Enterobacter aerogenes</i>	0.94	<i>Penicillium glabrum</i>	0.81
<i>Bacillus subtilis</i>	0.90	<i>Aspergillus flavus</i>	0.78
<i>Micrococcus lysodeketicus</i>	0.93	<i>Aspergillus niger</i>	0.77
<i>Staphylococcus aureus</i>	0.86	<i>Zygosaccharomyces rouxii</i> (osmophilic yeast)	0.62
<i>Halobacterium halobium</i> (halophilic bacterium)	0.75	<i>Xeromyces bisporus</i> (xerophilic fungi)	0.61

Table 2. Microbial Limit Testing Strategy for Representative Pharmaceutical and OTC Drug Products Based on Water Activity

Products	Water Activity (a_w)	Greatest Potential Contaminants	Testing Recommended
Nasal inhalant	0.99	Gram-negative bacteria	TAMC,* TCYMC, absence of <i>S. aureus</i> and <i>P. aeruginosa</i>
Hair shampoo	0.99	Gram-negative bacteria	TAMC, TCYMC, absence of <i>S. aureus</i> and <i>P. aeruginosa</i>
Antacid	0.99	Gram-negative bacteria	TAMC, TCYMC, absence of <i>E. coli</i> and <i>Salmonella</i> spp.
Topical cream	0.97	Gram-positive bacteria	TAMC, TCYMC, absence of <i>S. aureus</i> and <i>P. aeruginosa</i>
Oral liquid	0.90	Gram-positive bacteria and fungi	TAMC and TCYMC
Oral suspension	0.87	Fungi	TAMC and TCYMC
Topical ointment	0.55	None	Reduced testing
Lip balm	0.36	None	Reduced testing
Vaginal and rectal suppositories	0.30	None	Reduced testing
Compressed tablets	0.36	None	Reduced testing
Liquid-filled capsule	0.30	None	Reduced testing

* TAMC = Total aerobic microbial count; TCYMC = Total combined yeast and mold count.

NOTE—The water activities cited in Table 2 for the different dosage forms are representative, and companies are urged to test their individual products before developing a testing strategy.

Water activity, a_w , is the ratio of vapor pressure of H_2O in product (P) to vapor pressure of pure H_2O (P_o) at the same temperature. It is numerically equal to 1/100 of the relative humidity (RH) generated by the product in a closed system. RH can be calculated from direct measurements of partial vapor pressure or dew point or indirect measurement by sensors whose physical or electric characteristics are altered by the RH to which they are exposed.

The relationship between a_w and equilibrium relative humidity (ERH) is represented by the following equations:

$$a_w = P/P_o \text{ and } ERH(\%) = a_w \times 100$$

The a_w measurement may be conducted using the dew point/chilled mirror method.² A polished, chilled mirror is used as the condensing surface. The cooling system is electronically linked to a photoelectric cell into which light is reflected from the condensing mirror. An air stream, in equilibrium with the test sample, is directed at the mirror which

cools until condensation occurs on the mirror. The temperature at which this condensation begins is the dew point from which the ERH is determined. Sample preparation should be considered as it may affect the water activity level of the material tested. Commercially available instruments using the dew point/chilled mirror method or other technologies need to be evaluated for suitability, validated, and calibrated when used to make water activity determinations. These instruments are typically calibrated using saturated salt solutions at 25°, as listed in Table 3.

Table 3. Standard Saturated Salt Solutions Used to Calibrate Water Activity Determination Instruments

Saturated Salt Solutions	ERH (%)	a_w
Potassium sulfate (K_2SO_4)	97.3	0.973
Barium chloride ($BaCl_2$)	90.2	0.902
Sodium chloride (NaCl)	75.3	0.753

² AOAC International Official Method 978.18. In: *Official Methods of Analysis of AOAC International*, 17th edition, AOAC International, Gaithersburg, Maryland.

Table 3. Standard Saturated Salt Solutions Used to Calibrate Water Activity Determination Instruments (Continued)

Saturated Salt Solutions	ERH (%)	a _w
Magnesium nitrate [Mg(NO ₃) ₂]	52.9	0.529
Magnesium chloride (MgCl ₂)	32.8	0.328

<1113> MICROBIAL
CHARACTERIZATION,
IDENTIFICATION, AND STRAIN
TYPING

INTRODUCTION

Microorganisms, if detected in drug substances, excipients, water for pharmaceutical use, the manufacturing environment, intermediates, and finished drug products, typically undergo characterization. This may include identification and strain typing, as appropriate. [NOTE—A *Glossary of Terms* is provided at the end of this chapter.] Routine characterization of microorganisms may include the determination of colony morphology, cellular morphology (rods, cocci, cell groupings, modes of sporulation, etc.), Gram reaction or other differential staining techniques, and certain key biochemical reactions (e.g., oxidase, catalase, and coagulase activity) that can be diagnostic. Microbial characterization to this level is sufficient for many risk-assessment purposes in nonsterile pharmaceutical manufacturing operations and in some sterile product manufacturing environments.

In some cases a more definitive identification of the microorganisms yields genus- and species-level identification. Beyond this, available methodologies can perform strain-level identification, which can be useful in an investigation to determine the source of the microorganism. Identification is especially common when organisms are recovered at atypically high rates or in numbers that exceed recommended levels for specific categories of products. Additionally, microbial identification is useful in aseptic processing and is necessary where sterility test positives have occurred and in the assessment of contamination recovered from failed aseptic process simulations, i.e., media fills.

Microbiological identification systems are based on different analytical methodologies, and limitations may be inher-

ent to the method and/or arise from database limitations. Identification is accomplished by matching characteristics (genotypic and/or phenotypic) to an established standard (reference) organism such as a type strain. If a microorganism is not included in the database it will not be identified, so manufacturers should review the breadth of the database of the identification system they plan to use and its applicability to their needs. Users should consider which microbiological identification system(s) is (are) most applicable to their requirements. Bearing in mind both these limitations and the level of identification required (genus, species, strain), users also must select the appropriate technology to use in routine microbiological identification testing.

The need for microbial identification is specifically cited in USP general test chapter *Microbiological Examination of Non-sterile Products: Tests for Specified Microorganisms* <62>. This chapter indicates a requirement for confirmatory identification tests for organisms that grow on selective or diagnostic media and demonstrate defined morphological characteristics. Also, USP general test chapter *Sterility Tests* <71> allows for invalidation of the test, if after identification of the microorganisms isolated from the test, the growth of this (or these) species may be unequivocally ascribed to faults with respect to the material and/or the technique used in conducting the sterility test procedure. USP general information chapter *Microbiological Control and Monitoring of Aseptic Processing Environments* <1116> recommends that microbial isolates be identified at a rate sufficient to support the environmental monitoring program.

ISOLATION OF PURE CULTURES

The first step in identification is to isolate a pure culture for analysis. This is typically accomplished by successive streaking of the colony of interest in a quadrant pattern on appropriate general microbiological solid media with the objective of obtaining discreet colonies that usually yield pure cultures. This technique also allows phenotypic expression and growth of sufficient inoculum for succeeding identification procedures. Analysts should recognize that expression of the microbial phenotype (i.e., cell size and shape, sporulation, cellular composition, antigenicity, biochemical activity, and sensitivity to antimicrobial agents) may be affected by isolate origins, media selection, and growth conditions (see *Table 1*). Therefore, the preparatory media for identification and the number of subcultures may affect the results of phenotype identification methods.

In contrast, the microbial genotype generally is well conserved and unaffected by cultural conditions. Therefore, once the isolation of a pure, monoclonal colony is assured, the microorganism may be analyzed without concern over the most recent growth media or the viability of the isolate. *Table 2* lists genotypic characteristics that can be determined.

Table 1. Phenotypic Characteristics Used in Microbial Taxonomy

Categories	Characteristics
Culture	Colony morphology, colony color, shape and size, pigment production
Morphological	Cellular morphology, cell size, cell shape, flagella type, reserve material, Gram reaction, spore and acid-fast staining, mode of sporulation
Physiological	Oxygen tolerance, pH range, temperature optimum and range, salinity tolerance
Biochemical	Carbon utilization, carbohydrate oxidation or fermentation, enzyme patterns
Inhibition	Bile salt-tolerance, antibiotic susceptibility, dye tolerance
Serological	Agglutination, fluorescent antibody
Chemo-taxonomic	Fatty acid profile, microbial toxins, whole cell composition
Ecological	Origin of the organism

Table 2. Genotypic/Phylogenetic Characteristics That Can Be Used in Microbial Taxonomy

Categories	Characteristics
Genotypic	DNA base ratio (G + C content), restriction fragment patterns, and DNA probes
Phylogenetic	DNA–DNA hybridization, and 16S and 23S rRNA sequences

Bacterial taxonomy as described in *Bergey’s Manual of Systematic Bacteriology (Bergey’s Manual)*¹ is at present accomplished by comparative analysis of genetic material. When the DNA from an unknown organism is compared to the DNA from a known organism, the degree of relatedness can be determined. Genotypic identification (Table 2) is accomplished through the use of DNA hybridization, restriction fragment pattern comparisons, and/or DNA probes. For example, greater than 70% relatedness with DNA–DNA hybridization indicates the organisms are the same species. Phylogenetic analysis (Table 2) is typically performed by comparing the base sequence of a portion of the 16S ribosomal RNA gene for bacteria, or the 23S ribosomal RNA gene for fungi. Polymerase chain reaction (PCR) is used to amplify these genes, and the amplified region is then isolated and base sequenced using an electrophoretic or dideoxy chain termination method. Comparisons can be made using validated proprietary databases or those that are publicly available. [CAUTION: Publicly available databases may not be validated].

PRIMARY SCREENING AND CHARACTERIZATION

Microorganisms isolated on compendial media from samples of pharmaceutical ingredients, water for pharmaceutical use, the manufacturing environment, intermediates, and finished products may be physiologically stressed. The microorganisms will pass from a metabolic state suitable for survival under adverse ambient conditions to culture conditions that are far richer nutritionally and are at an optimal incubation temperature. This transition can be managed by careful handling of the isolates. In preparation for identification, individual representative colonies from the primary isolation media are streaked for monoclonal colonies onto solid media as described above. The first step is to determine the Gram reaction, cellular morphology, and in some cases diagnostic biochemical reactions of the bacteria isolates. This is a critical step for many phenotypic identification schemes. If the wrong characteristics are assigned to an isolate, subsequent testing may be conducted using the wrong microbial identification kit, resulting in an incorrect result. Several common preliminary screening tests are described below.

Gram Staining

Gram staining methods include the four-step method: crystal violet (primary stain), iodine (mordant), alcohol or alcohol–acetone (decolorizer), and safranin (counterstain). In the three-step method the decolorization and counterstaining steps are combined. Under optimal conditions, Gram-positive organisms retain the crystal violet stain and appear blue violet. Gram-negative organisms lose the crystal violet stain, so they contain only the counterstain safranin and appear red. Some bacteria may be Gram-variable. Common pitfalls in this method are that heat fixation may cause Gram-positive cells to stain Gram-negative, and older cultures may give Gram-variable reactions. Using too much decolorizer could result in a false Gram-negative result, and not using enough decolorizer may yield a false Gram-positive result. One variation that has advantages in some situations is to perform a methanol, rather than heat, fixation of the bacterial smear. In some cases alcohol fixation may give more consistent Gram stain results. In either method a

Gram-positive and a Gram-negative control should be included to allow identification of errors in staining. Because the Gram-staining reaction must be read under a microscope, cellular morphology can be simultaneously ascertained.

Spore Staining

Spore staining can be accomplished using a malachite green stain for bacterial spores. A positive control should be included to allow identification of errors in spore staining.

Biochemical Screening

Key biochemical screening tests include (1) the oxidase test to separate Gram-negative, rod-shaped bacteria into nonfermenters (oxidase positive) and enteric (oxidase negative) bacteria, (2) the catalase test to separate *Staphylococci* (catalase positive) from *Streptococci* (catalase negative), and (3) the coagulase test to separate *Staphylococci* into coagulase negative (presumptively nonpathogenic) and coagulase positive (more likely pathogenic) *Staphylococci*. For many types of investigations and routine surveying of manufacturing environmental bioburden, these few tests can provide sufficient information for ongoing evaluation. However, when circumstances dictate greater in-depth assessment, identification to the genus, species, or strain level can yield valuable insights about the nature and source of environmental bioburden. Also, microbial identification to the species and even strain level can be critical in assessing and mitigating risk from microbial contamination.

MICROBIAL IDENTIFICATION BY PHENOTYPIC METHODS

Phenotypic methods use expressed gene products to distinguish among different microorganisms. Generally, these require a relatively large number of cells in pure, monoclonal culture. Recovery and growth methods for microbial enumeration and identification are limited by the length of incubation and the fact that many organisms present in the environment are not recovered by general microbiological growth media. Additionally, freshly isolated, stressed microorganisms by subculture from primary recovery may not result in a full expression of phenotypic properties. However, methods based on carbon utilization and biochemical reaction, as well as fatty acid profiles by gas–liquid chromatography and whole-cell composition by MALDI–TOF mass spectrometry, are always based on inocula development for a specific identification system. These systems rely on specified culture media and incubation conditions to achieve consistent identification. Phenotypic microbial identification methods are successfully used in food, water, clinical, and pharmaceutical microbiological testing laboratories.² Phenotypic microbial identification methods provide information that enables microbiologists to make informed decisions regarding product risk and to recognize changes in environmental microflora. In many quality control investigations, phenotypic identification alone is sufficient and will enable scientists to conduct a thorough investigation and to recommend appropriate corrective actions as needed.

² O’Hara, C.M., M.P. Weinstein, and J.M. Miller. Manual and automated systems for detection and identification of microorganisms. *ASM Manual of Clinical Microbiology*, 8th Edition, 2003.

¹ *Bergey’s Manual of Systematic Bacteriology*, 2nd Edition, 2003.

MICROBIAL IDENTIFICATION BY GENOTYPIC METHODS

Genotypic microbial identification methods are theoretically more reliable because nucleic acid sequences are highly conserved in most microbial species. Applicable genotypic methods include DNA–DNA hybridization, PCR, 16S and 23S rRNA sequencing, multilocus sequence typing (MLST), pyrosequencing, DNA probes, and analytical ribotyping. These methods can be technically challenging for microbiologists. They also require more expensive analytical equipment and supplies. Often these analyses are conducted by contract laboratories, government laboratories, universities, research institutes, or specialized laboratories within industrial firms. Therefore, the use of genotypic identification methods is typically limited to critical microbiological investigations such as product failure investigations. Further, if strain-level identification is done in the course of an investigation, analysts must ensure that the method is appropriate.

DNA sequencing of the first 500 base pairs of the 16S rRNA sequence is useful for identification to the species level but may not provide sufficient power to resolve among closely related species or strains of the same species. In contrast, Southern hybridization of restriction endonuclease digests is powerful and can be effective in demonstrating differences between two strains. If the banding patterns appear identical, this shows only that restriction endonuclease has similar cleavage sites in that region of the two organisms. Demonstration that the two organisms are the same should include two or more different restriction endonuclease digests, each of which yields bands in the area of interest. All bands from the two organisms must be identical.

In contrast to microbial identification, nucleic acid-based methods can be used to screen for specific microorganisms. The steps associated with this activity are sample collection, nucleic acid extraction, target amplification, hybridization, and detection. The problem of amplifying DNA from non-viable bacterial cells can be overcome by using reverse transcription to convert rRNA that is transitional, hence related to viability, to DNA for PCR amplification. Issues include the detection of microbial variants, limits of detection, matrix effects, positive cutoff verification, instrument and system carry-over, diagnostic accuracy, and reproducibility.

VERIFICATION OF MICROBIAL IDENTIFICATION METHODS

Microbial identification tests include serological tests, chemical reagents, reference organisms, and instrumentation. The verification of an identification test system can include one of the following: (1) using an existing system for parallel testing of microbial isolates obtained from routine testing (the number of isolates tested may be as high as 50, and any discrepancies in identification can be arbitrated using a referee method); (2) testing 12–15 known representative stock cultures of different commonly isolated species for a total of 50 tests; or (3) confirming that 20–50 organism identifications, including 15–20 different species, agree with the results of a reference laboratory testing of split sample.³ In each case the appropriate quality control organisms, as recommended by the supplier and the compendia, should be included in the verification process.

With identification systems, verification of the identity of the species should be evaluated and the level of agreement should be considered. Typically greater than 90% agreement can be achieved with samples of microorganisms that

are appropriate for the identification system. Groups of organisms that are challenging to identify (e.g., nonfermenting bacteria, corynebacteria, and coagulase-negative *Staphylococci*) may be included, when appropriate, in the verification process but may yield lower levels of agreement.

The hierarchy of microbial identification errors in descending order of impact is (1) misidentification to genera, (2) misidentification to species, and (3) no identification. Misidentification could lead to inappropriate corrective and preventive actions and product disposition.

A microbial identification system may not be able to identify an isolate because the organism is not included in the database, the system parameters are not sufficiently comprehensive to identify the organism, the isolate may be nonreactive in the system, or the species may not have been taxonomically described. Such isolates can be sent to the supplier of the microbial identification system for additional study and, if appropriate, added to the database. Alternatively, genotypic identification tests can be conducted, and the species can be added to an in-house database. Misidentification is more difficult to determine, but any microbial identification should be reviewed for reasonableness in terms of the microorganism's morphology, physiological requirements, and source of isolation. Organisms identified only to genus may be common for the numerous non-pathogenic species of *Staphylococcus*, *Corynebacterium* (and other small pleomorphic Gram-positive rods), and *Micrococcus*.

The most important verification tests are accuracy and reproducibility. These measurements can be defined as follows:

Accuracy % = (Number of correct results/Total number of results) × 100

Reproducibility % = (Number of correct results in agreement/Total number of results) × 100

The user should establish suitable acceptance criteria for accuracy and reproducibility, taking into account method capability.

Other measurements are sensitivity, specificity, and positive and negative predictive value. These measurements are best illustrated by an example. A clinical microbiology laboratory compared the frequency of isolation of a DNA hybridization probe to a culture method for the sexually transmitted bacterium *Neisseria gonorrhoeae*.³ The frequency of isolation from clinical specimens was historically 10%. The lab ran 100 split samples, and the results are presented in Table 3.

Table 3. Comparison of the Distribution of Negative and Positive Results for the DNA Probe and Culture Methods

DNA Probe Results	Culture Results	
	Positive	Negative
Positive	9	2
Negative	1	88

Sensitivity = $[9/(9 + 1)] \times 100 = 90\%$

Specificity = $[88/(88 + 2)] \times 100 = 97.7\%$

Positive Predictive Value = $[9/(9 + 2)] \times 100 = 81.8\%$

Negative Predictive Value = $[88/(88 + 1)] \times 100 = 98.9\%$

Note that the positive predictive value (PPV) is not intrinsic to the test; it also depends on the prevalence of the microorganism in clinical samples. PPV is directly proportional to the prevalence of the disease or condition. In this example, if the group of people tested had included a higher proportion of people with infection, then the PPV would probably be higher and the negative predictive value (NPV) lower. If all persons in the group had infection, the PPV would be 100% and the NPV 0%. The mathematical derivation of these functions is outlined in Table 4.

³ Cumitech 31. *Verification and Validation of Procedures in the Clinical Microbiology Laboratory*. Elder, B.L., S.A. Hansen, J.A. Kellogg, F.J. Marsik, and R.J. Zabransky, ASM, February 1997.

Table 4. A Two-Row by Two-Column Contingency Table with Respect to the Reference Culture Method and the Alternate PCR Method (After ISO 5725-1 and 5725-2 2004)*

Culture	PCR		
	Positive	Negative	Sum
Positive	a True Positive	b False Negative	a + b
Negative	c False Positive	d True Negative	c + d
Sum	a + c	b + d	

* ISO 5725-1:1994 Accuracy (trueness and precision) of measurement methods and results—Part 1: General principles and definitions and ISO 5725-2:1994 Accuracy (trueness and precision) of measurement methods and results—Part 2: Basic methods for the determination of repeatability and reproducibility of standard measurement methods.

$$\text{Inclusivity (\%)} = [a/(a + b)] \times 100$$

$$\text{Exclusivity (\%)} = [d/(c + d)] \times 100$$

$$\text{Positive Predictivity (\%)} = [a/(a + c)] \times 100$$

$$\text{Negative Predictivity (\%)} = [d/(b + d)] \times 100$$

$$\text{Analytical Accuracy (\%)} = [(a + d)/(a + b + c + d)] \times 100$$

$$\text{Kappa Index} = 2(ad - bc)/[(a + c) \times (c + d) + (a + b) \times (b + d)]$$

Phylogenetic Considerations

The second edition of *Bergey's Manual* represented a major departure from the first edition, and also from the eighth and ninth editions of the *Manual of Determinative Bacteriology*. The organization of content in *Bergey's Manual* follows a phylogenetic framework, based on analysis of the nucleotide sequence of the ribosomal small subunit 16S RNA, rather than a phenotypic structure.

Phylogenetic trees or dendrograms show the closest genetically related organisms. The application of this technology has resulted in taxonomic revisions and the renaming of some well-known microorganisms; e.g., the fungus *A. niger* ATCC 16404 was renamed *A. brasiliensis*. In general, organisms with relatedness less than or equal to 97% are considered different genera and those with relatedness less than or equal to 99% are considered different species,⁴ but there are many exceptions to this generalization.

Differences in genotype and phenotype are relatively uncommon, e.g., same or very similar genotype shared by microorganisms with different phenotypes, similar phenotypes but different genotypes, and microorganisms that are genotypically too distant to be the same species or genus. The concept of polyphasic taxonomy⁵ that refers to assembly and use of many levels of information, e.g., microbial characterization, phenotypic and genotypic data, and origin of the microorganisms, can be successfully applied to microbial identification. This avoids decisions made solely using genotypic data that make no sense when the microbial characteristics, testing history, and source of isolation are considered.

GLOSSARY OF TERMS

Microbial classification: The arrangement of microorganisms into taxonomic groups based on their similarities and relationships.

Microbial identification: The determination of which broad group (e.g., bacteria, yeast, or mold) or narrow group (e.g., genus and/or species) to which a laboratory isolate belongs.

⁴ J.E. Clarridge III. The Impact of 16S rRNA Gene Sequencing Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases, *Clin. Microbiol. Rev.* 17 (2) 840–862, 2004.

⁵ Gillis, M., P. Vandamme, P. De Vos, J. Swings and K. Kersters. Polyphasic Taxonomy. *Bergey's Manual of Systematic Bacteriology*, 2nd Edition, 2003.

Microbial characterization: The use of colony growth, cellular morphology, differential staining, and key diagnostic features to characterize a laboratory isolate for trending and investigative purposes without identification, e.g., nonpathogenic *Staphylococci*.

Mol %GC: The molecular percentage of guanine–cytosine range within the chromosomal DNA. [NOTE—The %GC + %AT = 100%.]

Phylogenetic species: A species consisting of many strains including the type of strain that shares at least 70% total genome DNA–DNA hybridization and less than 5° ΔTm (difference in melting point of the hybrid).

Polyphasic taxonomy: Taxonomy that assembles and assimilates many levels of information from molecular, physiological, morphological, serological, or ecological sources to classify a microorganism.

Relatedness: The extent of relationship or similarity of two (or more) organisms on a Phylogenetic Tree or a Dendrogram.

rRNA Sequence: The DNA sequences that encode rRNA used in protein synthesis are highly conserved among microorganisms of a common ancestry. They are used to determine the phylogenetic distance between organisms and are useful in microbial taxonomy and identification.

Strain: A specific isolate of a species that is maintained in pure culture and is characterized. The type strain is representative of the species that provides a reference for the species based on its historic isolation, characterization, and deposition in recognized culture collections.

Strain typing: Strain typing is an integral part of epidemiological investigations in clinical and public health microbiology. Methods including pulsed-field gel electrophoresis, ribotyping, arbitrarily primed polymerized chain reaction, and whole genome ordered restriction or optical mapping can be used to demonstrate that microbial species are the same strain and most likely are from a common source.

<1116> MICROBIOLOGICAL CONTROL AND MONITORING OF ASEPTIC PROCESSING ENVIRONMENTS

Microbiologically controlled environments are used for a variety of purposes within the healthcare industry. This general information chapter provides information and recommendations for environments where the risk of microbial contamination is controlled through aseptic processing. Products manufactured in such environments include pharmaceutical sterile products, bulk sterile drug substances, sterile intermediates, excipients, and, in certain cases, medical devices. Aseptic processing environments are far more critical in terms of patient risk than controlled environments used for other manufacturing operations—for example, equipment and component preparation, limited bioburden control of nonsterile products, and processing of terminally sterilized products. In this chapter, the type of aseptic processing is differentiated by the presence or absence of human operators. An advanced aseptic process is one in which direct intervention with open product containers or exposed product contact surfaces by operators wearing conventional cleanroom garments is not required and never

permitted. [NOTE—A description of terms used in this chapter can be found in the *Appendix* at the end of the chapter.]

The guidance provided in this chapter and the monitoring parameters given for microbiological evaluation should be applied only to clean rooms, restricted-access barrier systems (RABS), and isolators used for aseptic processing. ISO-classified environments used for other purposes are not required to meet the levels of contamination control required for aseptically produced sterile products. The environments used for nonsterile applications require different microbial control strategies.

A large proportion of products labeled as sterile are manufactured by aseptic processing rather than terminal sterilization. Because aseptic processing relies on the exclusion of microorganisms from the process stream and the prevention of microorganisms from entering open containers during processing, product bioburden as well as the bioburden of the manufacturing environment are important factors governing the risk of unacceptable microbial contamination. The terms *aseptic* and *sterile* are not synonymous. *Sterile* means having a complete absence of viable microorganisms or organisms that have the potential to reproduce. In the purest microbiological sense, an *aseptic* process is one that prevents contamination by the exclusion of microorganisms. In contemporary aseptic healthcare-product manufacturing, *aseptic* describes the process for handling sterilized materials in a controlled environment designed to maintain microbial contamination at levels known to present minimal risk.

In any environment where human operators are present, microbial contamination at some level is inevitable. Even the most cautious clean-room environment design and operation will not eliminate the shedding of microorganisms if human operators are present. Thus, an expectation of zero contamination at all locations during every aseptic processing operation is technically not possible and thus is unrealistic. There are no means to demonstrate that an aseptic processing environment and the product-contact surfaces within that environment are sterile. Monitoring locations should be determined based upon an assessment of risk. Although manufacturers should review environmental monitoring results frequently to ensure that the facility operates in a validated state of control, monitoring results can neither prove nor disprove sterility. Because of the limitations of monitoring, manufacturers cannot rely directly on monitoring, statistics, or periodic aseptic-processing simulations to ensure a sterility assurance level.

Environmental monitoring is usually performed by personnel and thus requires operator intervention. As a result, environmental monitoring can both increase the risk of contamination and also give false-positive results. Thus, intensive monitoring is unwarranted, particularly in the ISO 5 environments that are used in the most critical zones of aseptic processing.

A number of sampling methods can be used to assess and control the microbiological status of controlled environments for aseptic processing. At present, nearly all of these methods rely on the growth and recovery of microorganisms, many of which can be in a damaged state caused by environmental stress and therefore may be difficult to recover. The numerical values for air, surface, and personnel monitoring included in this chapter are not intended to represent limits or specifications but are strictly informational. Because of the variety of microbiological sampling equipment and methods, it is not scientifically reasonable to suggest that the attainment of these values guarantees microbial control or that excursions beyond values in this chapter indicate a loss of control. The assessment of risks associated with manufacturing environments must be made over a significant period; and in each case, the contamination recovery rate metric should be established on the basis of a review of actual findings within the facility. The objective of each user should be to use contamination recovery rates to track ongoing performance and to refine the microbiological control program to foster improvements. When optimum operational conditions are achieved within a facility, con-

tamination recovery rate levels typically become relatively stable within a normal range of variability.

There are no standard methods for air sampling, and available literature indicates that air-sampling methods are highly variable. It should not be assumed that similar sample volumes taken by different methods will produce similar rates of recovery. Many factors can affect microbial recovery and survival, and different air sampler suppliers may have designed their systems to meet different requirements. Also, sample-to-sample variation in microbial sampling can be extensive. Limited data are available regarding the accuracy, precision, sensitivity, and limits of detection of monitoring methods used in the aseptic processing of healthcare products.

Surface sampling methods are also not standardized. Different media are employed, and in the case of swabs, different results have been reported for wet and dry swab methods and contact plates. Replicate sample contact plates should be expected to give similar results under identical conditions, but rates of recovery have been reported to be both lower than expected and highly variable. In general, surface monitoring has been found to recover <50%, even when used with relatively high inoculum levels on standardized coupons. In actual production environments where organisms are stressed to varying degrees, recovery rates may be lower.

ADVANCED ASEPTIC TECHNOLOGIES

Advanced aseptic technologies can be defined as those that do not rely on the direct intervention of human operators during processing. At present, technologies such as isolators, blow/fill/seal, and closed RABS (designs that are never opened during setup or operation) may be considered advanced aseptic technologies, provided that direct intervention by gowned personnel is disallowed during processing. In recent years, isolator technology has found a broad acceptance in healthcare manufacturing. Isolators and closed RABS effectively separate the operator from the critical aseptic processing environment. Because these systems substantially reduce contamination risk, their microbiological control levels are higher than those of conventional clean rooms that have comparable particulate air classification level, for example, ISO 5.

CLEAN ROOM CLASSIFICATION FOR ASEPTIC PROCESSING ENVIRONMENTS

The design and construction of clean rooms and controlled environments are covered in ISO 14644 series. This standard defines the performance of a clean environment with respect to the concentration of total particulates per unit volume. ISO 14644-1 stipulates the total particulate counts allowed for a clean environment to meet the defined air quality classifications. The reader is referred to this standard regarding the design characteristics and certification of clean environments.

Pharmaceutical manufacturers are concerned with nonviable particulate contamination in injectable products (see *Particulate Matter in Injections* (788)). Unlike microbial contamination in which experimental data suggest that humans are the only significant source, nonviable particulates can arise both from humans and from processing equipment. Studies indicate that gowned humans slough particulate and microbial contamination at a rather consistent rate. However, the relationship between microbial (viable) and nonviable contamination does not hold for particulates shed by processing equipment. Where equipment is the primary source of particulate matter, the resulting particulates are essentially all nonviable.

The argument that if fewer total particulates are present in a clean room, it is less likely that airborne microorganisms will be present is true only if human operators are the

source of particulate matter. It is not possible to clearly distinguish between background total particulate contamination generated largely by mechanical operations and the total particulates contributed by personnel. Thus, it is both commonplace and proper for clean-room environmental monitoring programs to consist of both a total particulate component and a microbiological component. Table 1 describes the clean room classifications commonly used in the pharmaceutical industry. In aseptic processing, clean environments of ISO 14644-1 Classes 5–8 are typically used.

Table 1. Airborne Total Particulate Cleanliness Classes^a

ISO Class ^b	Particles $\geq 0.5 \mu\text{m}/\text{m}^3$
ISO 5	3520
ISO 6	35,200
ISO 7	352,000
ISO 8	3,520,000

^a Taken from ISO International Standard 14644 Part 1, published by the International Organization for Standardization, May 1999.

^b The four ISO 14644-1 classes correspond closely to former U.S. Federal Standard 209E classifications. The relationships are ISO 5/Class 100, ISO 6/Class 1000, ISO 7/Class 10,000, and ISO 8/Class 100,000.

Isolators and closed RABS present a different picture, because personnel are excluded from the aseptic processing environment and manipulations are made using glove-and-sleeve assemblies and half-suits made of thick, flexible plastic (such as polyvinyl chloride or synthetic rubber). Personnel have far less effect on the microbial quality of the environment within an isolator enclosure than in clean room environments. Some users have chosen to operate RABS in a manner that allows open, direct human intervention. In an open operational state, these systems are more similar in operation to conventional clean rooms and therefore cannot be considered advanced aseptic processing systems. In an open RABS, the ability of operators to adversely affect microbial contamination risk is higher than with closed RABS or isolators.

Specifications for air changes per hour and air velocities are not included in ISO 14644, nor were they included in Federal Standard 209E. Typically, ISO Class 8/Class 100,000 rooms are designed to provide a minimum of 20 air changes per hour; ISO Class 7/Class 10,000 rooms are designed to provide more than 50 air changes per hour; and ISO Class 5/Class 100 clean rooms provide more than 100 air changes per hour. The design of some facility criteria may differ. By diluting and removing contaminants, large volumes of air are likely to reduce airborne contamination in aseptic production. Optimum conditions vary considerably, depending on process characteristics, particularly the amount of contamination derived from personnel. These specifications should be used only as a guide in the design and operation of clean rooms, because the precise correlations among air changes per hour, air velocity, and microbial control have not been satisfactorily established experimentally.

Manufacturers should maintain a predominantly unidirectional flow of air (either vertical or horizontal) in a staffed Class 5 clean room environment, particularly when products, product containers, and closures are exposed. In the evaluation of air movement within a clean room, studying airflow visually by smoke studies or other suitable means is probably more useful than using absolute measures of airflow velocity and change rates. Risk assessment models are another useful way of reducing contamination risk and should be considered.

Air velocity and change rates are far less important in isolators or closed RABS than in clean rooms because personnel are more carefully separated from the product, product containers, and closures. Air velocities substantially lower than those used in human-scale clean rooms have proved adequate in isolator systems and may be appropriate in RABS as

well. In zones within isolators where particulate matter poses a hazard to product quality, predominantly vertical or horizontal unidirectional airflow can be maintained. Experience has shown that well-controlled mixing or turbulent airflow is satisfactory for many aseptic processes and for sterility testing within isolators (see *Sterility Testing—Validation of Isolator Systems* (1208)).

IMPORTANCE OF A MICROBIOLOGICAL EVALUATION PROGRAM FOR CONTROLLED ENVIRONMENTS

Monitoring of total particulate count in controlled environments, even with the use of electronic instrumentation on a continuous basis, does not provide information on the microbiological content of the environment. The basic limitation of particulate counters is that they measure particles of $0.5 \mu\text{m}$ or larger. While airborne microorganisms are not free-floating or single cells, they frequently associate with particles of $10\text{--}20 \mu\text{m}$. Particulate counts as well as microbial counts within controlled environments vary with the sampling location and the activities being conducted during sampling. Monitoring the environment for nonviable particulates and microorganisms is an important control function because they both are important in achieving product compendial requirements for *Foreign and Particulate Matter* and *Sterility in Injections* (1).

Total particulate monitoring may provide a better means of evaluating the overall quality of the environment in isolators and closed RABS than in most conventional clean rooms. The superior exclusion of human-borne contamination provided by an isolator results in an increased proportion of nonviable particulates. Total particulate counting in an isolator is likely to provide an immediate indicator of changes in contamination level. Microbial monitoring programs should assess the effectiveness of cleaning and sanitization practices by and of personnel who could have an impact on the bioburden. Because isolators are typically decontaminated using an automatic vapor or gas generation system, microbial monitoring is much less important in establishing their efficiency in eliminating bioburden. These automatic decontamination systems are validated directly, using an appropriate biological indicator challenge, and are controlled to defined exposure parameters during routine use to ensure consistent decontamination.

Microbial monitoring cannot and need not identify and quantify all microbial contaminants in these controlled environments. Microbiological monitoring of a clean room is technically a semiquantitative exercise, because a truly quantitative evaluation of the environment is not possible, given the limitations in sampling equipment. Both the lack of precision of enumeration methods and the restricted sample volumes that can be effectively analyzed suggest that environmental monitoring is incapable of providing direct quantitative information about sterility assurance. Analysts should remember that no microbiological sampling plan can prove the absence of microbial contamination, even when no viable contamination is recovered. The absence of growth on a microbiological sample means only that growth was not discovered; it does not mean that the environment is free of contamination.

Routine microbial monitoring should provide sufficient information to demonstrate that the aseptic processing environment is operating in an adequate state of control. The real value of a microbiological monitoring program lies in its ability to confirm consistent, high-quality environmental conditions at all times. Monitoring programs can detect changes in the contamination recovery rate that may be indicative of changes in the state of control within the environment.

Environmental microbial monitoring and analysis of data by qualified personnel can assist in ensuring that a suitable state of control is maintained. The environment should be sampled during normal operations to allow the collection of

meaningful, process-related data. Microbial sampling should occur when materials are in the area, processing activities are ongoing, and a full complement of personnel is working within the aseptic processing environment.

Microbial monitoring of manufacturing clean rooms, RABS, and isolators should include compressed gases, surfaces, room or enclosure air, and any other materials and equipment that might produce a risk of contamination. The analysis of contamination trends in an aseptic environment has long been a component of the environmental control program. In aseptic processing environments and particularly in ISO Class 5 environments, contamination is infrequently observed. In isolator enclosures, contamination is rarer still because of superior exclusion of human-borne contamination. Because of the criticality of these environments, even minor changes in the contamination incident rates may be significant, and manufacturers should frequently and carefully review monitoring data. In less critical environments, microbial contamination may be higher, but changes in recovery rates should be noted, investigated, and corrected. Isolated recoveries of microorganisms should be considered a normal phenomenon in conventional clean rooms, and these incidents generally do not require specific corrective action, because it is almost certain that investigations will fail to yield a scientifically verifiable cause. Because sampling itself requires an aseptic intervention in conventional clean rooms, any single uncorrelated contamination event could be a false positive.

When contamination recovery rates increase from an established norm, process and operational investigation should take place. Investigations will differ depending on the type and processing of the product manufactured in the clean room, RABS, or isolator. Investigation should include a review of area maintenance documentation; sanitization/decontamination documentation; the occurrence of nonroutine events; the inherent physical or operational parameters, such as changes in environmental temperature and relative humidity; and the training status of personnel.

In closed RABS and isolator systems, the loss of glove integrity or the accidental introduction of material that has not been decontaminated are among the most probable causes of detectable microbial contamination. Following the investigation, actions should be taken to correct or eliminate the most probable causes of contamination. Because of the relative rarity of contamination events in modern facilities, the investigation often proves inconclusive. When corrective actions are undertaken, they may include reinforcement of personnel training to emphasize acceptable gowning and aseptic techniques and microbial control of the environment. Some additional microbiological sampling at an increased frequency may be implemented, but this may not be appropriate during aseptic processing because intrusive or overly intensive sampling may entail an increased contamination risk. When additional monitoring is desirable, it may be more appropriate during process simulation studies. Other measures that can be considered to better control microbial contamination include additional sanitization, use of different sanitizing agents, and identification of the microbial contaminant and its possible source.

In any aseptic environment, conventional or advanced, the investigation and the rationale for the course of action chosen as a result of the investigation must be carefully and comprehensively documented.

PHYSICAL EVALUATION OF CONTAMINATION CONTROL EFFECTIVENESS

Clean environments should be certified as described in ISO 14644 series in order to meet their design classification requirements. The design, construction, and operation of clean rooms vary greatly, so it is difficult to generalize requirements for parameters such as filter integrity, air velocity, air patterns, air changes, and pressure differential. In particularly critical applications such as aseptic processing, a

structured approach to physical risk assessment may be appropriate.

One such method has been developed by Ljungqvist and Reinmüller. This method, known as the L-R method, challenges the air ventilation system by evaluating both airflow and the ability of an environment to dilute and remove airborne particles. In the L-R method, a smoke generator allows analysts to visualize the air movements throughout a clean room or a controlled environment, including vortices or turbulent zones, and the airflow pattern can be fine-tuned to minimize these undesirable effects. Following visual optimization of airflow, particulate matter is generated close to the critical zone and sterile field. This evaluation is done under simulated production conditions but with equipment and personnel in place. This type of test can also be used to evaluate the ability of RABS and isolator systems, particularly around product exit ports in these systems, to resist the effects of contamination.

Visual evaluation of air movement within clean rooms is a subjective process. Complete elimination of turbulence or vortices is not possible in operating clean rooms that contain personnel and equipment. Air visualization is simply one step in the effort to optimize clean room operations and is not a definitive pass/fail test, because acceptable or unacceptable conditions are not readily definable.

Proper testing and optimization of the physical characteristics of the clean room, RABS, or isolator are essential before implementation of the microbiological monitoring program. Assurance that the clean room, RABS, or isolator is in compliance with its predetermined engineering specifications provides confidence that the ability of the facility systems and operating practices to control the bioburden and nonviable particulate matter are appropriate for the intended use. These tests should be repeated during routine certification of the clean room or advanced aseptic processing systems, and whenever significant changes are made to the operation, such as personnel flow, equipment operation, material flow, air-handling systems, or equipment layout.

TRAINING OF PERSONNEL

Good personnel performance plays an essential role in the control of contamination, proper training and supervision are central to contamination control. Aseptic processing is the most critical activity conducted in microbiological controlled environments, and manufacturers must pay close attention to details in all aspects of this endeavor. Rigorous discipline and strict supervision of personnel are essential in order to ensure a level of environmental quality appropriate for aseptic processing.

Training of all personnel working in controlled environments is critical. This training is equally important for personnel responsible for the microbial monitoring program, because contamination of the clean working area could inadvertently occur during microbial sampling. In highly automated operations, monitoring personnel may be the employees who have the most direct contact with the critical surfaces and zones within the processing area. Microbiological sampling has the potential to contribute to microbial contamination caused by inappropriate sampling techniques or by placing personnel in or near the critical zone. A formal training program is required to minimize this risk. This training should be documented for all personnel who enter controlled environments. Interventions should always be minimized, including those required for monitoring activities; but when interventions cannot be avoided, they must be conducted with aseptic technique that approaches perfection as closely as possible.

Management of the facility must ensure that personnel involved in operations in clean rooms and advanced aseptic processing environments are well versed in relevant microbiological principles. The training should include instruction about the basic principles of aseptic technique and should emphasize the relationship of manufacturing and handling

procedures to potential sources of product contamination. Those supervising, auditing, or inspecting microbiological control and monitoring activities should be knowledgeable about the basic principles of microbiology, microbial physiology, disinfection and sanitation, media selection and preparation, taxonomy, and sterilization. The staff responsible for supervision and testing should have academic training in medical or environmental microbiology. Sampling personnel as well as individuals working in clean rooms should be knowledgeable about their responsibilities in minimizing the release of microbial contamination. Personnel involved in microbial identification require specialized training about required laboratory methods. Additional training about the management of collected data must be provided. Knowledge and understanding of applicable standard operating procedures are critical, especially those procedures relating to corrective measures taken when environmental conditions require. Understanding of contamination control principles and each individual's responsibilities with respect to good manufacturing practices (GMPs) should be an integral part of the training program, along with training in conducting investigations and in analyzing data.

The only significant sources of microbial contamination in aseptic environments are the personnel. Because operators disperse contamination and because the ultimate objective in aseptic processing is to reduce end-user risk, only healthy individuals should be permitted access to controlled environments. Individuals who are ill must not be allowed to enter an aseptic processing environment, even one that employs advanced aseptic technologies such as isolators, blow/fill/seal, or closed RABS.

The importance of good personal hygiene and a careful attention to detail in aseptic gowning cannot be overemphasized. Gowning requirements differ depending on the use of the controlled environment and the specifics of the gowning system itself. Aseptic processing environments require the use of sterilized gowns with the best available filtration properties. The fullest possible skin coverage is desirable, and sleeve covers or tape should be considered to minimize leaks at the critical glove-sleeve junction. Exposed skin should never be visible in conventional clean rooms under any conditions. The personnel and gowning considerations for RABS are essentially identical to those for conventional clean rooms.

Once employees are properly gowned, they must be careful to maintain the integrity of their gloves, masks, and other gown materials at all times. Operators who work with isolator systems are not required to wear sterilized clean-room gowns, but inadequate aseptic technique and employee-borne contamination are the principal hazards to safe aseptic operations in isolators, as well as RABS, and in conventional clean rooms. Glove-and-sleeve assemblies can develop leaks that can allow the mechanical transfer of microorganisms to the product. A second glove, worn either under or over the primary isolator/RABS glove, can provide an additional level of safety against glove leaks or can act as a hygienic measure. Also, operators must understand that aseptic technique is an absolute requirement for all manipulations performed with gloves within RABS and isolator systems.

The environmental monitoring program, by itself, cannot detect all events in aseptic processing that might compromise the microbiological quality of the environment. Therefore, periodic media-fill or process simulation studies are necessary, as is thorough ongoing supervision, to ensure that appropriate operating controls and training are effectively maintained.

CRITICAL FACTORS IN THE DESIGN AND IMPLEMENTATION OF A MICROBIOLOGICAL ENVIRONMENTAL MONITORING PROGRAM

Since the advent of comprehensive environmental monitoring programs, their applications in capturing adverse

trends or drifts has been emphasized. In a modern aseptic processing environment—whether an isolator, RABS, or conventional clean room—contamination has become increasingly rare. Nevertheless, a monitoring program should be able to detect a change from the validated state of control in a facility and to provide information for implementing appropriate countermeasures. An environmental monitoring program should be tailored to specific facilities and conditions. It is also helpful to take a broad perspective in the interpretation of data. A single uncorrelated result on a given day may not be significant in the context of the technical limitations associated with aseptic sampling methods.

Selection of Growth Media

A general microbiological growth medium such as soybean-casein digest medium (SCDM) is suitable for environmental monitoring in most cases because it supports the growth of a wide range of bacteria, yeast, and molds. This medium can be supplemented with additives to overcome or to minimize the effects of sanitizing agents or of antibiotics. Manufacturers should consider the specific detection of yeasts and molds. If necessary, general mycological media such as Sabouraud's, modified Sabouraud's, or inhibitory mold agar can be used. In general, monitoring for strict anaerobes is not performed, because these organisms are unlikely to survive in ambient air. However, micro-aerophilic organisms may be observed in aseptic processing. Should anoxic conditions exist or if investigations warrant (e.g., identification of these organisms in sterility testing facilities or *Sterility Tests* (71) results), monitoring for micro-aerophiles and organisms that grow under low-oxygen conditions may be warranted. The ability of any media used in environmental monitoring, including those selected to recover specific types of organisms, must be evaluated for their ability to support growth, as indicated in (71).

Selection of Culture Conditions

Time and incubation temperatures are set once the appropriate media have been selected. Typically, for general microbiological growth media such as SCDM, incubation temperatures in the ranges of approximately 20°–35° have been used with an incubation time of not less than 72 hours. Longer incubation times may be considered when contaminants are known to be slow growing. The temperature ranges given above are by no means absolute. Mesophilic bacteria and mold common to the typical facility environment are generally capable of growing over a wide range of temperatures. For many mesophilic organisms, recovery is possible over a range of approximately 20°. In the absence of confirmatory evidence, microbiologists may incubate a single plate at both a low and a higher temperature. Incubating at the lower temperature first may compromise the recovery of Gram-positive cocci that are important because they are often associated with humans.

Sterilization processes for preparing growth media should be validated. When selective media are used for monitoring, incubation conditions should reflect published technical requirements. Contamination should not be introduced into a manufacturing clean room as a result of using contaminated sampling media or equipment. Of particular concern is the use of aseptically prepared sampling media. Wherever possible, sampling media and their wrappings should be terminally sterilized by moist heat, radiation, or other suitable means. If aseptically prepared media must be used, analysts must carry out preincubation and visual inspection of all sampling media before introduction into the clean room. The reader is referred to *Microbiological Best Laboratory Practices* (1117) for further information regarding microbiology laboratory operations and control.

ESTABLISHMENT OF SAMPLING PLAN AND SITES

During initial startup or commissioning of a clean room or other controlled environment, specific locations for air and surface sampling should be determined. Locations considered should include those in proximity of the exposed product, containers, closures, and product contact surfaces. In aseptic processing, the area in which containers, closures, and product are exposed to the environment is often called the *critical zone*—the critical zone is always ISO 5. For aseptic operations the entire critical zone should be treated as a sterile field. A nonsterile object, including the gloved hands of clean room personnel or an RABS/isolator glove, should never be brought into contact with a sterile product, container closure, filling station, or conveying equipment before or during aseptic processing operations. Operators and environmental monitoring personnel should never touch sterile parts of conveyors, filling needles, parts hoppers, or any other equipment that is in the product-delivery pathway. This means that surface monitoring on these surfaces is best done at the end of production operations.

The frequency of sampling depends on the manufacturing process conducted within an environment. Classified environments that are used only to provide a lower overall level of bioburden in nonsterile product manufacturing areas require relatively infrequent environmental monitoring. Classified environments in which closed manufacturing operations are conducted, including fermentation, sterile API processing, and chemical processes, require fewer monitoring sites and less frequent monitoring because the risk of microbial contamination from the surrounding environment is comparatively low. Microbiological monitoring of environments in which products are filled before terminal sterilization is generally less critical than the monitoring of aseptic processing areas. The amount of monitoring required in filling operations for terminal sterilization depends on the susceptibility of the product survival and the potential for proliferation of microbial contamination. The identification and estimated number of microorganisms that are resistant to the subsequent sterilization may be more critical than the microbiological monitoring of the surrounding manufacturing environments.

It is not possible to recommend microbial control levels for each type of manufacturing environment. The levels established for one ISO Class 7 environment, for example, may be inappropriate for another ISO Class 7 environment, depending on the production activities undertaken in each. The user should conduct a prospective risk analysis and develop a rationale for the sampling locations and frequencies for each controlled environment. The classification of a clean room helps establish control levels, but that does not imply that all rooms of the same classification should have the same control levels and the same frequency of monitoring. Monitoring should reflect the microbiological control requirements of manufacturing or processing activities. Formal risk assessment techniques can result in a scientifically valid contamination control program.

Table 2 suggests frequencies of sampling in decreasing order of frequency and in relation to the criticality or product risk of the area being sampled. This table distinguishes between aseptic processing where personnel are aseptically gownned and those where a lesser gownning is appropriate. Environmental monitoring sampling plans should be flexible with respect to monitoring frequencies, and sample plan locations should be adjusted on the basis of the observed rate of contamination and ongoing risk analysis. On the basis of long-term observations, manufacturers may increase or decrease sampling at a given location or eliminate a sampling location altogether. Oversampling can be as deleterious to contamination control as undersampling, and careful consideration of risk and reduction of contamination sources can guide the sampling intensity.

Table 2. Suggested Frequency of Sampling for Aseptic Processing Areas^a

Sampling Area/Location	Frequency of Sampling
Clean Room/RABS	
<i>Critical zone (ISO 5 or better)</i>	
Active air sampling	Each operational shift
Surface monitoring	At the end of the operation
<i>Aseptic area adjacent critical zone</i>	
All sampling	Each operating shift
<i>Other nonadjacent aseptic areas</i>	
All sampling	Once per day
Isolators	
<i>Critical zone (ISO 5 or better)</i>	
Active air sampling	Once per day
Surface monitoring	At the end of the campaign
<i>Nonaseptic areas surrounding the isolator</i>	
All sampling	Once per month

^a All operators are aseptically gownned in these environments (with the exception of background environments for isolators). These recommendations do not apply to production areas for nonsterile products or other classified environments in which fully aseptic gowns are not donned.

SELECTION OF SAMPLE SITES WITHIN CLEAN ROOMS AND ASEPTIC PROCESSING AREAS

ISO 14644 suggests a grid approach for the total particulate air classification of clean rooms. This approach is appropriate for certifying the total particulate air quality performance against its design objective. Grids may also have value in analyzing risk from microbial contamination, although in general, grids that have no personnel activity are likely to have low risk of contamination. Microbial contamination is strongly associated with personnel, so microbiological monitoring of unstaffed environments is of limited value.

Microbiological sampling sites are best selected with consideration of human activity during manufacturing operations. Careful observation and mapping of the clean room during the qualification phase can provide useful information concerning the movement and positioning of personnel. Such observation can also yield important information about the most frequently conducted manipulations and interventions.

The location and movement of personnel within the clean room correlate with contamination risk to the environment and to the processes conducted within that environment. Sample sites should be selected so that they evaluate the impact of personnel movement and work within the area, particularly interventions and manipulations within the critical zone.

The most likely route of contamination is airborne, so the samples most critical to risk assessment are those that relate to airborne contamination near exposed sterile materials. Other areas of concern are entry points where equipment and materials move from areas of lower classification to those of higher classification. Areas within and around doors and airlocks should be included in the monitoring scheme. It is customary to sample walls and floors, and indeed sampling at these locations can provide information about the effectiveness of the sanitization program. Sampling at these locations can take place relatively infrequently, because contamination there is unlikely to affect product. Operators should never touch floors and walls, so mechanical transmission of contamination from these surfaces to critical areas where product is exposed should not occur.

Manufacturers typically monitor surfaces within the critical zone, although this should be done only at the end of operations. Residues of media or diluent from wet swabs should

be avoided on surfaces, because they could lead to microbial proliferation. Also, cleaning surfaces to remove diluent or media requires personnel intervention and movements that can result in release of microbial contamination into the critical zone and can disrupt airflow.

MICROBIOLOGICAL CONTROL PARAMETERS IN CLEAN ROOMS, ISOLATORS, AND RABS

Since the early 1980s, manufacturers have established alert and action levels for environmental monitoring. In recent years the numerical difference between alert and action levels has become quite small, especially in ISO 5 environments. Growth and recovery in microbiological assays have normal variability in the range of $\pm 0.5 \log_{10}$. Studies on active microbiological air samplers indicate that variability of as high as tenfold is possible among commonly used sampling devices. As a result of this inherent variability and indeterminate sampling error, the supposed differences between, for example, an alert level of 1 cfu and an action level of 3 cfu are not analytically significant. Treating differences that are within expected, and therefore, normal ranges as numerically different is not scientifically valid and can result in unwarranted activities. In a practical sense, numerical values that vary by as much as five- to tenfold may not be significantly different.

Because of the limited accuracy and precision of microbial growth and recovery assays, analysts can consider the frequency with which contamination is detected rather than absolute numbers of cfu detected in any single sample. Also, a cfu is not a direct enumeration of microorganisms present but rather is a measure of contamination that may have originated from a clump of organisms.

Mean contamination recovery rates should be determined for each clean room environment, and changes in contamination recovery rate at a given site or within a given room may indicate the need for corrective action. Within the ISO 5 critical zone, airborne and surface contamination recovery rates of 1% or less should be attainable with current methods. Contamination recovery rates for closed RABS and isolator systems should be significantly lower still and can be expected to be <0.1%, on the basis of published monitoring results.

Contamination observed at multiple sites in an environment within a single sampling period may indicate increased risk to product and should be carefully evaluated. The appearance of contamination nearly simultaneously at multiple sites could also arise from poor sampling technique, so careful review is in order before drawing conclusions about potential loss of control. Resampling an environment several days after contamination is of little value, because the conditions during one sampling occasion may not be accurately duplicated during another.

Surface samples may also be taken from clean room garments. Personnel sampling should be emphasized during validation and is best done at the completion of production work in order to avoid adventitious contamination of the garments. In this case the average should be <1% for these sample sites as well. Gloves on closed RABS and isolators should meet the more rigorous expectation of <0.1% contamination recovery rates.

Because of the inherent variability of microbial sampling methods, contamination recovery rates are a more useful measure of trending results than is focusing on the number of colonies recovered from a given sample. *Table 3* provides recommended contamination recovery rates for aseptic processing environments. The incident rate is the rate at which environmental samples are found to contain microbial contamination. For example, an incident rate of 1% would mean that only 1% of the samples taken have any contamination regardless of colony number. In other words, 99% of the samples taken are completely free of contamination. Contamination recovery rates that are higher than those recommended in *Table 3* may be acceptable in rooms of

similar classification that are used for lower-risk activities. Action should be required when the contamination recovery rate trends above these recommendations for a significant time.

Table 3. Suggested Initial Contamination Recovery Rates in Aseptic Environments^a

Room Classification	Active Air Sample (%)	Settle Plate (9 cm) 4 h Exposure (%)	Contact Plate or Swab (%)	Glove or Garment (%)
Isolator/Closed RABS (ISO 5 or better)	<0.1	<0.1	<0.1	<0.1
ISO 5	<1	<1	<1	<1
ISO 6	<3	<3	<3	<3
ISO 7	<5	<5	<5	<5
ISO 8	<10	<10	<10	<10

^a All operators are aseptically gowned in these environments (with the exception of background environments for isolators). These recommendations do not apply to production areas for nonsterile products or other classified environments in which fully aseptic gowns are not donned.

Detection frequency should be based on actual monitoring data and should be retabulated monthly. Action levels should be based on empirical process capability. If detection frequencies exceed the recommendations in *Table 3* or are greater than established process capability, then corrective actions should be taken. Corrective actions may include but are not limited to the following:

- Revision of the sanitization program, including selection of antimicrobial agents, application methods, and frequencies
- Increased surveillance of personnel practices, possibly including written critiques of aseptic methods and techniques
- Review of microbiological sampling methods and techniques

When higher-than-typical recovery levels for glove and garment contamination are observed, additional training for gowning practices may be indicated.

SIGNIFICANT EXCURSIONS

Excursions beyond approximately 15 cfu recovered from a single ISO 5 sample, whether from airborne, surface, or personnel sources, should happen very infrequently. When such ISO 5 excursions do occur, they may be indicative of a significant loss of control when they occur within the ISO 5 critical zone in close proximity to product and components. Thus, any ISO 5 excursion >15 cfu should prompt a careful and thorough investigation.

A key consideration for an abnormally high number of recovered colonies is whether this incident is isolated or can be correlated with other recoveries. Microbiologists should review recovery rates for at least two weeks before the incident of abnormally high recovery so that they can be aware of other recoveries that might indicate an unusual pattern. Microbiologists should carefully consider all recoveries, including those that are in the more typical range of 1–5 cfu. The identity of the organisms recovered is an important factor in the conduct of this investigation.

In the case of an isolated single excursion, establishing a definitive cause probably will not be possible, and only general corrective measures can be considered. It is never wise to suggest a root cause for which there is no solid scientific evidence. Also, there should be an awareness of the variability of microbial analysis. Realistically, there is no scientific reason to treat a recovery of 25 cfu as statistically different

from a recovery of 15 cfu. A value of 15 cfu should not be considered significant in terms of process control, because realistically there is no difference between a recovery of 14 cfu and one of 15 cfu. Microbiologists should use practical scientific judgment in their approach to excursions.

FURTHER CONSIDERATIONS ABOUT DATA INTERPRETATION

In the high-quality environments required for aseptic processing, detection frequency typically is low. As can be seen from the rates recommended in *Table 3*, the majority of samples taken in an aseptic processing area will yield a recovery of zero contamination. In the most critical areas within an aseptic processing operation, it is expected that less than 1% of the samples will yield any recoverable contamination. In the most advanced of modern aseptic operations that use separative technologies such as isolators or closed RABS, the recovery rate will approach zero at all times.

The microbiologist responsible for environmental control or sterility assurance should not take this to mean that the environmental quality approaches sterility. The sensitivity of any microbial sampling system in absolute terms is not known. In environmental monitoring, a result of zero means only that the result is below the limit of detection of the analytical system. A false sense of security should not be derived from the infrequency of contamination recovery in aseptic processing.

Sterility assurance is best accomplished by a focus on human-borne contamination and the facility design features that best mitigate risk from this contamination. Greatest risk mitigation can be attained by reducing or eliminating human interventions through proper equipment design and by providing sufficient air exchanges per hour for the intended personnel population of the facility. Other risk mitigation factors include effective personnel and material movement and the proper control of temperature and humidity. Secondary factors for risk mitigation include cleaning and sanitization. Risk analysis models that analyze processes prospectively to reduce human-borne contamination risk by minimizing operator interventions are more powerful tools for sterility assurance than monitoring. Environmental monitoring cannot prove or disprove in absolute terms the sterility of a lot of product. Environmental monitoring can only assure those responsible for a process that a production system is in a consistent, validated state of control. Care should be taken to avoid drawing inappropriate conclusions from monitoring results.

SAMPLING AIRBORNE MICROORGANISMS

Among the most commonly used tools for monitoring aseptic environments are impaction and centrifugal samplers. A number of commercially available samplers are listed for informational purposes. The selection, appropriateness, and adequacy of using any particular sampler are the responsibility of the user.

Slit-to-Agar Air Sampler (STA): The unit is powered by an attached source of controllable vacuum. The air intake is obtained through a standardized slit below which is placed a slowly revolving Petri dish that contains a nutrient agar. Airborne particles that have sufficient mass impact the agar surface, and viable organisms are allowed to grow. A remote air intake is often used to minimize disturbance of unidirectional airflow.

Sieve Impactor: This apparatus consists of a container designed to accommodate a Petri dish that contains a nutrient agar. The cover of the unit is perforated with openings of a predetermined size. A vacuum pump draws a known volume of air through the cover, and airborne particles that contain microorganisms impact the agar medium in the Petri dish. Some samplers feature a cascaded series of sieves

that contain perforations of decreasing size. These units allow determination of the size range distribution of particulates that contain viable microorganisms based on the size of the perforations through which the particles landed on the agar plates.

Centrifugal Sampler: The unit consists of a propeller or turbine that pulls a known volume of air into the unit and then propels the air outward to impact on a tangentially placed nutrient agar strip set on a flexible plastic base.

Sterilizable Microbiological Atrium: The unit is a variant of the single-stage sieve impactor. The unit's cover contains uniformly spaced orifices approximately 0.25 inch in size. The base of the unit accommodates one Petri dish containing a nutrient agar. A vacuum pump controls the movement of air through the unit, and a multiple-unit control center as well as a remote sampling probe are available.

Surface Air System Sampler: This integrated unit consists of an entry section that accommodates an agar contact plate. Immediately behind the contact plate is a motor and turbine that pulls air through the unit's perforated cover over the agar contact plate and beyond the motor, where it is exhausted. Multiple mounted assemblies are also available.

Gelatin Filter Sampler: The unit consists of a vacuum pump with an extension hose terminating in a filter holder that can be located remotely in the critical space. The filter consists of random fibers of gelatin capable of retaining airborne microorganisms. After a specified exposure time, the filter is aseptically removed and dissolved in an appropriate diluent and then plated on an appropriate agar medium to estimate its microbial content.

Settling Plates: This method is still widely used as a simple and inexpensive way to qualitatively assess the environments over prolonged exposure times. Published data indicate that settling plates, when exposed for 4- to 5-hour periods, can provide a limit of detection for a suitable evaluation of the aseptic environment. Settling plates may be particularly useful in critical areas where active sampling could be intrusive and a hazard to the aseptic operation.

One of the major drawbacks of mechanical air samplers is the limited sample size of air being tested. When the microbial level in the air of a controlled environment is expected to contain extremely low levels of contamination per unit volume, at least 1 cubic meter of air should be tested in order to maximize sensitivity. Typically, slit-to-agar devices have an 80-L/min sampling capacity (the capacity of the surface air system is somewhat higher). If 1 cubic meter of air were tested, then it would require an exposure time of 15 min. It may be necessary to use sampling times in excess of 15 min to obtain a representative environmental sample. Although some samplers are reported to have high sampling volumes, consideration should be given to the potential for disruption of the airflow patterns in any critical area and to the creation of turbulence.

Technicians may wish to use remote sampling systems in order to minimize potential risks resulting from intervention by environmental samplers in critical zones. Regardless of the type of sampler used, analysts must determine that the extra tubing needed for a remote probe does not reduce the method's sensitivity to such an extent that detection of low levels of contamination becomes unlikely or even impossible.

SURFACE SAMPLING

Another component of the microbial-control program in controlled environments is surface sampling of equipment, facilities, and personnel. The standardization of surface sampling methods and procedures has not been as widely addressed in the pharmaceutical industry as has the standardization of air-sampling procedures. Surface sampling can be accomplished by the use of contact plates or by the swabbing method.

Contact plates filled with nutrient agar are used for sampling regular or flat surfaces and are directly incubated for the appropriate time and temperature for recovery of viable organisms. Specialized agar can be used for the recovery of organisms that have specific growth requirements. Microbial estimates are reported per contact plate.

The swabbing method can be used to supplement contact plates for sampling of irregular surfaces, especially irregular surfaces of equipment. The area that will be swabbed is defined with a sterile template of appropriate size. In general, it is in the range of 24–30 cm². After sample collection the swab is placed in an appropriate diluent or transport medium and is plated onto the desired nutrient agar. The microbial estimates are reported per swab of defined sampling area.

Surface monitoring is used as an environmental assessment tool in all types of classified environments. In ISO 5 environments for aseptic processing, surface monitoring is generally performed beside critical areas and surfaces. Component hoppers and feed chutes that contact sterile surfaces on closures and filling needles can be tested for microbial contamination. Often in conventional staffed clean rooms, these product contact surfaces are steam sterilized and aseptically assembled. The ability of operators to perform these aseptic manipulations are evaluated during process stimulations or media fills, although true validation of operator technique in this manner is not possible. Surface monitoring on surfaces that directly contact sterile parts or product should be done only after production operations are completed. Surface sampling is not a sterility test and should not be a criterion for the release or rejection of product. Because these samples must be taken aseptically by personnel, it is difficult to establish with certainty that any contamination recovered is product related.

CULTURE MEDIA AND DILUENTS

The type of medium, liquid or solid, used for sampling or plating microorganisms depends on the procedure and equipment used. Any medium used should be evaluated for suitability for the intended purpose. The most commonly used all-purpose solid microbiological growth medium is soybean–casein digest agar. As previously noted, this medium can be supplemented with chemicals that counteract the effect of various antimicrobials.

IDENTIFICATION OF MICROBIAL ISOLATES

A successful environmental control program includes an appropriate level of identification of the flora obtained by sampling. A knowledge of the flora in controlled environments aids in determining the usual microbial flora anticipated for the facility and in evaluating the effectiveness of the cleaning and sanitization procedures, methods, agents, and recovery methods. The information gathered by an identification program can be useful in the investigation of the source of contamination, especially when recommended detection frequencies are exceeded.

Identification of isolates from critical and immediately adjacent areas should take precedence over identification of microorganisms from noncritical areas. Identification methods should be verified, and ready-to-use kits should be qualified for their intended purpose.

CONCLUSION

Environmental monitoring is one of several key elements required in order to ensure that an aseptic processing area is maintained in an adequate level of control. Monitoring is a qualitative exercise, and even in the most critical applications such as aseptic processing, conclusions regarding lot acceptability should not be made on the basis of environmental sampling results alone. Environments that are essen-

tially free of human operators generally have low initial contamination rates and maintain low levels of microbial contamination. Human-scale clean rooms present a very different picture. Studies conclusively show that operators, even when carefully and correctly gowned, continuously slough microorganisms into the environment. Therefore, it is unreasonable to assume that samples producing no colonies, even in the critical zone or on critical surfaces, will always be observed. Periodic excursions are a fact of life in human-scale clean rooms, but the contamination recovery rate, particularly in ISO 5 environments used for aseptic processing, should be consistently low.

Clean-room operators, particularly those engaged in aseptic processing, must strive to maintain suitable environmental quality and must work toward continuous improvement of personnel operations and environmental control. In general, fewer personnel involved in aseptic processing and monitoring, along with reduction in interventions, reduces risk from microbial contamination.

APPENDIX

Airborne Particulate Count (also referred to as *Total Particulate Count*): The total number of particles of a given size per unit volume of air.

Airborne Viable Particulate Count (also referred to as *Total Airborne Aerobic Microbial Count*): The recovered number of colony-forming units (cfu) per unit volume of air.

Air Changes: The frequency per unit of time (minutes, hours, etc.) that the air within a controlled environment is replaced. The air can be recirculated partially or totally replaced.

Air Sampler: Devices or equipment used to sample a measured amount of air in a specified time to quantify the particulate or microbiological status of air in the controlled environment.

Aseptic: Technically, the absence of microorganisms, but in aseptic processing this refers to methods and operations that minimize microbial contamination in environments where sterilized product and components are filled and/or assembled.

Aseptic Processing: An operation in which the product is assembled or filled into its primary package in an ISO 5 or better environment and under conditions that minimize the risk of microbial contamination. The ultimate goal is to produce products that are as free as possible of microbial contamination.

Barrier System: Physical barriers installed within an aseptic processing room to provide partial separation between aseptically gowned personnel and critical areas subject to considerable contamination risk. Personnel access to the critical zone is largely unrestricted. It is subject to a high level disinfection.

Bioburden: Total number and identity of the predominant microorganisms detected in or on an article.

Clean Room: A room in which the concentration of airborne particles is controlled to meet a specified airborne particulate cleanliness Class. In addition, the concentration of microorganisms in the environment is monitored; each cleanliness Class defined is also assigned a microbial level for air, surface, and personnel gear.

Commissioning of a Controlled Environment: Certification by engineering and quality control that the environment has been built according to the specifications of the desired cleanliness Class and that, under conditions likely to be encountered under normal operating conditions (or worst-case conditions), it is capable of delivering an aseptic process. Commissioning includes media-fill runs and results of the environmental monitoring program.

Contamination Recovery Rate: The contamination recovery rate is the rate at which environmental samples

are found to contain any level of contamination. For example, an incident rate of 1% would mean that only 1% of the samples taken have any contamination regardless of colony number.

Controlled Environment: Any area in an aseptic process system for which airborne particulate and microorganism levels are controlled to specific levels, appropriate to the activities conducted within that environment.

Corrective Action: Actions to be performed that are according to standard operating procedures and that are triggered when certain conditions are exceeded.

Critical Zone: Typically the entire area where product and the containers and closures are exposed in aseptic processing.

Detection Frequency: The frequency with which contamination is observed in an environment. Typically expressed as a percentage of samples in which contamination is observed per unit of time.

Environmental Isolates: Microorganisms that have been isolated from the environmental monitoring program.

Environmental Monitoring Program: Documented program implemented via standard operating procedures that describes in detail the methods and acceptance criteria for monitoring particulates and microorganisms in controlled environments (air, surface, personnel gear). The program includes sampling sites, frequency of sampling, and investigative and corrective actions.

Equipment Layout: Graphical representation of an aseptic processing system that denotes the relationship between and among equipment and personnel. This layout is used in the *Risk Assessment Analysis* to determine sampling site and frequency of sampling based on potential for microbiological contamination of the product/container/closure system. Changes must be assessed by responsible managers, since unauthorized changes in the layout for equipment or personnel stations could result in increase in the potential for contamination of the product/container/closure system.

Isolator for Aseptic Processing: An aseptic isolator is an enclosure that is over-pressurized with HEPA filtered air and is decontaminated using an automated system. When operated as a closed system, it uses only decontaminated interfaces or rapid transfer ports (RTPs) for materials transfer. After decontamination they can be operated in an open manner with the ingress and/or egress of materials through defined openings that have been designed and validated to preclude the transfer of contamination. It can be used for aseptic processing activities or for asepsis and containment simultaneously.

Material Flow: The flow of material and personnel entering controlled environments should follow a specified and documented pathway that has been chosen to reduce or minimize the potential for microbial contamination of the product/closure/container systems. Deviation from the prescribed flow could result in increase in the potential for microbial contamination. Material/personnel flow can be changed, but the consequences of the changes from a microbiological point of view should be assessed by responsible managers and must be authorized and documented.

Media Fill: Microbiological simulation of an aseptic process by the use of growth media processed in a manner similar to the processing of the product and with the same container/closure system being used.

Media Growth Promotion: Procedure that references *Growth Promotion Test of Aerobes, Anaerobes, and Fungi in Sterility Tests* (71) to demonstrate that media used in the microbiological environmental monitoring program, or in *media-fill* runs, are capable of supporting growth of indicator microorganisms and of environmental isolates from samples obtained through the monitoring program or their corresponding ATCC strains.

Product Contact Areas: Areas and surfaces in a controlled environment that are in direct contact with ei-

ther products, containers, or closures and the microbiological status of which can result in potential microbial contamination of the product/container/closure system.

Restricted Access Barrier System (RABS): An enclosure that relies on HEPA filtered air over-spill to maintain separation between aseptically gownned personnel and the operating environment. It is subject to a high level of disinfection prior to use in aseptic process. It uses decontaminated (where necessary) interfaces or RTPs for materials transfer. It allows for the ingress and/or egress of materials through defined openings that have been designed and validated to preclude the transfer of contamination. If opened subsequent to decontamination, its performance capability is adversely impacted.

Risk Assessment Analysis: Analysis of the identification of contamination potentials in controlled environments that establish priorities in terms of severity and frequency and that will develop methods and procedures that will eliminate, reduce, minimize, or mitigate their potential for microbial contamination of the product/container/closure system.

Sampling Plan: A documented plan that describes the procedures and methods for sampling a controlled environment; identifies the sampling sites, the sampling frequency, and number of samples; and describes the method of analysis and how to interpret the results.

Sampling Sites: Documented geographical location, within a controlled environment, where sampling for microbiological evaluation is taken. In general, sampling sites are selected because of their potential for product/container-closure contacts.

Standard Operating Procedures: Written procedures describing operations, testing, sampling, interpretation of results, and corrective actions that relate to the operations that are taking place in a controlled environment and auxiliary environments. Deviations from standard operating procedures should be noted and approved by responsible managers.

Sterile or Aseptic Field: In aseptic processing or in other controlled environments, it is the space at the level of or above open product containers, closures, or product itself, where the potential for microbial contamination is highest.

Sterility: Within the strictest definition of sterility, an article is deemed sterile when there is complete absence of viable microorganisms. *Viable*, for organisms, is defined as having the capacity to reproduce. Absolute sterility cannot be practically demonstrated because it is technically unfeasible to prove a negative absolute. Also, absolute sterility cannot be practically demonstrated without testing every article in a batch. Sterility is defined in probabilistic terms, where the likelihood of a contaminated article is acceptably remote.

Swabs for Microbiological Sampling: Devices used to remove microorganisms from irregular or regular surfaces for cultivation to identify the microbial population of the surface. A swab is generally composed of a stick with an absorbent tip that is moistened before sampling and is rubbed across a specified area of the sample surface. The swab is then rinsed in a sterile solution to suspend the microorganisms, and the solution is transferred to growth medium for cultivation of the microbial population.

Trend Analysis: Data from a routine microbial environmental monitoring program that can be related to time, shift, facility, etc. This information is periodically evaluated to establish the status or pattern of that program to ascertain whether it is under adequate control. A trend analysis is used to facilitate decision-making for requalification of a controlled environment or for maintenance and sanitization schedules.

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cepted methods and adherence to good laboratory practices.

MEDIA PREPARATION AND QUALITY CONTROL

Media Preparation

Culture media are the basis for most microbiological tests. Safeguarding the quality of the media is therefore critical to the success of the microbiology laboratory. Media preparation, proper storage, and quality control testing can ensure a consistent supply of high-quality media.

It is important to choose the correct media or components in making media based on the use of accepted sources or references for formulas. The manufacturer's formula and instructions for preparation routinely accompany dehydrated media and ready-made media. Because different media types may have different preparation requirements (e.g., heating, additives, and pH adjustment), it is important to follow these instructions to ensure preparation of acceptable media quality. A certificate of analysis describing expiration dating and recommended storage conditions accompanies ready-made media, as well as the quality control organisms used in growth-promotion and selectivity testing of that media.

Water is the universal diluent for microbiological media. Purified Water is most often used for media preparation, but in certain cases the use of deionized or distilled water may be appropriate. Water of lesser quality should not be used for microbiological media preparation. The volume of the water used should be recorded.

Consistent preparation of media requires accurate weighing of dehydrated media or media constituents. A calibrated balance with the appropriate weight range for the ingredients should be used (See *Weighing on an Analytical Balance* (1251)). Clean weighing containers and tools (such as spatulas) should be used to prevent foreign substances from entering the formulation. The weight of the components should be recorded.

Dehydrated media should be thoroughly dissolved in water before dispensing and sterilization. If heating is necessary to help dissolve the media, care should be taken not to overheat media, because all culture media, to a greater or lesser extent, are heat-sensitive. Equipment used in the preparation of media should be appropriate to allow for controlled heating, constant agitation, and mixing of the media. Darkening of media (Maillard-type reaction or nonenzymatic browning) is a general indication of overheating. When adding required supplements to media, adequate mixing of the medium after adding the supplement should be performed.

Preparation of media in poorly cleaned glassware can allow inhibitory substances to enter the media. Inhibitory substances can come from detergent residue after cleaning glassware or from prior materials used in the glassware. Be sure that the cleaning process removes debris and foreign matter, and that the detergent is thoroughly rinsed out with Purified Water. See *Cleaning Glass Apparatus* (1051) for additional guidance.

Sterilization of media should be performed within the parameters provided by the manufacturer or validated by the user. Commercially prepared media should provide documentation of the sterilization method used. Autoclaving by moist heat is the preferred sterilization technique, except in instances when boiling is required in order to avoid deterioration of heat-labile components of the media. Sterilization by filtration may also be appropriate for some formulations.

The effects of the sterilization method and conditions on the media should be validated by sterility and growth-promotion testing of the media. In addition, if sterilized by moist heat, the autoclave cycle should be validated to en-

(1117) MICROBIOLOGICAL BEST LABORATORY PRACTICES

INTRODUCTION

Good laboratory practices in a microbiology laboratory consist of activities that depend on several principles: aseptic technique, control of media, control of test strains, operation and control of equipment, diligent recording and evaluation of data, and training of the laboratory staff. Because of the inherent risk of variability in microbiology data, reliability and reproducibility are dependent on the use of ac-

sure proper heat distribution for selected loads and volumes. Typically, manufacturers recommend using an autoclave cycle of 121° for 15 minutes using a validated autoclave. These conditions apply to time at temperature of the media. As container size and the load configuration of the autoclave will influence the rate of heating, longer cycles may be required for larger loads. However, the sterilization time will be dependent on the media volume and autoclave load. Sterilization cycles in which the autoclave is slow to come up to temperature may result in overheating of the media. Therefore, care must be taken to validate a sterilization cycle, balancing the need for sterile media against the tendency of the media to degrade under excessive heating. Storage of the media in the autoclave after the liquid cycle is completed is not recommended after cooling, as it may damage the media. Improper heating or sterilizing conditions—for commercially prepared or internally prepared media—may result in a difference in color change, loss of clarity, altered gel strength, or pH drift from the manufacturer's recommended range, as well as reduced growth-promotion activity and/or selectivity.

The pH of each batch of medium should be confirmed after it has cooled to room temperature (20°–25°) by aseptically withdrawing a sample for testing. Refrigerated purchased media should be allowed to warm up to ambient room temperature if it is to be checked for pH confirmation. A flat pH probe is recommended for agar surfaces, and an immersion probe is recommended for liquids. See *pH* (791) for guidance with pH measurement and instrument calibration. The pH of media should be in a range of ± 0.2 of the value indicated by the manufacturer, unless a wider range is acceptable by the validated method.

Prepared media should be checked by appropriate inspection of plates and tubes for the following:

- Cracked containers or lids
- Unequal filling of containers
- Dehydration resulting in cracks or dimpled surfaces on solid medium
- Hemolysis
- Excessive darkening or color change
- Crystal formation from possible freezing
- Excessive number of bubbles
- Microbial contamination
- Status of redox indicators (if appropriate)
- Lot number and expiration date checked and recorded
- Sterility of the media
- Cleanliness of plates (lid should not stick to dish)

Media Storage

It is prudent to consider how the manufacturer or supplier transports and stores media before distribution to the end user. Manufacturers of media should use transport and storage conditions that minimize the loss of moisture, control the temperature, prevent microbial contamination, and provide mechanical protection to the prepared media.

Media should be labeled properly with batch or lot numbers, preparation and expiration dates, and media identification. Media should be stored according to the manufacturer's instructions. Media prepared in house should be stored under validated conditions. Do not store agar at or below 0°, as freezing could damage the gel structure. Protect stored media from exposure to light and excessive temperature. Before prolonged storage, agar plates should be placed into a sealed package or container to retard moisture loss.

Remelting of an original container of solid media should be performed only once to avoid media whose quality is compromised by overheating or potential contamination. It is recommended that remelting be performed in a heated water bath or by using free-flowing steam. The use of microwave ovens and heating plates is common, but care should be taken to avoid damaging media by overheating and to avoid the potential injury to laboratory personnel from glass breakage and burns. The molten agar medium

should be held in a monitored water bath at a temperature of 45° to 50° for not more than 8 hours. Caution should be taken when pouring the media from a container immersed in a water bath to prevent water from the bath commingling with the poured sterile media. Wiping the exterior of the container dry before pouring may be advisable.

Disposal of used cultured media (as well as expired media) should follow local biological hazard safety procedures.

Quality Control Testing

Although growth media can be prepared in a laboratory from individual components, many laboratories, for ease of use, use dehydrated media or purchase commercially prepared media in plastic plates or glass containers. Manufacturers of media attempt to standardize raw materials from biological sources, but must constantly deal with unavoidable differences in raw materials obtained from natural sources, and therefore, lot-to-lot variability of media must be considered. In addition, the performance of media prepared in a laboratory or by a manufacturer is highly dependent on preparation and storage conditions. Improper media preparation can cause unsatisfactory conditions for microbial growth or recovery and unreliable results.

Therefore, quality control tests should be performed on all prepared media, including media associated with swabs or media in strips and other nontraditional formats. Tests routinely performed on in-house prepared media should include pH, growth promotion, inhibition, and indicative properties (as appropriate), and periodic stability checks to confirm the expiration dating.

When in-house prepared microbiological media are properly prepared and sterilized using a validated method, the growth-promotion testing may be limited to each incoming lot of dehydrated media, unless otherwise instructed by the relevant compendial method. If the media preparation procedure was not validated, then every batch of media should be subjected to growth-promotion testing. Test organisms may be selected from the appropriate compendial test chapter. In addition, microorganisms used in growth-promotion testing may be based on the manufacturer's recommendation for a particular medium, or may include representative environmental isolates (but these latter are not to be construed as compendial requirements).

Expiration dates on media should have supporting growth-promotion testing to indicate that the performance of the media still meets acceptance criteria up to and including the expiration date. The length of shelf life of a batch of media will depend on the stability of the ingredients and formulation under specified conditions, as well as the type of container and closure.

When a batch of media does not meet the requirements of growth-promotion testing, an investigation should be initiated to identify the cause. This investigation should include a corrective action plan to prevent the recurrence of the problem. Any batch of media that fails growth-promotion testing is unsuitable for use. [NOTE—Failed growth-promotion test results may not be used to negate positive test results.]

Some reagents are used for diagnostic purposes to help support identification of microbial organisms, e.g., Gram stain and oxidase test reagents. These may have attributes that can be quality control tested similar to microbiological media. Select the correct quality control standard microorganisms, following the manufacturer's instructions, and perform the testing before unknown sample diagnostic testing. All relevant diagnostic reagents should be subjected to incoming quality confirmation before use.

Special care should be taken with media that is used in sterility tests (see *Sterility Tests* (71) for requirements) and in environmental monitoring studies. Media used for environmental monitoring of critical areas should preferably be double-wrapped and terminally sterilized. If terminal sterilization is not performed, media should be subjected to

100% pre-incubation and inspection before use within a critical area. [NOTE—Growth-promotion testing for this media must be performed after the pre-incubation stage.] This will prevent extraneous contamination from being carried into controlled environments and will prevent false-positive results. A raised agar level for surface contact plates should be verified.

MAINTENANCE OF MICROBIOLOGICAL CULTURES

Biological specimens can be the most delicate standards to handle because their viability and characteristics are dependent on adequate handling and storage. Standardizing the handling and storage of cultures by the user laboratory should be done in a way that will minimize the opportunity for contamination or alteration of growth characteristics. The careful and consistent treatment of stock cultures is critically important to the consistency of microbiological test results. Cultures for use in compendial tests should be acquired from a national culture collection or a qualified secondary supplier. They can be acquired frozen, freeze-dried, on slants, or in ready-to-use forms. Confirmation of the purity of the culture and the identity of the culture should be performed before its use in quality control testing. Ready-to-use cultures should be subjected to incoming testing for purity and identity before use. The confirmation of identity for commonly used laboratory strains should ideally be done at the level of genus and species.

Preparation and resuscitation of cultures should follow the instructions of the supplier or a validated, established method. The "Seed-Lot" technique is recommended for storage of stock cultures.

The original sample from the national culture collection or a qualified secondary supplier is resuscitated and grown in an appropriate medium. Aliquots of this stock culture (the first transfer or passage) are suspended in a cryoprotective medium, transferred to vials, and frozen at -30° or below, until use. If stored at -70° , or in lyophilized form, strains may be kept indefinitely. These frozen stocks can then be used to inoculate monthly or weekly working cultures. Once opened, do not refreeze unused cell suspensions after culturing a working suspension. The unused portion should be discarded to minimize the risk of loss of viability and contamination of the stock.

The number of transfers of working control cultures should be tracked to prevent excessive subculturing that increases the risk of phenotypic alteration or mutation. The number of transfers allowable for specific compendial tests may be specified in that test. One passage is defined as the transfer of organisms from a viable culture to a fresh medium with growth of the microorganisms. Any form of subculturing is considered to be a transfer/passage.

LABORATORY EQUIPMENT

Most equipment (incubators, water baths, and autoclaves) is subject to standard validation practices of incoming qualification, operational qualification, and performance qualification. Additionally, periodic calibration (generally annually) is commonly required. New equipment, critical to the operation of the laboratory, should be qualified according to a protocol approved by the quality assurance unit (QAU). In addition, regular cleaning and sanitization of equipment such as incubators, refrigerators, and water baths should be performed to minimize the potential for contamination in the laboratory. Door seals of incubators and refrigerators should be cleaned and checked for state of repair.

Instruments (pH meters and spectrophotometers) used in a microbiology laboratory should be calibrated on a regular schedule and tested to verify performance on a routine basis. The frequency of calibration and performance verification will vary based on the type of instrument and the im-

portance of that equipment to the generation of data in the laboratory.

Equipment that is difficult to sanitize (such as refrigerators and incubators) should be dedicated to aseptic operations (such as storage of media for testing and incubation of sterility test samples) and live culture operations to minimize the potential for inadvertent contamination of the tests.

Autoclaves are central to the operation of the laboratory and must have proper validation in place to demonstrate adequate sterilization for a variety of operations. Autoclave resources must be available (and validated) to sterilize waste media (if performed in that laboratory) as well as the media prepared in that laboratory. The choice of one or several autoclaves is not driven by a need to separate aseptic and live operations (everything in the properly maintained autoclave is sterile after the cycle) but rather driven by resource considerations (see below).

LABORATORY LAYOUT AND OPERATIONS

Laboratory layout and design should carefully consider the requirements of good microbiological practices and laboratory safety. It is essential that cross-contamination of microbial cultures be minimized to the greatest extent possible, and it is also important that microbiological samples be handled in an environment that makes contamination highly unlikely.

In general, a laboratory should be divided into clean or aseptic areas and live culture areas. Areas in which environmental or sterile product samples are handled and incubated should be maintained completely free of live cultures, if possible. If complete separation of live and clean culture zones cannot be accomplished, then other barriers and aseptic practices should be employed to reduce the likelihood of accidental contamination. These barriers include protective clothing, sanitization and disinfection procedures, and biological safety cabinets designated for clean or aseptic operations only. Procedures for handling spills or mishaps with live cultures should be in place, and all relevant technical personnel should be trained regarding these methods.

Some samples will demonstrate microbial growth and require further laboratory analysis to identify the contaminants. When growth is detected, the sample should be taken from the clean section of the laboratory to the live culture section without undue delay. Subculturing, staining, microbial identification, or other investigational operations should be undertaken in the live culture section of the laboratory. If possible, any sample found to contain growing colonies should not be opened in the clean zone of the laboratory. Careful segregation of contaminated samples and materials will reduce false-positive results.

Staff engaged in sampling activities should not enter or work in the live culture handling section of a laboratory unless special precautions are taken, including wearing protective clothing and gloves and careful sanitizing of hands upon exiting. Ideally, staff assigned to sampling activities, particularly those in support of aseptic processing, should not work in the vicinity of live culture laboratory operations.

It is important to consider that microbial contamination of samples, which leads to false-positive results, is always possible unless careful aseptic precautions are taken. Facilities should be designed so that raw material and excipient sampling can be done under controlled conditions, including proper gowning and sterilized sampling equipment. It may not always be possible to sample utility systems, such as water systems, under full aseptic conditions; however, it should be noted that when samples are not taken aseptically, their reliability is inevitably compromised.

Environmental sampling methods should require minimal aseptic handling in loading and unloading sampling instruments. Whenever possible, sampling equipment should be loaded with its microbiological recovery media in the environment that is to be sampled.

All testing in laboratories used for critical testing procedures, such as sterility testing of final dosage forms, bulk product, seed cultures for biological production, or cell cultures used in biological production, should be performed under controlled conditions. Isolator technology is also appropriate for critical, sterile microbiological testing. Isolators have been shown to have lower levels of environmental contamination than manned clean rooms, and therefore, are generally less likely to produce false-positive results. Proper validation of isolators is critical both to ensure environmental integrity and to prevent the possibility of false-negative results as a result of chemical disinfection of materials brought into or used within isolators (see *Sterility Testing—Validation of Isolator Systems* (1208)).

SAMPLE HANDLING

Viable microorganisms in most microbiology samples, particularly water, environmental monitoring and bioburden samples, are sensitive to handling and storage conditions. Critical parameters in these conditions include product (or sample) composition, container composition, time of storage, and temperature of storage. Therefore, it is important to minimize the amount of time between the sampling event and the initiation of testing and to control, as much as possible, the conditions of storage. If the sample is to be transported to a distant location for testing, then the conditions of transport (time, temperature, etc.) should be qualified as suitable for that test and sample. Guidance for water testing in this regard can be found in *Water for Pharmaceutical Purposes* (1231). Product mixing before sampling may need to be evaluated and applied in order to ensure microbial dispersment and representation in the sample aliquot.

All microbiological samples should be taken using aseptic techniques, including those taken in support of nonsterile products. If possible, all microbiological samples should be taken under full aseptic conditions in specialized sampling areas. The areas should be as close to the point of use as possible to minimize contamination during transit.

Samples submitted to the microbiology laboratory should be accompanied by documentation detailing source of the sample, date the sample was taken, date of sample submission, person or department responsible for the submission, and any potentially hazardous materials associated with the sample. The testing department should acknowledge receipt of the sample and reconcile the identity and number of samples as part of this sample documentation.

MICROBIOLOGICAL MEDIA INCUBATION TIMES

Incubation times for microbiological tests of less than 3 days' duration should be expressed in hours: e.g., "Incubate at 30° to 35° for 18 to 72 hours". Tests longer than 72 hours' duration should be expressed in days: e.g., "Incubate at 30° to 35° for 3 to 5 days". For incubation times expressed in hours, incubate for the minimum specified time, and exercise good microbiological judgment when exceeding the incubation time. For incubation times expressed in days, incubations started in the morning or afternoon should generally be concluded at that same time of day.

TRAINING OF PERSONNEL

Each person engaged in each phase of pharmaceutical manufacture should have the education, training, and experience to do his or her job. The demands of microbiological testing require that the core educational background of the staff, supervisors, and managers be in microbiology or a closely related biological science. They should be assigned responsibilities in keeping with their level of skill and experience.

A coherent system of standard operating procedures (SOPs) is necessary to run the microbiology laboratory. These procedures serve two purposes in a training program. Firstly, these SOPs describe the methodology that the microbiologist will follow to obtain accurate and reproducible results, and so serve as the basis for training. Secondly, by tracking the procedures in which a particular microbiologist has demonstrated proficiency, the procedure number or title also serves to identify what training the microbiologist has received specific to his or her job function.

Training curricula should be established for each laboratory staff member specific to his or her job function. He or she should not independently conduct a microbial test until qualified to run the test. Training records should be current, documenting the microbiologist's training in the current revision to the particular SOP.

Periodic performance assessment is a wise investment in data quality. This performance testing should provide evidence of competency in core activities of the microbiology laboratory such as hygiene, plating, aseptic technique, documentation, and others as suggested by the microbiologist's job function.

Microbiologists with supervisory or managerial responsibilities should have appropriate education and in-house training in supervisory skills, laboratory safety, scheduling, budgeting, investigational skills, technical report writing, relevant SOPs, and other critical aspects of the company's processes as suggested in their role of directing a laboratory function.

Competency may be demonstrated by specific course work, relevant experience, and routinely engaging in relevant continuing education. Achieving certification through an accredited body is also a desirable credential. Further, it is expected that laboratory supervisors and managers have a demonstrated level of competence in microbiology at least as high as those they supervise. Expertise in microbiology can be achieved by a variety of routes in addition to academic course work and accreditation. Each company is expected to evaluate the credentials of those responsible for designing, implementing, and operating the microbiology program. Companies can thus ensure that those responsible for the program understand the basic principles of microbiology, can interpret guidelines and regulations based on good science, and have access to individuals with theoretical and practical knowledge in microbiology to provide assistance in areas in which the persons responsible for the program may not have adequate knowledge and understanding. It should be noted that microbiology is a scientifically based discipline that deals with biological principles substantially different from those of analytical chemistry and engineering disciplines. Many times it is difficult for individuals without specific microbiological training to make the transition.

LABORATORY RESOURCES

The laboratory management is responsible for ensuring that the laboratory has sufficient resources to meet the existing testing requirements. This requires some proficiency in budget management and in determining appropriate measures of laboratory performance. A measure of laboratory performance is the number of investigations performed on tests conducted by the laboratory, but this measure alone is not sufficient. In addition to tracking investigations, the period of time between sample submission and initiation of testing should be tracked, as well as the period of time between end of test and report release (or test closure). Significant delays in these measures are also indications of an under-resourced laboratory staff.

The laboratory management should have sufficient budget to meet testing requirements. Particular measures of budgetary requirements will be specific to the given laboratory, but budgetary considerations related directly to the

need of the laboratory for sufficient resources must be addressed to ensure reliable testing results.

DOCUMENTATION

Documentation should be sufficient to demonstrate that the testing was performed in a laboratory and by methods that were under control. This includes, but is not limited to, documentation of the following:

- Microbiologist training and verification of proficiency
- Equipment validation, calibration, and maintenance
- Equipment performance during test (e.g., 24-hour/7-day chart recorders)
- Media preparation, sterility checks, and growth-promotion and selectivity capabilities
- Media inventory and control testing
- Critical aspects of test conducted as specified by a procedure
- Data and calculations verification
- Reports reviewed by QAU or a qualified responsible manager
- Investigation of data deviations (when required)

MAINTENANCE OF LABORATORY RECORDS

Proper recording of data and studies is critical to the success of the microbiology laboratory. The over-riding principle is that the test should be performed as written in the SOP, the SOP should be written to reflect how the test is actually performed, and the laboratory notebook should provide a record of all critical details needed to reconstruct the details of the testing and confirm the integrity of the data. At a minimum, the laboratory write-up should include the following:

- Date
- Material tested
- Microbiologist's name
- Procedure number
- Document test results
- Deviations (if any)
- Documented parameters (equipment used, microbial stock cultures used, media lots used)
- Management/Second review signature

Every critical piece of equipment should be noted in the write-up, and all should be on a calibration schedule documented by SOP and maintenance records. Where appropriate, logbooks or forms should be available and supportive of the laboratory notebook records. Equipment temperatures (water baths, incubators, autoclaves) should be recorded and traceable.

The governing SOP and revision should be clearly noted in the write-up. Changes in the data should be crossed off with a single line and initialed. Original data should not be erased or covered over.

Test results should include the original plate counts, allowing a reviewer to recreate the calculations used to derive the final test results. Methods for data analysis should be detailed in cited SOPs. If charts or graphs are incorporated into laboratory notebooks, they should be secured with clear tape and should not be obstructing any data on the page. The chart or graph should be signed by the person adding the document, with the signature overlapping the chart and the notebook page. Lab notebooks should include page numbers, a table of contents for reference, and an intact timeline of use.

All laboratory records should be archived and protected against catastrophic loss. A formal record retention and retrieval program should be in place.

INTERPRETATION OF ASSAY RESULTS

Analytical microbiological assay results can be difficult to interpret for several important reasons: (1) Microorganisms are ubiquitous in nature, and common environmental contaminants—particularly organisms associated with humans—predominate in many types of microbiological analysis; (2) the analyst has the potential to introduce contaminating organisms during sample handling or processing in the laboratory; (3) microorganisms may not be homogeneously distributed within a sample or an environment; and (4) microbiological assays are subject to considerable variability of outcome. Therefore, apparent differences from an expected outcome may not be significant.

Because of these characteristics of microbiological analysis, laboratory studies should be conducted with the utmost care to avoid exogenous contamination as previously discussed in this chapter. Equally important, results must be interpreted from a broad microbiological perspective, considering not only the nature of the putative contaminant, but the likelihood of that organism(s) surviving in the pharmaceutical ingredient, excipient, or environment under test. In addition, the growth characteristics of the microorganism should be considered (especially in questions of the growth of filamentous fungi in liquid media).

When results are observed that do not conform to a compendial monograph or other established acceptance criteria, an investigation into the microbial data deviation (MDD) is required. There are generally two distinct reasons for the observation of microbial contamination that does not comply with a target or requirement: There may be either a laboratory error or laboratory environmental conditions that produced an invalid result, or the product contains a level of contamination or specific types of contaminants outside established levels or limits. In either case, laboratory management and, in most cases, the Quality Unit should be notified immediately.

A full and comprehensive evaluation of the laboratory situation surrounding the result should be undertaken. All microbiological conditions or factors that could bring about the observed condition should be fully considered, including the magnitude of the excursion compared to established limits or levels. In addition, an estimate of the variability of the assay may be required in order to determine whether the finding is significant.

The laboratory environment, the protective conditions in place for sampling, historical findings concerning the material under test, and the nature of the material, particularly with regard to microbial survival or proliferation in contact with the material, should be considered in the investigation. In addition, interviews with the laboratory analyst(s) may provide information regarding the actual conduct of the assay that can be valuable in determining the reliability of the result and in determining an appropriate course of action. If laboratory operations are identified as the cause of the non-conforming test outcome, then a corrective action plan should be developed to address the problem(s). Following the approval and implementation of the corrective action plan, the situation should be carefully monitored and the adequacy of the corrective action determined.

If assay results are invalidated on the basis of the discovery of an attributable error, this action must be documented. Laboratories also should have approved procedures for confirmatory testing (retesting), and if necessary,

resampling where specific regulatory or compendial guidance does not govern the conduct of an assay investigation.

<1118> MONITORING DEVICES— TIME, TEMPERATURE, AND HUMIDITY

This chapter provides background on the science and technology of temperature and humidity monitoring. It describes the available technologies and their performance characteristics, and it provides recommendations for verification and validation of performance. The shelf life of a drug is a function of the temperature and humidity conditions under which it is stored and transported as well as the chemical and physical properties of the drug substance and preparation. For this reason, the ability to monitor those conditions is important in the shipping and storage of temperature- and humidity sensitive preparations. Historic geographic and seasonal trends may be used as a planning tool in selecting among the types of temperature and humidity monitoring devices. Meteorological forecasts are available for any pertinent location.

TEMPERATURE MEASUREMENT TECHNOLOGIES

The devices described in this section are those most commonly used to monitor temperature in the storage and distribution of drugs in North America. The measurement of temperature at extremes, such as close to absolute zero or above those reasonably expected to be experienced by drugs, is not addressed.

Alcohol or Mercury Thermometers—These devices are based on the change in volume of a liquid as a function of temperature. Mercury thermometers are typically used in the ranges from 0° to 50° with a precision of about 0.1°. [NOTE—Some local regulations apply to mercury-based thermometers. Alcohol thermometers may have a precision as good as 0.01°, but they must be quite large to measure temperatures in ranges of more than a few degrees. Both types of thermometers may be designed to indicate the maximum and minimum temperatures measured. See *Thermometers* <21>.]

Chemical Device—This is a device based on a phase change or chemical reaction that occurs as a function of temperature. Examples include liquid crystals, waxes, and lacquers that change phase, and thereby their appearance, as a function of temperature. Such materials represent the least expensive form of temperature measurement, but they may be difficult to interpret.

Other types of chemical sensors include systems in which a reaction rate or diffusion process is used to deduce a temperature equivalent integrated over time rather than the temperature at a specific moment in time such as a spike or critical threshold, for which a separate device may be preferred. Thus, chemical sensors provide a measure of accumulated heat rather than instantaneous temperature. It should be noted that these devices are generally irreversible; once a color change or diffusion process has taken place, exposure to low temperatures will not restore the device to its original state. Accuracy and precision vary widely among different types, to differentiate often limited by their ability or their ability to visually interpret diffusion distances.

Infrared Device—This is a device based on measuring the IR radiation from the article whose temperature is being determined; the IR radiation varies as a function of the object's temperature. The advantage of the device is that the article may be at some distance from the IR sensor. However, IR devices are expensive compared to other temperature sensors.

Resistance Temperature Detector (RTD)—This is a device based on the change in electrical resistance of a material as a function of temperature. Precision and accuracy depend on the quality of the electronics used to measure the resistance. Therefore, although RTDs are among the most stable and accurate temperature sensors, their accuracy may change with the age and temperature of the device as its electronic components are affected. A particular type of RTD uses platinum or platinum alloy wire as the sensor. These are referred to as platinum resistance temperature detectors (PRT or PRTD).

Solid State Device—This is a device based on the effect of temperature on either an integrated circuit (see *Thermistor* below) or a micromechanical or microelectrical system. These devices can attain the highest precision available and also have the advantage of producing a digital output. Their accuracy is typically limited by the accuracy of the calibrating system employed.

Thermistor—This is a semiconductor device whose resistance varies with temperature. Thermistors are able to detect very small changes in temperature. They are accurate over a broad range of temperatures.

Thermocouple—This is a device based on the change in the junction potential of two dissimilar metals as a function of temperature. Many metal pairs may be used, with each pair providing a unique range, accuracy, and precision. Precision and accuracy depend on the quality of the electronics used to measure the voltage and the type of temperature reference used. Accuracy may be a function of temperature reference used. Thermocouples have relatively poor stability and low sensitivity, but are simple and cover a wide temperature range.

Thermomechanical Device—This is a device based on the change in volume of a solid material as a function of temperature. For example, a mechanical spring, which expands or contracts as a function of temperature, thus opening and closing an electrical circuit or moving a chart pen, is such a device. Precision may be as good as 0.05°, but in practice it is rarely better than 0.5°. Accuracy is often in the range of ±1.0°, but it may change with the age and temperature of the device.

TIME-TEMPERATURE INTEGRATORS

Time-temperature integrators, commonly referred to as TTIs, change color or physical appearance as a result of exposure to a temperature above a specific threshold for a specific time duration, and thus accumulate heat. TTIs are typically single use, disposable devices that react irreversibly. Once the color changes, it will not revert to the original one even if the temperature returns to the acceptable, normal range. The four basic types of chemical-based TTIs are described below.

Table 1 lists the four types of chemical TTIs presently in use. The closer the activation energy of the TTI's color change to the activation energy of the degradation process of the drug being monitored, the more accurately the TTI will reflect the status of the drug. In actual practice, the activation energy for degradation of a particular drug is not known precisely enough to enable selection of a particular type of TTI. The range of possible activation energies of a TTI is given in the table to provide a sense of the flexibility of that particular technology. A TTI with a range of possible activation energies can be configured to cover a wider range of time and temperature thresholds.

Table 1. Characteristics of TTI Technologies

Type	Storage	Activation Energy (kcal/mol)	Indication	Placement	Activation
Chemical-Physical	Controlled room temperature	13–80	Readable message or image	Primary label or primary package	Placement of activator tape over indicator
Polymerization	–44°	21 or 37	Readable message or image	Primary label or primary package	Removal from frozen environment
Diffusion	Controlled room temperature	9.8	Progressive color diffusion observed through clear window	Primary package	Removal of barrier film
Enzymatic	Controlled room temperature; cold for extended storage	8–30	Color change observed through clear window	Primary package	Breaking seal to mix liquids

An important characteristic of chemical TTIs is the precision with which the endpoint can be determined. It is difficult to quantify an indication such as a gradual color change. Accuracy may also vary widely with the control and quality of the manufacturing process. As discussed below in *Validation of Temperature and Humidity Monitoring Devices*, it is not possible to calibrate an individual chemical TTI because the test is, by the nature of the device, necessarily destructive. Chemical time–temperature indicators are relatively inexpensive and may be customized for a wide range of applications.

Chemical-Physical Based TTI—This type of TTI is based on a temperature-dependent diffusion/chemical reaction process. It consists of a pressure-sensitive tape structure, which is composed of an indicator tape and an activator tape. The indicator tape contains a dye dispersed in a polymer carrier. The activator is incorporated into an adhesive on the activator tape. Laminating the activator tape over the indicator tape causes activation. A color change or readable message occurs as the activator migrates into the indicator as a function of temperature and time. These TTIs can be manufactured to provide a wide array of time–temperature configurations. Also, because they can be made using a printing process, they can be directly integrated into a product label or provided as a stand-alone label if required.

Chemical Polymerization Based TTI—This type of TTI uses a polymerization process in which a color change occurs as a function of time and temperature. The color change happens when a small, colorless molecule polymerizes into a larger, colored molecule on exposure to temperatures above a specific threshold for a specified period of time. These TTIs can be applied as print process, permitting direct integration into a product label or stand-alone label. Since this type of TTI does not require activation, it must be shipped from the manufacturer on dry ice and stored at temperatures below freezing prior to use. Chemical polymerization based TTIs have somewhat limited selections of time–temperature threshold configurations.

Diffusion Based TTI—This type of TTI is composed of a color-dyed fat, an ester that diffuses along a porous filter paper strip or wick once the temperature exceeds the melting point of the ester. The distance the colored fat migrates is a function of the time the TTI is exposed to temperatures above the melting point of the ester. Removing a barrier film that separates the dyed fat from the wick activates these devices. They can be modified for various applications by selecting esters of different melting points, and by changing the length of the wick. These TTIs are contained within their own packaging and have limited time–temperature threshold configurations.

Enzyme Based TTI—This type of TTI uses an enzyme-catalyzed color generating reaction that occurs as a function of time and temperature. The color change is caused by esterase hydrolysis of a fatty substance, accompanied by a decrease in pH. The enzyme and the fatty substrate are in separate solutions in adjacent compartments. Breaking the

barrier between the two compartments and mixing the two solutions activates the device. Enzymatic reactions provide a wide variety of time–temperature configurations.

ELECTRONIC TIME-TEMPERATURE HISTORY RECORDERS

These devices, which may serve as an alternative to chemical-based TTIs, use one of the electronic temperature measurement technologies described above and create a record of the temperature history experienced by a device. Some are simple electronic devices that record and save temperature values representative of the cumulative temperature history over a period of time. These may be designated as electronic TTIs. They have the advantages of being able to calculate the Mean Kinetic Temperature (MKT) based on the measurements recorded and they can be calibrated.

Data Loggers—A more capable device records the temperature at very short intervals and is able to download the temperature history record to a peripheral system, such as a personal computer. Such devices may be termed electronic temperature data loggers. In addition, data loggers may record the humidity using sensors described below. Data loggers may be permanently fixed within a storage facility or they may be portable and travel with a product. Data loggers equipped with transmitting devices (hard-wire or radio transmission) can be used to monitor temperature and humidity of a product while in transit, with the ability to download the recorded data when the data loggers arrive at a destination.

RELATIVE HUMIDITY MEASUREMENT TECHNOLOGIES

Relative humidity may be defined as the ratio of the observed partial pressure of water vapor in a volume of air to the saturation pressure at that temperature. In other words, the relative humidity is the amount of water vapor present divided by the theoretical amount of moisture that could be held by that volume of air at a given temperature. Extensive tables of data are available. Devices for measuring relative humidity are called hygrometers. Several different technologies exist for measuring relative humidity.

Sling Psychrometer—The simplest type of hygrometer is based on the temperature difference observed between two identical thermometers, one ordinary, and one with a wet cloth wick over its bulb. The two thermometers are whirled at the end of a chain, and the evaporation of water from the wick cools the wet bulb thermometer. The temperature difference between the wet and dry thermometers is then compared to a table, specific to that psychrometer, based on dry bulb temperature, and the relative humidity is determined. The use of a sling psychrometer in a commercial setting is impractical.

Hair Hygrometer—This type of device is based on the fact that the length of a synthetic or human hair increases as a function of the relative humidity. This change is used to move an indicator or affect a strain gauge. A hair hygrometer can be accurate to $\pm 3\%$, but it is unable to respond to rapid changes in humidity and loses accuracy at very high or very low levels of relative humidity.

Infrared Hygrometer—This type of hygrometer determines relative humidity by comparing the absorption of two different wavelengths of IR radiation through air. One wavelength is absorbed by water vapor and the other is not. This type of hygrometer can accurately measure relative humidity in large or small volumes of air. It is sensitive to rapid changes of humidity and can be integrated with an electronic data handling system.

Dew Point Hygrometer—This type of device uses a chilled mirror to determine the dew point of an air sample. The dew point is the temperature at which water vapor in the air begins to condense, that is, the temperature at which the relative humidity is 100%. From this measurement and an accurate measurement of the ambient temperature, the relative humidity can be calculated. The dew point hygrometer is the standard against which most commercially available instruments are calibrated.

Capacitive Thin-Film Hygrometer—The principle of this type of hygrometer is that the dielectric of a nonconductive polymer changes in direct proportion to the relative humidity. This change is measured as a change in capacitance. This type of hygrometer is accurate to $\pm 3\%$.

Resistive Thin-Film Hygrometer—This type of hygrometer is similar to the capacitive thin-film type in that it uses the effect of changing relative humidity on an electrical circuit. In the resistive thin-film hygrometer the sensor is an organic polymer whose electrical resistance changes in logarithmic proportion to the relative humidity. This type of hygrometer is accurate to $\pm 5\%$.

VALIDATION OF TEMPERATURE AND HUMIDITY MONITORING DEVICES

Thermometers and hygrometers, used to provide data about the temperature and humidity exposure of a product, must be suitable for their intended use. Specifically, they must be appropriately validated. Validation is a process that assures the user of the monitoring device that the device has been tested prior to use either by the manufacturer or the user, to assess the measurement accuracy, measurement responsiveness, and time accuracy, where appropriate. Monitors used in manufacturing, storage, and transport of drugs should be properly qualified by their users to ensure that the monitors have been received and maintained in proper working order. Pharmacies and consumers may accept the validation performed by the manufacturer of the device.

Measurement Accuracy—For temperature and humidity monitoring devices, measurement accuracy refers to the closeness of the value obtained with a particular device to the true value being measured. In practice, this is determined by comparison with a device that has been calibrated against a standard that is obtained from or traceable to the National Institute of Standards and Technology (NIST).

Measurement Responsiveness—Any monitor takes time to respond to a change in the temperature or humidity. The more rapid the response, the clearer the picture of the environmental history of a monitored product will be. Measurement responsiveness may be defined as the time, $t_{1/2}$, required for a device to read a value of $(x + y)/2$ after an instantaneous change in the property being measured from x to y . Measurement responsiveness is typically defined for the operating range of a device.

Different levels of responsiveness are needed for different monitoring applications. For devices used to monitor stor-

age locations, where the temperature and humidity are unlikely to change rapidly, a $t_{1/2} \leq 15$ minutes may be appropriate. For devices used to monitor transport, where more rapid changes are possible, a $t_{1/2} \leq 5$ minutes may be needed.

Time Accuracy—Most commonly, time accuracy is expressed as a \pm percentage of total duration of the recording period. For pharmaceutical applications, a $\pm 0.5\%$ time accuracy is adequate.

Validation of Chemical-Based TTIs—This type of device presents a problem for validation because testing the individual device causes its destruction. For this reason, calibration of individual chemical-based TTIs against an NIST traceable standard is not possible. Ideally, chemical-based TTIs would be made using Good Manufacturing Practices, and their use in connection with monitoring the storage and transport environment of drugs would be appropriately regulated. In the absence of those conditions, the performance of a batch of these devices may be assessed statistically by subjecting an appropriately sized sample to elevated temperature conditions for a set period of time and observing the results. Appropriate acceptance criteria should be adopted.

THE USE OF HISTORIC TEMPERATURE DATA

It is clear that the type of temperature monitoring needed is a function of the environmental conditions that can be expected. Therefore, climatic data are useful when selecting the most appropriate local storage conditions and monitoring methods. For example, an inexpensive limit detector may be all that is needed when there is a low probability that excessive temperatures will be experienced. Alternatively, a data logger may be preferred when it would be useful to demonstrate that exposure to the highest temperatures was very brief.

It should be noted, however, that outside temperatures are not necessarily reliable indicators of the temperatures experienced by different items in the distribution chain. For example, recent studies reported significant departures from ambient temperatures on summer days for mailboxes, trucks, and warehouses. Detailed historical temperature data are available from the National Oceanic and Atmospheric Administration showing the daily mean maximum and minimum temperature on any given day of the year in a geographical region of interest (e.g., <http://www.cdc.noaa.gov/Usclimate/states.fast.html>).

<1119> NEAR-INFRARED SPECTROSCOPY

INTRODUCTION

Near-infrared (NIR) spectroscopy is a branch of vibrational spectroscopy that shares many of the principles that apply to other spectroscopic measurements. The NIR spectral region comprises two subranges associated with detectors used in the initial development of NIR instrumentation. The short-wavelength (Herschel or silicon region) extends from approximately 780 to 1100 nm ($12,821$ – 9000 cm^{-1}); and longer wavelengths, between 1100 and 2500 nm, compose the traditional (lead sulfide) NIR region. Applications of NIR spectroscopy use spectra displayed in either wavelength or wavenumber units. As is the case with other spectroscopy

measurements, interactions between NIR radiation and matter provide information that can be for both qualitative and quantitative assessment of the chemical composition of samples. In addition, qualitative and quantitative characterization of a sample's physical properties can be made because of the sample's influence on NIR spectra. Measurements can be made directly on samples in situ in addition to applications during standard sampling and testing procedures.

Applications of qualitative analysis include identification of raw material, in-process sample, or finished product. These applications often involve comparing an NIR spectrum from a sample to reference spectra and assessing similarities against acceptance criteria developed and validated for a specific application. In contrast, applications of quantitative analysis involve the development of a predictive relationship between NIR spectral attributes and sample properties. These applications typically use numerical models to quantitatively predict chemical and/or physical properties of the sample on the basis of NIR spectral attributes.

Vibrational spectroscopy in the NIR region is dominated by overtones and combinations that are much weaker than the fundamental mid-IR vibrations from which they originate. Because molar absorptivities in the NIR range are low, radiation can penetrate several millimeters into materials, including solids. Many materials, such as glass, are relatively transparent in this region. Fiber-optic technology is readily implemented in the NIR range, which allows monitoring of processes in environments that might otherwise be inaccessible.

The instrument qualification tests and acceptance criteria provided in this chapter may not be appropriate for all instrument configurations. In such cases, alternative instrument qualification and performance checks should be scientifically justified and documented. In addition, validation parameters discussed in this chapter may not be applicable for all applications of NIR spectroscopy. Validation parameters characterized for a specific NIR application should demonstrate suitability of the NIR application for its intended use.

Transmission and Reflection

The most common measurements performed in the NIR spectral range are transmission and reflection spectroscopy. Incident NIR radiation is absorbed or scattered by the sample and is measured as transmittance or reflectance, respectively. Transflection spectrometry is a hybrid of transmission and reflection wherein a reflector is placed behind the sample so that the optical path through the sample and back to the detector is doubled compared to a transmission measurement of a sample of the same thickness. Transflection is used to describe any double-pass transmission technique. The light may be reflected from a diffuse or specular (mirror) reflector placed behind the sample. This configuration can be adapted to share instrument geometry with certain reflection or fiber-optic probe systems in which the source and the detector are on the same side of the sample.

TRANSMITTANCE, T , is a measure of the decrease in radiation intensity as a function of wavelength when radiation is passed through a sample. The sample is placed in the optical beam between the source and the detector. The results of both transmission and transflection measurements are usually presented directly in terms of absorbance, i.e., $\log_{10}(1/T)$.

REFLECTANCE, R , is a measure of the ratio of the intensity of light reflected from the sample, I , to that reflected from a background or reference reflective surface, I_R . Most reflection measurements in the NIR are made of scattering samples such as powders and slurries. For such materials NIR radiation can penetrate a substantial distance into the sample, where it can be absorbed when the wavelength of the radiation corresponds to a transition between the ground vibrational state of the analyte and either a harmonic of a given vibrational mode (an *overtone*) or the sum of two or

more different modes (a *combination band*). Nonabsorbed radiation is scattered back from the sample to the detector. NIR reflection spectra are accessed by calculating and plotting $\log(1/R)$ versus wavelength. This logarithmic form is the pseudo-absorbance of the material and is commonly called absorbance.

Factors That Affect NIR Spectra

The following list is not exhaustive, but it includes many of the major factors that affect NIR spectra.

Sample Temperature—Sample temperature influences spectra obtained from aqueous solutions and other hydrogen-bonded liquids, and a difference of a few degrees may result in significant spectral changes. Temperature may also affect spectra obtained from less polar liquids, as well as solids that contain solvents and/or water.

Moisture and Solvent—Moisture and solvent present in the sample material and analytical system may change the spectrum of the sample. Both absorption by moisture and solvent and their influence on hydrogen bonding of the APIs and excipients can change the NIR spectrum.

Sample Thickness—Sample thickness is a known source of spectral variability and must be understood and/or controlled. The sample thickness in transmission mode is typically controlled by using a fixed optical path length for the sample. In diffuse reflection mode, the sample thickness is typically controlled by using samples that are "infinitely thick" relative to the detectable penetration depth of NIR light into a solid material. Here "infinite thickness" implies that the reflection spectrum does not change if the thickness of the sample is increased.

Sample Optical Properties—In solids, both surface and bulk scattering properties of calibration standards and analytical samples must be taken into account. Surface morphology and refractive index properties affect the scattering properties of solid materials. For powder materials, particle size and bulk density influence scattering properties and the NIR spectrum.

Polymorphism—Variation in crystalline structure (polymorphism) from materials with the same chemical composition can influence NIR spectral response. Different polymorphs and amorphous forms of solid material may be distinguished from one another on the basis of their NIR spectral properties. Similarly, different crystalline hydration or solvation states of the same material can display different NIR spectral properties.

Age of Samples—Samples may exhibit changes in their chemical, physical, or optical properties over time. Care must be taken to ensure that both samples and standards used for NIR analysis are suitable for the intended application.

INSTRUMENTATION

Apparatus

All NIR measurements are based on exposing material to incident NIR light radiation and measuring the attenuation of the emerging (transmitted, scattered, or reflected) light. Several spectrophotometers are available; they are based on different operating principles—for example: filters, grating-based dispersive, acousto-optical tunable filter (AOTF), Fourier-transform NIR (FT-NIR), and liquid crystal tunable filter (LCTF). Silicon, lead sulfide, indium gallium arsenide, and deuterated triglycine sulfate are common detector materials. Conventional cuvette sample holders, fiber-optic probes, transmission dip cells, and spinning or traversing sample holders are common examples of sample interfaces

for introducing the sample to the optical train of a spectrometer.

The selection of specific NIR instrumentation and sampling accessories should be based on the intended application, and particular attention should be paid to the suitability of the sampling interface for the type of sample that will be analyzed.

Near-Infrared Reference Spectra

NIR references, by providing known stable measurements to which other measurements can be compared, are used to minimize instrumental variations that would affect the measurement.

Transmittance—The measurement of transmittance requires a background reference spectrum for determining the absorption by the sample relative to the background. Suitable transmittance reference materials depend on the specific NIR application and include air, an empty cell, a solvent blank, or a reference sample.

Reflectance—The measurement of reflectance requires the measurement of a reference reflection spectrum to determine the attenuation of reflected light relative to the unattenuated incident beam. The reflectance spectrum is calculated as the ratio of the single-beam spectrum of the sample to that of the reference material. Suitable reflectance reference materials depend on the specific NIR application and include ceramic, perfluorinated polymers, gold, and other suitable materials.

Qualification of NIR Instruments

Qualification—Qualification of an NIR instrument can be divided into three elements: Installation Qualification (IQ); Operational Qualification (OQ); and Performance Qualification (PQ). For further discussion, see general information chapter *Analytical Instrument Qualification* (1058).

Installation Qualification—The IQ requirements help ensure that the hardware and software are installed to accommodate safe and effective use of the instrument at the desired location.

Operational Qualification—In operational qualification, an instrument's performance is characterized using standards to verify that the system operates within target specifications. The purpose of operational qualification is to demonstrate that instrument performance is suitable. Because there are so many different approaches for measuring NIR spectra, operational qualification using standards with known spectral properties is recommended. Using external traceable reference standard materials does not justify omitting the instrument's internal quality control procedures. As is the case with any spectroscopic device, wavelength uncertainty, photometric linearity, and noise characteristics of NIR instruments should be qualified against target specifications for the intended application.

Performance Qualification—Performance qualification demonstrates that the NIR measurement consistently operates within target specifications defined by the user for a specific application; it is often referred to as *system suitability*. Performance qualification for NIR measurements can include comparing a sample or standard spectrum to previously recorded spectra. Comparisons of spectra taken over time from identical and stable samples or reference standard materials can form the basis for evaluating the long-term stability of an NIR measurement system. The objective is to demonstrate that no abnormal wavelength shift or change in detector sensitivity has occurred during ongoing analysis.

Characterizing Instrument Performance—Specific procedures, acceptance criteria, and time intervals for characterizing NIR instrument performance depend on the instrument and intended application. Many NIR applications use previously validated models that relate NIR spectral response

to a physical or chemical property of interest. Demonstrating stable instrument performance over extended periods of time provides some assurance that reliable measurements can be taken from sample spectra using previously validated NIR models.

Wavelength Uncertainty—NIR spectra from sample and/or reference standard materials can be used to demonstrate an instrument's suitable wavelength dispersion performance against target specifications. The USP Near IR System Suitability Reference Standard or the National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 2036 for reflectance measurement and NIST SRM 2035 for transmittance measurement can be used for wavelength verification. Suitable materials for demonstrating wavelength dispersion performance include polystyrene, mixtures of rare earth oxides, and absorption by water vapor for instruments that use an interferometer for wavelength dispersion. With appropriate justification, alternative standards may be used. Wavelength uncertainty typically is characterized from a single spectrum (collected with the same spectral resolution to obtain the standard value) using a minimum of three peaks that cover a suitable spectral range of the instrument. Typical tolerances for agreement with standard values are ± 1.0 nm below 2000 nm and ± 1.5 nm from 2000 nm to 2500 nm. Alternative tolerances may be used when justified for specific applications.

Photometric Linearity and Response Stability—NIR spectra from samples and/or reference standard materials with known relative transmittance or reflectance can be used to demonstrate a suitable relationship between NIR light attenuation (due to absorption) and instrument response. For reflectance measurements, commercially available reflectance standards with known reflectance properties are often used. Spectra obtained from reflection standards are subject to variability as a result of the difference between the experimental conditions under which they were factory calibrated and those under which they are subsequently put to use. Hence, the reflectance values supplied with a set of calibration standards may not be useful in the attempt to establish an "absolute" calibration for a given instrument. Provided that (1) the standards do not change chemically or physically, (2) the same reference background is also used to obtain the standard values, and (3) the instrument measures each standard under identical conditions (including precise sample positioning), the reproducibility of the photometric scale will be established over the range of standards. Subsequent measurements on the identical set of standards give information on long-term stability. Photometric linearity is typically characterized using a minimum of four reference standards in the range from 10% to 90% reflection (or transmission). NIR applications based on measuring an absorbance larger than 1.0 may require standards with reflectivity properties between 2% and 5% reflection (or transmission) for characterizing instrument performance at low reflectance. The purpose is to demonstrate a linear relationship between NIR reflectance and/or transmittance and instrument response over the scanning range of the instrument. Typical tolerances for a linear relationship are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept of a plot of the measured photometric response versus standard photometric response. Alternative tolerances may occur when justified for specific applications.

Spectroscopic Noise—NIR instrument software may include built-in procedures to automatically determine system noise and to provide a statistical report of noise or S/N over the instrument's operating range. In addition, it may be desirable to supplement such checks with measurements that do not rely directly on manufacturer-supplied procedures. Typical procedures involve measuring spectra of traceable reference materials with high and low reflectance. Tolerances for these procedures should demonstrate suitable S/N for the intended application.

HIGH-FLUX NOISE—Instrument noise is evaluated at high-light flux by measuring reflectance or transmittance of the

reference standard, with the reference material (e.g., 99% reflection standard) acting as both the sample and the background reference.

LOW-FLUX NOISE—The same procedure may be used with a lower-reflectivity reference material (e.g., 10% reflectance standard) to determine system noise at reduced light flux. The source, optics, detector, and electronics make significant contributions to the noise under these conditions.

METHOD VALIDATION

Introduction

The objective of NIR method validation, as is the case with the validation of any analytical procedure, is to demonstrate that the measurement is suitable for its intended purpose. NIR spectroscopy is somewhat different from conventional analytical techniques because validation of the former generally is achieved by the assessment of chemometric parameters, but these parameters can still be related to the fundamental validation characteristics required for any analytical method.

Data pretreatment is often a vital step in the chemometric analysis of NIR spectral data. Data pretreatment can be defined as the mathematical transformation of NIR spectral data to enhance spectral features and/or remove or reduce unwanted sources of variation prior to using the spectrum. *Calibration* is the process of developing a mathematical relationship between NIR spectral response and properties of samples. Many suitable chemometric algorithms for data pretreatment and calibration exist; the selection should be based on sound scientific judgment and suitability for the intended application.

Validation Parameters

Performance characteristics that demonstrate the suitability of NIR methods are similar to those required for any analytical procedure. A discussion of the applicable general principles is found in *Validation of Compendial Procedures* (1225). These principles should be considered typical for NIR procedures, but exceptions should be dealt with on a case-by-case basis. For qualitative NIR methods, see chapter (1225), *Data Elements Required for Validation, Category IV* assays. For quantitative NIR methods, see chapter (1225), *Data Elements Required for Validation, Category I and Category II* assays. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the method. The samples for validation should be independent of the calibration set.

Specificity—The extent of specificity testing depends on the intended application. Demonstration of specificity in NIR methods is typically accomplished by using the following approaches:

Qualitative—Identification testing is a common application of qualitative NIR spectroscopy. Identification is achieved by comparing a sample spectrum to a reference spectrum or a library of reference spectra. The specificity of the NIR identification method is demonstrated by obtaining positive identification from samples coupled with negative results from materials that should not meet criteria for positive identification. Materials to demonstrate specificity should be based on sound scientific judgment and can include materials similar in visual appearance, chemical structure, or name.

Quantitative—Quantitative applications of NIR spectroscopy typically involve establishing a mathematical relationship between NIR spectral response and a physical or chemical property of interest. Demonstrating specificity against a physical or chemical property of interest is based on interpreting both NIR spectral attributes and chemometric pa-

rameters in terms of the intended application and may include the following:

- Spectral regions in the calibration model can be correlated to a known NIR spectral response associated with the property of interest.
- Wavelengths used by regression analysis for the calibration (e.g., for multiple linear regression [MLR] models) or the loading vector for each factor (e.g., for partial least squares [PLS] or principal component regression [PCR] models) can be examined to verify relevant spectroscopic information that is used for the mathematical model.
- Variation in spectra from samples for calibration can be examined and interpreted as expected spectral observations.
- Variation in material composition and sample matrix may be shown to have no significant effect on quantification of the property of interest within the specified method range.

Linearity—Quantitative NIR methods generally attempt to demonstrate a linear relationship between NIR spectral response and the property of interest. Although demonstrating a linear response is not required for all NIR applications, the model chosen, whether linear or not, should properly represent the relationship.

Validation of linearity in NIR methods may be accomplished by examining a plot of NIR spectral response versus actual or accepted values for the property of interest. Many statistical methods are available for evaluation of the goodness of fit of the linear relationship. Other applicable statistics and graphical methods may be as appropriate.

The correlation coefficient, r , may not be an informative measure of linearity. The square of the (Pearson) correlation coefficient is a measure of the fraction of the data's variation that is adequately modeled by the equation. Linearity depends on the standard error of the calibration equation (and hence the reference method) and on the range of the calibration data. Thus, although values very near 1.00, such as 0.99 or greater, typically indicate a linear relationship, lower values do not distinguish between nonlinearity and variability around the line.

Range—The specified range of an NIR method depends on the specific application. The range typically is established by confirming that the NIR method provides suitable measurement capability (accuracy and precision) when applied to samples within extreme limits of the NIR measurement. Controls must be used to ensure that results outside the validated range are not accepted. In certain circumstances, it may not be possible or desirable to extend the validated range to include sample variability outside the validated range. Extending the range of an NIR method requires demonstration of suitable measurement capability within the limits of the expanded range. Examples of situations in which only a limited sample range may be available are samples from a controlled manufacturing process and in-process samples. A limited method range does not preclude the use of an NIR method.

Accuracy—Accuracy in NIR methods is demonstrated by showing the closeness of agreement between the value that is accepted as either a conventional true value or an accepted reference value. Accuracy can be determined by direct comparison between NIR validation results and actual or accepted reference values. Suitable agreement between NIR and reference values is based on required measurement capability for a specific application. The purpose is to demonstrate a linear relationship between NIR results and actual values. Accuracy can be determined by agreement between the standard error of prediction (SEP) and the standard error of the reference method for validation. The error of the reference method may be known on the basis of historical data, through validation results specific to the reference method, or by calculating the standard error of the laboratory (SEL). Suitable agreement between SEP and SEL is

based on required measurement capability for a specific application.

Precision—The precision of an NIR method expresses the closeness of agreement between a series of measurements under prescribed conditions. Two levels of precision should be considered: repeatability and intermediate precision. The precision of an NIR method typically is expressed as the relative standard deviation of a series of NIR method results and should be suitable for the intended application. Demonstration of precision in NIR methods may be accomplished using the following approaches:

Repeatability—Repeatability can be demonstrated by the following:

- Statistical evaluation of a number of replicate measurements of the sample without repositioning the sample between each individual spectral acquisition, or
- Statistical evaluation of multiple NIR method results, each result from a replicate analysis of a sample subsequent to repositioning between spectral acquisitions

Intermediate Precision—Intermediate precision can be shown by the following:

- Statistical evaluation of a number of replicate NIR measurements of the same or similar samples in the *Repeatability* study by different analysts on different days.

Robustness—NIR measurement parameters selected to demonstrate robustness will vary depending on the application and the sample's interface with the NIR instrument. Critical measurement parameters associated with robustness often are identified and characterized during method development. Typical measurement parameters include the following:

- Effect of environmental conditions (e.g., temperature, humidity, and vibration)
- Effect of sample temperature
- Sample handling (e.g., probe depth, compression of material, sample depth/thickness, sample presentation)
- Influence of instrument changes (e.g., lamp change, warm-up time)

Ongoing Method Evaluation

Validated NIR methods should be subject to ongoing performance evaluation, which may include monitoring accuracy, precision, and other suitable method parameters. If performance is unacceptable, corrective action is necessary. It involves conducting an investigation to identify the cause of change in method performance and may indicate that the NIR method is not suitable for continued use. Improving the NIR method to meet measurement suitability criteria may require additional method development and documentation of validation experiments demonstrating that the improved method is suitable for the intended application. The extent of revalidation required depends on the cause of change in method performance and the nature of corrective action required in order to establish suitable method performance. Appropriate change controls should be implemented to document ongoing method improvement activities.

Revalidation of a qualitative model may be necessary as a result of the following:

- Addition of a new material to the spectral reference library
- Changes in the physical properties of the material
- Changes in the source of material supply
- Identification of previously unknown critical attribute(s) of material(s)

Revalidation of a quantitative model may be necessary as a result of the following:

- Changes in the composition of the test sample or finished product

- Changes in the manufacturing process
- Changes in the sources or grades of raw materials
- Changes in the reference analytical method
- Major changes in instrument hardware

Outliers—Sample spectra that produce an NIR response that differs from the qualitative or quantitative calibration model may produce an outlier. This does not necessarily indicate an out-of-specification result; but rather an outlier indicates that further testing of the sample may be required and is dependent on the particular NIR method. If subsequent testing of the sample by an appropriate method indicates that the property of interest is within specifications, then the sample meets its specifications. Outlier samples may be incorporated into an updated calibration model subsequent to execution and documentation of suitable validation studies.

Method Transfer

Controls and measures for demonstrating the suitability of NIR method performance following method transfer are similar to those required for any analytical procedure. Exceptions to general principles for conducting method transfer for NIR methods should be justified on a case-by-case basis. The transfer of an NIR method is often performed by using an NIR calibration model on a second instrument that is similar to the primary instrument used to develop and validate the method. When a calibration model is transferred to another instrument, procedures and criteria must be applied to demonstrate that the calibration model meets suitable measurement criteria on the second instrument. The selection of an appropriate calibration model transfer procedure should be based on sound scientific judgment.

GLOSSARY

ABSORBANCE, A , is represented by the equation:

$$A = -\log T = \log(1/T)$$

where T is the transmittance of the sample. Absorbance is also frequently given as:

$$A = \log(1/R)$$

where R is the reflectance of the sample.

BACKGROUND SPECTRUM is used for generating a sample spectrum with minimal contributions from instrument response. It is also referred to as a *reference spectrum* or *background reference*. The ratio of the sample spectrum to the background spectrum produces a transmittance or reflectance spectrum dominated by NIR spectral response associated with the sample. In reflection measurements, a highly reflective diffuse standard reference material is for the measurement of the background spectrum. For transmission measurement, the background spectrum may be measured with no sample present in the spectrometer or using a cell with the solvent blank or a cell filled with appropriate reference material.

CALIBRATION MODEL is a mathematical expression to relate the response from an analytical instrument to the properties of samples.

DIFFUSE REFLECTANCE is the ratio of the spectrum of radiated light penetrating the sample surface, interacting with the sample, passing back through the sample's surface, and reaching the detector to the background spectrum. This is the component of the overall reflectance that produces the absorption spectrum of the sample.

FIBER-OPTIC PROBES consist of two components: optical fibers that may vary in length and in the number of fibers and a terminus, which contains specially designed optics for examination of the sample matrix.

INSTALLATION QUALIFICATION is the documented collection of activities necessary to establish that an instrument is delivered as designed and specified, is properly installed in the selected environment, and that this environment is suitable for the instrument's intended purpose.

INSTRUMENT BANDWIDTH OR RESOLUTION is a measure of the ability of a spectrometer to separate radiation of similar wavelengths.

MULTIPLE LINEAR REGRESSION is a calibration algorithm to relate the response from an analytical instrument to the properties of samples. The distinguishing feature of this algorithm is the use of a limited number of independent variables. Linear-least-squares calculations are performed to establish a relationship between these independent variables and the properties of the samples.

OPERATIONAL QUALIFICATION is the process by which it is demonstrated and documented that an instrument performs according to specifications and that it can perform the intended task. This process is required following any significant change such as instrument installation, relocation, or major repair.

OVERALL REFLECTANCE is the sum of diffuse and specular reflectance.

PARTIAL LEAST SQUARES (PLS) is a calibration algorithm to relate instrument responses to the properties of samples. The distinguishing feature of this algorithm is that data concerning the properties of the samples for calibration are used in the calculation of the factors to describe instrument responses.

PERFORMANCE QUALIFICATION is the process of using one or more well-characterized and stable reference materials to verify consistent instrument performance. Performance qualification may employ the same or different standards for different performance characteristics.

PHOTOMETRIC LINEARITY, also referred to as *photometric verification*, is the process of verifying the response of the photometric scale of an instrument.

PRINCIPAL COMPONENT REGRESSION (PCR) is a calibration algorithm to relate the response from an analytical instrument to the properties of samples. This algorithm, which expresses a set of independent variables as a linear combination of factors, is a method of relating these factors to the properties of the samples for which the independent variables were obtained.

PSEUDO-ABSORBANCE, A , is represented by the equation:

$$A = -\log R = \log(1/R)$$

where R is the diffuse reflectance of the sample.

REFERENCE SPECTRUM—See *Background Spectrum*.

REFLECTANCE is described by the equation:

$$R = I/I_R$$

in which I is the intensity of radiation reflected from the surface of the sample and I_R is the intensity of radiation reflected from a background reference material and its incorporated losses due to solvent absorption, refraction, and scattering.

ROOT-MEAN-SQUARE (RMS) NOISE is calculated by the equation:

$$\text{RMS} = \sqrt{\frac{1}{N} \times \sum_i^N (A_i - \bar{A})^2}$$

in which A_i is the absorbance for each data point; \bar{A} is the mean absorbance over the spectral segment; and N is the number of points per segment.

SPECTRAL REFERENCE LIBRARY is a collection of spectra of known materials for comparison with unknown materials. The term is commonly used in connection with qualitative methods of spectral analysis (e.g., identification of materials).

SPECULAR (SURFACE) REFLECTANCE is the reflectance of the front surface of the sample.

STANDARD ERROR OF CALIBRATION (SEC) is a measure of the capability of a model to fit reference data. SEC is the standard deviation of the residuals obtained from comparing the known values for each of the calibration samples to the values that are calculated from the calibration. SEC should not be used as an assessment tool for the expected method accuracy (trueness and precision of prediction) of the predicted value of future samples. The method accuracy should generally be verified by calculating the standard error of prediction (SEP), using an independent validation set of samples. An accepted method is to mark a part of the calibration set as the validation set. This set is not fully independent but can be used as an alternative for the determination of the accuracy.

STANDARD ERROR OF CROSS-VALIDATION (SECV) is the standard deviation calculated using the leave-one-out method. In this method, one calibration sample is omitted from the calibration, and the difference is found between the value for this sample calculated from its reference value and the value obtained from the calibration calculated from all the other samples in the set. This process is repeated for all samples in the set, and the SECV is the standard deviation of the differences calculated for all the calibration samples. This procedure can also be performed with a group of samples. Instead of leaving the sample out, a group of samples is left out. The SECV is a measure of the model accuracy that one can expect when measuring future samples if not enough samples are available for the SEP to be calculated from a completely independent validation set.

STANDARD ERROR OF THE LABORATORY (SEL) is a calculation based on repeated readings of one or more samples to estimate the precision and/or accuracy of the reference laboratory method, depending on how the data were collected.

STANDARD ERROR OF PREDICTION (SEP) is a measure of model accuracy of an analytical method based on applying a given calibration model to the spectral data from a set of samples different from but similar to those used to calculate the calibration model. SEP is the standard deviation of the residuals obtained from comparing the values from the reference laboratory to those from the method under test for the specified samples. SEP provides a measure of the model accuracy expected when one measures future samples.

SURFACE REFLECTANCE, also known as *specular reflection*, is that portion of the radiation not interacting with the sample but simply reflecting back from the sample surface layer (sample-air interface).

TRANSFLECTION is a transmittance measurement technique in which the radiation traverses the sample twice. The second time occurs after the radiation is reflected from a surface behind the sample.

TRANSMITTANCE is represented by the equation:

$$T = I/I_0 \text{ or } T = 10^A$$

in which I is the intensity of the radiation transmitted through the sample; I_0 is the intensity of the radiant energy incident on the sample and includes losses due to solvent absorption, refraction, and scattering; and A is the absorbance.

<1120> RAMAN SPECTROSCOPY

INTRODUCTION

Raman spectroscopy shares many of the principles that apply to other spectroscopic measurements discussed in

Spectrophotometry and Light-Scattering <851>. Raman is a vibrational spectroscopic technique and is therefore related to infrared (IR) and near-infrared (NIR) spectroscopy. The Raman effect itself arises as a result of a change in the polarizability of molecular bonds during a given vibrational mode and is measured as inelastically scattered radiation.

A Raman spectrum is generated by exciting the sample of interest to a virtual state with a monochromatic source, typically a laser. Light elastically scattered (no change in wavelength) is known as Rayleigh scatter and is not of interest in Raman spectrometry, except for marking the laser wavelength. However, if the sample relaxes to a vibrational energy level that differs from the initial state, the scattered radiation is shifted in energy. This shift is commensurate with the energy difference between the initial and final vibrational states. This "inelastically scattered" light is referred to as Raman scatter. Only about one in 10^6 – 10^8 photons incident on the sample undergoes Raman scattering. Thus lasers are employed in Raman spectrometers. If the Raman-scattered photon is of lower energy, it is referred to as Stokes scattering. If it is of higher energy, it is referred to as anti-Stokes scattering. In practice, nearly all analytically useful Raman measurements make use of Stokes-shifted Raman scatter.

The appearance of a Raman spectrum is much like an infrared spectrum plotted linearly in absorbance. The intensities, or the number of Raman photons counted, are plotted against the shifted energies. The x-axis is generally labeled "Raman Shift/cm⁻¹" or "Wavenumber/cm⁻¹". The Raman shift is usually expressed in wavenumber and represents the difference in the absolute wavenumber of the peak and the laser wavenumber. The spectrum is interpreted in the same manner as the corresponding mid-infrared spectrum. The positions of the (Raman shifted) wavenumbers for a given vibrational mode are identical to the wavenumbers of the corresponding bands in an IR absorption spectrum. However, the stronger peaks in a Raman spectrum are often weak in an IR spectrum, and vice versa. Thus the two spectroscopic techniques are often said to be complementary.

Raman spectroscopy is advantageous because quick and accurate measurements can often be made without destroying the sample (solid, semisolid, liquid or, less frequently, gas) and with minimal or no sample preparation. The Raman spectrum contains information on fundamental vibrational modes of the sample that can yield both sample and process understanding. The signal is typically in the visible or NIR range, allowing efficient coupling to fiber optics. This also means that a signal can be obtained from any medium transparent to the laser light; examples are glass, plastics, or samples in aqueous media. In addition, because Raman spectra are ordinarily excited with visible or NIR radiation, standard glass/quartz optics may be used. From an instrumental point of view, modern systems are easy to use, provide fast analysis times (seconds to several minutes), and are reliable. However, the danger of using high-powered lasers must be recognized, especially when their wavelengths are in the NIR and, therefore, not visible to the eye. Fiber-optic probes should be used with caution and with reference to appropriate government regulations regarding lasers and laser classes.

In addition to "normal" Raman spectroscopy, there are several more specialized Raman techniques. These include resonance Raman (RR), surface-enhanced Raman spectroscopy (SERS), Raman optical activity (ROA), coherent anti-Stokes Raman spectroscopy (CARS), Raman gain or loss spectroscopy, and hyper-Raman spectroscopy. These techniques are not widely employed in pharmaceutical laboratories, and are not addressed in this general information chapter.

QUALITATIVE AND QUANTITATIVE RAMAN MEASUREMENTS

There are two general classes of measurements that are commonly performed by Raman spectrometry: qualitative and quantitative.

Qualitative Raman Measurements

Qualitative Raman measurements yield spectral information about the functional groups that are present in a sample. Because the Raman spectrum is specific for a given compound, qualitative Raman measurements can be used as a compendial ID test, as well as for structural elucidation.

Quantitative Raman Measurements

For instruments equipped with a detector that measures optical power (such as Fourier transform [FT]-Raman spectrometers), quantitative Raman measurements utilize the following relationship between signal, S_v , at a given wavenumber, v , and the concentration of an analyte, C :

$$S_v = K\sigma_v(v_L - v_\beta)^4 P_0 C$$

in which K is a constant that depends on laser beam diameter, collection optics, sample volume, and temperature; σ_v is the Raman cross section of the particular vibrational mode; v_L is the laser wavenumber; v_β is the wavenumber of the vibrational mode; and P_0 is the laser power. The Raman cross section, σ_v , is characteristic of the nature of the particular vibrational mode. The sample volume is defined by size of the focus of the laser beam at the sample, the optic being used for focusing, and the optical properties of the sample itself. Spot sizes at the sample can range from less than 1 μm for a microprobe to 6 mm for a large area sample system. For Raman spectrometers that measure the number of photons per second (such as charge-coupled device [CCD]-Raman spectrometers) the corresponding equation is:

$$S_v = K\sigma_v v_L (v_L - v_\beta)^3 P_0 C$$

From the above equations, it is apparent that peak signal is directly proportional to concentration. It is this relationship that is the basis for the majority of quantitative Raman applications.

FACTORS AFFECTING QUANTIFICATION

Sample-Based Factors

The most important sample-based factors that deleteriously affect quantitative Raman spectrometry are fluorescence, sample heating, absorption by the matrix or the sample itself, and the effect of polarization. If the sample matrix includes fluorescent compounds, the measured signal will usually contain a contribution from fluorescence. Fluorescence will be observed only if the laser excitation wavelength overlaps with an absorption band of a fluorescent compound. Fluorescence is typically observed as a broad sloping background underlying the Raman spectrum. Fluorescence can cause both a baseline offset and reduced signal-to-noise ratio. The wavelength range and intensity of the fluorescence is dependent on the chemical composition of the fluorescent material. Because fluorescence is generally a much more efficient process than Raman scattering, even very minor amounts of fluorescent impurities can lead to significant degradation of the Raman signal. Fluorescence can be reduced by using longer wavelength excitation sources such as 785 nm or 1064 nm. However, it should be

remembered that the strength of the Raman signal is proportional to $(\nu_L - \nu_B)^4$, so the advantage of using a long-wavelength excitation laser to minimize fluorescence is at least partially offset by the reduced strength of the Raman signal. The greatest signal-to-noise ratio will be obtained by balancing fluorescence rejection, signal strength, and detector response.

Fluorescence in solids can sometimes be mitigated by exposing the sample to the laser radiation for a period of time before measurement. This process is called photobleaching, and operates by degrading the highly absorbing species. Photobleaching is less effective in liquids, where the sample is mobile, or if the amount of fluorescent material is more than a trace.

Sample heating by the laser source can cause a variety of effects, such as physical form change (melting), polymorph conversion, or sample burning. The chance for sample heating is greatest when the spot size at the sample is the smallest, i.e., when a microprobe is being used. This is usually an issue for colored, highly absorbing species, or very small particles that have low heat transfer. The effects of sample heating are usually observable either as changes in the Raman spectrum over time or by visual inspection of the sample. Besides decreasing the laser flux, a variety of methods can be employed to diminish laser-induced heating, such as moving the sample or laser during the measurement or improving the heat transfer from the sample with thermal contact or liquid immersion.

Absorption of the Raman signal by the matrix or the sample itself can also occur. This problem is more prevalent with long-wavelength FT-Raman systems where the Raman signal can overlap with an NIR overtone absorption. This effect will be dependent on the optics of the system as well as on the sample presentation. Associated with this effect is variability from scattering in solids as a result of packing and particle-size differences. The magnitude of all of these effects, however, is typically less severe than in NIR because of the limited depth of penetration and the relatively narrower wavelength region sampled in Raman spectroscopy.

Finally, it should be recognized that laser radiation is polarized and the Raman spectra of crystalline materials and other oriented samples can differ significantly depending on the way that the sample is mounted. If the Raman spectrometer is capable of producing linearly polarized radiation at the sample then a polarization scrambler is recommended for routine sample analysis.

Sampling Factors

Raman spectroscopy is a zero-background technique, in that the signal at the detector is expected to be zero in the absence of a sample. This situation can be contrasted with absorption spectrometry, where the signal at the detector is at a maximum in the absence of a sample. Zero-background techniques are inherently sensitive because small changes in sample concentration lead to proportionate changes in the signal level. The instrument will also be sensitive to other sources of light that can cause sample-to-sample variations in the measured signal level. In addition, a large background signal caused by fluorescence will lead to an increased noise level (photon shot noise). Thus it may be very difficult to use the absolute Raman signal for direct determination of an analyte. Other potential sources of variation are changes in the sample opacity and heterogeneity, changes in the laser power at the sample, and changes in optical collection geometry or sample position. These effects can be minimized by sampling in a reproducible, representative manner. Careful design of the instrumentation can reduce these effects but they cannot be eliminated entirely.

Use of an internal reference standard is the most common and robust method of eliminating variations caused by absolute intensity fluctuations. There are several choices for this approach. An internal standard can be deliberately added, and isolated peaks from this standard can be em-

ployed; or a band due to a moiety such as an aromatic ring, the Raman cross-section of which does not change with the way the sample is prepared, can also be used. For solution spectra, an isolated solvent band can be employed because the solvent will remain relatively unchanged from sample to sample. Also, in a formulation, an excipient peak can be used if it is in substantial excess compared to the analyte. The entire spectrum can also be used as a reference, with the assumption that laser and sample-orientation changes will affect the entire spectrum equally.

A second important sampling-based factor to consider is spectral contamination. Raman scattering is a weak effect that can be masked by a number of external sources. Common contamination sources include sample-holder artifacts (container or substrate) and ambient light. Typically, these issues can be identified and resolved by careful experimentation.

APPARATUS

Components

All modern Raman measurements involve irradiating a sample with a laser, collecting the scattered radiation, rejecting the Rayleigh-scattered light, differentiating the Raman photons by wavelength, and detecting the resulting Raman spectrum. All commercial Raman instruments therefore share the following common features to perform these functions:

1. Excitation source (laser)
2. Sampling device
3. Device to filter/reject light scattered at the laser wavelength
4. Wavelength processing unit
5. Detector and electronics

EXCITATION SOURCE (LASER)

Table 1 identifies several common lasers used for pharmaceutical applications or Raman spectrometry. UV lasers have also been used for specialized applications but have various drawbacks that limit their utility for general analytical measurements. As more applications for UV lasers are described, it is likely that they may become more common for Raman spectrometry.

SAMPLING DEVICE

Several sampling arrangements are possible, including direct optical interfaces, microscopes, fiber optic-based probes (either noncontact or immersion optics), and sample chambers (including specialty sample holders and automated sample changers). The sampling optics can also be designed to obtain the polarization-dependent Raman spectrum, which often contains additional information. Selection of the sampling device will often be dictated by the analyte and sample. However, considerations such as sampling volume, speed of the measurement, laser safety, and reproducibility of sample presentation should be evaluated to optimize the sampling device for any given application.

FILTERING DEVICE

The intensity of scattered light at the laser wavelength (Rayleigh) is many orders of magnitude greater than the Raman signal and must be rejected prior to the detector. Notch filters are almost universally used for this purpose and provide excellent rejection and stability combined with small size. The traditional use of multistage monochromators for this purpose, although still viable, is now rare. In addition,

Table 1. Lasers Used in Pharmaceutical Applications

Laser λ , nm (nearest whole number)	Type	Typical Power at Laser	Wavelength Range, nm (Stokes Region, 100 cm^{-1} to 3000 cm^{-1} shift)	Comments
NIR Lasers				
1064	Solid state (Nd:YAG)	Up to 3 W	1075–1563	Commonly used in Fourier transform instruments
830	Diode	Up to 300 mW	827–980	Typically limited to 2000 cm^{-1} ; Raman shift because of CCD spectral response; less common than the other lasers
785	Diode	Up to 500 mW	791–1027	Most widely used dispersive Raman laser
Visible Lasers				
632.8	He–Ne	Up to 500 mW	637–781	Relatively small fluorescence risk
532	Doubled (Nd:YAG)	Up to 1 W	535–632.8	High fluorescence risk
514.5	Ar+	Up to 1 W	517–608	High fluorescence risk
488–632.8	Ar+	Up to 1 W	490–572	High fluorescence risk

various filters or physical barriers to shield the sample from external radiation sources (e.g., room lights, laser plasma lines) may be required depending on the collection geometry of the instrument.

WAVELENGTH PROCESSING UNIT

The wavelength scale may be encoded by either a scanning monochromator, a grating polychromator (in CCD-Raman spectrometers) or a two-beam interferometer (in FT-Raman spectrometers). A discussion of the specific benefits and drawbacks of each of the dispersive designs compared to the FT instrument is beyond the scope of this chapter. Any properly qualified instruments should be suitable for qualitative measurements. However, care must be taken when selecting an instrument for quantitative measurements, as dispersion and response linearity might not be uniform across the full spectral range.

DETECTOR

The silicon-based CCD array is the most common detector for dispersive instruments. The cooled array detector allows measurements over the spectral range from 4500 to 100 cm^{-1} Raman shift with low noise when most visible lasers, such as frequency-doubled neodymium-doped yttrium–aluminum–garnet (Nd:YAG) (532 nm) or helium–neon (632.8 nm) lasers, are used. When a 785-nm diode laser is used, the wavelength range is reduced to about 3100 to 100 cm^{-1} . The most commonly used CCD has its peak wavelength responsivity when matched to the commonly used 632.8-nm He–Ne gas laser or 785-nm diode laser. FT instruments typically use single-channel germanium or indium–gallium–arsenide (InGaAs) detectors responsive in the NIR to match the 1064-nm excitation of a Nd:YAG laser.

Calibration

Raman instrument calibration involves three components: primary wavelength (x-axis), laser wavelength, and intensity (y-axis).

PRIMARY WAVELENGTH (X-AXIS)

In the case of FT-Raman instruments, primary wavelength-axis calibration is maintained, at least to a first approximation, with an internal He–Ne laser. Most dispersive instruments utilize atomic emission lamps for primary wavelength-axis calibration. In all instruments suitable for analytical Raman measurements, the vendor will offer a procedure of

x-axis calibration that can be performed by the user. For dispersive Raman instruments, a calibration based on multiple atomic emission lines is preferred. The validity of this calibration approach can be verified subsequent to laser wavelength calibration by using a suitable Raman shift standard. For scanning dispersive instruments, calibration might need to be performed more frequently, and precision in both a scanning and static operation mode may need to be verified.¹

LASER WAVELENGTH

Laser wavelength variation can impact both the wavelength precision and the photometric (signal) precision of a given instrument. Even the most stable current lasers can vary slightly in their measured wavelength output. The laser wavelength must therefore be confirmed to ensure that the Raman shift positions are accurate for both FT-Raman or dispersive Raman instruments. A reference Raman shift standard material such as those outlined in ASTM E1840-96 (2002)¹ or other suitably verified materials can be utilized for this purpose. [NOTE—Reliable Raman shift standard values for frequently used liquid and solid reagents, required for wavenumber calibration of Raman spectrometers, are provided in the ASTM Standard Guide cited. These values can be used in addition to the highly accurate and precise low-pressure arc lamp emission lines that are also available for use in Raman instrument calibration.] Spectrometric grade material can be purchased from appropriate suppliers for this use. Certain instruments may use an internal Raman standard separate from the primary optical path. External calibration devices exactly reproduce the optical path taken by the scattered radiation. [NOTE—When chemical standards are used, care must be taken to avoid contamination and to confirm standard stability.]

Unless the instrument is of a continuous calibration type, the primary wavelength axis calibration should be performed, as per vendor procedures, just prior to measuring the laser wavelength. For external calibration, the Raman shift standard should be placed at the sample location and measured using appropriate acquisition parameters. The peak center of a strong, well-resolved band in the spectral region of interest should be evaluated. The position can be assessed manually or with a suitable, valid peak-picking algorithm. The software provided by the vendor might measure the laser wavelength and adjust the laser wavelength appropriately so that this peak is at the proper position. If the vendor does not provide this functionality, the laser wavelength should be adjusted manually. Depending on the

¹ ASTM E1840-96 (2002) *Standard Guide for Raman Shift Standards for Spectrometer Calibration*, ASTM International, 100 Barr Harbor Drive, PO Box C700, West Conshohocken, PA, USA 19428-2959.

type of laser, the laser wavelength can vary with temperature, current, and voltage. Wavelength tolerances can vary depending on the specific application.

SIGNAL LEVEL (Y-AXIS)

Calibration of the photometric axis can be critical for successful quantification by using certain analytical methods (chemometrics) and method transfer between instruments. Both FT-Raman and dispersive Raman spectrometers should undergo similar calibration procedures. The tolerance of photometric precision acceptable for a given measurement should be assessed during the method development stage.

To calibrate the photometric response of a Raman instrument, a broad-band emission source should be used. There are two accepted methods. *Method A* utilizes a tungsten white light source.² The output power of such sources is traceable to the National Metrology Institute (NMI). In the United Kingdom, the National Physical Laboratory also provides calibrated light bulbs. Several other vendors also provide NIST-traceable irradiance calibration standards. This method is applicable to all common laser excitation wavelengths listed in *Table 1*. In *Method B*, NIST standard reference materials (SRMs) are utilized.³ Several doped-glass fluorescence standards are currently available.

Method A—The source should be placed at the sample location with the laser off and the response of the detector measured (using parameters appropriate for the instrument). The output for the source used for calibration should be known. The ratio of the measured response to the true response should be determined and a correction file generated. This correction should be applied to all spectra acquired with the instrument. Most manufacturers will provide both appropriate calibration sources and software for this approach. If the manufacturer does not provide a procedure or method, the user can accomplish the task using a source obtained from NIST and appropriate software. If a manufacturer's method is used, attention must be paid to the calibration procedure and source validity. The user should obtain appropriate documentation from the manufacturer to ensure a qualified approach.

Method B—The fluorescence standard should be placed at the sample location. With the laser on, a spectrum of the SRM should be obtained (using parameters appropriate for the instrument). The output of the source used for calibration should be known. The ratio of the measured response to the true response should be determined and a correction file generated. This correction should be applied to all spectra acquired with the instrument. Most manufacturers will provide both appropriate calibration sources and software for this approach. If the manufacturer does not provide a procedure or method, the user can accomplish the task using a source obtained from NIST and appropriate software. If a manufacturer's method is used, attention must be paid to the calibration procedure and source validity. The user should obtain appropriate documentation from the manufacturer to ensure a qualified approach. [NOTE—*Method B* is currently appropriate for systems with 785-nm (SRM 2241), 532-nm (SRM 2242), and both 514.5-nm and 488-nm (SRM 2243) laser excitation. NIST is currently developing other SRMs that will be wavelength-specific for 1064-nm (SRM 2244) and 632.8-nm excitation (expected to be available in 2006).]

² NIST-traceable tungsten white light source statement: While the calibration of the Raman frequency (or Raman shift, cm^{-1}) axis using pure materials and an existing ASTM standard is well accepted, techniques for calibration of the Raman intensity axis are not. Intensity calibrations of Raman spectra can be accomplished with certified white light sources.

³ NIST SRM 2241: Ray KG, McCreery RL. Raman intensity correction standard for systems operating with 785-nm excitation. *Appl. Spectrosc.* 1997, 51, 108–116.

EXTERNAL CALIBRATION

Detailed functional validation employing external reference standards is recommended to demonstrate instrumental suitability for laboratory instruments, even for instruments that possess an internal calibration approach. The use of external reference standards does not obviate the need for internal quality control procedures; rather, it provides independent documentation of the fitness of the instrument to perform the specific analysis or purpose. For instruments installed in a process location or in a reactor where positioning of an external standard routinely is not possible, including those instruments that employ an internal calibration approach, the relative performance of an internal versus an external calibration approach should be periodically checked. The purpose of this test is to check for changes in components that might not be included in the internal calibration method (process lens, fiber-optic probe, etc.), e.g., photometric calibration of the optical system.

QUALIFICATION AND VERIFICATION OF RAMAN SPECTROMETERS

The suitability of a specific instrument for a given method is ensured by a thorough technology-suitability evaluation for the application; a routine, periodic instrument operational qualification; and the more frequent performance verification (see *Definition of Terms and Symbols*). The purpose of the technology-suitability evaluation is to ensure that the technology proposed is suitable for the intended application. The purpose of the instrument qualification is to ensure that the instrument to be used is suitable for its intended application and, when requalified periodically, continues to function properly over extended time periods. When the device is used for a specific qualitative or quantitative analysis, regular performance verifications are made. Because there are many different approaches to measuring Raman spectra, instrument operational qualification and performance verification often employ external standards that can be used on any instrument. As with any spectrometric device, a Raman instrument needs to be qualified for both wavenumber (x-axis and shift from the excitation source) and photometric (signal axis) precision.

In performance verification, a quality-of-fit to an initial scan or group of scans (often referred to in nonscanning instruments as an accumulation) included in the instrumental qualification can be employed. In such an analysis, it is assumed that reference standard spectra collected on a new or a newly repaired, properly operating instrument represent the best available spectra. Comparison of spectra taken over time on identical reference standards (either the original standard or identical new standards, if stability of the reference standards is a concern) forms the basis for evaluating the long-term stability of a Raman measurement system.

Frequency of Testing

Instrumental qualification is performed at designated intervals or following a repair or significant optical reconfiguration, such as the replacement of the laser, the detector or the notch or edge filters. Full instrument requalification might not be necessary when changing between sampling accessories such as a microprobe, a sample compartment, or a fixed fiber-optic probe. Performance verification tests may be sufficient in these cases; instrument-specific guidance from the vendor on qualification requirements should be followed. Tests include wavelength (x-axis and shift from the excitation source) and photometric (signal axis) precision. Instrument qualification tests require that specific application-dependent tolerances be met.

Performance verification is carried out on the instrument configured for the analytical measurements and is performed more frequently than instrument qualification. Per-

formance verification includes measurement of the wavelength uncertainty and intensity-scale precision. Wavelength precision and intensity-scale precision tests may be needed prior to any data collection on a given day. Performance is verified by matching the current spectra to those collected during the previous instrument qualification.

Instrument Operational Qualification

It is important to note that the acceptance specifications given in both the *Instrument Operational Qualification* and *Performance Qualification* sections are applicable for general use; specifications for particular instruments and applications can vary depending on the analysis method used and the desired accuracy of the final result. ASTM standard reference materials are also specified, with the understanding that under some circumstances (specifically remote on-line applications) calibration using one of these materials may be impractical, and other suitably verified materials can be employed. At this juncture it is important to note that specific parameters such as spectrometer noise, limits of detection (LOD), limits of quantification (LOQ), and acceptable spectral bandwidth for any given application should be included as part of the analytical method development. Specific values for tests such as spectrometer noise and bandwidth will be dependent on the instrument chosen and the purpose required. As a result, specific instrument tests for these parameters are not dictated in this information chapter.

WAVELENGTH (X-AXIS) ACCURACY

It is important to ensure the accuracy of the wavelength axis via calibration to maintain the integrity of Raman peak positions. Wavelength calibration of a Raman spectrometer consists of two parts: primary wavelength axis and laser wavelength calibration. After both the primary wavelength axis and the laser wavelength are calibrated, instrument wavelength uncertainty can be determined. This can be accomplished using a Raman shift standard such as the ASTM shift standards or other suitably verified material. Selection of a standard with bands present across the full Raman spectral range is recommended so that instrument wavelength uncertainty can be evaluated at multiple locations within the spectrum. The tolerance of wavelength precision that is required for a given measurement should be assessed during the method-development stage. [NOTE—For scanning dispersive instruments, calibration might need to be performed more frequently, and precision in both a scanning and static operation mode may need to be verified.]

PHOTOMETRIC PRECISION

Laser variation in terms of the total emitted photons occurring between two measurements can give rise to changes in the photometric precision of the instrument. Unfortunately, it is very difficult to separate changes in the photometric response associated with variations in the total emitted laser photons from the sample- and sampling-induced perturbations. This is one of the reasons why absolute Raman measurements are strongly discouraged and why the photometric precision specification is set relatively loosely. The tolerance of photometric precision required for a given measurement should be assessed during the method-development stage.

PERFORMANCE QUALIFICATION

The objective of performance qualification is to ensure that the instrument is performing within specified limits with respect to wavelength precision, photometric axis precision, and sensitivity. In certain cases when the instrument has been set up for a specific measurement (for example,

installed in a process reactor), it might no longer be possible or desirable to measure the wavelength and photometric (signal) qualification reference standards identified above. Provided instrument operational qualification has shown that the equipment is fit for use, a single external performance verification standard can be used to reverify function on a continuing basis (for example, a routinely used process solvent signal, for both wavelength and photometric precision, following reactor cleaning). The performance verification standard should match the format of the samples in the current analysis as closely as possible and use similar spectral acquisition parameters. Quantitative measurements of an external performance verification standard spectrum check both the wavelength (x-axis and laser wavelength) and the photometric (signal) precision. Favorable comparison of a series of performance verification spectra demonstrates proper continued operation of the instrument.

WAVELENGTH PRECISION

The wavelength precision should be measured by collecting data for a single spectrum of the selected Raman shift standard for a period equal to that used in the photometric consistency test. When appropriate, powdered samples should be repacked between each set of measurements. Peak positions across the spectral range of interest are used to calculate precision. Performance is verified by matching the current peak positions to those collected during the previous instrument qualification and should not vary with a standard deviation of more than $\pm 0.3 \text{ cm}^{-1}$, although this specification can be adjusted according to the required accuracy of the measurement.

PHOTOMETRIC PRECISION

The photometric precision should be measured by collecting data for a single spectrum of a suitably verified reference standard material for a specified time. After suitable baseline correction, the areas of a number of bands across the spectral range of interest should be calculated by means of an appropriate algorithm. The area of the strongest band is set to 1, and all other envelopes are normalized to this band. Performance is verified by matching the current band areas to the respective areas collected during the previous instrument qualification. The areas should vary by no more than 10%, although this specification can be adjusted according to the required accuracy of the measurement.

LASER POWER OUTPUT PRECISION AND ACCURACY

This test is applicable only to Raman instruments with automatic, internal laser power meters. Instruments without laser power measurement should utilize a calibrated laser power meter from a reputable supplier. The laser output should be set to a representative output, dictated by the requirements of the analytical measurement and the laser power measured. The output should be measured and checked against the output measured at instrument qualification. The power (in milliwatts or watts) should vary by no more than 25% compared to the qualified level. If the power varies by more than this amount, the instrument should be serviced (as this variation might indicate, among other things, a gross misalignment of the system or the onset of failure of the laser).

For instruments with an automatic, internal laser power meter, the accuracy of the values generated from the internal power meter should be compared to a calibrated external laser power meter at an interval of not more than 12 months. The internally calculated value should be compared to that generated by the external power meter. Performance is verified by matching the current value to that generated during the previous instrument qualification. The

manufacturer might provide software to facilitate this analysis. If the instrument design prevents the use of an external power meter, then the supplier should produce documentation to ensure the quality of the instrument and provide a recommended procedure for the above analysis to be accomplished during a scheduled service visit.

METHOD VALIDATION

Validation of Raman methods will follow the same protocols described in *Validation of Compendial Procedures* <1225> in terms of accuracy, precision, etc. However, several of these criteria are affected by variables specific to Raman spectrometry. Fluorescence is the primary variable that can affect the suitability of a method. The presence of fluorescent impurities in samples can be quite variable and have little effect on the acceptability of a material. The method must be flexible enough to accommodate different sampling regimes that may be necessary to minimize the effects of these impurities.

Detector linearity must be confirmed over the range of possible signal levels. Fluorescence might drive both the signal baseline and the noise higher than that used in the validation, in which case the fluorescence must be decreased, or the method modified to accommodate the higher fluorescence levels. This is also true for the precision, limit of detection, and limit of quantification of the method, as increased baseline noise will negatively impact all of these values. Because fluorescence can also affect quantification caused by baseline shifts, acceptable quantification at different levels of photobleaching, when used, should also be confirmed.

The impact of the laser on the sample must be determined. Visual inspection of the sample and qualitative inspection of the Raman spectrum for measurements with differing laser powers and exposure times will confirm that the sample is not being altered (other than by photobleaching). Specific variables to confirm in the spectrum are shifts in peak position, changes in peak height and band width, and unexpected changes in background intensity.

Method precision must also encompass sample position. The sample presentation is a critical factor for both solids and liquids, and must be either tightly controlled or accounted for in the calibration model. Sample-position sensitivity can often be minimized by appropriate sample preparation or sample holder geometry, but will vary from instrument to instrument based on excitation and collection optical configuration.

DEFINITION OF TERMS AND SYMBOLS

CALIBRATION MODEL is a mathematical expression that relates the response from an analytical instrument to the properties of samples.

INSTRUMENT BANDPASS (OR RESOLUTION) is a measure of the capability of a spectrometer to separate radiation of similar wavelengths.

OPERATIONAL QUALIFICATION is the process by which it is demonstrated and documented that the instrument performs according to specifications, and that it can perform the intended task. This process is required following any significant change such as instrument installation, relocation, major repair, etc.

PERFORMANCE QUALIFICATION is the process of using one or more well-characterized and stable reference materials to verify consistent instrument performance. Qualification may employ the same or different standards for different performance characteristics.

RAMAN SPECTRA⁴ are plots of the radiant energy, or number of photons, scattered by the sample through the indirect interaction between the molecular vibrations in the sample

and monochromatic radiation of frequency much higher than that of the vibrations. The abscissa is usually the difference in wavenumber between the incident and scattered radiation.

(NORMAL) RAMAN SCATTERING⁴ is the inelastic scattering of radiation that occurs because of changes in the polarizability, of the relevant bonds during a molecular vibration. Normal Raman spectra are excited by radiation that is not in resonance with electronic transitions in the sample.

RAMAN WAVENUMBER SHIFT⁴,

$$\Delta\tilde{\nu}$$

is the wavenumber of the exciting line minus the wavenumber of the scattered radiation. SI unit: m⁻¹. Common unit: cm⁻¹ = 100 m⁻¹.

$$\beta\Delta\tilde{\nu}$$

where β is the differential Raman cross section, is positive for Stokes scattering and negative for anti-Stokes scattering.

<1121> NOMENCLATURE

The *USP* (or *NF*) titles for monograph articles are legally recognized under the Federal Food, Drug, and Cosmetic Act as the designations for use in labeling the articles to which they apply.

The value of designating each drug by one and only one nonproprietary¹ name is important in terms of achieving simplicity and uniformity in drug nomenclature. In support of the U.S. Adopted Names program (see *Mission and Preface* in *USP-NF*), of which the U.S. Pharmacopeial Convention is a cosponsor, the USP Council of Experts gives consideration to the adoption of the U.S. Adopted Name, if any, as the official title for any compound that attains compendial recognition.

A compilation of the U.S. Adopted Names (USAN) published from the start of the USAN program in 1961, as well as other names for drugs, both current and retrospective, is provided in the *USP Dictionary of USAN and International Drug Names*. This publication serves as a book of names useful for identifying and distinguishing all kinds of names for drugs, whether public, proprietary, chemical, or code-designated names.²

A nonproprietary name of a drug serves numerous and varied purposes, its principal function being to identify the substance to which it applies by means of a designation that may be used by the professional and lay public free from the restrictions associated with registered trademarks. Teaching in the health sciences requires a common designation, especially for a drug that is available from several sources or is incorporated into a combination drug product; nonproprietary names facilitate communication among healthcare providers; nonproprietary names must be used as the titles of the articles recognized by official drug compendia; a nonproprietary name is essential to the pharmaceutical manufacturer as a means of protecting trademark rights in the brand name for the article concerned; and, finally, the manufacturer is obligated by federal law to include the established nonproprietary name in advertising and labeling.

Under the terms of the Drug Amendments of 1962 to the Federal Food, Drug, and Cosmetic Act, which became law

¹ The term "generic" has been widely used in place of the more accurate and descriptive term "nonproprietary" with reference to drug nomenclature.

² *USP Dictionary of USAN and International Drug Names* is obtainable on order from U.S. Pharmacopeia, Customer Service Department, 12601 Twinbrook Parkway, Rockville, MD 20852.

⁴ Chalmers, J., Griffiths, P., Eds. *Handbook of Vibrational Spectroscopy*; John Wiley & Sons, Ltd: New York, 2002.

October 10, 1962, the Secretary of Health and Human Services is authorized to designate an official name for any drug wherever deemed "necessary or desirable in the interest of usefulness and simplicity."³

The Commissioner of Food and Drugs and the Secretary of Health and Human Services published in the *Federal Register* regulations effective November 26, 1984, which state, in part:

"Sec. 299.4 Established names of drugs."

"(e) The Food and Drug Administration will not routinely designate official names under section 508 of the act. As a result, the established name under section 502(e) of the act will ordinarily be either the compendial name of the drug or, if there is no compendial name, the common and usual name of the drug. Interested persons, in the absence of the designation by the Food and Drug Administration of an official name, may rely on as the established name for any drug the current compendial name or the USAN adopted name listed in *USAN and the USP Dictionary of Drug Names*."⁴

It will be noted that the monographs on the biologics, which are produced under licenses issued by the Secretary of the U.S. Department of Health and Human Services, represent a special case. Although efforts continue toward achieving uniformity, there may be a difference between the respective title required by federal law and the USP title. Such differences are fewer than in past revisions of the Pharmacopeia. The USP title, where different from the FDA Center for Biologics Evaluation and Research title, does not necessarily constitute a synonym for labeling purposes; the conditions of licensing the biologic concerned require that each such article be designated by the name appearing in the product license issued to the manufacturer. Where a USP title differs from the title in the federal regulations, the former has been adopted with a view to usefulness, simplicity, and conformity with the principles governing the selection of monograph titles generally.

GENERAL NOMENCLATURE FORMS

Some monograph titles existing in the *USP-NF* do not conform to the formats outlined in this general information chapter. Typically, these monograph titles were adopted before the establishment of the title formats and nomenclature policies presented in this general information chapter. Such monograph titles may be subject to subsequent revision and should not be interpreted as precedents for other monograph titles.

Standardized forms of nomenclature have been devised in the interest of achieving uniformity for naming compendial articles. The general nomenclature forms that follow illustrate the terminology used throughout the official compendia for consistency in establishing titles of monographs on official pharmaceutical dosage forms and preparations. Examples are shown for the more frequently encountered categories of dosage forms.

For a variety of dosage forms, titles are in the following general form: [DRUG] [ROUTE OF ADMINISTRATION] [DOSAGE FORM].

Examples:

Calcium Carbonate Oral Suspension
Cetylpyridinium Chloride Topical Solution
Dexamethasone Ophthalmic Suspension
Epinephrine Bitartrate Ophthalmic Solution
Isosorbide Dinitrate Sublingual Tablets
Miconazole Nitrate Topical Powder
Triple Sulfa Vaginal Cream

The term "Vaginal Inserts", rather than "Vaginal Tablets", "Vaginal Capsules", or "Vaginal Suppositories" is used in the title of this type of vaginal preparation to avoid the potential for misuse of these products if the term "Tablets" or "Capsules" or "Suppositories" were to appear in the title.

³ F.D.&C. Act, Sec. 508 [358].

⁴ 53 Fed. Reg. 5369 (1988) amending 21 CFR § 299.4.

Example:

Clotrimazole Vaginal Inserts

The term for route of administration is omitted for those dosage forms for which the route of administration is understood. The general form then becomes simply [DRUG] [DOSAGE FORM]. Thus, capsules, tablets, and lozenges are administered via the oral route unless otherwise indicated by the title.

Examples:

Acetaminophen Capsules
Aminophylline Delayed-Release Tablets
Aspirin Extended-Release Tablets
Hexylresorcinol Lozenges
Meperidine Hydrochloride Tablets

Drugs that are injected may be administered via the intravenous, intramuscular, subcutaneous, etc., route; the route being specified in the labeling rather than in the name.

Examples:

Aurothioglucose Injectable Suspension
Epinephrine Injection
Fluorouracil Injection
Hydrocortisone Acetate Injectable Suspension
Phytonadione Injectable Emulsion

Creams, ointments, lotions, and pastes are applied topically, unless otherwise indicated by the name.

Examples:

Benzoyl Peroxide Lotion
Betamethasone Dipropionate Cream
Estradiol Vaginal Cream
Nystatin Ointment
Zinc Oxide Paste

The term "Suppositories" is used in the titles of preparations that are intended for rectal administration.

Example:

Aspirin Suppositories

The term "for" is included in names, as appropriate, of preparations for which a solid drug substance must be dissolved or suspended in a suitable liquid to obtain a dosage form, and the general form becomes [DRUG] for [ROUTE OF ADMINISTRATION] [DOSAGE FORM].

Examples:

Ampicillin for Oral Suspension
Epinephrine Bitartrate for Ophthalmic Solution
Nafcillin for Injection
Spectinomycin for Injectable Suspension

In some instances, the drug is supplied in one dosage form for the preparation of the intended dosage form.

Examples:

Aspirin Effervescent Tablets for Oral Solution
Methadone Hydrochloride Tablets for Oral Suspension
Papain Tablets for Topical Solution

Systems are preparations of drugs in carrier devices that are applied topically or inserted into body cavities, from which drugs are released gradually over extended times, after which the carrier device is removed. The general form for a system is [DRUG] [ROUTE] [SYSTEM].

Examples:

Nicotine Transdermal System
Progesterone Intrauterine Contraceptive System

Some drugs are available as concentrated solutions that are not intended for direct administration to humans or animals, but are to be diluted with suitable liquid vehicles to obtain the intended preparation. The general form for these preparations, which are not dosage forms, is [DRUG] [CONCENTRATE].

Examples:

Isosorbide Concentrate (used to prepare Isosorbide Oral Solution)
Glutaral Concentrate (used to prepare Glutaral Disinfectant Solution)

For products intended for parenteral administration, the use of the word "Concentrate" in the monograph title is restricted to one specific monograph, Potassium Chloride for Injection Concentrate. The word "Concentrate" should not appear in the monograph title for any other parenteral

product; rather, this issue is to be addressed in the product labeling.

Some drugs are supplied as preparations that may be intermediates used for convenience in formulating finished dosage forms. The general form for such preparations, which are not finished dosage forms, is [DRUG] [PREPARATION].

Examples:

Vitamin E Preparation
Cranberry Liquid Preparation

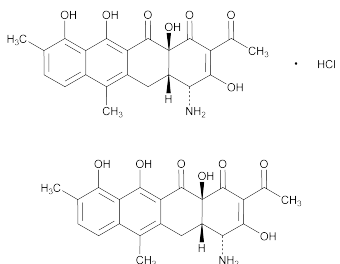
MONOGRAPH NAMING POLICY FOR SALT DRUG SUBSTANCES IN DRUG PRODUCTS AND COMPOUNDED PREPARATIONS

The titles of USP monographs for drug products and compounded preparations formulated with a salt of an acid or base use the name of the active moiety, as defined below. The strength of the product or preparation also is expressed in terms of the active moiety.

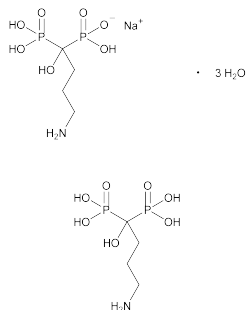
An active moiety is the molecule or ion, excluding those appended portions of the molecule that cause the drug to be an ester, salt (including a salt with hydrogen or coordination bonds), or other noncovalent derivative (such as a complex, chelate, or clathrate) of the molecule, responsible for the physiological or pharmacological action of the drug substance, without regard to the actual charged state of the molecule in-vivo.

For example, the active moiety of a hydrochloride salt of a base will be the free base and not the protonated form of the base. The active moiety of a metal acid salt will be the free acid.

i. Example: Chelocardin Hydrochloride active moiety is Chelocardin



ii. Example: Alendronate Sodium active moiety is Alendronic Acid



This Policy is followed by USP in naming drug products and compounded preparations that are newly recognized in

the USP. Revising existing monographs to conform to this Policy is not intended, except where the USP Council of Experts determines that, for reasons such as safety, a nomenclature change is warranted.

Related Issues

Labeling—The labeling clearly states the specific salt form of the active moiety that is present in the product/preparation, as this information may be useful to practitioners and patients. The names and strengths of both the active moiety and specific salt form (where applicable) are provided in the labeling.

Exceptions—In those rare cases in which the use of the specific salt form of the active moiety in the title provides vital information from a clinical perspective, an exception to this Policy may be considered. In such cases, where the monograph title contains the specific salt form of the active moiety, the strength of the product or preparation also is expressed in terms of the specific salt form.

POLICY FOR POSTPONEMENT SCHEDULES

It is the practice of USP to postpone the official dates of nomenclature and labeling revisions for a reasonable time primarily to allow for product label changes to be made and to allow health practitioners and consumers time to become familiar with the new terminology. A postponement period of 18 months is usually applied when only one or a small number of products is affected. A postponement period of 30 months is usually applied when names or labeling of multisource products or multiproduct lines of a firm's preparations are being changed. A postponement period of 60 months is usually applied for title and labeling changes that affect excipients, because such changes would require relabeling of very large numbers of prescription-only and OTC preparations.

There may be exceptions to this postponement schedule where a shorter time is needed in order to specify nomenclature and labeling changes in cases where public health and safety are a concern.

The assignment of a postponement schedule is handled by the USP Expert Committee on Nomenclature. The postponement schedules are presented below. USP's implementation of a postponement schedule is automatic, unless an exception is sought. Exceptions to the postponement schedule are rarely made, and must have suitable justification as well as the approval of the Expert Committee on Nomenclature. Any questions or concerns regarding this postponement schedule may be addressed to the USP staff liaison assigned to the Expert Committee on Nomenclature.

18 months—Schedule for title and labeling changes for a drug product. One or few companies are involved. *Example:* Sterile [Drug] change to [Drug] for Injection.

30 months—Schedule for title and labeling changes for prescription-only and OTC products.

1. Extensive product line for a company. *Examples:* syrups and elixirs.
2. Several companies are involved. *Examples:* syrups and elixirs; lotions; sunscreens.

60 months—Schedule for title and labeling changes for excipient monographs. Ingredient names in numerous multisource products are affected.

<1125> NUCLEIC ACID-BASED TECHNIQUES—GENERAL

SCOPE

Nucleic acid-based assays are used in a variety of settings, the most common of which include the detection of infectious agents (viruses, bacteria, etc.), and cellular materials, as well as disease profiling. More recently such assays have also been used for forensic purposes and for the detection of trace contamination in biological materials. The latter include pharmaceutical development applications, such as viral clearance and adventitious agent testing in vaccine seed lots and tissue culture cell banks. This chapter introduces a series of general information chapters that provide techniques that support procedures for the detection and analysis of nucleic acids (see *Figure 1*). The assays using these techniques may be presented in a USP general chapter or in a private specification.

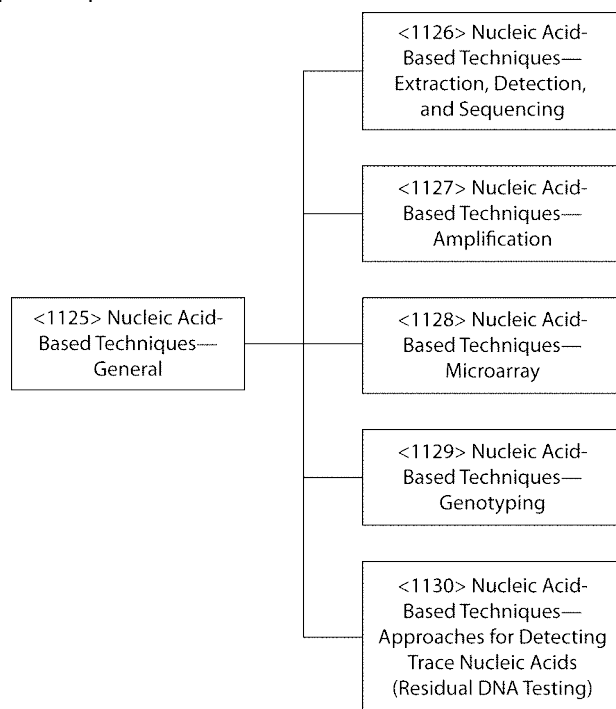


Figure 1

The major requirement for any nucleic acid analytical procedure is the availability of pure, intact nucleic acids for analysis. The information in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* <1126> discusses procedures available for nucleic acid extraction and handling. Hybridization is the core mechanism underlying many molecular biology techniques, and in addition to the detection of nucleic acids by absorbance and fluorescence measurements and size measurement by gel electrophoresis, this chapter

also covers blotting and identification of nucleic acid species by hybridization assays using labeled probes. Hybridization probes are oligonucleotides that have a sequence that is complementary to the target of interest. Probes contain radioactive, fluorescent, biotin, digoxigenin, or other tags that, upon binding of the probe to the target, allow visualization and identification of the target. Probes are capable of detecting target sequences that are present in concentrations too low to be detected by absorbance measurements or gel electrophoresis.

These analytical procedures require a minimum quantity of nucleic acid, typically in the nanogram to microgram range. However, in the vast majority of cases, e.g., in the detection of viruses or rare cellular RNA species, the nucleic acid under assay is present in minute quantities (in the picogram to femtogram range), and an amplification step must be performed before the nucleic acid can be detected and identified. The amplification step may be directed either at the signal used for detection (signal amplification), such as the branched DNA assay (bDNA assay), or at the target as in nucleic acid amplification technologies (NAT).

In 1983 a revolutionary yet simple process termed polymerase chain reaction (PCR) was developed for amplifying the number of specific nucleic acid fragments present in a sample, and in just a few years after its discovery PCR became the most frequently used procedure for amplifying nucleic acids, especially DNA. Since the inception of PCR, the number of applications has expanded rapidly, and the technique, which now includes quantitative and multiplex assays, is currently used in almost every field of research and development in biology and medicine. Numerous variations of assay procedures have been developed for specific analytes. The general information chapter, *Nucleic Acid-Based Techniques—Amplification* <1127>, describes amplification procedures used for DNA and RNA analysis as well as qualitative and quantitative NAT assays. Signal amplification procedures in which the signals, typically fluorescent signals, are used to detect the nucleic acid of interest, are not very common. The major signal amplification procedure, the branched DNA or bDNA assay, is used predominantly for viral nucleic acid detection.

Quality assurance aspects of the methodology are also covered, together with a summary of current regulatory requirements for NAT assays. The need for globally comparable, accurate, and reliable results in the diagnostics field has driven the quest for, and development of, national and international standards within an increasingly sophisticated and metrologically sound, highly developed international regulatory environment devoted to the highest standards of regulatory science. Because NAT has become the most widely used of nucleic acid techniques, the majority of guidance documents and standards are related to NAT. The general information chapter, *Nucleic Acid-Based Techniques—Microarrays* <1128>, addresses a still-emerging field that is of increasing relevance to molecular DNA analysis. Detailed treatment of various microarrays, including data analysis and validation, are excluded from <1128> at this time. The general information chapter, *Nucleic Acid-Based Techniques—Genotyping* <1129>, focuses on the specific modifications of the techniques that are necessary to enable detection of single base differences and common genetic variations, e.g., single nucleotide polymorphisms (SNPs). The final general information chapter in the series, *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* <1130>, describes residual DNA testing in the context of pharmaceutical manufacturing. Applications relevant to viral adventitious agents, however, are discussed in the general information chapter *Virology Test Methods* <1237>.

Two major uses of nucleic acid testing are excluded from this family of NAT chapters: viral testing for blood and blood product safety and genetic testing. The traditional perspective of USP is to develop public standards that can be applied to a particular final product without expressively defining a product and/or its production details. This chapter aims to specify when traditional methodologies or ex-

isting standards can be adapted. Novel methodologies for amplification and detection by NAT are also highlighted. As these new methodologies become mature and properly validated, they will be included in subsequent revisions.

Due to rapid development in the field, compendial and regulatory affairs scientists are advised to consult the current edition of *USP* and its *Supplements* regularly.

APPENDIX: REGULATIONS AND STANDARDS

Nucleic acid-based techniques have rapidly transformed almost every field of research, pharmaceutical development, and diagnostics. The need for globally comparable, accurate, and reliable results in the diagnostic field has driven the development of national and international standards as well as fostered a highly developed regulatory environment. Because NAT has become the most widely used of nucleic acid techniques, the majority of guidance documents and standards are related to NAT.¹ Virus-specific regulations and reference standards will be addressed in the Appendix to General Information chapter *Virology Test Methods* (1237). The following is a selective list of national guidance documents. For application-specific guidance the compendial user is referred back to the relevant regulatory agency for the most current guidance.

- FDA Center for Biologics Evaluation (CBER) "Review Criteria for Nucleic Acid Amplification-Based In Vitro Diagnostic Devices for Direct Detection of Infectious Microorganisms" (1993)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: Content and Format of Chemistry, Manufacturing and Controls Information and Establishment Description Information for a Biological In Vitro Diagnostic Product" (1999)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: In the Manufacture and Clinical Evaluation of In Vitro Tests to Detect Nucleic Acid Sequences of Human Immunodeficiency Viruses Types 1 and 2" (1999)
- FDA Center for Biologics Evaluation (CBER) "Guidance for Industry: Use of Nucleic Acid Tests on Pooled and Individual Samples from Donations of Whole Blood and Blood Components (including Source Plasma and Source Leukocytes) to Adequately and Appropriately Reduce the Risk of Transmission of HIV-1 and HCV" (2004)

GLOSSARY

3'-5' EXONUCLEASE ACTIVITY—Enzymatic activity to remove a mispaired nucleotide from the 3' end of the growing strand. The reaction is a hydrolysis of a phosphoester bond. The presence of a 3'-5' exonuclease, or proofreading, activity improves the fidelity of the polymerization.

5'-3' EXONUCLEASE ACTIVITY—Enzymatic activity to remove a mispaired nucleotide from the 5' end of a polynucleotide strand. This activity is actually that of a single-strand-dependent endonuclease and is needed to remove RNA primers of Okazaki fragments, the RNA strand in the intermediate DNA-RNA heteroduplex during reverse transcription, and during DNA repair.

ABSORBANCE [Symbol: A]—The logarithm, to the base 10, of the reciprocal of the transmittance (T). [NOTE—Descriptive terms used formerly include optical density, absorbancy, and extinction.]

ACCURACY—The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value.

¹ Reference materials for nucleic acid-based techniques are available from National Institute of Standards and Technology (NIST), <http://ts.nist.gov/measurementservices/referencematerials/index.cfm>.

ALLELE—One of two or more alternative forms of a gene at a given position (locus) on a chromosome, caused by a difference in the sequence of DNA.

AMPLICON—A short segment of DNA generated by the PCR process whose sequence is defined by forward and reverse primers. Sometimes referred to as an amplicon.

AMPLIFICATION—The enzymatic in vitro replication of a target nucleic acid.

ANNEALING—Hybridizing or binding of complementary nucleic acids, usually at an optimal temperature.

CONCATENATION—The process in which a DNA segment composed of repeated sequences is linked end-to-end.

COMPLEMENTARY DNA (CDNA)—DNA synthesized from an RNA template in an enzymatic reaction catalyzed by the enzyme reverse transcriptase.

DENATURATION—The process of separating double-stranded DNA into single strands by breaking the hydrogen bonds. This is typically accomplished by heating the DNA solution to temperatures greater than 90° or by treating it with strong alkali.

DEOXYRIBONUCLEIC ACID (DNA)—The genetic material that is passed from parent to daughter cells and propagates the characteristics of the species in the form of genes it contains and the proteins for which it codes. DNA contains the following four deoxyribonucleosides: dA, dC, dT, and dG.

DEOXYRIBONUCLEOTIDE TRIPHOSPHATE (dNTP)—A base that is added to a primer during the PCR that comprises the newly synthesized strand. Examples of dNTPs are dATP, dUTP, dCTP, dGTP, and dTTP.

DETECTION LIMIT—It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions.

DNA POLYMERASE—An enzyme that can synthesize new complementary DNA strands using a DNA template and primer. Several of these enzymes are commercially available, including *Taq* DNA polymerase and *rTth* DNA polymerase.

ENDONUCLEASE—An enzyme that cleaves phosphodiester bonds in a polynucleotide chain.

ENERGY TRANSFER—This describes the process in which an excited state of one molecular entity (the donor) is deactivated to a lower-lying state by transferring energy to a second molecular entity (the acceptor), which is thereby raised to a higher energy state.

EXTENSION—Refers to the elongation of the DNA chain that is being synthesized using the parent DNA strand as the template for synthesis of that daughter strand. This is a natural process that occurs during DNA replication. Extension occurs during the PCR process with DNA polymerases.

EXTINCTION COEFFICIENT [Symbol: ϵ]—The quotient of the absorbance (A) divided by the product of the concentration, expressed in moles/L, of the substance and the absorption path length, in cm. [NOTE—Terms formerly used include molar absorptivity index; molar absorptivity; and molar absorption coefficient.]

FIDELITY—Fidelity is a measure of the accuracy of nucleic acid replication. The polymerase enzyme used is only one of the elements that influences fidelity. Other elements include buffer conditions, thermal cycling parameters, number of cycles, efficiency of amplification, and the sequence of the DNA being copied.

FLUOROPHORE—A functional group in a molecule that makes the molecule fluorescent by absorbing energy of a specific wavelength and re-emits the energy at another wavelength.

FLUORESCENCE—The emission of one or more photons by a molecule or atom activated by the absorption of a quantum of electromagnetic radiation. X-rays, UV, visible light, and IR radiations may all stimulate fluorescence. For details on the spectroscopic measurement of fluorescence, see *Spectrophotometry and Light-Scattering* (851).

GENOME—The complete genetic complement or the complete set of instructions for reproducing an organism and carrying out its biological function in life. The DNA in our cells comprises our genome. When our cells divide, the complete genome in our cells is duplicated for transmission to each of the remaining daughter cells.

GENOTYPE—The genetic constitution of an organism as revealed by genetic or molecular analysis, i.e., the complete set of genes, both dominant and recessive, possessed by a particular cell or organism.

GENOTYPING—The process of assessing genetic variations present in an individual.

HAIRPIN—Antiparallel duplex structure that forms by pairing of inverted repeat sequences within a single-stranded nucleic acid. The helical section is called the stem and the unpaired base segment at the end of the structure is called the loop.

HOT-START PCR—Technique that is commonly used to improve the sensitivity and specificity of PCR amplification. A hot start is performed by withholding from the reaction mix a key component necessary for amplification until the reaction reaches a temperature above the optimal annealing temperature of the primers. The component withheld from the reaction mix can be primers, DNA polymerase, $MgCl_2$, or dNTPs.

HYBRIDIZATION—The process of forming a double-stranded nucleic acid molecule, for example between a nucleotide sequence (probe) and a target.

LIGATION—The process of joining two or more DNA fragments.

MELTING TEMPERATURE (T_m)—The temperature at which 50% of the DNA becomes single-stranded.

MICROARRAY—Sets of miniaturized chemical reaction areas that are used to test DNA fragments, antibodies, or proteins. Usually the probes are immobilized on a chip and hybridized with target.

MISMATCH—Unconventional base pairing (other than C with G, and A with T or U). A mismatched base pair has lower bonding energy and decreases the stability of the DNA molecule.

NUCLEIC ACID—Linear polymers of nucleotides, linked by 3', 5' phosphodiester linkages. In DNA, deoxyribonucleic acid, the sugar group is deoxyribose, and the bases consist of adenine, guanine, thymine, and cytosine. RNA, ribonucleic acid, has ribose as the sugar, and uracil replaces thymine.

OLIGONUCLEOTIDE—Linear sequence comprising as many as 25 nucleotides joined by phosphodiester bonds, generally used as a DNA synthesis primer.

PHOTOBLEACHING—Photobleaching is the irreversible destruction of a fluorophore in the excited state. Different fluorophores have different rates of photobleaching. For example, fluorescein photobleaches very easily. Often the rate of decomposition is proportional to the intensity of illumination. A simple and practical way to overcome this is to reduce the incident radiation.

POLYMERASE—An enzyme that catalyzes the synthesis of nucleic acids on pre-existing nucleic acid templates, assembling RNA from ribonucleotides or DNA from deoxyribonucleotides.

POLYMERASE CHAIN REACTION (PCR)—A laboratory technique that rapidly amplifies a specific region of double-stranded DNA, predetermined by the pair of primers used for amplification. Generally involves the use of a heat-stable DNA polymerase.

PRECISION—The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample.

PRIMER—Nucleic acid polymerases link a mononucleotide to a chain of nucleic acids, which is called the primer. RNA

polymerases are able to use a single nucleotide as a primer, but DNA polymerases always require an oligonucleotide.

PROBE—A specific DNA or RNA sequence that has been labeled by radioactive, fluorescent, or chemiluminescent tags and is used to detect complementary sequences by hybridization techniques such as blotting or colony hybridization. In addition, probes can also be used for quantitation of amplicons as described for quantitative PCR assays. A more detailed description of such probes is given in the general information chapter, *Nucleic Acid-Based Techniques—Amplification* (1127).

PROCESSIVITY—The ability of an enzyme to repetitively continue its catalytic function without dissociating from its substrate.

PROOFREADING ACTIVITY—Literally to read for the purpose of detecting errors for later correction. DNA polymerase has a 3' to 5' exonuclease activity that is used during polymerization to remove recently added nucleotides that are incorrectly paired.

QUANTITATION LIMIT—It is the lowest amount of analyte in a sample that can be determined with an acceptable precision and accuracy under the stated experimental conditions.

QUENCHING—The process of extinguishing, removing, or diminishing a physical property such as heat or light. Fluorescence quenching can be either collisional or static.

REVERSE TRANSCRIPTASE—An enzyme that requires a DNA primer and catalyzes the synthesis of a DNA strand from an RNA template. An enzyme that can use RNA as a template to synthesize DNA.

REVERSE TRANSCRIPTION (RT)—The process of making cDNA using an RNA template.

REAL-TIME PCR—May often be referred to as Quantitative PCR or Real-Time Quantitative PCR but not RT-PCR and is a procedure for simultaneous DNA quantitation and amplification. The generation of amplicons monitored as they are generated by the use of a fluorescent reporter system and captured by sophisticated instrumentation.

REAL-TIME (RT-PCR)—The combination of real-time PCR and reverse transcription PCR.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)—A variation of the PCR technique in which cDNA is made from RNA via reverse transcription. The cDNA is then amplified using standard PCR protocols.

RIBONUCLEIC ACID (RNA)—A type of nucleic acid composed of a specific sequence of ribonucleotides linked together. RNA contains the following four ribonucleosides: A, C, G, and U.

ROBUSTNESS—The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage.

rTth DNA POLYMERASE—Recombinant thermostable DNA polymerase originally isolated from the bacterium *Thermus thermophilus*. rTth has optimal activity at 70°–80° and survives the denaturation steps of PCR. In addition to DNA polymerase activity, it has efficient reverse transcriptase activity in the presence of manganese.

SPECIFICITY—The ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

TAQ DNA—Thermostable DNA polymerase that is originally isolated from the bacterium *Thermus aquaticus*, Taq has optimal activity at 70°–80° and is not degraded during the high-heat denaturation steps of PCR.

TEMPLATE—A master copy used to start the DNA or RNA replication process.

TRANSCRIPTION—The synthesis of RNA using a DNA template.

ABBREVIATIONS

AABB	American Association of Blood Banks
ACD	acid citrate dextrose
ASO	allele-specific oligonucleotides
bDNA	branched DNA assay
BMA	bone marrow aspirate
CE-LIF	capillary electrophoresis and laser-induced fluorescence
CCD	charge-coupled device
cDNA	complementary DNA
CPR	cyclic probe reaction
CsCl	cesium chloride
Ct	cycle threshold
DEPC	diethylpyrocarbonate
DHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DMSO	dimethyl sulfoxide
dNTP	dinucleotide triphosphate
DOP-PCR	degenerated oligonucleotide primed PCR
dsDNA	double-stranded DNA
ssDNA	single-stranded DNA
dUTP	2'-deoxyuridine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
ESI	electrospray ionization
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FEN	flap endonuclease
FISH	fluorescent in situ hybridization
FFPE	formalin-fixed paraffin embedded
FRET	fluorescence resonance energy transfer
GLP	good laboratory practice
HCV	hepatitis C virus
HIV	human immunodeficiency virus
ICH	International Conference on Harmonization
LAPS	light-addressable potentiometric sensor
LCR	ligase chain reaction
LED	light-emitting diode
LNA	locked nucleic acid
MALDI	matrix-assisted laser desorption-ionization
MDA	multiple-displacement amplification
MOPS	3-[N-morpholino]propanesulfonic acid
MS	mass spectrometry
mRNA	messenger RNA
NAT	nucleic acid amplification technologies
NASBA	nucleic acid sequence-based amplification
NTP	nucleotide triphosphate
OLA	oligonucleotide ligation assay
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEP	primer-extension-preamplification
PPi	pyrophosphate
QA	quality assurance
QC	quality control
RCA	rolling circle amplification
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcriptase

RT-PCR	reverse transcription-polymerase chain reaction
rTth	recombinant <i>Thermus thermophilus</i>
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
3SR	self-sustained sequence replication
SSCP	single-strand conformation polymorphism
STR	short tandem repeat
Taq	<i>Thermus aquaticus</i>
Tm	melting temperature; the temperature at which 50% of the double-stranded nucleic acid molecule becomes single-stranded
TMA	transcription-mediated amplification
TOF	time-of-flight
UNG	uracil-N-glycosylase
WGA	whole-genome amplification

(1126) NUCLEIC ACID-BASED TECHNIQUES—EXTRACTION, DETECTION, AND SEQUENCING

NUCLEIC ACID EXTRACTION

Introduction

The basic principles of nucleic acid amplification technology (NAT) and definitions of the various techniques are covered in *Nucleic Acid-Based Techniques—General* (1125). The current chapter covers general steps in the extraction and purification of nucleic acids from a variety of samples.

The expanding discipline of molecular biology in pharmaceutical and biomedical research and development is characterized by the rapid discovery of new markers for disease and technologies for their detection. Nucleic acid targets are isolated from a wide variety of specimens, and the quality and quantity of the extracted target are highly affected by specimen collection, handling, and choice of extraction procedure.

The analysis of complex organisms by molecular biological techniques requires the isolation of pure, high molecular weight genomic DNA and intact full-length RNA. The application of these techniques then allows the detection, identification, and characterization of the associated organism or adventitious agent. Recently developed tests employing purified human DNA enable genetic testing for the presence, predisposition, or carrier status of inherited diseases such as cystic fibrosis, hereditary hemochromatosis, or Tay-Sachs disease, to name a few examples, or the analysis of single nucleotide polymorphisms (SNPs).

DNase and RNase are the major sources of nucleic acid instability. Although both enzymes are ubiquitous and are easily released during nucleic acid extraction, RNases are far more stable and harder to inactivate than are DNases because they generally do not require co-factors in order to function. Minute amounts of RNase are sufficient to destroy RNA, so great care should be taken to avoid inadvertently introducing these enzymes into the sample during or after the isolation procedure. If RNA is collected for the specific application of gene expression analysis, researchers should

keep in mind that the sample collection process itself can alter the resulting expression profile.

Because of the ubiquity of RNases, measurement of intracellular RNA targets has lagged behind that of DNA targets in contributing to patient management and characterization of targets for pharmaceutical purposes. However, RNA represents the current status of the organism and is an important tool for correlating a phenotype with its associated genetic activity. The unstable nature of RNA has made standardization of NAT tests difficult, and false negative results can easily arise from a poorly handled sample because of target degradation rather than from the absence of disease or regulation of gene activity. Nevertheless, commercially available isolation and detection systems provide a high level of standardization and robustness, resulting in the implementation of RNA-based assays in recent years. The following sections discuss general steps in the extraction and purification of nucleic acids from a variety of samples, focusing on (1) collection, handling and storage of samples; (2) disruption of samples; (3) subsequent extraction and purification of nucleic acids; and (4) storage of purified nucleic acids.

Sample Source

The broad diversity of possible specimens requires different procedures for collection. For example, blood samples are collected in an appropriate anticoagulant- or additive-containing tube. Ethylenediaminetetraacetic acid (EDTA) and acid citrate dextrose (ACD) are the recommended anticoagulants for tests that require plasma or bone marrow aspirate (BMA) samples. When extraction from tissues is appropriate, the optimal amount of tissue is usually 1 to 2 g, depending on the type of tissue, because the amount of DNA and RNA per weight of tissue varies greatly from tissue to tissue. In general, more than 10 mg of tissue is required to obtain >10 µg of DNA or RNA. Because of the highly variable amounts and types of proteins and other contaminants present in different tissues, nucleic acid isolation protocols are tissue-specific, and a broad range of ready-to-use isolation systems are available from different manufacturers of kits for nucleic acid extraction. The tissue type also influences the stability of both DNA and RNA in specimens, and the two types of nucleic acid differ importantly with respect to sample preparation and downstream analysis. These issues are described later in the chapter.

Pre-Analytical Steps and Sample Collection

Although the genetic makeup of the organism remains mostly unchanged over time, the mRNA population represents the current status of a cell under any given set of conditions, and thus is highly dynamic. To prevent degradation of mRNA and/or to preserve the original transcription pattern of the cellular mRNA, tissue should be placed immediately on ice or snap-frozen in liquid nitrogen. However, freezing disrupts the cellular structure and releases RNases. Hence, for RNA isolation in general (mRNA, ribosomal RNA, viral RNA, etc.), thawing in an RNase-inactivating buffer is essential. A more convenient procedure employs a stabilizing agent at ambient temperature. Several reagents for different types of sample material (e.g., tissue or bacteria) are commercially available. Vanadium salts were once used to inhibit RNase activity, but they have been superseded by the use of chaotropic agents for the inhibition of RNase and stabilization of RNA. The sample can easily be collected in such reagents and stored for several days to weeks prior to RNA isolation.

For reliable gene-expression analysis, the immediate stabilization of the RNA expression pattern and of the RNA itself is an absolute prerequisite. Directly after the biological sample is harvested or extracted, changes in the gene-expression pattern occur because of specific and nonspecific RNA degradation as well as transcriptional induction. Such

changes in the gene-expression pattern should be avoided for all reliable quantitative gene-expression analyses, such as biochip and array analyses and quantitative reverse transcription-polymerase chain reaction (RT-PCR).

The use of gloves while handling reagents and RNA samples is mandatory to prevent RNase contamination arising from contact with the surface of the skin or from laboratory equipment. In order to create and maintain an RNase-free environment, laboratory personnel should treat water or buffer solutions with diethylpyrocarbonate (DEPC), which inactivates RNases by covalent chemical modification. Care should be taken because DEPC is irritating to the eyes, skin, and mucous membranes and is also a suspected carcinogen. Alternatively, commercially available RNase-free solutions and reagents may be used. Commercially available RNase inhibitor proteins are also available for use in reactions but with different levels of effectiveness with respect to various RNase types. However, it should be noted that DEPC cannot be used with Tris-buffered solutions. Many scientists recommend the use of disposable vessels when working with RNA. Nondisposable glassware should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240° for 4 or more hours before use (autoclaving alone will not fully inactivate many RNases). Alternatively, glassware can also be treated with DEPC. Nondisposable plasticware should be thoroughly rinsed with 0.1 M sodium hydroxide and 1 mM EDTA, followed by RNase-free water. Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases. The use of aerosol-resistant filter tips is also important for avoiding RNase contamination. These issues are not critical for DNA, and following the rules of Good Laboratory Practice (GLP) is generally sufficient for successful isolation of DNA.

As a general precaution, staff should follow all applicable safety precautions when handling tissue or body fluids (human or other). Some of these precautions (e.g., the use of disposable gloves) also prevent contamination of the sample. Applicable guidelines and standards for the collection and processing of human-derived materials have been published by the American Association of Blood Banks, the International Conference on Harmonization, and the FDA.

Sample Disruption and Homogenization

Prior to extraction, source material is disrupted and homogenized. Disruption is the complete breakage of cell walls and plasma membranes of solid tissues and cells in order to release all DNA and RNA contained in the specimen. This is usually done using a lysis buffer that also inactivates endogenous nucleases. In addition to disrupting tissues, homogenization shears high molecular weight DNA and cellular components. During RNA isolation, scientists often must reduce the viscosity of cell lysates (caused by the presence of high molecular weight DNA molecules) prior to final isolation in order to make the subsequent extraction steps easier and more efficient. Incomplete homogenization may interfere with subsequent RNA purification steps (e.g., inefficient binding of RNA to silica membranes) and therefore result in significantly reduced yields. A typical procedure to shear high molecular weight DNA and homogenize the sample is to repeatedly pass the lysate through a small-gauge needle. However, this procedure is time-consuming and is not suitable for high throughput of samples. Better procedures to achieve complete disruption and homogenization of cells and tissue include rapid agitation in the presence of beads and lysis buffer (bead milling) or rotor-stator homogenization.

During the bead milling process, disruption and simultaneous homogenization occur by the shearing and crushing action of the beads as they collide with the cells. Disruption efficiency is influenced by the size and composition of the beads, the speed and configuration of the agitator, the ratio of buffer to beads, the disintegration time, and the amount of starting material. These parameters must be determined

empirically for each application. For disruption with mortar and pestle, the samples should be frozen in liquid nitrogen and ground to a fine powder under liquid nitrogen. Standard safety precautions and the use of safety clothing to protect the skin and eyes should be employed when working with liquid nitrogen. Rotor-stator homogenizers are able to disrupt and homogenize animal and plant tissues within 5 to 90 seconds, depending on the sample. The rotor turns at very high speed, causing the sample to be disrupted by a combination of turbulence and mechanical shearing. Other alternatives are commercial spin-column homogenizers in combination with silica-membrane technology, which provide a fast and efficient way to homogenize cell and tissue lysates without cross-contamination of samples.

In order to achieve complete disruption, different sample types require different procedures. Cells from tissue culture grown as a monolayer or in suspension are easily disrupted by the addition of a lysis buffer that typically contains a mixture of an anionic detergent, a protease, and a chaotropic agent in a buffered salt solution. In contrast, nucleic acid isolation from fibrous tissues such as skeletal muscle, heart, and aorta can be difficult to disrupt because of the abundance of contractile proteins, connective tissue, and collagen. Fresh or frozen tissue samples should be cut into small pieces to aid lysis. Blood samples, including those treated to remove erythrocytes, can be efficiently lysed using a lysis buffer and a proteinase.

In general, the same procedures are applicable for extraction of DNA and RNA. For DNA isolation more gentle procedures are preferable, but during RNA isolation, cells and tissues can be disrupted using a mixer mill because there is no risk of shearing the RNA. Certain downstream applications require high molecular weight DNA, and care should be taken not to shear the DNA molecules and thus render the DNA unsuitable for further analysis.

Extraction and Purification

Although several procedures are available for nucleic acid extraction, the suitability of a procedure depends on the starting material, the type and purity of nucleic acid isolated, and possibly the downstream application. The principal procedures are described below; several commercial kits are available to accommodate different sample types and applications.

Phase Extraction—The original technique for extraction of DNA and RNA from lysed samples is phase extraction, which involves nucleic acid extraction using a mixture of phenol and chloroform. Depending on pH and salt concentration, either DNA or RNA partitions in the aqueous phase. At neutral/basic pH, the DNA remains in the aqueous phase, and RNA remains in the organic phase or in the interphase (with the proteins). However, at acidic pH, DNA in the sample is protonated, neutralizing the charge and causing it to partition into the organic phase. RNA, which remains charged, partitions in the aqueous phase. The two phases are separated by centrifugation, and the aqueous phase is re-extracted with a mixture of phenol and chloroform, followed by extraction with chloroform to remove any residual phenol. The nucleic acid is recovered from the aqueous phase by precipitation with alcohol. For RNA, this procedure is often combined with a protease digestion, alcohol or lithium chloride precipitation, and/or cesium chloride (CsCl) density gradients. A potential problem is contamination of the recovered DNA or RNA with organic solvents that may interfere with enzymatic downstream applications or spectrometry readouts.

Cesium Chloride Density Gradient Centrifugation—For the isolation of high molecular weight genomic DNA, CsCl density gradient centrifugation is the traditional procedure. Cells are lysed using a detergent, and the DNA is isolated from the lysate by alcohol precipitation. The DNA is then mixed with CsCl and ethidium bromide and centrifuged for several hours at a high g force (typically $100,000 \times g$). The

DNA band, which can be visualized under UV light as a result of the intercalation of the ethidium bromide with the DNA, is collected from the centrifuge tube, extracted with isopropanol to remove the ethidium bromide, and then precipitated with ethanol to recover the DNA. This procedure allows the isolation of high-quality DNA, but it is time consuming and also a safety concern because of the high quantity of EtBr involved.

Anion-Exchange Chromatography—An alternative procedure for the purification of high molecular weight genomic DNA is anion-exchange chromatography based on the interaction between the negatively charged phosphate groups of the nucleic acid and positively charged surface molecules on the anion-exchange resin. Binding occurs under low-salt conditions, and impurities such as RNA, cellular proteins, and metabolites are washed away using medium-salt buffers. Pure DNA is eluted with a high-salt buffer and is desalted and concentrated by alcohol precipitation. This procedure yields DNA of a purity and biological activity equivalent to two rounds of purification in CsCl gradients, but in much less time. The procedure also avoids the use of toxic substances, and it can be adapted for different scales of purification. DNA up to 150 kilobases (kb) in length may be isolated using this procedure. Several kits are available for the isolation of DNA based on anion-exchange technology, and procedures vary in processing times and the quality and size of the isolated DNA.

Silica Technology—The current procedure of choice for most applications is based on silica technology and can be used for isolation of full-length RNA or DNA with an average size of 20 to 50 kb. However, higher molecular weight DNA exceeding 100 kb is not efficiently extracted by this technology. The procedure relies on the selective adsorption of nucleic acids to silica in the presence of high concentrations of chaotropic salts. Although both types of nucleic acid adsorb to silica, the use of specific buffers in the lysis procedure ensures that only the desired nucleic acid is adsorbed while other nucleic acids, cellular proteins, and metabolites remain in solution. The contaminants are washed away, and high-quality RNA or DNA is eluted from the silica using a low-salt buffer. The silica matrix can be used as particles in suspension, in the form of magnetic beads, or as a membrane. This technique is suitable for high throughput, and several kits and automated systems are commercially available. However, these aqueous lysis buffers (in contrast to lysis buffers based on an organic solvent such as phenol) are not ideally suited for difficult-to-lyse samples (e.g., fatty tissues). Kits designed to facilitate lysis of fatty tissues and to inhibit RNases are available. Silica-based kits provide a fast and reliable procedure for both DNA and RNA purification and are commonly used for nucleic acid extraction.

Although these procedures yield pure nucleic acids, for some applications in which even trace contaminations with either RNA or DNA may interfere, pretreatment with DNase or RNase may be necessary. Alternatively, procedures that use specific probe capture may be used. Relevant applications requiring such ultra-pure nucleic acids are discussed in *Nucleic Acid-Based Techniques—Amplification* (1127).

Specific Applications for Hard-to-Extract Materials

Extraction from Formalin-Fixed and Paraffin-Embedded Biopsies—The nucleic acids in formalin-fixed paraffin embedded (FFPE) biopsies are usually heavily fragmented and chemically modified by formaldehyde. Although formaldehyde modification cannot be detected in standard quality control assays such as gel electrophoresis, formaldehyde modification does interfere with enzymatic analyses. Sufficient extraction and demodification for DNA can be achieved by prolonged digestion with protease, but this will lead to heavy fragmentation and degradation of RNA. Some isolation systems have been optimized to re-

verse as much formaldehyde modification as possible without further RNA degradation. Nevertheless, RNA purified from FFPE samples should not be used in downstream applications that require full-length RNA. Some applications may require modifications to allow the use of fragmented RNA (e.g., designing small amplicons for RT-PCR).

Extraction from Bacteria and Pathogens—Although Gram-negative bacteria are relatively easy to lyse, Gram-positive bacteria or yeasts typically need an enzymatic pretreatment to remove the cell wall for efficient lysis. This methodology can be applied only to DNA isolation because the enzymatic treatment will influence the expression profile of the organism, and therefore RNA isolation requires a more rapid lysis procedure. Another factor to consider is that microorganisms normally occur against the background of a host or an environmental matrix (e.g., soil), which makes detection by polymerase chain reaction (PCR) often difficult because of inhibitory components. This means that the isolation procedure has to be carefully adapted and optimized for the specific organism and sample type. Commercial kits are available, and most are based on the use of lysozyme for the removal of cell walls.

Special Considerations for Limited Sample Amounts—Multiple genetic testing techniques, including SNP analysis, short tandem repeat analysis, sequencing or genotyping using arrays, real-time PCR, and other procedures depend on the availability of high-quality DNA. Because human genomic DNA or samples of individual genotypes are often limited, a process to immortalize nucleic acid samples can overcome this limitation. Procedures applicable to genotyping are discussed in *Nucleic Acid-Based Techniques—Genotyping* (1129). Whole-genome amplification (WGA) has recently been employed to amplify limited genomic DNA from already purified DNA or directly from clinical or casework samples without any DNA purification. Two basic technologies for WGA are available and are PCR-based or rely on isothermal multiple-displacement amplification. These applications are described in more detail in *Nucleic Acid-Based Techniques—Amplification* (1127).

Sample Handling and Long-Term Storage

DNA is a relatively stable macromolecule, and once isolated it can be kept at 2° to 8° for at least 1 year. However, where DNA is present in very small quantities, such as in a test of residual DNA, it may be advisable to store the DNA at less than or equal to -20°. Generally, DNA is stored in solution. Distilled water can be used if DNA will be used for PCR and/or endonuclease digestion within a few days after its isolation. However, Tris-EDTA at pH 7.5–8.5 is the preferred buffer for DNA storage because DNA degradation can occur in water because of the limited buffering capacity of this medium. Purified nucleic acids retain recognizable characteristics during long-term storage, provided the samples are stored as frozen solutions. The DNA solution should be stored as a primary stock solution frozen at -80°. DNA can also be lyophilized and stored dry without the need for refrigeration. In some cases DNA can be stored for years on special filter papers that bind DNA and allow storage in a dried state at ambient temperature.

The ubiquity of RNases requires extra precautions when handling RNA. Isolated RNA should be kept on ice when aliquots are pipetted. Filter tips that prevent RNase carry-over from the pipette and sterile, disposable polypropylene tubes are recommended throughout the procedure because these tubes are generally RNase-free and do not require any pretreatment to inactivate RNases. Purified RNA can be stored at -20° or -80° in water. Under these conditions no degradation is normally detectable. Unlike DNA, RNA does not benefit from basic buffer solutions during long-term storage because of its sensitivity to alkaline conditions. Generally, if nucleic acid samples are required for multiple testing, RNA and DNA samples should be frozen in multiple aliquots at -80° for subsequent analysis in order to avoid

repeated freeze-thaw cycles that can lead to degradation, and also to minimize the possibility of contamination, which could result in analytical inaccuracy.

QUALITATIVE AND QUANTITATIVE EVALUATION OF NUCLEIC ACIDS

Introduction

This section describes procedures that assess the purity, integrity, and quantity of purified nucleic acids, including spectroscopic procedures, electrophoresis of nucleic acid fragments, and probe-based techniques. Detection and quantitation by amplification are discussed in *Nucleic Acid-Based Techniques—Amplification* (1127).

ABSORBANCE SPECTROSCOPY

The basic principles of spectroscopy are addressed in *Spectrophotometry and Light-Scattering* (851). For nucleic acids, absorbance is determined at 260 nm, but this procedure does not distinguish between DNA and RNA. Absorbance can also be used to estimate protein contamination in nucleic acids. Proteins maximally absorb at 280 nm, and nucleic acids maximally absorb at 260 nm. Thus the calculation of the A260/A280 ratio is used as an estimation of protein contamination in nucleic acid preparations. A ratio of 1.8 to 2.0 is considered desirable. As an example, double-stranded DNA has an extinction coefficient of 20 for 1 mg per mL of DNA at 260 nm and a coefficient of 10 at 280 nm. In contrast, for 1 mg per mL of protein, the extinction coefficients are on the order of 1 at 280 nm (depending on tyrosine and tryptophan content) and 0.57 at 260 nm. Thus a large protein contamination could exist at a 260/280 ratio of greater than 1.8 because of the lower sensitivity of protein absorbance. In addition, the change of absorbance of DNA with wavelength ($\Delta A/\Delta \lambda$) is steep at 280 nm, and this could lead to an incorrect determination if the spectrophotometer is out of calibration. The peak at 260 nm is broad, and thus readings are less sensitive to calibration issues.

Information on contamination by nonproteinaceous materials can be provided by a scan of DNA from 220 nm to 320 nm. Pure DNA has a mostly symmetric peak around 260 nm, zero absorbance at 320 nm, and a minimum at 230 nm. Absorbance rises again from 230 nm to 220 nm. Interfering substances can co-purify with DNA and absorb in the lower UV range (around 230 nm). These substances can interfere with and lead to an overestimation of DNA content, thus showing the utility of a scan—or at least a measurement of absorbance—at 230 nm in addition to 260 nm and 280 nm. Absorbance above 300 nm can arise from other contaminants and particulate matter. Common reagents used in the isolation of DNA, particularly solvents such as phenol and alcohols if they are not completely removed, can interfere with DNA absorbance measurements. Analysts should be aware of the limitations of this type of measurement. Finally, the absorbance of DNA and the 260/280 ratio is dependent on ionic strength—a difference as large as 30% can exist. Absorbance of genomic DNA is higher, and the 260/280 ratio is lower in pure water when compared with the same DNA in a buffer or a salt solution.

For the purposes of quantitation of nucleic acids, the respective extinction coefficients for DNA and RNA are used. An absorbance of 1 in a 1-cm cuvette corresponds to 50 μg per mL of double-stranded DNA [E (specific absorption coefficient) = 0.02 (μg per mL) $^{-1}$ cm $^{-1}$]. The specific absorption coefficient for RNA at 260 nm is $E = 0.025$ (μg per mL) $^{-1}$ cm $^{-1}$ (absorbance of 1.0 corresponds to 40 μg per mL), and for single-stranded DNA $E = 0.027$ (absorbance of 1.0 corresponds to 37 μg per mL). A solution of DNA is read against a blank of the same buffer solution in which the DNA is

dissolved. Ideally, readings should fall within a range of 0.1 to 1.0 absorbance for adequate linearity. Absorbance above 1.0 becomes increasingly nonlinear as the absorbance rises. The accuracy of readings below 0.1 (5 µg per mL DNA) depends on the quality and noise level of the spectrophotometer.

Fluorescence Protocols for DNA and RNA Quantitation

Cyanine dye derivatives are used for the quantitation of nucleic acids because they specifically interact with nucleic acids (DNA, RNA, and oligonucleotides) and fluoresce only upon binding. The exact mechanism of interaction is not always fully understood but may involve intercalation in double-stranded DNA and surface binding.

Measurements can be performed using a fluorometer or a plate reader. The sensitivity of fluorescence with these dyes is much higher than that of absorbance, which gives these dyes great utility when DNA concentration is low (down to 25 pg per mL). The dye must be protected from light to avoid photobleaching. Linearity is maintained over three to four orders of magnitude. Calf thymus and Lambda phage DNA are often used as calibrants to construct a standard curve. Some of these dyes have been optimized to bind double-stranded DNA or single-stranded RNA and oligonucleotides. A DNA-binding dye will also bind to single-stranded DNA and RNA but at low ionic strength, and the signal is about 10% or less than that seen with double-stranded DNA for an equivalent mass of material. Thus, this methodology is preferred for DNA measurements when no effort has been made to remove RNA from the preparation. Another fluorescent dye is available and is optimized for RNA measurements. Using two different concentrations of this dye, analysts can detect RNA in amounts as low as 1 ng per mL and as high as 1 µg per mL. The dye also fluoresces with DNA but does not display an equivalent ability to minimize binding by the use of particular conditions (e.g., with DNA and the double-strand binding dye). Quantitation may be affected by contaminating nucleic acid (e.g., DNA in an RNA preparation and vice versa). Treatment with a DNase is needed if DNA is present in the RNA preparation. Proteins are unlikely to interfere with these dyes, but some detergents as well as phenol result in loss of fluorescence. Nucleic acid extraction reagents should thus be checked for effect on subsequent fluorescent assays.

Bisbenzimidide fluorochrome dyes such as (2'-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1*H*-benzimidazole) represent another option for measuring DNA. Researchers have studied the binding of these dyes to the minor groove of DNA and have found that sequences of adenine or thymine in the DNA sequence provide a minor groove dimension that binds the dyes best. Thus the fluorescent signal can show DNA sequence dependence, and the calibrant DNA should have a nucleotide composition that is similar to that of the DNA to be measured. These dyes are not as sensitive as cyanine dyes but are more sensitive than absorbance measurements. Low dye concentrations and high ionic strength are required in order for analysts to distinguish double-stranded DNA from RNA. Low ionic strength conditions are required in order to differentiate double-stranded DNA from single-stranded DNA.

Detection by Size

Agarose Gel Electrophoresis—Agarose gel electrophoresis provides a simple and accurate procedure for separating nucleic acids by fragment size. The technique can be adapted to separate fragments over a large range of sizes and can be used in a preparative or analytical fashion. For example, gel electrophoresis can be used to verify that a product of a PCR reaction is of the correct size. DNA fragments can be retrieved from a gel slice and provide a suffi-

ciently pure PCR product for cloning or sequencing. The general integrity of an RNA preparation can be determined by gel electrophoresis as well. The stoichiometry of the nucleic acid fragment size (in base pairs) and negative charge from the phosphate provide the basis for the separation. With the exception of plasmids, electrophoresis is generally free of DNA conformation-induced effects. Supercoiled plasmid DNA will migrate ahead of linear or open-circle/nicked plasmid, which is useful for determining the conformation of a plasmid preparation. In contrast, denaturing gels are used for RNA because of RNA's tendency to form inter- and intramolecular secondary structures.

Agarose gel electrophoresis utilizes a horizontal setup wherein the gel is cast in a box and placed on a bridge between two buffer compartments that are filled with the buffer of choice. The gel is also covered with a thin layer (~1 mm) of buffer. Although the main electrical resistance resides in the gel itself, there is sufficient charge on the nucleic acids to move fragments through the gel toward the anode. The fragments move in proportion to size, the smallest moving the fastest. The parameters that most affect electrophoresis are gel pore size, buffer concentration, and the voltage gradient. The ability to separate the fragments of choice is largely a function of the gel pore size, which depends on agarose concentration. Generally the agarose concentration is in the range of 0.5% to 1.0% for DNA fragments of <100 to 25,000 base pairs, and the higher concentration is used when it is important to distinguish the smallest fragments. Lowering the agarose concentration in the gel results in the resolution of larger fragments but also in a loss of resolution of small fragments. For the largest fragments pulsed, (reversed)-field electrophoresis is utilized.

To achieve uniform electrophoresis, all of the agarose must be completely melted. Electrophoresis-grade agarose is dissolved in the same buffer that will be used for electrophoresis. The buffers most commonly used for DNA separations are TBE (tris-borate-EDTA) or TAE (tris-acetate-EDTA). TBE has a higher buffering capacity than TAE, but TAE should be used if the DNA is going to be retrieved from the gel. Denaturing RNA gels use MOPS buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0). Melting the agarose is conveniently achieved with the assistance of a microwave oven. The agarose will easily come to a boil, but this may not result in complete melting of the agarose, which may require bringing the solution to a boil several times, with intermittent mixing and holding periods, until the agarose is completely melted. Agarose particles transform from white to transparent before melting. Any partially melted agarose can be detected by swirling the flask while holding it up to the light. If the solution does not appear uniform, then it requires additional heating. The agarose is poured into the gel box after partial cooling but before setting up. Commercially available ready-to-use gels suitable for a particular application can also be used. For RNA-denaturing gels, formaldehyde is added under a fume hood to the melted agarose to a final concentration of 2.2 M or 6.7%. Before the agarose has hardened, the analyst places a comb in the gel to provide wells for the samples and size standards. Once solidified, the gel is placed in the electrophoresis box, and buffer is added until both sides are filled and there is a layer of buffer across the surface of the gel. Then 10X tracking buffer (40% sucrose with 0.25% bromphenol blue or 0.25% xylene cyanol or both) is added to each DNA sample to increase the sample density and to provide a tracking dye that is used to assess when the electrophoresis is finished. The increased density allows the sample to be transferred into the well and to remain there until it migrates into the gel during electrophoresis.

One or more lanes should be used for a DNA size standard containing fragments in the range that is relevant to the samples and agarose concentration. Size standards in various ranges are readily available. Bracketing the samples in wells between standards is useful to determine whether the electrophoresis gradient has been uniform over the width of the gel. However, in the case of eukaryotic RNA

preparations, the 18S and 28S ribosomal RNAs that are co-extracted from prominent bands (corresponding to 1900 and 4700 nucleotides) can also be used as size standards. In addition, the rRNA provides information on the RNA integrity because missing or fuzzy rRNA bands indicate problems with the quality of the RNA preparation. Once the wells are filled, the cover is placed over the gel box, and the box is connected to the power supply. The indicator dye in the tracking buffer added to the samples and size standard allows the easy determination of how far the electrophoresis has proceeded. Bromophenol blue will migrate with DNA fragments of <500 base pairs, and xylene cyanol will migrate with fragments of 5000 base pairs.

The power supply is frequently run under conditions of constant voltage (1 to 10 V per cm) of gel length. Elevated voltage can cause high current, resulting in the generation of damaging heat and exhaustion of the buffer.

Pulsed-Field Electrophoresis—This variation on agarose gel electrophoresis is used to separate a range of large DNA fragments and is most useful when resolution of 50,000 to 200,000 base-pair fragments is needed. The main difference is the addition of an alternating-field device that controls the power supply operating under constant voltage. Large fragments of DNA change conformation in order to move through the agarose pores, and the larger pieces take longer to readjust when the field is reversed and thus move more slowly than do smaller fragments. This allows resolution of fragments over the period of hours that the pulsed-field procedure operates. A commonly used ratio of forward to reverse is 3:1, and, in addition, the procedure typically calls for a stepwise increase in the unit time between reverses of the field. Electrophoresis may continue for 10 to 16 hours to avoid fluctuation in gel temperature, viscosity, and other properties that may cause artifacts.

Polyacrylamide Gel Electrophoresis (PAGE)—The format for performing PAGE is quite different from that for agarose gel electrophoresis. The general procedure for PAGE is described in *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* (1056). For resolution of small fragments of DNA in the 10 to 500 base-pair range, nondenaturing polyacrylamide gel electrophoresis is more suitable than agarose gel electrophoresis because separation of fragments of this size requires much smaller pore size than is achievable in agarose gels. The gel is prepared by polymerization of acrylamide monomers. The percentage of acrylamide dictates the range of fragment sizes that can be best resolved. For example, 20% acrylamide is suitable for the 10–100 base-pair range, and 5% acrylamide is useful in the 100–500 base-pair range. Commercially available ready-to-use polyacrylamide gels suitable for the particular size discrimination can also be used. The separated nucleic acids are visualized by staining with, for example, silver nitrate solution rather than with ethidium bromide or cyanine dye. However, staining with silver nitrate is laborious and time-consuming and not suitable for preparations that contain a large amount of protein, because proteins will also stain with silver nitrate.

Capillary Electrophoresis and Laser-Induced Fluorescence (CE-LIF)—CEF has been used for many years to separate DNA fragments (for the general principles of CE, see *Biotechnology-Derived Articles—Capillary Electrophoresis* (1053)). The procedure relies on a principle similar to that underlying agarose gel electrophoresis. CE can utilize the cross-linked buffer systems applied in gel electrophoresis, but the technique can also use polymer-containing solutions (e.g., polymethylcelluloses) that are designed to create pores that entangle proteins. These polymer solutions may be added to the capillary between injections, allowing a “fresh” gel prior to each run. In addition, capillaries can be used for more injections than are possible for polymerized gel-filled capillaries. The resolving power of the separation depends on the size of the pores, which is based on the composition of the gel. Kits are available to separate fragments into the desired size ranges. Fragment sizes outside

the resolution window can possibly be separated, but the separation may not be reliable or reproducible when the gel capability is exceeded.

Fragments can be detected by a variety of mechanisms. Detection utilizing UV absorbance is possible, but the preferred and most common detection procedure is laser-induced fluorescence (LIF). Fluorescence offers improvements over UV detection in terms of selectivity and sensitivity. In addition, the detection limits for fluorescence are two to three orders of magnitude better than those for UV. Although DNA is intrinsically fluorescent, the background fluorescence and complex laser spectroscopy required preclude routine use. The most common way to label DNA is described in the section above on fluorescent protocols for RNA and DNA quantitation. This system is widely employed because of its simplicity (the dyes are added to the sample or into the reaction buffer) and effectiveness. The advantages of CE include speed of analysis, sensitivity using minimum sample volumes, and the potential for automation. These are achieved mainly by the inherent miniaturization of the gel. Automated systems allow robust analysis of the quality, quantity, and fragment size of both RNA and DNA. CE applications have been especially important for evaluating the integrity of RNA because of the instability and progressive degradation of RNA caused by ubiquitous RNases, and new technologies that compare the ratios of 28S and 18S are improving the capabilities of these procedures.

FILTER HYBRIDIZATION AND IN VITRO LABELING OF PROBES

Introduction

Hybridization techniques were used early in molecular biology to identify individual nucleic acids and to estimate the degree of similarity between species. Hybridization is widely used in the procedures described in this and other chapters to visualize and identify nucleic acid sequences (see *Nucleic Acid-Based Techniques—Amplification* (1127), *Nucleic Acid-Based Techniques—Genotyping* (1129), and *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* (1130)). With the advent of restriction endonuclease digestion of DNA and electrophoretic separation by molecular mass, hybridization using labeled probes provided a way to visualize the organization of genes within a specific genome.

The hybridization techniques described are dot and slot blotting, Northern blotting, Southern blotting, in situ hybridization, and fluorescent in situ hybridization (FISH). All these techniques rely on the use of nucleic acid probes. Probes are oligonucleotides with specific DNA or RNA sequences that have been labeled with radioactive, fluorescent, chemiluminescent, chemical tags or enzymes (reporter molecules). Hybridized probes bind to complementary sequences on the target nucleic acids and are used to visualize and characterize targets, as described below.

Dot and Slot Blotting

Dot blotting is the simplest and quickest of the hybridization techniques. The nucleic acids are directly applied to a support membrane, which may be a nitrocellulose or nylon membrane, without prior separation of the nucleic acid species by agarose gel electrophoresis. The nucleic acids are spotted onto the filter using a micropipettor or an apparatus such as a dot blot or slot blot apparatus. This consists of a membrane frame with a membrane sandwiched in between the two pieces of the frame. The bottom frame plate is connected to a vacuum manifold, and the top piece of the frame has slots through which the nucleic acids are loaded. The samples are loaded under vacuum and pulled through the membrane by vacuum, with the nucleic acid binding to

the membrane, and then the filter is air-dried. The nucleic acids are fixed to the filter either by heating to 80° for nitrocellulose membranes or by exposure to UV light for a predetermined time for nylon filters. Hybridization with a labeled probe provides confirmation of the identity of the nucleic acid but does not provide any information about the number or sizes of the species. The nucleic acid species of interest can be quantitated by spotting known concentrations of the purified nucleic acid on the filter and comparing the signal generated by the unknown samples with those of the standard preparations.

Southern Blotting

Southern blotting refers to the transfer of DNA from an agarose or polyacrylamide gel to a nitrocellulose or nylon membrane. Small, single-stranded DNA probes can then be used to visualize and identify the DNA species of interest. Southern blot analysis is based on a transfer and immobilization methodology developed in 1975, coupled with the electrophoretic separation of fragmented DNA. More specifically, the procedure typically is used to identify specific nucleic acid sequences in the context of a defined genetic topography, such as a restriction endonuclease map. The position of genes within the viral genome can be accurately mapped using a variety of restriction endonucleases in combination with Southern blot analysis. The procedure requires that DNA be obtained in sufficient quantity for analysis. Fragmented DNA is separated according to size using agarose gel electrophoresis. Double-stranded DNA fragments must be denatured before they are transferred and immobilized on a membrane by capillary action. The immobilized DNA is then cross-linked to the filter, which may be composed of nitrocellulose or nylon, as described above. However, the use of positively charged nylon membranes eliminates the need to fix the DNA to the nylon membrane. Nitrocellulose membranes are more fragile and may be probed up to 3 times with separate probes. Nylon membranes are more robust and may be probed 10 to 12 times, but they may present more background noise, particularly when they are used with chromogenic probes.

Northern Blotting

Northern blot analysis comprises a series of steps for the separation, transfer, and immobilization of RNA in a manner similar to the treatment of DNA using Southern blot analysis. Denaturation of the RNA is required to reduce secondary structure to ensure that the RNA separates in the agarose uniformly according to length. Denaturation of RNA is accomplished either prior to electrophoresis using glyoxal or dimethyl sulfoxide (DMSO) or during electrophoresis by means of gels that contain formaldehyde. Transfer is achieved in a manner identical to that used for Southern blotting. However, in the case of Northern blotting, it is unnecessary to denature the RNA prior to transfer because denaturation is accomplished before electrophoretic separation of the RNA species. The immobilized RNA is cross-linked to the membrane in a manner similar to the cross-linking of DNA.

In Situ Hybridization and Fluorescent In Situ Hybridization (FISH)

Hybridization of a nucleic acid in situ classically refers to determining the location of that nucleic acid sequence in its natural state—in tissue, in individual cells, or on a chromosome. In situ hybridization probes are designed to bind to complementary nucleic acid sequences, whether they be DNA or RNA. The purpose of these hybridization procedures is to discover where in a tissue a gene is being expressed, in which case the target is RNA, or to map a specific DNA

sequence to its location on a chromosome, in which case the target is DNA.

Chromosome mapping of DNA sequences is accomplished by chemically attaching silver grains to the probe sequences and then counting the density of the grains in a metaphase chromosome spread. Although, historically, these procedures worked well, sensitivity was always an issue. The solution was to use a reporter that was more sensitive and safer than the other reporters, namely, fluorescence used in the technique of fluorescent in situ hybridization (FISH). FISH has an additional benefit in that the different colors available in fluorescence afford the ability to observe multiple hybridization events simultaneously, a feature not available with other detection systems.

Detection of DNA and RNA in Hybridization Assays Using Labeled Probes

Visualization and location of individual nucleic acid species of interest are achieved by the specific hybridization of DNA or RNA probes that are labeled for easy visualization. The filter or sample (fixed cells or tissues in the cases of in situ hybridization and FISH) is incubated with the labeled probe at an appropriate temperature and salt concentration that allows hybridization of desired stringency. This is followed by washing with buffers of varying detergent and salt concentrations and at varying temperatures in order to minimize background signal due to nonspecific hybridization. The labeling and types of probes are discussed below.

Probes can be RNA probes generated in vitro or DNA probes, either double-stranded fragments, plasmids, or single-stranded oligonucleotides containing moieties to facilitate detection of fragments that contain portions of the gene of interest. Probes can be labeled with radioactive tracers such as ^{32}P or ^{35}S by incorporation of a labeled nucleotide in the probe sequence or with a nonradioactive label such as biotin by incorporation of a modified base, such as adenine monophosphate linked to biotin. Radioactive probes are visualized with X-ray film placed over the blot. Biotin-labeled probes are detected with a conjugate of streptavidin-alkaline phosphatase. An enzymatic reaction is run with alkaline phosphatase and a substrate that yields an insoluble colored product at the site of the probe. Variations on nonradioactive probes utilize other modifications to the DNA and linked antibody-alkaline phosphatase, as well as chemiluminescent probes that are detected on film.

Nucleic acids can be synthesized and manipulated by either enzymatic or chemical means. These same systems can be used to modify nucleic acid structure and to introduce foreign moieties to create unique molecules that can provide an advantage to the detection of limiting viral nucleic acids against a background of host nucleic acids. The chemical synthesis of nucleic acids and their purification has become routine, and high-quality synthesis and purification are commonly achieved. Moreover, larger segments can be synthesized, and when even larger segments are required, the subsections can be designed for concatenation and ligation.

Custom synthesis of DNA oligonucleotides is readily achievable in the laboratory using commercially available reagents and equipment. Alternatively, probes can be custom ordered from numerous commercial providers. Size-exclusion procedures for purification generally are used to eliminate incomplete oligonucleotides. RNA oligonucleotides also may be chemically synthesized or generated in vitro using complementary cloned DNA fragments under the control of various prokaryotic RNA polymerase promoter sequences. The use of DNA probes is much more common, but there may be some applications in which the increased association of RNA-RNA or RNA-DNA hybrids is advantageous.

The principal procedures of labeling DNA are direct labeling using a kinase reaction to attach a labeled nucleotide to the end of each DNA strand, by incorporating labeled nucleotides into a nicked DNA by utilizing the DNA repair function of the Klenow fragment of *Escherichia coli* DNA

polymerase I enzyme (nick translation), and by PCR. This last procedure generates a relatively higher yield of internally labeled probe because each round of thermal cycling doubles the amount of labeled probe, whereas the former procedures result in a ratio of less than one probe molecule per template molecule. The PCR procedure also is used to generate unique probes with a variety of moieties located at the termini.

NUCLEIC ACID SEQUENCING

Introduction

The first DNA sequencing procedure, described in 1977, utilized chemical cleavage to specifically introduce chain breaks in a DNA sequence (Maxam and Gilbert sequencing). The procedure proved to be of significant utility in the early years of molecular biology, but it has not been used to perform high-volume sequencing and therefore is not discussed in detail here. The majority of sequencing performed today is based on the dideoxysequencing procedure, also described in 1977 (Sanger sequencing). This procedure fundamentally changed sequencing by exploiting the enzymatic specificity of polymerases that introduce strand interruptions at specific bases. This is the most widely recognized sequencing procedure and is considered a routine assay in molecular biology laboratories. Innovations in instrumentation, sample preparation and collection, data management, data analysis, and sequence assembly have relied on this sequencing procedure as their fundamental sequence generator.

High-throughput sequencing takes all the elements of the sequencing procedures and applies them to a mass collection of sequence data, typically for larger genomes, but high-throughput sequencing certainly may be used for smaller projects as well. Obtaining the final sequence information includes all processes associated with sample preparation, sequencing, data assembly, and data finishing. The technology to achieve these individual objectives includes the instrumentation, disposables, protocols, and procedures.

Sequencing Reaction

The dideoxysequencing procedure takes advantage of specificity of the Klenow enzyme to introduce chain-terminating nucleosides, called dideoxynucleotides, intermittently during the polymerase extension process. The sequencing of each sample requires four separate reactions (one for each base). The resulting mixture of various nucleotide chain lengths is then separated on the basis of individual molecular masses. The incorporation of radioactively labeled nucleotides during the sequencing reaction permits the detection of the nucleotide chains.

Improvements in biotechnology have led to the discovery of more robust enzymes with high fidelity, improved stability, and other attributes that have led to longer reads and improved sequence fidelity. These improvements have made possible the introduction of cycle sequencing, which is now commonly used. The principle of the cycle sequencing procedure is a combination of Sanger sequencing and aspects of PCR amplification, whereby dideoxynucleotides are incorporated into the amplified DNA. Cycle sequencing leads to a higher concentration of labeled fragments covering a wider range of sizes than does Sanger sequencing, leading in turn to a higher read length.

Separation Procedures for DNA Sequencing Fragments

The previous sections of this chapter deal with the treatment of intact DNA and RNA molecules; the following sections

address the challenges of separating the fragments that result from the sequencing reactions, notably slab gel sequencing and capillary electrophoresis. Subsequent sections address detection technologies and sequence integrity.

Slab Gel Sequencing

Polyacrylamide gel electrophoresis, frequently referred to as slab gel electrophoresis, was the first separation mechanism employed for the separation of DNA sequencing fragments. As described above, the electrophoretic separation of DNA fragments is driven by the size of the fragments in the reaction mixture. However, for slab gel sequencing the pore sizes are chosen so that single-base resolution for many hundreds of bases is possible. In addition to the polyacrylamide in the gel, a denaturant such as urea is frequently included to ensure denaturation of the fragments. Until the implementation of multicapillary sequencing systems, the separation power and throughput of slab gel separation mechanisms were often considered state of the art.

Capillary Electrophoresis Sequencing

As noted above, capillary electrophoresis offers significant advantages over gel-based separations. However, as with slab gel sequencing, the pore sizes are chosen so that single-base resolution for many hundreds of bases is possible. Multicapillary systems that utilize 8 to 384 capillaries are commercially available. These systems are the primary systems used for large-scale DNA sequencing, and, theoretically, they yield more than 1.1 billion base pairs of DNA sequences per year.

Detection

Radioactivity—The first detection strategies for DNA sequencing reactions utilized radioactive isotopes such as ^{32}P or ^{35}S , primarily because these were practical for gel separations. The advantages are that detection is universal, low limits of detection are possible, mobility shifts are eliminated, and fidelity differences for the DNA polymerases do not occur. Disadvantages include the high disposal and safety costs, the inability to multiplex (ultimately limiting throughput), and the need for 24 to 36 hours of exposure time (i.e., no real-time detection).

Fluorescence—Fluorescence dyes have largely replaced radioactive isotopes as detection tools during DNA sequencing, mainly because they do not have the disadvantages of radioactive probes. Because the dyes can be discriminated by means of their emission maxima, multiplexing is possible, so four sequencing reactions per sample can be replaced by a single reaction using four different labels. Thus a single lane can be used rather than the four separate lanes that were necessary with radioactive probes. Additional advantages are higher throughput and automated data collection in real time.

Mass Spectrometry—Mass spectrometry (MS) has revolutionized the field of biochemistry and has significant potential in the area of nucleic acid sequencing. Soft-ionization techniques such as electrospray ionization and matrix-assisted laser desorption-ionization have expanded the potential application of MS to DNA sequencing. MS offers some advantages over other detection methodologies, including speed of fragment detection (signal acquisition is in the range of microseconds versus hours for conventional approaches) and accuracy (e.g., the molecular mass of each fragment can be determined with a high degree of accuracy). The Sanger procedure makes use of mass differences of the fragments generated as part of the polymerization reaction. MS is sufficiently precise to resolve fragment sizes that differ by only one base pair. Unfortunately, the sensitiv-

ity of MS detection suffers as fragment length increases, and the 100-base-pair barrier has yet to be crossed.

More recently, other sequencing technologies have emerged that are based on massively parallel sequencing techniques that attempt to achieve low-cost sequencing. These techniques are based, for example, on solid-phase sequencing or they make use of highly parallel and miniaturized pyrosequencing, which is described in *Nucleic Acid-Based Techniques—Genotyping* (1129).

Sequence Integrity

A prerequisite for automated data collection and interpretation is that the data must be of good quality, which means minimizing human intervention and allowing the system to make base identifications following detection steps. It is a critical step to ensure accurate base identification by minimally sequencing both strands of the DNA several times. In addition, other tactics may be employed, such as using primers at different sequence positions, which can improve the accuracy of the developed consensus sequence. This task can be facilitated by the use of specialized software packages that are commercially available. More recent technology developments have produced alternative sequencing platforms that are more amenable to large-scale sequencing projects. These techniques include array-based platforms on which short stretches of target are sequenced on a chip that supplies raw data to sophisticated computational programs that reconstruct the sequence. Other sequencing approaches have been developed for the rapid sequencing of short nucleic acid sequences such as oligonucleotides of short PCR products. These technologies include MS-based and pyrosequencing platforms, the latter of which is described in *Nucleic Acid-Based Techniques—Genotyping* (1129).

(1127) NUCLEIC ACID-BASED TECHNIQUES—AMPLIFICATION

INTRODUCTION

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in *Nucleic Acid-Based Techniques—General* (1125). The current chapter covers major techniques that result in amplification of targeted nucleic acid sequences. The most common NAT assay is the polymerase chain reaction (PCR), which was first described by Kary Mullis. This procedure has been further refined to amplify a DNA fragment starting from RNA (reverse transcription-PCR, or RT-PCR). Initially, PCR was used in a qualitative manner to amplify and detect DNA molecules because its exquisite sensitivity paired with its high specificity made it a useful tool for the detection of nucleic acid targets. Since its inception, the number of PCR applications has expanded rapidly, and the technique, which now includes quantitative and multiplex assays, is currently used in almost every field of research and development in biology and medicine. In addition to the changes and improvements to the original design of the PCR procedure, alternatives to PCR are techniques used to amplify target nucleic acids to generate RNA instead of DNA amplicons. The most commonly used techniques are nucleic acid sequence-based amplification (NASBA) and the transcription-mediated amplification (TMA) which are described here in detail. In contrast to PCR, which relies on incubating

the sample at three different temperatures, NASBA and TMA are based on isothermal conditions.

In addition to amplification of the target nucleic acid, the amplification step also can be directed at the signal used for detection (signal amplification). The most commonly employed technique is the branched DNA (bDNA) assay, in which the signal, typically a fluorescent probe that binds to the target sequence, is amplified. The bDNA assay is used predominantly for viral nucleic acid detection and quantitation.

This chapter describes the main assay components necessary for a PCR procedure and includes a discussion of the general optimization of PCR assays. The various PCR assay formats, including PCR, nested PCR, and RT-PCR are covered, and a discussion of the detection of the resulting amplicons follows. Although all these assays are essentially qualitative procedures, they can be modified for semiquantitation, and the various modifications are described. For accurate and reliable quantitation, real-time PCR has now replaced the methods listed above; real-time PCR and real-time RT-PCR are described in the *NAT Assays* section. The same section includes a discussion about probes and dyes that are an essential component of real-time PCR and the methods of quantitation using the generation of standard curves. The next PCR technique discussed is multiplex PCR, which is used for simultaneous detection of multiple targets or for normalization of assay results. Apart from PCR, the major alternative NAT tests that are used routinely, primarily in blood screening and clinical diagnostic screening are NASBA and TMA. The final technique described is whole-genome amplification, wherein the complexities of amplification require modifications to the PCR procedures. The chapter concludes with a discussion about the evolution of instrumentation used in NAT assays and the quality assurance and quality control issues associated with NAT because this is probably one of the most highly regulated biological techniques, especially when applied to blood screening.

ASSAY COMPONENTS

Enzymes

The essential components for NAT assays—polymerases, reaction buffers which include desoxynucleotides, ions, primers, probes, and fluorescent dyes—can be chosen from a broad selection of commercially available NAT reagent kits and vendors. Polymerases suitable for NAT applications can, in principle, be grouped into *Taq* DNA polymerases or DNA I polymerases from other *Thermus* species that are polymerases with features that are similar to those of *Taq* DNA polymerase. In addition, so-called proofreading polymerases are available (e.g., from *Pyrococcus* species) that display a 3'–5' exonuclease activity capable of removing wrongly incorporated DNA bases from the growing DNA strand under amplification conditions. *Taq* DNA polymerase is the standard NAT enzyme and is the one most often used in NAT assays. Modifications of *Taq* DNA polymerase, such as deletions of the 5'–3' exonuclease domain (Klenow fragment, Stoffel fragment) or point mutations for improved incorporation of dideoxynucleotides are also employed (e.g., for PCR-based sequencing reactions). Proofreading DNA polymerases or mixtures of *Taq* DNA polymerase with a proofreading polymerase are used if either fidelity of the NAT product is critical (e.g., for DNA cloning experiments) or longer NAT products are to be amplified. For RT-PCR, a reverse transcriptase is necessary to first convert the RNA target to copy DNA (cDNA) that can subsequently be amplified. For TMA reverse transcriptase with an RNase H activity is needed to convert the RNA target to double-stranded template DNA, while for NASBA exogenous RNase H is added to the reaction mixture. Depending on the reaction environment, two types of enzymes can be used to gener-

ate cDNA: a reverse transcriptase isolated from retroviral sources or a DNA polymerase that can function both as reverse transcriptase and DNA polymerase. Finally, chemical modification of the polymerase, resulting in an inactive enzyme at temperatures below 90°, is now typically used to prevent mispriming of templates at sub-optimal temperatures (see section on *Assay Optimization*).

Reaction Buffers

Reaction buffers vary with respect to ion composition, pH, and additives and are sometimes specifically adopted for particular applications such as multiplex PCR, real-time PCR, RT-PCR, TMA and NASBA. An important component of the reaction mixture is Mg²⁺ ions, or, in the case of polymerases with both reverse transcriptase and DNA polymerase functions, such as *Thermus thermophilus* (*Tth*), Mn²⁺ ions. Other additives that enhance the sensitivity and specificity of the assay may be present. The concentration of the four deoxynucleotide triphosphates (dNTPs) must be optimized.

Primers

Primer sets are oligonucleotides with sequences that are designed specifically to prime the amplification of a portion of a target nucleic acid of interest. Synthetic oligonucleotide primers for both standard PCR and for real-time or quantitative PCR are designed for the specific recognition of and binding to a single DNA or RNA sequence. Such specificity is achieved through design that involves both the length and the sequence of the primers. Length and sequence specifications have separate criteria that must be simultaneously met in order for the primers to perform properly. The length of a primer is a statistical issue that relates to the issue of the minimum length of a specific sequence necessary to guarantee that the desired target sequence is unique, regardless of the size or complexity of the genome. As an example, in the case of the human genome, with its 3.2 billion DNA bases, that length is 17 bases. For this reason the vast majority of PCR primers are between 20 and 25 bases long. The specificity of a primer should be determined by comparison with sequences in all known databases. Tools available on the Web facilitate such comparisons.

In terms of primer sequence, the issues to consider are T_m (the temperature at which 50% of the double-stranded nucleic acid molecule becomes single-stranded) and secondary structure. Every DNA has its own characteristic T_m , determined by length, sequence composition, and reaction environment. PCR primers are designed to bind to a perfectly complementary DNA sequence via guanine-cytosine (G-C) and adenine-thymine (A-T) base pairing. The T_m of the two PCR primers used in a reaction should be as close as possible. In terms of secondary structure, the formation of secondary structures by intra- or intercomplementarity should be minimized. Interaction between different primers can result in primer-dimers that will compromise assay sensitivity and specificity. All of the design issues presented are accounted for in any one of the dozens of primer design software packages that are available and can be found on the Internet.

Assay Optimization

NAT assay optimization is necessary for successful amplification that is sensitive and specific. Parameters that should be optimized include the thermocycling conditions, both temperatures and cycling times (that depend to a large extent on the target, primer, and probe sequences), concentrations of template, concentrations of NAT reagents, sample matrix and the number of amplification cycles. In the case of multiplex PCR, a compromise among elements of the reaction conditions is usually necessary because of the difficulties in optimizing the conditions for all the primer

and probe sets. Recent changes have been made to improve sensitivity and specificity of NAT assays. One change is hot-start PCR, in which the addition of one of the essential components of the NAT assay, typically the DNA polymerase, is temporarily withheld. When this occurs during reaction setup, the initial nucleic acid denaturation step prevents nonspecific amplification due to mispriming at suboptimal temperatures. Early hot-start procedures made use of wax barriers that effectively separated essential components into two liquid phases that were mixed only when the wax melted. However, this procedure has been replaced by two important hot-start technologies that do not require physical separation of the components by inconvenient additional handling steps. In the first procedure, antibodies directed against the DNA polymerase are complexed with the enzyme and lose their binding avidity at elevated temperature at the start of the reaction. The second procedure uses chemical modification of the polymerase, resulting in an inactive enzyme. At temperatures above 90°, typically in the first denaturation step, the modifier dissociates from the enzyme, and the enzymatic activity is restored. The advantage of an antibody-mediated hot start is the immediate release of enzyme activity at the start of the reaction by a very short heat incubation step. However, antibody-mediated hot-start chemistries tend to be less stringent when compared with chemically activated enzymes if there is a large excess of active polymerase molecules.

NAT ASSAYS

This section describes the basic techniques of PCR, nested PCR, and RT-PCR and procedural modifications that allow semiquantitation.

Polymerase Chain Reaction

The PCR technique is based on a three-step process: denaturing double-stranded DNA (dsDNA) into single strands (ssDNA), annealing primers to the ssDNA, and enzymatic extension of primers that are complementary to the ssDNA templates. Each step is usually carried out at a different temperature. By cycling the temperature steps many times (usually 30 to 45 times), a billion-fold amplification of the target nucleic acid can be achieved, but the optimal number of cycles should be determined empirically. In some cases, especially where sensitivity is more important than false positive results due to excessive cycling, such as in blood screening, extra sensitivity can be gained by increasing the number of cycles to 60 to ensure that extremely low levels of target are detected. In a typical reaction, PCR product (amplicon) doubles at each cycle of amplification (exponential amplification). The increase in amplification in the early cycles follows a sigmoidal curve. In later cycles, the concentrations of the template strands and amplicons favor template strands re-annealing instead of PCR primer annealing to the template. At this point the concentration of the PCR product no longer doubles after each cycle, and the curve begins to plateau. A thermostable enzyme such as *Taq*-polymerase is a prerequisite because temperature cycling at 95° (the typical temperature step used to denature double-stranded templates) would inactivate a thermolabile polymerase.

NESTED PCR

An early variation of the PCR assay was nested PCR, which was designed to increase the assay's sensitivity and specificity. In this procedure amplicons from the initial PCR reaction are subjected to a second round of amplification using a different set of primers. This set of primers is specific to the amplicon sequence but is within the first set of primers (nested primers). The advantage of amplification with two sets of target-specific primers is increased specificity (any

nonspecific amplification during the first amplification round would be reduced) and increased sensitivity (due to initial amplification of the target in the first amplification round). In addition, amplification of a product of the expected size is taken as confirmation of the presence of the target. However, a major drawback of this procedure is the high likelihood of cross-contamination due to the increased manipulation of amplicons generated in the first round of amplification. The use of highly specific primers and probes and the optimization of reaction conditions have resulted in the diminished applications of this procedure for routine testing, but the procedure is sometimes used for samples that are difficult to amplify by conventional PCR.

RT-PCR

In amplifying RNA targets, analysts prepare cDNA before the amplification step (RT-PCR). One-step and two-step RT-PCR procedures are available. In one-step RT-PCR the reverse transcription of RNA into cDNA and the subsequent amplification step are carried out in a single reaction without intermediate procedures. Therefore the reaction mixture for one-step RT-PCR includes the gene-specific amplification primers that are used for both reverse transcription and amplification. The advantage of this procedure is the overall reduction in handling time, increased throughput, and reduced contamination risk because reopening the reaction vessel is not necessary. In contrast, in two-step RT-PCR the reverse transcription and amplification are performed as two separate steps. In general, random primers or oligo-d(T) primer rather than gene-specific primers are used for the reverse transcription step. An aliquot of the cDNA synthesis reaction is then transferred into the NAT reaction for subsequent amplification. The advantage of this procedure is the standardization of the reverse transcription reaction, which can be used as a single source for the analysis of multiple transcripts in gene expression analysis.

DETECTION OF AMPLICONS

Following amplification, analysts can employ a variety of procedures for detection of the amplicon as described in detail in the general information chapter, *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* (1126). These include agarose gel electrophoresis with ethidium bromide or other dyes, capillary electrophoresis, and laser-induced fluorescence and hybridization followed by chromogenic detection such as streptavidin horseradish peroxidase detection, chemiluminescence, or fluorescent detection using labeled probes.

Quantitation—The original PCR and RT-PCR assays were qualitative and detected amplicons at the end of the reaction. Such detection is not easy to quantitate because at this stage the amplification is in a plateau phase at the end of the assay, and the amount of amplicon is not necessarily directly related to the quantity of the starting template. Several approaches have been deployed to attempt to overcome the shortcoming of PCR to produce reliable, quantitative results. Initial attempts at quantitation relied on assessing the amount of amplified DNA during the early or exponential part of the assay, but this procedure was fraught with problems because the aliquots had to be taken from the reaction mixtures at regular intervals, thus greatly increasing the risk of cross-contamination. One of the earliest and most straightforward approaches to quantifying PCR products was to measure the amount of amplicons that were generated during the exponential phase of the reaction by comparing this to a serially diluted external control. Several aspects, including variability in sample preparation and variations in reaction conditions, however, hampered this approach. Because of the exponential amplification of NAT procedures, even small errors or variances can lead to distinct differences.

Compared with dilution procedures, competitive PCR proved to be a much more precise approach to achieving reliable estimates of the originally present target molecules. This procedure relies on the simultaneous co-amplification of a specific target sequence in the presence of increasing concentrations of an exogenous target molecule (control) which shares the primer binding sites with the target sequence but whose sequence is slightly modified or shortened in order to facilitate discrimination from wild-type amplicons. In addition, the concentration of the control is known. The close sequence homology and similar size of the control and target amplicons are designed to ensure that the template and internal control are amplified with comparable efficiency. The relative strength of the amplicon bands of template and control can be assayed, for example, on ethidium bromide-stained agarose gels, giving a relatively precise quantitation of the wild-type target. A drawback of this approach is that the internal control and the template should be present in the reaction in approximately the same quantity in order to yield correct results. The development of real-time, quantitative PCR has eliminated the variability associated with quantitative PCR, thus allowing the routine and reliable quantification of PCR products.

REAL-TIME PCR AND REAL-TIME RT-PCR

Although gene quantitation by quantitative PCR was a widely used procedure, its applications were expanded by the advent of real-time PCR and real-time RT-PCR. Real-time PCR displays the same advantages as standard quantitative PCR—sensitivity, specificity, and a wide dynamic range—but the real-time procedure offers the additional advantage of requiring no post amplification processing because it combines amplification and detection in a single step. Real-time PCR collects data throughout the amplification process by measuring a fluorescence signal created as amplification progresses. A multitude of fluorescence chemistries allows the correlation of generated PCR product to fluorescence intensity. In principle, fluorescence intensity will increase with every cycle performed. Once the intensity is greater than background fluorescence, the so-called cycle threshold (C_t) value is achieved. This value, which represents the first cycle in which there is a detectable increase in fluorescence above the background level, is used to measure relative or absolute target quantities. The C_t value is inversely proportional to the number of target molecules in the sample and thus provides a means to quantitate the amount of target in the starting material (i.e., the greater the number of target molecules present, the lower the C_t value).

The reaction conditions for real-time PCR applications have to take into account the presence of the probe(s) and will require optimization. The most commonly used probes currently are hydrolysis probes, although hybridization probes are an alternative. In most cases, the amplification and detection steps can be combined into a two-step cycling reaction, but these conditions have to be optimized. In contrast, DNA-binding dyes which may also be used for amplicon detection require separation of the annealing and extension steps since the dye binding occurs during the extension step which is usually done at 72°.

A fluorescent DNA intercalating dye is used for detection of the PCR product in real-time mode. This dye emits light when bound to double-stranded DNA and the subsequent increase in fluorescence can be detected by real-time PCR instruments. Dyes that bind to dsDNA bind not only to the specific PCR product but also to artifacts such as nonspecific PCR products and primer-dimers. Analysts have observed substantial differences in the specificity of dsDNA-binding dyes in use with real-time PCR kits. Therefore, some analysts recommend verifying the presence of a single PCR product by gel electrophoresis to determine the correct size of the PCR product. Also, a melting curve analysis is advisable to ensure the absence of artifacts that could contribute to the fluorescent signal and thereby lead to misinterpretation of

quantitative data. Alternatively, sequence-specific labeled probes can be employed. A wide variety of fluorescence-labeled probes and primers exist for use in real-time PCR and are described in the next section.

Real-Time PCR Probes—The difference between conventional PCR and real-time PCR is the presence of a third chemically synthesized oligonucleotide, the probe, which, for the most basic hybridization probes, contains some type of reporter molecule, usually a fluorescent molecule or fluorophore. Non-nucleic acid materials can be added to chemically synthesized DNAs that are then incorporated into oligonucleotide probes for real-time PCR. Other applications include hybridization probes such as those used for fluorescence in situ hybridization (FISH) and microarrays and probes designed to capture other nucleic acids. A challenge arises in using fluorescent probes for real-time PCR because the unbound or free probe is not removed before detection, thus requiring a means to distinguish between signal obtained from bound and free probe. In contrast, FISH assays involve washing away free probe following hybridization.

All of the issues associated with primer design for conventional PCR apply to real-time PCR primers as well as to the probe sequence. As a general rule only two additional considerations apply to the probe sequence. One of these is thermodynamic, and the other specifically concerns the reporter moieties themselves. Thermodynamically, a good probe molecule that is designed to bind in the sequence somewhere between the two PCR primers will have a T_m that is about 5° higher than that of the two primers. In the large majority of cases the amplicon will be between 100 and 500 DNA bases in length, although for real-time PCR a smaller amplicon between 100 and 150 bases long results in a more efficient reaction. Thus it is rarely a problem to find a sequence inside a PCR amplicon that meets the necessary criteria.

Current probe designs overcome the problems of background from unbound probe using simple hybridization probes. In the original design, two probes that hybridize to adjacent sequences on the target nucleic acid are labeled. The reporter moiety is a fluorescent molecule attached to the 3' end of the upstream probe sequence, and a second fluorescent molecule is attached to the 5' end of the second probe. Excitation of the 5' fluorophore with light energy of the proper wavelength results in absorption of that energy, followed by emission of light energy of a slightly longer or less energetic wavelength (Stoke's Law). This emitted energy then excites the 3' fluorophore if it is close enough to the emitter and compatible with it in the sense that the emitted energy from the 5' fluorophore can excite the 3' fluorophore. When this occurs, the observed fluorescent light wavelength will be that of the acceptor molecule and not that of the donor. Fluorescence absorption and emission spectra are readily available for all of the commonly used fluorophores, and the only applicable rules are that the two fluorescent molecules must be fewer than 40 DNA bases apart and that the emission spectrum of the donor must overlap the absorption spectrum of the acceptor. Thus hybridization of the two probes, also known as hybridization probes or FRET probes (Fluorescence Resonance Energy Transfer), results in the emission of a fluorescent signal by the acceptor, and the latter signal can be detected. In the absence of hybridization, the probes are sufficiently separated in solution so that energy transfer cannot occur, and only background fluorescence is emitted by the donor.

Issues of fluorophore compatibility have been resolved by the increased use of a special class of molecule called a quencher. Quenchers are fluorescent molecules that absorb fluorescence energy over a wide range of wavelengths. Instead of re-emitting that energy as light they simply dissipate it as heat. Thus, if a quencher molecule is placed at the 3' end of a probe and a fluorophore at the 5' end, the probe will remain dark even when excitation energy is present so long as the molecule remains intact (hydrolysis probes). These probes utilize the 5' nuclease activity of the

DNA polymerase to hydrolyze a probe bound to its target amplicon. Cleavage results in separation of the reporter and quencher and permits fluorescence of the reporter. This reduces much of the work of optimization of the assay conditions (since only a single probe is used) and background noise generated with two probes.

A variation on hydrolysis probes involves placing the reporter and quencher molecules on a single oligonucleotide that is constructed so that, in the unbound state, the quencher and reporter are in close proximity, resulting in efficient quenching of the reporter. When the probe hybridizes to its complementary sequence on the amplicon, the probe undergoes a conformational change that forces the quencher and reporter apart, permitting fluorescence of the reporter. A variation on these kinds of probes is a combined primer and probe in which, again, the quencher and reporter are in close proximity in the native probe, thus resulting in no signal. Priming and subsequent elongation of the primer-probe results in hybridization to the newly synthesized DNA strand, causing spatial separation of the quencher and reporter and resulting in the generation of a signal.

Probe Labeling—Modern synthetic oligonucleotide modification chemistries permit the manufacture of oligonucleotides with non-nucleic acid materials. Placement of modifications is carried out in one of two ways: during synthesis or after synthesis. For the former, modifications are constructed in such a way that they behave like the four DNA or RNA bases that are routinely placed in the sequence. The modification is then presented in the desired location during the synthesis as if it were just another base in the series. In the latter, usually employed when more than one modification occurs, the synthesis contains a linker, such as an amino group, to which the desired modification is then attached. This process is often called "hand-tagging."

Perhaps the best-known example of hand-tagging is the conventional dual-labeled probe used in real-time PCR. The quencher is placed at the 3' end of the sequence during synthesis, and the fluorescent reporter molecule is hand-tagged to an amino modification at the 5' end of the sequence after the synthesis is finished and has undergone purification. Some modifications, such as biotins, are designed so that multiple modifications can be carried out in a single synthesis. Thus, it is possible to modify a synthetic DNA or RNA sequence to contain a number of different non-nucleic acid molecules. A cost is associated with such modifications insofar as alterations often are achieved with a loss of mass due either to an inherently lower efficiency of modifications to bind to the oligonucleotide as compared with standard DNA or RNA bases or to the requirement that the synthesis must be purified before modification, after modification, or both.

The benefits of modifying synthetic DNAs or RNAs usually outweigh the costs. The standard, quenched, dual-labeled, real-time PCR probe has permitted precise quantification of gene expression. Fluorescently labeled DNA oligonucleotides are also essential components of in situ hybridizations and microarrays. Some modifications confer increased thermal stability when synthetic DNAs or RNAs are hybridized to complementary DNAs or RNAs by comparison with unmodified DNA-DNA and DNA-RNA duplexes. These analogues include peptide nucleic acids, 2'-fluoro N3-P5'-phosphoramidates, and 1', 5'-anhydrohexitol nucleic acids. Although such analogues succeed to varying degrees in achieving increased thermal stabilities, they fail to provide enhanced target recognition. Another approach is to use base analogues such as locked nucleic acid, which is an analogue that contains a 2'-O, 4'-C methylene bridge. This bridge restricts the flexibility of the ribofuranose ring and locks the structure into a rigid bicyclic formation, conferring enhanced hybridization performance and stability.

The modification of a probe typically is governed by its intended use. Generally, fluorescent reporters are used in real-time PCR and for in situ hybridization. The range of

available fluorescent reporters covers the spectrum from 517 nm to 778 nm. For hybridization probes, base modifications are preferred because these primarily alter thermodynamic interactions between bases, leading to improved specificity. Amino attachment groups, both with and without C-spacers, are used to attach other modifications to DNA sequences and to attach DNA sequences to solid surfaces such as glass slides. An example is the attachment of biotin molecules to DNA sequences. Biotin forms a strong bond with streptavidin-coated materials such as magnetic beads, allowing capture of specific nucleic acids that may themselves be hybridized to other molecules.

Quantitation—PCR products may be quantified using a standard curve drawn from replicate serial dilutions of a reference reagent or standard for the nucleic acid sequence of interest. The concentration of the nucleic acid in the reference reagent is known. Real-time PCR quantitation based on a standard curve may utilize plasmid DNA or other forms of DNA. However, the efficiency of PCR must be the same for the standards and the target samples. Performing PCR from purified targets can in some cases be more efficient than performing PCR with complex nucleic acid mixtures. The cycle threshold (Ct) values and concentrations of the dilutions of the reference reagent can be used to construct a standard curve from which the concentration of the unknown sample can be estimated. When the assay run conditions have been well standardized and the standard curve for a particular target has been well calibrated, in subsequent assay runs it may be sufficient to co-amplify only two dilutions of a reference reagent (usually dilutions containing known amounts of nucleic acid at high and low concentrations). These dilutions, or calibrators, can then be used to quantitate any unknown samples by comparison of the C_t values.

Multiplex PCR—Multiplex PCR describes the simultaneous amplification of several nucleic acid targets in a single assay reaction. This is a particularly demanding variation of PCR because it requires the use of a single set of reaction conditions for the amplification of multiple targets with different sequence characteristics. Additional complications can arise due to the increased chance of nonspecific amplification products arising from multiple primer interactions. In addition, the differing individual target amplification efficiencies can result in weaker reactions being out-competed by stronger, more efficient reactions.

Both qualitative and quantitative applications of multiplex PCR have been described in the literature, as have multiplex RT-PCR assays. Quantitative multiplex PCR relies on either the generation of multiple standard curves to enable quantitation of each target in the assay, or the inclusion of internal competitor sequences that can be used as calibrants.

Hybridization kinetics of primers and probes may be significantly different, even when designed using the same algorithm. This leaves the analyst with very limited room to optimize reaction conditions. However, optimization may include adjustment of DNA polymerase amount, Mg²⁺ to increase hybridization efficiency, or primer concentration. Especially in real-time PCR, optimization of primer concentration is critical for quantitative co-amplification of target genes. These are contained in the sample at significantly different amounts. Increasing hybridization efficiency of the primer-probe system can be achieved by providing sufficient reagents, such as Mg²⁺, as well as adding a “molecular crowding” reagent that increases the effective concentration of all reaction components in the mixture. Multiplex PCR is not only used for genotyping applications, but also for quantitative real-time PCR because it offers several advantages over standard single real-time PCR reactions. Some of these advantages are a minimized amount of sample used, increased precision through the use of an internal control (e.g., housekeeping gene) co-amplified with the target gene in the same reaction, no separate pipetting steps, and cost-effectiveness.

Most PCR assays, however, suffer from a common problem—that of minimizing differences in extractions or amplifications between different samples. Multiplex PCR is useful in cases where it is critical to ensure that variability in quantitation of different samples is not due to differences in nucleic extraction or amplification measurements (usually when one measures the production of an mRNA species). Certain precautions and techniques can be employed to minimize these challenges; they are discussed in the next section on normalization of assay results.

Normalization of Assay Results—To minimize the effects of assay variables, analysts sometimes use a relative quantitation procedure that normalizes the target transcript level to a control that can be employed and compared for all samples included in the gene expression study. Probably the most reliable and most frequently used relative quantitation procedure relies on the measurement of “housekeeping” or control genes to normalize the expression of the target gene in a multiplex PCR format. This procedure is preferred because the quantitation of both the housekeeping gene and the target gene are influenced by varying cDNA synthesis efficiencies or the presence of enzyme inhibitors contained in the sample. However, it should be noted that the efficiency of conversion of target RNA to cDNA is not necessarily consistent even within a single-tube reaction but is a function of primer design, target sequence, etc. which may differ between target and housekeeping genes. The selection of appropriate control genes can cause problems because they may not necessarily be equally expressed across all unknown samples and may vary under experimental conditions. Normalizing measurements to a set of housekeeping genes in order to avoid the problem of variability may circumvent this concern. Alternatively, analysts can establish a thorough evaluation of housekeeping genes that do not alter gene expression levels under the experimental conditions.

All the NAT techniques described thus far are variations on the PCR assay, which is the most widely used of the NAT techniques. However, isothermal assays that are based primarily on the amplification of RNA are used for routine purposes. This is known as the transcription-mediated amplification (TMA) assay, which is closely related to the nucleic acid sequence-based amplification (NASBA) assay. Both assays are described in more detail in the following section.

NUCLEIC ACID SEQUENCE-BASED AMPLIFICATION AND TRANSCRIPTION-MEDIATED AMPLIFICATION

Both NASBA and TMA rely on *in vitro* isothermal amplification for detection and amplification of nucleic acids, also referred to as self-sustained sequence replication or 3SR. The major difference between the assays is that NASBA uses three enzymes—reverse transcriptase (RT), RNA polymerase, and RNase H—whereas TMA uses only two enzymes: RT and RNA polymerase. The complete procedure generally is performed at 41° to 42° using two primers. Both NASBA and TMA are especially suited to amplifying RNA analytes, including rRNA, mRNA, pathogens that have RNA as their genetic material, as well as DNA targets.

One of the primers that has a promoter sequence for the RNA polymerase at the 5′ end binds to the RNA target and is extended via the DNA polymerase activity of the RT. The product of this reaction is an RNA–DNA hybrid. RNase H activity then specifically digests the RNA strand of the hybrid, leaving only the cDNA to which the second primer can bind. A complementary strand of DNA is then synthesized by the RT, resulting in a dsDNA molecule with a T7 promoter at the 5′ end. The T7 RNA polymerase then transcribes multiple copies of the RNA amplicon. The RNA copies may undergo the same cycle to create new duplex DNA molecules with a T7 promoter from which many molecules of RNA are transcribed. Thus, unlike the action of PCR, the amplicon amplified in this case is of an RNA species.

Some of the characteristics of this technology are that only relatively short target sequences can be amplified efficiently (around 100–250 nucleotides); it uses a single temperature, which eliminates the need for special thermocycling equipment; the fidelity of the technique is comparable to that of other amplification processes; and the RNA amplicons are exponentially amplified. Carryover contamination is minimized because of the labile nature of the RNA amplicon in the laboratory environment. Containment procedures built into the assay procedure further help to minimize contamination. Detection of amplicons is typically achieved by the use of labeled probes and, in TMA technology, a common method is detection of chemiluminescent signals from hybridized probes that remain intact during the subsequent alkaline hydrolysis step used to destroy free probe.

The NAT techniques described, both PCR and TMA, are optimized for amplifying specific, small fragments of a genome. In cases when whole genome amplification is desirable, such as for mutation analysis or identity testing, modifications of the PCR procedure are necessary in order to ensure adequate sequence representation of genetic loci, as described in the following section.

WHOLE GENOME AMPLIFICATION

Historically, whole genome amplification (WGA) has been performed using modified PCR procedures. These procedures have relied on the nonspecific amplification of the genome using primers that bind under low-stringency conditions to the DNA template. PCR-based approaches differ mainly in terms of the type of primer employed in the reaction: in primer-extension-preamplification (PEP), short 15 base random primers are used in an initial cycling reaction at low stringency to make multiple random copies of segments of the genome. This product is then used as target for the specific PCR reaction. Amplification bias of favorable sequence contexts leading to uneven representation of the genome is the major drawback of this technique. The generation of increasingly shorter fragments during each round of amplification is a further drawback. Another procedure called degenerate oligonucleotide primed-PCR (DOP-PCR) uses tagged primers and low stringency amplification for the first few cycles of amplification followed by an increase in annealing stringency in later cycles. The tagged primers are characterized by defined sequence tags at the 3' and 5' ends and a random sequence in the centre of the primer. Under the later, more stringent conditions, the target DNA fragments generated during the first cycles containing the amplification tag sequences are amplified preferentially without any further shortening of the fragment length. PCR-based WGA typically employs *Taq*-like polymerases that possess the disadvantage of introducing variations into the amplified DNA due to their relatively low processivity and fidelity which become compounded by the very high number of amplification cycles used in these methods. This may cause problems in downstream applications such as genotyping analysis. These limitations as well as the relatively poor sequence representation of genomic loci inherent to PCR-based WGA can be overcome by an isothermal reaction called multiple displacement amplification (MDA).

The enzyme that is used for MDA comprises a high processivity polymerase with proofreading and strand-displacement activity. The isothermal reaction is performed at 30° without any change in reaction temperature. The reaction starts with the annealing of multiple random primers to the target DNA and elongation of the primers using a DNA polymerase from the *Bacillus subtilis* phage Phi29. Because the polymerase is able to displace DNA strands in a 5'–3' direction, the polymerase reaction is not stopped when the elongating strands meet downstream DNA strands. The displaced DNA strand serves again as a target for multiple

primed elongation reactions so that the DNA template is amplified exponentially in a branched-like manner, yielding high molecular weight DNA with a good representation of the genomic loci. Compared with PCR-based WGA, the error rate is very low. In particular, the mutation rate of repetitive sequence structures is low because of the limited strand-displacement activity of Phi29-polymerase. This permits reliable genotyping of genomic DNA (e.g., SNP analysis, mutation analysis, identity testing, or analysis of case work samples) on different platforms such as real-time PCR or array analysis.

INSTRUMENTATION

The development of the numerous and varied NAT techniques described in this chapter has been facilitated by the evolution of instrumentation that has served to automate these complex procedures. A general description of the major changes in instrumentation is discussed in this section.

The continuous control of the temperature steps necessary to achieve exponential amplification for PCR assays is carried out by fully automated thermocyclers that consist of a heating block in which the temperature can be rapidly cycled. Temperature changes are induced by water, or more recently, by using the Peltier effect. These instruments may be coupled to a fluorometer apparatus if they are used for real-time PCR analysis. In the latter case certain instruments are equipped with a rotor device that is heated and cooled by air instead of a metal block that typically is used as a heating module. In the case of endpoint PCR, PCR products are usually analyzed according to size on agarose or polyacrylamide gels, or by capillary electrophoresis using fluorophore-labeled primers. They may also be analyzed by an array-based approach or other hybridization procedures.

Because no post-PCR processing or label-separation steps are required, real-time PCR assays are simple to perform, making them useful for high-throughput applications. Real-time PCR instruments combine the properties of a thermocycler and a fluorometer to allow determination of PCR products by fluorescence measurement. In each PCR cycle, either one or several fluorescence readouts are taken to monitor the PCR reaction for generation of amplicons, usually at the extension step of the PCR reaction.

Real-time PCR instruments vary with regard to simultaneous sample throughput (32–384 reaction vessels), sample volume (5–100 μ L), excitation source, and detector used. These compositions define the suitable range of fluorescent dyes for multiplex real-time PCR as well as size and heating/cooling principle (see above). The excitation source of real-time thermocyclers is either a laser-based system, halogen bulbs, or light-emitting diodes (LED). Optical filters are used to select the wavelength of interest. In most instruments, the emitted light is detected by a charge-coupled device (CCD) that consists of an array of light-sensitive cells. Light projected onto the CCD is converted to an electric charge, resulting in a signal that is proportional to the light intensity.

The versatility of the PCR assay has resulted in the widespread and diverse use of this technique. With the advent of real-time PCR, it has been possible to design high-throughput instrumentation for automated testing. Similarly, the TMA assay has also been automated. Such technology is used by laboratories doing high-throughput, highly regulated testing, typically blood screening for hepatitis C virus (HCV) or human immunodeficiency virus-1 (HIV-1) because automated tests are ideal in a regulated environment where minimum human intervention is required. The use of NAT in a highly regulated environment has resulted in the development of guidances for managing the quality assurance (QA) and quality control (QC) aspects of testing, as well as the validation of systems and assays as described in the following section.

QUALITY ASSURANCE AND QUALITY CONTROL FOR NAT

This section serves as a general guidance for the development of laboratory- and procedure-specific QC and QA procedures for NAT. Aspects such as waste management, management of radioactive material, or working with hazardous material are not covered. NAT is a technology that offers extreme sensitivity with its ability to generate millions of amplicons from as little as a single nucleic acid template, resulting in a detectable signal. The advantages of this technology can be offset by the necessity of establishing complex assay protocols and the requirement to follow carefully very stringent QC/QA protocols. Deviation from these protocols can cause major problems, such as false positive results due to the contamination of templates by amplicons generated in previous assay runs. Similarly, failure to control inhibitors could lead to suboptimal amplification and possible false negative results. Given the myriad factors that can greatly influence the outcome of a NAT assay, all aspects concerning NAT need to be covered by appropriate and stringent QC/QA procedures. This requires careful facility design, workflow, and selection of equipment suitable to the purpose. Data recording, record keeping, and data interpretation are other aspects that should be covered by QC/QA. Thus, QA for NAT assays includes assay validation, establishment of acceptance criteria and specifications, and adherence to good manufacturing/laboratory practices. These aspects are also described in this section. In addition, reference should be made to other published guidelines such as the ICH Guideline *Validation of Analytical Methods: Methodology* (Q2B) and the NCCLS Guidelines.

Laboratory QC/QA

An NAT laboratory should be designed and operated in a manner that prevents contamination of reactions with products from previous amplifications (carry-over) as well as cross-contamination between samples. Historically, the application of PCR required strict separation of the various steps of the assay in order to prevent cross-contamination of PCR by amplicons. This was necessary because early procedures for analysis of PCR products involved the transfer of the product, which potentially could lead to contamination. Therefore, in an open system the best measure to prevent contamination has been the strict separation of working areas for individual process steps. This includes individual areas for template preparation, master mix setup, distribution of the master mix to individual reaction wells and addition of template, space for cycling the PCR assays and, optionally, a separate work space for PCR product analysis. These requirements are not necessary with closed systems. With both open and closed systems it is still necessary to take additional precautions. These safety measures include UV illumination of work spaces overnight to inactivate residual DNA by crosslinking. In case of contamination, laboratory benches and pipettes can be decontaminated by cleaning with a 10% solution of commercial bleach, which usually contains about 5% sodium hypochlorite, taking appropriate safety measures such as wearing gloves and eye protection. Afterwards, benches and pipettes should be rinsed with distilled water. A unidirectional workflow will reduce the opportunity for contamination to occur. Also, no materials, supplies, or equipment should be exchanged between designated working areas or rooms.

Equipment QC/QA

Other good laboratory practices that are related to the prevention of carry-over contamination include the use of suitable and clean equipment. Generally, disposable consumables (tubes, pipette tips, etc.) are highly preferable to reusable equipment. The use of disposable tips containing

hydrophobic filters is another very effective measure to minimize cross-contamination. All samples, primer, probes, etc. must be labeled with relevant information such as identity of the content, date of use or preparation, expiration date, concentration, and storage information. Dedicated laboratory coats or disposable lab coats should be available in each room (or section) of the NAT laboratory. Appropriate gloves should be used during all processing steps to prevent sample contamination. The gloves should be changed frequently. Because heat sterilization does not completely destroy DNA, PCR products may lead to detectable contamination of, for example, glass surfaces. Following unique sterilization procedures for different materials such as waste and glass laboratory equipment is advisable.

Carry-Over Prevention with Uracil-*N*-Glycosylase

Contamination by PCR product carry-over can be mitigated by using the commercially available uracil-*N*-glycosylase (UNG) procedure. The procedure involves substituting 2'-deoxyuridine 5'-triphosphate (dUTP) for 2'-deoxythymidine 5'-triphosphate (dTTP) in the PCR setup and treating all PCR mixtures with UNG prior to PCR amplification, which can be easily incorporated as a first step into PCR cycling programs. Incorporating dUTP into the amplicon makes the PCR products biochemically distinct from the native DNA template. The enzyme UNG cleaves the deoxyuridine-containing PCR products by opening the deoxyribose ring at the C1 position. When the deoxyuridine-containing DNA is heated during the first thermal cycle, the amplicon DNA chain breaks at the position of the deoxyuridine at the alkaline pH of the PCR reaction mixture and thereby renders the carried-over PCR product nonamplifiable. Thus, any previously generated U-containing amplicon that might have contaminated another sample will become nonamplifiable. As a consequence, false positive results can be avoided. However, it should be noted that UNG has concentration limits above which it does not fully remove PCR carry-over products.

VALIDATION OF NAT SYSTEMS

Assay validation is achieved by

- (1) ensuring the quality and consistency of assay components, including primers, probes, and enzymes; (including shelf life and contamination control) and
- (2) establishing the performance characteristics of the NAT assay in terms of reproducibility, accuracy, ruggedness, robustness, specificity, precision, and analytical and clinical sensitivity.

The analytical sensitivity of an assay is defined as the minimum concentration of a reference reagent or standard detected by the test while the clinical sensitivity of a test is determined by testing clinical specimens and determining the 95% LOD. The clinical sensitivity of a test is not necessarily the same as the analytical sensitivity. The closer the reference or standard material is to the samples being tested the closer the correlation.

The principal steps of assay validation are

- (1) sample preparation;
- (2) consistent production of critical reagents;
- (3) use of controls, calibrators, and quantitation standards;
- (4) specimen and reagent stability;
- (5) functionality of instruments and software;
- (6) operator training; and
- (7) laboratory surveillance for proficiency.

Following assay validation, further QA is necessary to monitor specifications and functional characteristics that have been established by the use of well-characterized reagents of known potency.

Quality Control of Reagents

DNA Templates—The test specimens used are usually, but not limited to, whole blood, plasma, and serum. Specimen preparation is a key step in the NAT assay and has a major influence on the performance and variability of the assay. Specimen collection is the first step in sample preparation. QC/QA staff should carefully evaluate the effects on the integrity of DNA of collection tubes and temperatures during sample transport. To prevent cross-contamination during specimen collection, aseptic techniques should be used along with closed sampling systems in order to avoid specimen contamination. The use of appropriate sample handling techniques, temperature conditions, and anticoagulants or preservatives should help reduce the risk of contamination. Anticoagulants such as heparin or EDTA may interfere with the NAT assay.

Sample Extraction—The buffers, reagents, and detergent or chaotropic agents used for nucleic acid extraction should be evaluated for inhibitory effects on the NAT assay. Extraction controls, including spiked materials, should be included to monitor the efficiency and reproducibility of the extraction method. Reproducibility of the sample preparation method should be determined under the specimen processing conditions, including sample handling, storage, and shipping conditions. DNA is generally stable, but personnel should take care to avoid storage at refrigerated temperatures for extended periods of time to avoid sample degradation. Repeated freeze-thaw cycles can sometimes cause DNA fragmentation. In the case that the target is RNA, it should be noted that RNA is very unstable and specimens should be frozen.

Primers—Primers and probes should be qualified in terms of purity, identity, and functional potency. Purity can be assessed by use of HPLC or mass spectrometry; identity can be established by sequencing; and functionality can be established by the use of reference reagents. However, in many cases, these methods may not be available for in-house testing. In these cases, it may be sufficient to compare lot-to-lot variation of purity and functional potency using relevant methods available in-house coupled with the use of reference reagents.

DNA Polymerases—The functionality of enzymes should be determined using reference materials. Enzyme preparations should be tested for other enzymatic activities; for example, exonucleases and DNA- and RNA-dependent polymerase activities and specifications should be established. Lot-to-lot comparison, as well as comparison with the manufacturer's CoA should also be done. Storage conditions recommended by the manufacturer should be strictly followed, and appropriate controls should be used to monitor the stability of enzymes.

Run Controls

The use of controls affords the operator assurance that the assay has performed within accepted specifications. In PCR testing, several steps in the testing process, as outlined above, should be monitored and verified. Multiple controls or controls that serve multiple purposes may be needed for a PCR assay. Controls should reflect the specific technology under development but should typically allow monitoring of ultracentrifugation, extraction, amplification, hybridization, quantitation, contamination, etc. Controls should be similar to the specimen type whenever feasible, although spiked controls may be acceptable.

A negative control is one that does not contain the target sequence or pathogen that is being tested. It should resemble as closely as possible the sample matrix under testing. Multiple negative controls should be examined, including nontarget sequences and nucleic acid-free controls to monitor for false positives resulting from contamination. Because

of the high sensitivity of amplification assays, QC/QA personnel highly recommend that sponsors include control measures for the prevention of contamination events.

A positive control is one that contains the target sequence of interest. It should resemble as closely as possible the specimen matrix being tested and should contain an appropriate and defined amount of target sequences. (e.g., kit control).

Specifications for both positive and negative controls should be provided, as well as validation data supporting the proposed assay cut-off/reporting threshold value or the assay's limit of detection. The laboratory should define the source of the controls and calibrators and have a plan for their continued renewal. Controls can be infectious or non-infectious. In the latter case, validation of viral inactivation should be provided.

Reagent controls are often referred to as blanks and could include samples that have no target sequence, no enzyme, no primers, etc. These controls provide additional information about problems encountered in PCR assays.

An internal control is added to each specimen to ensure the overall validity of the individual test results. Internal controls are used to verify sample extraction, amplification, and detection.

External Quality Assessment and Proficiency Testing

Quality assessment of the laboratory is achieved by participation in periodic competency assessment and laboratory proficiency programs. The latter should include the testing of reference reagents and well-characterized panels to measure the technical proficiency of operators. Therefore, care should be taken to prevent cross-contamination, to monitor workflow, and to ensure careful specimen and test sample handling. Evaluation of operator proficiency should include participation in competency and quality assessment programs. Each operator in a particular laboratory should participate in such programs and should demonstrate comparable results.

Data Management

Complete and consistent documentation of all activities performed and all data generated is necessary. Such documentation does not only require the maintenance of records of the data generated through sample testing but also information about reagents and equipment calibration and maintenance. Moreover, any alteration in the assay procedure needs to be introduced through a planned change control process and documented in such a way that change can be assessed by an independent party.

<1128> NUCLEIC ACID-BASED TECHNIQUES—MICROARRAY

INTRODUCTION

Microarrays are microscopic spots of DNA (measured in micrometers) arranged in an ordered manner (columns and rows) on a planar surface so that each DNA spot can be uniquely identified to facilitate an accurate analysis of the data. The DNA spots, also called array elements, are specific DNA molecules of known or unknown sequences and can

be of similar or different nucleotide lengths. Samples of these mixtures are placed in fixed locations on the microarray.

Unlike conventional probes, which are a specific DNA or RNA sequence labeled with radioactive, fluorescent, or chemiluminescent tags (see *Nucleic Acid-Based Techniques—General* (1125), *Glossary*), the array elements are referred to as probes when the sequence information of the array elements is known, despite not being labeled. In this context, the target refers to labeled nucleic acids in solutions that are hybridized to the array elements or probes. The purpose of a microarray experiment is to identify the sequence of these labeled nucleic acids and/or determine their content. Compendial applications at this time are limited but may increase with wider use of microarrays in diagnostics and in drug discovery, development, registration, and control applications. When used for compendial purposes, standard assay development and validation approaches with availability of suitable reference materials are likely to apply.

Microarrays can range from hundreds to thousands of array elements (low density), tens to hundreds of thousands of array elements (high density), to millions of array elements (very high density). In addition to the use of planar surfaces for microarrays, the array elements can also be immobilized on individual support particles, such as beads. In these cases the array elements are identified by the particles themselves rather than specific locations on an array. The advantages of using microscopic spots on the array include high density, fast hybridization kinetics, and low sample volumes. Microarrays greatly speed up the acquisition of data, and in some cases increase the predictive power of results, by comparison with conventional nucleic acid-based assays. This is achieved by miniaturization, multiplexing, and parallel execution of nucleic acid-based tests that traditionally are performed in tubes, plates, or capillaries as described in general chapter (1125) (see also *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* (1126), *Nucleic Acid-Based Techniques—Amplification* (1127), *Nucleic Acid-Based Techniques—Genotyping* (1129), and *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* (1130)).

The principle of microarray analysis is the specific binding of the target DNA molecules to the probes or array elements. The ordered array of rows and columns of spots allows highly automated detection and analysis. DNA microarrays are manufactured, processed, detected, and analyzed in a number of different ways and have many applications. With the aid of computers, laboratory automation, and high-resolution detection devices, microarrays produce large amounts of data and are the analytical tool of choice to unravel the molecular complexity of DNA or expressed RNA.

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are described in chapter (1127). The present chapter covers the general field of microarrays, but detailed treatment of various application-specific microarrays, including data analysis and validation, are excluded from this chapter at this time. The following sections address the major applications of microarrays, sample processing, labeling, workflow, detection, and analysis of data. Several of these sections, for ex-

ample, sample preparation and labeling, overlap with chapters (1126) and (1127), and cross references are made accordingly. Finally, regulatory aspects of microarrays will be discussed.

GENERAL PRINCIPLES OF MICROARRAY EXPERIMENTS

Types and Applications

Microarrays are most widely used in three types of analysis: gene expression, microarray-based comparative genomic hybridization (or array comparative genome hybridization, aCGH), and single nucleotide polymorphism (SNP). In brief, gene expression microarrays generally measure messenger RNA in a cell; aCGH analyzes DNA copy number variations, chromosomal additions, and deletions in genomic DNA; and SNP microarrays are used in genotyping to analyze single nucleotide polymorphisms (see (1129)). Within each type of microarray, various platforms, both manual and with various levels of automation, are available. *Table 1* summarizes the three major types and most common applications, as well as the target for each application, the probe, and the complementary nucleic acid techniques (see (1126), (1127), and (1129)).

Gene Expression Microarrays: Gene expression microarrays are used to measure the relative level at which a certain gene is expressed. They are a powerful tool for target gene discovery, molecular tumor characterization, diagnosis, classification, treatment, and monitoring of diseases. Underlying molecular subgroups that are active in diseases have been identified by observing distinct and recurring gene expression subsets found within diseased tissues. Gene expression microarrays are also used to measure changes in gene expression over a given period of time, e.g., within various stages of a cell cycle or by identification of gene mutation(s) that lead to cancerous growth. Another application for gene expression microarrays is the development of new drugs, e.g. by measuring the down-regulation of a gene associated with a particular disease to monitor the effectiveness of a new drug. When the expression levels from a set of genes are measured, the term gene expression signature (biomarker or classifier) is used. Other examples of biomarkers or classifiers are drug activity classifiers that are used to diagnose the mechanism of action of a drug or toxicity classifiers that are used to diagnose and develop dosage parameters for a patient.

aCGH Microarrays: In contrast to gene expression microarrays, aCGH microarrays target segments of DNA rather than individual genes (this is, similar to chromosomal banding and traditional comparative genomic hybridization). In an aCGH microarray, the array elements, which are large pieces of genomic DNA or specially designed oligonucleotides, are used to identify a known chromosomal location or changes. The primary advantage of aCGH is the ability to detect DNA copy changes at multiple loci in a single assay and to do so at a much greater resolution compared

Table 1. Major Types and Applications of Microarrays

Types	Application	Target	Probe	Complementary Technology
Gene Expression	Gene Expression	mRNA	Oligonucleotide/ cDNA	qRT/PCR, Northern Blotting
aCGH	aCGH CNV	DNA	Oligonucleotide/ cDNA/Pac, Yac, Bac	Cytogenetic chromosome analysis
SNP	SNP Genotyping	DNA	Oligonucleotides	Sequencing
	SNP	Amplicons	Oligonucleotide	Sequencing
	SNP	Oligonucleotide	Amplicon	Sequencing

to traditional CGH. Depending on their design, aCGH microarrays provide distinct advantages over conventional cytogenetic analysis such as karyotyping and fluorescence in situ hybridization (FISH) because they have the potential to detect the majority of microscopic and submicroscopic chromosomal abnormalities. Compared to aCGH, these conventional cytogenetic techniques have low throughput, are labor-intensive, and often require specially trained staff to perform tests in a consistent manner. aCGH microarrays are also useful for the detection of cancer by monitoring the loci of oncogenes and tumor suppressor genes.

SNP Microarrays: SNP microarrays identify the presence of known sequence polymorphisms by analysis of the pattern of hybridization to a series of probes that are specifically complementary either to wild-type or mutant sequences. If the SNP or set of SNPs associated with a particular disease are known, SNP microarrays can be used to identify a disease in an individual. SNP microarrays provide an efficient and inexpensive tool for simultaneously studying multiple genetic variations in multiple samples.

Design of Microarrays

The following sections discuss the design of the three types of microarrays described above and the suitability of the materials used for the microarray probes for each of the three types.

Gene Expression Microarray: These microarrays are the most common type of microarray in use today. The array elements consist of either cDNA derived from mRNA of known genes but of unknown sequence, or oligonucleotides for which detailed sequence information is available. Oligonucleotides are preferred array elements because of the affordable cost of synthesis and the large amount of sequence information now available for specific genes or gene fragments. These can be arrayed in specific patterns to enable accurate analysis of related gene sequences and gene families in a single hybridization assay. The following general principles apply to oligonucleotide design for gene expression microarrays:

- (1) Oligonucleotides should be 25–70 mers.
- (2) Oligonucleotides should include appropriate controls (i.e., oligonucleotides corresponding to sequences from a different organism).
- (3) All oligonucleotides should map to within 1000 nucleotides of the 3' end of cDNAs and should correspond to the coding strand.
- (4) Sequence repeats, stretches of polyA, G, C, and T and extremes of T_m s should be avoided.
- (5) Oligonucleotides should be compared to sequences in existing databases to avoid cross-reactivity (less than 70% sequence identity with nontarget sequences is preferable).

In addition to oligonucleotides, PCR amplicons and double-stranded DNA (dsDNA) are also used as probes. However, the PCR amplicons require purification to remove enzymes, salts, nucleotides, and other contaminants from the amplification process that could interfere with the binding of the probes and could also inhibit hybridization. In addition, the preparation of dsDNA probes for spotting is labor intensive and expensive. Moreover, dsDNA probes can have repetitive sequences that compromise hybridization specificity. When sequence information is unavailable, dsDNA remain the probes of choice because unknown dsDNA probes can still be used to study gene expression.

aCGH Microarrays: These microarrays traditionally use bacterial artificial chromosomes (BACs) of 100–200 kilo-base pairs per DNA segment as the array elements. However, the large-scale DNA isolations or PCR amplifications of such large-insert clones are elaborate and time consuming. As is the case in expression profiling applications, aCGH microarrays have transitioned from dsDNA targets to oligonucleotide targets. Oligonucleotide libraries or ready-made

microarrays can now be purchased, saving considerable time and effort.

SNP Microarrays: Depending on the application, SNP microarrays can use both amplicons and oligonucleotides as probes. In one of the most common formats to detect mutations in a gene sequence, the probe is that of a single gene in which the sequence differs by a single nucleotide polymorphism from the sequence of the other probes for that gene in the same microarray. For the discrimination of only one mismatch, short oligonucleotide probes (15–30 bp) maximize the destabilization caused by mispairing and are therefore used for the detection of SNPs.

Manufacturing of Microarrays

Microarray elements are deposited onto a solid support, the most widely used of which is glass. Microarray manufacturing can be divided into two main categories, direct synthesis of the probes on the microarray (in situ) or synthesis of the probes before spotting on the microarray (ex situ). In situ synthesis is generally used for higher density microarrays but is limited to nucleotides of approximately 25–100 bases. With increasing nucleotide length, the likelihood of truncated products increases because of the limited stability of building oligonucleotides in situ. In contrast, ex situ microarray manufacturing can put any premade material into a microarray format, including oligonucleotides, PCR products (amplicons), complementary DNA (cDNAs), and BACs.

The main techniques for in situ synthesis are photolithography, maskless lithography, and ink jetting. Microarrays are generally manufactured commercially, although for a small number of low-density microarrays, the end user can manufacture the microarrays using a low-throughput microarray manufacturing robotic instrument (a personal microarrayer). However, only maskless lithography and ink jetting are available for end user manufacturing. In photolithography, a glass substrate containing a photomask, which is chemically prepared so that particular nucleotides bind to specific positions, is used to synthesize the oligonucleotides on the substrate. The masks predetermine which of the nucleotides are activated when flooded with one of the four types of nucleotides. The process is repeated until the required number of bases is synthesized. The manufacture of these microarrays uses computer algorithms and multiple spots to cover the gene of interest. Maskless lithography uses a digital micromirror device that uses a solid-state array of miniature aluminum mirrors to create virtual masks that replace the physical photomasks. A computer controls the desired pattern of UV light via individual mirrors. Each digital micromirror in turn controls the pattern of UV light projected onto the glass in the reaction chamber, which is coupled to a DNA synthesizer. The UV light selectively cleaves a UV-labile protecting group at the precise location where the next nucleotide will be coupled. The patterns are coordinated with the DNA synthesis chemistry in a parallel, combinatorial manner so that hundreds of thousands of unique oligonucleotides can be synthesized in a single microarray. Ink jetting is accomplished by building up the nucleotides, base-by-base, in repetitive print layers using standard phosphoramidite chemistry. Inkjet heads similar to those used in commercial inkjet printers are connected to bottles that contain the four different phosphoramidite nucleotides that make up the building blocks of in situ nucleic acid synthesis. The advantages of inkjetting and maskless lithography are flexibility in design and the ability to make small batches of arrays quickly.

The two main types of ex situ manufacturing techniques are microspotting pins (contact printing) and piezoelectric printing (noncontact). The technology excels at printing multiple probes many times over numerous surfaces with one small-volume loading of probe. Spot size and delivery volume are controlled by the size of the end of the tip, and many tip sizes are available. A piezoelectric printing mechanism uses a small dielectric crystal in contact with a glass

capillary that holds the sample fluid. Application of the voltage results in ejection of fluid from the tip, resulting in drop volumes from hundreds of picoliters to several microliters.

General Experimental Considerations

Regardless of the type and application, all microarray experiments have a similar workflow: amplification step, labeling, hybridization, and wash steps, followed by scanning, quantitation, and reporting. The experimental design determines the type of microarray used, number of spots required, and the specific sets of nucleic acids on the microarray. The experimental design also influences the platform used, such as the number of spots, surface type, nucleic acid type, throughput, resolution, and number of colors that can be detected in a single assay. Platforms can be open (support is available from multiple vendors) or closed (support from a single vendor). In general, experimental designs that require a high density of spots and quantitation are more difficult and expensive to implement than qualitative assays.

Microarray Sample Considerations

Sample extraction, isolation, and preparation should be carefully chosen in order not to alter the ability of the resulting target to hybridize to the microarray. In general, sample preparation issues are the same for microarrays as for other laboratory techniques such as qPCR (quantitative PCR) and sequencing (described in chapters (1126) and (1127)). RNA, cDNA, genomic DNA, and PCR products are some of the sample types analyzed with microarrays. In some genotyping applications, specific alleles are used both as array elements and targets.

As with any nucleic acid technique, the quality of the nucleic acid is critical for the microarray experiment. The nucleic acid should be pure, intact, and accurately quantitated before use (1126). In particular, the presence of contaminating DNA in total RNA samples may cause problems in microarray analysis because some labeling methods label both RNA and DNA with equal efficiency. For some applications in which even trace contaminants with either RNA or DNA may interfere, pretreatment with DNase or RNase may be necessary. For example, contaminating, labeled DNA can hybridize with microarray targets leading to high-level hybridization signals that are not derived from RNA transcripts, thus resulting in an inaccurate estimation of the target RNA concentration because both nucleic acid species are quantitated at the same wavelength.

A major consideration in any microarray experiment is the availability of adequate amounts of sample nucleic acid for analysis. For example, sample from laser-capture microdissection, needle tissue biopsies, or other small clinical samples do not yield sufficient RNA (for expression microarrays) or DNA (for aCGH microarrays) and must be amplified before analysis. It is critical that the amplification procedures for amplification of mRNA be so designed that the final mixture of amplicons accurately reflect the distribution of mRNA species in the sample. Uniform amplification of genomic DNA for aCGH microarrays can be achieved by the use of multiple displacement amplification (MDA), which overcomes the nonuniform amplification of genomic DNA that occurs in PCR-based amplification methods that use degenerate oligonucleotide primed PCR (DOP-PCR). For SNP arrays where specific alleles are the target of interest, nonuniform amplification is not an issue, and samples can be amplified (and labeled) by PCR, multiplex PCR, and WGA (see (1127)).

Microarray Labeling

The targets for a microarray are a population of nucleic acids that are extracted from a sample and are appropriately

labeled. Many methods can be used for labeling targets (see (1127)), but fluorescent labeling is the most widely used because it offers high sensitivity and a superior dynamic range. An added advantage is the ability to detect two or more signals in a single experiment. The method of labeling depends on the microarray type. The two methods used to fluorescently label targets for gene expression microarrays, direct and indirect labeling, have been described in (1127). In general, the second method (indirect labeling), in which the label is added via a linker, requires less starting material and is less expensive. Published reports have shown that this method yields results similar to those obtained from directly labeled samples. In microarray aCGH, a patient's DNA and reference DNA (300–1000 ng) are typically fluorescently labeled with red and green fluorescent dyes, respectively, often using a random priming protocol. Random prime labeling uses a high concentration of Klenow enzyme whereby genomic DNA is digested with restriction enzymes and hybridized with random primers. The primers are extended by the 5'–3' polymerase activity of Klenow, resulting in a strand displacement activity with the direct incorporation of labeled nucleotides. SNP microarrays using oligonucleotides as array elements are labeled using fluorescently labeled nucleotides in both single and multiplexed PCR reactions, followed by a purification step to remove unincorporated dyes. Where amplicons are used as array element, labeled oligonucleotide probes are synthesized using phosphoramidite chemistry.

Hybridization and Wash

Hybridization should be carried out under conditions that minimize annealing of noncomplementary fragments. The wash steps following a hybridization reaction are optimized to provide the highest possible specificity, signal-to-noise ratio, and reproducibility (see (1126)). Before hybridization, double-stranded probes and targets should be denatured, and nonspecific sites should be blocked. Microarray surface chemistries are designed to capture all nucleic acids with high efficiency, so the free-binding groups on the surface must be blocked or inactivated to prevent nonspecific binding of labeled material that could compromise the signal-to-noise ratio. Surfaces are blocked and washed with various aqueous-based buffers that typically include salts, detergents, and blocking agents such as low molecular weight, hydrolyzed proteins. The purpose of the posthybridization washes is to remove all unattached and nonspecifically bound label from the surface and probes. In general, both automated and manual washes are done in saline sodium citrate/sodium dodecyl sulfate (SSC/SDS) buffers of various concentrations and at different elevated temperatures depending on the stringency required. After the final wash step, microarrays using fluorescent targets are dried immediately by centrifugation or in a nitrogen stream. Hybridized microarrays must be stored in the dark and should be scanned as soon as possible. Some fluorescent dyes used in microarray analysis are subject to degradation by environmental ozone, and in these cases ozone levels in the experimental environment must be less than 5 parts per billion. Specialized ozone-free hoods are made to protect microarray dyes.

Microarray Detection

Regardless of the microarray type, each spot on a microarray represents a unique probe sequence to which a single, labeled target is bound, and this specific binding allows detection and quantitation of the target. This is achieved by the emission of light (photons) at a particular wavelength by the fluorescently labeled duplexes when the microarray is exposed to light of specific wavelength from an excitation source. The emitted fluorescent light is converted to electrical energy by a detector. The detector is either a photomultiplier tube (PMT) or a charge-coupled device (CCD) with

specially designed optical paths that collect the raw data from microarrays (scanning). The detector filters and optical paths are designed to detect specific fluorescent dyes at sufficient resolution while eliminating crosstalk when two or more dyes are used on a single microarray. The resulting signal is proportional to the number of photons emitted by the microarray. These signals are used to create a digitized image showing the presence and quantitation of specific targets.

Samples can be scanned from a single wavelength channel or can be sequentially scanned from two channels. For instance, for a single-channel microarray platform, a sample is typically labeled with a fluorophore that emits a signal in the red channel. For a dual-channel microarray format, a second sample can be labeled with a dye that emits in the green channel. Dual labeling is used in some experimental designs, such as expression microarrays, to measure the overexpression of a gene associated with a disease state. In such experiments, cDNAs derived from the mRNA of normal and diseased tissues are differentially labeled, mixed, and tested on the same slide in a competitive hybridization reaction. The resulting ratios of the two colors reflect the relative abundance of the labeled material within each sample. Similarly, calculating the fluorescent ratios from each target on an aCGH microarray allows the mapping of gains and losses for a chromosome of interest.

Microarray Image Processing

Most microarray scanners detect and acquire one, two, or more colors (via one, two, or more channels). The optical path of the system minimizes overlap between the spectra (crosstalk) and allows acquisition of two spectrally separate images. In many cases, the images are represented as a red and a green image. When two colors are used, the ratio of the two fluorescence images eliminates artifacts caused by regional bias and irregular spot size. When one color is used, the fluorescence signals from two or more microarrays are normalized and can be compared with each other. Diameters of spots printed on the arrays range from 10 μm to just under 1000 μm , and the resolution of scanners ranges from 1 to 50 μm . Thus, depending on scanner resolution, variable amounts of pixel data can be collected per scan over an entire microarray.

MICROARRAY IMAGE ANALYSIS

The analysis of scanned images usually involves three tasks: spot finding or gridding, image segmentation, and spot quantification.

Spots are initially assigned specific coordinates, and the process of spot finding or gridding can range from manual to fully automatic, depending on the image-processing software used. This takes into account the individual size and shape of each spot and adjusts for uneven rows and columns that may be produced by the printing process.

The process of segmentation partitions the entire image to foreground or background pixels and relies on the spatial and intensity properties of each pixel. There are four main types of signal segmentation that have been used for spotted arrays. The simplest method is spatial segmentation which places two circles (inner and outer circles) of fixed but different sizes over each spot to demarcate probe signal from the immediate background signal. On the one hand, because of the irregularity of spot sizes on some microarrays, the actual area inner circle may be larger than the diameter of a spot and thus will contain background pixels. On the other hand, artifacts and signal can be found in the area between the inner and outer circles and contribute to the background signal. The second method, intensity-based segmentation, distinguishes signal pixels from background pixels based on the spot intensities within a target region. In this case, a certain percentage of pixels within the top-

ranked intensities may be classified as signal pixels. The advantages of this method are simplicity and speed, but the drawback is the inability to distinguish between artifacts and signal and the tendency to detect low signals that are close to background. The third method is a statistical approach known as Mann-Whitney segmentation that combines information from spatial and intensity-based analysis. Here, background pixels located outside the inner circle set are used to determine a threshold intensity level for a signal within the inner circle. The limitation of this method is that a large amount of spot irregularities and artifacts can reduce its accuracy. The fourth method, the trimmed measurement segmentation method, also combines spatial and intensity information and measures signal distributions inside and outside the inner circle. The method trims the upper and lower extremes of each distribution to allow removal of signal from artifacts and incorrectly located background or foreground signal pixels.

The main assumption of spot quantification is that the total fluorescent intensity from a spot is proportional to the expression level of the labeled transcript. This is highly dependent on a number of factors, including target preparation, hybridization conditions, and signal detection within the linear dynamic range. If the amount of probe deposited during the microarray manufacturing procedure varies from spot to spot and from array to array, thus resulting in different sized spots, the sum or total signal intensity can be variable and inaccurate. To correct for this variation, microarrays should be spotted via homogenous surface chemistry that has a fixed binding capacity. This ensures the same amount of probe at each spot location. Alternatively, spots can be quantified by taking the mean, median, or mode of intensities of all signal pixels determined to be foreground signal. The more robust methods that protect against outlier signals are the trimmed mean (where a certain percentage of top and bottom signals are trimmed before calculation of the mean) and median signal intensities. When two different fluorophores are used, the intensity ratio can be used to correct for variable probe amounts and can be calculated from mean, median, and mode intensities from each channel.

MICROARRAY DATA ANALYSIS

Particularly dense formats of microarrays that contain tens of thousands to millions of probes per chip or slide generate a large volume of raw data per array, which requires the use of specialized data-analysis software. Microarray software programs are designed to extract primary data, normalize the data to remove the influence of experimental variation, and link probes to relevant gene and sequence-derived targets. Software programs are also available to apply statistical methods, analyze, visually display, and manage data in order to extract biologically meaningful information. The major parts of data analysis are normalization, background correction, and ratio calculation.

Normalization systematically adjusts microarray raw data in an effort to reduce the variability brought about by differences in the manufacture and processing of the microarrays and by technical variables so that true biological differences between samples can be detected. The wide range of normalization methods precludes a detailed discussion of the topic in this chapter, and currently there are no standards for normalization. Commonly used algorithms are selected based on the microarray type, the number of fluorophores used, and the samples being studied. Some methods are built into the manufacturer's software, but others are available from commercial sources or open-source software providers.

Background correction eliminates low levels of noise in microarrays stemming from both the inherent noise of the detection instruments and from the surface chemistry used in manufacturing. Several contaminants acquired from mi-

croarray processing can cause high levels of background that must be corrected before data analysis.

In two-color microarrays, the ratio of signal intensities of array elements of two co-hybridized samples is used as a relative measure of gene expression. In single-channel systems, the ratio can be calculated between signals taken from two different samples (one sample is a reference sample) hybridized on individual microarrays. Thus, the resulting data from microarrays does not represent an absolute quantification but rather a relative level of RNA or DNA against a reference sample or control.

Quality Control and Quality Assurance

As with any diagnostic assay, quality control and quality assurance are critically important. Microarrays must demonstrate robustness and reproducibility. The general quality control and assurance steps outlined in chapter (1127) for nucleic acids and NAT also apply to microarrays. Unlike other diagnostic tests, no reference reagents are available at present for quality control of microarrays, and regulatory guidance is emerging. FDA has issued a draft guideline titled "In Vitro Diagnostic Multivariate Index Assays." This guidance addresses the definition and regulatory status of a class of in vitro diagnostic devices referred to as in vitro diagnostic multivariate index assays (IVDMIAs), and microarrays fall into this category. The guidance addresses premarket pathways and postmarket requirements with respect to IVDMIAs.

Several unintended sources of variability that are specific to microarrays can extensively affect signal intensities and the accurate derivation of a true signal that accurately reflects the labeled transcript. A major source of variability is spot quality. Measurements of spot quality at the processing stage permit removal of spots with poor or questionable quality. Other sources of variability are artifacts, for example, regional shifts (rise or fall) in an array's overall signal that can be visualized within single chips or in-composite data derived from multiple chips. These changes can be distinguished from actual variability because they are nonrandom, and patterns can be detected by visualizing signals over the entire area of the chip. When dyes of different spectral properties are used to label two different samples in a competitive hybridization reaction using a single array, differences may arise because of labeling bias rather than gene expression level. For instance, the green channel may appear consistently brighter than the red channel despite the fact that there are no real differences in expression. Hybridization with reverse dyes can ensure detection and elimination of dye bias effects. As with any quantitative assay for RNA, the integrity of the sample affects its measurement, and sample quality is an important determinant for accuracy. For instance, because labeling is directed from the 3' to the 5' end but RNA degrades from the 5' end, degraded RNA leads to high 3'/5' ratios, resulting in nonuniform labeling across the entire transcript. Finally, variability can be introduced during the processing of microarrays, which is a relatively complex procedure that involves multiple steps such as labeling, hybridization, washing, and staining (technical variables). Such variability can mask true differences in the samples tested.

When used as a diagnostic test, the microarray should demonstrate robustness, reproducibility, a high degree of correlation to the original format, and reliable prognosis prediction. The microarray ideally should contain at least 2–3 replicate spots for each reporter gene to ensure intra-assay reproducibility. With a two-channel microarray, a reference sample pool can be hybridized in the complementary fluorescent channel so that data can be expressed as log ratios, which reduces the need for extensive normalization. Interassay reproducibility of test results and stability over time can be tracked by using a number of reference samples that, when labeled and hybridized, represent a spectrum of predictive endpoints (for instance, high risk, borderline risk, low risk) and that should fall within a

predetermined range of results. Failure of these controls should result in rejection of results of samples in the same assay run. If the assay is performed at many sites, site-to-site reproducibility is imperative and must be assessed. The reproducibility of the assay with regard to tissue extraction also must be determined, and the quality of tissue specimens or RNA should be specified clearly (for instance, percentage of tumor cells within a specimen).

In conclusion, microarray experiments should be carefully designed and conducted in order to minimize variability and to yield data that accurately relate to the samples analyzed. In addition, biomarkers of interest should be analyzed and verified using an alternative platform such as qRT-PCR that should be shown to be reproducibly detected in the same and different samples. The development of reference standards, especially when microarrays are used as diagnostic tests, is the next step to ensuring the quality and validation of microarray results. With the shift from custom-built to commercial microarrays, issues with reproducibility, standardization, and quality control have been largely addressed by the stringent quality controls used in commercial manufacturing.

(1129) NUCLEIC ACID-BASED TECHNIQUES—GENOTYPING

INTRODUCTION

This chapter outlines techniques for detecting single-base DNA differences and other types of polymorphic DNA sequences that occur in the three billion bases that make up the human genome. The most common genetic variation is a single nucleotide polymorphism (SNP), which is a simple change in one base of the gene sequence. SNPs occur on average every 1000 bases and account for a significant amount of inter-individual variability. SNPs can predispose individuals to disease or influence their response to a drug. Approximately 1.8 million human SNP loci have been identified, and more are likely to be discovered in the coming years.¹

Common approaches for detecting SNPs and other types of polymorphic DNA sequences are described in the following sections. These approaches encompass a variety of techniques, such as nucleic acid amplification techniques (NAT), real-time NAT, and microarrays, the principles of which are covered in more detail in related chapters. This chapter focuses on the specific modifications of the techniques that are necessary to enable detection of single base differences.

SNP Genotyping Technologies

Although the usefulness of studying SNPs for gene mapping and disease association studies is apparent, a single standardized procedure for SNP genotyping has not been adopted. Various approaches for performing SNP genotyping have been developed to meet a wide range of needs, including throughput capacity, ease of assay design, accuracy, and reliability. Available procedures can also be divided according to whether they are based on identifying known SNPs or whether they can be used to screen for unknown SNPs. To identify the most appropriate SNP genotyping procedure for a specific application, the throughput require-

¹ Database of Single Nucleotide Polymorphism (db SNP) Build 128 is available from National Center for Biotechnology Information (NCBI), <http://www.ncbi.nlm.nih.gov/projects/SNP/index.html>.

ments in terms of the number of SNPs to be analyzed per sample (multiplexing level) and the sample throughput need to be determined because different approaches may work best depending on these requirements.

Most procedures used for genotyping SNPs depend on polymerase chain reaction (PCR) amplification of the genomic regions that span the SNPs followed by the actual genotyping reaction. PCR provides the required sensitivity and specificity for distinguishing between heterozygous and homozygous genotypes in large, complex genomes. The difficulty of designing and carrying out multiplex PCR reactions limits the throughput of many of the current SNP genotyping assays. The following sections outline several of the major approaches currently in use for SNP genotyping. In many cases the underlying technology can be modified to meet the specific application requirements in terms of sample throughput and number of SNPs detected. In general, real-time PCR-based procedures are better suited to higher sample numbers, and array-based procedures are better suited to the simultaneous detection of many SNPs. Newer technologies based on multiplexed array formats are also emerging and will be suitable for high sample numbers and many SNP applications.

Sequencing

Sequencing is the definitive procedure for DNA analysis, and its use for SNP detection allows unambiguous identification of base changes (see *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* (1126) for nucleic acid sequencing). The standard technology is expensive, and the procedure is time consuming and labor intensive and suffers from low sample throughput. Sequencing is a useful confirmatory tool, and it has applications in situations when other technologies are not appropriate, but is not the most cost-effective solution for the majority of SNP genotyping applications that require the identification of only one or a few bases.

Restriction Fragment Length Polymorphism Analysis

The first widely used procedure for the detection of polymorphisms exploited alterations in restriction enzyme sites caused by SNPs, leading to the gain or loss of cutting events. PCR–restriction fragment length polymorphism (RFLP) analysis comprises PCR amplification of a fragment of interest and subsequent digestion with a restriction enzyme. The fragments produced are typically analyzed by a size fractionation procedure, usually gel electrophoresis. Because of its simplicity, the procedure has been and still is extensively used, although it entails certain limitations: only a subset of polymorphisms that reside in an endonuclease restriction site can be studied with the conventional procedure; incomplete digestion due to suboptimal processing can produce misleading digestion patterns; and the procedure is less amenable to automation than are other SNP genotyping procedures.

Probe Hybridization

The basis of many SNP genotyping procedures are DNA hybridizations that make use of the stronger binding of a DNA probe to a perfectly matched complementary target than to a target that contains a single base mismatch. The ability of hybridization with allele-specific oligonucleotides (ASO) to detect a single base mismatch was first shown in the late 1970s and subsequently was used to detect the sickle-cell mutation in the beta-globin gene by Southern blot hybridization. The invention of PCR facilitated the further development of probe-based assays for genotyping SNPs in complex genomes.

The thermal stability of a hybrid between an ASO probe and its SNP-containing target sequence is not only determined by the stringency of the reaction conditions but also by the secondary structure of the target sequence and the nucleotide sequence flanking the SNP. Therefore, prediction a priori of the reaction conditions or the sequence of the ASO probe that will allow optimal discrimination between two alleles using ASO hybridization is difficult. These parameters should be established empirically and separately for each SNP. Consequently, there is no single set of reaction conditions that would be optimal for genotyping all SNPs, which makes the design of multiplex assays based on hybridization with ASO probes an extremely difficult task.

One approach to counter the problem of assay design is to carry out multiplex ASO hybridization reactions on arrays that carry multiple probes for each SNP that will be analyzed. This involves using probe sets in which the SNP occurs at different positions along the probes. It becomes feasible to include large numbers of ASO probes per SNP when one uses high-density arrays that can carry as many as 10^6 probes per cm^2 .

Another approach is to use base analogues such as locked nucleic acid (LNA), which is described in detail in *Nucleic Acid-Based Techniques—Amplification* (1127). For applications that involve few SNPs but many samples, homogeneous real-time PCR approaches have been developed. These include the use of fluorescent probe chemistries such as hydrolysis probes, stem-loop probes, and FRET (fluorescence resonance energy transfer) hybridization probes. The principle of these assays is discussed in more detail in *Nucleic Acid-Based Techniques—Amplification* (1127). For SNP detection, the basis of many assays is the selective binding of the ASO probe to its perfectly matched target sequence, resulting in energy transfer and generation of a fluorescence signal. Probes designed with specific secondary structures tend to form a stem-loop structure that destabilizes mismatched hybrids, increasing their power of allele distinction as compared with that of linear ASO probes. Hydrolysis probes modified with minor groove-binder molecules that increase target affinity show improved powers of allele discrimination. The use of two probes, each labeled with a different reporter fluorophore, allows both SNP alleles to be detected in a single tube. Limited multiplexing can be achieved by using probes labeled with different fluorophores. In the fluorescent probe-based assays, the increase in fluorescence due to accumulating PCR product is usually monitored in real time in 96-well or 384-well microtiter plates. Alternatively, the fluorescence generated from the two alleles can be measured after completion of the PCR. In this case the results are expressed as a signal ratio that reflects the hybridization of the two oligonucleotides to the target sequence, and so differences in amplification efficiency between samples do not affect interpretation of the genotyping results.

A third approach involves heating the reaction after PCR has been completed in order to disassociate the probe from the target. Each duplex has its own specific T_m , which is defined as the temperature at which 50% of the DNA becomes single stranded. The T_m depends on the stability of the probe–target duplex. Perfectly matched probe–target duplexes have a greater stability and hence a higher T_m than does the same duplex containing a single base mismatch. By continuously monitoring the fluorescence during the heating phase, analysts generate a “melt curve” that measures the changes in fluorescence that result when the probe denatures, or “melts,” away from the amplicon. This approach can be used only for systems that do not rely on hydrolysis of the probe to generate a signal and is therefore not suitable for hydrolysis probe assays.

Because no post-PCR processing or label-separation steps are required, homogeneous real-time PCR assays are simple to perform, making them useful for high-throughput genotyping applications. The optimal probes must be designed individually for each SNP, and the assays are therefore most efficient when a limited number of SNPs is analyzed. The cost of probes modified with fluorescent and

quenching moieties may also be a limiting factor in the high-throughput application of the assays.

Primer Extension

In this technique, an oligonucleotide is used to prime DNA synthesis by a polymerase enzyme, as performed in a standard PCR or sequencing reaction. Variations of the technique exist. Allele-specific PCR uses two primers, each fully complementary to one of the SNP alleles, with the SNP position being at the 3' end of the primer, and with a common reverse PCR primer to selectively amplify the SNP alleles. Because only perfectly matched oligonucleotides will prime DNA polymerase extension, product will be detected only from the reaction containing the perfectly matched primer.

Agarose gel electrophoresis is used to detect the amplified products, although homogeneous, real-time, allele-specific PCR approaches have also been developed using primers labeled with different fluorophores or a fluorescent dye that intercalates with the double-stranded PCR products or by performing amplicon detection using probes such as hydrolysis and hairpin (stem-loop) probes. When using intercalating dyes or labeled allele-specific PCR primers without a consecutive target-specific detection reaction or size-separation step, one may find that the specificity of the procedure may be compromised owing to primer-dimers and other spurious amplification products that will not be distinguished from the actual PCR product. A limitation of all variants of allele-specific PCR is that the reaction conditions or primer design for selective allele amplification must be optimized empirically for each SNP. Like the hydrolysis and hairpin probe assays, the homogeneous allele-specific PCR procedures are best suited for the analysis of a limited number of SNPs in large sample collections. Array-based approaches for greater SNP multiplexing have also been developed.

In procedures based on single nucleotide primer extension (sometimes known as minisequencing), allele discrimination is based on the high accuracy of nucleotide incorporation by DNA polymerase. A primer is used, and its 3' end is positioned on the base just preceding the SNP to be tested. The DNA polymerase is then used to incorporate labeled ddNTPs, each labeled with different fluorescent dyes. After the labeled oligonucleotides are separated from the nonincorporated ddNTPs, the results can be scored on a fluorescence plate reader. In addition to fluorescent tags, ddNTPs may be labeled with biotin or haptens and then detected indirectly through antibodies conjugated to alkaline phosphatase or peroxidase using colorimetric or chemiluminiscent markers in ELISA formats.

Multiplexing of this procedure has also been described to reduce costs and improve throughput. In these procedures, the different loci genotyped simultaneously are separated either by gel electrophoresis or by hybridization to arrayed tags. Primer extension directly on a solid support such as a microarray is also possible. The immobilization of the single-stranded primers on the solid support may be through biotin-avidin-streptavidin reaction or covalently via 5' disulfide groups.

Mass spectrometry using techniques such as matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) can also be used to determine the identity of the ddNTP incorporated based on mass. A difficulty with MALDI-TOF MS is that the primer extension products must be rigorously purified before measurement to avoid background from biological material present in the sample. Such enzyme-assisted procedures have proven to be more robust and to provide more specific allele discrimination than does ASO hybridization at similar reaction conditions. These features are advantageous for high-throughput applications because the effort required for assay design and optimization is minimized.

Ligation

In the oligonucleotide ligation assay (OLA), oligonucleotides are designed so that they meet at the position of the SNP to be tested. Enzymatic joining, using a DNA ligase, occurs only when the match is perfect. The test is usually performed by designing two oligonucleotides specific for each allele and labeled differently on one side of the SNP, and one common oligonucleotide on the other. Detection of the alleles can be performed directly in the microplate wells by colorimetric approaches. Multiplexing and the use of gel separation have also been described.

OLA has also been used in microarray formats with one of the ligation probes immobilized or with immobilized single stem-loop probes. Alternatively, ligation can be carried out in solution followed by capture of the ligation products on microarrays or on microparticles that carry a generic set of oligonucleotides that are complementary to a "tag" sequence on one of the ligation probes. In practice, thermostable ligases are frequently used for genotyping SNPs in combination with PCR before allele-specific ligase detection reactions. Because the reaction mechanisms for PCR and ligation are different, the reagents for both reactions can be combined. This feature is used in a homogeneous, real-time PCR assay with ligase-mediated genotyping and detection by FRET. Compared with DNA-polymerase-assisted primer extension procedures, a drawback of the OLAs is that detection of each SNP requires three oligonucleotides, which increases the costs of these assays.

Padlock probes are linear oligonucleotides, the ends of which are complementary to the target and have a central stretch of random sequence. When perfectly hybridized to their target sequence, padlock probes can be circularized by ligation, whereas a mismatch with the target sequence prevents ligation. Circularized oligonucleotides can act as templates for DNA-polymerase-assisted rolling circle amplification (RCA). RCA can be used to amplify the ligated circularized padlock probes to a level required for detecting single-copy sequences. A homogeneous, isothermal assay for genotyping individual SNPs in a microtiter plate format has been devised by combining exponential amplification of ligated padlock probes using a branched rolling circle amplification reaction with detection by energy-transfer-labeled hairpin primers.

Displacement

The invader assay uses the property of flap endonucleases (FENs) for removing redundant portions (flap) from the 5' end of a downstream DNA fragment overlapping an upstream (invader) DNA fragment. An invader oligonucleotide is designed with its 3' end on the SNP to be tested. Two oligonucleotide signal probes are also designed, overlapping the polymorphic site and each corresponding to one of the alleles. After displacement of the signal probes by the invader probe, FEN-mediated cleavage occurs only for the perfectly matched allele-specific signal probe. Generation of the cleaved fragment is monitored by using it in a second reaction as an invader probe to cleave a FRET probe. This assay does not require PCR amplification of the locus to be tested, and scoring can be done using a simple fluorescence plate reader.

Pyrosequencing

In the pyrosequencing procedure, primer extension is monitored by enzyme-mediated luminometric detection of pyrophosphate (PPi), which is released on incorporation of deoxynucleotide triphosphates. The genotype of an SNP is deduced by sequential addition and degradation of the four nucleotides using apyrase in a dedicated instrument that operates in a 96-well or 384-well microtiter plate format. Using

pyrosequencing, the apparatus can determine short 30 to 50 bp sequences of DNA that flank an SNP. A limitation of the procedure is that the sequential identification of bases prevents genotyping of several SNPs per reaction in diploid genomes. An advantage of the procedure is that any new polymorphism will be detected. However, specific equipment is needed for the injection of the nucleotides.

Single-Strand Conformation Polymorphism and Heteroduplex Analysis

Single-strand conformation polymorphism (SSCP) and heteroduplex analysis were among the first procedures established for the detection of SNPs. Conventional SSCP analysis involves denaturing PCR-amplified fragments and subsequent formation of sequence-specific secondary and tertiary structures of the single strands during nondenaturing gel electrophoresis. The electrophoretic mobility then depends on the 3-D shape of the single-stranded molecules. One single base difference in DNA fragments of up to 300 bp will usually change the conformation in a way that can be detected by nondenaturing PAGE.

The traditional polyacrylamide gels and ^{32}P -labeled fragments are frequently being replaced by fluorescently labeled fragments and automated capillary electrophoresis. The simplicity of the procedure, combined with automation and short analysis time, contribute to high-throughput analysis at relatively low cost. If the denatured PCR products are allowed to slowly re-nature, they form DNA duplexes. The duplexes with the same sequence on both strands (homoduplexes) or with a single base pair mismatch on one strand (heteroduplexes) have different electrophoretic mobility in a native gel. In the case of a single base pair substitution, the heteroduplex can easily be separated from a homoduplex.

In other versions of the technique, denaturing high-performance liquid chromatography (DHPLC) is used for the separation of the heteroduplex and homoduplex strands. The mutation analysis with DHPLC can be almost totally automated with an autosampler on one end and a fraction collector on the other. Analysis is rapid (about 5 minutes per sample), and simple evaluation of data distinguishes between simple and multiple peaks in the elution profiles, allowing lengths as large as 1.5 kb of DNA to be analyzed. A disadvantage may be the recommended use of Pfu DNA polymerase, which, as a high-fidelity enzyme, allows sharper peaks but may be less successful in amplifying some regions.

Short Tandem Repeat Profiling

A short tandem repeat (STR) is a type of DNA polymorphism that occurs when a pattern of two or more nucleotides is repeated and the repeated sequences are directly adjacent to each other. The pattern can range in length from 2 to 10 bp (e.g., CATG_n in a genomic region) and is typically in the noncoding intronic, or upstream/downstream regions. By examining several STR loci and counting how many repeats of a specific STR sequence there are at a given locus, one can create a unique genetic profile of an individual. Currently more than 10,000 STR sequences in the human genome have been published. STR analysis has become the prevalent analysis procedure for determining genetic profiles in forensic cases. STR analysis in the field of forensics came into popularity in the mid to late 1990s. The STRs in use for forensic analysis are tetra- or penta-nucleotide repeats (4 or 5 repeat units) because these give a high degree of error-free data while being robust enough to survive degradation in nonideal conditions. Shorter repeat sequences tend to suffer from artifacts such as stutter and preferential amplification; several genetic diseases are associated with tri-nucleotide repeats, including Huntington's disease. Longer repeat sequences suffer more highly from envi-

ronmental degradation and do not amplify by PCR as well as do shorter sequences.

The analysis is performed by extracting nuclear DNA from the cells of a forensic sample of interest and then PCR amplifying specific polymorphic regions of the extracted sample. Once these sequences have been amplified, they are resolved either by gel electrophoresis or capillary electrophoresis, which allow the analyst to enumerate the repeats of the STR sequence in question. If the DNA is resolved by gel electrophoresis, the DNA can be visualized either by silver staining or an intercalating dye such as ethidium bromide or, as in most modern forensics labs, by fluorescent dyes. Instruments built to resolve STR fragments by capillary electrophoresis also use fluorescent dyes. In the United States, 13 core STR loci have been selected as the basis by which an individual genetic profile can be generated. These profiles are stored in local, state, and national DNA databanks such as the Combined DNA Index System (CODIS).

Forensic reference materials are available. The DNA Profiling Standard is composed of well-characterized human DNA in two forms: genomic DNA and DNA to be extracted from cells spotted onto filter paper.

ASSAY VALIDATION CONSIDERATIONS

The difficulty in reproducing and validating existing and emerging SNP genotyping assays due to factors such as variation in performance of PCR thermal cyclers, efficiency of different enzymes, personnel, and the presence of PCR inhibitors in the sample matrix (discussed in more detail in *Nucleic Acid-Based Techniques—Amplification* (1127) for general NAT assays) can hamper appropriate implementation of the technologies. Also, in the clinical laboratory the use of in-house assay formats often makes comparisons between laboratories difficult. Incorrect diagnosis of a genetic mutation can have significant consequences, so accuracy of 99.99% or higher is essential for such assays. To determine the accuracy of a technology, the new procedure should be validated on multiple samples in which the genotype has been previously determined with a gold standard procedure, such as sequencing. Even with the most accurate procedure of analysis, sample preparation and amplification and detection procedures must be optimized to eliminate any potential inaccuracies.

Some genotyping errors can be minimized by careful planning of the laboratory procedures, the inclusion of well-defined controls, and increased automation. However, errors due to the processes used for genotyping are sometimes difficult to overcome and need to be taken into account. The types of errors and the frequency with which they occur differ between different approaches. Situations in which preferential amplification of one allele or nonspecific probe hybridization occur can all result in SNP miscalls. Additional unanticipated polymorphisms present within the primer/probe sequences can lead to amplification bias, highlighting the need for careful assay design and validation using alternative techniques. Limited and degraded samples can also result in preferential allelic amplification due to chance PCR priming events at low copy number.

It is preferable to have a no-call result, which would require the test to be repeated, than a miscall that provides incorrect results that are subsequently reported. Performance of replicate assays may also help to ensure accuracy. Data interpretation can also affect accuracy. Wild-type, heterozygous, and homozygous mutant results should be clearly distinguished from one another, and a well-defined measure of uncertainty should be attributed to them. Proficiency testing schemes and ring-trials go some way toward ensuring that individual assays are fit for the purpose for which they are intended for specific applications and that the staff performing them are competent. Sharing of technical information for assay design and sample preparation will also help. The availability of reference panels of well-charac-

terized samples aids assay design and evaluation and allows sound interlaboratory comparisons to be made.

(1130) NUCLEIC ACID-BASED TECHNIQUES—APPROACHES FOR DETECTING TRACE NUCLEIC ACIDS (RESIDUAL DNA TESTING)

INTRODUCTION

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in *Nucleic Acid-Based Techniques—General* (1125). This chapter covers the analytical procedure used to quantify residual DNA in biopharmaceuticals.

Quantification of residual DNA impurities in biopharmaceuticals is based on safety concerns. The cells used to produce biopharmaceuticals can be sources of a range of complex, heterogeneous, and potentially unsafe impurities, and host cell DNA is among these. Much of the safety concern associated with residual DNA in biopharmaceuticals lies in the possibility that host cell DNA, particularly continuous-cell-line DNA, may result in tumors or adverse reactions. Cells used to produce biopharmaceuticals may possibly carry viruses or harbor harmful nucleic acid, and the residual DNA in a given biopharmaceutical product may be infectious. Although animal testing has shown that extraneous DNA can cause tumors or infections, no reports to date have demonstrated this risk in humans. Therefore, some regulatory agencies have allowed a target of 100 pg or less of residual DNA per dose in biopharmaceuticals, and levels up to 10 ng of residual DNA per dose may be considered, depending on the source of the residual DNA and the product's route of administration.

One can address residual DNA in biopharmaceutical processes in two ways: by validating clearance during process validation or by monitoring residual DNA levels by routine testing of the drug substance. The level of concern regarding residual DNA can be tied to the potential source of the residual DNA (e.g., infectious viral DNA) and the route of administration, so the residual DNA specification and procedure for monitoring DNA clearance for a given product should be developed in consultation with regulatory agencies. Regardless of whether routine testing of a drug product is used to determine residual DNA content or whether DNA clearance is demonstrated by process validation, analytical procedures for the quantification of residual DNA are required. The analytical procedures used to determine the residual DNA content of biopharmaceuticals can include hybridization, instrumentation based on DNA-binding protein, quantitative PCR (q-PCR), or other DNA amplification methods. The expectation is that the analytical procedure used to quantify residual DNA in biopharmaceuticals has a detection limit approximating 10 pg per dose. The assays based on hybridization, DNA-binding protein, and q-PCR are typically the techniques of choice because they can meet the sensitivity expectation.

SAMPLE PRETREATMENT

Analysis of residual DNA requires accurate quantification of pg levels of DNA in mg (or larger) quantities of product. The sample itself, whether it is a protein or other chemical

entity, can create sample matrix effects that must be overcome in order to yield a useful assay. Protein samples may require only digestion with proteinase (e.g., Proteinase K, Pronase) to allow the analytical method to quantitatively recover the residual DNA. Treating the sample with a detergent may be required to dissociate the residual DNA from the sample matrix. Traditionally, extraction methods based on phenol and chloroform, followed by ethanol precipitation, have been applied to the purification of DNA in molecular biology research. The phenol/chloroform extraction technique may be a useful pretreatment for residual DNA samples prior to analysis. Because of the typically low levels of residual DNA present in samples, quantitative DNA recovery with ethanol precipitation may be difficult. For this reason, a carrier molecule (e.g., glycogen) may be necessary to aid in DNA recovery if this technique is used.

A commercial kit is available¹ and has been used successfully for pretreatment of residual DNA samples. The commercial kit uses a chaotrope (sodium iodide) and a detergent (sodium *N*-lauroyl sarcosinate) to disrupt the association of the DNA with the sample. The DNA is then co-precipitated using glycogen as the carrier molecule in the presence of isopropanol.

Each of these pretreatment techniques may yield acceptable results, or analysts may combine the techniques to obtain acceptable recovery of the residual DNA from the sample. Sample extraction is an extra handling step that may cause the incomplete recovery of the residual DNA or may introduce environmental DNA into the sample, so great care must be taken during any sample manipulations. Addition of DNA-spiked samples in the residual DNA assay is a common practice. A recovery of 80% to 120% of the spiked DNA is an acceptance criterion often applied to residual DNA assays to ensure that the assay yields acceptable results. When sample characteristics (e.g., matrix effects, sample preparation method) make achieving a recovery acceptance criterion of 80–120% impractical, then correcting the observed DNA concentration by the load recovery percentage is also an acceptable approach. During the qualification of a residual DNA assay, some scientists treat the samples with DNase I to degrade the DNA in the sample in order to demonstrate that the assay response was due to DNA and not some other sample component.

HYBRIDIZATION-BASED RESIDUAL DNA ASSAY

The first residual DNA assays were based on DNA hybridization, wherein a DNA probe created from host cell DNA detects and quantifies the amount of complementary DNA present in the product under assay. Double-stranded host cell DNA consists of two complementary strands of DNA that are held together by hydrogen bonding. The double-stranded DNA in the test sample is denatured to single strands and immobilized to a membrane, typically a nitrocellulose or nylon membrane. The sample is probed using host cell DNA that has been denatured and labeled. The host cell DNA probe is not a specific sequence but is prepared by a random labeling procedure during which a radioactive or fluorescent label is introduced into the host cell DNA to produce the probe. When the denatured labeled DNA probe is brought into contact with the membrane-immobilized DNA, the probe will bind to complementary sequences of the host cell DNA. If the probe is radioactive, the membrane is placed against autoradiography film for a sufficient length of time, the film is developed, and a dark spot will be observed where the test DNA was immobilized. If the probe has a fluorescent label, the intensity of the spots is determined using a phosphor- or fluorescence-imaging system. The intensity of the spot is proportional to the amount of probe that was hybridized to the test DNA and therefore is proportional to the amount of residual DNA in

¹ DNA Extractor Kit, Wako Chemicals.

the sample. The intensity of the spot can be compared visually with the intensity of spots that correspond to a standard curve yielding semi-quantitative results (i.e., visual quantitation), or the intensity can be determined using an instrument (e.g., densitometer) to create a quantitative value that is compared with the values obtained from the standard curve.

DNA-BINDING PROTEIN-BASED RESIDUAL DNA ASSAY

Instrumentation is commercially available for the quantitation of residual DNA in biopharmaceuticals. The instrumentation requires reagents that use DNA-binding protein and antibodies targeted for DNA in a four-step analytical procedure. The *first* step requires that the DNA be denatured into single-stranded DNA by sample heating. The denatured DNA is mixed with a single reagent that contains DNA-binding protein that is conjugated with streptavidin and a monoclonal anti-DNA antibody that is conjugated to urease. The DNA-binding protein and the monoclonal antibody are specific for single-stranded DNA but do not have any sequence specificity. This liquid phase facilitates the formation of reaction complexes that contain DNA, streptavidin, and urease. During the *second* step the sample is filtered through a biotinylated membrane that binds to the streptavidin and captures the complexes on the membrane, which is washed to remove any reagents that are not bound to the membrane. During the *third* step the membrane is inserted into a sensor on the instrument, where the urease in the DNA complex reacts with a urea solution in the sensor, producing ammonia and a change in pH that is detected using a light-addressable potentiometric sensor (LAPS). The change in pH directly correlates with the amount of DNA in the sample. In the *fourth* step the raw data from the instrument are analyzed using the appropriate software to determine the residual DNA content of the sample.

QUANTITATIVE PCR-BASED RESIDUAL DNA ASSAY

Real-time q-PCR is a procedure that is well-adapted to fast sample throughput and has applications in many areas of biopharmaceutical manufacture (e.g., copy number detection, virus detection). The technique can quantify the amount of a nucleic acid target sequence in DNA from a variety of samples. The DNA probe used in the analysis is the key to the procedure. The probe has a reporter dye attached to one end and a quencher dye attached to the other end. A DNA primer is also added to the reaction. During the amplification reaction, DNA polymerase I attaches where the DNA primer binds to the single-stranded sample (template) DNA and moves along the sample DNA synthesizing new complementary DNA. While following the template DNA, DNA polymerase I cleaves any complementary DNA in the path. If DNA polymerase I encounters the labeled DNA probe it will cleave the reporter dye from the probe. The reporter dye is released into solution and, in the absence of the quencher dye, can be quantitated as a fluorescent measurement. Repeating the reaction cycle results in an amplification of the fluorescent signal. The number of cycles required for the fluorescent measurement to exceed a threshold value correlates to the amount of starting residual DNA in the sample. By comparing with a standard curve the fluorescence obtained from a sample, analysts can quantify the residual DNA in the sample.

PRACTICAL APPLICATIONS OF RESIDUAL DNA TESTING

Analysts choosing hybridization, DNA-binding protein, or q-PCR techniques for residual DNA analysis should consider how the assay will be used, the structure of the DNA available (e.g., fragment length), and regulatory issues. The cost of analysis can be significant and should be considered when evaluating an assay format. Traditionally, hybridization assays were performed using ^{32}P -labeled DNA and autoradiography. Because ^{32}P decays quickly, probes prepared with ^{32}P have a limited shelf life, and the precautions necessary for handling radioactive material can be cumbersome.

These issues with ^{32}P labeling may make fluorescence labeling of the hybridization probe a more desirable option. If the hybridization assay is assessed visually, this represents a semiquantitative assay, but if the intensity of the spots is determined using a densitometer or other image system, the results can be quantitative. DNA-binding protein assays and q-PCR give quantitative results. Quantitative assays are typically preferred instead of semiquantitative assays because the results are considered more accurate and precise, which allows better process monitoring and control.

Due to sample interference, a sample pretreatment step is often required to obtain accurate and reproducible results. Pretreatment steps can influence the recovery of DNA, so it is often necessary to design the assay with a spike-recovery control and an acceptance criterion to ensure assay performance. Commercial sources of host cell and vector DNA are typically not available to prepare in-house controls. In-house controls are usually prepared in the laboratory and quantified by UV spectroscopy, using standard techniques employed in molecular biology, to determine the DNA content and purity. Additionally, it is a good practice to evaluate in-house residual DNA controls by agarose gel electrophoresis to demonstrate that the DNA is of a proper size for the assay employed and has not degraded.

The hybridization assay uses genomic and/or vector DNA, labeled randomly throughout the DNA, as the hybridization probe reagent. For this reason the hybridization assay is specific for the source of DNA but is not specific for a given sequence. A synthesized probe, specific for a specific sequence, can be prepared and used in the hybridization assay if this level of specificity is desirable. The DNA-binding protein residual DNA assay is not sequence-specific and hence not specific for the host DNA. Therefore, laboratory personnel should avoid contaminating samples for this assay with environmental DNA before denaturing the DNA; otherwise the DNA result may be falsely elevated. The q-PCR probe is sequence-specific, which creates some special challenges for development of a q-PCR residual DNA assay. The q-PCR-specific sequence must be a stable sequence within a highly conserved region of DNA. The recovery of the probe target sequence must consistently represent the recovery of all the residual DNA. As a guideline, for a DNA fragment to be detected by hybridization, q-PCR, and DNA-binding protein assays, it must have no fewer than 50, 150, and 600 base pairs, respectively. A bioprocess typically may have operations that shear DNA into smaller fragments, and this must be taken into consideration when selecting an assay. Procedures exist to determine whether the DNA fragments in a sample are too small for adequate residual DNA recovery with a given assay. As noted, residual DNA assays are extremely sensitive. Detection limits as low as <1, 3, and 6 pg of DNA per sample have been reported for q-PCR, DNA-binding protein, and hybridization assays, respectively.

Although safety concerns regarding residual DNA impurities are not as prominent as they once were, the levels of residual DNA in any bioprocess remain a key quality attribute and help define the process.

Change to read:

(1136) ▲ PACKAGING AND REPACKAGING—SINGLE-UNIT CONTAINERS

SCOPE

This chapter provides guidance for the packaging and repackaging of single-unit containers, and for the use and application of unit-of-use packaging. Although the chapter is intended for use by drug manufacturers, repackagers, and pharmacists, the information in the chapter may also be useful for suppliers of packages and packaging components. For the definition of specific types of packaging, see *Packaging and Storage Requirements* (659).

SINGLE-UNIT CONTAINER

Single-unit containers that package a prescription drug to be dispensed directly to the patient are required to be child-resistant. Single-unit packaging intended for institutional or hospital use may or may not be required to be child-resistant. Single-unit containers that are child-resistant include supported blisters, such as separate, peel, push, and tear notch, and enclosed or in-card blisters, such as pull tabs and slide packs.

PACKAGING MATERIALS

Materials used to manufacture single-unit packaging containers include glass and plastic. Glass used as a primary packaging component should meet the requirements of *Containers—Glass* (660). Plastic materials as a primary packaging component should meet the requirements of *Containers—Plastics* (661). The test for moisture permeability may be carried out as described in general test chapter *Containers—Performance Testing* (671).

PACKAGING CLOSURE TYPES

Reclosables and nonreclosables may be used for solid, semisolid, and liquid dosage forms. Both must be packaged in compliance with the 16 CFR 1700.15 standards. The Poison Prevention Packaging Act (PPPA) of 1970 requires in certain cases the use of special packaging—child-resistant and senior-friendly. Child-resistant packaging protects children from serious injury or illness resulting from ingesting or handling hazardous products including drugs.

Because drugs packaged in unit-of-use packaging are intended to be dispensed to the consumer without repackaging by the pharmacist, the manufacturer or repackager is responsible for the special packaging of PPPA-regulated substances in unit-of-use containers (16 CFR 1701.1).

Reclosables

Reclosables are containers with suitable closures that may incorporate tamper evidence and child-resistance capabilities. Reclosables may be used for glass or plastic containers.

Nonreclosables

Nonreclosables are containers with closures that are non-reclosable, such as blisters, sachets, strips, and other single-

unit containers. Nonreclosables may include packs such as cold-formed foil blisters, foil strip packs, and PVC/Aclar® combining multilayer materials that are thermo-formed or cold-formed foil blisters. Nonreclosables may be child resistant depending on the intended use and place of use. Household nonreclosables are subject to the PPPA as defined in 16 CFR 1700.14. However, because of some unit-dose designs, not all unit-dose packages comply with the PPPA.

UNIT-OF-USE

Unit-of-use packaging, when provided by the manufacturer, offers some of the following attractive advantages. (1) A dosage form can be dispensed to a patient in the manufacturer's original container, a practice that recognizes that the suitability of the container has been established on the basis of the manufacturer's stability studies. (2) The counting and repackaging of dosage units in the pharmacy is eliminated, thereby reducing the possibility of human error. (3) The pharmacist is able to affix the label for the patient onto the unit-of-use package and is free to use the manufacturer's expiration date as the beyond-use date. (4) The number of dosage units in a single unit-of-use package may be determined on a case-by-case basis. (5) Patient compliance is improved. (6) The unit-of use package can protect against counterfeiting because traceability of product is ensured through bar coding techniques and National Drug Code (NDC) numbers.

Unit-of-use packaging, when provided by repackagers, offers the same attractive advantages as those offered by the manufacturer. However, unit-of-use repackagers should conform to all requirements as presented in *Good Repackaging Practices* (1178). There are a number of reasons why repackagers produce unit-of-use packaging, for example, (1) requests from institutions, (2) better inventory control, (3) reduced dispensing times, and (4) variations in some drug therapies.

The packaging of a unit-of-use system may be a multiple-unit or single-unit container. A unit-of-use system may contain a drug product in a liquid, semisolid, or solid dosage form (see also *FDA Guidance for Industry, Container Closure Systems for Packaging Human Drugs and Biologics*). [NOTE—The terms "unit-of-use package" and "unit-of-use container" may be used interchangeably.]

Unit-of-Use Labeling

The unit-of-use containers are labeled to include expiration dates, the manufacturer's lot number, the NDC designation, and bar codes as provided in the *Labeling* section of *General Notices and Requirements, Preservation, Packaging, Storage, and Labeling* and in *Good Repackaging Practices* (1178). Some of the advantages of having bar codes on the label include reduced medication errors, improved inventory control, and improved access to medication identity. The labeling covers information placed in the container by the manufacturer (see *General Notices and Requirements*). Acceptable labeling can range from full labeling, such as that for multiple-unit containers, to abbreviated labeling when the container is too small to include all of the text. Full labeling may also be provided on the carton if it is not present on the immediate container.

Unit-of-Use—Repackaging and Reprocessing

Unit-of-use containers are reprocessed or repackaged as instructed by the manufacturer. A unit-of-use package that is a blister package may not be reprocessed by a pharmacist once it has been deblistered from a unit-dose container (see *General Notices and Requirements* for application of the appropriate beyond-use date for a multiple-unit or unit-dose container). Deblistering is the process of removing medication from a blister-type container. However, under current

Good Manufacturing Practices (cGMPs) and tight quality controls, the manufacturer or contract repackager may repack and reprocess unit-of-use containers.

Information from Manufacturers

The manufacturer should provide appropriate stability information that can be used to determine appropriate labeling, storage, and shipping statements that will properly inform patients and practitioners. The manufacturer may make other assurances based on product information on packaging and distribution arrangements. In the event that a product is not to be repackaged, the manufacturer may so state in the labeling. The manufacturer also includes labeling and information necessary for optimal handling by the practitioner and the patient. The labeling and information should be bar coded to eliminate medication error and promote medication traceability.

Responsibility of the Dispenser—Labeling

The labeling on a unit-of-use container also includes a label added at the dispensing stage by the pharmacist. Prior to dispensing the unit-of-use package, the dispenser shall add label(s) that provide the following information:

1. The name of the patient;
2. The name and strength of the drug product, the directions for use as prescribed by a doctor or health-care provider, and the name of the prescriber; and
3. Any storage instruction, beyond-use date, and other information as deemed appropriate by federal and state laws.

In the pharmacy setting, pharmacists are encouraged to use bar codes, in conjunction with computerized prescription orders, to confirm that the right drug is being dispensed to the right patient. Bar coding would minimize errors and create an opportunity for medication traceability and accountability.

Quality Control of Packaging System

The packaging system shall meet the general considerations for system suitability, protection, safety, and performance characteristics as described in *FDA Guidance for Industry, Container Closure Systems for Packaging Human Drugs and Biologics*, and in *Containers—Glass* (660), *Containers—Plastics* (661), and *Containers—Performance Testing* (671).

REPACKAGING A SINGLE SOLID ORAL DRUG PRODUCT INTO A UNIT-DOSE CONTAINER

Repackaging of solid oral drug products, such as tablets and capsules, into unit-dose configurations is common practice both for the pharmacy that is dispensing drugs pursuant to a prescription and for the pharmaceutical repackaging firm. The following section contains minimum standards to be used as a guideline for repackaging practices.

Repackaging preparations into unit-dose configurations is an important aspect of pharmaceutical care and of optimization of patient compliance. For purposes of this chapter, there are two types of repackaging: the first involves pharmacies that dispense prescription drugs, and the second concerns commercial pharmaceutical repackaging firms.

Nomenclature and Definitions

Dispenser: A dispenser is a licensed or registered practitioner who is legally responsible for providing the patient with a preparation that is in compliance with a prescription or a medication order and contains a specific patient label.

In addition, dispensers may prepare limited quantities in anticipation of a prescription or medication order from a physician. Dispensers are governed by the board of pharmacy of the individual state.

Package: The term “package” is synonymous with the term “container”. See *Packaging and Storage Requirements* (659).

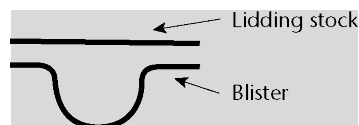
Pharmacy: A pharmacy is an establishment that is legally responsible for providing the patient with a drug preparation with a specific patient label, in compliance with a prescription or a medication order. The terms “dispenser” and “pharmacy” are used interchangeably.

Repackaging: Repackaging is the act of removing a preparation from its original primary container and placing it into another primary container, usually of smaller size.

Repackager: A repackager is an establishment that repackages drugs and sends them to a second location in anticipation of a need. Repackaging firms repackage preparations for distribution (e.g., for resale to distributors, hospitals, or other pharmacies), a function that is beyond the regular practice of a pharmacy. Distribution is not patient specific in that there are no prescriptions. Unlike dispensers, repackaging firms are required to register with the FDA and to comply with the Current Good Manufacturing Practice regulations in 21 CFR 210 and 211.

Materials

Blister packages offer a wide array of designs both in functionality and in appearance. Various packaging materials are used to create blisters that are tailored to provide optimum performance. The blister container consists of two components: the blister, which is the formed cavity that holds the product, and the lid stock, which is the material that seals to the blister, as shown below.



Schematic Presentation of a Typical Blister Pack

Because of the variety of blister films available, film selection should be based upon the degree of protection required. The choice of lid stock depends on how the blister is to be used, but generally the lid stock is made of aluminum foil. The material used to form the cavity is typically a plastic, which can be designed to protect the dosage form from moisture. There are widely varying degrees of moisture protection now available. For purposes of this general chapter, they are referred to as nominal, medium, high, and extreme moisture barrier properties.

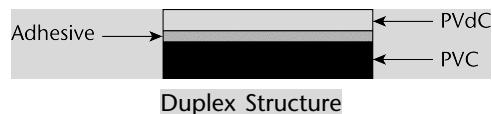
Polyvinyl chloride: The most commonly used blister material is polyvinyl chloride (PVC). This material, which provides a nominal or zero barrier to moisture, is used when the product does not require effective moisture protection. PVC is available in a range of gauges and can be made opaque or can be tinted with pigments to block out specific light wavelengths.

The thickness of the PVC used is determined by the depth and size of the cavity to be formed. Because the plastic thins during the blister-forming process, care should be taken to ensure that the finished blister provides sufficient protection from light (if required) and that it is strong enough to adequately protect the dosage form. Common gauges of PVC used in the pharmaceutical industry range from 7.5 to 15 mil (0.0075 to 0.015 inch).

Barrier films: Many drug preparations are extremely sensitive to moisture and therefore require high barrier films. Several materials may be used to provide moisture protec-

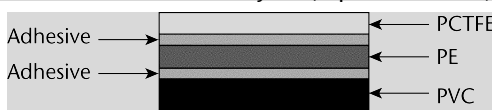
tion. Barrier films commonly used in the pharmaceutical industry are described below.

PVC/PCTFE laminations—Polychlorotrifluoroethylene (PCTFE) film¹ is a thermoplastic film made from polychlorotrifluoroethylene fluoropolymer. The PCTFE film is laminated to the PVC by an adhesive layer between the PVC and the PCTFE film (duplex structure),



Duplex Structure

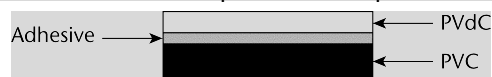
or by a layer of polyethylene (PE) between the PVC–adhesive and the PCTFE–adhesive layers (triplex structure).



Triplex Structure

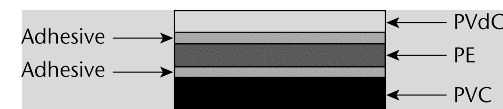
By using various gauges of PCTFE film, medium to extreme moisture barriers can be obtained.

PVC/PVdC laminations—PVC/PVdC is a film in which the PVC is coated with an emulsion of polyvinylidene chloride (PVdC), as shown in the duplex structure pictured below.



Duplex Structure

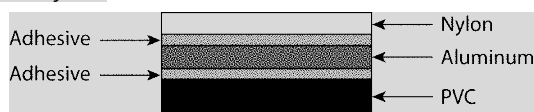
The PVdC layer is specified in g/m² and can be constructed to provide medium to high barrier protection. The coating weights commonly used in the pharmaceutical industry are 40, 60, and 90 g/m², and the film is offered with or without a middle layer of polyethylene (PE), as shown in the triplex structure below. The polyethylene is used with heavier coating weights, such as 60 and 90 g/m², to improve the thermoforming characteristics of the blister cavity.



Triplex Structure

Polypropylene—Because of its morphology, polypropylene (PP) serves as a good moisture barrier, its spherulitic structure creating an arduous path for water molecules to traverse. Although not commonly used as a pharmaceutical blister film in the United States, PP provides an economical alternative to medium barrier materials and is used in Europe as an alternative to PVC.

Cold form foil—This material is used for products that are extremely hygroscopic or light sensitive. It is an extreme moisture barrier and consists of three layers: PVC, aluminum foil, and nylon.



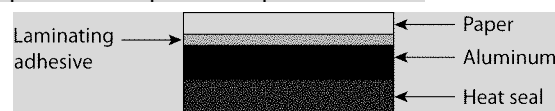
Cold Form Foil

Lid stock: The lid stock is sealed to the molded blister as described above. Different designs of lid stocks are available, and selection of a particular design depends on how

¹ PCTFE film is available from Allied Signal (as Aclar®) and from other sources.

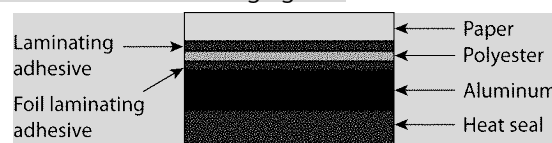
the package will be used. Standard designs—peelable, child-resistant peelable, and push-through—are described below. The primary component of lid stock is typically aluminum, and its gauge varies from 18–25 µm (0.0078–0.001 inch). The side of the aluminum foil laminate in contact with the product provides the heat-sealable layer that forms the seal to the blister material. The heat-seal coating should be capable of forming an adequate seal with the blister film to which it is intended to seal. The materials used in the makeup of the heat-seal layer meet the requirements of 21 CFR 175 and 177.

Peelable—Peelable foil, commonly used in an institutional setting, consists of several layers, as shown below, and can be peeled away from the blister. [NOTE—For child-resistant peelable foil, a layer of polyester with the appropriate adhesives would be added.] With the peelable-foil lid stock, which is used in conjunction with blister tooling, a three-step process is required to open the blister.



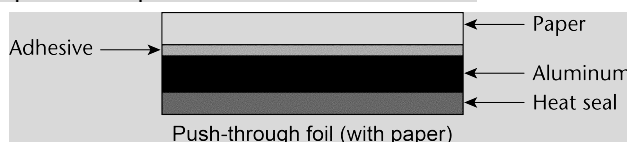
Peelable Foil Construction

First, the blister cavity must be separated from the rest of the blister card. Next, the paper and polyester layers are pulled back from an unsealed area. Finally, the product is pushed through the remaining aluminum foil. It is important to note that use of this type of foil structure helps make the package more child resistant. However, if child-resistant packaging is required, the package design should be tested in accordance with the protocol described in 16 CFR 1700, the Poison Prevention Packaging Act.

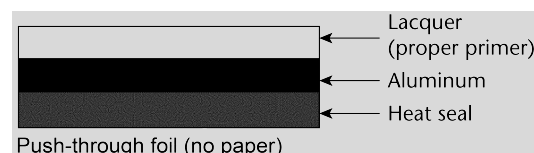


Child-Resistant Foil

Push-through—There are two commonly used types of push-through foil: one with a paper outer layer separated from the aluminum by a layer of adhesive, and one without paper. The paper outer layer serves as an aesthetic and makes it possible to print on the back of the blister.



Push-through foil (with paper)

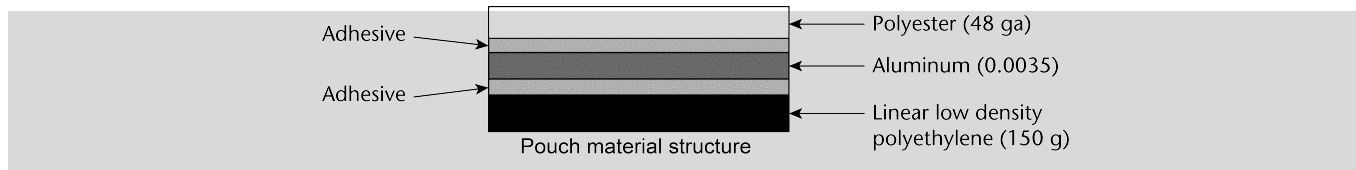


Push-through foil (no paper)

Other package styles: Other types of packages used for unit-dose packaging of solid dosage forms are strip packs, pouches, and sachets.

PROCESS

Unit-dose packages can be formed and sealed in a variety of ways. Larger scale repackagers may use thermoformers that accomplish these functions in-line, while smaller repackagers may purchase preformed blister material. This section



begins with an overview of the process involved in thermoforming a blister, the fundamental process that also applies to other unit-dose package types such as pouches. The overview is not intended to be all-encompassing, but it highlights the major operations along with their critical parameters.

Thermoforming a blister unit-dose package: The complete thermoforming process consists of four basic stations where the following operations occur: forming, filling, sealing, and finishing. Thermoforming requires the use of heat and air in forming the blister. The lid stock material is sealed to the blister cavity material for a defined time (the stroke of the machine) at the point where the heat plate closes on the two materials.

Forming station—Prior to entering the forming station, the blister material passes through a heating unit where the blister material is heated uniformly in stages to ensure proper formation. Because different plastics have different softening points, careful attention must be paid to determining the proper temperature of the heating station, which often has multiple temperature zones. The temperature, based on the blister material used and on the speed at which that material travels through the heating station, is a critical parameter for optimal performance. At the forming station, the blister material is heated to the point where the plastic softens sufficiently to allow the cavity to be formed. The blister material is drawn from a reel-mounted roll (referred to as the web) and pulled through the machine. A splicing table is located at the reel unwind to provide room for a second roll of blister material to be readily available for splicing and resumption of the packaging process. An unwind device may be installed to aid in moving the blister material from the roll as adjusted for a specific index.

Once the blister material is properly heated, compressed air is generally used to form the blister cavity. Upper and lower forming dies close on the blister material as air is introduced, forming a blister that corresponds to the size of the cavity. A plug assist may be necessary, depending on the material and size of the cavity. The plug assist ensures a uniform thinning of the blister material to optimize the protective characteristics of the formed material. Once the blister material is formed into the desired blister configuration, it is advanced to the filling station.

Filling station—The product is loaded into the blister cavity at this station. An automated filling device may be used, or the cavities may be hand filled. The critical parameter at this station is proper filling of the formed blisters.

Sealing station—At this station, the lid stock is sealed to the filled blister cavity, using heat and pressure for a defined dwell time. The critical parameters to be considered at this station are temperature, pressure, and dwell time.

The lid stock material is staged on a roll above the blister cavity and may be preprinted or printed online. Lot numbers and expiration dates may be applied at this point. Preprinted lid stock materials will require a print registration system to control the position of the printing relative to the blister cavity. The critical parameters at this part of the station include legible and correct labeling.

Finishing station—The finishing station encompasses all other steps in the packaging process, including embossing, perforation, and cutting. Embossing involves application of a lot number and expiration date to the package. Steel type is used to emboss information on the edges of the blister package. One of the critical parameters at this station is package integrity. It is important that the embossing, perforation, and cutting processes do not compromise the blister,

lid, or seal. The quality of the embossing is another critical parameter in the process. The embossing must be legible and correct, and must include all required information.

Pouch unit-dose packages: The pouch process is also a form, fill, and seal operation, but it does not provide a defined, formed cavity as does the thermoforming process. Although the equipment used to form pouch unit-dose packages may function differently from that described for thermoforming a blister, the main operations (form, fill, and seal) and critical parameters at those stations are quite similar. [NOTE—See the critical parameters defined in the section on thermoforming.]

The strip-pack process involves the drug product being dosed into a three-sided, formed pouch. Once filled with the drug, the machine seals the pouch, forming a strip of sealed unit-dose pouches. The basic flow of the process begins with the drug situated above the pouch material. One roll of strip-pack material is used to form the pouch. This is accomplished by moving the material over a device that forces the material to fold into two equal sides. The sides and bottom are sealed prior to dosing. The strip pack may be cut later during the equipment processing or roll continuously and be manually cut. Temperature and dwell time are the main critical factors for this equipment.

Preformed unit-dose packages: Preformed containers are sealed either by heat or by adhesion. Heat sealers may be manual units requiring hand pressure application or automated units that provide a more controlled pressure for sealing.

Heat sealing may be accomplished through the use of manual tabletop equipment, which is generally operated at a set pressure. Critical parameters with these devices are pressure and temperature control because undesirable variation in these parameters may yield inadequate seals.

Critical parameters: In order to ensure that the finished container performs as intended, qualification of critical parameters should be determined. Typically, validation of a packaging line consists of qualification of the installation, operation, and performance of a packaging system.

Installation qualification—Equipment should be installed and found to be in proper working condition prior to use.

Operational qualification—Operational qualification should be performed to establish that the equipment operates within the manufacturer's specified ranges. Incoming utilities for the equipment, such as air, electricity, etc., should be monitored and checked periodically.

Performance qualification—Performance qualification should be done to establish that the equipment performs properly with the required materials to produce a container that functions as intended. The critical parameters include forming temperature and pressure, sealing temperature and pressure, and dwell time at the seal station. Qualified ranges should be readily available in a reference source for the setup of equipment. Re-evaluation may be necessary with changes to equipment, materials, or process.

In-process inspections: Strict controls covering the packaging and labeling processes should be in place. The final container should be evaluated for performance in each of the stations previously described. Specifically, the formed container should be inspected visually to ensure that it is properly formed. Evaluation of the filling station should include a check to ensure that the unit dose is properly filled (i.e., that the correct product is present). The sealing station should be evaluated to ensure that a proper seal has been made and that the moisture permeation specifications of the sealed container have been met. A visual examination of the

package should be performed to ensure that the final steps of the packaging process are acceptable.

Repackagers and dispensers should use a standard inspection plan to verify the adequacy of the package. A visual inspection should be performed to verify that the correct product is in the proper packaging materials with correct labeling. Seal integrity should be evaluated, using vacuum testing,² helium testing, tear testing, and other testing methods suitable to establish whether seal integrity is maintained.

PERFORMANCE

The primary purpose of the unit-dose package used in the packaging of a drug preparation is to ensure that, until its intended expiration date, there is adequate protection from the environment as the dosage form is distributed and stored. It is also essential that the materials used do not interact with the dosage form.

When determining what type of package to use in the repackaging operation, consideration must be given to the dosage form's sensitivities (if any) to the storage and distribution environments (e.g., temperature, light, and moisture).

The properties of the finished container are defined by the materials used in constructing the unit-dose container, and by the process used to form and seal the container. As discussed in *Materials*, there is a wide variety of commercially available film structures that provide unit-dose containers with a range of moisture and light protection. Suppliers of these materials typically provide quantitative data, obtained from well-established test methods, to highlight the protective properties of their material. These data are based on flat sheets of the film, not on the formed container.

It is critical to understand that once the film is formed, protective properties change because the overall thickness of the film decreases as the blister cavity is formed. Usually the change is a decrease, especially in the case of barrier properties. However, the extent of change will vary with the type of film structure used and is also highly dependent on the container-forming process used (see *Process*). Further, a suboptimal seal on the formed container will decrease the protective properties of the container. Insufficient temperature, time, or pressure during a heat-seal operation may enable the passage of moisture or oxygen through the seal area over time, which may have an effect on the dosage form. In addition, if the seal area is designed with insufficient surface area, the same problem may occur. To ensure a good seal, a minimum sealing distance of 3 mm from the edge of the blister cavity to the nearest edge or perforation is recommended. Therefore, it is important to measure the performance of the formed and sealed container rather than the performance of the flat sheet.

Moisture is a critical factor in preparation integrity. *Containers—Performance Testing* (671) describes how to determine and classify moisture permeation rates. If the manufacturer's labeling includes "Protect From Moisture," the repackager shall utilize a high barrier film.

If light protection is required for a drug preparation, the repackager should follow the requirements for light transmission established under *Containers—Performance Testing* (671). Again, this testing should be conducted on the formed container, because the light-protective properties of the film are compromised once the film is thinned during the forming process. It is recommended that these tests, in

²Vacuum testing consists of placing samples from the packaging operation into a jar filled with water. A lid is placed over the samples to fully immerse them in the water. A container lid is applied to create a seal effective enough to create approximately 25 cm of vacuum. The vacuum pump is set, and the samples are tested for approximately 1 minute, removed from the water, wiped down, and opened to determine whether the inside of the unit-dose cavity or pouch is wet. This process should be adjusted until it is under control, and additional testing may be performed to ensure that the seal integrity is consistently acceptable. Wetness indicates a defective seal and therefore the potential for the drug to degrade when exposed to the atmosphere. Defective packages must be removed from further use.

conjunction with any guidance provided by the manufacturer, be considered appropriate for any container-closure system used in repackaging a drug preparation.

BEYOND-USE-DATE

In the absence of stability data for the drug product in the repackaged container, the beyond-use dating period is one year or the time remaining until the expiration date, whichever is shorter. If current stability data are available for the drug product in the repackaged container, the length of time established by the stability study may be used to establish the beyond-use date, but must not exceed the manufacturer's expiration date.

As stated in the *General Notices and Requirements*, the dispenser must maintain the facility where the dosage forms are packaged and stored at a temperature such that the mean kinetic temperature is not greater than 25°. The plastic material used in packaging the dosage forms must afford better protection than polyvinyl chloride, which does not provide adequate protection against moisture permeation. Records must be kept of the temperature of the facility where the dosage forms are stored, and of the plastic materials used in packaging.

MINIMUM REQUIREMENTS

The previous sections serve as a general introduction to repackaging by providing a basic understanding of materials selection, the form-fill-seal process, and the importance of performance of the sealed container. In this section, certain minimum requirements for repackaging, which must be met, are described in more detail.

Personnel: Each person with responsibility for the repackaging of a preparation shall have the education, training, and experience, or any combination thereof, to perform assigned functions in a manner such that the safety, identity, strength, quality, purity, potency, and pharmaceutical elegance of the drug dosage form are retained. Training should be documented.

Personnel engaged in the repackaging of a preparation shall wear clean clothing appropriate for the duties or processes performed.

Facility: The repackaging facility may require areas of low relative humidity, and temperature conditions should meet controlled room temperature requirements specified in *Packaging and Storage Requirements* (659).

Equipment: Equipment used in the repackaging of a preparation shall be of appropriate design and suitably located to facilitate operations for its intended use. Its design should allow for cleaning to preclude cross-contamination as well as for maintenance to be performed. Equipment shall be constructed so that those surfaces that contact components or a preparation are not reactive, additive, or absorptive. Any substances required for operation, such as lubricants or coolants, shall not come into contact with components or a preparation.

Equipment and utensils shall be cleaned, maintained, and sanitized at appropriate intervals to prevent malfunctions or contamination. Preventive maintenance should be performed at appropriate intervals in accordance with the equipment manufacturer's recommendation. Any instruments used to monitor critical parameters should be calibrated on a defined schedule.

Process: Steps should be taken to determine the critical process parameters (e.g., seal temperature, dwell time) in operating the equipment. Set points for these parameters should be documented and procedures established to ensure that they are adhered to each time the equipment is operated.

Labeling: The labeling requirements for a commercial repackager and a pharmacist are different. For example, the

commercial repackager must comply with 21 CFR 201.1, but the pharmacist or dispenser does not have to comply with this requirement. If stability data are unavailable, the dispenser shall repack only an amount of stock sufficient for a limited time and shall include product name and strength, lot number, manufacturer, and appropriate beyond-use date on the label. When quantities are repackaged in advance of immediate needs, each preparation must bear an identifying label, and the dispenser is required to maintain suitable repackaging records showing the name of the manufacturer, lot number, expiration date, date of repackaging, and designation of persons responsible for repackaging and for checking. The repackager or dispenser will use documented controls to prevent labeling errors.

Materials: The repackager or dispenser shall place an appropriate beyond-use date on the label and package in appropriate materials. Materials used by the repackager shall not be reactive, additive, or absorptive, and must meet the requirements described in 21 CFR 175 and 177.

Storage: The dispenser shall rotate and monitor stock closely to ensure that the dispensing of preparations is on a first-in-first-out (FIFO) basis. The repackager or dispenser shall store preparations under required environmental conditions (e.g., controlled room temperature with a mean kinetic temperature not higher than 25°).

Drug product: The repackager or dispenser shall examine preparations for evidence of instability such as change in color or odor, and shall exercise professional judgment as to the acceptability of a package.

Complaints: The repackager or dispenser will maintain written procedures describing the handling of written and oral complaints regarding a drug product and will ensure that complaints are investigated and appropriately resolved.

Returned goods: Policies and procedures relating to returned goods should be developed to ensure proper handling.

Reprocessing: Reprocessing of repackaged unit-dose containers (i.e., removing medication from one unit-dose container and placing it into another unit-dose container) shall not be done. However, reprocessing of the secondary package (e.g., removing the blister card from the cardboard carrier and placing the blister card into another cardboard carrier) is allowed provided the original beyond-use date is maintained, and provided the integrity of the blister is ensured.

Special considerations: If a product is known to be oxygen sensitive or if it exhibits extreme moisture or light sensitivity (e.g., cold form foil), it shall not be repackaged. If a product is refrigerated, it shall not be repackaged unless proper environmental conditions and suitable materials are available. Certain drug products (such as oncologic agents, hormones, or penicillin derivatives) require special handling because they are considered very potent or toxic, and because transfer of any portion of these products to another product could have deleterious effects.

REPACKAGING NONSTERILE SOLID AND LIQUID DOSAGE FORMS INTO SINGLE-UNIT CONTAINERS AND UNIT-DOSE CONTAINERS

The following guidance is intended for those engaged in pharmaceutical dispensing, and does not apply to commercial dispensing. An official dosage form is required to bear on its label an expiration date assigned for the particular formulation and package of the article. This date limits the time during which the product may be dispensed or used. Because the expiration date stated on the original manufacturer's container-closure system has been determined for the drug in that particular system and is not intended to apply to a product that has been repackaged in a different container, repackaged drugs dispensed pursuant to a prescription are exempt from using the expiration date from

the original manufacturer's package. However, under no circumstance should the repackaged pharmaceutical preparation's expiration date exceed the original manufacturer's expiration date. It is necessary, therefore, that other precautions be taken by the dispenser to preserve the strength, quality, and purity of drugs that are repackaged for ultimate distribution or sale to patients.

The following guidelines and requirements are applicable where official dosage forms are repackaged into single-unit or unit-dose containers or mnemonic packs for dispensing pursuant to prescription.

Labeling: It is the responsibility of the dispenser to place a suitable expiration date on the label, taking into account the nature of the drug repackaged, any packaging and expiration dating information in the manufacturer's product labeling, the characteristics of the containers, and the storage conditions to which the article may be subjected. Repackaged dosage forms must bear on their labels expiration dates as determined from information in the product labeling (see *General Notices and Requirements, Preservation, Packaging, Storage, and Labeling*). Each single-unit or unit-dose container bears a separate label, unless the device holding the unit-dose form does not allow for the removal or separation of the intact single-unit or unit-dose container therefrom.

Storage: Store the repackaged article in a humidity-controlled environment and at the temperature specified in the individual monograph or in the product labeling. For further directions, see *Packaging and Storage Requirements* (659).

A refrigerator or freezer shall not be considered to be a humidity-controlled environment. Drugs that are to be stored at a cold temperature in a refrigerator or freezer must be protected during storage in the refrigerator or freezer. An outer container may be necessary for such protection; it is recommended that the drug monograph be referenced for storage.

Reprocessing: Reprocessing of repackaged unit-dose containers (i.e., removing a dosage unit from one unit-dose container and placing it in another unit-dose container) shall not be done. However, reprocessing of the secondary package (e.g., removing the blister card from the cardboard carrier and placing the blister card into another cardboard carrier) is allowed provided that the original expiration date is maintained.

CUSTOMIZED PATIENT MEDICATION PACKAGES

In lieu of dispensing two or more prescribed drug products in separate containers, a pharmacist may, with the consent of the patient, the patient's caregiver, or a prescriber, provide a customized patient medication package (patient med pak).³

A patient med pak, i.e., a package prepared by a pharmacist for a specific patient, comprises a series of containers and contains two or more prescribed solid oral dosage forms. The patient med pak is so designed, or each container is so labeled, as to indicate the day and time, or period of time, that the container contents are to be taken.

It is the responsibility of the dispenser to instruct the patient or caregiver on the use of the patient med pak.

Label: The patient med pak shall bear a label stating the following:

1. The name of the patient;
2. A serial number for the patient med pak itself and a separate identifying serial number for each of the pre-

³It should be noticed that for patient med paks there is no special exemption from the requirements of the Poison Prevention Packaging Act. Thus, the patient med pak, if it does not meet child-resistant standards, shall be placed in an outer package that does comply, or the necessary consent of the purchaser or physician to dispense in a container not intended to be child-resistant shall be obtained.

scription orders for each of the drug products contained therein;

3. The name, strength, physical description or identification, and total quantity of each drug product contained therein;
4. The directions for use and cautionary statements, if any, contained in the prescription order for each drug product therein;
5. Any storage instructions or cautionary statements required by the official compendia;
6. The name of the prescriber of each drug product;
7. The date of preparation of the patient med pak and the beyond-use date or period of time assigned to the patient med pak (such beyond-use date or period of time shall be not longer than the shortest recommended beyond-use date for any dosage form included therein or not longer than 60 days from the date of preparation of the patient med pak, and shall not exceed the shortest expiration date on the original manufacturer's bulk containers for the dosage forms included therein); alternatively, the package label shall state the date of the prescription(s) or the date of preparation of the patient med pak, provided the package is accompanied by a record indicating the start date and the beyond-use date;
8. The name, address, and telephone number of the dispenser (and the dispenser's registration number where necessary); and
9. Any other information, statements, or warnings required for any of the drug products contained therein.

If the patient med pak allows for the removal or separation of the intact containers therefrom, each individual container shall bear a label identifying each of the drug products contained therein.

Labeling: The patient med pak shall be accompanied by a patient package insert, in the event that any medication therein is required to be dispensed with such insert as accompanying labeling. Alternatively, such required information may be incorporated into a single, overall educational insert provided by the pharmacist for the total patient med pak.

Packaging: In the absence of more stringent packaging requirements for any of the drug products contained therein, each container of the patient med pak shall comply with the moisture permeation requirements for a Class B single-unit or unit-dose container (see *Containers—Performance Testing* (671)). Each container shall be either nonreclosable or so designed as to show evidence of having been opened.

Guidelines: It is the responsibility of the dispenser, when preparing a patient med pak, to take into account any applicable compendial requirements or guidelines and the physical and chemical compatibility of the dosage forms placed within each container, as well as any therapeutic incompatibilities that may attend the simultaneous administration of the medications. In this regard, pharmacists are encouraged to report to USP headquarters any observed or reported incompatibilities. Once a medication has been placed in a patient med pak with another solid dosage form, it may not be returned to stock, redistributed, or resold if unused.

Recordkeeping: In addition to any individual prescription filing requirements, a record of each patient med pak shall be made and filed. Each record shall contain, as a minimum:

1. The name and address of the patient;
2. The serial number of the prescription order for each drug product contained therein;
3. The name of the manufacturer or labeler and lot number for each drug product contained therein;
4. Information identifying or describing the design, characteristics, or specifications of the patient med pak

sufficient to allow subsequent preparation of an identical patient med pak for the patient;

5. The date of preparation of the patient med pak and the beyond-use date that was assigned;
6. Any special labeling instructions; and
7. The name or initials of the pharmacist who prepared the patient med pak.

▲ USP36

Delete the following:

▲ (1146) PACKAGING PRACTICE—REPACKAGING A SINGLE SOLID ORAL DRUG PRODUCT INTO A UNIT-DOSE CONTAINER

INTRODUCTION

Repackaging of solid oral drug products, such as tablets and capsules, into unit-dose configurations is common practice both for the pharmacy that is dispensing drugs pursuant to a prescription and for the pharmaceutical repackaging firm. This general chapter contains minimum standards to be used as a guideline for repackaging practices. This guideline is not intended to replace or supplant the requirements of regulatory agencies.

Repackaging preparations into unit-dose configurations is an important aspect of pharmaceutical care and of optimization of patient compliance. For purposes of this chapter, there are two types of repackaging: the first involves pharmacies that dispense prescription drugs; the second concerns commercial pharmaceutical repackaging firms.

NOMENCLATURE AND DEFINITIONS

DISPENSER—A dispenser is a licensed or registered practitioner who is legally responsible for providing a preparation for patient use, with a specific patient label, pursuant to a prescription or a medication order. In addition, dispensers may prepare limited quantities in anticipation of a prescription or medication order from a physician. Dispensers are governed by the board of pharmacy of the individual state.

PACKAGE—The term "package" is synonymous with the term "container." See *Containers* in (659) *Packaging and Storage Requirements*.

PHARMACY—A pharmacy is an establishment that is legally responsible for providing the drug preparation for patient use, with a specific patient label, pursuant to a prescription or a medication order. The terms dispenser and pharmacy are used interchangeably.

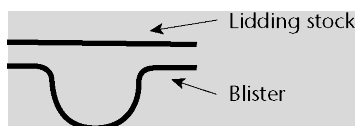
REPACKAGING—Repackaging is the act of removing a preparation from its original primary container and placing it into another primary container, usually of smaller size.

REPACKAGER—A repackager is an establishment that repackages drugs and sends them to a second location in anticipation of a need. Repackaging firms repackage preparations for distribution (e.g., for resale to distributors, hospitals, or other pharmacies), a function that is beyond the regular practice of a pharmacy. Distribution is not patient specific in that there are no prescriptions. Unlike dispensers, repackaging firms are required to register with the FDA and to

comply with the Current Good Manufacturing Practice regulations in 21 CFR 210 and 211.

MATERIALS

Blister packages offer a wide array of designs both in functionality and in appearance. Various packaging materials are used to create blisters that are tailored to provide optimum performance. The blister container consists of two components: the blister, which is the formed cavity that holds the product, and the lid stock, which is the material that seals to the blister, as shown below.



Schematic Presentation of a Typical Blister Pack

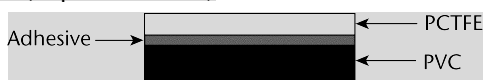
Because of the variety of blister films available, film selection should be based upon the degree of protection required. The choice of lid stock depends on how the blister is to be used, but generally the lid stock is made of aluminum foil. The material used to form the cavity is typically a plastic, which can be designed to protect the dosage form from moisture. There are widely varying degrees of moisture protection now available. For purposes of this general chapter, they are referred to as nominal, medium, high, and extreme moisture barrier properties.

Polyvinyl Chloride—The most commonly used blister material is polyvinyl chloride (PVC). This material, which provides a nominal or zero barrier to moisture, is used when the product does not require effective moisture protection. PVC is available in a range of gauges and can be made opaque or can be tinted with pigments to block out specific light wavelengths.

The thickness of the PVC used is determined by the depth and size of the cavity to be formed. Because the plastic thins during the blister-forming process, care should be taken to ensure that the finished blister provides sufficient protection from light (if required) and that it is strong enough to adequately protect the dosage form. Common gauges of PVC used in the pharmaceutical industry range from 7.5 to 15 mil (0.0075 to 0.015 inch).

Barrier Films—Many drug preparations are extremely sensitive to moisture and therefore require high barrier films. Several materials may be used to provide moisture protection. Barrier films commonly used in the pharmaceutical industry are described below.

PVC/PCTFE Laminations—Polychlorotrifluoroethylene (PCTFE) film¹ is a thermoplastic film made from polychlorotrifluoroethylene fluoropolymer. The PCTFE film is laminated to the PVC by an adhesive layer between the PVC and the PCTFE film (duplex structure)



Duplex Structure

or by a layer of polyethylene (PE) between the PVC-adhesive and the PCTFE-adhesive layers (triplex structure).



Triplex Structure

¹ PCTFE film is available from Allied Signal (as Aclar) and from other sources.

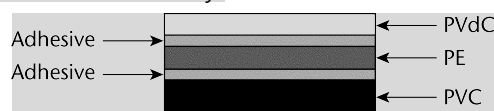
By using various gauges of the PCTFE film, *medium* to *extreme* moisture barriers can be obtained.

PVC/PVdC Laminations—PVC/PVdC is a film in which the PVC is coated with an emulsion of polyvinylidene chloride (PVdC).



Duplex Structure

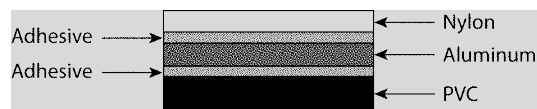
The PVdC layer is specified in g per m² and can be constructed to provide *medium* to *high* barrier protection. The coating weights commonly used in the pharmaceutical industry are 40, 60, and 90 g per m², and the film is offered with or without a middle layer of polyethylene (PE). The polyethylene is used with heavier coating weights, such as 60 and 90 g per m², to improve the thermoforming characteristics of the blister cavity.



Triplex Structure

Polypropylene—Because of its morphology, polypropylene (PP) serves as a good moisture barrier, its spherulitic structure creating an arduous path for water molecules to traverse. Although not commonly used as a pharmaceutical blister film in the U.S., PP provides an economical alternative to medium barrier materials and is used in Europe as an alternative to PVC.

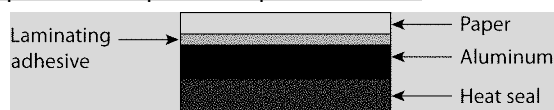
Cold Form Foil—This material is used for products that are extremely hygroscopic or light sensitive. It is an extreme moisture barrier and consists of three layers: PVC, aluminum foil, and nylon.



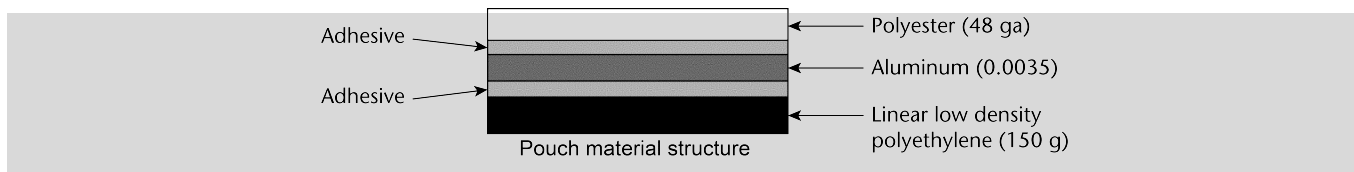
Cold Form Foil

Lid Stock—Lid stock is sealed to the molded blister as described above. Different designs of lid stocks are available, and selection of a particular design depends on how the package will be used. Standard designs—peelable, child-resistant peelable, and push-through—are described below. The primary component of lid stock is typically aluminum, and its gauge varies from 18 to 25 μ m (0.0078 to 0.001 inch). The side of the aluminum foil laminate in contact with the product provides the heat-sealable layer that forms the seal to the blister material. The heat-seal coating should be capable of forming an adequate seal with the blister film to which it is intended to seal. The materials used in the makeup of the heat-seal layer meet 21 CFR 175 and 177.

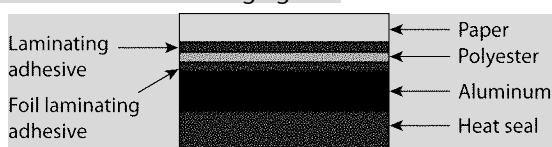
Peelable—Peelable foil, commonly used in an institutional setting, consists of several layers, as shown below, and can be peeled away from the blister. [NOTE—For child-resistant peelable foil, a layer of polyester with the appropriate adhesives would be added.] With the peelable foil lid stock, which is used in conjunction with blister tooling, a three-step process is required to open the blister.



Peelable Foil Construction

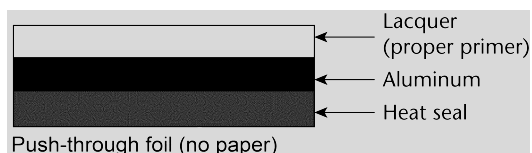
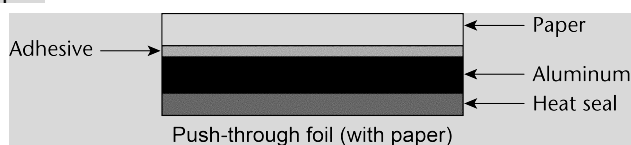


First, the blister cavity must be separated from the rest of the blister card. Next, the paper and polyester layers are pulled back from an unsealed area. Finally, the product is pushed through the remaining aluminum foil. It is important to note that use of this type of foil structure helps make the package more child resistant. However, if child-resistant packaging is required, the package design should be tested in accordance with the protocol described in 16 CFR 1700, the Poison Prevention Packaging Act.



Child-Resistant Foil

Push-Through—There are two commonly used types of push-through foil: one with a paper outer layer separated from the aluminum by a layer of adhesive and one without paper.



The paper outer layer serves as an aesthetic and makes it possible to print on the back of the blister.

Other Package Styles—Other types of packages used for unit-dose packaging of solid dosage forms are strip packs, pouches, and sachets.

PROCESS

Unit-dose packages can be formed and sealed in a variety of ways. Larger scale repackagers may use thermoformers that accomplish these functions in-line, while smaller repackagers may purchase preformed blister material. This section begins with an overview of the process involved in thermoforming a blister, the fundamental process that also applies to other unit-dose package types such as pouches. The overview is not intended to be all-encompassing, but it highlights the major operations along with their critical parameters.

Thermoforming a Blister Unit-Dose Package—The complete thermoforming process consists of four basic stations where the following operations occur: forming, filling, sealing, and finishing. Thermoforming requires the use of heat and air in forming the blister. The lid stock material is sealed to the blister cavity material for a defined time (the stroke of the machine) at the point where the heat plate closes on the two materials.

Forming Station—Prior to entering the forming station, the blister material passes through a heating unit where the blister material is heated uniformly in stages to ensure

proper formation. Because different plastics have different softening points, careful attention must be paid to determining the proper temperature of the heating station, which often has multiple temperature zones. The temperature, based on the blister material used and on the speed at which that material travels through the heating station, is a critical parameter for optimal performance. At the forming station the blister material is heated to the point where the plastic softens sufficiently to allow the cavity to be formed. The blister material is drawn from a reel-mounted roll (referred to as the web) and pulled through the machine. A splicing table is located at the reel unwind to provide room for a second roll of blister material to be readily available for splicing and resumption of the packaging process. An unwind device may be installed to aid in moving the blister material from the roll as adjusted for a specific index.

Once the blister material is properly heated, compressed air is generally used to form the blister cavity. Upper and lower forming dies close on the blister material as air is introduced, forming a blister that corresponds to the size of the cavity. A plug assist may be necessary, depending on the material and size of the cavity. The plug assist ensures a uniform thinning of the blister material to optimize the protective characteristics of the formed material. Once the blister material is formed into the desired blister configuration, it is advanced to the filling station.

Filling Station—The product is loaded into the blister cavity at this station. An automated filling device may be used, or the cavities may be hand filled. The critical parameter at this station is proper filling of the formed blisters.

Sealing Station—At this station, the lid stock is sealed to the filled blister cavity, using heat and pressure for a defined dwell time. The critical parameters to be considered at this station are temperature, pressure, and dwell time.

The lid stock material is staged on a roll above the blister cavity and may be preprinted or printed on-line. Lot numbers and expiration dates may be applied at this point. Preprinted lid stock materials will require a print registration system to control the position of the printing relative to the blister cavity. The critical parameters at this part of the station include legible and correct labeling.

Finishing Station—The finishing station encompasses all other steps in the packaging process, including embossing, perforation, and cutting. Embossing involves application of a lot number and expiration date to the package. Steel type is used to emboss information on the edges of the blister package. One of the critical parameters at this station is package integrity. It is important that the embossing, perforation, and cutting processes do not compromise the blister, lid, or seal. The quality of the embossing is another critical parameter in the process. The embossing must be legible, and correct and must include all required information.

Pouch Unit-Dose Packages—The pouch process is also a form, fill, and seal operation, but it does not provide a defined, formed cavity as does the thermoforming process. Although the equipment used to form pouch unit-dose packages may function differently from that described for thermoforming a blister, the main operations (form, fill, and seal) and critical parameters at those stations are quite similar. [NOTE—See the aforementioned critical parameters defined in the section on thermoforming.]

The strip-pack process involves the drug product being dosed into a three-sided, formed pouch. Once filled with the drug, the machine seals the pouch, forming a strip of sealed unit-dose pouches. The basic flow of the process begins with the drug situated above the pouch material. One

roll of strip-pack material is used to form the pouch. This is accomplished by moving the material over a device that forces the material to fold into two equal sides. The sides and bottom are sealed prior to dosing. The strip pack may be cut later during the equipment processing or roll continuously and be manually cut. Temperature and dwell time are the main critical factors for this equipment.

Preformed Unit-Dose Packages—Preformed containers are sealed either by heat or by adhesion. Heat sealers may be manual units requiring hand pressure application or automated units that provide a more controlled pressure for sealing.

Heat sealing may be accomplished through the use of manual tabletop equipment. This equipment is generally operated at a set pressure. Critical parameters with these devices are pressure and temperature control because undesirable variation in these parameters may yield inadequate seals.

Critical Parameters—In order to ensure that the finished container performs as intended, qualification of critical parameters should be determined. Typically, validation of a packaging line consists of qualification of the installation, operation, and performance of a packaging system.

Installation Qualification—Equipment should be installed and found to be in proper working condition prior to use.

Operational Qualification—Operational qualification should be performed to establish that the equipment operates within the manufacturer's specified ranges. Incoming utilities for the equipment, such as air, electricity, etc., should be monitored and checked periodically.

Performance Qualification—Performance qualification should be done to ensure that the equipment is performing properly with the required materials to produce a container that functions as intended. The critical parameters include forming temperature and pressure, sealing temperature and pressure, and dwell time at the seal station. Qualified ranges should be readily available in a reference source for the setup of equipment. Re-evaluation may be necessary with changes to equipment, materials, or the process.

In-Process Inspections—Strict controls covering the packaging and labeling processes should be in place. The final container should be evaluated for performance in each of the stations described above. Specifically, the formed container should be inspected visually to ensure that it is properly formed. Evaluation of the filling station should include a check to ensure that the unit dose is properly filled (i.e., that the correct product is present). The sealing station should be evaluated to ensure that a proper seal has been made and that the moisture permeation specifications of the sealed container have been met. A visual examination of the package should be performed to ensure that the final steps of the packaging process are acceptable.

Repackagers and dispensers should use a standard inspection plan to verify the adequacy of the package. A visual inspection should be performed to verify that the correct product is in the proper packaging materials with correct labeling. Seal integrity should be evaluated, using vacuum testing,² helium testing, tear testing, and other testing methods suitable to establish whether seal integrity is maintained.

² Vacuum testing consists of placing samples from the packaging operation into a jar filled with water. A lid is placed over the samples to fully immerse them in the water. A container lid is applied to create a seal effective enough to create approximately 25 cm of vacuum. The vacuum pump is set, and the samples are tested for approximately 1 minute, removed from the water, wiped down, and opened to determine whether the inside of the unit-dose cavity or pouch is wet. This process should be adjusted until it is under control, and additional testing may be performed to ensure that the seal integrity is consistently acceptable. Wetness indicates a defective seal and therefore the potential for the drug to degrade when exposed to the atmosphere. Defective packages must be removed from further use.

PERFORMANCE

The primary purpose of the unit-dose package used in the packaging of a drug preparation is to ensure that until its intended expiration date there is adequate protection from the environment as the dosage form is distributed and stored. It is also essential that the materials used do not interact with the dosage form.

When determining what type of package to use in the repackaging operation, consideration must be given to the dosage form's sensitivities (if any) to the storage and distribution environments (e.g., temperature, light, and moisture).

The materials used in constructing the unit-dose container as well as the process of forming and sealing the container all together define the properties of the finished container. As discussed in *Materials*, there is a wide variety of commercially available film structures that provide unit-dose containers with a range of moisture and light protection. Suppliers of these materials typically provide quantitative data, obtained from well-established test methods, to highlight the protective properties of their material. These data are based on flat sheets of the film, not on the formed container.

It is critical to understand that once the film is formed, protective properties change because the overall thickness of the film decreases as the blister cavity is formed. Usually the change is a decrease, especially in the case of barrier properties. However, the extent of change will vary with the type of film structure used and is also highly dependent on the container-forming process used (see *Process*). Further, a suboptimal seal on the formed container will decrease the protective properties of the container. Insufficient temperature, time, or pressure during a heat-seal operation may enable the passage of moisture or oxygen through the seal area over time, which may have an effect on the dosage form. In addition, if the seal area is designed with insufficient surface area, the same problem may occur. To ensure a good seal, a minimum sealing distance of 3 mm from the edge of the blister cavity to the nearest edge or perforation is recommended. Therefore, it is important to measure the performance of the formed and sealed container rather than the performance of the flat sheet.

Moisture is a critical factor in preparation integrity. *Containers—Performance Testing* (671) describes how to determine and classify moisture permeation rates. If the manufacturer's labeling includes "Protect From Moisture," the repackager shall utilize a high barrier film.

If light protection is required for a drug preparation, the repackager should follow the requirements for light transmission established under *Containers—Performance Testing* (671). Again, this testing should be conducted on the formed container, because the light protective properties of the film are compromised once the film is thinned during the forming process. It is recommended that these tests, in conjunction with any guidance provided by the manufacturer, be considered appropriate for any container-closure system used in repackaging a drug preparation.

BEYOND-USE DATE

In the absence of stability data for the drug product in the repackaged container, the beyond-use dating period is one year or the time remaining of the expiration date, whichever is shorter. If current stability data are available for the drug product in the repackaged container, the length of time established by the stability study may be used to establish the beyond-use date but must not exceed the manufacturer's expiration date.

As stated in the *General Notices and Requirements*, the dispenser must maintain the facility where the dosage forms are packaged and stored at a temperature such that the mean kinetic temperature is not greater than 25°. The plastic material used in packaging the dosage forms must afford better protection than polyvinyl chloride, which does

not provide adequate protection against moisture permeation. Records must be kept of the temperature of the facility where the dosage forms are stored, and of the plastic materials used in packaging.

MINIMUM REQUIREMENTS

The previous sections serve as a general introduction to repackaging by providing a basic understanding of materials selection, the form-fill-seal process, and the importance of performance of the sealed container. In this section, certain minimum requirements for repackaging, which must be met, are described in more detail.

Personnel—Each person with responsibility for the repackaging of a preparation shall have the education, training, and experience, or any combination thereof, to perform assigned functions in a manner such that the safety, identity, strength, quality, purity, potency, and pharmaceutical elegance of the drug dosage form are retained. Training should be documented.

Personnel engaged in the repackaging of a preparation shall wear clean clothing appropriate for the duties or processes performed.

Facility—The repackaging facility may require areas of low relative humidity, and temperature conditions should meet controlled room temperature requirements specified in the *General Notices*.

Equipment—Equipment used in the repackaging of a preparation shall be of appropriate design and suitably located to facilitate operations for its intended use. Its design should allow for cleaning to preclude cross-contamination as well as for maintenance to be performed. Equipment shall be constructed so that those surfaces that contact components or a preparation are not reactive, additive, or absorptive.

Any substances required for operation, such as lubricants or coolants, shall not come into contact with components or a preparation.

Equipment and utensils shall be cleaned, maintained, and sanitized at appropriate intervals to prevent malfunctions or contamination. Preventive maintenance should be performed at appropriate intervals in accordance with the equipment manufacturer's recommendation. Any instruments used to monitor critical parameters should be calibrated on a defined schedule.

Process—Steps should be taken to determine the critical process parameters (e.g., seal temperature, dwell time) in operating the equipment. Set points for these parameters should be documented and procedures established to ensure that they are adhered to each time the equipment is operated.

Labeling—The labeling requirements for a commercial repackager and a pharmacist are different. For example, the commercial repackager must comply with 21 CFR 201.1, but the pharmacist or dispenser does not have to comply with this requirement. If stability data are unavailable, the dispenser shall repackage only an amount of stock sufficient for a limited time and shall include product name and strength, lot number, manufacturer, and appropriate beyond-use date on the label. When quantities are repackaged in advance of immediate needs, each preparation must bear an identifying label, and the dispenser is required to maintain suitable repackaging records showing the name of the manufacturer, lot number, expiration date, date of repackaging, and designation of persons responsible for repackaging and for checking. The repackager or dispenser will use documented controls to prevent labeling errors.

Materials—The repackager or dispenser shall place an appropriate beyond-use date on the label and package in appropriate materials. Materials used by the repackager shall not be reactive, additive, or absorptive, and must meet the requirements described in 21 CFR 175 and 177.

Storage—The dispenser shall rotate and monitor stock closely to ensure that the dispensing of preparations is on a first-in-first-out (FIFO) basis. The repackager or dispenser shall store preparations under required environmental conditions (e.g., controlled room temperature with a mean kinetic temperature not higher than 25°).

Drug Product—The repackager or dispenser shall examine preparations for evidence of instability such as change in color or odor, and shall exercise professional judgment as to the acceptability of a package.

Complaints—The repackager or dispenser will maintain written procedures describing the handling of written and oral complaints regarding a drug product and will ensure that complaints are investigated and appropriately resolved.

Returned Goods—Policies and procedures relating to returned goods should be developed to ensure proper handling.

Reprocessing—Reprocessing of repackaged unit-dose containers (i.e., removing medication from one unit-dose container and placing it into another unit-dose container) shall not be done. However, reprocessing of the secondary package (e.g., removing the blister card from the cardboard carrier and placing the blister card into another cardboard carrier) is allowed provided the original beyond-use date is maintained, and provided the integrity of the blister is ensured.

Special Considerations—If a product is known to be oxygen sensitive or if it exhibits extreme moisture or light sensitivity (e.g., cold form foil), it shall not be repackaged. If a product is refrigerated, it shall not be repackaged unless proper environmental conditions and suitable materials are available. Certain drug products (such as oncologic agents, hormones, or penicillin derivatives) require special handling because they are considered very potent or toxic, and because transfer of any portion of these products to another product could have deleterious effects.

▲ USP36

<1151> PHARMACEUTICAL DOSAGE FORMS

GENERAL CONSIDERATIONS

This chapter provides general descriptions of and definitions for drug products, or dosage forms, commonly used to administer the active pharmaceutical ingredient (API). It discusses general principles involved in the manufacture or compounding of these dosage forms and recommendations for proper use and storage. A glossary is provided as a resource on nomenclature.

A dosage form is a combination of API and often excipients to facilitate dosing, administration, and delivery of the medicine to the patient. The design and testing of all dos-

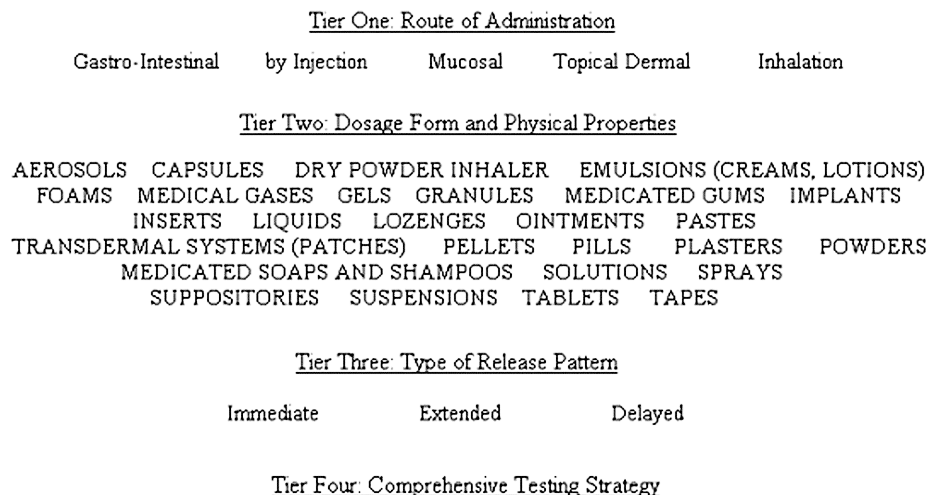


Figure 1. Compendial Taxonomy for Pharmaceutical Dosage Forms.

age forms target drug product quality.¹ A testing protocol must consider not only the physical, chemical, and biological properties of the dosage form as appropriate, but also the administration route and desired dosing regimen. The interrelationships of dosage forms and routes of administration have been summarized in the compendial taxonomy for pharmaceutical dosage forms (see *Figure 1*).² The organization of this general information chapter is by the physical attributes of each particular dosage form (*Tier Two*), generally without specific reference to route of administration. Information specific to route of administration is given when needed.

Tests to ensure compliance with Pharmacopeial standards for dosage form performance fall into one of the following areas.

Dose Uniformity (see also *Uniformity of Dosage Units* (905))—Consistency in dosing for a patient or consumer requires that the variation in the API content of each dosage unit be accurately controlled throughout the manufactured batch or compounded lot of drug product. Uniformity of dosage units typically is demonstrated by one of two procedures: content uniformity or weight variation. The procedure for content uniformity requires the assay of API content of individual units and that for weight variation uses the weight of the individual units to estimate their content. Weight variation may be used where the underlying distribution of API in the blend is presumed to be uniform and well-controlled, as in solutions. In such cases the content of API may be adequately estimated by the net weight. Content uniformity does not rely on the assumption of blend uniformity and can be applied in all cases. Successful development and manufacture of dosage forms requires careful

evaluation of API particle or droplet size, incorporation techniques, and excipient properties.

Stability—Drug product stability involves the evaluation of chemical stability, physical stability, and performance over time. The chemical stability of the API in the dosage form matrix must support the expiration dating for the commercially prepared dosage forms and a beyond-use date for a compounded dosage form. Test procedures for potency must be stability indicating (see *Validation of Compendial Procedures* (1225)). Degradation products should be quantified. In the case of dispersed or emulsified systems, consideration must be given to the potential for settling or separation of the formulation components. Any physical changes to the dosage form must be easily reversed (e.g., by shaking) prior to dosing or administration. For the example of tablets, capsules, and oral suspensions, in vitro release test procedures such as dissolution and disintegration provide a measure of continuing consistency in performance over time (see *Dissolution* (711), *Disintegration* (701), and *Drug Release* (724)).

Bioavailability (see also *In Vitro and In Vivo Evaluation of Dosage Forms* (1088) and *Assessment of Drug Product Performance—Bioavailability, Bioequivalence, and Dissolution* (1090))—Bioavailability is influenced by factors such as the method of manufacture or compounding, particle size, crystal form (polymorph) of the API, the properties of the excipients used to formulate the dosage form, and physical changes as the drug product ages. Assurance of consistency in bioavailability over time (bioequivalence) requires close attention to all aspects of the production (or compounding) and testing of the dosage form. With proper justification, in vitro release (e.g., disintegration and dissolution) testing may sometimes be used as a surrogate to demonstrate consistent availability of the API from the formulated dosage.

Manufacture—Although detailed instructions about the manufacture of any of these dosage forms are beyond the scope of this general information chapter, general manufacturing principles have been included, as well as suggested testing for proper use and storage. Information relative to extemporaneous compounding of dosage forms can be found in *Pharmaceutical Compounding—Nonsterile Preparations* (795) and *Pharmaceutical Compounding—Sterile Preparations* (797).

Route of Administration—The primary routes of administration for pharmaceutical dosage forms can be defined as mucosal, gastrointestinal, parenteral (by injection), inhalation, and topical/dermal, and each has subcategories as needed. Many tests used to ensure quality generally are ap-

¹ In the United States a drug with a name recognized in *USP–NF* must comply with compendial identity standards or be deemed adulterated, misbranded, or both. To avoid being deemed adulterated such drugs also must comply with compendial standards for strength, quality, or purity, unless labeled to show all respects in which the drug differs. See the Federal Food, Drug, and Cosmetic Act (FDCA), Sections 501(b) and 502(e)(3)(b), and Food and Drug Administration (FDA) regulations at 21 CFR 299.5. In addition, to avoid being deemed misbranded, drugs recognized in *USP–NF* also must comply with compendial standards for packaging and labeling, FDCA Section 502(g). “Quality” is used herein as suitable shorthand for all such compendial requirements. This approach also is consistent with U.S. and FDA participation in the International Conference on Harmonization (ICH). The ICH guideline on specifications, Q6A, notes that “specifications are chosen to confirm the quality of the drug substance and drug product...” and defines “quality” as “The suitability of either a drug substance or drug product for its intended use. This term includes such attributes as identity, strength, and purity.”

² Marshall K, Foster TS, Carlin HS, Williams RL. Development of a compendial taxonomy and glossary for pharmaceutical dosage forms. *Pharm Forum*. 2003;29(5):1742–1752.

plied across all of the administration routes, but some tests are specific for individual routes. For example, products intended for injection must be evaluated for *Sterility Tests* (71) and *Pyrogen Test* (151), and the manufacturing process (and sterilization technique) employed for parenterals (by injection) should ensure compliance with these tests. Tests for particulate matter may be required for certain dosage forms depending on the route of administration (e.g., by injection—*Particulate Matter in Injections* (788), or mucosal—*Particulate Matter in Ophthalmic Solutions* (789)). Additionally, dosage forms intended for the inhalation route of administration must be monitored for particle size and spray pattern (for a metered-dose inhaler or dry powder inhaler) and droplet size (for nasal sprays). Further information regarding administration routes and suggested testing can be found in the *Guide to General Chapters, Charts 4–8 and 10–13*.

An appropriate manufacturing process and testing regimen help ensure that a dosage form can meet the appropriate quality attributes for the intended route of administration.

Excess Volume in Injections—Each container of an Injection is filled with a volume in slight excess of the labeled “size” or the volume that is to be withdrawn. The excess volumes recommended in the accompanying table are usually sufficient to permit withdrawal and administration of the labeled volumes.

Labeled Size	Recommended Excess Volume	
	For Mobile Liquids	For Viscous Liquids
0.5 mL	0.10 mL	0.12 mL
1.0 mL	0.10 mL	0.15 mL
2.0 mL	0.15 mL	0.25 mL
5.0 mL	0.30 mL	0.50 mL
10.0 mL	0.50 mL	0.70 mL
20.0 mL	0.60 mL	0.90 mL
30.0 mL	0.80 mL	1.20 mL
50.0 mL or more	2%	3%

Labeling Statements—Some dosage forms or articles have mandatory labeling statements that are given in the Code of Federal Regulations (e.g., 21 CFR 201.320 and 21 CFR 369.21). The text of 21 CFR should be consulted to determine the current recommendations.

PRODUCT QUALITY TESTS, GENERAL

ICH Guidance Q6A (available at www.ich.org) recommends specifications (list of tests, references to analytical procedures, and acceptance criteria) to ensure that commercialized drug products are safe and effective at the time of release and over their shelf life. Tests that are universally applied to ensure safety and efficacy (and strength, quality, and purity) include description, identification, assay, and impurities.

Description—According to the ICH guidance a qualitative description (size, shape, color, etc.) of the dosage form should be provided. The acceptance criteria should include the final acceptable appearance. If any of these characteristics change during manufacturing or storage, a quantitative procedure may be appropriate. It specifies the content or the label claim of the article. This parameter is not part of the USP dosage form monograph because it is product specific. USP monographs define the product by specifying the range of acceptable assayed content of the API(s) present in the dosage form, together with any additional information about the presence or absence of other components, excipients, or adjuvants.

Identification—Identification tests are discussed in the *General Notices and Requirements*. Identification tests should establish the identity of the API(s) present in the drug prod-

uct and should discriminate between compounds of closely related structure that are likely to be present. Identification tests should be specific for the API(s). The most conclusive test for identity is the infrared absorption spectrum (see *Spectrophotometry and Light-Scattering* (851) and *Spectrophotometric Identification Tests* (197)). If no suitable infrared spectrum can be obtained, other analytical methods can be used. Near-infrared (NIR) or Raman spectrophotometric methods also could be acceptable as the sole identification method of the drug product formulation (see *Near-Infrared Spectrophotometry* (1119) and *Raman Spectroscopy* (1120)). Identification by a chromatographic retention time from a single procedure is not regarded as specific. The use of retention times from two chromatographic procedures for which the separation is based on different principles or a combination of tests in a single procedure can be acceptable (see *Chromatography* (621) and *Thin-Layer Chromatographic Identification Test* (201)).

Assay—A specific and stability-indicating test should be used to determine the strength (API content) of the drug product. Some examples of these procedures are *Antibiotics—Microbial Assays* (81), *Chromatography* (621), or *Assay for Steroids* (351). In cases when the use of a nonspecific assay is justified, e.g., *Titrimetry* (541), other supporting analytical procedures should be used to achieve specificity. When evidence of excipient interference with a nonspecific assay exists, a procedure with demonstrated specificity should be used.

Impurities—Process impurities, synthetic by-products, and other inorganic and organic impurities may be present in the API and excipients used in the manufacture of the drug product. These impurities are evaluated by tests in API and excipients monographs. Impurities arising from degradation of the drug substance or from the drug-product manufacturing process should be monitored. *Residual Solvents* (467) is applied to all products where relevant.

In some cases, testing for heavy metal impurities is appropriate. *Heavy Metals* (231) provides the current procedures and criteria.

In addition to the universal tests listed above, the following tests may be considered on a case-by-case basis.

Physicochemical Properties—Examples include *pH* (791), *Viscosity* (911), and *Specific Gravity* (841).

Particle Size—For some dosage forms, particle size can have a significant effect on dissolution rates, bioavailability, therapeutic outcome, and stability. Procedures such as *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* (601) and *Particle Size Distribution Estimation by Analytical Sieving* (786) could be used.

Uniformity of Dosage Units—See discussion of *Dose Uniformity* in the section *General Considerations* above.

Water Content—A test for water content is included when appropriate (see *Water Determination* (921)).

Microbial Limits—The type of microbial test(s) and acceptance criteria are based on the nature of the drug substance, method of manufacture, and the route of administration (see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61) and *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* (62)).

Antimicrobial Preservative Content—Acceptance criteria for preservative content in multidose products should be established. They are based on the levels of antimicrobial preservative necessary to maintain the product's microbiological quality at all stages throughout its proposed usage and shelf life (see *Antimicrobial Effectiveness Testing* (51)).

Antioxidant Content—If antioxidants are present in the drug product, tests of their content should be performed to maintain the product's quality at all stages throughout its proposed usage and shelf life.

Sterility—Depending on the route of administration—e.g., ophthalmic preparations, implants, aqueous-based

preparations for oral inhalation, and solutions for injection—sterility of the product is demonstrated as appropriate (see *Sterility Tests* (71)).

Dissolution—A test to measure release of the API(s) from the drug product normally is included for dosage forms such as tablets, capsules, suspensions, granules for suspensions, implants, transdermal delivery systems, and medicated chewing gums. Single-point measurements typically are used for immediate-release dosage forms. For modified-release dosage forms, appropriate test conditions and sampling procedures are established as needed (see *Dissolution* (711) and *Drug Release* (724)). In some cases, dissolution testing may be replaced by disintegration testing (see *Disintegration* (701)).

Breaking Force and Friability—These parameters are evaluated as in-process controls. Acceptance criteria depend on packaging, supply chain, and intended use (see *Tablet Friability* (1216) and *Tablet Breaking Force* (1217)).

Leachables—When evidence exists that leachables from the container-closure systems (e.g., rubber stopper, cap liner, or plastic bottle) have an impact on the safety or efficacy of the drug product, a test is included to evaluate the presence of leachables.

Other Tests—Depending on the type and composition of the dosage form, other tests such as alcohol content, redispersibility, particle size distribution, rheological properties, reconstitution time, endotoxins/pyrogens, particulate matter, functionality testing of delivery systems, delivered dose uniformity, viscosity, and osmolarity may be necessary.

DOSAGE FORMS

Aerosols

Aerosols are preparations packaged under pressure and contain therapeutic agent(s) and a propellant that are released upon actuation of an appropriate valve system. Upon actuation of the valve system, the API is released as a plume of fine particles or droplets. Only one dose is released from the preparation upon actuation of a metered valve. In the case of topical products and depending on the nature of the API and the conditions being treated, actuation of the valve may result in a metered release of a controlled amount of the formulation or the continuous release of the formulation as long as the valve is depressed.

In this chapter, the aerosol dosage form refers only to those products packaged under pressure that release a fine mist of particles or droplets when actuated (see *Glossary*). Other products that produce dispersions of fine droplets or particles will be covered in subsequent sections (e.g., *Inhalation Powders* and *Sprays*).

TYPICAL COMPONENTS

Typical components of aerosols are the formulation containing one or more API(s) and propellant, the container, the valve, and the actuator. Each component plays a role in determining various characteristics of the emitted plume, such as droplet or particle size distribution, uniformity of delivery of the therapeutic agent, delivery rate, and plume velocity and geometry. The metering valve and actuator act in tandem to generate the plume of droplets or particles. The metering valve allows measure of an accurate volume of the liquid formulation under pressure within the container. The actuator directs the metered volume to a small orifice that is open to the atmosphere. Upon actuation, the formulation is forced through the opening, forming the fine mist of particles that are directed to the site of administration.

Aerosol preparations may consist of either a two-phase (gas and liquid) or a three-phase (gas, liquid, and solid or liquid) formulation. The two-phase formulation consists of

API(s) dissolved in liquefied propellant. Co-solvents such as alcohol may be added to enhance the solubility of the API(s). Three-phase inhalation and nasal aerosol systems consist of suspended API(s) in propellant(s), co-solvents, and potentially other suitable excipients. The suspension or emulsion of the finely divided API typically is dispersed in the liquid propellant with the aid of suitable biocompatible surfactants or other excipients.

Propellants for aerosol formulations are typically low molecular weight hydrofluorocarbons or hydrocarbons that are liquid when constrained in the container, exhibit a suitable vapor pressure at room temperature, and are biocompatible and nonirritating. Compressed gases do not supply a constant pressure over use and typically are not used as propellants.

Metal containers can withstand the vapor pressure produced by the propellant. Excess formulation may be added to the container to ensure that the full number of labeled doses can be accurately administered. The container and closure must be able to withstand the pressures anticipated under normal use conditions as well as when the system is exposed to elevated temperatures.

TYPES OF AEROSOL DOSAGE FORMS

Aerosol dosage forms can be delivered via various routes. The container, actuator, and metering valve, as well as the formulation, are designed to target the site of administration.

Inhalation aerosols, commonly known as metered-dose inhalers (MDIs), are intended to produce fine particles or droplets for inhalation through the mouth and deposition in the pulmonary tree. The design of the delivery system is intended to release measured mass and appropriate quality of the active substance with each actuation.

Nasal aerosols, commonly known as nasal MDIs, produce fine particles or droplets for delivery through the nasal vestibule and deposition in the nasal cavity. Each actuation of the valve releases measured mass and appropriate quality of the active substance.

Lingual aerosols are intended to produce fine particles or droplets for deposition on the surface of the tongue. The design of the delivery system releases one dose with each actuation.

Topical aerosols produce fine particles or droplets for application to the skin.

Topical aerosol drug products may be designed, as needed, to deliver a metered amount of formulation upon actuation of the designed valve or continuous release of formulation during depressed status of the valve.

PACKAGING

The accuracy of a system's delivered dose is demonstrated at the range of pressures likely to be encountered as a result of ambient temperature variations or storage in a refrigerator. As an alternative, the system should include clear instructions for use to ensure the container and contents have been equilibrated to room temperature prior to use.

LABELING FOR PROPER USE

Refer to 21 CFR 201.320 and 21 CFR 369.21.

Many experts recommend the addition of a statement indicating that patients and/or consumers should seek advice and instruction from a health care professional about the proper use of the device.

Capsules

Capsules are solid dosage forms in which the API and excipients are enclosed within a soluble container or shell.

The shells may be composed of two pieces, a body and a cap, or they may be composed of a single piece. Two-piece capsules are commonly referred to as hard-shell capsules, and one-piece capsules are often referred to as soft-shell capsules. This distinction, although it is imprecise, reflects differing levels of plasticizers in the two compositions and the fact that one-piece capsules typically are more pliable than two-piece capsules.

The shells of capsules usually are made from gelatin. However, they also may be made from cellulose polymers or other suitable material. Most capsules are designed for oral administration. When no deliberate effort has been made to modify the API release rate, capsules are referred to as immediate-release.

Two-Piece or Hard-Shell Capsules—Two-piece capsules consist of two telescoping cap and body pieces in a range of standard sizes.

One-Piece or Soft-Shell Capsules—One-piece capsules typically are used to deliver an API as a solution or suspension. Liquid formulations placed into one-piece capsules may offer advantages by comparison with dry-filled capsules and tablets in achieving content uniformity of potent APIs or acceptable dissolution of APIs with poor aqueous solubility. Because the contact between the shell wall and its liquid contents is more intimate than in dry-filled capsules, undesired interactions may be more likely to occur (including gelatin crosslinking and pellicle formation).

Modified-Release Capsules—The release of APIs from capsules can be modified in several ways. There are two categories of modified-release capsule formulations recognized by the Pharmacopeia:

Delayed-Release Capsules—Capsules sometimes are formulated to include enteric-coated granules to protect acid-labile APIs from the gastric environment or to prevent adverse events such as irritation. Enteric-coated multiparticulate capsule dosage forms may reduce variability in bioavailability associated with gastric emptying times for larger particles (i.e., tablets) and to minimize the likelihood of a therapeutic failure when coating defects occur during manufacturing.

Extended-Release Capsules—Extended-release capsules are formulated in such a manner as to make the contained API available over an extended period of time following ingestion. Expressions such as “prolonged-action”, “repeat-action”, “controlled-release”, and “sustained-release” have also been used to describe such dosage forms. However, the term, extended-release, is used for Pharmacopeial purposes. Requirements for dissolution (see *Dissolution* (711)) typically are specified in the individual monograph.

Methods for modifying API release from capsules include coating the filled capsule shells or the contents in the case of dry-filled capsules.

PREPARATION

Two-Piece Capsules—Two-piece gelatin capsules usually are formed from blends of gelatins that have relatively high gel strength in order to optimize shell clarity and toughness or from hypromellose. They also may contain colorants such as D&C and FD&C dyes³ or various pigments, opaquing agents such as titanium dioxide, dispersing agents, plasticiz-

³ In 1960 Congress enacted the Color Additive Amendments, requiring FDA to regulate dyes, pigments, or other coloring agents in foods, drugs, and cosmetics separately from food additives. Under the law, color additives are deemed unsafe unless they are used in compliance with FDA regulations. The law provides a framework for the listing and certification of color additives. See FDCA section 721; see FDA regulations at 21 CFR Part 70. Colors must also be listed in pertinent FDA regulations for specific uses; the list of color additives for drugs that are exempt from certification is published at 21 CFR Part 73, Subpart B. FDA also conducts a certification program for batches of color additives that are required to be certified before sale; see 21 CFR Part 74 (Subpart B re: drugs). Regulations regarding certification procedures, general specifications, and the listing of certified provisionally listed colors are at 21 CFR Part 80. FDA maintains a color additives website with links to various legal and regulatory resources at: <http://www.cfsan.fda.gov/~dms/col-toc.html>.

ers, and preservatives. Gelatin capsule shells normally contain between 12% and 16% water.

The shells are manufactured in one set of operations and later filled in a separate manufacturing process. Two-piece shell capsules are made by a process that involves dipping shaped pins into gelatin or hypromellose solutions, followed by drying, cutting, and joining steps.

Powder formulations for two-piece gelatin capsules generally consist of the API and at least one excipient. Both the formulation and the method of filling can affect release of the API. In the filling operation, the body and cap of the shell are separated before filling. Following the filling operation, the machinery rejoins the body and cap and ensures satisfactory closure of the capsule by exerting appropriate force on the two pieces. The joined capsules can be sealed after filling by a band at the joint of the body and cap or by a designed locking joint between the cap and body. In compounding prescription practice, two-piece capsules may be hand-filled. This permits the prescriber the choice of selecting either a single API or a combination of APIs at the exact dose level considered best for an individual patient.

One-Piece Capsules—One-piece shell capsules are formed, filled, and sealed in a single process on the same machine and are available in a wide variety of sizes, shapes, and colors. The most common type of one-piece capsule is that produced by a rotary die process that results in a capsule with a seam. The soft gelatin shell is somewhat thicker than that of two-piece capsules and is plasticized by the addition of polyols such as glycerin, sorbitol, or other suitable material. The ratio of the plasticizer to the gelatin can be varied to change the flexibility of the shell depending on the nature of the fill material, its intended usage, or environmental conditions.

In most cases, one-piece capsules are filled with liquids. Typically, APIs are dissolved or suspended in a liquid vehicle. Classically, an oleaginous vehicle such as a vegetable oil was used. However, nonaqueous, water-miscible liquid vehicles such as the lower molecular weight polyethylene glycols now are more common. The physicochemical properties of the vehicle can be chosen to ensure stability of the API as well as to influence the release profile from the capsule shell.

Inhalation Powders

Inhalation Powders, commonly known as dry powder inhalers (DPIs), consist of a mixture of API(s) and typically the carrier; and all formulation components exist in a finely divided solid state packaged in a suitable container-closure system. The dose is released from the packaging by a mechanism and is mobilized into a fine dispersion upon oral inhalation by the patient.

TYPICAL COMPONENTS

The basic components of the DPI are the formulation consisting of the API(s) and typically the carrier, both in the dry state. The formulation may be packaged in pre-metered or device-metered units. Pre-metered DPIs contain a previously measured amount of formulation in individual units (e.g., capsules, blisters) that are inserted into the device before use. Pre-metered DPIs may also contain pre-metered dose units as ordered multidose assemblies in the delivery system. Pre-metered DPIs include a mechanism designed to pierce the capsule or open the unit-dose container and allow mobilization and aerosolization of the powder by the patient inhaling through the integral mouthpiece. Device-metered DPI(s) have an internal reservoir that contains a sufficient quantity of formulation for multiple doses that are metered by the device during actuation by the patient. To facilitate dosing compliance, device-metered DPIs incorporate a dosing administration information mechanism, such as a dose counter or a dose indicator system.

PACKAGING

For pre-metered DPIs packaged in blister units, the packs must be designed to allow individual unit cavities to be opened without compromising the seal of adjacent cavities. Package components must provide acceptable protection from humidity, light, and/or oxygen as appropriate. The components of the DPI container-closure system typically are made of plastic.

LABELING AND USE

Many experts recommend the addition of a statement indicating that patients and/or consumers should seek advice and instruction from a health care professional about the proper use of the device.

Emulsions (Creams and Lotions)

Creams—Creams are semisolid emulsion dosage forms. They often contain more than 20% water and volatiles and typically contain less than 50% hydrocarbons, waxes, or polyols as the vehicle for the API. Creams generally are intended for external application to the skin or to the mucous membranes. Creams have a relatively soft, spreadable consistency and can be formulated as either a water-in-oil emulsion (e.g., *Cold Cream* or *Fatty Cream* as in the *European Pharmacopoeia*) or as an oil-in-water emulsion (e.g., *Betamethasone Valerate Cream*). Creams generally are described as either nonwashable or washable, reflecting the fact that an emulsion with an aqueous external continuous phase is more easily removed than one with a nonaqueous external phase (water-in-oil emulsion). Where the term “cream” is used without qualification, a water-washable product is generally inferred.

Lotions—Lotions are an emulsified liquid dosage form generally intended for external application to the skin. Historically, some topical suspensions such as calamine lotion have been called lotions but that nomenclature is not currently preferred. Lotions share many characteristics with creams. The distinguishing factor is that they are more fluid than semisolid and thus pourable. Due to their fluid character, lotions are more easily applied to large skin surfaces than semisolid preparations. Lotions may contain antimicrobial agents as preservatives.

PREPARATION

Pharmaceutical Compounding—Nonsterile Preparations (795) provides general information regarding the preparation of emulsions.

Creams—Creams may be formulated from a variety of oils, both mineral and vegetable, and from fatty alcohols, fatty acids, and fatty esters. The solid excipients are melted at the time of preparation. Emulsifying agents include nonionic surfactants, detergents, and soaps. Soaps are usually formed from a fatty acid in the oil phase hydrolyzed by a base dissolved in the aqueous phase *in situ* during the preparation of creams.

Preparation usually involves separating the formula components into two portions: lipid and aqueous. The lipid portion contains all water-insoluble components and the aqueous portion the water-soluble components. Both phases are heated to a temperature above the melting point of the highest melting component. The phases then are mixed and the mixture is stirred until reaching ambient temperature or the mixture has congealed. Mixing generally is continued during the cooling process to promote uniformity. Traditionally, the aqueous phase is added to the lipid phase, but comparable results have been obtained with the reverse procedure. High-shear homogenation may be employed to

reduce particle or droplet size and improve the physical stability of the resultant dosage form.

The API(s) can be added to the phase in which it is soluble at the beginning of the manufacturing process, or it can be added after the cream is prepared by a suitable dispersion process such as levigation or milling with a roller mill. Creams usually require the addition of a preservative(s) unless they are compounded immediately prior to use and intended to be consumed in a relatively short period of time.

Lotions—Lotions usually are prepared by dissolving or dispersing the API into the more appropriate phase (oil or water), adding the appropriate emulsifying or suspending agents, and mixing the oil and water phases to form a uniform fluid emulsion.

LABELING AND PACKAGING

Some products may require labeling directions indicating to shake well prior to application and to avoid freezing. Storage limits must be specifically indicated to prevent melting of semisolid components. Instructions to ensure proper dosing and administration must accompany the product. Tight containers are used for preparation and storage to prevent loss by evaporation.

Veterinary Drugs and Drug Products Delivered in Animal Feeds

Medicated articles/feeds are preparations used in veterinary medicine to deliver the API(s) via the water or food given to animals. The medicated article/feed may be either a solid or liquid and sometimes is called a premix. Medicated articles/feeds are further subdivided into three types.

TYPE A MEDICATED ARTICLES

Type A medicated articles consist of a new animal drug(s) with or without a carrier (e.g., calcium carbonate, rice hull, corn, gluten) and with or without inactive ingredients. They are sold to licensed feed mills or producers and are intended to be further diluted by mixing into food or water prior to consumption by the animals. Because these preparations are not actually dosed to animals, they are not considered dosage forms.

TYPE B MEDICATED FEEDS

Type B medicated feeds are products that contain a type A medicated article, or another type B medicated feed, plus a substantial quantity of nutrients (not less than 25% of the total weight). Like type A medicated articles, type B medicated feeds are intended for mixture with food or water and additional nutrients, are not to be fed directly to the animals, and are not considered dosage forms.

TYPE C MEDICATED FEEDS

Type C medicated feeds are made from type A medicated articles or type B medicated feeds and are prepared at concentrations of the API appropriate for administration to animals by mixing in food or water. Administration of type C medicated feeds can be accomplished by blending directly into the feed; top-dressing the preparation onto the animal's normal daily rations; or heating, steaming, and extruding into pellets that are mixed or top-dressed onto the animal's food. Another form of type C medicated feeds is compressed or molded blocks from which animals receive the API or nutrients via licking the block.

PREPARATION

Type A medicated articles that are liquids are produced by mixing the API(s) with a suitable solvent (e.g., water or propylene glycol). The API(s) is usually dissolved to produce a solution, but suspension products also could be produced.

Type A medicated articles that are solids are produced by blending the API with excipients to provide a uniform dosage form when mixed with the animal's feed. Often the API is first mixed with an excipient (e.g., starch or sodium aluminosilicate) that has a similar particle size and can help distribute the API uniformly throughout the final drug product. This pre-blend is then mixed with bulking excipients (e.g., calcium carbonate or soybean hulls). Mineral oil may be added to aid uniform distribution, to prevent particle segregation during shipping, and to minimize formation of airborne API particles during production of type B or C medicated feeds.

Type B or C medicated feeds are produced at licensed feed mills or by farm producers. Type A medicated articles are added to the feeds (e.g., ground corn or oats) during the milling process of making feeds. Liquid type A medicated articles often are sprayed in at set rates, and solid type A medicated articles are added slowly to aid in creating uniform distribution in the feeds. Liquid type A medicated articles can also be mixed in with bulk water sources at prescribed amounts.

LABELING AND PACKAGING

Type A medicated articles or type B medicated feeds include special labeling to indicate that they should be used in the manufacture of animal feeds or added to the drinking water. The labels indicate that they are not to be fed directly to animals. Also included is a statement indicating "Not for Human Use". Type A medicated articles or type B medicated feeds are packaged either in paper bags, often with polyethylene liners for solids, and in plastic containers for liquids. Typical sizes are 50-lb bags or several-gallon containers.

Foams

Medicated foams are emulsions containing a dispersed phase of gas bubbles in a liquid continuous phase containing the API. Medicated foams are packaged in pressurized containers or special dispensing devices and are intended for application to the skin or mucous membranes. The medicated foam is formed at the time of application. Surfactants are used to ensure the dispersion of the gas and the two phases. Medicated foams have a fluffy, semisolid consistency and can be formulated to break to a liquid quickly or to remain as foam to ensure prolonged contact.

Medicated foams intended to treat severely injured skin or open wounds must be sterile.

PREPARATION

A foam may contain one or more APIs, surfactants, aqueous or nonaqueous liquids, and the propellants. If the propellant is in the internal (discontinuous) phase (i.e., is of the oil-in-water type), a stable foam is discharged. If the propellant is in the external (continuous) phase (i.e., is of the water-in-oil type), a spray or a quick-breaking foam is discharged. Quick-breaking foams formulated with alcohol create a cooling sensation when applied to the skin and may have disinfectant properties.

LABELING AND USE

Foams formulated with flammable components should be appropriately labeled. Labeling indicates that prior to dis-

persing, a foam drug product is shaken well to ensure uniformity. The instructions for use must clearly note special precautions that are necessary to preserve sterility. In the absence of a metering valve, delivered volume may be variable.

Medical Gases (Inhalation Materials)

Medical gases are products that are administered directly as a gas. A medical gas has a direct pharmacological action or acts as a diluent for another medical gas. Gases used as excipients for administration of aerosol products, as an adjuvant in packaging, or produced by other dosage forms, are not included in this definition.

Components—Medical gases may be single components or defined mixtures of components. Mixtures also can be extemporaneously prepared at the point of use.

Administration—Medical gases may be administered to the patient using several methods: nasal cannulas, face masks, atmospheric tents, and endotracheal tubes for the pulmonary route; hyperbaric chambers for the pulmonary and dermal routes of administration; jetted tubes that are directed at dental tissue to promote drying in preparation for fillings and crowns; tubes for expanding the intestines to facilitate medical imaging during colonoscopy; tubes for expanding the pelvis via transuterine inflation in preparation for fallopian tubal ligation; and tubes for expanding angioplasty devices. The dose of medical gas typically is metered by a volume rate of flow under ambient temperature and pressure conditions. Administration of a highly compressed gas generally requires a regulator to decrease the pressure, a variable-volume flow controller, and suitable tubing to conduct the gas to the patient. For pulmonary administration, the gas flow will be directed to the nose or mouth by a suitable device or into the trachea through a mechanical ventilator. When medical gases are administered chronically, provision for humidification is common. Care should be exercised to avoid microbial contamination.

STORAGE

Medical gases are stored in a compressed state in cylinders or other suitable containers. The containers must be constructed of materials that can safely withstand the expected pressure and must be impact resistant. In some cases each container holds a single defined dose (e.g., general anesthetics), but in other cases the container holds sufficient gas for extended administration.

SPECIAL CONSIDERATIONS

The container and system fittings should be appropriate for the medical gas. Adaptors should not be used to connect containers to patient-use supply system piping or equipment. Large quantities of gases such as oxygen or nitrogen can be stored in the liquid state in a cryogenic container and converted into a gas, as needed, by evaporation. Additional rules concerning the construction and use of cryogenic containers are promulgated by governmental agencies (e.g., U.S. Department of Commerce).

Containers, tubing, and administration masks employed for gases containing oxygen are free of any compound that would be sensitive to oxidation or that would be irritating to the respiratory tract.

A significant fraction of the dose of a medical gas may be released into the general vicinity of the patient due to incomplete absorption. Adequate ventilation may be necessary to protect health care workers and others from exposure to the gas (e.g., nitrous oxide).

LABELING

If required under the individual monograph, label to indicate the method of manufacture (such as oxygen via air liquefaction). When piped directly from the storage container to the point of use, the gas must be labeled for content at each outlet.

When oxygen is in use, a posted warning should indicate the necessity of extinguishing smoking materials and avoiding the use of open flames or other potential ignition sources.

Gels

Gels are semisolids consisting either of suspensions of small inorganic particles or of organic molecules interpenetrated by a liquid. Jellies are a type of gel that typically have a higher water content. Gels can be classed either as single-phase or two-phase systems.

A two-phase gel consists of a network of small discrete particles (e.g., *Aluminum Hydroxide Gel* or *Psyllium Hemicellulose*). Gels may be thixotropic, forming semisolids on standing and becoming less viscous on agitation. They should be shaken before use to ensure homogeneity and should be so labeled.

Single-phase gels consist of organic macromolecules uniformly distributed throughout a liquid in such a manner that no apparent boundaries exist between the dispersed macromolecules and the liquid. Single-phase gels may be made from natural or synthetic macromolecules (e.g., *Carbomer*, *Hydroxypropyl Methylcellulose*, or *Starch*) or natural gums (e.g., *Tragacanth*). The latter preparations are also called mucilages. Although these gels commonly are aqueous, alcohols and oils may be used as the continuous phase. For example, mineral oil can be combined with a polyethylene resin to form an oleaginous ointment base.

Gels can be administered by the topical or mucosal routes. Gels containing antibiotics administered by teat infusion can be used in veterinary medicine to treat mastitis.

PREPARATION

See *Pharmaceutical Compounding—Nonsterile Preparations* (795) for general procedures. Also see the information contained under *Suspensions* for the formulation and manufacture of gels containing inorganic components or APIs in the solid phase. See *Pharmaceutical Compounding—Sterile Preparations* (797) for general procedures for the preparation of sterile gels such as *Lidocaine Hydrochloride Jelly*.

Gels formed with large organic molecules may be formed by dispersing the molecule in the continuous phase (e.g., by heating starch), by cross-linking the dispersed molecules by changing the pH (as for *Carbomer Copolymer*), or by reducing the continuous phase (as for jellies formed with sucrose).

Care should be taken to ensure uniformity of the APIs by dispersing them by vigorous mixing or milling or by shaking if the preparation is less viscous.

PACKAGING AND STORAGE

Store in tight containers to prevent water loss. Avoid freezing.

Granules

Granules are solid dosage forms that are composed of agglomerations of smaller particles. These multicomponent compositions are prepared for oral administration and are used to facilitate flexible dosing regimens as granules or as suspensions, address stability challenges, allow taste masking, or facilitate flexibility in administration (for instance, to

pediatric patients, geriatric patients, or animals). Granular dosage forms may be formulated for direct oral administration and may facilitate compounding of multiple APIs by allowing compounding pharmacists to blend various granular compositions in the retail or hospital pharmacy. More commonly, granules are reconstituted to a suspension by the addition of water or a supplied liquid diluent immediately prior to delivery to the patient. Effervescent granules are formulated to liberate gas (carbon dioxide) upon addition of water. Common examples of effervescent granules include antacid and potassium supplementation preparations. Common therapeutic classes formulated as granule dosage forms include antibiotics, certain laxatives (such as senna extract products), electrolytes, and various cough and cold remedies that contain multiple APIs.

Granular dosage forms also are employed in veterinary medicine when they are often placed on top of or mixed with an animal's food. They are frequently provided with a measuring device to allow addition to feeds. The resultant mix facilitates dosing.

PREPARATION

Granules often are the precursors used in tablet compression or capsule filling. Although this application represents a pharmaceutical intermediate and not a final dosage form, numerous commercial products are based on granules. In the typical manufacture of granules, the API is blended with excipients (processing aids) and wetted with an appropriate pharmaceutical binding solution, solvent, or blend of solvents to promote agglomeration. This composition is dried and sized to yield the desired material properties.

Frequently, granules are used because the API is unstable in aqueous environments and cannot be exposed to water for periods sufficient to accommodate manufacture, storage, and distribution in a suspension. Preparation of the liquid dosage form from the granules immediately prior to dispensing allows acceptable stability for the duration of use. Granules manufactured for this purpose are packaged in quantities sufficient for a limited time period—usually one course of therapy that typically does not exceed two weeks. In addition to the API, other ingredients may be added to ensure acceptable stability (e.g., buffers, antioxidants, or chelating agents) or to provide color, sweetness, and flavor; and for suspensions, to provide acceptable viscosity to ensure adequate suspension of the particulate to enable uniform dosing.

Effervescent granules typically are formulated from sodium or potassium bicarbonate and an acid such as citric or tartaric acid. To prevent untimely generation of carbon dioxide, manufacturers should take special precautions to limit residual water in the product due to manufacture and to select packaging that protects the product from moisture. The manufacture of effervescent granules can require specialized facilities designed to maintain very low humidity (approximately 10% relative humidity). Effervescent powder mixtures are purposely formed into relatively coarse granules to reduce the rate of dissolution and provide a more controlled effervescence.

PACKAGING AND STORAGE

Granules for reconstitution may be packaged in unit-of-use containers or in containers with sufficient quantities to accommodate a typical course of therapy (frequently 10 days to two weeks with antibiotic products). Packaging should provide suitable protection from moisture. This is particularly true for effervescent granules. Granules may be stored under controlled room temperature conditions unless other conditions are specifically noted.

Many granule products specify refrigerated storage following reconstitution and direct the patient to discard unused

contents after a specified date that is based on the stability of the API in the reconstituted preparation.

LABELING AND USE

Effervescent granules (and tablets) are labeled to indicate that they are not to be swallowed directly.

Reconstitution of granules must ensure complete wetting of all ingredients and sufficient time and agitation to allow the soluble components to dissolve. Specific instructions for reconstitution provided by the manufacturer should be carefully followed.

Reconstituted suspensions should be thoroughly mixed or shaken before use to re-suspend the dispersed particulates. This is especially true of suspension preparations dosed from multiple-dose containers. For particularly viscous suspensions prone to air entrapment, instructions may advise the user how to shake the preparation to re-suspend settled particulates while minimizing air entrapment.

SPECIAL CONSIDERATIONS

For granules reconstituted to form suspensions for oral administration, acceptable suspension of the particulate phase depends on the particle size of the dispersed phase as well as the viscosity of the vehicle. Temperature can influence the viscosity, which influences suspension properties and the ease of removal of the dose from the bottle. In addition, temperature cycling can lead to changes in the particle size of the dispersed phase via Ostwald ripening. Thus, clear instructions should be provided regarding the appropriate storage temperature for the product.

Medicated Gums

Medicated gum is a semisolid confection that is designed to be chewed rather than swallowed. Medicated gums release the API(s) into the saliva. Medicated gums can deliver therapeutic agents for local action in the mouth (such as antibiotics to control gum disease) or for systemic absorption via the buccal or gastrointestinal routes (e.g., nicotine or aspirin). Most medicated gums are manufactured using the conventional melting process derived from the confectionary industry or alternatively may be directly compressed from gum powder. Medicated gums are formulated from insoluble synthetic gum bases such as polyisoprene, polyisobutylene, isobutyleneisoprene copolymer, styrene butadiene rubber, polyvinyl acetate, polyethylene, ester gums, or polyterpenes. Plasticizers and softeners such as propylene glycol, glycerin, oleic acid, or processed vegetable oils are added to keep the gum base pliable and to aid incorporation of the API(s), sweeteners, and flavoring agents. Sugars as well as artificial sweeteners and flavorings are incorporated to improve taste, and dyes may be used to enhance appearance. Some medicated gums are coated with magnesium stearate to reduce tackiness and improve handling during packaging. A preservative may be added.

PREPARATION

Melted Gum—The gum base is melted at a temperature of about 115° until it has the viscosity of thick syrup and at that point is filtered through a fine-mesh screen. This molten gum base is transferred to mixing tanks where the sweeteners, plasticizers, and typically the API are added and mixed. Colorings, flavorings, and preservatives are added and mixed while the melted gum is cooling. The cooled mixture is shaped by extrusion or rolling and cutting. Dosage units of the desired shape and potency are packaged individually. Additional coatings such as powder coatings to reduce tackiness or film or sugar coatings may be added to improve taste or facilitate bulk packaging.

Directly Compressed Gum—The gum base is supplied in a free-flowing granular powder form. The powder gum base is then dry blended with sweeteners, flavors, the API, and lubricant. The blend is then processed through a conventional tablet press and tableted into desired shapes. The resulting medicated gum tablets can be further coated with sugar or sugar-free excipients. These tablets can be packaged in blisters or bottles as needed.

SPECIAL CONSIDERATIONS

Medicated gums are typically dispensed in unit-dose packaging. The patient instructions also may include a caution to avoid excessive heat.

Implants

Implants are long-acting dosage forms that provide continuous release of the API often for periods of months to years. They are administered by the parenteral route. For systemic delivery they may be placed subcutaneously, or for local delivery they can be placed in a specific region in the body.

Several types of implants are available. Pellet implants are small, sterile, solid masses composed of an API with or without excipients. They are usually administered by means of a suitable special injector (e.g., trocar) or by surgical incision. Release of the API from pellets typically is controlled by diffusion and dissolution kinetics. The size of the pellets and rate of erosion will influence the release rate, which typically follows first-order kinetics. API release from pellets for periods of six months or more is possible. Pellet implants have been used to provide extended delivery of hormones such as testosterone or estradiol.

Resorbable microparticles are a type of implants that provide extended release of API over periods varying from a few weeks to months. They can be administered subcutaneously or intramuscularly for systemic delivery, or they may be deposited in a desired location in the body for site-specific delivery. Injectable resorbable microparticles (or microspheres) generally range from 20 to 100 µm in diameter. They are composed of an API dispersed within a biocompatible, bioresorbable polymeric excipient (matrix). Poly(lactide-co-glycolide) polymers have been used frequently. These excipients typically resorb by hydrolysis of ester linkages. The microparticles are administered by suspension in an aqueous vehicle followed by injection with a conventional syringe and needle. Release of the API from the microparticles begins after physiological fluid enters the polymer matrix, dissolving some of the API that then is released by a diffusion-controlled process. Drug release also can occur as the matrix erodes.

Polymer implants can be formed as a single-shaped mass such as a cylinder. The polymer matrix must be biocompatible, but it can be either biodegradable or nonbiodegradable. Shaped polymer implants are administered by means of a suitable special injector. Release kinetics typically are not zero-order, but zero-order kinetics are possible. API release can be controlled by the diffusion of the API from the bulk polymer matrix or by the properties of a rate-limiting polymeric membrane coating. Polymer implants are used to deliver potent small molecules like steroids (e.g., estradiol for cattle) and large molecules like peptides [e.g., luteinizing hormone-releasing hormone (LHRH)]. Example durations of API release are two and three months for biodegradable implants and one year for nonbiodegradable implants. An advantage of biodegradable implants is that they do not require removal after release of all API content. Nonbiodegradable polymer implants can be removed before or after API release is complete or may be left in situ. An implant can have a tab with a hole in it to facilitate suturing it in place, e.g., for an intravitreal implant for local ocular deliv-

ery. Such implants may provide therapeutic release for periods as long as 2.5 years.

Some implants are designed to form as a mass in situ. These implants are initially prepared as liquid formulations comprising polymer, API, and solvent for the polymer. The polymer solvent can be water or an organic solvent. After administration of the liquid formulation to a patient by subcutaneous or intramuscular administration, it forms a gel or a solid polymeric matrix that traps the API and extends the API release for days or months. In situ-forming implants also are used for local delivery of the API to treat periodontal disease. The implant is formed within the periodontal pocket.

Another type of implant can be fabricated from a metal such as titanium and plastic components. These implants are administered by means of a suitable injector or by surgical installation. A solution of API inside the implant, like an LHRH solution, is released via an osmotically driven pump inside the implant. Duration of release may be as long as one year or more. Release kinetics are zero order. After the API is delivered, metal-based implants are removed.

API-eluting stents combine the mechanical effect of the stent to maintain arterial patency with the prolonged pharmacologic effect of the incorporated API (to reduce restenosis, inhibit clot formation, or combat infection). As an example, a metal stent can be coated with a nonbiodegradable or biodegradable polymer-containing API. The resultant coating is a polymeric matrix that controls the extended release of the API.

PREPARATION

Pellet implants are made by API compression or molding. Cylindrical polymeric implants typically are made by melt extrusion of a blend of API and polymer, resulting in a rod that is cut into shorter lengths. Polymer implants also can be made by injection molding. Still other implants are assembled from metal tubes and injection-molded plastic components.

Sterility can be achieved by terminal sterilization or by employing aseptic manufacturing procedures.

PACKAGING AND STORAGE

All implants are individually packaged (typically in their injector or for veterinary use in cartridges that are placed in the injector guns), are sterile (except for some animal health products), and conform to the appropriate standards for injection. Biodegradable implants are protected from moisture so the polymer does not hydrolyze and alter drug release kinetics before use.

Inserts

Inserts are solid dosage forms that are inserted into a naturally occurring (nonsurgical) body cavity other than the mouth or rectum (see *Suppositories*). The API is delivered in inserts for local or systemic action. Inserts applied to the eye, such as *Pilocarpine Ocular System*, typically are sterile. Vaginal inserts for humans are usually globular or oviform and weigh about 5 g each. Vaginal inserts for cattle are T-shaped, are formed of polymer, are removable, and can be used for up to eight days. One veterinary application is for estrus synchronization. Inserts intended to dissolve in vaginal secretions usually are made from water-soluble or water-miscible vehicles such as polyethylene glycol or glycerinated gelatin. Vaginal inserts such as dinoprostone vaginal insert (e.g., see USP monograph *Dinoprostone Vaginal Suppositories*) are formulated to deliver medication to the cervix and to be removed or recovered once the API has been

released. Intrauterine inserts such as *Progesterone Intrauterine Contraceptive System* are used to deliver APIs locally to achieve efficacy while reducing side effects. Some intrauterine inserts are formulated to remain in the uterus for extended periods of time. An intra-urethral insert of alprostadil is available for the treatment of erectile dysfunction.

PREPARATION

For general considerations see *Pharmaceutical Compounding—Nonsterile Preparations* (795). Inserts vary considerably in their preparation. Inserts may be molded (using technology similar to that used to prepare lozenges, suppositories, or plastics), compressed from powders (as in tableting), or formulated as special applications of capsules (soft gelatin capsules and hard gelatin capsules have been employed for extemporaneously compounded preparations). Inserts may be formulated to melt at body temperature or disintegrate upon insertion. Design of the dosage form should take into consideration the fluid volume available at the insertion site and minimize the potential to cause local irritation. Most inserts are formulated to ensure retention at the site of administration.

STORAGE AND LABELING

Appropriate storage conditions must be clearly indicated in the labeling for all inserts, especially for those that are designed to melt at body temperature. Instructions to ensure proper dosing and administration must accompany the product.

Liquids

As a dosage form a liquid consists of a pure chemical in its liquid state. Examples include mineral oil, isoflurane, and ether. This dosage form term is not applied to solutions.

STORAGE AND LABELING

Storage, packaging, and labeling consider the physical properties of the material and are designed to maintain potency and purity.

Lotions

(See *Emulsions*.)

Lozenges

Lozenges are solid oral dosage forms that are designed to dissolve or disintegrate slowly in the mouth. They contain one or more APIs that are slowly liberated from the flavored and sweetened base. They are frequently intended to provide local action in the oral cavity or the throat but also include those intended for systemic absorption after dissolution. The typical therapeutic categories of APIs delivered in lozenges are antiseptics, analgesics, decongestants, antitussives, and antibiotics. Molded lozenges are called cough drops or pastilles. Lozenges prepared by compression or by stamping or cutting from a uniform bed of paste sometimes are known as troches. Troches are often produced in a circular shape.

Lozenges can be made using sugars such as sucrose and dextrose or can provide the benefits of a sugar-free formulation that is usually based on sorbitol or mannitol. Polyethylene glycols and hypromellose sometimes are included to slow the rate of dissolution.

MANUFACTURE

Excipients used in molded lozenge manufacture include gelatin, fused sucrose, sorbitol, or another carbohydrate base.

Molded lozenges using a sucrose or sorbitol base containing APIs such as phenol, dextromethorphan, fentanyl, and dyclonine hydrochloride and menthol are prepared by cooking the sugar (sucrose, corn syrup, and sorbitol) and water at about 150° to reduce the water content to less than 2%. The molten sugar solution is transferred to a cooling belt or cooling table, and medicaments, flavorings, and colorings are added and thoroughly mixed while cooling. Individual dosage units of the desired shape are formed by filling the molten mass into molds. These lozenges are quickly cooled in the molds to trap the base in the glassy state. Once formed, the lozenges are removed from the molds and packaged. Care is taken to avoid excessive moisture during storage to prevent crystallization of the sugar base.

Compressed lozenges are made using excipients that may include a filler, binder, sweetening agent, flavoring agent, and lubricant. Sugars such as sucrose, sorbitol, and mannitol often are included because they can act as filler and binder as well as serve as sweetening agents. Approved FD&C and D&C dyes or lakes (dyes adsorbed onto insoluble aluminum hydroxide) also may be present.

The manufacturing of compressed lozenges is essentially the same as that for conventional tableting, with the exception that a tablet press capable of making larger tablets and exerting greater force to produce harder tablets may be required (see *Tablets*).

The paste used to produce lozenges manufactured by stamping or cutting contains a moistening agent, sucrose, and flavoring and sweetening agents. The homogenous paste is spread as a bed of uniform thickness, and the lozenges are cut or stamped from the bed and are allowed to dry. Some lozenges are prepared by forcing dampened powders under low pressure into mold cavities and then ejecting them onto suitable trays for drying at moderate temperatures.

PACKAGING AND STORAGE

Many lozenges are sensitive to moisture, and typically a monograph indicates that the package or container type is well closed and/or moisture resistant. Storage instructions may include protection from high humidity.

Ointments

Ointments are semisolid preparations intended for external application to the skin or mucous membranes. APIs delivered in ointments are intended for local action or for systemic absorption. Ointments usually contain less than 20% water and volatiles and more than 50% hydrocarbons, waxes, or polyols as the vehicle. Ointment bases recognized for use as vehicles fall into four general classes: hydrocarbon bases, absorption bases, water-removable bases, and water-soluble bases.

Hydrocarbon Bases—Also known as oleaginous ointment bases, they allow the incorporation of only small amounts of an aqueous component. Ointments prepared from hydrocarbon bases act as occlusive dressings and provide prolonged contact of the API with the skin. They are difficult to remove and do not change physical characteristics upon aging.

Absorption Bases—Allow the incorporation of aqueous solutions. Such bases include only anhydrous components (e.g., *Hydrophilic Petrolatum*) or water-in-oil emulsions (e.g., *Lanolin*). Absorption bases are also useful as emollients.

Water-Removable Bases—Oil-in-water emulsions (e.g., *Hydrophilic Ointment*) are sometimes referred to as creams

(see *Emulsions*). They may be readily washed from the skin or clothing with water, making them acceptable for cosmetic reasons. Other advantages of the water-removable bases are that they can be diluted with water and that they favor the absorption of serous discharges in dermatological conditions.

Water-Soluble Bases—Also known as greaseless ointment bases, they are formulated entirely from water-soluble constituents. *Polyethylene Glycol Ointment* is the only official preparation in this group. They offer many of the advantages of the water-removable bases and, in addition, contain no water-insoluble substances such as petrolatum, anhydrous lanolin, or waxes. They are more correctly categorized as gels (see *Gels*).

The choice of an ointment base depends on the action desired, the characteristics of the incorporated API, and the latter's bioavailability if systemic action is desired. The product's stability may require the use of a base that is less than ideal in meeting other quality attributes. APIs that hydrolyze rapidly, for example, are more stable in hydrocarbon bases than in bases that contain water.

Ophthalmic ointments are intended for application directly to the eye or eye-associated structures such as the subconjunctival sac. They are manufactured from sterilized ingredients under aseptic conditions and meet the requirements under *Sterility Tests* (71). Ingredients meeting the requirements described under *Sterility Tests* (71) are used if they are not suitable for sterilization procedures. Ophthalmic ointments in multiple-dose containers contain suitable antimicrobial agents to control microorganisms that might be introduced during use unless otherwise directed in the individual monograph or unless the formula itself is bacteriostatic (see *Ophthalmic Ointments* (771), *Added Substances*). The finished ointment is free from large particles and must meet the requirements for *Leakage* and for *Metal Particles* under *Ophthalmic Ointments* (771). The immediate container for ophthalmic ointments is sterile at the time of filling and closing. The immediate containers for ophthalmic ointments are sealed and made tamper-proof so that sterility is ensured at time of first use.

A suitable ophthalmic ointment base is nonirritating to the eye and permits diffusion of the API throughout the secretions bathing the eye. Petrolatum is most commonly used as a base for ophthalmic APIs. Some absorption bases, water-removable bases, and water-soluble bases may be desirable for water-soluble APIs if the bases are nonirritating.

MANUFACTURE

Ointments typically are prepared by either direct incorporation into a previously prepared ointment base or by fusion (heating during the preparation of the ointment). A levigating agent is often added to facilitate the incorporation of the medicament into the ointment base by the direct incorporation procedure. In the fusion method, the ingredients are heated, often in the range of 60° to 80°. Homogenization is often necessary. The rate of cooling is an important manufacturing detail because rapid cooling can impart increased structure to the product of the fusion method.

PACKAGING AND STORAGE

Protect from moisture. For emulsified systems, temperature extremes can lead to physical instability of the preparation. When this is the case products should be clearly labeled to specify appropriate storage conditions. Ointments typically are packaged either in ointment jars or ointment tubes. Ointment jars are often used for more viscous ointments that do not require sterility. Ointment tubes typically are used for less viscous ointments and those such as ophthalmic ointments that require the maintenance of sterility. The package sizes for ophthalmic preparations are con-

trolled to minimize the likelihood of contamination and loss of sterility.

Pastes

Pastes are semisolid preparations of stiff consistency and contain a high percentage of finely dispersed solids. Pastes are intended for application to the skin, oral cavity, or mucous membranes. In veterinary practice, pastes are used for systemic delivery of APIs.

Pastes ordinarily do not flow at body temperature and thus can serve as occlusive, protective coatings. As a consequence, pastes are more often used for protective action than are ointments.

Fatty pastes that have a high proportion of hydrophilic solids appear less greasy and more absorptive than ointments. They are used to absorb serous secretions and are often preferred for acute lesions that have a tendency toward crusting, vesiculation, or oozing.

Dental pastes may be applied to the teeth, or alternatively they may be indicated for adhesion to the mucous membrane for a local effect (e.g., *Triamcinolone Acetonide Dental Paste*). Some paste preparations intended for animals are administered orally. The paste is squeezed into the mouth of the animal, generally at the back of the tongue, or is spread inside the mouth.

PREPARATION

Pastes can be prepared by direct incorporation or by fusion (the use of heat to soften the base). The solid ingredients often are incorporated following comminution and sieving. If a levigating agent is needed, a portion of the ointment base is often employed rather than a liquid.

LABELING AND STORAGE

Veterinary products should be labeled to ensure they are not administered to humans. Labeling should indicate the need for protection from heat.

Transdermal Systems (Patches)

Transdermal API delivery systems (TDSs) are discrete dosage forms that are designed to deliver the API(s) through intact skin to the systemic circulation. Typically, a TDS is composed of an outer covering (barrier), an API reservoir (possibly covered with a rate-controlling membrane), a contact adhesive applied to some or all parts of the system (to attach the TDS to the skin surface), and a protective layer that is removed before the patch is applied. The activity of a TDS is defined in terms of the release rate of the API(s) from the system. The total duration of drug release from the system and the system surface area also may be stated.

Most TDSs can be considered either matrix-type or reservoir-type systems. Matrix-type patches are often further divided into monolithic adhesive matrix or polymer matrix types. Reservoir-type systems include liquid reservoir systems and solid-state reservoir systems. Solid-state reservoir patches also include multilaminate adhesive and multilaminate polymer matrix systems.

Drug delivery from some TDSs is controlled by diffusion kinetics. The API diffuses from the drug reservoir directly or through the rate-controlling membrane and/or contact adhesive and then through the skin into the general circulation. Modified-release systems are generally designed to provide drug delivery at a constant rate so that a true steady-state blood concentration is achieved and maintained until the system is removed. Other TDSs work by active transport of the API. For example, iontophoretic transdermal delivery uses the electric current between two electrodes to enhance the movement of ionized APIs through the skin.

TDSs are applied to the body areas recommended by the labeling. The API content of the system provides a reservoir that, by design, maintains a constant API concentration at the system-skin interface. The dosing interval of the system is a function of the amount of API in the reservoir and the release rate. Some API concentration may remain in the reservoir at the end of the dosing interval, in particular for diffusion-controlled delivery mechanisms. [NOTE—Where the API is intended for local action, it may be embedded in adhesive on a cloth or plastic backing. This type of product is sometimes called a plaster or tape (see *Plasters and Tapes*).]

PREPARATION

TDSs require a backing, a means of storing the API for delivery to the skin, an adhesive to attach the system to the skin, and a removable release liner to protect the adhesive, API, and excipients before application. The backing has low moisture- and vapor-transmission rates to support product stability. The adhesive layer may contain the API and permeation enhancers in the case of matrix-type systems or multilaminate reservoir systems for which a priming dose is desired. Adhesive may be applied to the entire patch release surface or merely to the periphery. Liquid reservoir systems are often formed-filled-sealed between the backing and release-controlling materials. For monolithic adhesive matrix systems, the API and excipients are applied as a solution or suspension either to the backing or the release liner, and the solvent is allowed to evaporate.

PACKAGING AND STORAGE

Storage conditions are clearly specified because extreme temperature excursions can influence the performance of some systems.

LABELING

The labeling should clearly indicate any performance limitations of the system (e.g., influence of application site, hydration state, hair, or other variables).

Pellets

Pellets are dosage forms composed of small, solid particles of uniform shape sometimes called beads. Typically, pellets are nearly spherical but this is not required. Pellets may be administered by the oral (gastrointestinal) or by the injection route (see also *Implants*). Pellet formulations may provide several advantages including physical separation for chemically or physically incompatible materials, extended release of the API, or delayed release to protect an acid-labile API from degradation in the stomach or to protect stomach tissues from irritation. Extended-release pellet formulations may be designed with the API dispersed in a matrix, or the pellet may be coated with an appropriate polymer coating that modifies the drug-release characteristics. Alternatively, the pellet design may combine these two approaches. In the case of delayed-release formulations, the coating polymer is chosen to resist dissolution at the lower pH of the gastric environment but to dissolve in the higher pH intestinal environment. Injected or surgically administered pellet preparations (see *Implants*) are often used to provide continuous therapy for periods of months or years.

Pellet dosage forms may be designed as single or multiple entities. Often implanted pellets will contain the desired API content in one or several units. In veterinary practice, multiple pellets may be implanted in the ears of cattle, depending on animal size. Oral pellets typically are contained within hard gelatin capsules for administration. Although there are no absolute requirements for size, the useful size

range of pellets is governed by the practical constraints of the volume of commonly used capsules and the need to include sufficient numbers of pellets in each dose to ensure uniform dosing of the API. As a result, many pellets used for oral administration fall within a size range of 710 μm to 2.36 mm. Pellet formulations sometimes are used to minimize variability associated with larger dosage forms caused by gastric retention upon stomach emptying.

Enteric-coated (delayed-release) pellet formulations and some extended-release formulations are prepared by applying a coating to the formulated particles. The coating must be applied as a continuous film over the entire surface of each particle. Because a small population of imperfectly coated particles may be unavoidable, oral pellets are designed to require the administration of a large number in a single dose to minimize any adverse influence of imperfectly coated pellets on drug delivery.

PREPARATION

The desired performance characteristics determine the manufacturing method chosen. In general, pellet dosage forms are manufactured by wet extrusion processes followed by spheronization, by wet or dry coating processes, or by compression. Manufacture of pellets by wet coating usually involves the application of successive coatings upon nonpareil seeds. This manufacturing process frequently is conducted in fluid-bed processing equipment. Dry powder coating or layering processes often are performed in specialized rotor granulation equipment. The extent of particle growth achievable in wet coating processes is generally more limited than the growth that can be obtained with dry powder layering techniques, but either method allows the formulator to develop and apply multiple layers of coatings to achieve the desired release profile. The manufacture of pellets by compression is largely restricted to the production of material for subcutaneous implantation. This method of manufacture provides the necessary control to ensure dose uniformity and generally is better suited to aseptic processing requirements.

Alternatively, microencapsulation techniques can be used to manufacture pellets. Coacervation coating techniques typically produce coated particles that are much smaller than those made by other techniques.

PACKAGING AND STORAGE

Pellets for oral administration generally are filled into hard gelatin capsules and are placed in bottles or blister packages. The packaging provides suitable protection from moisture to ensure the stability of the pellet formulation as well as to preserve desirable moisture content of the capsule shells. Pellets for implantation are sterile and should be packaged in tight containers suitable for maintaining sterile contents. Pellets may be stored under controlled room temperature conditions unless other conditions are specifically noted.

LABELING AND USE

Pellets for oral administration that are formulated to provide delayed or extended release must be swallowed intact to ensure preservation of the desired release characteristics. These products should be labeled accordingly to ensure that the material is not crushed or chewed during administration.

Pills

Pills are API-containing small, round solid bodies intended for oral administration. At one time pills were the most extensively used oral dosage form, but they have been re-

placed by compressed tablets and capsules. Pills are distinguished from tablets because pills are usually prepared by a wet massing and molding technique, while tablets are typically formed by compression.

PREPARATION

Excipients are selected on the basis of their ability to produce a mass that is firm and plastic. The API is triturated with powdered excipients in serial dilutions to attain a uniform mixture. Liquid excipients that act to bind and provide plasticity to the mass are subsequently added to the dry materials. The mass is formed by kneading. The properties of firmness and plasticity are necessary to permit the mass to be worked and retain the shape produced. Cylindrical pill pipes are produced from portions of the mass. The pill pipe is cut into individual lengths corresponding to the intended pill size, and the pills are rolled to form the final shape. Pill-making machines can automate the preparation of the mass, production of pill piping, and the cutting and rolling of pills.

PACKAGING AND LABELING

Labeling and use instructions for pills are similar to those for tablets. Although many pills are resistant to breakage, some pills are friable. Appropriate handling guidelines should be provided in such cases in order to avoid breakage.

Plasters

A plaster is a semisolid substance for external application and usually is supplied on a support material. Plasters are applied for prolonged periods to provide protection, support, or occlusion (maceration).

Plasters consist of an adhesive layer that may contain active substances. This layer is spread uniformly on an appropriate support that is usually made of a rubber base or synthetic resin. Unmedicated plasters are designed to provide protection or mechanical support to the site of application. These plasters are neither irritating nor sensitizing to the skin.

Plasters are available in a range of sizes or cut to size to effectively provide prolonged contact to the site of application. They adhere firmly to the skin but can be peeled off the skin without causing injury.

One example of a plaster currently in use is salicylic acid plasters used for the removal of corns by the keratolytic action of salicylic acid.

PACKAGING AND STORAGE

Plasters are preserved in well-closed containers, preferably at controlled room temperature.

Powders

Powders are defined as a solid or a mixture of solids in a finely divided state intended for internal or external use. Powders used as pharmaceutical dosage forms may contain one or more APIs and can be mixed with water for oral administration or injection. Often pediatric antibiotics utilize a powder dosage form for improved stability. In some areas medicated powders are used for extemporaneous compounding of preparations for simultaneous administration of multiple APIs. Medicated powders also can be inhaled for pulmonary administration (see *Inhalation Powders*). Aerosolized powders for the lungs typically contain processing aids to improve flow and ensure uniformity (see *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers*).

(601)). Powders can also be used topically as a dusting powder.

Externally applied powders should have a particle size of 150 μm or less (typically in the 50- to 100- μm range) in order to prevent a gritty feel on the skin that could further irritate traumatized skin. Powders are grouped according to the following terms: very coarse, coarse, moderately coarse, fine, and very fine (see *Powder Fineness* (811)). The performance of powder dosage forms can be affected by the physical characteristics of the powder. Particle size can influence the dissolution rate of the particles and affect bioavailability. For dispersed delivery systems, particle size can influence the mixing and segregation behavior of the particle, which in turn affects the uniformity of the dosage form.

PREPARATION

Powder dosage forms can be produced by the combination of multiple components into a uniform blend. This can also involve particle size reduction, a process referred to as comminution. Mills and pulverizers are used to reduce the particle size of powders when necessary. As the particle size is decreased, the number of particles and the surface area increase, which can increase the dissolution rate and bioavailability of the API.

Blending techniques for powders include those used in compounding pharmacy such as spatulation and trituration (see *Pharmaceutical Compounding—Nonsterile Preparations* (795)). Industrial processes may employ sifting or tumbling the powders in a rotating container. One of the most common tumble blenders is a V-blender, which is available in a variety of scales suitable for small-scale and large-scale compounding and industrial production.

Powder flow can be influenced by both particle size and shape. Larger particles generally flow more freely than do fine particles. Powder flow is an important attribute that can affect the packaging or dispensing of a medicated powder.

PACKAGING AND STORAGE

Powders for pharmaceutical use can be packaged in multiple- or single-unit containers. Bulk containers have been used for antacid powders and for laxative powders. In these instances the patient dissolves the directed amount in water prior to administration. This type of multiple-unit packaging is acceptable for many APIs but should not be utilized for powders that require exact dosing. Multiple-unit powders for topical application often are packaged in a container with a sifter top.

Potent APIs in a powder dosage form are dispensed in unit-of-use allocations in folded papers, cellophane envelopes, or packets. Powder boxes are often used by the dispensing pharmacist to hold multiple doses of individual folded papers. Hygroscopic powders pose special challenges and typically are dispensed in moisture-resistant packaging.

LABELING

Typical warning statements include:

- External powders must indicate: "External Use Only".
- Oral powders should indicate: "For Oral Use Only".

Individual monographs specify the labeling requirements for powder dosage forms that are listed in *USP–NF*. Oral powders for reconstitution prior to dispensing typically have a limited shelf life (for example, two weeks), and the dispensed product should indicate a beyond-use date based on the date of the water addition. Pharmaceutical powders that are compounded indicate a beyond-use date. Compounded preparations typically are intended for immediate use and have short-term storage durations.

Medicated Soaps and Shampoos

Medicated soaps and shampoos are solid or liquid preparations intended for topical application to the skin or scalp followed by subsequent rinsing with water. Soaps and shampoos are emulsions or surface-active compositions that readily form emulsions or foams upon the addition of water followed by rubbing. Incorporation of APIs in soaps and shampoos combines the cleansing/degreasing abilities of the vehicle and facilitates the topical application of the API to affected areas, even large areas, of the body. The surface-active properties of the vehicle facilitate contact of the API with the skin or scalp. Medicated soap and shampoo formulations frequently contain suitable antimicrobial agents to protect against bacteria, yeast, and mold contamination.

PREPARATION

The preparation of medicated soaps and shampoos follows techniques frequently used for the preparation of emulsified systems. To ensure uniformity, the API(s) must be added to the vehicle prior to congealing (in the case of soaps) followed by thorough mixing. If the medication is present as a suspension, the particle size must be controlled to promote uniform distribution of the API and possibly optimize performance. Because soap manufacture frequently involves processing the ingredients at elevated temperature, care must be exercised to avoid excessive degradation of the API during processing.

PACKAGING AND STORAGE

Individual monographs specify the packaging and storage requirements for medicated soaps and shampoos in *USP–NF*.

LABELING AND USE

Medicated soaps and shampoos are clearly labeled to indicate "For External Use Only". The preparations also clearly advise the patient to discontinue use and consult a physician/veterinarian if skin irritation or inflammation occurs or persists following application.

Solutions

A solution is a preparation that contains one or more dissolved chemical substances in a suitable solvent or mixture of mutually miscible solvents. Because molecules of an API in solution are uniformly dispersed, the use of solutions as dosage forms generally provides assurance of uniform dosage upon administration and good accuracy when the solution is diluted or otherwise mixed.

Substances in solutions are more susceptible to chemical instability than they are in the solid state and dose-for-dose generally are heavier and more bulky than solid dosage forms. These factors increase the cost of packaging and shipping relative to that of solid dosage forms. Solution dosage forms can be administered by injection; inhalation; and the mucosal, topical/dermal, and gastrointestinal routes. Terminology for solutions in veterinary practice includes spot-ons or pour-ons that refer to solutions that are applied to an animal's skin for systemic absorption, dips that refer to solutions that are used for washing and disinfection (e.g., udders, eggs, and whole animals), and drenches that include solutions that are orally administered to livestock, usually with a dosing device. Solutions administered by injection are officially titled injections (see *Injections* (1)).

Solutions intended for oral administration usually contain flavorings and colorants to make the medication more attractive and palatable for the patient or consumer. When

needed, they also may contain stabilizers to maintain chemical and physical stability and preservatives to prevent microbial growth.

STORAGE AND USE

Light-resistant containers should be considered when photolytic chemical degradation is a potential issue. To prevent water or solvent loss, solutions are stored in tight containers. Instructions to ensure proper dosing and administration must accompany the product.

Sprays

Spray preparations may deliver either accurately metered or nonmetered amounts of formulation.

By definition and in accordance with the USP drug product monographs, a spray dosage form drug product delivers an accurately metered spray through the delivery system, i.e., device. A spray drug product is a preparation that contains an API(s) in either solution or suspension form, typically in presence of excipients for nasal sprays, and that is intended for administration using a predefined metered amount of formulation as a fine mist of aqueous droplets.

Alternatively, nonmetered spray drug products can be generated by package designs that do not accurately control the volume of formulation delivered. These preparations release the formulation as a fine mist of droplets upon physical manipulation of the package by the patient. This generally involves squeezing the sides of the container and expelling the formulation through the nozzle of the container.

Depending on the design of the formulation and the valve system, the droplets generated may be intended for immediate inhalation through the mouth and deposition in the pulmonary tree or for inhalation into the nose and deposition in the nasal cavity.

The mechanism for droplet generation and the intended use of the preparation distinguish various classes of sprays. A spray may be composed of a pump, container, actuator, valve, nozzle or mouthpiece in addition to the formulation containing the drug(s), solvent(s), and any excipient(s). The design of each component plays a role for the appropriate performance of the drug product and in determining the critical characteristics of the droplet size distribution. Droplet and particle size distributions, delivered dose uniformity, plume geometry, and droplet velocity are critical parameters that influence the efficiency of drug delivery. When the preparation is supplied as a multidose container, the addition of a suitable antimicrobial preservative may be necessary. Spray formulations intended for nasal or pulmonary administration have an aqueous base and are usually isotonic and may contain excipients to control pH and viscosity. Pulmonary spray preparations typically are solutions. Nasal spray preparations may be solutions, or suspensions intended for local or systemic effect. Nasal delivery may be used for APIs with high hepatic extraction ratios.

PACKAGING

Containers typically are made of a plastic, but metal or glass may be suitable.

The nasal spray pump is designed to allow convenient one-handed operation. The nasal spray nozzle is designed so that it fits comfortably into the vestibule of the nasal cavity and allows the plume to be directed toward the appropriate region of the cavity.

LABELING AND USE

Refer to CDER *Guidance for Industry: Nasal Spray and Inhalation Solution, Suspension, and Spray Drug Products—Chemistry, Manufacturing, and Controls Documentation*.

Many experts recommend the addition of a statement that patients should seek advice and instruction from a health care professional about the proper use of the device. Guidance should be provided about the proper care and cleaning of the device to prevent introduction of microbes into the pulmonary airways.

Suppositories

Suppositories are dosage forms adapted for application into the rectum. They usually melt, soften, or dissolve at body temperature. A suppository may have a local protectant or palliative effect or may deliver an API for systemic or local action.

Suppository bases typically include cocoa butter, glycerinized gelatin, hydrogenated vegetable oils, mixtures of polyethylene glycols of various molecular weights, and fatty acid esters of polyethylene glycol. The suppository base can have a notable influence on the release of the API(s). Although cocoa butter melts quickly at body temperature, it is immiscible with body fluids and this inhibits the diffusion of fat-soluble APIs to the affected sites. Polyethylene glycol is a suitable base for some antiseptics. In cases when systemic action is desired, incorporating the ionized rather than the nonionized form of the API may help maximize bioavailability. Although nonionized APIs partition more readily out of water-miscible bases such as glycerinized gelatin and polyethylene glycol, the bases themselves tend to dissolve very slowly, which slows API release. Cocoa butter and its substitutes (e.g., *Hard Fat*) perform better than other bases for allaying irritation in preparations intended for treating internal hemorrhoids. Suppositories for adults are tapered at one or both ends and usually weigh about 2 g each.

PREPARATION

Cocoa butter suppositories have cocoa butter as the base and can be made by incorporating the finely divided API into the solid oil at room temperature and suitably shaping the resulting mass or by working with the oil in the melted state and allowing the resulting suspension to cool in molds. A suitable quantity of hardening agents may be added to counteract the tendency of some APIs (such as chloral hydrate and phenol) to soften the base. The finished suppository melts at body temperature.

A variety of vegetable oils, such as coconut or palm kernel, modified by esterification, hydrogenation, or fractionation, are used as cocoa butter substitutes to obtain products that display varying compositions and melting temperatures (e.g., *Hydrogenated Vegetable Oil* and *Hard Fat*). These products can be designed to reduce rancidity while incorporating desired characteristics such as narrow intervals between melting and solidification temperatures and melting ranges to accommodate formulation and climatic conditions.

APIs can be incorporated into glycerinized gelatin bases by addition of the prescribed quantities to a vehicle consisting of about 70 parts of glycerin, 20 parts of gelatin, and 10 parts of water.

Several combinations of polyethylene glycols that have melting temperatures that are above body temperature are used as suppository bases. Because release from these bases depends on dissolution rather than on melting, there are significantly fewer problems in preparation and storage than is the case for melting-type vehicles. However, high concentrations of higher molecular weight polyethylene glycols may lengthen dissolution time, resulting in problems with retention.

Several nonionic surface-active agents closely related chemically to the polyethylene glycols can be used as suppository vehicles. Examples include polyoxyethylene sorbitan fatty acid esters and the polyoxyethylene stearates. These surfactants are used alone or in combination with other suppository vehicles to yield a wide range of melting tempera-

tures and consistencies. A notable advantage of such vehicles is their water dispersibility. However, care must be taken with the use of surfactants because they may either increase the rate of API absorption or interact with the API to reduce therapeutic activity.

Compounding suppositories using a suppository base typically involves melting the suppository base and dissolution or dispersion of the API in the molten base (see *Pharmaceutical Compounding—Nonsterile Preparations* (795)). When compounding suppositories, the manufacturer or compounding professional prepares an excess amount of total formulation to allow the prescribed quantity to be accurately dispensed. In compounding suppositories, avoid caustic or irritating ingredients, carefully select a base that will allow the API to provide the intended effect, and in order to minimize abrasion of the rectal membranes, reduce solid ingredients to the smallest reasonable particle size. A representative number of the compounded suppositories should be weighed to confirm that none is less than 90% or more than 110% of the average weight of all units in the batch.

STORAGE AND USE

Suppositories typically are provided in unit-dose packaging with storage instructions to prevent melting of the suppository base. Suppositories with cocoa butter base require storage in well-closed containers, preferably at a temperature below 30° (controlled room temperature). Glycerinated gelatin suppositories require storage in tight containers, preferably at a temperature below 2°. Although polyethylene glycol suppositories can be stored without refrigeration, they should be packaged in tightly closed containers.

Include instructions about insertion procedures to ensure ease of use and absorption. Labels on polyethylene glycol suppositories should contain directions that they be moistened with water before insertion.

Suspensions

A suspension is a biphasic preparation consisting of solid particles dispersed throughout a liquid phase. Suspension dosage forms may be formulated for specific routes of administration such as oral suspensions, topical suspensions, or suspensions for aerosols (see *Aerosols*). Some suspensions are prepared and ready for use, and others are prepared as solid mixtures intended for reconstitution with an appropriate vehicle just before use. The term “milk” is sometimes used for suspensions in aqueous vehicles intended for oral administration (e.g., *Milk of Magnesia*). The term “magma” is often used to describe suspensions of inorganic solids, such as clays in water, that display a tendency toward strong hydration and aggregation of the solid, giving rise to gel-like consistency and thixotropic rheological behavior (e.g., *Bentonite Magma*). The term “lotion” may refer to a suspension dosage form although the liquid phase in these preparations is commonly an emulsion intended for application to the skin (e.g., *Calamine Topical Suspension*; see *Emulsions*). Some suspensions are prepared in sterile form and are used as injectables (see *Injections* (1)). Other sterile suspensions are for ophthalmic or otic administration. Suspensions generally are not injected intravenously, epidurally, or intrathecally unless the product labeling clearly specifies these routes of administration.

Limited aqueous solubility of the API(s) is the most common rationale for developing a suspension. Other potential advantages of a suspension include taste masking and improved patient compliance because of the more convenient dosage form. When compared to solutions, suspensions have improved chemical stability. Ideally, a suspension should contain small uniform particles that are readily suspended and easily redispersed following settling. Unless the dispersed solid is colloidal, the particulate matter in a suspension likely will settle to the bottom of the container

upon standing. Such sedimentation may lead to caking and solidification of the sediment and difficulty in redispersing the suspension upon agitation. To prevent such problems, manufacturers commonly add ingredients to increase viscosity and the gel state of the suspension or flocculation, including clays, surfactants, polyols, polymers, or sugars. Frequently, thixotropic vehicles are used to counter particle-settling tendencies, but these vehicles must not interfere with pouring or redispersion. Additionally, the density of the dispersed phase and continuous phase may be modified to further control settling rate. For topical suspensions, rapid drying upon application is desirable.

The product is both chemically and physically stable throughout its shelf life. Temperature can influence the viscosity (and thus suspension properties and the ease of removing the dose from the bottle), and temperature cycling can lead to changes in the particle size of the dispersed phase via Ostwald ripening. When manufacturers conduct stability studies to establish product shelf life and storage conditions, they should cycle conditions (freeze/thaw) to investigate temperature effects.

Unless studies confirm that the formulation will not support microbial growth, suspensions should contain suitable antimicrobial agents to protect against bacterial, yeast, and mold contamination (see *Antimicrobial Effectiveness Testing* (51)) or other appropriate measures should be taken to avoid microbial contamination.

Suspensions for reconstitution are dry powder or granular mixtures that require the addition of water or a supplied formulated diluent before administration. This formulation approach is frequently used when the chemical or physical stability of the API or suspension does not allow sufficient shelf life for a preformulated suspension. Typically, these suspensions are refrigerated after reconstitution to increase their shelf life. For this type of suspension, the powder blend is uniform and the powder readily disperses when reconstituted. Taste of the reconstituted suspension is also an important attribute because many suspensions are used for pediatric populations.

Injectable suspensions generally are intended for either subcutaneous or intramuscular routes of administration and should have a controlled particle size, typically in the range of 5 µm or smaller. The rationale for the development of injectable suspensions includes poor API solubility, improved chemical stability, prolonged duration of action, and avoidance of first-pass metabolism. Care is needed in selecting the sterilization technique because it may affect product stability or alter the physical properties of the material.

PREPARATION

Suspensions are prepared by adding suspending agents or other excipients and purified water or oil to solid APIs and mixing to achieve uniformity. In the preparation of a suspension, the characteristics of both the dispersed phase and the dispersion medium should be considered. During development manufacturers should define an appropriate particle size distribution for the suspended material to minimize the likelihood of particle size changes during storage.

In some instances the dispersed phase has an affinity for the vehicle and is readily wetted upon its addition. For some materials the displacement of air from the solid surface is difficult, and the solid particles may clump together or float on top of the vehicle. In the latter case, a wetting agent is used to facilitate displacement of air from the powder surface. Surfactants, alcohol, glycerin, and other hydrophilic liquids can be used as wetting agents when an aqueous vehicle will be used as the dispersion phase. These agents function by displacing the air in the crevices of the particles and dispersing the particles. In the large-scale preparation of suspensions, wetting of the dispersed phase may be aided by the use of high-energy mixing equipment such as colloid mills or other rotor–stator mixing devices.

After the powder has been wetted, the dispersion medium (containing the soluble formulation components such as colorants, flavorings, and preservatives) is added in portions to the powder, and the mixture is thoroughly blended before subsequent additions of the vehicle. A portion of the vehicle is used to wash the mixing equipment free of suspended material, and this portion is used to bring the suspension to final volume and ensure that the suspension contains the desired concentration of solid matter. The final product may be passed through a colloid mill or other blender or mixing device to ensure uniformity. When necessary, preservatives are included in the formulation of suspensions to protect against bacterial and mold contamination.

Suspensions are shaken before the dose is dispensed. Because of the viscosity of many suspension vehicles, air entrainment may occur during dosing. The formulation process allows evaluation of this possibility; adjustments in vehicle viscosity or the incorporation of low levels of antifoaming agents are common approaches to minimize air entrainment. Alternatively, specific instructions for shaking the formulation may be provided to minimize air incorporation and ensure accurate dosing.

PACKAGING AND STORAGE

Individual monographs specify the packaging and storage requirements for suspension products. Typically, the monograph will indicate a container type such as tight, well-closed, or light-resistant and may indicate storage conditions such as controlled room temperature. For additional information about meeting packaging requirements listed in the individual monographs, refer to *Containers—Glass* (660), *Containers—Plastic* (661), *Containers—Performance Testing* (671), *Good Packaging Practices* (1177), and the *General Notices* for statements about preservation, packaging, storage, and labeling.

Acceptable suspension of the particulate phase depends on the particle size of the dispersed phase as well as the viscosity and density of the vehicle. Clear instruction is provided regarding the appropriate storage temperature for the product because temperature can influence the viscosity and density (that affect suspension properties and the ease of removal of the dose from the bottle), and temperature cycling can lead to changes in particle size of the dispersed phase. Suspensions require storage in tight containers. Avoid freezing.

LABELING AND USE

Instructions to ensure proper dosing and administration must accompany the product. When labeling a suspension, consider any air that might be entrained in the preparation as a result of shaking, and avoid such entrainment. Compounded suspensions should indicate a beyond-use date that is calculated from the time of compounding. Suspensions are shaken well before use to ensure uniform distribution of the solid in the vehicles.

Tablets

Tablets are solid dosage forms in which the API is blended with excipients and compressed into the final dosage. Tablets are the most widely used dosage form in the U.S. Tablet presses use steel punches and dies to prepare compacted tablets by the application of high pressures to powder blends or granulations. Tablets can be produced in a wide variety of sizes, shapes, and surface markings. Capsule-shaped tablets are commonly referred to as caplets. Specialized tablet presses may be used to produce tablets with multiple layers or with specially formulated core tablets placed in the interior of the final dosage form. These specialized tablet presentations can delay or extend the release

of the API(s) or physically separate incompatible APIs. Tablets may be coated by a variety of techniques to provide taste masking, protection of photo-labile API(s), extended or delayed release, or unique appearance (colors). When no deliberate effort has been made to modify the API release rate, tablets are referred to as immediate-release.

Tablet Triturates—Small, usually cylindrical, molded or compacted tablets. Tablet triturates traditionally were used as dispensing tablets in order to provide a convenient, measured quantity of a potent API for compounding purposes, but they are rarely used today.

Hypodermic Tablets—Molded tablets made from completely and readily water-soluble ingredients; formerly intended for use in making preparations for hypodermic injection. They may be administered orally or sublingually when rapid API availability is required, as in the case of *Nitroglycerin Sublingual Tablets*.

Bolus Tablets—Large, usually elongated, tablets intended for administration to large animals. Conventional tableting processes can be used to manufacture bolus tablets, but due to their size higher compression forces may be necessary.

Buccal Tablets—Intended to be inserted in the buccal pouch, where the API is absorbed directly through the oral mucosa. Few APIs are readily absorbed in this way (examples are nitroglycerin and certain steroid hormones).

Effervescent Tablets—Prepared by compaction and contain, in addition to the API(s), mixtures of acids (e.g., citric acid or tartaric acid) and carbonates and/or hydrogen carbonates. Upon contact with water, these formulations release carbon dioxide, producing the characteristic effervescent action.

Chewable Tablets—Formulated and manufactured to produce a pleasant-tasting residue in the mouth and to facilitate swallowing. Hard chewable tablets are typically prepared by compaction, usually utilizing mannitol, sorbitol, or sucrose as binders and fillers, and contain colors and flavors to enhance their appearance and taste. Soft chewable tablets are typically made by a molding or extrusion process, frequently with more than 10% water to help maintain a pliable, soft product. Hard chewable tablets in veterinary medicine often have flavor enhancers like brewer's yeast or meat/fish-based flavors.

Tablets for human use that include "Chewable" in the title must be chewed or crushed prior to swallowing to ensure reliable release of the API(s) or to facilitate swallowing. If tablets are designed so that they may be chewed (but chewing is not required for API release or ease of swallowing), the title should not include a reference to "chewable". In that case, the product may still be described as "chewable" in the ancillary labeling statement.

Tablets for veterinary use that are intended to be chewed will include "Chewable" in the title. However, it is understood that for veterinary products it is not possible to ensure that tablets are chewed prior to ingestion. Chewable tablets may be broken into pieces and fed to animals that normally swallow treats whole.

Modified-Release Tablets—There are two categories of modified-release tablet formulations recognized by the Pharmacopeia:

Delayed-Release Tablets—Tablets sometimes are formulated with enteric coatings to protect acid-labile APIs from the gastric environment or to prevent adverse events such as irritation.

Extended-Release Tablets—Extended-release tablets are formulated in such a manner as to make the API available over an extended period of time following ingestion. Expressions such as "prolonged-release", "repeat-action", "controlled-release", and "sustained-release" have also been used to describe such dosage forms. However, the term "extended-release" is used for Pharmacopeial purposes. Requirements for dissolution (see *Dissolution* (711)) typically are specified in the individual monographs.

Orally Disintegrating Tablets—Orally disintegrating tablets are intended to disintegrate rapidly within the mouth to provide a fine dispersion before the patient swallows the resulting suspension where the API is intended for gastrointestinal delivery and/or absorption. Some of these dosage forms have been formulated to facilitate rapid disintegration and are manufactured by conventional means or by using lyophilization or molding processes. Further details may be found in the *CDER Guidance for Industry: Orally Disintegrating Tablets*.

Sublingual Tablets—Sublingual tablets are intended to be inserted beneath the tongue, where the API is absorbed directly through the oral mucosa. As with buccal tablets, few APIs are extensively absorbed in this way, and much of the API is swallowed and is available for gastrointestinal absorption.

PREPARATION

Most compacted (compressed) tablets consist of the API(s) and a number of excipients. These excipients may include fillers (diluent), binders, disintegrating agents, lubricants, and glidants. Approved FD&C and D&C dyes or lakes, flavors, and sweetening agents also may be present.

Fillers or diluents are added when the quantity of API(s) is too small or the properties of the API do not allow satisfactory compaction in the absence of other ingredients. Binders impart adhesiveness to the powder blend and promote tablet formation and maintenance of API uniformity in the tableting mixture. Disintegrating agents facilitate reduction of the tablet into small particles upon contact with water or biological fluids. Lubricants reduce friction during the compaction and ejection cycles. Glidants improve powder fluidity, powder handling properties, and tablet weight control. Colorants are often added to tablet formulations for esthetic value or for product identification.

Tablets are prepared from formulations that have been processed by one of three general methods: wet granulation, dry granulation (roll compaction or slugging), and direct compression.

Wet Granulation involves the mixing of dry powders with a granulating liquid to form a moist granular mass that is dried and sized prior to compression. It is particularly useful in achieving uniform blends of low-dose APIs and facilitating the wetting and dissolution of poorly soluble, hydrophobic APIs.

Dry Granulations can be produced by passing powders between rollers at elevated pressure (roll compaction). Alternatively, dry granulation also can be carried out by the compaction of powders at high pressures on tablet presses, a process also known as slugging. In either case the compacts are sized before compression. Dry granulation improves the flow and handling properties of the powder formulation without involving moisture in the processing.

Direct Compression tablet processing involves dry blending of the API(s) and excipients followed by compression. The simplest manufacturing technique, direct compression is acceptable only when the API and excipients possess acceptable flow and compression properties without prior process steps.

Tablets may be coated to protect the ingredients from air, moisture, or light; to mask unpleasant tastes and odors; to improve tablet appearance; and to reduce dustiness. In addition, coating may be used to protect the API from acidic pH values associated with gastric fluids or to control the rate of drug release in the gastrointestinal tract.

The most common coating in use today is a thin film coating composed of a polymer that is derived from cellulose. Sugar coating is an alternative, less common approach. Sugar-coated tablets have considerably thicker coatings that are primarily sucrose with a number of inorganic diluents. A variety of film-coating polymers are available and enable the development of specialized release profiles. These formulations are used to protect acid-labile APIs from the acidic

stomach environment as well as to prolong the release of the API to reduce dosing frequency (see *Dissolution* (711) or *Disintegration* (701)).

PACKAGING, STORAGE, AND LABELING

Individual monographs specify the packaging and storage requirements for tablet products. Typically, the monograph will indicate the container type such as tight, well-closed, or light-resistant. For additional information on meeting USP packaging requirements, see *Containers—Glass* (660), *Containers—Plastic* (661), and *Containers—Performance Testing* (671). Effervescent tablets are stored in tightly closed containers or moisture-proof packs and are labeled to indicate that they should not be swallowed directly.

Tapes

A tape is a dosage form suitable for delivering APIs to the skin. It consists of an API(s) impregnated into a durable yet flexible woven fabric or extruded synthetic material that is coated with an adhesive agent. Typically the impregnated API is present in the dry state. The adhesive layer is designed to hold the tape securely in place without the aid of additional bandaging. Unlike transdermal patches, tapes are not designed to control the release rate of the API.

The API content of tapes is expressed as amount per surface area with respect to the tape surface exposed to the skin. The use of an occlusive dressing with the tape enhances the rate and extent of delivery of the API to deeper layers of the skin and may result in greater systemic absorption of the API.

LABELING, STORAGE, AND USE

Label to indicate "External Use Only". Tapes are stored in tight containers protected from light and moisture. To employ the tape, one cuts a patch slightly larger than the area that will be treated. The backing paper is removed from the adhesive side, and the tape is applied to the skin. To ensure optimal adhesion, the tape should not be applied to folds in the skin. To minimize systemic absorption and to ensure good adhesion, tapes should be applied to dry skin.

GLOSSARY

This glossary provides definitions for terms in use in medicine and serves as a source of official names for official articles, except when the definition specifically states that the term is not to be used in article names. Examples of general nomenclature forms for the more frequently encountered categories of dosage forms appear in *Nomenclature* (1121). In an attempt to be comprehensive, this glossary was compiled without the limits imposed by current preferred nomenclature conventions. To clearly identify/distinguish preferred from not preferred terms, entries indicate when a term is not preferred and direct the user to the current preferred term. When a term is described as an attribute of a dosage form, it is intended to distinguish the term from those used for actual dosage form titles. While attribute terms are typically not used as the official name for the dosage form, when they are used they identify a specialized presentation of the dosage form. For example, the attribute, chewable, may be used with the dosage form term, tablets, to identify a specific type of tablet that must be chewed prior to swallowing.

Aerosol: A dosage form consisting of a liquid or solid preparation packaged under pressure and intended for administration as a fine mist. The descriptive term aerosol also refers to the fine mist of small droplets or solid particles that are emitted from the product.

Aromatic Water (not preferred; see *Solution*): A clear, saturated, aqueous solution of volatile oils or other aromatic or volatile substances.

Aural (Auricular) (not preferred; see *Otic*): For administration into, or by way of, the ear.

Bead (not preferred; see *Pellets*): A solid dosage form in the shape of a small sphere. In most products a unit dose consists of multiple beads.

Blocks: A large veterinary product intended to be licked by animals and containing the API(s) and nutrients such as salts, vitamins, and minerals.

Bolus (not preferred; see *Tablet*): A large tablet intended for administration to large animals.

Caplet (not preferred; see *Tablet*): Tablet dosage form in the shape of a capsule.

Capsule: A solid dosage form in which the API, with or without other ingredients, is filled into either a hard or soft shell. Most capsule shells are composed mainly of gelatin.

Chewable: Attribute of a solid dosage form that is intended to be chewed or crushed before swallowing.

Coated: Attribute of a solid dosage form that is covered by deposition of an outer solid that is different in composition from the core material.

Colloidion (not preferred; see *Solution*): A preparation that is a solution dosage form composed of pyroxilin dissolved in a solvent mixture of alcohol and ether and applied externally.

Colloidal Dispersion: An attribute of a preparation or formulation in which particles of colloidal dimension (i.e., typically between 1 nm and 1 μ m) are distributed uniformly throughout a liquid.

Concentrate: A liquid or solid preparation of higher concentration and smaller volume than the final dosage form; usually intended to be diluted prior to administration. The term continues to be used for veterinary preparations but is being phased out of USP–NF titles for human applications.

Conventional-Release (not preferred; see *Immediate-Release*): Descriptive term for a dosage form in which no deliberate effort has been made to modify the release rate of the API. In the case of capsules and tablets, the inclusion or exclusion of a disintegrating agent is not interpreted as a modification. This term is not used in article names.

Cream: An emulsion dosage form often containing more than 20% water and volatiles and/or containing less than 50% hydrocarbons, waxes, or polyols as the vehicle for the API. Creams are generally intended for external application to the skin or mucous membranes.

Delayed-Release: A type of modified-release dosage form. A descriptive term for a dosage form deliberately modified to delay release of the API for some period of time after initial administration. For example, release of the API is prevented in the gastric environment but promoted in the intestinal environment; this term is synonymous with *Enteric-Coated* or *Gastro-Resistant*.

Dental: Descriptive term for a preparation that is applied to the teeth for localized action.

Dermal: A topical route of administration where the article is intended to reach or be applied to the dermis.

Dosage Form: A formulation that typically contains the API(s) and excipients in quantities and physical form designed to allow the accurate and efficient administration of the API to the human or animal patient. This term is not used in article names.

Dry Powder Inhaler: A device used to administer an inhalation powder in a finely divided state suitable for oral inhalation by the patient. This term is not used in article names.

Effervescent: Attribute of an oral dosage form, frequently tablets or granules, containing ingredients that, when in contact with water, rapidly release carbon dioxide. The dosage form is dissolved or dispersed in water to initiate the effervescence prior to ingestion.

Elixir (not preferred; see *Solution*): A preparation that typically is a clear, flavored, sweetened hydroalcoholic solution intended for oral use. The term should not be used for new articles in USP–NF but is commonly encountered in compounding pharmacy practice.

Emollient: Attribute of a cream or ointment indicating an increase in the moisture content of the skin following application of bland, fatty, or oleaginous substances. This term should not be used in article names.

Emulsion: A dosage form consisting of a two-phase system composed of at least two immiscible liquids, one of which is dispersed as droplets (internal or dispersed phase) within the other liquid (external or continuous phase), generally stabilized with one or more emulsifying agents. Emulsion is not used as a dosage form term if a more specific term is applicable (e.g., *Cream*, *Lotion*, or *Ointment*).

Enteric-Coated (not preferred; see *Delayed-Release*): Descriptive term for a solid dosage form in which a polymer coating has been applied to prevent the release of the API in the gastric environment.

Excipient: An ingredient of a dosage form other than an API. This term is not used in article names. The term, excipient, is synonymous with inactive ingredient.

Extended-Release: Descriptive term for a dosage form that is deliberately modified to protract the release rate of the API compared to that observed for an immediate-release dosage form. The term is synonymous with prolonged- or sustained-release. Many extended-release dosage forms have a pattern of release that begins with a "burst effect" that mimics an immediate release followed by a slower release of the remaining API in the dosage form.

Film: A term used to describe a thin, flexible sheet of material, usually composed of a polymer. Films are used in various routes of administration including as a means of oral administration of material in a rapidly dissolving form. The term, film, also may be used as an attribute when applied to solid oral dosage forms for taste masking, product identification, and aesthetic purposes.

Foam: An emulsion dosage form containing dispersed gas bubbles. When dispensed it has a fluffy, semisolid consistency.

Gas: One of the states of matter having no definite shape or volume and occupying the entire container when confined.

Gastro-Resistant (not preferred; see *Delayed-Release*): Descriptive term for a solid dosage form in which a polymer coating has been applied to prevent the release in the gastric environment.

Gel: A dosage form that is a semisolid dispersion of small inorganic particles or a solution of large organic molecules containing a gelling agent to provide stiffness. A gel may contain suspended particles.

Granules: A dosage form composed of dry aggregates of powder particles that may contain one or more APIs, with or without other ingredients. They may be swallowed as such, dispersed in food, or dissolved in water. Granules are frequently compacted into tablets or filled into capsules, with or without additional ingredients.

Gum: A dosage form in which the base consists of a pliable material that, when chewed, releases the API into the oral cavity.

Hard-Shell Capsule (not preferred; see *Capsules*): A type of capsule in which one or more APIs, with or without other ingredients, are filled into a two-piece shell. Most hard-shell capsules are composed mainly of gelatin and are fabricated prior to the filling operation.

Immediate-Release: Descriptive term for a dosage form in which no deliberate effort has been made to modify the API release rate. In the case of capsules and tablets, the inclusion or exclusion of a disintegrating agent is not interpreted as a modification. This term is not used in article names.

Implant: A dosage form that is a solid or semisolid material containing the API that is inserted into the body.

The insertion process is invasive, and the material is intended to reside at the site for a period consistent with the design release kinetics or profile of the API(s).

Inhalation (by inhalation): A route of administration for aerosols characterized by dispersion of the API into the airways during inspiration.

By Injection: A route of administration of a liquid or semisolid deposited into a body cavity, fluid, or tissue by use of a needle.

Insert: A solid dosage form that is inserted into a naturally occurring (nonsurgical) body cavity other than the mouth or rectum. It should be noted that a suppository is intended for application into the rectum and is not classified as an insert (see *Suppository*).

Intraocular: A route of administration to deliver a sterile preparation within the eye.

Irrigation: A sterile solution or liquid intended to bathe or flush open wounds or body cavities.

Jelly (not preferred; see *Gel*): A semisolid dispersion of small inorganic particles or a solution of large organic molecules containing a gelling agent to promote stiffness.

Liquid: A dosage form consisting of a pure chemical in its liquid state. This dosage form term should not be applied to solutions. The term is not used in article names. When liquid is used as a descriptive term, it indicates a material that is pourable and conforms to its container at room temperature.

Lotion: An emulsion liquid dosage form applied to the outer surface of the body. Historically, this term has also been applied to suspensions and solutions.

Lozenge: A solid dosage form intended to disintegrate or dissolve slowly in the mouth.

Modified-Release: A descriptive term for a dosage form with an API release pattern that has been deliberately changed from that observed for the immediate-release dosage form of the same API. This term is not used in article names.

Molded Tablet: A tablet that has been formed by dampening the ingredients and pressing into a mold, then removing and drying the resulting solid mass. This term is not used in article names.

Mouthwash (not preferred; see *Solution*): Term applied to a solution preparation used to rinse the oral cavity.

Nasal: Route of administration (mucosal) characterized by deposition in the nasal cavity for local or systemic effect.

Ocular (not preferred; see *Intraocular*): Route of administration indicating deposition of the API within the eye.

Ointment: A semisolid dosage form, usually containing less than 20% water and volatiles and more than 50% hydrocarbons, waxes, or polyols as the vehicle. This dosage form generally is for external application to the skin or mucous membranes.

Ophthalmic: A route of administration characterized by application of a sterile preparation to the external parts of the eye.

Orally Disintegrating: A descriptive term for a solid oral dosage form that disintegrates rapidly in the mouth prior to swallowing. The API is intended for gastrointestinal delivery and/or absorption. See also *CDER Guidance for Industry, Orally Disintegrating Tablets*.

Oro-Pharyngeal: A route of administration characterized by deposition of a preparation into the oral cavity and/or pharyngeal region to exert a local or systemic effect.

Otic: A route of administration (mucosal) characterized by deposition of a preparation into, or by way of, the ear. Sometimes referred to as *Aural* (*Aural* not preferred).

Paste: A semisolid dosage form containing a high percentage (e.g., 20%–50%) of finely dispersed solids with a stiff consistency. This dosage form is intended for application to the skin, oral cavity, or mucous membranes.

Patch (not preferred): Frequently used to describe a *Transdermal System*.

Pellet: A small solid dosage form of uniform, often spherical, shape. Spherical pellets are sometimes referred to as *Beads*. Pellets intended as implants must be sterile.

Periodontal: Descriptive term for a preparation that is applied around a tooth for localized action.

Pill (not preferred but frequently incorrectly used to describe a *Tablet*): A solid spherical pharmaceutical dosage form, usually prepared by a wet massing technique. This term is not used in article names.

Plaster: A dosage form containing a semisolid composition supplied on a support material for external application. Plasters are applied for prolonged periods of time to provide protection, support, or occlusion (for macerating action).

Powder: A dosage form composed of a solid or mixture of solids reduced to a finely divided state and intended for internal or external use.

Powder, Inhalation: A powder containing an API for oral inhalation. The powder is used with a device that aerosolizes and delivers an accurately metered amount.

Prolonged-Release (not preferred; see *Extended-Release*)

Rectal: A route of administration (mucosal) characterized by deposition into the rectum to provide local or systemic effect.

Semisolid: Attribute of a material characterized by a reduced ability to flow or conform to its container at room temperature. A semisolid does not flow at low shear stress and generally exhibits plastic flow behavior. This term is not used in article names.

Shampoo: A solution or suspension dosage form used to clean the hair and scalp. May contain an API intended for topical application to the scalp.

Soap: The alkali salt(s) of a fatty acid or mixture of fatty acids used to cleanse the skin. Soaps used as dosage forms may contain an API intended for topical application to the skin. Soaps have also been used as liniments and enemas.

Soft Gel Capsule (not preferred; see *Capsule*): A specific capsule type characterized by increased levels of plasticizers producing a more pliable and thicker-walled material than hard gelatin capsules. Soft gel capsules are further distinguished because they are single-piece sealed dosages. Frequently used for delivering liquid compositions.

Solution: A clear, homogeneous liquid dosage form that contains one or more chemical substances dissolved in a solvent or mixture of mutually miscible solvents.

Spirit (not preferred; see *Solution*): A liquid dosage form composed of an alcoholic or hydroalcoholic solution of volatile substances.

Spray: Attribute that describes the generation of droplets of a liquid or solution to facilitate application to the intended area.

Stent, Drug-Eluting: A specialized form of implant used for extended local delivery of the API to the immediate location of stent placement.

Strip (not preferred; see *Tape*): A dosage form or device in the shape of a long, narrow, thin solid material.

Sublingual: A route of administration (mucosal) characterized by placement underneath the tongue and for release of the API for absorption in that region.

Suppository: A solid dosage form in which one or more APIs are dispersed in a suitable base and molded or otherwise formed into a suitable shape for insertion into the rectum to provide local or systemic effect.

Suspension: A liquid dosage form that consists of solid particles dispersed throughout a liquid phase.

Syrup (not preferred; see *Solution*): A solution containing high concentrations of sucrose or other sugars. This term is commonly used in compounding pharmacy.

Tablet: A solid dosage form prepared from powders or granules by compaction.

Tape, Medicated: A dosage form or device composed of a woven fabric or synthetic material onto which an API is placed, usually with an adhesive on one or both sides to facilitate topical application.

Tincture (not preferred; see *Solution*): An alcoholic or hydroalcoholic solution prepared from vegetable materials or from chemical substances.

Topical: A route of administration characterized by application to the outer surface of the body.

Transdermal System: Dosage forms designed to deliver the API(s) through the skin into the systemic circulation. Transdermal systems are typically composed of an outer covering (barrier), a drug reservoir (that may incorporate a rate-controlling membrane), a contact adhesive to affix the transdermal system to the administration site, and a protective layer that is removed immediately prior to application of the transdermal system.

Troche (not preferred; see *Lozenge*): A solid dosage form intended to disintegrate or dissolve slowly in the mouth and usually prepared by compaction in a manner similar to that used for tablets.

Urethral: A route of administration (mucosal) characterized by deposition into the urethra.

Vaginal: A route of administration (mucosal) characterized by deposition into the vagina.

Vehicle: A term commonly encountered in compounding pharmacy that refers to a component for internal or external use that is used as a carrier or diluent in which liquids, semisolids, or solids are dissolved or suspended. Examples include water, syrups, elixirs, oleaginous liquids, solid and semisolid carriers, and proprietary products (see *Excipient*). This term is not used in article names.

Veterinary: Descriptive term for dosage forms intended for nonhuman use.

<1160> PHARMACEUTICAL CALCULATIONS IN PRESCRIPTION COMPOUNDING

INTRODUCTION

The purpose of this chapter is to provide general information to guide and assist pharmacists in performing the necessary calculations when preparing or compounding any pharmaceutical article (see *Pharmaceutical Compounding—Nonsterile Preparations* <795>, *Pharmaceutical Compounding—Sterile Preparations* <797>, and *Good Compounding Practices* <1075>) or when simply dispensing prescriptions (see *Stability Considerations in Dispensing Practice* <1191>).

Correct pharmaceutical calculations can be accomplished by using, for example, proper conversions from one measurement system to another and properly placed decimal points, by understanding the arithmetical concepts, and by paying close attention to the details of the calculations. Before proceeding with any calculation, pharmacists should do the following: (a) read the entire formula or prescription carefully; (b) determine which materials are needed; and then (c) select the appropriate methods of preparation and the appropriate calculation.

There are often several ways to solve a given problem. Logical methods that require as few steps as possible should be selected in order to ensure that calculations are done correctly. The best approach is the one that yields results

that are accurate and free of error. The pharmacist must double-check each calculation before proceeding with the preparation of the article or prescription order. One way of double-checking is by estimation. This involves rounding off the quantities involved in the calculation, and comparing the estimated result with the calculated value.

Finally, the following steps should be taken: the dosage of each active ingredient in the prescription should be checked; all calculations should be doubly checked, preferably by another pharmacist; and where instruments are used in compounding, they should be carefully checked to ascertain that they will function properly. See *USP* general chapters *Aerosols*, *Nasal Sprays*, *Metered-Dose Inhalers*, and *Dry Powder Inhalers* <601>, *Deliverable Volume* <698>, *Density of Solids* <699>, *Osmolality and Osmolarity* <785>, *pH* <791>, *Pharmaceutical Compounding—Nonsterile Preparations* <795>, *Pharmaceutical Compounding—Sterile Preparations* <797>, *Viscosity* <911>, *Specific Gravity* <841>, *Cleaning Glass Apparatus* <1051>, *Medicine Dropper* <1101>, *Prescription Balances and Volumetric Apparatus* <1176>, *Teaspoon* <1221>, *Weighing on an Analytical Balance* <1251>, and *Good Compounding Practices* <1075> for information on specific instruments.

BASIC MATHEMATICAL CONCEPTS

SIGNIFICANT FIGURES

Expressed values are considered significant to the last digit shown (see *Significant Figures and Tolerances* in the *General Notices*). Significant figures are digits with practical meaning. The accuracy of the determination is implied by the number of figures used in its expression. In some calculations zeros may not be significant. For example, for a measured weight of 0.0298 g, the zeros are not significant; they are used merely to locate the decimal point. In the example, 2980 g, the zero may also be used to indicate the decimal point, in which case the zero is not significant. Alternately, however, the zero may indicate that the weight is closer to 2981 g or 2979 g, in which case the zero is significant. In such a case, knowledge of the method of measurement would be required in order to indicate whether the zero is or is not significant. In the case of a volume measurement of 298 mL, all of the digits are significant. In a given result, the last significant figure written is approximate but all preceding figures are accurate. For example, a volume of 29.8 mL implies that 8 is approximate. The true volume falls between 29.75 and 29.85. Thus, 29.8 mL is accurate to the nearest 0.1 mL, which means that the measurement has been made within ± 0.05 mL. Likewise, a value of 298 mL is accurate to the nearest 1 mL and implies a measurement falling between 297.5 and 298.5, which means that the measurement has been made within ± 0.5 mL and is subject to a maximum error calculated as follows:

$$(0.5 \text{ mL}/298 \text{ mL}) \times 100\% = 0.17\%$$

A zero in a quantity such as 298.0 mL is a significant figure and implies that the measurement has been made within the limits of 297.95 and 298.05 with a possible error calculated as follows:

$$(0.05 \text{ mL}/298.0 \text{ mL}) \times 100\% = 0.017\%$$

EXAMPLES—

1. 29.8 mL = 29.8 ± 0.05 mL (accurate to the nearest 0.1 mL)
2. 29.80 mL = 29.80 ± 0.005 mL (accurate to the nearest 0.01 mL)
3. 29.800 mL = 29.800 ± 0.0005 mL (accurate to the nearest 0.001 mL)

The degree of accuracy in the last example is greatest. Thus, the number of significant figures provides an estimate both of true value and of accuracy.

EXAMPLES OF SIGNIFICANT FIGURES—

Measurement	Number of Significant Figures
2.98	3
2.980	4
0.0298	3
0.0029	2

Calculations—All figures should be retained until the calculations have been completed. Only the appropriate number of significant figures, however, should be retained in the final result.

Determining the number of significant figures—

Sums and Differences—When adding or subtracting, the number of decimal places in the result shall be the same as the number of decimal places in the component with the fewest decimal places.

EXAMPLE—

$$11.5 + 11.65 + 9.90 = 33.1$$

Products and Quotients—When multiplying or dividing, the result shall have no more significant figures than the measurement with the smallest number of significant figures entering into the calculation.

EXAMPLE—

$$4.266 \times 21 = 90$$

Rounding Off—For rules on rounding off measurements or calculated results, see *Interpretation of Requirements* under *Significant Figures and Tolerances* in the *General Notices*. Note, however, that in the example above, if 21 is an absolute number (e.g., the number of doses), then the answer, 89.586, is rounded off to 89.59 which has 4 significant figures.

LOGARITHMS

The logarithm of a number is the exponent or the power to which a given base must be raised in order to equal that number.

Definitions—

$$\text{pH} = -\log [\text{H}^+], \text{ and}$$

$$\text{pK}_a = -\log K_a$$

$\text{pH} = -\log [\text{H}^+]$, and $\text{pK}_a = -\log K_a$, where $[\text{H}^+]$ is the hydrogen ion concentration in an aqueous solution and K_a is the ionization constant of the acid in an aqueous solution. The $[\text{H}^+] =$ the antilogarithm of $(-\text{pH})$, and the $K_a =$ the antilogarithm of $(-\text{pK}_a)$.

The pH of an aqueous solution containing a weak acid may be calculated using the Henderson-Hasselbalch equation:

$$\text{pH} = \text{pK}_a + \log [\text{salt}]/[\text{acid}]$$

EXAMPLE—

A solution contains 0.020 moles per L of sodium acetate and 0.010 mole per L of acetic acid, which has a pK_a value of 4.76. Calculate the pH and the $[\text{H}^+]$ of the solution. Substituting into the above equation, $\text{pH} = 4.76 + \log (0.020/0.010) = 5.06$, and the $[\text{H}^+] =$ antilogarithm of $(-5.06) = 8.69 \times 10^{-6}$.

BASIC PHARMACEUTICAL CALCULATIONS

The remainder of this chapter will focus on basic pharmaceutical calculations. It is important to recognize the rules involved when adding, subtracting, dividing, and multiplying values. The interrelationships between various units within the different weighing and measuring systems are also important and have to be understood.

CALCULATIONS IN COMPOUNDING

The pharmacist must be able to calculate the amount or concentration of drug substances in each unit or dosage portion of a compounded preparation at the time it is dispensed. Pharmacists must perform calculations and measurements to obtain, theoretically, 100% of the amount of each ingredient in compounded formulations. Calculations must account for the active ingredient, or active moiety, and water content of drug substances, which includes that in the chemical formulas of hydrates. Official drug substances and added substances must meet the requirements under *Loss on Drying* <731>, which must be included in the calculations of amounts and concentrations of ingredients. The pharmacist should consider the effect of ambient humidity on the gain or loss of water from drugs and added substances in containers subjected to intermittent opening over prolonged storage. Each container should be opened for the shortest duration necessary and then closed tightly immediately after use.

The nature of the drug substance that is to be weighed and used in compounding a prescription must be known exactly. If the substance is a hydrate, its anhydrous equivalent weight may need to be calculated. On the other hand, if there is adsorbed moisture present that is either specified on a certificate of analysis or that is determined in the pharmacy immediately before the drug substance is used by the procedure under *Loss on Drying* <731>, this information must be used when calculating the amount of drug substance that is to be weighed in order to determine the exact amount of anhydrous drug substance required.

There are cases in which the required amount of a dose is specified in terms of a cation [e.g., Li^+ , netilmicin (n^+)], an anion [e.g., F^-], or a molecule (e.g., theophylline in aminophylline). In these instances, the drug substance weighed is a salt or complex, a portion of which represents the pharmacologically active moiety. Thus, the exact amount of such substances weighed must be calculated on the basis of the required quantity of the pharmacological moiety.

The following formula may be used to calculate the exact theoretical weight of an ingredient in a compounded preparation:

$$W = ab/de$$

in which W is the actual weighed amount; a is the prescribed or pharmacist-determined weight of the active or functional moiety of drug or added substance; b is the chemical formula weight of the ingredient, including waters of hydration for hydrous ingredients; d is the fraction of dry weight when the percent by weight of adsorbed moisture content is known from the loss on drying procedure (see *Loss on Drying* <731>); and e is the formula weight of the active or functional moiety of a drug or added substance that is provided in the formula weight of the weighed ingredient.

Example 1: Triturate Morphine Sulfate USP and Lactose NF to obtain 10 g in which there are 30 mg of Morphine Sulfate USP for each 200 mg of the morphine-lactose mixture. [NOTE—Clinical dosages of morphine mean Morphine Sulfate USP, which is the pentahydrate.]

Equation Factor	Numerical Value
W	weight, in g, of Morphine Sulfate USP
a	1.5 g of morphine sulfate pentahydrate in the prescription
b	759 g/mole
d	1.0
e	759 g/mole

$$W = (1.5 \text{ g} \times 759 \text{ g/mole}) / (1.0 \times 759 \text{ g/mole}) = 1.5 \text{ g}$$

Example 2: Accurately weigh an amount of Aminophylline USP to obtain 250 mg of anhydrous theophylline. [NOTE—The powdered aminophylline dihydrate weighed contains 0.4% w/w adsorbed moisture as stated in the Certificate of Analysis.]

Equation Factor	Numerical Value
W	weight, in mg, of Aminophylline USP (dihydrate)
a	250 mg of theophylline
b	456 g/mole
d	0.996
e	360 g/mole

$$W = (250 \text{ mg} \times 456 \text{ g/mole}) / (0.996 \times 360 \text{ g/mole}) = 318 \text{ mg}$$

Example 3: Accurately weigh an amount of Lithium Citrate USP (containing 2.5% moisture as stated in the Certificate of Analysis) to obtain 200 mEq of lithium (Li^+). [NOTE—One mEq of Li^+ is equivalent to 0.00694 g of Li^+ .]

Equation Factor	Numerical Value
W	weight, in g, of Lithium Citrate USP (tetrahydrate)
a	200 mEq of Li^+ or 1.39 g of Li^+
b	282 g/mole
d	0.975
e	$3 \times 6.94 \text{ g/mole}$ or 20.8 g/mole

$$W = (1.39 \text{ g} \times 282 \text{ g/mole}) / (0.975 \times 20.8 \text{ g/mole}) = 19.3 \text{ g}$$

Example 4: Accurately weigh an amount of Netilmicin Sulfate USP, equivalent to 2.5 g of netilmicin. [NOTE—Using the procedure under *Loss on Drying* (731), the Netilmicin Sulfate USP that was weighed lost 12% of its weight.]

Equation Factor	Numerical Value
W	weight, in g, of Netilmicin Sulfate USP
a	2.5 g
b	1442 g/mole
d	0.88
e	951 g/mole

$$W = (2.5 \text{ g} \times 1442 \text{ g/mole}) / (0.88 \times 951 \text{ g/mole}) = 4.31 \text{ g}$$

BUFFER SOLUTIONS

Definition—A buffer solution is an aqueous solution that resists a change in pH when small quantities of acid or base are added, when diluted with the solvent, or when the temperature changes. Most buffer solutions are mixtures of a weak acid and one of its salts or mixtures of a weak base

and one of its salts. Water and solutions of a neutral salt such as sodium chloride have very little ability to resist the change of pH and are not capable of effective buffer action.

Preparation, Use, and Storage of Buffer Solutions—

Buffer solutions for Pharmacopeial tests should be prepared using freshly boiled and cooled water (see *Standard Buffer Solutions* under *Buffer Solutions* in *Reagents, Indicators, and Solutions*). They should be stored in containers such as Type I glass bottles and used within 3 months of preparation.

Buffers used in physiological systems are carefully chosen so as not to interfere with the pharmacological activity of the medication or the normal function of the organism. Commonly used buffers in parenteral products, for example, are acetic, citric, glutamic, and phosphoric acids and their salts. Buffer solutions should be freshly prepared.

The Henderson-Hasselbalch equation, noted above, allows the pH of a buffer solution of a weak acid and its salt to be calculated. Appropriately modified, this equation may be applied to buffer solutions composed of a weak base and its salt.

Buffer Capacity—The buffer capacity of a solution is the measurement of the ability of that solution to resist a change in pH upon addition of small quantities of a strong acid or base. An aqueous solution has a buffer capacity of 1 when 1 L of the buffer solution requires 1 gram equivalent of strong acid or base to change the pH by 1 unit. Therefore, the smaller the pH change upon the addition of a specified amount of acid or base, the greater the buffer capacity of the buffer solution. Usually, in analysis, much smaller volumes of buffer are used in order to determine the buffer capacity. An approximate formula for calculating the buffer capacity is gram equivalents of strong acid or base added per L of buffer solution per unit of pH change, i.e., $(\text{Eq/L})/(\text{pH change})$.

EXAMPLE—

The addition of 0.01 g equivalents of sodium hydroxide to 0.25 L of a buffer solution produced a pH change of 0.50. The buffer capacity of the buffer solution is calculated as follows:

$$(0.01/0.25)/0.50 = 0.08(\text{Eq/L})/(\text{pH change})$$

DOSAGE CALCULATIONS

Special Dosage Regimens—Geriatric and pediatric patients require special consideration when designing dosage regimens. In geriatric patients, the organs are often not functioning efficiently as a result of age-related pharmacokinetic changes or disease. For these patients, modifications in dosing regimens are available in references such as *USP Drug Information*.

For pediatric patients, where organs are often not fully developed and functioning, careful consideration must be applied during dosing. Modifications in dosing regimens for pediatric patients are also available in references such as *USP Drug Information*. General rules for calculating doses for infants and children are available in pharmacy calculation textbooks. These rules are not drug-specific and should be used only in the absence of more complete information.

The usual method for calculating a dose for children is to use the information provided for children for the specific drug. The dose is frequently expressed as mg of drug per kg of body weight for a 24-hour period, and is then usually given in divided portions.

The calculation may be made using the following equation:

$$(\text{mg of drug per kg of body weight}) \times (\text{kg of body weight}) = \text{dose for an individual for a 24-hour period}$$

A less frequently used method of calculating the dose is based on the surface area of the individual's body. The dose

is expressed as amount of drug per body surface area in m², as shown in the equation below:

$$(\text{amount of drug per m}^2 \text{ of body surface area}) \times (\text{body surface area in m}^2) = \text{dose for an individual for a 24-hour period}$$

The body surface area (BSA) may be determined from nomograms relating height and weight in dosage handbooks. The BSA for adult and pediatric patients may also be determined using the following equations:

$$\text{BSA (m}^2\text{)} = \text{square root of } \{[\text{Height (in)} \times \text{Weight (lb)}]/3131\}$$

or

$$\text{BSA (m}^2\text{)} = \text{square root of } \{[\text{Height (cm)} \times \text{Weight (kg)}]/3600\}$$

EXAMPLE—

Rx for Spironolactone Suspension 25 mg/tsp. Sig: 9 mg BID for an 18 month-old child who weighs 22 lbs.

The *USP DI 2002*, 22nd ed., states that the normal pediatric dosing regimen for Spironolactone is 1 to 3 mg per kg per day. In this case, the weight of the child is 22 lbs, which equals 22 lbs/(2.2 lbs/kg) = 10 kg. Therefore the normal dose for this child is 10 to 30 mg per day and the dose ordered is 18 mg per day as a single dose or divided into 2 to 4 doses. The dose is acceptable based on published dosing guidelines.

PERCENTAGE CONCENTRATIONS

Percentage concentrations of solutions are usually expressed in one of three common forms:

$$\text{Volume percent (v/v)} = \text{Volume of solute/Volume of solution} \times 100\%$$

$$\text{Weight percent (w/w)} = (\text{Weight of solute} \times 100\%)/\text{Weight of solution}$$

$$\text{Weight in volume percent (w/v)} = (\text{Weight of solute (in g)}/\text{Volume of solution (in mL)}) \times 100\%$$

See also *Percentage Measurements* under *Concentrations* in the *General Notices*. The above three equations may be used to calculate any one of the three values (i.e., weights, volumes, or percentages) in a given equation if the other two values are known.

Note that weights are always additive, i.e., 50 g plus 25 g = 75 g. Volumes of two different solvents or volumes of solvent plus a solid solute are not strictly additive. Thus 50 mL of water + 50 mL of pure alcohol do not produce a volume of 100 mL. Nevertheless, it is assumed that in some pharmaceutical calculations, volumes are additive, as discussed below under *Reconstitution of Drugs Using Volumes Other than Those on the Label*.

EXAMPLES—

1. Calculate the percentage concentrations (w/w) of the constituents of the solution prepared by dissolving 2.50 g of phenol in 10.00 g of glycerin. Using the

weight percent equation above, the calculation is as follows.

$$\text{Total weight of the solution} = 10.00 \text{ g} + 2.50 \text{ g} = 12.50 \text{ g}$$

$$\text{Weight percent of phenol} = (2.50 \text{ g} \times 100\%)/12.50 \text{ g} = 20.0\% \text{ of phenol}$$

$$\text{Weight percent of glycerin} = (10 \text{ g} \times 100\%)/12.50 \text{ g} = 80.0\% \text{ of glycerin}$$

2. A prescription order reads as follows:
Eucalyptus Oil 3% (v/v) in Mineral Oil.
Dispense 30.0 mL.
What quantities should be used for this prescription?
Using the volume percent equation above, the calculation is as follows.
Amount of Eucalyptus Oil:

$$3\% = (\text{Volume of oil in mL}/30.0 \text{ mL}) \times 100\%$$

Solving the equation, the volume of oil = 0.90 mL.

Amount of Mineral Oil: To 0.90 mL of Eucalyptus Oil add sufficient Mineral Oil to prepare 30.0 mL.

3. A prescription order reads as follows:

Zinc oxide	7.5 g
Calamine	7.5 g
Starch	15 g
White petrolatum	30 g

Calculate the percentage concentration for each of the four components. Using the weight percent equation above, the calculation is as follows.

$$\text{Total weight} = 7.5 \text{ g} + 7.5 \text{ g} + 15 \text{ g} + 30 \text{ g} = 60.0 \text{ g}$$

$$\text{Weight percent of zinc oxide} = (7.5 \text{ g zinc oxide}/60 \text{ g ointment}) \times 100\% = 12.5\%$$

$$\text{Weight percent of calamine} = (7.5 \text{ g calamine}/60 \text{ g ointment}) \times 100\% = 12.5\%$$

$$\text{Weight percent of starch} = (15 \text{ g starch}/60 \text{ g ointment}) \times 100\% = 25\%$$

$$\text{Weight percent of white petrolatum} = (30 \text{ g white petrolatum}/60 \text{ g ointment}) \times 100\% = 50\%$$

SPECIFIC GRAVITY

The definition of specific gravity is usually based on the ratio of weight of a substance in air at 25° to that of the weight of an equal volume of water at the same temperature. The weight of 1 mL of water at 25° is approximately 1 g. The following equation may be used for calculations.

$$\text{Specific Gravity} = (\text{Weight of the substance})/(\text{Weight of an equal volume of water})$$

EXAMPLES—

1. A liquid weighs 125 g and has a volume of 110 mL.
What is the specific gravity?
The weight of an equal volume of water is 110 g.

Using the above equation,

$$\text{specific gravity} = 125 \text{ g}/110 \text{ g} = 1.14$$

2. Hydrochloric Acid NF is approximately a 37% (w/w) solution of hydrochloric acid (HCl) in water. How many grams of HCl are contained in 75.0 mL of HCl NF? (Specific gravity of Hydrochloric Acid NF is 1.18.) Calculate the weight of HCl NF using the above equation. The weight of an equal volume of water is 75 g.

$$\text{Specific Gravity } 1.18 = \frac{\text{weight of the HCl NF}}{75.0 \text{ g}}$$

Solving the equation, the weight of HCl NF is 88.5 g. Now calculate the weight of HCl using the weight percent equation.

$$37.0 \% \text{ (w/w)} = (\text{weight of solute g}/88.5 \text{ g}) \times 100$$

Solving the equation, the weight of the HCl is 32.7 g.

DILUTION AND CONCENTRATION

A concentrated solution can be diluted. Powders and other solid mixtures can be triturated or diluted to yield less concentrated forms. Because the amount of solute in the diluted solution or mixture is the same as the amount in the concentrated solution or mixture, the following relationship applies to dilution problems.

The quantity of *Solution 1* (Q_1) \times concentration of *Solution 1* (C_1) = the quantity of *Solution 2* (Q_2) \times concentration of *Solution 2* (C_2), or

$$(Q_1)(C_1) = (Q_2)(C_2)$$

Almost any quantity and concentration terms may be used. However, the units of the terms must be the same on both sides of the equation.

EXAMPLES—

1. Calculate the quantity (Q_2), in g, of diluent that must be added to 60 g of a 10% (w/w) ointment to make a 5% (w/w) ointment. Let

$$(Q_1) = 60 \text{ g}, (C_1) = 10\%, \text{ and } (C_2) = 5\%$$

Using the above equation,

$$60 \text{ g} \times 10\% = (Q_2) \times 5\% \text{ (w/w)}$$

Solving the above equation, the quantity of product needed, Q_2 , is 120 g. The initial quantity of product added was 60 g, and therefore an additional 60 g of diluent must be added to the initial quantity to give a total of 120 g.

2. How much diluent should be added to 10 g of a trituration (1 in 100) to make a mixture that contains 1 mg of drug in each 10 g of final mixture? Determine the final concentration by first converting mg to g. One mg of drug in 10 g of mixture is the same as 0.001 g in 10 g. Let

$$(Q_1) = 10 \text{ g}, (C_1) = (1 \text{ in } 100),$$

and

$$(C_2) = (0.001 \text{ in } 10)$$

Using the equation for dilution,

$$10 \text{ g} \times (1/100) = (Q_2) \text{ g} \times (0.001/10)$$

Solving the above equation,

$$(Q_2) = 1000 \text{ g}$$

Because 10 g of the final mixture contains all of the drug and some diluent, (1000 g – 10 g) or 990 g of diluent is required to prepare the mixture at a concentration of 0.001 g of drug in 10 g of final mixture.

3. Calculate the percentage strength of a solution obtained by diluting 400 mL of a 5.0% solution to 800 mL. Let

$$(Q_1) = 400 \text{ mL}, (C_1) = 5\%, \text{ and } (Q_2) = 800 \text{ mL}$$

Using the equation for dilution,

$$400 \text{ mL} \times 5\% = 800 \text{ mL} \times (C_2)\%$$

Solving the above equation,

$$(C_2) = 2.5\% \text{ (w/v)}$$

USE OF POTENCY UNITS

See *Units of Potency* in the *General Notices*.

Because some substances may not be able to be defined by chemical and physical means, it may be necessary to express quantities of activity in biological units of potency.

EXAMPLES—

1. One mg of Pancreatin contains not less than 25 USP Units of amylase activity, 2.0 USP Units of lipase activity, and 25 USP Units of protease activity. If the patient takes 0.1 g (100 mg) per day, what is the daily amylase activity ingested?

1 mg of Pancreatin corresponds to 25 USP Units of amylase activity.

100 mg of Pancreatin corresponds to

$$100 \times (25 \text{ USP Units of amylase activity}) = 2500 \text{ Units}$$

2. A dose of penicillin G benzathine for streptococcal infection is 1.2 million units intramuscularly. If a specific product contains 1180 units per mg, how many milligrams would be in the dose?

1180 units of penicillin G benzathine are contained in 1 mg.

1 unit is contained in 1/1180 mg.

1,200,000 units are contained in

$$(1,200,000 \times 1)/1180 \text{ units} = 1017 \text{ mg}$$

BASE VS SALT OR ESTER FORMS OF DRUGS

Frequently, for stability or other reasons such as taste or solubility, the base form of a drug is administered in an altered form such as an ester or salt. This altered form of the drug usually has a different molecular weight (MW), and at times it may be useful to determine the amount of the base form of the drug in the altered form.

EXAMPLES—

1. Four hundred milligrams of erythromycin ethylsuccinate (molecular weight, 862.1) is administered. Determine the amount of erythromycin (molecular weight, 733.9) in this dose.

862.1 g of erythromycin ethylsuccinate corresponds to 733.9 g of erythromycin.

1 g of erythromycin ethylsuccinate corresponds to (733.9/862.1) g of erythromycin.

- 0.400 g of erythromycin ethylsuccinate corresponds to $(733.9/862.1) \times 0.400$ g or 0.3405 g of erythromycin.
2. The molecular weight of testosterone cypionate is 412.6 and that of testosterone is 288.4. What is the dose of testosterone cypionate that would be equivalent to 60.0 mg of testosterone?
 288.4 g of testosterone corresponds to 412.6 g of testosterone cypionate.
 1 g of testosterone corresponds to $412.6/288.4$ g of testosterone cypionate.
 60.0 mg or 0.0600 g of testosterone corresponds to $(412.6/288.4) \times 0.0600 = 0.0858$ g or 85.8 mg of testosterone cypionate.

RECONSTITUTION OF DRUGS USING VOLUMES OTHER THAN THOSE ON THE LABEL

Occasionally it may be necessary to reconstitute a powder in order to provide a suitable drug concentration in the final product. This may be accomplished by estimating the volume of the powder and liquid medium required.

EXAMPLES—

1. If the volume of 250 mg of ceftriaxone sodium is 0.1 mL, how much diluent should be added to 500 mg of ceftriaxone sodium powder to make a suspension having a concentration of 250 mg per mL?

$$500 \text{ mg} \times (1 \text{ mL}/250 \text{ mg}) = 2 \text{ mL}$$

$$\text{Volume of 500 mg of ceftriaxone sodium} = 500 \text{ mg} \times (0.1 \text{ mL}/250 \text{ mg}) = 0.2 \text{ mL}$$

$$\text{Volume of the diluent required} = (2 \text{ mL of suspension}) - (0.2 \text{ mL of Ceftriaxone Sodium}) = 1.8 \text{ mL}$$

2. What is the volume of dry powder cefonicid, if 2.50 mL of diluent is added to 1 g of powder to make a solution having a concentration of 325 mg per mL?

$$\text{Volume of solution containing 1 g of the powder} = 1 \text{ g of cefonicid} \times (1000 \text{ mg}/1 \text{ g}) \times (1 \text{ mL of solution}/325 \text{ mg of cefonicid}) = 3.08 \text{ mL}$$

$$\text{Volume of dry powder cefonicid} = 3.08 \text{ mL of solution} - 2.50 \text{ mL of diluent} = 0.58 \text{ mL}$$

ALLIGATION ALTERNATE AND ALGEBRA

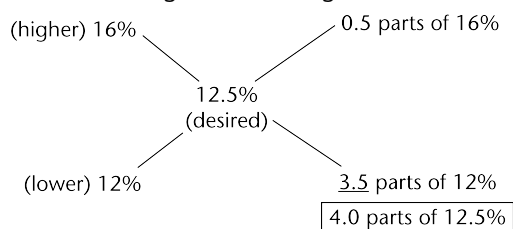
Alligation—Alligation is a rapid method of determining the proportions in which substances of different strengths are mixed to yield a desired strength or concentration. Once the proportion is found, the calculation may be performed to find the exact amounts of substances required. Set up the problem as follows.

- Place the desired percentage or concentration in the center.
- Place the percentage of the substance with the lower strength on the lower left-hand side.
- Place the percentage of the substance with the higher strength on the upper left-hand side.
- Subtract the desired percentage from the lower percentage, and place the obtained difference on the upper right-hand side.
- Subtract the higher percentage from the desired percentage, and place the obtained difference on the lower right-hand side.

The results obtained will determine how many parts of the two different percentage strengths should be mixed to produce the desired percentage strength of a drug mixture.

EXAMPLES—

1. How much ointment having a 12% drug concentration and how much ointment having a 16% drug concentration must be used to make 1 kg of a preparation containing a 12.5% drug concentration?



In a total of 4.0 parts of 12.5% product, 3.5 parts of 12% ointment and 0.5 parts of 16% ointment are needed.

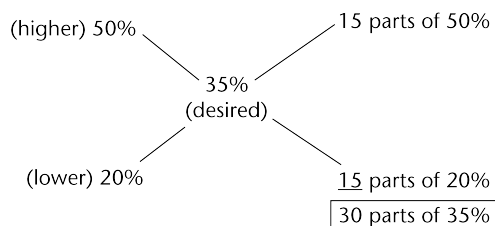
4 parts correspond to 1 kg or 1000 g.

1 part corresponds to 250 g.

3.5 parts correspond to 3.5×250 g or 875 g.

0.5 parts correspond to 0.5×250 g or 125 g.

2. How many mL of 20% dextrose in water and 50% dextrose in water are needed to make 750 mL of 35% dextrose in water?



In a total of 30 parts of 35% dextrose in water, 15 parts of 50% dextrose in water and 15 parts of 20% dextrose in water are required.

30 parts correspond to 750 mL.

15 parts correspond to 375 mL.

Thus use 375 mL of the 20% solution and 375 mL of the 50% solution to prepare the product.

Algebra—Instead of using alligation to solve the above problems, algebra may be used, following the scheme outlined below.

In order to represent the total quantity (weights, parts, or volumes) of the final mixture or solution, 1 or a specified quantity is used.

Let x be the quantity of one portion and $[1$ (or the specified amount) $- x]$ be the remaining portion. Set up the equation according to the statement below, and solve.

The amount of drug in one part plus the amount of drug in the other part equals the total amount in the final mixture or solution.

EXAMPLES—

1. How much ointment having a 12% drug concentration and how much ointment having a 16% drug concentration must be used to make 1 kg of a preparation containing a 12.5% drug concentration? Let 1 kg be the total quantity of ointment to be prepared, let x be the quantity, in kg, of the 12% ointment, and let $(1 - x)$ be the quantity in kg of the 16% ointment. The equation is as follows:

$$(12/100)x + (16/100)(1 - x) = (12.5/100)(1)$$

Solving the equation, x equals 0.875 kg of the 12% ointment and $(1 - x)$ equals $(1 - 0.875)$ or 0.125 kg of the 16% ointment.

2. How many mL of 20% dextrose in water and 50% dextrose in water are needed to make 750 mL of 35% dextrose in water?
Let x be the volume, in mL, of the 20% solution, and let $(750 - x)$ be the volume in mL of the 50% solution. The equation is as follows:

$$(20/100)x + (50/100)(750 - x) = (35/100)(750)$$

Solving the equation, x equals 375 mL of the 20% solution and $(750 - x)$ equals $(750 - 375)$ or 375 mL of the 50% solution.

MOLAR, MOLAL, AND NORMAL CONCENTRATIONS

See *Concentrations* in the *General Notices*.

Molarity—The molar concentration, M , of the solution is the number of moles of the solute contained in one L of solution.

Molality—The molal concentration, m , is the number of moles of the solute contained in one kilogram of solvent.

Normality—The normal concentration, N , of a solution expresses the number of milliequivalents (mEq) of solute contained in 1 mL of solution or the number of equivalents (Eq, gram-equivalent weight) of solute contained in 1 L of solution. When using normality, the pharmacist must apply quantitative chemical analysis principles using molecular weight (MW). Normality depends on the reaction capacity of a chemical compound and therefore the reaction capacity must be known. For acids and bases, reaction capacity is the number of accessible protons available from, or the number of proton binding sites available on, each molecular aggregate. For electron transfer reactions, reaction capacity is the number of electrons gained or lost per molecular aggregate.

EXAMPLES—

- How much sodium bicarbonate powder is needed to prepare 50.0 mL of a 0.07 N solution of sodium bicarbonate (NaHCO_3)? (MW of NaHCO_3 is 84.0 g per mol.)
In an acid or base reaction, because NaHCO_3 may act as an acid by giving up one proton, or as a base by accepting one proton, one Eq of NaHCO_3 is contained in each mole of NaHCO_3 . Thus the equivalent weight of NaHCO_3 is 84 g. [NOTE—The volume, in L, \times normality of a solution equals the number of equivalents in the solution.]
The number of equivalents of NaHCO_3 required = $(0.07 \text{ Eq/L})(50.0 \text{ mL}/1000 \text{ mL/L}) = 0.0035$ equivalents.
1 equivalent weight is 84.0 g.
 0.0035 equivalents equals $84.0 \text{ g/Eq} \times 0.0035 \text{ Eq} = 0.294 \text{ g}$.
- A prescription calls for 250 mL of a 0.1 N hydrochloric acid (HCl) solution. How many mL of concentrated hydrochloric acid are needed to make this solution? [NOTE—The specific gravity of concentrated hydrochloric acid is 1.18, the molecular weight is 36.46 and the concentration is 37.5% (w/w). Because hydrochloric acid functions as an acid and reacts by giving up one proton in a chemical reaction, 1 Eq is contained in each mole of the compound. Thus the equivalent weight is 36.46 g.]
The number of equivalents of HCl required is $0.250 \text{ L} \times 0.1 \text{ N} = 0.025$ equivalents.
1 equivalent is 36.46 g.
 0.025 equivalents correspond to $0.025 \text{ Eq} \times 36.46 \text{ g/Eq} = 0.9115 \text{ g}$.
37.5 g of pure HCl are contained in 100 g of concentrated HCl.
Thus 1 g of pure HCl is contained in $(100/37.5) \text{ g} = 2.666 \text{ g}$ of concentrated acid, and 0.9115 g is contained in $(0.9115 \times 2.666) \text{ g}$ or 2.43 g of concentrated acid.

In order to determine the volume of the supplied acid required, use the definition for specific gravity as shown below.

Specific gravity = (weight of the substance)/(weight of an equal volume of water).

$1.18 = 2.43 \text{ g}/(\text{weight of an equal volume of water})$.

The weight of an equal volume of water is 2.056 g or 2.06 g, which measures 2.06 mL. Thus, 2.06 mL of concentrated acid is required.

MILLIEQUIVALENTS AND MILLIMOLES

NOTE—This section addresses milliequivalents (mEq) and millimoles (mmol) as they apply to electrolytes for dosage calculations.

The quantities of electrolytes administered to patients are usually expressed in terms of mEq. This term must not be confused with a similar term used in quantitative chemical analysis as discussed above. Weight units such as mg or g are not often used for electrolytes because the electrical properties of ions are best expressed as mEq. An equivalent is the weight of a substance (equivalent weight) that supplies one unit of charge. An equivalent weight is the weight, in g, of an atom or radical divided by the valence of the atom or radical. A milliequivalent is one-thousandth of an equivalent (Eq). Because the ionization of phosphate depends on several factors, the concentration is usually expressed in millimoles, moles, or milliosmoles, which are described below. [NOTE—Equivalent weight (Eq.wt) = wt. of an atom or radical (ion) in g/valence (or charge) of the atom or radical. Milliequivalent weight (mEq.wt) = Eq.wt. (g)/1000.]

EXAMPLES—

- Potassium (K^+) has a gram-atomic weight of 39.10. The valence of K^+ is 1+. Calculate its milliequivalent weight (mEq wt).

$$\text{Eq wt} = 39.10 \text{ g}/1 = 39.10 \text{ g}$$

$$\text{mEq wt} = 39.10 \text{ g}/1000 = 0.03910 \text{ g} = 39.10 \text{ mg}$$

- Calcium (Ca^{2+}) has a gram-atomic weight of 40.08. Calculate its milliequivalent weight (mEq wt).

$$\text{Eq wt} = 40.08 \text{ g}/2 = 20.04 \text{ g}$$

$$\text{mEq wt.} = 20.04 \text{ g}/1000 = 0.02004 \text{ g} = 20.04 \text{ mg}$$

NOTE—The equivalent weight of a compound may be determined by dividing the molecular weight in g by the product of the valence of either relevant ion and the number of times this ion occurs in one molecule of the compound.

- How many milliequivalents of potassium ion (K^+) are there in a 250-mg Penicillin V Potassium Tablet? [NOTE—Molecular weight of penicillin V potassium is 388.48 g per mol; there is one potassium atom in the molecule; and the valence of K^+ is 1.]

$$\text{Eq wt} = 388.48 \text{ g}/[1(\text{valence}) \times 1 (\text{number of charges})] = 388.48 \text{ g}$$

$$\text{mEq wt} = 388.48 \text{ g}/1000 = 0.38848 \text{ g} = 388.48 \text{ mg}$$

$$(250 \text{ mg per Tablet})/(388.48 \text{ mg per mEq}) = 0.644 \text{ mEq of } \text{K}^+ \text{ per Tablet}$$

4. How many equivalents of magnesium ion and sulfate ion are contained in 2 mL of a 50% Magnesium Sulfate Injection? (Molecular weight of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ is 246.48 g per mol.)

Amount of magnesium sulfate in 2 mL of 50% Magnesium Sulfate Injection

$$2 \text{ mL of Injection} \times (50 \text{ g of magnesium sulfate} / 100 \text{ mL of Injection}) = 1 \text{ g}$$

$$\text{Eq wt of } \text{MgSO}_4 \cdot 7\text{H}_2\text{O} = \text{MW (g)} / (\text{valence of specified ion} \times \text{number of specified ions in one mole of salt}).$$

For the magnesium ion:

The number of equivalents is calculated as follows:

$$246.48 / [2(\text{valence}) \times 1 (\text{number of ions in the compound})] = 123.24 \text{ g/Eq of magnesium ion}$$

The number of equivalents in 1 g is $1 \text{ g} / 123.24 \text{ g/Eq} = 0.008114 \text{ Eq}$.

The number of mEq may be calculated as follows:

$$\text{The mEq wt} = \text{Eq wt (g)} / 1000 = (123.24 \text{ g/Eq}) / 1000 = 0.12324 \text{ g}$$

The number of milliequivalents of magnesium ion in 1 g is

$$1 \text{ g} / 0.12324 \text{ g/mEq} = 8.114 \text{ mEq}$$

For the sulfate ion:

The number of equivalents is calculated as follows:

$$246.48 / [2(\text{valence}) \times 1 (\text{number of ions in the compound})] = 123.24 \text{ g/Eq of sulfate ion}$$

The number of equivalents in 1 g is

$$1 \text{ g} / 123.24 \text{ g/Eq} = 0.008114 \text{ Eq}$$

The number of mEq may be calculated as follows:

$$\text{The mEq wt} = \text{Eq wt (g)} / 1000 = (123.24 \text{ g/Eq}) / 1000 = 0.12324 \text{ g}$$

The number of milliequivalents of sulfate ion in 1 g is

$$1 \text{ g} / 0.12324 \text{ g/mEq} = 8.114 \text{ mEq}$$

5. A vial of Sodium Chloride Injection contains 3 mEq of sodium chloride per mL. What is the percentage strength of this solution? (Molecular weight of sodium chloride is 58.44 g per mol.)

$$1 \text{ mEq} = 1 \text{ Eq} / 1000 = 58.44 \text{ g} / 1000 = 0.05844 \text{ g} = 58.44 \text{ mg}$$

$$\text{Amount of sodium chloride in 3 mEq per mL} = 58.44 \text{ mg per mEq} \times 3 \text{ mEq per mL} = 175.32 \text{ mg per mL}$$

$$175.32 \text{ mg} / 1 \text{ mL} = 17532 \text{ mg} / 100 \text{ mL} = 17.532 \text{ g} / 100 \text{ mL} = 17.5\%$$

Using mols and mmols—

A number of countries have adopted the International System of Units and no longer calculate doses using mEq as described above, but instead use the terms moles (mol) and millimoles (mmol). In *USP-NF* or in the *Pharmacists' Pharma-*

copeia the International System of Units is used except for the labeling of electrolytes.

Definitions—

A mole equals one gram atomic weight or gram molecular weight of a substance.

A millimole equals 1/1000 of a mole.

EXAMPLES—

1. Potassium (K) has a gram-atomic weight of 39.10.

Calculate its weight in millimoles (mmol).

The weight of one mole is 39.10 g and the weight in millimoles is

$$39.10 \text{ g} / 1000 = 0.0391 \text{ g or } 39.1 \text{ mg}$$

2. How many millimoles of Penicillin V are in a tablet that contains 250 mg of Penicillin V Potassium? (Molecular weight of penicillin V potassium is 388.48 g per mol.)

The weight of one mole is 388.48 and the weight in millimoles is

$$388.48 / 1000 = 0.38848 \text{ g or } 388.48 \text{ mg}$$

Thus there are $250 \text{ mg} / 388.48 \text{ mg/mmol} = 0.644 \text{ mmol}$ of Penicillin V ion per tablet.

ISOOSMOTIC SOLUTIONS

The following discussion and calculations have therapeutic implications in preparations of dosage forms intended for ophthalmic, subcutaneous, intravenous, intrathecal, and neonatal use.

Cells of the body, such as erythrocytes, will neither swell nor shrink when placed in a solution that is isotonic with the body fluids. However, the measurement of tonicity, a physiological property, is somewhat difficult. It is found that a 0.9% (w/v) solution of sodium chloride, which has a freezing point of -0.52° , is isotonic with body fluids and is said to be isoosmotic with body fluids. In contrast to isotonicity, the freezing point depression is a physical property. Thus many solutions that are isoosmotic with body fluids are not necessarily isotonic with body fluids, e.g., a solution of urea. Nevertheless many pharmaceutical products are prepared using freezing point data or related sodium chloride data to prepare solutions that are isoosmotic with the body fluids. A closely related topic is osmolality (see *Osmolality and Osmolarity* <785>).

Freezing point data or sodium chloride equivalents of pharmaceuticals and excipients (see *Table 1* below) may be used to prepare isoosmotic solutions, as shown in the examples below.

Table 1. Sodium Chloride Equivalents (E) and Freezing Point (FP) Depressions for a 1% Solution of the Drug or Excipient

Drug or Excipient	E	FP Depression
Atropine sulfate	0.13	0.075
Sodium chloride	1.00	0.576

EXAMPLE—

Determine the amount of sodium chloride required to prepare 60 mL of an isoosmotic solution of atropine sulfate 0.5% using the sodium chloride equivalent values and also the freezing point depression values.

Using the sodium chloride equivalent values—

The total amount of substances equivalent to sodium chloride (for a 0.9% solution) = $(0.9 \text{ g} / 100 \text{ mL}) \times 60 \text{ mL} = 0.54 \text{ g}$.

The amount of atropine sulfate required = $(0.5 \text{ g} / 100 \text{ mL}) \times 60 \text{ mL} = 0.3 \text{ g}$.

1 g of atropine sulfate is equivalent to 0.13 g of sodium chloride.

0.3 g atropine sulfate is equivalent to $0.3 \times 0.13 \text{ g} = 0.039 \text{ g}$ of sodium chloride.

Thus the required amount of sodium chloride is $0.54 - 0.039 = 0.501 \text{ g}$ or 0.50 g .

Using freezing point depression values—

The freezing point depression required is 0.52° .

A 1% solution of atropine sulfate causes a freezing point depression of 0.075° .

A 0.5% solution of atropine sulfate causes a freezing point depression of

$$0.075^\circ \times 0.5 = 0.0375^\circ$$

The additional freezing point depression required is

$$0.52^\circ - 0.0375^\circ = 0.482^\circ$$

A 1% solution of sodium chloride causes a freezing point depression of 0.576° .

A (1%/ 0.576) solution of sodium chloride causes a freezing point depression of 1° .

A $(1\% / 0.576) \times 0.482 = 0.836\%$ solution of sodium chloride causes a freezing point depression of 0.482° .

The required amount of sodium chloride is

$$(0.836 \text{ g}/100 \text{ mL}) \times 60 \text{ mL} = 0.502 \text{ g or } 0.50 \text{ g}$$

FLOW RATES IN INTRAVENOUS SETS

Some calculations concerning flow rates in intravenous sets are provided below. [NOTE—Examples below are *not* to be used for treatment purposes.]

EXAMPLES—

1. Sodium Heparin 8,000 units in 250 mL Sodium Chloride Injection 0.9% solution are to be infused over 4 hours. The administration set delivers 20 drops per mL.

What is the flow rate in mL per hour?

In 4 hours, 250 mL are to be delivered.

In 1 hour, $250 \text{ mL}/4 = 62.5 \text{ mL}$ are delivered.

What is the flow rate in drops per minute?

In 60 minutes, 62.5 mL are delivered.

In 1 minute, $62.5 \text{ mL}/60 = 1.04 \text{ mL}$ are delivered.

1 mL = 20 drops.

$1.04 \text{ mL} = 1.04 \times 20 \text{ drops} = 20.8 \text{ drops}$.

Thus in 1 minute, 20.8 or 21 drops are administered.

2. A 14.5 kg patient is to receive 50 mg of Sodium Nitroprusside in 250 mL of dextrose 5% in water (D5W) at the rate of $1.3 \mu\text{g}$ per kg per minute. The set delivers 50 drops per mL.

Calculate the flow rate in mL per hour.

The dose for 1 kg is $1.3 \mu\text{g}$ per minute.

The 14.5 kg patient should receive $14.5 \times 1.3 \mu\text{g} = 18.85 \mu\text{g}$ per minute.

50 mg or 50,000 μg of drug are contained in 250 mL of D5W.

$18.85 \mu\text{g}$ are contained in $250 \text{ mL} \times 18.85/50,000 = 0.09425 \text{ mL}$ D5W, which is administered every minute.

In 1 minute, 0.09425 mL are administered.

In 1 hour or 60 minutes, $60 \times 0.09425 \text{ mL} = 5.655$ or 5.7 mL are administered.

Calculate the flow rate in drops per minute.

1 mL corresponds to 50 drops per minute.

0.09425 mL corresponds to $0.09425 \times 50 = 4.712$ or 4.7 drops per minute.

TEMPERATURE

The relationship between Celsius degrees ($^\circ\text{C}$) and Fahrenheit degrees ($^\circ\text{F}$) is expressed by the following equation:

$$9 (^\circ\text{C}) = 5 (^\circ\text{F}) - 160$$

in which $^\circ\text{C}$ and $^\circ\text{F}$ are the numbers of Celsius degrees and Fahrenheit degrees, respectively.

EXAMPLES—

1. Convert 77°F to Celsius degrees.

$$9 (^\circ\text{C}) = 5 (^\circ\text{F}) - 160$$

$$^\circ\text{C} = [5 (^\circ\text{F}) - 160]/9 = [(5 \times 77) - 160]/9 = 25^\circ\text{C}$$

2. Convert 30°C to Fahrenheit degrees.

$$9 (^\circ\text{C}) = 5 (^\circ\text{F}) - 160$$

$$^\circ\text{F} = [9 (^\circ\text{C}) + 160]/5 = [(9 \times 30) + 160]/5 = 86^\circ\text{F}$$

The relationship between the Kelvin and the Celsius scales is expressed by the equation:

$$\text{K} = ^\circ\text{C} + 273.1$$

in which K and $^\circ\text{C}$ are the numbers of Kelvin degrees and Celsius degrees, respectively.

APPLICATION OF MEAN KINETIC TEMPERATURE

See *Good Storage and Distribution Practices for Drug Products* (1079) for the definition of mean kinetic temperature (MKT). MKT is usually higher than the arithmetic mean temperature and is derived from the Arrhenius equation. MKT addresses temperature fluctuations during the storage period of the product. The mean kinetic temperature, T_k , is calculated by the following equation:

$$T_k = \frac{\frac{-\Delta H}{R}}{\ln \left(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \dots + e^{-\Delta H/RT_n}}{n} \right)}$$

in which ΔH is the heat of activation, which equals 83.144 kJ per mol (unless more accurate information is available from experimental studies); R is the universal gas constant, which equals 8.3144×10^{-3} kJ per degree per mol; T_1 is the average temperature, in degrees Kelvin, during the first time period, e.g., the first week; T_2 is the average temperature, in degrees Kelvin, during the second time period, e.g., second week; and T_n is the average temperature, in degrees Kelvin during the n th time period, e.g., n th week, n being the total number of temperatures recorded. The mean kinetic temperature is calculated from average storage temperatures recorded over a one-year period, with a minimum of twelve equally spaced average storage temperature observations being recorded (see *Good Storage and Distribution Practices for Drug Products* (1079)). This calculation can be performed manually with a pocket calculator or electronically with computer software.

EXAMPLES—

1. The means of the highest and lowest temperatures for 52 weeks are 25 °C each. Calculate the MKT.

$$n = 52$$

$$\Delta H/R = 10,000 \text{ K}$$

$$T_1, T_2, \dots, T_n = 25^\circ\text{C} = 273.1 + 25 = 298.1 \text{ K}$$

$$R = 0.0083144 \text{ kJ K}^{-1}\text{mol}^{-1}$$

$$\Delta H = 83.144 \text{ kJ per mol}$$

$$T_K = \frac{\frac{-\Delta H}{R}}{\ln\left(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \dots + e^{-\Delta H/RT_n}}{n}\right)}$$

$$= -10,000\text{K}/(\ln[(52 \times e^{-\Delta H/R \times 298.1})/52])$$

$$= -10,000\text{K}/(\ln[(52 \times e^{-33.5458})/52])$$

$$-10,000\text{K}/-33.5458 = 298.1\text{K} = 25.0^\circ\text{C}$$

The calculated MKT is 25.0 °C. Therefore the controlled room temperature requirement is met by this pharmacy. [NOTE—If the averages of the highest and lowest weekly temperatures differed from each other and were in the allowed range of 15 °C to 30 °C (see <659> *Packaging and Storage Requirements*), then each average would be substituted individually into the equation. The remaining two examples illustrate such calculations, except that the monthly averages are used.]

2. A pharmacy recorded a yearly MKT on a monthly basis, starting in January and ending in December. Each month, the pharmacy recorded the monthly highest

temperature and the monthly lowest temperature, and the average of the two was calculated and recorded for the MKT calculation at the end of the year (see Table 2). From these data the MKT may be estimated or it may be calculated. If more than half of the observed temperatures are lower than 25 °C and a mean lower than 23 °C is obtained, the MKT may be estimated without performing the actual calculation.

- a. To estimate the MKT, the recorded temperatures are evaluated and the average is calculated. In this case, the calculated arithmetic mean is 22.9 °C. Therefore, the above requirements are met and it can be concluded that the mean kinetic temperature is lower than 25 °C. Therefore, the controlled room temperature requirement is met.
- b. The second approach is to perform the actual calculation.

$$n = 12$$

$$T_K = \frac{\frac{-\Delta H}{R}}{\ln\left(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \dots + e^{-\Delta H/RT_n}}{n}\right)}$$

$$= \frac{-10,000\text{K}}{\ln\left(\frac{1.710 \times 10^{-15} + 2.033 \times 10^{-15} + 1.710 \times 10^{-15} + \dots + 2.699 \times 10^{-15}}{12}\right)}$$

$$= -10,000\text{K}/\ln[(2.585 \times 10^{-14})/12]$$

$$-10,000\text{K}/-33.771 = 296.11\text{K} = 23.0^\circ\text{C}$$

The calculated MKT is 23.0 °C, so the controlled room temperature requirement is met. [NOTE—These data and calculations are used only as an example.]

3. An article was stored for one year in a pharmacy where the observed monthly average of the highest and lowest temperatures was 25 °C (298.1 K), except for one month with an average of 28 °C (301.1 K). Calculate the MKT of the pharmacy.

Table 2. Data for Calculation of MKT

n	Month	Lowest Temperature (in °C)	Highest Temperature (in °C)	Average Temperature (in °C)	Average Temperature (in K)	$\Delta H/RT$	$e^{-\Delta H/RT}$
1	Jan.	15	27	21	294.1	34.002	1.710×10^{-15}
2	Feb.	20	25	22.5	295.6	33.830	2.033×10^{-15}
3	Mar.	17	25	21	294.1	34.002	1.710×10^{-15}
4	Apr.	20	25	22.5	295.6	33.830	2.033×10^{-15}
5	May	22	27	24.5	297.6	33.602	2.551×10^{-15}
6	June	15	25	20	293.1	34.118	1.523×10^{-15}
7	July	20	26	23	296.1	33.772	2.152×10^{-15}
8	Aug.	22	26	24	297.1	33.659	2.411×10^{-15}
9	Sept.	23	27	25	298.1	33.546	2.699×10^{-15}
10	Oct.	20	28	24	297.1	33.659	2.411×10^{-15}
11	Nov.	20	24	22	295.1	33.887	1.919×10^{-15}
12	Dec.	22	28	25	298.1	33.546	2.699×10^{-15}

n = 12

$$T_k = \frac{\frac{-\Delta H}{R}}{\ln\left(\frac{e^{-\Delta H/RT_1} + e^{-\Delta H/RT_2} + \dots + e^{-\Delta H/RT_n}}{n}\right)}$$
$$= \frac{\frac{-\Delta H}{R}}{\ln\left(\frac{11 \times e^{-\Delta H/(R \times 298.1)} + 1 \times e^{-\Delta H/(R \times 301.1)}}{12}\right)}$$
$$= -10,000K/(\ln[(11 \times e^{-33.546} + 1 \times e^{-33.212})/12])$$
$$= -10,000K/(\ln[(2.9692 \times 10^{-14} + 3.7705 \times 10^{-15})/12])$$
$$= -10,000K/(\ln[(3.3463 \times 10^{-14})/12])$$
$$= -10,000K/[\ln(2.7886 \times 10^{-15})]$$
$$= -10,000K/-33.513 = 298.39K = 25.29^{\circ}C$$

The controlled room temperature requirement is not met because the calculated MKT exceeds 25 °C. (See Note in Example 2 above.)

4. Using the same calculation technique for controlled room temperature, the MKT for controlled cold temperatures can also be calculated.
- a. For example, if the mean of the highest and lowest temperatures for each week over a period of 52 weeks was 8 °C (i.e., the same mean for

each week), then the MKT can be calculated as follows:

$$T_k = -10,000/[\ln(52e^{-\Delta H/(R \times 281.1)})/52]$$
$$T_k = -10,000/[\ln(e^{-\Delta H/(R \times 281.1)})]$$
$$T_k = -10,000/[\ln(e^{-35.575})]$$
$$= -10,000/[\ln(3.548 \times 10^{-16})]$$
$$= -10,000/-35.575$$
$$T_k = 281.1K$$
$$C = 281.1 - 273.1$$
$$C = 8^{\circ}$$

- b. In another example, where a variety of average temperatures are used, as would be the case in reality, if the average of the highest and lowest temperatures ranges from 0° to 15 °C, then these averages would be individually substituted into the equation. For simplification of the mathematical process, 10 intervals are shown in Table 3 below. This illustration is intended for calculation of MKT at storage or in transit; i.e., during shipping or distribution of the critical drug product. These calculations can be performed manually or with a computer.

Table 3. Sample Data for MKT Calculations

Intervals	Low Temperature (in °C)	High Temperature (in °C)	Average Temperature (in °C)	Average Temperature (in K)	ΔH/RT	e ^{-ΔH/RT} × 10 ¹⁶
1	0	5	2.5	275.6	36.284	1.746
2	2	8	5	278.1	35.958	2.419
3	3	9	6	279.1	35.829	2.752
4	3	14	8.5	281.6	35.511	3.782
5	7	15	11	284.0	35.211	5.106
6	1	6	3.5	276.6	36.153	1.990
7	5	15	10	283.1	35.323	4.565
8	2	14	8	281.1	35.575	3.548
9	2	6	4	277.1	36.088	2.124
10	3	10	6.5	279.6	35.765	2.934

<1163> QUALITY ASSURANCE IN PHARMACEUTICAL COMPOUNDING

INTRODUCTION

The need for a quality assurance system is well documented in *United States Pharmacopeia (USP)* chapters for compounded preparations (see *Quality Control under Pharmaceutical Compounding—Nonsterile Preparations* <795> and *Quality Assurance (QA) Program under Pharmaceutical Compounding—Sterile Preparations* <797>). A quality assurance program is guided by written procedures that define responsibilities and practices that ensure compounded preparations are produced with quality attributes appropriate to meet the needs of patients and health care professionals. The authority and responsibility for the Quality Assurance program should be clearly defined and implemented and should include at least the following nine separate but integrated components: (1) training; (2) standard operating procedures (SOPs); (3) documentation; (4) verification; (5) testing; (6) cleaning, disinfecting, and safety; (7) containers, packaging, repackaging, labeling, and storage; (8) outsourcing, if used; and (9) responsible personnel.

The definition of compounding for the purpose of this chapter is defined in general test chapter <795>.

The safety, quality, and efficacy and/or benefit of compounded preparations depend on correct ingredients and calculations; accurate and precise measurements; appropriate formulation, facilities, equipment, and procedures; and prudent pharmaceutical judgment. As a final check, the compounder shall review each procedure in the compounding process. To ensure accuracy and completeness, the compounder shall observe the finished preparation to ensure that it appears as expected and shall investigate any discrepancies and take appropriate corrective action before the prescription is dispensed to the patient.

The water used in all aspects of compounding should meet the requirements of *Waters for Pharmaceutical Purposes* <1231>.

Radiopharmaceuticals and radiolabeled materials have unique characteristics requiring additional quality assurances described in *Positron Emission Tomography Drugs for Compounding, Investigational, and Research Uses* <823> and the *Radiopharmaceuticals as CSPs* section under <797>.

The responsibilities of the compounder and compounding personnel can be found in chapters <795> and <797>.

TRAINING

Personnel involved in nonsterile or sterile compounding require additional, specific training and periodic retraining beyond the training needed for routine dispensing duties. A thorough quality assurance program for compounded preparations requires documentation of both training and skill competency. In addition, the authority and responsibility for the QA program should be clearly defined as implemented. Training for nonsterile compounders should meet or exceed the standards set forth in <795>, and personnel training for sterile preparation compounders should meet or exceed the standards set forth in <797>.

STANDARD OPERATING PROCEDURES

SOPs for pharmaceutical compounding are documents that describe how to perform routine and expected tasks in

the compounding environment, including but not limited to procedures involving:

- Beyond-Use dating
- Chemical and physical stability
- Cleaning and disinfecting
- Component quality evaluation
- Compounding methods
- Dispensing
- Documentation
- Environmental quality and maintenance
- Equipment maintenance, calibration, and operation
- Formulation development
- Labeling
- Materials and final compounded preparation handling and storage
- Measuring and weighing
- Packaging and repackaging
- Patient monitoring, complaints, and adverse event reporting
- Patient or caregiver education and training
- Personnel cleanliness and garb
- Purchasing
- Quality Assurance and Continuous Quality Monitoring
- Safety
- Shipping
- Testing
- Training and retraining

SOPs are itemized instructions that describe when a task will be performed, how a task will be performed, who will perform the task, why the task is necessary, any limitations in performing the task, and what action to take when unacceptable deviations or discrepancies occur.

SOPs must be reviewed regularly and updated as necessary. Auditing and verifying compliance with established SOPs should be performed periodically. The SOP should be specific to each device and process used in compounding. Properly maintained and implemented SOPs are vital to preparation quality.

DOCUMENTATION

The purpose of documentation is to provide a record of all aspects of compounding operations and procedures that are described in this chapter, in <795>, and in <797>. Information on the compounding record should ideally be entered as the tasks are performed or as testing data is received. Compounding records should be reviewed for accuracy, completeness (as appropriate) and approved by QA personnel, prior to dispensing. Additionally, beyond-use dating and sterility studies, where appropriate, should be documented by reference to at least one of the following:

- Stability studies published in peer-reviewed literature,
- In-house or laboratory conducted stability and/or sterility studies,
- National compendia, or
- An extrapolation of above based on professional judgment.

VERIFICATION

Verification involves authoritatively signed assurance and documentation that a process, procedure, or piece of equipment is functioning properly and producing the expected results. The act of verification of a compounding procedure involves checking to ensure the calculations, weighing and measuring, order of mixing, and compounding techniques and equipment were appropriate and accurately performed. The quality of ingredients should be verified upon receipt (e.g., Certificate of Analysis, manufacturer's label on commercial products, etc.). Verification may require outside laboratory testing when in-house capabilities are not adequate. Equipment verification methods are sometimes available from manufacturers of the specific equipment or can be de-

veloped in-house. The responsibility for assuring that equipment performance is verified, including work completed by contractors, resides with the compounder.

See *Component Selection, Handling, and Storage* under (795).

TESTING

A quality assurance program for compounded preparations should include testing during the compounding process and of the finished compounded preparation, when appropriate, as described in chapters (795) and (797). The compounder should have a basic understanding of pharmaceutical analysis to ensure that valid results are obtained when tests are being conducted, whether they are done in-house or outsourced. Acceptance criteria shall be determined prior to testing. Testing every compounded preparation is neither practical nor officially required, but compounders should conduct visual inspections and know: (1) the importance of testing in the overall quality program in the compounding facility, (2) when to test, (3) what to test, (4) what appropriate method(s) and equipment to use, (5) how to interpret the results, (6) the limits of the test, and (7) specific actions required when a preparation does not meet specifications. Investigative and corrective action should extend to other preparations that may have been

associated with the specific failure or discrepancy. Testing may involve one or more quality attributes, and each test will have one or more acceptable procedures, usually with well-defined acceptance criteria.

The goal in testing is to determine accurately the adequacy of the compounding process and the quality of the preparation. Any testing procedure used should have accuracy, reproducibility, and specificity. No single testing procedure is suitable for all drugs or preparations because a number of factors determine the validity and reliability of results.

Compounding professionals have two options for the testing that is required for compounded preparations or their ingredients. Some testing methods can easily be performed at the compounding site, but some may need to be outsourced to a contract laboratory. Some testing methods can be conducted in-house by an individual who possesses a good understanding of pharmaceutical analysis and proper training. See *Table 1* for a list of compendial testing methods and *USP* chapters for reference.

If testing is done at the compounding site, appropriate equipment shall be obtained and qualified either by the manufacturer upon sale or by the compounding professional upon receipt and shall be maintained, calibrated, and used properly. If testing is outsourced, the compounding professional should determine what to outsource, how to select a laboratory, and should develop an ongoing relationship with the laboratories chosen. Contract laboratories shall follow

Table 1. U.S. Pharmacopeia Chapters for Selected Quality Testing Methods and Procedures

	Chapter Title	Chapter
General Testing		
Boiling point	Distilling Range	(721)
Density	Density of Solids	(699)
Ion selective potentiometry	—	—
Loss on drying	Loss on Drying	(731)
	Pharmaceutical Calculations in Prescription Compounding	(1160)
Melting point	Melting Range or Temperature	(741)
Osmolality and osmolarity	Pharmaceutical Calculations in Prescription Compounding	(1160)
	Osmolality and Osmolarity	(785)
Particle size	Powder Fineness	(811)
Particulate matter in injections	Particulate Matter in Injections	(788)
pH	pH	(791)
Refractive index	Refractive Index	(831)
Viscosity change	Viscosity	(911)
Volumetric	Prescription Balances and Volumetric Apparatus	(1176)
Weight	Prescription Balances and Volumetric Apparatus	(1176)
Spectroscopy		
Flame emission and atomic absorption spectroscopy	Spectrophotometry and Light-Scattering	(851)
Fluorescence/phosphorescence spectroscopy	Spectrophotometry and Light-Scattering	(851)
Infrared spectroscopy	Spectrophotometry and Light-Scattering	(851)
Ultraviolet/visible spectroscopy	Spectrophotometry and Light-Scattering	(851)
Chromatography		
Column chromatography (CC)	Chromatography	(621)
Gas chromatography (GC)	Chromatography	(621)
High-performance liquid chromatography (HPLC)	Chromatography	(621)
Paper chromatography (PC)	Chromatography	(621)
Thin-layer chromatography (TLC)	Chromatography	(621)
Microbiology		
Endotoxin testing	Bacterial Endotoxins Test	(85)
Microbial limit testing	Microbiological Examination of Nonsterile Products:	(61)
	Microbial Enumeration Tests	
	Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms	
Preservative effectiveness testing	Antimicrobial Effectiveness Testing	(51)
Sterility	Sterility Tests	(71)

standards set forth in *USP* general chapters, as appropriate, and preferably should be registered with the U.S. Food and Drug Administration (FDA).

Selection of a Testing Method—One general consideration in testing procedure selection is the type of information needed, such as quantitative (strength, concentration), semiquantitative (where a tolerance level is involved, as in endotoxin levels), or qualitative (presence/absence testing, including substance identification, sterility). Another consideration involves the physical and chemical characteristics of the analyte, including solubility, partition coefficient, dissociation constant (pKa), volatility, binding, and the quantity present. The testing method selected also depends upon factors such as sample handling/preparation/purification requirements; type of data needed; and accuracy, reproducibility, and specificity required.

The degree of quantitative measurement and specificity must be considered in the verification process. The typical analytical characteristics used in method verification include accuracy, precision, specificity, detection limit, quantitation limit, linearity, range, and ruggedness. Generally, the greater the level of accuracy, precision, or specificity required, the more sophisticated and expensive the testing methods needed. The methods used are also governed by the types of instrumentation available and the standards available for comparison.

Pharmaceutical analysis decisions include procedure selection, obtaining a representative sample (the number of preparation units selected to adequately represent the entire formulation, e.g., 10 randomly selected capsules from a preparation of 100 capsules), storage/shipping of the sample, sample preparation for analysis, the actual analysis, data acquisition, data treatment, and interpretation.

The compounding professional is responsible for implementing a program using selected testing methods for the preparations compounded in the facility. *USP* chapters on spectroscopy and chromatography methods are referenced in *Table 1*. Examples of general and microbiological testing methods are discussed later in this chapter. Examples of selected testing methods for bulk substances and various dosage forms (see *Pharmaceutical Dosage Forms* <1151>) are shown in *Table 2*.

Sampling Requirements—Before collecting samples for testing, compounding professionals should consider the following factors:

- Quantity of preparation being compounded, for a specific prescription versus in anticipation of prescriptions routinely received
- Number of samples needed
- Destructive or nondestructive testing
- Appropriate methods of obtaining representative samples
- Physical state of the samples (solid, liquid, or gas)
- Type of container required for collection and storage
- Any special handling and shipping requirements or restrictions (e.g., controlled drug substances, dangerous or hazardous chemicals, flammable or caustic substances, and refrigerated or frozen preparations)

Storage Requirements—Storage requirements for samples must be specified, including type of container, temperature, humidity, and light protection (see *General Notices and Requirements* and the *Containers, Packaging, Repackaging, Labeling, and Storage* section in this chapter).

The effect(s) that any substance has on the compounded preparation that may interfere or alter the results must be known beforehand. When sending a preparation to a contract laboratory, the compounder should provide the complete written formulation so that the laboratory can quickly determine if there may be any interfering substances present.

Data Interpretation Requirements—The collection of raw data from the testing process must be completed accurately. One must ensure that appropriate and valid descrip-

tive statistics (e.g., mean, standard deviation) are used to analyze the data and that the operating parameters of the analytical instruments are well-established. Reference values, if available, should be provided with the analytical results. A description of the analytical controls used by the laboratory is important for documentation, as is the source of reference standards used to establish standard curves.

Personnel Requirements and Considerations—If testing is done in-house, personnel involved in this activity must be appropriately trained and evaluated with documentation of the training and evaluation. If testing is outsourced, the compounder must be assured of the credentials, proper training, and continuing competency activities of the personnel in the contract laboratory.

PHYSICAL TESTING OF DOSAGE UNITS

NOTE: In this section the terms “unit” and “dosage unit” are synonymous. To ensure the consistency of dosage units, each unit in a batch should have a uniform weight within a narrow range. Dosage units are defined as dosage forms containing a single dose or a part of a dose in each unit. If multiple dose units are compounded in a batch formulation, the total number of units should not deviate outside of $\pm 10\%$ of the theoretical number of units.

WEIGHT ASSESSMENT

First, zero or tare the balance. During the compounding process intermediate weighing may be necessary to ensure that all substances have been included and weighed accurately.

At the end of the compounding process, for the dosage form and quantity designated, take care to preserve the integrity of each dosage unit during the following assessment procedures. Assume the concentration (weight of drug substance per weight of dosage unit) is uniform. The following are examples of weight assessment.

Hard Capsules—

- Zero or tare balance with an empty capsule.
- Accurately weigh each individual filled capsule from a representative sample of the finished batch (for example, a minimum of 5% of total capsules or 10 individual capsules, whichever is less) and record the weight of each finished capsule on the compounding record.
- Calculate the theoretical weight of a finished capsule's contents.
- Compare the actual content weight of each finished capsule in the representative sample with the theoretical weight of a finished capsule's contents.
- Determine if there is a deviation outside $\pm 10\%$ with any weight of a finished capsule's contents and the theoretical weight of a finished capsule, and if so,
 - Review the compounding record to ensure no steps were omitted.
 - Repeat with a larger representative sample of the finished batch (10% of total capsules or 20 individual capsules, whichever is less). Do not mix with the first batch tested.
- If a deviation outside of $\pm 10\%$ is discovered in the second representative sample, then destroy the batch.

Other Solids (Including Tablets, Suppositories, Inserts, and Lozenges)—

- Accurately weigh each individual dosage unit from a representative sample of the finished batch (for example, a minimum of 5% of total tablets or 10 individual tablets, whichever is less) and record the weight of each dosage unit on the compounding record.
- Calculate the theoretical weight of the dosage unit.

Table 2. Selected Compendial Testing Methods for Bulk Substances and Various Dosage Forms

Bulk Substances and Dosage Forms	Testing Method ^a													
	Wt	Vol	pH	Osm	RI	Sp Gr	MP	UV/Vis	HPLC	GC	IR	Sterile	Endotoxin	PM
Bulk substances	—	—	+	—	+	—	+	+	+	+	+	—	+ ^b	—
Capsules	+	—	—	—	—	—	—	—	+	+	—	—	—	—
Emulsions	+	+	+	—	—	+	—	—	+	+	—	—	—	—
Gels	+	+	+	—	+	+	—	—	+	+	—	—	—	—
Inhalations	+	+	+	+	+	+	—	+	+	+	—	+	+	—
Injections	+	+	+	+	+	+	—	+	+	+	—	+	+	+
Inserts	+	—	—	—	—	+	+	—	+	+	—	—	—	—
Irrigations	+	+	+	+	+	+	—	+	+	+	—	+	+	—
Lozenges	+	—	—	—	—	—	—	—	+	+	—	—	—	—
Nasals	+	+	+	+	+	+	—	+	+	+	—	* ^c	—	—
Ophthalmics	+	+	+	+	+	+	—	+	+	+	—	+	—	+ ^d
Otics	+	+	+	+	+	+	—	+	+	+	—	—	—	—
Powders	+	—	—	—	—	—	—	—	+	+	—	—	—	—
Semisolids	+	—	+	—	—	+	+	—	+	+	—	—	—	—
Solutions, nonsterile	+	+	+	+	+	+	—	+	+	+	—	—	—	—
Sterile implant gels	+	+	+	+	+	+	—	+	+	+	—	+	+	—
Sterile implant solids	+	+	—	—	—	—	+	+	+	+	—	+	+	—
Sticks	+	—	—	—	—	+	+	—	+	+	—	—	—	—
Suppositories	+	—	—	—	—	+	+	—	+	+	—	—	—	—
Suspensions, nonsterile	+	+	+	—	—	+	—	—	+	+	—	—	—	—
Tablets	+	—	—	—	—	—	—	—	+	+	—	—	—	—

^a Wt, weight; Vol, volume; Osm, osmolality/osmolarity; RI, refractive index; Sp Gr, specific gravity; MP, melting point; UV/Vis, ultraviolet/visible spectroscopy; HPLC, high-performance liquid chromatography; GC, gas chromatography; IR, infrared spectroscopy; PM, particulate matter; +, test applicable; —, test not applicable.

^b Endotoxin testing may be needed for bulk substances used in compounding some sterile preparations.

^c *, microbial limits (see *Microbiological Examination of Nonsterile Products: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use* (1111) and *Pharmaceutical Compounding—Sterile Preparations* (797)).

^d Solutions only, not suspensions or ointments.

- Compare the actual weight of each dosage unit in the representative sample with the theoretical weight of a dosage unit.
- Determine if there is a deviation outside $\pm 10\%$ with any weight of a finished dosage unit and the theoretical weight of a finished dosage unit, and if so,
 - Review the compounding record to ensure no steps were omitted.
 - Repeat with a larger representative sample of the finished batch (10% of total tablets or 20 individual tablets, whichever is less). Do not mix with the first batch tested.
- If a deviation outside of $\pm 10\%$ is discovered in the second representative sample, then destroy the batch.

Semi-Solids (Including Creams, Gels, and Ointments)—

- Accurately weigh an empty container and record the weight on the compounding record.
 - Fill an empty container with the final compounded preparation.
 - Calculate the theoretical weight of the compounded preparation.
 - Weigh the filled container.
 - Determine if there is a deviation outside of $\pm 10\%$, and if so, review the compounding record to ensure no steps were omitted. If the deviation cannot be explained, destroy the batch and prepare a new one.
- Additional Quality Assurance Checks Before Packaging Semi-Solids—*
- Visually inspect the preparation for foreign materials and expected appearance.
 - Measure pH, when applicable.

MICROBIOLOGICAL TESTING

Microbiological testing for pharmacy compounding includes sterility, endotoxin, preservative effectiveness testing, and microbial limit testing (see (797)).

Sterility Testing—Sterility tests may be conducted using commercial kits or by developing and verifying USP sterility testing protocols. Standards and procedures are explained in (71).

Endotoxin Testing—Endotoxin tests may be conducted using commercial kits or by purchasing the components separately. Endotoxin testing may be performed in-house with appropriate training and experience. See (85).

Preservative Effectiveness Testing—Preservative effectiveness testing may be conducted when preparing a frequently compounded formulation that contains a preservative. When such a test is performed, the results shall support the beyond-use-date (BUD) assigned to the compounded preparations. See (51).

Microbial Limit Testing—Microbial limit testing may be conducted to provide an estimate of the number of viable aerobic microorganisms (see (61)) or to demonstrate freedom from designated microbial species (see (62)).

CLEANING, DISINFECTING, AND SAFETY

This section applies to both equipment and facilities (see (795), (797), and *Disinfectants and Antiseptics* (1072)).

CONTAINERS, PACKAGING, REPACKAGING, LABELING, AND STORAGE

For storage, packaging, repackaging, and labeling of compounded preparations and repackaging of manufactured products (when defined as compounding in *USP*), refer to

USP General Notices and Requirements and the following general chapters:

- Containers—Glass <660>
- Containers—Plastic <661>
- Elastomeric Closures for Injections <381>
- Good Packaging Practices <1177>
- Good Repackaging Practices <1178>
- Good Storage and Shipping Practices <1079>
- Injections <1>
- Packaging and Repackaging—Single-Unit Containers <1136>
- Pharmaceutical Dosage Forms <1151>

OUTSOURCING

NOTE: This section addresses only the purchasing or selling of compounded preparations from pharmacy to pharmacy, not the outsourcing of analytical testing of compounded preparations.

For pharmacies that prepare outsourced compounded preparations or repackaged commercial products, documentation of beyond-use dating, as defined previously in the *Documentation* section of this chapter, is required and shall be provided upon request. In addition, documentation of compliance with USP chapters <795> and <797> is required and shall be provided upon request.

For facilities that receive outsourced compounded preparations or repackaged commercial products, documentation shall be on file for all BUDs assigned to those preparations or products.

RESPONSIBLE PERSONNEL

The responsibility and authority for a quality assurance program should be clearly defined and implemented. Personnel responsible for the quality assurance program should have the education, training, and experience necessary to perform the assigned functions. Quality assurance personnel should assure that documentation, verification, and testing are performed in accordance with written policies and procedures. If deviations from approved policies and procedures occur, it is the responsibility of the quality assurance personnel to investigate and to implement appropriate corrective action. Documentation of any investigations and corrective actions is the responsibility of the quality assurance personnel. Responsible personnel in the quality assurance program are essential in assuring the safety, identity, strength, quality, and purity of compounded drug preparations.

SUMMARY

A quality assurance program is necessary to ensure the quality of compounded preparations. A sound quality assurance program includes detailed SOPs, documentation, verification, analytical and microbiological testing as appropriate to particular compounded preparations, and responsible quality assurance personnel. Compounding professionals must determine the types of testing and degree of testing that will be a part of their quality assurance program. They

also must decide whether to perform testing in-house or outsource it to a contract laboratory.

<1171> PHASE-SOLUBILITY ANALYSIS

Phase-solubility analysis is the quantitative determination of the purity of a substance through the application of precise solubility measurements. At a given temperature, a definite amount of a pure substance is soluble in a definite quantity of solvent. The resulting solution is saturated with respect to the particular substance, but the solution remains unsaturated with respect to other substances, even though such substances may be closely related in chemical structure and physical properties to the particular substance being tested. Constancy of solubility, like constancy of melting temperature or other physical properties, indicates that a material is pure or is free from foreign admixture except in the unique case in which the percentage composition of the substance under test is in direct ratio to solubilities of the respective components. Conversely, variability of solubility indicates the presence of an impurity or impurities.

Phase-solubility analysis is applicable to all species of compounds that are crystalline solids and that form stable solutions. It is not readily applicable to compounds that form solid solutions with impurities.

The standard solubility method consists of six distinct steps: (1) mixing, in a series of separate systems, increasing quantities of material with measured, fixed amounts of a solvent; (2) establishment of equilibrium for each system at identical constant temperature and pressure; (3) separation of the solid phase from the solutions; (4) determination of the concentration of the material dissolved in the various solutions; (5) plotting the concentration of the dissolved materials per unit of solvent (y-axis or solution composition) against the weight of material per unit of solvent (x-axis or system composition); and (6) extrapolation and calculation.

Solvents

A proper solvent for phase-solubility analysis meets the following criteria: (1) The solvent is of sufficient volatility that it can be evaporated under vacuum, but is not so volatile that difficulty is experienced in transferring and weighing the solvent and its solutions. Normally, solvents having boiling points between 60° and 150° are suitable. (2) The solvent does not adversely affect the substance being tested. Solvents that cause decomposition or react with the test substance are not to be used. Solvents that solvate or form salts are to be avoided, if possible. (3) The solvent is of known purity and composition. Carefully prepared mixed solvents are permissible. Trace impurities may affect solubility greatly. (4) A solubility of 10 mg to 20 mg per g is optimal, but a wider working range can be used.

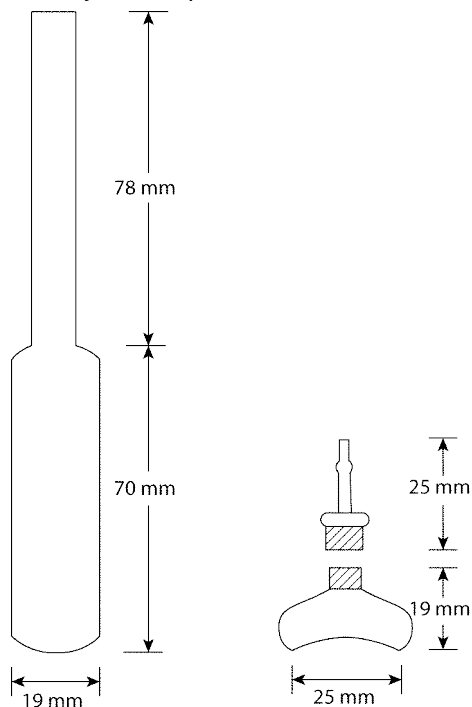
Apparatus*

Constant-Temperature Bath—Use a constant-temperature bath that is capable of maintaining the temperature within $\pm 0.1^\circ$ and that is equipped with a horizontal shaft capable of rotating at approximately 25 rpm. The shaft is equipped with clamps to hold the *Ampuls*. Alternatively, the bath may contain a suitable vibrator, capable of agitating

* Available from Hanson Research Corp., 19727 Bahama St., P. O. Box 35, Northridge, CA 91324.

the ampuls at 100 to 120 vibrations per second, and equipped with a shaft and suitable clamps to hold the ampuls.

Ampuls—Use 15-mL ampuls of the type shown in the accompanying illustration. Other containers may be used provided that they are leakproof and otherwise suitable.



Ampul (left) and Solubility Flask (right) Used in Phase-Solubility Analysis

Solubility Flasks—Use solubility flasks of the type shown in the accompanying illustration.

Procedure

NOTE—Make all weighings within $\pm 10 \mu\text{g}$.

System Composition—Weigh accurately, in g, not less than 7 scrupulously cleaned 15-mL ampuls. Weigh accurately, in g, increasingly larger amounts of the test substance into each of the ampuls. The weight of the test substance is selected so that the first ampul contains slightly less material than will go into solution in 5 mL of the selected solvent, the second ampul contains slightly more material, and each subsequent ampul contains increasingly more material than meets the indicated solubility. Transfer 5.0 mL of the solvent to each of the ampuls, cool in a dry ice-acetone mixture, and seal, using a double-jet air-gas burner and taking care to save all glass. Allow the ampuls and their contents to come to room temperature, and weigh the individual sealed ampuls with the corresponding glass fragments. Calculate the system composition, in mg per g, for each ampul by the formula:

$$1000(W_2 - W_1)/(W_3 - W_2)$$

in which W_2 is the weight of the ampul plus test substance, W_1 is the weight of the empty ampul, and W_3 is the weight of ampul plus test substance, solvent, and separated glass.

Equilibration—The time required for equilibration varies with the substance, the method of mixing (rotation or vibration), and the temperature. Normally, equilibrium is obtained more rapidly by the vibration method (1 to 7 days) than by the rotational method (7 to 14 days). In order to determine whether equilibration has been effected, 1 am-

pul, i.e., the next to the last in the series, may be warmed to 40° to produce a supersaturated solution. Equilibration is ensured if the solubility obtained on the supersaturated solution falls in line with the test specimens that approach equilibrium from an undersaturated solution.

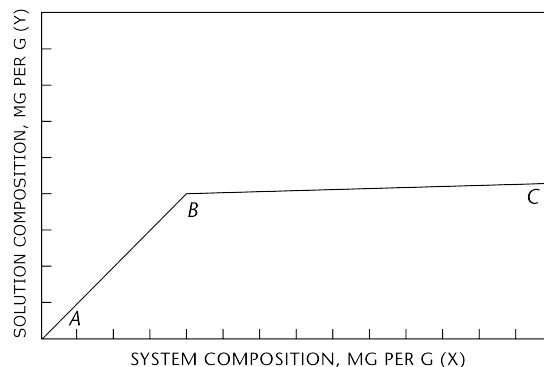
Solution Composition—After equilibration, place the ampuls vertically in a rack in the constant-temperature bath, with the necks of the ampuls above the water level, and allow the contents to settle. Open the ampuls, and remove a portion greater than 2 mL from each by means of a pipet equipped with a small pledget of cotton membrane or other suitable filter. Transfer a 2.0-mL aliquot of clear solution from each ampul to a marked, tared solubility flask, and weigh each flask plus its solution to obtain the weight of the solution. Cool the flasks in a dry ice-acetone bath, and then evaporate the solvent in vacuum. Gradually increase the temperature to a temperature consistent with the stability of the compound, and dry the residue to constant weight. Calculate the solution composition, in mg per g, by the formula:

$$1000(F_3 - F_1)/(F_2 - F_3)$$

in which F_3 is the weight of the flask plus residue, F_1 is the weight of the solubility flask, and F_2 is the weight of the flask plus solution.

Calculation

For each portion of the test substance taken, plot the solution composition as the ordinate and the system composition as the abscissa. As shown in the accompanying diagram,



Typical Phase-Solubility Diagram

the points for those containers, frequently only one, that represent a true solution fall on a straight line (AB) with a slope of 1, passing through the origin; the points corresponding to saturated solutions fall on another straight line (BC), the slope, S , of which represents the weight fraction of impurity or impurities present in the test substance. Failure of points to fall on a straight line indicates that equilibrium has not been achieved. A curve indicates that the material under test may be a solid solution. Calculate the percentage purity of the test substance by the formula:

$$100 - 100S.$$

The slope, S , may be calculated graphically or by least-squares treatment for best fit of the experimental values to a straight line.

The solubility of the main component is obtained by extending the solubility line (BC) through the y-axis. The point of interception on the y-axis is the extrapolated solubility, in mg per g, and is a constant for a given compound.

Purification Technique

Since the solvent phase in all combinations of solvent and solute that are used to construct segment BC of a phase-solubility diagram contains essentially all the impurities originally present in the substance under analysis, whereas the solid phase is essentially free from impurities, phase-solubility analysis can be used to prepare pure reference specimens of desired compounds as well as concentrates of impurities from substances otherwise considered pure. A simple modification of this technique can be used to accomplish these purposes with considerably less effort than is usually required for rigorous phase-solubility analysis.

In practice, a weighed amount of test specimen is suspended in a nonreactive solvent of suitable composition and amount so that about 10% of the material is dissolved at equilibrium. The suspension is sealed (a screw-cap vial is usually adequate) and shaken at room temperature until equilibrium is attained (usually 24 hours is sufficient for this purpose). The mother liquor is then drawn off and evaporated at or near room temperature to dryness. Since the mother liquor contained essentially all the impurities that were present in the specimen, the residue has been concentrated with respect to the impurities roughly in proportion to the ratio of the weight of specimen taken to the weight of solids dissolved in the volume of solvent used.

The undissolved crystals remaining after withdrawal of the mother liquor are usually sufficiently pure to be used as a reference standard after appropriate rinsing and drying.

〈1174〉 POWDER FLOW

The widespread use of powders in the pharmaceutical industry has generated a variety of methods for characterizing powder flow. Not surprisingly, scores of references appear in the pharmaceutical literature, attempting to correlate the various measures of powder flow to manufacturing properties. The development of such a variety of test methods was inevitable; powder behavior is multifaceted and thus complicates the effort to characterize powder flow. The purpose of this chapter is to review the methods for characterizing powder flow that have appeared most frequently in the pharmaceutical literature. In addition, while it is clear that no single and simple test method can adequately characterize the flow properties of pharmaceutical powders, this chapter proposes the standardization of test methods that may be valuable during pharmaceutical development.

Four commonly reported methods for testing powder flow are (1) angle of repose, (2) compressibility index or Hausner ratio, (3) flow rate through an orifice, and (4) shear cell. In addition, numerous variations of each of these basic methods are available. Given the number of test methods and variations, standardizing the test methodology, where possible, would be advantageous.

With this goal in mind, the most frequently used methods are discussed below. Important experimental considerations are identified and recommendations are made regarding standardization of the methods. In general, any method of measuring powder flow should be practical, useful, reproducible, sensitive, and yield meaningful results. It bears repeating that no one simple powder flow method will adequately or completely characterize the wide range of flow properties experienced in the pharmaceutical industry. An appropriate strategy may well be the use of multiple standardized test methods to characterize the various aspects of powder flow as needed by the pharmaceutical scientist.

ANGLE OF REPOSE

The angle of repose has been used in several branches of science to characterize the flow properties of solids. Angle of repose is a characteristic related to interparticulate friction or resistance to movement between particles. Angle of repose test results are reported to be very dependent upon the method used. Experimental difficulties arise as a result of segregation of material and consolidation or aeration of the powder as the cone is formed. Despite its difficulties, the method continues to be used in the pharmaceutical industry, and a number of examples demonstrating its value in predicting manufacturing problems appear in the literature.

The angle of repose is the constant, three-dimensional angle (relative to the horizontal base) assumed by a cone-like pile of material formed by any of several different methods (described briefly below).

Basic Methods for Angle of Repose

A variety of angle of repose test methods are described in the literature. The most common methods for determining the static angle of repose can be classified on the basis of the following two important experimental variables:

- (1) The height of the "funnel" through which the powder passes may be fixed relative to the base, or the height may be varied as the pile forms.
- (2) The base upon which the pile forms may be of fixed diameter or the diameter of the powder cone may be allowed to vary as the pile forms.

Variations in Angle of Repose Methods

In addition to the above methods, the following variations have been used to some extent in the pharmaceutical literature:

- *Drained angle of repose* is determined by allowing an excess quantity of material positioned above a fixed diameter base to "drain" from the container. Formation of a cone of powder on the fixed diameter base allows determination of the drained angle of repose.
- *Dynamic angle of repose* is determined by filling a cylinder (with a clear, flat cover on one end) and rotating it at a specified speed. The dynamic angle of repose is the angle (relative to the horizontal) formed by the flowing powder. The internal angle of kinetic friction is defined by the plane separating those particles sliding down the top layer of the powder and those particles that are rotating with the drum (with roughened surface).

Angle of Repose General Scale of Flowability

Although there is some variation in the qualitative description of powder flow using the angle of repose, much of the pharmaceutical literature appears to be consistent with the classification by Carr*, which is shown in Table 1. There are examples in the literature of formulations with an angle of repose in the range of 40° to 50° that were manufactured satisfactorily. When the angle of repose exceeds 50°, the flow is rarely acceptable for manufacturing purposes.

Table 1. Flow Properties and Corresponding Angles of Repose*

Flow Property	Angle of Repose (degrees)
Excellent	25–30
Good	31–35
Fair—aid not needed	36–40
Passable—may hang up	41–45

* Carr, R.L. Evaluating Flow Properties of Solids. *Chem. Eng.* 1965, 72, 163–168.

Table 1. Flow Properties and Corresponding Angles of Repose* (Continued)

Flow Property	Angle of Repose (degrees)
Poor—must agitate, vibrate	46–55
Very poor	56–65
Very, very poor	>66

Experimental Considerations for Angle of Repose

Angle of repose is not an intrinsic property of the powder; i.e., it is very much dependent upon the method used to form the cone of powder. The following important considerations are raised in the existing literature:

- The peak of the cone of powder can be distorted by the impact of powder from above. By carefully building the powder cone, the distortion caused by impact can be minimized.
- The nature of the base upon which the powder cone is formed influences the angle of repose. It is recommended that the powder cone be formed on a "common base," which can be achieved by forming the cone of powder on a layer of powder. This can be done by using a base of fixed diameter with a protruding outer edge to retain a layer of powder upon which the cone is formed.

Recommended Procedure for Angle of Repose

Form the angle of repose on a fixed base with a retaining lip to retain a layer of powder on the base. The base should be free of vibration. Vary the height of the funnel to carefully build up a symmetrical cone of powder. Care should be taken to prevent vibration as the funnel is moved. The funnel height should be maintained approximately 2–4 cm from the top of the powder pile as it is being formed in order to minimize the impact of falling powder on the tip of the cone. If a symmetrical cone of powder cannot be successfully or reproducibly prepared, this method is not appropriate. Determine the angle of repose by measuring the height of the cone of powder and calculating the angle of repose, α , from the following equation:

$$\tan(\alpha) = \text{height}/0.5 \text{ base}$$

COMPRESSIBILITY INDEX AND HAUSNER RATIO

In recent years the compressibility index and the closely related Hausner ratio have become the simple, fast, and popular methods of predicting powder flow characteristics. The compressibility index has been proposed as an indirect measure of bulk density, size and shape, surface area, moisture content, and cohesiveness of materials because all of these can influence the observed compressibility index. The compressibility index and the Hausner ratio are determined by measuring both the bulk volume and the tapped volume of a powder.

Basic Methods for Compressibility Index and Hausner Ratio

Although there are some variations in the method of determining the compressibility index and Hausner ratio, the basic procedure is to measure (1) the unsettled apparent volume, V_o , and (2) the final tapped volume, V_i , of the powder after tapping the material until no further volume

changes occur. The compressibility index and the Hausner ratio are calculated as follows:

$$\text{Compressibility Index} = 100 \times [(V_o - V_i)/V_o]$$

$$\text{Hausner Ratio} = (V_o/V_i)$$

Alternatively, the compressibility index and Hausner ratio may be calculated using measured values for bulk density (ρ_{bulk}) and tapped density (ρ_{tapped}) as follows:

$$\text{Compressibility Index} = 100 \times [(\rho_{\text{tapped}} - \rho_{\text{bulk}})/\rho_{\text{tapped}}]$$

$$\text{Hausner Ratio} = (\rho_{\text{tapped}}/\rho_{\text{bulk}})$$

In a variation of these methods, the rate of consolidation is sometimes measured rather than, or in addition to, the change in volume that occurs on tapping. For the compressibility index and the Hausner ratio, the generally accepted scale of flowability is given in Table 2*.

Table 2. Scale of Flowability*

Compressibility Index (%)	Flow Character	Hausner Ratio
≤10	Excellent	1.00–1.11
11–15	Good	1.12–1.18
16–20	Fair	1.19–1.25
21–25	Passable	1.26–1.34
26–31	Poor	1.35–1.45
32–37	Very poor	1.46–1.59
>38	Very, very poor	>1.60

Experimental Considerations for the Compressibility Index and Hausner Ratio

Compressibility index and Hausner ratio are not intrinsic properties of the powder; i.e., they depend on the methodology used. In the existing literature, there are discussions of the following important considerations affecting the determination of (1) the unsettled apparent volume, V_o , (2) the final tapped volume, V_i , (3) the bulk density, ρ_{bulk} , and (4) the tapped density, ρ_{tapped} :

- The diameter of the cylinder used
- The number of times the powder is tapped to achieve the tapped density
- The mass of material used in the test
- Rotation of the sample during tapping

Recommended Procedure for Compressibility Index and Hausner Ratio

Use a 250-mL volumetric cylinder with a test sample weight of 100 g. Smaller weights and volumes may be used, but variations in the method should be described with the results. An average of three determinations is recommended.

FLOW THROUGH AN ORIFICE

The flow rate of a material depends upon many factors, some of which are particle-related and some related to the process. Monitoring the rate of flow of material through an orifice has been proposed as a better measure of powder flowability. Of particular significance is the utility of monitoring flow continuously because pulsating flow patterns have been observed even for free flowing materials. Changes in flow rate as the container empties can also be observed. Empirical equations relating flow rate to the diameter of the

opening, particle size, and particle density have been determined. However, determining the flow rate through an orifice is useful only with free-flowing materials.

The flow rate through an orifice is generally measured as the mass per time flowing from any of a number of types of containers (cylinders, funnels, hoppers). Measurement of the flow rate can be in discrete increments or continuous.

Basic Methods for Flow Through an Orifice

There are a variety of methods described in the literature. The most common method for determining the flow rate through an orifice can be classified on the basis of three important experimental variables:

- (1) The type of container used to contain the powder. Common containers are cylinders, funnels, and hoppers from production equipment.
- (2) The size and shape of the orifice used. The orifice diameter and shape are critical factors in determining powder flow rate.
- (3) The method of measuring powder flow rate. Flow rate can be measured continuously using an electronic balance with some sort of recording device (strip chart recorder, computer). It can also be measured in discrete samples (for example, the time it takes for 100 g of powder to pass through the orifice to the nearest tenth of a second or the amount of powder passing through the orifice in 10 seconds to the nearest tenth of a gram).

Variations in Methods for Flow Through an Orifice

Either mass flow rate or volume flow rate can be determined. Mass flow rate is the easier of the methods, but it biases the results in favor of high-density materials. Because die fill is volumetric, determining volume flow rate may be preferable. A vibrator is occasionally attached to facilitate flow from the container; however, this appears to complicate interpretation of the results. A moving orifice device has been proposed to more closely simulate rotary press conditions. The minimum diameter orifice through which powder flows can also be identified.

General Scale of Flowability for Flow Through an Orifice

No general scale is available because flow rate is critically dependent on the method used to measure it. Comparison between published results is difficult.

Experimental Considerations for Flow Through an Orifice

Flow rate through an orifice is not an intrinsic property of the powder. It very much depends on the methodology used. Several important considerations affecting these methods are discussed in the existing literature:

- The diameter and shape of the orifice
- The type of container material (metal, glass, plastic)
- The diameter and height of the powder bed.

Recommended Procedure for Flow Through an Orifice

Flow rate through an orifice can be used only for materials that have some capacity to flow. It is not useful for cohesive materials. Provided that the height of the powder bed (the "head" of the powder) is much greater than the diameter of the orifice, the flow rate is virtually independent of the powder head. Use a cylinder as the container because

the cylinder material should have little effect on flow. This configuration results in flow rate being determined by the movement of powder over powder rather than powder along the wall of the container. Powder flow rate often increases when the height of the powder column is less than two times the diameter of the column. The orifice should be circular and the cylinder should be free of vibration. General guidelines for dimensions of the cylinder are as follows:

- Diameter of opening > 6 times the diameter of the particles
- Diameter of the cylinder > 2 times the diameter of the opening

Use of a hopper as the container may be appropriate and representative of flow in a production situation. It is not advisable to use a funnel, particularly one with a stem, because flow rate will be determined by the size and length of the stem as well as the friction between the stem and the powder. A truncated cone may be appropriate, but flow will be influenced by the powder-wall friction coefficient, making selection of an appropriate construction material an important consideration.

For the opening in the cylinder, use a flat-faced bottom plate with the option to vary orifice diameter to provide maximum flexibility and to better ensure a powder-over-powder flow pattern. Rate measurement can be either discrete or continuous. Continuous measurement using an electronic balance can more effectively detect momentary flow rate variations.

SHEAR CELL METHODS

In an effort to put powder flow studies and hopper design on a more fundamental basis, a variety of powder shear testers and methods that permit more thorough and precisely defined assessment of powder flow properties have been developed. Shear cell methodology has been used extensively in the study of pharmaceutical materials. From these methods, a wide variety of parameters can be obtained, including the yield loci representing the shear stress-shear strain relationship, the angle of internal friction, the unconfined yield strength, the tensile strength, and a variety of derived parameters such as the flow factor and other flowability indices. Because of the ability to more precisely control experimental parameters, flow properties can also be determined as a function of consolidation load, time, and other environmental conditions. The methods have been successfully used to determine critical hopper and bin parameters.

Basic Methods for Shear Cell

One type of shear cell is the cylindrical shear cell that is split horizontally, forming a shear plane between the lower stationary base and the upper moveable portion of the shear cell ring. After powder bed consolidation in the shear cell (using a well-defined procedure), the force necessary to shear the powder bed by moving the upper ring is determined. Annular shear cell designs offer some advantages over the cylindrical shear cell design, including the need for less material. A disadvantage, however, is that because of its design, the powder bed is not sheared as uniformly; i.e., material on the outside of the annulus is sheared more than material in the inner region. A third type of shear cell (plate-type) consists of a thin sandwich of powder between a lower stationary rough surface and an upper rough surface that is moveable.

All of the shear cell methods have their advantages and disadvantages, but a detailed review is beyond the scope of this chapter. As with the other methods for characterizing powder flow, many variations are described in the literature. A significant advantage of shear cell methodology in general is a greater degree of experimental control. The methodol-

ogy is rather time-consuming and requires significant amounts of material and a well-trained operator.

Recommendations for Shear Cell

The many existing shear cell configurations and test methods provide a wealth of data and can be used very effectively to characterize powder flow. They are also helpful in the design of equipment such as hoppers and bins. Because of the diversity of available equipment and experimental procedures, no specific recommendations regarding methodology are presented in this chapter. It is recommended that the results of powder flow characterization using shear cell methodology include a complete description of equipment and methodology used.

(1176) PRESCRIPTION BALANCES AND VOLUMETRIC APPARATUS

Prescription Balances

NOTE—Balances other than the type described herein may be used if these afford equivalent or better accuracy. This includes micro-, semimicro-, or electronic single-pan balances (see *Weights and Balances* (41)). Some balances offer digital or direct-reading features. All balances should be calibrated and tested frequently using appropriate test weights, both singly and in combination.

Description—A prescription balance is a scale or balance adapted to weighing medicinal and other substances required in prescriptions or in other pharmaceutical compounding. It is constructed so as to support its full capacity without developing undue stresses, and its adjustment is not altered by repeated weighings of the capacity load. The removable pans or weighing vessels should be of equal weight. The balance should have leveling feet or screws. The balance may feature dial-in weights and also a precision spring and dial instead of a weighbeam. A balance that has a graduated weighbeam must have a stop that halts the rider or poise at the zero reading. The reading edge of the rider is parallel to the graduations on the weighbeam. The distance from the face of the index plate to the indicator pointer or pointers should be not more than 1.0 mm, the points should be sharp, and when there are two, their ends should be separated by not more than 1.0 mm when the scale is in balance. The indicating elements and the lever system should be protected against drafts, and the balance lid should permit free movement of the loaded weighing pans when the lid is closed. The balance must have a mechanical arresting device.

Definitions—

Capacity—Maximum weight, including the weight of tares, to be placed on one pan. The *N.B.S. Handbook 44*, 4th ed., states: "In the absence of information to the contrary, the nominal capacity of a Class A balance shall be assumed to be 15.5 g ($\frac{1}{2}$ apothecaries' ounce)." Most of the commercially available Class A balances have a capacity of 120 g and bear a statement to that effect.

Weighbeam or Beam—A graduated bar equipped with a movable poise or rider. Metric graduations are in 0.01-g increments up to a maximum of 1.0 g.

Tare Bar—An auxiliary ungraduated weighbeam bar with a movable poise. It can be used to correct for variations in weighing glasses or papers.

Balance Indicator—A combination of elements, one or both of which will oscillate with respect to the other, to indicate the equilibrium state of the balance during weighing.

Rest Point—The point on the index plate at which the indicator or pointer stops when the oscillations of the balance cease; or the index plate position of the indicator or pointer calculated from recorded consecutive oscillations in both directions past the zero of the index plate scale. If the balance has a two-pointer indicating mechanism, the position or the oscillations of only one of the pointers need be recorded or used to determine the rest point.

Sensitivity Requirements (SR)—The maximum change in load that will cause a specified change, one subdivision on the index plate, in the position of rest of the indicating element or elements of the balance.

Class A Prescription Balance—A balance that meets the tests for this type of balance has a sensitivity requirement of 6 mg or less with no load and with a load of 10 g on each pan. The Class A balance should be used for all the weighing operations required in prescription compounding.

In order to avoid errors of 5% or more that might be due to the limit of sensitivity of the Class A prescription balance, do not weigh less than 120 mg of any material. If a smaller weight of dry material is required, mix a larger known weight of the ingredient with a known weight of dry diluent, and weigh an aliquot portion of the mixture for use.

Testing the Prescription Balance—A Class A prescription balance meets the following four basic tests. Use a set of test weights, and keep the rider on the weighbeam at zero unless directed to change its position.

1. **Sensitivity Requirement**—Level the balance, determine the rest point, and place a 6-mg weight on one of the empty pans. Repeat the operation with a 10-g weight in the center of each pan. The rest point is shifted not less than one division on the index plate each time the 6-mg weight is added.

2. **Arm Ratio Test**—This test is designed to check the equality of length of both arms of the balance. Determine the rest point of the balance with no weight on the pans. Place in the center of each pan a 30-g test weight, and determine the rest point. If the second rest point is not the same as the first, place a 20-mg weight on the lighter side; the rest point should move back to the original place on the index plate scale or farther.

3. **Shift Tests**—These tests are designed to check the arm and lever components of the balance.

A. Determine the rest point of the indicator without any weights on the pans.

B. Place one of the 10-g weights in the center of the left pan, and place the other 10-g weight successively toward the right, left, front, and back of the right pan, noting the rest point in each case. If in any case the rest point differs from the rest point determined in Step A, add a 10-mg weight to the lighter side; this should cause the rest point to shift back to the rest point determined in Step A or farther.

C. Place a 10-g weight in the center of the right pan, and place a 10-g weight successively toward the right, left, front, and back of the left pan, noting the rest point in each case. If in any case the rest point is different from that obtained with no weights on the pans, this difference should be overcome by addition of the 10-mg weight to the lighter side.

D. Make a series of observations in which both weights are simultaneously shifted to off-center positions on their pans: both toward the outside, both toward the inside, one toward the outside and the other toward the inside, both toward the back, and so on until all combinations have been checked. If in any case the rest point differs from that obtained with no weights on the pan, the addition of the

10-mg weight to the lighter side should overcome this difference.

A balance that does not meet the requirements of these tests must be adjusted.

4. *Rider and Graduated Beam Tests*—Determine the rest point for the balance with no weight on the pans. Place on the left pan the 500-mg test weight, move the rider to the 500-mg point on the beam, and determine the rest point. If it is different from the zero rest point, add a 6-mg weight to the lighter side. This addition should bring the rest point back to its original position or farther. Repeat this test, using the 1-g test weight and moving the rider to the 1-g division on the beam. If the rest point is different, it should be brought back at least to the zero rest point position by the addition of 6 mg to the lighter pan. If the balance does not meet this test, the weighbeam graduations or the rider must be corrected.

Metric or apothecaries' weights for use with a prescription balance should be kept in a special rigid and compartmentalized box and handled with plastic or plastic-tipped forceps to prevent scratching or soiling. For prescription use, analytical weights (Class P or better) are recommended. However, Class Q weights have tolerances well within the limits of accuracy of the prescription balance, and they retain their accuracy for a long time with proper care. Coin-type (or disk-shaped) weights should not be used.

Test weights consisting of two 20-g or two 30-g, two 10-g, one 1-g, one 500-mg, one 20-mg, one 10-mg, and one 6-mg (or suitable combination totaling 6 mg) weights, adjusted to N.B.S. tolerances for analytical weights (Class P or better) should be used for testing the prescription balances. These weights should be kept in a tightly closed box and should be handled only with plastic or plastic-tipped forceps. The set of test weights should be used only for testing the balance or constantly used weights. If properly cared for, the set lasts indefinitely.

Volumetric Apparatus

Pharmaceutical devices for measuring volumes of liquids, including burets, pipets, and cylinders graduated either in metric or apothecary units meet the standard specifications for glass volumetric apparatus described in NTIS COM-73-10504 of the National Technical Information Service.¹ Conical graduates meet the standard specifications described in N.B.S. Handbook 44, 4th Edition, of the National Institute of Standards and Technology.² Graduated medicine droppers meet the specifications (see *Medicine Dropper* <1101>). An acceptable ungraduated medicine dropper has a delivery end 3 mm in external diameter and delivers 20 drops of water, weighing 1 g at a temperature of 15°. A tolerance of $\pm 10\%$ of the delivery specification is reasonable.

Selection and Use of Graduates—

Capacity—The capacity of a graduate is the designated volume, at the maximum graduation, that the graduate will contain, or deliver, as indicated, at the specified temperature.

Cylindrical and Conical Graduates—The error in a measured volume caused by a deviation of ± 1 mm in reading the lower meniscus in a graduated cylinder remains constant along the height of the uniform column. The same deviation of ± 1 mm causes a progressively larger error in a conical graduate, the extent of the error being further dependent upon the angle of the flared sides to the perpendicular of the upright graduate. A deviation of ± 1 mm in the meniscus reading causes an error of approximately 0.5 mL in the measured volume at any mark on the uniform 100-mL cylinder graduate. The same deviation of ± 1 mm can cause an

error of 1.8 mL at the 100-mL mark on an acceptable conical graduate marked for 125 mL.

A general rule for selection of a graduate for use is to use the graduate with a capacity equal to or just exceeding the volume to be measured. Measurement of small volumes in large graduates tends to increase errors, because the larger diameter increases the volume error in a deviation of ± 1 mm from the mark. The relation of the volume error to the internal diameters of graduated cylinders is based on the equation $V = \pi r^2 h$. An acceptable 10-mL cylinder having an internal diameter of 1.18 cm holds 109 μ L in 1 mm of the column. Reading 4.5 mL in this graduate with a deviation of ± 1 mm from the mark causes an error of about $\pm 2.5\%$, and the same deviation in a volume of 2.2 mL in the same graduate causes an error of about $\pm 5\%$. Minimum volumes that can be measured within certain limits of error in graduated cylinders of different capacities are incorporated in the design details of graduates in N.B.S. Handbook 44, 4th ed., of the National Institute of Standards and Technology. Conical graduates having a capacity of less than 25 mL should not be used in prescription compounding.

<1177> GOOD PACKAGING PRACTICES

This chapter provides general guidance on packaging considerations for Pharmacopeial preparations that may be stored, transported, and distributed. It describes procedures that should be considered to ensure that proper packaging practices are maintained. It does not affect any applicable requirements under good manufacturing practices, state laws governing pharmacy, the *USP General Notices and Requirements* or monographs, or provisions under approved labeling.

Definitions for storage conditions and packaging are provided in <659> *Packaging and Storage Requirements*. All equipment used for recording, monitoring, and maintaining these temperature and humidity conditions should be calibrated on a regular basis. This calibration should be traceable to national or international standards (see also the general information chapter *Monitoring Devices—Time, Temperature, and Humidity* <1118>).

CONTAINERS

The monograph packaging and storage statement specifies that the container (primary package) should meet the requirements under *Containers—Glass* <660>, *Containers—Plastic* <661>, and *Containers—Performance Testing* <671>, which include the stipulations for determining if a container is "tight" or "well-closed." In most cases, compendial preparations are expected to be packaged in "tight" containers, especially if the article is moisture sensitive. In addition, where necessary, the packaging component should protect the preparation from light, reactive gases, solvent loss, microbial contamination, etc. "Tight" and "well-closed" containers are clearly defined in <659> *Packaging and Storage Requirements*, whereas testing protocol and moisture permeation limits to determine if the container meets either of these definitions can be found in *Containers—Glass* <660>, *Containers—Plastic* <661>, and *Containers—Performance Testing* <671> for single-unit and multiple-unit containers.

A packaging system is composed of a container system with its closure. This system may include several layers of protection for the Pharmacopeial preparation along with any sealing devices, delivery devices, labeling, and package

¹ NTIS COM-73-10504 is for sale by the National Technical Information Service, Springfield, VA 22151.

² N.B.S. Handbook 44, 4th ed. (1971), is for sale by the Superintendent of Documents, U. S. Government Printing Office, Washington, DC 20402.

inserts. The *General Notices* section also provides definitions for types of packaging systems that contain and protect a Pharmacopeial preparation (e.g., single-unit containers, unit-dose containers, etc.). Stability testing is conducted on the dosage forms packaged in the container–closure system proposed for marketing.

One type of permeation test for multiple-unit containers is described in *Containers—Performance Testing* (671). This test is intended for drug products being dispensed on prescription in vials with a container–closure system. The results of the test reflect the water vapor permeation through the container and through the closure. Limits have been established to define whether a container for dispensing has tight or well-closed characteristics with regard to water vapor permeation. FDA recommends that manufacturers perform this test on the container–closure system, although it is not specified in *USP*. In this particular test, the inner seal of the manufacturer's container–closure system is removed prior to testing.

Single-unit containers for capsules and tablets under *Containers—Performance Testing* (671) are measured for water vapor permeation according to the criteria for the four classes of containers (classes A–D).

The *USP* recognizes several official container materials that can be selected on the basis of their properties. Most containers are made of glass or plastic. Glass containers must be evaluated for chemical resistance and light transmission (if indicated) as described in *Containers—Glass* (660). In addition, injectable medication containers should be reviewed according to the section *Packaging* under *Injections* (1). Elastomeric closures should be evaluated separately as described in *Elastomeric Closures for Injections* (381). Plastic containers should be assessed using different criteria for the three types of plastics as described in the following sections under *Containers—Plastic* (661): *Polyethylene Containers* (PE) for dry oral solid dosage forms, *Polyethylene Terephthalate Bottles and Polyethylene Terephthalate G Bottles* (PET, PETG) for liquid oral dosage forms, and *Polypropylene Containers* (PP) for dry solid and liquid oral dosage forms. As articulated in these sections, plastics should undergo testing for light transmission (if appropriate), water vapor permeation (see also *Containers—Performance Testing* (671)), extraction physicochemical testing, and biological testing (see also *Biological Reactivity Tests, In Vitro* (87) and *Biological Reactivity Tests, In Vivo* (88)). For example, testing water vapor permeation for a PE container is conducted by sealing the container with heat-sealed foil laminate and measuring the water permeation in a humid atmosphere. Given that water vapor does not permeate the foil laminate, this test assesses only the properties of the container. The level of protection provided by a packaging system marketed with a heat-sealed foil laminate inner seal (prior to removal of the foil) is approximated by this test. However, in the case of a PET bottle for liquid preparations, water vapor permeation testing is done by filling containers with water and measuring the water loss rate in a dry atmosphere. Additional testing may be required for certain pharmaceutical dosage forms as well.

The container–closure system for the storage or shipment of a bulk liquid drug substance is typically plastic, stainless steel, a glass-lined metal container, or an epoxy-lined metal container with a rugged, tamper-resistant closure. Qualification of the container–closure system for these types of preparations includes evaluation for solvent and gas permeation, light transmittance, closure integrity, ruggedness in shipment, protection against microbial contamination through the closure, and compatibility and safety of the packaging components as appropriate (see *Containers—Glass* (660) and *Containers—Plastics* (661)).

Other information on container–closure systems may be found in FDA's *Guidance for Industry: Container Closure System for Packaging Human Drugs and Biologics*, www.fda.gov.

PACKAGING

Packaging for Pharmacopeial articles can be divided into categories according to terminology generally accepted by industry. As mentioned earlier, the *General Notices* section provides some definitions for different types of containers classified by their characteristics and uses. In addition, the ASTM Committee D10 on packaging publishes terminology, practices, test methods, specifications, guides, and classifications for testing and evaluating packaging (see ASTM D99695, "Standard Terminology of Packaging and Distribution Environments"). Under certain rules and guidelines (e.g., such as 49 CFR, Dangerous Goods, and others), however, alternate terminology is used for the components described below. For terminology pertaining to repackaging processes, refer to *Packaging and Repackaging—Single-Unit Containers* (1136).

Primary Container—This container is in direct contact with the Pharmacopeial preparation. The purpose of a primary container, also referred to as an immediate container, is to protect the preparation from environmental hazards during storage and handling. In some cases, the primary container is also a specialized delivery system, such as an aerosol or a metered-dose dispenser (see *Pharmaceutical Dosage Forms* (1151)). For the majority of oral dosage forms, the primary container consists of a cap and a bottle or a blister or pouch package that can be made from many different materials, including glass, plastic, single or laminated flexible materials, and metal. All components of the primary container must meet the requirements under 21 CFR for direct food contact and, where applicable, the *USP* requirements under *Containers—Glass* (660), *Containers—Plastic* (661), and *Containers—Performance Testing* (671). A full description of the primary container is included under the "Container/Closure System" section of the New Drug Application (NDA), Abbreviated New Drug Application (ANDA), or other classes of FDA submissions.

Critical Secondary Container—This container is not in direct contact with the article, but it provides essential product stability protection. For example, a primary container may be packed inside a critical secondary container such as a pouch to provide moisture, gas, light, or microbial protection not afforded by the primary container. A description of the critical secondary container is included under the "Container/Closure System" section of the NDA, ANDA, or other classes of FDA submissions.

Secondary Container—This container encloses one or more primary containers. A secondary container is not always present. If used, it is usually designed for the final market presentation. Secondary containers are often used simply to carry required labeling or to keep individual primary containers together with delivery systems or other add-on features. Secondary containers can also provide protection against damage in the handling and distribution system. The most common secondary container is the standard folding carton. Some products, such as syringes, may be placed in trays prior to packing in a carton. Secondary container materials are not included in the container and closure description and require neither stability studies nor prior approval when making a change in the materials used.

Additional Packaging—A wide variety of additional packaging, such as trays and display cartons, may be used to hold primary containers.

Unit of Sale—This may be an individual bottle, a carton containing one or more bottles, or a tray with multiple primary containers. A unit of sale may contain more than one item for individual sale. For example, a display tray may have multiples of a single article or a variety of related articles from a single manufacturer, each intended for individual sale. The individual item intended for sale is referred to as a stock-keeping-unit (SKU). SKUs are distinguished by a discrete National Drug Code (NDC). Over-the-counter (OTC) articles contain a Universal Product Code (UPC) for

all SKUs. A prescription SKU may be intended for final consumer use and may not be repackaged by a pharmacy. Such packages, often called “unit of issue” or “unit of use,” require child-resistant (CR) packaging as described under 16 CFR 1700, “Poison Prevention Packaging,” except for packages exempted by the Consumer Product Safety Commission. The CR feature is typically incorporated by the manufacturer (see *Packaging and Repackaging—Single-Unit Containers* <1136>). OTC articles are regulated under the same rule, but only if they contain certain active ingredients above specified limits. Any regulated product shipped via the United States Postal Service (USPS) must meet the USPS rules under 39 CFR 111.

Final Exterior Package—This is typically a corrugated fiberboard box (case) or a wrapper. The shipping case label is affixed to this outermost layer and incorporates all of the bar codes required by the National Wholesale Druggists’ Association (NWDA). This final package is normally shipped on pallets to distribution centers, wholesalers, and other large-volume customers. The manufacturer may or may not intend that this package enter the small-package-shipping environment as an individual unit without further protection.

Especially with fiberboard boxes, relative humidity (RH) may have a negative effect on the compression strength of the box, causing loads to shift and potentially damage the article or the outer and inner packaging. Articles stored in refrigerators or freezers, which are environments with high RH, are prone to this type of damage when stacked. The problem may be exacerbated by carton design, stacking pattern, or use of low edge-crush-test corrugated fiberboard. Computer programs are available to determine the acceptable stack height and patterns on the basis of carton weight, style, size, and material. If problems occur, the product manufacturer should be contacted. Source materials and reference information on corrugated fiberboard boxes can be found in the “Fiber Box Handbook” published by the Fiber Box Association.

A wholesaler or other reshipper should not assume that the package received from the manufacturer is suitable for reuse. Many packages are customized for very specific routes and modes of transportation and are not suitable for other applications. Like any other shipping container, insulated cartons and inner protective packaging can be damaged during transit, thus affecting package performance and possibly allowing damage to contents if reused.

ENVIRONMENTAL ISSUES

Packaging materials are regulated by a variety of federal, state, and local rules. In general, most pharmaceutical packaging containers can be recycled within local programs. The use of recycled material in primary containers is governed by the FDA, but it is generally not allowed. Pharmaceutical manufacturers commonly follow the most current Coalition of Northeastern Governors’ rules (e.g., Model Toxics in Packaging Legislation) regarding heavy metals in packaging and other environmental issues.

Certain classes of Pharmacopeial articles may require special handling. Such articles include products classified as (1) Dangerous Goods under the Department of Transportation (DOT), state, local, or carrier rules; (2) controlled drugs under the Drug Enforcement Administration (DEA); or (3) scheduled substances under state regulations.

LABELING

The labeling of shipping containers by manufacturers must be in compliance with the pertinent sections of FDA and DOT rules.

Dangerous Goods—The labeling of shipments classified as *Dangerous Goods*, including all information on the bill of lading or airway bill, must follow the instructions provided by the DOT, the International Air Transport Association

(IATA), and the carrier. The exterior package must carry all of the required standard symbols for the class of goods, and the shipping container must comply with the performance standards for the articles enclosed. The shipper of record is responsible for compliance with the Dangerous Goods requirements.

Controlled Substances—When Pharmacopeial preparations that contain DEA-scheduled controlled substances are distributed to a patient directly via the USPS, these articles must be marked and labeled in accordance with USPS Domestic Mail Manual, Regulation Article C023, Section 7.2.

<1178> GOOD REPACKAGING PRACTICES

INTRODUCTION

This chapter is intended to provide guidance to those engaged in repackaging of oral solid drug products; and the chapter provides information to any person who removes drugs from their original container–closure system (new primary package) and repackages them into a different container–closure system for sale and/or for distribution.

This chapter does not apply to pharmacists engaged in dispensing prescription drugs in accordance with state practice of pharmacy. The pharmacist needs to apply

- (1) the principal information provided in the USP general information chapter *Packaging—Unit-of-Use* <1136> and
- (2) other beyond-use date references in the subsection *Expiration Date and Beyond-Use Date* in the *Labeling* section under *General Notices and Requirements*.

DEFINITIONS

(659) *Packaging and Storage Requirements* provides definitions related to repackaging. For the purposes of this chapter, a repackager, a contract packager, and an equivalent container–closure system are defined as follows:

1. **Repackager**—A repackager is an establishment that repackages drugs and sends them to a second location in anticipation of a need. Repackaging firms repackage preparations for distribution (e.g., for resale to distributors, hospitals, or other pharmacies), a function that is beyond the regular practice of a pharmacy. Distribution is not patient-specific in that there are no prescriptions. Unlike dispensers, repackaging firms are required to register with the FDA and to comply with the Current Good Manufacturing Practice Regulations in 21 CFR 210 and 211.
2. **Contract Packager**—A contract packager is an establishment that is contracted to package or repack a drug product into a single- or multi-unit container. These containers should meet all of the applicable requirements in this chapter. A contract packager does not take ownership from the manufacturer and generally receives the assigned expiration date from the contractor.
3. **Equivalent Container–Closure System**—This term refers to a container–closure system that is at least as protective or more protective than the original container–closure system in terms of moisture vapor transmission rate (MVTR), oxygen transmission, light transmission, and compatibility of the container–

closure system with the drug product. System equivalency extends to any special protective materials, such as for light transmission, seals, or desiccants associated with the original container–closure system. These values may be determined by the repackager, or they may be obtained from the container–closure vendor for the specific container–closure system under consideration.

ESTABLISHING EXPIRATION DATE

In the absence of stability data, the following criteria should be considered by repackagers when assigning an expiration date.

Unit-Dose Packaging

1. The original container–closure system of the drug product to be used for repackaging must be received un-opened and show no outward signs of having been previously opened.
2. The unit-dose container–closure system must meet the testing requirements under *Containers—Performance Testing* (671) for either *Class A* or *Class B* containers.
3. The contents of the original bulk drug product to be repackaged are repackaged at one time unless the repackager has data and/or other scientific information from literature sources demonstrating that the drug product is not sensitive to exposure to moisture, oxygen, or light.
4. The unit-dose container–closure system must meet or exceed the original container's specification for light resistance.
5. The conditions of storage must meet the storage specifications provided in the *USP General Notices* and as described in the labeling of the original container–closure system received for repackaging. Where no specific storage conditions are specified, the product must be maintained at controlled room temperature and in a dry place during the repackaging process, including storage.
6. The expiration dating period used for the repackaged product does not exceed (1) 6 months from the date of repackaging; or (2) the manufacturer's expiration date; or (3) 25% of the time between the date of repackaging and the expiration date shown on the manufacturer's bulk article container of the drug being repackaged, whichever is earlier.
7. Nitroglycerin Sublingual Tablets or any other drug product known to have stability problems should not be repackaged. This would include any drug known to be oxygen-sensitive or one that exhibits extreme moisture or light sensitivity. In deciding whether a particular drug product is suitable for repackaging, the repackager should take into consideration any available information from the manufacturer, published literature, the USP, and the FDA.
8. Documentation must be maintained to demonstrate that the preceding criteria are met.
9. Documentation must be maintained that specifies the container–closure packaging material used in repackaging operations.
2. The original container–closure system of the drug product to be used for repackaging must be received un-opened and shows no outward signs of having been previously opened.
3. The contents of the original bulk drug product to be repackaged are repackaged at one time unless the repackager has data and/or other scientific information from literature sources demonstrating that the drug product is not sensitive to exposure to moisture, oxygen, or light.
4. The conditions of storage meet the storage specifications in the *USP General Notices* and as described in the labeling of the original container–closure system received for repackaging. When no specific storage conditions are specified, the product should be maintained at controlled room temperature and in a dry place during repackaging operations.
5. The type of container–closure system used for repackaging must be at least as protective or more protective than the original container–closure system in terms of moisture vapor transmission rate (MVTR), oxygen transmission, light transmission, and compatibility of the container–closure system with the drug product. System equivalency extends to any special protective materials, such as for light transmission, seals, or desiccants associated with the original container–closure system.
6. The container–closure system must meet or exceed the original container–closure system's results for light transmission.
7. Nitroglycerin Sublingual Tablets or any other drug product known to have stability problems should not be repackaged. This would include any drug known to be oxygen-sensitive or one that exhibits extreme moisture or light sensitivity. In deciding whether a particular drug product is suitable for repackaging, the repackager should take into consideration any available information from the manufacturer, published literature, the USP, and the FDA.
8. Documentation must be maintained to demonstrate that the preceding criteria are met.
9. Documentation must be maintained that specifies the container–closure packaging material used in repackaging operations.

REFERENCES FOR REPACKAGING REGULATIONS AND GUIDANCES

The references listed below are not meant to be all inclusive; specific repackaging operations may have additional requirements.

- **Food, Drug, and Cosmetic Act**
- **Food and Drug Administration Regulations and Guidances**
 - Enforcement Policy*: 21 CFR, Part 7
 - General Labeling Provisions*: 21 CFR, Part 201, Subpart A
 - Drug Establishment Registration and Listing*: 21 CFR, Part 207.20
 - Current Good Manufacturing Regulations*: 21 CFR, Parts 210–211
 - Special Requirements for Specific Human Drugs*: 21 CFR, Part 250
 - Controlled Substances*: 21 CFR, Part 1300
 - Potable Water*: 40 CFR, Part 141
 - FDA Compliance Policy Guides*, including the following:
 - Sub Chapter 430 Labeling and Repackaging
 - Sub Chapter 460 Pharmacy Issues
 - Sub Chapter 480 Stability/Expiration Dating
- **Applicable USP Chapters**
 - (660) *Containers—Glass*
 - (661) *Containers—Plastics*
 - (671) *Containers—Performance Testing*
 - (1079) *Good Storage and Shipping Practices*
 - (1136) *Packaging—Unit-of-Use*

Multiple-Unit Packaging

1. A repackager may use the manufacturer's original expiration date without additional stability testing if the drug product is repackaged into an equivalent container–closure system that is at least as protective as, or more protective than, the original system and complies with criteria established for equivalency.

(1180) HUMAN PLASMA

SCOPE

This chapter provides a consolidated source of information regarding human plasma, with emphasis on plasma for fractionation. Specifically, the chapter addresses plasma classification and nomenclature; collection and processing procedures required for ensuring product safety; details of specific plasmas; and quality systems relating to plasma collection. The chapter also includes, at the end of the text sections, a glossary; a list of abbreviations used in the chapter; and appendices that provide plasma definitions, donor selection criteria, and testing requirements.

Plasma originating from U.S.-licensed collection facilities provides the major supply for the global plasma derivative market. The U.S. Food and Drug Administration (FDA) regulates the collection and processing of plasma used for further manufacture. Title 21 of the Code of Federal Regulations (CFR) details Good Manufacturing Practice (GMP) requirements and product standards to protect the health of the blood donor and to ensure the safety and efficacy of blood products. CFR regulations are updated periodically, but between revisions, existing regulations may not address the most current issues and scientific developments. Therefore, the FDA periodically publishes guidance documents.¹ (For further information, see *Quality Systems*, below).

This chapter emphasizes U.S. practices, but because plasma and its derivatives are shipped globally, it is important to recognize that there are regional differences in recommendations, requirements, and regulations. Therefore this chapter provides information regarding the European Union (EU), the United Kingdom (UK), and Australia.

OVERVIEW

Composition of Plasma

Plasma constitutes approximately 55% of the total blood volume. It is a clear, straw-colored, complex liquid that is 7% protein, 91% water, and 0.9% mineral salts. The majority (approximately 70%) of total plasma protein is albumin. Additional plasma proteins relevant to fractionation include immunoglobulins, coagulation factors, fibrinolytic proteins, proteases, and protease inhibitors. These constituent plasma proteins can be isolated on the basis of the different solubility characteristics of each protein when subjected to specific conditions of pH, temperature, ionic strength, and ethanol concentration. The major products derived from fractionation are listed in *Table 1*.

Table 1. The Major Fractions and Products from the Cohn Process

Fraction	Product
Cryoprecipitate	Antihemophilic factor (FVIII)
Cryosupernatant	Antithrombin III, factor IX complex
Fraction I	Fibrinogen, factor XIII
Fraction II	Immune globulin G (IgG)
Fraction III	IgA, IgM, prothrombin, plasminogen
Fraction IV-1	Factor IX complex, activated factor IX complex
Fraction IV-4	Plasma protein fraction, alpha-1 proteinase inhibitor
Fraction V	Albumin

¹FDA. Blood Guidances. www.fda.gov/cber/blood/bldguid.htm. FDA Memoranda to Blood Establishments available at www.fda.gov/cber/memo.htm.

Plasma for Manufacture of Derivative Products

The two methods for collection of human plasma are automated apheresis (for definitions, see the *Glossary* preceding the appendices) and centrifugation of whole blood donations. Source Plasma collected by apheresis constitutes the majority of plasma used in the manufacture of plasma derivatives in the United States. Plasma collected for transfusion but not so used (i.e., recovered plasma) also may be used for manufacture. Flow charts delineating how apheresis and whole blood-derived plasma can be used in the manufacture of plasma derivatives in the United States and Europe are presented in *Figures 1* and *2*, respectively.

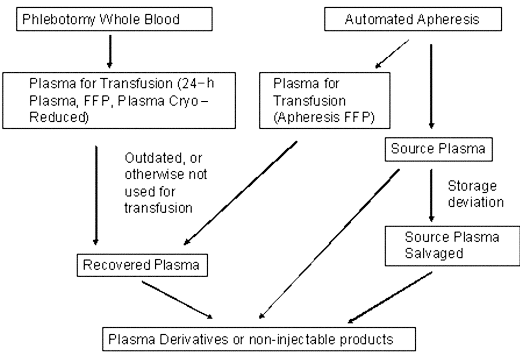


Figure 1. U.S. plasma derivative manufacture: FDA standards.

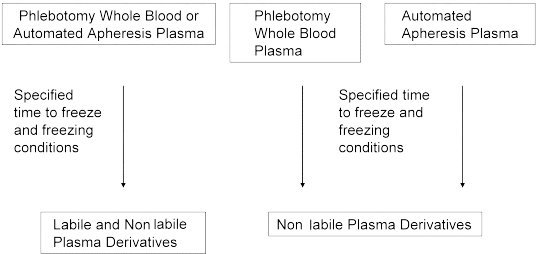


Figure 2. EU plasma derivative manufacture: EU standards.

Regardless of the collection method, plasma for fractionation should be a clear to slightly turbid liquid without visible sign of hemolysis; it may vary in color from light yellow to green; it should be $\pm 10\%$ of the stated volume; and it should show no sign of clots.

Source Plasma

Licensed Source Plasma may be manufactured only in collection centers that are approved by the FDA for the collection and distribution of Source Plasma in interstate commerce. Currently, federal regulations governing the manufacture of Source Plasma, including minimal requirements for donors, are found in 21 CFR 640, Subpart G. By definition, Source Plasma is plasma intended for further manufacture. Source Plasma donors can donate as often as twice a week and may be compensated. In addition to FDA requirements, most plasma collectors and fractionators also comply with voluntary standards established by the Plasma Protein Therapeutics Association (PPTA), a trade and standards-setting organization.² PPTA voluntary standards address several areas of donor, plasma unit, plasma pool, and

²PPTA. www.pptaglobal.org.

center management, and are designed to supplement existing regulatory requirements.

Plasma for Transfusion

Plasma for transfusion is not intended for further manufacture but for direct transfusion to patients. It may be collected by either whole blood or apheresis donation. In the United States, plasma for transfusion comes from unpaid volunteer donors. Blood collecting facilities that collect plasma for transfusion typically comply with requirements of both the CFR and a voluntary trade organization, the American Association of Blood Banks (AABB). Currently, sections 640.3 and 640.31 of 21 CFR outline requirements for donors of whole blood and therefore govern most donors of plasma for transfusion, regardless of the collection method. AABB voluntary standards include information contained in FDA regulations and guidance pertaining to blood and plasma, as well as additional standards.³ Plasma for transfusion may be converted to recovered plasma, an unlicensed product that may be used for further manufacture.

Plasma for Ancillary Use in Biologics Manufacturing

Human plasma and its derivatives are used in the manufacture of other biologic products. In this role, the plasma or plasma derivative falls into the category of ancillary use. This is defined as use of a reagent or material as a processing or purification aid or a reagent that exerts an effect on the therapeutic substance but is not intended to be part of the final product formulation (see the USP general information chapter *Ancillary Materials for Cell, Gene, and Tissue-Engineered Products* (1043)).

Human plasma is commonly used in manufacturing processes that involve primary human cells or cell lines intended for therapeutic applications. In these applications, plasma provides a source of protein and possibly other factors that enhance expansion and differentiation of cell populations. A variety of methods have been used to prepare human plasma and derivatives for ancillary use, but the practices are not standardized. Allogeneic plasma typically is obtained from either apheresis or whole blood, using citrate anticoagulation. Allogeneic plasma typically is collected from paid donors who, like Source Plasma donors, have been screened for the absence of transfusion-transmissible diseases. Preferred donors may be blood type group AB, because they lack anti-A and anti-B isohemagglutinins. Other preferred donors include untransfused males, because this group is unlikely to have human leukocyte antigen (HLA) antibodies that could react with cells in a given culture system. Serum is prepared either from nonanticoagulated whole blood that has been allowed to clot or by the addition of calcium to plasma obtained from citrated whole blood or apheresis. Heating plasma or serum to 56° inactivates heat-labile complement and other proteins.

Although there are no standardized specifications for plasma products used as ancillary materials, assays often include safety testing associated with general biologics (e.g., bacterial/fungal cultures, endotoxin, and mycoplasma). In addition, characterization of the products may include tests for irregular erythrocyte and HLA antibodies, osmolality, pH, total protein and immunoglobulin concentrations, hemoglobin concentration, and chemistries such as Na, K, Cl, Ca, and glucose.

In some instances, plasma from bovine sources has been used instead of human plasma. The differences between bovine and human plasma products include factors relevant to their efficacy as ancillary materials, as well as safety of the final manufactured product when administered to humans (see the USP general information chapter *Bovine Serum*

(1024)). Fetal bovine serum is preferred to human serum for some applications because it may be superior in promoting human cell growth in vitro. Safety considerations, especially the risk of transmissible spongiform encephalopathy and allergic reactions related to antibody antibodies, have led to recommendations that human plasma sources be used whenever possible instead of bovine sources. However, substitution of human for bovine plasma does not completely eliminate the risk of infectious and immunologic sequelae, even when human plasma is used as an ancillary material.

PLASMA COLLECTION AND PROCESSING

This section discusses the standard principles involved in plasma collection and the methods used to ensure the safety of the plasma and subsequently manufactured plasma derivatives. Principles for screening and testing of donors are presented in other parts of this chapter.

Collection

Source Plasma is collected by apheresis. Recovered plasma can be obtained either by apheresis or as a by-product of whole blood collection. Collection should take place via an FDA-approved, closed, sterile, pyrogen-free collection system that contains an anticoagulant. No antibacterial or antifungal agent should be added to the plasma. Donations must be collected aseptically. The skin of the donor must be aseptically prepared. Source Plasma is collected using 4% sodium citrate as the anticoagulant. The composition of sodium citrate is given in Table 2.

Three anticoagulant solutions are licensed in the United States for collection of whole blood: citrate phosphate dextrose (CPD), citrate phosphate double dextrose (CP2D), and citrate phosphate dextrose adenine (CPDA-1). The composition of blood collection bags containing these anticoagulants is shown in Table 3. Plasma for transfusion or further manufacture can be made from a unit of whole blood collected in any of the three anticoagulant solutions. Regulations relating to plasma make no distinction among the three anticoagulant solutions. Consequently, collection, storage, and transport requirements are identical regardless of the anticoagulant solution used in the primary collection.

Table 2. Anticoagulant Solution for Collection of Source Plasma by Apheresis (4% Sodium Citrate)

Volume	Sodium Citrate Dihydrate	Citric Acid Anhydrous	pH (25°)	Ratio of Solution to Whole Blood
250 mL or 500 mL	40 g/L	As required for pH adjustment	6.4–7.5	1:16

Table 3. Anticoagulant Solutions Used during Whole Blood Collection for Recovery of Plasma (500-mL collection bags)

Anticoagulant	CPD ^a	CP2D ^b	CPDA-1 ^c
Volume (mL)	70	63	63
Dextrose (mg)	1780	3220	2010

Note: Collection of Source Plasma typically involves the use of sodium citrate as the anticoagulant. The specification for sodium citrate is given in Table 2. Plasma for transfusion is stored at 2° to 8° after collection. Plasma collected by apheresis should be frozen immediately at –18° or colder.

^aCitrate phosphate dextrose.
^bCitrate phosphate double dextrose.
^cCitrate phosphate dextrose adenine.

³AABB. *Standards for Blood Banks and Transfusion Services*. 25th ed. Bethesda, MD: AABB; 2008.

Table 3. Anticoagulant Solutions Used during Whole Blood Collection for Recovery of Plasma (500-mL collection bags) (Continued)

Anticoagulant	CPD ^a	CP2D ^b	CPDA-1 ^c
Sodium citrate dihydrate (mg)	1840	1660	1660
Citric acid anhydrous (mg)	209	206	206
Monobasic sodium phosphate (mg)	155	140	140
Adenine (mg)	—	—	17.3
pH (25°)	5.3–5.9	5.3–5.9	5.3–5.9
Ratio of solution to whole blood	1.4:10	1.4:10	1.4:10

Note: Collection of Source Plasma typically involves the use of sodium citrate as the anticoagulant. The specification for sodium citrate is given in Table 2. Plasma for transfusion is stored at 2° to 8° after collection. Plasma collected by apheresis should be frozen immediately at –18° or colder.

^aCitrate phosphate dextrose.

^bCitrate phosphate double dextrose.

^cCitrate phosphate dextrose adenine.

Labeling

The labeling for Source Plasma should comply with 21 CFR 640.70 and 21 CFR 640.69(b). The labeling for whole blood should comply with 21 CFR 606.121 and 606.122, and with internal licenses.

A unique identification number is assigned so that the donation can be related to the individual donor records and test results. The origin of each donation in a plasma pool and the results of the corresponding donation and laboratory tests must be traceable while the required degree of confidentiality concerning the donor's identity is maintained. Whole blood must be labeled "This Product may transmit infectious agents" [21 CFR 121(c)(9)]. Source Plasma or recovered plasma must be labeled "Caution: For Manufacturing Use Only" if the product is intended for use in fractionation. For plasma to be used as a reagent or for in vitro use, the required labeling statement is "Caution: For Use in Manufacturing Noninjectable Products Only" [21 CFR 121(e)(5)(ii)].

Storage

Plasma for fractionation should be stored at or below –20°. The plasma can still be used for fractionation if its temperature exceeds –20° on (at most) one occasion for not more than 72 hours and if the plasma has been maintained at a temperature of –5° or lower at all times. Storage temperatures must be maintained during transport.

PLASMA SAFETY CONSIDERATIONS

Plasma is protected by five overlapping safeguards that the FDA has termed the "Five-Layer Safety Net": donor screening, blood testing, donor deferral, quarantine, and investigation. For guidance in this area, see FDA Publication No. FS 02-1 February 2002.

Voluntary measures that provide an additional margin of safety include recruitment and retention of suitable donors and inventory hold procedures. In the manufacture of plasma-derived products, steps taken for viral clearance are very important for ensuring safety.

None of these measures is sufficient by itself; the safety net is the overlapping combination of the activities.

Donor Screening

The selection of a suitable site for blood and plasma donation activities is a first and very important step to ensure safe donations. Areas with low disease prevalence are preferred as locations for donation centers, thereby reducing the likelihood of collecting plasma from an infected donor.

One of the PPTA voluntary standards is the viral marker standard, which obliges plasma centers to report viral marker rates for human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) in the donor populations. A center's rates are compared to the industry average. Alert limits are set to take into account the number of annual donations in the center. If a center exceeds the limit for any of these viruses or the aggregate of all three, the center must implement corrective actions that will bring the center into compliance with the standard.

Appropriate donor selection helps provide a safe plasma supply. A detailed donor history questionnaire in conjunction with a careful medical examination allows center personnel to recognize unsuitable donors whose behavior puts them at risk for transfusion-transmitted disease or who have underlying medical conditions that preclude donation.

An additional measure put in place by centers that collect Source Plasma is the PPTA National Donor Deferral Registry (NDDR). It lists donors throughout the United States who have been previously deferred from donation (although it provides no information about the reason for deferral). Other countries have different systems depending on their national regulations concerning personal data gathering. Any individual who tests positive for HIV, HBV, or HCV is entered into the national database (the National Donor Deferral Registry) used by all U.S. plasma centers that are certified under the International Quality Plasma Program (IQPP). All individuals who present at a U.S. plasma center for the first time are checked against the NDDR. In this manner, donors who have previously been deferred for positive test results at any participating facility can be identified and rejected quickly. This standard ensures that donors deferred for positive test results do not donate in other facilities.

A voluntary safety initiative, the Qualified Donor Standard, implemented by the plasma fractionation industry, builds on the fact that many plasmapheresis donors contribute plasma frequently. A donor who enters a plasmapheresis center for the first time is called an Applicant Donor, and the first donation is used for further manufacturing only if the donor returns a second time. Potential donors must pass two separate medical screenings and testing for HIV, HBV, and HCV on two different occasions. Only after satisfactory screenings and negative test results does that person become a Qualified Donor. If a donor does not return within 6 months, that person loses his/her Qualified Donor status and must qualify again. This standard means that plasma from a one-time-only donor (even when all test results are negative) cannot be used for further manufacture. This standard results in committed donors and eliminates the risk that plasma centers will accept so-called test seekers. The interval between permitted donations of whole blood is too long to allow a similar screening program, although quite a number of donors in whole-blood donor centers are regular and repetitive donors.

Another PPTA voluntary standard addresses donor management criteria. The Community-Based Donor Standard allows only donors who permanently reside within its defined donor recruitment area to donate at a given center. In addition, a Donor Education Standard requires new donors to engage in an educational program and follow-up assessment regarding HIV/acquired immune deficiency syndrome (AIDS) and activities that place them at risk for HIV/AIDS.

In addition to donor management strategies and standards, PPTA has issued a plasma unit management standard called Inventory Hold. This standard states that collected

plasma will be held in inventory for at least 60 days from the time of collection. This allows the retrieval of units as a result of post-donation information (information that was not known at the time of donation) that would have disqualified the donor. This information could include admitting high-risk behavior; becoming reactive for HIV, HBV, or HCV; or providing incorrect information about international travel.

Blood Testing

Testing of donations is an important safety measure both for plasma intended for transfusion and plasma intended for further manufacturing. Both enzyme-linked immunosorbent assays (ELISA) and nucleic acid amplification technologies (NAT) are used to screen donations for the presence of infectious disease. Automation provides the necessary throughput to screen every donation for a variety of potential pathogens.

Testing strategies differ depending on whether the plasma donation is intended for transfusion or further manufacture. Plasma for transfusion requires more extensive infectious disease testing, because there are no pathogen inactivation/removal technologies licensed for this product in the United States. On the other hand, plasma for further manufacture is subjected to several pathogen inactivation/removal steps during manufacture, thereby obviating the need for some disease testing. Table 4 outlines current infectious disease tests required by the FDA for plasma donations collected in the United States. Appendix 3 compares EU and U.S. disease testing and donor deferral requirements.

Table 4. FDA Disease Test Requirements for Plasma for Transfusion and Plasma for Further Manufacturing

Disease	Plasma for Transfusion	Plasma for Further Manufacturing ^a
Hepatitis B	Hepatitis B surface Antigen (HBsAg) Hepatitis B core antibody	HBsAg
Hepatitis C	Anti-HCV HCV RNA	Anti-HCV HCV RNA
HIV	Anti-HIV I/II HIV RNA	Anti-HIV I/II HIV RNA
Human T-lymphotropic virus (HTLV) I/II	Anti-HTLV I/II	Not required
Syphilis	Serologic test for syphilis, every donation	Serologic test for syphilis, every 4 months for donors only
West Nile virus (WNV) ^b	WNV RNA	Not required

^aThe FDA also encourages in-process NAT testing for parvovirus B19 and hepatitis A. HBV NAT testing also is performed on most Source Plasma.
^bTesting for WNV is recommended in an FDA draft guidance. The FDA is considering recommendations regarding testing for *Trypanosoma cruzi* (Chagas disease).

NAT, of which polymerase chain reaction (PCR) is the most widely used form, does not rely on the detection of antibodies produced by the infected host after exposure, but targets the nucleic acid of the infecting agent. By means of the selection of suitable priming molecules (the so-called primers), the assay is highly specific for the infecting virus (see the USP general information chapter *Nucleic Acid-Based Techniques—Amplification* (1127)). Through several cycles of amplification, the polymerase enzyme can repetitively generate copies of the targeted fragment of the viral nucleic acid, providing an exponential amplification of a very short

stretch of the viral deoxyribonucleic acid (DNA) [or ribonucleic acid (RNA)]. The exponential amplification leads to the generation of many copies of the target molecule and allows the subsequent detection of this virus-specific fragment, even if the original viral load was exceedingly low. This methodology has brought a new degree of safety.

NAT testing, because of its complexity and expense, is difficult to conduct on individual donations. Generally, aliquots from several donations are combined into a single pool, often called a minipool. Testing in pooled format remains more sensitive than serological ELISA screening of individual donations. In addition, the NAT principle circumvents several of the limitations in detecting pathogens by means of serological methods. Pooling can influence overall sensitivity, depending on the pool size and the analytical sensitivity of the NAT assay employed.

In many countries, the maximal load of a pathogen acceptable for a single donation defines the overall NAT sensitivity required. Assays of higher analytical sensitivity can use larger pools, but those of lower analytical sensitivity must test smaller pools in order to comply with regulations. The availability of commercial NAT test kits with defined analytical sensitivity has made minipools up to 512 very common, because these pool sizes, in combination with the analytical sensitivity of the assays used, comply with common regulations on overall sensitivity.

Effective NAT screening requires that the viral load of the plasma pool at the beginning of production be less than the inactivation and/or removal capacity of the process. Differences between the plasma transfusion and fractionation industries have led to different applications of NAT. For individual donations intended for transfusion, where there is no inactivation and/or removal process and where testing is the only method to interdict a contaminated donation, the safety of each individual donation must be ensured by testing with the most sensitive assays possible. Plasma intended for further manufacturing, in contrast, is pooled and serves as the starting material for a multistep process that has built-in pathogen inactivation methodologies. Therefore, NAT screening for plasma for further manufacture is focused on ensuring safe donations and limiting the viral load of the plasma pool to levels less than the known viral inactivation/removal capacity of the inactivation process.

To avoid the loss of large amounts of plasma from a reactive pool, the fractionation industry has implemented a prescreening strategy, the minipool screening concept mentioned earlier. Aliquots of plasma donations are combined to form minipools, and the minipools are tested by NAT. If a minipool is reactive for a virus tested, the individual donation that gave rise to this positive result can be identified and interdicted. The other donations demonstrated to be free of infection can be used for further manufacturing. The donations are then combined into a production pool, a sample of which is subjected to NAT testing as required by regulations.

As indicated, the NAT test portfolio is not uniform and depends on the intended use of the donation and the regulatory environment. Although screening for HCV RNA is done in most countries, screening for HIV is not universally required. NAT detection of HBV is used mainly for plasma for manufacturing. Screening for B19 virus or hepatitis A virus (HAV) is performed only on plasma for manufacturing. HBV screening using NAT detection is more widespread in European and Asian countries than in the United States.

Donor Deferral

A donor may be deferred from further donation as a result of answers provided on the donor history questionnaire, counseling of donors for reasons for deferral, a medical examination performed at the time of donation, or positive tests for infectious diseases. These processes ensure both the eligibility of the donor and the suitability of the donation. In the event that either is not acceptable, standard processes

permanently remove the donor and interdict unused units previously donated. The donor registries mentioned earlier are one means of ensuring that a donor deferred at one center cannot donate elsewhere.

Quarantine and Inventory Hold

Each individual unit of plasma, whether for transfusion or further manufacture, is held in quarantine until all the required tests have been completed. If all required tests have been performed and found acceptable, the unit can be released; if not, the unit must be destroyed. The plasma industry has voluntarily implemented the inventory hold protocol (also discussed in the previous section *Donor Screening*) for plasma for further manufacture. According to inventory hold requirements, during a 60-day hold period an individual plasma donation cannot be used for further manufacture. The rationale for the hold is that donors who have been recently infected with a pathogen may not have developed levels of antibody at the time of donation, thereby donating an infectious unit despite negative disease tests. The hold provides sufficient time for an infectious donor to develop levels of antibody that will be detected during a subsequent donation if the plasma was intended for transfusion. The 60-day hold also reduces the chance of releasing an infectious unit into the manufacturing process.

The introduction of NAT may have decreased the need for inventory hold, because NAT targets the infecting virus directly and thus does not rely on the time-delayed production of antibodies. Because NAT cannot detect all viruses and because even NAT has a certain (although very low) limit of detection, inventory hold is still of value and thus remains in place in the plasma fractionation industry.

Investigation

Each plasma donation must be traceable from donation to ultimate disposition in order to minimize the potential transmission of an infectious agent. Traceability encompasses all data concerning donation site, donor identifying information, test results, and data regarding transport, storage, and consignee(s).

Look-back is a process to identify and interdict (quarantine) previous donations from a donor who, at a subsequent donation event, has been found to be (1) infected with a transmissible agent or (2) unsuitable for donating plasma because of history, physical examination, or post-donation information. Although look-back strategies are similar in most countries, specific procedures may vary.

QUALITY SYSTEMS

The intent of this section is to outline the general principles and regulations that are the basis of quality systems relating to plasma collection. U.S. collection centers must follow cGMPs that originate in CFR and are elaborated in FDA regulations, guidance documents, and industry standards.

The GMP regulations specifically governing plasma are found in 21 CFR 600, Biological Products: General; and 606, Current Good Manufacturing Practice (cGMP) for Blood and Blood Components. More general cGMP regulations are found in 21 CFR 210, Current Good Manufacturing Practice in Manufacturing, Processing, Packing, or General Holding of Drugs, General; and 21 CFR 211, Current Good Manufacturing Practice for Finished Pharmaceuticals. Although quality systems regulations are part of 21 CFR for medical devices, they have been extended to other manufacturing as part of the "c" in cGMP and FDA Guidance documents.⁴

⁴FDA. Guidance for Industry: Quality Systems Approach to Pharmaceutical cGMP Regulations. 2006. Available at www.fda.gov/cber/gdlns/qualsystem.htm.

The quality system is divided into four major parts: management responsibility, resources, manufacturing operations, and evaluation activities. These are the foundation of the five manufacturing systems: production, facilities and equipment, laboratory control, materials, and packaging and labeling. The procedures of each system are designed to allow operations that facilitate implementation of cGMP requirements. In many instances, these requirements relate to providing facilities and expertise to achieve the requirements for the Five-Layer Safety Net (donor screening, blood testing, donor deferral, quarantine, and investigation), discussed above.

Management responsibilities include providing leadership; building a quality system for the organization that meets requirements; establishing policies, objectives, and plans; and reviewing the quality systems with defined frequency. Resources include having sufficient resources for operational activities, personnel development plans, adequate facilities, and suitable equipment; and controlling outsourced operations. Manufacturing includes designing, developing, and documenting product and processes, performing and monitoring operations, and addressing nonconformities. Evaluation activities include analyzing data for trends, conducting internal audits, and initiating corrective and preventive actions.

A number of required routine activities related to collection and release of plasma or normal recovered plasma from whole blood are linked to both cGMP guidelines and quality systems. These include the requirement for having SOPs to cover all aspects of collection, testing, and release. It is also necessary to validate equipment and systems used by the collection center, including temperature-controlled areas, laboratory equipment, water systems, and computer systems. The design and operation of the facility must be adequate to perform the tasks at hand and prevent cross-contamination. Plasma collection facilities must have an adequate number of knowledgeable and trained staff as well as procedures for acceptance and release of raw materials. To the extent possible, collection facilities must adhere to the GMP requirements for a pharmaceutical manufacturing facility.

GLOSSARY

Apheresis—A method of obtaining one or more blood components by machine processing of whole blood; the residual components of the blood are returned to the donor during or at the end of the process.

Blood component—A constituent of human blood: red cells, white cells, platelets, or plasma.

Blood establishment—Any structure or organization responsible for any aspect of the collection and testing of human blood or blood components, whatever their intended purpose, and their processing, storage, and distribution. Hospital transfusion services engaged only in compatibility testing and transfusion of blood and blood products are not included within the definition of blood establishment.

Blood product—Any therapeutic product derived from human blood or plasma.

Center—Collection site or location where blood or plasma is collected (and also may be processed and stored). *Center* is also applicable to a testing *Site* (see entry in this glossary).

Cryoprecipitate—A plasma component prepared from fresh-frozen plasma by freeze-thaw precipitation of proteins and subsequent concentration and resuspension of the precipitated proteins in a small volume of the plasma.

Deferral—Temporary or permanent suspension of the eligibility of an individual to donate blood or blood components.

Distribution—The act of delivery of blood and blood components to other blood establishments, hospital blood banks, and manufacturers of blood products.

Manufacturing pool—A combination of a specified number of plasma donations used as the first step in the manufacture of plasma derivatives.

Donation minipool—A combination of a small number of units or samples representative of donations used for pretesting prior to pooling units for manufacture.

Quarantine—The physical isolation of blood components or incoming materials/reagents over a variable period of time while awaiting acceptance, issuance, or rejection of the blood components or incoming material/reagents.

Site—Any location at which a blood establishment carries out blood collection, not including any location not owned or managed by the blood establishment at which blood is collected or any mobile blood collection unit.

Validation—The establishment of documented and objective evidence that the particular requirements for a specific intended use can be consistently fulfilled.

ABBREVIATIONS

AABB	American Association of Blood Banks
AIDS	Acquired immune deficiency syndrome
CFR	Code of Federal Regulations
CJD	Creutzfeldt-Jakob disease
CMV	Cytomegalovirus
CNS	Central nervous system
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EIA	Enzyme immunoassay
EU	European Union
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFP	Fresh-frozen plasma
FP24	Plasma frozen within 24 hours after phlebotomy
cGMP	Current Good Manufacturing Practice
HAV	Hepatitis A virus

ABBREVIATIONS (Continued)

HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HCT	Hematocrit
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HTLV	Human T-lymphotropic virus
IgA, IgG, IgM	Immunoglobulins A, G, and M, respectively
IQPP	International Quality Plasma Program
IU	International Unit
NAT	Nucleic acid amplification technology
NDDR	National Donor Deferral Registry
PCR	Polymerase chain reaction
PPTA	Plasma Protein Therapeutics Association
PRP	Platelet-rich plasma
RNA	Ribonucleic acid
SARS	Severe acute respiratory syndrome
TEP	Therapeutic exchange plasma
WBDP	Whole blood-derived plasma
WNV	West Nile virus

APPENDICES

Appendix 1

NOTE—The collection, processing, and uses of plasma have generated a large number of terms and definitions that reflect the diversity of operations. In addition to the FDA standards and terms, industrywide voluntary standards are discussed in the *Plasma Safety Considerations* section. Advisory Note: These terms are not meant as regulatory definitions, because plasma term definitions can vary from region to region and among industry sectors. The reader is advised to consult with regulatory authorities responsible for the region and industry sector. Often specific process variables must be considered.

Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions

Plasma Type and Agency or Agency Type	Specification
Recovered plasma CFR AABB Inter-region, Inter-sector	<p>Plasma derived from single units of whole blood as a by-product in the preparation of blood components from whole blood collection and intended for further manufacturing. Compliance Policy Guides Manual (CPG 71.34.12), Sec. 230.100.</p> <p>Plasma for use in manufacturing and prepared from allogenic donations. Plasma selected for manufacture that has been collected from whole blood or apheresis plasma collected for transfusion that has expired.</p> <p>Plasma separated from whole blood most often by manual centrifugation or by apheresis. The priority for the blood collected is usually for the production of red blood cells. However, the plasma can be suitable for further manufacture of biotherapeutics and transfusion. The time from collection to freezing can vary depending on the distance of collection and processing sites. Volunteer donors typically are used.</p>
Source Plasma CFR Inter-region, Inter-sector	<p>Fluid portion of human blood collected by plasmapheresis and intended as source material for further manufacturing use (21 CFR 640.60).</p> <p>Plasma separated from whole blood by plasmapheresis where the cellular components can be returned to the donor. The priority for the plasma usually is for further manufacture of biotherapeutic products. However, the plasma can be suitable for transfusion. It is rapidly frozen after collection.</p>
Fresh-frozen plasma (FFP) CFR AABB Inter-region, Inter-sector Council of Europe UK Australia	<p>Fresh-frozen plasma shall be prepared from blood collected by a single uninterrupted venipuncture with minimal damage to and minimal manipulation of the donor's tissue. The plasma shall be separated from the red blood cells and placed in a freezer within 8 hours or within the timeframe specified in the directions for use for the blood collecting, processing, and storage system and stored at -18° or colder [21 CFR 640.34(b)].</p> <p>Plasma separated from the blood of an individual donor and placed at -18° or colder within 6 to 8 hours of collection from the donor or within the timeframe specified by the manufacturer's instructions.</p> <p>Plasma that is collected and frozen quickly after preparation. Transfusion is the primary intended use. However, FFP can be suitable for further manufacture of biotherapeutic products.</p> <p>A component for transfusion or for fractionation prepared either from whole blood or from plasma collected by apheresis, frozen within a period of time and to a temperature that will adequately maintain labile coagulation factors in a functional state (Chapter 21).</p> <p>Supernatant plasma separated from a whole blood donation or plasma collected by apheresis, frozen, and stored.</p> <p>Plasma, Fresh Frozen is a component for transfusion or for fractionation prepared either from whole blood or from plasma collected by apheresis, frozen within a period of time and to a temperature that will adequately maintain the labile coagulation factors in a functional state. If prepared from whole blood, it should preferably be recovered within 6 hours, and not more than 18 hours after collection if the unit has been refrigerated. Plasma may also be collected up to 24 hours if the collected blood has been immediately cooled and maintained at 20°–24°. Separated plasma must be frozen to below -30° within one hour. Freezing of plasma collected by apheresis, as above, must commence within 6 hours of collection or within 24 hours if the collected blood has been immediately cooled and maintained at 20°–24°.</p>
Concurrent plasma Inter-region, Inter-sector	<p>Plasma collected concurrently with cellular components. Concurrent plasma may be suitable for transfusion or for further manufacture of biotherapeutics.</p>
Applicant Donor Inter-region, Inter-sector	<p>Source Plasma obtained during the first collection from a new donor. The plasma is reserved for testing, and any remainder or products derived from the remainder are not allowed for use in humans or are quarantined until the donor passes appropriate tests and returns for a second donation which also clears testing. At that time, both collections are reclassified as "Qualified".</p>
Platelet-rich plasma (PRP) CFR	<p>PRP shall be prepared from blood collected by a single uninterrupted venipuncture with minimal damage to and manipulation of the donor's tissue. The plasma shall be separated from the red blood cells by centrifugation within 4 hours after completion of the phlebotomy or within the timeframe specified in the directions for use for the blood collecting, processing, and storage system. The time and speed of the centrifugation shall have been shown to produce a product with at least 250,000 platelets per μL. The plasma shall be stored at a temperature between 20° and 24° immediately after filling the final container. A gentle and continuous agitation of the product shall be maintained throughout the storage period if stored at a temperature of 20° to 24° [21 CFR 640.34(d)].</p>

Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions (Continued)

Plasma Type and Agency or Agency Type	Specification
Inter-region, Inter-sector	Plasma that is a product of the first centrifugation of blood where it is separated from red cells. Platelets are fractionated into the plasma layer.
Platelet-poor plasma Inter-region, Inter-sector	Plasma that is further purified from platelets by a second centrifugation of PRP.
Cryo-poor plasma CFR	Plasma that remains after both platelets and cryoprecipitated AHF have been removed may be labeled "Plasma, Cryoprecipitate Reduced" [21CFR 640.34(e)(2)].
AABB Inter-region, Inter-sector	Plasma Cryoprecipitate Reduced; Fresh-frozen Plasma from which cryoprecipitate has been removed.
Council of Europe	Plasma that has been thawed by maintaining the temperature just above freezing (usually 4°). A large portion of certain plasma proteins (e.g., FVIII, cryoprecipitate, fibrinogen, fibronectin, or FXIII) has been precipitated from the plasma.
UK	Plasma, Fresh-Frozen, Cryoprecipitate-Depleted (Chapter 23). A component prepared from plasma by the removal of cryoprecipitate.
Australia	Plasma cryoprecipitate-depleted for transfusion means a plasma component prepared from a unit of plasma, fresh-frozen. It comprises the residual portion after the cryoprecipitate has been removed.
Cryo-rich plasma Inter-region, Inter-sector	Plasma, Fresh Frozen, Cryoprecipitate-Depleted is a component prepared from Fresh Frozen Plasma by the removal of cryoprecipitate. The content of albumin, immunoglobulin, and most clotting factors is maintained, but the levels of Factors V and VIII and fibrinogen are reduced. It can be stored for up to 36 months at below -25°.
Plasma for labile products Inter-region, Inter-sector	Plasma that has been thawed by gentle heat input (e.g., in a 37° water bath) where the cryoprecipitate remains dissolved.
Plasma for stable products Inter-region, Inter-sector	Plasma that has been collected and best maintains the activity and integrity of labile plasma proteins as exemplified by clotting Factor VIII. Generally the time from collection through processing to freezing is rapid.
Less than 6-hour plasma Inter-region, Inter-sector	Plasma that has been collected where conditions for preservation of labile products was not achieved, however conditions were sufficiently moderate so relatively stable products like IgG and albumin would not be impacted.
6- to 12-hour plasma Inter-region, Inter-sector	This is generally recovered plasma that has been collected, processed, and frozen prior to 6 hours after collection. This plasma generally is considered to be acceptable for the production of labile products.
12- to 24-hour plasma Inter-region, Inter-sector	Generally this is recovered plasma that has been collected, processed, and frozen more than 6 hours and less than 12 hours after collection. This plasma is generally considered to be acceptable for the production of labile products but is inferior to less than 6-hour plasma for this purpose.
Less than 12-hour plasma Inter-region, Inter-sector	Generally this is recovered plasma that has been collected, processed, and frozen more than 12 hours and less than 24 hours after collection. This plasma may be acceptable for the production of labile products but is inferior to less than 6-hour plasma and 6- to 12-hour plasma for this purpose.
More than 24-hour plasma Inter-region, Inter-sector	Generally this is recovered plasma that has been collected, processed, and frozen less than 12 hours after collection. This plasma may be acceptable for the production of labile products but is inferior to less than 6-hour plasma and 6- to 12-hour plasma for this purpose.
Pooled plasma Inter-region, Inter-sector	Generally this is recovered plasma that has been collected, processed, and frozen more than 24 hours after collection and usually less than 72 hours after collection. This plasma generally is not acceptable for the production of labile products.
Single-donor plasma Inter-region, Inter-sector	Plasma that has been pooled for manufacturing from several donors. Some plasma pools for further manufacture of biopharmaceutical products can be derived from several hundred to a few thousand donors.
	Plasma derived from a single donor. It can be a single unit or a pool of several units derived from multiple collections from the same donor.

Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions (Continued)

Plasma Type and Agency or Agency Type	Specification
Hyperimmune plasma Inter-region, Inter-sector	Plasma derived from donors with high titers to specific disease agents. Titers are elevated in these donors mostly as a result of immunization with a vaccine (e.g., hepatitis B, tetanus, or rabies) or exposure to disease agents (e.g., HCV or SARS). Hyperimmune plasma usually is intended for the preparation of IgG to provide passive immunity against target disease agents.
S/D plasma Inter-region, Inter-sector EU (PharmEuropa)	Plasma that has been treated with solvent/detergent, an inactivation method effective against envelope virus disease agents (e.g., HIV, HBV, or HCV). Some plasma protein components are inactivated or damaged by the process (e.g., alpha-1 proteinase inhibitor, Protein S, anti-plasmin, or FVIII). Human Plasma Pooled and Treated for Virus Inactivation is a frozen or freeze-dried, sterile, nonpyrogenic preparation obtained from human plasma derived from donors belonging to the same ABO blood group. The preparation is thawed or reconstituted before use to give a solution for infusion. The human plasma used complies with the monograph on Human Plasma for Fractionation.
Therapeutic exchange plasma (TEP) Inter-region, Inter-sector	Similar to Source Plasma in its collection. However, the donors are patients who are having their plasma replaced with electrolytes, protein solutions, or plasma from another donor. The objective usually is to remove disease elements from the patient's plasma. Generally, TEP is not advisable for further manufacture of biopharmaceutical products. However, there may be cases where a specialty product may propose a specific TEP as a source material.
Quarantine plasma Inter-region, Inter-sector	Plasma that has been collected and not had initial testing completed and/or stored as part of a controlled donor program. The donor is retested for disease agents (e.g., 6 months after collection). If the donor again is negative for the tested disease agents, then the plasma is released for use for further manufacture and/or use in humans.
Quarantine residual plasma Inter-region, Inter-sector	Plasma that has been collected and stored as part of a controlled donor program. The donor was not retested for disease agents (e.g., 6 months after collection). An example would be that the donor did not return to the collection facility to permit the later test. Quarantine Residual Plasma is not recommended for further manufacture and/or use in humans.
Salvaged plasma Inter-region, Inter-sector	Plasma that has experienced a storage or transport temperature deviation but may still be useful for the preparation of nonlabile products such as albumin or IgG.
Plasma for fractionation (Processing requirements) CFR Australia	Placed in a freezer within 8 hours and stored at -18° or colder. The liquid part of human blood remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure. It is intended for the manufacture of plasma-derived products. When the plasma is intended for the recovery of proteins that are labile in plasma, it is frozen rapidly to -25° or below within 24 hours of collection. For the recovery of nonlabile proteins, the plasma should be frozen to -20° or below as soon as possible and at the latest within 72 hours of collection. Frozen plasma is stored and transported in conditions designed to maintain the temperature at or below -20° . For accidental reasons, the storage temperature may rise to above -20° on one or more occasions during storage and transport, but the plasma is nevertheless considered suitable for fractionation if all the following conditions are fulfilled: the total period of time during which the temperature exceeds -20° does not exceed 72 hours; the temperature does not exceed -15° on more than one occasion; the temperature at no time exceeds -5° .

Appendix 1: Plasma Types and Specifications as Assigned by Regulatory Agencies in Selected Jurisdictions (Continued)

Plasma Type and Agency or Agency Type	Specification
EU (PharmEuropa)	<p>Human plasma for fractionation is the liquid part of human blood remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure. It is intended for the manufacture of plasma-derived products.</p> <p>When obtained by plasmapheresis or from whole blood (after separation from cellular elements), plasma intended for the recovery of proteins that are labile in plasma is frozen within 24 hours of collection by cooling rapidly in conditions validated to ensure that a temperature of -25° or below is attained at the core of each plasma unit within 12 hours after it is placed in the freezing apparatus.</p> <p>When obtained by plasmapheresis, plasma intended solely for the recovery of proteins that are not labile in plasma is frozen by cooling rapidly in a chamber at -20° or below as soon as possible and at the latest within 24 hours of collection.</p> <p>When obtained from whole blood, plasma intended solely for the recovery of proteins that are not labile in plasma is separated from cellular elements and is frozen in a chamber at -20° or below as soon as possible and at the latest within 72 hours of collection.</p> <p>Frozen plasma is stored and transported in conditions designed to maintain the temperature at or below -20°. For accidental reasons, the storage temperature may rise to above -20° on one or more occasions during storage and transport, but the plasma is nevertheless considered suitable for fractionation if all the following conditions are fulfilled: the total period of time during which the temperature exceeds -20° does not exceed 72 hours; the temperature does not exceed -15° on more than one occasion; the temperature at no time exceeds -5°.</p>
Plasma frozen within 24 hours after phlebotomy CFR	<p>Plasma manufactured from whole blood should be frozen within 24 hours after phlebotomy. Blood component must be labeled "Plasma Frozen Within 24 Hours after Phlebotomy."</p>

Appendix 2: Donor Criteria

Criterion	Region		
	United States	United Kingdom	Australia
General criteria for blood donation			
Appearance	Donor should appear to be in good health (AABB)	Donor should be in good health	Only donors in good health accepted
Underlying medical conditions	Most serious medical conditions are grounds for deferral under United States, EU, and UK guidelines. FDA guidelines require deferral only if a person has used bovine insulin manufactured from UK cattle. However, AABB guidelines require deferral for cancer, heart, liver, or lung disease, and bleeding tendency unless approved by a medical director. Cancer and cardiac disease and diabetes treated with insulin require permanent deferral under EU and UK guidelines	Most serious medical conditions are grounds for deferral. Cancer: accepted 5 years of remission. Cardiac disease: varies depending on clinical condition. Diabetes: acceptable if controlled.	Nothing specific noted
Age	≥16 or conform to applicable state law (AABB, whole blood)	Between 18 and 65; donation at 17 permitted if in accord with national legislation; first-time donors >60 only if permitted by physician	Whole blood: can start at 16–17 with consent of parents and continue to 80, but medical review required at >70. Apheresis: accept new donors 18–65, with medical evaluation required when >60. Existing donors require annual medical review if >65.
Weight	None stated; no more than 10.5 mL/kg may be withdrawn (AABB) FDA requires Source Plasma donors to weigh at least 110 lb	Whole blood: ≥50 kg Apheresis: no specific weight requirement	<45 kg—defer. Medical opinion required for unexplained weight loss
Blood pressure	Systolic ≤180 mm Hg Diastolic ≤100 mm Hg (AABB)	Systolic ≤180 mm Hg Diastolic ≤100 mm Hg	Acceptable ranges: Systolic 90–180 mm Hg. Diastolic 60–90 mm Hg. Hypertension 180–100: defer. Hypotension 90–60: defer.
Pulse	Between 50 and 100 beats per minute and regular; lower pulses acceptable at discretion of physician (AABB)	Between 50 and 100 beats per minute and regular	Regular pulse between 50 and 100: accept Pulse between 40 and 49: accept if donor is physically fit and not on medication
Temperature	≤37.5° taken orally at time of donation (AABB)	Donors who have had a temperature of ≥38° may not donate for 2 weeks	No requirement

Appendix 2: Donor Criteria (Continued)

Criterion	Region		
	United States	United Kingdom	European Union
HgB/HCT	≥125 g/L or HCT 38%	Males: ≥135 g/L Females: ≥125 g/L	Males: ≥135 g/L Females: ≥125 g/L
Skin examination	Free of infectious skin disease at site of phlebotomy; no skin punctures or scars indicative of addiction to self-injected narcotics (CFR) Free of infectious diseases (AABB)	Skin at venipuncture site should be clear of lesions, including eczema	There should be no skin disease at venipuncture site
Pregnancy	Defer for 6 weeks after delivery (AABB)	Defer 6 months after delivery	Defer for 1 week for every completed week of pregnancy
Underlying medical conditions that require deferral and that do not pose a risk of transfusion-transmissible infection			
Cancer	Permanent deferral unless deemed suitable by medical director (AABB)	Permanent deferral although physician may make exceptions. Permitted after cervical cancer or basal cell carcinoma if successfully treated	Malignant neoplasms, including leukemias and myeloproliferative disorders, are cause for permanent deferral; exceptions may be made for certain conditions after successful therapy
Cardiac disease	Free of major organ disease (heart, liver, and lungs) unless deemed suitable by medical director (AABB)	Permanent deferral for persons with a history of heart disease, especially coronary disease, angina pectoris, severe cardiac arrhythmia, arterial thrombosis, or recurrent venous thrombosis. 2-year deferral for rheumatic heart disease with no evidence of chronic disease	Permanent deferral for persons with active or past serious cardiovascular disease, except congenital abnormalities with complete cure
Cerebrovascular diseases	No specific guideline other than that the donor must be free of major organ disease (AABB)	Permanent deferral for history of cerebrovascular diseases	Permanent deferral for donors with a history of serious CNS disease
Epilepsy	No deferral	Must be free of epileptic attack for 3 years and have been taken off all medication	Permanent deferral unless at least 3 years have elapsed since the date that donor last took anticonvulsant medication and there has been no recurrence of symptoms

Whole blood: females, 120–165 g/L; males, 130–185 g/L.
Apheresis: females, 115–165 g/L; males, 125–185 g/L.
If high, defer for 6 months and test for ferritin.

Avoid venesection where there is evidence of inflammation or infection

Current: defer 9 months from estimated date of confinement.
After third-trimester delivery: defer 9 months.
Miscarriage or termination: defer 3, 6, or 9 months, respectively for 1st, 2nd, and 3rd trimester.

Permanent deferral for haematological malignancies.
Skin cancer—basal-cell carcinoma: accept.
Other cancers: defer 5 years after completion of treatment

Permanent deferral for arrhythmias, endocarditis, ischaemic heart disease, heart surgery, myocardial disease.
Accept: congenital heart disease if surgically corrected.
Heart murmurs: accept, subject to medical opinion.
Accept after full recovery: pericardial disease, rheumatic heart disease.

Permanent deferral

Defer for 2 years from last seizure

Appendix 2: Donor Criteria (Continued)

Criterion	Region			
	United States	United Kingdom	European Union	Australia
Gastrointestinal disease	No specific deferral; free of major organ disease unless deemed suitable by medical director (AABB)	No specific deferral	Deferral (not noted to be a permanent deferral) for disease that renders the individual liable to impaired iron absorption or blood loss	Ulcers: defer indefinitely
Genitourinary and renal disease	No specific deferral. Free of major organ disease unless deemed suitable by medical director (AABB)	Five-year deferral after complete recovery from acute glomerulonephritis	Permanent deferral for donor with serious genitourinary or renal disease	Permanent deferral: chronic pyelonephritis, chronic kidney infection, chronic dialysis. Accept if resolved: haematuria, acute kidney infection. Urinary catheter present: plasma only for fractionation if underlying condition acceptable. Acute dialysis: defer for 12 months. Acute glomerulonephritis: defer 5 years after recovery.
Diabetes	No specific deferral except that receipt of bovine insulin manufactured in the UK requires permanent deferral (FDA). Free of major organ disease unless deemed suitable by medical director	Permanent deferral if insulin therapy required	Permanent deferral for donors on insulin treatment	Permanent deferral if diabetes-associated complications present Accept if disease is controlled, even if patient is taking insulin
Respiratory disease	Free of acute respiratory disease (CFR). Free of major organ disease (lungs) unless deemed suitable by medical director (AABB)	Permanent deferral for chronic bronchitis; common cold acceptable	Permanent deferral for serious disease	Permanent deferral: chronic abscess, bronchiectasis, or emphysema with respiratory insufficiency. Accept if mild and controlled: asthma, bronchiectasis without respiratory insufficiency Plasma for fractionation only: chronic bronchitis. Acute bronchitis: defer 2 weeks after recovery and being off antibiotics for 5 days. Pleurisy, pneumonia: defer 4 weeks after recovery. Acute pulmonary embolism: defer 12 months after recovery
Hematologic disorders	Free of abnormal bleeding tendency unless determined suitable by medical director (AABB) Free of major organ disease (cancer) unless deemed suitable by medical director (AABB)	No specific deferral. Donors who are heterozygous for beta thalassemia eligible if Hgb values are within normal limits	Permanent deferral for donors with serious hematologic diseases	Permanent deferral: donors with serious hematologic diseases (sickle cell disease, thalassemia major). Accept: thalassemia minor. Defer: anaemia Plasma for fractionation only: elliptocytosis, glucose-6-phosphate dehydrogenase (G6PD) deficiency, spherocytosis. Apheresis only permitted for patients with G6PD deficiency

Appendix 2: Donor Criteria (Continued)

Criterion	Region		
	United States	United Kingdom	European Union
Immunologic disorders	No specific deferral. Free of major organ disease unless deemed suitable by medical director (AABB)	Permanent deferral for donors with autoimmune disease that affects more than one organ. Defer: documented history of anaphylaxis	Permanent deferral for donors with serious immunologic diseases
Metabolic disease	No specific deferral. Free of major organ disease unless deemed suitable by medical director (AABB)	No specific deferral	Permanent deferral for serious metabolic disease
Bone disease	No specific deferral. Free of major organ disease unless deemed suitable by medical director (AABB)	Two-year deferral after having been declared cured of osteomyelitis	No specific reference
Surgery	No specific deferral unless blood was transfused; in which case, a 12-month deferral applies (CFR)	Major surgery requires evaluation of risk for transfusion-transmissible disease	Minor (e.g., skin lesions, arthroscopy): defer until recovered. Routine minor (e.g., appendectomy, laparoscopy): defer for 2 months. Major surgery (if donor received autologous blood only): defer 6 months. Neurosurgery: medical assessment required.
Medications that require deferral			
Antibiotics	As defined by medical director (AABB)	Donors treated with any prescribed drug should be deferred for a period consistent with the pharmacokinetic properties of the drug	Defer for 2 weeks from full recovery or 1 week from cessation of antibiotic therapy, whichever is longer
Drugs with teratogenic potential	Etretinate: permanent deferral (FDA). Acitretin: 3-year deferral from last dose (FDA). Dutasteride: 6-month deferral from last dose (FDA). Isotretinoin: 1-month deferral from last dose (FDA). Finasteride: 1-month deferral from last dose (FDA).	Donors treated with drugs with proven teratogenic effect should be deferred for a period consistent with the pharmacokinetic properties of the drug	Raloxifene (Evista): defer for 6 months after completion of treatment. Acitretin (Neotigason): defer for 3 years after completion of treatment. Etretnate (Tigason): permanent deferral. Finasteride (Proscar): 7 days after completion of treatment. Isotretinoin: defer for 8 weeks after completion of treatment.
Growth hormone from human pituitary glands	Permanent deferral (FDA)		Permanent deferral

Appendix 2: Donor Criteria (Continued)

Criterion	Region		
	United States	United Kingdom	European Union
Other drugs	No other deferrals by FDA or AABB. Any other deferral at discretion of medical director of blood center (AABB). Warfarin: 7-day deferral for plasma donation (AABB).	Recommended that a list of commonly used drugs with rules for acceptability of donors, approved by the medical staff of the transfusion center, be available.	Other drugs acceptable as long as the underlying condition for which the drug is taken is acceptable.
Insulin	Permanent deferral: bovine insulin made in UK (FDA).	Permanent deferral if treated with insulin	Permanent deferral if treated with insulin
Immunizations			
Toxoids	No deferral	No deferral	No deferral
Licensed killed bacterial vaccines	No deferral	No deferral	No deferral
Licensed inactivated viral vaccines	No deferral	No deferral	No deferral except 1-week deferral after hepatitis B vaccination
Unlicensed killed vaccines	1-year deferral (AABB)	No deferral	Defer 3 months after vaccination
Inactivated rickettsial vaccines	No deferral	No deferral	
Live attenuated bacterial and viral vaccines	4 weeks for varicella and rubella 2 weeks for rubeola, yellow fever, mumps, polio (oral), typhoid (oral) (AABB)	4 weeks	8 weeks Plasma only for fractionation for 4 weeks after vaccination
Transfusion-transmissible infections that require deferral			
HIV infection/AIDS and sexual partners	Permanent deferral if present or past clinical or laboratory evidence of HIV infection/AIDS; positive EIA with positive or indeterminate confirmatory test; positive NAT test; clinical signs include unexplained weight loss, night sweats, blue or purple spots in mouth or on skin, white spots or unusual sores in the mouth, swollen lymph nodes for more than 1 month, persistent cough or shortness of breath, persistent diarrhea, fever for more than 10 days; sexual partners deferred for 1 year from time of last contact (FDA)	Permanent deferral for donors found to have a confirmed positive marker for HIV. Donors found to have a repeat positive marker for HIV that cannot be confirmed should be informed according to the nationally agreed algorithm.	Donors with HIV I or II must be permanently deferred Infection: permanent deferral. Relevant symptoms within the last 6 months: defer for 12 months. Sexual contact with HIV-positive partner: defer for 12 months after last sexual contact.

Appendix 2: Donor Criteria (Continued)

Criterion	Region		
	United States	United Kingdom	European Union
Hepatitis and sexual partners and household contacts	<p>Permanent deferral for the following:</p> <ul style="list-style-type: none"> History of viral hepatitis after the 11th birthday. Confirmed repeatedly reactive for HBsAg. Positive test for anti-HBc on more than 1 occasion (testing is not required for Source Plasma donors). Present or past laboratory or clinical evidence of infection with HCV. Sexual partners of patients with hepatitis deferred for 1 year from last contact; household contacts of persons with hepatitis B deferred for 1 year from last contact (CFR). 	<p>Permanent deferral for donors whose blood gives a positive reaction for the presence of HBsAg and/or anti-HCV.</p> <p>Donors with a history of jaundice or hepatitis may, at the discretion of the appropriate competent medical authority, be accepted as blood donors, provided that an approved test for HBsAg and anti-HCV is negative.</p>	<p>Permanent deferral for hepatitis B and C: donors with history of hepatitis B may donate after 12 months after recovery, provided that all markers are negative or core antibody positive. HBsAg is negative, and anti-HBs ≥ 100 IU/L; donors with documented current or past infection with hepatitis C are permanently deferred; donors with hepatitis A are deferred for 12 months.</p>
HTLV	<p>Present or past clinical or laboratory evidence of infection with HTLV I/II (positive EIA on 2 occasions).</p> <p>[HTLV not tested in Source Plasma donors] (FDA)</p>	<p>Permanent deferral for carriers of HTLV I/II</p>	<p>Permanent deferral for donors with HTLV I/II</p>
West Nile virus	<p>Donors with symptoms suspicious of or actual diagnosis of WNV deferred for 120 days. Donor testing positive on WNV NAT deferred for 120 days. Donor who develops symptoms of WNV within 2 weeks of donation should be deferred for 120 days. Donors implicated in possible transfusion-transmitted WNV infection should be deferred for 120 days (FDA).</p>	<p>Defer for 28 days after donor leaves an area with ongoing transmission to humans.</p>	<p>Defer for 6 months if donor was in area endemic for WNV and was diagnosed with or had symptoms consistent with WNV. Defer for 28 days after donor returns from endemic area, provided donor has no symptoms of WNV.</p>
			<p>Hepatitis B acute or past infection: defer for 12 months after recovery, then perform hepatitis testing.</p> <p>Hepatitis B chronic carrier: permanent deferral.</p> <p>Hepatitis B contact, sexual, mucosal, household: defer for 12 months from last exposure unless immune.</p> <p>Hepatitis B other contact: accept.</p> <p>Hepatitis C positive past infection: permanent deferral.</p> <p>Hepatitis C contact, sexual, mucosal, household: defer for 12 months from last exposure; other contact: accept.</p> <p>Infection: permanent deferral.</p> <p>Repeat reactive status: plasma only for fractionation.</p> <p>Sexual contact: defer for 12 months after last contact.</p> <p>Household contact: accept.</p> <p>Infection: defer for 3 months after full recovery.</p> <p>Area exposure: plasma only for fractionation for 8 weeks after leaving risk area.</p>

Appendix 2: Donor Criteria (Continued)

Criterion	Region		
	United States	United Kingdom	European Union
Chagas disease and babesiosis	<p>Permanent deferral for history of Chagas disease; current FDA regulations do not require testing for Chagas disease. However, although not required, most facilities collecting blood for transfusion perform EIA test for Chagas and permanently defer following positive test. (AABB) FDA probably will not require antibody screening for fractionated or recovered plasma used for further manufacture. FDA has granted exemptions and permitted the collection and distribution of Source Plasma for further manufacture into noninjectable products from a donor known to have Chagas disease (CFR).</p> <p>Permanent deferral for history of babesiosis (AABB)</p>	<p>Permanent deferral for individuals with Chagas disease or history of Chagas disease; blood of persons who were born or have been transfused in areas where the disease is endemic should be used only for plasma fractionation products unless a validated test for infection is negative</p>	<p>Permanent deferral for individuals in the following categories may donate 6 months after leaving an endemic area, provided that a validated test for Chagas disease is negative (if a validated test is positive or not performed, the donor is permanently deferred): born in South or Central America, mother born in South or Central America, transfused in South or Central America; lived or worked in a rural subsistence community in South or Central America for 4 weeks or more</p>
Creutzfeldt-Jakob disease (CJD) and variant CJD	<p>Permanent deferral if donor: has diagnosis of CJD or vCJD; has a blood relative diagnosed with CJD; received dura mater graft; received human-derived pituitary growth hormone; received bovine insulin made in UK; spent a cumulative 3 months in UK between 1980 and 1996; received a blood transfusion in the UK at any time since 1980; spent 6 months between 1980 and 1990 on a US military base in Northern Europe; spent 6 months between 1980 and 1996 on a US military base elsewhere in Europe; spent a cumulative 5 years in Europe (Source Plasma donors are not deferred for the latter) (FDA)</p>	<p>Permanent deferral if donor: treated with extracts derived from human pituitary glands; has been recipient of dura mater or corneal graft; has a family risk of CJD or any other TSE. For vCJD: Member states should determine on the basis of the prevalence of BSE within individual countries, of the endogenous exposure of the population to bovine products imported from countries with a high BSE prevalence, and of the incidence of cases of vCJD, what precautionary measures they may need to take to minimize the risk of transmission of vCJD via blood transfusion</p>	<p>Permanent deferral if donor: Diagnosed with CJD, vCJD, or any other prion-associated disease; has family risk of CJD; at increased risk from surgery, transfusion, or transplant of tissues or organs; received a dura mater graft; received a corneal, scleral, or ocular graft; received human-derived pituitary extract; received a blood transfusion in UK since 1980; received intravenous immunoglobulin (IVIg) of UK origin; donated unit of blood implicated in possible case of transfusion-related vCJD. Additionally, all plasma from British donors cannot be used for fractionation</p>
			<p>Infection: permanent deferral. Contact: accept. Chagas disease area resident: plasma only for fractionation, permanently. Chagas disease area visitor: plasma only for fractionation for 12 months after leaving endemic area</p>

Appendix 2: Donor Criteria (Continued)

Criterion		Region		
		United States	United Kingdom	European Union
Visceral leishmaniasis (Whole Blood)		Donors who have been to Iraq are deferred for 1 year. Permanent deferral for signs and symptoms of visceral leishmaniasis (FDA)	No specific deferral	Permanent deferral for visceral leishmaniasis
		Donors who have been to Iraq are deferred for 1 year. Permanent deferral for signs and symptoms of visceral leishmaniasis (FDA)	No specific deferral	Permanent deferral for visceral leishmaniasis
Medical conditions and behaviors that place an individual at risk for a transfusion-transmissible infection and require deferral.		No current deferral required for blood donation, but draft regulations for tissue and organs require permanent deferral.	Permanent deferral	Permanent deferral
Xenotransplant		No current deferral required for blood donation, but draft regulations for tissue and organs require permanent deferral.	Permanent deferral	Permanent deferral
		No current deferral required for blood donation, but draft regulations for tissue and organs require permanent deferral.	Permanent deferral	Permanent deferral
Blood transfusion organ and tissue transplant; treatment with plasma-derived clotting factor concentrates		One-year deferral (dura mater graft is permanent deferral); permanent deferral if patient received clotting factor concentrates and sexual partner deferred for 1 year after last contact	Six-month deferral: if NAT test for hepatitis C is negative, may donate after 4 months	Permanent deferral if donor ever received clotting factor concentrate or was transfused after 1 January 1980; 1 year for tissue or organ transplant
		One-year deferral (dura mater graft is permanent deferral); permanent deferral if patient received clotting factor concentrates and sexual partner deferred for 1 year after last contact	Six-month deferral: if NAT test for hepatitis C is negative, may donate after 4 months	Permanent deferral if donor ever received clotting factor concentrate or was transfused after 1 January 1980; 1 year for tissue or organ transplant
Surgery or use of endoscope with biopsy		No specific deferral criteria; general health and transfusion criteria apply	Six-month deferral for major surgery; stomach resection requires permanent deferral; 1-week deferral for minor surgery; 6-month deferral for endoscope with biopsy; if NAT test for hepatitis C is negative, may donate after 4 months	Six-month deferral for major surgery or procedure using an endoscope; 1-week deferral for minor surgery
		No specific deferral criteria; general health and transfusion criteria apply	Six-month deferral for major surgery; stomach resection requires permanent deferral; 1-week deferral for minor surgery; 6-month deferral for endoscope with biopsy; if NAT test for hepatitis C is negative, may donate after 4 months	Six-month deferral for major surgery or procedure using an endoscope; 1-week deferral for minor surgery
Nonsterile skin penetration or mucous membrane exposure to blood or body fluids not the donor's own		Twelve-month deferral	Six-month deferral; if NAT test for hepatitis C is negative, may donate after 4 months	Twelve-month deferral
		Twelve-month deferral	Six-month deferral; if NAT test for hepatitis C is negative, may donate after 4 months	Twelve-month deferral
Acupuncture, tattoo, body piercing, etc.		Twelve-month deferral unless performed by a state-regulated entity, using sterile needles and disposable dyes	Six-month deferral; if NAT test for hepatitis C is negative, may donate after 4 months; exception can be made according to national risk assessment	Twelve-month deferral; 6-month deferral if validated test for hepatitis B core antibody is negative; for acupuncture, no deferral if performed by state-regulated entity
		Twelve-month deferral unless performed by a state-regulated entity, using sterile needles and disposable dyes	Six-month deferral; if NAT test for hepatitis C is negative, may donate after 4 months; exception can be made according to national risk assessment	Twelve-month deferral; 6-month deferral if validated test for hepatitis B core antibody is negative; for acupuncture, no deferral if performed by state-regulated entity

Cutaneous: plasma only for fractionation, permanently
Visceral: permanent deferral
Contact: accept.

Permanent deferral for visceral leishmaniasis

Permanent deferral

Donors who have been to Iraq are deferred for 1 year. Permanent deferral for signs and symptoms of visceral leishmaniasis (FDA)

Permanent deferral

Blood transfusion homologous: defer 12 months
Coagulation factor, blood derived, short term: defer 12 months from last treatment.
Coagulation factor, blood derived, continuous: permanent deferral.
Human tissue recipients: Organ/haematological: permanent deferral.
Homologous, bone, tendon, skin: accept.
Collagen: accept.
Corneal: permanent deferral for iatrogenic cCJD risk.

Permanent deferral if donor ever received clotting factor concentrate or was transfused after 1 January 1980; 1 year for tissue or organ transplant

Six-month deferral: if NAT test for hepatitis C is negative, may donate after 4 months

One-year deferral (dura mater graft is permanent deferral); permanent deferral if patient received clotting factor concentrates and sexual partner deferred for 1 year after last contact

Permanent deferral

Blood transfusion homologous: defer 12 months
Coagulation factor, blood derived, short term: defer 12 months from last treatment.
Coagulation factor, blood derived, continuous: permanent deferral.
Human tissue recipients: Organ/haematological: permanent deferral.
Homologous, bone, tendon, skin: accept.
Collagen: accept.
Corneal: permanent deferral for iatrogenic cCJD risk.

Permanent deferral if donor ever received clotting factor concentrate or was transfused after 1 January 1980; 1 year for tissue or organ transplant

Six-month deferral: if NAT test for hepatitis C is negative, may donate after 4 months

One-year deferral (dura mater graft is permanent deferral); permanent deferral if patient received clotting factor concentrates and sexual partner deferred for 1 year after last contact

Permanent deferral

Defer 6 months

Six-month deferral for major surgery or procedure using an endoscope; 1-week deferral for minor surgery

Six-month deferral for major surgery; stomach resection requires permanent deferral; 1-week deferral for minor surgery; 6-month deferral for endoscope with biopsy; if NAT test for hepatitis C is negative, may donate after 4 months

No specific deferral criteria; general health and transfusion criteria apply

Defer 6 months

Defer 12 months

Twelve-month deferral

Six-month deferral; if NAT test for hepatitis C is negative, may donate after 4 months

Twelve-month deferral

Twelve-month deferral

If using single-use items: plasma only for fractionation for 12 months
If not single-use or unsure: defer for 12 months

Twelve-month deferral; 6-month deferral if validated test for hepatitis B core antibody is negative; for acupuncture, no deferral if performed by state-regulated entity

Six-month deferral; if NAT test for hepatitis C is negative, may donate after 4 months; exception can be made according to national risk assessment

Twelve-month deferral unless performed by a state-regulated entity, using sterile needles and disposable dyes

Twelve-month deferral

Appendix 2: Donor Criteria (Continued)

Criterion	Region			
	United States	United Kingdom	European Union	Australia
Injection of medications or steroids not prescribed by a physician	Permanent deferral; sexual partner deferred for 1 year	All blood donors should be provided with accurate and updated information about HIV transmission and AIDS so that persons who have unsafe sex practices or other risk behavior exposing them to potential infection will refrain from donating. The information provided may vary among countries according to local epidemiological data.	Permanent deferral if donor ever injected or has been injected with drugs; sexual partner deferred for 1 year	Permanent deferral
Males who have sexual contact with another male	Permanent deferral for sexual contact, even once, since 1977; female sexual partner deferred for 1 year from last contact	persons who have unsafe sex practices or other risk behavior exposing them to potential infection will refrain from donating. The information provided may vary among countries according to local epidemiological data.	Permanent deferral for oral or anal sexual contact even if protection used; female sexual partner deferred for 1 year from last contact.	Defer for 12 months after last sexual contact
Accepted money or drugs or other payment in exchange for sex since 1977	Permanent deferral; sexual partner deferred for 1 year from last contact		Permanent deferral; sexual partner deferred for 1 year from last contact.	Defer for 12 months after last sexual contact
Incarceration for more than 72 hours in the past year	One-year deferral		No deferral	Defer for 12 months after release
Born in or lived in Africa	Permanent deferral if born or lived in countries where HIV 1 subtype O is endemic (Cameroon, Central African Republic, Chad, Congo, Equatorial Guinea, Gabon, Niger, Nigeria); sexual partner deferred for 1 year from last contact unless tested with a test validated to detect Group O		No specific deferral for Africa; malaria rules apply; however, sexual partners of persons who were sexually active in areas where HIV is endemic deferred for 1 year from last contact	No specific deferral: donors who have visited a malaria-endemic area are subject to a plasma-only restriction period of at least 12 months. The restriction period is extended to 3 years if residence has been for 6 continuous months or more within the past 3 years. With a negative malaria test, the restriction period can be reduced to 4 months. Donors who have traveled to an HIV risk area must be asked if they had sexual contact with a resident of that area.

Appendix 3: Disease Testing

Disease Test		United States	European Union
Hepatitis			European Pharmacopoeia:
HBsAg		Permanent deferral for repeatedly reactive test results (FDA)	Human Plasma for Fractionation
Hepatitis B core antibody		Permanent deferral if reactive on 2 or more separate occasions; permanent deferral if core antibody positive results are coupled with prior or concurrent repeatedly reactive HBsAg test (FDA). Testing is not required for Source Plasma donors	Laboratory tests are carried out for each donation to detect the following viral markers: (1) antibodies against HIV-1 (anti-HIV-1). (2) antibodies against HIV-2 (anti-HIV-2). (3) hepatitis B surface antigen (HBsAg). (4) antibodies against hepatitis C virus (anti-HCV). If a repeat-reactive result is found in any of these tests, the donation is not accepted.
Hepatitis C antibody EIA		Permanent deferral if repeatedly reactive for hepatitis C antibody; may re-enter donor after 6 months if confirmatory test is negative (FDA)	Blood Directive 2002/98/EC (Annex IV): Basic Testing Requirements for Whole Blood and Plasma Donations
Hepatitis C NAT testing		Permanent deferral if positive on single testing (FDA)	The following tests must be performed for whole blood and apheresis donations, including autologous predeposit donations: ABO group (not required for plasma intended only for fractionation). Rh D group (not required for plasma intended only for fractionation). Testing for the following infections is required in donors: Hepatitis B (HBsAg), Hepatitis C (Anti-HCV), HIV 1 and 2 (Anti-HIV 1 and 2). Additional tests may be required for specific components or donors or epidemiological situations. Blood Directive 2004/33/EC (Annex III): Permanent Deferral Criteria (excerpt): Hepatitis B, except for HBsAg-negative persons who are demonstrated to be immune Hepatitis C HIV 1 or 2

Appendix 3: Disease Testing (Continued)

Disease Test		United States	European Union
HIV	HIV NAT	Permanent deferral if positive on single testing (FDA)	European Pharmacopoeia: Human Plasma for Fractionation Laboratory tests are carried out for each donation to detect the following viral markers: (1) antibodies against HIV-1 (anti-HIV-1) (2) antibodies against HIV-2 (anti-HIV-2) (3) hepatitis B surface antigen (HBsAg) (4) antibodies against hepatitis C virus (anti-HCV) If a repeat-reactive result is found in any of these tests, the donation is not accepted Blood Directive 2002/98/EC (Annex IV): Basic Testing Requirements for Whole Blood and Plasma Donations The following tests must be performed for whole blood and apheresis donations, including autologous predeposit donations: ABO group (not required for plasma intended only for fractionation) Rh D group (not required for plasma intended only for fractionation) Testing for the following infections are required in donors: Hepatitis B (HBsAg) Hepatitis C (Anti-HCV) HIV 1 or 2 (Anti-HIV 1 and 2) Additional tests may be required for specific components or donors or epidemiological situations. Blood Directive 2004/33/EC (Annex III), Permanent Deferral Criteria (excerpt) Hepatitis B, except for HBsAg-negative persons who are demonstrated to be immune Hepatitis C HIV 1 or 2
	HIV 1 or II Antibody (EIA)	Permanent deferral for repeatedly reactive HIV EIA test; may reenter after 6 months if confirmatory test negative (FDA). Testing is not required for Source Plasma donors	

<1181> SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) is an electron optical imaging technique that yields both topographic images and elemental information when used in conjunction with energy-dispersive X-ray analysis (EDX) or wavelength-dispersive X-ray spectrometry (WDS). SEM is useful for characterizing the size and morphology of microscopic specimens. Together, image and X-ray analyses are important for the identification of small particles. Elemental analyses using SEM/EDX or SEM/WDS are useful for qualitative and semi-quantitative determination of elemental content. Accurate quantitation is possible only for bulk samples with smooth surfaces and thus is not practical for particle specimens.

Typically, SEM analysis requires a small amount (10^{-10} to 10^{-12} g) of a solid specimen that is coated with a conductive substance to inhibit sample charging. The sample is placed in an evacuated chamber and scanned in a controlled raster pattern by an electron beam. Interaction of the electron beam with the specimen produces a variety of physical phenomena that, when detected, are used to form images and provide elemental information about the specimen. These phenomena include (1) emission of secondary electrons (SES), (2) reflection of backscattered electrons (BSES), (3) characteristic X-ray emission, (4) emission of Auger electrons, (5) cathodoluminescence (CL), (6) conduction of current, (7) charging from induced voltages (IVS) or adsorbed electrons (AES), (8) electron transmission, (9) heat generation, and (10) electromotive forces (see Figure 1).

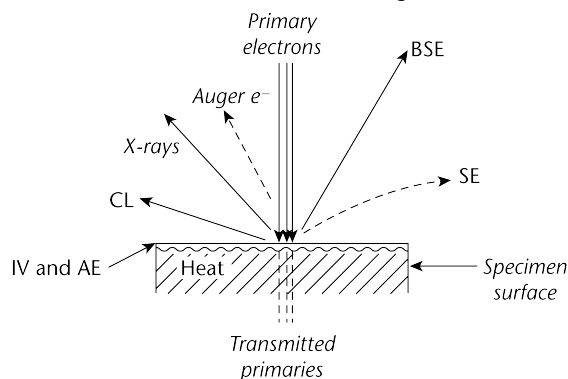


Figure 1. Interaction Diagram.

Of these, SES and BSES are the most important for constructing SEM images, and X-ray emission analyses are the most common methods for detecting the presence of particular elements. Use of ancillary instrumentation to detect the variety of phenomena other than (1), (2), or (3) above greatly increases the cost and complexity of the SEM system and will not be addressed here.

ELECTRON BEAM-SAMPLE INTERACTION

Imaging—Images are formed in a SEM system by detection and manipulation of electrons. SE are emitted from a specimen surface as the result of inelastic collisions between primary (incident) electrons (PE) and electrons within a specimen. When the energy imparted to a specimen electron exceeds the work function of a sample, that electron is emitted as an SE. Most SES have energies of 5 to 20 eV; electrons in this low-energy range can be efficiently collected, yielding high signal-to-noise images. Because such low-energy electrons can penetrate only short distances through the specimen, SES originate from within 2 to 30

nm of the surface and generate highly resolved images. The actual PES penetration depth is dependent on PES accelerating voltage, specimen elemental composition, specimen density, and specimen mounting angle. Excitation volumes of 0.5 to 5 μm in diameter are common.

Backscattered electrons are PES that have been reflected from the sample. The PES can undergo multiple collisions prior to exiting from the specimen; therefore, BSES have energies over a broad range and emerge from relatively deep penetration (≈ 0.1 to 5 μm) (see Figure 2).

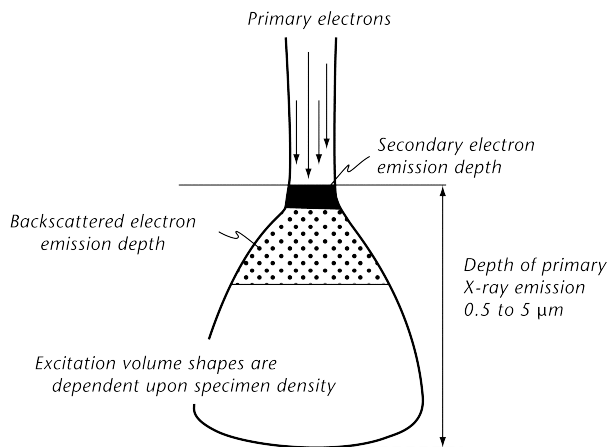


Figure 2. Bulk Penetration.

These high-energy (15 to 25 keV) BSES are collected less efficiently than SES, and they yield images with poorer resolution. The efficiency of BSES reflection is a function of the atomic number (Z) of the specimen atoms; thus, the contrast of BSES images depends on elemental composition. The penetration depth of all electrons is affected by elemental composition, specimen density, specimen tilt, and incident beam energy (accelerating voltage). For example, the SE images of sodium phosphate and zinc phosphate crystals are quite similar. However, the heavier nuclei of the zinc species produce more efficient BSE reflection and BSES images with higher contrast. BSE images of heavy- versus light-element phases, or mixtures of species, show dramatic contrast differences that are representative of elemental heterogeneity.

Although single-angstrom resolution is possible, practical SEM image resolution is limited to ≥ 100 Å (≥ 0.1 μm for X-ray images). These limits depend not only on instrument performance but also on operator acuity. Resolution is optimized under the following conditions: minimum working distance, high accelerating voltage, excellent grounding, excellent mechanical alignment, excellent electronic alignment, minimum incident spot diameter, minimum final aperture diameter, and cleanest column conditions. Sample preparations can be viewed in a variety of orientations and detector modes. Often the examination of a specimen at an oblique angle reveals features unobserved by an electron beam normal to the surface. This is especially true of specimens that have flat, featureless surfaces or that are poor conductors, e.g., glass surfaces. The PE accelerating voltage can be varied to change the PE penetration depth. This procedure is useful for characterizing specimens that are laminated or otherwise heterogeneous between surface and bulk content.

Coating a sample allows observation of a specimen's topography, undisturbed by flare and distortion caused by thermal effects and insufficient grounding. Coatings such as gold, gold-palladium, and carbon are often used because they are highly conductive, easy to apply, and relatively inert. Either evaporation or sputter-coating systems can be used to apply metal films; carbon films must be evaporated. Metal coatings give superior resolution, although their fluorescence can interfere with elemental analysis. Specimen

charging affects not only image quality but also X-ray fluorescence yield.

X-ray Emission Analysis—When a PE encounters an orbital electron in an atom, the resultant collision can either promote that orbital electron to a higher energy level or ionize the atom. Stabilization of an atom by relaxation of a higher energy electron to fill a vacancy results in the emission of an X-ray photon. These X-ray energies are discrete and element-specific; they equal the differences between the shell electron energies for the various shells of a given element. For instance, an ejected K-shell electron can be stabilized by a higher energy L-shell electron, yielding a net energy ($E_L - E_K$), which is specific for the X-ray photon energy of the elemental K line. X-ray emission lines are classified according to the electron shell in which the vacancy existed, e.g., K, L, M. The lines are further categorized according to the shell from which the relaxing electron originates. Thus, a $K\alpha$ X-ray line arises from a vacancy in a K-shell that is filled from an L-shell; a $K\beta$ X-ray line arises from a K-shell vacancy filled from an M-shell, and so on (see Figure 3).

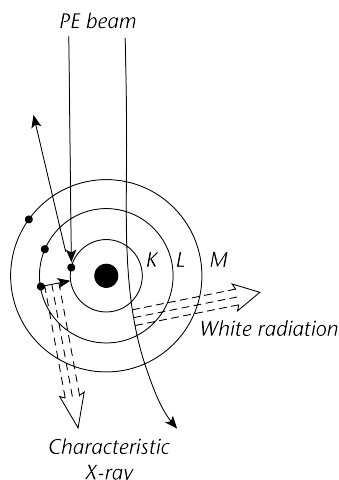


Figure 3. Atom Model.

Since each shell above K possesses a number of energy levels, electron transitions yield a number of lines, such as $K\alpha_1$, $K\alpha_2$, $K\beta_1$, and $K\beta_2$. The existence of several X-ray emission lines for each element (a few for $Z \leq 11$ and many for $Z \geq 11$) is useful in overcoming detection problems due to (1) interelement spectral interferences, e.g., titanium $K\alpha$ and barium $L\alpha$, (2) sample matrix effects on energy or intensity, and (3) insufficient PE energy to excite some elemental lines, e.g., lead, K lines.

The energies normally encountered in a SEM/EDX (or WDS) analysis range from 0.28 keV (≈ 447 nm) for carbon $K\alpha$ to the upper end of the instrument accelerating voltage, typically ≤ 40 keV (≤ 1 nm). The natural line width, which is inversely proportional to the lifetime of the upper electronic state, is governed primarily by the transition probabilities for X-ray emission and Auger electron emission. Interaction of X-ray photons with electrons within the specimen can result in Compton scattering to produce a broadened line shifted to lower energy. X-ray photons are also emitted as a result of inelastic acceleration of electrons by atomic nuclei within a specimen. These X-ray photons, termed bremsstrahlung or white radiation, have a broad, continuous energy distribution; and their characteristic lines are superimposed on this background signal.

For lighter elements, $Z \leq 11$, the low-energy X-ray photons originating from K-shell transitions can be detected only with wavelength-dispersive spectrometers or specially configured energy-dispersive detectors. All other elements emit easily detectable X-ray photons. Heavier elements, $Z \geq 16$, emit two or more detectable lines corresponding to

K- and L-shell transitions; and $Z > 57$ emit three or more detectable lines corresponding to K-, L-, and M-shell transitions. For a given element, X-ray intensities generally vary as follows: $K\alpha > K\beta > L\alpha > L\beta$, etc. (see Figure 4).

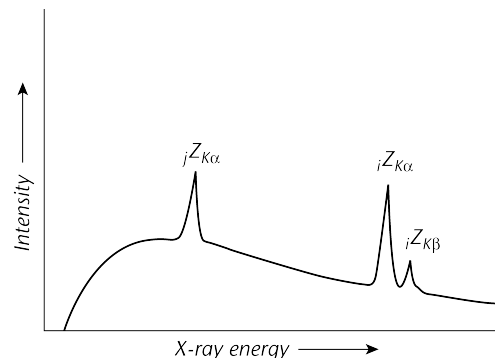


Figure 4. X-ray Spectrum.

The elemental content of a sample has a bearing on the selection of conditions for analysis. The most useful range of accelerating voltage is ≈ 3 to 20 kV; most elements of interest can be ionized by electrons with energies in this range. The energy required in order to excite X-ray emission from a given line is termed its critical excitation potential. The critical excitation potential for a K line can be approximated by the sum of the primary line energies ($K\alpha + L\alpha + M\alpha$). Selection of an accelerating voltage equal to 1.5 times this sum is usually sufficient for semiquantitative analyses. For example, copper has $K\alpha$ at 8.05 keV + $L\alpha$ at 0.93 keV = 8.98 keV: 1.5×8.98 keV = 13.47 keV. Selection of 15-kV accelerating voltage yields sufficient energy to ionize the K-shell of copper atoms and generate a useful analytical signal.

Interelement interferences originate from many effects. High-energy X-rays emitted from heavy atoms can ionize lighter elements to produce secondary X-ray emission from the lighter species. Lower high-Z element fluorescence and higher low-Z element fluorescence can be observed, in contrast to that expected from the PE-induced signal of a pure element. Conversely, X-ray emission from a light element may be absorbed by a heavier matrix to yield a negative bias in the light-element signal. These effects always exist in heterogeneous specimens and must be corrected for during any quantitative analysis. A common algorithm, ZAF, may be used to correct for Z-dependent interferences due to absorption and secondary X-ray emission.

APPARATUS

The SEM system consists of three electronic groups: (1) illumination, (2) optics, and (3) scanning control-display (see Figure 5).

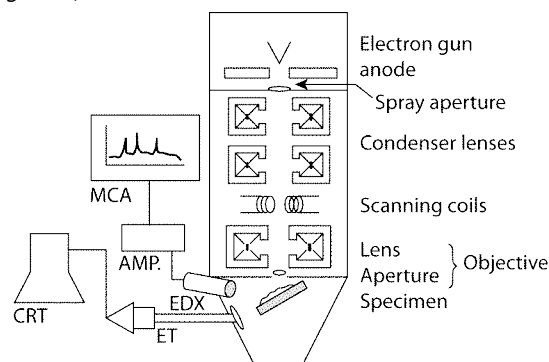


Figure 5. Optics Diagram.

The image is produced by mapping a specimen with an incident electron beam, rastered in a two-dimensional array. The electron beam is generated by emission from one of three types of sources, listed in order of increasing current density, vacuum requirements, and cost: (1) a tungsten filament cathode, (2) a LaB₆ cathode, or (3) a field emission gun. By far the most common SEM source is the tungsten filament, although the high current density of the LaB₆ source is especially useful where high resolution or detection of low-Z elements is required.

The optics consist of condenser and objective lenses, in conjunction with selected apertures. The size of the final aperture controls the beam diameter and, accordingly, the image resolution and total current at a specimen. Selection of an objective aperture is an important choice. Small apertures are required for high resolution and large apertures provide high current for optimal X-ray emission intensity. In many systems, the objective aperture can be adjusted during use with a sliding or rotating holder. Flexibility in trading resolution for specimen current is also important because sample characteristics affect these two criteria differently. This feature is beneficial to users requiring high magnification and elemental detection, especially of $Z \leq 11$ elements.

Image magnification is controlled by altering the area of the electron beam raster; smaller areas yield higher magnification, because the cathode-ray tube (CRT) area remains constant. An Everhart-Thornley (ET) detector is used for electron detection; the resultant images are most similar to those of reflected light microscopy. An ET detector consists of a Faraday cage and a scintillator disk connected by a light pipe to a photomultiplier tube. The Faraday cage serves three functions: (1) at positive bias it attracts SE; (2) at negative bias it repels SE to enable the ET detector to collect BSE signals alone; and (3) it shields the PE beam from the scintillator potential. Various scintillator coatings are used. For example, phosphorus-based coatings yield intense, high-contrast images. Aluminum-based coatings, although less sensitive, can withstand the high SE flux generated during elemental analyses. Solid-state detectors provide up to 10 times greater sensitivity for BSE collection. They can be placed at a variety of positions and distances with respect to a specimen.

Procedure

Preparation—Samples for analysis are easily prepared, especially with an optical aid or a stereomicroscope.

Particles—Place isolated or selected particles onto the SEM sample substrate (e.g., a pyrolytic carbon or metal pedestal). In all cases, the specimen should be attached to an exposed area of the substrate with a suitable liquid cement¹ (see Figure 6).

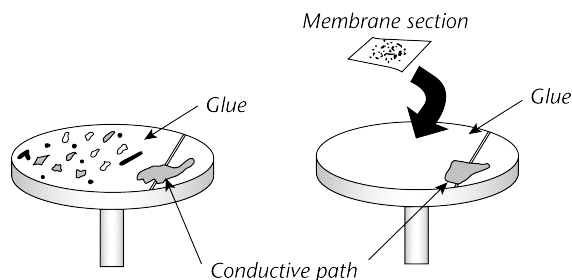


Figure 6. SEM Pedestal Mount.

Use silver or carbon paint to provide a conductive path between the cement surface and the substrate. A single prepa-

¹ Any "superglue," copper diallylphthalate, double-sided sticky tape, or other types of mounting resins can be used. The cement with the least residual organic phase will have the longest stability.

ration can accommodate from one to several hundred particles, placed in an identifiable pattern.

Particles isolated from liquid samples by membrane filtration can be examined by placing a filter membrane on a sample substrate, as described above (see Figure 6). This preparation is especially useful for examining a random sample of particles from a liquid. Membrane or film filters rather than depth filters are recommended, since small particles are easily lost in the open pores of a depth filter. Portions of a random isolate can be used for the SEM examination, and the remainder can be saved for other tests. Alternatively, particles can be dispersed in a solvent and concentrated onto a substrate. If a membrane filter is precoated and used to collect particles, a sample can be examined directly without further coating. This procedure alleviates excessive handling of a specimen and lessens exposure of the specimen to coater (sputter or evaporation) environments and to background conductive film signals.

Bulk Materials—Scatter a loose powder over double-sided sticky tape on a substrate; excess material can be blown off with a clean air jet. Liquid adhesives can be used instead of tape. However, rapid filming of an adhesive surface can occur and can hinder particle adhesion. Also, particles may sink into an adhesive before it dries. Pack loose powder into small holes cut into the surface of a metal pedestal. This technique is favored for semiquantitative analyses because it produces a relatively flat surface, is for practical purposes infinitely thick to the electron beam, provides effective grounding, and requires a minimal amount of sample.

Cement or clamp large materials directly onto the mounting substrate. Nearly any sample that will fit into a vacuum chamber, withstand evacuated conditions, and be effectively grounded is amenable to analysis. For all the above methods, specimen coating is required. In the absence of coating, a preparation must be viewed at sufficiently low energies, usually ≤ 5 kV, to yield suitable micrograph quality.

Analysis—The method of probing each particle and obtaining a composition is dependent on the level of information required. Several factors must be considered if semiquantitative analysis is desired. The presence of multiple elements, the type of elements contained in the specimen, and the size and surface characteristics of each particle are a few of the considerations.

Any specimen ≥ 10 μm in diameter can be probed quickly and without subsequent data reduction. Particles between 10 μm and 0.2 μm must be considered more carefully, since their size approaches that of the excitation volume of the probe. Elemental characterization of a particle should be conducted on a specimen sufficiently thick that the particle volume is equal to or greater than the volume of X-ray production. Depth of signal excitation (d_x) determines the volume of X-ray production. Conditions such as accelerating voltage, source current, and spot size that are appropriate for analyzing a given specimen volume can be determined empirically, if particles are mounted on a metal substrate from which fluorescence can be detected. Substrate fluorescence indicates that the excitation volume has exceeded the volume of the particle. Quantitatively, d_x depends on particle characteristics and SEM conditions as follows:

$$d_x = 0.033 \times (A_{\text{ave}}/dZ_{\text{ave}}) \times (E_0^{1.69} - E_c^{1.69})$$

where d_x is the depth of signal excitation, Z_{ave} is the average atomic number, A_{ave} is the average atomic mass, E_0 is the energy of incident electrons, E_c is the critical excitation energy of the measured X-ray line, and d is the density of the particle.

Elemental content is estimated directly from elemental line intensities. Use the following procedures.

(1) Mount the specimen(s) by any of the above procedures and ensure good electronic and physical alignment of the SEM.

(2) Tilt or align the specimen toward the detector window at an angle that optimizes collection of the X-rays, i.e., 45° for horizontal detectors.

(3) Select the symmetrical center of the particle and use the raster mode, rather than the point mode, for the analysis. This eliminates topographical variations and generates an average signal for the sample. Choose a magnification at which the raster is $\approx 50\%$ of the particle area to eliminate background signal from the substrate.

(4) Adjust the accelerating voltage to produce adequate signal-to-noise for detection of the element of choice.

(5) Integrate the signal as long as necessary to achieve statistically significant results. This period can be determined through analysis of reference materials using the SEM conditions of choice (40 seconds per particle is a good working rule).

(6) Ensure sufficient specimen current. A good working rule is 20% to 40% dead time, as measured by the detector multichannel analyzer (MCA).

(7) Subtract a background spectrum that is representative of the coating, chamber, and handling.

(8) Perform an analysis, using exactly the same conditions as above on a reference standard that contains the element of choice.

(9) Perform an analysis, using exactly the same conditions as above on an internal reference (such as the substrate or other metal), for use in normalizing the analytical conditions.

The weight percent of a given element (E) in the specimen can be calculated as

$$\text{Weight \%} = \left[\frac{(I_E/I_{\text{ref}})_{\text{Sample}}}{(I_E/I_{\text{ref}})_{\text{Standard}}} \right] \times C^{\text{Std}} \times 100\%$$

where I_E is the intensity of the element of interest, I_{ref} is the intensity of the internal reference element, and C^{Std} is the concentration of the element of interest in the standard specimen. More accurate quantitative analysis must take into account the effects of matrix type, of interelements, of counting times, and of takeoff angle. The ZAF algorithm used to correct for these effects involves multiplying the measured weight percent by a series of correction factors.

<1184> SENSITIZATION TESTING

INTRODUCTION

This chapter considers sensitization and hypersensitization in the context of medical devices and implants, and describes methodologies for testing such articles for their potential to cause sensitization.

There are four types of hypersensitization reactions according to the Gell and Coombs classification system. Type I reactions involve the fixation of IgE to mast cells that subsequently release pharmacologically active substances, such as histamine. Type II reactions are the result of IgG and/or IgM binding to target cells, followed by complement fixation and cell lysis. Type III reactions are caused by the presence of antigen-antibody complexes that cause physical damage such as kidney damage due to glomerular blockage. Type IV reactions are cell-mediated (involve the action of T cells and their interaction with the human lymphocyte antigens). Type IV reactions are also called delayed-type hypersensitivity reactions. *Table 1* below summarizes the types of reactions, the mediators of the reactions, and examples of representative diseases.

Table 1. The Four Types of Hypersensitization Reactions*, Mediators, and Disease Examples

Reaction Class	Mediators	Disease Examples
Type I	IgE molecules bound to mast cells interact with antigen to release pharmacologically active substances	Hay fever, bronchial asthma, other atopic reactions
Type II	IgM and/or IgG molecules interact with target cells, fix complement, cell lysis	Various drug allergies, erythroblastosis fetalis, hemolytic anemia, thrombocytopenia
Type III	Antigen-antibody complexes, complement	Arthus reaction, serum sickness, allergic glomerulonephritis
Type IV	T lymphocytes, antigen, monocytes, macrophages	Contact dermatitis

*According to Gell and Coombs classification scheme

A multi-step process, delineated in chapter *The Biocompatibility of Materials Used in Drug Containers, Medical Devices, and Implants* <1031> is followed in determining which, if any, toxicological tests need to be performed on a given article. In some cases, sufficient evidence to satisfy toxicology requirements may be available from previously marketed articles (See *Figure 1* in chapter <1031>). Important factors addressed in *Figure 1* (chapter <1031>) include the type and extent of contact with the body, the chemical composition, the manufacturing process, the sterilization process, and, as mentioned above, similarity to previously marketed articles.

If further toxicological testing is necessary, the classification of medical devices provided in *Table 2* from general information chapter <1031> is important, because the degree and extent of toxicological testing that is required is strongly influenced by the nature and duration of the bodily contact with the article. The classification derived from *Table 2* in chapter <1031>, coupled with the length of exposure to the article, is used in *Tables 3–5* of chapter <1031> to determine which toxicological tests need to be performed. *Table 2* below presents information extracted from *Tables 3–5* of chapter <1031> and indicates those circumstances for which sensitization testing should be considered.

Table 2. Articles For Which Sensitization Testing Should Be Considered Based on Article Category and Length of Exposure

Device Category	Body Contact	Contact Duration
Surface devices	Skin	A ^a , B ^b , C ^c
	Mucosal membrane	A, B, C
	Breached or compromised surfaces	A, B, C
External communicating devices	Blood path, indirect	A, B, C
	Tissue, bone, or dentin communicating	A, B, C
	Circulating blood	A, B, C
Implant devices	Tissue or bone	A, B, C
	Blood	A, B, C

^aA: limited (less than 24 hours)

^bB: prolonged (24 hours to 30 days)

^cC: permanent (more than 30 days)

There are nine test methodologies reviewed in this chapter. *Table 3* lists the methods and the species with which they are performed.

Table 3. Test Methodologies That May Be Used in Sensitization Testing, and Species Required for Test

Test	Species Used in Test
Magnusson & Kligman Maximization	Guinea pig
Standard Buehler	Guinea pig
Open Epicutaneous	Guinea pig
Freund's Complete Adjuvant	Guinea pig
Optimization	Guinea pig
Split Adjuvant	Guinea pig
Local Lymph Node Assay	Mouse
Mouse Ear Swelling	Mouse
Vitamin A Enhancement	Mouse

Given the preponderance of testing performed with either the *Magnusson & Kligman Guinea Pig Maximization Test* (GPMT) or *Buehler Tests* (BT), those tests will be reviewed in detail in this chapter. A brief summary of the remaining tests is provided as alternatives to the more frequently used procedures.

Each test should be periodically validated in the performing laboratory using positive controls such as hexyl cinnamic aldehyde, mercaptobenzothiazole, or benzocaine (positive controls recommended by the Organization for Economic Cooperation and Development [OECD]).

MAGNUSSON & KLIGMAN GUINEA PIG MAXIMIZATION TEST (GPMT)

Animals

Either male and female albino guinea pigs or both may be used. All animals should be in good health and weigh between 300 g and 500 g at the start of the experiment. The females should not be pregnant, nor should they have borne young previously. Prior to use, it is essential to acclimatize the animals to the laboratory conditions for at least 5 days. All animals should be handled in accordance with the guidelines in the appropriate regulatory requirements established for the humane treatment of animals. At least 10 test animals and 5 control animals should be used. To obtain sufficient analytical power (i.e., to detect weak sensitizers) it may be necessary to use 20 test animals and 10 control animals. Additional animals may be required to establish the proper doses to administer (see *Determination of Test Article Concentration*).

Housing and Feeding

The animal room should be held at 20 ± 3°, at 30% to 70% relative humidity, with 12 hours of light and dark. Animals may be housed individually or in group housing. Standard laboratory diets may be used (those satisfactory for guinea pigs ensure an adequate amount of ascorbic acid). Drinking water should be available ad libitum.

Animal Pretest Preparation

Animals should be randomized via a validated randomization method. For example, such methods may utilize random number tables or computer-generated random numbers. Sites on the animals intended for test article application (intrascapular region) should have the hair removed in a manner that does not abrade the skin. This may be accomplished via clipping, shaving, or with chemical depilatories. The chemical depilatory must not elicit irritation of its own. General observations of the animals prior to use in the test should be recorded, including any indication of ill health (do not use such animals in tests), and body weights.

Test Article Preparation¹

The use of this test requires that the test article can be injected intradermally. When the test article is not suitable for direct administration, extracts should be prepared according to the procedure provided in general chapter *Biological Reactivity Tests, In Vivo* (88).

Determination of Test Article Concentration

The purpose of this preliminary study is to determine the concentrations of *Test Article Preparation* to be used during the initial induction phase and the second challenge phase of a GPMT study. Two or three animals may be used for the concentration determination.

A range of concentrations of the test article, or extracts of the article, should be injected intradermally (0.1 mL per site), using the solvent that will be employed in the *Test Procedure*. The concentration that causes only mild to moderate irritation (no extensive skin destruction, with no evidence of overt systemic toxicity to the animals) should be used in the *Intradermal Injection Induction Phase* of the *Test Procedure*.

Using two or more animals, apply via occlusive dressings and patches, a range of concentrations of test article or extracts of the article. Remove the dressings/patches after 24 hours, and examine the sites for erythema. Choose the concentration that causes only slight erythema for the *Topical Application Induction Phase* of the *Test Procedure*. Use the highest concentration of test article or extract that does not cause erythema for the *Challenge Phase* of the *Test Procedure*. If the irritation threshold is not reached, then select the highest possible concentration for the *Topical Application Induction Phase* and *Challenge Phase* of the *Test Procedure*.

Test Procedure

INTRADERMAL INJECTION INDUCTION PHASE

This phase requires three pairs of injections administered intradermally, with the test and control injection of each pair on opposite sides intrascapularly. Each injection should contain 0.1 mL, with injection pairs 1 and 2 administered nearer to the head, and injection pair 3 administered slightly farther towards the tail. The pairs are nominally within an area of 8 cm². The pairs of injections consist of the following:

Injection pair 1:	A 1:1 (v/v) mixture of Freund's Complete Adjuvant (FCA), an oil–water emulsion containing mycobacteria, and the appropriate solvent/vehicle (see <i>Biological Reactivity Tests, In Vivo</i> (88)). Control animals receive a mixture of FCA and physiological saline (1:1).
Injection pair 2:	The <i>Test Article Preparation</i> in the concentration as specified in <i>Determination of Test Article Concentration</i> , using the appropriate solvent/vehicle. Control animals receive only the solvent/vehicle.
Injection pair 3:	The <i>Test Article Preparation</i> in the concentration as specified in <i>Determination of Test Article Concentration</i> in a 1:1 (v/v) mixture with FCA. Control animals receive an injection of a 1:1 (v/v) mixture of FCA and solvent/vehicle.

¹ For further information on sample preparation, see ANSI/AAMI/ISO/CEN Standard 10993–12—1996: Biological Evaluation of Medical Devices—Part 12: Sample Preparation and Reference Materials

TOPICAL APPLICATION INDUCTION PHASE

Seven days (± 1 day) after completion of the *Intradermal Injection Induction Phase*, administer the test sample by topical application to the intrascapular region of each animal. For both test and control animals, if the *Test Article Preparation* does not cause skin irritation, apply 10% sodium lauryl sulfate in petrolatum approximately 24 hours before the start of the *Topical Application Induction Phase* to induce a local irritation.

Test animals should have 2- \times 4-cm pieces of filter paper or absorbent gauze fully loaded with the *Test Article Preparation* (prepared within 24 hours of use) using the concentration selected in *Determination of Test Article Concentration* applied to each injection site. The filter paper or absorbent gauze should be secured to the animals using occlusive dressings. Control animals receive the same treatment, except that the appropriate solvent/vehicle is used instead of the test article.

Remove the dressings and patches approximately 48 hours after application.

CHALLENGE PHASE

This phase should occur 14 ± 1 days after the *Topical Application Induction Phase*. Hair should be removed from the test application sites. Filter paper patches or chambers are soaked with a freshly prepared *Test Article Preparation* in the concentration specified in *Determination of Test Article Concentration*. This is done for all test and control animals. The patches or chambers are secured with an occlusive dressing and removed after 24 ± 2 hours.

Observations

At approximately 24, 48, and 72 hours after removal of the challenge patches, the application sites should be examined for signs of reactions. Of particular importance are instances where the reaction of the test animals exceeds that of the control animals. All signs of reactivity should be recorded, with particular attention paid to signs of erythema and edema. A true edematous reaction will blanch under gentle pressure. The longer the period of blanching, the greater the severity of edema.

Interpretation

There is more than one way of evaluating and grading the results from GPMT. *Tables 4, 5, and 6* list details for three such grading systems. Grades of 1 or higher in the test animals, with grades of less than 1 in control animals, are indicative of sensitization. If control animals display grade 1 reactivity, and if the test animals display reactivity above the greatest reactivity seen in the control animals, sensitization due to the test article is again suspected. The percentages in *Table 4* need to be revised if there are only 10 test animals (i.e., the categories would be 0, <10%, 10%–30%, 31%–60%, 61%–80%, and 81%–100%). If there are 20 test animals, then multiples of 5% are appropriate.

Table 4. Classification Based on Percent of Responsive Test Animals

% of Positives in Test Group	Assigned Grade Class	
0	—	Nonsensitizer
<8	1	Weak
8–28	2	Mild
29–64	3	Moderate
65–80	4	Strong
81–100	5	Extreme

Table 5. Classification Based on Erythema and Edema Formation

Erythema and Eschar	Grade
No erythema	0
Slight or equivocal erythema	<1
Well-defined erythema	2
Moderate erythema	3
Severe erythema to slight eschar formation	4
Edema	
No edema	0
Slight or equivocal edema	<1
Well-defined edema	2
Moderate edema	3
Severe edema	4

Table 6. Classification Based on Erythema Formation Alone

Erythema formation	Grade
No erythema	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and swelling	3

The results should be submitted for statistical analysis (e.g., chi-square contingency table) to determine if the differences in scores between treated and control animals are significant. The response of the test group versus the control group should be compared statistically. (The Mann-Whitney U test can be used for the comparison.)

Rechallenge

The extent of any response in the negative control group, under experimental conditions, shows the irritation potential of the *Test Article Preparation*. In this case, test and control animals should be rechallenged 1 week later on the untreated side of the animal, with a reduced concentration of the *Test Article Preparation*. A sensitized guinea pig will react to some degree to both challenges. A weak reaction occurring at a single time point in only one challenge should cast strong doubt as to whether that guinea pig is truly sensitized.²

STANDARD BUEHLER TESTS (SBT)

Animals

See *Animals* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*.

Housing and Feeding

See *Housing and Feeding* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*.

Animal Pretest Preparation

See *Animal Pretest Preparation* under *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*. The fur of the guinea pig may be removed from one flank by clipping.

²Basketter D.A. Guinea pig predictive tests for contact hypersensitivity. In *Immunotoxicology and Immunopharmacology*, 2nd ed.; Dean, J.H., Luster, M.I., Munson, A.E., Kimber, I., Eds; Raven Press, Ltd: New York, 1994; pp 693–702.

Test Article Preparation

See *Test Article Preparation* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*.

Determination of Test Article Concentration

The purpose of this preliminary study is to determine the concentrations of *Test Article Preparation* to be used during the initial induction phase and the second challenge phase of an SBT study. Two or three animals may be used for the concentration determination.

A range of concentrations of the test article, or extracts of the article, should be applied using patches (for example, four 4 cm² absorbent pads) or chambers. The patches should be held in place using tape (if necessary) and occlusive dressings. The patches should be removed after approximately 6 hours, and any residues of the test chemical are removed from the test site. Observations are made at that time, and at 24 and 48 hours.

The concentration that causes only mild to moderate irritation (slight erythema, with no evidence of overt toxicity to the animals) and can be applied repeatedly to the same site should be used in the *Induction Phase* of the *Test Procedure*. Use the highest concentration of test article or extract that does not cause erythema for the *Challenge Phase* of the *Test Procedure*.

Test Procedure

INDUCTION PHASE

Apply 0.4 mL of the *Test Article Preparation* in an appropriate solvent/vehicle at the dose identified in *Determination of Test Article Concentration*. Use patches similar to those used in *Determination of Test Article Concentration*. The patches should be applied to one flank (hair clipped off) and held in place occlusively for 6 hours. The animals may need to be restrained to ensure occlusion. Patches and any visible residues should be removed after 6 hours. Control animals also receive patches, but these contain only the appropriate solvent/vehicle. This process should be repeated three times a week for both test and control animals on the same site for three consecutive weeks (weekly intervals are used in the modified Buehler Test).

CHALLENGE PHASE

This phase should be carried out 14 days after the last application of the *Induction Phase*. Clip the hair off the previously untested flank of each animal 24 hours before the challenge application. As in the *Induction Phase*, apply patches containing the test article (concentration specified in *Determination of Test Article Concentration*) or solvent/vehicle alone to the untested areas of the test and control animals. To obtain well-defined edges at the application sites, commercial chambers with a lipped edge are preferred. Secure the patches with occlusive dressings, and keep them in place for 6 hours. Remove all patches after 6 hours.

Observations

At 22 ± 2 hours after removal of the patches, the application sites should have the animal's fur removed via clipping or depilation. After approximately 2 more hours, grade the sites (*Tables 4, 5 or 6* may be employed). All signs of reactivity should be recorded, with particular attention paid to signs of erythema and edema. Repeat the grading once again after 24 to 48 hours more have elapsed. The response

of the test group versus the control group can be compared statistically. (The Mann-Whitney U test can be used for the comparison.)

Interpretation

The results should be submitted for a statistical analysis (e.g., chi-square contingency table) to determine if the differences in scores between treated and control animals are significant.

See *Interpretation* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*.

Rechallenge

See *Rechallenge* in the *Magnusson & Kligman Guinea Pig Maximization Test (GPMT)*.

OTHER SENSITIZATION TEST PROCEDURES

The *Magnusson & Kligman Guinea Pig Maximization Test* and the *Standard Buehler Tests* are the most frequently performed sensitization tests. However, there are a number of other methods that may be useful in the assessment of the potential for sensitization. Some may be applicable to both solid test articles and extracts, some only to extracts.

Where the use of guinea pigs is called for in the following tests, the animals and their housing should meet the requirements as specified for *Animals* in the *Magnusson & Kligman Guinea Pig Maximization Test*. The fur of the guinea pig should be removed from test sites as indicated for *Animal Pretest Preparation* in the *Magnusson & Kligman Guinea Pig Maximization Test*.

Draize Test

This was the first predictive test accepted by the regulatory agencies, and is still in use. The test uses guinea pigs and the test article is administered via intradermal injections.

TEST ARTICLE PREPARATION

This test requires that the test article be in the form of a solution that may be directly applied to the animal's skin. Therefore, extracts of the material would need to be made. See *Biological Reactivity Tests, In Vivo* (88) for information on the preparation procedure.

INDUCTION PHASE

One flank of each of 20 guinea pigs is shaved, then 0.05 mL of a 0.1% solution of test article is injected into the anterior flank. The next day, and then every other day thereafter up to day 20, 0.1 mL of the test article is injected into a new site on the same flank.

CHALLENGE PHASE

This phase begins 2 weeks after the final injection of the *Induction Phase*. The untreated flank is shaved, then 0.05 mL of test article is injected into each of the 20 guinea pigs. Twenty previously untreated animals serve as the controls, and receive injections of the test article as well.

OBSERVATIONS

The test sites of all control and test animals are evaluated for erythema at 24 and 48 hours after the challenge injections. The degree of reaction in test animals is compared to

the reaction in control animals. A larger and/or more intense response by the test animals versus the control animals is indicative of sensitization.

Open Epicutaneous Test

This test uses guinea pigs. The goal is to determine the dose required to induce sensitization by simulating human usage via topical application of the test article.

TEST MATERIAL PREPARATION

This test requires that the test article be in the form of a solution that may be directly applied to the animal's skin. Therefore, extracts of the material need to be made. See *Biological Reactivity Tests, In Vivo* (88) for information on the preparation procedure.

PRELIMINARY TESTING

A series of concentrations of test article is applied to 2 cm² areas of skin on the anterior flank of 6 to 8 guinea pigs (0.025 mL per application). The test sites should be examined for erythema 24 hours after test article administration. The highest concentration that does not cause irritation (maximum nonirritant concentration) and the lowest concentration causing erythema in approximately 25% of the animals (minimum irritant concentration) are determined.

INDUCTION PHASE

The test article (or control vehicle) is applied to 8 cm² areas of the flank skin of 6 to 8 guinea pigs daily for 3 weeks, or five times a week for 4 weeks. The amount per application is 0.01 mL. A set of increasing concentrations is again employed, ranging from the minimum irritant concentration using a stepwise progression. The test article should be applied to the same sites each time, unless irritation develops, in which case a new site on the same flank should be used. Control animals receive the same series of treatments using the vehicle instead of the test article.

CHALLENGE PHASE

Each animal is challenged on the untreated flank 24 to 72 hours after the last *Induction Phase* treatment using 0.025 mL applied to 2 cm² areas. A set of increasing concentrations is used, from minimum irritant concentration to the maximum nonirritant concentration, and five lower concentrations are also used.

OBSERVATIONS

The test sites are evaluated at 24, 48, and 72 hours post-treatment. The maximum concentration that does not cause irritation in the control group is determined. Animals from the test groups that develop inflammatory responses at concentrations lower than the maximum nonirritating concentration in the controls should be considered to be sensitized.

Freund's Complete Adjuvant Test

This test is based upon the use of intradermal injections using the test article in a mixture of Freund's complete adjuvant and distilled water (50:50).

TEST MATERIAL PREPARATION

Because this test uses intradermal injections, extracts of the test material need to be made in order to use this procedure. See *Biological Reactivity Tests, In Vivo* (88) for information on the extraction procedure.

PRELIMINARY TESTING

The minimum irritating and the maximum nonirritating concentrations are determined in the same manner as for *Preliminary Testing in the Open Epicutaneous Test*.

INDUCTION PHASE

The test area consists of six 2 cm² areas across the shoulders of the guinea pigs. Two groups of 10 to 20 guinea pigs each should be used. The test group animals are injected intradermally with 0.1 mL of a 5% solution of the test article extract in FCA/water. Control animals receive injections with FCA/water without the test article. These injections are repeated every 4 days until a total of three injections have been given.

CHALLENGE PHASE

This phase should begin 2 weeks after the last injection of the *Induction Phase*. Topical applications of 0.025 mL of test article at the minimum irritating and the maximum nonirritating concentrations, plus two lower concentrations, are administered to 2 cm² areas of the shaved flank. The test sites should remain uncovered.

OBSERVATIONS

The test sites are examined for the presence of erythema 24, 48 and 72 hours after the topical applications. The minimum nonirritating concentration in the control animals should be determined. Those test animals that display erythema at concentrations lower than the minimum nonirritating concentration in the control animals should be considered to be sensitized.

Optimization Test

This test has some similarities to the older *Draize Test*. Unlike the *Draize Test*, however, this test uses both intradermal and topical treatments, and includes adjuvant for some induction injections.

TEST MATERIAL PREPARATION

As with other test procedures that incorporate intradermal injections, the test article needs to be in a form suitable for injection. See *Biological Reactivity Tests, In Vivo* (88) for information on the extraction procedure.

INDUCTION PHASE

Twenty test and 20 control guinea pigs are used. A total of 10 intradermal injections should be given to each animal. Test animals receive 0.1 mL of a mixture of 0.1% test article and 0.9% saline (50:50) on day 1, with one injection into a shaved flank, and another into a portion of shaved dorsal skin. Two and 4 days later, one intradermal injection of the test article in saline is given to eight new dorsal sites. Every other day during weeks 2 and 3, the test article is injected intradermally into 10 sites over the shoulders in a 50:50 mixture of saline and FCA. The same sequence of injections

is given to the 20 control animals, except that no test article is included with the saline or saline/FCA injections.

CHALLENGE PHASE

Thirty-five days after the first injection, the animals are challenged topically with 0.1 mL of the 0.1% solution of test article in saline (for test animals). The control animals receive saline injections only. At 45 days after the first injection, a second topical challenge is given. A nonirritating concentration of test article (0.05 mL) is applied topically to a 1 cm² area of untreated skin. This site should then be covered with a 2 cm² piece of filter paper, after which an occlusive dressing should be applied. The patch should be removed after 24 hours.

OBSERVATIONS

Twenty-four hours after each injection during week 1, the thickness of a fold of skin over the injection sites for each animal should be measured using a caliper (mm), and the two largest cross-diameters of each erythematous reaction should be recorded (mm). The reaction volumes are calculated by multiplying the fold thickness by the products of the two cross-diameters (expressed as μL). The mean reaction (+1 SD) volume during week 1 should be calculated for each animal.

Challenge reaction volumes are calculated for each animal following the injections at day 35. If an animal develops a challenge reaction volume greater than its mean reaction volume + 1 SD, it should be considered sensitized.

Following the patch testing challenge, the test sites are evaluated for erythema and edema. Evaluations should be made using *Table 5*.

The number of positive animals should be compared statistically with the pseudopositive control animals. This should be done for both intradermal injection results and patch testing results. The Fisher exact test may be used.

The results from the intradermal injections and the patch testing, following separate statistical analysis, may be combined and evaluated using *Table 7* in order to classify a test article as a strong, moderate, or weak sensitizer; or not a sensitizer.

Table 7. Classification Scheme for Test Articles Based on the Optimization Test

Intradermal % of Positive Animals	Patch Test % of Positive Animals	Classification
$S^*, > 75$	and/or $S, > 50$	Strong sensitizer
$S, 50-75$	and/or $S, 30-50$	Moderate sensitizer
$S, 30-50$	$N.S.^*, 0-30$	Weak sensitizer
$N.S., 0-30$	$N.S., 0$	Not a sensitizer

*S = significant; N.S. = not significant

Split Adjuvant Test

This test makes use of both FCA and skin damage. The test article is applied topically.

TEST MATERIAL PREPARATION

Because this test employs topical test article applications, the article can be either in solid or liquid form. If extracts are to be made, see chapter *Biological Reactivity Tests, In Vivo* (88) for extraction procedures.

INDUCTION PHASE

Ten to 20 guinea pigs are used for both test and control groups. An area of back skin immediately behind the scapulas should be shaved to the extent that the skin becomes glistening. The shaved areas should then be treated with dry ice for 5 to 10 seconds. A dressing made of loose mesh gauze with stretch adhesive and a 2- × 2-cm opening should be placed over the treated area, then secured with adhesive tape. The test article (0.2 mL of viscous materials, 0.1 mL of liquids, or solid material) is placed within the opening in the dressing on top of the treated skin. Two layers of #2 filter paper should be placed over the test article, then backed by occlusive tape. Then the filter paper/occlusive backed material should be secured to the surrounding dressing with adhesive tape. After 2 days have passed, the filter paper should be lifted from the test sites, and the test article reapplied on the same site. The filter paper and backing should be secured once again. After 2 more days, the filter paper should be lifted and two injections of 0.075 mL of FCA should be administered into the edges of the test site. Then the test material is once again applied, and the filter paper/backing resecured. The test article should be reapplied once more on day 7 and the filter paper/backing resealed. On day 9, the filter paper and all associated dressing material should be removed.

CHALLENGE PHASE

On day 22 following the induction treatment, 0.5 mL of test material (or the solid article) should be applied to a 2- × 2-cm area of shaved midback. The test sites should be covered by filter paper and backed by adhesive tape. This should be held in place with an elastic bandage secured with adhesive tape. Control animals receive the same challenge phase treatment. The preparation should be removed after 24 hours.

OBSERVATIONS

Twenty-four, 48, and 72 hours after the removal of the challenge phase preparation, the test sites should be evaluated for erythema and edema. The grading scheme of *Table 5* could be employed.

Mouse Ear Swelling Test

There are a number of potential advantages in using mice versus guinea pigs for sensitization methods. The classic guinea pig tests tend to be costly and require a long time to complete. Moreover, with the dependence upon relatively subjective scoring based on edema and erythema, methodological robustness, and ruggedness may be questionable. This test uses mice and employs both topical exposures and injections.

ANIMALS

Female, 6- to 8-week old CF-1, Balb/c, or Swiss mice should be used. They may be group housed in direct bedding cages. Acclimatization should be for at least 5 to 7 days. Food (appropriate mouse feed) and water should be available ad libitum. No animals with damaged pinnae should be used in the study. The thickness of both ears of each animal should be measured and recorded at this time.

TEST MATERIAL PREPARATION

As with other test procedures that incorporate intradermal injections, the test article needs to be in a form suitable for

injection. See *Biological Reactivity Tests, In Vivo* (88) for information on the extraction procedure.

PRELIMINARY TESTING

The minimally irritating and maximally nonirritating concentrations of test article for this procedure should be determined. This is done by using four groups of two mice and examining the effects of at least four concentrations of test article.

INDUCTION PHASE

The abdomens of the animals should be shaved, then tape-stripped using a surgical adhesive tape until the test area is glistening. A single injection of 0.05 mL of FCA is subdivided into two injection sites administered intradermally within the shaved/stripped area, but along the borders. After the adjuvant injections, 100 μ L of test article (using the minimally irritating concentration) or vehicle (controls) is applied to the center of the shaved test areas. After the test areas dry, the mice should be returned to their cages. The tape stripping and application of test article (but not FCA) is repeated each day for the next 3 days.

CHALLENGE PHASE

This phase should occur 7 days after the final topical induction application. The test article (highest nonirritating concentration) should be applied topically (20 μ L) to one ear, while the opposite ear receives 10 μ L of vehicle alone. This should be done for both test and control animals.

OBSERVATIONS

The thickness of both ears of each animal should be recorded after 24 and 48 hours postchallenge. The measurements should be made with a caliper (a spring-loaded caliper is preferable). A sensitized animal is one in which the test article-treated ear is at least 20% thicker than its opposite ear. For the test to be valid, the test article-treated ears of control animals should not be more than 10% thicker than the opposite ears. If the control animal ears do not meet the requirements, the test should be repeated using lower concentrations.

Local Lymph Node Test

This test is based on the observation that exposure of the mice to sensitizers can cause hyperplasia of T cells within the auricular lymph nodes of mice. The method combines both in vivo and in vitro phases, and requires the use of radioisotopes. An unusual aspect of this test is that no challenge phase is required.

ANIMALS

Four groups of four mice at least, male or female CBA/ca mice (only one sex in a given test) between the ages of 8 to 12 weeks should be used.

TEST MATERIAL PREPARATION

Although in theory one could apply a solid test article to the dorsal surface of the ear of a mouse, in practice an extract of such an article should be used. See *Biological Reactivity Tests, In Vivo* (88) for information on the extraction procedure.

PRELIMINARY TESTING

A nontoxic concentration of test article should be used. If not already established, a preliminary test for overt toxicity may be required to establish a suitable dose.

INDUCTION PHASE

Twenty-five μ L of the appropriate test article concentration, or vehicle (controls), should be applied to the dorsal surface of each pinna for 3 consecutive days. Five days after the first treatment, the animals should be injected, via the tail vein, with 2.5 mL of phosphate buffered saline containing 20 μ Ci of 3 H-methyl thymidine. Five hours after the isotopic injection, the animals should be euthanized. The draining auricular lymph nodes should be removed from each animal of each test and control group. The nodes from all animals within a given group should be combined, such that a single cell suspension can be made from each group of animals. The cell suspension can be made by passing the nodes through a 200-mesh stainless steel gauze using a syringe plunger. The cells should then be centrifuged at 190 \times g for 10 minutes, resuspended in 3 mL of 5% trichloroacetic acid (TCA), and held overnight at 4°.

The resulting precipitate should be recovered by centrifugation, and the pelleted precipitate should be resuspended in 1 mL of 5% TCA. The suspension should then be placed in scintillation vials with 10 mL of scintillation fluid, and the disintegrations/minute (dpm) counted with a β -counter.

OBSERVATIONS

The ratio of dpm for each test group should be compared to the dpm for the control group. If the ratio equals or exceeds 3 for any test group, the concentration of test article used with that group may be considered to be sensitizing.

Vitamin A Enhancement Test

This test is similar to the *Mouse Ear Swelling Test* in that test articles are applied topically to the abdomen, with a challenge application to the ears, followed by measurements of ear thickness. A principal difference is the use of mouse feed supplemented with vitamin A acetate. The purpose of the supplementation is to increase the reactivity of the immune system, thereby increasing the potential sensitization reaction.

ANIMALS

Male, 3- to 4-week old Balb/c mice should be maintained on a diet supplemented with vitamin A acetate. The diet may be prepared by mixing each kg of feed with 0.477 g of gelatinized vitamin A acetate. The feed mixture should be used within 3 weeks of preparation. Mice intended for use in sensitization studies should have been on the supplemented diet for at least 4 weeks. The mice at the time of the sensitization study should therefore be between 7 and 10 weeks old. The thickness of both ears of each animal should be measured and recorded at this time.

TEST MATERIAL PREPARATION

Although, in theory, one could apply a solid test article to the dorsal surface of the ear of a mouse, in practice an extract of such an article should be used. See *Biological Reactivity Tests, In Vivo* (88) for information on the extraction.

PRELIMINARY TESTING

The maximally nonirritating dose and minimally irritating concentrations should be determined using separate groups of animals. This could be done as described for *Preliminary Testing in the Mouse Ear Swelling Test*.

INDUCTION PHASE

The fur of the abdomen and thorax of 10 mice per group should be shaved. Then 100 µL of test article (at the minimally irritating concentration) should be applied to the test areas on days 0, 2, 4, 7, and 11. Control animals receive 100 µL of vehicle alone on the same schedule.

CHALLENGE PHASE

This phase should occur 4 days after the final application of the *Induction Phase*. Twenty-five µL of test article (at the maximally nonirritating concentration) should be applied to each ear of each animal in the test and control groups.

OBSERVATIONS

Ear thickness for both ears of each animal should be recorded after 24 and 48 hours postchallenge. The measurements should be made with a caliper (a spring-loaded caliper is preferable). The percent increase in ear thickness should be calculated for each ear by subtracting the pretreatment measurement from the post-treatment measurement, dividing the result by the pretreatment measurement, then multiplying by 100. The response of the test group versus the control group should be compared statistically. (The Mann-Whitney U test could be used for the comparison.)

The results of individual animals should also be calculated. If an increase in ear thickness for an animal from the test group is at least 50% greater than the largest increase of a control animal, that is indicative of sensitization. As an overall evaluation, should the results of the study provide a significant result of the statistical test at $p < 0.01$ for the control versus test group comparisons, or if at least two test animals have ear thickness increases in excess of 50% of the maximum control thickness changes and the group comparison showed a $p < 0.05$, sensitization is indicated for the test article.

<1191> STABILITY
CONSIDERATIONS IN
DISPENSING PRACTICE

NOTE—Inasmuch as this chapter is for purposes of general information only, no statement in the chapter is intended to modify or supplant any of the specific requirements pertinent to Pharmacopeial articles, which are given elsewhere in this Pharmacopeia.

Aspects of drug product stability that are of primary concern to the pharmacist in the dispensing of medications are discussed herein.

Pharmacists should avoid ingredients and conditions that could result in excessive physical deterioration or chemical decomposition of drug preparations, especially when compounding (see *Pharmaceutical Compounding—Nonsterile*

Preparations <795>). The stability and clinical effect of manufactured dosage forms can be greatly compromised by seemingly negligible alterations or inappropriate prescription compounding. Pharmacists should establish and maintain compounding conditions that include the ensuring of drug stability to help prevent therapeutic failure and adverse responses.

Stability—Stability is defined as the extent to which a product retains, within specified limits, and throughout its period of storage and use (i.e., its shelf-life), the same properties and characteristics that it possessed at the time of its manufacture. Five types of stability generally recognized are shown in the accompanying table.

Criteria for Acceptable Levels of Stability

Type of Stability	Conditions Maintained Throughout the Shelf Life of the Drug Product
Chemical	Each active ingredient retains its chemical integrity and labeled potency, within the specified limits.
Physical	The original physical properties, including appearance, palatability, uniformity, dissolution, and suspendability, are retained.
Microbiological	Sterility or resistance to microbial growth is retained according to the specified requirements. Antimicrobial agents that are present retain effectiveness within the specified limits.
Therapeutic	The therapeutic effect remains unchanged.
Toxicological	No significant increase in toxicity occurs.

FACTORS AFFECTING PRODUCT STABILITY

Each ingredient, whether therapeutically active or pharmaceutically necessary, can affect the stability of drug substances and dosage forms. The primary environmental factors that can reduce stability include exposure to adverse temperatures, light, humidity, oxygen, and carbon dioxide. The major dosage form factors that influence drug stability include particle size (especially in emulsions and suspensions), pH, solvent system composition (i.e., percentage of “free” water and overall polarity), compatibility of anions and cations, solution ionic strength, primary container, specific chemical additives, and molecular binding and diffusion of drugs and excipients. In dosage forms, the following reactions usually cause loss of active drug content, and they usually do not provide obvious visual or olfactory evidence of their occurrence.

Hydrolysis—Esters and β -lactams are the chemical bonds that are most likely to hydrolyze in the presence of water. For example, the acetyl ester in aspirin is hydrolyzed to acetic acid and salicylic acid in the presence of moisture, but in a dry environment the hydrolysis of aspirin is negligible. The aspirin hydrolysis rate increases in direct proportion to the water vapor pressure in an environment.

The amide bond also hydrolyzes, though generally at a slower rate than comparable esters. For example, procaine (an ester) will hydrolyze upon autoclaving, but procainamide will not. The amide or peptide bond in peptides and proteins varies in the lability to hydrolysis.

The lactam and azomethine (or imine) bonds in benzodiazepines are also labile to hydrolysis. The major chemical accelerators or catalysts of hydrolysis are adverse pH and specific chemicals (e.g., dextrose and copper in the case of ampicillin hydrolysis).

Epimerization—Members of the tetracycline family are most likely to incur epimerization. This reaction occurs rapidly when the dissolved drug is exposed to a pH of an intermediate range (higher than 3), and it results in the steric rearrangement of the dimethylamino group. The epimer of

tetracycline, epitetraacycline, has little or no antibacterial activity.

Decarboxylation—Some dissolved carboxylic acids, such as *p*-aminosalicylic acid, lose carbon dioxide from the carboxyl group when heated. The resulting product has reduced pharmacological potency.

β -Keto decarboxylation can occur in some solid antibiotics that have a carbonyl group on the β -carbon of a carboxylic acid or a carboxylate anion. Such decarboxylations will occur in the following antibiotics: carbenicillin sodium, carbenicillin free acid, ticarcillin sodium, and ticarcillin free acid.

Dehydration—Acid-catalyzed dehydration of tetracycline forms epianhydrotetracycline, a product that both lacks antibacterial activity and causes toxicity.

Oxidation—The molecular structures most likely to oxidize are those with a hydroxyl group directly bonded to an aromatic ring (e.g., phenol derivatives such as catecholamines and morphine), conjugated dienes (e.g., vitamin A and unsaturated free fatty acids), heterocyclic aromatic rings, nitroso and nitrite derivatives, and aldehydes (e.g., flavorings). Products of oxidation usually lack therapeutic activity. Visual identification of oxidation, for example, the change from colorless epinephrine to its amber colored products, may not be visible in some dilutions or to some eyes.

Oxidation is catalyzed by pH values that are higher than optimum, polyvalent heavy metal ions (e.g., copper and iron), and exposure to oxygen and UV illumination. The latter two causes of oxidation justify the use of antioxidant chemicals, nitrogen atmospheres during ampul and vial filling, opaque external packaging, and transparent amber glass or plastic containers.

Photochemical Decomposition—Exposure to, primarily, UV illumination may cause oxidation (photo-oxidation) and scission (photolysis) of covalent bonds. Nifedipine, nitroprusside, riboflavin, and phenothiazines are very labile to photo-oxidation. In susceptible compounds, photochemical energy creates free radical intermediates, which can perpetuate chain reactions.

Ionic Strength—The effect of the total concentration of dissolved electrolytes on the rate of hydrolysis reactions results from the influence of ionic strength on interionic attraction. In general, the hydrolysis rate constant is inversely proportional to the ionic strength with oppositely charged ions (e.g., drug cation and excipient anions) and directly proportional to the ionic strength with ions of like charge. A reaction that produces an ion of opposite charge to the original drug ion because of the increasing ionic strength, can increase the drug hydrolysis rate as the reaction proceeds. High ionic strength of inorganic salts can also reduce the solubility of some other drugs.

pH Effect—The degradation of many drugs in solution accelerates or decelerates exponentially as the pH is decreased or increased over a specific range of pH values. Improper pH ranks with exposure to elevated temperature as a factor most likely to cause a clinically significant loss of drug, resulting from hydrolysis and oxidation reactions. A drug solution or suspension, for example, may be stable for days, weeks, or even years in its original formulation, but when mixed with another liquid that changes the pH, it degrades in minutes or days. It is possible that a pH change of only 1 unit (e.g., from 4 to 3 or 8 to 9) could decrease drug stability by a factor of 10 or greater.

A pH buffer system, which is usually a weak acid or base and its salt, is a common excipient used in liquid preparations to maintain the pH in a range that minimizes the drug degradation rate. The pH of drug solutions may also be either buffered or adjusted to achieve drug solubility. For example, pH in relation to pK_a controls the fractions of the usually more soluble ionized and less soluble nonionized species of weak organic electrolytes.

The influence of pH on the physical stability of two phase systems, especially emulsions, is also important. For example, intravenous fat emulsion is destabilized by acidic pH.

Interionic (Ion^{N+} – Ion^{N-}) Compatibility—The compatibility or solubility of oppositely charged ions depends mainly on the number of charges per ion and the molecular size of the ions. In general, polyvalent ions of opposite charge are more likely to be incompatible. Thus, an incompatibility is likely to occur upon the addition of a large ion with a charge opposite to that of the drug.

Solid State Stability—Solid state reactions are relatively slow; thus, stability of drugs in the solid state is rarely a dispensing concern. The degradation rate of dry solids is usually characterized by first-order kinetics or a sigmoid curve. Therefore, solid drugs with lower melting point temperatures should not be combined with other chemicals that would form a eutectic mixture.

When moisture is present, the solid drug decomposition may change to zero-order chemical kinetics because the rate is controlled by the relatively small fraction of the drug that exists in a saturated solution, which is located (usually imperceptibly) at the surface or in the bulk of the solid drug product.

Temperature—In general, the rate of a chemical reaction increases exponentially for each 10° increase in temperature. This relationship has been observed for nearly all drug hydrolysis and some drug oxidation reactions. The actual factor of rate increase depends on the activation energy of the particular reaction. The activation energy is a function of the specific reactive bond and the drug formulation (e.g., solvent, pH, additives). As an example, consider a hydrolyzable drug that is exposed to a 20° increase in temperature, such as that from cold to controlled room temperature (see *General Notices and Requirements*). The shelf life of the drug at controlled room temperature should be expected to decrease to one-fourth to one-twenty-fifth of its shelf life under refrigeration.

The pharmacist should also be aware that inappropriately cold temperatures may cause harm. For example, refrigeration may cause extreme viscosity in some liquid drugs and cause supersaturation in others. Freezing may either break or cause a large increase in the droplet size of emulsions; it can denature proteins; and in rare cases, it can cause less soluble polymorphic states of some drugs to form.

STABILITY STUDIES IN MANUFACTURING

The scope and design of a stability study vary according to the product and the manufacturer concerned. Ordinarily the formulator of a product first determines the effects of temperature, light, air, pH, moisture, trace metals, and commonly used excipients or solvents on the active ingredient(s). From this information, one or more formulations of each dosage form are prepared, packaged in suitable containers, and stored under a variety of environmental conditions, both exaggerated and normal. At appropriate time intervals, samples of the product are assayed for potency by use of a stability-indicating method, observed for physical changes, and, where applicable, tested for sterility and/or for resistance to microbial growth and for toxicity and bioavailability. Such a study, in combination with clinical and toxicological results, enables the manufacturer to select the optimum formulation and container and to assign recommended storage conditions and an expiration date for each dosage form in its package.

Responsibility of Pharmacists

Pharmacists help to ensure that the products under their supervision meet acceptable criteria of stability by (1) dispensing oldest stock first and observing expiration dates, (2) storing products under the environmental conditions stated in the individual monographs, labeling, or both, (3) observ-

ing products for evidence of instability, (4) properly treating and labeling products that are repackaged, diluted, or mixed with other products, (5) dispensing in the proper container with the proper closure, and (6) informing and educating patients concerning the proper storage and use of the products, including the disposition of outdated or excessively aged prescriptions.

Rotation of Stock and Observance of Expiration

Dates—Proper rotation of stock is necessary to ensure the dispensing of suitable products. A product that is dispensed infrequently should be closely monitored so that old stocks are given special attention, particularly with regard to expiration dates. The manufacturer can guarantee the quality of a product up to the time designated as its expiration date only if the product has been stored in the original container under recommended storage conditions.

Storage under Recommended Environmental

Conditions—In most instances, the recommended storage conditions are stated on the label, in which case it is imperative to adhere to those conditions. They may include a specified temperature range or a designated storage place or condition (e.g., "refrigerator," or "controlled room temperature") as defined in the *General Notices*. Supplemental instructions, such as a direction to protect the product from light, also should be followed carefully. Where a product is required to be protected from light and is in a clear or translucent container enclosed in an opaque outer covering, such outer covering is not to be removed and discarded until the contents have been used. In the absence of specific instructions, the product should be stored at controlled room temperature (see *Storage Temperature* in the *General Notices*). The product should be stored away from locations where excessive or variable heat, cold, or light prevails, such as those near heating pipes or fluorescent lighting.

Observing Products for Evidence of Instability—Loss of potency usually results from a chemical change, the most common reactions being hydrolysis, oxidation-reduction, and photolysis. Chemical changes may also occur through interaction between ingredients within a product, or rarely between product and container. An apparent loss of potency in the active ingredient(s) may result from diffusion of the drug into, or its combination with, the surface of the container-closure system. An apparent gain in potency usually is caused by solvent evaporation or by leaching of materials from the container-closure system.

The chemical potency of the active ingredient(s) is required to remain within the limits specified in the monograph definition. Potency is determined by means of an assay procedure that differentiates between the intact molecule and its degradation products. Chemical stability data should be available from the manufacturer. Although chemical degradation ordinarily cannot be detected by the pharmacist, excessive chemical degradation sometimes is accompanied by observable physical changes. In addition, some physical changes not necessarily related to chemical potency, such as change in color and odor, formation of a precipitate, or clouding of solution, may serve to alert the pharmacist to the possibility of a stability problem. It should be assumed that a product that has undergone a physical change not explained in the labeling may also have undergone a chemical change, and such a product is never to be dispensed. Excessive microbial growth, contamination, or both, may also appear as a physical change. A gross change in a physical characteristic such as color or odor is a sign of instability in any product. Other common physical signs of deterioration of dosage forms include the following.

Solid Dosage Forms—Many solid dosage forms are designed for storage under low-moisture conditions. They require protection from environmental water and therefore should be stored in tight containers (see *Containers* in the *General Notices*) or in the container supplied by the manufacturer. The appearance of fog or liquid droplets, or clumping of the product, inside the container signifies improper conditions. The presence of a desiccant inside the manufac-

turer's container indicates that special care should be taken in dispensing. Some degradation products, for example, salicylic acid from aspirin, may sublime and be deposited as crystals on the outside of the dosage form or on the walls of the container.

HARD AND SOFT GELATIN CAPSULES—Since the capsule formulation is encased in a gelatin shell, a change in gross physical appearance or consistency, including hardening or softening of the shell, is the primary evidence of instability. Evidence of release of gas, such as a distended paper seal, is another sign of instability.

UNCOATED TABLETS—Evidence of physical instability in uncoated tablets may be shown by excessive powder and/or pieces (i.e., crumbling as distinct from breakage) of tablet at the bottom of the container (from abraded, crushed, or broken tablets); cracks or chips in tablet surfaces; swelling; mottling; discoloration; fusion between tablets; or the appearance of crystals that obviously are not part of the tablet itself on the container walls or on the tablets.

COATED TABLETS—Evidence of physical instability in coated tablets is shown by cracks, mottling, or tackiness in the coating and the clumping of tablets.

DRY POWDERS AND GRANULES—Dry powders and granules that are not intended for constitution into a liquid form in the original container may cake into hard masses or change color, which may render them unacceptable.

POWDERS AND GRANULES INTENDED FOR CONSTITUTION AS SUSPENSIONS—Dry powders and granules intended for constitution into solutions or suspensions require special attention. Usually such forms are antibiotics or vitamins that are particularly sensitive to moisture. Since they are always dispensed in the original container, they generally are not subject to contamination by moisture. However, an unusual caked appearance necessitates careful evaluation, and the presence of a fog or liquid droplets inside the container generally renders the preparation unfit for use. Presence of an objectionable odor also may be evidence of instability.

EFFERVESCENT TABLETS, GRANULES, AND POWDERS—Effervescent products are particularly sensitive to moisture. Swelling of the mass or development of gas pressure is a specific sign of instability, indicating that some of the effervescent action has occurred prematurely.

Liquid Dosage Forms—Of primary concern with respect to liquid dosage forms are homogeneity and freedom from excessive microbial contamination and growth. Instability may be indicated by cloudiness or precipitation in a solution, breaking of an emulsion, nonresuspendable caking of a suspension, or organoleptic changes. Microbial growth may be accompanied by discoloration, turbidity, or gas formation.

SOLUTIONS, ELIXIRS, AND SYRUPS—Precipitation and evidence of microbial or chemical gas formation are the two major signs of instability.

EMULSIONS—The breaking of an emulsion (i.e., separation of an oil phase that is not easily dispersed) is a characteristic sign of instability; this is not to be confused with creaming, an easily redispersible separation of the oil phase that is a common occurrence with stable emulsions.

SUSPENSIONS—A caked solid phase that cannot be resuspended by a reasonable amount of shaking is a primary indication of instability in a suspension. The presence of relatively large particles may mean that excessive crystal growth has occurred.

TINCTURES AND FLUIDEXTRACTS—Tinctures, fluidextracts, and similar preparations usually are dark because they are concentrated, and thus they should be scrutinized carefully for evidence of precipitation.

STERILE LIQUIDS—Maintenance of sterility is of course critical for sterile liquids. The presence of microbial contamination in sterile liquids usually cannot be detected visually, but any haze, color change, cloudiness, surface film, particulate or flocculent matter, or gas formation is sufficient reason to suspect possible contamination. Clarity of sterile solutions in-

tended for ophthalmic or parenteral use is of utmost importance. Evidence that the integrity of the seal has been violated on such products should make them suspect.

Semisolids (Creams, Ointments, and Suppositories)—For creams, ointments, and suppositories, the primary indication of instability is often either discoloration or a noticeable change in consistency or odor.

CREAMS—Unlike ointments, creams usually are emulsions containing water and oil. Indications of instability in creams are emulsion breakage, crystal growth, shrinking due to evaporation of water, and gross microbial contamination.

OINTMENTS—Common signs of instability in ointments are a change in consistency and excessive “bleeding” (i.e., separation of excessive amounts of liquid) and formation of granules or grittiness.

SUPPOSITORIES—Excessive softening is the major indication of instability in suppositories, although some suppositories may dry out and harden or shrivel. Evidence of oil stains on packaging material should warn the pharmacist to examine individual suppositories more closely by removing any foil covering. As a general rule (although there are exceptions), suppositories should be stored in a refrigerator (see *Storage Temperature* in the *General Notices*).

Proper Treatment of Products Subjected to Additional Manipulations—In repackaging, diluting a product or mixing it with another product, the pharmacist may become responsible for its stability.

Repackaging—In general, repackaging is inadvisable. However, if repackaging is necessary, the manufacturer should be consulted concerning potential problems. In the filling of prescriptions, it is essential that suitable containers be used. Appropriate storage conditions and, when appropriate, an expiration date and beyond use date should be indicated on the label of the prescription container. Single-unit packaging calls for care and judgment and for strict observance of the following guidelines: (1) use appropriate packaging materials, (2) if stability data on the new package are not available, repackage at any one time only sufficient stock for a limited time, (3) include on the unit-dose label a lot number and an appropriate beyond-use date, (4) if a sterile product is repackaged from a multiple-dose vial into unit-dose (disposable) syringes, discard the latter if not used within 24 hours, unless data are available to support longer storage, (5) if quantities are repackaged in advance of immediate need, maintain suitable repackaging records showing name of manufacturer, lot number, date, and designation of persons responsible for repackaging and for checking (see *General Notices*), (6) if safety closures are required, use container closure systems that ensure compliance with compendial and regulatory standards for storage.

Dilution or Mixing—If a product is diluted, or if two products are mixed, the pharmacist should observe good professional and scientific procedures to guard against incompatibility and instability. For example, tinctures such as those of belladonna and digitalis contain high concentrations of alcohol to dissolve the active ingredient(s), and they may develop a precipitate if they are diluted or mixed with aqueous systems. Pertinent technical literature and labeling should be consulted routinely; it should be current literature, because at times formulas are changed by the manufacturer. If a particular combination is commonly used, consultation with the manufacturer(s) is advisable. Since the chemical stability of extemporaneously prepared mixtures is unknown, the use of such combinations should be discouraged; if such a mixture involves an incompatibility, the pharmacist might be responsible. Oral antibiotic preparations constituted from powder into liquid form should never be mixed with other products.

Combining parenteral products necessitates special care, particularly in the case of intravenous solutions, primarily because of the route of administration. This area of practice demands the utmost in care, aseptic technique, judgment, and diligence. Because of potential unobservable problems

with respect to sterility and chemical stability, all extemporaneous parenteral preparations should be used within 24 hours unless data are available to support longer storage.

Informing and Educating the Patient—As a final step in meeting responsibility for the stability of drugs dispensed, the pharmacist is obligated to inform the patient about the proper storage conditions (for example, in a cool, dry place—not in the bathroom) for both prescription and non-prescription products, and to suggest a reasonable estimate of the time after which the medication should be discarded. When beyond-use dates are applied, the pharmacist should emphasize to the patient that the dates are applicable only when proper storage conditions are observed. Patients should be encouraged to clean out their drug storage cabinets periodically.

<1195> SIGNIFICANT CHANGE GUIDE FOR BULK PHARMACEUTICAL EXCIPIENTS

BACKGROUND

This general information chapter was derived from an international guidance on the evaluation of the significance of changes involving the manufacture of bulk pharmaceutical excipients. It is intended to assist excipient manufacturers in determining the need for informing the excipient user and regulatory authorities about the nature of the change.

The chapter provides minimum recommendations when considering the effect of a change in the manufacturing process on the excipient. When deciding how to use this chapter, each manufacturer must consider how it may apply to that manufacturer's product and processes. The diversity of excipients means that some principles of this chapter may not be applicable to certain products and processes.

This chapter is divided into several sections. The first section provides the general guidance necessary for evaluating a change and determining the necessity of informing the user and/or regulatory authorities. One section provides criteria for determining whether a change will involve a significant risk. Also included is a decision tree that is useful in considering the potential effect of a change on excipient performance.

INTRODUCTION

Purpose

This document is meant to establish uniform considerations for evaluating the significance of changes involving the manufacture of bulk pharmaceutical excipients (BPEs). The purpose of the evaluation is to determine the need for informing the excipient user and regulatory authorities about the nature of the change.

Scope

The principles and information in this chapter can be applied to the manufacture of all bulk pharmaceutical excipients [referred to throughout this document as “excipient(s)”] intended for use in human drugs, veterinary drugs,

and biologics. The principles set forth here must be applied once it has been determined that a chemical is intended for use as a component of a drug product. As the excipient manufacturing process progresses, the degree of assurance concerning the quality of the product should increase and should be controlled and documented. However, at some logical processing step, as determined by the manufacturer, the GMP as described in *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* (1078) should be applied and maintained. Judgment, based on risk analysis and a thorough knowledge of the process, is required to determine from which processing step the GMPs should be implemented.

Principles Adopted

This chapter should be of international application, bearing in mind that pharmaceutical excipients are diverse and often have uses other than pharmaceutical applications. It provides minimum recommendations when considering the impact of a change on the excipient. As an international guidance document, it cannot specify all national legal requirements nor cover in detail the particular characteristics of every excipient.

When considering how to use this chapter, each manufacturer should consider how it may apply to that manufacturer's product and processes. The diversity of excipients means that some principles of the chapter may not be applicable to certain products and processes. The terminology "should" and "it is recommended" do not necessarily mean "must" as used in the application of this chapter.

Excipients may contain minor components that are known to be or might be necessary for the correct functioning of the excipient. The presence of these "essential concomitant components" in the excipient should not be construed as undesirable. These concomitant components are not considered part of the impurity profile, but should be considered separately. Water may be a concomitant component in some excipients, but may be included in the impurity profile for others. (See *Impurity Profile* in *Appendix C* for more information.)

Layout

This chapter is divided into several sections. The first section provides a background discussion necessary for evaluating a change and determining the necessity of informing the user and/or regulatory authorities. A second section provides criteria for determining the risk that a change will be significant, including guidance on development of an impurity profile. Also included are the following: The *Glossary* contains terms and definitions used in all sections of this document. *Appendix A* includes some examples of changes that would be classified into each of the three risk levels. *Appendix B* provides a decision tree useful in considering the potential impact of a change on excipient performance. *Appendix C* delineates the development of an impurity profile.

GENERAL GUIDANCE

Differentiation of Excipient Manufacture

Evaluating the impact of a change in the manufacture of an excipient could be more difficult than that for an active pharmaceutical ingredient (API). Although the API is seldom used for more than a handful of therapeutic purposes, the BPE is often used with a broad range of active ingredients and in a diverse range of finished dosage forms. Whereas the API is typically of high purity and well characterized by the quality control and analytical laboratory, the BPE is often a natural substance, mixture, or polymer, the chemical and

physical properties of which are more difficult to quantify. For a more thorough discussion of GMPs that apply to excipient manufacture, see the general information chapter *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* (1078).

Definition of Significant Change

Any change by the manufacturer of an excipient that alters an excipient's physical or chemical property outside the established limits, or that is likely to alter the excipient performance in the dosage form, is considered significant. Such changes may necessitate notifying the local regulatory authority if required. Regardless of whether there is a regulatory requirement, the manufacturer has an obligation to notify its users of a significant change so that the user can evaluate the impact of the change on the user's products. It is suggested that unless there is clear indication from evaluation of the change that it is not significant as stipulated by this general chapter, the pharmaceutical user should be notified.

The types of change that are considered here are changes to the following: site, scale, equipment, process, packaging and labeling, and specification (including raw materials).

The requirement for evaluating the impact of change on the excipient begins, at a minimum, with the raw materials for the first processing step from where GMP compliance begins. GMP requirements increase as the manufacturing process progresses. Thus, at some logical processing step, usually well before the final finishing operation, appropriate GMPs should be imposed and maintained throughout the remainder of the process. Methods such as HACCP (Hazard Analysis and Critical Control Point), FEMA (Failure Effects Mode Analysis), or a detailed process flow diagram may be used to identify the unit operations, required equipment, stages at which various substances are added, key steps in the process, critical parameters (time, temperature, pressure, etc.), and necessary monitoring points. Judgment, based on risk analysis and a thorough knowledge of the process, is required to determine at which processing step the GMP should be implemented.¹

It is important to give careful consideration to any processing changes that occur after the excipient has been synthesized or isolated but prior to packaging. However, it must be recognized that a change made earlier in the process can result in a change in the excipient functionality, and it is recommended that such changes also be considered.

SIGNIFICANT CHANGE

Evaluation Criteria

These criteria, in the form of questions, are presented for consideration when evaluating the impact of a change relating to excipient manufacture:

1. Has there been a change in the chemical properties or composition of the excipient as a result of the change?
2. Has there been a change in the physical properties of the excipient as a result of the change?
3. Has there been a change in the "essential concomitant components" profile for the excipient as a result of the change?
4. Has there been a change in the functionality of the excipient as a result of the change?
5. Where applicable, has the moisture level changed?
6. Where applicable, has the bioburden changed?
7. Has there been a change in the origin of any raw materials or contact packaging?

¹ See chapter (1078), *GMPs For Excipients*.

An affirmative answer to any of these questions indicates that the impact of the change on the excipient may lead to changes in its performance in the dosage form.

It is important to provide objective criteria for evaluating when a change has occurred in an excipient property or composition, in the essential concomitant components profile, in biological origin, or in its functionality. This enables the BPE manufacturer to evaluate the significance of the change on the excipient for the purpose of notifying the regulatory authorities and/or the user.

Determination of Significance

Criterion 1: Evaluation of the chemical properties or composition of an excipient should include, at a minimum, all monograph and manufacturer specification parameters. A comparison of these test results for the excipient before and after a change should be done to determine if there is a statistically significant difference.

Criterion 2: Physical properties should be considered based upon the physical form of the excipient and its functionality as known or as used by the end users. A physical property that is part of a mutually agreed-upon specification between the manufacturer and end user should also be evaluated. For example, a manufacturer of an excipient powder should consider measuring the impact of changes on such physical parameters as bulk density, surface area, particle shape, and particle size distribution. Liquid excipients might be evaluated for changes in their pH and viscosity. For all polymeric excipients, the effect of a change on a physical property, such as molecular weight distribution, should be considered.

Criterion 3: Objective criteria are also necessary when considering changes to the “essential concomitant components” profile for an excipient as a result of changes. The profile, as noted in *Appendix C*, contains the following:

- identified organic impurities,
- unidentified organic impurities at or above 0.10%, whether specified or not,²
- residual solvents, and
- inorganic impurities

The feasibility of developing an impurity profile varies with the composition and origin of the excipient. It is important to note that identifying and quantifying impurities in some excipients are extremely difficult. Thus, an excipient manufacturer may not have developed an impurity profile. In that case, it is important for excipient manufacturers to either document their efforts to identify and quantify the impurities that may be present to justify their limited results or to justify other means by which changes may be evaluated.

The significance of the change can be determined by comparing the impurity profile of the pre-change material with that of the post-change product. Therefore, once the profile has been developed, it should be reassessed following changes to the process. An impurity should be monitored as part of the profile if it is present at or greater than 0.10%, if it has an established physiological effect, or if it is known to be unsafe at a lower level.

The content of the impurity profile varies with the nature of the excipient, the raw materials used in its manufacture, and its chemical composition. Where possible, changes are considered significant whenever a new impurity is introduced at the 0.10% concentration or higher, or when an impurity previously present at or greater than 0.10% disappears. Changes to the quantity of an existing impurity specified in a monograph and reported on the Certificate of Analysis (COA) should be treated as a chemical property for the purposes of this evaluation.

² It is recognized that while desirable, it may not be possible to achieve this for all excipients, particularly those of a more complex chemical nature, e.g. natural polymers, for which there may be no adequate means of determining related substances. However, the impurity profile documentation should demonstrate why this was not achievable.

Changes in the levels of residual solvents should be considered when determining the significance of change. See *Residual Solvents* (467) for details.

Criterion 4: Objective criteria for evaluating changes to excipient functionality are desirable. However, the nature of this type of study can vary broadly based upon the excipient and its application in the dosage form. It must also be recognized that the excipient manufacturer does not always know each use of the excipient. Therefore this chapter cannot provide objective criteria for this study but stresses the importance of such a consideration by the manufacturer. If there is the potential that the functionality of the excipient may be affected by the change, users should be notified and material provided upon request so that they can determine the impact of the change in their finished pharmaceutical products.

Criterion 5: Often the excipient contains moisture, the presence of which can have an impact on excipient performance in the preparation of the pharmaceutical dosage form. Therefore a change in the moisture level beyond the range typical of production, even though within the compendial or specification limit, can affect its stability and/or end use.

Criterion 6: Change in the processing steps, raw materials, or equipment can adversely affect control of microorganisms in the excipient. Therefore the effect of the change on the bioburden should be evaluated, particularly for excipients susceptible to microbial growth.

Criterion 7: Change in the origin of a raw material or contact packaging can result in a change to the other six change criteria. Change in origin can involve the country of origin, geological origin, or species of origin for the raw material.

A change in the country of origin of a raw material or contact packaging material can affect the status of the excipient as it relates to the potential presence of bovine spongiform encephalopathies (BSEs), transmissible spongiform encephalopathy (TSE) material, or genetically modified organisms (GMOs). The country of origin of animal raw material, or of components used in the manufacture of the raw material, can result in noncompliance with relevant TSE regulations.^{3,4} Current information on BSE and related diseases can be accessed on the United States Department of Agriculture (USDA) website (usda.gov).

A change in the geological origin of a mineral-based excipient can alter the composition of the excipient. Geological formations containing the same mineral can differ in their chemical composition, crystalline structure, density, etc. A change in geological origin of raw material can affect the excipient's chemical or physical properties, the impurity profile, or excipient functionality.

A change to the species of origin for raw materials of either animal or vegetable origin can raise concern. Switching from one animal species to another can affect the status of the excipient as it relates to the presence of BSE or TSE material in the excipient, as noted above. Switching from animal-derived to plant-derived raw materials, although eliminating the issue of BSE or TSE material, raises the potential for the presence of plant-based allergenic material in the excipient. Switching from one plant species to another also can result in the possible presence of an allergen in the excipient. In addition to this issue with allergens, use of plant-derived raw materials can affect pharmaceutical manufacturers who have a concern about the presence of GMOs in the excipient.

³ European Pharmacopoeia, General Text 5.2.8, *Minimizing the Risk of Transmitting Animal Spongiform Encephalopathy Agents Via Medicinal Products*.

⁴ U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS), Federal Register: November 4, 2003, Volume 68, Number 213 (Proposed Rules), 9 CFR Parts 93, 94, and 95, *Bovine Spongiform Encephalopathy; Minimal Risk Regions and Importation of Commodities*.

Risk Levels

In the evaluation of the effect of changes on the excipient, it is recognized that even with objective criteria, some judgment may be necessary. To facilitate the decision as to the significance of a change and the likely effect on the dosage form, the types of changes are classified using three levels:

- Level 1: Minor Change
- Level 2: Might Be Significant
- Level 3: Always Significant

LEVEL 1: MINOR CHANGE

These changes are considered unlikely to affect the excipient's chemical or physical properties, impurity profile, or functionality. Such changes should be documented, but notifications to the users and regulatory authorities are not necessary.

LEVEL 2: MIGHT BE SIGNIFICANT

The effect of the change should be evaluated against *Criterion 1*, *Criterion 2*, and *Criterion 3* (chemical and physical properties and impurity profile) to determine its potential effect on excipient functionality. A change in the biological origin of a raw material should be considered with regard to TSE or GMO regulations. A Level 2 change should always be communicated to the users and regulatory authorities.

LEVEL 3: ALWAYS SIGNIFICANT

This type of change should always be communicated to the users and regulatory authorities. Shipment of the changed excipient to the user should not occur without consent from the user's company.

Protocol Design

There should be a written protocol for the evaluation of a change to determine whether it is significant. The protocol should describe the nature of the change, the reason it may be significant, the testing to be performed to evaluate the change, and the criteria for determining the significance. If the change is attributable to a new biological source for raw materials used in the manufacture of the excipient, it is recommended that the regulatory status of the raw material (i.e., BSE/TSE, GMO agents) be evaluated first. Then, where possible, the results from the testing of a minimum of 10 pre- and 3 post-change batches of excipient should be compared (see *Supporting Data*, below). If significant changes are seen, then an assessment of the significance should be made.

The manufacturer should test the excipient made after the change for all specification properties and compare the results to the historical data. A standard statistical test, such as a *t*-test of the means, should be used to compare the new data with the historical data. If when using an appropriate statistical analysis there is sufficient evidence that the populations are different at the 95% confidence interval, the change should be considered significant. As an additional check on consistency, it is also recommended that the new batch specification properties be plotted on standard Statistical Quality Control (SQC) control charts, along with standard batch results.

Supporting Data

It is preferable to use data to measure the effect of a change on the excipient. The comparison should begin with

chemical and physical properties, followed, where appropriate, by moisture, bioburden, impurity profile, and functionality. The manufacturer should use good judgment on sample comparisons for the other evaluations.

Chemical and physical properties lend themselves to quantitative measurement. Often these properties are part of the specification for the excipient. As such there should be a large body of test data to use for the properties affected for comparison to the corresponding data of the excipient made after the change.

Equivalence of impurity profiles is shown by comparing the data for the pre-change and post-change batches. If the following conditions are met, there has been no significant change in the impurity profile. [NOTE—*Residual Solvents* (467) notes that under certain circumstances an impurity concentration below 0.10% may be of concern and the excipient manufacturer should take this under consideration.]

1. No new impurity is present at or above 0.10%, nor has an impurity at this level disappeared that was previously in the impurity profile.
2. Residual solvent and impurities remain within the 95% confidence interval of the mean of the batches produced before the change.

TYPES OF CHANGES

Site Change

A change in site can involve either the production or packaging of the excipient or its quality control testing. If the proposed manufacturing site was never used to produce the excipient, then the change poses a greater risk of altering the excipient's performance and is considered a *Level 3* change. If the proposed site was used for this purpose within the past year and the process, equipment, utilities, and raw materials are all unchanged, the risk is considered minor and thus a *Level 1* change. However, if the excipient was produced before at the proposed site with the same process, equipment, utilities, and raw materials more than 1 year ago, the risk is moderate and thus *Level 2*.

If the change involves the quality control laboratory, then the impact hinges on the test method. If the method remains the same, the change is a *Level 1*, provided a formal method transfer or validation is conducted. If the new lab uses a different analytical technique or analytical equipment, then the change should be evaluated more carefully, as required by a *Level 2* change.

Scale

Manufacturers often find ways to increase the scale of production. If the excipient is being scaled up from pilot to production, the change is likely to be significant and thus a *Level 3*. When the change in scale results from the use of new and larger, or smaller, production equipment using the same operating principle, which is often the case in batch processing, the change is a *Level 2*. If the existing equipment is optimized to increase capacity without altering the process, often found in continuous processing, the change is considered minor and treated as *Level 1* provided that a comparison of pre- and post-change data shows no statistically significant difference. However, careful consideration should be given to changes that are made that can clearly affect the properties of the excipient.

Equipment

The evaluation of equipment change concerns the issue of whether it is equivalent to the equipment it replaces. Generally, equipment that is a replacement in kind is considered a minor *Level 1* change. If the new equipment is not a re-

placement in kind but is included in the process validation, then the change is still a *Level 1*. Otherwise the change is considered *Level 3*.

Manufacturing Process

A change in process often involves changes to the processing instructions, such as target levels for such parameters as temperature, pressure, and flow rate; the raw materials to be used; the sequence of operating steps; and the operation to be performed, including reprocessing. As illustrated in the decision tree in *Appendix B*, each type of process change can be further detailed.

If there is a change in a process parameter that is within the process validation, such as operating at a new target within the qualified range, then the change is a *Level 1*. However, if the process parameter is outside of the validation, then the change should be evaluated as a *Level 2*.

If minor changes are made to the processing steps, such as a small change outside of the validated range in the rate of addition of an ingredient, then the change is a *Level 2*. A major change, such as changing the point at which an ingredient is added to earlier or later in the process, is potentially significant and thus a *Level 3*.

Reprocessing of an excipient followed by a purification step, when not typical of the process, should be evaluated as a *Level 2* change. However, if no further purification of the bulk excipient occurs, this type of change is considered a *Level 3*.

Packaging and Labeling

These changes involve the package components meant for protection and distribution of the excipient. Any change in the package or packaging components such as the drum, box, liner, or tamper-evident seal that is a replacement in kind is a minor change (*Level 1*). Replacement in kind applies to containers constructed of the same materials and sealed in a similar manner, and liners made of the same components. Any change that is not a replacement in kind should be evaluated as a *Level 3*. Any change to labeling pertaining to the site of manufacture or testing, the biological origin, additives, or storage and handling conditions should be evaluated as a *Level 3*.

Specifications

Differences in raw materials can be further defined by the supplier used, their specifications, biological origin, country of origin for those derived from animals, or the addition to or removal of the raw material from the BPE process. If the new supplier provides its raw material against a specification essentially the same as that of the former supplier and the raw material method of manufacture is similar, the change is minor and treated as *Level 1*. However, if the specifications, biological origin, or country of origin changes, or the manufacturing process is different, then the change should be evaluated as potentially significant (*Level 2*). Also, any change of source for an animal-origin material should be treated as a *Level 2* change, if the source is determined to be not from a risk country as codified in 9 CFR 94.18. Finally, if the change in raw material involves the addition or removal of an ingredient from the process to produce or preserve the BPE or is otherwise used to produce the bulk excipient, the change is likely to be significant (*Level 3*). Similar consideration should be given for any change in origin of raw materials that results in a potential that the raw material might contain risk materials (i.e., BSE, TSE, allergens, or GMOs).

Changes are sometimes made to the excipient specification or the quality control test method. When changes are not the result of a monograph change, their significance should be evaluated. Such test or specification changes may

be made to the excipient product or to the intermediate component. [NOTE—In some circumstances, relaxing the specification may lower the quality if the specification is for a significant property; therefore any change needs to be evaluated and its significance needs to be documented.]

Changes to an excipient specification or test method are *Level 3* changes. For example, adding a new specification parameter for the purpose of improving the quality of the excipient through lot selection is potentially a very significant *Level 3* change. If the specification change relaxes a specification parameter, the effect on excipient quality should be evaluated as a *Level 2* change. An example of a minor change is the additional testing of the excipient initiated with the sole purpose of further characterizing the material without altering its quality, and is a *Level 1* risk; however, notification is supported.

If a specification for a raw material from the same supplier(s) is made more stringent, then the change is unlikely to be significant (*Level 1*), whereas if the specification becomes less stringent, then the change should be evaluated carefully (*Level 2*).

When a change is made that either increases or maintains the level of process control in the manufacturing process, it should be treated as a *Level 1*. If the change in process control relaxes the control, then the effect should be carefully evaluated as *Level 2*. An illustrative example is pH control. If a new pH meter allows for more precise measurement, the process control is improved and the change falls under *Level 1*. However, if the pH control is relaxed by using a less precise measuring device, the change is treated as *Level 2*.

Multiple Changes

Multiple changes involve more than one change occurring simultaneously. The risk level for consideration of the impact of the changes should be the highest level for any single change. However, the effect of the totality of changes should also be assessed, because this may suggest that the overall risk is higher.

REPORTING REQUIREMENTS

Documentation

It is recommended that the evaluation of changes to the excipient be documented, regardless of the level of change. The report should indicate the basis for evaluating the effect of the change on the excipient, the significance of the data used in reaching the conclusion, and the actions taken.

Where appropriate, the process validation should be updated to reflect the changed process. This is clearly indicated where the evaluation has led to the conclusion that the change should be considered significant.

Notification

The user should be given as much advance notification of impending change as possible. For *Level 3* changes in particular, the user may require time to complete the evaluation of the impact of the change on their formulations. During this period the user may request inventory of the excipient produced before the change was made. The manufacturer should plan for the change with this eventuality in mind.

Regardless of the apparent level of the change, changes that are found to meet the definition of significant change resulting from the evaluation require user notification.

Regulatory authorities often require notification of significant changes involving the manufacture of excipients. Such notification should be done as required by the applicable authority.

GLOSSARY

ACTIVE PHARMACEUTICAL INGREDIENT (API): Any substance or mixture of substances that is intended to be used in the manufacture of a drug (medicinal) product and that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or animals.

BATCH PROCESS: A manufacturing process that produces the excipient from a discrete supply of the raw materials that are present before the completion of the reaction.

BIOBURDEN: The nature and quantity of microorganisms present in the excipient.

BIOLOGICAL ORIGIN: Defined as either animal origin or nonanimal origin, based on the source of the raw material used in the manufacture of the excipient, and also includes materials that potentially come into contact with equipment used in the manufacture of other materials with animal-derived or GMO-derived components.

BOVINE SPONGIFORM ENCEPHALOPATHY (BSE): A pathological brain deterioration condition of cattle believed to be caused by a prion that can be transmitted to cause variant Creutzfeldt-Jakob disease (vCJD) in humans.

BULK PHARMACEUTICAL EXCIPIENT (BPE): See *Excipient*.

CHEMICAL PROPERTY: A quality parameter that is measured by chemical or physicochemical test methods.

CONCOMITANT COMPONENT: A substance found in an excipient that is not the intended chemical entity, but may be necessary for ensuring the proper performance of the excipient in its intended use, and is not an impurity or a foreign substance (formerly referred to as a minor component).

CONFIDENCE INTERVAL: A range, calculated from sample data, within which a population parameter, such as the population mean, is expected to lie, with a given level of confidence.

CONTINUOUS PROCESS: A manufacturing process that continually produces the excipient from a continuous supply of raw material.

DECISION TREE: A visual presentation of the sequence of events that can occur, including decision points.

DRUG SUBSTANCE: See *Active Pharmaceutical Ingredient*.

EQUIPMENT: The implements used in the manufacture of an excipient.

EXCIPIENT: Any substance, other than the drug substance, in a drug product that has been appropriately evaluated for safety and is included in a drug delivery system to either aid the processing of the drug product during its manufacture; protect, support or enhance stability, bioavailability, or patient acceptability; assist in product identification; or enhance any other attribute of the overall safety and effectiveness of the drug product during storage or use.

FOREIGN SUBSTANCE: A component that is present in the BPE but that is *not* introduced into the excipient as a consequence of its synthesis or purification and is not necessary to achieve the required functionality (formerly referred to as a contaminant).

FUNCTIONALITY: The set of performance criteria that the excipient is intended to meet when used in a formulation.

GENETICALLY MODIFIED ORGANISM (GMO): Living organisms such as animals, plants, or microbes with an altered genetic makeup produced using a special set of technologies.

IMPURITY: A component of an excipient that is not the intended chemical entity or a concomitant component but is present as a consequence of either the raw materials used or the manufacturing process and is not a foreign substance.

IMPURITY PROFILE: A description of the impurities present in the excipient.

MASS BALANCE: The sum of the quantifiable material present in the excipient.

PACKAGING: The container and its components that hold the excipient for transport to the user.

PHYSICAL PROPERTY: A quality parameter that can be measured solely by physical means.

PHYSIOLOGICAL EFFECT: Any effect on the normal health of the human body.

PROCESS: The set of operating instructions describing how the excipient is to be synthesized, isolated, purified, packaged, etc.

PROCESS PARAMETER: A measurable operating condition.

PROCESS STEP: An instruction to the BPE manufacturing personnel directing that an operation be done.

PROCESS VALIDATION: A documented program that provides a high degree of assurance that a specific process will consistently produce a result that will meet predetermined acceptance criteria.

RAW MATERIAL: Any substance used in the production of an excipient, excluding packaging materials.

REPLACEMENT IN KIND: Manufacturing equipment that uses the same operating principles and is of similar construction or packaging components made with the same materials of construction and sealed in a similar manner.

REPROCESSING: Introduction of previously processed material that did not conform to standards or specifications back into the process and with repetition of one or more necessary steps that are part of the normal manufacturing process.

RESIDUAL SOLVENT: An organic volatile chemical that is used or produced in the manufacture of excipients. The residual solvent is not completely removed by practical manufacturing techniques.

SCALE: An increase or decrease in the batch size in batch processing or the throughput capability for continuous processing, whether or not different equipment is used.

SIGNIFICANT CHANGE: A change that alters an excipient's physical or chemical property from the norm or that is likely to alter the excipient's performance in the dosage form.

SITE: A defined location of the equipment in which the excipient is manufactured. It may be within a larger facility. A change in site may be to a different part of the existing facility but in a different operational area or may be to a remote facility, including a contract manufacturer.

SOLVENT: A vehicle, other than water, used in the synthesis of the product that remains chemically unchanged.

SPECIFICATION: The quality parameters to which the excipient, component, or intermediate must conform and that serve as a basis for quality evaluation.

STATISTICAL QUALITY CONTROL (SQC): The plotting of sequential test results to show their variation relative to the specification range and their normal variation.

TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHY (TSE): Any agent that causes a symptomatic illness in animals or humans akin to BSE and variant Creutzfeldt-Jakob disease (vCJD), e.g., scrapie in sheep.

APPENDIX A: CHANGE LEVELS

For guidance, examples of changes that typically would be classified into the three change levels are provided.

Level 1

1. A processing parameter changed to a new set point that is within the process validation.
2. Use of alternate equipment that is listed as an alternate in a regulatory document (i.e., Drug Master File).
3. Use of equipment that is a replacement in kind. This is typically new equipment that uses the same operating principles as the equipment replaced.
4. Revision to a specification for one of the excipient's raw materials that involves more stringent quality or conformance to additional pharmacopeias.

5. Addition of a test parameter or tightening of an existing parameter to an excipient specification that is used for informational purposes only. This is not used for quality improvement or control purposes.
6. Improved environmental control to prevent cross-contamination of the excipient. An example of this is an improved packaging room or additional segregation of manufacturing equipment.

Level 2

1. A processing parameter changed to a new set point that is outside of the process validation.
2. A site change returning the manufacture of an excipient to a site previously used for this purpose more than 1 year ago.
3. Process control that is outside the normal limits of variability. An example of this is new process control equipment for control of excipient properties not previously controlled that create process adjustments.
4. A change in the handling, storage, or delivery of the excipient. An example of a handling change is the movement of a powder with new powder-conveying equipment. The storage of the excipient in bulk versus the shipping container is illustrative of a change in storage. The delivery of the excipient in temperature-controlled trucks versus uncontrolled trucks exemplifies a change in delivery but not vice versa.
5. A change in container size or shape.

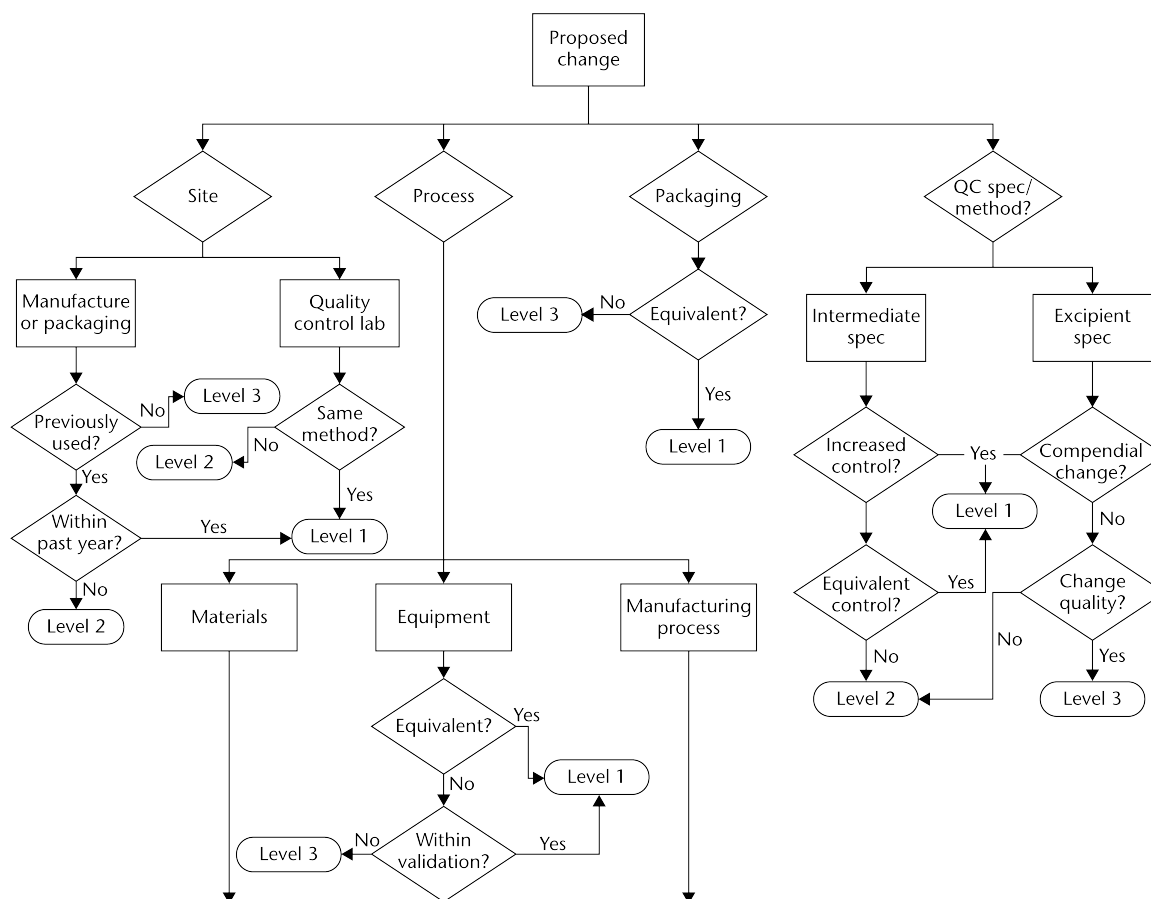
Level 3

1. Addition or removal of a chemical entity from the manufacturing process. An example would be the addition or removal of a preservative agent, buffering agent, stabilizer, or catalyst.
2. Manufacture at a new site never used for this purpose.
3. Revision to a sales specification made for the purpose of improving the quality of the excipient either through improved process control or lot selection.
4. Use of a new package, such as a drum of a different construction (i.e., plastic versus steel).
5. Revision of the product label.
6. Revision of the tamper-evident seal.
7. A change to the stated shelf life or retest interval.

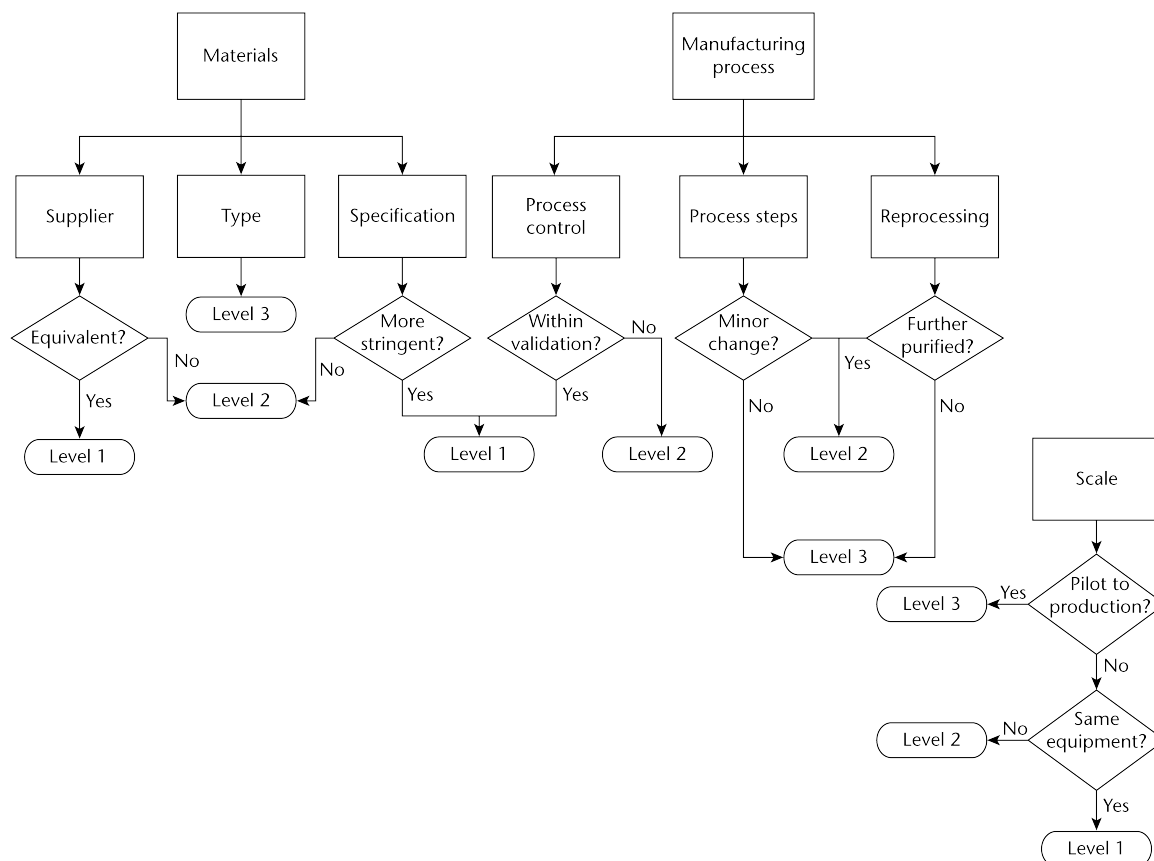
APPENDIX B: DECISION TREE

A decision tree has been developed to aid in classifying the change into levels. The diagram begins with the proposed change and guides the BPE maker to an indication of the likelihood that the change will affect the excipient user. The decision tree classifies the types of change that occur in excipient manufacture as involving the site of manufacture, the processing steps, packaging, and testing and quality control.

Appendix B: Decision tree



Appendix B : Decision tree (continued)

**APPENDIX C: IMPURITY PROFILE****Definition of Impurity Profile**

The impurity profile of an excipient may be defined as a description of the impurities present in a typical lot of excipient produced by a given manufacturing process. The impurity profile includes the identity of each major impurity or an appropriate qualitative description, such as peak retention time (if unidentified), the quantity of impurity observed expressed as a range, and the classification, as discussed below, of each identified impurity. Excipients frequently function because they are not “pure”. That is to say that often there are concomitant components that are necessary for the correct functioning of the excipient. These essential concomitant components should not be considered as part of the impurity profile but should be evaluated separately, if possible.

The composition of the impurity profile is dependent upon such variables as the raw materials, solvents, reagents, catalysts, and manufacturing process used in the excipient’s manufacture. Foreign substances, such as manufacturing aids that can be present in the excipient, should be controlled to a level that is unobjectionable.⁵

It is recognized that the presence of essential concomitant components is important to the performance of the excipient in the drug product. Therefore, the presence of these essential concomitant components in the excipient should neither be construed as being undesirable nor be confused with the presence of foreign substances or impurities.

⁵ Current USP General Notices.

It should be noted that in some excipients, water may be an essential concomitant component, necessary to achieve the desired functionality. For other excipients, water may be included in the impurity profile, if appropriate, and should be classified as an inorganic impurity in such circumstances.

Use of the Impurity Profile

The impurity profile, as used in this chapter, is meant to help determine the significance of a change. Impurities should be profiled by the excipient manufacturer if possible. This may be accomplished through knowledge of the starting materials and manufacturing process and subsequent application of validated analytical testing to provide a qualitative and/or quantitative result of the impurity profile.

Procedure for Development of an Impurity Profile

Because of the diverse nature of substances that may be incorporated as pharmaceutical excipients, including highly complex mixtures from animal, botanical, mineral, and/or synthetic sources, differing approaches to characterizing their properties may be required. It is recognized that the development of an impurity profile may not be technically feasible for certain excipients. In such cases, the manufacturer should document what method is being used to monitor the excipient for the effect of changes as noted in this chapter in *Evaluation Criteria and Determination of Significance in Significant Change*.

Classification of Impurities

Excipient impurities are classified as follows.

Organic Impurities: Any organic material that arises during the manufacturing process that is not listed as the intended excipient in the monograph or specification and is not a concomitant component or foreign substance. This may include starting materials, byproducts, intermediates, reagents, ligands, and catalysts.

Inorganic Impurities: Any inorganic material that arises during the manufacturing process that is not listed as the intended excipient in the monograph or specification and is not a concomitant component or foreign substance. This may include starting materials, byproducts, intermediates, reagents, ligands, and catalysts.

Residual Solvents: Solvents resulting from the incomplete removal of organic or inorganic liquids, regardless of the source. See *Residual Solvents* (467) for details. Note that the limits specified apply to the drug product as considered in *Option 2* and to the excipient as in *Option 1*. It should be noted that a residual solvent can also be classified as a concomitant component but still must be considered.

Impurity Profile

The characterization of the impurity profile of an excipient should be attempted by the manufacturer, where possible, by taking into account the manufacturing process and potential impurities anticipated as a consequence. A sensible approach includes control of all impurities that have known toxicological characteristics. The limits of these impurities may be based upon the usage of the drug product when so informed by the user and should comply with the requirements of ICH Q3B(R) *Impurities in Drug Products* and of *Residual Solvents* (467).

For the purpose of developing an impurity profile, excipients may be classified as those where purity can be directly measured and those where purity cannot be directly measured. Examples of the former are excipients whose monograph or specification includes a requirement for purity. Polymers or derivatives of naturally occurring products are often examples of excipients where purity cannot be directly measured.

The material to be used for the development of the impurity profile should be sampled using the same sampling technique and sampling point in the manufacturing process as the sample taken for use in the quality control release of the lot.

Excipients for Which Purity Can Be Measured

A mass balance is desirable, but it is recognized that a mass balance of 100% cannot generally be achieved because of the inherent limitation in accuracy and precision of the available tests, as well as the possible lack of suitable tests for some components. Mass balance of the excipient composition should be computed through the addition of the organic impurities, inorganic impurities, residual solvents, and excipient. If there are measurable essential concomitant components, they should be included with the excipient for purposes of this calculation. The purpose of calculating the mass balance is to estimate the amount of material not measured in the impurity profile. The excipient manufacturer should include in the report of the development of the impurity profile the mass balance achieved and what are thought to be the components not fully quantified.

Organic Impurities: Identify each impurity at or greater than 0.10% using appropriate analytical techniques. If organic impurities cannot be identified, a qualitative description, such as chromatographic retention time, should be as-

signed for all impurities at or greater than 0.10%. If direct measurement of organic impurities is not possible, total *Organic Impurities* can be reported as:

$$100 - (\text{Inorganic Impurities} + \text{Residual Solvents} + \text{Excipient})$$

Inorganic Impurities: Identify each impurity at or above 0.10% using appropriate analytical techniques. If direct measurement of inorganic impurities is not possible, total *Inorganic Impurities* may be estimated as:

$$100 - (\text{Organic Impurities} + \text{Residual Solvents} + \text{Excipient})$$

Residual Solvents: Report the solvents present by classification (see *Residual Solvents* (467)) and level.

Excipients for Which Purity Cannot Be Measured

Although a mass balance of the excipient composition of 100% is desirable, it is recognized that this goal is often technically difficult, if not impossible, to achieve. Therefore, manufacturers should include reports of the development of the impurity profile, the mass balance achieved, and what are thought to be the components not otherwise quantified.

For excipients produced by continuous chemical processing, it may not be possible to calculate a chemical mass balance, only an overall process balance.

Where direct measurement of the excipient purity is not feasible, techniques should be used to provide an estimate of excipient purity. This information is then applied in the equations listed above under *Excipients for Which Purity Can Be Measured*.

Documentation

The excipient supplier should develop documentation to support the development of an impurity profile. This documentation can be compiled in various ways by the supplier so that it can be retrieved to support the impurity profile. Documentation of an excipient impurity profile should include the following information:

1. Sampling plan
2. Analytical test methods
3. Identity and quantity of each component of the excipient, including both the excipient components and identified impurities
4. Discussion of the uncertainty in the measurement of each component of the excipient and impurity
5. Discussion of the mass balance

(1196) PHARMACOPEIAL HARMONIZATION

This general information chapter provides information about the concept of harmonization by the Pharmacopeial Discussion Group (PDG). The chapter provides: (1) the *PDG Policy Statement*; (2) the *PDG Working Procedures*; (3) a discussion; (4) a status report; and (5) a glossary.

HARMONIZATION POLICY

The following policy statement was approved by the PDG at its September 2002 meeting.

General Information

In 1989, the PDG was formed with representatives from the European Directorate for the Quality of Medicines in the Council of Europe, the United States Pharmacopeial Convention, Inc., and the Japanese Pharmacopoeia in the Ministry of Health and Welfare—now the Ministry of Health, Labor, and Welfare (MHLW). Since that time, the PDG generally meets twice a year to work on pharmacopeial harmonization topics. In May 2001, the PDG welcomed the World Health Organization as an observer. While not part of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), the PDG usually meets in conjunction with the ICH and provides the ICH Steering Committee with reports of its progress. To facilitate harmonization of some ICH Quality guidelines and the Quality section of the Common Technical Document, the PDG representatives sometimes attend ICH expert working group discussions as observers.

Purpose

A pharmacopeial monograph for an active ingredient or excipient, preparation, or other substance used in the manufacture or compounding of a medicinal product generally provides a name, definition, description, and sometimes packaging, labeling, and storage statements. Thereafter, the monograph provides tests, procedures, and acceptance criteria that constitute the specification. For frequently cited procedures, a monograph may refer to a general chapter for editorial convenience. The PDG works to harmonize excipient monographs and general chapters. This will reduce manufacturers' burden of performing analytical procedures in different ways, using different acceptance criteria. At all times, the PDG works to maintain an optimal level of science consistent with protection of the public health.

Definition of Harmonization

The PDG has defined harmonization of a pharmacopeial monograph or general chapter as follows:

"A pharmacopeial general chapter or other pharmacopeial document is harmonized when a pharmaceutical substance or product tested by the document's harmonized procedure yields the same results and the same accept/reject decision is reached."

When using a fully harmonized pharmacopeial monograph or general chapter, an analyst will perform the same procedures and reach the same accept/reject decisions irrespective of which PDG pharmacopeia is referenced. This approach is called interchangeability, and each pharmacopeia will identify, in an appropriate manner, such a monograph or general chapter.

When full harmonization of a pharmacopeial monograph or general chapter is not possible, the PDG works to harmonize it using an approach termed harmonization by attribute. In this approach, some elements of a monograph or general chapter may be harmonized, but others may not. When a monograph is harmonized by attribute, a combination of approaches is needed. For nonharmonized elements, reliance on the individual PDG pharmacopeia is necessary.

Process

Harmonization of pharmacopeial documents in the PDG is based upon decisions of the expert bodies of each pharmacopeia. The PDG works transparently in many ways, principally through the public notice and comment procedures of each pharmacopeia. The details are described below under *PDG Working Procedures*.

Implementation

The implementation of a harmonized document varies in the three PDG regions, depending upon their legal requirements, need for translation, and publication schedules. Each pharmacopeia generally allows some period of time after publication to implement official harmonized texts to allow manufacturers and other users to achieve conformity. Harmonization is not achieved until the text becomes official in all three pharmacopeias.

Revision of Harmonized Monographs

The pharmacopeias participating in the PDG have agreed not to revise unilaterally any harmonized document after publication. Should revisions be necessary for any appropriate reasons, the initiating pharmacopeia notifies the PDG, and revision proceeds according to the *PDG Working Procedures*.

PDG WORKING PROCEDURES

Working Procedures of the PDG were updated at the October 2006 PDG meeting.

General

Harmonization may be carried out retrospectively for existing monographs or chapters or prospectively for new monographs or chapters.

The three pharmacopeias have a commitment to respect the agreed working procedures and the associated time deadlines as an essential part of the harmonization procedure.

Harmonization of pharmacopeial documents in the PDG is performed on the basis of decisions of the expert bodies of each pharmacopeia. The PDG works transparently in many ways, but principally through the public notice and comment procedures of each pharmacopeia.

Where necessary, meetings of experts are held to identify potential solutions to difficult problems.

The specific stages of the PDG procedure (Process) involved in harmonization are described below.

Stage 1: Identification

On the basis of an inquiry among its users, the PDG identifies subjects to be harmonized among PDG pharmacopeias and nominates a coordinating pharmacopeia for each subject.

The PDG distributes the work by consensus among the three pharmacopeias and strives for a balance in the distribution of assignments to coordinating pharmacopeias.

Stage 2: Investigation

For a subject to be harmonized retrospectively, the coordinating pharmacopeia collects the information on the existing specifications in the three pharmacopeias, on the grades of products marketed, and on the potential analytical procedures.

The coordinating pharmacopeia prepares a draft monograph or chapter, accompanied by a report giving the rationale for the proposal with validation data.

Stage 2 ends with the proposal draft, which is mentioned in this procedure as a Stage 3 draft. The Stage 3 draft, accompanied by supporting comments or data that explain the reasons for each test procedure or limit proposed, is sent by the coordinating pharmacopeia to the secretariats of the other two PDG pharmacopeias.

Stage 3: Proposal for Expert Committee Review

The three pharmacopeias forward the Stage 3 draft to their expert committee (through meetings or consultation by correspondence).

Comments by the experts resulting from this preliminary survey are sent to their respective pharmacopeial secretariat, preferably within 2 months. However, the comment period should not exceed 4 months. Within 2 months of receipt of the comments, the pharmacopeial secretariat should consolidate the comments and forward them to the coordinating pharmacopeia.

The coordinating pharmacopeia reviews the comments received and prepares a harmonized document (Stage 4 draft) accompanied by a commentary discussing comments received about the previous text and providing reasons for action taken in response to those comments.

The Stage 4 draft, as far as possible written in global style—a style easily understood by a variety of readers—together with the commentary, is sent to the secretariats of the other pharmacopeias (end of Stage 3).

Stage 4: Official Inquiry

The Stage 4 draft and the commentary are published in the revision document or forum of each pharmacopeia in a section entitled International Harmonization. The draft is published in its entirety.

The corresponding secretariats may have to add information essential to the understanding of the implementation of the texts (e.g., the description of an analytical procedure or of reagents that do not exist in the pharmacopeia), and a translation is added by the European and Japanese Pharmacopeias. The style may be adapted to that of the pharmacopeia concerned, or global style may be used. The three pharmacopeias endeavor to publish the drafts simultaneously or as closely as possible.

Comments regarding this draft are sent by readers of the revision document to their respective pharmacopeial secretariat, preferably within 4 months and at most within 6 months of its publication.

Each pharmacopeia analyzes the comments received and submits its consolidated comments to the coordinating pharmacopeia within 2 months of the end of the review or comment period.

The coordinating pharmacopeia reviews the comments received and prepares a draft harmonized document (Stage 5A draft), accompanied by a commentary discussing comments received regarding the previous text and providing reasons for action taken in response to those comments.

The Stage 5A draft, together with the commentary, is sent to the secretariats of the other two PDG pharmacopeias.

Stage 5. Consensus

5A. PROVISIONAL

The Stage 5A draft is reviewed and commented on by the other two PDG pharmacopeias within 4 months of receipt. The three pharmacopeias shall do their utmost to reach full agreement at this stage to obtain a final consensus document.

If a consensus has not been reached, the coordinating pharmacopeia prepares a revised version (Stage 5A/2), taking into consideration relevant, substantiated comments on the Stage 5A document from the two other pharmacopeias. The revised document (Stage 5A/2), together with the commentary, is sent to the secretariats of the other two PDG

pharmacopeias. The revised document is reviewed and commented on by the other two PDG pharmacopeias, preferably within 2 months of receipt. This review or comment and revision process of the 5A document is repeated (Stage 5A/n) until the three PDG pharmacopeias reach a consensus or until the coordinating pharmacopeia considers that harmonization by attribute should be applied.

If the coordinating pharmacopeia considers certain attributes in the monograph or provisions in a general chapter (especially for retroactive harmonization) are such that it will not be possible to harmonize within a reasonable time period, harmonization by attribute will be applied. If harmonization by attribute is applied, a special cover page (see the table in the *Appendix*) indicating harmonization is included with the draft. The text contains harmonized attributes and provisions, and nonharmonized and local attributes are not included. The nonharmonized attributes are clearly indicated in the text as such. The table is prepared as follows: if three pharmacopeias agree on the attribute, there will be a (+) in all columns; if two pharmacopeias agree that the attribute should be included and have agreed on the method and limit, there will be a (+) in the column for those two pharmacopeias, and a (–) in the column for the pharmacopeia that will not stipulate the test.

For nonharmonized or local requirements, if three pharmacopeias agree that the attribute should be included, but have not come to agreement on the method or limit: state attribute under “nonharmonized attributes.” If only one pharmacopeia will include an attribute: state under “local requirement.”

If the Stage 5A draft is substantially different from the Stage 4 draft, the PDG may decide that it should be published again in the revision documents; the draft then reverts technically to Stage 4, revised.

5B. DRAFT SIGN-OFF

When agreement is reached, the 5B draft is sent by the coordinating pharmacopeia to the other pharmacopeias no later than 4 weeks before a PDG meeting for final confirmation. The document is then presented for sign-off at the PDG meeting. This document includes nonharmonized attributes clearly marked as such.

Stage 6: Regional Adoption and Implementation

NOTE—The last two stages of the implementation of the “harmonized” chapters and monographs take place independently according to the procedures established by each pharmacopeial organization.

6A. REGIONAL ADOPTION AND PUBLICATION

The document is submitted for adoption to the organization responsible for each pharmacopeia. Each pharmacopeia incorporates the harmonized draft according to its own procedures.

Adopted texts are published by the three pharmacopeias in their supplements, or where applicable, in a new edition.

If necessary, the Stage 5B draft may be adopted with some amendments (local requirements) corresponding to a general policy in the national or regional (European) area. If a pharmacopeia includes a local attribute after the sign-off of a text, it will inform the PDG. It is, however, preferred to include the nonharmonized text in Stage 5B as an alert to the other pharmacopeias that there will be some differences in text in the final document.

Users of the pharmacopeias are appropriately informed of the harmonization status of monographs and general chapters. In the *European Pharmacopoeia (EP)* and *USP–NF*, for general chapters, this is done via a preliminary paragraph.

For the *Japanese Pharmacopoeia (JP)*, a notification is made by the MHLW, and information is given in a general chapter.

6B. IMPLEMENTATION

The pharmacopeias will inform each other of the date of implementation in their particular region.

The date of implementation of a harmonized document varies in the three PDG regions depending on their legal requirements, need of translation, and publication schedules. Each pharmacopeia generally allows some period of time after publication for implementation to allow manufacturers and other users to achieve conformity. Harmonization is not achieved until the text becomes official in all three pharmacopeias.

6C. INDICATION OF HARMONIZATION

Each pharmacopoeia will introduce a statement indicating the harmonization status. EP and USP reference the corresponding text of the other PDG pharmacopeias. JP references the harmonized text. In case of residual differences, these are indicated by a specific symbol (black diamond ◆).

Harmonization is achieved when all pharmacopeias have highlighted harmonization and any residual differences, based on a general policy in the national or regional area.

Concurrent to Stages 6A, B, and C, a dialogue is opened between PDG and ICH Q4B Expert Working Group for the purpose of obtaining regulatory acceptance of the harmonized text. The coordinating pharmacopeia provides documents to ICH Q4B EWG as defined in the ICH Q4B Guideline.

Stage 7: Inter-Regional Acceptance

Following the Q4B evaluation process, a formal notification of regulatory acceptance is posted by ICH.

A topic-specific annex to the Q4B guideline for each monograph or chapter concerned is processed for publishing and implementation by each regional authority.

Revision

The procedure for the revision of harmonized monographs and chapters is as follows.

The pharmacopeias participating in the PDG have agreed not to revise unilaterally any harmonized document (monograph or chapter) after sign-off or after publication.

A pharmacopeia requesting the revision of a monograph or chapter shall apply the following criteria for justification of the revision:

- Public health and safety reasons.
- Insufficient supply of pharmacopeial-quality product on the market.
- Specified analytical reagents or equipment unavailable.
- New methods of preparation of products or reagents not covered by the current monograph.
- Analytical procedures capable of being replaced by more appropriate, accurate, or precise procedures.

The PDG as a whole has to agree to initiate the revision. A coordinating pharmacopeia will be nominated. The coordinating pharmacopeia will prepare a Stage 3 draft, based on the validation of data provided by the pharmacopeia requesting the revision.

The *PDG Working Procedures* will then be followed. The revisions of a sign-off document prepared for this or other reasons are indicated as revision 1, 2, 3, etc.

In case of health and safety issues, and whenever agreed to by the PDG, an accelerated procedure shall be applied (shortening or eliminating stages).

Discussion

Harmonization of general chapters and monographs benefits manufacturers of pharmaceutical products intended for human use, regulatory agencies, and ultimately, practitioners and patients. Benefits are derived from (1) reduced development effort; (2) simplification of regulatory filings; and (3) reduced release testing.

Pharmacopeial harmonization amplifies the work of the ICH, particularly for Quality topics. While the PDG is not part of the ICH, the PDG periodically provides updates to the ICH Steering Committee, and in the past participated in a joint task force. This task force focused on harmonization of general chapters considered important to the ICH harmonized document *Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (Q6A)*. USP also participates in the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products (VICH). As with the ICH, some of the quality guidelines developed in VICH depend upon harmonization of pharmacopeial general chapters. A major difference between the PDG and ICH/VICHs is that the ICH/VICH guidelines generally are applicable only to ingredients and drug products not previously registered in an ICH/VICH region or nation, whereas the PDG harmonization applies to all marketed products in the applicable region or nation.

In the case of harmonization by attribute, nonharmonization or divergence will be indicated in *USP–NF* and *EP* by the symbol ◆. For these nonharmonized attributes, reliance upon the individual pharmacopeia is required. A monograph or general chapter in one PDG pharmacopeia may unilaterally include additional local or national attributes that are not included in the corresponding text of the other pharmacopeias. Such text is not considered by the PDG to be a divergence from the PDG harmonized text.

As with other *USP–NF* revisions, draft harmonization texts are published for comment in *Pharmacopeial Forum*. Final harmonized official text in *USP–NF* is presented in the latest edition, *Supplement*, or *Interim Revision Announcement*. The current status of all harmonization projects appears in *Table 1* and *Table 2*. These status tables will be updated in subsequent editions of *USP–NF* and its *Supplements*.

In the U.S., cases of noncompliance or dispute are resolved through performance of the official procedure in *USP* or *NF*. If the procedure and its acceptance criteria are harmonized in the PDG, then a manufacturer may follow the relevant compendial instructions in *USP–NF*, *EP*, or *JP*.

Table 1. Status of Harmonization—Excipient Monographs

Excipient Name	Coordinating Pharmacopeia	Harmonization Stage
Alcohol (Rev 2)	EP	4
Benzyl Alcohol (Rev 1)	EP	6
Dehydrated Alcohol (Rev 2)	EP	4
Butylparaben	EP	6
Calcium Carbonate	USP	4
Calcium Disodium Edetate	JP	6
Calcium Phosphate Dibasic (and anhydrous)	JP	6
Carmellose Calcium (Rev 1)	USP	6
Carmellose Sodium	USP	4
Carmellose	JP	4
Cellulose Acetate (Rev 1)	USP	6

Table 1. Status of Harmonization—Excipient Monographs (Continued)

Excipient Name	Coordinating Pharmacopeia	Harmonization Stage
Cellulose Acetate Phthalate	USP	6
Microcrystalline Cellulose (Rev 1)	USP	6
Cellulose, Powdered (Rev 1)	USP	6
Citric Acid, Anhydrous (Rev 1)	EP	6
Citric Acid, Monohydrate (Rev 1)	EP	6
Copovidone	JP	4
Croscarmellulose Sodium	USP	6
Crospovidone	EP	4
Ethylcellulose	EP	6
Ethylparaben	EP	6
Gelatin	EP	3
Glucose Monohydrate/Anhydrous	EP	3
Glycerin	USP	3
Glyceryl Monostearate	USP	2
Hydroxyethyl Cellulose	EP	4/2
Hydroxypropyl Cellulose	USP	4
Hydroxypropyl Cellulose, Low Substituted	USP	4
Hydroxypropylmethyl Cellulose	JP	6
Hypromellose Phthalate	USP	6
Lactose, Anhydrous (Rev 2)	USP	6
Lactose, Monohydrate	USP	6
Magnesium Stearate	USP	5A
Mannitol	EP	3
Methylcellulose	JP	6
Methylparaben	EP	6
Petrolatum	USP	4
Petrolatum, White	USP	4
Polyethylene Glycol	USP	4
Polysorbate 80	EP	4 rev
Povidone	JP	6
Propylene Glycol	EP	4
Propylparaben	EP	6
Saccharin	USP	6
Saccharin, Calcium	USP	6
Saccharin, Sodium (Rev 1)	USP	6
Silicon Dioxide	JP	4 rev
Silicon Dioxide, Colloidal	JP	4 rev
Sodium Chloride (Rev 2)	EP	6
Sodium Lauryl Sulfate	USP	3
Sodium Starch Glycolate (Rev 1)	USP	6
Starch, Corn (Rev 2)	USP	6
Starch, Potato	EP	6
Starch, Pregelatinized	JP	3
Starch, Rice	EP	6
Starch, Wheat	EP	6
Stearic Acid	EP	5B
Sucrose	EP	4

Table 1. Status of Harmonization—Excipient Monographs (Continued)

Excipient Name	Coordinating Pharmacopeia	Harmonization Stage
Sterile Water for Injection in Containers	USP	3
Talc	EP	6
Titanium Dioxide	JP	5A2

Table 2. Status of Harmonization—General Chapters

Chapter Title	Coordinating Pharmacopeia	Harmonization Stage
Amino Acid Determination	USP	6
Bacterial Endotoxins (Rev 1)	JP	4 rev
Bulk Density and Tapped Density	EP	5A2
Conductivity	EP	2
Color (Instrumental Method)	EP	3
Density of Solids	EP	5B
Disintegration	USP	6
Dissolution (Rev 1)	USP	6
Capillary Electrophoresis	EP	6
Polyacrylamide Gel Electrophoresis	EP	6
Extractable Volume (Rev 1)	EP	6
Heavy Metals	USP	3
Inhalation	EP	4
Isoelectric Focusing	EP	6
Laser Diffraction Measure of Particle Size	EP	4
Limits for Nonsterile Products	EP	6
Microbial Contamination	EP	6
Tests for Specified Microorganisms	EP	6
Microbial Enumeration	EP	6
Optical Microscopy	USP	6
Particle Size Distribution Estimation by Analytical Sieving (Rev 1)	USP	5B
Particulate Contamination (Rev 1)	EP	6
Peptide Mapping	USP	6
Porosimetry by Mercury Intrusion	EP	4
Powder Fineness	USP	5A
Powder Flow	USP	6
Protein Determination	USP	6
Residue on Ignition (Rev 2)	JP	6
Specific Surface Area	EP	6
Sterility Tests	EP	6
Tablet Friability	USP	6
Thermal Behavior of Powders	EP	3
Uniformity of Content/Mass	USP	6
Uniformity of Delivered Dose of Inhalations	EP	2

Table 2. Status of Harmonization—General Chapters (Continued)

Chapter Title	Coordinating Pharmacopeia	Harmonization Stage
Water-Solid Interaction	EP	3
X-Ray Powder Diffraction	EP	4

GLOSSARY

Harmonized monograph or general chapter—Text that has reached Stage 5B and that has been countersigned by the three PDG pharmacopeias.

Total harmonization—A monograph or a general chapter that is identical in the three PDG pharmacopeias in terms of identical tests, test procedures, and acceptance criteria.

Harmonization by attributes—A monograph or a general chapter that contains a combination of harmonized and nonharmonized tests or sections.

Interchangeability—A test or a section of a monograph or a general chapter that is not the same among the PDG pharmacopeias, but the accept/reject decision is the same regardless of which one of the tests or sections of the PDG pharmacopeia is used.

Local or national divergence—A monograph or a general chapter in one of the PDG pharmacopeias contains specific attributes of local or national origin in addition to the PDG harmonized/nonharmonized attributes.

Nonharmonized attributes—Attributes that could not be agreed upon by the three PDG pharmacopeias because of regional differences, regulatory differences, nonavailability of reagents, etc.

APPENDIX

Example

Pharmacopeial Discussion Group

Sign-Off Document

Name: _____

Attributes	EP	JP	USP
1	+	+	+
2	+	+	–
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	–	+
8	+	+	+
9	+	+	+
10	+	–	+
11	+	+	+

Legend: + will adopt and implement; – will not stipulate
Nonharmonized attributes:

Reagents and reference materials: Each pharmacopeia will adapt the text to take account of local reference materials and reagent specifications.

Date:

Signatures: European Pharmacopoeia Japanese Pharmacopoeia United States Pharmacopeia

Add the following:

▲ (1197) GOOD DISTRIBUTION PRACTICES FOR BULK PHARMACEUTICAL EXCIPIENTS

SECTION 1. INTRODUCTION AND SCOPE

1.1 Introduction

Excipients are used in virtually all drug products and are essential to product performance and quality. Typically, excipients are manufactured and supplied so that they comply with compendial standards. The pharmaceutical excipient supply chain participants include manufacturers, distributors, brokers, suppliers, traders, transporters, forwarding agents, and repackagers. The quality of pharmaceutical excipients is affected by inadequate control of activities including distribution, packaging, repackaging, labeling, and storage. Improper or inadequately controlled trade and distribution practices can pose a significant risk to the quality of pharmaceutical excipients and can increase the risk of contamination, cross-contamination, adulteration, mix-ups, degradation, or changes in physical or chemical properties. To maintain the original and intended quality, all participants in the excipient supply chain should carry out their activities according to appropriate standards for good trade and distribution practices as discussed in this chapter.

NOTE: The Appendix consists of definitions and acronyms.

1.2 Scope

This general information chapter provides recommendations for those activities and practices that ensure good trade and distribution practices for pharmaceutical excipients in order to ensure their intended quality. These activities and practices include quality management, organization, documentation, premises, storage, equipment, stability, prevention of adulteration, importation, packaging, repackaging, labeling, dispatch, transport, returned goods, and compounding practices. In addition, personnel, authenticity of data, expiration dating, retesting, complaints and recalls, handling of nonconforming materials, internal/external/third-party audits, quality agreements, shelf life, traceability, economically motivated adulteration, and conformance to compendial monographs are included. The procedures outlined here are applicable to all persons and manufacturers involved in the handling of pharmaceutical excipients and apply to every step in the supply chain. This chapter covers all materials designated as, or intended for

use as pharmaceutical excipients, beginning with the point at which the starting material is designated for pharmaceutical use.

1.3 General Considerations

Manufacturers, distributors, users, regulators, and consumers expect pharmaceutical excipients to be manufactured, packed, stored, and transported in a manner that does not compromise their suitability for use in medicinal products for human or veterinary use. Because they are components of drug products, excipients are drugs within the meaning of the U.S. Federal Food, Drug, and Cosmetic Act (FD&C Act), and thus the U.S. Food and Drug Administration (FDA) definition of adulteration applies when an excipient is not fit for its intended use.

Excipients are a diverse group of materials. They can be of animal, mineral, synthetic, or vegetable origin, and they include materials that are solids, liquids, or gases. Excipients can be packed and transported in container sizes ranging from a few grams to a railroad tank car.

Because of their diverse nature and the number of ways in which excipients can be transported from the manufacturing site through the supply chain to the ultimate site of use, this general information chapter cannot provide exhaustive detail for specific materials and modes of transport. Rather, this chapter provides general guidance about what is expected of those people and organizations involved in the supply and distribution of pharmaceutical excipients intended for use in the manufacture of pharmaceutical finished products. Hence, there are instances when *USP–NF* chapters *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* (1078), *Bulk Pharmaceutical Excipients—Certificate of Analysis* (1080), and *Significant Change Guide for Bulk Pharmaceutical Excipients* (1195) provide a more detailed guide about what is expected in these specific areas.

Excipients also are used in a variety of industries. Although most drug substances typically are made exclusively for use in pharmaceutical finished products, the pharmaceutical use of an excipient may be only a small fraction of the total use of the material across all industries. This complicates the regulation of both the manufacture and the supply of pharmaceutical excipients. Excipients often are manufactured outside the United States, which further complicates the regulation of the manufacture and the supply of pharmaceutical excipients. Thus, all stages in the supply chain for the pharmaceutical excipient require transparency and proper flow of the necessary information regarding the excipient shipment. In addition, to ensure compliance with this chapter, suppliers of pharmaceutical excipients must follow all applicable national, regional, and local laws and regulations.

1.4 Pharmaceutical-Grade Excipients

Pharmaceutical excipients must be prepared according to the recognized principles of good manufacturing practices (GMPs) using ingredients that comply with specifications designed to ensure that the resulting substances meet the requirements of the compendial monograph (see *General Notices* 3.10 and chapter *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* (1078)).

USP or *NF* standards apply to any excipient marketed in the United States that is recognized in the compendium and is intended or labeled for use as an ingredient in a pharmaceutical product. The applicable standard applies to such articles whether or not the added designation "*USP*" or "*NF*" is used (see *General Notices* 3.10.10). An ingredient may include the designation "*USP*" or "*NF*" in conjunction with its official title or elsewhere on the label only when a monograph is provided in the compendium and the article complies with the monograph standards and other applicable standards in the compendium including, but not limited

to, the principles of GMP manufacture (see *General Notices* 3.20).

When *USP*- or *NF*-grade excipients are unavailable, manufacturers may consider one of the following if its intended use can be adequately justified: referencing other national pharmacopeias for pharmaceutical quality materials (e.g., *EP*, *JP*), or the use of food-grade materials that meet *USP–NF* specifications. The pharmaceutical manufacturer/user is responsible for the development and confirmation of suitable quality tests, procedures, and attributes to ensure that the material is appropriate for its intended use and that manufacturing is carried out under GMPs or a quality management system that demonstrates the same level of assurance of quality as that provided in *USP* (see (1078)). It is an unacceptable practice to upgrade technical- or industrial-grade material to pharmaceutical-grade quality based only on analytical results that show compliance with the requirements of a pharmacopeial monograph.

1.5 Authenticity of Data

In the United States, the responsibility for the quality of the components of a finished pharmaceutical product lies with the organization that guarantees the quality of the finished pharmaceutical product. Thus, an important consideration in the purchase and supply of a pharmaceutical excipient is confirmation that the material is what it purports to be, that it meets specifications, that it was manufactured under applicable GMPs, that it has not been tampered with in any way before arriving at the site of intended use, and that it is fit for its intended use. Certain paperwork should accompany all shipments of pharmaceutical excipients. This paperwork should include a bona fide and legible copy of a Certificate of Analysis (COA) (see *Bulk Pharmaceutical Excipients—Certificate of Analysis* (1080)).

When they receive a COA, manufacturers should take appropriate steps to verify the authenticity of the COA and the data contained therein. This has become particularly important in recent years because of instances of adulteration of excipients intended for use in the manufacture of pharmaceutical products. Steps to verify the authenticity of the COA should be taken at all stages in the supply chain.

Data on the COA can be verified in a number of ways, but the excipient user is responsible for confirming that the data are authentic by means of periodic verification of compliance with established specifications as stated in 21 Code of Federal Regulations Part 211 (21 CFR 211; see *Current Good Manufacturing Practice For Finished Pharmaceuticals*, <http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcrf/CFRSearch.cfm?CFRPart=211>, <http://www.fda.gov/downloads/AboutFDA/CentersOffices/CDER/UCM095852.txt>). In addition, other documents such as dispatch notes from previous stages in the supply chain can provide further evidence of the pedigree of the excipient shipment. Such documents are termed "pedigree documents".¹

This chapter may present additional challenges for certain excipient users, e.g., compounding pharmacies. However, those who compound still are obliged to take all reasonable steps to verify that excipients they receive are fit for their intended use. Part of this verification can include an examination of pedigree documents and a signed certificate of conformance (COC) from the suppliers. Information contained in the *USP–NF* monograph's labeling requirements, FDA's Inactive Ingredient Database, and the CFR provide specific information about the excipient's permitted use in FDA-approved products. All purchasers of pharmaceutical excipients should establish written procedures for the verification of data and verification that the excipient is fit for its intended purpose.

¹ IPEC. The IPEC Excipient Pedigree White Paper. Arlington, VA: IPEC; ND. Available at: http://ipecamericas.org/sites/default/files/Excipient_Pedigree.pdf (Accessed July 6, 2011).

SECTION 2: QUALITY, ORGANIZATION, AND DOCUMENTATION

2.1 Quality Management

A Quality Management System (QMS) is a tool by which all parties involved in the excipient supply chain maintain the quality of the excipient. A documented quality policy is the cornerstone of the QMS and formally describes the company's overall philosophy with regard to quality as authorized by top or senior management. Additionally, an appropriate QMS should include:

- An organizational structure capable of supporting the elements of the quality policy
- Documented procedures and relevant records that demonstrate that a product will meet established quality criteria. This is commonly known as quality assurance (QA)
- Established procedures for approving suppliers of starting materials and verifying that they continue to meet agreed-upon requirements
- A material-release testing procedure to confirm the quality of excipients for their intended purpose(s).

Manufacturers should prepare a Quality Manual. The Quality Manual describes the elements of the QMS and includes the quality organizational structure, written policies, procedures, and processes or references to them, and a description of departmental functions as they relate to the policies, procedures, and processes (see *Section 2.3 Documentation Requirements*). In implementing the QMS, companies must ensure that adequate qualified personnel are available to carry out the actions called for in the QMS and must avoid giving any one individual such extensive responsibilities that quality could be at risk.

COC to quality systems such as applicable International Organization for Standardization (ISO) guides or hazard analysis and critical control point (HACCP) analyses are not mandatory but provide assurance that products are produced and handled appropriately. However, certification to these quality systems should not be viewed as a substitute for the information contained in this chapter. In addition, internal audits should be conducted at regular intervals to confirm compliance with GMP (as applicable) and good distribution practices (GDP), and manufacturers should seek opportunities for improvement (see *Section 2.7 Audits: Internal, External, and Third-Party*).

All parties involved in the excipient supply chain share responsibility for the quality and safety of pharmaceutical excipients. These responsibilities should be delineated in a quality agreement between parties in the supply chain (see *Section 2.9 Quality Agreements*). All parties and their activities in the supply chain should be documented, and records should be maintained according to written procedures that ensure the traceability of all products acquired and distributed. All members of the supply chain have an obligation to protect excipients in their custody from deliberate economically motivated adulteration or deliberate introduction of foreign materials that could compromise the quality or performance of the excipient or adversely affect human or animal health.

2.2 Organization and Personnel

The organizational structure should be adequate and sufficiently staffed, and workers should be appropriately authorized for the activities they conduct. An organizational chart should delineate the responsibilities and interrelationships of personnel. Management ultimately is responsible for implementation of GDPs and on-going verification that the QMS is maintaining the intended excipient quality.

Individuals within the company should have clearly defined responsibilities that are documented in writing. All in-

dividuals should understand their responsibilities and should be suitably qualified to perform their assigned duties. Their qualifications should be assessed for adequacy for their responsibilities and should be documented. Qualifications can include a combination of formal education, training, and experience. This also extends to any contracted service providers. Procedures should be in place to ensure that permanent, temporary, and contract employees minimize the possibility that unauthorized individuals will handle products.

An employee at each supply chain site should be designated and given the authority and responsibility for the implementation and maintenance of the QMS. The designated employee should have sufficient authority, qualifications, and resources to perform this function, as well as to identify and correct deviations from the QMS. Management and other personnel must not be subject to conflicts of interest or other pressures that could have an adverse effect on their ability to perform their duties related to product quality.

Staff should be aware of the principles of GDP included in this chapter and should receive regular, on-going training relevant to their responsibilities and to general quality principles. All training should be conducted according to a written training plan, and records of this training should be maintained. Personnel who have special duties such as handling hazardous materials or supervising activities required by local legislation may require additional training, including specific hazard management. Effectiveness of training should be verified regularly.

Personnel working with open product must understand and maintain good hygiene, health, and sanitation practices. Staff should use appropriate, nonshedding, protective apparel that will protect the product from the sampler as well as the sampler from the hazards of the product. Established procedures should eliminate the potential for product contamination by personal items such as jewelry, food, drink, or tobacco products. Written procedures that address hygiene, health, sanitation, and protective apparel should be in place.

Each supply channel party should have in place disciplinary procedures to address situations when personnel involved in the handling of products are suspected of or are implicated in inappropriate or illegal activities.

Some quality-related duties may be contracted to third parties, persons, or entities outside of the direct employ of the supplier. The delegation of these activities should be documented in a quality agreement or contract with the third party, and the organization should confirm compliance with the principles of GDP by conducting periodic on-site audits of these third parties. Delegation to a third party does not remove the organization's overall responsibilities for these activities.

2.3 Documentation Requirements

2.3.1 GENERAL

Organizations should have in place a system to control documents and data that relate to the requirements of the QMS.

2.3.2 QUALITY MANUAL

Organizations also should maintain a quality manual that describes the QMS, the quality policy, and the company's commitment to applying the appropriate GDP and quality management standards contained in this chapter. This manual should include the scope of the QMS, reference(s) to supporting procedures, and a description of the interaction between quality management processes.

2.3.3 DOCUMENT CONTROL

Procedures for the identification, collection, indexing, filing, storage, withdrawal, archiving, maintenance, and disposition of controlled documents, including documents of external origin that are part of the QMS, should be established and maintained. Procedures used for the handling and distribution of excipients should be documented, implemented, and maintained. In addition, organizations should establish formal controls relating to procedure approval, revision, and distribution. These controls should provide assurance that the current version of a procedure is used throughout the operational areas and that previous revisions of documents have been removed or withdrawn.

Designated qualified personnel should review documents and subsequent changes to the documents before the latter are issued to the appropriate areas. Documents that influence product quality should be reviewed and approved by the quality unit. Controlled documents may include a unique identifier, date of issue, and revision number to facilitate identification of the most recent document. The department with the responsibility for issuing the documents should be identified. The reasons for changes and the implementation date should be documented.

Electronic documentation should meet the requirements stated above for the document control system. If electronic signatures are used, they should be controlled to provide security equivalent to that given by a hand-written signature. Electronic documents and signatures also may need to satisfy local regulatory requirements.

2.3.4 CONTROL OF RECORDS

Procedures for the identification, collection, indexing, filing, storage, maintenance, and disposition of records should be established and maintained. Records should be maintained to demonstrate achievement of the required quality and the effective operation of the QMS. Records should be legible and clearly linked with the product or process involved. Pertinent third-party quality data also should be an element of these records.

Entries in records should be clear and indelible and should be made directly after the person performs the activity and then should be signed and dated by the person who made the entry. Corrections to entries should be signed and dated, leaving the original entry legible and with an explanation for the change, especially if this may not be obvious to subsequent reviewers.

Records should be kept for a defined period that is appropriate for the excipient, its use, and its retest or re-evaluation date. Records should be stored and maintained in such a manner that they are readily retrievable and in facilities that provide a suitable environment to minimize deterioration or damage. Electronic records and automated data-capture systems should meet the requirements for controlled records as stated above.

2.3.5 CHANGE CONTROL

Procedures to evaluate and approve all changes, including evaluating the impact of the change on the quality of the excipient, should be established and maintained, for example, changes to:

- Authorized excipient manufacturer or packaging material supplier
- Manufacturing or packaging sites
- Excipient or packaging material specifications
- Test methods and laboratory
- Repackaging, labeling, and storage equipment
- Analytical equipment
- Repackaging, labeling, and storage processes

- Process and equipment changes at the original excipient manufacturer's site (see *Significant Change Guide for Bulk Pharmaceutical Excipients* (1195)).

An independent QA group should have the responsibility and authority for the final approval of any changes. The QA group may be part of another operational unit such as regulatory affairs or research and development.

Customers and, if applicable, regulatory authorities (e.g., those responsible for drug master files or certificates of suitability to the *European Pharmacopoeia*) should be notified of significant changes to established production and process control procedures that could affect excipient quality. The original manufacturer and downstream intermediaries (distributors and traders) should have excipient change control agreements in place defining the extent of notification by the original manufacturer in case of a change as described above. Each of the handling parties within the supply chain should have change control agreements to ensure that changes from the original excipient manufacturer are communicated to the end user. This change control agreement is part of the overall contractual agreements between the parties.

2.4 Complaints and Deviations

Customer complaints and information about possible defects should be systematically documented and investigated based on a written procedure with assigned responsibilities that describes the action that will be taken and includes the criteria on which a decision to recall a product should be based. Investigations should be formally conducted and written up in a timely manner to establish if the complaint is justified, to identify the origin or reason for the complaint (e.g., the repackaging procedure, the original manufacturing process, etc.), to identify root cause(s), to define any initial and follow-up action(s), and the method of communication (e.g., to the customer, original excipient manufacturer, authorities, etc.). Complaint records should be retained and regularly evaluated for trends, frequency, and criticality in order to identify possibly needed corrective or preventive actions.

Investigations should identify whether the reported defect is limited to a single batch of material or if other batches must be investigated. If additional batches are implicated, they should be identified and labeled accordingly (e.g., "under quarantine"). As necessary, appropriate follow-up action, possibly including a recall (as outlined in *Section 2.5 Recalls*), should be taken after investigation and evaluation of the complaint. Confirmed serious problems related to product quality (e.g., faulty manufacturing, packaging, or product deterioration) should be communicated upstream to the manufacturer and downstream to customer(s) in case they received material with the same batch number. A similar process should be implemented for the handling of deviations and product defects not identified by a customer complaint.

2.5 Recalls

Those involved in the excipient supply chain should have a system for recalling promptly and effectively any materials known or suspected to be defective. Entities involved in the supply chain should implement written procedures to manage excipient recall (retrieval) in a timely manner. The procedures should:

- Describe how the process of recall (retrieval) should be managed based on the risk involved
- Describe a decision-making process with defined responsibilities
- Define the functions involved in the process (e.g., QA, sales, logistics, senior management, competent authorities, etc.)

- Define the communication process and documentation to parties within the supply channel as well as to regulatory authorities
- Define the steps needed to retrieve the material.

If the original excipient manufacturer does not initiate a recall, it should be informed of the recall. Entities in the supply chain should have written procedures for the organization of any recall activity, and these should be regularly checked and updated. All recalled materials should be stored in a secure, segregated (quarantined) area while their disposition is decided. In the event of serious or potentially life-threatening situations, all customers and competent authorities in all countries to which an excipient potentially was distributed should be promptly informed of any intention to recall the excipient. All records should be readily available to the designated person(s) responsible for recalls. These records should contain sufficient information about materials supplied to customers (including exported materials). At regular intervals, QA groups in supply-chain organizations should evaluate the effectiveness of recall arrangements.

2.6 Handling of Nonconforming Materials

Nonconforming materials should be handled in accordance with a procedure that will prevent their inadvertent introduction or reintroduction into the market. They should be stored separately, either physically separated or under electronic control, to prevent their inadvertent introduction into commerce. Firms that conduct recalls should maintain records covering all activities, including destruction, disposal, return, and reclassification, and should perform an investigation to establish whether any other batches also are affected. They should document the investigation and actions taken to prevent recurrence of the problem. As necessary, firms should take corrective measures. Procedures should exist for the evaluation and subsequent disposition of nonconforming products, and the disposition of the material, including downgrading to other suitable purposes, should be documented. Nonconforming materials should never be blended with materials that comply with specifications.

2.7 Audits: Internal, External, and Third-Party

To verify compliance with the principles of GDP for pharmaceutical excipients, firms in the excipient supply chain should perform regularly scheduled internal audits in accordance with approved procedures. Firms should document audit findings and corrective actions and ensure that they are brought to the attention of responsible management. Accepted corrective actions should be completed in a timely and effective manner and should be conducted by designated, qualified individuals. Qualified individuals may be employees of the company, but they must be sufficiently removed from the function under audit so that their independence is not compromised.

Firms should perform external audits in accordance with approved procedures and schedules to assess the capability of suppliers to meet requirements for a product or service, as specified. A response to a questionnaire may be considered in the auditing process but generally does not take the place of an on-site inspection and should not be considered a substitute when an audit is required. Independent auditing organizations can perform third-party audits to determine the level of compliance or conformance to specified standards and regulations (e.g., GMP, GDP, and ISO).

2.8 Contract Activities

Any GDP-related activity that is delegated to another party should be agreed upon in writing in an approved contract with clearly defined responsibilities. The contract

should clearly establish which party is responsible for each applicable quality activity. Before entering into an agreement, the contract giver should evaluate the proposed contract acceptor's compliance with GDP as described in this general chapter. The evaluation should include an initial on-site audit of the contract acceptor's premises and quality system, giving special consideration to the prevention of cross-contamination and maintaining traceability. The contract also should include the responsibilities of the contract giver for measures to avoid the entrance of counterfeit or adulterated materials into the distribution chain.

There should be no gaps or unexplained lapses in the application of GDP. The contract acceptor should conduct periodic on-site auditing of contracted distribution activities with regard to the application of GDP by the contract giver. Subcontracting may be permissible under certain conditions, subject to approval by the original contract giver, especially for activities such as sampling, analysis, repacking, and labeling. If subcontracting occurs, the subcontractor should conform to the same GDP standards as the primary contract giver. The subcontractor also should permit an on-site audit by the contract acceptor's quality unit or its designee.^{2,3}

2.9 Quality Agreements

Quality agreements are legally binding and are mutually negotiated between parties involved in the supply chain for pharmaceutical excipients. The quality agreement identifies who is responsible for certain quality activities and how quality issues will be resolved between the parties. Although they are intended to address the parties' quality commitments, quality agreements are not designed to take the place of an audit.

Suppliers should have in place quality agreements between themselves and the parties with whom they do business. Original excipient manufacturers should have quality agreements in place with their direct customers and authorized distributors of their products. Distributors should also have agreements with end users and other parties in the supply chain to whom they supply products. All entities in the supply chain should fully understand which entity is responsible for the GDP-related activities (as outlined in this chapter) at each step in the supply chain.

Quality agreements should address the quality systems requirements, but they are not intended to list every element of the quality system. It is not necessary to reiterate agreement on every point of the quality system when the parties state general agreement on the applicable quality standard. Quality responsibilities included in a quality agreement should be those that may require action by one or both parties to the agreement.

A key element that must be defined in the quality agreement is the communication pathways and timing for quality events. Parties must be clear about their responsibility for notifying the next party in the supply chain and for notifying the applicable regulatory authorities in the case of a significant quality event. Many times a decision about who should notify the regulatory authority is a collaborative effort between the parties. Depending on the issue's impact, the timing of these notifications relative to the time of the incident should be specified within the quality agreement.

Both parties to the agreement are responsible for ensuring that the quality agreement is maintained as an accurate document throughout the life of the business relationship. Revisions to this document may be needed as regulatory requirements change, new products are supplied, or a new material risk arises. The parties should maintain a history of the revisions to the quality agreement.

²WHO. *Good Trade and Distribution Practices for Pharmaceutical Starting Materials*. Geneva; WHO: Technical Report Series, No. 917, 2003, Annex 2. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_917_annex2.pdf (Accessed June 30, 2011).

³WHO. *Finished Products: Good Distribution Practices for Pharmaceutical Products*. Geneva; WHO: Technical Report Series 957, 2010, Annex 5. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_957_eng.pdf (Accessed July 7, 2011).

SECTION 3: PREMISES, STORAGE, REPACKAGING, AND STABILITY

3.1 Buildings and Facilities

Organizations should establish operating procedures for the use of buildings and facilities, including the areas discussed below, and firms should consider protective measures to ensure the security of the grounds (e.g., fencing or perimeter walls).

The buildings and facilities used in the storage and handling of excipients should restrict access to allow entrance only by authorized persons to areas used for the manufacture, packaging, and holding. Organizations should take precautions to prevent unauthorized persons from entering limited-access areas. When the status of excipients requires protection from use (e.g., quarantine), organizations must have clearly marked limited-access controls in place, or they should use validated computerized systems to prevent material distribution before approved release.

Buildings should be of adequate size and capacity to allow the orderly flow of materials, proper storage and handling of materials, and appropriately controlled environmental conditions for the final dispatch of excipients into and out of the premises. Buildings should be maintained in a good state of repair. The construction materials must be easily cleanable and maintained, and buildings and facilities should be designed to prevent cross-contamination, product mix-ups, or the accumulation of filth or contaminating materials, particularly when excipients are exposed to the environment. Adequate storage space must be available for excipients that are highly sensitizing or toxic, and dedicated facilities may be necessary. Adequate procedures should be in place to ensure the cleaning, maintenance, and use of buildings and facilities.

Receiving and dispatch bays should be designed to protect the facilities and excipients during loading and unloading during adverse weather conditions. Incoming bay areas should be designed and equipped to allow containers to be cleaned before storage. A pest-control system should be in place to ensure that materials are protected from infestation by insects, rodents, animals, birds, or other vermin. There should be written procedures defining the adequate holding and storage of excipients, including pest-control processes. The pest-control materials must be safe and must be known not to cause contamination. Approved pesticides, insecticides, and rodenticides should be used and documented. Excipients that may contain contamination must be controlled to prevent cross-contamination in holding areas or the spread of contamination to other areas of the facility.

3.2 Warehousing and Storage

Written procedures should describe the receipt, storage, dispatch, and other handling of excipients, as well as the security measures necessary to prevent theft of materials or the introduction of counterfeit or adulterated materials into the supply chain. Buildings should be adequately lighted and should have proper utilities for the intended activities. They should be dry and controlled to appropriate environmental conditions. Buildings and facilities should store excipients in the proper environmental conditions. Temperature-controlled and -monitored storage should be available as required for any building used for holding excipients.

Warehousing and storage conditions for excipients should comply with the monograph specifications, as reflected in the excipient's container label. When specific storage conditions are required for excipients (e.g., temperature and humidity control), they should be provided in a controlled manner, monitored (e.g., by an alarm system or manual control), and recorded. Any automated system(s) used to monitor the environmental conditions for areas where excip-

ients are handled or stored must be validated. An approved document should indicate the location of each environmental monitoring device and the condition(s) it monitors. The locations for these devices or probes should reflect the extreme environmental conditions of the space as determined by an environmental mapping exercise. Excipients that present risks such as fire or explosion should be stored in safe, dedicated areas. Excipients that are sensitizing or toxic should be adequately and appropriately segregated, and warehouse and storage areas should be routinely cleaned, appropriately maintained, and free of pests.

Excipients should be stored in a manner that permits cleaning of the storage area and movement of materials. Pallets used to hold materials should not cause contamination, and required pallet quality and construction materials should be defined in writing. Pallets should be clean and in a good state of repair, and firms should appropriately track supplies to ensure adequate treatment of the wood materials. Wood pallets, if used, should comply with import requirements.

Organizations should have in place written procedures to ensure that the excipient will be supplied within its expiry or retest period and should have adequate controls to prevent the distribution of expired excipients. If no expiry date is applicable, the first-in-first-out principle should be used. Rejected excipients and other materials related to excipient quality (e.g., packaging components) should be so labeled or identified, and controls such as physical or electronic separation should prevent their use pending final disposition. During the warehousing or storage of excipients, any known broken or damaged containers should be withdrawn from usable stock, and the containers should be handled as rejected materials.

Materials quarantined pending a release decision should be labeled or identified (e.g., electronically) to prevent unauthorized use. These materials should be held from use, and written procedures should guide final disposition. There should be written procedures for the cleanup of any spillage to ensure complete removal of any risk of contamination.

3.2.1 ENVIRONMENTAL CONTROLS

When excipients require specific storage conditions to preserve their integrity and quality during the retest/re-evaluation or expiry interval, the storage conditions required should be stated on the label, labeling, or other literature, e.g., the Excipient Information Package⁴ or COA. Distributors should follow the information and requirements for environmental controls provided by the manufacturer and should provide appropriate controls and monitoring to ensure adherence to the stated storage conditions with appropriate documentation. Distributors also should maintain records to indicate the excipient was stored according to the manufacturer's recommendations and should conduct regular assessments to confirm that designated conditions are met.

If the manufacturer does not indicate specific storage conditions, the distributor should ensure that proper storage conditions are maintained to protect the packaging and labeling. Uncontrolled warehousing conditions vary with geographical location, particularly with latitude. If the excipient is shipped to geographical locations that have storage conditions well outside the conditions used in the manufacturer's stability study or justification for the absence of special storage conditions, then additional studies may be required to show stability at the new conditions. A warehouse-monitoring program should be established if the effects of the new environmental conditions are not known.

Outdoor storage of excipients (for example, bulk materials, flammable materials, acids, or other corrosive substances) is acceptable provided the containers give suitable protection against deterioration or contamination of their

⁴ IPEC. *The IPEC Excipient Information Package (EIP): Template and User Guide*. Arlington, VA: IPEC; 2011. <http://ipecamericas.org/ipec-store>.

contents, identifying labels remain legible, discharge ports have adequate protective closures, and the exteriors of moveable containers are adequately cleaned before opening and use.

3.3 Equipment

Equipment used in bulk transport, repackaging, labeling, testing, or storage of the excipient should be maintained in a good state of repair and should be of suitable size, construction, and location to facilitate cleaning, maintenance, and correct operation. Equipment should be verified before use to ensure that it is constructed, installed, and functioning as required for the excipient. When equipment is located outdoors, there should be suitable controls (e.g., closed systems or protective encasements) to minimize environmental risks to excipient quality.

When possible, dedicated equipment (e.g., bulk trucks, packaging equipment, storage tanks, pipework, hoses, and pumps) should be used in direct contact with the excipient. When non-dedicated equipment is used in direct contact with the excipient, validated cleaning procedures should be applied. A restricted prior-cargo list should be supplied to transport companies in case non-dedicated bulk transport equipment is used. Quality-critical measuring equipment and balances for the handling and testing of the excipient should be of appropriate range and precision. Such equipment should be identified.

3.3.1 EQUIPMENT CONSTRUCTION

Equipment in contact with an excipient should be constructed so that contact surfaces are not reactive, additive, or absorptive and thus do not alter the quality of the excipient. Substances required for operation, such as lubricants or coolants, preferably should not come into contact with excipients and packaging materials. When contact is possible, distributors should use materials of suitable quality that will not affect product quality. The choice of such materials should be justified.

Equipment should be designed to minimize the possibility of contamination from the environment and direct operator contact during activities such as unloading bulk trucks, use of transfer hoses (particularly those used for transfer of excipients), sampling, repackaging, and cleaning. Distributors should consider the sanitary design of equipment in contact with excipients. They should assess the suitability and integrity of seals in order to minimize the risk of contamination. Piping should be appropriately labeled to indicate the content and direction of flow.

3.3.2 EQUIPMENT MAINTENANCE

Documented procedures should be established and followed for maintenance of critical equipment used in the repackaging, labeling, testing, or storage of the excipient. Distributors should maintain records (e.g., logs, computer databases, or other appropriate documentation) of quality-critical equipment use and maintenance. Defective equipment should be removed or appropriately labeled to avoid misuse.

Quality-critical measuring equipment and balances should be controlled on a scheduled basis. This control should include:

- Calibration of instruments or other appropriate verification at suitable intervals, according to an established documented program
- Establishment of the equipment's limits of accuracy and precision
- Provisions for remedial action in the event that accuracy or precision requirements are not met.

Calibration standards should be traceable to recognized national or compendial standards as appropriate. Instru-

ments and equipment that do not meet established specifications should not be used, and an investigation should be conducted to determine the validity of the previous results since the last successful calibration. The current calibration or verification status of quality-critical equipment should be known to users and should be verifiable.

3.3.3 EQUIPMENT CLEANING

Cleaning equipment should be chosen and used so that it cannot be a source of contamination. Cleaning materials should be appropriate for the task, and their selection should be justified. Rotation of sanitizing and cleaning agents should be considered where appropriate. In order to avoid contamination with cleaning products or products previously processed in the equipment, written cleaning procedures should be established for equipment that comes in contact with the excipient. Cleaning procedures should contain sufficient detail to allow cleaning in a reproducible and effective manner. Cleaning and sanitation processes should be recorded, and evidence of their effectiveness should be provided, for example, by:

- Testing the final rinse after cleaning for residues of the previous product
- Checking the equipment after cleaning for residues of the previous product
- Testing each batch for residues of the previous product handled with the same equipment.

3.4 Sampling, Repackaging, and Labeling

To minimize risks associated with repackaging and labeling, appropriate GMPs should be applied (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* (1078)). For completeness, certain key activities and the necessary precautions are discussed below.

3.4.1 BLENDING, REPACKAGING, AND LABELING

Operations such as combining sublots into a homogeneous batch, repackaging, or labeling are manufacturing processes, and therefore distributors should follow appropriate GMPs (see (1078)):

- Processes whereby the excipient's packaging is opened and the excipient is exposed to the environment (for example, transferring excipient from one container to another, including from bulk equipment to storage tanks/silos or from storage tanks/silos into containers) are critical handling steps related to the integrity of the finished product. If only the secondary packaging is modified, operators should take appropriate care to maintain the integrity of the primary packaging and the excipient.
- Excipients may degrade because of exposure to the repackaging atmosphere (e.g., oxygen, humidity, light, and temperature).
- Excipients can be contaminated by foreign matter such as lubricants, cleaning materials, or other substances.
- Transparency to the customer that re-labeling, with or without opening the original excipient manufacturer's packaging, has occurred is critical to representation of the product quality and suitability for use.
- Transparency to the customer of data sources listed on certification documentation (labeling) is critical to representation of the product quality and suitability for use.

3.4.2 REPACKAGING AND LABELING BATCHES

Staff in the excipient supply chain should give special attention to the following points:

- All repackaging and labeling requirements should be defined in written procedures.
- Contamination, cross-contamination, and mix-ups should be avoided by the use of suitable equipment and cleaning procedures and with adequate labeling.
- Environmental conditions and repackaging procedures should be designed to avoid contamination and to maintain the integrity of the excipient during repackaging and labeling.
- Operators should consider the use of filtered air in the repackaging area if necessary for the product. The standard of filtration should be justified.
- Labels should be printed using a controlled process (see *Section 3.4.9 Repackaging and Labeling*).
- Personnel involved in repackaging processes should wear clean protective apparel such as head, face, hand, and arm coverings, as necessary, and should practice appropriate personal hygiene (e.g., hand disinfection following health requirements, health monitoring, and removal of jewelry). Personnel should be trained about special hygiene requirements, and this training should be documented.
- Repackaging areas should be cleaned and sanitized regularly.

Batch numbers should be assigned according to documented procedures. When staff assigns new batch numbers, they should ensure traceability to original batch numbers by proper documentation. Assigning one batch number to containers of different batches that comply with the same specification is an unacceptable practice (see also *Sections 3.4.3 Excipient Batch Homogeneity* and *3.4.4 Blended Excipients*).

- As part of the batch record, a copy of the information on the original labels should be retained (e.g., a photocopy). A sample of the new label also should be kept.
- All repackaging and labeling processes should be designed and carried out to avoid commingling, contamination, and mix-up and to ensure full traceability of the excipients back to the original excipient manufacturer and traceability downstream to the final customer. Responsible personnel should sufficiently record every completed step, along with the name of the operator and the date and time each step was completed, e.g., in the master batch manufacturing record, or by means of computerized systems.

3.4.3 EXCIPIENT BATCH HOMOGENEITY

Mixing to form a homogeneous batch is a manufacturing step and should be defined in a written procedure. A batch can be homogenous only when conforming materials are thoroughly mixed. The conformity of each batch with its specification should be confirmed before it is added. Mixing should always be controlled, and homogeneity should be verified and documented (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* (1078)). Blending of batches or lots of excipients that individually do not conform to specifications with other lots that do conform (in an attempt to salvage or hide adulterated or expired material) is not an acceptable practice. Only excipients from the same manufacturing site received by a distributor and shown to conform to the same specifications can be mixed. The customer should be informed that the material supplied is a mixture of the manufacturer's batches.

3.4.4 BLENDED EXCIPIENTS

The blending process should be verified to ensure that it does not influence the quality of the excipient. The blended excipient should be tested to ensure conformance to the specification and to provide data for the COA (see *Significant Change Guide for Bulk Pharmaceutical Excipients* (1195)). Under certain circumstances and with appropriate controls,

a COC can be used if the basis for the claim of conformity is traceable within the document. The blended batch referred to in the new certification document should be traceable to all the original certification documents and batch numbers (see (1078)).

3.4.5 CERTIFICATES OF ANALYSIS

The original excipient manufacturer's COA should be retained and made available to the user on request. The batch referred to in the COA delivered to the end user should be traceable to the original excipient manufacturer's COA. Quality documents accompanying deliveries should be subject to an agreement between the distributor and the final customer. For retesting, analytical methods of the original excipient manufacturer or pharmacopeial methods should be applied. When other methods are applied, these should be agreed upon by both parties.

3.4.6 CONTAINER-CLOSURE SYSTEMS

For repackaged material, the repackager is responsible for justifying the shelf life and repackaging conditions. The original manufacturer and the distributor should share information and agree about repackaging conditions and primary packaging materials. They should establish primary container-closure system material and packaging configuration specifications, and they should develop a written procedure that clearly defines packaging for each individual excipient based on its stability.

If the same types of primary container-closure system and packaging configuration are used for repackaging, then the new container-closure system and packaging configuration should be equivalent to that used by the original excipient manufacturer. The repackager and distributor should consider exposure of the excipient to the repackaging environment, and both can rely on the manufacturer's stability evaluation and thus assign the same shelf life for the excipient.

When the repackager's primary container-closure system's packaging configuration differs significantly from that of the original manufacturer [e.g., in terms of desiccants, permeability of the protective barrier layer (which may be either the primary or secondary container-closure system), or the headspace], the repackager must demonstrate that the new system is adequate to protect the excipient from contamination and deterioration for the shelf life (retest or expiration period) defined by the excipient manufacturer. Otherwise, the shelf life defined by the manufacturer cannot be transferred to the repackaged material. The need for stability studies should be confirmed (see *Sections 3.4.14 Stability and Expiration Dates* and *3.5 Retesting and Shelf Life*).

The container-closure system for the pharmaceutical excipient should protect the material from the time of packaging until its final use by the drug product manufacturer. The container-closure system should be designed to help prevent theft or adulteration by counterfeiting.

Storage and handling procedures should protect containers and closures and minimize the risk of contamination, damage or deterioration, and mix-ups (e.g., between containers that have different specifications but are similar in appearance).

3.4.7 RETURNED AND REUSED CONTAINERS

Returned containers may have unknown residues from uses other than the intended one. Therefore, use of new containers is recommended for excipients. If containers are reused, a rationale for the extent of cleaning should be justified and documented for specific excipients and different types of containers. Repackagers should collect evidence

that the quality of the material packed is not adversely affected by reuse of containers.

Distributors and customers should have an agreement defining the specific conditions for reuse (e.g., handling, sealing, and cleaning). If returnable excipient containers are reused, all previous labeling should be removed or obliterated.

3.4.8 ENVIRONMENTAL CONTROLS

Environmental controls should ensure that temperature, humidity, and cleanliness of air and equipment are appropriate to avoid any contamination to or deterioration of the excipient. The necessary environmental conditions for the repackaging of each excipient should be defined. Environmental control is a specialist subject, and experts should be consulted (see also *Section 2.6 Handling of Nonconforming Materials*).

3.4.9 REPACKAGING AND LABELING

Repackagers should implement procedures to ensure that the correct quantity of labels is printed and issued and that labels contain the necessary information. Sufficient cross-checks should be in place to ensure proper data transfer. Procedures should be in place to avoid mislabeling, and printing and use of labels should be restricted. All labeling operations (e.g., generating, printing, storage, use, and destruction) should be recorded. Labeled containers should be inspected, and surplus labels should be destroyed to avoid any misuse. If labels are not printed immediately before each specific labeling operation, the security of the label stock should be controlled, and access limitations should be defined. Repackaging and labeling facilities should be inspected immediately before use to ensure that all materials that are not required for the next repackaging operation have been removed.

3.4.10 REPACKAGED EXCIPIENTS—ACCOMPANYING DOCUMENTATION

Deliveries of repackaged excipients should be accompanied by information about the original manufacturing site (name and address) and repackaging and labeling sites. This information should be provided in the supplier certification documentation (e.g., COAs) or by other means (see *Section 4.8 Traceability*). The supplier should provide this information to the customer via official communications.

3.4.11 TESTING OF REPACKAGED EXCIPIENTS

Appropriate testing of repackaged excipients should be performed to demonstrate consistent excipient quality. Testing to the complete monograph may not be necessary, but the recipient should test defined key quality parameters that could be affected by the repackaging process. Recipients should consider the manufacturer's recommendations for key quality parameters, and until these tests have been performed the repackaged materials should be kept under quarantine and should be identified as quarantined material. The materials should comply with the defined specifications before they are released for distribution.

Excipient testing and release should be performed under the responsibility of the quality unit and should conform to written specifications and analytical test requirements. Repackagers should ensure that test data are recorded and that results are evaluated before release of the repackaged or transferred excipient.

The excipient cannot be upgraded as a result of any repackaging process. It is unacceptable to upgrade nonpharmaceutical grades to pharmaceutical grades on the basis of conforming analytical results, i.e., by testing to

pharmacopeial standards. Pharmaceutical grades can be achieved only when the excipient is originally produced and subsequently processed in accordance with GMPs (see *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* (1078)).

3.4.12 OFFICIAL PHARMACOPEIAL METHODS FOR RETESTING

For control of key parameters during repackaging or full retesting of excipients, official pharmacopeial methods or methods validated against the pharmacopeial methods should be used. Otherwise, repackagers should use the original excipient manufacturer's analytical methods. The methods used should be listed on the COA accompanying the excipient or should be made available to the customer by other documents. These documents should also reference any contract laboratory that is used to perform analyses. The COA should clearly identify which tests have not been performed on the repackaged or transferred batch but have been taken from the original manufacturer's COA.

3.4.13 SAMPLING

Excipient sampling must be done in a manner that prevents contamination, and dedicated sampling areas with adequate environmental controls are necessary. Areas for sampling should be designed to allow cleaning of the outside of the container before the container is opened. Adequate cleaning procedures should be in place for the sampling areas. Sampling tools should be dedicated to the sampling area and also to the specific material, or sampling tool cleaning must be validated to ensure no cross-contamination from the tool.

Any container opened for sampling should be marked with the date and name of the person who performs this operation. The amount of sample removed should be recorded.

If excipients are repackaged, processed, or packaged from bulk, retained samples representative of the excipient batch should be kept for at least one year after the expiration or re-evaluation date or for at least one year after distribution is complete, whichever is longer. The minimum sample size should be based on the amount required to perform at least two complete analyses. Sample storage conditions should prevent any contamination or deterioration and should comply with the label storage conditions (see general information chapter, *Bulk Powder Sampling Procedures* (1097)).

3.4.14 STABILITY AND EXPIRATION DATES

Excipient stability and expiration dating of excipients are primarily the responsibility of the original manufacturer. Whenever the original manufacturer's packaging is opened, the repacker is responsible for providing evidence that the excipient manufacturer's stability and expiration dating are still applicable.

If a distributor transfers an excipient to another container or repackages it, stability and shelf life (retest or expiry period) should be taken into account. The type of container, primary packaging materials, barrier packaging materials, packaging configuration, environmental exposure during repackaging, and storage conditions at the repackaging site also should be taken into account when the shelf life (retest or expiry period) is defined. The recommended expiration date provided by the original excipient manufacturer should not be extended without demonstrating sufficient stability to justify extended shelf life (retest or expiry period). If shelf life is extended beyond the original manufacturer's recommendation, the type of packaging, storage conditions, and stability-indicating analytical data should be clearly defined, and the repacker assumes the primary responsibility for the extension.

If special storage conditions (e.g., inert gas overlay, protection from light, heat, moisture, etc.) are needed, the restrictions should be indicated on the new labeling (see *Section 3.5 Retesting and Shelf Life*).

3.5 Retesting and Shelf Life

The organization's stated shelf life or retest/re-evaluation interval should be maintained for the excipient. Expiration or shelf life dates indicate the period beyond which the excipient should not be used or distributed. Retest/re-evaluation intervals indicate the period beyond which the excipient must be evaluated to determine continuing acceptability for use. The expiration/shelf life date provided by the original excipient manufacturer should not be extended without documentation from the manufacturer demonstrating sufficient stability to justify an extended shelf life. Such documentation should specify the type of container and storage conditions necessary to make this claim, and the distributor should have documentation that the excipient was stored in the stated container and under the necessary conditions.

Excipients without expiration, retest, re-evaluation, or shelf life dates should be accepted for use only if the manufacturing date can be confirmed and only if the excipient has been held and shipped under conditions that conform to the appropriate standards of GMP or GDP. Distribution of the excipient beyond the retest/re-evaluation period should be done only in consultation with the manufacturer and with the consent of the purchaser or recipient. If the distributor has the capabilities for sampling and performing the manufacturer's specified evaluation, then the distributor can perform the assessment. Sampled lots should be placed under quarantine to prevent shipping during the evaluation.

Distributors who do have capabilities for sampling according to the manufacturer's instructions but do not have testing or evaluation capabilities should send the samples to the manufacturer or a qualified third-party laboratory for retesting/re-evaluation. Excipient lots that conform to the manufacturer's criteria can be released from quarantine, and the distributor's supporting evaluation data should accompany the original excipient manufacturer's data to indicate the excipient's acceptability for use. If the distributor does not have the capability to sample or evaluate the excipient, it should not be shipped to customers beyond the end of the retest/re-evaluation interval. The excipient or a representative sample of the excipient can be returned to the manufacturer or a third party for retesting/re-evaluation. The excipient can be held by the distributor pending further results obtained from the representative sample.

If an excipient is transferred to another container or is repackaged by the distributor, the latter must conduct an assessment of the stability of the excipient to determine if the original excipient manufacturer's information can be carried forward. If the distributor uses the same type of packaging material that provides the same packaged environment (headspace, surface area, closure tightness, etc.) as that used by the original manufacturer and if the transfer or repackaging is performed in a manner that protects the excipient from adverse environmental effects that could affect the stability, then the original excipient manufacturer's shelf life/expiry date or retest/re-evaluation interval can be carried forward. If primary packaging material or barrier packaging material differs from the original excipient manufacturer's primary packaging material or if the packaged environment varies significantly, then an evaluation of the container and its closure system should demonstrate that it is adequate to protect the excipient from deterioration and contamination during the manufacturer's shelf life/expiry date or retest/re-evaluation interval. Otherwise, a stability assessment is necessary to determine the appropriate shelf life/expiry date or retest/re-evaluation interval for the repackaged excipient. Such assessments should be conducted according to the manufacturer's specifications and test methods.

3.6 Expiration Dates

Not all excipients have an expiration date, but if one is assigned it should be displayed on the container and should show the period during which the excipient is expected to remain within specifications if stored properly and after which it should not be used. It is established for every batch by adding the shelf life to the date manufacturing began. The expiration date is based on the type of container and storage conditions, so these parameters should be clearly defined. If special storage conditions are needed (e.g., protection from light, oxygen, heat, humidity, etc.), they should be indicated on the labeling because they could influence usability through the expiration date.⁵

The expiration dates for excipients should be established by documented stability tests or long-term stability data (see *Pharmaceutical Stability* (1150)). Occasionally, the expiration date may be established by reference to historical data. Stability involves not only the compendial requirements but also changes in performance properties. Excipient stability tests should determine whether possible degradation, changes in molecular weight and distribution, moisture gain or loss, viscosity changes, microbiological contamination, or other possible changes in excipients could occur when the excipient is stored in a specific container-closure type at specific storage conditions. Stability for repackaged excipients can be found under *Section 3.5. Retesting and Shelf Life*.

3.7 Labels, Icons, and Labeling

3.7.1 LABELS AND ICONS

Label-generating systems and processes should be secure, controlled, and documented. Appropriate verification records should be maintained, and each container should be appropriately identified and labeled. Labels applied to individual small containers should be clear, unambiguous, and permanently fixed in the company's established format. The information on the label should be indelible. Alternative methods can be used for bulk containers/transport and should be justified.

The label may include wording or depict icons to highlight storage and transportation handling requirements and hazards (e.g., avoid dropping, maintain specified environmental conditions, etc.). The use of symbols that are recognized by international organizations is recommended (see *Good Storage and Transportation Practices for Drug Products* (1079)). During international distribution, the proper language(s) should be used to ensure that handlers understand the requirements set forth on the label.

3.7.2 LABELING

The labeling (which includes both the label and any accompanying documents) should include at least the following information:

- Name of the excipient, including grade and reference to pharmacopeia, as relevant
- If applicable, the International Nonproprietary Name
- Amount (weight or volume)
- Batch number assigned by the original excipient manufacturer or the batch number assigned by the repacker if the material has been repacked and relabeled
- Retest date or expiry date (as applicable)
- Any specified storage conditions, as applicable
- Handling precautions, where necessary

⁵ IPEC. *The IPEC Excipient Stability Program Guide 2010*. Arlington, VA: IPEC; 2010. Available at: <http://ipecamericas.org/ipec-store> (Accessed July 12, 2011).

- Identification of the original manufacturing site as agreed with the pharmaceutical customer (see *Section 4.8 Traceability*)
- Name and contact details of the suppliers.

SECTION 4: RETURNED GOODS, DISPATCH, TRANSPORT, IMPORTATION, ADULTERATION, AND TRACEABILITY

4.1 Returned Goods

4.1.1 GENERAL

Return of goods by users to suppliers should be reviewed on a case-by-case basis. The distributor should facilitate a root cause analysis and investigation of complaints.

NOTE: Users should document the reason(s) for return of goods to the supplier.

Before returning the goods, if the user identifies unacceptable product quality the user should provide the supplier with the user's supporting documentation, such as tests and investigation results. If requested, the user also should provide product samples used for tests and investigations. The supplier should be provided an opportunity to conduct thorough investigations to confirm the validity of the user's quality complaint. While the investigation proceeds, the user should quarantine the material in accordance with internal standard operating procedures and should store the material in an area specifically designated for returns, with limited access to operations, and well-separated from incoming or released raw materials. The user should not reject the materials without supplier confirmation of quality issues.

Goods returned by the user because of excess inventory or other causes unrelated to quality can re-enter commerce within the specified shelf life, provided conditions of storage, transportation, and container integrity have been thoroughly reviewed by the supplier and the quality of the excipient has not been compromised in any way. A formal documented review of each returned container and container tamper-evidence device should be done to verify that these match the container configuration when the materials left the supplier's facility.

If the user opens a commercial packaging container for sampling or investigation (related or unrelated to quality issues) and whether any material was taken out or not, each container should be clearly labeled *Opened*. Written documentation should be provided to the supplier confirming that the container(s) were opened and resealed according to GMPs and describing the reasons for opening, amount withdrawn, and how the pack/container was resealed. Documentation of returned goods should contain a detailed description of all such events including repackaging. Returned excipient containers opened by the user should be clearly identified as such and should not be released as pharmaceutical excipients. In exceptional cases, the material can be released as excipient-grade product if a documented thorough investigation shows no risk of product contamination or deterioration. The quality department should release this material.

Users and suppliers should maintain records of all returned goods, including the product name (trade name and chemical name), batch or lot number, reason for the return, quantity returned, and investigation documentation when applicable. In addition, the supplier should record the final disposition of the material. If returned excipients have been held, stored, or shipped under conditions that could compromise product quality (including ingredients, containers, or labeling), the manufacturer should destroy the excipients. Exceptionally, manufacturers can release the excipients if

their examination, testing, and investigations prove that the material meets suitable standards of identity, quality, and purity and that GMPs and GDPs have not been compromised.

4.1.2 DISPOSITION OF RETURNED GOODS

The excipient manufacturer's quality unit should assess returned product. The options are:

- Return to commerce
- Regrade to a less stringent standard such as technical or industrial grade (non-GMP use)
- Destroy
- Reprocess.

Only containers that have not been opened should be considered for return to commerce without further action.

If the quality assessment of returned goods leads to their final destruction and if associated batches are potentially implicated, an appropriate investigation should be conducted and documented to show that the quality of the associated batches is not affected.

4.1.3 REPROCESSING

Returned goods may be reprocessed according to documented procedures approved in advance by the quality unit. Written procedures should define conditions for holding, testing, and acceptance of the material for reprocessing, as well as procedures for reprocessing, testing, and release of reprocessed material. Reprocessed material should meet appropriate standards, specifications, and characteristics and must be accompanied by a new COA that contains lot number, test results, monograph compliance information, and new release and retest dates. Manufacturers must clearly distinguish the reprocessed material in batch records that are specific to the reprocessed batch, including a new batch or lot number and other appropriate information. The reprocessing event need not be specified on the COA, but the original excipient manufacturer is responsible for ensuring that the reprocessing is performed in compliance with GMPs and that the material meets the established identity, quality, and purity criteria before the material enters commerce. Documentation must establish that the reprocessed material is at least as stable as the original material. Reprocessing is a manufacturing step, and the requirements of *Good Manufacturing Practices for Bulk Pharmaceutical Excipients* (1078) apply. The requirements of (1078) apply only to those intermediate supply chain entities that undertake reprocessing.

4.2 Shipping and Transportation

4.2.1 SHIPPING

The supplier (the manufacturer or distributor) of pharmaceutical excipients should ensure the integrity of the pharmaceutical excipient is maintained by the appropriate storage and transport conditions as described in product labeling. After training, staff should follow written procedures for shipping pharmaceutical excipients. These procedures include the requirement to follow the recommended storage and transportation requirements including temperature, humidity, or other special handling precautions.

Actions should be documented when they are performed. Shipping records for pharmaceutical excipients should provide for the following information:

- Date of shipment
- Name and address of the entity that accepted the materials for the transportation
- Mode of transportation
- Name, address, and status of the consignee

- Material name
- Quantity shipped
- Batch number and expiry date
- Required storage and transport conditions (refrigeration, freezing, or controlled room temperature required)
- Shipping code or identification number of the delivery order.

When regulatory actions such as FDA Field Alerts or drug product recalls occur, the excipient handler must be prepared to act promptly. Shipping documentation must be sufficient to allow adequate handling of any excipient associated with regulatory action. When reasonable, the shipping schedule for excipients should be documented, and responsibilities can be enumerated in a quality or collaborative agreement between entities to show ownership in the supply chain (Entity A to Entity B; Entity B to Entity C; etc.—see *Section 4.4 Packaging: Tamper-Evident Seals*). The buildings and facilities used to ship materials should be appropriate for their intended use in the storage and handling of excipients (see *Section 3.1 Buildings and Facilities*).

Before loading materials, shippers should inspect the container and vehicle to ensure cleanliness and other consignments (if the shipment is a part load) to ensure no form of contamination is likely to occur. This inspection should be documented according to a written procedure. Materials should not be offloaded into other containers or vehicles without the written permission of the material owner or consignee.

4.2.2 TRANSPORTATION

Materials should be transported in a manner that will ensure the maintenance of controlled conditions as specified by the manufacturer. The transport process should not adversely affect the materials or integrity of the packaging. The supplier of transport services must be provided with the required information in order to maintain specified conditions.

The pharmaceutical excipient manufacturer or supplier should agree with the purchaser for arranging transportation. If temperature-controlled transportation is contracted, the shipper must have a mechanism for noting and reporting temperature excursions. Labeling on containers and transportation documents should detail the environmental conditions in a manner that provides the transporter or receiver with knowledge and immediate identification of these conditions, if required. The responsibility for ensuring that the proper storage conditions are met rests with each entity that handles, stores, or transports the materials.

Pharmaceutical excipients should be stored and transported in such a way that the identity and integrity of the material are retained, the material does not contaminate and is not contaminated by other materials, and adequate precautions are taken against spillage, breakage, misappropriation, and theft. The required storage conditions for pharmaceutical excipients should be maintained within acceptable limits during transportation.

Excipients that are potentially dangerous because of the risk of fire or explosion (e.g., combustible liquids, solids, and pressurized gases) should be stored and transported in safe, dedicated, and secure areas, containers, and vehicles. In addition, applicable international agreements and federal regulations should be followed.

4.3 Tampering or Damaged Materials

Materials that are suspected of being tampered with or damaged must be quarantined immediately, and the manufacturer or distributor should be notified. The manufacturer or supplier of excipients should ask the transporter to return the material to the site of shipment origin if tampering or damage is suspected or confirmed. The supplier should make every effort to prevent these materials from being

used until an investigation is completed and the final disposition of the material is determined. Written procedures should guide treatment of excipients that have been tampered with or the identification and handling of damaged material.

4.4 Packaging: Tamper-Evident Seals

A tamper-evident package has one or more indicators or barriers to entry that, if breached or missing, can reasonably be expected to provide visible evidence that tampering has occurred. To reduce the likelihood of successful tampering and to increase the likelihood that any breach will be discovered, the package should be distinctive by design or should employ one or more indicators of or barriers to entry. The term *distinctive by design* means that the packaging cannot be duplicated with commonly available materials or by commonly available processes.

A tamper-evident package may involve an immediate container-closure system in direct contact with the contents (primary packaging), a secondary container-closure system not in direct contact with the contents (secondary packaging), or any combination of systems intended to provide visual evidence of package integrity. For primary packaging in direct contact with the excipient (e.g., paper bags), any leak or break should be considered tampering even if the leak or tear is simply accidental damage.

Visual examination of the packaging at each stage in the supply chain should provide evidence of repackaging or tampering with commercial packaging. In addition, the manufacturer's name and address, net weight of the material, material name, batch or packaging number, date of manufacture, and date of retest should be identified on a packaging label. The label should be prominently placed on the package and should be unaffected if the tamper-evident feature of the package is breached or missing.

The tamper-evident feature for excipient packaging should be designed so that it remains intact when handled in a reasonable manner from the time of packaging at the site of manufacture and throughout the supply chain—including but not limited to warehouse storage during various phases of the supply chain, transport, distribution, receipt, and storage at the user's facility until use for drug product manufacture.

The manufacturer should communicate tamper-evident features to the downstream members of the supply chain. If the latter observe any evidence that the tamper-evident feature or other part of the package has been compromised in any way, they should quarantine the material immediately and inform the supplier. Appropriate arrangements should be made with the supplier to return the material promptly with a description of the packaging breach. The user should ensure adequate protection of the breached packaging during shipment to the supplier and can send photographs of the breached packaging to aid the supplier's investigation.

The supplier is responsible for the integrity of packaging, including but not limited to its tamper-evident features, until ownership of the commercial packages is transferred to the user. Material returned because of breach in packaging should be thoroughly reviewed and investigated by the supplier. The material should not be returned to commerce until the supplier has established that the integrity, identity, quality, purity, and safety of the excipient have not been compromised. The documentation requirement should comply with GMP expectations as well as elements of documentation and investigation suggested in *Section 4.1 Returned Goods*.

4.5 Where Ownership Begins

The excipient user is responsible for purchased materials throughout the supply chain. The supply chain qualification is documented by audits and COAs for all parties involved in trade and distribution of the materials. Such supply chain

qualification and documentation supports the Excipient Pedigree and ownership of the excipient. The pedigree includes documentation of suitable excipient GMPs applied by the excipient manufacturer and suitable GDPs.

Ownership of the materials begins with the original excipient manufacturer and transfers to an intermediary or customer according to agreed-upon terms for insurance costs, transportation, and risk assumption. Such agreements are defined according to International Chamber of Commerce terms (Incoterms).⁶ Incoterms are a series of international sales terms that are used to divide transaction costs and responsibilities between buyer and seller and reflect state-of-the-art transportation practices.

4.6 Adulteration and Economically Motivated Adulteration

4.6.1 ADULTERATION

Adulteration is defined in the FD&C Act and 21 CFR in Sections 501(a)(2)(B) and 501(b)⁷ and 21 CFR 211 for finished pharmaceuticals and Sections 402(a)(3) and (4)⁸ and 21 CFR 110 for human food, respectively. These laws and regulations establish the minimum current GMP (cGMP) necessary to prevent adulteration for food products and finished pharmaceuticals, respectively. Excipients are derived from many sources, so end users must establish raw material specifications to ensure suitability for the safety and efficacy of the final product. Excipients for pharmaceutical use must be manufactured under appropriate GMPs and must meet the required chemical and physical specifications. In addition to specifications, excipient manufacturers and users have generally agreed quality attributes and limits defined by regulatory agencies, common industry practices, and pharmacopeial expectations. Adulteration or contamination of the products can be monitored and detected by many means including, but not limited to, compliance with these predefined quality expectations.

Adulteration occurs when any possible contamination of a product takes place, e.g., from foreign materials or undesirable microorganisms. The problem of adulteration can be addressed by standard practices supporting cGMPs, such as HACCP, Standard Operating Procedures, and staff training to control product safety and purity. This type of adulteration is the unforeseeable and unintentional type that can be controlled and, at worst, detected before the product leaves the manufacturer's site.

FDA specifies that a product can be considered adulterated when conditions *may* lead to adulteration because it is impossible to test every product for every conceivable contaminant. The safety and purity of substances require that manufacturers should build quality controls into the process rather than relying on QC testing.

4.6.2 ECONOMICALLY MOTIVATED ADULTERATION

Compared to simple adulteration, economically motivated adulteration is a much more significant problem because it requires a willful and knowing violation of regulations and standards designed to protect end user safety. It is the deliberate adulteration of an excipient for economic gain. Economically motivated adulteration can occur when a lower-cost material is substituted for a material of higher cost.

Every participant in the supply chain should take all reasonable precautions to prevent economically motivated adulteration.⁹

4.7 Importation

Excipients manufactured outside the United States are subject to US FDA and US Customs and Border Protection (CBP) regulations for importation into the United States. The Bioterrorism Act (Public Health Security and Bioterrorism Preparedness and Response Act of 2002, Title III—Protecting Safety and Security of Food and Drug Supply)¹⁰ has further formalized the requirements for importation of foods and drugs into the United States.

Overseas manufacturers of excipients used in drugs, food, and dietary supplements intended for human or veterinary use who intend to export products into the United States are required to follow the FDA, CBP, and Bioterrorism Act regulations. A streamlined process for the importation of excipients used in pharmaceuticals, food, and dietary supplements must be followed and implemented before the imports are allowed into the United States.

The manufacturing facilities and the manufacturers who produce the excipients should be registered with FDA. An FDA registration number is required for importation. In addition, FDA requires information about Prior Notice (PN) of Imports. Upon receipt of the information, FDA grants a PN confirmation number. FDA must confirm PN before the products are shipped, and the PN confirmation number must appear on the customs declaration that accompanies the package. PN information can be submitted electronically to FDA's Prior Notice Systems Interface (PNSI), a free Internet application that allows facilities to provide information regarding the expected imports. PN information also can be submitted via CBP's Automated Commercial System (ACS), a system that processes imports and obtains information needed to make decisions regarding articles entering the United States.

CBP processes imports of all goods for entry into United States, including but not limited to pharmaceuticals, food, and dietary supplements. CBP inspects but does not release products regulated by the FD&C Act and delegates the final release responsibility at the port of entry to the FDA for such materials. After reviewing the PN information, FDA may determine that the regulated articles should not be allowed into the United States or may allow conditional import of articles subject to testing and release at the port of entry. In addition, during FDA review at the port the regulated articles must meet all requirements of the FD&C Act and 21 CFR before they are released by FDA to the importer.

Importers of record (individuals or companies) for excipients can contract with a broker to transmit PN information and other documents for them. In this case, the submitter is the person responsible for providing the information, but the broker is the transmitter. Brokers are licensed private individuals or companies regulated by CBP and who aid importers and exporters in moving merchandise through CBP. Brokers provide the proper paperwork and payments to CBP for clients and charge a fee for this service. Before brokers apply for a license, they must pass the customs broker examination.

FDA currently uses its Operational and Administrative System for Import Support (OASIS)¹¹ for making its admissibility determinations to ensure the safety, efficacy, and quality of

⁶International Chamber of Commerce. Incoterms. <http://www.iccwbo.org/incoterms> (Accessed June 6, 2011).

⁷FD&C Act, Chapter V: Drugs and Devices. Sec. 501. [21 USC §351] Adulterated Drugs and Devices. <http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticAct/FDCAChapterVDrugsandDevices/ucm108055.htm>.

⁸FD&C Act, Chapter IV: Food. Sec. 402. [21 USC §342] Adulterated Food. <http://www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticAct/FDCAChapterIVFood/ucm107527.htm> (Accessed June 6, 2011).

⁹FDA. Public Meeting on Economically Motivated Adulteration. 2009. <http://www.fda.gov/NewsEvents/MeetingsConferencesWorkshops/ucm163619.htm> (Accessed June 6, 2011).

¹⁰FDA. Guidance for Industry: Questions and Answers Regarding the Interim Final Rule on Prior Notice of Imported Food, (Edition 2); Availability. 2004. <http://www.gpo.gov/fdsys/pkg/FR-2004-05-03/pdf/04-10023.pdf> (Accessed June 6, 2011).

¹¹FDA. Operational and Administrative System for Import Support (OASIS). 2009. <http://www.fda.gov/ForIndustry/ImportProgram/AdmissibilityDeterminationsforShipmentsOfForeign-originOASIS/ucm077691.htm> (Accessed June 6, 2011).

the foreign-origin products for which FDA has regulatory responsibility under the FD&C Act. OASIS is integrated with CBP's ACS and FDA's PNSI systems to receive information related to imported articles.

US Customs and FDA storage areas at the Port of Arrival may not strictly be in compliance with storage conditions required for certain excipients. Importers of record and brokers who represent importers must ensure that the products are released from Customs and FDA inspection as soon as possible. If release is delayed, FDA generally allows removal by Customs and FDA and quarantine in the importer's warehouse until release. FDA staff who review imports at the port of entry are trained to understand that pharmaceutical excipients must be stored under defined conditions. The manufacturer, the importer on record in the importing country, and brokers have a responsibility in working with Customs and FDA staff to ensure that the storage conditions do not adversely affect product quality during quarantine and review.

4.8 Traceability

4.8.1 TRACEABILITY

The pedigree of the excipient should be tracked from the manufacturer's storage through the final delivery to customers by means of recorded identification. The entire supply chain should provide full traceability (for example, via lot numbers and shipping documents) in order to allow fast and efficient investigation of any quality issue or product recall. Every entity in the supply chain also should take responsibility from the preceding supplier and pass the product to subsequent intermediaries down to the end user. Therefore, the original excipient manufacturer and subsequent handlers should always be traceable, and the information should be available both downstream and upstream in the supply chain. All parties to the excipient supply chain should ensure that the excipient is strictly handled according to GDP at every stage.

To ensure the integrity of the supply chain, intermediaries should use contracts, agreements, inspections, and audits downstream and upstream to monitor compliance with GDP principles. When multiple entities constitute the supply chain for each single batch of excipient, each entity should provide its own supplier's certification documentation (see *Appendix: Definitions and Acronyms*) that represents their manufacture or receipt of the excipient batch through release to the subsequent entity. The total of each entity's supplier certification documentation should represent the entire supply chain from original excipient manufacture through use in the final drug product.

4.8.2 TRACEABILITY-RELATED DOCUMENTS

To ensure traceability, all entities in the supply chain should have clear definitions about the shipping documents to be expected with every delivery. At a minimum the documents for every delivery should provide the following information:

- Name and grade of the excipient
- Lot number(s) assigned by the original excipient manufacturer (see *Section 3.4.2 Repackaging and Labeling Batches*)
- Quality and compliance data (e.g., COA) of the excipient
- Origin of the excipient (manufacturer and manufacturing site)
- Original excipient COA(s) (see *Section 3.4.5 Certificates of Analysis*)
- Entity and site of repackaging (when performed), including opening or relabeling the original excipient manufacturer's packaging for any purpose

- Date of shipment and carrier
- Consignor and consignee.

A copy of the COA also should accompany the shipment (see *Significant Change Guide for Bulk Pharmaceutical Excipients* (1195) and *Bulk Pharmaceutical Excipients—Certificate of Analysis* (1080)).

In the event of repackaging from the original excipient manufacturer's package into another container (including any breach or labeling that does not result in a new package), the identity and address of the repackaging entity should be included in the shipping documents.

Additional data resulting from analyses conducted by entities other than the original excipient manufacturer should be provided, along with a clear indication of the source. Quality documents should facilitate traceability back to the manufacturer with contact information. The COA issued by the manufacturer should indicate which results were obtained by testing the original material and which results were obtained by other means. A distributor should not change the original title and data of the COA or other quality documents. Whenever possible, the original excipient manufacturer's documentation should be used, or data transcription should be verified. The original manufacturing site should be identified on the COA.

If any lot mixing is carried out, COAs from manufacturers are no longer valid, and the distributor should perform analyses in its own laboratory or at an approved and qualified contract laboratory. The distributor should supply a COC, and if the blended lot has not been retested the distributor should inform the customer that the material is a mixture of different original excipient manufacturers' lots, provided that all other repackaging and storage activities are carried out according to GDP.

SECTION 5: EXCIPIENTS USED IN PHARMACY COMPOUNDING

Pharmacy compounding is defined in *USP* general chapter *Pharmaceutical Compounding—Nonsterile Preparations* (795). In certain instances *USP–NF* handles compounded preparations differently than commercially manufactured lots. For example, expiration dates are assigned to commercially manufactured products, and beyond-use dates are assigned to compounded preparations (see *General Notices 10.40.100.1 Compounded Preparations*). A similar situation is needed for bulk pharmaceutical Excipients as not all excipients that are useful in compounding are listed in official compendia (see *Pharmaceutical Compounding—Nonsterile Preparations* (795)).

State boards of pharmacy regulate pharmacy compounding. *USP* standards are provided in *Pharmaceutical Compounding—Nonsterile Preparations* (795), *Pharmaceutical Compounding—Sterile Preparations* (797), and *Quality Assurance in Pharmaceutical Compounding* (1163).

APPENDIX: DEFINITIONS AND ACRONYMS

Acceptance Criteria: The specifications and acceptance or rejection limits—such as acceptable quality level or unacceptable quality level with an associated sampling plan—that are necessary for making a decision to accept or reject a lot or batch of raw material, intermediate, packaging material, or excipient.

ACS: Automated Commercial System.

Adulterated Material: A material that either has been contaminated with a foreign material or has not been manufactured using GMP. This does not pertain to a material that simply does not meet physical or chemical specifications.

Audit: An assessment of a system or process to determine its compliance with the requirements of a particular stan-

dard of operation. See also External, Internal, and Third-Party Audit.

Batch (Lot): A defined quantity of excipient processed which can be expected to be homogeneous. In a continuous process, a batch corresponds to a defined portion of the production based on time or quantity (e.g., vessel's volume, one day's production, etc.).

Batch Number (Lot Number): A unique and distinctive combination of numbers and/or letters from which the complete history of the manufacture, processing, packaging, coding, and distribution of a batch can be determined.

Batch Process: A manufacturing process which produces the excipient from a discrete supply of raw materials processed through discrete unit operations in one mass.

Batch Record: Documentation that provides a history of the manufacture of a batch of excipient.

Blending (Mixing): Intermingling different conforming grades into a homogeneous lot.

Broker: An entity that acts as an intermediary between a buyer and a seller of products or services. Brokers neither buy nor take possession of the products or services.

Calibration: The demonstration that a particular instrument or measuring device produces results within specified limits by comparison with those produced by a reference or traceable standard over an appropriate range of measurements.

CBP: Customs and Border Protection.

CEP (Certificate of Suitability to the European Pharmacopoeia): Certification granted to individual manufacturers by the European Directorate for the Quality of Medicines when a specific excipient or active ingredient is judged to be in conformity with a *European Pharmacopoeia* monograph.

CFR: Code of Federal Regulations.

CFR (Cost and Freight, Named Destination): (Incoterm) Seller must pay the costs and freight to bring the goods to the port of destination. However, risk is transferred to the buyer once the goods have crossed the ship's rail (maritime transport only).

cGMP: Current good manufacturing practices.

CIF (Cost, Insurance, and Freight, Named Destination): (Incoterm) Same as CFR except that the seller must, in addition, procure and pay for insurance for the buyer.

CIP (Carriage and Insurance Paid, Named Destination): (Incoterm) The containerized transport or multimodal equivalent of CIF. Seller pays for carriage and insurance to the named destination, but risk passes when the goods are handed over to the first carrier.

Closed-Container Distributor (Pass-Through Distributor): A distributor who sells only products that are tested, packaged, and sealed in the containers provided by the original manufacturer.

Closed System: A system that is isolated from its surroundings by a boundary so that no material can be transferred across it.

COA (Certificate of Analysis): A document that reports the results of a test of a representative sample drawn from the batch of material that will be delivered.

COC (Certificate of Conformance): A document that certifies that the supplied goods or service meets the required specifications. Also known as Certificate of Conformity and Certificate of Compliance.

Commissioning: The introduction of equipment for use in a controlled manner.

Compounding: The preparation, mixing, assembling, altering, packaging, and labeling of a drug, drug-delivery device, or device in accordance with a licensed practitioner's prescription, medication order, or initiative based on the practitioner/patient/pharmacist/compounder relationship in the course of professional practice (defined in *USP* general

chapter *Pharmaceutical Compounding—Nonsterile Preparations* (795)).

Consignee/Consignor: Person or firm (usually the seller) who delivers a consignment to a carrier for transportation to a consignee (usually the buyer) named in the transportation documents.

Contamination: The undesired introduction of impurities of a chemical or microbiological nature or foreign matter into or onto a raw material, intermediate, or excipient during production, sampling, packaging or repackaging, storage, or transport.

Continuous Process: A manufacturing process that continuously produces the excipient from a continuous supply of raw material.

Contract Giver: A person or organization letting a contract.

Contract Acceptor: A person or organization accepting the terms of a contract and thereby agreeing to carry out the work or provide the services as specified in the contract.

Critical: A process step, process condition, test requirement, or other relevant parameter or item that must be controlled within predetermined criteria to ensure that the excipient meets its specification.

Critical to Quality: See *Quality, Critical*.

Cross-Contamination: Contamination of a material or product with another material or product.

Customer: The organization that receives the excipient once it has left the control of the excipient manufacturer; includes brokers, agents, and users.

Deviation: Departure from an approved instruction or established standard.

Distributor: An entity that buys products from a manufacturer, takes possession of those products, and resells them to another party or parties. An essential characteristic of a distributor is the order of these transactions. Distributors buy products (i.e., hold inventory) before making sales.

Drug Master File (DMF): Detailed information about the manufacture of an excipient that is submitted to the US FDA.

Drug (Medicinal) Product: The dosage form in the final immediate packaging intended for marketing.

Drug Substance: Any substance or mixture of substances that is intended for use in the manufacture of a drug product and that, when used in the production of a drug, becomes an active ingredient of the drug product. Such substances are intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of humans or animals.

Economically Motivated Adulteration: The fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production for economic gain.

Electronic Signature: A computer data compilation of any symbol or series of symbols, executed, adopted, or authorized by an individual and intended to be the legally binding equivalent of the individual's handwritten signature.

Excipient: Any substance, other than the active pharmaceutical ingredient or drug product, that has been appropriately evaluated for safety and is included in a drug delivery system to aid the processing of the drug delivery system during manufacture; to protect, support, or enhance stability, bioavailability, or patient acceptability; to assist in product identification; or to enhance any other attribute of the overall safety and effectiveness of the drug delivery system during storage or use.

Excipient Pedigree: Includes documentation of suitable excipient good manufacturing practices applied by the excipient manufacturer and suitable good distribution practices. See *IPEC Excipient Pedigree White Paper*.

External Audit: (See also *Audit, Internal*, and *Third-Party Audit*.) An audit carried out typically on behalf of an excipient manufacturer's customer by a person or organization that is not the manufacturer or the customer.

Expiry (Expiration) Date: The date designating the time during which the excipient is expected to remain within specifications and after which it should not be used.

FCA (Free Carrier, Named Place): The seller hands over the goods, cleared for export, into the custody of the first carrier (named by the buyer) at the named place. This term is suitable for all modes of transport, including carriage by air, rail, road, and containerized/multimodal transport (also called *roll on–roll off*).

FDA: Food and Drug Administration.

FD&C Act: Food, Drug, and Cosmetic Act.

FOB (Free on Board, Named Loading Port): The classic maritime trade term according to which the seller must load the goods on board the ship nominated by the buyer, and cost and risk are divided at ship's rail. The seller must clear the goods for export. The purchaser is then responsible for all further costs associated with transport, importation, and storage until the shipment reaches its destination. The term also is applied to air transport when the seller is not able to export the goods according to the time schedule detailed in the letter of credit. In this case the seller allows a deduction equivalent to the carriage by ship from the air carriage. FOB also can be qualified in other ways. For example, *FOB Factory Gate* means that title and responsibility change as soon as the shipment leaves the supplier's premises.

Forwarding Agents (Freight Forwarders): Agents who assist other organizations or individuals in moving cargo to a destination and are familiar with the import and export rules and regulations of their own and foreign countries, the methods of shipping, and the documents related to foreign trade.

Freight Forwarder: See *Forwarding Agent*.

GDP: Good distribution practices.

GMP: Good manufacturing practices.

Headspace: The volume left at the top of an almost-filled container before sealing.

HACCP (Hazard Analysis Critical Control Point): Hazard Analysis and Critical Control Points has seven principles established by the National Advisory Committee for Microbiological Criteria for Foods to control product safety.

Importer: Either the US owner or consignee at the time of entry into the United States or the US agent or representative of the foreign owner or consignee at the time of entry into the United States who is responsible for ensuring that goods offered for entry into the United States are in compliance with all laws affecting the importation.

Impurity: A component of an excipient that is not the intended chemical entity or a concomitant component but is present as a consequence of either the raw materials used or the manufacturing process and is not a foreign substance.

Independent: In the context of internal audits, the quality of being free from any influence, economic or otherwise, from the group, department, or organization under audit.

In-Process Control: Checks performed during production in order to monitor and if necessary to adjust the process to ensure that the material conforms to its specifications. The control of the environment or equipment also can be regarded as a part of in-process control.

In-Process Control/Testing: Checks performed during production to monitor and, if appropriate, to adjust the process to ensure that the intermediate or excipient conforms to its specification.

Intermediate: Material that must undergo further manufacturing steps before it becomes an excipient.

Internal Audit: An audit conducted by an employee of the organization or by an individual from outside the organiza-

tion, but on behalf of the organization, to determine the effectiveness of a system. (See: *Audit, External Audit*, and *Third-Party Audit*).

International Nonproprietary Name: International Nonproprietary Names (INN) facilitate the identification of pharmaceutical substances or active pharmaceutical ingredients. Each INN is a unique name that is globally recognized and is public property. A nonproprietary name also is known as a generic name.

ISO: International Organization for Standardization.

Lot: See *Batch*.

Labeling: The affixing to a container or vessel of a tag or document that contains information about that container and its contents.

Manufacturer/Manufacturing Process: All operations of receipt of materials, production, packaging, repackaging, labeling, relabeling, quality control, release, and storage of excipients and related controls.

Master Production Instruction (Master Production and Control Record): Documentation that describes the manufacture of the excipient from raw material to completion.

Material: A general term used to denote raw materials (starting materials, reagents, and solvents), process aids, intermediates, excipients, packaging, and labeling materials.

Nonconforming Material: A material that is deficient in a characteristic, product specification, process parameter, record, or procedure that renders its quality unacceptable, indeterminate, or not according to specified requirements.

OASIS: Operational and Administrative System for Import Support.

Original Excipient Manufacturer: Organization responsible for manufacturing, under appropriate GMPs, the excipient(s) distributed and addressed by this chapter.

Packaging/Repackaging Distributor: A distributor who transfers products from the original packaging or transport vessel(s) provided by the original manufacturer into alternative packaging and sells the products in the alternative packages. See *Distributor* and *Repackager*.

Primary Container–Closure System: The packaging components that come into direct contact with the excipient in the closed, sealed package during storage and transport.

Packaging Material: A material intended to protect an intermediate or excipient during storage and transport.

Packaging: The container and its components that hold the excipient for storage and transport to the customer.

Pass-Through Distributor: See *Closed-Container Distributor*.

PN: Prior notice.

PNSI: Prior Notice Systems Interface.

Primary, Secondary Packaging: See *Packaging/Repackaging Distributor* and *Primary Container–Closure System*. Packaging materials which do not come into contact with the excipient during the normal course of storage and transport of the excipient.

Production: Operations involved in the preparation of an excipient from receipt of raw materials through processing and packaging of the excipient.

QbD (Quality by Design): A systematic approach to pharmaceutical development that begins with predefined objectives and emphasizes product and process understanding and process control based on sound science and quality risk management. It means designing and developing products and manufacturing processes to ensure a predefined quality.

QMS: Quality management system.

Quality Assurance (QA): The total of the organized arrangements made to ensure that all excipients are of the quality required for their intended use and that quality systems are maintained. See *Quality Unit*.

Quality Control (QC): Checking or testing that specifications are met. See *Quality Unit*.

Quality, Critical: Describes a material, process step or process condition, test requirement, or any other relevant parameter that directly influences the quality attributes of the excipient and that must be controlled within predetermined criteria.

Quality Management System (QMS): Management system that directs and controls a pharmaceutical company with regard to quality.

Quality Manual: Describes the elements of the QMS and includes the quality organizational structure, written policies, procedures, and processes or references to them, and a description of departmental functions as they relate to the policies, procedures, and processes. Document specifying the quality management system of an organization.

Quality Unit: See also: *Quality Control* and *Quality Assurance*. A group within a larger organization that is responsible for monitoring and ensuring all aspects of quality. Current industry practice generally divides the responsibilities of the quality control unit (QCU), as defined in the cGMP regulations, between quality control (QC) and quality assurance (QA) functions. QC usually involves (1) assessing the suitability of incoming components, containers, closures, labeling, in-process materials, and the finished products; (2) evaluating the performance of the manufacturing process to ensure adherence to proper specifications and limits; and (3) determining the acceptability of each batch for release. QA primarily involves (1) review and approval of all procedures related to production and maintenance, (2) review of associated records, and (3) auditing and performing/evaluating trend analyses.

Quarantine: The status of materials isolated physically or by other effective means pending a decision about their subsequent approval or rejection.

Raw Material: A general term used to denote starting materials, reagents, and solvents intended for use in the production of intermediates or excipients.

Recall: See *Retrieval*.

Record: A document stating results achieved or providing evidence of activities performed. The medium can be paper, magnetic, electronic or optical, photographic, another medium, or a combination thereof.

Reevaluation Date (Retest Date, Re-evaluation Interval): The date when the material should be reexamined to ensure that it is still in conformity with the specification.

Recommended Re-evaluation Date: The date suggested by the supplier when the material should be re-evaluated to ensure continued compliance with specifications. It differs from the Expiration Date because the excipient can be re-evaluated to extend the length of time the material can be used, if supported by the results of the evaluation and appropriate stability data.

Repackager: A person or organization that takes an excipient from the original manufacturer's container and repackages it into different containers. See also *Distributor* and *Packaging/Repackaging Distributor*.

Repackaging: Removal of the excipient from its original container (combination of secondary and/or primary packaging), and transfer to another container.

Reprocessing: Introduction of previously processed material that did not conform to standards or specifications back into the process and repetition of one or more necessary steps that are part of the normal manufacturing process.

Retrieval (Recall): Process for the removal of an excipient from the distribution chain.

Reworking: Subjecting previously processed material that did not conform to standards or specifications to processing steps that differ from the normal process.

Secondary, Primary Packaging: See *Primary, Secondary Packaging*.

Senior Management: See *Top Management*.

Significant Change: A change that alters an excipient's physical or chemical property from the norm or that is likely to alter the excipient's performance in the dosage form.

Specification: The quality parameters to which the excipient, component, or intermediate must conform and that serve as a basis for quality evaluation.

Stability: Continued conformity of the excipient to its specifications.

Stable Process: A process whose output, regardless of the nature of the processing (batch or continuous), can be demonstrated by appropriate means to show a level of variability that consistently meets all aspects of the stated specification (both USP and customer specifications) and thus is acceptable for its intended use.

Subcontractor: A person or organization that undertakes work or services on behalf of a different person or organization that in turn is contracted to undertake work or provide services from the original contract giver.

Supplier's Certification Documentation: Specific information and data associated with a single batch of an excipient. Its accuracy is certified by the business entity that has had control of the same single batch of excipient. Supplier's Certification Documentation includes both quality and supply chain data and information. The methods and processes that derive the included data and information should be understood and controlled, and all data and information sources should be traceable. All entities that take possession and responsibility for the excipient batch should provide Supplier's Certification Documentation including the original excipient manufacturer, all distributors, and all repackagers. Special attention and clarity should be applied within the Supplier's Certification Documentation in any event that breaches the original manufacturer's packaging and/or labels (including addition of new labels).

Third-Party Audit: An audit conducted by an individual from outside the organization and who is neither a supplier nor customer of the organization, e.g., a certification body, to determine the effectiveness of a system.

Top Management: Person or group of people who direct and control an organization at the highest level. The highest level can be at either the site level or the corporate level and depends on how the quality management system is organized.

Traceability: Ability to determine the history, application, or location that is under consideration, e.g., origin of materials and parts, processing history, or distribution of the product after delivery.

Trader: An entity that buys products from a manufacturer, may or may not take possession of the products, and resells them to another party or parties.

NOTE: In the case of traders, the sale usually is made before product purchase.

User: A person or organization that uses pharmaceutical excipients to manufacture pharmaceutical intermediates or finished products.

Validation: A documented program that provides a high degree of assurance that a specific process, method, or system will consistently produce a result meeting predetermined acceptance criteria.▲ USP36

(1207) STERILE PRODUCT PACKAGING—INTEGRITY EVALUATION

This information chapter addresses the maintenance of the microbiological integrity of sterile product packaging until the time of use of its contents. The scope of application of this chapter includes the container and closure systems of drug products and the sterile barrier packaging of medical devices, including in vitro diagnostic products. Specially designed or novel container–closure and barrier packaging systems, which are generally more complex than classical packaging and delivery systems, are also discussed in this chapter. The establishment of sterile product integrity is necessary to ensure the maintenance of two extremely important product conditions: total product attributes within label claim specifications and product sterility prior to use.

Product package integrity testing continues throughout the life cycle of the product. Generally, this integrity testing should occur during three phases: (1) the initial development of the product packaging system, (2) routine manufacturing, and (3) shelf life stability assessments. Generally, during initial packaging development both physical and microbial studies are conducted to assess integrity. It is at this time that comparative information about physical and microbial challenge methods may be obtained.

During routine manufacturing, physical measurements may be conducted in accordance with an established sampling plan to determine whether the packaging or capping system or both are operating consistently within predetermined performance acceptance ranges. Packaging integrity tests conducted during shelf life stability assessments are physical tests that confirm the integrity of the packaging system supported by acceptance values established during packaging development. Microbial ingress testing may be unnecessary for shelf life stability assessment if comparative physical and microbial testing conducted during the packaging development stage has established physical acceptance values that would preclude microbial ingress. Reconfirmation of product packaging integrity should occur when there are major changes in the package design and materials or whenever there are changes in the manufacturing processing conditions, including sterilization conditions.

PRODUCT PACKAGE DEVELOPMENT PHASE

Testing during the product development phase is frequently intensive, because it establishes product design limitations before full-scale manufacturing occurs. At this phase, an appropriate product package design relative to the end use of the product is selected, and manufacturing process variables are defined. The effect of the design and process variables on the maintenance of package integrity is assessed. Approximate design tolerances are assessed to ensure sterility of the fluid pathway or contents during processing and prior to usage. Product packaging integrity evaluations should take into consideration the maximum stress conditions encountered during the manufacturing and sterilization processes. Testing should also validate the integrity of the design when exposed to anticipated extreme conditions of storage, shipment, and distribution. During this phase, physical methods are developed to assess the integrity of the proposed package, and they are used later in routine manufacturing testing or marketed product stability testing.

ROUTINE MANUFACTURING PHASE

During this phase, maintenance of specified production, engineering, and microbiological conditions is monitored through standard operating procedures. Physical testing methods, on-line or not, may be intermittently used to supplement process control measurements to ensure that product packaging values are maintained within acceptable limits established during the product development phase. During routine manufacturing, monitoring for continuous unit product integrity or finished product release package integrity may be unnecessary when critical production processes are well controlled. Microbiological testing of package integrity is usually not expected during routine manufacturing that operates within specification limits previously determined to produce acceptable packaging.

MARKETED PRODUCT STABILITY PHASE

Physical testing methods may be used to evaluate product packaging systems that are included in a marketed product shelf life stability program. Such systems should be evaluated at stability conditions, at the beginning of the product shelf life as well as at the expiration date of the product, and at other times and intervals defined by regulatory requirements or guidelines. Sole testing of product sterility will not ensure maintenance of product package integrity over the shelf life of the product when performed as part of the packaging stability program.

PHYSICAL AND MICROBIOLOGICAL TESTING

During the initial evaluation of product packaging, both physical and microbiological testing are frequently used.¹ Physical testing to assess product packaging integrity has a variety of advantages over microbial testing, depending on the test method and the packaging being evaluated. These advantages may include greater sensitivity, ease of use, rapid speed of testing, or lower cost. Physical testing can be used to evaluate product packaging throughout its life cycle to ensure that total product attributes are maintained within optimum predefined limits. Microbial ingress testing in concert with physical testing should also be considered at the initial stages of product packaging development. However, physical testing methods with sensitivity comparable to or greater than that of microbial methods are preferred for evaluating product packaging in a marketed product shelf life stability program. Physical testing methods provide rapid assessment of packaging integrity during routine testing of large numbers of product stability samples.

Comparison of Microbial and Physical Methods

A comparative evaluation should determine whether microbial intrusion or ingress into the product packaging could occur at those physical integrity test value ranges that have been deemed acceptable for the finished product. This determination should be based on a comparison between microbiological data and the values obtained from physical integrity test method. The comparison of physical integrity testing to microbial ingress testing for assessment of product packaging integrity can be obtained either by direct comparison or by studies that demonstrate that the physical test measures defects that are too small for microbial passage.

Due to its design or material composition, a product packaging system may not permit or yield graded physical test responses when a range of defects is created in the

¹ A review of physical and microbiological methods related to the evaluation of product packaging integrity appears in *Pharmaceutical Package Integrity*, Parenteral Drug Association's Technical Report No. 27, 1998.

packaging. In some situations, even upon creating artificial defects in a product packaging system, the resulting range of physical testing values may remain approximately the same. In other words, the test method may yield a qualitative rather than a quantitative measure of package leakage. Also, microbial ingress may not occur until exaggerated physical defects are created in the packaging. In such cases, a direct correlation of microbial ingress to a series of physical value ranges is not possible. When this occurs, the testing results become merely pass or fail results conducted on packaging with known defects.

Selection of Evaluation Methods

Physical and microbiological methods for product package integrity testing should take into consideration the design of the closure system; the manufacturing method, including the sterilization process; and the intended use of the product. Any particular physical or microbial method may not be applied to all product packaging systems.

During initial integrity evaluations of a packaging system, a number of physical tests may be used. These physical tests may include, but are not limited to, pressure and vacuum decay tests, dye immersion tests, liquid chemical tracer tests, gas ionization of evacuated containers, high-voltage leak detection of plastic or glass containers, visual examination for glass cracks, and gas leakage or package headspace analysis. Other tests that may be valuable in evaluating package seal quality include screw-cap removal torque, elastomeric stopper residual seal force, or heat seal strength.

Microbiological tests may include closure immersion testing for rigid container systems, inoculated shipment testing for certain packaging, and an aerobiological challenge for tortuous path barrier packaging.

Dual Function Container–Closure Systems²

Dual function container–closure systems are characterized by the addition of one or more intended functions to that of a container and require special consideration for integrity evaluation. For instance, in the case of small, flexible or rigid containers with an appended device component that allows direct patient injection and drug delivery, a delivery function is added to the container function. Thus, frequently, one compartment of the dual container–closure system is designed to contain the drug or solution prior to use or activation of the system—product containment compartment. Another compartment, different in function and design, either directly delivers the product from the container portion to a fluid pathway for direct injection of the patient or communicates with a sterile pathway of another access device. For example, a prefilled syringe contains a solution (container compartment) and a device component (delivery compartment) physically separated from the container compartment and used to directly administer the drug to the patient.

Therefore, dual container–closure systems typically have at least two compartments that require microbial barrier properties, and packaging integrity after sterilization and/or aseptic filling should be demonstrated for both compartments. In many cases, different portions of the dual system require different integrity testing methods. The selection of the integrity testing method is determined primarily on the basis of the intended objectives or performance requirements of the particular compartment. For example, the solution or drug-containing compartment of the dual container–closure system must be enclosed or sealed in a manner that precludes leakage of product or microbial ingress during and following the manufacturing process (see *Selection of Evaluation Methods*). On the other hand, the delivery portion of the dual container–closure system frequently contains a fluid pathway that is empty during the

sterilization or aseptic filling process and is intended to remain dry until the product container portion is activated prior to use. A covering, a sheath, or perhaps a cap designed to vent during sterilization and storage protects the delivery compartment from airborne microbial ingress throughout the life of the article. However, this portion of the device is frequently not designed to prevent liquid ingress. Liquid ingress can be precluded by secondary packaging or by the physical design of the system itself. Microbial integrity testing of the delivery portion of such a dual container–closure system may include a nonimmersion microbial method or physical integrity test. Microbial testing would include, for example, an aerosolized microbial challenge under defined pressure changes.

<1208> STERILITY TESTING— VALIDATION OF ISOLATOR SYSTEMS

This chapter provides guidelines for the validation of isolator systems for use in sterility testing of compendial articles. [NOTE—In the context of this chapter, “decontaminated” refers to an item or surface that has been subjected to a process that eliminates viable bioburden.]

Isolators—devices that create controlled environments in which to conduct Pharmacopeial sterility tests—have been used since the mid-1980s. An isolator is supplied with air through a HEPA or better air filter and is able to be reproducibly decontaminated. Closed isolators, which are systems with no direct opening to the external environment, are normally used for sterility testing, although open isolators which allow the egress of materials through a defined opening that precludes the entry of contamination by means of air overpressure may be used. Closed isolators use only decontaminated interfaces or a rapid-transfer port for the transfer of materials. Isolators are constructed of flexible plastics (such as polyvinyl chloride), rigid plastics, glass, or stainless steel.

Isolator systems protect the test article and supplies from contamination during handling by essentially eliminating direct contact between the analyst and the test articles. All transfers of material into and out of the isolator are accomplished in an aseptic fashion while maintaining complete environmental separation. Aseptic manipulations within the isolator are made with half-suits, which are flexible components of the isolator wall that allow the operator a full range of motion within the isolator, or by gloves and sleeves. Operators are not required to wear special clean-room clothing for conducting sterility tests within isolators; standard laboratory clothing is adequate, although a pair of sterile gloves is frequently worn under the isolator gloves as an added precaution against contamination entering the isolator enclosure and for hygiene purposes. The interior of the isolator is treated with sporicidal chemicals that result in the elimination of all viable bioburden on exposed surfaces.

ISOLATOR DESIGN AND CONSTRUCTION

Air Handling Systems

An isolator used for sterility testing is equipped with microbial retentive filters (HEPA filters or better are required). At rest, the isolator meets the particulate air-quality requirements for an ISO Class 5 area as defined in ISO 14644-1

²For more information, see ANSI/AAMI/ISO Standard 11607-2000, 2nd ed., Packaging for Terminally Sterilized Medical Devices.

through -3* (see *Microbiological Control and Monitoring of Aseptic Processing Environments* <1116>). However, the isolator need not meet Class 5 conditions during an operation that may generate particulates, and no requirements for air velocity or air exchange rate exist. The isolator should be sealed well enough during decontamination that the dissemination of sporicidal vapors or gases into the surrounding environment is kept to appropriately low levels. When direct openings to the outside environment exist, constant air overpressure conditions maintain sterile conditions within the isolator. In general, both open and closed isolators are maintained at positive pressure relative to the surrounding environment, and overpressures of 20 Pa or more are typical. The user should never exceed the maximum pressure recommended by the isolator manufacturer. Airflow within isolators used for sterility testing is either unidirectional or turbulent.

Transfer Ports and Doors

Isolators may be attached to a “pass-through” decontaminator or transfer isolator to enable the direct transfer of sterile media, sterile dilution fluids, and sterile supplies from the decontaminator into the isolator system. Rapid transfer ports (RTPs) enable two isolators, i.e., the work station and transfer isolator, to be connected to one another, so that supplies can be moved aseptically from one isolator to another. Aseptic connections between two isolators or an isolator and an RTP-equipped container can be made in unclassified environments using RTPs. The nonsterile surfaces of the RTP are connected using locking rings or flanges. A compressed gasket assembly provides an airtight seal, thereby preventing the ingress of microorganisms.

When the two RTP flanges are linked to form an airtight passage, a narrow band of gasket remains that could harbor microbial contamination. This exposed gasket should be routinely disinfected immediately after the connection is made, and before materials are transferred through the RTP. Good aseptic technique is used when transferring materials and care is taken not to touch the gasket with the materials being transferred or with the gloved hands.

Preventive maintenance and lubrication of the gasket assemblies on the flanges is performed according to the RTP manufacturer's recommendations. The RTP gaskets are changed at the recommended frequency and periodically checked for damage, because cut or torn gaskets cannot make a truly airtight seal.

Selection of a Location for the Isolator

Isolators for sterility testing need not be installed in a classified clean room, but it is important to place the isolator in an area that provides limited access to nonessential staff. The appropriate location provides adequate space around the isolator for moving transfer isolators, staging of materials, and general maintenance. No environmental monitoring of the surrounding room is required.

Temperature and humidity control in the room is important to operator safety and comfort and is critical for the effective utilization of certain decontamination technologies. Uniform temperature conditions in the room are desirable when temperature-sensitive decontamination methods are employed. Care should be taken in locating the isolator so that cold spots are avoided that might result in excessive condensation when condensing vapors are used for decontamination.

VALIDATION OF THE ISOLATOR SYSTEM

The isolator system must be validated before its use in sterility testing as part of a batch release procedure. To ver-

ify that the isolator system and all associated equipment are suitable for sterility tests, validation studies are performed in three phases: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). The following sections contain points to consider in the validation of isolator systems for sterility testing. The assignment of test functions to a particular phase of the validation program (i.e., IQ, OQ, and PQ) is not critical, as long as proper function of the isolator is demonstrated and documented before its use in compendial Assays.

Installation Qualification (IQ)

The IQ phase includes a detailed description of the physical aspects of the system, such as the dimensions, internal configuration, and materials of construction. The unit layout is diagrammed with interfaces and transfer systems clearly and dimensionally indicated. Compliance with design specifications for utility services, such as air supply, vacuum, external exhaust, and temperature and humidity control, is verified. Other equipment used with the isolator system is also described in detail; if any revisions to design specifications are made, these are included. Equipment manuals and copies are catalogued and stored where they can be retrieved and reviewed. Compliance of drawings to design specifications is verified. All drawings and process and instrumentation diagrams are catalogued, stored, and are retrievable.

All documentation is reviewed to verify that it precisely reflects the key attributes of the installed system. This establishes a general benchmark for the isolator system's compliance with design specifications and installation requirements.

Potential process-control or equipment problems that could cause system failure during operation are identified and documented during failure-mode analysis and hazard analysis. The system is modified, if necessary, to minimize the risk of failure, and critical control point methods are established.

The results of the IQ are summarized in an Installation Qualification Report. The following documentation is suggested.

Equipment—The equipment is listed with its relevant design specifications. The IQ verifies that equipment meeting the appropriate design specifications was received and that it was installed according to the manufacturer's requirements.

Construction Materials—The construction materials of critical system components are checked for compliance with design specifications. The compatibility of the intended decontamination method with the construction materials is verified.

Instruments—System instruments are listed with their calibration status.

Utility Specifications—All utilities required for operation—as defined in the operating manuals and process and instrumentation diagrams—are checked for availability and compliance with design specifications. Any connection between utility systems and the isolator system is inspected and conformance of these connections to specifications is verified.

Filter Certification—HEPA filters and other microbial retentive filters are tested and certified; copies of test results and certificates are included in the IQ summary. Purchase orders are reviewed and conformance of the air filtration system to specifications is verified.

Computer Software—All computer software associated with the isolator system is listed with its name, size, and file revision number. The master computer disks are checked for proper labeling and stored securely.

* International Organization for Standardization (ISO) International Standards 14644-1, -2, -3, and -7

Operational Qualification (OQ)

The OQ phase verifies that the isolator system operates in conformance to functional specifications.

Operational Performance Check—This test verifies that all alert and alarm functions comply with their functional specifications. The system's ability to comply with all set points and adjustable parameters is verified.

Isolator Integrity Check—The integrity of the isolator is maintained during all normal operating conditions. A leak test is performed to verify the compliance with the manufacturer's functional specifications and to ensure safety prior to charging the isolator with a decontaminating sporicidal chemical. To safeguard against adventitious contamination, isolators are operated at a suitable positive pressure during normal operation. Validation studies must show that the air pressure set point can be maintained and controlled during operation.

Decontamination Cycle Verification—A decontamination cycle that is the function of the decontamination equipment in concert with the isolator(s) is verified.

Different decontamination methods can be used to eliminate bioburden from isolator systems and supplies. Among the chemicals that have been used to treat isolators are peracetic acid, chlorine dioxide, ozone, and hydrogen peroxide; each has different requirements for exposure conditions and process control. It is critical to comply with the manufacturer's operational requirements for the selected decontamination method and to describe them in the functional specifications. The temperature inside the isolator is also important, particularly for hydrogen peroxide vapor decontamination, where it is critical to maintain the concentration relative to the condensation point. Some sterilization chemicals, such as chlorine dioxide and ozone, require the addition of moisture to the isolator prior to decontamination. When elevated relative humidity is required, the ability to control it must be verified during OQ.

It is also important to verify the concentration and distribution of the decontaminating chemical. When applied in gaseous or vapor form, the distribution may be evaluated using chemical indicators, spectroscopic methods, or electronic sensors.

Gas and vapor decontamination methods may require fans in the isolator to distribute the chemical evenly. The location and orientation of these fans are adjusted to ensure optimum air distribution. If the isolator utilizes a recirculating unidirectional airflow system, distribution fans may not be required, but this should be evaluated on a case-by-case basis. Because shelving units, equipment, glove-and-sleeve assemblies, and half-suits have an impact on distribution patterns, distribution checks are done with the isolator fully loaded with equipment and supplies, and the setup of these units is defined and documented.

Many installations use smaller transfer isolators as portable surface decontamination units. In these transfer isolators, test articles and supplies are treated chemically to eliminate bioburden before transfer through an RTP into the testing isolator. Its loading configuration is defined, and configuration drawings are reviewed and verified during the OQ. [NOTE—The decontaminating chemicals used in isolators work on the surfaces of materials; therefore, any surface that is occluded will not be treated and could contain viable bioburden. Special precautions should be in place for treating surfaces known to be occluded with a sporicide if such surfaces may be revealed during the conduct of sterility tests.]

Decontamination agents need to be removed from the isolator after the exposure period, which is accomplished by a current of fresh air provided either by the decontamination equipment or by utilizing the isolator air handling system. Aeration is accomplished either in an open loop, in which the gas is exhausted through a vent to the atmosphere, or in a closed loop, in which the chemical is removed and destroyed by the decontamination equipment.

The aeration system is checked; if an open-loop configuration is used, the external exhaust system's flow and safety are checked.

Decontamination Cycle Development—When the OQ is completed, decontamination cycle development is performed to establish the parameters necessary for process control during routine decontamination cycles. Any of the methods generally used in the industry for the validation of decontamination processes—including bioburden-based, fractional cycle, and overkill methods—are adequate. The decontamination process is challenged with biological indicators (BIs). The spore population and resistance of the BIs to the decontamination conditions being applied are known. Wherever possible, a D value estimate is done for each B1 system or, alternatively, a survivor curve for the BI system is obtained (see *Biological Indicators—Resistance Performance Tests* (55)); it is acceptable to obtain the D value from the BI vendor.

Performance Qualifications (PQ)

The PQ phase verifies that the system is functioning in compliance with its operator requirement specifications. At the completion of the PQ phase, the efficacy of the decontamination cycle and, if appropriate, the adequacy of decontaminating chemical venting are verified. All PQ data are adequately summarized, reviewed, and archived.

Cleaning Verification—In general, cleaning is not critical for sterility testing applications. However, residual products are a concern in multiproduct testing, particularly for aggressive antimicrobial agents, because these materials could interfere with the ability of subsequent tests to detect low levels of contamination in the product. Concerns about contamination with the product are heightened when it is an inherently antimicrobial powder, because powders are more readily disseminated. Cleaning to a level at which no visible contamination is present is adequate for sterility test isolator systems and is a suitable operator requirement specification. The cleaning method, frequency, equipment, and materials used to clean the isolator are documented.

Decontamination Validation—The interior surfaces of the isolator, the equipment within the isolator, and the materials brought into the isolator are treated to eliminate all bioburden. The decontamination methods used to treat isolators, test articles, and sterility testing supplies are capable of reproducibly yielding greater than a three-log reduction against highly resistant biological indicators (see *Biological Indicators for Sterilization* (1035)), as verified by the fraction negative or total kill analysis methods. Total kill analysis studies are suitable for BIs with a population of 10^3 spores per unit, while fraction negative studies are suitable for BIs with a population of 10^5 or greater. A sufficient number of BIs are used to prove statistical reproducibility and adequate distribution of the decontaminating agent. Particular attention is given to areas that pose problems relative to the concentration of the agent. A larger number of BIs may be required in isolators that are heavily loaded with equipment and materials. The ability of the process to reproducibly deliver a greater than three-log kill is confirmed in three consecutive validation studies.

The operator establishes a frequency for re-decontamination of the isolator. The frequency may be as short as a few days or as long as several weeks, depending on the sterility maintenance effort (see *Maintenance of Asepsis within the Isolator Environment*).

PACKAGE INTEGRITY VERIFICATION

Some materials are adversely affected by decontaminating agents, which can result in inhibition of microbial growth. Of concern are the penetration of decontaminating agents into product containers; accessory supplies such as filter sets and tubing; or any material that could come in contact with

product, media, or dilution fluids used in the sterility test. It is the responsibility of the operator to verify that containers, media, and supplies are unaffected by the decontamination process. Screw-capped tubes, bottles, or vials sealed with rubber stoppers and crimp overseals have proven very resistant to the penetration of commonly used decontaminating agents. Wrapping materials in metal foil or placing them in a sealed container will prevent contact with the decontaminating agent; however, these procedures may also result in some surfaces not being decontaminated. In some cases, the use of shorter duration decontamination cycles and reduced concentrations may be necessary to minimize penetration of decontaminating agents into the package or container. Cycles that provide a less than three-log kill of resistant BIs may be acceptable provided microbiological analysis of the environment proves that the isolator(s) are free of recoverable bioburden.

In many cases, the operator will choose to treat the surfaces of product containers under test with the decontaminating agent in order to minimize the likelihood of bioburden entering the isolator. It is the responsibility of the operator to demonstrate, via validation studies, that exposure of product containers to the decontaminating agent does not adversely affect the ability of the sterility test to detect low levels of contamination within these test articles. It is suggested that the ability of the package to resist contamination be examined using both chemical and microbiological test procedures. Bacteriostasis and fungistasis validation tests must be performed using actual test articles that have been exposed to all phases of the decontamination process (see *Sterility Tests* <71>). This applies to medicinal device packages as well as pharmaceutical container and closure systems.

Validation studies determine whether both sterility test media and environmental control media meet the requirements for *Growth Promotion Test of Aerobes, Anaerobes, and Fungi* under *Sterility Tests* <71>.

MAINTENANCE OF ASEPSIS WITHIN THE ISOLATOR ENVIRONMENT

The ability of the isolator system to maintain an aseptic environment throughout the defined operational period must be validated. In addition, a microbiological monitoring program must be implemented to detect malfunctions of the isolator system or the presence of adventitious contamination within the isolator. Microbiological monitoring usually involves a routine sampling program, which may include, for instance, sampling following decontamination on the first day of operation and sampling on the last day of the projected maintenance of asepsis period. Periodic sampling throughout the use period can be performed to demonstrate maintenance of asepsis within the isolator.

The surfaces within the isolator can be monitored using either contact plates for flat surfaces or swabs for irregular surfaces. However, because media residues could impose a risk on isolator asepsis, these tests are generally best done at the end of the test period. If performed concurrently with testing, care is used to ensure that any residual medium is removed from isolator surfaces, and that those surfaces are carefully cleaned and disinfected. Active air samples and settling plates may be used, but they may not be sufficiently sensitive to detect the very low levels of contamination present within the isolator enclosure.

A potential route for contamination to enter the isolator is during the introduction of supplies and samples into the

enclosure. Validating that all materials taken into the isolator enclosure are free of microbial contamination is critical, as is periodic inspection of gaskets to detect imperfections that could allow ingress of microorganisms. Gloves and half-suit assemblies are another potential source of microbial contamination. Gloves are of particular concern because they are used to handle both sterility testing materials and test articles. Resistance to puncture and abrasion should be considered in the selection of gloves and sleeves. Hypalon materials are resistant to both chemical sporicides used in the decontamination of isolators and to punctures and are available in several thicknesses to provide adequate tactile feel through the gloves while maintaining their integrity.

Very small leaks in gloves are difficult to detect until the glove is stretched during use. There are several commercially available glove leak detectors; the operator ensures that the detectors test the glove under conditions as close as possible to actual use conditions. Microbiological tests are used to supplement or substitute physical tests. [NOTE—Standard “finger dab plates” may not be sensitive enough to detect low levels of contamination. Submersion of the gloves in 0.1% peptone water followed by filtration of the diluent and plating on growth media can detect loss of integrity in the gloves that would otherwise go unnoticed.]

Continuous nonviable particulate monitoring within the isolator’s enclosure is ideal, because it can quickly detect filter failure. A second choice is periodic monitoring using a portable particle counter. Sampling for particles must be done in a manner that poses no risk to the maintenance of asepsis within the isolator.

INTERPRETATION OF STERILITY TEST RESULTS

A sterility test resulting in a false positive in a properly functioning and validated isolator is very unlikely if bioburden is eliminated from the isolator interior with a high degree of assurance; if gloves, sleeves, and half-suits are free of leaks; and if the RTPs are functioning properly. Nevertheless, isolators are mechanical devices and good aseptic techniques are still required. A decision to invalidate a false positive is made only after fully complying with the requirements of *Observation and Interpretation of Results* under *Sterility Tests* <71>.

TRAINING AND SAFETY

As with sterility testing conducted in conventional clean rooms, operators are trained in procedures that are specific to their isolator. Use of proper aseptic techniques is vital to the conduct of sterility tests in isolators, just as it is in clean rooms. Therefore, training in proper aseptic techniques is required for all sterility testing technicians. All training sessions and the evaluation of the operator’s performance are documented in the individual’s training record. Training of all personnel in the appropriate safety procedures necessary for the operation and maintenance of the isolation system is imperative.

Personnel safety in the use of a decontaminating agent must be assessed. Material Safety Data Sheets, or equivalent documents, are available in the immediate area where the decontaminating agent is being used. All storage and safety precautions are followed. An operational readiness inspection of the safety of the isolator and all associated equip-

ment is performed and documented prior to placing the unit in service.

(1209) STERILIZATION— CHEMICAL AND PHYSICOCHEMICAL INDICATORS AND INTEGRATORS

INTRODUCTION

The Federal Code of Regulations, Part 211 on Good Manufacturing Practices for Finished Pharmaceuticals in section 211.165 states: "There shall be appropriate laboratory testing, as necessary, of each batch of drug product required to be free of objectionable microorganisms." This statement has been interpreted to mean that an alternate laboratory control test for sterility is required for any batch load of a product that is parametrically released. An appropriate laboratory test for each batch may be a biological indicator, which is included in each batch of product that is terminally sterilized (see *Biological Indicators for Sterilization* (1035)), or a physicochemical indicator or integrator. This requirement may also be met by a primary product release system that includes the documented recording of thermometric measurements systems that are calibrated with a NIST traceability system and that demonstrate a $\pm 0.5^\circ$ performance capability.

The presence of this chapter in the *USP* does not mean that chemical indicators and integrators are primary release requirements for parametric released product. The recorded and documented measurements from established thermometric measurement systems and associated process controllers (that have been calibrated and used during initial and periodic validation studies, as well as in routine production) can be considered to be primary, product-release systems for parametric release.

PERFORMANCE

Performance standards within lots and between lots of physicochemical indicators or integrators from a given manufacturer should be consistent. They should not interact physically or chemically with any container or product when placed adjacent to the product for sterilization in the sterilizer load, and should not alter the strength, quality, or purity of the sterilized article. The safety of personnel handling the physicochemical indicators or integrators should also be assessed, and, if need be, appropriate precautions should be taken.

Similar to biological indicators, chemical indicators are considered Class II devices and require the indicator manufacturer to obtain a device 510K approval prior to commercial use.

PHYSICOCHEMICAL INDICATORS

Recorded process engineering data can be supplemented by the presence in each sterilized batch of a physicochemical indicator. A physicochemical indicator is defined as a device that responds in a measurable fashion to one or more critical sterilization parameters.

A number of different kinds of indicators dependent on chemical or physicochemical means have been developed for monitoring sterilization cycles. Some products are used in a sterilization apparatus for monitoring whether the contents have been exposed to a selected factor (i.e., temperature) of the particular sterilization cycle, but may not show the duration or intensity of such exposure. Chemical and physicochemical indicators are used to monitor a physical parameter of a sterilization apparatus and can be placed on the outside of the packages of articles to be sterilized, or they may be distributed within the sterilizer load. In the latter case, one can evaluate to some extent the effect of the packaging material and configuration of the load on the selected parameter.

PHYSICOCHEMICAL INTEGRATORS

A physicochemical integrator is defined as a device that responds to a sterilization process critical parameter, which results in a measurable or quantifiable value that can be correlated to some standard of microbial lethality. Physicochemical integrators have been designed to broadly match the predictable inactivation of those spore preparations in biological indicators that have high and defined resistance to the sterilizing agent.

The manufacturers of physicochemical integrators should provide data to demonstrate that the labeled performance characteristics tests of the integrators are met. Users of physicochemical integrators should verify that specific measured values directly correlate to successful microbial lethality in a validated sterilization cycle.

A physicochemical integrator indicates whether or not the critical combination of physical parameters of a validated sterilization cycle has been met or exceeded. The integrator is not generally used as a substitute for a biological indicator in the development and validation of sterilization cycles. An indication by a physicochemical integrator that the critical combination of physical parameters of a stated sterilization cycle has been achieved should not be considered equivalent to the inactivation of spores of a variety of biological indicators. However, the physicochemical integrator can detect whether the sterilization process has been continued for too long, at too high a temperature or gas concentration, or has been overexposed to radiation.

The interval between the lower range and the upper range of time, or any other designated set of parameters, resembles the survival time and kill time window characteristics of a biological indicator. This interval should not be wider than that desirable for the designated parameter, but may be narrower if the manufacturer is able to achieve consistent performance over a narrower range. Even where a sterilization apparatus with consistent performance is used, cases may occur where the determined performance characteristics of the integrator differ from the label claims. This could represent a difference between the user's apparatus performance and the manufacturer's apparatus used for verifying the label claims. Closer conformity to the label claims may also be shown with any highly developed apparatus, such as a BIER vessel.^{1,2} Hence, the integrator requires its own precautions in use and has appropriate interpretive criteria within its performance characteristics. Tests for performance characteristics of physicochemical integrators include determination under applicable defined conditions of (a) the maximum time of exposure at which none of the specimens indicates that adequate exposure to the cycle has occurred, and (b) a minimum time of exposure at which all specimens show that adequate exposure to the cycle has occurred. An intermediate time of exposure, where about half the number of specimens show adequate exposure, could indicate

¹ Standard for BIER/Steam Vessels, 27 March 1981, Association for the Advancement of Medical Instrumentation (AAMI), 3330 Washington Boulevard, Suite 400, Arlington, VA 22201-4598.

² Standard for BIER/EO Vessels, 27 March 1992, Association for the Advancement of Medical Instrumentation (AAMI), 3330 Washington Boulevard, Suite 400, Arlington, VA 22201-4598.

an approaching exposure endpoint for the physicochemical integrator.

Because an indicator reflects only the interaction of the physical parameters of sterilization, it will not be affected by some of the factors that may influence the resistance of the microbial load on the products to be sterilized (e.g., progeny resistance, spore population, inoculum substrate, oil, salts, proteins, or residues or configurations), all of which may protect a contaminated area from penetration by the sterilizing agent. (Hence, the inappropriateness of these devices for cycle development.) There are other factors, however, that may affect a biological indicator that could also affect a physicochemical integrator (e.g., interfering configuration of a pack in which the integrator was placed, variations in the applied timing or temperature control, or failure of the apparatus to reach the set temperature or meet other requirements).

Defective performance of the sterilization apparatus generally can be ascertained from gauges and from records of temperature, pressure, time of exposure, and gas concentration, whichever are applicable. The integrator can only indicate inadequate, adequate, or excessive exposure to a combination of critical sterilization parameters. Where an integrator shows inadequate exposure to the sterilization parameters, it is necessary to ascertain whether the gauges and recordings reflect accurately the sterilization conditions within the sterilization chamber. Variations between sterilization vessels, which might affect the efficiency of a selected sterilization cycle, might be detectable by parallel exposure of several integrators in a number of locations in each sterilizer load.

Physicochemical integrators for steam sterilization are designed to react predictably to a particular combination of physical sterilization parameters: temperature, steam pressure, and time of exposure. Deviation to some extent of one or more of these critical parameters, not compensated by modification of other parameters, causes the integrator to indicate failure to reach the preset integrated limits.

Physicochemical integrators for ethylene oxide sterilization are designed with similar general principles as the integrators for steam sterilization, but to react predictably to the particular combination of the physical sterilization parameters: humidity, temperature, sterilizing gas concentration, and time of exposure. Deviation to some extent of one or more of these critical parameters, not compensated by modification of other parameters, causes the integrator to indicate failure to reach the preset integrated limits.

Physicochemical integrators have been designed to match broadly the predictable inactivation of spore preparations that have a high and defined resistance to the sterilizing agent. For steam sterilization, a strain of *Bacillus stearothermophilus* is used (see *Biological Indicator for Steam Sterilization, Paper Carrier*), and for ethylene oxide sterilization, a strain of *Bacillus subtilis*, subspecies *niger*, is used (see *Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier*). Since there are no standard preparations of these strains, the performance characteristics of these physicochemical integrators must be interpreted in relation to a specific validated sterilization cycle.

Performance standards within lots and between lots of physicochemical integrators from a given manufacturer should be consistent. The integrators should not interact physically or chemically with container or product when placed adjacent to the product for sterilization in the sterilizer load, and shall not alter the strength, quality, or purity of the sterilized article beyond official requirements. Users should obtain information from integrator manufacturers on whether or not sterilization in the presence of physicochemical integrators may affect particular articles to be sterilized. In addition, safety in using these integrators should be verified.

Moist Heat Sterilization

The use of steam sterilization physicochemical integrators to supplement the information obtained through physical assessment of the critical operating parameters should be part of parametric release of moist heat sterilized products. These should be designed to enable the assurance that the lethality delivery specified for the process has been met or exceeded. The consistency of the performance of physicochemical integrators for moist heat sterilization should be ensured through the testing of performance characteristics that include testing of the performance of the indicator or integrator system at various pre-set selected moist heat process conditions.

Critical elements of this type of physicochemical integrator would include an organic compound sensitive to the combination of temperature and steam, a polymeric material penetrable by saturated steam, and a wicking device under the polymeric material that is in contact with the organic compound. As steam passes through the polymeric material, the organic compound melts in a predictable fashion in steps dependent on the steam temperature during the cycle. The liquefied material travels along the wick for a distance that can be measured on a scale. This organic compound has a stated melting temperature range. Some integrators may, for example, have melting ranges of 132.2° to 134.5° or of 137.0° to 142.0°. Other melting ranges could also be specified so as to indicate the sterilization parameters to which it has actually been exposed. The combination of the sterilization parameters, applied for the exposure time required for a stated moist heat sterilization cycle, is indicated on the front of the article by the linear travel of the melt.

These types of physicochemical integrators can also be used for so-called "flash" moist heat sterilization cycles in which the successive steps of the sterilization process are rapidly carried out in such a manner as to achieve the required lethality for the validated process. Other types of physicochemical integrators for moist heat sterilization can be used if they are also calibrated against a specified validated moist heat sterilization cycle.

Ethylene Oxide Sterilization

Physicochemical integrators for ethylene oxide sterilization should be designed to match broadly the predictable inactivation of spore preparations that have a high and defined resistance to ethylene oxide sterilization. The inactivation of spores of a strain of *Bacillus subtilis*, subspecies *niger*, can be used as a model, although other spores of relevant microorganisms can also be used. The critical elements of physicochemical integrators for ethylene oxide sterilization are a base with an organic compound along a linear indicator strip sensitive to a combination of temperature, humidity, and sterilizing gas concentration. Where the organic compound is exposed to a sterilizing gas mixture at a specific temperature and humidity, a chemical reaction triggers the appearance of a color along the linear indicator bar. This is dependent on the time of exposure under the conditions of ethylene oxide sterilization in a predictable fashion. The absence of fading or of decolorization of the indicator bar for a stated period after the sterilization cycle has been completed would confirm adequate humidification in the cycle. The integrator should be capable of detecting deviations from the prescribed parameters of temperature, sterilizing gas concentration, humidity, and time of exposure that may affect sterilization. It does not show the required reactions if exposed to reduced amounts of gas concentration, temperature, and humidity, even if exposed for prolonged periods.

Other types of physicochemical integrators for ethylene oxide sterilization based on different principles or mechanisms of integration of critical parameters of sterilization

could be used if they are also calibrated against a specified validated ethylene oxide sterilization cycle.

The consistency of performance of physicochemical integrators for ethylene oxide sterilization has to be ensured through the testing of performance characteristics at various pre-set selected times for a given ethylene oxide sterilization cycle.

(1211) STERILIZATION AND STERILITY ASSURANCE OF COMPENDIAL ARTICLES

This informational chapter provides a general description of the concepts and principles involved in the quality control of articles that must be sterile. Any modifications of or variations in sterility test procedures from those described under *Sterility Tests* (71) should be validated in the context of the entire sterility assurance program and are not intended to be methods alternative to those described in that chapter.

Within the strictest definition of sterility, a specimen would be deemed sterile only when there is complete absence of viable microorganisms from it. However, this absolute definition cannot currently be applied to an entire lot of finished compendial articles because of limitations in testing. The sterility of a lot purported to be sterile is therefore defined in probabilistic terms, where the likelihood of a contaminated unit or article is acceptably remote. Such a state of sterility assurance can be established only through the use of validated sterilization processes or aseptic processing, if any, under appropriate current good manufacturing practice, and not by reliance solely on sterility testing. The basic principles for validation and certification of a sterilizing process are enumerated as follows:

1. Establish that the process equipment has the capability of operating within the required parameters.
2. Demonstrate that the critical control equipment and instrumentation are capable of operating within the prescribed parameters for the process equipment.
3. Perform replicate cycles representing the required operational range of the equipment and employing actual or simulated product. Demonstrate that the processes have been carried out within the prescribed protocol limits and, finally, that the probability of microbial survival in the replicate processes completed is not greater than the prescribed limits.
4. Monitor the validated process during routine operation. Periodically as needed, requalify and recertify the equipment.
5. Complete the protocols, and document steps (1) through (4) above.

The principles and implementation of a program to validate an aseptic processing procedure are substantially more extensive than the validation of a sterilization process. In aseptic processing, the components of the final dosage form are sterilized separately and the finished article is assembled in an aseptic manner.

Proper validation of the sterilization process or the aseptic process requires a high level of knowledge of the field of sterilization and clean room technology. In order to comply with currently acceptable and achievable limits in sterilization parameters, it is necessary to employ appropriate instrumentation and equipment to control the critical parameters such as temperature, time, pressure, humidity, sterilizing gas concentration, and/or absorbed radiation. An important aspect of the validation program in many sterili-

zation procedures involves the employment of biological indicators (see *Biological Indicators* (1035)). The validated and certified process should be revalidated periodically; however, the revalidation program need not necessarily be as extensive as the original program.

A typical validation program, as outlined below, is one designed for the steam autoclave, but several of these principles may be applicable to the other sterilization procedures discussed in this informational chapter. The program comprises several stages.

The *installation qualification* stage is intended to establish that controls and other instrumentation are properly designed and calibrated. Documentation should be on file demonstrating the quality of the required utilities such as steam, water, and air. The *operational qualification* stage is intended to confirm that the empty chamber functions within the parameters of temperature at key chamber locations prescribed in the protocol. It is usually appropriate to develop heat profile records, i.e., simultaneous temperatures in the chamber employing multiple temperature-sensing devices. A typical acceptable range of temperature in the empty chamber is $\pm 1^\circ$ when the chamber temperature is not less than 121° . The *confirmatory* stage of the validation program is the actual sterilization of materials or articles. This determination requires the employment of temperature-sensing devices inserted into samples of the articles, as well as samples of the articles to which appropriate concentrations of suitable test microorganisms (biological indicators) have been added in operationally fully loaded autoclave configurations. The effectiveness of moist heat penetration into the actual articles and the time of the exposure are the two main factors that determine the lethality of the sterilization process. The *final* stage of the validation program requires the documentation of the supporting data developed in executing the program.

It is generally accepted that terminally sterilized injectable articles or critical devices purporting to be sterile, when sterilized, attain a 10^{-6} microbial survivor probability, i.e., assurance of less than or equal to 1 chance in 1 million that viable microorganisms are present in the sterilized article or dosage form. With heat-stable articles, the approach often is to exceed the critical time necessary to achieve the 10^{-6} microbial survivor probability (overkill) of presterilization bioburden that is considerably greater in population (typically 10^6) and resistance (typically D_{121} is equal to or greater than 1.0 minute) than the natural presterilization bioburden. However, with an article where extensive heat exposure may have a damaging effect, it will not be feasible to employ an overkill approach. In this latter instance, the development of the sterilization cycle depends heavily on knowledge of the population and resistance microbial burden of the product, based on examination, over a suitable time period, of a substantial number of lots of the presterilized product.

The D value is the time (in minutes) required to reduce the microbial population by 90% or 1 log cycle (i.e., to a surviving fraction of 1/10), at a specific lethal condition, such as, temperature. Therefore, where the D value of a BI preparation of, for example, *Geobacillus stearothermophilus* spores is 1.5 minutes under the process conditions defined, e.g., at 121° , if it is treated for 12 minutes under the same conditions, it can be stated that the lethality input is 8D. The effect of applying this input to the product would depend on the initial microbial burden. Assuming that its resistance to sterilization is equivalent to that of the BI, if the microbial burden of the product in question is 10^2 microorganisms, a lethality input of 2D yields a microbial burden of 1 (10^0 theoretical), and a further 6D yields a calculated microbial survivor probability of 10^{-6} . (Under the same conditions, a lethality input of 12D may be used in a typical "overkill" approach.) Generally, the survivor probability achieved for the article under the validated sterilization cycle is not completely correlated with what may occur with the BI. For valid use, therefore, it is essential that the resistance of the BI be greater than that of the natural microbial burden of the article sterilized. It is then appropriate to make a

worst-case assumption and treat the microbial burden as though its heat resistance were equivalent to that of the BI, although it is not likely that the most resistant of a typical microbial burden isolates will demonstrate a heat resistance of the magnitude shown by this species, frequently employed as a BI for steam sterilization. In the above example, a 12-minute cycle is considered adequate for sterilization if the product had a microbial burden of 10^2 microorganisms. However, if the indicator originally had 10^6 microorganisms content, actually a 10^{-2} probability of survival could be expected; i.e., 1 in 100 BIs may yield positive results. This type of situation may be avoided by selection of the appropriate BI. Alternatively, high content indicators may be used on the basis of a predetermined acceptable count reduction.

The D value for the *Geo bacillus stearothermophilus* preparation determined or verified for these conditions should be reestablished when a specific program of validation is changed. Determination of survival curves (see *Biological Indicators* (1035)), or what has been called the fractional cycle approach, may be employed to determine the D value of the biological indicator preferred for the specific sterilization procedure. The fractional cycle approach may also be used to evaluate the resistance of the microbial burden. Fractional cycles are studied either for microbial count-reduction or for fraction negative achievement. These numbers may be used to determine the lethality of the process under production conditions. The data can be used in qualified production equipment to establish appropriate sterilization cycles. A suitable biological indicator such as the *Geo bacillus stearothermophilus* preparation may be employed also during routine sterilization. Any microbial burden-based sterilization process requires adequate surveillance of the microbial resistance of the article to detect any changes, in addition to periodic surveillance of other attributes.

METHODS OF STERILIZATION

In this informational chapter, five methods of terminal sterilization, including removal of microorganisms by filtration and guidelines for aseptic processing, are described. Modern technological developments, however, have led to the use of additional procedures. These include blow-molding (at high temperatures), forms of moist heat other than saturated steam and UV irradiation, as well as on-line continuous filling in aseptic processing. The choice of the appropriate process for a given dosage form or component requires a high level of knowledge of sterilization techniques and information concerning any effects of the process on the material being sterilized.¹

¹Documents addressing the development and validation of sterilization cycles and related topics include, by the Parenteral Drug Association, Inc. (PDA), *Validation of Moist Heat Sterilization Processes: Cycle Design, Development, Qualification and Ongoing Control* (Technical Report No. 1); *Process Simulation for Aseptically Filled Products* (Technical Report No. 22); *Sterilizing Filtration of Liquids* (Technical Report No. 26); and *Validation of Dry Heat Processes Used for Sterilization and Depyrogenation* (Technical Monograph No. 3); and by the Pharmaceutical Manufacturers Association (PMA), *Validation of Sterilization of Large-Volume Parenterals—Current Concepts* (Science and Technology Publication No. 25). Other technical publications include Health Industry Manufacturers Association (HIMA), *Validation of Sterilization Systems* (Report No. 78-4.1); *Sterilization Cycle Development* (Report No. 78-4.2); *Industrial Sterility: Medical Device Standards and Guidelines* (Document #9, Vol. 1); and *Operator Training . . . for Ethylene Oxide Sterilization, for Steam Sterilization Equipment, for Dry Heat Sterilization Equipment, and for Radiation Sterilization Equipment* (Report Nos. 78-4.5 through 4.8). Recommended practice guidelines published by the Association for the Advancement of Medical Instrumentation (AAMI) include *Guideline for Industrial Ethylene Oxide Sterilization of Medical Devices—Process Design, Validation, Routine Sterilization* (No. OPEO-12/81) and *Process Control Guidelines for the Radiation Sterilization of Medical Devices* (No. RS-P 10/82). Additional radiation sterilization content can be found in ISO 11137—*Sterilization of Health Care Products—Requirements for Validation and Routine Control—Radiation Sterilization*. These more detailed publications should be consulted for more extensive treatment of the principles and procedures described in this chapter.

Steam Sterilization

The process of thermal sterilization employing saturated steam under pressure is carried out in a chamber called an autoclave. It is probably the most widely employed sterilization process. The basic principle of operation is that the air in the sterilizing chamber is displaced by the saturated steam, achieved by employing vents or traps. In order to displace air more effectively from the chamber and from within articles, the sterilization cycle may include air and steam evacuation stages. The design or choice of a cycle for given products or components depends on a number of factors, including the heat lability of the material, knowledge of heat penetration into the articles, and other factors described under the validation program (see above). Apart from that description of sterilization cycle parameters, using a temperature of 121°, the F_0 concept may be appropriate. The F_0 , at a particular temperature other than 121°, is the time (in minutes) required to provide the lethality equivalent to that provided at 121° for a stated time. Modern autoclaves generally operate with a control system that is significantly more responsive than the steam reduction valve of older units that have been in service for many years. In order for these older units to achieve the precision and level of control of the cycle discussed in this chapter, it may be necessary to upgrade or modify the control equipment and instrumentation on these units. This modification is warranted only if the chamber and steam jacket are intact for continued safe use and if deposits that interfere with heat distribution can be removed.

Dry-Heat Sterilization/Depyrogenation

The process of thermal sterilization of Pharmacopeial articles by dry heat may be carried out by a batch process in an oven designed expressly for that purpose or in a dry-heat tunnel in which glass containers move on a continuous basis through the system. A dry-heat sterilization/depyrogenation system is supplied with heated, HEPA filtered air, distributed uniformly throughout the unit by convection or radiation and employing a blower system with devices for sensing, monitoring, and controlling all critical parameters. A typical acceptable range in temperature in the empty chamber is $\pm 15^\circ$ when the unit is operating at not less than 250°.

In addition to the batch process described above, the continuous-tunnel system usually requires a much higher temperature than cited above for the batch process because of a much shorter dwell time. The continuous process also usually necessitates a rapid cooling stage prior to the aseptic filling operation. In the qualification and validation program, in view of the short dwell time, parameters for uniformity of the temperature, and particularly the dwell time, should be established.

Because depyrogenation is a more rigorous challenge for dry-heat processing systems than biological indicator inactivation, it is generally not necessary to include BIs when validating dry-heat processes if validation of depyrogenation is demonstrated. A 3 log cycle reduction or greater is a suitable acceptance criterion for depyrogenation and when successfully demonstrated will ensure not only adequate depyrogenation of compendial articles but also sterilization. Depyrogenation tests are typically done using articles inoculated with reference standard endotoxin. Articles are then evaluated after exposure for residual levels of endotoxin using *Limulus* lysate-based assays. For additional information on the endotoxin assay, see *Bacterial Endotoxins Test* (85).

Gas Sterilization

The choice of gas sterilization as an alternative to heat is frequently made when the material to be sterilized cannot withstand the high temperatures obtained in the steam ster-

ilization or dry-heat sterilization processes. The most commonly employed method of gaseous sterilization is ethylene oxide. Among the disadvantages of ethylene oxide are its highly flammable nature unless mixed with suitable inert gases, its mutagenic properties, and the possibility of toxic residues in treated materials, particularly those containing chloride ions. The sterilization process is generally carried out in a pressure and vacuum-rated chamber designed similarly to a steam autoclave but with the additional features (see below) unique to sterilizers employing this gas. Facilities employing this sterilizing agent should be designed to provide adequate post sterilization degassing, to enable microbial survivor monitoring, and to minimize exposure of operators to the potentially harmful gas.²

Validation of a sterilizing process employing ethylene oxide gas is accomplished along the lines discussed earlier. However, the program is more comprehensive than for the other sterilization procedures, because in addition to temperature, the humidity, vacuum/positive pressure, and ethylene oxide concentration also require appropriate parametric control. An important determination is to demonstrate that all critical process parameters in the chamber are adequate during the entire cycle. Because the sterilization parameters applied to the articles to be sterilized are critical variables, it is frequently advisable to precondition the load to achieve the required moisture content in order to minimize the time of holding at the required temperature before placement of the load in the ethylene oxide chamber. Validation is generally conducted employing product inoculated with appropriate BIs such as spore preparations of *Bacillus atrophaeus*. For validation they may be used in full chamber loads of product, or simulated product. The monitoring of moisture and gas concentration requires the utilization of sophisticated instrumentation that only knowledgeable and experienced individuals can calibrate, operate, and maintain. BIs may also be employed in monitoring routine runs.

As is indicated elsewhere in this chapter, the BI may be employed in a fraction negative mode to establish the ultimate microbiological survivor probability in designing an ethylene oxide sterilization cycle using inoculated product or inoculated simulated product.

One of the principal limiting factors of the ethylene oxide sterilization process is the limited ability of the gas to diffuse to the innermost product areas that require sterilization. Package design and chamber loading patterns therefore must be determined to allow for necessary gas penetration. The reader is referred to ISO 11135 for a complete description of process development, validation, and routine control of ethylene oxide sterilization processes.

Sterilization by Ionizing Radiation

The rapid proliferation of medical devices unable to withstand heat sterilization and the concerns about the safety of ethylene oxide have resulted in increasing applications of radiation sterilization. This method may also be applicable to active pharmaceutical ingredients and final dosage forms. The advantages of sterilization by irradiation include low chemical reactivity, low measurable residues, and the fact that there are fewer variables to control. In fact, radiation sterilization is unique in that the basis of control is essentially that of the absorbed radiation dose, which can be precisely measured. Dose-setting and dose-substantiation procedures are typically used to validate the radiation dose required to achieve a sterility assurance level. Irradiation causes only a minimal temperature rise but can affect certain grades and types of plastics and glass.

² See *Ethylene Oxide*, Encyclopedia of Industrial Chemical Analysis, 1971, 12, 317–340, John Wiley & Sons, Inc., and *Use of Ethylene Oxide as a Sterilant in Medical Facilities*, NIOSH Special Occupational Hazard Review with Control Recommendations, August 1977, U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Division of Criteria Documentation and Standards Development, Priorities and Research Analysis Branch, Rockville, MD.

The two types of ionizing radiation in use are radioisotope decay (gamma radiation) and electron-beam radiation. In either case the radiation dose established to yield the required degree of sterility assurance should be such that, within the range of minimum and maximum doses set, the properties of the article being sterilized are acceptable. The reader is referred to ISO 11137-1, -2, and -3 for a complete description of process development, validation, and routine control of ionizing radiation processes.

Sterilization by Filtration

The sterilization of fluids by filtration is a separative process and differs from the other methods of sterilization that rely on destructive mechanisms. Filtration through microbial retentive materials is frequently employed for the sterilization of heat-labile solutions by physical removal of the contained microorganisms. A filter assembly generally consists of a porous matrix integrated with or clamped into a housing. The effectiveness of a filter medium depends upon the pore size of the porous material, the prefiltration bioburden, and may depend upon adsorption of bacteria on or in the filter matrix or upon a sieving mechanism. There is some evidence to indicate that sieving is the more important component of the mechanism. While fiber-shedding filters are to be avoided unless no alternative filtration procedures are possible, it should be noted that in accordance with 21CFR 211.72, the use of asbestos-containing filters is prohibited. Where a fiber-shedding filter is required, it is obligatory that the process include a nonfiber-shedding filter introduced downstream or subsequent to the initial filtration step.

Filter Rating—The pore sizes of filter membranes are rated by a nominal rating that reflects the capability of the filter membrane to retain microorganisms of size represented by specified strains, not by determination of an average pore size and statement of distribution of sizes. Sterilizing filters cannot be narrowly defined because, depending upon the bioburden present in the fluid stream, different filters may be considered effective for sterilization. Currently a sterilizing filter can be defined as, “a filter that, when appropriately validated, will remove all microorganisms from a fluid stream, producing a sterile effluent”. The nominal ratings of sterilizing filters based on microbial retention properties differ when rating is done by other means, e.g., by retention of latex spheres of various diameters. It is the user’s responsibility to select a filter of correct rating for the particular purpose, depending on the nature of the product (especially considering its potential bioburden) to be filtered. It is not feasible to repeat the tests of filtration capacity in the user’s establishment. Microbial challenge tests are preferably performed under a manufacturer’s conditions on each lot of manufactured filter membranes.

The user must determine whether filtration parameters employed in manufacturing will significantly influence microbial retention efficiency. Some of the other important concerns in the validation of the filtration process include product compatibility, sorption of drug, preservative or other additives, and initial effluent endotoxin content.

Because the effectiveness of the filtration process is also influenced by the microbial burden of the solution to be filtered, determining the microbiological quality of solutions prior to filtration is an important aspect of the validation of the filtration process, in addition to establishing the other parameters of the filtration procedure, such as pressures, flow rates, and filter unit characteristics. Hence, another method of describing filter-retaining capability is the use of the log reduction value (LRV). For instance, a 0.2- μ m filter that can retain 10^7 microorganisms of a specified strain will have an LRV of not less than 7 under the stated conditions.

The housings and filter assemblies that are chosen should first be validated for compatibility and integrity by the user. While it may be possible to mix assemblies and filter membranes produced by different manufacturers, the compatibil-

ity of these hybrid assemblies should first be validated. Additionally, there are other tests to be made by the manufacturer of the membrane filter, which are not usually repeated by the user. These include microbiological challenge tests. Results of these tests on each lot of manufactured filter membranes should be obtained from the manufacturer by users for their records.

Filtration for sterilization purposes is usually carried out with assemblies having membranes of nominal pore size rating of 0.2 μm or less. A membrane filter assembly must be tested for initial integrity prior to use, provided that such test does not impair the safety, integrity, and validity of the system, and should be tested after the filtration process is completed to demonstrate that the filter assembly maintained its integrity throughout the entire filtration procedure. Typical use tests are the bubble point test, the diffusive airflow test, the pressure hold test, and the forward flow test. These tests should be correlated with microorganism retention.

Unidirectional Aseptic Processing

Although there is general agreement that sterilization of the final filled container as a dosage form or final packaged device is the preferred process for ensuring the minimal risk of microbial contamination in a lot, there is a substantial class of products that are not terminally sterilized but are prepared by a series of aseptic steps. These are designed to prevent the introduction of viable microorganisms into components, where sterile, or once an intermediate process has rendered the bulk product or its components free from viable microorganisms. The fundamental difference between aseptically produced sterile products and terminally sterilized products is the presence of a step that can be validated, whereby the final package is subjected to conditions shown to kill viable contaminants. Consequently, an aseptically filled product labeled as sterile must use a system of risk assessments to establish that an acceptable level of sterility assurance has been achieved. Current technology cannot provide an adequate safety assessment based on individual unit testing. In currently used methods of environmental monitoring, process simulations have not been shown to correlate directly with contaminated finished products. Finished product destructive testing (sterility tests) can only examine a very small percentage of a lot and are thus only capable of detecting grossly contaminated lots. This section provides a review of the principles involved in producing aseptically processed products with a minimal risk of microbial contamination in the finished lot of final dosage forms.

A product defined as aseptically processed is likely to consist of components that have been sterilized by one of the processes described earlier in this chapter. For example, the bulk product, if a filterable liquid, may have been sterilized by filtration. The final empty container components would probably be sterilized by heat, dry heat being employed for glass vials and an autoclave being employed for rubber closures. The areas of critical concern are the immediate microbial environment where these presterilized components are exposed during assembly to produce the finished dosage form and the aseptic filling operation.

The requirements for a properly designed, validated, and maintained filling or other aseptic processing facility are mainly directed to (1) an air environment that is suitably controlled with respect to viable and nonviable particulates, of a proper design to permit effective maintenance of air supply units, and (2) the provision of trained operating personnel who are adequately equipped and gowned. The desired environment may be achieved through the high level of air filtration technology now available, which contributes to the delivery of air of the requisite microbiological qual-

ity.³ The facilities include both primary (in the vicinity of the exposed article) and secondary (where the aseptic processing is carried out) barrier systems.

For a properly designed aseptic processing facility or aseptic filling area, consideration should be given to such features as nonporous and smooth surfaces, including walls and ceilings that can withstand routine decontamination; gowning rooms with adequate space for personnel and storage of sterile garments; adequate separation of preparatory rooms for personnel from final aseptic processing rooms, with the availability, if necessary, of devices such as airlocks and air showers; proper pressure differentials between rooms, the most positive pressure being in the aseptic processing rooms or areas; the employment of unidirectional airflow in the immediate vicinity of exposed product or components, and filtered air exposure thereto, with adequate air change frequency; appropriate humidity and temperature environmental controls; and a documented sanitization program. Proper training of personnel in hygienic and gowning techniques should be undertaken so that, for example, gowns, gloves, and other body coverings substantially cover exposed skin surfaces.

Certification and validation of the aseptic process and facility are achieved by establishing the efficiency of the filtration systems, by employing microbiological environmental monitoring procedures, and by processing of sterile culture medium as simulated product.

Monitoring of the aseptic facility should include periodic HEPA filter evaluation and testing, as well as routine particulate and microbiological environmental monitoring. Periodic media-fill or process-simulation testing should also be performed.

STERILITY TESTING OF LOTS

It should be recognized that the referee sterility test might not detect microbial contamination if present in only a small percentage of the finished articles in the lot because the specified number of units to be taken imposes a significant statistical limitation on the utility of the test results. This inherent limitation, however, has to be accepted, because current knowledge offers no nondestructive alternatives for ascertaining the microbiological quality of every finished article in the lot, and it is not a feasible option to increase the number of specimens significantly. For information regarding the conduct of the sterility test please see *Sterility Tests* (71).

(1216) TABLET FRIABILITY

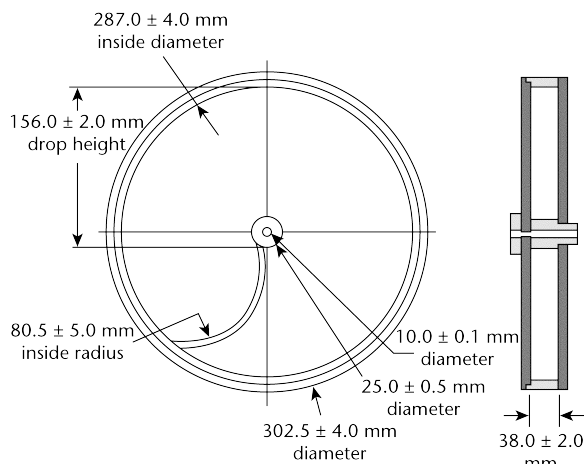
This general information chapter has been harmonized with the corresponding texts of the *European Pharmacopoeia* and the *Japanese Pharmacopoeia*. The harmonized texts of these three pharmacopoeias are therefore interchangeable, and the methods of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia* may be used for demonstration of compliance instead of the present *United States Pharmacopoeia* general information chapter method. These pharmacopoeias have undertaken not to make any unilateral change to this harmonized chapter.

This chapter provides guidelines for the friability determination of compressed, uncoated tablets. The test procedure

³ Available published standards for such controlled work areas include the following: (1) ISO 14644 1-7 Cleanrooms and Associated Controlled Environments. (2) NASA Standard for Clean Room and Work Stations for Microbially Controlled Environment, publication NHB5340.2, Aug. 1967. (3) Contamination Control of Aerospace Facilities, U.S. Air Force, T.O. 00-25-203, 1 Dec. 1972, change 1-1, Oct. 1974.

presented in this chapter is generally applicable to most compressed tablets. Measurement of tablet friability supplements other physical strength measurements, such as tablet breaking force.

Use a drum,* with an internal diameter between 283 and 291 mm and a depth between 36 and 40 mm, of transparent synthetic polymer with polished internal surfaces, and subject to minimum static build-up (see figure for a typical apparatus). One side of the drum is removable. The tablets are tumbled at each turn of the drum by a curved projection with an inside radius between 75.5 and 85.5 mm that extends from the middle of the drum to the outer wall. The outer diameter of the central ring is between 24.5 and 25.5 mm. The drum is attached to the horizontal axis of a device that rotates at 25 ± 1 rpm. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other.



Tablet Friability Apparatus

For tablets with a unit weight equal to or less than 650 mg, take a sample of whole tablets corresponding as near as possible to 6.5 g. For tablets with a unit weight of more than 650 mg, take a sample of 10 whole tablets. The tablets should be carefully dedusted prior to testing. Accurately weigh the tablet sample, and place the tablets in the drum. Rotate the drum 100 times, and remove the tablets. Remove any loose dust from the tablets as before, and accurately weigh.

Generally, the test is run once. If obviously cracked, cleaved, or broken tablets are present in the tablet sample after tumbling, the sample fails the test. If the results are difficult to interpret or if the weight loss is greater than the targeted value, the test should be repeated twice and the mean of the three tests determined. A maximum mean weight loss from the three samples of not more than 1.0% is considered acceptable for most products.

If tablet size or shape causes irregular tumbling, adjust the drum base so that the base forms an angle of about 10° with the horizontal and the tablets no longer bind together when lying next to each other, which prevents them from falling freely.

Effervescent tablets and chewable tablets may have different specifications as far as friability is concerned. In the case of hygroscopic tablets, an appropriate humidity-controlled environment is required for testing.

* The apparatus meeting these specifications is available from laboratory supply houses such as VanKel Technology Group, 13000 Weston Parkway, Cary, NC 27513, or from Erweka Instruments, Inc., 56 Quirk Road, Milford, CT 06460.

Drums with dual scooping projections, or an apparatus with more than one drum, for the running of multiple samples at one time, are also permitted.

(1217) TABLET BREAKING FORCE

INTRODUCTION

There are a variety of presentations for tablets as delivery systems for pharmaceutical agents, such as rapidly disintegrating, slowly disintegrating, eroding, chewable, and lozenge. Each of these presentations places a certain demand on the bonding, structure, and integrity of the compressed matrix. Tablets must be able to withstand the rigors of handling and transportation experienced in the manufacturing plant, in the drug distribution system, and in the field at the hands of the end users (patients/consumers). Manufacturing processes such as coating, packaging, and printing can involve considerable stresses, which the tablets must be able to withstand. For these reasons, the mechanical strength of tablets is of considerable importance and is routinely measured. Tablet strength serves both as a criterion by which to guide product development and as a quality control specification.

One commonly employed test of the ability of tablets to withstand mechanical stresses determines their resistance to chipping and surface abrasion by tumbling them in a rotating cylinder. The percentage weight loss after tumbling is referred to as the *friability* of the tablets. Standardized methods and equipment for testing friability have been provided in general chapter *Tablet Friability* (1216).

Another measure of the mechanical integrity of tablets is their *breaking force*, which is the force required to cause them to fail (i.e., break) in a specific plane. The tablets are generally placed between two platens, one of which moves to apply sufficient force to the tablet to cause fracture. For conventional, round (circular cross-section) tablets, loading occurs across their diameter (sometimes referred to as diametral loading), and fracture occurs in that plane.

The breaking force of tablets is commonly called *hardness* in the pharmaceutical literature; however, the use of this term is misleading. In material science, the term *hardness* refers to the resistance of a surface to penetration or indentation by a small probe. The term *crushing strength* is also frequently used to describe the resistance of tablets to the application of a compressive load. Although this term describes the true nature of the test more accurately than does *hardness*, it implies that tablets are actually crushed during the test, which often is not the case. Moreover, the term *strength* in this application can be questioned, because in the physical sciences that term is often used to describe a stress (e.g., tensile strength). Thus, the term *breaking force* is preferred and will be used in the present discussion.

TABLET BREAKING FORCE DETERMINATIONS

Early measuring devices were typically hand operated. For example, the Monsanto (or Stokes) hardness tester was based on compressing tablets between two jaws via a spring gauge and screw. In the Pfizer hardness tester, the vertically mounted tablet was squeezed in a device that resembled a pair of pliers. In the Strong Cobb hardness tester, the break-

ing load was applied through the action of a small hydraulic pump that was first operated manually but was later motorized. Problems associated with these devices were related to operator variability in rates of loading and difficulties in proper setup and calibration. Modern testers employ mechanical drives, strain gauge-based load cells for force measurements, and electronic signal processing, and therefore are preferred. However, several important issues must be considered when using them for the analytical determination of breaking force; these are discussed below.

Platens

The platens should be parallel. Their faces should be polished smooth and precision-ground perpendicularly to the direction of movement. Perpendicularity must be preserved during platen movement, and the mechanism should be free of any bending or torsion displacements as the load is applied. The contact faces must be larger than the area of contact with the tablet.

Rate and Uniformity of Loading

Either the rate of platen movement or the rate at which the compressive force is applied (i.e., the loading rate) should be constant. Maintaining a constant loading rate avoids the rapid buildup of compressive loads, which may lead to uncontrolled crushing or shear failure and greater variability in the measured breaking force. However, constant loading rate measurements may be too slow for real time monitoring of tablet production.

The rate at which the compressive load is applied can significantly affect results, because time-dependent processes may be involved in tablet failure (1). How a tablet matrix responds to differences in the loading rate depends on the mechanism of failure. At low strain rates, some materials may fail in a ductile manner, but brittle failure is more likely at faster strain rates. The transition from ductile to brittle failure is accompanied by an increase in the breaking force. Devices that simply crush tablets may produce deceptively reproducible data because they lack sensitivity.

The test must be run consistently with equipment which has been routinely calibrated. Changing from testing units of different designs or from different manufacturers will require comparison of data to ensure that the two units are subjecting the dosage form to similar stress in a similar manner. Currently available equipment provides a constant loading rate of 20 newtons (N) or less per second or a constant platen movement of 3.5 mm or less per second. Controlled and consistent breaking is an important test procedure attribute. To ensure comparability of results, testing must occur under identical conditions of loading rate or platen movement rate. Since there are certain advantages to each system of load application, both are found in practice. Because the particular testing situation and the type of tablet matrix being evaluated will pose different constraints, there is also no basis to declare an absolute preference for one system over the other. This general chapter proposes consideration of both approaches.

The different methods may lead to numerically different results for a particular tablet sample, requiring that the rate of load application or displacement must be specified along with the determined breaking force.

Dependence of Breaking Force on Tablet Geometry and Mass

Measurements of breaking force do not take into account the dimensions or shape of the tablet. Thicker tablets of the same material compressed under conditions identical to those of thinner tablets, with the same tooling shape and to the same peak force, will require greater breaking forces. Tablet orientation and failure should occur in a manner con-

sistent with those used during the development of the dosage form. For direct comparisons (i.e., without any normalizations of the data), breaking force measurements should be performed on tablets having the same dimensions, geometry, and consistent orientation in test equipment.

Tablet Orientation

Tablet orientation in diametral compression of round tablets without any scoring is unequivocal. That is, the tablet is placed between the platens so that compression occurs across a diameter. However, tablets with a unique or complex shape may have no obvious orientation for breaking force determination. Because the breaking force may depend on the tablet's orientation in the tester, to ensure comparability of results, it is best to settle on a standard orientation, preferably one that is most readily and easily reproduced by operators. In general, tablets are tested either across the diameter or parallel to the longest axis. Scored tablets have two orientation possibilities. When they are oriented with their scores perpendicular to the platen faces, the likelihood that tensile failure will occur along the scored line increases. This provides information about the strength of the matrix at the weakest point in the structure. When scored tablets are oriented with their scores parallel to the platen faces, more general information about the strength of the matrix is derived.

Capsule-shaped tablets or scored tablets may best be broken in a three-point flexure test (2). A fitting, which is either installed on the platens or substituted for the platens, supports the tablet at its ends and permits the breaking load to be applied to the opposite face at the unsupported midpoint of the tablet. The fittings are often available from the same source that supplies the hardness tester.

Units, Resolution, and Calibration

Modern breaking force testers are usually calibrated in kiloponds or newtons. The relationship between these units of force (3) is 1 kilopond (kp) = 1 kilogram-force (kgf) = 9.80 N. The test results should be expressed in standard units of force which facilitate communication. Some breaking force testers also will provide a scale in Strong Cobb units (SCU), a carryover from the days when Strong Cobb hardness testers were in common usage. The conversion between SCU and N or kp must be viewed with caution, because the SCU is derived from a hydraulic device and is a pressure.

Generally, contemporary breaking force testers use modern electronic designs with digital readouts. Some units also have an integral printer or may be interfaced with a printer. Breaking forces should be readable to within 1 N.

Breaking force testers should be calibrated periodically. The force sensor as well as the mechanics of the apparatus needs to be considered. For the force sensor, the complete measuring range (or, at a minimum, the range used for measuring the test sample) should be calibrated to a precision of 1 N, using either the static or dynamic method. Static calibration generally employs traceable counterweights; at least three different points are checked to assess linearity. Dynamic calibration makes use of a traceable reference-load cell that is compressed between the platens. The functional calibration of a breaking force test apparatus should also confirm that the velocity and the constancy of velocity for load application or displacement are within prescribed tolerances throughout the range of platen movement.

Sample Size

In order to achieve sufficient statistical precision for the determination of average breaking force, a minimum of 6 tablet samples should be tested. The average breaking force

alone may be adequate to fulfill the purpose of process or product quality control. In cases where breaking force may be particularly critical, the average plus individual breaking force values should be accessible.

TENSILE STRENGTH

The measurement of tensile strengths provides a more fundamental measure of the mechanical strength of the compacted material and takes into account the geometry of the tablet. If tablets fail in tension, the breaking force can be used to calculate the tensile strength. Unfortunately, this is practical only for simple shapes. If flat-faced round tablets (right circular cylinders) fail in tension, as indicated by a clean split into halves under diametral compression, the breaking force may be used to compute the tensile strength from the following equation (4), which applies only to cylindrical tablets:

$$\sigma_x = 2F/\pi DH$$

in which σ_x is the tensile strength, F is the breaking force, D is the tablet diameter, and H is the tablet thickness. Because only tablets that fail in tension are counted, the data are based on tablets that fail in a consistent way. Thus, reproducibility of data should be enhanced when compared to conventional breaking-strength testing. Moreover, the data will be normalized with respect to tablet dimensions, because both diameter and thickness are included in the equation. The derivation of this equation may be found in standard texts (5, 6); it is based on elastic theory and the following assumptions:

1. The tablet is an isotropic body
2. Hooke's law is obeyed
3. The modulus of elasticity in compression and in tension is the same
4. Ideal point loading occurs

The derivation has been extended to convex-faced tablets (7, 8):

$$\sigma_x = (10F/\pi D^2) \times [(2.84H/D) - (0.126H/W) + (3.15W/D) + 0.01]^{-1}$$

where σ_x is the tensile strength, F is the breaking force, D is the tablet diameter, H is the tablet thickness, and W is the central cylinder thickness (tablet wall height).

The slow and constant loading rate of modern motorized break force testers encourages tensile failure. However, ideal point loading may not occur, because of crushing and the induction of shear failure at the interface with the surface of the platens. The addition of padding to the platens helps prevent shear at contact points and promotes true tensile failure. On that basis, padding is strongly recommended when highly precise measurements are needed. Padding should be relatively thin so that any deviation from the assumption of true point-source force application will not be large. The padding should also collapse very easily so that its deformation does not become part of the force measured by the test apparatus. In more routine settings involving measurements on a large number of samples, the addition of padding could contribute to inaccuracies in measurement as powder from previously tested samples becomes embedded in the collapsible matrix and thereby alters its properties. Unless provisions for frequent and routine replacement of the padding are made, it can be considered acceptable to ignore the use of padding material to maintain constancy of the test conditions.

Bending or flexure of tablets is another option for determining the tensile strength of tablets. Under ideal loading conditions, a breaking load applied to the unsupported midpoint of one face will result in the generation of pure tensile stress in the opposite face. If the tablets are right circular cylinders and are subjected to three-point flexure, the ten-

sile strength may be estimated using the following equation (9):

$$\sigma_x = 3FL/2H^2D$$

in which L is the distance between supports, and the other terms are as defined above. The assumptions are the same as those for calculating tensile strength from diametral compression. However, tensile strengths determined by flexure and diametral compression may not agree, because of likely nonideal loading and the induction of shear failure during testing.

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<1222> TERMINALLY STERILIZED PHARMACEUTICAL PRODUCTS—PARAMETRIC RELEASE

INTRODUCTION

Parametric release is defined as the release of terminally sterilized batches or lots of sterile products based upon compliance with the defined critical parameters of sterilization without having to perform the requirements under *Sterility Tests* <71>. Parametric release is a possibility when the mode of sterilization is very well understood, the physical parameters of processing are well defined, predictable, and measurable, and the lethality of the cycle has been microbiologically validated through the use of appropriate biological indicators or, in the case of ionizing radiation, the appropriate microbiological and dosimetric tests. The use of parametric release for sterilization processes requires prior FDA approval. It should be expected that the regulatory agencies evaluating submissions including the use of parametric product release would insist upon a well supported

scientific rationale for the sterilization process and well documented validation data. The agencies would need assurance that any marketed sample of product will be sterile and if tested after release would pass the requirements for sterility as found in the general chapter *Sterility Tests* (71).

It is important to consider the limitations of the *Sterility Tests* (71) in the evaluation of terminally sterilized products. The sterility test described in general chapter (71) is limited in its sensitivity and is statistically ill-suited to the evaluation of terminally sterilized products given the exceedingly low probability of contaminated units. Therefore, *once a sterilization process is fully validated and operates consistently*, a combination of physical sterilization data such as accumulated lethality or dosimetry in combination with other methods, such as load monitors (e.g., biological indicators, thermochemical indicators, or physicochemical integrators), can provide more accurate information than the sterility test regarding the release of terminally sterilized product to the marketplace.

There are four modes of sterilization that theoretically and practically could qualify for parametric release: moist heat, dry heat, ethylene oxide, and ionizing radiation sterilization. This information chapter first will cover the general issues related to parametric release, regardless of the modes of sterilization, and then discuss some specific modes of sterilization. The chapter will *not* address the parametric release of terminally sterilized medical devices.

Terminally sterilized products represent the lowest risk category of sterile pharmaceutical products. Unlike products aseptically manufactured in a microbiologically controlled environment, terminally sterilized products are subjected to a sterilization process that imparts a measurable minimum sterility assurance level, or SAL. Because aseptic processing relies on exclusion of microbiological contamination and is not based upon lethality imparted on the product in its sealed container, it is not possible to estimate the SAL. It is important to note that in the case of aseptic processing, SAL can only be estimated from media fill contamination rates or other forms of risk assessment. In the case of terminal sterilization, it is possible to calculate a minimum SAL or Probability of Nonsterility (PNS) quite accurately. Therefore, the term SAL has different contextual meanings when used to describe aseptic rather than terminal processes, and it is important that this difference is fully understood by scientists and engineers working in the field of sterile product manufacturing and control. The terms PNS and SAL are often used interchangeably.

Terminally sterilized products must have a probability of nonsterility (PNS) of not more than one in a million units produced. This is often stated as a PNS or SAL of 10^{-6} , or the probability of product bioburden surviving the sterilization process in any single unit of product is less than one in one million. The proof that a terminally sterilized product complies with the 10^{-6} PNS can be accomplished by several different sterilization cycle development approaches. The proper application of these methods requires extensive scientific knowledge regarding the sterilization method selected for use with a specific product.

The strategies used to validate a terminal sterilization process development fall into three categories:

1. Bioburden-based process.
2. Biological indicator/bioburden combined process.
3. Overkill process.

The bioburden-based process requires extensive knowledge of product bioburden. It should be noted that several radiation dose-setting procedures involve establishing radiation processes on the basis of bioburden count and radiation resistance. This method requires that at least a 10^{-6} PNS be attained for bioburden by the sterilization process. This means that if the product bioburden action level is 10 microorganisms or one logarithm, at least seven logarithms of bioburden must be inactivated to assure a 10^{-6} PNS. The bioburden-based method requires the user to develop suitable critical control points within the process to control the bioburden titer. Products that readily permit bioburden sur-

vival require more controlled manufacturing environments and more precise in-process control. This process is better suited for cycle development for clean or ultra-clean products containing a consistently low level of colony forming units (cfu) per product unit with a low frequency of spore-forming microorganisms. Also, this process may be necessary to permit terminal sterilization of a product that may potentially lose key qualities or attributes as a result of a more rigorous sterilization process.

The microbiologist may find that formal hazard analysis procedures, such as Hazard Analysis Critical Control Point (HACCP), are useful in establishing appropriate manufacturing control conditions and in-process control parameters.

The biological indicator/bioburden combined process is generally used when the manufacturer desires a sterilization process that demonstrates the inactivation of high numbers of biological indicator microorganisms known to be resistant to the process. While the manufacturer may have preferred utilizing an overkill process, potential loss of some product attributes may occur in an overkill process thereby necessitating the use of a biological indicator/bioburden combined process. This process requires knowledge of the bioburden load on and in the product, and a database relative to the sterilization resistance of the bioburden. The relative resistance of the selected biological indicator to that of the bioburden must be established on or in the product. Frequently, biological indicators bearing approximately 10^6 spores with D_{121} -value > 1 minute are used in the development of such processes. Fractional exposure cycles are generally conducted to determine the relative sterilization resistance (or D value) between product inoculated with the biological indicator microorganism(s) and frequently encountered bioburden. This process is frequently used for sterilization cycle development by manufacturers of terminally sterilized parenteral products and for ethylene oxide sterilization of medical devices.

The overkill process is frequently used when the article being sterilized is completely inert to the sterilizing agent and sterilization cycle conditions without any concern for loss of product attributes or quality. When using this process, some bioburden knowledge should be available to ensure that the materials are not adulterated before sterilization. These data may include product bioburden count data and knowledge concerning the prevalence of spore formers. The database for this process need not be as extensive as bioburden data required for the bioburden process or the biological indicator/bioburden process. Generally, process-resistant biological indicators containing approximately 10^6 spores are used to establish the effectiveness of the sterilization process. However, a spore population of N_0 can be chosen to confirm adequate process lethality. Overkill is generally defined as a process that would deliver a minimum of F_0^1 of 12 minutes (see *Critical Operating Parameters* below) and is demonstrated biologically based upon the spore log reduction of calibrated biological indicators.

GENERAL REVIEW

Validation of Sterilization Process

Parametric release first requires that the chosen sterilization process be designed and validated to achieve a 10^{-6} PNS. Validation of most sterilization processes includes the validation of physical parameters of the process and of its microbiological effectiveness through the use of biological indicators. However, the use of biological indicators for establishing or periodically validating gamma radiation sterilization processes is uncommon. Widely recognized biological indicator organisms are used in the validation of moist heat

¹ F_0 is defined as the calculated equivalent time (in minutes) of process lethality to time at 121.1°, assuming a Z value of 10.0° in the product being sterilized.

processes because they provide a means of comparing physically measured lethality data with biological lethality. There should be a reasonable correlation between physically measured lethality data (F_0) and biological lethality as determined by the evaluation of the process with biological indicators.

The predictable effectiveness of bioburden-based terminal sterilization is based on the number and resistance of microorganisms on or in a product. For this reason, one component of parametric release is an active microbiology control program to monitor the count and sterilization resistance of product bioburden. Bioburden control and enumeration is of far less significance when the overkill process design is used. In many cases, overkill processes do not require extensive ongoing assessment of bioburden and require less in-process control of the manufacturing environment.

Sterilization Microbiology Control Program

The purpose of this control program is to ensure that the microbiological status of the product, *prior to being terminally sterilized*, has not significantly deviated from the established microbiological control level used for validation of the sterilization process. The microbiology control program includes the monitoring of the bioburden on or in the product and the monitoring of the microbiological status of any necessary containers, closures, or packaging materials. Also included is a program to evaluate the microbiological status of the environment where the product is processed. The control program is particularly important in cases where the terminal sterilization is not based on overkill, but rather on the bioburden or combined bioburden/biological indicator cycle development approach. In many cases, bioburden control and manufacturing environmental monitoring will not be required for overkill process designs, where the F_0 of the process is at least 12 minutes. In other cases, even when overkill processes are employed, some limited monitoring will be needed. Monitoring of overkill processes for bioburden is generally limited to those products that support microbial growth. Of particular concern in this case is the potential for the product to be contaminated with microbial toxins or to be degraded by microorganisms.

The frequency of monitoring will depend on the variations of bioburden from potential sources. The number of microorganisms, their identification, as well as their resistance to the specified sterilization mode should be considered when parametric release of terminally sterilized product is established. Resistance to a specified sterilization mode by different species can influence sterilization effectiveness and the determination of sterilization process conditions when using the bioburden or combined bioburden/biological indicator method of cycle development. In the bioburden approach to process development, indicator organisms more resistant than typical bioburden may be used, although extreme differentials in resistance are not required. Information on the performance of biological indicators may be found in the general chapter *Biological Indicators—Resistance Performance Tests* (55).

Change Control System

Changes introduced to the sterilization processing equipment could result in a significant departure of the initially validated parametric release process. It is, therefore, essential that a change control system be instituted. A change control system is a formal system with appropriate standard operating procedures, which would include approval of changes in the sterilization processing equipment. This system would assess all the changes in relation to the critical parameters included in parametric release. The change control system also includes technical and management review and criteria for acceptance or revision of changes. If a change would significantly affect any critical parameter, each parameter would have to be revalidated in terms of

sterility assurance of the pharmaceutical product to a minimum 10^{-6} PNS. Appropriate regulatory notification would also be part of the revalidation process.

Release Procedures

A quality assurance program should be established that describes in detail the batch or lot release steps for parametric release of sterilized products and the required documentation. Although the assessment of the sterility assurance of products is primarily based on measurement of physical process parameters, a number of areas should be reviewed, documented, and approved for the parametric release of these products. These areas may include the following: a review of batch records; a review of the ongoing microbiological environmental control program results and presterilization bioburden; and a review of records of thermographic data, load monitors, and results of critical and noncritical data that may have been used to demonstrate process control. It is also important to ensure that the sterilizer is current relative to calibration, maintenance, and revalidation.

The implementation and practice of parametric release is not an intermittent program. Once such a program is implemented, release of the sterilized product is made in accordance with the requirements of the regulatory approved program. Product release by other means is not acceptable if the predefined critical operational parameters are not achieved.

MODES OF STERILIZATION

Moist Heat Sterilization

Moist heat sterilization of pharmaceutical products includes several types of sterilizing environments and sterilizing media. Saturated steam, hot water spray, and submerged hot water processes are all considered as moist heat sterilizing environments. Different processes may be used to sterilize products by moist heat, and they include batch-type sterilizers and continuous-type sterilizers.

CRITICAL OPERATING PARAMETERS

A defined list of key process parameters and their respective operating limits are defined and established in the sterilization process specifications. Critical operating parameters are those that are absolutely essential to ensure product sterilization to a 10^{-6} PNS. Examples of critical operating parameters may include, but are not limited to, dwell time limits, minimum and maximum limits for process peak dwell temperature, average peak dwell temperature, and the results of the batch or lot release test that satisfies the requirements of CFR, Part 211 (e.g., a load monitor results from the laboratory). F_0 may be used as a critical parameter only when temperature and time relationships are well defined. Other measured parameters may be considered secondary (or noncritical) parameters and may include maximum and minimum time to peak dwell, chamber pressure, and if applicable, chamber water level, sterilizing water time above defined temperature limits, and recirculating water pump pressure differential.

Ethylene Oxide Sterilization

The application of parametric release of pharmaceutical products sterilized by ethylene oxide is more difficult than parametric release of products sterilized by moist heat processes. Critical parameters for ethylene oxide (ETO) steril-

ization are interrelated and more complex than moist heat processes.

CRITICAL OPERATING PARAMETERS

Critical parameters may include the following: temperature, amount of relative humidity present, ethylene oxide concentration, overall exposure time, product and load density, and gas permeability factors.

Parametric release of pharmaceutical products can be achieved if an automated measurement system for the critical parameters is employed and sterilization loads are closely defined and validated relative to product types, densities, packaging materials, and overall load configurations. An example of the measurement of critical factors that may be considered for parametric release would be the use of calibrated ETO pressure recordings to provide an estimate of ETO concentration during the process hold time or the use of direct measurement of ETO concentration by IR or gas chromatography. Because of variances that might occur in the key parameters during sterilization, parametric release is not widely used for products sterilized by ETO.

However, to ensure parametric release, in addition to the attainment of process parameters of the ethylene oxide sterilization, biological indicators (and their sterility testing after sterilization processing) or the use of physicochemical integrators for the ethylene oxide sterilization are often used as load monitors (critical parameters).

Radiation Sterilization

Two radiation sterilizing processes have been used: gamma and electron beam sterilization (i.e., ionizing radiation). Some pharmaceutical products, either in bulk or in their finished formats, have been sterilized by radiation. In discussing the critical parameters of radiation sterilization necessary for parametric release, it is customary to refer to parametric release as *dosimetric release*. Dosimetric release is provided by the use of a chemical dosimeter that measures the delivery of a minimum specified radiation dosage, which has been shown to provide sterilization of the product to a minimum 10^{-6} PNS.

The use of a dosimeter in ionizing radiation sterilization measures delivery of a minimum absorbed radiation dose to a pre-established low dose zone in the irradiated product carrier. This will require mapping of the profile of absorbed ionizing radiation across the density ranges processed in the product carrier. The lowest specified radiation dosage for the process is correlated to predictable bioburden reduction levels by any one of the three documented methods.² An alternative method may be considered whereby extensive product bioburden count and radiation resistance data are available. Dose verification studies would be conducted to ensure that the worst case bioburden load, relative to resistance and numbers, can be inactivated at the lowest dose zone in the carrier system to provide at least a 10^{-6} PNS. This method would of course require an ongoing program of bioburden assessment. The target for the radiation cycle is a minimum 10^{-6} PNS relative to the product bioburden. Dosimetric release of a radiation-sterilized product depends on the delivery of at least a minimum dosage; thus, the critical operational parameters that govern the delivery of that dosage must be within specified limits. These operational critical parameters may include the following: a stacking configuration within the radiation carrier, bulk density of the product, speed of the conveyor or carrier system, distance to the radiation source, duration of product exposure, and appropriate defined adjustments for a decaying radiation source. Demonstration of consistency in the absorbed radiation dosage at areas of minimum and maximum zones of radiation absorption within the fully loaded carriers on a

batch-to-batch basis is a necessary condition for dosimetric release of radiation-sterilized pharmaceutical products.

SUMMARY

The conversion to parametric release in lieu of product sterility testing as described in general chapter *Sterility Tests* <71> requires prior FDA approval. Parametric release is advantageous for terminally sterilized products. The extensiveness of data required to establish parametric release, compared to the general chapter <71> procedures, which lack sensitivity to very low levels of microbial contamination, can result in a more accurate and reliable assessment of the probability of nonsterility of product lots.

<1223> VALIDATION OF ALTERNATIVE MICROBIOLOGICAL METHODS

INTRODUCTION

The purpose of this chapter is to provide guidance for validating methods for use as alternatives to the official compendial microbiological methods. For microbial recovery and identification, microbiological testing laboratories sometimes use alternative test methods to those described in the general chapters for a variety of reasons, such as economics, throughput, and convenience. Validation of these methods is required. Some guidance on validation of the use of alternate methods is provided in the *Tests and Assays* section in the *General Notices and Requirements*. This section also notes that in the event of a dispute, only the result obtained by the compendial test is conclusive.

Validation studies of alternate microbiological methods should take a large degree of variability into account. When conducting microbiological testing by conventional plate count, for example, one frequently encounters a range of results that is broader (%RSD 15 to 35) than ranges in commonly used chemical assays (%RSD 1 to 3). Many conventional microbiological methods are subject to sampling error, dilution error, plating error, incubation error, and operator error.

Validation of Compendial Procedures <1225> defines characteristics such as accuracy, precision, specificity, detection limit, quantification limit, linearity, range, ruggedness, and robustness in their application to analytical methods. These definitions are less appropriate for alternate microbiological method validation as "at least equivalent to the compendial method" given the comparative nature of the question (see the *Tests and Assays—Procedures* section in *General Notices and Requirements*). The critical question is whether or not the alternate method will yield results equivalent to, or better than, the results generated by the conventional method.

Other industry organizations have provided guidance for the validation of alternate microbiological methods.* The suitability of a new or modified method should be demonstrated in a comparison study between the USP compendial method and the alternate method. The characteristics defined in this chapter may be used to establish this comparison.

² ANSI/AAMI/ISO 11137-1996, *Sterilization of Health Care Products—Requirements for Validation and Routine Control—Radiation Sterilization*, July 11, 1994.

* PDA Technical Report No. 33. The Evaluation, Validation and Implementation of New Microbiological Testing Methods. *PDA Journal of Pharmaceutical Science & Technology*. 54 Supplement TR#33 (3) 2000 and Official Methods Programs of AOAC International.

TYPES OF MICROBIOLOGICAL TESTS

It is critical to the validation effort to identify the portion of the test addressed by an alternate technology. For example, there is a variety of technologies available to detect the presence of viable cells. These techniques may have application in a variety of tests (e.g., bioburden, sterility test) but may not, in fact, replace the critical aspects of the test entirely. For example, a sterility test by membrane filtration may be performed according to the compendial procedure up to the point of combining the processed filter with the recovery media, and after that the presence of viable cells might then be demonstrated by use of some of the available technologies. Validation of this application would, therefore, require validation of the recovery system employed rather than the entire test.

There are three major types of determinations specific to microbiological tests. These include tests to determine whether microorganisms are present in a sample, tests to quantify the number of microorganisms (or to enumerate a specific subpopulation of the sample), and tests designed to identify microorganisms. This chapter does not address microbial identification.

Qualitative Tests for the Presence or Absence of Microorganisms

This type of test is characterized by the use of turbidity in a liquid growth medium as evidence of the presence of viable microorganisms in the test sample. The most common example of this test is the sterility test. Other examples of this type of testing are those tests designed to evaluate the presence or absence of a particular type of microorganism in a sample (e.g., coliforms in potable water and *E. coli* in oral dosage forms).

Quantitative Tests for Microorganisms

The plate count method is the most common example of this class of tests used to estimate the number of viable microorganisms present in a sample. The membrane filtration and Most Probable Number (MPN) multiple-tube methods are other examples of these tests. The latter was developed as a means to estimate the number of viable microorganisms present in a sample not amenable to direct plating or membrane filtration.

General Concerns

Validation of a microbiological method is the process by which it is experimentally established that the performance characteristics of the method meet the requirements for the intended application, in comparison to the traditional method. For example, it may not be necessary to fully validate the equivalence of a new quantitative method for use in the antimicrobial efficacy test by comparative studies, as the critical comparison is between the new method of enumeration and the plate count method (the current method for enumeration). As quantitative tests, by their nature, yield numerical data, they allow for the use of parametric statistical techniques. In contrast, qualitative microbial assays, e.g., the sterility test in the example above, may require analysis by nonparametric statistical methods. The validation of analytical methods for chemical assays follows well-established parameters as described in *Validation of Compendial Procedures* (1225). Validation of microbiological methods shares some of the same concerns, although consideration must be given to the unique nature of microbiological assays (see Table 1).

Table 1. Validation Parameters by Type of Microbiological Test

Parameter	Qualitative Tests	Quantitative Tests
Accuracy	No	Yes
Precision	No	Yes
Specificity	Yes	Yes
Detection limit	Yes	Yes
Quantification limit	No	Yes
Linearity	No	Yes
Operational range	No	Yes
Robustness	Yes	Yes
Repeatability	Yes	Yes
Ruggedness	Yes	Yes

VALIDATION OF QUALITATIVE TESTS FOR DEMONSTRATION OF VIABLE MICROORGANISMS IN A SAMPLE

Specificity

The specificity of an alternate qualitative microbiological method is its ability to detect a range of microorganisms that may be present in the test article. This concern is adequately addressed by growth promotion of the media for qualitative methods that rely upon growth to demonstrate presence or absence of microorganisms. However, for those methods that do not require growth as an indicator of microbial presence, the specificity of the assay for microbes assures that extraneous matter in the test system does not interfere with the test.

Limit of Detection

The limit of detection is the lowest number of microorganisms in a sample that can be detected under the stated experimental conditions. A microbiological limit test determines the presence or absence of microorganisms, e.g., absence of *Salmonella* spp. in 10 g. Due to the nature of microbiology, the limit of detection refers to the number of organisms present in the original sample before any dilution or incubation steps; it does not refer to the number of organisms present at the point of assay.

One method to demonstrate the limit of detection for a quantitative assay would be to evaluate the two methods (alternative and compendial) by inoculation with a low number of challenge microorganisms (not more than 5 cfu per unit) followed by a measurement of recovery. The level of inoculation should be adjusted until at least 50% of the samples show growth in the compendial test. It is necessary to repeat this determination several times, as the limit of detection of an assay is determined from a number of replicates (not less than 5). The ability of the two methods to detect the presence of low numbers of microorganisms can be demonstrated using the Chi square test. A second method to demonstrate equivalence between the two quantitative methods could be through the use of the Most Probable Number technique. In this method, a 5-tube design in a ten-fold dilution series could be used for both methods. These would then be challenged with equivalent inoculums (for example, a 10⁻¹, 10⁻², and 10⁻³ dilution from a stock suspension of approximately 50 cfu per mL to yield target inocula of 5, 0.5, and 0.05 cfu per tube) and the MPN of the original stock determined by each method. If the 95% confidence intervals overlapped, then the methods would be considered equivalent.

Ruggedness

The ruggedness of a qualitative microbiological method is the degree of precision of test results obtained by analysis of the same samples under a variety of normal test conditions, such as different analysts, instruments, reagent lots, and laboratories. Ruggedness can be defined as the intrinsic resistance to the influences exerted by operational and environmental variables on the results of the microbiological method. Ruggedness is a validation parameter best suited to determination by the supplier of the test method who has easy access to multiple instruments and batches of components.

Robustness

The robustness of a qualitative microbiological method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and provides an indication of its reliability during normal usage. Robustness is a validation parameter best suited to determination by the supplier of the test method. As there are no agreed upon standards for current methods, acceptance criteria are problematic and must be tailored to the specific technique. It is essential, however, that an estimate of the ruggedness of the alternate procedure be developed. The measure of robustness is not necessarily a comparison between the alternate method and the traditional, but rather a necessary component of validation of the alternate method so that the user knows the operating parameters of the method.

VALIDATION OF QUANTITATIVE ESTIMATION OF VIABLE MICROORGANISMS IN A SAMPLE

As colony-forming units follow a Poisson distribution, the use of statistical tools appropriate to the Poisson rather than those used to analyze normal distributions is encouraged. If the user is more comfortable using tools geared towards normally distributed data, the use of a data transformation is frequently useful. Two techniques are available and convenient for microbiological data. Raw counts can be transformed to normally distributed data either by taking the \log_{10} unit value for that count, or by taking the square root of count +1. The latter transformation is especially helpful if the data contain zero counts.

Accuracy

The accuracy of this type of microbiological method is the closeness of the test results obtained by the alternate test method to the value obtained by the traditional method. It should be demonstrated across the operational range of the test. Accuracy is usually expressed as the percentage of recovery of microorganisms by the assay method.

Accuracy in a quantitative microbiological test may be shown by preparing a suspension of microorganisms at the upper end of the range of the test, that has been serially diluted down to the lower end of the range of the test. The operational range of the alternate method should overlap that of the traditional method. For example, if the alternate method is meant to replace the traditional plate count method for viable counts, then a reasonable range might be from 10^0 to 10^6 cfu per mL. At least 5 suspensions across the range of the test should be analyzed for each challenge organism. The alternate method should provide an estimate of viable microorganisms not less than 70% of the estimate provided by the traditional method, or the new method should be shown to recover at least as many organisms as the traditional method by appropriate statistical analysis, an example being an ANOVA analysis of the \log_{10} unit trans-

forms of the data points. Note that the possibility exists that an alternate method may recover an apparent higher number of microorganisms if it is not dependent on the growth of the microorganisms to form colonies or develop turbidity. This is determined in the *Specificity* evaluation.

Precision

The precision of a quantitative microbiological method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of suspensions of laboratory microorganisms across the range of the test. The precision of a microbiological method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation). However, other appropriate measures may be applied.

One method to demonstrate precision uses a suspension of microorganisms at the upper end of the range of the test that has been serially diluted down to the lower end of the range of the test. At least 5 suspensions across the range of the test should be analyzed. For each suspension at least 10 replicates should be assayed in order to be able to calculate statistically significant estimates of the standard deviation or relative standard deviation (coefficient of variation). Generally, a RSD in the 15% to 35% range would be acceptable. Irrespective of the specific results, the alternate method should have a coefficient of variation that is not larger than that of the traditional method. For example, a plate count method might have the RSD ranges as shown in the following table.

Table 2. Expected RSD as a Function of cfu per Plate

cfu per Plate	Expected RSD
30–300	<15%
10–30	<25%
<10	<35%

Specificity

The specificity of a quantitative microbiological method is its ability to detect a panel of microorganisms suitable to demonstrate that the method is fit for its intended purpose. This is demonstrated using the organisms appropriate for the purpose of the alternate method. It is important to challenge the alternate technology in a manner that would encourage false positive results (specific to that alternate technology) to demonstrate the suitability of the alternate method in comparison to the traditional method. This is especially important with those alternate methods that do not require growth for microbial enumeration (for example, any that do not require enrichment or can enumerate microorganisms into the range of 1–50 cells).

Limit of Quantification

The limit of quantification is the lowest number of microorganisms that can be accurately counted. As it is not possible to obtain a reliable sample containing a known number of microorganisms, it is essential that the limit of quantification of an assay is determined from a number of replicates ($n > 5$) at each of at least 5 different points across the operational range of the assay. The limit of quantification should not be a number greater than that of the traditional method. Note that this may have an inherent limit due to the nature of bacterial enumeration and the Poisson distribution of bacterial counts (see *Validation of Microbial Recovery from Pharmacopeial Articles* <1227>). Therefore, the alternate method need only demonstrate that it is at least as sensitive as the traditional method to similar lower limits.

Linearity

The linearity of a quantitative microbiological test is its ability to produce results that are proportional to the concentration of microorganisms present in the sample within a given range. The linearity should be determined over the range of the test. A method to determine this would be to select at least 5 concentrations of each standard challenge microorganism and conduct at least 5 replicate readings of each concentration. An appropriate measure would be to calculate the square of the correlation coefficient, r^2 , from a linear regression analysis of the data generated above. While the correlation coefficient does not provide an estimate of linearity, it is a convenient and commonly applied measure to approximate the relationship. The alternate method should not have an r^2 value less than 0.95.

Limit of Detection

See *Limit of Detection* under *Validation of Qualitative Tests for Demonstration of Viable Microorganisms in a Sample*.

Range

The operational range of a quantitative microbiological method is the interval between the upper and lower levels of microorganisms that have been demonstrated to be determined with precision, accuracy, and linearity.

Ruggedness

See *Ruggedness* under *Validation of Qualitative Tests for Demonstration of Viable Microorganisms in a Sample*.

Robustness

See *Robustness* under *Validation of Qualitative Tests for Demonstration of Viable Microorganisms in a Sample*.

(1224) TRANSFER OF ANALYTICAL PROCEDURES

INTRODUCTION

Testing to the specification of an ancillary material, intermediate, and/or ingredient and product is critical in establishing the quality of a finished dosage form. The transfer of analytical procedures (TAP), also referred to as method transfer, is the documented process that qualifies a laboratory (the receiving unit) to use an analytical test procedure that originated in another laboratory (the transferring unit), thus ensuring that the receiving unit has the procedural knowledge and ability to perform the transferred analytical procedure as intended.

The purpose of this general information chapter is to summarize the types of transfers that may occur, including the possibility of waiver of any transfer, and to outline the potential components of a transfer protocol. The chapter does not provide statistical methods and does not encompass the transfer of microbiological or biological procedures.

TYPES OF TRANSFERS OF ANALYTICAL PROCEDURES

TAP can be performed and demonstrated by several approaches. The most common is comparative testing performed on homogeneous lots of the target material from standard production batches or samples intentionally prepared for the test (e.g., by spiking relevant accurate amounts of known impurities into samples). Other approaches include covalidation between laboratories, the complete or partial validation of the analytical procedures by the receiving unit, and the transfer waiver, which is an appropriately justified omission of the transfer process. The tests that will be transferred, the extent of the transfer activities, and the implementation strategy should be based on a risk analysis that considers the previous experience and knowledge of the receiving unit, the complexity and specifications of the product, and the procedure.

Comparative Testing

Comparative testing requires the analysis of a predetermined number of samples of the same lot by both the sending and the receiving units. Other approaches may be valid, e.g., if the receiving unit meets a predetermined acceptance criterion for the recovery of an impurity in a spiked product. Such analysis is based on a preapproved transfer protocol that stipulates the details of the procedure, the samples that will be used, and the predetermined acceptance criteria, including acceptable variability. Meeting the predetermined acceptance criteria is necessary to assure that the receiving unit is qualified to run the procedure.

Covalidation Between Two or More Laboratories

The laboratory that performs the validation of an analytical procedure is qualified to run the procedure. The transferring unit can involve the receiving unit in an interlaboratory covalidation, including them as a part of the validation team at the transferring unit and thereby obtaining data for the assessment of reproducibility. This assessment is made using a preapproved transfer or validation protocol that provides the details of the procedure, the samples to be used, and the predetermined acceptance criteria. The general chapter *Validation of Compendial Procedures* (1225) provides useful guidance about which characteristics are appropriate for testing.

Revalidation

Revalidation or partial revalidation is another acceptable approach for transfer of a validated procedure. Those characteristics described in (1225), which are anticipated to be affected by the transfer, should be addressed.

Transfer Waiver

The conventional TAP may be omitted under certain circumstances. In such instances, the receiving unit is considered to be qualified to use the analytical test procedures without comparison and generation of interlaboratory comparative data. The following examples give some scenarios that may justify the waiver of TAP:

- The new product's composition is comparable to that of an existing product and/or the concentration of active ingredient is similar to that of an existing product and is analyzed by procedures with which the receiving unit already has experience.
- The analytical procedure being transferred is described in the *USP-NF*, and is unchanged. Verification should apply in this case (see (1226)).

- The analytical procedure transferred is the same as or very similar to a procedure already in use.
- The personnel in charge of the development, validation, or routine analysis of the product at the transferring unit are moved to the receiving unit.

If eligible for transfer waiver, the receiving receiving unit should document it with appropriate justifications.

ELEMENTS RECOMMENDED FOR THE TRANSFER OF ANALYTICAL PROCEDURES

Several elements, many of which may be interrelated, are recommended for a successful TAP. When appropriate and as a part of pretransfer activities, the transferring unit should provide training to the receiving unit, or the receiving unit should run the procedures and identify any issues that may need to be resolved before the transfer protocol is signed. Training should be documented.

The transferring unit, often the development unit, is responsible for providing the analytical procedure, the reference standards, the validation reports, and any necessary documents, as well as for providing the necessary training and assistance to the receiving unit as needed during the transfer. The receiving unit may be a quality control unit, another intracompany facility, or another company such as a contract research organization. The receiving unit provides qualified staff or properly trains the staff before the transfer, ensures that the facilities and instrumentation are properly calibrated and qualified as needed, and verifies that the laboratory systems are in compliance with applicable regulations and in-house general laboratory procedures. Both the transferring and receiving units should compare and discuss data as well as any deviations from the protocol. This discussion addresses any necessary corrections or updates to the final report and the analytical procedure as necessary to reproduce the procedure.

A single lot of the article may be used for the transfer, because the aim of the transfer is not related to the manufacturing process but rather to the evaluation of the analytical procedure's performance at the receiving site.

PREAPPROVED PROTOCOL

A well-designed protocol should be discussed, agreed upon, and documented before the implementation of TAP. The document expresses a consensus between the parties, indicating an intended execution strategy, and should include each party's requirements and responsibilities. It is recommended that the protocol contain the following topics as appropriate: objective, scope, responsibilities of the transferring and receiving units, materials and instruments that will be used, analytical procedure, experimental design, and acceptance criteria for all tests and/or methods included in the transfer. Based on the validation data and procedural knowledge, the transfer protocol should identify the specific analytical performance characteristics (see <1225> and <1226>) that will be evaluated and the analysis that will be used to evaluate acceptable outcomes of the transfer exercise.

The transfer acceptance criteria, which are based on method performance and historical data from stability and release results, if available, should include the comparability criteria for results from all study sites. These criteria may be derived using statistical principles based on the difference between mean values and established ranges and should be accompanied by an estimation of the variability (e.g., percent relative standard deviation [%RSD] for each site), particularly for the intermediate precision %RSD of the receiving unit and/or a statistical method for the comparison of the means for assay and content uniformity tests. In instances of impurity testing, where precision may be poorer such as in the case of trace impurities, a simple descriptive approach can be used. Dissolution can be evaluated by a

comparison of the dissolution profiles using the similarity factor f_2 or by comparison of data at the specified time points. The laboratories should provide appropriate rationale for any analytical performance characteristic not included. The materials, reference standards, samples, instruments, and instrumental parameters that will be used should be described.

It is recommended that expired, aged, or spiked samples be carefully chosen and evaluated to identify potential problems related to differences in sample preparation equipment and to evaluate the impact of potential aberrant results on marketed products. The documentation section of the transfer protocol may include report forms to ensure consistent recording of results and to improve consistency between laboratories. This section should contain the additional information that will be included with the results, such as example chromatograms and spectra, along with additional information in case of a deviation. The protocol should also explain how any deviation from the acceptance criteria will be managed. Any changes to the transfer protocol following failure of an acceptance criterion must be approved before collection of additional data.

THE ANALYTICAL PROCEDURE

The procedure should be written with sufficient detail and explicit instructions, so that a trained analyst can perform it without difficulty. A pretransfer meeting between the transferring and receiving units is helpful to clarify any issues and answer any questions regarding the transfer process. If complete or partial validation data exist, they should be available to the receiving unit, along with any technical details required to perform the test in question. In some cases it may be useful for the individuals who were involved with the initial development or validation to be on site during the transfer. The number of replicates and injection sequences in the case of liquid or gas chromatography should be clearly expressed, and, in the case of dissolution testing, the number of individual dosage units should be stipulated.

TRANSFER REPORT

When the TAP is successfully completed, the receiving unit should prepare a transfer report that describes the results obtained in relation to the acceptance criteria, along with conclusions that confirm that the receiving unit is now qualified to run the procedure. Any deviations should be thoroughly documented and justified. If the acceptance criteria are met, the TAP is successful and the receiving unit is qualified to run the procedure. Otherwise, the procedure cannot be considered transferred until effective remedial steps are adopted in order to meet the acceptance criteria. An investigation may provide guidance about the nature and extent of the remedial steps, which may vary from further training and clarification to more complex approaches, depending on the particular procedure.

<1225> VALIDATION OF COMPENDIAL PROCEDURES

Test procedures for assessment of the quality levels of pharmaceutical articles are subject to various requirements. According to Section 501 of the Federal Food, Drug, and Cosmetic Act, assays and specifications in monographs of the United States Pharmacopeia and the National Formulary

constitute legal standards. The Current Good Manufacturing Practice regulations [21 CFR 211.194(a)] require that test methods, which are used for assessing compliance of pharmaceutical articles with established specifications, must meet proper standards of accuracy and reliability. Also, according to these regulations [21 CFR 211.194(a)(2)], users of analytical methods described in *USP–NF* are not required to validate the accuracy and reliability of these methods, but merely verify their suitability under actual conditions of use. Recognizing the legal status of *USP* and *NF* standards, it is essential, therefore, that proposals for adoption of new or revised compendial analytical procedures be supported by sufficient laboratory data to document their validity.

The text of this information chapter harmonizes, to the extent possible, with the Tripartite International Conference on Harmonization (ICH) documents *Validation of Analytical Procedures* and the *Methodology* extension text, which are concerned with analytical procedures included as part of registration applications submitted within the EC, Japan, and the USA.

SUBMISSIONS TO THE COMPENDIA

Submissions to the compendia for new or revised analytical procedures should contain sufficient information to enable members of the USP Council of Experts and its Expert Committees to evaluate the relative merit of proposed procedures. In most cases, evaluations involve assessment of the clarity and completeness of the description of the analytical procedures, determination of the need for the procedures, and documentation that they have been appropriately validated. Information may vary depending upon the type of method involved. However, in most cases a submission will consist of the following sections.

Rationale—This section should identify the need for the procedure and describe the capability of the specific procedure proposed and why it is preferred over other types of determinations. For revised procedures, a comparison should be provided of limitations of the current compendial procedure and advantages offered by the proposed procedure.

Proposed Analytical Procedure—This section should contain a complete description of the analytical procedure sufficiently detailed to enable persons “skilled in the art” to replicate it. The write-up should include all important operational parameters and specific instructions such as preparation of reagents, performance of system suitability tests, description of blanks used, precautions, and explicit formulas for calculation of test results.

Data Elements—This section should provide thorough and complete documentation of the validation of the analytical procedure. It should include summaries of experimental data and calculations substantiating each of the applicable analytical performance characteristics. These characteristics are described in the following section.

VALIDATION

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications. Typical analytical performance characteristics that should be considered in the validation of the types of procedures described in this document are listed in *Table 1*. Because opinions may differ with respect to terminology and use, each of the performance characteristics is defined in the next section of this chapter, along with a delineation of a typical method or methods by which it may be measured. The definitions refer to “test results.” The description of the analytical procedure should define what the test results for the procedure are. As noted in ISO 5725-1 and 3534-1, a test result is “the value

of a characteristic obtained by carrying out a specified test method. The test method should specify that one or a number of individual measurements be made, and their average, or another appropriate function (such as the median or the standard deviation), be reported as the test result. It may also require standard corrections to be applied, such as correction of gas volumes to standard temperature and pressure. Thus, a test result can be a result calculated from several observed values. In the simple case, the test result is the observed value itself.” A test result also can be, but need not be, the final, reportable value that would be compared to the acceptance criteria of a specification. Validation of physical property methods may involve the assessment of chemometric models. However, the typical analytical characteristics used in method validation can be applied to the methods derived from the use of the chemometric models.

Table 1. Typical Analytical Characteristics Used in Method Validation

Accuracy
Precision
Specificity
Detection Limit
Quantitation Limit
Linearity
Range
Robustness

The effects of processing conditions and potential for segregation of materials should be considered when obtaining a representative sample to be used for validation of procedures.

In the case of compendial procedures, revalidation may be necessary in the following cases: a submission to the USP of a revised analytical procedure; or the use of an established general procedure with a new product or raw material (see below in *Data Elements Required for Validation*).

The ICH documents give guidance on the necessity for revalidation in the following circumstances: changes in the synthesis of the drug substance; changes in the composition of the drug product; and changes in the analytical procedure.

Chapter (1225) is intended to provide information that is appropriate to validate a wide range of compendial analytical procedures. The validation of compendial procedures may use some or all of the suggested typical analytical characteristics used in method validation as outlined in *Table 1* and categorized by type of analytical method in *Table 2*. For some compendial procedures the fundamental principles of validation may extend beyond characteristics suggested in Chapter (1225). For these procedures the user is referred to the individual compendial chapter for those specific analytical validation characteristics and any specific validation requirements.

Analytical Performance Characteristics

ACCURACY

Definition—The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range. [A note on terminology: The definition of accuracy in (1225) and ICH Q2 corresponds to unbiasedness only. In the International Vocabulary of Metrology (VIM) and documents of the International Organization for Standardization (ISO), “accuracy” has a different meaning. In ISO, accuracy combines the concepts of unbiasedness (termed “trueness”) and precision.]

Determination—In the case of the assay of a drug substance, accuracy may be determined by application of the analytical procedure to an analyte of known purity (e.g., a Reference Standard) or by comparison of the results of the procedure with those of a second, well-characterized procedure, the accuracy of which has been stated or defined.

In the case of the assay of a drug in a formulated product, accuracy may be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure. If it is not possible to obtain samples of all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product (i.e., “to spike”) or to compare results with those of a second, well-characterized procedure, the accuracy of which has been stated or defined.

In the case of quantitative analysis of impurities, accuracy should be assessed on samples (of drug substance or drug product) spiked with known amounts of impurities. Where it is not possible to obtain samples of certain impurities or degradation products, results should be compared with those obtained by an independent procedure. In the absence of other information, it may be necessary to calculate the amount of an impurity based on comparison of its response to that of the drug substance; the ratio of the responses of equal amounts of the impurity and the drug substance (relative response factor) should be used if known.

Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals.

The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e., three concentrations and three replicates of each concentration).

Assessment of accuracy can be accomplished in a variety of ways, including evaluating the recovery of the analyte (percent recovery) across the range of the assay, or evaluating the linearity of the relationship between estimated and actual concentrations. The statistically preferred criterion is that the confidence interval for the slope be contained in an interval around 1.0, or alternatively, that the slope be close to 1.0. In either case, the interval or the definition of closeness should be specified in the validation protocol. The acceptance criterion will depend on the assay and its variability and on the product. Setting an acceptance criterion based on the lack of statistical significance of the test of the null hypothesis that the slope is 1.0 is not an acceptable approach.

Accuracy of physical property methods may be assessed through the analysis of standard reference materials, or alternatively, the suitability of the above approaches may be considered on a case-by-case basis.

PRECISION

Definition—The precision of an analytical procedure is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. Precision may be a measure of either the degree of reproducibility or of repeatability of the analytical procedure under normal operating conditions. In this context, reproducibility refers to the use of the analytical procedure in different laboratories, as in a collaborative study. Intermediate precision (also known as ruggedness) expresses within-laboratory variation, as on different days, or with different analysts or equipment within the same laboratory. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment.

Determination—The precision of an analytical procedure is determined by assaying a sufficient number of aliquots of a homogeneous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation (coefficient of variation). Assays in this context are independent analyses of samples that have been carried through the complete analytical procedure from sample preparation to final test result.

The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration) or using a minimum of six determinations at 100% of the test concentration.

SPECIFICITY

Definition—The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures. [NOTE—Other reputable international authorities (IUPAC, AOAC-I) have preferred the term “selectivity,” reserving “specificity” for those procedures that are completely selective.] For the tests discussed below, the above definition has the following implications:

Identification Tests: ensure the identity of the analyte.

Purity Tests: ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte (e.g., related substances test, heavy metals limit, organic volatile impurities).

Assays: provide an exact result, which allows an accurate statement on the content or potency of the analyte in a sample.

Determination—In the case of qualitative analyses (identification tests), the ability to select between compounds of closely related structure that are likely to be present should be demonstrated. This should be confirmed by obtaining positive results (perhaps by comparison to a known reference material) from samples containing the analyte, coupled with negative results from samples that do not contain the analyte and by confirming that a positive response is not obtained from materials structurally similar to or closely related to the analyte.

In the case of analytical procedures for impurities, specificity may be established by spiking the drug substance or product with appropriate levels of impurities and demonstrating that these impurities are determined with appropriate accuracy and precision.

In the case of the assay, demonstration of specificity requires that it can be shown that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay result is unaffected by the presence of these extraneous materials.

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure (e.g., a Pharmacopeial or other validated procedure). These comparisons should include samples stored under relevant stress conditions (e.g., light, heat, humidity, acid/base hydrolysis, and oxidation). In the case of the assay, the results should be compared; in the case of chromatographic impurity tests, the impurity profiles should be compared.

The ICH documents state that when chromatographic procedures are used, representative chromatograms should be presented to demonstrate the degree of selectivity, and peaks should be appropriately labeled. Peak purity tests (e.g., using diode array or mass spectrometry) may be used.

ful to show that the analyte chromatographic peak is not attributable to more than one component.

For validation of specificity for qualitative and quantitative determinations by spectroscopic methods, chapters related to topics such as near-infrared spectrophotometry, raman spectroscopy, and X-ray powder diffraction should be consulted.

DETECTION LIMIT

Definition—The detection limit is a characteristic of limit tests. It is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Thus, limit tests merely substantiate that the amount of analyte is above or below a certain level. The detection limit is usually expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

Determination—For noninstrumental procedures, the detection limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

For instrumental procedures, the same approach may be used as for noninstrumental procedures. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual detection limit. Rather, the detection limit is shown to be sufficiently low by the analysis of samples with known concentrations of analyte above and below the required detection level. For example, if it is required to detect an impurity at the level of 0.1%, it should be demonstrated that the procedure will reliably detect the impurity at that level.

In the case of instrumental analytical procedures that exhibit background noise, the ICH documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be detected is established. Typically acceptable signal-to-noise ratios are 2:1 or 3:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses. Whatever method is used, the detection limit should be subsequently validated by the analysis of a suitable number of samples known to be near, or prepared at, the detection limit.

QUANTITATION LIMIT

Definition—The quantitation limit is a characteristic of quantitative assays for low levels of compounds in sample matrices, such as impurities in bulk drug substances and degradation products in finished pharmaceuticals. It is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The quantitation limit is expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

Determination—For noninstrumental procedures, the quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision.

For instrumental procedures, the same approach may be used as for noninstrumental procedures. In the case of procedures submitted for consideration as official compendial procedures, it is almost never necessary to determine the actual quantitation limit. Rather, the quantitation limit is shown to be sufficiently low by the analysis of samples with known concentrations of analyte above and below the quantitation level. For example, if it is required that an analyte be assayed at the level of 0.1 mg per tablet, it

should be demonstrated that the procedure will reliably quantitate the analyte at that level.

In the case of instrumental analytical procedures that exhibit background noise, the ICH documents describe a common approach, which is to compare measured signals from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be quantified is established. A typically acceptable signal-to-noise ratio is 10:1. Other approaches depend on the determination of the slope of the calibration curve and the standard deviation of responses. Whatever approach is used, the quantitation limit should be subsequently validated by the analysis of a suitable number of samples known to be near, or prepared at, the quantitation limit.

LINEARITY AND RANGE

Definition of Linearity—The linearity of an analytical procedure is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Thus, in this section, "linearity" refers to the linearity of the relationship of concentration and assay measurement. In some cases, to attain linearity, the concentration and/or the measurement may be transformed. (Note that the weighting factors used in the regression analysis may change when a transformation is applied.) Possible transformations may include log, square root, or reciprocal, although other transformations are acceptable. If linearity is not attainable, a nonlinear model may be used. The goal is to have a model, whether linear or nonlinear, that describes closely the concentration-response relationship.

Definition of Range—The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written. The range is normally expressed in the same units as test results (e.g., percent, parts per million) obtained by the analytical procedure.

Determination of Linearity and Range—Linearity should be established across the range of the analytical procedure. It should be established initially by visual examination of a plot of signals as a function of analyte concentration of content. If there appears to be a linear relationship, test results should be established by appropriate statistical methods (e.g., by calculation of a regression line by the method of least squares). Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be submitted.

The range of the procedure is validated by verifying that the analytical procedure provides acceptable precision, accuracy, and linearity when applied to samples containing analyte at the extremes of the range as well as within the range.

ICH recommends that, for the establishment of linearity, a minimum of five concentrations normally be used. It is also recommended that the following minimum specified ranges should be considered:

Assay of a Drug Substance (or a finished product): from 80% to 120% of the test concentration.

Determination of an Impurity: from 50% to 120% of the acceptance criterion.

For Content Uniformity: a minimum of 70% to 130% of the test concentration, unless a wider or more appropriate range based on the nature of the dosage form (e.g., metered-dose inhalers) is justified.

For Dissolution Testing: $\pm 20\%$ over the specified range (e.g., if the acceptance criteria for a controlled-release prod-

uct cover a region from 30%, after 1 hour, and up to 90%, after 24 hours, the validated range would be 10% to 110% of the label claim).

The traditional definition of linearity, i.e., the establishment of a linear or mathematical relationship between sample concentration and response, is not applicable to particle size analysis. For particle size analysis, a concentration range is defined (instrument- and particle size-dependent) such that the measured particle size distribution is not affected by changes in concentration within the defined concentration range. Concentrations below the defined concentration range may introduce an error due to poor signal-to-noise ratio, and concentrations exceeding the defined concentration range may introduce an error due to multiple scattering.

ROBUSTNESS

Definition—The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage. Robustness may be determined during development of the analytical procedure.

SYSTEM SUITABILITY

If measurements are susceptible to variations in analytical conditions, these should be suitably controlled, or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness and ruggedness should be that a series of system suitability parameters is established to ensure that the validity of the analytical procedure is maintained whenever used. Typical variations are the stability of analytical solutions, different equipment, and different analysts. In the case of liquid chromatography, typical variations are the pH of the mobile phase, the mobile phase composition, different lots or suppliers of columns, the temperature, and the flow rate. In the case of gas chromatography, typical variations are different lots or suppliers of columns, the temperature, and the flow rate.

System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being evaluated. They are especially important in the case of chromatographic procedures. Submissions to the USP should make note of the requirements under the *System Suitability* section in the general test chapter *Chromatography* (621).

Data Elements Required for Validation

Compendial test requirements vary from highly exacting analytical determinations to subjective evaluation of attrib-

utes. Considering this broad variety, it is only logical that different test procedures require different validation schemes. This chapter covers only the most common categories of tests for which validation data should be required. These categories are as follows:

Category I—Analytical procedures for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products.

Category II—Analytical procedures for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. These procedures include quantitative assays and limit tests.

Category III—Analytical procedures for determination of performance characteristics (e.g., dissolution, drug release, etc.).

Category IV—Identification tests.

For each category, different analytical information is needed. Listed in *Table 2* are data elements that are normally required for each of these categories.

Already established general procedures (e.g., titrimetric determination of water, bacterial endotoxins) should be verified to establish their suitability for use, such as their accuracy (and absence of possible interference) when used for a new product or raw material.

When validating physical property methods, consider the same performance characteristics required for any analytical procedure. Evaluate use of the performance characteristics on a case-by-case basis, with the goal of determining that the procedure is suitable for its intended use. The specific acceptance criteria for each validation parameter should be consistent with the intended use of the method.

Physical methods may also be classified into the four validation categories. For example, validation of a quantitative spectroscopic method may involve evaluation of *Category I* or *Category II Analytical Performance Characteristics*, depending on the method requirements. Qualitative physical property measurements, such as particle size, surface area, bulk and tapped density, which could impact performance characteristics, often best fit in *Category III*. *Category IV Analytical Performance Characteristics* usually applies to validation of qualitative identification spectroscopic methods. However, the various techniques may be used for different purposes, and the specific use of the method and characteristics of the material being analyzed should be considered when definitively applying a category to a particular type of method.

The validity of an analytical procedure can be verified only by laboratory studies. Therefore, documentation of the successful completion of such studies is a basic requirement for determining whether a procedure is suitable for its intended application(s). Current compendial procedures are also subject to regulations that require demonstration of suitability under actual conditions of use (see *Verification of Compendial Procedures* (1226) for principles relative to the verification of compendial procedures). Appropriate documentation should

Table 2. Data Elements Required for Validation

Analytical Performance Characteristics	Category I	Category II		Category III	Category IV
		Quantitative	Limit Tests		
Accuracy	Yes	Yes	*	*	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	*	Yes
Detection Limit	No	No	Yes	*	No
Quantitation Limit	No	Yes	No	*	No
Linearity	Yes	Yes	No	*	No
Range	Yes	Yes	*	*	No

*May be required, depending on the nature of the specific test.

accompany any proposal for new or revised compendial analytical procedures.

<1226> VERIFICATION OF COMPENDIAL PROCEDURES

The intent of this general information chapter is to provide general information on the verification of compendial procedures that are being performed for the first time to yield acceptable results utilizing the personnel, equipment, and reagents available. This chapter is not intended for retroactive application to already successfully established laboratory procedures. The chapter *Validation of Compendial Procedures* (1225) provides general information on characteristics that should be considered for various test categories and on the documentation that should accompany analytical procedures submitted for inclusion in *USP–NF*. Verification consists of assessing selected analytical performance characteristics, such as those that are described in chapter <1225>, to generate appropriate, relevant data rather than repeating the validation process.

Users of compendial analytical procedures are not required to validate these procedures when first used in their laboratories, but documented evidence of suitability should be established under actual conditions of use. In the United States, this requirement is established in 21 CFR 211.194(a)(2) of the current Good Manufacturing Practice regulations, which states that the “suitability of all testing methods used shall be verified under actual conditions of use.”

Verification of microbiological procedures is not covered in this chapter because it is covered in USP general test chapters *Antimicrobial Effectiveness Testing* (51), *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), *Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms* (62), *Sterility Tests* (71), and in general information chapter *Validation of Microbial Recovery from Pharmacopoeial Articles* (1227).

VERIFICATION PROCESS

The verification process for compendial test procedures is the assessment of whether the procedure can be used for its intended purpose, under the actual conditions of use for a specified drug substance and/or drug product matrix.

Users should have the appropriate experience, knowledge, and training to understand and be able to perform the compendial procedures as written. Verification should be conducted by the user such that the results will provide confidence that the compendial procedure will perform suitably as intended.

If the verification of the compendial procedure is not successful, and assistance from USP staff has not resolved the problem, it may be concluded that the procedure may not be suitable for use with the article being tested in that laboratory. It may then be necessary to develop and validate an alternate procedure as allowed in the *General Notices*. The alternate procedure may be submitted to USP, along with the appropriate data, to support a proposal for inclusion or replacement of the current compendial procedure.

VERIFICATION REQUIREMENTS

Verification requirements should be based on an assessment of the complexity of both the procedure and the material to which the procedure is applied. Although complete revalidation of a compendial method is not required to verify the suitability of a procedure under actual conditions of use, some of the analytical performance characteristics listed in chapter <1225>, *Table 2*, may be used for the verification process. Only those characteristics that are considered to be appropriate for the verification of the particular procedure need to be evaluated. The process of assessing the suitability of a compendial analytical test procedure under the conditions of actual use may or may not require actual laboratory performance of each analytical performance characteristic. The degree and extent of the verification process may depend on the level of training and experience of the user, on the type of procedure and its associated equipment or instrumentation, on the specific procedural steps, and on which article(s) are being tested.

Verification should assess whether the compendial procedure is suitable for the drug substance and/or the drug product matrix, taking into account the drug substance's synthetic route, the method of manufacture for the drug product, or both, if applicable. Verification should include an assessment of elements such as the effect of the matrix on the recovery of impurities and drug substances from the drug product matrix, as well as the suitability of chromatographic conditions and column, the appropriateness of detector signal response, etc.

As an example, an assessment of specificity is a key parameter in verifying that a compendial procedure is suitable for use in assaying drug substances and drug products. For instance, acceptable specificity for a chromatographic method may be verified by conformance with system suitability resolution requirements (if specified in the procedure). However, drug substances from different suppliers may have different impurity profiles that are not addressed by the compendial test procedure. Similarly, the excipients in a drug product can vary widely among manufacturers and may have the potential to directly interfere with the procedure or cause the formation of impurities that are not addressed by the compendial procedure. In addition, drug products containing different excipients, antioxidants, buffers, or container extractives may affect the recovery of the drug substance from the matrix. In these cases, a more thorough assessment of the matrix effects may be required to demonstrate suitability of the procedure for the particular drug substance or product. Other analytical performance characteristics such as an assessment of the limit of detection or quantitation and precision for impurities procedures may be useful to demonstrate the suitability of the compendial procedure under actual conditions of use.

Verification is not required for basic compendial test procedures that are routinely performed unless there is an indication that the compendial procedure is not appropriate for the article under test. Examples of basic compendial procedures include, but are not limited to, loss on drying, residue on ignition, various wet chemical procedures such as acid value, and simple instrumental determinations such as pH measurements. However, for the application of already established routine procedures to compendial articles tested for the first time, it is recommended that consideration be

given to any new or different sample handling or solution preparation requirements.

<1227> VALIDATION OF MICROBIAL RECOVERY FROM PHARMACOPeIAL ARTICLES

This chapter provides guidelines for the validation of methods for the estimation of the number of viable microorganisms, for the detection of indicators or objectionable microorganisms, for the validation of microbiological methods used in antimicrobial effectiveness testing, and for the sterility testing of Pharmacopeial articles. It is generally understood that if a product possesses antimicrobial properties because of the presence of a specific preservative or because of its formulation, this antimicrobial property must be neutralized to recover viable microorganisms. This neutralization may be achieved by the use of a specific neutralizer, by dilution, by a combination of washing and dilution, or by any combination of these methods.

The tests under *Antimicrobial Effectiveness Testing* <51>, *Sterility Tests* <71>, and *Microbial Enumeration Tests* <61> and *Tests for Specified Microorganisms* <62> require the validation of recovery methods. To ensure that the results of the tests are credible, neutralization of antimicrobial properties of the test solution is required before estimating the number of viable microorganisms.

INFLUENTIAL FACTORS

Several factors affect the measurement of a test solution's antimicrobial activity, and these must be considered in the validation design. They include the nature of the microorganisms used as challenge organisms, the preparation of the inoculum of challenge organisms, the specific conditions of the test, and the conditions of recovery. These factors also affect the validation of recovery methods for aqueous or nonaqueous products, irrespective of their antimicrobial properties; thus, all test methods should be validated with these factors in mind.

The nature of the challenge microorganism exerts a strong effect upon the response to the antimicrobial agent, and so upon the neutralization required for recovery. Represented among these organisms in compendial tests are Gram-positive bacteria, Gram-negative bacteria, yeasts, and molds. Each organism to be used in the test must be included in the validation.

The preparation of the inoculum of challenge microorganisms also affects the testing of products having antimicrobial

properties. The growth and preparation of the challenge organism determines the physiological state of the cell. This state has a direct influence on the results of any test of antimicrobial efficacy. Microbial tests do not use individual cells; rather, populations of cells are harvested for study. The data generated from these studies are less variable if the cell populations are homogeneous. Liquid cultures or confluent growths on solid medium are best suited for reproducible culture preparation. The conditions of organism preparation and storage must be standardized for the neutralizer evaluation and should reflect the conditions of the antimicrobial assay.

The specific conditions of the test, including buffers used, water, light conditions, and temperature, must be reproduced in the validation study. All test conditions also should be standardized and performed in the validation study exactly as performed in the test.

The conditions of microbial recovery are among the most crucial in accurately estimating the number of microorganisms present in a test solution. The first consideration is the recovery medium used to support the growth of survivors. This concern is discussed in detail below. The second consideration is the incubation conditions. Optimal conditions for growth must be present to ensure complete growth and reproducible results.

METHODS OF NEUTRALIZING ANTIMICROBIAL PROPERTIES

Three common methods are used to neutralize antimicrobial properties of a product: (1) chemical inhibition, (2) dilution, and (3) filtration and washing.

Chemical Inhibition

Table 1 shows known neutralizers for a variety of chemical antimicrobial agents and the reported toxicity of some chemical neutralizers to specific microorganisms. However, despite potential toxicity, the convenience and quick action of chemical inhibitors encourage their use. Chemical inhibition of bactericides is the preferred method for the antimicrobial efficacy test. The potential of chemical inhibitors should be considered in the membrane filtration and the direct transfer sterility tests. Antibiotics may not be susceptible to neutralization by chemical means, but rather by enzymatic treatment (e.g., penicillinase). These enzymes may be used where required.

Dilution

A second approach to neutralizing antimicrobial properties of a product is by dilution, because the concentration of a chemical bactericide exerts a large effect on its potency. The relationship between concentration and antimicrobial effect differs among bactericidal agents but is constant for a

Table 1. Some Common Neutralizers for Chemical Biocides

Neutralizer	Biocide Class	Potential Action of Biocides
Bisulfate	Glutaraldehyde, Mercurials	Non-Sporing Bacteria
Dilution	Phenolics, Alcohol, Aldehydes, Sorbate	—
Glycine	Aldehydes	Growing Cells
Lecithin	Quaternary Ammonium Compounds (QACs), Parabens, Bis-biquanides	Bacteria
Mg ⁺² or Ca ⁺² ions	EDTA	—
Polysorbate	QACS, Iodine, Parabens	—
Thioglycollate	Mercurials	Staphylococci and Spores
Thiosulfate	Mercurials, Halogens, Aldehydes	Staphylococci

particular antimicrobial agent. This relationship is exponential in nature, with the general formula:

$$C \cdot t = k$$

in which C is the concentration; t is the time required to kill a standard inoculum; k is a constant; and the concentration exponent, η , is the slope of the plot of $\log t$ versus $\log C$. Antimicrobial agents with high η values are rapidly neutralized by dilution, whereas those with low η values are not good candidates for neutralization by dilution.

Membrane Filtration

An approach that is often used, especially in sterility testing, is neutralization by membrane filtration. This approach relies upon the physical retention of the microorganism on the membrane filter, with the antimicrobial agent passing through the filter into the filtrate. The filter is then incubated for recovery of viable microorganisms. However, filtration alone may not remove sufficient quantities of the bactericidal agent to allow growth of surviving microorganisms. Adherence of residual antimicrobial agents to the filter membrane may cause growth inhibition. Filtration through a low-binding filter material, such as polyvinylidene difluoride, helps to minimize this growth inhibition. Additionally, the preservative may be diluted or flushed from the filter by rinsing with a benign fluid, such as diluting *Fluid A* (see *Diluting and Rinsing Fluids for Membrane Filtration* under *Sterility Tests* (71) for diluting fluid compositions). Chemical neutralizers in the rinsing fluid can ensure that any antimicrobial residue on the membrane does not interfere with the recovery of viable microorganisms.

VALIDATION OF NEUTRALIZATION METHODS—RECOVERY COMPARISONS

A validated method for neutralizing the antimicrobial properties of a product must meet two criteria: neutralizer efficacy and neutralizer toxicity. The validation study documents that the neutralization method employed is effective in inhibiting the antimicrobial properties of the product (neutralizer efficacy) without impairing the recovery of viable microorganisms (neutralizer toxicity). Validation protocols may meet these two criteria by comparing recovery results for treatment groups.

The first is the test group, in which the product is subjected to the neutralization method, then a low level of challenge microorganism [less than 100 colony-forming units (cfu)] is inoculated for recovery. The second is the peptone control group, in which the neutralization method is used with peptone, or diluting *Fluid A* (see *Sterility Tests* (71)), as the test solution. The third is the viability group, in which the actual inoculum is used without exposure to the neutralization scheme. Similar recovery between the test group and the peptone group demonstrates adequate neutralizer efficacy; similar recovery between the peptone group and the viability group demonstrates adequate neutralizer toxicity.

In principle, the protocol must show that recovery of a low inoculum (less than 100 cfu) is not inhibited by the test sample and the neutralization method. Validation protocols may meet these two criteria by comparing recovery among three distinct test groups: (1) neutralized product with inoculum, (2) challenge inoculum control in buffered solution, and (3) inoculum in the absence of product or neutralizer. This can be established by directly comparing the result in the treated solution (1) to the inoculum (3) above. If the growth on the treated solution is not comparable to the growth on the inoculum group, it should be determined whether the neutralization method itself is toxic to the microorganisms.

Recovery on Agar Medium

In the tests under *Antimicrobial Effectiveness Testing* (51) and *Microbial Enumeration Tests* (61) and *Tests for Specified Microorganisms* (62), the number of viable challenge microorganisms in the product is estimated at various time intervals by calculating the concentration of cfu per mL by the plate count method. A design for validating neutralization would incorporate the treatment groups as described under *Validation of Neutralization Methods—Recovery Comparisons*. At least three independent replicates of the experiment should be performed, and each should demonstrate that the average number of cfu recovered from the challenge product is not less than 70% of that recovered from the inoculum control.

If a greater number of replicates is required in the validation study, the comparisons may be evaluated by transforming the numbers of cfu to their logarithmic values and analyzing the data statistically by the Student t test (pairwise comparisons) or by analysis of variance (ANOVA) (for comparing all groups). If ANOVA is used, and significant differences among the populations are determined, a test such as Dunnett's test may be used, with the peptone group used as the control group.

Recovery by Membrane Filtration

This validation follows the procedure described for *Validation Test* under *Sterility Tests* (71), with the exception of plating on solid medium to quantitate recovery. Three 100-mL rinses are assumed, but the volume and number of rinses are subject to validation. Each validation run should be performed independently at least three times.

In the test solution group, the product is filtered through the membrane filter, followed by two 100-mL portions of diluting-neutralizing fluid. After the second rinse has been filtered, a final 100-mL portion containing less than 100 cfu of the specific challenge microorganism is passed through the filter. This filter is then placed on the appropriate agar recovery medium and incubated for recovery.

The inoculum is directly plated onto the solid medium. It is possible that filtration will lead to reduced recovery of the challenge microorganism, either through inherent toxicity of the membrane or by adherence of the microorganism to the filtration vessel walls. A control group can be used to evaluate this component of membrane filtration validation. Diluting *Fluid A* is used as the dilution medium without exposing the filter to the product. After addition of the low-level inoculum to the final rinse, the filter is plated as above. Technique-specific loss of microorganisms can be estimated by comparing the recovery in the diluting *Fluid A* group to the inoculum count.

It is assumed in this discussion that the test sample can be filtered. If it is necessary to solubilize the test sample, the effects of the solubilization method on viable microorganisms must be determined. This situation can occur when testing ointments, suspensions, or other articles.

The method can be considered validated if the recovery rate in the three independent replicates is similar for the test solution and the diluting *Fluid A* control.

Recovery in Liquid Medium

It is assumed in *Direct Inoculation of the Culture Medium* in the section *Test for Sterility of the Product to be Examined* under *Sterility Tests* (71) that the recovery medium will allow for growth of all surviving microorganisms. The broth in that test must serve both to neutralize any antimicrobial properties of the test solution and to support the growth of the microorganisms. The treatment groups described under *Validation of Neutralization Methods—Recovery Comparisons* above can be used for validation of the recovery method,

with the proportions of product and recovery medium varied to achieve adequate neutralization. The method can be considered validated if all groups show copious growth within 7 days for all microorganisms.

RECOVERY OF INJURED MICROORGANISMS

The validation studies described above use challenge microorganisms that have never been exposed to antimicrobial agents, and thus are not identical to organisms seen in antimicrobial effectiveness testing or when a sterility test is performed on a preserved product. If the use of alternative media is desired, the recovery of injured microorganisms should be addressed in the validation study. This may be done by directly comparing the recovery of each challenge microorganism on the preferred medium and on the alternative medium, after exposure to the product. This exposure should include at least two time periods showing survival of less than 100 cfu per mL, unless the rate of kill of the antimicrobial agent is such that no recovery is possible even if the microorganism is plated within minutes of exposure. This comparison should be performed at least three times. The alternative medium is validated if the recovery seen on that medium is no less than that seen on the preferred medium, within an error of 0.5 log units.

ESTIMATING THE NUMBER OF COLONY-FORMING UNITS

The accuracy of any estimate of viable cfu is affected by the number plated. As the number of viable cells plated increases, crowding effects decrease the accuracy of the count, reducing the estimate. As the number decreases, random error plays an increasing role in the estimate.

The accepted range for countable colonies on a standard agar plate is between 25 and 250 for most bacteria and *Candida albicans*. This range was established in the food industry for counting coliform bacteria in milk. This range is acceptable for compendial organisms, except for fungi. It is not optimal for counting all environmental isolates. The recommended counting range for *Aspergillus niger* is between 8 and 80 cfu per plate. The use of membrane filtration to recover challenge microorganisms, or the use of environmental isolates as challenge microorganisms in antimicrobial effectiveness testing, requires validation of the countable range. This validation may be performed by statistical comparison of estimated cfu from successive pairs in a dilution series. Prepare a suspension so that plating will provide approximately 1000 cfu per plate, and then dilute twofold to a theoretical concentration of approximately 1 cfu per plate. Plate all dilutions in the series in duplicate, and incubate for recovery under the conditions of the *Antimicrobial Effectiveness Testing* 〈51〉. Compare the estimates of cfu per mL from paired tubes in the dilution series by the formula:

$$\frac{|2L_{cfu} - H_{cfu}|}{\sqrt{2L_{cfu} + H_{cfu}}} \leq 1.96$$

in which L_{cfu} is the number of colonies on the plate with the lower count (greater dilution), and H_{cfu} is the number of colonies on the plate with the higher count (lesser dilution). The estimates of the cfu per mL provided by L_{cfu} and H_{cfu} should agree within the limits of the formula with a critical value of 1.96. The upper limit of plate counts is then defined as the number (H_{cfu}) that reproducibly passes this test. This study should be independently repeated a sufficient number of times to establish an upper limit of cfu for the particular plating conditions.

There is a lower limit at which the ability of the antimicrobial effectiveness test to recover microorganisms becomes untenable. If the first plating is performed with 1 mL of a 10^{-1} dilution, cfu in the range of 1 to 10 per mL would not

be seen. On this dilution plating, only the lower number of cfu may be reduced to 3, allowing as few survivors as 30 cfu per mL to be reported.

Lower counting thresholds for the greatest dilution plating in series must be justified. Numbers of colonies on a plate follow the Poisson distribution, so the variance of the mean value equals the mean value of counts. Therefore, as the mean number of cfu per plate becomes lower, the percentage error of the estimate increases (see Table 2). Three cfu per plate at the 10^{-1} dilution provide an estimate of 30 cfu per mL, with an error of 58% of the estimate.

Table 2. Error as a Percentage of Mean for Plate Counts

cfu per Plate	Standard Error	Error as % of Mean
30	5.48	18.3
29	5.39	18.6
28	5.29	18.9
27	5.20	19.2
26	5.10	19.6
25	5.00	20.0
24	4.90	20.4
23	4.80	20.9
22	4.69	21.3
21	4.58	21.8
20	4.47	22.4
19	4.36	22.9
18	4.24	23.6
17	4.12	24.3
16	4.00	25.0
15	3.87	25.8
14	3.74	26.7
13	3.61	27.7
12	3.46	28.9
11	3.32	30.2
10	3.16	31.6
9	3.00	33.3
8	2.83	35.4
7	2.65	37.8
6	2.45	40.8
5	2.24	44.7
4	2.00	50.0
3	1.73	57.7
2	1.41	70.7
1	1.00	100.0

〈1230〉 WATER FOR HEMODIALYSIS APPLICATIONS

GENERAL PURIFICATION CONSIDERATIONS

Chemical and microbial components that can be found in drinking water meeting U.S. Environmental Protection Agency National Primary Drinking Water Regulations (or equivalent) may have the potential to produce significant negative effects in patients undergoing hemodialysis. It is, therefore, necessary to subject the water to further treatment to reduce these components to acceptable levels. The

Water for Hemodialysis monograph provides bacterial and chemical tests that are required to ensure patient safety. Additional testing is recommended as follows:

- (1) Excess levels of aluminum, fluorides, and chlorine may be found seasonally in drinking water as a result of chemicals used in water treatment. These components should be monitored in Water for Hemodialysis being produced in accordance with established standard operating procedures. The maximum acceptable levels of these and other elements and compounds, as proposed by AAMI (Association for the Advancement of Medical Instrumentation) are listed in Table 1. These attributes should be periodically monitored to ensure they are being controlled by the routine testing performed in accordance with the Water for Hemodialysis monograph.
- (2) A comprehensive validation testing of the system producing Water for Hemodialysis should be performed initially and periodically thereafter to ensure that the water treatment equipment and system sanitization processes are functioning properly.

Table 1. Maximum Allowable Chemical Levels in Water for Hemodialysis	
(water used to prepare dialysate and concentrates from powder at a dialysis facility and to reprocess dialyzers for multiple use)*	
Element or Compound	Maximum Concentration (mg/L)
Contaminants with documented toxicity in hemodialysis	
Aluminum	0.01
Chloramines	0.1
Free chlorine	0.5
Copper	0.1
Fluoride	0.2
Lead	0.005
Nitrate (as N)	2
Sulfate	100
Zinc	0.1
Contaminants normally included in dialysate	
Calcium	2 (0.1 mEq/L)
Magnesium	4 (0.3 mEq/L)
Potassium	8 (0.2 mEq/L)
Sodium	70 (3.0 mEq/L)
Other contaminants	
Antimony	0.006
Arsenic	0.005
Barium	0.1
Beryllium	0.0004
Cadmium	0.001
Chromium	0.014
Mercury	0.0002
Selenium	0.09
Silver	0.005
Thallium	0.002

* Reprinted with permission from ANSI/AAMI RD62: 2006, "Water treatment equipment for hemodialysis applications", ©Association for the Advancement of Medical Instrumentation, Arlington, VA.

The chemical limits included in Table 1 have been recognized by federal government agencies as standards for Water for Hemodialysis. Written standard operating procedures for water testing should be established by the physician in charge or the designated facility manager. The test frequency decision should be based upon historical data analysis, the quality of the source water as reported by the

municipal water treatment facility or public health agency in the area, etc. Records should be maintained to document levels and any necessary remedial action taken promptly.

Chemical analysis of water components listed should be performed using methods referenced in the American Public Health Association's *Standard Methods for the Examination of Water and Wastewater*, 21st Edition,¹ those referenced in the U.S. Environmental Protection Agency's *Methods for the Determination of Metals in Environmental Samples*,² or equivalent methods as referenced in ANSI/AAMI RD 62:2006.

MICROBIAL CONSIDERATIONS

The Water for Hemodialysis monograph includes total aerobic microbial count (TAMC) limits of 100 cfu/mL and endotoxin limits of 1 USP Endotoxin Unit/mL. In addition, the absence of *Pseudomonas aeruginosa* should be routinely determined because this is an opportunistic pathogen hazardous to acutely ill hemodialysis patients. Both the high microbial counts and the presence of *Pseudomonas aeruginosa* can be associated with inadequate water system maintenance and sanitization. Sampling the water should be done at all use points where the water enters the dialysis equipment. Samples should be assayed within 30 minutes of collection or immediately refrigerated and then assayed within 24 hours of collection. The microbial enumeration and absence tests are performed using procedures found in the USP general test chapters *Microbial Enumeration Tests* (61) and *Tests for Specified Microorganisms* (62). Quantification of bacterial endotoxins is performed using procedures found in the USP general test chapter *Bacterial Endotoxins Test* (85).

Because of the incubation time required to obtain definitive microbiological results, water systems should be microbiologically monitored to confirm that they continue to produce water of acceptable quality. "Alert" and "Action Levels" are therefore necessary for the monitoring and control of the system. An Alert Level constitutes a warning and does not require a corrective action. An Action Level indicates a drift from normal operating conditions and requires that corrective action be taken to bring the process back into the normal operating range. Exceeding an Alert or Action Level does not imply that water quality has been compromised. The maximum recommended Action Level for a total viable microbial count in the product water should be no greater than 25 cfu/mL, and the maximum recommended Action Level for bacterial endotoxins should be no greater than 0.25 USP Endotoxin Unit/mL. As with all process control values, Action and Alert Levels should be established from normal system monitoring trends and process capabilities in a fashion that allows remedial actions to occur in response to process control level excursions well before specifications are exceeded (also see *Microbial Considerations* under Water for Pharmaceutical Purposes (1231)).

(1231) WATER FOR PHARMACEUTICAL PURPOSES

INTRODUCTION

Water is widely used as a raw material, ingredient, and solvent in the processing, formulation, and manufacture of

¹ American Public Health Association, Washington, DC 20005.
² U.S. Environmental Protection Agency Publication EPA-600-R-94-111, Cincinnati, OH.

pharmaceutical products, active pharmaceutical ingredients (APIs) and intermediates, compendial articles, and analytical reagents. This general information chapter provides additional information about water, its quality attributes that are not included within a water monograph, processing techniques that can be used to improve water quality, and a description of minimum water quality standards that should be considered when selecting a water source.

This information chapter is not intended to replace existing regulations or guides that already exist to cover USA and International (ICH or WHO) GMP issues, engineering guides, or other regulatory (FDA, EPA, or WHO) guidances for water. The contents will help users to better understand pharmaceutical water issues and some of the microbiological and chemical concerns unique to water. This chapter is not an all-inclusive writing on pharmaceutical waters. It contains points that are basic information to be considered, when appropriate, for the processing, holding, and use of water. It is the user's responsibility to assure that pharmaceutical water and its production meet applicable governmental regulations, guidances, and the compendial specifications for the types of water used in compendial articles.

Control of the chemical purity of these waters is important and is the main purpose of the monographs in this compendium. Unlike other official articles, the bulk water monographs (*Purified Water* and *Water for Injection*) also limit how the article can be produced because of the belief that the nature and robustness of the purification process is directly related to the resulting purity. The chemical attributes listed in these monographs should be considered as a set of minimum specifications. More stringent specifications may be needed for some applications to ensure suitability for particular uses. Basic guidance on the appropriate applications of these waters is found in the monographs and is further explained in this chapter.

Control of the microbiological quality of water is important for many of its uses. Most packaged forms of water that have monograph standards are required to be sterile because some of their intended uses require this attribute for health and safety reasons. USP has determined that a microbial specification for the bulk monographed waters is inappropriate, and it has not been included within the monographs for these waters. These waters can be used in a variety of applications, some requiring extreme microbiological control and others requiring none. The needed microbial specification for a given bulk water depends upon its use. A single specification for this difficult-to-control attribute would unnecessarily burden some water users with irrelevant specifications and testing. However, some applications may require even more careful microbial control to avoid the proliferation of microorganisms ubiquitous to water during the purification, storage, and distribution of this substance. A microbial specification would also be inappropriate when related to the "utility" or continuous supply nature of this raw material. Microbial specifications are typically assessed by test methods that take at least 48 to 72 hours to generate results. Because pharmaceutical waters are generally produced by continuous processes and used in products and manufacturing processes soon after generation, the water is likely to have been used well before definitive test results are available. Failure to meet a compendial specification would require investigating the impact and making a pass/fail decision on all product lots between the previous sampling's acceptable test result and a subsequent sampling's acceptable test result. The technical and logistical problems created by a delay in the result of such an analysis do not eliminate the user's need for microbial specifications. Therefore, such water systems need to be operated and maintained in a controlled manner that requires that the system be validated to provide assurance of operational stability and that its microbial attributes be quantitatively monitored against established alert and action levels that would provide an early indication of system control. The issues of water system validation and alert/action levels and specifications are included in this chapter.

SOURCE OR FEED WATER CONSIDERATIONS

To ensure adherence to certain minimal chemical and microbiological quality standards, water used in the production of drug substances or as source or feed water for the preparation of the various types of purified waters must meet the requirements of the National Primary Drinking Water Regulations (NPDWR) (40 CFR 141) issued by the U.S. Environmental Protection Agency (EPA) or the drinking water regulations of the European Union or Japan, or the WHO drinking water guidelines. Limits on the types and quantities of certain organic and inorganic contaminants ensure that the water will contain only small, safe quantities of potentially objectionable chemical species. Therefore, water pretreatment systems will only be challenged to remove small quantities of these potentially difficult-to-remove chemicals. Also, control of objectionable chemical contaminants at the source-water stage eliminates the need to specifically test for some of them (e.g., trihalomethanes and heavy metals) after the water has been further purified.

Microbiological requirements of drinking water ensure the absence of coliforms, which, if determined to be of fecal origin, may indicate the potential presence of other potentially pathogenic microorganisms and viruses of fecal origin. Meeting these microbiological requirements does not rule out the presence of other microorganisms, which could be considered undesirable if found in a drug substance or formulated product.

To accomplish microbial control, municipal water authorities add disinfectants to drinking water. Chlorine-containing and other oxidizing substances have been used for many decades for this purpose and have generally been considered to be relatively innocuous to humans. However, these oxidants can interact with naturally occurring organic matter to produce disinfection by-products (DBPs), such as trihalomethanes (THMs, including chloroform, bromodichloromethane, and dibromochloromethane) and haloacetic acids (HAAs, including dichloroacetic acid and trichloroacetic acid). The levels of DBPs produced vary with the level and type of disinfectant used and the levels and types of organic materials found in the water, which can vary seasonally.

Because high levels of DBPs are considered a health hazard in drinking water, drinking water regulations mandate their control to generally accepted nonhazardous levels. However, depending on the unit operations used for further water purification, a small fraction of the DBPs in the starting water may carry over to the finished water. Therefore, the importance of having minimal levels of DBPs in the starting water, while achieving effective disinfection, is important.

DBP levels in drinking water can be minimized by using disinfectants such as ozone, chloramines, or chlorine dioxide. Like chlorine, their oxidative properties are sufficient to damage some pretreatment unit operations and must be removed early in the pretreatment process. The complete removal of some of these disinfectants can be problematic. For example, chloramines may degrade during the disinfection process or during pretreatment removal, thereby releasing ammonia, which in turn can carry over to the finished water. Pretreatment unit operations must be designed and operated to adequately remove the disinfectant, drinking water DBPs, and objectionable disinfectant degradants. A serious problem can occur if unit operations designed to remove chlorine were, without warning, challenged with chloramine-containing drinking water from a municipality that had been mandated to cease use of chlorine disinfection to comply with ever tightening EPA Drinking Water THM specifications. The dechlorination process might incompletely remove the chloramine, which could irreparably damage downstream unit operations, but also the release of ammonia during this process might carry through pretreatment and prevent the finished water from passing compendial conductivity specifications. The purification process must be

reassessed if the drinking water disinfectant is changed, emphasizing the need for a good working relationship between the pharmaceutical water manufacturer and the drinking water provider.

TYPES OF WATER

There are many different grades of water used for pharmaceutical purposes. Several are described in *USP* monographs that specify uses, acceptable methods of preparation, and quality attributes. These waters can be divided into two general types: bulk waters, which are typically produced on site where they are used; and sterile waters, which are produced, packaged, and sterilized to preserve microbial quality throughout their packaged shelf life. There are several specialized types of sterile waters, differing in their designated applications, packaging limitations, and other quality attributes.

There are also other types of water for which there are no monographs. These are all bulk waters, with names given for descriptive purposes only. Many of these waters are used in specific analytical methods. The associated text may not specify or imply certain quality attributes or modes of preparation. These nonmonographed waters may not necessarily adhere strictly to the stated or implied modes of preparation or attributes. Waters produced by other means or controlled by other test attributes may equally satisfy the intended uses for these waters. It is the user's responsibility to ensure that such waters, even if produced and controlled exactly as stated, be suitable for their intended use. Wherever the term "water" is used within these compendia without other descriptive adjectives or clauses, the intent is that water of no less purity than *Purified Water* be used.

What follows is a brief description of the various types of pharmaceutical waters and their significant uses or attributes. *Figure 1* may also be helpful in understanding some of the various types of waters.

Bulk Monographed Waters and Steam

The following waters are typically produced in large volume by a multiple-unit operation water system and distributed by a piping system for use at the same site. These particular pharmaceutical waters must meet the quality attributes as specified in the related monographs.

Purified Water—*Purified Water* (see the *USP* monograph) is used as an excipient in the production of nonparenteral preparations and in other pharmaceutical applications, such as cleaning of certain equipment and nonparenteral product-contact components. Unless otherwise specified, *Purified Water* is also to be used for all tests and assays for which water is indicated (see *General Notices and Requirements*). *Purified Water* is also referenced throughout the *USP-NF*. Regardless of the font and letter case used in its spelling, water complying with the *Purified Water* monograph is intended. *Purified Water* must meet the requirements for ionic and organic chemical purity and must be protected from microbial contamination. The minimal quality of source or feed water for the production of *Purified Water* is *Drinking Water*. This source water may be purified using unit operations that include deionization, distillation, ion exchange, reverse osmosis, filtration, or other suitable purification procedures. Purified water systems must be validated to reliably and consistently produce and distribute water of acceptable chemical and microbiological quality. Purified water systems that function under ambient conditions are particularly susceptible to the establishment of tenacious biofilms of microorganisms, which can be the source of undesirable levels of viable microorganisms or endotoxins in the effluent water. These systems require frequent sanitization and microbiological monitoring to ensure water of appropriate microbiological quality at the points of use.

The *Purified Water* monograph also allows bulk packaging for commercial use elsewhere. In contrast to *Sterile Purified Water*, bulk packaged *Purified Water* is not required to be sterile. Because there is potential for microbial contamination and other quality changes in this bulk packaged non-sterile water, this form of *Purified Water* should be prepared and stored in a fashion that limits microbial growth and/or is simply used in a timely fashion before microbial proliferation renders it unsuitable for its intended use. Also depending on the material used for packaging, there could be extractable compounds leaching into the water from the packaging. Though this article is required to meet the same chemical purity limits as the bulk water, packaging extractables will render the packaged water less pure than the bulk water. The nature of these impurities may even render the water an inappropriate choice for some applications. It is the user's responsibility to ensure fitness for use of this packaged article when used in manufacturing, clinical, or analytical applications where the pure bulk form of the water is indicated.

Water for Injection—*Water for Injection* (see the *USP* monograph) is used as an excipient in the production of parenteral and other preparations where product endotoxin content must be controlled, and in other pharmaceutical applications, such as cleaning of certain equipment and parenteral product-contact components. The minimum quality of source or feed water for the generation of *Water for Injection* is *Drinking Water* as defined by the U.S. Environmental Protection Agency (EPA), EU, Japan, or WHO. This source water may be pretreated to render it suitable for subsequent distillation (or whatever other validated process is used according to the monograph). The finished water must meet all of the chemical requirements for *Purified Water* as well as an additional bacterial endotoxin specification. Because endotoxins are produced by the kinds of microorganisms that are prone to inhabit water, the equipment and procedures used by the system to purify, store, and distribute *Water for Injection* must be designed to minimize or prevent microbial contamination as well as remove incoming endotoxins from the starting water. *Water for Injection* systems must be validated to reliably and consistently produce and distribute this quality of water.

The *Water for Injection* monograph also allows bulk packaging for commercial use. In contrast to *Sterile Water for Injection*, bulk packaged *Water for Injection* is not required to be sterile. However, to preclude significant changes in its microbial and endotoxins content during storage, this form of *Water for Injection* should be prepared and stored in a fashion that limits microbial growth and/or is simply used in a timely fashion before microbial proliferation renders it unsuitable for its intended use. Also depending on the material used for packaging, there could be extractable compounds leaching into the water from the packaging. Though this article is required to meet the same chemical purity limits as the bulk water, packaging extractables will render the packaged water less pure than the bulk water. The nature of these impurities may even render the water an inappropriate choice for some applications. It is the user's responsibility to ensure fitness for use of this packaged article when used in manufacturing, clinical, or analytical applications where the purer bulk form of the water is indicated.

Water for Hemodialysis—*Water for Hemodialysis* (see the *USP* monograph) is used for hemodialysis applications, primarily the dilution of hemodialysis concentrate solutions. It is produced and used on site and is made from EPA *Drinking Water* that has been further purified to reduce chemical and microbiological components. It may be packaged and stored in unreactive containers that preclude bacterial entry. The term "unreactive containers" implies that the container, especially its water contact surfaces, is not changed in any way by the water, such as by leaching of container-related compounds into the water or by any chemical reaction or corrosion caused by the water. The water contains no added antimicrobials and is not intended for injection. Its

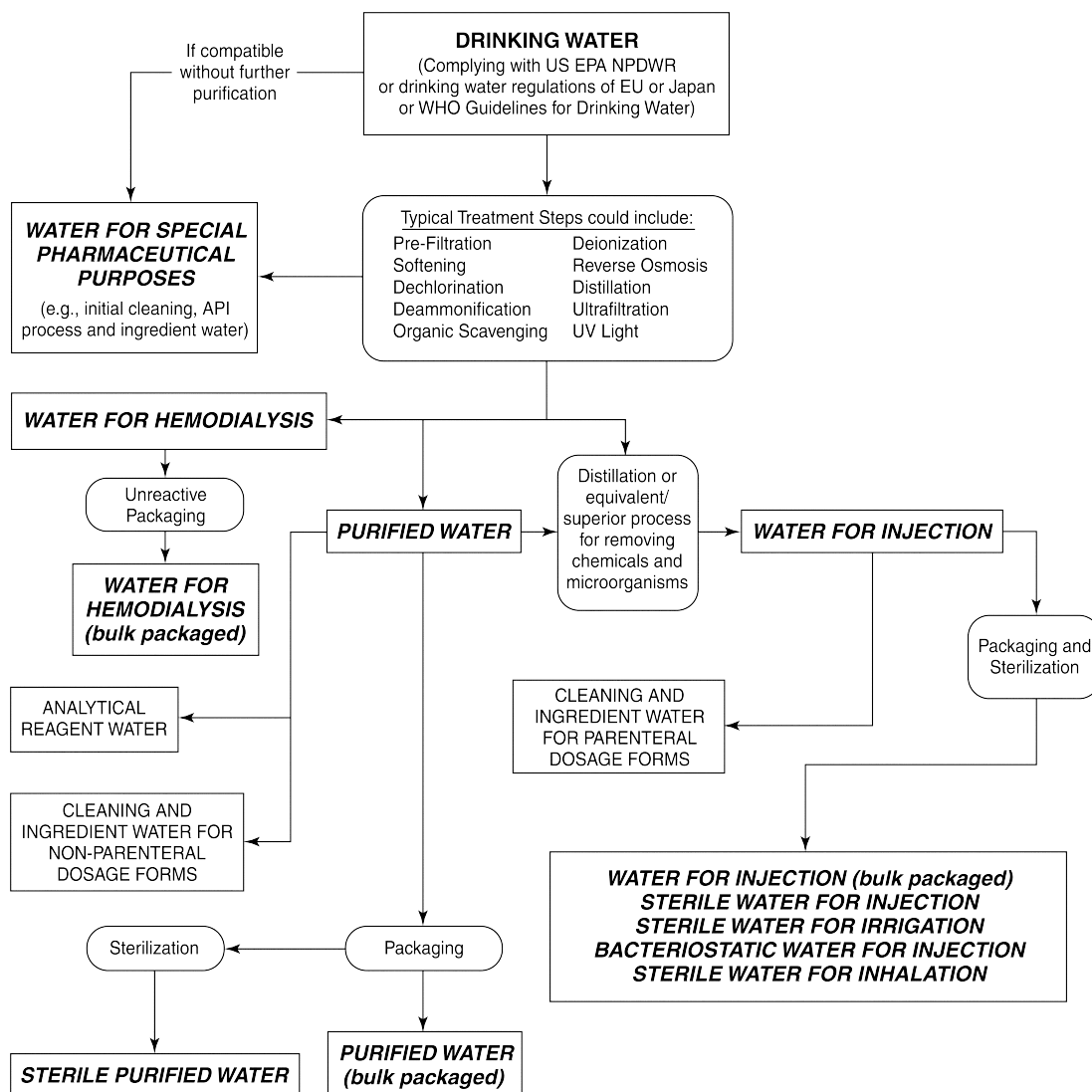


Figure 1. Water for pharmaceutical purposes.

attributes include specifications for *Water conductivity*, *Total organic carbon* (or oxidizable substances), *Microbial limits*, and *Bacterial endotoxins*. The *Water conductivity* and *Total organic carbon* attributes are identical to those established for *Purified Water* and *Water for Injection*; however, instead of total organic carbon, the organic content may alternatively be measured by the test for *Oxidizable substances*. The *Microbial limits* attribute for this water is unique among the "bulk" water monographs, but is justified on the basis of this water's specific application that has microbial content requirements related to its safe use. The *Bacterial endotoxins* attribute is likewise established at a level related to its safe use.

Pure Steam—*Pure Steam* (see the USP monograph) is also sometimes referred to as "clean steam". It is used where the steam or its condensate would directly contact official articles or article-contact surfaces, such as during their preparation, sterilization, or cleaning where no subse-

quent processing step is used to remove any codeposited impurity residues. These *Pure Steam* applications include but are not limited to porous load sterilization processes, product or cleaning solutions heated by direct steam injection, or humidification of processes where steam injection is used to control the humidity inside processing vessels where the official articles or their in-process forms are exposed. The primary intent of using this quality of steam is to ensure that official articles or article-contact surfaces exposed to it are not contaminated by residues within the steam.

Pure Steam is prepared from suitably pretreated source water analogously to either the pretreatment used for *Purified Water* or *Water for Injection*. The water is vaporized with suitable mist elimination, and distributed under pressure. The sources of undesirable contaminants within *Pure Steam* could arise from entrained source water droplets, anticorrosion steam additives, or residues from the steam production and distribution system itself. The attributes in the *Pure*

Steam monograph should detect most of the contaminants that could arise from these sources. If the official article exposed to potential *Pure Steam* residues is intended for parenteral use or other applications where the pyrogenic content must be controlled, the *Pure Steam* must additionally meet the specification for *Bacterial Endotoxins* (85).

These purity attributes are measured on the condensate of the article, rather than the article itself. This, of course, imparts great importance to the cleanliness of the *Pure Steam* condensate generation and collection process because it must not adversely impact the quality of the resulting condensed fluid.

Other steam attributes not detailed in the monograph, in particular, the presence of even small quantities of noncondensable gases or the existence of a superheated or dry state, may also be important for applications such as sterilization. The large release of energy (latent heat of condensation) as water changes from the gaseous to the liquid state is the key to steam's sterilization efficacy and its efficiency, in general, as a heat transfer agent. If this phase change (condensation) is not allowed to happen because the steam is extremely hot and in a persistent superheated, dry state, then its usefulness could be seriously compromised. Noncondensable gases in steam tend to stratify or collect in certain areas of a steam sterilization chamber or its load. These surfaces would thereby be at least partially insulated from the steam condensation phenomenon, preventing them from experiencing the full energy of the sterilizing conditions. Therefore, control of these kinds of steam attributes, in addition to its chemical purity, may also be important for certain *Pure Steam* applications. However, because these additional attributes are use-specific, they are not mentioned in the *Pure Steam* monograph.

Note that less pure "plant steam" may be used for steam sterilization of nonproduct contact nonporous loads, for general cleaning of nonproduct contact equipment, as a nonproduct contact heat exchange medium, and in all compatible applications involved in bulk pharmaceutical chemical and API manufacture.

Finally, owing to the lethal properties of *Pure Steam*, monitoring of microbial control within a steam system is unnecessary. Therefore, microbial analysis of the steam condensate is unnecessary.

Sterile Monographed Waters

The following monographed waters are packaged forms of either *Purified Water* or *Water for Injection* that have been sterilized to preserve their microbiological properties. These waters may have specific intended uses as indicated by their names and may also have restrictions on packaging configurations related to those uses. In general, these sterile packaged waters may be used in a variety of applications in lieu of the bulk forms of water from which they were derived. However, there is a marked contrast between the quality tests and purities for these bulk versus sterile packaged waters. These quality tests and specifications for sterile packaged waters have diverged from those of bulk waters to accommodate a wide variety of packaging types, properties, volumes, and uses. As a result, the inorganic and organic impurity specifications and levels of the bulk and sterile packaged forms of water are not equivalent as their name similarities imply. The packaging materials and elastomeric closures are the primary sources of these impurities, which tend to increase over these packaged articles' shelf lives. Therefore, due consideration must be given to the chemical purity suitability at the time of use of the sterile packaged forms of water when used in manufacturing, analytical, and cleaning applications in lieu of the bulk waters from which these waters were derived. It is the user's responsibility to ensure fitness for use of these sterile packaged waters in these applications. Nevertheless, for the applications discussed below for each sterile packaged water, their respec-

tive purities and packaging restrictions generally render them suitable by definition.

Sterile Purified Water—*Sterile Purified Water* (see the *USP* monograph) is *Purified Water*, packaged and rendered sterile. It is used in the preparation of nonparenteral compendial dosage forms or in analytical applications requiring *Purified Water* where access to a validated *Purified Water* system is not practical, where only a relatively small quantity is needed, where *Sterile Purified Water* is required, or where bulk packaged *Purified Water* is not suitably microbiologically controlled.

Sterile Water for Injection—*Sterile Water for Injection* (see the *USP* monograph) is *Water for Injection* packaged and rendered sterile. It is used for extemporaneous prescription compounding and as a sterile diluent for parenteral products. It may also be used for other applications where bulk *Water for Injection* or *Purified Water* is indicated but where access to a validated water system is either not practical or where only a relatively small quantity is needed. *Sterile Water for Injection* is packaged in single-dose containers not larger than 1 L in size.

Bacteriostatic Water for Injection—*Bacteriostatic Water for Injection* (see the *USP* monograph) is sterile *Water for Injection* to which has been added one or more suitable antimicrobial preservatives. It is intended to be used as a diluent in the preparation of parenteral products, most typically for multi-dose products that require repeated content withdrawals. It may be packaged in single-dose or multiple-dose containers not larger than 30 mL.

Sterile Water for Irrigation—*Sterile Water for Irrigation* (see the *USP* monograph) is *Water for Injection* packaged and sterilized in single-dose containers of larger than 1 L in size that allows rapid delivery of its contents. It need not meet the requirement under small-volume injections in the general test chapter *Particulate Matter in Injections* (788). It may also be used in other applications that do not have particulate matter specifications, where bulk *Water for Injection* or *Purified Water* is indicated but where access to a validated water system is not practical, or where somewhat larger quantities than are provided as *Sterile Water for Injection* are needed.

Sterile Water for Inhalation—*Sterile Water for Inhalation* (see the *USP* monograph) is *Water for Injection* that is packaged and rendered sterile and is intended for use in inhalators and in the preparation of inhalation solutions. It carries a less stringent specification for bacterial endotoxins than *Sterile Water for Injection* and therefore is not suitable for parenteral applications.

Nonmonographed Manufacturing Waters

In addition to the bulk monographed waters described above, nonmonographed waters can also be used in pharmaceutical processing steps such as cleaning, synthetic steps, or a starting material for further purification. The following is a description of several of these nonmonographed waters as cited in various locations within these compendia.

Drinking Water—This type of water can be referred to as Potable Water (meaning drinkable or fit to drink), National Primary Drinking Water, Primary Drinking Water, or National Drinking Water. Except where a singular drinking water specification is stated (such as the NPDWR [U.S. Environmental Protection Agency's National Primary Drinking Water Regulations as cited in 40 CFR Part 141]), this water must comply with the quality attributes of either the NPDWR, or the drinking water regulations of the European Union or Japan, or the WHO Drinking Water Guidelines. It may be derived from a variety of sources including a public water utility, a private water supply (e.g., a well), or a combination of these sources. *Drinking Water* may be used in the early stages of cleaning pharmaceutical manufacturing equipment and product-contact components. *Drinking Water* is also the minimum quality of water that should be



* Drinking water is water complying with US EPA NPDRW or drinking water regulations of EU or Japan or WHO drinking water guidelines.

** Water for sterile APIs or dosage forms must first be rendered sterile if there is not a subsequent sterilization step in the process where used.

*** See guidance in this chapter where waters other than purified water are required by some USP tests and assays.

Note: All water systems should be validated with whatever microbial control is needed to suit the intended purposes of the water.

Figure 2. Selection of water for pharmaceutical purposes.

used for the preparation of official substances and other bulk pharmaceutical ingredients. Where compatible with the processes, the allowed contaminant levels in *Drinking Water* are generally considered safe for use for official substances and other drug substances. Where required by the processing of the materials to achieve their required final purity, higher qualities of water may be needed for these manufacturing steps, perhaps even as pure as *Water for Injection* or *Purified Water*. Such higher purity waters, however, might require only selected attributes to be of higher purity than *Drinking Water* (see Figure 2). *Drinking Water* is the prescribed source or feed water for the production of bulk monographed pharmaceutical waters. The use of *Drinking Water* specifications establishes a reasonable set of maximum allowable levels of chemical and microbiological contaminants with which a water purification system will be challenged. As seasonal variations in the quality attributes of the *Drinking Water* supply can occur, due consideration to its synthetic and cleaning uses must be given. The processing steps in the production of pharmaceutical waters must be designed to accommodate this variability.

Hot Purified Water—This water is used in the preparation instructions for *USP–NF* articles and is clearly intended to be *Purified Water* that has been heated to an unspecified

temperature in order to enhance solubilization of other ingredients. There is no upper temperature limit for the water (other than being less than 100°), but for each monograph there is an implied lower limit below which the desired solubilization effect would not occur.

Nonmonographed Analytical Waters

Both *General Notices and Requirements* and the introductory section to *Reagents, Indicators, and Solutions* clearly state that where the term “water,” without qualification or other specification, is indicated for use in analyses, the quality of water shall be *Purified Water*. However, numerous such qualifications do exist. Some of these qualifications involve methods of preparation, ranging from specifying the primary purification step to specifying additional purification. Other qualifications call for specific attributes to be met that might otherwise interfere with analytical processes. In most of these latter cases, the required attribute is not specifically tested. Rather, a further “purification process” is specified that ostensibly allows the water to adequately meet this required attribute.

However, preparation instructions for many reagents were carried forward from the innovator’s laboratories to the orig-

inally introduced monograph for a particular *USP–NF* article or general test chapter. The quality of the reagent water described in these tests may reflect the water quality designation of the innovator's laboratory. These specific water designations may have originated without the innovator's awareness of the requirement for *Purified Water* in *USP–NF* tests. Regardless of the original reason for the creation of these numerous special analytical waters, it is possible that the attributes of these special waters could now be met by the basic preparation steps and current specifications of *Purified Water*. In some cases, however, some of the cited post-processing steps are still necessary to reliably achieve the required attributes.

Users are not obligated to employ specific and perhaps archaically generated forms of analytical water where alternatives with equal or better quality, availability, or analytical performance may exist. The consistency and reliability for producing these alternative analytical waters should be verified as producing the desired attributes. In addition, any alternative analytical water must be evaluated on an application-by-application basis by the user to ensure its suitability. Following is a summary of the various types of nonmonographed analytical waters that are cited in the *USP–NF*.

Distilled Water—This water is produced by vaporizing liquid water and condensing it in a purer state. It is used primarily as a solvent for reagent preparation, but it is also specified in the execution of other aspects of tests, such as for rinsing an analyte, transferring a test material as a slurry, as a calibration standard or analytical blank, and for test apparatus cleaning. It is also cited as the starting water to be used for making *High-Purity Water*. Because none of the cited uses of this water imply a need for a particular purity attribute that can only be derived by distillation, water meeting the requirements for *Purified Water* derived by other means of purification could be equally suitable where *Distilled Water* is specified.

Freshly Distilled Water—Also called “recently distilled water”, it is produced in a similar fashion to *Distilled Water* and should be used shortly after its generation. This implies the need to avoid endotoxin contamination as well as any other adventitious forms of contamination from the air or containers that could arise with prolonged storage. It is used for preparing solutions for subcutaneous test animal injections as well as for a reagent solvent in tests for which there appears to be no particularly high water purity needed that could be ascribable to being “freshly distilled”. In the test animal use, the term “freshly distilled” and its testing use imply a chemical, endotoxin, and microbiological purity that could be equally satisfied by *Water for Injection* (though no reference is made to these chemical, endotoxin, or microbial attributes or specific protection from recontamination). For nonanimal uses, water meeting the requirements for *Purified Water* derived by other means of purification and/or storage periods could be equally suitable where “recently distilled water” or *Freshly Distilled Water* is specified.

Deionized Water—This water is produced by an ion-exchange process in which the contaminating ions are replaced with either H^+ or OH^- ions. Similarly to *Distilled Water*, *Deionized Water* is used primarily as a solvent for reagent preparation, but it is also specified in the execution of other aspects of tests, such as for transferring an analyte within a test procedure, as a calibration standard or analytical blank, and for test apparatus cleaning. Also, none of the cited uses of this water imply any needed purity attribute that can only be achieved by deionization. Therefore, water meeting the requirements for *Purified Water* that is derived by other means of purification could be equally suitable where *Deionized Water* is specified.

Freshly Deionized Water—This water is prepared in a similar fashion to *Deionized Water*, though as the name suggests, it is to be used shortly after its production. This implies the need to avoid any adventitious contamination that could occur upon storage. This water is indicated for use as

a reagent solvent as well as for cleaning. Due to the nature of the testing, *Purified Water* could be a reasonable alternative for these applications.

Deionized Distilled Water—This water is produced by deionizing (see *Deionized Water*) *Distilled Water*. This water is used as a reagent in a liquid chromatography test that requires a high purity. Because of the importance of this high purity, water that barely meets the requirements for *Purified Water* may not be acceptable. *High-Purity Water* (see below) could be a reasonable alternative for this water.

Filtered Water—This water is *Purified Water* that has been filtered to remove particles that could interfere with the analysis where this water is specified. It is sometimes used synonymously with *Particle-Free Water* and *Ultra-Filtered Water* and is cited in some monographs and general chapters as well as in *Reagents*. Depending on its location, it is variously defined as water that has been passed through filters rated as 1.2- μm , 0.22- μm , or 0.2- μm ; or unspecified pore size. Even though the water names and the filter pore sizes used to produce these waters are inconsistently defined, the use of 0.2- μm pore size filtered *Purified Water* should be universally acceptable for all applications where *Particle-Free Water*, *Filtered Water*, or *Ultra-Filtered Water* are specified.

High-Purity Water—The preparation of this water is defined in *Containers—Glass* (660). It is water that may be prepared by deionizing previously distilled water, and then filtering it through a 0.45- μm rated membrane. This water must have an in-line conductivity of not greater than 0.15 $\mu S/cm$ (not less than 6.67 Megohm-cm) at 25°. For the sake of purity comparison, the analogous Stage 1 and 2 conductivity requirements for *Purified Water* at the same temperature are 1.3 $\mu S/cm$ and 2.1 $\mu S/cm$, respectively. The preparation specified in *Containers—Glass* (660) uses materials that are highly efficient deionizers and that do not contribute copper ions or organics to the water, assuring a very high quality water. If the water of this purity contacts the atmosphere even briefly as it is being used or drawn from its purification system, its conductivity will immediately degrade, by as much as about 1.0 $\mu S/cm$, as atmospheric carbon dioxide dissolves in the water and equilibrates to bicarbonate ions. Therefore, if the analytical use requires that water purity remains as high as possible, its use should be protected from atmospheric exposure. This water is used as a reagent, as a solvent for reagent preparation, and for test apparatus cleaning where less pure waters would not perform acceptably. However, if a user's routinely available *Purified Water* is filtered and meets or exceeds the conductivity specifications of *High-Purity Water*, it could be used in lieu of *High-Purity Water*.

Ammonia-Free Water—Functionally, this water must have a negligible ammonia concentration to avoid interference in tests sensitive to ammonia. It has been equated with *High-Purity Water* that has a significantly tighter Stage 1 conductivity specification than *Purified Water* because of the latter's allowance for a minimal level of ammonium among other ions. However, if the user's *Purified Water* were filtered and met or exceeded the conductivity specifications of *High-Purity Water*, it would contain negligible ammonia or other ions and could be used in lieu of *High-Purity Water*.

Carbon Dioxide-Free Water—The introductory portion of the *Reagents, Indicators, and Solutions* section defines this water as *Purified Water* that has been vigorously boiled for at least 5 minutes, then cooled and protected from absorption of atmospheric carbon dioxide. Because the absorption of carbon dioxide tends to drive down the water pH, most of the uses of *Carbon Dioxide-Free Water* are either associated as a solvent in pH-related or pH-sensitive determinations or as a solvent in carbonate-sensitive reagents or determinations. Another use of this water is for certain optical rotation and color and clarity of solution tests. Though it is possible that this water is indicated for these tests simply because of its purity, it is also possible that the pH effects of carbon dioxide-containing water could interfere with the results of

these tests. A third plausible reason that this water is indicated is that outgassing air bubbles might interfere with these photometric-type tests. The boiled water preparation approach will also greatly reduce the concentrations of many other dissolved gases along with carbon dioxide. Therefore, in some of the applications for *Carbon Dioxide-Free Water*, it could be the inadvertent deaeration effect that actually renders this water suitable. In addition to boiling, deionization is perhaps an even more efficient process for removing dissolved carbon dioxide (by drawing the dissolved gas equilibrium toward the ionized state with subsequent removal by the ion-exchange resins). If the starting *Purified Water* is prepared by an efficient deionization process and protected after deionization from exposure to atmospheric air, water that is carbon dioxide-free can be effectively made without the application of heat. However, this deionization process does not deaerate the water, so if *Purified Water* prepared by deionization is considered as a substitute water in a test requiring *Carbon Dioxide-Free Water*, the user must verify that it is not actually water akin to *Deaerated Water* (discussed below) that is needed for the test. As indicated in *High-Purity Water*, even brief contact with the atmosphere can allow small amounts of carbon dioxide to dissolve, ionize, and significantly degrade the conductivity and lower the pH. If the analytical use requires the water to remain as pH-neutral and as carbon dioxide-free as possible, even the analysis should be protected from atmospheric exposure. However, in most applications, atmospheric exposure during testing does not significantly affect its suitability in the test.

Ammonia- and Carbon Dioxide-Free Water—As implied by the name, this water should be prepared by approaches compatible with those mentioned for both *Ammonia-Free Water* and *Carbon Dioxide-Free Water*. Because the carbon dioxide-free attribute requires post-production protection from the atmosphere, it is appropriate to first render the water ammonia-free using the *High-Purity Water* process followed by the boiling and carbon dioxide-protected cooling process. The *High-Purity Water* deionization process for creating *Ammonia-Free Water* will also remove the ions generated from dissolved carbon dioxide and ultimately, by forced equilibration to the ionized state, all the dissolved carbon dioxide. Therefore, depending on its use, an acceptable procedure for making *Ammonia- and Carbon Dioxide-Free Water* could be to transfer and collect *High-Purity Water* in a carbon dioxide intrusion-protected container.

Deaerated Water—This water is *Purified Water* that has been treated to reduce the content of dissolved air by “suitable means”. In the *Reagents* section, approaches for boiling, cooling (similar to *Carbon Dioxide-Free Water* but without the atmospheric carbon dioxide protection), and sonication are given as applicable for test uses other than dissolution and drug release testing. Though *Deaerated Water* is not mentioned by name in *Dissolution* <711>, suggested methods for deaerating dissolution media (which may be water) include warming to 41°, vacuum filtering through a 0.45-µm rated membrane, and vigorously stirring the filtrate while maintaining the vacuum. This chapter specifically indicates that other validated approaches may be used. In other monographs that also do not mention *Deaerated Water* by name, degassing of water and other reagents is accomplished by sparging with helium. *Deaerated Water* is used in both dissolution testing as well as liquid chromatography applications where outgassing could either interfere with the analysis itself or cause erroneous results due to inaccurate volumetric withdrawals. Applications where ambient temperature water is used for reagent preparation, but the tests are performed at elevated temperatures, are candidates for outgassing effects. If outgassing could interfere with test performance, including chromatographic flow, colorimetric or photometric measurements, or volumetric accuracy, then *Deaerated Water* should probably be used, whether called for in the analysis or not. The above deaeration approaches might not render the water “gas-free”. At

best, they reduce the dissolved gas concentrations so that outgassing caused by temperature changes is not likely.

Recently Boiled Water—This water may include recently or freshly boiled water (with or without mention of cooling in the title), but cooling prior to use is clearly intended. Occasionally it is necessary to use when hot. *Recently Boiled Water* is specified because it is used in a pH-related test or carbonate-sensitive reagent, in an oxygen-sensitive test or reagent, or in a test where outgassing could interfere with the analysis, such as specific gravity or an appearance test.

Oxygen-Free Water—The preparation of this water is not specifically described in the compendia. Neither is there an oxygen specification or analysis mentioned. However, all uses involve analyses of materials that could be sensitive to oxidation by atmospheric oxygen. Procedures for the removal of dissolved oxygen from solvents, though not necessarily water, are mentioned in *Polarography* <801> and *Spectrophotometry and Light-Scattering* <851>. These procedures involve simple sparging of the liquid with an inert gas such as nitrogen or helium, followed by inert gas blanketing to prevent oxygen reabsorption. The sparging times cited range from 5 to 15 minutes to an unspecified period. Some *Purified Water* and *Water for Injection* systems produce water that is maintained in a hot state and that is inert gas blanketed during its preparation and storage and distribution. Though oxygen is poorly soluble in hot water, such water may not be oxygen-free. Whatever procedure is used for removing oxygen should be verified as reliably producing water that is fit for use.

Water for BET—This water is also referred to as LAL reagent water. This is often *Water for Injection*, which may have been sterilized. It is free from a level of endotoxin that would yield any detectable reaction or interference with the *Limulus Amoebocyte Lysate* reagent used in the *Bacterial Endotoxins Test* <85>.

Organic-Free Water—This water is defined by *Residual Solvents* <467> as producing no significantly interfering gas chromatography peaks. Referenced monographs specify using this water as the solvent for the preparation of standard and test solutions for the *Residual solvents* test.

Lead-Free Water—This water is used as a transferring diluent for an analyte in a *Lead* <251> test. Though no specific instructions are given for its preparation, it must not contain any detectable lead. *Purified Water* should be a suitable substitute for this water.

Chloride-Free Water—This water is specified as the solvent for use in an assay that contains a reactant that precipitates in the presence of chloride. Though no specific preparation instructions are given for this water, its rather obvious attribute is having a very low chloride level in order to be unreactive with this chloride sensitive reactant. *Purified Water* could be used for this water but should be tested to ensure that it is unreactive.

Hot Water—The uses of this water include solvents for achieving or enhancing reagent solubilization, restoring the original volume of boiled or hot solutions, rinsing insoluble analytes free of hot water soluble impurities, solvents for reagent recrystallization, apparatus cleaning, and as a solubility attribute for various *USP-NF* articles. In only one monograph is the temperature of “hot” water specified; so in all the other cases, the water temperature is less important, but should be high enough to achieve the desirable effect. In all cases, the chemical quality of the water is implied to be that of *Purified Water*.

VALIDATION AND QUALIFICATION OF WATER PURIFICATION, STORAGE, AND DISTRIBUTION SYSTEMS

Establishing the dependability of pharmaceutical water purification, storage, and distribution systems requires an appropriate period of monitoring and observation. Ordinar-

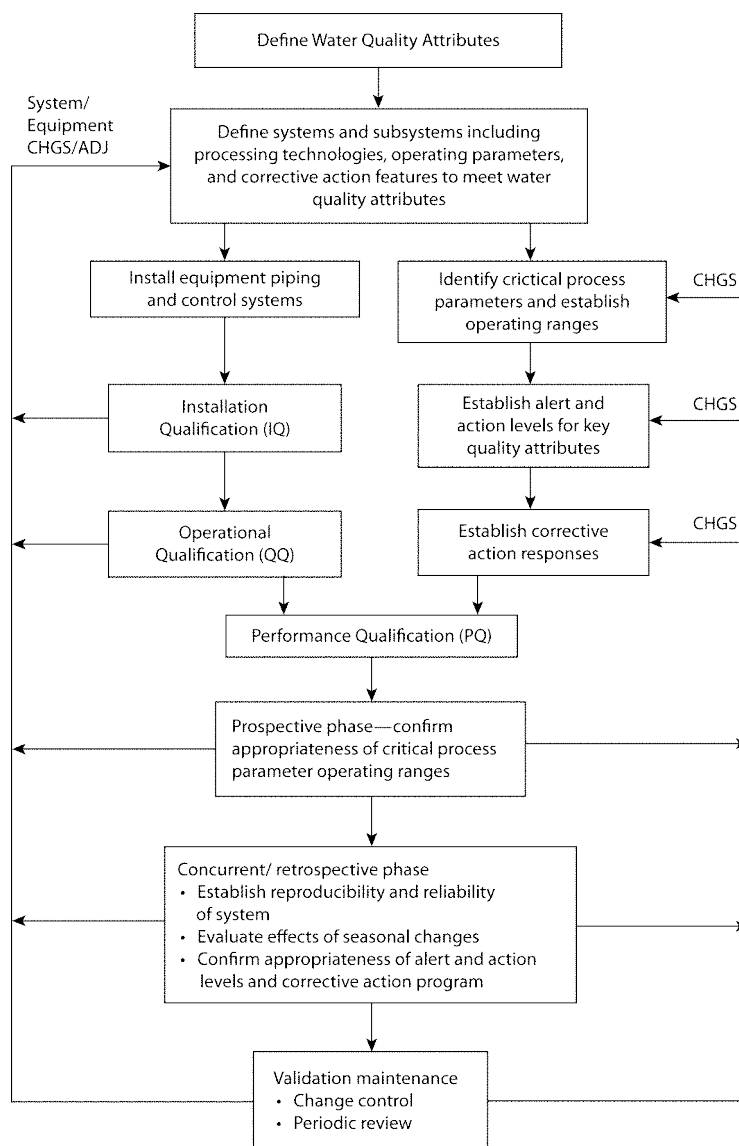


Figure 3. Water system validation life cycle.

ily, few problems are encountered in maintaining the chemical purity of *Purified Water* and *Water for Injection*. Nevertheless, the advent of using conductivity and TOC to define chemical purity has allowed the user to more quantitatively assess the water's chemical purity and its variability as a function of routine pretreatment system maintenance and regeneration. Even the presence of such unit operations as heat exchangers and use point hoses can compromise the chemical quality of water within and delivered from an otherwise well-controlled water system. Therefore, an assessment of the consistency of the water's chemical purity over time must be part of the validation program. However, even with the most well controlled chemical quality, it is often more difficult to consistently meet established microbiological quality criteria owing to phenomena occurring during and after chemical purification. A typical program involves intensive daily sampling and testing of major process points for at least one month after operational criteria have been established for each unit operation, point of use, and sampling point.

An overlooked aspect of water system validation is the delivery of the water to its actual location of use. If this transfer process from the distribution system outlets to the water use locations (usually with hoses) is defined as outside

the water system, then this transfer process still needs to be validated to not adversely affect the quality of the water to the extent it becomes unfit for use. Because routine microbial monitoring is performed for the same transfer process and components (e.g., hoses and heat exchangers) as that of routine water use (see *Sampling Considerations*), there is some logic to including this water transfer process within the distribution system validation.

Validation is the process whereby substantiation to a high level of assurance that a specific process will consistently produce a product conforming to an established set of quality attributes is acquired and documented. Prior to and during the very early stages of validation, the critical process parameters and their operating ranges are established. A validation program qualifies and documents the design, installation, operation, and performance of equipment. It begins when the system is defined and moves through several stages: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). A graphical representation of a typical water system validation life cycle is shown in *Figure 3*.

A validation plan for a water system typically includes the following steps: (1) establishing standards for quality attributes of the finished water and the source water; (2) defining

suitable unit operations and their operating parameters for achieving the desired finished water quality attributes from the available source water; (3) selecting piping, equipment, controls, and monitoring technologies; (4) developing an IQ stage consisting of instrument calibrations, inspections to verify that the drawings accurately depict the final configuration of the water system and, where necessary, special tests to verify that the installation meets the design requirements; (5) developing an OQ stage consisting of tests and inspections to verify that the equipment, system alerts, and controls are operating reliably and that appropriate alert and action levels are established (This phase of qualification may overlap with aspects of the next step.); and (6) developing a prospective PQ stage to confirm the appropriateness of critical process parameter operating ranges (During this phase of validation, alert and action levels for key quality attributes and operating parameters are verified.); (7) assuring the adequacy of ongoing control procedures, e.g., sanitization frequency; (8) supplementing a validation maintenance program (also called continuous validation life cycle) that includes a mechanism to control changes to the water system and establishes and carries out scheduled preventive maintenance including recalibration of instruments (In addition, validation maintenance includes a monitoring program for critical process parameters and a corrective action program.); (9) instituting a schedule for periodic review of the system performance and requalification; and (10) completing protocols and documenting Steps 1 through 9.

PURIFIED WATER AND WATER FOR INJECTION SYSTEMS

The design, installation, and operation of systems to produce *Purified Water* and *Water for Injection* include similar components, control techniques, and procedures. The quality attributes of both waters differ only in the presence of a bacterial endotoxin requirement for *Water for Injection* and in their methods of preparation, at least at the last stage of preparation. The similarities in the quality attributes provide considerable common ground in the design of water systems to meet either requirement. The critical difference is the degree of control of the system and the final purification steps needed to ensure bacterial and bacterial endotoxin removal.

Production of pharmaceutical water employs sequential unit operations (processing steps) that address specific water quality attributes and protect the operation of subsequent treatment steps. A typical evaluation process to select an appropriate water quality for a particular pharmaceutical purpose is shown in the decision tree in *Figure 2*. This diagram may be used to assist in defining requirements for specific water uses and in the selection of unit operations. The final unit operation used to produce *Water for Injection* is limited to distillation or other processes equivalent or superior to distillation in the removal of chemical impurities as well as microorganisms and their components. Distillation has a long history of reliable performance and can be validated as a unit operation for the production of *Water for Injection*, but other technologies or combinations of technologies can be validated as being equivalently effective. Other technologies, such as ultrafiltration following another chemical purification process, may be suitable in the production of *Water for Injection* if they can be shown through validation to be as effective and reliable as distillation. The advent of new materials for older technologies, such as reverse osmosis and ultrafiltration, that allow intermittent or continuous operation at elevated, microbial temperatures, show promise for a valid use in producing *Water for Injection*.

The validation plan should be designed to establish the suitability of the system and to provide a thorough understanding of the purification mechanism, range of operating conditions, required pretreatment, and the most likely modes of failure. It is also necessary to demonstrate the effectiveness of the monitoring scheme and to establish the

documentation and qualification requirements for the system's validation maintenance. Trials conducted in a pilot installation can be valuable in defining the operating parameters and the expected water quality and in identifying failure modes. However, qualification of the specific unit operation can only be performed as part of the validation of the installed operational system. The selection of specific unit operations and design characteristics for a water system should take into account the quality of the feed water, the technology chosen for subsequent processing steps, the extent and complexity of the water distribution system, and the appropriate compendial requirements. For example, in the design of a system for *Water for Injection*, the final process (distillation or whatever other validated process is used according to the monograph) must have effective bacterial endotoxin reduction capability and must be validated.

UNIT OPERATIONS CONCERNS

The following is a brief description of selected unit operations and the operation and validation concerns associated with them. Not all unit operations are discussed, nor are all potential problems addressed. The purpose is to highlight issues that focus on the design, installation, operation, maintenance, and monitoring parameters that facilitate water system validation.

Prefiltration

The purpose of prefiltration—also referred to as initial, coarse, or depth filtration—is to remove solid contaminants down to a size of 7 to 10 μm from the incoming source water supply and protect downstream system components from particulates that can inhibit equipment performance and shorten their effective life. This coarse filtration technology utilizes primarily sieving effects for particle capture and a depth of filtration medium that has a high “dirt load” capacity. Such filtration units are available in a wide range of designs and for various applications. Removal efficiencies and capacities differ significantly, from granular bed filters such as multimedia or sand for larger water systems, to depth cartridges for smaller water systems. Unit and system configurations vary widely in type of filtering media and location in the process. Granular or cartridge prefilters are often situated at or near the head of the water pretreatment system prior to unit operations designed to remove the source water disinfectants. This location, however, does not preclude the need for periodic microbial control because biofilm can still proliferate, although at a slower rate in the presence of source water disinfectants. Design and operational issues that may impact performance of depth filters include channeling of the filtering media, blockage from silt, microbial growth, and filtering-media loss during improper backwashing. Control measures involve pressure and flow monitoring during use and backwashing, sanitizing, and replacing filtering media. An important design concern is sizing of the filter to prevent channeling or media loss resulting from inappropriate water flow rates as well as proper sizing to minimize excessively frequent or infrequent backwashing or cartridge filter replacement.

Activated Carbon

Granular activated carbon beds adsorb low molecular weight organic material and oxidizing additives, such as chlorine and chloramine compounds, removing them from the water. They are used to achieve certain quality attributes and to protect against reaction with downstream stainless steel surfaces, resins, and membranes. The chief operating concerns regarding activated carbon beds include the propensity to support bacteria growth, the potential for hydraulic channeling, the organic adsorption capacity, appropriate water flow rates and contact time, the inability to be

regenerated in situ, and the shedding of bacteria, endotoxins, organic chemicals, and fine carbon particles. Control measures may involve monitoring water flow rates and differential pressures, sanitizing with hot water or steam, backwashing, testing for adsorption capacity, and frequent replacement of the carbon bed. If the activated carbon bed is intended for organic reduction, it may also be appropriate to monitor influent and effluent TOC. It is important to note that the use of steam for carbon bed sanitization is often incompletely effective due to steam channeling rather than even permeation through the bed. This phenomenon can usually be avoided by using hot water sanitization. It is also important to note that microbial biofilm development on the surface of the granular carbon particles (as well as on other particles such as found in deionizer beds and even multimedia beds) can cause adjacent bed granules to "stick" together. When large masses of granules are agglomerated in this fashion, normal backwashing and bed fluidization flow parameters may not be sufficient to disperse them, leading to ineffective removal of trapped debris, loose biofilm, and penetration of microbial controlling conditions (as well as regenerant chemicals as in the case of agglomerated deionizer resins). Alternative technologies to activated carbon beds can be used in order to avoid their microbial problems, such as disinfectant-neutralizing chemical additives and regenerable organic scavenging devices. However, these alternatives do not function by the same mechanisms as activated carbon, may not be as effective at removing disinfectants and some organics, and have a different set of operating concerns and control measures that may be nearly as troublesome as activated carbon beds.

Additives

Chemical additives are used in water systems (a) to control microorganisms by use of sanitants such as chlorine compounds and ozone, (b) to enhance the removal of suspended solids by use of flocculating agents, (c) to remove chlorine compounds, (d) to avoid scaling on reverse osmosis membranes, and (e) to adjust pH for more effective removal of carbonate and ammonia compounds by reverse osmosis. These additives do not constitute "added substances" as long as they are either removed by subsequent processing steps or are otherwise absent from the finished water. Control of additives to ensure a continuously effective concentration and subsequent monitoring to ensure their removal should be designed into the system and included in the monitoring program.

Organic Scavengers

Organic scavenging devices use macroreticular weakly basic anion-exchange resins capable of removing organic material and endotoxins from the water. They can be regenerated with appropriate biocidal caustic brine solutions. Operating concerns are associated with organic scavenging capacity, particulate, chemical and microbiological fouling of the reactive resin surface, flow rate, regeneration frequency, and shedding of resin fragments. Control measures include TOC testing of influent and effluent, backwashing, monitoring hydraulic performance, and using downstream filters to remove resin fines.

Softeners

Water softeners may be located either upstream or downstream of disinfectant removal units. They utilize sodium-based cation-exchange resins to remove water-hardness ions, such as calcium and magnesium, that could foul or interfere with the performance of downstream processing equipment such as reverse osmosis membranes, deionization devices, and distillation units. Water softeners can also be used to remove other lower affinity cations, such as the am-

monium ion, that may be released from chloramine disinfectants commonly used in drinking water and which might otherwise carryover through other downstream unit operations. If ammonium removal is one of its purposes, the softener must be located downstream of the disinfectant removal operation, which itself may liberate ammonium from neutralized chloramine disinfectants. Water softener resin beds are regenerated with concentrated sodium chloride solution (brine). Concerns include microorganism proliferation, channeling caused by biofilm agglomeration of resin particles, appropriate water flow rates and contact time, ion-exchange capacity, organic and particulate resin fouling, organic leaching from new resins, fracture of the resin beads, resin degradation by excessively chlorinated water, and contamination from the brine solution used for regeneration. Control measures involve recirculation of water during periods of low water use, periodic sanitization of the resin and brine system, use of microbial control devices (e.g., UV light and chlorine), locating the unit upstream of the disinfectant removal step (if used only for softening), appropriate regeneration frequency, effluent chemical monitoring (e.g., hardness ions and possibly ammonium), and downstream filtration to remove resin fines. If a softener is used for ammonium removal from chloramine-containing source water, then capacity, contact time, resin surface fouling, pH, and regeneration frequency are very important.

Deionization

Deionization (DI), and continuous electrodeionization (CEDI) are effective methods of improving the chemical quality attributes of water by removing cations and anions. DI systems have charged resins that require periodic regeneration with an acid and base. Typically, cationic resins are regenerated with either hydrochloric or sulfuric acid, which replace the captured positive ions with hydrogen ions. Anionic resins are regenerated with sodium or potassium hydroxide, which replace captured negative ions with hydroxide ions. Because free endotoxin is negatively charged, there is some removal of endotoxin achieved by the anionic resin. Both regenerant chemicals are biocidal and offer a measure of microbial control. The system can be designed so that the cation and anion resins are in separate or "twin" beds or they can be mixed together to form a mixed bed. Twin beds are easily regenerated but deionize water less efficiently than mixed beds, which have a considerably more complex regeneration process. Rechargeable resin canisters can also be used for this purpose.

The CEDI system uses a combination of mixed resin, selectively permeable membranes, and an electric charge, providing continuous flow (product and waste concentrate) and continuous regeneration. Water enters both the resin section and the waste (concentrate) section. As it passes through the resin, it is deionized to become product water. The resin acts as a conductor enabling the electrical potential to drive the captured cations and anions through the resin and appropriate membranes for concentration and removal in the waste water stream. The electrical potential also separates the water in the resin (product) section into hydrogen and hydroxide ions. This permits continuous regeneration of the resin without the need for regenerant additives. However, unlike conventional deionization, CEDI units must start with water that is already partially purified because they generally cannot produce *Purified Water* quality when starting with the heavier ion load of unpurified source water.

Concerns for all forms of deionization units include microbial and endotoxin control, chemical additive impact on resins and membranes, and loss, degradation, and fouling of resin. Issues of concern specific to DI units include regeneration frequency and completeness, channeling caused by biofilm agglomeration of resin particles, organic leaching from new resins, complete resin separation for mixed bed regeneration, and mixing air contamination (mixed beds). Control

measures vary but typically include recirculation loops, effluent microbial control by UV light, conductivity monitoring, resin testing, microporous filtration of mixing air, microbial monitoring, frequent regeneration to minimize and control microorganism growth, sizing the equipment for suitable water flow and contact time, and use of elevated temperatures. Internal distributor and regeneration piping for mixed bed units should be configured to ensure that regeneration chemicals contact all internal bed and piping surfaces and resins. Rechargeable canisters can be the source of contamination and should be carefully monitored. Full knowledge of previous resin use, minimum storage time between regeneration and use, and appropriate sanitizing procedures are critical factors ensuring proper performance.

Reverse Osmosis

Reverse osmosis (RO) units employ semipermeable membranes. The "pores" of RO membranes are actually intersegmental spaces among the polymer molecules. They are big enough for permeation of water molecules, but too small to permit passage of hydrated chemical ions. However, many factors including pH, temperature, and differential pressure across the membrane affect the selectivity of this permeation. With the proper controls, RO membranes can achieve chemical, microbial, and endotoxin quality improvement. The process streams consist of supply water, product water (permeate), and wastewater (reject). Depending on source water, pretreatment and system configuration variations and chemical additives may be necessary to achieve desired performance and reliability.

A major factor affecting RO performance is the permeate recovery rate, that is, the amount of the water passing through the membrane compared to the amount rejected. This is influenced by the several factors, but most significantly by the pump pressure. Recoveries of 75% are typical, and can accomplish a 1 to 2 log purification of most impurities. For most feed waters, this is usually not enough to meet *Purified Water* conductivity specifications. A second pass of this permeate water through another RO stage usually achieves the necessary permeate purity if other factors such as pH and temperature have been appropriately adjusted and the ammonia from chloraminated source water has been previously removed. Increasing recoveries with higher pressures in order to reduce the volume of reject water will lead to reduced permeate purity. If increased pressures are needed over time to achieve the same permeate flow, this is an indication of partial membrane blockage that needs to be corrected before it becomes irreversibly fouled, and expensive membrane replacement is the only option.

Other concerns associated with the design and operation of RO units include membrane materials that are extremely sensitive to sanitizing agents and to particulate, chemical, and microbial membrane fouling; membrane and seal integrity; the passage of dissolved gases, such as carbon dioxide and ammonia; and the volume of wastewater, particularly where water discharge is tightly regulated by local authorities. Failure of membrane or seal integrity will result in product water contamination. Methods of control involve suitable pretreatment of the influent water stream, appropriate membrane material selection, integrity challenges, membrane design and heat tolerance, periodic sanitization, and monitoring of differential pressures, conductivity, microbial levels, and TOC.

The development of RO units that can tolerate sanitizing water temperatures as well as operate efficiently and continuously at elevated temperatures has added greatly to their microbial control and to the avoidance of biofouling. RO units can be used alone or in combination with DI and CEDI units as well as ultrafiltration for operational and quality enhancements.

Ultrafiltration

Ultrafiltration is a technology most often employed in pharmaceutical water systems for removing endotoxins from a water stream. It can also use semipermeable membranes, but unlike RO, these typically use polysulfone membranes whose intersegmental "pores" have been purposefully exaggerated during their manufacture by preventing the polymer molecules from reaching their smaller equilibrium proximities to each other. Depending on the level of equilibrium control during their fabrication, membranes with differing molecular weight "cutoffs" can be created such that molecules with molecular weights above these cutoffs ratings are rejected and cannot penetrate the filtration matrix.

Ceramic ultrafilters are another molecular sieving technology. Ceramic ultrafilters are self supporting and extremely durable, backwashable, chemically cleanable, and steam sterilizable. However, they may require higher operating pressures than membrane type ultrafilters.

All ultrafiltration devices work primarily by a molecular sieving principle. Ultrafilters with molecular weight cutoff ratings in the range of 10,000 to 20,000 Da are typically used in water systems for removing endotoxins. This technology may be appropriate as an intermediate or final purification step. Similar to RO, successful performance is dependent upon pretreatment of the water by upstream unit operations.

Issues of concern for ultrafilters include compatibility of membrane material with heat and sanitizing agents, membrane integrity, fouling by particles and microorganisms, and seal integrity. Control measures involve filtration medium selection, sanitization, flow design (dead end vs. tangential), integrity challenges, regular cartridge changes, elevated feed water temperature, and monitoring TOC and differential pressure. Additional flexibility in operation is possible based on the way ultrafiltration units are arranged such as in a parallel or series configurations. Care should be taken to avoid stagnant water conditions that could promote microorganism growth in back-up or standby units.

Charge-Modified Filtration

Charge-modified filters are usually microbially retentive filters that are treated during their manufacture to have a positive charge on their surfaces. Microbial retentive filtration will be described in a subsequent section, but the significant feature of these membranes is their electrostatic surface charge. Such charged filters can reduce endotoxin levels in the fluids passing through them by their adsorption (owing to endotoxin's negative charge) onto the membrane surfaces. Though ultrafilters are more often employed as a unit operation for endotoxin removal in water systems, charge-modified filters may also have a place in endotoxin removal particularly where available upstream pressures are not sufficient for ultrafiltration and for a single, relatively short term use. Charge-modified filters may be difficult to validate for long-term or large-volume endotoxin retention. Even though their purified standard endotoxin retention can be well characterized, their retention capacity for "natural" endotoxins is difficult to gauge. Nevertheless, utility could be demonstrated and validated as short-term, single-use filters at points of use in water systems that are not designed for endotoxin control or where only an endotoxin "polishing" (removal of only slight or occasional endotoxin levels) is needed. Control and validation concerns include volume and duration of use, flow rate, water conductivity and purity, and constancy and concentration of endotoxin levels being removed. All of these factors may have to be evaluated and challenged prior to using this approach, making this a difficult-to-validate application. Even so, there may still be a possible need for additional backup endotoxin testing both upstream and downstream of the filter.

Microbial-Retentive Filtration

Microbial-retentive membrane filters have experienced an evolution of understanding in the past decade that has caused previously held theoretical retention mechanisms to be reconsidered. These filters have a larger effective "pore size" than ultrafilters and are intended to prevent the passage of microorganisms and similarly sized particles without unduly restricting flow. This type of filtration is widely employed within water systems for filtering the bacteria out of both water and compressed gases as well as for vent filters on tanks and stills and other unit operations. However, the properties of the water system microorganisms seem to challenge a filter's microbial retention from water with phenomena absent from other aseptic filtration applications, such as filter sterilizing of pharmaceutical formulations prior to packaging. In the latter application, sterilizing grade filters are generally considered to have an assigned rating of 0.2 or 0.22 μm . This rather arbitrary rating is associated with filters that have the ability to retain a high level challenge of a specially prepared inoculum of *Brevundimonas* (formerly *Pseudomonas*) *diminuta*. This is a small microorganism originally isolated decades ago from a product that had been "filter sterilized" using a 0.45- μm rated filter. Further study revealed that a percentage of cells of this microorganism could reproducibly penetrate the 0.45- μm sterilizing filters. Through historic correlation of *B. diminuta* retaining tighter filters, thought to be twice as good as 0.45- μm filter, assigned ratings of 0.2 or 0.22 μm with their successful use in product solution filter sterilization, both this filter rating and the associated high level *B. diminuta* challenge have become the current benchmarks for sterilizing filtration. New evidence now suggests that for microbial-retentive filters used for pharmaceutical water, *B. diminuta* may not be the best model microorganism.

An archaic understanding of microbial retentive filtration would lead one to equate a filter's rating with the false impression of a simple sieve or screen that absolutely retains particles sized at or above the filter's rating. A current understanding of the mechanisms involved in microbial retention and the variables that can affect those mechanisms has yielded a far more complex interaction of phenomena than previously understood. A combination of simple sieve retention and surface adsorption are now known to contribute to microbial retention.

The following all interact to create some unusual and surprising retention phenomena for water system microorganisms: the variability in the range and average pore sizes created by the various membrane fabrication processes, the variability of the surface chemistry and three-dimensional structure related to the different polymers used in these filter matrices, and the size and surface properties of the microorganism intended to be retained by the filters. *B. diminuta* may not be the best challenge microorganisms for demonstrating bacterial retention for 0.2- to 0.22- μm rated filters for use in water systems because it appears to be more easily retained by these filters than some water system flora. The well-documented appearance of water system microorganisms on the downstream sides of some 0.2- to 0.22- μm rated filters after a relatively short period of use seems to support that some penetration phenomena are at work. Unknown for certain is if this downstream appearance is caused by a "blow-through" or some other pass-through phenomenon as a result of tiny cells or less cell "stickiness", or by a "growth through" phenomenon as a result of cells hypothetically replicating their way through the pores to the downstream side. Whatever is the penetration mechanism, 0.2- to 0.22- μm rated membranes may not be the best choice for some water system uses.

Microbial retention success in water systems has been reported with the use of some manufacturers' filters arbitrarily rated as 0.1 μm . There is general agreement that for a given manufacturer, their 0.1- μm rated filters are tighter than their

0.2- to 0.22- μm rated filters. However, comparably rated filters from different manufacturers in water filtration applications may not perform equivalently owing to the different filter fabrication processes and the nonstandardized microbial retention challenge processes currently used for defining the 0.1- μm filter rating. It should be noted that use of 0.1- μm rated membranes generally results in a sacrifice in flow rate compared to 0.2- to 0.22- μm membranes, so whatever membranes are chosen for a water system application, the user must verify that the membranes are suitable for their intended application, use period, and use process, including flow rate.

For microbial retentive gas filtrations, the same sieving and adsorptive retention phenomena are at work as in liquid filtration, but the adsorptive phenomenon is enhanced by additional electrostatic interactions between particles and filter matrix. These electrostatic interactions are so strong that particle retention for a given filter rating is significantly more efficient in gas filtration than in water or product solution filtrations. These additional adsorptive interactions render filters rated at 0.2 to 0.22 μm unquestionably suitable for microbial retentive gas filtrations. When microbially retentive filters are used in these applications, the membrane surface is typically hydrophobic (non-wettable by water). A significant area of concern for gas filtration is blockage of tank vents by condensed water vapor, which can cause mechanical damage to the tank. Control measures include electrical or steam tracing and a self-draining orientation of vent filter housings to prevent accumulation of vapor condensate. However, a continuously high filter temperature will take an oxidative toll on polypropylene components of the filter, so sterilization of the unit prior to initial use, and periodically thereafter, as well as regular visual inspections, integrity tests, and changes are recommended control methods.

In water applications, microbial retentive filters may be used downstream of unit operations that tend to release microorganisms or upstream of unit operations that are sensitive to microorganisms. Microbial retentive filters may also be used to filter water feeding the distribution system. It should be noted that regulatory authorities allow the use of microbial retentive filters within distribution systems or even at use points if they have been properly validated and are appropriately maintained. A point-of-use filter should only be intended to "polish" the microbial quality of an otherwise well-maintained system and not to serve as the primary microbial control device. The efficacy of system microbial control measures can only be assessed by sampling the water upstream of the filters. As an added measure of protection, in-line UV lamps, appropriately sized for the flow rate (see *Sanitization*), may be used just upstream of microbial retentive filters to inactivate microorganisms prior to their capture by the filter. This tandem approach tends to greatly delay potential microbial penetration phenomena and can substantially extend filter service life.

Ultraviolet Light

The use of low-pressure UV lights that emit a 254-nm wavelength for microbial control is discussed under *Sanitization*, but the application of UV light in chemical purification is also emerging. This 254-nm wavelength is also useful in the destruction of ozone. With intense emissions at wavelengths around 185 nm (as well as at 254 nm), medium pressure UV lights have demonstrated utility in the destruction of the chlorine-containing disinfectants used in source water as well as for interim stages of water pretreatment. High intensities of this wavelength alone or in combination with other oxidizing sanitants, such as hydrogen peroxide, have been used to lower TOC levels in recirculating distribution systems. The organics are typically converted to carbon dioxide, which equilibrates to bicarbonate, and incompletely oxidized carboxylic acids, both of which can easily be removed by polishing ion-exchange resins. Areas of concern

include adequate UV intensity and residence time, gradual loss of UV emissivity with bulb age, gradual formation of UV-absorbing film at the water contact surface, incomplete photodegradation during unforeseen source water hyper-chlorination, release of ammonia from chloramine photodegradation, unapparent UV bulb failure, and conductivity degradation in distribution systems using 185-nm UV lights. Control measures include regular inspection or emissivity alarms to detect bulb failures or film occlusions, regular UV bulb sleeve cleaning and wiping, downstream chlorine detectors, downstream polishing deionizers, and regular (approximately yearly) bulb replacement.

Distillation

Distillation units provide chemical and microbial purification via thermal vaporization, mist elimination, and water vapor condensation. A variety of designs is available including single effect, multiple effect, and vapor compression. The latter two configurations are normally used in larger systems because of their generating capacity and efficiency. Distilled water systems require different feed water controls than required by membrane systems. For distillation, due consideration must be given to prior removal of hardness and silica impurities that may foul or corrode the heat transfer surfaces as well as prior removal of those impurities that could volatilize and condense along with the water vapor. In spite of general perceptions, even the best distillation process cannot afford absolute removal of contaminating ions and endotoxin. Most stills are recognized as being able to accomplish at least a 3 to 4 log reduction in these impurity concentrations. Areas of concern include carry-over of volatile organic impurities such as trihalomethanes (see *Source and Feed Water Considerations*) and gaseous impurities such as ammonia and carbon dioxide, faulty mist elimination, evaporator flooding, inadequate blowdown, stagnant water in condensers and evaporators, pump and compressor seal design, pinhole evaporator and condenser leaks, and conductivity (quality) variations during start-up and operation.

Methods of control may involve preliminary decarbonation steps to remove both dissolved carbon dioxide and other volatile or noncondensable impurities; reliable mist elimination to minimize feedwater droplet entrainment; visual or automated high water level indication to detect boiler flooding and boil over; use of sanitary pumps and compressors to minimize microbial and lubricant contamination of feedwater and condensate; proper drainage during inactive periods to minimize microbial growth and accumulation of associated endotoxin in boiler water; blow down control to limit the impurity concentration effect in the boiler to manageable levels; on-line conductivity sensing with automated diversion to waste to prevent unacceptable water upon still startup or still malfunction from getting into the finished water distribute system; and periodic integrity testing for pinhole leaks to routinely assure condensate is not compromised by nonvolatilized source water contaminants.

Storage Tanks

Storage tanks are included in water distribution systems to optimize processing equipment capacity. Storage also allows for routine maintenance within the pretreatment train while maintaining continuous supply to meet manufacturing needs. Design and operation considerations are needed to prevent or minimize the development of biofilm, to minimize corrosion, to aid in the use of chemical sanitization of the tanks, and to safeguard mechanical integrity. These considerations may include using closed tanks with smooth interiors, the ability to spray the tank headspace using sprayballs on recirculating loop returns, and the use of heated, jacketed/insulated tanks. This minimizes corrosion and biofilm development and aids in thermal and chemical sanitization. Storage tanks require venting to compensate for the dynamics of changing water levels. This can be ac-

complished with a properly oriented and heat-traced filter housing fitted with a hydrophobic microbial retentive membrane filter affixed to an atmospheric vent. Alternatively, an automatic membrane-filtered compressed gas blanketing system may be used. In both cases, rupture disks equipped with a rupture alarm device should be used as a further safeguard for the mechanical integrity of the tank. Areas of concern include microbial growth or corrosion due to irregular or incomplete sanitization and microbial contamination from unalarmed rupture disk failures caused by condensate-occluded vent filters.

Distribution Systems

Distribution system configuration should allow for the continuous flow of water in the piping by means of recirculation. Use of nonrecirculating, dead-end, or one-way systems or system segments should be avoided whenever possible. If not possible, these systems should be periodically flushed and more closely monitored. Experience has shown that continuously recirculated systems are easier to maintain. Pumps should be designed to deliver fully turbulent flow conditions to facilitate thorough heat distribution (for hot water sanitized systems) as well as thorough chemical sanitant distribution. Turbulent flow also appear to either retard the development of biofilms or reduce the tendency of those biofilms to shed bacteria into the water. If redundant pumps are used, they should be configured and used to avoid microbial contamination of the system.

Components and distribution lines should be sloped and fitted with drain points so that the system can be completely drained. In stainless steel distribution systems where the water is circulated at a high temperature, dead legs and low-flow conditions should be avoided, and valved tie-in points should have length-to-diameter ratios of six or less. If constructed of heat tolerant plastic, this ratio should be even less to avoid cool points where biofilm development could occur. In ambient temperature distribution systems, particular care should be exercised to avoid or minimize dead leg ratios of any size and provide for complete drainage. If the system is intended to be steam sanitized, careful sloping and low-point drainage is crucial to condensate removal and sanitization success. If drainage of components or distribution lines is intended as a microbial control strategy, they should also be configured to be completely dried using dry compressed air (or nitrogen if appropriate employee safety measures are used). Drained but still moist surfaces will still support microbial proliferation. Water exiting from the distribution system should not be returned to the system without first passing through all or a portion of the purification train.

The distribution design should include the placement of sampling valves in the storage tank and at other locations, such as in the return line of the recirculating water system. Where feasible, the primary sampling sites for water should be the valves that deliver water to the points of use. Direct connections to processes or auxiliary equipment should be designed to prevent reverse flow into the controlled water system. Hoses and heat exchangers that are attached to points of use in order to deliver water for a particular use must not chemically or microbiologically degrade the water quality. The distribution system should permit sanitization for microorganism control. The system may be continuously operated at sanitizing conditions or sanitized periodically.

INSTALLATION, MATERIALS OF CONSTRUCTION, AND COMPONENT SELECTION

Installation techniques are important because they can affect the mechanical, corrosive, and sanitary integrity of the system. Valve installation attitude should promote gravity drainage. Pipe supports should provide appropriate slopes

for drainage and should be designed to support the piping adequately under worst-case thermal and flow conditions. The methods of connecting system components including units of operation, tanks, and distribution piping require careful attention to preclude potential problems. Stainless steel welds should provide reliable joints that are internally smooth and corrosion-free. Low-carbon stainless steel, compatible wire filler, where necessary, inert gas, automatic welding machines, and regular inspection and documentation help to ensure acceptable weld quality. Follow-up cleaning and passivation are important for removing contamination and corrosion products and to re-establish the passive corrosion resistant surface. Plastic materials can be fused (welded) in some cases and also require smooth, uniform internal surfaces. Adhesive glues and solvents should be avoided due to the potential for voids and extractables. Mechanical methods of joining, such as flange fittings, require care to avoid the creation of offsets, gaps, penetrations, and voids. Control measures include good alignment, properly sized gaskets, appropriate spacing, uniform sealing force, and the avoidance of threaded fittings.

Materials of construction should be selected to be compatible with control measures such as sanitizing, cleaning, and passivating. Temperature rating is a critical factor in choosing appropriate materials because surfaces may be required to handle elevated operating and sanitization temperatures. Should chemicals or additives be used to clean, control, or sanitize the system, materials resistant to these chemicals or additives must be utilized. Materials should be capable of handling turbulent flow and elevated velocities without wear of the corrosion-resistant film such as the passive chromium oxide surface of stainless steel. The finish on metallic materials such as stainless steel, whether it is a refined mill finish, polished to a specific grit, or an electropolished treatment, should complement system design and provide satisfactory corrosion and microbial activity resistance as well as chemical sanitizability. Auxiliary equipment and fittings that require seals, gaskets, diaphragms, filter media, and membranes should exclude materials that permit the possibility of extractables, shedding, and microbial activity. Insulating materials exposed to stainless steel surfaces should be free of chlorides to avoid the phenomenon of stress corrosion cracking that can lead to system contamination and the destruction of tanks and critical system components.

Specifications are important to ensure proper selection of materials and to serve as a reference for system qualification and maintenance. Information such as mill reports for stainless steel and reports of composition, ratings, and material handling capabilities for nonmetallic substances should be reviewed for suitability and retained for reference. Component (auxiliary equipment) selection should be made with assurance that it does not create a source of contamination intrusion. Heat exchangers should be constructed to prevent leakage of heat transfer medium to the pharmaceutical water and, for heat exchanger designs where prevention may fail, there should be a means to detect leakage. Pumps should be of sanitary design with seals that prevent contamination of the water. Valves should have smooth internal surfaces with the seat and closing device exposed to the flushing action of water, such as occurs in diaphragm valves. Valves with pocket areas or closing devices (e.g., ball, plug, gate, globe) that move into and out of the flow area should be avoided.

SANITIZATION

Microbial control in water systems is achieved primarily through sanitization practices. Systems can be sanitized using either thermal or chemical means. Thermal approaches to system sanitization include periodic or continuously circulating hot water and the use of steam. Temperatures of at least 80° are most commonly used for this purpose, but

continuously recirculating water of at least 65° has also been used effectively in insulated stainless steel distribution systems when attention is paid to uniformity and distribution of such self-sanitizing temperatures. These techniques are limited to systems that are compatible with the higher temperatures needed to achieve sanitization. Although thermal methods control biofilm development by either continuously inhibiting their growth or, in intermittent applications, by killing the microorganisms within biofilms, they are not effective in removing established biofilms. Killed but intact biofilms can become a nutrient source for rapid biofilm regrowth after the sanitizing conditions are removed or halted. In such cases, a combination of routine thermal and periodic supplementation with chemical sanitization might be more effective. The more frequent the thermal sanitization, the more likely biofilm development and regrowth can be eliminated. Chemical methods, where compatible, can be used on a wider variety of construction materials. These methods typically employ oxidizing agents such as halogenated compounds, hydrogen peroxide, ozone, peracetic acid, or combinations thereof. Halogenated compounds are effective sanitizers but are difficult to flush from the system and may leave biofilms intact. Compounds such as hydrogen peroxide, ozone, and peracetic acid oxidize bacteria and biofilms by forming reactive peroxides and free radicals (notably hydroxyl radicals). The short half-life of ozone in particular, and its limitation on achievable concentrations require that it be added continuously during the sanitization process. Hydrogen peroxide and ozone rapidly degrade to water and oxygen; peracetic acid degrades to acetic acid in the presence of UV light. In fact, ozone's ease of degradation to oxygen using 254-nm UV lights at use points allow it to be most effectively used on a continuous basis to provide continuously sanitizing conditions.

In-line UV light at a wavelength of 254 nm can also be used to continuously "sanitize" water circulating in the system, but these devices must be properly sized for the water flow. Such devices inactivate a high percentage (but not 100%) of microorganisms that flow through the device but cannot be used to directly control existing biofilm upstream or downstream of the device. However, when coupled with conventional thermal or chemical sanitization technologies or located immediately upstream of a microbially retentive filter, it is most effective and can prolong the interval between system sanitizations.

It is important to note that microorganisms in a well-developed biofilm can be extremely difficult to kill, even by aggressive oxidizing biocides. The less developed and therefore thinner the biofilm, the more effective the biocidal action. Therefore, optimal biocide control is achieved by frequent biocide use that does not allow significant biofilm development between treatments.

Sanitization steps require validation to demonstrate the capability of reducing and holding microbial contamination at acceptable levels. Validation of thermal methods should include a heat distribution study to demonstrate that sanitization temperatures are achieved throughout the system, including the body of use point valves. Validation of chemical methods require demonstrating adequate chemical concentrations throughout the system, exposure to all wetted surfaces, including the body of use point valves, and complete removal of the sanitant from the system at the completion of treatment. Methods validation for the detection and quantification of residues of the sanitant or its objectionable degradants is an essential part of the validation program. The frequency of sanitization should be supported by, if not triggered by, the results of system microbial monitoring. Conclusions derived from trend analysis of the microbiological data should be used as the alert mechanism for maintenance. The frequency of sanitization should be established in such a way that the system operates in a state of microbiological control and does not routinely exceed alert levels (see *Alert and Action Levels and Specifications*).

OPERATION, MAINTENANCE, AND CONTROL

A preventive maintenance program should be established to ensure that the water system remains in a state of control. The program should include (1) procedures for operating the system, (2) monitoring programs for critical quality attributes and operating conditions including calibration of critical instruments, (3) schedule for periodic sanitization, (4) preventive maintenance of components, and (5) control of changes to the mechanical system and to operating conditions.

Operating Procedures—Procedures for operating the water system and performing routine maintenance and corrective action should be written, and they should also define the point when action is required. The procedures should be well documented, detail the function of each job, assign who is responsible for performing the work, and describe how the job is to be conducted. The effectiveness of these procedures should be assessed during water system validation.

Monitoring Program—Critical quality attributes and operating parameters should be documented and monitored. The program may include a combination of in-line sensors or automated instruments (e.g., for TOC, conductivity, hardness, and chlorine), automated or manual documentation of operational parameters (such as flow rates or pressure drop across a carbon bed, filter, or RO unit), and laboratory tests (e.g., total microbial counts). The frequency of sampling, the requirement for evaluating test results, and the necessity for initiating corrective action should be included.

Sanitization—Depending on system design and the selected units of operation, routine periodic sanitization may be necessary to maintain the system in a state of microbial control. Technologies for sanitization are described above.

Preventive Maintenance—A preventive maintenance program should be in effect. The program should establish what preventive maintenance is to be performed, the frequency of maintenance work, and how the work should be documented.

Change Control—The mechanical configuration and operating conditions must be controlled. Proposed changes should be evaluated for their impact on the whole system. The need to requalify the system after changes are made should be determined. Following a decision to modify a water system, the affected drawings, manuals, and procedures should be revised.

SAMPLING CONSIDERATIONS

Water systems should be monitored at a frequency that is sufficient to ensure that the system is in control and continues to produce water of acceptable quality. Samples should be taken from representative locations within the processing and distribution system. Established sampling frequencies should be based on system validation data and should cover critical areas including unit operation sites. The sampling plan should take into consideration the desired attributes of the water being sampled. For example, systems for *Water for Injection* because of their more critical microbiological requirements, may require a more rigorous sampling frequency.

Analyses of water samples often serve two purposes: in-process control assessments and final quality control assessments. In-process control analyses are usually focused on the attributes of the water within the system. Quality control is primarily concerned with the attributes of the water delivered by the system to its various uses. The latter usually employs some sort of transfer device, often a flexible hose, to bridge the gap between the distribution system use-point valve and the actual location of water use. The issue of sample collection location and sampling procedure is often hotly debated because of the typically mixed use of the data

generated from the samples, for both in-process control and quality control. In these single sample and mixed data use situations, the worst-case scenario should be utilized. In other words, samples should be collected from use points using the same delivery devices, such as hoses, and procedures, such as preliminary hose or outlet flushing, as are employed by production from those use points. Where use points per se cannot be sampled, such as hard-piped connections to equipment, special sampling ports may be used. In all cases, the sample must represent as closely as possible the quality of the water used in production. If a point of use filter is employed, sampling of the water prior to and after the filter is needed because the filter will mask the microbial control achieved by the normal operating procedures of the system.

Samples containing chemical sanitizing agents require neutralization prior to microbiological analysis. Samples for microbiological analysis should be tested immediately, or suitably refrigerated to preserve the original microbial attributes until analysis can begin. Samples of flowing water are only indicative of the concentration of planktonic (free floating) microorganisms present in the system. Biofilm microorganisms (those attached to water system surfaces) are usually present in greater numbers and are the source of the planktonic population recovered from grab samples. Microorganisms in biofilms represent a continuous source of contamination and are difficult to directly sample and quantify. Consequently, the planktonic population is usually used as an indicator of system contamination levels and is the basis for system *Alert* and *Action Levels*. The consistent appearance of elevated planktonic levels is usually an indication of advanced biofilm development in need of remedial control. System control and sanitization are key in controlling biofilm formation and the consequent planktonic population.

Sampling for chemical analyses is also done for in-process control and for quality control purposes. However, unlike microbial analyses, chemical analyses can be and often are performed using on-line instrumentation. Such on-line testing has unequivocal in-process control purposes because it is not performed on the water delivered from the system. However, unlike microbial attributes, chemical attributes are usually not significantly degraded by hoses. Therefore, through verification testing, it may be possible to show that the chemical attributes detected by the on-line instrumentation (in-process testing) are equivalent to those detected at the ends of the use point hoses (quality control testing). This again creates a single sample and mixed data use scenario. It is far better to operate the instrumentation in a continuous mode, generating large volumes of in-process data, but only using a defined small sampling of that data for QC purposes. Examples of acceptable approaches include using highest values for a given period, highest time-weighted average for a given period (from fixed or rolling sub-periods), or values at a fixed daily time. Each approach has advantages and disadvantages relative to calculation complexity and reflection of continuous quality, so the user must decide which approach is most suitable or justifiable.

CHEMICAL CONSIDERATIONS

The chemical attributes of *Purified Water* and *Water for Injection* in effect prior to USP 23 were specified by a series of chemistry tests for various specific and nonspecific attributes with the intent of detecting chemical species indicative of incomplete or inadequate purification. While these methods could have been considered barely adequate to control the quality of these waters, they nevertheless stood the test of time. This was partly because the operation of water systems was, and still is, based on on-line conductivity measurements and specifications generally thought to preclude the failure of these archaic chemistry attribute tests.

USP moved away from these chemical attribute tests to contemporary analytical technologies for the bulk waters *Purified Water* and *Water for Injection*. The intent was to up-

grade the analytical technologies without tightening the quality requirements. The two contemporary analytical technologies employed were TOC and conductivity. The TOC test replaced the test for *Oxidizable substances* that primarily targeted organic contaminants. A multistaged *Conductivity* test which detects ionic (mostly inorganic) contaminants replaced, with the exception of the test for *Heavy metals*, all of the inorganic chemical tests (i.e., *Ammonia*, *Calcium*, *Carbon dioxide*, *Chloride*, *Sulfate*).

Replacing the heavy metals attribute was considered unnecessary because (a) the source water specifications (found in the NPDWR) for individual *Heavy metals* were tighter than the approximate limit of detection of the *Heavy metals* test for *USP XXII Water for Injection* and *Purified Water* (approximately 0.1 ppm), (b) contemporary water system construction materials do not leach heavy metal contaminants, and (c) test results for this attribute have uniformly been negative—there has not been a confirmed occurrence of a singular test failure (failure of only the *Heavy metals* test with all other attributes passing) since the current heavy metal drinking water standards have been in place. Nevertheless, since the presence of heavy metals in *Purified Water* or *Water for Injection* could have dire consequences, its absence should at least be documented during new water system commissioning and validation or through prior test results records.

Total solids and *pH* were the only tests not covered by conductivity testing. The test for *Total solids* was considered redundant because the nonselective tests of conductivity and TOC could detect most chemical species other than silica, which could remain undetected in its colloidal form. Colloidal silica in *Purified Water* and *Water for Injection* is easily removed by most water pretreatment steps and even if present in the water, constitutes no medical or functional hazard except under extreme and rare situations. In such extreme situations, other attribute extremes are also likely to be detected. It is, however, the user's responsibility to ensure fitness for use. If silica is a significant component in the source water, and the purification unit operations could be operated or fail and selectively allow silica to be released into the finished water (in the absence of co-contaminants

detectable by conductivity), then either silica-specific or a total solids type testing should be utilized to monitor and control this rare problem.

The *pH* attribute was eventually recognized to be redundant to the conductivity test (which included *pH* as an aspect of the test and specification); therefore, *pH* was dropped as a separate attribute test.

The rationale used by USP to establish its *Purified Water* and *Water for Injection* conductivity specifications took into consideration the conductivity contributed by the two least conductive former attributes of *Chloride* and *Ammonia*, thereby precluding their failure had those wet chemistry tests been performed. In essence, the *Stage 3* conductivity specifications (see *Water Conductivity, Bulk Water* (645)) were established from the sum of the conductivities of the limit concentrations of chloride ions (from pH 5.0 to 6.2) and ammonia ions (from pH 6.3 to 7.0), plus the unavoidable contribution of other conductivity-contributing ions from water (H^+ and OH^-), dissolved atmospheric CO_2 (as HCO_3^-), and an electro-balancing quantity of either Na^+ or Cl^- , depending on the pH-induced ionic imbalance (see *Table 1*). The *Stage 2* conductivity specification is the lowest value on this table, 2.1 $\mu S/cm$. The *Stage 1* specifications, designed primarily for on-line measurements, were derived essentially by summing the lowest values in the contributing ion columns for each of a series of tables similar to *Table 1*, created for each 5° increment between 0° and 100°. For example purposes, the italicized values in *Table 1*, the conductivity data table for 25°, were summed to yield a conservative value of 1.3 $\mu S/cm$, the *Stage 1* specification for a nontemperature compensated, nonatmosphere equilibrated water sample that actually had a measured temperature of 25° to 29°. Each 5° increment in the table was similarly treated to yield the individual values listed in the table of *Stage 1* specifications (see *Water Conductivity, Bulk Water* (645)).

As stated above, this rather radical change to utilizing a conductivity attribute as well as the inclusion of a TOC attribute allowed for on-line measurements. This was a major philosophical change and allowed major savings to be realized by industry. The TOC and conductivity tests can also

Table 1. Contributing Ion Conductivities of the Chloride–Ammonia Model as a Function of pH
(in atmosphere-equilibrated water at 25°)

pH	Conductivity ($\mu S/cm$)						Combined Conductivities	Stage 3 Limit
	H^+	OH^-	HCO_3^-	Cl^-	Na^+	NH_4^+		
5.0	3.49	0	0.02	1.01	0.19	0	4.71	4.7
5.1	2.77	0	0.02	1.01	0.29	0	4.09	4.1
5.2	2.20	0	0.03	1.01	0.38	0	3.62	3.6
5.3	1.75	0	0.04	1.01	0.46	0	3.26	3.3
5.4	1.39	0	0.05	1.01	0.52	0	2.97	3.0
5.5	1.10	0	0.06	1.01	0.58	0	2.75	2.8
5.6	0.88	0	0.08	1.01	0.63	0	2.60	2.6
5.7	0.70	0	0.10	1.01	0.68	0	2.49	2.5
5.8	0.55	0	0.12	1.01	0.73	0	2.41	2.4
5.9	0.44	0	0.16	1.01	0.78	0	2.39	2.4
6.0	0.35	0	0.20	1.01	0.84	0	2.40	2.4
6.1	0.28	0	0.25	1.01	0.90	0	2.44	2.4
6.2	0.22	0	0.31	1.01	0.99	0	2.53	2.5
6.3	0.18	0	0.39	0.63	0	1.22	2.42	2.4
6.4	0.14	0.01	0.49	0.45	0	1.22	2.31	2.3
6.5	0.11	0.01	0.62	0.22	0	1.22	2.18	2.2
6.6	0.09	0.01	0.78	0	0.04	1.22	2.14	2.1
6.7	0.07	0.01	0.99	0	0.27	1.22	2.56	2.6
6.8	0.06	0.01	1.24	0	0.56	1.22	3.09	3.1
6.9	0.04	0.02	1.56	0	0.93	1.22	3.77	3.8
7.0	0.03	0.02	1.97	0	1.39	1.22	4.63	4.6

be performed “off-line” in the laboratories using collected samples, though sample collection tends to introduce opportunities for adventitious contamination that can cause false high readings. The collection of on-line data is not, however, without challenges. The continuous readings tend to create voluminous amounts of data where before only a single data point was available. As stated under *Sampling Considerations*, continuous in-process data is excellent for understanding how a water system performs during all of its various usage and maintenance events in real time, but is too much data for QC purposes. Therefore, a justifiable fraction or averaging of the data can be used that is still representative of the overall water quality being used.

Packaged/sterile waters present a particular dilemma relative to the attributes of conductivity and TOC. The package itself is the source of chemicals (inorganics and organics) that leach over time into the packaged water and can easily be detected by the conductivity and TOC tests. The irony of organic leaching from plastic packaging is that before the advent of bulk water TOC testing when the *Oxidizable substances* test was the only “organic purity” test for both bulk and packaged/sterile water monographs in *USP*, that test’s insensitivity to many of the organic leachables from plastic and elastomeric packaging materials was largely unrealized, allowing organic levels in packaged/sterile water to be quite high (possibly many times the TOC specification for bulk water). Similarly, glass containers can also leach inorganics, such as sodium, which are easily detected by conductivity, but poorly detected by the former wet chemistry attribute tests. Most of these leachables are considered harmless by current perceptions and standards at the rather significant concentrations present. Nevertheless, they effectively degrade the quality of the high-purity waters placed into these packaging systems. Some packaging materials contain more leachables than others and may not be as suitable for holding water and maintaining its purity.

The attributes of conductivity and TOC tend to reveal more about the packaging leachables than they do about the water’s original purity. These currently “allowed” leachables could render the sterile packaged versions of originally equivalent bulk water essentially unsuitable for many uses where the bulk waters are perfectly adequate.

Therefore, to better control the ionic packaging leachables, *Water Conductivity* <645> is divided into two sections. The first is titled *Bulk Water*, which applies to *Purified Water*, *Water for Injection*, *Water for Hemodialysis*, and *Pure Steam*, and includes the three-stage conductivity testing instructions and specifications. The second is titled *Sterile Water*, which applies to *Sterile Purified Water*, *Sterile Water for Injection*, *Sterile Water for Inhalation*, and *Sterile Water for Irrigation*. The *Sterile Water* section includes conductivity specifications similar to the *Stage 2* testing approach because it is intended as a laboratory test, and these sterile waters were made from bulk water that already complied with the three-stage conductivity test. In essence, packaging leachables are the primary target “analytes” of the conductivity specifications in the *Sterile Water* section of *Water Conductivity* <645>. The effect on potential leachables from different container sizes is the rationale for having two different specifications, one for small packages containing nominal volumes of 10 mL or less and another for larger packages. These conductivity specifications are harmonized with the *European Pharmacopoeia* conductivity specifications for *Sterile Water for Injection*. All monographed waters, except *Bacteriostatic Water for Injection*, have a conductivity specification that directs the user to either the *Bulk Water* or the *Sterile Water* section of *Water Conductivity* <645>. For the sterile packaged water monographs, this water conductivity specification replaces the redundant wet chemistry limit tests intended for inorganic contaminants that had previously been specified in these monographs.

Controlling the organic purity of these sterile packaged waters, particularly those in plastic packaging, is more challenging. Though the TOC test can better detect and therefore be better used to monitor and control these impurities

than the current *Oxidizable substances* test, the latter has many decades-old precedents and flexibility with the variety of packaging types and volumes applicable to these sterile packaged waters. Nevertheless, TOC testing of these currently allowed sterile, plastic-packaged waters reveals substantial levels of plastic-derived organic leachables that render the water perhaps orders of magnitude less organically pure than typically achieved with bulk waters. Therefore, usage of these packaged waters for analytical, manufacturing, and cleaning applications should only be exercised after suitability of the waters’ purity for the application has been assured.

MICROBIAL CONSIDERATIONS

The major exogenous source of microbial contamination of bulk pharmaceutical water is source or feed water. Feed water quality must, at a minimum, meet the quality attributes of Drinking Water for which the level of coliforms are regulated. A wide variety of other microorganisms, chiefly Gram-negative bacteria, may be present in the incoming water. These microorganisms may compromise subsequent purification steps. Examples of other potential exogenous sources of microbial contamination include unprotected vents, faulty air filters, ruptured rupture disks, backflow from contaminated outlets, unsanitized distribution system “openings” including routine component replacements, inspections, repairs, and expansions, inadequate drain and air-breaks, and replacement activated carbon, deionizer resins, and regenerant chemicals. In these situations, the exogenous contaminants may not be normal aquatic bacteria but rather microorganisms of soil or even human origin. The detection of nonaquatic microorganisms may be an indication of a system component failure, which should trigger investigations that will remediate their source. Sufficient care should be given to system design and maintenance in order to minimize microbial contamination from these exogenous sources.

Unit operations can be a major source of endogenous microbial contamination. Microorganisms present in feed water may adsorb to carbon bed, deionizer resins, filter membranes, and other unit operation surfaces and initiate the formation of a biofilm. In a high-purity water system, biofilm is an adaptive response by certain microorganisms to survive in this low nutrient environment. Downstream colonization can occur when microorganisms are shed from existing biofilm-colonized surfaces and carried to other areas of the water system. Microorganisms may also attach to suspended particles such as carbon bed fines or fractured resin particles. When the microorganisms become planktonic, they serve as a source of contamination to subsequent purification equipment (compromising its functionality) and to distribution systems.

Another source of endogenous microbial contamination is the distribution system itself. Microorganisms can colonize pipe surfaces, rough welds, badly aligned flanges, valves, and unidentified dead legs, where they proliferate, forming a biofilm. The smoothness and composition of the surface may affect the rate of initial microbial adsorption, but once adsorbed, biofilm development, unless otherwise inhibited by sanitizing conditions, will occur regardless of the surface. Once formed, the biofilm becomes a continuous source of microbial contamination.

ENDOTOXIN CONSIDERATIONS

Endotoxins are lipopolysaccharides found in and shed from the cell envelope that is external to the cell wall of Gram-negative bacteria. Gram-negative bacteria that form biofilms can become a source of endotoxins in pharmaceutical waters. Endotoxins may occur as clusters of lipopolysaccharide molecules associated with living microorganisms, fragments of dead microorganisms or the polysaccharide

slime surrounding biofilm bacteria, or as free molecules. The free form of endotoxins may be released from cell surfaces of the bacteria that colonize the water system, or from the feed water that may enter the water system. Because of the multiplicity of endotoxin sources in a water system, endotoxin quantitation in a water system is not a good indicator of the level of biofilm abundance within a water system.

Endotoxin levels may be minimized by controlling the introduction of free endotoxins and microorganisms in the feed water and minimizing microbial proliferation in the system. This may be accomplished through the normal exclusion or removal action afforded by various unit operations within the treatment system as well as through system sanitization. Other control methods include the use of ultrafilters or charge-modified filters, either in-line or at the point of use. The presence of endotoxins may be monitored as described in the general test chapter *Bacterial Endotoxins Test* (85).

MICROBIAL ENUMERATION CONSIDERATIONS

The objective of a water system microbiological monitoring program is to provide sufficient information to control and assess the microbiological quality of the water produced. Product quality requirements should dictate water quality specifications. An appropriate level of control may be maintained by using data trending techniques and, if necessary, limiting specific contraindicated microorganisms. Consequently, it may not be necessary to detect all of the microorganisms species present in a given sample. The monitoring program and methodology should indicate adverse trends and detect microorganisms that are potentially harmful to the finished product, process, or consumer. Final selection of method variables should be based on the individual requirements of the system being monitored.

It should be recognized that there is no single method that is capable of detecting all of the potential microbial contaminants of a water system. The methods used for microbial monitoring should be capable of isolating the numbers and types of organisms that have been deemed significant relative to in-process system control and product impact for each individual system. Several criteria should be considered when selecting a method to monitor the microbial content of a pharmaceutical water system. These include method sensitivity, range of organisms types or species recovered, sample processing throughput, incubation period, cost, and methodological complexity. An alternative consideration to the use of the classical "culture" approaches is a sophisticated instrumental or rapid test method that may yield more timely results. However, care must be exercised in selecting such an alternative approach to ensure that it has both sensitivity and correlation to classical culture approaches, which are generally considered the accepted standards for microbial enumeration.

Consideration should also be given to the timeliness of microbial enumeration testing after sample collection. The number of detectable planktonic bacteria in a sample collected in a scrupulously clean sample container will usually drop as time passes. The planktonic bacteria within the sample will tend to either die or to irretrievably adsorb to the container walls reducing the number of viable planktonic bacteria that can be withdrawn from the sample for testing. The opposite effect can also occur if the sample container is not scrupulously clean and contains a low concentration of some microbial nutrient that could promote microbial growth within the sample container. Because the number of recoverable bacteria in a sample can change positively or negatively over time after sample collection, it is best to test the samples as soon as possible after being collected. If it is not possible to test the sample within about 2 hours of collection, the sample should be held at refrigerated temperatures (2° to 8°) for a maximum of about 12 hours to maintain the microbial attributes until analysis. In situations

where even this is not possible (such as when using off-site contract laboratories), testing of these refrigerated samples should be performed within 48 hours after sample collection. In the delayed testing scenario, the recovered microbial levels may not be the same as would have been recovered had the testing been performed shortly after sample collection. Therefore, studies should be performed to determine the existence and acceptability of potential microbial enumeration aberrations caused by protracted testing delays.

The Classical Culture Approach

Classical culture approaches for microbial testing of water include but are not limited to pour plates, spread plates, membrane filtration, and most probable number (MPN) tests. These methods are generally easy to perform, are less expensive, and provide excellent sample processing throughput. Method sensitivity can be increased via the use of larger sample sizes. This strategy is used in the membrane filtration method. Culture approaches are further defined by the type of medium used in combination with the incubation temperature and duration. This combination should be selected according to the monitoring needs presented by a specific water system as well as its ability to recover the microorganisms of interest: those that could have a detrimental effect on the product or process uses as well as those that reflect the microbial control status of the system.

There are two basic forms of media available for traditional microbiological analysis: "high nutrient" and "low nutrient". High-nutrient media such as plate count agar (TGYA) and m-HPC agar (formerly m-SPC agar), are intended as general media for the isolation and enumeration of heterotrophic or "copiotrophic" bacteria. Low-nutrient media such as R2A agar and NWRI agar (HPCA), may be beneficial for isolating slow growing "oligotrophic" bacteria and bacteria that require lower levels of nutrients to grow optimally. Often some facultative oligotrophic bacteria are able to grow on high nutrient media and some facultative copiotrophic bacteria are able to grow on low-nutrient media, but this overlap is not complete. Low-nutrient and high-nutrient cultural approaches may be concurrently used, especially during the validation of a water system, as well as periodically thereafter. This concurrent testing could determine if any additional numbers or types of bacteria can be preferentially recovered by one of the approaches. If so, the impact of these additional isolates on system control and the end uses of the water could be assessed. Also, the efficacy of system controls and sanitization on these additional isolates could be assessed.

Duration and temperature of incubation are also critical aspects of a microbiological test method. Classical methodologies using high nutrient media are typically incubated at 30° to 35° for 48 to 72 hours. Because of the flora in certain water systems, incubation at lower temperatures (e.g., 20° to 25°) for longer periods (e.g., 5 to 7 days) can recover higher microbial counts when compared to classical methods. Low-nutrient media are designed for these lower temperature and longer incubation conditions (sometimes as long as 14 days to maximize recovery of very slow growing oligotrophs or sanitant injured microorganisms), but even high-nutrient media can sometimes increase their recovery with these longer and cooler incubation conditions. Whether or not a particular system needs to be monitored using high- or low-nutrient media with higher or lower incubation temperatures or shorter or longer incubation times should be determined during or prior to system validation and periodically reassessed as the microbial flora of a new water system gradually establish a steady state relative to its routine maintenance and sanitization procedures. The establishment of a "steady state" can take months or even years and can be perturbed by a change in use patterns, a change in routine and preventative maintenance or sanitiza-

tion procedures, and frequencies, or any type of system intrusion, such as for component replacement, removal, or addition. The decision to use longer incubation periods should be made after balancing the need for timely information and the type of corrective actions required when an alert or action level is exceeded with the ability to recover the microorganisms of interest.

The advantages gained by incubating for longer times, namely recovery of injured microorganisms, slow growers, or more fastidious microorganisms, should be balanced against the need to have a timely investigation and to take corrective action, as well as the ability of these microorganisms to detrimentally affect products or processes. In no case, however, should incubation at 30° to 35° be less than 48 hours or less than 96 hours at 20° to 25°.

Normally, the microorganisms that can thrive in extreme environments are best cultivated in the laboratory using conditions simulating the extreme environments from which they were taken. Therefore, thermophilic bacteria might be able to exist in the extreme environment of hot pharmaceutical water systems, and if so, could only be recovered and cultivated in the laboratory if similar thermal conditions were provided. Thermophilic aquatic microorganisms do exist in nature, but they typically derive their energy for growth from harnessing the energy from sunlight, from oxidation/reduction reactions of elements such as sulfur or iron, or indirectly from other microorganisms that do derive their energy from these processes. Such chemical/nutritional conditions do not exist in high purity water systems, whether ambient or hot. Therefore, it is generally considered pointless to search for thermophiles from hot pharmaceutical water systems owing to their inability to grow there.

The microorganisms that inhabit hot systems tend to be found in much cooler locations within these systems, for example, within use-point heat exchangers or transfer hoses. If this occurs, the kinds of microorganisms recovered are usually of the same types that might be expected from ambient water systems. Therefore, the mesophilic microbial cultivation conditions described later in this chapter are usually adequate for their recovery.

“Instrumental” Approaches

Examples of instrumental approaches include microscopic visual counting techniques (e.g., epifluorescence and immunofluorescence) and similar automated laser scanning approaches and radiometric, impedometric, and biochemically based methodologies. These methods all possess a variety of advantages and disadvantages. Advantages could be their precision and accuracy or their speed of test result availability as compared to the classical cultural approach. In general, instrument approaches often have a shorter lead time for obtaining results, which could facilitate timely system control. This advantage, however, is often counterbalanced by limited sample processing throughput due to extended sample collection time, costly and/or labor-intensive sample processing, or other instrument and sensitivity limitations.

Furthermore, instrumental approaches are typically destructive, precluding subsequent isolate manipulation for characterization purposes. Generally, some form of microbial isolate characterization, if not full identification, may be a required element of water system monitoring. Consequently, culturing approaches have traditionally been preferred over instrumental approaches because they offer a balance of desirable test attributes and post-test capabilities.

Suggested Methodologies

The following general methods were originally derived from *Standard Methods for the Examination of Water and Wastewater*, 17th Edition, American Public Health Association, Washington, DC 20005. Even though this publication has

undergone several revisions since its first citation in this chapter, the methods are still considered appropriate for establishing trends in the number of colony-forming units observed in the routine microbiological monitoring of pharmaceutical waters. It is recognized, however, that other combinations of media and incubation time and temperature may occasionally or even consistently result in higher numbers of colony-forming units being observed and/or different species being recovered.

The extended incubation periods that are usually required by some of the alternative methods available offer disadvantages that may outweigh the advantages of the higher counts that may be obtained. The somewhat higher baseline counts that might be observed using alternate cultural conditions would not necessarily have greater utility in detecting an excursion or a trend. In addition, some alternate cultural conditions using low-nutrient media tend to lead to the development of microbial colonies that are much less differentiated in colonial appearance, an attribute that microbiologists rely on when selecting representative microbial types for further characterization. It is also ironical that the nature of some of the slow growers and the extended incubation times needed for their development into visible colonies may also lead to those colonies being largely nonviable, which limits their further characterization and precludes their subculture and identification.

Methodologies that can be suggested as generally satisfactory for monitoring pharmaceutical water systems are as follows. However, it must be noted that these are not referee methods nor are they necessarily optimal for recovering microorganisms from all water systems. The users should determine through experimentation with various approaches which methodologies are best for monitoring their water systems for in-process control and quality control purposes as well as for recovering any contraindicated species they may have specified.

<i>Drinking Water:</i>	Pour Plate Method or Membrane Filtration Method ¹
	Sample Volume—1.0 mL minimum ² Growth Medium—Plate Count Agar ³ Incubation Time—48 to 72 hours minimum Incubation Temperature—30° to 35°
<i>Purified Water:</i>	Pour Plate Method or Membrane Filtration Method ¹

¹ A membrane filter with a rating of 0.45 μm is generally considered preferable even though the cellular width of some of the bacteria in the sample may be narrower than this. The efficiency of the filtration process still allows the retention of a very high percentage of these smaller cells and is adequate for this application. Filters with smaller ratings may be used if desired, but for a variety of reasons the ability of the retained cells to develop into visible colonies may be compromised, so count accuracy must be verified by a reference approach.

² When colony counts are low to undetectable using the indicated minimum sample volume, it is generally recognized that a larger sample volume should be tested in order to gain better assurance that the resulting colony count is more statistically representative. The sample volume to consider testing is dependent on the user's need to know (which is related to the established alert and action levels and the water system's microbial control capabilities) and the statistical reliability of the resulting colony count. In order to test a larger sample volume, it may be necessary to change testing techniques, e.g., changing from a pour plate to a membrane filtration approach. Nevertheless, in a very low to nil count scenario, a maximum sample volume of around 250 to 300 mL is usually considered a reasonable balance of sample collecting and processing ease and increased statistical reliability. However, when sample volumes larger than about 2 mL are needed, they can only be processed using the membrane filtration method.

³ Also known as Standard Methods Agar, Standard Methods Plate Count Agar, or TGYA, this medium contains tryptone (pancreatic digest of casein), glucose and yeast extract.

<i>Drinking Water:</i>	Pour Plate Method or Membrane Filtration Method ¹
	Sample Volume—1.0 mL minimum ² Growth Medium—Plate Count Agar ³ Incubation Time—48 to 72 hours minimum Incubation Temperature—30° to 35°
<i>Water for Injection:</i>	Membrane Filtration Method ¹
	Sample Volume—100 mL minimum ² Growth Medium—Plate Count Agar ³ Incubation Time—48 to 72 hours minimum Incubation Temperature—30°C to 35°C

¹ A membrane filter with a rating of 0.45 µm is generally considered preferable even though the cellular width of some of the bacteria in the sample may be narrower than this. The efficiency of the filtration process still allows the retention of a very high percentage of these smaller cells and is adequate for this application. Filters with smaller ratings may be used if desired, but for a variety of reasons the ability of the retained cells to develop into visible colonies may be compromised, so count accuracy must be verified by a reference approach.

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³ Also known as Standard Methods Agar, Standard Methods Plate Count Agar, or TGYA, this medium contains tryptone (pancreatic digest of casein), glucose and yeast extract.

IDENTIFICATION OF MICROORGANISMS

Identifying the isolates recovered from water monitoring methods may be important in instances where specific waterborne microorganisms may be detrimental to the products or processes in which the water is used. Microorganism information such as this may also be useful when identifying the source of microbial contamination in a product or process. Often a limited group of microorganisms is routinely recovered from a water system. After repeated recovery and characterization, an experienced microbiologist may become proficient at their identification based on only a few recognizable traits such as colonial morphology and staining characteristics. This may allow for a reduction in the number of identifications to representative colony types, or, with proper analyst qualification, may even allow testing short cuts to be taken for these microbial identifications.

ALERT AND ACTION LEVELS AND SPECIFICATIONS

Though the use of alert and action levels is most often associated with microbial data, they can be associated with any attribute. In pharmaceutical water systems, almost every quality attribute, other than microbial quality, can be very rapidly determined with near-real time results. These short-delay data can give immediate system performance feedback, serving as ongoing process control indicators. However, because some attributes may not continuously be monitored or have a long delay in data availability (like microbial monitoring data), properly established *Alert and Action Levels* can serve as an early warning or indication of a

potentially approaching quality shift occurring between or at the next periodic monitoring. In a validated water system, process controls should yield relatively constant and more than adequate values for these monitored attributes such that their *Alert and Action Levels* are infrequently broached.

As process control indicators, alert and action levels are designed to allow remedial action to occur that will prevent a system from deviating completely out of control and producing water unfit for its intended use. This "intended use" minimum quality is sometimes referred to as a "specification" or "limit". In the opening paragraphs of this chapter, rationale was presented for no microbial specifications being included within the body of the bulk water (*Purified Water* and *Water for Injection*) monographs. This does not mean that the user should not have microbial specifications for these waters. To the contrary, in most situations such specifications should be established by the user. The microbial specification should reflect the maximum microbial level at which the water is still fit for use without compromising the quality needs of the process or product where the water is used. Because water from a given system may have many uses, the most stringent of these uses should be used to establish this specification.

Where appropriate, a microbial specification could be qualitative as well as quantitative. In other words, the number of total microorganisms may be as important as the number of a specific microorganism or even the absence of a specific microorganism. Microorganisms that are known to be problematic could include opportunistic or overt pathogens, nonpathogenic indicators of potentially undetected pathogens, or microorganisms known to compromise a process or product, such as by being resistant to a preservative or able to proliferate in or degrade a product. These microorganisms comprise an often ill-defined group referred to as "objectionable microorganisms". Because objectionable is a term relative to the water's use, the list of microorganisms in such a group should be tailored to those species with the potential to be present and problematic. Their negative impact is most often demonstrated when they are present in high numbers, but depending on the species, an allowable level may exist, below which they may not be considered objectionable.

As stated above, alert and action levels for a given process control attribute are used to help maintain system control and avoid exceeding the pass/fail specification for that attribute. Alert and action levels may be both quantitative and qualitative. They may involve levels of total microbial counts or recoveries of specific microorganisms. Alert levels are events or levels that, when they occur or are exceeded, indicate that a process may have drifted from its normal operating condition. Alert level excursions constitute a warning and do not necessarily require a corrective action. However, alert level excursions usually lead to the alerting of personnel involved in water system operation as well as QA. Alert level excursions may also lead to additional monitoring with more intense scrutiny of resulting and neighboring data as well as other process indicators. Action levels are events or higher levels that, when they occur or are exceeded, indicate that a process is probably drifting from its normal operating range. Examples of kinds of action level "events" include exceeding alert levels repeatedly; or in multiple simultaneous locations, a single occurrence of exceeding a higher microbial level; or the individual or repeated recovery of specific objectionable microorganisms. Exceeding an action level should lead to immediate notification of both QA and personnel involved in water system operations so that corrective actions can immediately be taken to bring the process back into its normal operating range. Such remedial actions should also include efforts to understand and eliminate or at least reduce the incidence of a future occurrence. A root cause investigation may be necessary to devise an effective preventative action strategy. Depending on the nature of the action level excursion, it may also be necessary to evaluate its impact on the water uses during that time.

Impact evaluations may include delineation of affected batches and additional or more extensive product testing. It may also involve experimental product challenges.

Alert and action levels should be derived from an evaluation of historic monitoring data called a trend analysis. Other guidelines on approaches that may be used, ranging from “inspectional” to statistical evaluation of the historical data have been published. The ultimate goal is to understand the normal variability of the data during what is considered a typical operational period. Then, trigger points or levels can be established that will signal when future data may be approaching (alert level) or exceeding (action level) the boundaries of that “normal variability”. Such alert and action levels are based on the control capability of the system as it was being maintained and controlled during that historic period of typical control.

In new water systems where there is very limited or no historic data from which to derive data trends, it is common to simply establish initial alert and action levels based on a combination of equipment design capabilities but below the process and product specifications where water is used. It is also common, especially for ambient water systems, to microbiologically “mature” over the first year of use. By the end of this period, a relatively steady state microbial population (microorganism types and levels) will have been allowed or promoted to develop as a result of the collective effects of routine system maintenance and operation, including the frequency of unit operation rebeddings, backwashings, regenerations, and sanitizations. This microbial population will typically be higher than was seen when the water system was new, so it should be expected that the data trends (and the resulting alert and action levels) will increase over this “maturation” period and eventually level off.

A water system should be designed so that performance-based alert and action levels are well below water specifications. With poorly designed or maintained water systems, the system owner may find that initial new system microbial levels were acceptable for the water uses and specifications, but the mature levels are not. This is a serious situation, which if not correctable with more frequent system maintenance and sanitization, may require expensive water system renovation or even replacement. Therefore, it cannot be overemphasized that water systems should be designed for ease of microbial control, so that when monitored against alert and action levels, and maintained accordingly, the water continuously meets all applicable specifications.

An action level should not be established at a level equivalent to the specification. This leaves no room for remedial system maintenance that could avoid a specification excursion. Exceeding a specification is a far more serious event than an action level excursion. A specification excursion may trigger an extensive finished product impact investigation, substantial remedial actions within the water system that may include a complete shutdown, and possibly even product rejection.

Another scenario to be avoided is the establishment of an arbitrarily high and usually nonperformance based action level. Such unrealistic action levels deprive users of meaningful indicator values that could trigger remedial system maintenance. Unrealistically high action levels allow systems to grow well out of control before action is taken, when their intent should be to catch a system imbalance before it goes wildly out of control.

Because alert and action levels should be based on actual system performance, and the system performance data are generated by a given test method, it follows that those alert and action levels should be valid only for test results generated by the same test method. It is invalid to apply alert and action level criteria to test results generated by a different test method. The two test methods may not equivalently recover microorganisms from the same water samples. Similarly invalid is the use of trend data to derive alert and action levels for one water system, but applying those alert and action levels to a different water system. Alert and action levels are water system and test method specific.

Nevertheless, there are certain maximum microbial levels above which action levels should never be established. Water systems with these levels should unarguably be considered out of control. Using the microbial enumeration methodologies suggested above, generally considered maximum action levels are 100 cfu per mL for *Purified Water* and 10 cfu per 100 mL for *Water for Injection*. However, if a given water system controls microorganisms much more tightly than these levels, appropriate alert and action levels should be established from these tighter control levels so that they can truly indicate when water systems may be starting to trend out of control. These in-process microbial control parameters should be established well below the user-defined microbial specifications that delineate the water's fitness for use.

Special consideration is needed for establishing maximum microbial action levels for *Drinking Water* because the water is often delivered to the facility in a condition over which the user has little control. High microbial levels in *Drinking Water* may be indicative of a municipal water system upset, broken water main, or inadequate disinfection, and therefore, potential contamination with objectionable microorganisms. Using the suggested microbial enumeration methodology, a reasonable maximum action level for *Drinking Water* is 500 cfu per mL. Considering the potential concern for objectionable microorganisms raised by such high microbial levels in the feedwater, informing the municipality of the problem so they may begin corrective actions should be an immediate first step. In-house remedial actions may or may not also be needed, but could include performing additional coliform testing on the incoming water and pre-treating the water with either additional chlorination or UV light irradiation or filtration or a combination of approaches.

(1235) VACCINES FOR HUMAN USE—GENERAL CONSIDERATIONS

INTRODUCTION

Vaccines have been used for centuries to immunize individuals against pathogenic organisms with the goal of preventing the associated disease. Vaccines are biological products that contain antigens capable of inducing a specific and active acquired immune response in the body. Antigens present in vaccines are processed by specialized cells in the body's immune system, resulting in the development of blood proteins known as antibodies (i.e., humoral immunity) or specialized lymphocytes (i.e., cell-mediated immunity) or both. Therefore immune responses may be antibody mediated, cell mediated, or both. Thus, antigens are critical for vaccine function and generally consist of a portion of the pathogenic organism, or an attenuated form of the whole microorganism. In the case of DNA-based vaccines (currently under development), the vaccine would contain nucleotide sequences (genetic material) that encode microbial antigens.

Examples of types of licensed vaccines appear in *Appendix 1*. A current list of vaccines licensed in the United States is posted at www.fda.gov/cber/.

Vaccines can be of various types, depending on their design and processes involved in their manufacture. Vaccines for human use may contain whole killed or attenuated organisms (e.g., bacteria or viruses) or contain antigens de-

rived from portions of a pathogen, either by partitioning and purification or derived using recombinant technology (Table 1). Some polysaccharide vaccines are conjugated to a carrier in order to enhance their immune response.

Table 1. Bacterial and Viral Vaccines

Live attenuated whole cell or virus ^a
Inactivated/killed ^b
Whole cell or virus ^c
Recombinant proteins ^d
Subunit ^e
Polysaccharides
Proteins
Modified toxins

^a Live attenuated bacterial or viral vaccines are weakened (attenuated) forms of a pathogen. They contain antigens that are similar to disease-causing microbes. They may be derived from the pathogen itself, or from a different organism that contains antigens that cross-react with the virulent microbe (e.g., vaccinia and variola).

^b Inactivated bacterial and viral vaccines are produced by growing cells of disease-causing bacteria or viruses in cell substrates and subsequently inactivating them to prevent replication in the recipient.

^c Inactivated/killed whole-cell or virus vaccines consist of the entire microorganisms after they have been inactivated. These preparations may or may not be partially or completely purified.

^d Recombinant protein viral and bacterial vaccines are derived from host cells that have been transformed with expression vectors that carry genes that encode antigenic material from infectious agents. The expression cells are grown in bioreactors to produce the recombinant antigenic material.

^e Subunit vaccines are extracts from inactivated/killed viruses or bacteria. Subunit-type vaccines generally undergo some degree of purification.

In addition to antigen(s), vaccines may contain several other components, such as adjuvants that enhance the immune response to the vaccine antigen, preservatives to prevent bacterial or fungal contamination of multiple-dose vials, or other excipients needed for pharmaceutical manufacturing or vaccine stabilization. Residual components from the manufacturing process also may be present in vaccine preparations. Examples of these categories are listed in Table 2.

Table 2. Vaccine Components

Antigens
Whole organisms
Components/subunits
Recombinant proteins
Adjuvants
Aluminum salts
Antimicrobial preservatives
Thimerosal
2-Phenoxyethanol
Benzethonium chloride
Phenol
Stabilizers
Salts
Amino acids
Sugars
Proteins
Other
Manufacturing residuals
Cell-derived residuals
Materials of animal origin
Antibiotic residuals
Inactivating chemical agents
Other

Different vaccine antigens are often combined in one final formulation in order to elicit immunity against multiple diseases and to reduce the number of separate administrations needed to achieve immunity to the various vaccine antigens.

Despite the multiple forms vaccines may take, several common features characterize the manufacture and testing of vaccines. This chapter focuses on commonalities throughout the manufacturing process, from raw material qualifications to final release tests.

Regulations and Standards

Vaccines are regulated by FDA as biological products. The general requirements are listed in national laws and international guidances. For the U.S., national requirements are codified in 21 CFR, the 200 and 600 sections, with additional recommendations available in FDA *Points to Consider* and *Guidance* documents (www.fda.gov). International guidances are available from the International Conference on Harmonization (ICH) (www.ich.org; see *Appendix 2*) and the World Health Organization. New methodologies are continually being developed and validated and will be included in *USP* as they become available. Reference standards are available from USP and FDA.

OVERALL MANUFACTURING PLAN

When considering the overall plan for manufacturing a vaccine, manufacturers need to consider the following factors:

- Physical facilities;
- Raw materials and process aids;
- Actual manufacturing process, including
 - a. initial process (production of virus/bacteria and recombinant materials);
 - b. downstream processes (purification or chemical modification, if applicable);
- Antigen modifications such as conjugation or toxoiding;
- Storage of process intermediates and final bulk;
- In-process and final product testing regimens and control schemes;
- Addition of adjuvants, if applicable;
- Formulation and filling;
- Container–closure system; and
- Stability program that supports the dating period of the product.

Quality systems are needed to support the following manufacturing process development: specifications for raw materials, process intermediates, and final product; change control; and failure investigations and complaints. All of these elements are important in the life cycle of the vaccine product.

The overall goal of a comprehensive manufacturing program is to consistently produce a vaccine that is safe and effective. Concurrently with clinical development of the vaccine, the manufacturing process is refined and the process and testing methods are validated for consistency. This includes systems to control changes to the process or inputs. Manufacturers should expect that changes will be required during the vaccine’s manufacturing life cycle, and manufacturers necessarily will use data from development and routine manufacturing to assess the process as well as proposed changes. The manufacturers should adopt systems that continually evaluate all aspects of manufacturing to identify unanticipated changes in vaccine quality and to assess them as quickly as possible.

Manufacturing Facilities and Systems

Manufacturers should have a general layout of manufacturing facilities, including diagrams that show the following: flow of raw materials and process inputs; movement of

product, intermediates, waste streams, and personnel; and air flows and pressurization levels. These diagrams assist in minimizing the risk of potential product contamination from various sources. These sources can include cross-contamination from other products, contamination from different batches of the same product, and extraneous contamination from microorganisms and personnel. Evaluation of the flow diagrams can assist with strategies for development of engineering controls, personnel procedures, and monitoring systems to enable compliance with Good Manufacturing Practices (GMPs). Analysis of potential risks may also provide insights about what information should be recorded in batch documentation to facilitate consistent manufacture and also to facilitate failure investigations. Together, physical facilities, procedures, personnel, training, and quality systems make up the GMP environment in which a vaccine will be produced.

Manufacturing Process

The manufacturing process includes process inputs such as raw materials and processing aids and unit operations comprising both the initial and downstream processing steps. A process flow map for the manufacturing process is useful and assists in validation of the manufacturing process. This map shows all unit operations, the inputs to each operation, and the outputs to subsequent manufacturing steps. Analytical testing done at relevant steps and the specifications required to proceed to the next stage of processing may be added to the map. A process map also supports a processing space to facilitate a rugged process, i.e., one based on suitable characterization studies to establish boundaries within which manufacturing can occur to promote unchanged safety and efficacy outcomes.

The process flow map should include all steps from making the seed/cell bank (described below) to formulation and filling of the final product. The validation strategy should include the steps that require validation, along with identification of the process space, associated critical process parameters (CPP), and critical quality attributes (CQA). The critical process parameters are those that directly affect core quality attributes needed to successfully manufacture a batch of product. Some manufacturers identify other processing parameters that are important for processing but do not affect critical quality attributes. These important but noncritical factors help identify the process development space, can contribute to the development of a rugged process, or can be useful when the company assesses processing deviations. The concepts of quality by design and exploration of the process space are relatively new to the biologics/vaccine industry but are becoming considerations for the overall development-planning process.

Manufacturing Surveillance

Manufacturing surveillance is the continual observation of how the process and the resulting product are performing. This section is not exhaustive; rather, the points raised here outline the types of considerations recommended for a manufacturer during development of a vaccine. Manufacturing surveillance includes the following:

- Periodic review of the performance of the manufacturing process;
- Analytical assays;
- Stability programs;
- Product complaints;
- Adverse event reports;
- Product failure investigations;
- Atypical or deviation events.

Taken as a whole, these activities allow a manufacturer to assess the state of the process and product and to evaluate which, if any, operations need to be modified. These same systems also provide a surveillance matrix to evaluate changes. In any of these programs it is also valuable to de-

velop additional characterization assays that are not used for process intermediate or product release purposes but may be used for further evaluation when additional information is needed or desired. These additional assays for characterization are often based on different underlying analytical procedures to provide different ways to evaluate materials.

Routine surveillance processes are increasingly implemented to attempt to detect changes in processes before any critical quality attributes are adversely affected. Not all vaccine processes can be characterized to the same extent or level (e.g., a live virus vaccine vs. a recombinant protein vaccine), and statistical tools are often used to determine alert or action levels in surveillance programs. Exceeding these levels requires the manufacturer to evaluate the situation but does not necessarily signal product failure.

GMP manufacturing entails facility design, process development, quality systems, and manufacturing surveillance. Together these systems help the manufacturer to control the production of a vaccine. As noted, many types of vaccine are marketed, and each has its unique features and therefore requires different plans for each of the steps mentioned in this section.

SEED LOT SYSTEMS

Seed lots are the stocks of specific strains of bacteria, viruses, or biotechnology-engineered cells used to express vaccine antigens. All seed lots should be documented in terms of their isolation, derivation (or construction, in the case of recombinant vector or engineered cells), and passage history. The purpose of a seed-lot system, which typically includes master and working stock seeds, and associated master and working cell banks, is to help ensure the consistency of vaccine manufacturing. The use of master and working seed lots provides a method to limit the replication of the seed and to minimize the possibility of genetic variation.

A master seed lot is a physically homogeneous preparation derived from an original seed processed at one time and passaged for a limited number of times. The master seed lot is characterized for its biological, biochemical and genetic characteristics, and to ensure its purity, its freedom from adventitious agents, and its clinical ability to produce an effective vaccine.

Cultures from the working seed lot should have the same characteristics as the master seed lot from which they are derived. For influenza vaccines, which may be reformulated with new virus antigens each year, certified seed lots can be obtained from national regulatory agencies.

A working seed lot is derived from the master seed within a limited number of passages. The working seed is tested to ensure its purity, freedom from adventitious agents, and biochemical properties. The working seed is used for production of vaccine without intervening passages.

Bacterial Vaccine Seed Lot System

In the bacterial seed lot system, a master seed is subcultured to produce a working seed one passage beyond the master seed. An aliquot of the working seed is then expanded to produce a vaccine lot. The strain(s) used for the master seed lots are identified by historical records that include information about their origin. Information about the bacterial seed lot system should include source, passage history, and raw materials to which it was exposed, with specific emphasis on raw materials of ruminant origin. Seeds should be stored at an appropriate temperature in more than one location within a facility or at a distant site in order to decrease catastrophic risk.

Identity tests may include inoculation onto suitable biochemical media, Gram stains, genotype, and serological identification with suitable specific antisera. Special tests

may be added, for example, to show culture viability but also lack of virulence.

Purity of the bacterial strains used for seed lots is verified by methods of suitable sensitivity to ensure that no adventitious agents are present. These purity tests often are performed in the presence of the seed under conditions where growth is inhibited by the presence or the absence of specific nutrients. Streaking can also be used to show that the cultured seed is a pure culture.

Viral Vaccine Seed Lot System

The derivation and passage history of viral seeds should be recorded in detail. Any manipulation of the viral phenotype (e.g., cold adaptation, development of temperature sensitivity, or attenuation of virulence) or intentional genetic manipulations (e.g., reassortment or recombination) should be documented.

These viral seeds are commonly differentiated into a master viral seed and working viral seeds or working viral stock. Viral seeds should be stored at cryogenic temperatures to promote stability and in more than one location within a facility or at a distant site to decrease catastrophic risk. Manufacturers should assess the following characteristics of the viral seed stock:

- Growth characteristics on the intended production cell substrate;
- Tissue tropism;
- Genetic markers;
- Identity (for recombinant vectors);
- Viability during storage;
- Genetic stability through production;
- Attenuation properties;
- Purity;
- Absence of adventitious agents. If attenuation or derivation is achieved by passage through different species, the viral seed should be assessed for absence of adventitious agents common to those species.

The master viral seed should be extensively characterized to demonstrate the stability of genotype and phenotype for a number of passages beyond the level used in production. Generally, during assessment of genetic stability, a master seed undergoes a minimum of five passes beyond the passage that will produce the final vaccine.

Tests should be performed for identity (e.g., sequencing the entire virus or a portion of it), adventitious agents, viral phenotype, genetic stability, and, if applicable, agents that might be present in the seed as a result of its passage history. Viral phenotype can be assessed further for tissue tropism, attenuation properties, and temperature sensitivity. Not all of these tests may be necessary for every viral seed strain.

In some cases the viral seeds may have a broad host range and therefore may require neutralization of the vaccine virus before they are tested for adventitious agent(s). If possible, testing for adventitious agents should be done without neutralization in order to avoid an antiserum that may inadvertently neutralize an adventitious agent present in the seed. Sometimes it is not possible to effectively neutralize a viral seed, and in such cases alternative strategies can be used. For example, the test can be performed in a cell substrate that does not permit replication by the vaccine virus. However, such a substitution of the substrate cell may compromise the test's sensitivity for detection of other adventitious agents. Therefore, the tests may be supplemented with use of polymerase chain reaction (PCR) assays.

Assessment of neurovirulence may be appropriate if the virus is known to be neurotropic. Manufacturers should consult with regulators about appropriate animal models, methods, and scoring systems for this assessment before they initiate such studies. For viruses that are neurovirulent or may revert to neurovirulence (e.g., polioviruses), it may be necessary to assess neurovirulence beyond the master seed.

If the master viral seed is well characterized, the working viral seed may not require extensive characterization. For example, it may not be necessary to repeat testing for all the relevant viruses from the derivation history.

Systems for Biotechnology-Engineered Vaccines

For a vaccine produced via a biotechnology-engineered cell-expression system, a master seed lot or a master cell bank will be established during product development. The seed lot or cell banks should be homogenous, which is often accomplished by limiting dilutions. The seed lot or cell bank system should be characterized in a manner analogous to that used for the cell substrate discussed in the next section, and additional tests can be used to demonstrate the genetic stability of the expression system.

FERMENTATION AND CELL CULTURE MEDIA

A medium is the material in which an organism is grown and amplified in quantity to produce mass material for vaccine production. Its composition is diverse and depends on the cell types that the medium supports, ranging from well-defined chemical media to chemically undefined media that contain natural components such as sera from animal origin (see *Bovine Serum* (1024)). Culture media should be suitable for their intended purpose and should be free from adventitious agents and known undesirable components such as toxins, allergens, and similar compounds. If undefined ingredients are necessary, the amount should be kept below levels that are demonstrated to be safe for the final product.

Fermentation Media for Bacterial Growth

The nutrients consist of materials like proteins, sugars, inorganic trace elements, amino acids, and vitamins needed for bacterial growth. The protein component may be as simple as free casein (milk protein), or it can be as complex as extracts from bacterial, plant, or animal sources. Any fermentation nutrients of animal origin are sourced carefully and tested for adventitious agents. The composition of a medium is often customized to optimize product quality attributes. Medium components that are known to cause allergic reactions should be avoided.

Media for Cell Culture for Viral Vaccines

The types and composition of media used for isolation and all subsequent culture of components of viral vaccines need to be recorded in detail. Chemically defined media without materials of animal origin are preferred. The medium should be tested for sterility and suitability for the cells used in product production. If materials of animal origin are used, they are assessed for freedom from adventitious agents. If human albumin is used in a U.S.-licensed vaccine, it must be licensed by FDA. The final product should be within specified limits of residual medium components such as serum, antibiotics, selection agents or reagents added for growth enhancement.

Media for Biotechnology-Engineered Cells

The requirement for media used for the fermentation and propagation of biotechnology-engineered cells is the same as that noted above for bacterial fermentation and cell culture growth.

PROPAGATION AND HARVEST

The propagation and harvest phases follow the manufacturing process from the initiation of cell growth in the working cell bank to the separation of the crude drug substance. In addition, in these manufacturing process steps, raw materials, media, and solutions should be qualified for their intended use. Batch numbers should be clearly assigned as needed, and the relationship between component harvests and batches of individual drug substances should be recorded clearly.

Propagation and Harvest for Bacterial Vaccines

Propagation of bacteria for bacterial vaccines is performed under specified conditions for the inoculum preparation and the fermentation phases. In-process monitoring and testing should be conducted for quality assurance. All controls and testing performed after production (e.g., purity, viability, antigen yield, and phenotypic identity) should be documented. The first step of drug-substance recovery is harvesting from the bioreactor. A variety of equipment is available, and the process equipment used depends on the nature of the process. Procedures should be established to ensure containment and prevention of contamination during harvesting and to monitor bioburden (including acceptance criteria) or sterility. The storage conditions and the stability time limit for the harvest material should be described. For most bacterial vaccines, an inactivation step is necessary. Personnel involved in bacterial inactivation should consider the following: how cell culture purity is verified after inactivation, whether culture purity should be defined before inactivation, choice of the inactivation agent, and validation of the procedure(s).

Propagation and Harvest for Viral Vaccines

The manufacturing of viral vaccines using eukaryotic cell culture includes a two-phase production process. The first is the expansion of the cell cultures used as a substrate for viral replication. The second phase includes the initial virus infection and subsequent replication and virus production.

Cell Substrate Growth Phase—The cell substrate expansion process for viral production is the phase designed to prepare the cells in a physiological state appropriate to sustain virus growth. Cell substrates often require complex animal-derived supplements such as serum. The source and testing requirements of bovine serum are subject to regulatory requirements (see *Bovine Serum* (1024)).

Virus Production Phase—Relatively few cell types have been used as substrates in U.S.-licensed viral vaccines, but these include primary cells (e.g., certain cells derived from monkey, chick, or mouse tissue), diploid cell lines (e.g., WI-38, MRC-5, or FRhL-2), and continuous cell lines (e.g., Vero). Vaccine manufacturers have optimized nutrient requirements, growth factors, and serum concentration to support robust growth and strong virus productivity for these cell lines.

PURIFICATION

The objective of the purification steps is to remove as much as possible of the impurities in the initial harvest and to maximize the purity of the final vaccine product. Process residuals may consist of materials from the culture medium and/or cellular components. Purification procedures should be optimized and validated. When applicable, viral clearance steps (viral removal or inactivation) should be included and validated using relevant model viruses. Special considerations are observed depending on the types of vaccines and production system used, as discussed below.

Bacterial Fermentation

Bacterial fermentations are typically highly productive and yield large amounts of biomass. For bacterial subunit products or recombinant components expressed by bacteria, fermentation can produce very high concentrations of the desired active ingredient. Manufacturers should initiate culture purity testing before further processing.

Live Bacterial Vaccines—Live bacterial vaccines such as *Bacillus Calmette-Guérin* (BCG) and *Salmonella typhi* Ty21a are relatively fragile as pharmaceutical products and therefore tolerate only fairly gentle purification approaches. If osmotic and shear forces are constrained, then the integrity of the bacteria usually can be maintained.

Inactivated Bacterial Vaccines—At present no inactivated whole-cell bacterial vaccines are licensed for use in the U.S.

Purified Bacterial Antigens—Purification of bacterial components (e.g., proteins, toxins, and polysaccharides) generally requires cell disruption. More selective purification methods can be used to remove culture media and bacterial impurities and to achieve high purity of the target bacterial component.

Biotechnology-Engineered Cells

Of special concern in the purification of recombinant-derived vaccine components is the issue of residual host cell components that could produce an adverse immunogenic response in patients. This response could be exacerbated by the presence of vaccine adjuvants.

Recombinant Virus-Like Particles (VLP)—Formation of VLPs can coincidentally result in incorporation of host cell components (e.g., DNA) into the quaternary structure of the molecular assembly, resulting in a class of impurities that has a tight association with the active pharmaceutical ingredient. As a result, modern approaches to VLP production in some cases include a disassembly step that dissociates impurities from the viral proteins. This procedure is followed by a reassembly step that reforms the VLPs in the absence of the host components. Liquid-phase extractions and chromatographic procedures can be used to provide high-purity components for use in vaccine products with no substantial risk of carrying over significant residual host components.

Viral Vaccines Derived from Cell Culture

Viral Vaccines Derived from Continuous Cell Lines—If a continuous cell line (e.g., Vero) is used for vaccine production, a validated filtration step is necessary to separate virus from intact cells. The quantity and size of any residual host cell DNA also should be determined (see general information chapter *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* (1130)). Currently, 10 ng of host cell DNA is permitted per dose of a parenterally administered vaccine, and regulatory agencies continue to consider on a case-by-case basis the level of risk posed by host cell DNA for vaccines that are administered by other routes (e.g., nasal or oral). Multiple purification methods to reduce the size and amount of residual host-cell DNA present in the vaccine are desirable and include steps such as treatment with DNase, diafiltration, ultrafiltration, and column chromatography.

Viral Vaccines Derived from Human Diploid Cell Culture—FDA has licensed several vaccines made using human diploid cells. The two most commonly used diploid cell lines are MRC-5 and WI-38, both of which are derived from human embryonic cells and have the normal diploid number of human chromosomes. They are widely used to manufacture vaccines because they have been shown to have no tumorigenic or oncogenic potential and have been

shown to be susceptible to a wide range of human viruses. However, unlike continuous cell lines that can be passaged indefinitely, human diploid cell lines are capable of attaining only a certain number of population doublings, after which they experience a rapid decline in their ability to proliferate. This issue is managed by freezing multiple aliquots of master and working cell banks.

Viral Vaccines Derived from Primary Cell Culture—Like diploid cells, primary cells normally are not tumorigenic or oncogenic. However, when primary cells are used to manufacture live vaccines, the donor animals from which the primary cells are obtained are extensively tested for a variety of pathogens before being used. For example, chicken flocks used to prepare chicken embryo kidney cells undergo extensive serological testing for adventitious agents before the flock can be used to prepare the cells. Some of these tests are described in the Code of Federal Regulations (CFR, see the sections listed in *Appendix 2*) and the USP general information chapter *Virology Test Methods* (1237).

Viral Vaccines Derived from Chicken Eggs

The embryonated chicken egg is a highly productive growth substrate for certain viruses, such as those used to make vaccines for yellow fever and several influenza vaccines. In the case of influenza vaccines, vaccine virus is harvested from egg allantoic fluid. In the case of yellow fever vaccine, the vaccine virus is harvested from embryo tissues. Therefore, residual egg or embryo components are special considerations in vaccine purification.

Egg-based vaccine production, like all biomass expansions, requires care and quality control of the virus seed lots and egg substrates to avoid contamination with other organisms.

Live Attenuated Virus Vaccines—Viruses for live vaccines (e.g., yellow fever or live influenza) are produced using Specific Pathogen-Free (SPF) eggs. These eggs are produced by chicken flocks that are regularly screened for avian pathogens (e.g., avian leukosis virus) and are maintained using appropriate animal husbandry practices. To preserve the infectivity and antigenic integrity of the vaccine viruses while removing egg-derived components, relatively simple, mild methods (e.g., zonal sucrose gradient centrifugation and diafiltration) are used for vaccine virus concentration, purification, and buffer exchange.

Inactivated Whole Virus Vaccines—Viruses for inactivated vaccines can be produced using non-SPF eggs because of required chemical inactivation steps in the manufacturing process. Because the vaccine virus needs to be retained intact while removing egg-derived components and inactivating chemicals, relatively mild purification and concentration methods (e.g., zonal sucrose gradient centrifugation) are used. If chemical agents are used in the process, they should be minimized in the final product to below prespecified levels.

Split Virus and Purified Subunit Vaccines—Viruses for split virus and purified subunit influenza vaccines are produced in non-SPF embryonated eggs. Inactivation and purification of vaccine viruses are achieved by chemical treatment (e.g., formaldehyde or β -propiolactone) and zonal sucrose gradient centrifugation, respectively. Split virus vaccines are prepared by disruption of vaccine virus particles using a detergent (e.g., sodium deoxycholate) that preserves antigenic integrity.

INTERMEDIATES

Intermediates are defined here as the unformulated active (immunogenic) drug substances that are processed before final formulation and can be stored for long periods of time before further processing. These intermediates can be stored and should be included in a formal stability program. Exam-

ples of intermediates include bulk polysaccharides, purified recombinant proteins (concentrates), and conjugates.

Production of Intermediates

Intermediates are manufactured from starting materials by one or a combination of different processes (e.g., fermentation, cultivation, isolation, or synthesis). Subsequent steps of the procedure involve preparation, characterization, and purification, eventually resulting in the drug substance. Quality systems documents are adopted for production and all applicable information should be recorded in a controlled document (i.e., a batch record). When applicable, stability studies and release tests should be performed before proceeding to the next steps (see below).

Tests for Intermediates

The quality attributes of the intermediate are commonly tested in conjunction with further processing. Characterization beyond release testing should be considered. Characterization methods can use appropriately qualified procedures. Some tests are routinely performed before the intermediates are converted to the final bulk, depending on individual vaccines.

If intermediates need to be stored and/or subsequently shipped to a different location for further processing, the stability of these materials should be demonstrated. Stability tests can be a combination of both physicochemical analysis and biological assays.

FINAL BULK

Final bulk is the bulk drug product that contains the drug substance(s), excipients, and other ingredients at desired concentrations and is ready for filling into individual containers.

Production of Final Bulk

Appropriately controlled amounts of all ingredients are blended to uniformity to produce the final bulk. The processing may include one or more steps such as buffer exchange and addition of diluents, bulking agent, stabilizing excipients, adjuvants, and preservatives. Final bulk may be prepared aseptically or processing may include a sterilization step.

Tests for Final Bulk

The quality attributes of the final bulk should be tested. Appropriate testing should be performed with respect to identity, purity, potency, sterility (see *Sterility Tests* (71)), and antimicrobial effectiveness (see *Antimicrobial Effectiveness Testing* (51)). Tests demonstrating safety, if applicable, are performed. The list includes, for example, tests for the absence of adventitious agents, mycoplasma, and other microorganisms.

Testing is required for specific process-related and product-related impurities, depending on the vaccines being manufactured. In addition, tests are required for the bulking agent, stabilizing excipients, adjuvants, and/or preservatives, if used. All the testing should be done according to respective standard operating procedures (SOPs), and all tests should have specifications (or provisional specifications, where applicable).

Stability Test for Final Bulk

If final bulks are stored and/or subsequently shipped to a different location for further processing, the stability of these

materials should be demonstrated. Stability tests can be a combination of both physicochemical analysis and biological assays. Implementation of a stability program is required for formal stability studies, and the studies should be executed according to a protocol that contains detailed information about types of tests, including specifications, testing intervals, and data and analysis.

FINAL CONTAINER

A final container of vaccine contains the active ingredient(s) (i.e., antigen(s)) as well as additional components, such as stabilizers, adjuvants, or antimicrobial preservatives. They also may include residual materials from the manufacturing process.

Excipients and Other Additives

In addition to specific antigens, vaccines often include excipients and other additives that are intentionally added to the vaccine by the manufacturer for a specific purpose. These include adjuvants, antimicrobial preservatives, and stabilizers. Vaccines also contain manufacturing residuals, which are trace amounts of various components used during manufacturing. Thus, the combinations of these components comprise and define the complete vaccine product. Manufacturers must adhere to regulations governing permissible limits of such components, as indicated in the product's license.

Adjuvants—Adjuvants are agents incorporated into vaccine formulations to enhance and increase the immune responses generated by the vaccine antigens. Specifically, they can increase the amount of antibody produced, direct the immune response (Th1 or Th2), increase the duration of antibody presence (persistence), or produce a combination of these effects.

Aluminum compounds have long been the most widely used adjuvants worldwide. Two methods traditionally have been used for combining aluminum adjuvant to antigen to form aluminum-adsorbed vaccines. The first involves the addition of the antigen solution to preformed aluminum precipitate. The second involves the addition of an antigen to aluminum in solution and the addition of a compound that will coprecipitate the aluminum salt and the antigen *in situ*. Solutions of aluminum potassium sulfate, known as alum or aluminum chloride, have been used together with phosphate salts as precipitating agents. A number of aluminum adjuvant formulations are used in vaccines.

Tests for aluminum are based on metal detection tests described in the general test chapter *Aluminum* (206). Regulations limit the amount of aluminum permitted in a dose of vaccine. The Code of Federal Regulations [21 CFR 610.15(a), *Ingredients, preservatives, diluents, adjuvants*] states that "the amount of aluminum in the recommended individual dose of a biological product shall not exceed:

1. 0.85 milligrams if determined by assay;
2. 1.14 milligrams if determined by calculation on the basis of the amount of aluminum compound added; or
3. 1.25 milligrams determined by assay provided that the data demonstrating that the amount of aluminum used is safe and necessary to produce the intended effect and are submitted to and approved by the Director, CBER [Center for Biologics Evaluation and Research at FDA]."

The third criterion above aligns U.S. regulations with World Health Organization guidance for aluminum content in a single human dose of a vaccine product.

Note that adjuvants are not licensed by themselves; they do not constitute a product. Rather, a vaccine consisting of specific antigen(s) and an adjuvant are licensed together as a drug product.

Antimicrobial Preservatives—In the case of multiple-dose containers, antimicrobial preservatives are added to inhibit the growth of microorganisms that may be introduced from repeated puncture of multidose vials. With certain exceptions, a preservative is required to be present in vaccines marketed in multidose containers [21 CFR 610.15(a)]. Exceptions include yellow-fever vaccine; measles, mumps, and rubella (MMR); and dried vaccines when the accompanying diluent contains a preservative.

The microbial preservatives currently used in vaccines are thimerosal, 2-phenoxyethanol, benzethonium chloride, and phenol. These agents must pass the appropriate antimicrobial effectiveness test, as described in *Antimicrobial Effectiveness Testing* (51). Antimicrobial test challenges should be conducted as part of the normal formal stability program, including at expiration date. Various tests for preservatives can be found in *Antimicrobial Agents—Content* (341).

Stabilizers—The primary purpose of stabilizers is to protect certain vaccines from adverse conditions such as heat or to serve as a cryopreservative during the lyophilization process, usually the freezing step. The particular materials chosen for this purpose include sugars (e.g., sucrose or lactose), amino acids (e.g., glycine or glutamic acid [monosodium salt]), glycerol, and proteins (e.g., human serum albumin [HSA] or gelatin). Materials should be customized to a specific vaccine formulation and selected with patient safety in mind.

When a protein is chosen as a stabilizer, two main safety concerns arise. One stems from the source of the protein: animal or human origin raises the possibility of the presence of an adventitious agent. The second concern is the possibility of an allergic reaction in persons sensitized to that protein. This should be evaluated as part of the clinical program during vaccine development. At present two proteins are used as stabilizers for vaccines: HSA and gelatin. FDA requires that any serum-derived albumin used in manufacturing be U.S.-licensed HSA. FDA guidance further recommends that a statement indicating the source and related risks appear in the "Warnings" section of the labeling for HSA-containing products.

Gelatin or processed gelatin also is used as a vaccine stabilizer. The gelatin source may be either bovine or porcine. Although the conditions of manufacturing gelatin are harsh (i.e., the product is subjected to extremes of heat and pH), there remains a concern with bovine sources about the presence of the transmissible spongiform encephalopathy (TSE) agent, because this agent is known to resist such conditions. Therefore, if gelatin added to a vaccine or used in manufacturing is from a bovine source, the material should have the appropriate documentation certifying that it comes from a country or region that is in compliance with TSE guidance for industry.

Manufacturing Residuals—Vaccines may contain residual amounts of any of the materials used in the manufacturing process. These materials are termed manufacturing residuals. As a general principle, it is not possible to remove a particular substance completely, nor is it possible to conclusively demonstrate that a particular substance has been completely removed. Therefore the goal is to reduce these substances to an undetectable level, using a sensitive and validated analytical methodology. Some products are tested for pyrogenic substances as a manufacturing residual (see *Pyrogen Test* (151)); and, if the product is freeze-dried, it should be tested for residual moisture (see *Loss on Drying* (731)). Residual levels of manufacturing materials, including, if applicable, inactivating agents, should be justified. The release specifications of these components are required as part of the approved license.

Cell-Derived Residuals—Live attenuated bacterial vaccines are not usually subject to a high degree of postexpansion purification. But killed bacterial component vaccines typically undergo significant purification to reduce cell-derived residuals. Common cellular components to be reduced are proteins, nucleic acids, and polysaccharides. Assays for

these components are routinely conducted, if appropriate, to ensure purity. A common residual in bacterial vaccines made from Gram-negative bacteria is lipopolysaccharide (LPS), commonly known as endotoxin. Endotoxin testing is performed during the manufacturing process for any Gram-negative bacterial vaccine. In the case of Gram-positive bacterial vaccines, the endotoxin testing should be conducted to ensure that no contaminants from Gram-negative bacterial growth are present. Also, there must be a release specification for this residual. Two tests are currently used to detect LPS in biological products, the *Limulus* amoebocyte lysate (LAL) test (see *Bacterial Endotoxins Test* (85)) and the rabbit pyrogen test (see *Pyrogen Test* (151)). The *Limulus* lysate that is used to test for bacterial endotoxin in FDA-regulated products is itself a U.S.-licensed product. The rabbit pyrogenicity test requires the use of animals and is more difficult to perform; therefore, it is not employed to the extent that the LAL test is used.

Viral vaccine manufacturing requires cell substrates to produce the viruses, which are then taken through purification processes. Generally, killed viral vaccines are more highly purified than are live attenuated ones. Depending on the method used to manufacture the vaccine, manufacturers work with FDA to develop prudent specifications for the final vaccine. Animal-derived host cells have been used extensively in vaccine manufacturing, particularly viral vaccines. For example, influenza and yellow fever vaccines are produced, respectively, in egg allantoic fluid and chicken embryos. Mumps, measles, and some rabies vaccines are produced in chick embryo cells. The labels of these products must state that residual chicken proteins may be present in the final vaccine, and the label may indicate how much is present. Further, the label also urges practitioner caution when vaccinating a person with known hypersensitivity to eggs.

Two U.S.-licensed hepatitis B vaccines are based on recombinant DNA-derived proteins expressed in yeast cultures. In both cases, the labels notify health care professionals that yeast protein may be present in the vaccine and recommend that suitable precautions should be exercised. In the case of live viral vaccines, considerations may be given to the reduction of cellular residual materials (e.g., host DNA, proteins).

Materials of Animal Origin—Some raw materials and reagents, such as gelatin, calf serum (see *Bovine Serum* (1024)), or trypsin for vaccine manufacturing raise concerns regarding the potential presence of adventitious agents. Raw materials should be sourced from countries acceptable to FDA. Additionally, manufacturers should test these materials when possible to minimize the risks of contamination with adventitious agents. Reduction of serum components (e.g., BSA) should be considered in processing.

Antibiotic Residuals—Some antibiotics (but not penicillin) can be used in minimal amounts in the manufacturing process for viral vaccines, according to 21 CFR 610.15(c). Those that have been used include gentamicin, streptomycin, neomycin, and polymyxin B. There is no requirement for tests of residual levels of these antibiotics in the final vaccine. However, according to 21 CFR 610.61(m), the calculated amount expected to remain as a residual in the final vaccine, based on the amount added and the dilution factor in the manufacturing process, must be stated on the product label.

Inactivating Chemical Agents—Several chemical agents have been used to inactivate bacteria and viruses or to detoxify toxins in vaccine production processes. Formaldehyde and β -propiolactone are the most commonly used inactivating agents. Other less often used inactivating agents include glutaraldehyde and hydrogen peroxide. As a manufacturing residual, the inactivating agent should be removed from the final product as thoroughly as possible. The upper limit for formaldehyde is generally 0.02%, equivalent to 0.1 mg per 0.5-mL vaccine dose. The limit for β -propiolactone should be below the limit of detection.

EVALUATING THE STABILITY OF VACCINES

The stability of vaccine products depends on the nature of a vaccine antigen, the product formulation, and the control of vaccine storage prior to use.

Vaccine products are evaluated with programs that include real-time long-term storage under prescribed conditions. The use of extreme temperatures to potentially accelerate degradation may help manufacturers understand the stability of the product.

Vaccine products, like all pharmaceutical products, should be evaluated to define suitable conditions for storage (21 CFR 610.50 and 610.53). General principles of stability testing for biological products are described in *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* (1049). Typically these concerns are focused on the final vaccine product, but evaluations also are needed for bulk intermediates to justify the conditions under which they are held. In both cases manufacturers define in advance the conditions to which the product will be exposed (e.g., temperature, light, and humidity) and the time range during which the product will be exposed to those conditions. Stability studies should evaluate all storage conditions to which the product or intermediate is likely to be exposed during production, handling, shipping, and storage so that appropriate time limits can be placed on the exposure to those conditions.

The primary criteria for defining the storage conditions for these intermediates and the final products are generally focused on acceptable maintenance of potency; but, as discussed below, there often are other attributes that need to be considered.

Evaluation of the stability of vaccine products has three general purposes. First, the products are shown to maintain an acceptable analytical profile throughout manufacture and use to preserve safety and effectiveness. Second, stability studies across several product batches provide an effective way to characterize the inherent properties of the product. This in turn leads to the third use, demonstrating manufacturing consistency in the product.

Stability Protocols

The overall experimental plan for evaluating the stability profile of a given set of product or intermediate batches typically includes specific definition of the conditions under which the samples will be stored and why these conditions are relevant, the length of time the samples will be stored at each condition, when samples will be tested during this time course, and the analytical measurements at each time point. Additionally, these stability protocols include itemization of the analytical procedures to be used. For stability studies that occur early in product development, the studies may be conducted to confirm the suitability of the product formulation and/or storage conditions. Later in development, stability studies are typically conducted to provide data supporting product dating period or intermediate hold time, to provide more elaborate product characterization, and to evaluate manufacturing consistency. These latter studies define product end-expiry specifications that allow definitions of acceptable and unacceptable product. Unacceptable product is defined as product that is no longer acceptable for use in clinical studies or for commercial use (e.g., because of degradation or loss of potency). Stability studies should be conducted over a duration sufficient to determine the point of loss of acceptable potency or other relevant parameters.

Analytical Measurements

Manufacturers should consider the rigor of the analytical method(s) used to evaluate the stability of complex products and improve their understanding of the parameters

that are critical to immunogenicity (including stability-indicator parameters). Selection of the stability-indicator parameters varies with each vaccine's unique characteristics.

The primary parameter that reflects stability for most vaccines is the potency assay (see *Potency Tests in Lot Release Testing*, below). This assay can take many forms, depending on individual vaccines (e.g., an infectivity assay for a live virus vaccine or a measure of the proportion of conjugated polysaccharide for a polysaccharide–protein conjugate vaccine). The potency assay is generally the key analytical result predicting whether a vaccine remains suitable for use and whether it will produce the expected clinical response. Other analytical measurements can provide important supplemental data, particularly those that have a clear link to the potency of the product. Examples include degradation profile, dissociation of a carrier protein from conjugated vaccines, and dissociation of an adjuvant from an antigen complex. Additionally, other common assays typically are performed as part of the stability study and may address physical or chemical changes in the product that may or may not affect its potency (e.g., general safety, degree of aggregation, pH, moisture, container, preservative, and enclosure).

Formal Evaluation of Stability Data and Product Expiry Dating

Vaccines must remain within potency specifications at the expiration date, provided that the product was stored under the normal conditions specified. Manufacturers should conduct stability studies to determine those storage conditions and that dating period to demonstrate that the product remains within the potency specifications. Manufacturers should conduct stability studies on a continuing basis. If a major manufacturing process changes, additional stability studies should be conducted to verify that there is no adverse impact on the stability profile. Under certain conditions such as process changes, accelerated stability studies could be conducted. An accelerated study involving temperatures both higher and lower than routine can evaluate the impact of temperature excursions on products. A similar evaluation should be done for product intermediates to establish how long a given intermediate can be held under defined conditions before it is processed further or discarded.

NOMENCLATURE

There are no uniform systems for naming new vaccines. 21 CFR 299 describes the cooperation of the FDA and the U.S. Adopted Names Council (USAN) in naming drugs, including vaccines. USAN is a private organization sponsored by the American Medical Association, USP, and the American Pharmacists Association. Section 262 in Title 42 of the Public Health Service Act requires that each package of the biological product be plainly marked with the proper name (name designated in the license 21 CFR 600.3) of the biological product contained in the package.

LABELING

Vaccine product labeling is regulated in compliance with 21 CFR 201 and 610. Requirements are set for container labeling and package labeling.

Container Label

Provisions are made for the following labels:

- Full label;
- Partial label; and
- No label on the container itself when the containers cannot support a label that includes all required information

and should be placed in a package that does include all required information.

The label should be affixed to the container in a manner that allows visual inspection of the contents for the full length or circumference of the container. If no package exists, the container bears all of the information required for the package label.

The full container label normally contains the following:

- Proper name of the product;
- Name, address, and license number of the manufacturer;
- Lot number or other lot identification;
- Expiration date;
- Recommended individual dose, for multiple-dose containers;
- The phrase *Rx only* for prescription biologicals; and
- Any applicable cautionary statements.

Package Label

In addition to the information required on the container label, the package label should describe the following:

- Any preservative used and its concentration, or the words *no preservative* if no preservative is used and its absence is a safety factor;
- Number of containers, if more than one; or
- Amount of product in the container, expressed as number of doses, volume, units of potency, weight, and equivalent volume (for dried product to be reconstituted); or
- A combination of the above to provide an accurate description of the contents, as applicable;
- Recommended storage temperature;
- The words *shake well*, *do not freeze*, or the equivalent, as well as other instructions when indicated by the character of the product;
- Recommended individual dose, for multiple-dose containers;
- Recommended route of administration, or reference to such directions in an enclosed circular;
- Presence of known sensitizing substances;
- Type of antibiotics added during manufacture and the amount calculated to remain in the final product;
- Inactive ingredients, when they constitute a safety factor or are referenced to an enclosed circular;
- Adjuvant, if present;
- Source of the product, when this may be a factor in safe administration;
- Identity of each microorganism used in manufacture and, if applicable, the production medium and the method of inactivation or reference to an enclosed circular;
- Minimum potency in terms of official standard of potency, or the words *no U.S. standard of potency*.

Prescribing Information

Detailed information about a vaccine appears in its prescribing information, commonly called the package insert. Increasingly, vaccines are distributed with patient package inserts written in lay language. Prescribing information (21 CFR 201.56 and 201.57) includes the following:

- Highlights of prescribing information
- Product names, other required information
- Boxed warning
- Recent major changes
 - a. Indications and usage
 - b. Dosage and administration
 - c. Dosage forms and strengths
 - d. Contraindications
 - e. Warnings and precautions
 - f. Adverse reactions
 - g. Drug interactions

- h. Use in specific populations (e.g., pregnancy, nursing mothers, pediatric, geriatric)
- i. Drug abuse and dependence
- j. Overdosage
- k. Description
- l. Clinical pharmacology
- m. Nonclinical toxicology
- n. Clinical studies
- o. References
- p. How supplied/storage and handling
- q. Patient counseling information

LOT RELEASE TESTING

General Principles

Manufacturers perform all appropriate tests for the licensed specifications for the product, according to 21 CFR 610.1 and 610.2. Samples of each licensed lot and protocols containing the manufacturers' test results are submitted to FDA. After FDA evaluates the protocol to ensure that the product specifications are met, and after satisfactory confirmatory testing, FDA approves the release of the lot if all tests meet the standards of safety, purity, and potency established for the particular vaccine product. After approval is granted, the manufacturer distributes and markets the product.

Guidelines are available regarding alternatives to lot release and a surveillance system. All of these variations are subject to the regulations in 21 CFR 610.2 that allow FDA to require that samples of any lot of licensed product (e.g., vaccine), together with the protocols showing results of applicable tests, be sent to FDA.

Common Tests

The tests common to all lots of all products include tests for potency, general safety, sterility, purity, identity, and constituent materials. The manufacturer completes these tests for conformity with standards applicable to each product. The results of all tests are considered, except when a test has been invalidated as a result of causes unrelated to the product (21 CFR 610.1).

Potency Tests (Vaccine-Specific)—The basic definition and requirements for vaccine potency and potency assays are provided in 21 CFR 600.3 and 610.10. A vaccine potency assay should indicate the therapeutic activity of the drug product as indicated by appropriate laboratory tests or by adequately developed and controlled clinical data. Potency may be expressed in terms of units by reference to a standard. Product potency tests vary with vaccine product types (e.g., viral, bacterial, live attenuated, inactivated, or polysaccharide). As a result, potency assays for vaccines span a variety of approaches to the expression of potency. In vitro potency tests for live virus may include plaque formation assays, endpoint dilution assays (e.g., the tissue culture infective dose [TCID₅₀], virus neutralization assays, or quantitative polymerase chain reaction [PCR] assays). Quantitative colony formation assays are used for live attenuated bacterial vaccines. Animal challenge tests for immunogenicity assays of potency, such as those for diphtheria and tetanus (U.S. Department of Health, Education, and Welfare, 1953; see *Appendix 2*), or rabies and anthrax show in vivo response. Antigenicity assays use enzyme-linked immunosorbent assays (ELISA), e.g., with hepatitis A or rate nephelometry and rocket immunoelectrophoresis (e.g., with pneumococcal polysaccharides). The potency tests for bacterial vaccines, such as the meningococcal polysaccharides, pneumococcal polysaccharides, or *Haemophilus b* protein conjugate vaccines use chemical and physical chemical assays. In the case of pure polysaccharide vaccines, the concentration or quantity of the vaccine component (polysac-

charide) and its quality (e.g., size) have been shown to be indicative of human immune response.

Assay precision and reproducibility vary with the different methodologies that are used in potency assays, ranging from the high accuracy and precision of chemical tests at one end of the spectrum to bioassays at the other end. The general test chapter *Design and Analysis of Biological Assays* (111) provides guidance for bioassays and applies to vaccine potency assays. Other tests should be validated as described in the general information chapter *Validation of Compendial Procedures* (1225).

Release Tests—Official release of vaccines by the vaccine regulatory authority may be based on either the bulk or the final container. It is highly desirable to perform potency tests on the final container. However, under certain circumstances this may not be practical or even possible; thus, a case-by-case approach would be required. The choice of whether to test the bulk or the final container derives from a number of considerations, such as the quantity of vaccine available for tests at the different manufacturing stages. For certain vaccines, both bulk and final container receive official release. The potency test is generally required for the final container. If it is not feasible to perform the potency test on the final drug product, the test is performed on the bulk material.

General Safety—For biological products that are intended for administration to humans, manufacturers perform a general safety test in order to detect any extraneous toxic contaminants. Procedures and exceptions are specified in 21 CFR 610.11.

Sterility—A sterility test of each lot of each product is conducted according to procedures described in *Sterility Tests* (71) and 21 CFR 610.12 for both bulk and final container material.

Bacterial Endotoxins—Each lot of final containers of a vaccine intended for use by injection is tested for bacterial endotoxins, as indicated in *Bacterial Endotoxins Test* (85).

Purity—Vaccines need to be free of extraneous material. Approved vaccine license applications indicate extraneous materials that are unavoidable in the manufacturing process for a specific product. The application may indicate test results and allowable limits for such materials, according to procedures described in 21 CFR 610.13.

Residual Moisture—Each lot of dried product is tested for residual moisture [see 21 CFR 610.13 (a), *Loss on Drying* (731), and FDA's *Guideline for the Determination of Residual Moisture in Dried Biological Products* (see *Appendix 2*)].

Pyrogens—Each lot of final containers of a vaccine intended for use by injection is tested for pyrogenic substances, as indicated in *Pyrogen Test* (151) and 21 CFR 610.13 (b).

Identity—The contents of a final container of each filling of each lot are tested for identity after labeling is completed. Identity is established by physical or chemical characteristics of the vaccine, inspection by macroscopic or microscopic methods, specific cultural tests, or in vivo or in vitro immunological tests. In large part, identity tests are performed to distinguish the subject vaccine from other materials manufactured at the same site (21 CFR 610.14).

Constituent Materials—Ingredients, preservatives, diluents, adjuvants, extraneous protein, cell culture-produced vaccines, and antibiotics are tested according to 21 CFR 610.15.

Permissible Combinations

Formulations that combine several vaccines must be licensed as combinations (21 CFR 610.17). The potency of each vaccine in the combination is individually tested and must meet the specifications in the context of the final combined product; other appropriate quality tests apply as well. For vaccines that are physically combined in clinical loca-

tions just before administration to a patient, prescribing information should describe specific procedures to follow in those settings.

Quality

In general, quality control systems for vaccine manufacture are identical to those routinely employed for production of other pharmaceuticals. These include raw material testing and release, manufacturing, process-control documentation, and aseptic processing. Manufacturers formally assign responsibility to designated staff for maintaining the continued safety, purity, and potency of the product and for ensuring compliance with applicable product and establishment standards, along with compliance with current GMPs. Analysts use reference standards and validated methods to determine active ingredients, residuals, and impurities. Manufacturers determine product safety in a variety of ways that may include the use of experimental animals, procedures to demonstrate product sterility, and tests to ensure product potency. The complexity of the quality control systems for vaccines lies in the variety of methods used to produce and control production. Lot release testing proceeds according to 21 CFR 610.2 and involves evaluating lots for safety, purity, and potency before release. Manufacturers follow FDA and applicable international standards for testing and validation. The basic considerations for validation are included in *Validation of Compendial Procedures* (1225), in addition to guidance documents issued by FDA and the International Conference on Harmonization (ICH) (see *Appendix 2*).

Alternative Tests

Modification of test methods or manufacturing processes as licensed may be permitted if the regulatory authority can be assured that the modifications cause no reduction in safety, purity, potency, and effectiveness of the biological product. It may be necessary for the manufacturer to file the proposed changes prior to implementation (21 CFR 601.12 and 21 CFR 610.9).

APPENDIX 1. TYPES OF VACCINES CURRENTLY LICENSED IN THE U.S. (EXAMPLES)

- Bacterial, live attenuated (e.g., *Salmonella typhi*)
- Bacterial, polysaccharide (e.g., meningococcal, pneumococcal)
- Bacterial, polysaccharide-protein conjugate (e.g., meningococcal, pneumococcal)
- Bacterial, toxoid (e.g., diphtheria, tetanus)
- Bacterial, extracts (e.g., pertussis, anthrax)
- Viral, live attenuated (e.g., influenza, measles, mumps, rubella)
- Viral, whole inactivated (e.g., rabies)
- Viral, subunit (e.g., influenza, hepatitis B, human papillomavirus)

APPENDIX 2. SELECTED REGULATORY DOCUMENTS

- 21 CFR 201.
- 21 CFR 299.
- 21 CFR 600.
- 21 CFR 610.
- Section 262 in Title 42 of the Public Health Service Act
- Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration (FDA). *Guidance for Industry—Revised Preventive Measures to Reduce the Possible Risk of Transmission of Creutzfeldt-Jakob Disease (CJD) and Variant Creutzfeldt-Jakob Disease (vCJD)*

by *Blood and Blood Products* (January 2002). <http://www.fda.gov/cber/gdlns/cjdvcjd.pdf>

- Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration (FDA). *Guideline for the Determination of Residual Moisture in Dried Biological Products* (January 1990). <http://www.fda.gov/cber/gdlns/moisture.pdf>
- Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration (FDA). *Guidance on Alternatives to Lot Release for Licensed Biological Products*. Federal Register 1993;58(137): 38771–38773. <http://www.fda.gov/cber/gdlns/alttrntvlot071493.pdf>
- Center for Biologics Evaluation and Research (CBER), U.S. Food and Drug Administration (FDA). *Guidance For Industry—Characterization and Qualification of Cell Substrates and Other Biological Starting Materials Used in the Production of Viral Vaccines for the Prevention and Treatment of Infectious Diseases* (September 2006). <http://www.fda.gov/cber/gdlns/vaccsubstrates.pdf>
- FDA periodically issues or updates Guidance for Industry and posts these documents at <http://www.fda.gov/cber/guidelines.htm>
- Department of Health, Education, and Welfare (now the National Institutes of Health). *Minimum Requirements for Immune Serum Globulin (Human)*. 3rd rev. Bethesda, MD: Department of Health, Education, and Welfare, 1953.
- International Conference on Harmonization (ICH). Q2(R1). *Validation of Analytical Procedures: Text and Methodology*. Geneva, Switzerland: ICH, 2005. <http://www.ich.org/LOB/media/MEDIA417.pdf>

GLOSSARY

1. **Acceptance criteria**—The product specifications and acceptance or rejection criteria, with an associated sampling plan, necessary for making a decision to accept or reject a lot or batch (or any other convenient subgroups of manufactured units).
2. **Active ingredient**—Any component intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of man or other animals. The term includes those components that may undergo chemical change in the manufacture of the drug product and may be present in the drug product in a modified form intended to furnish the specified activity or effect.
3. **Adventitious agent**—A microorganism (e.g., bacteria, fungi, mycoplasma, spiroplasma, mycobacteria, rickettsia, viruses, protozoa, parasites, TSE agent) that is inadvertently introduced into the production of a biological product.
4. **Batch**—A specific quantity of a drug or other material intended to have uniform character and quality, within specified limits, and produced according to a single manufacturing order during the same cycle of manufacture.
5. **Biological product**—Any virus, therapeutic serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment or cure of diseases or injuries of man.
6. **Cell bank**—Vials of cells of uniform composition (not necessarily clonal) derived from a single tissue or cell, aliquoted into appropriate storage containers, and stored under appropriate conditions.
7. **Cell line**—Cells that have been propagated in culture since establishment of a primary culture and have survived through crisis and senescence. Such surviving cells are immortal and will not senesce. Diploid

cell strains have been established from primary cultures and expanded into cell banks, but have not passed through crisis and are not immortal.

8. **Characterization**—Determination of the properties of a substance.
9. **Component**—Any ingredient intended for use in the manufacture of a drug product, including those that may not appear in such drug product.
10. **Container** (also final container)—The immediate unit, bottle, vial, ampule, tube, or other receptacle containing the product as distributed for sale, barter, or exchange.
11. **Control**—Having responsibility for maintaining the continued safety, purity, and potency of the product and for compliance with applicable product and establishment standards, and for compliance with current good manufacturing practices.
12. **Control cells**—Cells that are split off from the production culture and maintained in parallel under the same conditions and using the same reagents (e.g., culture medium) to perform quality control tests on cells that have not been exposed to the vaccine virus (which may interfere with some tests).
13. **Dating period**—The period beyond which the product cannot be expected beyond reasonable doubt to yield its specific results.
14. **Diploid**—Having the expected number of chromosomes for a species (i.e., two of each autosomal chromosome and two sex chromosomes).
15. **Drug product**—A finished dosage form (e.g., solution, suspension) that contains an active drug ingredient generally in association with inactive ingredients.
16. **End-of-production cells**—Cells harvested at the end of a production run or cells cultured from the master cell bank or working cell bank to a passage level or population doubling level comparable to or beyond the highest level reached in production.
17. **End-of-production passage level**—The maximal passage level achieved during manufacturing at final vaccine harvest. Cells may be evaluated at this level or beyond.
18. **Endogenous virus**—A virus whose genome is present in an integrated form in a cell substrate by heredity. Endogenous viral sequences may or may not encode for an intact or infectious virus.
19. **Expiration date**—The calendar month and year, and where applicable, the day and hour, that the dating period ends.
20. **Filling**—A group of final containers identical in all respects, which have been filled with the same product from the same bulk lot without any change that will affect the integrity of the filling assembly.
21. **Final bulk**—The stage of vaccine production directly prior to filling of individual vials.
22. **Free of and freedom from**—For a substance to be considered free of a contaminant, an assay must demonstrate that a defined quantity of the substance is negative for that contaminant to a defined level of sensitivity. The level of assay sensitivity is defined by the choice of assay and can be determined experimentally using standardized reagents. Alternatively, a validated process that is known to remove a contaminant to a defined level may be used to demonstrate freedom from that contaminant.
23. **Harvest**—Collection of material at the end of vaccine virus propagation in cell culture, from which vaccine will be prepared. This material may be the culture supernatant, the cells themselves (often in disrupted form), or some combination thereof.
24. **Inactive ingredient**—Any component other than an active ingredient.
25. **In-process material**—Any material fabricated, compounded, blended, or derived by chemical reaction that is produced for, and used in, the preparation of the drug product.
26. **Intermediates**—Unformulated active ingredients that are processed before final formulation and can be stored for long periods of time before further processing.
27. **Label**—Any written, printed, or graphic matter on the container or package or any such matter clearly visible through the immediate carton, receptacle, or wrapper.
28. **Latent virus**—A virus that is present in a cell, without evidence of active replication, but with the potential to reactivate, is considered to be microbiologically latent.
29. **Lot**—A batch, or a specific identified portion of a batch, having uniform character and quality within specified limits; or, in the case of a drug product produced by continuous process, it is a specific identified amount produced in a unit of time or quantity in a manner that assures its having uniform character and quality within specified limits.
30. **Lot number, control number, or batch number**—Any distinctive combination of letters, numbers, or symbols, or any combination of them, from which the complete history of the manufacture, processing, packing, holding, and distribution of a batch or lot of drug product or other material can be determined.
31. **Manufacture**—All steps in the propagation or manufacture and preparation of products. Includes, but is not limited to, filling, testing, labeling, packaging, quality control, and storage by the manufacturer.
32. **Manufacturer**—Any legal person or entity engaged in the manufacture of a product subject to license under the Public Health Service (PHS) Act. Manufacturer also includes any legal person or entity who is an applicant for a license where the applicant assumes responsibility for compliance with the applicable product and establishment standards.
33. **Master cell bank**—A bank of a cell substrate from which all subsequent cell banks used for vaccine production will be derived. The master cell bank represents a characterized collection of cells derived from a single tissue or cell.
34. **Master virus seed**—A viral seed of a selected vaccine virus from which all future vaccine production will be derived, either directly, or via working virus seeds.
35. **Oncogenicity**—The property of certain biological agents (e.g., viruses) or materials (e.g., nucleic acids) that are capable of immortalizing cells and endowing them with the capacity to form tumors. Oncogenicity is distinct from tumorigenicity.
36. **Package**—The immediate carton, receptacle, or wrapper, including all labeling matter therein and thereon, and the contents of the one or more enclosed containers. If no package is used, the container shall be deemed to be the package.
37. **Passage level**—The number of times, since establishment from a primary cell culture, a culture has been split or reseeded.
38. **Population doubling level**—The number of times, since establishment from a primary cell culture, a culture has doubled in number of cells.
39. **Potency**—The therapeutic activity of the drug product as indicated by appropriate laboratory tests or by adequately developed and controlled clinical data. Potency may be expressed in terms of units by reference to a standard.
40. **Primary cells**—Cells placed into culture immediately after an embryo, tissue, or organ is removed from an animal or human and homogenized, minced, or otherwise separated into a suspension of cells. Primary cells may be maintained in medium, but are not passaged (split).
41. **Process**—A manufacturing step that is performed on the product itself which may affect its safety, purity, or potency, in contrast to such manufacturing steps

which do not affect intrinsically the safety, purity, or potency of the product.

42. **Proper name**—The name, designated in the license, to be used on each package of the product.
43. **Purity**—Relative freedom from extraneous matter in the finished product, whether or not harmful to the recipient or deleterious to the product. Purity includes but is not limited to relative freedom from residual moisture or other volatile substances and pyrogenic substances.
44. **Qualification**—Determination of the suitability of a material for manufacturing based on its characterization.
45. **Safety**—The relative freedom from harmful effect to persons affected, directly or indirectly, by a product when prudently administered, taking into consideration the character of the product in relation to the condition of the recipient at the time.
46. **Specification**—The quality standard (i.e., tests, analytical procedures, acceptance criteria) provided in an approved application to confirm the quality of products, intermediates, raw materials, reagents, components, in-process materials, container-closure systems, and other materials used in the production of a product.
47. **Standards**—Specifications and procedures applicable to an establishment or to the manufacture or release of products, which are prescribed in this subchapter or established in the biologics license application and designed to ensure the continued safety, purity, and potency of such products.
48. **Sterility**—Freedom from viable contaminating microorganisms, as determined by tests prescribed by the FDA.
49. **Tumorigenic**—A property of certain cell types to form tumors when inoculated into animals (generally a syngeneic, an immunosuppressed allogeneic, or an immunosuppressed xenogeneic host). These tumors may be at the injection site or a different site and may also metastasize to other sites.
50. **Tumorigenicity**—The process by which immortalized cells form tumors when inoculated into animals. Tumorigenicity is distinct from oncogenicity.
51. **Unacceptable product**—Product that is no longer acceptable for use in clinical studies or for commercial use (e.g., because of degradation or loss of potency).
52. **Validation**—The performance characteristics of an analytical procedure, based on the demonstration that the procedure is suitable for its intended purpose or use. Validation of a process is the determination of the extent to which a process meets the requirements for the various performance characteristics and the demonstration that the process uniformly performs to defined characteristics. Validation is generally performed in accordance with *Validation of Compendial Procedures* <1225> and the relevant ICH guidelines.
53. **Viral clearance**—The combination of the physical removal of viral particles and the reduction of viral infectivity through inactivation.
54. **Virus seed or viral seed**—A live viral preparation of uniform composition (not necessarily clonal) derived from a single culture process, aliquoted into appropriate storage containers, and stored under appropriate conditions.
55. **Working cell bank**—A cell bank derived by propagation of cells from the master cell bank under defined conditions and used to initiate production cell cultures on a lot-by-lot basis.
56. **Working virus seed**—A viral seed derived by propagation of virus from the master virus seed under defined

conditions and used to initiate production cell cultures lot-by-lot.

<1237> VIROLOGY TEST METHODS

INTRODUCTION

This chapter describes virology test methods applicable to the development of biological product drugs, such as recombinant proteins, subunit vaccines, therapeutic monoclonal antibodies, and growth hormones. Several topics are excluded from the scope of this chapter:

- Blood- and plasma-derived products as well as whole blood and plasma products used directly in transplantation or infusion. However, the basic principles, strategies, and testing methods for ensuring virus-free products are applicable.
- Methodologies for the safety testing of live viral vaccines.
- Specific methods for viral clearance studies, which are described in the *USP* general information chapter *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* (1050).

Virology test methods have historically been employed in the clinical settings of disease diagnosis, intervention, and containment; but the development of biological (biologics and biotechnology-derived) products and therapies for human or animal use has created the need for sensitive viral detection assays for use in the GMP production and testing of biological products. This need is not limited to the production of viral vaccines, but also applies to the development and manufacture of recombinant proteins, cell and gene therapies, and other products.

Sensitive virology test methods for quality control of biological products are necessary for several reasons. The production of biological products often requires a variety of raw materials and processing reagents of animal origin that have varying potential for introducing viral contaminants. The production of biological products may allow the replication of adventitious agents during processing, and therefore these materials must be prescreened to avoid the opportunity for contamination of the product. Another point to consider regarding screening these materials is that the product may not be compatible with processing methods used to eliminate or inactivate these adventitious agents. Because of the nature of the biological products, the production process needs to include appropriate testing regimens that monitor the possible introduction of adventitious agents and/or viral agents into the systems used. For these reasons, sensitive viral detection methods are required not only for the release testing of biological drug products, but also during the intermediate stages of processing, process development, and routine manufacture. Important stages for consideration include the development of cell substrates and banks, raw materials of animal origin, process intermediates, and critical excipients when derived from animal tissues. This strategy should be augmented with viral clearance and inactivation studies whenever possible.

For products intended to contain live viruses (e.g., infectious oncolytic viruses and live viral vector products used for gene therapy), the cell- and animal-based infectivity methods discussed in this chapter may be useful only following neutralization of the specific viral entity contained in the product for any product that is intended to contain live vi-

ruses (e.g., infectious oncolytic viruses and live viral vector products used for gene therapy). Alternatively, selection of appropriate indicator cell lines or animal models in which the specific viral entity is known not to replicate can be considered. It should also be expected that assay systems based on detection of viral particles or viral components will indicate the presence of the viral entity itself in such products, but may not indicate the viability of the virus. The remainder of the chapter is divided into three sections discussing assays for the three topics: (1) *Detection of Viable Viruses*, (2) *Detection of Viral Components*, and (3) *Detection of Antibodies to Viral Antigens*. The chapter covers the classic virology methods that are still routinely used, as well as modern molecular and immunological approaches. The methods described in these sections may possess different sensitivities to diverse viruses; they are therefore intended to complement each other to provide a science-based foundation for the detection of adventitious viruses. Multiple methods may be used in complementary fashion to improve the pathogen safety margin of a product. Identification of viruses detected in cell-based assays on the basis of cytopathic effects often depends on the use of molecular and immunological analyses; these analyses are therefore relevant both to viral detection and to subsequent viral identification. The chapter provides an overview of the detection and analysis of the most important groups of viruses as well as the most commonly used techniques. Tests specific to individual vaccines or biological products are excluded, because they are expected to be included in monographs for such products.

Methods that are well established with little variation in practice are described in more detail, whereas methods that are more flexible are described in general terms, both in the performance of the tests and in considerations for acceptance. Relevant regulatory references are given in the *Appendix*. Relevant USP general chapters should be consulted with regard to bioassay design, data analysis, interpretation, and assay validation.

DETECTION OF VIABLE VIRUSES

Infectious virus particles contaminating biologics and biotechnology-derived products are of great safety concern, because they have the potential for causing serious, possibly life-threatening, infections in the patients treated. This is particularly true if the patients are immunocompromised. Although complete assurance of viral safety for finished biological products can never be realized, a significant safety margin can be established through viral detection methods applied to unprocessed bulk and raw materials before purification in combination with purification processes that demonstrate the ability to inactivate or remove potential viral contaminants present at levels too low to detect. (See *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* (1050) for information on viral clearance and inactivation methods.) For cell and gene therapy products that lack extensive purification steps, final products may be directly tested for the presence of relevant contaminating viruses. This section describes two broad systems for the detection of infectious virus: cell culture-based infectivity assays and *in vivo* infectivity assays. These systems may possess complementary sensitivities for viruses, and as a result, both methods may be used as limit tests for cell bank and raw material characterization and for lot release testing of biologics and biotechnology-derived products. Considerations for optimizing sample preparation for these tests are discussed, followed by a description of the more commonly employed detection assays. Finally, to ensure the reliability of experimental results, quality control issues in general and detection limit estimation in particular are discussed.

Sample Selection of and Preparation for Cell- and Animal-Based Virus Detection Assays

The requirements for selection, preparation, and storage of test samples for viral detection methods (cell- and animal-based) are dictated by the lability of the viruses being detected. The ability of a virus to remain infectious in the absence of a host cell is highly variable. Virus infectivity also may differ in sensitivity to repeated freezing and thawing cycles.

Sample preparation typically involves storage of test samples at low temperatures (ideally -60° or below) as soon as practicable upon collection. When intended for use in a viral screening assay, aliquots of samples should be prepared to avoid multiple freezing and thawing. Samples intended for viral infectivity assays are typically shipped with sufficient dry ice to last several days more than the expected time required for transit. When received at the testing laboratory, the sample should be examined to verify that it is still frozen, and appropriate documentation should be completed. For any storage or hold condition, the impact of the condition on viral viability should be empirically assessed and sufficient cold chain management ensured.

Typical sample types for viral detection assays are described below.

Cell Lysates—Test samples derived from cell substrates (master and working cell banks, end-of-production cell samples) are prepared in a manner that allows sampling of both the cells (for cell-associated viruses) and the conditioned medium (for virus shed into the medium). To achieve this, a culture of the cells is sampled. A cell suspension of $\sim 10^7$ cells per mL in conditioned medium is prepared and frozen (ideally at -60° or below). Because this medium does not contain cryopreservative, the majority of the cells will lyse upon thawing of the sample, releasing the cell-associated virus. Low-speed centrifugation will remove larger cellular debris and yield a supernatant that may be inoculated directly onto detector cells in cell-based viral infectivity assays. A similar sample is prepared for *in vivo* viral adventitious agent testing. In this case, however, the test sample is thawed and injected without clarification into the various animal systems via the various described routes.

Biotechnology Bulk Harvest (Unprocessed Bulk Harvest) Samples—Routine lot testing of bulk harvest samples is mandatory for most types of biologics (see *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* (1050)). The sampling must be done at the unprocessed bulk harvest stage, because downstream purification processes may remove or inactivate any viruses that might contaminate the starting materials. The harvest sample from the bioreactor should be collected and stored without further manipulation, as soon as practicable, at -60° or below. To prevent multiple freeze and thaw cycles, individual aliquots should be prepared for each individual assay to be performed. Additional aliquots should be retained in case repeat testing is required. Depending on the nature of the manufacturing process, the bulk harvest samples may contain varying quantities of the production substrate cells. Because the bulk harvest does not contain cryopreservatives, the majority of the cells present will lyse upon thawing of the sample, releasing the cell-associated virus. Low-speed centrifugation to clarify the sample will result in a supernatant that may be inoculated directly onto detector cells in cell-based viral infectivity assays. There may be instances where the test sample is cytotoxic to the detector cells of the cell-based assays and procedural modifications may be required to deal with this. A similar sample is prepared for *in vivo* viral safety testing. In this case, however, the test sample is thawed and injected without clarification into the various animal systems via the various described routes.

Raw Materials of Animal Origin—Ingredients of animal origin used in the manufacture of biological products for human or veterinary use must be tested for species-specific viruses of concern as described in 9 CFR 113.53 (see also the USP general information chapter *Bovine Serum* (1024), being prepared for future publication). The raw materials may be stored under a variety of conditions, as appropriate to the raw material. Sample preparation and method of application to the test system depend on the nature of the sample. The possibility that animal-derived raw materials may contain bacterial or fungal contaminants should be considered. In some cases, it may be necessary to treat the samples with antibiotics or to filter the samples (0.22 or 0.45 micron pore size) prior to inoculation in order to prevent bacterial or fungal outgrowth in the test system. Animal sera are typically received frozen and are thawed and incorporated into the growth medium at an appropriate concentration (typically 15%, v/v) as a means of exposing the detector cells. Powdered trypsin (not less than 5 grams, as per 9 CFR 113.53) is suspended in a suitable diluent, such as phosphate-buffered saline, and is then subjected to high-speed centrifugation to pellet any virions that may be present. The concentrated pellet is resuspended in phosphate-buffered saline, and the resulting material is used to inoculate appropriate detector cells. Medium additives, such as bovine thrombin, may be incorporated into the growth medium at a predetermined multiple of the nominal concentration to be used in the manufacturing process. The resulting growth medium containing the additives is then used as a means of exposing the detector cells to the test material. The exact multiple to be used in such testing may be limited by such factors as solubility in growth medium or cytotoxicity to the detector cells. These factors should be assessed in advance of testing. The principle of using higher concentrations in the detection method than during processing should be followed, within the bounds of indicator cell toxicity, as a means to increase sensitivity to detection.

Whole Cells—Intact viable cells are used as the test sample in certain viral detection assays. Because the test cells may attach and proliferate in the culture vessel along with the detector cells, assays using this type of sample are referred to as cocultivation assays. The requirements for the specific assay may vary in relative proportions of detector and test cells, viability of the test cells, or the confluency of test cells at the time of collection.

Cell Culture-Based Viral Detection Methods

To ensure the absence of adventitious viral agents, cell culture-based viral detection assays are used for a variety of purposes, including but not limited to clinical diagnostic procedures; evaluation of raw materials and cell substrates; assessments of the viral identity, the purity, and the potency of virus seed stocks; and lot release testing of unprocessed bulk harvests during biologics production. An important distinction between cell-based assays and direct detection assays (see the section *Detection of Viral Components*) is that the former will detect only replicating virus, whereas the latter will detect viral antigens, viral genomic material, and the like, which may or may not be indicative of the presence of replicating virus. Similarly, detection of circulating antibodies directed against viral antigens (discussed in the section *Detection of Antibodies to Viral Antigens*), may be indicative of either a current or a past infection of an animal and does not necessarily indicate that the animal is currently harboring an infection.

Infectious viruses detected in cells or in cell-derived materials fall into two broad categories, based on the expectations of the analyst. Endogenous viruses are those normally detected in the cells as a result of the integration of the viral genomic material into the host cell DNA. Exogenous viruses

are those not normally present in the cells but found as a result of a viral infection of the cells.

The underlying assumption for all cell-based viral detection methods is the ability of viruses to replicate in an appropriate host cell. Viruses lack the cellular machinery required for producing their own genomic material and structural proteins, and they must therefore enter and subordinate a host cell for this purpose. Cell-based viral infectivity assays use indicator (detector) cells that serve as host cells for viable virions present in test samples.

Cell-based infectivity assays may be placed in three broad categories on the basis of types of viruses to be detected: (1) retroviral assays, (2) virus-specific assays, and (3) viral screening assays. The types of endpoints used to detect the viruses may differ by category. Although screening assays are typically not optimized for single viral entities, the virus-specific assays and titration assays, as well as some of the retroviral assays, may be optimized to some extent for specific viruses. Accurate titration of stock viruses that are used as positive controls or are used to determine the detection limit of an assay is critical.

The regulatory guidance underlying the various viral safety tests depends on the nature of the samples to be evaluated, and analysts are referred for more detail to documentation relevant to their own regulatory environments.

GENERAL REQUIREMENTS FOR CELL CULTURE-BASED ASSAYS

Detector Cells and the Concept of Viral Host Range—

The range of viruses detectable using a cell-based infectivity assay depends on a number of factors, including the type of host cell(s) used as the indicator (detector) cultures and the detection endpoints used in the assay. Viruses differ in their abilities to infect specific host cell types. Most viruses exhibit at least some degree of host cell tropism (i.e., ability to infect a specific species or tissue type). This attribute is typically due to a requirement for interaction of a virion with a specific cell membrane receptor during the process of infection of the host cell. A cell susceptible to infection and capable of production of progeny by a given virus is referred to as *permissive* for that virus; cells not supporting viral proliferation are referred to as *nonpermissive*, or *restricted*, for that virus. As a consequence of the differences in host cell tropism, assays intended to screen for a wide range of viruses must include multiple detector cell types. For the same reasons, design of a cell-based infectivity assay for a specific virus must include a detector cell known to be permissive for that virus.

Virus Susceptibility of Common Cell Lines—For most endpoint assays used to determine whether a host cell is infected with a virus, a monolayer culture is preferable to a semiaherent or suspension culture. For instance, cytopathic effect and hemadsorption are visualized microscopically. Cells that are not adherent have little morphology to evaluate, and hemadsorption cannot be properly evaluated in a suspension culture. For this reason, some regulatory documents pertaining to cell-based virus infectivity assays stipulate the use of monolayer detector cultures.

A list of commonly employed indicator cell lines and their application in viral screening assays is provided in *Table 1*. Regarding the viral tropism of these cells, "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals" (1993) and ICH's Q5A (R1) guidelines (for these references, see *Appendix*) require that human diploid cells such as MRC-5 and WI-38, which are permissive for a range of viruses of human concern, and monolayer cultures of the same species as that of the cell substrate used to produce the product are included in the viral screening test for biologics destined for use in humans.

Table 1. Indicator (Detector) Cell Lines Used for Adventitious Viral Screening Assays

Cell Line ^a	Origin	Endpoint(s) ^b	Target virus(es)
<i>Cell lines with relatively broad viral tropism:</i>			
BHK-21	Syrian hamster	CPE, HAd, HA	Insect-borne viruses (arboviruses)
Vero	African green monkey	CPE, HAd, HA	Viruses infectious to humans, primates
<i>For processes involving human cell substrates:</i>			
HeLa	Human	CPE, HAd, HA	Viruses infectious to humans
MRC-5	Human	CPE, HAd, HA	Viruses infectious to humans
<i>For processes involving Chinese hamster cell substrates:</i>			
CHO-K1	Chinese hamster	CPE, HAd, HA	Viruses infectious to Chinese hamsters
<i>For processes involving mouse cell substrates:</i>			
MEF	Mouse	CPE, HAd, HA	Viruses infectious to mouse cells
NIH/3T3	Mouse	CPE, HAd, HA	Viruses infectious to mouse cells
<i>For processes involving bovine cell substrates or bovine raw materials:^c</i>			
MDBK	Bovine	CPE, HAd, HA	Bovine viruses
BT	Bovine	CPE, HAd, HA	Bovine viruses
EBTr	Bovine	CPE, HAd, HA	Bovine viruses

^a Examples of cell lines used for viral screening assays are shown. MRC-5 and Vero, or cells with similar host ranges, are used in all assays. Depending on the cell substrate used to manufacture a biologic, additional cell lines are also used in the screening assay. In addition, a bovine cell might be included if bovine serum was used in the manufacturing process.

^b CPE, cytopathic effect; HAd, hemadsorption; HA, hemagglutination (optional).

^c Inclusion of a bovine cell in a virus screen should not be construed as a replacement for or alternative to a raw materials test. Raw materials testing is driven in the U.S. by 9 CFR 113.47 and 113.52, and cell lines used for this testing are described in Table 2.

A list of commonly employed indicator cell lines and their application in raw materials testing assays is provided in Table 2.

Table 2. Indicator (Detector) Cell Lines Used in Raw Material Testing

Cell Line ^a	Assay Type	Endpoint(s) ^b	Animal Origin of Raw Material
Vero	Isolation/detection	CPE, HAd, IFA	All sources
BT	Isolation/detection	CPE, HAd, IFA	Bovine; all sources (BVDV) ^c
EBTr	Isolation/detection	CPE, HAd, IFA	Bovine; all sources (BVDV) ^c
MDBK	Isolation/detection	CPE, HAd, IFA	Bovine; all sources (BVDV) ^c
PT-1	Isolation/detection	CPE, HAd, IFA	Porcine
PK-1	Isolation/detection	CPE, HAd, IFA	Porcine

^a The requirement (9 CFR 113.47 and 113.52) for evaluating raw materials of animal origin is to use (1) Vero cells, (2) a bovine cell for detecting BVDV, and (3) a cell line of the same species of origin as the raw material for detecting viruses of concern from that species. Examples are given of some cell lines that are used in the industry.

^b CPE, cytopathic effect; HAd, hemadsorption; IFA, immunofluorescent antibody staining.

^c As per 9 CFR 113.47, raw materials of any animal origin are to be tested for bovine viral diarrhea virus (BVDV).

Table 2. Indicator (Detector) Cell Lines Used in Raw Material Testing (Continued)

Cell Line ^a	Assay Type	Endpoint(s) ^b	Animal Origin of Raw Material
MDCK	Isolation/detection	CPE, HAd, IFA	Canine
GT	Isolation/detection	CPE, HAd, IFA	Caprine

^a The requirement (9 CFR 113.47 and 113.52) for evaluating raw materials of animal origin is to use (1) Vero cells, (2) a bovine cell for detecting BVDV, and (3) a cell line of the same species of origin as the raw material for detecting viruses of concern from that species. Examples are given of some cell lines that are used in the industry.

^b CPE, cytopathic effect; HAd, hemadsorption; IFA, immunofluorescent antibody staining.

^c As per 9 CFR 113.47, raw materials of any animal origin are to be tested for bovine viral diarrhea virus (BVDV).

There may be very specific requirements for detector cells for certain viruses. For instance, assays intended to detect infectious HIV use human peripheral blood lymphocytes and involve a p24 antigen capture enzyme immunoassay endpoint. A list of commonly employed indicator cell lines and their application in the detection of specific viruses is provided in Table 3.

Table 3. Indicator (Detector) Cell Lines Used for Detection of Specific Virus(es)

Cell Line ^a	Assay Type	Endpoint(s) ^b	Target Virus
324K	Isolation/detection	CPE, HAd, IFA	Murine minute virus
A9	Isolation/detection	CPE, HAd, IFA	Murine minute virus
BHK-21	Isolation/detection	CPE, HAd	Arboviruses ^c
MRC-5 ^d	Isolation/detection	CPE	Human cytomegalovirus

^a Examples of cell lines used for optimizing the detection of specific viruses or virus types are shown. In many cases, the assay methodologies must also be optimized for detection of the target viruses.

^b CPE, cytopathic effect; HAd, hemadsorption; IFA, immunofluorescent antibody staining.

^c Insect-borne viruses as a group are referred to as arboviruses. This term has no taxonomic significance.

^d Other human diploid cell lines such as WI-38 are also suitable. Assay duration must be 28 days at a minimum.

Growth Requirements for Detector Cells—Viral proliferation within a permissive host cell may be dependent on the rate of host cell proliferation. This is especially true for viruses that display cell-cycle dependence for generation of viral progeny. For most detection assays, detector cultures are seeded at a density intended to achieve a cell monolayer in exponential growth. This corresponds to a cell confluency of 50% or less (optimal cell densities may depend on the assay type and the detector cell to be used) at the time of inoculation of the cultures with virus or test sample. For the same reasons, the assay design may include provision for detector cell subculture (the collection of cells from the original culture and seeding of a predetermined fraction of these into a new flask). Alternatively, a passage may be performed, consisting of collection of conditioned medium from the original culture and inoculation of this material onto a secondary detector cell culture that is in log-phase growth. The frequency of subculture/passage required in an assay is determined largely by the rate of growth of the detector cell. Incorporation of these steps into detection assay designs helps to ensure that the conditions remain optimal for amplification of viral progeny within the host cells.

Need for Detector Cell Identification and Banking—

Detector cells used for cell-based viral detection assays are a critical reagent for ensuring viral safety, viral potency, viral identity, and viral clearance capacity in purification schemes. In many cases this testing is intended to support GMP processes; therefore, the detector cell banks may need to be prepared and qualified in much the same manner as other critical reagents. The details of the viral safety evaluation methods are described in *Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin* (1050). Regardless of specific compliance requirements, periodic identification and qualification of the detector cell banks to be used subsequently for viral infectivity assays is good practice. The following should be regarded as minimal quality control testing for such detector cell banks: sterility, mycoplasma and viral screening, and cell identity by DNA fingerprinting, karyology, or isoenzyme analysis. In addition, specific assays for bovine and porcine viruses may be required if bovine or porcine raw materials were used in preparation of the banks. *Bovine Serum* (1024) and relevant serum-type specific ancillary materials monographs should be consulted when serum products are used in cell growth media.

Endpoints for Detection of Viral Infection

The various endpoints used to identify infection of a detector cell with a virus include the following:

- Visual observation of cytopathic effects
- Hemagglutination or hemadsorption of erythrocytes
- Immunofluorescent staining
- Cocultivation with other types of detector cells
- Quantitative polymerase chain reaction (qPCR) for direct detection of viral genomic sequences
- Electron microscopic analysis of viral pellets or fixed cells for visual observation of viral particles
- Biochemical endpoints such as reverse transcriptase assays, which detect virus-specific enzymatic activity

These various endpoints are used in a complementary fashion, because a given virus may not cause a positive response in each endpoint. For instance, some viruses can grow to high titers without producing visible cytopathic effects and so must be detected using other endpoints. Polymerase chain reaction (PCR) and electron microscopic analysis per se are not capable of distinguishing viable from nonviable viruses. However, when used in conjunction with cell culture growth kinetics, these approaches can be powerful orthogonal detection methods to demonstrate the increase of viral replication and therefore viable virus. The failure to observe viral particles in electron microscopic analysis of fixed cells should not be considered absolute proof of the absence of infectious virus in the cells. In a general sense, the same is true for each of the detection endpoints discussed above. Each endpoint has a detection limit below which a virus may be present but not detected.

Viral Cytopathic Effects—Visually observable manifestations of the infection of susceptible host cells with certain types of viruses are collectively referred to as viral cytopathic effects (CPE). Although CPE may be considered an indirect detection of viral infection, in the context of specific host cells they can have distinctive morphological manifestations. These may include the appearance of inclusion bodies, abnormal cell morphology, changes in culture confluency, cell death and cell lysis, and others. The nature of the CPE observed may depend on the host cell and the infecting virus. In addition, for a virus that normally causes CPE, there may exist variants that do not cause CPE. CPE can be differentiated from cytotoxic effect by the tendency of the former to exhibit progression irreversibly with time, whereas the latter may be reversible. In some cases structural proteins of viruses may cause cytotoxic effects similar to the cytopathic effects of the infectious virus. Differentiating the cytotoxic effect of such proteins from the cytopathic effect of infectious virus may require observation of the culture over time

to determine whether the effect progresses or the cells appear to recover. Alternatively, cell-free passage of the original culture onto fresh detector cells can be used to differentiate these two apparently similar manifestations. The cytotoxicity associated with the structural proteins in the absence of infectious virus would not be expected to pass to the secondary culture.

Giemsa staining may optimize the ability of the operators to visualize certain inclusion bodies (clusters of viral particles) that are characteristic of viral cytopathic effect and is required by 9 CFR 113.53 in assays used to demonstrate that bovine, porcine, equine, and ovine raw materials are free of species-specific viruses.

Detection of Hemagglutinating Viruses—A characteristic of certain viruses (referred to as hemagglutinating viruses) is that one or more of their viral proteins cause hemagglutination of one or more types of erythrocytes. Hemagglutination is an interaction between viral proteins or *hemagglutinins* and erythrocytes, leading to adhesion of the erythrocytes to surfaces, cells, and each other. This property forms the basis of two endpoint procedures that are employed in cell-based viral infectivity assays: *hemadsorption* and *hemagglutination*.

Hemadsorption is performed by adding a suspension of one or more erythrocyte types directly to the monolayer culture of detector cells. If viral proteins of a hemagglutinating virus are expressed from infected cell membranes, the susceptible erythrocytes will bind tightly to the cell membranes. Noninfected cells do not display this binding; therefore, the technique can be used to visualize a focus of infected cells against a background of uninfected cells. For this reason, this particular endpoint may display greater detection sensitivity than other assay endpoints, such as cytopathic effect or hemagglutination. In the advanced stages of infection, binding of erythrocytes to cells, to each other, and to open plastic surfaces in the culture vessel may be observed.

The hemagglutination procedure is performed on the conditioned medium and is essentially an evaluation for free virus or viral hemagglutinins in solution. An aliquot of the conditioned medium from a detector culture is combined, in a microwell plate having v-bottomed wells, with one or more types of erythrocytes. After an appropriate amount of time the plates are evaluated. Absence of hemagglutination is reflected by a well-defined pellet (button) of erythrocytes sedimenting to the bottom of the well. In comparison, hemagglutination is reflected by the absence of a button, or by a button with irregular shape. Scoring the latter represents an opportunity for operator subjectivity. In addition, this endpoint can be considered the least sensitive in detection assays, because it is dependent on achieving a sufficient concentration of viral hemagglutinins in solution. For these reasons, hemadsorption is typically viewed as the more useful and reliable of the techniques for detecting hemagglutinating viruses.

The responses obtained in detection assays using hemadsorption and hemagglutination endpoints are highly dependent on the virus being assayed, as well as on the types of erythrocytes used. Many of the viruses of concern to the biotechnology industry do not cause hemadsorption and hemagglutination, or their hemagglutinins react with red blood cell types not commonly used in detection assays.

Detection by Immunofluorescent Antibody (IFA)

Staining—Certain cell-based viral detection assays are intended to detect specific viral entities and are required for raw materials tests derived from bovine-, porcine-, equine-, and ovine-derived materials (9 CFR 113.53). In order to achieve this, the assays must be optimized with respect to host cell selection, study design, and sample preparation. Specificity of detection is also conferred through use of IFA staining techniques. Primary antisera or monoclonal antibodies directed against the viral antigens of interest are used, either in direct staining applications or in conjunction with a fluorochrome-conjugated secondary antiserum. The

immunostained detector cell monolayers are then visualized with an epifluorescence microscope to reveal the presence of reactive infected cells.

Design of Cell-Based Viral Assays

VIRAL DETECTION ASSAYS

Detector cell cultures are seeded and allowed to incubate for the appropriate amount of time. Viral samples may be inoculated directly into the medium of the mitotic phase of cell culture, but more typically the medium is removed from the overnight detector cell culture and is replaced with the test sample. For the latter method, the test sample must therefore be approximately isotonic, and cytotoxic agents such as selection agents must be maintained within levels tolerable to the detector cell. For test samples comprised of live cells, the sample must be subjected to freeze-thaw before inoculation in order to lyse the sample cells. If the cells are not lysed, a cocultivation involving the detector and sample cells will result. The latter is part of the design for cocultivation assays. But for most of the cell-based infectivity assays such a cocultivation is not intended and could adversely impact the sensitivity of the assay. The test sample is typically allowed to adsorb to the detector cell monolayers for an appropriate amount of time. It is then removed and replaced with the growth medium suitable for the detector cell. Once inoculated, the detection assay involves incubation of the detector cells for a prescribed amount of time, with periodic refeeding as necessary. Endpoint evaluations as described above are performed according to the study design. Variations of the given procedure may be used for exposing detector cells to raw materials. For raw materials, detector cell exposure may consist of incorporation of the test materials into the growth medium used to maintain the detector cells throughout the assay.

VIRAL TITRATION ASSAYS

Viral titration assays are designed to generate quantitative information about the virus of interest. These assays do not quantify absolute numbers of viral particles; rather, the results are expressed in terms of infectious units. An infectious unit is the amount of virus required to establish a productive infection, and several categories of titration assays are used. They vary on the basis of the endpoint used to demonstrate infection and include viral plaque titration or plaque-forming units (units: PFU per mL); 50% tissue culture infectious dose (units: TCID₅₀ per mL); and 50% fluorescent antibody infectious dose (units: FAID₅₀ per mL). The amount of a hemagglutinating virus in a sample can also be expressed in terms of hemagglutinating titer (units: endpoint dilution, i.e., the greatest dilution of the sample which still results in a positive hemagglutination, or HA, response).

Regardless of the endpoint used, a typical titration assay design consists of a sequence of sample dilutions based on 0.5 log₁₀ or 1 log₁₀ increments. The various dilutions of the sample are then applied to an appropriate number of replicate permissive detector cell monolayers. After a suitable incubation time, the monolayers are scored directly for cytopathic effect (TCID₅₀ assay), fixed and processed for immunostaining and scored for reactive cells (FAID₅₀ assay), or overlaid with agarose and processed for plaque generation PFU assay. TCID₅₀ and FAID₅₀ titers are typically calculated using published formulas, such as Spearman-Kärber and Reed-Muench. Assay controls for such quantitative assessments should routinely include a reference sample of known potency.

Detection of Retroviruses

Retroviruses represent a special case for cell-based viral detection assay because of the occurrence of retroviral infection in the absence of responses to the typical endpoints discussed. In order to detect retroviruses, scientists can employ a number of different endpoints. A list of commonly employed indicator cell lines and associated endpoints, and their application in retrovirus detection assays, is provided in Table 4.

Table 4. Indicator (Detector) Cell Lines Used in Retrovirus Infectivity Testing

Cell Line	Assay Type	Endpoint(s) ^a	Target Virus
SC-1/XC	Isolation/direct detection	Plaques (XC); RT	Ecotropic murine retrovirus
Balb/C	Isolation/direct detection	Plaques (XC); RT	B-Tropic murine retrovirus
NIH/3T3	Isolation/direct detection	Plaques (XC); RT	N-Tropic murine retrovirus
Mink Lung	Isolation	RT; mink S+L-	Xenotropic, amphotropic murine retrovirus
Mink S+L-	Direct detection	Foci	Xenotropic, amphotropic murine retrovirus
Feline S+L-	Direct detection	Foci	Xenotropic, amphotropic murine retrovirus, gibbon ape leukemia virus (GALV), and RD-114 feline retrovirus
<i>Mus dunni</i>	Isolation, cocultivation	RT; S+L-	Murine retroviruses and retroviruses infectious to humans
QT6 ^b	Isolation, cocultivation	RT	Avian retroviruses
RD	Isolation, cocultivation	RT; S+L-	Retroviruses infectious to humans
MRC-5	Isolation, cocultivation	RT; S+L-	Retroviruses infectious to humans
WI-38	Isolation, cocultivation	RT; S+L-	Retroviruses infectious to humans
293	Isolation, cocultivation	RT; S+L-	Retroviruses infectious to humans
A549	Isolation, cocultivation	RT; S+L-	Retroviruses infectious to humans

^a The endpoint assays include the following: RT, reverse transcriptase; PERT, product enhanced reverse transcriptase (including also product enhanced reverse transcriptase and real-time quantitative product enhanced reverse transcriptase assays); and EIA, enzyme immunoassay.

^b A quail cell; primary fibroblast cultures of chicken or turkey origin are also sometimes used.

Table 4. Indicator (Detector) Cell Lines Used in Retrovirus Infectivity Testing (Continued)

Cell Line	Assay Type	Endpoint(s) ^a	Target Virus
Raji	Isolation, cocultivation	RT	Retroviruses infectious to humans
Human PBMC	Isolation, cocultivation	RT, HIV p24 EIA	HIV and other human retroviruses

^a The endpoint assays include the following: RT, reverse transcriptase; PERT, product enhanced reverse transcriptase (including also product enhanced reverse transcriptase and real-time quantitative product enhanced reverse transcriptase assays); and EIA, enzyme immunoassay.

^b A quail cell; primary fibroblast cultures of chicken or turkey origin are also sometimes used.

Retroviral infection is dependent on the presence of receptors on the host cell membranes. The presence of such receptors confers host cell tropism.

DESIGN FOR RETROVIRUS INFECTIVITY ASSAYS

Two types of infectivity assays are used, depending on the nature of the test material. For materials other than intact cells, the test material is inoculated onto one or more of a variety of detector cells, and the latter are then passaged as required to amplify any virus present. Because of the nature of retroviral replication, cytopathic effects typically do not occur during infection, although there are some exceptions. Before the first subculture and at the end of the final passage, one or more endpoint assays are employed to detect the presence of a retrovirus. For test materials in the form of intact cells, detector cells are seeded and subsequently inoculated with the test cells, resulting in a cocultivation. The cultures are passaged five or more times. Before the first subculture and at the end of the final passage, one or more endpoint assays are employed to detect the presence of a retrovirus.

ENDPOINT ASSAYS FOR RETROVIRUS DETECTION

Endpoint assays may be classified as direct, which lead to distinct morphological changes in the detector cells; or indirect, as measured by the detection of biochemical, molecular, or immunological markers for infection.

XC-Plaque Assay—The XC-plaque assay was developed as a direct means of detecting infectious murine retroviruses. Detection of the retrovirus is accomplished by UV-irradiating the detector cells used to amplify the virus and overlaying the irradiated detector cells with a specific rat cell (XC). The presence of infectious murine retroviruses in the detector cells is reflected by the formation of distinctive syncytia in the XC monolayer, which are easily visualized when the cultures are fixed and stained with a suitable dye such as crystal violet.

The N/B tropism of an ecotropic murine virus may be determined by inoculating Balb/c and NIH Swiss detector cells with the isolate, performing one or two passages on each cell line, and comparing the XC-plaque titer post passage to that determined for the initial isolate.

Mink and Feline S+L[−] Focus Assays—The S+L[−] focus endpoint was developed to facilitate direct detection of infectious murine xenotropic and amphotropic viruses. The test sample may be inoculated directly into cultures of the S+L[−] cells, or, alternatively, may be amplified first by inoculation into mink lung, human, or *Mus dunni* detector cells. Cell-free supernatants from the detector cell cultures are used to inoculate the S+L[−] cells. The latter are infected with a sarcoma virus that is replication-defective, requiring the presence of a helper leukemia virus to render it capable of

causing transformation of the host cell. The presence of infectious retrovirus virus in the detector cultures is reflected by the formation of characteristic focal areas of cell transformation in the S+L[−] cells caused by the rescued sarcoma virus.

Detection of Retroviral Reverse Transcriptase—Assays designed to measure reverse transcriptase (RT) activity are useful as an indirect detection method, because the enzyme is indicative of the presence of all retroviruses, whether infectious or not. The RT enzyme is encoded for in the retroviral genome and is used by the virus to transcribe genetic information in viral genomic RNA into proviral DNA.

Radiolabeled Nucleotide Incorporation Assay—The earliest methods for measuring RT activity were based on the measurement of ³²P- or ³H-labeled nucleotide incorporation into the complementary cDNA product, using an appropriate RNA template. Incorporation of radiolabeled nucleotide at levels higher than a predetermined threshold is interpreted as evidence of the presence of retroviral RT activity. The contributions of cellular DNA polymerases can be ruled out through use of a dual template assay (having both RNA and DNA templates) or inclusion of activated calf thymus DNA.

Product-Enhanced Reverse Transcriptase (PERT) or Quantitative PERT (Q-PERT)—Polymerase chain reaction amplification has been used to increase the sensitivity of RT activity measurement. RT activity is detected by PCR amplification of complementary DNA, newly synthesized from an RNA template by reverse transcriptase. The assay may be performed with a gel endpoint (PERT) or as a quantitative assay (Q-PERT). This method has increasingly gained acceptance by regulatory agencies. For more details on PCR-based techniques, see the USP general information chapter *Nucleic Acid Based Techniques—Amplification* (1127).

Electron Microscopy—Transmission electron microscopy (TEM) may be used to detect and enumerate viral particles within cells. In addition, the technique allows for differentiation of types A, B, C, and D retroviruses based on morphological considerations and can be used to localize viral particles within the cell. As a technique for identification of viruses (including RNA and DNA viruses in general), TEM of sectioned cells is extremely valuable. The cells, typically sampled during the log phase of growth, are pelleted by low-speed centrifugation, and the cell pellet is fixed with a suitable fixative. The fixed cell pellet is embedded, sectioned, stained, and observed with TEM. Size (diameter) of the particles, morphology, presence or absence of surface features such as envelopes and spikes, and location within the cell can be determined with this technique. Such information is important for the identification of a virus. However, failure to observe viral particles with this method does not conclusively demonstrate the lack of viral contamination in the sample.

Biological fluids may also be evaluated by TEM, primarily to determine particle size and concentration. The cell-free supernatant is subjected to ultracentrifugation to pellet any virus present. The resulting pellet is fixed with a suitable fixative. A predetermined number of grid spaces containing representative areas of thin sections of the pellet are evaluated for particles. The enumeration results obtained may show a high degree of variability, and failure to observe particles does not imply that none were present in the sample. Molecular (quantitative PCR and quantitative PERT) endpoints have also been used as alternative methods for estimation of viral particle load in samples.

Antigen-Capture Enzyme Immunoassay—Specific viral proteins (e.g., HIV p24 antigen or avian leucosis viral envelope proteins) may be detected as a means of determining the presence of a retrovirus. Viral antigens are captured by specific antibodies coated onto microtiter plate wells and are detected by the addition of a second labeled antibody and appropriate substrate.

Assays Designed to Detect Specific Viruses

Additional methodologies have been developed to allow detection of specific viruses or groups of viruses. These types of assays are often used for raw material evaluation. In some cases, these specific assays were developed because the target viruses do not cause endpoint responses in the viral screening assays. In contrast to screening assays, specific virus assays are typically optimized for detection of the target virus or viruses. This optimization takes into account the lability of the virus, the host range, the possible endpoint responses elicited, and any special requirements of the target virus. The use of well-characterized viruses as positive controls in such assays provides assurance that the methodologies are suitable for the target virus or viruses. Spiking of the test sample matrix with the positive control virus enables the investigator to assess the potential for matrix interference and to assess the limit of detection for the method. Such considerations are not applicable to screening assays. Specific virus testing for bovine- and porcine-derived raw materials is discussed below. Evaluation of caprine, ovine, equine, canine, and feline raw materials is also stipulated in 9 CFR section 113.47. This section should be consulted with respect to the viruses of concern, and 9 CFR 113.52 should be consulted for methodology. A list of commonly employed indicator cell lines and their application in raw materials testing assays is provided in Table 2. A list of commonly employed indicator cell lines and their application in detection of specific viruses is also provided (see Table 3).

Detection of Bovine Virus Contamination—Raw materials of bovine origin include such commonly employed medium components as fetal bovine and calf serum, serum albumin, collagen, thrombin, and trypsin. Each of these additives represents a route of entry for adventitious viral contaminants into a cell culture or manufacturing process. Requirements for evaluation of such materials ensure the absence of contaminating viruses.

For the details on testing for bovine serum and its derivatives, see future general chapter *Bovine Serum* (1024).

Detection of Porcine Viral Contaminants—Raw materials of porcine origin include trypsin as well as other cell culture reagents. The specific porcine viruses of concern in the United States are stipulated in 9 CFR 113.47 and include porcine parvovirus, porcine adenovirus, transmissible gastroenteritis virus, and porcine hemagglutinating encephalitis virus. In addition, porcine raw materials must also be evaluated for the presence of bovine viral diarrhea virus (BVDV), reovirus, and rabies virus. Porcine tissues intended for xenotransplantation into humans also are routinely evaluated for the porcine endogenous retrovirus (PERV). The host cells typically used in the detection of porcine viruses are porcine testicle or porcine kidney, a bovine cell, and Vero cells. The methodology described in 9 CFR 113.52 is analogous to that for evaluation of bovine raw materials and includes provision for multiple subcultures, for Giemsa staining of fixed cells, for hemadsorption testing, and for use of specific immunostaining of fixed cells.

Cell-Based Detection of Murine Minute Virus—Murine minute virus (MMV) is a mouse parvovirus that has been detected in biologics manufacturing involving Chinese hamster cell substrates. As with other parvoviruses, MMV represents a special case in that the virus is difficult to inactivate using typical cleaning agents and is capable of surviving for prolonged periods of time on surfaces. Cell-based assays for MMV involve detector cell lines that are especially susceptible to this virus, such as 324K (a human cell) and A9 (a murine cell). Optimization for detection of a parvovirus also includes provision for detector cell subcultures to remain in log-phase division for a significant portion of the incubation period. Endpoints for detection of MMV include one or more of the following: cytopathic effect, hemagglutination of mouse and guinea pig erythrocytes, immunostaining, and polymerase chain reaction.

Cell-Based Detection of Insect-Borne Viruses—Insect-borne viruses include both viruses infectious only for insect cells (e.g., baculovirus) and those transmitted to mammalian cells via insect vectors (arboviruses). Detection of the former may be accomplished using an insect cell as a detector cell. Suitable substrates might include cells of *Spodoptera*, *Trichoplusia*, *Drosophila*, mosquito, or other insect origin. Such cells are typically cultured at lower temperatures (25° to 28°) relative to mammalian cells, and many of these cultures are suspension or semiadherent at best. Endpoints may include cytopathic effect, electron microscopy, and PCR.

Of more relevance to patient safety is the detection of arboviruses (insect-borne viruses infectious to animals and humans). This may be accomplished using a suitable mammalian detector cell. The Syrian hamster kidney cell (BHK-21) is a cell line that has shown susceptibility to a wide range of arboviruses. This cell line grows in a monolayer culture, and the endpoints that may be used include cytopathic effect, hemadsorption and hemagglutination, and PCR.

Cell-Based Detection of Human Cytomegalovirus—Human cytomegalovirus (CMV) is a slow-growing virus of special concern for biologics produced using human cell substrates. It may be detected in cell-based assays using human diploid detector cells such as WI-38 or MRC-5, provided that sufficiently long durations of incubation are employed (28 or more days). The endpoints include cytopathic effects and immunostaining and/or PCR.

IN VIVO METHODS

Intact and susceptible animals may serve as potential host organisms for detecting viruses in test samples. In this case, viral proliferation in the tissues of the host animal may be reflected as adverse health effects (including death) that can be monitored and recorded. Viral detection assays based on intact animals are intended to complement in vitro assays, because some viruses that do not cause a response in the in vitro assays may be detectable in the animal systems (and vice versa). Viral safety studies employing live animals must be performed in accordance with applicable regional guidelines for the ethical use of animals, using laboratories that are accredited for the housing of the animals.

In Vivo Viral Screen—The in vivo viral screen is used primarily for cell bank, viral seed stock, and viral vaccine testing and is considered to complement the in vitro virus screening assay. Multiple animal species, as well as multiple injection routes, are employed to provide a broad range of host tissues and possible responses. A list of commonly used host animals, routes of inoculation, and target viruses are shown in Table 5.

Table 5. In Vivo Viral Screening Assays

Host Animal	Route of Inoculation	Target Virus
Suckling mouse	Intraperitoneal injection	Arboviruses
	Intracranial injection	Coxsackie A and B
	Per os injection	Herpes simplex Type 1 and 2
		Togaviruses
		Junin
Adult mouse		Herpes B
	Intraperitoneal injection	Rhabdoviruses
	Intracranial injection	Togaviruses
	Per os injection	Lymphocytic choriomeningitis virus (LCMV)

Table 5. In Vivo Viral Screening Assays (Continued)

Host Animal	Route of Inoculation	Target Virus
Guinea pig	Intraperitoneal injection	Rhabdoviruses
	Intracranial injection	LCMV
		Lassa
		Junin
		Marburg
		Ebola
Embryonated hens' eggs		Vaccinia viruses
	Allantoic	Arboviruses
	Yolk sac	Equine encephalomyelitis viruses
	Chorio-allantoic membrane	Herpes viruses
		Influenza
		Mumps
		Newcastle disease
		Parainfluenza Types 1 and 2
		Rabies
		Vaccinia
		Variola
		Lymphogranuloma venereum
		Ornithosis

Following injection of the test sample, each animal model is monitored for an appropriate period of time that allows for the observation of clinical signs of viral infection. Any abnormality is investigated to determine the cause of the effect.

The suckling mice are observed for an appropriate period of time. Pooled homogenates from any surviving animals are then passaged into additional litters of suckling mice. The latter are observed for an additional period of time.

The guinea pigs are observed for clinical signs of viral infection and for injection site lesions. Necropsy for gross tubercular lesions is performed for certain types of test samples.

Allantoic fluids from eggs can be tested for hemagglutination of chicken, guinea pig, and human type-O erythrocytes. Additional fluids are pooled for each treatment group (test article and control), and these are passaged (inoculated) into a new group of embryonated eggs. Following an appropriate incubation period (typically measured in days), the allantoic fluids are again tested for hemagglutination of chicken, guinea pig, and human type-O erythrocytes. Following injection by the yolk sac route, the eggs are incubated for at least 9 days and are assessed for viability. The yolk sacs are then harvested and pooled for each group (test article and control), and a 10% solution of the resulting material is inoculated by the same route into a new group of embryonated eggs. The eggs are again incubated for an appropriate period of time (days) and are assessed for viability.

IN VIVO ASSAYS INTENDED TO DETECT SPECIFIC VIRUSES

Some in vivo assays are designed to detect, if not specific viruses, at least specific sets of viruses. The antibody production assays use the production of a humoral immune response in susceptible host animals inoculated with test samples. Viral antibody-free animals of the various species are injected with the test sample. At the end of an appropriate incubation period, one or more of a variety of endpoint assays may be performed to detect the generation of a humoral antibody response in the animal sera. Production in

the animal of antibodies directed against a specific virus provides evidence of the presence of viral antigen or infectious virus in the test sample. This type of assay is typically used to ensure that rodent cell banks and viral seed stocks are free of adventitious viruses. Three antibody production assays, along with the route of injection and target viruses, are summarized in Table 6.

Table 6. In Vivo Antibody Production Assays

Antibody Production Assay	Route of Injection	Target Virus
Mouse antibody production (MAP) assay	Intranasal	Ectromelia
	Intraperitoneal	Hantaan
	Intracranial	Mouse K
		Lactate dehydrogenase elevating virus
		Lymphocytic choriomeningitis virus (LCMV)*
		Murine minute virus
		Mouse adenovirus
		Mouse cytomegalovirus
		Mouse encephalomyelitis virus type II
		Mouse hepatitis virus
Mouse antibody production (MAP) assay		Epizootic diarrhea of infant mice
		Pneumonia virus of mice
		Polyomavirus
		Reovirus type 3
		Sendai
		Mouse thymic virus
		Lymphocytic choriomeningitis virus (LCMV)*
Hamster antibody production (HAP) assay	Intranasal	Polyomavirus
	Intraperitoneal	Reovirus Type 3
	Intracranial	Sendai
		Simian virus 5
Rat antibody production (RAP) assay	Intranasal	Hantaan
	Intraperitoneal	Kilham rat virus
	Intracranial	Mouse encephalomyelitis virus type II
		Polyomavirus
		Reovirus type 3
		Sendai
		Toolan's H1 virus
		Rat coronavirus/sialodacryoadenitis virus

* A group of test sample-injected mice is challenged with a known lethal dose of authentic LCMV. If this group of mice does not die from the challenge dose, a second group with twice the number of mice is used for a repeat challenge. If there are survivors in this group, the test sample is considered positive for LCMV.

Considerations for Validation, Matrix Qualification, and Quality Control of Cell- and Animal-Based Test Systems

Viral detection assays used to ensure the viral safety of human and animal therapeutics are expected to have undergone validation. The approach to the validation depends on the nature of the assay and associated regulatory compliance level.

Any assay should be sufficiently developed that it can be performed with an appropriate set of predetermined system

suitability and acceptance criteria. These criteria usually include the use of relevant negative and positive controls but may also include requirements for linearity and meeting of a predetermined detection limit. The results constituting a positive or negative response in the assay should be established prior to execution of the validation. An assay used under GMP compliance is expected to have been validated according to appropriate guidelines.

An assay used to ensure the safety of a commercially marketed biological product must be further characterized for suitability in the presence of the specific product matrix. The matrix qualification study should address the potential for specific interference with the viral detection endpoints used in the assay, and typically involves spiking of one or more model viruses into the product matrix at levels approaching the limit of detection to ensure the absence of interference.

For quantitative detection assays, the detection limit should be probed. This usually involves spiking of the model virus(es) at decreasing amounts into medium or the product matrix. The lowest spiking level of the virus reliably detected is used as an approximation of the actual limit of detection of the assay. Experimental error for these cell culture-based assays is usually expected to be in the range of 0.5 to 1 log₁₀. The determination of a detection limit is less meaningful for limit tests and viral screening assays in general. For the latter, knowledge of the detection limit for one virus does not imply a similar limit for another virus. Since screening assays are not optimized for a specific virus, the limit of detection for the assay can vary greatly from one virus to another.

Animal-based viral detection systems are generally not subject to the requirements for validation, matrix qualification, use of positive controls, and determination of detection limit that regulatory agencies expect of cell-based and biochemical tests. The use of animals for safety testing is subject to the regional guidelines for the ethical use of animals, and the kinds of activities listed above generally are not considered appropriate use of animals. However, negative control animals are included in these assays, and retrospective validation or gap analysis based on historic incidence of system suitability failures or positive findings is sometimes possible.

DETECTION OF VIRAL COMPONENTS

Direct detection of viral components can provide a direct measurement of viral levels in a sample preparation. It has also become primarily important for detection or identification of viruses in biological products or in the raw materials used in their manufacture. Systems capable of identifying components unique to specific phases associated with viral latency and replication are now available. During interaction with their host cells, viruses may incorporate modified host molecules during the production of new intact virus particles, or they may induce discernable changes in host cell makeup or function.

Most immunological methods and reagents currently available detect the constituents of intact virions. It is the relative abundance of these proteins that makes them most amenable to the development of antibody-based reagents. Abundance also makes them optimal targets for detection of the virus. Recently developed targeting and detection reagents are aimed at minor viral components that may be found only during specific phases of replication. These allow a more detailed analysis of the stage of viral infection. The basic methodology for the detection of viral antigens is well established, but more recent innovations in materials and reagent development have broadened its application.

Developments in the targeting and detection of viral nucleic acid components have led to enzyme-based systems for the amplification of nucleic acids in vitro and in situ (see *Nucleic Acid-Based Techniques—Amplification* (1127)). The potential specificity of this detection method allows the exami-

nation of biological systems with a high degree of confidence for the presence or absence of a specific targeted virus. A wide variety of reagents, technology platforms, and methodologies are available. The aim of this section is to elaborate on the most common practices and platforms used in the detection of viral components.

Sample Selection and Preparation for the Detection of Viral Components

This subsection addresses general considerations for various types of test samples and the most common assay targets (viral proteins and nucleic acids). The target proteins may have varying levels of posttranslational modification (e.g., glycosylation, phosphorylation), and the target nucleic acids may be either RNA or DNA, single or double stranded. Therefore, it is important to have a basic understanding of the physicochemical nature of the virus under study so that the sample handling procedures support the detection of the target component. For detailed considerations regarding the extraction of nucleic acids, see the USP general information chapter *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* (1126).

Types of Samples

Cellular—When viral components are associated with intact cells, samples must be generated either as whole cell lysates or as subcellular fractions. Maintaining the temperature of the test samples at or near freezing (0° to 4°) during processing and limiting the time that samples are held in an unfrozen state will reduce the potential for loss of target antigens and nucleic acids due to cytosolic enzymes (proteases, nucleases) present in the cell lysates. Reagents that can inactivate or limit the activity of such enzymes may be used to prevent degradation of the target components, especially when exceptionally labile samples must be handled at room temperature. Centrifugation of intact cells allows for some additional manipulation of the sample matrix. The growth medium can be discarded and the cells suspended in a buffer formulated to enhance the recovery and detection of the targeted viral component. Collection and storage parameters should also account for the presence of cellular DNA. This can increase the viscosity of the sample, rendering it difficult to pipet.

Tissue Culture Supernatant—Depending on the stage of the infection and the type of virus involved, the conditioned medium may represent a preferred test sample. An advantage is that the presence of cellular debris can usually be reduced through use of a low-speed centrifugation (clarification) step. The main disadvantage is the potentially low concentration of the analyte, and therefore concentration of the sample may be required.

Process Intermediate (Unprocessed Bulk Harvest)—In general, viral safety lot release testing is done at the bulk harvest stage prior to any purification. This is true regardless of whether the assay detects infectious virus or viral components. The presence of host cell DNA may need to be assessed in the case of biologics manufactured in animal cells.

Sample Stability and Matrix Effect

Sample stability is a key element in the successful detection of viral components. Protein structure can be altered by numerous environmental factors, including pH, ionic strength, solvents, detergents, temperature, and free radicals. In addition, complex biological matrices frequently contain proteolytic enzymes that can alter or destroy key antigenic features of a protein or peptide. Sample collection, storage, and handling must allow maintenance of the antigenic features targeted by reagent antibodies.

Conformational changes affecting the opportunity for antigen detection are difficult to address. Depending on the reagents required for detection, conformational changes may be required for antigen detection. For example, if antibodies are produced for an antigen detection system using native viral antigen, then unmasking and maintaining the conformation of the antigen throughout the sample preparation is essential. Conversely, if peptide fragments are used to produce antibody, then a denaturation step may be required to allow for effective antigen detection.

Conditions associated with sample preparation must be investigated in a combinatorial fashion whereby one parameter or component is varied while all others remain fixed. In this way, the formulation of lysis and processing buffers can be optimized for pH, ionic strength, and types of detergents and denaturants. The sample preparation steps must condition the targeted antigen in order to obtain the form most readily recognized by the reagent antibody.

The stability of nucleic acids in test samples is largely affected by nuclease activities present in the sample and the degree of protection provided by the intact structure of the virus particle. Encapsidated nucleic acids are particularly stable as long as the integrity of the capsid is maintained. Viral capsids are vulnerable to proteolytic digestion. Virus particles stored at ambient temperature as part of a complex biological matrix are especially susceptible to degradation by proteases. Storage at refrigerated temperatures (2° to 8°) for short periods of time or at temperatures below freezing can be used to limit proteolytic activity. When samples are stored at frozen temperatures, freeze-thaw cycles should be limited. Under conditions where an individual sample must be accessed multiple times, preparation of aliquots is advisable.

Sample Collection—In a biotechnology setting, sample collection is dictated by sampling plans that are established to meet regulatory requirements. Nucleic acid testing in association with an amplification step has the potential of detecting a virus at the earlier stages of infection. The use of nucleic acid amplification methods reduces the dependence on timing and the amount of material required, because the amplification process effectively boosts assay sensitivity by increasing the amount of target relative to background.

General aspects of nucleic acid sample preparation and stability are discussed in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* (1126). The following section specifically addresses the unique aspects of viral sample selection and preparation.

Sample Storage—Conditions for sample storage should be consistent with maintaining the antigenic properties of targeted viral proteins and/or preserving the nucleic acid content of the sample. The duration and temperature of storage is dictated also by cycle times associated with testing.

Immune Complex Disruption—The masking of antigen epitopes may occur when other proteins associate at or near the epitope targeted by reagent antibodies. For viral antigens this may occur when the antigen comprises the structural component of the virus. Such viral antigens are likely to retain strong affinities for other viral proteins or the ability to exist in multimeric form under normal conditions for detection. Another obstacle for epitope recognition is the naturally occurring immune complex when reagent antibodies have been developed to detect native protein in a blood plasma matrix. Immune complexes consisting of viral antigens and host antibodies are normal in such physiologic samples. The stronger the host immune response, the more likely masking of antigen due to immune complex formation will occur. Methods aimed at the preparation of blood and plasma samples for detection should address the presence of preexisting immune complexes and incorporate steps designed to disrupt such complexes to improve the opportunity for viral antigen detection.

Detection of Viral Antigens

Viral capsid proteins are common targets for antigenic detection methods. Structural proteins that make up the framework of the viral core are often some of the most abundant viral proteins produced during viral replication. In nonenveloped viruses, the core structural proteins are likely to provide the dominant antigenic features. When the virus is enveloped, proteins associated with the envelope often provide key antigenic features. This section examines methods commonly used to detect viral antigens and addresses considerations aimed at optimizing formation of the appropriate immune complex.

ASSAYS USED FOR THE DETECTION OF VIRAL ANTIGENS

Immunologic methodologies used to detect viral antigens are based on the specificity and affinity of the antibody and viral antigen interaction. Of the various platforms available, one commonly employed in viral safety testing is immunofluorescent antibody staining. This lends some degree of viral specificity to the cell-based methods described in the first part of this chapter. Other techniques include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, and Western blotting. The principles and general methods for these assays will be described in the USP general information chapters *Immunological Test Methods—General Considerations* (1102), *Immunological Test Methods—Reagent Development* (1103), *Immunological Test Methods—Immunoassay Methodologies* (1104), and *Immunological Test Methods—Assay Design, Quality Control, and Data Analysis* (1105), being prepared for future publication. The following sections will generally address aspects specific to their use in virology.

Immunofluorescence Assay—The immunofluorescence assay (also referred to as immunofluorescent antibody staining) is used to detect viral proteins expressed in various cellular compartments. Since the technique can detect viral antigens within single cells, it confers a high degree of sensitivity and enables infection to be detected at a very early stage. The technique is often employed as a detection endpoint for cell-based viral infectivity assays to provide additional sensitivity and specificity (see tests for raw materials as described in 9 CFR 113.53). In addition, the technique is employed for verifying the identity of viral stocks and is useful as a means of identifying viruses detected in viral screening assays.

Enzyme-Linked Immunosorbent Assay (ELISA)—ELISA is best suited for detection of soluble antibodies and antigens in a variety of test samples. Sensitivity, quantification, robustness, ease of experimentation, and readily available inexpensive reagents make it adaptable to a high throughput environment. For viral antigen detection, a sandwich ELISA assay is commonly used. A viral antigen specific antibody (preferably a monoclonal antibody with high affinity) is first immobilized onto a solid phase. The test sample is then incubated for a predetermined period under appropriate conditions. After washing, a second antibody that recognizes the viral antigen is incubated. The second antibody is linked to either an enzyme or a chromogenic reagent that emits signal with an appropriate substrate and can be recorded with an appropriate instrument. The assay can be performed in a qualitative or a quantitative manner. For a qualitative ELISA assay, sufficient replicates of both positive and negative control samples are required in order to determine the appropriate cut-off value and the assay acceptance criteria. A mean value of a test sample that is equal to or greater than the cut-off value is considered positive. For a quantitative ELISA assay, an additional standard curve with a positive reference standard material of known quantity must be established. The number of replicates should be adequate to determine the assay variation and linearity. The

quantity of test sample can be calculated against the standard curve.

Radioimmunoassay (RIA)—The radioimmunoassay is a versatile quantitative immunoassay that can be used to detect substances including viral antigens and antibodies. It even can be applied to nonprotein molecules as long as an antibody that specifically binds the test substance is available. Radioimmunoassays can be customized in different formats to suit specific test requirements. Many of the considerations taken into account with other immunological assays are applicable to the RIA. In general, radioimmunoassays can be divided into two major categories: solution (homologous) and solid-phase radioimmunoassays. Both methods have been successfully used to detect and quantify a variety of viral antigens or components of viruses, such as hepatitis A, B, and C; human and murine retroviruses; adenovirus; avian C-type virus; rubella virus; and respiratory syncytial virus.

Western Blotting (Immunoblotting)—Western blotting, also known as immunoblotting, is used to identify specific antigens in the presence of other, potentially cross-reactive antigens. In this case, the specificity required is obtained by combining the antigen-antibody reaction with some form of separation (typically electrophoresis). Depending on the visualization methods employed (including digital methods such as densitometry), this method can be quite sensitive and even semiquantitative. One advantage of this approach is that in addition to detecting the antigen using an immunological approach, data on the approximate mass of the target protein may be obtained. A potential caveat is that most test proteins are denatured during this procedure and that antibody that depends on epitope conformation may not recognize the linear epitopes.

DETECTION OF VIRAL NUCLEIC ACIDS

The detection of viral nucleic acids provides another route for the determination of viral loads and for establishing the identity of a contaminant. Nucleic acids, like protein antigens, are essential components of viruses, and detectable quantities are usually indicative of viral presence. Detection assays can be designed and developed in some cases to parse viremia into phases, especially when the differentiation of nucleic acids along functional forms and configurations can provide clear insight into viral activity. Assays can be designed to determine whether viral DNA has been integrated into the host genome or still is encapsidated. Early viremia may be detected as viral mRNA transcripts prior to the accumulation of detectable viral particles. Nucleic acids may be the only detectable viral component of viruses that do not replicate well in tissue culture systems. Such systems may fail to produce mature virus particles, but the detection of viral transcripts can provide insight into whether the virus has the ability to infect the cell. Nucleic acid testing represents the most useful endpoint for the detection of certain viruses failing to cause responses using typical endpoints.

SAMPLE PREPARATION: SPECIAL CONSIDERATIONS FOR NUCLEIC ACID TESTING

The degradation of nucleic acids in samples can be limited through proper handling and storage practices and even enhanced by closely linking sample collection and preparation steps. In addition to preparing nucleic acids for further processing, denaturation is an important step toward stabilizing nucleic acids where storage temperatures extend above 0°.

Denaturation and Dissociation of Virions (Viral Lysis)—Chaotropic detergents and salts can be important agents for disrupting and removing viral proteins that make up the viral capsid. Their addition can provide a useful first step when concentration of virions is not necessary or even

possible. In sufficient quantity they rapidly denature the entire contents of a biological sample, essentially fixing nucleic acid content through the inactivation of nucleases and other proteins that may affect sample stability. Saturated solutions containing guanidium salts, such as guanidine hydrochloride or guanidine isothiocyanate, are commonly used for the dissociation of viral nucleic acids from protein components. These solutions may be used alone or in combination with ionic detergents and other denaturants such as phenol. The main advantage of guanidinium salts is that they are readily removed during the concentration of viral nucleic acids using ethanol or isopropanol. Urea may also be used as a mild denaturing agent, although it does not perform as effectively as guanidine in its ability to disrupt virus particles.

Deproteinization—The removal of proteins during the processing of samples for the detection of viral nucleic acids is helpful in ensuring the reproducibility and robustness of assays, particularly those that rely on amplification to detect exceptionally low quantities of nucleic acids. Several strategies may be used to facilitate deproteinization of the sample; they are discussed in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* (1126).

Recovery of Viral Nucleic Acids—Separation and recovery of extracted viral nucleic acid are important steps in the testing of nucleic acids. Nucleic acid yield and purity obtained at this step are critical determinants of assay robustness. Poor nucleic acid recovery and limited purification may inhibit amplification and detection reaction resulting in poor assay sensitivity. The development of high-yield, high-purity recovery steps is an important goal in the optimization of nucleic acid detection methods. Details on the general aspects of extraction and detection of nucleic acids are discussed in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* (1126). However, if no inhibitory components are identified in the system, direct testing on an aliquot of a sample may increase the assay sensitivity.

Isolation of Viral DNA—Integrated viral genomes must be recovered and processed along with the genome of the host cell. The viral genome is analyzed within the context of the host genome, and therefore similarities between virus and host genomes must be accounted for to ensure that assay results are specific for the virus and do not simply reflect the presence of cross-reacting host genome sequences. Viral genomes that exist as episomal entities require similar consideration, but there may be opportunities during sample preparation to limit the amount of host nucleic acid present in the preparation. Sedimentation gradients or silica-based separation chromatography can sometimes be used to enrich episomal nucleic acids through size-related exclusion or partitioning of processed nucleic acids.

The recovery of encapsidated viral genomes may allow for larger amounts of material, especially if the target virus has accumulated in large quantities in the system being monitored. When the amount of material available for recovery is low or the processing of large amounts of the material is impractical, the process of isolating and conditioning the virus particles, and subsequently the encapsidated genome, must be compatible with the assay system that will be used. Portions of the viral genome may need to be amplified, or the genome itself may be captured in an elaborate process that allows detection through the generation of an amplified signal. A standard method for isolating viral genomes may need to be modified appropriately to ensure that materials used in the preparation do not interfere with steps conducted later in the process.

Viral genomes that consist of RNA are typically converted to a DNA intermediate that is easier to handle and store. Methods aimed at the isolation of viral genomes consisting of RNA, however, require similar considerations concerning the localization of the genome: replicating RNA viral genomes may be recovered as part of a preparation of host total RNA or even mRNA if the genome contains polyA sequences. RNA lends itself more effectively to hybrid capture methods during isolation, and hybrid capture can be used

to enrich RNA preparations specifically for RNA viral genomes. RNA genomes can be extracted from virus particles in much the way that DNA viral genomes are extracted. RNA genomes are converted to complementary (cDNA) sequences using retroviral RT. Storage is of greater concern for naked viral RNAs. Storage of RNA usually requires temperatures below -20° .

DETECTION OF VIRAL GENOME VERSUS VIRAL TRANSCRIPTS

Viral genomes exist as either DNA or RNA, or sometimes both: in the case of retroviruses the integrated genome is DNA, whereas the encapsidated form is RNA. The ability to differentiate among the various forms of viral nucleic acids can help to elucidate the course of specific viral infections. Assays for nucleic acid activity can differentiate readily between integrated and encapsidated genomes when the form of the viral nucleic acid varies between states, as in the case of retroviruses. Incorporation of specific nucleases into the assay methodology can be used to reduce or eliminate one form over the other. If viral genomes are known to integrate at specific sites within the host genome, primers and probes can be developed around the integration site and incorporate significant elements of both host and viral genomes. Some viral mRNAs contain splice sites, and the differentiation of spliced nucleic acid sequences from unspliced sequences creates a unique mechanism for determining the status of nucleic acid localization and infection.

Characterization of DNA Viral Genomes—Methods for the recovery and preparation of viral genomes for characterization depend on the state of the viral genome. If the genome has been incorporated into a cellular compartment, the recovery and preparation strategy must take into account the cellular components that make up the sample matrix. If the viral genome targeted for analysis is the encapsidated form, the methods must focus on recovery of the virus particle and must include additional steps aimed at extracting the nucleic acid from the individual particles. Identification and characterization of viral genomes require specific complementary nucleic acid probes and primers whose sequence will be dictated by available information about the sequence of the targeted viral nucleic acids and the type of assay that will be used. Determination of the sequence of the viral nucleic acid of interest usually provides the most unambiguous means for characterization. However, a number of methods can be used as simple indicators for the presence or absence of specific sequence-based characteristics. For example, melting curve profiles using short oligonucleotide sequences can be used to establish whether a specific viral genotype is present.

Identification and Genotype Analysis—Nucleic acid testing is often used to identify viral isolates obtained from viral screening assays or to provide identity for viral stocks. Methods used for the identification of viral genomes are not unique to other applications in the field of molecular biology. Typically, an amplification step is required in order to achieve quantities for analysis. Amplified sequencing of the amplicons or application of a standard hybridization technique may be employed for more detail as to the nature of the amplified signal. For more details, refer to *Nucleic Acid-Based Techniques—Amplification* <1127>.

Hybridization Techniques—A variety of hybridization techniques are used to detect viral nucleic acid sequences, including Southern blot, Northern blot, DNase/RNase protection, in situ hybridization, microarray technology, and other techniques. The description of these methods, which is well beyond the scope of this chapter, can be found in *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* <1126>.

DETECTION OF ANTIBODIES TO VIRAL ANTIGENS

A variety of methods are available for detection and quantification of antibodies to viral agents, including neutralization, complement fixation, and immunoassays based on enzyme- or fluorescently labeled reagents. Although many details of the immunological methods mentioned above are beyond the scope of the chapter, this section addresses specific application aspects of viral antibody detection, including preparation and storage of test samples, common assay methods and platforms available, and specific examples of how these assays may be used to measure antibodies to specific viral agents.

Viral Structure Relative to Antigenic Composition and Selection of Antibody Assay

Mammalian viruses vary considerably in their nucleic acid content and thus the number of antigenic, virus-specific proteins produced.

Many viral proteins or glycoproteins are highly antigenic and induce a potent humoral immune response during natural infection, whether in humans or in animal models. In most cases, the immune system responds when the virus and its antigens appear in the extracellular fluid or on the infected cell membranes. The degree to which viral antigens are expressed is governed by the intracellular replication and protein synthesis of viruses in host organ tissues and by the several possible types of virus–host cell interaction. Antibodies produced as a result of natural viral infection are likely to represent the broadest response to antigens in their native state.

When selecting, developing, or evaluating an assay method for measurement of antibodies to viral proteins, the analyst must take into account the source of the antibodies and the method by which they were obtained or prepared. Details will be presented in *Immunological Test Methods—Reagent Development* <1103> and *Immunological Test Methods—Immunoassay Methodologies* <1104>.

General Considerations Regarding Sample Preparation for Antibody Detection of Viral Antigens

Antibodies are relatively stable, but care must be taken to ensure the integrity of the test antibodies during sample preparation and storage. Serologic tests may be developed to measure antibodies to viral agents in unfractionated biological fluids. The possibility for matrix interference with the antibody detection method should be considered.

In general, biological test samples should be clarified by centrifugation or filtration, depending on their intended use. Serum samples should not be hemolyzed, lipemic, or icteric. In some cases the specimen should also be heat-treated to inactivate endogenous complement and other components.

Test samples should be processed as soon as possible. When it is necessary to store samples, most test samples should be stored at -20° for short-term storage and below -80° for long-term storage. For all samples, the stability of the material needs to be assessed experimentally. Aliquots of appropriate volume should be prepared in accordance with test procedures to avoid unnecessary freeze-thaw cycles.

Antibody Methods

This section discusses the primary methods for detecting antibodies directed against viral antigens. The assay methods often include a variety of alternative formats for the detection of antibody. Only the more commonly used formats for antibody detection are discussed in this section. Some methods, including fluorescent antibody assays and

enzyme immunoassays, are widely applicable to the detection of antibodies to many different viral agents; others are limited to selected viruses having certain properties (e.g., hemagglutinins).

IMMUNOFLUORESCENCE MICROSCOPY FOR ANTIBODY DETECTION

When fluorescein isothiocyanate (FITC) is chemically coupled to an antibody molecule, the resulting *FITC-labeled antibody* can be used as a secondary antibody probe to detect the presence of a primary, virus-specific antibody bound to a virus-infected cell on a microscope slide (indirect immunofluorescence).

The indirect immunofluorescence or indirect fluorescent antibody (IFA) assay is one of the most basic and useful methods for detection of antibodies to viruses. The assay can be used to detect both virus-specific IgG- and IgM-class antibodies. When the assay is used to detect IgM antibodies, it usually requires the physical removal or inactivation/binding of IgG-class antibodies. In the absence of this step, the presence of IgM-specific antibody may be masked by excess IgG-specific antibody competing for primary binding sites on the substrate surface. IFA assays may be qualitative or quantitative.

The IFA for antibody to viral agents requires the use of virus-infected cells expressing viral antigens in cellular membranes. Viral stocks are prepared, titered, and used to infect permissive cells in tissue culture. The cells are harvested at appropriate times, washed, and spotted onto multiwell microscope slides at an appropriate density. Control slides are also prepared with noninfected cells. The slides are allowed to air-dry and then fixed in cold acetone. The fixed slides can then be stored under appropriate conditions for extended time periods. The stability of the viral antigens over time should be confirmed.

The test article to be examined for the presence of virus-specific antibodies can be applied to the slide, followed by an appropriate secondary antibody conjugated with a fluorescent tag that can be visualized under a fluorescent microscope. IgG- or IgM-specific antibodies can be distinguished by using the appropriately prepared secondary antibody.

Reading and correctly interpreting endpoints of IFA slides for antibody detection requires an experienced analyst, particularly when cellular location and fluorescent-staining patterns are critical for a specific virus. Such interpretation requires the use of appropriate controls and scoring or intensity of fluorescence. This is highly dependent on the quality of reagents, the consistency of the fluorescent microscopy and light source being used, and the experience of the analyst.

ENZYME IMMUNOASSAY FOR ANTIBODY DETECTION

The EIA and variations of it are the most widely used methods for the detection of viral antibodies in serum and other biological products. The most commonly used EIA for antibody detection is referred to as a noncompetitive solid phase EIA for antibody detection. The typical configuration of an EIA for antibody involves coating tubes or microwell plates with viral antigen(s), the addition of test serum or product to the tubes or wells, the binding of specific antibody in serum or product to antigen, and the detection of bound antibody by addition of a second antibody with binding affinity to the primary antibody, which is labeled to allow for its detection.

The assays can be specific to IgG- or IgM- class antibody or may detect total antibody. Assays for IgM may achieve improved specificity when performed as *IgM-capture* assays. These assays involve the use of plates or wells coated with anti-IgM antibody to capture total IgM in serum or product as a first step. Subsequently, viral antigen is added; it binds to the plate only if virus-specific IgM antibody has initially

been captured, and it is detected by addition of a second labeled antibody specific to viral antigen. The assay is most often performed as a qualitative measure of the presence of an antibody for a specific virus. Sufficient replicates of both positive and negative control samples are required in order to determine the appropriate cut-off value and the assay acceptance criteria. A mean value of a test sample equal to or greater than the cut-off value is considered positive.

COMPLEMENT FIXATION TEST

Complement fixation has selective value in allowing for simultaneous assay of antibodies to a wide variety of viral agents. The procedure involves multiple variables consisting of two pairs of antigen-antibody reactions. The first reaction, between a known virus antigen and a specific antibody in the test sample, takes place in the presence of a predetermined amount of exogenous complement. The complement is removed by the antigen-antibody complex. The second antigen-antibody reaction consists of sheep red blood cells (SRBCs) and hemolysin (antibody against SRBC). When this indicator system is added to the reaction mixture, the sensitized SRBCs will lyse only in the presence of free complement. The extent of lysis of SRBCs is inversely correlated with the amount of the antibody in the test article.

The experimental procedure involves the optimal titration of concentrations of hemolytic serum, complement, and viral antigen, using chessboard format. If used as the test sample, human serum should be inactivated at 56° for 30 minutes to inactivate the endogenous complement activity. A number of important controls must be run along with the test, and results must be within limits before the test can be properly interpreted. These include the sensitivity of SRBCs to lysis and complement concentration used. The relative amount of virus-specific antibody present can be determined by testing serial dilutions of the serum or product. The *complement-fixing titer* is the reciprocal of the highest dilution that prevents 50% hemolysis.

NEUTRALIZATION FOR ANTIBODY DETECTION

Neutralization for the measurement of antibodies to viral agents is still one of the most valuable assays available because of its high specificity and its ability to detect neutralizing antibodies. Neutralization is defined as the loss of viral infectivity through the binding of specific antibodies to viral coat proteins (or envelope glycoproteins) on the surface of the infectious viral particle. The assay may be used to measure the presence of antibodies to a known virus in a serum or product sample, or conversely to identify an unknown virus by using a serum or product sample containing known antibodies.

Before performing a neutralization assay to measure the presence of antibodies in serum or product, a known virus must first be grown and titrated in the test system in which the neutralization assay will be performed. For viruses prepared in cell culture, this usually involves inoculating susceptible cultures with relatively low multiplicity of infection (MOI; <1 PFU per cell) and harvesting the infected cells when about 50% to 75% cytopathic effect (CPE) is demonstrated. The virus preparation is then titrated by preparing serial multifold dilutions and inoculating replicate tubes or plate cultures with a fixed volume of the virus preparation. The endpoint of the titration is the dilution of the virus that will infect 50% of the cell cultures inoculated. This endpoint is said to contain one 50% tissue culture infective dose (TCID₅₀) in the volume used. If the test system involves animal lethality, the endpoint is referred as one 50% lethal dose (LD₅₀). The amount of virus used in the neutralization assay to follow is typically standardized to contain 100 TCID₅₀ or LD₅₀.

The test or host system used in neutralization assays is chosen on the basis of the specific virus to be tested and its

ability to replicate in the system. The commonly used host systems include cell culture, embryonated chicken eggs, and mice. Cell culture is usually the preferred test system, because the viruses used in the neutralization assay usually readily replicate and produce CPE. Susceptible host cells are grown in monolayers in dishes or multiwell plate cultures. After the virus/neutralizing serum mixture is added, the cultures are overlaid with agar-containing medium to restrict spread of CPE and allow development of viral plaques. The prevention of plaque development is indicative of the presence of neutralizing antibody. Alternatively, neutralization can be performed in tube monolayer cultures or even in suspension tissue culture. Embryonated eggs may be used when the virus to be used or tested does not produce plaques in tissue culture systems. The route of inoculation and the endpoint depend on the virus.

Neutralization assays may be set up in various ways, depending on the specific virus of interest and the serum or product to be tested for neutralizing activity. In general, a fixed amount of infectious virus is preincubated with undiluted and serial dilutions of serum or product to be tested for neutralizing activity and separately with preimmune serum or control product; this approach is referred to as the *constant virus-varying serum* method. Following preincubation, the mixtures are separately injected or added to the test system. Reduction in infectivity between test and control serum or product is scored in various ways, depending on the test system. The endpoint of the assay is generally defined as the highest dilution of the serum or product that neutralizes one-half of the initial viral inoculum, as calculated by Reed-Muench or the Spearman-Kärber method.

The titer of neutralizing antibody in the test serum or product is the reciprocal of the highest dilution that completely inhibits CPE or other virus effect in the test system. This dilution is said to contain 1 neutralizing antibody unit per unit volume used in the titration. When a serum or product known to contain neutralizing antibody is used in an assay to determine the identity of an unknown virus, 20 neutralizing antibody units in a fixed volume are generally used in the assay. Positive and negative control sera must give expected reactivity in the assay.

HEMAGGLUTINATION INHIBITION (HAI)

A number of enveloped viruses, including the influenza and parainfluenza viruses, acquire protein receptors capable of binding RBCs (hemagglutinins) of various animal species on their surface as they bud through infected cell plasma membranes during viral maturation. In addition, some nonenveloped viruses such as adenoviruses and certain enteroviruses have hemagglutinin proteins in their outer capsid. This property allows for detection of a specific virus in a sample if a known specific antibody to the virus is available. Alternatively, the presence of antibody specific to the virus can be detected and quantitated by its ability to inhibit hemagglutination. This is the principle of the hemagglutination inhibition (HAI) test.

The HAI test for antibody is performed by making serial dilutions of the specimen to be tested and mixing the dilutions with a fixed amount of the virus or specific viral hemagglutinin protein in a tube or microtitration plate format. Indicator RBCs from the appropriate animal species are added, the suspension is mixed, and the tubes or plates are allowed to stand for a predetermined period. If specific antibody is present, the virus will bind and the RBCs will not agglutinate; they will settle to the bottom of the tube or plate and form an RBC "button". If specific antibody is absent, the RBCs will be agglutinated by the virus and form a diffuse film. The titer of the serum or product is the reciprocal of the dilution that completely inhibits agglutination.

HAI is very useful for subtyping influenza virus isolates. A number of factors contribute to the potential variability of

the HAI test. Certain serum samples and products may contain nonspecific inhibitors of RBC agglutinins, which may yield false-positive results. A number of procedures have been developed to remove such inhibitors, including adsorption and heat inactivation procedures. Specimens may also contain RBC agglutinins other than specific antibody, and these may contribute to false-negative results. Appropriate preparation and titration of reagents, including RBCs and viral hemagglutinin stocks and suspensions, is critical. In addition, controls for nonspecific agglutination or inhibitors of agglutination must be included in every assay.

WESTERN BLOT (OR IMMUNOBLOT) ASSAY FOR ANTIBODY DETECTION

The immunoblot, or Western blot, assay is a technique for the simultaneous detection of antibodies to various protein antigens of a given virus. The term *recombinant immunoblot assay* (RIBA) is applicable when the starting protein mixtures are recombinant proteins obtained from prokaryotic or eukaryotic expression systems instead of crude or partially purified virus from infected cells. The method is often used diagnostically as a supplementary or confirmatory test in situations where an initial assay for antibody lacks sufficient specificity or is known to be prone to false-positive results. This is especially important when the test is being used to diagnose an infection of clinical significance such as HIV or HCV infection.

A number of commercial immunoblot kits are available, particularly for viruses such as HIV and HCV; several have regulatory approval for diagnostic use. Alternatively, viral antigen preparations may be produced in-house or purchased, along with other reagents required for the assays. Careful control and/or sourcing of these reagents are critical to ensuring that compliance requirements are maintained.

For selected viral agents, there are generally accepted interpretive standards for the analysis of reactivity or positive results in an immunoblot assay. However, the presence of nonspecific bands may be due to antibody reactivity to cellular protein antigens caused by autoimmune diseases and/or the use of crude virus-infected cell proteins as antigen in the assay. Indeterminate reactions may also occur if only a limited number of specific antibody bands are observed.

Appropriate positive and negative control sera must be included in each assay and reactivity must be scored for both the presence and the intensity of expected protein bands.

Application of the Antibody Detection Methods to Specific Viruses

Human blood-borne pathogens that may be present in infectious form in human donated blood used directly in the production of biological products are a concern because they may present a risk of transmission to others. Testing for virus-specific antibodies in donated blood serves as a screening procedure for the elimination of suspect units. Alternatively, the viruses may represent important agents for which human vaccines have been or are being developed. Thus the ability to detect virus-specific antibodies in an immunized individual or animal model may be important for demonstrating the efficacy of the vaccine. Currently, in the United States, a number of FDA-approved screening or definitive tests may be conducted on donated units of blood for evidence of the presence of agents of infectious diseases, including hepatitis B and C viruses, human immunodeficiency virus, and West Nile virus. In addition, plasma sent for fractionation before production of plasma-derived products is required to be tested for hepatitis A virus (HAV) and human parvovirus B-19.

GLOSSARY

Acceptance Criteria—Anticipated results, which may be numerical limits, ranges, or other characterization for the tests described. They establish the standards to which a drug substance or drug product should conform in order to be considered acceptable for its intended use.

Adventitious Agent—Acquired accidental contaminant in a cell line such as viruses and toxins; the agent is often infectious.

Amplicon—A segment of DNA generated by the PCR process whose sequence is defined by forward and reverse primers.

Antibody—An infection-fighting protein molecule that binds, neutralizes, and helps destroy foreign microorganisms or toxins. Also known as immunoglobulins, antibodies are produced by the immune system in response to antigens.

Antigen—Any agent that induces the production of an antibody and reacts specifically with it.

Assay Validation—A formal, archived demonstration of the analytical performance of an assay that provides justification for use of the assay for an intended purpose and a range of acceptable potency values.

Bioassay—Analytical method that uses living animals, cells, tissues, or organisms as test subjects.

Biologics—Products such as antitoxins, antivenins, blood, blood derivatives, immune serums, immunologic diagnostic aids, toxoids, vaccines, and related articles that are produced under license in accordance with the terms of the federal Public Health Service Act (58 Stat. 682) approved July 1, 1944, as amended, have long been known as “biologics.” However, in Table III, Part F, of the Act, the term “biological products” is applied to the group of licensed products as a whole. For Pharmacopeial purposes, the term “biologics” refers to those products that must be licensed under the Act and comply with Food and Drug Regulations—Code of Federal Regulations, Title 21 Parts 600–680, pertaining to federal control of these products (other than certain diagnostic aids), as administered by the Center for Biologics Evaluation and Research or, in the case of the relevant diagnostic aids, by the Center for Devices and Radiological Health of the federal Food and Drug Administration. [Definition from *Biologics* (1041), *USP–NF* vol. 30 (2007), p. 414.]

Biotechnology-Derived Product—Macromolecular article derived from biotechnology processes such as recombinant DNA (rDNA) technology, hybridoma technology, and the like.

Bulk Harvest—See *Unprocessed Bulk Harvest*.

Capsid—The outer protein shell of a virus particle.

Cell Bank—A defined population of cells, such as an immortalized cell line, grown by a defined process and cryopreserved in a defined process and within a defined passage number range. The assumption is that each vial from a cell bank is comparable and that when thawed and added to a manufacturing vessel (or an analytical assay), it will perform in a consistent way.

Chaotropic—A reagent that causes molecular structure to be disrupted; in particular, those formed by noncovalent forces such as hydrogen bonding, van der Waals interactions, and the hydrophobic effect.

Complement—A group of proteins in the blood that work in concert with other immune system proteins and cells (such as antibodies) in attaching foreign substances.

cDNA—Complementary DNA. Two strands of nucleic acid that can hybridize by specific base pairing between the nucleotides.

Confluency—Refers to the point when 100% of the surface area of the vessel is covered in cells.

Cryopreservative—Reagent used to keep a cell alive in deep-frozen condition (usually in liquid nitrogen).

Cytopathic—Damaging to cells, causing them to exhibit signs of disease or cell death.

ELISA—Enzyme-linked immunosorbent assay. A biochemical technique used to detect the presence of an antibody or an antigen in a sample.

Endpoint Assay—An analytical method that measures the amount of accumulated product at the end of the assay.

Epitope—A molecular region on the surface of an antigen that is recognized by an antibody and can combine with the specific antibody produced by such a response; also called a determinant or an antigenic determinant.

Glycoprotein—Protein that contains sugar side chains added as a posttranslational process; the presence of sugar side chains often affects activity, antigenicity, and in vivo stability.

Host Cell Tropism—The range of susceptible cells that a particular microorganism can infect.

ICH—The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use.

Limit of Detection (LOD)—The lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as a concentration at a specified signal-to-noise ratio, usually 2 or 3.

Mycoplasma—Parasitic microorganism that infects mammalian cells, possessing some characteristics of both bacteria and viruses. Prokaryotic microorganisms belong to the family *Mycoplasmataceae*, with no cell walls. They may grow attached or close to cell surfaces in the cytoplasm and subtly change the properties of the cells.

Passage—An operational procedure used to feed cultured cells, usually by providing fresh medium and dilution of cells in a new culture vessel. The number of such operations is referred to as the passage number. It is not the same as cell generation number, which is strictly related to cell doubling time.

qPCR—Quantitative polymerase chain reaction. A modification of the polymerase chain reaction used to measure the quantity of DNA, complementary DNA, or ribonucleic acid present in a sample. Like other forms of polymerase chain reaction, the process is used to amplify DNA samples via the enzyme DNA polymerase.

Raw Materials—All components used to manufacture a drug substance or drug product; regulated by 21 CFR 211.

RT-PCR—Reverse transcriptase polymerase chain reaction. A variation of the PCR technique in which cDNA is made from RNA via reverse transcription. The cDNA is then amplified using standard PCR protocols.

Serotype—The kind of microorganism as characterized by testing for recognizable antigens on the surface of cells of the microorganism.

Spiking—Adding a known amount of analyte from a laboratory standard acting as a tracer to check a method for recovery or accuracy.

Syncytium—A multinucleated mass of cytoplasm that is not separated into individual cells.

System Suitability—The checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution, and reproducibility are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability *sample*, which is a mixture of main components and expected by-products.

TCID₅₀—50% tissue culture infective dose. The level of dilution of a virus at which half of a series of laboratory wells contain active, growing virus.

Unprocessed Bulk Harvest—The pooled harvests of cell culture fluids that constitute a homogeneous mixture for manufacture into a unique lot of product.

APPENDIX: RELEVANT REGULATORY REFERENCES

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3. 9 CFR 113.53.
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6. 21 CFR 211.
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10. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Q5A (R1): Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin. 1997.
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<1238> VACCINES FOR HUMAN USE—BACTERIAL VACCINES

INTRODUCTION

An overview of vaccines for human use is presented in *Vaccines for Human Use—General Considerations* (1235). Bacterial vaccines can be derived from whole cells, either killed or attenuated in their ability to cause disease, or from some component(s) of the intact cell that are important for virulence or damage to the host. Another subset of bacterial vaccines, derived from toxins, is the toxoids. Bacterial vaccine products can be mixtures of components from different species, from different strains or different serotypes of the same species, or from different components from cells of the same species.

The simplest bacterial vaccines consist of the purified cell-surface capsular polysaccharides (CPS) from organisms such as *Salmonella enterica* serovar Typhi, various meningococcal serogroups, or pneumococcal serotypes that cause meningitis, otitis media, acute respiratory infections, and pneumonia. Although the typhoid vaccine consists of a single polysaccharide, the meningococcal vaccines contain as many as four serogroup-specific CPS, and the pneumococcal vaccine contains 23 serotypes.

The immunological response to meningococcal and pneumococcal polysaccharides, and to the capsular polysaccharide from *Haemophilus influenzae* type b (Hib), is improved by covalent attachment of the CPS or an oligosaccharide derived from it to a suitable carrier protein. The immunological response to these glycoconjugate vaccines is elicited via immunologic pathways different from those induced by purified polysaccharides, creates a T-cell-dependent response, and establishes immunological memory. The carrier proteins are typically bacterial toxoids or bacterial outer membrane protein vesicles but may also be from other sources. For

these products, anti-CPS antibodies appear to be sufficient to protect against disease, although the glycoconjugate vaccines also may reduce carriage of the organisms in the nasopharynx. Due to the complexity of their manufacturing processes, glycoconjugate vaccine products tend to contain fewer serotype or serogroup components than do the related purified polysaccharide vaccines.

Many bacterial pathogens, including those that cause diphtheria and tetanus, produce toxins that kill tissue. Immunological neutralization of these toxins is sufficient to prevent disease. These subunit vaccines consist of chemically detoxified toxins (toxoids) purified from culture supernatant and are capable of eliciting neutralizing antibodies against the native toxin. Other types of purified subunit and purification processes may be developed.

Although earlier pertussis vaccines consisted of myriad chemically inactivated whole-cell and toxin components, current acellular products contain various combinations of specific purified proteins, sometimes toxoided (e.g., fimbriae and other cell-surface protein components). Compared to older products, these vaccines apparently produce protection by a different mode of action but have a lower incidence of adverse events. A combination of diphtheria and tetanus toxoids and an acellular pertussis vaccine form the core components of many polyvalent pediatric and adult combination vaccines. To these may also be added an Hib glycoconjugate, hepatitis B, and/or inactivated poliovirus immunogens.

Live attenuated bacterial vaccines are currently limited to *Bacillus Calmette-Guérin* (BCG), which protects against tuberculosis when administered through the skin, and the *S. typhi* Ty21a construct, which is an oral vaccine against typhoid fever.

The immune response against these bacterial polysaccharide and protein antigens can be increased by inclusion of adjuvants. The primary adjuvant licensed in the United States is based on aluminum salts such as aluminum hydroxide and aluminum phosphate, although development and characterization of new adjuvants is an active area of research.

RAW MATERIALS

Raw materials can directly affect the identity, strength, purity, and quality of bacterial vaccines. A consistent manufacturing process critically depends on use of consistent raw materials (e.g., during seed banking, fermentation, harvest, purification, and formulation; see *Vaccines for Human Use—General Considerations* (1235)). Raw materials for bacterial growth media typically consist of both well-defined chemical entities (e.g., amino acids, carbohydrates, vitamins, minerals) and more complex components (e.g., protein hydrolysates, yeast extracts, peptones). Manufacturers should consider the source of each of these raw materials to ensure that they come from reliable vendors who adhere to cGMP quality standards and can assure a long-term supply. Manufacturers should communicate with raw material vendors in order to avoid any changes in the sourcing or manufacture of components and to avoid supply shortages. Without such communications, the consistency of the fermentation process and the supply of the vaccine can be adversely affected. Consistent raw materials are particularly critical for more complex fermentation components such as yeast extract or peptones for which changes may be difficult to detect but are likely to have a direct effect on fermentation.

Accurate records of the composition and source of the culture medium used in seed banking and routine fermentation should be maintained and also document release criteria for raw materials or components. Manufacturers should determine if any of their raw materials are derived from animal origin. If additives from animal sources are added to the culture medium, they should be certified to be free from contaminants and adventitious agents such as those that cause bovine spongiform encephalopathy or transmissible

spongiform encephalopathy. Vendors/manufacturers should provide information about the identity and source of additives and should test for adventitious agents. Use of antibiotics should be minimal or should be avoided to ensure that no unwanted antibiotics are included in the drug product, unless they are intentionally used in manufacturing (e.g., as selective markers).

As manufacturers scale up fermentation to pilot production (i.e., within tenfold of final manufacturing scale), they also should ensure, to the extent possible, the availability of multiple sources for all raw materials. This will ensure that supply or business instabilities at one vendor do not become the limiting factor in vaccine manufacture.

CELL BANKS

Source and History

The source of cells used in cell banks should be documented. The original isolate should include, when possible, the age, sex, and species of the donor; the donor's medical history; and, if available, culture history including methods used for the isolation of the substrate bacteria.

The source of cells from which the strain was derived is to be stated, and relevant references from the scientific literature should be cited. The source should generate a sufficient amount of antigen(s) to meet the medical need. Information obtained directly from the source laboratory is preferred. When this is not available, literature references can be used to provide bacterial classification (i.e., genus, species, and strain designation) and specific phenotypic and/or genotypic trait. For microbial-expression systems such as *E. coli* or *S. pneumoniae*, the manufacturer should describe the method used to prepare the DNA coding for the protein, including the cell and origin of the source nucleic acid. All propagations carried out with the original isolate should be documented and should include, as applicable, the method used for subculture, any use of animal-derived material, record of subcultivations, and storage conditions. Constituents of the culture medium must be described, in particular, materials of human or animal origin such as serum, enzymes, hydrolysates, or other living cells.

For microbial-expression systems, the steps in the assembly of the expression construct must be described in detail. This description should include the source and function of the component parts of the expression construct (e.g., origins of replication, antibiotic resistance genes, promoters, enhancers, and whether or not the protein is synthesized as a fusion protein). Manufacturers should provide restriction endonuclease digestion maps that illustrate the sites used in preparing the expression construct and sites used in identification of DNA fragments.

A complete nucleotide sequence analysis of the expression construct's coding region for the protein of interest should be performed. The sequence analysis should be provided and should include a complete annotation designating all of the important sequence features. The copy number and physical state of the expression construct should be determined.

Cell Bank Lineage and Genealogy

A flow chart can be used to demonstrate the preparation of the cell bank lineage from the original source, through preliminary cell banks (or process development cell banks, as applicable), to the Master Cell Bank (MCB) and production Working Cell Banks (WCB).

Manufacturers should describe their strategy for providing a continued supply of cells from their cell bank(s), including the lot size and anticipated use rate of the cell bank(s) for production, the expected intervals between generation of new cell bank(s), and the criteria for qualification of cell

bank(s). If multiple WCBs were used for clinical trials, process validation, or commercial supplies, flow charts can help illustrate the common source (i.e., MCB) from which the WCB were derived.

Once an MCB is produced, a cell bank system should be generated to prevent unwanted drift that might ensue from repeated subcultures or multiple generations. The system should ensure that an adequate supply of equivalent cells exists over the entire life span of the product. Ordinarily, the cell bank system consists of two tiers: an MCB and a series of WCB derived from the MCB. When additional tiers of WCBs are prepared, manufacturers should clearly identify the generation that will be used for WCB.

Cell Bank Manufacture

Generally, the MCB is made from a preliminary cell bank derived from the original source or directly from an initial clone. Manufacturers generally prepare cells for banking by expanding cultures in a progressively greater number of vessels or in larger vessels until a pool of cells is obtained. If manufacturers use more than one vessel, they can ensure the uniform composition of the contents by combining the cells from all of the culture vessels.

For microbial-expression systems, a single host cell that contains the expression construct is propagated to generate the MCB. Manufacturers should document the cell cloning history and method of transferring the expression construct into the host cell. They also should completely describe methods and criteria used to amplify the expression construct and to select the cell clone for production.

The process for WCB used in clinical trials and for commercial supply should be similar to the MCB process. A WCB is derived from one or more containers of the MCB and is typically used to directly provide cells for the manufacturing process. Additional WCB are generated from the MCB as needed.

Preferably the MCB and WCB should be prepared in a similar manner, but the MCB and WCB may differ in certain respects (e.g., culture components and culture conditions). Similarly, the culture conditions used to prepare the MCB and WCB may differ from those used for the production process or between clinical trial materials or commercial supply. The preparation procedures for all cell culture processes must be described, and details of process changes must be documented. Comparability of product quality must be demonstrated when process changes occur between WCBs.

Cell banks should be made under cGMP because they are expected to last for the lifetime of the product. The facility should be operated to minimize the chance of microbial contamination and have in place procedures to prevent cross-contamination with other materials. Critical equipment used in the preparation of cell banks should be qualified. Manufacturers should establish the cell bank in a suitably controlled environment to protect both the cell bank and personnel handling it. During the establishment of the cell bank, no other living infectious material (e.g., viruses, cell lines, or cell strains) can be handled simultaneously in the same area.

Cell Bank Validation

The cell banking process should be considered a unit operation and should be validated. The process begins with the MCB vial and the cell bank process validated for preparing WCB. The suitability of WCB for intended use should be further demonstrated by the consistency and quality of successive product batches. Qualified banks should be used for process validation of fermentation, drug substance, etc. If this is not possible, then manufacturers should perform a small-scale demonstration of the appropriateness of the cell bank. The basic principles of process validation apply, in-

cluding use of validated analytical methods and stability evaluation.

Manufacturers should describe the methods used to preserve cell banks, including the cryoprotectant and media used. Storage containers (e.g., vials, ampules, and other appropriate vessels) and closure systems should be described. Container–closure systems should incorporate materials and designs that withstand storage and retrieval without breakage or leakage and are physically and chemically compatible with the stored material.

Cell Bank Testing

A newly prepared cell bank (MCB or WCB) should be evaluated by a series of appropriate release and characterization tests on an aliquot of the cell bank or on cultures derived from it, as appropriate. The amount of testing required for an MCB may influence that required for subsequent WCBs, and the extent of testing both may influence the testing needed for production cell cultures. Manufacturers should evaluate all cell banks, including bacterial cultures or recombinant bacterial expression systems, for identity, culture purity, and viability. Additionally, manufacturers should evaluate the genetic stability and consistent productivity of all cell lines.

To confirm identity, manufacturers should perform appropriate tests to determine that the banked cells are what they are represented to be. Either phenotypic or genotypic characteristics can be used in identity testing to classify bacterial strains to species level, and when applicable, supplementary serological tests can be performed. For most microbial cells and transfected cells, analysis of growth on selective media is usually adequate to confirm host cell identity. Where a variety of strains can be used, biological characterization methods such as phage typing should be considered as supplementary tests. Expression of the desired product is also considered adequate to confirm the identity of the microbial expression system.

It must also be demonstrated that cell banks are biologically pure (i.e., free from adventitious microbial agents). Testing for adventitious agents should include tests for bacteria, fungi, mycoplasmas and viruses, as applicable.

Additionally, all cell banks should be tested to confirm the viability of the cells. Viable cell counts or growth tests should be performed to demonstrate that the cell culture has sufficient viability and is suitable for its subsequent intended use.

Evaluation of genetic stability and persistence of productivity is a reflection of how many doublings the cells can tolerate without compromising their genetic integrity (e.g., plasmid retention) and productivity (e.g., mass of product per cell). Such testing is critical to ensure that the cell line performs reliably in the full course of the production process from the initial MCB stage through the longest production intended. As part of this evaluation, manufacturers should document the number of passages from the original source, the number of subcultivations from the original source to the MCB, from the MCB to the WCB, and from the WCB to the final bulk. The earliest and latest culture states (e.g., MCB and end production) should be evaluated to ensure that the desired characteristics persist. Such a demonstration of cell line stability is commonly performed once for each product marketing application.

Characterization tests may be useful for demonstrating that the cell bank is composed of cells with the intended phenotypic/genotypic characteristics. Such tests can include cellular and colony morphology (i.e., use of selective and/or differential media), biochemical profiles (enzymatic activity or substrate utilization), immunological identity, characteristic growth, and antibiotic susceptibility.

Additionally, for recombinant bacterial expression cell lines (e.g., *E. coli*) molecular characterization testing can include DNA sequencing of the target gene sequence along with the flanking regions, expression construct retention, and

plasmid copy number. Analysis of the expression construct at the nucleic acid level should be performed with consideration that this verifies only the coding sequence of a recombinant gene. Restriction endonuclease mapping or other suitable techniques should be used to analyze the expression construct for insertions or deletions and for the number of integration sites. For extrachromosomal expression systems, the percent of host cells that retain the expression construct should be determined under selected and non-selected growth conditions. For cells with chromosomal copies of the expression construct, the nucleotide sequence encoding the product could be verified by recloning and sequencing of chromosomal copies.

Much of this testing should be conducted on the MCB if possible, which will preclude the need to repeat much of the testing on each WCB or production lot, although sometimes redundant testing (on both MCB and WCB) may be desirable.

Limited identity testing is generally performed on each WCB if extensive identity testing was performed on the MCB. For recombinant products, the identity of the WCB should be assessed by restriction endonuclease mapping of the expression construct for copy number and for insertions or deletions. In addition, where appropriate, the WCB should be identified by phenotypic characterization (e.g., auxotrophy, antibiotic resistance).

For each lot of WCB derived from the MCB, manufacturers should routinely test for contaminants that may have been introduced from the culture medium during preparation. Purity tests like those performed on the MCB to test for adventitious agents may be performed on the WCB.

Characteristics of the recombinant protein product can also be applied (see below) as another means of defining the ultimate output of the cell line.

In the event that a new MCB is needed, the testing performed on a new MCB should be the same as that performed on the original MCB unless justified. If a new MCB is to be generated by expression construct transfer into host cells followed by clonal selection, then acceptance criteria for both the new clone and the protein produced by the clone should be described and justified.

Cell Bank Storage

In both MCB and WCB of the same product, similar containers (such as cryovials) are generally used and are treated identically during storage.

The location, identity, and detailed inventory of individual ampules of cells should be thoroughly documented with procedures that allow the cell bank containers to be traced. Labeling should clearly indicate the biological name of the components, unique container number, lot or batch number if applicable, and the type of bank (such as MCB or WCB). The label must withstand storage and retrieval without loss of integrity or information.

Cell banks should be established, stored, and used in a way that minimizes the risk of contamination or cross-contamination by other cell types that may be present in storage. Once issued, banked materials cannot be returned to the controlled storage area. Access to banked material must be controlled by a strict inventory-control system with limited access by authorized individuals only.

Bacterial cell banks should be stored in either the liquid or vapor phase of liquid nitrogen or in mechanical freezers (generally $\leq -60^{\circ}$). Storage conditions (generally $\leq -60^{\circ}$) may be acceptable when supported by data that demonstrate that a minimum level of cell viability is maintained and is adequate for production use. Storage temperature and other critical storage conditions should be maintained within validated limits. Temperatures must be continuously monitored and recorded, preferably on an alarm system. Shipping containers used to transport cryopreserved cell banks to offsite storage facilities or manufacturing facilities

must be validated, and shipping qualification must be performed before use.

Because of more frequent usage of WCBs and to protect the MCB, the WCB should be stored separately from the MCB. Cell banks may also be stored in two or more widely separate areas within the production facility, as well as at a distant site in order to avoid loss of the cell bank (e.g., caused by equipment malfunctions or disaster at the site). When stored in different locations, the cell banks must be stored under the same conditions.

As part of a disaster recovery plan, the manufacturer should document the steps and timeline needed to restart production of new cell banks and/or contingency plans for continued manufacturing production.

Storage Stability

MCB and WCB should be placed in a stability program. Evidence for banked cell stability under defined storage conditions usually is generated during production of clinical trial material or commercial material from the banked cells. Data from the determination of cell viability when the preserved cells are reconstituted for production of clinical trial supplies can verify that the revived cells have survived the preservation process. Data from the preparation of clinical materials are used to demonstrate that the revived cells can be used to prepare the desired product.

Enough MCB material for the lifetime of the product should be put on stability (enough WCB should be put on stability to support the lifetime of the WCB). This can be a large volume because the product lifetime can be quite long (e.g., 50 years). During the preparation of the MCB, the lot size should be large enough to allow adequate inventory to support the lifetime of the stability study as well as production for the life of the product. Time points for such a long-term study might include 0, 6, and 12 months, and then perhaps every 1 to 3 years thereafter. Typically, no expiration dating is used for cell banks because stability studies are used to confirm the suitability of the material. Greater reliance is placed on the successful (and typical) culture of the cells themselves. The proposed monitoring should be documented in pre-approved protocols. The time points can be reduced (e.g., increase the time between time points) if data indicate stability. In addition, time points can be added if sufficient material is available and the data suggest that more monitoring is needed. The stability plan depends on the use rate in manufacturing.

FERMENTATION

Production of the drug substance for a bacterial vaccine requires a fermentation process that is consistent and sufficiently productive to support commercial production. The approach to achieving this has become fairly standardized and provides a relatively high probability of success for early batch production to support a development program. It is still a significant challenge to achieve sufficient productivity to support commercial manufacture of a licensed product. Directly following any fermentation process is the harvest process, which serves as a transition step between biomass expansion and downstream process steps. For purposes of this chapter, harvest will be considered as an extension of the fermentation process.

Fermentation Starting Materials: Cell Inoculum

The cell inoculum for the fermentation process is the single most important component for establishing a reproducible fermentation process. In early development before finalizing fermentation conditions, manufacturers typically must generate an interim source of this inoculum, a Process Development Cell Bank (PDCB). The origin of the PDCB should be a clonal isolate of the original transfected or isolated

strain that demonstrates suitable growth properties and produces the antigen of interest in sufficient quantity and quality for the intended purpose. The use of a clonal isolate ensures that the genetic starting point for each batch is the same and that subtle variations in process conditions will not inadvertently allow one population versus another to dominate the culture. That is, the PDCB is used for fermentation development to ensure that variations in the fermentation conditions can be interpreted without the overlay of competition between populations of transfectants.

Initial development of the fermentation process, preferably with the PDCB, typically precedes production of the MCB and WCB. Best practice is to derive these cell banks from the same clonal isolate as the PDCB in order to reduce the need for a second cycle of fermentation development when the WCB is deployed. Substitution of a WCB for the PDCB at the final stages of fermentation development is common practice, but care must be taken to constrain such experiments to optimization of the fermentation process. More detail is found in the cell banks section above.

Fermentation Hardware

The biomass production process typically begins with a small-volume inoculum in an initial fermentation volume that is 20- to 100-fold larger than the initial inoculum volume. This initial passage is often followed by one or more intermediate fermentations that expand the production volume by 20- to 100-fold at each step until the production fermentation volume (typically 500–3000 L) is reached. Routine manufacture at these scales requires well-controlled fermentation conditions and physical facilities that meet the economic and cGMP needs for a successful product.

Bacterial fermentations have traditionally been carried out in glass, glass-lined, or passivated stainless steel fermenters that comply with cGMP requirements, particularly when using large fermenters (e.g., those with >1000 L working volume) because of containment issues with such large volumes of liquid. Traditional fermentation systems require hard-piped control systems that meet the need for clean-in-place and steam-in-place capability. The bioburden and complexity of the facility are increased if the fermentation operations must accommodate multiple product lines as well.

Smaller fermentation batches are increasingly performed in disposable bioreactors such as single-use bags with completely disposable product contact surfaces, including sensors and probes. These systems are becoming readily available, are less expensive, and are more flexible than fixed equipment and meet the needs of the competitive business and evolving cGMP expectations and requirements. A note of caution is warranted, though, because this disposable technology can lead to changes in the material of product contact surfaces. Such changes then require re-evaluation and sometimes revalidation of the manufacturing process for late-stage development and commercial products. Thus the reduced cleaning burden may bring an increase in the need for extractability and leachability studies.

Harvest Hardware

Harvest of the fermentation product can focus on recovery of either the wet cell mass from which the product will be extracted or the fermentation broth from which the product will be directly purified. In the former case, centrifugal separation is typically employed. Production-scale centrifuges can be either closed operations with a fixed volume of input and manual recovery of the pellet or continuous-flow operations that automatically eject and recover the clarified supernatant and/or the accumulated pellet. Although centrifuges are efficient in harvesting a fermentation product, shear forces can have significant effects on the product stream (e.g., lysed cells, sheared molecules in solution). Alternatively, and particularly when the product is se-

creted into the solution rather than retained in the cells, membrane filtration systems may be used to clarify the product stream for subsequent purification. Tangential-flow and depth filtration systems can be effective means of recovering soluble product with less concern about shear forces.

In all cases, monitoring the processing of the fermentation output and solids removal from the liquid matrix can be simple but effective means to monitor process consistency and comparability. Off-line tools such as high performance liquid chromatography (HPLC), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), or Western blot analyses can track product integrity issues such as aggregation or proteolysis.

Process Development

A productive, robust fermentation process is the result of careful consideration of a broad collection of variables, considered alone as well as in conjunction with product design and downstream processing. Fermentation process variables include chemical inputs (e.g., carbon sources, minerals, vitamins, trace elements, antifoam, and gases), physical inputs (e.g., temperature, mixing, and pH), and biological processes (e.g., nutrient utilization rates, metabolite levels, and inductors [quantity/type/addition duration]).

Product Requirements

A critical consideration in process development is anticipating how much of the product will be needed. Too little product caused by operating at too small a scale imposes supply constraints and often postlicensure urgency to scale up the process. In contrast, too much product results in excess inventory, expiring lots, infrequent manufacturing (itself a problem), and generally poor economics. A clear market evaluation is needed before one designs the manufacturing process or commits to a process for scale-up or -down.

Process Design

If one has a reasonably defined production need and an initial estimate of product yield, one can extrapolate the scale of the fermentation from the volume (yield), production frequency, and the expected productivity. Commercial fermentation of bacterial cultures is routinely carried out in volumes as large as 3000 L, but larger volumes are also in use. A few large lots per year can be advantageous for a very robust process but may be limited by downstream process capabilities and/or the stability of the fermentation product as a production intermediate. An additional consideration may be the difficulty in generating enough lots to ensure that the fermentation process is indeed robust. Failure of a large lot carries important financial and inventory risks.

A large number of lots can impose logistical problems if turn-around time is too tight or coordination of downstream events becomes too complex. Logistics includes quality control testing, which depends on the number rather than the size of lots. Production that involves a large number of smaller lots can also require blending of multiple intermediate lots in order to produce a final drug product lot. This can cause challenges if product-related problems occur and may entail root-cause investigations. In general, appropriate fermentation sizing results in a process that has a turn-around time of less than a week, that can be accommodated with one or a few purification runs, and that results in one to several fills of final product after each purification cycle.

Early Development Considerations

During early development of a biological product, the most important fermentation considerations are an appropriate, well-defined MCB and a fermentation process that is reasonably productive, reproducible, and scalable. The latter is often underestimated when one considers the physical, chemical, and biological control of the process as process volumes change by orders of magnitude.

The mechanics of the fermentation process are an important consideration. Fermentations are typically studied in shake flask experiments or even microscale reactors that can readily accommodate many experiments conducted in parallel. Although this is attractive for initial identification of process conditions, the ultimate culture vessel should be a controlled fermenter where growth conditions can be controlled and monitored in a more rigorous and complete manner. Manufacturers should begin work in small-scale fermenters as early as possible to ensure that robust, controlled experiments can be run to refine the initial fermentation conditions.

Fermentation harvest processes also should be scalable. Although it is possible to scale centrifugation conditions, it is a challenge to maintain equivalent centrifugal conditions, particularly in a flow-through mode. Filtration processes can usually be scaled more predictably provided the membrane manufacturer is anticipating the needs of the process development scientist.

When manufacturers define a process, they should evaluate its robustness by purposeful deviations such as changes in sources of raw materials and time and temperature limits of unit operations. Such evaluations better define the rationale for setting process limits and for knowing which are most critical to the success of the manufacturing process.

Process Monitoring

On the basis of early development process characterization data, manufacturers should be able to identify key analytical measures that, if applied to all lots, can either verify the correct progression of the process or serve as a sentinel to determine whether a specific batch is showing signs of deviating from the typical profile. In the absence of such data, an aberrant process may go unnoticed or may not be detected until testing of a process intermediate shows either an out-of-trend or out-of-specification result.

For a fermentation process, many critical variables (e.g., optical density, pH, and specific nutrient levels) can be measured online and in real time to potentially allow intervention to bring a given process back into normal range or at least to identify the point in the process at which the deviation occurred. Such data can be valuable in identifying potential process improvements. Conversely, in the absence of such data troubleshooting can be a challenging and protracted process.

Scale-up

Just as early development requires a focus on small-scale operations, scale-up becomes essential at some point to ensure that sufficiently large lots can be made to meet program needs. As these needs become increasingly complex, larger lots are essential to ensure that multiple experiments and observations can be tied to the same lot of product, which in turn is critical to understanding critical process and product variables. If proper process engineering considerations were taken into account at the smaller production scale, scale-up can usually be done in increments of ten-fold in volume with reasonable expectation that significant process performance or product changes will not be seen. This approach may require adjustments at an intermediate scale if the initial fermentation was based on too small a volume

or if the final production scale is very large. Again, process monitoring data can be very helpful in evaluating the success of the scaled-up process.

If clinical development studies are performed at less than full manufacturing scale, as they usually are, manufacturers will be obliged to relate the comparability of the process performance and the product characteristics at the different scales. Analytical data can be compelling, but in their absence or in the presence of differences, manufacturers must demonstrate that scale-related differences are not clinically significant. However, the use of comparability protocols for scale changes will have to be approved by the local regulatory authority. In order to avoid fixing something that is not broken, analysts must take care to isolate differences caused by fermentation scale-up from changes caused by harvest or purification scale-up. One way to accomplish this is to compare process intermediates obtained, as possible, during the fermentation and harvest processes. As an example, online monitoring of fermentation conditions such as pH or glucose level can be used to demonstrate similarity during the time course of the fermentation. Similarly, measurements at the end of the fermentation process (e.g., final cell density, cell viability) and intermediate measurements during harvest (e.g., turbidity of clarified broth, wet cell mass in the pellet) provide useful information for evaluating the similarity or differences during scale-up.

PURIFICATION

A general overview of purification for bacterial derived vaccines is presented in *USP Vaccines for Human Use—General Considerations* (1235). In addition to a description of critical processing equipment, reagents, and processing steps, manufacturers should provide the rationale for the purification process chosen for component antigens recovered from the crude harvest. As with the other processes, analysts should consider the source of all raw materials and ensure that they come from reliable vendors who adhere to cGMP and can ensure a long-term supply. The cGMPs will apply to late-stage clinical supplies and commercial materials. The removal of nonproduct-related impurities (e.g., processing reagents, endotoxin, contaminating cell proteins or nucleic acids, and other residual contaminants) should be verified.

The drug substance can be one of several types of compounds: e.g., polysaccharides (wild type or modified), proteins (wild type, mutant, toxoids, or recombinant), or products of conjugation of polysaccharides and proteins, or products of conjugation of peptides and proteins.

To define and control purification processes for drug substance and drug products, the manufacturer should establish targets for process parameters and tolerances for all critical process steps including yields, activity, and purity to ensure efficacy, safety, and consistency of the final product. Requirements for pooling, if applicable, should be established. The requirements and conditions for storage of intermediates, bulks, and final containers must be established by an official stability program. The use, reuse, regeneration, and cleaning of all drug product/drug substance contact equipment (e.g., filters, chromatographic columns and resins, tanks, and process lines) should be validated. In addition, extractable/leachable studies should be performed for all product contact equipment (e.g., disposable bag systems, chromatographic column resins, and process lines).

Polysaccharide Purification

The purification steps for polysaccharides depend on the phenotype (e.g., gram negative or positive), polysaccharide presentation (e.g., membrane bound or excreted within the supernatant), and the chemical nature of the polysaccharide itself [e.g., idealized backbone linkages (glycosidic bond, phosphodiester bond), overall charge, types of charge

groups, and types of side group modifications (O-acetyl, uronic acid, sialic acid, N-acetyl, pyruvate, or O-methyl)]. Polysaccharide harvest methods determine clarification and downstream purification requirements. Clarification methods depend on whether it is necessary to perform cell lysis or only to separate cell-free broth from cellular debris. Harvest techniques include centrifugation, depth filtration, tangential-flow filtration, microfiltration, sizing filtration, or a combination of techniques. The culture may be inactivated or residual contaminants removed by selective precipitation (e.g., protein denaturation) using heat or chemical treatments (e.g., salts, detergents, enzymes, or phenol). This may require cold-storage settling before clarification.

Methods for postclarification polysaccharide precipitation are used both for isolation and purification. Fractional precipitation methods are based on overall charge or cationic binding affinities of the polysaccharide (e.g., alcoholic precipitation, ion-exchange chromatography). Agents such as cationic detergents, salts, and solvents can be used to differentially precipitate charged species from uncharged molecules. Precipitation can be performed in stepwise fashion to remove residual impurities from polysaccharide or by a series of precipitations to achieve the desired purity. Polysaccharide may be contained in either the precipitate or supernatant depending on the charge and nature of the polysaccharide. Precipitation is followed by isolation steps such as centrifugation and/or filtration during which either the precipitate is discarded or resuspended in a secondary precipitating agent until the polysaccharide is recovered. Extraction with solvents such as phenol is sometimes used to remove impurities.

An alternative and additional approach to selective precipitation methods is the use of chromatographic methods. Ion-exchange chromatography (e.g., DEAE Sepharose), hydrophobic-interaction chromatography (HIC), and gel-permeation chromatography separately and in combination have been used successfully to purify polysaccharides. At neutral pH the charge on acidic polysaccharides can be used on anion exchangers to separate acidic polysaccharides from impurities. Ion exchangers can also be used to purify neutral polysaccharides in flow-through mode, binding impurities while neutral polysaccharides flow through the column. HIC can also be used to bind impurities while the polysaccharide passes in the flow-through fraction. If there are no base-labile groups, the charge on neutral and acidic polysaccharides can be modified by addition of base to ionize hydroxyls before chromatography. The basic conditions used control the level of N-acetylation, and the polysaccharides can be re-acetylated as needed. Borate salts can be used to enhance separation during ion-exchange chromatography. A combination of precipitation, filtration, and chromatographic procedures can also be used.

Diafiltrations, ultrafiltrations, and intermediate drying steps can be used as needed to concentrate polysaccharides while removing low molecular weight impurities or replacing processing salts and solvents. The precipitates or column fractions can be further purified using suitable methods (e.g., enzyme treatments, solvent extractions, or column chromatography) to remove impurities such as nucleic acids, proteins, and lipopolysaccharides. A preliminary side group modification can also be included in the purification process (e.g., de-O-acetylation, partial depyruvylation).

The final purification step can consist of buffer exchange and filtration followed by storage of purified liquid polysaccharide (frozen) or additional final precipitation and washing of the precipitate with solvent before drying followed by storage. Drying can be performed via several types of processes (e.g., drying under vacuum or in desiccators or by lyophilization). Drying of polysaccharides can be performed in desiccators (at various temperatures) and can include several steps of grinding or fluffing and return to the desiccators for further drying. Lyophilization of polysaccharides is possible with appropriate controls if the process requires retention of bound water. Some polysaccharides may require a residual amount of moisture to maintain stability over

time. The polysaccharide is then stored under suitable conditions to avoid moisture uptake.

In-process Controls

Manufacturers identify critical process steps and perform appropriate tests to monitor the purification process. Among the latter are filter integrity tests, *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), *Bacterial Endotoxins Test* (85), and other suitable tests for residues of reagents (e.g., residual reagents, solvents, enzymes, or cations) used in purification. Polysaccharide size can be influenced throughout the production process from fermentation conditions to drying conditions or by the action of mechanical stirrers, impellers, or filtration devices. If molecular size is a critical quality attribute, analysts can perform an in-process test for size (e.g., high performance size-exclusion chromatography coupled with multi-angle laser light scattering [HPSEC-MALLS]) at the appropriate process steps to monitor and control polysaccharide size. In order to demonstrate process performance and reliability, manufacturers should characterize inherent residual contaminants (e.g., protein, DNA, and endotoxins). When validation studies have demonstrated removal of residual reagents, testing of purified polysaccharides can be omitted. If material must be sterile, analysts can perform *Sterility Tests* (71).

Protein Purification

The classes of bacterial protein vaccines include toxoids, nontoxoids (e.g., pertussis antigens), naturally occurring mutants (e.g., CRM197) as carrier proteins, and engineered recombinant products.

Toxoids. At the end of fermentation, toxin-containing culture medium should be separated aseptically from the bacterial mass as soon as possible or placed in a cold room until separation can be effected. The toxin content (Lf/mL) is checked by flocculation assay using the appropriate anti-toxin standard to monitor production consistency (culture should contain NLT 40 Lf/mL). The toxin is purified first to remove any components that could cause adverse reactions in humans. A typical process includes depth filtration followed by 0.2- μ m filtration to assist in removal of cellular debris. Following preliminary purification, the toxin is then detoxified with formaldehyde or glutaraldehyde or any suitable chemical reagent by a method that avoids both destruction of the immunogenic potency of the toxoid and reversion of the toxoid to toxin, particularly on exposure to heat. Some toxoids require a single addition of formaldehyde, but others can require multiple additions. Alternatively the toxin could be detoxified and then purified or partially purified by depth filtration, detoxified by addition of an appropriate aldehyde, filtered using 0.2- μ m filtration, and then pooled. The pooled toxoid solution is further purified by clarification with activated carbon, followed by multiple ammonium sulfate precipitation steps that further fractionate and concentrate the toxoid. Typical additional purification steps include concentration, diafiltration, and/or chromatography. Purification before detoxification results in a purer product and can be advantageous if the toxoid is to be used as the protein component of a protein-carbohydrate conjugate (because copurifying high molecular weight glycan will be removed before detoxification).

During detoxification and purification, endotoxin testing according to *Bacterial Endotoxins Test* (85), and formaldehyde, protein, and irreversibility testing are performed to control and ensure consistency of the purification process. If material must be sterile, *Sterility Tests* (71) can be performed.

Proteins/Recombinant Proteins. Proteins used to make vaccines can be recombinant (in their native state or engineered to modify certain amino acids), or they can be naturally occurring mutants that have no wild-type activity yet

are capable of inducing the appropriate immune response. Proteins are harvested from the fermenter and are extracted (e.g., by mechanical and chemical disruption) then purified by suitable methods, typically consisting of filtration-concentration steps (e.g., ultrafiltration, tangential-flow filtration, diafiltration, centrifugation, selective precipitation, and even direct capture using expanded-bed chromatography or big-bead technologies). The enriched protein solution can be further purified using appropriate filtration and chromatographic steps. For all equipment that contacts drug substances (e.g., chromatographic resins, membranes, disposable bag systems, or process lines), manufacturers should assess extractables and leachables. Analysts should determine column resin life for all chromatographic systems used in the purification (including number of uses, reconditioning requirements, and storage conditions).

The type of chromatography used to purify proteins depends on the physical/chemical properties of the desired protein as well as those of other molecular entities in the harvest culture. As an example, CRM197 can be purified using a multistep chromatographic process: Production material is first diafiltered and then is separated by ion-exchange chromatography (DEAE-Sepharose) in order to purify the target protein from other molecular entities present in the purification stream. The peak of interest is collected, and ammonium sulfate is added, followed by 0.22- μ m filtration to condition the material before loading on the hydrophobic-interaction chromatography column (Phenyl Sepharose) for purification of the target protein based on its surface hydrophobicity. The peak fraction is then diluted with Water for Injection and is separated onto a ceramic hydroxyapatite column to further purify the target protein based on its surface charge. The eluted peak is then buffer exchanged into the storage buffer by ultrafiltration/diafiltration using cross-flow membrane filtration followed by 0.22- μ m filtration to yield the sterile purified concentrate.

In-process control of protein purification includes monitoring specific protein content and critical process steps as well as monitoring removal of unwanted fermentation and purification components. The pH is critical for ion-exchange chromatography, and therefore pH should be monitored. For steps designed to remove endotoxin, procedures in *Bacterial Endotoxins Test* (85) are used to monitor column eluants. Bioburden is monitored according to *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61) after filtration and chromatography steps. If material must be sterile, the sterility test in *Sterility Tests* (71) should be performed.

Polysaccharide-Protein Conjugates. Polysaccharide preparation and activation polysaccharides used in conjugation reactions vary in size from native high molecular weight polysaccharide to oligosaccharides produced by controlled depolymerization. Some polysaccharides can also be modified (e.g., de-O-acetylated, partially depyruvylated). Sizing/depolymerization of polysaccharides is performed in a variety of ways (e.g., acid/base catalysis, chemical oxidation/reduction, microfluidization, or mechanical treatment). Activation of polysaccharides can be performed by several different methods depending on the lability of particular epitopes under differing depolymerization conditions or the type of conjugate desired (e.g., neoglycoconjugate or lattice-type conjugate, use of a linker molecule or direct conjugation, or reductive amination). Appropriately sized and/or activated polysaccharides are purified by suitable methods that typically consist of various combinations of concentration and filtration (ultrafiltration/diafiltration) and chromatographic methods (size-exclusion chromatography, HIC).

The in-process testing performed to monitor the depolymerization and activation process depends on the process used. Typical control tests are pH monitoring and temperature monitoring of the sizing and activation reactions. The size of the polysaccharide during depolymerization can be followed by an appropriate chromatographic procedure (e.g., HPLC-SEC RI or MALLS). Testing of depolymerized

polysaccharide for select functional groups (e.g., *O*-acetyl, *N*-acetyl, or pyruvyl groups) may be required and can be determined by nuclear magnetic resonance (see *Nuclear Magnetic Resonance* <761>). In-process testing of the activated polysaccharide depends on the activation process used. For example, if reductive amination is used to attach a linker to the depolymerized polysaccharide, the control testing would include measurement of reducing activity (e.g., available reducing sugars), polysaccharide content (e.g., for determining the loading ratio in conjugation), and total and free linker content (e.g., for determining the number of active sites for conjugation). Depending on the activation and conjugation process used (i.e., immediate conjugation after activation), consistency in degree of polysaccharide activation may also be demonstrated as part of process validation or reflected by characteristics of the final conjugate bulk. The concentration/filtration steps of the purification process are monitored for conductivity to ensure removal of salts.

Conjugation. The conjugation chemistry used determines the type of conjugate made (i.e., neoglycoconjugate or lattice). The conjugate is obtained by the covalent binding of activated polysaccharides to the carrier protein. Conjugates are purified by suitable methods designed to remove residual reagents used for conjugation as well as to remove unreacted polysaccharide and protein. The removal of residual reagents is confirmed by suitable tests or by validation of the purification process. Suitable tests are carried out to determine residues of reagents used during inactivation and purification. When validation studies have demonstrated removal of residual reagents, the test on conjugate polysaccharides can be omitted.

Appropriate chromatographic procedures (HIC, SEC) and/or filtration (ultrafiltration/diafiltration, tangential-flow filtration) are used to remove the unreacted polysaccharides, protein, residual chemicals, and salts that are used in conjugation or that are by-products of conjugation. Bioburden testing (see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* <61>) is performed before sterile filtration.

INTERMEDIATES

An intermediate or process intermediate in vaccine manufacture is the reaction product of each step in the process except the last one, which forms the final product. Examples of intermediates are bulk-purified polysaccharides, proteins, and activated polysaccharides that conjugate to protein.

Most vaccine production processes are stepwise and take more than one elementary step to complete. An intermediate is produced from raw materials at one or more process steps (e.g., bacterial growth, extraction and purification, and chemical modification), eventually resulting in the drug substance. The identification of the key intermediates, their production, and sampling for analytical tests must be defined in controlled documents (e.g., batch records, analytical protocols).

Intermediates can be stored for considerable periods of time before further processing and can be included in a formal stability program (see *Storage Stability*, above). Stability studies in normal or accelerated conditions should be performed to define maximal hold time for intermediates and when significant process changes are implemented.

From raw material to finished drug substance, testing throughout the process ensures a quality product. Testing of intermediates is a key quality control step to ensure their identity and purity. The quality attributes of the intermediate are commonly tested in conjunction with further processing, and their release testing should be considered. Standard operating procedures (SOPs) must be properly defined for the analytical control tests. Because of their critical role in the production process, some key intermediates could be included in formal release testing, in addition to the intermediates identified for in-process testing.

Examples of tests for structural characterization of carbohydrate-based intermediates include the following:

- identity and *O*-acetylation level (nuclear magnetic resonance)
- molecular size and polydispersity (SEC–UV, SEC–Refractive Index, SEC–MALLS, SEC–Fluorescence)
- saccharide content [colorimetric assays, high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD), -Fluorescence]
- specific rotation, see *Optical Rotation* <781>
- saccharide content, *O*-acetyl content (colorimetric assays)
- counterion content, e.g., Na⁺ and Ca²⁺ (inductively coupled plasma–mass spectroscopy, atomic absorption).

Examples of tests for the purity of carbohydrate-based intermediates based on estimates of the product- and process-related impurities include the following:

- endotoxin content (*Bacterial Endotoxins Test* <85>)
- proteins, nucleic acids, proteins, cetavlon (colorimetric assays)
- water content (Karl Fischer titration; see *Water Determination* <921>)
- volatile substances (thermogravimetry)
- organic solvents, e.g. ethanol, phenol, acetone, DMSO, or ethyl acetate (gas chromatography–flame ionization detection; see *Residual Solvents* <467>)
- bioburden (total viable aerobic count of microbial contamination; see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* <61>)

Examples of tests for structural characterization and purity estimation of protein-based intermediates include the following:

- identity and molecular size (SDS–PAGE, Western Blot, SEC–UV, SEC–Fluorescence, SEC–MALLS, reverse phase chromatography)
- endotoxin content (see *Bacterial Endotoxins Test* <85>)
- residual nucleic acids [colorimetric assay; see *Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing)* <1130>]
- bioburden (total viable aerobic count of microbial contamination; see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* <61>)
- other proteins as impurities (SDS–PAGE, Western Blot, SEC–UV, SEC–Fluorescence, reversed-phase chromatography)

The tests previously reported for protein-based intermediates are also applicable when the protein-based product is defined as the drug substance. In addition to the examples reported above, many other methodologies can be applied for the identity and purity evaluation.

Stability tests for intermediates can include physicochemical methods (see section on *Intermediates*, above), formally included within an analytical panel for the stability study. In addition, biological and immunochemical tests [e.g., enzyme-linked immunosorbent assay (ELISA)] can be included. Bioburden and endotoxin testing may not be required at each level (each intermediate, drug substance) provided testing is performed at sufficient steps in the overall production process. Bioburden is typically performed prior to sterile filtration via in-process testing. If intermediates must be stored and/or subsequently shipped to a different location for further processing, the stability of these materials must be demonstrated.

DRUG SUBSTANCE

The drug substance is the final bulk that contains the antigen at the desired concentration and is ready for the addition of other ingredients (e.g., diluents, bulking agents, stabilizing excipients, adjuvants, or preservatives) to produce the finished dosage formulation.

The drug substance is the final product of the antigen manufacture process, before the formulation of the final vaccine dosage. The final bulk may be prepared aseptically

or may include a sterilization step. Sampling for analytical tests for release and stability studies (see *Storage Stability*, above) must be defined in controlled documents (e.g., batch records, analytical protocols).

Drug substances can be stored for a considerable period of time before further processing, but if it is stored the drug substance must be included in a formal stability program (see *Storage Stability*, above). Stability studies in normal or accelerated conditions should be performed to define maximal hold times. A stability program is required for formal stability studies, and the studies must be executed according to a protocol that contains detailed information about types of tests, specifications, testing intervals, and time points.

Testing of the drug substance must be performed to ensure its identity and purity. All the testing must be done according to established SOPs, and all tests must have specifications (or provisional specifications, where applicable).

Examples of tests for structural characterization of carbohydrate-based products include the following:

- identity and O-acetylation level [nuclear magnetic resonance (NMR)]
- total and free saccharide content [HPAEC-PAD, capillary electrophoresis (CE), or colorimetric assays]
- total and free protein content (colorimetric assay, SEC with UV, RI, or fluorescence detection, CE)
- O-acetyl content (colorimetric assays)
- molecular size (SEC-UV, -RI, -Fluorescence, or -MALLS)

Examples of tests for the purity of carbohydrate-based drug substances based on estimating the product- and process-related impurities include the following:

- endotoxin content (*Bacterial Endotoxins Test* <85>)
- process-related residuals not shown to be removed by process validation (e.g., cyanide, iodate, bromide, ammonium sulfate, or organic solvents)
- Bioburden (total viable aerobic count of microbial contamination; see *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* <61>)

In addition to the examples reported above, many other methodologies can be applied for identity and purity evaluation. For instance, specific impurities that must be measured are determined by negotiations between manufacturers and the national drug regulatory agency during the licensure process. Bioburden and endotoxin testing may not be required at each level (each intermediate, drug substance) provided testing is performed at sufficient steps in the overall production process. Bioburden is typically performed prior to sterile filtration via in-process testing.

All the results must be reported in a controlled document. Stability tests can include both physicochemical methods (see stability information, above) and biological/immunochemical tests (e.g. ELISA and SBA; see *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products* <1049>).

DRUG PRODUCT AND LOT RELEASE

General principles are described in *Vaccines for Human Use—General Considerations* <1235>, which outlines the lot release procedure in accordance with 21 CFR 610.1 and 21 CFR 610.2. For products that will be used in the United States, samples and protocols containing all appropriate tests must be submitted to FDA for review and/or testing. If FDA determines that the lot meets the standards of safety, purity, and potency required for the particular vaccine, the lot is approved for release, distribution, and marketing.

Tests required for each lot-release protocol include potency, general safety, sterility, purity, identity, and constituent materials. Potency and potency-related tests are different for each bacterial vaccine. The inclusion of these tests makes each bacterial vaccine lot-release protocol unique.

The contents of a final container of each filling of each lot are tested for identity after labeling is completed. Identity is

established either by physical or chemical characteristics of the vaccine, inspection by macroscopic or microscopic methods, specific cultural tests, or in vivo or in vitro immunological tests. In large part, identity testing is performed to distinguish the vaccine from other materials that are manufactured at the same site (21 CFR 610.14). The same tests that establish identity may also be appropriate for defining the quantity of immunogen present in the final vial. This is especially important for carbohydrate-based vaccines that are dosed by mass and for which physicochemical measures of antigen quality are used.

Immunochemical methods, which include immunoprecipitation methods and immunoelectrophoretic methods, have been useful. Immunoprecipitation methods, flocculation and precipitation, can be carried out in solution or in a gel matrix and involve mixing the antigen with an appropriate antibody, leading to the formation of flocculating or precipitating aggregates that can be detected visually or by light scattering (light scattering or nephelometry). The ratio of reactants must be varied to optimize the detected response. In solution this can be achieved by titrating one reactant with the other, and increased sensitivity can be obtained by using antigen- or antibody-coated particles (e.g., latex) as reactants. In gel systems, a gradient is created as one or more of the reactants diffuse, creating a visible line where precipitation occurs. Immunoelectrophoresis (IE) is a qualitative technique that combines two methods: gel electrophoresis followed by immunodiffusion. Crossed IE is a modification of the IE method that is suitable both for qualitative and quantitative analysis. Visualization and characterization of immunoprecipitation lines can be performed by selective or nonselective stains, fluorescence, enzyme or isotope labeling, or other relevant techniques. Selective staining methods are usually performed for characterization of nonprotein substances in the precipitates. In translucent gels, such as agar or agarose, the precipitation line becomes clearly visible in the gel provided that the concentration of each of the reactants is appropriate.

Where multiple active components are present as a result of copurification (e.g., certain acellular pertussis vaccines), the manufacturer must demonstrate that the composition of the product is consistent between batches, unless this has been validated during the development of the manufacturing process.

For certain vaccines, notably those that use purified polysaccharide immunogens, identity and immunogen quantity can be demonstrated using one or more chemical and physicochemical approaches such as colorimetric determinations of different groups of sugar residues expected to be present, chromatography, or high-field nuclear magnetic resonance spectroscopy.

A number of classical colorimetric assays for quantification of various classes of sugar have been used to define the composition of polysaccharides used as vaccines, including the orcinol assay for ribose, phosphorus, sialic acid, uronic acids, and aminosugars. In general, these approaches have been superseded by chromatographic methods, including gas chromatography and HPAEC, which is widely used to determine Hib PRP glycoconjugate immunogens in monovalent or combination vaccines and in meningococcal conjugate immunogens.

Immunochemical methods that have been used to quantify polysaccharide antigens include (a) rocket immuno-electrophoresis and (b) rate nephelometry. Electroimmunoassay, also called rocket immuno-electrophoresis, is a quantitative method to determine antigens that differ in charge from the antibodies. Electrophoresis of the antigen to be determined is carried out in a gel that contains a lower concentration of the corresponding antibody. Nephelometry methods have been used to quantify antigen in pneumococcal conjugate vaccines.

The consistency of the molecular size and molecular size distribution of polysaccharide- and carbohydrate-containing conjugate vaccines can be determined by gel-permeation chromatography on appropriate resins calibrated with suitable

ble molecular weight markers or coupled to laser light-scattering equipment that indicates absolute molecular weight if a value for the refractive index increment (dn/dc) is known. Measurement of the molecular size of formulated conjugates may not be feasible for multivalent glycoconjugate vaccines. Integrity of the conjugate may be demonstrated by alternative, product-specific methods. Another alternative to demonstrate integrity of the glycoconjugates in the final product is measurement of molecular size as part of the stability studies at monovalent conjugate bulk prior to formulation of the multivalent vaccine.

Unless the contrary has been validated, manufacturers should demonstrate that reversion to toxicity has not occurred (and will not occur over the shelf life) for a product derived from or containing a toxoid material. This may require the use of a cell line or an *in vivo* test, although enzymatic approaches are being validated.

An antigenic purity test is an assay that assesses the quantity of antigen and is used for diphtheria and tetanus toxoid vaccines. The antigen content is determined by a flocculation assay.

The manufacturer should prove a high and consistent level of immunogen adsorption to any solid-phase adjuvant (such as aluminum phosphate or aluminum hydroxide) that is consistent with the release specification.

For certain vaccines such as the anthrax vaccine and toxoid vaccines, the manufacturer is required to demonstrate that the vaccine is protective against disease or death in animal models challenged with a predefined dose of the target pathogen. This generally requires definition of the animal model, route of administration, vaccine dilutions required, a means to observe effects, and a reference vaccine against which effects are compared. The data should be analyzed appropriately (see *Analysis of Biological Assays* <1034>).

Stability-indicating assays are those used to determine the stability of the product. Of primary importance is the potency assay, although glycan degradation may be important in glycoconjugate vaccines.

Other Vaccine Components and Vaccine Properties. Aluminum compounds are the primary adjuvants used in vaccines in the United States. General chapter *Vaccines for Human Use—General Considerations* <1235> provides provisions of the 21 CFR 610.15 governing the use of aluminum and amounts allowed. The adjuvants widely used in bacterial vaccines include aluminum potassium sulfate (alum), aluminum phosphate, aluminum hydroxide, and combinations of these compounds. Bacterial vaccines formulated with such adjuvants are referred to as adsorbed vaccines, and this term may be included in the official name of the vaccine. Other adjuvant systems may be evaluated. Aluminum is quantitated using colorimetric, titrimetric, emission or atomic absorption spectroscopy, or inductively coupled plasma-mass spectrometry.

For regulations regarding residual manufacturing reagents, see the FDA's 1999 *Guidance for Industry: Content and Format of Chemistry, Manufacturing, and Controls Information and Establishment Description Information for a Vaccine or Related Product*. Manufacturing reagents such as formaldehyde and glutaraldehyde sometimes are used in inactivation, the toxoid-making processes, or elsewhere during manufacture and may be present in residual amounts in the final product. Limits of formaldehyde and other residuals must be minimized in accordance with the approved product license.

Common preservatives used in bacterial vaccines include thimerosal, phenol, 2-phenoxyethanol, and benzalkonium chloride. *Vaccines for Human Use—General Considerations* <1235> and 21 CFR 610.15 provide additional information about the minimization of thimerosal content and the production of thimerosal-free vaccines. Limits and content specifications are set for each bacterial vaccine in the product license.

Each lot of final containers of a vaccine intended for use by injection is tested for bacterial endotoxins as indicated in *Bacterial Endotoxins Test* <85>.

Each lot of final containers of a vaccine intended for use by injection may be tested for pyrogenic substances as indicated in *Pyrogen Test* <151> and 21 CFR 610.14.

Each lot of dried product shall be tested for residual moisture (see *Loss on Drying* <731> and FDA Guideline for the Determination of Residual Moisture in Dried Biological Products, January 1990). Residual moisture should be determined for lyophilized vaccines.

A general safety test is performed on biological products intended for administration to humans with the purpose of detecting extraneous toxic contaminants. Procedures and exceptions are specified in 21 CFR 610.11.

Excipient identity and quantity, preservatives, diluents, adjuvants, extraneous protein; and cell culture-produced vaccines and antibiotics are tested according to 21 CFR 610.15 and/or appropriate guidance documents.

"Free" or unconjugated saccharide in glycoconjugate vaccines is considered undesirable and is subject to limit specifications. As an alternative to controlling free or unconjugated saccharide, integrity of the conjugates in the final product may be demonstrated via an appropriate method, said method depending on the properties of the final product (composition, adsorption, etc.). A test method that measures the increase in the amount of free saccharide is a stability-indicating method. The methods adopted depend on separation of saccharide from conjugate and application of the methods above to quantify the unconjugated saccharide. Separation methods used include membrane separation (such as dialysis), use of hydrophobic media to specifically trap the conjugate, solvent extraction, and selective immunochemical precipitation of the conjugate using anticarrier antibodies.

The sterility of each lot of each product is conducted according to procedures described in *Sterility Tests* <71> and 21 CFR 610.12 for both bulk and final container material.

Information insert (Label). Vaccine product labeling is regulated in compliance with 21 CFR 201 and 610. Requirements are set both for container labeling and package labeling.

OTHER REQUIREMENTS

Retention samples are held by the manufacturer for at least six months after the expiration date. Enough material of each lot of each product is held for examination and testing for safety and potency (see 21 CFR 600.13).

Records are maintained concurrently with each step in the manufacture and distribution of product such that at any time successive steps of manufacture and distribution may be traced (see 21 CFR 600.12).

For storage conditions, see 21 CFR 610.50 and 53.

For shelf life/expiry date, see 21 CFR 610.50 and 53.

<1241> WATER–SOLID INTERACTIONS IN PHARMACEUTICAL SYSTEMS

INTRODUCTION

This general chapter is harmonized with the corresponding texts of the *European Pharmacopoeia* and/or the *Japanese Pharmacopoeia*. These pharmacopoeias have undertaken not to make any unilateral change to this harmonized chapter.

Portions of the present general chapter text that are national USP text, and therefore not part of the harmonized text, are marked with symbols (♦) to specify this fact.

Pharmaceutical solids as raw materials or as constituents of dosage forms most often come in contact with water during processing and storage. This may occur (a) during crystallization, lyophilization, wet granulation, or spray drying; and (b) because of exposure upon handling and storage to an atmosphere containing water vapor or exposure to other materials in a dosage form that contain water capable of distributing it to other ingredients. Some properties known to be altered by the association of solids with water include rates of chemical degradation in the “solid-state”, crystal growth and dissolution, dispersibility and wetting, powder flow, lubricity, powder compactibility, compact hardness, and microbial contamination.

Although precautions can be taken when water is perceived to be a problem, i.e., eliminating all moisture, reducing contact with the atmosphere, or controlling the relative humidity of the atmosphere, such precautions generally add expense to the process with no guarantee that during the life of the product further problems associated with moisture will be avoided. It is also important to recognize that there are many situations where a certain level of water in a solid is required for proper performance, e.g., powder compaction. It is essential for both reasons, therefore, that as much as possible is known about the effects of moisture on solids before strategies are developed for their handling, storage, and use.

Some of the more critical pieces of required information concerning water–solid interactions are:

- total amount of water present;
- the extent to which adsorption and absorption occur;
- whether or not hydrates form;
- specific surface area of the solid, as well as such properties as degree of crystallinity, degree of porosity, and glass transition and melting temperature;
- site of water interaction, the extent of binding, and the degree of molecular mobility;
- effects of temperature and relative humidity;
- essentially irreversible hydration;
- kinetics of moisture uptake;
- various factors that might influence the rate at which water vapor can be taken up by a solid;
- for water-soluble solids capable of being dissolved by the sorbed water, under which conditions dissolution will take place.

PHYSICAL STATES OF SORBED WATER

Water can physically interact with solids in different ways. It can interact at the surface (adsorption) or it can penetrate the bulk solid structure (absorption). When both adsorption and absorption occur, the term sorption is often used. Adsorption is particularly critical in affecting the properties of solids when the specific surface area is large. Large values of specific surface area are seen with solids having very small particles, as well as with solids having a high degree of intraparticle porosity. Absorption is characterized by an association of water per gram of solid that is much greater than that which can form a monomolecular layer on the available surface, and an amount that is generally independent of the specific surface area.

Most crystalline solids will not absorb water into their bulk structures because of the close packing and high degree of order of the crystal lattice. Indeed, it has been shown that the degree of absorption into solids exhibiting partial crystallinity and partial amorphous structure is often inversely proportional to the degree of crystallinity. With some crystalline solids, however, crystal hydrates may form. These hydrates may exhibit a stoichiometric relationship, in terms of water molecules bound per solid molecule, or they may be non-stoichiometric. Upon dehydration, crystal hydrates may either retain their original crystal structure, lose their

crystallinity and become amorphous, or transform into a new anhydrous or less-hydrated crystal form.

Amorphous or partially amorphous solids are capable of taking up significant amounts of water because there is sufficient molecular disorder in the solid to permit penetration, swelling, or dissolution. Such behavior is observed with most amorphous polymers and with small-molecular-mass solids rendered amorphous during preparation, e.g., by lyophilization, or after milling. The introduction of defects into highly crystalline solids will also produce this behavior. The greater the chemical affinity of water for the solid, the greater the total amount that can be absorbed. When water is absorbed by amorphous solids, the bulk properties of the solid can be significantly altered. It is well established, for example, that amorphous solids, depending on the temperature, can exist in at least one of two states: “glassy” or “fluid”; the temperature at which one state transforms into the other is the glass transition temperature, T_G .

Water absorbed into the bulk solid structure, by virtue of its effect on the free volume of the solid, can act as an efficient plasticizer and reduce the value of T_G . Because the rheological properties of “fluid” and “glassy” states are quite different, i.e., the “fluid” state exhibits much less viscosity as the temperature rises above the glass transition point, it is not surprising that a number of important bulk properties dependent on the rheology of the solid are affected by moisture content. Because amorphous solids are metastable relative to the crystalline form of the material, with small-molecular-mass materials, it is possible for absorbed moisture to initiate reversion of the solid to the crystalline form, particularly if the solid is transformed by the sorbed water to a “fluid” state. This is the basis of “cake collapse” often observed during the lyophilization process. An additional phenomenon noted specifically with water-soluble solids is their tendency to deliquesce, i.e., to dissolve in their own sorbed water, at relative humidities, RH_i , in excess of the relative humidity of a saturated solution of the solid, RH_0 . Deliquescence arises because of the high water solubility of the solid and the significant effect it has on the colligative properties of water. It is a dynamic process that continues to occur as long as RH_i is greater than RH_0 .

The key to understanding the effects water can have on the properties of solids, and vice versa, rests with an understanding of the location of the water molecule and its physical state. More specifically, water associated with solids can exist in a state that is directly bound to the solid, as well as in a state of mobility approaching that of bulk water. This difference in mobility has been observed through such measurements as heat of sorption, freezing point, nuclear magnetic resonance, dielectric properties, and diffusion.

Such changes in mobility have been interpreted as arising because of changes in the thermodynamic state of water as more and more water is sorbed. Thus, water bound directly to a solid is often thought as unavailable to affect the properties of the solid, whereas larger amounts of sorbed water may become more clustered and form water more like that exhibiting solvent properties. In the case of crystal hydrates, the combination of intermolecular forces (hydrogen bonding) and crystal packing can produce very strong water–solid interactions. Recognizing that the presence of water in an amorphous solid can affect the glass transition temperature and hence the physical state of the solid, at low levels of water, most polar amorphous solids are in a highly viscous glassy state because of their high values of T_G . Hence, water is “frozen” into the solid structure and is rendered immobile by the high viscosity, e.g., 10^{13} Pa · s. As the amount of water sorbed increases and T_G decreases, approaching ambient temperatures, the glassy state approaches that of a “fluid” state and water mobility along with the mobility of the solid itself increases significantly. At high RH , the degree of water plasticization of the solid can be sufficiently high so that water and the solid can now achieve significant amounts of mobility. In general, therefore, this picture of the nature of sorbed water helps to explain the rather significant effect moisture can have on a

number of bulk properties of solids such as chemical reactivity and mechanical deformation. It suggests strongly that methods of evaluating chemical and physical stability of solids and solid dosage forms take into account the effects water can have on the solid when it is sorbed, particularly when it enters the solid structure and acts as a plasticizer.

Rates of Water Uptake

The rate and extent to which solids exposed to the atmosphere might either sorb or desorb water vapor can be a critical factor in the handling of solids. Even the simple act of weighing out samples of solid on an analytical balance and the exposure, therefore, of a thin layer of powder to the atmosphere for a few minutes can lead to significant error in, for example, the estimation of loss on drying values. It is well established that water-soluble solids exposed to relative humidities above that exhibited by a saturated solution of that solid will spontaneously dissolve via deliquescence and continue to dissolve over a long time period. The rate of water uptake in general depends on a number of parameters not found to be critical in equilibrium measurements because rates of sorption are primarily mass-transfer controlled with some contributions from heat-transfer mechanisms. Thus, factors such as vapor diffusion coefficients in air and in the solid, convective airflow, and the surface area and geometry of the solid bed and surrounding environment, can play an important role. Indeed, the method used to make measurements can often be the rate-determining factor because of these environmental and geometric factors.

DETERMINATION OF SORPTION–DESORPTION ISOTHERMS

Principle

The tendency to take up water vapor is best assessed by measuring sorption or desorption as a function of relative humidity, at constant temperature, and under conditions where sorption or desorption is essentially occurring independently of time, i.e., equilibrium. Relative humidity, RH, is defined by the following equation:

$$RH = (P_c \times 100)/P_0$$

P_c = pressure of water vapor in the system

P_0 = saturation pressure of water vapor under the same conditions

The ratio P_c/P_0 is referred to as the relative pressure. Sorption or water uptake is best assessed starting with dried samples and subjecting them to a known relative humidity. Desorption is studied by beginning with a system already containing sorbed water and reducing the relative humidity. As the name indicates, the sorption–desorption isotherm is valid only for the reference temperature, hence a special isotherm exists for each temperature. Ordinarily, at equilibrium, moisture content at a particular relative humidity must be the same, whether determined from sorption or desorption measurements. However, it is common to see sorption–desorption hysteresis.

Methods

Samples may be stored in chambers at various relative humidities. The mass gained or lost for each sample is then measured. The major advantage of this method is convenience, while the major disadvantages are the slow rate of reaching constant mass, particularly at high relative humidities, and the error introduced in opening and closing the

chamber for weighing. Dynamic gravimetric water sorption systems allow the on-line weighing of a sample in a controlled system to assess the interaction of the material with moisture at various programmable levels of relative humidity at a constant temperature. The major benefit of a controlled system is that isothermal conditions can be more reliably established and that the dynamic response of the sample to changing conditions can be monitored (see Figure 1). Data points for the determination of the sorption isotherm (e.g., from 0% to approximately 95% RH, noncondensing) are only taken after a sufficiently constant signal indicates that the sample has reached equilibrium at a given level of humidity. In some cases (e.g., deliquescence), the maximum time may be restricted although the equilibrium level is not reached. The apparatus must adequately control the temperature to ensure a good baseline stability as well as accurate control of the relative humidity generation. The required relative humidities can be generated, e.g., by accurately mixing dry and saturated vapor gas with flow controllers. The electrostatic behavior of the powder must also be considered. The verification of the temperature and the relative humidity (controlled with, for example, a certified hygrometer, certified salt solutions, or deliquescence points of certified salts over an adequate range) must be consistent with the instrument specification. The balance must provide a sufficient mass resolution and long-term stability.

It is also possible to measure amounts of water uptake not detectable gravimetrically using volumetric techniques. In the case of adsorption, to improve sensitivity, the specific surface area of the sample can be increased by reducing particle size or by using larger samples to increase the total area. It is important, however, that such comminution of the solid does not alter the surface structure of the solid or render it more amorphous or otherwise less ordered in crystallinity. For absorption, where water uptake is independent of specific surface area, only increasing sample size will help. Increasing sample size, however, will increase the time to establish some type of equilibrium. To establish accurate values, it is important to get desolvation of the sample as thoroughly as possible. Higher temperatures and lower pressures (vacuum) facilitate this process; however, care must be taken to note any adverse effects this might have on the solid such as dehydration, chemical degradation, or sublimation. Using higher temperatures to induce desorption, as in a thermogravimetric apparatus, likewise must be carefully carried out because of these possible pitfalls.

Report and Interpretation of the Data

Sorption data are usually reported as a graph of the apparent mass change in percent of the mass of the dry sample as a function of relative humidity or time. Sorption isotherms are reported both in tabular form and as a graph. The measurement method must be traceable with the data.

Adsorption–desorption hysteresis can be interpreted, for example, in terms of the porosity of the sample, its state of agglomeration (capillary condensation), the formation of hydrates, polymorphic change, or liquefying of the sample. Certain types of systems, particularly those with microporous solids and amorphous solids, are capable of sorbing large amounts of water vapor. Here, the amount of water associated with the solid as relative humidity is decreased, is greater than the amount that originally sorbed as the relative humidity was increased. For microporous solids, vapor adsorption–desorption hysteresis is an equilibrium phenomenon associated with the process of capillary condensation. This takes place because of the high degree of irregular curvature of the micropores and the fact that they “fill” (adsorption) and “empty” (desorption) under different equilibrium conditions. For nonporous solids capable of absorbing water, hysteresis occurs because of a change in the degree of vapor–solid interaction due to a change in the equilibrium state of the solid, e.g., conformation of polymer

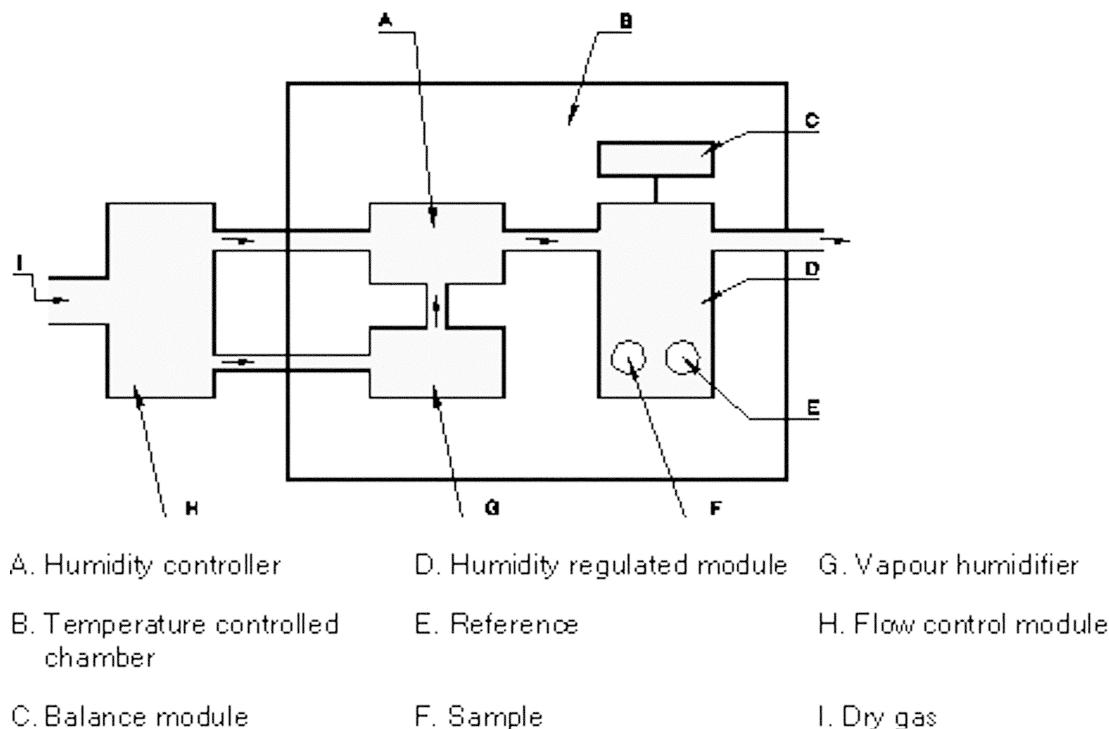


Figure 1. Example of an apparatus for the determination of the water sorption (other designs are possible).

chains, or because the time scale for structural equilibrium is longer than the time scale for water desorption. In measuring sorption–desorption isotherms, it is therefore important to establish that something close to an equilibrium state has been reached. Particularly with hydrophilic polymers at high relative humidities, the establishment of water sorption or desorption values independent of time is quite difficult, because one is usually dealing with a polymer plasticized into its “fluid” state, where the solid is undergoing significant change.

In the case of crystal hydrate formation, the plot of water uptake versus pressure or relative humidity will in these cases exhibit a sharp increase in uptake at a particular pressure and the amount of water taken up will usually exhibit a stoichiometric mole:mole ratio of water to solid. In some cases, however, crystal hydrates will not appear to undergo a phase change or the anhydrous form will appear amorphous. Consequently, water sorption or desorption may appear more like that seen with adsorption processes. X-ray crystallographic analysis and thermal analysis are particularly useful for the study of such systems.

For situations where water vapor adsorption occurs predominantly, it is very helpful to measure the specific surface area of the solid by an independent method and to express adsorption as mass of water sorbed per unit area of solid surface. This can be very useful in assessing the possible importance of water sorption in affecting solid properties. For example, 0.5% m/m uptake of water could hardly cover the bare surface of 100 m²/g, while for 1.0 m²/g this amounts to 100 times more surface coverage. In the case of pharmaceutical solids that have a specific surface area in the range of 0.01 m²/g to 10 m²/g, what appears to be low water content could represent a significant amount of water for the available surface. Because the “dry surface area” is not a factor in absorption, sorption of water with amorphous or partially amorphous solids can be expressed on the basis of unit mass corrected for crystallinity, when the crystal form does not sorb significant amounts of water relative to the amorphous regions.

DETERMINATION OF THE WATER ACTIVITY

Principle

Water activity, A_w , is the ratio of vapor pressure of water in the product (P) to saturation pressure of water vapor (P_0) at the same temperature. It is numerically equal to 1/100 of the relative humidity (RH) generated by the product in a closed system. RH can be calculated from direct measurements of partial vapor pressure or dew point, or from indirect measurement by sensors whose physical or electric characteristics are altered by the RH to which they are exposed. Ignoring activity coefficients, the relationship between A_w and equilibrium relative humidity (ERH) are represented by the following equations:

$$A_w = P/P_0$$

$$\text{ERH}(\%) = A_w \times 100$$

Method

The water activity is determined by placing the sample in a small airtight cup inside which the equilibrium between the water in the solid and the headspace can be established. The volume of the headspace must be small in relation to the sample volume in order not to change the sorption state of the sample during the test. The equilibration as a thermodynamic process takes time but may be accelerated by forced circulation within the cell. The acquired water activity value is only valid for the simultaneously determined temperature. This requires a precise temperature-measuring device as part of the equipment. Furthermore, the probe must be thermally insulated to guarantee a constant temperature during the test. The sensor measuring the humidity of the headspace air above the sample is a key component.

Theoretically, all types of hygrometers can be used, but for analytical purposes miniaturization and robustness are a precondition. The A_w measurement may be conducted using the dew point/chilled mirror method.¹ A polished, chilled mirror is used as a condensing surface. The cooling system is electronically linked to a photoelectric cell into which light is reflected from the condensing mirror. An air stream, in equilibrium with the test sample, is directed at the mirror which cools until condensation occurs on the mirror. The temperature at which this condensation begins is the dew point from which the ERH is determined. Commercially available instruments using the dew point/chilled mirror method or other technologies need to be evaluated for suitability, validated, and calibrated when used to make water activity determinations.

These instruments are typically calibrated over an adequate range, for example, using some saturated salt solutions at 25° such as those listed in Table 1.

Table 1. Standard Saturated Salt Solutions

Saturated Salt Solutions at 25°	ERH (%)	A_w
Potassium sulfate (K_2SO_4)	97.3	0.973
Barium chloride ($BaCl_2$)	90.2	0.902
Sodium chloride (NaCl)	75.3	0.753
Magnesium nitrate ($Mg(NO_3)_2$)	52.9	0.529
Magnesium chloride ($MgCl_2$)	32.8	0.328
Lithium chloride ($LiCl_2$)	11.2	0.112

<1251> WEIGHING ON AN ANALYTICAL BALANCE

Weighing is a frequent step in analytical procedures, and the balance is an essential piece of laboratory equipment in most analyses. In spite of this, weighing is a common source of error that can be difficult to detect in the final analytical results. The procedure described here applies directly to electronic balances; therefore, certain portions of the procedure are not applicable to other types of balance. The weighing procedure can be separated into three basic steps: planning, checking the balance, and weighing the material.

PLANNING

The initial step is to assemble the proper equipment, such as containers for weighing, receiving vessels, forceps, pipets, spatulas of proper size, and so forth. Use containers of size such that the loading capacity of the balance is not exceeded. Make sure that the containers selected to receive the weighed material are clean and dry. Assemble the necessary chemicals if solutions or reagents are required.

Preparation of the material to be weighed is often necessary. The material may require grinding or drying. Some materials may have been heated or stored in a refrigerator. Materials must be brought to the temperature of the balance before they are weighed. To avoid condensation of moisture, refrigerated materials must be allowed to come to room temperature before the container is opened.

¹ AOAC International Official Method 978.18.

CHECKING THE BALANCE

In the next step it is important to remember that, unless the balance is checked before each weighing operation is performed, errors can easily occur, resulting in faulty analytical data. The balance user should check the *Balance Environment*, *Calibration*, and *Balance Uncertainties*. Do not assume that the balance has been left in the proper operating condition by the previous user.

Balance Environment

The balance is placed in a suitable location with sufficiently low levels of vibration and air current. It must have a constant electrical supply. The balance and the surrounding work area have to be kept neat and tidy. It is good practice to use a camel's hair brush or its equivalent to dust the balance pan before any weighing so as to remove any materials that may have been left by the previous operator. [NOTE—Individuals must clean up debris, dispose of any spilled materials or paper, and remove the vessels and apparatus used in making the measurements.] When a balance is moved, it must be allowed to adjust to the temperature of its new environment and be recalibrated.

Calibration

If necessary, turn on the power, and allow the balance to equilibrate for at least 1 hour before proceeding with the calibration. (Microbalances may require up to 24 hours to reach equilibrium.) If the balance power has gone off and then has come back on, as in a power outage, certain types of balance may display a message indicating that the balance must be calibrated before a weighing is made. If the operator touches the balance bar, the message may be cleared and the balance may display zeros; however, the balance will not give the correct weighing until it has been calibrated. Electronic analytical balances have an internal calibration system based on an applied load. The calibration applies for the current ambient temperature.

Balance Uncertainties

DRIFT REDUCTION

Drift is one of the most common errors, and it is also one of the easiest to reduce or eliminate. Balance drift can be present without the operators being aware of the problem. Check the sample, the balance, and the laboratory environment for the following causes of errors, and eliminate them:

1. A balance door is open.
2. Temperatures of the balance and the material to be weighed are not the same.
3. The sample is losing or gaining weight.
4. The balance has been recently moved but has not been allowed to equilibrate to its surroundings or has not been recalibrated.
5. Air currents are present in the laboratory.
6. Temperatures in the laboratory vary.
7. The balance is not properly leveled.
8. Laboratory operations are causing vibration.
9. Hysteresis of the mechanical parts occurs during weighing.

MECHANICAL HYSTERESIS

Hysteresis in the balance is caused by excessive stretching of the springs, and it is primarily due to overloading or to the accidental dropping of an object onto the pan. Microbalances are very sensitive to overload and shock.

When using a microbalance, set the lever to the rest position when adding or removing material; turn the lever to the weigh position to register the weight. In some cases, drift due to hysteresis can be eliminated by allowing the balance to stand without weighing long enough for it to recover. If stretching of the springs is excessive, an expensive balance overhaul may be needed. In the case of electronic force restoration balances, springs are replaced by flexures, and the term *creep* is more appropriate than *hysteresis*.

QUALITY ASSURANCE PROCEDURE FOR MEASUREMENT OF BALANCE DRIFT

Over an extended period of time, balance drift and other day-to-day variations are monitored by weighing a fixed check-weight on a regular basis; this check should be performed after the balance has been calibrated at the ambient laboratory temperature. The check should be made before the first weighing of the day or after any event that might disturb the balance's calibration (power failure, moving the balance to a new location, etc.). The check-weight may be any object whose mass remains constant and does not exceed the load limit of the balance. A balance weight makes a reliable check-weight. Each balance should be provided with a check-weight, which should be stored in a protective container near the balance.

Perform the following procedures to reduce balance errors and the possibility of an incorrect reading because of drift:

1. Make certain that the electrical power to the balance is on and that the level bubble is in the center of the indicator.
2. Calibrate the analytical balance or the microbalance. [NOTE—Some balances have a calibration lever, which must be returned fully to its original weighing position. Do not depend upon any prior calibration.]
3. The first person to use the balance each day should weigh the check-weight and record the weight in the log book for comparison with previous readings. If a deviation greater than those indicated below for *Analytical Balances* and *Microbalances* is observed, the balance should be reported for service. [NOTE—Check-weights tend to gain weight upon standing because of mishandling and exposure to contaminants in the atmosphere. These weights can be cleaned by wiping with a lint-free cloth moistened with a small amount of an appropriate solvent such as diethyl ether.]

Analytical Balances—Select a check-weight of an appropriate mass to examine an analytical balance. If possible, set the balance to read to 5 decimal places. Follow the manufacturer's operating instructions. Pick up the check-weight with a forceps, place it carefully on the balance pan, and weigh it. [NOTE—Do not drop the weight on the balance pan, because damage to the balance could result.] Place the weight in the center of the pan to eliminate corner-weighing differences. The accuracy of the weight is not important; the only factor of interest is whether any drift has occurred. If no drift has taken place, the value should remain constant. Periodic weighing of a fixed weight will determine whether the boards (or knife edges in mechanical balances) in the instrument are defective. The check for drift at the most sensitive position will show whether a problem exists; the variation in the observed weight does not exceed ± 0.2 mg. For example, with a 20-g weight, if the mean value of the readings were 19.9984, the tolerance would be from 19.9982 to 19.9986 g. Thus, several readings must be taken before one can establish a tolerance. [NOTE—The check-weight need not be of high accuracy, but it is essential that its mass remain constant. In addition, the tolerance does not correspond to the value of 0.1%, specified under *Weights and Balances* (41), for weighing material accurately.

Rather, the tolerance is purposefully tight to reveal possible drift or calibration errors; this tolerance is readily achievable with modern electronic balances.]

Microbalances—Proceed as directed for *Analytical Balances*, but use a check-weight appropriate for the particular balance. For example, a 100-mg check-weight might be selected for a balance that has a load limit of 150 mg; or a 10-mg check-weight might be used for an ultramicrobalance with a load limit of 15 mg. (The operator must know the maximum capacity of the balance to select the correct check-weight.) The balance indicates the weight in milligrams. Record the weight as soon as the reading is stable for a few seconds. The variation in weighings ought to be within a range commensurate with the specifications given by the balance manufacturer, but not greater than 0.1% of the amount of material typically weighed on the particular balance. For example, if 10-mg samples are routinely weighed, the variation in the weighings of the check-weight cannot exceed 0.01 mg.

WEIGHING THE MATERIAL

In this final step, select the number of decimal places required for the analytical procedure. In most pharmaceutical analyses small quantities of material are used, requiring the balance reading to be set to the fifth decimal place to achieve the necessary accuracy. Weighing read with four decimal places is preferred for weighing near-gram quantities. Do not allow the material to remain on the balance for an extended period of time because changes, caused by interaction with atmospheric water or carbon dioxide, may take place.

Load Limit

Select the appropriate balance for the quantity and accuracy needed. Each balance has a load limit, which should not be exceeded. Each balance manufacturer supplies the maximum loading condition, and this limit varies with the type of balance. The operator should know this limit so that the balance will not be damaged. [NOTE—Electronic balances operate on a "load cell" principle that produces an electrical output proportional to the movement of the strain gauge and is linear over the range.]

Receivers

The proper receiver for the material must be selected. The receiver's weight plus the weight to be measured must not exceed the maximum load for the balance; the size and shape of the receiver should permit it to fit into the space and on the balance pan without interfering with any operation. It is important that the receiver be clean and dry. Common receivers are weighing bottles, weighing funnels, flasks, and weighing paper. The correct receiver depends upon the quantity and type of material (liquid, solid, or powder) to be weighed. All other things being equal, a vessel of low mass should be chosen when small amounts of material are to be weighed. It is recommended that gloves, forceps, or another type of gripping device be used when handling receivers, because oils from the hands will add weight.

The weighing funnel is often the most satisfactory receiver, because it can function as both a weighing dish and a transfer funnel, allowing easy transfer to volumetric flasks. Weighing funnels come in various sizes; the size suitable for the operation should be selected.

Weighing paper may be used for solids. Paper receivers must be handled by hand, and great care must be used to prevent spills.

Weighing by Difference

Weighing is usually done by difference. The following methods are acceptable for good analytical results.

METHOD 1

Tare the empty receiver as follows. Place the receiver on the balance in the center of the pan, and press the appropriate tare key on the balance. This operation electrically sets the signal from the strain gauge to zero so that the weight of the receiver is no longer indicated. Add the material to the receiver, and record the weight. Transfer the weighed material to the final flask or receiver; then reweigh the original weighing receiver by placing it in the same position on the pan. [NOTE—Do not change the set tare of the balance between these two weighings.] The second weight represents the untransferred material and is subtracted from the total material weight to determine the weight of the transferred material.

METHOD 2

If the empty receiver is not going to be tared, add the material to the receiver, and place the receiver on the balance in the center of the pan. Record the weight, and transfer the weighed material to the final flask or receiver; then reweigh the original weighing receiver by returning it to the same position on the pan. The second weight represents the sum of the weights of the receiver and the untransferred material; subtract this sum from the sum of the total material weight and the receiver weight to determine the weight of the transferred material.

METHOD 3

This method may be described as quantitative transfer. The material is added to the tared receiver, the amount is determined by difference, and then the whole amount is transferred quantitatively (e.g., by using a solvent) to the final receiver.

Materials-Handling Safety Procedures

The operator must be familiar with precautions described in the Material Safety Data Sheet for the substance before weighing it. Hazardous materials must be handled in an enclosure having appropriate air filtration. Many substances are extremely toxic, are possibly allergenic, and may be liquids or finely divided particles. A mask that covers the nose and mouth should be used to prevent any inhalation of chemical dust. Gloves should be used to prevent any contact with the skin. [NOTE—The use of gloves is good practice for handling any chemical. If it is necessary to handle the container being weighed, the operator should put on gloves, not only for self-protection but also to prevent moisture and oils from being deposited on the weighed container.] During a weighing, the operator may be exposed to high concentrations of the pure substance; therefore, the operator must carefully consider these possibilities at all times.

Weighings are made on many different types of materials, such as large solids, finely divided powders, and liquids (viscous or nonviscous, volatile or nonvolatile). Each type of material requires its own special handling.

Weighing Solids

Solids come in two forms: large chunks, with or without powdery surface, and finely divided powders or small crys-

tals. If large chunks with a powdery surface are to be weighed, at least a piece of weighing paper must be placed on the balance pan to protect it from damage. Large nonreactive chunks that have no powdery surface may be placed directly on the pan (for example, a coated tablet). [NOTE—Solid pieces must be handled with forceps, never by hand.]

STATIC CHARGE

Fine powders have a tendency to pick up static charge, which will cause the particles to fly around. This static charge must be eliminated before a suitable weighing can be made. An antistatic device may be used to minimize this problem. [NOTE—Such devices may use piezoelectric components or a very small amount of a radioactive element (typically polonium) to generate a stream of ions that dissipate the static charge when passed over the powder to be weighed.] The static charge depends upon the relative humidity of the laboratory, which in turn depends upon the atmospheric conditions. In certain conditions, static charge is caused by the type of clothing worn by the operator; this charge causes large errors in the weighing when discharged.

WEIGHING PROCEDURE

Place the receiver on the balance pan, close the balance door, and weigh as indicated for *Weighing by Difference*, with the following additions. Carefully add the powdered material from a spatula until the desired amount is added. Use care to avoid spilling. Close the balance door, and record the weight as soon as the balance shows a stable reading.

SPILLS

If solids are spilled, remove the receiver, and sweep out all of the spilled material from the balance. The spilled material must be properly disposed of and must not be swept out onto the balance table where other operators may come in contact with the chemical. Then either start the process over or reweigh the remaining material. [NOTE—Never return any excess material to the original container. Any excess material must be disposed of in a proper manner.]

Weighing Liquids

Liquids may be volatile or nonvolatile and viscous or nonviscous. Each type requires special attention.

WEIGHING PROCEDURE

Weigh as directed for *Weighing by Difference*, with the following additions. Liquids should always be weighed into a container that can be closed so that none of the material is lost. It is best if the liquid can be added to its receiving container outside the balance because of the possibility of a spill. [NOTE—Liquids spilled within the balance housing can cause serious damage to the balance, and they may be difficult to remove.]

Nonviscous liquids can be handled with a Pasteur capillary pipet equipped with a small rubber bulb such as a medicine dropper bulb. The liquid is discharged into its receiver, the top is closed or stoppered, and the receiver and contents are weighed. Small quantities of viscous liquids can be handled by touching a glass stirring rod to the surface of the liquid and then carefully touching the rod to the side of the receiving vessel, which allows some of the material to be transferred.

Weighing Corrosive Materials

Many chemicals, such as salts, are corrosive, and materials of this nature should not be spilled on the balance pan or inside the balance housing. Extreme care is essential when materials of this nature are being weighed.

CONCLUSION

By carefully following the procedures outlined above, laboratory personnel will eliminate many errors that might be introduced into weighing procedures. However, it is important for each balance to be serviced and calibrated regularly by a specially trained internal or external service person. The balance should be tested using weights traceable to standardization by the National Institute of Standards and Technology. No repairs should be made to any balance by anyone other than a qualified maintenance person.

(1265) WRITTEN PRESCRIPTION DRUG INFORMATION— GUIDELINES

The purpose of these guidelines—comprising format, content, and accessibility of prescription drug leaflets—is to help ensure that leaflets are useful. In this context, “useful” means that recipients receive, understand, and are motivated to apply written information about their medicines to achieve maximum benefit and minimize harm. Dispensers, prescribers, health care providers who counsel patients about their medicines, and the patients themselves are intended to be the primary beneficiaries for these guidelines.

CRITERIA (from the Keystone Action Plan¹)

Written prescription medicine information should be based on the following criteria:

1. Scientifically accurate,
2. Unbiased in content and tone,
3. Sufficiently specific and comprehensive,
4. Presented in an understandable and legible format that is readily comprehensible to consumers,
5. Timely and up-to-date, and
6. Useful.

FORMAT GUIDELINES

1. Group all information from the same category, using brief, clear titles and bullets or subheadings as needed. Avoid symbols and subheadings not directly connected to the information they mark.
2. Be consistent in the placement and labeling of categories of information in all leaflets.
3. Provide information at the sixth-grade reading level or below, if possible (never above eighth-grade level).

¹ In December 1996, the “Action Plan for the Provision of Useful Prescription Medicine Information” was presented to the Secretary of Health and Human Services. The plan, commonly known as the “Keystone Plan,” described certain criteria for written prescription medicine information. These criteria are described in detail in the action plan, which can be found at www.fda.gov/cder/offices/ods/keystone.pdf.

Do not exclude information to achieve a lower reading level.

4. Use simple, common, accurate terms (for example, use “noise in the ears”, not “tinnitus”).
5. Use direct language that avoids words with opposite meanings (for example, use “decrease blood pressure”, not “increase low blood pressure effect”).
6. Provide reasons for instructions (for example, “take with food to avoid upset stomach”).
7. Emphasize the most important information. Clearly distinguish warnings from instructions or from other text that may be misinterpreted as warnings.
8. Accompany each pictogram, if used, with corresponding text placed close to the pictogram. Use the simplest pictograms possible. For pictograms intended to prompt patients to ask questions or inform health care providers, add text such as “Tell Doctor” or “Ask Pharmacist”.
9. Make text readable by using 12-point or larger type, both uppercase and lowercase letters, an easy-to-read font (for example, a serif font), and adequate space between lines and paragraphs. To call attention to important information, use a larger, boldface type.
10. Evaluate format by performing tests of readability, comprehension, memory, problem solving, and behavioral efficacy and intention, using representative samples of the target population.

CONTENT GUIDELINES

1. Provide enough detail to facilitate correct use, achieve maximum benefit, and minimize harm, including a statement that identifies activities (such as driving or sunbathing) that the patient should avoid.
2. Write text that is unbiased in content and tone and scientifically accurate. The uses described should be consistent with FDA-approved labeling or otherwise permitted by FDA, or should appear in federally recognized drug compendia. Distinguish unlabeled from labeled use.
3. For drugs sold under a brand name, provide both brand and generic names, and include a pronunciation guide for each.
4. Describe the drug and its dosage form. Include indications and contraindications, specific directions for use, what to do if a dose is missed, and what to do in the event of an overdose or poisoning.
5. Do not use abbreviations.
6. Indicate the intended type of benefit (for example, “cure”, “prevention”, “to help relieve symptoms”). Indicate how—and how soon—the patient should recognize the benefit and what to do if none is observed.
7. Give a balanced evaluation of risks and benefits.
8. List side effects, in order of severity, such as “serious”, “most common”, and other similar type groupings. It may not be appropriate to provide sufficient detail for the patient to be able to monitor serious or common side effects. Provide guidance to consult the doctor or pharmacist, and indicate that not all the side effects are listed.
9. List sufficiently specific and comprehensive information that includes the provision of all important risk information. Patients should be advised to be sure to inform the provider about all the medicines they are taking.
10. Indicate the potential for therapeutic duplication if the drug is available under multiple names or over-the-counter, or if the active ingredient is contained in other products.
11. If known, include a statement concerning the safety of use in the presence of other conditions and during pregnancy or breast-feeding. Direct affected patients to discuss their condition with health care providers.

If the safety of use during pregnancy or breast-feeding has not been established, say so.

12. State whether safety and efficacy have been established in pediatric, geriatric, and other special populations. Patients should be encouraged to discuss with their health care provider any recommendations for dosage adjustment.
13. Illustrate information with diagrams when appropriate. Label the diagram components (for example, device parts) if they are not obvious. The words on the label should be prominently placed thereon with such conspicuousness and in such terms as to render them likely to be read and understood by the ordinary individual under customary conditions of purchase and use.
14. Include the following:
 - a. A statement that the product is to be used only by the person for whom it was prescribed,
 - b. Storage information,
 - c. A completeness disclaimer advising the patient to discuss this issue with the health care provider,
 - d. The publisher of the leaflet and the date the leaflet was developed or revised,
 - e. Sources of in-depth information and answers to questions, and
 - f. Other relevant general statements.
15. The patient should be advised about risks of developing dependence on, or tolerance to, the medication.

ACCESSIBILITY GUIDELINES

1. Write text that is relevant to the intended use of the drug.
2. Design the leaflets to be easy to recognize, consistent in format, and easy to store and retrieve.
3. Supplement the leaflets with oral counseling of patients, including children, the elderly, and caregivers.
4. Include a statement asking the patient to reread the leaflet.
5. Distribute the leaflets with all prescription medicines to consumers (namely, persons independently responsible for any aspect of medicine use or for giving medicines to others).
6. Produce leaflets in Spanish, English, or other languages; and establish criteria for producing them in other languages and for special populations (for example, children, visually handicapped) [NOTE—Ideally, prescription drug information leaflets would be customized for the patient's condition and for other relevant information (for example, gender, age, or physical limitations), and would be available in the patient's primary language. Currently, such customization is neither feasible nor practical, but it remains a goal.]

(1601) PRODUCTS FOR NEBULIZATION—CHARACTERIZATION TESTS

Products used for nebulization and intended for pulmonary delivery are characterized using the following tests:

- *Drug Substance Delivery Rate and Total Drug Substance Delivered;*

— *Aerodynamic Assessment of Nebulized Aerosols.*

These tests standardize the approach to the assessment of the dose that would be delivered to a patient but are not intended to provide assessment of the nebulizer device itself.¹ The mass rather than the number-weighted size distribution is more appropriate to evaluate product performance. Drug substance mass as a function of aerodynamic diameter is more indicative of therapeutic effect within the respiratory tract.

DRUG SUBSTANCE DELIVERY RATE AND TOTAL DRUG SUBSTANCE DELIVERED

These tests are performed to assess the rate of delivery to the patient and the total drug substance delivered to a patient using standardized conditions of volumetric flow rate. Breath-enhanced and breath-actuated nebulizers should be evaluated by a breathing simulator because the output of these types of device is highly dependent on inhalation flow rate. The methodology below describes the use of a standard breathing pattern defined for adults. Should a particular product for nebulization be indicated only for pediatric, i.e., neonate, infant, or child use, then pediatric breathing pattern(s) must be used.² Breathing patterns are used, rather than continuous flow rates, to provide a more appropriate measure of the mass of drug substance that would be delivered to patients.

Drug substance delivery rate and total drug substance delivered are appropriate characteristics because they allow the mass delivered to be characterized in a standard way regardless of the nebulizer used. Accordingly, the test methodology described below (a) measures the mass of drug substance delivered in the first period (typically 1 min) consequently giving an assessment of drug substance delivery rate and (b) captures the total drug substance mass delivered.

Apparatus

Breathing Simulator—A commercially available breathing simulator that is able to generate the breathing profiles specified in *Table 1* is used for the test. The breathing profile indicated for adults is used unless the medicinal product is specifically intended for use in pediatrics, when alternate patterns should be used, as indicated in *Table 1*.

Filter System—A suitably validated low-resistance filter, capable of quantitatively collecting the aerosol and enabling recovery of the drug substance with an appropriate solvent, is used for the test. The dead volume of the filter casing does not exceed 10% of the tidal volume used in the breath simulation.

Procedure

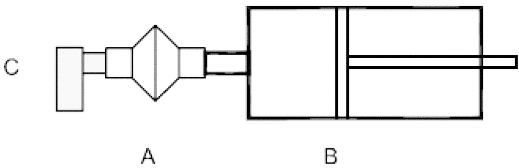
Attach the filter (contained in the filter holder) (A) to the breath simulator (B) according to *Figure 1*. Fill the nebulizer (C) with the volume of the drug product as specified in the patient instructions. Attach the mouthpiece of the nebulizer to the inhalation filter using a mouthpiece adapter if required, ensuring that connections are airtight. Position the nebulizer in the same orientation as intended for use. This

¹ European Standard 13544-1:2001. *Respiratory Therapy Equipment*. Part 1: Nebulizing systems and their components. European Committee for Standardization. Brussels, Belgium. 2001.

² Suitable breathing patterns for pediatric use may be found, for example, in Canadian Standard CAN/CSA/Z264.1-02:2002, *Spacers and Holding Chambers for Use with Metered Dose Inhalers*. Canadian Standards Association, Mississauga, Canada 2002.

Table 1. Breathing Simulator Specification

Item	Specification			
	Adult	Neonate	Infant	Child
Total volume	500 mL	25 mL	50 mL	155 mL
Frequency	15 cycles/min	40 cycles/min	30 cycles/min	25 cycles/min
Waveform	sinusoidal	sinusoidal	sinusoidal	sinusoidal
Inhalation:exhalation ratio	1:1	1:3	1:3	1:2



A. inhalation filter and filter holder B. breathing simulator C. nebulizer

Figure 1. Experimental Set-Up for Breathing Simulator Testing.

may require tilting the breathing simulator and filter holder. Set the breathing simulator to generate the specified breathing pattern.

Start the breathing simulator and at the beginning of an inhalation cycle, start the nebulizer. Operate the nebulizer for a defined initial time period. The length of the time interval ensures that sufficient drug substance is deposited on the inhalation filter for quantitative analysis. A time of 60 ± 1 s typically enables direct determination of the drug substance delivery rate. The time chosen, usually 60 ± 1 s, must allow sufficient drug substance deposition on the inhalation filter to allow quantitative analysis. If the quantity of drug substance deposited on the inhalation filter in 60 s is insufficient for this analysis, the length of the time interval for aerosol collection can be increased. If the filter is soaked with the product, this time can be decreased. At the end of this initial period, stop the nebulizer.

Place a fresh filter and filter holder in position and continue until nebulization ceases. Interrupt nebulization and exchange filters, if necessary, to avoid filter saturation.

Results

Using a suitable method of analysis, determine the mass of drug substance collected on the filters and filter holders during each time interval. Determine the drug substance delivery rate by dividing the mass of drug substance collected on the first inhalation filter by the time interval used for collection. Determine the total mass of drug substance delivered by summing the mass of drug substance collected on all inhalation filters.

AERODYNAMIC ASSESSMENT OF NEBULIZED AEROSOLS

Nebulized products need to be size-characterized at flow rates lower than the range that is typically used for powder inhalers and metered-dose inhalers. The CEN standard recommends a flow rate of 15 L/min because this value represents a good approximation to the mid-inhalation flow rate achievable by a healthy adult breathing at 500 mL tidal volume.

Although low-angle laser light-scattering instruments (laser diffractometers) can provide rapid size-distribution measurements of nebulizer-generated aerosols, these techniques do not detect the drug substance. Rather, they measure the

size distribution of the droplets irrespective of their content. This may not be a problem with homogeneous solutions, but it can result in significant error if the product to be nebulized is a suspension, or if droplet evaporation is significant, as can be the case with certain nebulizer types. Cascade impactors enable the aerosol to be characterized unambiguously in terms of the mass of drug substance as a function of aerodynamic diameter. Laser diffraction may be used if validated against a cascade impaction method.

Apparatus 5 (see general information chapter *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* <601>), a cascade impactor, has been calibrated at 15 L/min specifically to meet the recommendation of the CEN Standard and is therefore used for this test.³ Determining mass balance in the same way as for powder inhalers and metered-dose inhalers is not straightforward because the dose is being captured as a continuous output and hence is not included. Recovery experiments must be performed as part of method development and validation. Control of evaporation of droplets produced by nebulizers may be critical to avoid bias in the droplet size assessment process. Evaporation can be minimized by cooling the impactor to a temperature of about 5°, typically achieved by cooling the impactor in a refrigerator for about 90 min.

Typically, at least after each day of use, the apparatus must be fully cleaned, including the inter-stage passageways, because of the greater risk of corrosion caused by the condensation/accumulation of saline-containing droplets on inter-stage metalwork associated with cooling the impactor. All surfaces of the apparatus should be dried after each test, e.g. with compressed air. [NOTE—The micro-orifice collector (MOC) should not be dried with compressed air.]

Apparatus

A detailed description of Apparatus 5 and the induction port is contained in <601>, and includes details of critical dimensions and the qualification process for the impactor (stage mensuration).

A back-up filter in addition to the MOC must be used to ensure quantitative recovery of drug substance from the nebulized aerosol at the specified flow rate of 15 L/min. The filter is located below the MOC (internal filter option), or a

³ Marple VA, Olson BA, Santhanakrishnan K, et al. Next generation pharmaceutical impactor: A new impactor for pharmaceutical inhaler testing. Part III: Extension of archival calibration to 15 L/min. *J Aerosol Med* 2004; 17(4):335–343.

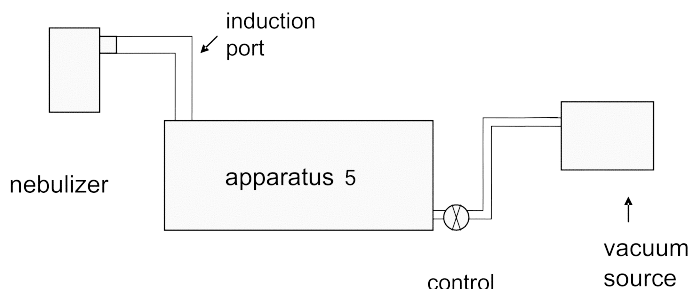


Figure 2. Apparatus 5 for Measuring the Size Distribution of Products for Nebulization.

filter in holder external to the impactor is used to capture any fine droplets that pass beyond the last size fractionating stage. A pre-separator is not used for testing nebulizer-generated aerosols.

Method Validation

Impactor Stage Overloading—During method development and validation, confirm that the volume of liquid sampled from the nebulizer does not overload the impactor. Visual inspection of the collection surfaces on stages collecting most of the droplets may reveal streaking if overloading has occurred. This phenomenon is usually also associated with an increase in mass of drug substance collected on the final stage and back-up filter. Reducing the sampling period (T_0) is the most effective way to avoid overloading in any given system, balancing overloading with analytical sensitivity.

Re-entrainment—Droplet bounce and re-entrainment are less likely with nebulizer-produced droplets than with solid particles from inhalers, thus coating would not normally be required.

Procedure

Pre-cool the assembled impactor and induction port in a refrigerator (set at about 5°) for not less than 90 min, and start the determination within about 5 min of impactor removal from the refrigerator. Other methods that maintain the impactor at a constant temperature (e.g. use of a cooling cabinet) can also be employed when validated.

Set up the nebulizer with a supply of driving gas (usually air or oxygen), or use a compressor at the pressure and flow rate specified by the manufacturer of the nebulizer. Ensure that the gas supply line does not become detached from the nebulizer when under pressure. Fill the nebulizer with the volume of the medicinal product as specified in the patient instructions.

Remove the impactor from the refrigerator. Attach the induction port to the impactor, and connect the outlet of the impactor/external filter to a vacuum source that is capable of withdrawing air through the system at 15 L/min, as specified in Figure 2. Turn on the flow through the impactor.

Connect a flow meter, calibrated for the volumetric flow leaving the meter, to the induction port. Adjust the flow control valve located between the impactor and the vacuum source to achieve a steady flow through the system at 15 L/min ($\pm 5\%$). Remove the flow meter.

Position the nebulizer in the same orientation as intended for use, then attach the mouthpiece of the nebulizer to the induction port, using a mouthpiece adapter if required. Switch on the flow/compressor for the nebulizer. Sample for a predetermined time (T_0). Once determined, this time (T_0) must be defined and used in the analytical method for a particular drug product to ensure that mass fraction data can be compared. At the end of the sampling period, switch

off the driving gas flow/compressor to the nebulizer, remove the nebulizer from the induction port, and switch off the flow from the vacuum source to the impactor. Dismantle the impactor and, using a suitable method of analysis, determine the mass of drug substance collected in the induction port on each stage and on the back-up filter as described for Apparatus 5 (see <601>). Add the mass of drug substance collected in the MOC to that deposited on the back-up filter/external filter and treat as a single sample for the purpose of subsequent calculations.

Calculate the fine particle mass of the drug substance based on the predetermined time (T_0). Calculate the mass fraction ($F_{m,comp}$) of the drug substance deposited on each component of the impactor, commencing with the induction port and proceeding in order through the impactor, using the following expression:

$$F_{m,comp} = m_{comp}/M$$

m_{comp} = mass associated with the component under evaluation;

M = total mass collected by the system.

Present $F_{m,comp}$ in order of location within the measurement equipment, beginning at the induction port and ending with the back-up filter of the impactor (see Figure 3). $F_{m,comp}$ values for adjacent stages of the impactor may be combined in order to report the mass fraction collected on a group of stages as a single value.

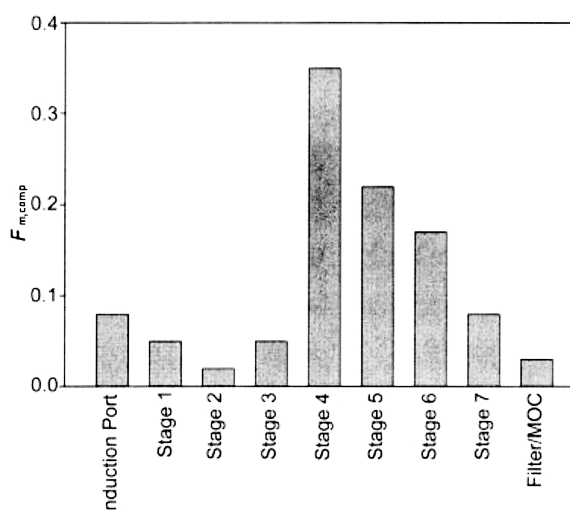


Figure 3. Example of Mass Fraction of Droplets Presented in Terms of Location within the Sampling System.

Determine the cumulative mass-weighted particle-size distribution of the aerosol size-fractionated by the impactor in accordance with the procedure given in <601>. Starting at

the filter, derive a cumulative mass versus effective cut-off diameter of the respective stages (see Table 2 for the appropriate cut-off diameters at 15 L/min). Plot the cumulative fraction of drug substance versus cut-off diameter in a suitable format, e.g., logarithmic or log-probability format. Where appropriate, use this plot to determine by interpolation the fraction either less than a given size or between an upper and lower size limit.

Table 2. Cut-off Sizes for Apparatus 5 at 15 L/min

Stage	Cut-off Diameter (μm)
1	14.1
2	8.61
3	5.39
4	3.30
5	2.08
6	1.36
7	0.98

If necessary, and as appropriate, use this plot to determine values for the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD).

<1644> THEORY AND PRACTICE OF ELECTRICAL CONDUCTIVITY MEASUREMENTS OF SOLUTIONS

This general chapter provides information in support of instrumental methods for procedures that measure electrical conductivity. Pharmaceutical applications include: chemical dosing, cleaning in place, fermentation control, and liquid mixing verification, among others. Although the general chapter focuses on aqueous systems, conductivity measurements can be extended to organic fluids. The general chapter also focuses on contacting conductivity measurements and does not cover applications which may use noncontacting inductive conductivity. After an introduction, the general chapter covers the following major topics: theory of operation, operational considerations, calibration, and operation for at-line, in-line, and off-line measurement procedures.

INTRODUCTION

Conductivity is the measurement of the ability of a fluid to conduct electricity via its chemical ions. The ability of any ion to electrically conduct is directly related to its ion mobility. Some of the common applications of conductivity measurements include water treatment and purification, clean-in-place process fluid management, fermentation process monitoring, dosing applications, nutrient media preparation, buffer production (e.g., distribution and dilution for dialysis and chromatography applications), chromatography detection of gradient and eluent, active pharmaceutical ingredient chemical synthesis, and concentration determination of basic chemicals. Fluids should be measured in a single homogeneous phase—i.e., conductivity should not be applied to mixed immiscible fluids unless they are separated. Electrical conductivity measurements cannot be applied to solids or gases, but they can be applied to the condensate of gases.

Besides its use to monitor ionic concentrations of process fluids, conductivity is also useful for the detection of ionic impurities in compendial waters (see *Water Conductivity* <645>) and for the detection of ionic impurities in organic matrices.

The measurement is non-ion-specific, and all ions respond with different efficiency or equivalent conductance, λ . Despite the lack of ionic specificity, conductivity is a valuable laboratory and process tool for measurement and control of total ionic content because it is proportional to the sum of the concentration of each ionic species (anions and cations) as described in Equation 1:

$$\kappa = 1000 \sum_i^{\text{all ions}} C_i \lambda_i$$

where κ is the conductivity (S/cm), C_i is the concentration of chemical ion i (mole/L), and λ_i is the specific conductance of ion i (S · cm²/mole). Though S/m is the appropriate SI unit for conductivity (i.e., the base SI units are the ampere and the meter) units of S/cm historically have been selected as the accepted unit of expression.

At low ion concentrations (typically <10⁻³ mole/L), the conductivity-concentration relationship is linear and valid because λ is constant for each ion, but there are three notable exceptions to this strict linearity and proportionality. First, at higher concentrations (approximately 10⁻³ to 1 mole/L) small negative deviations from linearity (<5% per decade) arise because of the decrease in λ for each ion, and the negative deviations vary from ion to ion. Second, at higher concentrations for weak acids and bases the extent of dissociation into ions decreases depending on their dissociation constants. As the concentration of a weak acid/base increases, the conjugate cation/anion concentration increases as the square root of the acid/base concentration. Third, at high concentrations (>20%) of certain strong acids such as HNO₃ and H₂SO₄, the negative deviations persist, and, in some cases, the conductivity decreases with increasing concentration. The conductivity of high-concentration acid systems is well documented.

Another variable that influences conductivity measurements is the fluid temperature. A stricter expression of Equation 1 is shown below as Equation 2:

$$\kappa(T) = 1000 \sum_i^{\text{all ions}} C_i(T) \lambda_i(T)$$

where the conductivity measurement, ion concentrations, and specific ion conductances are temperature (T) dependent. As fluid temperature increases, the ions become more efficient electrical conduits, making this physicochemical phenomenon the predominant reason for the temperature-compensation requirement when testing conductive fluids. The specific ion conductance of all ions increases with increasing temperature. In addition, the concentration of ions can also change as a function of temperature. For example, the auto-dissociation constant of water, K_w , increases with temperature from 0° to 100°, resulting in the increased production of H⁺ and OH⁻.

Strictly speaking, it is challenging to temperature-compensate perfectly the conductivity measurement to a reference temperature unless the ionic species are well known. In many applications the ionic species are well known, and in most other cases simple assumptions make this issue less demanding. Temperature compensation is discussed in further detail in *Temperature Compensation*, below.

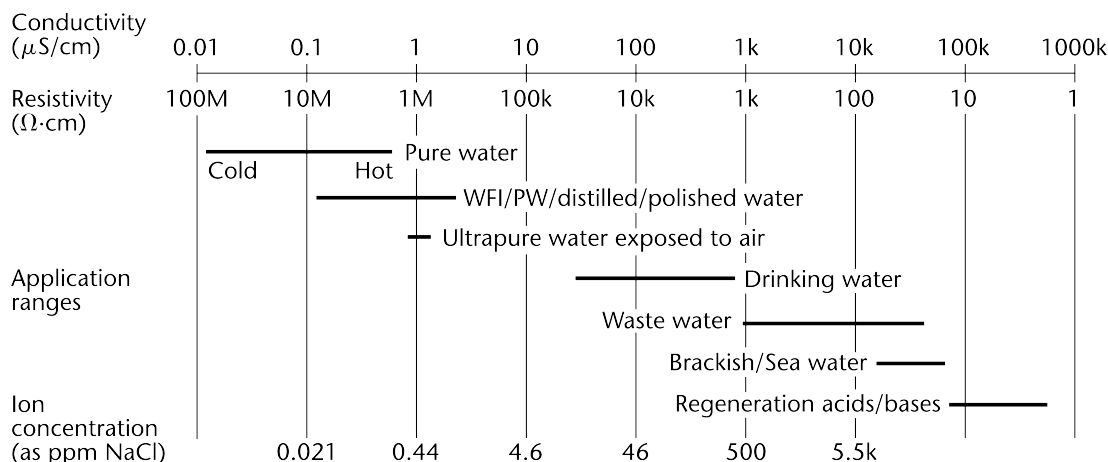


Figure 1. Relationships among conductivity, resistivity, and some example process fluids of various grades of purity.

THEORY OF OPERATION

Alternating Current Measurement Method

Conductivity is measured by applying a voltage (or current) between two conducting electrodes and measuring the resistance of the fluid using Ohm's Law. Various methods are used to apply the voltage/current, but all have the property of using an alternating voltage/current (AC) in order to minimize polarization (or collection of ions) at the electrodes or any electrolytic reaction. If a direct voltage/current (DC) is used, then the positive ions will collect at the negative electrode, and negative ions will collect at the positive electrode. The collection of ions at the electrode prevents the flow of current and adversely affects the accuracy and stability of the conductivity measurement. The measuring frequency of the AC signal depends on the technology and can range from as low as 30 Hz in low-conducting fluids and up to 4 kHz in highly conductive fluids. The specific frequencies are not relevant to operation of the system because the drive frequency is embedded in the instrument's measurement systems and is integrally linked to the supplier's measurement technology. This chapter does not seek to evaluate different measurement technologies because they are usually microprocessor-controlled systems and are proprietary.

The two-electrode AC measurement technique is valid for use with all concentrations of ionic species ranging from acids and bases (high conductivity) to *Water for Injection* and *Purified Water* (low conductivity) and even to organic, weakly ionic species such as alcohols and glycols. The measurement can be sensitive to ion concentrations as low as 0.05 μg/L. For high ion concentrations, analysts can use an alternative 4-electrode measurement method in which the current is applied between two of the electrodes and the voltage is measured between the two other electrodes. Ions are driven to the current electrodes while the voltage electrodes make the measurement with limited polarization effects.

Units of Expression

There is no difference in the physical measurement of conductivity and resistivity—they are multiplicative inverse measurements of each other. Therefore, if one measurement is known, then the other value is readily calculated by taking the reciprocal of the numerical value and the units. There is also no difference in the instrumentation or the sensors. The only difference is how the measured value is reported or displayed for the convenience of the analyst. For

example, $18.2 \text{ M}\Omega \cdot \text{cm} = 0.0550 \text{ }\mu\text{S/cm}$ and $5.23 \text{ k}\Omega \cdot \text{cm} = 0.191 \text{ mS/cm} = 191 \text{ }\mu\text{S/cm}$. Although the proper SI units are $\Omega \cdot \text{m}$ or S/m , the traditionally used units are $\Omega \cdot \text{cm}$ or S/cm .

Figure 1 shows relationships among conductivity, resistivity, and some example process fluids of various grades of purity. The conductivity of fluids in pharmaceutical systems varies over approximately 8 orders of magnitude. In high-purity water systems, the quantity of ions present in *Purified Water* or *Water for Injection* is very low, resulting in a conductivity $<5 \text{ }\mu\text{S/cm}$ and often approaching $0.055 \text{ }\mu\text{S/cm}$ or less. [NOTE—The conductivity of the purest waters at temperatures less than 25° is less than $0.055 \text{ }\mu\text{S/cm}$.] In drinking waters, the conductivity may vary from 30 to 2000 $\mu\text{S/cm}$. In chromatographic separations, the conductivity of the eluent may vary from 0.1 to 100 mS/cm . For hot concentrated acids, the conductivity may be as high as 1 S/cm .

Cell Constant Determination

The purpose of the sensor's cell constant is to normalize the conductance/resistance measurement for the geometrical construction of two electrodes. When two electrodes are placed in a conducting fluid and a voltage is applied to them, there is a conductance (resistance) between the electrodes. If the electrodes are placed farther apart, the conductance decreases (resistance increases). If the area of the electrodes increases, then the conductance increases (resistance decreases). In both cases, the ion concentrations between the electrodes do not change, but the geometrical construction of the sensor (cell constant) alters the measured conductance (resistance). The conductivity of a solution (κ , S/cm) is related to the conductance, G (siemens) according to Equation 3:

$$\kappa = G \times \left(\frac{d}{A} \right) = G \times \theta$$

where A is the area of the conducting electrodes (cm^2) and d is the distance between the electrodes (cm). The other common unit of expression is the reciprocal of conductivity, or resistivity, ρ ($\Omega \cdot \text{cm}$), as described in Equation 4:

$$\rho = \frac{1}{\kappa} = \frac{1}{G \times \theta} = \frac{R}{\theta}$$

where R is the resistance of the fluid between the electrodes ($1 \text{ }\Omega = 1 \text{ S}^{-1}$). The geometrical ratio, d/A (or θ), is known as the cell constant (cm^{-1}) of the sensor.

Determination of the cell constant by the direct measurement of d and A is impractical because of variations in the geometrical configurations and the nonuniformity of the electric field between the electrodes. Practically, the cell constant is determined by the measurement of aqueous solutions of known conductivity. See *Calibration* below.

Temperature Compensation

Temperature compensation is a typical requirement for most conductivity measurements, although there are exceptions such as those contained in *Water Conductivity* <645>. As noted previously, the conductivity of a fluid is related to its temperature. As the temperature increases, ions become more mobile and the conductivity increases. The effect of temperature depends on the type and concentration of the ion, but for most solutions $>10 \mu\text{S}/\text{cm}$ the impact of temperature is in the range of $1.9\%/^{\circ}$ to $2.2\%/^{\circ}$. For strong acids, this may be as low as $1.5\%/^{\circ}$. For high-purity compendial waters, the temperature coefficient varies from $2.0\%/^{\circ}$ to $7.5\%/^{\circ}$ depending on the temperature and purity of the water. For each case described here, some knowledge of the type of impurity is needed in order to ensure adequate temperature compensation. If the conductivity vs. temperature function is linear and the temperature coefficient is constant, the equation that relates the compensated conductivity to the non-temperature-compensated conductivity is described in *Equation 5*:

$$\kappa_{25} = \frac{\kappa_T}{[1 + \alpha(T - 25)]}$$

where T is the measured temperature, κ_{25} is the conductivity compensated to 25° , κ_T is the conductivity at T , and α is 0.02 for a temperature coefficient of $2\%/^{\circ}$.

Most conductivity measurement systems measure the uncompensated conductivity/resistivity and the temperature, and the temperature-compensated conductivity is determined via mathematical algorithms (e.g., by application of *Equation 5*) in the microprocessor of the transmitter. Depending on the application and knowledge of the content of the fluid, different compensation algorithms may be available. For most process control applications, temperature compensation is recommended because when the uncompensated conductivity changes it is impractical to distinguish whether this change is caused by temperature fluctuation or a change in ionic content. Temperature compensation allows the analyst to distinguish between changes in temperature and ionic content. Compensation to a reference temperature of 25° is standard practice, but some methods specify temperature compensation to 20° .

Most conductivity sensors have temperature devices such as a platinum RTD (resistance temperature device) or NTC (negative temperature coefficient) thermistor embedded inside the sensor, although external temperature measurement is possible.

OPERATIONAL CONSIDERATIONS

System Components

The usual components of a conductivity measurement system are the sensor, the transmitter, and the cable that connects the sensor to the transmitter. The sensor is the device that is in direct contact with the fluid. The sensor consists of the electrodes, usually an embedded temperature device, and a process connection (e.g., a tri-clamp if the sensor is intended for in-process sanitary applications). Traditionally, sensors are passive electromechanical devices and do not contain measurement circuitry. The transmitter is the device that measures the resistance between the sensor's

electrodes, measures the resistance of the temperature-measuring device, converts the resistance measurement to a conductivity (or resistivity), and performs temperature corrections to compensate the signal to a reference temperature. The cable connects the sensor to the transmitter. In process control or monitoring systems, the cable allows the sensor to be attached to a process tank or pipe, and the transmitter can be located at a control panel or other remote location, if necessary. The distance between the sensor and the transmitter may affect measurement accuracy caused by the added cable resistance and the susceptibility of the cable to external noise. Therefore, this distance should be considered during selection of the measurement system. In some conductivity measurement systems, the measurement circuitry is directly attached to the conductivity sensor, allowing digital transfer of the measurement results to a remote display at greater distances than traditional measuring systems with wire transmission of analog signals.

Materials of Construction

The sensor's materials of construction are critical when the sensor may be in contact directly or indirectly with the product. For most laboratory applications, materials of construction are less critical. All sensors contain measuring electrodes and an insulating material between the electrodes. Technically, the only requirements are for electrodes to be electrically conducting and to be able to withstand the physical and chemical environment. Electrodes can be made of various grades of stainless steel, titanium, graphite, and many other metals. The insulating materials must isolate the electrodes from each other so that only the fluid conductivity is measured. Insulating materials are usually made of an inert polymer, epoxy, or ceramic.

If the sensor is connected directly to a process vessel or the piping system, then the sensor's materials of construction must meet the thermal and hydraulic (pressure) requirements of the process system. The sensor must have a proper process connection to the vessel/piping. Depending on the application, the sensor also may be required to meet biological compatibility, material, or hygienic design requirements. The sensor must not degrade during installation and operation. The sensor must be able to withstand any other processes that the system may encounter such as clean-in-place or steam-in-place processes. Otherwise analysts may need to remove the sensor from the process. When immersion of the sensor into the process places the process at risk, the sensor should be installed in a housing that is attached to the sidestream. In this case, a fraction of process fluid flows to the housing and sensor and then to drain. This allows indirect measurement of the process fluid without harm to the process or product.

If the conductivity measurement is performed off-line, e.g., in a laboratory environment, then the operating considerations are reduced because the thermal, hydraulic, and other product-contact concerns are reduced or eliminated.

CALIBRATION

The process of calibrating a complete conductivity measurement system generally consists of three parts. First, the transmitter's electronic circuitry is calibrated. Second, the temperature sensor device is calibrated. Third, the cell constant of the conductivity sensor is determined. In each case, verification may precede any calibration to determine if an adjustment is necessary. In most cases for microprocessor-controlled instrumentation, there is no formal mechanical or electronic adjustment of these subsystems. Instead, these adjustments are usually made in software-controlled calibrations that are computed automatically by the transmitter.

A calibrated transmitter should be used in order to calibrate the temperature sensor and cell constant. The transmitter used to calibrate the temperature sensor and cell

constant can be the transmitter used in normal operation, or another transmitter can be used instead. Because of differences in a supplier's wiring and electronic compatibility, it may be necessary to use the same type of transmitter.

As is the case for all instrument-based methods, calibration frequency depends on many factors. Depending on the type of conductivity sensor and transmitter, calibration cycles vary from weekly to annually based on manufacturers' recommendations, historical performance of the instrumentation, internal requirements, and the criticality of the application. For robust process instrumentation, typical calibration cycles for the electronics and the sensor take place approximately every 12 months. When the sensor's cell constant can be altered by the fluid or the process conditions, more frequent calibration may be needed.

Instrument Calibration

The transmitter's electronic circuitry is calibrated by disconnecting the sensor from the transmitter, connecting precision resistors (resistors of known value) to the transmitter, and comparing the traceable resistance value to the measured resistance value. The resistance values should be traceable to a competent national authority. The resistance values should be selected so that they are in the range of (1) the measurement capability of the transmitter, and (2) the resistance that will be measured during operation. The transmitter may have multiple circuits internally, so verification of the appropriate circuit (or all circuits) and measurement range is necessary. Comparison of the measured resistance to the actual resistance verifies if the transmitter is properly calibrated. The resulting difference must be within a predetermined $x_1\%$ of the actual resistance, where x_1 indicates the desired conductivity circuit electronics performance. Typical conductivity circuit electronics performance is usually in the range of 2% or less of the target value. Otherwise, adjustment of the resistance measurement circuit is recommended.

An example of the transmitter's measurement electronics calibration process follows: A typical operating range for a clean-in-place process may be in the 50 to 75 mS/cm range. A sensor with a cell constant of 5.0 cm^{-1} is used in this example. Based on Equation 4, this requires a measuring resistance of 67 to 100 Ω . To verify the conductivity measuring circuit during calibration, analysts should use a resistor(s) with a traceable value in or near this range. When compendial waters are used, typical measurements are in the range of 1 to 20 $\text{M}\Omega \cdot \text{cm}$ and use sensors with a cell constant of 0.1 cm^{-1} . This computes to a resistance of 0.1 to 2 $\text{M}\Omega$. Resistors with a traceable value in this range should be used. If the recorded values are within the range of the pre-established acceptance criteria ($x_1\%$), then adjustment is not required.

Temperature Measurement and Sensor Calibration

If a temperature measurement circuit is integrated into the transmitter and is used as part of the measurement system, then verification and/or calibration of this circuit is required. Depending on the type of temperature device in the sensor, an appropriate signal source (e.g., resistance) should be input to the transmitter. Comparison of the measured temperature and the simulated temperature verifies if the temperature measurement circuit is properly calibrated. The resulting difference must be within x_2° of the simulated temperature where x_2 indicates the desired temperature circuit electronics performance. Typical temperature circuit electronics performance usually is in the range of $\pm 1^\circ$ or less. If the difference meets the pre-established acceptance criteria, no further action is required. Otherwise, adjustment of the temperature measurement circuit is recommended using the protocol in the transmitter's calibration function.

If a temperature sensor is integrated into the conductivity sensor, the temperature sensor can be calibrated by comparison of the sensor's temperature measurement to a reference system. This is accomplished by immersing the process and reference sensors in the same fluid. The reference system can be another traceable temperature measurement device or a fluid system of known temperature such as boiling water (corrected for elevation, if this degree of accuracy is necessary) or an ice-water bath.

The accuracy of the calibration is related to the accuracy of the reference sensor, the thermal homogeneity of the fluid, and the elimination or reduction of any artifacts that can negatively influence accuracy. For example, if the sensor is not fully immersed in the fluid, thermal conduction from the ambient environment can alter the temperature measurement. Careful insulation of the sensor from the ambient environment may improve the accuracy of temperature measurements, particularly if the reference temperature is substantially different than the ambient temperature.

Comparison of the measured temperature and the reference temperature verifies if the temperature sensor is properly calibrated. The resulting difference must be within x_3° of the reference temperature where x_3 indicates the desired temperature accuracy that is required for the process. Temperature sensor accuracy is usually in the range of $\pm 2^\circ$ or less. If the difference meets the pre-established acceptance criteria, no further action is required. Otherwise, adjustment of the temperature sensor calibration factors is recommended using the protocol in the sensor's calibration function.

Cell Constant Calibration

The cell constant of the sensor is determined by comparing the conductivity measurement of the system to that from a reference conductivity system and adjusting the cell constant. Place the sensor in a reference solution of known conductivity whose value is traceable to a competent authority. The reference solution can be a solution of known conductivity in one of three ways:

- (1) The solution can be produced according to a standard method that is traceable to a competent authority. One method is to prepare one of the solutions listed in ASTM D1125. Another method is to use ultra-pure deionized water with no exposure to air whose conductivity is known.
- (2) The solution can be procured from a third-party supplier with traceability to an acceptable authority. Cell constant calibration should be performed in the recommended temperature range of the reference solution.
- (3) The solution can be any fluid whose conductivity is known by measurement from an alternative and traceable reference conductivity measurement system.

In all three cases, regardless of the type of calibration methodology, there are some fundamental requirements. The sensor's cell constant should be calibrated at a conductivity that is in the measurement range of the measuring system. For example, if the system is designed to measure in the 0–100 $\mu\text{S}/\text{cm}$ range, then use of a 1000 $\mu\text{S}/\text{cm}$ reference solution is not advised.

Because the cell constant is a geometrical property of the sensor and is a constant, the determination and/or calibration of the cell constant does not need to be in the operational range of the pharmaceutical process as long as the conductivity is in the operational range of the measurement system. Also, because the cell constant is a geometrical property of the sensor, a single-point calibration typically is sufficient. In some applications, a 2-point calibration can provide improved accuracy at very high conductivity. Depending on the reference solution, cell constant calibration can be performed with or without temperature compensation. For reference solutions exceeding 10 $\mu\text{S}/\text{cm}$, if temperature compensation is needed a temperature coefficient (α)

of 2%/° is sufficient over the range of $25 \pm 10^\circ$ unless otherwise specified.

Adjustment of the cell constant is recommended if the difference between the measured and reference conductivity exceeds $x_r\%$ of the reference conductivity where x_r indicates the conductivity accuracy that is required for the process. Typical conductivity accuracy is 5% or less. If the accuracy measurement meets the pre-established acceptance criteria, no further action is required. Otherwise, adjustment of the sensor's cell constant is recommended using the protocol within the sensor's calibration function.

OPERATION

In-line, At-line, and Off-line Measurements

Depending on the application, in-line, at-line, and off-line measurements have specific universal requirements. Each has advantages and disadvantages depending on the application, but the fundamental technologies (AC measurement) supporting each measurement type are constant. The primary differences between the in-line/at-line and the off-line systems are sensor features related to process robustness and transmitter features related to output functions, external communication capabilities, and system installation costs. In-line and at-line systems have added benefits of real-time measurements and continuous data acquisition. They also have added one-time electrical and plumbing installation costs, and the instrumentation is fixed in a specific location for a single process and purpose. Also, sample is diverted to drain, resulting in product loss. Note that the fluid flow velocity should be high enough that the velocity does not affect the measurement. Off-line (or laboratory-based) systems have the benefit of measuring many sample types under controlled conditions. Off-line batch testing has added costs associated with cleaning containers and collecting samples as well as risks of sample contamination.

In-line conductivity measurements are suitable when the following conditions are met: (1) there is a need for or value of real-time, continuous data; (2) the sensor and the application/fluid are compatible and cause no harm to each other; and (3) atmospheric contamination must be avoided. In these cases, continuous process control, decisions, and intervention are available.

At-line (or sidestream) conductivity measurements are used when the following conditions are met: (1) a need exists for or value of real-time, continuous data; (2) the sensor and the application are compatible with each other; (3) the sensor can cause harm to the application and/or fluid; and (4) atmospheric contamination must be avoided. In such cases the measurement is made by delivering the sample from the tank/piping using hydraulic pressure (flow) to the conductivity sensor, and the fluid passes through the sensor and goes to drain. In these cases, continuous process control, decisions, and intervention are available and have no effect on product or application. The only requirement is positive pressure upstream from the sensor to prevent backstreaming of the sample fluid back into the process.

Off-line conductivity measurements are used when the following conditions are met: (1) no need exists for or value of real-time or continuous data; (2) the sensor and the application are compatible with each other, but the sensor can cause harm to the application and/or fluid; and (3) sampling needs are infrequent or are needed on a limited basis.

Sample Preparation and Off-line Measurement

When performing off-line measurements, analysts must ensure that the container used to collect the fluid sample(s) is sufficiently clean so that the container does not alter the result. Collect an amount of fluid needed for rinsing and measuring. For samples such as *Purified Water* where the

analysis can be affected by gasses, containers should be filled completely to reduce headspace. The volume of fluid needed to make a measurement depends on the design of the sensor and can range from <10 mL to 1 L. Transfer a portion of the sample fluid to the clean measuring container to rinse the container walls. Discard the fluid. The sample container also can be used as the measuring vessel. The sensor should be rinsed with a suitable quality of water or other appropriate fluid before use so the measurement is not affected and then rinsed at least once with the solution to be measured, which then is discarded. This rinse with sample solution removes any residue or fluids with which the conductivity sensor previously was in contact. The exact procedure varies depending on the solution tested. For example, when testing very low conductivity samples such as *Purified Water*, analysts must do more rinsing to remove any remaining residue from the sensor. Maximum holding time, temperature, and container type must be controlled for some low-conductivity samples, e.g., high-purity waters, because of the potential impact of ionic leachables from certain containers.

Transfer the fluid to the measuring container, immerse the sensor into the fluid, and ensure acceptable clearance around the sensor's measurement area. Stir or agitate the sample to prevent bubbles from attaching to the electrodes and disturbing the measurement. If a temperature-compensated measurement is used, then select the appropriate temperature-compensation algorithm in the transmitter, and adjust the sample temperature to the recommended range, if necessary, using an appropriate temperature bath. Otherwise, disable the temperature compensation. Verify that the temperature is sufficiently stable (<0.25° change per min), and record the conductivity reading and the temperature, if necessary.

In-line Measurement

For in-line testing, the sensor is installed into the process piping or vessel. The orientation of the sensor relative to the flow of the fluid is critical to ensure that (1) particles and sediment do not collect between the measuring electrodes, and (2) no air pockets are trapped between the measuring electrodes. Both factors can adversely affect the measurement accuracy. Depending on the electrode design, manufacturers usually provide recommendations. Ensure the sensor is clean before installation.

After the sensor is immersed in the closed process, such as a piping system or tank, the sensor does not require removal and cleaning before a measurement. If a temperature-compensated measurement is used, then select the appropriate temperature-compensation algorithm. Otherwise, disable the temperature compensation. Although temperature stability is desirable, it may not be possible depending on the temperature control of the process. Record the conductivity reading and the temperature, if necessary.

At-line Measurement

For at-line testing, a piece of tubing is used to connect the process vessel/piping to the sensor and its housing. The tubing should be cleaned or flushed with an appropriate cleaning agent to remove any impurities that could alter the conductivity measurement. The tubing can be made of metal or plastic depending on the application and chemical compatibility. Installation of the sensor into a housing permits fluid to be directed into the sensor and then to drain. Installation considerations are similar to those for in-line installations. Ensure the sensor and housing are clean before installation.

After the sensor and housing are connected to the sidestream, the sensor does not require removal and cleaning before a measurement. If a temperature-compensated measurement is used, then select the appropriate temperature-compensation algorithm. Otherwise, disable the tem-

perature compensation. Although temperature stability is desirable, it may not be possible depending on the temperature control of the process. Record the conductivity reading and the temperature, if necessary.

Other Considerations

In all cases mentioned above, the use of abrasive materials to clean the sensor typically is discouraged. Two reasons to avoid abrasive cleaning materials are that (1) the passive layer of stainless steel sensors (when used) can be destroyed, and (2) the surface of the measurement area can affect the measurement accuracy. For these reasons, the use of appropriate chemically compatible fluids is preferred instead of mechanical methods for cleaning.

Unlike the requirements for many electrochemical measurements, the need for flowing, circulating, or agitating fluid is not a fundamental requirement for a conductivity measurement. In general, there is no difference in the conductivity measurement of a static or a flowing sample, but two conditions must be met for static samples: First, bubbles cannot be allowed to collect on the measuring area of the electrodes because they can interfere with the flow of current conductivity measurement. Second, if sample homogeneity is affected by the lack of agitation or circulation, then the conductivity measurement may not represent the conductivity of the bulk fluid.

In all cases, the installation of the sensor should take into consideration wall effects from the vessel, piping, or laboratory container. If the proximity of the wall interferes with the electromagnetic field for the conductivity measurement, then the measurement could be positively or negatively altered. Some 2-electrode sensor designs, such as coaxial concentric electrodes, are not affected by nearby objects. The sensor's installation instructions may indicate if this must be considered.

If unstable conductivity readings are observed, some common causes could be inadequate grounding of the water system, electronic noise from pumps and other high-frequency generators, or internal leakage of the sensor. Various diagnostic approaches are available and can help identify the cause.

Add the following:

▲ (1761) APPLICATIONS OF NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

PRINCIPLES OF NMR

Nuclear magnetic resonance (NMR) spectroscopy is an analytical technique based on the magnetic properties of

certain atomic nuclei. NMR is similar to other types of spectroscopy in that absorption of electromagnetic energy at characteristic frequencies provides analytical information. NMR differs from other types of spectroscopy because the discrete energy levels between which the transitions take place are created by placing the nuclei in a magnetic field of strength H_0 . Although the initial field strength of the applied field is H_0 , when the sample is inserted into the magnet, the field strength throughout the sample becomes B_0 , defined as follows:

$$B_0 = \mu_s H_0 \quad [1]$$

in which μ_s is the magnetic susceptibility of the sample.

Atomic nuclei behave as if they were spinning on the nuclear axis. The angular momentum, ρ , of the nucleus is characterized by a spin quantum number (I). The maximum observable component of the angular vector, ρ , is

$$\rho = \hbar/2\pi = I\hbar \quad [2]$$

in which \hbar is Planck's constant and \hbar is modified Planck's constant.

Table 1 shows the values of I as a function of the mass number and the atomic number.

Table 1. Nuclear Spin Values as a Function of Mass and Atomic Numbers

Mass Number	Atomic Number	Nuclear Spin (I)
Odd	Even or odd	1/2, 3/2, 5/2 ...
Even	Even	0
Even	Odd	1, 2, 3 ...

The angular momentum creates a magnetic moment, μ , which is parallel to and directly proportional to ρ .

$$\mu = \gamma\rho = \gamma\hbar I \quad [3]$$

where γ is the magnetogyric ratio and is a constant for all nuclei of a given isotope, regardless of their position in a molecule.

Nuclei that have a spin quantum number $I \neq 0$, when placed in an external uniform static magnetic field, align with respect to the field in $(2I + 1)$ possible orientations. Thus, for nuclei with $I = 1/2$, which includes most isotopes of analytical significance (Table 2), there are two possible orientations, corresponding to two different energy states. The energies of these two states are $\pm \mu B_0$, and their separation is

$$E = \mu B_0 - (-\mu B_0) = 2\mu B_0 \quad [4]$$

with more nuclei populating the lower energy state ($-\mu B_0$) than the higher energy state ($+\mu B_0$). The populations are in accordance with the Boltzmann distribution:

$$N_+/N_- = \exp(-E/kT) \quad [5]$$

where N_+ and N_- are the populations of the high and low energy states, respectively; k is the Boltzmann constant; and T is the temperature in K.

Table 2. Properties of Some Nuclei Amenable to NMR Study

Nucleus	<i>I</i>	Natural Abundance (%)	Sensitivity	Resonance Frequency (MHz) at		
				1.4093 T ^a	7.0463 T	11.7440 T
¹ H	1/2	99.98	1.00	60.000	300.000	500.000
¹³ C	1/2	1.108	0.0159	15.087	75.432	125.721
¹⁹ F	1/2	100	0.83	56.446	282.231	470.385
³¹ P	1/2	100	0.0663	24.289	121.442	202.404
¹¹ B	(3/2)	80.42	0.17	19.250	96.251	160.419

^a T = tesla: 1.4093 T = 14.093 kilogauss.

A nuclear resonance is the transition between these states, and upward as well as downward transitions are possible. In a static magnetic field the nuclear magnetic axis precesses (Larmor precession) about the B_0 axis. The precessional angular velocity is often referred to as the Larmor frequency, ω_0 , and is related to B_0 :

$$\begin{aligned}
 E &= h\nu = 2\mu B_0 \\
 &= 2\gamma\hbar B_0 \\
 &= \gamma\hbar B_0 \\
 \nu &= \gamma B_0 / 2\pi \\
 \omega_0 &= \gamma B_0 \quad [6]
 \end{aligned}$$

If energy from an oscillating radio-frequency (rf) field is introduced, then resonance is achieved when the rf frequency is the same as the precessional angular velocity. Although the probability of an upward transition is equal to that of a downward transition, more upward transitions take place than downward transitions because N_- is greater than N_+ . Hence, an overall absorption of energy takes place. As shown in Table 2, the resonance frequency of a nucleus increases in direct proportion with the increase of the magnetic field strength.

NMR is a technique of high specificity but relatively low sensitivity. The basic reason for the low sensitivity is the comparatively small difference in energy between the upper and lower energy states (0.08 Joules at 1.5 to 2.0 T field strength), which results in a population difference between the two levels of only a few parts per million. Another important aspect of the NMR phenomenon, with negative effects on sensitivity, is the long lifetime of most nuclei in the excited state. Long lifetimes affect the design of the NMR analytical test, especially in repetitive pulsed experiments. Simultaneous acquisition of the entire range of resonant frequencies instead of frequency-swept spectra can give increased sensitivity per unit time.

All characteristics of the signal—chemical shift, multiplicity, linewidth, coupling constants, relative intensity, and relaxation time contribute analytical information. The analytical usefulness of NMR arises from the observation that the same types of nuclei, when located in different molecular environments, exhibit different resonance frequencies. The reason for this difference is that the effective field associated with a particular nucleus is a composite of the external field provided by the instrument and the field generated by the circulation of the surrounding electrons. The latter field is generally opposed to the external field and lowers the overall field strength at the nuclear site. The phenomenon is termed *shielding*. Hence, the more shielded nuclei have lower Larmor frequencies.

It is not convenient to accurately measure the absolute values of transition frequencies, as is done with other spectroscopic procedures. However, it is convenient to accurately measure the difference in frequencies between two resonance signals. The position of a signal in an NMR spec-

trum is described by its separation from another resonance signal arbitrarily taken as standard. This separation is called *chemical shift*, which may be expressed in units of magnetic field or in frequency units that are readily interconverted by the equation for the resonance condition, Equation 6. This equation shows that when the separation is expressed in frequency units, it is directly proportional to the field strength. It is more convenient, therefore, to express the chemical shift in terms of the dimensional unit δ , which is independent of the field strength, and is defined by

$$\delta = (v_{ss} - v_{rs})/v_0 \quad [7]$$

where v_{ss} is the test substance resonance frequency, in Hz; v_{rs} is the reference resonance frequency in Hz; and v_0 is the instrument frequency, in MHz. When v_0 is expressed in units of MHz, then Equation 7 is expressed in units as parts per million (ppm). Hence, it is common to use the unit ppm to express the chemical shift difference between the resonance peak of the test substance and that of the reference.

By using this equation, one can use (with appropriate caution) the chemical shift of any known species (such as the residual ¹H-containing species in a deuterated solvent) as a chemical shift reference. This equation, now in common use, is applicable to nearly all nuclei.

Tetramethylsilane (TMS) is the most widely used chemical shift reference for proton and carbon spectra. It is chemically inert, exhibits only one peak (which is more shielded than most signals), and is volatile, which allows ready specimen recovery. Either 2,2,3,3- d_4 sodium 3-(trimethylsilyl)propionate (TMSP) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) is used as an NMR reference for aqueous solutions. The resonance frequency of the TMSP or DSS methyl groups closely approximates that of the TMS signal. Where the use of an internal NMR reference material is not desirable, an external reference may be used, such as a reference standard in a separate NMR tube.

Conventional NMR spectra are shown with shielding increasing and chemical shift decreasing from left to right because less shielded nuclei have higher Larmor frequencies than do more shielded nuclei. Resonances on the left are said to be deshielded (i.e., they show lower electron density). Resonance peaks appearing at the right are termed more shielded (i.e., they show greater electron density) than those appearing at the left. Resonances from the more shielded and the less shielded nuclei often are inappropriately called the high-field or upfield peaks and the low-field or downfield peaks, respectively, as a result of the outdated method of acquiring data by sweeping the magnetic field. Today, the overwhelming majority of spectra are acquired on a pulsed Fourier transform (FT) spectrometer, which sweeps neither the magnetic field nor the transmitter frequency. Therefore, it is more appropriate to refer to resonances at the left side of the spectrum as high-frequency or deshielded resonances and those on the right as low-frequency or shielded resonances.

The coupling between two nuclei can be described in terms of the spin-spin coupling constant, J , which is the separation (in Hertz) between the individual peaks of the multiplet. When two nuclei interact and cause reciprocal

splitting, the measured coupling constants in the two resulting multiplets are equal. Furthermore, J is independent of magnetic field strength.

Coupled spin systems are usually referred to as weak or strong. These terms depend on the separation of the Larmor frequencies of the coupled nuclei compared to the coupling constant between them. Both of these values are easily measured from the spectrum. For a weakly coupled system, the separation expressed in Hz ($\Delta\nu$) is large compared to J , which is always expressed in Hz. Thus, the ratio of the two is dimensionless. For a weakly coupled system, the ratio is large. Typically, spectroscopists consider a ratio above 10 to be weak. Only weakly coupled spin systems produce first-order spectra, which are comparatively easy to analyze. The number of individual peaks that are expected to be present in a multiplet and the relative peak intensities are predictable. The number of peaks is determined by $2nI + 1$, where n is the number of identical nuclei on adjacent groups that are active in splitting and I is the spin of those nuclei causing the splitting. For protons this becomes $(n + 1)$ peaks. In general, the relative intensity of each peak in the multiplet follows the coefficient of the binomial expansion $(a + b)^n$. These coefficients can conveniently be found by use of Pascal's triangle, which produces the following relative areas for the specified multiplets: doublet, 1:1; triplet, 1:2:1; quartet, 1:3:3:1; quintet, 1:4:6:4:1; sextet, 1:5:10:10:5:1; and septet, 1:6:15:20:15:6:1. Two examples of first-order spectra arising from weak coupling are shown in Figure 1.

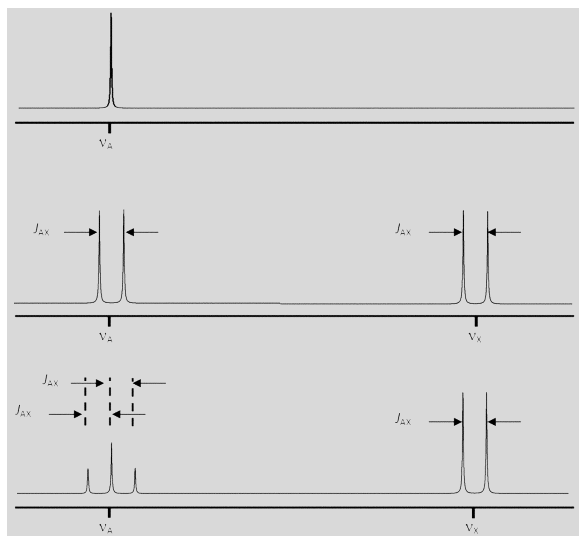


Figure 1. Diagrammatic representation of simple spectra resulting from weakly coupled spin systems.

Coupling may occur between ^1H and other nuclei, such as ^{19}F , ^{13}C , and ^{31}P . This type of coupling can frequently be observed between nuclei separated by 1–5 bonds.

Magnetically active nuclei with $I \geq 1$, such as ^{14}N , possess a nuclear quadrupole moment, which produces line broadening of the signals from neighboring nuclei.

Another characteristic of an NMR signal is its relative intensity, which has wide analytical applications. In carefully designed experiments, the area or intensity of a signal is directly proportional to the number of protons that give rise to the signal. As a result, NMR can be used for quantitation (see *Quantitative Analysis* in this chapter and in *Nuclear Magnetic Resonance, Qualitative and Quantitative NMR Analysis* (761)). NMR spectra may contain spinning side bands,

peaks that appear symmetrically located around each signal. These signals are due to the failure to optimize the off-axis (x and y) shims. The homogeneity of modern superconducting magnets, coupled with computer shimming techniques, reduce the need for sample spinning and completely eliminate these sidebands.

NMR SPECTROMETERS

Introduction

NMR spectrometers have evolved since the first commercial instrument, a Varian HR-30 that operated at 30 MHz, was produced in 1952. Initially NMR spectrometers used a data acquisition technique known as continuous wave (CW), which was based on sweeping the magnetic field. The limitations of a CW spectrometer include low sensitivity and long analysis time.

Today's spectrometers operate at frequencies up to 1 GHz and apply an rf pulse to the sample to produce a time-domain signal known as a free induction decay (FID), which is then Fourier transformed into a frequency-domain signal. This technique is known as FT NMR spectroscopy. Current NMR spectrometers are composed of several key components: the magnet, the probe, the console, and the computer.

The instruments are described by the approximate resonance frequency of the ^1H resonance, e.g., 600 MHz, or by their field strength, e.g., 14.1 T.

The Magnet

Until the early 1970s, NMR magnets were either ferromagnetic-core electromagnets or permanent magnets that operated at field strengths of 1.41–2.35 T, corresponding to ^1H resonance frequencies of 60, 80, 90, and 100 MHz. The first NMR magnets based on superconducting magnets were introduced in the 1960s and allowed access to much higher field strengths that currently are as high as 23.5 T (1 GHz).

The superconducting magnet is the most expensive component of an NMR spectrometer and can cost up to several million dollars. These magnets consist of miles of Nb_3Sn or NbTi wire. When these materials are wound into a solenoid that is immersed in liquid helium at 4.2 K, they become superconducting. That is, an electrical current can be induced in them by an energizing power supply, and that current will persist for many years even after the power supply is removed. This essentially constant electrical current is used to generate high static magnetic fields that can be several times higher than those obtained with ferromagnetic-core magnets. Ensuring that the superconducting coil is immersed in liquid helium at all times is the only maintenance needed to sustain these high fields.

Figure 2 contains a diagram of a typical superconducting magnet. The superconducting solenoid is immersed in a liquid-helium Dewar at 4.2 K. This unit is itself contained in a liquid-nitrogen Dewar at 77.4 K. Each Dewar is surrounded by a vacuum space and reflective film coating to prevent outside heat of the laboratory from entering the helium Dewar. The central core or room-temperature bore provides room for the stack that contains the shim coils for room-temperature shimming. Finally the probe is inserted into the stack. Samples are injected or ejected from the probe by means of a jet of filtered and dried air or nitrogen.

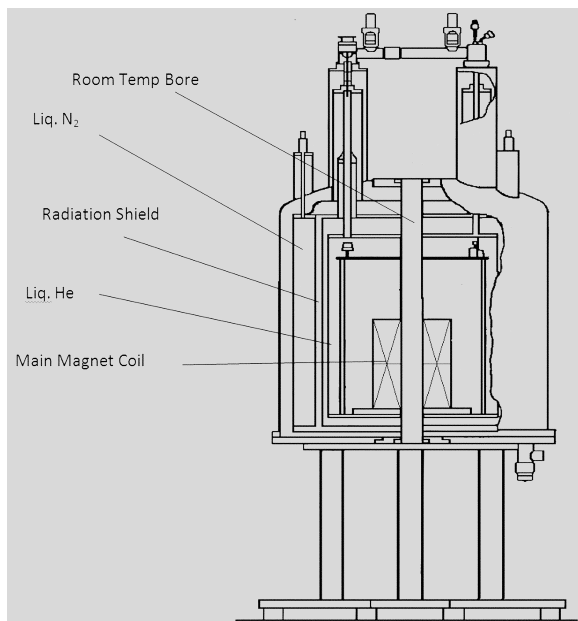


Figure 2. Schematic representation of a superconducting magnet.

An important recent advance in magnet technology has been the construction of shielded magnets with stray fields that can extend only one or two meters from the center of the magnet in three dimensions, thereby making magnet siting a far easier task than it was with unshielded magnets.

In addition to the main solenoid, the helium Dewar also contains several other superconducting coils that are used to shim the main magnetic field as a first step in attaining very high field homogeneity. Further shimming is accomplished by 20–30 shim coils in the room-temperature shim stack that is inserted in the bore of the magnet. These coils operate at ambient temperature and are used to generate small magnetic fields that oppose and cancel inhomogeneities caused by the surroundings, the probe, or the sample itself. Computer software has taken over a large amount of the tedious job of shimming the magnet homogeneity, a critical task for obtaining good NMR data. Using the lock signal from the sample, spectroscopists can generate a field map for each of the shim coils. Using this map, the computer then calculates the amount of current that should be applied to each of the shim coils to maximize the magnet homogeneity. Typically this operation takes less than a minute for shimming the on-axis (z_1 to z_6) shim coils. The off-axis (x/y) inhomogeneities can be compensated for in a similar manner but usually in a longer period of time (15–20 minutes) because of the larger number of off-axis coils.

The Probe

The NMR probe may be the most important part of the instrument. The probe consists of one or two rf coils. Each coil, which is inductive (L), is in a circuit that contains several tunable capacitive (C) elements. These elements are adjusted to enable the probe to transmit and receive at the Larmor frequency, ν_0 . For a given nucleus, the probe tuning is determined by $[\nu = 1/(2\pi(LC)^{1/2})]$. A pulse of rf at the Larmor frequency results in an applied magnetic field (B_1). To induce a transition, B_1 must be applied perpendicular to the static field generated by the magnet superconducting coil. The rf coil not only transmits the excitation pulse but is also electronically switched to receive the rf signal from the sample.

The most common NMR sample tube is the 5-mm (od) NMR tube. However, probes have been designed in many forms. Some probes can accommodate 10- or 20-mm tubes for samples that are not in limited supply, such as petroleum and polymers. For limited amounts of precious samples, probes have been made to accept tubes as small as 1 mm, which can accommodate solutions as small as 5 μ L. Also, flow probes are available to obtain data directly from a liquid chromatography effluent.

Probes are available in a large number of coil configurations. The most common probe usually contains a broadband observe coil that is tunable over a wide range of frequencies (^3P to ^{109}Ag), a decoupler (^1H) coil, and a coil tuned to deuterium (^2H) for field-frequency lock. Usually, the decoupler coil is double-tuned for both ^1H and ^2H . Probe configurations can include as many as four channels for multidimensional work with biological macromolecules.

The probe can come in a format where the X-nucleus observe-coil (e.g., ^{13}C) is wound closest to the sample for highest sensitivity, and the decoupler coil (^1H) is farther away. The inverse configuration is also available to provide the maximum signal-to-noise (S/N) ratio for ^1H , the detected nucleus in two-dimensional (2-D) indirect heteronuclear experiments, such as heteronuclear single-quantum coherence spectroscopy (HSQC) and heteronuclear multiple-bond correlation (HMBC) where the X-nucleus is indirectly detected via the ^1H frequency. Recent advances in probe technology have resulted in a single probe combining the sensitivity of the direct and inverse detection probes described above. Another advancement in probe design is the cryogenically cooled probe wherein the rf coils and their preamplifiers are held at close to liquid helium temperatures (20 K). Because rf electronics generate lower noise levels at colder temperatures, S/N ratios can be increased by at least a factor of four in these probes. Because the S/N ratio of a spectrum is given by $n^{1/2}$, where n is the number of acquisitions, an enhancement by a factor of four to the initial S/N ratio translates to a savings of 16 in time or to a four-fold reduction in sample size.

Probes can also be equipped with gradient coils that can apply a magnetic field gradient in the z only or x , y , and z directions. These gradients are used to study diffusion or, more commonly, to be integral parts of pulse sequences because they provide an efficient means for selecting specific coherences in 2-D experiments.

In addition to the electronic coils, probes usually come equipped with a heater coil that enables variable temperature work from -100° to $+150^\circ$. Probes also have gas outlets to allow sample insertion and ejection from the probe as well as sample spinning.

For solid-state samples the probes come with cylindrical rotors that are filled with the sample and capped. The entire rotor can then be oriented at an angle of 54.74° (known as the magic angle) relative to the magnetic field direction and spun at rates up to 70 kHz. Rapid rotation at this angle helps remove the chemical shift anisotropy and dipolar coupling so that the very broad resonances observed in the solid state can be reduced considerably.

The Console and Computer

The NMR console has the primary function of generating the various Larmor frequencies required for a given experiment, amplifying and transmitting these frequencies to the probe, and detecting the resulting signals that are transmitted from the probe so that they can be used to create an NMR spectrum. In addition to these primary functions, the console performs many more operations, all of which are computer-controlled. Figure 3 contains a schematic diagram illustrating the various components of the current majority of spectrometer consoles.

The computer continually controls the signal to the lock transmitter (A) so the resonance of the lock material can be detected in the lock receiver to maintain field/frequency

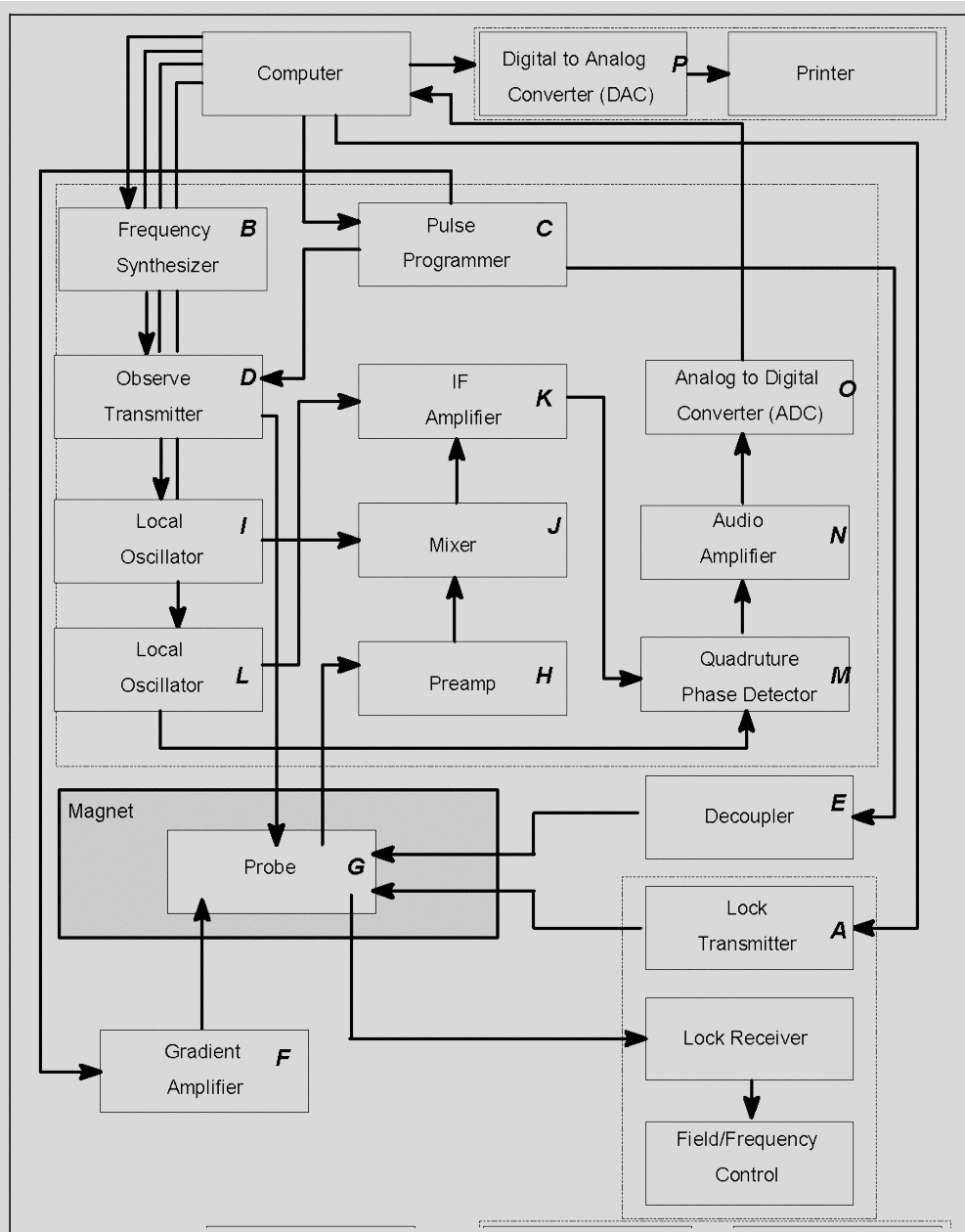


Figure 3. Schematic diagram of an NMR spectrometer.

control. The computer also controls the frequency synthesizer (**B**) that generates the various frequencies that are close to the Larmor frequencies of the observed or perturbed nuclei. For a specific experiment, the computer triggers the pulse programmer (**C**), which, in turn, sends timing signals (pulse width, phase, and shape) to the observer transmitter (**D**), the decoupler (**E**), and the gradient amplifier (**F**), depending on which of these units are needed for that experiment. Once the observed nucleus in the probe (**G**) is excited by a pulse from the observed transmitter, the resultant rf signal is amplified in the preamplifier (**H**). Then the signal is mixed (**J**) with a local oscillator (**I**) to generate a lower rf frequency, called the intermediate frequency (IF) that is further amplified (**K**). A second mixing stage, this time with a different local oscillator (**L**), results in an audio signal that is then detected in quadrature (**M**) before being amplified (**N**), converted (**O**) from an analog to a digital signal, and stored

in the computer. After the signal is processed to create a spectrum, the digital data can be converted back to an analog signal in a digital-to-analog converter (**P**) and printed.

The output from the phase-sensitive detector (**M**) is the free induction decay (FID), which is a time-domain signal, $f(t)$.

When two detectors are used (**M**) with their reference frequencies shifted from each other by 90° , frequencies that are positive with respect to the reference can be distinguished from those that are negative. This system is referred to as quadrature phase-sensitive detection (QPD). Each detector produces an FID, but they will always be 90° out of phase with respect to each other. One FID is called the real FID, and the other is called the imaginary FID. The Fourier transform that is performed in this case is called a complex Fourier transform. The combination of the real and imaginary FIDs yields the frequency spectrum, $F(\omega)$.

$$F(\omega) = \int_{-\infty}^{+\infty} f(t) \exp^{-i\omega t} dt \quad [8]$$

In addition to the Fourier transform process, the computer is also used for post-acquisition processing of the data. The frequency-domain spectrum that results from the complex Fourier transform can then be phased, baseline corrected to remove distortions, integrated to obtain peak areas, and peak picked to provide chemical shift information. The computer is also capable of providing spectra calculated from chemical shift and coupling values, curve-fitting resonances, and deconvoluting complex overlapped peaks. Finally, the digital data can be converted to their analog form by a DAC (O) and printed.

RELAXATION

NMR includes two types of relaxation: *Spin-Spin Relaxation*, sometimes referred to as *Transverse Relaxation*, or T_2 Relaxation, and *Spin-Lattice Relaxation*, sometimes referred to as *Longitudinal Relaxation*, or T_1 Relaxation. At least two mechanisms contribute to *Transverse Relaxation*: loss of signal due to B_0 inhomogeneity and the natural relaxation that would take place even in a perfectly homogeneous field. The combined effects of these two mechanisms produce a new time constant for the relaxation, which is referred to as T_2^* .

Spin-Spin Relaxation (Transverse Relaxation)

After an rf pulse, the component of M_0 in the (x, y) plane, M_{xy} , will gradually decay toward zero. The process is first order, and as it is in other types of first-order processes, the instantaneous rate of decay of M_{xy} is directly proportional to its displacement from equilibrium. The further M_{xy} is displaced from zero, the faster it decays, and as it approaches zero it decays more and more slowly. Hence, Equation 9 applies.

$$dM_{xy}/dt \propto (-M_{xy}) \quad [9]$$

The process is analogous to the decay of a radioactive element. However, spectroscopists do not speak of the rate of decay of an FID in terms of its half-life. Instead, they speak of its 1/e-life, that is, how long it takes for the FID to decay to a value that equals 1/e of its original value at time zero. Standard mathematical manipulations of Equation 9 yield

$$M_{xy} = M_0 \exp(-t/T_2^*) \quad [10]$$

where M_0 is the equilibrium distribution given by Equation 9, and T_2^* is the rate constant for the decay. T_2^* is a measure of how fast the signal decays, not how long it takes to de-

cay. The rate of decay is fastest immediately after the pulse because then it is furthest from its equilibrium position, zero. If the rate remained constant at this initial rate, then the signal would be completely decayed after one T_2^* .

Table 3 was prepared from Equation 10. It shows the percentage of M_{xy} remaining as a function of time after a 90° pulse. In Table 3 time is given as the number of T_2^* s.

M_{xy} asymptotically approaches zero, and it would take infinitely long for complete decay, but the decay is normally considered to be complete when the time has reached 3 to 5 times T_2^* . Hence, these times are commonly used as acquisition times. If acquisition times that are shorter than 3 times T_2^* are used, the FID is truncated and subsequent Fourier transform leads to visible baseline artifacts.

The decay of the FID produces the peak width in the final spectrum. The faster the decay, the broader is the line. The mathematical relationship is:

$$\Delta\nu_{1/2} = 1/(\pi T_2^*) \quad [11]$$

where $\Delta\nu_{1/2}$ is the width of the peak at its half-height.

Spin-Lattice Relaxation (Longitudinal Relaxation)

After an rf pulse, nuclei are excited from the low-energy state into the high-energy state. The nuclei will eventually relax back to establish the Boltzmann distribution (see Equation 5), and this process is called spin-lattice relaxation. The recovery process is first-order, and as it is in other types of first-order processes, the instantaneous rate of growth of M_z is directly proportional to its displacement from equilibrium. The farther M_z is displaced from M_0 , the faster it grows back, and as it approaches M_0 it grows back more and more slowly. Hence, Equation 12 applies.

$$dM_z/dt \propto (M_0 - M_z) \quad [12]$$

Standard mathematical manipulations yield

$$M_z = M_0(1 - \exp(-t/T_1)) \quad [13]$$

Note that T_1 is a measure of how fast M_z grows back to M_0 —it is not how long it takes to grow back. Table 4 was prepared from Equation 13 and shows the percentage of recovery of M_z as a function of time after a 90° pulse. In this table, time is given as the number of T_1 s.

As M_z asymptotically approaches M_0 , it takes infinitely long for 100% recovery. However, recovery is normally considered to be complete when it has reached 99%. Hence, relaxation delays of $5T_1$ are commonly used in pulse sequences. The rate of return to M_0 is fastest immediately after the pulse because that is when M_0 is farthest from its equilibrium position. If the rate remained constant at this initial rate, then full recovery would be achieved after one T_1 .

Table 3. Percent of M_{xy} Remaining as a Function of Time, in Units of T_2^*

Time/ T_2^*	0.0	0.5	1.0	1.5	2.0	3.0	4.0	5.0
% Remaining	100.0	60.7	36.8	22.3	13.5	5.0	1.8	0.7

Table 4. Percent Recovery of M_z as a Function of Time, in Units of T_1

Time/ T_1	0.0	0.5	1.0	1.5	2.0	3.0	4.0	5.0
% Recovery	0.0	39.3	63.2	77.7	86.5	95	98.2	99.3

TIP ANGLE

During an rf pulse, a magnetic field (B_1) is applied to the sample. The magnetization vector M precesses about B_1 according to

$$\omega_1 = \gamma B_1 \quad [14]$$

where ω_1 is the precessional frequency and B_1 is the strength of the magnetic field applied to the sample. During the time that the pulse is applied, M precesses to an angle (α) given by the precessional rate multiplied by the width of the pulse, in time, (PW). Then,

$$PW \times \omega_1 = \alpha = PW \times \gamma B_1 \quad [15]$$

Tip angles typically are expressed in units of degrees or in radians. For example, a 90° pulse is sometimes referred to as a $\pi/2$ pulse.

Optimum Tip Angle or Ernst Angle

Time averaging to improve the S/N ratio is accomplished by a delay-pulse-acquire sequence that is repeated as necessary. Suppose that during the first pulse, M_0 precesses 30° about B_1 before the pulse is turned off. At this time the magnitude of M_z is $M_0 \cos 30^\circ$. At the end of the pulse, M_z will begin to grow back toward its equilibrium value, M_0 . Typically, the second pulse is applied before M_z reaches M_0 , driving M_z down even farther away from M_0 . Consequently, M_z begins to grow back faster because it is even farther away from its equilibrium value. After 6–10 pulses, M_z will grow back an amount that is equal to the incremental displacement caused by each succeeding pulse and will reach a new equilibrium or steady state. Each succeeding pulse continues to tip this new steady-state value of M_z by 30° , resulting in signal intensity for each pulse.

The position of the steady-state equilibrium is determined by three factors: the T_1 , the tip angle, and the time between pulses. On the one hand, for a given T_1 and time between pulses, if the tip angle is too large then the steady-state value of M_z will be close to the origin, affording only a small signal. On the other hand, if the tip angle is too small, e.g., 5° , then the steady-state value will be large, but with a small angle the value of $M_z \sin 5^\circ$ will also be small, again affording a small signal. The optimum angle is frequently called the Ernst angle, which is given by:

$$\cos \alpha_{\text{opt}} = \exp(-PR/T_1) \quad [16]$$

$$\alpha_{\text{opt}} = \arccos[\exp(-PR/T_1)] \quad [17]$$

where PR is the time between pulses, or pulse-repetition time. This time is the sum of the acquisition time used to collect the FID plus any relaxation delay used. This angle provides a reasonably large steady-state value combined with a reasonably large angle and will produce the best S/N ratio per unit time. The value of T_1 to be used in Equation 17 should be for the longest relaxing nucleus in the molecule.

RELAXATION DELAY

Surprisingly, the optimum S/N ratio per unit time is obtained when no relaxation delay is used for a given T_1 . That is when PR equals to AT , the acquisition time. However, because the nuclei in most molecules do not have the same T_1 values, the relative intensity relationship will be lost.

For typical quantitation experiments, a relaxation delay is used and should be at least five times the longest T_1 expected for any of the nuclei in the molecule; and the pulse width should be set to 90° . Further details are provided in the section *Quantitative Applications*.

RESOLUTION

In NMR spectroscopy the typical definition of resolution is the ability to distinguish between two closely spaced resonance peaks in a spectrum. The industry standard for measuring resolution is to measure the width of a single peak, in units of Hz, at the half-height of the peak.

The uncertainty principle determines the best resolution that can be achieved in an NMR spectrum. The maximum resolution possible, or the minimum separation that can be observed between two frequencies, $\Delta\nu$, in a spectrum is equal to the reciprocal of the acquisition time, AT , of the FID.

$$\Delta\nu = 1/\Delta t = 1/AT \quad [18]$$

The time set by the spectrometer operator should not exceed the required AT , because this would result in the collection of only noise after the signal has decayed to near zero. Collecting this noise does not improve the resolution.

POSTACQUISITION DATA PROCESSING

The final appearance of the spectrum can usually be improved by applying a variety of mathematical procedures to the FID before the Fourier transform is performed. The two most common procedures are multiplying the FID by a mathematical function, generally known as a *window function*; or appending zeros to the end of the FID, generally known as *zero filling*.

Window Functions

Two types are generally used: one for increasing the resolution and another for increasing the S/N ratio.

INCREASING THE RESOLUTION

The decay of the signal produces a peak width in the spectrum, and if this decay can be removed, then the resonance peak would consist of a single point, i.e., an infinitely narrow peak. The decay of the signal can be represented by $\exp(-t/T_2^*)$. Hence the full equation representing the decaying signal is

$$A(t) = A_0 \exp(-t/T_2^*) \cos(\omega t + \theta) \quad [19]$$

If the FID is multiplied by an increasing function that exactly cancels the decay, then the peak width will be removed. This can be achieved by multiplying the FID by $\exp(t/T_2^*)$. Then Equation 19 becomes

$$A(t) = A_0 \exp(0) \cos(\omega t + \theta) = A_0 \cos(\omega t + \theta) \quad [20]$$

Unfortunately, the application of this function as described above will also disproportionately increase the noise power at the tail of the FID. The S/N ratio in the final spectrum is so poor that this function is not used without modifications. Typically, the beginning of the FID, where the S/N ratio is better, is multiplied up but then the tail of the FID, where S/N ratio is poorer, is multiplied down. Two commonly used functions that accomplish this are the Gaussian function and the transform of reversed-added FIDs (TRAF) function for resolution. The latter is sometimes given the name TRAFR by instrument manufacturers. It affords the same resolution enhancement as the Gaussian function but with much less degradation in overall S/N ratio. Numerous other window functions have been proposed though not always widely used. It should also be noted that in the quantitative experiments increasing the resolution should be used with caution because it may change the accuracy of signal integration in the spectrum.

INCREASING THE S/N RATIO

The overall S/N ratio in the spectrum can be increased by weighting the points at the beginning of the FID more highly than at the tail. This is because the S/N ratio is the highest in the beginning and the lowest at the tail. The weighting is often accomplished by multiplying the raw FID by a function that decreases with time. A popular function that gives the greatest increase in the S/N ratio is called the *matched filter*. It weights each point in the FID by an amount proportional to the S/N ratio at that point. A function that accomplishes this must match the decay. Hence, the FID is multiplied by $\exp(-t/T_2^*)$.

The penalty for the use of the matched filter is a loss of resolution that equals a doubling of the peak width. When the original decay is multiplied by the matched filter, then the new decay is given by the following:

$$\frac{\exp(-t/T_2^*) \times \exp(-t/T_2^*)}{\exp(-2t/T_2^*)} = \exp(-t/0.5T_2^*) \quad [21]$$

The FID then appears to have decayed with a T_2^* equal to one-half of the original, and according to *Equation 11* and *18* the peak width will double. Multiplying the FID by a steeper decay in an attempt to weight the beginning points more results only in less of an increase in S/N ratio and a greater increase in peak width.

Another function that accomplishes the same increase in S/N ratio, but without any change in resolution, is the TRAF function for sensitivity, which is sometimes given the name TRAFS by instrument manufacturers.

Zero Filling

Spectroscopists can improve the overall appearance of the spectrum considerably by appending zeros to the end of the FID before the Fourier transform is performed. This process results in placing more points on every resonance peak in the spectrum. The most common procedure is to append a number of zeros equal to the number used to collect the FID. Adding more zeros will result in only a very slight further improvement.

Although all of the peaks are better defined with zero filling, the resolution is not increased. For example, in a case where the separation between two lines is closer than the peak widths, only a single broadened line will result. Zero filling will not resolve these peaks—it will only place more points on an already broadened line. Only an increase in the acquisition time of the signal or the use of a window function could resolve the peaks.

Zero filling has a beneficial effect on quantitation. Integration of a digital spectrum is accomplished by taking the intensity at a given point and adding to it the intensity at successive points. If the number of data points is insufficient to depict the actual peak shape, the resultant integral of that peak will not be accurately determined. Therefore, zero filling until each peak is represented by at least 7–10 data points results in a more accurate integration. To obtain reliable peak representation and quantitative peak integration there should be at least 4–5 data points above the full width at half height of a peak.

GENERAL PROCEDURE FOR STRUCTURE IDENTIFICATION

NMR spectroscopy is a powerful technique for structure identification because of its specificity of detecting certain nuclei such as ^1H , ^{13}C , ^{31}P , and ^{19}F . Typically, a routine identification test can be performed by ^1H NMR spectroscopy in a short period of time for simple molecules. The basis for identification is provided by a comparison of the signals from the test sample with the expected signals from a qualified reference standard. A positive identification can be con-

cluded when the chemical shifts, multiplicities, and coupling constants of the spectrum of the test sample match those of the reference standard or, in the case of a *USP* monograph, the values listed in the monograph.

Data may be made unacceptable for analysis if incorrect sample preparation or poor adjustment of spectrometer parameters leads to poor resolution, decreased sensitivity, and spectral artifacts. It is preferable that the operator be familiar with the basic theory of NMR and operation of the spectrometer. Frequent checks of instrument performance are essential.

The procedures discussed here refer specifically to ^1H and ^{13}C NMR, but they are applicable, with modification, to other nuclei. The discussion assumes that the NMR spectra are obtained from solutions in suitable solvents.

Selection of Solvent

Deuterated solvents are usually used to prepare solutions for NMR analysis because they are readily available, have greatly reduced ^1H signals from solvents in ^1H spectra, and have the added advantage of providing a lock signal. Select a solvent whose residual ^1H signals will not interfere with signals of the analyte. If a residual ^1H solvent peak might interfere with any signals from the sample solution and another solvent is not possible, then the ^2H isotopic purity of the solvent should be as high as possible. Some solvents (e.g., D_2O or CD_3OD) have labile protons that can enter into fast exchange reactions with the labile protons in the analyte. This may eliminate resonance signals from $-\text{COOH}$, $-\text{OH}$, and $-\text{NH}_2$ structural groups. The most commonly used solvents for proton and carbon NMR are listed in *Table 5* along with their residual ^1H and ^{13}C chemical shifts as well as the multiplicities of these resonances caused by coupling to deuterium.

Table 5. Solvents Commonly Used for $^1\text{H}/^{13}\text{C}$ NMR Chemical Shifts

Solvent	Residual $^1\text{H}/^{13}\text{C}$ Signal ($\delta^{\text{a,b}}$) and Multiplicity	
	^1H	^{13}C
CDCl_3	7.27	77.23 (3)
CD_3OD	3.35, 4.78	49.15 (7)
$(\text{CD}_3)_2\text{CO}$	2.05	206.68 (1) 29.92 (7)
D_2O	4.7 ^c	—
$(\text{CD}_3)_2\text{SO}$	2.50	39.51 (7)
C_6D_6	7.20	128.39 (3)
Dioxane- d_8	3.55	66.66 (5)
$\text{CD}_3\text{CO}_2\text{D}$	2.05, 11.65 ^c	178.99 (1) 20.0 (7)
$(\text{CD}_3)_2\text{NCDO}$	2.77, 2.93, 8.05	163.15 (3) 34.89 (7) 29.76 (7)

^a Chemical shifts were measured at 295 K.

^b δ in ppm relative to TMS at 0 ppm.

^c Labile hydrogen.

Sample Preparation

For *USP* procedures, directions are usually given in individual monographs. The solute concentration depends on the objective of the experiment. Typically, NMR sample solutions are prepared so that they contain from a few to 50 mg/mL. Detection of minor contaminants may require higher concentrations. In some cases such as polymers, even higher concentrations can be used. The solutions are prepared in separate vials and are transferred to the NMR tube. The volume required depends on the size of the NMR tube and on the geometry of the probe. The level of the solution

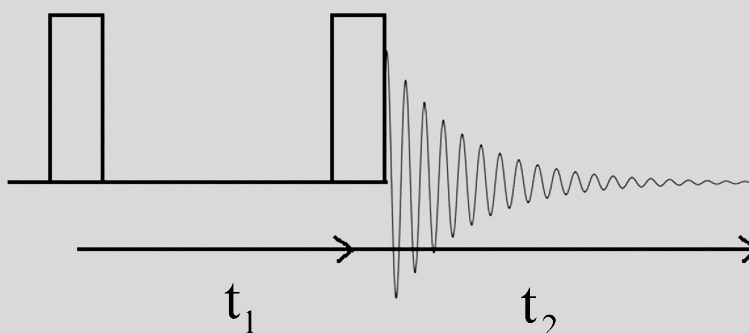


Figure 4. Pulse sequence for a COSY experiment.

in the tube must be high enough to extend beyond the coils when the tube is inserted in the instrument probe.

The NMR tubes must meet narrow tolerance specifications in diameter, wall thickness, concentricity, and camber. The most widely used tubes have a 5- or 10-mm outside diameter (OD) and a length between 15 and 20 cm, but 1- and 3-mm (OD) NMR tubes are becoming more common, and tubes as large as 20 mm (OD) have been used.

Procedure

The NMR tube is placed in a probe located in the magnetic field. Although samples traditionally have been spun to average the nonradial field gradients, the quality of the shim coils no longer makes spinning a requirement, and, in the case of many 2-D experiments, the sample should not be spun. The magnetic field's homogeneity is optimized by shimming, a function that is largely being taken over by the computer in most modern spectrometers. Probe tuning is optimized for the frequency being observed and is matched to the impedance of the spectrometer.

The computer serves to control all operations of the spectrometer from running the pulse program to storing and processing the data. The experimental setup involves selecting values for a large number of variables, including the spectral width to be examined, the duration of the excitation pulse (PW), the time interval over which data will be acquired (AT), the number of transients to be accumulated, and the delay between one acquisition and the next (relaxation delay). The acquisition time for one transient is on the order of seconds. The number of transients is a function of the specimen concentration, the type of nucleus, and the objective of the experiment and can vary from a few for most ^1H experiments to several thousand for ^{13}C spectra. At the end of the experiment, the signal (FID) is stored in digitized form in the computer memory and may be displayed on the monitor. The signal can be processed mathematically to enhance either the resolution or the sensitivity, and it can be Fourier-transformed into a frequency-domain spectrum, which can be further analyzed to obtain peak positions (chemical shifts) and intensities.

Structure Elucidation by NMR

The simplest case of using NMR spectroscopy to elucidate an unknown structure is to obtain a match with a spectrum from a reference standard or from a database. The informational content of an NMR spectrum is sufficient for deduc-

ing structures of organic molecules even when qualified reference standards or spectra are not available. Relatively simple structures can be identified using chemical shifts, coupling patterns, and intensities obtained from one-dimensional (1-D) ^1H and ^{13}C spectra. For more complex structures, spectroscopists may have to obtain two-dimensional (2-D) spectra from experiments that have been developed to determine homo- or heteronuclear connectivities.

Two-dimensional spectra are characterized by two frequency axes. The intensity, which is mathematically another dimension, is not considered to be a dimension in 2-D NMR because it is not an axis that spreads out the chemical shift. All modern 2-D experiments consist of at least two pulses separated by a time period, labeled t_1 , the evolution period, and a period of time used for collecting the signals, labeled t_2 , the detection period. A CORrelation SpectroscopY (COSY) sequence is the simplest of all and is shown in Figure 4.

A series of such sequences is performed using an incremental increase in the evolution period. Fourier transforms of the FIDs produced during each of the t_2 detection periods are stacked, a second Fourier transform is performed along this t_1 new axis, resulting in a plot of amplitude along two frequency axes, F_2 and F_1 .

The next simplest addition to the COSY sequence is another pulse. The Nuclear Overhauser Effect SpectroscopY (NOESY) sequence is an example and is shown in Figure 5.

The evolution period is again the time between the first two pulses and is incremented as in the COSY experiment. The time between the second and third pulses is fixed and is not incremented. This period of time is labeled τ_m and is called the mixing time. Cross-polarization occurs during this period.

The COSY and NOESY sequences described above are the two simplest 2-D experiments to perform. Many other experiments have been developed and contain a very complicated series of pulses with different pulse widths, time delays, gated decoupling, and pulsed field gradients. Some of these experiments are described below.

Strategies for Establishment of Homonuclear Connectivities

Assigning signals based on chemical shifts, spin multiplicity, and coupling constants serves as a starting point for structural elucidation. Structure elucidation is simplified if one can establish molecular connectivity between homonuclear spins. This can be done using correlations via

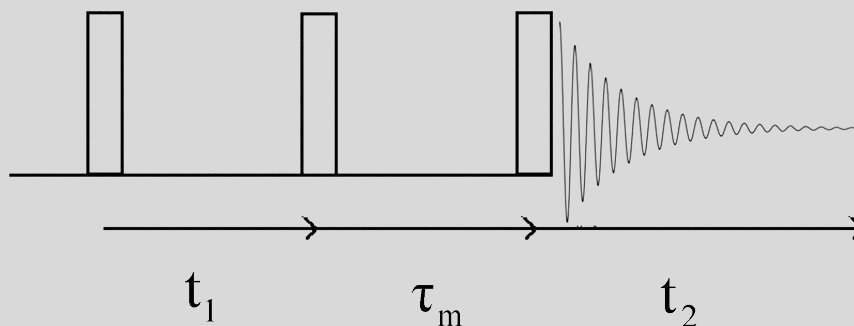


Figure 5. Pulse sequence for a NOESY experiment.

bonds (scalar coupling, sometimes referred to as J coupling) or via spatial (dipolar coupling) interactions. This section describes NMR techniques that can be used for the study of homonuclear connectivity. Popular experiments in this category are COSY, total correlated spectroscopy (TOCSY), NOESY, and rotating frame Overhauser effect spectroscopy (ROESY).

COSY

COSY has become a routine 2-D ^1H NMR experiment that can quickly provide the proton–proton connectivity for spin systems connected by two or three chemical bonds. In a COSY spectrum, the contour plot typically shows diagonal and off-diagonal cross-peaks. The diagonal peaks correspond to the places in 2-D space where chemical shifts of the same nucleus in the 1-D ^1H NMR spectrum intersect. The off-diagonal cross-peaks occur in 2-D space where the chemical shift of one nucleus intersects the chemical shift of a different nucleus to which it is coupled. The COSY spectrum enables the identification of scalar-coupled spins that are in geminal or vicinal positions. Generally, one starts the assignment process by selecting a resonance in the COSY spectrum that has already been identified in the 1-D ^1H NMR spectrum. Then, the off-diagonal peaks between this resonance and any others determine the resonances of the neighboring protons to which it is coupled. The neighboring protons identified in this way then serve as the next points to examine for cross-peaks to other protons, and so forth. The process continues until all coupled spin systems are identified.

Thus, a COSY experiment can provide useful information about proton–proton connectivity for various fragments of the molecules under examination. The relationship between these fragments may be difficult to establish because of an interruption of the coupling between the two fragments. For instance, if two segments are connected through quaternary carbons or heteronuclei, the very small four-bond coupling between two protons on the two separate fragments usually cannot be detected by COSY. Basic COSY experiments are usually processed using magnitude mode, which results in broad bases to the peaks. The diagonal peaks may be so broad that spin systems with close chemical shifts may not be observable because the broadness of the diagonal peaks will cause them to overlap the off-diagonal peaks. An example of a COSY experiment, specifically a gradient, or gCOSY, is shown in Figure 6.

A variety of COSY experiments have been devised to improve upon the original experiment. Perhaps the most important change has been to use gradients to acquire a gCOSY spectrum. The classic COSY experiment uses a 90°

pulse to generate transverse magnetization and relies on elaborate phase cycling to cancel unwanted signals over many scans, resulting in long experiment times. In a gCOSY experiment, which requires a probe capable of a pulsed field gradient (PFG), a magnetic field gradient pulse dephases any coherent magnetization in the xy plane. If a second gradient pulse of the proper strength is applied in the opposite direction, it will cause any dephased double-quantum magnetization to refocus. Hence, only those signals will be received. The strengths of the gradients can be used to select single-, double- or triple-quantum coherences.

The gradient pulses in a gCOSY experiment can be used to prevent the refocusing of magnetizations that cause the artifacts in the classic COSY experiment. Hence, instead of requiring a minimum of eight phase-cycled acquisitions for each data increment in the second dimension, a gCOSY spectrum requires only a single acquisition per increment. For samples that are sufficiently concentrated to produce an acceptable S/N in only one acquisition, this greatly shortens the experimental time.

Phase-sensitive COSY experiments have been developed to overcome the problem of overlap with closely spaced chemical shifts. A phase-sensitive COSY results in pure absorptive peaks with narrower peaks than are generated by the classic-magnitude COSY. These narrower peaks allow better resolution of resonances that are close to diagonal peaks.

Double-quantum filtered COSY (DQF-COSY) was developed to overcome the problem caused by intense signals from functional groups such as methyls. The singlets from these groups do not provide useful connectivity information, but their intensity often limits the dynamic range of the experiment, making it difficult to observe other weaker signals. The pulse sequences of DQF-COSY detect the spin systems that have only double-quantum transitions. Isolated singlets are not selected and thus are filtered out of the final 2-D spectrum. In addition, a reduction in the overall intensity of the diagonal signals is achieved with an increase in the intensities of off-diagonal signals.

TOCSY (OR HOHAHA—HOMONUCLEAR HARTMANN HAHN)

The ^1H – ^1H TOCSY experiment is closely related to COSY but differs because it yields correlations for every spin in a coupled network. This is especially useful when multiplets overlap or there is extensive strong coupling. For example, consider the network $-\text{CHa}-\text{CHb}-\text{CHc}-\text{CHd}-\text{CHE}-$, where each CHn stands for a spin that is coupled through three bonds to the adjacent spin. A COSY spectrum would show correlations for each adjacent pair of hydrogens. On the one hand, the Hb resonance would show connectivities to Ha

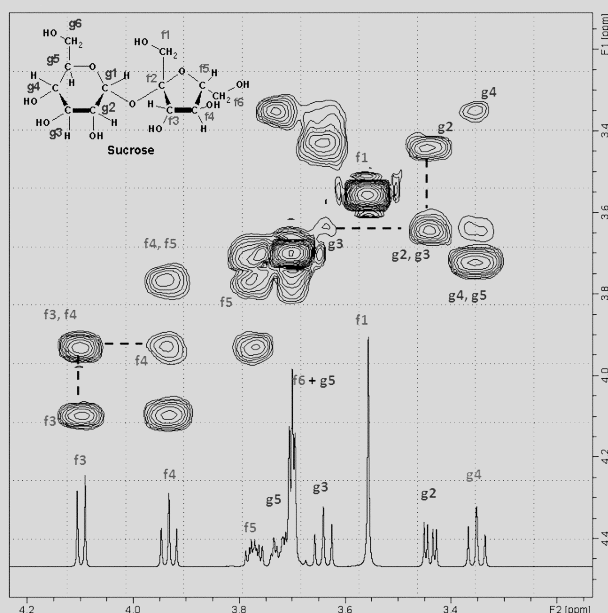


Figure 6. Partial COSY spectrum of sucrose. Dotted lines show how two off-diagonal (f3, f4 and g2, g3) contours are used to determine which nuclei are coupled to each other.

and Hc but not to Hd. A partial correlation is revealed for each CHn. On the other hand, a TOCSY spectrum would show all off-diagonal contours for every spin in this network. That is, for every peak in this coupling network there would be off-diagonal contours corresponding to CHa, CHb, CHc, CHd, and CHe. Thus, a TOCSY spectrum, such as the one shown in Figure 7, affirmatively identifies all of the spins within the same coupling network. This pattern is easily recognized, especially when there is extensive overlap with other coupled networks. However, a TOCSY experiment cannot establish connectivity between separate networks that are interrupted by heteronuclear atoms, quaternary carbons, or a carbon bearing only exchangeable protons. A TOCSY experiment is useful for the study of large molecules with many separated coupling networks such as peptides, proteins, oligosaccharides, and polysaccharides.

NOESY

The NOESY experiment gives correlations between protons that are close to each other in space even though they may not be connected by bonds. These through-space correlations are made via spin-lattice relaxation. Dipole interactions between protons close in space generate NOE transfers, and the magnetization is aligned along the z axis (B_0), producing positive or negative intensity changes that yield cross-peaks that are not normally observable in a COSY spectrum. The sign of the NOESY peaks depends on the size and mobility of the molecule under study. Used in combination with other techniques, a NOESY experiment can establish spatial relations for particular spins and can provide critical information about ring structures and conformations.

^1H – ^1H ROESY

The ROESY experiment is similar to a NOESY experiment insofar as it also provides correlations between protons that are close to each other in space, whether or not they are connected via bonds. A ROESY spectrum yields through-space correlations via spin-spin relaxation in the rotating frame. The ROESY experiment utilizes a spin-lock sequence as a mixing time during which NOE transfer occurs among

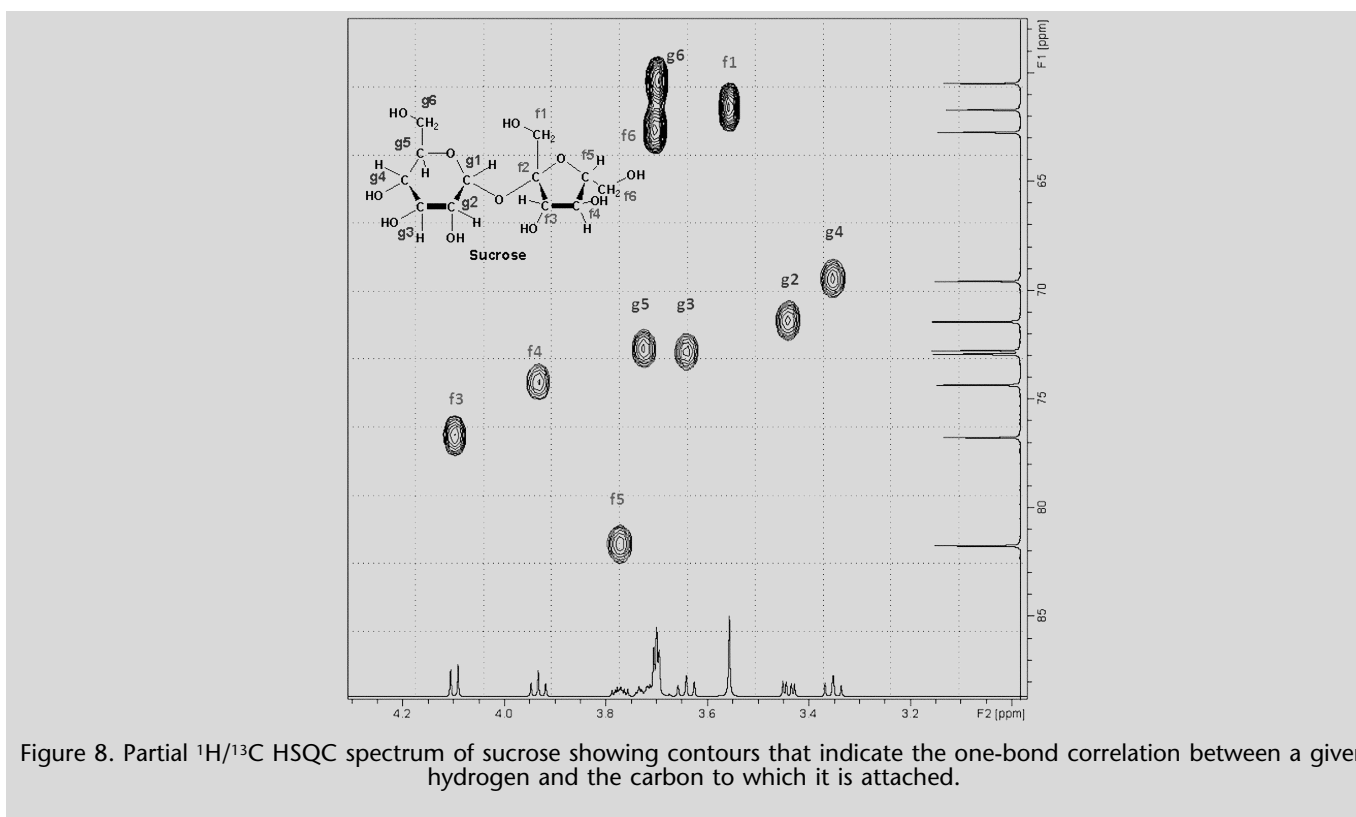
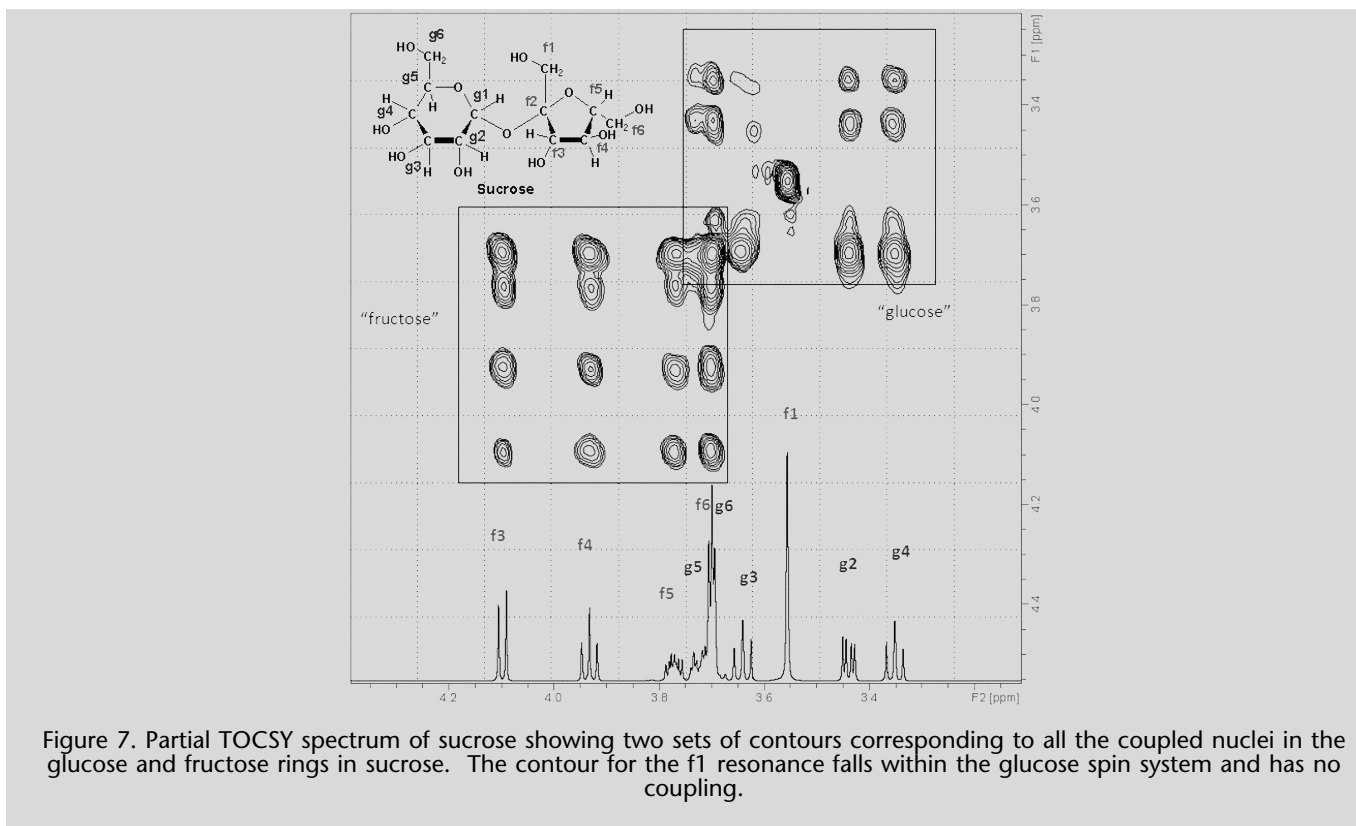
all components of the spins locked in the xy plane. In contrast to a NOESY experiment, in which NOE transfer occurs while magnetizations are aligned along the z axis (B_0) producing positive or negative intensity changes, the ROESY experiment depends on NOE transfers occurring in the rotating frame under the influence of a B_1 magnetic field. This always results in positive signals no matter how large the molecule or whether its motion is fast or slow. Therefore, a ROESY experiment frequently will provide through-space correlations when the same correlations in a NOESY experiment cannot be detected because of molecule mobility.

INCREDIBLE NATURAL ABUNDANCE DOUBLE QUANTUM TRANSFER EXPERIMENT

The Incredible Natural Abundance Double Quantum Transfer Experiment (INADEQUATE) uses a double quantum coherence to provide information about ^{13}C nuclei directly coupled to other ^{13}C nuclei. Thus it provides the same sort of information that is available using a COSY experiment for proton couplings. Because of the low probability of two ^{13}C nuclei being attached to each other (only 1 in 10,000 molecules), this technique is usually one of last resort. The ever-increasing sensitivity of cryogenically cooled probes used in modern instrumentation makes this experiment practical in some cases.

Strategies for Establishment of Heteronuclear Connectivities

Although homonuclear ^1H – ^1H connectivity is one important aspect of structure elucidation of organic molecules, the establishment of heteronuclear connectivities is equally important, although somewhat more difficult to obtain given the lower abundance of most heteroatoms. If one has partial assignments in either the ^1H or ^{13}C spectrum, the knowledge of this connectivity leads to a much fuller assignment of both spectra. Heteronuclear 2-D spectra do not exhibit a diagonal as is seen in homonuclear correlations. Rather, cross-peaks occur at the point of intersection of the



^1H and ^{13}C chemical shifts in the 2-D space as shown in Figure 8.

Heteronuclear 2-D spectra are designed so that ^1H is the detected nucleus and are usually acquired using inverse-

detection probes because the ^1H coil is wound closer to the sample than the broadband coil. This results in a better filling factor and a greater sensitivity for the ^1H coil. It should be noted that a newer configuration of coils has been devel-

oped that provides the same sensitivity but does not utilize the inverse-detection coil arrangement. Typical heteronuclear 2-D experiments include Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple Quantum Coherence (HMQC), and Heteronuclear Multiple Bond Correlation (HMBC) experiments.

HMQC

The 2-D HMQC experiment provides information about correlation between protons and their attached heteronuclei via the heteronuclear scalar coupling. The sequence selects double quantum coherence transfer between scalar-coupled spins (^{13}C - ^1H).

HSQC

HSQC spectroscopy is also an inverse chemical shift correlation experiment that yields the same information as HMQC, i.e., the identification of directly bonded hydrogen-carbon interactions. The correlation between heteronuclei is detected via the selection of single quantum coherence transfer using the insensitive nuclear enhancement by polarization transfer (INEPT) sequences. The main advantage of using this sequence instead of the HMQC sequence is that the F_1 domain does not contain any proton-proton couplings. Hence, the resolution is improved.

An interesting modification of this sequence is an edited HSQC experiment. This is a phase-sensitive experiment that not only gives one-bond correlations between hydrogen and carbon but also gives methyl and methine correlation peaks that are 180° out of phase with methylene resonances.

HMBC

HMBC spectroscopy is a modified version of HSQC and is suitable for determining long-range (> 1 -bond) ^1H - ^{13}C connectivity. Long-range heteronuclear correlation spectroscopy can yield signals for those nuclei that are separated by 2-4 bonds. This experiment, in conjunction with the other 2-D experiments discussed above, allows one to define the structure of a molecule in great detail.

QUANTITATIVE APPLICATIONS

NMR is one of the most useful techniques for quantitative analysis in chemistry. If appropriate experimental conditions are chosen, the relative intensities of resonances are proportional to the population of the nuclei causing those resonances. NMR experiments can be designed for relative or absolute quantitation, either with an internal standard or without one.

Experimental Design for Quantitative NMR

Design for quantitation involves the elimination or precise measurement of differences in intensities due to spin-lattice relaxation and NOE. The spin-lattice relaxation time (T_1) for all resonances used in the procedure can be measured with an inversion recovery pulse sequence. If a 90° pulse is used for excitation, quantitation at the level of 99.3% may be achieved with a recycle time, T_r (the sum of the relaxation delay and acquisition time), of $5 \times T_1$, and improved to even higher levels by using longer recycle times or shorter pulses. The general equation for the degree of quantitation, Q , of a resonance as a function of the pulse angle, α , and T_r , and T_1 is given by:

$$Q = \frac{1 - e^{-T_r/T_1}}{1 - [e^{-T_r/T_1} \cdot \cos(\alpha)]} \quad [22]$$

If a 45° excitation pulse is used, with a $T_r = 5T_1$, $Q = 0.998$. However, the accuracy of quantitation in the final spectrum depends not only on the Q values of the resonances but also on the accuracy of the integration method and on the S/N ratio in the spectrum. Hence, Q values somewhat less than unity may be warranted and other angles and recycle times should be used.

The minimization of systematic quantitation bias should be sufficient for the intended use of the procedure. Alternatively, quantitative procedures may be developed using conditions for which Q is not unity for some or all resonances, provided the value of Q is precisely known and corrected for.

For quantitative methods using heteronuclei, the possibility of differential NOEs should be avoided by using a T_r at least 5 times the longest T_1 value and by using inverse gated decoupling (decoupler gated on only during the acquisition time). Preferably, 90° pulses should be used. For quantitative methods, relaxation agents are often used for shortening the T_1 values of heteronuclei.

The reproducibility of an NMR method depends on a variety of acquisition and processing parameters, all of which should be described in the procedure. These include pulse angle, acquisition time, relaxation delay, spectral width, number of points in the FID, number of acquisitions, number of points used (if any) for zero filling, line broadening, baseline correction, integral breaks, and temperature. For best reproducibility, integral breaks should be specified to 0.01 ppm for ^1H NMR methods and to 0.1 ppm for ^{13}C methods.

Quantitative analysis, as well as detection of trace impurities, has markedly improved with modern instrumentation. Stronger magnetic fields and improved probe technology have enhanced the sensitivity of NMR procedures in recent years.

SOLID-STATE NMR

The analytical usefulness of solid-state NMR spectroscopy for studying solid materials lies in the fact that the same types of nuclei in different *solid-state* environments exhibit different resonance frequencies. Applications of this technique in pharmaceutical analysis range from solid form (polymorph, solvate) identification and quantitation in bulk drug substances to physical/chemical profiling of dosage forms. The technique has the unique ability to probe electronic environments of specific nuclei in the solid state over a large timescale without the requirement of single-crystal substrates or even homogeneous samples. Methods and procedures presented herein are directed at observing ^{13}C , the most popular NMR nucleus for solids. The concepts may be equally applied to other relevant spin-1/2 nuclei such as low-natural-abundance ^{15}N as well as high-natural-abundance ^{31}P .

Cross-Polarization Magic Angle Spinning (CPMAS) NMR Technique

The basic principles of NMR are the same for solution and solid-state measurements, but conventional solution-phase 1-D NMR data acquisition techniques do not normally produce detectable spectra for solid samples because of low sensitivity and extensive line broadening. The sensitivity of the solid-state experiment is low for ^{13}C based on its 1.1% natural abundance and long spin-lattice (T_1) relaxation times. Line broadening arises primarily from dipole-dipole interactions and chemical shift anisotropy (CSA), which are not averaged to zero because of the fixed orientation of molecules in a packed solid sample vs the rapid molecular

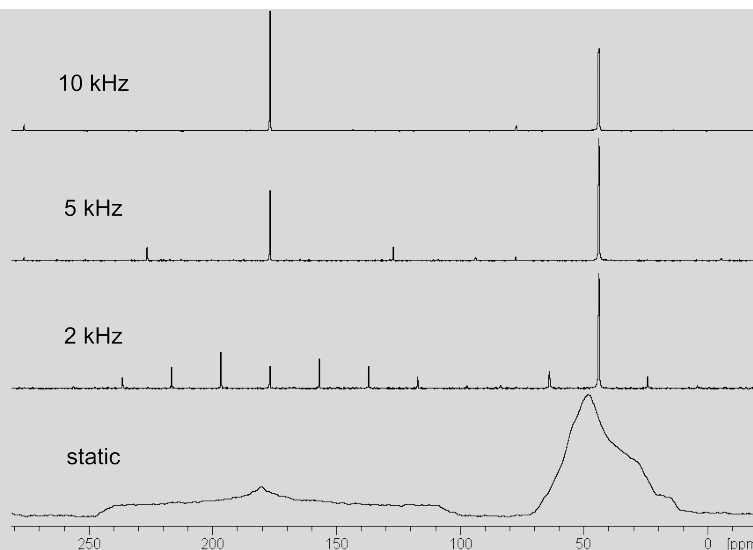


Figure 9. ^{13}C CPMAS NMR spectra of polycrystalline α -glycine collected at different sample spinning (MAS) rates. Spinning sidebands appear in solid-state NMR spectra when the sample spinning frequency is less than the width of the CSA pattern.

tumbling of the molecules when they are in solution. If not averaged, CSA results in the simultaneous observation of all different orientations of molecules with respect to the applied magnetic field. CSA patterns may span the width of an entire liquid spectrum. Three modifications to standard solution methods—cross-polarization (CP), magic angle spinning (MAS), and high-power ^1H decoupling—are routinely used in combination to obtain high-resolution solid-state NMR spectra.

CROSS-POLARIZATION (CP)

CP addresses the low sensitivity associated with collecting NMR spectra of dilute spin-1/2 nuclei such as ^{13}C . CP is a double-resonance procedure wherein abundant ^1H and rare ^{13}C spins are brought into resonance by simultaneously applying two spin-locking rf fields ($B_{1\text{H}}$ and $B_{1\text{C}}$), the magnitude of which will satisfy the Hartmann–Hahn matching condition,

$$\gamma_{\text{H}}B_{1\text{H}} = \gamma_{\text{C}}B_{1\text{C}} \quad [23]$$

During this contact time, polarization transfer occurs allowing the rare ^{13}C spins to take on the magnetization and relaxation behavior of the abundant ^1H spins, leading to a sensitivity enhancement (up to four-fold based on the ratio of the ^1H and ^{13}C magnetogyric ratios) and a reduction in the pulse repetition time. Reducing the pulse repetition time allows a greater number of acquisitions to be accumulated per unit time, which yields a better S/N ratio. In instances where it may be difficult or impossible to record CP spectra because of weak ^{13}C – ^1H coupling or short spin-lattice relaxation times in the rotating frame ($T_{1\rho\text{H}}$), direct polarization (Bloch decay) may be the only approach to recording solid-state NMR spectra.

MAGIC ANGLE SPINNING (MAS)

Line broadening in solid-state NMR is eliminated or averaged by both MAS and high-power ^1H decoupling. MAS involves mechanically rotating the sample at an angle of 54.7° (the magic angle) relative to the static magnetic field in order to simulate rapid molecular tumbling in solution. Rotating a solid sample at the magic angle to minimize line broadening requires that the sample be spun faster (in Hz) than the width of the CSA. High spinning rates are possible

with current MAS probe technology, but complications can arise with CP and may require techniques such as ramped-amplitude CP (RAMP-CP) and variable-amplitude CP (VACP) to improve the efficiency of magnetization transfer from ^1H . Additionally, MAS can raise the sample temperature significantly if it is not controlled, and pressures at the periphery of the rotor may be thousands of times the ambient pressure. These stresses may induce phase transformations, loss of solvent, and other effects.

SIDEBAND SUPPRESSION

As shown in Figure 9, slower spinning rates can be used to avoid compromising the solid sample, but when CSA is incompletely averaged, spinning sidebands will appear in solid-state NMR spectra. These artifacts are separated from the centerbands by integer multiples of the spinning rate (in Hz) and can be readily identified as the peaks that shift in spectra acquired at different spinning speeds. Spinning sideband manifolds contain useful information but can interfere with the signals of interest and may be particularly problematic when one uses higher field instruments. The total suppression of spinning sidebands (TOSS) procedure is commonly used to eliminate spinning sidebands from solid-state NMR spectra.

HIGH-POWER ^1H DECOUPLING

High-power ^1H decoupling is used to further reduce the line broadening from dipolar coupling to ^1H spins in the solid state. Specialized hardware is required to deliver the rf power needed for ^1H decoupling in solids, an rf power that is two orders of magnitude greater than that required to remove scalar coupling in liquids. CW decoupling is commonly used in solid-state NMR, although two-pulse phase-modulated and small phase incremental alteration (SPINAL-64) decoupling are increasingly used to improve the sensitivity and resolution of dilute spin spectra.

Typically only ^1H – ^{13}C heteronuclear dipolar interactions are significant when one acquires solid-state ^{13}C NMR spectra of organic materials. Homonuclear ^{13}C – ^{13}C dipole–dipole and scalar coupling interactions are negligible. Because of their low natural abundance, the probability that two ^{13}C nuclei are in close proximity is very small. However, homonuclear ^{13}C – ^{13}C dipolar coupling can be a concern for ^{13}C -labeled substrates.

EXPERIMENTAL SETUP

The basic setup for a CPMAS experiment will necessarily include magic angle setting, shimming, pulse calibration, Hartmann–Hahn matching, and spectral referencing, each of which is typically conducted on standard samples. Accurate measurement of the pulse lengths and associated rf power levels is essential for solid-state NMR experiments. Setting up the magic angle, shimming a CPMAS probe, and measuring its sensitivity for different tuning ranges are parts of probe setup and performance assessment. Shimming a solid-state NMR probe for MAS is more complicated than that for a solution probe because shim gradients are designed for the vertical orientation of solution NMR tubes. Solid-state NMR probes have no ²H lock channel, so shimming must be performed manually. Because of the intrinsically broad peaks, shimming is not nearly as critical in solid-state NMR as it is in solution NMR.

Standard compounds used for setting the magic angle and optimizing pulse lengths and the associated rf power levels for CP are listed in Table 6. KBr is commonly used for magic angle adjustments, observing the ⁷⁹Br resonance and adjusting the sample spinning angle to maximize (to 10 ms and beyond) the duration of the rotational echo train in the FID. Liquid samples can be used to shim solid-state NMR probes, although solid adamantane is commonly used for this purpose. Adamantane, glycine, and hexamethylbenzene (HMB) are commonly used for Hartmann–Hahn matching and testing sensitivity. Typically, the reference sample for testing sensitivity is permanently packed into a rotor to ensure that the same amount of sample is used.

Solid-state NMR spectrometers are generally used without field/frequency locking, so the resulting chemical shifts are less accurate than those for solutions. Calibration of the chemical shift can be done using either a primary or secondary standard. Spectral referencing is typically performed by sample replacement (external referencing). Standard compounds commonly used for spectral referencing in solid-state NMR are listed in Table 7. Note that glycine is polymorphic, so its crystal form should be ensured before its use in referencing spectra.

General Test Procedure

Spectrometer performance should be demonstrated first for a reference sample as described in *Cross-Polarization Magic Angle Spinning (CPMAS) NMR Technique, Experimental*

Setup. The magic angle, pulse lengths, and associated rf power levels for CP that are established using the reference compounds are sample independent. To obtain a CP spectrum of the sample, only the recycle delay needs to be chosen, followed by the contact time. When quantitative signal intensities are not required, an optimum recycle delay, i.e., one that affords the best S/N ratio, is 1.2*T*_{1H}, and the contact time is generally that which provides the optimum S/N ratio or that which best shows the features of most interest. For quantitative CP, a recycle delay of at least five times the longest *T*_{1H} of a heterogeneous mixture is suggested to ensure full relaxation, and a full analysis of the CP signal as a function of contact time must be conducted. See the subsection *Quantitative Analysis*.

SAMPLE PREPARATION

Sample-handling procedures used in solid-state NMR are substantially different from those used in liquid NMR. Solid samples are packed in ceramic rotors that are capped with fluted drive tips specifically designed for MAS. Fine powders are typically tamped into MAS rotors, although solid plugs, e.g., whole tablets, can be cut to fit the exact inner dimensions of the rotor and can be inserted directly into the rotor. Crushing or grinding may be used to reduce the sample to a fine powder, but caution should be used in order not to induce phase transformations. Depending on the compressibility of the powder and the rotor volume, 40–400 mg of material is typically required to fill a sample rotor.

Physical Characterization

Specific components in heterogeneous systems can be searched based on unique nuclei or different NMR relaxation properties. The identification of crystalline and amorphous materials can be accomplished by comparison of the solid-state NMR spectrum of the sample preparation with that of a reference standard. Chemical shifts and relative peak intensities can be used in the comparison. Amorphous materials generally give good MAS spectra with broader peaks than those seen for crystalline materials. Highly crystalline samples typically give ¹³C linewidths of the order of a few tens of Hz. The shape of the signals generally is between Lorentzian and Gaussian, a fact that should be recognized in deconvoluting overlapped spectra.

Table 6. Standard Samples Commonly Used for Setting Up CPMAS Experiments

Setup Procedure	Nucleus	Standard Sample(s)
Setting the magic angle	⁷⁹ Br	KBr
Shimming	¹³ C	adamantane
Pulse calibration	¹ H, ¹³ C	adamantane, HMB
Hartmann-Hahn matching	¹ H/ ¹³ C	adamantane, HMB
Sensitivity	¹³ C	HMB, adamantane, α-glycine

Table 7. Reference Compounds Commonly Used for Solid-State NMR

Nucleus	Primary Standard	Secondary Standard(s)	Chemical Shift in ppm from Primary Standard
¹³ C	TMS	HMB adamantane α-glycine	17.35 (CH ₃) 38.48 (CH ₂) 176.45 (carboxyl)
¹⁵ N	nitromethane	NH ₄ ¹⁵ NO ₃ ¹⁵ NH ₄ Cl α-glycine- ¹⁵ N	–5.1 (NO ₃) –338.1 –349.5
³¹ P	85% H ₃ PO ₄	CaHPO ₄ · 2H ₂ O (brushite)	1.4
²⁹ Si	TMS	tetramethylsilylmethane	–1.4
¹⁹ F	CF ₃ Cl	perfluorobenzene	–166.4

Relaxation

Relaxation parameters of interest in solids include spin-lattice relaxation (T_1), spin-lattice relaxation in the rotating frame ($T_{1\rho}$), spin-spin relaxation (T_2), and cross-relaxation (T_{CP}). In organic solids, ^1H spin diffusion is generally efficient so that pure compounds normally give single values for each of the relaxation times, T_1 and $T_{1\rho}$. For CP experiments, T_{1H} is used to establish the recycle delay between acquisitions. T_{1H} relaxation times can be measured using either progressive saturation or inversion recovery pulse sequences. In CP experiments, T_{CP} and $T_{1\rho H}$ characterize the magnetization build-up and decay, respectively. $T_{1\rho H}$ is measured via the ^{13}C signal using a delayed contact CP pulse sequence that has a variable delay time before the CP contact.

Quantitative Analysis

To quantitatively assess solid-state NMR spectra under CP conditions, extra measures must be taken. In addition to ensuring that the sample spinning axis is precisely set to the magic angle (54.7°) to minimize CSA broadening, the temperature and spin rate must be carefully controlled and the MAS probe properly tuned. Suggested recycle delays of $5T_{1H}$ are allotted between successive pulses to ensure that the magnetization has returned to its full equilibrium value. Both T_{CP} and $T_{1\rho H}$ relaxation must then be accounted for in the selection of the contact time. Typically, the CP contact time chosen is that which provides maximum sensitivity for the signals of interest. Quantitative analysis can be performed using either internal or external referencing methods. The use of internal standards can compensate for variability in sample volume and B_1 inhomogeneity throughout the sample.

By properly setting data acquisition parameters (recycle time, pulse widths, contact time, Hartmann–Hahn match, and decoupling power for each chemical system), signals can be obtained that are proportional to the number of nuclei producing them. For quantitative analysis, integrated signal intensities should be used rather than peak heights because linewidths in solid-state spectra often vary. When spinning sidebands are not eliminated by MAS, the intensity of the spinning sideband manifold must be added to the centerband intensity.

Spectral Editing

A key step in the analysis of any NMR spectrum is the assignment of individual resonances to unique phases and, in some cases, to specific atoms in the molecule. Special pulse sequences are available and may assist in simplifying CP/MAS spectra and assigning signals. Dipolar dephasing, also known as nonquaternary suppression or interrupted decoupling, yields spectra that typically contain signals only from quaternary and methyl carbons. Spectral subtraction of dipolar dephasing spectra from normal CP spectra or short contact time CP may be used to produce subspectra that contain signals from methylene and methine carbons only. Polarization-inversion techniques can be used to identify methylene and methine carbons.

LOW-FIELD NMR

Low field NMR (LF-NMR), sometimes referred to as time domain NMR (TD-NMR), experiments are performed by measuring relaxation, relaxometry, or diffusion. Instrumentation for these applications is based on low-field permanent magnet technologies that operate in the 2–25 MHz frequency range. Inexpensive stationary bench-top and portable TD-NMR spectrometers are commercially available. Typical bore sizes are 10–50 mm in diameter. A recent

development in spectrometer design uses a mobile mouse probe as an alternative to a stationary magnet. This design allows analysis of samples of unrestricted size.

Most TD-NMR applications are based on simple pulsing sequences, including FID, Hahn–echo, Carr–Purcell–Meiboom–Gill, and solid–echo acquisition. The choice of pulse sequence depends on the physical and chemical properties of the sample as well as the information that is desired from the experiment. These systems can be used to measure longitudinal (spin-lattice, T_1) and transverse (spin-spin, T_2) relaxation times. Diffusion properties of compounds can be exploited using pulsed field gradient (PFG-NMR) experiments.

The classical application of relaxometry is for the determination of food product components based on differences in longitudinal and transverse relaxation times of water, fats, and proteins.

Relaxivity

The magnitude of a substance's capacity to enhance the relaxation rate of a nucleus is referred to as relaxivity, expressed in units of $\text{sec}^{-1}\text{mM}^{-1}$. An example of such a substance commonly used in NMR spectroscopy is paramagnetic chromium acetylacetonate. Paramagnetic species are used in the medical industry as contrasting agents for magnetic resonance imaging. The relaxivity of a substance is determined experimentally by measuring the spin-lattice relaxation time (T_1) of a test substance and plotting $1/T_1$ against the concentration in units of mM (mmol/L). The slope of the curve is the numerical relaxivity. ▲ USP36

(1788) METHODS FOR THE DETERMINATION OF PARTICULATE MATTER IN INJECTIONS AND OPHTHALMIC SOLUTIONS

Methodology for the determination of particulate matter in injections is contained in USP (788), which has been harmonized with the European Pharmacopoeia and the Japanese Pharmacopoeia. Sections on instrument standardization for light obscuration and other method details were excluded from the harmonized chapter. Chapter (789) *Particulate Matter in Ophthalmic Solutions* has not been harmonized. Chapter (1788) includes important instrument standardization and calibration information applicable to (788) and (789); it also includes recommendations for sample handling, laboratory environment, operator training and general advice applicable to the microscopic method.

Chapter 1 requires injections to be essentially free from particulate matter that can be observed on visual inspection. The term “essentially free” has been difficult to define because particle detectability is influenced by their number and size, among other factors. The absolute limit of visibility, or detectability, is equivocal and depends upon the test conditions and the nature of the particulate matter. The lower end of the visible range certainly crosses over sub-visible detection capabilities in (788) and (789). Literature reports visibility extending to 50 μm , 100 μm , and 150 μm size (see *References 1 and 2*), and membrane assay can isolate and size particles to 1000 μm and larger.

Chapter (788) specifies limits for the sub-visible particulate matter content in injections in two size thresholds. Likewise, Chapter (789) establishes particle content expectations for ophthalmic solutions in two (Light Obscuration, or LO) or three (Membrane Microscope, or MM) size thresholds. The tests described in (788) and (789) are physical limit tests performed for the purpose of enumerating sub-visible particles [particulate matter] within specific size ranges (see Figure 1).

Chapter (788) states, "Particulate matter consists of mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions." Using the (788) test methods, any semi-solid to solid material (and even immiscible liquids) that trigger LO detector response above a selected size threshold will be tabulated.

There are two general categories of particulate matter sources: extrinsic and intrinsic. LO and MM methods will detect and tabulate particles of both categories. Extrinsic material is additive, foreign, and unchanging, and not part of the formulation, package, or assembly process.

Examples of extrinsic material include fibers, cellulosic matter, vegetative matter, corrosion products, paint/coatings, and building materials such as gypsum, concrete, metal, and plastic. Extrinsic particles are additive and generally non-changing over the life of the product, unless by fragmentation, swelling (hydration), or degradation. Fragments of rubber, plastic, metal, and glass are examples of extrinsic particulate matter deposited in the product during assembly or not removed in the container preparation process. However, if these typically extrinsic types have come from the specific package and/or process in a more consistent or chronic manner, then one may consider their presence to be an intrinsic variety, with a similar level of concern.

Intrinsic material is associated with the package, formulation ingredients and process or assembly process. Intrinsic material may also be extraneous material carried by the package or process and insufficiently removed. Intrinsic material may indeed change upon aging, due to concentration change, degradation, and acceleration of reaction.

Intrinsic sources are inherent in the product and process—formulation, package, and commercial assembly steps. Intrinsic sources represent a variety of phenomena yielding unwanted substances, such as: (a) extraction, (b) leaching, (c) degradation of ingredient (active or excipient), (d) change of ingredient by precipitation/salt form/crystalline form, (e) change of package physical integrity, (f) change of impurity level, (g) change of micellar association, (h) oligamerization, and (i) package- and process-related materials not removed during product assembly. Combinations of all of the above and physical phenomena such as aggregation, sedimentation, and coalescence by matrix (oils, semi-solids) may bring smaller particles ($<10\ \mu\text{m}$) into the detection zone of the test method ($\geq 10\ \mu\text{m}$). Intrinsic sources of detectable particulate matter are of great concern, since the substance may be present, however, not evident until particles form over time, even long after lot release.

The intrinsic categorization should be recognized as different from inherent formulation character. Solution properties such as a slight haze or faint coloration of high concentration formulae and protein formulations are typical examples of an inherent characteristic of the product fluid, and while the condition may cause difficulties in inspection or LO assay, are not particle-related.

Certain solution formulations may not be easily analyzed by LO. The LO method may encounter problems with a product that does not have clarity and a viscosity approximating those of water. Further, formulation characteristics such as color, high viscosity or inherent formulation properties, such as shear-induced changes, may generate erroneous LO data. Similarly, products that produce air or gas bubbles when drawn into the LO sensor, such as bicarbonate-buffered formulations may generate erroneous data. For these product types the MM method may have to be used. Documentation demonstrating that the LO procedure

is incapable of testing the test article or produces invalid results may aid regulatory filing strategy. It is expected that most test articles will meet the requirements on the basis of the LO test alone; however, it may be necessary to assay some test articles by the LO method followed by the MM method in order to reach a conclusion.

There may be a desire to test lower volumes of certain products, due to limited sample, high product cost, low container volume, or due to special fluid delivery characteristics. Examples include biopharmaceuticals, low-volume parenteral and ophthalmic products and formulations in novel packages intended for specific medical targets. The expectation is limits compliance for these products; however, one may employ methods validated by the manufacturer to demonstrate conformance with the test limits. Special low-volume "sippers" for LO sampling and the pooling of multiple containers may be necessary for these package presentations. Consider this example: a low-volume ($100\ \mu\text{L}$) product is packaged in a pre-filled sterile syringe. The nature of the package allows simple delivery of the solution product and may be used for direct sampling, but the $100\ \mu\text{L}$ volume precludes the pooling of the larger volumes ($\sim 25\ \text{mL}$) for the LO method. Direct sampling to a small membrane for microscopical counting and evaluating single and pooled package particle content may be the optimal means to collect data. Also in this example, careful statistical evaluation of the batch population using small sample volumes (but not doses) will be necessary to validate product acceptability.

Size domains considered in USP <1>, <788>, <789>

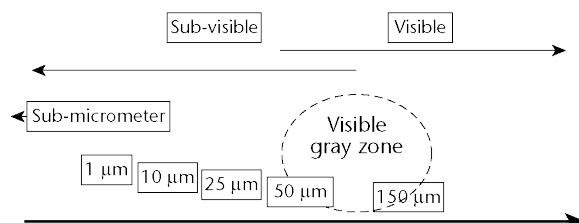


Figure 1. Increasing Probability of Visual Detection.

LIGHT OBSCURATION PARTICLE COUNT TEST

Test Apparatus

The apparatus is a liquid-borne particle counting system that uses a light-obscuration sensor with a suitable sample-feeding device to deliver controlled aliquots of sample for analysis. Suspended particles in the sample fluid flowing between a light source and sensor produce changes in signal that are correlated to particle dimension. Due to the nature of the detection and counting system, air bubbles and immiscible liquids may block sufficient light to be recorded along with the target suspended particles. These artifacts must be diminished through proper preparation techniques. Solutions with excessive immiscible liquids may not be amenable to LO analysis. A variety of suitable devices of this type are commercially available. It is the responsibility of those performing the test to ensure that the operating parameters of the instrumentation are appropriate to the required accuracy and precision of the test result, the artifacts and interferences inherent in certain products and with certain methods of preparation are eliminated or accommodated. An example is a protein formulation that may form shear-induced semi-solids due to mixing and counted as "particles." Adequate training must be provided for those responsible for the technical performance of the test.

It is important to note that for Pharmacopeial applications the ultimate goal is that the particle counter reproducibly size and count particles present in the material under investigation. The instruments available range from systems where calibration and other components of standardization must be carried out by manual procedures to sophisticated systems incorporating hardware- and software-based functions for the standardization procedures. Thus, it is not possible to specify exact methods to be followed for standardization of the instrument, and it is necessary to emphasize the required end result of a standardization procedure rather than a specific method for obtaining this result. This section is intended to emphasize the criteria that must be met by a system rather than specific methods to be used in their determination. It is the responsibility of the user to apply the various methods of standardization applicable to a specific instrument. Critical operational criteria consist of the following.

Sensor Concentration Limits—Use an instrument that has a concentration limit (the maximum number of particles per mL) identified by the manufacturer that is greater than the concentration of particles in the test specimen to be counted. The vendor-certified concentration limit for a sensor is specified as that count level at which coincidence counts due to the simultaneous presence of two or more particles in the sensor view volume compose less than 10% of the counts collected for 10- μ m particles.

Sensor Dynamic Range—The dynamic range of the instrument used (range of sizes of particles that can be accurately sized and counted) must include the smallest particle size to be enumerated in the products.

Instrument Standardization Tests

The following discussion of instrument standardization emphasizes performance criteria rather than specific methods for calibrating or standardizing a given instrument system. This approach is particularly evident in the description of calibration, where allowance must be made for manual methods as well as those based on firmware, software, or the use of electronic testing instruments. Appropriate instrument qualification is essential to performance of the test according to requirements. Since different brands of instruments may be used in the test, the user is responsible for ensuring that the counter used is operated according to the manufacturer's specific instruction; the principles to be followed to ensure that instruments operate within acceptable ranges are defined below. The following information for instrument standardization helps ensure that the sample volume accuracy, sample flow rate, particle size response curve, sensor resolution, and count accuracy are appropriate to performance of the test. Conduct these procedures at intervals of not more than six months.

SAMPLE VOLUME ACCURACY

Since the particle count from a sample aliquot varies directly with the volume of fluid sampled, it is important that the sampling accuracy is known to be within a certain range. For a sample volume determination, determine the dead (tare) volume in the sample feeder with particle-free water.¹ Transfer a volume of particle-free water that is greater than the sample volume to a container, and weigh. Using the sample feeding device, withdraw a volume that is appropriate for the specific sampler, and again weigh the container. Determine the sample volume by subtracting the tare volume from the combined sample plus tare volumes. Verify that the value obtained is within 5% of the appropriate sample volume for the test. Alternatively, the sample volume may be determined using a suitable Class A graduated cylinder (see *Volumetric Apparatus* (31)). [NOTE—Instru-

ments of this type require a variable tare volume. This is the amount of sample withdrawn before counting. This volume may be determined for syringe-operated samplers by setting the sample volume to zero and initiating sampling, so that the only volume of solution drawn is the tare. Subtract the tare volume from the total volume of solution drawn in the sampling cycle to determine the sample volume.]

SAMPLE FLOW RATE

Verify that the flow rate is within the manufacturer's specifications for the sensor used. This may be accomplished by using a calibrated stopwatch to measure the time required for the instrument to withdraw and count a specific sample volume (i.e., the time between beginning and ending of the count cycle as denoted by instrument indicator lights or other means). Sensors may be operated accurately over a range of flow rates. Perform the *Test Procedure* below at the same flow rate as that selected for calibration of the instrument.

CALIBRATION

USP (788) specifies the use of dispersions of spherical particles of known sizes between 10 μ m and 25 μ m in particle-free water. More options follow:

Manual Method—Calibrate the instrument with a minimum of three calibrators, such as near-mono-size polystyrene spheres having diameters of about 10, 15, and 25 μ m, in an aqueous particle-free vehicle. The calibrator spheres must have a mean diameter of within 5% of the nominal diameters and be standardized against materials traceable to NIST standard reference materials.² The total number of spheres counted must be within the sensor's concentration limit. Prepare suspensions of the calibrator spheres in water at a concentration of 1000 to 5000 particles per mL, and determine the channel setting that corresponds to the highest count setting for the sphere distribution. This is determined by using the highest count threshold setting to split the distribution into two bins containing equal numbers of counts, with the instrument set in the differential count mode (moving window half-count method). Use only the central portion of the distribution in this calculation to avoid including asymmetrical portions of the peak. The portion of the distribution, which must be divided equally, is the count window. The window is bounded by threshold settings that will define a threshold voltage window of $\pm 20\%$ around the mean diameter of the test spheres. The window is intended to include all single spheres, taking into account the standard deviation of the spheres and the sensor resolution, while excluding noise and aggregates of spheres. The value of 20% was chosen on the basis of the worst-case sensor resolution of 10% and the worst-case standard deviation of the spheres of 10%. Since the thresholds are proportional to the cross-sectional area of the spheres (and all particles) rather than the diameter, the lower and upper voltage settings are determined by the equations:

$$V_L = 0.64V_S$$

in which V_L is the lower voltage setting, and V_S is the voltage at the peak center, and

$$V_U = 1.44V_S$$

in which V_U is the upper voltage setting. Once the center peak thresholds are determined, use these thresholds for the standards to create a regression of log voltage versus log particle size, from which the instrument settings for the 10- and 25- μ m sizes can be determined.

² ASTM standard F658-00a provides useful discussions pertaining to calibration procedures applying near-monosize latex spheres.

¹ Passed through a filter having a nominal pore size of 1.2 μ m or finer.

Automated Method—The calibration (size response) curve may be determined for the instrument-sensor system by the use of validated software routines offered by instrument vendors; these may be included as part of the instrument software or used in conjunction with a microcomputer interfaced to the counter. The use of these automated methods is appropriate if the vendor supplies written certification that the software provides a response curve equivalent to that attained by the manual method and if the automated calibration is validated as necessary by the user.

Electronic Method—Using a multichannel peak height analyzer, determine the center channel of the particle counter pulse response for each standard suspension. This peak voltage setting becomes the threshold used for calculation of the voltage response curve for the instrument. The standard suspensions used for the calibration are run in order, and median pulse voltages for each are determined. These thresholds are then used to generate the size response curve manually or via software routines. The thresholds determined from the multichannel analyzer data are then transferred to the counter to complete the calibration.

SENSOR RESOLUTION

The particle size resolution of the instrumental particle counter is dependent upon the sensor used and may vary with individual sensors of the same model. Determine the resolution of the particle counter for 10- μ m particles, using the 10- μ m calibrator spheres. The relative standard deviation of the size distribution of the standard particles used is not more than 5%. Acceptable methods of determining particle size resolution are (1) manual determination of the amount of peak broadening due to instrument response; (2) using an electronic method of measuring and sorting particle sensor voltage output with a multichannel analyzer; and (3) automated methods.

Manual Method—Adjust the particle counter to operate in the cumulative mode or total count mode. Refer to the calibration curve obtained earlier, and determine the threshold voltage for the 10- μ m spheres. Adjust 3 channels of the counter to be used in the calibration procedure as follows:

Channel 1 is set for 90% of the threshold voltage.

Channel 2 is set for the threshold voltage.

Channel 3 is set for 110% of the threshold voltage.

Draw a sample through the sensor, observing the count in *Channel 2*. When the particle count in that channel has reached approximately 1000, stop counting, and observe the counts in *Channels 1* and *3*. Check to see if the *Channel 1* count and *Channel 3* count are $1.68 \pm 10\%$ and $0.32 \pm 10\%$, respectively, of the count in *Channel 2*. If not, adjust *Channel 1* and *Channel 3* thresholds to meet these criteria. When these criteria have been satisfied, draw a sample of suspension through the counter until the counts in *Channel 2* have reached approximately 10,000, or until an appropriate volume (e.g., 10 mL) of the sphere suspension has been counted. Verify that *Channel 1* and *Channel 3* counts are $1.68 \pm 3\%$ and $0.32 \pm 3\%$, respectively, of the count in *Channel 2*. Record the particle size for the thresholds just determined for *Channels 1, 2, and 3*. Subtract the particle size for *Channel 2* from the size for *Channel 3*. Subtract the particle size for *Channel 1* from the size for *Channel 2*. The values so determined are the observed standard deviations on the positive and negative side of the mean count for the 10- μ m standard. One commonly used method for calculating the percentage of resolution of the sensor is the following:

$$\% \text{ resolution} = (100/D) \times [(S_{\text{Obs}})^2 - (S_{\text{Std}})^2]^{1/2}$$

in which S_{Obs} is the highest observed standard deviation determined for the sphere standard; S_{Std} is the supplier's reported standard deviation for the spheres; and D is the diameter, in μ m, of the spheres as specified by the supplier. The resolution is not more than 10%.

Automated Method—Software that allows for the automated determination of sensor resolution is available for some counters. This software may be included in the instrument or used in conjunction with a microcomputer interfaced to the counter. The use of these automated methods is appropriate if the vendor supplies written certification that the software provides a resolution determination equivalent to the manual method and if the automated resolution determination is validated as necessary by the user.

Electronic Method—Record the voltage output distribution of the particle sensor, using a multichannel analyzer while sampling a suspension of the 10- μ m particle size standard. To determine resolution, move the cursor of the multichannel analyzer up and down the electric potential scale from the median pulse voltage to identify a channel on each side of the 10- μ m peak that has approximately 61% of the counts observed in the center channel. Use of the counter size response curve to convert the mV values of these two channels to particle sizes provides the particle size at within one standard deviation of the 10- μ m standard. Use these values to calculate the resolution as described under *Manual Method*.

PARTICLE COUNTING ACCURACY—SYSTEM SUITABILITY

Determine the particle counting accuracy of the instrument, using *Method 1* (for sensors requiring the moving window half-count (MWHC) method for calibration), *Method 2* (for multichannel sensors), or *Method 3* for any instrument (manual comparison to membrane microscope method).

Method 1—MWHC Instruments

Procedure—Prepare the suspension and blank using the USP Particle Count RS. With the instrument set to count in the cumulative (total) mode, collect counts at settings of greater than or equal to 10 μ m and greater than or equal to 15 μ m. Prepare the blank and suspension sample in the same manner. Degas the mixture by one of three means: by sonication (at 80 to 120 watts) for about 30 seconds, by allowing to stand, or by vacuum. Gently stir the contents by hand-swirling or by mechanical means, taking care not to introduce air bubbles or contamination. Stir continuously throughout the analysis. Withdraw directly from the container three consecutive volumes. Historically, these have been volumes of not less than 5 mL each, due to instrument limitations and the desire to maximize sample volume. However, where desired, volumes may be utilized that meet the standardization criteria and address the sensitivities of the formulation. Obtain the particle counts, and discard the data from the first portion. [NOTE—Complete the procedure within five minutes.] Repeat the procedure, using the suspension in place of the blank. From the averages of the counts resulting from the analysis of the two portions of the suspension at greater than or equal to 10 μ m and from the analysis of the two portions of the blank at greater than or equal to 10 μ m, calculate the number of particles in each mL by the formula:

$$(P_s - P_b)/V$$

in which P_s is the average particle count obtained from the suspension; P_b is the average particle count obtained from the blank; and V is the average volume, in mL, of the 4 portions tested. Repeat the calculations, using the results obtained at the setting of not less than 15 μ m.

Interpretation—The MWHC instrument meets the requirements for *Particle Counting Accuracy* if the count obtained at ≥ 10 μ m and the ratio of the counts obtained at greater than or equal to 10 μ m to those obtained at greater than or equal to 15 μ m conform to the values that accompany the USP Particle Count RS. If the instrument does not meet the requirements for *Particle Counting Accuracy*, and adequate test volumes remain, repeat the procedure with them; if in-

sufficient volumes remain, prepare new suspension and blank, and then repeat the procedure. If the results of the second test are within the limits given above, the instrument meets the requirements of the test for *Particle Counting Accuracy*. If on the second attempt the system does not meet the requirements of the test, determine and correct the source of the failures, and retest the instrument.

Method 2—Multichannel Instruments

Procedure—Use one of three standards: (1) a dilution of the USP Particle Count Reference Standard (USP PCRS); (2) commercial preparation of standard calibrator spheres of nominal diameter 15 to 30 μm in a suspension containing between 50 and 200 particles per mL, certified by the manufacturer; or (3) a laboratory-prepared suspension of standard calibrator spheres having a nominal diameter of 15 to 30 μm , containing between 50 and 200 particles per mL. Use of non-USP standards 2 and 3 is acceptable when they are compliant with USP standardization criteria: five successive counts are not more than $\pm 10\%$ of stated size.

Degas the suspension by one of three means: by sonication (at 80 to 120 watts) for about 30 seconds, by allowing to stand, or by vacuum. Gently stir the contents by hand-swirling or by mechanical means, taking care not to introduce air bubbles or contamination. Stir continuously throughout the analysis, and perform five counts on 5-mL volumes of the suspension, using the particle counter 10- μm size threshold. Obtain the mean cumulative particle count per mL.

Interpretation—The instrument meets the requirements for *Particle Counting Accuracy* if the count obtained at greater than or equal to 10 μm conforms to the values that accompany the USP Particle Count RS. If the instrument does not meet the requirements for *Particle Counting Accuracy*, repeat the procedure. If the results of the second test are within the limits given above, the instrument meets the requirements of the test for *Particle Counting Accuracy*. If on the second attempt the system does not meet the requirements of the test, determine and correct the source of the failures, and retest the instrument.

Method 3—Alternate Manual Method

Procedure—Prepare a suspension of standard calibrator spheres having a nominal diameter of 15 to 30 μm , containing between 50 and 200 particles per mL. Degas the suspension by one of three means: by sonication (at 80 to 120 watts) for about 30 seconds, by allowing to stand, or by vacuum. Gently stir the contents by hand-swirling or by mechanical means, taking care not to introduce air bubbles or contamination. Stir continuously throughout the analysis and perform five counts on 5-mL volumes of the suspension, using the particle counter 10- μm size threshold. Obtain the mean cumulative particle count per mL. Pipette a volume of this suspension containing 250 to 500 particles into a filter funnel prepared as described for *Microscope Particle Count Test, Filtration Apparatus*, below. After drying the membrane, count the total number of standard spheres collected on the membrane filter. This count should be within 20% of the mean instrumental count per mL for the suspension.

Test Environment

Specimens must be cleaned to the extent that the level of particles added by testing has a negligible effect on the outcome of the test.

Cleanse glassware, closures, and other required equipment, preferably by immersing and cleaning the items using warm, nonionic detergent solution. Rinse in flowing tap water, and then rinse again in flowing filtered water. Organic solvents may also be used to facilitate cleaning. [NOTE—These steps describe one way to clean equipment; alternatively, particulate-free equipment may be obtained from a suitable vendor.] Preferably, the test specimen, glass-

ware, closures, and other required equipment are then finally rinsed with filtered water, using a hand-held pressure nozzle with final filter or other appropriate filtered water source within an environment protected by high-efficiency particulate air (HEPA) filters. While conducting the assay, non-shedding garments and powder-free gloves are worn within the HEPA environment. Perform the test in an environment that does not contribute any significant amount of particulate matter.

To collect blank counts, use a cleaned vessel of the type and volume representative of that to be used in the test. Place a 50-mL or more volume of filtered water in the vessel, and agitate the water sample in the cleaned glassware by inversion or swirling. [NOTE—A smaller volume, consistent with the article to be counted, can be used.] Degas by the same method to be used for the product samples, by one of three means; sonication (at 80 to 120 watts) for about 30 seconds, by vacuum, or by allowing to stand. Swirl the vessel containing the water sample by hand or agitate by mechanical means to suspend particles.

As described in (788): *Determine the particulate matter in 5 samples of filtered water, each of 5 ml. If the number of particles of 10 μm or greater size exceeds 25 for the combined 25 ml (NMT 1/mL), the precautions taken for the tests are not sufficient.*

It is recommended that when utilizing the test for the (789) method, the blank test should be considered failed, if in addition, the number of particles of 25 μm or greater in size exceeds 3.

Test Procedure

TEST PREPARATION

Prepare the test specimens in the following sequence. Outside of the unidirectional airflow cabinet to be used for the test, remove outer closures, sealing bands, but not the sealing closure. If shedding is noted to be an issue, remove or tape over the product labels as well. Place the samples in the test cabinet, and rinse the exteriors of the containers with filtered water as directed under *Test Environment*. Protect the containers from environmental contamination until analyzed. After proper mixing, open and withdraw, pour or otherwise sample the contents of the containers under test in a manner least likely to generate particles that could enter the test specimen. Contents of containers with removable stoppers may be poured out directly after removing the closures. Sampling devices having a needle to penetrate the unit closure may also be employed. Products packaged in flexible plastic containers may be sampled by cutting the medication or administration port tube or a corner from the unit with a suitably cleaned razor blade or scissors. Dry or lyophilized products may be constituted using their internal diluent, by removing the closure to add supplied product diluent or by injecting filtered water via hypodermic syringe. If test specimens are to be pooled, remove the closure and empty the contents into a clean container.

NUMBER OF TEST SPECIMENS

USP (788) provides the sampling plan according to product volume. For all products, regardless of volume, comprehensive experience regarding the integrity and consistency of the batch is gained throughout development, allowing the proper sampling plans to be applied in commercial production that ensure sample selection is representative of batch quality. All batches must have sampling plans that accommodate desired statistical measures of batch quality and facilitate process control.

PRODUCT DETERMINATION

Depending upon the dosage form being tested, proceed as directed under the appropriate category below.

Liquid Preparations—

Volume in Container Less Than 25 mL—Prepare the containers as directed under *Test Preparation*. Mix and suspend the particulate matter in each unit by inverting the unit 20 times. [NOTE—Because of the small volume of some products, it may be necessary to agitate the solution more vigorously to suspend the particles properly.] Open and combine the contents of 10 or more units in a cleaned container to obtain a volume of not less than 25 mL. Degas the pooled solution by one of three means: sonication for about 30 seconds, or by vacuum, or by allowing the solution to stand.

Gently stir the contents of the container by hand-swirling or by mechanical means, taking care not to introduce air bubbles or contamination. Remove four portions, that conform to the volumes utilized in the IST, and count the number of particles equal to or greater than 10 μm and 25 μm . Disregard the result obtained for the first portion. [NOTE—For low-volume products, a pool of 15 or more units may be necessary to achieve a pool volume sufficient for four 5-mL sample aliquots. Smaller sample aliquots (i.e., less than 5 mL) can be used if the assay result obtained with the smaller aliquots is validated to give an assessment of batch suitability equivalent to that obtained with the 5-mL aliquots specified above.]

Volume in Container 25 mL or More—Prepare the containers as directed under *Test Preparation*. Mix and suspend the particulate matter in each unit by inverting the unit 20 times prior to opening the container for degassing. Degas the solution by one of three means: by sonication for about 30 seconds, or by vacuum, or by allowing the solution to stand. When sampling, ensure that the counter probe can be inserted into the middle of the solution. Gently stir the contents of the unit by hand-swirling or by mechanical means. Remove four portions, each of not less than 5 mL, and count the number of particles equal to or greater than 10 μm and 25 μm . Disregard the result obtained for the first portion.

Dry or Lyophilized Preparations—Prepare the containers as directed under *Test Preparation*. Open each container, taking care not to contaminate the opening or cover. Constitute as directed by the labeling, according to the *Test Preparation*. Alternately, depending on the experiment, use:

- filtered water or
- an appropriate laboratory-filtered diluent if suitable.

Replace the closure, and manually agitate the container sufficiently to ensure dissolution of the drug. [NOTE—For some dry or lyophilized products, it may be necessary to let the containers stand for a suitable interval, and then agitate again to effect complete dissolution.] After the drug in the constituted sample is completely dissolved, degas the solution by sonication for about 30 seconds, or by exposing to vacuum, or by allowing the solution to stand. When sampling, ensure that the counter draw or sipping probe can be inserted into the middle of the solution. Gently stir the contents of the unit by hand-swirling or by mechanical means to mix and suspend any particulate matter. Proceed as directed for the appropriate unit volume under *Liquid Preparations*, and analyze by withdrawing a minimum of four portions, each of not less than 5 mL, and count the number of particles equal to or greater than 10 μm and 25 μm . Disregard the result obtained for the first portion.

Products Packaged with Dual Compartments Constructed to Hold the Drug Product and a Solvent in Separate Compartments—Prepare the units to be tested as directed under *Test Preparation* and according to product insert directions. Mix each unit as directed in the labeling, activating and agitating so as to ensure thorough mixing of

the separate components and drug dissolution. Open and degas the units or pooled specimen to be tested by one of three means: sonication, or by vacuum, or by allowing the solution to stand. Proceed as directed for the appropriate unit volume under *Liquid Preparations*, mix and suspend the particulate matter present in each unit by inversion or swirling or by mechanical means and analyze by withdrawing a minimum of four portions, each of not less than 5 mL, and count the number of particles equal to or greater than 10 μm and 25 μm . Disregard the result obtained for the first portion.

Products Labeled “Pharmacy Bulk Package Not for Direct Infusion”—Proceed as directed for *Liquid Preparations* where the volume is 25 mL or more. Calculate the test result on a portion that is equivalent to the maximum dose given in the labeling. For example, if the total bulk package volume is 100 mL and the maximum dose volume is 10 mL, then the average LO particle count per mL would be multiplied by 10 to obtain the test result based on the 10-mL maximum dose. [NOTE—For the calculations of test results, consider this maximum dose portion to be the equivalent of the contents of one full container.]

LO Calculations

Note that the particle limits must be reported as all particles $\geq 10 \mu\text{m}$ and all particles $\geq 25 \mu\text{m}$. If the instrument has been configured to count in differential bins, such as $\geq 10\text{--}25 \mu\text{m}$, $\geq 25\text{--}50 \mu\text{m}$, $\geq 50 \mu\text{m}$, etc., all bins $\geq 10 \mu\text{m}$ must be added to yield total $\geq 10 \mu\text{m}$ count; all bins $\geq 25 \mu\text{m}$ need to be added to yield total count $\geq 25 \mu\text{m}$.

For example, the analyst has counted the test samples in eight bins: a) $\geq 10\text{--}15 \mu\text{m}$, b) $\geq 15 \mu\text{m}\text{--}25 \mu\text{m}$, c) $\geq 25 \mu\text{m}\text{--}40 \mu\text{m}$, d) $\geq 40 \mu\text{m}\text{--}75 \mu\text{m}$, e) $\geq 75 \mu\text{m}\text{--}100 \mu\text{m}$ and f) $\geq 100 \mu\text{m}$. They would then calculate $P_{\geq 10}$ as:

$$P_{\geq 10} = P_{\geq 10\text{--}15 \mu\text{m}} + P_{\geq 15\text{--}25 \mu\text{m}} + P_{\geq 25\text{--}40 \mu\text{m}} + P_{\geq 40\text{--}75 \mu\text{m}} + P_{\geq 75\text{--}100 \mu\text{m}} + P_{\geq 100 \mu\text{m}}$$

Pooled Samples—Average the counts from the two or more aliquot portions analyzed. Calculate the number of particles in each container by the formulae:

$$P_{\Sigma 10} V_T / V_A n$$

$$P_{\Sigma 25} V_T / V_A n$$

in which $P_{\Sigma 10}$ is the average particle count per threshold obtained from all portions analyzed and $P_{\Sigma 25}$ is the average particle count per threshold obtained from all portions $\geq 25 \mu\text{m}$ analyzed. V_T is the volume of pooled sample, in mL; V_A is the volume, in mL, of each portion analyzed; and n is the number of containers pooled.

Individual Samples—Average the counts obtained for the 5-mL or greater aliquot portions from each separate unit analyzed, and calculate the number of particles in each container by the formulae:

$$P_{\Sigma 10} V / V_A$$

$$P_{\Sigma 25} V / V_A$$

in which $P_{\Sigma 10}$ is the average particle count per threshold obtained from all portions analyzed; and $P_{\Sigma 25}$ is the average particle count per threshold obtained from all portions $\geq 25 \mu\text{m}$ analyzed. V is the volume, in mL, of the tested unit; and V_A is the volume, in mL, of each portion analyzed.

Individual Unit Samples—Average the counts obtained for the two or more 5-mL aliquot portions taken from the solu-

tion unit. Calculate the number of particles in each mL of product solution taken by the formulae:

$$P_{\Sigma 10}/V$$

$$P_{\Sigma 25}/V$$

in which $P_{\Sigma 10}$ is the average particle count per threshold obtained from all portions analyzed and $P_{\Sigma 25}$ is the average particle count per threshold obtained from all portions $\geq 25 \mu\text{m}$ analyzed. V is the volume, in mL, of the portion taken.

For all types of product, if the tested material has been diluted to decrease the viscosity, the dilution factor must be accounted for in the calculation of the final test result. For all test results, the particle count $\geq 10 \mu\text{m}$ represents all threshold bin counts.

MEMBRANE MICROSCOPE PARTICLE COUNT TEST

The microscope particulate matter test may be applied to both large-volume and small-volume parenteral injections and to ophthalmic solution products as well. This test enumerates essentially solid³ particulate matter $\geq 10 \mu\text{m}$ in these products, after collection, rinsing and drying on a microporous membrane filter. Since a wide range of test aliquots may be utilized, particle counts may be determined on a per-volume or per-container basis without dilution or extrapolation.

In the performance of the membrane microscope assay, one estimates the size of retained solids viewed at 100 \times magnification, tabulating them into specific size categories. In this process, one may encounter materials on the membrane surface that do not appear solid or substantial, showing little or no surface relief such as a "stain" or discontinuity on the membrane. Chapter 〈788〉 advises not to attempt to size or enumerate such semi-solid particles, due to historical comment from LVP terminal sterilization manufacturers that encountered stain-like brown residues after heat sterilization of Dextrose solutions. However, if not sampling a carbohydrate solution or similarly-performing formulation, recognizing the presence of such material is adds a measure of formulation robustness. Consistent evidence of such materials may be indication that further development research is warranted to understand their content. The nature of these materials and subsequent decision to count or investigate must be based upon product formulation experience. Interpretation of microscopical enumeration may be aided by testing a sample of the solution by the LO particle count or a validated, alternate method.

The Test Apparatus is described in 〈788〉. Additional comments are:

- Use a compound binocular microscope that corrects for changes in interpupillary distance by maintaining a constant tube length.
- The objective must be of 10 \times nominal magnification, a planar achromat or better in quality, with a minimum 0.25 numerical aperture.
- The objective must be compatible with an episcopic illuminator attachment.
- The eyepieces must be matched. In addition, one eyepiece must be designed to accept and focus an eyepiece graticule. The microscope must have a mechanical stage capable of holding and traversing the entire filtration area of a 25-mm or 47-mm membrane filter.
- Two illuminators are required. Both illuminators must be of sufficient output to provide a bright and even source of illumination and may be equipped with blue daylight filters to decrease operator fatigue during use.

- The USP graticule as described in 〈788〉 is used.

Stage Micrometer—Graduated in 10- μm increments, utilized each day-of-use. For initial calibration, utilize a stage micrometer that is certified by NIST to verify the USP graticule installation. Thereafter, for daily calibration/verification, one may utilize a commercial stage micrometer graduated in 10- μm increments to verify proper setup.

Filtration Apparatus—Use a filter funnel suitable for the volume to be tested, generally having an inner diameter of about 16 mm for 25-mm membranes or about 37 mm for 47-mm membranes. The funnel is made of plastic, glass, or stainless steel. Use a filter support made of stainless steel screen or sintered glass as the filtration diffuser. A solvent dispenser capable of delivering solvents filtered through a membrane filter at a range of pressures from 10 psi to 80 psi.

Membranes—As described by 〈788〉; however, finer pore size selections will have smoother surfaces, facilitating the microscopical examination; however, may impede more viscous sample fluid during the assay.

Test Environment

Following discussion is recommended operational detail to enhance the conductance of the MM assay.

It is ideal to use two unidirectional airflow hood (UAFH) or other unidirectional airflow enclosures, one for "wet" sample preparations, and the other an enclosure for the microscope counting phase. The UAFH having a capacity sufficient to envelop the area in which the analysis is prepared. The UAFH provides HEPA-filtered air which typically contains not more than 100 particles (0.5 μm or larger) per cubic foot. A blank determination is necessary at the beginning of each test sequence to verify minimal contribution from the background, equipment and personnel operations. What is the definition of a test sequence? Should it be one per shift, one per product family, one per series of filtrations (manifold) or one per sample? Any of these definitions may be suitable, dependant upon the operational needs of the lab system. The ability to clean glassware between samples, the number of different products being run, and the volume of samples through the lab will determine the appropriate control. However, consider the blank to be a system suitability check, and if it fails, all samples run prior to it up to the previous blank, are suspect.

To determine the blank count, duplicate the sample preparation process, in regard to the apparatus and membrane types. Assemble a clean filtration apparatus with a fresh membrane, rinse the interior with filtered water to drain, then deliver a 50-mL or more volume of filtered water to the filtration funnel while applying vacuum, and draw the entire volume of water through the membrane filter. Remove the membrane from the filter funnel base, and place onto a holding device as will be used for test specimens; typically atop a strip of double-sided tape on a microscope slide or in a commercial membrane holder or Petri dish. After allowing the membrane to dry (it must be counted dry), examine the entire filtration area microscopically at a magnification of 100 \times . If not more than 20 particles $\geq 10 \mu\text{m}$ and not more than 5 particles $\geq 25 \mu\text{m}$ or larger are present within the filtration area, the background particle level is sufficiently low for performance of the microscope assay for 〈788〉. If particle load exceeds these limits, repeat the procedure.

There is value in further limiting the background for both 〈788〉 and 〈789〉 testing in regard to good laboratory practice, and more specifically in regard to the 〈789〉 $\geq 25 \mu\text{m}$ and $\geq 50 \mu\text{m}$ limits, which may be considered more restrictive than injectable limits in consideration of total particle content allowed for the (usually) small unit volumes. Compare total particle load for a small SVI, small LVI versus a 5-mL ophthalmic product in Table 1.

³ Soft particles and semi-solid substances may also be retained.

Table 1. Comparison of Total At-Limit Load for Selected Products.

Size Limit	Blank Count	SVI, 5 mL	LVI, 125 mL	Ophthalmic Product, 5 mL
≥10 μm	20	3000 particles	1500 particles	250 particles
≥25 μm	5	300 particles	250 particles	25 particles
≥50 μm	not defined	N/A	N/A	10 particles

Therefore, in smaller volume (789) applications and in low-particle count injectable product, the laboratory should strive for consistent and lower blank counts such as NMT $5 \geq 10 \mu\text{m}$, NMT $1 \geq 25 \mu\text{m}$ and none $\geq 50 \mu\text{m}$ per blank.

Throughout the operational procedure (in the HEPA environment), it is preferable to use powder-free gloves, and low-shedding clothing. Prior to conducting the test, clean the work surfaces of the unidirectional flow enclosure with an appropriate filtered solvent. Glassware and equipment should be rinsed successively with a warm, residue-free solution of detergent, hot water, filtered distilled or deionized water, and isopropyl alcohol. [NOTE—Prior to use, pass the distilled or deionized water and the isopropyl alcohol through membrane filters having a nominal pore size of $0.2 \mu\text{m}$ or finer.] Perform the rinsing in the unidirectional airflow enclosure. Allow the glassware and filtration apparatus to dry in the unidirectional airflow enclosure, upstream of all other operations. Preferably, the enclosure is located in a separate room that is supplied with filtered air-conditioned air and maintained under positive pressure with respect to the surrounding areas.

MICROSCOPE PREPARATION

The microscope optical alignment and illumination are critical for success of this method. Although it is not difficult to differentiate a $10 \mu\text{m}$ from a $25 \mu\text{m}$ particle at $100\times$ with reflected light, the decision regarding the boundary at each size category will be difficult with poor equipment, maintenance or optical alignment. Also operator fatigue is caused by poor microscope alignment. We will have to make decisions such as “is this particle $9 \mu\text{m}$ or $11 \mu\text{m}$?” and “is that particle $24 \mu\text{m}$ or $26 \mu\text{m}$?” Optimized system resolution, that is, the ability to discern discrete points of minimal separation, relies upon good optical systems, aligned well. Factors including instrument cleanliness, resolution, e.g., objective N.A.,⁴ focus of both eyepieces and the graticule will play significant roles in attaining best images. In consideration of optimizing the use of the binocular compound microscope, it is best to utilize operators familiar with the instrument, and comfortable with alignment. The operator conducting the method should align the optics and illumination for their use, with supervisory/trainer approval.

It is recommended to start with alignment of the microscope for a typical transmitted illumination observation using a known sample. Any specimen familiar to the operator will suffice; however, a common particle count reference standard suspension such as the USP PCRS is a recommended selection, since it is also utilized in method system suitability evaluation. A drop of the USP PCRS is placed between a glass microscope slide and cover slip and viewed microscopically.⁵ With appropriate interpupillary distance and a comfortable sitting position at the microscope, the operator examines the fields of suspended spheres. One should observe the small standard spheres crisply in a combined field (with ease) for both eyes. One attains crisp focus

⁴N.A. is numerical aperture, an indicator of optical light-gathering capability, and thus resolution. High N.A. correlates to high resolution.

⁵The microscope objective requires a defined cover slip thickness, nominally $170 \mu\text{m}$, or No. 1 $\frac{1}{2}$.

and ease of view after separate focal adjustment of each eyepiece focus for a single point on the specimen.

Rotate the graticule in the right microscope eyepiece so that the linear scale is located at the bottom of the field of view, bringing the graticule into sharp focus by adjusting the right eyepiece diopter ring while viewing an out-of-focus specimen. Focus the microscope on a specimen, looking through the right eyepiece only. Then, looking through the left eyepiece, adjust the left eyepiece diopter to bring the specimen into sharp focus.

When the operator is not comfortable using the microscope or does not attain an equivalent crisp focus for each eye in a merged field of view, the counting will become a difficult experience and fatigue and flawed size comparison will result.

Nothing is better for preparing the operator for counting particles than to examine a test membrane as a positive control. Seasoned microscopists may not require this step, but for new operators or individuals conducting many different types of methods in the modern laboratory, familiarization is a prudent exercise. A filter membrane of the type being used for the method, such as a 25-mm color-contrast, plain $0.45\text{-}\mu\text{m}$ nominal pore size, containing particles, is a good choice. This may be a sample from previous method that contains a variety of particle types, or one prepared for familiarization. This positive test control will contain natural particles (flakes, threads, equant particles, various colors/opacity, a range of sizes, etc.) to effectively refresh the operator's sensitivity and facilitate microscope and illumination alignment for optimal viewing.

One would examine the membrane preparation, locate a typical array of particles and first bring the illumination into good alignment:

1. Adjust the external, incident illumination at an oblique angle ($10\text{--}20^\circ$ to the method) so that an even ellipse of reflected light is visible on the membrane and an even illumination evident through the eyepiece field of view (even across the full field). Shadows will be evident from larger particles, such as those with z axis $> 5 \mu\text{m}$ (z axis is the microscope optical axis).
2. Adjust the internal episcopic brightfield illuminator to yield an even illumination at a high setting on the transformer control, but more importantly, when dialing down the illumination one observes the evident shadow from the larger particles. In this manner, the high reflectivity of flat, glassy particles (find one) and the distinct shadows of more equant ($x:y:z \sim 1:1:1$) particles is evident.

USING THE CIRCULAR DIAMETER GRATICULE

The USP graticule is specifically fabricated for each microscope. The relative error of the graticule used must be $\pm 2\%$ and is initially measured with an NIST-certified stage micrometer. To accomplish this, align the graticule micrometer scale with the stage micrometer so that they are parallel. (Compare the scales, using as large a number of graduations on each as possible.) Read the number of graticule scale divisions, GSD, compared to stage micrometer divisions, SMD. Calculate the relative error by the formula:

$$100[(\text{GSD} - \text{SMD})/\text{SMD}]$$

A relative error of $\pm 2\%$ is acceptable and verifies good alignment, focus and proper magnification. Thereafter, a day-of-use verification by the microscope operator with the NIST stage micrometer or commercial stage micrometer is sufficient to demonstrate proper setup.

The basic technique of measurement applied with the use of the circular diameter graticule is to count all particles $10 \mu\text{m}$ and larger, further categorizing in $\geq 10 \mu\text{m}$ and $\geq 25 \mu\text{m}$. The circular zone or graticule field of view is a useful zone for active sizing and counting. Particles are compared to the

linear scale and/or circles to determine their size in equivalent circular diameter. This is conducted by transforming mentally the image of each particle into a circle and then comparing to the 10- and 25- μm graticule reference circles. The sizing process is carried out without superimposing the particle on the reference circles; particles are not moved from their locations within the graticule field of view (the large circle) for comparison to the reference circles. Compare the area of the particle being sized to that of the black or transparent circles. Use the area of the clear graticule reference circles to size white or transparent particles. Use the area of the black reference circles to size dark particles. The intent of comparing particles to an equivalent circular diameter is correlation to the LO particle sizing methodology, for which many manufacturers have extensive databases. In practice, particles with nearly circular areas will correlate well with the graticule circle diameters. For particles with one long axis, such as rods and needles, the conversion to circular area will produce more significant bias to smaller estimated sizes. It may be simpler, and most conservative, to count particles in longest chord. To use an extreme example, the total count of mono-dispersions of fine needle crystals would vary greatly dependant upon the size determination utilized.

In order to properly focus the ocular lenses and attain balanced single-field view, each operator must bring the USP graticule lines into sharp focus by adjusting the eyepiece diopter ring (it helps to have an "infinite" view, or out-of-focus specimen). Next, focus the microscope on a specimen, through this same eyepiece, and then looking only through the other eyepiece, adjust its diopter ring to bring the specimen into sharp focus. The USP graticule and specimen particles are now in focus on a well-balanced illumination field.

Preparation of Filtration Apparatus and Test Preparations are covered by (788). Further, prepare the test specimens in the following sequence. Outside of the unidirectional airflow cabinet to be used for the test, remove outer closures, sealing bands, and remove or tape over labels. Rinse the exteriors of the containers with filtered water as directed under Test Environment. Protect the containers from environmental contamination until analyzed.

Within the HEPA cabinet, open and withdraw, pour, or otherwise sample the contents of the containers under test in a manner least likely to generate particles that could enter the test specimen. Contents of containers with removable stoppers may be withdrawn directly by removing the closures. Sampling devices having a needle to penetrate the unit closure may also be employed. Products packaged in flexible plastic containers may be sampled by cutting the medication or administration port tube or a corner from the unit with a suitably cleaned razor blade or scissors.

For all products, regardless of volume, comprehensive experience regarding the integrity and consistency of the batch is gained throughout development, allowing the proper sampling plans to be applied in commercial production that ensure sample selection is representative of batch quality. All batches must have sampling plans that accommodate desired statistical measures of batch quality and facilitate process control.

PRODUCT PARTICLE COUNT DETERMINATION

Depending upon the dosage form being tested, proceed as directed under the appropriate category below.

Liquid Preparations—Thoroughly mix the units to be tested by inverting 20 times. Open the units in a manner consistent with the generation of the lowest possible numbers of background particles. For products less than 25 mL in volume, one may open them and drain to the filtration barrel individually, or combine the contents of 10 or more units in a cleaned container. [NOTE—When pooling containers, these must be included in the blank determination step.] Filter large-volume injection units individually. Small-

volume injection units having a volume of 25 mL or more may be filtered individually.

Transfer to the filtration funnel the total volume of a solution pool or of a single unit, and apply vacuum. If the volume of solution to be filtered exceeds the volume of the filtration funnel, add, stepwise, a portion of the solution until the entire volume is filtered. It is prudent to maintain the liquid volume in the filtration funnel above one-half of the funnel volume between refills, especially if the partial count procedure is to be used (see *Enumeration of Particles, Partial Count Procedure*, below). [NOTE—This is necessary in order to ensure even distribution of particles on the analytical membrane.] After the last addition of solution, begin rinsing the walls of the funnel by directing a low-pressure stream of filtered water in a circular pattern along the walls of the funnel, and stop rinsing the funnel before the volume falls below about one-fourth of the fill level. Maintain the vacuum until all the liquid in the funnel is gone.

Remove the filtration funnel from the filtration base while maintaining vacuum, then turn the vacuum off, and remove the filter membrane with non-serrated forceps. Place the filter in the prepared holder and label with sample identification. Allow the filter to air-dry in the unidirectional airflow enclosure with the cover ajar.

Dry or Lyophilized Preparations—Prepare the containers as directed under *Test Preparation*. Open each container, taking care not to contaminate the opening or cover. Constitute as directed by the labeling, according to the *Test Preparation*. Alternately, depending on the experiment, use:

- filtered water or an appropriate laboratory-filtered diluent if suitable.

Products Packaged with Dual Compartments Constructed to Hold the Drug Product and a Solvent in Separate Compartments—Activate each unit as directed in the labeling, agitating the contents sufficiently to ensure thorough mixing of the separate components, and then proceed as directed for *Liquid Preparations*.

Pharmacy Bulk Packages or Multiple-Dose Containers—For *Products Labeled "Pharmacy Bulk Package—Not for Direct Infusion"* or for multiple-dose containers, proceed as directed for *Liquid Preparations*, filtering the total unit volume.

Calculate the test result on a portion that is equal to the maximum dose given in the labeling. Consider this portion to be the equivalent of the contents of one full container. For example, if the total bulk package volume is 100 mL and the maximum dose listed is 10 mL, the microscope total unit volume count test result would be multiplied by 0.1 to obtain the test result for the 10-mL dose volume. [NOTE—For calculation of the test result, consider this portion to be the equivalent of the contents of one full container.]

Enumeration of Particles

The microscope test described in this section is flexible in that typical artifacts such as air and immiscible liquids do not interfere with the final count. The method has a broad size detection and counting range, if applying the partial count procedure. This method may be used where all particles on an analysis membrane surface are counted or where only those particles on some fractional area of a membrane surface are counted.

TOTAL COUNT PROCEDURE

The microscope method can be tedious (boring), imprecise (poor agreement within and between labs) and particle sizing can be inaccurate for non-spherical or equant particle shapes. Operator fatigue is promoted by poor ergonomic fit (chair height), by poor or imbalanced ocular focus and by excessive eye movement. Restricting the eye movement to a field-defining graticule such as the USP counting graticule restricts eye movement to the central one-third field of

view. This significantly limits eye movement and thus fatigue.

Sample size is an important consideration in counting precision. Care must be taken to sample many containers within a batch for good representation of the particle distribution. Accordingly, the portion of the individual package sampled is key. Particles may float or settle. Sampling only the first 25 mL of a LVP or sampling without adequate and recent mixing is a mistake, and will lead to serious undercounting. Sampling whole, well-mixed containers with the particles in suspension is the best approach.

Counting the isolated particles is an important parameter. Counting all of the particles retained on the membrane is certainly the best approach, and then the simple problem is determining the correct size for placement into the threshold bins, 10 μm and 25 μm . This will be increasingly important for methods utilizing additional bins for population determination, such as 5 μm , 50 μm , 100 μm , etc. Note that the particle limits for (788) and (789) must be reported as all particles $\geq 10 \mu\text{m}$ and all particles $\geq 25 \mu\text{m}$. If the lab method has been configured to count in several bins, such as $\geq 10\text{--}25 \mu\text{m}$, $\geq 25\text{--}50 \mu\text{m}$, $\geq 50 \mu\text{m}$, etc., all bins $\geq 10 \mu\text{m}$ must be added to yield total $\geq 10 \mu\text{m}$ count; all bins $\geq 25 \mu\text{m}$ need to be added to yield total count $\geq 25 \mu\text{m}$. Using a number of narrow size bins may be beneficial in product improvement efforts to separate particle groups.

In performance of a total count, the graticule field of view (GFOV) is defined by the large circle of the graticule, and the vertical crosshair is used as a counting target. Scan the membrane in paths that cover the effective filtration area (EFA), adjoining but not overlapping previous scan paths. Repeat this procedure, tabulating particle counts minimally in the $\geq 10 \mu\text{m}$ –25 μm and $\geq 25 \mu\text{m}$ thresholds, moving across the membrane until all particles on the membrane within the EFA are counted. Record the total number of particles that are $\geq 10 \mu\text{m}$ –25 μm and the number that are $\geq 25 \mu\text{m}$ or larger.

For large-volume products, calculate the particle count, in particles per mL, for each unit tested by the formulas:

$$P_{\geq 10}/V$$

$$P_{\geq 25}/V$$

in which $P_{\geq 10}$ is the total particle count obtained from all portions analyzed, $P_{\geq 25}$ is the total particle count obtained from all portions $\geq 25 \mu\text{m}$ analyzed, and V is the volume, in mL, of the solution tested.

For example, the analyst has counted the test samples in four bins: (a) $\geq 10\text{--}25 \mu\text{m}$, (b) $\geq 25 \mu\text{m}$ –50 μm , (c) $\geq 50 \mu\text{m}$ –100 μm , and (d) $\geq 100 \mu\text{m}$. They would then calculate as:

$$P_{\geq 10} = P_{\geq 10\text{--}25 \mu\text{m}} + P_{\geq 25 \mu\text{m}\text{--}50 \mu\text{m}} + P_{\geq 50 \mu\text{m}\text{--}100 \mu\text{m}} + P_{\geq 100 \mu\text{m}}$$

For small-volume products, calculate the particle count, in particles per container, by the formulas:

$$P_{\geq 10}/n$$

$$P_{\geq 25}/n$$

in which $P_{\geq 10}$ is the total particle count obtained from all portions analyzed, $P_{\geq 25}$ is the total particle count obtained from all portions $\geq 25 \mu\text{m}$ analyzed, and n is the number of units pooled (1 in the case of an individual unit).

PARTIAL COUNT PROCEDURE

When encountering a membrane full of particles, the task of counting all of them, properly, is daunting. Consider that an SVP with an at-limit content of small particles, sampled in a 10-vial pool would have 30,000 10- μm particles on the

membrane. Partial or statistical counting of the membrane effective filtration area may be the only means to attain reasonable results. Partial counting should not be used to reduce count times, just as a means to estimate the total load on a high-count isolate. A field-defining device, such as grids on the membrane surface or an ocular graticule field of view have been used reliably. An ocular graticule provides a sharp boundary for area definition. Gridded membrane lines are rather broad and have ink-spatter that may be taken for particulate matter.

Which portions and how much of the EFA should be counted? In consideration of 25-mm membranes, the EFA is 16-mm diameter using typical commercial filtration funnels, and thus $(\pi \times r^2) = 201 \text{ mm}^2$. Based upon earlier proposals from the HIMA committee and discussion by Draftz (see *Reference 3*), acceptable confidence intervals (Poisson distribution, 2 standard deviations) dictate that for samples with less than 1000 particles, the imprecision of statistical counting is objectionable. Full count is recommended for such samples. For samples with more than 1000 particles on the isolate membrane, using a 25-mm membrane, a reasonable estimate of particle population is attained using 20 GFOV. If a smaller confidence interval about the result is desired, a larger number of fields and particles may be counted.

For 47-mm membranes, the EFA is 37 mm. These larger diameter membranes may be selected for formulations needing more membrane surface area (having slow flow characteristics through 25-mm membranes) the EFA $= (\pi \times r^2) = (\pi \times 18.5 \text{ mm}^2) = 1075 \text{ mm}^2$. Thus, for 47-mm membrane EFAs, many more GFOVs must be counted to attain similar confidence. Using 100 GFOVs for partial counting of 47-mm membranes provides similar statistical confidence to the 20 GFOV/25-mm approach. Accordingly, when a particle load of 1000 or less is present, a full count is recommended.

When a partial count of particles on a membrane is to be performed, the analyst must first ensure that an even distribution of particles is present on the membrane. This is assessed by rapid scanning at 50 \times to qualitatively scan for heterogeneity or clumps of particles. If heterogeneity is observed, one should perform a full count on the membrane. Next, count the 10- μm or larger particles in one GFOV at the edge of the filtration area as well as one in the center of the membrane. The number of $\geq 10\text{--}\mu\text{m}$ or larger particles in the GFOV with the highest total particle count must not be more than twice that of the GFOV with the lowest particle count. Fully count the membrane failing these criteria.

To perform a partial count of the particles on a membrane, include all particles $\geq 10\text{--}25 \mu\text{m}$ and $\geq 25 \mu\text{m}$ within the GFOV and those that are in contact with the right side of the GFOV circle. Do not count particles outside of the GFOV. Ignore those that touch the left side of the GFOV circle. The dividing line between right and left sides of the GFOV circle is the vertical cross hair and is a useful counting line. [NOTE—Make the best possible judgment on particle size without changing the membrane position, microscope magnification or illumination.]

Start at the center edge of the filtration area and begin counting adjacent GFOVs. When the other edge of the filtration area is reached, move one GFOV toward the top of the filter and continue counting GFOVs by moving in the opposite direction. Moving from one GFOV to the next can be accomplished by one of two methods. One method is to define a landmark (particle or surface irregularity in the filter) and move over one GFOV in relation to the landmark. A second method is to use the vernier on the microscope method to move 1 mm between GFOVs. To facilitate the latter, adjust the microscope x- and y-method positioning controls to a whole number at the starting position at the center right edge of the filtration area, then each GFOV will be one whole division of movement of the x-method positioning control. If the top of the filtration area is reached before the desired number of GFOVs is reached, begin again at the right center edge of the filtration area one GFOV

lower than the first time. This time move downward on the membrane when the end of a row of GFOVs is reached. Continue as before until the number of GFOVs is complete.

For large-volume products, extrapolate the total count of particles per mL by the formulas:

$$P_{\Sigma 10} A_T / A_P V$$

$$P_{\Sigma 25} A_T / A_P V$$

in which $P_{\Sigma 10}$ is the total particle count obtained from all fields of view and all size thresholds; $P_{\Sigma 25}$ is the total particle count obtained from all fields of view and all size thresholds $\geq 25 \mu\text{m}$; A_T is the filtration area, in mm^2 , of the membrane (inner filtration barrel diameter); A_P is the partial area counted, in mm^2 , based on the number of graticule fields counted (GFOV area \times number of GFOV counted); and V is the volume, in mL, of solution filtered.

For a solution pool (for small-volume product units containing less than 25 mL) or for a single unit of a small-volume product, extrapolate the total count of particles per unit by the formulas:

$$P_{\Sigma 10} A_T / A_P n$$

$$P_{\Sigma 25} A_T / A_P n$$

in which $P_{\Sigma 10}$ is the total particle count obtained from all fields of view and all size thresholds, $P_{\Sigma 25}$ is the total particle

count obtained from all fields of view and all size thresholds $\geq 25 \mu\text{m}$, and n is the number of units counted (1 in the case of an individual unit). For all types of product, if the tested material has been diluted to decrease viscosity, the dilution factor must be accounted for in the calculation of the final test result.

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Dietary Supplements

General Chapters Information

(2021) MICROBIAL ENUMERATION TESTS— NUTRITIONAL AND DIETARY SUPPLEMENTS

INTRODUCTION

This chapter provides tests for the estimation of the number of viable aerobic microorganisms present in nutritional supplements of all kinds, from raw materials to the finished forms. Alternative methods may be substituted for the tests, provided that they have been properly validated as giving equivalent or better results. In preparing for and in applying the tests, observe aseptic precautions in handling the specimens. The term “growth” is used in a special sense herein, i.e., to designate the presence and presumed proliferation of viable microorganisms.

PREPARATORY TESTING

The validity of the results of the tests set forth in this chapter rests largely upon the adequacy of a demonstration that the test specimens to which they are applied do not, of themselves, inhibit the multiplication, under the test conditions, of microorganisms that may be present. Therefore, preparatory to conducting the tests on a regular basis and as circumstances require subsequently, inoculate diluted specimens of the material to be tested with separate viable cultures of the challenge microorganisms.

For the *Soybean–Casein Digest Agar* used for *Total Aerobic Microbial Counts*, inoculate duplicate plates with 25 to 250 cfu of *Staphylococcus aureus* (ATCC¹ No. 6538), *Escherichia coli* (ATCC No. 8739), and *Bacillus subtilis* (ATCC No. 6633) to demonstrate a greater than 70% bioburden recovery in comparison to a control medium. For the *Sabouraud Dextrose Agar* used for *Total Combined Yeast and Mold Counts*, inoculate duplicate plates with 25 to 250 cfu of *Candida albicans* (ATCC No. 10231) and *Aspergillus niger* (ATCC No. 16404) to demonstrate a greater than 70% bioburden recovery in comparison to a control medium. For *Enterobacterial Probable Number Determinations (Bile-Tolerant Gram-Negative Bacteria)*, appropriate dilutions of *Escherichia coli* (ATCC No. 8739) and *Salmonella typhimurium* (ATCC No. 13311) are used. Failure of the organism(s) to grow in the relevant medium invalidates that portion of the examination and ne-

¹Available from ATCC, 10801 University Boulevard, Manassas, VA 20110-2209. Equivalent microorganisms, provided that they are from a national collection repository, can be used in lieu of ATCC strains. However, the viable microorganisms used in the test must not be more than five passages removed from the original ATCC or national collection culture.

cessitates a modification of the procedure by (1) an increase in the volume of diluent, the quantity of test material remaining the same, or by (2) the incorporation of a sufficient quantity of suitable inactivating agent(s) in the diluents, or by (3) an appropriate combination of modifications to (1) and (2) so as to permit growth of the inoculum.

The following are examples of ingredients and their concentrations that may be added to the culture medium to neutralize inhibitory substances present in the sample: soy lecithin, 0.5%; and polysorbate 20, 4.0%. Alternatively, repeat the test as described in the preceding paragraph, using *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium* to demonstrate neutralization of preservatives or other antimicrobial agents in the test material. Where inhibitory substances are contained in the product and the latter is soluble, a suitable, validated adaptation of a procedure set forth under *Procedures* using the *Membrane Filtration Method* may be used.

If, in spite of the incorporation of suitable inactivating agents and a substantial increase in the volume of diluent, it is still not possible to recover the viable cultures described above, and where the article is not suitable for the employment of membrane filtration, it can be assumed that the failure to isolate the inoculated organism is attributable to the bactericidal or bacteriostatic activity of such magnitude that treatments are not able to remove the activity. This information serves to indicate that the article is not likely to allow proliferation or contamination with the given species of microorganism. Monitoring should be continued in order to determine the inhibitory range and bactericidal activity of the article.

BUFFER SOLUTION AND MEDIA

Culture media may be prepared as follows, or dehydrated culture media may be used provided that, when reconstituted as directed by the manufacturer or distributor, they have similar ingredients and/or yield media comparable to those obtained from the formulas given herein.

In preparing media by the formulas set forth herein, dissolve the soluble solids in the water, using heat if necessary to effect complete solution, and add solutions of hydrochloric acid or sodium hydroxide in quantities sufficient to yield the desired pH in the medium when it is ready for use. Determine the pH at $25 \pm 2^\circ$.

Where agar is called for in a formula, use agar that has a moisture content of not more than 15%. Where water is called for in a formula, use *Purified Water*.

pH 7.2 Phosphate Buffer

Prepare a stock solution by dissolving 34 g of monobasic potassium phosphate in about 500 mL of water contained in a 1000-mL volumetric flask. Adjust to a pH of 7.2 ± 0.1 by the addition of sodium hydroxide TS (about 175 mL), add water to volume, and mix. Dispense and sterilize. Store

under refrigeration. For use, dilute the stock solution with water in the ratio of 1 to 800, dispense as desired, and sterilize.

Media

Prepare media for the tests as described below. Alternatively, dehydrated formulations may be used provided that, when reconstituted as directed by the manufacturer or distributor, they meet the requirements of the *Growth Promotion Testing*. Unless otherwise indicated elsewhere in this chapter, media are sterilized in autoclaves using a validated process. The exposure time within the autoclave at 121° will depend on the volume of media to be sterilized. Thus, for example, a 500-mL volume would need to be autoclaved using a temperature and time relationship that will ensure that the medium has attained at least an F_0 of 12–15 in the sterilization process. However, the appropriate time and temperature duration for sterilizing prepared media at any given volume should be confirmed by a thermal penetration study using a thermocouple or thermoprobe placed within the liquid medium.

FLUID CASEIN DIGEST–SOY LECITHIN–POLYSORBATE 20 MEDIUM

Pancreatic Digest of Casein	20 g
Soy Lecithin	5 g
Polysorbate 20	40 mL
Water	960 mL

Dissolve pancreatic digest of casein and soy lecithin in 960 mL of water, heating in a water bath at 48° to 50° for about 30 minutes to effect solution. Add 40 mL of polysorbate 20. Mix, dispense as desired, and sterilize.

SOYBEAN–CASEIN DIGEST–AGAR MEDIUM

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean Meal	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Water	1000 mL

pH after sterilization: 7.3 ± 0.2 .

FLUID SOYBEAN–CASEIN DIGEST MEDIUM

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean Meal	3.0 g
Sodium Chloride	5.0 g
Dibasic Potassium Phosphate	2.5 g
Dextrose	2.5 g
Purified Water	1000 mL

Dissolve the solids in the water, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with 1 N sodium hydroxide so that after sterilization it will have a pH of 7.3 ± 0.2 . Filter, if necessary, and dispense into suitable containers. Sterilize at a temperature and time relationship that will ensure that the medium has attained at least an F_0 of 12–15 in the sterilization process, or by a validated filtration process.

SABOURAUD DEXTROSE–AGAR MEDIUM

Dextrose	40.0 g
Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1)	10.0 g
Agar	15.0 g
Water	1000 mL

Mix, and boil to effect solution.
pH after sterilization: 5.6 ± 0.2 .

VIOLET-RED BILE AGAR WITH GLUCOSE AND LACTOSE

Yeast Extract	3.0 g
Pancreatic Digest of Gelatin	7.0 g
Bile Salts	1.5 g
Lactose	10.0 g
Sodium Chloride	5.0 g
D-Glucose Monohydrate	10.0 g
Agar	15.0 g
Neutral Red	30 mg
Crystal Violet	2 mg
Water	1000 mL

Adjust the pH so that it is 7.4 ± 0.2 after heating. Heat to boiling, but do not heat in an autoclave. Pour onto plates.

MOSSEL–ENTEROBACTERIACEAE ENRICHMENT BROTH

Pancreatic Digest of Gelatin	10.0 g
D-Glucose Monohydrate	5.0 g
Dehydrated Ox Bile	20.0 g
Monobasic Potassium Phosphate	2.0 g
Dibasic Potassium Phosphate	8.0 g
Brilliant Green	15 mg
Water	1000 mL

Suspend the solids in water, and heat to boiling for 1 to 2 minutes. Transfer 120-mL portions to 250-mL volumetric flasks or 9-mL portions to test tubes, all being capped with cotton plugs or loose-fitting caps. Heat on a steam bath for 30 minutes. Adjust the pH so that it is 7.2 ± 0.2 after heating.

GROWTH PROMOTION TESTING

Each lot of dehydrated medium bearing the manufacturer's identifying number or each lot of medium prepared from basic ingredients must be tested for its growth-promoting qualities. Cultures of *Staphylococcus aureus* (ATCC No. 6538), *Escherichia coli* (ATCC No. 8739), *Bacillus subtilis* (ATCC No. 6633), *Candida albicans* (ATCC No. 10231), and *Aspergillus niger* (ATCC No. 16404) are used. A 10^{-3} dilution of a 24-hour broth culture of the microorganism to the first dilution (in pH 7.2 Phosphate Buffer or Fluid Soybean–Casein Digest Medium) may be used as the inocula. Serially streak plates of the media with the appropriate inocula to obtain isolated colonies to demonstrate the growth-promotion qualities of the Soybean–Casein Digest and Sabouraud Dextrose Agar media. Inoculate the Fluid Soybean–Casein Digest Medium and Mossel–Enterobacteriaceae Enrichment Broth with

10 to 100 cfu of the appropriate challenge organisms to demonstrate their growth-promotion qualities.

SAMPLING

Provide 10-mL or 10-g specimens for the tests called for in the individual monograph.

PROCEDURE

Prepare the specimen to be tested by a treatment that is appropriate to its physical characteristics and that does not alter the number and kind of microorganisms originally present, in order to obtain a solution or suspension of all or part of it in a form suitable for the test procedure(s) to be carried out.

For a solid that dissolves to an appreciable extent but not completely, reduce the substance to a moderately fine powder, suspend it in the vehicle specified, and proceed as directed under *Total Aerobic Microbial Count*.

For a fluid specimen that consists of a true solution, or a suspension in water or a hydroalcoholic vehicle containing less than 30% of alcohol, and for a solid that dissolves readily and practically completely in 90 mL of pH 7.2 Phosphate Buffer or the media specified, proceed as directed under *Total Aerobic Microbial Count*.

For water-immiscible products, prepare a suspension with the aid of a minimal quantity of a suitable, sterile emulsifying agent (such as one of the polysorbates), using a mechanical blender and warming to a temperature not exceeding 45°, if necessary, and proceed with the suspension as directed under *Total Aerobic Microbial Count*.

Total Aerobic Microbial Count

For specimens that are freely soluble, use the *Membrane Filtration Method* or *Plate Method*. For specimens that are sufficiently soluble or translucent to permit use of the *Plate Method*, use that method; otherwise, use the *Multiple-Tube Method*. With either method, first dissolve or suspend 10.0 g of the specimen if it is a solid, or 10 mL, accurately measured, if the specimen is a liquid, in pH 7.2 Phosphate Buffer, *Fluid Soybean–Casein Digest Medium*, or *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium* to make 100 mL. For viscous specimens that cannot be pipeted at this initial 1:10 dilution, dilute the specimen until a suspension is obtained, i.e., 1:50 or 1:100, etc., that can be pipeted. Perform the test for absence of inhibitory (antimicrobial) properties as described under *Preparatory Testing* before the determination of *Total Aerobic Microbial Count*. Add the specimen to the medium not more than 1 hour after preparing the appropriate dilutions for inoculation.

Membrane Filtration Method

Dilute the fluid further, if necessary, so that 1 mL will be expected to yield between 30 and 300 colonies. Pipet 1 mL

of the final dilution into 5 to 10 mL of pH 7.2 Phosphate Buffer, *Fluid Soybean–Casein Digest Medium*, or *Fluid Casein Digest–Soy Lecithin–Polysorbate 20 Medium*. Wash each membrane with an appropriate amount of one of the above diluents. Transfer each membrane to a Petri dish containing *Soybean–Casein Digest Agar Medium*, previously solidified at room temperature. Incubate the plates at a temperature between 30° and 35° for 48 to 72 hours. Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of microorganisms per g or per mL of specimen. If no microbial colonies are recovered from the dishes representing the initial 1:10 dilution of the specimen, express the results as “less than 10 microorganisms per g or per mL of specimen.”

Plate Method

Dilute the fluid further, if necessary, so that 1 mL will be expected to yield between 30 and 300 colonies. Pipet 1 mL of the final dilution onto each of two sterile Petri dishes. Promptly add to each dish 15 to 20 mL of *Soybean–Casein Digest–Agar Medium*, previously melted and cooled to about 45°. Cover the Petri dishes, mix the sample with agar by gently tilting or rotating the dishes, and allow the contents to solidify at room temperature. Invert the Petri dishes and incubate for 48 to 72 hours. Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of microorganisms per g or per mL of specimen. If no microbial colonies are recovered from the dishes representing the initial 1:10 dilution of the specimen, express the results as “less than 10 microorganisms per g or per mL of specimen.”

Multiple-Tube Method

Into each of 14 test tubes of similar size, place 9.0 mL of sterile *Fluid Soybean–Casein Digest Medium*. Arrange 12 of the tubes in four sets of three tubes each. Put aside one set of three tubes to serve as the controls. Into each of three tubes of one set (“100”) and into a fourth tube (A) pipet 1 mL of the solution or suspension of the specimen, and mix. Pipet 1 mL from tube A into the one remaining tube (B), not included in a set, and mix. These two tubes contain 100 mg or 100 µL and 10 mg or 10 µL of the specimen, respectively. Into each of the second set (“10”) of three tubes pipet 1 mL from tube A, and into each tube of the third set (“1”) pipet 1 mL from tube B. Discard the unused contents of tubes A and B. Close well, and incubate all of the tubes. Following incubation, examine the tubes for growth: the three control tubes remain clear, and the observations in the tubes containing the specimen, when interpreted by reference to *Table 1*, indicate the most probable number of microorganisms per g or per mL.

Table 1. Most Probable Count by Multiple-Tube Method

Observed Combinations of Numbers of Tubes Showing Growth in Each Set			Most Probable Number of Microorganisms per g or per mL	
Number of mg or µL of specimen per tube				
100	10	1		
3	3	3		more than 1100
3	3	2		1100
3	3	1		500
3	3	0		200
3	2	3		290
3	2	2		210
3	2	1		150

Table 1. Most Probable Count by Multiple-Tube Method (Continued)

Observed Combinations of Numbers of Tubes Showing Growth in Each Set			Most Probable Number of Microorganisms per g or per mL
Number of mg or μ L of specimen per tube			
100	10	1	
3	2	0	90
3	1	3	160
3	1	2	120
3	1	1	70
3	1	0	40
3	0	3	95
3	0	2	60
3	0	1	40
3	0	0	23
2	2	0	21
2	1	1	20
2	1	0	15
2	0	1	14
2	0	0	9
1	2	0	11
1	1	0	7
1	0	0	4
0	1	0	3
0	0	0	<3

Total Combined Molds and Yeasts Count

Procedure—Proceed as directed for *Membrane Filtration Method* or *Plate Method* under *Total Aerobic Microbial Count*, except to use the same amount of *Sabouraud Dextrose–Agar Medium* instead of *Soybean–Casein Digest–Agar Medium* and to incubate the plates for 5 to 7 days at 20° to 25°.

Retest—For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10-g specimen, a retest on an additional 10-g specimen from the original sample and a 10-g specimen from the new sample of the nutritional supplement may be conducted. Proceed as directed under *Procedure*.

Enterobacterial Count (Bile-Tolerant Gram-Negative Bacteria)

Dissolve or suspend the sample in a sufficient volume of pH 7.2 Phosphate Buffer or Fluid Soybean–Casein–Digest Medium and dilute with Fluid Soybean–Casein–Digest Medium to 100 mL. Pre-incubate for 2 to 5 hours at 20°–25° in Soybean–Casein Digest Broth diluent; inoculate suitable quantities of *Mossel–Enterobacteriaceae Enrichment Broth* to contain 0.1, 0.01, or 0.001 g or mL of the product. Incubate at 30°–35° for 24 to 48 hours. Subculture onto a plate of *Violet-Red Bile Agar* with *Glucose* and *Lactose*, and incubate at 30°–35° for 18 to 24 hours. Growth of well developed, generally red or reddish, colonies of Gram-Negative bacteria reveal the presence of enterobacteria. Determine the most probable number of microorganisms per g or per mL by reference to *Table 2*.

Table 2. The Most Probable Enterobacterial Count

Observed Presence of Enterobacteria			
Number of g of specimen per tube			
0.1	0.01	0.001	Most Probable Number of Enterobacteria per g
+	+	+	more than 100
+	+	–	fewer than 100 but more than 10
+	–	–	fewer than 10 but more than 1
–	–	–	fewer than 1

(2022) MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS— NUTRITIONAL AND DIETARY SUPPLEMENTS

INTRODUCTION

Good manufacturing practices require that objectionable organisms be absent from nonsterile nutritional and dietary products. A microorganism can be considered objectionable if it represents a potential health hazard to the user who is using the product as directed, or if it is capable of growing in the product. Objectionable microorganisms are defined as contaminants that, depending on the microbial species, number of organisms, dosage form, intended use, and patient population, would adversely affect product safety. Additionally, microorganisms may be deemed objectionable if they adversely affect product stability or if they may damage the integrity of the container closure system.

This chapter describes the testing of nutritional and dietary articles for specified microorganisms, which are specified in the individual monographs or whose absence is recommended by the guidance under *Microbiological Attributes of Nonsterile Nutritional and Dietary Supplements* (2023). When objectionable microorganisms are not specified in the individual monograph, it is the manufacturers' responsibility to determine which microorganisms in their products are objectionable. It is not intended that all nonsterile nutritional and dietary articles be tested for the absence of all of the microorganisms mentioned in this chapter, nor is the testing of relevant microorganisms restricted to those presented in this chapter.

Alternative microbiological, physicochemical, and biotechnological methods, including automated methods, may be substituted for these tests, provided they have been validated as being equivalent in their suitability for determining compliance.

BUFFER AND MEDIA

General Considerations

See *Buffer Solution and Media* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* (2021). The appropriateness of each medium for the intended purpose is to be assessed. Control sets of *Fluid Soybean–Casein Digest Medium* for *Preparatory Testing* are also used to assess the appropriateness of these media in the growth promotion of the specified microorganisms. For other media, streak agar plates to obtain isolated colonies of appropriate microorganisms, and inoculate the fluid media with the appropriate microorganisms at a final concentration of less than 100 cfu per mL. Observe the growth to establish the appropriateness of the media.

Buffer

Buffer Stock Solution and pH 7.2 Phosphate Buffer—Proceed as directed under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* (2021).

Media

FLUID SOYBEAN–CASEIN DIGEST MEDIUM

Prepare as directed under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* (2021).

MANNITOL–SALT–AGAR MEDIUM

Pancreatic Digest of Casein	5.0 g
Peptic Digest of Animal Tissue	5.0 g
Beef Extract	1.0 g
D-Mannitol	10.0 g
Sodium Chloride	75.0 g
Agar	15.0 g
Phenol Red	0.025 g
Water	1000 mL

Mix, then heat with frequent agitation, and boil for 1 minute to effect solution.
pH after sterilization: 7.4 ± 0.2 .

FLUID TETRATHIONATE MEDIUM

Pancreatic Digest of Casein	2.5 g
Peptic Digest of Animal Fat	2.5 g
Bile Salts	1.0 g
Calcium Carbonate	10.0 g
Sodium Thiosulfate	30.0 g
Water	1000 mL

Heat to boiling. Do not autoclave; use the same day. Immediately before use, add a solution prepared by dissolving 5 g of potassium iodide and 6 g of iodine in 20 mL of water. Then add 10 mL of a solution of brilliant green (1 in 1000), and mix. Do not heat after adding the brilliant green solution.

BRILLIANT GREEN–AGAR MEDIUM

Yeast Extract	3.0 g
Peptic Digest of Animal Tissue	5.0 g
Pancreatic Digest of Casein	5.0 g
Lactose	10.0 g
Sodium Chloride	5.0 g
Sucrose	10.0 g
Phenol Red	80.0 g
Agar	20.0 g
Brilliant Green	12.5 mg
Water	1000 mL

Boil for 1 minute. Sterilize just prior to use, melt, pour into Petri dishes, and allow to cool.
pH after sterilization: 6.9 ± 0.2

XYLOSE–LYSINE–DESOXYCHOLATE–AGAR MEDIUM

Xylose	3.5 g
L-Lysine	5.0 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium Chloride	5.0 g
Yeast Extract	3.0 g
Phenol Red	80 mg
Agar	13.5 g
Sodium Desoxycholate (as Bile Salts)	2.5 g
Sodium Thiosulfate	6.8 g
Ferric Ammonium Citrate	800 mg
Water	1000 mL

Heat, with swirling, just to the boiling point. Do not over-heat or sterilize. Transfer at once to a water bath maintained at about 50°, and pour into Petri plates as soon as the *Medium* has cooled.

Final pH: 7.4 ± 0.2.

HEKTOEN ENTERIC AGAR MEDIUM

Protease Peptone	12.0 g
Yeast Extract	3.0 g
Lactose	12.0 g
Sucrose	2.0 g
Salicin	9.0 g
Bile Salts No. 3	9.0 g
Sodium Chloride	5.0 g
Sodium Thiosulfate	5.0 g
Ferric Ammonium Citrate	1.5 g
Acid Fuchsin	0.1 g
Bromothymol Blue	65 mg
Agar	14.0 g
Water	1000 mL

Mix, and allow to stand for 10 minutes. Heat gently, and allow to boil for a few seconds to dissolve the agar. Do not sterilize. Cool to 60°, and pour into Petri dishes.

Final pH: 7.5 ± 0.2.

TRIPLE SUGAR–IRON–AGAR MEDIUM

Pancreatic Digest of Casein	10.0 g
Pancreatic Digest of Animal Tissue	10.0 g
Lactose	10.0 g
Sucrose	10.0 g
Dextrose	1.0 g
Ferrous Ammonium Sulfate	200 mg
Sodium Chloride	5.0 g
Sodium Thiosulfate	200 mg
Agar	13.0 g
Phenol Red	25 mg
Water	1000 mL

pH after sterilization: 7.3 ± 0.2.

MACCONKEY AGAR MEDIUM

Pancreatic Digest of Gelatin	17.0 g
Pancreatic Digest of Casein	1.5 g
Peptic Digest of Animal Tissue	1.5 g
Lactose	10.0 g
Bile Salts Mixture	1.5 g
Sodium Salts Mixture	5.0 g
Agar	13.5 g
Neutral Red	30 mg
Crystal Violet	1.0 mg
Water	1000 mL

Boil for 1 minute to effect solution.
pH after sterilization: 7.1 ± 0.2.

LEVINE EOSIN–METHYLENE BLUE–AGAR MEDIUM

Pancreatic Digest of Gelatin	10.0 g
Dibasic Potassium Phosphate	2.0 g
Agar	15.0 g
Lactose	10.0 g
Eosin Y	400 mg
Methylene Blue	65 mg
Water	1000 mL

Dissolve pancreatic digest of gelatin, dibasic potassium phosphate, and agar in water, with warming, and allow to cool. Just prior to use, liquefy the gelled agar solution, and add the remaining ingredients, as solutions, in the following amounts: for each 100 mL of the liquefied agar solution, add 5 mL of lactose solution (1 in 5), 2 mL of the eosin Y solution (1 in 50), and 2 mL of methylene blue solution (1 in 300). Mix. The finished *Medium* may not be clear.

pH after sterilization: 7.1 ± 0.2.

BAIRD–PARKER AGAR MEDIUM

Pancreatic Digest of Casein	10.0 g
Beef Extract	5.0 g
Yeast Extract	1.0 g
Lithium Chloride	5.0 g
Agar	20.0 g
Glycine	12.0 g
Sodium Pyruvate	10.0 g
Water	950 mL

Heat with frequent agitation, and boil for 1 minute. Sterilize, cool to between 45° and 50°, and add 10 mL of sterile potassium tellurite solution (1 in 100) and 50 mL of egg yolk emulsion prepared as follows. Disinfect the surface of whole-shell eggs, aseptically crack the eggs, transfer intact yolks to a sterile graduated cylinder, add sterile saline TS to obtain a 3 to 7 ratio of egg yolk to saline, add to a sterile blender cup, and mix at high speed for 5 seconds. Mix all ingredients well but gently, and pour into plates.

pH after sterilization: 6.8 ± 0.2.

VOGEL–JOHNSON AGAR MEDIUM

Pancreatic Digest of Casein	10.0 g
Yeast Extract	5.0 g
Mannitol	10.0 g
Dibasic Potassium Phosphate	5.0 g
Lithium Chloride	5.0 g
Glycine	10.0 g
Agar	16.0 g
Phenol Red	25.0 mg
Water	1000 mL

Boil for 1 minute. Sterilize, cool to between 45° and 50°, and add 20 mL of sterile potassium tellurite solution (1 in 100).

pH after sterilization: 7.2 ± 0.2.

FLUID SELENITE–CYSTINE MEDIUM

Pancreatic Digest of Casein	5.0 g
Lactose	4.0 g
Sodium Phosphate	10.0 g
Sodium Acid Selenite	4.0 g
L-Cystine	10.0 g
Water	1000 mL

Mix, and heat to effect solution. Then heat in flowing stream for 15 minutes. Do not sterilize.

Final pH: 7.0 ± 0.2.

REINFORCED MEDIUM FOR CLOSTRIDIA

Beef Extract	10.0 g
Peptone	10.0 g
Yeast Extract	3.0 g
Soluble Starch	1.0 g
Glucose Monohydrate	5.0 g
Cysteine Hydrochloride	0.5 g
Sodium Chloride	5.0 g
Sodium Acetate	3.0 g
Agar	0.5 g
Water	1000 mL

Dissolve agar in water by heating to boiling, while stirring continuously. Adjust the pH if necessary, and sterilize.

pH after sterilization: 6.8 ± 0.2.

COLUMBIA AGAR

Pancreatic Digest of Casein	10.0 g
Meat Peptic Digest	5.0 g
Heart Pancreatic Digest	3.0 g
Yeast Extract	5.0 g
Cornstarch	1.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Water	1000 mL

Dissolve agar in water by heating to boiling and with continuous stirring. If necessary, adjust the pH. Sterilize, and

allow to cool to 45° to 50°. Add, when necessary, gentamicin sulfate, equivalent to about 20 mg of gentamicin base, and pour into Petri dishes.

Pre-reduction of the medium is recommended. pH after sterilization: 7.3 ± 0.2.

RAPPAPORT VASSILIADIS SALMONELLA ENRICHMENT BROTH

Soya Peptone	4.5 g
Magnesium Chloride Hexahydrate	29.0 g
Sodium Chloride	8.0 g
Dipotassium Phosphate	0.4 g
Potassium Dihydrogen Phosphate	0.6 g
Malachite Green	0.036 g
Purified Water	1000 mL

Dissolve, warming slightly. Sterilize in an autoclave using a validated cycle, at a temperature not exceeding 115°.

The pH is 5.2 ± 0.2 at 25° after heating and autoclaving.

PREPARATORY TESTING

Proceed as directed for *Preparatory Testing* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* (2021).

For enrichment broth, selective media, and differential media use an inoculating loop to transfer the inoculum of each test organism to the plated or liquid media being tested. If a plated medium is being tested, streak the surface of plate with the loop in four directions to obtain a pattern of isolated colonies. Incubate the media, and examine the plated or liquid media for the characteristic growth of the inocula (See *Tables 1, 2, 3, and 4*).

SAMPLING

Proceed as directed for *Sampling* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* (2021).

TEST PROCEDURES

Test Preparation—Prepare as directed for *Sampling*. Transfer to a suitable container with 100 mL of *Fluid Soybean–Casein Digest Medium (FSCD)*. Mix by shaking gently. [NOTE—On the basis of results for *Preparatory Testing*, modify the *Test Preparation* as appropriate.]

Test for Absence of *Staphylococcus aureus*

Incubate at 30° to 35° for 18 to 24 hours. Streak a loopful from *FSCD* onto the surface of one or more of the following media: *Vogel–Johnson Agar Medium (VJ Agar)*, *Mannitol–Salt–Agar Medium (MS–Agar)*, and *Baird–Parker Agar Medium (BP Agar)*. Cover the Petri plates, invert them, and incubate at 30° to 35° for 24 to 48 hours.

Examine the plates of *VJ Agar*, *MS–Agar*, and/or *BP Agar*, and interpret the results with reference to *Table 1*: if no plate contains colonies having the characteristics described, the test specimen meets the requirement for the absence of *Staphylococcus aureus*. If characteristic colonies are present, perform coagulase test as follows. Transfer representative colonies to separate tubes containing 0.5 mL of rabbit plasma, horse plasma, or any other mammalian plasma. Incubate in a water bath at 37°. Examine for coagulation after 3 hours of incubation and at suitable intervals up to

24 hours. Comparing with positive and negative controls, the absence of a coagulase reaction indicates the absence of *Staphylococcus aureus* in the tested article.

Table 1. Characteristics of *Staphylococcus aureus* on Specified Agar Media

Agar Medium	Colonial Morphology	Gram Stain
<i>Vogel–Johnson</i>	Black surrounded by yellow zone	(+), cocci
<i>Mannitol–Salt</i>	Yellow colonies with yellow zone	(+), cocci
<i>Baird–Parker</i>	Black, shiny surrounded by 2–5-mm clear zones	(+), cocci

Test for Absence of *Salmonella* Species

Incubate at 30° to 35° for 18 to 24 hours. From *FSCD*, pipet a 1-mL aliquot into 10 mL of *Rappaport Vassiliadis Salmonella Enrichment Broth*, mix, and incubate at 30° to 35° for 18 to 24 hours. Streak a loopful from both incubated media onto individual surfaces of one or more of following media: *Brilliant Green Agar Medium (BG-Agar)*, *Xylose–Lysine–Desoxycholate–Agar Medium (XLDC-Agar)*, and *Hektoen Enteric Agar Medium (HE Agar)*. Cover, invert the plates, and incubate at 30° to 35° for 24 to 48 hours. Examine the inoculated plates of *BG-Agar*, *XLDC-Agar*, and/or *HE Agar*, and interpret the results with reference to *Table 2*: if no colonies having the characteristics described are observed, the test specimen meets the requirement for the absence of *Salmonella* species. If colonies with characteristics described in *Table 2* are present, the suspect colonies are transferred to a slant of *Triple Sugar–Iron–Agar Medium (TSI)* using an inoculating wire, by first streaking the surface of the slant, and then stabbing the wire well beneath the surface. Incubate at 30° to 35° for 24 to 48 hours. If the tubes do not have red alkaline slants and yellow acid butts, with or without concomitant blackening of the butts from hydrogen sulfide production, the test specimen meets the requirement for the absence of *Salmonella* species.

Table 2. Characteristics of *Salmonella* Species on Specified Agar Media

Agar Medium	Colonial Morphology	Gram Stain
<i>Brilliant Green</i>	Small, transparent and colorless; or opaque, pink or white (often surrounded by pink to red zone)	(–), rods
<i>Xylose–Lysine–Desoxycholate</i>	Red, with or without black centers	(–), rods
<i>Hektoen Enteric</i>	Blue-green, with or without black centers	(–), rods

Test for Absence of *Escherichia coli*

Incubate at 30° to 35° for 24 to 48 hours. From *FSCD*, pipet a 1-mL aliquot into a container containing 10 mL of *MacConkey Broth*, mix, and incubate at 42° to 44° for 24 to 48 hours. Streak a loopful from both incubated media onto individual surfaces of *MacConkey Agar Medium (MC Agar)*, and incubate at 30° to 35° for 18 to 24 hours. Examine the inoculated *MC Agar* plate, and interpret the results with reference to *Table 3*: if no colonies having the characteristics described are observed, the test specimen meets the requirement for the absence of *Escherichia coli*. Suspect colo-

nies showing the characteristics described in *Table 3* are transferred individually, using an inoculating loop, to the surface of a plate with *Levine Eosin–Methylene Blue–Agar Medium (LEMB-Agar)*. If a large number of suspect colonies are to be transferred, divide the surface of each plate into quadrants, each quadrant being inoculated with a different colony. Cover the plates, invert, and incubate at 30° to 35° for 24 to 48 hours. If none of the colonies exhibit a characteristic metallic sheen under reflected light, and if none exhibit a blue-black appearance under transmitted light, the test specimen meets the requirement for the absence of *Escherichia coli*.

Table 3. Characteristics of *Escherichia coli* on MacConkey Agar Medium

Colonial Morphology	Gram Stain
Brick red, may have surrounding zone of precipitated bile	(–), rods

Test for Absence of *Clostridium* Species

Test Preparation—Prepare as directed for *Sampling*. [NOTE—On the basis of results for *Preparatory Testing*, modify the *Test Preparation* as appropriate.]

Procedure—Take two equal portions of the *Test Preparation*, heat one to 80° for 10 minutes, and cool rapidly. Transfer 10 mL of each portion to separate containers, each containing 100 mL of *Reinforced Medium for Clostridia*, and incubate under anaerobic conditions at 35° to 37° for 48 hours. After incubation, subculture each specimen on *Columbia Agar Medium* to which gentamicin has been added, and incubate under anaerobic conditions at 35° to 37° for 48 hours. Examine the plates, and interpret with reference to *Table 4*: if no growth of microorganisms is detected, the test specimen meets the requirement for the absence of *Clostridium* species.

Table 4. Characteristics of *Clostridium* Species on Specified Media

Medium	Gram Stain	Catalase
<i>Reinforced Medium for Clostridia</i>	(+), rods	
<i>Columbia Agar</i>	(+), rods	Negative

If growth occurs, subculture each distinct colony on *Columbia Agar Medium*, and separately incubate in aerobic and in anaerobic conditions at 35° to 37° for 48 hours. The occurrence of only anaerobic growth of gram-positive bacilli, giving a negative catalase reaction, indicates the presence of *Clostridium sporogenes*. To perform the catalase test, transfer discrete colonies to glass slides, and apply a drop of dilute hydrogen peroxide solution: the reaction is negative if no gas bubbles evolve. If the test specimen exhibits none of these characteristics, it meets the requirement for the absence of *Clostridium* species.

Retest

For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10 g specimen, a retest on a 25 g specimen of the nutritional or dietary supplement may be conducted. Proceed as directed under *Procedure*, but make allowances for the larger specimen size.

(2023) MICROBIOLOGICAL ATTRIBUTES OF NONSTERILE NUTRITIONAL AND DIETARY SUPPLEMENTS

The raw materials, pharmaceutical ingredients, and active ingredients used in the manufacture of nutritional and dietary articles may range from chemically synthesized vitamins to plant extracts and animal byproducts, and these ingredients are typically not sterile. Considerable experience has accrued with these highly refined plant- and animal-derived pharmaceutical ingredients, such as microcrystalline cellulose, modified starch, lactose, and magnesium stearate, and their microbiological attributes are well established. Botanicals may be microbiologically contaminated at any point during cultivation, harvesting, processing, packing, and distribution. Major sources of microbial contamination are associated with human or animal feces used as plant manure, contaminated irrigation water and/or process water, and poor worker hygiene and sanitation practices during harvesting, sorting, processing, packaging, and transportation. Furthermore, it is essential that microbiological contamination be minimized during the manufacture of nonsterile dietary supplements. To achieve this, Good Manufacturing Practices are employed and adequate microbiological specifications are established.

Microbiological process control, control of the bioburden of raw materials, and control of the manufacturing process to minimize cross-contamination are necessary to guarantee acceptable microbial quality in the final dosage forms. Because nonaqueous or dry dosage forms do not support microbial growth because of low water activity, the microbial quality of such articles is a function of the microorganisms introduced through ingredients or during processing. In addition to considering the intended use of the product, the frequency of microbial testing for the finished nonsterile dietary supplement would be a function of the historical microbial testing database of that product, knowledge of the manufacturing processes, the susceptibility of the formulation to microbial proliferation, and the demonstrated effectiveness of programs controlling the raw materials.

FORMULATION AND PROCESS DESIGN

From a microbiological perspective, the development of the formulation of nutritional or dietary supplements includes an evaluation of raw materials and their suppliers and the contribution made to the products by each ingredient and the manufacturing processes. Characterization of these elements allows the adequacy of the manufacturing process to be demonstrated. For example, if a product is formulated with an ingredient of botanical or animal origin known to possess a high, variable, or unpredictable level of microbiological contamination, it is necessary to ensure that the microbiological monitoring identifies ingredients that have an inappropriate bioburden level and that a premanufacturing process such as drying, extraction, heat treatment, irradiation, or gaseous sterilization treatment will inactivate or remove any objectionable contaminant possibly present.

However, the selected treatment technique should not have any adverse effects. The treatment of raw materials by irradiation and ethylene oxide may cause unwanted changes affecting the safety and efficacy of the raw material. For instance, when treated by ethylene oxide, crude extracts containing alkaloids have shown reduced contents of alkaloids. Dry heat treatment has been used for inactivation as well, but it requires further evaluation because it may adversely affect stability and degradation of the raw material.

With regard to the design of the manufacturing process, appropriate consideration should be given to the microbiological effect of wet granulation manufacturing processes. Wetting of a dry powder can result in increased levels of microorganisms if the granulation is stored prior to drying. However, it is recognized that the pressure and temperature associated with compression of tablets will decrease microbial counts. Antimicrobial activity is also achieved, especially with aqueous preparations, by the addition of chemicals that have known antimicrobial properties and that are compatible with the formulation.

However, antimicrobial preservation is not a substitute for Good Manufacturing Practices. A process has to be designed to minimize the microbiological population. Operating procedures and temperatures and time limits, including holding times, are established to protect the product from microbiological contamination and growth. All processes have to be validated for their intended purposes. Moreover, in-process manufacturing and testing controls necessary for microbiological quality should be identified and implemented.

FACILITIES, EQUIPMENT, WATER, AND SANITIZATION

Facilities—The facilities, including the building and the heating, ventilation, and air-conditioning (HVAC) systems, should be designed to minimize microbiological contamination. The design of facilities used for the manufacture of supplements and their operating parameters should be documented, and the documentation should include, when appropriate, HVAC filter types, space pressure differentials, temperature, and relative humidity and air changes. Dry products processed in a dry environment do not possess a high potential for increased microbial levels. However, some control is warranted to minimize microbiological and chemical contamination. Potentially problematic areas are those that utilize *Purified Water* for wet granulation, batching liquid products, and film-coating tablets, because water encourages microbial growth.

Equipment—Equipment used for the processing of semi-solid and dry supplements should be designed to promote sanitary conditions, to be self-drying, and to be easy to clean. Dryers, ovens, wet granulation equipment, bulk tanks, and equipment for preparation of coating solutions are periodically evaluated to ensure that cleaning procedures are adequate.

Water—As one of the major components in nutritional and dietary supplement manufacturing processes, water deserves a special consideration in the microbiological control of these articles. It is a growth medium for a variety of microorganisms that present a threat to product quality, safety, preservation, and stability. Water may even act as a carrier of objectionable microorganisms. In view of this, water used in manufacturing is *Purified Water*. For the manufacture of raw materials, process water that meets specific microbiological objectives and U.S. Environmental Protection Agency National Drinking Water standards or equivalent European and Japanese standards may be used.

Cleaning and Sanitization—Detailed and specific cleaning and sanitization procedures should be evaluated, developed, and validated, with special attention given to product contact surfaces. Personnel should possess sufficient knowledge of these procedures.

SUPPLEMENT COMPONENTS

Raw materials, excipients, and active substances as components of nutritional and dietary supplements can be a primary source of microbiological contamination. Specifications should be developed and sampling plans and test procedures should be employed to guarantee the desired micro-

biological attributes of these materials. The nature and extent of microbiological testing should be based upon a knowledge of the material’s origin, its manufacturing process, its use, and historical data and experience. For instance, materials of animal or botanical origin that are not highly refined might require special, more frequent testing than synthetic products.

Since members of the family *Enterobacteriaceae* are a major component of the normal epiphytic and endophytic microflora (e.g., members of genera *Klebsiella*, *Enterobacter*, and *Erwinia*) and have been associated with the seeds, pods, roots, leaves, and stems of plants of economic importance, *Coliform* or *Enterobacteriaceae* counts will not be an appropriate general microbiological criterion for botanicals. However, when it is considered advantageous, *Coliform* or *Enterobacteriaceae* counts may be included in the individual monographs. Typically on new leaves, bacteria predominate in the microflora, while yeast and filamentous fungi succeed bacteria and become dominant late in the growing season. With dried botanicals, the bacterial population will tend to change from Gram-negative bacteria to Gram-positive spore formers and fungi. Refinement of botanicals from chopped or powdered plant material to powdered extracts using alcoholic, alkaline, acid hydro-alcoholic, or aqueous extracting materials will reduce the likelihood of vegetative microorganisms within the botanical material. The classification of botanical materials is contained in *Table 1*.

MICROBIOLOGICAL TESTING

Frequency of Sampling and Testing

Microbiological attribute sampling and testing plans vary widely. In some cases no sampling or testing is necessary; in

other cases periodic monitoring is warranted; and yet for some articles each batch requires sampling and testing. The design of the sampling and testing plans and the kind of attributes examined depend on the application and the type of the product, the potential for contamination from components and processing, the growth promotion or inhibition properties of the formulation, and the target population for the supplement. For example, a powdered botanical may have highly variable microbiological attributes so that an incoming batch would be sampled and composite testing would not be advised, while a highly refined botanical extract may not require routine microbial testing. Similarly, products with a low water activity will not be susceptible to microbial growth during their shelf life provided they are protected from elevated humidity by their containers.

Microbial Enumeration Tests

See the *Introduction* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* (2021). These tests provide meaningful information regarding the microbiological acceptability of excipients, active substances, and nonsterile supplement formulations. If the individual monograph does not specify microbial enumeration limits, the guidance provided in this chapter is used. Acceptable general limits of microbial levels for raw materials, excipients, and botanical products are shown in *Table 2*; and those for raw materials, excipients, active ingredients, and other nonsterile finished articles that are nutritional supplements but do not contain botanicals are shown in *Table 3*.

Table 1. Definitions of a Range of Botanical Materials

Botanical Preparation	Definition
Chopped or Powdered Botanicals	Hand-picked portions of the botanical (e.g., leaves, flowers, roots, tubers, etc.) that are air dried, and chopped, flaked, sectioned, ground, or pulverized to the consistency of a powder.
Botanical Extracts	Extracts are solids or semisolid preparations of a botanical that are prepared by percolation, filtration, and concentration by evaporation of the percolate. The extracting material may be alcoholic, alkaline, acid hydro-alcoholic, or aqueous in nature. Typically an extract is 4 to 10 times as strong as the original botanical. The extracts may be semisolids or dry powders termed powdered extracts.
Tinctures	Tinctures are solutions of botanical substances in alcohol obtained by extraction of the powdered, flaked, or sectioned botanical.
Infusions	Infusions are solutions of botanical principles obtained by soaking the powdered botanical in hot or cold water for a specified time and straining. Typically infusions are 5% in strength.
Decoctions	Decoctions are solutions of botanicals prepared by boiling the material in water for at least 15 minutes and straining. Typically decoctions are 5% in strength.
Fluidextracts	A fluidextract is an alcoholic liquid extract made by percolation of a botanical so that 1 mL of the fluidextract represents 1 g of the botanical.
Botanicals to be treated with boiling water before use	Dried botanicals to which boiling water is added immediately prior to consumption.

Table 2. Recommended Microbial Limits for Botanical Ingredients and Products

Material	Recommended Microbial Limit Requirements (cfu/g or mL)
Dried or Powdered Botanicals	Total Aerobic Microbial Count NMT 10 ⁵
	Total Combined Yeast & Mold Count NMT 10 ³
	Bile-tolerant Gram-negative Bacteria NMT 10 ³
	Absence of <i>Salmonella</i> spp. & <i>E. coli</i> in 10 g

Table 2. Recommended Microbial Limits for Botanical Ingredients and Products (Continued)

Material	Recommended Microbial Limit Requirements (cfu/g or mL)
Powdered Botanical Extracts	Total Aerobic Microbial Count NMT 10 ⁴
	Total Combined Yeast & Mold Count NMT 10 ³
	Absence of <i>Salmonella</i> spp. & <i>E. coli</i> in 10 g
Tinctures	Total Aerobic Microbial Count NMT 10 ⁴
	Total Combined Yeast & Mold Count NMT 10 ³
Fluidextracts	Total Aerobic Microbial Count NMT 10 ⁴
	Total Combined Yeast & Mold Count NMT 10 ³
Infusions/Decoctions	Total Aerobic Microbial Count NMT 10 ²
	Total Combined Yeast & Mold Count NMT 10
Nutritional Supplements with Botanicals	Total Aerobic Microbial Count NMT 10 ⁴
	Total Combined Yeast & Mold Count NMT 10 ³
	Absence of <i>Salmonella</i> spp. & <i>E. coli</i> in 10 g
Botanicals to be treated with boiling water before use	Total Aerobic Microbial Count NMT 10 ⁵
	Total Combined Yeast & Mold Count NMT 10 ³
	Absence of <i>E. coli</i> in 10 g

Table 3. Recommended Microbial Limits for Dietary Supplement Ingredients and Products

Material	Recommended Microbial Limit Requirements (cfu/g or mL)
Other raw materials and dietary supplement ingredients	Total Aerobic Microbial Count NMT 10 ³
	Total Combined Yeast & Mold Count NMT 10 ²
	Absence of <i>E. coli</i> in 10 g
Nutritional supplements with synthetic or highly refined ingredients	Total Aerobic Microbial Count NMT 10 ³
	Total Combined Yeast & Mold Count NMT 10 ²
	Absence of <i>E. coli</i> in 10 g

Absence of Objectionable Microorganisms

See Introduction under Microbiological Procedures for Absence of Specified Microorganisms—Nutritional and Dietary Supplements (2022). Absence of one or more species of objectionable microorganisms is required in some individual monographs.

Test for Aflatoxins—Dietary and nutritional articles containing botanical products with a history of mycotoxin contamination are also typically tested for aflatoxins, especially if the material is obtained from roots or rhizomes. See Articles of Botanical Origin (561) for the details of a test for aflatoxins. Where necessary, this test is included in the individual monograph.

Solid Oral Dosage Forms—Among all dosage forms, solid oral dosage forms present the lowest microbiological risk because of their method of manufacture, low water activity, and route of administration. When justified, reduced microbiological testing may be appropriate.

Other Concerns—The presence of some microorganisms in articles can be an indicator of processes that are not under microbiological control. For example, Purified Water used at some stage of the manufacture of these products might contain a typical flora of Gram-negative microorganisms. As with pharmaceutical products, inadequate processing of water and poor maintenance of water systems may result in the contamination of processed formulations by Gram-negative microorganisms.

(2030) SUPPLEMENTAL INFORMATION FOR ARTICLES OF BOTANICAL ORIGIN

This general chapter provides information about several aspects of botanical articles not covered in USP standards monographs. Although the standards in the monographs address the quality issues associated with botanical plant materials, extracts, and preparations of Pharmacopeial articles, there is a need to develop appropriate information to optimize the pre-harvest conditions for appropriate growth and the post-harvest handling to achieve consistent quality with minimum variations in the composition of chemical constituents.

PROTOCOL CONTENTS

- Black Cohosh (*Actaea racemosa* L.)
- Ginger (*Zingiber officinale* Roscoe)
- Valerian (*Valeriana officinalis* L.)
- Elm (*Ulmus rubra* Muhlenberg)

GENERAL GUIDANCES

It is recommended that, at a minimum, growers and others involved in the handling and distribution of botanical products should become familiar with and follow the WHO Guidelines for Good Agricultural and Collection Practices (GACP) for Medicinal Plants (found at <http://www.who.int/medicinedocs/collect/edweb/pdf/s4928e/s4928e.pdf>).

Commercial trade in natural products occurs in a global market. Material of domestic origin must be produced in

compliance with all federal laws of the United States. Material of foreign origin, imported into the U.S., must be produced and transported in compliance with the laws of the U.S., the country of origin, and relevant international treaties. These include, but may not be limited to, the following:

1. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) is an international agreement between governments. Its aim is to ensure that international trade in specimens of wild animals and plants does not threaten their survival. Information about CITES is available at <http://www.cites.org>.
2. The Convention on Biological Diversity (CBD) establishes three main goals: the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of the benefits from the use of genetic resources. Each country that has ratified and is a party to the Convention is responsible for implementation by means of national enabling legislation that can differ from country to country.
3. The Endangered Species Act (ESA) was originally adopted in 1973. The ESA is a law that aims to protect species of fish, wildlife, and plants believed to be threatened with extinction. The ESA is administered primarily by the U.S. Fish and Wildlife Service. Full text of the act is available at: <http://epw.senate.gov/esa73.pdf>.

Provided below is additional information not covered in the compendial specifications: compendial history; sources; collection and cultivation, including common adulterants; and drying, storing, and shipping. This information is provided to complement the standards for quality control in the monographs for botanical articles.

Compendial History—The focus in this section is on historical compendial use that has strong validity, with only brief reference to anecdotal use. This is important information because traditional use is one of the elements taken into consideration to support the safety and the presumptions of benefits of botanical dietary supplements.

Sources—Included here is the point of origin of the botanical; it also encompasses cultivation (defined as agricultural growing) and wildcrafting (defined as collected in the wild), along with a listing of the primary geographical (native) areas of production.

Collection and Cultivation—This section discusses wildcrafting, the conservation of restricted and rare species, and the trend to cultivation as an ecological alternative; such optimal harvesting and collection practices serve to preserve the integrity of species and botanical products. It is divided into three subsections:

1. Collection (conservation and ecology)
2. Cultivation Practices
3. Optimal Times for Harvest

Post-Harvest Handling (Optimal Handling and Processing Practices, Drying, Storage, and Shipping)—Important factors regarding storage of herbal products and how they should be maintained include the following.

1. **Light:** Protection from light is important for botanical articles. Light accelerates numerous chemical processes that may lead to degradation or changes in the constituents of the articles.
2. **Temperature:** Storage temperatures in this *Pharmacopeia* are defined in the *General Notices*. Excessive heat may affect the content of volatile constituents (essential oils) and accelerate degradation processes. However, heat treatments are sometimes useful in the maintenance of the article's quality and can be used in drying, reducing microbial load, and inhibiting certain enzymes. Heat application during these processes must be carefully controlled to achieve the desired balance between degradation and quality conservation.

3. **Humidity:** Moisture in the articles may allow certain enzymes such as glycosidases to become active, hence degrading constituents. High humidity also increases the danger of microbial proliferation. As a rule, it is advisable to store botanical articles below 60% relative humidity. Although controlled humidity and temperature warehouses are now required in many good manufacturing practices for natural products, much of the world still lacks access to these facilities.
4. **Degree of Comminution:** The degree of comminution plays a role in determining the stability of the botanical articles during storage. The increased surface area in fine powders allows oxidation and other degradation processes to occur more extensively and rapidly than in the case of a whole article. Plants containing tannins, bitter substances, and essential oils are particularly sensitive to the degree of comminution. In general, dried crude botanicals should be stored in a minimally processed form.
5. **Containers:** Appropriate containers are defined in this *Pharmacopeia* in the *General Notices*.

Constituents—Where known, the substances mainly responsible for the activity of the product are listed, along with other compounds contained in the plant.

SUPPLEMENTAL INFORMATION AND GENERAL GUIDANCE PROTOCOLS

Black Cohosh *Actaea racemosa* L. [*Cimicifuga racemosa* (L.) Nutt.] (Fam. Ranunculaceae)

Botanical Identification—*Actaea racemosa* L. Herbaceous perennial from rhizome.

Stem: Erect, solitary, to 2.5 m tall, glabrous.

Leaves: Basal and cauline, alternate, 2–4-ternately compound, petioles 15 to 60 cm long, bases clasping stem; leaflets 20 to 70; terminal leaflet of central division 3-lobed, 6 to 15 cm long, 6 to 16.5 cm wide, with 3 prominent veins arising from base; subterminal leaflets with blades ovate-lanceolate to obovate, 4 to 12 cm long and 3 to 8 cm wide; margins toothed to deeply incised; green above, paler below; glabrous or rarely pubescent along veins of undersurface.

Inflorescence: Terminal panicle of 4 to 9 slender branches, each 7 to 60 cm long, pubescent; 1 bract subtending each pedicel.

Flowers: Perfect, radially symmetric; sepals 4, greenish-white, caducous; petals 0; staminodes (1–) 4 (–8), petaloid, cream-colored, 2 to 3 mm long, clawed, apex bifid; stamens 55 to 110; pistils 1 (–3), glabrous to pubescent, ovary superior, style short, stigma 0.5 mm wide.

Fruit: Many-seeded follicle, 5 to 10 mm long, ovoid, laterally compressed with curved, stout beak (persistent style), pubescent; seeds hemispheric, brown, scales lacking.

Chromosome number: $n = 8$.

There are currently two varieties of *A. racemosa* recognized on the basis of differences in leaf morphology: var. *racemosa* and var. *dissecta*. The former variety has triternate-pinnate leaves with serrate margins, while the latter has quadraternate-pinnate leaves that are deeply incised with serrate lobes. Variety *dissecta* is only known from very few herbarium specimens, all of which were collected well over 100 years ago, making this taxon of uncertain taxonomic significance.

Compendial History—Black cohosh appeared on the secondary list of substances in the first *United States Pharmacopeia* (USP) of 1820, where it was listed as an anti-inflammatory and antispasmodic. It soon rose to the primary list in 1830, a position it held until the 10th decennial revision of

1920. Black cohosh appeared in the first edition of the *United States Dispensatory (USD)* in 1833 and remained through 1955 for a total of 122 years. Carrying forward the traditional Native American use of black cohosh for women's ailments and Barton's use for throat complaints, current therapeutics finds the plant used in a number of preparations for coughs and for gynecological disorders. In 2001, both the rhizome and the dry rhizome extract of black cohosh were proposed once again for inclusion in the *United States Pharmacopeia–National Formulary (USP–NF)*. (See revised proposal on page 1455 of *PF 28(5)* [Sept.–Oct. 2002].) The monograph became official in the *Second Supplement to USP 30–NF 25*.

Constituents—Major constituents of black cohosh are triterpene glycosides principally as beta-xylopyranosides and alpha-arabinopyranosides. The aglycones are mostly derived from acteol and cimigenol. The nomenclature of these compounds is quite confusing in the literature, with different names often given to the same compounds. A cyclopropane ring is a common feature of these compounds, which are structurally related to cycloartenol. The isoflavone formononetin has been reported in some publications; however, recent evidence indicates its absence in the roots and rhizomes of *Actaea racemosa*. Other constituents include tannins, resin, fatty acids, starch, sugars, and aromatic acids including ferulic acid, isoferulic acid, caffeic acid, and salicylic acid.

Sources and Distribution—

Sources—Black cohosh can be found in moist deciduous forests, ravines, moist meadows, creek margins, and mountainous terrain. Black cohosh flowers from June to September and is native to eastern North America from Ontario south to Georgia and west to Missouri. The entire supply of black cohosh comes from the United States. The major producers of black cohosh are Kentucky and Tennessee, with additional supplies coming from Georgia, Michigan, North Carolina, Ohio, South Carolina, Virginia, West Virginia, and Wisconsin. Although there are reports of black cohosh being grown in China and India for export, the true identity of the cultivated material has not been verified and may well be an Asian species of *Actaea* such as *A. cimicifuga* (syn. *Cimicifuga foetida*). The vast majority of the commercial black cohosh is wild harvested. Concern over the conservation of black cohosh due to increasing demand makes this species a good candidate for cultivation.

Distribution—North America (Ontario; Georgia, Kentucky, Michigan, Missouri, North Carolina, Ohio, South Carolina, Tennessee, Virginia, West Virginia, and Wisconsin); China; and India.

Collection and Cultivation—

Collection (Conservation and Ecology)—Traditionally, black cohosh has been harvested after plants become reproductive, which occurs anywhere from 2 to 8 years of age in cultivated plants, depending on growing techniques (see *Cultivation Practices*). A portion of the rhizome with a visible bud on it should be left in the ground to resprout the following year. There is no published information on the relationship between the constituent profile of the rhizome and its age, growing conditions, or place of origin, although such studies are underway. The impact of harvest on wild populations of black cohosh is currently unknown, and sources differ in their opinion about it. Whereas some maintain that current levels of harvest threaten the viability of wild populations, others feel that sustainable harvesting is possible at current levels of demand. A study of sustainable harvest limits is currently underway. The regulatory status regarding the trade of black cohosh is under review by CITES. Refraining from harvesting plants until after they have set seed and leaving a portion of the rhizome in the ground to resprout are key components to sustainable harvesting.

Cultivation Practices—Black cohosh is grown from rhizome cuttings or seeds and requires some shading, depending on

altitude and other environmental conditions. If grown from rhizome cuttings, a plant takes 2 to 3 years to become reproductive; grown from seed sown in the greenhouse and then planted, takes 4 to 6 years; direct-seeded may take from 6 to 8 years. Preliminary work indicates that black cohosh can be propagated successfully using in vitro techniques.

Optimal Times for Harvest—Rhizomes and roots should be harvested in autumn when the plant is dormant. At that time the underground portions of the plant have lower moisture content than in other seasons. Fall harvesting also allows plants to produce mature seeds before being uprooted.

Post-Harvest Handling—

Optimal Handling and Processing Practices—Rhizomes with roots may be processed fresh or dried. They should be thoroughly washed directly after harvest and then laid out to dry. Freshly harvested roots should be solid but not woody.

Drying—Rhizomes with roots are cut and air-dried at 35° to 45°. They are fully dried when they are brittle and snap easily and when no moisture is evident in cross section, either visibly or to the touch.

Storage—Follow general guidelines for storage by packing in airtight containers protected from light, heat, moisture, and insect infestation.

Adulterants and Contaminants—Other species of *Actaea*, especially yellow cohosh (*A. podocarpa* syn. *Cimicifuga americana*), have commonly been mixed with *A. racemosa* because of similarity in aboveground appearance and common growing habitat between species. The two species can be distinguished by differences in their freshly harvested underground parts: the fresh rhizome of *A. podocarpa* has a distinct yellowish hue, whereas that of *A. racemosa* is black. The rhizomes of both species are far more difficult to tell apart when dry because *A. podocarpa* darkens upon drying. The underground portions of baneberry (*Actaea pachypoda* and *A. rubra*) occur as occasional adulterants of black cohosh supplies. Fruiting plants of baneberry may be distinguished from black cohosh by their fleshy white or red poisonous berries, which contrast with the dry follicles of black cohosh. No information was available on how to distinguish the underground portions of black cohosh and baneberry from each other. According to one herb dealer, the roots of baneberry are smaller than those of black cohosh, and therefore are not often harvested by wildcrafters. In the Pacific Northwest, *Actaea elata* (syn. *Cimicifuga elata*) is collected for medicinal use.

Ginger *Zingiber officinale* Roscoe (Fam. Zingiberaceae)

Botanical Identification—*Zingiber officinale* Roscoe. Herbaceous perennial from tuberous rhizome, aromatic because of the presence of volatile oils.

Stem: Erect, unbranched pseudostem formed by the tight overlap of sheathing leaf bases; 0.9 to 1.5 m tall.

Leaf: Simple, alternate and two-ranked, sessile or petioles short with bases sheathing the stem and a ligule where the leaf base meets the stem; blade linear to narrowly lanceolate, 15 to 25 cm long, 1.5 to 3 cm wide; margin entire; glabrous to pubescent.

Inflorescence: Terminal spike, 3.5 to 8 cm long, 1.5 to 2 cm wide, with conspicuous spirally arranged primary bracts; usually borne on specialized leafless stems.

Flower: Perfect, bilaterally symmetric; calyx tubular with 3 lobes; corolla tube 2 to 2.5 cm long with lanceolate apical lobes, 1.5 to 2 cm long, 2 to 3.5 mm wide, greenish yellow; stamen 1, anther cream-colored with dark purple, elongated connective grasping upper part of style; staminodes 4, petaloid, 2 fused into an erect, ovate-oblong lip that is dull pur-

ple with cream mottling; ovary inferior; style 1, slender, exerted beyond connective.

Fruit: Loculicidal capsule; seeds shiny black with a white aril.

Chromosome number: n = 11.

There are several different varieties and forms of ginger. The varying morphological characteristics of these are displayed in *Table 1*.

Compendial History—Ginger was official in the *United States Pharmacopoeia* from the first edition of 1820 through the fourteenth revision of 1950, often appearing in multiple preparations. It also appeared in all editions of the *United States Dispensatory* from 1833 through the final edition of 1973, where it was described as “a stimulant and carminative that has been used for treatment of dyspepsia and flatulent colic”.

Constituents—The essential oils and the pungent principles make up some of the major components of the rhizome of ginger: 4.0% to 10.0% of the rhizome consists of an oleoresin composed of nonvolatile, pungent principles (phenols such as gingerols and their related dehydration products, shogaols); nonpungent fats and their waxes. The essential oil (1% to 3%) contains sesquiterpenes and monoterpenes, mainly geranial and nerals. Generally, but not always, sesquiterpenes predominate (30% to 70%), such as zingiberene, sesquiphellandrene, and beta-bisabolene, which decompose on drying and storage. The nonvolatile pungent principles include the phenylalkanones, the gingerols, and the phenylalkanonols, shogaols with varying chain lengths.

Sources and Distribution—

Sources—Ginger is cultivated in most tropical and subtropical countries to greater or lesser degrees. The world production is estimated to be 100,000 tons. China and India are reported to be the primary areas of production. Approximately 5000 tons of ginger are imported into the United States. An estimated 80% of this comes from China. In China, Sichuan and Guizhou provinces reportedly produce the largest quantities and highest quality. It is also produced in Guangdong, Hubei, Shandong, Shanxi, and Zhejiang provinces. Most of the dried ginger from China available in the United States has had the cortex scraped or rubbed off before it is dried. This gives it a whitish appearance. The freshly dug root is soaked overnight in water, scraped with a knife to remove the outer cortex, and then sun-dried. It has been reported that high arsenic levels in

the soil of Changning County of Hunan Province, China, has negatively affected ginger yields.

In India, ginger is grown on a large scale in the warm, moist regions of Madras and Cochin, and to a lesser extent in Bengal and the Punjab. Varieties grown in Bengal are reportedly the highest quality material in India. Other areas of production include Africa (Nigeria and Sierra Leone), Australia, the East Indies, Fiji, Hawaii, and Jamaica. The morphological characteristics of ginger cultivated in these different areas are outlined in *Table 1*.

In older literature, Jamaican ginger is reported to be the highest quality and the most aromatic, though supplies are limited.

Distribution—Most tropical and subtropical countries, such as Australia, China (Guangdong, Guizhou, Hubei, Shandong, Shanxi, Sichuan, and Zhejiang provinces), India (Bengal, Cochin, Malabar), the East Indies, Fiji, Jamaica, Japan, Nigeria, and Sierra Leone. Hawaii in the United States.

Collection and Cultivation—

Collection (Conservation and Ecology)—When the stems wither and are white, the rhizomes are ready for collection. Usually ginger is harvested after 6 months of growth at the earliest, and sometimes not until as late as 20 months; or to obtain larger roots, it is harvested in January or February of the second year of growth. In tropical and subtropical areas, roots are harvested as early as 4 months of growth, because they tend to become fibrous and tough as they get older. As ginger matures, it becomes more fibrous and stronger in flavor. Ginger harvest can be described in three stages:

1. Ginger that has been harvested early is known as green ginger and is traded as fresh ginger. It is succulent and tender, mellow, and mildly aromatic with a floral or lemony aroma and mild flavor.
2. Ginger harvested a few months later is more fibrous and drier and is collected for drying and may be sold as a full-flavored, pungent dried whole ginger.
3. The last harvest is usually around 9 months and yields the strongest ginger, which is quite dry and also richest in pungent components. This ginger is dried and then ground into powder.

Cultivation Practices—Ginger is a perennial herb that grows well at subtropical temperatures where the rainfall is at least 1.98 meters per year. The plant is sterile and is grown by vegetative means. Selected pieces of rhizome (“seed pieces” or “setts”), each bearing a bud, are planted in holes or trenches. Ideally the soil should be well-drained,

Table 1. Morphological and Key Characteristics of Ginger from Different Areas of Production

Source	Form	Aroma	Color (External)
Africa	Flat surfaces, mostly peeled, starchy and fibrous; 9 cm long, 1.5 cm wide	Poor quality is recognized by its camphoraceous aroma	Uncut surface dark grayish-brown; cut surface brownish-black
Australia		Citrus-like	Buff
Bengal	Flat surfaces, scraped		Gray-brown
China	Short stumpy lobes, unscraped, mostly sliced	Strong, floral to citrus	Pale brown
Cochin	Lateral surfaces lacking cork	Strong, floral to citrus	Cream color with numerous black resin dots
Jamaica (unbleached)	Up to 12 cm long, 1 cm wide; surfaces completely peeled; starchy and fibrous thin cortex	Delicate, citrus-like	All surfaces yellow-brown
Japan	Up to 7 cm long, 12 mm wide; flat surfaces usually completely peeled; starchy and fibrous thick cortex	Bergamot-like	Externally gray-white to light grayish-brown, often with white powder from being coated with lime
Malabar (Cochin and Calcutta)	Cork layer completely removed, mostly treated with chalk	Citrus-like	Almost white
Nigeria	Smaller in size than other varieties, rather less deeply scraped	Delicate	Somewhat darker than other varieties

rich clay loam. The growing conditions resemble those of potato cultivation. Mulching or manuring is necessary because the plant rapidly exhausts the soil of nutrients.

Ginger is susceptible to waterlogging and root rot. Preventive methods include using only the cleanest ginger for planting and washing it with fungicide before planting. A study growing ginger hydroponically yielded up to 125 tons per hectare in 6 to 7 months compared to 35 tons per hectare when grown in soil.

Optimal Times for Harvest: typically in December or January.

Post-Harvest Handling—

Optimal Handling and Processing Practices—After harvesting, the rhizome is cleaned and stripped of its stems and roots. Each area processes its ginger differently after harvest. This results in the different quality and commercial grades available on the market. Green ginger consists of the rhizomes sent to market without drying. Unscraped or partially scraped varieties are traded as coated or black ginger. These roots have been scalded with boiling water and dried quickly. When dry, black ginger breaks with a horny, blackish, somewhat diaphanous fracture, due to the pasty condition of the starch. White ginger is bleached, usually by rubbing with chalk or lime, to lighten its color and to prevent insect infestation. Preserved ginger consists of soft, yellowish-brown pieces obtained by steeping the fresh ginger in hot syrup and carefully bottling. It is soft, brown-yellow and translucent. When baked, ginger loses its pungency and acquires a bitter taste.

Drying—In general, after harvest, the fresh roots are washed, and the whole dark outer skin, consisting of cork and a little underlying parenchyma, is scraped away. Scraping speeds up the drying time of the crude drug. However, excessive scraping can result in lower concentrations of essential oil that is lost with the discarded epidermal tissue. After scraping, the rhizomes are then laid out on clean floors and dried in the sun for 7 to 10 days. During this time they are occasionally turned and are piled up every night. If the fresh rhizomes are too fleshy or moist, drying will take longer and the product will end up looking shriveled. To obtain a whiter product, the ginger is moistened after 5 or 6 days and dried for another 2 days, at which time it is ready for export. Dried ginger is more pungent and stronger in taste than fresh ginger.

Storage—Store in a tightly closed container, protected from light and moisture, in a cool area. A study was done on ginger harvested after 8, 9.5, 11, or 12 months. Samples were stored at 10° to 15° and 45% to 55% relative humidity or 25° to 30° and 75% relative humidity for 0, 4, or 8 weeks. Oil and oleoresin yields increased with the age of the ginger. Room temperature storage had adverse effects, but refrigerated storage for up to 4 weeks had no effect on quality. When stored for extended periods of time, ground ginger loses its pungency.

Adulterants—Because ginger is so characteristic, unintentional adulterants are rare. However, in East Asia sometimes the much larger *Zedoary cassumer* and *Zedoary zerumbet*, along with *Alpinia allughas*, are used and found in European commerce. They are easy to distinguish because of their characteristic aromas. Occasionally, Chinese sugar-candied “ginger” is prepared from *Alpinia galangal*.

In older literature, other herbs have reportedly been used as adulterants. These include various species of *Curcuma*, *Capsicum*, and Grains of Paradise (*Amomum melegueta*) added to exhausted material in order to enhance color and pungency.

Ginger powder is sometimes adulterated with plant starches such as those from wheat middlings, potatoes, corn, barley, rice, legumes, acorns, flaxseed meal, mannihot, oil cakes from linseed, rapeseed, mustard, almond meal, palm kernel or olives, hazelnut shells, and mineral additives.

These may be easily identified microscopically. The extent of this type of adulteration in trade is unknown.

Exhausted material should be considered an adulterant.

Valerian *Valeriana officinalis* L. (Fam. Valerianaceae)

Botanical Identification—*Valeriana officinalis* L. Herbaceous perennial, rhizomatous.

Stem: Solitary, hollow, 15 to 150 cm.

Leaf: Basal and cauline, opposite, oddly once pinnately lobed, lobes 11 to 21 lanceolate, entire or dentate, basal leaves petiolate, cauline leaves subsessile to clasping.

Inflorescence: Compound cyme, terminal or axillary, many pale pink to white, strongly scented flowers.

Flower: Calyx 5-lobed, lobes inconspicuous in flower, becoming elongate and pappus-like in fruit, corolla funnel-form, slightly saccate at the base, 5-lobed, tube 4 mm, lobes 1 mm, stamens 3, filaments attached to corolla tube alternate to corolla lobes, ovary inferior, trilobulate, uniovulate, only 1 locule fertile, stigma tripartite.

Fruit: Achene crowned by persistent calyx, lanceolate-oblong, 4.5 to 5 mm, hairy or glabrous. Populations of *V. officinalis* range in ploidy level from diploid to tetraploid or octaploid. British *V. officinalis* is usually octaploid, and central European supplies are tetraploid.

There are three subspecies of *V. officinalis*: ssp. *officinalis*, ssp. *collina* (Wallr.) Nyman, and ssp. *sambucifolia* (Mikan fil.) Celak. All three of these subspecies, as well as the other European species of valerian, *V. repens* Host, have been considered acceptable source material for medicinal preparations.

Macroscopic Identification—Various chemotypes will have slightly different characteristics. When dried, the whole rhizome is up to 50 mm long and up to 30 mm in diameter, obconical to cylindrical, with an elongated or compressed base. It has a yellowish-brown to dark brown exterior with a circular stem and leaf scars. The rhizome contains numerous thick, light to dark brown rootlets that are located around a thin ligneous cord. The root is longitudinally wrinkled, approximately 100 mm long and 1 to 3 mm in diameter, almost cylindrical, and almost the same color as the rhizome. In longitudinal section, the pith exhibits a central cavity transversely by septa. The stolons are 20 to 50 mm long, pale yellowish grey with prominent nodes separated by longitudinally striated internodes. It is commonly sliced in half for ease of cleaning. The rootlets, which contain the majority of the essential oil, are brittle and break in short, horny fractures and are whitish or yellowish internally. Aroma: when dried properly, *V. officinalis* L., s.l. has only a very faint characteristic, valeric acid-like aroma that becomes stronger as it ages. Improperly dried or old material possesses a strong and characteristic odor due to the enzymatic hydrolysis of esters of the valepotriates (isovaleric acid and hydroxyvaleric acid). Taste: mildly sweet and camphoraceous with a slightly bitter and spicy aftertaste.

Compendial History—Valerian was official in the *United States Pharmacopoeia* from the first edition of 1820 through the eleventh revision of 1930, often appearing in multiple preparations. At its peak from 1850 through 1880 it appeared six to seven times in different preparations. Valerian is among the top 30 most listed botanicals in the history of the *USP*. The root of valerian has been used as a sedative and spasmolytic in Europe since the 16th century.

Constituents—Major constituents of valerian have been identified as sesquiterpenes of volatile oils and iridoids (epoxy-triesters) known as valepotriates. The total content of volatile oil varies widely within a single species and between different species. European *Valeriana officinalis* L. usually contains 0.1% to 2.8% volatile oil. The oil consists of mixtures of monoterpene and sesquiterpene derivatives. The amount of valepotriates present also varies widely between species

and genera and even within a species, generally ranging from 0.5% to 1.2%. Valepotriates are particularly unstable; they decompose easily under the effect of moisture, temperatures above 40°, or acidity (pH <3).

Valerian also contains small amounts of aliphatic acids, alkaloids, amino acids, phenolic acids, flavonoids, free fatty acids, sugars, and salts. Valerian constituents that have possible sedative effects include acetoxyvalerenic acid, 1-acevaltrate, baldrinal, didrovaltrate, hydroxyvalerenic acid, kessane derivatives, valeranone, valerenal, valerenic acid, and valtrate.

Sources and Distribution—

Sources—Valerian is found in damp or dry meadows, scrub, or woods in most of Europe, although rare in the south, and it is cultivated and naturalized in North America. Valerian is cultivated in Belgium, Britain, Eastern Europe, France, Germany, Japan, the Netherlands, North America, and Russia. The majority of standardized extract products and crude cut and sifted material on the domestic market are prepared from European supplies. A large number of liquid extracts are prepared from domestically cultivated material. Many species other than *V. officinalis* are reported to be traded as medicinal valerian. These include *V. edulis* Nutt. ex Torr. & A. Gray, *V. corneana* Briq. k, *V. stubendorfi* Kreyer ex Kom., *V. amurensis* P. Smirn. ex Kom., *V. hardwickii* Wall., *V. exaltata* Mikan, and *V. wallichii* DC. syn. *V. jatamansi* Jones.* The most frequently used North American species include *V. sitchensis* Bong and *V. edulis* Nutt.* = *V. edulis* Nutt. ex Torr. & Gray ssp. *procera*. Other species reported to be used locally include *V. arizonica* Gray, *V. capitata* Pall ex Link., *V. dioica* L., and *V. scouleri* Rydb. Detailed chemical analyses of most American species are lacking. A limited number of assays of material cultivated in the Pacific Northwest show varying levels of essential oil ranging from 0.4% to 1.3%. Valerenic acid and valepotriates have been found to be present in fresh and dry samples of *V. sitchensis* Bong. *V. sitchensis* Bong exhibits a strong pungency when fresh. High quality material is reported to contain from 1.0% to 1.5% essential oil, ≥30% extractable matter, and ≥0.5% valerenic acid.

Distribution—Europe (Belgium, Britain, Eastern Europe, France, Germany, the Netherlands), Japan, North America, and Russia.

Collection and Cultivation—

Collection (Conservation and Ecology)—The majority of valerian in trade comes from cultivated material. Harvest times will vary geographically. The composition of the essential oil varies greatly among different populations of the same subspecies and even between the same population of plants from year to year. Essential oil content also varies with genotypes, harvest times, growing conditions, age of root, drying techniques, and method of analysis. It has been reported that valerian harvested in higher elevations, grown in dryer regions, or cultivated in phosphate-rich soil yields relatively high levels of essential oil.

Older literature reports that valerian should be harvested in the fall, between August and September, preferably in the second year of growth. Analyses of material cultivated in the Netherlands report that the majority of constituents, including the essential oil and valerenic acid, were highest in roots harvested in the first year of growth, with essential oil being highest in September and November (1.2% to 2.1%). The next highest level of essential oil was reported for material harvested in March (0.9% to 1.6%). Valerenic acid and its derivatives were found to be highest in February and March (0.7% to 0.9%), followed by material harvested in September (0.5% to 0.7%) and then in January (0.3% to 0.4%). From a commercial standpoint, it is more cost effective to harvest the roots in the same year the plants are sown than in the second year.

**V. wallichii* DC. and *V. edulis* Nutt. reportedly are lacking in valerenic acid and its derivatives.

Cultivation Practices—Sowing seeds has been reported to be preferred over planting of seedlings. Best results were achieved by flat field planting at row spacings of 50 cm and a seed rate of 3 kg per hectare. Cutting off the flowering tops before the plant has set seed causes the rhizome to develop more fully.

Optimal Times for Harvest—Wagner reports that harvest should take place in the morning during relatively cool weather, a general recommendation for roots rich in essential oils.

Post-Harvest Handling—

Optimal Handling and Processing Practices—The essential oil is located in the hypodermis of the rhizome in large thin-walled cells. Therefore, care must be taken not to damage these cells during handling. Excess washing of the roots can result in a significant reduction of extractive matter. Because of the sensitivity of volatile oils to heat, it is necessary to minimize the amount of time generated in the grinding or powdering process by doing small lots at a time, with frequent interruptions in run times, or by using a cryogenic grinder.

Drying—For maximum preservation of the essential oils, valerian should be dried at 40° with a flow rate of 0.05 kg per sec per m². Alternatively, drying at 20° for approximately 10 days, shade drying at approximately 45°, low temperature vacuum-drying, and freeze-drying are also reported to be appropriate drying techniques.

Careless or prolonged drying produces a darker color in the roots and results in the hydrolysis of the isovalerianic esters and the liberation of isovaleric and hydroxyisovaleric acid. This produces the characteristic valerianic aroma. Properly dried valerian will produce this same aroma over time.

Storage—Store in closed containers protected from light, air, and moisture. Hydroxyvalerenic acid, a decomposition product of acetoxyvalerenic acid, is formed when the herb is stored at too high humidity.

Improper storage conditions can cause significant deterioration of the material. Although the essential oil is relatively stable, it can evaporate with excessive exposure to air. The essential oil can degrade quickly in powdered material. In powdered root, the essential oil content can decrease by 50% within 6 months.

Valepotriates are sensitive to humidity, temperatures above 40°, and acid media (pH <3) and are generally not detected in commercial products after 60 days.

Adulterants—Other species of valerian: An unidentified *Apiaceae* species may be found in valerian trade. Adulteration of valerian in the American market is not common. Many species other than *V. officinalis* are reported to be traded as medicinal valerian. These include *V. edulis* Nutt. ex Torr. & A. Gray, *V. coreana* Briq. k, *V. stubendorfi* Kreyer ex Kom., *V. amurensis* P. Smirn. ex Kom., *V. hardwickii* Wall., *V. exaltata* Mikan, and *V. wallichii* DC. syn. *V. jatamansi* Jones.

Elm *Ulmus rubra* Muhlenberg [*Ulmus fulva* Michaux] (Fam. Ulmaceae)

Botanical Identification—*Ulmus rubra* Muhlenberg; tree to 35 m high, with spreading branches and open flat crown; preparations derived from inner bark. *U. rubra* appears to be more closely related to the introduced Asian species *U. pumila* L. than to other native American species of *Ulmus*; where the two co-occur, interbreeding is common.

Trunk: 18 to 35 m high, to 1 m in diameter; the trunk rises free of branches until about 5 to 6 m.

Branches: Erect, spreading; young twigs are scabrous-pubescent.

Bark: Dark brown to reddish-brown, deeply furrowed. Inner bark is whitish (outer surface yellow-orange; inner surface pale yellow), fragrant (upon powdering, a distinctive fenugreek-like odor) and very mucilaginous upon chewing or moistening.

Leaves: Alternate; simple; petiolate with petiole (3–)5–7(–9) mm long; 7–18(–23) cm long, 5–10(–15) cm broad; elliptical to ovate, oblong, or obovate with oblique base and acuminate apex; margins serrate toward base, elsewhere doubly serrate; upper surface scabrous, rough; lower surface tomentose; secondary veins parallel, slightly curved, running to tips of marginal teeth.

Inflorescence: Axillary fascicles, roughly hemispherical, to 1.5(–2.5) cm in diameter.

Flowers: Small, perfect; pedicels 1–2(–3) mm long; calyx campanulate, 5–9-lobed at apex, about 2.6–3.5 mm in diameter, reddish-pubescent; petals absent; stamens 5–9, exserted at flowering; styles 2. Flowers occur before the leaves from March through early May.

Fruit: Winged samara, yellowish, irregularly suborbicular or occasionally broadly elliptical or obovate, 10–20 mm in diameter, reddish-pubescent over seed; wing papery-textured.

Compendial History—Slippery elm (*Ulmus*) inner bark appeared in the list of materia medica in the first *United States Pharmacopoeia (USP)* of 1820 and remained official until it was removed from *USP XI* (1936). The *USP 1820* included instructions for the preparation of *Infusion of Slippery Elm*: “Take of Slippery elm, sliced, one ounce. Boiling water, one pint. Infuse for twelve hours in a covered vessel, near the fire with frequent agitation, and strain.” Immediately following its removal from *USP XI* (official: June 1, 1936), *Slippery Elm Bark* became an official monograph in the sixth edition of the *National Formulary (NF)*, official: June 1, 1936) until its elimination from the 11th edition (official: October 1, 1961). It became official again, as *Elm*, on November 15, 1995, in the *USP* section of the *Third Supplement to the United States Pharmacopoeia–National Formulary (USP 23–NF 18)*. A revision was published in the *Seventh Supplement* on November 15, 1997.

In 1982, Elm Bark appeared in the Food and Drug Administration (FDA) Advance Notice of Proposed Rulemaking (ANPR) for the establishment of a therapeutic monograph for oral health care drug products for over-the-counter (OTC) human use. In the ANPR (1982) as well as in the subsequent tentative final monograph of 1988 and in the amendment to the monograph of 1991, Elm bark was classified as a Category I (Generally Recognized as Safe and Effective (GRASE)) OTC oral demulcent active ingredient, and appropriate standards were urged to be developed in the official compendia.

Aside from *USP–NF*, the monograph of *Elm* had already appeared in the second edition of *The Dispensatory of the United States of America* (1834), and its last appearance was in the 25th edition, 1960.

Constituents—Constituents of relevance for conformance to *Identification A* under *Elm* are mucilaginous substances. Elm inner bark mucilage is readily extractable by water and consists principally of a polysaccharide which on hydrolysis yields D-galactose, D-methyl galactose, L-rhamnose, and glucose. Borohydride reduction of the periodate-oxidized polysaccharide affords, on partial hydrolysis with hot acid, three oligosaccharides: O-(3-O-methyl-β-D-galactopyranosyl)-(1→4)-O-(3-O-methyl-β-D-galactopyranosyl)-(1→4)-L-rhamnose, O-(3-O-methyl-β-D-galactopyranosyl)-(1→4)-L-rhamnose, and O-(3-O-methyl-β-D-galactopyranosyl)-(1→4)-3-O-methyl-D-galactose.

Other Elm constituents are traces of tannins, including proanthocyanidins, some starch, traces of oxalate salts, beta-sitosterol, and minerals.

Sources and Distribution—

Sources—Slippery elm bark is harvested from wild populations in eastern Canada and the United States, from southern Quebec west to North Dakota, south to south-central Texas, and Florida. It is common throughout eastern, southern, and midwestern U.S., and it grows in more than 25 states. An increasing amount of the commercial supply is being collected according to sustainable wild resource man-

agement plans as a condition of organic certification for wild crops. Conversely, Dutch elm disease has had a significant negative impact on elm populations, from 1930 when it was first found in the United States affecting over 50% of elm trees in the northern states.

Distribution—Canada (New Brunswick, Ontario, Quebec), the United States (Alabama, Arkansas, Connecticut, Delaware, the District of Columbia, Florida, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Maine, Maryland, Massachusetts, Michigan, Minnesota, Mississippi, Missouri, New Hampshire, New Jersey, New York, North Carolina, North Dakota, Ohio, Oklahoma, Pennsylvania, Rhode Island, South Carolina, South Dakota, Tennessee, Texas, Virginia, Vermont, Wisconsin, and West Virginia).

Collection and Cultivation—

Collection (Conservation and Ecology)—“Natural” (both inner and outer bark) and “rossed” (inner bark only) are harvested, but only the rossed bark will conform to the standards of the *USP* monograph. Harvesting should occur on dry, preferably warm days with no chance of precipitation, and it usually commences in late morning after the morning dew and humidity pass. This is because post-harvest processing (rossing) generally takes place at the collection site outdoors and humidity can damage the quality of the inner bark. Because the inner bark contains polysaccharide mucilage, when it comes in contact with moisture, it begins to gel.

The bark, from trees of a minimum age of 10 years (some harvesters recommend selecting at least 12- to 15-year-old trees) is obtained mainly by pruning or trimming the lower limbs and branches, but can also be obtained from the bole (trunk) and, very rarely, even from the roots in cases where the entire tree is felled. While the inner bark of the root reportedly contains more mucilage than that of the trunk or branches, sustainable wild resource management dictates harvesting only the branches of mature trees. In practice, commercial bark collectors are more likely to select branches from trees that are at least 30 to 50 years old in order to obtain a high enough yield. After pruning, the tree will heal over where harvested and continue to grow, but the trunk and/or branches should never be girdled, because this will kill the tree. Girdling is the stripping away of bark from the trunk or a branch all the way around. In practice, the most sustainable method of harvest from mature healthy trees is to prune off entire lower branches in a way that will not harm the tree. If sawn properly so that rainwater will not drop directly into the exposed cut area, the tree will grow over the cut area within a couple of years. The proper cut, which is made just outside the branch collar and the branch bark ridge, does the least amount of damage to the trees.

Although elm bark should not be gathered from already dead trees, selective collection from dying trees—for example, those affected with Dutch elm disease—is feasible. The fungus that causes Dutch elm disease, *Ophiostoma ulmi* and *Ophiostoma novo-ulmi* (syn: *Graphium ulmi* or *Ceratocystis ulmi*), is carried from tree to tree by the European elm bark beetle (*Scolytus multistriatus*), which arrived in North America on a ship carrying logs from Europe around 1930, and to a much lesser extent by the native elm bark beetle (*Hylurgopinus rufipes*). Elms appear to succumb to the disease at about 10 years of age and then die off in a two-year period. Once a tree is diagnosed with Dutch elm disease, healthy bark can still be harvested for about two years, up until the tree is near death. To monitor how long harvesting can continue, one can use a drawknife to take samples. Once the inner bark is showing increasingly noticeable black streaking, and as more and more limbs die and debarking is evident, the tree is near death. The near-dead tree can be dropped, and the remaining healthy bark can be stripped from the entire tree. Discolored (black-streaked) inner bark should be separated out and discarded. Pruning lower limbs from healthy trees and/or selectively harvesting entire older trees that are near death can both be acceptable methods

as part of a sustainable resource management plan for a specific area of forest under organic supervision.

Following organic production system rules (e.g., for harvest site selection, absence of prohibited substances, testing of soil and water, disallowed inputs, periodic residue testing, documentation control, independent inspection) will more likely result in botanical raw material that meets the general requirements for pesticide residue and heavy metals limits, among other potential contaminants. Certified organic production of wild elm bark requires producers to promote ecological balance and conserve biodiversity. Wild collected elm bark that is to be certified organic must be harvested from a designated area that has had no prohibited substance applied to it for a period of 3 years immediately preceding the harvest, and must be harvested in a manner that ensures that such harvesting or gathering will not be destructive to the environment and will sustain the growth and production of the wild crop. Wild crop producers must comply with the same organic system plan requirements and conditions, as applicable to their operation, as their counterparts who produce cultivated crops. The producer of organic wild harvested elm bark must initiate practices to support biodiversity and avoid, to the extent practicable, any activities that would diminish it. Production practices must maintain or improve the natural resources of the operation, including soil, water, wetlands, woodlands, and wildlife. This is accomplished, in part, by developing and executing a resource management plan that requires wild harvest from stable populations, minimizing disruption of priority species/sensitive habitats, avoiding erosion, allowing reestablishment, and monitoring wild crop sustainability.

Cultivation Practices—Even though the commercial supply is harvested from wild populations, slippery elm trees can be propagated by cuttings or by seed. For propagation by seed, the ripe seeds are collected from April to June from healthy and successful (dominant) trees from an area similar to the proposed planting site. A ripeness indicator is when the samaras (fruits) are green. It is best to collect seed from trees within 160 km north or south of the planting site, as potential for success is optimal within this range from the parents. Twenty-five seeds per square foot can be scattered, 0.6 cm deep. Slippery elm may be sown as in its normal cycle in the spring in a raised peat moss soil and sand bed. The seedbeds may need a wire top to protect young seedlings. Germination rate is 10% to 25%, with light germination in summer and increased germination the following spring. The young trees can be transplanted into tree tubes within the first month of germination and field planted after one or two years, depending on the size of the tree tube. The tree saplings must be watered during times of drought and routinely checked for insect predation and indications of fertilization needs.

Optimal Times for Harvest—Harvest should preferably occur in the spring (March to May), but can also take place in the autumn. In the spring, bark is harvested from mature trees (minimum 10 years) when the sap begins to rise.

Post-Harvest Handling—

Optimal Handling and Processing Practices—To produce pharmacopeial quality elm inner bark, the outer corky layer of bark must be removed, exposing the inner bark. If post-harvest processing occurs at the wild collection site, the pruned limbs and branches should be placed onto clean tarps and not directly on the ground. The very small branches with leaves are stripped off the pruned limbs by hand and discarded. To optimize conformance to standards for composition, identity, purity, and quality (e.g., NMT 2% of adhering outer bark, NMT 2% foreign organic matter, NMT 10% total ash, and NMT 0.65% acid-insoluble ash), a clean bark rosser (hand tool with handle and knife blade)

should be used to shave off the outer bark. The rough, scaly matter on the surface of the bark is called *ross*, and to *ross* bark is to scrape or shave the outer bark from the limb. An experienced rosser can visually discern that at least 98% of the outer bark has been shaved off. The inner bark is white in color (in the spring; reddish later in the season) in obvious visible contrast to the brown outer bark layer. After most of the outer bark is rossed off, greater care must be exercised to very carefully slice off the remaining thin layer of outer bark so as not to waste any of the inner bark in the process. After removal of the outer bark, the inner bark can then be removed in strips, squares, or chips. An incision is made with a clean knife down the center of the limb. Then a clean crowbar is slipped underneath the incision in order to lift and peel the inner bark off from the cambium. The strips of inner bark are stacked on a clean tarp and later bundled for transport to the drying facility.

Drying—*Elm USP* requires a loss on drying limit of NMT 12%. So long as rain is not expected, fresh elm inner bark can be sun-cured within a temperature range of 32° to 60°. Drying can also be carried out in a warm room with airflow or in a greenhouse. Greenhouse drying takes about 3 to 4 days. Drying indoors can take 5 to 7 days, depending on the heat source. Drying at commercial scale, however, is done typically in enclosed drying chambers, in which time and temperature can be better controlled. The strips of elm bark are placed onto a clean screen floor and dried over about 2 days' time at about 50° with fan-forced heat through the floor. Because of additional phytosanitary requirements for export of tree barks to Europe, higher heat exposure is necessary, usually at least 65° but up to 93° for up to two days. Post-drying, the strips of inner bark can be cut or sawn into pieces of equal length and bound into bundles with wire. The bundles usually consist of flat, oblong pieces, about 30 cm in length and from 10 to 15 cm in width. The bark strips can be stored this way until further processing (e.g., cutting or powdering) is scheduled.

Storage—To maintain pharmacopeial purity and quality (e.g., to prevent accumulation of excess moisture), dried elm inner bark should be preserved in well-closed containers, and stored in a cool, dry place.

Adulterants and Contaminants—Common contaminants that could cause a material not to conform with the identification tests in the *Elm* monograph in *USP* would include other plant parts: for example, greater than 2% outer bark, which lacks mucilage. Insufficient shaving or rossing of outer bark could cause the material to exceed the monograph limit of NMT 2% of adhering outer bark. Other possible contaminants would include visible discolored inner bark, although no maximum limit has been established (for example, inner bark with visible black streaking obtained from a diseased tree). Powdered bark can also be adulterated with cornmeal, rice flour, starch, or other starchy substances. Consequences of contamination with outer bark or adulteration with flour or starch are lower mucilage content, lower swelling index value, and correspondingly less of a therapeutic demulcent effect that is mucilage-dependent. Excess outer bark could also cause the material to fail the quantitative standard of NMT 10% total ash. Methods to determine the presence of adulterants include microscopic examination in order to determine the presence of excess outer bark or any other adulterant and the concentration of mucilage cells. The *Elm* mucilage test (*Identification A*) as well as a modified swelling volume test (based on the test in the *USP* monograph *Psyllium Husk*) may also be useful to investigate if adulteration is suspected.

(2040) DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS

Change to read:

INTRODUCTION

This general chapter is provided to determine compliance with the disintegration and dissolution standards for dietary supplements where stated in the individual monographs.

For the purposes of this chapter, dietary supplement dosage forms have been divided into three categories: *Vitamin–Mineral Dosage Forms*, *Botanical Dosage Forms*, and *Dietary Supplements Other Than Vitamin–Mineral and Botanical Dosage Forms*. *Vitamin–Mineral Dosage Forms* includes articles prepared with vitamins, minerals, or combinations of these dietary ingredients, ▲ as described in Table 1.▲ ^{USP36} *Botanical Dosage Forms* comprises formulations containing ingredients of botanical origin, including plant materials and extracts. *Dietary Supplements Other Than Vitamin–Mineral and Botanical Dosage Forms* encompasses dietary supplements formulated with lawfully recognized dietary ingredients that are different from those pertaining to the two foregoing categories (e.g., amino acids, chondroitin, and glucosamine.)

Where a dietary supplement represents a combination of the categories mentioned above, and there is a difference between the requirements for the individual categories, the more stringent requirement applies. ▲ [NOTE—“More stringent requirement” means stricter acceptance criteria and/or milder operational conditions.]

Disintegration and dissolution tests as described in this chapter are quality-control tools to assess performance characteristics of dietary supplement finished dosage forms. These performance standards are intended to detect problems that may arise due to use or misuse, or changes in coatings, lubricants, disintegrants, and other components. These performance tests are also intended to detect manufacturing process issues such as over-compression and over-drying that would affect the release characteristics of the final dosage forms. These tests are not intended to be used as a demonstration or as a surrogate for in vivo absorption, bioavailability, or effectiveness, unless an in vitro–in vivo correlation (IVIVC) has been established.▲ ^{USP36}

Change to read:

DISINTEGRATION

This test is provided to determine whether dietary supplement tablets or capsules disintegrate within the prescribed time when placed in a liquid medium at the experimental conditions presented below. Compliance with the limits on *Disintegration* stated in the individual monographs for dietary supplements is required except where the label states that the products are intended for use as troches, are to be chewed, or are designed as extended-release dosage forms. Dietary supplements claiming to be extended-release dosage forms must comply with standards other than disintegration to verify that the release of the dietary ingredients from the dosage form is for a defined period of time. Dietary supplements claiming to be extended-release dosage forms shall

not be labeled as in compliance with USP unless a USP monograph exists for such product. Determine the type of units under test from the labeling and from observation, and apply the appropriate procedure to 6 or more units.

For purposes of this test, disintegration does not imply complete solution of the unit or even of its active constituent. Complete disintegration is defined as that state in which any residue of the unit, except fragments of insoluble coating or capsule shell, remaining on the screen of the test apparatus or adhering to the lower surface of the disk, if used, is a soft mass having no palpably firm core.

Apparatus

Apparatus A: Use the *Apparatus* described under *Disintegration* (701) for tablets or capsules that are not greater than 18 mm long. For larger tablets or capsules, use *Apparatus B*.

Apparatus B: The apparatus¹ consists of a basket-rack assembly, a 1000-mL low-form beaker for the immersion fluid, a thermostatic arrangement for heating the fluid between 35° and 39°, and a device for raising and lowering the basket in the immersion fluid at a constant frequency rate between 29 and 32 cycles per min through a distance of NLT 53 mm and NMT 57 mm. The volume of the fluid in the vessel is such that at the highest point of the upward stroke the wire mesh remains at least 15 mm below the surface of the fluid and descends to NLT 25 mm from the bottom of the vessel on the downward stroke. At no time should the top of the basket-rack assembly become submerged. The time required for the upward stroke is equal to the time required for the downward stroke, and the change in stroke direction is a smooth transition rather than an abrupt reversal of motion. The basket-rack assembly moves vertically along its axis. There is no appreciable horizontal motion or movement of the axis from the vertical.

Basket-Rack Assembly: The basket-rack assembly (see Figure 1) consists of three open-ended transparent tubes, each 77.5 ± 2.5 mm long and having an inside diameter of 32.0–34.6 mm and a wall 2.0–3.0 mm thick; the tubes are held in a vertical position by two plastic plates, each 97 ± 2 mm in diameter and 7.5–10.5 mm in thickness, with three holes, 36.0–40.6 mm in diameter, equidistant from the center of the plate and equally spaced from one another. Attached to the undersurface of the lower plate is 10-mesh No. 23 (0.025-inch) W. and M. gauge woven stainless-steel wire cloth having a plain square weave. The parts of the apparatus are assembled and rigidly held by means of three bolts passing through the two plastic plates. A suitable means is provided to suspend the basket-rack assembly from the raising and lowering device, using a point on its axis.

The design of the basket-rack assembly may be varied somewhat, provided that the specifications for the glass tubes and the screen mesh size are maintained.

Beaker: Low form, 1000 mL; the difference between the diameter of the plastic plates, which hold the tubes in a vertical position, and the inside diameter of the beaker should be NMT 6 mm.²

Disks: Each tube is provided with a perforated cylindrical disk 15.3 ± 0.15 mm thick and 31.4 ± 0.13 mm in diameter. The disk is made of a suitable, transparent plastic material having a specific gravity of between 1.18 and 1.20. Seven 3.15 ± 0.1-mm ▲ in diameter,▲ ^{USP36} holes extend between the ends of the cylinder, ▲ one of the holes being in the center and the other six parallel with it and spaced

¹ An apparatus and disks meeting these specifications are available from Varian Inc., 13000 Weston Parkway, Cary, NC 27513, or from laboratory supply houses.

² 1000-mL low-form beakers, designed in compliance with the current ASTM E 960 Type I or Type II or ISO 3819 specifications, meet the size requirements.

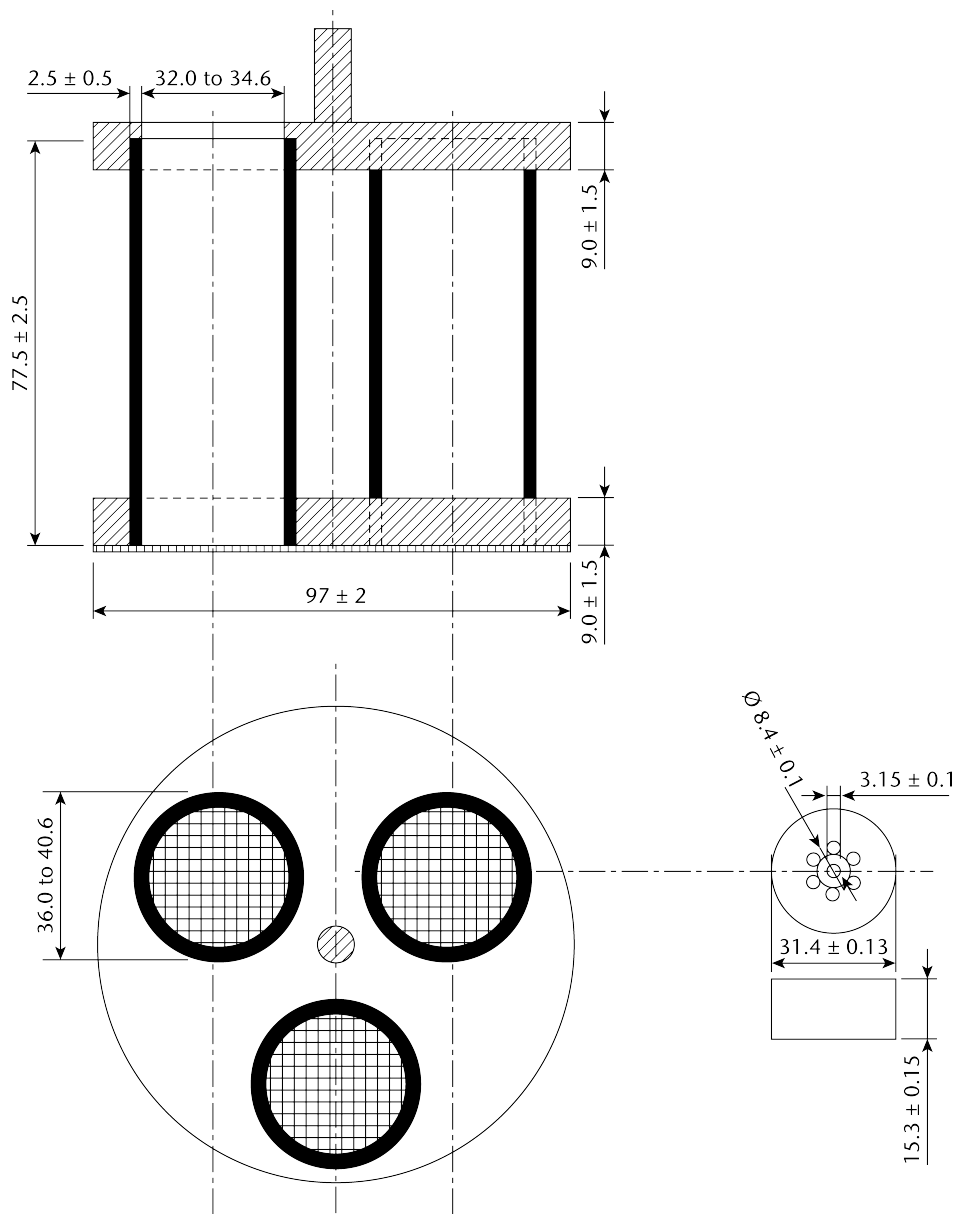


Figure 1. Basket-rack assembly, *Disintegration, Apparatus B* (dimensions in mm).

equally tangent to a circle with a radius of 4.2-mm from the center of the disc.▲▲³ USP36 All surfaces of the disk are smooth.³

Procedure

▲ Test 6 dosage forms as described below for each dosage type. [NOTE—Two basket arrangements for a total of 6 tubes are necessary for *Apparatus B*.] If 1 or 2 dosage forms fail to disintegrate completely, repeat the test on 12 additional dosage forms.▲▲³ USP36

Uncoated Tablets: Place 1 tablet in each of the tubes of the basket and, if prescribed, add a disk to each tube. Operate the apparatus, using water or the specified medium as the immersion fluid, maintained at $37 \pm 2^\circ$. At the end of 30 min, lift the basket from the fluid, and observe the tablets. ▲▲³ USP36

³ The use of automatic detection using modified disks is permitted where the use of disks is specified or allowed. Such disks must comply with the requirements for density and dimensions given in this chapter.

Plain Coated Tablets: Place 1 tablet in each of the tubes of the basket and, if the tablet has a soluble external sugar coating, immerse the basket in water at room temperature for 5 min. Then, if prescribed, add a disk to each tube, and operate the apparatus, using water or the specified medium as the immersion fluid, maintained at $37 \pm 2^\circ$. At the end of 30 min, lift the basket from the fluid, and observe the tablets. ▲▲³ USP36

Delayed-Release (Enteric-Coated) Tablets: ▲ Omit the use of a disk.▲▲³ USP36 Place 1 tablet in each of the 6 tubes of the basket, and if the tablet has a soluble external sugar coating, immerse the basket in water at room temperature for 5 min. Then operate the apparatus using simulated gastric fluid TS maintained at $37 \pm 2^\circ$ as the immersion fluid. After 1 h of operation in simulated gastric fluid TS, lift the basket from the fluid, and observe the tablets: the tablets show no evidence of disintegration, cracking, or softening. Operate the apparatus, using simulated intestinal fluid TS, maintained at $37 \pm 2^\circ$, as the immersion fluid for the time specified in the monograph. Lift the basket from the fluid, and observe the tablets. ▲▲³ USP36

Delayed-Release (Enteric-Coated) Soft Shell Capsules:

Place 1 softgel capsule in each of the 6 tubes of the basket. ▲▲ *USP36* Omit the use of a disk. Operate the apparatus using simulated gastric fluid TS maintained at $37 \pm 2^\circ$ as the immersion fluid. After 1 h of operation in simulated gastric fluid TS, lift the basket from the fluid and observe the softgels: the softgels show no evidence of disintegration or rupture permitting the escape of the contents. Operate the apparatus with disks, using simulated intestinal fluid TS, maintained at $37 \pm 2^\circ$, as the immersion fluid ▲ for NMT 60 min. ▲ *USP36* Lift the basket from the fluid, and observe the capsules. ▲▲ *USP36*

Buccal Tablets: Apply the test for *Uncoated Tablets*. After 4 h, lift the basket from the fluid, and observe the tablets. ▲▲ *USP36*

Sublingual Tablets: Apply the test for *Uncoated Tablets*. At the end of the time limit specified in the individual monograph, ▲ observe the tablets. ▲ *USP36*

Hard Shell Capsules: Apply the test for *Uncoated Tablets*, using as the immersion fluid, maintained at $37 \pm 2^\circ$, a 0.05 M acetate buffer prepared by mixing 2.99 g of sodium acetate trihydrate and 1.66 mL of glacial acetic acid with water to obtain a 1000-mL solution having a pH of 4.50 ± 0.05 . Attach a removable wire cloth, as described in *Basket-Rack Assembly*, to the surface of the upper plate of the basket-rack assembly. At the end of 30 min, lift the basket from the fluid, and observe the capsules. ▲▲ *USP36*

Soft Shell Capsules: Proceed as directed in *Rupture Test for Soft Shell Capsules*.

Use of Disks

Vitamin–Mineral Dosage Forms: Add a disk to each tube unless otherwise specified ▲ in the *Procedure* above or ▲ *USP36* in the individual monograph.

Botanical Dosage Forms: Omit the use of disks unless otherwise specified ▲ in the *Procedure* above or ▲ *USP36* in the individual monograph.

Dietary Supplements Other Than Vitamin–Mineral and Botanical Dosage Forms: Omit the use of disks unless otherwise specified ▲ above or ▲ *USP36* in the individual monograph.

▲ Tolerances

All of the 6 dosage forms initially tested or NLT 16 of a total of 18 dosage forms tested disintegrate completely. ▲ *USP36*

RUPTURE TEST FOR SOFT SHELL CAPSULES

Medium: Water; 500 mL.

Apparatus: Use *Apparatus 2* as described in *Dissolution* (711), operating at 50 rpm.

Time: 15 min

Procedure: Place 1 capsule in each vessel, and allow the capsule to sink to the bottom of the vessel before starting rotation of the blade. Use sinkers if the capsules float. Observe the capsules, and record the time taken for each capsule shell to rupture.

Tolerances: The requirements are met if all of the capsules tested rupture in NMT 15 min. If 1 or 2 of the capsules rupture in more than 15 but NMT 30 min, repeat the test on 12 additional capsules: NMT 2 of the total of 18 capsules tested rupture in more than 15 but NMT 30 min. For soft gelatin capsules that do not conform to the above rupture test acceptance criteria, repeat the test with the addition of purified pepsin to the *Medium* that results in an activity of 750,000 Units or less per 1000 mL.

Change to read:**DISSOLUTION**

This test is provided to determine compliance with the *Dissolution* requirements where stated in the individual monograph for dietary supplements, except where the label states that tablets are to be chewed. ▲ The operative assumption inherent in this test is that if the index vitamin or mineral or marker compound(s) for a botanical is dissolved within the time frame and under conditions specified, the dosage form does not suffer from formulation or manufacturing-related problems affecting the adequate release of the active ingredients.

Apparatus▲ *USP36*

See *Dissolution* (711) for description of apparatus used, *Apparatus Suitability Test*, and other related information.

▲▲ *USP36* Figure 2 shows the schematic view of a flow-through cell ▲ (USP Apparatus 4) specifically intended for dissolution of lipid-filled soft shell capsules. ▲ *USP36* The lower part (1) is made up of two adjacent chambers connected to an overflow device. The dissolution medium passes through chamber A and is subjected to an upward flow. The flow in chamber B is directed downward to a small-size bore exit that leads upward to a filter assembly. The middle part (2) of the cell has a cavity designed to collect lipophilic excipients that float on the dissolution medium. A metal grid serves as a rough filter. The upper part (3) holds a filter unit for paper, glass fiber, or cellulose filters.

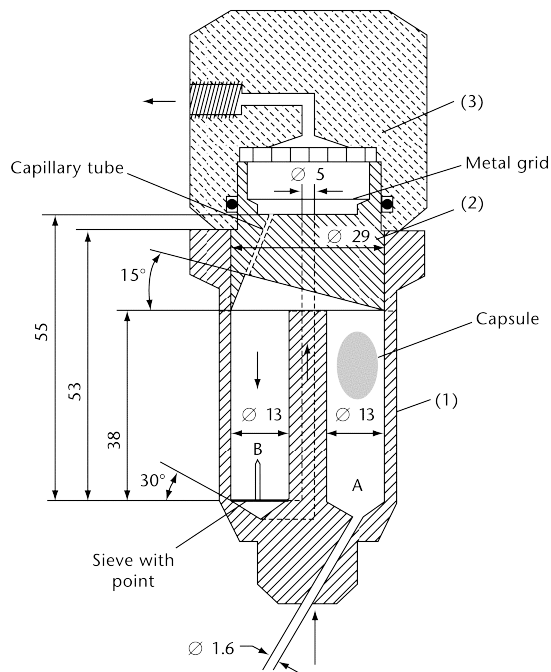


Figure 2. Flow-through cell designed for lipid-filled soft gelatin capsules (dimensions in mm).

Of the types of apparatus described in (711), use the one specified in the individual monograph.

For hard or soft gelatin capsules and gelatin-coated tablets that do not conform to the dissolution specification, repeat the test as follows. Where water or a medium with a pH of less than 6.8 is specified as the *Medium* in the individ-

ual monograph, the same *Medium* specified may be used with the addition of purified pepsin that results in an activity of 750,000 Units or less per 1000 mL. For media with a pH of 6.8 or greater, pancreatin can be added to produce NMT 1750 USP Units of protease activity per 1000 mL.

▲▲ USP36

Vitamin–Mineral Dosage Forms

▲ All dietary supplement tablets or capsules containing folic acid are subject to the dissolution test and criteria described in this chapter for folic acid. This test is required because of the importance of the relationship between folate deficiency and the risk of neural tube defects. Dietary supplements tablets or capsules containing water-soluble vitamins, minerals, or their combination are subject to the dissolution test and criteria described in this chapter for index vitamins, index minerals, or both. Dietary supplement tablet and hard shell capsule with solid contents dosage forms containing vitamin A are subject to the dissolution test and criteria described in this chapter for vitamin A. Dissolution standards were not established and therefore not applicable to vitamin A in dietary supplement soft shell capsules filled with liquids. *Table 1* summarizes the dissolution requirements for the assigned USP classes of dietary supplements. Vitamin–mineral combinations that don’t belong to any of the USP classes listed in the *Table 1* are subject to the dissolution test and criteria specified in the individual monographs.

Table 1. Dietary Supplements—Vitamin–Mineral Dosage Forms

USP Class	Ingredients	Dissolution Requirement for Tablets and Hard Shell Capsules With Solid Contents	Dissolution Requirement for Soft Shell Capsules Filled With Liquids
I	Oil-Soluble Vitamins	vitamin A (if present)	not applicable
II	Water-Soluble Vitamins	one index water-soluble vitamin and folic acid (if present)	one index water-soluble vitamin and folic acid (if present)
III	Water-Soluble Vitamins with Minerals	one index water-soluble vitamin, one index element, and folic acid (if present)	one index water-soluble vitamin, one index element, and folic acid (if present)
IV	Oil- and Water-Soluble Vitamins	vitamin A (if present), one index water-soluble vitamin, and folic acid (if present)	one index water-soluble vitamin and folic acid (if present)
V	Oil- and Water-Soluble Vitamins with Minerals	vitamin A (if present), one index water-soluble vitamin, one index element, and folic acid (if present)	one index water-soluble vitamin, one index element, and folic acid (if present)

Table 1. Dietary Supplements—Vitamin–Mineral Dosage Forms (Continued)

USP Class	Ingredients	Dissolution Requirement for Tablets and Hard Shell Capsules With Solid Contents	Dissolution Requirement for Soft Shell Capsules Filled With Liquids
VI	Minerals	one index element	one index element
VII	Oil-Soluble Vitamins with Minerals	vitamin A (if present) and one index element	one index element

▲ USP36

SELECTION OF INDEX ▲ WATER-SOLUBLE▲ USP36 VITAMINS AND INDEX ELEMENTS

Compliance with the dissolution requirements for dietary supplements representing combinations of water-soluble vitamins (*Water-Soluble Vitamins Capsules* and *Water-Soluble Vitamins Tablets*) and combinations of oil- and water-soluble vitamins (*Oil- and Water-Soluble Vitamins Capsules* and *Oil- and Water-Soluble Vitamins Tablets*) is determined by measuring the dissolution of a single index vitamin from the water-soluble vitamins present. Riboflavin is the index vitamin when present in the formulation. For formulations that do not contain riboflavin, pyridoxine is the index vitamin. If neither riboflavin nor pyridoxine is present in the formulation, the index vitamin is niacinamide (or niacin), and in the absence of niacinamide (or niacin), the index vitamin is thiamine. If none of the above four water-soluble vitamins is present in the formulation, the index vitamin is ascorbic acid.

Compliance with the dissolution requirements for dietary supplements representing combinations of minerals (*Minerals Capsules* and *Minerals Tablets*) is determined by measuring the dissolution of only one index element. Iron is the index element when present in the formulation. For formulations that do not contain iron, the index element is calcium. If neither iron nor calcium is present, the index element is zinc, and in the absence of all three of these elements, magnesium is the index element.

Compliance with the dissolution requirements for dietary supplements representing combinations of water-soluble vitamins and minerals (*Water-Soluble Vitamins with Minerals Capsules* and *Water-Soluble Vitamins with Minerals Tablets*) and combinations of oil- and water-soluble vitamins and minerals (*Oil- and Water-Soluble Vitamins with Minerals Capsules* and *Oil- and Water-Soluble Vitamins with Minerals Tablets*) is determined by measuring the dissolution of one index water-soluble vitamin and one index element, designated according to the respective hierarchies described above.

DISSOLUTION CONDITIONS FOR VITAMIN A TABLETS

NOTE—Perform this test under light conditions that minimize photodegradation.

Medium: 1% (w/v) sodium ascorbate and 1% (w/v) octoxynol 9 in 0.05 M phosphate buffer pH 6.8; 900 mL

Apparatus 2: 75 rpm

Time: 45 min

DISSOLUTION CONDITIONS FOR FOLIC ACID

NOTE—Perform this test under light conditions that minimize photodegradation.

Test 1

Medium: Water; 900 mL. If the units tested do not meet the requirements for dissolution in water, test 6 additional dosage units for dissolution in a medium of 900 mL of 0.05 M pH 6.0 citrate buffer solution, prepared by mixing 9.5 mL of 0.1 M citric acid monohydrate and 40.5 mL of 0.1 M sodium citrate dihydrate in a 100-mL volumetric flask, diluting with water to volume, mixing, and adjusting to a pH of 6.0 by using either 0.1 M hydrochloric acid or 0.1 M sodium hydroxide solution.

Apparatus 1: 100 rpm, for capsules

Apparatus 2: 75 rpm, for tablets

Time: 1 h

Test 2: If the product complies with this test, the labeling indicates that it meets USP *Dissolution Test 2*.

Medium: 45 mM citrate buffer, pH 6.0; 250 mL

Apparatus 3: 30 dpm

Screen (Top & Bottom): 56-mesh

Time: 1 h

NOTE—Compliance with the dissolution requirements for folic acid does not exempt the product from dissolution testing of the pertinent index vitamin or the corresponding index mineral.

DISSOLUTION CONDITIONS FOR INDEX WATER-SOLUBLE VITAMINS AND INDEX MINERALS

Test 1

Medium: 0.1 N hydrochloric acid; 900 mL

Apparatus 1: 100 rpm, for capsules

Apparatus 2: 75 rpm, for tablets

Time: 1 h

For formulations containing 25 mg or more of the index vitamin, riboflavin, use the following conditions:

Medium: 0.1 N hydrochloric acid; 1800 mL

Apparatus 1: 100 rpm, for capsules

Apparatus 2: 75 rpm, for tablets

Time: 1 h

Test 2 (Not suitable for Minerals)

If the product complies with this test, the labeling indicates that it meets USP *Dissolution Test 2*.

Medium: 45 mM citrate buffer, pH 6.0; 250 mL

Apparatus 3: 30 dpm

Screen (Top & Bottom): 56-mesh

Time: 1 h

NOTE—Compliance with dissolution requirements for the pertinent index vitamin or index mineral does not exempt the product from dissolution testing of folic acid, if present.

PROCEDURES

In the following procedures, combine equal volumes of the filtered solutions of the 6 individual specimens withdrawn, and determine the amount of vitamin A, folic acid, or the index vitamin or element dissolved, based on the average of 6 units tested. Make any necessary modifications including concentration of the analyte in the volume of *Sample solution* taken. Use the *Medium* for preparation of the *Standard solution* and dilution, if necessary, of the *Sample solution*.

Vitamin A: Determine the percentage of retinyl acetate or retinyl palmitate dissolved by using the following procedure.

Sample solution: Withdraw a portion of the solution under test, pass through a suitable filter of 0.45- μ m pore size, and use the pooled sample as the test specimen.

Standard solution: Dissolve a suitable amount of USP Retinyl Acetate RS or USP Retinyl Palmitate RS in isopropyl

alcohol, and dilute with *Medium* to obtain a concentration similar to that expected in the *Sample solution*. [NOTE—The amount of alcohol should be 5%–10%.]

Solution A: Methanol and water (90:10)

Solution B: Methanol and isopropyl alcohol (55:45)

Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	100	0
8	0	100
13	0	100
13.1	100	0
15	100	0

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm \times 10-cm; 3- μ m packing L1

Flow rate: 1.0 mL/min

Injection volume: 50 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 1.5 for retinyl acetate and NMT 2.0 for retinyl palmitate

Relative standard deviation: NMT 2.0%

Analysis

Samples: Appropriate *Standard solution* and *Sample solution*

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the appropriate *Standard solution*

C_S = concentration of retinol ($C_{20}H_{30}O$) in the appropriate *Standard solution* (μ g/mL)

V = volume of *Medium*, 900 mL

L = label claim of vitamin A, as retinol ($C_{20}H_{30}O$) (μ g/Tablet)

Folic Acid: Determine the amount of $C_{19}H_{19}N_7O_6$ dissolved by using the procedure set forth in the assay for *Folic Acid in Oil- and Water-Soluble Vitamins with Minerals Tablets*, in comparison with a *Standard solution* having a known concentration of USP Folic Acid RS in the same *Medium*.

Niacin or Niacinamide, Pyridoxine, Riboflavin, and Thiamine: Determine the amount of the designated index vitamin dissolved by using the procedure set forth in the *Assay for Niacin or Niacinamide, Pyridoxine Hydrochloride, Riboflavin, and Thiamine in Water-Soluble Vitamins Tablets*.

Ascorbic Acid: Determine the amount of $C_6H_8O_6$ dissolved by adding 10 mL of 1.0 N sulfuric acid and 3 mL of starch TS to 100.0 mL of *Sample solution*, and titrating immediately with 0.01 N iodine VS. Perform a blank determination, and make any necessary correction.

Iron, Calcium, Magnesium, and Zinc: Determine the amount of the designated index element dissolved by using the procedure set forth in the appropriate assay in *Minerals Capsules*.

TOLERANCES

The requirements are met if NLT 75% of the labeled content of vitamin A, NLT 75% of the labeled content of folic

acid, and NLT 75% of the labeled content of the index vitamin or the index element from the units tested is dissolved.

Botanical Dosage Forms

Compliance with dissolution requirements necessitates the testing of 6 dosage units individually, or testing 2 or more dosage units in each of the 6 vessels of the dissolution apparatus, and measuring the dissolution of one or more index/marker compound(s) or the extract specified in the individual monograph.

PROCEDURES

Combine equal volumes of the filtered solutions of the 6 or more individual specimens withdrawn, and use the pooled sample as the *Sample solution*. Determine the average amount of index or marker compound(s) or the extract dissolved in the pooled sample by the procedure specified in the individual monograph. Make any necessary modifications, including concentration of the analyte in the volume of the *Sample solution* taken. Use the *Medium* for preparation of the *Standard solution* and dilution, if necessary, of the *Sample solution*.

TOLERANCES

Unless otherwise specified in the individual monograph, the requirements are met if NLT 75% of the labeled content of the index or marker compound(s) or the extract from the units tested is dissolved in 1 h.

Dietary Supplements Other Than Vitamin–Mineral and Botanical Dosage Forms

Unless otherwise stated in the individual monographs for dietary supplement dosage forms in this category, compliance requires the testing of 6 individual units, measuring the dissolution of the dietary ingredient as the average of the 6 units tested.

PROCEDURES

Combine equal volumes of the filtered solutions of the 6 specimens withdrawn, and use the pooled sample as the *Sample solution*. Determine the average amount of dietary ingredient dissolved in the pooled sample by the procedure specified in the individual monograph. Make any necessary modifications, including concentration of the analyte in the volume of the *Sample solution* taken. Use the *Medium* for preparation of the *Standard solution* and for dilution, if necessary, of the *Sample solution*.

TOLERANCES

Because of the diversity of chemical characteristics and solubilities of dietary ingredients pertaining to this category,

general tolerances cannot be established. See individual monographs for *Tolerances*.

(2091) WEIGHT VARIATION OF DIETARY SUPPLEMENTS

The following tests provide limits for the permissible variations in the weights of individual tablets or capsules, expressed in terms of the allowable deviation from the average weight of a sample. Separate procedures and limits are described herein for capsules, uncoated tablets, and coated tablets that are intended for use as dietary supplements.

CAPSULES

Capsules meet the requirements of the following test with respect to variation in weight of contents.

Hard Capsules

Weigh 20 intact capsules individually, and determine the average weight. The requirements are met if each of the individual weights is within the limits of 90% and 110% of the average weight.

If not all of the capsules fall within the aforementioned limits, weigh the 20 capsules individually, taking care to preserve the identity of each capsule, and remove the contents of each capsule with the aid of a small brush or pledget of cotton. Weigh the emptied shells individually, and calculate for each capsule the net weight of its contents by subtracting the weight of the shell from the respective gross weight. Determine the average net content from the sum of the individual net weights. Then determine the difference between each individual net content and the average net content: the requirements are met if (a) not more than 2 of the differences are greater than 10% of the average net content and (b) in no case is the difference greater than 25%.

If more than 2 but not more than 6 capsules deviate from the average between 10% and 25%, determine the net contents of an additional 40 capsules, and determine the average content of the entire 60 capsules. Determine the 60 deviations from the new average: the requirements are met if (a) in not more than 6 of the 60 capsules does the difference exceed 10% of the average net content and (b) in no case does the difference exceed 25%.

Soft Capsules

Proceed as directed under *Hard Capsules*, but determine the net weight of the contents of individual capsules as follows. Weigh the intact capsules individually to obtain their gross weights, taking care to preserve the identity of each capsule. Then cut open the capsules by means of a suitable clean, dry cutting instrument, such as scissors or a sharp open blade, and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature over a period of about 30 minutes, taking precautions to avoid uptake or loss of moisture. Weigh the individual shells, and calculate the net contents. The requirements are as stated under *Hard Capsules*.

TABLETS

Tablets conform to the criteria given in the accompanying table.

Uncoated Tablets and Film-Coated Tablets

Weigh individually 20 whole tablets, and calculate the average weight. The requirements are met if the weights of not more than 2 of the tablets differ from the average weight by more than the percentage listed in the accompanying table and no tablet differs in weight by more than double that percentage.

Coated Tablets (Other Than Film-Coated Tablets)

Weigh individually 20 whole tablets, and calculate the average weight. If the coated tablets do not conform to the criteria in the accompanying table, place 20 tablets in a beaker of water at 37°, and swirl gently for not more than 5 minutes. Examine the cores for evidence of disintegration and repeat the procedure for a shorter time if disintegration has begun. Dry the cores at 50° for 30 minutes. Accurately weigh 20 individual tablet cores, and calculate the average weight.

The requirements are met if the weights of not more than 2 of the tablets differ from the average weight by more than the percentage listed in the accompanying table and no tablet differs in weight by more than double that percentage.

Criteria

Weight Variation Tolerances for Uncoated Tablets, Film-Coated Tablets, and Coated Tablets (Other Than Film-Coated Tablets)

Average Weight of Tablet, mg	Percentage Difference
130 or less	10
From 130 through 324	7.5
More than 324	5

(2750) MANUFACTURING PRACTICES FOR DIETARY SUPPLEMENTS

GENERAL PROVISIONS

The principles included in this chapter contain recommended minimum current good manufacturing practices for the methods to be used in, and the facilities and controls to be used for, the manufacture, holding, packaging, labeling, and distribution of dietary ingredients and dietary supplements. These principles are set forth to ensure that such products meet the requirements of safety, have the identity and strength, and meet the quality and purity characteristics that they are represented to possess.

Excluded from this chapter are establishments engaged solely in the harvesting, storage, or distribution of one or

more “raw agricultural commodities” as defined in Section 201(r) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 321(r)), which are ordinarily cleaned, prepared, treated, or otherwise processed before being marketed to the consuming public.

The requirements pertaining to holding dietary ingredients and dietary supplements do not apply to holding those dietary supplements at a retail establishment for the sole purpose of direct retail sale to individual consumers. A retail establishment does not include a warehouse or other storage facility for a retailer or a warehouse or other storage facility that sells directly to individual consumers.

A glossary of terms used in this chapter is presented at the end.

ORGANIZATION AND PERSONNEL

Responsibilities of a Quality Control Unit

A quality control unit shall be established that has the responsibility and authority to approve or reject all raw materials, product containers, closures, in-process materials, packaging material, labeling, and finished dietary supplements, and the authority to review production records to ensure that no errors have occurred or, if errors have occurred, that they have been fully investigated. The quality control unit should be responsible for approving or rejecting products manufactured, processed, packed, or held under contract by another company.

Adequate laboratory facilities for the testing and approval (or rejection) of raw materials, product containers, closures, packaging materials, in-process materials, dietary ingredients, and dietary supplements should be available to the quality control unit.

The quality control unit should have the responsibility for approving or rejecting all procedures or specifications that impact on the identity, strength, quality, and purity of the dietary supplement. All responsibilities and procedures applicable to the quality control unit shall be in writing.

The designated person within the Quality Control Unit who conducts a material review and makes the disposition decision must, at the time of performance, document that material review and disposition decision.

Personnel Qualifications

Each person engaged in the manufacture of dietary ingredients and dietary supplements should have the proper education, training, and experience (or any combination thereof) needed to perform the assigned functions. Training should be in the particular operation(s) that the employee performs as they relate to the employee’s functions.

Appropriate documentation of training shall be retained by the company.

Each person responsible for supervising the manufacture of a dietary ingredient, a dietary supplement, or both should have the proper education, training, and experience (or any combination thereof) to perform assigned functions in such a manner as to provide assurance that the product has the safety, identity, strength, quality, and purity that it is represented to possess.

An adequate number of qualified personnel to perform and supervise the manufacture of each dietary ingredient, dietary supplement, or both product should be provided.

Personnel Responsibilities

The company management shall take all reasonable measures and precautions to ensure the following:

1. *Disease control.* Any person who, by medical examination or supervisory observation, is shown to have, or

appears to have, an illness, open lesion, including boils, sores, or infected wounds, or any other abnormal source of microbial contamination by which there is a reasonable possibility of an in-process or finished dietary ingredient or dietary supplement becoming adulterated, or processing equipment, utensils, or packaging materials becoming contaminated, shall be excluded from any operations which may be expected to result in such adulteration or contamination until the condition is corrected. Personnel shall be instructed to report such health conditions to their supervisors.

2. **Cleanliness.** All persons working in direct contact with raw materials, in-process or finished dietary ingredients and dietary supplements, processing equipment, utensils, or packaging materials shall conform to hygienic practices while on duty to the extent necessary to protect against adulteration or contamination of such materials. The methods for maintaining cleanliness include, but are not limited to, the following:

- Wearing outer garments suitable to the operation in a manner that protects against the adulteration of raw materials or of in-process or finished dietary ingredients and dietary supplements, or contamination of processing equipment, utensils, or packaging materials;
- Maintaining adequate personal cleanliness;
- Removing cosmetics from parts of the body that may contact raw materials, in-process or finished dietary ingredients and dietary supplements, equipment, utensils, or containers;
- Washing hands thoroughly (and sanitizing if necessary to protect against contamination with undesirable microorganisms) in an adequate hand-washing facility before starting work, after each absence from the work station, and at any other time when the hands may have become soiled or contaminated;
- Removing all unsecured jewelry and other objects that might fall into raw materials, in-process or finished dietary ingredients and dietary supplements, equipment, or containers, and removing hand jewelry that cannot be adequately sanitized during periods in which in-process or finished product is manipulated by hand. If such hand jewelry and cosmetics cannot be removed, they may be covered by material that can be maintained in an intact, clean, and sanitary condition and that effectively protects against the adulteration of dietary ingredients and dietary supplements or contamination of processing equipment, utensils or packaging materials;
- Maintaining gloves, if they are used in raw materials or in in-process or finished product handling, in an intact, clean, and sanitary condition. The gloves should be of a material that adequately protects the product from contamination;
- Wearing, where appropriate, in an effective manner, hair nets, caps, beard covers, or other effective hair restraints;
- Storing clothing or other personal belongings in areas other than where in-process or finished product is exposed or where processing equipment or utensils are washed;
- Confining the following actions to areas other than where in-process or finished product may be stored or exposed, or where processing equipment or utensils are washed: eating food, chewing gum, drinking beverages, or using tobacco; and
- Taking any other necessary precautions to protect against adulteration of raw materials or of in-

process or finished product, or contamination of processing equipment, utensils, or packaging materials with microorganisms or foreign substances, including, but not limited to, perspiration, hair, cosmetics, tobacco, chemicals, and medicines applied to the skin.

GROUND, BUILDING, AND FACILITIES

Grounds

The grounds about a dietary ingredient manufacturing plant and a dietary supplement manufacturing plant under the control of the operator shall be kept in a condition that will protect against the adulteration of dietary ingredients and dietary supplements. The methods for adequate maintenance of grounds include, but are not limited to, the following:

- Properly storing equipment, removing litter and waste, and cutting weeds or grass within the immediate vicinity of the plant building or structures that may constitute an attractant, breeding place, or harborage for pests;
- Maintaining roads, yards, and parking lots so that they do not constitute a source of adulteration in areas where product is exposed;
- Adequately draining areas that may contribute to product adulteration by seepage, foot-borne filth, or providing a breeding place for pests; and
- Operating systems for waste treatment and disposal in an adequate manner so that they do not constitute a source of adulteration in areas where product is exposed. If the plant grounds are bordered by grounds not under the operator's control and not maintained in the manner described above, care shall be exercised in the plant by inspection, extermination, or other means to exclude pests, dirt, and filth that may be a source of product adulteration.

Building Design

Any building or buildings used in the manufacture of a dietary ingredient, a dietary supplement, or both should be of suitable size and shall be constructed in such a manner that floors, walls, and ceilings may be adequately cleaned and kept clean and in good repair; that drips or condensates from fixtures, ducts, and pipes do not adulterate raw materials or in-process or finished dietary ingredients and dietary supplements, or contaminate product containers, utensils, or packaging materials; and that aisles or working spaces are provided between equipment and walls and are adequately unobstructed and of adequate width to permit employees to perform their duties and to protect against adulterating in-process or finished product, or contaminating processing equipment with clothing or personal contact. Adequate screening or other protection against pests and insects should be installed, where necessary. The building should have adequate space for the orderly placement of equipment and materials to prevent mixups between different raw materials, product containers, closures, labeling, in-process materials, or finished products, and to prevent contamination. The flow of raw materials, product containers, closures, labeling, in-process materials, and products through the building or buildings should be designed to prevent contamination.

Operations should be performed within specifically defined areas of adequate size to prevent contamination or mixups or adulteration of in-process or finished dietary ingredients and dietary supplements, or contamination of processing equipment, utensils, or packaging materials with microorganisms, chemicals, filth, or other extraneous materials. The potential for mixups and product adulteration may be

reduced by adequate product safety controls and operating practices or effective design, including the separation of operations in which contamination is likely to occur, by one or more of the following means: location, time, partition, air-flow, enclosed systems, or other effective means. There should be separate or defined areas as follows:

1. An area for the receipt, identification, storage, and withholding from use of components, product containers, closures, and labeling, pending the appropriate sampling, testing, or examination by the quality control unit before release for manufacturing or packaging;
2. An area for the storage of released components, product containers, closures, and labeling;
3. An area for storage of in-process materials;
4. An area for manufacturing and processing operations;
5. An area for packaging and labeling operations; and
6. An area for control and laboratory operations.

Any building used in the manufacture of a dietary ingredient or a dietary supplement shall permit the taking of proper precautions to protect dietary ingredients or dietary supplements in outdoor bulk fermentation vessels by any effective means, including the following:

- (i) Using protective coverings,
- (ii) Controlling areas over and around the vessels to eliminate harborages for pests,
- (iii) Checking on a regular basis for pests and pest infestation, and
- (iv) Skimming the fermentation vessels, as necessary.

Lighting

Adequate lighting shall be provided in all areas and should not expose bulk or finished product to adulteration or contamination. Adequate lighting should be provided in hand-washing areas, dressing and locker rooms, and toilet rooms, and in all areas where product is examined, processed, or stored and where equipment or utensils are cleaned; and such lighting should provide safety-type light bulbs, fixtures, skylights, or other glass suspended over exposed product in any step of preparation or otherwise protect against product adulteration in case of glass breakage.

Ventilation, Air Filtration, Air Heating, and Cooling

Adequate ventilation shall be provided, as well as equipment for adequate control over microorganisms, dust, humidity, and temperature when used in the manufacture of a dietary ingredient and a dietary supplement to minimize odors and vapors (including steam and noxious fumes) in areas where they may adulterate dietary ingredients and dietary supplements; and to locate and operate fans and other air-blowing equipment in a manner that minimizes the potential for adulterating raw materials, in-process or finished dietary ingredients and dietary supplements, or contaminating processing equipment, utensils, or packaging materials.

Plumbing

The plumbing in the physical plant must be of an adequate size and design and be adequately installed and maintained to:

- Carry sufficient amounts of water to the required locations throughout the physical plant;
- Properly convey sewage and liquid disposable waste from the physical plant; and
- Avoid being a source of contamination to components, raw materials, dietary ingredients, dietary supplements, water supplies, or any contact surface, or creating an unsanitary condition.

Potable water at a suitable temperature, and under pressure as needed, should be supplied in a plumbing system free of defects that could contribute contamination to any dietary ingredients and dietary supplements. Potable water should meet the standards prescribed in the Environmental Protection Agency's Primary Drinking Water Regulations (40 CFR Part 141) or any state or local drinking water requirements that are more stringent. Water not meeting such standards should not be permitted in the potable water system for *Purified Water*. If potable water is to be used as a raw material, it should be further purified to satisfy compendial requirements.

Drains should be of adequate size and, where connected directly to a sewer, should have an air break or other mechanical device to prevent back-siphonage.

Sewage and Refuse

Sewage, trash, and other refuse in and from the building and immediate premises shall be disposed of in a safe and sanitary manner.

Washing and Toilet Facilities

Adequate washing facilities shall be provided, including hot and cold water, soap or detergent, air driers or single-service towels, and clean toilet facilities easily accessible to working areas.

General Maintenance and Sanitation

Any building used in the manufacture of a dietary ingredient, a dietary supplement, or both should be maintained in a clean and sanitary condition and shall be kept in repair sufficient to prevent raw materials and in-process or finished dietary ingredients and dietary supplements from becoming adulterated. It shall be free of infestation by rodents, birds, insects, and other vermin. Trash and organic waste matter shall be held and disposed of in a timely and sanitary manner.

Cleaning compounds and sanitizing agents used in cleaning and sanitizing procedures shall be free from undesirable microorganisms and shall be safe and adequate under the conditions of use. Compliance with this requirement may be verified by any effective means, including purchase of these substances under a supplier's guarantee or certification, or examination of these substances for contamination. Only the following toxic materials may be used or stored in a plant where product is processed or exposed:

1. Those required to maintain clean and sanitary conditions;
2. Those necessary for use in laboratory testing procedures;
3. Those necessary for plant and equipment maintenance and operation; and
4. Those necessary for use in the plant's operations.

Written procedures assigning responsibility for sanitation and describing in sufficient detail the cleaning schedules, methods, equipment, and materials to be used in cleaning the building and facilities shall be required.

Toxic cleaning compounds, sanitizing agents, and pesticide chemicals shall be identified, used, held, and stored in a manner that protects against adulteration of raw materials or of in-process or finished product, or contamination of processing equipment or packaging materials. All relevant regulations promulgated by other federal, state, and local government agencies for the application, use or holding of these products should be followed.

No pests shall be allowed in any area of a dietary ingredient manufacturing plant and a dietary supplement manufacturing plant. Effective measures shall be taken to exclude pests from the processing areas and to protect against the adulteration by pests of product on the premises. The use of

insecticides or rodenticides is permitted only under precautions and restrictions that will protect against the adulteration of raw materials, in-process or finished product, or contamination of processing equipment, utensils, or packaging materials.

Written procedures are also required for use of suitable rodenticides, insecticides, fungicides, fumigating agents, and cleaning and sanitizing agents. These procedures should be designed to prevent the contamination of equipment, raw materials, product containers, closures, packaging, labeling materials, or products. Rodenticides, insecticides, and fungicides should be registered and used in accordance with the Federal Insecticide, Fungicide, and Rodenticide Act.

Sanitation procedures shall apply to work performed by contractors or temporary employees as well as work performed by full-time employees during the ordinary course of operations.

EQUIPMENT AND UTENSILS

Equipment and utensils used in the manufacture of dietary ingredients and dietary supplements shall be of appropriate design or selection, adequate size, and suitably located to facilitate operations for its intended use and for its cleaning and maintenance and to ensure that the specifications of dietary ingredients and dietary supplements are correct and are met.

Equipment and utensils include, but are not limited to, the following:

- Equipment used to hold or convey;
- Equipment used to measure;
- Equipment using compressed air or gas;
- Equipment used to carry out processes in closed pipes and vessels; and
- Equipment used in automatic, mechanical, or electronic systems.

Construction

All equipment and utensils shall be:

- Constructed so that surfaces that contact raw materials, in-process materials, or finished products are not reactive, additive, or absorptive so as to alter the safety, identity, strength, quality, or purity of the product beyond the established requirements;
- Made of nontoxic materials;
- Designed and constructed to withstand the environment in which they are used; the action of raw materials, in-process materials, dietary ingredients, or dietary supplements; and, if applicable, cleaning compounds and sanitizing agents; and
- Maintained to protect raw materials, in-process materials, dietary ingredients, and dietary supplements from being contaminated by any source.

Equipment and utensils must have seams that are smoothly bonded or maintained to minimize the accumulation of dirt, filth, organic material, particles of raw materials, in-process materials, dietary ingredients, or dietary supplements, or any other extraneous materials or contaminants, in order to minimize the opportunity for growth of microorganisms.

Each freezer, refrigerator, and cold storage compartment used to hold raw materials, in-process materials, dietary ingredients, or dietary supplements

- Must be fitted with an indicating thermometer, temperature-measuring device, or temperature-recording device that shows, indicates, and records, or allows for recording by hand, the temperature accurately within the compartment; and
- Must have an automated device for regulating temperature or an automated alarm system to indicate a significant temperature change in a manual operation.

The design, construction, and use of equipment and utensils shall preclude the adulteration of raw materials, packaging materials, in-process materials, or finished product with any substances required for operation, such as:

- Lubricants,
- Fuel,
- Coolants,
- Metal or glass fragments,
- Filth or any other extraneous material,
- Contaminated water, or
- Any other contaminants.

Instruments or controls used in the manufacturing, packaging, labeling, or holding of a dietary ingredient, a dietary supplement, or both; and instruments or controls that are used to measure, regulate, or record temperatures, hydrogen-ion concentration (pH), water activity, or other conditions, and to control or prevent the growth of microorganisms or other contamination must be:

- Accurate and precise,
- Adequately maintained, and
- Adequate in number for their designated uses.

For any automated, mechanical, or electronic equipment that is used to manufacture, package, label, or hold a dietary ingredient, a dietary supplement, or both:

- The suitability of the equipment must be determined by ensuring that the equipment is capable of operating satisfactorily within the operating limits required by the process;
- The equipment must be routinely calibrated, inspected, or checked to ensure proper performance. The quality control unit must approve these calibrations, inspections, or checks;
- The appropriate controls for automated, mechanical, and electronic equipment (including software for a computer-controlled process) must be established and used to ensure that any changes to the manufacturing, packaging, labeling, holding, or other operations are approved by the quality control unit and instituted only by authorized personnel; and
- The appropriate controls must be established and used to ensure that the equipment functions in accordance with its intended use. These controls must be approved by the quality control unit.

Compressed air or other gases introduced mechanically into or onto raw materials, in-process materials, dietary ingredients, dietary supplements, or contact surfaces, or that are used to clean any contact surface, must be treated in such a way that the raw material, in-process material, dietary ingredient, dietary supplement, or contact surface is not contaminated.

Cleaning and Maintenance

Equipment and utensils shall be cleaned, maintained, and sanitized at adequate intervals, between the manufacture of different batches of the same product and between the manufacture of different products, to prevent malfunctions or contamination that would alter the safety, identity, strength, quality, or purity of the product beyond the established requirements.

In wet processing during manufacturing, all contact surfaces must be cleaned and sanitized, as necessary, to protect against the introduction of microorganisms into components, dietary ingredients, or dietary supplements. When cleaning and sanitizing is necessary, all contact surfaces must be cleaned and sanitized before use and after any interruption during which the contact surface may have become contaminated.

In a continuous production operation or in back-to-back consecutive operations, which involve different batches of the same dietary ingredient or dietary supplement, the contact surfaces must be adequately cleaned and sanitized.

The surfaces that do not come into direct contact with raw materials, in-process materials, dietary ingredients, or

dietary supplements must be cleaned as frequently as necessary to protect against contaminating raw materials, in-process materials, dietary ingredients, or dietary supplements.

Single-service articles (such as utensils intended for one-time use, paper cups, and paper towels) must be:

- Stored in appropriate containers; and
- Handled, dispensed, used, and disposed of in a manner that protects against contamination of raw materials, in-process materials, dietary ingredients, dietary supplements, or any contact surface.

Cleaning compounds and sanitizing agents must be adequate for their intended use and safe under their conditions of use.

The portable equipment and utensils that have contact surfaces must be cleaned, sanitized, and then stored in a location and manner that protects them from contamination.

Written procedures for cleaning and maintaining equipment, including utensils, used in the manufacture of a product should be established and followed. These procedures should include, but are not necessarily limited to, the following:

- Assignment of responsibility for cleaning and maintaining equipment;
- Maintenance and cleaning schedules, including, where adequate, sanitizing schedules;
- A description in sufficient detail of the methods, equipment, and materials used in cleaning and maintenance operations, and the methods of disassembling and reassembling equipment, as necessary, to ensure proper cleaning and maintenance;
- Removal or obliteration of previous batch identification;
- Identification and protection of clean equipment from contamination before use;
- Inspection of equipment for cleanliness immediately before use;
- Regular calibration and inspection of equipment, or checking machines, to ensure proper performance and function must be conducted:
 - (a) Before first use; and
 - (b) At frequency specified in writing by supporting references.
- Instruments or controls that cannot be adjusted to agree with the reference standard must be repaired or replaced.

A written record of calibration, inspection, maintenance of equipment, and major equipment cleaning and use shall be maintained in individual equipment logs that show the date, product, and lot number of each batch processed. The persons performing the cleaning shall record in the log that the work was performed. Entries in the log should be in chronological order.

The following is specified in order to keep records related to automated, or electric equipment:

- There must be backup file(s) of current software programs (and of outdated software that is necessary to retrieve records that are required to be retained, in accordance with the section *Records and Reports* in this chapter, when current software is not able to retrieve such records) and of data entered into computer systems used to manufacture, package, label, or hold dietary supplements.
 - (a) A backup file (e.g., a hard copy of data entered, diskettes, tapes, microfilm, or compact disks) must be an exact and complete record of the data entered.
 - (b) Backup software programs and data must be kept secure from alterations, inadvertent erasures, or loss.

RAW MATERIALS, PRODUCT CONTAINERS, AND CLOSURES

Written procedures describing in sufficient detail the receipt, identification, storage, handling, sampling, testing, and approval or rejection of raw materials, product containers, and closures should be provided.

Raw materials, product containers, and closures at all times should be handled and stored in a manner to prevent contamination.

Raw agricultural materials that contain soil or other contaminants shall be washed or cleaned as necessary. Water used for washing, rinsing, or conveying raw agricultural materials shall be safe and of adequate sanitary quality. Notwithstanding the general requirement for potable water, water may be reused for washing, rinsing, or conveying raw agricultural materials if it does not increase the level of contamination of such materials.

Bagged or boxed raw materials of product containers or closures should be stored off the floor and suitably spaced to permit cleaning and inspection.

Each lot should be appropriately identified as to its status (i.e., quarantined, approved, or rejected).

Receipt and Storage of Untested Raw Materials, Product Containers, and Closures

Written procedures shall be established and followed describing the receipt, identification, examination, handling, and sampling of raw materials. Upon receipt and before acceptance, each container or grouping of containers of raw materials, product containers, and closures should be examined visually for appropriate labeling as to contents, container damage, or broken seals, and for contamination. They are then stored under quarantine until they have been tested or examined, as appropriate, and released.

Raw materials shall be held in bulk, or in containers designed and constructed so as to protect against adulteration, and shall be held at such temperature and relative humidity and in such a manner as to prevent a dietary ingredient or dietary supplement from becoming adulterated. Frozen raw materials and other ingredients shall be kept frozen. If thawing is required prior to use, it shall be done in a manner that prevents the raw materials and other ingredients from becoming adulterated within the meaning of the Act.

Testing and Approval or Rejection

Each lot of raw materials, product containers, and closures should be sampled, tested, or examined, as appropriate, and released for use by the quality control unit. Based upon adequate process verification, in-process controls and statistical confidence, a skip-lot testing plan is an alternative to fully testing every batch provided that at least one identity test is conducted. An appropriate amount of each lot of raw materials should be reserved for 3 years beyond the shelf life appearing on the label of finished dietary supplements in which the raw materials were used. If adverse event reports are received (see the subsection *Adverse Event Reports*), the reserved raw materials should be kept for 6 years (serious events) or 3 years (nonserious events) from the date the first report is received.

Representative samples should be collected for testing or examination. Sampling of botanicals should be in compliance with the provisions set in *Articles of Botanical Origin* (561). The number of containers sampled, and the amount of material taken from each container, should be based upon appropriate criteria such as statistical criteria for raw material variability, confidence levels, and degree of precision desired, the past quality history of the supplier, and the quantity needed for analysis and reserve where required.

The following procedures should be used to collect the samples:

- The containers of raw materials selected should be cleaned, where necessary, by adequate means.
- The containers should be opened, sampled, and resealed in a manner designed to prevent contamination of their contents and contamination of other raw materials, product containers, or closures.
- These containers should be identified so that the following information can be determined: name of the material sampled, the lot number, the container from which the sample was taken, the date on which the sample was taken, and the name of the person who collected the sample.

Use the following procedure to examine and test the samples:

- At least one test should be conducted to verify the identity of each raw material of a product even in cases where skip-lot testing is used. Such tests may include any appropriate test with established sufficient specificity to determine identity, including chemical and laboratory tests, gross organoleptic analysis, microscopic identification, or analysis of constituent markers.
- Each raw material should be tested for conformity with all appropriate written specifications for purity, strength, and quality. However, a report of analysis may be accepted from the supplier of a raw material, provided that the manufacturer establishes the reliability of the supplier's analyses and provided that at least one identity test is conducted on such raw material by the manufacturer.
- Containers and closures should be tested for conformance with all appropriate written procedures. However, a certificate of testing may be accepted from the supplier, provided that at least a visual identification is conducted on such containers or closures by the manufacturer.
- Each lot of a raw material, rework, product container, or closure that is liable to contamination with filth, insect infestation, or other extraneous adulterant should be examined against established specifications for such contamination and shall comply with any applicable Food and Drug Administration regulations and guidelines. Skip-lot examination should not apply in such cases.
- Each lot of a raw material that is liable to microbiological contamination that is objectionable in view of its intended use shall be subjected to microbiological tests before use. Raw materials either shall not contain levels of microorganisms that may produce food poisoning or other disease in humans, or shall be otherwise treated during manufacturing operations so that they no longer contain levels that would cause the product to be adulterated within the meaning of the Act. In lieu of such testing by the manufacturer, a guarantee or certification of analysis may be accepted from the supplier of a component provided that the manufacturer establishes the reliability of the supplier's analysis.
- Raw materials and other ingredients susceptible to adulteration with aflatoxin, other natural toxins, pesticides, or heavy metals shall comply with current Food and Drug Administration regulations, guidelines, and action levels for poisonous or deleterious substances and the requirements in *Articles of Botanical Origin* (561), or in each monograph, before these materials or ingredients are incorporated into a finished dietary ingredient or dietary supplement. Compliance with this requirement may be accomplished by analyzing these materials and ingredients for aflatoxins and other natural toxins; or, in lieu of such testing by the manufacturer, a guarantee or certification of analysis may be accepted from the supplier of a raw material

provided that the manufacturer establishes the reliability of the supplier's analysis.

- Any lot of raw material, product container, or closure that meets the appropriate written specifications of identity, strength, quality, and purity and related tests may be approved and released for use. Any lot of such material that does not meet such specifications should be rejected.

Use of Approved Raw Materials, Product Containers, and Closures

Raw materials, product containers, and closures approved for use should be rotated so that the oldest approved stock is used first. Deviation from the requirement is permitted if such deviation is temporary and adequate.

Retesting of Approved Raw Materials, Product Containers, and Closures

Raw materials, product containers, and closures should be retested or reexamined, as appropriate, for identity, strength, quality, and purity and approved or rejected by the quality control unit after a specified time in storage or as necessary, e.g., after exposure to air, heat, or other conditions that might adversely affect the raw material, product container, or closure or after storage of active and inactive ingredients and in-process materials for long periods of time.

Rejected Raw Materials, Product Containers, and Closures

Rejected raw materials, product containers, and closures should be identified and controlled under a quarantine system that prevents their use in manufacturing or processing operations for which they are unsuitable.

PRODUCTION AND PROCESS CONTROLS

Written Procedures

Written procedures should be provided for production and process controls designed to ensure that the dietary ingredients and dietary supplements have the identity, strength, quality, and purity they are represented to possess. These procedures should be drafted, reviewed, and approved by the appropriate organizational units and reviewed and approved by the quality control unit. These production and process control procedures should be followed in the execution of the various production and process control functions and should be documented at the time of performance. Any deviation from the written procedures should be recorded and justified.

- (1) All operations in the receiving, inspecting, transporting, segregating, preparing, manufacturing, packaging, and storing of dietary ingredients and dietary supplements shall be conducted in accordance with adequate sanitation principles.
- (2) All reasonable precautions shall be taken to ensure that production procedures do not contribute adulteration from any source. Chemical, microbial, or extraneous-material testing procedures shall be used where necessary to identify sanitation failures or possible product adulteration.
- (3) All product that has become contaminated to the extent that it is adulterated within the meaning of the Act shall be rejected, or if permissible, treated or processed to eliminate the contamination.

- (4) All product manufacturing, including packaging and storage, shall be conducted under such conditions and controls as are necessary to minimize the potential for the growth of microorganisms, or for the adulteration of raw materials, in-process materials, and finished product.
- (5) Measures taken to destroy microorganisms, reduce the microbial load, or prevent the growth of undesirable microorganisms, particularly those of public health significance, shall be adequate under the conditions of manufacture, handling, and distribution to prevent dietary supplements and ingredients from being adulterated within the meaning of the Act. These measures shall also comply with current regulations affecting dietary supplement products and ingredients.
- (6) Work-in-process shall be handled in a manner that protects against adulteration.
- (7) In-process material must be held under appropriate conditions of temperature, humidity, and light.
- (8) Effective measures shall be taken to protect finished dietary ingredients and dietary supplements from adulteration by raw materials, in-process materials, or refuse. When raw materials, in-process materials or refuse are unprotected, they shall not be handled simultaneously in a receiving, loading, or shipping area if that handling could result in adulterated dietary ingredients and dietary supplements. Dietary ingredients and dietary supplements transported by conveyor shall be protected against adulteration as necessary.
- (9) Effective measures shall be taken as necessary to protect against the inclusion of metal or other extraneous material in product. Compliance with this requirement may be accomplished by using sieves, traps, magnets, electronic metal detectors, or other suitable effective means.
- (10) Mechanical manufacturing steps such as cutting, sorting, inspecting, shredding, drying, grinding, blending, and sifting shall be performed so as to protect dietary ingredients and dietary supplements against adulteration. Compliance with this requirement may be accomplished by providing adequate physical protection of dietary ingredients and dietary supplements from contact with adulterants. Protection may be provided by adequate cleaning and sanitizing of all processing equipment between each manufacturing step.
- (11) Heat blanching, when required in the preparation of a dietary ingredient or a dietary supplement, should be effected by heating the product to the required temperature, holding it at this temperature for the required time, and then either rapidly cooling the material or passing it to subsequent manufacturing without delay. Thermophilic growth and contamination in blanchers should be minimized by the use of adequate operating temperatures and by periodic cleaning. Where the blanched product is washed before filling, potable water shall be used.
- (12) Intermediate or dehydrated dietary ingredients and dietary supplements that rely on the control of water (a_w) for preventing the growth of undesirable microorganisms shall be processed to and maintained at a safe moisture level. Compliance with this requirement may be accomplished by any effective means, including employment of one or more of the following practices:
 - (i) Monitoring the water activity (a_w) of the material;
 - (ii) Controlling the soluble solids–water ratio in finished product; and
 - (iii) Protecting finished product from moisture pickup, by use of a moisture barrier or by other means, so that the water activity (a_w) of the product does not increase to an unsafe level.
- (13) Dietary ingredients and dietary supplements that rely principally on the control of pH for preventing the growth of undesirable microorganisms shall be monitored and maintained at an appropriate pH. Compliance with this requirement may be accomplished by any effective means, including employment of one or more of the following practices:
 - (i) Monitoring the pH and water activity, if appropriate, of raw materials, in-process material, and finished product; and
 - (ii) Controlling the amount of acid added to the product.
- (14) When ice is used in contact with dietary ingredients and dietary supplements, it shall be made from potable water, and shall be used only if it has been manufactured in accordance with current good manufacturing practice in manufacturing, packing, or holding human food as outlined in 21 CFR Part 110.

Charge-In of Raw Materials

Written production and control procedures should include the following, which are designed to ensure that the dietary supplements have the identity, strength, quality, and purity they are represented to possess:

- The batch should be formulated with the intent to provide not less than 100 percent of the labeled or established amount of dietary ingredient.
- Raw materials for product manufacturing should be weighed, measured, or subdivided as appropriate and the appropriate signatures recorded in the batch record.
- Actual yields and percentages of theoretical yield should be determined at appropriate phases of processing.

Material scheduled for rework shall be identified as such.

Equipment Identification

All compounding and storage containers, processing lines, and major equipment used during the production of a batch of a product should be properly identified to indicate their contents and, when necessary, the phase of processing of the batch.

Sampling and Testing of In-Process Materials, Dietary Ingredients, and Dietary Supplements

To ensure batch uniformity and integrity of dietary supplements, written procedures should be established and followed that describe the in-process controls and tests or examinations to be conducted on appropriate samples of in-process materials. Based upon process verification, in-process controls, and statistical confidence, a skip-lot testing plan is an alternative to testing every batch of finished products provided that at least one representative measure is performed. Control procedures should be established to monitor the output of those manufacturing processes that may be responsible for causing variability in the characteristics of in-process material and the finished product. Such control procedures may include, but are not limited to, the following, where appropriate:

- Friability
- Weight variation
- Disintegration time
- Dissolution time
- Clarity, completeness, or pH of solutions
- Blend uniformity

In-process specifications for such characteristics should be consistent with finished product specifications. Examination and testing of samples should ensure that the in-process material and dietary supplement conform to the established specifications.

In-process materials should be tested for identity, strength, quality, and purity as adequate, and approved or rejected by the quality control unit during the production process, e.g., at commencement or completion of significant phases or after storage for long periods.

Rejected or adulterated in-process materials should be identified and controlled under a quarantine system designed to prevent their use in manufacturing or processing operations for which they are unsuitable and to prevent the adulteration of other products.

LABELING AND PACKAGING

Materials Examination and Usage Criteria

Written procedures should be provided describing in sufficient detail the receipt, identification, storage, handling, sampling, examination, testing of labeling and packaging materials, or products received for packaging or labeling. Each immediate container or grouping of immediate containers in a shipment of product received for packaging or labeling, or of packaging and labeling materials, must be visually examined for appropriate content label, container damage, or broken seals to determine whether the container condition may have resulted in contamination or deterioration of the received product. The supplier's invoice, guarantee, or certification in a shipment of the received product must be visually examined to ensure that the received product is consistent with the purchase order. Labeling and packaging materials or products received for packaging or labeling should be quarantined until:

- Representative samples of each unique shipment, and of each unique lot within each unique shipment, of received product for packaging or labeling, or of packaging and labeling materials, are collected;
- The quality control unit reviews and approves the documentation to determine whether the received product for packaging or labeling, or packaging and labeling materials, meets the specifications; and
- The quality control unit approves the received product for packaging or labeling, or packaging and labeling materials, and releases for use from quarantine.

Those that do not meet such specifications should be identified and rejected to prevent their use in operations for which they are unsuitable.

A record should be kept of each shipment received of each different labeling and packaging material, or each different received product for packaging or labeling, indicating receipt, date of examination or testing, and whether accepted or rejected.

Each unique lot within each unique shipment of received product for packaging or labeling, or of packaging and labeling materials, must be identified in a manner that allows the recipient to trace the lot to the supplier, the date received, the name of the received product, the status of the received product (e.g., quarantined, approved, or rejected), and to the product that was packaged or labeled and distributed.

This unique identifier must be used whenever the disposition of each unique lot within each unique shipment of the received product for packaging or labeling, or of packaging and labeling materials, is recorded.

Labels and other labeling materials for each different product, strength, product type, or quantity of contents should be stored separately under conditions that will protect against contamination and deterioration and avoid mix-ups. Only authorized personnel should have access to the storage area.

Packaging and labels must be held under appropriate conditions so that the packaging and labels are not adversely affected (e.g. contamination, deterioration).

Gang printing of labeling to be used for different products or different strengths of the same product (or labeling of the same size and identical or similar format or color schemes) should be minimized. If gang printing is employed, packaging and labeling operations should provide for special control procedures, taking into consideration sheet layout, stacking, cutting, and handling during and after printing.

Printing devices on, or associated with, manufacturing lines used to imprint labeling upon the product unit label or case should be monitored to ensure that all imprinting conforms to the print specified in the batch production record.

Obsolete and outdated labels, labeling, other packaging materials, and products received for packaging or labeling should be destroyed and documented.

Labeling Issuance

Strict control should be exercised over labeling issued for use in product labeling operations. The control procedures employed should be in writing with sufficient detail.

Labeling materials issued for a batch should be carefully examined for identity and conformity to the labeling specified in the master and batch production records.

Procedures should be used to reconcile the quantities of labeling issued, used, and returned, and should require evaluation of discrepancies found. If discrepancies are found between the quantity of product finished and the quantity of labeling issued and are outside preset limits based on historical operating data, such discrepancies should be investigated.

Returned labeling should be maintained and sorted in a manner to prevent mixups and provide proper identification.

All excess labeling bearing lot or control numbers should be destroyed and documented.

Operations

Written procedures designed to ensure that correct labels, labeling, and packaging materials are used for dietary supplements should incorporate the following features:

- Prevention of mixups and cross-contamination by physical or spatial separation from operations on other products;
- Identification of the product with a lot or control number;
- Examination of packaging and labeling materials for suitability and correctness before packaging operations, and documentation of such examination in the batch production record; and
- Inspection of the packaging and labeling facilities immediately before use to ensure that all products have been removed from previous operations. Inspection should also be made to ensure that packaging and labeling materials not suitable for subsequent operations have been removed. Results of the inspection should be documented in the batch production records.

Relabeling and Repackaging

- Dietary ingredients and dietary supplements may be repackaged or relabeled only after the quality control unit has approved such repackaging or relabeling.
- A representative sample of each batch of repackaged or relabeled dietary ingredients and dietary supplements must be examined to determine whether the repackaged or relabeled dietary ingredients and dietary supplements meet all established specifications.
- The quality control unit must approve or reject each batch of repackaged or relabeled dietary ingredients

and dietary supplements before its release for distribution.

Tamper-Resistant Packaging

REQUIREMENTS

Each manufacturer and packer who packages a dietary supplement for retail sale shall package the product in a tamper-resistant package, if this product is accessible to the public while held for sale. A tamper-resistant package is one having an indicator or barrier to entry which, if breached or missing, can reasonably be expected to provide visible evidence to consumers that tampering has occurred. To reduce the likelihood of substitution of a tamper-resistant feature after tampering, the indicator or barrier to entry is required to be distinctive by design or by the use of an identifying characteristic (e.g., a pattern, name, registered trademark, logo, or picture). For purposes of this section, the term "distinctive by design" means that the packaging cannot be duplicated with commonly available materials or through commonly available processes. A tamper-resistant package may involve an immediate-container and closure system, or secondary-container or carton system, or any combination of systems intended to provide a visual indication of package integrity. The tamper-resistant feature should be designed to remain intact when handled in a reasonable manner during manufacture, distribution, and retail display.

LABELING

Each retail package of a dietary supplement covered by this section shall bear a statement that is prominently placed so that consumers are alerted to the specific tamper-resistant feature of the package. The labeling statement should be so placed that it will be unaffected if the tamper-resistant feature of the packaging is breached or missing. If the tamper-resistant feature chosen to meet the requirement above is one that uses an identifying characteristic, that characteristic should be referred to in the labeling statement. For example, the labeling statement on a bottle with a shrink band could say "For your protection, this bottle has an imprinted seal around the neck."

Dietary Supplement Examination

Packaged and labeled products should be examined during finishing operations to ensure that containers and packages in the lot have the correct label. A representative sample of units should be collected at the completion of finishing operations and visually examined for correct labeling. Results of these examinations should be recorded in the batch production or control records.

Contact Information

The manufacturer, packer, or distributor of dietary supplements is required to comply with the current labeling requirements in the law that also include a domestic address or phone number through which an adverse event report for a dietary supplement may be received.

Shelf Life

Dietary supplements should bear a date indicative of their shelf life, determined by appropriate testing, to ensure that they meet applicable standards of identity, strength, quality, and purity at or before the labeled shelf-life date.

Shelf life should be related to any storage conditions stated on the labeling.

HOLDING AND DISTRIBUTION

Warehousing Procedures

Storage and transportation of finished product shall be under conditions that will protect product against physical, chemical, and microbial adulteration as well as against deterioration of the product and the container.

Written procedures describing the warehousing of dietary supplements should be established and followed and should include the following:

- Quarantine of finished products before disposition by the quality control unit; and
- Storage of finished products under appropriate conditions of temperature, humidity, and light so that the identity, strength, quality, and purity of the products are not affected.

Distribution Procedures

Written procedures describing the distribution of dietary supplements shall be established and followed and should include the following:

- A procedure whereby the oldest approved stock of a product is distributed first. (Deviation from this requirement is permitted if such deviation is temporary and adequate.)
- A system by which the distribution of each lot of product can be readily determined to facilitate its recall if necessary.

QUALITY CONTROL OPERATIONS

The establishment of any specifications, standards, sampling plans, test procedures, or other laboratory control mechanisms required by this chapter, including any change in such specifications, standards, sampling plans, test procedures, or other laboratory control mechanisms, shall be drafted by the appropriate organizational unit and reviewed and approved by the quality control unit. The requirements in this section should be followed and documented at the time of performance. Any deviation from the written specifications, standards, sampling plans, test procedures, or other laboratory control mechanisms shall be recorded and justified.

Quality control operations include the establishment of scientifically sound and appropriate specifications, standards, sampling plans, and test procedures designed to ensure that raw materials, product containers, closures, in-process materials, labeling, products received for labeling and packaging operations as dietary supplements, and finished products conform to adequate standards of identity, strength, quality, and purity. These controls include the following:

- Determination of conformance to appropriate written specifications for the acceptance of each lot within each shipment of raw materials, product containers, closures, and labeling used in the manufacture of dietary ingredients and dietary supplements, and of products received for labeling and packaging operations as dietary supplements. (The specifications include a description of the sampling and testing procedures used. Samples should be representative and adequately identified. Such procedures also require appropriate retesting of any raw material, product container, or closure that is subject to deterioration.) Based upon adequate process verification, in-process controls, and statistical confidence, a skip-lot testing plan is an alternative to testing every batch, excluding raw materials, which require 100% identity testing.
- Determination of conformance to written specifications and a description of sampling and testing procedures

for in-process materials. (Such samples should be representative and properly identified.)

- Determination of conformance to written descriptions of sampling procedures and appropriate specifications for finished products. (Such samples should be representative and properly identified.)
- The calibration of instruments, at suitable intervals, in accordance with an established written program containing specific directions, schedules, limits for accuracy and precision, and provisions for remedial action in the event that accuracy and/or precision limits are not met. Instruments not meeting established specifications shall not be used until repaired.

Testing and Release for Distribution

There should be appropriate laboratory determination of satisfactory conformance to specifications for the finished product, including the identity and strength prior to release. Based upon adequate process verification, in-process controls, or statistical confidence, a skip-lot or composite testing plan is an alternative to testing every batch.

There should be appropriate laboratory testing, as necessary, of each batch of dietary supplement required to be free of objectionable microorganisms. The accuracy, linearity, sensitivity, specificity, and precision of test methods employed by the firm, when they differ from compendial methods, should be established and documented.

Written procedures should describe any sampling and testing plans, which should include the method of sampling and the number of units per batch to be tested.

Products failing to meet established standards or specifications and any other relevant quality control criteria should be rejected. Rejected or adulterated dietary ingredients and dietary supplements shall be identified, stored, and disposed of in a manner that protects against the adulteration of the other products. Reprocessing may be performed. Prior to acceptance and use, reprocessed material must meet established standards, specifications, and any other relevant criteria. Written procedures shall be established and followed prescribing the method for reprocessing batches or operations start-up materials that do not conform to finished goods standards or specifications. Finished goods manufactured using such materials shall meet all established purity, composition, and quality standards.

Stability Testing

There should be a written protocol designed to assess the stability characteristics of dietary supplements. The results of such testing should be used in determining appropriate storage conditions and shelf life. This protocol should include the following:

- Sample size and test intervals based on statistical criteria for each attribute should be examined to ensure valid estimates of stability;
- Storage conditions for samples retained for testing;
- Reliable, meaningful, and specific test methods should be used; and
- The dietary supplement should be tested in the same type of container-closure system as that in which the dietary supplement is marketed.

An adequate number of batches of each dietary supplement should be tested to determine an adequate shelf life, and a record of these data should be maintained. Accelerated studies combined with basic stability information on the raw materials, dietary supplements, and container-closure systems may be used to support tentative shelf life if full shelf-life studies are not available. Simplified stability testing procedures may be used where data from similar product formulations are available to support a shelf-life estimation of a new product. Where data from accelerated studies are used to project a tentative shelf life date that is beyond a date supported by actual shelf-life studies, stability

studies should be conducted, including dietary supplement testing at appropriate intervals, until the tentative shelf life is verified or the adequate shelf life is determined.

Reserve Samples

An appropriately identified reserve sample that is representative of each lot or batch of dietary supplement should be retained and stored under conditions consistent with product labeling until at least 3 years after the shelf life of the product. The reserve sample should be stored in the same immediate container-closure system in which the finished product is marketed or in one that has essentially the same characteristics. The reserve sample consists of at least twice the quantity necessary to perform all the required tests. If an adverse event report is received, the reserve samples of dietary supplements and dietary ingredients from the same lot or batch must be analyzed by an appropriate procedure to confirm their identity and determine any adulteration or contamination. The recovered samples associated with adverse event reports from consumers, distributors, or both should also be analyzed, following the same method used for the reserved samples, if available. The results should be reported with other required information to the federal authority, using the required form. The reserve samples from a particular lot or batch associated with an adverse event report should be held for 6 years (serious events) or 3 years (nonserious events) from the date when the first adverse event report is received by the manufacturer, packer, or distributor.

RECORDS AND REPORTS

Any record for production, control, quality control operations, or distribution that is required to be maintained and is specifically associated with a batch of a product should be retained for at least 3 years after the shelf life of the batch.

Records should be maintained for all raw materials, product containers, closures, and labeling for at least 3 years after the shelf life of the last lot of product incorporating the raw material or using the container, closure, or labeling.

Master Production and Control Records

To ensure uniformity from batch to batch, master production and control records for each product should be prepared, dated, and signed by one person and independently checked, dated, and signed by a second person from the quality control unit.

Master production and control records should include the following:

- The name and strength of the product;
- The name and weight or measure of each dietary ingredient per unit or portion or per unit of weight or measure of the product, and a statement of the total weight or measure of any dosage unit;
- A complete list of raw materials designated by names or codes sufficiently specific to indicate any special quality characteristic;
- An accurate statement of the weight or measure of each raw material, using the same weight system (metric, avoirdupois, or apothecary) for each raw material;
- A statement concerning any calculated excess of raw material;
- A statement of theoretical weight or measure at appropriate phases of processing;
- A statement of theoretical yield, including the maximum and minimum percentages of theoretical yield beyond which investigation is required;
- A description of the product containers, closures, and packaging materials, including a specimen or copy of each label and all other labeling signed and dated by

- the person or persons responsible for approval of such labeling or, in lieu of specimens or copies of each label or other labeling, a positive identification of all labeling used; and
- Complete manufacturing and control instructions, testing procedures, acceptance limits, special notations, and precautions to be followed.
- Specific actions necessary in order to perform and verify points, steps, or stages in the manufacturing process where control is necessary to ensure the quality of dietary ingredients and dietary supplements, and to ensure that dietary ingredients and dietary supplements are packaged and labeled as specified in the master production record.
 - (a) Such specific actions must include verifying the weight or measure of any component and verifying the addition of any component; and
 - (b) For manual operations, such specific actions must include:
 - (i) One person weighing or measuring a component and another person verifying the weight or measure; and
 - (ii) One person adding the component and another person verifying the addition.
- Corrective action plans for use when a specification is not met.

Batch Production and Control Records

Batch production and control records should be prepared for each batch of product produced and should include complete information relating to the production and control of each batch. These records should be reviewed and signed by a second person from the quality control unit. These records should include accurate reproduction of the appropriate master production or control record and documentation that each significant step in the manufacture, processing, packing, or holding of the batch was accomplished, including the following:

- Dates;
- Identity of individual major equipment and lines used;
- Specific identification of each batch of raw material or in-process material used;
- Weights and measures of raw materials used in the course of processing;
- In-process and laboratory control results;
- Inspection of the packaging and labeling areas before and after use;
- A statement of the actual yield and a statement of the percentage of theoretical yield at appropriate phases of processing;
- Description of product containers and closures used;
- Complete labeling control records, including:
 - (a) The unique identifier assigned to packaging and labels used, the quantity of the packaging and labels used, and, when label reconciliation is required, reconciliation of any discrepancies between issuance and use of labels; and
 - (b) An actual or representative label, or a cross-reference to the physical location of the actual or representative label specified in the master manufacturing record;
- Any sampling performed;
- Identification of the persons performing and directly supervising or checking any step in the operation;
- Any investigation made;
- The results of any tests or examinations conducted on packaged and labeled dietary supplements (including repackaged or relabeled dietary supplements), or a cross-reference to the physical location of such results;
- Documentation at the time of performance that quality control unit:
 - (a) Reviewed the batch production record, including:

- (i) Review of any required monitoring operation, and
- (ii) Review of the results of any tests and examinations, including tests and examinations conducted on components, in-process materials, finished batches of dietary supplements, and packaged and labeled dietary ingredients and dietary supplements;
- (b) Approved or rejected any reprocessing or repackaging;
- (c) Approved and released, or rejected, the batch for distribution, including any reprocessed batch; and
- (d) Approved and released, or rejected, the packaged and labeled dietary supplement, including any repackaged or relabeled dietary supplement;
- Documentation at the time of performance of any required material review and disposition decision; and
- Documentation at the time of performance of any reprocessing.

Records for Raw Materials, Packaging, and Labels and for Product Received for Packaging or Labeling as a Dietary Supplement

The following records must be made and retained:

- Written procedures for fulfilling the requirements for raw materials, packaging, and labels and for product received for packaging or labeling;
- Receiving records (including records such as certificates of analysis, suppliers' invoices, and suppliers' guarantees) for components, packaging, and labels and for products received for packaging or labeling; and
- Documentation that the requirements of *Raw Materials, Labeling and Packaging* were met:
 - (a) The person who performs the required operation must document, at the time of performance, that the required operation was performed; and
 - (b) The documentation must include:
 - (i) The date of receipt of the raw materials, packaging, labels, or products received for packaging or labeling as a dietary supplement;
 - (ii) The initials of the person performing the required operation;
 - (iii) The results of any tests or examinations conducted on raw materials, packaging, or labels, and of any visual examination of product received for packaging or labeling as a dietary supplement; and
 - (iv) Any material review and disposition decision conducted on raw materials, packaging, labels, or products received for packaging or labeling as a dietary supplement.

Laboratory Records

Laboratory records should include complete data derived from all tests necessary to ensure compliance with established specifications and standards, including examinations and assays, as follows:

- A description of the sample received for testing with identification of source (that is, location from where sample was obtained), quantity, lot number or other distinctive code, and date sample was taken.
- A statement of each method used in the testing of the sample.
- A statement of the weight or measure of sample used for each test, where appropriate.
- A complete record of all data secured in the course of each test, including all graphs, charts, and spectra from laboratory instrumentation, properly identified to show the specific raw material, product container,

closure, in-process material, or finished product, and lot tested.

- A record of all calculations performed in connection with the test, including units of measure, conversion factors, and equivalency factors.
- A statement of the results of tests and how the results compare with established standards of identity, strength, quality, and purity for the raw material, product container, closure, in-process material, or finished product tested.
- The initials or signature of the person who performs each test and the date(s) the tests were performed.

Complete records should be maintained of any modification of an established method employed in testing. Such records should include the reason for the modification and data to verify that the modification produced results that are at least as accurate and reliable for the material being tested as the established method.

Complete records should be maintained of any testing and standardization of laboratory reference standards, reagents, and standard solutions, the periodic calibration of laboratory instruments, and all stability testing performed. Any deviation should be reviewed and signed by the management of the quality control unit.

Quality Control Operation Records

The following records must be made and retained:

- Written procedures for the responsibilities of the quality control operations, including written procedures for conducting a material review and making a disposition decision and written procedures for approving or rejecting any reprocessing;
- Written documentation, at the time of performance, that quality control unit performed the review, approval, or rejection requirements, by recording the following:
 - (i) Date on which the review, approval, or rejection was performed; and
 - (ii) Signature of the person performing the review, approval, or rejection; and
- Documentation of any material review and disposition decision and follow-up. Such documentation must be included in the appropriate batch production record and must include:
 - (i) Description of the investigation into the cause of the deviation from the specification or the unanticipated occurrence;
 - (ii) Evaluation of whether the deviation or unanticipated occurrence has resulted in, or could lead to, a failure to ensure the quality of the dietary supplement or a failure to package and label the dietary supplement as specified in the master manufacturing record;
 - (iii) Identification of the action(s) taken to correct, and prevent a recurrence of, the deviation or the unanticipated occurrence;
 - (iv) Explanation of the actions taken with the raw material, dietary supplement, packaging, or label;
 - (v) A scientifically valid reason for any reprocessing of a dietary supplement that is rejected or any treatment or in-process adjustment of a component that is rejected; and
 - (vi) The signatures of (1) the individual(s) designated to perform the quality control operation, who have conducted the material review and made the disposition decision; and in addition, (2) each qualified individual who has provided information relevant to that material review and disposition decision.

Distribution Records

Distribution records should contain the name and strength of the product, name and address of the consignee, date and quantity shipped, and lot or control number of the finished product.

Record Keeping

The manufacturer, packer, or distributor of dietary supplements must keep all required records, as shown in this chapter, for 3 years beyond the shelf life of dietary supplements associated with those records. If adverse event reports are received, those records must be kept for additional 6 years from the date when the first report is received. All records must be accessible by the regulatory authority when requested.

Records must be kept as original records, as true copies (such as photocopies, microfilm, microfiche, or other accurate reproductions of the original records), or as electronic records.

All electronic records must comply with part 11 of Code of Federal Regulations Title 21 (21 CFR part 11).

If reduction techniques are used, such as microfilming, suitable reader and photocopying equipment must be readily available to auditors and inspectors.

Complaint Files

Written procedures describing the handling of all written and oral complaints regarding a dietary supplement shall be established and followed. These procedures should include provisions for review by the quality control unit of any complaint involving the possible failure of a product to meet any of its specifications and a determination as to the need for an investigation.

Each complaint should be recorded in a file designed especially for dietary supplement complaints. Written records should be maintained until at least 3 years after the shelf life of the product, or 3 years after the date when the complaint was received, whichever is longer.

The written record should include the following information, where known: the name and strength of the product, lot number, name of complainant, nature of complaint, and reply to complainant.

If an investigation is necessary, the written record should include the findings of the investigation and follow-up.

The review and investigation of the product complaint by a qualified person, the review by quality control unit about whether to investigate a product complaint, and the findings and follow-up action of any investigation performed must extend to all relevant batches and records.

Adverse Event Reports

Adverse event reports include reports on any health-related adverse event associated with the use of a dietary supplement that is adverse. It includes both nonserious and serious adverse event reports.

The manufacturer, packer, or distributor of a dietary supplement (called the *responsible person*) whose name appears on the label shall be responsible for keeping reports of all nonserious adverse events along with any related records (e.g., records of communications with the person who reported the nonserious event). All such records of nonserious adverse events should be kept for 6 years.

The responsible person whose name appears on the label shall also be responsible for reporting any serious adverse event reported to it, and associated with a dietary supplement that is marketed and used in the same country, to the regulatory authority as soon as appropriate, but no later than 15 business days after receipt of the report, using the

appropriate form as defined by the regulation (<http://www.fda.gov/Food/DietarySupplements/Alerts/ucm111110.htm>). A serious adverse event is an event that results in any of the following:

1. Death,
2. A life-threatening experience,
3. Inpatient hospitalization,
4. A persistent or significant disability or inability,
5. A congenital anomaly or birth defect, or
6. A condition that requires, according to reasonable medical judgment, a medical or surgical intervention to prevent one of the five outcomes listed above.

A retailer whose name appears on the label as a distributor may, by agreement, authorize the manufacturer or packer to submit the required reports to the regulatory authority, as long as the retailer directs all received adverse event reports to the manufacturer or packer. Each serious adverse event report should include a copy of the product's label, the information described in the preceding section *Complaint Files*, and if possible, the contact information of the complainant; daily intake; alcohol consumption and amount; use of prescription medicine and OTC medicine, including a daily dose; and other medical information. The information associated with personal identification and medical records should be obtained only for the reports and kept safe from disclosure. Any new medical information that is related to an already submitted serious adverse event report that is received within 1 year of the initial report shall be submitted to the regulatory authority as soon as appropriate, but no later than 15 business days after receipt of the information. The records related to each report of a serious adverse event received by the manufacturer, packer, or retailer should be maintained for 6 years. The authorized person who is designated by the regulatory authority should be permitted access to those records.

RETURNED AND SALVAGED PRODUCTS

Returned Dietary Supplements

Returned products should be identified as such and held. If the conditions under which returned dietary ingredients and dietary supplements have been held, stored, or shipped before or during their return, or if the condition of the product, its container, carton, or labeling, as a result of storage or shipping, casts doubt on the safety, identity, strength, quality, or purity of the product, the returned product should be destroyed unless examination, testing, or other investigations prove the product meets appropriate standards of safety, identity, strength, quality, or purity. The returned products associated with adverse events must be destroyed after a sufficient sample of products is stored for the purpose of further investigation only. The products related to the adverse event that have been returned should be kept for 6 years (serious events) or 3 years (nonserious events) from the date when the first report is received. A product may be reprocessed provided that the subsequent product meets adequate standards, specifications, and characteristics. Records of returned products should be maintained and should include the name and label potency of the product, lot number (or control number or batch number), reason for the return, quantity returned, date of disposition, and ultimate disposition of the returned product. If the reason for a product being returned implicates associated batches, an appropriate investigation is necessary.

Dietary Supplement Salvaging

Products that have been involved in adverse events or subjected to improper storage conditions, including extremes in temperature or humidity, smoke, fumes, pressure, age, or radiation due to natural disasters, fires, accidents, or

equipment failures should not be salvaged and returned to the marketplace. Whenever there is a question whether products have been subjected to such conditions, salvaging operations may be conducted only if there is (a) evidence from laboratory tests and assays that the products meet all applicable standards of identity, strength, quality, and purity, and (b) evidence that the products and their associated packaging were not subjected to improper storage conditions as a result of the disaster or accident. Organoleptic examinations should be accepted only as supplemental evidence that the dietary supplement meets appropriate standards of identity, strength, quality, and purity. Records including name, lot number, and disposition should be maintained for salvaged products. If the products are involved in adverse events, the instructions described in the preceding section *Records and Reports* should be followed.

Defect Action Levels

Some dietary ingredients and dietary supplements, even when produced under current good manufacturing practice, contain natural or unavoidable defects that at low levels are not hazardous to health. The Food and Drug Administration establishes maximum levels for these defects in dietary ingredients and dietary supplements produced under current good manufacturing practice and uses these levels in deciding whether to recommend regulatory action.

Defect action levels are established for dietary ingredients and dietary supplements whenever it is necessary and feasible to do so. These levels are subject to change upon the development of new technology or the availability of new information.

Compliance with defect action levels does not excuse violation of the requirement in section 402(a)(4) of the Act that dietary ingredients and dietary supplements shall not be prepared, packed, or held under unsanitary conditions or the requirements in this part that manufacturers, distributors, and holders of both dietary ingredients and dietary supplements shall observe current good manufacturing practice. Evidence indicating that such a violation exists causes a dietary ingredient and a dietary supplement to be adulterated within the meaning of the Act, even though the amounts of natural or unavoidable defects are lower than the currently established defect action levels. The manufacturer, distributor, and holder of a dietary ingredient or a dietary supplement shall at all times utilize quality control operations that reduce natural or unavoidable defects to the lowest level currently feasible.

The mixing of a dietary ingredient or dietary supplement containing defects above the current defect action level with another lot of dietary ingredient or dietary supplement is not permitted and renders the final product adulterated within the meaning of the Act, regardless of the defect level of the final product.

A compilation of the current defect action levels for natural or unavoidable defects in dietary ingredients and dietary supplements that present no health hazard may be obtained upon request from the Industry Activities Staff (HFS-565), Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, 5100 Paint Branch Parkway, College Park, MD 20740-3835.

GLOSSARY

Act means Federal Food, Drug and Cosmetic Act (United States Code [U.S.C.] Title 21, Chapter 9).

Acceptance criteria are the product specifications and acceptance or rejection criteria, such as acceptable quality level and unacceptable quality level, with an associated sampling plan, that are necessary for making a decision to accept or reject a lot or batch (or any other convenient subgroups of manufactured units).

Adequate means that which is needed to accomplish the intended purpose in keeping with good public health practice.

Adverse event means any health-related event that is adverse and that is associated with the use of a dietary supplement.

Adverse event report means a report of an adverse event (see definition above). (See also *Serious adverse event report*.)

Batch is a specific quantity of a finished product or other material that is intended to have uniform character and quality, within specified limits, and is produced according to a single manufacturing order during the same cycle of manufacture.

Blanching means a prepackaging heat treatment of a dietary ingredient and a dietary supplement for a sufficient time and at a sufficient temperature to partially or completely inactivate the naturally occurring enzymes and to effect other physical or biochemical changes in the product.

Composition is (1) the identity of a dietary ingredient or dietary supplement, and (2) the concentration of a dietary ingredient (e.g., weight or other unit of use/weight or volume), or the potency or activity of one or more dietary ingredients, as indicated by appropriate procedures.

Dietary ingredient is an ingredient intended for use or used in a dietary supplement that is

- a vitamin;
- a mineral;
- an herb or other botanical;
- an amino acid;
- a dietary substance for use by humans to supplement the diet by increasing the total dietary intake, or a concentrate, metabolite, constituent, extract; or
- a combination of any of the foregoing ingredients.

Dietary supplement is a product (other than tobacco) that is intended to supplement the diet and that bears or contains one or more of the following dietary ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by humans to supplement the diet by increasing the total daily intake, or a concentrate, metabolite, constituent, extract or combination of these ingredients, that is intended for ingestion in a pill, capsule, tablet or liquid form, that is not represented for use as a conventional food or as the sole item of a meal or diet, and that is labeled as a dietary supplement, and includes products such as new drug, certified antibiotic, or licensed biologic that was marketed as a dietary supplement or food before approval, certification, or license unless a sanitary authority waives this provision.

Inactive ingredient is any raw material other than a dietary ingredient.

In-process material is any material fabricated, compounded, blended, ground, extracted, sifted, sterilized, or processed in any other way that is produced for, and used in, the preparation of the dietary supplement.

Lot is a batch, or a specific identified portion of a batch, having uniform character and quality within specified limits.

Lot number, control number, or batch number is any distinctive combination of letters, numbers, or symbols, or any combination of them from which the complete history of the manufacture, processing, packing, holding, and distribution of a batch or lot of finished dietary ingredient, dietary supplement, or other material can be determined.

Manufacture or manufacturing includes all operations associated with the production of dietary ingredients and dietary

supplements, including packaging and labeling operations, testing, and quality control of a dietary ingredient or dietary supplement.

Microorganisms means yeast, molds, bacteria, and viruses and includes, but is not limited to, species having public health significance. The term “undesirable microorganisms” includes those microorganisms that are of public health significance, that subject a dietary ingredient or a dietary supplement to decomposition, that indicate that a dietary ingredient or dietary supplement is contaminated with filth, or that otherwise may cause a dietary ingredient or a dietary supplement to be adulterated within the meaning of the Act. Occasionally in these regulations, the adjective “microbial” is used instead of an adjectival phrase containing the word “microorganism.”

Pest refers to any objectionable animals or insects including, but not limited to, bird, rodents, flies, and larvae.

Plant means the building or facility or parts thereof, used for or in connection with the manufacturing, packaging, labeling, or holding of a dietary ingredient and a dietary supplement.

Process evaluation is a set of tests performed on a process intended to evaluate its capacity to consistently produce the results that it is intended for.

Quality control operation is a planned and systematic procedure for taking all actions necessary to prevent a dietary ingredient and a dietary supplement from being adulterated.

Quality control unit is any person or organizational element designated by the firm to be responsible for the duties relating to quality control operations.

Raw material is any ingredient intended for use in the manufacture of a dietary ingredient or dietary supplement, including those that may not appear in such finished product. (A dietary ingredient is a raw material when considering the manufacture of a dietary supplement.)

Representative sample is a sample that consists of a number of units that are drawn based on rational criteria such as random sampling and is intended to ensure that the sample accurately portrays the material being sampled.

Rework is a clean, unadulterated material that has been removed from processing for reasons other than unsanitary conditions or that has been successfully reconditioned by reprocessing and that is suitable for use in the manufacture of a dietary ingredient or a dietary supplement.

Sanitizing is to adequately treat equipment, containers, or utensils by a process that is effective in destroying vegetative cells of microorganisms of public health significance and in substantially reducing other undesirable microorganisms but without affecting the product or its safety for the consumer.

Serious adverse event report means a report of an adverse event that is termed *serious* because it meets certain criteria (see the subsection *Adverse Event Reports*). The Dietary Supplement and Nonprescription Drug Consumer Protection Act requires manufacturers and distributors of dietary supplements and OTC drugs to report all serious adverse events to the Secretary of the Food and Drug Administration (FDA). This is an entirely new requirement for dietary supplements.

Shall is used to state requirements that must be met under the provisions of this guideline.

Shelf life is the period of time after manufacturing in which the dietary supplement is ensured to meet applicable standards of identity, strength, quality, and purity.

Shelf-life (Use by) date is the date beyond which the dietary supplement is no longer ensured to meet applicable standards of identity, strength, quality, and purity.

Should is used to state recommended or advisory procedures or identify recommended equipment.

Skip-lot testing (or sampling) is a reduced level of testing (or sampling) for a particular specified parameter(s) based upon one or more of the following:

- Statistical analysis of an adequate quantity of historical test data;

- Statistical confidence in the capability of the manufacturing process as determined by suitable verification; or

- Ongoing monitoring of the process using recognized statistical process control (SPC) techniques.

Strength means the concentration of the active substance (weight/weight, weight/volume, or unit of use/volume or weight basis); and/or the potency, that is, the activity of the product as indicated by appropriate laboratory tests.

Water activity (a_w) is a measure of the free moisture in a dietary ingredient or dietary supplement and is the quotient of the water vapor pressure of the substance divided by the vapor pressure of pure water at the same temperature.

Reagents, Indicators and Solutions

This section deals with the reagents and solutions required in conducting the Pharmacopeial and the National Formulary tests and assays.

As is stated in the *General Notices*, listing of reagents, indicators, and solutions in the Pharmacopeia in no way implies that they have therapeutic utility; thus, any reference to the USP in their labeling is to include the term "reagent" or "reagent grade."

Reagents required in the tests and assays for the Pharmacopeial and National Formulary articles are listed in this section, generally with specifications appropriate to their intended uses. Exceptions to the latter include those reagents for which corresponding specifications are presented in the current edition of *Reagent Chemicals*, published by the American Chemical Society, and reagents for which specifications could not be drafted in time for inclusion here. Thus, where it is directed to "Use ACS reagent grade," it is intended that a grade meeting the corresponding specifications of the current edition of *ACS Reagent Chemicals* shall be used. Where no such specifications exist, and where it is directed to "Use a suitable grade," the intent is that a suitable reagent grade available commercially shall be used. Occasionally, additional test(s) augment the designation "suitable grade," as indicated in the text. Listed also are some, but not all, reagents that are required only in determining the quality of other reagents. For those reagents that are not listed, satisfactory specifications are available in standard reference works.

In those instances in which a reagent required in a Pharmacopeial or National Formulary test or assay need not be of analytical reagent quality, it suffices to refer to the monograph for that article appearing in this Pharmacopeia or the National Formulary or the current edition of the Food Chemicals Codex (FCC). In such cases it is to be understood that the specifications are minimum requirements and that any substance meeting more rigid specifications for chemical purity is suitable.

Where the name of a reagent specified in a test or assay is the same as the title of a USP or NF article, and it does not appear among the following *Reagent Specifications*, a substance meeting the requirements of the USP or NF monograph is to be used (e.g., *Benzocaine*, USP; or *Propylparaben*, NF). However, reference is specifically made, under *Reagent Specifications*, to a reagent bearing the name of a USP or NF article: (1) where there are requirements for a reagent in addition to the USP or NF monograph requirements (e.g., *Sodium Salicylate*, USP; or *Isopropyl Myristate*, NF), (2) where a source other than the USP or NF monograph is specified (e.g., *Lactose*, ACS reagent; or *Hydrochloric Acid*, ACS reagent), (3) where complete reagent specifications differ from the USP or NF monograph standards (e.g., *Calcium Lactate*; or *Thymol*), or (4) where a standard material is included among the reagent specifications (e.g., *Calcium Carbonate*, primary standard; or *Sodium Carbonate*, primary standard).

Reagents and solutions should be preserved in tight containers made of resistant glass or other suitable material. Directions for storage in light-resistant containers should be carefully observed.

Stoppers and stopcocks brought into contact with substances capable of attacking or penetrating their surfaces may be given a protective coating of a thin film of a suitable lubricant unless specifically interdicted.

Where a particular brand or source of a material or piece of equipment, or the name and address of a manufacturer, is mentioned, this identification is furnished solely for informational purposes as a matter of convenience, without implication of approval, endorsement, or certification.

Atomic absorption and flame photometry require the use of a number of metal-ion standard solutions. While the individual monographs usually provide directions for preparation of these solutions, use of commercially prepared standardized solutions of the appropriate ions is permissible, provided that the analyst confirms the suitability of the solutions and has data to support their use.

Reagents are substances used either as such or as constituents of solutions.

Indicators are reagents used to determine the specified endpoint in a chemical reaction, to measure hydrogen-ion concentration (pH), or to indicate that a desired change in pH has been effected. They are listed together with indicator test papers.

Buffer Solutions are referred to separately.

Colorimetric Solutions, abbreviated "CS," are solutions used in the preparation of colorimetric standards for comparison purposes.

Test Solutions, abbreviated "TS," are solutions of reagents in such solvents and of such definite concentrations as to be suitable for the specified purposes.

Volumetric Solutions, abbreviated "VS" and known also as **Standard Solutions**, are solutions of reagents of known concentration intended primarily for use in quantitative determinations. Concentrations are usually expressed in terms of normality.

Water—As elsewhere in the Pharmacopeia, where "water", without qualification, is mentioned in the tests for reagents or in directions for preparing test solutions, etc., Purified Water (USP monograph) is always to be used. *Carbon dioxide-free water* is Purified Water that has been boiled vigorously for 5 minutes or more and allowed to cool while protected from absorption of carbon dioxide from the atmosphere, or Purified Water that has a resistivity of not less than 18 Mohm-cm. *Deaerated water*, for purposes other than dissolution and drug release testing, is Purified Water that has been treated to reduce the content of dissolved air by suitable means, such as by boiling vigorously for 5 minutes and cooling or by the application of ultrasonic vibration. *Particle-free water* is water that has been passed through a 0.22- μ m filter.

Organic-free water is Purified Water that produces no significantly interfering peaks when chromatographed as it is indicated in *Identification*, *Control*, and *Quantification of Residual Solvents* under *Residual Solvents* (467).

Chromatographic Solvents and Carrier Gases—The chromatographic procedures set forth in the Pharmacopeia may require use of solvents and gases that have been especially purified for such use. The purpose may be (a) to exclude certain impurities that interfere with the proper conduct of the test procedure, or (b) to extend the life of a column by reducing the build-up of impurities on the column. Where solvents and gases are called for in chromatographic procedures, it is the responsibility of the analyst to ensure the suitability of the solvent or gas for the specific use. Solvents and gases suitable for specific high-pressure or other chromatographic uses are available as specialty products from various reagent supply houses, although there is

no assurance that similar products from different suppliers are of equivalent suitability in any given procedure. The reagent specifications provided herein are for general analytical

uses of the solvents and gases and not for chromatographic uses for which the especially purified specialty products may be required.

Reagents

For the purposes of the following specifications, these definitions apply: A *blank* consists of the same quantities of the same reagents treated in the same manner as the specimen under test. A *control* is a blank to which has been added the limiting quantity of the substance being tested for, or is a specified comparison solution prepared as directed in the particular test.

The values given in boldface type following chemical symbols and formulas represent, respectively, atomic and molecular weights of the substances concerned.

Color and turbidity comparisons are to be made in color-comparison tubes that are matched as closely as possible in internal diameter and in all other respects, as directed for *Visual Comparison* under *Spectrophotometry and Light-scattering* (851). Such tubes frequently are called "Nessler tubes."

In making visual comparisons of the densities of turbid fluids, compensate for differences in color, if necessary, by viewing the turbidity through a column of water, the depth of which is determined by the volume specified in the individual reagent specification. Place the water in color-comparison tubes, and hold one of the tubes above the control tube and the other below the specimen tube.

Where an expression such as "Retain the filtrate" appears it is to be understood, unless otherwise indicated, that the washings of the residue are not to be added to the filtrate obtained. In the test heading, *Calcium, magnesium, and R₂O₃ precipitate*, the expression *R₂O₃* is intended to indicate the residue on ignition from compounds precipitated upon the addition of ammonium hydroxide, such as *Fe₂O₃* and *Al₂O₃*.

GENERAL TESTS FOR REAGENTS

The following general test methods are provided for the examination of reagents to determine their compliance with the specifications of the individual reagents and are to be used unless it is otherwise directed in such specifications.

Boiling or Distilling Range for Reagents

Use the following procedure for determining the boiling or distilling range of reagents, unless otherwise directed in the individual specifications:

APPARATUS—Use apparatus similar to that specified for *Distilling Range—Method I* (721), except that the distilling flask is to be of 250-mL capacity, to have a short neck, and to be connected to the condenser by means of a three-way connecting tube fitted with standard-taper ground joints.

PROCEDURE—Place the distilling flask in an upright position in the perforation in the asbestos board, and connect it to the condenser.

Measure 100 mL of the liquid to be tested in a graduated cylinder, and transfer to the boiling flask together with some device to prevent bumping. Use the cylinder as the receiver for the distillate. Insert the thermometer, and heat so as to distill at the rate of 3 mL to 5 mL per minute. Make a preliminary trial, if necessary, to determine the adjustment for the proper rate of heating. Read the thermometer when about 20 drops have distilled and thereafter at volumes of distillate of 5, 10, 40, 50, 60, 90, and 95 mL. Continue the distillation until the dry point is reached.

The *Boiling or Distilling Range* is the interval between the temperatures when 1 mL and 95 mL, respectively, have distilled.

Arsenic in Reagents

Select reagents for this test for a low arsenic content, so that a blank test results in either no stain or one that is barely perceptible.

APPARATUS—Prepare a generator by fitting a 1-hole rubber stopper into a wide-mouth bottle of about 60-mL capacity. Through the perforation insert a vertical exit tube about 12 cm in total length and 1 cm in diameter along the entire upper portion (for about 8 cm) and constricted at its lower extremity to a tube about 4 cm in length and about 5 mm in diameter. The smaller portion of the tube should extend to just slightly below the stopper. Place washed sand or a pledget of purified cotton in the upper portion to about 3 cm from the top of the tube. Moisten the sand or cotton uniformly with lead acetate TS, and remove any excess or adhering droplets of the latter from the walls of the tube. Into the upper end of this tube fit a second glass tube 12 cm in length, having an internal diameter of 2.5 to 3 mm, by means of a rubber stopper. Just before running the test, place a strip of mercuric bromide test paper (see under *Indicator and Test Papers*) in this tube, crimping the upper end of the strip so that it will remain in position about 2 cm above the rubber stopper. Clean and dry the tube thoroughly each time it is used.

STANDARD ARSENIC SOLUTION—Use *Standard Preparation* prepared as directed under *Arsenic* (211).

TEST PREPARATION—Add 1 mL of sulfuric acid to 5 mL of a solution of the chemical substance (1 in 25), unless another quantity is directed in the individual reagent specification. Omit its addition entirely in the case of inorganic acids. Unless especially directed otherwise, add 10 mL of sulfurous acid. Evaporate the liquid in a small beaker, on a steam bath, until it is free from sulfurous acid and has been reduced to about 2 mL in volume. Dilute with water to 5 mL to obtain the *Test Preparation*. Substances subjected to special treatments specified in the individual reagent specification may be used directly as the *Test Preparation*.

[NOTE—Solutions prepared by the dissolving of the chemical substances in dilute acids are not considered to have undergone special treatment.]

STANDARD STAIN—Place in the generator bottle 5 mL of potassium iodide TS, 2.0 mL of *Standard Arsenic Solution*, 5 mL of acid stannous chloride TS, and 28 mL of water. Add 1.5 g of granulated zinc (in No. 20 powder), and immediately insert the stopper containing the exit tube. Keep the generator bottle immersed in water at 25° during the period of the test to moderate the reaction so that the stain will take the form of a distinctive band to facilitate the comparison of color intensity. When evolution of hydrogen has continued for 1 hour, remove the mercuric bromide test paper for comparison. This stain represents 2 µg of arsenic.

PROCEDURE—Pipet into the generator bottle 5 mL of potassium iodide TS and 5 mL of the *Test Preparation*, and add 5 mL of acid stannous chloride TS. Set the apparatus aside at room temperature for a period of 10 minutes, then add 25 mL of water and 1.5 g of granulated zinc (in No. 20 powder), and proceed as directed under *Standard Stain*. Remove the mercuric bromide test paper, and compare the stain upon it with the *Standard Stain*: the stain produced by the chemical tested does not exceed the standard stain in length or in intensity of color, indicating not more than 10 parts of arsenic per million parts of the substance being tested. Since light, heat, and moisture cause the stain to

fade rapidly, place the papers in clean, dry tubes, and make comparisons promptly.

INTERFERING CHEMICALS—*Antimony*, if present in the substance being tested, produces a gray stain. *Sulfites*, *sulfides*, *thiosulfates*, and other compounds that liberate hydrogen sulfide or sulfur dioxide when treated with sulfuric acid must be oxidized by means of nitric acid and then reduced by means of sulfur dioxide as directed under *Test Preparation* before they are placed in the apparatus. Certain *sulfur compounds*, as well as *phosphine*, give a bright yellow band on the test paper. If *sulfur compounds* are present, the lead acetate-moistened cotton or sand will darken. In that case, repeat the operation as directed under *Test Preparation* upon a fresh portion of the solution being tested and use greater care in effecting the complete removal of the sulfurous acid. In testing hypophosphites, observe special care to oxidize completely the solution being tested as directed, otherwise the evolution of phosphine may result in a yellow stain which may be confused with the orange-yellow color produced by arsine. The stain produced by phosphine may be differentiated from that given by arsine by means of moistening it with 6 N ammonium hydroxide. A stain caused by arsine becomes dark when so treated, but a stain produced by phosphine does not materially change in color.

Chloride in Reagents

STANDARD CHLORIDE SOLUTION—Dissolve 165.0 mg of dried sodium chloride in water to make 1000.0 mL. This solution contains the equivalent of 0.10 mg of chlorine (Cl) in each mL.

PROCEDURE—Neutralize, if alkaline, a solution of the quantity of the reagent indicated in the test in 25 mL of water, or a solution prepared as directed in the test, with nitric acid, litmus paper being used as the indicator, and add 3 mL more of nitric acid. Filter the solution, if necessary, through a filter paper previously washed with water until the paper is free from chloride, and add 1 mL of silver nitrate TS. Mix, and allow to stand for 5 minutes protected from direct sunlight. Compare the turbidity, if any, with that produced in a control made with the same quantities of the same reagents as in the final test and a volume of *Standard Chloride Solution* equivalent to the quantity of chloride (Cl) permitted by the test. Adjust the two solutions with water to the same volume before adding the silver nitrate TS, and compare the turbidities.

In *testing barium salts*, neutralize, if alkaline, the solution containing the reagent, with nitric acid, and add only 3 drops more of nitric acid. Conduct the remainder of the test as described previously.

In *testing salts giving colored solutions*, dissolve 2 g of the reagent in 25 mL of water, and add 3 mL of nitric acid. Filter the solution, if necessary, through a filter paper previously washed with water, and divide the filtrate into two equal portions. Treat one portion with 1 mL of silver nitrate TS, allow to stand for 10 minutes, and, if any turbidity is produced, filter it through a washed filter paper until clear, and use the filtrate as a blank. Treat the other portion with 1 mL of silver nitrate TS, mix, and allow to stand for 5 minutes protected from direct sunlight. Compare the turbidity with that produced in the blank by the addition of a volume of *Standard Chloride Solution* equivalent to the quantity of chloride (Cl) permitted in the test, both solutions being adjusted with water to the same volume.

Flame Photometry for Reagents

The use of flame photometric procedures to determine traces of calcium, potassium, sodium, and strontium is called for in some of the reagent specifications. The suitability of such determinations depends upon the use of adequate apparatus, and several instruments of suitable selectiv-

ity are available. The preferred type of flame photometer is one that has a red-sensitive phototube, a multiplier phototube, a monochromator, an adjustable slit-width control, a selector switch, and a sensitivity control. Other types of photometers may be used, provided the operator has proved that the instrument will determine accurately the amount of impurities permitted in the reagent to be tested.

The flame photometric procedures depend upon the use of semi-internal standards, and thus require both a *Sample Solution* and a *Control Solution*. For the *Sample Solution*, a specified weight of specimen is dissolved and diluted to a definite volume. For the *Control Solution*, the same amount of specimen is dissolved, the limiting amounts of the suspected impurities are added, and the solution is then diluted to the same definite volume as the *Sample Solution*. The flame photometer is set as directed in the general procedures and then adjusted to give an emission reading as near 100% transmittance as is possible with the *Control Solution* at the wavelength specified for the particular impurity concerned. With the instrument settings left unchanged, the emission from the *Sample Solution* is read at the same wavelength and at a specified background wavelength. The background reading is then used to correct the observed emission of the *Sample Solution* for the emission due to the specimen and the solvent. The specimen being tested contains less than the specified limit of impurity if the difference between the observed background and total emissions for the *Sample Solution* is less than the difference between the observed emissions for the *Control Solution* and the *Sample Solution* at the wavelength designated for the particular impurity.

CALCIUM IN REAGENTS

Standard Calcium Solution—Dissolve 250 mg of calcium carbonate in a mixture of 20 mL of water and 5 mL of diluted hydrochloric acid, and when solution is complete, dilute with water to 1 L. This solution contains 0.10 mg of calcium (Ca) per mL.

Procedure—Use the *Sample Solution* and the *Control Solution* prepared as directed in the individual test procedure.

Set the slit-width control of a suitable flame photometer at 0.03 mm, and set the selector switch at 0.1. Adjust the instrument to give the maximum emission with the *Control Solution* at the 422.7-nm calcium line, and record the transmittance. Without changing any of the instrument settings, record the transmittance for the emission of the *Sample Solution* at 422.7 nm. Change the monochromator to the wavelength specified in the individual test procedure, and record the background transmittance for the background emission of the *Sample Solution*: the difference between the transmittances for the *Sample Solution* at 422.7 nm and at the background wavelength is not greater than the difference between transmittances observed at 422.7 nm for the *Sample Solution* and the *Control Solution*.

POTASSIUM IN REAGENTS

Standard Potassium Solution—Dissolve 191 mg of potassium chloride in a few mL of water, and dilute with water to 1 L. Dilute a portion of this solution with water in the ratio of 1 to 10 to obtain a concentration of 0.01 mg of potassium (K) per mL.

Procedure—Use the *Sample Solution* and the *Control Solution* prepared as directed in the individual test procedure.

[NOTE—In testing calcium salts, use an oxyhydrogen burner.]

Set the slit-width control of a suitable flame photometer equipped with a red-sensitive detector at 0.1 mm, unless otherwise directed, and set the selector switch at 0.1. Adjust the instrument to give the maximum emission with the *Control Solution* at the 766.5-nm potassium line, and record the transmittance. Without changing any of the instrument settings, record the transmittance for the emission of the *Sample Solution* at 766.5 nm. Change the monochromator to 750 nm, and record the background transmittance for the background emission of the *Sample Solution*: the difference

between the transmittances for the *Sample Solution* at 766.5 nm and 750 nm is not greater than the difference between transmittances observed at 766.5 nm for the *Sample Solution* and the *Control Solution*.

SODIUM IN REAGENTS—

Standard Sodium Solution—Dissolve 254 mg of sodium chloride in a few mL of water, and dilute with water to 1 L. Dilute a portion of this solution with water in the ratio of 1 to 10 to obtain a concentration of 0.01 mg of sodium (Na) per mL.

Procedure—Use the *Sample Solution* and the *Control Solution* prepared as directed in the individual test procedure.

Set the slit-width control of a suitable flame photometer at 0.01 mm, and set the selector switch at 0.1. Adjust the instrument to give the maximum emission with the *Control Solution* at the 589-nm sodium line, and record the transmittance. Without changing any of the instrument settings, record the transmittance for the emission of the *Sample Solution* at 589 nm. Change the monochromator to 580 nm, and record the background transmittance for the background emission of the *Sample Solution*: the difference between the transmittances for the *Sample Solution* at 589 and 580 nm is not greater than the difference between transmittances observed at 589 nm for the *Sample Solution* and the *Control Solution*.

STRONTIUM IN REAGENTS—

Standard Strontium Solution—Dissolve 242 mg of strontium nitrate in a few mL of water, and dilute with water to 1 L. Dilute a portion of this solution with water in the ratio of 1 to 10 to obtain a concentration of 0.01 mg of strontium (Sr) per mL.

Procedure—Use the *Sample Solution* and the *Control Solution* prepared as directed in the individual test procedure.

Set the slit-width control of a suitable flame photometer at 0.03 mm, and set the selector switch at 0.1. Adjust the instrument to give the maximum emission with the *Control Solution* at the 460.7-nm strontium line, and record the transmittance. Without changing any of the instrument settings, record the transmittance for the emission of the *Sample Solution* at 460.7 nm. Change the monochromator to the wavelength specified in the individual test procedure, and record the background transmittance for the background emission of the *Sample Solution*: the difference between the transmittances for the *Sample Solution* at 460.7 nm and at the background wavelength is not greater than the difference between transmittances observed at 460.7 nm for the *Sample Solution* and the *Control Solution*.

Heavy Metals in Reagents

STANDARD LEAD SOLUTION—Use *Standard Lead Solution* (see *Heavy Metals* (231)). Each mL of this solution contains the equivalent of 0.01 mg of Pb.

PROCEDURE—Unless otherwise directed, test for heavy metals as follows:

(a) If the heavy metals limit is 0.0005% (5 ppm), dissolve 6.0 g of the specimen in water to make 42 mL.

(b) If the heavy metals limit is 0.001% (10 ppm) or more, or in the event of limited solubility, use 4 g, and dissolve in water to make 40 mL, warming, if necessary, to aid solution.

For the control, transfer 7 mL of the solution from (a) to a color-comparison tube, and add a volume of *Standard Lead Solution* equivalent to the amount of lead permitted in 4 g of the reagent. Dilute with water to 35 mL, and add diluted acetic acid, or ammonia TS, until the pH is about 3.5, determined potentiometrically, then dilute with water to 40 mL, and mix. Transfer the remaining 35 mL of the solution from (a) to a color-comparison tube closely matching that used for the control, and add diluted acetic acid, or ammonia TS, until the pH is about 3.5, determined potentiometrically, then dilute with water to 40 mL, and mix. Then to each

tube add 10 mL of hydrogen sulfide TS, mix, and compare the colors by viewing through the color-comparison tube downward against a white surface. The color in the test specimen is not darker than that of the control.

If the solution of the reagent is prepared as in (b), use for the control 10 mL of the solution, and add to it a volume of *Standard Lead Solution* equivalent to the amount of lead permitted in 2 g of the reagent. Dilute the remaining 30 mL of solution (b) with water to 35 mL, and proceed as directed in the preceding paragraph, beginning with "add diluted acetic acid, or ammonia TS," in the second sentence.

If the reagent to be tested for heavy metals is a salt of an aliphatic organic acid, substitute 1 N hydrochloric acid for the diluted acetic acid specified in the foregoing method.

Insoluble Matter in Reagents

Dissolve the quantity of reagent specified in the test in 100 mL of water, heat to boiling unless otherwise directed, in a covered beaker, and warm on a steam bath for 1 hour. Filter the hot solution through a tared sintered-glass crucible of fine porosity. Wash the beaker and the filter thoroughly with hot water, dry at 105°, cool in a desiccator, and weigh.

Loss on Drying for Reagents

Determine as directed under *Loss on Drying* (731).

Nitrate in Reagents

STANDARD NITRATE SOLUTION—Dissolve 163 mg of potassium nitrate in water, add water to make 100 mL, and dilute 10 mL of this solution with water to 1 liter, to obtain a solution containing the equivalent of 0.01 mg of NO₃ per mL.

BRUCINE SULFATE SOLUTION—Dissolve 600 mg of brucine sulfate in 600 mL of nitrate-free, dilute sulfuric acid (2 in 3) that previously has been cooled to room temperature, and dilute with the acid to 1 L. [NOTE—Prepare the nitrate-free sulfuric acid by adding 4 parts of sulfuric acid to 1 part of water, heating the solution to dense fumes of sulfur trioxide, and cooling. Repeat the dilution and heating three or four times.]

SAMPLE SOLUTION—To the weight of sample specified in the individual reagent specification, dissolved in the designated volume of water, add *Brucine Sulfate Solution* to make 50 mL.

CONTROL SOLUTION—To a volume of *Standard Nitrate Solution* equivalent to the weight of nitrate (NO₃) specified in the individual reagent specification, add the weight of sample specified in the individual reagent specification and then add *Brucine Sulfate Solution* to make 50 mL.

BLANK SOLUTION—Use 50 mL of *Brucine Sulfate Solution*.

PROCEDURE—Heat the *Sample Solution*, *Control Solution*, and *Blank Solution* in a boiling water bath for 10 minutes, then cool rapidly in an ice bath to room temperature. Adjust a suitable spectrophotometer to zero absorbance at 410 nm with the *Blank Solution*. Determine the absorbance of the *Sample Solution*, note the result, and adjust the instrument to zero absorbance with the *Sample Solution*. Determine the absorbance of the *Control Solution*: the absorbance reading for the *Sample Solution* does not exceed that for the *Control Solution*.

Nitrogen Compounds in Reagents

PROCEDURE—Unless otherwise directed, test for nitrogen compounds as follows: Dissolve the specified quantity of test specimen in 60 mL of ammonia-free water in a Kjeldahl flask

connected through a spray trap to a condenser, the end of which dips below the surface of 10 mL of 0.1 N hydrochloric acid. Add 10 mL of freshly boiled sodium hydroxide solution (1 in 10) and 500 mg of aluminum wire, in small pieces, to the Kjeldahl flask, and allow to stand for 1 hour, protected from loss of, and exposure to, ammonia. Distill 35 mL, and dilute the distillate with water to 50 mL. Add 2 mL of freshly boiled sodium hydroxide solution (1 in 10), mix, add 2 mL of alkaline mercuric-potassium iodide TS, and again mix: the color produced is not darker than that of a control containing the amount of added N (as ammonium chloride) specified in the individual test procedure.

Phosphate in Reagents

STANDARD PHOSPHATE SOLUTION—Dissolve 143.3 mg of dried monobasic potassium phosphate, KH_2PO_4 , in water to make 1000.0 mL. This solution contains the equivalent of 0.10 mg of phosphate (PO_4) in each mL.

PHOSPHATE REAGENT A—Dissolve 5 g of ammonium molybdate in 1 N sulfuric acid to make 100 mL.

PHOSPHATE REAGENT B—Dissolve 200 mg of *p*-methylaminophenol sulfate in 100 mL of water, and add 20 g of sodium bisulfite. Store this reagent in well-filled, tightly stoppered bottles, and use within one month.

PROCEDURE—[NOTE—The tests with the specimen and the control are made preferably in matched color-comparison tubes.] Dissolve the quantity of the reagent specified in the test, or the residue obtained after the prescribed treatment, in 20 mL of water, by warming, if necessary, add 2.5 mL of dilute sulfuric acid (1 in 7), and dilute with water to 25 mL. (If preferable, the test specimen or the residue may be dissolved in 25 mL of approximately 0.5 N sulfuric acid.) Then add 1 mL each of *Phosphate Reagents A* and *B*, mix, and allow to stand at room temperature for 2 hours. Compare any blue color produced with that produced in a control made with the same quantities of the same reagents as in the test with the specimen, and a volume of *Standard Phosphate Solution* equivalent to the quantity of phosphate (PO_4) designated in the reagent specifications.

Residue on Ignition in Reagents

PROCEDURE—Unless otherwise directed, determine the residue on ignition as follows: Weigh accurately 1 to 2 g of the substance to be tested in a suitable crucible that previously has been ignited, cooled, and weighed. Ignite the substance, gently and slowly at first and then at a more rapid rate, until it is thoroughly charred, if organic in nature, or until it is completely volatilized, if inorganic in nature. If the use of sulfuric acid is specified, cool the crucible, add the specified amount of acid, and ignite the crucible gently until fumes no longer are evolved. Then ignite the crucible at $800 \pm 25^\circ$, cool in a suitable desiccator, and weigh. If the use of sulfuric acid is not specified, the crucible need not be cooled but can be ignited directly at $800 \pm 25^\circ$ once the charring or volatilization is complete. Continue the ignition until constant weight is attained, unless otherwise specified.

Conduct the ignition in a well-ventilated hood, but protected from air currents, and at as low a temperature as is possible to effect the complete combustion of the carbon. A muffle furnace may be used, if desired, and its use is recommended for the final ignition at $800 \pm 25^\circ$.

Sulfate in Reagents

STANDARD SULFATE SOLUTION—Dissolve 181.4 mg of potassium sulfate (dried at 105° for 2 hours) in water to make 1000 mL. This solution contains the equivalent of 0.10 mg of sulfate (SO_4) per mL.

PROCEDURE—

Method I—Neutralize, if necessary, a solution of the quantity of the reagent or residue indicated in the test in 25 mL of water, or a solution prepared as directed in the test, with hydrochloric acid or with ammonia TS, litmus paper being used as the indicator, and add 1 mL of 1 N hydrochloric acid. Filter the solution, if necessary, through a filter paper previously washed with water, and add 2 mL of barium chloride TS. Mix, allow to stand for 10 minutes, and compare the turbidity, if any, with that produced in a control containing the same quantities of the same reagents used in the test and a quantity of *Standard Sulfate Solution* equivalent to the quantity of sulfate (SO_4) permitted in the test. Adjust the two solutions with water to the same volume before adding the barium chloride TS.

Method II—Heat to boiling the solution, prepared as directed in the individual test procedure, or the filtrate designated in the procedure, and add 5 mL of barium chloride TS. Then digest the solution on a steam bath for 2 hours, and allow to stand overnight. If any precipitate is formed, filter the solution through paper, wash the residue with hot water, and transfer the paper containing the residue to a tared crucible. Char the paper, without burning, and ignite the crucible and its contents to constant weight. Perform a blank determination concurrently with the test specimen determination, and subtract the weight of residue obtained from that obtained in the test specimen determination to obtain the weight of residue attributable to the sulfate content of the specimen.

REAGENT SPECIFICATIONS

Absolute Ether—See *Ethyl Ether, Anhydrous*.

Absorbent Cotton—Use *Purified Cotton* (USP monograph).

Acetal, $\text{C}_6\text{H}_{14}\text{O}_2$ —**118.2**—Use a suitable grade.

Acetaldehyde (*Ethanal*; *Acetic Aldehyde*), CH_3CHO —**44.05** [75-07-0]—Colorless liquid. Miscible with water and with alcohol. Use ACS reagent grade.

Acetanilide (*Phenylacetamide*; *Antifebrin*), $\text{C}_8\text{H}_9\text{NO}$ —**135.16** [103-84-4]—White, shiny crystals, usually in scales, or a white, crystalline powder. Is stable in air. Freely soluble in alcohol and in chloroform; soluble in boiling water, in ether, and in glycerin; slightly soluble in water.

Melting range (741°): between 114° and 116° .

Reaction—Its saturated solution is neutral to litmus.

Loss on drying (731°)—Dry it over sulfuric acid for 2 hours: it loses not more than 0.5% of its weight.

Residue on ignition (Reagent test): not more than 0.05%.

Acetic Acid (*6 N Acetic Acid*)—Use *Acetic Acid* (NF monograph) or prepare a suitable dilution of glacial acetic acid in such a way as to obtain a final concentration of acetic acid between 36.0% and 37.0%, by weight.

Acetic Acid, Diluted (*1 N Acetic Acid*)—Dilute 60.0 mL of glacial acetic acid with water to make 1000 mL.

Residue on evaporation—Evaporate 50 mL on a steam bath, and dry the residue at 105° for 2 hours: the residue weighs not more than 1 mg (0.002%).

Chloride (Reagent test)—Five mL shows not more than 0.01 mg of Cl (2 ppm).

Sulfate (Reagent test, *Method I*)—Ten mL shows not more than 0.5 mg of SO_4 (50 ppm).

Heavy metals (Reagent test)—Evaporate 20 mL on a steam bath to dryness. Add to the residue 2 mL of the acid, dilute with water to 25 mL, and add 10 mL of hydrogen sulfide TS: any brown color produced is not darker than that

of a control containing 0.04 mg of added Pb and 2 mL of the diluted acetic acid (2 ppm).

Acetic Acid, Glacial, CH_3COOH —**60.05** [64-19-7]—Use ACS reagent grade.

Acetic Anhydride (*Acetic Oxide; Acetyl Oxide*), $(\text{CH}_3\text{CO})_2\text{O}$ —**102.09** [108-24-7]—Use ACS reagent grade.

Acetone (*Propanone; Dimethylformaldehyde*), CH_3COCH_3 —**58.08** [67-64-1]—Use ACS reagent grade. [NOTE—For UV spectrophotometric determinations, use ACS reagent grade Acetone Suitable for Use in UV Spectrophotometry.]

Acetone, Anhydrous, CH_3COCH_3 —**58.08**—Use ACS reagent grade Acetone.

Acetone, Neutralized—To a suitable quantity of acetone add 2 or 3 drops of phenolphthalein TS and a sufficient amount of 0.02 or 0.01 N sodium hydroxide to produce a faint pink color. Prepare neutralized acetone just prior to use.

Acetonitrile (*Methyl Cyanide; Cyanomethane*), CH_3CN —**41.05** [75-05-8]—Use ACS reagent grade.

Acetonitrile, Spectrophotometric—Use ACS reagent grade, which meets also the requirements of the following test.

Spectral purity—Measure in a 1-cm cell between 250 nm and 280 nm, with a suitable spectrophotometer, against air as the blank: its absorbance is not more than 0.01.

Acetophenone (*Phenylethanone; Phenyl Methyl Ketone*), $\text{CH}_3\text{COC}_6\text{H}_5$ —**120.15** [98-86-2]—Liquid. Slightly soluble in water, freely soluble in alcohol and in ether.

Melting range (741): between 19° and 20°.

Refractive index (831): about 1.534 at 20°.

Specific gravity (841): about 1.03.

p-Acetotoluidide, $\text{C}_9\text{H}_{11}\text{NO}$ —**149.19** [103-89-9]—White to off-white powder.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μm layer of phase G2; the injection port temperature is maintained at 230°; the detector temperature is maintained at 300°; and the column temperature is maintained at 130° and programmed to rise 10° per minute to 280°. The area of the $\text{C}_9\text{H}_{11}\text{NO}$ peak is not less than 98.5% of the total peak area.

Melting range (741): between 145° and 151°.

Acetylacetone (*2,4-Pentanedione; Diacetyl methane*), $\text{C}_5\text{H}_8\text{O}_2$ —**100.12** [123-54-6]—Clear, colorless to slightly yellow, flammable liquid. Soluble in water; miscible with alcohol, with chloroform, with acetone, with ether, and with glacial acetic acid.

Assay—Not less than 98% of $\text{C}_5\text{H}_8\text{O}_2$, a suitable gas chromatograph equipped with a flame-ionization detector being used and helium being used as the carrier gas. The following conditions have been found suitable: a 3-mm \times 1.83-m stainless steel column containing 10% phase G43 on support S1A; the injection port and detector temperatures are maintained at 250° and 310°, respectively; the column temperature is programmed to rise 8° per minute, from 50° to 220°.

Refractive index (831): between 1.4505 and 1.4525, at 20°.

Acetyl Chloride, CH_3COCl —**78.50** [75-36-5]—Clear, colorless liquid. Is decomposed by water and by alcohol. Miscible with benzene and with chloroform. Use ACS reagent grade.

Specific gravity (841): about 1.1.

Acetylcholine Chloride (*Trimethylethanaminium Chloride; Acecoline*), $[\text{CH}_3\text{COOCH}_2\text{CH}_2\text{N}(\text{CH}_3)_3]\text{Cl}$ —**181.66** [60-31-1]—White, crystalline powder. Very deliquescent; very soluble in water; freely soluble in alcohol.

Melting range (741)—When previously dried at 110° in a capillary tube for 1 hour, it melts between 149° and 152°.

Reaction—A solution (1 in 10) is neutral to litmus.

Residue on ignition (Reagent test): negligible, from 200 mg.

Solubility in alcohol—A solution of 500 mg in 5 mL of alcohol is complete and colorless.

Percent of acetyl (CH_3CO)—Weigh accurately about 400 mg, previously dried at 105° for 3 hours, and dissolve in 15 mL of water in a glass-stoppered conical flask. Add 40.0 mL of 0.1 N sodium hydroxide VS, and heat on a steam bath for 30 minutes. Insert the stopper, allow to cool, add phenolphthalein TS, and titrate the excess alkali with 0.1 N sulfuric acid VS. Determine the exact normality of the 0.1 N sodium hydroxide by titrating 40.0 mL after it has been treated in the same manner as in the test. Each mL of 0.1 N sodium hydroxide is equivalent to 4.305 mg of CH_3CO . Between 23.2% and 24.2% is found.

Percent of chlorine (Cl)—Weigh accurately about 400 mg, previously dried at 105° for 3 hours, and dissolve in 50 mL of water in a glass-stoppered, 125-mL flask. Add with agitation 30.0 mL of 0.1 N silver nitrate VS, then add 5 mL of nitric acid and 5 mL of nitrobenzene, shake, add 2 mL of ferric ammonium sulfate TS, and titrate the excess silver nitrate with 0.1 N ammonium thiocyanate VS: each mL of 0.1 N silver nitrate is equivalent to 3.545 mg of Cl. Between 19.3% and 19.8% of Cl is found.

3-Acetylthio-2-methylpropanoic Acid, $\text{C}_6\text{H}_{10}\text{O}_3\text{S}$ —**162.21**—Use a suitable grade.

[NOTE—A suitable grade is available as β -(Acetylmercapto)isobutyric Acid, catalog number 39059, from Senn Chemicals AG www.sennchem.com.]

N-Acetyl-L-tyrosine Ethyl Ester, $\text{C}_{13}\text{H}_{17}\text{NO}_4$ —**251.28**—Determine the suitability of the material as directed in the *Assay* under *Chymotrypsin* (USP monograph).

Acrylic Acid (*2-Propenoic Acid; Vinylformic Acid*), $\text{C}_3\text{H}_4\text{O}_2$ —**72.06** [79-10-7]—Colorless liquid. Miscible with water, with alcohol, and with ether.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)), equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μm layer of phase G2; the injection port temperature is maintained at 150°; the detector temperature is maintained at 300°; and the column temperature is maintained at 50° and programmed to rise 10° per minute to 200°. The area of the $\text{C}_3\text{H}_4\text{O}_2$ peak is not less than 99% of the total peak area.

Refractive index (831): between 1.419° and 1.423° at 20°.

Activated Alumina—See *Alumina, Activated*.

Activated Charcoal—See *Charcoal, Activated*.

Activated Magnesium Silicate—See *Magnesium Silicate, Activated*.

Adamantane, $\text{C}_{10}\text{H}_{16}$ —**136.23** [281-23-2]

Melting range (741): between 270° and 271°.

Adenine Sulfate, $(\text{C}_5\text{H}_5\text{N}_5)_2 \cdot \text{H}_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$ —**404.36**—White crystals or crystalline powder. Melts, after drying at 110°, at about 200° with some decomposition. One g dissolves in about 160 mL of water; less soluble in alcohol. Soluble in solutions of sodium hydroxide. It is not precipitated from solution by iodine TS or mercuric-potassium iodide TS, but a precipitate is produced with trinitrophenol TS.

Residue on ignition (Reagent test): negligible, from 100 mg.

Water—Dry it at 105° to constant weight: it loses not more than 10.0% of its weight.

Adipic Acid (*Hexanedioic Acid; 1,4-Butanedicarboxylic Acid*), $\text{C}_6\text{H}_{10}\text{O}_4$ —**146.14** [124-04-9]—Colorless to white, crystalline powder. Slightly soluble in water and in cyclohexane; soluble in alcohol, in methanol, and in acetone; practically insoluble in benzene and in petroleum benzin.

Assay—Weigh accurately about 0.3 g, and dissolve in 50 mL of alcohol. Add 25 mL of water, mix, and titrate with 0.5 N sodium hydroxide VS to a pH of 9.5. Perform a blank determination, and make any necessary correction. Each mL of 0.5 N sodium hydroxide is equivalent to 36.54 mg of $C_6H_{10}O_4$. Not less than 98% is found.

Melting range (741): between 151° and 155°, but the range between beginning and end of melting does not exceed 2°.

Agar—Use *Agar* (NF monograph). When used for bacteriological purposes, it is to be dried to a water content of not more than 20%.

Agarose [9012-36-6]—Polysaccharide consisting of 1,3-linked β -D-galactopyranose and 1,4-linked 3,6-anhydro- α -L-galactopyranose. Use a suitable grade.

Air-Helium Certified Standard—A mixture of 1.0% air in industrial grade helium. It is available from most suppliers of specialty gases.

Albumin Bovine Serum [9048-46-8]—Almost colorless to faintly yellow powder. Not less than 95% pure. Solubility, 40 mg in 1 mL of water. Molecular weight is approximately 66,000. Use a suitable grade. Store between 2° and 8°.

Alcohol, Ethanol, Ethyl Alcohol, C_2H_5OH —46.07 [64-17-5]—Use a suitable grade with a content of NLT 92.3% and NMT 93.8%, by weight, corresponding to NLT 94.9% and NMT 96% by volume, at 15.56°.

Alcohol, 70 Percent, 80 Percent, and 90 Percent—Prepare by mixing alcohol and water in the proportions given, the measurements being made at 25°.

Percent by Volume of C_2H_5OH at 15.56°	Specific Gravity at 25°	Relative Proportions		Volume in mL of Alcohol, 94.9% v/v, Required for 100 mL
		Alcohol, mL	Water, mL	
70	0.884	38.6	15	73.7
80	0.857	45.5	9.5	84.3
90	0.827	51	3	94.8

The proportions of alcohol and water taken to prepare these or any other percentage (v/v) solutions may be determined as follows. Calculate the amount, in mL, of water to be mixed with 100 mL of alcohol taken by the formula:

$$[94.9(d/c) - 0.8096]100$$

in which 94.9 is the percentage (v/v) of C_2H_5OH in alcohol, 0.8096 is the specific gravity of 94.9% alcohol, d is the specific gravity, obtained from the Alcoholometric Table (see *Reference Tables*), of the solution containing $c\%$ (v/v) of C_2H_5OH , and 100 is the volume, in mL, of alcohol taken.

Alcohol, Absolute, C_2H_5OH —46.07—Use ACS reagent grade Ethyl Alcohol, Absolute.

Alcohol, Aldehyde-free—Dissolve 2.5 g of lead acetate in 5 mL of water, add the solution to 1000 mL of alcohol contained in a glass-stoppered bottle, and mix. Dissolve 5 g of potassium hydroxide in 25 mL of warm alcohol, cool the solution, and add it slowly, without stirring, to the alcohol solution of lead acetate. After 1 hour shake the mixture vigorously, allow it to stand overnight, decant the clear liquid, and recover the alcohol by distillation.

Alcohol, Amyl—See *Amyl Alcohol*.

Alcohol, Dehydrated (Absolute Alcohol), C_2H_5OH —46.07—Use ACS reagent grade Ethyl Alcohol, Absolute.

Alcohol, Dehydrated Isopropyl—See *Isopropyl Alcohol, Dehydrated*.

Alcohol, Denaturated—It is ethyl alcohol to which has been added some substance or substances which, while allowing the use of the alcohol in most applications, renders it entirely unfit for consumption as a beverage. The most common denaturants used, either alone or in combination, are the following: methanol, camphor, aldehyd, amyl alco-

hol, gasoline, isopropanol, terpineol, benzene, castor oil, acetone, nicotine, aniline dyes, ether, cadmium iodide, pyridine bases, sulfuric acid, kerosene, and diethyl phthalate. Use a suitable grade.

Alcohol, Diluted—Use *Diluted Alcohol* (NF monograph).

Alcohol, Isobutyl—See *Isobutyl Alcohol*.

Alcohol, Isopropyl—See *Isopropyl Alcohol*.

Alcohol, Methyl—See *Methanol*.

Alcohol, Neutralized—To a suitable quantity of alcohol add 2 or 3 drops of phenolphthalein TS and just sufficient 0.02 N or 0.1 N sodium hydroxide to produce a faint pink color. Prepare neutralized alcohol just prior to use.

Alcohol, n -Propyl—See *n-Propyl Alcohol*.

Alcohol, Secondary Butyl—See *Butyl Alcohol, Secondary*.

Alcohol, Tertiary Butyl—See *Butyl Alcohol, Tertiary*.

Aldehyde Dehydrogenase—A white powder. One mg contains not less than 2 enzyme activity units.

Assay—Transfer about 20 mg, accurately weighed, to a 200-mL volumetric flask, dissolve in 1 mL of water, dilute with an ice-cold solution of bovine serum albumin (1 in 100) to volume, and mix. Use this solution as the *Assay preparation*. Dissolve 3.3 g of potassium pyrophosphate, 15 mg of dithiothreitol, and 40 mg of edetate disodium in 70 mL of water, adjust with citric acid monohydrate solution (2.1 in 10) to a pH of 9.0 ± 0.1 , dilute with water to 100 mL, and mix to obtain a pH 9.0 buffer. Dissolve an accurately weighed quantity of β -nicotinamide adenine dinucleotide (β -NAD) in water to obtain a β -NAD solution having a known concentration of about 20 mg per mL. Pipet 0.1 mL of the *Assay preparation* into a 1-cm spectrophotometric cell. Pipet 0.1 mL of water into a second 1-cm spectrophotometric cell to provide the reagent blank. Add 2.5 mL of pH 9.0 buffer, 0.2 mL of β -NAD solution, and 0.1 mL of pyrazole solution (0.68 in 100) to each cell, and mix. Stopper the cells, and allow to stand for 2 minutes at $25 \pm 1^\circ$. Add 0.01 mL of acetaldehyde solution (0.3 in 100) to each cell, and mix. Stopper the cells, and determine the absorbance of the solution obtained from the *Assay preparation* at a wavelength of 340 nm, using the solution obtained from the reagent blank as the reference. Calculate the change, ΔA , in absorbance per minute for the solution obtained from the *Assay preparation*, starting at the point when the absorbance and time relationship becomes linear. One enzyme activity unit is defined as the amount of enzyme that oxidizes 1 μ mol of acetaldehyde per minute when the test is conducted under the conditions described herein. Calculate the enzyme activity units in each mg of aldehyde dehydrogenase taken by the formula:

$$[(2.91)(200)/(6.3)(0.1)(1000)](\Delta A/W)$$

in which ΔA is as defined above and W is the weight, in g, of aldehyde dehydrogenase taken.

Alkaline Phosphatase Enzyme—See *Phosphatase Enzyme, Alkaline*.

Alkylphenoxypolyethoxyethanol—A nonionic surfactant. Use a suitable grade.

[NOTE—A suitable grade is available commercially as "Triton X-100" from Sigma-Aldrich, www.sigma-aldrich.com.]

Alpha-Chymotrypsin—25 kDa [9004-07-3]—Use a suitable salt-free grade for protein sequencing.

[NOTE—A suitable grade is available as catalog number 4423 from www.sigma-aldrich.com.]

Alpha-Cyclodextrin Hydrate (Alpha-Schardinger Dextrin; Cyclohexaamylose), $C_{36}H_{60}O_{30} \cdot xH_2O$ [51211-51-9]—Use a suitable grade with a content of NLT 98%.

[NOTE—A suitable grade is available as catalog number 22729 from www.acros.com.]

Alpha-(2-(methylamino)ethyl)benzyl alcohol—Use a suitable grade.

Alphanaphthol—See *1-Naphthol*.

Alprenolol Hydrochloride, $C_{15}H_{23}NO_2 \cdot HCl$ —285.8 [13707-88-5]—Use a suitable grade.

Alum (Ammonium Alum, Aluminum Ammonium Sulfate), $AlNH_4(SO_4)_2 \cdot 12H_2O$ —453.33 [7784-26-1]—Large, color-

less crystals or crystalline fragments or a white powder. Soluble in 7 parts of water and in about 0.5 part of boiling water; insoluble in alcohol. Use ACS reagent grade.

Ammonium Alum—See *Alum*.

Alumina—See *Aluminum Oxide, Acid-washed*.

Alumina, Activated (*Aluminum Oxide*), [1344-28-1]—Use a suitable grade.

Alumina, Anhydrous (*Aluminum Oxide; Alumina specially prepared for use in chromatographic analysis*) [1344-28-1]—A white or practically white powder, 80- to 200-mesh. It does not soften, swell, or decompose in water. It is not acid-washed. Store it in well-closed containers.

Aluminon (*Aurin Tricarboxylic Acid, [tri]Ammonium Salt*), $C_{22}H_{23}N_3O_9$ —**473.43** [569-58-4]—Yellowish-brown, glassy powder. Freely soluble in water. Use ACS reagent grade.

Aluminum, Al—At. Wt. 26.98154 [7429-90-5]—Use ACS reagent grade, which also meets the requirements of the following test.

Arsenic—Place 750 mg in a generator bottle (see *Arsenic in Reagents under General Tests for Reagents*), omitting the pledget of cotton. Add 10 mL of water and 10 mL of sodium hydroxide solution (3 in 10), and allow the reaction to proceed for 30 minutes: not more than a barely perceptible stain is produced on the mercuric bromide test paper.

Aluminum Oxide, Acid-Washed (*Alumina specially prepared for use in chromatographic analysis*) [1344-28-1]—White or practically white powder or fine granules. Very hygroscopic. Store in tight containers.

pH of Slurry—The pH of a well-mixed slurry of 5 g in 150 mL of ammonia-free and carbon dioxide-free water, after 10 minutes' standing, is between 3.5 and 4.5.

Loss on ignition—Weigh accurately about 1 g, and ignite, preferably in a muffle furnace at 800° to 825°, to constant weight: it loses not more than 5.0% of its weight.

Silica—Fuse 500 mg with 10 g of potassium bisulfate for 1 hour in a platinum crucible, cool, and dissolve in hot water: not more than a small amount of insoluble matter remains.

Suitability for chromatographic adsorption—Dissolve 50 mg of o-nitroaniline in benzene to make 50.0 mL. Dilute 10 mL of the resulting solution with benzene to 100.0 mL, and mix (*Solution A*).

Weigh quickly about 2 (± 0.005) g of specimen in a glass-stoppered weighing bottle, and rapidly transfer it to a dry, glass-stoppered test tube. Add 20.0 mL of *Solution A*, insert the stopper, shake vigorously for 3 minutes, and allow to settle.

Pipet 10 mL of the clear supernatant into a 100-mL volumetric flask, dilute with benzene to volume, and mix (*Solution B*).

Determine the absorbances of *Solutions A* and *B* at 395 nm, with a suitable spectrophotometer, using benzene as the blank. Calculate the quantity, in mg, adsorbed per g of test specimen by the formula:

$$[2(1 - A_B/A_A)]/W$$

in which A_A and A_B are the absorbances of *Solutions A* and *B*, respectively; and W is the weight, in g, of the aluminum oxide. Not less than 0.3 mg of o-nitroaniline is adsorbed for each g of the aluminum oxide.

Aluminum Potassium Sulfate, $AlK(SO_4)_2 \cdot 12H_2O$ —**474.39** [10042-67-1]—Use ACS reagent grade.

Amaranth, $C_{20}H_{11}N_2Na_3O_{10}S_3$ —**604.48** [915-67-3]—A deep brown or dark reddish-brown fine powder. Use a suitable grade.

Aminoacetic Acid (*Glycine*), NH_2CH_2COOH —**75.07**

[56-40-6]—White, crystalline powder. Very soluble in water; slightly soluble in alcohol.

Nitrogen content (Reagent test)—Determine by the Kjeldahl method, using a test specimen previously dried at 105° for 2 hours: between 18.4% and 18.8% of N is found, corresponding to not less than 98.5% of $C_2H_5NO_2$.

Insoluble matter (Reagent test): not more than 1 mg, from 10 g (0.01%).

Residue on ignition (Reagent test): not more than 0.05%.

Chloride (Reagent test)—One g shows not more than 0.1 mg of Cl (0.01%).

Sulfate (Reagent test, *Method I*)—Two g shows not more than 0.1 mg of SO_4 (0.005%).

Heavy metals (Reagent test): 0.001%, 5 mL of 1 N hydrochloric acid VS being used to acidify the solution of the test specimen.

Iron (241)—One g, dissolved in 47 mL of water containing 3 mL of hydrochloric acid, shows not more than 0.01 mg of Fe (0.001%).

4-Aminoantipyrine, $C_{11}H_{13}N_3O$ —**203.24** [83-07-8]—Light yellow, crystalline powder. A 500-mg portion dissolves completely in 30 mL of water and yields a clear solution.

Melting range (741): between 108° and 110°.

p-Aminobenzoic Acid—See *Para-aminobenzoic Acid*.

2-Aminobenzonitrile (*Anthranilonitrile*), $C_7H_6N_2$ —**118.14** [1885-29-6]—Use 2-Aminobenzonitrile 98%.

Melting range (741): between 49° and 52°.

4-Amino-6-chloro-1,3-benzenedisulfonamide, $C_6H_8ClN_3O_4S_2$ —**285.73** [121-30-2]—White powder. Insoluble in water and in chloroform; soluble in ammonia TS.

Residue on ignition (Reagent test): not more than 2 mg from 2 g (0.1%).

Absorbance—A 1 in 200,000 solution in methanol exhibits absorbance maxima at about 223 nm, 265 nm, and 312 nm. Its absorptivity (see *Spectrophotometry and Light-Scattering* (851)) at 265 nm is about 64.0.

4-Amino-2-chlorobenzoic Acid, $C_6H_3Cl(NH_2)(COOH)$ —**171.58** [2457-76-3]—White crystals or white, crystalline powder.

Melting range (741): between 208° and 212°.

2-Amino-5-chlorobenzophenone, $C_{13}H_9ClNO$ —**231.68** [719-59-5]—Use USP 2-Amino-5-chlorobenzophenone RS.

7-Aminodesacetoxycephalosporanic Acid, $C_8H_{10}N_2O_3S$ —**214.2**—Light yellow powder.

Ordinary impurities (466)—

Test solution: 1 N ammonium hydroxide.

Standard solution: 1 N ammonium hydroxide.

Eluant: 0.5 N sodium chloride.

Visualization: 1.

2-Aminoethyl Diphenylborinate—See *Diphenylborinic Acid, Ethanolamine Ester*.

1-(2-Aminoethyl)piperazine, $C_6H_{15}N_3$ —**129.20** [140-31-8]—Viscous, colorless liquid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with G2. The injection port temperature is maintained at 280°; the column temperature is maintained at 180° and programmed to rise 10° per minute to 280° and held there for 10 minutes. The detector temperature is maintained at 300°. The area of the main peak is not less than 97% of the total peak area.

Refractive index (831): between 1.4978 and 1.5010 at 20°.

Aminoguanidine Bicarboxylate (*Aminoguanidine Hydrogen Carbonate*), $\text{CH}_6\text{N}_4 \cdot \text{H}_2\text{CO}_3$ —**136.11** [2582-30-1]—White powder.

Assay—Dissolve about 34 mg, accurately weighed, in 50 mL of glacial acetic acid. Titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary corrections. Each mL of 0.1 N perchloric acid is equivalent to 13.61 mg of $\text{CH}_6\text{N}_4 \cdot \text{H}_2\text{CO}_3$. Not less than 98.5% is found.

Melting point (741): about 170°, with decomposition.

2-Aminoheptane (*2-Heptylamine*; *1-Methylhexylamine*), $\text{C}_7\text{H}_{17}\text{N}$ —**115.22** [123-82-0]—Use a suitable grade with a content of not less than 99%.

N-Aminohexamethyleneimine (*N-Aminohomopiperidine*, *1-Aminohomopiperidine*), $\text{C}_6\text{H}_{14}\text{N}_2$ —**114.19** [5906-35-4]—Colorless liquid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with G2. The injection port temperature is maintained at 180°; the column temperature is maintained at 80° and programmed to rise 10° per minute to 230° and then maintained at 230° for 5 minutes. The detector temperature is maintained at 300°. The area of the main peak is not less than 95% of the total peak area.

Refractive index (831): between 1.4840 and 1.4860 at 20°.

4-Amino-3-hydroxy-1-naphthalenesulfonic Acid, $\text{C}_{10}\text{H}_9\text{NO}_4\text{S}$ —**239.25** [116-63-2]—Light purple powder. Use ACS reagent grade.

8-Amino-6-methoxyquinoline (*6-Methoxy-8-aminoquinoline*), $\text{C}_{10}\text{H}_{10}\text{N}_2\text{O}$ —**174.2** [90-52-8]—Use a suitable grade with a content of not less than 98.0%.

[NOTE—A suitable grade is available from www.3bmedical.com, catalog number 3B3-002598.]

1,2,4-Aminonaphtholsulfonic Acid, $\text{C}_{10}\text{H}_9\text{NO}_4\text{S}$ —**239.25**—White to slightly brownish pink powder. Sparingly soluble in water.

Sensitivity—Dissolve 100 mg in 50 mL of freshly prepared sodium bisulfite solution (1 in 5), warming if necessary to effect solution, and filter. Add 1 mL of the filtrate to a solution prepared by adding 2 mL of dilute sulfuric acid (1 in 6) and 1 mL of *Phosphate Reagent A* (see *Reagent test*) to 20 mL of a 1 in 100 dilution of *Standard Phosphate Solution* (see *Reagent test*): a distinct blue color develops within 5 minutes.

Solubility in sodium carbonate solution—Dissolve 100 mg in 3 mL of sodium carbonate TS, and add 17 mL of water: not more than a trace remains undissolved.

Residue on ignition (*Reagent test*)—To 1 g add 0.5 mL of sulfuric acid, and ignite at 800 \pm 25° to constant weight: the residue weighs not more than 5 mg (0.5%).

Sulfate (*Reagent test, Method I*)—Heat 500 mg with a mixture of 25 mL of water and 2 drops of hydrochloric acid on a steam bath for 10 minutes. Cool, dilute with water to 200 mL, and filter: 20 mL of the filtrate shows not more than 0.25 mg of SO_4 (0.5%).

2-Aminophenol (*o-Aminophenol*; *2-Hydroxyaniline*), $\text{C}_6\text{H}_7\text{NO}$ —**109.13** [95-55-6]—Off-white powder. Use a suitable grade with a content of not less than 99%.

m-Aminophenol (*3-Amino-1-Hydroxybenzene*), $\text{C}_6\text{H}_7\text{NO}$ —**109.13** [591-27-5]—Cream-colored to pale yellow flakes.

Sparingly soluble in cold water; freely soluble in hot water, in alcohol, and in ether.

Assay—Dissolve about 1.5 g, accurately weighed, in about 400 mL of water in a 500-mL volumetric flask, dilute with water to volume, and mix. Transfer 25.0 mL of this solution to an iodine flask, add 50.0 mL of 0.1 N bromine VS, dilute with 50 mL of water, add 5 mL of hydrochloric acid, and immediately insert the stopper in the flask. Shake for 1 minute, allow to stand for 2 minutes, and add 5 mL of potassium iodide TS through the slightly loosened stopper. Shake thoroughly, allow to stand for 5 minutes, remove the stopper, and rinse it and the neck of the flask with 20 mL of water, adding the rinsing to the flask. Titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. From the volume of 0.1 N sodium thiosulfate used, calculate the volume, in mL, of 0.1 N bromine consumed by the test specimen. Each mL of 0.1 N bromine is equivalent to 1.819 mg of $\text{C}_6\text{H}_7\text{NO}$: not less than 99.5% is found.

Melting range (741): between 121° and 123°.

Loss on drying (731)—Dry it over calcium chloride for 4 hours: the loss in weight is negligible.

Residue on ignition (*Reagent test*): negligible, from 2 g.

p-Aminophenol (*p-Hydroxyaniline*), $\text{C}_6\text{H}_7\text{NO}$ —**109.13** [123-30-8]—Fine, yellowish, crystalline powder. Slightly soluble in water and in alcohol. Use a suitable grade with a content of not less than 99%.

3-Amino-1-propanol, $\text{H}_2\text{N}(\text{CH}_2)_3\text{OH}$ —**75.11** [156-87-6]—Liquid.

Boiling range (*Reagent test*): between 184° and 188°.

Refractive index (831): between 1.461 and 1.463 at 20°.

3-Aminopropionic Acid (β -Alanine), $\text{NH}_2\text{CH}_2\text{CH}_2\text{COOH}$ —**89.09** [107-95-9]—Use a suitable grade.

3-Aminosalicylic Acid, $\text{C}_7\text{H}_7\text{NO}_3$ —**153.14** [570-23-0]—Tan-grey powder. Use a suitable grade with a content of not less than 97%.

Ammonia Detector Tube—A fuse-sealed glass tube so designed that gas may be passed through it and containing suitable absorbing filters and support media for the indicator bromophenol blue.

Measuring range: 5 to 70 ppm.

[NOTE—Available from Draeger Safety, Inc., www.draeger.com, or from Gastec Corp., www.gastec.co.jp, distributed in the USA by www.nextteq.com.]

Ammonia Solution, Diluted—Use *Ammonia TS*.

Ammonia Water, 25 Percent [1336-21-6]—Use a suitable grade.

Ammonia Water, Stronger (*Ammonium Hydroxide*)—[1336-21-6]—Use ACS reagent grade Ammonium Hydroxide.

Ammonium Acetate, $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ —**77.08** [631-61-8]—Use ACS reagent grade.

Ammonium Bicarbonate (*Ammonium Hydrogen Carbonate*), NH_4HCO_3 —**79.06** [1066-33-7]—Use a suitable grade with a content of NLT 99.0%.

Ammonium Bisulfate (*Ammonium Hydrogen Sulfate*), NH_4HSO_4 —**115.11** [7803-63-6]—White crystals. Freely soluble in water; practically insoluble in alcohol, in acetone, and in pyridine.

Assay—Dissolve about 300 mg, accurately weighed, in 50 mL of a mixture of water and alcohol (25:25). Titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination and make any necessary correction. Each mL of 0.1 N sodium hydrox-

ide is equivalent to 11.51 mg of NH_4HSO_4 . Not less than 98% is found.

Ammonium Bromide, NH_4Br —**97.94** [12124-97-9]—Use ACS reagent grade.

Ammonium Carbonate (*Hartshorn Salt*) [506-87-6]—Use ACS reagent grade.

Ammonium Chloride (*Salmiac*), NH_4Cl —**53.49** [12125-02-9]—Use ACS reagent grade.

Ammonium Citrate, Dibasic (*Citric Acid Diammonium Salt*), $(\text{NH}_4)_2\text{HC}_6\text{H}_5\text{O}_7$ —**226.18** [3012-65-5]—Use ACS reagent grade.

Ammonium Dihydrogen Phosphate—See *Ammonium Phosphate, Monobasic*.

Ammonium Fluoride, NH_4F —**37.04** [12125-01-8]—Use ACS reagent grade.

Ammonium Formate (*Formic Acid Ammonium Salt*), CH_3NO_2 —**63.06** [540-69-2]—Use a suitable grade.

Ammonium Hydroxide (*Ammonium Aqueous*), [1336-21-6]—Use ACS reagent grade.

Ammonium Hydroxide, 6 N—Prepare by diluting 400 mL of *Ammonia Water, Stronger* (see *Reagents* section) with water to make 1000 mL.

Ammonium Molybdate, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ —**1235.86** [12054-85-2]—Use ACS reagent grade.

Ammonium Nitrate, NH_4NO_3 —**80.04** [6484-52-2]—Use ACS reagent grade.

Ammonium Oxalate, $(\text{NH}_4)_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$ —**142.11** [6009-70-7]—Use ACS reagent grade.

Ammonium Persulfate (*Ammonium Peroxydisulfate*), $(\text{NH}_4)_2\text{S}_2\text{O}_8$ —**228.20** [7727-54-0]—Use ACS reagent grade Ammonium Peroxydisulfate.

Ammonium Phosphate, Dibasic (*Diammonium Hydrogen Phosphate*), $(\text{NH}_4)_2\text{HPO}_4$ —**132.06** [7783-28-0]—Use ACS reagent grade.

Ammonium Phosphate, Monobasic (*Ammonium Dihydrogen Phosphate*), $\text{NH}_4\text{H}_2\text{PO}_4$ —**115.03** [7722-76-1]—Use ACS reagent grade.

Ammonium Pyrrolidinedithiocarbamate (*1-pyrrolidine-carbodithioic acid, ammonium salt*), $\text{C}_5\text{H}_{12}\text{N}_2\text{S}_2$ —**164.29** [5108-96-3]—Use a suitable grade.

Ammonium Reineckate (*Reinecke Salt*), $\text{NH}_4[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4] \cdot \text{H}_2\text{O}$ —**354.44** [13573-16-5]—Dark red crystals or red, crystalline powder. Moderately soluble in cold water; more soluble in hot water. Gradually decomposes in solution.

Sensitivity—Dissolve 50 mg in 10 mL of water. Add 0.2 mL of the solution to 1 mL of a solution of 10 mg of choline chloride in 20 mL of water, and shake gently: a distinct precipitate forms within 5 to 10 seconds.

Ammonium Sulfamate, $\text{NH}_4\text{OSO}_2\text{NH}_2$ —**114.13** [7773-06-0]—Use ACS reagent grade.

Ammonium Sulfate, $(\text{NH}_4)_2\text{SO}_4$ —**132.14** [7783-20-2]—Use ACS reagent grade.

Ammonium Thiocyanate (*Ammonium Rhodanide*), NH_4SCN —**76.12** [1762-95-4]—Use ACS reagent grade.

Ammonium Vanadate (*Ammonium Metavanadate*), NH_4VO_3 —**116.98** [7803-55-6]—White, crystalline powder. Slightly soluble in cold water; soluble in hot water and in dilute ammonia TS.

Assay—Weigh accurately about 500 mg, transfer to a suitable container, add 30 mL of water and 2 mL of dilute sulfuric acid (1 in 4), swirl to dissolve, and pass sulfur dioxide gas through the solution until reduction is complete and the solution is bright blue in color. Boil gently while passing

a stream of carbon dioxide through the solution to remove any excess sulfur dioxide, then cool, and titrate with 0.1 N potassium permanganate VS. Each mL of 0.1 N potassium permanganate consumed is equivalent to 11.7 mg of NH_4VO_3 . Not less than 98.0% is found.

Solubility in ammonium hydroxide—Dissolve 1 g in a mixture of 3 mL of ammonium hydroxide and 50 mL of warm water: the solution is clear and colorless.

Carbonate—To 500 mg add 1 mL of water and 2 mL of diluted hydrochloric acid: no effervescence is produced.

Chloride—Dissolve 250 mg in 40 mL of hot water, add 2 mL of nitric acid, and allow to stand for 1 hour. Filter, and to the filtrate add 0.5 mL of silver nitrate TS: any turbidity produced does not exceed that of a blank containing 0.5 mg of added Cl (0.2%).

Sulfate—Dissolve 500 mg in 50 mL of hot water, and add 2 mL of diluted hydrochloric acid and 1.5 g of hydroxylamine hydrochloride. Heat at 60° for 3 minutes, filter, cool, and add to the filtrate 2 mL of barium chloride TS: no turbidity or precipitate is produced within 30 minutes.

Amyl Acetate (*Isoamyl Acetate*), $\text{CH}_3\text{CO}_2\text{C}_5\text{H}_{11}$ —**130.18** [2308-18-1]—Clear, colorless liquid. Slightly soluble in water. Miscible with alcohol, with amyl alcohol, with benzene, and with ether.

Specific gravity (841): about 0.87.

Boiling range (Reagent test, *Method I*): not less than 90°, between 137° and 142°.

Solubility in diluted alcohol—A 1.0-mL portion dissolves in 20 mL of diluted alcohol to form a clear solution.

Acidity—Add 5.0 mL to 40 mL of neutralized alcohol, and, if the pink color is discharged, titrate with 0.10 N sodium hydroxide: not more than 0.20 mL is required to restore the pink color (about 0.02% as CH_3COOH).

Water—A 5-mL portion gives a clear solution with 5 mL of carbon disulfide.

Amyl Alcohol (*Isoamyl Alcohol*), $\text{C}_5\text{H}_{11}\text{OH}$ —**88.15** [598-75-4]—Use ACS reagent grade Isopentyl Alcohol.

tert-Amyl Alcohol, $\text{C}_5\text{H}_{12}\text{O}$ —**88.15** [75-85-4]—Clear, colorless, flammable, volatile liquid.

Specific gravity (841): about 0.81.

Boiling range (Reagent test): not less than 95°, between 100° and 103°.

Residue on evaporation—Evaporate 50 mL (40 g) on a steam bath, and dry at 105° for 1 hour: the residue weighs not more than 1.6 mg (0.004%).

Acids and esters—Dilute 20 mL with 20 mL of alcohol, add 5.0 mL of 0.1 N sodium hydroxide VS, and reflux gently for 10 minutes. Cool, add 2 drops of phenolphthalein TS, and titrate the excess sodium hydroxide with 0.1 N hydrochloric acid VS: not more than 0.75 mL of the 0.10 N sodium hydroxide is consumed, correction being made for the amount consumed in a blank (0.06% as amyl acetate).

Aldehydes—Shake 5 mL with 5 mL of potassium hydroxide solution (30 in 100) in a glass-stoppered cylinder for 5 minutes, and allow to separate: no color develops in either layer.

α -Amylase—Use a suitable grade. It can be from vegetal or animal or microbiological origin.

(E)-Anethole (*1-Methoxy-4-(1-propenyl)benzene*), $\text{C}_{10}\text{H}_{12}\text{O}$ —**148.20** [4180-23-8]—Use a suitable grade of transomer.

Anhydrous Alumina—See *Alumina, Anhydrous*.

Anhydrous Barium Chloride—See *Barium Chloride, Anhydrous*.

Anhydrous Calcium Chloride—See *Calcium Chloride, Anhydrous*.

Anhydrous Cupric Sulfate—See *Cupric Sulfate, Anhydrous*.

Anhydrous Dibasic Sodium Phosphate—See *Sodium Phosphate, Dibasic, Anhydrous*.

Anhydrous Magnesium Perchlorate—See *Magnesium Perchlorate, Anhydrous*.

Anhydrous Magnesium Sulfate—See *Magnesium Sulfate, Anhydrous*.

Anhydrous Methanol—See *Methanol, Anhydrous*.

Anhydrous Potassium Carbonate—See *Potassium Carbonate, Anhydrous*.

Anhydrous Sodium Acetate—See *Sodium Acetate, Anhydrous*.

Anhydrous Sodium Carbonate—See *Sodium Carbonate, Anhydrous*.

Anhydrous Sodium Sulfate—See *Sodium Sulfate, Anhydrous*.

Anhydrous Sodium Sulfite—See *Sodium Sulfite, Anhydrous*.

Aniline, $C_6H_5NH_2$ —**93.13** [62-53-3]—Use ACS reagent grade.

Aniline Blue (*Certified Biological Aniline Blue*)

[8004-91-9]—A water-soluble dye consisting of a mixture of the tri-sulfonates of triphenylparosaniline and of diphenylrosaniline.

Aniline Sulfate, $C_{12}H_{14}N_2 \cdot H_2SO_4$ —**284.33** [542-16-5]—Use a suitable grade.

Anion-Exchange Resin, Chloromethylated Polystyrene-Divinylbenzene—Strongly basic, cross-linked resin containing quaternary ammonium groups. It consists of small, moist, yellow beads having a characteristic amine odor. It is available in the chloride form which can be converted to the hydroxide form by regeneration with sodium hydroxide solution (1 in 4). For satisfactory regeneration a contact time of about 25 minutes is required, after which it must be washed with water until neutral. Suitable for use in column chromatography.

[NOTE—A suitable resin is "Amberlite IRA-400," available from Sigma-Aldrich, www.sigma-aldrich.com.]

Anion-Exchange Resin, Strong, Lightly Cross-Linked, in the Chloride Form—Use a suitable grade.

[NOTE—A suitable resin is "AG 1-X4, catalog number 140-1331," produced by BioRad Laboratories, www.bio-rad.com.]

Anion-Exchange Resin, Styrene-Divinylbenzene—Strongly basic, cross-linked resin containing quaternary ammonium groups and about 8% of divinylbenzene. It is available in the chloride form in the 50- to 100-, 100- to 200-, and 200- to 400-mesh sizes. It can be converted to the hydroxide form by regeneration with a sodium hydroxide solution (5 in 100). Insoluble in water, in methanol, and in acetonitrile. Suitable for use in column chromatography.

[NOTE—A suitable resin is Dowex 1X8, produced by Dow Chemical Co. (www.dow.com) and available through Sigma-Aldrich (www.sigma-aldrich.com).]

Anion-Exchange Resin, 50- to 100-Mesh, Styrene-Divinylbenzene—Strongly basic, cross-linked resin containing quaternary ammonium groups and about 4% of divinylbenzene. It consists of tan-colored beads that may be relatively free flowing. It is available in the chloride form which can be converted to the hydroxide form by regeneration with a sodium hydroxide solution (5 in 100). For satisfactory regeneration a contact time of at least 30 minutes is required after which it must be washed free of excess alkali. Insoluble in water, in methanol, and in acetonitrile. Suitable for use in column chromatography.

[NOTE—A suitable resin is "Dowex 1X4," available from Sigma-Aldrich, www.sigma-aldrich.com.]

Moisture content of fully regenerated and expanded resin—Transfer 10 to 12 mL of the resin (as received) to a flask,

and convert it completely to the chloride form by stirring with 150 mL of hydrochloric acid (5 in 100) for not less than 30 minutes. Decant the acid, and wash the resin in the same manner with distilled water until the wash water is neutral to litmus.

Transfer 5 to 7 mL of the regenerated resin to a glass filtering crucible, and remove only the excess surface water by very careful suction filtration. Transfer the conditioned, dried resin to a tared weighing bottle, and weigh. Dry in a vacuum oven at 100° to 105° and at a pressure of 50 mm of mercury for 16 hours. Transfer from the vacuum oven to a desiccator, and cool to room temperature. Reweigh. The loss in weight is between 50% and 65%.

Total new volume capacity—Transfer 2.5 to 3 mL of the conditioned, undried (See *Moisture content*, above) resin to a 5-mL graduated cylinder, and fill it with water. Remove any air bubbles from the resin bed with a stainless steel wire, and settle the resin to its minimum volume by tapping the graduated cylinder. Record the volume of the resin.

Transfer the resin with 100 mL of water to a 250-mL flask. Add 2 mL of sulfuric acid, heat to 70° to 80°, and hold at that temperature for 5 minutes with occasional stirring (do not boil). Cool to room temperature, and add 2.5 mL of nitric acid (1 in 2), 2 mL of ferric ammonium sulfate TS, and 0.20 mL of 0.1 N ammonium thiocyanate. Titrate with 0.1 N silver nitrate VS until the solution turns colorless, and add a measured excess (1 to 5 mL). Heat to boiling to coagulate the silver chloride precipitate. Cool to room temperature, add 10 mL of nitrobenzene, shake vigorously, and titrate the excess silver nitrate with 0.1 N ammonium thiocyanate VS.

$$(\text{net mL AgNO}_3 \times N)/(\text{mL of resin}) = \text{mEq/mL}$$

The total exchange capacity of the regenerated, wet resin is more than 1.0 mEq per mL.

Wet screen analysis—The purpose of this test is to identify properly the mesh size of the resin. To obtain an accurate screen analysis requires special apparatus and technique.

Add 150 mL of resin to 200 mL of distilled water in an appropriate bottle, and allow it to stand at least 4 hours to completely swell the resin.

Transfer by means of a graduated cylinder 100 mL of settled and completely swollen resin to the top screen of a series (20-, 50-, 100-mesh) of 20.3-cm brass screens. Thoroughly wash the resin on each screen with a stream of distilled water until the resin is completely classified, collecting the wash water in a suitable container. Wash the beads remaining on the respective screens back into the 100-mL cylinder, and record the volume of settled resin on each screen: not less than 80% of the resin is between 50- and 100-mesh.

p-Anisaldehyde (*4-Methoxybenzaldehyde*), $C_8H_8O_2$ —**136.15** [123-11-5]—Clear, colorless liquid.

Boiling temperature: 248°.

Density: between 1.119 and 1.123.

Refractive index (831): between 1.5725 and 1.5730 at 20°.

p-Anisidine, C_7H_9NO —**123.06** [104-94-9]—Brown crystals. Use a suitable grade.

Anisole, $CH_3OC_6H_5$ —**108.14** [100-66-3]—Colorless liquid.

Assay—Inject an appropriate specimen (about 0.5 μ L) into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, nitrogen being used as the carrier gas. The following conditions have been found suitable: a 30-m capillary column is coated with phase G3; the injection port and detector temperatures are maintained at 140° and 300°, respectively; the column temperature is maintained at 70° and programmed to rise 10° per minute to 170°. The area of the anisole peak is not less than 99% of the total peak area.

Refractive index (831): 1.5160 at 20°.

Anthracene, $C_{14}H_{10}$ —**178.23** [120-12-7]—White to off-white crystals or platelets. Darkens in sunlight. Insoluble in water; sparingly soluble in alcohol, in benzene, and in chloroform.

Melting range (741): between 215° and 218°.

Anthrone, $C_{14}H_{10}O$ —**194.23** [90-44-8]—Use ACS reagent grade.

Anti-D Reagent—The reagent can be monoclonal (low protein) or polyclonal (high-protein) and must be obtained from manufacturers or suppliers licensed by the Center for Biologics Evaluation and Research, Food and Drug Administration. The use of reagents from an unlicensed manufacturer or supplier may invalidate the results. Note that this reagent is different from Anti-D (Rh_o) Reagent. Consult manufacturer's package insert to ensure that the reagent is suitable for the Weak Anti-D test and does not contain other antibodies that will react when antihuman immunoglobulin is added.

[NOTE—There are many manufacturers and suppliers of these reagents that are licensed by the Center for Biologics Evaluation and Research, Food and Drug Administration. Some examples of licensed manufacturers or suppliers are the following: Gamma Biologics, Houston, TX; and Ortho Diagnostics, Raritan, NJ.]

Anti-D (Rh_o) Reagent—The reagent can be monoclonal or polyclonal and must be obtained from manufacturers or suppliers licensed by the Center for Biologics Evaluation and Research, Food and Drug Administration for use in microplate tests. The use of reagents from an unlicensed manufacturer or supplier may invalidate the results. Note that this reagent is different from Anti-D Reagent. Consult manufacturer's package insert to ensure that it is Anti-D (Rh_o) Reagent and not Anti-D Reagent.

[NOTE—There are many manufacturers and suppliers of these reagents that are licensed by the Center for Biologics Evaluation and Research, Food and Drug Administration. Some examples of licensed manufacturers or suppliers are the following: Gamma Biologics, Houston, TX; and Ortho Diagnostics, Raritan, NJ.]

Antifoam Reagent—A 10% silicone-glycol emulsion, white in appearance. Nonionic but miscible with cool water.

[NOTE—A suitable grade is available as "Antifoam Reagent," catalog number 2210, from Dow Corning Corporation, www.dowcorning.com.]

Antihuman Globulin Reagent—The reagent can be poly-specific or anti-immunoglobulin (Anti-IgG) and must be obtained from manufacturers or suppliers licensed by the Center for Biologics Evaluation and Research, Food and Drug Administration. The use of reagents from an unlicensed manufacturer or supplier may invalidate the results.

[NOTE—There are many manufacturers and suppliers of these reagents that are licensed by the Center for Biologics Evaluation and Research, Food and Drug Administration. Some examples of licensed manufacturers or suppliers are the following: Gamma Biologics, Houston, TX; and Ortho Diagnostics, Raritan, NJ.]

Antimony Pentachloride, $SbCl_5$ —**299.02** [7647-18-9]—Clear, reddish-yellow, oily, hygroscopic, caustic liquid. Fumes in moist air and solidifies by absorption of one molecule of water. Is decomposed by water; soluble in dilute hydrochloric acid and in chloroform. Boils at about 92° at a pressure of 30 mm of mercury and has a specific gravity of about 2.34 at 25°.

[CAUTION—Antimony pentachloride causes severe burns, and the vapor is hazardous.]

Assay ($SbCl_5$)—Accurately weigh a glass-stoppered, 125-mL flask, quickly introduce about 0.3 mL of the test specimen, and reweigh. Dissolve with 20 mL of diluted hydrochloric acid (1 in 5), and add 10 mL of potassium iodide solution (1 in 10) and 1 mL of carbon disulfide. Titrate the liberated iodine with 0.1 N sodium thiosulfate VS. The brown color will gradually disappear from the solution, and the last traces of free iodine will be collected in the carbon disulfide, giving a pink color. When this pink color disap-

pears the endpoint has been reached. Each mL of 0.1 N sodium thiosulfate is equivalent to 14.95 mg of $SbCl_5$; not less than 99.0% of $SbCl_5$ is found.

Sulfate (Reagent test, *Method II*)—Dissolve 4.3 mL (10 g) in the minimum volume of hydrochloric acid, dilute with water to 150 mL, neutralize with ammonium hydroxide, and filter. To the filtrate add 2 mL of hydrochloric acid: the solution, 10 mL of barium chloride TS being used, yields not more than 1.3 mg of residue, correction being made for a complete blank test (0.005%).

Arsenic—Add 10 mL of a recently prepared solution of 20 g of stannous chloride in 30 mL of hydrochloric acid to 100 mg of specimen dissolved in 5 mL of hydrochloric acid. Mix, transfer to a color-comparison tube, and allow to stand for 30 minutes. Any color in the solution of the specimen should not be darker than that in a control containing 0.02 mg of arsenic (As), which has been treated in the same manner as the test specimen, when viewed downward over a white surface (0.02% of As).

Substances not precipitated by hydrogen sulfide (as SO_4)—Dissolve 0.90 mL (2 g) in 5 mL of hydrochloric acid, and dilute with 95 mL of water. Precipitate the antimony completely with hydrogen sulfide, allow the precipitate to settle, and filter, being careful not to transfer much of the precipitate to the filter paper. (Retain the precipitate.) To 50 mL of the filtrate, add 0.5 mL of sulfuric acid, evaporate in a tared porcelain crucible to dryness, and ignite at $800 \pm 25^\circ$ for 15 minutes. (Retain the residue.) The weight of the ignited residue should not be more than 0.0010 g greater than the weight obtained in a complete blank test (0.10%).

Iron (241)—To the residue from the test for *Substances not precipitated by hydrogen sulfide* add 2 mL of hydrochloric acid and 5 drops of nitric acid, and evaporate on a steam bath to dryness. Take up the residue in 2 mL of hydrochloric acid, and dilute with water to 47 mL: the solution shows not more than 0.01 mg of Fe (0.001%).

Other heavy metals (as Pb)—Dissolve the precipitate on the filter paper from the test for *Substances not precipitated by hydrogen sulfide*, with 75 mL of a solution containing 6 g of sodium sulfide and 4 g of sodium hydroxide dissolved in and diluted with water to 100 mL. Collect the filtrate in the original flask containing the remainder of the sulfide precipitate. Warm the solution to dissolve the soluble sulfides, and allow the insoluble sulfides to settle. Filter, wash thoroughly with hydrogen sulfide TS, and dissolve any precipitate remaining on the filter paper with 10 mL of hot diluted hydrochloric acid. Dilute the filtrate with water to 50 mL. Neutralize a 25-mL portion of this solution with 1 N sodium hydroxide, and add 1 mL of 1 N acetic acid and 10 mL of hydrogen sulfide TS. Any brown color should not exceed that produced by 0.05 mg of lead ion in an equal volume of solution containing 1 mL of 1 N acetic acid and 10 mL of hydrogen sulfide TS (0.005%).

Antimony Trichloride (*Antimonous Chloride*), $SbCl_3$ —**228.12** [10025-91-9]—Use ACS reagent grade.

Antithrombin III—Antithrombin III human (heparin cofactor, factor II_a inhibitor, and factor X_a inhibitor) is a serine protease inhibitor. It is a glycoprotein having a molecular weight of 58,000 Da.

One Antithrombin III Unit is the amount found in 1 mL of normal human plasma. The potency of antithrombin III is not less than 4.0 Antithrombin III Units per mg of protein when tested in the presence of heparin. It exhibits 90% homogeneity when tested by SDS-PAGE.

Antithrombin III for test or assay purposes contains no detectable heparin. Test as follows. To a solution containing 1 Antithrombin III Unit per mL, add 1 μ L of toluidine blue solution. In the presence of heparin, the color changes from blue to purple.

Aprobarbital, $C_{10}H_{14}N_2O_3$ —**210.23** [77-02-1]—Fine, white crystalline powder. Slightly soluble in cold water; soluble in alcohol, in chloroform, and in ether.

Assay—Dissolve about 200 mg, previously dried at 105° for 2 hours and accurately weighed, in 20 mL of dimethylformamide in a 100-mL conical flask. Add 4 drops of thymol blue solution (1 in 200 in methanol), and titrate with 0.1 N lithium methoxide VS using a 10-mL buret, a magnetic stirrer, and a cover for the flask to protect against atmospheric carbon dioxide. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N lithium methoxide is equivalent to 21.02 mg of $C_{10}H_{14}N_2O_3$. Between 98.5% and 101.0% of $C_{10}H_{14}N_2O_3$ is found.

Melting range (741): between 140° and 143°.

Arsenazo III Acid, $C_{22}H_{18}As_2N_4O_{14}S_2$ —**776.38** [1668-00-4]—Brown powder. Stable in air. Store at room temperature in a dry area.

Melting temperature (741): greater than 320°.

Arsenic Trioxide, As_2O_3 —**197.84** [1327-53-3]—Use ACS reagent grade.

[NOTE—Arsenic Trioxide of a quality suitable as a primary standard is available from the National Institute of Standards and Technology, Office of Standard Reference Materials, www.nist.gov, as standard sample No. 83.]

L-Asparagine (*L*-2-Aminosuccinamic Acid), $COOHCH(NH_2)CH_2CONH_2 \cdot H_2O$ —**150.13** [70-47-3]—Colorless crystals. One g dissolves in 50 mL of water; soluble in acids and in alkalis; insoluble in alcohol and in ether. Its neutral or alkaline solutions are levorotatory; its acid solutions are dextrorotatory.

Specific rotation (781): between +31° and +33°, determined in a solution in diluted hydrochloric acid containing the equivalent of 5 g (on the anhydrous basis, as determined by drying at 105° for 5 hours) in each 100 mL.

Residue on ignition (Reagent test): not more than 0.1%.

Chloride (Reagent test)—One g shows not more than 0.03 mg of Cl (0.003%).

Sulfate (Reagent test, *Method I*)—One g shows not more than 0.05 mg of SO_4 (0.005%).

Heavy metals (Reagent test): 0.002%.

Nitrogen content, Method II (461): between 18.4% and 18.8% of N is found.

L-Aspartic Acid, $C_4H_7NO_4$ —**133.1** [56-84-8]—White to off-white powder. Use a suitable grade.

Azure A, $C_{14}H_{14}ClN_3S$ —**291.80** [531-53-3]—Use a suitable grade.

Bacterial Alkaline Protease Preparation—Use a suitable grade.

[NOTE—A suitable grade is commercially available as “Protex 6L” from Genencor, www.genencor.com, or as “Optimase Enzyme” from Solvay Enzymes Inc., www.solvaypharmaceuticals.com.]

Barbital Sodium, $C_8H_{11}N_2NaO_3$ —**206.2** [144-02-5]—White, crystalline powder or colorless crystals. Freely soluble in water; slightly soluble in alcohol. Use a suitable reagent grade.

Barbituric Acid, $C_4H_4N_2O_3$ —**128.09** [65-52-7]—Faintly beige powder. Soluble in water, in alcohol, in chloroform, and in ether. Melts with decomposition at 251.6°. Use a suitable grade.

Barium Acetate, $C_4H_6BaO_4$ —**255.43** [543-80-6]—Use ACS reagent grade.

Barium Chloride, $BaCl_2 \cdot 2H_2O$ —**244.26**

[10326-27-9]—Use ACS reagent grade.

Barium Chloride, Anhydrous, $BaCl_2$ —**208.23** [10361-37-2]—This may be made by drying barium chloride in thin layers at 125° until the loss in weight between two successive, 3-hour drying periods does not exceed 1%.

Barium Chloride Dihydrate—Use *Barium Chloride*.

Barium Hydroxide, $Ba(OH)_2 \cdot 8H_2O$ —**315.46**

[12230-71-6]—Use ACS reagent grade.

Barium Nitrate, $Ba(NO_3)_2$ —**261.34** [10022-31-8]—Use ACS reagent grade.

Basic Fuchsin—See *Fuchsin, Basic*.

Beclomethasone $C_{22}H_{29}ClO_5$ —**408.92** [4419-39-0]—Use a suitable grade with a content of not less than 99%.

Beef Extract—A concentrate from beef broth obtained by extraction from fresh, sound, lean beef by means of cooking with water and evaporating the broth at a low temperature, usually in vacuum, until a thick, pasty residue results. Yellowish brown to dark brown, slightly acid, pasty mass having an agreeable meat-like odor. Store it in tight, light-resistant containers.

For the following tests, prepare a *test solution* by dissolving 25 g in water to make 250 mL of a practically clear and practically sediment-free solution.

Assay for nitrogen content of alcohol-soluble substances—Place a portion of the alcohol filtrate and washings remaining from the test for *Alcohol-insoluble substances*, corresponding to 1 g of the alcohol-soluble solids, in a 500-mL Kjeldahl flask. Add about 10 g of powdered potassium sulfate and 20 mL of sulfuric acid. Heat the mixture at a low temperature until frothing ceases, then raise the temperature and boil until the mixture acquires a pale yellow color or becomes practically colorless. Cool the flask, add about 250 mL of water, and cautiously add sodium hydroxide solution (3 in 10) until the contents are alkaline, then add 5 mL more. Connect the flask at once by means of a spray trap to a condenser, the lower outlet tube of which dips beneath the surface of 50.0 mL of 0.1 N sulfuric acid VS contained in a receiving flask. Distill the mixture until about 100 mL of distillate has been collected in the acid. Add methyl red TS, and titrate the excess acid with 0.1 N sodium hydroxide VS. Each mL of 0.1 N sulfuric acid is equivalent to 1.401 mg of N. Not less than 60 mg of nitrogen is found.

Assay for nitrogen as ammonia—To 100 mL of *test solution*, contained in a 500-mL Kjeldahl flask, add 5 g of barium carbonate and 100 mL of water, and by means of a spray trap, connect the flask to a condenser, the lower outlet tube of which dips beneath the surface of 50.0 mL of 0.1 N sulfuric acid VS contained in a receiving flask. Distill the mixture until about 100 mL of distillate has been collected, add methyl red TS, and titrate the excess acid with 0.1 N sodium hydroxide VS. Each mL of 0.1 N sulfuric acid is equivalent to 1.703 mg of NH_3 . The amount of ammonia found does not exceed 0.35% of the total solids in the portion of *test solution* taken.

Total solids—Distribute 10 mL of *test solution* over clean, dry sand or asbestos, tared in a porcelain dish, and dry at 105° for 16 hours: the residue weighs not less than 750 mg (75%).

Residue on ignition—Incinerate the residue obtained in the test for *Total solids* by heating the dish to a dull-red heat: the residue does not exceed 30% of the total solids.

Chlorides calculated as sodium chloride—Dissolve the ash obtained in the test for *Residue on ignition* in about 50 mL of water, and carefully transfer to a 100-mL volumetric flask. Add to the solution a few drops of nitric acid and 10.0 mL of 0.1 N silver nitrate VS. Add water to volume, and mix. Filter into a dry flask through a dry filter, rejecting the first 10 mL of the filtrate. To 50.0 mL of the subsequent filtrate add 1 mL of ferric ammonium sulfate TS, and titrate with 0.1 N ammonium thiocyanate VS. Each mL of 0.1 N silver nitrate is equivalent to 5.844 mg of NaCl. The weight of chlorides calculated as sodium chloride obtained, when multiplied by 2, does not exceed 6% of the total solids.

Alcohol-insoluble substances—Transfer 25 mL of *test solution* to a 100-mL conical flask, add 50 mL of alcohol, and shake thoroughly. Collect the precipitate on a counterpoised filter, wash it three times with a mixture of 2 volumes of

alcohol and 1 volume of water, and dry at 105° for 2 hours: the weight of the precipitate, representing the alcohol-insoluble solids, does not exceed 10% of the total solids in the portion of test solution taken.

Nitrate—Boil 10 mL of test solution for 1 minute with 1.5 g of activated charcoal, add water to replace that lost by evaporation, filter, and add 1 drop of the filtrate to 3 drops of a 1 in 100 solution of diphenylamine in sulfuric acid: no blue color is produced.

Benzaldehyde, C_7H_6O —**106.12** [100-52-7]—Colorless, strongly refractive liquid. Soluble in water; miscible with alcohol, with ether, and with fixed and volatile oils.

Assay—Pipet about 1 mL into a tared, glass-stoppered weighing bottle, and weigh accurately. Loosen the stopper, and transfer both the weighing bottle and its contents to a 250-mL conical flask containing 25 mL of a hydro-alcoholic solution of hydroxylamine hydrochloride (prepared by dissolving 34.7 g of hydroxylamine hydrochloride in 160 mL of water, then adding alcohol to make 1000 mL, and neutralizing to bromophenol blue by the addition of sodium hydroxide TS). Using a graduated cylinder to measure the volume, rinse the sides of the flask with an additional 50 mL of this reagent solution. Allow the solution to stand for 10 minutes, add 1 mL of bromophenol blue TS, and titrate the liberated hydrochloric acid with 1 N sodium hydroxide VS. Perform a blank determination with the same quantities of the same reagents, and make any necessary correction. Each mL of 1 N sodium hydroxide consumed is equivalent to 106.1 mg of C_7H_6O . Not less than 98% is found.

Specific gravity (841): between 1.041 and 1.046.

Refractive index (831): between 1.5440 and 1.5465 at 20°.

Hydrocyanic acid—Shake 0.5 mL with 5 mL of water, add 0.5 mL of sodium hydroxide TS and 0.1 mL of ferrous sulfate TS, and warm the mixture gently. Add a slight excess of hydrochloric acid: no greenish-blue color or blue precipitate is observed within 15 minutes.

Benzalkonium Chloride—Use *Benzalkonium Chloride* (NF monograph).

Benzamidinium Hydrochloride Hydrate (*Benzenecarboximidamide Monohydrochloride, Hydrate*), $C_7H_8N_2 \cdot HCl \cdot xH_2O$ —**156.6** [206752-36-5]—White to off-white powder. Use a suitable grade.

[NOTE—A suitable grade is available from Sigma-Aldrich, www.sigma-aldrich.com.]

Benzanilide, $C_{13}H_{11}NO$ —**197.23** [93-98-1]—Off-white, light gray to grayish-green powder. Insoluble in water; sparingly soluble in alcohol; slightly soluble in ether.

Melting range (741): between 162° and 165°.

Solubility in acetone—A 1.0-g portion dissolves completely in 50 mL of acetone to yield a clear solution.

Benzene, C_6H_6 —**78.11** [71-43-2]—Use ACS reagent grade.

Benzenesulfonamide, $C_6H_5SO_2NH_2$ —**157.19** [98-10-2]—White to pale beige crystals.

Melting range (741): between 150° and 153°.

Benzenesulfonyl Chloride, $C_6H_5SO_2Cl$ —**176.62** [98-09-9]—Colorless, oily liquid. Insoluble in cold water; soluble in alcohol and in ether. Solidifies at 0°.

Melting range (741): between 14° and 17°.

Boiling range (Reagent test): between 251° and 252°.

Benzhydrol (α -Phenylbenzenemethanol), $C_{13}H_{12}O$ —**184.23** [91-01-0]—White to pale yellow crystals. Very slightly soluble in water; soluble in alcohol, in ether, and in chloroform.

Melting range (741): between 65° and 67°, but the range between beginning and end of melting does not exceed 2°.

Benzoic Acid, C_6H_5COOH —**122.12** [65-85-0]—Use ACS reagent grade.

[NOTE—Benzoic Acid of a quality suitable as a primary standard is available from the National Institute of Standards

and Technology, Office of Standard Reference Materials, www.nist.gov, as standard sample No. 350.]

Benzophenone, $(C_6H_5)_2CO$ —**182.22** [119-61-9]—White, crystalline powder.

Melting range (741): between 47° and 49°.

p-Benzoquinone (*Quinone*), $C_6H_4O_2$ —**108.09** [106-51-4]—Dark yellow powder having a green cast. Slightly soluble in water; soluble in alcohol, in ether, and in fixed alkali solutions. May darken on standing. Darkened material may be purified by sublimation in vacuum.

Melting range (741): between 113° and 115°.

Benzoyl Chloride, C_6H_5COCl —**140.57** [98-88-4]—Use ACS reagent grade.

N-Benzoyl-L-arginine Ethyl Ester Hydrochloride, $C_{15}H_{22}N_4O_3 \cdot HCl$ —**342.82**—Determine suitability for use as a substrate as directed under *Crystallized Trypsin* (USP monograph).

3-Benzoylbenzoic Acid, $C_{14}H_{10}O_3$ —**226.23** [579-18-0]—White to off-white powder.

Assay—Prepare a mixture of 1% trifluoroacetic acid in water and 1% trifluoroacetic acid in acetonitrile (55:45) for the mobile phase. Inject about 20 μ L into a suitable liquid chromatograph (see *Chromatography* (621)) equipped with a 230-nm detector and a 4.6-mm \times 15-cm column that contains packing L1. The flow rate is about 1.5 mL per minute. The area of the $C_{14}H_{10}O_3$ peak is not less than 98.5% of the total peak area.

Benzoylformic Acid (*Phenylglyoxylic Acid*), $C_6H_5COCO_2H$ —**150.14** [611-73-4]—Powder. Soluble in methanol.

Melting range (741): between 62° and 67°.

Benzphetamine Hydrochloride, $C_{17}H_{21}N \cdot HCl$ —**275.82** [5411-22-3]—White to off-white, crystalline powder. Freely soluble in water, in alcohol, and in chloroform; slightly soluble in ether.

Assay—Dissolve about 500 mg, accurately weighed, in a mixture of 50 mL of glacial acetic acid and 10 mL of mercuric acetate TS, add 1 drop of crystal violet TS, and titrate with 0.1 N perchloric acid VS to a blue-green endpoint. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 27.58 mg of $C_{17}H_{21}N \cdot HCl$. Between 98.0% and 101.0%, calculated on the dried basis, is found.

Melting range (741): between 152° and 158°.

Specific rotation (781): between +22° and +26°, determined in a solution containing 200 mg in 10 mL, the specimen having been previously dried in vacuum at 60° for 3 hours.

Loss on drying (731)—Dry it in vacuum at 60° for 3 hours: it loses not more than 1% of its weight.

Residue on ignition (281): not more than 0.2%.

2-Benzylaminopyridine, $C_{12}H_{12}N_2$ —**184.24** [6935-27-9]—Use a suitable grade.

1-Benzylimidazole, $C_{10}H_{10}N_2$ —**158.20** [4238-71-5]—White crystals.

Assay—Transfer about 40 mg, accurately weighed, to a 100-mL beaker. Dissolve in 50 mL of glacial acetic acid. Titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically using a combination calomel-platinum electrode. Perform a blank determination and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 15.82 mg of $C_{10}H_{10}N_2$. Not less than 99% is found.

Benzyltrimethylammonium Chloride, $C_6H_5CH_2N(CH_3)_3Cl$ —**185.69** [56-93-9]—Available as a 60% aqueous solution. Is clear and is colorless or not more than slightly yellow.

Assay—Pipet 2 mL into a 50-mL volumetric flask, and add water to volume. Pipet 20 mL of the solution into a 125-mL conical flask, add about 30 mL of water, then add 0.25 mL of dichlorofluorescein TS, and titrate with 0.1 N silver nitrate

VS. Each mL of 0.1 N silver nitrate is equivalent to 18.57 mg of $C_6H_5CH_2N(CH_3)_3Cl$. Between 59.5% and 60.5% is found.

Beta-lactamase—Beta-lactamase is an enzyme produced by a variety of bacteria, but is usually obtained from culture filtrates of a strain of *Bacillus cereus*. It has the specific property of inactivating penicillins and cephalosporins by splitting the bond linking the nitrogen of the thiazolidine to the adjacent carbonyl carbon.

It occurs in the form of small, brown, easily pulverizable pieces or granules. Freely soluble in water, forming a slightly opalescent solution that is practically neutral to litmus paper. Is precipitated from its water solutions by acetone, by alcohol, and by dioxane, and is inactivated by contact with these solvents. Is rapidly inactivated by ethyl acetate and is irreversibly destroyed at a temperature of about 80°.

Beta-lactamase is assayed by a procedure depending upon a determination of the amount of penicillin G potassium or penicillin G sodium destroyed at a pH of 7.0 in a solution of such concentration that the inactivation proceeds as a zero-order reaction.

Betanaphthol—See 2-Naphthol.

Bibenzyl (Dibenzyl), $C_{14}H_{14}$ —**182.26** [103-29-7]—Colorless crystals. Freely soluble in chloroform and in ether; sparingly soluble in alcohol; practically insoluble in water.

Melting range (741): between 53° and 55°.

Bile Salts—A concentrate of beef bile, the principal constituent of which is sodium desoxycholate, determined as cholic acid. Soluble in water and in alcohol; the solutions foam strongly when shaken.

Insoluble substances—Dissolve 5 g in 100 mL of dilute alcohol (84 in 100), warming if necessary to aid solution. Filter within 15 minutes through a tared filter, and wash with small portions of the dilute alcohol until the last washing is colorless or practically so, then dry the residue at 105° for 1 hour, and weigh: the weight of the residue does not exceed 0.1%.

Assay—

STANDARD CHOLIC ACID SOLUTION—Dissolve 50.0 mg of cholic acid, accurately weighed, in dilute acetic acid (6 in 10) to make 100 mL, and mix. Store in a refrigerator.

PROCEDURE—Dissolve 1.0 g, accurately weighed, in 50 mL of dilute acetic acid (6 in 10). Filter the solution, if necessary, into a 100-mL volumetric flask, wash the original container and the filter with small portions of dilute acetic acid (6 in 10), add the same acetic acid to volume, and mix. Dilute 10 mL of this solution, accurately measured, with dilute acetic acid (6 in 10) to make 100 mL, and mix.

Pipet 1 mL each of the *Standard Cholic Acid Solution* and the solution of the Bile Salts into two matched test tubes. To each tube add 1 mL, accurately measured, of freshly prepared furfural solution (1 in 100), immediately place the tubes in an ice-bath for 5 minutes, then add to each tube 13 mL, accurately measured, of dilute sulfuric acid, made by cautiously mixing 50 mL of sulfuric acid with 65 mL of water. Mix the contents of the tubes, and place them in a water bath maintained at a temperature of 70° for 10 minutes. Immediately transfer the tubes to an ice-bath for 2 minutes, then determine the absorbance of each solution at the wavelength of maximum absorbance at about 670 nm, with a suitable spectrophotometer. Calculate the quantity, in mg, of cholic acid ($C_{24}H_{40}O_5$) in the weight of the Bile Salts taken by the formula:

$$500(A_u/A_s)$$

in which A_u and A_s are the absorbances of the solutions from the Bile Salts and the *Standard Cholic Acid Solution*, respectively. Not less than 45% of cholic acid is found.

Biphenyl, $C_{12}H_{10}$ —**154.21** [92-52-4]—Colorless to white crystals or crystalline powder. Insoluble in water; soluble in alcohol and in ether. Boils at about 254°.

Melting range (741): between 68° and 72°.

2,2'-Bipyridine (α,α' -Dipyridyl), $C_{10}H_8N_2$ —**156.18** [366-18-7]—White or pink, crystalline powder. Soluble in water and in alcohol. Melts at about 69°, and boils at about 272°.

Sensitivity—Prepare the following solutions: (A)—Dissolve 350 mg of ferrous ammonium sulfate in 50 mL of water containing 1 mL of sulfuric acid, and add 500 mg of hydrazine sulfate, then add water to make 500 mL. For use, dilute this solution with water in the ratio of 1 in 100 mL. (B)—Dissolve 8.3 g of sodium acetate and 12 mL of glacial acetic acid in water to make 100 mL. Add 1 mL of a solution of the specimen (1 in 1000) to a mixture of 10 mL of water and 1 mL of each of solutions A and B: a pink color results immediately.

Solubility—A 100-mg portion dissolves completely in 10 mL of water.

Residue on ignition (Reagent test): not more than 0.2%.

4,4'-Bis(4-amino-1-naphthylazo)-2,2'-stilbenedisulfonic Acid, $C_{34}H_{26}N_6O_6S_2$ —**678.74** [5463-64-9]—Use a suitable grade.

[NOTE—A suitable grade is available from TCI America, www.tciamerica.com.]

Bis(2-ethylhexyl) Maleate, $C_{20}H_{36}O_4$ —**340.50** [142-16-5]—Colorless to pale yellow, clear liquid. Miscible with acetone and with alcohol. Specific gravity about 0.945.

Assay—Place about 2.5 g, accurately weighed, in a 250-mL flask, add 50.0 mL of 0.5 N alcoholic potassium hydroxide VS, and reflux for 45 minutes. Cool, add 0.5 mL of phenolphthalein TS, and titrate the excess alkali with 0.5 N hydrochloric acid VS. Perform a blank determination at the same time, using the same amount of 0.5 N alcoholic potassium hydroxide (see *Residual Titrations under Titrimetry* (541)). The difference, in mL, between the volumes of 0.5 N hydrochloric acid consumed in the test titration and blank titration, multiplied by 85.1, represents the quantity, in mg, of bis(2-ethylhexyl) maleate in the portion taken. Not less than 97% is found.

Bis(2-ethylhexyl) Phthalate, $C_{6}H_4-1,2-[COOCH_2(C_2H_5)CH(CH_2)_3CH_3]_2$ —**390.56** [117-81-7]—Colorless to light yellow liquid.

Refractive index (831): between 1.4855 and 1.4875, at 20°.

Bis(2-ethylhexyl) Sebacate (Diocetyl Sebacate), $C_8H_{17}OOC(CH_2)_8COOC_8H_{17}$ —**426.67** [122-62-3]—Pale straw-colored liquid. Insoluble in water. Refractive index about 1.448. Suitable for use in gas chromatography.

Specific gravity, 20°/20°(841): between 0.913 and 0.917.

Boiling range: between 243° and 248° at 5 mm of mercury.

[NOTE—A suitable grade is "Diocetyl Sebacate," available from Sigma-Aldrich, www.sigma-aldrich.com.]

Bis(2-ethylhexyl)phosphoric Acid [Bis(2-ethylhexyl)Phosphate], $[CH_3(CH_2)_3CH(CH_2)_3CH_2]_2HPO_4$ —**322.42** [298-07-7]—Light yellow, viscous liquid. Insoluble in water; freely soluble in chloroform and in ethyl acetate. Refractive index: about 1.443. Specific gravity: about 0.997.

Assay—Dissolve about 250 mg, accurately weighed, in 50 mL of dimethylformamide, add 3 drops of a 1 in 100 solution of thymol blue TS in dimethylformamide, and titrate with 0.1 N sodium methoxide VS to a blue endpoint. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N sodium methoxide is equivalent to 32.24 mg of $(C_8H_{17})_2HPO_4$. Between 95% and 105% is found.

Solubility—One volume dissolves in 9 volumes of chloroform to yield a clear solution, and 1 volume dissolves in 9 volumes of ethyl acetate to yield a clear solution.

Color—A 1 in 100 solution in chloroform exhibits an absorptivity of not more than 0.03 at 420 nm.

Bis(4-sulfobutyl) Ether Disodium, $\text{Na}_2\text{C}_8\text{H}_{16}\text{O}_7\text{S}_2$ —**334.32**—Use a suitable grade with a content of NLT 95%.

[NOTE—A suitable grade is available as catalog number RM-969-C50 from www.cydexpharma.com.]

Bismuth Nitrate Pentahydrate, $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ —**485.07** [10035-06-0]—Use ACS reagent grade.

Bismuth Subnitrate (*Bismuth(III) Nitrate Basic*), $\text{Bi}_2\text{O}(\text{OH})(\text{NO}_3)_4$ —**1461.99** [1304-85-4]—Use *Bismuth Subnitrate* (USP monograph).

Bismuth Sulfite Agar—Use a suitable grade.

Bis(trimethylsilyl)acetamide (*N,O-Bis(trimethylsilyl)acetamide*; *BSA*), $\text{CH}_3\text{CON}[\text{Si}(\text{CH}_3)_3]_2$ —**203.43** [10416-59-8]—Clear, colorless liquid. Readily hydrolyzes when exposed to moist air. Handle under nitrogen, and store in a cool place.

Assay—Not less than 90% of $\text{CH}_3\text{CON}[\text{Si}(\text{CH}_3)_3]_2$, a suitable gas chromatograph equipped with a thermal conductivity detector being used. The following conditions are suitable and provide a retention time of approximately 15 minutes.

COLUMN: 3-mm \times 1.83-m stainless steel containing 5% phase G1 on support S1A.

INJECTION TEMPERATURE: 160°.

COLUMN TEMPERATURE: 90°, programmed to rise 4° per minute to 160°.

CARRIER GAS: Helium.

Refractive index (831): between 1.4150 and 1.4170 at 20°.

Bis(trimethylsilyl)trifluoroacetamide (*N,O-Bis(trimethylsilyl)trifluoroacetamide*; *BSTFA*), $\text{CF}_3\text{CON}[\text{Si}(\text{CH}_3)_3]_2$ —**257.40** [25561-30-2]—Clear, colorless liquid. Readily hydrolyzes when exposed to moist air. Store in a cool place.

Assay—Not less than 98% of $\text{CF}_3\text{CON}[\text{Si}(\text{CH}_3)_3]_2$, a suitable gas chromatograph equipped with a thermal conductivity detector being used. The following conditions are suitable and provide a retention time of approximately 15 minutes.

COLUMN: 3-mm \times 1.83-m stainless steel containing 5% phase G1 on support S1A.

INJECTION TEMPERATURE: 170°.

COLUMN TEMPERATURE: 70°, programmed to rise 4° per minute to 140°.

CARRIER GAS: Helium.

Refractive index (831): between 1.3820 and 1.3860 at 20°.

Bis(trimethylsilyl)trifluoroacetamide with Trimethylchlorosilane [25561-30-2]—Use a suitable grade.

[NOTE—A suitable grade is available from Sigma-Aldrich, www.sigma-aldrich.com.]

Blood (for carbon monoxide test in gases)—Use *oxalated* or *defibrinated blood* of dogs, sheep, cattle, or human beings within 24 hours after bleeding. Prepare *oxalated blood* by adding 10 mg of sodium oxalate to each mL of the freshly drawn blood.

Blood Group A₁ Red Blood Cells and Blood Group B Red Blood Cells—These cells must be obtained from manufacturers or suppliers licensed by the Center for Biologics Evaluation and Research/Food and Drug Administration. The use of reagents from an unlicensed manufacturer or supplier may invalidate the results. Generally, they are available as part of a kit for ABO Blood Group testing. The cells licensed for use in test tubes can also be used in the microtiter plate method described in the monographs of *Red Blood Cells* and *Whole Blood*.

[NOTE—There are many manufacturers and suppliers of these reagents that are licensed by the Center for Biologics Evaluation and Research/Food and Drug Administration. Some examples of licensed manufacturers or suppliers are the following: Gamma Biologics, Houston, TX; and Ortho Diagnostics, Raritan, NJ.]

Anti-A Blood Grouping Reagent, Anti-B Blood Grouping Reagent, and Anti-AB Blood Grouping Reagent—The reagents can be monoclonal or polyclonal and must be obtained from manufacturers or suppliers licensed by the Center for Biologics Evaluation and Research, Food and Drug Administration for use in microplate tests.

The use of reagents from an unlicensed manufacturer or supplier may invalidate the results. Generally, all three reagents are available as part of a kit.

[NOTE—There are many manufacturers and suppliers of these reagents that are licensed by the Center for Biologics Evaluation and Research, Food and Drug Administration. Some examples of licensed manufacturers or suppliers are the following: Gamma Biologics, Houston, TX; and Ortho Diagnostics, Raritan, NJ.]

Blue Tetrazolium (*3,3'-(3,3'-Dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis[2,5-diphenyl-2H-tetrazolium]dichloride*), $\text{C}_{40}\text{H}_{32}\text{Cl}_2\text{N}_8\text{O}_2$ —**727.64** [1871-22-3]—Lemon-yellow crystals. Slightly soluble in water; freely soluble in chloroform and in methanol; insoluble in acetone and in ether.

Solubility in methanol—Dissolve 1 g in 100 mL of methanol: complete solution results, and the solution is clear.

Color—Transfer a portion of the methanol solution obtained in the preceding test to a 1-cm cell, and determine its absorbance at 525 nm, against water as the blank: the absorbance does not exceed 0.20.

Molar absorptivity (851)—Its molar absorptivity in methanol, at 252 nm, is not less than 50,000.

Suitability test—

STANDARD PREPARATION—Dissolve in alcohol a suitable quantity of USP Hydrocortisone RS, previously dried at 105° for 3 hours and accurately weighed, and prepare by stepwise dilution a solution containing about 10 μg per mL.

PROCEDURE—Pipet 10-, 15-, and 20-mL portions of *Standard Preparation* into separate, glass-stoppered, 50-mL conical flasks. Add 10 mL and 5 mL, respectively, of alcohol to the flasks containing the 10- and 15-mL portions of *Standard Preparation*, and swirl to mix. To each of the flasks, and to a fourth flask containing 20 mL of alcohol, add 2.0 mL of a solution prepared by dissolving 50 mg of blue tetrazolium in 10 mL of alcohol, mix, and then add 2.0 mL of a solution prepared by diluting 1 mL of tetramethylammonium hydroxide TS with alcohol to 10 mL. Mix, allow the flasks to stand in the dark for 90 minutes, and determine the absorbances of the three solutions of the steroid standard at 525 nm, with a suitable spectrophotometer, using the solution in the fourth flask as the blank. Plot the absorbances on the abscissa and the amount of hydrocortisone on the ordinate scale of arithmetic coordinate paper, and draw the curve of best fit: the absorbance of each solution is proportional to the concentration, and the absorbance of the solution containing 200 μg of hydrocortisone is not less than 0.50.

Boric Acid, H_3BO_3 —**61.83** [10043-35-3]—Use ACS reagent grade.

(-)-Bornyl Acetate (*1,7,7-Trimethylbicyclo[2,2,1]-heptan-2-ol acetate*), $\text{C}_{12}\text{H}_{20}\text{O}_2$ —**196.29** [5655-61-8]—Use a suitable grade.

Boron Trifluoride, BF_3 —**67.81** [7637-07-2]—Use a suitable grade.

14% Boron Trifluoride-Methanol [373-57-9]—Use a suitable grade.

Bovine Collagen—Use a suitable grade that contains less than 1 μg glycosaminoglycan per mg.

[NOTE—A suitable grade is available from Sigma-Aldrich, www.sigma-aldrich.com.]

7 Percent Bovine Serum Albumin Certified Standard—Available from the National Institute of Standards and Technology, www.nist.gov, as SRM 927.

Branched Polymeric Sucrose—about 400 kDa [26873-85-8]—White to off-white powder. Synthetic polymer made by the copolymerization of sucrose and epichlorohydrin. Use a suitable grade.

[NOTE—A suitable grade is available commercially as "Ficoll" from Pharmacia Fine Chemicals, Inc., 800 Centennial Ave., Piscataway, NJ 08854.]

Brilliant Green (*Malachite Green G*), $C_{27}H_{34}N_2O_4S$ —**482.64** [3051-11-4]—Glistening, golden-yellow crystals. Soluble in water and in alcohol. Absorption maximum: 623 nm.

Bromelain—A proteolytic enzyme isolated from pineapple. Use a suitable grade.

Bromine, Br—At. Wt. 79.904 [7726-95-6]—Use ACS reagent grade.

α -Bromo-2'-acetophenone (*Bromomethyl 2-naphthyl ketone*), $C_{12}H_9BrO$ —**249.10**—Tannish-pink crystals.

Melting range (741): between 81° and 83°.

***p*-Bromoaniline**, C_6H_6BrN —**172.02** [106-40-1]—White to off-white crystals. Insoluble in water; soluble in alcohol and in ether.

Assay—Transfer about 650 mg, accurately weighed, to a suitable container, and dissolve in 50 mL of glacial acetic acid TS. Add crystal violet TS, and titrate with 0.1 N perchloric acid VS. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 17.20 mg of C_6H_6BrN . Not less than 98% is found.

Melting range (741): between 60° and 65°, within a 2° range.

Bromofluoromethane—Use a suitable grade.

***N*-Bromosuccinimide**, $C_4H_4BrNO_2$ —**177.98** [128-08-5]—White to off-white crystals or powder. Freely soluble in water, in acetone, and in glacial acetic acid. [CAUTION—Highly irritating to eyes, skin, and mucous membranes.]

Assay—Transfer 200 mg, accurately weighed, to a conical flask, add 25 mL of 0.5 N alcoholic potassium hydroxide, cover with a watch glass, heat to boiling, and boil for 5 minutes. Cool, transfer the solution to a beaker, rinsing the flask with water until the total volume of solution plus rinsings is about 100 mL, and add 10 mL of glacial acetic acid. Insert suitable electrodes, and titrate with 0.1 N silver nitrate VS, determining the endpoint potentiometrically. Each mL of 0.1 N silver nitrate is equivalent to 17.80 mg of $C_4H_4BrNO_2$. Not less than 98% is found.

Bromine Sulfate, $(C_{23}H_{26}N_2O_4)_2 \cdot H_2SO_4 \cdot 7H_2O$ —**1013.11** [5787-00-8]—Use ACS reagent grade.

Buffers—See *Buffer Solutions* under *Solutions*.

1,3-Butanediol (*1,3-Butylene Glycol*), $C_4H_{10}O_2$ —**90.12** [107-88-0]—Viscous, colorless liquid. Very hygroscopic. Soluble in water, in alcohol, in acetone, and in methyl ethyl ketone; practically insoluble in aliphatic hydrocarbons, in benzene, and in toluene.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 3-mm \times 1.8-m stainless steel column containing 20% phase G16 on support S1A; the injection port temperature is maintained at 265°; the column temperature is maintained at 150° and programmed to rise 8° per minute to 210°. The area of the butanediol peak is not less than 98% of the total peak area.

Refractive index (831): between 1.4390 and 1.4410 at 20°.

2,3-Butanedione (*Diacetyl*), $CH_3COCOCH_3$ —**86.09** [431-03-8]—Bright yellow to yellowish-green liquid. Soluble

in water. Miscible with alcohol and with ether. Boils at about 88°.

Assay—

HYDROXYLAMINE HYDROCHLORIDE SOLUTION—Dissolve 20 g of hydroxylamine hydrochloride in 40 mL of water, and dilute with alcohol to 400 mL. Add, with stirring, 300 mL of 0.5 N alcoholic potassium hydroxide VS, and filter. Discard after 2 days.

PROCEDURE—Transfer about 1 g, accurately weighed, to a glass-stoppered, 250-mL flask, add 75.0 mL of *Hydroxylamine hydrochloride solution*, and insert the stopper in the flask. Reflux the mixture for 1 hour, then cool to room temperature. Add bromophenol blue TS, and titrate with 0.5 N hydrochloric acid VS to a greenish-yellow endpoint. [NOTE—Alternatively, the solution may be titrated potentiometrically to a pH of 3.4.] Perform a blank test with the same quantities of reagent used for the test specimen, and make any necessary correction. Each mL of 0.5 N hydrochloric acid is equivalent to 43.05 mg of $CH_3COCOCH_3$. Not less than 97% of $CH_3COCOCH_3$ is found.

Congealing temperature (651): between -2.0° and -5.5°.

Refractive index (831): between 1.3935 and 1.3965, at 20°.

Specific gravity (841): about 0.98.

1-Butanesulfonic Acid Sodium Salt (*Sodium 1-butanefulfonate*), $C_4H_9NaO_3S$ —**160.16** [2386-54-1]—Use a suitable grade with a content of not less than 99.0%.

1,4-Butane Sultone (*4-Hydroxybutane-1-sulfonic Acid delta-sultone*), $C_4H_8O_3S$ —**136.17** [1633-83-6]—Use a suitable grade with a content of NLT 99%.

Butanol—See *Butyl Alcohol*.

Butyl Acetate, Normal, $CH_3COO(CH_2)_3CH_3$ —**116.16** [123-86-4]—Use ACS reagent grade.

Butyl Alcohol (*1-Butanol; Normal Butyl Alcohol*), $CH_3(CH_2)_3CH_2OH$ —**74.12** [71-36-3]—Use ACS reagent grade.

Butyl Alcohol, Normal—See *Butyl Alcohol*.

Butyl Alcohol, Secondary (*2-Butanol*), $CH_3CH_2CH(OH)CH_3$ —**74.12** [78-92-2]—Use a suitable grade with a content of NLT 99%.

Butyl Alcohol, Tertiary, $(CH_3)_3COH$ —**74.12** [75-65-0]—Use ACS reagent grade *tert*-Butyl Alcohol.

Butyl Benzoate, $C_{11}H_{14}O_2$ —**178.23** [136-60-7]—Thick, oily, colorless to pale yellow liquid. Practically insoluble in water; soluble in alcohol and in ether.

Assay—When examined by gas-liquid chromatography, it shows a purity of not less than 98%. The following conditions have been found suitable for assaying it: use a 3-mm \times 1.8-m stainless steel column packed with liquid phase G4 on support S1A. Helium is the carrier gas, the injection port temperature is maintained at 180°, the column temperature is maintained at 190°, and the flame-ionization detector is maintained at 280°. The retention time is about 15 minutes.

Refractive index (831): between 1.4980 and 1.5000, at 20°.

***n*-Butyl Chloride** (*1-Chlorobutane*), C_4H_9Cl —**92.57** [109-69-3]—Clear, colorless, volatile liquid. [CAUTION—Highly flammable.] Practically insoluble in water. Miscible with alcohol and with ether. Use HPLC grade.

Butyl Ether (**n*-Dibutyl Ether*), $C_8H_{18}O$ —**130.23** [142-96-1]—Use a suitable grade.

Butyl Methacrylate, $C_8H_{14}O_2$ —**142.20** [97-88-1]—Use a suitable grade.

***tert*-Butyl Methyl Ether**, $C_5H_{12}O$ —**88.15** [1634-04-4]—Colorless liquid.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 100°; the detector temperature is maintained at 300°; and the column temperature is maintained at ambient tempera-

ture and programmed to rise 10° per minute to 150°. The area of the C₅H₁₂O peak is not less than 99.8% of the total peak area.

Refractive index (831): between 1.367 and 1.371 at 20°.

n-Butylamine, CH₃CH₂CH₂CH₂NH₂—**73.14**

[109-73-9]—Colorless to pale yellow, flammable liquid. Miscible with water, with alcohol, and with ether. Store in tight containers. Specific gravity: about 0.740.

Distilling range, Method I (721)—Not less than 95% distills between 76° and 78°.

Water, Method I (921): not more than 1.0%, determined by the *Titrimetric Method*.

Chloride (Reagent test)—One g (1.5 mL) shows not more than 0.01 mg of Cl (0.001%).

Acidic impurities—To 50 mL add 5 drops of a saturated solution of azo violet in benzene, and titrate quickly with 0.1 N sodium methoxide VS to a deep blue endpoint, observing precautions to prevent absorption of atmospheric carbon dioxide as by use of an atmosphere of nitrogen: not more than 1.0 mL of 0.1 N sodium methoxide is required for neutralization.

tert-Butylamine, C₃H₉CNH₂—**73.14** [75-64-9]—Liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm × 30-m capillary column coated with a 1-μm layer of phase G2; the injection port temperature is maintained at 230°; the detector temperature is maintained at 300°; the column temperature is maintained at 130° and programmed to rise 10° per minute to 280°. The area of the C₃H₉CNH₂ peak is not less than 99.5% of the total peak area.

Refractive index (831): between 1.3770 and 1.3790 at 20°.

4-(Butylamino)benzoic Acid, C₁₁H₅NO₂—**193.25**

[4740-24-3]—Use a suitable grade.

[NOTE—A suitable grade is available from Sigma-Aldrich, Inc., P.O. Box 2060, Milwaukee, WI 53201; www.sigma-aldrich.com.]

n-Butylboronic Acid (1-Butaneboronic Acid),

C₄H₉B(OH)₂—**101.94** [4426-47-5]—Use a suitable grade.

[NOTE—This reagent is usually shipped and stored under water. Before use, remove any excess water by light vacuum filtration. A suitable grade is available from Sigma-Aldrich, www.sigma-aldrich.com.]

tert-Butyldimethylchlorosilane in N-Methyl-N-tert-butyldimethylsilyltrifluoroacetamide, (1 in 100)—Use a suitable grade.

[NOTE—A suitable grade is available as 99% MTBSTFA, 1% TBDMCS from Regis Chemical Company, 8210 Austin Ave., P.O. Box 519, Morton Grove, IL 60053.]

4-tert-Butylphenol, C₁₀H₁₄O—**150.22** [98-54-4]—

White, crystalline flakes or needles. Practically insoluble in water; soluble in alcohol and in ether.

Melting range (741): between 98° and 101°.

t-Butylthiol (tert-Butylthiol; tert-Butyl Mercaptan; 2-Methyl-2-propanethiol; TBM), (CH₃)₃CSH—**90.19** [75-66-1]—Use a suitable grade with a content of NLT 98.0%.

[NOTE—A suitable grade is available as catalog number 20230 at www.sigma-aldrich.com.]

Butyraldehyde (Butanal), C₄H₈O—**72.11** [123-72-8]—

Use a suitable grade, purified by redistillation, with a content of not less than 99.5%.

Butyric Acid, C₄H₈O₂—**88.11** [107-92-6]—Clear, colorless to faint yellow liquid. Miscible with water and with methanol.

Assay—Weigh accurately about 500 mg, transfer to a suitable container, add 30 mL of water, and mix. Add 40 mL of water, and mix. Add phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS. Each mL of 0.1 N sodium hydroxide is equivalent to 8.81 mg of C₄H₈O₂; not less than 99.0% of C₄H₈O₂ is found.

Refractive index (831): about 1.398 at 20°.

Butyrolactone (Dihydro-2-(3H)-furanone, γ-butyrolactone)

—**86.1** [96-48-0]—Clear, colorless to practically colorless, oily liquid. Miscible with water. Soluble in methanol and in ether.

Boiling range (721): between 193° and 208°.

Refractive index (831): about 1.435, at 20°.

Specific gravity (841): between 1.128 and 1.135.

Butyrophenone (Phenyl Propyl Ketone), C₁₀H₁₂O—**148.21** [495-40-9]—Use a suitable grade with a content of not less than 98.0%.

Cadmium Acetate, C₄H₆CdO₄ · 2H₂O—**266.53**

[543-90-8]—Colorless, transparent to translucent crystals. Freely soluble in water; soluble in alcohol.

Insoluble matter (Reagent test): not more than 1 mg, from 20 g (0.005%).

Chloride (Reagent test)—One g shows not more than 0.01 mg of Cl (0.001%).

Sulfate (Reagent test, *Method II*)—Dissolve 10 g in 100 mL of water, add 1 mL of hydrochloric acid, and filter: the residue weighs not more than 1.2 mg more than the residue obtained in a complete blank test (0.005%).

Substances not precipitated by hydrogen sulfide—Dissolve 2 g in a mixture of 135 mL of water and 15 mL of 1 N sulfuric acid, heat to boiling, and pass a rapid stream of hydrogen sulfide through the solution as it cools. Filter, and to 75 mL of the clear filtrate add 0.25 mL of sulfuric acid, then evaporate to dryness, and ignite gently: the residue weighs not more than 1 mg (0.1%).

Cadmium Nitrate, Cd(NO₃)₂ · 4H₂O—**308.48**

[10325-94-7]—Colorless, hygroscopic crystals. Very soluble in water; soluble in alcohol.

Insoluble matter (Reagent test): not more than 1 mg, from 20 g (0.005%).

Chloride (Cl) (Reagent test)—One g shows not more than 0.01 mg of Cl (0.001%).

Sulfate (Reagent test, *Method II*)—Evaporate a mixture of 12 g of specimen and 25 mL of hydrochloric acid on a steam bath to dryness. Add another 15 mL of hydrochloric acid, and again evaporate to dryness. Dissolve the residue in 100 mL of water, filter, and add 1 mL of hydrochloric acid: the residue weighs not more than 1.0 mg more than the residue obtained in a blank test (0.003%).

Copper (Cu)—Dissolve 0.5 g in 10 mL of water, add 10 mL of *Ammonium Citrate Solution* (see *Lead* (251)), and adjust the reaction to a pH of about 9 by the addition of 1 N ammonium hydroxide (about 30 mL). Add 1 mL of sodium diethyldithiocarbamate solution (1 in 1000), and mix. Add 5 mL of amyl alcohol, shake for about 1 minute, and allow the layers to separate: any yellow color in the amyl alcohol layer is not darker than that of a blank to which 0.01 mg of Cu has been added (0.002%).

Iron (Fe)—Dissolve 1 g in 15 mL of water, add 2 mL of hydrochloric acid, and boil for 2 minutes. Cool, and add about 30 mg of ammonium persulfate and 15 mL of a solution of potassium thiocyanate in normal butyl alcohol (made by dissolving 10 g of potassium thiocyanate in 10 mL of water, warming the solution to about 30°, diluting with normal butyl alcohol to 100 mL, and shaking until clear). Shake vigorously for 30 seconds, and allow the layers to separate: any red color in the clear alcoholic layer is not darker than that of a blank to which 0.01 mg of Fe has been added (0.001%).

Lead (Pb)—Dissolve 1.0 g in 10 mL of water, add 0.2 mL of glacial acetic acid, and filter if necessary. To a 7-mL portion of water add 0.2 mL of glacial acetic acid and 3 mL of

Standard Lead Solution (see *Lead* <251>), and mix, to provide a blank. Then add to each solution 1.0 mL of potassium chromate solution (1 in 10), and mix: after 5 minutes, the test solution is not more turbid than the blank (0.003%).

Substances not precipitated by hydrogen sulfide—Dissolve 2 g in 145 mL of water, add 5 mL of sulfuric acid (1 in 10), heat to boiling, and pass a rapid stream of hydrogen sulfide through the solution as it cools. Filter, and to 75 mL of the clear filtrate add 0.25 mL of sulfuric acid, then evaporate to dryness, and ignite gently: the residue weighs not more than 1 mg (0.1%).

Calcium Acetate, $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{H}_2\text{O}$ —**176.18** [5743-26-0]—White, crystalline granules or powder. Soluble in about 3 parts of water; slightly soluble in alcohol. Use ACS reagent grade.

Calcium Carbonate, CaCO_3 —**100.09** [471-34-1]—Use ACS reagent grade.

[NOTE—Calcium Carbonate of a quality suitable as a primary standard is available from the National Institute of Standards and Technology, Office of Standard Reference Materials, www.nist.gov, as standard sample No. 915.]

Calcium Carbonate, Chelometric Standard, CaCO_3 —**100.09** [471-34-1]—Use ACS reagent grade.

Calcium Caseinate—White or slightly yellow, nearly odorless, powder. Insoluble in cold water, but forms a milky solution when suspended in water, stirred, and heated.

Residue on ignition (Reagent test)—Ignite 5 g at 550°: the residue weighs between 150 and 300 mg (3.0% to 6.0%).

Calcium—Treat the residue from the preceding test with 10 mL of diluted hydrochloric acid, filter, and to the clear filtrate add 5 mL of ammonium oxalate TS: it shows a white precipitate upon standing.

Loss on drying <731>—Dry it in vacuum at 70° to constant weight: it loses not more than 7.0% of its weight.

Fat—Suspend 1.0 g in 5 mL of alcohol in a Mojonnier flask, add 0.8 mL of stronger ammonia water and 9 mL of water, and shake. Add a second 5-mL portion of alcohol, then add successive portions of 25 mL each of ether and solvent hexane, shaking after each addition by inverting the flask 30 times. Centrifuge, decant the solvent layer, evaporate it at a low temperature, and dry on a steam bath: the residue weighs not more than 20 mg (2.0%).

Nitrogen content, Method I <461>—Between 12.5% and 14.3% of N is found, calculated on the anhydrous basis.

Suspensibility in water—Place 2 g in a beaker, and add cool water slowly with stirring to form a thin, smooth paste. Add additional water to make a total of 100 mL. Stir, and heat to 80°: a milky suspension is formed that does not settle after standing for 2 hours.

Calcium Chloride, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ —**147.01** [10035-04-8]—Use ACS reagent grade Calcium Chloride Dihydrate.

Calcium Chloride, Anhydrous (for drying), CaCl_2 —**110.98** [10043-52-4]—Use ACS reagent grade Calcium Chloride Desiccant.

Calcium Citrate, $\text{Ca}_3(\text{C}_6\text{H}_5\text{O}_7)_2 \cdot 4\text{H}_2\text{O}$ —**570.49** [813-94-5]—A white, crystalline powder. Slightly soluble in water; freely soluble in 3 N hydrochloric acid and in 2 N nitric acid; insoluble in alcohol. To 15 mL of hot 2 N sulfuric acid add in small portions and with stirring about 500 mg of calcium citrate. Boil the mixture for 5 minutes, and filter while hot: the cooled filtrate responds to the identification test for *Citrate* <191>.

Assay—Accurately weigh about 400 mg of the salt, previously dried at 150° to constant weight, and transfer to a 250-mL beaker. Dissolve the test specimen in 150 mL of water containing 2 mL of 3 N hydrochloric acid, add 15 mL of 1 N sodium hydroxide and 250 mg of hydroxy naphthol blue, and titrate with 0.05 M edetate disodium VS until the solution turns deep blue. Each mL of 0.05 M edetate disodium is equivalent to 8.307 mg of $\text{Ca}_3(\text{C}_6\text{H}_5\text{O}_7)_2$: between 97.5% and 101% is found.

Calcium oxide and carbonate—Triturate 1 g of calcium citrate with 5 mL of water for 1 minute: the mixture does not turn red litmus blue. Then add 5 mL of warm 3 N hydrochloric acid: only a few isolated bubbles escape.

Hydrochloric acid-insoluble matter—Dissolve 5 g by heating with a mixture of 10 mL of hydrochloric acid and 50 mL of water for 30 minutes: not more than 2.5 mg of insoluble residue remains (0.05%).

Loss on drying <731>—Dry it at 150° to constant weight: it loses between 12.2% and 13.3% of its weight.

Arsenic <211>—Proceed with 0.50 g as directed for organic compounds (6 ppm of As).

Heavy metals, Method I <231>: 0.002%.

Calcium Hydroxide [1305-62-0]—Use ACS reagent grade.

Calcium Lactate, $(\text{CH}_3\text{CHOHCOO})_2\text{Ca} \cdot 5\text{H}_2\text{O}$ —**308.29** [814-80-2]—White granules or powder. Is somewhat efflorescent and at 120° becomes anhydrous. One g dissolves in 20 mL of water; practically insoluble in alcohol. Store it in tight containers.

Assay—Accurately weigh about 500 mg, previously dried at 120° for 4 hours, transfer to a suitable container, and dissolve in 150 mL of water containing 2 mL of diluted hydrochloric acid. Add 15 mL of sodium hydroxide TS and 300 mg of hydroxy naphthol blue indicator, and titrate with 0.05 M edetate disodium VS until the solution is deep blue. Each mL of 0.05 M edetate disodium is equivalent to 10.91 mg of $\text{C}_6\text{H}_{10}\text{CaO}_6$. Not less than 98% is found.

Loss on drying <731>—Dry it at 120° for 4 hours: it loses between 25.0% and 30.0% of its weight.

Acidity—Add phenolphthalein TS to 20 mL of a 1 in 20 solution, and titrate with 0.10 N sodium hydroxide: not more than 0.50 mL is required to produce a pink color.

Heavy metals (Reagent test)—Dissolve 1 g in 2.5 mL of diluted hydrochloric acid, dilute with water to 40 mL, and add 10 mL of hydrogen sulfide TS: any brown color produced is not darker than that of a control containing 0.02 mg of added Pb (0.002%).

Magnesium and alkali salts—Mix 1 g with 40 mL of water, carefully add 5 mL of hydrochloric acid, heat the solution, boil for 1 minute, and add rapidly 40 mL of oxalic acid TS. Add immediately to the warm mixture 2 drops of methyl red TS, then add ammonia TS dropwise, from a buret, until the mixture is just alkaline. Cool to room temperature, transfer to a 100-mL graduated cylinder, dilute with water to 100 mL, mix, and allow to stand for 4 hours or overnight. Filter, and transfer to a platinum dish 50 mL of the clear filtrate, to which has been added 0.5 mL of sulfuric acid. Evaporate the mixture on a steam bath to a small bulk. Carefully heat over a free flame to dryness, and continue heating to complete decomposition and volatilization of ammonium salts. Finally ignite the residue at 800 ± 25° for 15 minutes: the residue weighs not more than 5 mg (1%).

Volatile fatty acid—Stir about 500 mg with 1 mL of sulfuric acid, and warm: the mixture does not emit an odor of volatile fatty acid.

Calcium Nitrate, $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ —**236.15** [13780-06-8]—Use ACS reagent grade.

Calcium Pantothenate, Dextro—Use *Calcium Pantothenate* (USP monograph).

Calcium Sulfate, $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ —**172.17** [7778-18-9]—Use ACS reagent grade.

Calconcarboxylic Acid (2-Naphthalenecarboxylic acid, 3-hydroxy-4-[(2-hydroxy-4-sulfo-1-naphthalenyl)azo]; *Calcon-3-carboxylic Acid*; *Cal-Red*), $\text{C}_{21}\text{H}_{14}\text{N}_2\text{O}_7\text{S}$ —**438.42** [3737-95-9]—Use a suitable grade.

Calconcarboxylic Acid Triturate—Mix 1 part of calconcarboxylic acid with 99 parts of sodium chloride.

Test for sensitivity—Dissolve 50 mg of calconcarboxylic acid triturate in a mixture of 2 mL 10 N sodium hydroxide and 100 mL of water. The solution is blue but becomes violet on addition of 1 mL of a 10 g per L solution of magnesium sulfate and 0.1 mL of a 1.5 g per L solution of calcium

chloride and turns pure blue on addition of 0.15 mL of 0.01 M sodium edetate.

Calf Thymus DNA—Use a suitable grade. [NOTE—A suitable grade is commercially available from Worthington Biochemical Corp., www.worthington-biochem.com.]

dl-Camphene, $C_{10}H_{16}$ —**136.24** [79-92-5]—Use a suitable grade.

[NOTE—A suitable grade is available as camphene, 95%, catalog number 45,606-5, from Sigma-Aldrich, www.sigma-aldrich.com.]

d-10-Camphorsulfonic Acid [(1S)-(+)-10-Camphorsulfonic acid; (1S)-Camphor-10-sulfonic acid; (+)-Camphor-10-sulfonic acid (β)], $C_{10}H_{16}O_4S$ —**232.30** [3144-16-9]—Use a suitable grade.

[NOTE—A suitable grade is available as catalog number C2107 from www.sigma-aldrich.com.]

dl-10-Camphorsulfonic Acid, [Camphor-10-sulfonic acid (β)]; [(RS)-10-Camphorsulfonic acid], $C_{10}H_{16}O_4S$ —**232.30** [5872-08-2]—Is optically inactive.

Use a suitable grade.

[NOTE—A suitable grade is available as catalog number 147923 from www.sigma-aldrich.com.]

Canada Balsam [8007-47-4]—A natural product derived from the resin of *Abies balsamea*. Use a suitable grade.

Canola Oil [120962-03-0]—Use a suitable grade.

Capric Acid (Decanoic Acid), $C_{10}H_{20}O_2$ —**172.26** [334-48-5]—White, solidified melt or fragments. Soluble in alcohol, in chloroform, and in ether; practically insoluble in water.

Assay—Inject an appropriate sample dissolved in acetone into a gas chromatograph (see *Chromatography* (621)) that is equipped with a flame-ionization detector and contains a 0.53-mm × 30-m capillary column coated with a layer of phase G25. The carrier gas is helium, flowing at a rate of 9 mL per minute. The chromatograph is programmed as follows. Initially the column temperature is equilibrated at 150°, then the temperature is increased at a rate of 10° per minute to 250°. The injection port temperature is maintained at 240°, and the detector temperature at 265°. The area of the capric acid peak is not less than 98.5% of the total peak area.

Melting range (741): between 30° and 33°.

Carbazole, $C_{12}H_9N$ —**167.21** [86-74-8]—Off-white to tan powder.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm × 30-m capillary column coated with a 1-μm layer of phase G2; the injection port temperature is maintained at 280°; the detector temperature is maintained at 300°; and the column temperature is maintained at 280°. The area of the $C_{12}H_9N$ peak is not less than 95.5% of the total peak area.

Carbon Dioxide Detector Tube—A fuse-sealed glass tube so designed that gas may be passed through it. Contains suitable absorbing filters and support media for the indicators hydrazine and crystal violet.

Measuring range: 0.01 to 0.3 Vol.-%.

[NOTE—Available from Draeger Safety, Inc., www.draeger.com, or from Gastec Corp., www.gastec.co.jp, distributed in the USA by www.nextteq.com.]

Carbon Disulfide, Chromatographic—Use a suitable grade.

Carbon Disulfide, CS [75-15-0]—Use ACS reagent grade.

Carbon Monoxide Detector Tube—A fuse-sealed glass tube so designed that gas may be passed through it. Contains suitable absorbing filters and support media for the indicators iodine pentoxide and selenium dioxide and fuming sulfuric acid.

Measuring range: 5 to 150 ppm.

[NOTE—Available from Draeger Safety, Inc., www.draeger.com, or from Gastec Corp., www.gastec.co.jp, distributed in the USA by www.nextteq.com.]

Carbon Tetrachloride, CCl_4 —**153.82** [56-23-5]—Use a grade meeting the specifications of ACS *Reagent Chemicals*, 8th Edition.

Carboxylate (Sodium Form) Cation-exchange Resin (50- to 100-mesh)—See *Cation-Exchange Resin, Carboxylate (Sodium Form) (50- to 100-mesh)*.

Carboxymethoxylamine Hemihydrochloride, $2(C_2H_5NO_3) \cdot HCl$ —**218.59** [2921-14-4]—White, crystalline powder. Use a suitable grade.

Carmine (Alum Lake of Carminic Acid), $C_{22}H_{20}O_{13} \cdot xAl$ [1390-65-4]—Red powder. Use a suitable grade.

(R)-(-)-Carvone (2-Methyl-5-(1-methylethenyl)-2-cyclohexene-1-one), $C_{10}H_{14}O$ —**150.22** [6485-40-1]—Use a suitable grade.

Casein [9000-71-9]—White or slightly yellow, granular powder. Insoluble in water and in other neutral solvents; readily dissolved by ammonia TS and by solutions of alkali hydroxides, usually forming a cloudy solution.

Residue on ignition (Reagent test)—Ignite 2 g; the residue weighs not more than 20 mg (1.0%).

Loss on drying (731)—Dry it at 105° to constant weight; it loses not more than 10.0% of its weight.

Alkalinity—Shake 1 g with 20 mL of water for 10 minutes, and filter: the filtrate is not alkaline to red litmus paper.

Soluble substances—When the filtrate from the *Alkalinity* test is evaporated and dried at 105°, the residue weighs not more than 1 mg (0.1%).

Fats—Dissolve 1 g in a mixture of 10 mL of water and 5 mL of alcoholic ammonia TS, and shake out with two 20-mL portions of solvent hexane. Evaporate the hexane at a low temperature, and dry at 80°: the weight of the residue does not exceed 5 mg (0.5%).

Nitrogen content, Method I (461): between 15.2% and 16.0% of N is found, on the anhydrous basis.

Where vitamin-free casein is required, use casein that has been rendered free from the fat-soluble vitamins by continuous extraction with hot alcohol for 48 hours followed by air-drying to remove the solvent.

Casein, Hammersten [9000-71-9]. [NOTE—A suitable grade is available from www.emdchemicals.com, catalog number CX0525-1.]

Catechol (o-Dihydroxybenzene; Pyrocatechol), $C_6H_4(OH)_2$ —**110.11** [120-80-9]—White crystals, which become discolored on exposure to air and light. Readily soluble in water, in alcohol, in benzene, in ether, in chloroform, and in pyridine, forming clear solutions.

Use a suitable grade with a content of not less than 99%.

Cation-Exchange Resin—Use a suitable grade.

[NOTE—A suitable grade is available commercially as “Dowex 50-W-X8-100,” from Sigma-Aldrich, www.sigma-aldrich.com.]

Cation-Exchange Resin, Carboxylate (Sodium Form) (50- to 100-mesh)—Use a suitable grade.

[NOTE—A suitable grade is available as “Bio-Rex 70” from BioRad Laboratories, www.bio-rad.com.]

Cation-Exchange Resin, Polystyrene—Use a suitable grade.

[NOTE—A suitable grade is available as Dowex-50X2-100, from Sigma-Aldrich, www.sigma-aldrich.com.]

Cation-Exchange Resin, Styrene-Divinylbenzene—A strongly acidic, cross-linked sulfonated resin containing about 2% of divinylbenzene. It is available in the hydrogen form in the 50- to 100-, 100- to 200-, and 200- to 400-mesh sizes. It can be regenerated to the hydrogen form by treating with a hydrochloric acid solution (5 in 100). For satisfactory regeneration, a contact time of at least 30 minutes is required after which it must be washed free of excess acid. It is insoluble in water, in methanol, and in acetonitrile. Suitable for use in column chromatography.

Moisture content of fully regenerated and expanded resin—Transfer 10 to 12 mL of the resin (as received) to a flask, and convert it completely to the hydrogen form by stirring with 150 mL of hydrochloric acid solution (5 in 100) for not less than 30 minutes. Decant the acid, and wash the resin in the same manner with water until the wash water is neutral to litmus (pH 3.5).

Transfer 5 to 7 mL of the regenerated resin to a glass filtering crucible, and remove only the excess surface water by very careful suction filtration. Transfer the conditioned resin to a tared weighing bottle, and weigh. Dry in a vacuum oven at a pressure of 50 mm of mercury at 100° to 105° for 16 hours. Transfer from the vacuum oven to a desiccator, cool to room temperature, and weigh again. The loss in weight is between 75% and 83%.

Total wet volume capacity—Transfer 3 to 5 mL of the regenerated, undried (See *Moisture content* above) resin to a 5-mL graduated cylinder, and fill it with water. Remove any air bubbles from the resin bed with a stainless steel wire, and settle the resin to its minimum volume by tapping the graduated cylinder. Record the volume of the resin.

Transfer the resin to a 400-mL beaker. Add about 5 g of sodium chloride, and titrate, stirring well, with 0.1 N sodium hydroxide to the blue endpoint of bromothymol blue (pH 7.0).

$$(\text{net mL NaOH} \times N)/(\text{mL of resin}) = \text{mEq/mL}$$

The total wet volume capacity of the resin is more than 0.6 mEq per mL.

Wet screen analysis—The purpose of this test is to properly identify the mesh size of the resin. To obtain an accurate screen analysis would require a special apparatus and technique.

Add 150 mL of resin to 200 mL of water in an appropriate bottle, and allow it to stand at least 4 hours to completely swell the resin.

Transfer, by means of a graduated cylinder, 100 mL of settled and completely swollen resin to the top screen of a series of the designated U.S. Standard 20.3-cm brass screens. Thoroughly wash the resin on each screen with a stream of water until the resin is completely classified, collecting the wash water in a suitable container. Wash the beads remaining on the respective screens back into the 100-mL graduate, and record the volume of settled resin on each screen. At least 70% of the resin will be within the specific mesh size.

[NOTE—A suitable resin is Dowex 50WX2, produced by Dow Chemical Co. (www.dow.com) and also available through Sigma-Aldrich (www.sigma-aldrich.com).]

Cation-Exchange Resin, Styrene-Divinylbenzene, Strongly Acidic—Use a suitable grade.

[NOTE—A suitable grade is available commercially as "Dowex 50-W-X8-100," from Sigma-Aldrich, www.sigma-aldrich.com.]

Cation-Exchange Resin, Sulfonic Acid—Use a suitable grade.

[NOTE—A suitable grade is available commercially as "Amberlyst 15" or as "Dowex 50-W-X2" from Sigma-Aldrich, www.sigma-aldrich.com.]

Cedar Oil (for clearing microscopic sections) [8000-27-9]—A selected, distilled oil from the wood of the red cedar, *Juniperus virginiana* Linné (Fam. Pinaceae), should be used for this purpose. Refractive index: about 1.504 at 20°. For use with homogeneous immersion lenses, a specially prepared oil having a refractive index of 1.5150 ± 0.0002 at 20° is required.

Cellulose, Chromatographic—Use a suitable grade.

[NOTE—A suitable grade is available from EMD Chemicals, www.emdchemicals.com.]

Cellulose, Microcrystalline—Use Cellulose, Microcrystalline, FCC.

Cellulose Mixture, Chromatographic—Use a suitable grade.

[NOTE—A suitable grade is available commercially, in precoated plate form, with fluorescent indicator, from EMD Chemicals, www.emdchemicals.com.]

Ceric Ammonium Nitrate, $\text{Ce}(\text{NO}_3)_4 \cdot 2\text{NH}_4\text{NO}_3$ —548.22—Use ACS reagent grade.

Ceric Ammonium Sulfate, $\text{Ce}(\text{SO}_4)_2 \cdot 2(\text{NH}_4)_2\text{SO}_4 \cdot 2\text{H}_2\text{O}$ —632.55—Yellow to yellowish-orange crystals. Dissolves slowly in water, but more rapidly when mineral acids are present. Use ACS reagent grade.

Ceric Sulfate, $\text{Ce}(\text{SO}_4)_2$ with a variable amount of water—(anhydrous) 332.24 [13590-82-4]—It may also contain sulfates of other associated rare earth elements. Yellow to orange-yellow crystals or crystalline powder. Practically insoluble in cold water; slowly soluble in cold dilute mineral acids, but more readily soluble when heated with these solvents.

Assay—Weigh accurately about 800 mg, transfer to a flask, add 25 mL of water and 3 mL of sulfuric acid, and warm until dissolved. Cool, and add 60 mL of a mixture of 1 volume of phosphoric acid and 20 volumes of water. Add 25 mL of potassium iodide solution (1 in 10), insert the stopper in the flask, and allow to stand for 15 minutes. Replace the air over the solution with carbon dioxide, and while continuing the flow of carbon dioxide into the flask, titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. Each mL of 0.1 N sodium thiosulfate is equivalent to 33.22 mg of $\text{Ce}(\text{SO}_4)_2$. Not less than 80.0% is found.

Chloride (Reagent test)—Dissolve 1 g in a mixture of 5 mL of nitric acid and 4 mL of water. Filter, if necessary, and dilute with water to 20 mL. To 10 mL of the dilution add 1 mL of silver nitrate TS, allow to stand for 10 minutes, and filter until clear. To the remaining 10 mL of test solution add 1 mL of silver nitrate TS: any turbidity produced does not exceed that in a control prepared by adding 0.05 mg of Cl to the filtrate obtained from the first 10 mL of test solution (0.01%).

Heavy metals—Heat 500 mg with a mixture of 10 mL of water and 0.5 mL of sulfuric acid until solution is complete. Cool, dilute with water to 50 mL, and bubble hydrogen sulfide gas through the solution until it is saturated: the precipitate that is formed is white or not darker than pale yellow.

Iron—Dissolve 100 mg in a mixture of 5 mL of water and 2 mL of hydrochloric acid, warming if necessary, and cool. Transfer to a glass-stoppered cylinder, dilute with water to 25 mL, and add 5 mL of ammonium thiocyanate TS and 25 mL of ether. Shake gently, but well, and allow the layers to separate: any pink color in the ether layer is not darker than that of a control, similarly prepared, containing 0.02 mg of added Fe (0.02%).

Cesium Chloride, CsCl —168.36 [7647-17-8]—A white powder. Very soluble in water; freely soluble in methanol; practically insoluble in acetone. Use a suitable grade.

Cetrimide—See *Cetyltrimethylammonium Bromide*.

Cetyltrimethylammonium Bromide (CTAB; *Cetrimide*; *Hexadecyltrimethylammonium Bromide*), $\text{C}_{19}\text{H}_{42}\text{BrN}$ —364.46 [57-09-0]—Use a suitable grade. For any chromatographic application, use a suitable grade with a content of not less than 99.0%.

Cetyltrimethylammonium Chloride, 25 Percent in Water, $\text{C}_{19}\text{H}_{42}\text{ClN}$ —320.00—Use a suitable grade.

Charcoal, Activated (*Activated Carbon*; *Decolorizing Carbon*) C—12.01 [7440-44-0]—A fine, black powder, which is the residue from the destructive distillation of various organic materials, treated to increase its high capacity for adsorbing organic coloring substances, as well as nitrogenous bases. Use *Activated Charcoal* (USP monograph).

Chenodeoxycholic Acid, $\text{C}_{24}\text{H}_{40}\text{O}_4$ —392.57 [474-25-9]—White to off-white powder.

Assay—When tested by thin-layer chromatography, with the use of plates coated with chromatographic reversed-phase C18 mixture, a developing system consisting of 1 N acetic acid in methanol and 1 N acetic acid (19:1), and sprayed with a mixture of sulfuric acid and methanol (1:1),

heated at 110° for 20 minutes, and examined visually and under long-wavelength UV light, a single spot is exhibited.

Melting range (741): between 165° and 168°.

Chloramine T (*Sodium p-Toluenesulfonchloramide*), $C_7H_7ClNNaO_2S \cdot 3H_2O$ —**281.69** [7080-50-4]—Use ACS reagent grade.

Chlorine, Cl_2 —**70.9** [7782-50-5]—Greenish-yellow gas. High-purity grade available from most suppliers of specialty gases.

Chlorine Detector Tube—A fuse-sealed glass tube so designed that gas may be passed through it and containing suitable absorbing filters and support media for the indicator o-tolidine.

Measuring range: 0.2 to 3 ppm.

[NOTE—Available from Draeger Safety, Inc., www.draeger.com, or from Gastec Corp., www.gastec.co.jp, distributed in the USA by www.nextteq.com.]

m-Chloroacetanilide, C_8H_8ClNO —**169.61**—Off-white to beige granules.

Assay—

MOBILE PHASE—Prepare a mixture of acetonitrile and water (22:3).

PROCEDURE—Inject about 20 μ L into a suitable liquid chromatograph (see *Chromatography* (621)) equipped with a 254-nm detector and a 4.6-mm \times 15-cm column that contains packing L1. The flow rate is about 1.5 mL per minute. The area of the C_8H_8ClNO peak is not less than 99.9% of the total peak area.

Melting range (741): between 79° and 80°.

p-Chloroacetanilide, C_8H_8ClNO —**169.61**—White or pale yellow, needle-shaped crystals or crystalline powder. Insoluble in water; soluble in alcohol and in ether.

Solubility—One g dissolves in 30 mL of alcohol to form a clear solution.

Melting range (741): between 178° and 181°.

Residue on ignition (Reagent test): not more than 0.1%.

1-Chloroadamantane, $C_{10}H_{15}Cl$ —**170.68** [935-56-8]—White crystalline solid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 250°; the detector temperature is maintained at 300°; and the column temperature is maintained at 150° and programmed to rise 10° per minute to 280°. The area of the $C_{10}H_{15}Cl$ peak is not less than 97.5% of the total peak area.

2-Chloro-4-aminobenzoic Acid—See *4-Amino-2-chlorobenzoic Acid*.

5-Chloro-2-aminobenzophenone—See *2-Amino-5-chlorobenzophenone*.

3-Chloroaniline, C_6H_6ClN —**127.57** [108-42-9]—Colorless to light brown liquid. Soluble in acid and in most organic solvents; practically insoluble in water.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 250°; the detector temperature is maintained at 300°; and the column temperature is maintained at 150° and programmed to rise 10° per minute to 280°. The area of the C_6H_6ClN peak is not less than 99% of the total peak area.

Refractive index (831): between 1.592 and 1.596 at 20°.

p-Chloroaniline, (*4-Chloroaniline*), C_6H_6ClN —**127.57** [106-47-8]—Use a suitable grade.

Chlorobenzene, C_6H_5Cl —**112.56** [108-90-7]—Clear, colorless liquid. Insoluble in water; soluble in alcohol, in

benzene, in chloroform, and in ether. Use ACS reagent grade.

4-Chlorobenzoic Acid, C_6H_4COOH —**156.57** [74-11-3]—White, crystalline solid.

Assay—Dissolve about 700 mg, accurately weighed, in a mixture of 100 mL of hot alcohol and 50 mL of water. Titrate with 0.5 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.5 N sodium hydroxide is equivalent to 78.28 mg of C_6H_4COOH . Not less than 98% is found.

Solubility—One g dissolved in 25 mL of 0.5 N sodium hydroxide yields a clear and complete solution.

m-Chlorobenzoic Acid (*3-Chlorobenzoic Acid*), $C_7H_5ClO_2$ —**156.57** [535-80-8]—Use a suitable grade.

4-Chlorobenzophenone, $C_{13}H_9ClO$ —**216.66** [134-85-0]—Use a suitable grade.

1-Chlorobutane—See *n-Butyl Chloride*.

2-Chloroethanol (*Ethylene Chlorohydrin*), C_2H_5ClO —**80.51** [107-07-3]—Use a suitable grade with a content of NLT 99%.

2-Chloroethylamine Monohydrochloride, $C_2H_6ClN \cdot HCl$ —**115.99**—Off-white powder.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of a phase consisting of 14% cyanopropylphenyl-86% dimethylpolysiloxane; the injection port temperature is maintained at 150°; the detector temperature is maintained at 300°; and the column temperature is maintained at 50° and programmed to rise 10° per minute to 200°. The area of the $C_2H_6ClN \cdot HCl$ peak is not less than 99% of the total peak area.

Melting range (741): between 150° and 246°.

Chloroform, $CHCl_3$ —**119.38** [67-66-3]—Use ACS reagent grade.

Chloroform, Alcohol-Free—Use a suitable grade that does not contain alcohol as a stabilizer.

Chloroform, Methyl—See *Methyl Chloroform*.

Chlorogenic Acid, $C_{16}H_{18}O_9$ —**354.31** [327-97-9]—White to off-white powder. Use a suitable grade.

Assay—When tested by thin-layer chromatography (see *Chromatography* (621)) with the use of plates coated with chromatographic silica gel mixture and a developing system consisting of a mixture of butyl alcohol, water, and acetic acid (60:25:15), and examined under short-wavelength UV light, a single spot is exhibited, with trace impurities.

Chloromethylated Polystyrene-Divinylbenzene Anion-exchange Resin—See *Anion-exchange Resin, Chloromethylated Polystyrene-Divinylbenzene*.

1-Chloronaphthalene (α -*Chloronaphthalene*), $C_{10}H_7Cl$ —**162.62** [90-13-1]—Colorless to light yellow liquid.

Assay—Use a gas chromatograph equipped with a flame-ionization detector. The following conditions have been found suitable: a 3.2-mm \times 1.83-m stainless steel column is packed with 7% phase G2 on support S1A; the injection port temperature is maintained at 250° and the detector temperature at 310°; and the column temperature is programmed to increase at a rate of 10° per minute from 50° to 250°. Not less than 90% of $C_{10}H_7Cl$ is found, of which not more than 10% is 2-chloronaphthalene.

Refractive index (831): between 1.6320 and 1.6340, at 20°.

4-Chloro-1-naphthol, $C_{10}H_7ClO$ —**178.6** [604-44-4]—A white to off-white powder, with a melting point between 118° and 120°. Use a suitable grade. Store below 0°.

2-Chloronicotinic Acid, $C_6H_4ClNO_2$ —**157.55** [2942-59-8]—Off-white powder.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas.

The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 280°; the detector temperature is maintained at 300°; and the column temperature is maintained at 180° and programmed to rise 10° per minute to 280°. The area of the $C_6H_4ClNO_2$ peak is not less than 98% of the total peak area.

2-Chloro-4-nitroaniline, 99%, $C_6H_5ClN_2O_2$ —**172.57**
[121-87-9]—White to off-white powder.

Melting range (741): between 107° and 109°.

Chloroplatinic Acid, $H_2PtCl_6 \cdot 6H_2O$ —**517.90**
[18497-13-7]—Use ACS reagent grade Chloroplatinic Acid Hexahydrate.

5-Chlorosalicylic Acid, $C_7H_5ClO_3$ —**172.57**
[321-14-2]—White to off-white powder.

Assay—When tested by thin-layer chromatography (see *Chromatography* (621)) with the use of plates coated with chromatographic silica gel mixture and a developing system consisting of a mixture of cyclohexane, chloroform, and acetic acid (14:4:2), and examined visually and under long-wavelength UV light or iodine spray, a single spot is exhibited.

Melting range (741): between 172° and 178°.

1-Chloro-2,2,2-trifluoroethylchlorodifluoromethyl Ether—Use a suitable grade.

Chlorotrimethylsilane (*Trimethylsilyl Chloride*), C_3H_9ClSi —**108.64** [75-77-4]—Clear, colorless to light yellow liquid. Fumes when exposed to moist air.

[CAUTION]—It reacts vigorously with water, alcohols, and other hydrogen donors. Store in tight glass containers.]

Refractive index (831): between 1.3850 and 1.3890 at 20°.

Chlortetracycline Hydrochloride—Use *Chlortetracycline Hydrochloride* (USP monograph).

Cholestane, $C_{27}H_{48}$ —**372.67** [481-21-0]—Use a suitable grade.

Cholesterol, $C_{27}H_{46}O$ —**386.66** [57-88-5]—Use a suitable grade (*NF* monograph).

Cholesteryl Benzoate, $C_{34}H_{50}O_2$ —**490.76** [604-32-0]—Use a suitable grade.

Cholesteryl *n*-Heptylate—Use a suitable grade.

Choline Chloride, $HOCH_2CH_2N(CH_3)_3Cl$ —**139.62**
[67-48-1]—White crystals or crystalline powder. Very soluble in water. Is hygroscopic. Store in tight containers.

Assay—Transfer about 100 mg, previously dried at 105° for 2 hours and accurately weighed, to a beaker, add 20 mL of water and 1 drop of aluminum chloride solution (1 in 10), and mix. Add slowly 20 mL of a freshly prepared, filtered sodium tetraphenylborate solution (1 in 50), and allow the mixture to stand for 30 minutes with occasional swirling. Pass through a medium-porosity, sintered-glass filter, and wash the beaker and the precipitate with four 10-mL portions of water. The weight of the precipitate, determined after drying at 105° for 2 hours, and multiplied by 0.3298, gives the equivalent weight of $C_5H_{14}ClNO$. Not less than 99.5% is found.

Residue on ignition (281): not more than 0.1%.

Chromatographic Fuller's Earth—See *Fuller's Earth, Chromatographic*.

Chromatographic *n*-Heptane—See *n-Heptane, Chromatographic*.

Chromatographic Magnesium Oxide—See *Magnesium Oxide, Chromatographic*.

Chromatographic Reagents—See *Reagents, Chromatographic Reagents*.

[NOTE—Listings of the numerical designations for phases (G), packings (L), and supports (S), together with corre-

sponding brand names, are published periodically in *Pharmacopeial Forum* as a guide for the chromatographer.]

Chromatographic Silica Gel—See *Silica Gel, Chromatographic*.

Chromatographic Silica Gel Mixture—See *Silica Gel Mixture, Chromatographic*.

Chromatographic Siliceous Earth—See *Siliceous Earth, Chromatographic*.

Chromatographic Siliceous Earth, Silanized—See *Siliceous Earth, Chromatographic, Silanized*.

Chromatographic Solvent Hexane—See *Hexane, Solvent, Chromatographic*.

Chromium Potassium Sulfate Dodecahydrate, $CrK(SO_4)_2 \cdot 12H_2O$ —**499.40** [10279-63-7]—Use ACS reagent grade.

Chromium Trioxide, CrO_3 —**99.99** [1333-82-0]—Use ACS reagent grade.

Chromogenic Substrate for Amidolytic Test—Synthetic molecules consisting of tripeptides or tetrapeptides coupled to a chromophore. The terminal amino acid is specific for the protease utilized. The synthetic peptides mimic the peptide sequence (specific to the activated coagulation factor) of the active site on the natural substrate. The coagulation factor catalyzes the splitting of the chromophore (*p*-nitroaniline) from the peptide. The amount of release can be measured directly in a spectrophotometer, because the maximum absorbance spectra of the bound and free chromophores differ. The released chromophore is a colored compound; the complete substrate itself is colorless.

Molecular weights of the various substrates range from about 600 to 750 Da. Solubility in aqueous solutions can vary. Not all substrates are of equal sensitivity, and incubation periods may have to be extended.

Chromotrope 2R, $C_{16}H_{10}N_2Na_2O_8S_2$ —**468.4**
[4197-07-3]—Red powder or crystals. Use a suitable grade.

Chromotropic Acid (4,5-Dihydroxy-2,7-naphthalenedisulfonic Acid), $C_{10}H_8O_8S_2 \cdot 2H_2O$ —**356.33**
[148-25-4], for the anhydrous form—Use a suitable grade.

Chromotropic Acid Disodium Salt (4,5-Dihydroxy-2,7-naphthalenedisulfonic Acid, Disodium Salt), $C_{10}H_6O_8Na_2S_2 \cdot 2H_2O$ —**400.29** [5808-22-0]—Use ACS reagent grade.

Cinchonidine, $C_{19}H_{22}N_2O$ —**294.39** [485-71-2]—White crystals, crystalline or granular powder. Soluble in alcohol and in chloroform; practically insoluble in water.

Assay—Dissolve about 125 mg, accurately weighed, in 50 mL of glacial acetic acid. Add a few drops of *p*-naphtholbenzein TS, and titrate with 0.1 N perchloric acid VS. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 14.72 mg of $C_{19}H_{22}N_2O$. Not less than 99.0% is found.

Loss on drying (731)—Dry it at 105° to constant weight; it loses not more than 1.0% of its weight.

Melting range (741): between 200° and 205°.

Specific rotation (781): between −105° and −115°, calculated on the dried basis, determined in a solution in alcohol containing 10 mg per mL.

Cinchonine, $C_{19}H_{22}N_2O$ —**294.39** [118-10-5]—White crystals, crystalline or granular powder. Slightly soluble in chloroform, sparingly soluble in alcohol, and practically insoluble in water.

Assay—Dissolve about 125 mg, accurately weighed, in 50 mL of glacial acetic acid. Add a few drops of *p*-naphtholbenzein TS, and titrate with 0.1 N perchloric acid VS. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 14.72 mg of $C_{19}H_{22}N_2O$. Not less than 99.0% is found.

Loss on drying (731)—Dry it at 105° to constant weight; it loses not more than 1.0% of its weight.

Melting range (741): between 255° and 261°.

Specific rotation (781): between +219° and +229°, calculated on the dried basis, determined in a solution in alcohol containing 50 mg per 10 mL.

Citric Acid—Use *Citric Acid Monohydrate* (USP monograph).

Citric Acid, Anhydrous [77-92-9]—Use *Anhydrous Citric Acid* (USP monograph).

Cobalt Chloride (*Cobaltous Chloride*), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ —**237.93** [7791-13-1]—Use ACS reagent grade.

Cobalt Nitrate, $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ —**291.03**

[10026-22-9]—Use ACS reagent grade.

Cobaltous Acetate (*Cobalt Acetate*), $\text{Co}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 4\text{H}_2\text{O}$ —**249.08** [71-48-7]—Red, needlelike crystals. Soluble in water and in alcohol. Use ACS reagent grade.

Cobaltous Chloride—See *Cobalt Chloride*.

Coenzyme Q9 (*Ubiquinone 45*), $\text{C}_{54}\text{H}_{82}\text{O}_4$ —**795.2** [303-97-9]—Yellow to yellow-orange powder. Clear yellow solution, at a concentration of 1 mg per mL in a mixture of chloroform and ethanol (9:1).

Absorptivity—Its absorptivity at 275 nm, in alcohol solution, is about 1700.

Collagen—Use a suitable grade.

[NOTE—A suitable grade is acid-soluble Collagen Type I from calf skin, and is commercially available from Sigma-Aldrich Corp., www.sigma-aldrich.com; catalog number C3511.]

Rat Tail Collagen—Use a suitable grade.

[NOTE—A suitable grade is available from BD Biosciences, www.bdbiosciences.com.]

Collagenase—Use a suitable grade.

[NOTE—A suitable grade is commercially available as Collagenase Type 2, CLS-2, from Worthington Biochemical Corp., www.worthington-biochem.com.]

Compactin, $\text{C}_{23}\text{H}_{34}\text{O}_5$ —**390.52** [73573-88-3]—Use a suitable grade.

[NOTE—A suitable grade is available from Sigma-Aldrich, www.sigma-aldrich.com.]

Congo Red, $\text{C}_{32}\text{H}_{22}\text{N}_6\text{Na}_2\text{O}_6\text{S}_2$ —**696.67** [573-58-0]—A dark red or reddish-brown powder. Decomposes on exposure to acid fumes. Its solutions have a pH of about 8 to 9.5. One g dissolves in about 30 mL of water. Is slightly soluble in alcohol.

Loss on drying (731)—Dry it at 105° for 4 hours: it loses not more than 3.0% of its weight.

Residue on ignition—Accurately weigh about 1 g, previously dried at 105° for 4 hours, and place it in a porcelain dish or crucible. Carefully ignite until well charred, cool, add 2 mL of sulfuric acid, and carefully ignite until the residue is white or practically so. Cool, add 0.5 mL of sulfuric acid and 1 mL of nitric acid, evaporate, and again ignite to constant weight: the weight of the sodium sulfate so obtained is between 20.0% and 24.0% of the weight of the dried specimen taken.

Sensitiveness—To 50 mL of carbon dioxide-free water add 0.1 mL of congo red solution (1 in 1000). The red color of the solution is changed to violet by the addition of 0.05 mL of 0.10 N hydrochloric acid and is restored by the subsequent addition of 0.05 mL of 0.10 N sodium hydroxide.

Coomassie Blue G-250 (*Coomassie Brilliant Blue G-250*, *Serva Blue G*), $\text{C}_{47}\text{H}_{48}\text{N}_3\text{O}_7\text{S}_2\text{Na}$ —**854.0** [6104-58-1]—A dark blue powder. Soluble in water. Use a suitable grade. Store between 15° and 30°.

Coomassie Brilliant Blue R-250, $\text{C}_{45}\text{H}_{44}\text{N}_3\text{O}_7\text{S}_2\text{Na}$ —**825.97** [6104-58-1]—Brown powder.

Copper, Cu—**At. Wt. 63.546** [7440-50-8]—Use ACS reagent grade.

Cortisone, $\text{C}_{21}\text{H}_{28}\text{O}_5$ —**360.44** [53-06-5]—White, crystalline powder. Practically insoluble in water; sparingly solu-

ble in alcohol and in acetone. Melts at about 220°, with decomposition.

Absorption maximum—The UV absorption spectrum of a 1 in 100,000 solution in alcohol shows a maximum at about 238 nm.

Specific rotation (781): about +209°, determined in a 1 in 100 solution in alcohol.

Cotton, Absorbent—Use *Purified Cotton* (USP monograph).

m-Cresol Purple, $\text{C}_{21}\text{H}_{18}\text{O}_5\text{S}$ —**382.43** [2303-01-7]—Use a suitable grade.

Cupric Acetate, $\text{Cu}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{H}_2\text{O}$ —**199.65** [6046-93-1]—Use ACS reagent grade.

Cupric Chloride, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ —**170.48** [7447-39-4]—Bluish-green deliquescent crystals. Freely soluble in water; soluble in alcohol; slightly soluble in ether. Use ACS reagent grade.

Cupric Citrate (*[Citrate(4-)]dicopper*), $\text{Cu}_2\text{C}_6\text{H}_4\text{O}_7$ —**315.18** [866-82-0]—Use a suitable grade.

Cupric Nitrate [3251-23-8]—Use ACS reagent grade *Cupric Nitrate Hydrate*.

Cupric Nitrate Hydrate, $\text{Cu}(\text{NO}_3)_2 \cdot 2.5\text{H}_2\text{O}$ —**232.59** [3252-23-8]; $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ —**241.60** [10031-43-3]—Use ACS reagent grade.

[NOTE—This reagent is available containing either 2.5 or 3 molecules of water of hydration.]

Cupric Sulfate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ —**249.69**—Use ACS reagent grade.

Cupric Sulfate, Anhydrous, CuSO_4 —**159.61**

[7758-98-7]—A white or grayish-white powder free from a blue tinge. Upon the addition of a small quantity of water, it becomes blue. Soluble in water. Store in tight containers.

Chloride (Reagent test)—One g shows not more than 0.02 mg of Cl (0.002%).

Substances not precipitated by hydrogen sulfide—Determine as directed for ACS reagent grade of *Cupric Acetate*: the residue weighs not more than 6 mg (0.15%).

Cupriethylenediamine Hydroxide Solution, 1.0 M—Use a suitable grade.

[NOTE—A suitable grade is available from GFS Chemicals, www.gfschemicals.com.]

Cyanoacetic Acid, $\text{C}_3\text{H}_3\text{NO}_2$ —**85.06** [372-09-8]—White to light yellow, crystalline solid. Very soluble in water.

Assay—Dissolve about 300 mg, accurately weighed, in 25 mL of water and 25 mL of alcohol. Titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination and make any necessary corrections. Each mL of 0.1 N sodium hydroxide is equivalent to 85.06 mg of $\text{C}_3\text{H}_3\text{NO}_2$. Not less than 99% is found.

Cyanogen Bromide, BrCN—**105.92** [506-68-3]—Colorless crystals. Volatilizes at room temperature. Its vapors are highly irritating and very toxic. Melts at about 52°. Freely soluble in water and in alcohol. Store in tight containers in a cold place.

Solubility—Separate 1-g portions dissolve completely in 10 mL of water and in 10 mL of alcohol, respectively, to yield colorless solutions.

4-Cyanophenol (*4-Hydroxybenzonitrile*), $\text{C}_6\text{H}_4\text{CNOH}$ —**119.12** [767-00-0]—Use 95 percent reagent.

Cyclam (*1,4,8,11-Tetraazacyclotetradecane*), $\text{C}_{10}\text{H}_{24}\text{N}_4$ —**200.33** [295-37-4]—Use 98 percent reagent.

α -Cyclodextrin (*Cyclomaltose*; *Schardinger α -Dextrin*), $\text{C}_{36}\text{H}_{60}\text{O}_{30}$ —**972.86** [10016-20-3]—Use a suitable grade with a content of not less than 98.0%.

[NOTE—A suitable grade is available from Fluka, www.sigma-aldrich.com, catalog number 28705.]

Add the following:

▲ **β-Cyclodextrin** (*β-Schardinger dextrin*; *Cycloheptaamylose*), $C_{42}H_{70}O_{35} \cdot xH_2O$ —**1134.98** (anhydrous) [68168-23-0]—Use a suitable hydrate grade.▲ *USP36*
Cyclohexane, C_6H_{12} —**84.16** [110-82-7]—Use ACS reagent grade.

Cyclohexanol, $C_6H_{12}O$ —**100.16** [108-93-0]—A clear liquid. Freely soluble in water. Miscible with alcohol, with ethyl acetate, and with aromatic hydrocarbons.

Assay—When examined by gas-liquid chromatography, using suitable gas chromatographic apparatus and conditions, it shows a purity of not less than 98%.

Melting temperature: about 23°.

Specific gravity: about 0.962, at 20°.

(1,2-Cyclohexylenedinitrilo)tetraacetic Acid (*trans*-1,2-Diaminocyclohexane-*N,N,N',N'*-tetraacetic Acid), $C_{14}H_{22}N_2O_8 \cdot H_2O$ —**364.35**—Use ACS reagent grade.

Cyclohexylmethanol, $C_7H_{14}O$ —**114.19**—Use a suitable grade.

L-Cystine, $HOOC(NH_2)CHCH_2S-SCH_2CH(NH_2)COOH$ —**240.30** [58-89-3]—A white, crystalline powder. Very slightly soluble in water; soluble in dilute mineral acids and in solutions of alkali hydroxides; insoluble in alcohol and in other organic solvents.

Specific rotation (781): between -215° and -225°, determined in a 2 in 100 solution of test specimen, previously dried over silica gel for 4 hours, in dilute hydrochloric acid (1 in 10) at a temperature of 20°.

Loss on drying (731)—Dry it over silica gel for 4 hours: it loses not more than 0.2% of its weight.

Residue on ignition (Reagent test): not more than 0.1%.

DEAE-Agarose [57407-08-6]—Agarose beads chemically bonded with diethylaminoethane and suspended in a 20% ethanol solution in water.

[NOTE—Commercially available as DEAE-Sephrose.]

Decanol (*n-Decyl Alcohol*), $C_{10}H_{22}O$ —**158.28** [112-30-1]—A clear, viscous liquid. *Specific gravity*: about 0.83 at 20°. Solidifies at about 6.5°. Insoluble in water; soluble in alcohol and in ether.

Assay—When examined by gas-liquid chromatography, using suitable gas chromatographic apparatus and conditions, it shows a purity of not less than 99%.

Decyl Sodium Sulfate, $C_{10}H_{21}NaO_4S$ —**260.33**—White, crystalline solid.

Assay—Transfer about 1 g, accurately weighed, to a suitable, tared crucible, moisten with a few drops of sulfuric acid, and ignite gently to constant weight. Each mg of residue is equivalent to 3.662 mg of $C_{10}H_{21}NaO_4S$. Not less than 95% is found.

Dehydrated Alcohol—See *Alcohol, Dehydrated*.

Deoxyadenosine Triphosphate, $C_{10}H_{16}N_5O_{12}P_3$ —**491.18** [1927-31-7]—Use a suitable grade.

[NOTE—A suitable grade is available from either BD Biosciences, www.bdbiosciences.com or Applied Biosystems, www.appliedbiosystems.com.]

Deoxycytidine Triphosphate, $C_9H_{16}N_3O_{13}P_3$ —**467.16** [2056-98-6]—Use a suitable grade.

[NOTE—A suitable grade is available from either BD Biosciences, www.bdbiosciences.com or Applied Biosystems, www.appliedbiosystems.com.]

Deoxyguanosine Triphosphate, $C_{10}H_{16}N_5O_{13}P_3$ —**507.18** [2564-35-4]—Use a suitable grade.

[NOTE—A suitable grade is available from either BD Biosciences, www.bdbiosciences.com, or Applied Biosystems, www.appliedbiosystems.com.]

Deoxyribonucleic Acid Polymerase—Thermostable, recombinant DNA polymerase. Use a suitable grade.

[NOTE—A suitable grade is available from Applied Biosystems, www.appliedbiosystems.com.]

Deoxythymidine Triphosphate, $C_{10}H_{17}N_2O_{14}P_3$ —**482.17**—Use a suitable grade.

[NOTE—A suitable grade is available from either BD Biosciences, www.bdbiosciences.com or Applied Biosystems, www.appliedbiosystems.com.]

Deuterated Methanol (*Methanol- ^{12}C - d_4* , *Methyl- ^{12}C - d_3 alcohol- d_1*)—**36.1** [811-98-3]—The degree of deuteration is not less than 99.8%. Is a clear colorless liquid miscible with water, with alcohol, and with methylene chloride; density at 20°: 0.888 g/mL; refractive index at 20° (D-line): 1.326; boiling point 65.4° (760 mm Hg).

Deuterated Water—See *Deuterium Oxide*.

Deuterium Chloride (*Deutero Hydrochloric Acid*), DCl—**37.47** [7698-05-7]—Toxic gas. Use a suitable grade with a degree of deuteration of NLT 99%.

Deuterium Oxide, D_2O —**20.032** [7789-20-0]—Use a suitable grade having a minimum isotopic purity of 99.8 atom % of deuterium.

Deuteriochloroform, $CDCl_3$ —**120.38**—Use a suitable grade.

Devarda's Alloy (*Devarda's Metal*) [8049-11-4]—A gray powder composed of 50 parts of copper, 45 parts of aluminum, and 5 parts of zinc.

Dextran, High Molecular Weight [9004-54-0]—A dextran molecular weight standard having a weight-average molecular weight, M_w , of 1 to 2×10^6 Da and a weight-average molecular weight to number-average molecular weight ratio, M_w / M_n , of 1.0 to 1.8.

[NOTE—A suitable grade is available from American Polymer Standards Corporation, www.ampolymer.com.]

Dextrin, $(C_6H_{10}O_5)_n \cdot xH_2O$ [9004-53-9]—A white amorphous powder. Slowly soluble in cold water; more readily soluble in hot water; insoluble in alcohol.

Insoluble matter—Boil 1 g with 30 mL of water in a small flask: the solution is colorless and clear, or not more than opalescent.

Loss on drying (731)—Dry it at 105° to constant weight: it loses not more than 10.0% of its weight.

Residue on ignition (Reagent test)—Ignite 1 g with 0.5 mL of sulfuric acid: the residue weighs not more than 5 mg (0.5%).

Chloride (Reagent test)—Dissolve 3 g in 75 mL of boiling water, cool, dilute with water to 75 mL, and filter if necessary. To 25 mL of the filtrate add 2 mL of nitric acid and 1 mL of silver nitrate TS, and allow to stand for 5 minutes: any turbidity produced is not greater than that of a control containing 0.02 mg of added Cl (0.002%).

Sulfate (Reagent test, *Method I*)—To a 25-mL portion of the filtrate from the preceding test add 0.5 mL of diluted hydrochloric acid and 2 mL of barium chloride TS, and allow to stand for 10 minutes: any turbidity produced is not greater than that of a control containing 0.2 mg of added SO_4 (0.02%).

Alcohol-soluble substances—Boil 1 g with 20 mL of alcohol for 5 minutes under a reflux condenser, and filter while hot. Evaporate 10 mL of the filtrate on a steam bath, and dry at 105°: the residue weighs not more than 5 mg (1%).

Reducing sugars—Shake 2 g with 100 mL of water for 10 minutes, and filter until clear. To 50 mL of the filtrate add 50 mL of alkaline cupric tartrate TS, and boil for 3 minutes. Filter through a tared filtering crucible, wash with water, then with alcohol, and finally with ether, and dry at 105° for 2 hours: the precipitate of cuprous oxide weighs not more than 115 mg (corresponding to about 5% of reducing sugars as dextrose).

Dextro Calcium Pantothenate—Use *Calcium Pantothenate* (USP monograph).

Dextrose, Anhydrous, $C_6H_{12}O_6$ —**180.16**—Use ACS reagent grade D-Glucose, Anhydrous.

Diacetyl—See *2,3-Butanedione*.

3,3'-Diaminobenzidine Hydrochloride, $(NH_2)_2C_6H_3C_6H_3(NH_2)_2 \cdot 4HCl$ —**360.11** [7411-49-6]—White to yellowish-tan (occasionally purple), needle-shaped crystals. Soluble in water. Stable in organic solvents but un-

stable in aqueous solution at room temperature. Store aqueous solutions in a refrigerator.

Insoluble matter—Dissolve 2 g in 100 mL of water, without heating, and filter immediately: the insoluble residue does not exceed 1 mg (0.05%).

Residue on ignition (Reagent test): not more than 1 mg, from 2 g (0.05%).

Suitability test for detection of selenium—Dissolve 1.633 g of selenious acid (H_2SeO_3) in water, and dilute with water to 1 L. Dilute 10 mL of this solution with water to 1 L, to make a solution containing 0.010 mg of Se per mL. Place 1 mL of the resulting solution in a 100-mL beaker, add 2 mL of formic acid solution (1 in 7), and dilute with water to 50 mL. Add 2 mL of 3,3'-diaminobenzidine hydrochloride solution (1 in 200), and allow to stand for 30 to 50 minutes. Adjust with 6 N ammonium hydroxide to a pH between 6 and 7. Transfer to a 125-mL separator, add 10.0 mL of toluene, and shake vigorously for 30 seconds: a distinct yellow color is produced in the toluene layer. A blank containing diaminobenzidine hydrochloride but no selenium standard, treated in the same manner, shows no color in the toluene layer.

2,3-Diaminonaphthalene, $\text{C}_{10}\text{H}_{10}\text{N}_2$ —**158.20** [771-97-1]—Use a suitable grade.

Diatomaceous Earth [91053-39-3]—Use a suitable grade. [NOTE—A suitable grade is available as Celite 545-AW.]

Diatomaceous Earth, Flux-Calcined [91053-39-3]—Use a suitable grade.

[NOTE—A suitable grade is "Chromosorb W, AW-DMCS," available from Grace, www.grace.com.]

Diatomaceous Earth, Silanized [91053-39-3]—Use a suitable grade.

[NOTE—Suitable grades are available commercially as "Anachrome Q," "Gas-Chrom Q," and "Varaport 30."]

Diatomaceous Silica, Calcined [68855-54-9]—A form of silica (SiO_2) consisting of fused frustules and fragments of diatoms. It is an amorphous, fine, light pink or white powder. Insoluble in water, in acids, and in dilute solutions of alkali hydroxides.

Loss on ignition—Accurately weigh about 4 g, and ignite to constant weight: it loses not more than 10.0% of its weight.

Organic impurities—It does not darken appreciably upon ignition.

Loss on drying (731)—Dry it at 110° for 2 hours: it loses not more than 2.0% of its weight.

[NOTE—Suitable grades are "Chromosorb P" and "Chromosorb W," available from Grace, www.grace.com.]

Diaveridine (5-([3,4-Dimethoxyphenyl]methyl)-2,4-pyrimidinediamine), $\text{C}_{13}\text{H}_{16}\text{N}_4\text{O}_2$ —**260.3** [5355-16-8]—Use a suitable grade. [NOTE—A suitable grade is available as catalog number 46174 from www.sigma-aldrich.com.]

Dibasic Ammonium Citrate—See *Ammonium Citrate, Dibasic*.

Dibasic Ammonium Phosphate—See *Ammonium Phosphate, Dibasic*.

Dibasic Potassium Phosphate—See *Potassium Phosphate, Dibasic*.

Dibenzyl—See *Bibenzyl*.

2,6-Dibromoquinone-chlorimide (2,6-Dibromo-N-chloro-p-benzoquinone Imine; DBQ Reagent), $\text{O}:\text{C}_6\text{H}_2\text{Br}_2:\text{NCl}$ —**299.35** [537-45-1]—A yellow, crystalline powder. Insoluble in water; soluble in alcohol and in dilute alkali hydroxide solutions.

Melting range (741): between 82° and 84°.

Solubility in alcohol—A solution of 100 mg in 10 mL of alcohol is not more than faintly turbid.

Residue on ignition (Reagent test)—Ignite 500 mg with 0.5 mL of sulfuric acid: the residue weighs not more than 1 mg (0.2%).

Sensitiveness—To 10 mL of a water solution containing 0.01 mg of phenol add 0.3 mL of a sodium borate buffer

(made by dissolving 2.84 g of crystallized sodium borate in 90 mL of warm water, adding 8.2 mL of 1 N sodium hydroxide, and diluting with water to 100 mL) and 0.1 mL of a solution of 10 mg of the test specimen in 20 mL of alcohol: a distinct blue color develops within 10 minutes.

Dibutyl Phthalate, $\text{C}_{16}\text{H}_{22}\text{O}_4$ —**278.34** [84-74-2]—Clear, colorless liquid.

Assay—Accurately weigh about 2 g into a suitable flask, add 25.0 mL of 1 N sodium hydroxide and 30 mL of isopropyl alcohol, and mix. Digest the mixture at a temperature near boiling for 30 minutes, then cool in a water bath to room temperature. Add phenolphthalein TS, and titrate with 1 N sulfuric acid VS to the disappearance of the pink color. Perform a complete blank determination, and make any necessary correction. Each mL of 1 N sulfuric acid consumed is equivalent to 139.2 mg of $\text{C}_{16}\text{H}_{22}\text{O}_4$. Not less than 98% is found.

Refractive index (831): between 1.491 and 1.493 at 20°.

Acid content—Accurately weigh about 10 g, and dissolve in 100 mL of an alcohol-ether mixture (1:1). Add phenolphthalein TS, and titrate immediately with 0.05 N alcoholic potassium hydroxide VS. Each mL of 0.05 N alcoholic potassium hydroxide is equivalent to 4.15 mg of phthalic acid: not more than 0.02% is found.

Dibutylamine, $\text{C}_8\text{H}_{19}\text{N}$ —**129.24** [111-92-2]—Colorless liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μm layer of phase G2; the injection port temperature is maintained at 200°; the detector temperature is maintained at 300°; and the column temperature is maintained at 100° and programmed to rise 10° per minute to 200°. The area of the $\text{C}_8\text{H}_{19}\text{N}$ peak is not less than 99% of the total peak area.

Refractive index (831): between 1.415 and 1.419 at 20°.

Dibutylammonium Phosphate—Use a suitable grade.

[NOTE—A suitable grade is available as PIC Reagent D4 from Waters Corporation, www.waters.com.]

1,3-Dicaffeoylquinic Acid (Cynarin; (1R,3R,4S,5R)-1,3-Bis-[[3-(3,4-dihydroxyphenyl)propenoyl]oxy]-4,5-dihydroxycyclohexanecarboxylic Acid), $\text{C}_{25}\text{H}_{24}\text{O}_{12}$ —**516.45** [30964-13-7]—Use a suitable grade.

[NOTE—A suitable grade is available as catalog number 3991 from www.chromadex.com.]

Dichloroacetic Acid, $\text{C}_2\text{H}_2\text{Cl}_2\text{O}_2$ —**128.9** [79-43-6]—Colorless liquid. Miscible with water, with alcohol, and with ether. Use a suitable grade.

2,5-Dichloroaniline, $\text{Cl}_2\text{C}_6\text{H}_3\text{NH}_2$ —**162.02** [95-82-9]—White, needle-like crystals. Slightly soluble in water; soluble in alcohol and in ether.

Melting range, Class I (741): between 49° and 50°.

2,6-Dichloroaniline, $\text{C}_6\text{H}_3\text{Cl}_2\text{N}$ —**162.02** [608-31-1]—Off-white powder.

Melting range (741): between 38° and 41°.

o-Dichlorobenzene, $\text{C}_6\text{H}_4\text{Cl}_2$ —**147.00** [95-50-1]—Clear liquid, having a light yellowish-brown tint (about APHA 20). Practically insoluble in water. Miscible with alcohol and with ether. Boils at about 180°.

Assay—When examined by gas-liquid chromatography, with the use of suitable apparatus and conditions, it shows a purity of not less than 98%.

Density: between 1.299 and 1.301.

Refractive index (831): between 1.548 and 1.550 at 25°.

Residue on evaporation—Evaporate 80 mL on a steam bath, and dry at 105° for 1 hour: the residue weighs not more than 50 mg (0.005%).

Acidity—Add phenolphthalein TS to 25 mL of methanol, and titrate with 0.02 N alcoholic potassium hydroxide VS

until a faint pink color persists for 15 seconds. Pipet 25 mL of test specimen into the solution; mix, avoiding exposure to the atmosphere; and titrate with 0.02 N alcoholic potassium hydroxide VS: not more than 2.2 mL is required to restore the pink color (about 0.005%).

1,2-Dichloroethane—See *Ethylene Dichloride*.

Dichlorofluorescein, $C_{20}H_{10}Cl_2O_5$ —**401.20** [76-54-0]—[NOTE—This specification covers both the 4,5- and 2,7-isomers of dichlorofluorescein, either of which is suitable for the preparation of dichlorofluorescein TS.] A weak orange-colored, crystalline powder. Sparingly soluble in water; soluble in alcohol and in solutions of alkali hydroxides.

Residue on ignition (Reagent test)—Ignite 200 mg with 5 drops of sulfuric acid: the residue weighs not more than 1 mg (0.5%).

Sensitiveness—Dissolve 100 mg in 60 mL of alcohol, add 2.5 mL of 0.1 N sodium hydroxide, and dilute with water to 100 mL. Add 1 mL of this solution to a solution of potassium iodide prepared by dissolving 100 mg of potassium iodide, previously dried at 105° to constant weight and accurately weighed, in 50 mL of water containing 1 mL of glacial acetic acid, and titrate with 0.1 N silver nitrate VS until the color of the precipitate changes from pale yellowish-orange to pink. The volume of 0.1 N silver nitrate consumed is not more than 0.10 mL greater than the calculated volume, the calculated volume being based upon the KI content of the dried specimen as determined in the *Assay* under *Potassium Iodide* (USP monograph).

Dichlorofluoromethane, $CHCl_2F$ —**102.92** [75-43-4]—Colorless gas.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a thermal-conductivity detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.53-mm \times 30-m capillary column coated with a 5- μ m layer of phase G2; the injection port temperature is maintained at 200°; the detector temperature is maintained at 200°; and the column temperature is maintained at 0° and programmed to rise 5° per minute to 40°, and then to rise 10° per minute to 180°. The area of the $CHCl_2F$ peak is not less than 98% of the total peak area.

2,6-Dichloroindophenol Sodium—See *2,6-Dichlorophenol-indophenol Sodium*.

Dichloromethane—Use *Methylene Chloride*.

2,4-Dichloro-1-naphthol, $C_{10}H_6OCl_2$ —**213.06** [2050-76-2]—Light tan powder.

Melting range (741): between 103° and 107°, but the range between beginning and end of melting does not exceed 2°.

2,6-Dichlorophenol-indophenol Sodium (*2,6-Dichloroindophenol Sodium*), $O:C_6H_2Cl_2:NC_6H_4ONa$ with about $2H_2O$ —**290.08** (anhydrous) [620-45-1]—Use ACS reagent grade.

2,6-Dichlorophenylacetic Acid, $C_8H_6Cl_2O_2$ —**205.04** [6575-24-2]—White powder.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 250°; the detector temperature is maintained at 300°; and the column temperature is maintained at 150° and programmed to rise 10° per minute to 280°. The area of the $C_8H_6Cl_2O_2$ peak is not less than 97% of the total peak area.

2,6-Dichloroquinone-chlorimide (*2,6-Dichloro-N-chloro-p-benzoquinone Imine*), $O:C_6H_2Cl_2:NCl$ —**210.44**—Pale yellow,

crystalline powder. Insoluble in water; soluble in alcohol and in dilute alkali hydroxide solutions.

Melting range (741): between 65° and 67°.

Solubility in alcohol—A solution of 100 mg in 10 mL of alcohol is complete and clear.

Residue on ignition—Ignite 500 mg with 0.5 mL of sulfuric acid: the residue weighs not more than 1 mg (0.2%).

Sensitiveness—It meets the requirements of the test for *Sensitiveness* under *2,6-Dibromoquinone-chlorimide*.

Dicyclohexyl, (*Bicyclohexyl*) $C_{12}H_{22}$ —**166.31**

[92-51-3]—Use a suitable grade.

Dicyclohexyl Phthalate, $C_{20}H_{26}O_4$ —**330.42** [84-61-7].

Melting range (741): between 62° and 66°.

Dicyclohexylamine, $(C_6H_{11})_2NH$ —**181.32** [101-83-7]—

Clear, strongly alkaline liquid. Sparingly soluble in water.

Miscible with common organic solvents. Use a suitable grade with a content of not less than 98%.

Diethylamine, $(C_2H_5)_2NH$ —**73.14** [109-89-7]—Colorless, flammable, strongly alkaline liquid. Miscible with water and with alcohol. Forms a hydrate with water. *May be irritating to skin and mucous membranes*. Store in well-closed containers. Use ACS reagent grade.

Diethylamine Phosphate (*Phosphoric Acid: Diethylamine*), $C_4H_{11}N \cdot H_3PO_4$ —**171.13** [68109-72-8]—Use a suitable grade. [NOTE—A suitable grade is available from www.richmanchemical.com.]

N,N-Diethylaniline, $C_6H_5N(C_2H_5)_2$ —**149.23** [91-66-7]—Light yellow to amber liquid.

Assay—Inject an appropriate specimen (about 0.2 μ L) into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas flowing at about 40 mL per minute. The following conditions have been found suitable: a 3-mm \times 1.8-m stainless steel column containing 20% phase G16 on support S1A; the injection port temperature is maintained at 250°; the column temperature is maintained at 140° and programmed to rise 6° per minute to 200°. The detector temperature is maintained at 310°. The area of the *N,N*-diethylaniline peak having a retention time of about 4.9 minutes is not less than 99% of the total peak area.

Refractive index (831): between 1.5405 and 1.5425 at 20°.

Diethylene Glycol, (*Bis(2-hydroxyethyl) Ether; Diglycol*;

2-Hydroxyethyl Ether; 2,2'-Oxydiethanol), $C_4H_{10}O_3$ —**106.12**

[111-46-6]—A colorless to faintly yellow, viscous, hygroscopic liquid. Miscible with water, with alcohol, with ether, and with acetone. Insoluble in benzene and in carbon tetrachloride. Use a suitable grade with a content of not less than 99.0%.

Di(ethylene glycol) Methyl Ether (*2-(2-Methoxyethoxy)ethanol; Methyl diglycol*), $C_5H_{12}O_3$ —**120.15**

[111-77-3]—Use a suitable grade.

Diethylene Glycol Succinate Polyester, $(OCH_2CH_2OCH_2CH_2OOCCH_2CH_2COO)_n$ [26183-02-8]—Clear, viscous liquid. Soluble in chloroform. Is stabilized by modification of the diethylene glycol succinate polyester, to render it suitable for use in gas-liquid chromatography to a temperature of 200°.

[NOTE—A suitable grade is available from Alltech, www.alltechweb.com.]

Diethylenetriamine, $C_4H_{13}N_3$ —**103.17** [111-40-0]—Colorless liquid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with G2. The injection port temperature is maintained at 200°; the column temperature is maintained at 100° and programmed to rise 10° per minute to 250° and held there for 5 minutes; and the detector temperature is maintained at 300°. The area of the main peak is not less than 95% of the total peak area.

Refractive index (831): between 1.4815 and 1.4845 at 20°.

Di(2-ethylhexyl)phthalate [*Bis(2-ethylhexyl)phthalate*], $C_{24}H_{38}O_4$ —**390.56** [117-81-7]—Use a suitable grade.

Diethylpyrocarbonate, $C_6H_{10}O_5$ —**162.14** [1609-47-8]—Clear, colorless liquid. Use a suitable grade.

Diethyl Sulfone (*Ethyl Sulfone*), $(C_2H_5)_2SO_2$ —**122.19** [597-35-3]—Use a suitable grade with NLT 97%.

Digitonin, $C_{56}H_{92}O_{29}$ —**1229.31** [11024-24-1]—White, crystalline powder. Almost insoluble in water; soluble in warm alcohol, and in glacial acetic acid and in 75% acetic acid; insoluble in chloroform and in ether. Melts at about 230°, with decomposition.

Specific rotation (781): between -47° and -49°, determined in a solution in 75% acetic acid containing 100 mg per mL.

Solubility in alcohol—A solution of 500 mg in 20 mL of warm alcohol is colorless and complete.

Loss on drying (731)—Dry it at 105° to constant weight: it loses not more than 6% of its weight.

Residue on ignition (Reagent test): not more than 0.3%.
Digoxigenin ($3\beta,12\beta,14\beta,21$ -Tetrahydroxy-20(22)-norcholenic Acid Lactone; $3\beta,12\beta,14$ -Trihydroxy- $5\beta,20(22)$ -cardenolide; $5\beta,20(22)$ -Cardenolide- $3\beta,12\beta,14$ -triol; *Lanadigenin*), $C_{23}H_{34}O_5$ —**390.51** [1672-46-4]—Use a suitable grade.

Dihydroquinidine Hydrochloride, $C_{20}H_{27}ClN_2O_2$ —**362.89** [1476-98-8]—Rhombic plates. Freely soluble in methanol and in chloroform.

Assay—

MOBILE PHASE—Prepare a mixture of water, acetonitrile, diethylamine, and methanesulfonic acid (860:100:20:20).

PROCEDURE—Inject about 20 μ L into a suitable liquid chromatograph (see *Chromatography* (621)) equipped with a 235-nm detector and a 4.6-mm \times 15-cm column that contains packing L1. The flow rate is about 1.5 mL per minute. The area of the $C_{20}H_{27}ClN_2O_2$ peak is not less than 97.5% of the total peak area.

Dihydroquinine (*Hydroquinine*), $C_{20}H_{26}N_2O_2$ —**326.43** [522-66-7]—Freely soluble in acetone, in alcohol, and in chloroform; almost insoluble in water.

Assay—

MOBILE PHASE—Prepare a mixture of water, acetonitrile, diethylamine, and methanesulfonic acid (860:100:20:20) and methanol (82:18).

PROCEDURE—Inject about 20 μ L into a suitable liquid chromatograph (see *Chromatography* (621)) equipped with a 235-nm detector and a 4.6-mm \times 15-cm column that contains packing L1. The flow rate is about 1.5 mL per minute. The area of the $C_{20}H_{26}N_2O_2$ peak is not less than 97.5% of the total peak area.

2,5-Dihydroxybenzoic Acid, $C_7H_6O_4$ —**154.12** [303-07-1]—Off-white powder. Freely soluble in alcohol yielding a clear, very pale yellow solution.

Assay—Dissolve about 75 mg, accurately weighed, in 30 mL of methanol. Slowly add 40 mL of water. Titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination, and make

any necessary correction. Each mL of 0.1 N sodium hydroxide is equivalent to 15.41 mg of $C_7H_6O_4$. Not less than 99% is found.

Melting range (741): about 207°, with decomposition.

2,7-Dihydroxynaphthalene—See *2,7-Naphthalenediol*.

Diiodofluorescein, $C_{20}H_{10}I_2O_5$ —**584.10** [31395-16-1]—Orange-red powder. Slightly soluble in water; soluble in alcohol and in solutions of alkali hydroxides.

Residue on ignition—Ignite 200 mg with 5 drops of sulfuric acid: the weight of the residue does not exceed 1.0 mg (0.5%).

Sensitiveness—Accurately weigh about 100 mg of potassium iodide, previously dried at 105° to constant weight, and dissolve it in 50 mL of water. Add 1 mL of diiodofluorescein TS prepared from the test specimen and 1 mL of glacial acetic acid, and titrate with 0.1 N silver nitrate VS until the color of the precipitate changes from brownish-red to a bluish-red. The volume of 0.1 N silver nitrate consumed is not in excess of 0.10 mL over the calculated volume, based on the KI content of the dried potassium iodide determined as follows. Dissolve about 500 mg of potassium iodide, accurately weighed, in about 10 mL of water, and add 35 mL of hydrochloric acid and 5 mL of chloroform. Titrate with 0.05 M potassium iodate VS until the purple color of iodine disappears from the chloroform. Add the last portions of the iodate solution dropwise, agitating vigorously and continuously. After the chloroform has been decolorized, allow the mixture to stand for 5 minutes. If the chloroform develops a purple color, titrate further with the iodate solution. Each mL of 0.05 M potassium iodate is equivalent to 16.60 mg of KI.

Diisodecyl Phthalate [*Bis(isodecyl)phthalate*], $C_{28}H_{46}O_4$ —**446.66** [26761-40-0]—Use a suitable grade.

Diisopropyl Ether (*Isopropyl Ether*), $[(CH_3)_2CH]_2O$ —**102.17** [108-20-3]—Colorless, mobile liquid. Slightly soluble in water. Miscible with alcohol and with ether.

[CAUTION]—It is highly flammable. Do not use where it may be ignited. Do not evaporate to the point of near dryness, since it tends to form explosive peroxides.]

Specific gravity: between 0.716 and 0.720.

Distilling range, Method II (721)—Not less than 95% distills between 65° and 70°.

Peroxides—To 10 mL, contained in a clean, glass-stoppered cylinder previously rinsed with a portion of the ether under examination, add 1 mL of freshly prepared potassium iodide solution (1 in 10). Shake, and allow to stand for 1 minute: no yellow color is observed in either layer (about 0.001% as H_2O_2).

Residue on evaporation—[NOTE—If peroxide is present, do not carry out this procedure.] Evaporate 14 mL (10 g) from a tared shallow dish, and dry at 105° for 1 hour: the residue weighs not more than 1 mg (0.01%).

Acidity—Add 2 drops of bromothymol blue TS to 10 mL of water in a glass-stoppered, 50-mL flask, and titrate with 0.010 N sodium hydroxide until a blue color persists after vigorous shaking. Add 5 mL of diisopropyl ether, and titrate with 0.010 N sodium hydroxide: not more than 0.30 mL is required to restore the blue color (0.005% as CH_3COOH).

[NOTE—For spectrophotometric determinations, use diisopropyl ether that meets the following additional requirement:]

Absorbance—Its absorbance at 255 nm, in a 10-mm quartz cell, does not exceed 0.2, water being used as the blank.

Diisopropylamine, $[(CH_3)_2CH]_2NH$ —**101.19** [108-18-9]—Colorless liquid.

Assay—Not less than 98% of $C_6H_{15}N$ is found, a suitable gas chromatograph equipped with a flame-ionization detector being used. The following conditions have been found suitable: a 3.2-mm \times 1.83-m stainless steel column is packed with a cross-linked polystyrene support; the injection port temperature is maintained at 250° and the detector

temperature at 310°; the column temperature is programmed to rise at 10° per minute from 50° to 220°.

Refractive index (831): between 1.3915 and 1.3935, at 20°.

Diisopropylethylamine (*N,N*-Diisopropylethylamine), $C_8H_{19}N$ —**129.24** [7087-68-5]—Clear, colorless liquid. Soluble in glacial acetic acid.

Assay—Accurately weigh about 500 mg, dissolve in 50 mL of glacial acetic acid, mix, add crystal violet TS, and titrate with 0.1 N perchloric acid VS. Each mL of 0.1 N perchloric acid is equivalent to 12.92 mg of $C_8H_{19}N$. Not less than 98% is found.

Refractive index (831): between 1.4125 and 1.4145 at 20°.

1,2-Dilinoleoyl-3-oleoyl-rac-glycerol, $C_{57}H_{100}O_6$ —**881.4** [2190-21-8]—Use a suitable grade.

1,2-Dilinoleoyl-3-palmitoyl-rac-glycerol, $C_{55}H_{98}O_6$ —**855.4** [2190-15-0]—Use a suitable grade.

Diluted Acetic Acid—See *Acetic Acid, Diluted*.

Diluted Alcohol—Use *Diluted Alcohol* (NF monograph).

Diluted Hydrochloric Acid—See *Hydrochloric Acid, Diluted*.

Diluted Nitric Acid—See *Nitric Acid, Diluted*.

Diluted Sulfuric Acid—See *Sulfuric Acid, Diluted*.

Dimethicone, viscosity 500 centistokes (*Poly(dimethylsiloxane), viscosity 500 centistokes*), $[-Si(CH_3)_2O-]_n$ [63148-62-9]—Use a suitable grade.

2,5-Dimethoxybenzaldehyde, $C_9H_{10}O_3$ —**166.17** [93-02-7]—Off-white crystals.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, nitrogen being used as the carrier gas. The following conditions have been found suitable: a 0.3-mm \times 30-m capillary column coated with phase G1; the injection port temperature is maintained at 270°; the detector temperature is maintained at 300°; the column temperature is maintained at 150° and programmed to rise 10° per minute to 270°. The area of the main peak is not less than 97% of the total peak area.

Melting range (741): between 50° and 52°.

1,2-Dimethoxyethane, $C_4H_{10}O_2$ —**90.12** [110-71-4]—Clear, colorless liquid. Miscible with water and with alcohol. Soluble in hydrocarbon solvents. May form peroxides on standing.

Boiling range (Reagent test)—Not less than 95% distills between 83° and 86°.

Refractive index (831): between 1.379 and 1.381, at 20°.

Acidity—To 20 mL add bromophenol blue TS, and titrate with 0.020 N sodium hydroxide: not more than 2.0 mL is consumed (about 0.015% as CH_3COOH).

Water, Method I (921): not more than 0.2%.

Dimethoxymethane (*Formaldehyde Dimethyl Acetal, Methylal*), $C_3H_8O_2$ —**76.10** [109-87-5]—Use a suitable grade.

(3,4-Dimethoxyphenyl)acetone (*Homoveratrol*), $C_{10}H_{11}NO_2$ —**177.20** [93-17-4]—Off-white fibers.

Melting range (741): between 65° and 67°.

Dimethyl Phthalate, $C_{10}H_{10}O_4$ —**194.19** [131-11-3]—Viscous, colorless liquid.

Assay—

MOBILE PHASE—Prepare a filtered and degassed mixture of chromatographic *n*-heptane and *n*-propyl alcohol (HPLC grade) (97:3). Make adjustments if necessary (see *System Suitability* under *Chromatography* (621)).

STANDARD SOLUTION—Dissolve a suitable quantity of dimethyl phthalate in *Mobile phase* to obtain a solution having a known concentration of about 0.26 mg per mL.

PROCEDURE—Inject about 20 μ L into a suitable liquid chromatograph (see *Chromatography* (621)) equipped with a 238-nm detector and a 4.6-mm \times 25-cm column that contains packing L10. The flow rate is about 1.0 mL per minute.

The area of the dimethyl phthalate peak is not less than 99% of the total peak area.

Refractive index (831): between 1.514 and 1.518 at 20°.

Dimethyl Sulfone (*Methyl Sulfone*), $(CH_3)_2SO_2$ —**94.13** [67-71-0]—White crystals.

Melting range (741): between 109° and 111°.

Dimethyl Sulfoxide (*Methyl Sulfoxide*), $(CH_3)_2SO$ —**78.13**—Use ACS reagent grade methyl sulfoxide.

Dimethyl Sulfoxide, Spectrophotometric Grade [67-68-5]—Use methyl sulfoxide ACS spectrophotometric reagent grade.

***N,N*-Dimethylacetamide**, C_4H_9NO —**87.12**

[127-19-5]—Clear, colorless liquid. Miscible with water and with many organic solvents. Use a suitable HPLC or spectroscopic grade.

***p*-Dimethylaminoazobenzene** (*Methyl Yellow, Butter Yellow*), $C_6H_5N:NC_6H_4N(CH_3)_2$ —**225.29** [60-11-7]—Yellow leaflets or yellow, crystalline powder.

Solubility—Insoluble in water; sparingly soluble in chloroform, in ether, or in fatty oils. Dissolve 100 mg in 20 mL of alcohol: the solution is complete or practically so and clear.

Melting range (741): between 115° and 117°.

Residue on ignition (281): not more than 0.1%.

Sensitiveness—Add 0.05 mL of an alcohol solution (1 in 200) and 2 g of ammonium chloride to 25 mL of carbon dioxide-free water: the lemon-yellow color of the solution is changed to orange by the addition of 0.05 mL of 0.1 N hydrochloric acid and restored on the subsequent addition of 0.05 mL of 0.1 N sodium hydroxide.

***p*-Dimethylaminobenzaldehyde**, $(CH_3)_2NC_6H_4CHO$ —**149.19** [100-10-7]—Use ACS reagent grade.

***p*-Dimethylaminocinnamaldehyde**, $(CH_3)_2NC_6H_4CH:CHCHO$ —**175.23**—Orange-yellow powder. Soluble in acetone, in alcohol, and in benzene.

Melting range (741): between 132° and 136°.

2-Dimethylaminoethyl Methacrylate, $C_8H_{15}NO_2$ —**157.2** [2867-47-2]—Use a suitable grade.

Dimethylaminophenol (*meta isomer*), $C_8H_{11}NO$ —**137.18**—Purplish-black, gray, or tan-colored, crystalline solid.

Melting range (741): between 83° and 85°.

2,6-Dimethylaniline, C_8H_9N —**121.18** [87-62-7]—Yellow liquid.

Refractive index (831): about 1.5609 at 20°.

***N,N*-Dimethylaniline**, C_8H_9N —**121.18** [121-69-7]—Light yellow liquid. Clear, colorless liquid when freshly distilled, but acquiring a reddish to reddish-brown color. Specific gravity: about 0.960. Freezing point about 2°. Insoluble in water; soluble in alcohol, in chloroform, in ether, and in dilute mineral acids.

Assay—Inject an appropriate specimen (about 0.2 μ L) into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas flowing at about 40 mL per minute. The following conditions have been found suitable: a 3-mm \times 1.8-m stainless steel column containing 20% phase G16 on support S1A; the injection port temperature is maintained at 250°; the column temperature is maintained at 50° and programmed to rise 10° per minute to 200°. The detector temperature is maintained at 310°. The area of the *N,N*-dimethylaniline peak having a retention time of about 11.5 minutes is not less than 99% of the total peak area.

Refractive index (831): between 1.5571 and 1.5591 at 20°.

Boiling range (Reagent test)—Distill 100 mL: the difference between the temperatures observed, when 1 mL and 95 mL have distilled, is not more than 2.5°. Its boiling temperature at a pressure of 760 mm of mercury is 194.2°.

Hydrocarbons—Dissolve 5 mL in a mixture of 10 mL of hydrochloric acid and 15 mL of water: a clear solution results and it remains clear on cooling to about 10°.

Aniline or monomethylaniline—Place 5 mL in a glass-stoppered flask, add 5 mL of a solution of acetic anhydride in benzene (1 in 10), mix, and allow to stand for 30 minutes. Add 30.0 mL of 0.5 N sodium hydroxide VS, shake the mixture, add phenolphthalein TS, and titrate with 0.5 N hydrochloric acid VS. Perform a blank determination, and make any necessary correction. Not more than 0.30 mL of 0.5 N sodium hydroxide is consumed by the test specimen.

3,4-Dimethylbenzophenone, $C_{15}H_{14}O$ —**210.27**

[2571-39-3]—White chunks melting at about 45°.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with phase G1; the detector temperature and the injection port temperature are maintained at 300°; the column temperature is maintained at 180° and programmed to rise at the rate of 10° per minute to 280° and held at that temperature for 10 minutes. The area of the main peak is not less than 99% of the total peak area.

5,5-Dimethyl-1,3-cyclohexanedione, $C_8H_{12}O_2$ —**140.18**

[126-81-8]—White, crystalline solid. Slightly soluble in water; soluble in alcohol, in methanol, in chloroform, and in acetic acid.

Melting range (741): between 148° and 150°.

N,N-Dimethyldodecylamine (1-(Dimethylamino)decane), $C_{14}H_{29}NO$ —**185.35** [1120-24-7]—Use a suitable grade with a content of not less than 98%.

1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide (Polybrene), [28728-55-4]—This is a positively charged polymer. It is available as an off-white, crystalline solid or powder and is extremely hygroscopic. Use a suitable reagent grade.

[NOTE—Commercially available as Polybrene.]

N,N-Dimethyldodecylamine-N-oxide (Lauryldimethylamine-N-oxide), $CH_3(CH_2)_{11}NO(CH_3)_2$ —**229.40**

[1643-20-5]—Use a suitable grade. [NOTE—N,N-Dimethyldodecylamine-N-oxide, 0.1 N in Water and N,N-Dimethyldodecylamine-N-oxide, 30 Percent are suitable grades as well.]

Dimethylethyl(3-hydroxyphenyl)ammonium Chloride—See *Edrophonium Chloride*.

Dimethylformamide (N,N-Dimethylformamide), $HCON(CH_3)_2$ —**73.09** [68-12-2]—Use ACS reagent grade.

N,N-Dimethylformamide Diethyl Acetal—**147.22** [1188-33-6]—Use a suitable grade.

[NOTE—A suitable grade is available from Sigma-Aldrich, www.sigma-aldrich.com.]

1,3-Dimethyl-2-imidazolidinone, $C_5H_{10}N_2O$ —**114.15** [80-73-9]—Use a suitable grade.

1,9-Dimethyl-methylene Blue, $C_{36}H_{46}Cl_4N_6OS_2Zn$ —**850.1** [23481-50-7]—Dark green powder. Use a suitable grade.

[NOTE—A suitable grade is available from Sigma-Aldrich, www.sigma-aldrich.com.]

N,N-Dimethyl-1-naphthylamine, $C_{12}H_{13}N$ —**171.24**

[86-56-6]—Pale yellow to yellow, aromatic liquid. Soluble in alcohol and in ether.

Assay—Transfer about 250 mg, accurately weighed, to a suitable beaker, add 100 mL of glacial acetic acid, and dissolve by stirring. When solution is complete, titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 17.12 mg of $C_{12}H_{13}N$. Not less than 98% is found.

Refractive index (831): between 1.6210° and 1.6230° at 20°, sodium light being used.

Sulfanilamide test—Dissolve 20 mg of USP Sulfanilamide RS in 100 mL of water to obtain the *Sulfanilamide solution*. Into two 150-mL beakers pipet 1.0 mL and 2.5 mL of the *Sulfanilamide solution*, respectively. Dilute with water to 90 mL. To provide a blank, place 90 mL of water in a third beaker. To each beaker add 8.0 mL of trichloroacetic acid solution (3 in 20) and 1.0 mL of sodium nitrite solution (1 in 1000). Stir the solutions for 5 minutes, then add 10 mL of acetate buffer TS, and 1.0 mL of a 1 in 1000 solution of N,N-dimethyl-1-naphthylamine in alcohol. The pH is about 5 to 6, using pH paper. Stir for an additional 5 minutes, then add 20 mL of glacial acetic acid. The pH is about 3 to 4, using pH paper. In comparison with the blank, the beaker containing 1.0 mL of the *Sulfanilamide solution* shows a pink color, while the other beaker shows a deep pink to red color.

N,N-Dimethyloctylamine, $C_{10}H_{23}N$ —**157.30**

[7378-99-6]—Colorless liquid.

Refractive index (831): 1.4243 at 20°.

2,5-Dimethylphenol, $C_8H_{10}O$ —**122.16** [95-87-4]—Use a suitable grade.

2,6-Dimethylphenol, $(CH_3)_2C_6H_3OH$ —**122.16** [576-26-1]—White to pale yellow, crystalline solid.

Assay—Inject a 1 in 3 solution of it in xylene into a suitable gas chromatograph equipped with a flame-ionization detector, helium being used as the carrier gas at a flow rate of about 40 mL per minute. The following conditions have been found suitable: a 3.2-mm \times 1.83-m stainless steel column packed with 10% phase G25 on support S1A; the injection port temperature is maintained at about 250° and the detector temperature at about 310°; the column temperature is programmed to rise at 8° per minute from 100° to 200°. Similarly inject a specimen of xylene. The area of the $C_8H_{10}O$ peak is not less than 98% of the total peak area corrected for xylene.

Melting range (741): between 44° and 46°.

3,5-Dimethylphenol, $C_8H_{10}O$ —**122.16** [108-68-9]—Use a suitable grade.

[NOTE—A suitable grade is available as product number 5600 from www.alfa.com.]

N,N-Dimethyl-p-phenylenediamine Dihydrochloride, $(CH_3)_2NC_6H_4NH_2 \cdot 2HCl$ —**209.12** [99-89-9]—Nearly white, fine, crystalline, hygroscopic solid that may have a pinkish cast. Freely soluble in water; soluble in alcohol.

Assay—Transfer about 400 mg, accurately weighed, to a 250-mL beaker, and dissolve in about 75 mL of water. Titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically. Each mL of 0.1 N sodium hydroxide is equivalent to 10.46 mg of $C_8H_{12}N_2 \cdot 2HCl$. Not less than 98% is found.

Solubility—A solution of 1 g in 10 mL of water produces not more than a slight haze.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide, $C_{18}H_{16}N_5SBr$ —**414.3** [298-93-1]—Yellow to orange powder. Use a suitable grade.

Dimethyltin Dibromide, $C_2H_6Br_2Sn$ —**308.59** [2767-47-7]—Use a suitable grade.

m-Dinitrobenzene, $C_6H_4(NO_2)_2$ —**168.11** [99-65-0]—Pale yellow crystals or crystalline powder. Almost insoluble in cold water; slightly soluble in hot water. Soluble in chloroform and in benzene; sparingly soluble in alcohol. Is volatile in steam.

Melting range (741): between 89° and 92°.

Residue on ignition (Reagent test): not more than 0.5%.

3,5-Dinitrobenzoyl Chloride, $C_7H_3ClN_2O_5$ —**230.56**

[99-33-2]—Pale yellow, crystalline powder. Freely soluble in dilute sodium hydroxide solutions; soluble in alcohol.

[CAUTION—Corrosive, moisture-sensitive, lachrymator, and possible mutagen. Store under nitrogen.]

Melting range (741): between 69° and 71°.

Solubility in sodium hydroxide—A solution of 500 mg in 25 mL of 1 N sodium hydroxide is clear or not more than faintly turbid.

Residue on ignition—Ignite 1 g with 0.5 mL of sulfuric acid: the residue weighs not more than 1 mg (0.1%).

2,4-Dinitrochlorobenzene, $C_6H_3(NO_2)_2Cl$ —**202.55** [97-00-7]—Yellow to brownish-yellow crystals. Insoluble in water; soluble in hot alcohol, in ether, and in benzene.

Melting range (741): between 51° and 53°.

Residue on ignition—Ignite 500 mg with 5 drops of sulfuric acid: the residue weighs not more than 1 mg (0.2%).

2,4-Dinitrofluorobenzene (1-Fluoro-2,4-dinitrobenzene), $C_6H_3FNO_2$ —**186.10** [70-34-8]—Light yellow solid. Use a suitable grade.

2,4-Dinitrophenylhydrazine, $2,4-C_6H_3(NO_2)_2NHNH_2$ —**198.14** [119-26-6]—Orange-red crystals, which under the microscope appear individually to be lemon-yellow, lath-like needles. Very slightly soluble in water; slightly soluble in alcohol; moderately soluble in dilute inorganic acids. Use a suitable grade with a content of not less than 97%.

Diocetyl Sodium Sulfosuccinate—Use *Docosate Sodium*.

Dioxane (Diethylene Dioxide; 1,4-Dioxane), $C_4H_8O_2$ —**88.11** [123-91-1]—Use ACS reagent grade.

Diphenyl Ether (Phenyl Ether), $(C_6H_5)_2O$ —**170.21** [101-84-8]—A colorless liquid. Insoluble in water; soluble in glacial acetic acid and in most organic solvents. Boils at about 259°.

Melting range (741): between 26° and 28°.

Diphenylamine, $(C_6H_5)_2NH$ —**169.22** [122-39-4]—Use ACS reagent grade.

Diphenylborinic Acid, Ethanolamine Ester, (2-Aminoethyl Diphenylborinate) $C_{14}H_{16}BNO$ —**225.09**—White, crystalline powder. Use a suitable grade.

Melting range (741): between 192° and 194°.

Diphenylcarbazine, $(C_6H_5NHNH)_2CO$ —**242.28** [140-22-7]—Use ACS reagent grade 1,5-Diphenylcarbohydrazide.

Diphenylcarbazon [Diphenylcarbazon compd. with *s*-Diphenylcarbazol (1:1)], $C_6H_5NHNHCON:NC_6H_5 \cdot C_6H_5NHNHCONHNHC_6H_5$ —**482.54** [538-62-5]—Use ACS reagent grade Diphenylcarbazon Compound with *s*-Diphenylcarbazide (1:1).

2,2-Diphenylglycine, $C_{14}H_{13}NO_2$ —**227.26** [3060-50-2]—Off-white powder. Melts at about 244°, with decomposition.

Assay—Dissolve about 115 mg, accurately weighed, in 30 mL of methanol. Slowly add about 20 mL of water, heating slightly if necessary for complete solution. Titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination and make any necessary correction. Each mL of 0.1 N sodium hydroxide is equivalent to 22.73 mg of $C_{14}H_{13}NO_2$. Not less than 98.0% is found.

Dipicrylamine—See *Hexanitrodiphenylamine*.

Dipropyl Phthalate, $C_{14}H_{18}O_4$ —**250.29** [131-16-8]—Viscous, colorless liquid.

Assay—

MOBILE PHASE—Prepare a mixture of acetonitrile and water (52:48).

PROCEDURE—Inject about 20 μ L into a suitable liquid chromatograph (see *Chromatography* (621)) equipped with a 230-nm detector and a 4.6-mm \times 15-cm column that contains packing L1. The flow rate is about 2.0 mL per minute. The area of the $C_{14}H_{18}O_4$ peak is not less than 99% of the total peak area.

Refractive index (831): between 1.495 and 1.499 at 20°.

4,4'-Dipyridyl, $C_{10}H_8N_2$ —**156.18** [553-26-4]—Use a suitable grade. [NOTE—A suitable grade is available from Sigma-Aldrich Catalog number 289426.]

α, α' -Dipyridyl—See 2,2'-Bipyridine.

Disodium Chromotropate (4,5-Dihydroxy-2,7-naphthalenedisulfonic Acid, Disodium Salt), $C_{10}H_6O_8S_2Na_2 \cdot 2H_2O$ —**400.29**—Use ACS reagent grade Chromotropic Acid Disodium Salt.

Disodium Ethylenediaminetetraacetate—See *Edetate Disodium*.

Disodium Phosphate—See *Sodium Phosphate*.

5,5'-Dithiobis(2-nitrobenzoic Acid) (3-Carboxy-4-nitrophenyl disulfide; Ellman's reagent), $C_{14}H_8N_2O_8S_2$ —**396.35** [69-78-3]—Yellow powder, melting at about 242°. Sparingly soluble in alcohol.

Dithiothreitol (Cleland's Reagent; Threo-1,4-dimercapto-2,3-butanediol; DTT), $HSCH_2CH(OH)CH(OH)CH_2SH$ —**154.25** [3483-12-3]—Slightly hygroscopic needles from ether. Freely soluble in water, in alcohol, in acetone, in ethyl acetate, and in ether.

Melting range (741): between 42° and 44°.

Dithizone (Diphenylthiocarbazone; Phenylazothioformic Acid 2-Phenylhydrazide), $C_6H_5N:NCSNHNHC_6H_5$ —**256.33** [60-10-6]—Use ACS reagent grade.

1-Dodecanol (Dodecyl Alcohol), $CH_3(CH_2)_{11}OH$ —**186.33** [112-53-8]—A clear, colorless liquid. Crystallizes as leaflets from dilute alcohol solution. Use ACS reagent grade.

Dodecyl Alcohol—See 1-Dodecanol.

Dodecyl Lithium Sulfate (Lithium Dodecyl Sulfate, Lithium Lauryl Sulfate), $C_{12}H_{25}LiO_4S$ —**272.3** [2044-56-6]—White to off-white powder, clear to slightly hazy, colorless to faint yellow solution in water at 50 mg per mL at ambient temperature. The UV absorbance of a 0.1 M solution is less than 0.05 at both 260 and 280 nm. The pH of a 0.1 M solution in water is 7.0 ± 0.5 . Use a suitable grade.

Dodecyl Sodium Sulfonate (Sodium 1-Dodecanesulfonate; 1-Dodecanesulfonic Acid, Sodium Salt), $C_{12}H_{25}SO_3Na$ —**272.38** [2386-53-0]—White, powdery solid. One g dissolves in 50 mL of warm water to yield a clear, colorless solution. Use a suitable grade.

Melting point (741): higher than 300°.

3-(Dodecyltrimethylammonio)propanesulfonate (Lauryl sulfobetaine, *N,N*-dimethyl-*N*-dodecyl-*N*-(3-sulfopropyl) ammonium betaine), $C_{17}H_{37}NO_3S$ —**335.54** [14933-08-5]—Use a suitable grade.

Dodecyltriethylammonium Phosphate, 0.5 M, $[C_{12}H_{25}N \cdot (C_2H_5)_3]_3PO_4$ —**906.52**—Use a suitable grade.

[NOTE—A suitable grade is available as Ion Pair Cocktail Q12 (catalogue number 404031) from Regis Technologies, Inc., www.registech.com.]

Dodecyltrimethylammonium Bromide (Lauryltrimethylammonium bromide), $CH_3(CH_2)_{11}N(CH_3)_3Br$ —**308.3** [1119-94-4]—Use a suitable grade.

[NOTE—A suitable grade is available as catalog number D5047 from Sigma-Aldrich, www.sigma-aldrich.com.]

Drabkin's Reagent—The reagent consists of 100 parts of sodium bicarbonate, 20 parts of potassium ferricyanide, and 5 parts of potassium cyanide. [CAUTION—The reagent is HIGHLY TOXIC. Very toxic by inhalation, in contact with skin, and if swallowed, and there is a risk of serious damage to eyes. Wear suitable protective clothing, gloves, and eye and face protection. Do not mix with acids. Contact with acids liberates a very toxic gas. If ingested, perform gastric lavage, and call a physician.]

[NOTE—The reagent can be obtained from many manufacturers and suppliers. Some examples of manufacturers or suppliers are the following: Sigma Chemical Co., St. Louis, MO; and CIMA Scientific, Dallas, TX.]

Dried Peptone—See *Peptone, Dried*.

Earth, Chromatographic, Silanized, Acid-Base Washed—Use a suitable grade.

[NOTE—A suitable chromatographic grade is "Gas-Chrom Q," available from Alltech, www.alltechweb.com.]

Edetate Disodium (Disodium Ethylenediaminetetraacetate) $C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$ —**372.24**—Use ACS reagent grade (Ethylenedinitrilo)tetraacetic Acid Disodium Salt Dihydrate.

n-Eicosane, $C_{20}H_{42}$ —**282.55** [112-95-8]—White, crystalline solid.

Melting range (741): between 37° and 39°.

Eicosanol [629-96-9]—Use a suitable grade.

Eosin Y (Eosin Yellowish Y) (Certified Biological Eosin Y; Sodium Tetrabromofluorescein), $C_{20}H_6Br_4Na_2O_5$ —**691.85** [17372-87-1]—Red to brownish-red pieces or powder. Use ACS reagent grade.

Epiandrosterone (*trans*-Androsterone, 5α -Androstan-3 β -ol-17-one), $C_{19}H_{30}O_2$ —**290.44** [481-29-8]—Use a suitable grade.

[NOTE—A suitable grade is available as catalog number E3375 from www.sigma-aldrich.com.]

Equilenin, $C_{18}H_{18}O_2$ —**266.33** [517-09-9]—Colorless or white crystals or crystalline powder. Insoluble in water; soluble in chloroform and in dioxane; moderately soluble in alcohol.

Melting range, Class II (741): between 256° and 260°.

Specific rotation (781): between +85° and +88°, determined in a solution in dioxane containing 75 mg of equilenin in each 10 mL.

Absorption maxima—An alcohol solution exhibits absorption maxima at 231, 282, 325, and 340 nm.

α -Ergocryptine, $C_{32}H_{41}N_5O_5$ —**575.70** [511-09-1]—Use a suitable grade.

Eriochrome Black T-Sodium Chloride Indicator—Mix 0.1 g of eriochrome black T and 10 g of sodium chloride, and triturate until the mixture becomes homogenous.

Eriochrome Cyanine R, $C_{23}H_{15}Na_3O_9S$ —**536.40** [3564-18-9]—Dark, red-brown powder. Freely soluble in water; insoluble in alcohol.

Solubility—200 mg in 100 mL of water yields a solution that remains clear and free from undissolved matter for 30 minutes.

Loss on drying (731)—Dry it in vacuum over silica gel to constant weight: it loses not more than 2% of its weight.

Residue on ignition (Reagent test)—0.5 g, treated with 1 mL of sulfuric acid and 2 mL of nitric acid, yields between 42.0% and 44.0% of the dry weight (theoretical yield is 42.9% of Na_2SO_4).

Sensitiveness—Add 2 mL of a solution (1 in 1000) to 1 mL of aluminum sulfate solution (1 in 10,000), heat at $37 \pm 3^\circ$ for 5 minutes, cool, and add 1 mL of sodium acetate TS: a strong red to red-violet color is produced in not more than 5 minutes.

Escin [6805-41-0]—Use a suitable grade.

Ethanesulfonic Acid, $C_2H_5SO_3H$ —**110.13** [594-45-6]—Colorless to light yellow liquid. Soluble in water.

Assay—Accurately weigh about 300 mg, dissolve in 30 mL of water, add phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS. Each mL of 0.1 N sodium hydroxide is equivalent to 11.013 mg of $C_2H_5SO_3H$: between 94.0% and 106.0% is found.

Refractive index (831): between 1.432 and 1.436 at 20°.

Ether—See *Ethyl Ether*.

Ether, Absolute—See *Ethyl Ether, Anhydrous*.

Ether, Diphenyl—See *Diphenyl Ether*.

Ether, Isopropyl—See *Diisopropyl Ether*.

Ether, Nonyl Phenyl Polyethylene Glycol—See (*p*-tert-Octylphenoxy)nonaethoxyethanol.

Ether, Peroxide-Free (*Diethyl Ether; Ether*), $(C_2H_5)_2O$ —**74.12**—Use ACS reagent grade.

Peroxide—Transfer 8 mL of potassium iodide and starch TS to a 12-mL ground glass-stoppered cylinder about 15 mm in diameter. Fill completely with the substance under test, mix, and allow to stand protected from light for 5 minutes. No color develops. Alternatively, peroxide test strips may be used.

[NOTE—Suitable peroxide test strips can be obtained from EMD Chemicals, www.emdchemicals.com, or from J. T. Baker, www.mallbaker.com.]

Ethidium Bromide, $C_{21}H_{20}N_3Br$ —**394.3** [1239-45-8]—Purple to purple-red powder. Use a suitable grade.

4'-Ethoxyacetophenone, $C_{10}H_{12}O_2$ —**164.20** [1676-63-7]—White to tan crystals.

Assay—Dissolve about 50 mg in 1 mL of ether. Inject about 1 μ L of this solution into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector and a 0.25-mm \times 30-m capillary column containing stationary phase G1. The carrier gas is helium. The chromatograph is programmed as follows. Initially, the column temperature is equilibrated at 180°, then the temperature is increased at a rate of 10° per minute to 280°, and maintained at 280° for 10 minutes. The injection port temperature is maintained at 280°, and the detector is maintained at 300°. The area of the 4'-ethoxyacetophenone peak is not less than 97.5% of the total peak area.

Melting range (741): between 34° and 39°.

2-Ethoxyethanol (*Ethylene Glycol Monoethyl Ether*), $C_4H_{10}O_2$ —**90.12** [110-80-5]—Clear, colorless liquid. Miscible with water, with alcohol, with ether, and with acetone.

Specific gravity (841): about 0.93.

Boiling range (Reagent test)—Not less than 95% distills between 133° and 135°.

Ethyl Acetate, $CH_3COOC_2H_5$ —**88.11** [141-78-6]—Use ACS reagent grade.

Ethyl Acrylate [140-88-5]—Use a suitable grade.

Ethyl Alcohol (*Alcohol; Ethanol*), C_2H_5OH —**46.07**—Use *Alcohol*.

Ethyl Arachidate, $C_{22}H_{44}O_2$ —**340.6** [18281-05-5]—Use a suitable grade.

Ethyl Benzoate, $C_9H_{10}O_2$ —**150.17** [93-89-0]—Clear, colorless liquid. Practically insoluble in water; miscible with alcohol, with chloroform, and with ether.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)), helium being used as the carrier gas. The following conditions have been found suitable: a 3-mm \times 2.4-m stainless steel column containing 20% phase G16 on support S1A; the injection port, column, and detector temperatures are maintained at 180°, 195°, and 250°, respectively. The area of the ethyl benzoate peak is not less than 98% of the total peak area.

Refractive index (831): between 1.5048 and 1.5058 at 20°.

Ethyl Cyanoacetate, $CNCH_2COOC_2H_5$ —**113.11**

[105-56-6]—Colorless to pale yellow liquid. Slightly soluble in water. Miscible with alcohol and with ether. At atmospheric pressure it boils between 205° and 209°, with decomposition. At a pressure of 10 mm of mercury it distills at about 90°.

Specific gravity (841): between 1.057 and 1.062.

Acidity—Dissolve 2 mL in 25 mL of neutralized alcohol, add phenolphthalein TS, and titrate with 0.10 N sodium hydroxide: not more than 1.5 mL is required to produce a pink color.

Ethyl Ether (*Diethyl Ether; Ether*), $(C_2H_5)_2O$ —**74.12** [60-29-7]—Use ACS reagent grade.

Ethyl Ether, Anhydrous (*Diethyl Ether, Anhydrous; Ether, Absolute*), $(C_2H_5)_2O$ —**74.12** [60-29-7]—Use ACS reagent grade.

Ethyl Salicylate, $C_9H_{10}O_3$ —**166.17** [118-61-6]—Colorless liquid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 10-m capillary column coated with a 1- μ m layer of methylsilicone; the injection port temperature is maintained at 240°; the detector temperature is maintained at 300°; the column temperature is maintained at 150° and programmed to rise 10° per minute to 250°. The area of

the ethyl salicylate peak is not less than 99% of the total peak area.

Refractive index (831): between 1.5216 and 1.5236 at 20°.

2-Ethylaminopropiophenone Hydrochloride, $C_6H_5COCH(CH_3)NHC_2H_5 \cdot HCl$ —**213.70** [51553-17-4]—Use a suitable grade.

4-Ethylbenzaldehyde, $C_2H_5C_6H_4CHO$ —**134.18** [4748-78-1]—Colorless to pale yellow liquid.

Assay—Dissolve about 600 mg, accurately weighed, in a mixture of 100 mL of alcohol and 25 mL of 1 M hydroxylamine hydrochloride in a beaker. Cover the beaker with a watch glass. Heat gently until condensate begins to form on the watch glass. Allow to cool for about 30 minutes. Titrate with 0.5 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.5 N sodium hydroxide is equivalent to 67.09 mg of $C_2H_5C_6H_4CHO$. Not less than 98% is found.

Ethylbenzene, C_8H_{10} —**106.17** [100-41-4]—Not less than 99.5%.

Ethylene Dichloride (1,2-Dichloroethane), $C_2H_4Cl_2$ —**98.96** [107-06-2]—Use ACS reagent grade 1,2-Dichloroethane.

Ethylene Glycol, $HOCH_2CH_2OH$ —**62.07** [107-21-1]—Clear, colorless, slightly viscous, hygroscopic liquid. Slightly soluble in ether; practically insoluble in benzene. Miscible with water and with alcohol.

Specific gravity (841): about 1.11.

Boiling range (Reagent test): between 194° and 200°.

Residue on ignition—Evaporate 100 mL (110 g) in a tared evaporating dish over a flame until the vapors continue to burn after the flame is removed. Allow the vapors to burn until the specimen is consumed. Ignite at $800 \pm 25^\circ$ for 1 hour, cool, and weigh: the residue weighs not more than 5.5 mg (0.005%).

Acidity—Add 0.2 mL of phenol red TS to 50 mL of water, and titrate with 0.1 N sodium hydroxide to a red endpoint. Add 50 mL (55 g) of ethylene glycol, and titrate with 0.1 N sodium hydroxide: not more than 1 mL is required to restore the red color (0.01% as CH_3COOH).

Chloride (Reagent test)—A 4.5-mL (5-g) portion shows not more than 0.025 mg of Cl (5 ppm).

Water, Method I (921): not more than 0.20%.

Ethylene Glycol Monoethyl Ether—See 2-Ethoxyethanol.

Ethylene Oxide in Methylene Chloride (50 mg/mL)—Use a suitable grade.

[NOTE—A suitable grade is available from Sigma Aldrich Corporation, www.sigma-aldrich.com.]

Ethylenediamine (1,2-Diaminoethane) $C_2H_8N_2$ —**60.10** [107-15-3]—Use a suitable grade with a content of not less than 99%.

N-Ethylmaleimide (1-Ethyl-1H-pyrrole 2,5-Dione), $C_6H_7NO_2$ —**125.12** [128-53-0]—Use a suitable grade.

Melting range (741): between 43° and 47°.

2-Ethyl-2-methylsuccinic Acid, $C_7H_{12}O_4$ —**160.17**—White crystals.

Assay—Transfer about 80 mg, accurately weighed, to a suitable beaker, add 30 mL of methanol, and dissolve by stirring. Slowly add 40 mL of deionized water. [NOTE—If cloudiness appears when adding the deionized water, dissolve in 50 mL of methanol only.] When solution is complete, titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank titration, and make any necessary correction. Each mL of 0.1 N sodium hydroxide is equivalent to 16.02 mg of $C_7H_{12}O_4$. Not less than 98.5% of $C_7H_{12}O_4$ is found.

Melting point (741): between 101° and 103°.

1-Ethylquinadine Iodide, $C_{12}H_{14}IN$ —**299.15** [606-55-3]—Yellow-green solid. Sparingly soluble in water.

Assay—Dissolve about 290 mg, accurately weighed, in 100 mL of water, and add 10 mL of glacial acetic acid. Titrate with 0.1 N silver nitrate VS, determining the endpoint

potentiometrically, using a silver-ion selective electrode and a calomel reference electrode containing 1 M potassium nitrate. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N silver nitrate is equivalent to 29.92 mg of $C_{12}H_{14}IN$: not less than 97.0% is found.

Factor X_a (Activated Factor X) for Anti-Factor X_a Test—Factor X_a is the proteolytic enzyme derived from bovine plasma, and it cleaves prothrombin to form thrombin. It is a glycoprotein having a molecular weight of 40,000. One Factor X_a Unit is the amount of activated endogenous Factor X (55,000 Da when nonactivated) that is contained in 1 mL of normal plasma. Russell's viper venom is used to activate the enzyme, and is then removed. The preparation is stabilized and lyophilized.

Factor X_a is free of thrombin at the concentration used in the test. When tested against pure fibrinogen, no clotting takes place within 24 hours. It has not less than 40 Factor X_a Units per mg of protein, and exhibits 90% homogeneity when tested by disk electrophoresis.

One Factor X_a Unit per mL gives a 15-second clotting time when tested in the following manner. Mix 0.1 mL of a saturated solution of cephalin (derived from rabbit brain-acetone powder or from an equivalent amount of rabbit brain thromboplastin) in 0.1 mL of citrated bovine plasma and 0.1 mL of 0.025 M calcium chloride. Immediately add 0.1 mL of a solution of factor X_a (1 in 10), and incubate at 37°.

Fast Blue B Salt, $C_{14}H_{12}N_4O_2 \cdot ZnCl_4$ —**475.47** [91-91-8]—Green powder.

Loss on drying (731)—Dry it in vacuum at 110° for 1 hour: it loses not more than 5.0% of its weight.

Absorbance—Dissolve 50 mg in 100 mL of water. In a second container dissolve 100 mg of 2-naphthol in 100 mL of 2-methoxyethanol. Pipet 5 mL of the test solution and 10 mL of the 2-naphthol solution into a 100-mL volumetric flask, and dilute with acetone to volume. For the blank, pipet 5 mL of water and 10 mL of 2-naphthol solution into a second 100-mL volumetric flask, and dilute with acetone to volume. Determine the absorbance of the test solution in a 1-cm cell at the wavelength of maximum absorbance at about 545 nm, with a suitable spectrophotometer, using the blank to set the instrument: the absorbance is not less than 0.80.

Fast Blue BB Salt, $(C_{17}H_{18}ClN_3O_3)_2 \cdot ZnCl_2$ —**831.89** [15710-69-7]—Yellow powder melting at about 162°, with decomposition. Sparingly soluble in water.

Chloride—Transfer about 80 mg, accurately weighed, to a suitable beaker. Add 25 mL of acetone, 25 mL of water, and 500 mg of sodium nitrate. Stir until solution is complete. Titrate with 0.01 N silver nitrate VS, determining the endpoint potentiometrically. Perform a blank determination and make any necessary correction. Not less than 15.0% of chloride is found.

Fast Green FCF, $C_{37}H_{34}N_2Na_2O_{10}S_3$ —**808.86** [2353-45-9]—Red to brown-violet powder or crystals. Soluble in water; sparingly soluble in alcohol. Use a suitable grade.

FD&C Blue No. 1 (Brilliant Blue), $C_{37}H_{34}N_2O_9S_3Na_2$ —**792.86** [3844-45-9]—Use a suitable grade.

Ferric Ammonium Citrate—Thin, transparent, garnet-red scales or granules or brownish-yellow powder. Is deliquescent and is affected by light. Very soluble in water; insoluble in alcohol.

Assay—Accurately weigh about 1 g, dissolve in 25 mL of water in a glass-stoppered flask, add 5 mL of hydrochloric acid and 4 g of potassium iodide, insert the stopper in the flask, and allow to stand in the dark for 15 minutes. Add 100 mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 5.585 mg of Fe: between 16.5% and 18.5% is found.

Ferric citrate—To 250 mg dissolved in 25 mL of water add 1 mL of potassium ferrocyanide TS: no blue precipitate is formed.

Tartrate—Dissolve 1 g in 10 mL of water, add 1 mL of potassium hydroxide TS, boil to coagulate the ferric hydroxide, adding more potassium hydroxide TS, if necessary, to precipitate all of the iron, filter, and slightly acidify the filtrate with glacial acetic acid. Add 2 mL of glacial acetic acid, and allow to stand for 24 hours: no crystalline white precipitate is formed.

Lead (251)—Dissolve 1.0 g in 30 mL of water, add 5 mL of dilute nitric acid (1 in 21), boil gently for 5 minutes, cool, and dilute with water to 50 mL: 20 mL of the solution shows not more than 0.008 mg of Pb (0.002%).

Ferric Ammonium Sulfate, $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ —**482.19**—Use ACS reagent grade.

Ferric Chloride, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ —**270.29** [10025-77-1]—Use ACS reagent grade.

Ferric Nitrate, $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ —**404.00** [10421-48-4]—Use ACS reagent grade.

Ferric Sulfate, $\text{Fe}_2(\text{SO}_4)_3 \cdot x\text{H}_2\text{O}$ [10028-22-5]—Grayish-white, hygroscopic powder, or fawn-colored pearls, slowly soluble in water.

Assay—Accurately weigh about 700 mg, and dissolve it in a mixture of 50 mL of water and 3 mL of hydrochloric acid in a glass-stoppered flask. Add 3 g of potassium iodide, and allow to stand in the dark for 30 minutes. Then dilute with 100 mL of water, and titrate with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. Each mL of 0.1 N sodium thiosulfate is equivalent to 5.585 mg of Fe: not less than 21.0% and not more than 23.0% is found.

Insoluble matter (Reagent test)—A 10-g portion, dissolved in a mixture of 100 mL of water and 5 mL of sulfuric acid, shows not more than 2 mg of insoluble matter (0.02%).

Chloride—Dissolve 1 g by warming with a mixture of 10 mL of water and 1 mL of nitric acid, add 4 mL of additional nitric acid, and dilute with water to 50 mL. To 25 mL add 1 mL of phosphoric acid and 1 mL of silver nitrate TS. Any turbidity does not exceed that produced in a control containing 0.01 mg of chloride ion (Cl), 1 mL of nitric acid, 1 mL of phosphoric acid, and 1 mL of silver nitrate TS (0.002%).

Ferrous iron—Dissolve 4 g by warming with 50 mL of dilute sulfuric acid (1 in 10), cool, and titrate with 0.1 N potassium permanganate: not more than 0.16 mL is required to produce a permanent pink color (0.02% as Fe+2). [NOTE—Because the reagents used in the tests for *Copper* and *Zinc* may contain excessive amounts of copper and zinc, they should first be purified by extracting with *Dithizone Extraction Solution* (see *Lead (251)*).]

Copper—Dissolve 1.2 g in 100 mL of water. To 10 mL add 50 mL of a solution containing 5 g of ammonium tartrate and 5 mL of ammonium hydroxide. Add 10 mL of *Standard Dithizone Solution* (see *Lead (251)*), shake for 2 minutes, draw off the dithizone layer, and compare the pink color with that in a control containing 6 µg of copper ion (Cu) and treated exactly as the 10-mL portion of test solution. If the color in the test solution is less than that in the control, then the test specimen contains less than the limit of both *Copper* and *Zinc*. If the color in the test solution is more than that in the control, add 15 mL of dilute hydrochloric acid (1 in 250), and shake for 2 minutes. Draw off the dithizone solution, and shake with a second 15 mL of dilute hydrochloric acid (1 in 250) for 2 minutes. Draw off the dithizone, combine the two acid extracts, and reserve for the *Zinc* test. Any pink color in the dithizone solution is not darker than that in the control solution treated exactly as the test solution (0.005%).

Zinc—To the combined acid extracts saved from the *Copper* test, add 0.5 M sodium acetate to bring the pH between 5.0 and 5.5, and then add 1 mL of 0.1 N sodium thiosulfate. Add 10 mL of *Standard Dithizone Solution* (see *Lead (251)*), shake for 2 minutes, and allow the layers to separate. Draw off the dithizone, and discard the water layer. Any pink color is not greater than that in a control prepared by adding 0.006 mg of zinc ion (Zn) to the combined acid extracts from the control used in the test for *Copper* (0.005%).

Nitrate—Dissolve 10 g in 100 mL of dilute sulfuric acid (1 in 100), heat to boiling, and pour, slowly, into a mixture of 140 mL of water and 50 mL of stronger ammonia TS. Filter through a folded filter while still hot, wash with hot water until the volume of the filtrate is 300 mL, mix, and cool. To 15 mL of this solution add 1 mL of sodium chloride solution (1 in 200), 0.10 mL of indigo carmine TS, and 15 mL of sulfuric acid. The blue color is not completely discharged at the end of 5 minutes (0.01%).

Substances not precipitated by ammonia—Evaporate to dryness 30 mL of the filtrate obtained in the test for *Nitrate*, and ignite gently: the weight of residue does not exceed 1 mg (0.10%).

Ferrocypen (*Dicyano-bis (1,10-phenantroline) iron (II) Complex*), $(\text{C}_{12}\text{H}_8\text{N}_2)_2\text{Fe}(\text{CN})_2$ —**468.3**—Brown to black powder. Dark red solution at 10 mg per mL in glacial acetic acid. Soluble in chloroform and in water at 5 mg per mL, yielding a clear purple solution in chloroform and a clear orange solution in water.

Water, Method I (921): not more than 10%.

Ferrous Ammonium Sulfate, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ —**392.14**—Use ACS reagent grade.

Ferrous Sulfate, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ —**278.02** [7782-63-0]—Use ACS reagent grade.

Ferulic Acid (4-Hydroxy-3-methoxycinnamic acid), $\text{C}_{10}\text{H}_{10}\text{O}_4$ —**194.19** [1135-24-6]—Use a suitable grade.

Fibroblast Growth Factor-2—Use a suitable grade.

[NOTE—A suitable grade can be obtained from Roche Diagnostics Corporation, www.rosche-diagnostics.com.]

Filter Paper, Quantitative—For the *Mercuric Bromide Test Paper* used in testing for arsenic, use Swedish O filter paper or other makes of like surface, quality, and ash.

Fluorene, $\text{C}_{13}\text{H}_{10}$ —**166.22** [86-73-7]—White to off-white crystals or powder. Soluble in benzene, in carbon disulfide, in ether, and in hot alcohol; freely soluble in glacial acetic acid.

Solubility test—One g dissolves in 10 mL of acetone to yield a clear and complete solution.

Melting range (741): between 113° and 117°, within a 2° range.

9-Fluorenylmethyl Chloroformate, $\text{C}_{15}\text{H}_{11}\text{ClO}_2$ —**258.70** [28920-43-6]—Clear, colorless solid. Melts at about 62°.

Fluorescamine, $\text{C}_{17}\text{H}_{10}\text{O}_4$ —**278.26** [38183-12-9]—White to off-white powder. Very slightly soluble in water; freely soluble in methylene chloride; soluble in alcohol; slightly soluble in chloroform.

Assay—Dissolve about 600 mg in 75 mL of dimethylformamide, and titrate with 0.1 N lithium methoxide to a blue endpoint, using 1% thymol blue in dimethylformamide as the indicator. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N lithium methoxide is equivalent to 27.83 mg of $\text{C}_{17}\text{H}_{10}\text{O}_4$. Not less than 99% is found.

Loss on drying (731)—Dry it at 105° for 4 hours: it loses not more than 0.5% of its weight.

4'-Fluoroacetophenone, $\text{FC}_6\text{H}_4\text{COCH}_3$ —**138.14** [403-42-9]—Colorless liquid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 200°; the detector temperature is maintained at 250°; the column temperature is maintained at 100° and programmed to rise 10° per minute to 250°. The area of the $\text{C}_6\text{H}_4\text{COCH}_3$ peak is not less than 99% of the total peak area.

Refractive index (831): 1.510 at 20°.

Formaldehyde Solution, HCHO —(30.03) and water—Use ACS reagent grade.

Formamide, HCONH_2 —45.04 [75-12-7]—Use ACS reagent grade.

Preparation for Digitoxin Assay—To ensure freedom from ammonia, treat Formamide as follows. Shake a suitable quantity of formamide with about 10% of its weight of anhydrous potassium carbonate for 15 minutes, and filter. Distill the filtrate in an all-glass apparatus under vacuum at a pressure of about 25 mm of mercury or less. Reject the first portion of distillate containing water, and collect the fraction that boils at about 115° at a pressure of 25 mm of mercury or at 101° at a pressure of 12 mm of mercury. Store in tight containers, protected from light.

Formamide, Anhydrous, HCONH_2 —45.04 [75-12-7]—Use formamide that has a water content of less than 0.1%.

Formic Acid, HCOOH —46.03 [64-18-6]—Use ACS reagent grade Formic Acid, 88 Percent.

Formic Acid, 96 Percent, HCOOH —46.03 [64-18-6]—Use ACS reagent grade Formic Acid, 96 Percent.

Formic Acid, Anhydrous—Use ACS reagent grade Formic Acid, 96 Percent.

Fuchsin, Basic (*Basic Red 9*, *Parafuchsin Hydrochloride*), $\text{C}_{19}\text{H}_{17}\text{N}_3$ —323.82 [569-61-9]—Use a suitable grade.

Fuller's Earth, Chromatographic—(*Very Fine and Moderately Coarse*)—Gray or grayish-white powder or granules consisting mainly of hydrous aluminum-magnesium silicate.

Powder fineness—See *Powder Fineness* (811).

Soluble matter—Twenty g, treated with 50 mL of cold water and filtered, yields not more than 60 mg of residue upon evaporation of the filtrate (0.3%). A second 20-g portion, treated with 50 mL of cold alcohol and filtered, yields not more than 14 mg upon evaporation of the filtrate (0.07%).

Loss on drying (731)—Dry it at 105° for 6 hours: it loses between 7.0% and 10.0% of its weight.

[NOTE—Adjust the water content, if necessary, by drying in vacuum at room temperature, restoring the water required, and equilibrating by shaking for 2 hours.]

Fuming Nitric Acid—See *Nitric Acid, Fuming*.

Fuming Sulfuric Acid—See *Sulfuric Acid, Fuming*.

Furfural (*2-Furancarboxyaldehyde*; *2-Furaldehyde*), $\text{C}_4\text{H}_3\text{OCHO}$ —96.08 [98-01-1]—Use ACS reagent grade.

G Designations—See phases for gas chromatography under *Reagents, Chromatographic Columns*.

Gadolinium (Gd III) Acetate Hydrate, $(\text{CH}_3\text{CO}_2)_3\text{Gd} \cdot x\text{H}_2\text{O}$ —334.38 [100587-93-7]—White, crystalline, hygroscopic powder. Irritant. Use a suitable grade.

Geneticin (G418; *O*-2-Amino-2,7-dideoxy-*D*-glycero- α -*D*-glucoheptopyranosyl-(1-4)-*O*-(3-deoxy-4-*C*-methyl-3-(methylamino)- β -*D*-arabinopyranosyl-(1-6)-*D*-streptamine), $\text{C}_{20}\text{H}_{40}\text{N}_4\text{O}_{10}$ —496.55 [49863-47-0]—Use a suitable grade, cell culture tested.

Girard Reagent T—See *Trimethylacetylhydrazide Ammonium Chloride*.

Gitoxin, $\text{C}_{41}\text{H}_{64}\text{O}_{14}$ —780.94 [4562-36-1]—White, crystalline powder. Practically insoluble in water, in chloroform, and in ether; slightly soluble in pyridine and in diluted alcohol. Melts at about 250°, with decomposition.

Specific rotation (781): between +3.8° and +4.8°, determined in a solution of pyridine containing 10 mg per mL, with the use of a mercury light at 546.1 nm; between +21° and +25°, determined in a solution of equal parts of chloroform and methanol containing 5 mg per mL, with the use of sodium light.

Suitability—Dissolve 10 mg each of USP Digitoxin RS, previously dried, USP Digoxin RS, previously dried, and gitoxin, respectively, in separate 5-mL portions of a mixture of 2 parts of chloroform and 1 part of methanol, and dilute each with additional solvent mixture to 10 mL. Then proceed as directed in the *Identification test* under *Digoxin*. The chromatogram of gitoxin shows one fluorescent spot, located between the digoxin and digitoxin spots.

Glacial Acetic Acid—See *Acetic Acid, Glacial*.

Glass Wool—Fine threads of glass.

Acid-soluble substances—Boil 1 g for 30 minutes with 30 mL of diluted hydrochloric acid, and filter. Evaporate the filtrate, and dry the residue at 105° to constant weight: the residue weighs not more than 5 mg (0.5%).

Heavy metals—Boil 2 g with a mixture of 25 mL each of diluted nitric acid and water for 5 minutes, and filter. Evaporate one-half of the filtrate to dryness, dissolve the residue in 10 mL of water to which 3 drops of hydrochloric acid have been added, filter if necessary, and add an equal volume of hydrogen sulfide TS to the filtrate: no darkening is produced.

D-Gluconic Acid, 50 Percent in Water, $\text{C}_6\text{H}_{12}\text{O}_7$ —196.16 [526-95-4]—Pale yellow liquid.

Assay—Dilute about 200 mg of the solution, accurately weighed, with 30 mL of water. Titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination and make any necessary correction. Each mL of 0.1 N sodium hydroxide is equivalent to 19.62 mg of $\text{C}_6\text{H}_{12}\text{O}_7$. Not less than 49.0% is found.

Refractive index (831): between 1.4160 and 1.4180 at 20°.

Specific rotation (781): between +9.9° and +11.9°, determined as is, at 20°.

Glucose, $\text{C}_6\text{H}_{12}\text{O}_6$ —180.2 [50-99-7]—Use a suitable grade. A white, crystalline powder. Freely soluble in water; sparingly soluble in alcohol.

D-Glucuronolactone, $\text{C}_6\text{H}_8\text{O}_6$ —176.12 [32449-92-6]—Use a suitable grade.

Glutamic Acid, $\text{C}_5\text{H}_9\text{NO}_4$ —147.13 [56-86-0]—Use a suitable grade.

L-Glutamic Acid, $\text{C}_5\text{H}_9\text{NO}_4$ —147.1 [56-86-0]—White powder or white powder with a faint yellow cast. Use a suitable grade.

L-Glutamine, $\text{C}_5\text{H}_{10}\text{N}_2\text{O}_3$ —146.15 [56-85-9]—White, crystalline powder. Use a suitable grade.

Glycerin (*Glycerol*) [56-81-5]—Use ACS reagent grade Glycerol.

Glycolic Acid, $\text{C}_2\text{H}_4\text{O}_3$ —76.05 [79-14-1]—White crystalline powder or chunks.

Assay—

SILYLATING REAGENT: Pyridine, hexamethyldisilazane, chlorotrimethylsilane (9:3:1)

SAMPLE PREPARATION: Weigh approximately 25 mg (about 3 drops) of the sample into a test tube. Add 2 mL of the *Silylating reagent* and cap the test tube. Mix well and allow to incubate at room temperature for a minimum of 2 h. A white precipitate of ammonium chloride will form. Centrifuge and use the clear supernatant for injection.

PROCEDURE: Inject an appropriate volume of the *Sample preparation* into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2. The injection port temperature is maintained at 250°; the detector temperature is maintained at 300°; the column temperature is main-

tained at 100° and programmed to rise 10° per min to 250°. The area of the peak corresponding to $C_2H_4O_3$ is NLT 98.5% of the total peak area.

Gold Chloride (*Chlorauric Acid*), $HAuCl_4 \cdot 3H_2O$ —**393.83** [16903-35-8]—Use ACS reagent grade.

Guaiaol (*o*-Methoxyphenol), $C_7H_8O_2$ —**124.14** [95-05-1]—Colorless to yellowish, refractive liquid. Soluble in about 65 parts of water; soluble in sodium hydroxide solution; miscible with alcohol, with chloroform, with ether, and with glacial acetic acid.

Assay—When examined by gas-liquid chromatography, it shows a purity of not less than 98%. The following conditions have been found suitable for assaying it: a 3-mm \times 1.8-m stainless steel column containing liquid phase G16 on 60- to 80-mesh support S1A. Helium is the carrier gas, the injection port temperature is maintained at 180°, the column temperature is maintained at 200°, and the flame-ionization detector is maintained at 280°. The retention time is about 8 minutes.

Refractive index (831): between 1.5430 and 1.5450, at 20°.

Guanidine Hydrochloride, (*Aminoformamidinium Hydrochloride*; *Aminomethanamidinium Hydrochloride*), $CH_5N_3 \cdot HCl$ —**95.53** [50-01-1]—White, crystalline powder. Freely soluble in water and in alcohol. Use a suitable grade with a content not less than 99%.

Guanidine Isothiocyanate, $C_2H_6N_4S$ —**118.2** [593-84-0]—White powder or colorless crystals. Use a suitable grade.

Guanine Hydrochloride, $C_5H_5N_5O \cdot HCl \cdot H_2O$ —**205.60** [635-39-2]—White, crystalline powder. Melts above 250°, with decomposition. Slightly soluble in water and in alcohol; soluble in acidulated water and in sodium hydroxide TS. Its solutions are not precipitated by iodine TS or by mercuric-potassium iodide TS, but form a precipitate with trinitrophenol TS.

Residue on ignition (Reagent test): negligible, from 100 mg.

Loss on drying (731)—Dry it at 105° to constant weight: it loses not more than 10.0% of its weight.

Hematein, $C_{16}H_{12}O_6$ —**300.26** [475-25-2]—Prepared from logwood extract or from hematoxylin by treatment with ammonia and exposure to air. Reddish-brown crystals with a yellowish-green metallic luster. Very slightly soluble in water (about 1 in 1700); slightly soluble in alcohol and in ether; insoluble in benzene and in chloroform; freely soluble in diluted ammonia solution to form a solution of dusky purplish-red color and in an aqueous solution of sodium hydroxide (1 in 50), to form a solution of bright red color, viewed in each case through a layer 1 cm in depth. Melts at a temperature above 200° and tends to decompose at 250°.

Hematoxylin (*Hydroxybrasilin*), $C_{16}H_{14}O_6 \cdot 3H_2O$ —**356.32** [517-28-2]—A crystalline substance derived from the heartwood of *Haematoxylon campechianum* Linné (Fam. Leguminosae). Colorless to yellow prisms. Very slightly soluble in cold water and in ether; rapidly soluble in hot water and in hot alcohol. When exposed to light, it acquires a red color and yields a yellow solution. Dissolves in ammonia TS and in solutions of alkali hydroxides and carbonates. When dissolved in solutions of the following salts, it develops the colors indicated: in alum solution, a red color; in stannous chloride solution, a rose color; and in solutions of cupric salts, a greenish-gray color. It gradually turns black in potassium dichromate solution. Store hematoxylin and its solutions protected from light and air.

Hemoglobin, Bovine [9008-02-0]—Use a suitable grade.

[NOTE—A suitable grade is available as Bovine Hemoglobin substrate powder from Sigma-Aldrich, www.sigma-aldrich.com.]

1-Heptadecanol, $C_{17}H_{36}O$ —**256.48** [1454-85-9]—Use a suitable grade.

Heptafluorobutyric Acid, $C_4F_7O_2H$ —**214.04** [375-22-4]—Use a suitable grade.

Heptakis-(2,6-di-O-methyl)- β -cyclodextrin (*2,6-Di-O-methyl- β -cyclodextrin*; *Dimethyl- β -cyclodextrin*) $C_{56}H_{98}O_{35}$ —**1331.36** [51166-71-3]—Use a suitable grade.

n-Heptane—Use *n-Heptane*, *Chromatographic*.

n-Heptane, Chromatographic C_7H_{16} —**100.21** [142-82-5]—Clear, colorless, volatile, flammable liquid consisting essentially of C_7H_{16} . Practically insoluble in water; soluble in absolute alcohol. Miscible with ether, with chloroform, with benzene, and with most fixed and volatile oils. Use a suitable grade, chromatographic or HPLC, with a content of not less than 99%.

Heptyl p-Hydroxybenzoate (*Heptyl 4-Hydroxybenzoate*; *N-Heptyl 4-Hydroxybenzoate*; *Benzoic Acid, 4-Hydroxy-, Heptyl Ester*), $C_{14}H_{20}O_3$ —**236.31** [1085-12-7]—Use a suitable grade with a content of NLT 98%.

Hexadecyl Hexadecanoate (*Hexadecyl Palmitate*; *Cetyl Palmitate*), $C_{32}H_{64}O_2$ —**480.85** [540-10-3]—Use a suitable grade.

[NOTE—Suitable grades are available commercially as Hexadecyl Palmitate and Palmitic Acid Palmityl Ester from Sigma-Aldrich, www.sigma-aldrich.com, and Cetyl Palmitate, Catalog number C1203, from Spectrum Chemical Mfg. Corp., www.spectrumchemical.com.]

Hexadecyltrimethylammonium Bromide—See *Cetyltrimethylammonium Bromide*.

Hexadimethrine Bromide, $(C_{13}H_{30}Br_2N_2)_n$ [28728-55-4]—White to off-white powder, hygroscopic, amorphous polymer. Soluble in water up to 10% to give a colorless to light yellow solution. Use a suitable grade.

Hexamethyldisilazane, $C_6H_{19}NSi_2$ —**161.39** [999-97-3]—Clear, colorless liquid.

Assay—When examined by gas-liquid chromatography, it shows a purity of not less than 95%. The following conditions have been found suitable for assaying the article: A 2-mm \times 1.8-m glass column packed with phase G3 on support S1. Helium, flowing at a rate of about 40 mL per minute, is the carrier gas; the detector temperature is about 310°; the injection port temperature is about 100°; and the column temperature is programmed to start at 35°, hold for 5 minutes, then rise at a rate of 8° per minute to 200°. A flame-ionization detector is employed.

Residue after evaporation—Transfer 200 g to a tared dish, and evaporate on a steam bath to dryness. Dry the residue at 105° for 1 hour, cool, and weigh: not more than 0.0025% of residue is found.

Hexamethyleneimine (*Homopiperidine*), $C_6H_{12}NH$ —**99.17** [111-49-9]—Colorless to nearly colorless liquid.

Refractive index (831): between 1.4640 and 1.4660 at 20°.

Hexamethylenetetramine—See *Methenamine*.

n-Hexane, C_6H_{14} —**86.18** [110-54-3] (for use in spectrophotometry)—Use *Hexanes*.

Hexane, Solvent (*Petroleum Benzin*; *Petroleum Ether*, *Ligroin*) [8032-32-4]—Clear, volatile liquid. Practically insoluble in water; soluble in absolute alcohol. Miscible with ether, with chloroform, with benzene, and with most fixed and volatile oils.

[CAUTION—It is dangerously flammable. Keep it away from flames, and store in tight containers in a cool place.]

Use ACS reagent grade Petroleum Ether.

Hexane, Solvent, Chromatographic—Use ACS HPLC reagent grade.

Hexanes (suitable for use in UV spectrophotometry); usually a mixture of several isomers of hexane (C_6H_{14}), predomi-

nantly *n*-hexane, and methylcyclopentane (C_6H_{12})—Use ACS spectrophotometric reagent grade.

Hexanitrodiphenylamine (*Dipicrylamine*), $C_{12}H_5N_7O_{12}$ —**439.21** [131-73-7]—Yellow-gold powder or prisms. *Explosive*. Usually contains about 15% of water as a safety precaution. Insoluble in water, in alcohol, in acetone, and in ether; soluble in glacial acetic acid and in alkalis.

Water, Method I (921): not more than 16%.

Hexanophenone, $C_{12}H_{16}O$ —**176.25** [942-92-7]—Yellow liquid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G3; the injection port temperature is maintained at 280°; the detector temperature is maintained at 300°; the column temperature is maintained at 180° and programmed to rise 10° per minute to 280°. The area of the $C_{12}H_{16}O$ peak is not less than 98% of the total peak area.

Refractive index (831): 1.511 ± 0.002 at 20°.

Hexylamine (*1-Aminohexane*), $C_6H_{15}N$ —**101.19** [111-26-2]—Use a suitable grade with a content of not less than 99%.

Histamine Dihydrochloride, $C_5H_9N_3 \cdot 2HCl$ —**184.07**—Use USP Histamine Dihydrochloride RS.

L-Histidine Hydrochloride Monohydrate, $C_6H_9N_3O_2 \cdot HCl \cdot H_2O$ —**209.63** [5934-29-2]—Use a suitable grade.

Horseradish Peroxidase Conjugated to Goat Anti-Mouse IgG—Affinity purified polyclonal antibody to Mouse Immune globulin (IgG) heavy and light chains (whole IgG) produced in Goat and labeled with horseradish peroxidase. Available either as a lyophilized powder or as a solution in a suitable buffer, generally 10 mM sodium phosphate, pH 7.4, containing a suitable preservative, such as 0.01% thimerosal, and an inactive protein(s) to prevent adsorption on the surface of the container. Use a suitable grade. Store at -20°.

Hydrazine Dihydrochloride, $(NH_2)_2 \cdot 2HCl$ —**104.97** [5341-61-7]—White powder.

Assay—Dissolve about 34 mg, accurately weighed, in 50 mL of water. Add carefully while stirring, 1 g of sodium bicarbonate. [CAUTION—There may be a rapid evolution of carbon dioxide.] Titrate with 0.1 N iodine solution, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary corrections. Each mL of 0.1 N iodine solution is equivalent to 2.63 mg of $(NH_2)_2 \cdot 2HCl$. Not less than 98% is found.

Hydrazine Hydrate, 85% in Water, $(NH_2)_2 \cdot H_2O$ —**50.06** [7803-57-8]—Colorless liquid.

Assay—Transfer 600 mg, accurately weighed, to a 100-mL volumetric flask. Dilute with water to volume, and mix. Pipet 10 mL into a suitable beaker, and add 1.0 g of sodium bicarbonate and 50.0 mL of 0.1 N iodine VS. Titrate the excess iodine with 0.1 N sodium thiosulfate VS, using starch TS as the indicator. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N iodine is equivalent to 1.252 mg of $(NH_2)_2 \cdot H_2O$. Not less than 83% is found.

Hydrazine Sulfate, $(NH_2)_2 \cdot H_2SO_4$ —**130.12** [10034-93-2]—Use ACS reagent grade. [CAUTION—Great care should be taken in handling hydrazine sulfate because it is a suspected carcinogen.]

Hydrindantin (*2,2'-Dihydroxy-2,2'-biindan-1,1',3,3'-tetraone*), $C_{18}H_{10}O_6$ —**322.27** [5103-42-4]—Sparingly soluble in hot water; soluble in methoxyethanol. When heated above 200°, it becomes reddish brown.

Melting range (741): between 249° and 254°.

Hydriodic Acid, HI—**127.91** [10034-85-2]—Use ACS reagent grade (containing not less than 47.0% of HI).

[NOTE—For methoxy determination (see *Methoxy Determination* (431)), use hydriodic acid ACS reagent grade 55%.

Use this grade also for alkoxyl determinations in assays in the individual monographs.]

Hydrobromic Acid, HBr—**80.91** [10035-10-6]—Use ACS reagent grade.

Hydrochloric Acid, HCl—**36.46** [7647-01-0]—Use ACS reagent grade.

Hydrochloric Acid, Diluted (10 percent) [7647-01-0]—Prepare by mixing 226 mL of hydrochloric acid with sufficient water to make 1000 mL.

Hydrofluoric Acid, HF—**20.01** [7664-39-3]—Use ACS reagent grade.

Hydrogen Peroxide, H_2O_2 —**34.01** [7722-84-1]—Use ACS reagent grade with an assay content between 29.0% and 32.0%.

Hydrogen Peroxide, 10 Percent, H_2O_2 —**34.01**—Dilute 30 mL of 30 percent hydrogen peroxide with water to 100 mL.

Hydrogen Peroxide, 30 Percent, Unstabilized H_2O_2 —**34.01** [7722-84-1]—Use ACS reagent grade, with an assay content between 29.0% and 32.0%, without an added stabilizer.

Hydrogen Peroxide, 50 Percent in Water, H_2O_2 —**34.01** [7722-84-1]—Use a suitable grade.

Hydrogen Peroxide Solution—Use *Hydrogen Peroxide Topical Solution*.

Hydrogen Sulfide, H_2S —**34.08** [7783-06-4]—Colorless, poisonous gas, heavier than air. Soluble in water. Is generated by treating ferrous sulfide with diluted sulfuric or diluted hydrochloric acid. Other sulfides yielding hydrogen sulfide with diluted acids may be used. Is also available in compressed form in cylinders.

Hydrogen Sulfide Detector Tube—A fuse-sealed glass tube so designed that gas may be passed through it and containing suitable absorbing filters and support media for the indicator, the latter consisting of a suitable lead salt.

Measuring range: 1 to 20 ppm.

[NOTE—Available from Draeger Safety, Inc., www.draeger.com, or from Gastec Corp., www.gastec.co.jp, distributed in the USA by www.nextteq.com.]

Hydroquinone, $C_6H_4(OH)_2$ —**110.11** [123-31-9]—Fine, colorless or white, needle crystals. Darkens on exposure to air and light. Soluble in water, in alcohol, and in ether.

Assay—Accurately weigh about 250 mg, and dissolve in a mixture of 100 mL of water and 10 mL of 0.1 N sulfuric acid in a 250-mL conical flask. Add 3 drops of a 1 in 100 solution of diphenylamine in sulfuric acid, and titrate with 0.1 N ceric sulfate VS until the solution is red-violet in color. Each mL of 0.1 N ceric sulfate is equivalent to 5.506 mg of $C_6H_4(OH)_2$. Not less than 99% is found.

Melting range (741): between 172° and 174°.

Hydroxy Naphthol Blue (*1-(2-Naphtholazo-3,6-disulfonic Acid)-2-naphthol-4-sulfonic Acid, Disodium Salt*), $C_{20}H_{12}N_2O_{11}S_3Na_2$ —**598.50** [165660-27-5]—Deposited on crystals of sodium chloride in the concentration of about 1%. Use ACS reagent grade.

3'-Hydroxyacetophenone, $C_8H_8O_2$ —**136.15** [121-71-1]—Light brown powder chips and chunks. Melts at about 96°. Sparingly soluble in chloroform, yielding a clear, light yellow solution.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with G1; the detector and the injection port temperatures are maintained at 300°; the column temperature is maintained at 180° and programmed to rise 10° per minute to 280° and held at that temperature for 10 minutes. The area of the main peak is not less than 97% of the total peak area.

4'-Hydroxyacetophenone, $HOC_6H_4COCH_3$ —**136.15** [99-93-4]—Gray powder, melting at about 109°.

p-Hydroxybenzoic Acid, $C_7H_6O_3$ —**138.12** [99-96-7]—White crystals.

Assay—Transfer about 700 mg, accurately weighed, to a suitable container, and dissolve in 50 mL of acetone. Add 100 mL of water, mix, and titrate with 0.5 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.5 N sodium hydroxide is equivalent to 69.06 mg of $C_7H_6O_3$; not less than 97% is found.

Melting range (741): over a range of 2° that includes 216°.

4-Hydroxybenzoic Acid Isopropyl Ester, $HOC_6H_4COOCH(CH_3)_2$ —**180.18** [4191-73-5]—Use a suitable grade.

[NOTE—A suitable grade is available from TCI America, www.tciamerica.com.]

Melting range (741): between 84° and 87°.

2-Hydroxybenzyl Alcohol, $C_7H_8O_2$ —**124.14** [90-01-7]—Off-white flakes. Very soluble in alcohol, in chloroform, and in ether; soluble in 15 parts water and in benzene.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)), equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 250°; the detector temperature is maintained at 300°; and the column temperature is maintained at 150° and programmed to rise 10° per minute to 280°. The area of the $C_7H_8O_2$ peak is not less than 99% of the total peak area.

Melting range (741): between 83° and 85°.

4-Hydroxybutane-1-sulfonic Acid (4-Hydroxy-1-butanefulfonic Acid), $C_4H_{10}O_4S$ —**154.19** [26978-64-3]—Use a suitable grade with a content of NLT 95%. [NOTE—A suitable grade is available as catalog number RM-967-C50 from www.cydexpharma.com.]

N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), $C_8H_{18}N_2O_4S$ —**238.3** [7365-45-9]—Use a suitable grade.

Hydroxylamine Hydrochloride, $NH_2OH \cdot HCl$ —**69.49** [5470-11-1]—Use ACS reagent grade.

10 β -Hydroxynorandrostenedione (10 β -Hydroxy-19-norandrost-4-ene-3,17-dione), $C_{18}H_{24}O_3$ —**288.38**—Use a suitable grade.

4-(4-Hydroxyphenyl)-2-butanone, $C_{10}H_{12}O_2$ —**164.20** [5471-51-2]—White powder.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G43; the injection port temperature is maintained at 280°; the detector temperature is maintained at 300°; the column temperature is maintained at 180° and programmed to rise 10° per minute to 280°. The area of the $C_{10}H_{12}O_2$ peak is not less than 98.5% of the total peak area.

Melting range (741): between 81° and 87°.

3-Hydroxyphenyldimethylethyl Ammonium Chloride [Dimethylethyl(3-hydroxyphenyl)ammonium Chloride]—Use Edrophonium Chloride.

D- α -4-Hydroxyphenylglycine, $C_8H_9NO_3$ —**167.16** [22818-40-2]—Shiny leaflets. Sparingly soluble in water, in alcohol, in acetone, in ether, in chloroform, in ethyl acetate, in benzene, and in glacial acetic acid; soluble in alkalis and in mineral acids; freely soluble in warm 20% v/v hydrochloric acid.

Melting range (741): between 220° and 247°, with decomposition.

2'-(4-Hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole Trihydrochloride Pentahydrate—**623.97** [23491-44-3]—Dark yellow to tan powder with a green cast. Use a suitable grade.

Hydroxypropyl- β -cyclodextrin (Hydroxypropylbetadex), $C_{42}H_{70}O_{35}(C_3H_6O)_x$ with $x = 7$ molar substitution

[94035-02-6]—Use a suitable grade with a substitution degree between 0.40 and 1.50.

8-Hydroxyquinoline (Oxine), C_9H_7NO —**145.16**

[148-24-3]—Use ACS reagent grade 8-Quinolinol.

Hypophosphorous Acid, 50 Percent (Hypophosphorous Acid), HPH_2O_2 —**66.00** [6303-21-5]—A colorless to faintly yellow liquid. Miscible with water and with alcohol.

Assay—Accurately weigh about 4 mL, dilute with 25 mL of water, add methyl red TS, and titrate with 1 N sodium hydroxide VS: each mL of 1 N sodium hydroxide is equivalent to 66.00 mg of HPH_2O_2 . Not less than 48% is found.

Chloride—Add 0.2 mL to a mixture of 10 mL of silver nitrate TS and 5 mL of nitric acid, and heat until brown fumes are no longer evolved: any white, insoluble residue remaining is negligible.

Phosphate—Dilute 1 mL with water to 50 mL, render alkaline with ammonia TS, filter if a precipitate is formed, and add to the filtrate 5 mL of magnesia mixture TS: not more than a slight precipitate is formed within 5 minutes.

Sulfate (Reagent test, *Method I*)—Dilute 1 mL with water to 50 mL: 20 mL of the solution shows not more than 0.2 mg of SO_4 .

Hypoxanthine, $C_5H_4N_4O$ —**136.11** [68-94-0]—White to yellowish-white powder. Soluble in 1 N sodium hydroxide. Use a suitable grade.

IgG-Coated Red Cells—Red cells coated with human immunoglobulin (IgG). The reagent must be obtained from manufacturers or suppliers licensed by the Center for Biologics Evaluation and Research, Food and Drug Administration. The use of reagents from an unlicensed manufacturer or supplier may invalidate the results.

[NOTE—There are many manufacturers and suppliers of these reagents that are licensed by the Center for Biologics Evaluation and Research, Food and Drug Administration. Some examples of licensed manufacturers or suppliers are the following: Gamma Biologics, Houston, TX; and Ortho Diagnostics, Raritan, NJ.]

Imidazole, $C_3H_4N_2$ —**68.08** [288-32-4]—White to light yellow crystals. Freely soluble in water. Use ACS reagent grade.

Indene, C_9H_8 —**116.16** [95-13-6]—Colorless liquid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 10-m capillary column coated with a 1- μ m layer of methylsilicone; the injection port temperature is maintained at 200°; the detector temperature is maintained at 300°; the column temperature is maintained at 100° and programmed to rise 10° per minute to 250°. The area of the indene peak is not less than 99% of the total peak area.

Refractive index (831): between 1.5749 and 1.5769 at 20°.

Indicators—See separate subsection.

Indigo Carmine—Use Indigotindisulfonate Sodium.

Indole (2,3-Benzopyrrole), C_8H_7N —**117.14**

[120-72-9]—Use a suitable grade.

Indole-3-carboxylic Acid, $C_9H_7NO_2$ —**161.2**

[771-50-6]—Use a suitable grade.

Inosine, $C_{10}H_{12}N_4O_5$ —**268.23** [58-63-9]—White, crystalline powder.

Melting point (741): about 90°.

Inositol (Hexahydroxycyclohexane), $C_6H_6(OH)_6$ —**180.16** [87-89-8]—Fine, white crystals or a white, crystalline powder; stable in air. Its solutions are neutral to litmus. Optically inactive. One g dissolves in 5.7 mL of water. Slightly soluble in alcohol; insoluble in ether and in chloroform. Store in well-closed containers.

Melting range (741): between 223° and 226°.

Loss on drying (731)—Dry it at 105° for 4 hours: it loses not more than 0.5% of its weight.

Residue on ignition (Reagent test): not more than 0.1%.

Iobenguane Sulfate (*m*-Iodobenzylguanidine Hemisulfate Salt), $C_8H_{10}IN_3 \cdot \frac{1}{2}H_2SO_4$ —**324.1**—White powder. Freely soluble in methanol.

Assay—When tested by thin-layer chromatography, with the use of plates coated with chromatographic silica gel mixture, a developing system consisting of a mixture of butyl alcohol, water, and acetic acid (60:25:15), and examined under short-wavelength UV light, not more than a single impurity spot of not more than 0.5% is observed.

Iodic Acid, HIO_3 —**175.91** [7782-68-5]—Use ACS reagent grade.

Iodine, I_2 —**253.81** [7553-56-2]—Use ACS reagent grade.

Iodine Monobromide, IBr —**206.81** [7789-33-5]—Black, gray, or blue-purple crystals, crystalline needles, or crystalline chunks.

Assay—Place about 100 mL of acetic acid in a 150-mL beaker. Separately dissolve 2 g of potassium iodide in a minimum volume of water, add this solution to the acetic acid, and mix. Transfer about 200 mg of Iodine Monobromide, accurately weighed, to the beaker containing the potassium iodide and acetic acid mixture, and stir to dissolve. Titrate immediately with 0.1 N sodium thiosulfate VS, determining the endpoint potentiometrically (see *Titrimetry* (541)). Perform a blank determination, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 20.681 mg of IBr . Not less than 97.5% is found.

Iodine Monochloride, ICl —**162.36** [7790-99-0]—Use ACS reagent grade.

Iodoethane, C_2H_5I —**155.9** [75-03-6]—Use a suitable grade.

p-Iodonitrotetrazolium Violet, (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride), $C_{19}H_{13}ClIN_5O_2$ —**505.70**—Light yellow powder.

Assay—When tested by thin-layer chromatography, with the use of plates coated with chromatographic silica gel mixture and a developing system consisting of a mixture of amyl alcohol, formic acid, and water (8:1:1), sprayed with 0.1% sodium thiosulfate solution, and examined under short-wavelength UV light, a single spot is exhibited, with trace impurities.

Melting point (741): 240°, with decomposition.

Ion-Exchange Resin—An intimate mixture of 4 parts of a strongly acidic cation-exchanger in the hydrogen form (produced by sulfonation of a styrene-divinylbenzene copolymer, representing 8 to 10% divinylbenzene) and 6 parts of a strongly basic anion-exchanger in the hydroxyl form (produced by amination with trimethylamine of a chloromethylated styrene-divinylbenzene copolymer, representing 3 to 5% divinylbenzene).

[NOTE—A suitable resin is “Amberlite MB-150,” available from Sigma-Aldrich, www.sigma-aldrich.com.]

Iron Wire, Fe—**At. Wt. 55.847**—Use a suitable grade.

Isoamyl Alcohol—Use *Amyl Alcohol*.

Isobutyl Acetate, $C_6H_{12}O_2$ —**116.16** [110-19-0]—Clear, colorless liquid. Slightly soluble in water. Miscible with alcohol.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with G2. The injection port temperature is maintained at 130°; the column temperature is maintained at 30° and programmed to rise 10° per minute to 180° and held there for 10 minutes. The detector temperature is maintained at 300°. The area of the main peak is not less than 99% of the total peak area.

Specific gravity (841): between 0.863 and 0.868.

Refractive index (831): between 1.3900 and 1.3920 at 20°.

Isobutyl Alcohol (2-Methyl-1-propanol), $(CH_3)_2CHCH_2OH$ —**74.12** [78-83-1]—Use ACS reagent grade.

4-Isobutylacetophenone, $C_{12}H_{16}O$ —**176**—Pale yellow liquid. Soluble in chloroform, in glycerols, in alcohols, in ether, and in fatty oils; insoluble in water. Use a suitable grade.

[NOTE—A suitable grade is available from TCI America, www.tciamerica.com.]

N-Isobutylpiperidone, $C_9H_{17}NO$ —**155.24**—Use a suitable grade.

Isoflupredone Acetate (9- α -Fluoroprednisolone Acetate), $C_{23}H_{29}FO_6$ —**420.47**—Use Isoflupredone Acetate USP.

Isomaltotriose (α -D-Glucosyl-(1-6)- α -D-glucosyl-(1-6)- α -D-glucose), $C_{18}H_{32}O_{16}$ —**504.4** [3371-50-4]—White lyophilized powder. Use a suitable grade.

Isonicotinic Acid, $C_6H_5NO_2$ —**123.11** [52-22-1]—Use a suitable grade.

Isonicotinic Acid Hydrazide—Use *Isoniazid*.

Isooctane—See 2,2,4-Trimethylpentane.

Isopropyl Acetate, $C_5H_{10}O_2$ —**102.13** [108-21-4]—Use a suitable grade.

Isopropyl Alcohol (2-Propanol), $(CH_3)_2CHOH$ —**60.10** [67-63-0]—Use ACS reagent grade.

[NOTE—For use in assays and tests involving UV spectrophotometry, use ACS reagent grade Isopropyl Alcohol Suitable for Use in UV Spectrophotometry.]

Isopropyl Alcohol, Dehydrated [67-63-0]—Use Isopropyl Alcohol that previously has been dried by being shaken with a suitable molecular sieve capable of adsorbing water, and filtered.

Isopropyl Ether—See *Diisopropyl Ether*.

Isopropyl Iodide (2-Iodopropane), C_3H_7I —**169.99** [75-30-9]—Use a suitable grade.

Isopropyl Myristate, $C_{17}H_{34}O_2$ —**270.45** [110-27-0]—Use *Isopropyl Myristate* (NF monograph). For use as a solvent in sterility test procedures, Isopropyl Myristate conforms to the following additional specification:

pH of water extract—Transfer 100 mL to a 250-mL centrifuge bottle, add 10 mL of twice-distilled water, close the bottle with a suitable closure, and shake vigorously for 60 minutes. Centrifuge the mixture at 1800 rpm for 20 minutes, aspirate the upper (isopropyl myristate) layer, and determine the pH of the residual water layer: the pH is not less than 6.5.

Isopropyl Myristate not conforming to the test for *pH of water extract* may be rendered suitable for use in sterility test procedures as follows:

Using a 20-mm \times 20-cm glass column, add activated alumina, and tamp down to a height of 15 cm. Pass 500 mL of the isopropyl myristate through the column, using a slight positive pressure to maintain an even flow, and use the eluate collected directly in the sterility test procedure.

Isopropyl Salicylate, $C_6H_4OHCOOCH(CH_3)_2$ —**180.20**—Colorless liquid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 2-mm \times 1.8-m glass column packed with 7% phase G2 on support S1A; the injection port temperature is maintained at 250°; the detector temperature is maintained at 310°; the column temperature is maintained at 50° and programmed to rise 10° per minute to 250°. The area of the main peak is not less than 97% of the total peak area.

Isopropylamine (2-Aminopropane), $C_3H_7NH_2$ —**59.11** [75-31-0]—Clear, colorless, flammable liquid. Miscible with water, with alcohol, and with ether.

Assay—Transfer about 0.2 g, accurately weighed, to a suitable container, add 50 mL of water, and mix. Titrate with 0.1 N hydrochloric acid VS, using a mixture of bromocresol green TS and methyl red TS (5:1) as indicator. Each

mL of 0.1 N hydrochloric acid is equivalent to 59.11 mg of C_3H_9N . Not less than 98% is found.

Boiling range (Reagent test)—Not less than 95% distills between 31° and 33°.

Refractive index (831): between 1.3743 and 1.3753, at 20°.

Isorhamnetin, $C_{16}H_{12}O_7$ —**316.27** [418-19-3]—Yellow powder.

Melting point: greater than 300°.

Isovaleric Acid (3-Methylbutanoic Acid, Isovaleric Acid, Isopropylacetic Acid), $C_5H_{10}O_2$ —**102.13** [503-74-2]—Use a suitable grade.

Kaempferol, $C_{15}H_{10}O_6$ —**286.24** [520-18-3]—Light yellow to yellow powder. It is a clear, bright yellow solution in alcohol.

Kerosene [8008-20-6]—A mixture of hydrocarbons, chiefly of the methane series. A clear, colorless liquid. Specific gravity: about 0.80. Distills between 180° and 300°.

L Designations—See packings for high-pressure liquid chromatography under *Reagents, Chromatographic Columns*.

Lactose, $C_{12}H_{22}O_{11} \cdot H_2O$ —**360.31** [64-42-3]—Use ACS reagent grade.

Alpha Lactose Monohydrate (α -D-Lactose Monohydrate), $C_{12}H_{22}O_{11} \cdot H_2O$ —**360.31**—White powder. The β -D-lactose content is less than 3%.

Assay—Inject an appropriate derivatized specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 250°; the detector temperature is maintained at 280°; the column temperature is maintained at 230° and programmed to rise 4° per minute to 280°. The area of the $C_{12}H_{22}O_{11} \cdot H_2O$ peak is not less than 97% of the total peak area.

Beta Lactose (β -D-Lactose), $C_{12}H_{22}O_{11}$ —**342.30**—White to faint yellow powder. The α -D-lactose content is not more than 35%.

Assay—Inject an appropriate derivatized specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G43; the injection port temperature is maintained at 250°; the detector temperature is maintained at 250°; the column temperature is maintained at 20° and programmed to rise 8° per minute to 280°. The area of the $C_{12}H_{22}O_{11}$ peak is not less than 99% of the total peak area.

Lanthanum Alizarin Complexan Mixture—Use a suitable grade.

Lanthanum Chloride, $LaCl_3 \cdot (6-7)H_2O$ [10025-84-0]—This reagent is available in degrees of hydration ranging from 6 to 7 molecules of water. Use ACS reagent grade.

Lanthanum Nitrate Hexahydrate, $La(NO_3)_3 \cdot 6H_2O$ —**433.01** [10277-43-7]—Use a suitable grade with a content of NLT 99.9%.

Lanthanum Oxide, La_2O_3 —**325.82** [1312-81-8]—An almost white, amorphous powder, practically insoluble in water. It dissolves in dilute solutions of mineral acids and absorbs atmospheric carbon dioxide. Use Atomic Absorption Spectroscopy grade.

Lauryl Dimethyl Amine Oxide (*N,N*-Dimethyldodecylamine-*N*-oxide), $C_{14}H_{31}NO$ [1643-20-5]—**229.41**—Use a suitable grade.

[NOTE—A suitable grade is available from Fluka, catalog number 40234, www.sigma-aldrich.com.]

Lead Acetate, $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ —**379.33** [6080-56-4]—Use ACS reagent grade.

Lead Acetate Paper—Immerse filter paper weighing about 80 g per square meter in a mixture of diluted acetic

acid and lead acetate TS (1:10). Remove, dry, and cut into 15-mm \times 40-mm strips.

Lead Monoxide (*Litharge*), PbO —**223.20**

[1317-36-8]—Heavy, yellowish or reddish-yellow powder. Insoluble in water and in alcohol; soluble in acetic acid, in diluted nitric acid, and in warm solutions of the fixed alkali hydroxides.

Assay—Accurately weigh about 300 mg, freshly ignited in a muffle furnace at $600 \pm 50^\circ$, and dissolve it by warming with 10 mL of water and 1 mL of glacial acetic acid. Dilute with 75 mL of water, heat to boiling, add 50.0 mL of 0.1 N potassium dichromate VS, and boil for 2 to 3 minutes. Cool, transfer to a 200-mL volumetric flask with the aid of water, dilute with water to volume, mix, and allow to settle. Withdraw 100.0 mL of the clear liquid, and transfer to a glass-stoppered flask. Add 10 mL of diluted sulfuric acid and 1 g of potassium iodide, insert the stopper, mix gently, and allow to stand for 10 minutes. Then titrate the liberated iodine, representing the excess of dichromate, with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached: each mL of 0.1 N potassium dichromate is equivalent to 7.440 mg of PbO . Not less than 98% is found.

Insoluble in acetic acid—Dissolve 2 g in 30 mL of dilute glacial acetic acid (1 in 2), boil gently for 5 minutes, filter, wash the residue with diluted acetic acid, and dry at 105° for 2 hours: the residue weighs not more than 10 mg (0.5%).

Substances not precipitated by hydrogen sulfide—Completely precipitate the lead from the filtrate obtained in the test for *Insoluble in acetic acid* by passing hydrogen sulfide into it, filter, and wash the precipitate with 20 mL of water. To one-half of the mixed filtrate and washings add 5 drops of sulfuric acid, evaporate to dryness, and ignite at $800 \pm 25^\circ$ for 15 minutes: the residue weighs not more than 5 mg (0.5%).

Volatile substances—Accurately weigh about 5 g, and heat strongly in a covered porcelain crucible: it loses not more than 2.0% of its weight.

Lead Nitrate, $Pb(NO_3)_2$ —**331.21** [10099-74-8]—Use ACS reagent grade.

Lead Perchlorate, $Pb(ClO_4)_2 \cdot 3H_2O$ —**460.15**—Use ACS reagent grade.

Lead Tetraacetate, $C_8H_{12}O_8Pb$ —**443.38** [546-67-8]—Colorless to pinkish crystals. Soluble in hot glacial acetic acid, in chloroform, in nitrobenzene, and in tetrachloroethane. Hydrolyzed by water, producing brown lead dioxide and acetic acid. Unstable in air.

Melting range (741): between 175° and 180°.

Linalool (3,7-Dimethyl-1,6-octadien-3-ol), $C_{10}H_{18}O$ —**154.25** [78-70-6]—Use a suitable grade.

Linoleic Acid, $C_{18}H_{32}O_2$ —**280.4** [60-33-3]—Clear, colorless liquid. Use a suitable grade.

α -Lipoic Acid, $C_8H_{14}O_2S_2$ —**206.3** [1077-28-7]—Yellow powder. Use a suitable grade.

Liquid Petrolatum—Use *Mineral Oil*.

Lithium Chloride, $LiCl$ —**42.39** [7447-41-8]—Use ACS reagent grade.

Lithium Hydroxide, $LiOH \cdot H_2O$ —**41.96** [1310-65-2]—Use ACS reagent grade.

Lithium Metaborate, $LiBO_2$ —**49.75** [13453-69-5]—Use ACS reagent grade.

Lithium Nitrate, $LiNO_3$ —**68.95** [7790-69-4]—Colorless crystals. Use a suitable grade labeled to contain not less than 97.0%.

Lithium Perchlorate, $LiClO_4$ —**106.39** [7791-03-9]—Use ACS reagent grade.

Lithium Sulfate, $Li_2SO_4 \cdot H_2O$ —**127.96** [10377-48-7]—Use ACS reagent grade.

Lithocholic Acid, $C_{24}H_{40}O_3$ —**376.57** [434-13-9]—White powder.

Assay—When tested by thin-layer chromatography, with the use of plates coated with chromatographic silica gel

mixture, a developing system consisting of a mixture of toluene, 1,4-dioxane, and acetic acid (15.2:4.2:0.6), and sprayed with a mixture of sulfuric acid and methanol (1:1), heated at 110° for 20 minutes, and examined visually and under long-wavelength UV light, a single spot is exhibited.

Melting range (741): between 184° and 186°.

Litmus [1393-92-6]—A blue pigment prepared from various species of *Rocella* DeCandolle, *Lecanora* Acharius, or other lichens (Fam. Parmeliaceae).

Description—Cubes, masses, fragments, or granules, of an indigo blue or deep violet color. Has the combined odor of indigo and violets, and tinges the saliva a deep blue. The indicator substances it contains are soluble in water and less soluble or insoluble in alcohol.

Ash—It yields not more than 60.0% of ash.

Locust Bean Gum—A gum obtained from the ground endosperms of *Ceratonia siliqua* Linné Taub. (Fam. Leguminosae). Use Locust Bean Gum, FCC.

L-Lysine (2,6-Diaminohexanoic Acid), $C_6H_{14}N_2O_2$ —**146.19** [56-87-1]—Crystalline needles or hexagonal plates. Soluble in water; very slightly soluble in alcohol; insoluble in ether.

Specific rotation (781): between +25.5° and +26.0°.

Test solution: 20 mg per mL, in dilute hydrochloric acid (1 in 2).

Nitrogen content, Method I (461): between 18.88% and 19.44% of N is found, corresponding to not less than 98.5% of $C_6H_{14}N_2O_2$, the test specimen previously having been dried at 105° for 2 hours.

Magnesium, Mg—**24.305** [7439-95-4]—Silvery metal in ribbon form. Reacts slowly with water at room temperature. Dissolves readily in dilute acids with the liberation of hydrogen.

Assay—Transfer 1 g, accurately weighed, to a 250-mL volumetric flask, and dissolve in a mixture of 15 mL of hydrochloric acid and 85 mL of water. When solution is complete, dilute with water to volume, and mix. Pipet 25 mL of the dilution into a 400-mL beaker, dilute with water to 250 mL, add 20 mL of ammonia-ammonium chloride TS and a few mg of eriochrome black T trituration, and titrate with 0.1 M edetate disodium VS to a blue endpoint. Each mL of 0.1 M edetate disodium VS is equivalent to 2.430 mg of Mg. Not less than 99% is found.

Magnesium Acetate, $Mg(C_2H_3O_2)_2 \cdot 4H_2O$ —**214.45** [142-72-3]—Use ACS reagent grade.

Magnesium Chloride, $MgCl_2 \cdot 6H_2O$ —**203.30** [7786-30-3]—Use ACS reagent grade.

Magnesium Nitrate, $Mg(NO_3)_2 \cdot 6H_2O$ —**256.41** [10377-60-3]—Use ACS reagent grade.

Magnesium Oxide, MgO —**40.30** [1309-48-4]—Use ACS reagent grade.

Magnesium Oxide, Chromatographic—Use a suitable grade.

Magnesium Perchlorate, Anhydrous, $Mg(ClO_4)_2$ —**223.21** [10034-81-8]—Use ACS reagent grade.

Magnesium Silicate, Activated—Use a suitable grade.

Magnesium Silicate, Chromatographic—Extremely white, hard, powdered (60- to 100-mesh) magnesia-silica gel. Suitable for use as an adsorbant in column chromatography.

Magnesium Sulfate, $MgSO_4 \cdot 7H_2O$ —**246.48** [10034-99-8]—Use ACS reagent grade.

Magnesium Sulfate, Anhydrous, $MgSO_4$ —**120.37**

[7487-88-9]—Anhydrous Magnesium Sulfate may be prepared as follows. Place a suitable quantity of magnesium sulfate (see above), preferably powdered, in a shallow vessel, and expose to a temperature of about 80° for several hours with occasional stirring. Then heat at 275° to 300° until the weight is practically constant. Transfer the product

while still warm to tight containers, as the anhydrous salt is very hygroscopic.

Malachite Green G—See *Brilliant Green*.

Maleic Acid, $C_4H_4O_4$ —**116.07** [110-16-7]—White, crystalline powder. Soluble in 1.5 parts of water, in 2 parts of alcohol, and in 12 parts of ether.

Assay—Dissolve about 2 g, accurately weighed, in 100 mL of water and titrate with 1 N sodium hydroxide VS, using phenolphthalein TS as the indicator. Each mL of 1 N sodium hydroxide is equivalent to 58.04 mg of $C_4H_4O_4$; not less than 99% of $C_4H_4O_4$, calculated on the dried basis, is found.

Loss on drying—Dry it in vacuum over phosphorus pentoxide for 2 hours: it loses not more than 1.5% of its weight.

Residue on ignition (281): not more than 0.1%.

Maltotriose, $C_{18}H_{32}O_{16}$ —**504.44** [1109-28-0]—Use a suitable grade with a content of not less than 95%.

Manganese Dioxide—See *Manganese Dioxide, Activated*. **Manganese Dioxide, Activated** (*Manganese (IV) Oxide, Activated*), MnO_2 —**86.94** [1313-13-9]—Use a suitable grade.

Melamine (2,4,6-Triamino-1,3,5-triazine), $C_3H_6N_6$ —**126.1** [108-78-1]—Use a suitable grade.

2-Mercaptoethanol (β -Mercaptoethanol), C_2H_5OS —**78.13** [60-24-2]—Use a suitable grade.

Mercuric Acetate, $Hg(C_2H_3O_2)_2$ —**318.68**

[1600-27-7]—Use ACS reagent grade.

Mercuric Bromide, $HgBr_2$ —**360.40** [7789-47-1]—Use ACS reagent grade.

Mercuric Chloride, $HgCl_2$ —**271.50** [7487-94-7]—Use ACS reagent grade.

Mercuric Iodide, Red, HgI_2 —**454.40** [7774-29-0]—Use ACS reagent grade.

Mercuric Nitrate, $Hg(NO_3)_2 \cdot xH_2O$ —**342.62**

[10045-94-0]—Use ACS reagent grade. This reagent is available as either the mono- or dihydrate.

Mercuric Oxide, Yellow, HgO —**216.59**

[21908-53-2]—Use ACS reagent grade.

Mercuric Sulfate, $HgSO_4$ —**296.65** [7783-35-9]—Use ACS reagent grade.

Mercuric Thiocyanate, $Hg(SCN)_2$ —**316.76**

[592-85-8]—White, crystalline powder. Very slightly soluble in water; soluble in solutions of sodium chloride; slightly soluble in alcohol and in ether.

Mercurous Nitrate Dihydrate (*Mercury (I) Nitrate Dihydrate*), $Hg_2(NO_3)_2 \cdot 2H_2O$ —**561.22**—Use ACS reagent grade.

Mercury, Hg —**At. Wt. 200.59** [7439-97-6]—Use ACS reagent grade.

Mesityl Oxide, $C_6H_{10}O$ —**98.14** [141-79-7]—Colorless liquid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 150°; the detector temperature is maintained at 300°; the column temperature is maintained at 50° and programmed to rise 10° per minute to 200°. The area of the $C_6H_{10}O$ peak is not less than 98% of the total peak area.

Refractive index (831): between 1.443 and 1.447 at 20°.

Metanil Yellow (*Acid Yellow 36*;

3-(4-Anilinophenylazo)benzenesulfonic Acid Sodium Salt), $C_{18}H_{14}N_3NaO_3S$ —**375.38** [587-98-4]—Use a suitable grade with a dye content of NLT 70%.

Metaphenylenediamine Hydrochloride (*Metaphenylenediamine Dihydrochloride*), $C_6H_4(NH_2)_2 \cdot 2HCl$ —**181.06**—White or slightly reddish-white, crystalline powder. Easily soluble in water. On exposure to light it acquires a reddish color. Store it protected from light.

Solubility—A solution of 1 g in 200 mL of water is colorless.

Residue on ignition (Reagent test)—Ignite 1 g with 0.5 mL of sulfuric acid: the residue weighs not more than 1 mg (0.1%).

[NOTE—Metaphenylenediamine hydrochloride solution can be decolorized by treatment with a small quantity of activated charcoal.]

Metaphosphoric Acid (*Vitreous Sodium Acid Metaphosphate*), HPO_3 —**79.98** [37267-86-0]—Use ACS reagent grade.

Methacrylic Acid [79-41-4]—Use a suitable grade.

Methanesulfonic Acid, $\text{CH}_3\text{O}_3\text{S}$ —**96.11** [75-75-2]—Use a suitable grade.

Methanol (*Methyl Alcohol*), CH_3OH —**32.04** [67-56-1]—Use ACS reagent grade.

Methanol, Aldehyde-Free, CH_3OH —**32.04**—Dissolve 25 g of iodine in 1 L of methanol and pour the solution, with constant stirring, into 400 mL of 1 N sodium hydroxide. Add 150 mL of water, and allow to stand for 16 hours. Filter, and boil under a reflux condenser until the odor of iodoform disappears. Distill the solution by fractional distillation. It contains not more than 0.001% of aldehydes and ketones.

Methanol, Anhydrous—Use *Methanol*.

Methanol, Spectrophotometric—Use ACS reagent grade Methanol Suitable for Use in UV Spectrophotometry.

Methenamine (*Hexamethylenetetramine*; *Urotropine*; *Uritone*; *Hexamine*), $\text{C}_6\text{H}_{12}\text{N}_4$ —**140.19** [100-97-0]—Use ACS reagent grade Hexamethylenetetramine.

7-Methoxycoumarin (*Herniarin*; *Methyl Umbelliferol Ether*), $\text{C}_{10}\text{H}_8\text{O}_3$ —**176.17** [531-59-9]—Use a suitable grade with a content of not less than 98%.

Methoxyethanol (*Ethylene Glycol Monomethyl Ether*; *2-Methoxyethanol*), $\text{CH}_3\text{OCH}_2\text{CH}_2\text{OH}$ —**76.09** [109-86-4]—Use ACS reagent grade.

2-Methoxyethanol (*Ethylene Glycol Monomethyl Ether*; *Methoxyethanol*), $\text{CH}_3\text{OCH}_2\text{CH}_2\text{OH}$ —**76.09** [109-86-4]—See *Methoxyethanol*.

Add the following:

▲ **5-Methoxy-1H-benzimidazole-2-thiol** (*5-Methoxy-2-benzimidazolethiol*), $\text{C}_8\text{H}_8\text{N}_2\text{OS}$ —**180.23** [37052-78-1]—Use a suitable grade with a content of NLT 99.0%.▲ *USP36*

5-Methoxy-2-methyl-3-indoleacetic Acid, $\text{C}_{12}\text{H}_{13}\text{NO}_3$ —**219.24** [2882-15-7]—Off-white powder.

Assay—Transfer about 110 mg, accurately weighed, to a 100-mL beaker. Add 30 mL of methanol and dissolve by stirring. Add 40 mL of water, and mix. Titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination and make any necessary correction. Each mL of 0.1 N sodium hydroxide is equivalent to 21.92 mg of $\text{C}_{12}\text{H}_{13}\text{NO}_3$. Not less than 98% is found.

Melting range (741): between 161° and 168°, but the range between beginning and end of melting does not exceed 3°.

Methoxyphenylacetic Acid (*α-Methoxyphenylacetic Acid*), $\text{C}_9\text{H}_{10}\text{O}_3$ —**166.2** [7021-09-2]—Use a suitable grade.

Methyl Acetate, $\text{C}_3\text{H}_6\text{O}_2$ —**74.08** [74-20-9]—Colorless liquid. Soluble in water. Miscible with alcohol and with ether.

Specific gravity (841): about 0.933.

Refractive index (831): between 1.3615 and 1.3625 at 20°.

Boiling range (Reagent test)—Not less than 95% distills between 57° and 58°.

Methyl 4-Aminobenzoate, $\text{C}_8\text{H}_9\text{NO}_2$ —**151.16** [619-45-4]—Off-white powder.

Assay—Dissolve about 38 mg, accurately weighed, in 50 mL of glacial acetic acid. Titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction.

Each mL of 0.1 N perchloric acid is equivalent to 15.12 mg of $\text{C}_8\text{H}_9\text{NO}_2$. Not less than 99.0% is found.

Melting range (741): between 108° and 110°.

Methyl Arachidate (*Eicosanoic acid, methyl ester*), $\text{C}_{21}\text{H}_{42}\text{O}_2$ —**326.56** [1120-28-1]—Off-white flakes.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a thermal-conductivity detector, helium being used as the carrier gas. The following conditions have been found suitable: a 2.0-mm × 1.8-m glass column packed with 5% G2 phase on support S1A; the injection port temperature is maintained at 300°; the detector temperature is maintained at 230° and programmed to rise 3° per minute to 280°. The area of the $\text{C}_{21}\text{H}_{42}\text{O}_2$ peak is not less than 99% of the total peak area.

Melting range (741): between 46° and 51°.

Methyl Behenate, $\text{C}_{23}\text{H}_{46}\text{O}_2$ —**354.61** [929-77-1]—White powder.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a thermal conductivity detector, helium being used as the carrier gas. The following conditions have been found suitable: a 2.0-mm × 1.8-m glass column packed with 5% G3 phase on support S1A; the injection port temperature is maintained at 300°; the detector temperature is maintained at 300°; the initial temperature of the oven is 220°, which is held for 2 minutes, and then programmed to rise 3° per minute to attain a final temperature of 270°, which is held for 10 minutes. The area of the $\text{C}_{23}\text{H}_{46}\text{O}_2$ peak is not less than 98% of the total peak area.

Melting range (741): between 54° and 56°.

Methyl Benzenesulfonate, $\text{C}_7\text{H}_8\text{O}_3\text{S}$ —**172.20** [80-18-2]—Use a suitable grade.

Methyl Caprate, $\text{C}_{11}\text{H}_{22}\text{O}_2$ —**186.29** [110-42-9]—Colorless liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm × 30-m capillary column coated with a 1-μm layer of phase G2; the injection port temperature is maintained at 250°; the detector temperature is maintained at 300°; the column temperature is maintained at 150° and programmed to rise 10° per minute to 280°. The area of the $\text{C}_{11}\text{H}_{22}\text{O}_2$ peak is not less than 98.5% of the total peak area.

Methyl Caprylate, $\text{C}_9\text{H}_{18}\text{O}_2$ —**158.24** [111-11-5]—Colorless liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm × 30-m capillary column coated with a 1-μm layer of phase G2; the injection port temperature is maintained at 230°; the detector temperature is maintained at 300°; the column temperature is maintained at 130° and programmed to rise 10° per minute to 280°. The area of the $\text{C}_9\text{H}_{18}\text{O}_2$ peak is not less than 98.5% of the total peak area.

Methyl Carbamate, $\text{C}_2\text{H}_5\text{NO}_2$ —**75.07** [598-55-0]—White crystals. Freely soluble in water.

Melting range (741): between 54° and 56°.

Methyl Chloroform (*Methylchloroform*; *1,1,1-Trichloroethane*), CH_3CCl_3 —**133.40** [71-55-6]—Use ACS reagent grade.

Methyl Erucate, $\text{C}_{23}\text{H}_{44}\text{O}_2$ —**352.59** [1120-34-9]—Colorless liquid.

Methyl Ethyl Ketone, $\text{CH}_3\text{COC}_2\text{H}_5$ —**72.11** [78-93-3]—Use ACS reagent grade 2-butanone.

Methyl Green (*Methyl Green Zinc Chloride Double Salt*; *Ethyl Green Zinc Chloride Double Salt*; *C.I. 42590*), $\text{C}_{27}\text{H}_{35}\text{Cl}_2\text{N}_3 \cdot \text{ZnCl}_2$ —**608.78** [7114-03-6]—Use a suitable grade for microscopy.

Methyl Heptadecanoate, $\text{C}_{18}\text{H}_{36}\text{O}_2$ —**284.48** [1731-92-6]—White, crystalline flakes.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G8; the injection port temperature is maintained at 220°; the detector temperature is maintained at 220°; the column temperature is maintained at 180° and programmed to rise 4° per minute to 220°. The area of the $C_{18}H_{36}O_2$ peak is not less than 99% of the total peak area.

Melting range (741): between 31° and 32°.

Methyl Iodide, (Iodomethane), CH_3I —**141.94**

[74-88-4]—Colorless, heavy, transparent liquid. Slightly soluble in water. Miscible with alcohol, with ether, and with solvent hexane. Turns brown on exposure to light as a result of liberation of iodine.

Use a suitable grade with a content of not less than 99%.

Methyl Isobutyl Ketone—See 4-Methyl-2-pentanone.

Methyl Laurate, $C_{13}H_{26}O_2$ —**214.34** [110-82-0]—Colorless liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 280°; the detector temperature is maintained at 300°; the column temperature is maintained at 180° and programmed to rise 10° per minute to 280°. The area of the $C_{13}H_{26}O_2$ peak is not less than 99.45% of the total peak area.

Methyl Lignocerate, $C_{25}H_{50}O_2$ —**382.66** [2442-49-1]—White crystals.

Methyl Linoleate, $C_{19}H_{34}O_2$ —**294.47** [112-63-0]—Colorless liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 300°; the detector temperature is maintained at 300°; the column temperature is maintained at 200° and programmed to rise 10° per minute to 300°. The area of the $C_{19}H_{34}O_2$ peak is not less than 99% of the total peak area.

Methyl Linolenate, $C_{19}H_{32}O_2$ —**292.46** [301-00-8]—Colorless liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase 14% cyanopropylphenyl-86% dimethylpolysiloxane; the injection port temperature is maintained at 280°; the detector temperature is maintained at 300°; the column temperature is maintained at 180° and programmed to rise 10° per minute to 280°. The area of the $C_{19}H_{32}O_2$ peak is not less than 99% of the total peak area.

Refractive index (831): between 1.469 and 1.473 at 20°.

Methyl Methacrylate [80-62-6]—Use a suitable grade.

Methyl Myristate, $C_{15}H_{30}O_2$ —**242.40** [124-10-7]—Colorless liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas.

The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 300°; the detector temperature is maintained at 300°; the column temperature is maintained at 200° and programmed to rise 10° per minute to 300°. The area of the $C_{15}H_{30}O_2$ peak is not less than 99% of the total peak area.

Refractive index (831): between 1.434 and 1.438 at 20°.

Methyl Oleate, $C_{19}H_{36}O_2$ —**296.49** [112-62-9]—Colorless liquid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 300°; the detector temperature is maintained at 300°; the column temperature is maintained at 230° and programmed to rise 10° per minute to 280°. The area of the $C_{19}H_{36}O_2$ peak is not less than 99% of the total peak area.

Refractive index (831): 1.452 at 20°.

Methyl Palmitate, $C_{17}H_{34}O_2$ —**270.45** [112-39-0]—White solid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 300°; the detector temperature is maintained at 300°; the column temperature is maintained at 200° and programmed to rise 10° per minute to 300°. The area of the $C_{17}H_{34}O_2$ peak is not less than 96.5% of the total peak area.

Methyl Red (2-[4-Dimethylaminophenylazo]benzoic Acid; C. I. Acid Red 2) $C_{15}H_{15}N_3O_2$, free acid—**269.30**

[493-52-7] $C_{15}H_{14}N_3O_2Na$, sodium salt—**291.28**

[845-10-3]—Use ACS reagent grade. The free acid is recommended for nonaqueous titrations, particularly when an aprotic solvent is used. The sodium salt is recommended for titrations in aqueous media and also for nonaqueous titrations where the medium is an amphiprotic solvent. The hydrochloride salt is recommended for titrations in aqueous media and amphiprotic solvents.

Methyl Stearate, $C_{19}H_{38}O_2$ —**298.50** [112-61-8]—Off-white, crystalline solid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 300°; the detector temperature is maintained at 300°; the column temperature is maintained at 200° and programmed to rise 10° per minute to 300°. The area of the $C_{19}H_{38}O_2$ peak is not less than 99% of the total peak area.

Melting range (741): between 40° and 42°.

Methyl Sulfoxide [67-68-5]—See Dimethyl Sulfoxide.

Methyl Yellow (p-Dimethylaminoazobenzene), $C_{14}H_{15}N_3$ —**225.3** [60-11-7]—Use a suitable grade.

Methylamine, 40 Percent in Water, CH_3N —**31.06** [74-89-5]—Colorless liquid.

Assay—Using a syringe, transfer about 0.5 mL of a well-shaken specimen to 100 mL of water at a point below the surface of the water. Determine the weight of the specimen by weighing the syringe before and after the transfer. Mix, and titrate with 0.5 N hydrochloric acid VS, determining the endpoint potentiometrically, using a silver-silver chloride pH electrode and a calomel reference electrode. Perform a blank determination, and make any necessary correction. Each mL of 0.5 N hydrochloric acid is equivalent to 15.53 mg of CH_3N : between 39.0% and 41.0% is found.

Refractive index (831): between 1.3680 and 1.3710, at 20°.

p-Methylaminophenol Sulfate, $(p\text{-CH}_3\text{NHC}_6\text{H}_4\text{OH})_2 \cdot \text{H}_2\text{SO}_4$ —**344.38** [55-55-0]—Use ACS reagent grade.

4-Methylbenzophenone, $\text{C}_{14}\text{H}_{12}\text{O}$ —**196.25** [134-84-9]—Use a suitable grade.

Melting range (741): between 56.5° and 57°.

Methylbenzothiazolone Hydrazone Hydrochloride, $\text{C}_8\text{H}_{10}\text{ClN}_3\text{S} \cdot \text{H}_2\text{O}$ —**233.7** [38894-11-0] (monohydrate form)—**215.70** (anhydrous form); [149022-15-1] (hydrate form)—An almost white or yellowish, crystalline powder.

Suitability for determination of aldehydes—To 2 mL of aldehyde-free methanol add 60 μL of a 1 g per L solution of propionaldehyde in aldehyde-free methanol and 5 mL of a 4 g per L solution of methylbenzothiazolone hydrazone hydrochloride. Mix, and allow to stand for 30 minutes. Prepare a blank, omitting the propionaldehyde solution. Add 25.0 mL of a 2 g per L solution of ferric chloride to the test solution and to the blank, dilute with acetone to 100.0 mL, and mix. The absorbance of the test solution, measured at 660 nm using the blank as compensation liquid, is not less than 0.62.

Use a suitable grade with a content of not less than 97%.

(R)-(+)-alpha-Methylbenzyl Isocyanate ((R)-(+)-1-Phenylethyl Isocyanate), $\text{C}_9\text{H}_9\text{NO}$ —**147.17** [33375-06-3]—Use a suitable grade with a content of NLT 99.0%.

(S)-(-)-alpha-Methylbenzyl Isocyanate $\text{C}_9\text{H}_9\text{NO}$ —**147.18** [14649-03-7]—Use a suitable grade.

Methylene Blue, $\text{C}_{16}\text{H}_{18}\text{ClN}_3\text{S} \cdot 3\text{H}_2\text{O}$ —**373.90** [7220-79-3]—Dark green crystals or a crystalline powder, having a bronzelike luster. One g dissolves in about 25 mL of water and in about 65 mL of alcohol. Soluble in chloroform. Use a suitable grade with a dye content of not less than 85%.

Methylene Chloride (Dichloromethane), CH_2Cl_2 —**84.93** [75-09-2]—Use ACS reagent grade Dichloromethane.

5,5'-Methylenedisalicylic Acid (3,3'-Methylene-bis[6-hydroxybenzoic Acid]), $\text{C}_{15}\text{H}_{12}\text{O}_6$ —**288.25** [122-25-8]—Use a suitable grade.

3-O-Methylestrone, $\text{C}_{19}\text{H}_{24}\text{O}_2$ —**284.39**—Use a suitable grade.

[NOTE—Commercially available as catalog number 1883-5 from Research Plus, Inc., P.O. Box 324, Bayonne, NJ 07002, fax number 908-754-2901, Web site: www.researchplus.com.]

2-Methyl-5-nitroimidazole, $\text{C}_4\text{H}_4\text{N}_3\text{O}_2$ —**127.10** [88052-22-2]—Use a suitable grade.

[NOTE—Available as Catalog No. 13,625-5 from Sigma-Aldrich, www.sigma-aldrich.com.]

N-Methyl-N-nitroso-p-toluenesulfonamide (p-Tolylsulfonfylmethylnitrosamide), $\text{C}_8\text{H}_{10}\text{N}_2\text{O}_3\text{S}$ —**214.24**—Light yellow crystals or powder. Insoluble in water; soluble in benzene, in carbon tetrachloride, and in chloroform.

Melting range (741): between 59° and 63°, but the range between beginning and end of melting does not exceed 2°.

4-Methylpentan-2-ol, $\text{C}_6\text{H}_{14}\text{O}$ —**102.2**—Use a suitable grade.

2-Methylpentane (2-Methyl-pentane; 1,1-Dimethylbutane; Isohexane), C_6H_{14} —**86.18** [107-83-5]—Use a suitable grade with a content of not less than 99.0%.

4-Methyl-2-pentanone (Methyl Isobutyl Ketone), $(\text{CH}_3)_2\text{CHCH}_2\text{COCH}_3$ —**100.16** [108-10-1]—Use ACS reagent grade.

2-Methyl-2-propyl-1,3-propanediol, $\text{C}_7\text{H}_{16}\text{O}_2$ —**132.20** [78-26-2]—White crystals, melting at about 58°.

N-Methylpyrrolidine (1-Methylpyrrolidine), $\text{C}_4\text{H}_8\text{NCH}_3$ —**85.15** [120-94-5]—Use a suitable grade.

Mineral Acid—Use Hydrochloric Acid or Sulfuric Acid.

5,800, 23,700, and 100,000 Molecular Weight (MW)

Pullulan Standards (a commercial Pullulan Standard set contains standards having several molecular weights: 5,800; 12,000; 24,000; 48,000; 100,000; 186,000; 380,000; and 750,000) [9057-02-7]—Use a suitable grade. Each individual Pullulan Standard with a different molecular weight, such as 5,800, 24,000, or 100,000, is equivalently used.

[NOTE—The standard set is available from Polymer Laboratories (www.polymerlabs.com), Sigma-Aldrich (www.sigma-aldrich.com), and Waters (www.waters.com).]

Molybdic Acid (85 Percent Molybdic Acid) [7782-91-4]—Use ACS reagent grade.

Monobasic Potassium Phosphate—See Potassium Phosphate, Monobasic.

Monobasic Sodium Phosphate—See Sodium Phosphate, Monobasic.

Monochloroacetic Acid (Chloroacetic Acid, Chloroethanoic Acid), CH_2ClCOOH —**94.50** [79-11-8]—Use ACS reagent grade.

Monoethanolamine, (2-Aminoethanol) $\text{C}_2\text{H}_7\text{NO}$ —**61.08**—Use ACS reagent grade.

Morin (Morin Hydrate; 2',3,4',5,7-Pentahydroxyflavone Monohydrate), $\text{C}_{15}\text{H}_{10}\text{O}_7 \cdot \text{H}_2\text{O}$ —**320.25** [480-16-0]—Use a suitable grade.

Morpholine (Tetrahydro-1,4-oxazine), $\text{C}_4\text{H}_9\text{NO}$ —**87.12** [110-91-8]—Use ACS reagent grade.

Naphthalene, C_{10}H_8 —**128.17** [91-20-3]—Monoclinic prismatic plates, or white scales or powder. Use a suitable grade with a content of not less than 98%.

1,3-Naphthalenediol (Naphthoresorcinol), $\text{C}_{10}\text{H}_6(\text{OH})_2$ —**160.17** [132-86-5]—Grayish-white to tan crystals or powder. Freely soluble in methanol; sparingly soluble in water, in alcohol, and in ether.

Melting range (741): between 122° and 127°.

Solubility in methanol—Dissolve 500 mg in 50 mL of methanol: the solution is clear and complete.

2,7-Naphthalenediol (2,7-Dihydroxynaphthalene), $\text{C}_{10}\text{H}_8\text{O}_2$ —**160.17** [582-17-2]—Off-white to yellow, crystalline solid or powder. Dissolves in acetone.

Melting range (741): between 187° and 191°.

2-Naphthalenesulfonic Acid, $\text{C}_{10}\text{H}_8\text{O}_3\text{S} \cdot \text{H}_2\text{O}$ —**226.25** [120-18-3]—Off-white to light gray crystals. Soluble in water.

Assay—Dissolve about 1 g, accurately weighed, in 100 mL of water, add phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N sodium hydroxide is equivalent to 22.63 mg of $\text{C}_{10}\text{H}_8\text{O}_3\text{S} \cdot \text{H}_2\text{O}$. Not less than 98.0% is found.

Melting range (741): between 122° and 126°, but the range between beginning and end of melting does not exceed 2°.

1-Naphthol (*Alphanaphthol*), $C_{10}H_7OH$ —**144.17** [90-15-3]—Colorless or slightly pinkish crystals or crystalline powder.

2-Naphthol (*Betanaphthol*), $C_{10}H_7OH$ —**144.17** [135-19-3]—White leaflets or crystalline powder. Discolors on exposure to light. Very slightly soluble in water; soluble in alcohol, in ether, in chloroform, and in solutions of alkali hydroxides.

Melting range (741): between 121° and 123°.

Solubility in alcohol—A solution of 1 g in 10 mL of alcohol is complete and colorless or practically so.

Residue on ignition (Reagent test): not more than 0.05%.

Acidity—Shake 1 g with 50 mL of water occasionally during 15 minutes, and filter: the filtrate is neutral to litmus.

1-Naphthol—Boil 100 mg with 10 mL of water until dissolved, cool, and filter. Add to the filtrate 0.3 mL of 1 N sodium hydroxide and 0.3 mL of 0.1 N iodine: no violet color is produced.

Insoluble in ammonia (naphthalene, etc.)—Shake 500 mg with 30 mL of ammonia TS: the 2-naphthol dissolves completely and the solution is not darker than pale yellow.

Naphthol Dipotassium Disulfonate (*2-Naphthol-6,8-dipotassium Disulfonate*), $C_{10}H_6K_2O_7S_2$ —**380.48** [842-18-2]—Use a suitable grade.

[NOTE—A suitable grade is available as “2-naphthyl-6,8-disulfonic acid dipotassium salt” from Pfaltz and Bauer, Inc., www.pfaltzandbauer.com.]

Naphthol Disodium Disulfonate (*2-Naphthol-3,6-disodium Disulfonate*), $C_{10}H_6Na_2O_7S_2$ —**348.26**—Use a suitable grade.

p-Naphtholbenzein, $C_{27}H_{18}O_2$ —**374.43** [145-50-6]—Red-brown powder. Use a suitable grade.

β-Naphthoquinone-4-sodium Sulfonate, $C_{10}H_5NaO_5S$ —**260.20**—Yellow to orange-yellow crystals or crystalline powder. Soluble in about 10 parts of water; insoluble in alcohol.

Loss on drying (731)—Dry it in vacuum at about 50°: it loses not more than 2.0% of its weight.

Residue on ignition (Reagent test)—Ignite 1 g of dried sample with 3 mL of sulfuric acid: the residue weighs between 265 and 280 mg (between 26.5% and 28.0%).

Naphthoresorcinol (*1,3-Dihydroresorcinol*), $C_{10}H_8O_2$ —**160.17** [132-86-5]—Use a suitable grade.

2-Naphthyl Chloroformate (*Chloroformic Acid 2-Naphthyl Ester*), $ClCOOC_{10}H_7$ —**206.62** [7693-50-7]—Use a suitable grade.

[NOTE—A suitable grade is available from TCI America, www.tciamerica.com.]

1-Naphthylamine, $C_{10}H_9N$ —**143.19** [134-32-7]—Use a suitable grade.

1-Naphthylamine Hydrochloride, $C_{10}H_7NH_2 \cdot HCl$ —**179.65** [552-46-5]—White, crystalline powder that turns bluish upon exposure to light and air. Soluble in water, in alcohol, and in ether.

A 1 in 100 solution, make slightly acid with acetic acid, gives a violet color with 5 drops of ferric chloride TS. A 1 in 40 solution in diluted acetic acid is colorless and not more than slightly opalescent.

Residue on ignition (Reagent test)—Ignite 200 mg with a few drops of sulfuric acid: the weight of the residue is negligible.

N-(1-Naphthyl)ethylenediamine Dihydrochloride, $C_{10}H_7NH(CH_2)_2NH_2 \cdot 2HCl$ —**259.17** [1465-25-4]—Use ACS reagent grade.

Neutralized Alcohol—See *Alcohol, Neutralized*.

Nickel, Ni—**58.6934** [7440-02-0]—Use a suitable grade.

Nickel-Aluminum Catalyst—Use a suitable grade.

[NOTE—A suitable grade is “Raney Nickel, Active Catalyst,” available as “aluminum-nickel alloy,” catalog number

72240, available from Fluka Chemical Corp., fax 1-800-962-9591, Web site: www.sigma-aldrich.com.]

Nickel Sulfate, $NiSO_4 \cdot 6H_2O$ —**262.85** [7786-81-4]—Use ACS reagent grade.

Nickel (II) Sulfate Heptahydrate, $NiSO_4 \cdot 7H_2O$ —**280.9** [10101-98-1]—Use a suitable grade.

β-Nicotinamide Adenine Dinucleotide, $C_{21}H_{27}N_7O_{14}P_2$ —**663.4** [53-84-9]—White, very hygroscopic powder. Freely soluble in water.

Assay—Dissolve 17.9 g of anhydrous dibasic sodium phosphate in water to make 500 mL (*Solution A*). Dissolve 6.8 g of monobasic potassium phosphate in water to make 500 mL (*Solution B*). To a volume of *Solution A*, add *Solution B* until the mixture is adjusted to a pH of 7.0 (about 2:1 by volume of *Solutions A* and *B*) to obtain a pH 7.0 Buffer. Transfer about 25 mg of β-nicotinamide adenine dinucleotide, accurately weighed, to a 25-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Transfer 0.2 mL of this solution to a 10-mL volumetric flask, dilute with pH 7.0 Buffer to volume, and mix. Use this solution as the *Assay preparation*. Determine the absorbances of the *Assay preparation* and the pH 7.0 Buffer in 1-cm cells at a wavelength of 260 nm, using water as the reference. Calculate the quantity, in mg, of $C_{21}H_{27}N_7O_{14}P_2$ in the portion of β-nicotinamide adenine dinucleotide taken by the formula:

$$(0.6634/17.6)(10/0.2)(25)(A_A - A_B)$$

in which A_A and A_B are the absorbances of the *Assay preparation* and the pH 7.0 Buffer, respectively. Not less than 94.5% is found.

Nicotinamide Adenine Dinucleotide Phosphate-adenosine-5'-triphosphate Mixture—Use a suitable grade.

Suitability—When used in the assay of lactulose, determine that a suitable absorbance-versus-concentration slope is obtained, using USP Lactulose RS, the reagent blank absorbance being not more than 0.020. The commercially available reagent contains 64 mg of nicotinamide adenine dinucleotide phosphate and 160 mg of adenosine-5'-triphosphate per vial. The mixture is buffered and stabilized. For use in the Assay of lactulose it is diluted with water to 100 mL.

Nicotinic Acid—Use *Niacin* (USP monograph).

Ninhydrin, $C_9H_4O_3 \cdot H_2O$ —**178.14** [485-47-2]—Use ACS reagent grade.

Nitric Acid, HNO_3 —**63.01** [7697-37-2]—Use ACS reagent grade.

Nitric Acid, Diluted (10 percent HNO_3) [7697-37-2]—Dilute 143 mL of nitric acid with water to 1000 mL.

Nitric Acid, Fuming (90 Percent Nitric Acid), HNO_3 —**63.01** [7697-37-2]—Use ACS reagent grade Nitric Acid, 90 Percent.

Nitric Acid, Lead-Free—Use ACS reagent grade.

Lead—To 100 g add 0.1 g of anhydrous sodium carbonate and evaporate to dryness. Dissolve the residue in water, heating slightly, and dilute with the same solvent to 50.0 mL. Determine the lead content by atomic absorption spectrophotometry (see *Spectrophotometry and Light-Scattering* (851)) measuring the absorbance at 283.3 nm or 217.0 nm using a lead hollow-cathode lamp and an air-acetylene flame. It contains not more than 0.1 ppm of lead (Pb).

Nitric Oxide-Nitrogen Dioxide Detector Tube—A fuse-sealed glass tube so designed that gas may be passed through it and containing suitable absorbing filters and support media for an oxidizing layer and the indicator diphenyl benzidine.

Measuring range: 0.5 to 10 ppm.

[NOTE—Available from Draeger Safety, Inc., www.draeger.com, or from Gastec Corp., www.gastec.co.jp, distributed in the USA by www.nextteq.com.]

Nitrilotriacetic Acid, $\text{N}(\text{CH}_2\text{COOH})_3$ —**191.14** [139-13-9]—Use ACS reagent grade.

4'-Nitroacetophenone (*p'*-Nitroacetophenone), $\text{C}_8\text{H}_7\text{NO}_3$ —**165.15** [100-19-6]—Yellow crystals.

Assay—Inject an appropriate ether solution of the specimen (about 0.5 μL) into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a thermal conductivity detector, helium being used as the carrier gas. The following conditions have been found suitable: a 4-mm \times 1.8-m stainless steel column containing 10% phase G1 on support S1A; the injection port and detector temperatures are maintained at 200° and 300°, respectively; the column temperature is maintained at 170° and programmed to rise 3° per minute to 220°. The area of the 4'-nitroacetophenone peak is not less than 97% of the total peak area.

Melting range (741): between 78° and 80°.

o-Nitroaniline, $\text{NO}_2\text{C}_6\text{H}_4\text{NH}_2$ —**138.12** [88-74-4]—Orange-yellow crystals. Slightly soluble in cold water; soluble in hot water; freely soluble in alcohol and in chloroform. It forms water-soluble salts with mineral acids.

Melting range (741): between 71° and 72°.

p-Nitroaniline, $\text{NO}_2\text{C}_6\text{H}_4\text{NH}_2$ —**138.12** [100-01-6]—Bright yellow, crystalline powder. Insoluble in water; soluble in alcohol and in ether.

Melting range (741): between 146° and 148°.

Solubility—Separate 1-g portions dissolve in 30 mL of alcohol and in 40 mL of ether, respectively, to yield solutions that are clear or practically so.

Residue on ignition (Reagent test): not more than 0.2%.

Nitrobenzene, $\text{C}_6\text{H}_5\text{NO}_2$ —**123.11** [98-95-3]—Use ACS reagent grade.

p-Nitrobenzenediazonium Tetrafluoroborate, $\text{NO}_2\text{C}_6\text{H}_4\text{N}_2\text{BF}_4$ —**236.92** [456-27-9]—Yellow-gold crystals. Soluble in acetonitrile. [CAUTION—Shock-sensitive; keep refrigerated.]

Assay—Transfer about 30 mg, accurately weighed, to a low-actinic, 100-mL volumetric flask. Dissolve in 0.01 N hydrochloric acid, dilute with 0.01 N hydrochloric acid to volume, and mix. Using low-actinic glassware, dilute 2.0 mL of the resulting solution with spectrophotometric grade methanol to 50.0 mL. Measure the absorbance of this solution in a 1-cm cell at about 255 nm, using methanol as the blank. Calculate the absorptivity of the solution by dividing the measured absorbance by the concentration in g per mL. Calculate the assay value by the formula:

$$100a/59.4$$

in which *a* is the absorptivity of the solution: not less than 95.0% is found.

p-Nitrobenzyl Bromide, $\text{NO}_2\text{C}_6\text{H}_4\text{CH}_2\text{Br}$ —**216.03**—Almost white to pale yellow crystals, darkening on exposure to light. Practically insoluble in water; freely soluble in alcohol, in ether, and in glacial acetic acid. Store in tight, light-resistant containers.

Melting range (741): between 98° and 100°.

Solubility—Separate 200-mg portions yield clear solutions in 5 mL of alcohol and in 5 mL of glacial acetic acid.

Residue on ignition (Reagent test): negligible, from 200 mg.

4-(p-Nitrobenzyl)pyridine, $\text{C}_{12}\text{H}_{10}\text{N}_2\text{O}_2$ —**214.22** [1083-48-3]—Yellow crystals. Soluble in acetone. Use a suitable grade with a content of not less than 98%.

Nitromethane, CH_3NO_2 —**61.04** [75-52-5]—Use ACS reagent grade.

5-Nitro-1,10-phenanthroline, $\text{C}_{12}\text{H}_7\text{N}_3\text{O}_2$ —**225.20** [4199-88-6]—White powder. Soluble in water.

Melting range (741): between 198° and 200°.

Suitability as redox indicator—Dissolve 25 mg in a minimum volume of diluted sulfuric acid, add 10 mg of ferrous

sulfate, and dilute with water to 100 mL: the solution is deep red in color and exhibits an absorption maximum at 510 nm. To 1.0 mL of the solution add 1.0 mL of 0.01 M ceric sulfate: the red color is discharged.

Nitroso R Salt (1-Nitroso-2-naphthol-3,6-disodium Disulfonate), $\text{NOC}_{10}\text{H}_4\text{OH}(\text{SO}_3\text{Na})_2$ —**377.26** [525-05-3]—Yellow crystals or crystalline powder. One g dissolves in about 40 mL of water; insoluble in alcohol.

Sensitiveness—Dissolve 500 mg of sodium acetate in a solution of 0.4 mg of cobaltous chloride (0.1 mg of cobalt) in 5 mL of water. Add 1 mL of diluted acetic acid, and follow with 1 mL of a solution of the nitroso R salt (1 in 500): a red color, which is produced at once, persists when the solution is boiled with 1 mL of hydrochloric acid for 1 minute.

1-Nitroso-2-naphthol, $\text{C}_{10}\text{H}_7\text{NO}_2$ —**173.17** [131-91-9]—Brown to yellowish-brown powder. Insoluble in water; soluble in alcohol, in benzene, in ether, in carbon tetrachloride, and in acetic acid.

Assay—Transfer about 250 mg, previously dried over silica gel to constant weight and accurately weighed, to a glass-stoppered flask, and dissolve in 10 mL of sodium hydroxide solution (1 in 10). Cool the solution in an ice bath, add dilute sulfuric acid (1 in 6) until a slight, permanent precipitate is formed and the solution is slightly acid, then add 3 g of potassium iodide, shake to dissolve, add 20 mL of dilute sulfuric acid (1 in 6), immediately insert the stopper in the flask, and allow to stand in the dark for 2 hours. Titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. Perform a complete blank determination, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 8.66 mg of $\text{C}_{10}\text{H}_7\text{NO}_2$: not less than 95.0% is found.

Melting range (741): between 109° and 111°.

Residue on ignition (Reagent test): not more than 0.2%.

Nitrous Oxide Certified Standard [10024-97-2]—A container of 99.9% nitrous oxide. It is available from most suppliers of specialty gases.

Nonadecane, $\text{C}_{19}\text{H}_{40}$ —**268.52** [629-92-5]—White solid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a thermal conductivity detector, helium being used as the carrier gas. The following conditions have been found suitable: 3-mm \times 1.8-m stainless steel column containing 5% phase G2 on support S1AB; the injection port temperature is maintained at 330°; the detector temperature is maintained at 300°; and the oven temperature is held initially at 190° and allowed to rise gradually to 250°. The area of the nonadecane peak is not less than 99% of the total peak area.

Melting range (741): between 31.5° and 33.5°.

Nonanoic Acid, $\text{C}_9\text{H}_{18}\text{O}_2$ —**158.24** [112-05-0]—Clear, colorless to faint yellow liquid. Miscible with water and with methanol.

Assay—Accurately weigh about 500 mg, transfer to a suitable container, add 30 mL of water, and mix. Add 40 mL of water, and mix. Add phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS. Each mL of 0.1 N sodium hydroxide is equivalent to 15.82 mg of $\text{C}_9\text{H}_{18}\text{O}_2$: not less than 96.0% of $\text{C}_9\text{H}_{18}\text{O}_2$ is found.

Refractive index (831): about 1.432 at 20°.

Nonionic Wetting Agent—Use a suitable amphoteric surfactant.

[NOTE—A suitable grade is commercially available as Triton X-100 or Octoxynol 9.]

Nonoxynol-9 (Igepal CO-630, Semicid, Staycept, Tergitol TP-9, Sterox), $(\text{C}_2\text{H}_4\text{O})_n\text{C}_{15}\text{H}_{24}\text{O}$ —[26027-38-3]—Use a suitable grade.

1-Nonyl Alcohol (1-Nonanol), $\text{CH}_3(\text{CH}_2)_8\text{OH}$ —**144.25** [143-08-8]—Colorless liquid.

Assay—Not less than 97% of $C_9H_{20}O$ is found, a suitable gas chromatograph equipped with a flame-ionization detector and helium being used as the carrier gas at a flow rate of about 40 mL per minute. The following conditions have been found suitable: a 3.2-mm \times 1.83-m stainless steel column packed with 20% phase G16 on support S1A; the injection port, column, and detector temperatures are maintained at about 250°, 160°, and 310°, respectively.

Refractive index (831): between 1.432 and 1.434 at 20°.

***n*-Nonylamine** (1-Aminononane), $C_9H_{21}N$ —**143.27** [112-20-9]—Use a suitable grade.

Nonylphenol Polyoxyethylene Ether, $(CH_3)_3CCH_2C(CH_3)_2CH_2C_6H_4O(CH_2CH_2O)_xH$, where *x* is approximately 40 to 1900—2100 [9016-45-9]—White solid; melts at approximately 44°. Use a suitable grade.

Nonylphenoxypoly(ethyleneoxy)ethanol—Clear, viscous, pale yellow liquid. May exhibit slight solidification on cooling; warming with agitation will restore to original condition. Density: about 1.06. Soluble in alcohol, in xylene, and in water. Suitable for use in gas-liquid chromatography.

[NOTE—A suitable grade is "Igepal CO 710," available from General Aniline and Film Corp., 140 West 51st St., New York, NY 10020.]

Normal Butyl Alcohol—See *Butyl Alcohol*.

Normal Butyl Nitrite—See *n*-Butyl Nitrite.

Normal Butylamine—See *n*-Butylamine.

***n*-Octadecane**, $CH_3(CH_2)_{16}CH_3$ —**254.49** [593-45-3]—Use a gas chromatographic standard with a content of not less than 99.5%.

Octadecyl Silane [18623-11-5]—This reagent is formed *in situ* by reaction of the column support with a suitable silylating agent such as octadecyl trichlorosilane.

Octanesulfonic Acid Sodium Salt (*Sodium 1-octanesulfonate*), $C_8H_{17}NaO_3S$ —**216.27** [5324-84-5]—Use a suitable grade with a content of not less than 99.0%.

1-Octanol (*Alcohol C8*; *Capryl Alcohol*; *Octyl Alcohol*), $C_8H_{18}O$ —**130.23** [111-87-5]—Use ACS reagent grade.

Octanophenone, $C_{14}H_{20}O$ —**204.31** [1674-37-9]—Colorless liquid.

Assay—

MOBILE PHASE—Prepare a filtered and degassed mixture of acetonitrile and water (7:3).

PROCEDURE—Inject about 20 μ L into a suitable liquid chromatograph (see *Chromatography* (621)) equipped with a 254-nm detector and a 4.6-mm \times 15.0-cm column that contains packing L1. The flow rate is about 2 mL per minute. The area of the $C_{14}H_{20}O$ peak is not less than 99% of the total peak area.

Refractive index (831): 1.5043 at 20°.

Octoxynol 9—See *Nonionic Wetting Agent*.

(*p*-tert-Octylphenoxy) nonaethoxyethanol, $C_{34}H_{62}O_{11}$ —**646.85**—Use a suitable grade.

[NOTE—A suitable grade is available commercially as "Tergitol Nonionic NPX," and as "Triton N101," from reagent suppliers.]

(*p*-tert-Octylphenoxy)polyethoxyethanol—Use a suitable grade.

[NOTE—A suitable grade is available commercially as "Triton 100" from reagent suppliers.]

Octyl Sulfate, Sodium Salt, $C_8H_{17}O_4SNa$ —**232.27**—White powder.

Solubility—A 2-g portion dissolves in 100 mL of water.

Melting range (741): between 195° and 197°, with decomposition.

Odorless Absorbent Paper—See *Filter Paper, Quantitative*.

Olefin Detector Tube—A fuse-sealed glass tube so designed that gas may be passed through it and containing suitable absorbing filters and support media for the indicator in a stabilized form of permanganate.

Measuring range: 0.06 to 3.2 Vol.-% Propylene, 0.04 to 2.4 Vol.-% Butylene.

[NOTE—Available from Draeger Safety, Inc., www.draeger.com.]

Oligo-deoxythymidine—Polymeric length: 18. Use a suitable grade.

[NOTE—A suitable grade is available from BD Biosciences, www.bdbiosciences.com.]

Orange G (*the sodium salt of azobenzene-beta-naphthol disulfonic acid*), $C_{16}H_5N_2O_6Na$ —**452.37** [1936-15-8]—Orange to brick-red powder or dark red crystals. Readily soluble in water, yielding an orange-yellow solution; slightly soluble in alcohol; insoluble in ether and in chloroform. The addition of tannic acid TS to its 1 in 500 solution causes no precipitation (*acid color*). The addition of hydrochloric acid to a mixture of 500 mg of zinc dust and 10 mL of its 1 in 500 solution produces decolorization. When filtered, the colorless filtrate, on standing exposed to air, does not regain its original color (*presence of azo-group*). When heated, orange G does not deflagrate (distinction from *nitro colors*). The addition of barium or calcium chloride TS to a concentrated solution of orange G produces a colored, crystalline precipitate. The addition of hydrochloric acid to its 1 in 500 solution produces no change; the addition of sodium hydroxide TS to a similar solution produces a yellowish red to a Bordeaux color but no precipitation. Orange G dissolves in sulfuric acid with an orange to yellowish-red color. No change in color results upon diluting the solution cautiously with water.

Orcinol (*5-Methylresorcinol*), $C_7H_8O_2 \cdot H_2O$ —**142.15** [6153-39-5]—White to light tan crystals.

Assay—Transfer about 60 mg, accurately weighed, to a 100-mL volumetric flask, dissolve in methanol, dilute with methanol to volume, and mix. Transfer 5.0 mL of this solution to a 50-mL volumetric flask and dilute with methanol to volume, and mix. Using a suitable spectrophotometer, 1-cm cells, and methanol as the blank, record the absorbance of the solution at the wavelength of maximum absorbance at about 273 nm. From the observed absorbance, calculate the absorptivity (see *Spectrophotometry and Light-scattering* (851)): the absorptivity is not less than 13.2, corresponding to not less than 98% of $C_7H_8O_2 \cdot H_2O$.

Melting range (741): between 58° and 61°.

Orthophenanthroline—See *1,10-Phenanthroline*.

Osmium Tetroxide (*Osmic Acid*; *Perosmic Anhydride*), OsO_4 —**254.23** [20816-12-0]—Use ACS reagent grade.

Oxalic Acid, $H_2C_2O_4 \cdot 2H_2O$ —**126.07** [6153-56-6]—Use ACS reagent grade.

3,3'-Oxydipropionitrile, $O(CH_2CH_2CN)_2$ —**124.14** [1656-48-0]—Clear, colorless to slightly yellow liquid. Refractive index: about 1.446 at 20°.

Boiling range: between 174° and 176° at 10 mm of mercury.

Oxygen-Helium Certified Standard—A mixture of 1.0% oxygen in industrial grade helium. It is available from most suppliers of specialty gases.

Packings for High-Pressure Liquid Chromatography—See packings for high-pressure liquid chromatography in the *Chromatographic Reagents* section under *Chromatography* (621).

Palladium Catalyst—Use a suitable grade.

[NOTE—A suitable grade is available commercially as "Palladium Catalyst, Type I (5% Palladium on Calcium Carbonate)," from Engelhard Industries, Inc., fax number (864) 885-1375.]

Palladium Chloride, $PdCl_2$ —**177.33** [7647-10-1]—Brown, crystalline powder. Soluble in water, in alcohol, in acetone, and in diluted hydrochloric acid.

Assay—Dissolve 80 mg, accurately weighed, in 10 mL of diluted hydrochloric acid, dilute with water to 50 mL, and add 25 mL of a 1 in 100 solution of dimethylglyoxime in alcohol. Allow to stand for 1 hour, and filter. Check for complete precipitation with the dimethylglyoxime solution. Ignite the precipitate in a tared platinum crucible at 850° for 2 hours, cool, and weigh the palladium. The weight of the

residue is not less than 59.0% of the weight of the test specimen.

Palladous Chloride—See *Palladium Chloride*.

Pancreatic Digest of Casein (a bacteriological peptone; *Tryptone*)—A grayish-yellow powder, having a characteristic, but not putrescent, odor. Freely soluble in water; insoluble in alcohol and in ether.

Nitrogen content (Reagent test)—Determine by the Kjeldahl method: 9.0%–14.0% is found.

Loss on drying (731)—Dry it at 100° to constant weight: it loses not more than 7.0% of its weight.

Residue on ignition (281)—Ignite 500 mg with 1 mL of sulfuric acid: the residue weighs not more than 75 mg (15%).

Microbial content—NMT 10,000 cfu/g.

Bacteriological test—Prepare medium of the following composition:

2% of digest, 0.5% of sodium chloride, and 1.5% of agar in purified water.

Adjust with diluted hydrochloric acid or diluted sodium hydroxide to a pH of 7.2–7.4. Autoclave at 121° for 15 min.

Growth-supporting properties—Slants of the above medium, inoculated with *Escherichia coli* ATCC 25922, *Enterobacter aerogenes* ATCC 13048, *Salmonella enterica* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, and *Staphylococcus epidermidis* ATCC 12228, show characteristic growth after incubation for 24 h.

The above medium, to which 5% of sheep blood or rabbit blood has been added, and which has been inoculated and poured into Petri dishes, shows characteristic alpha or beta zones around colonies of *Streptococcus pneumoniae* ATCC 6305 and *Streptococcus pyogenes* ATCC 49117, recognizable within 24 h and fully developed after 48 h of incubation.

The above medium, to which 10% of sheep blood or rabbit blood has been added, and which then has been heated to 80°–90° until the blood has turned chocolate-brown, permits the growth of *Neisseria gonorrhoeae* ATCC 19424 colonies within 48 h when incubated in an atmosphere containing 10% of carbon dioxide.

Pancreatin [8049-47-6]—Use a grade of pancreatin which meets the USP requirements for amylase, lipase, and protease activities specified for the official substance.

Papaic Digest of Soybean Meal—A soluble nutrient material prepared by the action of the enzyme papain on soybean meal followed by suitable purification and concentration. It contains fermentable carbohydrates.

Loss on drying (731)—Dry it at 100° to constant weight: it loses not more than 7.0% of its weight.

Residue on ignition (Reagent test)—Ignite 500 mg with 1 mL of sulfuric acid: the residue weighs not more than 75 mg (15.0%).

Coagulable protein—Heat a filtered solution (1 in 20) to boiling: no precipitate forms.

Microbial content—NMT 10⁴ cfu/g

Nitrogen content (Reagent test)—Determine by the Kjeldahl method, using a test specimen previously dried at 105° to constant weight: not less than 8.5% is found.

Paper, Odorless Absorbent—See *Filter Paper, Quantitative*.

Para-aminobenzoic Acid (*p*-Aminobenzoic Acid), H₂NC₆H₄COOH—**137.14** [150-13-0]—White or slightly yellow crystals or crystalline powder, becoming discolored on exposure to air or light. One g dissolves in 170 mL of water, in 9 mL of boiling water, in 8 mL of alcohol, and in 50 mL of ether. Freely soluble in solutions of alkali hydroxides and carbonates; soluble in warm glycerin; sparingly soluble in diluted hydrochloric acid; slightly soluble in chloroform. Store in tight, light-resistant containers.

Assay—Accurately weigh about 300 mg, previously dried at 105° for 2 hours, and transfer to a beaker or casserole.

Add 5 mL of hydrochloric acid and 50 mL of water, and stir until dissolved. Cool to about 15°, add about 25 g of crushed ice, and slowly titrate with 0.1 M sodium nitrite VS until a glass rod dipped into the titrated solution produces an immediate blue ring when touched to starch iodide paper. When the titration is complete, the endpoint is reproducible after the mixture has been allowed to stand for 1 minute. Each mL of 0.1 M sodium nitrite is equivalent to 13.71 mg of C₇H₇NO₂. Not less than 98.5% is found.

Melting range (741): between 186° and 189°.

Loss on drying (731)—Dry it at 105° for 2 hours: it loses not more than 0.2% of its weight.

Residue on ignition (Reagent test): not more than 0.1%.

Paraformaldehyde, (CH₂O)_n [30525-89-4]—Fine, white powder.

Assay—Transfer about 1 g, accurately weighed, to a 250-mL conical flask containing 50.0 mL of 1 N sodium hydroxide VS, and mix by swirling. Immediately, and slowly, add 50 mL of hydrogen peroxide TS, previously neutralized to bromothymol blue, through a small funnel placed in the neck of the flask. After the reaction moderates, rinse the funnel and inner wall of the flask with water, allow the solution to stand for 30 minutes, add bromothymol blue TS, and titrate the excess alkali with 1 N sulfuric acid VS. Each mL of 1 N sodium hydroxide is equivalent to 30.03 mg of HCHO: not less than 95% is found.

Residue on ignition: not more than 0.1%.

Solubility in ammonia—Dissolve 5 g in 50 mL of ammonia TS: a practically clear, colorless solution results.

Reaction—Shake 1 g with 20 mL of water for about 1 minute, and filter: the filtrate is neutral to litmus.

Pectate Lyase [9015-75-2]—An enzyme obtained from *Aspergillus* sp. Light brown, viscous liquid. Specific gravity is about 1.5. It is readily soluble in water. It is supplied at approximately 14 units per mL (at pH 8.0 in Tris-HCl buffer [50 mM of Tris(hydroxymethyl)aminomethane containing 1 mM of CaCl₂, pH 8.0] in a solution of 50% glycerol and 0.02% sodium azide. One unit is defined as the enzyme activity that produces 1 μmol of unsaturated product per minute.

Activity—

PECTIN SOLUTION—Transfer a quantity of Pectin, equivalent to 0.05 g on the dried basis, to a 100-mL volumetric flask. [NOTE—Pectin has a molecular weight of 103,000 Da; its degree of esterification (percentage of galacturonic acid groups substituted with methyl) is 12.] Moisten with 0.1 mL of 2-propanol. Add 50 mL of water to the flask, and mix the solution with a magnetic stirrer. Use 0.5 N sodium hydroxide to adjust the solution to a pH of 12. Stop the stirrer, and allow the solution to stand undisturbed at room temperature for 15 minutes. Adjust the solution with 0.5 N hydrochloric acid to a pH of 8.0. Dilute with water to volume.

TRIS BUFFER SOLUTION—Transfer 6.055 g of Tris(hydroxymethyl)aminomethane and 0.147 g of calcium chloride (CaCl₂ · H₂O) to a 1000-mL volumetric flask containing 950 mL of water, and mix. Adjust the solution with 1 N hydrochloric acid to a pH of 8.0. Dilute with water to volume.

DILUTED PECTATE LYASE—Transfer 0.5 mL of Pectate Lyase to a 50-mL volumetric flask, dilute with *Tris buffer solution* to volume, and mix.

PROCEDURE—Add the solutions set forth in the table below to quartz cuvettes.

Label	Tris buffer solution (mL)	Pectin solution (mL)	Diluted pectate lyase (mL)	Water (mL)
Enzyme blank	0.5	1.0	0	1.0
Test blank	0.5	0	0.5	1.5
Test solution	0.5	1.0	0.5	0.5

Perform the test on the solutions so obtained, using a suitable UV-Vis spectrophotometer (see *Spectrophotometry and Light-Scattering* (851)) and using water as the blank. Mix the solutions well at time 0, and immediately measure the absorbances at 235 nm. Record the value for the *Enzyme blank*, A_{0-EB} ; for the *Test blank*, A_{0-TB} ; and for the *Test solution*, A_{0-TS} . After incubation at room temperature for 30 minutes, determine the absorbance again at 235 nm for the *Enzyme blank*, A_{30-EB} ; for the *Test blank*, A_{30-TB} ; and for the *Test solution*, A_{30-TS} . One unit is defined as the enzymatic activity that produces 1 μmol of unsaturated product from pectin per minute. Calculate the Pectate Lyase activity, in units per mL, using the following formula:

$$50(10^3)[(A_{30-TS} - A_{30-EB} - A_{30-TB}) - (A_{0-TS} - A_{0-EB} - A_{0-TB})]/30\epsilon_{235}L$$

in which 50 is the volume, in mL, of *Diluted pectate lyase*; 10^3 is the unit conversion factor; 30 is the time, in minutes, of the reaction; ϵ_{235} is the molar extinction coefficient, in $\text{M}^{-1}\text{cm}^{-1}$, of the reaction product ($4600 \text{ M}^{-1} \text{ cm}^{-1}$); and L is the path length, in cm, of the reaction cuvette (1 cm). Alternatively, these solutions, after being mixed in the cuvettes, can be immediately measured at 235 nm continuously in a recording UV-Vis spectrophotometer set up for kinetic assays. The result is obtained by correcting the blank determination, using the *Enzyme blank* and the *Test blank*.

Penicillinase—See *Beta-lactamase*.

Pentadecane, $\text{C}_{15}\text{H}_{32}$ —**212.41** [629-62-9]—Colorless liquid.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μm layer of phase G2; the injection port temperature is maintained at 280°; the detector temperature is maintained at 300°; and the column temperature is maintained at 180° and programmed to rise 10° per minute to 280°. The area of the $\text{C}_{15}\text{H}_{32}$ peak is not less than 99% of the total peak area.

Refractive index (831): between 1.430 and 1.434 at 20°.

Add the following:

▲ **Pentafluoropropanoic Acid** (*Pentafluoropropionic Acid*), $\text{C}_3\text{HF}_5\text{O}_2$ —**164.03** [422-64-0]—Use a suitable grade with a content of NLT 97%.▲ USP36

Pentane (*n*-Pentane), C_5H_{12} —**72.15** [109-66-0]—Clear, colorless, flammable liquid. Very slightly soluble in water. Miscible with alcohol, with ether, and with many organic solvents. Specific gravity: about 0.62.

Boiling range (Reagent test)—Not less than 95% distills between 34° and 36°.

1-Pentanesulfonic Acid Sodium Salt—See *Sodium 1-Pentanesulfonate*.

2-Pentanone, $\text{C}_5\text{H}_{10}\text{O}$ —**86.13** [107-87-9]—Use a suitable grade.

Pepsin [9001-75-6]—Use *Pepsin (Enzyme Preparations)* FCC, having an activity of 1.0 to 1.17 Pepsin units per mg. Pepsin of higher activity may be reduced to this activity by admixture with pepsin of lower activity or with lactose.

Pepsin, Purified—A white or yellowish-white powder, spongy mass, or translucent scales or granules. Freely soluble in water, producing more or less opalescence; practically insoluble in alcohol, in chloroform, and in ether. Purified Pepsin used in the second tier of the *Dissolution* test has an activity that is determined by the following method.

Activity—

PEPSIN SOLUTION—Transfer about 2.5 mg of Purified Pepsin, accurately measured, to a 100-mL volumetric flask, dilute with 10 mM hydrochloric acid to volume, and mix. [NOTE—Prepare immediately before use.]

2.0% HEMOGLOBIN SOLUTION—Dissolve and dilute 2.5 g of bovine hemoglobin with water to 100 mL. Dilute 80 mL of this solution with 0.3 M hydrochloric acid to a volume of 100 mL.

TRICHLOROACETIC ACID SOLUTION—Dilute 5 g of trichloroacetic acid with water to 100 mL.

TEST SOLUTION—Transfer 5.0 mL of 2.0% *Hemoglobin solution* to a suitable container equilibrated at 37°. Add 1.0 mL of *Pepsin solution*, mix by swirling, and incubate at 37° for 10 minutes. Immediately add 10.0 mL of *Trichloroacetic acid solution*, mix by swirling, and incubate at 37° for 5 minutes. Pass through a filter having a 0.8- μm or finer porosity.

CONTROL SOLUTION—Transfer 5.0 mL of 2.0% *Hemoglobin solution* to a suitable container equilibrated at 37°. Mix by swirling, and incubate at 37° for 10 minutes. Immediately add 10.0 mL of *Trichloroacetic acid solution* and 1.0 mL of *Pepsin solution*, mix by swirling, and incubate at 37° for 5 minutes. Pass through a filter having a 0.8- μm or finer porosity.

PROCEDURE—Determine the absorbances of the *Test solution* and *Control solution*, in 1-cm cells, at a wavelength of about 280 nm, using water as the reference. Calculate the activity of the portion of Purified Pepsin taken by the formula:

$$10,000(A_U - A_C)$$

in which A_U and A_C are the absorbances of the *Test solution* and the *Control solution*, respectively.

Peptic Digest of Animal Tissue (a bacteriological peptone)—Tan powder, having a characteristic, but not putrescent, odor. Soluble in water; insoluble in alcohol and in ether. An autoclaved solution (2 in 100) is clear and is neutral or nearly so in its reaction.

Nitrogen content—Determine by the Kjeldahl method, using a test specimen previously dried at 105° to constant weight: between 9.0% and 14.0% of nitrogen is found.

Residue on ignition (281)—Ignite 500 mg with 1 mL of sulfuric acid: the residue weighs not more than 75 mg (15%).

Loss on drying (731)—Dry it at 100° to constant weight: it loses not more than 7.0% of its weight.

Coagulable protein—Heat a filtered solution (1 in 20) to boiling: no precipitate forms.

Microbial content—NMT 10^4 cfu/g

Bacteriological test (for growth promotion and freedom from fermentable carbohydrates)—

Medium: 2% of digest and sufficient phenol red TS to give a perceptible color in water; adjust the pH to 7.2–7.4.

Place 10 mL of *Medium* in test tubes containing a Durham fermentation tube. Autoclave at 121° for 15 min. After autoclaving and standing for 24 h, the *Medium* is clear.

In separate tubes for each organism, inoculate 10 mL of *Medium* with *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 12953. Incubate at $35 \pm 2^\circ$ for 48 h.

It meets the following criteria for bacteria-nutrient properties: *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 12953 growth evident, no acid or only a trace in the inner tube, and no gas produced after incubation at 48 h.

Peptone, Dried (*Meat Peptone*)—Reddish-yellow to brown powder, having a characteristic, but not putrescent, odor. Soluble in water, forming a yellowish-brown solution having a slight acid reaction; insoluble in alcohol and in ether.

Nitrogen content (Reagent test)—Determine by the Kjeldahl method, using a test specimen previously dried at 105° to constant weight: between 12% and 18% of nitrogen is found.

Residue on ignition (Reagent test)—Ignite 500 mg with 1 mL of sulfuric acid: the residue weighs NMT 75 mg (15.0%).

Loss on drying (731)—Dry it at 105° to constant weight: it loses not more than 7.0% of its weight.

Coagulable protein—Heat a filtered solution (1 in 20) to boiling: no precipitate forms.

Microbial content—NMT 10⁴cfu/g

Perchloric Acid (70 Percent Perchloric Acid), HClO₄—**100.46** [7601-90-3]—Use ACS reagent grade (containing between 69.0% and 72.0% of HClO₄).

Periodic Acid, H₅IO₆—**227.94** [10450-60-9]—White to pale yellow crystals. Very soluble in water. Undergoes slow decomposition to iodic acid. Use ACS reagent grade.

Petroleum Benzin—See *Hexane, Solvent*.

Phases for Gas Chromatography—See phases for gas chromatography in the *Chromatographic Columns* section under *Chromatography* (621).

Phenacetin [62-44-2]—Use a suitable grade.

1,10-Phenanthroline (*Orthophenanthroline*), C₁₂H₈N₂ · H₂O—**198.22** [5144-89-8]—Use ACS reagent grade.

o-Phenanthroline Monohydrochloride Monohydrate, C₁₂H₈N₂ · HCl · H₂O—**234.69** [3829-86-5]—Use a suitable grade.

Phenol [108-95-2]—Use ACS reagent grade.

Phenol Red, Sodium, C₁₉H₁₃O₅Na—**376.4** [34487-61-1]—Red to brown powder. Use ACS reagent grade.

Phenolsulfonphthalein—Use *Phenol Red* (see *Indicators* under *Indicators and Indicator Test Papers*).

Phenoxybenzamine Hydrochloride [*N*-(2-Chloroethyl)-*N*-(1-methyl-2-phenoxyethyl)benzylamine Hydrochloride], C₁₈H₂₂ClNO · HCl—**340.29** [63-92-3]—White, crystalline powder.

Melting range (741): between 137° and 140°.

Absorptivity—Its absorptivity, 1%, 1 cm, in the range of 272 nm to 290 nm, in chloroform solution is about 178.

3-Phenoxybenzoic Acid, C₁₃H₁₀O₃—**214.22** [3739-38-6]—Use a suitable grade.

Melting range (741): between 149° and 150°.

2-Phenoxyethanol, C₆H₅OCH₂CH₂OH—**138.16** [122-99-6]—Colorless, slightly viscous liquid. Soluble in water. Miscible with alcohol, with acetone, and with glycerin. Density: about 1.107.

Assay—To 2 g, accurately weighed, add 10 mL of a freshly prepared solution made by dissolving 25 g of acetic anhydride in 100 g of anhydrous pyridine. Swirl to mix the liquids, heat on a steam bath for 45 minutes, add 10 mL of water, heat for 2 additional minutes, and cool. Add 10 mL of normal butyl alcohol, shake vigorously, add phenolphthalein TS, and titrate with 1 N sodium hydroxide VS. Perform a blank test using the same quantities of the same reagents, and in the same manner, and make any necessary correction. Each mL of 1 N sodium hydroxide is equivalent to 138.2 mg of C₈H₁₀O₂. Not less than 99% is found.

Phenol—Add 0.2 mL of it to 20 mL of water, mix, and to 5 mL of the mixture add 0.2 mL of Millon's reagent. Warm the solution at 60° for 90 seconds, and allow to stand: no pink or red color is produced within 1 minute.

Phenyl Ether—See *Diphenyl Ether*.

Phenyl Isocyanate, C₆H₅NCO—**119.12** [103-71-9]—Clear, colorless to straw-yellow liquid of medium volatility. **[CAUTION—Phenyl Isocyanate is a violent lacrimator, and the vapor is highly toxic. Handle with care.]**

Assay—Transfer 250 mg, accurately weighed, to a glass-stoppered, 250-mL flask. Exercise care to avoid loss by volatilization, and avoid breathing the vapor. Add 20 mL of butylamine solution (25 g of butylamine diluted to 1000 mL with dioxane previously dried over potassium hydroxide pellets), insert the stopper in the flask, and allow to stand for 15 minutes. Add a few drops of methyl red TS and 25 mL of water, and titrate the excess amine with 0.1 N sulfuric acid VS. Perform a blank titration on 20 mL of the butylamine solution (see *Residual Titrations* (541)). Subtract the volume of 0.1 N sulfuric acid consumed in the test specimen titration from that consumed in the blank titration. Each mL of

0.1 N sulfuric acid, representing this difference, is equivalent to 11.91 mg of C₆H₅NCO: not less than 97.0% of C₆H₅NCO is found.

2-Phenylacetamide (*α-Phenylacetamide*), C₈H₉NO—**135.16** [103-81-1]—Bimorphous plates or leaflets. Slightly soluble in water. Use a suitable grade.

Melting range (741): between 156° and 158°.

dl-Phenylalanine, C₉H₁₁NO₂—**165.19** [150-30-1]—Use a suitable grade.

o-Phenylenediamine Dihydrochloride, C₆H₈N₂ · 2HCl—**181.1**—White powder.

Assay—When tested by thin-layer chromatography, with the use of plates coated with chromatographic silica gel mixture and a developing system consisting of a mixture of butyl alcohol, water, and acetic acid (12:5:3), and examined under short-wavelength UV light, a single spot is exhibited, with trace impurities.

p-Phenylenediamine Dihydrochloride—See *p-Phenylene-diamine Hydrochloride*.

p-Phenylenediamine Hydrochloride (1,4-Diaminobenzene Dihydrochloride), C₆H₈N₂ · 2HCl—**181.06**—White to pale tan crystals or crystalline powder, turning red on exposure to air. Freely soluble in water; slightly soluble in alcohol and in ether. Preserve in well-closed containers, protected from light.

Insoluble matter—Dissolve 1 g in 10 mL of water: the solution is clear and complete.

Molar absorptivity (see *Spectrophotometry and Light-scattering* (851))—Dissolve 60 mg in 100.0 mL of water, and mix. Pipet 2 mL of this solution into a 50-mL volumetric flask, dilute with pH 7 buffer solution to volume, and mix. The molar absorptivity of this solution, at 239 nm, is not less than 9000.

Phenylglycine (D(-)-2-Phenylglycine), (C₆H₅CH(NH₂)COOH)—**151.17** [875-74-1]—Use a suitable grade.

Phenylhydrazine, C₆H₅NHNH₂—**108.14** [100-63-0]—A colorless, or slightly yellowish, highly refractive liquid. **[NOTE—Protect from light, and distill under reduced pressure shortly prior to use.]**

Congearing temperature (651): not below 16°.

Insoluble matter—Shake 1 mL with 20 mL of diluted acetic acid: the resulting solution is clear or practically so.

Residue on ignition (Reagent test)—Ignite 1 mL with 0.5 mL of sulfuric acid: the residue weighs not more than 1 mg (0.1%).

Phenylhydrazine Hydrochloride, C₆H₅NHNH₂ · HCl—**144.60** [59-88-1]—White or yellowish crystals or powder. Soluble in water and in alcohol. Store in tight containers, protected from light. Use a suitable grade with a content of not less than 99%.

Phenylmethylsulfonyl Fluoride, C₇H₇FO₂S—**174.2** [329-98-6]—White to faint yellow powder. Use a suitable grade.

[NOTE—A suitable grade is available from Sigma-Aldrich, www.sigma-aldrich.com.]

3-Phenylphenol (*m*-Phenylphenol), C₆H₅C₆H₄OH—**170.21** [580-51-8]—White to off-white, crystalline powder.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm × 30-m capillary column coated with G1; the injection port temperature is maintained at 250°; the column temperature is maintained at 150° and programmed to rise 15° per minute to 250°; and the detector temperature is maintained at 310°. The area of the 3-phenylphenol peak is not less than 98% of the total peak area.

Melting range (741): between 76° and 79°.

Phloroglucinol, C₆H₃(OH)₃ · 2H₂O—**162.14** [6099-90-7]—White or yellowish-white crystals or a crystalline powder. Soluble in alcohol and in ether; slightly soluble in water.

Insoluble in alcohol—Dissolve 1 g in 20 mL of alcohol: a clear and complete solution results.

Melting range, Class Ia (741): between 215° and 219°.

Residue on ignition (Reagent test)—Ignite 1 g with 0.5 mL of sulfuric acid: the residue weighs not more than 1 mg (0.1%).

Diresorcinol—Heat to boiling a solution of 100 mg in 10 mL of acetic anhydride, cool the solution, and superimpose it upon 10 mL of sulfuric acid: no violet color appears at the zone of contact of the liquids.

Phloxine B (*Acid red 92; Eosin 10B; 2',4',5',7'-tetrabromo-4,5,6,7-tetrachlorofluorescein disodium salt*) $C_{20}H_2Br_4Cl_4Na_2O_5$ —**829.63** [18472-87-2]—Use a suitable grade with a dye content of not less than 80%, certified by the Biological Stain Commission.

Phosphatase Enzyme, Alkaline—Use a suitable grade from intestinal origin.

[NOTE—A suitable grade is available from Worthington Biochemical Corp., www.worthington-biochem.com.]

Phosphatic Enzyme—An enzyme preparation of microbial origin, high in both phosphatase and amylase activity, the former being the property that renders it suitable for use in the liberation of thiamine from its orthophosphate and pyrophosphate esters. Light cream-colored or slightly gray powder. Freely soluble in water. It hydrolyzes 300 times its weight of starch in 30 minutes.

Amylase activity—Place in a test tube 5 mL of a 1 in 50 solution of soluble starch in 0.2 M, pH 5 sodium acetate buffer (containing 1.6 g of anhydrous sodium acetate in each L and sufficient glacial acetic acid to adjust to a pH of 5), and add 4 mL of water. Mix, and place in a water bath at 40°. Add 1 mL of a solution containing 0.3 mg of the phosphatic enzyme, mix, and note the exact time. After 30 minutes remove 1.0 mL of the mixture, and add it to 5.0 mL of 0.0005 N iodine in a 20- × 150-mm test tube: a clear, red color results.

Phosphomolybdic Acid, approximately $20MoO_3 \cdot P_2O_5 \cdot 51H_2O$ —**3939.49** [11104-88-4]—Use ACS reagent grade.

Phosphoric Acid, H_3PO_4 —**98.00** [7664-38-2]—Use ACS reagent grade.

Phosphorous Acid (*Phosphonic Acid*), H_3O_3P —**82.00** [13598-36-2]—Use a suitable grade with a content of not less than 99%.

Phosphorus, Red, P—At. Wt. 30.97376—A dark red powder. Insoluble in water and in dilute acids; soluble in dehydrated alcohol.

Yellow phosphorus—Shake 20 g with 75 mL of carbon disulfide in a glass-stoppered vessel, and allow to stand in the dark overnight. Filter, and wash the residue with carbon disulfide until the filtrate, collected in a graduated cylinder, measures 100 mL. Evaporate the solvent to 10 mL by immersing the cylinder in hot water. Dip a strip of cupric sulfate test paper in the remaining solvent: no more color is produced than in a similar strip dipped into 10 mL of solution in carbon disulfide containing 3 mg of yellow phosphorus (0.015% as P).

Soluble substances—Digest 2 g with 30 mL of acetic acid on a steam bath for 15 minutes. Cool, dilute with water to 40 mL, and filter. Evaporate 20 mL of the filtrate on a steam bath, and dry at 105° for 2 hours: the residue weighs not more than 6 mg (0.6%).

Phosphorus Pentoxide (*Phosphoric Anhydride*), P_2O_5 —**141.94** [1314-56-3]—Use ACS reagent grade.

Phosphotungstic Acid, approximately $24WO_3 \cdot P_2O_5 \cdot 51H_2O$ —**6624.84**—White or yellowish-green crystals or a crystalline powder. Soluble in water, in alcohol, and in ether.

Insoluble matter (Reagent test): not more than 1 mg, from 5 g (0.02%).

Chloride (Reagent test)—One g shows not more than 0.3 mg of Cl (0.03%).

Nitrate—Dissolve 500 mg in 10 mL of water, and add about 10 mg of sodium chloride, 0.1 mL of indigo carmine TS, and 10 mL of sulfuric acid: the blue color does not disappear within 1 minute (about 0.01%).

Sulfate (Reagent test, *Method I*)—A 500-mg portion shows not more than 0.1 mg of SO_4 (0.02%).

o-Phthalaldehyde (*Phthalic Dicarboxaldehyde*), $C_6H_4(CHO)_2$ —**134.13** [643-79-8]—Use a suitable grade.
Phthalazine, $C_8H_6N_2$ —**130.15** [253-52-1]—Yellow to tan crystals.

Melting range (741): between 89° and 92°.

Phthalic Acid, $C_8H_6O_4$ —**166.13** [88-99-3]—Use ACS reagent grade.

Phthalic Anhydride, $C_8H_4O_3$ —**148.12** [85-44-9]—Use ACS reagent grade.

Phthalimide, $C_8H_5NO_2$ —**147.13** [85-41-6]—White powder.

Assay—

MOBILE PHASE—Prepare a mixture of isooctane and methyl-tert-butyl ether (88:12).

PROCEDURE—Inject about 20 μ L into a suitable liquid chromatograph (see *Chromatography* (621)) equipped with a 230-nm detector and a 4.6-mm × 15-cm column that contains packing L3. The flow rate is about 2 mL per minute. The area of the $C_8H_5NO_2$ peak is not less than 99% of the total peak area.

Melting range (741): between 233° and 235°, with decomposition.

2-Picoline, C_6H_7N —**93.13** [109-06-8]—Colorless to yellowish liquid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 2-mm × 2-m glass column packed with 20% liquid phase G16 on 80- to 100-mesh support S1C; the injection port temperature is maintained at 140°; the detector temperature is maintained at 300°; the column temperature is maintained at 90° and programmed to rise 3° per minute to 140°. The area of the C_6H_7N peak is not less than 98% of the total peak area.

Refractive index (831): 1.500 ± 0.002 at 20°.

Picric Acid (*2,4,6-Trinitrophenol; Trinitrophenol*), $C_6H_2(OH)(NO_2)_3$ —**229.10** [88-89-1]—Use ACS reagent grade.

Picrolonic Acid (*3-Methyl-4-nitro-1-(p-nitrophenyl)-5-pyrazolone*), $C_{10}H_8N_4O_5$ —**264.19** [550-74-3]—Yellow to brownish-yellow, crystalline powder. Slightly soluble in water; soluble in alcohol, in chloroform, in ether, in benzene, and in solutions of alkali hydroxides.

Melting range (741): between 115° and 117°.

Residue on ignition (Reagent test): negligible, from 200 mg.

Sensitiveness—Dissolve 25 mg in 10 mL of warm water containing 0.1 mL of glacial acetic acid, and filter the solution, if necessary. Dissolve 100 mg of calcium chloride in 250 mL of water, and mix. Heat 1 mL of the calcium chloride solution in a test tube to about 60°, then add to it 1 mL of the picrolonic acid solution: a bulky precipitate forms in 5 minutes or less.

Pipemidic Acid (*8-Ethyl-3,8-dihydro-5-oxo-2-(1-piperazinyl)pyrido[2,3-d]pyrimidine-6-carboxylic acid*), $C_{14}H_{17}N_5O_3$ —**303.3** [51940-44-4]—Use a suitable grade.

Piperazine (*Diethylenediamine*), $C_4H_{10}N_2 \cdot 6H_2O$ —**194.23**—Use a suitable grade.

Piperidine, $C_5H_{11}N$ —**85.15** [110-89-4]—Colorless liquid. Miscible with water and with alcohol. Specific gravity: about 0.860.

Congealing range (651): between 12° and 15°.

Boiling range (Reagent test)—Not less than 95% distills between 104° and 106°.

Refractive index: about 1.454.

Platinic Chloride (*Chloroplatinic Acid*), $\text{H}_2\text{PtCl}_6 \cdot 6\text{H}_2\text{O}$ —**517.90** [18497-13-7]—Use ACS reagent grade Chloroplatinic Acid.

Polydimethylsiloxane, *viscosity 0.65 centistokes* (*Hexamethyldisiloxane*), $(\text{CH}_3)_3\text{SiOSi}(\text{CH}_3)_3$ —**162.38** [107-46-0]—Liquid. Freezes at about 0°.

Refractive index (831): about 1.3770.

Specific gravity (841): about 0.760.

Polyethylene Glycol 200, $\text{H}(\text{OCH}_2\text{CH}_2)_n\text{OH}$ in which the average value of n is 4—average molecular weight 200 [25322-68-3]—Clear, colorless or almost colorless, viscous, hygroscopic liquid. Very soluble in acetone and in alcohol; practically insoluble in ether and in fatty oils. Use a suitable grade.

Refractive index (831): 1.4590 at 20°.

Density: 1.127 at 25°.

Viscosity: 4.3 centistokes at 98.9°.

Polyethylene Glycol 600 [25322-68-3]—A clear, practically colorless, viscous liquid condensation polymer represented by $\text{H}(\text{OCH}_2\text{CH}_2)_n\text{OH}$, in which n varies from 12 to 14. Its average molecular weight is about 600.

It meets the requirements of all of the tests under *Polyethylene Glycol* (NF monograph) except *Limit of ethylene glycol and diethylene glycol*.

Polyethylene Glycol 20,000 [25322-68-3]—Molecular weight range: 15,000–20,000. Hard, white, waxy solid, usually supplied in flake form. Soluble in water with subsequent gel formation.

Viscosity of 25% solution (911)—Add 50.0 g of test specimen to a 250-mL wide-mouth, screw-cap jar containing 150.0 g of water. Attach the cap securely to the jar, and roll on a mechanical roller until the test specimen is completely dissolved, in 2 to 4 hours. Allow the solution to stand until all air bubbles have disappeared. Another 2 to 4 hours may be required. Adjust the temperature of the solution to $37.8 \pm 0.1^\circ$, and determine the kinematic viscosity on a suitable viscometer of the Ubbelohde type. The viscosity is not less than 100 centistokes.

pH (791): between 6.5 and 8.0 in a solution (1 in 20). [NOTE—A five-fold dilution of the test solution prepared for the *Viscosity of 25% solution* test may be used.]

Residue on ignition (281): not more than 0.7%, the use of sulfuric acid being omitted.

Polyoxyethylene (23) Lauryl Ether (*Brij-35*)—Use a suitable grade.

[NOTE—A suitable grade is available commercially as “Brij-35.”]

Polyoxyethylene (20) Sorbitan Monolaurate [9005-64-5]—Viscous, light yellow to yellow-green liquid. Use a suitable grade.

Polysaccharide Molecular Weight Standards—Polymaltotriose polymers of different weight-average molecular weight, M_w , values ranging from 5,000 to 400,000 Da. [NOTE—A suitable set is available from Shodex (www.shodex.com) as Kit P-82.]

Polystyrene Cation-Exchange Resin—See *Cation-Exchange Resin, Polystyrene*.

Polytef—Use *Poly(tetrafluoroethylene)*.

Polyvinyl Alcohol, $(\text{C}_2\text{H}_4\text{O})_n$ [9002-89-5]—White powder. Soluble in water; insoluble in organic solvents.

pH (791): between 5.0 and 8.0, in a solution (1 in 25).

Loss on drying—Dry it at 110° to constant weight: it loses not more than 5% of its weight.

Residue on ignition: not more than 0.75%.

[NOTE—Suitable grades are available as catalog number U 232, from J.T. Baker Chemical Co., www.jtbaker.com.]

Potassium Acetate, $\text{KC}_2\text{H}_3\text{O}_2$ —**98.14** [127-08-2]—Use ACS reagent grade.

Potassium Alum—Use *Potassium Alum* [see *Potassium Alum* (USP monograph)].

Potassium Arsenate Monobasic, KH_2AsO_4 —**180.03** [7784-41-0]—Use a suitable grade with a content of NLT 98%.

Potassium Bicarbonate, KHCO_3 —**100.12** [298-14-6]—Use ACS reagent grade.

Potassium Biphosphate—See *Potassium Phosphate, Monobasic*.

Potassium Biphthalate (*Acid Potassium Phthalate; Phthalic Acid Monopotassium Salt; Potassium Hydrogen Phthalate Acidimetric Standard*), $\text{KHC}_6\text{H}_4(\text{COO})_2$ —**204.22** [877-24-7]—Use ACS reagent grade Potassium Hydrogen Phthalate, Acidimetric Standard.

Potassium Bisulfate, KHSO_4 —**136.17** [7646-93-7]—Fused, white, deliquescent masses or granules. Very soluble in water. When ignited, it evolves SO_3 and H_2O , changing first to potassium pyrosulfate, then to sulfate.

Acidity—Dissolve 4 g, accurately weighed, in 50 mL of water, add phenolphthalein TS, and titrate with 1 N alkali: it contains between 34% and 36%, calculated as H_2SO_4 .

Insoluble matter and ammonium hydroxide precipitate—Dissolve 10 g in 100 mL of water, add methyl red TS, render slightly alkaline with ammonia TS, boil for 1 minute, and digest on a steam bath for 1 hour. Pass through a tared filtering crucible, wash thoroughly, and dry at 105° for 2 hours: the precipitate weighs not more than 1 mg (0.01%).

For the following tests, prepare a *Test solution* as follows. Dissolve 6 g in 45 mL of water, add 2 mL of hydrochloric acid, boil gently for 10 minutes, cool, and dilute with water to 60 mL.

Heavy metals (Reagent test)—To 30 mL of *Test solution* add phenolphthalein TS, and neutralize with ammonia TS. Add 0.5 mL of glacial acetic acid, dilute with water to 40 mL, and add 10 mL of hydrogen sulfide TS: any brown color produced is not darker than that of a control containing 10 mL of *Test solution* and 0.02 mg of added Pb (0.001%).

Iron (241)—To 5 mL of *Test solution* add 2 mL of hydrochloric acid, and dilute with water to 47 mL: the solution shows not more than 0.01 mg of Fe (0.002%).

Potassium Bromate, KBrO_3 —**167.00** [7758-01-2]—Use ACS reagent grade.

Potassium Bromide, KBr —**119.00** [7758-02-3]—Use ACS reagent grade.

Potassium Carbonate—See *Potassium Carbonate, Anhydrous*.

Potassium Carbonate, Anhydrous, K_2CO_3 —**138.21** [584-08-7]—Use ACS reagent grade.

Potassium Chlorate, KClO_3 —**122.55** [3811-04-9]—Use ACS reagent grade.

Potassium Chloride, KCl —**74.55** [7447-40-7]—Use ACS reagent grade.

Potassium Chloroplatinate, K_2PtCl_6 —**485.99**—Heavy, yellow powder. Soluble in hydrochloric acid and in nitric acid.

Assay—Accurately weigh about 300 mg, transfer to a 600-mL beaker, add 20 mL of hydrochloric acid, and heat gently if necessary to achieve complete solution. Add zinc granules, slowly, until no more dissolves, then add 2 mL of hydrochloric acid, and digest for 1 hour on a steam bath to coagulate the reduced platinum. Add more acid, if necessary, to ensure that all of the zinc has dissolved. Filter through paper, rinsing the beaker with diluted hydrochloric acid until all of the precipitate is transferred to the filter, then wash with several small portions of water. Ignite the filter in a tared crucible at $800 \pm 25^\circ$ to constant weight.

Each mg of residue is equivalent to 1.0 mg of platinum. Not less than 40% is found.

Potassium Chromate, K_2CrO_4 —**194.19** [7789-00-6]—Use ACS reagent grade.

Potassium Cyanide, KCN —**65.12** [151-50-8]—Use ACS reagent grade.

Potassium Dichromate, $K_2Cr_2O_7$ —**294.18** [7778-50-9]—Use ACS reagent grade.

[NOTE—Potassium dichromate of a quality suitable as a primary standard is available from the National Institute of Standards and Technology, Washington, DC, www.nist.gov, as standard sample No. 136.]

Potassium Ferricyanide, $K_3Fe(CN)_6$ —**329.24** [13746-66-2]—Use ACS reagent grade.

Potassium Ferrocyanide, $K_4Fe(CN)_6 \cdot 3H_2O$ —**422.39** [14459-95-1]—Use ACS reagent grade.

Potassium Hyaluronate—White to cream-colored powder. Freely soluble in water. Store in a tight container, in a refrigerator.

Inhibitor content—Prepare as directed in the Assay under *Hyaluronidase for Injection* (USP monograph) a quantity of *Standard solution* containing 1 USP Hyaluronidase Unit in each mL, and a similar quantity of acetate-buffered *Standard solution* using as the solvent 0.1 M, pH 6 sodium acetate buffer (prepared by diluting the 0.2 M buffer prepared as directed below with an equal volume of water). Prepare from the potassium hyaluronate under test 10 mL of *Potassium hyaluronate stock solution*, and dilute 2 mL of it with the specified *Phosphate buffer solution* to make a *Hyaluronate solution*. In the same way, and concurrently, dilute a second 2-mL portion of the stock solution with 0.2 M, pH 6 sodium acetate buffer (containing 16.4 g of anhydrous sodium acetate and 0.45 mL of glacial acetic acid in each 1000 mL).

Place 0.50-mL portions of the *Hyaluronate solution* in each of four 16- × 100-mm test tubes, and place 0.50-mL portions of the acetate-buffered *Hyaluronate solution* in two similar tubes. To two of the four tubes containing *Hyaluronate solution* add 0.50 mL of *Diluent for hyaluronidase solutions*, prepared as directed in the Assay under *Hyaluronidase for Injection* (USP monograph). To the remaining two tubes, on a rigid schedule, at 30-second intervals, add 0.50 mL of *Standard solution*. Similarly, to the two tubes containing acetate-buffered *Hyaluronate solution* add at 30-second intervals 0.50-mL portions of acetate-buffered *Standard solution*. Then proceed as directed in the second paragraph for *Procedure*, beginning with "Mix the contents," as far as "Plot the average." The reduction in absorbance of acetate-buffered *Hyaluronate solution* is not less than 25% of that observed in the *Hyaluronate solution*.

Turbidity production—The average absorbance of the solutions in the two tubes containing *Hyaluronate solution* and *Diluent for hyaluronidase solutions* prepared in the test for *Inhibitor content* is not less than 0.26 at a wavelength of 640 nm in a suitable spectrophotometer using a 1-cm cell.

Potassium Hydrogen Sulfate, $KHSO_4$ —**136.17**—White crystals. Soluble in water.

Melting point (741): about 197°.

Potassium Hydroxide, KOH —**56.11** [1310-58-3]—Use ACS reagent grade.

Potassium Iodate, KIO_3 —**214.00** [7758-05-6]—Use ACS reagent grade.

Potassium Iodide, KI —**166.00** [7681-11-0]—Use ACS reagent grade.

Potassium Metabisulfite (*Potassium Disulfite*; *Potassium Pyrosulfite*), $K_2S_2O_5$ —**222.32** [16731-55-8]—Use a suitable grade with a content of not less than 98%.

Potassium Nitrate, KNO_3 —**101.10** [7757-79-1]—Use ACS reagent grade.

Potassium Nitrite, KNO_2 —**85.10** [7758-09-0]—Use ACS reagent grade.

Potassium Perchlorate, $KClO_4$ —**138.55** [7778-74-7]—Use ACS reagent grade.

Potassium Periodate (*Potassium meta-Periodate*), KIO_4 —**230.00** [7790-21-8]—Use ACS reagent grade.

Potassium Permanganate, $KMnO_4$ —**158.03** [7722-64-7]—Use ACS reagent grade.

Potassium Persulfate, $K_2S_2O_8$ —**270.32** [7727-21-1]—Use ACS reagent grade Potassium Peroxydisulfate.

Potassium Phosphate, Dibasic (*Dipotassium Hydrogen Phosphate*; *Dipotassium Phosphate*), K_2HPO_4 —**174.18** [7758-11-4]—Use ACS reagent grade.

Potassium Phosphate, Dibasic, Trihydrate (*Dipotassium Hydrogen Phosphate Trihydrate*; *Dipotassium Phosphate*), $K_2HPO_4 \cdot 3H_2O$ —**228.22** [16788-57-1]—Use a suitable grade with a content of NLT 99.0%.

Potassium Phosphate, Monobasic (*Potassium Biphosphate*; *Potassium Dihydrogen Phosphate*), KH_2PO_4 —**136.09** [7778-77-0]—Use ACS reagent grade.

[NOTE—Certified Potassium Dihydrogen Phosphate is available from the National Institute of Standards and Technology, Washington, DC, www.nist.gov, as standard sample No. 186.]

Potassium Phosphate, Tribasic, K_3PO_4 —**212.27** [7778-53-2]—Deliquescent, orthorhombic crystals. Use ACS reagent grade.

Potassium Pyroantimonate (*Potassium hexahydroxyantimonate*), $KSb(OH)_6$ —**262.90** [12208-13-8]—White crystals or a white, crystalline powder. Sparingly soluble in water. Use a suitable grade.

Potassium Pyrophosphate, $K_4P_2O_7$ —**330.34** [7320-34-5]—Colorless, deliquescent granules. Freely soluble in water; insoluble in alcohol.

Potassium Pyrosulfate [7790-62-7]—Usually available as a mixture of potassium pyrosulfate ($K_2S_2O_7$) and potassium bisulfate ($KHSO_4$). Use ACS reagent grade.

Potassium Sodium Tartrate, $KNaC_4H_4O_6 \cdot 4H_2O$ —**282.22** [6381-59-5]—Use ACS reagent grade.

Potassium Sulfate, K_2SO_4 —**174.26** [7778-80-5]—Use ACS reagent grade.

Potassium Tellurite (*Potassium Tellurate IV*), K_2TeO_3 —**253.79** [7790-58-1]—White, granular powder. Soluble in water. Its solution is alkaline.

Assay—Weigh accurately about 120 mg, transfer to a beaker, and dissolve in a mixture of 10 mL of nitric acid, 10 mL of sulfuric acid, and 25 mL of water. Heat to boiling, and boil until copious fumes of sulfur trioxide are evolved. Cool, cautiously add 100 mL of water, heat to boiling, add 6 g of sodium fluoride, and titrate the hot solution with 0.1 N potassium permanganate VS. Each mL of 0.1 N potassium permanganate is equivalent to 12.69 mg of K_2TeO_3 . Not less than 98% is found.

Chloride (Reagent test)—One g shows not more than 0.1 mg of Cl (0.01%).

Potassium Thiocyanate, $KSCN$ —**97.18** [333-20-0]—Use ACS reagent grade.

Potato Starch—See *Starch, Potato*.

Propionaldehyde, C_3H_6O —**58.08** [123-38-6]—Use a suitable grade.

Propionic Anhydride, $C_6H_{10}O_3$ —**130.14** [123-62-6]—Colorless liquid. Is decomposed by water. Soluble in methanol, in alcohol, in ether, and in chloroform.

Assay—Accurately weigh about 350 mg into a tared, glass-stoppered flask containing 50 mL of dimethylformamide previously neutralized to the thymol blue endpoint with 0.1 N sodium methoxide in methanol VS. Titrate with 0.1 N sodium methoxide in methanol VS to the thymol blue endpoint. Perform a blank determination, and make any

necessary correction. Each mL of 0.1 N sodium methoxide is equivalent to 13.014 mg of $C_6H_{10}O_3$. Not less than 97.0% is found.

Refractive index (831): between 1.4035 and 1.4045 at 20°.

Propiophenone, $C_9H_{10}O$ —**134.18** [93-55-0]—Use a suitable grade.

iso-Propyl Alcohol—See *Isopropyl Alcohol*.

n-Propyl Alcohol (1-Propanol), $CH_3CH_2CH_2OH$ —**60.10** [71-23-8]—Use ACS reagent grade.

Propylamine Hydrochloride (1-Propanamine hydrochloride; n-Propylamine hydrochloride), $C_3H_9N \cdot HCl$ —**95.57** [556-53-6]—Use a suitable grade with a content of not less than 99%.

Protein Molecular Weight Standard—Also known as protein molecular weight markers (for SDS-PAGE) and consists of a mixture of several proteins of well-defined molecular weights. The products are generally available in a suitable buffer containing a suitable reducing agent (generally, 100 mM DTT), a preservative (for example, sodium azide), and 50% glycerol to prevent freezing. Use a suitable grade. Store at -20°.

Protein Standard Solution (8 g/dL)—A solution containing 5 g of Albumin Human and 3 g of human gamma globulin per dL.

[NOTE—A suitable grade is available as Protein Standard Solution, catalog number 540-10, from Sigma-Aldrich, www.sigma-aldrich.com.]

Protocatechuic Acid (3,4-Dihydroxybenzoic acid), $C_7H_6O_4$ —**154.12** [99-50-3]—Use a suitable grade.

Pullulanase (Amylopectin-6-gluconohydrolase) [9075-68-7]—An enzyme obtained from *Klebsiella pneumoniae*. It contains not less than 30 units per mg of protein. One unit defined for enzymatic activity will liberate 1.0 μ mol of maltotriose (measured as glucose) from pullulan per minute at pH 5.0 at 30°. It can be suspended in 3.2 M ammonium sulfate solution, pH 6.2.

Measurement of relative pullulanase activity—

DETERMINATION OF PULLULANASE ACTIVITY—

Substrate—Dissolve pullulan¹ in water to make a 1.25% (w/v) solution. [NOTE—Add pullulan to the water. Clumping will occur if water is added to pullulan.]

Buffer solution A, pH 5.0—Add 0.1 M disodium phosphate (27 g of dibasic sodium phosphate in each L of the solution) to 0.1 M citric acid (21 g of citric acid in each L of the solution) to adjust pH to 5.0.

Buffer solution B, pH 6.0—Add diluted acetic acid to 1 M sodium acetate (136 g of sodium acetate in each L of solution) to adjust the pH to 6.0. Dilute with water to prepare the final buffer solution as 0.01 M acetic acid buffer, pH 6.0.

Somogyi reagent—Add 54 g of disodium phosphate heptahydrate or 28 g of anhydrous disodium hydrogen phosphate and 40 g of potassium sodium tartrate to about 650 mL of water or about 700 mL for anhydrous disodium hydrogen phosphate. Add 100 mL of 1 N sodium hydroxide to this solution and mix. Add 80 mL of 10% cupric sulfate to the solution, and mix. Heat until any solid is completely dissolved. Add 180 g of anhydrous sodium sulfate to the solution and adjust the volume to 1 L. Allow the solution to stand at room temperature for 1 or 2 days to let insoluble matter precipitate. Filter the solution with standard filter paper, and keep the solution in a brown bottle with a ground-glass stopper.

Nelson reagent—Dissolve 50 g of ammonium molybdate in 900 mL of water. Add 42 g of sulfuric acid, and mix. Dissolve 6 g of sodium arsenate or 3.6 g of monobasic potassium arsenate in 50 g of water. Allow the solution to stand in a brown bottle with a ground-glass stopper at 37° for 1 or 2 days.

¹A suitable supplier for pullulan is www.hayashibara-intl.com.

Glucose standard solution—Dry anhydrous glucose crystals under less than 50-mm Hg at 60° for 5 hours, and calculate the water content. Transfer 10.00 g of dried glucose to a 1-L volumetric flask, dissolve in and dilute with water to volume, and mix. Transfer 10.0 mL to a 1-L volumetric flask and dilute to volume with water. Each mL contains 100 μ g of glucose.

Pullulanase diluent—Dilute pullulanase with *Buffer solution B*, pH 6.0 to prepare a solution having the enzyme activity of about 0.2 units per mL. [NOTE—The measurement range is between 0.1 and 0.4 units per mL.] Record the dilution factor (F_{PD}). This diluent is used as a diluted enzyme solution.

Procedure—Transfer 4 mL of *Substrate* to a test tube and add 0.5 mL of *Buffer solution A*, pH 5.0, mix, and incubate at 30°. Add 0.5 mL of *Pullulanase diluent*, and mix thoroughly. After 30 seconds, transfer 1 mL of this solution to a test tube labeled as "Pullulan test solution 1", and add 2 mL of *Somogyi reagent*, and mix. After 30 minutes and 30 seconds, transfer 1 mL of the mixture of *Substrate* and *Pullulanase diluent* to a second test tube labeled as "Pullulan test solution 2", add 2 mL of *Somogyi reagent*, and mix. In a third test tube labeled as "Standard blank", mix 2 mL of *Somogyi reagent* and 1 mL of water. In a fourth test tube labeled as "Glucose standard solution", mix 2 mL of *Somogyi reagent* and 1 mL of *Glucose standard solution*, and add 1 mL of water. Incubate the fourth test tube in a boiling water bath for exactly 10 minutes. Remove the tube and allow it to cool in cold running water. Add 2 mL of *Nelson reagent*, mix well, and allow the solution to stand for at least 15 minutes. Add 5 mL of water to each of the four test tubes, and mix thoroughly. Determine the absorbance at 520 nm of the Standard blank, A_{blank} , of the *Glucose standard solution*, A_{Std} , of the Pullulan test solution 1, A_0 , and of the Pullulan test solution 2, A_{30} , using water as the blank. One unit is defined as the enzymatic activity that produces 1 μ mol of maltotriose (measured as glucose) from pullulan per minute. Calculate the pullulanase activity, PA, in units per mL, using the formula:

$$PA = [(A_{30} - A_0)/(A_{Std} - A_{blank})] \times 0.185 \times F_{PD}$$

MEASUREMENT OF PROTEIN AMOUNT (MEASURED AS ALBUMINOID AMOUNT) FOR THE CALCULATION OF SPECIFIC ACTIVITY—

Reagent A—Prepare a solution having known concentrations of about 0.1 N sodium hydroxide and about 0.2 M sodium carbonate.

Reagent B—Transfer 0.5 g of cupric sulfate and 1.0 g of sodium citrate dihydrate to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

Lowry solution—Mix *Reagent A* and *Reagent B* at the proportion of 50:1.

Diluted Folin-Ciocalteu's phenol reagent (for albuminoid quantification)—Prepare a two-fold dilution of 2 N Folin-Ciocalteu's phenol reagent commercially available or prepare a solution by making an appropriate dilution from Folin-Ciocalteu Phenol TS (see *Method 2* in *Biotechnology-Derived Articles—Total Protein Assay* (1057)).

Bovine serum albumin standard stock solution—Transfer 0.05 g of bovine serum albumin to a 500-mL volumetric flask, dissolve in and dilute with water to volume, and mix. It contains 100 μ g of bovine serum albumin per mL.

Standard solutions—Using appropriate dilutions of *Bovine serum albumin standard stock solution* in water, prepare five *Standard solutions* having concentration equally spaced between 5 and 100 μ g of bovine serum albumin per mL.

Test solution—Dilute pullulanase with *Buffer solution B*, pH 6.0 in order to obtain a solution having a concentration between 60 and 70 μ g of albuminoid per mL. [NOTE—Water can be used as diluent.] Record the dilution factor, F_{TS} .

Blank solution—Use water.

Procedure—To 0.3 mL in separate tubes of the *Standard solutions*, the *Test solution*, and the *Blank solution*, add 3 mL of *Lowry solution*, and mix. Allow to incubate at room temperature for 10 minutes. Add 0.3 mL of *Diluted Folin-Ciocalteu's phenol reagent* to each tube, mix immediately, and allow to stand at room temperature for 60 minutes. Determine the absorbances of the *Standard solutions* and the *Test solution* at the wavelength of maximum absorbance at about 750 nm, using the *Blank solution* as the blank.

Calculation—[NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using linear regression method, plot the absorbances of the *Standard solutions* versus the protein (bovine serum albumin) concentrations, in μg per mL, and determine the best fit curve. Using the plot, determine the concentration, $C_{\text{albuminoid}}$, in μg per mL, of protein (albuminoid amount) in the *Test solution*. Calculate the albuminoid concentration, in mg per mL, in the pullulanase taken by the formula:

$$C_{\text{protein}} = (C_{\text{albuminoid}} \times F_{\text{TS}})/1000$$

Calculate the specific activity, SA, in units per mg, of pullulanase using the formula:

$$\text{SA} = \text{PA}/C_{\text{protein}}$$

Pumice—A substance of volcanic origin consisting chiefly of complex silicates of aluminum and alkali metals. Occurs as very light, hard, rough, porous, gray masses, or as a gray-colored powder. Is insoluble in water and is not attacked by diluted acids.

Acid- and water-soluble substances—Boil 2.0 g of powdered pumice with 50 mL of diluted hydrochloric acid under a reflux condenser for 30 minutes. Cool, and filter. To half of the filtrate add 5 drops of sulfuric acid, evaporate to dryness, ignite, and weigh: the residue weighs not more than 60 mg (6.0%).

Purine, $\text{C}_5\text{H}_4\text{N}_4$ —**120.11** [120-73-0]—White to off-white powder.

Melting range (741): between 214° and 217°.

A single spot is exhibited when it is examined by thin-layer chromatography, with the use of plates coated with chromatographic silica gel mixture and a developing system consisting of butyl alcohol, water, and glacial acetic acid (60:25:15).

Putrescine Dihydrochloride, $\text{C}_4\text{H}_{12}\text{N}_2 \cdot 2\text{HCl}$ —**161.07** [333-93-7]—White, crystalline powder. Use a suitable grade.

Pyrazole, $\text{C}_3\text{H}_4\text{N}_2$ [288-13-1]—White to pale yellow crystals or crystalline powder. Soluble in water, in alcohol, and in ether.

Melting range (741): between 67° and 71°.

Pyrene, $\text{C}_{16}\text{H}_{10}$ —**202.25** [129-00-0]—White to light yellow crystals.

Assay—Transfer about 9 mg, accurately weighed, to a 100-mL volumetric flask, dissolve in methanol, dilute with methanol to volume, and mix. Transfer 2.0 mL of this solution to a 100-mL volumetric flask, dilute with methanol to volume, and mix. Using a suitable spectrophotometer, 1-cm cells, and methanol as the blank, record the absorbance of the solution at the wavelength of maximum absorbance at about 238 nm. From the observed absorbance, calculate the absorptivity (see *Spectrophotometry and Light-scattering* (851)): the absorptivity is not less than 432.9, corresponding to not less than 98% of $\text{C}_{16}\text{H}_{10}$.

Melting range (741): between 149° and 153° over a 2° range.

Pyridine, $\text{C}_5\text{H}_5\text{N}$ —**79.10** [110-86-1]—Use ACS reagent grade.

Pyridine, Dried [110-86-1]—Use ACS reagent grade.

Pyridoxal Hydrochloride, $\text{C}_8\text{H}_9\text{NO}_3 \cdot \text{HCl}$ —**203.62** [65-22-5]—White to slightly yellow crystals or crystalline powder. Gradually darkens on exposure to air or sunlight. One g dissolves in about 2 mL of water and in about 25 mL of alcohol. Insoluble in acetone, in chloroform, and in ether. Its solutions are acid (pH about 3).

Melting range (741): between 171° and 175° with some decomposition.

Residue on ignition (Reagent test): not more than 0.1%.

Loss on drying (731)—Dry it at 105° for 2 hours: it loses not more than 0.5% of its weight.

Nitrogen content (Reagent test)—Determine by the Kjeldahl method, using a test specimen previously dried at 105° for 2 hours: between 6.7% and 7.1% of N is found.

Chloride content—Accurately weigh about 500 mg, previously dried at 105° for 2 hours, and dissolve in 50 mL of water. Add 3 mL of nitric acid and 50.0 mL of 0.1 N silver nitrate VS, then add 5 mL of nitrobenzene, shake for about 2 minutes, add ferric ammonium sulfate TS, and titrate the excess silver nitrate with 0.1 N ammonium thiocyanate VS: each mL of 0.1 N silver nitrate is equivalent to 3.545 mg of Cl. Between 17.2% and 17.7% is found.

Pyridoxal 5-Phosphate, $4\text{-CHOC}_5\text{HN-2-CH}_3, 3\text{-OH}, 5\text{-CH}_2\text{PO}_4\text{H}_2 \cdot \text{H}_2\text{O}$ —**265.16** [41468-25-1]—Light yellow powder. Use a suitable grade.

Pyridoxamine Dihydrochloride, $\text{C}_8\text{H}_{12}\text{N}_2\text{O}_2 \cdot 2\text{HCl}$ —**241.11** [524-36-7]—White to slightly yellow crystals or crystalline powder. Gradually darkens on exposure to air or sunlight. One g dissolves in about 1 mL of water and in about 60 mL of alcohol. Insoluble in chloroform and in ether. Its solutions are acid.

Melting range (741): between 225° and 230°, with some decomposition.

Residue on ignition (Reagent test): not more than 0.15%.

Loss on drying (731)—Dry it at 105° for 2 hours: it loses not more than 0.5% of its weight.

Nitrogen content (Reagent test)—Determine by the Kjeldahl method, using a test specimen previously dried at 105° for 2 hours: between 11.3% and 11.8% of N is found.

Chloride content—Determine as directed in the test for *Chloride content* under *Pyridoxal Hydrochloride*: between 29.1% and 29.6% of Cl is found.

1-(2-Pyridylazo)-2-naphthol, $\text{C}_{15}\text{H}_{11}\text{N}_3\text{O}$ —**249.27** [85-85-8]—Stable, orange-red crystals. Soluble in alcohol and in hot solutions of dilute alkalis; slightly soluble in water.

Melting range (741): between 140° and 142°.

Sensitiveness—Add 0.1 mL of a 1 in 1000 solution of it in alcohol to a mixture of 10 mL of water and 1 mL of a buffer solution prepared by mixing 80 mL of 0.2 M acetic acid and 20 mL of sodium acetate solution (8.2 in 100), and mix. To this solution add 1 mL of a mixture of 1 mL of cupric sulfate TS and 2 mL of water, and mix: the color changes from yellow to red.

4-(2-Pyridylazo)resorcinol (PAR), $\text{C}_{11}\text{H}_9\text{N}_3\text{O}_2$, free acid; $\text{C}_{11}\text{H}_8\text{N}_3\text{NaO}_2$, monosodium salt—**215.21**, free acid—**237.21**, monosodium salt [1141-59-9], free acid; 16593-81-0, monosodium salt—Use ACS reagent grade.

3-(2-Pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5',5''-disulfonic Acid, Disodium Salt (3-(2-Pyridyl)-5,6-bis(5-sulfo-2-furyl)-1,2,4-triazine, Disodium Salt Hydrate), $\text{C}_{16}\text{H}_8\text{N}_4\text{Na}_2\text{O}_8\text{S}_2$ —**494.37** [79551-14-7]—Use a suitable grade.

[NOTE—A suitable grade is available as product number P4272 from Sigma-Aldrich, 1-800-558-9160; www.sigma-aldrich.com.]

Pyrogallol, $C_6H_3(OH)_3$ —**126.11** [87-66-1]—Use ACS reagent grade.

Pyrrole, C_4H_5N —**67.09** [109-97-7]—Clear liquid, colorless when freshly distilled, becoming yellow in a few days. Specific gravity: about 0.94. Insoluble in water; soluble in alcohol, in benzene, and in ether.

Boiling range (Reagent test)—Not less than 90% distills between 128° and 132°.

Pyruvic Acid, $CH_3COCOOH$ —**88.06** [127-17-3]—Colorless to light yellow liquid. Miscible with water, with alcohol, and with ether.

Refractive index (831): about 1.43 at 20°.

Assay—Accurately weigh about 1 g, transfer to a suitable container, and add 100 mL of water. Mix, add phenolphthalein TS, and titrate with 0.5 N sodium hydroxide VS. Each mL of 0.5 N sodium hydroxide is equivalent to 44.03 mg of $CH_3COCOOH$: not less than 98% of $CH_3COCOOH$ is found.

Quantitative Filter Paper—See *Filter Paper, Quantitative*.

Quinhydrone, $C_6H_4(OH)_2 \cdot C_6H_4O_2$ —**218.21** [106-34-3]—Green crystals having a metallic luster. Slightly soluble in cold water; soluble in hot water, in alcohol, and in ether.

Assay—Transfer about 450 mg, accurately weighed, to a glass-stoppered flask, add 50 mL of 1 N sulfuric acid and 3 g of potassium iodide, insert the stopper in the flask, and shake until dissolved. Titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. Each mL of 0.1 N sodium thiosulfate is equivalent to 5.405 mg of quinone ($C_6H_4O_2$). Between 49.0% and 51.0% is found.

Alcohol-insoluble matter—Dissolve 10 g in 100 mL of hot alcohol, filter through a suitable tared crucible of fine porosity, and wash with hot alcohol until the last washing is colorless. Dry at 105°, cool in a desiccator, and weigh: the residue weighs not more than 1.0 mg (0.010%).

Residue on ignition (Reagent test): not more than 0.050%, a 2.0-g test specimen being used. Save the residue.

Sulfate—Transfer 1 g to a platinum crucible, add 10 mL of hot water and 0.5 g of sodium carbonate, evaporate to dryness, and ignite, protected from the sulfur in the flame, until the residue is nearly white. Cool, add 20 mL of water and 1 mL of 30 percent hydrogen peroxide, boil gently for a few minutes, add 2 mL of hydrochloric acid, and evaporate on a steam bath to dryness. Cool, dissolve the residue in 20 mL of water, filter, and to the filtrate add 1 mL of 1 N hydrochloric acid and 3 mL of barium chloride TS: any turbidity produced within 10 minutes does not exceed that in a control containing 0.2 mg of added SO_4 and 0.5 mg of sodium carbonate, 1 mL of 30 percent hydrogen peroxide, and 2 mL of hydrochloric acid previously evaporated on a steam bath to dryness (0.02%).

Heavy metals—To the residue retained from the test for *Residue on ignition* add 2 mL of hydrochloric acid and 0.5 mL of nitric acid, and evaporate on a steam bath to dryness. Dissolve the residue in 30 mL of hot water containing 1 mL of 1 N hydrochloric acid, cool, dilute with water to 40 mL, and mix. Dilute 20 mL of this solution (retain the rest of the solution) with water to 25 mL, adjust to a pH between 3.0 and 4.0 by the addition of 1 N acetic acid or 6 N ammonium hydroxide as necessary, dilute with water to 40 mL, and add 10 mL of freshly prepared hydrogen sulfide TS: any brown color produced does not exceed that in a control containing 0.02 mg of added Pb (0.002%).

Iron (241)—To 10 mL of the solution retained from the test for *Heavy metals* add 2 mL of hydrochloric acid, and

dilute with water to 47 mL: the solution shows not more than 0.01 mg of Fe (0.002%).

Quinone—See *p-Benzoquinone*.

Rayon—Use *Purified Rayon* (General Monograph).

Direct Red 80, $C_{45}H_{26}N_{10}Na_6O_{21}S_6$ —**1373.07** [2610-10-8]—Red powder. Soluble in water; poorly soluble in alcohol. Use a suitable grade.

Red-Cell Lysing Agent—The reagent is available as a solution containing 0.33% potassium cyanide and 0.11% sodium nitroprusside and a quarternary ammonium salt as the surface active agent (5.5%).

[NOTE—The reagent is manufactured by Coulter Electronics Diagnostics, Hialeah, FL and is available from many suppliers under the name of Zapoglobin® (or Zap-oglobin®).]

Red Phosphorus—See *Phosphorus, Red*.

Resazurin (Sodium), $C_{12}H_6N_4NaO_4$ —**251.17** [62758-13-8]—A brownish-purple, crystalline powder. One g dissolves in 100 mL of water, forming a deep-violet-colored solution.

Hydrogen sulfide and other compounds containing the thiol group decolorize solutions of resazurin sodium, forming dihydroresorufin. When the decolorized solution is shaken in the presence of air, a rose color develops as a result of the formation of resorufin.

Retinyl Palmitate, $C_{36}H_{60}O_2$ —**524.9**—Yellow liquid.

Assay—

Mobile phase—Prepare a mixture of acetonitrile and tetrahydrofuran (55:15).

Procedure—Inject about 10 μ L into a suitable liquid chromatograph (see *Chromatography* (621)) equipped with a 320-nm detector and a 4.6-mm \times 15-cm column that contains packing L1. The flow rate is about 1 mL per minute. The area of the $C_{36}H_{60}O_2$ peak is not less than 93% of the total peak area.

Reverse Transcriptase—Use a suitable grade.

[NOTE—A suitable grade is available from BD Biosciences, www.bdbiosciences.com.]

Rhodamine B (Tetraethylrhodamine), $C_{28}H_{31}ClN_2O_3$ —**479.01** [81-88-9]—Green crystals or a reddish-violet powder. Very soluble in water, yielding a bluish-red solution that is strongly fluorescent when dilute. Very soluble in alcohol; slightly soluble in dilute acids and in alkali solutions. In strong acid solution, it forms a pink complex with antimony that is soluble in isopropyl ether.

Clarity of solution—Its solution (1 in 200) is complete and clear.

Residue on ignition (Reagent test)—Ignite 1 g with 1 mL of sulfuric acid: the residue weighs not more than 2 mg (0.2%).

Rhodamine 6G, $C_{28}H_{31}ClN_2O_3$ —**479.02** [989-38-8]—Use a suitable grade.

Ribonuclease Inhibitor—Use a suitable grade.

[NOTE—A suitable grade is available from BD Biosciences, www.bdbiosciences.com.]

Rose Bengal Sodium (Disodium Salt of 4,5,6,7-Tetrachloro-2',4',5',7'-tetraiodofluorescein), $C_{20}H_2Cl_4I_4Na_2O_5$ —**1017.64** [632-69-9]—Fine, rose-colored crystals or crystalline powder. Soluble in water. [NOTE—Render commercially available material suitably pure by the following treatment. Dissolve 8 g in 200 mL of water, and adjust to a pH between 10 and 11, using short-range pH indicator paper. Add 200 mL of acetone, while stirring gently, then add dilute hydrochloric acid (1 in 10), while continuing to stir, until the pH of the solution reaches 4.0. Add 400 mL more of water, with stirring, and continue the stirring for 5 minutes. Filter the crystals on a filtering funnel, and return the crystals to the beaker used for crystallization. Recrystallize three more times in the same manner, and dry the crystals at 110° for 12 hours. Store in an amber bottle in a refrigerator at a temperature between 2° and 8°. Prepare this reagent fresh monthly.]

Chromatographic purity—Dissolve 100 mg of rose bengal sodium, prepared as described above, in 100 mL of water,

and apply 10 μ L of the solution on suitable chromatographic paper. Develop the chromatogram by ascending chromatography, using a mixture of 1 part of dilute alcohol (1 in 4) and 1 part of dilute stronger ammonia water (1 in 12). Examine the chromatogram in daylight and under UV light (360 nm): no colored or fluorescent spot is visible other than the rose bengal sodium spot.

[NOTE—A suitable grade is available commercially as "Silica Gel H."]

Ruthenium Red (*Ruthenium Oxichloride, Ammoniated*), $\text{Ru}_2(\text{OH})_2\text{Cl}_4 \cdot 7\text{NH}_3 \cdot 3\text{H}_2\text{O}$ —**551.23** [11103-72-3]—A brownish-red to dark purple powder. Soluble in water.

S Designations—See supports for gas chromatography under *Reagents, Chromatographic Columns*.

Saccharose—Use *Sucrose* (NF monograph).

Safranin O [477-73-6]—Dark red powder consisting of a mixture of 3,7-diamino-2,8-dimethyl-5-phenylphenazinium chloride, $\text{C}_{20}\text{H}_{19}\text{ClN}_4$ —350.85, and 3,7-diamino-2,8-dimethyl-5-o-tolylphenazinium chloride, $\text{C}_{21}\text{H}_{21}\text{ClN}_4$ —364.88—Sparingly soluble in 70 percent alcohol yielding a clear red solution with a yellowish-red fluorescence.

Identification—

A: To 10 mL of a 0.5% w/v solution add 5 mL of hydrochloric acid: a bluish violet solution is produced.

B: To 10 mL of a 0.5% w/v solution add 5 mL of sodium hydroxide solution (1 in 5): a brownish-red precipitate is produced.

C: To 100 mg add 5 mL of sulfuric acid: a green solution is produced, which, on dilution, changes to blue and finally to red.

Absorption characteristics—Dissolve 50 mg in 250 mL of 50 percent alcohol. Dilute 3 mL of this solution with 50 percent alcohol to 200 mL. Determine the absorbance, in a 1-cm cell, with a suitable spectrophotometer. The absorbance maximum is in the range of 530 to 533 nm; the ratio $(P - 15)/(P + 15)$ is between 1.10 and 1.32, in which P is the wavelength of maximum absorbance.

[NOTE—A suitable grade is available as catalog number 10,214-8 from Sigma-Aldrich, www.sigma-aldrich.com.]

Salicylaldazine, $\text{C}_{14}\text{H}_{12}\text{N}_2\text{O}_2$ —**240.26**—Use a suitable grade or prepare as follows. Dissolve 300 mg of hydrazine sulfate in 5 mL of water, add 1 mL of glacial acetic acid and 2 mL of a freshly prepared 1 in 5 solution of salicylaldehyde in isopropyl alcohol, mix, and allow to stand until a yellow precipitate is formed. Extract the mixture with two 15-mL portions of methylene chloride. Combine the methylene chloride extracts, and dry over anhydrous sodium sulfate. Decant the methylene chloride solution, and evaporate it to dryness. Recrystallize the residue of salicylaldazine from a mixture of warm toluene and methanol (60:40) with cooling. Filter, and dry the crystals in vacuum.

Melting range (741°): between 213° and 219°, but the range between beginning and end of melting does not exceed 1°.

Chromatography—Proceed as directed in *Limit of hydrazine* under *Povidone*: the chromatogram shows only one spot.

Salicylaldehyde, (*2-Hydroxybenzaldehyde*), $2\text{-HOC}_6\text{H}_4\text{CHO}$ —**122.12** [90-02-8]—Clear, colorless to yellowish-green liquid. Specific gravity: about 1.17. Slightly soluble in water; soluble in alcohol and in ether. May contain a stabilizer.

Assay—When examined by gas-liquid chromatography, using suitable apparatus and conditions, it shows a purity of not less than 98%.

Sand, Standard 20- to 30-Mesh—Silica sand, composed almost entirely of naturally rounded grains of nearly pure quartz. Predominantly graded to pass an 850- μ m (No. 20) sieve (85 to 100 percentage passing) and be retained on a 600- μ m (No. 30) sieve (0 to 5 percentage passing).

[NOTE—A suitable grade is available as Ottawa Standard Sand from Thomas Scientific, 99 High Hill Road at I-295, P.O. Box 99, Swedesboro, NJ 08085-0099.]

Sand, Washed—It may be prepared as follows. Digest clean, hard sand at room temperature with a mixture of 1 part of hydrochloric acid and 2 parts of water (about 13% of HCl) for several days, or at an elevated temperature for several hours. Collect the sand on a filter, wash with water until the washings are neutral and show only a slight reaction for chloride, and finally dry. Washed sand meets the following tests.

Substances soluble in hydrochloric acid—Digest 10 g with a mixture of 10 mL of hydrochloric acid and 40 mL of water on a steam bath for 4 hours, replacing from time to time the water lost by evaporation. Filter, and to 25 mL of the filtrate add 5 drops of sulfuric acid, evaporate, and ignite to constant weight: the residue weighs not more than 8 mg (0.16%).

Chloride (Reagent test)—Shake 1 g with 20 mL of water for 5 minutes, filter, and add to the filtrate 1 mL of nitric acid and 1 mL of silver nitrate TS: any turbidity produced corresponds to not more than 0.03 mg of Cl (0.003%).

Sawdust, Purified—It may be prepared as follows. Extract sawdust in a percolator, first with sodium hydroxide solution (1 in 100), and then with dilute hydrochloric acid (1 in 100) until the acid percolate gives no test for alkaloid with mercuric-potassium iodide TS or with iodine TS. Then wash with water until free from acid and soluble salts, and dry. Purified sawdust meets the following test.

Alkaloids—To 5 g of purified sawdust contained in a flask add 50 mL of a mixture of 2 volumes of ether and 1 volume of chloroform and 10 mL of ammonia TS, and shake frequently for 2 hours. Decant 20 mL of the clear, ether-chloroform liquid, and evaporate to dryness. Dissolve the residue in 2 mL of dilute hydrochloric acid (1 in 12), and divide into two portions. To 1 portion add mercuric-potassium iodide TS, and to the other add iodine TS: no turbidity is produced in either portion.

Scandium Oxide, Sc_2O_3 —**137.91** [12060-01-1]—Fine, white powder.

Secondary Butyl Alcohol—See *Butyl Alcohol, Secondary*.

Selenious Acid (*Selenous Acid*), H_2SeO_3 —**128.97**

[7783-00-8]—Colorless or white crystals, efflorescent in dry air and hygroscopic in moist air. Soluble in water and in alcohol.

Assay—Accurately weigh about 100 mg, transfer to a glass-stoppered flask, and dissolve in 50 mL of water. Add 10 mL of potassium iodide solution (3 in 10) and 5 mL of hydrochloric acid, mix, insert the stopper in the flask, and allow to stand for 10 minutes. Dilute with 50 mL of water, add 3 mL of starch TS, and titrate with 0.1 N sodium thiosulfate VS until the color is no longer diminished, then titrate with 0.1 N iodine VS to a blue color. Subtract the volume of 0.1 N iodine solution from the volume of 0.1 N sodium thiosulfate to give the volume of 0.1 N thiosulfate equivalent to selenious acid. Each mL of 0.1 N sodium thiosulfate is equivalent to 3.225 mg of H_2SeO_3 ; not less than 93% is found.

Insoluble matter—Dissolve 1 g in 5 mL of water: the solution is clear and complete.

Residue on ignition (Reagent test): not more than 1.0 mg (0.01%), from 10 g.

Selenate and sulfate—Dissolve 500 mg in 10 mL of water, and add 0.1 mL of hydrochloric acid and 1 mL of barium chloride TS: no turbidity or precipitate is formed within 10 minutes.

Selenium, Se—**At. Wt. 78.96** [7782-49-2]—Dark-red amorphous, or bluish-black, crystalline powder. Soluble in solutions of sodium and potassium hydroxides or sulfides;

insoluble in water. Use a suitable grade with a content of not less than 99.99%.

Selenomethionine, $C_5H_{11}NO_2Se$ —**196.11**
[1464-42-2]—[CAUTION—Handle with care, as this reagent is highly toxic.]

Assay—Weigh accurately about 750 mg, dissolve in 100 mL of methanol, add crystal violet TS, and titrate with 0.1 N perchloric acid to a blue-green endpoint. Each mL of 0.1 N perchloric acid is equivalent to 19.61 mg of $C_5H_{11}NO_2Se$: between 97.0% and 103.0%, calculated on the as-is basis, is found.

Melting range (741): about 260°, with decomposition.

Nitrogen content (461)—Determine by the Kjeldahl method: between 6.8% and 7.4%, calculated on the as-is basis, is found.

Silica, Calcined Diatomaceous—See *Diatomaceous Silica, Calcined*.

Silica, Chromatographic, Silanized, Flux-Calcined, Acid-washed—Use a suitable grade.

[NOTE—Suitable grades are available commercially as “Aeropak 30,” “Diatoport S,” and “Gas-Chrom Z.”]

Silica Gel—An amorphous, partly hydrated SiO_2 occurring in glassy granules of varying size. When used as a desiccant, it frequently is coated with a substance that changes color when the capacity to absorb water is exhausted. Such colored products may be regenerated (i.e., may regain their capacity to absorb water) by being heated at 110° until the gel assumes the original color.

For use as a desiccant, use ACS Silica Gel Desiccant grade.

Silica Gel, Binder-Free—Silica gel for chromatographic use formulated without a binder, since only activated forms of the silica gel are used as the binding agent.

[NOTE—A suitable grade is available commercially as “Silica Gel H.”]

Silica Gel, Chromatographic—Use a suitable grade.

[NOTE—A suitable grade is available commercially as “Silica Gel G.”]

Silica Gel, Octadecylsilanized Chromatographic—Use a suitable grade.

[NOTE—A suitable grade is available commercially as “Reversed Phase Uniplates” from Analtech, www.analtech.com.]

Silica Gel, Porous—Use a grade suitable for high-pressure liquid chromatography.

[NOTE—A suitable grade for reverse phase high-pressure liquid chromatography is available as “LiChrosorb SI60, Reverse Phase.”]

Silica Gel-Impregnated Glass Microfiber Sheet—Use a suitable grade.

[NOTE—A suitable grade is available commercially as “Seprachrom” Chamber with Type SG ITLC, Product No. 51923, from Gelman Instrument Co., Ann Arbor, MI 48106.]

Silica Gel Mixture, Chromatographic—A mixture of silica gel with a suitable fluorescing substance.

[NOTE—A suitable grade is available commercially as “Silica Gel GF 254.”]

Silica Gel Mixture, Chromatographic, with Chemically Bound Amino Groups—Use a suitable grade.

Silica Gel Mixture, Dimethylsilanized, Chromatographic—Use a suitable grade.

[NOTE—A suitable grade is available as “Silica Gel 60 silanized RP-2 F₂₅₄,” from EMD Chemicals, www.emdchemicals.com.]

Silica Gel Mixture, Octadecylsilanized Chromatographic—Use a suitable grade.

[NOTE—A suitable grade is available commercially as KC-18F from Whatman Chemical Separation, Inc., 9 Bridewell Place, Clifton, NJ 07014.]

Silica Gel Mixture, Octylsilanized, Chromatographic—Use a mixture of RP-8 chromatographic silica gel with a suitable fluorescing substance agent.

Silica Microspheres—Use a suitable grade.

[NOTE—A suitable grade, in a controlled-diameter, spherical, porous form, is available commercially as “Zorbax Sil,” from Agilent, www.agilent.com.]

Siliceous Earth, Chromatographic—

For gas chromatography, use a specially prepared grade meeting the following general description: Purified siliceous earth of suitable mesh size that has been acid- and/or base-washed. It may or may not be silanized.

For column partition chromatography, it is essential that the material be free from interfering substances. If such interferences are known or thought to be present, purify the material as follows: Place a pledget of glass wool in the base of a chromatographic column having a diameter of 100 mm or larger, and add *Purified Siliceous Earth* (NF monograph) to a height equal to 5 times the diameter of the column. Add a volume of hydrochloric acid equivalent to one-third the volume of siliceous earth, and allow the acid to percolate into the column. Wash the column with methanol, using small volumes at first to rinse the walls of the column, and continue washing with methanol until the last washing is neutral to moistened litmus paper. Extrude the washed column into shallow dishes, heat on a steam bath to remove the excess methanol, and dry at 105° until the material is powdery and free from traces of methanol. Store the dried material in well-closed containers.

[NOTE—A suitable grade is “Chromosorb W-AW.”]

[NOTE—Suitable silanized grades for gas chromatography are “Gas Chrom Q,” and “Chromosorb W (AW-DMCS-treated).”]

[NOTE—A suitable grade for column chromatography is acid-washed “Celite 545,” available from Sigma-Aldrich, www.sigma-aldrich.com.]

Siliceous Earth, Chromatographic, Silanized—Place about 450 g of purified siliceous earth in a large, open, glass crystallizing dish in a vacuum desiccator containing 30 mL of a suitable silane, e.g., a mixture of 1 volume of dimethyldichlorosilane and 1 volume of trimethylchlorosilane, or a mixture of 1 volume of methyltrichlorosilane and 2 volumes of dimethyldichlorosilane. Apply vacuum intermittently for several hours, until no liquid silane remains. Float the treated purified siliceous earth on water, and gently agitate to allow any uncoated particles to sink. Skim the silanized material off the surface, wash it on a sintered-glass funnel with warm methanol until the filtrate no longer is acidic, and dry at 110°.

Silicic Acid, $SiO_2 \cdot xH_2O$ —(anhydrous) **60.08**

[1343-98-2]—White, amorphous powder. Insoluble in water and in acids; soluble in hot solutions of strong alkalis.

Residue on ignition (Reagent test): not less than 80.0%.

Nonvolatile with hydrofluoric acid—Heat 500 mg with 1 mL of sulfuric acid and 10 mL of hydrofluoric acid in a platinum crucible to dryness, and ignite to constant weight: the weight of the residue does not exceed 1.0 mg (0.2%).

Chloride (Reagent test)—One g shows not more than 0.05 mg of Cl (0.005%).

Sulfate (Reagent test)—Boil 2 g with 20 mL of dilute hydrochloric acid (1 in 40), filter, neutralize the filtrate with ammonia TS, and dilute with water to 20.0 mL. A 10-mL aliquot of the solution shows not more than 0.1 mg of SO_4 (0.01%).

Heavy metals (Reagent test)—Boil 2.5 g with 50 mL of dilute hydrochloric acid (1 in 10) for 5 minutes, filter while hot, and evaporate the filtrate on a steam bath to dryness. Take up the residue in 20 mL of dilute hydrochloric acid (1 in 500), digest for 5 minutes, cool, add water to make 100 mL, and filter. To 40 mL of the filtrate add 10 mL of hydrogen sulfide TS: any color produced is not darker than that produced by adding 10 mL of hydrogen sulfide TS to a control containing 0.03 mg of Pb (0.003%).

Iron (241)—To 20 mL of the filtrate obtained in the test for *Heavy metals* add 1 mL of hydrochloric acid, and dilute

with water to 47 mL: the solution shows not more than 0.015 mg of Fe (0.003%).

Silicic Acid—Impregnated Glass Microfilament Sheets with Fluorescent Indicator—Use a suitable grade.

[NOTE—One example of a suitable grade is "ITLC Type SAF" sheets, available from Gelman Instrument Co., 600 South Wagner Rd., Ann Arbor, MI 48106.]

Silicon Carbide, SiC—40.10 [409-21-2]—In small clean chips, suitable for use in promoting ebullition.

Silicone (75 Percent Phenyl, Methyl)—Use a suitable grade.

[NOTE—A suitable grade is available as "OV-25."]

Silicotungstic Acid, *n*-Hydrate (*Tungstosilicic Acid*), $\text{H}_4\text{Si}(\text{W}_3\text{O}_{10})_4 \cdot n\text{H}_2\text{O}$ —**2878.17** (anhydrous) [12520-88-6]—Green powder.

Assay—Dissolve about 1 g, accurately weighed, in 25 mL of dilute hydrochloric acid (1 in 5). Add 50 mL of a solution of 5 g of cinchonine in dilute hydrochloric acid (1 in 2). Warm on a steam bath for about 30 minutes. Cool, filter through a tared crucible, and ignite at 800° to constant weight. The weight of the residue multiplied by 1.047 is equal to the weight of silicotungstic acid dihydrate in the sample taken. Not less than 98% is found.

Silver Diethyldithiocarbamate, $(\text{C}_2\text{H}_5)_2\text{NCS}_2\text{Ag}$ —**256.14** [1470-61-7]—Use ACS reagent grade.

Silver Nitrate, AgNO_3 —**169.87** [7761-88-8]—Use ACS reagent grade.

Silver Oxide, Ag_2O —**231.74** [20667-12-3]—Brownish-black, heavy powder. Slowly decomposes on exposure to light. Absorbs carbon dioxide when moist. Practically insoluble in water; freely soluble in dilute nitric acid and in ammonia; insoluble in alcohol. Store in well-closed containers; do not expose to ammonia fumes or easily oxidizable substances.

Assay—Dissolve about 500 mg, previously dried at 120° for 3 hours and accurately weighed, in a mixture of 20 mL of water and 5 mL of nitric acid. Dilute with 100 mL of water, add 2 mL of ferric ammonium sulfate TS, and titrate with 0.1 N ammonium thiocyanate VS to a permanent reddish-brown color. Each mL of 0.1 N ammonium thiocyanate is equivalent to 11.59 mg of Ag_2O : not less than 99.7% of Ag_2O is found.

Loss on drying—Dry it at 120° for 3 hours: it loses not more than 0.25% of its weight.

Nitrate—To 500 mg add 30 mg of sodium carbonate and 2 mL of phenoldisulfonic acid TS, mix, and heat on a steam bath for 15 minutes. Cool, cautiously add 20 mL of water, render alkaline with ammonia TS, and dilute with water to 30 mL: any color produced by the test solution is not darker than that produced in a control containing 0.01 mg of NO_3 (0.002%).

Substances insoluble in nitric acid—Dissolve 5 g in a mixture of 5 mL of nitric acid and 10 mL of water, dilute with water to about 65 mL, and filter any undissolved residue on a tared filtering crucible (retain the filtrate for the test for *Substances not precipitated by hydrochloric acid*). Wash the crucible with water until the last washing shows no opalescence with 1 drop of hydrochloric acid, and dry at 105° to constant weight: the residue weighs not more than 1 mg (0.02%).

Substances not precipitated by hydrochloric acid—Dilute the filtrate obtained in the test for *Substances insoluble in nitric acid* with water to 250 mL, heat to boiling, and add, dropwise, sufficient hydrochloric acid to precipitate all of the silver (about 5 mL), avoiding any great excess. Cool, dilute with water to 300 mL, and allow to stand overnight. Filter, evaporate 200 mL of the filtrate in a suitable tared porcelain dish to dryness, and ignite: the residue weighs not more than 1.7 mg (0.05%).

Alkalinity—Heat 2 g with 40 mL of water on a steam bath for 15 minutes, cool, and dilute with water to 50 mL. Filter, discarding the first 10 mL of the filtrate. To 25 mL of the subsequent filtrate add 2 drops of phenolphthalein TS, and

titrate with 0.02 N hydrochloric acid VS to the disappearance of any pink color: not more than 0.20 mL is required (0.016% as NaOH).

β -Sitosterol, (22:23 *Dihydrostigmasterol*), $\text{C}_{29}\text{H}_{50}\text{O}$ —**414.7** [83-46-5]—White powder. Soluble in chloroform. Store in a freezer.

Specific rotation (781S): between -33° and -39° , determined in a solution containing 0.5 g of test specimen per mL of chloroform.

Water, Method I (921): not more than 6%.

Soda Lime—Use *Soda Lime* (NF monograph).

Sodium, Na—At. Wt. 22.98977 [7440-23-5]—Use ACS reagent grade.

Sodium Acetate, $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ —**136.08** [6131-90-4]—Use ACS reagent grade Sodium Acetate Trihydrate.

Sodium Acetate, Anhydrous, $\text{NaC}_2\text{H}_3\text{O}_2$ —**82.03** [127-09-3]—Use ACS reagent grade.

Sodium Alizarinsulfonate (*Alizarin Red S*; *Alizarin Sodium Monosulfonate*), $\text{C}_{14}\text{H}_7\text{NaO}_7\text{S} \cdot \text{H}_2\text{O}$ —**360.27**—Yellow-brown or orange-yellow powder. Freely soluble in water, with production of a yellow color; sparingly soluble in alcohol.

Sensitiveness—Add 3 drops of a solution of it (1 in 100) to 100 mL of water, and add 0.25 mL of 0.02 N sodium hydroxide: a red color is produced. Add 0.25 mL of 0.02 N hydrochloric acid: the original yellow color returns.

Sodium Ammonium Phosphate (*Microcosmic Salt*), $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$ —**209.07**—Colorless crystals or white granules. Freely soluble in water; insoluble in alcohol. Effloresces in air and loses ammonia.

Insoluble matter and ammonium hydroxide precipitate—Dissolve 10 g in 100 mL of water, add 10 mL of ammonia TS, and heat on a steam bath for 1 hour. If any precipitate is formed, filter, wash well with water, and ignite: the ignited precipitate weighs not more than 1 mg (0.01%).

Chloride (Reagent test)—One g shows not more than 0.02 mg of Cl (0.002%).

Heavy metals—Dissolve 3 g in 25 mL of water, add 15 mL of 1 N sulfuric acid, then add 10 mL of hydrogen sulfide TS: any brown color developed in 1 minute is not darker than that of a control containing 3 mL of *Standard Lead Solution* (see (231)) and 0.5 mL of 1 N sulfuric acid (0.001%).

Nitrate—Dissolve 1 g in 10 mL of water, add 0.1 mL of indigo carmine TS, then add, with stirring, 10 mL of sulfuric acid: the blue color persists for 10 minutes (about 0.005%).

Sulfate (Reagent test, *Method II*)—Dissolve 10 g in 100 mL of water, add 5 mL of hydrochloric acid, and filter if necessary: the filtrate yields not more than 5 mg of residue (0.02%).

Sodium Arsenate (*Arsenic Acid Sodium Salt*), $\text{Na}_2\text{HASO}_4 \cdot 7\text{H}_2\text{O}$ —**312.01** [10048-95-0]—Use ACS reagent grade.

Sodium Arsenite, NaAsO_2 —**129.91** [7784-46-5]—White, crystalline powder. Soluble in water; slightly soluble in alcohol.

Assay—Transfer about 5.5 g, accurately weighed, to a 500-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Pipet 25 mL of this solution into a suitable container, add 50 mL of water and 5 g of dibasic sodium phosphate, swirl to dissolve, and titrate with 0.1 N iodine VS, adding 3 mL of starch TS as the endpoint is approached. Each mL of 0.1 N iodine is equivalent to 3.746 mg of As. Between 57.0% and 60.5% is found (equivalent to 98.8% to 104.9% of NaAsO_2).

Chloride (Reagent test)—One g shows not more than 0.10 mg of Cl (0.01%).

Heavy metals—Dissolve 200 mg in 8 mL of dilute hydrochloric acid (3 in 8), and evaporate on a steam bath to dryness. Dissolve the residue in 5 mL of dilute hydrochloric acid (2 in 5), and again evaporate to dryness. Dissolve the residue in 10 mL of water, and add 2 mL of diluted acetic acid and 10 mL of hydrogen sulfide TS. Any brown color

produced is not darker than that of a control containing 0.01 mg of added Pb (0.005%).

Iron—Dissolve 1 g in 20 mL of dilute hydrochloric acid (1 in 5), and add, dropwise, a slight excess of bromine TS. Boil the solution to remove the excess bromine, cool, dilute with water to 40 mL, and add 10 mL of ammonium thiocyanate solution (3 in 10). Any red color produced is not darker than that of a control containing 0.02 mg of added Fe (0.02%).

Sulfide—Dissolve 1 g in 20 mL of water, and add 5 drops of lead acetate TS: no brown color is produced (about 0.0005%).

Sulfate (Reagent test, *Method II*)—Dissolve 5 g in 100 mL of water, add methyl orange TS, neutralize with 1 N hydrochloric acid, add 3 mL of the acid in excess, and filter: the filtrate yields not more than 3 mg of residue (0.02%).

Sodium Azide, NaN_3 —**65.01** [26628-22-8]—White powder.

Assay—[**CAUTION**—Sodium azide is a potent poison. Its conjugate acid HN_3 is more toxic than hydrogen cyanide and is readily liberated from neutral aqueous solutions. Contact of NaN_3 or hydrazoic acid (HN_3) with certain metals may produce explosive salts. Work in a well-ventilated hood, and handle the sample with care.] Dissolve about 100 mg, accurately weighed, in 50 mL of water, and add 3 drops of phenolphthalein. Adjust the pH, if necessary, to 7.0, and add 35.0 mL of 0.1 N perchloric acid. Pipet, while stirring, 2.5 mL of 1.0 M sodium nitrite into the solution, and stir for 15 seconds. Titrate rapidly to the phenolphthalein endpoint with 0.1 N sodium hydroxide. The endpoint should be reached in less than 4 minutes after addition of perchloric acid because HN_3 is readily volatile. Calculate the percentage of azide by the formula:

$$[(N_p)(V_p) - (N_s)(V_s)](65.01)(100)/2C$$

where N_p is the normality of perchloric acid solution; V_p is the volume of perchloric acid, in mL, taken; N_s is the normality of sodium hydroxide solution; V_s is the volume, in mL, of sodium hydroxide taken; 65.01 is the molecular weight of sodium azide; and C is the weight, in mg, of sodium azide. Not less than 98.5% of NaN_3 is found.

Sodium Bicarbonate, NaHCO_3 —**84.01** [144-55-8]—Use ACS reagent grade.

Sodium Biphenyl, $\text{C}_{12}\text{H}_9\text{Na}$ —**176.19**—Available as a solution in 2-ethoxyethyl ether, or in 1,2-dimethoxyethane (diethylene glycol diether).

Activity—Place 20 mL of dry toluene in a titration flask equipped with a magnetic stirring bar and a stopper having a hole through which the delivery tip of a weight buret may be inserted. Add a quantity of sodium biphenyl sufficient to produce a blue color in the mixture, and titrate with amyl alcohol, contained in a weight buret, to the disappearance of the blue color. (Disregard the amounts of sodium biphenyl and amyl alcohol used in this adjustment.) Weigh accurately the weight buret containing the amyl alcohol. Transfer the contents of a vial of well-mixed test specimen to the titration flask, and titrate quickly with the amyl alcohol to the disappearance of the blue color. Weigh the buret to determine the weight of amyl alcohol consumed, and calculate the activity, in mEq/vial, by the formula:

$$\text{Result} = 11.25W$$

in which W is the weight of amyl alcohol consumed. NLT 10% activity is found.

Iodine content—Add 10 mL to 5 mL of toluene contained in a 125-mL separator fitted with a suitable inert plastic stopcock, and shake vigorously for 2 min. Extract gently with three 10-mL portions of dilute phosphoric acid (1 in 3), combining the lower phases in a 125-mL iodine flask. Add sodium hypochlorite TS, dropwise, to the combined extracts until the solution turns brown, then add 0.5 mL in excess. Shake intermittently for 3 min, add 5 mL of freshly prepared, saturated phenol solution, mix, and allow to stand for 1 min, accurately timed. Add 1 g of potassium iodide, shake for 30 s, add 3 mL of starch TS, and titrate with 0.1 N sodium thiosulfate VS: NMT 0.1 mL of 0.1 N sodium thiosulfate is consumed.

[NOTE—A suitable grade is available as catalog number 277134 from www.sigma-aldrich.com or as catalog number 54101 from www.gfschemicals.com.]

Sodium Biphosphate, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ —**137.99**—Use ACS reagent grade Sodium Phosphate, Monobasic.

Sodium Bisulfite [7631-90-5]—This reagent is usually a mixture of sodium bisulfite and sodium metabisulfite [7681-57-4]. Use ACS reagent grade Sodium Bisulfite.

Sodium Bitartrate, $\text{NaHC}_4\text{H}_4\text{O}_6 \cdot \text{H}_2\text{O}$ —**190.08** [6131-98-2]—White crystals or a crystalline powder. Soluble in cold water.

Assay—Dissolve about 500 mg, accurately weighed, in 30 mL of water, add phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS: each mL of 0.1 N sodium hydroxide is equivalent to 19.01 mg of $\text{NaHC}_4\text{H}_4\text{O}_6 \cdot \text{H}_2\text{O}$. Between 99% and 100.5% is found.

Insoluble matter (Reagent test): not more than 1 mg, from 10 g (0.01%).

Chloride (Reagent test)—One g shows not more than 0.2 mg of Cl (0.02%).

Heavy metals (Reagent test)—Dissolve 4 g in 25 mL of water, add 2 drops of phenolphthalein TS, and then add ammonia TS, dropwise, until the solution is slightly pink. Add 4 mL of 1 N hydrochloric acid, dilute with water to 40 mL, and add 10 mL of hydrogen sulfide TS: any brown color produced is not darker than that of a control containing 0.04 mg of added Pb (0.001%).

Sulfate (Reagent test, *Method I*)—One g shows not more than 0.2 mg of SO_4 (0.02%).

Sodium Borate (*Borax*; *Sodium Tetraborate*), $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ —**381.37** [1303-96-4]—Use ACS reagent grade.

[NOTE—Certified Borax is available from the National Institute of Standards and Technology, Washington, DC, www.nist.gov, as standard sample No. 187.]

Sodium Borohydride, NaBH_4 —**37.83** [16940-66-2]—White, crystalline solid. Freely soluble in water; soluble (with reaction) in methanol. Its solutions are rapidly decomposed by boiling.

Assay—

POTASSIUM IODATE SOLUTION (0.25 N)—Dissolve 8.917 g, previously dried at 110° to constant weight and accurately weighed, in water to make 1000.0 mL.

PROCEDURE—Dissolve about 500 mg, accurately weighed, in 125 mL of sodium hydroxide solution (1 in 25) in a 250-mL volumetric flask, dilute with the sodium hydroxide solution to volume, and mix. Pipet 10 mL of the solution into a 250-mL iodine flask, add 35.0 mL of *Potassium iodate solution*, and mix. Add 2 g of potassium iodide, mix, add 10 mL of dilute sulfuric acid (1 in 10), insert the stopper in the flask, and allow to stand in the dark for 3 minutes. Titrate the solution with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. Calculate

the amount, in mg, of NaBH₄ in the specimen titrated by the formula:

$$[(35.0)(0.25)] - 0.1V)4.729$$

in which V is the volume, in mL, of 0.1 N sodium thiosulfate used in the titration. Not less than 98% is found.

Sodium Bromide, NaBr—**102.89** [7647-15-6]—Use ACS reagent grade.

Sodium Carbonate—Use *Sodium Carbonate, Anhydrous*.

Sodium Carbonate, Anhydrous, Na₂CO₃—**105.99** [497-19-8]—Use ACS reagent grade.

Sodium Carbonate, Monohydrate, Na₂CO₃ · H₂O—**124.00** [5968-11-6]—Use ACS reagent grade.

Sodium Chloride, NaCl—**58.44** [7647-14-5]—Use ACS reagent grade.

Sodium Chloride Solution, Isotonic—Use *Saline TS*.

Sodium Cholate Hydrate (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid sodium salt; cholic acid sodium salt), C₂₄H₃₉NaO₅ · xH₂O—**430.55** (anhydrous) [206986-87-0]—It can be from ox or sheep bile. Use a suitable grade with a content of not less than 99%.

Sodium Chromate, Na₂CrO₄ · 4H₂O—**234.03** [7755-11-3]—Lemon-yellow crystals. Soluble in water.

Assay—Accurately weigh about 300 mg, and dissolve in 10 mL of water contained in a 500-mL flask. Add 3 g of potassium iodide and 10 mL of diluted sulfuric acid, and dilute with 350 mL of oxygen-free and carbon dioxide-free water. Titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. Each mL of 0.1 N sodium thiosulfate consumed is equivalent to 7.802 mg of Na₂CrO₄ · 4H₂O. Not less than 99% is found.

Insoluble matter (Reagent test): not more than 1 mg, from 20 g dissolved in 150 mL of water (0.005%).

Aluminum—Dissolve 20 g in 140 mL of water, filter, and add 5 mL of glacial acetic acid to the filtrate. Add stronger ammonia water until alkaline, and digest for 2 hours on a steam bath. Pass through hardened filter paper, wash thoroughly, ignite, and weigh: the residue weighs not more than 0.8 mg (0.002%).

Calcium—Determine as directed in the test for calcium for ACS reagent grade Potassium Chromate (0.005%).

Chloride—Determine as directed in the test for chloride for ACS reagent grade Potassium Chromate (about 0.005%).

Sulfate—Determine as directed in the test for sulfate for ACS reagent grade Potassium Dichromate, but add 4.5 mL of hydrochloric acid to the water used to dissolve the test specimen: the residue weighs not more than 2.4 mg (0.01%).

Sodium Chromotropate—See *Chromotropic Acid*.

Sodium Citrate Dihydrate (2-Hydroxy-1,2,3-propanetricarboxylic Acid, Trisodium Salt, Dihydrate), Na₃C₆H₅O₇ · 2H₂O—**294.10** [6132-04-3]—Use ACS reagent grade.

Sodium Cobaltinitrite, Na₃Co(NO₂)₆—**403.94** [13600-98-1]—Use ACS reagent grade.

Sodium Cyanide, NaCN—**49.01** [1433-33-9]—Use ACS reagent grade.

Sodium 1-Decanesulfonate (1-Decanesulfonic Acid Sodium Salt), C₁₀H₂₁NaO₃S—**244.33** [13419-61-9]—Use a suitable grade for ion pair chromatography with a content of not less than 99.0%.

Sodium Desoxycholate—Use *Bile Salts*.

Sodium Dichromate, Na₂Cr₂O₇ · 2H₂O (for chromic acid cleaning mixture)—**298.00** [7789-12-0]—Use ACS reagent grade.

Sodium Diethyldithiocarbamate, (C₂H₅)₂NCS₂Na · 3H₂O—**225.31** [20624-25-3]—Use ACS reagent grade.

Sodium 2,2-dimethyl-2-silapentane-5-sulfonate—See *Sodium 3-(trimethylsilyl)-1-propane sulfonate*.

Sodium Dithionite—Use *Sodium Hydrosulfite*.

Sodium Dodecyl Sulfate (*Sodium Lauryl Sulfate*), C₁₂H₂₅SO₄Na—**288.38** [151-21-3]—Light yellow, crystalline powder.

Sodium Ferrocyanide, Na₄Fe(CN)₆ · 10H₂O—**484.06** [13601-19-9]—Yellow crystals or granules. Freely soluble in water.

Assay—Dissolve 2 g, accurately weighed, in 400 mL of water, add 10 mL of sulfuric acid, and titrate with 0.1 N potassium permanganate VS. Each mL of 0.1 N potassium permanganate is equivalent to 48.41 mg of Na₄Fe(CN)₆ · 10H₂O. Not less than 98% is found.

Insoluble matter (Reagent test): not more than 1 mg, from 10 g (0.01%).

Chloride (Reagent test)—Dissolve 1 g in 75 mL of water, add a solution prepared by dissolving 1.2 g of cupric sulfate in 25 mL of water, mix, and allow to stand for 15 minutes. To 20 mL of the decanted, clear liquid add 2 mL of nitric acid and 1 mL of silver nitrate TS: any turbidity produced does not exceed that of a control containing 0.02 mg of Cl, 2 mL of nitric acid, 1 mL of silver nitrate TS, and sufficient cupric sulfate to match the color of the Test solution.

Sulfate—Dissolve 5 g in 100 mL of water without heating, filter, and to the filtrate add 0.25 mL of glacial acetic acid and 5 mL of barium chloride TS: no turbidity is produced in 10 minutes (about 0.01% as SO₄).

Sodium Fluorescein, C₂₀H₁₀Na₂O₅—**376.28**—Orange-red, hygroscopic powder. Freely soluble in water; slightly soluble in alcohol. Its water solution is yellowish red in color and exhibits a strong yellowish green fluorescence that disappears when the solution is acidified and reappears when the solution is neutralized or made basic.

Loss on drying (731)—Dry it at 120° to constant weight: it loses not more than 7.0% of its weight.

Sodium Fluoride, NaF—**41.99** [7681-49-4]—Use ACS reagent grade.

Sodium Glycocholate, C₂₆H₄₂NNaO₆—**487.60** [863-57-0]—White to tan powder. Is hygroscopic. Freely soluble in water and in alcohol.

Specific rotation (781): between +28° and +31°, calculated on the dried basis (it is rendered anhydrous by drying at 100° for 2 hours), determined at 20° in a solution containing 10 mg per mL.

Nitrogen, Method I (461): between 2.6% and 3.2% of N is found, calculated on the dried basis.

Sodium 1-Heptanesulfonate (1-Heptanesulfonic Acid Sodium Salt), C₇H₁₅NaO₃S—**202.25** [22767-50-6]—Use a suitable grade.

Sodium 1-Heptanesulfonate Monohydrate, C₇H₁₅NaO₃S · H₂O—**220.26** [22767-50-6]—Use a suitable grade.

Sodium 1-Hexanesulfonate (1-Hexanesulfonic Acid Sodium Salt), C₆H₁₃NaO₃S—**188.22** [2832-45-3]—Use a suitable grade.

Sodium 1-Hexanesulfonate Monohydrate, C₆H₁₃NaO₃S · H₂O—**206.23** [2832-45-3]—Use a suitable grade.

Sodium Hydrogen Sulfate (*Sodium Bisulfate*), NaHSO₄—**120.06** [7681-38-1]—Freely soluble in water; very soluble in boiling water. It decomposes in alcohol into sodium sulfate and free sulfuric acid. Use a suitable reagent grade.

Melting point (741): about 315°.

Sodium Hydrosulfite (*Sodium Dithionite*), Na₂S₂O₄—**174.11** [7775-14-6]—White or grayish-white crystalline powder. Soluble in water; slightly soluble in alcohol. Gradually oxidizes in air, more readily when in solution, to bisulfite, acquiring an acid reaction. Is affected by light.

Assay—Accurately weigh about 1 g, dissolve it in a mixture of 10 mL of formaldehyde TS and 10 mL of water contained in a small glass-stoppered flask, and allow to stand for 30 minutes with frequent agitation. Transfer the solution to a 250-mL volumetric flask, add 150 mL of water and 3 drops of methyl orange TS, and then add, dropwise, 1 N sulfuric acid to a slightly acid reaction. Dilute with water to 250 mL, and mix. To 50.0 mL of the dilution add 2 drops of phenolphthalein TS and just sufficient 0.1 N sodium hydroxide to produce a slight, pink color, then titrate with 0.1 N iodine, adding 3 mL of starch TS as the indicator. Then discharge the blue color of the solution with 1 drop of 0.1 N sodium thiosulfate, and titrate with 0.1 N sodium hydroxide VS to a pink color: each mL of 0.1 N sodium hydroxide is equivalent to 3.482 mg of $\text{Na}_2\text{S}_2\text{O}_4$. Not less than 88% is found.

Sulfide—Add sodium hydroxide solution (1 in 10) to lead acetate TS until the precipitate dissolves. Add 5 drops of this solution to a solution of 1 g of the sodium hydrosulfite in 10 mL of water: no immediate darkening is observed.

Heavy metals—Dissolve 1 g in 10 mL of water, add 10 mL of hydrochloric acid, and evaporate on a steam bath to dryness. Dissolve the residue in 20 mL of water and 0.5 mL of diluted hydrochloric acid, filter, and add to the filtrate 10 mL of hydrogen sulfide TS: no darkening is produced. Render the solution alkaline with ammonia TS: a slight, greenish color may be produced, but not a dark or white precipitate.

Suitability for riboflavin assay—To each of 2 or more tubes add 10 mL of water and 1.0 mL of a standard riboflavin solution containing 20 µg of riboflavin in each mL, and mix. To each tube add 1.0 mL of glacial acetic acid, mix, add with mixing, 0.5 mL of potassium permanganate solution (1 in 25), and allow to stand for 2 minutes. Then to each tube add, with mixing, 0.5 mL of hydrogen peroxide TS: the permanganate color is destroyed within 10 seconds. Shake the tubes vigorously until excess oxygen is expelled. If gas bubbles remain on the sides of tubes after foaming has ceased, remove the bubbles by tipping the tubes so that the solution flows slowly from end to end. In a suitable fluorometer, measure the fluorescence of the solution. Then add, with mixing, 8.0 mg of sodium hydrosulfite: the riboflavin is completely reduced in not more than 5 seconds.

Sodium Hydroxide, NaOH —**40.00** [1310-73-2]—Use ACS reagent grade.

Sodium Hypochlorite Solution [7681-52-9]—A solution of sodium hypochlorite (NaOCl) in water. Usually yellow to yellowish-green in color. Has an odor of chlorine. Is affected by light and gradually deteriorates. Store it in light-resistant containers, preferably below 25°. [**CAUTION**—This solution is corrosive and may evolve gases that are corrosive and toxic. It is a powerful oxidant that can react violently with reducing agents. Is irritating and corrosive to skin and mucous membranes.]

Assay—Transfer about 3 mL to a tared, glass-stoppered iodine flask, and weigh accurately. Add 50 mL of water, 2 g of potassium iodide, and 10 mL of acetic acid, insert the stopper in the flask, and allow to stand in the dark for 10 minutes. Remove the stopper, rinse the walls of the flask with a few mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is neared. Each mL of 0.1 N sodium thiosulfate consumed is equivalent to 3.723 mg of NaOCl : not less than 5.25% is found. If it is desired to calculate the percentage of available chlorine, note that each mL of 0.1 N sodium thiosulfate consumed is equivalent to 3.545 mg of available chlorine.

Calcium—Transfer 10.0 g to a 150-mL beaker, dissolve in 10 mL of water, and add 5 mL of hydrochloric acid and 2 g of potassium iodide. Heat the mixture for 5 minutes, cool, and add 2 mL of 30 percent hydrogen peroxide. Evaporate to dryness, cool, and add 2 mL of hydrochloric acid and 2 mL of 30 percent hydrogen peroxide. Rinse the inner walls of the beaker with a few mL of water, and evaporate to

dryness. Take up the residue in 20 mL of water, and filter if necessary. To the filtrate add ammonium hydroxide until the solution is just alkaline, then add 4 drops of ammonium hydroxide and 5 mL of ammonium oxalate TS: any turbidity produced within 15 minutes does not exceed that in a blank containing 0.1 mg of added Ca carried through the entire procedure (0.001%).

Phosphate (Reagent test)—Transfer 2 g to a beaker, and add 5 mL of hydrochloric acid and 2 g of potassium iodide. Heat the solution for 5 minutes, and cool. Add 2 mL of 30 percent hydrogen peroxide, and evaporate the solution to dryness. Rinse the walls of the beaker with a few mL of water, and add 2 mL of hydrochloric acid and 2 mL of 30 percent hydrogen peroxide. Evaporate again to dryness: the residue shows not more than 0.01 mg of PO_4 (5 ppm).

Sodium Iodate, NaIO_3 —**197.9** [7681-55-2]—White to yellowish-white powder. Use a suitable reagent grade.

Sodium Lauryl Sulfate—See *Sodium Dodecyl Sulfate*.

Sodium Metabisulfite, $\text{Na}_2\text{S}_2\text{O}_5$ —**190.11**

[7681-57-4]—Use ACS reagent grade.

Sodium Metaperiodate, NaIO_4 —**213.89**

[7790-28-5]—Use ACS reagent grade Sodium Periodate.

Sodium Methoxide, CH_3ONa —**54.02** [124-41-4]—

Fine, white powder. Reacts violently with water with evolution of heat. Soluble in alcohol and in methanol.

Assay—Transfer about 220 mg to a tared, glass-stoppered flask, and weigh accurately. Dissolve the test specimen in about 10 mL of methanol, then add 100 mL of water slowly, with stirring. Add phenolphthalein TS, and titrate with 0.1 N hydrochloric acid VS to a colorless endpoint: each mL of 0.1 N hydrochloric acid VS is equivalent to 5.402 mg of CH_3ONa . Not less than 98.0% is found.

Sodium Molybdate, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ —**241.95**

[7631-95-0]—Use ACS reagent grade.

Sodium Nitrate, NaNO_3 —**84.99** [7631-99-4]—Use

ACS reagent grade.

Sodium Nitrite, NaNO_2 —**69.00** [7632-00-0]—Use ACS

reagent grade.

Sodium Nitroferricyanide (*Sodium Nitroprusside*), $\text{Na}_2\text{Fe}(\text{NO})(\text{CN})_5 \cdot 2\text{H}_2\text{O}$ —**297.95** [13755-38-9]—Use ACS reagent grade.

Sodium 1-Octanesulfonate—See *Octanesulfonic Acid Sodium Salt*.

Sodium Oxalate, $\text{Na}_2\text{C}_2\text{O}_4$ —**134.00** [62-76-0]—Use ACS reagent grade.

[NOTE—Sodium Oxalate of a quality suitable as a primary standard is available from the Office of Standard Reference Materials, National Institute of Standards and Technology, Washington, DC, www.nist.gov, as standard sample No. 40.]

Sodium (tri) Pentacyanoamino Ferrate [*Trisodium Aminepentacyanoferrate* (3-)], $\text{Na}_3[\text{Fe}(\text{CN})_5\text{NH}_3]$ —**271.93** [14099-05-9]—Yellow to tan powder. Soluble in water.

Solubility—Dissolve 500 mg in 50 mL of water, and allow to stand for 1 hour: the solution is clear and free from foreign matter.

Sensitivity—

1,1-DIMETHYLHYDRAZINE STANDARD SOLUTION—Place 500 mL of water in a 1-L volumetric flask, and add from a buret 1.27 mL of anhydrous 1,1-dimethylhydrazine. Dilute with water to volume, and mix. Pipet 10 mL of this solution into a 100-mL volumetric flask, and dilute with water to volume. Each mL of this solution contains the equivalent of 100 µg of 1,1-dimethylhydrazine.

BUFFER SOLUTION—Transfer 4.8 g of citric acid monohydrate to a 1-L volumetric flask, dissolve in water, add 14.6 g of sodium phosphate, swirl to dissolve, and dilute with water to volume.

TEST PREPARATION—Dissolve 100 mg of sodium (tri)pentacyanoamino ferrate in 100 mL of water.

PROCEDURE—Into each of five 25-mL volumetric flasks pipet 0 mL, 0.25 mL, 0.50 mL, 1.0 mL, and 1.5 mL, respectively, of 1,1-Dimethylhydrazine standard solution; to each

add 15 mL of *Buffer solution*, and swirl to mix. To each flask, add by pipet 2 mL of *Test preparation*, mix, dilute with *Buffer solution* to volume, and allow to stand for 1 hour. Using a suitable spectrophotometer, 1-cm cells, and the solution containing no 1,1-Dimethylhydrazine standard solution as the blank, determine the absorbances of the remaining solutions at 500 nm. Plot the observed absorbance as the ordinate versus the concentration of standard as the abscissa on coordinate paper, and draw the curve of best fit. The plot is linear and the absorbance of the 150- μ g solution is not less than 0.65.

Sodium 1-Pentanesulfonate (1-Pentanesulfonic Acid Sodium Salt), $C_5H_{11}NaO_3S \cdot H_2O$ —**192.21** [207605-40-1]—Use a suitable grade with a content of NLT 98.0%.

Sodium 1-Pentanesulfonate, Anhydrous (1-Pentanesulfonic Acid Sodium Salt, Anhydrous), $C_5H_{11}SO_3Na$ —**174.19** [22767-49-3]—Use a suitable grade with a content of NLT 98%.

Sodium Perchlorate, $NaClO_4 \cdot H_2O$ —**140.46** [7791-07-3]—Use ACS reagent grade.

Sodium Peroxide, Na_2O_2 —**77.98** [1313-60-6]—Use ACS reagent grade.

Sodium Phosphate, Dibasic (Disodium Phosphate; Disodium Hydrogen Phosphate; Sodium Phosphate, Dibasic, Heptahydrate), $Na_2HPO_4 \cdot 7H_2O$ —**268.07** [7782-85-6]—Use ACS reagent grade Sodium Phosphate, Dibasic, Heptahydrate.

Sodium Phosphate, Dibasic, Anhydrous (Anhydrous Disodium Hydrogen Phosphate) (for buffer solutions), Na_2HPO_4 —**141.96** [7558-79-4]—Use ACS reagent grade Sodium Phosphate, Dibasic, Anhydrous.

Sodium Phosphate, Dibasic, Dihydrate (Sodium Monohydrogen Phosphate; Disodium Hydrogen Phosphate), $Na_2HPO_4 \cdot 2H_2O$ —**177.99** [10028-24-7]—Use a suitable grade with a content of not less than 99.5%. [NOTE—A suitable grade is available from www.emdchemicals.com, catalog number SX0713.]

Sodium Phosphate Dibasic, Dodecahydrate (Disodium Hydrogen Phosphate, Dodecahydrate), $Na_2HPO_4 \cdot 12H_2O$ —**358.14** [10039-32-4]—Use a suitable grade with a content of between 98.0% and 102.0% of $Na_2HPO_4 \cdot 12H_2O$.

Sodium Phosphate, Dibasic, Heptahydrate (Disodium Hydrogen Phosphate Heptahydrate; Disodium Phosphate), $Na_2HPO_4 \cdot 7H_2O$ —**268.07** [7782-85-6]—Use ACS reagent grade.

Sodium Phosphate, Monobasic (Sodium Biphosphate; Sodium Dihydrogen Phosphate; Acid Sodium Phosphate; Monosodium Orthophosphate), $NaH_2PO_4 \cdot H_2O$ —**137.99** [10049-21-5]—Use ACS reagent grade.

Sodium Phosphate, Monobasic, Anhydrous (Sodium Biphosphate; Sodium Dihydrogen Phosphate; Acid Sodium Phosphate; Monosodium Orthophosphate), NaH_2PO_4 —**119.98** [7558-80-7]—Use a suitable grade with a content of not less than 99.0%.

Sodium Phosphate, Monobasic, Dihydrate (Sodium Dihydrogen Phosphate, Dihydrate), $NaH_2PO_4 \cdot 2H_2O$ —**156.01** [13472-35-0]—Use a suitable grade with a content of not less than 99.0%.

Sodium Phosphate, Tribasic, $Na_3PO_4 \cdot 12H_2O$ —**380.12** [10101-89-0]—Use ACS reagent grade.

Sodium Phosphite Pentahydrate (Disodium Hydrogen Phosphite), $Na_2HPO_3 \cdot 5H_2O$ —**216.04** [13517-23-2]—Use a suitable grade with a content of not less than 98%.

[NOTE—A suitable grade is available from Fluka, www.sigma-aldrich.com, catalog number 04283.]

Sodium Pyrophosphate, $Na_4P_2O_7 \cdot 10H_2O$ —**446.06** [13472-36-1]—Use ACS reagent grade.

Sodium Pyruvate, CH_3COCO_2Na —**110.04** [113-24-6]—White to practically white powder or crystalline solid. Soluble in water.

Assay—Transfer about 300 mg, accurately weighed, to a high-form titration beaker, add 150 mL of glacial acetic acid, and stir until dissolved. Titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically, using a

glass electrode and a calomel electrode modified to use 0.1 N tetramethylammonium chloride in methanol as the electrolyte. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 11.00 mg of CH_3COCO_2Na : not less than 98.0% is found.

Solubility—Dissolve 1.5 g in 25 mL of water: the solution is clear and complete.

Free acid—Dissolve 10 g in 150 mL of water, and titrate with 0.5 N sodium hydroxide VS, determining the endpoint potentiometrically: not more than 2.8 mL of 0.5 N sodium hydroxide is consumed (about 1% as $C_3H_4O_3$).

Sodium Salicylate [54-21-7]—It complies with the specifications under *Sodium Salicylate* (USP monograph), and in addition meets the requirements of the following test.

Nitrate—Dissolve 100 mg in 5 mL of water, and superimpose the solution upon 5 mL of sulfuric acid: no brownish-red color appears at the junction of the two liquids.

Sodium Selenite, Na_2SeO_3 —**172.94** [10102-18-8]—White, odorless, crystalline powder, usually partially hydrated. Freely soluble in water; insoluble in alcohol.

Assay—Accurately weigh about 180 mg, previously dried at 120° to constant weight, and dissolve it in 50 mL of water in a glass-stoppered flask. Add, successively, 3 g of potassium iodide and then 5 mL of hydrochloric acid, insert the stopper, and allow to stand for 10 minutes. Add 50 mL of water, 50.0 mL of 0.1 N sodium thiosulfate VS, and 3 mL of starch TS, and immediately titrate with 0.1 N iodine VS to a blue color. Perform a blank determination. The difference in volumes of 0.1 N iodine is equivalent to 4.323 mg of Na_2SeO_3 . Between 98% and 101% is found.

Solubility—One g in 10 mL of water shows not more than a faint haze.

Carbonate—To 500 mg add 1 mL of water and 2 mL of diluted hydrochloric acid: no effervescence is produced.

Chloride (Reagent test)—A 500-mg portion shows not more than 0.05 mg of Cl (0.01%).

Nitrate (Reagent test)—A 200-mg portion dissolved in 3 mL of water shows not more than 0.02 mg of NO_3 (0.01%).

Selenate and sulfate (as SO_4)—To 500 mg in a small evaporating dish add 20 mg of sodium carbonate and 10 mL of hydrochloric acid. Slowly evaporate the solution on a steam bath under a hood to dryness. Wash the sides of the dish with 5 mL of hydrochloric acid, and again evaporate to dryness. Dissolve the residue in a mixture of 15 mL of hot water and 1 mL of hydrochloric acid. Proceed as directed under *Sulfate in Reagents* (Reagent test, *Method I*), beginning with "Filter the solution." The test specimen shows no more turbidity than that produced by 0.15 mg of SO_4 (0.03%).

Sodium Sulfate (Glauber's Salt), $Na_2SO_4 \cdot 10H_2O$ —**322.20** [7727-73-3]—Use ACS reagent grade.

Sodium Sulfate, Anhydrous, Na_2SO_4 —**142.04** [7757-82-6]—Use ACS reagent grade.

For use in assaying alkaloids by gas-liquid chromatography, it conforms to the following additional test.

Suitability for alkaloid assays—Transfer about 10 mg of atropine, accurately weighed, to a 25-mL volumetric flask, dissolve in and dilute with alcohol to volume. Pipet 3 mL of the solution into each of two 60-mL separators, and add to each 10 mL of water, 1 mL of 1 N sodium hydroxide, and 10 mL of chloroform. Shake thoroughly, and allow the layers to separate. Filter the organic phase from one separator through phase-separating paper, previously washed with 5 mL of chloroform, supported in a funnel, and collect the filtrate in a suitable container. Add 10 mL of chloroform to the separator, shake thoroughly, and filter the organic layer through the same phase-separating paper, collecting and combining the filtrates in the same container. Designate the combined filtrates as *Solution A*. Filter the organic phase from the second separator through 30 g of the Anhydrous

Sodium Sulfate, supported on a pledget of glass wool in a small funnel, and previously washed with chloroform, and collect the filtrate in a suitable container. Add 10 mL of chloroform to the separator, shake thoroughly, and filter the organic layer through the same portion of anhydrous sodium sulfate, collecting and combining the two filtrates in the same container. Designate the combined filtrates as *Solution B*. Evaporate the two solutions in vacuum to a volume of about 1 mL. Inject an accurately measured volume of *Solution A* into a suitable gas chromatograph, and record the peak height. Repeat the determination with a second accurately measured volume of *Solution A*, record the peak height, and obtain the average of the two results. In a similar manner, determine the peak height of two portions of *Solution B*, and obtain the average of the results. The average value obtained for *Solution B* is within 5.0% of the value obtained for *Solution A*.

Under typical conditions, the gas chromatograph contains a 4-mm \times 1.2-m glass column packed with 3% phase G3 on packing S1A. After curing and conditioning, the column temperature is maintained at 210°, the injector port temperature at 225°, and the detector block temperature at 240° during the determinations. The carrier gas is helium, flowing at a rate of 60 mL per minute.

Sodium Sulfate Decahydrate—Use *Sodium Sulfate*.

Sodium Sulfide, $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ —**240.18** [1313-84-4]—Use ACS reagent grade.

Sodium Sulfite—Use *Sodium Sulfite, Anhydrous*.

Sodium Sulfite, Anhydrous (*Exsiccated Sodium Sulfite*), Na_2SO_3 —**126.04** [7753-83-7]—Use ACS reagent grade.

Sodium p-Sulfophenylazochromotrope [*Trisodium Salt of 4,5-Dihydroxy-3-(p-sulfophenylazo)-2,7-naphthalenedisulfonic Acid*], $\text{C}_{16}\text{H}_9\text{N}_2\text{Na}_3\text{O}_{11}\text{S}_3 \cdot 3\text{H}_2\text{O}$ —**624.47**—Bright red powder. Very soluble in water; insoluble in alcohol. Combines with zirconium oxychloride to form a soluble pink zirconium lake.

[NOTE—The reagent is available as Catalog No. 7309 from Distillation Products Industries, Eastman Organic Chemicals Dept., Rochester, NY 14650. A procedure for its preparation is described in *Z. Anal. Chem.*, 146, 417 (1955).]

Sodium Tartrate, $\text{Na}_2\text{C}_4\text{H}_4\text{O}_6 \cdot 2\text{H}_2\text{O}$ —**230.08** [6106-24-7]—Use ACS reagent grade.

Sodium Tetraphenylborate, $\text{NaB}(\text{C}_6\text{H}_5)_4$ —**342.22** [143-66-8]—Use ACS reagent grade.

Sodium Tetraphenylboron—See *Sodium Tetraphenylborate*.

Sodium Thioglycolate (*Sodium Thioglycollate*), $\text{HSCH}_2\text{COONa}$ —**114.10** [367-51-1]—A white, crystalline powder. Very soluble in water; slightly soluble in alcohol. Is hygroscopic, and oxidizes in air. Store in tight, light-resistant containers. It should not be used if it is pale yellow or darker in color.

Assay—Accurately weigh about 250 mg, and dissolve in 50 mL of oxygen-free water. Add 5 mL of diluted hydrochloric acid, boil for 2 minutes, cool, and titrate the solution with 0.1 N iodine VS, adding 3 mL of starch TS toward the end: each mL of 0.1 N iodine is equivalent to 11.41 mg of $\text{HSCH}_2\text{COONa}$. Not less than 75% is found.

Insoluble matter—A solution of 1 g in 10 mL of water is clear, and practically complete.

Sulfide—Dissolve 500 mg in 10 mL of water in a small flask, add 2 mL of hydrochloric acid, then place a strip of filter paper, moistened with lead acetate TS, over the mouth of the flask, and bring the solution to a boil: the lead acetate paper is not darkened.

Sodium Thiosulfate, $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ —**248.19** [10102-17-7]—Use ACS reagent grade.

Sodium L-Thyroxine—Use *Levothyroxine Sodium* (USP monograph).

Sodium 3-(trimethylsilyl)-1-propane sulfonate (*Sodium 2,2-dimethyl-2-silapentane-5-sulfonate*), $\text{C}_6\text{H}_{15}\text{SiNaO}_3\text{S}$ —**218.32**—Use a suitable grade.

Sodium Tungstate, $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ —**329.85** [10213-10-2]—Use ACS reagent grade.

Soluble Starch—See *Starch, Soluble*.

Solvent Hexane—See *Hexane, Solvent*.

Sorbitol—Use *Sorbitol* (NF monograph).

Stachyose Hydrate, $\text{C}_{24}\text{H}_{42}\text{O}_{21} \cdot x\text{H}_2\text{O}$ —**666.58** [10094-58-3]—Use a suitable grade.

Standard Sand, 20- to 30-Mesh—See *Sand, Standard 20- to 30-mesh*.

Stannous Chloride, $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ —**225.65** [10025-69-1]—Use ACS reagent grade.

Starch, Potato—The starch separated from the tubers of *Solanum tuberosum* Linné (Fam. Solanaceae). A more or less finely granular powder, consisting of starch grains of characteristic shape and appearance when examined microscopically.

Starch, Soluble (*for iodimetry*) [9005-84-9]—Use ACS reagent grade.

Starch, Soluble, Purified—White, amorphous powder; under microscopic examination it shows the characteristic form of potato starch. Soluble in hot water; very slightly soluble in alcohol.

TEST SOLUTION FOR DETERMINATION OF PH AND SENSITIVITY—Stir 2.0 g in 10 mL of water, add boiling water to make 100 mL, and boil for 2 minutes. The hot solution is almost clear. On cooling, the solution may become opalescent or turbid, but does not gel. Use it as the *Test solution*.

pH (791)—The pH of the *Test solution* is between 6.0 and 7.5.

Sensitivity—Mix 2.5 mL of *Test solution*, 97.5 mL of water, and 0.50 mL of 0.010 N iodine: a distinct blue color results, and it disappears upon the addition of 0.50 mL of 0.010 N sodium thiosulfate.

Absorbance—Prepare a pH 5.3 buffer solution by dissolving 43.5 g of sodium acetate (trihydrate) and 4.5 mL of glacial acetic acid in water, transferring the resultant solution to a 250-mL volumetric flask, adding water to volume, and mixing.

Dissolve 1.00 g of Soluble Purified Starch in 2.5 mL of the buffer solution by warming, transfer to a 100-mL volumetric flask, add water to volume, and mix. Add 0.50 mL of this solution to a 100-mL volumetric flask containing about 75 mL of water, 1 mL of 1 N hydrochloric acid, and 1.5 mL of 0.020 N iodine, swirling the flask during the addition. Add water to volume, mix, and allow to stand in the dark for 1 hour. The absorbance of this solution, measured at 575 nm in a 1-cm cell against a blank, is between 0.5 and 0.6.

Reducing substances—Shake 10.0 g with 100 mL of water for 15 minutes, and allow to settle for about 12 hours. Filter a portion of the supernatant through fine sintered glass. To 50 mL of the filtrate add 50 mL of alkaline cupric tartrate TS, and boil for 1 to 2 minutes. Filter the resulting cuprous oxide, wash it with hot water and then with alcohol, and dry it at 105° for 2 hours: not more than 47 mg is found, corresponding to 0.7% of reducing sugars as maltose.

Loss on drying (731)—Dry it at 105° for 2 hours: it loses not more than 10% of its weight.

Residue on ignition (281): not more than 0.5%.

Stearic Acid, $\text{C}_{18}\text{H}_{36}\text{O}_2$ —**284.48** [57-11-4]—Hard, white crystals or amorphous, white powder. Freely soluble in chloroform and in ether; soluble in alcohol and in solvent hexane.

Congealing temperature (651): between 67° and 69°.

Acid value (401): between 196 and 199.

Iodine value (401): not more than 1.

Saponification value (401): between 197 and 200.

Palmitic acid—Determine as directed in the Assay under *Stearic Acid* (NF monograph): not more than 5.0% is found.

Stearyl Alcohol (1-Octadecanol), $C_{18}H_{38}O$ —**270.49** [112-92-5]—White flakes, granules, or crystals. Soluble in alcohol, in ether, in acetone, and in benzene; insoluble in water.

Melting range (741): between 56° and 58°.

Other requirements—It conforms to the tests for *Acid value*, *Iodine value*, and *Hydroxyl value* under *Stearyl Alcohol* (NF monograph).

Stronger Ammonia Water—See *Ammonia Water*, *Stronger*.

Strontium Acetate, $Sr(CH_3COO)_2 \cdot \frac{1}{2}H_2O$ —**214.72** [543-94-2]—White, crystalline powder. Soluble in 3 parts of water; slightly soluble in alcohol.

Assay—Ignite about 3 g, accurately weighed, in a platinum crucible, protecting from sulfur in the flame. Cool, transfer the crucible with the residue to a beaker, and add 50 mL of water and 40.0 mL of 1 N hydrochloric acid VS. Boil gently for 30 minutes or longer, if necessary; filter; wash with hot water until the washings are neutral; add methyl red TS; and titrate the excess acid with 1 N sodium hydroxide VS. Each mL of 1 N hydrochloric acid is equivalent to 107.4 mg of $Sr(CH_3COO)_2 \cdot \frac{1}{2}H_2O$: not less than 99% is found.

Insoluble matter (Reagent test): not more than 2 mg, from 10 g (0.02%).

Free alkali or free acid—Dissolve 3 g in 30 mL of water, and add 3 drops of phenolphthalein TS: no pink color is produced. Titrate with 0.1 N sodium hydroxide VS to a pink color: not more than 0.30 mL of the 0.1 N sodium hydroxide is required.

Barium—Dissolve 1 g in 10 mL of water, and add 1 drop of glacial acetic acid and 5 drops of potassium dichromate solution (1 in 10): no turbidity is produced within 2 minutes (about 0.02%).

Calcium—Ignite 1 g until completely carbonized. Warm the residue with a mixture of 3 mL of nitric acid and 10 mL of water, filter, wash with 5 mL of water, and evaporate the filtrate on a steam bath to dryness. Powder the residue, and dry it at 120° for 3 hours. Reflux the dried powder with 15 mL of dehydrated alcohol for 10 minutes, cool in ice, and filter. Repeat the extraction with 10 mL of dehydrated alcohol. Evaporate the combined filtrates to dryness, add 0.5 mL of sulfuric acid, and ignite: the weight of the residue is not more than 10 mg (0.3% of Ca).

Chloride (Reagent test)—One g shows not more than 0.1 mg of Cl (0.01%).

Heavy metals (Reagent test): 0.001%.

Iron (241)—Dissolve 1.0 g in 45 mL of water, and add 2 mL of hydrochloric acid: the solution shows not more than 0.01 mg of Fe (0.001%).

Alkali salts—Dissolve 2 g in 80 mL of water, heat to boiling, add an excess of ammonium carbonate TS, boil for 5 minutes, dilute with water to 100 mL, and filter. Evaporate 50 mL of the filtrate, and ignite: the residue, after correcting for the ignition residue from half the volume of the clear ammonium carbonate TS used above, is not more than 3 mg (0.3%).

Nitrate—Dissolve 1 g in 10 mL of water, add 0.10 mL of indigo carmine TS, and then add 10 mL of sulfuric acid: the blue color persists for 5 minutes (about 0.01% of NO_3).

Strontium Hydroxide (*Strontium Hydroxide Octahydrate*), $Sr(OH)_2 \cdot 8H_2O$ —**265.76** [18480-07-4]—White, crystalline, free-flowing powder. Sparingly soluble in water. May absorb carbon dioxide from the air. Keep tightly closed.

Assay and carbonate—Accurately weigh about 5 g, dissolve in 200 mL of warm carbon dioxide-free water in a glass-stoppered, 500-mL flask, add phenolphthalein TS, and titrate with 1 N hydrochloric acid VS to determine the hydroxide alkalinity. Then add methyl orange TS, and titrate with 1 N hydrochloric acid VS. Each mL of 1 N hydrochloric acid required to reach the phenolphthalein endpoint is equivalent to 132.9 mg of $Sr(OH)_2 \cdot 8H_2O$, and each additional mL of 1 N hydrochloric acid VS required to reach the methyl orange endpoint is equivalent to 73.8 mg of $SrCO_3$. Not less than 95.0% of $Sr(OH)_2 \cdot 8H_2O$ and not more than 3.0% of $SrCO_3$ are found.

Chloride (Reagent test)—Dissolve 1.0 g in 100 mL of water, and filter if necessary: 1.0 mL of the solution shows not more than 0.01 mg of Cl (0.1%).

Calcium (Reagent test)—

TEST SOLUTION—Dissolve 5.0 g in water, and dilute with water to 100 mL.

SAMPLE SOLUTION—Dilute 10.0 mL of the *Test solution* with water to 100 mL.

CONTROL SOLUTION—To 10.0 mL of the *Test solution* add 0.50 mg of calcium ion (Ca), and dilute with water to 100 mL.

PROCEDURE—Determine the background emission at 416.7 nm: the limit is 0.1%.

Iron—Dissolve 1 g in warm water, and dilute with water to 100 mL. To 20 mL of this solution add 2 mL of hydrochloric acid and 0.1 mL of 0.1 N potassium permanganate, allow to stand for 5 minutes, and add 3 mL of ammonium thiocyanate solution (3 in 10). Any red color produced is not darker than that of a control containing 0.03 mg of added Fe (0.015%).

Heavy metals—Dissolve 2.0 g in 14 mL of dilute hydrochloric acid (1 in 6), and evaporate on a steam bath to dryness. Take up the residue in 25 mL of water, filter, and dilute with water to 100 mL (*Test solution*). To 5.0 mL of the *Test solution* add 0.02 mg of lead (Pb), and dilute with water to 30 mL, to provide the standard. For the test specimen, use 30 mL of the *Test solution*. Adjust each solution with diluted acetic acid or ammonia TS to a pH between 3.0 and 4.0 (using short-range pH paper), dilute with water to 40 mL, and add 10 mL of freshly prepared hydrogen sulfide TS: any brown color developed in the sample solution is not darker than that in the control solution (0.004%).

Strychnine Sulfate, $(C_{21}H_{22}N_2O_2)_2 \cdot H_2SO_4 \cdot 5H_2O$ —**856.98** [60-41-3]—Colorless or white crystals, or a white, crystalline powder. Its solutions are levorotatory. One g dissolves in about 35 parts of water, in 85 mL of alcohol, and in about 220 mL of chloroform. Insoluble in ether.

Solubility—A solution of 500 mg in 25 mL of water is complete, clear, and colorless.

Residue on ignition (Reagent test): not more than 0.1%.

Brucine—To 100 mg add 1 mL of dilute nitric acid (1 in 2): a yellow color may be observed, but not a red or reddish-brown color.

Styrene-Divinylbenzene Anion-exchange Resin, 50- to 100-Mesh—See *Anion-exchange Resin, 50- to 100-Mesh*, *Styrene-Divinylbenzene*.

Styrene-Divinylbenzene Cation-exchange Resin, Strongly Acidic—See *Cation-exchange Resin, Styrene-Divinylbenzene, Strongly Acidic*.

Styrene-Divinylbenzene Copolymer Beads—Neutral, porous, cross-linked beads, 200–400 mesh, molecular weight operating range up to 2,000 (based on beads fully swollen in benzene). Suitable for use in the gel permeation separation of lipophilic polymers and other solutes requiring organic eluant.

[NOTE—A suitable grade is available commercially as “BioBeads S-X” from Bio-Rad, www.bio-rad.com.]

Succinic Acid, $C_4H_6O_4$ —**118.09** [771-50-6]—Use ACS reagent grade.

Sudan III, $C_{22}H_{16}N_4O$ —**352.39** [85-86-9]—Red to red-brown powder. Use a suitable grade.

Assay—When tested by thin-layer chromatography (see *Chromatography* (621)) with the use of plates coated with chromatographic silica gel mixture and a developing system consisting of a mixture of hexane and ethyl acetate (80:20), and examined under short-wavelength UV light, a single spot is exhibited, with trace impurities.

Sudan IV, $C_{24}H_{20}N_4O$ —**380.44** [85-83-6]—Brown to reddish-brown powder.

Assay—Transfer about 25 mg, accurately weighed, to a 100-mL volumetric flask. Dissolve in chloroform, dilute with chloroform to volume, and mix. Dilute 2.0 mL of the resulting solution with chloroform to 50.0 mL. Determine the absorbance of this solution in 1-cm cells at the wavelength of maximum absorbance at about 520 nm, with a suitable spectrophotometer, using chloroform as the blank. Calculate the percentage of Sudan IV in the test specimen taken by the formula:

$$(100A)/(85C)$$

in which A is the absorbance at 520 nm and C is the concentration of the test specimen in g per L. Not less than 90% is found.

Loss on drying (731)—Dry it at 105° for 2 hours: it loses not more than 10% of its weight.

Sulfamerazine (4-Amino-N-(4-methyl-2-pyrimidinyl)-benzenesulfonamide), $C_{11}H_{12}N_4O_2S$ —**264.30** [127-79-7]—Use a suitable grade with a content of NLT 99.0%.

Sulfamic Acid, HSO_3NH_2 —**97.09** [5329-14-6]—Use ACS reagent grade.

Sulfanilamide, $C_6H_8N_2O_2S$ —**172.21** [63-74-1]—Use USP Sulfanilamide Melting Point RS.

Sulfanilic Acid, $p\text{-NH}_2C_6H_4SO_3H \cdot H_2O$ —**191.21** [121-57-3]—Use ACS reagent grade.

Sulfatase Enzyme Preparation—Use a suitable grade.

[NOTE—A suitable grade is available commercially under catalog number S-9626 from Sigma-Aldrich, Web site: www.sigma-aldrich.com.]

Sulfathiazole Sodium (4-Amino-N-2-thiazolylbenzenesulfonamide Sodium Salt), $C_9H_8N_3NaO_2S_2$ —**277.29** [144-74-1]—Use a suitable grade.

Sulfonic Acid Cation-exchange Resin—See *Cation-exchange Resin, Sulfonic Acid*.

Sulfosalicylic Acid, $C_6H_3(COOH)(OH)(SO_3H) \cdot 1,2,5 \cdot 2H_2O$ —**254.22** [97-05-2]—Use ACS reagent grade.

Sulfur—Use *Precipitated Sulfur* (USP monograph).

Sulfur Dioxide Detector Tube—A fuse-sealed glass tube so designed that gas may be passed through it and containing suitable absorbing filters and support media for an iodine-starch indicator.

Measuring range: 1 to 25 ppm.

[NOTE—Available from Draeger Safety, Inc., www.draeger.com, or from Gastec Corp., www.gastec.co.jp, distributed in the USA by www.nextteq.com.]

Sulfuric Acid, H_2SO_4 —**98.08** [7664-93-9]—Use ACS reagent grade.

Sulfuric Acid, Diluted (10 percent)—Cautiously add 57 mL of sulfuric acid to about 100 mL of water, cool to room temperature, and dilute with water to 1000 mL.

Sulfuric Acid, Fluorometric—Use ACS reagent grade Sulfuric Acid that conforms to the following additional test:

Fluorescence—Using a suitable fluorometer having a sharp cut-off 360-nm excitation filter and a sharp cut-off 415-nm excitation filter, determine the fluorescence of the sulfuric acid in a cuvette previously rinsed with water followed by several portions of the acid under examination: the fluores-

cence does not exceed that of quinine sulfate solution (1 in 1,600,000,000), similarly measured.

Sulfuric Acid, Fuming, H_2SO_4 plus free SO_3 [8014-95-7]—having a nominal content of 15%, 20%, or 30% of free SO_3 —Use ACS reagent grade (containing between 15.0% and 18.0%, between 20.0% and 23.0%, or between 30.0% and 33.0% of free SO_3).

Sulfuric Acid, Nitrogen Free, H_2SO_4 —**98.08** [7664-93-9]—Use a suitable grade.

[NOTE—A suitable grade is available as Sulfuric Acid, Ultrex II, catalog number 6902-05 from www.mallbaker.com.]

Sulfurous Acid, H_2SO_3 —**82.08** [7782-99-2]—A water solution of sulfur dioxide. Use ACS reagent grade.

Sunflower Oil [8001-21-6]—Use a suitable grade.

Supports for Gas Chromatography—See supports for gas chromatography in the *Chromatographic Reagents* section under *Chromatography* (621).

Tannic Acid (Tannin) [1401-55-4]—Use ACS reagent grade.

Tartaric Acid, $H_2C_4H_4O_6$ —**150.09**—Use ACS reagent grade.

Tertiary Butyl Alcohol—See *Butyl Alcohol, Tertiary*.

Testosterone Benzoate, $C_{26}H_{32}O_2$ —**376.53**—Use a suitable grade.

2',4',5',7'-Tetrabromofluorescein (Eosin Y, Eosin Yellowish, Eosin Bromo ES, Solvent Red 43, Acid Red 87), $C_{20}H_8Br_4O_5$ —**167.8** [630-20-6]—Dark red to brown powder. Solubility 0.1% in water (clear orange solution). Use a suitable grade.

Tetrabromophenolphthalein Ethyl Ester, $C_{22}H_{14}Br_4O_4$ —**661.96**—Use ACS reagent grade.

Tetrabutylammonium Bromide, $(C_4H_9)_4NBr$ —**322.37** [1643-19-2]—Use ACS reagent grade.

Tetrabutylammonium Hydrogen Sulfate, $C_{16}H_{37}NO_4S$ —**339.54** [32503-27-8]—White, crystalline powder. Soluble in alcohol yielding a slightly hazy, colorless solution.

Assay—Dissolve about 170 mg, accurately weighed, in 40 mL of water. Titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination and make any necessary correction. Each mL of 0.1 N sodium hydroxide is equivalent to 33.95 mg of $C_{16}H_{37}NO_4S$. Not less than 97.0% is found.

Melting range (741): between 169° and 173°.

Tetrabutylammonium Hydrogen Sulfate Ion Pairing Reagent—A mixture of tetrabutylammonium hydrogen sulfate and phosphate buffer. Use a suitable grade.

[NOTE—A suitable grade is available as catalog number WAT084189 from the Waters Corporation (www.waters.com).]

0.4 M Aqueous Tetrabutylammonium Hydroxide, $C_{16}H_{37}NO$ —**259.47** [2052-49-5]—Use a suitable grade.

[NOTE—A suitable grade is available as catalog number 420120025 from www.fishersci.com.]

Tetrabutylammonium Hydroxide, 1.0 M in Methanol [2052-49-5]—Use a suitable grade.

Tetrabutylammonium Hydroxide 30-Hydrate, $C_{16}H_{37}NO \cdot 30H_2O$ —**799.93** [2052-49-5]—Use a suitable grade with a content of not less than 98.0%.

Tetrabutylammonium Hydroxide, 40 Percent in Water, $[CH_3(CH_2)_3]_4NOH$ —**259.47** [2052-49-5]—Use a suitable grade.

Tetrabutylammonium Iodide, $(C_4H_9)_4NI$ —**369.37** [311-28-4]—White, shiny, crystalline flakes. Soluble in alcohol and in ether; slightly soluble in water.

Assay—Dissolve 370 mg, accurately weighed, in 60 mL of acetone with vigorous stirring. Stir the solution by mechanical means, add 10 mL of 16% sulfuric acid, and titrate with 0.1 N silver nitrate VS, determining the endpoint potentiometrically, using a glass-silver electrode system, and adding the titrant in 0.1-mL increments as the endpoint is approached. Perform a blank determination, and make any necessary corrections. Each mL of 0.1 N silver nitrate is

equivalent to 36.94 mg of $(C_4H_9)_4NI$: not less than 99.0% is found.

Tetrabutylammonium Phosphate, $(C_4H_9)_4NH_2PO_4$ —**339.46** [5574-97-0]—White to off-white powder. Soluble in water.

Assay—Dissolve about 1.5 g, accurately weighed, in 100 mL of water. Without delay, titrate with 0.5 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.5 N sodium hydroxide is equivalent to 169.7 mg of $(C_4H_9)_4NH_2PO_4$. Not less than 97.0% is found.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, ^{13}C -labeled, $^{13}C_{12}H_4Cl_4O_2$ —**333.84**—Clear, colorless liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with an electron-capture detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 300°; the detector temperature is maintained at 300°; and the column temperature is maintained at 70° and programmed to rise 15° per minute to 300°. The area of the $^{13}C_{12}H_4Cl_4O_2$ peak is not less than 99.0% of the total peak area.

[NOTE—A suitable grade is available from Cambridge Isotopes Laboratories (www.isotope.com).]

2,3,7,8-Tetrachlorodibenzofuran, ^{13}C -labeled, $^{13}C_{12}H_4Cl_4O$ —**317.84**—Clear, colorless liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with an electron-capture detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.32-mm \times 60-m capillary column coated with a 1- μ m layer of phase G27; the injection port temperature is maintained at 300°; the detector temperature is maintained at 350°; and the column temperature is maintained at 70° and programmed to rise 15° per minute to 275°. The area of the $^{13}C_{12}H_4Cl_4O$ peak is not less than 99.0% of the total peak area.

[NOTE—A suitable grade is available from Cambridge Isotopes Laboratories (www.isotope.com).]

1,1,2,2-Tetrachloroethane, $C_2H_2Cl_4$ —**167.8** [630-20-6]—Colorless clear liquid. Specific gravity: 1.553. Refractive index at 20° is 1.481. Use a suitable grade.

Tetracosane, $C_{24}H_{50}$ —**338.66** [646-31-1]—White powder.

Melting range (741): between 51° and 53°.

Tetradecane, $C_{14}H_{30}$ —**198.39** [629-59-4]—Clear, colorless liquid.

Assay—When examined by gas-liquid chromatography, it shows a purity of not less than 98%. The following conditions have been found suitable for assaying the reagent: a 3-mm \times 2.4-m stainless steel column packed with phase G16 on support S1; the carrier gas is helium, flowing at a rate of 27.5 mL per minute; the column temperature is maintained at 250°, the injection port is maintained at 200°, and the detector is maintained at 280°. A flame-ionization detector is employed.

Melting range, Class II (741): between 4° and 8°, within a 2° range.

Refractive index (831): between 1.4280 and 1.4300 at 20°.

Tetraethylammonium Perchlorate, $(C_2H_5)_4NClO_4$ —**229.70**—White crystals. Soluble in water. Use a suitable grade.

Tetraethylene Glycol, $C_8H_{18}O_5$ —**194.23** [112-60-7]—Nearly colorless liquid. Refractive index: about 1.46.

Assay—When examined by gas-liquid chromatography, using suitable gas chromatographic apparatus and conditions, it shows a purity of not less than 90%.

Boiling range (Reagent test): between 177° and 187°, at a pressure of 9 mm of Hg.

Tetraethylenepentamine, $C_8H_{23}N_5$ —**189.31** [112-57-2]—Colorless liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 250°; the detector temperature is maintained at 300°; and the column temperature is maintained at 150° and programmed to rise 10° per minute to 280°. The area of the $C_8H_{23}N_5$ peak is not less than 30% of the total peak area.

Refractive index (831): between 1.503 and 1.507 at 20°.

Tetraheptylammonium Bromide, $(C_7H_{15})_4NBr$ —**490.70** [4368-51-8]—White, flaky powder.

Melting range (741): between 89° and 91°.

Tetrahexylammonium Hydrogen Sulfate, $C_{24}H_{53}NO_4S$ —**451.75** [32503-34-7]—Use a suitable grade with a content of not less than 98.0%.

Delta-8-tetrahydrocannabinol (Δ -8-Tetrahydrocannabinol), $C_{21}H_{30}O_2$ —**314.47** [5957-75-5]—Use a suitable grade which may be a solid material or a solution in methanol. [NOTE—A suitable grade of a methanolic solution (1 mg/mL) is available from www.cerilliant.com, catalog number T-032.]

Tetrahydrofuran, C_4H_8O —**72.11** [109-99-9]—Use ACS reagent grade.

Tetrahydrofuran, Peroxide-Free, C_4H_8O —**72.11**—Use ACS reagent grade.

Peroxide—Transfer 8 mL of potassium iodide and starch TS to a 12-mL ground glass-stoppered cylinder about 15 mm in diameter. Fill completely with the substance under test, mix, and allow to stand protected from light for 5 minutes. No color develops.

Tetrahydrofuran, Stabilizer-Free—Use a suitable grade.

Tetrahydro-2-furancarboxylic Acid (\pm Tetrahydro-2-furoic Acid), $C_5H_8O_3$ —**116.12** [16874-33-2]—Use a suitable grade with a content of not less than 97%. [NOTE—A suitable grade is available from www.sigma-aldrich.com, catalog number 341517.]

N-(2-Tetrahydrofuroyl)piperazine (1-[(2-Tetrahydro-2-furanyl)carbonyl]piperazine), $C_9H_{16}N_2O_2$ —**184.23**—Use a suitable grade.

[NOTE—A suitable grade can be obtained from EMS-DÖTTIKON, www.ems-dottikon.ch.]

1,2,3,4-Tetrahydronaphthalene, $C_{10}H_{12}$ —**132.21** [119-64-2]—Colorless liquid.

Refractive index (831): 1.5401 at 20°.

Tetramethylammonium Bromide, $(CH_3)_4NBr$ —**154.05** [64-20-0]—Use ACS reagent grade.

Tetramethylammonium Chloride, $(CH_3)_4NCl$ —**109.60** [75-57-0]—Colorless crystals. Soluble in water and in alcohol; insoluble in chloroform.

Assay—Transfer about 200 mg, accurately weighed, to a beaker, add 50 mL of water and 10 mL of diluted nitric acid, swirl to dissolve the test specimen, add 50.0 mL of 0.1 N silver nitrate VS, and mix. Add 2 mL of ferric ammonium sulfate TS and 5 mL of nitrobenzene, shake, and titrate the excess silver nitrate with 0.1 N ammonium thiocyanate VS: each mL of 0.1 N silver nitrate is equivalent to 10.96 mg of $(CH_3)_4NCl$. Not less than 98% is found.

Tetramethylammonium Hydroxide, $(CH_3)_4NOH$ —**91.15** [75-59-2]—Available as an approximately 10% or approximately 25% aqueous solution, or as the crystalline pentahydrate. Is clear and colorless. Tetramethylammonium hydroxide is a stronger base than ammonia and rapidly absorbs carbon dioxide from the air. Store in tight containers.

Assay—Accurately weigh a glass-stoppered flask containing about 15 mL of water. Add a quantity of a solution of tetramethylammonium hydroxide, equivalent to about

200 mg of $(\text{CH}_3)_4\text{NOH}$, and again weigh. Add methyl red TS, and titrate the solution with 0.1 N hydrochloric acid VS: each mL of 0.1 N hydrochloric acid is equivalent to 9.115 mg of $(\text{CH}_3)_4\text{NOH}$.

Residue on evaporation—Evaporate 5 mL of solution on a steam bath, and dry at 105° for 1 hour: the weight of the residue is equivalent to not more than 0.02% of the weight of the test specimen.

Ammonia and other amines—Accurately weigh a quantity of solution, corresponding to about 300 mg of $(\text{CH}_3)_4\text{NOH}$, in a low-form weighing bottle tared with 5 mL of water. Add a slight excess of 1 N hydrochloric acid (about 4 mL), evaporate on a steam bath to dryness, and dry at 105° for 2 hours: the weight of the tetramethylammonium chloride so obtained, multiplied by 0.8317, represents the quantity, in mg, of $(\text{CH}_3)_4\text{NOH}$ in the portion of test specimen taken and corresponds to within 0.2% above or below that found in the Assay.

Tetramethylammonium Hydroxide, Pentahydrate, $(\text{CH}_3)_4\text{NOH} \cdot 5\text{H}_2\text{O}$ —**181.23** [10424-65-4]—White to off-white crystals. Is hygroscopic. Strong base. Keep well-closed. Soluble in water and in methanol.

Assay—Accurately weigh about 800 mg, dissolve in 100 mL of water, and titrate with 0.1 N hydrochloric acid VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N hydrochloric acid is equivalent to 18.22 mg of $(\text{CH}_3)_4\text{NOH} \cdot 5\text{H}_2\text{O}$: not less than 98% is found.

Tetramethylammonium Hydroxide Solution in Methanol [75-59-2]—A solution in methanol of tetramethylammonium hydroxide [$(\text{CH}_3)_4\text{NOH}$ —91.15]. Is generally available in concentrations of 10% and 25%. The following specifications apply specifically to the 25% concentration; for other concentrations, appropriate adjustments in the procedures may be necessary.

Assay—Accurately weigh about 1 g of the solution, and dilute with water to about 50 mL. Add phenolphthalein TS, and titrate with 0.1 N hydrochloric acid VS to the disappearance of the pink color: each mL of 0.1 N hydrochloric acid VS is equivalent to 91.15 mg of $(\text{CH}_3)_4\text{NOH}$. Between 23% and 25% is found.

Clarity—A portion of it in a test tube is clear, or only slightly turbid, when viewed transversely.

Tetramethylammonium Nitrate, $(\text{CH}_3)_4\text{NNO}_3$ —**136.15** [1941-24-8]—White crystals. Freely soluble in water.

Tetramethylbenzidine, (4-(4-Amino-3,5-dimethylphenyl)-2,6-dimethylaniline; 3,3',5,5'-Tetramethylbenzidine; 3,3',5,5'-Tetramethyl-[1,1'-biphenyl] 4,4'-diamine) $\text{C}_{16}\text{H}_{20}\text{N}_2$ —**240.34** [54827-17-7]—Use a suitable grade.

4,4'-Tetramethyldiaminodiphenylmethane [(4,4'-Methylenebis(N,N-dimethylaniline)], $[(\text{CH}_3)_2\text{NC}_6\text{H}_4]_2\text{CH}_2$ —**254.38** [101-61-1]—Off-white crystals.

Melting range (741): between 87° and 90° .

Tetramethylsilane, $(\text{CH}_3)_4\text{Si}$ —**88.23** [75-76-3]—Use ACS reagent grade.

Tetrapropylammonium Chloride, $\text{C}_{12}\text{H}_{28}\text{ClN}$ —**221.82** [5810-42-4]—Use a suitable grade with a content of not less than 98.0%.

Tetrasodium Ethylenediaminetetraacetate ((Ethylenedinitrilo)tetraacetic Acid Tetrasodium Salt), $\text{C}_{10}\text{H}_{12}\text{N}_2\text{Na}_4\text{O}_8$ —**380.17**—Fine, white, crystalline powder. Soluble in water.

Loss on drying (731)—Dry it at 105° for 4 hours: it loses not more than 8% of its weight.

Thallous Chloride, TlCl —**239.84**—Fine, white, crystalline powder. Soluble in about 260 parts of cold water and in about 70 parts of boiling water; insoluble in alcohol. *Poisonous; use with adequate ventilation.*

Assay—Dissolve about 500 mg, accurately weighed, in a mixture of 80 mL of water and 0.5 mL of sulfuric acid. When dissolution is complete, add 20 mL of hydrochloric

acid. Heat to 60° and maintain this temperature while titrating with 0.1 N ceric sulfate VS, determining the endpoint potentiometrically, using silver-silver chloride and platinum electrodes. Each mL of 0.1 N ceric sulfate is equivalent to 11.99 mg of TlCl . Not less than 99% is found.

Theobromine, $\text{C}_7\text{H}_8\text{N}_4\text{O}_2$ —**180.17** [83-67-0]—White, crystalline solid. Very slightly soluble in water and in alcohol; almost insoluble in benzene, in ether, and in chloroform.

Assay—Dissolve about 34 mg, accurately weighed, in 50 mL of glacial acetic acid. Titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 18.02 mg of $\text{C}_7\text{H}_8\text{N}_4\text{O}_2$. Not less than 95% is found.

Thiazole Yellow (CI Direct Yellow 9; Clayton Yellow; Titan Yellow), $\text{C}_{28}\text{H}_{19}\text{N}_5\text{Na}_2\text{O}_6\text{S}_4$ —**695.74** [1829-00-1]—Yellowish-brown powder. Soluble in water and in alcohol to yield in each instance a yellow solution; soluble in dilute alkali to yield a brownish-red solution. Protect from light.

Solubility—A 200-mg portion mixed with 50 mL of water shows not more than a faint haze.

Residue on ignition—Accurately weigh about 1.5 g, previously dried at 105° for 2 hours, and ignite until thoroughly charred. Cool, add 2 mL of nitric acid and 2 mL of sulfuric acid, ignite gently to expel excess acids, then at 600° to 800° to constant weight: the residue of sodium sulfate (Na_2SO_4) is between 19.8% and 21.5% of the weight of the test specimen (theory is 20.4%).

Sensitiveness to magnesium—Add 0.2 mL of a solution (1 in 10,000) and 2 mL of 1 N sodium hydroxide to a mixture of 9.5 mL of water and 0.5 mL of a solution prepared by dissolving 1.014 g of clear crystals of magnesium sulfate in water, diluting with water to 100 mL, then diluting 10 mL of the resulting solution with water to 1 L: a distinct pink color is produced within 10 minutes.

Thioacetamide, $\text{C}_2\text{H}_5\text{NS}$ —**75.13** [62-55-5]—Use ACS reagent grade.

2-Thiobarbituric Acid, $\text{C}_4\text{H}_4\text{N}_2\text{O}_2\text{S}$ —**144.15** [504-17-6]—White leaflets. Slightly soluble in water.

Melting temperature (741): 236° , with decomposition.

2,2'-Thiodiethanol, $(\text{HOCH}_2\text{CH}_2)_2\text{S}$ —**122.19** [111-48-8]—Pale yellow to colorless liquid.

Assay—Not less than 98% of $\text{C}_4\text{H}_4\text{N}_2\text{O}_2\text{S}$ is found, a suitable gas chromatograph equipped with a flame-ionization detector being used. The following conditions have been found suitable: a 4.0-mm \times 1.83-m glass column is packed with 10% phase G25 on support S1A; the column, injection port, and detector temperatures are maintained at 200° , 250° , and 310° , respectively.

Refractive index (831): between 1.4250 and 1.4270, at 20° .

Thioglycolic Acid, HSCH_2COOH —**92.12** [68-11-1]—A colorless or nearly colorless liquid. Miscible with water. Soluble in alcohol.

Sensitiveness—Mix 1 mL with 2 mL of stronger ammonia water, and dilute with water to 20 mL. Add 1 mL of this solution to a mixture of 20 mL of water and 0.1 mL of dilute ferric chloride TS (1 in 100), then add 5 mL of ammonia TS: a distinct pink color is produced.

Thionine Acetate, $\text{C}_{12}\text{H}_9\text{N}_3\text{S} \cdot \text{C}_2\text{H}_4\text{O}_2$ —**287.34** [78338-22-4]—Use a suitable grade.

Thiourea, $(\text{NH}_2)_2\text{CS}$ —**76.12** [62-56-6]—Use ACS reagent grade.

Thorium Nitrate, $\text{Th}(\text{NO}_3)_4 \cdot 4\text{H}_2\text{O}$ —**552.12** [13823-29-5]—Use ACS reagent grade.

Thrombin Human (Factor II_a)—**~33,600** [9002-04-4]—A preparation of a serine protease (enzyme) that converts human fibrinogen into fibrin. It is obtained from human plasma and may be prepared by precipitation with suitable salts and organic solvents under controlled

conditions of pH, ionic strength, and temperature. A yellowish-white powder, freely soluble in a 9 g per L solution of sodium chloride, which forms a cloudy, pale yellow solution. Store in a sealed, sterile container under nitrogen, protected from light, at a temperature below 0°. One unit corresponds to the amount of enzyme that hydrolyzes 1 μ mol of Tos-Gly-Pro-Arg-4-nitroaniline acetate per minute at a pH of 8.4 and a temperature of 37°.

Thromboplastin [9035-58-9]—Buff-colored powder, or opalescent or turbid suspension. It exhibits thrombokinase activity derived from the acetone-extracted brain and/or lung tissue of freshly killed rabbits. It may contain sodium chloride and calcium chloride in suitable proportions, and it may contain a suitable antimicrobial agent. It is used in suspension form for the determination of the prothrombin time and activity of blood. Its thrombokinase activity is such that it gives a clotting time of 11 to 16 seconds with normal human plasma and the proper concentration of calcium ions. Store in tight containers, preferably at a temperature below 5°.

Loss on drying (731)—[NOTE—This test is applicable only to the dry form.] Dry it in vacuum at 60° for 6 hours: it loses not more than 5.0% of its weight.

Thymidine, $C_{10}H_{14}N_2O_5$ —**242.2** [50-89-5]—White powder. Use a suitable grade.

Thymol, $C_6H_3[CH_3][OH][CH(CH_3)_2]_{1,3,4}$ —**150.22** [89-83-8]—Colorless, often large, crystals, or a white, crystalline powder. Is affected by light. Has greater density than water, but when liquefied by fusion is less dense than water. Its alcohol solutions are neutral to litmus. One g dissolves in about 1000 mL of water, in 1 mL of alcohol, in 1 mL of chloroform, in 1.5 mL of ether, and in about 2 mL of olive oil. Soluble in glacial acetic acid and in fixed or volatile oils. Store in tight, light-resistant containers.

Melting range (741): between 48° and 51°, but when melted it remains liquid at a considerably lower temperature.

Nonvolatile matter—Volatilize 2 g on a steam bath, and dry at 105° to constant weight: the residue weighs not more than 1 mg (0.05%).

Thyroglobulin, [9010-34-8]—A protein having a molecular weight of 670 kDa. Available as a slightly beige, freeze-dried powder made from bovine or porcine thyroid gland. Use a suitable grade.

Tin, Sn—At. Wt. **118.71** [7440-31-5]—Use ACS reagent grade.

Titanium Tetrachloride, $TiCl_4$ —**189.68** [7550-45-0]—Clear, colorless liquid. Fumes in air. [CAUTION—It reacts violently with water.]

Assay—Accurately weigh 0.75 g into 100 mL of 2 N sulfuric acid contained in a Smith weighing buret. Pour the solution through a zinc–mercury reduction column into 50 mL of 0.1 N ferric ammonium sulfate VS. Elute with 100 mL of 2 N sulfuric acid and 100 mL of water. Add 10 mL of phosphoric acid, and titrate with 0.1 N potassium permanganate VS. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N potassium permanganate is equivalent to 18.97 mg of $TiCl_4$. Not less than 99.5% is found.

Boiling range (Reagent test): between 135° and 140°.

Titanium Trichloride (*Titanous Chloride*), $TiCl_3$ —**154.23** [7705-07-9]—Black, hygroscopic powder, unstable in air. Soluble in water, the solution depositing titanic acid on exposure to air. Is available usually as 15% to 20%, dark violet-blue, aqueous solutions. Store the solution in tightly closed, glass-stoppered bottles, protected from light.

o-Tolidine (4,4'-Diamino-3,3'-dimethylbiphenyl), $(NH_2)(CH_3)_2C_6H_3 \cdot C_6H_3(CH_3)(NH_2)_{4,3,3',4'}$ —**212.29** [119-93-7]—White to reddish crystals or crystalline powder. Slightly soluble in water; soluble in alcohol, in ether, and in dilute acids. Preserve in well-closed containers, protected from light. [CAUTION—Avoid contact with o-tolidine and mixtures containing o-tolidine, and conduct all tests in a well-ventilated fume hood.]

Melting range (741): between 129° and 131°.

Tolualdehyde (*o-Tolualdehyde*), C_8H_8O —**120.15** [529-20-4]—Use a suitable grade.

p-Tolualdehyde, C_8H_8O —**120.15** [104-87-0]—Colorless to yellow, clear liquid.

Assay—When examined by gas-liquid chromatography, it shows a purity of not less than 98%. The following conditions have been found suitable for assaying the article: A 3-mm \times 1.8-m stainless steel column packed with a 5% phase G4 on support S1. Nitrogen, having a flow rate of about 12 mL per minute, is the carrier gas, the detector and column temperature are about 125°, and the injection port temperature is about 205°. A flame-ionization detector is employed and the specimen is a 5% solution in carbon disulfide.

Refractive index (831): between 1.544 and 1.546, at 20°.

Toluene (*Toluol*), $C_6H_5CH_3$ —**92.14** [108-88-3]—Use ACS reagent grade.

p-Toluenesulfonic Acid, $CH_3C_6H_4SO_3H \cdot H_2O$ —**190.22** [6192-52-5]—Use ACS reagent grade.

p-Toluenesulfonyl-L-arginine Methyl Ester Hydrochloride (*N α -p-Tosyl-L-arginine methyl ester hydrochloride; TAME*), $C_{14}H_{22}N_4O_4S \cdot HCl$ —**378.88** [1784-03-8]—Determine its suitability as directed in the test for *Trypsin* under *Chymotrypsin* (USP monograph).

p-Toluic Acid, $CH_3C_6H_4COOH$ —**136.15** [99-94-5]—White, crystalline powder. Sparingly soluble in hot water; very soluble in alcohol, in methanol, and in ether.

Assay—Transfer about 650 mg, accurately weighed, to a suitable container, dissolve in 125 mL of alcohol, add 25 mL of water, and mix. Titrate with 0.5 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.5 N sodium hydroxide is equivalent to 68.07 mg of $C_8H_8O_2$: not less than 98% is found.

Melting range (741): over a range of 2° that includes 181°.

o-Toluidine (*2-Aminotoluene; 2-Methylaniline*), $C_6H_4(CH_3)(NH_2)_{1,2}$ —**107.15** [95-53-4]—Light yellow liquid becoming reddish brown on exposure to air and light. Soluble in alcohol, in ether, and in dilute acids; slightly soluble in water. Preserve in well-closed containers, protected from light.

Specific gravity (841): 1.008 at 20°.

Boiling range (Reagent test): between 200° and 202°.

p-Toluidine, C_7H_9N —**107.15** [106-49-0]—White to beige crystals or flakes.

Freely soluble in alcohol, in acetone, in methanol, and in dilute acids; slightly soluble in water.

Assay—Dissolve 400 mg, accurately weighed, in 100 mL of glacial acetic acid, and titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 10.72 mg of $CH_3C_6H_4NH_2$. Not less than 98%, calculated on the dried basis, is found.

Loss on drying—Weigh accurately about 1 g, and dry at 30° to constant weight: it loses not more than 2% of its weight.

Toluidine Blue, $(C_{15}H_{16}ClN_3S)_2 \cdot ZnCl_2$ —**747.95** [6586-04-5]—Use a suitable grade.

Toluidine Blue O, $C_{15}H_{16}N_3S$ —**305.8** [92-31-9]—Use a suitable grade.

***n*-Triacontane**, $C_{30}H_{62}$ —**422.81** [638-68-6]—Use a suitable grade.

2,4,6-Triamino-5-nitrosopyrimidine, $C_4H_6N_6O$ —**154.13**—Pink powder.

Assay—Dissolve about 34 mg, accurately weighed, in 50 mL of glacial acetic acid. Titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary corrections. Each mL of 0.1 N perchloric acid is equivalent to 15.41 mg of $C_4H_6N_6O$. Not less than 97% is found.

Tributyl Phosphate (*Tri-*n*-butyl Phosphate*), $(C_4H_9)_3PO_4$ —**266.31** [126-73-8]—Clear, almost colorless liquid. Slightly soluble in water. Miscible with common organic solvents. Specific gravity: about 0.976.

Refractive index (831): between 1.4205 and 1.4225.

Tributylethylammonium Hydroxide, $C_{14}H_{33}NO$ —**231.42**—Use a suitable grade.

Tributylin (*Glyceryl Tributrylate*), $C_{15}H_{26}O_6$ —**302.36** [60-01-5]—Colorless, oily liquid. Insoluble in water; very soluble in alcohol and in ether.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, nitrogen being used as the carrier gas. The following conditions have been found suitable: a 3-mm \times 1.8-m stainless steel column containing phase G4 on support S1A; the injection port temperature is maintained at 270°; and the detector temperature is maintained at 300°. The area of the tributyrin peak is not less than 98% of the total peak area.

Refractive index (831): between 1.4345 and 1.4365 at 20°.

Acid content—Transfer 1.0 g, accurately weighed, to a beaker, add 75 mL of methanol, and dissolve by stirring. When dissolution is complete, add 25 mL of water, and titrate with 0.05 N potassium hydroxide VS, using phenolphthalein TS as the indicator. Perform a blank determination, and make any necessary correction. Each mL of 0.05 N potassium hydroxide is equivalent to 88.1 mg of butyric acid: not more than 0.5% is found.

Trichloroacetic Acid, CCl_3COOH —**163.39** [76-03-9]—Use ACS reagent grade.

Trichloroethane—See *Methyl Chloroform*.

Trichlorofluoromethane, CCl_3F —**137.37** [75-69-4]—Colorless liquid.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a thermal conductivity detector, helium being used as the carrier gas. The following conditions have been found suitable: a 2.0-mm \times 1.8-m glass column packed with 10% G1 phase on support S1A; the injection port temperature is maintained at 50°; the detector temperature is maintained at 300°; and the column temperature is maintained at 0° and programmed to rise 3° per minute to 50°. The area of the CCl_3F peak is not less than 99% of the total peak area.

Refractive index (831): between 1.380 and 1.384 at 20°.

Trichlorotrifluoroethane—Use a suitable grade.

[NOTE—A suitable preparation, listed as "Freon-TF aerosol," is available from E. I. du Pont de Nemours and Co., Wilmington, DE 19898.]

***n*-Tricosane**, $C_{23}H_{48}$ —**324.63** [638-67-5]—Colorless or white, more or less translucent mass, showing a crystalline structure. Has a slightly greasy feel. Insoluble in water and in alcohol; soluble in chloroform, in ether, in volatile oils, and in most warm fixed oils; slightly soluble in dehydrated alcohol. Boils at about 380°.

Melting range (741): between 47° and 49°.

Suitability—Determine its suitability for use in the test for *Related compounds* under *Propoxyphene Hydrochloride* (USP monograph) as follows. Dissolve a suitable quantity in chloroform to yield a solution containing 20 μ g per mL. Following the directions given in the test for *Related compounds* under *Propoxyphene Hydrochloride*, inject a suitable volume of the solution into the chromatograph, and record the chromatogram. Concomitantly record the chromatogram from the *Standard preparation* prepared as directed in the test for *Related compounds*: only one main peak is obtained from the *n*-tricosane solution, and no minor peaks are observed at, or near, the peak positions obtained for propoxyphene, acetoxy, or carbinol in the chromatogram from the *Standard preparation*.

Triethanolamine—Use *Trolamine* (NF monograph).

Triethylamine, $(C_2H_5)_3N$ —**101.19** [121-44-8]—Colorless liquid. Slightly soluble in water. Miscible with alcohol, with ether, and with cold water. Store in well-closed containers. Use a suitable grade with a content of not less than 99.5%.

Triethylamine Hydrochloride, $C_6H_{15}N \cdot HCl$ —**137.65** [554-68-7]—White to off-white powder.

Assay—Transfer about 35 mg, accurately weighed, to a suitable beaker, add 50 mL of glacial acetic acid, and dissolve by stirring. Add 5 mL of mercuric acetate TS, with stirring. When solution is complete, titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically. Perform a blank titration, and make any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 13.77 mg of $C_6H_{15}N \cdot HCl$. Not less than 97.5% of $C_6H_{15}N \cdot HCl$ is found.

Melting point (741): between 256° and 259°, with decomposition.

Triethylamine Phosphate (*Triethylammonium Phosphate*), $C_6H_{15}N \cdot H_3O_4P$ —**199.19**—Use a suitable grade.

[NOTE—A suitable grade is available from www.tciamerica.com, catalog number T1300.]

Triethylene Glycol, $C_6H_{14}O_4$ —**150.17** [112-27-6]—Colorless to pale yellow liquid. Is hygroscopic. Miscible with water, with alcohol, and with toluene.

Assay—Inject an appropriate test specimen into a suitable gas chromatograph equipped with a flame-ionization detector (see *Chromatography* (621)), helium being used as the carrier gas. The following conditions have been found suitable: a 3-mm \times 1.85-m stainless steel column packed with support S2; the injection port, column, and detector temperatures are maintained at 250°, 230°, and 310°, respectively. The area of the $C_6H_{14}O_4$ peak is not less than 97% of the total peak area.

Refractive index (831): between 1.4550 and 1.4570, at 20°.

Triethylenediamine (*1,4-Diazobicyclo[2.2.2]octane*), $C_6H_{12}N_2$ —**112.17** [280-57-9]—Use a suitable grade with a content of not less than 98%.

Trifluoroacetic Acid, $C_2HF_3O_2$ —**114.02** [76-05-1]—Colorless liquid. Miscible with ether, with acetone, with ethanol, with benzene, with carbon tetrachloride, and with hexane.

Assay—Dissolve about 300 mg, accurately weighed, in 25 mL of water and 25 mL of alcohol. Titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary corrections. Each mL of 0.1 N sodium hydroxide is equivalent to 11.40 mg of $C_2HF_3O_2$. Not less than 99% is found.

Trifluoroacetic Anhydride, $(F_3CCO)_2O$ —**210.03** [407-25-0]—Colorless liquid. Boils between 40° and 42°. Extremely volatile. Avoid exposure to air or water.

Assay—Transfer about 0.8 g, accurately weighed, to a glass-stoppered flask containing 50 mL of methanol. Add 500 mg of phenolphthalein, and titrate with 0.1 N sodium

methoxide VS to a pink endpoint. Calculate A by the formula:

$$V/W$$

in which V is the volume, in mL, of 0.1 N sodium methoxide and W is the weight, in mg, of test specimen. To a second glass-stoppered flask containing 50 mL of a mixture of dimethylformamide and water (1:1) transfer 0.4 g, accurately weighed, of the specimen under test, add 500 mg of phenolphthalein, and titrate with 0.1 N sodium hydroxide VS to a pink endpoint. Calculate B by the formula:

$$V^1/W^1$$

in which V¹ is the volume, in mL, of 0.1 N sodium hydroxide and W¹ is the weight, in mg, of test specimen. Calculate the percentage of (F₃CCO)₂O by the formula:

$$2100.3(B - A)$$

Not less than 97% is found. If 2A is greater than B, calculate the percentage of F₃CCOOH by the formula:

$$1140.3(2A - B)$$

α,α,α-Trifluoro-p-cresol (4-hydroxybenzotrifluoride, 4-trifluoromethylphenol), C₇H₅F₃O—**162.11** [402-45-9]

Melting range (741): between 48° and 52°.

2,2,2-Trifluoroethanol, CF₃CH₂OH—**100.04** [75-89-8]—Colorless liquid.

Assay—Inject an appropriate specimen into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm × 30-m capillary column coated with a 1-μm layer of phase G2; the injection port temperature is maintained at 100°; the detector temperature is maintained at 150°; and the column temperature is maintained at 0° and programmed to rise 10° per minute to 150°. The area of the CF₃CH₂OH peak is not less than 99% of the total peak area.

Boiling range: between 77° and 80°.

2,2,2-Trifluoroethyldifluoromethyl Ether (Difluoromethyl-2,2,2-trifluoroethyl ether), C₃H₃F₅O—**150.05**—Clear liquid. Use a suitable grade.

Boiling range: between 28° and 30°.

[NOTE—A suitable grade is available from PCR Incorporated, P.O. Box 1466, Gainesville, FL 32602. Tel: 904-376-8246. The catalogue number is 17151-2.]

(m-Trifluoromethylphenyl) Trimethylammonium Hydroxide in Methanol—Use a suitable grade.

[NOTE—A suitable grade is available as "Meth-Prep II" from Alltech, www.alltechweb.com.]

5-(Trifluoromethyl)uracil, C₅H₃F₃N₂O₂—**180.08** [54-20-6]—White to off-white powder.

Assay—When tested by thin-layer chromatography, with the use of plates coated with chromatographic silica gel mixture, a developing system consisting of chloroform, methanol, and acetic acid (17:2:1), and examined visually and under long-wavelength UV light, a single spot is exhibited.

Trifluorovinyl Chloride Polymer (Fluorolube; 1-Chloro-1,2,2-trifluoro-ethene Homopolymer), (C₂ClF₃)_x—Use a suitable grade.

[NOTE—A suitable grade is available as catalog number Z123552 from www.sigma-aldrich.com.]

Trimethylacetylhydrazide Ammonium Chloride (Betaine Hydrazide Chloride; Girard Reagent T), [(CH₃)₃N⁺CH₂CONHNH₂]Cl—**167.64** [123-46-6]—Colorless or white crystals. Freely soluble in water. One g dissolves in about 25 mL of alcohol. Insoluble in chloroform and in ether. Hygroscopic.

Melting range (741): between 185° and 192°, determined after recrystallization from hot alcohol, if necessary.

Residue on ignition (Reagent test)—Ignite 1 g with 0.5 mL of sulfuric acid: the residue weighs not more than 10 mg (1%).

Trimethylchlorosilane—See *Chlorotrimethylsilane*.

2,2,4-Trimethylpentane (Isooctane), C₈H₁₈—**114.23** [540-84-1]—Use ACS reagent grade.

2,4,6-Trimethylpyridine (5-Collidine), C₈H₁₁N—**121.18** [108-75-8]—Clear, colorless liquid. Soluble in cold water and less soluble in hot water; soluble in alcohol, in chloroform, and in methanol. Miscible with ether.

Assay—Inject an appropriate test specimen into a suitable gas chromatograph (see *Chromatography* (621)), helium being used as a carrier gas. The following conditions have been found suitable: a 3-mm × 1.85-m stainless steel column containing phase G16 on support S1A; the injection port, column, and detector temperatures are maintained at 180°, 165°, and 270°, respectively; and a flame-ionization detector is used. The area of the C₈H₁₁N peak is not less than 98% of the total peak area.

Refractive index (831): between 1.4970 and 1.4990, at 20°.

N-(Trimethylsilyl)-imidazole, C₆H₁₂N₂Si—**140.26** [18156-74-6]—A clear, colorless, to light yellow liquid.

Refractive index (831): between 1.4744 and 1.4764 at 20°.

Trimethyltin Bromide, C₃H₉BrSn—**243.72** [1066-44-0]—Use a suitable grade.

2,4,6-Trinitrobenzenesulfonic Acid, C₆H₂(NO₂)₃SO₃H · 3H₂O—**347.21** [2508-19-2]—Pale yellow to tan crystals. Use a suitable grade. Also available as a 5% (w/v) or a 1 M aqueous solution.

Trinitrophenol—See *Picric Acid*.

Trioctylphosphine Oxide, C₂₄H₅₁PO—**386.63**

[78-50-2]—White, crystalline powder. Insoluble in water; soluble in organic solvents.

Melting range (741): between 54° and 56°.

1,3,5-Triphenylbenzene, (C₆H₅)₃C₆H₃—**306.41** [612-71-5]—White to off-white powder.

Melting range (741): between 172° and 175°.

Triphenylmethane, C₁₉H₁₆—**244.34** [519-73-3]—Light brown powder.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm × 30-m capillary column coated with a 1-μm layer of phase G2; the injection port temperature is maintained at 300°; the detector temperature is maintained at 300°; and the column temperature is maintained at 200° and programmed to rise 10° per minute to 300°. The area of the C₁₉H₁₆ peak is not less than 99% of the total peak area.

Melting range (741): between 92° and 94°.

Triphenylmethanol, C₁₉H₁₆O—**260.34** [76-84-6]—White to off-white powder.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm × 30-m capillary column coated with a 1-μm layer of phase G2; the injection port temperature is maintained at 280°; the detector temperature is maintained at 300°; and the column temperature is maintained at 180°. The area of the C₁₉H₁₆O peak is not less than 96.5% of the total peak area.

Triphenyltetrazolium Chloride, C₁₉H₁₅ClN₄—**334.80**

[298-96-4]—White to yellowish, crystalline powder. Soluble in about 10 parts of water and of alcohol; slightly soluble in acetone; insoluble in ether. Usually contains solvent of crystallization, and when dried at 105° it melts at about 240°, with decomposition.

Solubility—Separate 100-mg portions dissolve completely in 10 mL of water and in 10 mL of alcohol, respectively, to yield solutions that are clear, or practically so.

Loss on drying (731)—Dry it at 105° to constant weight: it loses not more than 5.0% of its weight.

Residue on ignition (Reagent test): negligible, from 100 mg.

Sensitiveness—Dissolve 10 mg in 10 mL of dehydrated alcohol (A). Then dissolve 10 mg of dextrose in 20 mL of dehydrated alcohol (B). To 0.2 mL of B add 1 mL of dehydrated alcohol and 0.5 mL of dilute tetramethylammonium hydroxide TS (1 volume diluted with 9 volumes of dehydrated alcohol), then add 0.2 mL of A: a pronounced red color develops within about 10 minutes.

Tris(2-aminoethyl)amine, $C_6H_{18}N_4$ —**146.23**

[4097-89-6]—Yellow liquid. Soluble in methanol.

Assay—Dissolve about 80 mg in 30 mL of methanol. Add 40 mL of water, and titrate with 1 N hydrochloric acid, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary correction. Each mL of 1 N hydrochloric acid is equivalent to 48.75 mg of $C_6H_{18}N_4$. Not less than 98.0% is found.

Refractive index (831): between 1.4956 and 1.4986 at 20°.

Tris(hydroxymethyl)aminomethane [77-86-1]—Use ACS reagent grade—See also *Tromethamine*.

Tris(hydroxymethyl)aminomethane Acetate, $C_4H_{11}O_3 \cdot NCH_3$ —**181.19** [6850-28-8]—White powder with lumps. Use a suitable grade.

Tris(hydroxymethyl)aminomethane Hydrochloride, $C_4H_{11}NO_3 \cdot HCl$ —**157.60** [1185-53-1]—Colorless crystals. Use a suitable grade.

N-Tris(hydroxymethyl)methylglycine, $C_6H_{13}NO_5$ —**179.2** [5704-04-1]—White, crystalline powder. Use a suitable grade.

Tritirachium Album Proteinase K—Use a suitable grade.

[NOTE—A suitable grade can be obtained from Qiagen, Inc., www.qiagen.com.]

Tromethamine [*Tris(hydroxymethyl)aminomethane*; *THAM*; 2-Amino-2-(hydroxymethyl)-1,3-propanediol], $C_4H_{11}NO_3$ —**121.14**—Use ACS reagent grade *Tris(hydroxymethyl)aminomethane*.

Tropaeolin OO (*Acid Orange 5*), $C_{18}H_{14}N_3NaO_3S$ —**375.38** [554-73-4]—Orange-yellow scales, or yellow powder. Soluble in water.

pH range: from 1.4 (red) to 2.6 (yellow).

Tropic Acid, $C_9H_{10}O_3$ —**166.18** [529-64-6]—Use a suitable grade.

Tropine, $C_8H_{15}NO$ —**141.2** [120-29-6]—Use a suitable grade.

Trypan Blue (*Direct Blue 14*), $C_{34}H_{24}N_6Na_4O_{14}S_4$ —**960.8** [72-57-1]—Use a suitable grade.

Tryptone—Use *Pancreatic Digest of Casein*.

L-Tryptophane, $C_{11}H_{12}N_2O_2$ —**204.23** [73-22-3]—White or not more than slightly yellow leaflets or powder. One g dissolves in about 100 mL of water; soluble in dilute acids and in solutions of the alkali hydroxides; slightly soluble in alcohol.

Assay—Accurately weigh about 300 mg, dissolve in a mixture of 3 mL of formic acid and 50 mL of glacial acetic acid, add 2 drops of crystal violet TS, and titrate with 0.1 N perchloric acid VS to a green endpoint. Each mL of 0.1 N perchloric acid is equivalent to 20.42 mg of $C_{11}H_{12}N_2O_2$. Between 98.0% and 102.0%, calculated on the dried basis, is found.

Specific rotation (781): between -30.0° and -33.0° , determined in a solution containing 1.0 g of test specimen, previously dried at 105° for 3 hours, in 100 mL.

Loss on drying (731)—Dry it at 105° for 3 hours: it loses not more than 0.3% of its weight.

Residue on ignition (Reagent test): not more than 0.1%.

Tyrosine—Dissolve 100 mg in 3 mL of diluted sulfuric acid, add 10 mL of mercuric sulfate TS, and heat on a steam bath for 10 minutes. Filter, wash with 5 mL of mercuric sul-

fate TS, and add to the combined filtrate 0.5 mL of sodium nitrite solution (1 in 20): no red color is produced within 15 minutes.

Tuberculin, Purified Protein Derivative (*Tuberculin PPD*)—Derived from the human strain of *Mycobacterium tuberculosis*, and available either as a solution or as a lyophilized powder. For lyophilized powder, reconstitute according to the manufacturer's instructions using the diluent provided by the manufacturer. Solutions may contain a stabilizer and a preservative. One Tuberculin Unit (TU) is equivalent to 0.02 μ g of Tuberculin PPD.

Tubocurarine Chloride (*7',12'-Dihydroxy-6,6'-dimethoxy-2,2',2'-trimethyltubocurarium Chloride*), $C_{37}H_{42}Cl_2N_2O_6$ —**681.65** [6989-98-6]—Use a suitable grade with an assay result between 98.0% and 102.0%.

[NOTE—A suitable grade is available from Acros Organics, catalog number 24349 at www.acros.com.]

Tungstic Acid, H_2WO_4 —**249.85** [7783-03-1]—Use a suitable grade with a content of not less than 99%.

L-Tyrosine Disodium, $C_9H_9NO_3Na_2$ —**225.2**

[69849-45-6]—Off-white to tan powder. Use a suitable grade.

Uracil, $C_4H_4N_2O_2$ —**112.09** [66-22-8]—White to cream-colored, crystalline powder. Melts above 300°. One g dissolves in about 500 mL of water; less soluble in alcohol; soluble in ammonia TS and in sodium hydroxide TS. Its solutions yield no precipitate with the usual alkaloidal precipitants.

Residue on ignition (Reagent test): negligible, from 100 mg.

Loss on drying (731)—Dry it at 105° for 2 hours: it loses not more than 2% of its weight.

Uranyl Acetate (*Uranium Acetate*), $UO_2(C_2H_3O_2)_2 \cdot 2H_2O$ —**424.15** [541-09-3]—Use ACS reagent grade.

Urea, NH_2CONH_2 —**60.06** [57-13-6]—Use ACS reagent grade.

Urethane (*Ethyl carbamate*), $C_3H_7NO_2$ —**89.09**

[51-79-6]—White powder with chunks. Freely soluble in water.

Melting range (741): between 48° and 50°.

Uridine, $C_9H_{12}N_2O_6$ —**244.20** [58-96-8]—White powder.

Assay—

MOBILE PHASE—Prepare a mixture of methanol and 0.2 M ammonium acetate (10:90), and adjust with phosphoric acid to a pH of 7.0.

TEST SOLUTION: 0.5 mg per mL in water.

PROCEDURE—Inject about 20 μ L of the *Test solution* into a liquid chromatograph (see *Chromatography* (621)), equipped with a 280-nm detector and a 4.6-mm \times 15-cm column that contains packing L1. The flow rate is about 2.0 mL per minute. The area of the $C_9H_{12}N_2O_6$ peak is not less than 99% of the total peak area.

Melting range (741): between 166° and 171°.

Valeric Acid, $C_5H_{10}O_2$ —**102.13** [109-52-4]—Clear, colorless liquid.

Assay—Accurately weigh about 500 mg, transfer to a suitable container, add 30 mL of water, and mix. Add 40 mL of water, and mix. Add phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS. Each mL of 0.1 N sodium hydroxide is equivalent to 10.21 mg of $C_5H_{10}O_2$: not less than 99.0% of $C_5H_{10}O_2$ is found.

Valerophenone, $C_{11}H_{14}O$ —**162.23** [1009-14-9]—Colorless liquid.

Assay—Inject an appropriate specimen into a suitable gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a capillary column coated with a 1- μ m layer of phase G2; the injection port temperature is maintained at 250°; the detector temperature is maintained at 300°; and the column temperature is maintained at 150° and programmed to rise 10°

per minute to 300°. The area of the $C_{11}H_{14}O$ peak is not less than 98% of the total peak area.

Refractive index (831): 1.5149 at 20°.

Boiling range: between 105° and 107°, at a pressure of 5 mm of mercury.

Vanadium Pentoxide, V_2O_5 —**181.88** [1314-62-1]—Fine, yellow to orange-yellow powder. Slightly soluble in water; soluble in concentrated acids and in alkalis; insoluble in alcohol.

Assay—Transfer about 400 mg, accurately weighed, to a 500-mL conical flask, and add 150 mL of water and 30 mL of dilute sulfuric acid (1 in 2). Boil the solution on a hot plate for 5 minutes, add 50 mL of water, and continue boiling until a yellow solution is obtained. Transfer the hot plate and the flask to a well-ventilated hood, and bubble sulfur dioxide gas through the solution for 10 minutes, or until the solution is a clear, brilliant blue color. Rinse the gas delivery tube into the flask with a few mL of water, then bubble carbon dioxide gas through the solution for 30 minutes while continuing to boil the solution gently. Cool the solution to about 80°, and titrate with 0.1 N potassium permanganate VS to a yellow-orange endpoint. Perform a complete blank determination, and make any necessary correction. Each mL of 0.1 N potassium permanganate is equivalent to 9.095 mg of V_2O_5 . Not less than 99.5% is found.

Vanadyl Sulfate, $VOSO_4 \cdot xH_2O$ (anhydrous)—**163.00** [27774-13-6]—Blue, hygroscopic crystals. Slowly and usually incompletely soluble in water.

Assay—Accurately weigh about 400 mg of the dried test specimen obtained in the test for *Water*, and transfer with 15 to 20 mL of water into a beaker. Add 3 mL of sulfuric acid, cover the beaker with a watch glass, and heat on a steam bath until all dissolves. Cool, dilute with 125 mL of water, and titrate with 0.1 N potassium permanganate VS to the production of a pinkish color that persists for 1 minute: each mL of 0.1 N potassium permanganate is equivalent to 16.30 mg of $VOSO_4$. Not less than 97% is found.

Water—Dry about 1 g, accurately weighed, at 220° to constant weight: it loses not more than 50.0% of its weight.

Pentavalent vanadium—Heat 1 g, accurately weighed, with 50 mL of water and 5 mL of hydrochloric acid in a flask until dissolved. Cool, add 2 g of potassium iodide, insert the stopper, and allow to stand for 30 minutes. Add 50 mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the indicator. Correct for the volume of thiosulfate consumed by a blank. Each mL of 0.1 N thiosulfate is equivalent to 5.095 mg of vanadium (V). Not more than 0.5% is found, calculated on the dried basis.

Substances not precipitated by ammonia—Dissolve 1.0 g by heating with 20 mL of water and 2 mL of hydrochloric acid. Dilute with water to about 75 mL, and neutralize to litmus paper with ammonia TS. Transfer the solution to a cylinder, slowly add 5 mL of ammonia TS and sufficient water to make 100 mL, and allow to stand overnight. Decant 50 mL of the supernatant through a filter, add 5 drops of sulfuric acid, evaporate to dryness, and ignite: the residue weighs not more than 10 mg (2.0%).

Vinyl Acetate, $CH_3COOCH=CH_2$ —**86.09** [108-05-4]—Liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of G2; the injection port temperature is maintained at 100°; the detector temperature is maintained at 300°; and the column temperature is maintained at 100° and programmed to rise 10° per minute to 150°. The area of the

$CH_3COOCH=CH_2$ peak is not less than 99% of the total peak area.

2-Vinylpyridine, C_7H_7N —**105.14** [100-69-6].

Boiling range: between 79° and 82°, at 29 mm of mercury.

Density: 0.975 at 25°.

Refractive index (831): about 1.5490 at 20°.

Vinylpyrrolidinone (1-Vinyl-2-pyrrolidinone; 1-Vinyl-2-pyrrolidone; N-Vinylpyrrolidinone; N-Vinylpyrrolidone), C_6H_9NO —**111.14** [88-12-4]—Colorless liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of G2; the injection port temperature is maintained at 250°; the detector temperature is maintained at 300°; and the column temperature is maintained at 100° and programmed to rise 10° per minute to 250°. The area of the C_6H_9NO peak is not less than 99.0% of the total peak area.

Water, Method 1 (921): not more than 0.1%, determined on 2.5 g, using a mixture of 50 mL of methanol and 10 mL of butyrolactone as the solvent.

[NOTE—A suitable grade is available from Merck KGaA/EMD chemicals, catalogue number 8.08518.0250, www.emdchemicals.com.]

Washed Sand—See *Sand, Washed*.

Water, Ammonia-Free, H_2O —**18.02**—Use *High-Purity Water* as defined for *Chemical Resistance* under *Containers—Glass* (660).

Water, Carbon Dioxide-Free—See *Water*, in the introductory section.

Water, Deaerated—See *Water*, in the introductory section.

Water Vapor Detector Tube—A fuse-sealed glass tube so designed that gas may be passed through it and containing suitable absorbing filters and support media for the indicator, which consists of a selenium sol in suspension in sulfuric acid.

Measuring range: 5 to 250 mg per cubic meter.

[NOTE—Available from Draeger Safety, Inc., www.draeger.com, or from Gastec Corp., www.gastec.co.jp, distributed in the USA by www.nextteq.com.]

Wright's Stain [68988-92-1]—A mixture of methylene blue, methylene azure, and the eosinates of both, available as a solid and as a solution in methanol. Use a suitable grade. [NOTE—If a solid is used, dissolve 6.0 g of Wright's stain powder (CAS# 68988-92-1, dark green powder) and 0.6 g of Giemsa stain powder (CAS# 51811-82-6, dark green to black powder or crystals) in 1000 mL of methanol. Stir overnight, and filter before use.]

Xanthine, $C_5H_4N_4O_2$ —**152.11** [69-89-6]—White, crystalline powder. Decomposes on heating. Slightly soluble in water and in alcohol; soluble in sodium hydroxide TS; sparingly soluble in diluted hydrochloric acid. When subjected to the murexide reaction, a purple color is produced with the ammonia, but on the subsequent addition of fixed alkali hydroxides, the color is not discharged but is changed to violet.

Residue on ignition (Reagent test): negligible, from 100 mg.

Loss on drying (731)—Dry it at 105° for 2 hours: it loses not more than 1% of its weight.

Xanthidrol, $C_{13}H_{10}O_2$ —**198.22** [90-46-0]—Pale yellow, crystalline powder. Insoluble in water; soluble in alcohol, in chloroform, and in ether. Soluble in glacial acetic acid, forming a practically colorless solution; but when the powder is treated with diluted hydrochloric acid, a lemon-yellow color is produced.

Melting range (741): between 121° and 123°.

Residue on ignition—Ignite 500 mg with 0.5 mL of sulfuric acid: the residue weighs not more than 10 mg (2.0%).

Xylene, C_8H_{10} —**106.17** [1330-20-7]—Use ACS reagent grade.

m-Xylene, $C_6H_4(CH_3)_2$ —**106.17** [108-38-3]—Clear, colorless, flammable liquid. Insoluble in water; miscible with alcohol and with ether. Use a suitable grade.

o-Xylene, C_8H_{10} —**106.17** [95-47-6]—Clear, colorless, mobile, flammable liquid. Insoluble in water; miscible with alcohol and with ether.

Assay—When examined by gas-liquid chromatography, it shows a purity of not less than 95%. The following conditions have been found suitable for assaying the substance: A 3-mm \times 1.8-m stainless steel column packed with 1.75% hydrated aluminum silicate plus 5.0% diisodecyl phthalate on support S1. Helium, having a flow rate of about 27.5 mL per minute is the carrier gas, the detector temperature is about 280°, the injection port temperature is about 180°, and the column temperature is 80°. A flame-ionization detector is employed.

Refractive index (831): between 1.5040 and 1.5060, at 20°.

p-Xylene, C_8H_{10} —**106.17** [106-42-3]—Colorless liquid.

Assay—Inject an appropriate volume into a gas chromatograph (see *Chromatography* (621)) equipped with a flame-ionization detector, helium being used as the carrier gas. The following conditions have been found suitable: a 0.25-mm \times 30-m capillary column coated with a 1- μ m layer of phase G14; the column temperature is maintained at 50° and programmed to rise 10° per minute to 100°; the injection port is maintained at 130°; and the detector is maintained at 300°. The area of the C_8H_{10} peak is not less than 99% of the total peak area.

Refractive index (831): between 1.493 and 1.497 at 20°.

Xylene Cyanole FF, $C_{25}H_{27}N_2NaO_6S_2$ —**538.61** [2650-17-1]—Gray-blue to dark blue powder. Soluble in water.

Assay—Transfer about 50 mg, accurately weighed, to a 100-mL volumetric flask, dissolve in water, dilute with water to volume, and mix. Transfer 2.0 mL of the solution to a 50-mL volumetric flask, dilute with pH 7.0 phosphate buffer to volume (See *Solutions*, in this section), and mix. Using a suitable spectrophotometer, 1-cm cells, and water as the blank, record the absorbance of the solution at the wavelength of maximum absorbance at about 614 nm. From the observed absorbance, calculate the absorptivity (see *Spectrophotometry and Light-scattering* (851)): the absorptivity is not less than 55.9, corresponding to about 83% of $C_{25}H_{27}N_2NaO_6S_2$.

Loss on drying (731)—Dry it at 110° to constant weight: it loses not more than 6.0% of its weight.

Xylose, $C_5H_{10}O_5$ —**150.13** [58-86-6]—Use a suitable grade.

Yeast Extract—A water-soluble, peptone-like derivative of yeast cells (*Saccharomyces*) prepared under optimum condi-

tions, clarified, and dried to a reddish-yellow to brown powder, having a characteristic but not putrescent odor. Soluble in water, forming a yellow to brown solution, having a slightly acid reaction. Contains no added carbohydrate. One g represents not less than 7.5 g of yeast.

Loss on drying (731)—Dry it at 105° to constant weight: it loses not more than 6% of its weight.

Residue on ignition—Ignite 500 mg with 1 mL of sulfuric acid: the residue weighs not more than 75 mg (15%).

Coagulable protein—Heat a filtered solution (1 in 20) to boiling: no precipitate is formed.

Chloride (Reagent test)—It shows not more than 5% of Cl, calculated as sodium chloride.

Nitrogen content (Reagent test)—Determine by the Kjeldahl method, using a test specimen previously dried at 105° to constant weight: between 7.2% and 13.0% of N is found.

Microbial content—NMT 10^4 cfu/g

Yellow Mercuric Oxide—See *Mercuric Oxide, Yellow*.

Zinc, Zn—**At. Wt. 65.39** [7440-66-6]—Use ACS reagent grade.

Zinc, Activated—Place zinc pellets in a conical flask, and add a sufficient quantity of a 50-ppm solution of chloroplatinic acid to cover all pellets. Allow the metal to remain in contact with the solution for 10 min, drain, wash, and dry immediately.

Assay—To 5 g of activated zinc add 15 mL of hydrochloric acid, 25 mL of water, 0.1 mL of stannous chloride solution (235 mg/mL in hydrochloric acid), and 5 mL of potassium iodide solution (166 mg/mL in water). No stain is produced when in contact with mercuric bromide paper. Repeat the test for arsenic, using the same reagents and adding a solution containing 1 μ g of arsenic. An appreciable stain appears when in contact with mercuric bromide paper.

Zinc Acetate, $Zn(CH_3COO)_2 \cdot 2H_2O$ —**219.51** [557-34-6]—Use ACS reagent grade.

Zinc Amalgam—Add 54 g of mossy or granular zinc to 100 mL of mercury in a beaker. Heat, with stirring, on a hot plate under a hood [Caution—mercury vapor is extremely toxic] until solution of the zinc is complete or practically so. Allow to cool to room temperature, and if necessary add sufficient mercury to prevent solidification of the amalgam. Transfer the amalgam to a glass-stoppered bottle, and shake a few times with dilute hydrochloric acid (1 in 2), to remove any zinc oxide formed.

Zinc Chloride, Anhydrous, Powdered—Use *Zinc Chloride* (USP monograph) that has been dried and powdered.

Zinc Sulfate Heptahydrate, $ZnSO_4 \cdot 7H_2O$ —**287.54** [7446-20-0]—Odorless crystals, granules, or powder.

Melting point (741): 100°.

Density: 1.97.

Zirconyl Chloride, Octahydrate, Basic—Use *Zirconium Oxychloride*.

Zirconyl Nitrate, $ZrO(NO_3)_2$ —**231.23** [13826-66-9]—Use a suitable grade.

Indicators and Indicator Test Papers

INDICATORS

Indicators are required in the Pharmacopeial tests and assays either to indicate the completion of a chemical reaction in volumetric analysis or to indicate the hydrogen-ion concentration (pH) of solutions. The necessary solutions of indicators are listed among the Test Solutions, abbreviated "TS."

Solutions of indicators of the basic type and of the phthalins are prepared by dissolving in alcohol. With indicators

containing an acidic group, the acid must first be neutralized with sodium hydroxide as follows:

Triturate 100 mg of the indicator in a smooth-surfaced mortar with the volume of 0.05 N sodium hydroxide specified in the directions for preparing its Test Solution, or with the equivalent of 0.02 N sodium hydroxide. When the indicator has dissolved, dilute the solution with carbon dioxide-free water to 200 mL (0.05%). Store the solutions in suitably resistant containers, protected from light.

Listed in ascending order of the lower limit of their range, useful pH indicators are: thymol blue, pH 1.2–2.8; methyl yellow, pH 2.9–4.0; bromophenol blue, pH 3.0–4.6; bromocresol green, pH 4.0–5.4; methyl red, pH 4.2–6.2; bromocresol purple, pH 5.2–6.8; bromothymol blue, pH 6.0–7.6; phenol red, pH 6.8–8.2; thymol blue, pH 8.0–9.2; and thymolphthalein, pH 8.6–10.0.

Alphazurine 2G—Use a suitable grade.

Azo Violet [4-(*p*-Nitrophenylazo)resorcinol], $C_{12}H_9N_3O_4$ —**259.22**—Red powder. It melts at about 193°, with decomposition.

Bismuth Sulfite—Use a suitable grade.

Brilliant Green—See *Brilliant Green* in the section *Reagents*.

Brilliant Yellow (C.I. 24890), $C_{26}H_{18}N_4Na_2O_8S$ —**592.49**—Orange to rust-colored powder. Soluble in water.

Loss on drying (731)—Dry it in vacuum at 60° for 1 hour: it loses not more than 5% of its weight.

Bromocresol Blue—Use *Bromocresol Green*.

Bromocresol Green (*Bromocresol Blue*; *Tetrabromo-m-cresol-sulfonphthalein*), $C_{21}H_{14}Br_4O_5S$ —**698.01**—White or pale buff-colored powder. Slightly soluble in water; soluble in alcohol and in solutions of alkali hydroxides. Transition interval: from pH 4.0 to 5.4. Color change: from yellow to blue.

Bromocresol Green Sodium Salt—Use a suitable grade.

Bromocresol Purple (*Dibromo-o-cresolsulfonphthalein*), $C_{21}H_{16}Br_2O_5S$ —**540.22**—White to pink, crystalline powder. Insoluble in water; soluble in alcohol and in solutions of alkali hydroxides. Transition interval: from pH 5.2 to 6.8. Color change: from yellow to purple.

Bromocresol Purple Sodium Salt, $C_{21}H_{15}Br_2O_5SNa$ —**562.20**—Black powder. Soluble in water. Transition interval: from pH 5.0 to 6.8. Color change: from greenish yellow to purple-violet.

Melting range (741): between 261° and 264°.

Bromophenol Blue (3',3'',5',5''-*Tetrabromophenol-sulfonphthalein*), $C_{19}H_{10}Br_4O_5S$ —**669.96**—Pinkish crystals. Insoluble in water; soluble in alcohol and in solutions of alkali hydroxides. Transition interval: from pH 3.0 to 4.6. Color change: from yellow to blue.

Bromophenol Blue Sodium—The sodium salt of 3',3'',5',5'' (*Tetrabromophenolsulfonphthalein*), $C_{19}H_9Br_4O_5SNa$ —**646.36**—Pinkish crystals. Soluble in water and in alcohol. Transition interval: from pH 3.0 to 4.6. Color change: from yellow to blue.

Bromothymol Blue (3',3''-*Dibromothymolsulfonphthalein*), $C_{27}H_{28}Br_2O_5S$ —**624.38**—Cream-colored powder. Insoluble in water; soluble in alcohol and in solutions of alkali hydroxides. Transition interval: from pH 6.0 to 7.6. Color change: from yellow to blue.

Congo Red—See *Congo Red* in the section *Reagents*.

Cresol Red (*o-Cresolsulfonphthalein*), $C_{21}H_{18}O_5S$ —**382.43**—Red-brown powder. Slightly soluble in water; soluble in alcohol and in dilute solutions of alkali hydroxides. Transition interval: from pH 7.2 to 8.8. Color change: from yellow to red.

Crystal Violet (*Hexamethyl-p-rosaniline Chloride*), $C_{25}H_{30}ClN_3$ —**407.98**—Dark-green crystals. Slightly soluble in water; sparingly soluble in alcohol and in glacial acetic acid. Its solutions are deep violet in color.

Sensitiveness—Dissolve 100 mg in 100 mL of glacial acetic acid, and mix. Pipet 1 mL of the solution into a 100-mL volumetric flask, and dilute with glacial acetic acid to volume: the solution is violet-blue in color and does not show a reddish tint. Pipet 20 mL of the diluted solution into a beaker, and titrate with 0.1 N perchloric acid VS, adding the perchloric acid slowly from a microburet: not more than 0.10 mL of 0.1 N perchloric acid is required to produce an emerald-green color.

4,5-Dihydroxy-3-(*p*-sulfophenylazo)-2,7-naphthalenedisulfonic Acid, Trisodium Salt—See 2-(4-Sulfophenylazo)-1,8-dihydroxy-3,6-naphthalenedisulfonic Acid, Trisodium Salt.

Eosin Y (*Indicator grade Eosin Y, Sodium tetrabromofluorescein*), $C_{20}H_6Br_4Na_2O_5$ —**691.86** [17372-87-1]—Red to brownish-red pieces or powder. One g dissolves in about 2 mL of water and in 50 mL of alcohol. Dye content about 80%.

Eriochrome Black T [*Sodium 1-(1-Hydroxy-2-naphthylazo)5-nitro-2-naphthol-4-sulfonate*], $C_{20}H_{12}N_3NaO_7S$ —**461.38**—Brownish-black powder having a faint, metallic sheen. Soluble in alcohol, in methanol, and in hot water.

Sensitiveness—To 10 mL of a 1 in 200,000 solution in a mixture of equal parts of methanol and water add sodium hydroxide solution (1 in 100) until the pH is 10: the solution is pure blue in color and free from cloudiness. Add 0.01 mg of magnesium ion (Mg): the color of the solution changes to red-violet, and with the continued addition of magnesium ion it becomes wine-red.

Eriochrome Black T Trituration—Grind 200 mg of eriochrome black T to a fine powder with 20 g of potassium chloride.

Litmus—Blue powder, cubes, or pieces. Partly soluble in water and in alcohol. Transition interval: from approximately pH 4.5 to 8. Color change: from red to blue. Litmus is unsuitable for determining the pH of alkaloids, carbonates, and bicarbonates.

Malachite Green Oxalate, $[C_{23}H_{25}N_2^+]_2 \cdot [C_2HO_4^-]_2 \cdot C_2H_2O_4$ —**927.00**—The oxalate salt, crystallized with oxalic acid, of a triphenylmethane dye. Dark-green powder, having a metallic luster. Sparingly soluble in water; soluble in glacial acetic acid. Transition interval: from pH 0.0 to 2.0. Color change: from yellow to green.

Methyl Orange (*Helianthin* or *Tropaeolin D*), $C_{14}H_{14}N_3NaO_3S$ —**327.33**—The sodium salt of dimethylaminoazobenzene sulfonic acid or dimethylaminoazobenzene sodium sulfonate. An orange-yellow powder or crystalline scales. Slightly soluble in cold water; readily soluble in hot water; insoluble in alcohol. Transition interval: from pH 3.2 to 4.4. Color change: from pink to yellow.

Methyl Red (2-[4-(*Dimethylamino*)phenyl]azo]benzoic Acid Hydrochloride), $2-[4-(CH_3)_2NC_6H_4N:N]C_6H_4COOH \cdot HCl$ —**305.76**—Dark-red powder or violet crystals. Sparingly soluble in water; soluble in alcohol. Transition interval: from pH 4.2 to 6.2. Color change: from red to yellow.

Methyl Red Sodium—The sodium salt of 2-[4-(dimethylamino)phenyl]azo]benzoic acid. $2-[4-(CH_3)_2NC_6H_4N:N]C_6H_4COONa$ —**291.28**—Orange-brown powder. Freely soluble in cold water and in alcohol. Transition interval: from pH 4.2 to 6.2. Color change: from red to yellow.

Methyl Yellow (*p*-*Dimethylaminoazobenzene*), $C_{14}H_{15}N_3$ —**225.29**—Yellow crystals, melting between 114° and 117°. Insoluble in water; soluble in alcohol, in benzene, in chloroform, in ether, in dilute mineral acids, and in oils. Transition interval: from pH 2.9 to 4.0. Color change: from red to yellow.

p-Naphtholbenzein (4-[α -(4-Hydroxy-1-naphthyl)benzylidene]-1(4*H*)-naphthalenone), (4-HOC₁₀H₆) $C(C_{10}H_6-4:O)(C_6H_5)$ —**374.43**—Reddish brown powder. Insoluble in water; soluble in alcohol, in benzene, in ether, and in glacial acetic acid. Transition interval: from pH 8.8 to 10.0. Color change: from orange to green.

Neutral Red (3-Amino-7-dimethylamino-2-methylphenazine Monohydrochloride), $C_{15}H_{16}N_4 \cdot HCl$ —**288.78**—Reddish to olive-green, coarse powder. Sparingly soluble in water and in alcohol. Transition interval: from pH 6.8 to 8.0. Color change: from red to orange.

Nile Blue Hydrochloride (*Nile Blue A, as the hydrochloride*; 5-Amino-9-(diethylamino)benzo[*a*]phenoxazin-7-ium chloride), $C_{20}H_{20}ClN_3O$ —**353.85**—Slightly soluble in alcohol and in glacial acetic acid. Transition interval: from pH 9.0 to 13.0. Color change: from blue to pink.

Oracet Blue B (Solvent Blue 19)—A mixture of 1-methylamino-4-anilinoanthraquinone ($C_{21}H_{16}N_2O_2$) and 1-amino-4-anilinoanthraquinone ($C_{20}H_{14}N_2O_2$). Where used for titration in non-aqueous media, it changes from blue (basic) through purple (neutral) to pink (acidic).

Phenol Red [4,4'-(3*H*-2,1-Benzoxathiol-3-ylidene)diphenol, *S,S*-Dioxide], $C_{19}H_{14}O_5S$ —**354.38**—Crystalline powder, varying in color from bright to dark red. Very slightly soluble in water; freely soluble in solutions of alkali carbonates and hydroxides; slightly soluble in alcohol. Transition interval: from pH 6.8 to 8.2. Color change: from yellow to red.

Phenolphthalein [3,3-Bis(*p*-hydroxyphenyl)phthalide], $C_{20}H_{14}O_4$ —**318.32**—White or faintly yellowish-white, crystalline powder. Insoluble in water; soluble in alcohol. Transition interval: from pH 8.0 to 10.0. Color change: from colorless to red.

Quinaldine Red (5-Dimethylamino-2-styrylethylquinolinium iodide), $C_{21}H_{23}IN_2$ —**430.33**—Dark blue-black powder. Sparingly soluble in water; freely soluble in alcohol. Melts at about 260°, with decomposition. Transition interval: from pH 1.4 to 3.2. Color change: from colorless to red.

2-(4-Sulfophenylazo)-1,8-dihydroxy-3,6-naphthalenedisulfonic Acid, Trisodium Salt (4,5-Dihydroxy-3-(*p*-sulfophenylazo)-2,7-naphthalenedisulfonic Acid, Trisodium Salt), $C_{16}H_9N_2O_{11}S_3Na_3$ —**570.42**—Red powder. Soluble in water.

Thymol Blue (Thymolsulfonphthalein), $C_{27}H_{30}O_5S$ —**466.59**—Dark-colored, crystalline powder. Slightly soluble in water; soluble in alcohol and in dilute alkali solutions. *Acid*—Transition interval: from pH 1.2 to 2.8. Color change: from red to yellow. *Alkaline*—Transition interval: from pH 8.0 to 9.2. Color change: from yellow to blue.

Thymolphthalein, $C_{28}H_{30}O_4$ —**430.54**—White to slightly yellow, crystalline powder. Insoluble in water; soluble in alcohol and in solutions of alkali hydroxides. Transition interval: from pH 9.3 to 10.5. Color change: from colorless to blue.

Xylenol Orange, (N,N'-[3*H*-2,1-Benzoxathiol-3-ylidenebis-[(6-hydroxy-5-methyl-3,1-phenylene)methylene]]bis[N-(carboxymethyl)glycine]*S,S*-dioxide), $C_{31}H_{28}N_2Na_4O_{13}S$ —**760.58**—Orange powder. Soluble in alcohol and in water. In acid solution, it is lemon-yellow in color, and its metal complexes are intensely red. It yields a distinct endpoint where a metal such as bismuth, cadmium, lanthanum, lead, mercury, scandium, thorium, or zinc is titrated with edetate disodium.

INDICATORS AND TEST PAPERS

Indicator and test papers are strips of paper of suitable dimension and grade (see *Filter Paper, Quantitative*, in the section *Reagents*) impregnated with an indicator or a reagent that is sufficiently stable to provide a convenient form of the impregnated substance. Some test papers may be obtained from commercial sources of laboratory supplies. Those required in Pharmacopeial tests and assays may be prepared as directed in the following paragraphs, by means of the solutions specified, or to meet the tests set forth herein under the individual titles.

Treat strong, white filter paper with hydrochloric acid, and wash with water until the last washing no longer shows an acid reaction to methyl red. Then treat with ammonia TS, and wash again with water until the last washing is not alkaline to phenolphthalein.

After thorough drying, saturate the paper with the proper strength of indicator solutions, and carefully dry in still air, unless otherwise specified, by suspending it from rods of glass or other inert material in a space free from acid, alkali, and other fumes.

Cut the paper into strips of convenient size, and store the papers in well-closed containers, protected from light and moisture.

Cupric Sulfate Test Paper—Use cupric sulfate TS.

Lead Acetate Test Paper—Usually about 6 × 80 mm in size. Use lead acetate TS, and dry the paper at 100°, avoiding contact with metal.

Litmus Paper, Blue—Usually about 6 × 50 mm in size. It meets the requirements of the following tests.

Phosphate (Reagent test)—Cut 5 strips into small pieces, mix with 500 mg of magnesium nitrate in a porcelain crucible, and ignite. To the residue add 5 mL of nitric acid, and evaporate to dryness: the residue shows not more than 0.02 mg of PO_4 .

Residue on ignition—Ignite carefully 10 strips of the paper to constant weight: the weight of the residue corresponds to not more than 0.4 mg per strip of about 3 square cm.

Rosin acids—Immerse a strip of the blue paper in a solution of 100 mg of silver nitrate in 50 mL of water: the color of the paper does not change in 30 seconds.

Sensitiveness—Drop a 10- to 12-mm strip into 100 mL of 0.0005 *N* acid contained in a beaker, and stir continuously: the color of the paper is changed within 45 seconds. The 0.0005 *N* acid is prepared by diluting 1 mL of 0.1 *N* hydrochloric acid with freshly boiled and cooled purified water to 200 mL.

Litmus Paper, Red—Usually about 6 × 50 mm in size. Red litmus paper meets the requirements of the tests for *Phosphate*, *Residue on ignition*, and *Rosin acids*, under *Litmus Paper, Blue*.

Sensitiveness—Drop a 10- to 12-mm strip into 100 mL of 0.0005 *N* sodium hydroxide contained in a beaker, and stir continuously: the color of the paper changes within 30 seconds. The 0.0005 *N* sodium hydroxide is prepared by diluting 1 mL of 0.1 *N* sodium hydroxide with freshly boiled and cooled purified water to 200 mL.

Mercuric Bromide Test Paper—Place a 50 mg/mL solution of mercuric bromide in dehydrated alcohol in a dish, and immerse in it pieces of white filter paper weighing 80 g/m² (speed of filtration = filtration time expressed in s for 100 mL of water at 20° with a filter surface of 10 cm² and a constant pressure of 6.7 kPa; 40–60 s), each measuring 1.5 cm by 20 cm and folded in the middle. Allow the excess of liquid to drain, and allow the paper to dry, protected from light, suspended over a nonmetallic thread. Discard 1 cm from each end of each strip, and cut the remainder into 1.5-cm squares or discs of 1.5-cm diameter. Store in a glass-stoppered container wrapped with black paper.

Methyl Green-Iodomercurate Paper—Immerse thin strips of suitable filter paper in a 40 g per L solution of methyl green, and allow to air-dry. Immerse the strips for 1 hour in a solution containing 140 g per L of potassium iodide and 200 g per L of mercuric iodide. Wash with water until the washings are practically colorless, and allow to air-dry. Store protected from light, and use within 48 hours.

Methyl Yellow Paper—Use a 1 in 2000 solution of methyl yellow in alcohol.

pH Indicator Paper, Short-Range—Use a suitable grade.

Phenolphthalein Paper—Use a 1 in 1000 solution of phenolphthalein in diluted alcohol.

Starch Iodate Paper—Use a mixture of equal volumes of starch TS and potassium iodate solution (1 in 20).

Starch Iodide Paper—Use a solution of 500 mg of potassium iodide in 100 mL of freshly prepared starch TS.

Thiazole Yellow Paper—Use a 1 in 2000 solution of thiazole yellow in water.

Turmeric Paper—Use a solution prepared as follows: Macerate 20 g of powdered turmeric, the dried root of *Curcuma longa* Linné (Fam. Zingiberaceae), with four 100-mL portions of cold water, decanting the clear liquid portion each time and discarding it. Dry the residue at a temperature not over 100°. Macerate with 100 mL of alcohol for several days, and filter.

Sensitiveness—Dip a strip of the paper, of about 1.5-cm length, in a solution of 1.0 mg of boric acid in 5 mL of water, previously mixed with 1 mL of hydrochloric acid. After 1 minute remove the paper from the liquid, and allow it to dry: the yellow color changes to brown. Then moisten the paper with ammonia TS: the color of the paper changes to greenish black.

Solutions

BUFFER SOLUTIONS

The successful completion of many Pharmacopeial tests and assays requires adjustment to or maintenance of a specified pH by the addition of buffer solutions. In pH measurements, standard buffer solutions are required for reference purposes. For convenience, the preparation of these solutions is in some instances described in the sections in which their use is specified; i.e., five separate phosphate buffers are described under *Antibiotics—Microbial Assays* (81), and several miscellaneous single-purpose solutions are described in the individual monographs.

A solution is said to be buffered if it resists changes in the activity of an ion on the addition of substances that are expected to change the activity of that ion. Buffers are substances or combinations of substances that impart this resistance to a solution. Buffered solutions are systems in which the ion is in equilibrium with substances capable of removing or releasing the ion.

Buffer capacity refers to the amount of material that may be added to a solution without causing a significant change in ion activity. It is defined as the ratio of acid or base added (in gram-equivalents per liter) to the change in pH (in pH units). The capacity of a buffered solution is adjusted to the conditions of use, usually by adjustment of the concentrations of buffer substances.

Buffers are used to establish and maintain an ion activity within narrow limits. The most common systems are used (a) to establish hydrogen-ion activity for the calibration of pH meters, (b) in the preparation of dosage forms that approach isotonicity, (c) in analytical procedures, and (d) to maintain stability of various dosage forms. Buffers used in physiological systems are carefully chosen so as not to interfere with pharmacological activity of the medicament or normal function of the organism. It is essential that buffers used in chemical analysis be compatible with the substance determined and the reagents used.

Standard Buffer Solutions—Standard solutions of definite pH are readily available in buffer solutions prepared from the appropriate reagents. In addition, buffer solutions, buffer

tablets, and buffer solids may be obtained from commercial sources in convenient prepackaged form. Such preparations are available for the entire working range in pharmaceutical analysis, but are not recommended for pH meter standardization (see *pH* (791)).

The required reagents are described in the section, *Reagents*. Previously dry the crystalline reagents, except the boric acid and sodium acetate trihydrate, at 110° to 120° for 1 hour.

[NOTE—Where water is specified for solution or dilution of test substances in pH determinations, use carbon dioxide-free water.]

Store the prepared solutions in chemically resistant, tight containers such as Type I glass bottles. Use the solutions within 3 months.

Standard Buffer Solutions for various ranges between pH 1.2 and 10.0 may be prepared by appropriate combinations of the solutions described herein, used in the proportions shown in the accompanying table. The volumes shown in the table are for 200 mL of buffer solution, except that the volumes shown for *Acetate Buffer* are used to prepare 1000 mL of buffer solution.

1. *Hydrochloric Acid, 0.2 M*, and *Sodium Hydroxide, 0.2 M*—Prepare and standardize as directed under *Volumetric Solutions*.
2. *Potassium Biphthalate, 0.2 M*—Dissolve 40.85 g of potassium biphthalate [$\text{KHC}_8\text{H}_4(\text{COO})_2$] in water, and dilute with water to 1000 mL.
3. *Potassium Phosphate, Monobasic 0.2 M*—Dissolve 27.22 g of monobasic potassium phosphate (KH_2PO_4) in water, and dilute with water to 1000 mL.
4. *Boric Acid and Potassium Chloride, 0.2 M*—Dissolve 12.37 g of boric acid (H_3BO_3) and 14.91 g of potassium chloride (KCl) in water, and dilute with water to 1000 mL.
5. *Potassium Chloride, 0.2 M*—Dissolve 14.91 g of potassium chloride (KCl) in water, and dilute with water to 1000 mL.
6. *Acetic Acid, 2 N*—Prepare and standardize as directed under *Volumetric Solutions*.

Composition of Standard Buffer Solutions											
<i>Hydrochloric Acid Buffer</i>											
Place 50 mL of the potassium chloride solution in a 200-mL volumetric flask, add the specified volume of the hydrochloric acid solution, then add water to volume.											
pH	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0	2.1	2.2
0.2 M HCl, mL	85.0	67.2	53.2	41.4	32.4	26.0	20.4	16.2	13.0	10.2	7.8

<i>Acid Phthalate Buffer</i>											
Place 50 mL of the potassium biphthalate solution in a 200-mL volumetric flask, add the specified volume of the hydrochloric acid solution, then add water to volume.											
pH	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0	
0.2 M HCl, mL	49.5	42.2	35.4	28.9	22.3	15.7	10.4	6.3	2.9	0.1	

<i>Neutralized Phthalate Buffer</i>											
Place 50 mL of the potassium biphthalate solution in a 200-mL volumetric flask, add the specified volume of the sodium hydroxide solution, then add water to volume.											
pH		4.2	4.4	4.6	4.8	5.0	5.2	5.4	5.6	5.8	
0.2 M NaOH, mL		3.0	6.6	11.1	16.5	22.6	28.8	34.1	38.8	42.3	

Phosphate Buffer												
Place 50 mL of the monobasic potassium phosphate solution in a 200-mL volumetric flask, add the specified volume of the sodium hydroxide solution, then add water to volume.												
pH	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.2	7.4	7.6	7.8	8.0
0.2 M NaOH, mL	3.6	5.6	8.1	11.6	16.4	22.4	29.1	34.7	39.1	42.4	44.5	46.1

Alkaline Borate Buffer												
Place 50 mL of the boric acid and potassium chloride solution in a 200-mL volumetric flask, add the specified volume of the sodium hydroxide solution, then add water to volume.												
pH	8.0	8.2	8.4	8.6	8.8	9.0	9.2	9.4	9.6	9.8	10.0	
0.2 M NaOH, mL	3.9	6.0	8.6	11.8	15.8	20.8	26.4	32.1	36.9	40.6	43.7	

Acetate Buffer											
Place the specified amount of sodium acetate $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ in a 1000-mL volumetric flask, add the specified volume of the acetic acid solution, then add water to volume, and mix.											
pH	4.1	4.3	4.5	4.7	4.9	5.1	5.2	5.3	5.4	5.5	
pH (measured)	4.10	4.29	4.51	4.70	4.90	5.11	5.18	5.30	5.40	5.48	
$\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$, g	1.5	1.99	2.99	3.59	4.34	5.08	5.23	5.61	5.76	5.98	
2 N CH_3COOH , mL	19.5	17.7	14.0	11.8	9.1	6.3	5.8	4.4	3.8	3.0	

COLORIMETRIC SOLUTIONS (CS)

(For the Preparation of Matching Fluids, see *Color and Achromicity* (631).)

These solutions are used in the preparation of the colorimetric standards for certain drugs, and for the carbonization tests with sulfuric acid that are specified in several monographs. Store the solutions in suitably resistant, tight containers.

Comparison of colors as directed in the Pharmacopeial tests preferably is made in matched color-comparison tubes or in a suitable colorimeter under conditions that ensure that the colorimetric reference solution and that of the specimen under test are treated alike in all respects. The comparison of colors is best made in layers of equal depth, and viewed transversely against a white background (see also *Visual Comparison under Spectrophotometry and Light-Scattering* (851)). It is particularly important that the solutions be compared at the same temperature, preferably 25°.

Cobaltous Chloride CS—Dissolve about 65 g of cobaltous chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) in enough of a mixture of 25 mL of hydrochloric acid and 975 mL of water to make 1000 mL. Pipet 5 mL of this solution into a 250-mL iodine flask, add 5 mL of hydrogen peroxide TS and 15 mL of sodium hydroxide solution (1 in 5), boil for 10 minutes, cool, and add 2 g of potassium iodide and 20 mL of dilute sulfuric acid (1 in 4). When the precipitate has dissolved, titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the indicator. Perform a blank determination with the same quantities of the same reagents, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 23.79 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. Adjust the final volume of the solution by the addition of enough of the mixture of hydrochloric acid and water so that each mL contains 59.5 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$.

Cupric Sulfate CS—Dissolve about 65 g of cupric sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in enough of a mixture of 25 mL of hydrochloric acid and 975 mL of water to make 1000 mL. Pipet 10 mL of this solution into a 250-mL iodine flask, add 40 mL of water, 4 mL of acetic acid, 3 g of potassium iodide, and 5 mL of hydrochloric acid, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the indicator. Perform a blank determination with the same quantities of the same reagents, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 24.97 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Adjust the final volume of the solution by the addition of enough of the mixture of hydrochloric acid and water so that each mL contains 62.4 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

Ferric Chloride CS—Dissolve about 55 g of ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in enough of a mixture of 25 mL of hydrochloric acid and 975 mL of water to make 1000 mL. Pipet 10 mL of this solution into a 250-mL iodine flask, add 15 mL of water, 3 g of potassium iodide, and 5 mL of hydrochloric acid, and allow the mixture to stand for 15 minutes. Dilute with 100 mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the indicator. Perform a blank determination with the same quantities of the same reagents, and make any necessary correction. Each mL of 0.1 N sodium thiosulfate is equivalent to 27.03 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. Adjust the final volume of the solution by the addition of enough of the mixture of hydrochloric acid and water so that each mL contains 45.0 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$.

INDICATOR SOLUTIONS

See *TEST SOLUTIONS*.

TEST SOLUTIONS (TS)

Certain of the following test solutions are intended for use as acid-base indicators in volumetric analyses. Such solutions should be so adjusted that when 0.15 mL of the indicator solution is added to 25 mL of carbon dioxide-free water, 0.25 mL of 0.02 N acid or alkali, respectively, will produce the characteristic color change. Similar solutions are intended for use in pH measurement. Where no special directions for their preparation are given, the same solution is suitable for both purposes.

Where it is directed that a volumetric solution be used as the test solution, standardization of the solution used as TS is not required.

In general, the directive to prepare a solution "fresh" indicates that the solution is of limited stability and must be prepared on the day of use.

For the preparation of Test Solutions, use reagents of the quality described under *Reagents*.

Acetaldehyde TS—Mix 4 mL of acetaldehyde, 3 mL of alcohol, and 1 mL of water. Prepare this solution fresh.

Acetate Buffer TS—Dissolve 320 g of ammonium acetate in 500 mL of water, add 5 mL of glacial acetic acid, dilute with water to 1000.0 mL, and mix. This solution has a pH between 5.9 and 6.0.

Acetic Acid, Glacial, TS—Determine the water content of a specimen of glacial acetic acid by the *Titrimetric Method* (see *Water Determination* (921)). If the acid contains more than 0.4% of water, add a few mL of acetic anhydride, mix, allow to stand overnight, and again determine the water

content. If the acid contains less than 0.02% of water, add sufficient water to make the final concentration between 0.02% and 0.4%, mix, allow to stand overnight, and again determine the water content. Repeat the adjustment with acetic anhydride or water, as necessary, until the resulting solution shows a water content of not more than 0.4%.

Acetic Acid, Strong, TS—Add 300.0 mL of glacial acetic acid, and dilute with water to 1000 mL. This solution contains about 30% (v/v) of CH_3COOH and has a concentration of about 5 N.

Acetic Acid–Ammonium Acetate Buffer TS—Dissolve 77.1 g of ammonium acetate in water, add 57 mL of glacial acetic acid, and dilute with water to 1000 mL.

Acetone, Buffered, TS—Dissolve 8.15 g of sodium acetate and 42 g of sodium chloride in about 100 mL of water, and add 68 mL of 0.1 N hydrochloric acid and 150 mL of acetone. Mix, and dilute with water to 500 mL.

Acid Ferric Chloride TS—Mix 60 mL of glacial acetic acid with 5 mL of sulfuric acid, add 1 mL of ferric chloride TS, mix, and cool.

Acid Ferrous Sulfate TS—See *Ferrous Sulfate, Acid, TS*.

Acid Stannous Chloride TS—See *Stannous Chloride, Acid, TS*.

Change to read:

Acid Stannous Chloride TS, Stronger—See **Stannous Chloride, Acid, Stronger, TS**. • (ERR 1-Jul-2012)

Albumen TS—Carefully separate the white from the yolk of a strictly fresh hen's egg. Shake the white with 100 mL of water until mixed and all but the chalaza has undergone solution; then filter. Prepare the solution fresh.

Alcohol–Phenol TS—Dissolve 780 mg of phenol in alcohol to make 100 mL.

Alcoholic TS—It contains 95 parts of specially denaturated alcohol 3A with 5 parts of isopropyl alcohol. The final concentrations are approximately 90% alcohol, 5% methanol, and 5% isopropanol.

[NOTE—A suitable grade is available as Reagent alcohol, catalog number R8382, available at www.sigma-aldrich.com.]

Alcoholic Ammonia TS—See *Ammonia TS, Alcoholic*.

Alcoholic Mercuric Bromide TS—See *Mercuric Bromide TS, Alcoholic*.

Alcoholic Potassium Hydroxide TS—See *Potassium Hydroxide TS, Alcoholic*.

Alkaline Cupric Citrate TS—See *Cupric Citrate TS, Alkaline*.

Alkaline Cupric Citrate TS 2—See *Cupric Citrate TS 2, Alkaline*.

Alkaline Cupric Iodide TS—See *Cupric Iodide TS, Alkaline*.

Alkaline Cupric Tartrate TS (Fehling's Solution)—See *Cupric Tartrate TS, Alkaline*.

Alkaline Mercuric–Potassium Iodide TS—See *Mercuric–Potassium Iodide TS, Alkaline*.

Alkaline Picrate TS—See *Picrate TS, Alkaline*.

Alkaline Sodium Hydrosulfite TS—See *Sodium Hydrosulfite TS, Alkaline*.

Amaranth TS—Dissolve 20 mg of amaranth in 10 mL of water.

Aminonaphtholsulfonic Acid TS—Accurately weigh 5 g of sodium sulfite, 94.3 g of sodium bisulfite, and 700 mg of 1,2,4-aminonaphtholsulfonic acid, and mix. Prepare aminonaphtholsulfonic acid TS fresh on the day of use by dissolving 1.5 g of the dry mixture in 10 mL of water.

Ammonia TS—It contains between 9.5% and 10.5% of NH_3 . Prepare by diluting 350 mL of *Ammonia Water, Stronger* (see in the section, *Reagents*) with water to make 1000 mL.

Ammonia TS 2—Prepare by diluting 13.5 mL of *Ammonia Water, Stronger* (see *Reagent Specifications* in the section *Reagents*) with water to make 100 mL.

Ammonia TS, Alcoholic—A solution of ammonia gas in alcohol. Clear, colorless liquid having a strong odor of am-

monia. Specific gravity: about 0.80. It contains between 9% and 11% of NH_3 . Store it in alkali-resistant containers, in a cold place.

Ammonia TS, Stronger—Use *Ammonia Water, Stronger* (see in the section *Reagents*).

Ammonia–Ammonium Chloride Buffer TS—Dissolve 67.5 g of ammonium chloride in water, add 570 mL of ammonium hydroxide, and dilute with water to 1000 mL.

Ammonia–Cyanide TS—Dissolve 2 g of potassium cyanide in 15 mL of ammonium hydroxide, and dilute with water to 100 mL.

Ammoniacal Potassium Ferricyanide TS—Dissolve 2 g of potassium ferricyanide in 75 mL of water, add 25 mL of ammonium hydroxide, and mix.

Ammoniated Cupric Oxide TS—See *Cupric Oxide, Ammoniated, TS*.

Ammonium Acetate TS—Dissolve 10 g of ammonium acetate in water to make 100 mL.

Ammonium Carbonate TS—Dissolve 20 g of ammonium carbonate and 20 mL of ammonia TS in water to make 100 mL.

Ammonium Carbonate TS 2—Prepare a 158-mg/mL solution of ammonium carbonate in water.

Ammonium Chloride TS—Dissolve 10.5 g of ammonium chloride in water to make 100 mL.

Ammonium Chloride–Ammonium Hydroxide TS—Mix equal volumes of water and ammonium hydroxide, and saturate with ammonium chloride.

Ammonium Molybdate TS—Dissolve 6.5 g of finely powdered molybdic acid in a mixture of 14 mL of water and 14.5 mL of ammonium hydroxide. Cool the solution, and add it slowly, with stirring, to a well-cooled mixture of 32 mL of nitric acid and 40 mL of water. Allow to stand for 48 hours, and filter through a fine-porosity, sintered-glass crucible. This solution deteriorates upon standing and is unsuitable for use if, upon the addition of 2 mL of dibasic sodium phosphate TS to 5 mL of the solution, an abundant yellow precipitate does not form at once or after slight warming. Store it in the dark. If a precipitate forms during storage, use only the clear supernatant.

Ammonium Oxalate TS—Dissolve 3.5 g of ammonium oxalate in water to make 100 mL.

Ammonium Phosphate, Dibasic, TS (Ammonium Phosphate TS)—Dissolve 13 g of dibasic ammonium phosphate in water to make 100 mL.

Ammonium Polysulfide TS—Yellow liquid, made by saturating ammonium sulfide TS with sulfur.

Ammonium Pyrrolidinedithiocarbamate, Saturated, TS—Add about 10 g of ammonium pyrrolidinedithiocarbamate to a 1000-mL volumetric flask, and dilute with water to volume.

Ammonium Reineckate TS—Shake about 500 mg of ammonium reineckate with 20 mL of water frequently during 1 hour, and filter. Use within 2 days.

Ammonium Sulfide TS—Use ACS reagent-grade Ammonium Sulfide Solution.

Ammonium Thiocyanate TS—Dissolve 8 g of ammonium thiocyanate in water to make 100 mL.

Ammonium Vanadate TS—Dissolve 2.5 g of ammonium vanadate in 500 mL of boiling water, cool, and add 20 mL of nitric acid. Mix, cool, and add water to make 1 L. Store in polyethylene containers.

Anthrone TS—Within 12 hours of use, rapidly dissolve 35 mg of anthrone in a hot mixture of 35 mL of water and 65 mL of sulfuric acid. Immediately cool in an ice bath to room temperature, and filter through glass wool. Allow the solution to stand at room temperature for 30 minutes before use.

Antimony Trichloride TS—Dissolve 20 g of antimony trichloride in chloroform to make 100 mL. Filter if necessary.

Barium Chloride TS—Dissolve 12 g of barium chloride in water to make 100 mL.

Barium Hydroxide TS—A saturated solution of barium hydroxide in recently boiled water. Prepare the solution fresh.

Barium Nitrate TS—Dissolve 6.5 g of barium nitrate in water to make 100 mL.

Betanaphthol TS—See *2-Naphthol TS*.

Biuret Reagent TS—Dissolve 1.5 g of cupric sulfate and 6.0 g of potassium sodium tartrate in 500 mL of water in a 1000-mL volumetric flask. Add 300 mL of carbonate-free sodium hydroxide solution (1 in 10), dilute with carbonate-free sodium hydroxide solution (1 in 10) to 1000 mL, and mix.

Blue Tetrazolium TS—Dissolve 500 mg of blue tetrazolium in alcohol to make 100 mL.

Brilliant Blue G TS—Transfer 25 mg of brilliant blue G to a 100-mL volumetric flask, add 12.5 mL of alcohol and 25 mL of phosphoric acid, dilute with water to volume, and mix.

Bromine TS (Bromine Water)—A saturated solution of bromine, prepared by agitating 2 to 3 mL of bromine with 100 mL of cold water in a glass-stoppered bottle, the stopper of which should be lubricated with petrolatum. Store it in a cold place, protected from light.

Bromine-Sodium Acetate TS—Dissolve 100 g of sodium acetate in 1000 mL of glacial acetic acid, add 50 mL of bromine, and mix.

p-Bromoaniline TS—Add 8 g of *p*-bromoaniline to a mixture of 380 mL of thiourea-saturated glacial acetic acid, 10 mL of sodium chloride solution (1 in 5), 5 mL of oxalic acid solution (1 in 20), and 5 mL of dibasic sodium phosphate solution (1 in 10) in a low-actinic glass bottle. Mix, and allow to stand overnight before using. Protect from light, and use within 7 days.

Bromocresol Blue TS—Use *Bromocresol Green TS*.

Bromocresol Green TS—Dissolve 50 mg of bromocresol green in 100 mL of alcohol, and filter if necessary.

Bromocresol Green-Methyl Red TS—Dissolve 0.15 g of bromocresol green and 0.1 g of methyl red in 180 mL of alcohol, and dilute with water to 200 mL.

Bromocresol Purple TS—Dissolve 250 mg of bromocresol purple in 20 mL of 0.05 N sodium hydroxide, and dilute with water to 250 mL.

Bromophenol Blue TS—Dissolve 100 mg of bromophenol blue in 100 mL of diluted alcohol, and filter if necessary.

Bromothymol Blue TS—Dissolve 100 mg of bromothymol blue in 100 mL of diluted alcohol, and filter if necessary.

Buffered Acetone TS—See *Acetone, Buffered, TS*.

Calcium Chloride TS—Dissolve 7.5 g of calcium chloride in water to make 100 mL.

Calcium Hydroxide TS—Use *Calcium Hydroxide Topical Solution* (USP monograph).

Calcium Sulfate TS—A saturated solution of calcium sulfate in water.

Ceric Ammonium Nitrate TS—Dissolve 6.25 g of ceric ammonium nitrate in 10 mL of 0.25 N nitric acid. Use within 3 days.

Chloral Hydrate TS—Dissolve 50 g of chloral hydrate in a mixture of 15 mL of water and 10 mL of glycerin.

Chlorine TS (Chlorine Water)—A saturated solution of chlorine in water. Place the solution in small, completely filled, light-resistant containers. Chlorine TS, even when kept from light and air, is apt to deteriorate. Store it in a cold, dark place. For full strength, prepare this solution fresh.

Chromotropic Acid TS—Dissolve 50 mg of chromotropic acid or its disodium salt in 100 mL of 75% sulfuric acid, which may be made by cautiously adding 75 mL of sulfuric acid to 33.3 mL of water.

Cobalt-Uranyl Acetate TS—Dissolve, with warming, 40 g of uranyl acetate in a mixture of 30 g of glacial acetic acid and sufficient water to make 500 mL. Similarly, prepare a solution containing 200 g of cobaltous acetate in a mixture of 30 g of glacial acetic acid and sufficient water to make 500 mL. Mix the two solutions while still warm, and cool to 20°. Maintain the temperature at 20° for about 2 hours to separate the excess salts from solution, and then pass through a dry filter.

Cobaltous Chloride TS—Dissolve 2 g of cobaltous chloride in 1 mL of hydrochloric acid and sufficient water to make 100 mL.

Congo Red TS—Dissolve 500 mg of congo red in a mixture of 10 mL of alcohol and 90 mL of water.

m-Cresol Purple TS—Dissolve 0.10 g of metacresol purple in 13 mL of 0.01 N sodium hydroxide, dilute with water to 100 mL, and mix.

Cresol Red TS—Triturate 100 mg of cresol red in a mortar with 26.2 mL of 0.01 N sodium hydroxide until solution is complete, then dilute the solution with water to 250 mL.

Cresol Red-Thymol Blue TS—Add 15 mL of thymol blue TS to 5 mL of cresol red TS, and mix.

Crystal Violet TS—Dissolve 100 mg of crystal violet in 10 mL of glacial acetic acid.

Cupric Acetate TS—Dissolve 100 mg of cupric acetate in about 5 mL of water to which a few drops of acetic acid have been added. Dilute to 100 mL, and filter, if necessary.

Cupric Acetate TS, Stronger (Barfoed's Reagent)—Dissolve 13.3 g of cupric acetate in a mixture of 195 mL of water and 5 mL of acetic acid.

Cupric-Ammonium Sulfate TS—To cupric sulfate TS add ammonia TS, dropwise, until the precipitate initially formed is nearly but not completely dissolved. Allow to settle, and decant the clear solution. Prepare this solution fresh.

Cupric Citrate TS—Dissolve 25 g of cupric sulfate, 50 g of citric acid, and 144 g of anhydrous sodium carbonate in water, and dilute with water to 1000 mL.

Cupric Citrate TS, Alkaline—With the aid of heat, dissolve 173 g of dihydrated sodium citrate and 117 g of monohydrated sodium carbonate in about 700 mL of water, and filter through paper, if necessary, to obtain a clear solution. In a separate container dissolve 17.3 g of cupric sulfate in about 100 mL of water, and slowly add this solution, with constant stirring, to the first solution. Cool the mixture, add water to make 1000 mL, and mix.

Cupric Citrate TS 2, Alkaline—With the aid of heat, dissolve about 173 g of sodium citrate dihydrate and 117 g of sodium carbonate monohydrate in about 700 mL of water, and filter. In a second flask, dissolve about 27.06 g of cupric sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in about 100 mL of water. Slowly combine the two solutions while stirring, and dilute with water to 1000 mL.

Cupric Iodide TS, Alkaline—Dissolve 7.5 g of cupric sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in about 100 mL of water. In a separate container dissolve 25 g of anhydrous sodium carbonate, 20 g of sodium bicarbonate, and 25 g of potassium sodium tartrate in about 600 mL of water. With constant stirring, add the cupric sulfate solution to the bottom of the alkaline tartrate solution by means of a funnel that touches the bottom of the container. Add 1.5 g of potassium iodide, 200 g of anhydrous sodium sulfate, 50 to 150 mL of 0.02 M potassium iodate, and sufficient water to make 1000 mL.

Cupric Oxide, Ammoniated, TS (Schweitzer's Reagent)—Dissolve 10 g of cupric sulfate in 100 mL of water, add sufficient sodium hydroxide solution (1 in 5) to precipitate the copper hydroxide, collect the latter on a filter, and wash free from sulfate with cold water. Dissolve the precipitate, which must be kept wet during the entire process, in the minimum quantity of ammonia TS necessary for complete solution.

Cupric Sulfate TS—Dissolve 12.5 g of cupric sulfate in water to make 100 mL.

Cupric Tartrate TS, Alkaline (Fehling's Solution)—

The Copper Solution (A)—Dissolve 34.66 g of carefully selected, small crystals of cupric sulfate, showing no trace of efflorescence of adhering moisture, in water to make 500 mL. Store this solution in small, tight containers.

The Alkaline Tartrate Solution (B)—Dissolve 173 g of crystallized potassium sodium tartrate and 50 g of sodium hydroxide in water to make 500 mL. Store this solution in small, alkali-resistant containers.

For use, mix exactly equal volumes of *Solutions A and B* at the time required.

Delafield's Hematoxylin TS—Prepare 400 mL of a saturated solution of ammonium alum (*Solution A*). Dissolve 4 g of hematoxylin in 25 mL of alcohol, mix it with *Solution A*, and allow it to stand for 4 days in a flask closed with a pledget of purified cotton and exposed to light and air (*Solution B*). Then filter *Solution B*, and add to it a *Solution C* consisting of a mixture of 100 mL of glycerin and 100 mL of methanol. Mix, and allow the mixture to stand in a warm place, exposed to light, for 6 weeks until it becomes dark-colored. Store in tightly stoppered bottles.

For use in staining endocrine tissue, dilute this test solution with an equal volume of water.

Denaturated Alcoholic TS—A specially denaturated alcohol containing either rubber hydrocarbon solvent of heptane or toluene. [NOTE—A suitable grade is available from www.lyondell.com or from www.sasol.com, as Ethanol SDA 2B HEP 200, or Ethanol SDA 2B TOL 200, or Ethanol SDA 2B TOL 190, or Alcohol SDA 2B-2.]

Denigès' Reagent—See *Mercuric Sulfate TS*.

Diazobenzenesulfonic Acid TS—Place in a beaker 1.57 g of sulfanilic acid, previously dried at 105° for 3 hours, add 80 mL of water and 10 mL of diluted hydrochloric acid, and warm on a steam bath until dissolved. Cool to 15° (some of the sulfanilic acid may separate but will dissolve later), and add slowly, with constant stirring, 6.5 mL of sodium nitrite solution (1 in 10). Then dilute with water to 100 mL.

Dichlorofluorescein TS—Dissolve 100 mg of dichlorofluorescein in 60 mL of alcohol, add 2.5 mL of 0.1 N sodium hydroxide, mix, and dilute with water to 100 mL.

2,7-Dihydroxynaphthalene TS—Dissolve 100 mg of 2,7-dihydroxynaphthalene in 1000 mL of sulfuric acid, and allow the solution to stand until the yellow color disappears. If the solution is very dark, discard it and prepare a new solution from a different supply of sulfuric acid. This solution is stable for approximately 1 month if stored in a dark bottle.

Diiodofluorescein TS—Dissolve 500 mg of diiodofluorescein in a mixture of 75 mL of alcohol and 30 mL of water.

Diluted Lead Subacetate TS—See *Lead Subacetate TS, Diluted*.

p-Dimethylaminobenzaldehyde TS—Dissolve 125 mg of p-dimethylaminobenzaldehyde in a cooled mixture of 65 mL of sulfuric acid and 35 mL of water, and add 0.05 mL of ferric chloride TS. Use within 7 days.

Dinitrophenylhydrazine TS—Carefully mix 10 mL of water and 10 mL of sulfuric acid, and cool. To the mixture, contained in a glass-stoppered flask, add 2 g of 2,4-dinitrophenylhydrazine, and shake until dissolved. To the solution add 35 mL of water, mix, cool, and filter.

Diphenylamine TS—Dissolve 1.0 g of diphenylamine in 100 mL of sulfuric acid. The solution should be colorless.

Diphenylcarbazone TS—Dissolve 1 g of crystalline diphenylcarbazone in 75 mL of alcohol, then add alcohol to make 100 mL. Store in a brown bottle.

Dithizone TS—Dissolve 25.6 mg of dithizone in 100 mL of alcohol. Store in a cold place, and use within 2 months.

Dragendorff's TS—Mix 850 mg of bismuth subnitrate with 40 mL of water and 10 mL of glacial acetic acid (*Solution A*). Dissolve 8 g of potassium iodide in 20 mL of water (*Solution B*). Mix equal portions of *Solution A* and *Solution B* to obtain a stock solution, which can be stored for several months in a dark bottle. Mix 10 mL of the stock solution with 20 mL of glacial acetic acid, and dilute with water to make 100 mL.

Edetate Disodium TS—Dissolve 1 g of edetate disodium in 950 mL of water, add 50 mL of alcohol, and mix.

Eosin Y TS (adsorption indicator)—Dissolve 50 mg of eosin Y in 10 mL of water.

Eriochrome Black TS—Dissolve 200 mg of eriochrome black T and 2 g of hydroxylamine hydrochloride in methanol to make 50 mL.

Eriochrome Cyanine TS—Dissolve 750 mg of eriochrome cyanine R in 200 mL of water, add 25 g of sodium chloride, 25 g of ammonium nitrate, and 2 mL of nitric acid, and dilute with water to 1000 mL.

Fehling's Solution—See *Cupric Tartrate TS, Alkaline*.

Ferric Ammonium Sulfate TS—Dissolve 8 g of ferric ammonium sulfate in water to make 100 mL.

Ferric Chloride TS—Dissolve 9 g of ferric chloride in water to make 100 mL.

Ferrioin TS—Dissolve 0.7 g of ferrous sulfate and 1.76 g of o-phenanthroline monohydrochloride monohydrate in water, and dilute with water to 100 mL.

Ferrous Sulfate TS—Dissolve 8 g of clear crystals of ferrous sulfate in about 100 mL of recently boiled and thoroughly cooled water. Prepare this solution fresh.

Ferrous Sulfate, Acid, TS—Dissolve 7 g of ferrous sulfate crystals in 90 mL of recently boiled and thoroughly cooled water, and add sulfuric acid to make 100 mL. Prepare this solution immediately prior to use.

Folin-Ciocalteu Phenol TS—Into a 1500-mL flask introduce 100 g of sodium tungstate, 25 g of sodium molybdate, 700 mL of water, 50 mL of phosphoric acid, and 100 mL of hydrochloric acid. Gently reflux the mixture for about 10 hours, and add 150 g of lithium sulfate, 50 mL of water, and a few drops of bromine. Boil the mixture, without the condenser, for about 15 minutes, or until the excess bromine is expelled. Cool, dilute with water to 1 L, and filter: the filtrate has no greenish tint. Before use, dilute 1 part of the filtrate with 1 part of water. When used for protein determination (i.e., Lowry assay), this reagent must be further diluted (1:5) with water. See *Method 2* in *Total Protein Assay* under *Biotechnology-Derived Articles—Total Protein Assay* (1057).

Formaldehyde TS—Use *Formaldehyde Solution* (see in the section *Reagents*).

Fuchsin-Pyrogallol TS—Dissolve 100 mg of basic fuchsin in 50 mL of water that previously has been boiled for 15 minutes and allowed to cool slightly. Cool, add 2 mL of a saturated solution of sodium bisulfite, mix, and allow to stand for not less than 3 hours. Add 0.9 mL of hydrochloric acid, mix, and allow to stand overnight. Add 100 mg of pyrogallol, shake until solution is effected, and dilute with water to 100 mL. Store in an amber-colored glass bottle in a refrigerator.

Fuchsin-Sulfurous Acid TS—Dissolve 200 mg of basic fuchsin in 120 mL of hot water, and allow the solution to cool. Add a solution of 2 g of anhydrous sodium sulfite in 20 mL of water, then add 2 mL of hydrochloric acid. Dilute the solution with water to 200 mL, and allow to stand for at least 1 hour. Prepare this solution fresh.

Gastric Fluid, Simulated, TS—Dissolve 2.0 g of sodium chloride and 3.2 g of purified pepsin, that is derived from porcine stomach mucosa, with an activity of 800 to 2500 units per mg of protein, in 7.0 mL of hydrochloric acid and sufficient water to make 1000 mL. [NOTE—Pepsin activity is described in the *Food Chemicals Codex* specifications under *General Tests and Assays*.] This test solution has a pH of about 1.2.

Gelatin TS (for the assay of *Corticotropin Injection*)—Dissolve 340 g of acid-treated precursor gelatin (Type A) in water to make 1000 mL. Heat the solution in an autoclave at 115° for 30 minutes after the exhaust line temperature has reached 115°. Cool the solution, and add 10 g of phenol and 1000 mL of water. Store in tight containers in a refrigerator.

Glacial Acetic Acid TS—See *Acetic Acid, Glacial, TS*.

Glucose Oxidase-Chromogen TS—A solution containing, in each mL, 0.5 μ mol of 4-aminoantipyrine, 22.0 μ mol of sodium p-hydroxybenzoate, not less than 7.0 units of glucose oxidase, and not less than 0.5 units of peroxidase, and buffered to a pH of 7.0 \pm 0.1.

Suitability—When used for determining glucose in Inulin, ascertain that no significant color results by reaction with fructose, and that a suitable absorbance-versus-concentration slope is obtained with glucose.

[NOTE—A suitable grade is available, as a concentrate, from Worthington Diagnostics, Division of Millipore Corp., www.millipore.com.]

Glycerin Base TS—To 200 g of glycerin add water to bring the total weight to 235 g. Add 140 mL of 1 N sodium hydroxide and 50 mL of water.

Gold Chloride TS—Dissolve 1 g of gold chloride in 35 mL of water.

Hydrogen Peroxide TS—Use *Hydrogen Peroxide Topical Solution* (USP monograph).

Hydrogen Sulfide TS—A saturated solution of hydrogen sulfide, made by passing H_2S into cold water. Store it in small, dark amber-colored bottles, filled nearly to the top. It is unsuitable unless it possesses a strong odor of H_2S , and unless it produces at once a copious precipitate of sulfur when added to an equal volume of ferric chloride TS. Store in a cold, dark place.

Hydroxylamine Hydrochloride TS—Dissolve 3.5 g of hydroxylamine hydrochloride in 95 mL of 60% alcohol, and add 0.5 mL of bromophenol blue solution (1 in 1000 of alcohol) and 0.5 N alcoholic potassium hydroxide until a greenish tint develops in the solution. Then add 60% alcohol to make 100 mL.

8-Hydroxyquinoline TS—Dissolve 5 g of 8-hydroxyquinoline in alcohol to make 100 mL.

Indigo Carmine TS (*Sodium Indigotindisulfonate TS*)—Dissolve a quantity of sodium indigotindisulfonate, equivalent to 180 mg of $\text{C}_{16}\text{H}_8\text{N}_2\text{O}_2(\text{SO}_3\text{Na})_2$, in water to make 100 mL. Use within 60 days.

Indophenol-Acetate TS (for the assay of *Corticotropin Injection*)—To 60 mL of standard dichlorophenol-indophenol solution (see in the section *Volumetric Solutions*) add water to make 250 mL. Add to the resulting solution an equal volume of sodium acetate solution freshly prepared by dissolving 13.66 g of anhydrous sodium acetate in water to make 500 mL and adjusting with 0.5 N acetic acid to a pH of 7. Store in a refrigerator, and use within 2 weeks.

Intestinal Fluid, Simulated, TS—Dissolve 6.8 g of monobasic potassium phosphate in 250 mL of water, mix, and add 77 mL of 0.2 N sodium hydroxide and 500 mL of water. Add 10.0 g of pancreatin, mix, and adjust the resulting solution with either 0.2 N sodium hydroxide or 0.2 N hydrochloric acid to a pH of 6.8 ± 0.1 . Dilute with water to 1000 mL.

Iodine TS—Use 0.1 N Iodine (see in the section *Volumetric Solutions*).

Iodine, Diluted TS—Transfer 10.0 mL of 0.1 N iodine VS to a 100-mL volumetric flask, dilute with water to volume, and mix.

Iodine Monochloride TS—Dissolve 10 g of potassium iodide and 6.44 g of potassium iodate in 75 mL of water in a glass-stoppered container. Add 75 mL of hydrochloric acid and 5 mL of chloroform, and adjust to a faint iodine color (in the chloroform) by adding dilute potassium iodide or potassium iodate solution. If much iodine is liberated, use a stronger solution of potassium iodate than 0.01 M at first, making the final adjustment with the 0.01 M potassium iodate. Store in a dark place, and readjust to a faint iodine color as necessary.

Iodine and Potassium Iodide TS 1—Dissolve 500 mg of iodine and 1.5 g of potassium iodide in 25 mL of water.

Iodine and Potassium Iodide TS 2—Dissolve 12.7 g of iodine and 20 g of potassium iodide in water, and dilute with water to 1000.0 mL. To 10.0 mL of this solution, add 0.6 g of potassium iodide, and dilute with water to 100.0 mL. Prepare immediately before use.

Iodine and Potassium Iodide TS 3—Dissolve 0.127 g of iodine and 0.20 g of potassium iodide in water, and dilute with water to 10.0 mL.

Iodobromide TS—Dissolve 20 g of iodine monobromide in glacial acetic acid to make 1000 mL. Store in glass containers, protected from light.

Iodochloride TS—Dissolve 16.5 g of iodine monochloride in 1000 mL of glacial acetic acid.

Iodoplatinate TS—Dissolve 300 mg of platonic chloride in 97 mL of water. Immediately prior to use, add 3.5 mL of potassium iodide TS, and mix.

Iron-Phenol TS (*Iron-Kober Reagent*)—Dissolve 1.054 g of ferrous ammonium sulfate in 20 mL of water, and add 1 mL of sulfuric acid and 1 mL of 30 percent hydrogen peroxide. Mix, heat until effervescence ceases, and dilute with water to 50 mL. To 3 volumes of this solution contained in a volumetric flask add sulfuric acid, with cooling, to make 100 volumes. Purify phenol by distillation, discarding the first 10% and the last 5%, collecting the distillate, with exclusion of moisture, in a dry, tared glass-stoppered flask of about twice the volume of the phenol. Solidify the phenol in an ice bath, breaking the top crust with a glass rod to ensure complete crystallization. Weigh the flask and its contents, add to the phenol 1.13 times its weight of the iron-sulfuric acid solution prepared as directed, insert the stopper in the flask, and allow to stand, without cooling but with occasional mixing, until the phenol is liquefied. Shake the mixture vigorously until mixed, allow to stand in the dark for 16 to 24 hours, and again weigh the flask and its contents. To the mixture add 23.5% of its weight of a solution of 100 volumes of sulfuric acid in 110 volumes of water, mix, transfer to dry glass-stoppered bottles, and store in the dark, protected from atmospheric moisture. Use within 6 months. Dispense the reagent from a small-bore buret, arranged to exclude moisture, capable of delivering 1 mL in 30 seconds or less, and having no lubricant, other than reagent, on its stopcock. Wipe the buret tip with tissue before each addition.

Iron Salicylate TS—Dissolve 500 mg of ferric ammonium sulfate in 250 mL of water containing 10 mL of diluted sulfuric acid, and add water to make 500 mL. To 100 mL of the resulting solution add 50 mL of a 1.15% solution of sodium salicylate, 20 mL of diluted acetic acid, and 80 mL of a 13.6% solution of sodium acetate, then add water to make 500 mL. Store in a well-closed container. Protect from light. Use within 2 weeks.

Lanthanum Nitrate TS—Dissolve 5.0 g of lanthanum nitrate hexahydrate in 100 mL of water.

Lead Acetate TS—Dissolve 9.5 g of clear, transparent crystals of lead acetate in recently boiled water to make 100 mL. Store in well-stoppered bottles.

Lead Acetate TS, Alcoholic—Dissolve 2 g of clear, transparent crystals of lead acetate in alcohol to make 100 mL. Store in tight containers.

Lead Subacetate TS—Dissolve 40.0 g of lead acetate in 90 mL of carbon dioxide-free water. Adjust with 10 M sodium hydroxide to a pH of 7.5, centrifuge, and use the clear supernatant. It contains NLT 16.7% (w/w) and NMT 17.4% (w/w) of Pb in a form corresponding to the formula $\text{C}_8\text{H}_{14}\text{O}_{10}\text{Pb}_3$. The solution remains clear when stored in a well-closed container.

Lead Subacetate TS, Diluted—Dilute 3.25 mL of lead subacetate TS with water, recently boiled and cooled, to make 100 mL. Store in small, well-filled, tight containers.

Litmus TS—Digest 25 g of powdered litmus with three successive 100-mL portions of boiling alcohol, continuing each extraction for about 1 hour. Filter, wash with alcohol, and discard the alcohol filtrate. Macerate the residue with about 25 mL of cold water for 4 hours, filter, and discard the filtrate. Finally digest the residue with 125 mL of boiling water for 1 hour, cool, and filter.

Locke-Ringer's Solution—See *Locke-Ringer's TS*.

Locke-Ringer's TS (*Locke-Ringer's Solution*)—

Sodium Chloride	9.0 g
Potassium Chloride	0.42 g
Calcium Chloride	0.24 g
Magnesium Chloride	0.2 g
Sodium Bicarbonate	0.5 g
Dextrose	0.5 g
Water, recently distilled from a hard-glass flask, a sufficient quantity to make	1000 mL

Prepare fresh each day. The constituents (except the dextrose and the sodium bicarbonate) may be made up in stock solutions and diluted as needed.

Magnesia Mixture TS—Dissolve 5.5 g of magnesium chloride and 7 g of ammonium chloride in 65 mL of water, add 35 mL of ammonia TS, set the mixture aside for a few days in a well-stoppered bottle, and filter. If the solution is not perfectly clear, filter it before using.

Magnesium Sulfate TS—Dissolve 12 g of crystals of magnesium sulfate, selected for freedom from efflorescence, in water to make 100 mL.

Malachite Green TS—Dissolve 1 g of malachite green oxalate in 100 mL of glacial acetic acid.

Mallory's Stain—Dissolve 500 mg of water-soluble aniline blue, 2 g of orange G, and 2 g of oxalic acid in 100 mL of water.

Mayer's Reagent—See *Mercuric-Potassium Iodide TS*.

Mercuric Acetate TS—Dissolve 6.0 g of mercuric acetate in glacial acetic acid to make 100 mL. Store in tight containers, protected from direct sunlight.

Mercuric-Ammonium Thiocyanate TS—Dissolve 30 g of ammonium thiocyanate and 27 g of mercuric chloride in water to make 1000 mL.

Mercuric Bromide TS, Alcoholic—Dissolve 5 g of mercuric bromide in 100 mL of alcohol, employing gentle heat to facilitate solution. Store in glass containers, protected from light.

Mercuric Chloride TS—Dissolve 6.5 g of mercuric chloride in water to make 100 mL.

Mercuric Iodide TS (Valser's Reagent)—Slowly add potassium iodide solution (1 in 10) to red mercuric iodide until almost all of the latter is dissolved, and filter off the excess. A solution containing 10 g of potassium iodide in 100 mL dissolves approximately 14 g of HgI_2 at 20°.

Mercuric Nitrate TS—Dissolve 40 g of mercuric oxide (red or yellow) in a mixture of 32 mL of nitric acid and 15 mL of water. Store in glass containers, protected from light.

Mercuric-Potassium Iodide TS (Mayer's Reagent)—Dissolve 1.358 g of mercuric chloride in 60 mL of water. Dissolve 5 g of potassium iodide in 10 mL of water. Mix the two solutions, and dilute with water to 100 mL.

Mercuric-Potassium Iodide TS, Alkaline (Nessler's Reagent)—Dissolve 143 g of sodium hydroxide in 700 mL of water. Dissolve 50 g of red mercuric iodide and 40 g of potassium iodide in 200 mL of water. Pour the iodide solution into the hydroxide solution, and dilute with water to 1000 mL. Allow to settle, and use the clear supernatant.

Mercuric Sulfate TS (Denigès' Reagent)—Mix 5 g of yellow mercuric oxide with 40 mL of water, and while stirring slowly add 20 mL of sulfuric acid, then add another 40 mL of water, and stir until completely dissolved.

Mercurous Nitrate TS—Dissolve 15 g of mercurous nitrate in a mixture of 90 mL of water and 10 mL of diluted nitric acid. Store in dark, amber-colored bottles in which a small globule of mercury has been placed.

Metaphenylenediamine Hydrochloride TS—Dissolve 1 g of metaphenylenediamine hydrochloride in 200 mL of water. The solution must be colorless when used. If necessary, decolorize by heating with activated charcoal.

Metaphosphoric-Acetic Acids TS—Dissolve 15 g of metaphosphoric acid in 40 mL of glacial acetic acid and sufficient water to make 500 mL. Store in a cold place, and use within 2 days.

Methoxyphenylacetic TS—Dissolve 2.7 g of methoxyphenylacetic acid in 6 mL of *Tetramethylammonium Hydroxide TS*, and add 20 mL of dehydrated alcohol. Store in a polyethylene container.

Methyl Orange TS—Dissolve 100 mg of methyl orange in 100 mL of water, and filter if necessary.

Methyl Purple TS—Use *Methyl Red-Methylene Blue TS*.

Methyl Red TS—Dissolve 100 mg of methyl red in 100 mL of alcohol, and filter if necessary.

Methyl Red TS 2—To 1.86 mL of 0.1 M sodium hydroxide and 50 mL of alcohol, add 50 mg of methyl red, and dilute with water to 100 mL.

Methyl Red TS, Methanolic—Dissolve 1 g of methyl red in 100 mL of methanol, and filter, if necessary. Store protected from light, and use within 21 days.

Methyl Red-Methylene Blue TS—Add 10 mL of methyl red TS to 10 mL of methylene blue TS, and mix.

Methyl Violet TS—Use *Crystal Violet TS*.

Methyl Yellow TS—Prepare a solution containing 0.10 mg per mL in alcohol.

Methyl Yellow-Methylene Blue TS—Dissolve 1 g of methyl yellow and 100 mg of methylene blue in 125 mL of methanol.

3-Methyl-2-benzothiazolinone Hydrazone Hydrochloride TS—Dissolve 0.1 g of 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate in 10 mL of water, dilute the resulting solution with methanol to 100 mL, and mix.

Methylene Blue TS—Dissolve 125 mg of methylene blue in 100 mL of alcohol, and dilute with alcohol to 250 mL.

Methylthionine Perchlorate TS—To 500 mL of potassium perchlorate solution (1 in 1000) add dropwise, with constant shaking, methylene blue solution (1 in 100) until a slight, permanent turbidity results. Allow the precipitate to settle, decant the supernatant through paper, and use only the clear solution.

Millon's Reagent—To 2 mL of mercury in a conical flask add 20 mL of nitric acid. Shake the flask under a hood to break up the mercury into small globules. After about 10 minutes, add 35 mL of water, and, if a precipitate or crystals appear, add sufficient dilute nitric acid (1 in 5, prepared from nitric acid from which the oxides have been removed by blowing air through it until it is colorless) to dissolve the separated solid. Add sodium hydroxide solution (1 in 10) dropwise, with thorough mixing, until the curdy precipitate that forms after the addition of each drop no longer redissolves but is dispersed to form a suspension. Add 5 mL more of the dilute nitric acid, and mix. Prepare this solution fresh.

Molybdo-phosphotungstate TS (Folin-Denis Reagent)—To about 350 mL of water contained in a round-bottom flask add 50 g of sodium tungstate, 12 g of phosphomolybdic acid, and 25 mL of phosphoric acid. Boil the mixture under a reflux condenser for 2 hours, then cool, dilute with water to 500 mL, and mix. Store in tight containers, protected from light, and in a cold place.

1-Naphthol Reagent—Dissolve 1 g of 1-naphthol in 25 mL of methanol. Prepare this solution fresh.

1-Naphthol TS—Use *1-Naphthol Reagent*.

2-Naphthol TS (Betanaphthol TS)—Dissolve 1 g of 2-naphthol in 100 mL of sodium hydroxide solution (1 in 100).

p-Naphtholbenzein TS—Dissolve 250 mg of p-naphtholbenzein in 100 mL of glacial acetic acid.

N-(1-Naphthyl)ethylenediamine Dihydrochloride TS—Dissolve 100 mg of N-(1-naphthyl)ethylenediamine dihydrochloride in 100 mL of a mixture of 7 parts of acetone and 3 parts of water.

Nessler's Reagent—See *Mercuric-Potassium Iodide TS, Alkaline*.

Neutral Red TS—Dissolve 100 mg of neutral red in 100 mL of 50% alcohol.

Nickel Standard Solution TS—Dissolve 4.78 g of nickel (II) sulfate heptahydrate in water, and dilute with water to 1000 mL. Immediately prior to use, dilute 10.0 mL of the solution so obtained with water to 1000 mL. Suitable nickel standard solutions are also available commercially.

Ninhydrin TS—Use *Triketohydrindene Hydrate TS*.

p-Nitroaniline TS—To 350 mg of p-nitroaniline add 1.5 mL of hydrochloric acid, and mix. Dilute with water to 50 mL, mix, and allow to settle. Place 5 mL of the clear supernatant in a 100-mL volumetric flask, and immerse it in an ice bath. While it is in the ice bath, add 1 mL of hydrochloric acid, then add, in small portions, 2 mL of sodium

nitrite solution (1 in 100), dilute with water to volume, and mix.

Nitrophenanthroline TS—Dissolve 150 mg of 5-nitro-1,10-phenanthroline in 15 mL of freshly prepared ferrous sulfate solution (1 in 140).

Oracet Blue B TS—A 1 in 200 solution of oracet blue B in glacial acetic acid.

Orthophenanthroline TS—Dissolve 150 mg of orthophenanthroline in 10 mL of a solution of ferrous sulfate, prepared by dissolving 700 mg of clear crystals of ferrous sulfate in 100 mL of water. The ferrous sulfate solution must be prepared immediately before dissolving the orthophenanthroline. Store in well-closed containers.

Oxalic Acid TS—Dissolve 6.3 g of oxalic acid in water to make 100 mL.

Palladium Chloride TS, Buffered—Weigh 500 mg of palladium chloride into a 250-mL beaker, add 5 mL of concentrated hydrochloric acid, and warm the mixture on a steam bath. Add 200 mL of hot water in small increments with continued heating until solution is complete. Transfer the solution to a 250-mL volumetric flask, and dilute with water to volume. Transfer 50 mL to a 100-mL volumetric flask. Add 10 mL of 1 M sodium acetate and 9.6 mL of 1 N hydrochloric acid. Dilute with water to volume.

Perchloric Acid TS—Dilute 8.5 mL of perchloric acid with water to 100 mL.

Phenol TS—Dissolve 1.2 g of phenol in alcohol to make 10 mL. Prepare weekly.

Phenol Red TS (*Phenolsulfonphthalein TS*)—Dissolve 100 mg of phenolsulfonphthalein in 100 mL of alcohol, and filter if necessary.

pH 4.7 Phenol Red TS—Dissolve 33 mg of phenolsulfonphthalein in 1.5 mL of 2 N sodium hydroxide solution, dilute with water to 100 mL, and mix (*Solution A*). Dissolve 25 mg of ammonium sulfate in 235 mL of water, add 105 mL of 2 N sodium hydroxide solution and 135 mL of 2 N acetic acid, and mix (*Solution B*). Add 25 mL of *Solution A* to *Solution B*, and mix. If necessary, adjust the pH of this solution to 4.7.

Phenoldisulfonic Acid TS—Dissolve 2.5 g of phenol in 15 mL of sulfuric acid in a flask of suitable capacity. Add 7.5 mL of fuming sulfuric acid, stir well, and heat at 100° for 2 hours. Transfer the product, while still fluid, to a glass-stoppered bottle, and, when desired for use, warm in a water bath until liquefied.

Phenolphthalein TS—Dissolve 1 g of phenolphthalein in 100 mL of alcohol.

Phenylhydrazine Acetate TS—Dissolve 10 mL of phenylhydrazine and 5 mL of glacial acetic acid in water to make 100 mL.

Phenylhydrazine-Sulfuric Acid TS—Dissolve 65 mg of phenylhydrazine hydrochloride in 100 mL of a cooled mixture of equal volumes of sulfuric acid and water.

Phloroglucinol TS—Dissolve 500 mg of phloroglucinol in 25 mL of alcohol. Store in tight containers, protected from light.

Phosphatic Enzyme TS—Dissolve 5 g of phosphatic enzyme in water to make 50 mL. Prepare this solution fresh.

Phosphomolybdic Acid TS—Dissolve 20 g of phosphomolybdic acid in alcohol to make 100 mL. Filter the solution, and use only the clear filtrate.

Phosphotungstic Acid TS—Dissolve 1 g of phosphotungstic acid in water to make 100 mL.

Picrate TS, Alkaline—Mix 20 mL of trinitrophenol solution (1 in 100) with 10 mL of sodium hydroxide solution (1 in 20), dilute with water to 100 mL, and mix. Use within 2 days.

Picric Acid TS—See *Trinitrophenol TS*.

Platinic Chloride TS—Dissolve 2.6 g of platinic chloride in water to make 20 mL.

Platinum-Cobalt TS—Dissolve 1.246 g of potassium chloroplatinate (K_2PtCl_6) and 1.000 g of cobalt chloride ($CoCl_2 \cdot 6H_2O$) in water, add 100 mL of hydrochloric acid, and dilute with water to 1 L.

Potassium Acetate TS—Dissolve 10 g of potassium acetate in water to make 100 mL.

Potassium-Bismuth Iodide TS—Dissolve 12.5 g of tartaric acid in 25 mL of water, then dissolve 1.06 g of bismuth subnitrate in this mixture (*Solution A*). Dissolve 20 g of potassium iodide in 25 mL of water (*Solution B*). Dissolve 100 g of tartaric acid in 450 mL of water (*Solution C*). Add *Solutions A* and *B* to *Solution C*, and mix.

Potassium Carbonate TS—Dissolve 7 g of anhydrous potassium carbonate in water to make 100 mL.

Potassium Chromate TS—Dissolve 10 g of potassium chromate in water to make 100 mL.

Potassium Dichromate TS—Dissolve 7.5 g of potassium dichromate in water to make 100 mL.

Potassium Ferricyanide TS—Dissolve 1 g of potassium ferricyanide in 10 mL of water. Prepare this solution fresh.

Potassium Ferrocyanide TS—Dissolve 1 g of potassium ferrocyanide in 10 mL of water. Prepare this solution fresh.

Potassium Hydroxide TS—Dissolve 6.5 g of potassium hydroxide in water to make 100 mL.

Potassium Hydroxide TS, Alcoholic—Use 0.5 N Potassium Hydroxide, Alcoholic (see in the section *Volumetric Solutions*).

Potassium Hydroxide TS 2, Alcoholic—Dissolve 130 g of potassium hydroxide, with cooling, in 200 mL of water. Add alcohol to 1000 mL. Store in a well-stoppered dark glass bottle.

Potassium Iodide TS—Dissolve 16.5 g of potassium iodide in water to make 100 mL. Store in light-resistant containers.

Potassium Iodide and Starch TS—Dissolve 0.75 g of potassium iodide in 100 mL of water. Heat to boiling, and add, with stirring, a solution of 0.5 g of soluble starch in 35 mL of water. Boil for 2 minutes, and allow to cool.

Sensitivity—Mix 15 mL in 0.05 mL of glacial acetic acid and 0.3 mL of diluted iodine TS: a blue color is produced.

Potassium Iodoplatinate TS—Dissolve 200 mg of platinic chloride in 2 mL of water, mix with 25 mL of potassium iodide solution (1 in 25), and add water to make 50 mL.

Potassium Permanganate TS—Use 0.1 N Potassium Permanganate (see in the section *Volumetric Solutions*).

Potassium Pyroantimonate TS—Dissolve 2 g of potassium pyroantimonate in 85 mL of hot water. Cool quickly, and add 50 mL of a solution containing 50 mg/mL of potassium hydroxide in water and 1 mL of sodium hydroxide solution (8.5 in 100). Allow to stand for 24 h, filter, and dilute with water to 150 mL.

Potassium Sulfate TS—Dissolve 1 g of potassium sulfate in water to make 100 mL.

Potassium Thiocyanate TS—Dissolve 9.7 g of potassium thiocyanate in water to make 100 mL.

Pyridine-Pyrazolone TS—To 100 mL of a saturated solution of 1-phenyl-3-methyl-2-pyrazolone-5-one add 20 mL of a 1 in 1000 solution of 3,3'-dimethyl-1,1'-diphenyl-[4,4'-bi-2-pyrazoline]-5,5'-dione in pyridine. Store in a dark bottle, and use within 3 days.

Pyrogallol TS, Alkaline—Dissolve 500 mg of pyrogallol in 2 mL of water. Dissolve 12 g of potassium hydroxide in 8 mL of water. The solutions should be freshly prepared and mixed immediately before use.

Quinaldine Red TS—Dissolve 100 mg of quinaldine red in 100 mL of alcohol.

Quinone TS—Dissolve 500 mg of *p*-benzoquinone in 2.5 mL of glacial acetic acid, and dilute with alcohol to 50 mL. Prepare this solution fresh daily.

Resorcinol TS—Dissolve 1 g of resorcinol in hydrochloric acid to make 100 mL.

Ruthenium Red TS—Dissolve 10 g of lead acetate in water, dilute with water to 100 mL, and add 80 mg of ruthenium red. The solution is wine-red in color. [NOTE—If necessary, add additional ruthenium red to obtain a wine-red color.]

Saline TS—Dissolve 9.0 g of sodium chloride in water to make 1000 mL.

[NOTE—Where pyrogen-free saline TS is specified in this Pharmacopeia, saline TS that has met the requirements of the *Pyrogen Test* (151) is to be used.]

Saline TS, Pyrogen-Free—See *Saline TS*.

Schweitzer's Reagent—See *Cupric Oxide, Ammoniated, TS*.

Silver–Ammonia–Nitrate TS—Dissolve 1 g of silver nitrate in 20 mL of water. Add ammonia TS, dropwise, with constant stirring, until the precipitate is almost but not entirely dissolved. Filter, and store in tight, light-resistant containers.

Silver–Ammonium Nitrate TS—See *Silver–Ammonia–Nitrate TS*.

Silver Diethyldithiocarbamate TS—Dissolve 1 g of silver diethyldithiocarbamate in 200 mL of pyridine from a freshly opened bottle or that which has been recently distilled. Store in light-resistant containers, and use within 30 days.

Silver Nitrate TS—Use 0.1 N *Silver Nitrate* (see in the section *Volumetric Solutions*).

Simulated Gastric Fluid TS—See *Gastric Fluid, Simulated, TS*.

Simulated Intestinal Fluid TS—See *Intestinal Fluid, Simulated, TS*.

Sodium Acetate TS—Dissolve 13.6 g of sodium acetate in water to make 100 mL.

Sodium Alizarinsulfonate TS—Dissolve 100 mg of sodium alizarinsulfonate in 100 mL of water, and filter.

Sodium Aminoacetate TS (*Sodium Glycinate TS*)—Dissolve 3.75 g of aminoacetic acid in about 500 mL of water, add 2.1 g of sodium hydroxide, and dilute with water to 1000 mL. Mix 9 mL of the resulting solution with 1 mL of dilute glacial acetic acid (1 in 300). This test solution has a pH between 10.4 and 10.5.

Sodium Bisulfite TS—Dissolve 10 g of sodium bisulfite in water to make 30 mL. Prepare this solution fresh.

Sodium Bitartrate TS—Dissolve 1 g of sodium bitartrate in water to make 10 mL. Prepare this solution fresh.

Sodium Carbonate TS—Dissolve 10.6 g of anhydrous sodium carbonate in water to make 100 mL.

Sodium Chloride TS, Alkaline—Dissolve 2 g of sodium hydroxide in 100 mL of water, saturate the solution with sodium chloride, and filter.

Sodium Citrate TS—Dissolve 73.5 g of sodium citrate dihydrate in water to make 250 mL.

Sodium Citrate TS, Alkaline—Dissolve 50 g of sodium citrate dihydrate and 2.5 g of sodium hydroxide in water to make 250 mL.

Sodium Cobaltinitrite TS—Dissolve 10 g of sodium cobaltinitrite in water to make 50 mL, and filter if necessary.

Sodium Fluoride TS—Dry about 500 mg of sodium fluoride at 200° for 4 hours. Accurately weigh 222 mg of the dried material, and dissolve in water to make 100.0 mL. Pipet 10 mL of this solution into a 1-L volumetric flask, and dilute with water to volume. Each mL of this solution corresponds to 0.01 mg of fluorine (F).

Sodium Hydrosulfite TS, Alkaline—Dissolve 25 g of potassium hydroxide in 35 mL of water, and 50 g of sodium hydrosulfite in 250 mL of water. When the test solution is required, mix 40 mL of the hydroxide solution with the 250 mL of the hydrosulfite solution. Prepare this solution fresh.

Sodium Hydroxide TS—Dissolve 4.0 g of sodium hydroxide in water to make 100 mL.

Sodium Hydroxide TS 2—Transfer 8.5 g of sodium hydroxide to a 100-mL volumetric flask, and dissolve in and dilute with water to volume.

Sodium Hydroxide TS 3—Prepare a 420-mg/mL solution of sodium hydroxide in water.

Sodium Hypobromite TS—To a solution of 20 g of sodium hydroxide in 75 mL of water add 5 mL of bromine. After solution has taken place, dilute with water to 100 mL. Prepare this solution fresh.

Sodium Hypochlorite TS—Use *Sodium Hypochlorite Solution* (see in the section *Reagent Specifications*).

Sodium Iodoxyhydroxyquinolinesulfonate TS—Dissolve 8.8 g of iodoxyhydroxyquinoline sulfonic acid in 200 mL of water, and add 6.5 mL of 4 N sodium hydroxide. Dilute with water to 250 mL, mix, and filter.

Sodium Nitroferricyanide TS—Dissolve 1 g of sodium nitroferricyanide in water to make 20 mL. Prepare this solution fresh.

Dibasic Sodium Phosphate TS—Dissolve 12 g of dibasic sodium phosphate in water to make 100 mL.

Sodium Phosphotungstate TS—To a solution of 20 g of sodium tungstate in 100 mL of water add sufficient phosphoric acid to impart a strongly acid reaction to litmus, and filter. When required for use, decant the clear solution from any sediment that may be present. Store in tight, light-resistant containers.

Sodium Sulfide TS—Dissolve 1 g of sodium sulfide in water to make 10 mL. Prepare this solution fresh.

Sodium Tartrate TS—Dissolve 11.5 g of sodium tartrate in water to make 100 mL.

Sodium Tetraphenylboron TS—Dissolve 1.2 g of sodium tetraphenylboron in water to make 200 mL. If necessary, stir for 5 minutes with 1 g of aluminum oxide, and filter to clarify.

Sodium Thioglycolate TS—Dissolve 1.5 g of sodium thioglycolate in 450 mL of water, and add 50 mL of alcohol. Use within 3 days.

Sodium Thiosulfate TS—Use 0.1 N *Sodium Thiosulfate* (see in the section *Volumetric Solutions*).

Standard Lead Solution—See under *Heavy Metals* (231).

Stannous Chloride, Acid, TS—Dissolve 8 g of stannous chloride in 500 mL of hydrochloric acid. Store in glass containers, and use within 3 months.

Stannous Chloride, Acid, Stronger, TS—Dissolve 40 g of stannous chloride in 100 mL of hydrochloric acid. Store in glass containers, and use within 3 months.

Starch TS—Mix 1 g of soluble starch with 10 mg of red mercuric iodide and sufficient cold water to make a thin paste. Add 200 mL of boiling water, and boil for 1 minute with continuous stirring. Cool, and use only the clear solution. [NOTE—Commercially available, stabilized starch indicator solutions may be used, including mercury-free solutions preserved with other compounds such as salicylic acid.]

Starch, Iodide-Free, TS—Mix 1 g of soluble starch with sufficient cold water to make a thin paste. While stirring, add 100 mL of boiling water, and allow to cool. Prepare this solution immediately before use. Iodide-free starch TS shows a blue color when 20 mL of potassium iodide solution (1 in 400) and 0.05 mL of an iodine–potassium iodide solution (prepared by dissolving 127 mg of iodine and 800 mg of potassium iodide in water and diluting with water to 100 mL) are added to 1 mL of the iodide-free starch TS.

Starch Iodide Paste TS—Heat 100 mL of water in a 250-mL beaker to boiling, add a solution of 0.75 g of potassium iodide in 5 mL of water, then add 2 g of zinc chloride dissolved in 10 mL of water, and, while the solution is boiling, add, with stirring, a smooth suspension of 5 g of soluble starch in 30 mL of cold water. Continue to boil for 2 minutes, then cool. Store in well-closed containers in a cold place.

Starch iodide paste TS must show a definite blue streak when a glass rod, dipped in a mixture of 1 mL of 0.1 M sodium nitrite, 500 mL of water, and 10 mL of hydrochloric acid, is streaked on a smear of the paste.

Starch–Potassium Iodide TS—Dissolve 500 mg of potassium iodide in 100 mL of freshly prepared starch TS. Prepare this solution fresh.

Stronger Cupric Acetate TS—See *Cupric Acetate TS, Stronger*.

Sudan III TS—Dissolve 0.05 g of Sudan III in 25 mL of alcohol, with warming if necessary. Cool, add 25 mL of glycerin, and mix. Filter if undissolved material persists.

Sudan IV TS—Dissolve 0.5 g of Sudan IV in chloroform to make 100 mL.

Sulfanilic Acid TS—Dissolve 800 mg of sulfanilic acid in 100 mL of acetic acid. Store in tight containers.

Diazotized Sulfanilic Acid TS—Dissolve 0.9 g of sulfanilic acid in 9 mL of hydrochloric acid with warming, and dilute with water to 100 mL. Cool 10 mL of this solution in iced water, and add 10 mL of a sodium nitrite solution (4.5 in 100) previously cooled in iced water. Allow to stand at 0° for at least 15 minutes (the solution may be kept for 3 days at this temperature). Immediately before use, add 20 mL of sodium carbonate solution (1 in 10).

Sulfanilic-1-Naphthylamine TS—Dissolve 500 mg of sulfanilic acid in 150 mL of acetic acid. Dissolve 100 mg of 1-naphthylamine hydrochloride in 150 mL of acetic acid, and mix the two solutions. The pink color that may develop on standing can be removed by treatment with zinc.

Sulfanilic- α -Naphthylamine TS—See *Sulfanilic-1-Naphthylamine TS*.

Sulfomolybdic Acid TS—Dissolve, with the aid of heat, 2.5 g of ammonium molybdate in 20 mL of water, add 50 mL of 12 N sulfuric acid, and dilute with water to 100 mL. Store this solution in a polyethylene container.

Sulfuric Acid TS—Add a quantity of sulfuric acid of known concentration to sufficient water to adjust the final concentration to between 94.5% and 95.5% (w/w) of H₂SO₄.

[NOTE—Since the acid concentration may change upon standing or upon intermittent use, the concentration should be checked frequently and solutions assaying more than 95.5% or less than 94.5% discarded.]

Sulfuric Acid-Formaldehyde TS—Add 1 drop of formaldehyde TS to each mL of sulfuric acid, and mix. Prepare this solution fresh.

Tannic Acid TS—Dissolve 1 g of tannic acid in 1 mL of alcohol, and dilute with water to 10 mL. Prepare this solution fresh.

Tartaric Acid TS—Dissolve 3 g of tartaric acid in water to make 100 mL. Prepare this solution fresh.

Tetrabromophenolphthalein Ethyl Ester TS—Dissolve 100 mg of tetrabromophenolphthalein ethyl ester in 90 mL of glacial acetic acid, and dilute with glacial acetic acid to 100 mL. Prepare this solution fresh.

Tetramethylammonium Hydroxide TS—Use an aqueous solution containing, in each 100 mL, the equivalent of 10 g of anhydrous tetramethylammonium hydroxide.

Thioacetamide TS—Dissolve 4 g of thioacetamide in 100 mL of water.

Thioacetamide-Glycerin Base TS—Mix 0.2 mL of thioacetamide TS and 1 mL of glycerin base TS, and heat in a boiling water bath for 20 seconds. Use the mixture immediately.

Thorium Nitrate TS—Dissolve 1 g of thorium nitrate in water to make 100 mL. Filter, if necessary.

Thymol Blue TS—Dissolve 100 mg of thymol blue in 100 mL of alcohol, and filter if necessary.

Thymolphthalein TS—Dissolve 100 mg of thymolphthalein in 100 mL of alcohol, and filter if necessary.

Titanium Trichloride TS—Dissolve 15 g of titanium trichloride in 100 mL of 10% hydrochloric acid solution.

Titanium Trichloride-Sulfuric Acid TS—Mix carefully 20 mL of titanium trichloride TS in 13 mL of sulfuric acid. Add sufficient 30% hydrogen peroxide to produce a yellow color. Heat until white fumes are evolved, allow to cool, and dilute with water. Repeat the evaporation and addition of water until a colorless solution is obtained. Dilute with water to 100 mL.

p-Toluenesulfonic Acid TS—Dissolve 2 g of *p*-toluenesulfonic acid in 10 mL of a mixture of 7 parts of acetone and 3 parts of water.

Triketohydrindene Hydrate TS (Ninhydrin TS)—Dissolve 200 mg of triketohydrindene hydrate in water to make 10 mL. Prepare this solution fresh.

Trinitrophenol TS (Picric Acid TS)—Dissolve the equivalent of 1 g of anhydrous trinitrophenol in 100 mL of hot water. Cool the solution, and filter if necessary.

Triphenyltetrazolium Chloride TS—Dissolve 500 mg of triphenyltetrazolium chloride in dehydrated alcohol to make 100 mL.

Xylenol Orange TS—Dissolve 100 mg of xylenol orange in 100 mL of alcohol.

Zinc Uranyl Acetate TS—Dissolve 50 g of uranyl acetate in a mixture of 15 mL of glacial acetic acid and water to make 500 mL. Then dissolve 150 g of zinc acetate in a mixture of 15 mL of glacial acetic acid and water to make 500 mL. Mix the two solutions, allow to stand overnight, and pass through a dry filter, if necessary.

VOLUMETRIC SOLUTIONS

Normal Solutions—Normal solutions are solutions that contain 1 gram equivalent weight of the active substance in each 1000 mL of solution; that is, an amount equivalent to 1.0079 g of hydrogen or 7.9997 g of oxygen. Normal solutions and solutions bearing a specific relationship to normal solutions, and used in volumetric determinations, are designated as follows: normal, 1 N; double-normal, 2 N; half-normal, 0.5 N; tenth-normal, 0.1 N; fiftieth-normal, 0.02 N; hundredth-normal, 0.01 N; thousandth-normal, 0.001 N.

Molar Solutions—Molar solutions are solutions that contain, in 1000 mL, 1 gram-molecule of the reagent. Thus, each liter of a molar solution of sulfuric acid contains 98.07 g of H₂SO₄ and each liter of a molar solution of potassium ferricyanide contains 329.25 g of K₃Fe(CN)₆. Solutions containing, in 1000 mL, one-tenth of a gram-molecule of the reagent are designated "tenth-molar," 0.1 M; and other molarities are similarly indicated.

Empirical Solutions—It is frequently difficult to prepare standard solutions of a desired theoretical normality, and this is not essential. A solution of approximately the desired normality is prepared and standardized by titration against a primary standard solution. The normality factor so obtained is used in all calculations where such empirical solutions are employed. If desired, an empirically prepared solution may be adjusted downward to a given normality provided it is strong enough to permit dilution.

All volumetric solutions, whether made by direct solution or by dilution of a stronger solution, must be thoroughly mixed by shaking before standardization. As the strength of a standard solution may change upon standing, the factor should be redetermined frequently.

When solutions of a reagent are used in several normalities, the details of the preparation and standardization are usually given for the normality most frequently required. Stronger or weaker solutions are prepared and standardized in the same general manner as described, using proportionate amounts of the reagent. It is possible in many instances to prepare lower normalities accurately by making an exact dilution of a stronger solution. Volumetric solutions prepared by dilution should be restandardized either as directed for the stronger solution or by comparison with another volumetric solution having a known ratio to the stronger solution.

Dilute solutions that are not stable, as, for instance, potassium permanganate 0.01 N and more dilute sodium thiosulfate, are preferably prepared by exactly diluting the higher normality with thoroughly boiled and cooled water on the same day they are required for use.

Blank Determinations—Where it is directed that "any necessary correction" be made by a blank determination, the determination is to be conducted with the use of the same quantities of the same reagents treated in the same manner as the solution or mixture containing the portion of the substance under assay or test, but with the substance itself omitted. Appropriate blank corrections are to be made for all Pharmacopeial titrimetric assays (see *Titrimetry* (541)).

All Pharmacopeial assays that are volumetric in nature indicate the weight of the substance being assayed to which each mL of the primary volumetric solution is equivalent. In general, these equivalents may be derived by simple calculation.

tion from the data given under *Molecular Formulas and Weights*, in the *Reference Tables*.

Preparation and Methods of Standardization of Volumetric Solutions

The following directions give only one method for standardization, but other methods of standardization, capable of yielding at least the same degree of accuracy, may be used. The values obtained in the standardization of volumetric solutions are valid for all Pharmacopeial uses of these solutions, regardless of the instrumental or chemical indicators employed in the individual monographs. Where the apparent normality or molarity of a titrant depends upon the special conditions of its use, the individual monograph sets forth the directions for standardizing the reagent in the specified context. For those salts that usually are available as certified primary standards, or that are available as highly purified salts of primary standard quality, it is permissible to prepare solutions by accurately weighing a suitable quantity of the salt and dissolving it to produce a specific volume of solution of known concentration. Acetic, hydrochloric, and sulfuric acids may be standardized against a sodium hydroxide solution that recently has been standardized against a certified primary standard.

All volumetric solutions, if practicable, are to be prepared, standardized, and used at the standard temperature of 25°. If a titration is carried out with the volumetric solution at a markedly different temperature, standardize the volumetric solution used as the titrant at that different temperature, or make a suitable temperature correction.

Acetic Acid, Double-Normal (2 N)

$\text{C}_2\text{H}_4\text{O}_2$, **60.05**

120.10 g in 1000 mL

Add 116 mL of glacial acetic acid to sufficient water to make 1000 mL after cooling to room temperature.

Ammonium Thiocyanate, Tenth-Normal (0.1 N)

NH_4SCN , **76.12**

7.612 g in 1000 mL

Dissolve about 8 g of ammonium thiocyanate in 1000 mL of water, and standardize the solution as follows.

Accurately measure about 30 mL of 0.1 N silver nitrate VS into a glass-stoppered flask. Dilute with 50 mL of water, then add 2 mL of nitric acid and 2 mL of ferric ammonium sulfate TS, and titrate with the ammonium thiocyanate solution to the first appearance of a red-brown color.

$$N = \frac{\text{mL AgNO}_3 \times N \text{ AgNO}_3}{\text{mL NH}_4\text{SCN Solution}}$$

If desirable, 0.1 N ammonium thiocyanate may be replaced by 0.1 N potassium thiocyanate where the former is directed in various tests and assays.

Bismuth Nitrate, 0.01 M

$\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$, **485.07**

1000 mL of this solution contains 4.851 g of bismuth nitrate pentahydrate

Dissolve 4.86 g of bismuth nitrate pentahydrate in 60 mL of dilute nitric acid, add 0.01 N nitric acid to make 1000 mL, and standardize the solution as follows.

Accurately measure 25 mL of the prepared bismuth nitrate solution, add 50 mL of water and 1 drop of xylenol orange TS, and titrate the solution with 0.01 M edetate disodium VS until the red color changes to yellow. Calculate the molarity factor.

Bromine, Tenth-Normal (0.1 N)

Br, **79.90**

7.990 g in 1000 mL

Dissolve 3 g of potassium bromate and 15 g of potassium bromide in water to make 1000 mL, and standardize the solution as follows.

Accurately measure about 25 mL of the solution into a 500-mL iodine flask, and dilute with 120 mL of water. Add 5 mL of hydrochloric acid, insert the stopper in the flask, and shake it gently. Then add 5 mL of potassium iodide TS, again insert the stopper, shake the mixture, allow it to stand for 5 minutes, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached.

Preserve in dark amber-colored, glass-stoppered bottles.

$$N = \frac{\text{mL Na}_2\text{S}_2\text{O}_3 \times N \text{ Na}_2\text{S}_2\text{O}_3}{\text{mL Br}_2 \text{ Solution}}$$

Ceric Ammonium Nitrate, Twentieth-Normal (0.05 N)

$\text{Ce}(\text{NO}_3)_4 \cdot 2\text{NH}_4\text{NO}_3$, **548.22**

2.741 g in 100 mL

Dissolve 2.75 g of ceric ammonium nitrate in 1 N nitric acid to obtain 100 mL of solution, and filter. Standardize the solution as follows.

Accurately measure 10 mL of freshly standardized 0.1 N ferrous ammonium sulfate VS into a flask, and dilute with water to about 100 mL. Add 1 drop of nitrophenanthroline TS, and titrate with the ceric ammonium nitrate solution to a colorless endpoint.

$$N = \frac{\text{mL Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \times N \text{ Fe}(\text{NH}_4)_2(\text{SO}_4)_2}{\text{mL Ce}(\text{NO}_3)_4 \cdot 2\text{NH}_4\text{NO}_3}$$

Ceric Sulfate, Tenth-Normal (0.1 N)

$\text{Ce}(\text{SO}_4)_2$, **332.24**

33.22 g in 1000 mL

Use commercially available volumetric standard solution. Standardize the solution as follows.

Accurately weigh about 0.2 g of sodium oxalate, primary standard, dried according to the instructions on its label, and dissolve in 75 mL of water. Add, with stirring, 2 mL of sulfuric acid that has previously been mixed with 5 mL of water, mix well, add 10 mL of hydrochloric acid, and heat to between 70° and 75°. Titrate with 0.1 N ceric sulfate to a permanent slight yellow color. Each 6.700 mg of sodium oxalate is equivalent to 1 mL of 0.1 N ceric sulfate.

$$N = \frac{\text{mg Na}_2\text{C}_2\text{O}_4}{67.00 \times \text{mL Ce}(\text{SO}_4)_2 \text{ solution}}$$

Cupric Nitrate, Tenth-Normal (0.1 N)

$\text{Cu}(\text{NO}_3)_2 \cdot 2.5\text{H}_2\text{O}$, **232.59**

23.26 g in 1000 mL

$\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$, **241.60**

24.16 g in 1000 mL

Dissolve 23.3 g of cupric nitrate 2.5 hydrate, or 24.2 g of the trihydrate, in water to make 1000 mL. Standardize the solution as follows.

Transfer 20.0 mL of the solution to a 250-mL beaker. Add 2 mL of 5 M sodium nitrate, 20 mL of ammonium acetate TS, and sufficient water to make 100 mL. Titrate with 0.05 M edetate disodium VS. Determine the endpoint potentiometrically using a cupric ion-double junction reference

electrode system. Perform a blank determination, and make any necessary correction.

$$N = \frac{\text{mL edetate disodium (corrected for the blank)} \times M \text{ edetate disodium}}{20.0}$$

Standard Dichlorophenol–Indophenol Solution

To 50 mg of 2,6-dichlorophenol–indophenol sodium that has been stored in a desiccator over soda lime add 50 mL of water containing 42 mg of sodium bicarbonate, shake vigorously, and when the dye is dissolved, add water to make 200 mL. Filter into an amber, glass-stoppered bottle. Use within 3 days and standardize immediately before use. Standardize the solution as follows.

Accurately weigh 50 mg of USP Ascorbic Acid RS, and transfer to a glass-stoppered, 50-mL volumetric flask with the aid of a sufficient volume of metaphosphoric–acetic acids TS to make 50 mL. Immediately transfer 2 mL of the ascorbic acid solution to a 50-mL conical flask containing 5 mL of the metaphosphoric–acetic acids TS, and titrate rapidly with the dichlorophenol–indophenol solution until a distinct rose-pink color persists for at least 5 seconds. Perform a blank titration by titrating 7 mL of the metaphosphoric–acetic acids TS plus a volume of water equal to the volume of the dichlorophenol solution used in titrating the ascorbic acid solution. Express the concentration of the standard solution in terms of its equivalent in mg of ascorbic acid.

Edetate Disodium, Twentieth-Molar (0.05 M)

$\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_8 \cdot 2\text{H}_2\text{O}$, 372.24
18.61 g in 1000 mL

Dissolve 18.6 g of edetate disodium in water to make 1000 mL, and standardize the solution as follows.

Accurately weigh about 200 mg of chelometric standard calcium carbonate, previously dried at 110° for 2 hours and cooled in a desiccator, or dried according to the label instructions, transfer to a 400-mL beaker, add 10 mL of water, and swirl to form a slurry. Cover the beaker with a watch glass, and introduce 2 mL of diluted hydrochloric acid from a pipet inserted between the lip of the beaker and the edge of the watch glass. Swirl the contents of the beaker to dissolve the calcium carbonate. Wash down the sides of the beaker, the outer surface of the pipet, and the watch glass with water, and dilute with water to about 100 mL. While stirring the solution, preferably with a magnetic stirrer, add about 30 mL of the edetate disodium solution from a 50-mL buret. Add 15 mL of sodium hydroxide TS and 300 mg of hydroxy naphthol blue, and continue the titration with the edetate disodium solution to a blue endpoint.

$$M = \frac{(\text{g CaCO}_3)(1000)}{100.09 \times \text{mL EDTA}}$$

Ferric Ammonium Sulfate, Tenth-Normal (0.1 N)

$\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 482.19
48.22 g in 1000 mL

Dissolve 50 g of ferric ammonium sulfate in a mixture of 300 mL of water and 6 mL of sulfuric acid, dilute with water to 1000 mL, and mix. Standardize the solution as follows.

Accurately measure about 40 mL of the solution into a glass-stoppered flask, add 5 mL of hydrochloric acid, mix, and add a solution of 3 g of potassium iodide in 10 mL of water. Insert the stopper, allow to stand for 10 minutes, then titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. Correct for a blank run on the same quantities of the same reagents.

Store in tight containers, protected from light.

$$N = \frac{\text{mL Na}_2\text{S}_2\text{O}_3 \times N \text{ Na}_2\text{S}_2\text{O}_3}{\text{mL FeNH}_4(\text{SO}_4)_2}$$

Ferrous Ammonium Sulfate, Tenth-Normal (0.1 N)

$\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 392.14
39.21 g in 1000 mL

Dissolve 40 g of ferrous ammonium sulfate in a previously cooled mixture of 40 mL of sulfuric acid and 200 mL of water, dilute with water to 1000 mL, and mix. On the day of use, standardize the solution as follows.

Accurately measure 25 to 30 mL of the solution into a flask, add 2 drops of orthophenanthroline TS, and titrate with 0.1 N ceric sulfate VS until the red color is changed to pale blue.

$$N = \frac{\text{mL Ce}^{\text{IV}} \times N \text{ Ce}^{\text{IV}}}{\text{mL Fe}^{\text{II}} \text{ Solution}}$$

Hydrochloric Acid, Alcoholic, Tenth-Molar (0.1 M)

HCl, 36.46

Dilute 9.0 mL of hydrochloric acid to 1000 mL with aldehyde-free alcohol.

Hydrochloric Acid, Half-Normal (0.5 N)

HCl, 36.46

18.23 g in 1000 mL

To a 1000-mL volumetric flask containing 40 mL of water slowly add 43 mL of hydrochloric acid. Cool, and add water to volume. Standardize the solution as follows.

Accurately weigh about 2.5 g of tromethamine, dried according to the label instructions or, if this information is not available, dried at 105° for 3 h. Dissolve in 50 mL of water, and add 2 drops of bromocresol green TS. Titrate with 0.5 N hydrochloric acid to a pale yellow endpoint. Each 60.57 mg of tromethamine is equivalent to 1 mL of 0.5 N hydrochloric acid.

$$N = \frac{\text{mg tromethamine}}{121.14 \times \text{mL HCl}}$$

Hydrochloric Acid, Half-Normal (0.5 N) in Methanol

HCl, 36.46

18.23 g in 1000 mL

To a 1000-mL volumetric flask containing 40 mL of water slowly add 43 mL of hydrochloric acid. Cool, and add methanol to volume. Standardize the solution as follows.

Accurately weigh about 2.5 g of tromethamine, dried according to the label instructions or, if this information is not available, dried at 105° for 3 h. Proceed as directed under *Hydrochloric Acid, Normal (1 N)*, beginning with "Dissolve in 50 mL of water."

$$N = \frac{\text{mg tromethamine}}{121.14 \times \text{mL HCl}}$$

Hydrochloric Acid, Normal (1 N)

HCl, 36.46

36.46 g in 1000 mL

Dilute 85 mL of hydrochloric acid with water to 1000 mL. Standardize the solution as follows.

Accurately weigh about 5.0 g of tromethamine, dried according to the label instructions or, if this information is not available, dried at 105° for 3 h. Dissolve in 50 mL of water, and add 2 drops of bromocresol green TS. Titrate with 1 N

hydrochloric acid to a pale yellow endpoint. Each 121.14 mg of tromethamine is equivalent to 1 mL of 1 N hydrochloric acid.

$$N = \frac{\text{mg tromethamine}}{121.14 \times \text{mL HCl}}$$

Iodine, Tenth-Normal (0.1 N)

I, 126.90

12.69 g in 1000 mL

Dissolve about 14 g of iodine in a solution of 36 g of potassium iodide in 100 mL of water, add 3 drops of hydrochloric acid, dilute with water to 1000 mL, and standardize the solution as follows.

Transfer 25.0 mL of the iodine solution to a 250-mL flask, dilute with water to 100 mL, add 1 mL of 1 N hydrochloric acid, swirl gently to mix, and titrate with 0.1 N sodium thiosulfate VS until the solution has a pale yellow color. Add 2 mL of starch TS and continue titrating until the solution is colorless.

Preserve in amber-colored, glass-stoppered bottles.

$$N = \frac{\text{mL Na}_2\text{S}_2\text{O}_3 \times N \text{ Na}_2\text{S}_2\text{O}_3}{25}$$

Iodine, Twentieth-Normal (0.05 N)

I, 126.90

6.33 g in 1000 mL

Dissolve about 6.5 g of iodine in a solution of 18 g of potassium iodide in 100 mL of water, add 3 drops of hydrochloric acid, dilute with water to 1000 mL, and standardize the solution as follows.

Transfer 50.0 mL of the iodine solution to a 250-mL flask, dilute with water to 100 mL, add 1 mL of 1 N hydrochloric acid, swirl gently to mix, and titrate with 0.1 N sodium thiosulfate VS until the solution has a pale yellow color. Add 2 mL of starch TS, and continue titrating until the solution is colorless.

$$N = \frac{\text{mL Na}_2\text{S}_2\text{O}_3 \times N \text{ Na}_2\text{S}_2\text{O}_3}{50}$$

Iodine, Hundredth-Normal (0.01 N)

I, 126.90

1.269 g in 1000 mL

Dissolve about 1.4 g of iodine in a solution of 3.6 g of potassium iodide in 100 mL of water, add 3 drops of hydrochloric acid, dilute with water to 1000 mL, and standardize the solution as follows.

Transfer 100.0 mL of iodine solution to a 250-mL flask, add 1 mL of 1 N hydrochloric acid, swirl gently to mix, and titrate with 0.1 N sodium thiosulfate VS until the solution has a pale yellow color. Add 2 mL of starch TS, and continue titrating until the solution is colorless.

Preserve in amber-colored, glass-stoppered bottles.

$$N = \frac{\text{mL Na}_2\text{S}_2\text{O}_3 \times N \text{ Na}_2\text{S}_2\text{O}_3}{100}$$

Lead Nitrate, Hundredth-Molar (0.01 M)

Pb(NO₃)₂, 331.21

3.312 g in 1000 mL

Xylenol Orange Triturate—Triturate 1 part of xylenol orange with 99 parts of potassium nitrate.

0.1 M Lead Nitrate—Dissolve 33 g of lead nitrate in 1000 mL of water. Standardize the solution as follows. To 20.0 mL of the lead nitrate solution add 300 mL of water. Add about 50 mg of *Xylenol Orange Triturate*, and add methenamine until the solution becomes violet-pink. Titrate with 0.1 M edetate disodium VS to the yellow endpoint. Calculate the molarity.

Dilute 50.0 mL of 0.1 M Lead Nitrate to 500.0 mL with water.

Lead Perchlorate, Tenth-Molar (0.1 M)

Pb(ClO₄)₂ · 3H₂O, 460.15

46.01 g in 1000 mL

Dissolve 46 g of lead perchlorate in water, and dilute with water to 1000.0 mL. Accurately weigh about 150 mg of sodium sulfate, previously dried at 105° for 4 hours, and dissolve in 50 mL of water. Add 50 mL of a mixture of water and formaldehyde (1:1), and stir for about 1 minute. Determine the endpoint potentiometrically using a lead ion selective electrode. Perform a blank determination, and make any necessary corrections. Each 14.204 mg of sodium sulfate is equivalent to 1 mL of 0.1 M lead perchlorate.

$$M = \frac{\text{mg sodium sulfate}}{142.04 \times \text{mL lead perchlorate}}$$

Lead Perchlorate, Hundredth-Molar (0.01 M)

Pb(ClO₄)₂, 406.10

Accurately pipet 100 mL of commercially available 0.1 M lead perchlorate solution into a 1000-mL volumetric flask, add a sufficient quantity of water to make 1000 mL, and standardize the solution as follows.

Accurately pipet 50 mL of 0.01 M lead perchlorate solution, as prepared above, into a 250-mL conical flask. Add 3 mL of aqueous hexamethylenetetramine solution (2.0 g per 100 mL) and 4 drops of 0.5% xylenol orange indicator prepared by adding 500 mg of xylenol orange to 10 mL of alcohol and diluting with water to 100 mL. (Omit the alcohol if the sodium salt of the indicator is used). Titrate with 0.05 M edetate disodium VS to a yellow endpoint.

$$M = \frac{\text{mL edetate disodium} \times M \text{ edetate disodium}}{50.0}$$

Lithium Methoxide, Fiftieth-Normal (0.02 N) in Methanol

CH₃LiO, 37.97

759.6 mg in 1000 mL

Dissolve 0.12 g of freshly cut lithium metal in 150 mL of methanol, cooling the flask during addition of the metal. When the reaction is complete, add 850 mL of methanol, and mix. Store the solution preferably in the reservoir of an automatic delivery buret suitably protected from carbon dioxide and moisture. Standardize the solution by titration against benzoic acid as described under *Sodium Methoxide, Tenth-Normal (0.1 N) (in Toluene)*, but use only 100 mg of benzoic acid. Each 2.442 mg of benzoic acid is equivalent to 1 mL of 0.02 N lithium methoxide.

[NOTE—Restandardize the solution frequently.]

$$N = \frac{\text{mg benzoic acid}}{121.1 \times \text{mL lithium methoxide (corrected for the blank)}}$$

Lithium Methoxide, Tenth-Normal (0.1 N) in Chlorobenzene

CH₃OLi, 37.97

3.798 g in 1000 mL

Dissolve 700 mg of freshly cut lithium metal in 150 mL of methanol, cooling the flask during addition of the metal. When the reaction is complete, add 850 mL of chlorobenzene. If cloudiness or precipitation occurs, add sufficient methanol to clarify the solution. Store preferably in the reservoir of an automatic delivery buret suitably protected from carbon dioxide and moisture. Standardize the solution by titration against benzoic acid as described under *Sodium Methoxide, Tenth-Normal (0.1 N) (in Toluene)*.

[NOTE—Restandardize the solution frequently.]

$$N = \frac{\text{mg benzoic acid}}{121.1 \times \text{mL lithium methoxide (corrected for the blank)}}$$

Lithium Methoxide, Tenth-Normal (0.1 N) in Methanol

CH_3OLi , 37.97

3.798 g in 1000 mL

Dissolve 700 mg of freshly cut lithium metal in 150 mL of methanol, cooling the flask during addition of the metal. When the reaction is complete, add 850 mL of methanol. If cloudiness or precipitation occurs, add sufficient methanol to clarify the solution. Store preferably in the reservoir of an automatic delivery buret suitably protected from carbon dioxide and moisture. Standardize the solution by titration against benzoic acid as described under *Sodium Methoxide, Tenth-Normal (0.1 N) (in Toluene)*.

[NOTE—Restandardize the solution frequently.]

$$N = \frac{\text{mg benzoic acid}}{121.1 \times \text{mL lithium methoxide (corrected for the blank)}}$$

Lithium Methoxide, Tenth-Normal (0.1 N) in Toluene

CH_3OLi , 37.97

3.798 g in 1000 mL

Dissolve 700 mg of freshly cut lithium metal in 150 mL of methanol, cooling the flask during addition of the metal. When reaction is complete, add 850 mL of toluene. If cloudiness or precipitation occurs, add sufficient methanol to clarify the solution. Store preferably in the reservoir of an automatic delivery buret suitably protected from carbon dioxide and moisture. Standardize the solution by titration against benzoic acid as described under *Sodium Methoxide, Tenth-Normal (0.1 N) (in Toluene)*.

[NOTE—Restandardize the solution frequently.]

$$N = \frac{\text{mg benzoic acid}}{121.1 \times \text{mL lithium methoxide (corrected for the blank)}}$$

Magnesium Chloride, 0.01 M

$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 203.30

2.0330 g in 1000 mL

Dissolve about 2.04 g of magnesium chloride in 1000 mL of freshly boiled and cooled water, and standardize the solution as follows.

Accurately measure 25 mL of the prepared magnesium chloride solution. Add 50 mL of water, 3 mL of ammonia-ammonium chloride buffer TS and 0.04 g of eriochrome black T-sodium chloride reagent. Titrate with 0.05 M edetate disodium VS until the red-purple color of the solution changes to blue-purple.

$$M = \frac{(\text{mL edetate disodium VS}) \times (M \text{ edetate disodium})}{\text{mL magnesium chloride}}$$

Mercuric Nitrate, Tenth-Molar (0.1 M)

$\text{Hg}(\text{NO}_3)_2$, 324.60

32.46 g in 1000 mL

Dissolve about 35 g of mercuric nitrate in a mixture of 5 mL of nitric acid and 500 mL of water, and dilute with water to 1000 mL. Standardize the solution as follows.

Transfer an accurately measured volume of about 20 mL of the solution to a conical flask, and add 2 mL of nitric acid and 2 mL of ferric ammonium sulfate TS. Cool to below 20°, and titrate with 0.1 N ammonium thiocyanate VS to the first appearance of a permanent brownish color.

$$M = \frac{\text{mL NH}_4\text{SCN} \times N \text{ NH}_4\text{SCN}}{\text{mL Hg}(\text{NO}_3)_2 \times 2}$$

Oxalic Acid, Tenth-Normal (0.1 N)

$\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$, 126.07

6.303 g in 1000 mL

Dissolve 6.45 g of oxalic acid in water to make 1000 mL. Standardize by titration against freshly standardized 0.1 N potassium permanganate VS as directed under *Potassium Permanganate, Tenth-Normal (0.1 N)*.

Preserve in glass-stoppered bottles, protected from light.

$$N = \frac{\text{mL KMnO}_4 \times N \text{ KMnO}_4}{\text{mL H}_2\text{C}_2\text{O}_4}$$

Perchloric Acid, Tenth-Normal (0.1 N) in Dioxane

Mix 8.5 mL of perchloric acid with sufficient dioxane to make 1000 mL. Standardize the solution as follows.

Accurately weigh about 700 mg of potassium biphthalate, previously crushed lightly and dried at 120° for 2 hours, and dissolve in 50 mL of glacial acetic acid in a 250-mL flask. Add 2 drops of crystal violet TS, and titrate with the perchloric acid solution until the violet color changes to bluish green. Carry out a blank determination. Each 20.423 mg of potassium biphthalate is equivalent to 1 mL of 0.1 N perchloric acid.

$$N = \frac{\text{g KHC}_8\text{H}_4\text{O}_4}{0.20423 \times \text{mL HClO}_4 \text{ solution (corrected for the blank)}}$$

Perchloric Acid, Tenth-Normal (0.1 N) in Glacial Acetic Acid

HClO_4 , 100.46

10.05 g in 1000 mL

[NOTE—Where called for in the tests and assays, this volumetric solution is specified as "0.1 N perchloric acid." Thus, where 0.1 N or other strength of this volumetric solution is specified, the solution in glacial acetic acid is to be used, unless the words "in dioxane" are stated. [See also *Perchloric Acid, Tenth-Normal (0.1 N) in Dioxane*.]]

Mix 8.5 mL of perchloric acid with 500 mL of glacial acetic acid and 21 mL of acetic anhydride, cool, and add glacial acetic acid to make 1000 mL. Alternatively, the solution may be prepared as follows. Mix 11 mL of 60 percent perchloric acid with 500 mL of glacial acetic acid and 30 mL of acetic anhydride, cool, and add glacial acetic acid to make 1000 mL.

Allow the prepared solution to stand for 1 day for the excess acetic anhydride to be combined, and determine the water content by *Method I* (see *Water Determination* (921)), except to use a test specimen of about 5 g of the 0.1 N perchloric acid that is expected to contain approximately 1 mg of water and the *Reagent* (see *Reagent* under *Method Ia* in *Water Determination* (921)) diluted such that 1 mL is equivalent to about 1 to 2 mg of water. If the water content exceeds 0.5%, add more acetic anhydride. If the solution contains no titratable water, add sufficient water to obtain a content of between 0.02% and 0.5% of water. Allow the solution to stand for 1 day, and again titrate the water con-

tent. The solution so obtained contains between 0.02% and 0.5% of water, indicating freedom from acetic anhydride.

Standardize the solution as follows.

Accurately weigh about 700 mg of potassium biphthalate, previously crushed lightly and dried at 120° for 2 hours, and dissolve it in 50 mL of glacial acetic acid in a 250-mL flask. Add 2 drops of crystal violet TS, and titrate with the perchloric acid solution until the violet color changes to blue-green. Deduct the volume of the perchloric acid consumed by 50 mL of the glacial acetic acid. Each 20.423 mg of potassium biphthalate is equivalent to 1 mL of 0.1 N perchloric acid.

$$N = \frac{\text{g KHC}_8\text{H}_4\text{O}_4}{0.20423 \times \text{mL HClO}_4 \text{ solution (corrected for the blank)}}$$

Potassium Arsenite, Tenth-Normal (0.1 N)

KAsO₂, 146.02

7.301 g in 1000 mL

Dissolve 4.9455 g of arsenic trioxide primary standard, previously dried at 105° for 1 hour, in 75 mL of 1 N potassium hydroxide. Add 40 g of potassium bicarbonate, dissolved in about 200 mL of water, and dilute with water to 1000.0 mL.

Potassium Bromate, Tenth-Normal (0.1 N)

KBrO₃, 167.00

2.784 g in 1000 mL

Dissolve 2.784 g of potassium bromate in water to make 1000 mL, and standardize the solution as follows.

Transfer an accurately measured volume of about 40 mL of the solution to a glass-stoppered flask, add 3 g of potassium iodide, and follow with 3 mL of hydrochloric acid. Allow to stand for 5 minutes, then titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. Correct for a blank run on the same quantities of the same reagents, and calculate the normality.

$$N = \frac{\text{mL Na}_2\text{S}_2\text{O}_3 \times N \text{ Na}_2\text{S}_2\text{O}_3}{\text{mL KBrO}_3 \text{ Solution}}$$

Potassium Bromide-Bromate, Tenth-Normal (0.1 N)

Dissolve 2.78 g of potassium bromate (KBrO₃) and 12.0 g of potassium bromide (KBr) in water, and dilute with water to 1000 mL. Standardize by the procedure set forth for *Potassium Bromate, Tenth-Normal (0.1 N)*.

$$N = \frac{\text{mL Na}_2\text{S}_2\text{O}_3 \times N \text{ Na}_2\text{S}_2\text{O}_3}{\text{mL KBrO}_3/\text{KBr}}$$

Potassium Dichromate, Tenth-Normal (0.1 N)

K₂Cr₂O₇, 294.18

4.903 g in 1000 mL

Dissolve about 5 g of potassium dichromate in 1000 mL of water. Standardize the solution as follows.

Transfer 25.0 mL of this solution to a glass-stoppered, 500-mL flask, add 2 g of potassium iodide (free from iodate), dilute with 200 mL of water, add 5 mL of hydrochloric acid, allow to stand for 10 minutes in a dark place, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. Carry out a blank determination.

$$N = \frac{\text{mL Na}_2\text{S}_2\text{O}_3 \times N \text{ Na}_2\text{S}_2\text{O}_3}{25.0}$$

Potassium Ferricyanide, Twentieth-Molar (0.05 M)

K₃Fe(CN)₆, 329.24

16.46 g in 1000 mL

Dissolve about 17 g of potassium ferricyanide in water to make 1000 mL. Standardize the solution as follows.

Transfer 50.0 mL of this solution to a glass-stoppered, 500-mL flask, dilute with 50 mL of water, add 10 mL of potassium iodide TS and 10 mL of dilute hydrochloric acid, and allow to stand for 1 minute. Then add 15 mL of zinc sulfate solution (1 in 10), and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached.

Protect from light, and restandardize before use.

$$M = \frac{\text{mL Na}_2\text{S}_2\text{O}_3 \times N \text{ Na}_2\text{S}_2\text{O}_3}{50.0}$$

Potassium Hydroxide, Alcoholic, Half-Normal (0.5 N)

28.06 g in 1000 mL

Dissolve about 34 g of potassium hydroxide in 20 mL of water, and add aldehyde-free alcohol to make 1000 mL. Allow the solution to stand in a tightly stoppered bottle for 24 hours. Then quickly decant the clear supernatant into a suitable, tight container, and standardize the solution as follows.

Accurately measure about 25 mL of 0.5 N hydrochloric acid VS. Dilute with 50 mL of water, add 2 drops of phenolphthalein TS, and titrate with the alcoholic potassium hydroxide solution until a permanent, pale pink color is produced.

[NOTE—Store in tightly stoppered bottles, protected from light.]

$$N = \frac{\text{mL HCl} \times N \text{ HCl}}{\text{mL KOH}}$$

Potassium Hydroxide, Alcoholic, Tenth-Molar (0.1 M)

KOH, 56.11

Dilute 20 mL of 0.5 M alcoholic potassium hydroxide to 100.0 mL with aldehyde-free alcohol.

Potassium Hydroxide, Methanolic, Tenth-Normal

(0.1 N)

5.612 g in 1000 mL

Dissolve about 6.8 g of potassium hydroxide in 4 mL of water, and add methanol to make 1000 mL. Allow the solution to stand in a tightly stoppered bottle for 24 hours. Then quickly decant the clear supernatant into a suitable, tight container, and standardize the solution as follows.

Accurately measure about 25 mL of 0.1 N hydrochloric acid VS. Dilute with 50 mL of water, add 2 drops of phenolphthalein TS, and titrate with the methanolic potassium hydroxide solution until a permanent, pale pink color is produced.

[NOTE—Store in tightly stoppered bottles, protected from light.]

$$N = \frac{\text{mL HCl} \times N \text{ HCl}}{\text{mL KOH}}$$

Potassium Hydroxide, Normal (1 N)

KOH, 56.11

56.11 g in 1000 mL

Dissolve 68 g of potassium hydroxide in about 950 mL of water. Add a freshly prepared saturated solution of barium hydroxide until no more precipitate forms. Shake the mixture thoroughly, and allow it to stand overnight in a stoppered bottle. Decant the clear liquid, or filter the solution in

a tight, polyolefin bottle, and standardize by the procedure set forth for *Sodium Hydroxide, Normal (1 N)*.

$$N = \frac{g \text{ KHC}_8\text{H}_4\text{O}_4}{0.20423 \times \text{mL KOH}}$$

Potassium Iodate, Twentieth-Molar (0.05 M)

KIO₃, 214.00

10.70 g in 1000 mL

Dissolve 10.700 g of potassium iodate, previously dried at 110° to constant weight, in water to make 1000.0 mL. Standardize the solution as follows: to 15.0 mL of solution in a 250-mL iodine flask, add 3 g of potassium iodide and 3 mL of hydrochloric acid previously diluted with 10 mL of water. Stopper immediately, and allow to stand in the dark for 5 minutes. Then add 50 mL of cold water, and titrate the liberated iodine with freshly standardized 0.1 N sodium thiosulfate. Add 3 mL of starch indicator solution near the end of the titration, and continue to the absence of the blue-starch-iodine complex.

$$M = \frac{\text{mL} \times N \text{ Na}_2\text{S}_2\text{O}_3}{\text{mL KIO}_3 \times 6}$$

Potassium Permanganate, Tenth-Normal (0.1 N)

KMnO₄, 158.03

3.161 g in 1000 mL

Dissolve about 3.3 g of potassium permanganate in 1000 mL of water in a flask, and boil the solution for about 15 minutes. Insert the stopper in the flask, allow it to stand for at least 2 days, and filter through a fine-porosity, sintered-glass crucible. If necessary, the bottom of the sintered-glass crucible may be lined with a pledget of glass wool. Standardize the solution as follows.

Accurately weigh about 200 mg of sodium oxalate, dried according to the instructions on its label, and dissolve it in 250 mL of water. Add 7 mL of sulfuric acid, heat to about 70°, and then slowly add the permanganate solution from a buret, with constant stirring, until a pale pink color, which persists for 15 seconds, is produced. The temperature at the conclusion of the titration should be not less than 60°. Calculate the normality. Each 6.700 mg of sodium oxalate is equivalent to 1 mL of 0.1 N potassium permanganate.

Since potassium permanganate is reduced on contact with organic substances such as rubber, the solution must be handled in apparatus entirely of glass or other suitably inert material. It should be frequently restandardized. Store in glass-stoppered, amber-colored bottles.

$$N = \frac{g \text{ Na}_2\text{C}_2\text{O}_4}{\text{mL KMnO}_4 \text{ solution} \times 0.06700}$$

Potassium Thiocyanate, Tenth-Normal (0.1 N)

KSCN, 97.18

9.72 g in 1000 mL

Weigh exactly 9.72 g of potassium thiocyanate, previously dried for 2 hours at 110°, transfer to a 1-L volumetric flask, dilute with water to volume, and mix well. Standardize as follows: transfer 40.0 mL of freshly standardized 0.1 N silver nitrate VS to a 250-mL Erlenmeyer flask; and add 100 mL of water, 1 mL of nitric acid, and 2 mL of ferric ammonium sulfate TS. Titrate with the potassium thiocyanate solution, with agitation, to a permanent light pinkish-brown color of the supernatant.

$$N = \frac{\text{mL} \times N \text{ AgNO}_3}{\text{mL KSCN}}$$

Silver Nitrate, Tenth-Normal (0.1 N)

AgNO₃, 169.87

16.99 g in 1000 mL

Dissolve about 17.5 g of silver nitrate in 1000 mL of water, and standardize the solution as follows.

Transfer about 100 mg, accurately weighed, of reagent-grade sodium chloride, previously dried at 110° for 2 hours, to a 150-mL beaker, dissolve in 5 mL of water, and add 5 mL of acetic acid, 50 mL of methanol, and about 0.5 mL of eosin Y TS. Stir, preferably with a magnetic stirrer, and titrate with the silver nitrate solution.

$$N = \frac{\text{mg NaCl}}{\text{mL AgNO}_3 \times 58.44}$$

Sodium Arsenite, Twentieth-Molar (0.05 M)

NaAsO₂, 129.91

6.496 g in 1000 mL

Transfer 4.9455 g of arsenic trioxide, which has been pulverized and dried at 100° to constant weight, to a 1000-mL volumetric flask, dissolve it in 40 mL of 1 N sodium hydroxide, and add 1 N sulfuric acid or 1 N hydrochloric acid until the solution is neutral or only slightly acid to litmus. Add 30 g of sodium bicarbonate, dilute with water to volume, and mix.

Sodium Hydroxide, Alcoholic, Tenth-Normal (0.1 N)

NaOH, 40.00

To 250 mL of alcohol add 2 mL of a 50% (w/v) solution of sodium hydroxide.

Dissolve about 200 mg of benzoic acid, accurately weighed, in 10 mL of alcohol and 2 mL of water. Add 2 drops of phenolphthalein TS, and titrate with the alcoholic sodium hydroxide solution until a permanent pale pink color is produced.

$$N = \frac{\text{mg benzoic acid}}{122.1 \times \text{mL sodium hydroxide}}$$

Sodium Hydroxide, Normal (1 N)

NaOH, 40.00

40.00 g in 1000 mL

Dissolve 162 g of sodium hydroxide in 150 mL of carbon dioxide-free water, cool the solution to room temperature, and filter through hardened filter paper. Transfer 54.5 mL of the clear filtrate to a tight, polyolefin container, and dilute with carbon dioxide-free water to 1000 mL.

Accurately weigh about 5 g of potassium biphthalate, previously crushed lightly and dried at 120° for 2 hours, and dissolve in 75 mL of carbon dioxide-free water. Add 2 drops of phenolphthalein TS, and titrate with the sodium hydroxide solution to the production of a permanent pink color. Each 204.22 mg of potassium biphthalate is equivalent to 1 mL of 1 N sodium hydroxide.

$$N = \frac{g \text{ KHC}_8\text{H}_4\text{O}_4}{0.20422 \times \text{mL NaOH solution}}$$

[NOTES—(1) Solutions of alkali hydroxides absorb carbon dioxide when exposed to air. They should be preserved in bottles having well-fitted, suitable stoppers, provided with a tube filled with a mixture of sodium hydroxide and lime (soda-lime tubes) so that air entering the container must pass through this tube, which will absorb the carbon dioxide. (2) Prepare solutions of lower concentration (e.g., 0.1 N, 0.01 N) by quantitatively diluting accurately measured volumes of the 1 N solution with sufficient carbon dioxide-free water to yield the desired concentration.]

Restandardize the solution frequently.

Sodium Methoxide, Half-Normal (0.5 N) in Methanol

CH_3ONa , 54.02
27.01 g in 1000 mL

Weigh 11.5 g of freshly cut sodium metal, and cut into small cubes. Place about 0.5 mL of anhydrous methanol in a round-bottom, 250-mL flask equipped with a ground-glass joint, add 1 cube of the sodium metal, and, when the reaction has ceased, add the remaining sodium metal to the flask. Connect a water-jacketed condenser to the flask, and slowly add 250 mL of anhydrous methanol, in small portions, through the top of the condenser. Regulate the addition of the methanol so that the vapors are condensed and do not escape through the top of the condenser. After addition of the methanol is complete, connect a drying tube to the top of the condenser, and allow the solution to cool. Transfer the solution to a 1-L volumetric flask, dilute with anhydrous methanol to volume, and mix. Standardize the solution as follows.

Accurately measure about 20 mL of freshly standardized 1 N hydrochloric acid VS into a 250-mL conical flask, add 0.25 mL of phenolphthalein TS, and titrate with the sodium methoxide solution to the first appearance of a permanent pink color.

$$N = \frac{\text{mL HCl} \times N \text{ HCl}}{\text{mL sodium methoxide}}$$

Sodium Methoxide, Tenth-Normal (0.1 N) in Toluene

CH_3ONa , 54.02
5.402 g in 1000 mL

Cool in ice-water 150 mL of methanol contained in a 1000-mL volumetric flask, and add, in small portions, about 2.5 g of freshly cut sodium metal. When the metal has dissolved, add toluene to make 1000 mL, and mix. Store preferably in the reservoir of an automatic delivery buret suitably protected from carbon dioxide and moisture. Standardize the solution as follows.

Accurately weigh about 400 mg of primary standard benzoic acid, and dissolve in 80 mL of dimethylformamide in a flask. Add 3 drops of a 1 in 100 solution of thymol blue in dimethylformamide, and titrate with the sodium methoxide to a blue endpoint. Correct for the volume of the sodium methoxide solution consumed by 80 mL of the dimethylformamide. Each 12.21 mg of benzoic acid is equivalent to 1 mL of 0.1 N sodium methoxide.

$$N = \frac{\text{mg benzoic acid}}{122.1 \times \text{mL sodium methoxide (corrected for the blank)}}$$

[NOTES—(1) To eliminate any turbidity that may form following dilution with toluene, add methanol (25 to 30 mL usually suffices) until the solution is clear. (2) Restandardize the solution frequently.]

Sodium Nitrite, Tenth-Molar (0.1 M)

NaNO_2 , 69.00
6.900 g in 1000 mL

Dissolve 7.5 g of sodium nitrite in water to make 1000 mL, and standardize the solution as follows.

Accurately weigh about 500 mg of USP Sulfanilamide RS, previously dried at 105° for 3 hours, and transfer to a suitable beaker. Add 20 mL of hydrochloric acid and 50 mL of water, stir until dissolved, and cool to 15°. Maintaining the temperature at about 15°, titrate slowly with the sodium nitrite solution, placing the buret tip below the surface of the solution to preclude air oxidation of the sodium nitrite, and stir the solution gently with a magnetic stirrer, but avoid pulling a vortex of air beneath the surface. Use the indicator specified in the individual monograph, or, if a potentiometric procedure is specified, determine the endpoint electrometrically, using platinum–calomel or platinum–platinum electrodes. When the titration is within

1 mL of the endpoint, add the titrant in 0.1-mL portions, and allow 1 minute between additions. Each 17.22 mg of sulfanilamide is equivalent to 1 mL of 0.1000 M sodium nitrite.

$$M = \frac{\text{mg of sulfanilamide}}{172.22 \times \text{mL NaNO}_2}$$

Sodium Tetraphenylboron, Fiftieth-Molar (0.02 M)

$\text{NaB}(\text{C}_6\text{H}_5)_4$, 342.22
6.845 g in 1000 mL

Dissolve an amount of sodium tetraphenylboron, equivalent to 6.845 g of $\text{NaB}(\text{C}_6\text{H}_5)_4$, in water to make 1000 mL, and standardize the solution as follows.

Pipet two 75-mL portions of the solution into separate beakers, and to each add 1 mL of acetic acid and 25 mL of water. To each beaker add, slowly and with constant stirring, 25 mL of potassium biphthalate solution (1 in 20), and allow to stand for 2 hours. Filter one of the mixtures through a filtering crucible, and wash the precipitate with cold water. Transfer the precipitate to a container, add 50 mL of water, shake intermittently for 30 minutes, filter, and use the filtrate as the saturated potassium tetraphenylborate solution in the following standardization procedure. Filter the second mixture through a tared filtering crucible, and wash the precipitate with three 5-mL portions of saturated potassium tetraphenylborate solution. Dry the precipitate at 105° for 1 hour. Each g of potassium tetraphenylborate (KTPB) is equivalent to 955.1 mg of sodium tetraphenylboron.

$$M = \frac{\text{g KTPB} \times 0.9551}{342.22 \times 0.075}$$

[NOTE—Prepare this solution just before use.]

Sodium Thiosulfate, Tenth-Normal (0.1 N)

$\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, 248.19
24.82 g in 1000 mL

Dissolve about 26 g of sodium thiosulfate and 200 mg of sodium carbonate in 1000 mL of recently boiled and cooled water. Standardize the solution as follows.

Accurately weigh about 210 mg of primary standard potassium dichromate, previously pulverized and dried according to the instructions on its label, if necessary, and dissolve in 100 mL of water in a glass-stoppered, 500-mL flask. Swirl to dissolve the solid, remove the stopper, and quickly add 3 g of potassium iodide, 2 g of sodium bicarbonate, and 5 mL of hydrochloric acid. Insert the stopper gently in the flask, swirl to mix, and allow to stand in the dark for exactly 10 minutes. Rinse the stopper and the inner walls of the flask with water, and titrate the liberated iodine with the sodium thiosulfate solution until the solution is yellowish green in color. Add 3 mL of starch TS, and continue the titration until the blue color is discharged. Perform a blank determination.

Restandardize the solution as frequently as supported by laboratory stability data. In the absence of such data, restandardize the solution weekly.

$$N = \frac{\text{mg K}_2\text{Cr}_2\text{O}_7}{49.04 \times \text{mL Na}_2\text{S}_2\text{O}_3}$$

Sulfuric Acid, Half-Normal (0.5 N) in Alcohol

H_2SO_4 , 98.08
24.52 g in 1000 mL

Add slowly, with stirring, 13.9 mL of sulfuric acid to a sufficient quantity of dehydrated alcohol to make 1000 mL.

Cool, and standardize against tromethamine as described under *Hydrochloric Acid, Half-Normal (0.5 N) in Methanol*.

$$N = \frac{\text{mg tromethamine}}{121.14 \times \text{mL H}_2\text{SO}_4}$$

Sulfuric Acid, Normal (1 N)

H_2SO_4 , **98.08**
49.04 g in 1000 mL

Add slowly, with stirring, 27 mL of sulfuric acid to a sufficient quantity of water to make 1000 mL. Cool and standardize against tromethamine as described under *Hydrochloric Acid, Normal (1 N)*.

Tetrabutylammonium Hydroxide, Tenth-Normal (0.1 N)

$(\text{C}_4\text{H}_9)_4\text{NOH}$, **259.47**
25.95 g in 1000 mL

Dissolve 40 g of tetra-*n*-butylammonium iodide in 90 mL of anhydrous methanol in a glass-stoppered flask. Place in an ice bath, add 20 g of powdered silver oxide, insert the stopper in the flask, and agitate vigorously for 60 minutes. Centrifuge a few mL, and test the supernatant for iodide (see *Iodide* (191)). If the test is positive, add an additional 2 g of silver oxide, and continue to allow to stand for 30 minutes with intermittent agitation. When all of the iodide has reacted, filter through a fine-porosity, sintered-glass funnel. Rinse the flask and the funnel with three 50-mL portions of anhydrous toluene, adding the rinsings to the filtrate. Dilute with a mixture of three volumes of anhydrous toluene and 1 volume of anhydrous methanol to 1000 mL, and flush the solution for 10 minutes with dry, carbon dioxide-free nitrogen. [NOTE—If necessary to obtain a clear solution, further small quantities of anhydrous methanol may be added.] Store in a reservoir protected from carbon dioxide and moisture, and discard after 60 days. Alternatively, the solution may be prepared by diluting a suitable volume of commercially available tetrabutylammonium hydroxide solution in methanol with a mixture of 4 volumes of anhydrous toluene and 1 volume of anhydrous methanol. [NOTE—If necessary to obtain a clear solution, further small quantities of methanol may be added.]

Standardize the solution on the day of use as follows.

Dissolve about 400 mg of primary standard benzoic acid, accurately weighed, in 80 mL of dimethylformamide, add 3 drops of a 1 in 100 solution of thymol blue in dimethylformamide, and titrate to a blue endpoint with the tetrabutylammonium hydroxide solution, delivering the titrant from a buret equipped with a carbon dioxide absorption trap. Perform a blank determination, and make any necessary correction. Each mL of 0.1 N tetrabutylammonium hydroxide is equivalent to 12.21 mg of benzoic acid.

$$N = \frac{\text{mg benzoic acid}}{122.1 \times \text{mL } (\text{C}_4\text{H}_9)_4\text{NOH}}$$

**Tetrabutylammonium Hydroxide in Methanol/
Isopropyl Alcohol, 0.1 N**

Prepare as described for *Tetrabutylammonium Hydroxide, Tenth-Normal (0.1 N)* using isopropyl alcohol instead of toluene, and standardize as described. Alternatively, the solution may be prepared by diluting a suitable volume of commercially available tetrabutylammonium hydroxide solution in methanol with 4 volumes of anhydrous isopropyl alcohol.

$$N = \frac{\text{mg benzoic acid}}{122.1 \times \text{mL } (\text{C}_4\text{H}_9)_4\text{NOH}}$$

Tetramethylammonium Bromide, Tenth-Molar (0.1 M)

$(\text{CH}_3)_4\text{NBr}$, **154.05**
15.41 g in 1000 mL

Dissolve 15.41 g of tetramethylammonium bromide in water to make 1000 mL, and standardize the solution as follows.

Transfer an accurately measured volume of about 40 mL of the solution to a beaker, add 10 mL of diluted nitric acid and 50.0 mL of 0.1 N silver nitrate VS, and mix. Add 2 mL of ferric ammonium sulfate TS, and titrate the excess silver nitrate with 0.1 N ammonium thiocyanate VS.

$$M = \frac{\text{mL AgNO}_3 \times N \text{ AgNO}_3}{\text{mL } (\text{CH}_3)_4\text{NBr}}$$

Tetramethylammonium Chloride, Tenth-Molar (0.1 M)

$(\text{CH}_3)_4\text{NCl}$, **109.60**
10.96 g in 1000 mL

Dissolve 10.96 g of tetramethylammonium chloride in water to make 1000 mL, and standardize the solution as follows.

Transfer an accurately measured volume of about 40 mL of the solution to a flask, add 10 mL of diluted nitric acid and 50.0 mL of 0.1 N silver nitrate VS, and mix. Add 5 mL of nitrobenzene and 2 mL of ferric ammonium sulfate TS, shake, and titrate the excess silver nitrate with 0.1 N ammonium thiocyanate VS.

$$M = \frac{\text{mL AgNO}_3 \times N \text{ AgNO}_3}{\text{mL } (\text{CH}_3)_4\text{NCl}}$$

Titanium Trichloride, Tenth-Normal (0.1 N)

TiCl_3 , **154.23**
15.42 g in 1000 mL

Add 75 mL of titanium trichloride solution (1 in 5) to 75 mL of hydrochloric acid, dilute to 1000 mL, and mix. Standardize the solution as follows, using the special titration apparatus described.

Apparatus—Store the titanium trichloride solution in the reservoir of a closed-system titration apparatus in an atmosphere of hydrogen.

Use a wide-mouth, 500-mL conical flask as the titration vessel, and connect it by means of a tight-fitting rubber stopper to the titration buret, an inlet tube for carbon dioxide, and an exit tube. Arrange for mechanical stirring. All joints must be air-tight. Arrange to have both the hydrogen and the carbon dioxide pass through wash bottles containing titanium trichloride solution (approximately 1 in 50) to remove any oxygen.

If the solution to be titrated is to be heated before or during titration, connect the titration flask with an upright reflux condenser through the rubber stopper.

Standardization—Place an accurately measured volume of about 40 mL of 0.1 N ferric ammonium sulfate VS in the titration flask, and pass in a rapid stream of carbon dioxide until all the air has been removed. Add the titanium trichloride solution from the buret until near the calculated endpoint (about 35 mL), then add through the outlet tube 5 mL of ammonium thiocyanate TS, and continue the titration until the solution is colorless.

$$N = \frac{\text{mL FeNH}_4(\text{SO}_4)_2 \times N \text{ FeNH}_4(\text{SO}_4)_2}{\text{mL TiCl}_3}$$

Zinc Sulfate, Twentieth-Molar (0.05 M)

$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, **287.56**
14.4 g in 1000 mL

Dissolve 14.4 g of zinc sulfate in water to make 1 L. Standardize the solution as follows.

Accurately measure about 10 mL of 0.05 M edetate disodium VS into a 125-mL conical flask, and add, in the order given, 10 mL of acetic acid–ammonium acetate buffer TS,

50 mL of alcohol, and 2 mL of dithizone TS. Titrate with the zinc sulfate solution to a clear, rose-pink color.

$$M = \frac{\text{mL edetate disodium} \times M \text{ edetate disodium}}{\text{mL ZnSO}_4}$$

Chromatographic Columns

The following list of packings (L), phases (G), and supports (S) is intended to be a convenient reference for the chromatographer. [NOTE—Particle sizes given in this listing are those generally provided. Where other, usually finer, sizes are required, the individual monograph specifies the desired particle size. Within any category of packings or phases listed below, there may be a wide range of columns available. Where it is necessary to define more specifically the chromatographic conditions, the individual monograph so indicates.]

Packings

L1—Octadecyl silane chemically bonded to porous or nonporous silica or ceramic microparticles, 1.5 to 10 µm in diameter, or a monolithic silica rod.

L2—Octadecyl silane chemically bonded to silica gel of a controlled surface porosity that has been bonded to a solid spherical core, 30 to 50 µm in diameter.

L3—Porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod.

L4—Silica gel of controlled surface porosity bonded to a solid spherical core, 30 to 50 µm in diameter.

L5—Alumina of controlled surface porosity bonded to a solid spherical core, 30 to 50 µm in diameter.

L6—Strong cation-exchange packing—sulfonated fluorocarbon polymer coated on a solid spherical core, 30 to 50 µm in diameter.

L7—Octylsilane chemically bonded to totally porous or superficially porous silica particles, 1.5–10 µm in diameter, or a monolithic silica rod.

L8—An essentially monomolecular layer of aminopropylsilane chemically bonded to totally porous silica gel support, 1.5 to 10 µm in diameter.

L9—Irregular or spherical, totally porous silica gel having a chemically bonded, strongly acidic cation-exchange coating, 3 to 10 µm in diameter.

L10—Nitrile groups chemically bonded to porous silica particles, 1.5 to 10 µm in diameter.

L11—Phenyl groups chemically bonded to porous silica particles, 1.5 to 10 µm in diameter.

L12—A strong anion-exchange packing made by chemically bonding a quaternary amine to a solid silica spherical core, 30 to 50 µm in diameter.

L13—Trimethylsilane chemically bonded to porous silica particles, 3 to 10 µm in diameter.

L14—Silica gel having a chemically bonded, strongly basic quaternary ammonium anion-exchange coating, 5 to 10 µm in diameter.

L15—Hexylsilane chemically bonded to totally porous silica particles, 3 to 10 µm in diameter.

L16—Dimethylsilane chemically bonded to porous silica particles, 5 to 10 µm in diameter.

L17—Strong cation-exchange resin consisting of sulfonated cross-linked styrene–divinylbenzene copolymer in the hydrogen form, 6 to 12 µm in diameter.

L18—Amino and cyano groups chemically bonded to porous silica particles, 3 to 10 µm in diameter.

L19—Strong cation-exchange resin consisting of sulfonated cross-linked styrene–divinylbenzene copolymer in the calcium form, about 9 µm in diameter.

L20—Dihydroxypropane groups chemically bonded to porous silica or hybrid particles, 1.5 to 10 µm in diameter.

L21—A rigid, spherical styrene–divinylbenzene copolymer 3 to 10 µm in diameter.

L22—A cation-exchange resin made of porous polystyrene gel with sulfonic acid groups, about 10 µm in size.

L23—An anion-exchange resin made of porous polymethacrylate or polyacrylate gel with quaternary ammonium groups, 7–12 µm in size.

L24—A semi-rigid hydrophilic gel consisting of vinyl polymers with numerous hydroxyl groups on the matrix surface, 32 to 63 µm in diameter. [NOTE—Available as YMC-Pack PVA-SIL manufactured by YMC Co., Ltd. and distributed by Waters Corp. (www.waters.com).]

L25—Packing having the capacity to separate compounds with a molecular weight range from 100–5000 (as determined by polyethylene oxide), applied to neutral, anionic, and cationic water-soluble polymers. A polymethacrylate resin base, cross-linked with polyhydroxylated ether (surface contained some residual carboxyl functional groups) was found suitable.

L26—Butyl silane chemically bonded to totally porous silica particles, 1.5 to 10 µm in diameter.

L27—Porous silica particles, 30 to 50 µm in diameter.

L28—A multifunctional support, which consists of a high purity, 100 Å, spherical silica substrate that has been bonded with anionic exchanger, amine functionality in addition to a conventional reversed phase C8 functionality.

L29—Gamma alumina, reverse-phase, low carbon percentage by weight, alumina-based polybutadiene spherical particles, 5 µm in diameter with a pore volume of 80 Å.

L30—Ethyl silane chemically bonded to totally porous silica particles, 3 to 10 µm in diameter.

L31—A hydroxide-selective, strong anion-exchange resin—quaternary amine bonded on latex particles attached to a core of 8.5-µm macroporous particles having a pore size of 2000 Å and consisting of ethylvinylbenzene cross-linked with 55% divinylbenzene.

L32—A chiral ligand-exchange packing—L-proline copper complex covalently bonded to irregularly shaped silica particles, 5 to 10 µm in diameter.

L33—Packing having the capacity to separate dextrans by molecular size over a range of 4,000 to 500,000 Da. It is spherical, silica-based, and processed to provide pH stability. [NOTE—Available as TSK-GEL G4000SWxl from Tosoh Bioscience (www.tosohbioscience.com).]

L34—Strong cation-exchange resin consisting of sulfonated cross-linked styrene–divinylbenzene copolymer in the lead form, 7 to 9 µm in diameter.

L35—A zirconium-stabilized spherical silica packing with a hydrophilic (diol-type) molecular monolayer bonded phase having a pore size of 150 Å.

L36—A 3,5-dinitrobenzoyl derivative of L-phenylglycine covalently bonded to 5-µm aminopropyl silica.

L37—Packing having the capacity to separate proteins by molecular size over a range of 2,000 to 40,000 Da. It is a polymethacrylate gel.

L38—A methacrylate-based size-exclusion packing for water-soluble samples.

L39—A hydrophilic polyhydroxymethacrylate gel of totally porous spherical resin.

L40—Cellulose tris-3,5-dimethylphenylcarbamate coated porous silica particles, 5 to 20 μm in diameter.

L41—Immobilized α_1 -acid glycoprotein on spherical silica particles, 5 μm in diameter.

L42—Octylsilane and octadecylsilane groups chemically bonded to porous silica particles, 5 μm in diameter.

L43—Pentafluorophenyl groups chemically bonded to silica particles by a propyl spacer, 1.5 to 10 μm in diameter.

L44—A multifunctional support, which consists of a high purity, 60 Å, spherical silica substrate that has been bonded with a cationic exchanger, sulfonic acid functionality in addition to a conventional reversed phase C8 functionality.

L45—Beta cyclodextrin bonded to porous silica particles, 5 to 10 μm in diameter.

L46—Polystyrene/divinylbenzene substrate agglomerated with quaternary amine functionalized latex beads, about 9 to 11 μm in diameter.

L47—High-capacity anion-exchange microporous substrate, fully functionalized with trimethylamine groups, 8 μm in diameter. [NOTE—Available as CarboPac MA1 and distributed by Dionex Corp. (www.dionex.com).]

L48—Sulfonated, cross-linked polystyrene with an outer layer of submicron, porous, anion-exchange microbeads, 10 to 15 μm in diameter.

L49—A reversed-phase packing made by coating a thin layer of polybutadiene onto spherical porous zirconia particles, 3 to 10 μm in diameter. [NOTE—Available as Zirchrom PBD from www.zirchrom.com.]

L50—Multifunction resin with reversed-phase retention and strong anion-exchange functionalities. The resin consists of ethylvinylbenzene, 55% cross-linked with divinylbenzene copolymer, 3 to 15 μm in diameter, and a surface area not less than 350 m^2 per g. Substrate is coated with quaternary ammonium functionalized latex particles consisting of styrene cross-linked with divinylbenzene. [NOTE—Available as OmniPac PAX-500 and distributed by Dionex Corp. (www.dionex.com).]

L51—Amylose tris-3,5-dimethylphenylcarbamate-coated, porous, spherical, silica particles, 5 to 10 μm in diameter. [NOTE—Available as Chiralpak AD from Chiral Technologies, Inc., (www.chiraltech.com).]

L52—A strong cation-exchange resin made of porous silica with sulfopropyl groups, 5 to 10 μm in diameter. [NOTE—Available as TSK-GEL IC-Cation-SW from Tosoh Bioscience (www.tosohbioscience.com).]

L53—Weak cation-exchange resin consisting of ethylvinylbenzene, 55% cross-linked with divinylbenzene copolymer, 3 to 15 μm diameter. Substrate is surface grafted with carboxylic acid and/or phosphoric acid functionalized monomers. Capacity not less than 500 μEq /column. [NOTE—Available as IonPac CS14 distributed by Dionex Corp. (www.dionex.com).]

L54—A size exclusion medium made of covalent bonding of dextran to highly cross-linked porous agarose beads, about 13 μm in diameter.

[NOTE—Available as Superdex Peptide HR 10/30 from www.gelifesciences.com.]

L55—A strong cation-exchange resin made of porous silica coated with polybutadiene-maleic acid copolymer, about 5 μm in diameter. [NOTE—Available as IC-Pak C M/D from Waters Corp. (www.waters.com).]

L56—Propyl silane chemically bonded to totally porous silica particles, 3 to 10 μm in diameter. [NOTE—Available as Zorbax SB-C3 from Agilent Technologies (www.agilent.com/chem).]

L57—A chiral-recognition protein, ovomucoid, chemically bonded to silica particles, about 5 μm in diameter, with a pore size of 120 Å. [NOTE—Available as Ultron ES-OVM from Agilent Technologies (www.agilent.com/chem).]

L58—Strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in

the sodium form, about 6 to 30 μm in diameter. [NOTE—Available as Aminex HPX-87N from Bio-Rad Laboratories, (2000/01 catalog, #125-0143) www.bio-rad.com.]

L59—Packing for the size-exclusion separation of proteins (separation by molecular weight) over the range of 5 to 7000 kDa. The packing is a spherical 1.5- to 10- μm silica or hybrid packing with a hydrophilic coating.

L60—Spherical, porous silica gel, 10 μm or less in diameter, the surface of which has been covalently modified with alkyl amide groups and endcapped. [NOTE—Available as Supelcosil LC-ABZ from Supelco (www.sigmaaldrich.com/supelco).]

L61—A hydroxide selective strong anion-exchange resin consisting of a highly cross-linked core of 13- μm microporous particles having a pore size less than 10 Å units and consisting of ethylvinylbenzene cross-linked with 55% divinylbenzene with a latex coating composed of 85-nm diameter microbeads bonded with alkanol quaternary ammonium ions (6%). [NOTE—Available as Ion Pac AS-11 and AG-11 from Dionex (www.dionex.com).]

L62—C30 silane bonded phase on a fully porous spherical silica, 3 to 15 μm in diameter.

L63—Glycopeptide teicoplanin linked through multiple covalent bonds to a 100-Å units spherical silica. [NOTE—Available as Astec Chirobiotic T from Supelco (www.sigmaaldrich.com).]

L64—Strongly basic anion-exchange resin consisting of 8% cross-linked styrene-divinylbenzene copolymer with a quaternary ammonium group in the chloride form, 45 to 180 μm in diameter. [NOTE—A suitable grade is available as AG 1-X8 resin chloride form from www.discover.bio-rad.com.]

L65—Strongly acidic cation-exchange resin consisting of 8% sulfonated cross-linked styrene-divinylbenzene copolymer with a sulfonic acid group in the hydrogen form, 45 to 250 μm in diameter. [NOTE—A suitable grade is available as AG 50W-X2 resin hydrogen form from www.discover.bio-rad.com.]

L66—A crown ether coated on a 5- μm particle size silica gel substrate. The active site is (S)-18-crown-6-ether. [NOTE—Available as Crownpak CR(+) from Daicel (www.daicel.com).]

L67—Porous vinyl alcohol copolymer with a C18 alkyl group attached to the hydroxyl group of the polymer, 2 to 10 μm in diameter. [NOTE—Available as apHera C18 from Supelco (www.sigmaaldrich.com).]

L68—Spherical, porous silica, 10 μm or less in diameter, the surface of which has been covalently modified with alkyl amide groups and not endcapped. [NOTE—Available as SUPELCOSIL SUPLEX pKb-100 from Supelco (www.sigmaaldrich.com).]

L69—Ethylvinylbenzene/divinylbenzene substrate agglomerated with quaternary amine functionalized 130-nm latex beads, about 6.5 μm in diameter. [NOTE—Available as CarboPac PA20 from www.dionex.com.]

L70—Cellulose tris(phenyl carbamate) coated on 5- μm silica. [NOTE—Available as Chiralcel OC-H from www.chiraltech.com.]

L71—A rigid, spherical polymetacrylate, 4 to 6 μm in diameter. [NOTE—Available as RSpak DE-613 from www.shodex.com.]

L72—(S)-phenylglycine and 3,5-dinitroaniline urea linkage covalently bonded to silica.

[NOTE—Available as Sumichiral OA-3300, distributed by www.phenomenex.com.]

L73—A rigid spherical polydivinylbenzene particle, 5 to 10 μm in diameter. [NOTE—Available as Jordi-Gel DBV from www.jordiflp.com.]

L74—A strong anion-exchange resin consisting of a highly cross-linked core of 7- μm macroporous particles having a 100-Å average pore size and consisting of ethylvinylbenzene cross-linked with 55% divinylbenzene and an anion-exchange layer grafted to the surface, which is functionalized with alkyl quaternary ammonium ions. [NOTE—Available as IonPac AS14A from Dionex (www.dionex.com).]

L75—A chiral-recognition protein, bovine serum albumine (BSA), chemically bonded to silica particles, about 7 µm in diameter, with a pore size of 300 Å.

Phases

- G1**—Dimethylpolysiloxane oil.
G2—Dimethylpolysiloxane gum.
G3—50% Phenyl-50% methylpolysiloxane.
G4—Diethylene glycol succinate polyester.
G5—3-Cyanopropylpolysiloxane.
G6—Trifluoropropylmethylpolysiloxane.
G7—50% 3-Cyanopropyl-50% phenylmethylsilicone.
G8—80% Bis(3-cyanopropyl)-20% 3-cyanopropylphenylpolysiloxane (percentages refer to molar substitution).
G9—Methylvinylpolysiloxane.
G10—Polyamide formed by reacting a C₃₆ dicarboxylic acid with 1,3-di-4-piperidylpropane and piperidine in the respective mole ratios of 1.00:0.90:0.20.
G11—Bis(2-ethylhexyl) sebacate polyester.
G12—Phenyldiethanolamine succinate polyester.
G13—Sorbitol.
G14—Polyethylene glycol (av. mol. wt. of 950 to 1050).
G15—Polyethylene glycol (av. mol. wt. of 3000 to 3700).
G16—Polyethylene glycol compound (av. mol. wt. about 15,000). A high molecular weight compound of polyethylene glycol with a diepoxide linker. [NOTE—Available commercially as Polyethylene Glycol Compound 20M, or as Carbowax 20M, from suppliers of chromatographic reagents.]
G17—75% Phenyl-25% methylpolysiloxane.
G18—Polyalkylene glycol.
G19—25% Phenyl-25% cyanopropyl-50% methylsilicone.
G20—Polyethylene glycol (av. mol. wt. of 380 to 420).
G21—Neopentyl glycol succinate.
G22—Bis(2-ethylhexyl) phthalate.
G23—Polyethylene glycol adipate.
G24—Diisodecyl phthalate.
G25—Polyethylene glycol compound TPA. A high molecular weight compound of a polyethylene glycol and a diepoxide that is esterified with terephthalic acid.
 [NOTE—Available commercially as Carbowax 20M-TPA from suppliers of chromatographic reagents.]
G26—25% 2-Cyanoethyl-75% methylpolysiloxane.
G27—5% Phenyl-95% methylpolysiloxane.
G28—25% Phenyl-75% methylpolysiloxane.
G29—3,3'-Thiodipropionitrile.
G30—Tetraethylene glycol dimethyl ether.
G31—Nonylphenoxypoly(ethyleneoxy)ethanol (av. ethyleneoxy chain length is 30); Nonoxynol 30.
G32—20% Phenylmethyl-80% dimethylpolysiloxane.
G33—20% Carborane-80% methylsilicone.
G34—Diethylene glycol succinate polyester stabilized with phosphoric acid.
G35—A high molecular weight compound of a polyethylene glycol and a diepoxide that is esterified with nitroterephthalic acid.
G36—1% Vinyl-5% phenylmethylpolysiloxane.
G37—Polyimide.
G38—Phase G1 containing a small percentage of a tailing inhibitor.
 [NOTE—A suitable grade is available commercially as "SP2100/0.1% Carbowax 1500" from Supelco, Inc. (www.sigmaaldrich.com/supelco).]

G39—Polyethylene glycol (av. mol. wt. about 1500).

G40—Ethylene glycol adipate.

G41—Phenylmethyldimethylsilicone (10% phenyl-substituted).

G42—35% phenyl-65% dimethylpolysiloxane (percentages refer to molar substitution).

G43—6% cyanopropylphenyl-94% dimethylpolysiloxane (percentages refer to molar substitution).

G44—2% low molecular weight petrolatum hydrocarbon grease and 1% solution of potassium hydroxide.

G45—Divinylbenzene-ethylene glycol-dimethylacrylate.

G46—14% Cyanopropylphenyl-86% methylpolysiloxane.

G47—Polyethylene glycol (av. mol. wt. of about 8000).

G48—Highly polar, partially cross-linked cyanopolysiloxane.

Supports

[NOTE—Unless otherwise specified, mesh sizes of 80 to 100 or, alternatively, 100 to 120 are intended.]

S1A—Siliceous earth for gas chromatography has been flux-calcined by mixing diatomite with Na₂CO₃ flux and calcining above 900°. The siliceous earth is acid-washed, then water-washed until neutral, but not base-washed. The siliceous earth may be silanized by treating with an agent such as dimethyldichlorosilane [NOTE—Unless otherwise specified in the individual monograph, silanized support is intended.] to mask surface silanol groups.

S1AB—The siliceous earth as described above is both acid- and base-washed. [NOTE—Unless otherwise specified in the individual monograph, silanized support is intended.]

S1C—A support prepared from crushed firebrick and calcined or burned with a clay binder above 900° with subsequent acid-wash. It may be silanized.

S1NS—The siliceous earth is untreated.

S2—Styrene-divinylbenzene copolymer having a nominal surface area of less than 50 m² per g and an average pore diameter of 0.3 to 0.4 µm.

S3—Copolymer of ethylvinylbenzene and divinylbenzene having a nominal surface area of 500 to 600 m² per g and an average pore diameter of 0.0075 µm.

S4—Styrene-divinylbenzene copolymer with aromatic -O and -N groups, having a nominal surface area of 400 to 600 m² per g and an average pore diameter of 0.0076 µm.

S5—40- to 60-mesh, high-molecular weight tetrafluorethylene polymer.

S6—Styrene-divinylbenzene copolymer having a nominal surface area of 250 to 350 m² per g and an average pore diameter of 0.0091 µm.

S7—Graphitized carbon having a nominal surface area of 12 m² per g.

S8—Copolymer of 4-vinyl-pyridine and styrene-divinylbenzene.

S9—A porous polymer based on 2,6-diphenyl-*p*-phenylene oxide.

S10—A highly polar cross-linked copolymer of acrylonitrile and divinylbenzene.

S11—Graphitized carbon having a nominal surface area of 100 m² per g modified with small amounts of petrolatum and polyethylene glycol compound.

S12—Graphitized carbon having a nominal surface area of 100 m² per g.

Reference Tables

CONTAINERS FOR DISPENSING CAPSULES AND TABLETS

The following table is provided as a reminder for the pharmacist engaged in the typical dispensing situation who already is acquainted with the *Packaging and Storage* requirements set forth in the individual monographs. It lists the capsules and tablets that are official in the *United States Pharmacopeia* and indicates the relevant tight (T), well-closed (W), and light-resistant (LR) specifications applicable to containers in which the drug that is repackaged should be dispensed.

This table is not intended to replace, nor should it be interpreted as replacing, the definitive requirements stated in the individual monographs.

Container Specifications for Capsules and Tablets

Monograph Title	Container Specification
Abacavir Tablets	W
Acebutolol Hydrochloride Capsules	T
Acepromazine Maleate Tablets	T, LR
Acetaminophen Capsules	T
Acetaminophen Tablets, Extended-Release	T
Acetaminophen Tablets	T
Acetaminophen and Aspirin Tablets	T
Acetaminophen, Aspirin, and Caffeine Tablets	T
Acetaminophen and Caffeine Tablets	T
Acetaminophen and Salts of Chlorpheniramine, Dextromethorphan, and Phenylpropanolamine, Capsules Containing at Least Three of the Following—	T
Acetaminophen and Salts of Chlorpheniramine, Dextromethorphan, and Phenylpropanolamine, Tablets Containing at Least Three of the Following—	T
Acetaminophen and Salts of Chlorpheniramine, Dextromethorphan, and Pseudoephedrine, Capsules Containing at Least Three of the Following—	T
Acetaminophen and Salts of Chlorpheniramine, Dextromethorphan, and Pseudoephedrine, Tablets Containing at Least Three of the Following—	T
Acetaminophen, Chlorpheniramine Maleate, and Dextromethorphan Hydrobromide Tablets	T
Acetaminophen and Codeine Phosphate Capsules	T, LR
Acetaminophen and Codeine Phosphate Tablets	T, LR
Acetaminophen and Diphenhydramine Citrate Tablets	T
Acetaminophen, Diphenhydramine Hydrochloride, and Pseudoephedrine Hydrochloride Tablets	T
Acetaminophen and Pseudoephedrine Hydrochloride Tablets	T
Acetaminophen and Tramadol Hydrochloride Tablets	T
Acetazolamide Tablets	T
Acetohexamide Tablets	W
Acetohydroxamic Acid Tablets	T
Acitretin Capsules	W, LR

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Acyclovir Capsules	T
Acyclovir Tablets	T
Albendazole Tablets	T
Albuterol Tablets	T, LR
Alendronate Sodium Tablets	T
Add the following:	
▲ Alfuzosin Hydrochloride Tablets, Extended-Release	LR▲ ^{USP36}
Allopurinol Tablets	W
Alprazolam Tablets	T, LR
Alprazolam Tablets, Extended-Release	T, LR
Alprazolam Tablets, Orally Disintegrating	T
Altretamine Capsules	T, LR
Alumina and Magnesia Tablets	W
Alumina, Magnesia, and Calcium Carbonate Tablets	W
Alumina, Magnesia, Calcium Carbonate, and Simethicone Tablets	W
Alumina, Magnesia, and Simethicone Tablets	W
Alumina and Magnesium Carbonate Tablets	T
Alumina, Magnesium Carbonate, and Magnesium Oxide Tablets	T
Alumina and Magnesium Trisilicate Tablets	W
Aluminum Carbonate Gel, Dried Basic, Capsules	W
Aluminum Carbonate Gel, Dried Basic, Tablets	W
Aluminum Hydroxide Gel, Dried, Capsules	W
Aluminum Hydroxide Gel, Dried, Tablets	W
Amantadine Hydrochloride Capsules	T
Amiloride Hydrochloride Tablets	W
Amiloride Hydrochloride and Hydrochlorothiazide Tablets	W
Aminobenzoate Potassium Capsules	W
Aminobenzoate Potassium Tablets	W
Aminocaproic Acid Tablets	T
Aminogluthethimide Tablets	T, LR
Aminopentamide Sulfate Tablets	W
Aminophylline Tablets	T
Aminophylline Tablets, Delayed-Release	T
Aminosalicylate Sodium Tablets	T, LR
Aminosalicylic Acid Tablets	T, LR
Amitriptyline Hydrochloride Tablets	W
Amlodipine Besylate Tablets	T, LR
Ammonium Chloride Tablets, Delayed-Release	T
Amodiaquine Hydrochloride Tablets	T
Amoxapine Tablets	W
Amoxicillin Capsules	T
Amoxicillin Tablets	T
Amoxicillin and Clavulanate Potassium Tablets	T
Amphetamine Sulfate Tablets	W
Ampicillin Capsules	T
Ampicillin Tablets	T
Anagrelide Capsules	T, LR
Anileridine Hydrochloride Tablets	T, LR
Apomorphine Hydrochloride Tablets	T, LR

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Arginine Capsules	T, LR
Arginine Tablets	T, LR
Ascorbic Acid Tablets	T, LR
Aspirin Capsules	T
Aspirin Capsules, Delayed-Release	T
Aspirin Tablets	T
Aspirin Tablets, Buffered	T
Aspirin Tablets, Delayed-Release	T
Aspirin Tablets, Effervescent for Oral Solution	T
Aspirin Tablets, Extended-Release	T
Aspirin, Alumina, and Magnesia Tablets	T
Aspirin, Alumina, and Magnesium Oxide Tablets	T
Aspirin, Caffeine, and Dihydrocodeine Bitartrate Capsules	T
Aspirin and Codeine Phosphate Tablets	W, LR
Aspirin, Codeine Phosphate, Alumina, and Magnesia Tablets	W, LR
Astemizole Tablets	T
Atenolol Tablets	W
Atenolol and Chlorthalidone Tablets	W
Atropine Sulfate Tablets	W
Azatadine Maleate Tablets	W
Azathioprine Tablets	LR
Azithromycin Capsules	W
Azithromycin Tablets	T
Bacampicillin Hydrochloride Tablets	T
Baclofen Tablets	W
Balsalazide Disodium Capsules	T
Barium Sulfate Tablets	W
Belladonna Extract Tablets	T, LR
Benazepril Hydrochloride Tablets	W
Bendroflumethiazide Tablets	T
Benzonatate Capsules	T, LR
Benztropine Mesylate Tablets	W
Beta Carotene Capsules	T, LR
Betamethasone Tablets	T
Betaxolol Tablets	T
Bethanechol Chloride Tablets	T
Bicalutamide Tablets	T
Biperiden Hydrochloride Tablets	T
Bisacodyl Tablets	T
Bisacodyl Tablets, Delayed-Release	W
Bismuth Subsalicylate Tablets	T
Bisoprolol Fumarate Tablets	T, LR
Bisoprolol Fumarate and Hydrochlorothiazide Tablets	W
Black Cohosh Tablets	T, LR
Bromocriptine Mesylate Capsules	T, LR
Bromocriptine Mesylate Tablets	T, LR
Brompheniramine Maleate Tablets	T
Bumetanide Tablets	T, LR
Bupropion Hydrochloride Tablets, Extended-Release	W
Buspirone Hydrochloride Tablets	T, LR
Busulfan Tablets	W
Butabarbital Sodium Tablets	W
Butalbital, Acetaminophen, and Caffeine Capsules	T
Butalbital, Acetaminophen, and Caffeine Tablets	T
Butalbital and Aspirin Tablets	T
Butalbital, Aspirin, and Caffeine Capsules	T
Butalbital, Aspirin, and Caffeine Tablets	T

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Butalbital, Aspirin, Caffeine, and Codeine Phosphate Capsules	T, LR
Cabergoline Tablets	T, LR
Calcifediol Capsules	T, LR
Calcium with Vitamin D Tablets	T, LR
Calcium Acetate Tablets	W
Calcium Carbonate Tablets	W
Calcium Carbonate and Magnesia Tablets	W
Calcium Carbonate and Magnesia Chewable Tablets	W
Calcium Citrate Tablets	W
Calcium and Magnesium Carbonates Tablets	W
Calcium Gluconate Tablets	W
Calcium Lactate Tablets	T
Calcium Pantothenate Tablets	T
Calcium Phosphate, Dibasic Tablets	W
Capecitabine Tablets	T
Captopril Tablets	T
Captopril and Hydrochlorothiazide Tablets	T
Carbamazepine Tablets	T
Carbamazepine Tablets, Extended-Release	T
Carbenicillin Indanyl Sodium Tablets	T
Carbidopa and Levodopa Tablets	W, LR
Carbinoxamine Maleate Tablets	T, LR
Urea C14 Capsules	T
Carboxymethylcellulose Sodium Tablets	T
Carisoprodol Tablets	W
Carisoprodol and Aspirin Tablets	W
Carisoprodol, Aspirin, and Codeine Phosphate Tablets	W
Carprofen Tablets	T
Carteolol Hydrochloride Tablets	T
Carvedilol Tablets	T, LR
Cascara Tablets	T, W
Castor Oil Capsules	T
Cat's Claw Capsules	T, LR
Cat's Claw Tablets	T, LR
Cefaclor Capsules	T
Cefaclor Tablets, Chewable	T
Cefaclor Tablets, Extended-Release	T, LR
Cefadroxil Capsules	T
Cefadroxil Tablets	T
Cefdinir Capsules	T, LR
Cefixime Tablets	T
Cefpodoxime Proxetil Tablets	T
Cefprozil Tablets	T
Cefuroxime Axetil Tablets	W
Cephalexin Capsules	T
Cephalexin Tablets	T
Cephadrine Capsules	T
Cephadrine Tablets	T
Cetirizine Hydrochloride Tablets	W
Cetirizine Hydrochloride and Pseudoephedrine Hydrochloride Tablets, Extended-Release	W
Chloral Hydrate Capsules	T
Chlorambucil Tablets	W, LR
Chloramphenicol Capsules	T
Chloramphenicol Tablets	T
Chlordiazepoxide Tablets	T, LR
Chlordiazepoxide and Amitriptyline Hydrochloride Tablets	T, LR
Chlordiazepoxide Hydrochloride Capsules	T, LR

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Chlordiazepoxide Hydrochloride and Clidinium Bromide Capsules	T, LR
Chloroquine Phosphate Tablets	W
Chlorothiazide Tablets	W
Chlorpheniramine Maleate Capsules, Extended-Release	T
Chlorpheniramine Maleate Tablets	T
Chlorpheniramine Maleate and Phenylpropanolamine Hydrochloride Capsules, Extended-Release	T, LR
Chlorpheniramine Maleate and Phenylpropanolamine Hydrochloride Tablets, Extended-Release	T, LR
Chlorpheniramine Maleate and Pseudoephedrine Hydrochloride Capsules, Extended-Release	T, LR
Chlorpromazine Hydrochloride Tablets	W, LR
Chlorpropamide Tablets	W
Chlortetracycline Hydrochloride Tablets	T, LR
Chlorthalidone Tablets	W
Chlorzoxazone Tablets	T
Chlorzoxazone and Acetaminophen Capsules	T
Chondroitin Sulfate Sodium Tablets	T, LR
Chromium Picolinate Tablets	W
Cimetidine Tablets	T, LR
Cilostazol Tablets	T, LR
Cinoxacin Capsules	W
Ciprofloxacin Tablets	W
Citalopram Tablets	W
Clarithromycin Tablets	T
Clarithromycin Tablets, Extended-Release	W
Clemastine Fumarate Tablets	W
Clindamycin Hydrochloride Capsules	T
Clofazimine Capsules	W
Clofibrate Capsules	W, LR
Clomiphene Citrate Tablets	W
Clomipramine Hydrochloride Capsules	W
Clonazepam Tablets	T, LR
Clonazepam Tablets, Orally Disintegrating	W, LR
Clonidine Hydrochloride Tablets	W
Clonidine Hydrochloride and Chlorthalidone Tablets	W
Clopidogrel Tablets	W
Clorazepate Dipotassium Tablets	T, LR
Clotrimazole Tablets, Vaginal	W
Red Clover Tablets	T, LR
Cloxacillin Sodium Capsules	T
Clozapine Tablets	W
Cyanocobalamin Co 57 Capsules	W, LR
Cyanocobalamin Co 58 Capsules	W, LR
Cocaine Hydrochloride Tablets for Topical Solution	W, LR
Codeine Phosphate Tablets	W, LR
Codeine Sulfate Tablets	W
Colectipol Hydrochloride Tablets	T
Cortisone Acetate Tablets	W
Cromolyn Sodium for Inhalation (in Capsules)	T, LR
Cryptocodinium cohnii Oil Capsules	T, LR
Curcuminoids Capsules	W, LR
Curcuminoids Tablets	W, LR
Cyclizine Hydrochloride Tablets	T, LR
Cyclobenzaprine Hydrochloride Tablets	W
Cyclophosphamide Tablets	T

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Cycloserine Capsules	T
Cyclosporine Capsules	T
Cyproheptadine Hydrochloride Tablets	W
Danazol Capsules	W
Dantrolene Sodium Capsules	T
Dapsone Tablets	W, LR
Dehydrocholic Acid Tablets	W
Demeclocycline Hydrochloride Capsules	T, LR
Demeclocycline Hydrochloride Tablets	T, LR
Desipramine Hydrochloride Tablets	T
Desogestrel and Ethinyl Estradiol Tablets	W
Dexamethasone Tablets	W
Dexchlorpheniramine Maleate Tablets	T
Dextroamphetamine Sulfate Capsules	T
Dextroamphetamine Sulfate Tablets	W
Diazepam Capsules	T, LR
Diazepam Capsules, Extended-Release	T, LR
Diazepam Tablets	T, LR
Diazoxide Capsules	W
Dichlorphenamide Tablets	W
Diclofenac Potassium Tablets	T, LR
Diclofenac Sodium Tablets, Delayed-Release	T, LR
Diclofenac Sodium Tablets, Extended-Release	W
Dicloxacillin Sodium Capsules	T
Dicyclomine Hydrochloride Capsules	W
Dicyclomine Hydrochloride Tablets	W
Add the following:	
▲ Didanosine Capsules, Delayed-Release	W ▲ USP36
Didanosine Tablets for Oral Suspension	T
Diethylcarbamazine Citrate Tablets	T
Diethylpropion Hydrochloride Tablets	W
Diethylstilbestrol Tablets	W
Diffunisal Tablets	W
Digitalis Capsules	T
Digitalis Tablets	T
Digitoxin Tablets	W
Digoxin Tablets	T
Dihydrotachysterol Capsules	W, LR
Dihydrotachysterol Tablets	W, LR
Dihydroxyaluminum Sodium Carbonate Tablets	W
Diltiazem Hydrochloride Tablets	T, LR
Diltiazem Hydrochloride Capsules, Extended-Release	T
Dimenhydrinate Tablets	W
Diphenhydramine Hydrochloride Capsules	T
Diphenhydramine and Pseudoephedrine Capsules	T
Diphenoxylate Hydrochloride and Atropine Sulfate Tablets	W, LR
Dipyridamole Tablets	T, LR
Dirithromycin Tablets, Delayed-Release	T
Disopyramide Phosphate Capsules	W
Disopyramide Phosphate Capsules, Extended-Release	W
Disulfiram Tablets	T, LR
Divalproex Sodium Capsules, Delayed-Release	T, LR
Divalproex Sodium Tablets, Delayed-Release	T, LR
Divalproex Sodium Tablets, Extended-Release	W
Docusate Calcium Capsules	T
Docusate Potassium Capsules	T
Docusate Sodium Capsules	T

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Docusate Sodium Tablets	W
Dolasetron Mesylate Tablets	W
Donepezil Hydrochloride Tablets	W
Donepezil Hydrochloride Tablets, Orally Disintegrating	W
Doxazosin Tablets	T
Doxepin Hydrochloride Capsules	W
Doxycycline Capsules	T, LR
Doxycycline Hyclate Capsules	T, LR
Doxycycline Hyclate Capsules, Delayed-Release	T, LR
Doxycycline Hyclate Tablets	T, LR
Doxycycline Hyclate Tablets, Delayed-Release	T, LR
Add the following:	
▲ Doxycycline Tablets	T, LR ▲ <i>USP36</i>
Doxylamine Succinate Tablets	W, LR
Dronabinol Capsules	W, LR
Drospirenone and Ethinyl Estradiol Tablets	W
Duloxetine Capsules, Delayed-Release	T
Dydrogesterone Tablets	W
Dyphylline Tablets	T
Dyphylline and Guaifenesin Tablets	T
Efavirenz Capsules	W
Enalapril Maleate Tablets	W
Enalapril Maleate and Hydrochlorothiazide Tablets	W
Entacapone Tablets	LR
Ephedrine Sulfate Capsules	T, LR
Ergocalciferol Capsules	T, LR
Ergocalciferol Tablets	T, LR
Ergoloid Mesylates Capsules	T, LR
Ergoloid Mesylates Tablets	T, LR
Ergoloid Mesylates Tablets, Sublingual	T, LR
Ergonovine Maleate Tablets	W
Ergotamine Tartrate Tablets	W, LR
Ergotamine Tartrate Tablets, Sublingual	W, LR
Ergotamine Tartrate and Caffeine Tablets	W, LR
Erythromycin Capsules, Delayed-Release	T
Erythromycin Tablets	T
Erythromycin Tablets, Delayed-Release	T
Erythromycin Estolate Capsules	T
Erythromycin Estolate Tablets	T
Erythromycin Ethylsuccinate Tablets	T
Erythromycin Stearate Tablets	T
Escitalopram Tablets	W
Esomeprazole Magnesium Capsules, Delayed-Release	T
Estazolam Tablets	T, LR
Estradiol Tablets	T, LR
Estradiol and Norethindrone Acetate Tablets	W
Estrogens Tablets, Conjugated	W
Estrogens Tablets, Esterified	W
Estropipate Tablets	W
Ethacrynic Acid Tablets	W
Ethambutol Hydrochloride Tablets	W
Ethchlorvynol Capsules	T, LR
Ethinyl Estradiol Tablets	W
Ethionamide Tablets	W
Ethosuximide Capsules	T
Ethotoin Tablets	T
Ethinodiol Diacetate and Ethinyl Estradiol Tablets	W

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Ethinodiol Diacetate and Mestranol Tablets	W
Etidronate Disodium Tablets	T
Etodolac Capsules	T
Etodolac Tablets	T
Etodolac Tablets, Extended-Release	W
Famotidine Tablets	W, LR
Felbamate Tablets	W
Felodipine Tablets, Extended-Release	T
Fenofibrate Capsules	W
Fenofibrate Tablets	W
Fenoprofen Calcium Capsules	W
Fenoprofen Calcium Tablets	W
Ferrous Fumarate Tablets	T
Ferrous Fumarate and Docusate Sodium Tablets, Extended-Release	W
Ferrous Gluconate Capsules	T
Ferrous Gluconate Tablets	T
Ferrous Sulfate Tablets	T
Fexofenadine Hydrochloride Capsules	T, LR
Fexofenadine Hydrochloride Tablets	W
Fexofenadine Hydrochloride and Pseudoephedrine Hydrochloride Tablets, Extended-Release	W
Finasteride Tablets	T, LR
Fish Oil Containing Omega-3 Acids Capsules	T, LR
Fish Oil Containing Omega-3 Acids Capsules, Delayed-Release	T, LR
Flavoxate Hydrochloride Tablets	W, LR
Flecainide Acetate Tablets	W
Fluconazole Tablets	W
Flucytosine Capsules	T, LR
Fludrocortisone Acetate Tablets	W
Fluoxetine Capsules	T, LR
Fluoxetine Capsules, Delayed-Release	T
Fluoxetine Tablets	T
Fluoxymesterone Tablets	W
Fluphenazine Hydrochloride Tablets	T, LR
Flurazepam Hydrochloride Capsules	T, LR
Flurbiprofen Tablets	W
Flutamide Capsules	W, LR
Fluvastatin Capsules	T, LR
Fluvoxamine Maleate Tablets	T
Folic Acid Tablets	W
Fosinopril Sodium Tablets	T
Fosinopril Sodium and Hydrochlorothiazide Tablets	T
Furazolidone Tablets	T, LR
Furosemide Tablets	W, LR
Gabapentin Capsules	W
Gabapentin Tablets	W
Galantamine Tablets	W
Garlic Tablets, Delayed-Release	T
Gemfibrozil Capsules	T
Gemfibrozil Tablets	T
Ginger Capsules	W
Ginkgo Capsules	T, LR
Ginkgo Tablets	T, LR
American Ginseng Capsules	T, LR
American Ginseng Tablets	T, LR
Asian Ginseng Capsules	T, LR
Asian Ginseng Tablets	T, LR
Glimepiride Tablets	W

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Glipizide Tablets	T
Glipizide and Metformin Hydrochloride Tablets	W
Glucosamine Tablets	T, LR
Glucosamine and Chondroitin Sulfate Tablets	T, LR
Glucosamine and Methylsulfonylmethane Tablets	T, LR
Glucosamine, Chondroitin Sulfate Sodium, and Methylsulfonylmethane Tablets	T, LR
Glyburide Tablets	W
Glyburide and Metformin Hydrochloride Tablets	T, LR
Glycopyrrolate Tablets	T
Granisetron Hydrochloride Tablets	W, LR
Griseofulvin Capsules	T
Griseofulvin Tablets	T
Griseofulvin Tablets, Ultramicrosize	W
Guaifenesin Capsules	T
Guaifenesin Tablets	T
Guaifenesin and Pseudoephedrine Hydrochloride Capsules	T, LR
Guaifenesin, Pseudoephedrine Hydrochloride, and Dextromethorphan Hydrobromide Capsules	T, LR
Guanabenz Acetate Tablets	T, LR
Guanadrel Sulfate Tablets	T, LR
Guanethidine Monosulfate Tablets	W
Guanfacine Tablets	T, LR
Guggul Tablets	W, LR
Halazone Tablets for Solution	T, LR
Haloperidol Tablets	T, LR
Hexylresorcinol Lozenges	W
Homatropine Methylbromide Tablets	T, LR
Hydralazine Hydrochloride Tablets	T, LR
Hydrochlorothiazide Capsules	W
Hydrochlorothiazide Tablets	W
Hydrocodone Bitartrate Tablets	T, LR
Hydrocodone Bitartrate and Acetaminophen Tablets	T, LR
Hydrocodone Bitartrate and Homatropine Methylbromide Tablets	T, LR
Hydrocortisone Tablets	W
Hydroflumethiazide Tablets	T
Hydromorphone Hydrochloride Tablets	T, LR
Hydroxychloroquine Sulfate Tablets	T, LR
Hydroxyurea Capsules	T
Hydroxyzine Hydrochloride Tablets	T
Hydroxyzine Pamoate Capsules	W
Hyoscyamine Tablets	W, LR
Hyoscyamine Sulfate Tablets	T, LR
Ibuprofen Tablets	W
Ibuprofen and Pseudoephedrine Hydrochloride Tablets	T
Imipramine Hydrochloride Tablets	T
Indapamide Tablets	W
Indomethacin Capsules	W
Indomethacin Capsules, Extended-Release	W
Sodium Iodide I 123 Capsules	W
Sodium Iodide I 131 Capsules	W
Iodoquinol Tablets	W
Iopanoic Acid Tablets	T, LR
Iopodate Sodium Capsules	T
Irbesartan Tablets	W
Irbesartan and Hydrochlorothiazide Tablets	W
Isoniazid Tablets	W, LR
Isopropamide Iodide Tablets	W

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Isoproterenol Hydrochloride Tablets	W, LR
Isosorbide Dinitrate Capsules, Extended-Release	W
Isosorbide Dinitrate Tablets	W
Isosorbide Dinitrate Tablets, Chewable	W
Isosorbide Dinitrate Tablets, Extended-Release	W
Isosorbide Dinitrate Tablets, Sublingual	W
Isosorbide Mononitrate Tablets	T
Isosorbide Mononitrate Tablets, Extended-Release	T
Isotretinoin Capsules	T
Isoxsuprine Hydrochloride Tablets	T
Isradipine Capsules	T
Ivermectin Tablets	W
Ivermectin and Pyrantel Pamoate Tablets	T, LR
Kanamycin Sulfate Capsules	T
Ketoconazole Tablets	W
Ketoprofen Capsules, Extended-Release	T
Ketorolac Tromethamine Tablets	W
Labetalol Hydrochloride Tablets	T, LR
Lamivudine and Zidovudine Tablets	W, LR
Lamotrigine Tablets	W
Lamotrigine Tablets for Oral Suspension	T, LR
Lansoprazole Capsules, Delayed-Release	T
Leflunomide Tablets	T, LR
Letrozole Tablets	T
Leucovorin Calcium Tablets	W, LR
Levamisole Hydrochloride Tablets	W
Levetiracetam Tablets	T
Levocarnitine Tablets	T
Levodopa Capsules	T, LR
Levodopa Tablets	T, LR
Levonorgestrel and Ethinyl Estradiol Tablets	W
Livorphanol Tartrate Tablets	W
Levothyroxine Sodium Tablets	T, LR
Lincomycin Hydrochloride Capsules	T
Alpha Lipoic Acid Capsules	W
Alpha Lipoic Acid Tablets	W
Liothyronine Sodium Tablets	T
Liotrix Tablets	T
Lisinopril Tablets	T
Lisinopril and Hydrochlorothiazide Tablets	W
Lithium Carbonate Capsules	W
Lithium Carbonate Tablets	W
Lithium Carbonate Tablets, Extended-Release	W
Loperamide Hydrochloride Capsules	W
Loracarbef Capsules	W
Loratadine Tablets	T
Loratadine Tablets, Orally Disintegrating	T
Lorazepam Tablets	T, LR
Losartan Potassium Tablets	T, LR
Losartan Potassium and Hydrochlorothiazide Tablets	T, LR
Lovastatin Tablets	W
Loxapine Capsules	T
Lysine Hydrochloride Tablets	W
Magaldrate Tablets	W
Magaldrate and Simethicone Tablets	W
Magnesia Tablets	W
Magnesia and Alumina Tablets	W
Magnesium Gluconate Tablets	W

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Magnesium Oxide Capsules	W
Magnesium Oxide Tablets	W
Magnesium Salicylate Tablets	T
Magnesium Trisilicate Tablets	W
Maprotiline Hydrochloride Tablets	W
Mazindol Tablets	T
Mebendazole Tablets	W
Mecamylamine Hydrochloride Tablets	W
Meclizine Hydrochloride Tablets	W
Meclofenamate Sodium Capsules	T, LR
Medroxyprogesterone Acetate Tablets	W
Mefenamic Acid Capsules	T
Mefloquine Hydrochloride Tablets	T, LR
Megestrol Acetate Tablets	W
Add the following:	
▲ Melatonin Tablets	T, LR ▲ <i>USP36</i>
Meloxicam Tablets	W
Melphalan Tablets	W, LR
Menadiol Sodium Diphosphate Tablets	W, LR
Meperidine Hydrochloride Tablets	W, LR
Mephénytoin Tablets	W
Mephobarbital Tablets	W
Meprobamate Tablets	W
Mercaptopurine Tablets	W
Mesalamine Capsules, Extended-Release	T, LR
Mesalamine Tablets, Delayed-Release	T
Mesoridazine Besylate Tablets	W, LR
Metaproterenol Sulfate Tablets	W, LR
Metformin Hydrochloride Tablets	T
Metformin Hydrochloride Tablets, Extended-Release	W, LR
Methacycline Hydrochloride Capsules	T, LR
Methadone Hydrochloride Tablets	W
Methamphetamine Hydrochloride Tablets	T, LR
Methazolamide Tablets	W
Methdilazine Hydrochloride Tablets	T, LR
Methenamine Tablets	W
Methenamine Hippurate Tablets	W
Methenamine Mandelate Tablets	W
Methenamine Mandelate Tablets, Delayed-Release	W
Methimazole Tablets	W, LR
Methocarbamol Tablets	T
Methotrexate Tablets	W
Methoxsalen Capsules	T, LR
Methscopolamine Bromide Tablets	T
Methsuximide Capsules	T
Methyclothiazide Tablets	W
Methylcellulose Tablets	W
Methyldopa Tablets	W
Methyldopa and Chlorothiazide Tablets	W
Methyldopa and Hydrochlorothiazide Tablets	W
Methylergonovine Maleate Tablets	T, LR
Methylphenidate Hydrochloride Tablets	T
Methylphenidate Hydrochloride Tablets, Extended-Release	T
Methylprednisolone Tablets	T
Methylsulfonylmethane Tablets	T, LR
Methyltestosterone Capsules	W
Methyltestosterone Tablets	W

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Methysergide Maleate Tablets	T
Metoclopramide Tablets	T, LR
Metolazone Tablets	T, LR
Metoprolol Succinate Tablets, Extended-Release	T
Metoprolol Tartrate Tablets	T, LR
Metoprolol Tartrate and Hydrochlorothiazide Tablets	T, LR
Metronidazole Capsules	W, LR
Metronidazole Tablets	W, LR
Metirapone Tablets	T, LR
Metirosine Capsules	W
Mexiletine Hydrochloride Capsules	T
Midodrine Hydrochloride Tablets	W
Minerals Capsules	T, LR
Minerals Tablets	T, LR
Minocycline Hydrochloride Capsules	T, LR
Minocycline Hydrochloride Tablets	T, LR
Minoxidil Tablets	T
Mirtazapine Tablets	T, LR
Mirtazapine Tablets, Orally Disintegrating	LR
Mitotane Tablets	T, LR
Modafinil Tablets	T
Molindone Hydrochloride Tablets	T, LR
Moricizine Hydrochloride Tablets	T
Morphine Sulfate Capsules, Extended-Release	T, LR
Mycophenolate Mofetil Capsules	W, LR
Mycophenolate Mofetil Tablets	W, LR
Nabumetone Tablets	W
Nadolol Tablets	T
Nadolol and Bendroflumethiazide Tablets	T
Nafcillin Sodium Capsules	T
Nafcillin Sodium Tablets	T, LR
Nalidixic Acid Tablets	T
Naltrexone Hydrochloride Tablets	T
Naproxen Tablets	W
Naproxen Tablets, Delayed-Release	W
Naproxen Sodium Tablets	W
Naratriptan Tablets	T
Nateglinide Tablets	T
Nefazodone Hydrochloride Tablets	T
Neomycin Sulfate Tablets	T
Neostigmine Bromide Tablets	T
Nevirapine Tablets	W
Niacin Tablets	W
Niacin Tablets, Extended-Release	T
Niacinamide Tablets	T
Nifedipine Capsules	T, LR
Nifedipine Tablets, Extended-Release	T, LR
Nitrofurantoin Capsules	T
Nitrofurantoin Tablets	T, LR
Nitroglycerin Tablets	T
Nitroglycerin Tablets, Sublingual	T
Norethindrone Tablets	W
Norethindrone and Ethinyl Estradiol Tablets	W
Norethindrone and Mestranol Tablets	W
Norethindrone Acetate Tablets	W
Norethindrone Acetate and Ethinyl Estradiol Tablets	W
Norfloxacin Tablets	W

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Norgestimate and Ethinyl Estradiol Tablets	W
Norgestrel Tablets	W
Norgestrel and Ethinyl Estradiol Tablets	W
Nortriptyline Hydrochloride Capsules	T
Nystatin Tablets	T, LR
Nystatin Tablets, Vaginal	W, LR
Ofloxacin Tablets	W
Olanzapine Tablets	T, LR
Olanzapine and Fluoxetine Capsules	T
Oleovitamin A and D Capsules	T, LR
Omega-3 Acids Ethyl Esters Capsules	T, LR
Omeprazole Capsules, Delayed-Release	T, LR
Ondansetron Tablets	T, LR
Ondansetron Tablets, Orally Disintegrating	LR
Orbifloxacin Tablets	T
Orlistat Capsules	T
Orphenadrine Citrate Tablets, Extended-Release	T, LR
Oseltamivir Phosphate Capsules	W
Oxacillin Sodium Capsules	T
Oxandrolone Tablets	T, LR
Oxaprozin Tablets	T, LR
Oxazepam Capsules	W
Oxazepam Tablets	W
Oxprenolol Hydrochloride Tablets	W, LR
Oxprenolol Hydrochloride Tablets, Extended-Release	W, LR
Oxtriphylline Tablets	T
Oxtriphylline Tablets, Delayed-Release	T
Oxtriphylline Tablets, Extended-Release	T
Oxybutynin Chloride Tablets	T, LR
Oxybutynin Chloride Tablets, Extended-Release	T
Oxycodone Hydrochloride Tablets	T, LR
Oxycodone Hydrochloride Tablets, Extended-Release	T, LR
Oxycodone and Acetaminophen Capsules	T, LR
Oxycodone and Acetaminophen Tablets	T, LR
Oxycodone and Aspirin Tablets	T, LR
Oxymetholone Tablets	W
Oxytetracycline Tablets	T, LR
Oxytetracycline and Nystatin Capsules	T, LR
Oxytetracycline Hydrochloride Capsules	T, LR
Oxytetracycline Hydrochloride and Polymyxin B Sulfate Tablets, Vaginal	W
Pancreatin Tablets	T
Pancrelipase Capsules	T
Pancrelipase Capsules, Delayed-Release	T
Pancrelipase Tablets	T
Pantoprazole Sodium Tablets, Delayed-Release	W
Papain Tablets for Topical Solution	T, LR
Papaverine Hydrochloride Tablets	T
Paromomycin Sulfate Capsules	T
Paroxetine Tablets	T
Penbutolol Sulfate Tablets	W, LR
Penicillamine Capsules	T
Penicillamine Tablets	T
Penicillin G Benzathine Tablets	T
Penicillin G Potassium Tablets	T
Penicillin V Tablets	T
Penicillin V Potassium Tablets	T
Pentazocine and Acetaminophen Tablets	T, LR

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Pentazocine and Aspirin Tablets	T, LR
Pentazocine and Naloxone Tablets	T, LR
Pentoxifylline Tablets, Extended-Release	W
Perphenazine Tablets	T, LR
Perphenazine and Amitriptyline Hydrochloride Tablets	W
Phenazopyridine Hydrochloride Tablets	T
Phendimetrazine Tartrate Capsules	T
Phendimetrazine Tartrate Tablets	W
Phenelzine Sulfate Tablets	T
Phenmetrazine Hydrochloride Tablets	T
Phenobarbital Tablets	W
Phenoxybenzamine Hydrochloride Capsules	W
Phensuximide Capsules	T
Phentermine Hydrochloride Capsules	T
Phentermine Hydrochloride Tablets	T
Phenylbutazone Tablets	T
Phenylpropanolamine Hydrochloride Capsules	T, LR
Phenylpropanolamine Hydrochloride Capsules, Extended-Release	T, LR
Phenylpropanolamine Hydrochloride Tablets	T, LR
Phenylpropanolamine Hydrochloride Tablets, Extended-Release	T, LR
Phenytoin Tablets	W
Phenytoin Sodium Capsules, Extended	T
Phenytoin Sodium Capsules, Prompt	T
Phytonadione Tablets	W, LR
Pilocarpine Hydrochloride Tablets	T
Pimozide Tablets	T, LR
Pindolol Tablets	W, LR
Pioglitazone Tablets	T
Piperazine Citrate Tablets	T
Piroxicam Capsules	T, LR
Potassium Bicarbonate Effervescent Tablets for Oral Solution	T
Potassium Bicarbonate and Potassium Chloride Effervescent Tablets for Oral Solution	T
Potassium and Sodium Bicarbonates and Citric Acid Effervescent Tablets for Oral Solution	T
Potassium Chloride Capsules, Extended-Release	T
Potassium Chloride Tablets, Extended-Release	T
Potassium Chloride, Potassium Bicarbonate, and Potassium Citrate Effervescent Tablets for Oral Solution	T
Potassium Citrate Tablets	W
Potassium Citrate Tablets, Extended-Release	T
Potassium Gluconate Tablets	T
Potassium Iodide Tablets	T
Potassium Iodide Tablets, Delayed-Release	T
Potassium Perchlorate Capsules	T, LR
Pravastatin Sodium Tablets	T
Praziquantel Tablets	T
Prazosin Hydrochloride Capsules	W, LR
Prednisolone Tablets	W
Prednisone Tablets	W
Primaquine Phosphate Tablets	W, LR
Primidone Tablets	W
Probenecid and Colchicine Tablets	W, LR
Probuco Tablets	W, LR
Procainamide Hydrochloride Capsules	T
Procainamide Hydrochloride Tablets	T
Procarbazine Hydrochloride Capsules	T, LR

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Prochlorperazine Maleate Tablets	W
Procyclidine Hydrochloride Tablets	T
Promazine Hydrochloride Tablets	T, LR
Promethazine Hydrochloride Tablets	T, LR
Propantheline Bromide Tablets	W
Propoxyphene Hydrochloride Capsules	T
Propoxyphene Hydrochloride and Acetaminophen Tablets	T
Propoxyphene Hydrochloride, Aspirin, and Caffeine Capsules	T
Propoxyphene Napsylate Tablets	T
Propoxyphene Napsylate and Acetaminophen Tablets	T
Propoxyphene Napsylate and Aspirin Tablets	T
Propranolol Hydrochloride Capsules, Extended-Release	W
Propranolol Hydrochloride Tablets	W, LR
Propranolol Hydrochloride and Hydrochlorothiazide Capsules, Extended-Release	W
Propranolol Hydrochloride and Hydrochlorothiazide Tablets	W
Propylthiouracil Tablets	W
Protriptyline Hydrochloride Tablets	T
Pseudoephedrine Hydrochloride Tablets	T
Pseudoephedrine Hydrochloride Tablets, Extended-Release	T
Pygeum Capsules	T
Pyrazinamide Tablets	W
Pyridostigmine Bromide Tablets	T
Pyridoxine Hydrochloride Tablets	W
Pyrilamine Maleate Tablets	W
Pyrimethamine Tablets	T, LR
Pyrvinium Pamoate Tablets	T, LR
Quazepam Tablets	W
Quinapril Tablets	W
Quinidine Gluconate Tablets, Extended-Release	W, LR
Quinidine Sulfate Capsules	T, LR
Quinidine Sulfate Tablets	W, LR
Quinidine Sulfate Tablets, Extended-Release	W, LR
Quinine Sulfate Capsules	T
Quinine Sulfate Tablets	W
Raloxifene Hydrochloride Tablets	T
Ramipril Capsules	W
Ranitidine Tablets	T, LR
Rauwolfia Serpentina Tablets	T, LR
Reserpine Tablets	T, LR
Reserpine and Chlorothiazide Tablets	T, LR
Reserpine, Hydralazine Hydrochloride, and Hydrochlorothiazide Tablets	T, LR
Reserpine and Hydrochlorothiazide Tablets	T, LR
Ribavirin Tablets	T
Riboflavin Tablets	T, LR
Rifabutin Capsules	W
Rifampin Capsules	T, LR
Rifampin and Isoniazid Capsules	T, LR
Rifampin, Isoniazid, and Pyrazinamide Tablets	T, LR
Rifampin, Isoniazid, Pyrazinamide, and Ethambutol Hydrochloride Tablets	T, LR
Riluzole Tablets	W, LR
Rimantadine Hydrochloride Tablets	T, LR
Risedronate Sodium Tablets	W
Risperidone Tablets, Orally Disintegrating	W, LR

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Risperidone Tablets	T, LR
Ritodrine Hydrochloride Tablets	T
Rivastigmine Tartrate Capsules	T
Ropinirole Tablets	W
Add the following:	
▲ Rufinamide Tablets	T ▲ USP36
Saccharin Sodium Tablets	W
Salsalate Capsules	T
Salsalate Tablets	T
Saquinavir Capsules	T
Saw Palmetto Capsules	T, LR
Schizochytrium Oil Capsules	T, LR
Scopolamine Hydrobromide Tablets	T, LR
Secobarbital Sodium Capsules	T
Secobarbital Sodium and Amobarbital Sodium Capsules	W
Selegiline Hydrochloride Tablets	T, LR
Sennosides Tablets	W
Sertraline Tablets	W
Simethicone Capsules	W
Simethicone Tablets	W
Simvastatin Tablets	T
Sodium Bicarbonate Tablets	W
Sodium Chloride Tablets	W
Sodium Chloride Tablets for Solution	W
Sodium Chloride and Dextrose Tablets	W
Sodium Fluoride Tablets	T
Sodium Salicylate Tablets	W
Sotalol Hydrochloride Tablets	W, LR
Soy Isoflavones Capsules	T, LR
Soy Isoflavones Tablets	T, LR
Spironolactone Tablets	T, LR
Spironolactone and Hydrochlorothiazide Tablets	T, LR
Stanozolol Tablets	T, LR
Stavudine Capsules	T
Sulfadiazine Tablets	W, LR
Sulfadimethoxine Tablets	T, LR
Sulfadoxine and Pyrimethamine Tablets	W, LR
Sulfamethizole Tablets	W
Sulfamethoxazole Tablets	W, LR
Sulfamethoxazole and Trimethoprim Tablets	W, LR
Sulfapyridine Tablets	W, LR
Sulfasalazine Tablets	W
Sulfasalazine Tablets, Delayed-Release	W
Sulfipyrazone Capsules	W
Sulfipyrazone Tablets	W
Sulfisoxazole Tablets	W, LR
Sulindac Tablets	W
Sumatriptan Tablets	W
Tacrolimus Capsules	T
Add the following:	
▲ Tadalafil Tablets	T ▲ USP36
Tamoxifen Citrate Tablets	W, LR
Tamsulosin Hydrochloride Capsules	T
Telmisartan Tablets	W
Telmisartan and Hydrochlorothiazide Tablets	W
Temazepam Capsules	W, LR
Terazosin Capsules	W, LR
Terazosin Tablets	W, LR

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Terbinafine Tablets	W, LR
Terbutaline Sulfate Tablets	T
Testolactone Tablets	T
Tetracycline Hydrochloride Capsules	T, LR
Tetracycline Hydrochloride Tablets	T, LR
Tetracycline Hydrochloride and Novobiocin Sodium Tablets	T
Tetracycline Hydrochloride, Novobiocin Sodium, and Prednisolone Tablets	T
Tetracycline Hydrochloride and Nystatin Capsules	T, LR
Thalidomide Capsules	W
Theophylline Capsules	W
Theophylline Capsules, Extended-Release	W
Theophylline Tablets	W
Theophylline, Ephedrine Hydrochloride, and Phenobarbital Tablets	T
Theophylline and Guaifenesin Capsules	T
Theophylline Sodium Glycinate Tablets	W
Thiabendazole Tablets	T
Thiamine Hydrochloride Tablets	T, LR
Thiethylperazine Maleate Tablets	T, LR
Thioguanine Tablets	T
Thioridazine Hydrochloride Tablets	T, LR
Thiothixene Capsules	W, LR
Thyroid Tablets	T
Ticlopidine Hydrochloride Tablets	W
Timolol Maleate Tablets	W
Timolol Maleate and Hydrochlorothiazide Tablets	W, LR
Tizanidine Tablets	T
Tocainide Hydrochloride Tablets	W
Tolazamide Tablets	T
Tolazoline Hydrochloride Tablets	W
Tolbutamide Tablets	W
Tolcapone Tablets	T
Tolmetin Sodium Capsules	T
Tolmetin Sodium Tablets	W
Topiramate Tablets	T
Tramadol Hydrochloride Tablets	T
Tramadol Hydrochloride Tablets, Extended-Release	T
Trandolapril Tablets	T
Tranylcypromine Tablets	W
Trazodone Hydrochloride Tablets	T, LR
Triamcinolone Tablets	W
Triamterene Capsules	T, LR
Triamterene and Hydrochlorothiazide Capsules	T, LR
Triamterene and Hydrochlorothiazide Tablets	T, LR
Triazolam Tablets	T, LR
Trichlormethiazide Tablets	T
Trientine Hydrochloride Capsules	T
Trifluoperazine Hydrochloride Tablets	W, LR
Triflupromazine Hydrochloride Tablets	W, LR
Trihexyphenidyl Hydrochloride Capsules, Extended-Release	T
Trihexyphenidyl Hydrochloride Tablets	T
Trimeprazine Tartrate Tablets	W, LR
Trimethobenzamide Hydrochloride Capsules	W
Trimethoprim Tablets	T, LR
Trioxsalen Tablets	W, LR

Container Specifications for Capsules and Tablets (Continued)

Monograph Title	Container Specification
Tripelennamine Hydrochloride Tablets	W
Triple Sulfa Tablets, Vaginal	W, LR
Tripolidine Hydrochloride Tablets	T, LR
Tripolidine and Pseudoephedrine Hydrochlorides Tablets	T, LR
Trisulfapyrimidines Tablets	W
Troleandomycin Capsules	T
Add the following:	
▲ Trosium Chloride Tablets	T, LR▲ USP36
Ubidecarenone Capsules	T, LR
Ubidecarenone Tablets	T, LR
Ursodiol Capsules	W
Ursodiol Tablets	W
Valacyclovir Tablets	T
Valerian Tablets	T, LR
Valganciclovir Tablets	T
Valproic Acid Capsules	T
Valsartan Tablets	T
Valsartan and Hydrochlorothiazide Tablets	T
Vancomycin Hydrochloride Capsules	T
Venlafaxine Tablets	W
Verapamil Hydrochloride Capsules, Extended-Release	T, LR
Verapamil Hydrochloride Tablets	T, LR
Verapamil Hydrochloride Tablets, Extended-Release	T, LR
Vitamin A Capsules	T, LR
Vitamin A Tablets	T, LR
Vitamin E Capsules	T
Oil-Soluble Vitamins Capsules	T, LR
Oil-Soluble Vitamins Tablets	T, LR
Add the following:	
▲ Oil-Soluble Vitamins with Minerals Capsules	T, LR▲ USP36
Add the following:	
▲ Oil-Soluble Vitamins with Minerals Tablets	T, LR▲ USP36
Oil- and Water-Soluble Vitamins Capsules	T, LR
Oil- and Water-Soluble Vitamins Tablets	T, LR
Oil- and Water-Soluble Vitamins with Minerals Capsules	T, LR
Oil- and Water-Soluble Vitamins with Minerals Tablets	T, LR
Water-Soluble Vitamins Capsules	T, LR
Water-Soluble Vitamins Tablets	T, LR
Water-Soluble Vitamins with Minerals Capsules	T, LR
Water-Soluble Vitamins with Minerals Tablets	T, LR
Warfarin Sodium Tablets	T, LR
Zalcitabine Tablets	T, LR
Zaleplon Capsules	LR
Zidovudine Capsules	T, LR
Zidovudine Tablets	T, LR
Zinc Citrate Tablets	W
Zinc Gluconate Tablets	T, LR
Zinc Sulfate Tablets	W
Zolpidem Tartrate Tablets	W
Zolpidem Tartrate Tablets, Extended-Release	W
Zonisamide Capsules	T, LR

DESCRIPTION AND SOLUBILITY

Description and Relative Solubility of USP and NF Articles

The "description" and "solubility" statements pertaining to an article (formerly included in the individual monograph) are general in nature. The information is provided for those who use, prepare, and dispense drugs, solely to indicate descriptive and solubility properties of an article complying with monograph standards. The properties are not in themselves standards or tests for purity even though they may indirectly assist in the preliminary evaluation of the integrity of an article.

Taste and Odor

Organoleptic characteristics are indicated in many instances because they may be useful and descriptive properties of substances. However, they are not meant to be applied as tests for identifying materials.

The inclusion of odor or taste among other descriptive properties may aid in identifying the causative agent following accidental exposure to or contact with a substance. This information is provided as a warning or to make an individual aware of sensations that may be encountered. The use of odor or taste as a test for identification or content is strongly discouraged.

The characteristic odor of a volatile substance becomes apparent immediately on opening a container of it. The odor may be agreeable (e.g., Peppermint Oil), unpleasant (e.g., Sulfur Dioxide), or potentially hazardous on prolonged exposure (e.g., Coal Tar). Moreover, an unexpected odor may be encountered if the characteristics of a substance are not known or if a container is incorrectly labeled. Consequently, containers of such substances should be opened cautiously, preferably in a well-ventilated fume hood. A characteristic taste or sensation produced in the oral cavity likewise is apparent if traces of residue materials on fingers are inadvertently brought into contact with the tongue or adjacent mucosal tissues.

Solubility

Only where a special, quantitative solubility test is given in the individual monograph, and is designated by a test heading, is it a test for purity.

The approximate solubilities of Pharmacopeial and National Formulary substances are indicated by the descriptive terms in the accompanying table. The term "miscible" as used in this Pharmacopeia pertains to a substance that yields a homogeneous mixture when mixed in any proportion with the designated solvent.

Descriptive Term	Parts of Solvent Required for 1 Part of Solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1000
Very slightly soluble	From 1000 to 10,000
Practically insoluble, or Insoluble	10,000 and over

Soluble Pharmacopeial and National Formulary articles, when brought into solution, may show traces of physical impurities, such as minute fragments of filter paper, fibers,

and other particulate matter, unless limited or excluded by definite tests or other specifications in the individual monographs.

Abacavir Sulfate: White to off-white powder. Soluble in water, in ethyl acetate, in absolute alcohol, and in methanol.

Acacia: Is practically odorless and produces a mucilaginous sensation on the tongue. Insoluble in alcohol. Optical rotation varies depending on the source of Acacia. For example, specific rotation values, calculated on the anhydrous basis and determined on a 1.0% (w/v) solution, usually are between -25° and -35° for *Acacia senegal* and between $+35^{\circ}$ and $+60^{\circ}$ for *Acacia seyal*. *NF category:* Emulsifying and/or solubilizing agent; suspending and/or viscosity-increasing agent; tablet binder.

Acebutolol Hydrochloride: White or almost white, crystalline powder. Soluble in alcohol and in water; very slightly soluble in acetone and in methylene chloride; practically insoluble in ether. Melts at about 141° to 144° .

Acesulfame Potassium: A white, crystalline powder or colorless crystals. Soluble in water; very slightly soluble in acetone and in alcohol. *NF category:* Sweetening agent.

Acetaminophen: White, odorless, crystalline powder, having a slightly bitter taste. Freely soluble in alcohol; soluble in boiling water and in 1 N sodium hydroxide.

Acetazolamide: White to faintly yellowish-white, crystalline, odorless powder. Sparingly soluble in practically boiling water; slightly soluble in alcohol; very slightly soluble in water.

Acetic Acid: Clear, colorless liquid, having a strong, characteristic odor, and a sharply acid taste. Specific gravity is about 1.045. Miscible with water, with alcohol, and with glycerin. *NF category:* Acidifying agent; buffering agent.

Glacial Acetic Acid: Clear, colorless liquid, having a pungent, characteristic odor and, when well diluted with water, an acid taste. Boils at about 118° . Specific gravity is about 1.05. Miscible with water, with alcohol, and with glycerin. *NF category:* Acidifying agent.

Acetohexamide: White, crystalline, practically odorless powder. Soluble in pyridine and in dilute solutions of alkali hydroxides; slightly soluble in alcohol and in chloroform; practically insoluble in water and in ether.

Acetohydroxamic Acid: White, slightly hygroscopic, crystalline powder. Melts, after drying at about 80° for 2 to 4 hours, at about 88° . Freely soluble in water and in alcohol; very slightly soluble in chloroform.

Acetone: Transparent, colorless, mobile, volatile liquid, having a characteristic odor. A solution (1 in 2) is neutral to litmus. Miscible with water, with alcohol, with ether, with chloroform, and with most volatile oils. *NF category:* Solvent.

Acetylcholine Chloride: White or off-white crystals or crystalline powder. Very soluble in water; freely soluble in alcohol; insoluble in ether. Is decomposed by hot water and by alkalies.

Acetylcysteine: White, crystalline powder, having a slight acetic odor. Freely soluble in water and in alcohol; practically insoluble in chloroform and in ether.

Acetyltributyl Citrate: Clear, practically colorless, oily liquid. Freely soluble in alcohol, in isopropyl alcohol, in acetone, and in toluene; insoluble in water. *NF category:* Plasticizer.

Acetyltriethyl Citrate: Clear, practically colorless, oily liquid. Freely soluble in alcohol, in isopropyl alcohol, in ace-

tone, and in toluene; insoluble in water. *NF category*: Plasticizer.

Acitretin: Yellow or greenish, crystalline powder. Sparingly soluble in tetrahydrofuran; slightly soluble in acetone and in alcohol; very slightly soluble in cyclohexane; practically insoluble in water.

Acyclovir: White to off-white, crystalline powder. Melts at temperatures higher than 250°, with decomposition. Soluble in diluted hydrochloric acid; slightly soluble in water; insoluble in alcohol.

Add the following:

• **Adapalene**: White or almost white powder. Soluble in tetrahydrofuran; sparingly soluble in ethanol; practically insoluble in water. ● (RB 1-Dec-2012)

Ademetionine Disulfate Tosylate: White powder. Freely soluble in water.

Adenine: White crystals or crystalline powder. Is odorless and tasteless. Sparingly soluble in boiling water; slightly soluble in alcohol; very slightly soluble in water; practically insoluble in ether and in chloroform.

Adenosine: White, odorless, crystalline powder. Slightly soluble in water; practically insoluble in alcohol. Melts at about 235°.

Adipic Acid: A white, crystalline powder. Freely soluble in alcohol and in methanol; soluble in boiling water and in acetone; slightly soluble in water. *NF category*: Buffering agent.

Agar: Odorless or has a slight odor, and produces a mucilaginous sensation on the tongue. Soluble in boiling water; insoluble in cold water. *NF category*: Suspending and/or viscosity-increasing agent.

Alamic Acid: *NF category*: Suspending and/or viscosity-increasing agent.

Alanine: White, odorless crystals or crystalline powder, having a slightly sweet taste. Freely soluble in water; slightly soluble in 80% alcohol; insoluble in ether.

Albendazole: White to faintly yellowish powder. Freely soluble in anhydrous formic acid; very slightly soluble in ether and in methylene chloride; practically insoluble in alcohol and in water.

Albumin Human: Practically odorless, moderately viscous, clear, brownish fluid.

rAlbumin Human: Clear, slightly viscous, and colorless to yellow amber in color. *NF category*: Vehicle (sterile).

Albuterol: White, crystalline powder. Soluble in alcohol; sparingly soluble in water. Melts at about 156°.

Albuterol Sulfate: White or practically white powder. Freely soluble in water; slightly soluble in alcohol, in chloroform, and in ether.

Alcohol: Clear, colorless, mobile, volatile liquid. Has a characteristic odor and produces a burning sensation on the tongue. Is readily volatilized even at low temperatures, and boils at about 78°. Is flammable. Miscible with water and with practically all organic solvents. *NF category*: Solvent.

Dehydrated Alcohol: Clear, colorless, mobile, volatile liquid. Has a characteristic odor and produces a burning sensation on the tongue. Is readily volatilized even at low temperatures, and boils at about 78°. Is flammable. Miscible with water and with practically all organic solvents.

Diluted Alcohol: Clear, colorless, mobile liquid, having a characteristic odor and producing a burning sensation on the tongue. *NF category*: Solvent.

Rubbing Alcohol: Transparent, colorless, or colored as desired, mobile, volatile liquid. Has an extremely bitter taste and, in the absence of added odorous constituents, a characteristic odor. Is flammable.

Alendronate Sodium: White, free-flowing powder. Soluble in water; very slightly soluble in dimethyl sulfoxide, in

methyl alcohol, and in propylene glycol; practically insoluble in acetone, in acetonitrile, in alcohol, in chloroform, and in isopropyl alcohol.

Alfadex: A white or almost white, amorphous or crystalline powder. Freely soluble in water and in propylene glycol; practically insoluble in ethanol and in methylene chloride.

Alfentanil Hydrochloride: White to almost white powder. Freely soluble in methanol, in alcohol, and in chloroform; soluble in water; sparingly soluble in acetone. Melting point range, crystals from acetone: 136° – 143° (anhydrous) and reported as crystals from aqueous hydrochloric acid: 116° – 126° (monohydrate).

Alfentanil Injection: Clear, colorless solution.

Alfuzosin Hydrochloride: White to almost white powder, slightly hygroscopic. Freely soluble in water; sparingly soluble in alcohol; practically insoluble in methylene chloride.

Alginic Acid: White to yellowish white, fibrous powder. Is odorless, or practically odorless, and is tasteless. Soluble in alkaline solutions; insoluble in water and in organic solvents. *NF category*: Suspending and/or viscosity-increasing agent; tablet binder; tablet disintegrant.

Alkyl (C12-15) Benzoate: Clear, practically colorless, oily liquid. Soluble in acetone, in alcohol, in isopropyl alcohol, in ethyl acetate, in isopropyl myristate, in isopropyl palmitate, in lanolin, in mineral oil, in vegetable oils, and in volatile silicones; insoluble in water, in glycerin, and in propylene glycol. *NF category*: Vehicle (oleaginous); emollient.

Allantoin: White, crystalline powder. Slightly soluble in water; very slightly soluble in alcohol. Melts at about 225°, with decomposition.

Allopurinol: Fluffy white to off-white powder, having only a slight odor. Soluble in solutions of potassium and sodium hydroxides; very slightly soluble in water and in alcohol; practically insoluble in chloroform and in ether.

Allyl Isothiocyanate: Colorless to pale yellow, very refractive, liquid. Pungent irritating odor, acrid taste. [Caution: Lachrymator.] Miscible with alcohol, with carbon disulfide, and with ether. Slightly soluble in water.

Almond Oil: Clear, pale straw-colored or colorless, oily liquid, having a bland taste. Remains clear at –10°, and does not congeal until cooled to almost –20°. Slightly soluble in alcohol. Miscible with ether, with chloroform, with benzene, and with solvent hexane. *NF category*: Flavors and perfumes; vehicle (oleaginous).

Aloe: Has a characteristic, somewhat sour and disagreeable, odor.

Alprazolam: A white to off-white, crystalline powder. Melts at about 225°. Freely soluble in chloroform; soluble in alcohol; sparingly soluble in acetone; slightly soluble in ethyl acetate; insoluble in water.

Alprostadil: A white to off-white, crystalline powder. Melts at about 110°. Freely soluble in alcohol; soluble in water and in acetone; slightly soluble in ethyl acetate; very slightly soluble in chloroform and in ether.

Altretamine: White, crystalline powder. Soluble in chloroform; insoluble in water.

Ammonium Alum: Large, colorless crystals, crystalline fragments, or white powder. Is odorless, and has a sweetish, strongly astringent taste. Its solutions are acid to litmus. Very soluble in boiling water; freely soluble in water; freely soluble in glycerin; insoluble in alcohol.

Potassium Alum: Large, colorless crystals, crystalline fragments, or white powder. Is odorless, and has a sweetish, strongly astringent taste. Its solutions are acid to litmus. Very soluble in boiling water; freely soluble in water; freely soluble in glycerin; insoluble in alcohol.

Aluminum Acetate Topical Solution: Clear, colorless liquid having a faint odor of acetic acid, and a sweetish, astringent taste. Specific gravity is about 1.02.

Aluminum Chloride: White, or yellowish-white, deliquescent, crystalline powder. Is practically odorless, and has a sweet, very astringent taste. Its solutions are acid to litmus. Very soluble in water; freely soluble in alcohol; soluble in glycerin.

Aluminum Hydroxide Gel: White, viscous suspension, from which small amounts of clear liquid may separate on standing.

Dried Aluminum Hydroxide Gel: White, odorless, tasteless, amorphous powder. Soluble in dilute mineral acids and in solutions of fixed alkali hydroxides; insoluble in water and in alcohol.

Aluminum Monostearate: Fine, white to yellowish-white, bulky powder, having a faint, characteristic odor. Insoluble in water, in alcohol, and in ether. *NF category:* Suspending and/or viscosity-increasing agent.

Aluminum Oxide: Occurs as a white or almost white, amorphous powder. It is very slightly soluble in dilute mineral acids and in solutions of alkali hydroxides. It is practically insoluble in water.

Aluminum Phosphate Gel: White, viscous suspension from which small amounts of water separate on standing.

Aluminum Subacetate Topical Solution: Clear, colorless or faintly yellow liquid, having an odor of acetic acid and an acid reaction to litmus. Gradually becomes turbid on standing, through separation of a more basic salt.

Aluminum Sulfate: White, crystalline powder, shining plates, or crystalline fragments. Is stable in air. Is odorless, and has a sweet taste, becoming mildly astringent. Freely soluble in water; insoluble in alcohol.

Amantadine Hydrochloride: White or practically white, crystalline powder, having a bitter taste. Freely soluble in water; soluble in alcohol and in chloroform.

Amifostine: White, crystalline powder. Freely soluble in water.

Amikacin: White, crystalline powder. Sparingly soluble in water.

Amikacin Sulfate: White, crystalline powder. Freely soluble in water.

Amiloride Hydrochloride: Yellow to greenish-yellow, odorless or practically odorless powder. Freely soluble in dimethyl sulfoxide; sparingly soluble in methanol; slightly soluble in water; insoluble in ether, in ethyl acetate, in acetone, and in chloroform.

Amino Methacrylate Copolymer: Colorless to yellowish granules. Soluble in acetone, in isopropyl alcohol, and in diluted acids; practically insoluble in water. The solutions are clear to slightly cloudy. *NF category:* Coating agent; polymer membrane; tablet binder.

Aminobenzoate Potassium: White, crystalline powder. The pH of a 1 in 100 solution in water is about 7. Very soluble in water; soluble in alcohol; practically insoluble in ether.

Aminobenzoic Acid: White or slightly yellow, odorless crystals or crystalline powder. Discolors on exposure to air or light. Freely soluble in alcohol and in solutions of alkali hydroxides and carbonates; sparingly soluble in ether; slightly soluble in water and in chloroform.

Aminobenzoic Acid Topical Solution: Straw-colored solution having the odor of alcohol.

Aminocaproic Acid: Fine, white, crystalline powder. Is odorless, or practically odorless. Its solutions are neutral to litmus. Melts at about 205°. Freely soluble in water, in acids, and in alkalies; slightly soluble in methanol and in alcohol; practically insoluble in chloroform and in ether.

Aminogluthethimide: Fine, white, or creamy white, crystalline powder. Soluble in most organic solvents; very slightly soluble in water. Forms water-soluble salts with strong acids.

Aminohippuric Acid: White, crystalline powder. Discolors on exposure to light. Melts at about 195°, with decomposition. Freely soluble in alkaline solutions, with some decomposition, and in diluted hydrochloric acid; sparingly soluble in water and in alcohol; very slightly soluble in benzene, in carbon tetrachloride, in chloroform, and in ether.

Aminopentamide Sulfate: White, crystalline powder. Freely soluble in water and in alcohol; very slightly soluble in chloroform; practically insoluble in ether.

Aminophylline: White or slightly yellowish granules or powder, having a slight ammoniacal odor and a bitter taste. Upon exposure to air, it gradually loses ethylenediamine and absorbs carbon dioxide with the liberation of free theophylline. Its solutions are alkaline to litmus. One g dissolves in 25 mL of water to give a clear solution; 1 g dissolved in 5 mL of water crystallizes upon standing, but redissolves when a small amount of ethylenediamine is added. Insoluble in alcohol and in ether.

Aminophylline Tablets: May have a faint ammoniacal odor.

Aminosalicylate Sodium: White to cream-colored, crystalline powder. Is practically odorless, and has a sweet, saline taste. Its solutions decompose slowly and darken in color. Freely soluble in water; sparingly soluble in alcohol; very slightly soluble in ether and in chloroform.

Aminosalicylic Acid: White or practically white, bulky powder, that darkens on exposure to light and air. Is odorless, or has a slight acetous odor. Soluble in alcohol; slightly soluble in water and in ether; practically insoluble in benzene.

Amiodarone Hydrochloride: White or almost white, fine, crystalline powder. Freely soluble in methylene chloride; soluble in methanol; sparingly soluble in alcohol; very slightly soluble in water.

Amitriptyline Hydrochloride: White or practically white, odorless or practically odorless, crystalline powder or small crystals. Freely soluble in water, in alcohol, in chloroform, and in methanol; insoluble in ether.

Amlodipine Besylate: A white or almost white powder. Freely soluble in methanol; sparingly soluble in alcohol; slightly soluble in 2-propanol and in water.

Strong Ammonia Solution: Clear, colorless liquid, having an exceedingly pungent, characteristic odor. Specific gravity is about 0.90. *NF category:* Alkalizing agent.

Aromatic Ammonia Spirit: Practically colorless liquid when recently prepared, but gradually acquiring a yellow color on standing. Has the taste of ammonia, has an aromatic and pungent odor, and is affected by light. Specific gravity is about 0.90.

Ammonio Methacrylate Copolymer: Colorless, clear to white-opaque granules or a white powder, both with a faint amine-like odor. Soluble to freely soluble in methanol, in alcohol, and in isopropyl alcohol, each of which contains small amounts of water; soluble to freely soluble in acetone, in ethyl acetate, and in methylene chloride. The solutions are clear to slightly cloudy. Insoluble in petroleum ether and in water. *NF category:* Coating agent; tablet binder; polymer membrane.

Ammonio Methacrylate Copolymer Dispersion: Milky-white liquids of low viscosity with a faint characteristic odor. Miscible with water in any proportion, the milky-white appearance being retained. A clear or slightly cloudy solution is obtained on mixing one part with five parts of acetone, alcohol, or isopropyl alcohol. When mixed with methanol in a ratio of 1:5, Ammonio Methacrylate Copolymer Dispersion Type A dissolves completely, and Ammonio Methacrylate Copolymer Dispersion Type B dissolves only partially. *NF category:* Coating agent; polymer membrane; tablet binder.

Ammonium Carbonate: White powder, or hard, white or translucent masses, having a strong odor of ammonia, without empyreuma, and a sharp, ammoniacal taste. Its solutions are alkaline to litmus. On exposure to air, it loses

ammonia and carbon dioxide, becoming opaque, and is finally converted into friable porous lumps or a white powder of ammonium bicarbonate. Freely soluble in water, but is decomposed by hot water. *NF category:* Alkalizing agent; buffering agent.

Ammonium Chloride: Colorless crystals or white, fine or coarse, crystalline powder. Has a cool, saline taste, and is somewhat hygroscopic. Freely soluble in water and in glycerin, and even more so in boiling water; sparingly soluble in alcohol.

Ammonium Molybdate: Colorless or slightly greenish or yellowish crystals. Soluble in water; practically insoluble in alcohol.

Ammonium Phosphate: Colorless or white granules or powder, having a saline taste. Freely soluble in water; practically insoluble in acetone and in alcohol. *NF category:* Buffering agent.

Ammonium Sulfate: Colorless or white crystals or granules that decompose at temperatures above 280°. One g is soluble in about 1.5 mL of water. It is insoluble in alcohol. The pH of a 0.1 M solution is between 4.5 and 6.0.

Amobarbital Sodium: White, friable, granular powder. Is odorless, has a bitter taste, and is hygroscopic. Its solutions decompose on standing, heat accelerating the decomposition. Very soluble in water; soluble in alcohol; practically insoluble in ether and in chloroform.

Amodiaquine: Very pale yellow to light tan-yellow, odorless powder. Sparingly soluble in 1.0 N hydrochloric acid; slightly soluble in alcohol; practically insoluble in water.

Amodiaquine Hydrochloride: Yellow, crystalline powder. Is odorless and has a bitter taste. Soluble in water; sparingly soluble in alcohol; very slightly soluble in benzene, in chloroform, and in ether.

Amoxapine: White to yellowish crystalline powder. Freely soluble in chloroform; soluble in tetrahydrofuran; sparingly soluble in methanol and in toluene; slightly soluble in acetone; practically insoluble in water.

Amoxicillin: White, practically odorless, crystalline powder. Slightly soluble in water and in methanol; insoluble in benzene, in carbon tetrachloride, and in chloroform.

Amphetamine Sulfate: White, odorless, crystalline powder, having a slightly bitter taste. Its solutions are acid to litmus, having a pH of 5 to 6. Freely soluble in water; slightly soluble in alcohol; practically insoluble in ether.

Amphotericin B: Yellow to orange powder; odorless or practically so. Soluble in dimethylformamide, in dimethyl sulfoxide, and in propylene glycol; slightly soluble in methanol; insoluble in water, in anhydrous alcohol, in ether, in benzene, and in toluene.

Amphotericin B for Injection: It yields a colloidal dispersion in water.

Ampicillin: White, practically odorless, crystalline powder. Slightly soluble in water and in methanol; insoluble in benzene, in carbon tetrachloride, and in chloroform.

Ampicillin Sodium: White to off-white, odorless or practically odorless, crystalline powder. Is hygroscopic. Very soluble in water and in isotonic sodium chloride and dextrose solutions.

Amprolium ($C_{14}H_{19}ClN_4 \cdot HCl$): White to light yellow powder. Freely soluble in water, in methanol, in alcohol, and in dimethylformamide; sparingly soluble in dehydrated alcohol; practically insoluble in isopropyl alcohol, in butyl alcohol, and in acetone.

Amyl Nitrite: Clear, yellowish liquid, having a peculiar, ethereal, fruity odor. Is volatile even at low temperatures, and is flammable. Boils at about 96°. Practically insoluble in water. Miscible with alcohol and with ether.

Amylene Hydrate: Clear, colorless liquid, having a camphoraceous odor. Its solutions are neutral to litmus. Freely

soluble in water. Miscible with alcohol, with chloroform, with ether, and with glycerin. *NF category:* Solvent.

Anagrelide Hydrochloride: Off-white to pale pinkish powder. Sparingly soluble in dimethylsulfoxide and dimethylformamide; very slightly soluble in water.

Anastrozole: White to off-white crystalline powder. Very soluble in acetonitrile; freely soluble in methanol, in acetone, in alcohol, and in tetrahydrofuran.

Anethole: Colorless or faintly yellow liquid at or above 23°. Has a sweet taste and the aromatic odor of anise. Is affected by light. Freely soluble in alcohol; very slightly soluble in water. Readily miscible with ether and with chloroform. *NF category:* Flavors and perfumes.

Anileridine: White to yellowish-white, odorless to practically odorless, crystalline powder. Is oxidized on exposure to air and light, becoming darker in color. It exhibits polymorphism, and of two crystalline forms observed, one melts at about 80° and the other at about 89°. Freely soluble in alcohol and in chloroform; soluble in ether, although it may show turbidity; very slightly soluble in water.

Anileridine Hydrochloride: White or nearly white, odorless, crystalline powder. Is stable in air. Melts at about 270°, with decomposition. Freely soluble in water; sparingly soluble in alcohol; practically insoluble in ether, and in chloroform.

Antazoline Phosphate: White to off-white, crystalline powder, having a bitter taste. Soluble in water; sparingly soluble in methanol; practically insoluble in benzene and in ether.

Anthralin: Yellowish-brown, crystalline powder. Is odorless and tasteless. Soluble in chloroform, in acetone, in benzene, and in solutions of alkali hydroxides; slightly soluble in alcohol, in ether, and in glacial acetic acid; insoluble in water.

Anticoagulant Citrate Dextrose Solution: Clear, colorless, odorless liquid. Is dextrorotatory.

Anticoagulant Citrate Phosphate Dextrose Solution: Clear, colorless to slightly yellow, odorless liquid. Is dextrorotatory.

Anticoagulant Sodium Citrate Solution: Clear and colorless liquid.

Antihemophilic Factor: White or yellowish powder. On constitution is opalescent with a slight blue tinge or is a yellowish liquid.

Cryoprecipitated Antihemophilic Factor: Yellowish, frozen solid. On thawing becomes a very viscous, yellow, gummy liquid.

Antimony Potassium Tartrate: Colorless, odorless, transparent crystals, or white powder. The crystals effloresce upon exposure to air and do not readily rehydrate even on exposure to high humidity. Its solutions are acid to litmus. Freely soluble in boiling water; soluble in water and in glycerin; insoluble in alcohol.

Antimony Sodium Tartrate: Colorless, odorless, transparent crystals, or white powder. The crystals effloresce upon exposure to air. Freely soluble in water; insoluble in alcohol.

Antipyrine: Colorless crystals, or white, crystalline powder. Is odorless and has a slightly bitter taste. Its solutions are neutral to litmus. Very soluble in water; freely soluble in alcohol and in chloroform; sparingly soluble in ether.

Antivenin (Crotalidae) Polyvalent: Solid exhibiting the characteristic structure of a freeze-dried solid; light cream in color.

Antivenin (Micrurus Fulvius): Solid exhibiting the characteristic structure of a freeze-dried solid; light cream in color.

Apomorphine Hydrochloride: Minute, white or grayish-white, glistening crystals or white powder. Is odorless. It gradually acquires a green color on exposure to light and air. Its solutions are neutral to litmus. Soluble in water at

80°; sparingly soluble in water and in alcohol; very slightly soluble in chloroform and in ether.

Apraclonidine Hydrochloride: White to off-white, odorless to practically odorless powder. Soluble in methanol; sparingly soluble in water and in alcohol; insoluble in chloroform, in ethyl acetate, and in hexanes.

Arginine: White, practically odorless crystals. Freely soluble in water; sparingly soluble in alcohol; insoluble in ether.

Arginine Hydrochloride: White crystals or crystalline powder, practically odorless. Freely soluble in water.

Aromatic Elixir: *NF category:* Vehicle (flavored and/or sweetened).

Arsanilic Acid: White to off-white, crystalline powder. Melts at about 232°. Soluble in hot water, in amyl alcohol, and in solutions of alkali carbonates; sparingly soluble in concentrated mineral acids; slightly soluble in cold water, in alcohol, and in acetic acid; insoluble in acetone, in benzene, in chloroform, in ether, and in dilute mineral acids.

Articaine Hydrochloride: White or almost white, crystalline powder. Freely soluble in water and in alcohol.

Ascorbic Acid: White or slightly yellow crystals or powder. On exposure to light it gradually darkens. In the dry state, is reasonably stable in air, but in solution rapidly oxidizes. Melts at about 190°. Freely soluble in water; sparingly soluble in alcohol; insoluble in chloroform, in ether, and in benzene. *NF category:* Antioxidant.

Ascorbyl Palmitate: White to yellowish white powder, having a characteristic odor. Soluble in alcohol; very slightly soluble in water and in vegetable oils. *NF category:* Antioxidant.

Asparagine: White crystals or a crystalline powder. Soluble in water; practically insoluble in alcohol and in ether. Its solutions are acid to litmus. It melts at about 234°.

Aspartame: White, odorless, crystalline powder, having a sweet taste. Sparingly soluble in water; slightly soluble in alcohol. Melts at about 246°. The pH of an 8 in 1000 solution is about 5. *NF category:* Sweetening agent.

Aspartame Acesulfame: White, odorless, crystalline powder. Slightly soluble in water and in ethanol. *NF category:* Sweetening agent.

Aspartic Acid: White or almost white, crystalline powder, or colorless crystals. Soluble in dilute solutions of alkali hydroxides and in dilute mineral acids; slightly soluble in water; practically insoluble in alcohol and in ether.

Aspirin: White crystals, commonly tabular or needle-like, or white, crystalline powder. Is odorless or has a faint odor. Is stable in dry air; in moist air it gradually hydrolyzes to salicylic and acetic acids. Freely soluble in alcohol; soluble in chloroform and in ether; sparingly soluble in absolute ether; slightly soluble in water.

Atenolol: White or practically white, odorless powder. Melting point 146° – 148° (crystals from ethyl acetate). Freely soluble in methanol; sparingly soluble in alcohol; slightly soluble in water and in isopropanol.

Atorvastatin Calcium: White to off-white crystalline powder. Freely soluble in methanol; slightly soluble in alcohol; very slightly soluble in distilled water, in pH 7.4 phosphate buffer, and in acetonitrile; insoluble in aqueous solutions of pH 4 and below.

Atovaquone: Yellow powder. Freely soluble in *N*-methyl-2-pyrrolidone and in tetrahydrofuran; soluble in chloroform; sparingly soluble in acetone, in di-*n*-butyl adipate, in dimethyl sulfoxide, and in polyethylene glycol 400; slightly soluble in alcohol, in 1,3-butanediol, in ethyl acetate, in glycerin, in octanol, and in polyethylene glycol 200; very slightly soluble in 0.1 N sodium hydroxide; insoluble in water.

Atracurium Besylate: White to yellowish-white powder, slightly hygroscopic. Very soluble in acetonitrile, in alcohol, and in methylene chloride; soluble in water.

Atropine: White crystals, usually needle-like, or white, crystalline powder. Its saturated solution is alkaline to phenolphthalein TS. Is optically inactive, but usually contains some levorotatory hyoscyamine. Freely soluble in alcohol and in chloroform; soluble in glycerin and in ether; slightly soluble in water; sparingly soluble in water at 80°.

Atropine Sulfate: Colorless crystals, or white, crystalline powder. Odorless; effloresces in dry air; is slowly affected by light. Very soluble in water; freely soluble in alcohol and even more so in boiling alcohol; freely soluble in glycerin.

Activated Attapulgate: Cream-colored, micronized, nonswelling powder, free from gritty particles. The high heat treatment used in its preparation causes it to yield only moderately viscous aqueous suspensions, its dispersion consisting mainly of particle groups. Insoluble in water. *NF category:* Suspending and/or viscosity-increasing agent.

Colloidal Activated Attapulgate: Cream-colored, micronized, nonswelling powder, free from gritty particles. Yields viscous aqueous suspensions, as a result of dispersion into its constituent ultimate particles. Insoluble in water. *NF category:* Suspending and/or viscosity-increasing agent.

Aurothioglucose: Yellow, odorless or practically odorless powder. Is stable in air. An aqueous solution is unstable on long standing. The pH of its 1 in 100 solution is about 6.3. Freely soluble in water; practically insoluble in acetone, in alcohol, in chloroform, and in ether.

Azatadine Maleate: White to light cream-colored, odorless powder. Melts at about 153°. Freely soluble in water, in alcohol, in chloroform, and in methanol; practically insoluble in benzene and in ether.

Azathioprine: Pale yellow, odorless powder. Soluble in dilute solutions of alkali hydroxides; sparingly soluble in dilute mineral acids; very slightly soluble in alcohol and in chloroform; insoluble in water.

Azathioprine Sodium for Injection: Bright yellow, hygroscopic, amorphous mass or cake.

Azithromycin: White or almost white powder. Freely soluble in anhydrous ethanol and in methylene chloride; practically insoluble in water.

Aztreonam: White, odorless, crystalline powder. Soluble in dimethylformamide and in dimethyl sulfoxide; slightly soluble in methanol; very slightly soluble in dehydrated alcohol; practically insoluble in ethyl acetate, in chloroform, and in toluene.

Bacampicillin Hydrochloride: White or practically white powder. Is hygroscopic. Freely soluble in alcohol and in chloroform; soluble in methylene chloride and in water; very slightly soluble in ether.

Bacitracin: White to pale buff powder, odorless or having a slight odor. Is hygroscopic. Its solutions deteriorate rapidly at room temperature. Is precipitated from its solutions and is inactivated by salts of many of the heavy metals. Freely soluble in water; soluble in alcohol, in methanol, and in glacial acetic acid, the solution in the organic solvents usually showing some insoluble residue; insoluble in acetone, in chloroform, and in ether.

Bacitracin Zinc: White to pale tan powder, odorless or having a slight odor. Is hygroscopic. Sparingly soluble in water.

Baclofen: White to off-white, crystalline powder. Is odorless or practically so. Slightly soluble in water; very slightly soluble in methanol; insoluble in chloroform.

Balsalazide Disodium: Orange to yellow powder. Freely soluble in water and in isotonic saline; sparingly soluble in methanol and in alcohol; practically insoluble in all other organic solvents.

Adhesive Bandage: The compress of Adhesive Bandage is substantially free from loose threads or ravelings. The adhesive strip may be perforated, and the back may be coated with a water-repellent film.

Gauze Bandage: One continuous piece, tightly rolled, in various widths and lengths and substantially free from loose threads and ravelings.

Barium Hydroxide Lime: White or grayish-white granules. May have a color if an indicator has been added. *NF category:* Sorbent, carbon dioxide.

Barium Sulfate: Fine, white, odorless, tasteless, bulky powder, free from grittiness. Practically insoluble in water, in organic solvents, and in solutions of acids and of alkalis.

Barium Sulfate for Suspension: White or colored, bulky or granular powder.

BCG Vaccine: White to creamy white, dried mass, having the characteristic texture of material dried in the frozen state.

Beclomethasone Dipropionate: White to cream white, odorless powder. Very soluble in chloroform; freely soluble in acetone and in alcohol; very slightly soluble in water.

Behenoyl Polyoxylglycerides: Waxy solid or fine powder. Soluble in methylene chloride; insoluble in alcohol; dispersible in water. *NF category:* Tablet and/or capsule lubricant.

Belladonna Leaf: When moistened, its odor is slight, somewhat tobacco-like. Its taste is somewhat bitter and acrid.

Benazepril Hydrochloride: White to off-white, crystalline powder. Soluble in water, in methanol, and in alcohol.

Bendroflumethiazide: White to cream-colored, finely divided, crystalline powder. Is odorless, or has a slight odor. Melts at about 220°. Freely soluble in alcohol and in acetone; practically insoluble in water.

Benoxinate Hydrochloride: White, or slightly off-white, crystals or crystalline powder. Is odorless, or has a slight characteristic odor, has a salty taste, and exhibits local anesthetic properties when placed upon the tongue. Its solutions are neutral to litmus, and it melts at about 158°. Very soluble in water; freely soluble in chloroform and in alcohol; insoluble in ether.

Bentonite: Very fine, odorless, pale buff or cream-colored to grayish powder, free from grit. Has a slightly earthy taste. Is hygroscopic. Insoluble in water, but swells to approximately twelve times its volume when added to water; insoluble in, and does not swell in, organic solvents. *NF category:* Suspending and/or viscosity-increasing agent.

Purified Bentonite: Odorless, tasteless, fine (micronized) powder or small flakes that are creamy when viewed on their flat surfaces and tan to brown when viewed on their edges. Insoluble in water and in alcohol. Swells when added to water or glycerin. *NF category:* Suspending and/or viscosity-increasing agent.

Bentonite Magma: *NF category:* Suspending and/or viscosity-increasing agent.

Change to read:

Benzaldehyde: Colorless, strongly refractive liquid.

▲ *NF31* Is affected by light. Slightly soluble in water. Miscible with alcohol, with ether, and with fixed and volatile oils. ▲ The specific gravity is 1.041–1.046 at 25° (see *Specific Gravity* (841)), and the refractive index is 1.544–1.546 at 20° (see *Refractive Index* (831)). ▲ *NF31* *NF category:* Flavors and perfumes.

Benzaldehyde Elixir, Compound: *NF category:* Flavored and/or sweetened vehicle.

Benzalkonium Chloride: White or yellowish-white, thick gel or gelatinous pieces. Usually has a mild, aromatic odor. Its aqueous solution has a bitter taste, foams strongly when shaken, and usually is slightly alkaline. Very soluble in water and in alcohol. Anhydrous form freely soluble in benzene, and slightly soluble in ether. *NF category:* Antimicrobial preservative; wetting and/or solubilizing agent.

Benzalkonium Chloride Solution: Clear liquid; colorless or slightly yellow unless a color has been added. Has an aromatic odor and a bitter taste. *NF category:* Antimicrobial preservative.

Benzethonium Chloride: White crystals, having a mild odor. Its solution (1 in 100) is slightly alkaline to litmus. Soluble in water, in alcohol, and in chloroform; slightly soluble in ether. *NF category:* Antimicrobial preservative; wetting and/or solubilizing agent.

Benzethonium Chloride Solution: Odorless, clear liquid, slightly alkaline to litmus.

Benzethonium Chloride Tincture: Clear liquid, having the characteristic odor of acetone and of alcohol.

Benzocaine: Small, white crystals or white, crystalline powder. Is odorless, is stable in air, and exhibits local anesthetic properties when placed upon the tongue. Freely soluble in alcohol, in chloroform, and in ether; sparingly soluble in almond oil and in olive oil; very slightly soluble in water. Dissolves in dilute acids.

Benzoic Acid: White crystals, scales, or needles. Has a slight odor, usually suggesting benzaldehyde or benzoin. Somewhat volatile at moderately warm temperatures. Freely volatile in steam. Freely soluble in alcohol, in chloroform, and in ether; slightly soluble in water. *NF category:* Antimicrobial preservative.

Benzoin: Sumatra Benzoin has an aromatic and balsamic odor. When heated it does not emit a pinaceous odor. When Sumatra Benzoin is digested with boiling water, the odor suggests cinnamates or storax. Its taste is aromatic and slightly acrid. Siam Benzoin has an agreeable, balsamic, vanilla-like odor. Its taste is aromatic and slightly acrid.

Benzonate: Clear, pale yellow, viscous liquid, having a faint, characteristic odor. Has a bitter taste, and exhibits local anesthetic properties when placed upon the tongue. Miscible with water in all proportions. Freely soluble in chloroform, in alcohol, and in benzene.

Hydrous Benzoyl Peroxide: White, granular powder, having a characteristic odor. Soluble in acetone, in chloroform, and in ether; sparingly soluble in water and in alcohol.

Benzoyl Peroxide Gel: A soft, white gel, having a characteristic odor.

Benzoyl Peroxide Lotion: White, viscous, creamy lotion, having a characteristic odor.

Benzotropine Mesylate: White, slightly hygroscopic, crystalline powder. Very soluble in water; freely soluble in alcohol; very slightly soluble in ether.

Benzyl Alcohol: Clear, colorless, oily liquid. Boils at about 206°, without decomposition. Is neutral to litmus. Freely soluble in 50% alcohol; sparingly soluble in water. Miscible with alcohol, with ether, and with chloroform. The specific gravity is between 1.042 and 1.047. *NF category:* Antimicrobial preservative.

Benzyl Benzoate: Clear, colorless, oily liquid having a slight aromatic odor and producing a sharp, burning sensation on the tongue. Practically insoluble in water and in glycerin. Miscible with alcohol, with ether, and with chloroform. *NF category:* Solvent.

Beta Carotene: Red or reddish-brown to violet-brown crystals or crystalline powder. Soluble in carbon disulfide, in benzene, and in chloroform; sparingly soluble in ether, in solvent hexane, and in vegetable oils; practically insoluble in methanol and in alcohol; insoluble in water and in acids and in alkalis.

Betadex: White, practically odorless, fine crystalline powder having a slightly sweet taste. Sparingly soluble in water. *NF category:* Sequestering agent.

Betadex Sulfobutyl Ether Sodium: White to off-white, amorphous powder. Freely soluble in water; sparingly soluble in methanol; practically insoluble in ethanol, in *n*-hexane, in 1-butanol, in acetonitrile, in 2-propanol, and in ethyl

acetate. *NF category:* Complexing agent; sequestering agent; wetting and/or solubilizing agent.

Betahistine Hydrochloride: White to almost yellow, crystalline powder. Very hygroscopic. Melts between 151° and 154°. Very soluble in water; freely soluble in alcohol; practically insoluble in isopropyl alcohol.

Betaine Hydrochloride: White, crystalline powder. Soluble in water and in alcohol; practically insoluble in chloroform and in ether.

Betamethasone: White to practically white, odorless, crystalline powder. Melts at about 240°, with some decomposition. Sparingly soluble in acetone, in alcohol, in dioxane, and in methanol; very slightly soluble in chloroform and in ether; insoluble in water.

Betamethasone Acetate: White to creamy white, odorless powder. Sinters and resolidifies at about 165°, and remelts at about 200° or 220°, with decomposition (see *Melting Range or Temperature* (741)). Freely soluble in acetone; soluble in alcohol and in chloroform; practically insoluble in water.

Betamethasone Benzoate: White to practically white, practically odorless powder. Melts at about 220°, with decomposition. Soluble in alcohol, in methanol, and in chloroform; insoluble in water.

Betamethasone Dipropionate: White to cream-white, odorless powder. Freely soluble in acetone and in chloroform; sparingly soluble in alcohol; insoluble in water.

Betamethasone Sodium Phosphate: White to practically white, odorless powder. Is hygroscopic. Freely soluble in water and in methanol; practically insoluble in acetone and in chloroform.

Betamethasone Valerate: White to practically white, odorless powder. Melts at about 190°, with decomposition. Freely soluble in acetone and in chloroform; soluble in alcohol; slightly soluble in benzene and in ether; practically insoluble in water.

Betaxolol Hydrochloride: White, crystalline powder. Freely soluble in water, in alcohol, in chloroform, and in methanol.

Bethanechol Chloride: Colorless or white crystals or white, crystalline powder, usually having a slight, amine-like odor. Is hygroscopic. Exhibits polymorphism, and of two crystalline forms observed, one melts at about 211° and the other melts at about 219°. Freely soluble in water and in alcohol; insoluble in chloroform and in ether.

Bicalutamide: Fine, white to off-white powder. Freely soluble in tetrahydrofuran and in acetone; soluble in acetonitrile; sparingly soluble in methanol; slightly soluble in alcohol.

Biotin: Practically white, crystalline powder. Very slightly soluble in water and in alcohol; insoluble in other common organic solvents.

Biperiden: White, practically odorless, crystalline powder. Freely soluble in chloroform; sparingly soluble in alcohol; practically insoluble in water.

Biperiden Hydrochloride: White, practically odorless, crystalline powder. Melts at about 275°, with decomposition. Is optically inactive. Sparingly soluble in methanol; slightly soluble in water, in ether, in alcohol, and in chloroform.

Bisacodyl: White to off-white, crystalline powder, in which the number of particles having a longest diameter smaller than 50 µm predominate. Soluble in chloroform and in benzene; sparingly soluble in alcohol and in methanol; slightly soluble in ether; practically insoluble in water.

Milk of Bismuth: Thick, white, opaque suspension that separates on standing. Is odorless and practically tasteless. Miscible with water and with alcohol.

Bismuth Citrate: White, amorphous or crystalline powder. Stable in air. Melts at about 300°, with decomposition.

Soluble in ammonia TS and in solutions of alkali citrates; insoluble in water and in alcohol.

Bismuth Subcarbonate: White or almost white powder. Practically insoluble in water, in alcohol, and in ether. Dissolves in dilute acids with effervescence.

Bismuth Subgallate: Amorphous, bright yellow powder. Is odorless and tasteless. Is stable in air, but is affected by light. Dissolves readily with decomposition in warm, moderately dilute hydrochloric, nitric, or sulfuric acid; readily dissolved by solutions of alkali hydroxides, forming a clear, yellow liquid that rapidly assumes a deep red color. Practically insoluble in water, in alcohol, in chloroform, and in ether; insoluble in very dilute mineral acids.

Bismuth Subnitrate: White, slightly hygroscopic powder. Practically insoluble in water and in alcohol; readily dissolved by hydrochloric acid or by nitric acid.

Bismuth Subsalcylate: Fine to off-white, microcrystalline, odorless, tasteless powder. Practically insoluble in water, in alcohol, and in ether. Reacts with alkalies and mineral acids.

Bisoprolol Fumarate: White, crystalline powder. Very soluble in water and in methanol; freely soluble in chloroform, in glacial acetic acid, and in alcohol; slightly soluble in acetone and in ethyl acetate.

Bleomycin Sulfate: Cream-colored, amorphous powder. Very soluble in water.

Anti-A Blood Grouping Serum: Liquid Serum is a clear or slightly opalescent fluid unless artificially colored blue. Dried Serum is light yellow to deep cream color, unless artificially colored as indicated for liquid Serum. The liquid Serum may develop slight turbidity on storage. The dried Serum may show slight turbidity upon reconstitution for use.

Anti-B Blood Grouping Serum: Liquid Serum is a clear or slightly opalescent fluid unless artificially colored yellow. Dried Serum is light yellow to deep cream color, unless artificially colored as indicated for liquid Serum. The liquid Serum may develop a slight turbidity on storage. The dried Serum may show slight turbidity upon reconstitution for use.

Blood Grouping Serums Anti-D, Anti-C, Anti-E, Anti-c, Anti-e: The liquid Serums are clear, slightly yellowish fluids, that may develop slight turbidity on storage. The dried Serums are light yellow to deep cream color.

Blood Group Specific Substances A, B, and AB: Clear solution that may have a slight odor because of the preservative.

Red Blood Cells: Dark red in color when packed. May show a slight creamy layer on the surface and a small supernatant layer of yellow or opalescent plasma. Also supplied in deep-frozen form with added cryoprotective substance to extend storage time.

Whole Blood: Deep red, opaque liquid from which the corpuscles readily settle upon standing for 24 to 48 hours, leaving a clear, yellowish or pinkish supernatant layer of plasma.

Boric Acid: Colorless, odorless scales of a somewhat pearly luster, or crystals, or white powder that is slightly unctuous to the touch. Is stable in air. Freely soluble in glycerin, in boiling water, and in boiling alcohol; soluble in water and in alcohol. *NF category:* Buffering agent.

Botulism Antitoxin: Transparent or slightly opalescent liquid, practically colorless, and practically odorless or having an odor because of the antimicrobial agent.

Bretylum Tosylate: White, crystalline powder. Is hygroscopic. Freely soluble in water, in methanol, and in alcohol; practically insoluble in ether, in ethyl acetate, and in hexane.

Brinzolamide: White or almost white powder. Slightly soluble in alcohol and in methanol; insoluble in water.

Bromocriptine Mesylate: White or slightly colored, fine crystalline powder, odorless or having a weak, characteristic odor.

Bromodiphenhydramine Hydrochloride: White to pale buff, crystalline powder, having no more than a faint odor. Freely soluble in water and in alcohol; soluble in isopropyl alcohol; insoluble in ether and in solvent hexane.

Brompheniramine Maleate: White, odorless, crystalline powder. Freely soluble in water; soluble in alcohol and in chloroform; slightly soluble in ether and in benzene.

Budesonide: White to off-white, odorless, crystalline powder. Freely soluble in chloroform; sparingly soluble in alcohol; practically insoluble in water and in heptane.

Bumetanide: Practically white powder. Soluble in alkaline solutions; slightly soluble in water.

Bupivacaine Hydrochloride: White, odorless, crystalline powder. Melts at about 248°, with decomposition. Freely soluble in water and in alcohol; slightly soluble in chloroform and in acetone.

Bupivacaine Hydrochloride Injection: Clear, colorless solution.

Bupivacaine Hydrochloride and Epinephrine Injection: Clear, colorless solution.

Bupropion Hydrochloride: White powder. Soluble in water, in 0.1 N hydrochloric acid, and in alcohol.

Busulfan: White, crystalline powder. Sparingly soluble in acetone; slightly soluble in alcohol; very slightly soluble in water.

Buspiron Hydrochloride: White crystalline powder. Very soluble in water; freely soluble in methanol and in methylene chloride; sparingly soluble in ethanol and in acetonitrile; very slightly soluble in ethyl acetate; practically insoluble in hexanes.

Butabarbital: White, odorless, crystalline powder. Soluble in alcohol, in chloroform, in ether, and in solutions of alkali hydroxides and carbonates; very slightly soluble in water.

Butabarbital Sodium: White powder, having a bitter taste. Freely soluble in water and in alcohol; practically insoluble in absolute ether.

Butalbital: White, crystalline, odorless powder, having a slightly bitter taste. Is stable in air. Its saturated solution is acid to litmus. Freely soluble in alcohol, in ether, and in chloroform; soluble in boiling water, and in solutions of fixed alkalis and alkali carbonates; slightly soluble in cold water.

Butamben: White, crystalline powder. Is odorless and tasteless. Soluble in dilute acids, in alcohol, in chloroform, in ether, and in fixed oils; very slightly soluble in water. Is slowly hydrolyzed when boiled with water.

Butane: Colorless, flammable gas (boiling temperature is about -0.5°). One volume of water dissolves 0.15 volume, and 1 volume of alcohol dissolves 18 volumes at 17° and 770 mm; 1 volume of ether or chloroform at 17° dissolves 25 or 30 volumes, respectively. Vapor pressure at 21° is about 1620 mm of mercury (17 psig). *NF category:* Aerosol propellant.

Butoconazole Nitrate: White to off-white, crystalline powder. Melts at about 160°. Sparingly soluble in methanol; slightly soluble in acetonitrile, in acetone, in dichloromethane, and in tetrahydrofuran; very slightly soluble in ethyl acetate; practically insoluble in water.

Butorphanol Tartrate: White powder. Its solutions are slightly acidic. Melts between 217° and 219°, with decomposition. Soluble in dilute acids; sparingly soluble in water; slightly soluble in methanol; insoluble in alcohol, in chloroform, in ethyl acetate, in ethyl ether, and in hexane.

Butyl Alcohol: Clear, colorless, mobile liquid, having a characteristic, penetrating vinous odor. Soluble in water. Miscible with alcohol, with ether, and with many other organic solvents. *NF category:* Solvent.

Butylated Hydroxyanisole: White or slightly yellow, waxy solid, having a faint, characteristic odor. Freely soluble in alcohol, in propylene glycol, in chloroform, and in ether; insoluble in water. *NF category:* Antioxidant.

Butylated Hydroxytoluene: White, crystalline solid, having a faint, characteristic odor. Freely soluble in alcohol, in chloroform, and in ether; insoluble in water and in propylene glycol. *NF category:* Antioxidant.

Butylparaben: Small, colorless crystals or white powder. Freely soluble in acetone, in alcohol, in ether, and in propylene glycol; very slightly soluble in water and in glycerin. *NF category:* Antimicrobial preservative.

Cabergoline: White or almost white, crystalline powder. Freely soluble in alcohol (96%); slightly soluble in 0.1 M hydrochloric acid; very slightly soluble in hexane; practically insoluble in water.

Caffeine: White powder or white, glistening needles, usually matted together. Is odorless and has a bitter taste. Its solutions are neutral to litmus. The hydrate is efflorescent in air. Freely soluble in chloroform; sparingly soluble in water and in alcohol; slightly soluble in ether.

Calamine: Pink, odorless, practically tasteless, fine powder. Soluble in mineral acids; insoluble in water.

Calcitriol: White or almost white crystals. Freely soluble in alcohol; soluble in ether and in fatty oils; practically insoluble in water. It is sensitive to air, heat, and light.

Calcium Acetate: White, odorless or almost odorless, hygroscopic, crystalline powder. When heated to above 160°, it decomposes to calcium carbonate and acetone. Freely soluble in water; slightly soluble in methanol; practically insoluble in acetone, in dehydrated alcohol, and in benzene.

Calcium Ascorbate: White to slightly yellow, practically odorless powder. Freely soluble in water (approximately 50 g per 100 mL); slightly soluble in alcohol; insoluble in ether.

Calcium Carbonate: Fine, white, odorless, tasteless, microcrystalline powder. Is stable in air. Practically insoluble in water. Its solubility in water is increased by the presence of any ammonium salt or of carbon dioxide. The presence of any alkali hydroxide reduces its solubility. Insoluble in alcohol. Dissolves with effervescence in 1 N acetic acid, in 3 N hydrochloric acid, and in 2 N nitric acid. *NF category:* Tablet and/or capsule diluent.

Calcium Chloride: White, hard, odorless fragments or granules. Is deliquescent. Very soluble in boiling water; freely soluble in water, in alcohol, and in boiling alcohol. *NF category:* Desiccant.

Calcium Citrate: White, odorless, crystalline powder. Freely soluble in diluted 3 N hydrochloric acid and in diluted 2 N nitric acid; slightly soluble in water; insoluble in alcohol.

Calcium Gluceptate: White to faintly yellow, amorphous powder. Is stable in air, but the hydrous forms may lose part of their water of hydration on standing. Freely soluble in water; insoluble in alcohol and in many other organic solvents.

Calcium Gluconate: White, crystalline, odorless, tasteless granules or powder. Is stable in air. Its solutions are neutral to litmus. Freely soluble in boiling water; sparingly (and slowly) soluble in water; insoluble in alcohol.

Calcium Hydroxide: White powder. Has an alkaline, slightly bitter taste. Soluble in glycerin and in syrup; slightly soluble in water; very slightly soluble in boiling water; insoluble in alcohol.

Calcium Hydroxide Solution: Clear, colorless liquid having an alkaline taste. Is alkaline to litmus.

Calcium Lactate: White, practically odorless granules or powder. The pentahydrate is somewhat efflorescent and at 120° becomes anhydrous. The pentahydrate is soluble in water; it is practically insoluble in alcohol.

Calcium Levulinate: White, crystalline or amorphous, powder, having a faint odor suggestive of burnt sugar. Has a bitter, salty taste. Freely soluble in water; slightly soluble in alcohol; insoluble in ether and in chloroform.

Calcium Pantothenate: Slightly hygroscopic, white powder. Is odorless and has a bitter taste. Freely soluble in water; soluble in glycerin; practically insoluble in alcohol, in chloroform, and in ether.

Racemic Calcium Pantothenate: White, slightly hygroscopic powder, having a faint, characteristic odor, and a bitter taste. Is stable in air. Its solutions are neutral or alkaline to litmus. Is optically inactive. Freely soluble in water; soluble in glycerin; practically insoluble in alcohol, in chloroform, and in ether.

Dibasic Calcium Phosphate: White, odorless, tasteless powder. Is stable in air. Soluble in 3 N hydrochloric acid and in 2 N nitric acid; practically insoluble in water; insoluble in alcohol. *NF category:* Tablet and/or capsule diluent.

Tribasic Calcium Phosphate: White, odorless, tasteless powder. Is stable in air. Freely soluble in 3 N hydrochloric acid and in 2 N nitric acid; practically insoluble in water; insoluble in alcohol. *NF category:* Tablet and/or capsule diluent.

Calcium Polycarbophil: White to creamy white powder. Insoluble in water, in dilute acids, in dilute alkalies, and in common organic solvents.

Calcium Propionate: Occurs as a white crystalline solid. One g dissolves in about 3 mL of water. *NF category:* Antimicrobial preservative.

Calcium Saccharate: White, odorless, tasteless, crystalline powder. Soluble in dilute mineral acids and in solutions of calcium gluconate; slightly soluble in boiling water; very slightly soluble in alcohol, and in cold water; practically insoluble in ether and in chloroform.

Calcium Silicate: White to off-white, free-flowing powder that remains so after absorbing relatively large amounts of water or other liquids. Insoluble in water. Forms a gel with mineral acids. *NF category:* Glidant and/or anticaking agent.

Calcium Stearate: Fine, white to yellowish-white, bulky powder having a slight, characteristic odor. Is unctuous, and is free from grittiness. Insoluble in water, in alcohol, and in ether. *NF category:* Tablet and/or capsule lubricant.

Calcium Sulfate: Fine, white to slightly yellow-white, odorless powder. Soluble in 3 N hydrochloric acid; slightly soluble in water. *NF category:* Desiccant; tablet and/or capsule diluent.

Calcium Undecylenate: Fine, white powder, having a characteristic odor and no grit. Slightly soluble in hot alcohol; practically insoluble in water, in ether, in chloroform, in acetone, and in cold alcohol.

Camphor: Colorless or white crystals, granules, or crystalline masses; or colorless to white, translucent, tough masses. Has a penetrating, characteristic odor and a pungent, aromatic taste. Specific gravity is about 0.99. Slowly volatilizes at ordinary temperatures. Slightly soluble in water; very soluble in alcohol, in chloroform, and in ether; freely soluble in carbon disulfide, in solvent hexane, and in fixed and volatile oils.

Candelilla Wax: A hard, yellowish-brown-opaque to translucent wax. Its specific gravity is about 0.983. Soluble in chloroform and in toluene; insoluble in water.

Candesartan Cilexetil: White to off-white powder. Sparingly soluble in methanol; practically insoluble in water.

Canola Oil: Clear, pale yellow, slightly viscous liquid. Practically insoluble in water and in alcohol. Miscible with light petroleum (bp: 40° to 60°). *NF category:* Solvent; vehicle (oleaginous).

Capecitabine: White to off-white crystalline powder. Freely soluble in methanol; soluble in acetonitrile and in alcohol; sparingly soluble in water.

Capreomycin Sulfate: White to practically white, amorphous powder. Freely soluble in water; practically insoluble in most organic solvents.

Add the following:

▲ **Caprylic Acid:** Clear, colorless or slightly yellowish, oily liquid. Very soluble in acetone and in alcohol; very slightly soluble in water. It dissolves in dilute solutions of alkali hydroxides. *NF category:* Emulsifying and/or solubilizing agent.▲ *NF31*

Caprylocaproyl Polyoxylglycerides: Pale yellow, oily liquids. Dispersible in hot water; freely soluble in methylene chloride. *NF category:* Ointment base; solvent.

Capsaicin: Off-white powder. Melts at about 65°. Soluble in alcohol, in benzene, in chloroform; slightly soluble in carbon disulfide; practically insoluble in cold water.

Capsicum Oleoresin: Dark red, oily liquid. Soluble in alcohol, in acetone, in ether, in chloroform, and in volatile oils; soluble with opalescence in fixed oils.

Captopril: White to off-white, crystalline powder, which may have a characteristic, sulfide-like odor. Melts in the range of 104° to 110°. Freely soluble in water, in methanol, in alcohol, and in chloroform.

Caramel: Thick, dark brown liquid having the characteristic odor of burnt sugar, and a pleasant, bitter taste. One part dissolved in 1000 parts of water yields a clear solution having a distinct yellowish-orange color. The color of this solution is not changed and no precipitate is formed after exposure to sunlight for 6 hours. When spread in a thin layer on a glass plate, it appears homogeneous, reddish-brown, and transparent. Miscible with water. Soluble in dilute alcohol up to 55% (v/v). Immiscible with ether, with chloroform, with acetone, with benzene, and with solvent hexane. *NF category:* Color.

Carbachol: White powder. Freely soluble in water; sparingly soluble in alcohol; practically insoluble in chloroform and in ether.

Carbamazepine: White to off-white powder. Soluble in alcohol and in acetone; practically insoluble in water.

Carbamide Peroxide Topical Solution: Clear, colorless, viscous liquid, having a characteristic odor and taste.

Carbenicillin Disodium: White to off-white, crystalline powder. Freely soluble in water; soluble in alcohol; practically insoluble in chloroform and in ether.

Carbenicillin Indanyl Sodium: White to off-white powder. Soluble in water and in alcohol.

Carbidopa: White to creamy white, odorless or practically odorless, powder. Freely soluble in 3 N hydrochloric acid; slightly soluble in water, and in methanol; practically insoluble in alcohol, in acetone, in chloroform, and in ether.

Carbinoxamine Maleate: White, odorless, crystalline powder. Very soluble in water; freely soluble in alcohol and in chloroform; very slightly soluble in ether.

Carbol-Fuchsin Topical Solution: Dark purple liquid, which appears purplish red when spread in a thin film.

Carbomer 910: White, fluffy powder, having a slight, characteristic odor. Is hygroscopic. The pH of a 1 in 100 dispersion is about 3. When neutralized with alkali hydroxides or with amines, it dissolves in water, in alcohol, and in glycerin. *NF category:* Suspending and/or viscosity-increasing agent.

Carbomer 934: See *Carbomer 910*.

Carbomer 934P: See *Carbomer 910*.

Carbomer 940: See *Carbomer 910*.

Carbomer 941: See *Carbomer 910*.

Carbomer 1342: See *Carbomer 910*.

Carbomer Copolymer: White, hygroscopic powder. It swells in water when a dispersion of it is neutralized with

sodium hydroxide to a pH within the range of 7.3 to 7.8. *NF category:* Emulsifying and/or solubilizing agent; suspending and/or viscosity increasing agent; tablet binder.

Carbomer Homopolymer: White, fluffy hygroscopic powder, having a slight, characteristic odor. The pH of a 1 in 100 dispersion in water is about 3. When neutralized with alkali hydroxides or with amines, it swells giving the appearance of dissolving in water; when neutralized with lower amines and alkanolamines, it swells giving the appearance of dissolving in methanol or glycerin; when neutralized with ethoxylated long-chain (C_{14} – C_{18}) amines, it swells giving the appearance of dissolving in ethanol. *NF category:* Tablet binder; suspending and/or viscosity-increasing agent.

Carbomer Interpolymer: White, hygroscopic powder. It swells in water when a dispersion of it is neutralized with sodium hydroxide to a pH within the range of 5.5 to 9. *NF category:* Emulsifying and/or solubilizing agent; suspending and/or viscosity increasing agent; tablet binder.

Carbon Dioxide: Odorless, colorless gas. Its solutions are acid to litmus. One L at 0° and at a pressure of 760 mm of mercury weighs 1.977 g. One volume dissolves in about 1 volume of water. *NF category:* Air displacement.

Carboprost Tromethamine: White to off-white powder. Soluble in water.

Carboxymethylcellulose Calcium: White to yellowish-white powder. Is hygroscopic. Practically insoluble in alcohol, in acetone, in ether, in chloroform, and in benzene. It swells with water to form a suspension; the pH of the suspension, obtained by shaking 1 g with 100 mL of water, is between 4.5 and 6.0. *NF category:* Suspending and/or viscosity-increasing agent.

Carboxymethylcellulose Sodium: White to cream-colored powder or granules. The powder is hygroscopic. Is easily dispersed in water to form colloidal solutions. Insoluble in alcohol, in ether, and in most other organic solvents. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; tablet binder.

Carboxymethylcellulose Sodium 12: Colorless or white to off-white powder or granules. Is odorless. Water solubility depends on degree of substitution (easily dispersed in water at all temperatures, forming a clear, colloidal solution). Insoluble in acetone, in alcohol, in ether, and in toluene. *NF category:* Suspending and/or viscosity-increasing agent.

Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium: White or slightly yellowish or grayish, odorless, slightly hygroscopic granular or fibrous powder. Soluble in water; insoluble in alcohol. *NF category:* Coating agent; suspending and/or viscosity-increasing agent.

Low-Substituted Carboxymethylcellulose Sodium: A white or almost white powder or short fibers. Practically insoluble in acetone, in alcohol, and in toluene. It swells in water to form a gel.

Carisoprodol: White, crystalline powder, having a mild, characteristic odor and a bitter taste. Freely soluble in alcohol, in chloroform, and in acetone; very slightly soluble in water.

Carmellose: White powder. Practically insoluble in ethanol (99.5%). Swells with water to form a suspension. Becomes viscous in sodium hydroxide TS. Is hygroscopic. *NF category:* Suspending and/or viscosity increasing agent.

Carmustine: Light yellow powder. Freely soluble in ether.

Carprofen: White crystalline powder. Freely soluble in ether, in acetone, in ethyl acetate, and in sodium hydroxide TS or sodium carbonate TS; practically insoluble in water.

Carrageenan: Yellowish or tan to white, coarse to fine powder. Is practically odorless and has a mucilaginous taste. Soluble in water at a temperature of about 80°, forming a viscous, clear or slightly opalescent solution that flows readily. Disperses in water more readily if first moistened with alcohol, with glycerin, or with a saturated solution of su-

crose in water. *NF category:* Suspending and/or viscosity-increasing agent.

Carvedilol: White or nearly white, crystalline powder. Slightly soluble in alcohol; practically insoluble in water and in dilute acids.

Casanthranol: Light tan to brown, amorphous, hygroscopic powder. Freely soluble in water, with some residue; partially soluble in methanol and in hot isopropyl alcohol; practically insoluble in acetone.

Cascara Sagrada: Has a distinct odor and a bitter and slightly acid taste.

Castor Oil: Pale yellowish or almost colorless, transparent, viscid liquid. Has a faint, mild odor; is free from foreign and rancid odor; and has a bland, characteristic taste. Soluble in alcohol. Miscible with dehydrated alcohol, with glacial acetic acid, with chloroform, and with ether. *NF category:* Plasticizer.

Hydrogenated Castor Oil: White, crystalline wax. Insoluble in water and in most common organic solvents. *NF category:* Stiffening agent.

Cefaclor: White to off-white, crystalline powder. Slightly soluble in water; practically insoluble in methanol, in chloroform, and in benzene.

Cefadroxil: White to off-white, crystalline powder. Slightly soluble in water; practically insoluble in alcohol, in chloroform, and in ether.

Cefamandole Nafate: White, odorless, crystalline solid. Soluble in water and in methanol; practically insoluble in ether, in chloroform, in benzene, and in cyclohexane.

Cefazolin: White to slightly off-white, odorless, crystalline powder. Melts at about 198° to 200°, with decomposition. Soluble in dimethylformamide and in pyridine; sparingly soluble in acetone; slightly soluble in alcohol, in methanol, and in water; very slightly soluble in ethyl acetate, in isopropyl alcohol, and in methyl isobutyl ketone; practically insoluble in benzene, in chloroform, in ether, and in methylene chloride.

Cefazolin Sodium: White to off-white, practically odorless, crystalline powder, or white to off-white solid. Freely soluble in water, in saline TS, and in dextrose solutions; very slightly soluble in alcohol; practically insoluble in chloroform and in ether.

Cefdinir: White to light-yellow crystalline powder. Sparingly soluble in 0.1 M phosphate buffer (pH 7) solution; practically insoluble in water, in alcohol, and in diethyl ether.

Cefepime Hydrochloride: White to off-white, crystalline, nonhygroscopic solid. Freely soluble in water.

Cefepime for Injection: White to pale yellow powder. Freely soluble in water.

Cefixime: White to light yellow, crystalline powder. Soluble in methanol and in propylene glycol; slightly soluble in alcohol, in acetone, and in glycerin; very slightly soluble in 70% sorbitol and in octanol; practically insoluble in ether, in ethyl acetate, in hexane, and in water.

Cefmenoxime Hydrochloride: White to light orange-yellow crystals or crystalline powder. Freely soluble in formamide; slightly soluble in methanol; very slightly soluble in water; practically insoluble in dehydrated alcohol and in ether.

Cefmetazole Sodium: White solid. Very soluble in water and in methanol; soluble in acetone; practically insoluble in chloroform.

Cefonicid Sodium: White to off-white solid. Freely soluble in water, in 0.9% sodium chloride solution, and in 5% dextrose solution; soluble in methanol; very slightly soluble in dehydrated alcohol.

Cefoperazone Sodium: White to pale buff crystalline powder. Freely soluble in water; soluble in methanol; slightly soluble in dehydrated alcohol; insoluble in acetone, in ethyl acetate, and in ether.

Ceforanide: White to off-white powder. Very soluble in 1 N sodium hydroxide; practically insoluble in water, in methanol, in chloroform, and in ether.

Cefotaxime Sodium: Off-white to pale yellow crystalline powder. Freely soluble in water; practically insoluble in organic solvents.

Cefoxitin Sodium: White to off-white, granules or powder, having a slight characteristic odor. Is somewhat hygroscopic. Very soluble in water; soluble in methanol; sparingly soluble in dimethylformamide; slightly soluble in acetone; insoluble in ether and in chloroform.

Cefpodoxime Proxetil: White to light brownish-white powder. Odorless or having a faint odor, and has a bitter taste. Freely soluble in dehydrated alcohol; soluble in acetonitrile and in methanol; slightly soluble in ether; very slightly soluble in water.

Ceftazidime: White to cream-colored, crystalline powder. Soluble in alkali and in dimethyl sulfoxide; slightly soluble in dimethylformamide, in methanol, and in water; insoluble in acetone, in alcohol, in chloroform, in dioxane, in ether, in ethyl acetate, and in toluene.

Ceftizoxime Sodium: White to pale yellow crystalline powder. Freely soluble in water.

Ceftriaxone Sodium: White to yellowish-orange crystalline powder. Freely soluble in water; sparingly soluble in methanol; very slightly soluble in alcohol.

Cefuroxime Axetil: White to almost white powder. The amorphous form is freely soluble in acetone; soluble in chloroform, in ethyl acetate, and in methanol; slightly soluble in dehydrated alcohol; insoluble in ether and in water. The crystalline form is freely soluble in acetone; sparingly soluble in chloroform, in ethyl acetate, and in methanol; slightly soluble in dehydrated alcohol; insoluble in ether and in water.

Cefuroxime Sodium: White or faintly yellow powder. Freely soluble in water; soluble in methanol; very slightly soluble in alcohol, in ether, in ethyl acetate, and in chloroform.

Celecoxib: White or almost white, crystalline or amorphous powder. Soluble to freely soluble in ethanol; soluble in methylene chloride; practically insoluble in water.

Cellulurate: Fine white or almost white powder or granules. Available in a range of viscosities, acetyl and butyl contents. Slightly hygroscopic; soluble in acetone, in methylene chloride, in pyridine, and in dimethyl sulfoxide; practically insoluble in water and in alcohol. *NF category:* Coating agent; polymer membrane.

Cellacefate: Free-flowing, white powder. May have a slight odor of acetic acid. Soluble in acetone and in dioxane; insoluble in water and in alcohol. *NF category:* Coating agent.

Cellulose Acetate: Fine, white powder or free-flowing pellets. Available in a range of viscosities and acetyl contents. High viscosity, which reflects high molecular weight, decreases solubility slightly. High acetyl content cellulose acetates generally have more limited solubility in commonly used organic solvents than low acetyl content cellulose acetates, but are more soluble in methylene chloride. All acetyl content cellulose acetates are soluble in dioxane and in dimethylformamide; insoluble in alcohol and in water. *NF category:* Coating agent; polymer membrane.

Microcrystalline Cellulose: Fine, white or almost white powder. It consists of free-flowing, nonfibrous particles. Practically insoluble in sodium hydroxide solution (1 in 20); insoluble in water, in dilute acids, and in most organic solvents. *NF category:* Tablet binder; tablet disintegrant; tablet and/or capsule diluent.

Silicified Microcrystalline Cellulose: White or almost white, very fine to moderately fine powder. It is a free-flowing material that may be compacted into self-binding tablets that disintegrate rapidly in water. Slightly soluble in sodium hydroxide solution (1 in 20); practically insoluble in

water, in acetone, in ethanol, in toluene, and in diluted acid. *NF category:* Tablet binder; tablet disintegrant; tablet and/or capsule diluent.

Microcrystalline Cellulose and Carboxymethylcellulose Sodium: Tasteless, odorless, white to off-white, coarse to fine powder. Swells in water, producing, when dispersed, a white, opaque dispersion or gel. Insoluble in organic solvents and in dilute acids. *NF category:* Suspending and/or viscosity-increasing agent.

Oxidized Cellulose: In the form of gauze or lint. Is slightly off-white in color, is acidic to the taste, and has a slight, charred odor. Soluble in dilute alkalies; insoluble in water and in acids.

Oxidized Regenerated Cellulose: A knit fabric, usually in the form of sterile strips. Slightly off-white, having a slight odor. Soluble in dilute alkalies; insoluble in water and in dilute acids.

Powdered Cellulose: White or almost white powder. Exhibits degrees of fineness ranging from a free-flowing dense powder to a coarse, fluffy, nonflowing material. Slightly soluble in sodium hydroxide solution (1 in 20); insoluble in water, in dilute acids, and in nearly all organic solvents. *NF category:* Filtering aid; sorbent; tablet and/or capsule diluent.

Cellulose Sodium Phosphate: Free-flowing cream-colored, odorless, tasteless powder. Insoluble in water, in dilute acids, and in most organic solvents.

Cephalexin: White to off-white, crystalline powder. Slightly soluble in water; practically insoluble in alcohol, in chloroform, and in ether.

Cephalexin Hydrochloride: White to off-white, crystalline powder. Soluble to the extent of 10 mg per mL in water, in acetone, in acetonitrile, in alcohol, in dimethylformamide, and in methanol; practically insoluble in chloroform, in ether, in ethyl acetate, and in isopropyl alcohol.

Cephalothin Sodium: White to off-white, practically odorless, crystalline powder. Freely soluble in water, in saline TS, and in dextrose solutions; insoluble in most organic solvents.

Cephapirin Benzathine: White, crystalline powder. Soluble in 0.1 N hydrochloric acid; practically insoluble in water, in ether, and in toluene; insoluble in alcohol.

Cephapirin Sodium: White to off-white, crystalline powder, odorless or having a slight odor. Very soluble in water; insoluble in most organic solvents.

Cephradine: White to off-white, crystalline powder. Sparingly soluble in water; very slightly soluble in alcohol and in chloroform; practically insoluble in ether.

Cetirizine Hydrochloride: White to almost white powder. Freely soluble in water; practically insoluble in acetone and in methylene chloride.

Cetostearyl Alcohol: Unctuous, white flakes or granules, having a faint, characteristic odor, and a bland, mild taste. Soluble in alcohol and in ether; insoluble in water. *NF category:* Stiffening agent.

Cetrimonium Bromide: A white to creamy white, voluminous, free-flowing powder, with a characteristic faint odor and bitter, soapy taste. Freely soluble in water and in alcohol; practically insoluble in ether. *NF category:* Antimicrobial preservative.

Cetyl Alcohol: Unctuous, white flakes, granules, cubes, or castings. Has a faint characteristic odor and a bland, mild taste. Usually melts in the range between 45° and 50°. Soluble in alcohol and in ether, the solubility increasing with an increase in temperature; insoluble in water. *NF category:* Stiffening agent.

Cetyl Esters Wax: White to off-white, somewhat translucent flakes, having a crystalline structure and a pearly luster when caked. Has a faint odor and a bland, mild taste, free from rancidity, and has a specific gravity of about 0.83 at 50°. Soluble in boiling alcohol, in ether, in chloroform,

and in fixed and volatile oils; slightly soluble in cold solvent hexane; practically insoluble in cold alcohol; insoluble in water. *NF category:* Stiffening agent.

Cetyl Palmitate: White crystals or flakes. Freely soluble in alcohol and in ether; practically insoluble in water. *NF category:* Stiffening agent.

Cetylpyridinium Chloride: White powder, having a slight, characteristic odor. Very soluble in water, in alcohol, and in chloroform; slightly soluble in benzene and in ether. *NF category:* Antimicrobial preservative; wetting and/or solubilizing agent.

Cetylpyridinium Chloride Topical Solution: Clear liquid. Is colorless unless a color has been added; has an aromatic odor and a bitter taste.

Activated Charcoal: Fine, black, odorless, tasteless powder, free from gritty matter. *NF category:* Sorbent.

Chitosan: White or almost white powder or granules. Soluble in aqueous solutions of glycolic acid, of formic acid, of acetic acid, of hydrochloric acid, and of lactic acid; practically insoluble in organic solvents and in water. *NF category:* Coating agent; film-forming agent; suspending and/or viscosity-increasing agent; vehicle (solid carrier).

Chloral Hydrate: Colorless, transparent, or white crystals having an aromatic, penetrating, and slightly acrid odor, and a slightly bitter, caustic taste. Melts at about 55°, and slowly volatilizes when exposed to air. Very soluble in water and in olive oil; freely soluble in alcohol, in chloroform, and in ether.

Chlorambucil: Off-white, slightly granular powder. Freely soluble in acetone; soluble in dilute alkali; very slightly soluble in water.

Chloramphenicol: Fine, white to grayish-white or yellowish-white, needle-like crystals or elongated plates. Its solutions are practically neutral to litmus. Is reasonably stable in neutral or moderately acid solutions. Its alcohol solution is dextrorotatory and its ethyl acetate solution is levorotatory. Freely soluble in alcohol, in propylene glycol, in acetone, and in ethyl acetate; slightly soluble in water.

Chloramphenicol Palmitate: Fine, white, unctuous, crystalline powder, having a faint odor and a bland, mild taste. Freely soluble in acetone and in chloroform; soluble in ether; sparingly soluble in alcohol; very slightly soluble in solvent hexane; insoluble in water.

Chloramphenicol Sodium Succinate: Light yellow powder. Freely soluble in water and in alcohol.

Chlordiazepoxide: Yellow, practically odorless, crystalline powder. Is sensitive to sunlight. Melts at about 240°. Sparingly soluble in chloroform and in alcohol; insoluble in water.

Chlordiazepoxide Hydrochloride: White or practically white, odorless, crystalline powder. Is affected by sunlight. Soluble in water; sparingly soluble in alcohol; insoluble in solvent hexane.

Chlorhexidine Acetate: A white or almost white, microcrystalline powder. Soluble in alcohol; sparingly soluble in water; slightly soluble in glycerol and in propylene glycol.

Chlorhexidine Gluconate Solution: Almost colorless or pale yellow, clear liquid. Miscible with glacial acetic acid and with water; miscible with three times its volume of acetone and with five times its volume of dehydrated alcohol; further addition of acetone or dehydrated alcohol yields a white turbidity.

Chlorhexidine Hydrochloride: White or almost white, crystalline powder. Sparingly soluble in propylene glycol and in water; very slightly soluble in alcohol.

Chlorobutanol: Colorless to white crystals, having a characteristic, somewhat camphoraceous, odor and taste. Anhydrous form melts at about 95°, and hydrous form melts at about 76°. Freely soluble in alcohol, in ether, in chloroform, and in volatile oils; soluble in glycerin; slightly soluble in water. *NF category:* Antimicrobial preservative.

Chlorocresol: Colorless or practically colorless crystals or crystalline powder, having a characteristic, nontarry odor. Is volatile in steam. Very soluble in alcohol; soluble in ether, in terpenes, in fixed oils, and in solutions of alkali hydroxides; slightly soluble in water and more soluble in hot water. *NF category:* Antimicrobial preservative.

Chloroprocaine Hydrochloride: White, crystalline powder. Is odorless, and is stable in air. Its solutions are acid to litmus. Exhibits local anesthetic properties when placed upon the tongue. Soluble in water; slightly soluble in alcohol; very slightly soluble in chloroform; practically insoluble in ether.

Chloroquine: White or slightly yellow, crystalline powder. Is odorless, and has a bitter taste. Soluble in dilute acids, in chloroform, and in ether; very slightly soluble in water.

Chloroquine Hydrochloride Injection: Colorless liquid.

Chloroquine Phosphate: White, crystalline powder. Is odorless, has a bitter taste, and is discolored slowly on exposure to light. Its solutions have a pH of about 4.5. Exists in two polymorphic forms, one melting between 193° and 195° and the other between 210° and 215° (see *Melting Range or Temperature* (741)); mixture of the forms melts between 193° and 215°. Freely soluble in water; practically insoluble in alcohol, in chloroform, and in ether.

Chlorothiazide: White or practically white, crystalline, odorless powder. Melts at about 340°, with decomposition. Freely soluble in dimethylformamide and in dimethyl sulfoxide; slightly soluble in methanol and in pyridine; practically insoluble in ether, in benzene, and in chloroform; very slightly soluble in water.

Chloroxylenol: White crystals or crystalline powder, having a characteristic odor. Is volatile in steam. Freely soluble in alcohol, in ether, in terpenes, in fixed oils, and in solutions of alkali hydroxides; very slightly soluble in water.

Chlorpheniramine Maleate: White, odorless, crystalline powder. Its solutions have a pH between 4 and 5. Freely soluble in water; soluble in alcohol and in chloroform; slightly soluble in ether and in benzene.

Chlorpromazine: White, crystalline solid, having an amine-like odor. Darkens on prolonged exposure to light. Melts at about 60°. Freely soluble in alcohol, in benzene, in chloroform, in ether, and in dilute mineral acids; practically insoluble in water and in dilute alkali hydroxides.

Chlorpromazine Hydrochloride: White or slightly creamy white, odorless, crystalline powder. Darkens on prolonged exposure to light. Very soluble in water; freely soluble in alcohol and in chloroform; insoluble in ether and in benzene.

Chlorpropamide: White, crystalline powder, having a slight odor. Soluble in alcohol; sparingly soluble in chloroform; practically insoluble in water.

Chlortetracycline Hydrochloride: Yellow, crystalline powder. Is odorless, and has a bitter taste. Is stable in air, but is slowly affected by light. Soluble in solutions of alkali hydroxides and carbonates; sparingly soluble in water; slightly soluble in alcohol; practically insoluble in acetone, in chloroform, in dioxane, and in ether.

Chlorthalidone: White to yellowish-white, crystalline powder. Melts at a temperature above 215°, with decomposition. Soluble in methanol; slightly soluble in alcohol; practically insoluble in water, in ether, and in chloroform.

Chlorzoxazone: White or practically white, practically odorless, crystalline powder. Soluble in solutions of alkali hydroxides and ammonia; sparingly soluble in alcohol, in isopropyl alcohol, and in methanol; slightly soluble in water.

Cholecalciferol: White, odorless crystals. Is affected by air and by light. Melts at about 85°. Soluble in alcohol, in chloroform, and in fatty oils; insoluble in water.

Cholesterol: White or faintly yellow, practically odorless, pearly leaflets, needles, powder, or granules. Ac-

quires a yellow to pale tan color on prolonged exposure to light. Soluble in acetone, in chloroform, in dioxane, in ether, in ethyl acetate, in solvent hexane, and in vegetable oils; sparingly soluble in dehydrated alcohol; slightly (and slowly) soluble in alcohol; insoluble in water. *NF category:* Emulsifying and/or solubilizing agent.

Cholestyramine Resin: White to buff-colored, hygroscopic, fine powder. Is odorless or has not more than a slight amine-like odor. Insoluble in water, in alcohol, in chloroform, and in ether.

Choline Bitartrate: White, hygroscopic, crystalline powder. Clear, colorless liquid in solution. Melts between 148° and 153°. Is odorless, or may have a faint trimethylamine odor. Freely soluble in water; slightly soluble in alcohol; insoluble in ether and in chloroform.

Choline Chloride: Colorless or white crystals or crystalline powder, usually having a slight odor of trimethylamine. Clear and colorless in solution. Hygroscopic. Soluble in alcohol and in water.

Sodium Chromate Cr 51 Injection: Clear, slightly yellow solution.

Chromic Chloride: Dark green, odorless, slightly deliquescent crystals. Soluble in water and in alcohol; slightly soluble in acetone; practically insoluble in ether.

Chymotrypsin: White to yellowish-white, crystalline or amorphous, odorless, powder. An amount equivalent to 100,000 USP Units is soluble in 10 mL of water and in 10 mL of saline TS.

Ciclopirox: White to slightly yellowish-white, crystalline powder. Freely soluble in ethanol and in methylene chloride; soluble in ether; slightly soluble in water.

Ciclopirox Olamine: White to slightly yellowish-white, crystalline powder. Very soluble in alcohol and in methylene chloride; slightly soluble in water; practically insoluble in cyclohexane.

Cilastatin Sodium: White to tan-colored powder. Soluble in water and in methanol.

Cilostazol: White to off-white crystals. Freely soluble in chloroform; slightly soluble in methanol and in alcohol; practically insoluble in water.

Cimetidine: White to off-white, crystalline powder; odorless, or having a slight mercaptan odor. Freely soluble in methanol; soluble in alcohol and in polyethylene glycol 400; sparingly soluble in isopropyl alcohol; slightly soluble in water and in chloroform; practically insoluble in ether.

Cinoxacin: White to yellowish-white, crystalline solid. Is odorless, and has a bitter taste and a lingering aftertaste. Soluble in alkaline solution; insoluble in water and in most common organic solvents.

Ciprofloxacin Hydrochloride: Faintly yellowish to light yellow crystals. Sparingly soluble in water; slightly soluble in acetic acid and in methanol; very slightly soluble in dehydrated alcohol; practically insoluble in acetone, in acetonitrile, in ethyl acetate, in hexane, and in methylene chloride.

Cisapride: White or almost white powder. Freely soluble in dimethylformamide; soluble in methylene chloride; sparingly soluble in methanol; practically insoluble in water.

Change to read:

Citalopram Hydrobromide: White to almost white, crystalline powder. Soluble in alcohol; sparingly soluble in water ▲ and in dehydrated alcohol.▲ *USP36*

Anhydrous Citric Acid: Colorless, translucent crystals, or white, granular to fine, crystalline powder. Melts at about 153°, with decomposition. Very soluble in water; freely soluble in alcohol; very slightly soluble in ether. *NF category:* Acidifying agent; buffering agent.

Citric Acid Monohydrate: Colorless, translucent crystals, or white, granular to fine, crystalline powder. Efflores-

cent in dry air. Very soluble in water; freely soluble in alcohol; very slightly soluble in ether. *NF category:* Acidifying agent; buffering agent.

Clarithromycin: White to off-white, crystalline powder. Soluble in acetone; slightly soluble in dehydrated alcohol, in methanol, and in acetonitrile, and in phosphate buffer at pH values of 2 to 5; practically insoluble in water.

Clavulanate Potassium: White to off-white powder. Is moisture-sensitive. Freely soluble in water, but stability in aqueous solution is not good, optimum stability at a pH of 6.0 to 6.3; soluble in methanol, with decomposition.

Clemastine Fumarate: White to off-white, odorless powder. Its solutions are acid to litmus. Slightly soluble in methanol; very slightly soluble in water, and in chloroform.

Clenbuterol Hydrochloride: White or almost white, crystalline powder. Soluble in water and in alcohol; slightly soluble in acetone. It melts with decomposition at 173°.

Clidinium Bromide: White to nearly white, practically odorless, crystalline powder. Is optically inactive. Melts at about 242°. Soluble in water and in alcohol; slightly soluble in benzene and in ether.

Clindamycin Hydrochloride: White or practically white, crystalline powder. Is odorless or has a faint mercaptan-like odor. Is stable in the presence of air and light. Its solutions are acidic and are dextrorotatory. Freely soluble in water, in dimethylformamide, and in methanol; soluble in alcohol; practically insoluble in acetone.

Clindamycin Palmitate Hydrochloride: White to off-white amorphous powder, having a characteristic odor. Very soluble in ethyl acetate and in dimethylformamide; freely soluble in water, in benzene, in ether, in chloroform, and in alcohol.

Clindamycin Phosphate: White to off-white, hygroscopic, crystalline powder. Is odorless or practically odorless, and has a bitter taste. Freely soluble in water; slightly soluble in dehydrated alcohol; very slightly soluble in acetone; practically insoluble in chloroform, in benzene, and in ether.

Clioquinol: Voluminous, spongy, yellowish-white to brownish-yellow powder, having a slight, characteristic odor. Darkens on exposure to light. Melts at about 180°, with decomposition. Soluble in hot ethyl acetate and in hot glacial acetic acid; practically insoluble in water and in alcohol.

Clobetasol Propionate: White to cream, crystalline powder. Soluble in acetone, in dimethyl sulfoxide, in chloroform, in methanol, and in dioxane; sparingly soluble in ethanol; slightly soluble in benzene and in diethyl ether; practically insoluble in water.

Clocortolone Pivalate: White to yellowish-white, odorless powder. Melts at about 230°, with decomposition. Freely soluble in chloroform and in dioxane; soluble in acetone; sparingly soluble in alcohol; slightly soluble in benzene and in ether.

Clofazimine: Dark red crystals. Melts at about 217°, with decomposition. Soluble in chloroform and in benzene; sparingly soluble in alcohol, in acetone, and in ethyl acetate; practically insoluble in water.

Clofibrate: Colorless to pale yellow liquid having a characteristic odor. Soluble in acetone, in alcohol, in benzene, and in chloroform; insoluble in water.

Clomiphene Citrate: White to pale yellow, essentially odorless powder. Freely soluble in methanol; sparingly soluble in alcohol; slightly soluble in water and in chloroform; insoluble in ether.

Clomipramine Hydrochloride: White to faintly yellow, crystalline powder. Very soluble in water.

Clonazepam: Light yellow powder, having a faint odor. Sparingly soluble in acetone and in chloroform; slightly soluble in alcohol and in ether; insoluble in water.

Clonidine: White to almost white, crystalline powder. Melting point is about 130°. Freely soluble in methanol and in alcohol.

Change to read:

Clopidogrel Bisulfate: White to off-white powder.

▲ Freely soluble at pH 1; practically insoluble at neutral pH. ▲ *USP36*

Cloprostenol Sodium: White or almost white, amorphous powder. Is hygroscopic. Freely soluble in water, in alcohol, and in methanol; practically insoluble in acetone.

Clorazepate Dipotassium: Light yellow, crystalline powder. Darkens on exposure to light. Soluble in water but, upon standing, may precipitate from the solution; slightly soluble in alcohol and in isopropyl alcohol; practically insoluble in acetone, in benzene, in chloroform, in ether, and in methylene chloride.

Clorsulon: White to off-white powder. Freely soluble in acetonitrile and in methanol; slightly soluble in water; very slightly soluble in methylene chloride.

Clotrimazole: White to pale yellow, crystalline powder. Melts at about 142°, with decomposition. Freely soluble in methanol, in acetone, in chloroform, and in alcohol; practically insoluble in water.

Cloxacillin Benzathine: White or almost white, almost odorless, crystals or crystalline powder. Soluble in chloroform and in methanol; sparingly soluble in acetone; slightly soluble in water, in alcohol, and in isopropyl alcohol.

Cloxacillin Sodium: White, odorless, crystalline powder. Freely soluble in water; soluble in alcohol; slightly soluble in chloroform.

Clozapine: Yellow, crystalline powder. Soluble in chloroform, in acetone, and in alcohol; sparingly soluble in acetonitrile; insoluble in water.

Coal Tar: Nearly black, viscous liquid, heavier than water, having a characteristic, naphthalene-like odor, and producing a sharp, burning sensation on the tongue. Slightly soluble in water, to which it imparts its characteristic odor and taste and a faintly alkaline reaction; partially soluble in acetone, in alcohol, in carbon disulfide, in chloroform, in ether, in methanol, and in solvent hexane; soluble in benzene and nitrobenzene.

Cyanocobalamin Co 57 Capsules: May contain a small amount of solid or solids, or may appear empty.

Cyanocobalamin Co 57 Oral Solution: Clear, colorless to pink solution.

Cocaine: Colorless to white crystals or white, crystalline powder. Is levorotatory in 3 N hydrochloric acid solution. Its saturated solution is alkaline to litmus. Very soluble in warm alcohol; freely soluble in alcohol, in chloroform, and in ether; soluble in olive oil; sparingly soluble in mineral oil; slightly soluble in water.

Cocaine Hydrochloride: Colorless crystals or white, crystalline powder. Very soluble in water; freely soluble in alcohol; soluble in chloroform and in glycerin; insoluble in ether.

Coccidioidin: Clear, practically colorless or amber-colored liquid.

Cocoa Butter: Yellowish-white solid, having a faint, agreeable odor, and a bland, chocolate-like taste if the cocoa butter is obtained by pressing. If obtained by extraction, the taste is bland. Is usually brittle at temperatures below 25°. Freely soluble in ether and in chloroform; soluble in boiling dehydrated alcohol; slightly soluble in alcohol. *NF category:* Suppository base.

Coconut Oil: Clear, white to light yellow-tan, viscous liquid. Freely soluble in methylene chloride and in light petroleum (bp: 65° to 70°); very slightly soluble in alcohol;

practically insoluble in water. *NF category:* Coating agent; emulsifying and/or solubilizing agent.

Hydrogenated Coconut Oil: White to yellowish, fatty solid to semi-solid. Freely soluble in ether; very slightly soluble in alcohol; practically insoluble in water. *NF category:* Coating agent; tablet binder; tablet and/or capsule lubricant.

Cod Liver Oil: Thin, oily liquid, having a characteristic, slightly fishy but not rancid odor, and a fishy taste. Freely soluble in ether, in chloroform, in carbon disulfide, and in ethyl acetate; slightly soluble in alcohol.

Codeine: Colorless or white crystals or white, crystalline powder. It effloresces slowly in dry air, and is affected by light. In acid or alcohol solutions it is levorotatory. Its saturated solution is alkaline to litmus. Very soluble in chloroform; freely soluble in alcohol; sparingly soluble in ether; slightly soluble in water. When heated in an amount of water insufficient for complete solution, it melts to oily drops that crystallize on cooling.

Codeine Phosphate: Fine, white, needle-shaped crystals, or white, crystalline powder. Is odorless. Is affected by light. Its solutions are acid to litmus. Very soluble in hot water; freely soluble in water; slightly soluble in alcohol but more so in boiling alcohol.

Codeine Sulfate: White crystals, usually needle-like, or white, crystalline powder. Is affected by light. Freely soluble in water at 80°; soluble in water; very slightly soluble in alcohol; insoluble in chloroform and in ether.

Colchicine: Pale yellow to pale greenish-yellow, amorphous scales, or powder or crystalline powder. Is odorless or nearly so, and darkens on exposure to light. Freely soluble in alcohol and in chloroform; soluble in water; slightly soluble in ether.

Colestipol Hydrochloride: Yellow to orange beads. Swells but does not dissolve in water or dilute aqueous solutions of acid or alkali. Insoluble in the common organic solvents.

Colistimethate Sodium: White to slightly yellow, odorless, fine powder. Freely soluble in water; soluble in methanol; insoluble in acetone and in ether.

Colistin Sulfate: White to slightly yellow, odorless, fine powder. Freely soluble in water; slightly soluble in methanol; insoluble in acetone and in ether.

Collodion: Clear, or slightly opalescent, viscous liquid. Is colorless, or slightly yellowish, and has the odor of ether.

Flexible Collodion: Clear, or slightly opalescent, viscous liquid. Is colorless or slightly yellow, and has the odor of ether. The strong odor of camphor becomes noticeable as the ether evaporates.

Copovidone: White to yellowish-white powder or flakes. Is hygroscopic. Freely soluble in water, in alcohol, and in methylene chloride; practically insoluble in ether. *NF category:* Tablet binder; coating agent.

Corn Oil: Clear, light yellow, oily liquid, having a faint, characteristic odor and taste. Slightly soluble in alcohol. Miscible with ether, with chloroform, with benzene, and with solvent hexane. *Specific gravity* (841): Between 0.914 and 0.921. *NF category:* Solvent; vehicle (oleaginous).

Corn Syrup: Clear, white to light yellow, viscous liquid. Is miscible in all proportions with water. *NF category:* Suspending and/or viscosity-increasing agent; sweetening agent; tablet and/or capsule diluent; tablet binder; tonicity agent.

Corn Syrup Solids: Sweet, white to light yellow powder or granules. Soluble in water. *NF category:* Coating agent; flavored and/or sweetened vehicle; humectant; solid carrier; suspending and/or viscosity-increasing agent; sweetening agent; tablet and/or capsule diluent; tablet binder; tonicity agent.

Corticotropin Injection: Colorless or light straw-colored liquid.

Corticotropin for Injection: White or practically white, soluble, amorphous solid having the characteristic appearance of substances prepared by freeze-drying.

Repository Corticotropin Injection: Colorless or light straw-colored liquid, which may be quite viscid at room temperature. Is odorless or has an odor of an antimicrobial agent.

Corticotropin Zinc Hydroxide Injectable Suspension: Flocculent, white, aqueous suspension, free from large particles following moderate shaking.

Cortisone Acetate: White or practically white, odorless, crystalline powder. Is stable in air. Melts at about 240°, with some decomposition (see *Melting Range or Temperature* <741>). Freely soluble in chloroform; soluble in dioxane; sparingly soluble in acetone; slightly soluble in alcohol; insoluble in water.

Purified Cotton: White, soft, fine filament-like hairs appearing under the microscope as hollow, flattened, and twisted bands, striate and slightly thickened at the edges. Is practically odorless and practically tasteless. Soluble in ammoniated cupric oxide TS; insoluble in ordinary solvents.

Cottonseed Oil: Pale yellow, oily liquid. Is odorless or nearly so, and has a bland taste. At temperatures below 10° particles of solid fat may separate from the Oil, and at about 0° to -5° the Oil becomes a solid or nearly so. *Specific gravity* (841): Between 0.915 and 0.921. Slightly soluble in alcohol. Miscible with ether, with chloroform, with solvent hexane, and with carbon disulfide. *NF category:* Solvent; vehicle (oleaginous).

Hydrogenated Cottonseed Oil: A white mass or powder that melts to a clear, pale yellow liquid when heated. Freely soluble in methylene chloride and in toluene; very slightly soluble in alcohol; practically insoluble in water.

Creatinine: White crystals or crystalline powder; odorless. Soluble in water; slightly soluble in alcohol; practically insoluble in acetone, in ether, and in chloroform. *NF category:* Bulking agent for freeze-drying.

Cresol: Colorless, or yellowish to brownish-yellow, or pinkish, highly refractive liquid, becoming darker with age and on exposure to light. Has a phenol-like, sometimes empyreumatic odor. A saturated solution of it is neutral or only slightly acid to litmus. Sparingly soluble in water, usually forming a cloudy solution; dissolves in solutions of fixed alkali hydroxides. Miscible with alcohol, with ether, and with glycerin. *NF category:* Antimicrobial preservative.

Cromolyn Sodium: White, odorless, crystalline powder. Is tasteless at first, with a slightly bitter aftertaste. Is hygroscopic. Soluble in water; insoluble in alcohol and in chloroform.

Cromolyn Sodium for Inhalation: White to creamy white, odorless, hygroscopic, and very finely divided powder.

Croscarmellose Sodium: White, free-flowing powder. Partially soluble in water; insoluble in alcohol, in ether, and in other organic solvents. *NF category:* Tablet disintegrant.

Crospovidone: White to creamy-white, hygroscopic powder, having a faint odor. Insoluble in water and in ordinary organic solvents. *NF category:* Tablet disintegrant.

Crotamiton: Colorless to slightly yellowish oil, having a faint amine-like odor. Soluble in alcohol and in methanol.

Cupric Chloride: Bluish green, deliquescent crystals. Freely soluble in water; soluble in alcohol; slightly soluble in ether.

Cupric Sulfate: Deep blue, triclinic crystals or blue, crystalline granules or powder. It effloresces slowly in dry air. Its solutions are acid to litmus. Very soluble in boiling water; freely soluble in water and in glycerin; slightly soluble in alcohol.

Cyanocobalamin: Dark red crystals or amorphous or crystalline red powder. In the anhydrous form, it is very hygroscopic and when exposed to air it may absorb about

12% of water. Soluble in alcohol; sparingly soluble in water; insoluble in acetone, in chloroform, and in ether.

Cyclandelate: White, crystalline powder. Very soluble in acetonitrile, in alcohol, and in ether; practically insoluble in water. Melts at about 58°.

Cyclizine Hydrochloride: White, crystalline powder or small, colorless crystals. Is odorless or nearly so, and has a bitter taste. Melts indistinctly at about 285°, with decomposition. Sparingly soluble in chloroform; slightly soluble in water and in alcohol; insoluble in ether.

Cyclobenzaprine Hydrochloride: White to off-white, odorless, crystalline powder. Freely soluble in water, in alcohol, and in methanol; sparingly soluble in isopropanol; slightly soluble in chloroform and in methylene chloride; insoluble in hydrocarbons.

Cyclopentolate Hydrochloride: White, crystalline powder, which upon standing develops a characteristic odor. Its solutions are acid to litmus. Melts at about 138°, the melt appearing opaque. Very soluble in water; freely soluble in alcohol; insoluble in ether.

Cyclophosphamide: White, crystalline powder. Liquefies upon loss of its water of crystallization. Soluble in water and in alcohol.

Cyclopropane: Colorless gas having a characteristic odor. Has a pungent taste. One L at a pressure of 760 mm and a temperature of 0° weighs about 1.88 g. One volume dissolves in about 2.7 volumes of water at 15°. Freely soluble in alcohol; soluble in fixed oils.

Cycloserine: White to pale yellow, crystalline powder. Is odorless or has a faint odor. Is hygroscopic and deteriorates upon absorbing water. Its solutions are dextrorotatory. Freely soluble in water.

Cyclosporine: White to almost white powder. Soluble in acetone, in alcohol, in methanol, in ether, in chloroform, and in methylene chloride; slightly soluble in saturated hydrocarbons; practically insoluble in water.

Cyproheptadine Hydrochloride: White to slightly yellow, odorless or practically odorless, crystalline powder. Freely soluble in methanol; soluble in chloroform; sparingly soluble in alcohol; slightly soluble in water; practically insoluble in ether.

Cyromazine: White or off-white, odorless, crystalline powder. Slightly soluble in methanol and in water.

Cysteine Hydrochloride: White crystals or crystalline powder. Soluble in water, in alcohol, and in acetone.

Cytarabine: Odorless, white to off-white, crystalline powder. Freely soluble in water; slightly soluble in alcohol and in chloroform.

Dactinomycin: Bright red, crystalline powder. Is somewhat hygroscopic and is affected by light and by heat. Freely soluble in alcohol; soluble in water at 10° and slightly soluble in water at 37°; very slightly soluble in ether.

Danazol: White to pale yellow, crystalline powder. Melts at about 225°, with some decomposition. Freely soluble in chloroform; soluble in acetone; sparingly soluble in alcohol and in benzene; slightly soluble in ether; practically insoluble or insoluble in water and in hexane.

Change to read:

Dantrolene Sodium: Fine orange to orange-brown powder. Sparingly soluble in ▲ dimethylformamide and in glycerine; sparingly soluble to practically insoluble in acetone.▲ USP36

Dapsone: White or creamy white, crystalline powder. Is odorless and has a slightly bitter taste. Freely soluble in alcohol; soluble in acetone and in dilute mineral acids; very slightly soluble in water.

Daunorubicin Hydrochloride: Orange-red, crystalline, hygroscopic powder. Freely soluble in water and in metha-

nol; slightly soluble in alcohol; very slightly soluble in chloroform; practically insoluble in acetone.

Deferoxamine Mesylate: White to off-white powder. Freely soluble in water; slightly soluble in methanol.

Dehydrocholic Acid: White, fluffy, odorless powder, having a bitter taste. Soluble in glacial acetic acid and in solutions of alkali hydroxides and carbonates; sparingly soluble in chloroform (the solutions in alcohol and in chloroform usually are slightly turbid); slightly soluble in alcohol and in ether; practically insoluble in water.

Dehydroacetic Acid: White or nearly white, crystalline powder. Soluble in aqueous solutions of alkalies; very slightly soluble in water. One g of sample dissolves in about 35 mL of alcohol and in 5 mL of acetone. *NF category:* Antimicrobial preservative.

Demecarium Bromide: White or slightly yellow, slightly hygroscopic, crystalline powder. Freely soluble in water and in alcohol; soluble in ether; sparingly soluble in acetone.

Demeclocycline: Yellow, crystalline, odorless powder, having a bitter taste. Soluble in alcohol; sparingly soluble in water. Dissolves readily in 3 N hydrochloric acid and in alkaline solutions.

Demeclocycline Hydrochloride: Yellow, crystalline, odorless powder, having a bitter taste. Sparingly soluble in water and in solutions of alkali hydroxides and carbonates; slightly soluble in alcohol; practically insoluble in acetone and in chloroform.

Denatonium Benzoate: Very soluble in chloroform and in methanol; freely soluble in water and in alcohol; very slightly soluble in ether. *NF category:* Alcohol denaturant.

Desipramine Hydrochloride: White to off-white, crystalline powder. Melts at about 213°. Freely soluble in methanol and in chloroform; soluble in water and in alcohol; insoluble in ether.

Desmopressin Acetate: White, fluffy powder. Soluble in water, in alcohol, and in acetic acid.

Desoximetasone: White to practically white, odorless, crystalline powder. Freely soluble in alcohol, in acetone, and in chloroform; insoluble in water.

Desoxycholic Acid: Occurs as a white, crystalline powder. Freely soluble in alcohol; soluble in acetone and in solutions of alkali hydroxides and carbonates; slightly soluble in chloroform and in ether; practically insoluble in water. *NF category:* Emulsifying and/or solubilizing agent.

Desoxycorticosterone Acetate: White or creamy white, crystalline powder. Is odorless, and is stable in air. Sparingly soluble in alcohol, in acetone, and in dioxane; slightly soluble in vegetable oils; practically insoluble in water.

Dexamethasone: White to practically white, odorless, crystalline powder. Is stable in air. Melts at about 250°, with some decomposition. Sparingly soluble in acetone, in alcohol, in dioxane, and in methanol; slightly soluble in chloroform; very slightly soluble in ether; practically insoluble in water.

Dexamethasone Acetate: Clear, white to off-white, odorless powder. Freely soluble in methanol, in acetone, and in dioxane; practically insoluble in water.

Dexamethasone Sodium Phosphate: White or slightly yellow, crystalline powder. Is odorless or has a slight odor of alcohol, and is exceedingly hygroscopic. Freely soluble in water; slightly soluble in alcohol; very slightly soluble in dioxane; insoluble in chloroform and in ether.

Dexbrompheniramine Maleate: White, odorless, crystalline powder. Exists in two polymorphic forms, one melting between 106° and 107° and the other between 112° and 113°. Mixtures of the forms may melt between 105° and 113°. The pH of a solution (1 in 100) is about 5. Freely soluble in water; soluble in alcohol and in chloroform.

Dexchlorpheniramine Maleate: White, odorless, crystalline powder. Freely soluble in water; soluble in alcohol and in chloroform; slightly soluble in benzene and in ether.

Dexpanthenol: Clear, viscous, somewhat hygroscopic liquid, having a slight, characteristic odor. Some crystallization may occur on standing. Freely soluble in water, in alcohol, in methanol, and in propylene glycol; soluble in chloroform and in ether; slightly soluble in glycerin.

Dextran 1: A white to off-white powder. Is hygroscopic. Very soluble in water; sparingly soluble in alcohol.

Dextrates: Free-flowing, porous, white, odorless, spherical granules consisting of aggregates of microcrystals, having a sweet taste and producing a cooling sensation in the mouth. May be compressed directly into self-binding tablets. Freely soluble in water (heating increases its solubility in water); soluble in dilute acids and alkalies and in basic organic solvents such as pyridine; insoluble in the common organic solvents. *NF category:* Sweetening agent; tablet and/or capsule diluent.

Dextrin: Free-flowing, white, yellow, or brown powder. Its solubility in water varies; it is usually very soluble, but often contains an insoluble portion. *NF category:* Tablet binder; tablet and/or capsule diluent.

Dextroamphetamine Sulfate: White, odorless, crystalline powder. Soluble in water; slightly soluble in alcohol; insoluble in ether.

Dextromethorphan: Practically white to slightly yellow, odorless, crystalline powder. Eleven mg of Dextromethorphan is equivalent to 15 mg of dextromethorphan hydrobromide monohydrate. Freely soluble in chloroform; practically insoluble in water.

Dextromethorphan Hydrobromide: Practically white crystals or crystalline powder, having a faint odor. Melts at about 126°, with decomposition. Freely soluble in alcohol and in chloroform; sparingly soluble in water; insoluble in ether.

Dextrose: Colorless crystals or white, crystalline or granular powder. Is odorless, and has a sweet taste. Very soluble in boiling water; freely soluble in water; soluble in boiling alcohol; slightly soluble in alcohol. *NF category:* Sweetening agent; tonicity agent; vehicle (flavored and/or sweetened).

Dextrose Excipient: Colorless crystals or white, crystalline or granular powder. Is odorless and sweet-tasting. Very soluble in boiling water; freely soluble in water; sparingly soluble in boiling alcohol; slightly soluble in alcohol. *NF category:* Sweetening agent; tablet and/or capsule diluent.

Diacetylated Monoglycerides: Clear liquid. Very soluble in 80% (w/w) aqueous alcohol, in vegetable oils, and in mineral oils; sparingly soluble in 70% alcohol. *NF category:* Plasticizer.

Diatrizoate Meglumine: White, odorless powder. Freely soluble in water.

Diatrizoate Meglumine Injection: Clear, colorless to pale yellow, slightly viscous liquid.

Diatrizoate Meglumine and Diatrizoate Sodium Injection: Clear, colorless to pale yellow, slightly viscous liquid. May crystallize at room temperature or below.

Diatrizoate Sodium: White, odorless powder. Soluble in water; slightly soluble in alcohol; practically insoluble in acetone and in ether.

Diatrizoate Sodium Injection: Clear, colorless to pale yellow, slightly viscous liquid.

Diatrizoate Sodium Solution: Clear, pale yellow to light brown liquid.

Diatrizoic Acid: White, odorless powder. Soluble in dimethylformamide and in alkali hydroxide solutions; very slightly soluble in water and in alcohol.

Diazepam: Off-white to yellow, practically odorless, crystalline powder. Freely soluble in chloroform; soluble in alcohol; practically insoluble in water.

Diazoxide: White or cream-white crystals or crystalline powder. Very soluble in strong alkaline solutions; freely solu-

ble in dimethylformamide; sparingly soluble to practically insoluble in water and in most organic solvents.

Dibucaine: White to off-white powder, having a slight, characteristic odor. Darkens on exposure to light. Soluble in 1 N hydrochloric acid and in ether; slightly soluble in water.

Dibucaine Hydrochloride: Colorless or white to off-white crystals or white to off-white, crystalline powder. Is odorless, is somewhat hygroscopic, and darkens on exposure to light. Its solutions have a pH of about 5.5. Freely soluble in water, in alcohol, in acetone, and in chloroform.

Dibutyl Phthalate: A clear, oily liquid, colorless or very slightly yellow. Practically insoluble in water. Miscible with alcohol and with ether.

Dibutyl Sebacate: Colorless, oily liquid of very mild odor. Soluble in alcohol, in isopropyl alcohol, and in mineral oil; very slightly soluble in propylene glycol; practically insoluble in water and in glycerin. *NF category:* Plasticizer.

Dichloralphenazone: White, microcrystalline powder. Has a slight odor characteristic of chloral hydrate. Decomposed by dilute alkali, liberating chloroform. Freely soluble in water, in alcohol, and in chloroform; soluble in dilute acids.

Dichlorodifluoromethane: Clear, colorless gas, having a faint, ethereal odor. Its vapor pressure at 25° is about 4880 mm of mercury (80 psig). *NF category:* Aerosol propellant.

Dichlorotetrafluoroethane: Clear, colorless gas, having a faint, ethereal odor. Its vapor pressure at 25° is about 1620 mm of mercury (17 psig). Usually contains between 6% and 10% of its isomer, CCl₂F-CF₃. *NF category:* Aerosol propellant.

Diclazuril: White to yellow powder. Sparingly soluble in dimethylformamide; practically insoluble in water, in alcohol, and in methylene chloride.

Diclofenac Potassium: White to off-white or slightly yellowish crystalline powder, slightly hygroscopic. Freely soluble in methanol; soluble in alcohol; sparingly soluble in water; slightly soluble in acetone.

Diclofenac Sodium: White to off-white, hygroscopic, crystalline powder. Melts at about 284°. Freely soluble in methanol; soluble in ethanol; sparingly soluble in water; practically insoluble in chloroform and in ether.

Dicloxacillin Sodium: White to off-white, crystalline powder. Freely soluble in water.

Dicyclomine Hydrochloride: Fine, white, crystalline powder. Is practically odorless and has a very bitter taste. Freely soluble in alcohol and in chloroform; soluble in water; very slightly soluble in ether.

Dicyclomine Hydrochloride Injection: Colorless solution, which may have the odor of a preservative.

Didanosine: White to off-white, crystalline powder. Very soluble in dimethyl sulfoxide; practically insoluble or insoluble in acetone and in methanol.

Dienestrol: Colorless, white or practically white, needle-like crystals, or white or practically white, crystalline powder. Is odorless. Soluble in alcohol, in acetone, in ether, in methanol, in propylene glycol, and in solutions of alkali hydroxides; slightly soluble in chloroform and in fatty oils; practically insoluble in water.

Diethanolamine: White or clear, colorless crystals, deliquescent in moist air; or colorless liquid. Miscible with water, with alcohol, with acetone, with chloroform, and with glycerin. Slightly soluble to insoluble in benzene, in ether, and in petroleum ether. *NF category:* Alkalizing agent; emulsifying and/or solubilizing agent.

Diethylcarbamazine Citrate: White, crystalline powder. Melts at about 136°, with decomposition. Is odorless or has a slight odor; is slightly hygroscopic. Very soluble in water; sparingly soluble in alcohol; practically insoluble in acetone, in chloroform, and in ether.

Diethylene Glycol Monoethyl Ether: Clear, colorless liquid. Is hygroscopic. Miscible with water, with acetone, and with alcohol; partially miscible with vegetable oils; immiscible with mineral oils. Specific gravity about 0.991. *NF category:* Ointment base; solvent.

Diethylene Glycol Stearates: White or almost white, waxy solid. Soluble in acetone and in hot alcohol; practically insoluble in water. *NF category:* Emulsifying and/or solubilizing agent.

Diethyl Phthalate: Colorless, practically odorless, oily liquid. Insoluble in water. Miscible with alcohol, with ether, and with other usual organic solvents. *NF category:* Plasticizer.

Diethylpropion Hydrochloride: White to off-white, fine crystalline powder. Is odorless, or has a slight characteristic odor. It melts at about 175°, with decomposition. Freely soluble in water, in chloroform, and in alcohol; practically insoluble in ether.

Add the following:

▲ **Diethyl Sebacate:** A colorless to slightly yellow liquid. Miscible with alcohol, with ether, with other organic solvents, and with most fixed oils; insoluble or practically insoluble in water. *NF category:* Flavors and perfumes. ▲ *NF31*

Diethylstilbestrol: White, odorless, crystalline powder. Soluble in alcohol, in chloroform, in ether, in fatty oils, and in dilute alkali hydroxides; practically insoluble in water.

Diethyltoluamide: Colorless liquid, having a faint, pleasant odor. Boils at about 111° under a pressure of 1 mm of mercury. Practically insoluble in water and in glycerin. Miscible with alcohol, with isopropyl alcohol, with ether, with chloroform, and with carbon disulfide.

Diflorasone Diacetate: White to pale yellow, crystalline powder. Soluble in methanol and in acetone; sparingly soluble in ethyl acetate; slightly soluble in toluene; very slightly soluble in ether; insoluble in water.

Diflunisal: White to off-white, practically odorless powder. Freely soluble in alcohol and in methanol; soluble in acetone and in ethyl acetate; slightly soluble in chloroform, in carbon tetrachloride, and in methylene chloride; insoluble in hexane and in water.

Digitoxin: White or pale buff, odorless, microcrystalline powder. Sparingly soluble in chloroform; slightly soluble in alcohol; very slightly soluble in ether; practically insoluble in water.

Digoxin: Clear to white, odorless crystals or white, odorless crystalline powder. Freely soluble in pyridine; slightly soluble in diluted alcohol and in chloroform; practically insoluble in water and in ether.

Dihydroergotamine Mesylate: White to slightly yellowish powder, or off-white to faintly red powder, having a faint odor. Soluble in alcohol; slightly soluble in water and in chloroform.

Dihydrostreptomycin Sulfate: White or almost white, amorphous or crystalline powder. Amorphous form is hygroscopic. Freely soluble in water; practically insoluble in acetone, in chloroform, and in methanol.

Dihydroxycholesterol: Colorless or white, odorless crystals, or white, odorless, crystalline powder. Freely soluble in ether and in chloroform; soluble in alcohol; sparingly soluble in vegetable oils; practically insoluble in water.

Dihydroxyacetone: White to off-white crystalline powder. The monomeric form is freely soluble in water, in alcohol, and in ether. The dimeric form is freely soluble in water; soluble in alcohol; and sparingly soluble in ether.

Dihydroxyaluminum Aminoacetate: White, odorless powder having a faintly sweet taste. Soluble in dilute mineral acids and in solutions of fixed alkalies; insoluble in water and in organic solvents.

Dihydroxyaluminum Aminoacetate Magma: White, viscous suspension, from which small amounts of water may separate on standing.

Dihydroxyaluminum Sodium Carbonate: Fine, white, odorless powder. Soluble in dilute mineral acids with the evolution of carbon dioxide; practically insoluble in water and in organic solvents.

Diloxanide Furoate: White or almost white, crystalline powder. Freely soluble in chloroform; slightly soluble in alcohol and in ether; very slightly soluble in water.

Diltiazem Hydrochloride: White, odorless, crystalline powder or small crystals. Freely soluble in chloroform, in formic acid, in methanol, and in water; sparingly soluble in dehydrated alcohol; insoluble in ether. Melts at about 210°, with decomposition.

Dimenhydrinate: White, crystalline, odorless powder. Freely soluble in alcohol and in chloroform; sparingly soluble in ether; slightly soluble in water.

Dimercaprol: Colorless or practically colorless liquid, having a disagreeable, mercaptan-like odor. Soluble in water, in alcohol, in benzyl benzoate, and in methanol.

Dimercaprol Injection: Yellow, viscous solution having a pungent, disagreeable odor. Specific gravity is about 0.978.

Dimethicone: A clear, colorless, and odorless liquid. Soluble in chlorinated hydrocarbons, in benzene, in toluene, in xylene, in *n*-hexane, in petroleum spirits, in ether, and in amyl acetate; very slightly soluble in isopropyl alcohol; insoluble in water, in methanol, in alcohol, and in acetone. *NF* category: Antifoaming agent; water repelling agent.

Dimethyl Sulfoxide: Clear, colorless, odorless, hygroscopic liquid. Melts at about 18.4°. Boils at about 189°. Soluble in water; practically insoluble in acetone, in alcohol, in benzene, in chloroform, and in ether.

Dinoprostone: White to off-white, crystalline powder. Freely soluble in acetone, in alcohol, in ether, in ethyl acetate, in isopropyl alcohol, in methanol, and in methylene chloride; soluble in toluene and in diisopropyl ether; practically insoluble in hexanes.

Dinoprost Tromethamine: White to off-white, crystalline powder. Very soluble in water; freely soluble in dimethylformamide; soluble in methanol; slightly soluble in chloroform.

Dioxybenzone: Yellow powder. Freely soluble in alcohol and in toluene; practically insoluble in water.

Diphenhydramine Hydrochloride: White, odorless, crystalline powder. Slowly darkens on exposure to light. Its solutions are practically neutral to litmus. Freely soluble in water, in alcohol, and in chloroform; sparingly soluble in acetone; very slightly soluble in benzene and in ether.

Diphenoxylate Hydrochloride: White, odorless, crystalline powder. Its saturated solution has a pH of about 3.3. Freely soluble in chloroform; soluble in methanol; sparingly soluble in alcohol and in acetone; slightly soluble in water and in isopropanol; practically insoluble in ether and in solvent hexane.

Diphtheria and Tetanus Toxoids Adsorbed: Turbid, and white, slightly gray, or slightly pink suspension, free from evident clumps after shaking.

Dipivefrin Hydrochloride: White, crystalline powder or small crystals, having a faint odor. Very soluble in water.

Dipyridamole: Intensely yellow, crystalline powder or needles. Very soluble in methanol, in alcohol, and in chloroform; slightly soluble in water; very slightly soluble in acetone and in ethyl acetate.

Dirithromycin: White or practically white powder. Very soluble in methanol and in methylene chloride; very slightly soluble in water.

Disopyramide Phosphate: White or practically white, odorless powder. Melts at about 205°, with decomposition.

Freely soluble in water; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

Disulfiram: White to off-white, odorless, crystalline powder. Soluble in acetone, in alcohol, in carbon disulfide, and in chloroform; very slightly soluble in water.

Divalproex Sodium: White to off-white powder. Very soluble in chloroform; freely soluble in methanol and in ethyl ether; soluble in acetone; practically insoluble in acetonitrile.

Dobutamine Hydrochloride: White to practically white, crystalline powder. Soluble in alcohol and in pyridine; sparingly soluble in water and in methanol.

Docetaxel: White or almost white, crystalline powder. Freely soluble in acetone; soluble in methanol; practically insoluble in water.

Docosate Calcium: White, amorphous solid, having the characteristic odor of octyl alcohol. It is free of the odor of other solvents. Very soluble in alcohol, in polyethylene glycol 400, and in corn oil; very slightly soluble in water.

Docosate Potassium: White, amorphous solid, having a characteristic odor suggestive of octyl alcohol. Very soluble in solvent hexane; soluble in alcohol and in glycerin; sparingly soluble in water.

Docosate Sodium: White, wax-like, plastic solid, having a characteristic odor suggestive of octyl alcohol, but no odor of other solvents. Very soluble in solvent hexane; freely soluble in alcohol and in glycerin; sparingly soluble in water. *NF* category: Wetting and/or solubilizing agent.

Dofetilide: White to off-white powder. Soluble in 0.1 N sodium hydroxide, in acetone, and in 0.1 N hydrochloric acid; very slightly soluble in water and in isopropyl alcohol.

Dolasetron Mesylate: White to off-white powder. Freely soluble in water and in propylene glycol; slightly soluble in alcohol and in saline TS.

Donepezil Hydrochloride: White crystalline powder. Freely soluble in chloroform; soluble in water and glacial acetic acid; slightly soluble in alcohol and acetonitrile; practically insoluble in ethyl acetate and *n*-hexane.

Dopamine Hydrochloride: White to off-white, crystalline powder. May have a slight odor of hydrochloric acid. Melts at about 240°, with decomposition. Freely soluble in water and in aqueous solutions of alkali hydroxides; soluble in methanol; insoluble in ether and in chloroform.

Dorzolamide Hydrochloride: White to off-white, crystalline powder. Soluble in water.

Doxapram Hydrochloride: White to off-white, odorless, crystalline powder. Melts at about 220°. Soluble in water and in chloroform; sparingly soluble in alcohol; practically insoluble in ether.

Doxazosin Mesylate: White to tan-colored powder. Freely soluble in formic acid; very slightly soluble in methanol and in water.

Doxorubicin Hydrochloride: Red-orange, hygroscopic, crystalline or amorphous powder. Soluble in water, in isotonic sodium chloride solution, and in methanol; practically insoluble in chloroform, in ether, and in other organic solvents.

Doxycycline: Yellow, crystalline powder. Freely soluble in dilute acid and in alkali hydroxide solutions; very slightly soluble in alcohol and in water; practically insoluble in chloroform and in ether.

Doxycycline Hyclate: Yellow, crystalline powder. Soluble in water and in solutions of alkali hydroxides and carbonates; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

Doxylamine Succinate: White or creamy white powder, having a characteristic odor. Very soluble in water and in alcohol; freely soluble in chloroform; very slightly soluble in ether and in benzene.

Dronabinol: Light yellow resinous oil that is sticky at room temperature and hardens upon refrigeration. Insoluble in water.

Droperidol: White to light tan, amorphous or microcrystalline powder. Freely soluble in chloroform; slightly soluble in alcohol and in ether; practically insoluble in water. Melts at about 145°.

Drospirenone: White to off-white powder. Freely soluble in methylene chloride; soluble in acetone and in methanol; sparingly soluble in ethyl acetate and in alcohol; practically insoluble in hexane and in water.

Duloxetine Hydrochloride: White to brownish-white solid. Slightly soluble in water.

Absorbable Dusting Powder: White, odorless powder.

Dyclonine Hydrochloride: White crystals or white crystalline powder, which may have a slight odor. Exhibits local anesthetic properties when placed upon the tongue. Soluble in water, in acetone, in alcohol, and in chloroform.

Dydrogesterone: White to pale yellow, crystalline powder. Sparingly soluble in alcohol; practically insoluble in water.

Dyphylline: White, odorless, extremely bitter, amorphous or crystalline solid. Freely soluble in water; sparingly soluble in alcohol and in chloroform; practically insoluble in ether.

Ecamsule Solution: Clear yellow liquid.

Echothiophate Iodide: White, crystalline, hygroscopic solid having a slight mercaptan-like odor. Its solutions have a pH of about 4. Freely soluble in water and in methanol; soluble in dehydrated alcohol; practically insoluble in other organic solvents.

Echothiophate Iodide for Ophthalmic Solution: White, amorphous powder.

Econazole Nitrate: White or practically white, crystalline powder, having not more than a slight odor. Soluble in methanol; sparingly soluble in chloroform; slightly soluble in alcohol; very slightly soluble in water and in ether.

Edetate Calcium Disodium: White, crystalline granules or white, crystalline powder. Is odorless, is slightly hygroscopic, and has a faint, saline taste. Is stable in air. Freely soluble in water. *NF category:* Chelating agent; complexing agent.

Edetate Disodium: White, crystalline powder. Soluble in water. *NF category:* Chelating agent; complexing agent.

Edetic Acid: White, crystalline powder. Melts above 220°, with decomposition. Soluble in solutions of alkali hydroxides; very slightly soluble in water. *NF category:* Chelating agent; complexing agent.

Edrophonium Chloride: White, odorless, crystalline powder. Its solution (1 in 10) is practically colorless. Very soluble in water; freely soluble in alcohol; insoluble in chloroform and in ether.

Efavirenz: White to slightly pink crystalline powder. Soluble in methanol; practically insoluble in water.

Emedastine Fumarate: White to faintly yellow, crystalline powder. Soluble in water.

Emetine Hydrochloride: White or very slightly yellowish, odorless, crystalline powder. Is affected by light. Freely soluble in water and in alcohol.

Enalapril Maleate: Off-white, crystalline powder. Melts at about 144°. Freely soluble in methanol and in dimethylformamide; soluble in alcohol; sparingly soluble in water; slightly soluble in semipolar organic solvents; practically insoluble in nonpolar organic solvents.

Enalaprilat: White to nearly white, hygroscopic, crystalline powder. Sparingly soluble in methanol and in dimethylformamide; slightly soluble in water and in isopropyl alcohol; very slightly soluble in acetone, in alcohol, and in hexane; practically insoluble in acetonitrile and in chloroform.

Enflurane: Clear, colorless, stable, volatile liquid, having a mild, sweet odor. Is nonflammable. Slightly soluble in water. Miscible with organic solvents, with fats, and with oils.

Enrofloxacin: Pale yellow to light yellow crystalline powder. Very slightly soluble in water at pH 7.

Entacapone: Greenish yellow to yellow powder. Sparingly soluble in acetone and in methanol; slightly soluble in ethanol, chloroform, isopropanol, and ether; very slightly soluble in toluene; practically insoluble in water.

Ephedrine: Unctuous, practically colorless solid or white crystals or granules. Gradually decomposes on exposure to light. Melts between 33° and 40°, the variability in the melting point being the result of differences in the moisture content, anhydrous Ephedrine having a lower melting point than the hemihydrate of Ephedrine. Its solutions are alkaline to litmus. Soluble in water, in alcohol, in chloroform, and in ether; sparingly and slowly soluble in mineral oil, the solution becoming turbid if the Ephedrine contains more than about 1% of water.

Ephedrine Hydrochloride: Fine, white, odorless crystals or powder. Is affected by light. Freely soluble in water; soluble in alcohol; insoluble in ether.

Ephedrine Sulfate: Fine, white, odorless crystals or powder. Darkens on exposure to light. Freely soluble in water; sparingly soluble in alcohol.

Ephedrine Sulfate Nasal Solution: Clear, colorless solution. Is neutral or slightly acid to litmus.

Epinephrine: White to practically white, odorless, microcrystalline powder or granules, gradually darkening on exposure to light and air. With acids, it forms salts that are readily soluble in water, and the base may be recovered by the addition of ammonia water or alkali carbonates. Its solutions are alkaline to litmus. Very slightly soluble in water and in alcohol; insoluble in ether, in chloroform, and in fixed and volatile oils.

Epinephrine Injection: Practically colorless, slightly acid liquid. Gradually turns dark on exposure to light and air.

Epinephrine Inhalation Solution: Practically colorless, slightly acid liquid. Gradually turns dark on exposure to light and air.

Epinephrine Nasal Solution: Nearly colorless, slightly acid liquid. Gradually turns dark on exposure to light and air.

Epinephrine Ophthalmic Solution: Colorless to faint yellow solution. Gradually turns dark on exposure to light and air.

Epinephrine Bitartrate: White, or grayish-white or light brownish-gray, odorless, crystalline powder. Slowly darkens on exposure to air and light. Its solutions are acid to litmus, having a pH of about 3.5. Freely soluble in water; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

Epinephrine Bitartrate for Ophthalmic Solution: White to off-white solid.

Epinephryl Borate Ophthalmic Solution: Clear, pale yellow liquid, gradually darkening on exposure to light and air.

Epirubicin Hydrochloride: Orange-red powder. Soluble in water and in methanol; slightly soluble in anhydrous ethanol; practically insoluble in acetone.

Eprinomectin: White to off-white powder. Insoluble in cold water.

Ergocalciferol: White, odorless crystals. Is affected by air and by light. Soluble in alcohol, in chloroform, in ether, and in fatty oils; insoluble in water.

Ergocalciferol Oral Solution: Clear liquid having the characteristics of the solvent used in preparing the Solution.

Ergoloid Mesylates: White to off-white, microcrystalline or amorphous, practically odorless powder. Soluble in meth-

anol and in alcohol; sparingly soluble in acetone; slightly soluble in water.

Ergonovine Maleate: White to grayish-white or faintly yellow, odorless, microcrystalline powder. Darkens with age and on exposure to light. Sparingly soluble in water; slightly soluble in alcohol; insoluble in ether and in chloroform.

Ergotamine Tartrate: Colorless crystals or white to yellowish-white, crystalline powder. Is odorless. Melts at about 180°, with decomposition. One g dissolves in about 3200 mL of water; in the presence of a slight excess of tartaric acid 1 g dissolves in about 500 mL of water. Slightly soluble in alcohol.

Erythorbic Acid: White or slightly yellow crystals or powder. It gradually darkens when exposed to light. In the dry state, it is reasonably stable in air, but in solution, it rapidly deteriorates in the presence of air. It melts between 164° and 171° with decomposition. One g is soluble in about 2.5 mL of water and in about 20 mL of alcohol. Slightly soluble in glycerin. *NF category:* Antimicrobial preservative; antioxidant.

Erythritol: White or almost white, crystalline powder or free-flowing granules. It is stable to heat and is nonhygroscopic. Freely soluble in water; very slightly soluble in alcohol. *NF category:* Humectant; sweetening agent.

Erythromycin: White or slightly yellow, crystalline powder. Is odorless or practically odorless. Soluble in alcohol, in chloroform, and in ether; slightly soluble in water.

Erythromycin Estolate: White, crystalline powder. Is odorless or practically odorless, and is practically tasteless. Soluble in alcohol, in acetone, and in chloroform; practically insoluble in water.

Erythromycin Ethylsuccinate: White or slightly yellow crystalline powder. Is odorless or practically odorless, and is practically tasteless. Freely soluble in alcohol, in chloroform, and in polyethylene glycol 400; very slightly soluble in water.

Erythromycin Gluceptate: Colorless to white crystals. Slightly hygroscopic. Freely soluble in water, in alcohol, in methanol, in dioxane, and in propylene glycol; slightly soluble in acetone and in chloroform; practically insoluble in ether, in carbon tetrachloride, in benzene, and in toluene.

Erythromycin Lactobionate for Injection: White or slightly yellow crystals or powder, having a faint odor. Its solution (1 in 20) is neutral or slightly alkaline. Freely soluble in water, in alcohol, and in methanol; slightly soluble in acetone and in chloroform; practically insoluble in ether.

Erythromycin Stearate: White or slightly yellow crystals or powder. Is odorless or may have a slight, earthy odor, and has a slightly bitter taste. Soluble in alcohol, in chloroform, in methanol, and in ether; practically insoluble in water.

Esmolol Hydrochloride: White to off-white crystalline powder. Very soluble in water; freely soluble in alcohol.

Escitalopram Oxalate: Fine, white to slightly yellow powder. Freely soluble in methanol and in dimethyl sulfoxide; sparingly soluble in water and in alcohol; very slightly soluble in ethyl acetate and in isopropyl alcohol; insoluble in heptane.

Esomeprazole Magnesium: White to slightly colored powder. Soluble in methanol; slightly soluble in water; practically insoluble in heptane.

Estazolam: White to pale yellowish-white crystal. Soluble in methanol and in acetic anhydride; sparingly soluble in ethanol; practically insoluble in water and in ether.

Estradiol: White or creamy white, small crystals or crystalline powder. Is odorless, and is stable in air. Is hygroscopic. Soluble in alcohol, in acetone, in dioxane, in chloroform, and in solutions of fixed alkali hydroxides; sparingly soluble in vegetable oils; practically insoluble in water.

Estradiol Benzoate: White to off-white, crystalline powder. Soluble in alcohol and in acetone; slightly soluble in diethyl ether; insoluble in water.

Estradiol Cypionate: White to practically white, crystalline powder. Is odorless or has a slight odor. Soluble in alcohol, in acetone, in chloroform, and in dioxane; sparingly soluble in vegetable oils; insoluble in water.

Estradiol Valerate: White, crystalline powder. Is usually odorless but may have a faint, fatty odor. Soluble in castor oil, in methanol, in benzyl benzoate, and in dioxane; sparingly soluble in sesame oil and in peanut oil; practically insoluble in water.

Estriol: White to practically white, odorless, crystalline powder. Melts at about 280°. Soluble in acetone, in chloroform, in dioxane, in ether, and in vegetable oils; sparingly soluble in alcohol; insoluble in water.

Conjugated Estrogens: Conjugated Estrogens obtained from natural sources is a buff-colored, amorphous powder, odorless or having a slight, characteristic odor. The synthetic form is a white to light buff, crystalline or amorphous powder, odorless or having a slight odor.

Synthetic Conjugated Estrogens: A white to light buff, crystalline or amorphous powder that is odorless or has a slight odor.

Esterified Estrogens: White or buff-colored, amorphous powder, odorless or having a slight, characteristic odor.

Estrone: Small, white crystals or white to creamy white, crystalline powder. Is odorless, and is stable in air. Melts at about 260°. Soluble in alcohol, in acetone, in dioxane, and in vegetable oils; slightly soluble in solutions of fixed alkali hydroxides; practically insoluble in water.

Estopipate: White to yellowish-white, fine, crystalline powder. Is odorless, or may have a slight odor. Melts at about 190° to a light brown, viscous liquid, which solidifies on further heating and finally melts at about 245°, with decomposition. Soluble in warm water; very slightly soluble in water, in alcohol, in chloroform, and in ether.

Ethacrynic Acid: White or practically white, odorless or practically odorless, crystalline powder. Freely soluble in alcohol, in chloroform, and in ether; very slightly soluble in water.

Ethambutol Hydrochloride: White, crystalline powder. Freely soluble in water; soluble in alcohol and in methanol; slightly soluble in ether and in chloroform.

Ethchlorvynol: Colorless to yellow, slightly viscous liquid, having a characteristic pungent odor. Darkens on exposure to light and air. Immiscible with water; miscible with most organic solvents.

Ether: Colorless, mobile, volatile liquid, having a characteristic sweet, pungent odor. Is slowly oxidized by the action of air and light, with the formation of peroxides. It boils at about 35°. Soluble in water and in hydrochloric acid. Miscible with alcohol, with benzene, with chloroform, with solvent hexane, with methylene chloride, and with fixed and volatile oils.

Ethinyl Estradiol: White to creamy white, odorless, crystalline powder. Soluble in alcohol, in chloroform, in ether, in vegetable oils, and in solutions of fixed alkali hydroxides; insoluble in water.

Ethiodized Oil Injection: Straw-colored to amber-colored, oily liquid. It may possess an alliaceous odor. Soluble in acetone, in chloroform, in ether, and in solvent hexane; insoluble in water.

Ethionamide: Bright yellow powder, having a faint to moderate sulfide-like odor. Soluble in methanol; sparingly soluble in alcohol and in propylene glycol; slightly soluble in water, in chloroform, and in ether.

Ethopabate: White to pinkish-white, odorless or practically odorless powder. Soluble in acetonitrile, in acetone, in dehydrated alcohol, and in methanol; sparingly soluble in isopropyl alcohol, in dioxane, in ethyl acetate, and in meth-

ylene chloride; slightly soluble in ether; very slightly soluble in water.

Ethosuximide: White to off-white, crystalline powder or waxy solid, having a characteristic odor. Freely soluble in water and in chloroform; very soluble in alcohol and in ether; very slightly soluble in solvent hexane.

Ethotoin: White, crystalline powder. Freely soluble in dehydrated alcohol and in chloroform; soluble in ether; insoluble in water.

Ethyl Acetate: Transparent, colorless liquid, having a fragrant, refreshing, slightly acetous odor, and a peculiar, acetous, burning taste. Soluble in water. Miscible with alcohol, with ether, with fixed oils, and with volatile oils. *NF category:* Flavors and perfumes; solvent.

Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion: Milky-white liquid of low viscosity with a faint, characteristic odor. It is miscible with water in any proportion; the milky-white appearance is retained. A clear or slightly opalescent, viscous solution is obtained on mixing one part with five parts of acetone, alcohol, or isopropyl alcohol; the polymer substance first precipitates, but then dissolves in the excess organic solvent. When mixed with 1 N sodium hydroxide in a ratio of 1:2, the dispersion does not dissolve; the milky-white appearance is retained. *NF category:* Coating agent; polymer membrane; tablet binder.

Ethyl Chloride: Colorless, mobile, very volatile liquid at low temperatures or under pressure, having a characteristic, ethereal odor. It boils between 12° and 13°, and its specific gravity at 0° is about 0.921. When liberated at room temperature from its sealed container, it vaporizes immediately. It burns with a smoky, greenish flame, producing hydrogen chloride. Freely soluble in alcohol and in ether; slightly soluble in water.

Ethyl Oleate: Mobile, practically colorless liquid, having an agreeable taste. Insoluble in water. Miscible with vegetable oils, with mineral oil, with alcohol, and with most organic solvents. *NF category:* Vehicle (oleaginous).

Ethyl Maltol: White, crystalline powder having a cotton-candy odor and a sweet, fruitlike flavor in dilute solution. One g dissolves in about 55 mL of water, 10 mL of alcohol, 17 mL of propylene glycol, and 5 mL of chloroform. It melts at about 90°. *NF category:* Vehicle (flavored and/or sweetened).

Ethyl Vanillin: Fine, white or slightly yellowish crystals. Its taste and odor are similar to the taste and odor of vanillin. Is affected by light. Its solutions are acid to litmus. Freely soluble in alcohol, in chloroform, in ether, and in solutions of alkali hydroxides; sparingly soluble in water at 50°. *NF category:* Flavors and perfumes.

Ethylcellulose: Free-flowing, white to light tan powder. It forms films that have a refractive index of about 1.47. Its aqueous suspensions are neutral to litmus. Ethylcellulose containing less than 46.5% of ethoxy groups is freely soluble in tetrahydrofuran, in methyl acetate, in chloroform, and in mixtures of aromatic hydrocarbons with alcohol; Ethylcellulose containing not less than 46.5% of ethoxy groups is freely soluble in alcohol, in methanol, in toluene, in chloroform, and in ethyl acetate; insoluble in water, in glycerin, and in propylene glycol. *NF category:* Coating agent; tablet binder.

Ethylcellulose Dispersion Type B: Off-white and slightly viscous liquid. Soluble in alcohol, in methyl alcohol, in toluene, in chloroform, and in ethyl acetate; insoluble in water, in glycerin, and in propylene glycol. *NF category:* Coating agent; film-forming agent.

Ethylenediamine: Clear, colorless or only slightly yellow liquid, having an ammonia-like odor and a strong alkaline reaction. Miscible with water and with alcohol.

Ethylene Glycol Stearates: White or almost white, waxy solid. Soluble in acetone and in hot alcohol; practically insoluble in water. *NF category:* Emulsifying and/or solubilizing agent.

Ethylene Glycol and Vinyl Alcohol Graft Copolymer:

White or slightly yellowish powder. Very soluble in water; practically insoluble in anhydrous alcohol, and in acetone. It dissolves in dilute acids and dilute solutions of alkali hydroxides. *NF category:* Coating agent; tablet binder.

Ethylparaben: Small, colorless crystals or white powder. Freely soluble in acetone, in alcohol, in ether, and in propylene glycol; slightly soluble in water and in glycerin. *NF category:* Antimicrobial preservative.

Ethynodiol Diacetate: White, odorless, crystalline powder. Is stable in air. Very soluble in chloroform; freely soluble in ether; soluble in alcohol; sparingly soluble in fixed oils; insoluble in water.

Etidronate Disodium: White powder, which may contain lumps. Freely soluble in water; practically insoluble in alcohol.

Etomidate: White or almost white powder. Freely soluble in alcohol and in methylene chloride; very slightly soluble in water.

Etoposide: Fine, white to off-white, crystalline powder. Sparingly soluble in methanol; slightly soluble in alcohol, in chloroform, in ethyl acetate, and in methylene chloride; very slightly soluble in water.

Eugenol: Colorless or pale yellow liquid, having a strongly aromatic odor of clove and a pungent, spicy taste. Upon exposure to air, it darkens and thickens. Is optically inactive. Slightly soluble in water. Miscible with alcohol, with chloroform, with ether, and with fixed oils.

Famotidine: White to pale yellowish-white, crystalline powder. Is sensitive to light. Freely soluble in dimethylformamide and in glacial acetic acid; slightly soluble in methanol; very slightly soluble in water; practically insoluble in acetone, in alcohol, in chloroform, in ether, and in ethyl acetate.

Hard Fat: White mass; almost odorless and free from rancid odor; greasy to the touch. On warming, melts to give a colorless or slightly yellowish liquid. When the molten material is shaken with an equal quantity of hot water, a white emulsion is formed. Freely soluble in ether; slightly soluble in alcohol; practically insoluble in water. *NF category:* Stiffening agent; suppository base.

Felbamate: White to off-white powder. Freely soluble in dimethyl sulfoxide; sparingly soluble in methanol; slightly soluble in acetonitrile; very slightly soluble in water.

Felodipine: Light yellow to yellow, crystalline powder. Freely soluble in acetone and in methanol; very slightly soluble in heptane; insoluble in water.

Fenbendazole: White to off-white powder. Sparingly soluble in dimethylformamide; very slightly soluble in methanol; practically insoluble in water.

Fenofibrate: White or almost white, crystalline powder. Very soluble in methylene chloride; slightly soluble in alcohol; practically insoluble in water.

Fenoldopam Mesylate: White to off-white powder. Soluble in water.

Fenoprofen Calcium: White, crystalline powder. Slightly soluble in *n*-hexanol, in methanol, and in water; practically insoluble in chloroform.

Fentanyl Citrate: White, crystalline powder or white, glistening crystals. Melts at about 150°, with decomposition. Soluble in methanol; sparingly soluble in water; slightly soluble in chloroform.

Ferric Oxide: Powder exhibiting two basic colors (red and yellow), or other shades produced on blending the basic colors. Insoluble in water and in organic solvents; dissolves in hydrochloric acid upon warming, a small amount of insoluble residue usually remaining. *NF category:* Color.

Ferric Subsulfate Solution: Reddish-brown liquid, odorless or nearly so. Acid to litmus, and is affected by light. Specific gravity is about 1.548.

Ferric Sulfate: Grayish-white or yellowish powder or fawn-colored pearls. Hygroscopic. Slightly soluble in water and in ethanol (96%); practically insoluble in acetone and in ethyl acetate. Hydrolyzes slowly in aqueous solution.

Ferrosoferric Oxide: Black powder. Dissolves in hydrochloric acid upon warming, a small amount of insoluble residue usually remaining; insoluble in water and in organic solvents. *NF category:* Color.

Ferrous Fumarate: Reddish-orange to red-brown, odorless powder. May contain soft lumps that produce a yellow streak when crushed. Slightly soluble in water; very slightly soluble in alcohol. Its solubility in dilute hydrochloric acid is limited by the separation of fumaric acid.

Ferrous Gluconate: Yellowish-gray or pale greenish-yellow, fine powder or granules, having a slight odor resembling that of burned sugar. Its solution (1 in 20) is acid to litmus. Soluble in water, with slight heating; practically insoluble in alcohol.

Ferrous Sulfate: Pale, bluish-green crystals or granules. Is odorless and is efflorescent in dry air. Oxidizes readily in moist air to form brownish yellow basic ferric sulfate. Its solution (1 in 10) is acid to litmus, having a pH of about 3.7. Very soluble in boiling water; freely soluble in water; insoluble in alcohol.

Dried Ferrous Sulfate: Grayish-white to buff-colored powder, consisting primarily of $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ with varying amounts of $\text{FeSO}_4 \cdot 4\text{H}_2\text{O}$. Slowly soluble in water; insoluble in alcohol.

Ferumoxides Injection: Black to reddish-brown, aqueous colloid. It is stable for 24 hours after dilution.

Add the following:

▲ **Fexofenadine Hydrochloride:** White to off-white powder. Freely soluble in methanol; slightly soluble in water; very slightly soluble in acetone. ▲ *USP36*

Finasteride: White to off-white, crystalline solid. Melts at about 257°. Freely soluble in chloroform and in alcohol; very slightly soluble in water.

Fish Oil Containing Omega-3 Acids: Pale yellow liquid. Very soluble in acetone and in heptane; slightly soluble in anhydrous alcohol; practically insoluble in water.

Flavoxate Hydrochloride: White or almost white, crystalline powder. Slightly soluble in alcohol, in water, and in methylene chloride.

Flecainide Acetate: White to slightly off-white, crystalline powder. Freely soluble in alcohol; soluble in water. pK_a is 9.3.

Fluconazole: White or almost white, crystalline powder. Freely soluble in methanol; soluble in alcohol and in acetone; sparingly soluble in isopropanol and in chloroform; slightly soluble in water; very slightly soluble in toluene.

Flucytosine: White to off-white, crystalline powder. Is odorless or has a slight odor. Sparingly soluble in water; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

Fludarabine Phosphate: White to off-white, crystalline, hygroscopic powder. Freely soluble in dimethylformamide; slightly soluble in water and in 0.1 M hydrochloric acid; practically insoluble in ethanol.

Fludrocortisone Acetate: White to pale yellow crystals or crystalline powder. Is odorless or practically odorless. Is hygroscopic. Sparingly soluble in alcohol and in chloroform; slightly soluble in ether; insoluble in water.

Flumazenil: White to off-white powder. Slightly soluble in acidic aqueous solutions; practically insoluble in water.

Flumethasone Pivalate: White to off-white, crystalline powder. Slightly soluble in methanol; very slightly soluble in chloroform and in methylene chloride; insoluble in water.

Flunisolide: White to creamy-white, crystalline powder. Melts at about 245°, with decomposition. Soluble in acetone; sparingly soluble in chloroform; slightly soluble in methanol; practically insoluble in water.

Flunixin Meglumine: White to off-white crystalline powder. Soluble in water, in alcohol, and in methanol; practically insoluble in ethyl acetate.

Fluocinolone Acetonide: White or practically white, odorless, crystalline powder. Is stable in air. Melts at about 270°, with decomposition. Soluble in methanol; slightly soluble in ether and in chloroform; insoluble in water.

Fluocinonide: White to cream-colored, crystalline powder, having not more than a slight odor. Sparingly soluble in acetone and in chloroform; slightly soluble in alcohol, in methanol, and in dioxane; very slightly soluble in ether; practically insoluble in water.

Fluorescein: Yellowish-red to red, odorless powder. Soluble in dilute alkali hydroxides; insoluble in water.

Fluorescein Sodium: Orange-red, hygroscopic, odorless powder. Freely soluble in water; sparingly soluble in alcohol.

Fluorescein Sodium Ophthalmic Strip: Each Strip is a dry, white piece of paper, one end of which is rounded and is uniformly orange-red in color because of the fluorescein sodium impregnated in the paper.

Fluorometholone: White to yellowish-white, odorless, crystalline powder. Melts at about 280°, with some decomposition. Slightly soluble in alcohol; very slightly soluble in chloroform and in ether; practically insoluble in water.

Fluorouracil: White to practically white, practically odorless, crystalline powder. Decomposes at about 282°. Sparingly soluble in water; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

Fluoxetine Hydrochloride: White to off-white crystalline powder. Freely soluble in alcohol and in methanol; sparingly soluble in water and in dichloromethane; practically insoluble in ether.

Fluoxymesterone: White or practically white, odorless, crystalline powder. Melts at about 240°, with some decomposition. Sparingly soluble in alcohol; slightly soluble in chloroform; practically insoluble in water.

Fluphenazine Enanthate: Pale yellow to yellow-orange, clear to slightly turbid, viscous liquid, having a characteristic odor. Is unstable in strong light, but stable to air at room temperature. Freely soluble in alcohol, in chloroform, and in ether; insoluble in water.

Fluphenazine Hydrochloride: White or nearly white, odorless, crystalline powder. Melts, within a range of 5°, at a temperature above 225°. Freely soluble in water; slightly soluble in acetone, in alcohol, and in chloroform; practically insoluble in benzene and in ether.

Flurandrenolide: White to off-white, fluffy, crystalline powder. Is odorless. Freely soluble in chloroform; soluble in methanol; sparingly soluble in alcohol; practically insoluble in water and in ether.

Flurazepam Hydrochloride: Off-white to yellow, crystalline powder. Is odorless, or has a slight odor, and its solutions are acid to litmus. Melts at about 212°, with decomposition. Freely soluble in water and in alcohol; slightly soluble in isopropyl alcohol and in chloroform.

Flurbiprofen: White, crystalline powder. Freely soluble in acetone, in dehydrated alcohol, in ether, and in methanol; soluble in acetonitrile; practically insoluble in water. Optically inactive (1 in 50 solution in dehydrated alcohol).

Flutamide: Pale yellow, crystalline powder. Freely soluble in acetone, in ethyl acetate, and in methanol; soluble in chloroform and in ether; practically insoluble in mineral oil, in petroleum ether, and in water.

Fluticasone Propionate (micronized): Fine, white powder.

Fluvastatin Sodium: White to pale yellow, brownish-pale yellow, or reddish-pale yellow, hygroscopic powder. Soluble in alcohol, in methanol, and in water.

Fluvoxamine Maleate: White to off-white, crystalline powder. Freely soluble in alcohol and in chloroform; sparingly soluble in water; and practically insoluble in diethyl ether.

Folic Acid: Yellow, yellow-brownish, or yellowish-orange, odorless, crystalline powder. It readily dissolves in dilute solutions of alkali hydroxides and carbonates. Soluble in hot, 3 N hydrochloric acid, in hot, 2 N sulfuric acid, in hydrochloric acid, and in sulfuric acid, yielding very pale yellow solutions; very slightly soluble in water; insoluble in alcohol, in acetone, in chloroform, and in ether.

Folic Acid Injection: Clear, yellow to orange-yellow, alkaline liquid.

Formaldehyde Solution: Clear, colorless or practically colorless liquid, having a pungent odor. The vapor from it irritates the mucous membrane of the throat and nose. On long standing, especially in the cold, it may become cloudy because of the separation of paraformaldehyde. This cloudiness disappears when the solution is warmed. Miscible with water and with alcohol.

Formoterol Fumarate Dihydrate: White or almost white or slightly yellow powder. Freely soluble in dimethyl sulfoxide and in acetic acid; soluble in methanol; slightly soluble in water and in 2-propanol; practically insoluble in acetonitrile and in diethyl ether.

Foscarnet Sodium: White to almost white, crystalline powder. Soluble in water; practically insoluble in alcohol.

Fosphenytoin Sodium: White to pale yellow solid. Freely soluble in water.

Fructose: Colorless crystals or as a white, crystalline powder. Is odorless, and has a sweet taste. Freely soluble in water; soluble in alcohol and in methanol. *NF category:* Sweetening agent; tablet and/or capsule diluent.

Basic Fuchsin: Dark green powder or greenish glistening crystalline fragments, having a bronze-like luster and not more than a faint odor. Soluble in water, in alcohol, and in amyl alcohol; insoluble in ether.

Fulvestrant: White powder. Freely soluble in alcohol.

Fumaric Acid: White, odorless granules or crystalline powder. Soluble in alcohol; slightly soluble in water and in ether; very slightly soluble in chloroform. *NF category:* Acidifying agent.

Furazolidone: Yellow, odorless, crystalline powder. Is tasteless at first, then a bitter aftertaste develops. Practically insoluble in water, in alcohol, and in carbon tetrachloride.

Furosemide: White to slightly yellow, odorless, crystalline powder. Freely soluble in acetone, in dimethylformamide, and in solutions of alkali hydroxides; soluble in methanol; sparingly soluble in alcohol; slightly soluble in ether; very slightly soluble in chloroform; practically insoluble in water.

Furosemide Injection: Clear, colorless solution.

Gabapentin: White to off-white, crystalline solid. Freely soluble in water and in alkaline and acidic solutions.

Gadodiamide: White, odorless powder. Freely soluble in water and in methanol; soluble in ethyl alcohol; slightly soluble in acetone and in chloroform.

Gadoteridol: White to off-white, crystalline, odorless powder. Freely soluble in water and in methyl alcohol; soluble in isopropyl alcohol. Melts at about 300°.

Gadoversetamide: White, odorless powder. Freely soluble in water.

Galactose: A white, crystalline or finely granulated powder. Soluble in water; very slightly soluble in alcohol. *NF category:* Sweetening agent.

Galantamine Hydrobromide: White to almost white powder. Soluble in 0.1 N sodium hydroxide; sparingly solu-

ble in water; very slightly soluble in alcohol; insoluble in *n*-propanol.

Gallamine Triethiodide: White, odorless, amorphous powder. Is hygroscopic. Very soluble in water; sparingly soluble in alcohol; very slightly soluble in chloroform.

Gamma Cyclodextrin: White or almost white, amorphous or crystalline powder. Freely soluble in water and in propylene glycol; very slightly soluble in alcohol. *NF category:* Sequestering agent; emulsifying and/or solubilizing agent.

Ganciclovir: White to off-white, crystalline powder.

Ganciclovir for Injection: White to off-white powder. Soluble in water.

Petrolatum Gauze: The petrolatum recovered by draining in the *Assay* is a white or faintly yellowish, unctuous mass, transparent in thin layers even after cooling to 0°.

Gelatin: Sheets, flakes, or shreds, or coarse to fine powder. Is faintly yellow or amber in color, the color varying in depth according to the particle size. Has a slight, characteristic bouillon-like odor in solution. Is stable in air when dry, but is subject to microbial decomposition when moist or in solution. Gelatin has any suitable strength that is designated by Bloom Gelometer number (see *Gel Strength of Gelatin* (1081)). Type A Gelatin exhibits an isoelectric point between pH 7 and pH 9, and Type B Gelatin exhibits an isoelectric point between pH 4.7 and pH 5.2. Soluble in hot water, in 6 N acetic acid, and in a hot mixture of glycerin and water; insoluble in cold water, but swells and softens when immersed in it, gradually absorbing from 5 to 10 times its own weight of water, in alcohol, in chloroform, in ether, and in fixed and volatile oils. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; tablet binder.

Absorbable Gelatin Film: Light amber, transparent, pliable film which becomes rubbery when moistened. Insoluble in water.

Absorbable Gelatin Sponge: Light, nearly white, nonelastic, tough, porous, hydrophilic solid. Insoluble in water.

Gellan Gum: Off-white powder. Soluble in hot or in cold deionized water. *NF category:* Suspending and/or viscosity-increasing agent.

Gemcitabine Hydrochloride: White to off-white solid. Soluble in water; slightly soluble in methanol; practically insoluble in alcohol and in polar organic solvents.

Gemfibrozil: White, waxy, crystalline solid. Soluble in alcohol, in methanol, and in chloroform; practically insoluble in water.

Gentamicin Sulfate: White to buff powder. Freely soluble in water; insoluble in alcohol, in acetone, in chloroform, in ether, and in benzene.

Gentamicin Injection: Clear, slightly yellow solution, having a faint odor.

Gentian Violet: Dark green powder or greenish, glistening pieces having a metallic luster, and having not more than a faint odor. Soluble in alcohol, in glycerin, and in chloroform; sparingly soluble in water; insoluble in ether.

Gentian Violet Cream: Dark purple, water-washable cream.

Gentian Violet Topical Solution: Purple liquid, having a slight odor of alcohol. A dilution (1 in 100), viewed downward through 1 cm of depth, is deep purple in color.

Powdered Asian Ginseng Extract: Pale yellow-brown, hygroscopic, powdery or easily pulverizable mass. Soluble in water, forming a slightly cloudy solution.

Glaze, Pharmaceutical: Denatured alcohol solution. *NF category:* Coating agent.

Glimepiride: White to almost white powder. Soluble in dimethylformamide; sparingly soluble in methylene chloride; slightly soluble in methanol; practically insoluble in water.

Glipizide: White to off-white powder. Freely soluble in dimethylformamide; soluble in 0.1 N sodium hydroxide; slightly soluble in methylene chloride.

Immune Globulin: Transparent or slightly opalescent liquid, either colorless or of a brownish color due to denatured hemoglobin. Is practically odorless. May develop a slight, granular deposit during storage.

Rh₀(D) Immune Globulin: Transparent or slightly opalescent liquid. Is practically colorless and odorless. May develop a slight, granular deposit during storage.

Glucagon: Fine, white or faintly colored, crystalline powder. Is practically odorless and tasteless. Soluble in dilute alkali and acid solutions; insoluble in most organic solvents.

Glucagon for Injection: White, odorless powder.

Glucanolate: Fine, white, practically odorless, crystalline powder. Melts at about 153°, with decomposition. Freely soluble in water; sparingly soluble in alcohol; insoluble in ether.

Liquid Glucose: Colorless or yellowish, thick, syrupy liquid. Odorless or nearly odorless, and has a sweet taste. Sparingly soluble in alcohol. Miscible with water. *NF category:* Tablet binder.

L-Glutamic Acid Hydrochloride: A white, crystalline powder. 1 g dissolves in about 3 mL of water. It is almost insoluble in alcohol and in ether. Its solutions are acid to litmus. *NF category:* Flavors and perfumes.

Glutamine: White crystals or crystalline powder. Soluble in water; practically insoluble in alcohol and in ether.

Glutaral Concentrate: Clear, colorless or faintly yellow liquid, having a characteristic, irritating odor.

Glycerin: Clear, colorless, syrupy liquid, having a sweet taste. Has not more than a slight characteristic odor, which is neither harsh nor disagreeable. Is hygroscopic. Its solutions are neutral to litmus. Insoluble in chloroform, in ether, and in fixed and volatile oils. Miscible with water and with alcohol. *NF category:* Humectant; plasticizer; solvent; tonicity agent.

Glyceryl Behenate: Fine powder, having a faint odor. Melts at about 70°. Soluble in chloroform; practically insoluble in water and in alcohol.

Glyceryl Distearate: Hard, waxy mass or powder or white or almost white flakes. Soluble in methylene chloride and in tetrahydrofuran; slightly soluble in hot alcohol; insoluble in water. *NF category:* Emulsifying and/or solubilizing agent.

Glyceryl Monolinoleate: Amber, oily liquids that may be partially solidified at room temperature. Freely soluble in methylene chloride; soluble in tetrahydrofuran; practically insoluble in water. *NF category:* Emulsifying and/or solubilizing agent.

Glyceryl Monooleate: Amber, oily liquids that may be partially solidified at room temperature. Freely soluble in methylene chloride; soluble in tetrahydrofuran; practically insoluble in water. *NF category:* Emulsifying and/or solubilizing agent.

Glyceryl Monostearate: White to yellowish wax-like solid; or white to yellowish wax-like beads, flakes, or powder. Slight, agreeable, fatty odor and taste. Is affected by light. Dissolves in hot organic solvents such as alcohol, minerals or fixed oils, benzene, ether, and acetone. Insoluble in water, but it may be dispersed in hot water with the aid of a small amount of soap or other suitable surface-active agent. *NF category:* Emulsifying and/or solubilizing agent.

Add the following:

▲ **Glyceryl Tristearate:** White, solid, microcrystalline powder. Soluble in hot alcohol, in acetone, and in chloro-

form; very slightly soluble in cold alcohol, in ether, and in petroleum ether; insoluble in water. *NF category:* Tablet and/or capsule lubricant; emulsifying and/or solubilizing agent. ▲ *NF31*

Glycine: White, odorless, crystalline powder, having a sweetish taste. Its solutions are acid to litmus. Freely soluble in water; very slightly soluble in alcohol and in ether.

Glycopyrrolate: White, odorless, crystalline powder. Soluble in water and in alcohol; practically insoluble in chloroform and in ether.

Gonadorelin Acetate: White to slightly yellowish powder. Soluble in water; sparingly soluble in methanol.

Chorionic Gonadotropin: White or practically white, amorphous powder. Freely soluble in water.

Chorionic Gonadotropin for Injection: White or practically white, amorphous solid having the characteristic appearance of substances prepared by freeze-drying.

Gramicidin: White or practically white, odorless, crystalline powder. Soluble in alcohol; insoluble in water.

Granisetron Hydrochloride: White or almost white powder. Freely soluble in water; sparingly soluble in methylene chloride; slightly soluble in methanol.

Green Soap: Soft, unctuous, yellowish-white to brownish or greenish yellow, transparent to translucent mass. Has a slight, characteristic odor, often suggesting the oil from which it was prepared. Its solution (1 in 20) is alkaline to bromothymol blue TS.

Griseofulvin: White to creamy white, odorless powder, in which particles of the order of 4 µm in diameter predominate. Soluble in acetone, in dimethylformamide, and in chloroform; sparingly soluble in alcohol; very slightly soluble in water.

Guaifenesin: White to slightly gray, crystalline powder. May have a slight characteristic odor. Soluble in water, in alcohol, in chloroform, and in propylene glycol; sparingly soluble in glycerin.

Guanabenz Acetate: White or almost white powder having not more than a slight odor. Soluble in alcohol and in propylene glycol; sparingly soluble in water and in 0.1 N hydrochloric acid.

Guanadrel Sulfate: White to off-white, crystalline powder. Melts at about 235°, with decomposition. Soluble in water; sparingly soluble in methanol; slightly soluble in alcohol and in acetone.

Guanethidine Monosulfate: White to off-white, crystalline powder. Very soluble in water; sparingly soluble in alcohol; practically insoluble in chloroform.

Guar Gum: White to yellowish-white, practically odorless powder. Dispersible in hot or cold water, forming a colloidal solution. *NF category:* Suspending and/or viscosity-increasing agent; tablet binder.

Gutta Percha: Lumps or blocks of variable size; externally brown or grayish-brown to grayish-white in color; internally reddish yellow or reddish gray and having a laminated or fibrous appearance. Is flexible but only slightly elastic. Has a slight, characteristic odor and a slight taste. Soluble in chloroform; partly soluble in benzene, in carbon disulfide, and in turpentine oil; insoluble in water.

Halazone: White, crystalline powder, having a characteristic chlorine-like odor. Is affected by light. Melts at about 194°, with decomposition. Soluble in glacial acetic acid; very slightly soluble in water and in chloroform. Dissolves in solutions of alkali hydroxides and carbonates with the formation of a salt.

Halazone Tablets for Solution: Soluble in water.

Halcinonide: White to off-white, odorless, crystalline powder. Soluble in acetone and in chloroform; slightly soluble in alcohol and in ethyl ether; insoluble in water and in hexanes.

Halobetasol Propionate: White to off-white powder. Freely soluble in dichloromethane and in acetone; practically insoluble in water.

Haloperidol: White to faintly yellowish, amorphous or microcrystalline powder. Its saturated solution is neutral to litmus. Soluble in chloroform; sparingly soluble in alcohol; slightly soluble in ether; practically insoluble in water.

Haloperidol Decanoate: A white or almost white powder. Very soluble in alcohol, in methanol, and in methylene chloride; practically insoluble in water.

Halothane: Colorless, mobile, nonflammable, heavy liquid, having a characteristic odor resembling that of chloroform. Its taste is sweet and produces a burning sensation. Slightly soluble in water. Miscible with alcohol, with chloroform, with ether, and with fixed oils.

Helium: Colorless, odorless, tasteless gas, which is not combustible and does not support combustion. Very slightly soluble in water. At 0° and at a pressure of 760 mm of mercury, 1000 mL of the gas weighs about 180 mg.

Heparin Sodium: White or pale-colored, amorphous powder. Is odorless or practically so, and is hygroscopic. Soluble in water.

Hexachlorophene: White to light tan, crystalline powder. Is odorless or has only a slight, phenolic odor. Freely soluble in acetone, in alcohol, and in ether; soluble in chloroform and in dilute solutions of fixed alkali hydroxides; insoluble in water.

Hexachlorophene Liquid Soap: Clear, amber-colored liquid, having a slight, characteristic odor. Its solution (1 in 20) is clear and has an alkaline reaction.

Hexylene Glycol: Clear, colorless, viscous liquid. Absorbs moisture when exposed to moist air. Miscible with water and with many organic solvents, including alcohol, ether, chloroform, acetone, and hexanes. *NF category:* Humectant; solvent.

Histamine Phosphate: Colorless, odorless, long prismatic crystals. Is stable in air but is affected by light. Its solutions are acid to litmus. Freely soluble in water.

Histidine: White, odorless crystals, having a slightly bitter taste. Soluble in water; very slightly soluble in alcohol; insoluble in ether.

Histoplasmin: Clear, red liquid. Miscible with water.

Homatropine Hydrobromide: White crystals, or white, crystalline powder. Slowly darkens on exposure to light. Freely soluble in water; sparingly soluble in alcohol; slightly soluble in chloroform; insoluble in ether. Melts between 214° and 217°, with slight decomposition.

Homatropine Methylbromide: White, odorless powder. Slowly darkens on exposure to light. Melts at about 190°. Very soluble in water; freely soluble in alcohol and in acetone containing about 20% of water; practically insoluble in ether and in acetone.

Hydralazine Hydrochloride: White to off-white, odorless, crystalline powder. Melts at about 275°, with decomposition. Soluble in water; slightly soluble in alcohol; very slightly soluble in ether.

Hydrochloric Acid: Colorless, fuming liquid having a pungent odor. It ceases to fume when it is diluted with 2 volumes of water. Specific gravity is about 1.18. *NF category:* Acidifying agent.

Diluted Hydrochloric Acid: Colorless, odorless liquid. Specific gravity is about 1.05. *NF category:* Acidifying agent.

Hydrochlorothiazide: White, or practically white, practically odorless, crystalline powder. Freely soluble in sodium hydroxide solution, in *n*-butylamine, and in dimethylformamide; very slightly soluble in water; sparingly soluble in methanol; insoluble in ether, in chloroform, and in dilute mineral acids.

Hydrocodone Bitartrate: Fine, white crystals or a crystalline powder. Is affected by light. Soluble in water; slightly soluble in alcohol; insoluble in ether and in chloroform.

Hydrocortisone: White to practically white, odorless, crystalline powder. Melts at about 215°, with decomposition. Sparingly soluble in acetone and in alcohol; slightly soluble in chloroform; very slightly soluble in water and in ether.

Hydrocortisone Acetate: White to practically white, odorless, crystalline powder. Melts at about 200°, with decomposition. Slightly soluble in alcohol and in chloroform; insoluble in water.

Hydrocortisone Butyrate: White to practically white, practically odorless, crystalline powder. Freely soluble in chloroform; soluble in methanol, in alcohol, and in acetone; slightly soluble in ether; practically insoluble in water.

Hydrocortisone Sodium Phosphate: White to light yellow, odorless or practically odorless, powder. Is exceedingly hygroscopic. Freely soluble in water; slightly soluble in alcohol; practically insoluble in chloroform, in dioxane, and in ether.

Hydrocortisone Sodium Succinate: White or nearly white, odorless, hygroscopic, amorphous solid. Very soluble in water and in alcohol; very slightly soluble in acetone; insoluble in chloroform.

Hydroflumethiazide: White to cream-colored, finely divided, odorless, crystalline powder. Freely soluble in acetone; soluble in alcohol; very slightly soluble in water.

Hydrogen Peroxide Concentrate: Clear, colorless liquid. Is acid to litmus. Slowly decomposes, and is affected by light.

Hydrogen Peroxide Solution: Clear, colorless liquid, odorless, or having an odor resembling that of ozone. Is acid to litmus and to the taste and produces a froth in the mouth. Rapidly decomposes when in contact with many oxidizing as well as reducing substances. When rapidly heated, it may decompose suddenly. Is affected by light. Specific gravity is about 1.01.

Hydromorphone Hydrochloride: Fine, white or practically white, odorless, crystalline powder. Is affected by light. Freely soluble in water; sparingly soluble in alcohol; practically insoluble in ether.

Hydroquinone: Fine white needles. Darkens upon exposure to light and air. Freely soluble in water, in alcohol, and in ether.

Hydroxocobalamin: Dark red crystals or red crystalline powder. Is odorless, or has not more than a slight acetone odor. The anhydrous form is very hygroscopic. Sparingly soluble in water, in alcohol, and in methanol; practically insoluble in acetone, in ether, in chloroform, and in benzene.

Hydroxyamphetamine Hydrobromide: White, crystalline powder. Its solutions are slightly acid to litmus, having a pH of about 5. Freely soluble in water and in alcohol; slightly soluble in chloroform; practically insoluble in ether.

Hydroxychloroquine Sulfate: White or practically white, crystalline powder. Is odorless, and has a bitter taste. Its solutions have a pH of about 4.5. Exists in two forms, the usual form melting at about 240° and the other form melting at about 198°. Freely soluble in water; practically insoluble in alcohol, in chloroform, and in ether.

Hydroxyethyl Cellulose: White to light tan, practically odorless and tasteless, hygroscopic powder. Soluble in hot water and in cold water, giving a colloidal solution; practically insoluble in alcohol and in most organic solvents. *NF category:* Suspending and/or viscosity-increasing agent.

Hydroxyprogesterone Caproate: White or creamy white, crystalline powder. Is odorless or has a slight odor. Soluble in ether; slightly soluble in benzene; insoluble in water.

Hydroxypropyl Betadex: White or almost white, amorphous or crystalline powder. Freely soluble in water and in propylene glycol. *NF category:* Sequestering agent.

Hydroxypropyl Cellulose: White to cream-colored, practically odorless and tasteless, granular solid or powder.

Is hygroscopic after drying. Soluble in cold water, in alcohol, in chloroform, and in propylene glycol, giving a colloidal solution; insoluble in hot water. *NF category:* Coating agent; suspending and/or viscosity-increasing agent.

Low-Substituted Hydroxypropyl Cellulose: White to yellowish-white, practically odorless and tasteless, fibrous or granular powder. Is hygroscopic. Practically insoluble in alcohol and in ether. Dissolves in a solution of sodium hydroxide (1 in 10), and produces a viscous solution. Swells in water, in sodium carbonate TS, and in 2 N hydrochloric acid. The pH of the suspension, obtained by shaking 1.0 g with 100 mL of water, is between 5.0 and 7.5. *NF category:* Tablet binder; tablet disintegrant.

Hydroxyurea: White to off-white powder. Is somewhat hygroscopic, decomposing in the presence of moisture. Melts at a temperature exceeding 133°, with decomposition. Freely soluble in water and in hot alcohol.

Hydroxyzine Hydrochloride: White, odorless powder. Melts at about 200°, with decomposition. Very soluble in water; soluble in chloroform; slightly soluble in acetone; practically insoluble in ether.

Hydroxyzine Pamoate: Light yellow, practically odorless powder. Freely soluble in dimethylformamide; practically insoluble in water and in methanol.

Hymetellose: A white, yellowish-white or grayish-white powder or granules. Hygroscopic after drying. Dissolves in cold water, giving a colloidal solution. Insoluble in hot water, in acetone, in alcohol, in ether, and in toluene.

Hyoscyamine: White, crystalline powder. Is affected by light. Its solutions are alkaline to litmus. Freely soluble in alcohol, in chloroform, and in dilute acids; sparingly soluble in ether; slightly soluble in water and in benzene.

Hyoscyamine Hydrobromide: White, odorless crystals or crystalline powder. The pH of a solution (1 in 20) is about 5.4. Is affected by light. Freely soluble in water, in alcohol, and in chloroform; very slightly soluble in ether.

Hyoscyamine Sulfate: White or almost white, crystalline powder or colorless needles. Is deliquescent and is affected by light. The pH of a solution (1 in 100) is about 5.3. Very soluble in water; freely soluble in alcohol; practically insoluble in ether. Melts at a temperature not less than 200°.

Hypophosphorous Acid: Colorless or slightly yellow, odorless liquid. Specific gravity is about 1.13. *NF category:* Antioxidant.

Hypromellose: White to slightly off-white, fibrous or granular powder. Swells in water and produces a clear to opalescent, viscous, colloidal mixture. Insoluble in dehydrated alcohol, in ether, and in chloroform. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; tablet binder.

Hypromellose 2208: White to slightly off-white, fibrous or granular powder. Swells in water and produces a clear to opalescent, viscous, colloidal mixture. Insoluble in dehydrated alcohol, in ether, and in chloroform. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; tablet binder.

Hypromellose 2906: White to slightly off-white, fibrous or granular powder. Swells in water and produces a clear to opalescent, viscous, colloidal mixture. Insoluble in dehydrated alcohol, in ether, and in chloroform. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; tablet binder.

Hypromellose 2910: White to slightly off-white, fibrous or granular powder. Swells in water and produces a clear to opalescent, viscous, colloidal mixture. Insoluble in dehydrated alcohol, in ether, and in chloroform. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; tablet binder.

Hypromellose Acetate Succinate: White to yellowish-white powder or pills. Odorless, or has a faint, acetic acid-like odor, and tasteless. Practically insoluble in water, in de-

hydrated alcohol, in xylene, and in hexane. On the addition of a mixture of dehydrated alcohol and dichloromethane (1:1) or acetone, a clear or turbid viscous liquid is produced. Dissolves in 1 N sodium hydroxide. Slightly hygroscopic. *NF category:* Coating agent; tablet binder.

Hypromellose Phthalate: White powder or granules. Is odorless and tasteless. Practically insoluble in water, in dehydrated alcohol, and in hexane. Produces a viscous solution in a mixture of methanol and dichloromethane (1:1), or in a mixture of dehydrated alcohol and acetone (1:1). Dissolves in 1 N sodium hydroxide. *NF category:* Coating agent.

Ibuprofen: White to off-white, crystalline powder, having a slight, characteristic odor. Very soluble in alcohol, in methanol, in acetone, and in chloroform; slightly soluble in ethyl acetate; practically insoluble in water.

Ichthammol: Reddish-brown to brownish-black, viscous fluid, having a strong, characteristic, empyreumatic odor. Miscible with water, with glycerin, and with fixed oils and fats. Partially soluble in alcohol and in ether.

Idarubicin Hydrochloride: Red-orange to red-brown powder. Soluble in methanol; slightly soluble in water; insoluble in acetone and in ethyl ether.

Idoxuridine: White, crystalline, practically odorless powder. Slightly soluble in water and in alcohol; practically insoluble in chloroform and in ether.

Ifosfamide: White, crystalline powder. Melts at about 40°. Very soluble in alcohol, in ethyl acetate, in isopropyl alcohol, in methanol, and in methylene chloride; freely soluble in water; very slightly soluble in hexanes.

Imidurea: White, odorless, tasteless powder. Soluble in water and in glycerin; sparingly soluble in propylene glycol; insoluble in most organic solvents.

Imipenem: White to tan-colored crystalline powder. Slightly soluble in water and in methanol.

Imipramine Hydrochloride: White to off-white, odorless or practically odorless, crystalline powder. Freely soluble in water and in alcohol; soluble in acetone; insoluble in ether and in benzene.

Inamrinone: Pale yellow to tan powder. It is odorless or has a faint odor. Slightly soluble in methanol; practically insoluble or insoluble in chloroform and in water.

Indapamide: White to off-white, crystalline powder. Melts between 167° and 170°. Soluble in methanol, in alcohol, in acetonitrile, in glacial acetic acid, and in ethyl acetate; very slightly soluble in ether and in chloroform; practically insoluble in water.

Indigotindisulfonate Sodium: Dusky, purplish-blue powder, or blue granules having a coppery luster. Is affected by light. Its solutions have a blue or bluish purple color. Slightly soluble in water and in alcohol; practically insoluble in most other organic solvents.

Indinavir Sulfate: White or almost white, hygroscopic powder. Freely soluble in water; soluble in methanol; practically insoluble in heptane.

Indocyanine Green: Olive-brown, dark green, blue-green, dark blue, or black powder. Is odorless or has a slight odor. Its solutions are deep emerald-green in color. The pH of a solution (1 in 200) is about 6. Its aqueous solutions are stable for about 8 hours. Soluble in water and in methanol; practically insoluble in most other organic solvents.

Indomethacin: Pale yellow to yellow-tan, crystalline powder, having not more than a slight odor. Is sensitive to light. Melts at about 162°. Exhibits polymorphism. Sparingly soluble in alcohol, in chloroform, and in ether; practically insoluble in water.

Influenza Virus Vaccine: Slightly turbid liquid or suspension, which may have a slight yellow or reddish tinge and may have an odor because of the preservative.

Inositol: White or almost white, crystalline powder. Very soluble in water; practically insoluble in alcohol absolute and in ether.

Insulin: White or practically white crystals. Soluble in solutions of dilute acids and alkalis.

Insulin Injection: The Injection containing, in each mL, not more than 100 USP Units is a clear, colorless or almost colorless liquid; the Injection containing, in each mL, 500 Units may be straw-colored. Contains between 0.1% and 0.25% (w/v) of either phenol or cresol. Contains between 1.4% and 1.8% (w/v) of glycerin.

Insulin Lispro: White or practically white crystals. Soluble in solutions of dilute acids and alkalis.

Isophane Insulin Suspension: White suspension of rod-shaped crystals, free from large aggregates of crystals following moderate agitation. Contains either (1) between 1.4% and 1.8% (w/v) of glycerin, between 0.15% and 0.17% (w/v) of metacresol, and between 0.06% and 0.07% (w/v) of phenol; or (2) between 1.4% and 1.8% (w/v) of glycerin and between 0.20% and 0.25% (w/v) of phenol. Contains between 0.15% and 0.25% (w/v) of dibasic sodium phosphate. When examined microscopically, the insoluble matter in the Suspension is crystalline, and contains not more than traces of amorphous material.

Insulin Zinc Suspension: Practically colorless suspension of a mixture of characteristic crystals predominantly between 10 and 40 μm in maximum dimension and many particles that have no uniform shape and do not exceed 2 μm in maximum dimension. Contains between 0.15% and 0.17% (w/v) of sodium acetate, between 0.65% and 0.75% (w/v) of sodium chloride, and between 0.09% and 0.11% (w/v) of methylparaben.

Extended Insulin Zinc Suspension: Practically colorless suspension of a mixture of characteristic crystals the maximum dimension of which is predominantly between 10 and 40 μm . Contains between 0.15% and 0.17% (w/v) of sodium acetate, between 0.65% and 0.75% (w/v) of sodium chloride, and between 0.09% and 0.11% (w/v) of methylparaben.

Prompt Insulin Zinc Suspension: Practically colorless suspension of particles that have no uniform shape and the maximum dimension of which does not exceed 2 μm . Contains between 0.15% and 0.17% (w/v) of sodium acetate, between 0.65% and 0.75% (w/v) of sodium chloride, and between 0.09% and 0.11% (w/v) of methylparaben.

Inulin: White, friable, chalk-like, amorphous, odorless, tasteless powder. Soluble in hot water; slightly soluble in cold water and in organic solvents.

Iodine: Heavy, grayish-black plates or granules, having a metallic luster and a characteristic odor. Freely soluble in carbon disulfide, in chloroform, in carbon tetrachloride, and in ether; soluble in alcohol and in solutions of iodides; sparingly soluble in glycerin; very slightly soluble in water.

Iodine Topical Solution: Transparent, reddish-brown liquid, having the odor of iodine.

Strong Iodine Solution: Transparent liquid having a deep brown color and having the odor of iodine.

Iodine Tincture: Transparent liquid having a reddish-brown color and the odor of iodine and of alcohol.

Sodium Iodide I 123 Capsules: Capsules may contain a small amount of solid or solids, or may appear empty.

Sodium Iodide I 123 Solution: Clear, colorless solution. Upon standing, both the Solution and the glass container may darken as a result of the effects of the radiation.

Iodinated I 125 Albumin Injection: Clear, colorless to slightly yellow solution. Upon standing, both the Albumin and the glass container may darken as a result of the effects of the radiation.

Iodinated I 131 Albumin Injection: Clear, colorless to slightly yellow solution. Upon standing, both the Albumin and the glass container may darken as a result of the effects of the radiation.

Iodinated I 131 Albumin Aggregated Injection: Dilute suspension of white to faintly yellow particles, which may

settle on standing. The glass container may darken on standing, as a result of the effects of the radiation.

Sodium Rose Bengal I 131 Injection: Clear, deep-red solution.

Iodohippurate Sodium I 131 Injection: Clear, colorless solution. Upon standing, both the Injection and the glass container may darken as a result of the effects of the radiation.

Sodium Iodide I 131 Capsules: May contain a small amount of solid or solids, or may appear empty.

Sodium Iodide I 131 Solution: Clear, colorless solution. Upon standing, both the Solution and the glass container may darken as a result of the effects of the radiation.

Iodipamide: White, practically odorless, crystalline powder. Slightly soluble in alcohol; very slightly soluble in water, in chloroform, and in ether.

Iodipamide Meglumine Injection: Clear, colorless to pale yellow, slightly viscous liquid.

Iodixanol: White to off-white, amorphous, odorless, hygroscopic powder. Freely soluble in water.

Iodoform: Lustrous greenish yellow powder, or lustrous crystals. It is slightly volatile even at ordinary temperatures, and distills slowly with steam. Freely soluble in ether and in chloroform; soluble in boiling alcohol; sparingly soluble in alcohol, in glycerin, and in olive oil; practically insoluble in water. Melts to a brown liquid at about 115°, and decomposes at a higher temperature, emitting vapors of iodine.

Iodoquinol: Light yellowish to tan, microcrystalline powder not readily wetted by water. Is odorless or has a faint odor; is stable in air. Melts with decomposition. Sparingly soluble in alcohol and in ether; practically insoluble in water.

Iohexol: White to off-white, hygroscopic, odorless powder. Very soluble in water and in methanol; practically insoluble or insoluble in ether and in chloroform.

Iohexol Injection: Clear, colorless to pale yellow liquid.

Iopamidol: Practically odorless, white to off-white powder. Very soluble in water; sparingly soluble in methanol; practically insoluble in alcohol and in chloroform.

Iopanoic Acid: Cream-colored powder. Is tasteless or practically so, and has a faint, characteristic odor. Is affected by light. Soluble in alcohol, in chloroform, and in ether, and in solutions of alkali hydroxides and carbonates; insoluble in water.

Iophendylate: Colorless to pale yellow, viscous liquid, the color darkening on long exposure to air. Is odorless or has a faintly ethereal odor. Freely soluble in alcohol, in benzene, in chloroform, and in ether; very slightly soluble in water.

Iophendylate Injection: Colorless to pale yellow, viscous liquid, the color darkening on long exposure to air. Is odorless or has a faintly ethereal odor. Freely soluble in alcohol, in benzene, in chloroform, and in ether; very slightly soluble in water.

Iopromide: White to slightly yellow powder. Freely soluble in water and in dimethyl sulfoxide; practically insoluble in alcohol, in acetone, and in ether.

Iothalamate Meglumine Injection: Clear, colorless to pale yellow, slightly viscous liquid.

Iothalamate Meglumine and Iothalamate Sodium Injection: Clear, colorless to pale yellow, slightly viscous liquid.

Iothalamate Sodium Injection: Clear, colorless to pale yellow, slightly viscous liquid.

Iothalamic Acid: White, odorless powder. Soluble in solutions of alkali hydroxides; slightly soluble in water and in alcohol.

Ioxilan: White to off-white, practically odorless powder. Soluble in water and in methanol.

Iloxilan Injection: Clear, colorless to pale yellow liquid.
Powdered Ipecac: Pale brown, weak yellow, or light olive-gray powder.

Ipodate Sodium: White to off-white, odorless, fine, crystalline powder. Freely soluble in water, in alcohol, and in methanol; very slightly soluble in chloroform.

Ipratropium Bromide: White to off-white, crystalline powder. Freely soluble in methanol; soluble in water; slightly soluble in alcohol.

Irbesartan: White to off-white, crystalline powder. Slightly soluble in alcohol and in methylene chloride; practically insoluble in water.

Irinotecan Hydrochloride: Pale yellow to yellow crystalline powder. Sparingly soluble in water and in alcohol; slightly soluble in most organic solvents.

Iron Dextran Injection: Dark brown, slightly viscous liquid.

Iron Sorbitex Injection: Clear liquid, having a dark brown color.

Isobutane: Colorless, flammable gas (boiling temperature is about -11°). Vapor pressure at 21° is about 2950 mm of mercury (31 psig). *NF category:* Aerosol propellant.

Isoetharine Inhalation Solution: Colorless or slightly yellow, slightly acid liquid, gradually turning dark on exposure to air and light.

Isoetharine Hydrochloride: White to off-white, odorless, crystalline solid. Melts between 196° and 208° , with decomposition. Soluble in water; sparingly soluble in alcohol; practically insoluble in ether.

Isoetharine Mesylate: White or practically white, odorless crystals having a salty, bitter taste. Freely soluble in water; soluble in alcohol; practically insoluble in acetone and in ether.

Isflurane: Clear, colorless, volatile liquid, having a slight odor. Boils at about 49° . Insoluble in water. Miscible with common organic solvents and with fats and oils.

Isflophate: Clear, colorless or faintly yellow liquid. Its vapor is extremely irritating to the eye and mucous membranes. Is decomposed by moisture, with the formation of hydrogen fluoride. Specific gravity is about 1.05. Soluble in alcohol and in vegetable oils; sparingly soluble in water.

Isoleucine: White, practically odorless crystals, having a slightly bitter taste. Soluble in water; slightly soluble in hot alcohol; insoluble in ether.

Isomethoprene Mucate: White, crystalline powder. Freely soluble in water; soluble in alcohol; practically insoluble in chloroform and in ether.

Isoniazid: Colorless or white crystals or white, crystalline powder. Is odorless and is slowly affected by exposure to air and light. Freely soluble in water; sparingly soluble in alcohol; slightly soluble in chloroform; very slightly soluble in ether.

Isoniazid Injection: Clear, colorless to faintly greenish-yellow liquid. Gradually darkens on exposure to air and light. Tends to crystallize at low temperatures.

Isoopropamide Iodide: White to pale yellow, crystalline powder, having a bitter taste. Freely soluble in chloroform and in alcohol; sparingly soluble in water; very slightly soluble in benzene and in ether.

Isopropyl Alcohol: Transparent, colorless, mobile, volatile liquid, having a characteristic odor and a slightly bitter taste. Is flammable. Miscible with water, with alcohol, with ether, and with chloroform. *NF category:* Solvent.

Azeotropic Isopropyl Alcohol: Transparent, colorless, mobile, volatile liquid, having a characteristic odor and a slightly bitter taste. Is flammable. Miscible with water, with alcohol, with ether, and with chloroform.

Isopropyl Myristate: Clear, practically colorless, oily liquid. Is practically odorless, and congeals at about 5° . Freely

soluble in 90% alcohol; insoluble in water, in glycerin, and in propylene glycol. Miscible with most organic solvents and with fixed oils. *NF category:* Vehicle (oleaginous).

Isopropyl Palmitate: Colorless, mobile liquid having a very slight odor. Soluble in acetone, in castor oil, in chloroform, in cottonseed oil, in ethyl acetate, in alcohol, and in mineral oil; insoluble in water, in glycerin, and in propylene glycol. *NF category:* Vehicle (oleaginous).

Isoproterenol Inhalation Solution: Colorless or practically colorless, slightly acid liquid, gradually turning dark on exposure to air and light.

Isoproterenol Hydrochloride: White to practically white, odorless, crystalline powder, having a slightly bitter taste. Gradually darkens on exposure to air and light. Its solutions become pink to brownish pink on standing exposed to air, doing so almost immediately when rendered alkaline. Its solution (1 in 100) has a pH of about 5. Freely soluble in water; sparingly soluble in alcohol and less soluble in dehydrated alcohol; insoluble in chloroform and in ether.

Isoproterenol Hydrochloride Injection: Colorless or practically colorless liquid, gradually turning dark on exposure to air and light.

Isoproterenol Sulfate: White to practically white, odorless, crystalline powder. It gradually darkens on exposure to air and light. Its solutions become pink to brownish pink on standing exposed to air, doing so almost immediately when rendered alkaline. A solution (1 in 100) has a pH of about 5. Freely soluble in water; very slightly soluble in alcohol, in benzene, and in ether.

Isosorbide Concentrate: Colorless to slightly yellow liquid. Soluble in water and in alcohol.

Diluted Isosorbide Dinitrate: Ivory-white, odorless powder. [NOTE—Undiluted isosorbide dinitrate occurs as white, crystalline rosettes.] Undiluted isosorbide dinitrate is very soluble in acetone; freely soluble in chloroform; sparingly soluble in alcohol; very slightly soluble in water.

Isotretinoin: Yellow crystals. Soluble in chloroform; sparingly soluble in alcohol, in isopropyl alcohol, and in polyethylene glycol 400; practically insoluble in water.

Isoxsuprine Hydrochloride: White, odorless, crystalline powder, having a bitter taste. Melts at about 200° , with decomposition. Sparingly soluble in alcohol; slightly soluble in water.

Iradipine: Yellow, fine crystalline powder.

Itraconazole: A white or almost white powder. Freely soluble in methylene chloride; sparingly soluble in tetrahydrofuran; very slightly soluble in alcohol; practically insoluble in water.

Ivermectin: White to yellowish-white, crystalline powder. Slightly hygroscopic. Freely soluble in methanol and in methylene chloride; soluble in acetone and in acetonitrile; practically insoluble in hexane and in water.

Juniper Tar: Dark brown, clear, thick liquid, having a tarry odor and a faintly aromatic, bitter taste. Sparingly soluble in solvent hexane; very slightly soluble in water. One volume dissolves in 9 volumes of alcohol. Dissolves in 3 volumes of ether, leaving only a slight, flocculent residue. Miscible with amyl alcohol, with chloroform, and with glacial acetic acid.

Kanamycin Sulfate: White, odorless, crystalline powder. Freely soluble in water; insoluble in acetone, in ethyl acetate, and in benzene.

Kaolin: Soft, white or yellowish-white powder or lumps. Has an earthy or clay-like taste and, when moistened with water, assumes a darker color and develops a marked clay-like odor. Insoluble in water, in cold dilute acids, and in solutions of alkali hydroxides. *NF category:* Tablet and/or capsule diluent.

Ketamine Hydrochloride: White, crystalline powder, having a slight, characteristic odor. Freely soluble in water

and in methanol; soluble in alcohol; sparingly soluble in chloroform.

Ketorolac Tromethamine: White to off-white, crystalline powder. Melts between 165° and 170°, with decomposition. Freely soluble in water and in methanol; slightly soluble in alcohol, in dehydrated alcohol, and in tetrahydrofuran; practically insoluble in acetone, in dichloromethane, in toluene, in ethyl acetate, in dioxane, in hexane, in butyl alcohol, and in acetonitrile.

Labetalol Hydrochloride: White to off-white powder. Melts at about 180°, with decomposition. Soluble in water and in alcohol; insoluble in ether and in chloroform.

Alpha-Lactalbumin: Free-flowing, slightly hygroscopic light cream-colored powder. Freely soluble in water; soluble in wide pH ranges; insoluble in methanol, in alcohol, in ether, and in acetone. *NF category:* Buffering agent; bulking agent for freeze-drying; coating agent; complexing agent; emulsifying and/or solubilizing agent; stiffening agent; suspending and/or viscosity-increasing agent; tablet binder; tablet and/or capsule diluent; vehicle.

Lactic Acid: Colorless or yellowish, practically odorless, syrupy liquid. Is hygroscopic. When it is concentrated by boiling, lactic acid lactate is formed. Specific gravity is about 1.20. Insoluble in chloroform. Miscible with water, with alcohol, and with ether. *NF category:* Buffering agent.

Lactitol: A white or light brown, odorless crystal. Has a mild, sweet taste, and no aftertaste. *NF category:* Flavors and perfumes; tablet and/or capsule diluent.

Lactobionic Acid: White or almost white, crystalline powder with a melting point of about 125° with decomposition. Freely soluble in water; slightly soluble in glacial acetic acid, in anhydrous ethanol, and in methanol. *NF category:* Antioxidant.

Anhydrous Lactose: White or almost white powder. Freely soluble in water; practically insoluble in alcohol. *NF category:* Tablet and/or capsule diluent.

Lactose Monohydrate: White, free-flowing powder. Freely but slowly soluble in water; practically insoluble in alcohol. *NF category:* Tablet and/or capsule diluent.

Lactulose Concentrate: Colorless to amber syrupy liquid, which may exhibit some precipitation and darkening upon standing. Miscible with water.

Lamivudine: White to off-white solid. Soluble in water. Melts at about 176°.

Lamotrigine: A white to pale cream-colored powder. Slightly soluble in 0.1 N hydrochloric acid, in acetone, in methanol, and in water.

Lanolin: Yellow, tenacious, unctuous mass, having a slight, characteristic odor. Freely soluble in ether and in chloroform; soluble in hot alcohol; sparingly soluble in cold alcohol; insoluble in water, but mixes without separation with about twice its weight of water. *NF category:* Ointment base.

Lanolin Alcohols: Hard, waxy, amber solid, having a characteristic odor. Freely soluble in chloroform, in ether, and in petroleum ether; slightly soluble in alcohol; insoluble in water. *NF category:* Emulsifying and/or solubilizing agent.

Lansoprazole: White to brownish-white powder. Freely soluble in dimethylformamide; practically insoluble in water. Melts at about 166°, with decomposition.

Lauroyl Polyoxylglycerides: Pale yellow, waxy liquids. Freely soluble in methylene chloride. Dispersible in hot water. *NF category:* Ointment base; solvent.

Lecithin: The consistency of both natural grades and refined grades of lecithin may vary from plastic to fluid, depending upon free fatty acid and oil content, and upon the presence or absence of other diluents. Its color varies from light yellow to brown, depending on the source, on crop variations, and on whether it is bleached or unbleached. It is odorless or has a characteristic, slight nut-like odor and a bland taste. Practically insoluble in water, but it readily

hydrates to form emulsions. The oil-free phosphatides are soluble in fatty acids, but are practically insoluble in fixed oils. When all phosphatide fractions are present, lecithin is sparingly soluble in alcohol and practically insoluble in acetone. *NF category:* Emulsifying and/or solubilizing agent.

Leflunomide: White to almost white powder. Freely soluble in methanol, in alcohol, in 2-propanol, in ethyl acetate, in acetone, and in acetonitrile; practically insoluble in water.

Letrozole: White to yellowish, crystalline powder. Freely soluble in dichloromethane; slightly soluble in alcohol; practically insoluble in water.

Leucine: White, practically odorless, tasteless crystals. Sparingly soluble in water; insoluble in ether.

Leucovorin Calcium: Yellowish-white or yellow, odorless powder. Very soluble in water; practically insoluble in alcohol.

Leucovorin Calcium Injection: Clear, yellowish solution.

Levamisole Hydrochloride: White or almost white, crystalline powder. Freely soluble in water; soluble in alcohol; slightly soluble in methylene chloride; practically insoluble in ether.

Levetiracetam: White to almost white powder. Very soluble in water; soluble in acetonitrile; practically insoluble in hexane.

Levmetamfetamine: Clear, practically colorless liquid.

Levobunolol Hydrochloride: White crystalline, odorless powder. Soluble in water and in methanol; slightly soluble in alcohol and in chloroform.

Levocarnitine: White crystals or crystalline powder. Hygroscopic. Freely soluble in water, and in hot alcohol; practically insoluble in acetone, in ether, and in benzene.

Levodopa: White to off-white, odorless, crystalline powder. In the presence of moisture, is rapidly oxidized by atmospheric oxygen and darkens. Freely soluble in 3 N hydrochloric acid; slightly soluble in water; insoluble in alcohol.

Levofloxacin: Light yellowish-white to yellow-white crystals or crystalline powder. Soluble in dimethylsulfoxide and in acetic acid; sparingly soluble in water, in acetone, and in methanol; practically insoluble in glycerin and in *n*-octanol.

Levonordefrin: White to buff-colored, odorless, crystalline solid. Melts at about 210°. Freely soluble in aqueous solutions of mineral acids; slightly soluble in acetone, in chloroform, in alcohol, and in ether; practically insoluble in water.

Levonorgestrel: White or practically white, odorless powder. Soluble in chloroform; slightly soluble in alcohol; practically insoluble in water.

Levorphanol Tartrate: Practically white, odorless, crystalline powder. Sparingly soluble in water; slightly soluble in alcohol; insoluble in chloroform and in ether. Melts, in a sealed tube, at about 110°, with decomposition.

Levothyroxine Sodium: Light yellow to buff-colored, odorless, tasteless, hygroscopic powder. Is stable in dry air but may assume a slight pink color upon exposure to light. The pH of a saturated solution is about 8.9. Soluble in solutions of alkali hydroxides and in hot solutions of alkali carbonates; slightly soluble in alcohol; very slightly soluble in water; insoluble in acetone, in chloroform, and in ether.

Lidocaine: White or slightly yellow, crystalline powder. Has a characteristic odor and is stable in air. Very soluble in alcohol and in chloroform; freely soluble in benzene and in ether; practically insoluble in water. Dissolves in oils.

Lidocaine Hydrochloride: White, odorless, crystalline powder, having a slightly bitter taste. Very soluble in water and in alcohol; soluble in chloroform; insoluble in ether.

Lime: Hard, white or grayish-white masses or granules, or white or grayish white powder. Is odorless. Slightly soluble in water; very slightly soluble in boiling water.

Lincomycin Hydrochloride: White or practically white, crystalline powder. Is odorless or has a faint odor. Is stable in the presence of air and light. Its solutions are acid and dextrorotatory. Freely soluble in water; soluble in dimethylformamide; very slightly soluble in acetone.

Lincomycin Hydrochloride Injection: Clear, colorless to slightly yellow solution, having a slight odor.

Lincomycin Hydrochloride Soluble Powder: White to off-white, or light tan free-flowing, fine powder.

Lindane: White, crystalline powder, having a slight, musty odor. Freely soluble in chloroform; soluble in dehydrated alcohol; sparingly soluble in ether; slightly soluble in ethylene glycol; practically insoluble in water.

Linoleoyl Polyoxylglycerides: Amber, oily liquids. May develop deposit after prolonged storage periods at 20°. Freely soluble in methylene chloride; practically insoluble but dispersible in water. *NF category:* Ointment base; solvent.

Liothyronine Sodium: Light tan, odorless, crystalline powder. Slightly soluble in alcohol; very slightly soluble in water; practically insoluble in most other organic solvents.

Lisinopril: White, crystalline powder. Melts at about 160°, with decomposition. Soluble in water; sparingly soluble in methanol; practically insoluble in alcohol, in acetone, in acetonitrile, and in chloroform.

Lithium Carbonate: White, granular, odorless powder. Sparingly soluble in water; very slightly soluble in alcohol. Dissolves, with effervescence, in dilute mineral acids.

Lithium Citrate: White, odorless, deliquescent powder or granules, having a cooling, faintly alkaline taste. Freely soluble in water; slightly soluble in alcohol.

Loperamide Hydrochloride: White to slightly yellow powder. Melts at about 225°, with some decomposition. Freely soluble in methanol and in chloroform; slightly soluble in water and in dilute acids; very slightly soluble in isopropyl alcohol.

Lopinavir: White powder. Freely soluble in methanol and alcohol; soluble in isopropanol; practically insoluble in water.

Loratadine: White to off-white powder. Freely soluble in acetone, in chloroform, in methanol, and in toluene; insoluble in water.

Lorazepam: White or practically white, practically odorless powder. Sparingly soluble in alcohol; slightly soluble in chloroform; insoluble in water.

Losartan Potassium: White to off-white powder. Freely soluble in water; sparingly soluble in isopropyl alcohol; slightly soluble in acetonitrile.

Lovastatin: White to off-white, crystalline powder. Freely soluble in chloroform; soluble in acetone, in acetonitrile, and in methanol; sparingly soluble in alcohol; practically insoluble in hexane; insoluble in water.

Loxapine Succinate: White to yellowish, crystalline powder. Is odorless.

Lutein: Red, crystalline powder. Soluble in ethanol, in ethyl acetate, and in methylene chloride; partially soluble in hexane.

Lysine Acetate: White, odorless crystals or crystalline powder, having an acid taste. Freely soluble in water.

Lysine Hydrochloride: White, odorless powder. Freely soluble in water.

Mafenide Acetate: White to pale yellow, crystalline powder. Freely soluble in water.

Magaldrate: White, odorless, crystalline powder. Soluble in dilute solutions of mineral acids; insoluble in water and in alcohol.

Milk of Magnesia: White, opaque, more or less viscous suspension from which varying proportions of water usually separate on standing. pH is about 10.

Magnesium Aluminometasilicate: White powder or granules having an amorphous structure. Very slightly soluble in acids and in alkalis; practically insoluble in water and in alcohol.

Magnesium Aluminosilicate: White powder or granules having an amorphous structure. Very slightly soluble in acids and in alkalis; practically insoluble in water and in alcohol.

Magnesium Aluminum Silicate: Odorless, tasteless, fine (micronized) powder, small cream to tan granules, or small flakes that are creamy when viewed on their flat surfaces and tan to brown when viewed on their edges. Insoluble in water and in alcohol. Swells when added to water or glycerin. *NF category:* Suspending and/or viscosity-increasing agent.

Magnesium Carbonate: Light, white, friable masses or bulky, white powder. Is odorless, and is stable in air. Practically insoluble in water to which, however, it imparts a slightly alkaline reaction; insoluble in alcohol, but is dissolved by dilute acids with effervescence.

Magnesium Chloride: Colorless, odorless, deliquescent flakes or crystals, which lose water when heated to 100° and lose hydrochloric acid when heated to 110°. Very soluble in water; freely soluble in alcohol.

Magnesium Citrate Oral Solution: Colorless to slightly yellow, clear, effervescent liquid, having a sweet, acidulous taste and a lemon flavor.

Magnesium Gluconate: Colorless crystals or white powder or granules. Is odorless and tasteless. Freely soluble in water; very slightly soluble in alcohol; insoluble in ether.

Magnesium Hydroxide: Bulky, white powder. Soluble in dilute acids; practically insoluble in water and in alcohol.

Magnesium Oxide: Very bulky, white powder or relatively dense, white powder or granulated powder. Soluble in dilute acids; practically insoluble in water; insoluble in alcohol.

Magnesium Phosphate: White, odorless, tasteless powder. Soluble in diluted mineral acids; practically insoluble in water.

Magnesium Salicylate: White, odorless, efflorescent, crystalline powder. Freely soluble in methanol; soluble in alcohol and in water; slightly soluble in ether.

Magnesium Silicate: Fine, white, odorless, tasteless powder, free from grittiness. Insoluble in water and in alcohol. Is readily decomposed by mineral acids. *NF category:* Glidant and/or anticaking agent.

Magnesium Stearate: Very fine, light, white powder, slippery to touch. Insoluble in water, in alcohol, and in ether. *NF category:* Tablet and/or capsule lubricant.

Magnesium Sulfate: Small, colorless crystals, usually needle-like, with a cooling, saline, bitter taste. It effloresces in warm, dry air. Very soluble in boiling water; freely soluble in water; freely (and slowly) soluble in glycerin; sparingly soluble in alcohol.

Magnesium Trisilicate: Fine, white, odorless, tasteless powder, free from grittiness. Insoluble in water and in alcohol. Is readily decomposed by mineral acids.

Malathion: Clear, colorless, or slightly yellowish liquid, having a characteristic odor. Congeals at about 2.9°. Slightly soluble in water. Miscible with alcohols, with esters, with ketones, with ethers, with aromatic and alkylated aromatic hydrocarbons, and with vegetable oils.

Maleic Acid: White, crystalline powder. Freely soluble in water and in alcohol; sparingly soluble in ether.

Malic Acid: White or practically white, crystalline powder or granules, having a strongly acid taste. Melts at about 130°. Very soluble in water; freely soluble in alcohol. *NF category:* Acidifying agent.

Maltitol: White, crystalline powder. Very soluble in water; practically insoluble in ethanol. *NF category:* Humectant; sweetening agent; tablet and/or capsule diluent.

Maltodextrin: White, hygroscopic powder or granules. Freely soluble or readily dispersible in water; slightly soluble to insoluble in anhydrous alcohol. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; tablet binder; and/or capsule diluent.

Maltol: A white, crystalline powder having a characteristic caramel-butterscotch odor, suggestive of a fruity-strawberry aroma in dilute solution. One g dissolves in about 82 mL of water, in 21 mL of alcohol, in 80 mL of glycerin, and in 28 mL of propylene glycol. *NF category:* Flavors and perfumes.

Maltose: Maltose occurs in either the anhydrous state or as a monohydrate. It is a white, crystalline powder, odorless, and has a sweet taste. Very slightly soluble in ethanol; freely soluble in water; slightly soluble in methanol; practically insoluble in ether.

Mangafodipir Trisodium: Pale yellow crystals or crystalline powder. Freely soluble in water; sparingly soluble in methanol; slightly soluble in chloroform; very slightly soluble in alcohol and in acetone.

Manganese Chloride: Large, irregular, pink, odorless, translucent crystals. Soluble in water and in alcohol; insoluble in ether.

Manganese Chloride for Oral Solution: Off-white to tan-colored powder with a strawberry odor. Soluble in water.

Manganese Sulfate: Pale red, slightly efflorescent crystals, or purple, odorless powder. Soluble in water; insoluble in alcohol.

Mannitol: White, crystalline powder or free-flowing granules. Is odorless and has a sweet taste. Freely soluble in water; soluble in alkaline solutions; slightly soluble in pyridine; very slightly soluble in alcohol; practically insoluble in ether. *NF category:* Sweetening agent; tablet and/or capsule diluent; tonicity agent; bulking agent for freeze-drying.

Maprotiline Hydrochloride: Fine, white to off-white, crystalline powder. Is practically odorless. Freely soluble in methanol and in chloroform; slightly soluble in water; practically insoluble in isooctane.

Mazindol: White to off-white, crystalline powder, having not more than a faint odor. Slightly soluble in methanol and in chloroform; insoluble in water.

Measles Virus Vaccine Live: Solid having the characteristic appearance of substances dried from the frozen state. Undergoes loss of potency on exposure to sunlight. The Vaccine is to be constituted with a suitable diluent just prior to use.

Measles, Mumps, and Rubella Virus Vaccine Live: Solid having the characteristic appearance of substances dried from the frozen state. The Vaccine is to be constituted with a suitable diluent just prior to use. Constituted vaccine undergoes loss of potency on exposure to sunlight.

Measles and Rubella Virus Vaccine Live: Solid having the characteristic appearance of substances dried from the frozen state. The Vaccine is to be constituted with a suitable diluent just prior to use. Constituted vaccine undergoes loss of potency on exposure to sunlight.

Mebendazole: White to slightly yellow powder. Is almost odorless. Melts at about 290°. Freely soluble in formic acid; practically insoluble in water, in dilute solutions of mineral acids, in alcohol, in ether, and in chloroform.

Mechlorethamine Hydrochloride: White, crystalline powder. Is hygroscopic.

Meclizine Hydrochloride: White or slightly yellowish, crystalline powder. Has a slight odor and is tasteless. Slightly soluble in dilute acids and in alcohol; practically insoluble in water and in ether; freely soluble in chloroform, in pyridine, and in acid-alcohol-water mixtures.

Meclofenamate Sodium: A white to creamy white, odorless to almost odorless, crystalline powder. Freely soluble in water, the solution sometimes being somewhat turbid

due to partial hydrolysis and absorption of carbon dioxide; soluble in methanol; slightly soluble in chloroform; practically insoluble in ether. The solution is clear above pH 11.5.

Medroxyprogesterone Acetate: White to off-white, odorless, crystalline powder. Melts at about 205°. Is stable in air. Freely soluble in chloroform; soluble in acetone and in dioxane; sparingly soluble in alcohol and in methanol; slightly soluble in ether; insoluble in water.

Mefenamic Acid: White to off-white, crystalline powder. Melts at about 230°, with decomposition. Soluble in solutions of alkali hydroxides; sparingly soluble in chloroform; slightly soluble in alcohol and in methanol; practically insoluble in water.

Mefloquine Hydrochloride: White or slightly yellow, crystalline powder. It exhibits polymorphism. Freely soluble in methanol; soluble in alcohol; very slightly soluble in water.

Megestrol Acetate: White to creamy white, tasteless and essentially odorless, crystalline powder. Very soluble in chloroform; soluble in acetone; sparingly soluble in alcohol; slightly soluble in ether and in fixed oils; insoluble in water. Is unstable under aqueous conditions at pH 7 or above.

Meglumine: White to faintly yellowish-white, odorless crystals or powder. Freely soluble in water; sparingly soluble in alcohol.

Melengestrol Acetate: White to light yellow, crystalline powder. Freely soluble in chloroform and in ethyl acetate; slightly soluble in alcohol; insoluble in water.

Meloxicam: Pale yellow powder. Soluble in dimethylformamide; slightly soluble in acetone; very slightly soluble in methanol and in alcohol; practically insoluble in water.

Melphalan: Off-white to buff powder, having a faint odor. Melts at about 180°, with decomposition. Soluble in dilute mineral acids; slightly soluble in alcohol and in methanol; practically insoluble in water, in chloroform, and in ether.

Menadiol Sodium Diphosphate: White to pink powder, having a characteristic odor. Is hygroscopic. Its solutions are neutral or slightly alkaline to litmus, having a pH of about 8. Very soluble in water; insoluble in alcohol.

Menadione: Bright yellow, crystalline, practically odorless powder. Is affected by sunlight. Soluble in vegetable oils; sparingly soluble in chloroform and in alcohol; practically insoluble in water.

Menthol: Colorless, hexagonal crystals, usually needle-like, or in fused masses, or crystalline powder. Has a pleasant, peppermint-like odor. Very soluble in alcohol, in chloroform, in ether, and in solvent hexane; freely soluble in glacial acetic acid, in mineral oil, and in fixed and volatile oils; slightly soluble in water. *NF category:* Flavors and perfumes.

Meperidine Hydrochloride: Fine, white, crystalline, odorless powder. The pH of a solution (1 in 20) is about 5. Very soluble in water; soluble in alcohol; sparingly soluble in ether.

Mephobarbital: White, odorless, crystalline powder, having a bitter taste. Its saturated solution is acid to litmus. Soluble in chloroform and in solutions of fixed alkali hydroxides and carbonates; slightly soluble in water, in alcohol, and in ether.

Mepivacaine Hydrochloride: White, odorless, crystalline solid. The pH of a solution (1 in 50) is about 4.5. Freely soluble in water and in methanol; very slightly soluble in chloroform; practically insoluble in ether.

Meprobamate: White powder, having a characteristic odor and a bitter taste. Freely soluble in acetone and in alcohol; slightly soluble in water; practically insoluble or insoluble in ether.

Mercaptopurine: Yellow, odorless or practically odorless, crystalline powder. Melts at a temperature exceeding 308°, with decomposition. Soluble in hot alcohol and in

dilute alkali solutions; slightly soluble in 2 N sulfuric acid; insoluble in water, in acetone, and in ether.

Ammoniated Mercury: White, pulverulent pieces or white, amorphous powder. Is odorless, and is stable in air, but darkens on exposure to light. Readily soluble in warm hydrochloric, nitric, and acetic acids; insoluble in water, and in alcohol.

Meropenem: Colorless to white crystals. Soluble in dimethylformamide and in 5% dibasic potassium phosphate solution; sparingly soluble in water and in 5% monobasic potassium phosphate solution; very slightly soluble in alcohol; practically insoluble in acetone and in ether.

Mesalamine: Light tan to pink colored, needle-shaped crystals. Color may darken on exposure to air. Is odorless or may have a slight characteristic odor. Soluble in dilute hydrochloric acid and in dilute alkali hydroxides; slightly soluble in water; very slightly soluble in methanol, in dehydrated alcohol, and in acetone; practically insoluble in *n*-butyl alcohol, in chloroform, in ether, in ethyl acetate, in *n*-hexane, in methylene chloride, and in *n*-propyl alcohol.

Mesna: White or slightly yellow crystalline powder; hygroscopic. Freely soluble in water; slightly soluble in alcohol; practically insoluble in cyclohexane.

Mesoridazine Besylate: White to pale yellowish powder, having not more than a faint odor. Melts at about 178°, with decomposition. Freely soluble in water, in chloroform, and in methanol.

Mestranol: White to creamy white, odorless, crystalline powder. Freely soluble in chloroform; soluble in dioxane; sparingly soluble in dehydrated alcohol; slightly soluble in methanol; insoluble in water.

Metaproterenol Sulfate: White to off-white, crystalline powder. Freely soluble in water.

Metformin Hydrochloride: White, crystalline powder. Freely soluble in water; slightly soluble in alcohol; practically insoluble in acetone and in methylene chloride.

Methacholine Chloride: Colorless or white crystals, or white, crystalline powder. Is odorless or has a slight odor, and is very hygroscopic. Its solutions are neutral to litmus. Very soluble in water; freely soluble in alcohol and in chloroform.

Methacrylic Acid Copolymer: White powder having a faint, characteristic odor. The polymer is soluble in diluted alkali, in simulated intestinal fluid TS, and in buffer solutions of pH 7 and above. The solubility between pH 5.5 and pH 7 depends on the content of methacrylic acid units in the copolymer. The polymer is freely soluble to soluble in methanol, in alcohol, in isopropyl alcohol, and in acetone, each of which contains not less than 3% of water; insoluble in water, in diluted acids, in simulated gastric fluid TS, and in buffer solutions of up to pH 5. *NF category:* Coating agent.

Methacrylic Acid Copolymer Dispersion: Milky-white liquid of low viscosity. It is miscible with water in any proportion; the milky-white appearance is retained. A clear or slightly opalescent, viscous solution is obtained on mixing one part with five parts of acetone, alcohol, or isopropyl alcohol; the polymer substance is first precipitated, but then dissolves in the excess organic solvent. A clear or slightly opalescent, viscous solution is obtained on mixing one part with two parts of 1 N sodium hydroxide.

Methacrylic Acid and Ethyl Acrylate Copolymer: White powder having a faint, characteristic odor. Soluble to freely soluble in methanol, in alcohol, in isopropyl alcohol, and in acetone, each of which contains not less than 3% of water; soluble in diluted alkali, in simulated intestinal fluid TS, and in buffer solutions of pH 7 and above; insoluble in water, in diluted acids, in simulated gastric fluid TS, and in buffer solutions of up to pH 5. The solubility between pH 5.5 and pH 7 depends on the content of methacrylic acid units in the copolymer. *NF category:* Coating agent; film-forming agent.

Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer: White or almost white, free-flowing powder. Freely soluble in alcohol, in methanol, and in a 40 g/L solution of sodium hydroxide; soluble in solutions at pH values above pH 5.5 under salt formation; practically insoluble in ethyl acetate and in acidic aqueous solutions. *NF category:* Coating agent; film-forming agent.

Methacrylic Acid and Methyl Methacrylate Copolymer: White powder having a faint, characteristic odor. Soluble to freely soluble in methanol, in alcohol, in isopropyl alcohol, and in acetone, each of which contains not less than 3% of water; soluble in diluted alkali, in simulated intestinal fluid TS, and in buffer solutions of pH 7 and above; insoluble in water, in diluted acids, in simulated gastric fluid TS, and in buffer solutions of up to pH 5. The solubility between pH 5.5 and pH 7 depends on the content of methacrylic acid units in the copolymer. *NF category:* Coating agent; film-forming agent.

Methacycline Hydrochloride: Yellow to dark yellow, crystalline powder. Soluble in water.

Methadone Hydrochloride: Colorless crystals or white, crystalline, odorless powder. Freely soluble in alcohol and in chloroform; soluble in water; practically insoluble in ether and in glycerin.

Methadone Hydrochloride Oral Concentrate: Clear to slightly hazy, syrupy liquid.

Methamphetamine Hydrochloride: White crystals or white, crystalline powder. Is odorless or practically so. Its solutions have a pH of about 6. Freely soluble in water, in alcohol, and in chloroform; very slightly soluble in absolute ether.

Methazolamide: White or faintly yellow, crystalline powder having a slight odor. Melts at about 213°. Soluble in dimethylformamide; slightly soluble in acetone; very slightly soluble in water and in alcohol.

Methdilazine Hydrochloride: Light tan, crystalline powder, having a slight, characteristic odor. Freely soluble in water, in alcohol, and in chloroform.

Methenamine: Colorless, lustrous crystals or white, crystalline powder. Is practically odorless. When brought into contact with fire, it readily ignites, burning with a smokeless flame. It sublimes at about 260°, without melting. Its solutions are alkaline to litmus. Freely soluble in water; soluble in alcohol and in chloroform.

Methenamine Mandelate: White, crystalline powder. Has a sour taste and is practically odorless. Its solutions have a pH of about 4. Melts at about 127°, with decomposition. Very soluble in water; soluble in alcohol and in chloroform; slightly soluble in ether.

Methimazole: White to pale buff, crystalline powder, having a faint, characteristic odor. Its solutions are practically neutral to litmus. Freely soluble in water, in alcohol, and in chloroform; slightly soluble in ether.

Methionine: White crystals, having a characteristic odor and taste. Soluble in water, in warm dilute alcohol, and in dilute mineral acids; insoluble in ether, in absolute alcohol, in benzene, and in acetone (L-form).

Methocarbamol: White powder, odorless, or having a slight characteristic odor. Melts at about 94°, or, if previously ground to a fine powder, melts at about 90°. Soluble in alcohol only with heating; sparingly soluble in water and in chloroform; insoluble in benzene and in *n*-hexane.

Methohexital: White to faintly yellowish-white, crystalline, odorless powder. Slightly soluble in alcohol, in chloroform, and in dilute alkalies; very slightly soluble in water.

Methohexital Sodium for Injection: White to off-white, hygroscopic powder. Is essentially odorless.

Methotrexate: Orange-brown, or yellow, crystalline powder. Freely soluble in dilute solutions of alkali hydroxides and carbonates; slightly soluble in 6 N hydrochloric acid;

practically insoluble in water, in alcohol, in chloroform, and in ether.

Methotrimeprazine: Fine, white, practically odorless, crystalline powder. Melts at about 126°. Freely soluble in chloroform, in ether, and in boiling alcohol; sparingly soluble in methanol and in alcohol at 25°; practically insoluble in water.

Methoxsalen: White to cream-colored, fluffy, needle-like crystals. Is odorless. Freely soluble in chloroform; soluble in boiling alcohol, in acetone, in acetic acid, in propylene glycol, and in benzene; sparingly soluble in boiling water and in ether; practically insoluble in water.

Methoxsalen Topical Solution: Clear, colorless liquid.

Methoxyflurane: Clear, practically colorless, mobile liquid, having a characteristic odor. Boils at about 105°. Miscible with alcohol, with acetone, with chloroform, with ether, and with fixed oils.

Methsuximide: White to grayish white, crystalline powder. Is odorless, or has not more than a slight odor. Very soluble in chloroform; freely soluble in alcohol and in ether; slightly soluble in hot water.

Methylclothiazide: White or practically white, crystalline powder. Is odorless, or has a slight odor. Freely soluble in acetone and in pyridine; sparingly soluble in methanol; slightly soluble in alcohol; very slightly soluble in water, in chloroform, and in benzene.

Methyl Alcohol: Clear, colorless liquid, having a characteristic odor. Is flammable. Miscible with water, with alcohol, with ether, with benzene, and with most other organic solvents. *NF category:* Solvent.

Methyl Benzylidene Camphor: A white, fine crystalline powder. Very soluble in chloroform; freely soluble in alcohol; practically insoluble in water.

Methyl Isobutyl Ketone: Transparent, colorless, mobile, volatile liquid, having a faint ketonic and camphoraceous odor. Slightly soluble in water. Miscible with alcohol, with ether, and with benzene. *NF category:* Alcohol denaturant; solvent.

Methyl Salicylate: Colorless, yellowish, or reddish liquid, having the characteristic odor and taste of wintergreen. It boils between 219° and 224°, with some decomposition. Soluble in alcohol and in glacial acetic acid; slightly soluble in water. *NF category:* Flavors and perfumes.

Methylbenzethonium Chloride: White, hygroscopic crystals, having a mild odor. Its solutions are neutral or slightly alkaline to litmus. Very soluble in water, in alcohol, and in ether; practically insoluble in chloroform.

Methylcellulose: White, fibrous powder or granules. Its aqueous suspensions are neutral to litmus. It swells in water and produces a clear to opalescent, viscous, colloidal suspension. Soluble in glacial acetic acid and in a mixture of equal volumes of alcohol and chloroform; insoluble in alcohol, in ether, and in chloroform. *NF category:* Coating agent; suspending and/or viscosity-increasing agent; tablet binder.

Methyldopa: White to yellowish-white, odorless, fine powder, which may contain friable lumps. Very soluble in 3 N hydrochloric acid; sparingly soluble in water; slightly soluble in alcohol; practically insoluble in ether.

Methyldopate Hydrochloride: White or practically white, odorless or practically odorless, crystalline powder. Freely soluble in water, in alcohol, and in methanol; slightly soluble in chloroform; practically insoluble in ether.

Methylene Blue: Dark green crystals or crystalline powder having a bronze-like luster. Is odorless or practically so, and is stable in air. Its solutions in water and in alcohol are deep blue in color. Soluble in water and in chloroform; sparingly soluble in alcohol.

Methylene Chloride: Clear, colorless, mobile liquid, having an odor resembling that of chloroform. Miscible with

alcohol, with ether, and with fixed and volatile oils. *NF category:* Solvent.

Methylergonovine Maleate: White to pinkish-tan, microcrystalline powder. Is odorless. Slightly soluble in water and in alcohol; very slightly soluble in chloroform and in ether.

Methylparaben: White, crystalline powder or colorless crystals. Freely soluble in alcohol and in methanol; slightly soluble in water. *NF category:* Antimicrobial preservative.

Methylparaben Sodium: White, hygroscopic powder. Freely soluble in water; sparingly soluble in alcohol; insoluble in fixed oils. *NF category:* Antimicrobial preservative.

Methylphenidate Hydrochloride: White, odorless, fine, crystalline powder. Its solutions are acid to litmus. Freely soluble in water and in methanol; soluble in alcohol; slightly soluble in chloroform and in acetone.

Methylprednisolone: White to practically white, odorless, crystalline powder. Melts at about 240°, with some decomposition (see *Melting Range or Temperature* <741>). Sparingly soluble in alcohol, in dioxane, and in methanol; slightly soluble in acetone and in chloroform; very slightly soluble in ether; practically insoluble in water.

Methylprednisolone Acetate: White or practically white, odorless, crystalline powder. Melts at about 225°, with some decomposition (see *Melting Range or Temperature* <741>). Soluble in dioxane; sparingly soluble in acetone, in alcohol, in chloroform, and in methanol; slightly soluble in ether; practically insoluble in water.

Methylprednisolone Hemisuccinate: White or nearly white, odorless or nearly odorless, hygroscopic solid. Freely soluble in alcohol; soluble in acetone; very slightly soluble in water.

Methylprednisolone Sodium Succinate: White or nearly white, odorless, hygroscopic, amorphous solid. Very soluble in water and in alcohol; very slightly soluble in acetone; insoluble in chloroform.

Methylpyrrolidone: A clear, colorless liquid. Miscible with water and with most organic solvents including alcohol, ketones, and aromatic and chlorinated hydrocarbons. Boiling point: about 202°. Refractive index: about 1.469. *NF category:* Solvent.

Methylsulfonylmethane: White powder or flake crystal. Melts at about 109°. Freely soluble in water, in methanol, in alcohol, and in acetone; sparingly soluble in ether.

Methyltestosterone: White or creamy white crystals or crystalline powder. Is odorless and is stable in air, but is slightly hygroscopic. Is affected by light. Soluble in alcohol, in methanol, in ether, and in other organic solvents; sparingly soluble in vegetable oils; practically insoluble in water.

Methysergide Maleate: White to yellowish-white or reddish-white, crystalline powder. Is odorless or has not more than a slight odor. Slightly soluble in water and in alcohol; very slightly soluble in chloroform; practically insoluble in ether.

Metoclopramide Hydrochloride: White or practically white, crystalline, odorless or practically odorless powder. Very soluble in water; freely soluble in alcohol; sparingly soluble in chloroform; practically insoluble in ether.

Metoprolol Succinate: White to off-white powder. Freely soluble in water; soluble in methanol; sparingly soluble in alcohol; slightly soluble in isopropyl alcohol.

Metoprolol Tartrate: White, crystalline powder. Very soluble in water; freely soluble in methylene chloride, in chloroform, and in alcohol; slightly soluble in acetone; insoluble in ether.

Metronidazole: White to pale yellow, odorless crystals or crystalline powder. Is stable in air, but darkens on exposure to light. Soluble in dilute hydrochloric acid (1 in 2); sparingly soluble in water and in alcohol; slightly soluble in ether and in chloroform.

Metronidazole Benzoate: White to slightly yellow, crystalline powder. Freely soluble in methylene chloride; soluble in acetone; slightly soluble in alcohol; very slightly soluble in ethyl ether; practically insoluble in water.

Metyrapone: White to light amber, fine, crystalline powder, having a characteristic odor. Darkens on exposure to light. Soluble in methanol and in chloroform; sparingly soluble in water. It forms water-soluble salts with acids.

Mexiletine Hydrochloride: White powder. Freely soluble in dehydrated alcohol and in water; slightly soluble in acetonitrile; practically insoluble in ether. Optically inactive (1 in 20 solution in water).

Mezlocillin Sodium: White to pale yellow, crystalline powder. Freely soluble in water.

Mibolerone: White to off-white powder. Slightly soluble in chloroform, in dioxane, and in methylene chloride; practically insoluble in water (0.0454 mg per mL at 37°).

Miconazole: White to pale cream powder. Melts in the range of 78° to 88°. May exhibit polymorphism. Freely soluble in alcohol, in methanol, in isopropyl alcohol, in acetone, in propylene glycol, in chloroform, and in dimethylformamide; soluble in ether; insoluble in water.

Miconazole Nitrate: White or practically white, crystalline powder, having not more than a slight odor. Melts in the range of 178° to 183°, with decomposition. Freely soluble in dimethyl sulfoxide; soluble in dimethylformamide; sparingly soluble in methanol; slightly soluble in alcohol, in chloroform, and in propylene glycol; very slightly soluble in water and in isopropyl alcohol; insoluble in ether.

Midazolam: White or yellowish powder. The hydrochloride salt of midazolam is soluble in aqueous solutions. Insoluble in water.

Midodrine Hydrochloride: White crystalline powder. Soluble in water; sparingly soluble in methanol.

Milrinone: White to tan, crystalline solid. Is hygroscopic. Freely soluble in dimethyl sulfoxide; very slightly soluble in methanol; practically insoluble in water and in chloroform.

Mineral Oil: Colorless, transparent, oily liquid, free or practically free from fluorescence. Is odorless and tasteless when cold, and develops not more than a faint odor of petroleum when heated. Soluble in volatile oils; insoluble in water and in alcohol. Miscible with most fixed oils but not with castor oil. *NF category:* Solvent; vehicle (oleaginous).

Light Mineral Oil: Colorless, transparent, oily liquid, free, or practically free, from fluorescence. Is odorless and tasteless when cold, and develops not more than a faint odor of petroleum when heated. Soluble in volatile oils; insoluble in water and in alcohol. Miscible with most fixed oils, but not with castor oil. *NF category:* Tablet and/or capsule lubricant; vehicle (oleaginous).

Minocycline Hydrochloride: Yellow, crystalline powder. Soluble in solutions of alkali hydroxides and carbonates; sparingly soluble in water; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

Minoxidil: White to off-white, crystalline powder. Melts in the approximate range of between 248° and 268°, with decomposition. Soluble in alcohol and in propylene glycol; sparingly soluble in methanol; slightly soluble in water; practically insoluble in chloroform, in acetone, in ethyl acetate, and in hexane.

Mirtazapine: White to creamy white, crystalline powder. Freely soluble in methanol and in toluene; soluble in ethyl ether; sparingly soluble in *n*-hexane; practically insoluble in water.

Misoprostol: Clear, colorless or light yellow viscous liquid. Very slightly soluble in water.

Mitomycin: Blue-violet, crystalline powder. Soluble in acetone, in methanol, in butyl acetate, and in cyclohexanone; slightly soluble in water.

Mitotane: White, crystalline powder, having a slight, aromatic odor. Soluble in alcohol, in ether, in solvent hexane, and in fixed oils and fats; practically insoluble in water.

Mitoxantrone Hydrochloride: Dark blue powder. Sparingly soluble in water; slightly soluble in methanol; practically insoluble in acetone, in acetonitrile, and in chloroform.

Modafinil: White to off-white, crystalline powder. Sparingly soluble in methanol; slightly soluble in absolute alcohol; very slightly soluble in water.

Mometasone Furoate: White to off-white powder. Melts at about 220°, with decomposition. Soluble in acetone and in methylene chloride.

Monensin Sodium: Off-white to tan, crystalline powder. Soluble in chloroform and in methanol; slightly soluble in water; practically insoluble in solvent hexane.

Mono- and Di-glycerides: Vary in consistency from yellow liquids, through ivory-colored plastics, to ivory white-colored solids (bead or flake forms). Soluble in alcohol, in ethyl acetate, in chloroform, and in other chlorinated hydrocarbons; insoluble in water. *NF category:* Emulsifying and/or solubilizing agent.

Monobenzene Ointment: Dispersible with, but not soluble in, water.

Monoethanolamine: Clear, colorless, moderately viscous liquid, having a distinctly ammoniacal odor. Miscible with water, with acetone, with alcohol, with glycerin, and with chloroform. Immiscible with ether, with solvent hexane, and with fixed oils, although it dissolves many essential oils. *NF category:* Emulsifying and/or solubilizing agent.

Monoglyceride Citrate: Soft white to ivory-colored, waxy solid with a lard-like consistency and bland odor. Dispersible in most common fat solvents and in alcohol. Insoluble in water.

Monosodium Glutamate: White, practically odorless, free-flowing crystals or crystalline powder. Freely soluble in water; sparingly soluble in alcohol. May have either a slightly sweet or a slightly salty taste. *NF category:* Flavors and perfumes.

Monothioglycerol: Colorless or pale yellow, viscous liquid, having a slight sulfidic odor. Is hygroscopic. Freely soluble in water; insoluble in ether. Miscible with alcohol. *NF category:* Antioxidant.

Montelukast Sodium: White or almost white, hygroscopic powder. Freely soluble to very soluble in alcohol; freely soluble in water and in methylene chloride.

Morantel Tartrate: A white or pale yellow, crystalline powder. Very soluble in water and in alcohol; practically insoluble in ethyl acetate.

Moricizine Hydrochloride: White to off-white, crystalline powder. Melts at about 189°, with decomposition. Soluble in water and in alcohol.

Morphine Sulfate: White, feathery, silky crystals, cubical masses of crystals, or white, crystalline powder. Is odorless, and when exposed to air it gradually loses water of hydration. Darkens on prolonged exposure to light. Freely soluble in hot water; soluble in water; slightly soluble in alcohol but more so in hot alcohol; insoluble in chloroform and in ether.

Moxifloxacin Hydrochloride: Slightly yellow to yellow powder or crystals. Soluble in 0.1 N sodium hydroxide; sparingly soluble in water and in methanol; slightly soluble in 0.1 N hydrochloric acid, in dimethylformamide, and in alcohol; practically insoluble in methylene chloride, in acetone, in ethyl acetate, and in toluene; insoluble in *tert*-butyl methyl ether and *n*-heptane.

Mumps Skin Test Antigen: Slightly turbid liquid.

Mumps Virus Vaccine Live: Solid having the characteristic appearance of substances dried from the frozen state. The Vaccine is to be constituted with a suitable diluent just prior to use. Constituted vaccine undergoes loss of potency on exposure to sunlight.

Mupirocin: White to off-white, crystalline solid. Freely soluble in acetone, in chloroform, in dehydrated alcohol, and in methanol; slightly soluble in ether; very slightly soluble in water.

Mycophenolate Mofetil: White or almost white, crystalline powder. Its melting range is between 94° and 98°. Freely soluble in acetone; soluble in methanol; sparingly soluble in dehydrated alcohol; slightly soluble in water.

Myristic Acid: Hard, white or faintly yellow, somewhat glossy, crystalline solid or white or yellow-white powder. Soluble in alcohol, in chloroform, and in ether; practically insoluble in water. *NF category:* Antifoaming agent.

Nabumetone: A white, or almost white, crystalline powder. Freely soluble in acetone; sparingly soluble in alcohol and in methanol; practically insoluble in water.

Nadolol: White to off-white, practically odorless, crystalline powder. Freely soluble in alcohol and in methanol; soluble in water at pH 2; slightly soluble in chloroform, in methylene chloride, in isopropyl alcohol, and in water (between pH 7 and pH 10); insoluble in acetone, in benzene, in ether, in hexane, and in trichloroethane.

Nafcillin Sodium: White to yellowish-white powder, having not more than a slight characteristic odor. Freely soluble in water and in chloroform; soluble in alcohol.

Nalidixic Acid: White to very pale yellow, odorless, crystalline powder. Soluble in chloroform, in methylene chloride, and in solutions of fixed alkali hydroxides and carbonates; slightly soluble in acetone, in alcohol, in methanol, and in toluene; very slightly soluble in ether and in water.

Naloxone Hydrochloride: White to slightly off-white powder. Its aqueous solution is acidic. Soluble in water, in dilute acids, and in strong alkali; slightly soluble in alcohol; practically insoluble in ether and in chloroform.

Naloxone Hydrochloride Injection: Clear, colorless liquid.

Nandrolone Decanoate: Fine, white to creamy white, crystalline powder. Is odorless, or may have a slight odor. Soluble in chloroform, in alcohol, in acetone, and in vegetable oils; practically insoluble in water.

Naphazoline Hydrochloride: White, crystalline powder. Is odorless and has a bitter taste. Melts at a temperature of about 255°, with decomposition. Freely soluble in water and in alcohol; very slightly soluble in chloroform; practically insoluble in ether.

Naproxen: White to off-white, practically odorless, crystalline powder. Soluble in chloroform, in dehydrated alcohol, and in alcohol; sparingly soluble in ether; practically insoluble in water.

Naproxen Sodium: White to creamy crystalline powder. Soluble in water and in methanol; sparingly soluble in alcohol; very slightly soluble in acetone; and practically insoluble in chloroform and in toluene. Melts at about 255°, with decomposition.

Narasin: White to off-white, crystalline powder. Melts at about 217°, with decomposition. Soluble in methanol and in water.

Naratriptan Hydrochloride: White to pale yellow solid. Soluble in water.

Natamycin: Off-white to cream-colored powder, which may contain up to 3 moles of water. Soluble in glacial acetic acid and in dimethylformamide; slightly soluble in methanol; practically insoluble in water.

Nateglinide: White powder. Freely soluble in methanol and in alcohol; soluble in ether; sparingly soluble in acetone, in toluene, and in octanol; practically insoluble in water.

Nefazodone Hydrochloride: Nonhygroscopic, white powder. Freely soluble in chloroform; soluble in propylene glycol; slightly soluble in polyethylene glycol and in water.

Neomycin Sulfate: White to slightly yellow powder, or cryodesiccated solid. Is odorless or practically so and is hygroscopic. Its solutions are dextrorotatory. Freely soluble in

water; very slightly soluble in alcohol; insoluble in acetone, in chloroform, and in ether.

Netilmicin Sulfate: White to pale yellowish-white powder. Freely soluble in water; practically insoluble in dehydrated alcohol and in ether.

Nevirapine: White to off-white, odorless to nearly odorless, crystalline powder. Slightly soluble in alcohol and in methanol; practically insoluble in water. Hydrus form also slightly soluble in propylene glycol.

Niacin: White crystals or crystalline powder. Is odorless, or has a slight odor. Melts at about 235°. Freely soluble in boiling water, in boiling alcohol, and in solutions of alkali hydroxides and carbonates; sparingly soluble in water; practically insoluble in ether.

Niacinamide: White, crystalline powder. Is odorless or practically so, and has a bitter taste. Its solutions are neutral to litmus. Freely soluble in water and in alcohol; soluble in glycerin.

Nifedipine: Yellow powder. Is affected by exposure to light. Freely soluble in acetone; practically insoluble in water.

Nimodipine: Light yellow or yellow, crystalline powder, affected by light. Freely soluble in ethyl acetate; sparingly soluble in alcohol; practically insoluble in water. Exhibits polymorphism.

Nitric Acid: Highly corrosive fuming liquid, having a characteristic, highly irritating odor. Stains animal tissues yellow. Boils at about 120°. Specific gravity is about 1.41. *NF category:* Acidifying agent.

Nitrofurantoin: Lemon-yellow, odorless crystals or fine powder. Has a bitter aftertaste. Soluble in dimethylformamide; very slightly soluble in water and in alcohol.

Nitrofurazone: Lemon yellow, odorless, crystalline powder. Darkens slowly on exposure to light. Melts at about 236°, with decomposition. Soluble in dimethylformamide; slightly soluble in propylene glycol and in polyethylene glycol mixtures; very slightly soluble in alcohol and in water; practically insoluble in chloroform and in ether.

Nitrofurazone Ointment: Yellow, opaque, and water-miscible, and has ointment-like consistency.

Nitrofurazone Topical Solution: Light yellow, clear, somewhat viscous liquid, having a faint characteristic odor. Miscible with water.

Nitrogen: Colorless, odorless, tasteless gas. Is nonflammable and does not support combustion. One L at 0° and at a pressure of 760 mm of mercury weighs about 1.251 g. One volume dissolves in about 65 volumes of water and in about 9 volumes of alcohol at 20° and at a pressure of 760 mm of mercury. *NF category:* Air displacement.

Diluted Nitroglycerin: When diluted with lactose, it is a white, odorless powder. When diluted with propylene glycol or alcohol, it is a clear, colorless, or pale yellow liquid. [NOTE—Undiluted nitroglycerin occurs as a white to pale yellow, thick, flammable, explosive liquid.] Undiluted nitroglycerin is soluble in methanol, in alcohol, in carbon disulfide, in acetone, in ethyl ether, in ethyl acetate, in glacial acetic acid, in benzene, in toluene, in nitrobenzene, in phenol, in chloroform, and in methylene chloride; slightly soluble in water.

Nitromersol: Brownish yellow to yellow granules or brownish yellow to yellow powder. Is odorless and tasteless and is affected by light. Soluble in solutions of alkalis and of ammonia by opening of the anhydride ring and the formation of a salt; very slightly soluble in water, in alcohol, in acetone, and in ether.

Nitromersol Topical Solution: Clear, reddish-orange solution. Is affected by light.

Nitrous Oxide: Colorless gas, without appreciable odor or taste. One L at 0° and at a pressure of 760 mm of mercury weighs about 1.97 g. One volume dissolves in about 1.4 volumes of water at 20° and at a pressure of 760 mm of

mercury. Freely soluble in alcohol; soluble in ether and in oils.

Nizatidine: Off-white to buff crystalline solid. Freely soluble in chloroform; soluble in methanol; sparingly soluble in water.

Nonoxynol 9: Clear, colorless to light yellow, viscous liquid. Soluble in water, in alcohol, and in corn oil. *NF category:* Wetting and/or solubilizing agent.

Norepinephrine Bitartrate: White or faintly gray, odorless, crystalline powder. Slowly darkens on exposure to air and light. Its solutions are acid to litmus, having a pH of about 3.5. Freely soluble in water; slightly soluble in alcohol; practically insoluble in chloroform and in ether. Melts between 98° and 104°, without previous drying of the specimen, the melt being turbid.

Norepinephrine Bitartrate Injection: Colorless or practically colorless liquid, gradually turning dark on exposure to air and light.

Norethindrone: White to creamy white, odorless, crystalline powder. Is stable in air. Soluble in chloroform and in dioxane; sparingly soluble in alcohol; slightly soluble in ether; practically insoluble in water.

Norethindrone Acetate: White to creamy white, odorless, crystalline powder. Very soluble in chloroform; freely soluble in dioxane; soluble in ether and in alcohol; practically insoluble in water.

Norfloxacin: White to pale yellow, crystalline powder. Sensitive to light and moisture. Freely soluble in acetic acid; sparingly soluble in chloroform; slightly soluble in acetone, in water, and in alcohol; very slightly soluble in methanol and in ethyl acetate; insoluble in ether.

Norgestimate: White to pale yellow powder. Very to freely soluble in methylene chloride; sparingly soluble in acetonitrile; insoluble in water.

Norgestrel: White or practically white, practically odorless, crystalline powder. Freely soluble in chloroform; sparingly soluble in alcohol; insoluble in water.

Nortriptyline Hydrochloride: White to off-white powder, having a slight, characteristic odor. Its solution (1 in 100) has a pH of about 5. Soluble in water and in chloroform; sparingly soluble in methanol; practically insoluble in ether, in benzene, and in most other organic solvents.

Noscapine: Fine, white or practically white, crystalline powder. Freely soluble in chloroform; soluble in acetone; slightly soluble in alcohol and in ether; practically insoluble in water.

Novobiocin Calcium: White or yellowish-white, odorless, crystalline powder. Freely soluble in alcohol and in methanol; sparingly soluble in acetone and in butyl acetate; slightly soluble in water and in ether; very slightly soluble in chloroform.

Novobiocin Sodium: White or yellowish-white, odorless, hygroscopic, crystalline powder. Freely soluble in water, in alcohol, in methanol, in glycerin, and in propylene glycol; slightly soluble in butyl acetate; practically insoluble in acetone, in chloroform, and in ether.

Nystatin: Yellow to light tan powder, having an odor suggestive of cereals. Is hygroscopic, and is affected by long exposure to light, heat, and air. Freely soluble in dimethylformamide and in dimethyl sulfoxide; sparingly to slightly soluble in methanol, in *n*-propyl alcohol, and in *n*-butyl alcohol; practically insoluble in water and in alcohol; insoluble in chloroform and in ether.

Octoxynol 9: Clear, pale yellow, viscous liquid, having a faint odor and a bitter taste. Soluble in benzene and in toluene; practically insoluble in solvent hexane. Miscible with water, with alcohol, and with acetone. *NF category:* Wetting and/or solubilizing agent.

Octyldodecanol: Clear water-white, free-flowing liquid. Soluble in alcohol and in ether; insoluble in water. *NF category:* Vehicle (oleaginous).

Octyl Methoxycinnamate: Pale yellow oil. Insoluble in water.

Ofloxacin: Pale yellowish-white to light yellowish-white crystals or crystalline powder. Sparingly soluble in chloroform; slightly soluble in alcohol, in methanol, and in water.

Hydrophilic Ointment: *NF category:* Ointment base.

White Ointment: *NF category:* Ointment base.

Yellow Ointment: *NF category:* Ointment base.

Olanzapine: A yellow crystalline solid. Soluble in *n*-propanol; sparingly soluble in acetonitrile; slightly soluble in methanol and in dehydrated alcohol; practically insoluble in water.

Oleic Acid: Colorless to pale yellow, oily liquid when freshly prepared, but on exposure to air it gradually absorbs oxygen and darkens. Has a characteristic, lard-like odor and taste. When strongly heated in air, it is decomposed with the production of acid vapors. Practically insoluble in water. Miscible with alcohol, with chloroform, with ether, with benzene, and with fixed and volatile oils. *NF category:* Emulsifying and/or solubilizing agent.

Oleovitamin A and D: Yellow to red, oily liquid, practically odorless or having a fish-like odor, and having no rancid odor or taste. Is a clear liquid at temperatures exceeding 65°, and may crystallize on cooling. Is unstable in air and in light. Very soluble in ether and in chloroform; soluble in dehydrated alcohol and in vegetable oils; insoluble in water and in glycerin.

Oleovitamin A and D Capsules: The oil contained in Oleovitamin A and D Capsules is a yellow to red, oily liquid, practically odorless or having a fish-like odor, and having no rancid odor or taste. Is a clear liquid at temperatures exceeding 65°, and may crystallize on cooling. Is unstable in air and in light.

Oleoyl Polyoxylglycerides: Amber, oily liquids. May develop deposit after prolonged storage at 20°. Freely soluble in methylene chloride; practically insoluble but dispersible in water. *NF category:* Ointment base; solvent.

Oleyl Alcohol: Clear, colorless to light yellow, oily liquid. Has a faint characteristic odor and a bland taste. Soluble in alcohol, in ether, in isopropyl alcohol, and in light mineral oil; insoluble in water. *NF category:* Emulsifying and/or solubilizing agent.

Oleyl Oleate: Clear, colorless to light yellow liquid. Has a faint characteristic odor. Slightly soluble in alcohol. Miscible with chloroform and with ether. *NF category:* Emollient; emulsifying and/or solubilizing agent.

Olive Oil: Pale yellow, or light greenish-yellow, oily liquid, having a slight, characteristic odor and taste, with a faintly acrid aftertaste. Slightly soluble in alcohol. Miscible with ether, with chloroform, and with carbon disulfide. *Specific gravity* (841): Between 0.910 and 0.915. *NF category:* Vehicle (oleaginous).

Olmesartan Medoxomil: White to off-white crystalline powder. Sparingly soluble in methanol; practically insoluble in water.

Olopatadine Hydrochloride: White crystalline powder. Very soluble in formic acid; sparingly soluble in water; very slightly soluble in dehydrated alcohol.

Omeprazole: White to off-white powder. Melts between 150° and 160°, with decomposition. Soluble in dichloromethane; sparingly soluble in methanol and in alcohol; very slightly soluble in water.

Omeprazole Magnesium: White to off-white powder. Sparingly soluble in methanol; slightly soluble in alcohol; very slightly soluble in water and in dichloromethane.

Ondansetron: White to off-white powder. Very soluble in acid solutions; sparingly soluble in water.

Ondansetron Hydrochloride: White to off-white powder. Soluble in methanol; sparingly soluble in water and in alcohol; slightly soluble in isopropyl alcohol and in dichloro-

methane; very slightly soluble in acetone, in chloroform, and in ethyl acetate.

Opium: Has a very characteristic odor and a very bitter taste.

Powdered Opium: Light brown or moderately yellowish-brown powder.

Orbifloxacin: White to pale yellow crystals or crystalline powder. Odorless. Soluble in acetic acid; very slightly soluble in methanol, in water, and in chloroform; practically insoluble in ethanol and in diethyl ether.

Orlistat: White to off-white fine powder or fine powder with lumps. Freely soluble in chloroform; very soluble in methanol and in alcohol; practically insoluble in water.

Orphenadrine Citrate: White, practically odorless, crystalline powder, having a bitter taste. Sparingly soluble in water; slightly soluble in alcohol; insoluble in chloroform, in benzene, and in ether.

Osetamivir Phosphate: White to off-white powder. Freely soluble in water; soluble in methanol, in dimethyl sulfoxide, and in propylene glycol; sparingly soluble in dimethylformamide; slightly soluble in alcohol; very slightly soluble in isopropyl alcohol and in polyethylene glycol 400; practically insoluble in acetonitrile, in acetone, in dichloromethane, and in *n*-hexane.

Oxacillin Sodium: Fine, white, crystalline powder, odorless or having a slight odor. Freely soluble in water, in methanol, and in dimethyl sulfoxide; slightly soluble in absolute alcohol, in chloroform, in pyridine, and in methyl acetate; insoluble in ethyl acetate, in ether, in benzene, and in ethylene chloride.

Oxacillin Sodium for Injection: Fine, white, crystalline powder, odorless or having a slight odor. Freely soluble in water, in methanol, and in dimethyl sulfoxide; slightly soluble in absolute alcohol, in chloroform, in pyridine, and in methyl acetate; insoluble in ethyl acetate, in ether, in benzene, and in ethylene chloride.

Oxaliplatin: White to off-white crystalline powder. Slightly soluble in water; very slightly soluble in methanol; practically insoluble in alcohol.

Oxandrolone: White, odorless, crystalline powder. Is stable in air, but darkens on exposure to light. Melts at about 225°. Freely soluble in chloroform; sparingly soluble in alcohol and in acetone; practically insoluble in water.

Oxaprozin: White to yellowish-white, crystalline powder.

Oxazepam: Creamy white to pale yellow powder. Is practically odorless. Slightly soluble in alcohol and in chloroform; very slightly soluble in ether; practically insoluble in water.

Oxcarbazepine: Light orange to creamish white or off-white powder. Soluble in acetic acid; sparingly soluble in chloroform; practically insoluble in water.

Oxfendazole: White or almost white powder. Slightly soluble in alcohol and in methylene chloride; practically insoluble in water.

Oxprenolol Hydrochloride: White, crystalline powder. Freely soluble in alcohol, in chloroform, and in water; sparingly soluble in acetone; practically insoluble in ether.

Oxtriphylline: White, crystalline powder, having an amine-like odor. A solution (1 in 100) has a pH of about 10.3. Freely soluble in water and in alcohol; very slightly soluble in chloroform.

Oxybenzone: Pale yellow powder. Freely soluble in alcohol and in toluene; practically insoluble in water.

Oxybutynin Chloride: White, crystalline, practically odorless powder. Very soluble in methanol and in chloroform; freely soluble in water and in alcohol; soluble in acetone; slightly soluble in ether; very slightly soluble in hexane.

Oxycodone Hydrochloride: White to off-white, hygroscopic crystals or powder. Is odorless. Soluble in water; slightly soluble in alcohol.

Oxygen: Colorless, odorless, tasteless gas, which supports combustion more energetically than does air. One L at 0° and at a pressure of 760 mm of mercury weighs about 1.429 g. One volume dissolves in about 32 volumes of water and in about 7 volumes of alcohol at 20° and at a pressure of 760 mm of mercury.

Oxymetazoline Hydrochloride: White to practically white, fine crystalline powder. Is hygroscopic. Melts at about 300°, with decomposition. Soluble in water and in alcohol; practically insoluble in benzene, in chloroform, and in ether.

Oxymetholone: White to creamy white, crystalline powder. Is odorless, and is stable in air. Freely soluble in chloroform; soluble in dioxane; sparingly soluble in alcohol; slightly soluble in ether; practically insoluble in water.

Oxymorphone Hydrochloride: White or slightly off-white, odorless powder. Darkens on exposure to light. Its aqueous solutions are slightly acidic. Freely soluble in water; sparingly soluble in alcohol and in ether.

Oxyquinoline Sulfate: Yellow powder. Melts at about 185°. Very soluble in water; freely soluble in methanol; slightly soluble in alcohol; practically insoluble in acetone and in ether. *NF category:* Complexing agent.

Oxytetracycline: Pale yellow to tan, odorless, crystalline powder. Is stable in air, but exposure to strong sunlight causes it to darken. It loses potency in solutions of pH below 2, and is rapidly destroyed by alkali hydroxide solutions. Freely soluble in 3 N hydrochloric acid and in alkaline solutions; sparingly soluble in alcohol; very slightly soluble in water.

Oxytetracycline Calcium: Yellow to light brown, crystalline powder. Insoluble in water.

Oxytetracycline Hydrochloride: Yellow, odorless, crystalline powder, having a bitter taste. Is hygroscopic. Decomposes at a temperature exceeding 180°, and exposure to strong sunlight or to temperatures exceeding 90° in moist air causes it to darken. Its potency is diminished in solutions having a pH below 2, and is rapidly destroyed by alkali hydroxide solutions. Freely soluble in water, but crystals of oxytetracycline base separate as a result of partial hydrolysis of the hydrochloride; sparingly soluble in alcohol and in methanol, and even less soluble in dehydrated alcohol; insoluble in chloroform and in ether.

Paclitaxel: White to off-white powder. Soluble in alcohol; insoluble in water.

Padimate O: A light yellow, mobile liquid having a faint, aromatic odor. Soluble in alcohol, in isopropyl alcohol, and in mineral oil; practically insoluble in water, in glycerin, and in propylene glycol.

Palm Oil: White to yellowish, fatty solid to semisolid. Insoluble in water. *NF category:* Coating agent; emulsifying and/or solubilizing agent.

Hydrogenated Palm Oil: White to yellowish, fatty solid to semi-solid. Freely soluble in ether; very slightly soluble in alcohol; practically insoluble in water. *NF category:* Coating agent; tablet binder; tablet and/or capsule lubricant.

Palm Kernel Oil: White to yellowish, fatty solid. Insoluble in water. *NF category:* Coating agent; emulsifying and/or solubilizing agent.

Palmitic Acid: Hard, white or faintly yellow, somewhat glossy crystalline solid, or white or yellowish-white powder. It has a slight characteristic odor and taste. Soluble in alcohol, in ether, and in chloroform; practically insoluble in water.

Pamidronate Disodium: White, crystalline powder. Soluble in water and in 2 N sodium hydroxide; sparingly soluble in 0.1 N hydrochloric acid and in 0.1 N acetic acid; practically insoluble in organic solvents.

Pancreatin: Cream-colored, amorphous powder, having a faint, characteristic, but not offensive odor. It hydrolyzes fats to glycerol and fatty acids, changes protein into proteoses and derived substances, and converts starch into dextrins and sugars. Its greatest activities are in neutral or faintly alkaline media; more than traces of mineral acids or large amounts of alkali hydroxides make it inert. An excess of alkali carbonate also inhibits its action.

Pancrelipase: Cream-colored, amorphous powder, having a faint, characteristic, but not offensive odor. Pancrelipase hydrolyzes fats to glycerol and fatty acids, changes protein into proteoses and derived substances, and converts starch into dextrins and sugars. Its greatest activities are in neutral or faintly alkaline media; more than traces of mineral acids or large amounts of alkali hydroxides make it inert. An excess of alkali carbonate also inhibits its action.

Pancrelipase Capsules: The contents of Capsules conform to the *Description* under *Pancrelipase*, except that the odor may vary with the flavoring agent used.

Pancuronium Bromide: White, yellowish-white, or slightly pink, crystalline powder. Is hygroscopic. Freely soluble in water, in methylene chloride, and in alcohol.

Panthanol: White to creamy white, crystalline powder having a slight, characteristic odor. Freely soluble in water, in alcohol, and in propylene glycol; soluble in chloroform and in ether; slightly soluble in glycerin.

Pantoprazole Sodium: White to off-white powder. Freely soluble in water, in methanol, and in dehydrated alcohol; practically insoluble in hexane and in dichloromethane.

Papain: White to light tan, amorphous powder. Soluble in water, the solution being colorless to light yellow and more or less opalescent; practically insoluble in alcohol, in chloroform, and in ether.

Papaverine Hydrochloride: White crystals or white, crystalline powder. Is odorless, and has a slightly bitter taste. Is optically inactive. Its solutions are acid to litmus. Melts at about 220°, with decomposition. Soluble in water and in chloroform; slightly soluble in alcohol; practically insoluble in ether.

Parachlorophenol: White or pink crystals having a characteristic phenolic odor. When undiluted, it whitens and cauterizes the skin and mucous membranes. Melts at about 42°. Very soluble in alcohol, in glycerin, in chloroform, in ether, and in fixed and volatile oils; soluble in petrolatum; sparingly soluble in water and in liquid petrolatum.

Paraffin: Colorless or white, more or less translucent mass showing a crystalline structure. Is odorless and tasteless, and is slightly greasy to the touch. Freely soluble in chloroform, in ether, in volatile oils, and in most warm fixed oils; slightly soluble in dehydrated alcohol; insoluble in water and in alcohol. *NF category:* Stiffening agent.

Synthetic Paraffin: Very hard, white, practically tasteless and odorless wax. Contains mostly long-chain, unbranched, saturated hydrocarbons, with a small amount of branched hydrocarbons. Is represented by the formula C_nH_{2n+2} , in which n may range from 20 to about 100. The average molecular weight may range from 400 to 1400. Slightly soluble in aromatic and normal paraffinic solvents; very slightly soluble in aliphatic, oxygenated, and halogenated hydrocarbon solvents; insoluble in water. *NF category:* Stiffening agent.

Paraldehyde: Colorless, transparent liquid. Has a strong, characteristic but not unpleasant or pungent odor, and a disagreeable taste. Specific gravity is about 0.99. Soluble in water, but less soluble in boiling water. Miscible with alcohol, with chloroform, with ether, and with volatile oils.

Paricalcitol: White to almost white powder. Soluble in alcohol; insoluble in water.

Paromomycin Sulfate: Creamy white to light yellow powder. Is odorless or practically odorless, and is very hy-

groscopic. Very soluble in water; insoluble in alcohol, in chloroform, and in ether.

Paroxetine Hydrochloride: White to off-white solid. Soluble in methanol and in alcohol; slightly soluble in water.

Peanut Oil: Colorless or pale yellow, oily liquid with a bland taste. May have a characteristic, nutty odor. Very slightly soluble in alcohol. Miscible with ether, with chloroform, and with carbon disulfide. *Specific gravity* (841): Between 0.912 and 0.920. *Refractive index* (831): Between 1.462 and 1.464 at 40°. *NF category:* Solvent; vehicle (oleaginous).

Pectin: Coarse or fine powder, yellowish-white in color, almost odorless, and having a mucilaginous taste. Soluble in 20 parts of water, forming a viscous, opalescent, colloidal solution that flows readily and is acid to litmus; practically insoluble in alcohol or in diluted alcohol and in other organic solvents. Pectin dissolves in water more readily if first moistened with alcohol, glycerin, or simple syrup, or if first mixed with 3 or more parts of sucrose. *NF category:* Suspending and/or viscosity-increasing agent.

Penbutolol Sulfate: White to off-white, crystalline powder. Melts at about 217°, with decomposition. Soluble in water and in methanol.

Penicillamine: White or practically white, crystalline powder, having a slight, characteristic odor. Freely soluble in water; slightly soluble in alcohol; insoluble in chloroform and in ether.

Penicillin G Benzathine: White, odorless, crystalline powder. Sparingly soluble in alcohol; very slightly soluble in water.

Penicillin G Potassium: Colorless or white crystals, or white, crystalline powder. Is odorless or practically so, and is moderately hygroscopic. Its solutions are dextrorotatory. Its solutions retain substantially full potency for several days at temperatures below 15°, but are rapidly inactivated by acids, by alkali hydroxides, by glycerin, and by oxidizing agents. Very soluble in water, in saline TS, and in dextrose solutions; sparingly soluble in alcohol.

Penicillin G Procaine: White crystals or white, very fine, microcrystalline powder. Is odorless or practically odorless, and is relatively stable in air. Its solutions are dextrorotatory. Is rapidly inactivated by acids, by alkali hydroxides, and by oxidizing agents. Soluble in alcohol and in chloroform; slightly soluble in water.

Penicillin G Sodium: Colorless or white crystals or white to slightly yellow, crystalline powder. Is odorless or practically odorless, and is moderately hygroscopic. Its solutions are dextrorotatory. Is relatively stable in air, but is inactivated by prolonged heating at about 100°, especially in the presence of moisture. Its solutions lose potency fairly rapidly at room temperature, but retain substantially full potency for several days at temperatures below 15°. Its solutions are rapidly inactivated by acids, by alkali hydroxides, by oxidizing agents, and by penicillinase.

Penicillin V: White, odorless, crystalline powder. Freely soluble in alcohol and in acetone; very slightly soluble in water; insoluble in fixed oils.

Penicillin V Benzathine: Practically white powder, having a characteristic odor. Sparingly soluble in chloroform; slightly soluble in alcohol and in ether; very slightly soluble in water.

Penicillin V Potassium: White, odorless, crystalline powder. Very soluble in water; slightly soluble in alcohol; insoluble in acetone.

Pentamidine Isethionate: White or almost white powder or colorless crystals, hygroscopic. Freely soluble in water; sparingly soluble in alcohol; practically insoluble in methylene chloride.

Pentazocine: White or very pale, tan-colored powder. Freely soluble in chloroform; soluble in alcohol, in acetone, and in ether; sparingly soluble in benzene and in ethyl acetate; practically insoluble in water.

Pentazocine Hydrochloride: White, crystalline powder. It exhibits polymorphism, one form melting at about 254° and the other at about 218°. Freely soluble in chloroform; soluble in alcohol; sparingly soluble in water; very slightly soluble in acetone and in ether; practically insoluble in benzene.

Pentetic Acid: White, odorless or almost odorless powder. Melts with foaming and degradation at 220°.

Pentobarbital: White to practically white, fine, practically odorless powder. May occur in a polymorphic form that melts at about 116°. This form gradually reverts to the more stable higher-melting form upon being heated at about 110°. Very soluble in alcohol, in methanol, in ether, in chloroform, and in acetone; soluble in benzene; very slightly soluble in water and in carbon tetrachloride.

Pentobarbital Sodium: White, crystalline granules or white powder. Is odorless or has a slight characteristic odor, and has a slightly bitter taste. Its solutions decompose on standing, heat accelerating the decomposition. Very soluble in water; freely soluble in alcohol; practically insoluble in ether.

Pentoxifylline: White to almost white crystalline powder. Freely soluble in chloroform and in methanol; soluble in water; sparingly soluble in alcohol; slightly soluble in ether.

Peppermint: Has an aromatic, characteristic odor and a pungent taste, and produces a cooling sensation in the mouth. *NF category:* Flavors and perfumes.

Peppermint Oil: Colorless or pale yellow liquid, having a strong, penetrating, characteristic odor and a pungent taste, followed by a sensation of cold when air is drawn into the mouth. *NF category:* Flavors and perfumes.

Peppermint Spirit: A clear, colorless liquid with a peppermint fragrance. Freely soluble in methanol and in diethyl ether; soluble in water. *NF category:* Flavors and perfumes.

Peppermint Water: *NF category:* Vehicle (flavored and/or sweetened).

Perflubron: Clear, colorless, practically odorless liquid.

Pergolide Mesylate: White to off-white powder. Sparingly soluble in methanol; slightly soluble in water, in dehydrated alcohol, and in chloroform; very slightly soluble in acetone; practically insoluble in ether.

Perphenazine: White to creamy white, odorless powder. Freely soluble in alcohol and in chloroform; soluble in acetone; practically insoluble in water.

Pertussis Immune Globulin: Transparent or slightly opalescent liquid, practically colorless, free from turbidity or particles, and practically odorless. May develop a slight, granular deposit during storage. Is standardized for agglutinating activity with the U.S. Standard Antipertussis Serum.

Petrolatum: Unctuous yellowish to light amber mass, having not more than a slight fluorescence even after being melted. Is transparent in thin layers. Is free or practically free from odor and taste. Freely soluble in benzene, in carbon disulfide, in chloroform, and in turpentine oil; soluble in ether, in solvent hexane, and in most fixed and volatile oils; practically insoluble in cold and hot alcohol and in cold dehydrated alcohol; insoluble in water. *NF category:* Ointment base.

Hydrophilic Petrolatum: *NF category:* Ointment base.

White Petrolatum: White or faintly yellowish, unctuous mass, transparent in thin layers even after cooling to 0°. Freely soluble in benzene, in carbon disulfide, and in chloroform; soluble in ether, in solvent hexane, and in most fixed and volatile oils; slightly soluble in cold or hot alcohol, and in cold dehydrated alcohol; insoluble in water. *NF category:* Ointment base.

Phenazopyridine Hydrochloride: Light or dark red to dark violet, crystalline powder. Is odorless, or has a slight odor. Melts at about 235°, with decomposition. Slightly soluble in water, in alcohol, and in chloroform.

Phendimetrazine Tartrate: White, odorless, crystalline powder. Freely soluble in water; sparingly soluble in warm alcohol; insoluble in chloroform, in acetone, in ether, and in benzene. Phendimetrazine base is extracted by organic solvents from alkaline solution.

Phenelzine Sulfate: White to yellowish white powder, having a characteristic odor. Freely soluble in water; practically insoluble in alcohol, in chloroform, and in ether.

Pheniramine Maleate: White, crystalline powder having a faint amine-like odor. Soluble in water and in alcohol.

Phenmetrazine Hydrochloride: White to off-white, crystalline powder. Very soluble in water; freely soluble in alcohol and in chloroform.

Phenobarbital: White, odorless, glistening, small crystals, or white, crystalline powder, which may exhibit polymorphism. Is stable in air. Its saturated solution has a pH of about 5. Soluble in alcohol, in ether, and in solutions of fixed alkali hydroxides and carbonates; sparingly soluble in chloroform; very slightly soluble in water.

Phenobarbital Sodium: Flaky crystals, or white, crystalline granules, or white powder. Is odorless, has a bitter taste, and is hygroscopic. Its solutions are alkaline to phenolphthalein TS, and decompose on standing. Very soluble in water; soluble in alcohol; practically insoluble in ether and in chloroform.

Phenol: Colorless to light pink, interlaced or separate, needle-shaped crystals, or white to light pink, crystalline mass. Has a characteristic odor. Is liquefied by warming and by the addition of 10% of water. Boils at about 182°, and its vapor is flammable. Gradually darkens on exposure to light and air. Very soluble in alcohol, in glycerin, in chloroform, in ether, and in fixed and volatile oils; soluble in water; sparingly soluble in mineral oil. *NF category:* Antimicrobial preservative.

Liquefied Phenol: Colorless to pink liquid, which may develop a red tint upon exposure to air or light. Has a characteristic, somewhat aromatic odor. It whitens and cauterizes the skin and mucous membranes. Specific gravity is about 1.065. Miscible with alcohol, with ether, and with glycerin. A mixture of equal volumes of Liquefied Phenol and glycerin is miscible with water.

Camphorated Phenol Topical Gel: Clear, colorless, oily gel.

Phenolsulfonphthalein: A bright-red to dark-red, crystalline powder. Slightly soluble in alcohol; very slightly soluble in water.

Phenoxyethanol: A colorless, slightly viscous liquid. Slightly soluble in water, in peanut oil, and in olive oil. Miscible with acetone, with alcohol, and with glycerol. *NF category:* Antimicrobial preservative.

Phensuximide: White to off-white, crystalline powder. Is odorless, or has not more than a slight odor. Very soluble in chloroform; soluble in alcohol; slightly soluble in water.

Phentermine Hydrochloride: White, odorless, hygroscopic, crystalline powder. Soluble in water and in the lower alcohols; slightly soluble in chloroform; insoluble in ether.

Phentolamine Mesylate: White or off-white, odorless, crystalline powder. Its solutions are acid to litmus, having a pH of about 5, and slowly deteriorate. Melts at about 178°. Freely soluble in water and in alcohol; slightly soluble in chloroform.

Phenylalanine: White, odorless crystals, having a slightly bitter taste. Sparingly soluble in water; very slightly soluble in methanol, in alcohol, and in dilute mineral acids.

Phenylbenzimidazole Sulfonic Acid: White to ivory-colored, odorless powder. Soluble in alcohol; practically insoluble in oily solvents and in water. Its salts are freely soluble in water.

Phenylbutazone: White to off-white, odorless, crystalline powder. Freely soluble in acetone and in ether; soluble in alcohol; very slightly soluble in water.

Phenylephrine Bitartrate: White or almost white powder or colorless crystals. Freely soluble in water.

Phenylephrine Hydrochloride: White or practically white, odorless crystals, having a bitter taste. Freely soluble in water and in alcohol.

Phenylephrine Hydrochloride Nasal Solution: Clear, colorless or slightly yellow, odorless liquid. Is neutral or acid to litmus.

Phenylephrine Hydrochloride Ophthalmic Solution: Clear, colorless or slightly yellow liquid, depending on the concentration.

Phenylethyl Alcohol: Colorless liquid, having a rose-like odor and a sharp, burning taste. Very soluble in alcohol, in fixed oils, in glycerin, and in propylene glycol; sparingly soluble in water; slightly soluble in mineral oil. *NF category:* Antimicrobial preservative.

Phenylmercuric Acetate: White to creamy white, crystalline powder, or small white prisms or leaflets. Is odorless. Soluble in alcohol and in acetone; slightly soluble in water. *NF category:* Antimicrobial preservative.

Phenylmercuric Nitrate: White, crystalline powder. Is affected by light. Its saturated solution is acid to litmus. Slightly soluble in alcohol and in glycerin; very slightly soluble in water. It is more soluble in the presence of either nitric acid or alkali hydroxides. *NF category:* Antimicrobial preservative.

Phenylpropanolamine Bitartrate: White, crystalline powder.

Phenylpropanolamine Hydrochloride: White, crystalline powder, having a slight aromatic odor. Is affected by light. Freely soluble in water and in alcohol; insoluble in ether.

Phenyltoloxamine Citrate: White, crystalline powder. Very soluble in boiling water; slightly soluble in cold water and in alcohol; practically insoluble in cold acetone, in ethyl ether, and in toluene.

Phenytol: White, odorless powder. Melts at about 295°. Soluble in hot alcohol; slightly soluble in cold alcohol, in chloroform, and in ether; practically insoluble in water.

Phenytol Sodium: White, odorless powder. Is somewhat hygroscopic and on exposure to air gradually absorbs carbon dioxide. Freely soluble in water, the solution usually being somewhat turbid due to partial hydrolysis and absorption of carbon dioxide; soluble in alcohol; practically insoluble in ether and in chloroform.

Sodium Phosphate P 32 Solution: Clear, colorless solution. Upon standing, both the Solution and the glass container may darken as a result of the effects of the radiation.

Phosphoric Acid: Colorless, odorless liquid of syrupy consistency. Specific gravity is about 1.71. Miscible with water and with alcohol. *NF category:* Acidifying agent; buffering agent.

Diluted Phosphoric Acid: Clear, colorless, odorless liquid. Specific gravity is about 1.057. *NF category:* Acidifying agent.

Physostigmine: White, odorless, microcrystalline powder. Acquires a red tint when exposed to heat, light, air, or contact with traces of metals. Melts at a temperature not lower than 103°. Very soluble in chloroform and in dichloromethane; freely soluble in alcohol; soluble in benzene and in fixed oils; slightly soluble in water.

Physostigmine Salicylate: White, shining, odorless crystals or white powder. Acquires a red tint when exposed to heat, light, air, or contact with traces of metals for long periods. Melts at about 184°. Freely soluble in chloroform; soluble in alcohol; sparingly soluble in water; slightly soluble in ether.

Physostigmine Sulfate: White, odorless, microcrystalline powder. Is deliquescent in moist air and acquires a red tint when exposed to heat, light, air, or contact with traces of metals for long periods. Melts at about 143°. Freely solu-

ble in water; very soluble in alcohol; very slightly soluble in ether.

Phytonadione: Clear, yellow to amber, very viscous, odorless or practically odorless liquid, having a specific gravity of about 0.967. Is stable in air, but decomposes on exposure to sunlight. Soluble in dehydrated alcohol, in benzene, in chloroform, in ether, and in vegetable oils; slightly soluble in alcohol; insoluble in water.

Pilocarpine: A viscous, oily liquid, or crystals melting at about 34°. Exceedingly hygroscopic. Soluble in water, in alcohol, and in chloroform; sparingly soluble in ether and in benzene; practically insoluble in petroleum ether.

Pilocarpine Hydrochloride: Colorless, translucent, odorless, faintly bitter crystals. Is hygroscopic and is affected by light. Its solutions are acid to litmus. Very soluble in water; freely soluble in alcohol; slightly soluble in chloroform; insoluble in ether.

Pilocarpine Nitrate: Shining, white crystals. Is stable in air but is affected by light. Its solutions are acid to litmus. Freely soluble in water; sparingly soluble in alcohol; insoluble in chloroform and in ether.

Pimozide: White, crystalline powder. Freely soluble in chloroform; slightly soluble in ether and in alcohol; insoluble in water.

Pindolol: White to off-white, crystalline powder, having a faint odor. Slightly soluble in methanol; very slightly soluble in chloroform; practically insoluble in water.

Pioglitazone Hydrochloride: White crystals or crystalline powder. Soluble in dimethylformamide; slightly soluble in dehydrated alcohol; very slightly soluble in acetone and in acetonitrile; practically insoluble in water; insoluble in ether.

Piperacillin: White to off-white, crystalline powder. Very soluble in methanol; slightly soluble in isopropyl alcohol; very slightly soluble in ethyl acetate and in water.

Piperacillin Sodium: White to off-white solid. Freely soluble in water and in alcohol.

Piperazine: White to slightly off-white lumps or flakes, having an ammoniacal odor. Soluble in water and in alcohol; insoluble in ether.

Piperazine Adipate: White crystalline powder. Soluble in water; practically insoluble in alcohol.

Piperazine Citrate: White, crystalline powder, having not more than a slight odor. Its solution (1 in 10) has a pH of about 5. Soluble in water; insoluble in alcohol and in ether.

Piperazine Dihydrochloride: White crystalline powder. Soluble in water.

Piperazine Phosphate: White crystalline powder. Sparingly soluble in water; practically insoluble in alcohol.

Piroxicam: Off-white to light tan or light yellow, odorless powder. Forms a monohydrate that is yellow. Slightly soluble in alcohol and in aqueous alkaline solutions; very slightly soluble in water, in dilute acids, and in most organic solvents.

Plantago Seed: All varieties are practically odorless and have a bland, mucilaginous taste.

Plicamycin: Yellow, odorless, hygroscopic, crystalline powder. Freely soluble in ethyl acetate; slightly soluble in water and in methanol; very slightly soluble in alcohol.

Podophyllum: Has a slight odor and a disagreeably bitter and acrid taste.

Podophyllum Resin: Amorphous powder, varying in color from light brown to greenish yellow, turning darker when subjected to a temperature exceeding 25° or when exposed to light. Has a slight, peculiar, faintly bitter taste. Its alcohol solution is acid to moistened litmus paper. Soluble in alcohol with a slight opalescence; partially soluble in ether and in chloroform.

Polacrilin Potassium: White to off-white, free-flowing powder. Has a faint odor or is odorless. Insoluble in water and in most liquids. *NF category:* Tablet disintegrant.

Poliovirus Vaccine Inactivated: Clear, reddish-tinged or yellowish liquid, that may have a slight odor because of the preservative.

Poloxalene: Colorless or pale yellow liquid. Soluble in water, in chloroform, and in ethylene dichloride.

Poloxamer: *NF category:* Emulsifying and/or solubilizing agent; wetting and/or solubilizing agent.

Poloxamer 124: Colorless liquid, having a mild odor. When solidified, it melts at about 16°. Freely soluble in water, in alcohol, in isopropyl alcohol, in propylene glycol, and in xylene.

Poloxamer 188: White, prilled or cast solid. Is odorless, or has a very mild odor. Melts at about 52°. Freely soluble in water and in alcohol.

Poloxamer 237: White, prilled or cast solid. Is odorless, or has a very mild odor. Melts at about 49°. Freely soluble in water and in alcohol; sparingly soluble in isopropyl alcohol and in xylene.

Poloxamer 338: White, prilled or cast solid. Is odorless, or has a very mild odor. Melts at about 57°. Freely soluble in water and in alcohol; sparingly soluble in propylene glycol.

Poloxamer 407: White, prilled or cast solid. Is odorless, or has a very mild odor. Melts at about 56°. Freely soluble in water, in alcohol, and in isopropyl alcohol.

Polycarbophil: White to creamy white granules, having a characteristic, ester-like odor. Swells in water to a range of volumes, depending primarily on the pH. Insoluble in water, in dilute acids, in dilute alkalies, and in common organic solvents.

Hydrogenated Polydecene: Clear, colorless, odorless, tasteless liquid. Very slightly soluble in water. *NF category:* Emollient; ointment base; solvent; vehicle (oleaginous).

Polydextrose: Off-white to light tan-colored solid. Very soluble in water; soluble in alcohol; slightly soluble in glycerin and in propylene glycol. *NF category:* Bulking agent; humectant.

Hydrogenated Polydextrose: Off-white to light tan-colored solid. Very soluble in water; soluble in alcohol; slightly soluble in glycerin and in propylene glycol. *NF category:* Bulking agent; coating agent; humectant; tablet binder; suspending and/or viscosity-increasing agent.

Polyethylene Glycol: Polyethylene Glycol is usually designated by a number that corresponds approximately to its average molecular weight. As the average molecular weight increases, the water solubility, vapor pressure, hygroscopicity, and solubility in organic solvents decrease, while congealing temperature, specific gravity, flash point, and viscosity increase. Liquid grades occur as clear to slightly hazy, colorless or practically colorless, slightly hygroscopic, viscous liquids, having a slight, characteristic odor, and a specific gravity at 25° of about 1.12. Solid grades occur as practically odorless and tasteless, white, waxy, plastic material having a consistency similar to beeswax, or as creamy white flakes, beads, or powders. The accompanying table states the approximate congealing temperatures that are characteristic of commonly available grades. Liquid grades are miscible with water; solid grades are freely soluble in water; and all are soluble in acetone, in alcohol, in chloroform, in ethylene glycol monoethyl ether, in ethyl acetate, and in toluene; all are insoluble in ether and in hexane. *NF category:* Coating agent; plasticizer; solvent; suppository base; tablet and/or capsule lubricant.

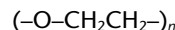
Nominal Molecular Weight Polyethylene Glycol	Approximate Congealing Temperature (°)
300	-11
400	6
600	20
900	34

Nominal Molecular Weight Polyethylene Glycol	Approximate Congealing Temperature (°)
1000	38
1450	44
3350	56
4500	58
8000	60

Polyethylene Glycol Monomethyl Ether: Polyethylene Glycol Monomethyl Ether is usually designated by a number that corresponds approximately to its average molecular weight. As the average molecular weight increases, the water solubility, vapor pressure, hygroscopicity, and solubility in organic solvents decrease, while congealing temperature, specific gravity, flash point, and viscosity increase. Liquid grades occur as clear to slightly hazy, colorless or practically colorless, slightly hygroscopic, viscous liquids, having a slight, characteristic odor, and a specific gravity at 25° of about 1.09 – 1.10. Solid grades occur as practically odorless and tasteless, white, waxy, plastic material having a consistency similar to beeswax, or as creamy white flakes, beads, or powders. The accompanying table states the approximate congealing temperatures that are characteristic of commonly available grades. Liquid grades are miscible with water; solid grades are freely soluble in water; and all are soluble in acetone, in alcohol, in chloroform, in ethylene glycol monoethyl ether, in ethyl acetate, and in toluene; all are insoluble in ether and in hexane. *NF category:* Ointment base; solvent; plasticizer.

Nominal Molecular Weight Polyethylene Glycol Monomethyl Ether	Approximate Congealing Temperature (°)
350	-7
550	17
750	28
1000	35
2000	51
5000	59
8000	60
10000	61

Polyethylene Oxide: Polyethylene oxide resins are high molecular weight polymers having the common structure:



in which n , the degree of polymerization, varies from about 2000 to over 100,000. Polyethylene oxide, being a polyether, strongly hydrogen bonds with water. It is nonionic and undergoes salting-out effects associated with neutral molecules in solutions of high dielectric media. Salting-out effects manifest themselves in depressing the upper temperature limit of solubility, and in reducing the viscosity of both dilute and concentrated solutions of the polymers. All molecular weight grades are powdered or granular solids. They are soluble in water but, because of the high solution viscosities obtained (see *table*), solutions over 1% in water may be difficult to prepare. The water solubility, hygroscopicity, solubility in organic solvents, and melting point do not vary in the specified molecular weight range. At room temperature polyethylene oxide is miscible with water in all proportions. At concentrations of about 20% polymer in water, the solutions are nontacky, reversible, elastic gels. At higher concentrations, the solutions are tough, elastic materials with the water acting as a plasticizer. Polyethylene oxide is also freely soluble in acetonitrile, in ethylene dichloride, in trichloroethylene, and in methylene chloride. Heating may be required to obtain solutions in many other organic solvents. It is insoluble in aliphatic hydrocarbons, in ethylene glycol, in di-

ethylene glycol, and in glycerol. *NF category:* Suspending and/or viscosity-increasing agent; tablet binder.

Approximate Molecular Weight	Typical Solution Viscosity (cps), 25°	
	5% Solution	1% Solution
100,000	40	
200,000	100	
300,000	800	
400,000	3000	
600,000	6000	
900,000	15,000	
4,000,000		3500
5,000,000		5500

Polyethylene 50 Stearate: *NF category:* Emulsifying and/or solubilizing agent.

Polyglyceryl 3 Diisostearate: Viscous liquid. Soluble in alcohol, in methylene chloride, in mineral oil, and in vegetable oils; insoluble in water. *NF category:* Emulsifying and/or solubilizing agent; ointment base.

Polyglyceryl Dioleate: Viscous liquid. Soluble in methylene chloride, in mineral oil, and in vegetable oils; sparingly soluble in alcohol; insoluble in water. *NF category:* Emulsifying and/or solubilizing agent.

Polyisobutylene: Low molecular-weight grades are soft and gummy; high molecular-weight grades are tough and elastic. All grades are light in color, odorless, and tasteless. Soluble in diisobutylene, in toluene, and in chloroform; insoluble in water.

Polymyxin B Sulfate: White to buff-colored powder. Is odorless or has a faint odor. Freely soluble in water; slightly soluble in alcohol.

Polyoxyl Lauryl Ether: A material with 3–5 oxyethylene units per molecule is a colorless liquid. Soluble or dispersible in alcohol; practically insoluble in water and in hexane. A material with 9–23 oxyethylene units per molecule is a white, waxy mass. Soluble or dispersible in water; soluble in alcohol; practically insoluble in hexane. *NF category:* Emulsifying and/or solubilizing agent.

Polyoxyl Oleate: A slightly yellowish, viscous liquid. Dispersible in water and in oils. Soluble in alcohol and in isopropyl alcohol. Miscible with fatty oils and with waxes. Its refractive index is about 1.466.

Polyoxyl 10 Oleyl Ether: White, soft semisolid, or pale yellow liquid, having a bland odor. Soluble in water and in alcohol. Dispersible in mineral oil and in propylene glycol, with possible separation on standing. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Polyoxyl 15 Hydroxystearate: Yellowish to white waxy mass. Very soluble in water; soluble in alcohol and in 2-propanol; insoluble in mineral oil. It solidifies at 25°. *NF category:* Tablet and/or capsule lubricant; wetting and/or solubilizing agent; vehicle (oleaginous).

Polyoxyl 20 Cetostearyl Ether: Cream-colored, waxy, unctuous mass, melting, when heated, to a clear brownish-yellow liquid. Soluble in water, in alcohol, and in acetone; insoluble in solvent hexane. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Polyoxyl 35 Castor Oil: Yellow, oily liquid, having a faint, characteristic odor and a somewhat bitter taste. Very soluble in water, producing a practically odorless and colorless solution; soluble in alcohol and in ethyl acetate; insoluble in mineral oils. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Polyoxyl 40 Hydrogenated Castor Oil: White to yellowish paste or pasty liquid, having a faint odor and a slight taste. Very soluble in water, producing a practically tasteless,

odorless, and colorless solution; soluble in alcohol and in ethyl acetate; insoluble in mineral oils. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Polyoxyl 40 Stearate: Waxy, white to light tan solid. Is odorless or has a faint, fat-like odor. Soluble in water, in alcohol, in ether, and in acetone; insoluble in mineral oil and in vegetable oils. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Polyoxyl Stearate: White or slightly yellowish waxy mass. Soluble in alcohol and in isopropyl alcohol. Polyoxyl stearate corresponding to a product with 6–8 units of ethylene oxide per molecule is soluble in fatty oils and in waxes; practically insoluble in water. Polyoxyl stearate corresponding to a product with 20–100 units of ethylene oxide per molecule is soluble in water; practically insoluble in fatty oils and in waxes. *NF category:* Emulsifying and/or solubilizing agent; wetting and/or solubilizing agent.

Polyoxyl Stearyl Ether: A white to yellowish-white, waxy, unctuous mass, pellets, microbeads, or flakes. Polyoxyl Stearyl Ether with 2 oxyethylene units per molecule is soluble in alcohol, with heating, and in methylene chloride; practically insoluble in water. Polyoxyl Stearyl Ether with 10 oxyethylene units per molecule is soluble in water and in alcohol. Polyoxyl Stearyl Ether with 20 oxyethylene units per molecule is soluble in water, in alcohol, and in methylene chloride. After melting, it solidifies at about 45°.

Polysorbate 20: Lemon to amber liquid having a faint characteristic odor. Soluble in water, in alcohol, in ethyl acetate, in methanol, and in dioxane; insoluble in mineral oil. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Polysorbate 40: Yellow liquid having a faint, characteristic odor. Soluble in water and in alcohol; insoluble in mineral oil and in vegetable oils. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Polysorbate 60: Lemon- to orange-colored, oily liquid or semi-gel having a faint, characteristic odor. Soluble in water, in ethyl acetate, and in toluene; insoluble in mineral oil and in vegetable oils. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Polysorbate 80: Lemon- to amber-colored, oily liquid having a faint, characteristic odor and a warm, somewhat bitter taste. Very soluble in water, producing an odorless and practically colorless solution; soluble in alcohol and in ethyl acetate; insoluble in mineral oil. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Polyvinyl Acetate: White or off-white powder or colorless granules or beads. Freely soluble in ethyl acetate; soluble in alcohol, in acetone, and in chloroform; practically insoluble in water. It is hygroscopic and swells in water. *NF category:* Coating agent; desiccant; tablet binder.

Polyvinyl Acetate Dispersion: Opaque, white or off-white, slightly viscous liquid. Miscible with water and with ethanol. It is sensitive to spoilage by microbial contaminants. *NF category:* Coating agent.

Polyvinyl Acetate Phthalate: Free-flowing white powder. May have a slight odor of acetic acid. Soluble in methanol and in alcohol; insoluble in water, in methylene chloride, and in chloroform. *NF category:* Coating agent.

Polyvinyl Alcohol: White to cream-colored granules, or white to cream-colored powder. Is odorless. Freely soluble in water at room temperature. Solution may be effected more rapidly at somewhat higher temperatures. *NF category:* Suspending and/or viscosity-increasing agent.

Sulfurated Potash: Irregular, liver-brown pieces when freshly made, changing to a greenish yellow. Has an odor of hydrogen sulfide and a bitter, acrid, and alkaline taste, and

decomposes on exposure to air. A solution (1 in 10) is light brown in color and is alkaline to litmus. Freely soluble in water, usually leaving a slight residue. Alcohol dissolves only the sulfides.

Potassium Acetate: Colorless, monoclinic crystals or white, crystalline powder having a saline and slightly alkaline taste. Is odorless, or has a faint acetous odor. Deliquesces on exposure to moist air. Very soluble in water; freely soluble in alcohol.

Potassium Alginate: White to yellow, fibrous or granular powder. Dissolves in water to form a viscous, colloidal solution; insoluble in alcohol and in hydroalcoholic solutions in which the alcohol content is greater than 30% by weight; insoluble in chloroform, in ether, and in acids having a pH lower than about 3.

Potassium Benzoate: White, odorless, or practically odorless, granular or crystalline powder. Is stable in air. Freely soluble in water; soluble in 90% alcohol; sparingly soluble in alcohol. *NF category:* Antimicrobial preservative.

Potassium Bicarbonate: Colorless, transparent, monoclinic prisms or as a white, granular powder. Is odorless, and is stable in air. Its solutions are neutral or alkaline to phenolphthalein TS. Freely soluble in water; practically insoluble in alcohol.

Potassium Bitartrate: Colorless or slightly opaque crystals, or white, crystalline powder. A saturated solution is acid to litmus. Soluble in boiling water; slightly soluble in water; very slightly soluble in alcohol.

Potassium Bromide: White, crystalline powder or colorless, cubical crystals. Freely soluble in water and in glycerol; slightly soluble in alcohol.

Potassium Chloride: Colorless, elongated, prismatic, or cubical crystals, or white, granular powder. Is odorless, has a saline taste, and is stable in air. Its solutions are neutral to litmus. Freely soluble in water; insoluble in alcohol. *NF category:* Tonicity agent.

Potassium Citrate: Transparent crystals or white, granular powder. Is odorless, has a cooling, saline taste, and is deliquescent when exposed to moist air. Freely soluble in water; very slightly soluble in alcohol. *NF category:* Buffering agent.

Potassium Gluconate: White to yellowish-white, crystalline powder or granules. Is odorless, has a slightly bitter taste, and is stable in air. Its solutions are slightly alkaline to litmus. Freely soluble in water; practically insoluble in dehydrated alcohol, in ether, in benzene, and in chloroform.

Potassium Hydroxide: White or practically white, fused masses, or small pellets, or flakes, or sticks, or other forms. Is hard and brittle and shows a crystalline fracture. Exposed to air, it rapidly absorbs carbon dioxide and moisture, and deliquesces. Very soluble in boiling alcohol; freely soluble in water, in alcohol, and in glycerin. *NF category:* Alkalizing agent.

Potassium Iodide: Hexahedral crystals, either transparent and colorless or somewhat opaque and white, or a white, granular powder. Is slightly hygroscopic. Its solutions are neutral or alkaline to litmus. Very soluble in water and even more soluble in boiling water; freely soluble in glycerin; soluble in alcohol.

Potassium Iodide Oral Solution: Clear, colorless, odorless liquid, having a characteristic, strongly salty taste. Is neutral or alkaline to litmus. Specific gravity is about 1.70.

Potassium Metabisulfite: White or colorless, free-flowing crystals, crystalline powder, or granules, usually having an odor of sulfur dioxide. Gradually oxidizes in air to the sulfate. Its solutions are acid to litmus. Soluble in water; insoluble in alcohol. *NF category:* Antioxidant.

Potassium Metaphosphate: White, odorless powder. Soluble in dilute solutions of sodium salts; insoluble in water. *NF category:* Buffering agent.

Potassium Nitrate: White, crystalline powder or colorless crystals. Very soluble in boiling water; freely soluble in water; soluble in glycerin; practically insoluble in alcohol.

Potassium Permanganate: Dark purple crystals, almost opaque by transmitted light and of a blue metallic luster by reflected light. Its color is sometimes modified by a dark bronze-like appearance. Is stable in air. Freely soluble in boiling water; soluble in water.

Dibasic Potassium Phosphate: Colorless or white, somewhat hygroscopic, granular powder. The pH of a solution (1 in 20) is about 8.5 to 9.6. Freely soluble in water; very slightly soluble in alcohol. *NF category:* Buffering agent.

Monobasic Potassium Phosphate: Colorless crystals or white, granular or crystalline powder. Is odorless, and is stable in air. The pH of a solution (1 in 100) is about 4.5. Freely soluble in water; practically insoluble in alcohol. *NF category:* Buffering agent.

Potassium Sodium Tartrate: Colorless crystals or white, crystalline powder, having a cooling, saline taste. As it effloresces slightly in warm, dry air, the crystals are often coated with a white powder. Freely soluble in water; practically insoluble in alcohol.

Potassium Sorbate: White crystals or powder, having a characteristic odor. Melts at about 270°, with decomposition. Freely soluble in water; soluble in alcohol. *NF category:* Antimicrobial preservative.

Povidone: White to slightly creamy white powder. Is hygroscopic. Freely soluble in water, in methanol, and in alcohol; slightly soluble in acetone; practically insoluble in ether. *NF category:* Suspending and/or viscosity-increasing agent; tablet binder.

Povidone-Iodine: Yellowish-brown to reddish-brown, amorphous powder, having a slight, characteristic odor. Its solution is acid to litmus. Soluble in water and in alcohol; practically insoluble in chloroform, in carbon tetrachloride, in ether, in solvent hexane, and in acetone.

Povidone-Iodine Topical Aerosol Solution: The liquid obtained from Povidone-Iodine Topical Aerosol Solution is transparent, having a reddish brown color.

Pralidoxime Chloride: White to pale-yellow, crystalline powder. Is odorless and is stable in air. Freely soluble in water.

Pramipexole Dihydrochloride: White to almost white crystalline powder. Freely soluble in water; soluble in methanol; slightly soluble in alcohol; practically insoluble in methylene chloride.

Pramoxine Hydrochloride: White to practically white, crystalline powder, having a numbing taste. May have a slight aromatic odor. The pH of a solution (1 in 100) is about 4.5. Freely soluble in water and in alcohol; soluble in chloroform; very slightly soluble in ether.

Pravastatin Sodium: White to yellowish white, hygroscopic powder. Freely soluble in water and in methanol; soluble in dehydrated alcohol; practically insoluble in acetonitrile and in chloroform.

Praziquantel: White or practically white, crystalline powder; odorless or having a faint characteristic odor. Freely soluble in alcohol and in chloroform; very slightly soluble in water.

Prazosin Hydrochloride: White to tan powder. Slightly soluble in water, in methanol, in dimethylformamide, and in dimethylacetamide; very slightly soluble in alcohol; practically insoluble in chloroform and in acetone.

Prednicarbate: White to almost white, crystalline powder. Freely soluble in acetone and in alcohol; sparingly soluble in propylene glycol; practically insoluble in water.

Prednisolone: White to practically white, odorless, crystalline powder. Melts at about 235°, with some decomposition (see *Melting Range or Temperature* (741)). Soluble in methanol and in dioxane; sparingly soluble in acetone and

in alcohol; slightly soluble in chloroform; very slightly soluble in water.

Prednisolone Acetate: White to practically white, odorless, crystalline powder. Melts at about 235°, with some decomposition (see *Melting Range or Temperature* (741)). Slightly soluble in acetone, in alcohol, and in chloroform; practically insoluble in water.

Prednisolone Hemisuccinate: Fine, creamy white powder with friable lumps; practically odorless. Melts at about 205°, with decomposition. Freely soluble in alcohol; soluble in acetone; very slightly soluble in water.

Prednisolone Sodium Phosphate: White or slightly yellow, friable granules or powder. Is odorless or has a slight odor. Is slightly hygroscopic. Freely soluble in water; soluble in methanol; slightly soluble in alcohol and in chloroform; very slightly soluble in acetone and in dioxane.

Prednisolone Sodium Succinate for Injection: Creamy white powder with friable lumps, having a slight odor.

Prednisolone Tebutate: White to slightly yellow, free-flowing powder, which may show some soft lumps. Is odorless or has not more than a moderate, characteristic odor. Is hygroscopic. Freely soluble in chloroform and in dioxane; soluble in acetone; sparingly soluble in alcohol and in methanol; very slightly soluble in water.

Prednisone: White to practically white, odorless, crystalline powder. Melts at about 230°, with some decomposition (see *Melting Range or Temperature* (741)). Slightly soluble in alcohol, in chloroform, in dioxane, and in methanol; very slightly soluble in water.

Prilocaine: White or almost white powder or crystal aggregates. Very soluble in alcohol and in acetone; slightly soluble in water.

Prilocaine Hydrochloride: White, odorless, crystalline powder, having a bitter taste. Freely soluble in water and in alcohol; slightly soluble in chloroform; very slightly soluble in acetone; practically insoluble in ether.

Primaquine Phosphate: Orange-red, crystalline powder. Is odorless and has a bitter taste. Its solutions are acid to litmus. Melts at about 200°. Soluble in water; insoluble in chloroform and in ether.

Primidone: White, crystalline powder. Is odorless and has a slightly bitter taste. Slightly soluble in alcohol; very slightly soluble in water and in most organic solvents.

Probucol: White to off-white, crystalline powder. Freely soluble in chloroform and in *n*-propyl alcohol; soluble in alcohol and in solvent hexane; insoluble in water.

Probenecid: White or practically white, fine, crystalline powder. Is practically odorless. Soluble in dilute alkali, in chloroform, in alcohol, and in acetone; practically insoluble in water and in dilute acids.

Procainamide Hydrochloride: White to tan, crystalline powder. Is odorless. Its solution (1 in 10) has a pH between 5 and 6.5. Very soluble in water; soluble in alcohol; slightly soluble in chloroform; very slightly soluble in benzene and in ether.

Procainamide Hydrochloride Injection: Colorless, or having not more than a slight yellow color.

Procaine Hydrochloride: Small, white crystals or white, crystalline powder. Is odorless. Exhibits local anesthetic properties when placed on the tongue. Freely soluble in water; soluble in alcohol; slightly soluble in chloroform; practically insoluble in ether.

Procaine Hydrochloride Injection: Clear, colorless liquid.

Prochlorperazine: Clear, pale yellow, viscous liquid. Is sensitive to light. Freely soluble in alcohol, in chloroform, and in ether; very slightly soluble in water.

Prochlorperazine Edisylate: White to very light yellow, odorless, crystalline powder. Its solutions are acid to litmus. Freely soluble in water; very slightly soluble in alcohol; insoluble in ether and in chloroform.

Prochlorperazine Maleate: White or pale yellow, practically odorless, crystalline powder. Its saturated solution is acid to litmus. Slightly soluble in warm chloroform; practically insoluble in water and in alcohol.

Procyclidine Hydrochloride: White, crystalline powder, having a moderate, characteristic odor. Melts at about 225°, with decomposition. Soluble in water and in alcohol; insoluble in ether and in acetone.

Progesterone: White or creamy white, odorless, crystalline powder. Is stable in air. Soluble in alcohol, in acetone, and in dioxane; sparingly soluble in vegetable oils; practically insoluble in water.

Proguanil Hydrochloride: White, crystalline powder. Sparingly soluble in alcohol; slightly soluble in water; practically insoluble in methylene chloride.

Proline: White, odorless crystals, having a slightly sweet taste. Freely soluble in water and in absolute alcohol; insoluble in ether, in butanol, and in isopropanol.

Promazine Hydrochloride: White to slightly yellow, practically odorless, crystalline powder. It oxidizes upon prolonged exposure to air and acquires a blue or pink color. Freely soluble in water and in chloroform.

Promethazine Hydrochloride: White to faint yellow, practically odorless, crystalline powder. Slowly oxidizes, and acquires a blue color, on prolonged exposure to air. Freely soluble in water, in hot dehydrated alcohol, and in chloroform; practically insoluble in ether, in acetone, and in ethyl acetate.

Propafenone Hydrochloride: White powder. Soluble in methanol and in hot water; slightly soluble in alcohol and in chloroform; very slightly soluble in acetone; insoluble in diethyl ether and in toluene.

Propane: Colorless, flammable gas (boiling temperature is about -42°). One hundred volumes of water dissolves 6.5 volumes at 17.8° and 753 mm pressure; 100 volumes of anhydrous alcohol dissolves 790 volumes at 16.6° and 754 mm pressure; 100 volumes of ether dissolves 926 volumes at 16.6° and 757 mm pressure; 100 volumes of chloroform dissolves 1299 volumes at 21.6° and 757 mm pressure. Vapor pressure at 21° is about 10290 mm of mercury (108 psig). *NF category:* Aerosol propellant.

Propantheline Bromide: White or practically white crystals. Is odorless and has a bitter taste. Melts at about 160°, with decomposition. Very soluble in water, in alcohol, and in chloroform; practically insoluble in ether and in benzene.

Proparacaine Hydrochloride: White to off-white, or faintly buff-colored, odorless, crystalline powder. Its solutions are neutral to litmus. Soluble in water, in warm alcohol, and in methanol; insoluble in ether and in benzene.

Proparacaine Hydrochloride Ophthalmic Solution: Colorless or faint yellow solution.

Propionic Acid: Oily liquid having a slight pungent, rancid odor. Miscible with water and with alcohol and various other organic solvents. *NF category:* Acidifying agent.

Propofol: Clear, colorless to slightly yellowish liquid. Very soluble in methanol and in ethanol; slightly soluble in cyclohexane and in isopropyl alcohol; very slightly soluble in water.

Propoxycaïne Hydrochloride: White, odorless, crystalline solid, which discolors on prolonged exposure to light and air. The pH of a solution (1 in 50) is about 5.4. Freely soluble in water; soluble in alcohol; sparingly soluble in ether; practically insoluble in acetone and in chloroform.

Propoxyphene Hydrochloride: White, crystalline powder. Is odorless, and has a bitter taste. Freely soluble in water; soluble in alcohol, in chloroform, and in acetone; practically insoluble in benzene and in ether.

Propoxyphene Napsylate: White powder, having essentially no odor, but having a bitter taste. Soluble in meth-

anol, in alcohol, in chloroform, and in acetone; very slightly soluble in water.

Propranolol Hydrochloride: White to off-white, crystalline powder. Is odorless and has a bitter taste. Melts at about 164°. Soluble in water and in alcohol; slightly soluble in chloroform; practically insoluble in ether.

Propyl Gallate: White, crystalline powder having a very slight, characteristic odor. Freely soluble in alcohol; slightly soluble in water. *NF category:* Antioxidant.

Propylene Glycol: Clear, colorless, viscous liquid having a slight, characteristic taste. Is practically odorless. Absorbs moisture when exposed to moist air. Miscible with water, with acetone, and with chloroform. Soluble in ether and will dissolve many essential oils, but is immiscible with fixed oils. *NF category:* Humectant; plasticizer; solvent.

Propylene Glycol Alginate: White to yellowish fibrous or granular powder. Practically odorless and tasteless. Soluble in water, in solutions of dilute organic acids, and, depending on the degree of esterification, in hydroalcoholic mixture containing up to 60% by weight of alcohol to form stable, viscous colloidal solutions at a pH of 3. *NF category:* Suspending and/or viscosity-increasing agent.

Propylene Glycol Dicaprylate/Dicaprate: Clear, colorless or slightly yellow oily liquid at 20°. Soluble in fatty oils and in light petroleum; slightly soluble in dehydrated alcohol; practically insoluble in water. *NF category:* Emulsifying and/or solubilizing agent; vehicle.

Propylene Glycol Dilaurate: Clear, oily liquid at 20°. Colorless or slightly yellow. Very soluble in alcohol, in methanol, and in methylene chloride; practically insoluble in water.

Propylene Glycol Monocaprylate: Clear, colorless, or slightly yellow, oily liquid at 20°. Very soluble in alcohol, in chloroform, and in methylene chloride; practically insoluble in water. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule diluent; vehicle.

Propylene Glycol Monolaurate: Clear, oily liquid at 20°. Colorless or slightly yellow. Very soluble in alcohol, in methanol, and in methylene chloride; practically insoluble in water.

Propylene Glycol Monostearate: White, wax-like solid or as white, wax-like beads or flakes. Has a slight, agreeable, fatty odor and taste. Soluble in organic solvents such as alcohol, mineral or fixed oils, benzene, ether, and acetone; insoluble in water, but may be dispersed in hot water with the aid of a small amount of soap or other suitable surface-active agent. *NF category:* Emulsifying and/or solubilizing agent.

Propylhexedrine: Clear, colorless liquid, having a characteristic, amine-like odor. Volatilizes slowly at room temperature. Absorbs carbon dioxide from the air, and its solutions are alkaline to litmus. Boils at about 205°. Very slightly soluble in water. Miscible with alcohol, with chloroform, and with ether.

Propylidone: White or almost white, crystalline powder. Is odorless or has a faint odor. Soluble in acetone, in alcohol, and in ether; practically insoluble in water.

Propylparaben: Small, colorless crystals or white powder. Freely soluble in alcohol and in ether; slightly soluble in boiling water; very slightly soluble in water. *NF category:* Antimicrobial preservative.

Propylparaben Sodium: White powder. Is odorless and hygroscopic. Freely soluble in water; sparingly soluble in alcohol; insoluble in fixed oils. *NF category:* Antimicrobial preservative.

Propylthiouracil: White, powdery, crystalline substance. Is starch-like in appearance and to the touch, and has a bitter taste. Soluble in ammonium hydroxide and in alkali hydroxides; sparingly soluble in alcohol; slightly soluble in water, in chloroform, and in ether.

Protamine Sulfate Injection: Colorless solution, which may have the odor of a preservative.

Protamine Sulfate for Injection: White, odorless powder, having the characteristic appearance of solids dried from the frozen state.

Protein Hydrolysate Injection: Yellowish to reddish-amber, transparent liquid.

Protriptyline Hydrochloride: White to yellowish powder. Is odorless, or has not more than a slight odor. Melts at about 168°. Freely soluble in water, in alcohol, and in chloroform; practically insoluble in ether.

Pseudoephedrine Hydrochloride: Fine, white to off-white crystals or powder, having a faint characteristic odor. Very soluble in water; freely soluble in alcohol; slightly soluble in chloroform.

Pseudoephedrine Sulfate: White crystals or crystalline powder. Is odorless. Freely soluble in alcohol.

Pullulan: White powder. Freely soluble in water; practically insoluble in dehydrated alcohol. *NF category:* Bulking agent for freeze-drying; coating agent; plasticizer; polymer membrane; sequestering agent; suspending and/or viscosity-increasing agent; tablet binder; tablet and/or capsule diluent; tablet disintegrant; wetting and/or solubilizing agent.

Pumice: Very light, hard, rough, porous, grayish masses or gritty, grayish powder. Is odorless and tasteless, and is stable in air. Practically insoluble in water; is not attacked by acids.

Pyrantel Pamoate: Yellow to tan solid. Soluble in dimethyl sulfoxide; slightly soluble in dimethylformamide; practically insoluble in water and in methanol.

Pyrazinamide: White to practically white, odorless or practically odorless, crystalline powder. Sparingly soluble in water; slightly soluble in alcohol, in ether, and in chloroform.

Pyrethrum Extract: Pale yellow liquid having a bland, flowery odor. Soluble in mineral oil and in most organic solvents; insoluble in water. *Pyrethrins I* denotes the group containing pyrethrin 1, cinerin 1, and jasmolin 1; *Pyrethrins II* denotes the group containing pyrethrin 2, cinerin 2, and jasmolin 2.

Pyridostigmine Bromide: White or practically white, crystalline powder, having an agreeable, characteristic odor. Is hygroscopic. Freely soluble in water, in alcohol, and in chloroform; slightly soluble in solvent hexane; practically insoluble in ether.

Pyridoxine Hydrochloride: White to practically white crystals or crystalline powder. Is stable in air, and is slowly affected by sunlight. Its solutions have a pH of about 3. Freely soluble in water; slightly soluble in alcohol; insoluble in ether.

Pyrilamine Maleate: White, crystalline powder, usually having a faint odor. Its solutions are acid to litmus. Very soluble in water; freely soluble in alcohol and in chloroform; slightly soluble in ether and in benzene.

Pyrimethamine: White, odorless, crystalline powder. Slightly soluble in acetone, in alcohol, and in chloroform; practically insoluble in water.

Pyrvinium Pamoate: Bright orange or orange-red to practically black, crystalline powder. Freely soluble in glacial acetic acid; slightly soluble in chloroform and in methoxyethanol; very slightly soluble in methanol; practically insoluble in water and in ether.

Pyrvinium Pamoate Oral Suspension: Dark red, opaque suspension of essentially very fine, amorphous particles or aggregates, usually less than 10 µm in size. Larger particles, some of which may be crystals, up to 100 µm in size also may be present.

Quazepam: Off-white to yellowish powder.

Quinapril Hydrochloride: White to off-white powder, with a pink cast at times. Freely soluble in aqueous solvents.

Quinidine Gluconate: White powder. Is odorless and has a very bitter taste. Freely soluble in water; slightly soluble in alcohol.

Quinidine Sulfate: Fine, needle-like, white crystals, frequently cohering in masses, or fine, white powder. Is odorless, and darkens on exposure to light. Its solutions are neutral or alkaline to litmus. Soluble in alcohol; sparingly soluble in chloroform; slightly soluble in water; insoluble in ether.

Quinine Sulfate: White, fine, needle-like crystals, usually lusterless, making a light and readily compressible mass. Is odorless. It darkens on exposure to light. Its saturated solution is neutral or alkaline to litmus. Freely soluble in alcohol at 80°, and in a mixture of 2 volumes of chloroform and 1 volume of dehydrated alcohol; sparingly soluble in water at 100°; slightly soluble in water, in alcohol, and in chloroform; very slightly soluble in ether.

Rabies Immune Globulin: Transparent or slightly opalescent liquid, practically colorless and practically odorless. May develop a slight, granular deposit during storage.

Rabies Vaccine: White to straw-colored, amorphous pellet, which may or may not become fragmented when shaken.

Racemethionine: Almost white, crystalline powder or small flakes. Sparingly soluble in water; very slightly soluble in alcohol. It dissolves in dilute acids and in dilute solutions of alkali hydroxides. It melts at about 270°. *NF category:* Antioxidant; buffering agent; flavors and perfumes.

Racpinephrine: White to nearly white, crystalline, odorless powder, gradually darkening on exposure to light and air. With acids, it forms salts that are readily soluble in water, and the base may be recovered by the addition of ammonium hydroxide. Very slightly soluble in water and in alcohol; insoluble in ether, in chloroform, and in fixed and volatile oils.

Racpinephrine Hydrochloride: Fine, white, odorless powder. Darkens on exposure to light and air. Its solutions are acid to litmus. Melts at about 157°. Freely soluble in water; sparingly soluble in alcohol.

Raloxifene Hydrochloride: Almost white to pale yellow powder. Freely soluble in dimethylsulfoxide; sparingly soluble in methanol; slightly soluble in alcohol; very slightly soluble in water, in isopropyl alcohol, and in octanol; practically insoluble in ether and in ethyl acetate.

Ramipril: White to almost white crystalline powder. Freely soluble in methanol; sparingly soluble in water.

Ranitidine Hydrochloride: White to pale yellow, crystalline, practically odorless powder. Is sensitive to light and moisture. Melts at about 140°, with decomposition. Very soluble in water; sparingly soluble in alcohol.

Fully Hydrogenated Rapeseed Oil: White, waxy solid. Insoluble in water and in alcohol. *NF category:* Coating agent; stiffening agent.

Superglycerinated Fully Hydrogenated Rapeseed Oil: White solid. Insoluble in water and in alcohol. *NF category:* Coating agent; emulsifying and/or solubilizing agent; stiffening agent.

Purified Rayon: White, lustrous or dull, fine, soft, filamentous fibers, appearing under the microscope as round, oval, or slightly flattened translucent rods, straight or crimped, striate and with serrate cross-sectional edges. Is practically odorless and practically tasteless. Very soluble in ammoniated cupric oxide TS and in dilute sulfuric acid (3 in 5); insoluble in ordinary solvents.

Repaglinide: White to off-white solid. Melts at about 132° to 136°. Soluble in methanol.

Reserpine: White or pale buff to slightly yellowish, odorless, crystalline powder. Darkens slowly on exposure to light, but more rapidly when in solution. Freely soluble in acetic acid and in chloroform; slightly soluble in benzene;

very slightly soluble in alcohol and in ether; insoluble in water.

Resorcinol: White, or practically white, needle-shaped crystals or powder. Has a faint, characteristic odor and a sweetish, followed by a bitter, taste. Acquires a pink tint on exposure to light and air. Its solution (1 in 20) is neutral or acid to litmus. Freely soluble in water, in alcohol, in glycerin, and in ether; slightly soluble in chloroform.

Ribavirin: White, crystalline powder. Freely soluble in water; slightly soluble in dehydrated alcohol.

Riboflavin: Yellow to orange-yellow, crystalline powder having a slight odor. Melts at about 280°. Its saturated solution is neutral to litmus. When dry, it is not appreciably affected by diffused light, but when in solution, light induces quite rapid deterioration, especially in the presence of alkalies. Soluble in dilute solutions of alkalies; very slightly soluble in water, in alcohol, and in isotonic sodium chloride solution; insoluble in ether and in chloroform.

Riboflavin 5'-Phosphate Sodium: Fine, orange-yellow, crystalline powder, having a slight odor. Sparingly soluble in water. When dry, it is not affected by diffused light, but when in solution, light induces deterioration rapidly. Is hygroscopic.

Rifabutin: Amorphous red-violet powder. Soluble in chloroform and in methanol; sparingly soluble in alcohol; very slightly soluble in water.

Rifampin: Red-brown, crystalline powder. Freely soluble in chloroform; soluble in ethyl acetate and in methanol; very slightly soluble in water.

Riluzole: White to slightly yellow powder or crystalline powder. Freely soluble in acetonitrile, in alcohol, and in methylene chloride; slightly soluble in hexane; very slightly soluble in water.

Rimexolone: White to off-white powder. Freely soluble in chloroform; sparingly soluble in methanol.

Risedronate Sodium: White to off-white powder. Soluble in water and in aqueous solutions; insoluble in common organic solvents.

Risperidone: White or almost white powder. Soluble in methylene chloride; sparingly soluble in alcohol; practically insoluble in water.

Ritodrine Hydrochloride: White to nearly white, odorless or practically odorless, crystalline powder. Melts at about 200°. Freely soluble in water and in alcohol; soluble in *n*-propyl alcohol; practically insoluble in ether.

Ritonavir: White to light tan powder. Freely soluble in methanol and in methylene chloride; very slightly soluble in acetonitrile; practically insoluble in water.

Rivastigmine Tartrate: White to off-white powder. Very soluble in water and in methanol; very slightly soluble in ethyl acetate.

Rizatriptan Benzoate: White to almost white crystalline powder. Soluble in water; sparingly soluble in alcohol; slightly soluble in methylene chloride.

Rocuronium Bromide: Almost white or pale yellow. Slightly hygroscopic powder. Freely soluble in water and in dehydrated alcohol.

Ropinirole Hydrochloride: Pale cream to yellow powder. Soluble in water.

Ropivacaine Hydrochloride: White, crystalline powder. Soluble in water.

Rose Oil: Colorless or yellow liquid, having the characteristic odor and taste of rose. At 25° is a viscous liquid. Upon gradual cooling, changes to a translucent, crystalline mass, easily liquefied by warming. *NF category:* Flavors and perfumes.

Rose Water Ointment: *NF category:* Ointment base.

Stronger Rose Water: Practically colorless and clear, having the pleasant odor and taste of fresh rose blossoms. Is

free from empyreuma, mustiness, and fungal growths. *NF category:* Flavors and perfumes.

Change to read:

Rosiglitazone Maleate: White to off-white solid.
 ▲ USP36 Sparingly soluble in alcohol; slightly soluble in methylene chloride; practically insoluble to very slightly soluble in water.

Roxarsone: Pale yellow, crystalline powder. Freely soluble in acetic acid, in acetone, in alkalis, in methanol, and in dehydrated alcohol; soluble in boiling water; sparingly soluble in dilute mineral acids; slightly soluble in cold water; insoluble in ether and in ethyl acetate. Puffs up and deflates on heating.

Rubella Virus Vaccine Live: Solid having the characteristic appearance of substances dried from the frozen state. Undergoes loss of potency on exposure to sunlight. The Vaccine is to be constituted with a suitable diluent just prior to use.

Add the following:

▲ **Rufinamide:** White, crystalline neutral powder. Slightly soluble in tetrahydrofuran and in methanol; very slightly soluble in alcohol and in acetonitrile; practically insoluble in water. ▲ USP36

Saccharin: White crystals or white, crystalline powder. Is odorless or has a faint, aromatic odor. In dilute solution, it is intensely sweet. Its solutions are acid to litmus. Soluble in boiling water; sparingly soluble in alcohol; slightly soluble in water, in chloroform, and in ether. Is readily dissolved by dilute solutions of ammonia, by solutions of alkali hydroxides, and by solutions of alkali carbonates with the evolution of carbon dioxide. *NF category:* Sweetening agent.

Saccharin Calcium: White crystals or white, crystalline powder. Is odorless, or has a faint, aromatic odor, and has an intensely sweet taste even in dilute solutions. Its dilute solution is about 300 times as sweet as sucrose. Freely soluble in water. *NF category:* Sweetening agent.

Saccharin Sodium: White crystals or white, crystalline powder. Is odorless, or has a faint, aromatic odor, and has an intensely sweet taste even in dilute solutions. Its dilute solution is about 300 times as sweet as sucrose. When in powdered form, it usually contains about one-third the theoretical amount of water of hydration as a result of efflorescence. Freely soluble in water; sparingly soluble in alcohol. *NF category:* Sweetening agent.

Saccharin Sodium Oral Solution: Clear, colorless, odorless liquid, having a sweet taste.

Safflower Oil: Light yellow oil. Thickens and becomes rancid on prolonged exposure to air. Insoluble in water. Miscible with ether and with chloroform. *NF category:* Vehicle (oleaginous).

Salicylamide: White, practically odorless, crystalline powder. Freely soluble in ether and in solutions of alkalis; soluble in alcohol and in propylene glycol; slightly soluble in water and in chloroform.

Salicylic Acid: White crystals, usually in fine needles, or fluffy, white, crystalline powder. Has a sweetish, followed by an acid, taste and is stable in air. The synthetic form is white and odorless. When prepared from natural methyl salicylate, it may have a slightly yellow or pink tint, and a faint, mint-like odor. Freely soluble in alcohol and in ether; soluble in boiling water; sparingly soluble in chloroform; slightly soluble in water and in benzene.

Salmeterol Xinafoate: White to off-white powder. Soluble in methanol; slightly soluble in alcohol, in isopropanol, and in chloroform; practically insoluble in water (pH 8), and in saline solution (0.9% w/w).

Scopolamine Hydrobromide: Colorless or white crystals or white, granular powder. Melts at about 197°, with decomposition. Is odorless, and slightly efflorescent in dry air. Freely soluble in water; soluble in alcohol; slightly soluble in chloroform; insoluble in ether.

Secobarbital: White, amorphous or crystalline, odorless powder, having a slightly bitter taste. Its saturated solution has a pH of about 5.6. Freely soluble in alcohol, in ether, and in solutions of fixed alkali hydroxides and carbonates; soluble in chloroform; very slightly soluble in water.

Secobarbital Sodium: White powder. Is odorless, has a bitter taste, and is hygroscopic. Its solutions decompose on standing, heat accelerating the decomposition. Very soluble in water; soluble in alcohol; practically insoluble in ether.

Selegiline Hydrochloride: White, odorless, crystalline powder. Freely soluble in water, in chloroform, and in methanol.

Selenium Sulfide: Reddish-brown to bright orange powder, having not more than a faint odor. Practically insoluble in water and in organic solvents.

Sennosides: Brownish powder.

Serine: White, odorless crystals, having a sweet taste. Soluble in water; practically insoluble in absolute alcohol and in ether.

Sertraline Hydrochloride: White or off-white crystalline powder. Sparingly soluble in absolute alcohol; slightly soluble in water, in acetone, and in isopropanol.

Sesame Oil: Pale yellow, oily liquid. Is practically odorless, and has a bland taste. Slightly soluble in alcohol. Miscible with ether, with chloroform, with solvent hexane, and with carbon disulfide. *NF category:* Solvent, vehicle (oleaginous).

Sevoflurane: Clear, colorless, volatile, nonflammable liquid. Slightly soluble in water. Miscible with alcohol, with chloroform, and with ether.

Shellac: *Orange Shellac*—Thin, hard, brittle, transparent, pale lemon-yellow to brownish orange flakes, having little or no odor; *Bleached Shellac*—Opaque, amorphous cream to yellow granules or coarse powder, having little or no odor. Soluble (very slowly) in alcohol, 85% to 95% (w/w), in ether, 13% to 15%, in benzene, 10% to 20%, in petroleum ether, 2% to 6%; soluble in aqueous solutions of ethanalamines, alkalis, and borax; sparingly soluble in oil of turpentine; insoluble in water. *NF category:* Coating agent.

Sibutramine Hydrochloride Monohydrate: White to cream crystalline powder. Slightly soluble in pH 5.2 water.

Sildenafil Citrate: White or almost white slightly hygroscopic crystalline powder. Slightly soluble in water and in methanol; practically insoluble in hexane.

Dental-Type Silica: Fine, white, hygroscopic, odorless, amorphous powder, in which the diameter of the average particles ranges between 0.5 and 40 µm. Soluble in hot solutions of alkali hydroxides; insoluble in water, in alcohol, and in acid (except hydrofluoric acid). *NF category:* Glidant and/or anticaking agent; suspending and/or viscosity-increasing agent.

Hydrophobic Colloidal Silica: Light, fine, white or almost white, amorphous powder, not wettable by water. Disolves slowly in hot solutions of alkali hydroxides. Practically insoluble in water and in mineral acids, except hydrofluoric acid. *NF category:* Glidant and/or anticaking agent; suspending and/or viscosity-increasing agent.

Purified Siliceous Earth: Very fine, white, light gray, or pale buff mixture of amorphous powder and lesser amounts of crystalline polymorphs, including quartz and cristobalite. Is gritty, readily absorbs moisture, and retains about four times its weight of water without becoming fluid. Insoluble in water, in acids, and in dilute solutions of the alkali hydroxides. *NF category:* Filtering aid; sorbent.

Silicon Dioxide: Fine, white, hygroscopic, odorless, amorphous powder, in which the diameter of the average

particles ranges between 2 and 10 μm . Soluble in hot solutions of alkali hydroxides; insoluble in water, in alcohol, and in other organic solvents. *NF category*: Desiccant; suspending and/or viscosity-increasing agent.

Colloidal Silicon Dioxide: Light, white, nongritty powder of extremely fine particle size (about 15 nm). Soluble in hot solutions of alkali hydroxides; insoluble in water and in acid (except hydrofluoric). *NF category*: Glidant and/or anti-caking agent; suspending and/or viscosity-increasing agent.

Silver Nitrate: Colorless or white crystals. The pH of its solutions is about 5.5. On exposure to light in the presence of organic matter, it becomes gray or grayish black. Very soluble in water and even more so in boiling water; freely soluble in boiling alcohol; sparingly soluble in alcohol; slightly soluble in ether.

Toughened Silver Nitrate: White, crystalline masses generally molded as pencils or cones. It breaks with a fibrous fracture. Its solutions are neutral to litmus. It becomes gray or grayish black upon exposure to light. Soluble in water to the extent of its nitrate content (there is always a residue of silver chloride); partially soluble in alcohol; slightly soluble in ether.

Simethicone: Translucent, gray, viscous fluid. The liquid phase is soluble in chloroform, in ether, and in benzene, but silicon dioxide remains as a residue in these solvents. Insoluble in water and in alcohol. *NF category*: Antifoaming agent; water repelling agent.

Simvastatin: White to off-white powder. Freely soluble in chloroform, in methanol, and in alcohol; sparingly soluble in propylene glycol; very slightly soluble in hexane; practically insoluble in water.

Smallpox Vaccine: Liquid Vaccine is a turbid, whitish to greenish suspension, which may have a slight odor due to the antimicrobial agent. Dried Vaccine is a yellow to grayish pellet, which may or may not become fragmented when shaken.

Soda Lime: White or grayish-white granules. May have a color if an indicator has been added. *NF category*: Sorbent, carbon dioxide.

Sodium Acetate: Colorless, transparent crystals, or white, granular crystalline powder, or white flakes. Is odorless or has a faint acetous odor, and has a slightly bitter, saline taste. Is efflorescent in warm, dry air. Very soluble in water; soluble in alcohol. *NF category*: Buffering agent.

Sodium Alginate: Practically odorless and tasteless, coarse or fine powder, yellowish white in color. Soluble in water, forming a viscous, colloidal solution; insoluble in alcohol and in hydroalcoholic solutions in which the alcohol content is greater than about 30% by weight, in chloroform, in ether, and in acids when the pH of the resulting solution becomes lower than about 3. *NF category*: Suspending and/or viscosity-increasing agent.

Sodium Ascorbate: White or very faintly yellow crystals or crystalline powder. Is odorless or practically odorless. Is relatively stable in air. On exposure to light it gradually darkens. Freely soluble in water; very slightly soluble in alcohol; insoluble in chloroform and in ether.

Sodium Benzoate: White, odorless or practically odorless, granular or crystalline powder. Is stable in air. Freely soluble in water; soluble in 90% alcohol; sparingly soluble in alcohol. *NF category*: Antimicrobial preservative.

Sodium Bicarbonate: White, crystalline powder. Is stable in dry air, but slowly decomposes in moist air. Its solutions, when freshly prepared with cold water, without shaking, are alkaline to litmus. The alkalinity increases as the solutions stand, as they are agitated, or as they are heated. Soluble in water; insoluble in alcohol. *NF category*: Alkalizing agent.

Sodium Bisulfite: White, crystalline powder. Freely soluble in cold water and in hot water; sparingly soluble in alcohol. *NF category*: Antioxidant.

Sodium Borate: Colorless, transparent crystals or white, crystalline powder. Is odorless. Its solutions are alkaline to phenolphthalein TS. As it effloresces in warm, dry air, the crystals are often coated with white powder. Freely soluble in boiling water and in glycerin; soluble in water; insoluble in alcohol. *NF category*: Alkalizing agent.

Sodium Bromide: White, crystalline powder or colorless, cubical crystals. Freely soluble in water; soluble in alcohol.

Sodium Butyrate: Clear, colorless, hygroscopic powder. Soluble in water and in methanol. Melting range is about 250° to 253°.

Sodium Caprylate: A white, crystalline powder. Very soluble or freely soluble in water; freely soluble in acetic acid; sparingly soluble in alcohol; practically insoluble in acetone.

Sodium Carbonate: Colorless crystals, or white, crystalline powder or granules. Is stable in air under ordinary conditions. When exposed to dry air above 50°, the hydrous salt effloresces and, at 100°, becomes anhydrous. Very soluble in boiling water; freely soluble in water. *NF category*: Alkalizing agent.

Sodium Cetostearyl Sulfate: A white or pale yellow, amorphous or crystalline powder. Soluble in hot water giving an opalescent solution; slightly soluble in alcohol; practically insoluble in cold water.

Sodium Chloride: Colorless, cubic crystals or white crystalline powder. Has a saline taste. Freely soluble in water; soluble in glycerin; slightly soluble in alcohol. *NF category*: Tonicity agent.

Sodium Chloride Inhalation Solution: Clear, colorless solution.

Bacteriostatic Sodium Chloride Injection: Clear, colorless solution, odorless or having the odor of the bacteriostatic substance. *NF category*: Vehicle (sterile).

Sodium Chloride Irrigation: Clear, colorless solution.

Sodium Citrate: Colorless crystals, or white, crystalline powder. Hydrous form very soluble in boiling water; freely soluble in water; insoluble in alcohol. *NF category*: Buffering agent.

Sodium Citrate and Citric Acid Oral Solution: Clear solution having the color of any added preservative or flavoring agents.

Sodium Dehydroacetate: White or practically white, odorless powder, having a slight characteristic taste. Freely soluble in water, in propylene glycol, and in glycerin. *NF category*: Antimicrobial preservative.

Sodium Fluoride: White, odorless powder. Soluble in water; insoluble in alcohol.

Sodium Formaldehyde Sulfoxylate: White crystals or hard, white masses, having the characteristic odor of garlic. Freely soluble in water; slightly soluble in alcohol, in ether, in chloroform, and in benzene. *NF category*: Antioxidant.

Sodium Hydroxide: White, or practically white, fused masses, in small pellets, in flakes, or sticks, and in other forms. Is hard and brittle and shows a crystalline fracture. Exposed to the air, it rapidly absorbs carbon dioxide and moisture. Freely soluble in water and in alcohol. *NF category*: Alkalizing agent.

Sodium Hypochlorite Solution: Clear, pale greenish-yellow liquid, having the odor of chlorine. Is affected by light.

Sodium Iodide: Colorless, odorless crystals, or white, crystalline powder. Is deliquescent in moist air, and develops a brown tint upon decomposition. Very soluble in water; freely soluble in alcohol and in glycerin.

Sodium Lactate Solution: Clear, colorless or practically colorless, slightly viscous liquid, odorless or having a slight, not unpleasant odor. Miscible with water. *NF category*: Buffering agent.

Sodium Lauryl Sulfate: Small, white or light yellow crystals having a slight, characteristic odor. Freely soluble in water, forming an opalescent solution. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Sodium Metabisulfite: White crystals or white to yellowish, crystalline powder, having the odor of sulfur dioxide. Freely soluble in water and in glycerin; slightly soluble in alcohol. *NF category:* Antioxidant.

Sodium Monofluorophosphate: White to slightly gray, odorless powder. Freely soluble in water.

Sodium Nitrite: White to slightly yellow, granular powder, or white or practically white, opaque, fused masses or sticks. Has a mild, saline taste and is deliquescent in air. Its solutions are alkaline to litmus. Freely soluble in water; sparingly soluble in alcohol.

Sodium Nitrite Injection: Clear, colorless liquid.

Sodium Nitroprusside: Reddish-brown, practically odorless, crystals or powder. Freely soluble in water; slightly soluble in alcohol; very slightly soluble in chloroform; insoluble in benzene.

Dibasic Sodium Phosphate (dried): White powder that readily absorbs moisture. Freely soluble in water; insoluble in alcohol. *NF category:* Buffering agent.

Dibasic Sodium Phosphate (heptahydrate): Colorless or white, granular or caked salt. Effloresces in warm, dry air. Its solutions are alkaline to phenolphthalein TS, a 0.1 M solution having a pH of about 9. Freely soluble in water; very slightly soluble in alcohol. *NF category:* Buffering agent.

Monobasic Sodium Phosphate: Colorless crystals or white, crystalline powder. Is odorless and is slightly deliquescent. Its solutions are acid to litmus and effervesce with sodium carbonate. Freely soluble in water; practically insoluble in alcohol. *NF category:* Buffering agent.

Tribasic Sodium Phosphate: The formula for a crystalline material is approximately $4(\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O})\text{NaOH}$. It occurs as white, odorless crystals or granules or as a crystalline powder. Freely soluble in water; insoluble in alcohol. The pH of a 1 in 100 solution is between 11.5 and 12.0.

Sodium Polystyrene Sulfonate: Golden brown, fine powder. Is odorless and has a characteristic taste. Insoluble in water.

Sodium Propionate: Colorless, transparent crystals or granular, crystalline powder. Is odorless, or has a faint acetic-butyric odor and is deliquescent in moist air. Very soluble in water; soluble in alcohol. *NF category:* Antimicrobial preservative.

Sodium Salicylate: Amorphous or microcrystalline powder or scales. Is colorless, or has not more than a faint, pink tinge. Is odorless, or has a faint, characteristic odor, and is affected by light. A freshly made solution (1 in 10) is neutral or acid to litmus. Very soluble in boiling water and in boiling alcohol; freely (and slowly) soluble in water and in glycerin; slowly soluble in alcohol.

Sodium Starch Glycolate: White, tasteless, odorless, relatively free-flowing powder; available in several different viscosity grades. A 2% (w/v) dispersion in cold water settles, on standing, in the form of a highly hydrated layer. *NF category:* Tablet disintegrant.

Sodium Stearate: Fine, white powder, soapy to the touch, usually having a slight, tallow-like odor. Is affected by light. Its solutions are alkaline to phenolphthalein TS. Slowly soluble in cold water and in cold alcohol; readily soluble in hot water and in hot alcohol. *NF category:* Emulsifying and/or solubilizing agent.

Sodium Stearyl Fumarate: Fine, white powder. Slightly soluble in methanol; practically insoluble in water. *NF category:* Tablet and/or capsule lubricant.

Sodium Sulfate: Large, colorless, odorless, transparent crystals, or a granular powder. Effloresces rapidly in air, liquefies in its water of hydration at about 33°, and loses all of

its water of hydration at about 100°. Freely soluble in water; soluble in glycerin; insoluble in alcohol.

Sodium Sulfite: Colorless crystals. Freely soluble in water; very slightly soluble in alcohol. *NF category:* Antioxidant.

Sodium Tartrate: Transparent, colorless, odorless crystals. Freely soluble in water; insoluble in alcohol. *NF category:* Sequestering agent.

Sodium Thiosulfate: Large, colorless crystals or coarse, crystalline powder. Is deliquescent in moist air and effloresces in dry air at temperatures exceeding 33°. Its solutions are neutral or faintly alkaline to litmus. Very soluble in water; insoluble in alcohol. *NF category:* Antioxidant.

Sorbic Acid: Free-flowing, white, crystalline powder, having a characteristic odor. Soluble in alcohol and in ether; slightly soluble in water. *NF category:* Antimicrobial preservative.

Sorbitan Monolaurate: Yellow to amber-colored, oily liquid, having a bland, characteristic odor. Soluble in mineral oil; slightly soluble in cottonseed oil and in ethyl acetate; insoluble in water. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Sorbitan Monooleate: Viscous, yellow to amber-colored, oily liquid, having a bland, characteristic odor. Insoluble in water and in propylene glycol. Miscible with mineral and vegetable oils. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Sorbitan Monopalmitate: Cream-colored, waxy solid having a faint fatty odor. Soluble in warm absolute alcohol; soluble, with haze, in warm peanut oil and in warm mineral oil; insoluble in water. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Sorbitan Monostearate: Cream-colored to tan, hard, waxy solid, having a bland odor and taste. Soluble, with haze, above 50° in mineral oil and in ethyl acetate; insoluble in cold water and in acetone. Dispersible in warm water. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Sorbitan Sesquioleate: Viscous, yellow to amber-colored, oily liquid. Soluble in alcohol, in isopropyl alcohol, in cottonseed oil, and in mineral oil; insoluble in water and in propylene glycol. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Sorbitan Trioleate: Yellow to amber-colored, oily liquid. Soluble in methyl alcohol, in alcohol, in isopropyl alcohol, in corn oil, in cottonseed oil, and in mineral oil; insoluble in water, in ethylene glycol, and in propylene glycol. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant; wetting and/or solubilizing agent.

Sorbitol: D-Sorbitol occurs as white granules, powder, or crystalline masses. Is odorless, and has a sweet taste with a cold sensation. Very soluble in water; sparingly soluble in alcohol; and practically insoluble in ethyl ether. Is hygroscopic. *NF category:* Humectant; sweetening agent; tablet and/or capsule diluent.

Sorbitol Solution: Clear, colorless, syrupy liquid. Is odorless and has a sweet taste. It sometimes separates into crystalline masses. Miscible with water, with alcohol, with glycerin, and with propylene glycol. Is neutral to litmus. *NF category:* Sweetening agent; vehicle (flavored and/or sweetened).

Sorbitol Sorbitan Solution: A clear, colorless to pale yellow, syrupy liquid. Is odorless and has a sweet taste. Insoluble in mineral oil and in vegetable oil. Miscible with water, with alcohol, with glycerin, and with propylene glycol. *NF category:* Humectant; plasticizer.

Sotalol Hydrochloride: White to off-white powder. Freely soluble in water; soluble in alcohol; very slightly soluble in chloroform.

Soybean Oil: Clear, pale yellow, oily liquid having a characteristic odor and taste. Insoluble in water. Miscible with ether and with chloroform. *Specific gravity* (841): between 0.916 and 0.922. *Refractive index* (831): between 1.465 and 1.475. *NF category:* Vehicle (oleaginous).

Hydrogenated Soybean Oil: A white mass or powder that melts to a clear, pale yellow liquid when heated. Freely soluble in methylene chloride, in hexane after heating, and in toluene; very slightly soluble in alcohol; practically insoluble in water. *NF category:* Emollient.

Spectinomycin Hydrochloride: White to pale-buff crystalline powder. Freely soluble in water; practically insoluble in alcohol, in chloroform, and in ether.

Spironolactone: Light cream-colored to light tan, crystalline powder. Has a faint to mild mercaptan-like odor; is stable in air. Freely soluble in benzene and in chloroform; soluble in ethyl acetate and in alcohol; slightly soluble in methanol and in fixed oils; practically insoluble in water.

Squalane: Colorless, practically odorless transparent oil. Slightly soluble in acetone; very slightly soluble in absolute alcohol; insoluble in water. Miscible with ether and with chloroform. *NF category:* Ointment base; vehicle (oleaginous).

Stannous Chloride: White, crystalline powder or colorless crystals, efflorescent in air. Freely soluble in water (the solution becomes cloudy after standing or on dilution) and in alcohol. Dissolves in dilute hydrochloric acid. *NF category:* Emulsifying agent; antioxidant.

Stannous Fluoride: White, crystalline powder, having a bitter, salty taste. Melts at about 213°. Freely soluble in water; practically insoluble in alcohol, in ether, and in chloroform.

Stanozolol: Odorless, crystalline powder, occurring in two forms: as needles, melting at about 155°, and as prisms, melting at about 235°. Soluble in dimethylformamide; sparingly soluble in alcohol and in chloroform; slightly soluble in ethyl acetate and in acetone; very slightly soluble in benzene; insoluble in water.

Starch: Irregular, angular, white masses or fine powder. Is odorless, and has a slight, characteristic taste. Insoluble in cold water and in alcohol. *NF category:* Tablet and/or capsule diluent; tablet disintegrant; tablet and/or capsule lubricant.

Corn Starch: Irregular, angular, white masses or fine powder. Is odorless, and has a slight, characteristic taste. Insoluble in cold water and in alcohol. *NF category:* Tablet and/or capsule diluent; tablet disintegrant; tablet binder; suspending and/or viscosity-increasing agent.

Hydroxypropyl Corn Starch: White or slightly yellowish powder. Practically insoluble in cold water and in alcohol. *NF category:* Tablet binder; tablet and/or capsule diluent; tablet disintegrant; suspending and/or viscosity-increasing agent.

Pregelatinized Hydroxypropyl Corn Starch: White or slightly yellowish powder. It swells in water and produces a clear or translucent, viscous, colloidal mixture. *NF category:* Suspending and/or viscosity-increasing agent; tablet binder; tablet and/or capsule diluent; tablet disintegrant.

Pea Starch: White or almost white, very fine powder. Practically insoluble in cold water and in alcohol. *NF category:* Suspending and/or viscosity-increasing agent; tablet binder; tablet and/or capsule diluent; tablet disintegrant.

Hydroxypropyl Pea Starch: White or slightly yellowish powder. Practically insoluble in cold water and in alcohol. *NF category:* Tablet binder; tablet and/or capsule diluent; tablet disintegrant; suspending and/or viscosity-increasing agent.

Pregelatinized Hydroxypropyl Pea Starch: White or slightly yellowish powder. It swells in water and produces a clear or translucent, viscous, colloidal mixture. *NF category:* Suspending and/or viscosity-increasing agent; tablet binder; tablet and/or capsule diluent; tablet disintegrant.

Potato Starch: Irregular, angular, white masses or fine powder. Is odorless, and has a slight, characteristic taste. Insoluble in cold water and in alcohol. *NF category:* Tablet and/or capsule diluent; tablet disintegrant; tablet binder; suspending and/or viscosity-increasing agent.

Hydroxypropyl Potato Starch: White or slightly yellowish powder. Practically insoluble in cold water and in alcohol. *NF category:* Tablet binder; tablet and/or capsule diluent; tablet disintegrant; suspending and/or viscosity-increasing agent.

Pregelatinized Hydroxypropyl Potato Starch: White or slightly yellowish powder. It swells in water and produces a clear or translucent, viscous, colloidal mixture. *NF category:* Suspending and/or viscosity-increasing agent; tablet binder; tablet and/or capsule diluent; tablet disintegrant.

Pregelatinized Starch: Moderately coarse to fine, white to off-white powder. Is odorless and has a slight, characteristic taste. Slightly soluble to soluble in cold water; insoluble in alcohol. *NF category:* Tablet binder; tablet and/or capsule diluent; tablet disintegrant.

Pregelatinized Modified Starch: Moderately coarse to fine, white to off-white powder. Is odorless and has a slight, characteristic taste. Soluble to slightly soluble in cold water; insoluble in alcohol. *NF category:* Tablet binder; tablet and/or capsule diluent; tablet disintegrant.

Tapioca Starch: Irregular, angular, white to pale yellow masses or fine powder. Insoluble in cold water and in alcohol. *NF category:* Suspending and/or viscosity-increasing agent; tablet binder; tablet and/or capsule diluent; tablet disintegrant.

Wheat Starch: Irregular, angular, white masses or fine powder. Is odorless and has a slight, characteristic taste. Insoluble in cold water and in alcohol. *NF category:* Tablet and/or capsule diluent; tablet disintegrant; tablet binder; suspending and/or viscosity-increasing agent.

Hydrogenated Starch Hydrolysate: Concentrated, aqueous solution or spray-dried or dried powder. Very soluble in water; insoluble in alcohol. *NF category:* Sweetening agent; humectant; tablet binder; tablet and/or capsule diluent.

Stavudine: White to off-white, crystalline powder. Soluble in water, in dimethylacetamide, and in dimethyl sulfoxide; sparingly soluble in methanol, in alcohol, and in acetonitrile; slightly soluble in dichloromethane; insoluble in hexane.

Stearic Acid: Hard, white or faintly yellowish, somewhat glossy and crystalline solid, or white or yellowish-white powder. Its odor and taste are slight, suggesting tallow. Freely soluble in chloroform and in ether; soluble in alcohol; practically insoluble in water. *NF category:* Emulsifying and/or solubilizing agent; tablet and/or capsule lubricant.

Purified Stearic Acid: Hard, white or faintly yellowish, somewhat glossy and crystalline solid, or white or yellowish-white powder. Its odor and taste are slight, suggesting tallow. Freely soluble in chloroform and in ether; soluble in alcohol; practically insoluble in water. *NF category:* Tablet and/or capsule lubricant.

Stearoyl Polyoxylglycerides: Pale yellow, waxy solids. Dispersible in warm water and in warm paraffin. Freely soluble in methylene chloride; soluble in warm methanol. *NF category:* Ointment base; solvent.

Stearyl Alcohol: Unctuous, white flakes or granules. Has a faint, characteristic odor and a bland, mild taste. Soluble in alcohol and in ether; insoluble in water. *NF category:* Stiffening agent.

Storax: Semiliquid, grayish to grayish-brown, sticky, opaque mass depositing on standing a heavy dark brown

layer (Levant Storax); or semisolid, sometimes a solid mass, softened by gently warming (American Storax). Is transparent in thin layers, has a characteristic odor and taste, and is more dense than water. Soluble, usually incompletely, in an equal weight of warm alcohol, in acetone, in carbon disulfide, and in ether, some insoluble residue usually remaining; insoluble in water.

Streptomycin Sulfate: White or practically white powder. Is odorless or has not more than a faint odor. Is hygroscopic, but is stable in air and on exposure to light. Its solutions are acid to practically neutral to litmus. Freely soluble in water; very slightly soluble in alcohol; practically insoluble in chloroform.

Streptomycin Sulfate Injection: Clear, colorless to yellow, viscous liquid. Is odorless or has a slight odor.

Strontium Chloride: Colorless, odorless crystals or white granules. Effloresces in air; deliquesces in moist air. Very soluble in water; soluble in alcohol.

Succinic Acid: White, odorless crystals. Freely soluble in boiling water; soluble in water, in alcohol, and in glycerin. *NF category:* Buffering agent.

Succinylcholine Chloride: White, odorless, crystalline powder. Its solutions have a pH of about 4. The dihydrate form melts at about 160°; the anhydrous form melts at about 190°, and is hygroscopic. Freely soluble in water; slightly soluble in alcohol and in chloroform; practically insoluble in ether.

Sucralose: White to off-white, crystalline powder. Freely soluble in water, in methanol, and in alcohol; slightly soluble in ethyl acetate. *NF category:* Sweetening agent.

Sucrose: White, crystalline powder or lustrous, dry, colorless or white crystals. Very soluble in water; slightly soluble in alcohol; practically insoluble in dehydrated alcohol. *NF category:* Coating agent; sweetening agent; tablet and/or capsule diluent.

Sucrose Palmitate: White or almost white, unctuous powder. Sparingly soluble in ethanol (96%); very slightly soluble in water. *NF category:* Suspending and/or viscosity-increasing agent.

Sucrose Octaacetate: White, practically odorless powder, having an intensely bitter taste. Is hygroscopic. Very soluble in methanol and in chloroform; soluble in alcohol and in ether; very slightly soluble in water. *NF category:* Alcohol denaturant.

Sucrose Stearate: White or almost white, unctuous powder. Sparingly soluble in ethanol (96%); very slightly soluble in water. *NF category:* Tablet and/or capsule lubricant; emulsifying and/or solubilizing agent.

Sufentanil Citrate: White powder. Freely soluble in methanol; soluble in water; sparingly soluble in acetone, in alcohol, and in chloroform. Melts between 133° and 140°.

Compressible Sugar: Practically white, crystalline, odorless powder, having a sweet taste. Is stable in air. The sucrose portion of Compressible Sugar is very soluble in water. *NF category:* Sweetening agent; tablet and/or capsule diluent.

Confectioner's Sugar: Fine, white, odorless powder, having a sweet taste. Is stable in air. The sucrose portion of Confectioner's Sugar is soluble in cold water. Confectioner's Sugar is freely soluble in boiling water. *NF category:* Sweetening agent; tablet and/or capsule diluent.

Sugar Spheres: Hard, brittle, free-flowing, spherical masses ranging generally in size from 10- to 60-mesh. Usually white, but may be colored. Solubility in water varies according to the sugar-to-starch ratio. *NF category:* Vehicle (solid carrier).

Sulbactam Sodium: White to off-white, crystalline powder. Freely soluble in water and in dilute acid; sparingly soluble in acetone, in ethyl acetate, and in chloroform.

Sulconazole Nitrate: White to off-white, crystalline powder. Melts at about 130°, with decomposition. Freely

soluble in pyridine; sparingly soluble in methanol; slightly soluble in alcohol, in chloroform, in acetone, and in methylene chloride; very slightly soluble in water, in toluene, and in dioxane.

Sulfabenzamide: Fine, white, practically odorless powder. Soluble in alcohol, in acetone, and in sodium hydroxide TS; insoluble in water and in ether.

Sulfacetamide: White, crystalline powder, odorless and having a characteristic sour taste. Its aqueous solutions are sensitive to light, and are unstable when acidic or strongly alkaline. Freely soluble in dilute mineral acids and in solutions of potassium and sodium hydroxides; soluble in alcohol; slightly soluble in water and in ether; very slightly soluble in chloroform; practically insoluble in benzene.

Sulfacetamide Sodium: White, crystalline powder. Is odorless and has a bitter taste. Freely soluble in water; sparingly soluble in alcohol; practically insoluble in chloroform and in ether.

Sulfadiazine: White or slightly yellow powder. Is odorless or nearly odorless and is stable in air, but slowly darkens on exposure to light. Freely soluble in dilute mineral acids, in solutions of potassium and sodium hydroxides, and in ammonia TS; sparingly soluble in alcohol and in acetone; slightly soluble in human serum at 37°; practically insoluble in water.

Silver Sulfadiazine: White to creamy-white, crystalline powder, odorless to having a slight odor. Is stable in air, but turns yellow on exposure to light. Freely soluble in 30% ammonium solution; slightly soluble in acetone; practically insoluble in alcohol, in chloroform, and in ether. Decomposes in moderately strong mineral acids.

Sulfadiazine Sodium: White powder. On prolonged exposure to humid air it absorbs carbon dioxide with the liberation of sulfadiazine and becomes incompletely soluble in water. Its solutions are alkaline to phenolphthalein. Is affected by light. Freely soluble in water; slightly soluble in alcohol.

Sulfadimethoxine: Practically white, crystalline powder. Soluble in 2 N sodium hydroxide; sparingly soluble in 2 N hydrochloric acid; slightly soluble in alcohol, in ether, in chloroform, and in hexane; practically insoluble in water.

Sulfamethazine: White to yellowish-white powder, which may darken on exposure to light. Has a slightly bitter taste and is practically odorless. Soluble in acetone; slightly soluble in alcohol; very slightly soluble in water and in ether.

Sulfamethizole: White crystals or powder, having a slightly bitter taste. Is practically odorless, and has no odor of hydrogen sulfide. Freely soluble in solutions of ammonium, potassium, and sodium hydroxides; soluble in dilute mineral acids and in acetone; sparingly soluble in alcohol; very slightly soluble in water, in chloroform, and in ether; practically insoluble in benzene.

Sulfamethoxazole: White to off-white, practically odorless, crystalline powder. Freely soluble in acetone and in dilute solutions of sodium hydroxide; sparingly soluble in alcohol; practically insoluble in water, in ether, and in chloroform.

Sulfapyridine: White or faintly yellowish-white crystals, granules, or powder. Is odorless or practically odorless, and is stable in air, but slowly darkens on exposure to light. Freely soluble in dilute mineral acids and in solutions of potassium and sodium hydroxides; sparingly soluble in acetone; slightly soluble in alcohol; very slightly soluble in water.

Sulfasalazine: Bright yellow or brownish-yellow, odorless, fine powder. Melts at about 255°, with decomposition. Soluble in aqueous solutions of alkali hydroxides; very slightly soluble in alcohol; practically insoluble in water, in ether, in chloroform, and in benzene.

Sulfathiazole: Fine, white or faintly yellowish-white, practically odorless powder. Soluble in acetone, in dilute

mineral acids, in solutions of alkali hydroxides, and in 6 N ammonium hydroxide; slightly soluble in alcohol; very slightly soluble in water.

Sulfinpyrazone: White to off-white powder. Soluble in alcohol and in acetone; sparingly soluble in dilute alkali; practically insoluble in water and in solvent hexane.

Sulfisoxazole: White to slightly yellowish, odorless, crystalline powder. Soluble in boiling alcohol and in 3 N hydrochloric acid; very slightly soluble in water.

Sulfisoxazole Acetyl: White or slightly yellow, crystalline powder. Sparingly soluble in chloroform; slightly soluble in alcohol; practically insoluble in water.

Precipitated Sulfur: Very fine, pale yellow, amorphous or microcrystalline powder. Is odorless and tasteless. Very soluble in carbon disulfide; slightly soluble in olive oil; very slightly soluble in alcohol; practically insoluble in water.

Sublimed Sulfur: Fine, yellow, crystalline powder, having a faint odor and taste. Sparingly soluble in olive oil; practically insoluble in water and in alcohol.

Sulfur Dioxide: Colorless, nonflammable gas, possessing a strong, suffocating odor characteristic of burning sulfur. Under pressure, it condenses readily to a colorless liquid that boils at -10° and has a density of approximately 1.5. At 20° and at standard pressure, approximately 36 volumes dissolve in 1 volume of water and approximately 114 volumes dissolve in 1 volume of alcohol. Soluble also in ether and in chloroform. *NF category:* Antioxidant.

Sulfuric Acid: Clear, colorless, oily liquid. Miscible with water and with alcohol with the generation of much heat. Is very caustic and corrosive. Specific gravity is about 1.84. *NF category:* Acidifying agent.

Sulindac: Yellow, crystalline powder, which is odorless or practically so. Slightly soluble in methanol, in alcohol, in acetone, and in chloroform; very slightly soluble in isopropanol and in ethyl acetate; practically insoluble in hexane and in water.

Sulisobenzone: Light tan powder, with a melting point of about 145° . Freely soluble in methanol, in alcohol, and in water; sparingly soluble in ethyl acetate.

Sumatriptan: White to pale yellow powder. Very slightly soluble in water.

Sumatriptan Succinate: White or almost white powder. Freely soluble in water; sparingly soluble in methanol; practically insoluble in methylene chloride.

Suprofen: White to off-white powder, odorless to having a slight odor. Sparingly soluble in water.

Syrup: *NF category:* Sweetening agent; tablet binder; vehicle (flavored and/or sweetened).

Tacrine Hydrochloride: White powder. Freely soluble in water, in 0.1 N hydrochloric acid, in pH 4.0 acetate buffer, in phosphate buffer (pH between 7.0 and 7.4), in methanol, in dimethylsulfoxide, in alcohol, and in propylene glycol; sparingly soluble in linoleic acid and in polyethylene glycol 400.

Add the following:

▲ **Tadalafil:** White or almost white powder. Freely soluble in dimethyl sulfoxide; slightly soluble in methylene chloride; practically insoluble in water. ▲ *USP36*

Tagatose: White or almost white crystals, having a sweet taste. Very soluble in water; very slightly soluble in alcohol. *NF category:* Sweetening agent; humectant.

Talc: Very fine, white or grayish-white, crystalline powder. Is unctuous, adheres readily to the skin, and is free from grittiness. *NF category:* Glidant and/or anticaking agent; tablet and/or capsule lubricant.

Tamoxifen Citrate: White, fine, crystalline powder. Soluble in methanol; very slightly soluble in water, in acetone,

in chloroform, and in alcohol. Melts at about 142° , with decomposition.

Tamsulosin Hydrochloride: White or almost white crystalline powder. Melts with decomposition at approximately 230° . Freely soluble in formic acid; sparingly soluble in methanol; slightly soluble in water and in dehydrated alcohol; practically insoluble in ether.

Tannic Acid: Amorphous powder, glistening scales, or spongy masses, varying in color from yellowish-white to light brown. Is odorless or has a faint, characteristic odor, and has a strongly astringent taste. Very soluble in water, in acetone, and in alcohol; freely soluble in diluted alcohol; slightly soluble in dehydrated alcohol; practically insoluble in benzene, in chloroform, in ether, and in solvent hexane; 1 g dissolves in about 1 mL of warm glycerin.

Tartaric Acid: Colorless or translucent crystals or white, fine to granular, crystalline powder. Is odorless, has an acid taste, and is stable in air. Very soluble in water; freely soluble in alcohol. *NF category:* Acidifying agent.

Taurine: White crystals or crystalline powder. Soluble in water.

Tazobactam: White to pale yellow, nonhygroscopic, crystalline powder. Soluble in dimethylformamide; slightly soluble in water, in methanol, in acetone, and in alcohol; very slightly soluble in ethyl acetate, in ethyl ether, and in chloroform; insoluble in hexane.

Technetium Tc 99m Aggregated Albumin Injection: Milky suspension, from which particles settle upon standing.

Technetium Tc 99m Pentetate Injection: Clear, colorless solution.

Sodium Pertechnetate Tc 99m Injection: Clear, colorless solution.

Technetium Tc 99m (Pyro- and trimeta-) Phosphates Injection: Clear solution.

Technetium Tc 99m Sulfur Colloid Injection: Colloidal dispersion. Slightly opalescent, colorless to light tan liquid.

Telmisartan: White or slightly yellowish, crystalline powder. Sparingly soluble in methylene chloride; slightly soluble in methanol; practically insoluble in water. It dissolves in 1 M sodium hydroxide.

Temazepam: White or nearly white, crystalline powder. Sparingly soluble in alcohol; very slightly soluble in water. Melts between 157° and 163° , within a 3° range.

Temozolomide: White to light pink/light tan powder. Soluble in dimethyl sulfoxide; sparingly soluble in water; practically insoluble in toluene.

Terazosin Hydrochloride: White to pale yellow, crystalline powder. Freely soluble in isotonic saline solution; soluble in methanol and in water; slightly soluble in alcohol and in 0.1 N hydrochloric acid; very slightly soluble in chloroform; practically insoluble in acetone and in hexanes.

Terbinafine Hydrochloride: White or off-white powder. Freely soluble in dehydrated alcohol and in methanol; slightly soluble in acetone; very slightly or slightly soluble in water.

Terbutaline Sulfate: White to gray-white, crystalline powder. Is odorless or has a faint odor of acetic acid. Soluble in water and in 0.1 N hydrochloric acid; slightly soluble in methanol; insoluble in chloroform.

Terconazole: White to off-white powder. Freely soluble in methylene chloride; soluble in acetone; sparingly soluble in alcohol; practically insoluble in water. It exhibits polymorphism.

Terpin Hydrate: Colorless, lustrous crystals or white powder. Has a slight odor, and effloresces in dry air. A hot solution (1 in 100) is neutral to litmus. When dried in vacuum at 60° for 2 hours, it melts at about 103° . Very soluble in boiling alcohol; soluble in alcohol; sparingly soluble in boiling water; slightly soluble in water, in chloroform, and in ether.

Testolactone: White to off-white, practically odorless, crystalline powder. Melts at about 218°. Soluble in alcohol and in chloroform; slightly soluble in water and in benzyl alcohol; insoluble in ether and in solvent hexane.

Testosterone: White or slightly creamy white crystals or crystalline powder. Is odorless, and is stable in air. Freely soluble in dehydrated alcohol and in chloroform; soluble in dioxane and in vegetable oils; slightly soluble in ether; practically insoluble in water.

Testosterone Cypionate: White or creamy white, crystalline powder. Is odorless or has a slight odor, and is stable in air. Freely soluble in alcohol, in chloroform, in dioxane, and in ether; soluble in vegetable oils; insoluble in water.

Testosterone Enanthate: White or creamy white, crystalline powder. Is odorless or has a faint odor characteristic of heptanoic acid. Very soluble in ether; soluble in vegetable oils; insoluble in water.

Testosterone Propionate: White or creamy white crystals or crystalline powder. Is odorless and is stable in air. Freely soluble in alcohol, in dioxane, in ether, and in other organic solvents; soluble in vegetable oils; insoluble in water.

Tetanus Immune Globulin: Transparent or slightly opalescent liquid, practically colorless and practically odorless. May develop a slight granular deposit during storage.

Tetanus Toxoid: Clear, colorless to brownish-yellow, or slightly turbid liquid, free from evident clumps or particles, having a characteristic odor or an odor of formaldehyde.

Tetanus Toxoid Adsorbed: Turbid, white, slightly gray, or slightly pink suspension, free from evident clumps after shaking.

Tetanus and Diphtheria Toxoids Adsorbed for Adult Use: Turbid, white, slightly gray, or cream-colored suspension, free from evident clumps after shaking.

Tetracaine: White or light yellow, waxy solid. Soluble in alcohol, in ether, in benzene, and in chloroform; very slightly soluble in water.

Tetracaine Hydrochloride: Fine, white, crystalline, odorless powder. Has a slightly bitter taste followed by a sense of numbness. Its solutions are neutral to litmus. Melts at about 148°, or may occur in either of two other polymorphic modifications that melt at about 134° and 139°, respectively. Mixtures of the forms may melt within the range of 134° to 147°. Is hygroscopic. Very soluble in water; soluble in alcohol; insoluble in ether and in benzene.

Tetracycline: Yellow, odorless, crystalline powder. Is stable in air, but exposure to strong sunlight causes it to darken. It loses potency in solutions of pH below 2, and is rapidly destroyed by alkali hydroxide solutions. Freely soluble in dilute acid and in alkali hydroxide solutions; sparingly soluble in alcohol; very slightly soluble in water; practically insoluble in chloroform and in ether.

Tetracycline Hydrochloride: Yellow, odorless, crystalline powder. Is moderately hygroscopic. Is stable in air, but exposure to strong sunlight in moist air causes it to darken. It loses potency in solution at a pH below 2, and is rapidly destroyed by alkali hydroxide solutions. Soluble in water and in solutions of alkali hydroxides and carbonates; slightly soluble in alcohol; practically insoluble in chloroform and in ether.

Tetrahydrozoline Hydrochloride: White, odorless solid. Melts at about 256°, with decomposition. Freely soluble in water and in alcohol; very slightly soluble in chloroform; practically insoluble in ether.

Thalidomide: White to off-white powder. Very soluble in dimethylformamide, in dioxane, and in pyridine; sparingly soluble in acetone, in butyl acetate, in ethanol, in ethyl acetate, in glacial acetic acid, in methanol, and in water; practically insoluble in benzene, in chloroform, and in ether.

Theophylline: White, odorless, crystalline powder, having a bitter taste. Is stable in air. Freely soluble in solutions of alkali hydroxides and in ammonia; sparingly soluble in alcohol, in chloroform, and in ether; slightly soluble in water, but more soluble in hot water.

Theophylline Sodium Glycinate: White, crystalline powder having a slight ammoniacal odor and a bitter taste. Freely soluble in water; very slightly soluble in alcohol; practically insoluble in chloroform.

Thiabendazole: White to practically white, odorless or practically odorless powder. Slightly soluble in acetone and in alcohol; very slightly soluble in chloroform and in ether; practically insoluble in water.

Thiacetarsamide: White to yellowish, crystalline powder. Soluble in warm dehydrated alcohol and in warm methanol; sparingly soluble in cold dehydrated alcohol, in cold methanol, and in cold water; more soluble in water above 90°; insoluble in warm isopropyl alcohol. pK_a is 4.

Thiamine Hydrochloride: White crystals or crystalline powder, usually having a slight, characteristic odor. When exposed to air, the anhydrous product rapidly absorbs about 4% of water. Melts at about 248°, with some decomposition. Freely soluble in water; soluble in glycerin; slightly soluble in alcohol; insoluble in ether and in benzene.

Thiamine Mononitrate: White crystals or crystalline powder, usually having a slight, characteristic odor. Slightly soluble in water; slightly soluble in alcohol; very slightly soluble in chloroform.

Thiethylperazine Maleate: Yellowish, granular powder. Is odorless or has not more than a slight odor. Melts at about 183°, with decomposition. Slightly soluble in methanol; practically insoluble in water and in chloroform.

Thimerosal: Light cream-colored, crystalline powder, having a slight characteristic odor. Is affected by light. The pH of a solution (1 in 100) is about 6.7. Freely soluble in water; soluble in alcohol; practically insoluble in ether. *NF* category: Antimicrobial preservative.

Thimerosal Topical Solution: Clear liquid, having a slight characteristic odor. Is affected by light.

Thimerosal Tincture: Transparent, mobile liquid, having the characteristic odor of alcohol and acetone. Is affected by light.

Thioguanine: Pale yellow, odorless or practically odorless, crystalline powder. Freely soluble in dilute solutions of alkali hydroxides; insoluble in water, in alcohol, and in chloroform.

Thiopental Sodium: White to off-white, crystalline powder, or yellowish-white to pale greenish-yellow, hygroscopic powder. May have a disagreeable odor. Its solutions are alkaline to litmus. Its solutions decompose on standing, and on boiling precipitation occurs. Soluble in water and in alcohol; insoluble in benzene, in absolute ether, and in solvent hexane.

Thiopental Sodium for Injection: White to off-white, crystalline powder, or yellowish-white to pale greenish-yellow, hygroscopic powder. May have a disagreeable odor. Its solutions are alkaline to litmus. Its solutions decompose on standing, and on boiling precipitation occurs.

Thioridazine: White to slightly yellow, crystalline or micronized powder, odorless or having a faint odor. Very soluble in chloroform; freely soluble in dehydrated alcohol and in ether; practically insoluble in water.

Thioridazine Hydrochloride: White to slightly yellow, granular powder, having a faint odor and a very bitter taste. Freely soluble in water, in methanol, and in chloroform; insoluble in ether.

Thiostrepton: White to off-white, crystalline solid. Soluble in glacial acetic acid, in chloroform, in dimethylformamide, in dimethyl sulfoxide, in dioxane, and in pyridine; practically insoluble in water, in the lower alcohols, in

nonpolar organic solvents, and in dilute aqueous acids or alkali.

Thiotepa: Fine, white, crystalline flakes, having a faint odor. Freely soluble in water, in alcohol, in chloroform, and in ether.

Thiotepa for Injection: White powder.

Thiothixene: White to tan, practically odorless crystals. Is affected by light. Very soluble in chloroform; slightly soluble in methanol and in acetone; practically insoluble in water.

Thiothixene Hydrochloride: White, or practically white, crystalline powder, having a slight odor. Is affected by light. Soluble in water; slightly soluble in chloroform; practically insoluble in benzene, in acetone, and in ether.

Threonine: White, odorless crystals, having a slightly sweet taste. Freely soluble in water; insoluble in absolute alcohol, in ether, and in chloroform.

Thrombin: White to grayish, amorphous substance dried from the frozen state.

Thymol: Colorless, often large, crystals, or white, crystalline powder, having an aromatic, thyme-like odor and a pungent taste. Is affected by light. Its alcohol solution is neutral to litmus. Freely soluble in alcohol, in chloroform, in ether, and in olive oil; soluble in glacial acetic acid and in fixed and volatile oils; very slightly soluble in water. *NF category:* Antimicrobial preservative; flavors and perfumes.

Thyroid: Yellowish to buff-colored, amorphous powder, having a slight, characteristic, meat-like odor and a saline taste.

Tiagabine Hydrochloride: White to off-white powder. Freely soluble in methanol and in alcohol; soluble in isopropanol; very slightly soluble in chloroform; sparingly soluble in water; practically insoluble in *n*-heptane.

Tiamulin: A sticky, translucent yellowish mass, slightly hygroscopic. Very soluble in dichloromethane; freely soluble in dehydrated alcohol; practically insoluble in water.

Ticarcillin Disodium: White to pale yellow powder, or white to pale yellow solid. Freely soluble in water.

Ticlopidine: White or almost white crystalline powder. Sparingly soluble in water and in alcohol; very slightly soluble in ethyl acetate.

Tiletamine Hydrochloride: White to off-white, crystalline powder. Freely soluble in water and in 0.1 N hydrochloric acid; soluble in methanol; slightly soluble in chloroform; practically insoluble in ether.

Tilmicosin: White to off-white, amorphous solid. Slightly soluble in water and in *n*-hexane.

Timolol Maleate: White to practically white, odorless or practically odorless powder. Soluble in water, in alcohol, and in methanol; sparingly soluble in chloroform and in propylene glycol; insoluble in ether and in cyclohexane.

Tinidazole: Almost white or pale yellow, crystalline powder. Soluble in acetone and in methylene chloride; sparingly soluble in methanol; practically insoluble in water.

Titanium Dioxide: White, odorless, tasteless powder. Its 1 in 10 suspension in water is neutral to litmus. Insoluble in water, in hydrochloric acid, in nitric acid, and in 2 N sulfuric acid. Dissolves in hydrofluoric acid and in hot sulfuric acid. Is rendered soluble by fusion with potassium bisulfate or with alkali carbonates or hydroxides. *NF category:* Coating agent.

Tizanidine Hydrochloride: Almost white to slightly yellow, crystalline powder. Slightly soluble in water and in methanol.

Tobramycin: White to off-white, hygroscopic powder. Freely soluble in water; very slightly soluble in alcohol; practically insoluble in chloroform and in ether.

Tobramycin Sulfate Injection: Clear, colorless solution.

Tocainide Hydrochloride: Fine, white, odorless powder. Freely soluble in water and in alcohol; practically insoluble in chloroform and in ether.

Tocopherol: Clear, colorless to yellow, yellowish-brown, or greenish-yellow, viscous oil. Is odorless. Soluble in oils, in fats, in acetone, in alcohol, in chloroform, in ether, and in alcohol; insoluble in water. *NF category:* Antioxidant.

Tocopherols Excipient: Brownish-red to red, clear, viscous oil, having a mild, characteristic odor and taste. May show a slight separation of waxlike constituents in microcrystalline form. Oxidizes and darkens slowly in air and on exposure to light, particularly in alkaline media. Soluble in alcohol; insoluble in water. Miscible with acetone, with chloroform, with ether, and with vegetable oils. *NF category:* Antioxidant.

Tolazamide: White to off-white, crystalline powder, odorless or having a slight odor. Melts with decomposition in the approximate range of 161° to 173°. Freely soluble in chloroform; soluble in acetone; slightly soluble in alcohol; very slightly soluble in water.

Tolazoline Hydrochloride: White to off-white, crystalline powder. Its solutions are slightly acid to litmus. Freely soluble in water and in alcohol.

Tolbutamide: White, or practically white, crystalline powder. Is slightly bitter and practically odorless. Soluble in alcohol and in chloroform; practically insoluble in water.

Tolbutamide Sodium: White to off-white, practically odorless, crystalline powder, having a slightly bitter taste. Freely soluble in water; soluble in alcohol and in chloroform; very slightly soluble in ether.

Tolcapone: Yellow, fine powder or fine powder with lumps. Freely soluble in acetone and in tetrahydrofuran; soluble in methanol and in ethyl acetate; sparingly soluble in chloroform and in dichloromethane; insoluble in water and in *n*-hexane.

Tolmetin Sodium: Light yellow to light orange, crystalline powder. Freely soluble in water and in methanol; slightly soluble in alcohol; very slightly soluble in chloroform.

Tolnaftate: White to creamy white, fine powder, having a slight odor. Freely soluble in acetone and in chloroform; sparingly soluble in ether; slightly soluble in alcohol; practically insoluble in water.

Tolu Balsam: Brown or yellowish-brown, plastic solid, transparent in thin layers and brittle when old, dried, or exposed to cold temperatures. Has a pleasant, aromatic odor resembling that of vanilla, and a mild, aromatic taste. Soluble in alcohol, in chloroform, and in ether, sometimes with slight residue or turbidity; practically insoluble in water and in solvent hexane. *NF category:* Flavors and perfumes.

Topiramate: White to off-white powder. Freely soluble in dichloromethane.

Torsemide: White to off-white, crystalline powder. Slightly soluble in 0.1 N sodium hydroxide, in 0.1 N hydrochloric acid, in alcohol, and in methanol; very slightly soluble in acetone and in chloroform; practically insoluble in water and in ether.

Tragacanth: Is odorless, and has an insipid, mucilaginous taste. *NF category:* Suspending and/or viscosity-increasing agent.

Tramadol Hydrochloride: White, crystalline powder. Freely soluble in water and in methanol; very slightly soluble in acetone.

Trandolopril: White or almost white powder. Freely soluble in methylene chloride; sparingly soluble in absolute alcohol; practically insoluble in water.

Tranexamic Acid: White, crystalline powder. Freely soluble in water and in glacial acetic acid; practically insoluble in acetone and in alcohol.

Tranlycypromine Sulfate: White or almost white crystalline powder. Freely soluble in water; very slightly soluble in alcohol and in ether; practically insoluble in chloroform.

Travoprost: Clear, colorless, viscous oil. Insoluble in water.

Trazodone Hydrochloride: White to off-white, crystalline powder. Sparingly soluble in chloroform and in water. Melts between 231° and 234° when the melting point determination is carried out in an evacuated capillary tube; otherwise melts with decomposition over a broad range below 230°.

Trehalose: White, odorless, nonhygroscopic crystalline powder. Soluble in water, solubility increases with temperature; practically insoluble in dehydrated alcohol. Trehalose is typically used in the dihydrate form. *NF category:* Bulking agent for freeze drying; sweetening agent; tablet binder; tablet and/or capsule diluent; tablet disintegrant; vehicle (flavored and/or sweetened).

Tretinoin: Yellow to light-orange, crystalline powder. Slightly soluble in alcohol, in chloroform, and in methanol; insoluble in water.

Triacetin: Colorless, somewhat oily liquid having a slight, fatty odor and a bitter taste. Soluble in water; slightly soluble in carbon disulfide. Miscible with alcohol, with ether, and with chloroform. *NF category:* Plasticizer.

Triamcinolone: White or practically white, odorless, crystalline powder. Slightly soluble in alcohol and in methanol; very slightly soluble in water, in chloroform, and in ether.

Triamcinolone Acetonide: White to cream-colored, crystalline powder, having not more than a slight odor. Sparingly soluble in dehydrated alcohol, in chloroform, and in methanol; practically insoluble in water.

Triamcinolone Diacetate: Fine, white to off-white, crystalline powder, having not more than a slight odor. Soluble in chloroform; sparingly soluble in alcohol and in methanol; slightly soluble in ether; practically insoluble in water.

Triamcinolone Hexacetonide: White to cream-colored powder. Soluble in chloroform; slightly soluble in methanol; practically insoluble in water.

Triamterene: Yellow, odorless, crystalline powder. Soluble in formic acid; sparingly soluble in methoxyethanol; very slightly soluble in acetic acid, in alcohol, and in dilute mineral acids; practically insoluble in water, in benzene, in chloroform, in ether, and in dilute alkali hydroxides.

Triazolam: White to off-white, practically odorless, crystalline powder. Soluble in chloroform; slightly soluble in alcohol; practically insoluble in ether and in water.

Tributyl Citrate: Clear, practically colorless, oily liquid. Freely soluble in alcohol, in isopropyl alcohol, in acetone, and in toluene; insoluble in water. *NF category:* Plasticizer.

Trichlorfon: White crystalline powder. Very soluble in methylene chloride; freely soluble in acetone, in alcohol, in benzene, in chloroform, in ether, and in water; very slightly soluble in hexane and in pentane. Decomposed by alkali. Melts at about 78° with decomposition.

Trichlormethiazide: White or practically white, crystalline powder. Is odorless, or has a slight characteristic odor. Melts at about 274°, with decomposition. Freely soluble in acetone; soluble in methanol; sparingly soluble in alcohol; very slightly soluble in water, in ether, and in chloroform.

Trichloromonofluoromethane: Clear, colorless gas, having a faint, ethereal odor. Its vapor pressure at 25° is about 796 mm of mercury (1 psig). *NF category:* Aerosol propellant.

Triclosan: Fine, whitish, crystalline powder. Melts at about 57°. Soluble in methanol, in alcohol, and in acetone; slightly soluble in hexane; practically insoluble in water.

Trientine Hydrochloride: White to pale yellow, crystalline powder. Melts at about 117°. Freely soluble in water;

soluble in methanol; slightly soluble in alcohol; insoluble in chloroform and in ether.

Triethyl Citrate: Practically colorless, oily liquid. Soluble in water. Miscible with alcohol and with ether. *NF category:* Plasticizer.

Trifluoperazine Hydrochloride: White to pale yellow, crystalline powder. Is practically odorless, and has a bitter taste. Melts at about 242°, with decomposition. Freely soluble in water; soluble in alcohol; sparingly soluble in chloroform; insoluble in ether and in benzene.

Triflupromazine: Viscous, light amber-colored, oily liquid, which crystallizes on prolonged standing into large, irregular crystals. Practically insoluble in water.

Triflupromazine Hydrochloride: White to pale tan, crystalline powder, having a slight, characteristic odor. Melts between 170° and 178°. Soluble in water, in alcohol, and in acetone; insoluble in ether.

Trifluridine: Odorless, white powder appearing under the microscope as rodlike crystals; melts at 175°, with sublimation.

Medium-Chain Triglycerides: Colorless or slightly yellowish, oily liquid. Practically insoluble in water. Miscible with alcohol, with methylene chloride, with hexane, and with fatty oils.

Trihexyphenidyl Hydrochloride: White or slightly off-white, crystalline powder, having not more than a very faint odor. Melts at about 250°. Soluble in alcohol and in chloroform; slightly soluble in water.

Timeprazine Tartrate: White to off-white, odorless, crystalline powder. Freely soluble in water and in chloroform; soluble in alcohol; very slightly soluble in ether and in benzene.

Trimethobenzamide Hydrochloride: White, crystalline powder having a slight phenolic odor. Soluble in water and in warm alcohol; insoluble in ether and in benzene.

Trimethoprim: White to cream-colored, odorless crystals, or crystalline powder. Soluble in benzyl alcohol; sparingly soluble in chloroform and in methanol; slightly soluble in alcohol and in acetone; very slightly soluble in water; practically insoluble in ether and in carbon tetrachloride.

Trimethoprim Sulfate: White to off-white, crystalline powder. Soluble in water, in alcohol, in dilute mineral acids, and in fixed alkalies.

Trimipramine Maleate: White to almost white crystalline powder. Slightly soluble in water and in alcohol.

Trioxsalen: White to off-white or grayish, odorless, crystalline solid. Melts at about 230°. Sparingly soluble in chloroform; slightly soluble in alcohol; practically insoluble in water.

Tripelennamine Hydrochloride: White, crystalline powder. Slowly darkens on exposure to light. Its solutions are practically neutral to litmus. Freely soluble in water, in alcohol, and in chloroform; slightly soluble in acetone; insoluble in benzene, in ether, and in ethyl acetate.

Tripolidine Hydrochloride: White, crystalline powder, having no more than a slight, but unpleasant, odor. Its solutions are alkaline to litmus, and it melts at about 115°. Soluble in water, in alcohol, and in chloroform; insoluble in ether.

Trolamine: Colorless to pale yellow, viscous, hygroscopic liquid having a slight, ammoniacal odor. Soluble in chloroform. Miscible with water and with alcohol. *NF category:* Alkalinizing agent; emulsifying and/or solubilizing agent.

Troleandomycin: White, odorless, crystalline powder. Freely soluble in alcohol; soluble in chloroform; slightly soluble in ether and in water.

Tromethamine: White, crystalline powder, having a slight, characteristic odor. Freely soluble in water.

Tropicamide: White or practically white, crystalline powder, odorless or having not more than a slight odor.

Freely soluble in chloroform and in solutions of strong acids; slightly soluble in water.

Tropium Chloride: Colorless or white to slightly yellow crystalline powder. Very soluble in water; freely soluble in methanol.

Crystallized Trypsin: White to yellowish white, odorless, crystalline or amorphous powder.

Tryptophan: White to slightly yellowish-white crystals or crystalline powder, having a slightly bitter taste. Soluble in hot alcohol and in dilute hydrochloric acid.

Tuberculin: Old Tuberculin is a clear, brownish liquid, which is readily miscible with water and has a characteristic odor. Purified Protein Derivative (PPD) of Tuberculin is a very slightly opalescent, colorless solution. Old Tuberculin and PPD concentrates contain 50% of glycerin for use with various application devices. Old Tuberculin and PPD are also dried on the tines of multiple-puncture devices.

Tubocurarine Chloride: White or yellowish-white to grayish-white, crystalline powder. Melts at about 270°, with decomposition. Soluble in water; sparingly soluble in alcohol.

Tylosin: White to buff-colored powder. Freely soluble in methanol; soluble in alcohol, in amyl acetate, in chloroform, and in dilute mineral acids; slightly soluble in water.

Tylosin Tartrate: Almost white or slightly yellow, hygroscopic powder. Freely soluble in water and in dichloromethane; slightly soluble in alcohol. It dissolves in dilute solutions of mineral acids.

Tyloxapol: Viscous, amber liquid, having a slight, aromatic odor. May exhibit a slight turbidity. Slowly but freely miscible with water. Soluble in glacial acetic acid, in benzene, in toluene, in carbon tetrachloride, in chloroform, and in carbon disulfide. *NF category:* Wetting and/or solubilizing agent.

Tyrosine: White, odorless, tasteless crystals or crystalline powder. Very slightly soluble in water; insoluble in alcohol and in ether.

Ubidecarenone: Yellow to orange, crystalline powder. Melts at about 48°. Soluble in ether; very slightly soluble in dehydrated alcohol; practically insoluble in water.

Undecylenic Acid: Clear, colorless to pale yellow liquid having a characteristic odor. Practically insoluble in water. Miscible with alcohol, with chloroform, with ether, with benzene, and with fixed and volatile oils.

Urea: Colorless to white, prismatic crystals, or white, crystalline powder, or small white pellets. Is practically odorless, but may gradually develop a slight odor of ammonia upon long standing. Its solutions are neutral to litmus. Freely soluble in water and in boiling alcohol; practically insoluble in chloroform and in ether.

Urea C 13: See *Urea*.

Ursodiol: White or almost white, crystalline powder. Freely soluble in alcohol and in glacial acetic acid; sparingly soluble in chloroform; slightly soluble in ether; practically insoluble in water.

Vaccinia Immune Globulin: Transparent or slightly opalescent liquid. Is practically colorless and practically odorless. May develop a slight, granular deposit during storage.

Valacyclovir Hydrochloride: White to off-white powder. Soluble in water; insoluble in dichloromethane.

Powdered Valerian Extract: Brown, hygroscopic, powdery or easily pulverizable mass. Soluble in water to form a slightly cloudy solution; sparingly soluble in 70 percent alcohol; practically insoluble in alcohol.

Valganciclovir Hydrochloride: White to off-white powder. Very slightly soluble in alcohol; practically insoluble in 2-propanol, in hexane, in acetone, and in ethyl acetate.

Valine: White, odorless, tasteless crystals. Soluble in water; practically insoluble in ether, in alcohol, and in acetone.

Valproic Acid: Colorless to pale yellow, slightly viscous, clear liquid, having a characteristic odor. Refractive index: about 1.423 at 20°. Freely soluble in 1 N sodium hydroxide, in methanol, in alcohol, in acetone, in chloroform, in benzene, in ether, and in *n*-heptane; slightly soluble in water and in 0.1 N hydrochloric acid.

Valrubicin: Orange to orange-red, crystalline powder. Soluble in methylene chloride, in dehydrated alcohol, in methanol, and in acetone; very slightly soluble in water, in hexane, and in petroleum ether.

Valsartan: White or almost white, hygroscopic powder. Freely soluble in anhydrous ethanol; sparingly soluble in methylene chloride; practically insoluble in water.

Vancomycin Hydrochloride: White, almost white, or tan to brown, free-flowing powder, odorless, and having a bitter taste. Freely soluble in water; insoluble in ether and in chloroform.

Vanillin: Fine, white to slightly yellow crystals, usually needle-like, having an odor and taste suggestive of vanilla. Is affected by light. Its solutions are acid to litmus. Freely soluble in alcohol, in chloroform, in ether, and in solutions of the fixed alkali hydroxides; soluble in glycerin and in hot water; slightly soluble in water. *NF category:* Flavors and perfumes.

Vasopressin Injection: Clear, colorless or practically colorless liquid, having a faint, characteristic odor.

Vecuronium Bromide: White or creamy white crystals, or a crystalline powder. Sparingly soluble in alcohol; slightly soluble in water and in acetone.

Hydrogenated Vegetable Oil: Type I Hydrogenated Vegetable Oil—Fine, white powder, beads, or small flakes. Type II Hydrogenated Vegetable Oil—Plastic (semi-solid) or flakes having a softer consistency than Type I. Soluble in hot isopropyl alcohol, in hexane, and in chloroform; insoluble in water. *NF category:* Type I Hydrogenated Vegetable Oil—Tablet and/or capsule lubricant; Type II Hydrogenated Vegetable Oil—Ointment base.

Venlafaxine Hydrochloride: Off-white to white crystalline powder. Soluble in methanol and in water.

Verapamil Hydrochloride: White or practically white, crystalline powder. Is practically odorless and has a bitter taste. Freely soluble in chloroform; soluble in water; sparingly soluble in alcohol; practically insoluble in ether.

Vidarabine: White to off-white powder. Slightly soluble in dimethylformamide; very slightly soluble in water.

Vinblastine Sulfate: White or slightly yellow, odorless, amorphous or crystalline powder. Is hygroscopic. Freely soluble in water.

Vincristine Sulfate: White to slightly yellow, odorless, amorphous or crystalline powder. Is hygroscopic. Freely soluble in water; soluble in methanol; slightly soluble in alcohol.

Vincristine Sulfate for Injection: Yellowish-white solid, having the characteristic appearance of products prepared by freeze-drying.

Vinorelbine Tartrate: White to yellow or light brown, amorphous powder. Freely soluble in water.

Vitamin A: In liquid form, a light-yellow to red oil that may solidify upon refrigeration. In solid form, has the appearance of any diluent that has been added. May be practically odorless or may have a mild fishy odor, but has no rancid odor or taste. Is unstable to air and light. In liquid form, very soluble in chloroform and in ether; soluble in absolute alcohol and in vegetable oils; insoluble in water and in glycerin. In solid form, may be dispersible in water.

Vitamin E: Practically odorless and tasteless. The alpha tocopherols and alpha tocopheryl acetates occur as clear, yellow, or greenish yellow, viscous oils. *d*-Alpha tocopheryl

acetate may solidify in the cold. Alpha tocopheryl acid succinate occurs as a white powder; the *d*-isomer melts at about 75°, and the *dl*-form melts at about 70°. The alpha tocopherols are unstable to air and light, particularly when in alkaline media. The esters are stable to air and light, but are unstable to alkali; the acid succinate is also unstable when held molten. Alpha tocopheryl acid succinate is very soluble in chloroform; soluble in alcohol, in ether, in acetone, and in vegetable oils; slightly soluble in alkaline solutions; insoluble in water. The other forms of Vitamin E are insoluble in water; soluble in alcohol; miscible with ether, with acetone, with vegetable oils, and with chloroform.

Vitamin E Preparation: The liquid forms are clear, yellow to brownish red, viscous oils. The solid forms are white to tan-white granular powders. The liquid forms are soluble in alcohol; insoluble in water. Miscible with ether, with acetone, with vegetable oils, and with chloroform. The solid forms disperse in water to give cloudy suspensions.

Voriconazole: White to almost white powder. Freely soluble in acetone and in methylene chloride; very slightly soluble in water.

Warfarin Sodium: White, odorless, amorphous or crystalline powder, having a slightly bitter taste. Is discolored by light. Very soluble in water; freely soluble in alcohol; very slightly soluble in chloroform and in ether.

Water for Injection: Clear, colorless, odorless liquid. *NF category:* Solvent.

Bacteriostatic Water for Injection: Clear, colorless liquid, odorless or having the odor of the antimicrobial substance. *NF category:* Vehicle (sterile).

Sterile Water for Inhalation: Clear, colorless solution.

Sterile Water for Injection: Clear, colorless, odorless liquid. *NF category:* Solvent.

Sterile Water for Irrigation: Clear, colorless, odorless liquid. *NF category:* Solvent.

Purified Water: Clear, colorless, odorless liquid. *NF category:* Solvent.

Carnauba Wax: Light brown to pale yellow, moderately coarse powder or flakes, possessing a characteristic bland odor, and free from rancidity. Specific gravity is about 0.99. Freely soluble in warm benzene; soluble in warm chloroform and in warm toluene; slightly soluble in boiling alcohol; insoluble in water. *NF category:* Coating agent.

Emulsifying Wax: Creamy white, wax-like solid, having a mild, characteristic odor. Freely soluble in ether, in chloroform, in most hydrocarbon solvents, and in aerosol propellants; soluble in alcohol; insoluble in water. *NF category:* Emulsifying and/or solubilizing agent; stiffening agent.

Microcrystalline Wax: White or cream-colored, odorless, waxy solid. Soluble in chloroform, in ether, in volatile oils, and in most warm fixed oils; sparingly soluble in dehydrated alcohol; insoluble in water. *NF category:* Coating agent.

White Wax: Yellowish-white solid, somewhat translucent in thin layers. Has a faint, characteristic odor, and is free from rancidity. Specific gravity is about 0.95. Sparingly soluble in cold alcohol; insoluble in water. Boiling alcohol dissolves the cerotic acid and a portion of the myricin, which are constituents of White Wax. Completely soluble in chloroform, in ether, and in fixed and volatile oils. Partly soluble in cold benzene and in cold carbon disulfide; completely soluble in these liquids at about 30°. *NF category:* Stiffening agent.

Yellow Wax: Solid varying in color from yellow to grayish brown. Has an agreeable, honey-like odor. Is somewhat brittle when cold, and presents a dull, granular, noncrystalline fracture when broken. It becomes pliable from the heat of the hand. Specific gravity is about 0.95. Sparingly soluble in cold alcohol; insoluble in water. Boiling alcohol dissolves the cerotic acid and a portion of the myricin, that are constituents of Yellow Wax. Soluble in chloroform, in ether, in fixed oils, and in volatile oils; sparingly soluble in cold ben-

zene and in cold carbon disulfide; soluble in these liquids at about 30°. *NF category:* Stiffening agent.

Wheat Bran: Light tan powder having a characteristic aroma. Practically insoluble in cold water and in alcohol. Available in a variety of particle sizes depending upon the degree of milling to which it is subjected. Color and flavor development variable, depending on the extent to which it is heat-stabilized.

Xanthan Gum: Cream-colored powder. Its solutions in water are neutral to litmus. Soluble in hot or cold water. *NF category:* Suspending and/or viscosity-increasing agent.

Xenon Xe 127: Clear, colorless gas.

Xenon Xe 133 Injection: Clear, colorless solution.

Xylazine: Colorless to white crystals. Sparingly soluble in dilute acid, in acetone, and in chloroform; insoluble in dilute alkali.

Xylazine Hydrochloride: Colorless to white crystals. Sparingly soluble in dilute acid, in acetone, and in methanol; insoluble in dilute alkali.

Xylitol: White crystals or crystalline powder. It has a sweet taste and produces a cooling sensation in the mouth. One g dissolves in about 0.65 mL of water. Sparingly soluble in alcohol. Crystalline xylitol has a melting range between 92° and 96°.

Xylometazoline Hydrochloride: White to off-white, odorless, crystalline powder. Melts above 300°, with decomposition. Freely soluble in alcohol; soluble in water; sparingly soluble in chloroform; practically insoluble in benzene and in ether.

Xylose: Colorless needles or white, crystalline powder. Is odorless, and has a slightly sweet taste. Very soluble in water; slightly soluble in alcohol.

Yellow Fever Vaccine: Slightly dull, light-orange colored, flaky or crustlike, desiccated mass.

Yohimbine Hydrochloride: White to yellow powder. Melts at about 295°, with decomposition. Soluble in boiling water; slightly soluble in water and in alcohol.

Yttrium Chloride: Colorless, deliquescent crystals. Soluble in water and in alcohol.

Zalcitabine: White to off-white, crystalline powder. Soluble in water and in methanol; sparingly soluble in alcohol, in acetonitrile, in chloroform, and in methylene chloride; slightly soluble in cyclohexane.

Zaleplon: White to off-white powder. Sparingly soluble in alcohol; slightly soluble in propylene glycol; practically insoluble in water.

Zein: White to yellow powder. Soluble in aqueous alcohols, in glycols, in ethylene glycol ethyl ether, in furfuryl alcohol, in tetrahydrofurfuryl alcohol, in aqueous alkaline solutions of pH 11.5 or greater, and in acetone-water mixtures between the limits of 60% and 80% of acetone by volume; insoluble in water, in acetone, and in all anhydrous alcohols except methanol. *NF category:* Coating agent.

Zidovudine: White to yellowish powder. Melts at about 124°. Exhibits polymorphism. Soluble in alcohol; sparingly soluble in water.

Zileuton: White to off-white powder.

Zinc Acetate: White crystals or granules, having a slight acetous odor and an astringent taste. Is slightly efflorescent. Freely soluble in water and in boiling alcohol; slightly soluble in alcohol.

Zinc Chloride: White or practically white, odorless, crystalline powder, or white or practically white crystalline granules. May also be in porcelain-like masses or molded into cylinders. Is very deliquescent. A solution (1 in 10) is acid to litmus. Very soluble in water; freely soluble in alcohol and in glycerin. Its solution in water or in alcohol is usually slightly turbid, but the turbidity disappears when a small quantity of hydrochloric acid is added.

Zinc Gluconate: White or practically white powder or granules. Soluble in water; very slightly soluble in alcohol.

Zinc Oxide: Very fine, odorless, amorphous, white or yellowish white powder, free from gritty particles. It gradually absorbs carbon dioxide from air. Soluble in dilute acids; insoluble in water and in alcohol.

Zinc Stearate: Fine, white, bulky powder, free from grittiness. Has a faint, characteristic odor. Is neutral to moistened litmus paper. Insoluble in water, in alcohol, and in ether. *NF category:* Tablet and/or capsule lubricant.

Zinc Sulfate: Colorless, transparent prisms, or small needles. May occur as a white, granular, crystalline powder. Is odorless and is efflorescent in dry air. Its solutions are acid to litmus. Very soluble in water (heptahydrate); freely soluble in water (monohydrate) and in glycerin (heptahydrate); practically insoluble in alcohol (monohydrate); insoluble in alcohol (heptahydrate).

Zinc Undecylenate: Fine, white powder. Practically insoluble in water and in alcohol.

Ziprasidone Hydrochloride: White to slightly pink powder. Very soluble in methanol; slightly soluble in isopropyl alcohol, and in hot tetrahydrofuran; practically insoluble in water.

Zolazepam Hydrochloride: White to off-white, crystalline powder. Freely soluble in water and in 0.1 N hydrochloric acid; soluble in methanol; slightly soluble in chloroform; practically insoluble in ether.

Zolpidem Tartrate: White to off-white powder, hygroscopic. Sparingly soluble in methanol; slightly soluble in water; practically insoluble in methylene chloride.

Zonisamide: White to off-white powder. Freely soluble in dimethylformamide; soluble in methanol.

SOLUBILITIES

Approximate Solubilities of USP and NF Articles

Solute (1 g)	Name and Volume, in mL, of Solvent					
	Water	Boiling Water	Alcohol	Chloroform	Ether	Other
Acenocoumarol	67,000		280	130	1800	
Acetaminophen		20	10			1 N sodium hydroxide, 15
Acetohexamide			230	210		
Acetylcysteine	5		4			
Acetyldigitoxin	6100		62.5	12	>10,000	
Ammonium Alum	7	0.5				
Aluminum Chloride	0.9		4			
Aluminum Sulfate	1					
Amantadine Hydrochloride	2.5		5.1	18		polyethylene glycol 400, 70
Amaranth	15					
Aminocaproic Acid	3					methanol, 450
Aminohippuric Acid	45		50			3 N hydrochloric acid, 5
Aminosalicylate Sodium	2					
Ammonium Carbonate	4					
Amodiaquine Hydrochloride	25		78	>10,000	>10,000	benzene, 10,000
Anethole ¹			2			
Anileridine	>10,000		2	1		
Anileridine Hydrochloride	5		80	>10,000	>10,000	
Antimony Potassium Tartrate	12	3				glycerin, 15
Apomorphine Hydrochloride	50		50			water at 80°, 20
Apraclonidine Hydrochloride	34		74	>10,000		methanol, 13; ethyl acetate, >10,000; hexanes, >10,000
Ascorbic Acid	3		40			
Ascorbyl Palmitate	>1000		125	>1000	>1000	
Aspirin	300		5	17	10 to 15	
Atropine	460		2	1	25	water at 80°, 90
Atropine Sulfate	0.5	2.5	5			glycerin, 2.5
Bendroflumethiazide			23		200	
Benoxinate Hydrochloride	0.8		2.6	2.5	>10,000	
Benzalkonium Chloride (anhydrous)					100	benzene, 6
Gamma Benzene Hexachloride				3.5	40	dehydrated alcohol, 20
Benzethonium Chloride	<1		<1	<1	6000	
Benzocaine	2500		5	2	4	almond oil or olive oil, 30–50
Benzoic Acid	300		3	5	3	
Benzonatate	<1		<1	<1	<1	
Betadex	54					
Betamethasone	5300		65	325		warm alcohol, 15; methanol, 3
Betamethasone Acetate	2000		9	16		
Betamethasone Sodium Phosphate	2		470	>10,000	>10,000	
Betamethasone Valerate	10,000		16	<10	400	
Bisacodyl	>10,000		210	2.5	275	
Boric Acid	18	4	18			boiling alcohol, 6; glycerin, 4
Bromodiphenhydramine Hydrochloride	<1		2	2	3500	isopropyl alcohol, 31
Brompheniramine Maleate	5		15	15		
Busulfan						acetone, 45
Butabarbital Sodium	2		7	7000	10,000	
Butamben	7000					
Butylated Hydroxyanisole			4.0	2.0	1.2	
Butylated Hydroxytoluene			4.0	1.1	1.1	
Caffeine (hydrous)	50		75	6	600	

¹ Solubility data for compounds that ordinarily are liquids at 25° are expressed in terms of the ratio of the *volume* of solute to the *volume* of solvent; i.e., 1 mL dissolved in _____ mL of solvent.

² Liquid phase only; silicon dioxide remains as a residue in these solvents.

Approximate Solubilities of USP and NF Articles (Continued)

Solute (1 g)	Name and Volume, in mL, of Solvent					
	Water	Boiling Water	Alcohol	Chloroform	Ether	Other
Calcium Chloride	0.7	0.2	4			boiling alcohol, 2
Calcium Gluconate	30 (slowly)	5				
Calcium Hydroxide	630	1300				
Calcium Lactate	20					
Calcium Pantothenate	3					
Calcium Sulfate	375	485				
Camphor	800		1	0.5	1	
Carbinoxamine Maleate	<1		1.5	1.5	8300	
Carisoprodol	2083		2.5	2.3		acetone, 2.5
Cephaloridine	5		1000	10,000	10,000	
Cetylpyridinium Chloride	4.5		2.5	4.5		
Chloral Betaine	1		4	>10,000	>10,000	0.1 N hydrochloric acid, 1; 0.1 N sodium hydroxide, 1
Chloral Hydrate	0.25		1.3	2	1.5	
Chlorambucil						acetone, 2
Chloramphenicol	400					
Chlordiazepoxide	>10,000		50	6250	130	
Chlorobutanol	125		1			glycerin, 10
Chlorocresol	260		0.4			
Chloroprocaine Hydrochloride	20		100			
Chlorpheniramine Maleate	4		10	10		
Chlorpromazine			3	2	3	benzene, 2
Chlorpromazine Hydrochloride	1		1.5	1.5		
Chlortetracycline Hydrochloride	75		560			
Cholesterol			100 (slowly)			dehydrated alcohol, 50
Citric Acid	0.5		2		30	
Clindamycin Palmitate Hydrochloride			3			ethyl acetate, 9
Clindamycin Phosphate	2.5		>1000	>1000	>1000	
Clioquinol	>100,000		3500	120	4500	
Cocaine	600		7	1	3.5	olive oil, 12; liquid petrolatum, 80–100
Cocaine Hydrochloride	0.5		3.5	15		
Codeine	120		2	0.5	50	
Codeine Phosphate	2.5		325			boiling alcohol, 125; water at 80°, 0.5
Codeine Sulfate	30		1300			water at 80°, 6.5
Colchicine	25				220	
Cortisone Acetate			350	4		acetone, 75; dioxane, 30
Cupric Sulfate	3	0.5	500			glycerin, 3
Cyanocobalamin	80					
Cyclizine Hydrochloride	115		115	75		
Cyproheptadine Hydrochloride	275		35	26		methanol, 1.5
Dehydrocholic Acid			100	35	2200 (15°)	acetic acid at 15°, 135; acetone at 15°, 130; benzene at 15°, 960; ethyl acetate at 15°, 135
Demeclocycline			200			methanol, 40
Demeclocycline Hydrochloride	60					methanol, 50
Denatonium Benzoate	20		2.4	2.9	5000	
Desipramine Hydrochloride	12		14	3.5	>10,000	
Dexamethasone Sodium Phosphate	2					
Dexbrompheniramine Maleate	1.2		2.5	2	3000	
Dexchlorpheniramine Maleate	1.1		2	1.7	2500	
Dextroamphetamine Sulfate	10		800			
Dextromethorphan Hydrobromide	65					

¹ Solubility data for compounds that ordinarily are liquids at 25° are expressed in terms of the ratio of the *volume* of solute to the *volume* of solvent; i.e., 1 mL dissolved in _____ mL of solvent.

² Liquid phase only; silicon dioxide remains as a residue in these solvents.

Approximate Solubilities of USP and NF Articles (Continued)

Solute (1 g)	Name and Volume, in mL, of Solvent					
	Water	Boiling Water	Alcohol	Chloroform	Ether	Other
Dextrose	1		100			
Diazepam	333		16	2	39	
Dibucaïne	4600		0.7	0.5	1.4	
Dicyclomine Hydrochloride	13		5	2	770	glacial acetic acid, 2
Diethylpropion Hydrochloride	0.5		3	3		
Digitoxin			150	40		
Dihydroergotamine Mesylate	125		90	175	2600	
Dimercaprol	20					
Dimethisterone			3	0.7		
Diphenhydramine Hydrochloride	1		2	2		acetone, 50
Disulfiram	>5000		30		15	
Docusate Calcium	3300		>1	>1	>1	
Docusate Sodium	70 (slowly)					
Doxapram Hydrochloride	50					
Doxylamine Succinate	1		2	2	370	
Droperidol	10,000		140	4	500	
Dyclonine Hydrochloride	60		24	2.3	>10,000	hexane, >10,000
Dydrogesterone	>10,000		40	2	200	
Echothiophate Iodide	1					dehydrated alcohol, 25; methanol, 3
Edrophonium Chloride	0.5		5			
Enalaprilat	200		>1000			dimethylformamide, 40; methanol, 68
Ephedrine	20		0.2			
Ephedrine Hydrochloride	3		14			
Ephedrine Sulfate	1.3		90			
Epinephrine Bitartrate	3					
Ergotamine Tartrate	500		500			
Erythromycin	1000					
Erythromycin Estolate			20	10		acetone, 15
Estradiol			28	435	150	
Estradiol Cypionate	>10,000		40	7	2800	
Estrone			250 (15°)	110 (15°)		boiling alcohol, 50; boiling chloroform, 80; acetone at 50°, 50; boiling acetone, 33; boiling benzene, 145
Estropipate	>2000		>2000	>2000	>2000	warm alcohol, 500
Ethacrynic Acid			1.6	6	3.5	
Ether ¹	12					
Ethyl Vanillin	100 (50°)		2			
Ferrous Gluconate	5					
Ferrous Sulfate	1.5	0.5				
Flumethasone Pivalate	>10,000		89	350	2800	
Fluocinolone Acetonide	>1000		45	25	350	
Fluorometholone	>10,000		200	2200	>10,000	
Fluphenazine Enanthate			<1	<1	2	
Fluphenazine Hydrochloride	1.4		6.7			
Flurandrenolide			72	10		methanol, 25
Flurazepam Hydrochloride	2		4	90	5000	methanol, 3; isopropanol, 69; benzene, 2500; petroleum ether, 5000
Fluroxene	220					
Fructose			15			methanol, 14
Gentian Violet			10			glycerin, 15
Glyceryl Monostearate				10.0	100.0	methanol, 100; isopropyl alcohol, 33
Glycine	4		1254			water at 50°, 2.6; at 75°, 1.9; at 100°, 1.5

¹ Solubility data for compounds that ordinarily are liquids at 25° are expressed in terms of the ratio of the *volume* of solute to the *volume* of solvent; i.e., 1 mL dissolved in _____ mL of solvent.

² Liquid phase only; silicon dioxide remains as a residue in these solvents.

Approximate Solubilities of USP and NF Articles (Continued)

Solute (1 g)	Name and Volume, in mL, of Solvent					
	Water	Boiling Water	Alcohol	Chloroform	Ether	Other
Glycopyrrolate	4.2		30	260	35,000	
Guaifenesin	60–70					
Halazone	>1000		140	>1000	>2000	
Haloperidol	>10,000		60	15	200	
Heparin Sodium	20					
Histamine Phosphate	4					
Homatropine Hydrobromide	6		40	420		
Hydralazine Hydrochloride	25		500			
Hydrocortisone			40			acetone, 80
Hydrocortisone Acetate			230	200		
Hydrocortisone Sodium Phosphate	1.5					
Hydroflumethiazide	>5000		39	>5000	2500	
Hydromorphone Hydrochloride	3					
Hydroquinone	17		4	51	16.5	
Hydroxocobalamin	50		100	10,000	10,000	
Hydroxyzine Hydrochloride	1		4.5	13	>1000	
Hydroxyzine Pamoate	>1000		700	>1000	>1000	dimethylformamide, 10; 10 N sodium hydroxide, 3.5
Hyoscyamine Hydrobromide			2.5	1.7	2300	
Hyoscyamine Sulfate	0.5		5			
Ichthammol	10					
Indigotindisulfonate Sodium	100					
Indomethacin			50	30	40	
Iodine	3000		13			carbon disulfide, 4; glycerin, 80
Ipodate Sodium	<1		2			dimethylacetamide, 2; dimethylformamide, 3.5; dimethylsulfoxide, 3.5
Isoniazid	8		50			
Isopropamide Iodide	50		10	5		
Isopropyl Alcohol	<1		<1	<1	<1	
Isoproterenol Hydrochloride	3		50			
Isoproterenol Sulfate	4		>2000	>2000	>2000	
Isoxsuprine Hydrochloride	500		100	>10,000	>10,000	0.1 N hydrochloric acid, 2500; 0.1 N sodium hydroxide, 100
Ketamine Hydrochloride	4		14	60	>10,000	methanol, 6; absolute alcohol, 60
Lactose	5 (slowly)	2.6				
Levorphanol Tartrate	50		120			
Levothyroxine Sodium	700		300			
Lisinopril	10		>10,000	>10,000	>10,000	methanol, 70
Magnesium Hydroxide	>10,000		>10,000	>10,000	>10,000	
Magnesium Sulfate	0.8	0.5				glycerin, 1 (slowly)
Mannitol	5.5					
Menadione			60			benzene, 10; vegetable oils, 50
Mesoridazine Besylate	1		11	3	6300	
Methacholine Chloride	1.2		1.7	2.1		
Methacrylic Acid Copolymer						water in methanol (≥3 in 100), 10; water in alcohol (≥3 in 100), 10; water in isopropyl alcohol (≥3 in 100), 10; water in acetone (≥3 in 100), 10
Methacycline Hydrochloride	100		300	>1000	>1000	0.1 N sodium hydroxide, 25
Methdilazine Hydrochloride	2		2	6	>10,000	0.1 N hydrochloric acid, 1; 0.1 N sodium hydroxide, 1
Methenamine	1.5		12.5	10	320	
Methenamine Mandelate			10	20	350	
Methimazole	5		5	4.5	125	

¹ Solubility data for compounds that ordinarily are liquids at 25° are expressed in terms of the ratio of the *volume* of solute to the *volume* of solvent; i.e., 1 mL dissolved in _____ mL of solvent.

² Liquid phase only; silicon dioxide remains as a residue in these solvents.

Approximate Solubilities of USP and NF Articles (Continued)

Solute (1 g)	Name and Volume, in mL, of Solvent					
	Water	Boiling Water	Alcohol	Chloroform	Ether	Other
Methocarbamol	40 (20°)					
Methotrimeprazine	10		10	2		methanol, 10
Methoxyflurane	500		<1	<1	<1	
Methsuximide	350		3	<1	2	
Methyclothiazide	>10,000		92.5	>10,000	2700	
Methylbenzethonium Chloride	0.8		0.9	>10,000	0.7	
Methylene Blue	25		65			
Methylergonovine Maleate	100		175	1900	8400	
Methylparaben	400		3		10	water at 80°, 50
Methylprednisolone	10,000		100	800	800	
Methylprednisolone Acetate	1500		400	250	1500	
Methylprednisolone Sodium Succinate	1.5		12	>10,000	>10,000	
Methysergide Maleate	200		165	3400	>10,000	
Miconazole	>100,000		9.5	2	15	isopropyl alcohol, 4; propylene glycol, 9; methanol, 5.3
Miconazole Nitrate	6250		312	525	50,000	isopropyl alcohol, 1408; propylene glycol, 119; methanol, 75
Morphine Sulfate	16		570			alcohol at 60°, 240; water at 80°, 1
Nalidixic Acid	>1000		910	29	>1000	
Neomycin Sulfate	1					
Niacin	60					
Niacinamide	1.5	10	5.5			
Nifedipine	>10,000					acetone, 10
Nitrofurazone	4200		590			propylene glycol, 350
Nitromersol	>2000		>2000	>2000	>2000	
Norepinephrine Bitartrate	2.5		300			
Norethindrone Acetate	>10,000		10	<1	18	dioxane, 2
Nortriptyline Hydrochloride	90		30	20		methanol, 10
Oxandrolone	5200		57	<5	860	acetone, 69
Oxazepam	>10,000		220	270	2200	
Oxtriphylline	1					
Oxymetazoline Hydrochloride	6.7		3.6	862		
Oxymetholone	>10,000		40	5	82	dioxane, 14
Oxymorphone Hydrochloride	4		100	>1000	>1000	methanol, 25
Oxytetracycline	4150			>10,000	6250	absolute alcohol, 66
Oxytetracycline Calcium	>1000		>1000	>1000	>1000	0.1 N sodium hydroxide, 15
Papaverine Hydrochloride	30		120			
Paraldehyde ¹	10	17				
Paramethasone Acetate				50		methanol, 40
Paromomycin Sulfate	<1		>10,000	>10,000	>10,000	
Pectin	20					
Penicillin G Benzathine	5000		65			
Sterile Penicillin G Procaine	250		30	60		
Sterile Penicillin G Sodium	40					
Penicillin V Benzathine	3200		330	42	910	acetone, 37
Penicillin V Potassium			150			
Pentazocine	>1000		11	2	42	
Pentazocine Hydrochloride	30		20	4	>10,000	
Pentobarbital	>2000		4.5	4.0	10	
Pentolinium Tartrate	0.5		475	>1000	>2000	
Perphenazine			7			acetone, 13
Phenazopyridine Hydrochloride	<10	20	59	331	>5000	cold water, 300; glycerin, 100
Phenindamine Tartrate	40		350			
Phenmetrazine Hydrochloride	0.4		2	2		

¹ Solubility data for compounds that ordinarily are liquids at 25° are expressed in terms of the ratio of the *volume* of solute to the *volume* of solvent; i.e., 1 mL dissolved in _____ mL of solvent.

² Liquid phase only; silicon dioxide remains as a residue in these solvents.

Approximate Solubilities of USP and NF Articles (Continued)

Solute (1 g)	Name and Volume, in mL, of Solvent					
	Water	Boiling Water	Alcohol	Chloroform	Ether	Other
Phenobarbital	1000		10			
Phenol	15					mineral oil, 70
Phentolamine Mesylate	1		4	700		
Phenylethyl Alcohol	60		<1	<1	<1	alcohol solution (1 in 2), 2; diethylphthalate, <1; benzyl benzoate, <1
Phenylmercuric Acetate	180		225	6.8	200	
Phenylmercuric Nitrate	600					
Phenylpropanolamine Hydrochloride	1.1		7.4	4100		
Physostigmine Salicylate	75		16	6	250	
Physostigmine Sulfate	4		0.4		1200	
Pilocarpine Hydrochloride	0.3		3	360		
Pilocarpine Nitrate	4		75			
Piminodine Esylate	>1000		6	2	>1000	
Pimozide	>10,000		1000	10	1000	acetone, 100; methanol, 1000; 0.1 N hydrochloric acid, >1000
Polyethylene Glycol 1540	1			3		absolute alcohol, 100
Polyethylene Glycol 4000	4		2.5	2		
Potash, Sulfurated	2					
Potassium Acetate	0.5	0.2	3			
Potassium Alum	7	0.3				
Potassium Benzoate	2		75			alcohol solution (9 in 10), 50
Potassium Chloride	2.8	2				
Potassium Citrate	1					glycerin, 2.5
Potassium Gluconate	3					
Potassium Hydroxide	1		3			glycerin, 2.5
Potassium Iodide	0.7	0.5	22			glycerin, 2
Potassium Permanganate	15	3.5				
Potassium Sodium Tartrate	1					
Potassium Sorbate	4.5		35	>1000	>1000	
Pramoxine Hydrochloride				35		
Prednisolone			30	180		acetone, 50
Prednisolone Acetate			120			
Prednisolone Hemisuccinate	4170		6.3	1064	248	
Prednisolone Sodium Phosphate	4					methanol, 13
Prednisone			150	200		
Prilocaine Hydrochloride	3.5		4.2	175		
Primaquine Phosphate	15					
Primidone	2000		200			
Procaine Hydrochloride	1		15			
Prochlorperazine Edisylate	2		1500			
Prochlorperazine Maleate			1200			
Procyclidine Hydrochloride	35		9	6	11,000	
Promazine Hydrochloride	3					
Propoxycaïne Hydrochloride	2		10	>10,000	80	
Propoxyphene Napsylate	10,000		15	10		
Propylhexedrine	>500		0.4	0.2	0.1	
Propylparaben	2500	400	1.5		3	
Protriptyline Hydrochloride	2		3.5	2.5	>10,000	
Pseudoephedrine Hydrochloride	0.5		3.6	91	7000	
Pyrazinamide	67			135	1000	absolute alcohol, 175; methanol, 72
Pyridoxine Hydrochloride	5		115			
Pyrilamine Maleate	0.5		3	2		absolute alcohol, 15
Pyrimethamine			200	125		

¹ Solubility data for compounds that ordinarily are liquids at 25° are expressed in terms of the ratio of the *volume* of solute to the *volume* of solvent; i.e., 1 mL dissolved in _____ mL of solvent.

² Liquid phase only; silicon dioxide remains as a residue in these solvents.

Approximate Solubilities of USP and NF Articles (Continued)

Solute (1 g)	Name and Volume, in mL, of Solvent					
	Water	Boiling Water	Alcohol	Chloroform	Ether	Other
Pyrrocaine Hydrochloride	1.5		12	8		
Quinidine Sulfate	100		10	15		
Quinine Sulfate	500		120			
Reserpine			1800	6		
Resorcinol	1		1			
Rotoxamine Tartrate	10		100	>10,000	>10,000	
Saccharin	290	25	31			
Saccharin Calcium	2.6		4.7			
Saccharin Sodium	1.5		50			
Salicylic Acid	460	15	3	45	3	benzene, 135
Scopolamine Hydrobromide	1.5		20			
Secobarbital						0.5 N sodium hydroxide, 8.5
Selenium Sulfide				161	1667	
Sennosides	35		2100	3700	6100	
Silver Nitrate	0.4	0.1	30			boiling alcohol, 6.5
Simethicone ²	>10,000			10 ²	10 ²	benzene, 10 ² ; absolute alcohol, >10,000
Sodium Acetate	0.8	0.6	19			
Sodium Ascorbate	1.3					
Sodium Benzoate	2		75			90 percent alcohol, 50
Sodium Bicarbonate	12					
Sodium Bisulfite	4					
Sodium Borate	16	1				glycerin, 1
Sodium Carbonate	3	1.8				
Sodium Chloride	2.8	2.7				glycerin, 10
Sodium Citrate (hydrous)	1.5	0.6				
Sodium Fluoride	25					
Sodium Formaldehyde Sulfoxylate	3.4		510	175	180	
Sodium Hydroxide	1					
Sodium Iodide	0.6		2			glycerin, 1
Sodium Lauryl Sulfate	10					
Sodium Nitrite	1.5					
Sodium Phosphate, Dried	8					
Sodium Propionate	1	0.65	24	>10,000	>10,000	
Sodium Thiosulfate	0.5					
Sorbic Acid	1000		10	15	30	absolute alcohol, 8; methanol, 8; propylene glycol, 19
Sorbitol	0.45					
Stanozolol	>1000		41	74	370	
Stearic Acid			20	2	3	
Stibophen	1		>10,000	>10,000	10,000	
Succinylcholine Chloride	1		350			
Sucrose	0.5	0.2	170			
Sucrose Octaacetate	1100		11			acetone, 0.3; benzene, 0.6; toluene, 0.5
Sulconazole Nitrate	3333		100	333		pyridine, 10; methanol, 71; acetone, 130; methylene chloride, 286; toluene, 2000; dioxane, 2000
Sulfacetamide Sodium	2.5					
Sulfadiazine	13,000					human serum at 37°, 620
Sulfadiazine Sodium	2					
Sulfadimethoxine			200			2 N hydrochloric acid, 50
Sulfaethidole	>3000		75	1300	1700	methanol, 51; acetone, 13; benzene, 2277
Sulfamethizole	2000		38	1900	1900	acetone, 13
Sulfamethoxazole	3400		50	1000	1000	carbon disulfide, 2 (slowly and usually incompletely)

¹ Solubility data for compounds that ordinarily are liquids at 25° are expressed in terms of the ratio of the *volume* of solute to the *volume* of solvent; i.e., 1 mL dissolved in _____ mL of solvent.

² Liquid phase only; silicon dioxide remains as a residue in these solvents.

Approximate Solubilities of USP and NF Articles (Continued)

Solute (1 g)	Name and Volume, in mL, of Solvent					
	Water	Boiling Water	Alcohol	Chloroform	Ether	Other
Sulfapyridine	3500		440			acetone, 65
Sulfasalazine	>10,000		2900	>10,000	>10,000	methanol, 1500
Sulfisoxazole	7700					boiling alcohol, 10
Sulfisoxazole Acetyl			176	35	1064	methanol, 203
Precipitated Sulfur						carbon disulfide, 2 (slowly and usually incompletely); olive oil, 100
Tartaric Acid	0.8	0.5	3		250	methanol, 1.7
Terpin Hydrate	200	35	13	140	140	boiling alcohol, 3
Testolactone	4050					
Testosterone				2	100	absolute alcohol, 6
Tetracaine	>1000		5	2	2	
Tetracycline	2500		50			
Tetracycline Hydrochloride	10		100			
Tetrahydrozoline Hydrochloride	3.5		7.5	>1000	>1000	
Theophylline Sodium Glycinate	6					
Thiamine Hydrochloride	1		170			
Thiamine Mononitrate	44					
Thiethylperazine Maleate	1700		530	>10,000	>10,000	
Thimerosal	1		12			
Thioguanine			7700			
Thiotepa	13		8.3	1.9	4.1	
Thiothixene	>10,000			2	120	absolute alcohol, 110
Thiothixene Hydrochloride	8			280	>10,000	absolute alcohol, 270
Thymol	1000		1	1	1.5	olive oil, 2
Tolazoline Hydrochloride	<1		2	3	>10,000	
Triamcinolone Diacetate			13	80		methanol, 40
Triamterene						formic acid, 30; 2-methoxyethanol, 85
Triazolam	>10,000		1000	25	>10,000	0.1 N hydrochloric acid, 600
Trichlormethiazide	1100		48	5000	1400	dioxane, 9.1; dimethylformamide, 4.35
Trichloroethylene	>10,000					
Triethylenemelamine	2.5		13	3.6		methanol, 8; acetone, 9.5; benzene, 18
Trifluoperazine Hydrochloride	3.5		11	100		
Triflupromazine Hydrochloride	<1		<1	1.7		
Trimeprazine Tartrate	2		20	5	1800	
Trimethobenzamide Hydrochloride	2		59	67	720	
Trioxsalen			1150	84		methylenedichloride, 43; 4-methyl-2-pentanone, 100
Tripelennamine Hydrochloride	1		6	6		acetone, 350
Tripolidine Hydrochloride	2.1		1.8	1	2000	
Tromethamine	1.8		45.5	>10,000		
Tubocurarine Chloride	20		45			
Urea	1.5		10			boiling alcohol, 1
Vanillin	100					glycerin, 20; water at 80°, 20
Xylometazoline Hydrochloride	35					
Zinc Acetate	2.5		30			
Zinc Chloride	0.5		1.5			glycerin, 2
Zinc Sulfate	0.6					glycerin, 2.5

¹ Solubility data for compounds that ordinarily are liquids at 25° are expressed in terms of the ratio of the *volume* of solute to the *volume* of solvent; i.e., 1 mL dissolved in _____ mL of solvent.

² Liquid phase only; silicon dioxide remains as a residue in these solvents.

ATOMIC WEIGHTS

Standard Atomic Weights of the Elements, Recommended by the Commission on Atomic Weights and Isotopic Abundances of the International Union of Pure and Applied Chemistry (2007) (©2008 IUPAC)

Standard atomic weights 2007 [In alphabetical order: scaled to $A_r(12\text{C}) = 12$, where ^{12}C is a neutral atom in its nuclear and electronic ground state.]

The atomic weights of many elements are not invariant but depend on the origin and treatment of the material. The standard values of $A_r(\text{E})$ and the uncertainties (in parentheses, following the last significant figure to which they are attributed) apply to elements of natural terrestrial origin. The footnotes to this Table elaborate the types of variation which may occur for individual elements and which may be larger than the listed uncertainties of values of $A_r(\text{E})$. Names of elements with atomic numbers 112, 113, 114, 115, 116, and 118 are temporary.

Name	Atomic Symbol	Atomic Number	Atomic Weight	Footnotes
Actinium*	Ac	89		
Aluminum	Al	13	26.9815386(8)	
Americium*	Am	95		
Antimony (Stibium)	Sb	51	121.760(1)	q
Argon	Ar	18	39.948(1)	q, r
Arsenic	As	33	74.92160(2)	
Astatine*	At	85		
Barium	Ba	56	137.327(7)	
Berkelium*	Bk	97		
Beryllium	Be	4	9.012182(3)	
Bismuth	Bi	83	208.98040(1)	
Bohrium*	Bh	107		
Boron	B	5	10.811(7)	q, m, r
Bromine	Br	35	79.904(1)	
Cadmium	Cd	48	112.411(8)	q
Caesium (Cesium)	Cs	55	132.9054519(2)	
Calcium	Ca	20	40.078(4)	q
Californium*	Cf	98		
Carbon	C	6	12.0107(8)	q, r
Cerium	Ce	58	140.116(1)	q
Chlorine	Cl	17	35.453(2)	m
Chromium	Cr	24	51.9961(6)	
Cobalt	Co	27	58.933195(5)	
Copper	Cu	29	63.546(3)	r
Curium*	Cm	96		
Darmstadtium*	Ds	110		
Dubnium*	Db	105		
Dysprosium	Dy	66	162.500(1)	q
Einsteinium*	Es	99		
Erbium	Er	68	167.259(3)	q
Europium	Eu	63	151.964(1)	q
Fermium*	Fm	100		
Fluorine	F	9	18.9984032(5)	
Francium*	Fr	87		
Gadolinium	Gd	64	157.25(3)	q
Gallium	Ga	31	69.723(1)	
Germanium	Ge	32	72.64(1)	
Gold	Au	79	196.966569(4)	
Hafnium	Hf	72	178.49(2)	

*Element has no stable nuclides. One or more well-known isotopes are given in the accompanying table with the appropriate relative atomic mass and half-life. However, three such elements (Th, Pa, and U) do have a characteristic terrestrial isotopic composition, and for these an atomic weight is tabulated.

†Commercially available Li materials have atomic weights that are known to range between 6.939 and 6.996; if a more accurate value is required, it must be determined for the specific material.

‡Geological specimens are known in which the element has an isotopic composition outside the limits for normal material. The difference between the atomic weight of the element in such specimens and that given in the Table may exceed the stated uncertainty.

§Modified isotopic compositions may be found in commercially available material because it has been subjected to an undisclosed or inadvertent isotopic fractionation. Substantial deviations in atomic weight of the element from that given in the Table can occur.

¶Range in isotopic composition of normal terrestrial material prevents a more precise $A_r(\text{E})$ being given; the tabulated $A_r(\text{E})$ value should be applicable to any normal material.

Name	Atomic Symbol	Atomic Number	Atomic Weight	Footnotes
Hassium*	Hs	108		
Helium	He	2	4.002602(2)	g, r
Holmium	Ho	67	164.93032(2)	
Hydrogen	H	1	1.00794(7)	g, m, r
Indium	In	49	114.818(3)	
Iodine	I	53	126.90447(3)	
Iridium	Ir	77	192.217(3)	
Iron	Fe	26	55.845(2)	
Krypton	Kr	36	83.798(2)	g, m
Lanthanum	La	57	138.90547(7)	g
Lawrencium*	Lr	103		
Lead	Pb	82	207.2(1)	g, r
Lithium	Li	3	6.941(2) [†]	g, m, r
Lutetium	Lu	71	174.9668(1)	g
Magnesium	Mg	12	24.3050(6)	
Manganese	Mn	25	54.938045(5)	
Meitnerium*	Mt	109		
Mendelevium*	Md	101		
Mercury	Hg	80	200.59(2)	
Molybdenum	Mo	42	95.96(2)	g
Neodymium	Nd	60	144.242(3)	g
Neon	Ne	10	20.1797(6)	g, m
Neptunium*	Np	93		
Nickel	Ni	28	58.6934(4)	
Niobium	Nb	41	92.90638(2)	
Nitrogen	N	7	14.0067(2)	g, r
Nobelium*	No	102		
Osmium	Os	76	190.23(3)	g
Oxygen	O	8	15.9994(3)	g, r
Palladium	Pd	46	106.42(1)	g
Phosphorus	P	15	30.973762(2)	
Platinum	Pt	78	195.084(9)	
Plutonium*	Pu	94		
Polonium*	Po	84		
Potassium (Kalium)	K	19	39.0983(1)	
Praseodymium	Pr	59	140.90765(2)	
Promethium*	Pm	61		
Protactinium*	Pa	91	231.03588(2)	
Radium*	Ra	88		
Radon*	Rn	86		
Rhenium	Re	75	186.207(1)	
Rhodium	Rh	45	102.90550(2)	
Roentgenium*	Rg	111		
Rubidium	Rb	37	85.4678(3)	g
Ruthenium	Ru	44	101.07(2)	g
Rutherfordium*	Rf	104		
Samarium	Sm	62	150.36(2)	g
Scandium	Sc	21	44.955912(6)	
Seaborgium*	Sg	106		
Selenium	Se	34	78.96(3)	
Silicon	Si	14	28.0855(3)	r

*Element has no stable nuclides. One or more well-known isotopes are given in the accompanying table with the appropriate relative atomic mass and half-life. However, three such elements (Th, Pa, and U) do have a characteristic terrestrial isotopic composition, and for these an atomic weight is tabulated.

[†]Commercially available Li materials have atomic weights that are known to range between 6.939 and 6.996; if a more accurate value is required, it must be determined for the specific material.

[‡]Geological specimens are known in which the element has an isotopic composition outside the limits for normal material. The difference between the atomic weight of the element in such specimens and that given in the *Table* may exceed the stated uncertainty.

[§]Modified isotopic compositions may be found in commercially available material because it has been subjected to an undisclosed or inadvertent isotopic fractionation. Substantial deviations in atomic weight of the element from that given in the *Table* can occur.

^{||}Range in isotopic composition of normal terrestrial material prevents a more precise Ar(E) being given; the tabulated Ar(E) value should be applicable to any normal material.

Name	Atomic Symbol	Atomic Number	Atomic Weight	Footnotes
Silver	Ag	47	107.8682(2)	g
Sodium (Natrium)	Na	11	22.98976928(2)	
Strontium	Sr	38	87.62(1)	g, r
Sulfur	S	16	32.065(5)	g, r
Tantalum	Ta	73	180.94788(2)	
Technetium*	Tc	43		
Tellurium	Te	52	127.60(3)	g
Terbium	Tb	65	158.92535(2)	
Thallium	Tl	81	204.3833(2)	
Thorium*	Th	90	232.03806(2)	g
Thulium	Tm	69	168.93421(2)	
Tin	Sn	50	118.710(7)	g
Titanium	Ti	22	47.867(1)	
Tungsten (Wolfram)	W	74	183.84(1)	
Ununbium*	Uub	112		
Ununhexium*	Uuh	116		
Ununoctium*	Uuo	118		
Ununpentium*	Uup	115		
Ununquadium*	Uuq	114		
Ununtrium*	Uut	113		
Uranium*	U	92	238.02891(3)	g, m
Vanadium	V	23	50.9415(1)	
Xenon	Xe	54	131.293(6)	g, m
Ytterbium	Yb	70	173.054(5)	g
Yttrium	Y	39	88.90585(2)	
Zinc	Zn	30	65.38(2)	
Zirconium	Zr	40	91.224(2)	g

*Element has no stable nuclides. One or more well-known isotopes are given in the accompanying table with the appropriate relative atomic mass and half-life. However, three such elements (Th, Pa, and U) do have a characteristic terrestrial isotopic composition, and for these an atomic weight is tabulated.

[†]Commercially available Li materials have atomic weights that are known to range between 6.939 and 6.996; if a more accurate value is required, it must be determined for the specific material.

^gGeological specimens are known in which the element has an isotopic composition outside the limits for normal material. The difference between the atomic weight of the element in such specimens and that given in the *Table* may exceed the stated uncertainty.

^mModified isotopic compositions may be found in commercially available material because it has been subjected to an undisclosed or inadvertent isotopic fractionation. Substantial deviations in atomic weight of the element from that given in the *Table* can occur.

^rRange in isotopic composition of normal terrestrial material prevents a more precise Ar(E) being given; the tabulated Ar(E) value should be applicable to any normal material.

Relative Atomic Masses and Half-Lives of Selected Radionuclides (© 1998 IUPAC).

[Abbreviations for units are: a = year; d = day; h = hour; min = minute; s = second; ms = millisecond. Names of elements with atomic numbers 110, 111, and 112 are temporary.]

Atomic Number	Name	Symbol	Mass Number	Relative Atomic Mass	Half-Life and Uncertainty	Unit
43	Technetium	Tc	97	96.9064	$2.6 \pm 0.4 \times 10^6$	a
			98	97.9072	$4.2 \pm 0.3 \times 10^6$	a
			99	98.9063	$2.1 \pm 0.3 \times 10^5$	a
61	Promethium	Pm	145	144.9127	17.7 ± 0.4	a
			147	146.9151	2.623 ± 0.003	a
84	Polonium	Po	209	208.9824	102 ± 5	a
			210	209.9828	138.4 ± 0.1	d
85	Astatine	At	210	209.9871	8.1 ± 0.4	h
			211	210.9875	7.21 ± 0.01	h
86	Radon	Rn	211	210.9906	14.6 ± 0.2	h
			220	220.0114	55.6 ± 0.1	s
			222	222.0176	3.823 ± 0.004	d
87	Francium	Fr	223	223.0197	22.0 ± 0.1	min
88	Radium	Ra	223	223.0185	11.43 ± 0.01	d
			224	224.0202	3.66 ± 0.02	d
			226	226.0254	1599 ± 4	a
			228	228.0311	5.75 ± 0.03	a
89	Actinium	Ac	227	227.0277	21.77 ± 0.02	a
90	Thorium	Th	230	230.0331	$7.54 \pm 0.03 \times 10^4$	a
			232	232.0380	$1.40 \pm 0.01 \times 10^{10}$	a
			231	231.0359	$3.25 \pm 0.01 \times 10^4$	a
			233	233.0396	$1.592 \pm 0.002 \times 10^5$	a
91	Protactinium	Pa	234	234.0409	$2.455 \pm 0.006 \times 10^5$	a
			235	235.0439	$7.04 \pm 0.01 \times 10^8$	a
			236	236.0456	$2.342 \pm 0.004 \times 10^7$	a
			238	238.0508	$4.47 \pm 0.02 \times 10^9$	a
			237	237.0482	$2.14 \pm 0.01 \times 10^6$	a
			239	239.0529	2.355 ± 0.006	d
			238	238.0496	87.7 ± 0.1	a
94	Plutonium	Pu	239	239.0522	$2.410 \pm 0.003 \times 10^4$	a
			240	240.0538	$6.56 \pm 0.01 \times 10^3$	a
			241	241.0568	14.4 ± 0.1	a
			242	242.0587	$3.75 \pm 0.02 \times 10^5$	a
			244	244.0642	$8.00 \pm 0.09 \times 10^7$	a
			241	241.0568	432.7 ± 0.6	a
			243	243.0614	$7.37 \pm 0.02 \times 10^3$	a
96	Curium	Cm	243	243.0614	29.1 ± 0.1	a
			244	244.0627	18.1 ± 0.1	a
			245	245.0655	$8.48 \pm 0.06 \times 10^3$	a
			246	246.0672	$4.76 \pm 0.04 \times 10^3$	a
			247	247.0703	$1.56 \pm 0.05 \times 10^7$	a
			248	248.0723	$3.48 \pm 0.06 \times 10^5$	a
			247	247.0703	$1.4 \pm 0.3 \times 10^3$	a
97	Berkelium	Bk	249	249.0750	$3.20 \pm 0.06 \times 10^2$	d
			249	249.0748	351 ± 2	a
98	Californium	Cf	250	250.0764	13.1 ± 0.1	a
			251	251.0796	$9.0 \pm 0.5 \times 10^2$	a
			252	252.0816	2.64 ± 0.01	a
			252	252.0830	472 ± 2	d
99	Einsteinium	Es	252	252.0830	100.5 ± 0.2	d
100	Fermium	Fm	257	257.0951	78 ± 2	min
101	Mendelevium	Md	256	256.0941	51.5 ± 0.3	d
			258	258.0984		d

^a The uncertainties of these elements are asymmetric.

^b The value given is determined from only a few decays.

Atomic Number	Name	Symbol	Mass Number	Relative Atomic Mass	Half-Life and Uncertainty	Unit
102	Nobelium	No	259	259.1011	58±5	min
103	Lawrencium	Lr	262	262.110	3.6±0.3	min
104	Rutherfordium	Rf	261	261.1088	1.3 ^a	min
105	Dubnium	Db	262	262.1141	34±5	s
106	Seaborgium	Sg	266	266.1219	21 ^a	s
107	Bohrium	Bh	264	264.1247	0.44 ^a	s
108	Hassium	Hs	269	269.1341	9.35 ^{a,b}	s
109	Meitnerium	Mt	268	268.1388	70 ^{a,b}	ms
110	Ununnilium	Uun	271	271.1461	1.1 ^{a,b}	ms
111	Unununium	Uuu	272	272.1535	1.5 ^{a,b}	ms
112	Ununbium	Uub	277		0.24 ^{a,b}	ms

^a The uncertainties of these elements are asymmetric.

^b The value given is determined from only a few decays.

ALCOHOLOMETRIC TABLE

Based on data appearing in the National Bureau of Standards Bulletin, vol. 9, pp. 424–425 (publication of the National Institute of Standards and Technology).

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Percentage of C ₂ H ₅ OH		Specific gravity in air		Percentage of C ₂ H ₅ OH		Specific gravity in air	
By volume at 15.56°C	By weight	At 25°/25°	At 15.56°/15.56°	By weight	By volume at 15.56°C	At 25°/25°	At 15.56°/15.56°
0	0.00	1.0000	1.0000	0	0.00	1.0000	1.0000
1	0.80	0.9985	0.9985	1	1.26	0.9981	0.9981
2	1.59	0.9970	0.9970	2	2.51	0.9963	0.9963
3	2.39	0.9956	0.9956	3	3.76	0.9945	0.9945
4	3.19	0.9941	0.9942	4	5.00	0.9927	0.9928
5	4.00	0.9927	0.9928	5	6.24	0.9911	0.9912
6	4.80	0.9914	0.9915	6	7.48	0.9894	0.9896
7	5.61	0.9901	0.9902	7	8.71	0.9879	0.9881
8	6.42	0.9888	0.9890	8	9.94	0.9863	0.9867
9	7.23	0.9875	0.9878	9	11.17	0.9848	0.9852
10	8.05	0.9862	0.9866	10	12.39	0.9833	0.9839
11	8.86	0.9850	0.9854	11	13.61	0.9818	0.9825
12	9.68	0.9838	0.9843	12	14.83	0.9804	0.9812
13	10.50	0.9826	0.9832	13	16.05	0.9789	0.9799
14	11.32	0.9814	0.9821	14	17.26	0.9776	0.9787
15	12.14	0.9802	0.9810	15	18.47	0.9762	0.9774
16	12.96	0.9790	0.9800	16	19.68	0.9748	0.9763
17	13.79	0.9778	0.9789	17	20.88	0.9734	0.9751
18	14.61	0.9767	0.9779	18	22.08	0.9720	0.9738
19	15.44	0.9756	0.9769	19	23.28	0.9706	0.9726
20	16.27	0.9744	0.9759	20	24.47	0.9692	0.9714
21	17.10	0.9733	0.9749	21	25.66	0.9677	0.9701
22	17.93	0.9721	0.9739	22	26.85	0.9663	0.9688
23	18.77	0.9710	0.9729	23	28.03	0.9648	0.9675
24	19.60	0.9698	0.9719	24	29.21	0.9633	0.9662
25	20.44	0.9685	0.9708	25	30.39	0.9617	0.9648
26	21.29	0.9673	0.9697	26	31.56	0.9601	0.9635
27	22.13	0.9661	0.9687	27	32.72	0.9585	0.9620
28	22.97	0.9648	0.9676	28	33.88	0.9568	0.9605
29	23.82	0.9635	0.9664	29	35.03	0.9551	0.9590
30	24.67	0.9622	0.9653	30	36.18	0.9534	0.9574
31	25.52	0.9609	0.9641	31	37.32	0.9516	0.9558
32	26.38	0.9595	0.9629	32	38.46	0.9498	0.9541
33	27.24	0.9581	0.9617	33	39.59	0.9480	0.9524
34	28.10	0.9567	0.9604	34	40.72	0.9461	0.9506
35	28.97	0.9552	0.9590	35	41.83	0.9442	0.9488
36	29.84	0.9537	0.9576	36	42.94	0.9422	0.9470
37	30.72	0.9521	0.9562	37	44.05	0.9402	0.9451
38	31.60	0.9506	0.9548	38	45.15	0.9382	0.9432
39	32.48	0.9489	0.9533	39	46.24	0.9362	0.9412
40	33.36	0.9473	0.9517	40	47.33	0.9341	0.9392
41	34.25	0.9456	0.9501	41	48.41	0.9320	0.9372
42	35.15	0.9439	0.9485	42	49.48	0.9299	0.9352
43	36.05	0.9421	0.9469	43	50.55	0.9278	0.9331
44	36.96	0.9403	0.9452	44	51.61	0.9256	0.9310
45	37.87	0.9385	0.9434	45	52.66	0.9235	0.9289
46	38.78	0.9366	0.9417	46	53.71	0.9213	0.9268
47	39.70	0.9348	0.9399	47	54.75	0.9191	0.9246
48	40.62	0.9328	0.9380	48	55.78	0.9169	0.9225
49	41.55	0.9309	0.9361	49	56.81	0.9147	0.9203
50	42.49	0.9289	0.9342	50	57.83	0.9124	0.9181
51	43.43	0.9269	0.9322	51	58.84	0.9102	0.9159
52	44.37	0.9248	0.9302	52	59.85	0.9079	0.9137

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Percentage of C ₂ H ₅ OH		Specific gravity in air		Percentage of C ₂ H ₅ OH		Specific gravity in air	
By volume at 15.56°C	By weight	At 25°/25°	At 15.56°/15.56°	By weight	By volume at 15.56°C	At 25°/25°	At 15.56°/15.56°
53	45.33	0.9228	0.9282	53	60.85	0.9056	0.9114
54	46.28	0.9207	0.9262	54	61.85	0.9033	0.9092
55	47.25	0.9185	0.9241	55	62.84	0.9010	0.9069
56	48.21	0.9164	0.9220	56	63.82	0.8987	0.9046
57	49.19	0.9142	0.9199	57	64.80	0.8964	0.9024
58	50.17	0.9120	0.9177	58	65.77	0.8941	0.9001
59	51.15	0.9098	0.9155	59	66.73	0.8918	0.8978
60	52.15	0.9076	0.9133	60	67.79	0.8895	0.8955
61	53.15	0.9053	0.9111	61	68.64	0.8871	0.8932
62	54.15	0.9030	0.9088	62	69.59	0.8848	0.8909
63	55.17	0.9006	0.9065	63	70.52	0.8824	0.8886
64	56.18	0.8983	0.9042	64	71.46	0.8801	0.8862
65	57.21	0.8959	0.9019	65	72.38	0.8777	0.8839
66	58.24	0.8936	0.8995	66	73.30	0.8753	0.8815
67	59.28	0.8911	0.8972	67	74.21	0.8729	0.8792
68	60.33	0.8887	0.8948	68	75.12	0.8706	0.8768
69	61.38	0.8862	0.8923	69	76.02	0.8682	0.8745
70	62.44	0.8837	0.8899	70	76.91	0.8658	0.8721
71	63.51	0.8812	0.8874	71	77.79	0.8634	0.8697
72	64.59	0.8787	0.8848	72	78.67	0.8609	0.8673
73	65.67	0.8761	0.8823	73	79.54	0.8585	0.8649
74	66.77	0.8735	0.8797	74	80.41	0.8561	0.8625
75	67.87	0.8709	0.8771	75	81.27	0.8537	0.8601
76	68.98	0.8682	0.8745	76	82.12	0.8512	0.8576
77	70.10	0.8655	0.8718	77	82.97	0.8488	0.8552
78	71.23	0.8628	0.8691	78	83.81	0.8463	0.8528
79	72.38	0.8600	0.8664	79	84.64	0.8439	0.8503
80	73.53	0.8572	0.8636	80	85.46	0.8414	0.8479
81	74.69	0.8544	0.8608	81	86.28	0.8389	0.8454
82	75.86	0.8516	0.8580	82	87.08	0.8364	0.8429
83	77.04	0.8487	0.8551	83	87.89	0.8339	0.8404
84	78.23	0.8458	0.8522	84	88.68	0.8314	0.8379
85	79.44	0.8428	0.8493	85	89.46	0.8288	0.8354
86	80.66	0.8397	0.8462	86	90.24	0.8263	0.8328
87	81.90	0.8367	0.8432	87	91.01	0.8237	0.8303
88	83.14	0.8335	0.8401	88	91.77	0.8211	0.8276
89	84.41	0.8303	0.8369	89	92.52	0.8184	0.8250
90	85.69	0.8271	0.8336	90	93.25	0.8158	0.8224
91	86.99	0.8237	0.8303	91	93.98	0.8131	0.8197
92	88.31	0.8202	0.8268	92	94.70	0.8104	0.8170
93	89.65	0.8167	0.8233	93	95.41	0.8076	0.8142
94	91.03	0.8130	0.8196	94	96.10	0.8048	0.8114
95	92.42	0.8092	0.8158	95	96.79	0.8020	0.8086
96	93.85	0.8053	0.8118	96	97.46	0.7992	0.8057
97	95.32	0.8011	0.8077	97	98.12	0.7962	0.8028
98	96.82	0.7968	0.8033	98	98.76	0.7932	0.7998
99	98.38	0.7921	0.7986	99	99.39	0.7902	0.7967
100	100.00	0.7871	0.7936	100	100.00	0.7871	0.7936

INTRINSIC VISCOSITY TABLE

This table is based on data appearing in Table 3 of ASTM Test Method D 1795 (publication of the American Society for Testing and Materials).

Intrinsic Viscosity, $[\eta]c$, at Different Values of Relative Viscosity, $\eta_{rel}^{A,B}$

η_{rel}	$[\eta]c$									
	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
1.1	0.098	0.106	0.115	0.125	0.134	0.143	0.152	0.161	0.170	0.180
1.2	0.189	0.198	0.207	0.216	0.225	0.233	0.242	0.250	0.259	0.268
1.3	0.276	0.285	0.293	0.302	0.310	0.318	0.326	0.334	0.342	0.350
1.4	0.358	0.367	0.375	0.383	0.391	0.399	0.407	0.414	0.422	0.430
1.5	0.437	0.445	0.453	0.460	0.468	0.476	0.484	0.491	0.499	0.507
1.6	0.515	0.522	0.529	0.536	0.544	0.551	0.558	0.566	0.573	0.580
1.7	0.587	0.595	0.602	0.608	0.615	0.622	0.629	0.636	0.642	0.649
1.8	0.656	0.663	0.670	0.677	0.683	0.690	0.697	0.704	0.710	0.717
1.9	0.723	0.730	0.736	0.743	0.749	0.756	0.762	0.769	0.775	0.782
2.0	0.788	0.795	0.802	0.809	0.815	0.821	0.827	0.833	0.840	0.846
2.1	0.852	0.858	0.864	0.870	0.876	0.882	0.888	0.894	0.900	0.906
2.2	0.912	0.918	0.924	0.929	0.935	0.941	0.948	0.953	0.959	0.965
2.3	0.971	0.976	0.983	0.988	0.994	1.000	1.006	1.011	1.017	1.022
2.4	1.028	1.033	1.039	1.044	1.050	1.056	1.061	1.067	1.072	1.078
2.5	1.083	1.089	1.094	1.100	1.105	1.111	1.116	1.121	1.126	1.131
2.6	1.137	1.142	1.147	1.153	1.158	1.163	1.169	1.174	1.179	1.184
2.7	1.190	1.195	1.200	1.205	1.210	1.215	1.220	1.225	1.230	1.235
2.8	1.240	1.245	1.250	1.255	1.260	1.265	1.270	1.275	1.280	1.285
2.9	1.290	1.295	1.300	1.305	1.310	1.314	1.319	1.324	1.329	1.333
3.0	1.338	1.343	1.348	1.352	1.357	1.362	1.367	1.371	1.376	1.381
3.1	1.386	1.390	1.395	1.400	1.405	1.409	1.414	1.418	1.423	1.427
3.2	1.432	1.436	1.441	1.446	1.450	1.455	1.459	1.464	1.468	1.473
3.3	1.477	1.482	1.486	1.491	1.496	1.500	1.504	1.508	1.513	1.517
3.4	1.521	1.525	1.529	1.533	1.537	1.542	1.546	1.550	1.554	1.558
3.5	1.562	1.566	1.570	1.575	1.579	1.583	1.587	1.591	1.595	1.600
3.6	1.604	1.608	1.612	1.617	1.621	1.625	1.629	1.633	1.637	1.642
3.7	1.646	1.650	1.654	1.658	1.662	1.666	1.671	1.675	1.679	1.683
3.8	1.687	1.691	1.695	1.700	1.704	1.708	1.712	1.715	1.719	1.723
3.9	1.727	1.731	1.735	1.739	1.742	1.746	1.750	1.754	1.758	1.762
4.0	1.765	1.769	1.773	1.777	1.781	1.785	1.789	1.792	1.796	1.800
4.1	1.804	1.808	1.811	1.815	1.819	1.822	1.826	1.830	1.833	1.837
4.2	1.841	1.845	1.848	1.852	1.856	1.859	1.863	1.867	1.870	1.874
4.3	1.878	1.882	1.885	1.889	1.893	1.896	1.900	1.904	1.907	1.911
4.4	1.914	1.918	1.921	1.925	1.929	1.932	1.936	1.939	1.943	1.946
4.5	1.950	1.954	1.957	1.961	1.964	1.968	1.971	1.975	1.979	1.982
4.6	1.986	1.989	1.993	1.996	2.000	2.003	2.007	2.010	2.013	2.017
4.7	2.020	2.023	2.027	2.030	2.033	2.037	2.040	2.043	2.047	2.050
4.8	2.053	2.057	2.060	2.063	2.067	2.070	2.073	2.077	2.080	2.083
4.9	2.087	2.090	2.093	2.097	2.100	2.103	2.107	2.110	2.113	2.116
5.0	2.119	2.122	2.125	2.129	2.132	2.135	2.139	2.142	2.145	2.148
5.1	2.151	2.154	2.158	2.160	2.164	2.167	2.170	2.173	2.176	2.180
5.2	2.183	2.186	2.190	2.192	2.195	2.197	2.200	2.203	2.206	2.209
5.3	2.212	2.215	2.218	2.221	2.224	2.227	2.230	2.233	2.236	2.240
5.4	2.243	2.246	2.249	2.252	2.255	2.258	2.261	2.264	2.267	2.270
5.5	2.273	2.276	2.279	2.282	2.285	2.288	2.291	2.294	2.297	2.300
5.6	2.303	2.306	2.309	2.312	2.315	2.318	2.320	2.324	2.326	2.329
5.7	2.332	2.335	2.338	2.341	2.344	2.347	2.350	2.353	2.355	2.358
5.8	2.361	2.364	2.367	2.370	2.373	2.376	2.379	2.382	2.384	2.387
5.9	2.390	2.393	2.396	2.400	2.403	2.405	2.408	2.411	2.414	2.417

^A Swedish Method CCA 27:57, Karin Wilson, *Svensk Papperstidning*, Vol. 60, 1957, pp. 513 to 521.

^B Derived from the equation: $\eta_{rel} - 1 = \eta_{sp} = [\eta]c e^{K'[\eta]c}$, where $K' = 0.30$.

Intrinsic Viscosity, $[\eta]c$, at Different Values of Relative Viscosity, $\eta_{rel}^{A,B}$ (Continued)

η_{rel}	$[\eta]c$									
	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
6.0	2.419	2.422	2.425	2.428	2.431	2.433	2.436	2.439	2.442	2.444
6.1	2.447	2.450	2.453	2.456	2.458	2.461	2.464	2.467	2.470	2.472
6.2	2.475	2.478	2.481	2.483	2.486	2.489	2.492	2.494	2.497	2.500
6.3	2.503	2.505	2.508	2.511	2.513	2.516	2.518	2.521	2.524	2.526
6.4	2.529	2.532	2.534	2.537	2.540	2.542	2.545	2.547	2.550	2.553
6.5	2.555	2.558	2.561	2.563	2.566	2.568	2.571	2.574	2.576	2.579
6.6	2.581	2.584	2.587	2.590	2.592	2.595	2.597	2.600	2.603	2.605
6.7	2.608	2.610	2.613	2.615	2.618	2.620	2.623	2.625	2.627	2.630
6.8	2.633	2.635	2.637	2.640	2.643	2.645	2.648	2.650	2.653	2.655
6.9	2.658	2.660	2.663	2.665	2.668	2.670	2.673	2.675	2.678	2.680
7.0	2.683	2.685	2.687	2.690	2.693	2.695	2.698	2.700	2.702	2.705
7.1	2.707	2.710	2.712	2.714	2.717	2.719	2.721	2.724	2.726	2.729
7.2	2.731	2.733	2.736	2.738	2.740	2.743	2.745	2.748	2.750	2.752
7.3	2.755	2.757	2.760	2.762	2.764	2.767	2.769	2.771	2.774	2.776
7.4	2.779	2.781	2.783	2.786	2.788	2.790	2.793	2.795	2.798	2.800
7.5	2.802	2.805	2.807	2.809	2.812	2.814	2.816	2.819	2.821	2.823
7.6	2.826	2.828	2.830	2.833	2.835	2.837	2.840	2.842	2.844	2.847
7.7	2.849	2.851	2.854	2.856	2.858	2.860	2.863	2.865	2.868	2.870
7.8	2.873	2.875	2.877	2.879	2.881	2.884	2.887	2.889	2.891	2.893
7.9	2.895	2.898	2.900	2.902	2.905	2.907	2.909	2.911	2.913	2.915
8.0	2.918	2.920	2.922	2.924	2.926	2.928	2.931	2.933	2.935	2.937
8.1	2.939	2.942	2.944	2.946	2.948	2.950	2.952	2.955	2.957	2.959
8.2	2.961	2.963	2.966	2.968	2.970	2.972	2.974	2.976	2.979	2.981
8.3	2.983	2.985	2.987	2.990	2.992	2.994	2.996	2.998	3.000	3.002
8.4	3.004	3.006	3.008	3.010	3.012	3.015	3.017	3.019	3.021	3.023
8.5	3.025	3.027	3.029	3.031	3.033	3.035	3.037	3.040	3.042	3.044
8.6	3.046	3.048	3.050	3.052	3.054	3.056	3.058	3.060	3.062	3.064
8.7	3.067	3.069	3.071	3.073	3.075	3.077	3.079	3.081	3.083	3.085
8.8	3.087	3.089	3.092	3.094	3.096	3.098	3.100	3.102	3.104	3.106
8.9	3.108	3.110	3.112	3.114	3.116	3.118	3.120	3.122	3.124	3.126
9.0	3.128	3.130	3.132	3.134	3.136	3.138	3.140	3.142	3.144	3.146
9.1	3.148	3.150	3.152	3.154	3.156	3.158	3.160	3.162	3.164	3.166
9.2	3.168	3.170	3.172	3.174	3.176	3.178	3.180	3.182	3.184	3.186
9.3	3.188	3.190	3.192	3.194	3.196	3.198	3.200	3.202	3.204	3.206
9.4	3.208	3.210	3.212	3.214	3.215	3.217	3.219	3.221	3.223	3.225
9.5	3.227	3.229	3.231	3.233	3.235	3.237	3.239	3.241	3.242	3.244
9.6	3.246	3.248	3.250	3.252	3.254	3.256	3.258	3.260	3.262	3.264
9.7	3.266	3.268	3.269	3.271	3.273	3.275	3.277	3.279	3.281	3.283
9.8	3.285	3.287	3.289	3.291	3.293	3.295	3.297	3.298	3.300	3.302
9.9	3.304	3.305	3.307	3.309	3.311	3.313	3.316	3.318	3.320	3.321
10	3.32	3.34	3.36	3.37	3.39	3.41	3.43	3.45	3.46	3.48
11	3.50	3.52	3.53	3.55	3.56	3.58	3.60	3.61	3.63	3.64
12	3.66	3.68	3.69	3.71	3.72	3.74	3.76	3.77	3.79	3.80
13	3.80	3.83	3.85	3.86	3.88	3.89	3.90	3.92	3.93	3.95
14	3.96	3.97	3.99	4.00	4.02	4.03	4.04	4.06	4.07	4.09
15	4.10	4.11	4.13	4.14	4.15	4.17	4.18	4.19	4.20	4.22
16	4.23	4.24	4.25	4.27	4.28	4.29	4.30	4.31	4.33	4.34
17	4.35	4.36	4.37	4.38	4.39	4.41	4.42	4.43	4.44	4.45
18	4.46	4.47	4.48	4.49	4.50	4.52	4.53	4.54	4.55	4.56
19	4.57	4.58	4.59	4.60	4.61	4.62	4.63	4.64	4.65	4.66

^A Swedish Method CCA 27:57, Karin Wilson, *Svensk Papperstidning*, Vol. 60, 1957, pp. 513 to 521.

^B Derived from the equation: $\eta_{rel} - 1 = \eta_{sp} = [\eta]c^{K[\eta]c}$, where $K = 0.30$.

THERMOMETRIC EQUIVALENTS

Fahrenheit to Centigrade (Celsius) Scale

$(^{\circ}\text{F} - 32) (5 / 9) = ^{\circ}\text{C}$									
$^{\circ}\text{F}$	$^{\circ}\text{C}$	$^{\circ}\text{F}$	$^{\circ}\text{C}$	$^{\circ}\text{F}$	$^{\circ}\text{C}$	$^{\circ}\text{F}$	$^{\circ}\text{C}$	$^{\circ}\text{F}$	$^{\circ}\text{C}$
0	-17.78	51	10.56	101	38.33	151	66.11	201	93.89
1	-17.22	52	11.11	102	38.89	152	66.67	202	94.44
2	-16.67	53	11.67	103	39.44	153	67.22	203	95.
3	-16.11	54	12.22	104	40.	154	67.78	204	95.56
4	-15.56	55	12.78	105	40.56	155	68.33	205	96.11
5	-15.	56	13.33	106	41.11	156	68.89	206	96.67
6	-14.44	57	13.89	107	41.67	157	69.44	207	97.22
7	-13.89	58	14.44	108	42.22	158	70.	208	97.78
8	-13.33	59	15.	109	42.78	159	70.56	209	98.33
9	-12.78	60	15.56	110	43.33	160	71.11	210	98.89
10	-12.22	61	16.11	111	43.89	161	71.67	211	99.44
11	-11.67	62	16.67	112	44.44	162	72.22	212	100.
12	-11.11	63	17.22	113	45.	163	72.78	213	100.56
13	-10.56	64	17.78	114	45.56	164	73.33	214	101.11
14	-10.	65	18.33	115	46.11	165	73.89	215	101.67
15	-9.44	66	18.89	116	46.67	166	74.44	216	102.22
16	-8.89	67	19.44	117	47.22	167	75.	217	102.78
17	-8.33	68	20.	118	47.78	168	75.56	218	103.33
18	-7.78	69	20.56	119	48.33	169	76.11	219	103.89
19	-7.22	70	21.11	120	48.89	170	76.67	220	104.44
20	-6.67	71	21.67	121	49.44	171	77.22	221	105.
21	-6.11	72	22.22	122	50.	172	77.78	222	105.56
22	-5.56	73	22.78	123	50.56	173	78.33	223	106.11
23	-5.	74	23.33	124	51.11	174	78.89	224	106.67
24	-4.44	75	23.89	125	51.67	175	79.44	225	107.22
25	-3.89	76	24.44	126	52.22	176	80.	226	107.78
26	-3.33	77	25.	127	52.78	177	80.56	227	108.33
27	-2.78	78	25.56	128	53.33	178	81.11	228	108.89
28	-2.22	79	26.11	129	53.89	179	81.67	229	109.44
29	-1.67	80	26.67	130	54.44	180	82.22	230	110.
30	-1.11	81	27.22	131	55.	181	82.78	231	110.56
31	-0.56	82	27.78	132	55.56	182	83.33	232	111.11
32	0.	83	28.33	133	56.11	183	83.89	233	111.67
33	0.56	84	28.89	134	56.67	184	84.44	234	112.22
34	1.11	85	29.44	135	57.22	185	85.	235	112.78
35	1.67	86	30.	136	57.78	186	85.56	236	113.33
36	2.22	87	30.56	137	58.33	187	86.11	237	113.89
37	2.78	88	31.11	138	58.89	188	86.67	238	114.44
38	3.33	89	31.67	139	59.44	189	87.22	239	115.
39	3.89	90	32.22	140	60.	190	87.78	240	115.56
40	4.44	91	32.78	141	60.56	191	88.33	241	116.11
41	5.	92	33.33	142	61.11	192	88.89	242	116.67
42	5.56	93	33.89	143	61.67	193	89.44	243	117.22
43	6.11	94	34.44	144	62.22	194	90.	244	117.78
44	6.67	95	35.	145	62.78	195	90.56	245	118.33
45	7.22	96	35.56	146	63.33	196	91.11	246	118.89
46	7.78	97	36.11	147	63.89	197	91.67	247	119.44
47	8.33	98	36.67	148	64.44	198	92.22	248	120.
48	8.89	99	37.22	149	65.	199	92.78	249	120.56
49	9.44	100	37.78	150	65.56	200	93.33	250	121.11
50	10.								

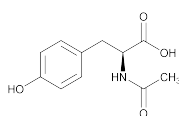
Centigrade (Celsius) to Fahrenheit Scale

$(9 / 5)^{\circ}\text{C} + 32 = ^{\circ}\text{F}$									
$^{\circ}\text{C}$	$^{\circ}\text{F}$	$^{\circ}\text{C}$	$^{\circ}\text{F}$	$^{\circ}\text{C}$	$^{\circ}\text{F}$	$^{\circ}\text{C}$	$^{\circ}\text{F}$	$^{\circ}\text{C}$	$^{\circ}\text{F}$
-20	-4.0	21	69.8	61	141.8	101	213.8	141	285.8
-19	-2.2	22	71.6	62	143.6	102	215.6	142	287.6
-18	-0.4	23	73.4	63	145.4	103	217.4	143	289.4
-17	1.4	24	75.2	64	147.2	104	219.2	144	291.2
-16	3.2	25	77.	65	149.	105	221.	145	293.
-15	5.	26	78.8	66	150.8	106	222.8	146	294.8
-14	6.8	27	80.6	67	152.6	107	224.6	147	296.6
-13	8.6	28	82.4	68	154.4	108	226.4	148	298.4
-12	10.4	29	84.2	69	156.2	109	228.2	149	300.2
-11	12.2	30	86.	70	158.	110	230.	150	302.
-10	14.	31	87.8	71	159.8	111	231.8	151	303.8
-9	15.8	32	89.6	72	161.6	112	233.6	152	305.6
-8	17.6	33	91.4	73	163.4	113	235.4	153	307.4
-7	19.4	34	93.2	74	165.2	114	237.2	154	309.2
-6	21.2	35	95.	75	167.	115	239.	155	311.
-5	23.	36	96.8	76	168.8	116	240.8	156	312.8
-4	24.8	37	98.6	77	170.6	117	242.6	157	314.6
-3	26.6	38	100.4	78	172.4	118	244.4	158	316.4
-2	28.4	39	102.2	79	174.2	119	246.2	159	318.2
-1	30.2	40	104.	80	176.	120	248.	160	320.
0	32.	41	105.8	81	177.8	121	249.8	161	321.8
1	33.8	42	107.6	82	179.6	122	251.6	162	323.6
2	35.6	43	109.4	83	181.4	123	253.4	163	325.4
3	37.4	44	111.2	84	183.2	124	255.2	164	327.2
4	39.2	45	113.	85	185.	125	257.	165	329.
5	41.	46	114.8	86	186.8	126	258.8	166	330.8
6	42.8	47	116.6	87	188.6	127	260.6	167	332.6
7	44.6	48	118.4	88	190.4	128	262.4	168	334.4
8	46.4	49	120.2	89	192.2	129	264.2	169	336.2
9	48.2	50	122.	90	194.	130	266.	170	338.
10	50.	51	123.8	91	195.8	131	267.8	171	339.8
11	51.8	52	125.6	92	197.6	132	269.6	172	341.6
12	53.6	53	127.4	93	199.4	133	271.4	173	343.4
13	55.4	54	129.2	94	201.2	134	273.2	174	345.2
14	57.2	55	131.	95	203.	135	275.	175	347.
15	59.	56	132.8	96	204.8	136	276.8	176	348.8
16	60.8	57	134.6	97	206.6	137	278.6	177	350.6
17	62.6	58	136.4	98	208.4	138	280.4	178	352.4
18	64.4	59	138.2	99	210.2	139	282.2	179	354.2
19	66.2	60	140.	100	212.	140	284.	180	356.
20	68.								

Dietary Supplements

Official Monographs

N-Acetyltyrosine



$C_{11}H_{13}NO_4$ 223.2
N-Acetyl-L-tyrosine;
 (2*S*)-2-(Acetylamino)-3-(4-hydroxyphenyl)propanoic acid
 [537-55-3].

DEFINITION

N-Acetyltyrosine contains NLT 98.5% and NMT 101.0% of $C_{11}H_{13}NO_4$, as *N*-acetyl-L-tyrosine, calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B. OPTICAL ROTATION**, *Specific Rotation* (781S)
Sample solution: 10 mg/mL
Acceptance criteria: NLT +46.0° and NMT +49.0°, determined at 20°
- **C.** The R_f value of the principal spot of the *Sample solution* in the test for *Organic Impurities* corresponds to that of *Standard solution 1*.

ASSAY

PROCEDURE

Sample solution: Dissolve about 180 mg of *N*-Acetyltyrosine, weighed, in 50 mL of carbon dioxide-free water.

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N sodium hydroxide VS

Endpoint detection: Potentiometric

Equivalency: Each mL of 0.1 N sodium hydroxide VS is equivalent to 22.32 mg of $C_{11}H_{13}NO_4$.

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 0.1%
- **CHLORIDE AND SULFATE**, *Chloride* (221): NMT 200 ppm. A 0.7-g portion shows no more chloride than corresponds to 0.40 mL of 0.01 N hydrochloric acid.
- **CHLORIDE AND SULFATE**, *Sulfate* (221): NMT 200 ppm. A 1.2-g portion shows no more sulfate than corresponds to 0.25 mL of 0.020 N sulfuric acid.
- **IRON** (241): NMT 20 ppm
- **HEAVY METALS**, *Method 1* (231): NMT 10 ppm

Organic Impurities

PROCEDURE

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Standard stock solution 1: 8 mg/mL of USP *N*-Acetyl-L-tyrosine RS in a mixture of water, glacial acetic acid, and alcohol (3:3:94)

Standard solution 1: Dilute *Standard stock solution 1* with alcohol to obtain a solution having a known concentration of about 0.4 mg/mL.

Standard solution 2: 0.8 mg/mL of USP L-Tyrosine RS dissolved in a mixture of glacial acetic acid and water (1:1), and diluted with alcohol

Sample solution: Transfer 0.8 g of *N*-Acetyltyrosine to a 10-mL volumetric flask, dissolve in 6 mL of a mixture of glacial acetic acid and water (1:1), and dilute with alcohol to volume.

Application volume: 5 μ L

Developing solvent system: A mixture of ammonia and 2-propanol (3:7)

Spray reagent: Dissolve 0.2 g of ninhydrin in 100 mL of a mixture of butanol and 2 N acetic acid (95:5).

Analysis: Proceed as directed for *Chromatography* (621), *Thin-Layer Chromatography*. After air-drying the plate, repeat the development process. After air-drying a second time, spray with *Spray reagent*, and heat between 100° and 105° for about 15 min.

Acceptance criteria: Any secondary spot from the *Sample solution* is not larger or more intense than the principal spot from *Standard solution 1*, except for the spot corresponding to tyrosine, which is not larger or more intense than the principal spot from *Standard solution 2*.

Individual impurities: NMT 0.5%

Limit of tyrosine: NMT 1.0%

SPECIFIC TESTS

- **LOSS ON DRYING** (731): Dry a sample at 105° for 3 h: it loses NMT 0.1% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.
- **USP REFERENCE STANDARDS** (11)
 USP *N*-Acetyl-L-tyrosine RS
 USP L-Tyrosine RS

Alpha Lipoic Acid—see *Alpha Lipoic Acid under L*.

Ademetionine Disulfate Tosylate—see *S-adenosyl-L-methionine Disulfate Tosylate*

Alanine—see *Alanine General Monographs*

American Ginseng

DEFINITION

American Ginseng consists of the dried roots of *Panax quinquefolius* L. (Fam. Araliaceae). It contains NLT 4.0% of total ginsenosides, calculated on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: 20 mg/mL of USP Powdered American Ginseng Extract RS in methanol

Standard solution B: 20 mg/mL of USP Powdered Asian Ginseng Extract RS in methanol

Sample solution: Transfer about 1.0 g of finely powdered American Ginseng to a 25-mL flask fitted with a reflux condenser. Add 10.0 mL of a mixture of methanol and water (7:13), and heat under reflux for 15 min. Cool, filter, and dilute the filtrate with methanol to 10.0 mL.

Adsorbent: 0.25-mm layer of silica gel, typically 20 cm long (TLC plates)

Application volume: 20 μ L

Developing solvent system A: Chloroform, methanol, and water (13:7:2). Use the lower phase.

Developing solvent system B: Butyl alcohol, ethyl acetate, and water (4:1:5). Use the upper phase.

Spray reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, add 85 mL of methanol, mix, and carefully add 5 mL of sulfuric acid.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop in a chamber containing *Developing solvent system A* until the solvent front has moved 10.5 cm from the origin. Remove the plates, and allow to dry. Turn the plates 90°, and develop in a chamber containing *Developing solvent system B* until the solvent front has moved 10.5 cm from the origin. Remove the plates, and allow to dry. Spray with *Spray reagent*. Heat the plates at 105°–110° for 10 min, and examine.

Suitability requirements: The order, from top to bottom, of ginsenosides on the chromatographic plates is: Rg₂ (on left) and Rg₁ (on right), Rf, Re, Rd, Rc, Rb₂ (on left) and Rb₁ (on right), and Ro. Ginsenosides Rg₂, Rg₁, Rf, Re, and Rd are found on the upper half of the plates; the remaining ginsenosides are found on the lower half after chromatographing with *Developing solvent system B*. *Standard solution A* does not exhibit a spot for ginsenoside Rf. *Standard solution B* exhibits a spot for ginsenoside Rf.

Acceptance criteria: The spots from the *Sample solution* correspond to those from *Standard solution A*.

- **B.** The retention times of the peaks for ginsenosides Rg₁, Re, Rb₁, Rb₂, Rc₂, and Rd of the *Sample solution* correspond to those of *Standard solution A*, as obtained in the test for *Content of Ginsenosides*. The ratio of the peak responses for ginsenosides Rb₂ to Rb₁ is less than 0.4, and the ratio of the peak responses for ginsenosides Rg₁ to Rb₁ is less than 0.3. The chromatogram shows no significant peak at the retention time corresponding to that for ginsenoside Rf of *Standard solution B*, as obtained in the test for *Content of Ginsenosides*.

COMPOSITION

• CONTENT OF GINSENOSES

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Diluent: Alcohol and water (4:6)

Standard solution A: Transfer a quantity of USP Powdered American Ginseng Extract RS, equivalent to about 2 mg of ginsenoside Rb₁, to a suitable container, and dissolve in 10.0 mL of *Diluent*.

Standard solution B: Transfer a quantity of USP Powdered Asian Ginseng Extract RS, equivalent to about 2 mg of ginsenoside Rg₁, to a suitable container, and dissolve in 10.0 mL of *Diluent*.

Sample solution: Reduce 100 g of American Ginseng to a powder, and transfer about 1.0 g of the powder, accurately weighed, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of *Diluent* and a few grains of pumice, boil on a water bath under reflux for 1 h, cool, and filter. Wash the flask and the residue with 20 mL of *Diluent*, and pass through the same filter. Combine the filtrates, and evaporate in a rotary evaporator at 50° to dryness. Dissolve the residue in 10.0 mL of *Diluent*.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Analytical column: 4.6-mm \times 15-cm; 3- μ m packing L1

Guard column: 4.6-mm \times 2.0-cm; packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the 6 major ginsenosides, in replicate injections

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd in the *Standard solutions* and the *Sample solution* by comparing the chromatograms with the Reference Chromatogram provided with USP Powdered American Ginseng Extract RS, and measure the peak responses.

Calculate the percentages of individual ginsenosides in the portion of American Ginseng taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of ginsenoside Rg₁, Re, Rb₁, Rc, Rb₂, or Rd from the *Sample solution*

- r_s = peak response of ginsenoside Rg₁, Re, Rb₁, Rc, Rb₂, or Rd from the appropriate *Standard solution*
- C_s = concentration of ginsenoside Rg₁, Re, Rb₁, Rc, Rb₂, or Rd in the appropriate *Standard solution* (mg/mL)
- V = volume of the *Sample solution* (mL)
- W = weight of American Ginseng taken to prepare the *Sample solution* (mg)

Calculate the percentage of total ginsenosides in the portion of American Ginseng taken by adding the individual percentages.

Acceptance criteria: NLT 4.0% of total ginsenosides on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method III* (231): NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10⁴ cfu/g. The total combined molds and yeasts count does not exceed 100 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Fusiform or cylindrical roots, sometimes branched, typically 1–10 cm, sometimes up to 20 cm, in length and up to 2.5 cm in diameter at the crown, with one or more stem scars. Externally pale yellow to golden, rough-textured, with prominent horizontal rings and fine longitudinal ridges as a result of drying. Root scars or fine rootlets are present. If stem base is present, scales are thin and perishing (differs from *P. ginseng*, in which scales at base of stem are fleshy and persistent). Fracture is short; fractured surface is white to ivory, with distinct aromatic odor and rings of secretory canals present in secondary phloem.

Histology

Transverse section of root: Multiple layers of thin-walled cork cells are present. Secondary phloem is characterized by conspicuous air lacunae; abundant, starch-containing storage parenchyma; few sieve elements, found in small groupings; and rings of schizogenous secretory canals. Each secretory canal is lined with 6–8 epithelial cells that lack starch. Xylem is characterized by abundant starch-containing storage parenchyma and a few tracheary elements, composed of nonlignified tracheids and slightly lignified spiral or reticulated vessels lacking secretory canals and found in isolation or in small groupings. Druse crystals are sometimes present within vascular parenchyma cells. Diarch or triarch primary xylem is in center of root.

- **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* (561): NMT 2.0%
- **LOSS ON DRYING** (731): Dry 1 g of it, finely powdered, at 105° for 2 h: it loses NMT 10.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 8%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store protected from heat.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.

• USP REFERENCE STANDARDS (11)

USP Powdered American Ginseng Extract RS
USP Powdered Asian Ginseng Extract RS

Powdered American Ginseng

DEFINITION

Powdered American Ginseng is American Ginseng reduced to a fine or a very fine powder. It contains NLT 4.0% of total ginsenosides, calculated on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: 20 mg/mL of USP Powdered American Ginseng Extract RS in methanol

Standard solution B: 20 mg/mL of USP Powdered Asian Ginseng Extract RS in methanol

Sample solution: Transfer about 1.0 g of Powdered American Ginseng to a 25-mL flask fitted with a reflux condenser. Add 10.0 mL of a mixture of methanol and water (7:13), and heat under reflux for 15 min. Cool, filter, and dilute the filtrate with methanol to 10.0 mL.

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 20 µL

Developing solvent system A: Chloroform, methanol, and water (13:7:2). Use the lower phase.

Developing solvent system B: Butyl alcohol, ethyl acetate, and water (4:1:5). Use the upper phase.

Spray reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, add 85 mL of methanol, mix, and carefully add 5 mL of sulfuric acid.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop in a chamber containing *Developing solvent system A* until the solvent front has moved 10.5 cm from the origin. Remove the plates, and allow to dry. Turn the plates 90°, and develop in a chamber containing *Developing solvent system B* until the solvent front has moved 10.5 cm from the origin. Remove the plates, and allow to dry. Spray with *Spray reagent*. Heat the plates at 105°–110° for 10 min, and examine.

Suitability requirements: The order, from top to bottom, of ginsenosides on the chromatographic plates is: Rg₂ (on left) and Rg₁ (on right), Rf, Re, Rd, Rc, Rb₂ (on left) and Rb₁ (on right), and Ro. Ginsenosides Rg₂, Rg₁, Rf, Re, and Rd are found on the upper half of the plates; the remaining ginsenosides are found on the lower half after chromatographing with *Developing solvent system B*. *Standard solution A* does not exhibit a spot for ginsenoside Rf. *Standard solution B* exhibits a spot for ginsenoside Rf.

Acceptance criteria: The spots from the *Sample solution* correspond to those from *Standard solution A*.

- **B.** The retention times of the peaks for ginsenosides Rg₁, Re, Rb₁, Rb₂, Rc₂, and Rd of the *Sample solution* correspond to those of *Standard solution A*, as obtained in the test for *Content of Ginsenosides*. The ratio of the peak responses for ginsenosides Rb₂ to Rb₁ is less than 0.4, and the ratio of the peak responses for ginsenosides Rg₁ to Rb₁ is less than 0.3. The chromatogram shows no significant peak at the retention time corresponding to that for ginsenoside Rf of *Standard solution B*, as obtained in the test for *Content of Ginsenosides*.

COMPOSITION**• CONTENT OF GINSENOSES****Solution A:** Water**Solution B:** Acetonitrile and water (4:1)**Mobile phase:** See Table 1.**Table 1**

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Diluent: Alcohol and water (4:6)**Standard solution A:** Transfer a quantity of USP Powdered American Ginseng Extract RS, equivalent to about 2 mg of ginsenoside Rb₁, to a suitable container, and dissolve in 10.0 mL of *Diluent*.**Standard solution B:** Transfer a quantity of USP Powdered Asian Ginseng Extract RS, equivalent to about 2 mg of ginsenoside Rg₁, to a suitable container, and dissolve in 10.0 mL of *Diluent*.**Sample solution:** Transfer about 1.0 g of Powdered American Ginseng, accurately weighed, to a 100-mL, round-bottom flask fitted with a reflux condenser. Add 50 mL of *Diluent* and a few grains of pumice, boil on a water bath under reflux for 1 h, cool, and filter. Wash the flask and the residue with 20 mL of *Diluent*, and pass through the same filter. Combine the filtrates, and evaporate in a rotary evaporator at 50° to dryness. Dissolve the residue in 10.0 mL of *Diluent*.**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 203 nm**Analytical column:** 4.6-mm × 15-cm; 3-μm packing L1**Guard column:** 4.6-mm × 2.0-cm; packing L1**Column temperature:** 25°**Flow rate:** 1.5 mL/min**Injection size:** 10 μL**System suitability****Sample:** *Standard solution B***Suitability requirements****Chromatogram similarity:** The chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.**Relative standard deviation:** NMT 2.0%, determined for the sum of the peak areas for the 6 major ginsenosides, in replicate injections**Analysis****Samples:** *Standard solution A*, *Standard solution B*, and *Sample solution*Identify ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd in the *Standard solutions* and the *Sample solution* by comparing the chromatograms with the Reference Chromatogram provided with USP Powdered American Ginseng Extract RS, and measure the peak responses.

Calculate the percentages of individual ginsenosides in the portion of Powdered American Ginseng taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

 r_U = peak response of ginsenoside Rg₁, Re, Rb₁, Rc, Rb₂, or Rd from the *Sample solution* r_S = peak response of ginsenoside Rg₁, Re, Rb₁, Rc, Rb₂, or Rd from the appropriate *Standard solution* C_S = concentration of ginsenoside Rg₁, Re, Rb₁, Rc, Rb₂, or Rd in the appropriate *Standard solution* (mg/mL) V = volume of the *Sample solution* (mL) W = weight of Powdered American Ginseng taken to prepare the *Sample solution* (mg)

Calculate the percentage of total ginsenosides in the portion of Powdered American Ginseng taken by adding the individual percentages.

Acceptance criteria: NLT 4.0% of total ginsenosides on the dried basis**CONTAMINANTS****• HEAVY METALS, Method III (231):** NMT 20 ppm**• ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis (561):** Meets the requirements**SPECIFIC TESTS****• BOTANIC CHARACTERISTICS:** Pale yellowish-brown powder with a slightly aromatic odor. Oval parenchymatous cells packed with starch granules and occasional druse crystals of calcium oxalate. Yellowish-brown secretory vessels with yellowish-brown contents.**• ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter (561):** NMT 2.0%**• LOSS ON DRYING (731):** Dry 1 g of it, finely powdered, at 105° for 2 h: it loses NMT 10.0% of its weight.**• ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 8%**ADDITIONAL REQUIREMENTS****• PACKAGING AND STORAGE:** Store in tight containers, protected from light, moisture, and heat.**• LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.**• USP REFERENCE STANDARDS (11)**

USP Powdered American Ginseng Extract RS

USP Powdered Asian Ginseng Extract RS

Powdered American Ginseng Extract**DEFINITION**Powdered American Ginseng Extract is prepared from the pulverized dried roots of *Panax quinquefolius* L. (Fam. Araliaceae), using suitable solvents, and dried to a powder. It contains NLT 10.0% of total ginsenosides, calculated on the anhydrous basis. The ratio of starting crude plant material to Powdered American Ginseng Extract is between 3:1 and 7:1.**IDENTIFICATION****• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST****Standard solution A:** 20 mg/mL of USP Powdered American Ginseng Extract RS in methanol**Standard solution B:** 20 mg/mL of USP Powdered Asian Ginseng Extract RS in methanol**Sample solution:** 20 mg/mL in methanol**Adsorbent:** 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)**Application volume:** 20 μL**Developing solvent system A:** Chloroform, methanol, and water (13:7:2). Use the lower phase.**Developing solvent system B:** Butyl alcohol, ethyl acetate, and water (4:1:5). Use the upper phase.**Spray reagent:** Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, add 85 mL of methanol, mix, and carefully add 5 mL of sulfuric acid.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop in a chamber containing *Developing solvent system A* until the solvent front has moved 10.5 cm from the origin. Remove the plates, and allow to dry. Turn the plates 90°, and develop in a chamber containing *Developing solvent system B* until the solvent front has moved 10.5 cm from the origin. Remove the plates, and allow to dry. Spray with *Spray reagent*. Heat the plates at 105°–110° for 10 min, and examine.

Suitability requirements: The order, from top to bottom, of ginsenosides on the chromatographic plates is: Rg₂ (on left) and Rg₁ (on right), Rf, Re, Rd, Rc, Rb₂ (on left) and Rb₁ (on right), and Ro. Ginsenosides Rg₂, Rg₁, Rf, Re, and Rd are found on the upper half of the plates; the remaining ginsenosides are found on the lower half after chromatographing with *Developing solvent system B*. *Standard solution A* does not exhibit a spot for ginsenoside Rf. *Standard solution B* exhibits a spot for ginsenoside Rf.

Acceptance criteria: The spots from the *Sample solution* correspond to those from *Standard solution A*.

- **B.** The retention times of the peaks for ginsenosides Rg₁, Re, Rb₁, Rb₂, Rc, and Rd of the *Sample solution* correspond to those of *Standard solution A*, as obtained in the test for *Content of Ginsenosides*. The ratio of the peak responses for ginsenosides Rb₂ to Rb₁ is less than 0.4, and the ratio of the peak responses for ginsenosides Rg₁ to Rb₁ is less than 0.3. The *Sample solution* shows no significant peak at the retention time corresponding to that for ginsenoside Rf of *Standard solution B*, as obtained in the test for *Content of Ginsenosides*.

COMPOSITION**• CONTENT OF GINSENOIDES**

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Diluent: Alcohol and water (4:6)

Standard solution A: Transfer a quantity of USP Powdered American Ginseng Extract RS, equivalent to about 2 mg of ginsenoside Rb₁, to a suitable container, and dissolve in 10.0 mL of *Diluent*.

Standard solution B: Transfer a quantity of USP Powdered Asian Ginseng Extract RS, equivalent to about 2 mg of ginsenoside Rg₁, to a suitable container, and dissolve in 10.0 mL of *Diluent*.

Sample solution: Transfer a quantity of Powdered American Ginseng Extract, equivalent to about 5 mg of ginsenosides, to a suitable container. Dissolve in 10.0 mL of *Diluent*, sonicating for 10 min, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Analytical column: 4.6-mm × 15-cm; 3-μm packing L1

Guard column: 4.6-mm × 2.0-cm; packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the 6 major ginsenosides, in replicate injections

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd in the *Standard solutions* and the *Sample solution* by comparing the chromatograms with the Reference Chromatogram provided with USP Powdered American Ginseng Extract RS, and measure the peak responses.

Calculate the percentages of individual ginsenosides in the portion of Powdered American Ginseng Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times P$$

r_U = peak response of ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, or Rd from the *Sample solution*

r_S = peak response of ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, or Rd from the appropriate *Standard solution*

C_S = concentration of ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, or Rd in the appropriate *Standard solution* (mg/mL)

C_U = concentration of Powdered American Ginseng Extract in the *Sample solution* (mg/mL)

P = labeled amount, in percentage, of each relevant ginsenoside in USP Powdered American Ginseng Extract RS

Calculate the percentage of total ginsenosides in the portion of Powdered American Ginseng Extract taken by adding the individual percentages.

Acceptance criteria: NLT 10.0% of total ginsenosides on the anhydrous basis

CONTAMINANTS

- **HEAVY METALS**, *Method II* <231>: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10⁴ cfu/g. The total combined molds and yeasts count does not exceed 10³ cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **WATER DETERMINATION**, *Method I* <921>: NMT 7.0%
- **BOTANICAL EXTRACTS**, *Residue on Evaporation* (565): Meets the requirements
- **ALCOHOL DETERMINATION**, *Method II* <611>: NMT 0.25%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. Label it to indicate the content of total ginsenosides, the extracting solvent used for preparation, and the ratio of the starting crude plant material to the Powdered Extract. It meets the labeling requirements under *Botanical Extracts* (565).

- **USP REFERENCE STANDARDS** <11>
USP Powdered American Ginseng Extract RS
USP Powdered Asian Ginseng Extract RS

American Ginseng Capsules

DEFINITION

American Ginseng Capsules contain Powdered American Ginseng Extract. Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of Extract, calculated as the sum of ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** <201>

Sample solution

Soft-shell gelatin Capsules: Transfer a portion of the contents of the Capsules, equivalent to 100 mg of Powdered Extract, to a separatory funnel containing 30 mL of a mixture of hexanes, methanol, and water (20:15:10), dissolve in this mixture, and collect the lower layer. Wash the upper layer with three 15-mL portions of a mixture of methanol and water (15:10), and combine the washings with the lower layer. Evaporate to dryness under vacuum at 45°–50°. Dissolve the residue in 5 mL of methanol.

Hard-shell gelatin Capsules: Transfer a portion of the contents of the Capsules, equivalent to 100 mg of Powdered Extract, to a conical flask. Extract at 55° with three 20-mL portions of a mixture of methanol and water (2:8). Evaporate the combined extracts to dryness under vacuum at 45°–50°. Dissolve the residue in 5 mL of methanol.

Standard solution A: 20 mg/mL of USP Powdered American Ginseng Extract RS in methanol

Standard solution B: 20 mg/mL of USP Powdered Asian Ginseng Extract RS in methanol

Application volume: 20 µL

Developing solvent system A: The lower phase of a mixture of chloroform, methanol, and water (13:7:2)

Developing solvent system B: The upper phase of a mixture of butyl alcohol, ethyl acetate, and water (4:1:5)

Spray reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, add 85 mL of methanol, mix, carefully add 5 mL of sulfuric acid, and mix.

Analysis

Samples: *Sample solution*, *Standard solution A*, and *Standard solution B*

Develop the chromatograms in a chamber containing *Developing solvent system A* until the solvent front has moved 10.5 cm from the origin. Remove the plate from the chamber, and allow to dry. Turn the plate 90°, and develop in a chamber containing *Developing solvent system B* until the solvent front has moved 10.5 cm from the origin. Remove the plate from the chamber, and allow to dry. Spray with *Spray reagent*. Heat the plate at 105°–110° for 10 min, and examine. The order, from top to bottom, of ginsenosides on the plates is Rg₂ (on left) and Rg₁ (on right), Rf, Re, Rd, Rc, Rb₂ (on left) and Rb₁ (on right), and Ro.

Ginsenosides Rg₂, Rg₁, Rf, Re, and Rd are found on the upper half of the plates; the remaining ginsenosides are found on the lower half after chromatographing with *Developing solvent system B*.

Acceptance criteria: *Standard solution A* does not exhibit a spot for ginsenoside Rf. *Standard solution B* exhibits a spot for ginsenoside Rf. The spots from the *Sample solution* correspond to those from *Standard solution A*.

- **B.** The retention times of the peaks for ginsenosides Rg₁, Re, Rb₁, Rb₂, Rc, and Rd in the chromatogram of the

Sample solution correspond to those from the *Standard solution*, as obtained in the test for *Content of Ginsenosides*. The ratio of the peak response for Rb₂ to the peak response for Rb₁ is less than 0.4; and the ratio of the peak response for Rg₁ to the peak response for Rb₁ is less than 0.3. There is no significant peak at the retention time corresponding to that of ginsenoside Rf in the *System suitability solution*, as obtained in the test for *Content of Ginsenosides*.

STRENGTH

- **CONTENT OF GINSENOSES**

Method 1

Diluent: Water and alcohol (3:2)

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Standard solution: A solution of USP Powdered American Ginseng Extract RS in *Diluent* containing the equivalent of 0.2 mg/mL of ginsenoside Rb₁

Sample solution (soft-gelatin Capsules): Open NLT 20 Capsules, transfer the contents to a suitable container, and mix to homogenize. Transfer a portion, expected to contain an amount of Extract equivalent to 12 mg of ginsenosides, to a suitable flask with a stopper. Add 5.0 mL of tetrahydrofuran, and sonicate for 5 min. Add 25.0 mL of a mixture of methanol and water (4:6), and shake for 50 min in an automatic shaker. Transfer 15.0 mL of the obtained emulsion to a centrifuge tube with a stopper, add 800 mg of sodium chloride, shake for 30 s, and centrifuge to obtain a clear upper phase. Dilute 1.0 mL of the upper phase with 4 mL of water in a suitable tube, and transfer the solution to a column containing 360 mg of packing L2 that has been previously treated with 3.0 mL of methanol followed by 8.0 mL of water. [NOTE—Elute slowly, not faster than 1 drop/s, in all elution steps. Do not use vacuum.] Rinse the tube with 5 mL of water, transfer to the column taking the precaution of slow elution, and discard the eluate. Repeat the elution with 5 mL of a mixture of methanol and water (4:6), and discard the eluate. Elute the ginsenosides with 5.0 mL of methanol. Evaporate the solution under a stream of nitrogen at 40° (50 min), and dissolve the residue with 1.0 mL of a solution of acetonitrile and water (1:4).

System suitability solution: 24 mg/mL of USP Powdered Asian Ginseng Extract RS in *Diluent*. Filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Column

Guard column: 4.6-mm × 2.0-cm; packing L1

Analytical column: 4.6-mm × 15-cm; 3-µm packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *System suitability solution* (inject 20 µL)

Suitability requirements

Chromatogram similarity: The *System suitability solution* chromatogram is similar to the Reference Chro-

matogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the six major ginsenosides, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Identify ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd in the *Standard solution* and the *Sample solution* by comparing the chromatograms with the Reference Chromatogram provided with USP Powdered American Ginseng Extract RS being used, and measure the peak responses.

Calculate the quantity, in mg, of each relevant ginsenoside (Rg₁, Re, Rb₁, Rc, Rb₂, and Rd) in the portion of Capsule contents taken:

$$\text{Result} = 0.3 \times (r_U/r_S) \times C_S \times P$$

r_U = peak area for each relevant ginsenoside from the *Sample solution*

r_S = peak area for each relevant ginsenoside from the *Standard solution*

C_S = concentration of USP Powdered American Ginseng Extract RS in the *Standard solution* (mg/mL)

P = labeled amount, in percentage, of each relevant ginsenoside in the USP Powdered American Ginseng Extract RS lot being used

Calculate the content of total ginsenosides, T, in mg, by adding the amounts of individual ginsenoside.

Calculate the percentage of Powdered Extract with respect to the label claim:

$$\text{Result} = T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

T = content of total ginsenosides in the portion of Capsule contents taken (mg)

A_{WT} = average weight of Capsule contents (mg/Capsule)

W = weight of the portion of Capsule contents taken (mg)

L_E = content of total ginsenosides, mg, in 100 mg of the Extract used to prepare the Capsules

L = amount of Extract per Capsule according to label claim (mg/Capsule)

Method 2

Diluent, Solution A, Solution B, Mobile phase, System suitability solution, Chromatographic system, and Suitability requirements: Proceed as directed under *Method 1*.

Solvent A: Upper phase of a mixture consisting of hexane, methanol, and water (4:3:2)

Solvent B: Lower phase of a mixture consisting of hexane, methanol, and water (4:3:2)

Standard solution: A solution of USP Powdered American Ginseng Extract RS in *Diluent* containing the equivalent of 1 mg/mL of ginsenoside Rb₁

Sample solution A (for soft-gelatin Capsules): Open NLT 20 Capsules and transfer the contents to a suitable container. Mix to homogenize and transfer a portion, expected to contain an amount of Extract equivalent to 15 mg of total ginsenosides, to a 50-mL flask. Add 10.0 mL of *Solvent A*, and sonicate for 3–5 min at 25°–30°. Transfer the solution to a 125-mL separatory funnel. To the residue add 10 mL of *Solvent B*, and sonicate for 3–5 min at 25°–30°. Transfer the solution to the same separatory funnel. Repeat the above procedure twice (the total volume will be about 60 mL). Shake, and then allow the phases to separate. Collect the combined lower phase in a round-bottom flask, and wash the combined upper phase twice with 10 mL of *Solvent B*. Evaporate the combined lower phase to dryness under vacuum at 45°–50°. Transfer the residue to a 10-mL volumetric flask using small

volumes of methanol, and dilute with methanol to volume.

Sample solution B (for hard-gelatin Capsules): Weigh the contents of NLT 20 Capsules, and composite the contents. Transfer a portion of the composite, expected to contain an amount of Extract equivalent to 15 mg of total ginsenosides, to a conical flask. Add 15 mL of methanol, and shake to mix. Sonicate the mixture at 25°–30° for 30 min. Cool, pass through filter paper, and return the residue to the conical flask. Add another 15 mL of methanol, sonicate the mixture at 25°–30° for 30 min, and filter. Wash the residue with three 15-mL portions of methanol. Evaporate the combined extracts and washing to dryness under vacuum at 45°–50°. Transfer the residue to a 10-mL volumetric flask using small volumes of methanol, and dilute with methanol to volume.

Analysis

Samples: *Standard solution* and *Sample solution*

Identify ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd in the *Standard solution* and the *Sample solution* by comparing the chromatograms with the Reference Chromatogram provided with USP Powdered American Ginseng Extract RS, and measure the peak responses.

Calculate the quantity, in mg, of each relevant ginsenoside (Rg₁, Re, Rb₁, Rc, Rb₂, and Rd) in the portion of Capsule contents taken:

$$\text{Result} = 0.1 \times (r_U/r_S) \times C_S \times P$$

r_U = peak area for each relevant ginsenoside from the *Sample solution*

r_S = peak area for each relevant ginsenoside from the *Standard solution*

C_S = concentration of USP Powdered American Ginseng Extract RS in the *Standard solution* (mg/mL)

P = labeled amount, in percentage, of each relevant ginsenoside in the USP Powdered American Ginseng Extract RS lot being used

Calculate the content of total ginsenosides, T, in mg, by adding the amounts of individual ginsenoside.

Calculate the percentage of Powdered Extract with respect to the label claim:

$$\text{Result} = T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

T = content of total ginsenosides in the portion of Capsule contents taken (mg)

A_{WT} = average weight of Capsule contents (mg/Capsule)

W = weight of the portion of Capsule contents taken (mg)

L_E = content of total ginsenosides, mg, in 100 mg of the Extract used to prepare the Capsules

L = amount of Extract per Capsule according to label claim (mg/Capsule)

Acceptance criteria: 90.0%–110.0% of Extract, calculated as the sum of ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd

PERFORMANCE TESTS

• DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS

(2040): Meet the requirements for *Disintegration*

• WEIGHT VARIATION OF DIETARY SUPPLEMENTS (2091):

Meet the requirements

CONTAMINANTS

• MICROBIAL ENUMERATION TESTS (2021):

The total aerobic microbial count does not exceed 10⁴ cfu/g. The total combined molds and yeasts count does not exceed 10³ cfu/g.

• ABSENCE OF SPECIFIED MICROORGANISMS (2022):

Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. Store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Capsules were prepared. The label also indicates the amount of Extract, in mg/Capsule. Label the Capsules to indicate the percentage of ginsenosides in the Extract contained in the Capsules. For soft-gelatin Capsules, state the method for *Content of Ginsenosides* with which the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** (11)
 - USP Powdered American Ginseng Extract RS
 - USP Powdered Asian Ginseng Extract RS

American Ginseng Tablets

DEFINITION

American Ginseng Tablets contain Powdered American Ginseng Extract. Tablets contain NLT 90.0% and NMT 110.0% of Extract, calculated as the sum of ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** (201)

Standard solution A: 20 mg/mL of USP Powdered American Ginseng Extract RS in methanol

Standard solution B: 20 mg/mL of USP Powdered Asian Ginseng Extract RS in methanol

Sample solution: Transfer a quantity of finely powdered Tablets, equivalent to 100 mg of Extract, to a conical flask. Extract at 55° with three 20-mL portions of a mixture of methanol and water (2:8). Evaporate the combined extracts to dryness under vacuum at 45°–50°. Dissolve the residue in 5 mL of methanol.

Application volume: 20 µL

Developing solvent system A: The lower phase of a mixture of chloroform, methanol, and water (13:7:2)

Developing solvent system B: The upper phase of a mixture of butyl alcohol, ethyl acetate, and water (4:1:5)

Spray reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, add 85 mL of methanol, mix, carefully add 5 mL of sulfuric acid, and mix.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Proceed as directed in the chapter. Develop in a chamber containing *Developing solvent system A* until the solvent front has moved 10.5 cm from the origin. Remove the plates from the chamber, and allow to dry. Turn the plates 90°, and develop in a chamber containing *Developing solvent system B* until the solvent front has moved 10.5 cm from the origin. Remove the plates from the chamber, and allow to dry. Spray with *Spray reagent*. Heat the plates at 105°–110° for 10 min, and examine. The order, from top to bottom, of ginsenosides on the plates is Rg₂ (on left) and Rg₁ (on right), Rf, Re, Rd, Rc, Rb₂ (on left) and Rb₁ (on right), and Ro. Ginsenosides Rg₂, Rg₁, Rf, Re, and Rd are found on the upper half of the plates; the remaining ginsenosides are found on the lower half after chromatographing with *Developing solvent system B*.

Acceptance criteria: The chromatogram of *Standard solution A* does not exhibit a spot for ginsenoside Rf. *Standard solution B* exhibits a spot for ginsenoside Rf. The spots from the *Sample solution* correspond to those from *Standard solution A*.

- **B.** The retention times of the peaks for ginsenosides Rg₁, Re, Rb₁, Rb₂, Rc₂, and Rd in the chromatogram of the

Sample solution correspond to those from the *Standard solution*, as obtained in the test for *Content of Ginsenosides*. The ratio of the peak response for Rb₂ to the peak response for Rb₁ is less than 0.4; and the ratio of the peak response for Rg₁ to the peak response for Rb₁ is less than 0.3. The *Sample solution* chromatogram shows no significant peak at the retention time corresponding to that of ginsenoside Rf in the *System suitability solution*, as obtained in the test for *Content of Ginsenosides*.

STRENGTH

- **CONTENT OF GINSENOSES**

Diluent: Water and alcohol (3:2)

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Standard solution: A solution of USP Powdered American Ginseng Extract RS in *Diluent* containing the equivalent of 1 mg/mL of ginsenoside Rb₁

Sample solution: Accurately weigh and finely powder NLT 20 Tablets. Transfer to a conical flask an accurately weighed portion of the powder expected to contain an amount of Extract equivalent to 15 mg of total ginsenosides, add 15 mL of methanol, and shake to mix. Sonicate the mixture at 25°–30° for 30 min. Cool, pass through filter paper, and return the residue to the conical flask. Add another 15 mL of methanol, sonicate the mixture at 25°–30° for 30 min, and filter. Wash the residue with three 15-mL portions of methanol. Evaporate the combined extracts and washings to dryness under vacuum at 45°–50°. Transfer the residue to a 10.0-mL volumetric flask, using small volumes of methanol, and dilute with methanol to volume.

System suitability solution: 24 mg/mL of USP Powdered Asian Ginseng Extract RS in *Diluent*. Filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Column

Guard column: 4.6-mm × 2.0-cm; packing L1

Analytical column: 4.6-mm × 15-cm; 3-µm packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *System suitability solution* (inject 20 µL)

Suitability requirements

Chromatogram similarity: The *System suitability solution* chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the six major ginsenosides, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*
Identify ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd in the *Standard solution* and *Sample solution* by comparing the chromatograms with the Reference Chromatogram provided with USP Powdered American Ginseng

Extract RS lot being used, and measure the peak responses.

Calculate the quantity, in mg, of each relevant ginsenoside (Rg₁, Re, Rb₁, Rc, Rb₂, and Rd) in the portion of Tablets taken:

$$\text{Result} = 0.1 \times (r_U/r_S) \times C_S \times P$$

- r_U = peak area for each relevant ginsenoside from the *Sample solution*
 r_S = peak area for each relevant ginsenoside from the *Standard solution*
 C_S = concentration of USP Powdered American Ginseng Extract RS in the *Standard solution* (mg/mL)
 P = labeled amount, in percentage, of each relevant ginsenoside in the USP Powdered American Ginseng Extract RS lot being used
 Calculate the content of total ginsenosides, T , in mg, by adding the amounts of individual ginsenoside.
 Calculate the percentage of Powdered Extract with respect to the label claim:

$$\text{Result} = T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

- T = content of total ginsenosides in the portion of Tablets taken (mg)
 A_{WT} = average weight of Tablets (mg/Tablet)
 W = weight of the portion of Tablets taken (mg)
 L_E = content of total ginsenosides, mg, in 100 mg of the Extract used to prepare the Tablets
 L = amount of Extract per Tablet according to label claim (mg/Tablet)
Acceptance criteria: 90.0%–110.0% of Powdered Extract, calculated as the sum of ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS <2040>:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS <2021>:** The total aerobic microbial count does not exceed 10⁴ cfu/g. The total combined molds and yeasts count does not exceed 10³ cfu/g. Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. Store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. The label also indicates the amount of Extract, in mg/Tablet. Label the Tablets to indicate the percentage of total ginsenosides in the Extract contained in the Tablets.
- **USP REFERENCE STANDARDS <11>**
 USP Powdered American Ginseng Extract RS
 USP Powdered Asian Ginseng Extract RS

Asian Ginseng

DEFINITION

Asian Ginseng consists of the dried roots of *Panax ginseng* C.A. Mey. (Fam. Araliaceae). It contains NLT 0.2% of ginsenoside Rg₁ and NLT 0.1% of ginsenoside Rb₁, both calculated on the dried basis.

IDENTIFICATION

A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 5 mg/mL each of arbutin and escin, in methanol

Sample solution: 1.0 g of finely powdered Asian Ginseng in a 25-mL flask fitted with a reflux condenser. Add 10.0 mL of a mixture of methanol and water (7:3), and heat under reflux for 15 min. Cool, filter, and dilute the filtrate with methanol to 10.0 mL.

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 20 µL, as bands

Developing solvent system: The upper layer of a mixture of butyl alcohol, ethyl acetate, and water (10:2.5:5) in an unsaturated chamber

Spray reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, add 85 mL of methanol, mix, and carefully add 5 mL of sulfuric acid, and mix.

Analysis

Samples: *Standard solution* and *Sample solution*
 Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, mark the solvent front, and allow the plate to dry. Spray with *Spray reagent*. Heat the plate at 105°–110° for 10 min, and examine the plate.

System suitability: The *Standard solution* chromatogram shows, in the upper third, a brown zone corresponding to arbutin, and in the lower third, a gray zone corresponding to escin.

Acceptance criteria: The *Sample solution* exhibits violet-gray zones corresponding to ginsenoside Rg₁ in the upper portion and to ginsenoside Re in the middle and in between the zones corresponding to arbutin and escin in the *Standard solution*. A violet-gray zone corresponding to ginsenoside Rb₁ is located at the same R_F value as the gray zone corresponding to escin in the *Standard solution*. Other, less intense bands may be observed between the zones due to ginsenosides Rb₁ and Re, and the zone closest to the origin corresponds to ginsenoside Rc. Other spots may be visible in the lower third of the chromatogram.

- **B.** The retention times of the peaks for ginsenosides Rg₁, Re, Rf, Rb₁, Rc, and Rd in the *Sample solution* chromatogram correspond to those in the *Standard solution*, as obtained in the test for *Content of Ginsenosides Rb₁ and Rg₁*. The ratio of the peak area for ginsenoside Rb₂ to the peak area for ginsenoside Rb₁ is NLT 0.4 (differentiation from American Ginseng).

COMPOSITION

CONTENT OF GINSENOIDES Rb₁ AND Rg₁

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Diluent: Alcohol and water (4:6)

Standard solution: Transfer a quantity of USP Powdered Asian Ginseng Extract RS, equivalent to 2 mg of ginsenoside Rg₁, to a suitable container, and dissolve in 10.0 mL of *Diluent*. [NOTE—The concentrations of ginsenoside Rg₁ and ginsenoside Rb₁ in this solution are not

expected to be equal and are determined on the basis of the labeled quantities present in USP Powdered Asian Ginseng Extract RS.]

Sample solution: Reduce 100 g of Asian Ginseng to a powder, and transfer about 1.0 g of the powder, accurately weighed, to a 100-mL, round-bottom flask fitted with a reflux condenser. Add 50 mL of *Diluent* and a few grains of pumice, and boil on a water bath under reflux for 1 h. Cool, and filter. Wash the flask and the residue with 20 mL of *Diluent*, and pass through the same filter. Combine the filtrates, and evaporate in a rotary evaporator at 50° to dryness. Dissolve the residue in 10.0 mL of *Diluent*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Analytical column: 4.6-mm × 15-cm; 3-μm packing L1

Guard column: 4.6-mm × 2.0-cm; packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the 6 major ginsenosides, in replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of ginsenosides Rb₁ and Rg₁ in the portion of Asian Ginseng taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of ginsenoside Rg₁ or ginsenoside Rb₁ from the *Sample solution*

r_S = peak response of ginsenoside Rg₁ or ginsenoside Rb₁ from the *Standard solution*

C_S = concentration of ginsenoside Rg₁ or ginsenoside Rb₁ in the *Standard solution* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Asian Ginseng taken to prepare the *Sample solution* (mg)

Acceptance criteria

Ginsenoside Rg₁: NLT 0.2% on the dried basis

Ginsenoside Rb₁: NLT 0.1% on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method III* <231>: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Methods for Pesticide Residues Analysis* <561>: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 10⁴ cfu/g. The total combined molds and yeasts count does not exceed 100 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

SPECIFIC TESTS

BOTANIC CHARACTERISTICS

Macroscopic: Fusiform or cylindrical roots, with distinct aromatic odor, sometimes branched, typically 1–10 cm,

sometimes up to 20 cm in length and up to 2.5 cm in diameter at the crown, with one or more stem scars. Externally pale yellow to golden, rough textured in the lower part, with prominent horizontal rings and fine longitudinal ridges as a result of drying. Root scars or fine rootlets are present. Fractures are short, with the fractured surface, white to ivory, exposing a ring of secretory canals present in secondary phloem.

Histology

Transverse section of root: Multiple layers of thin-walled cork cells present. Secondary phloem characterized by conspicuous air lacunae, abundant starch-containing storage parenchyma, few sieve elements, and rings of schizogenous secretory canals. Xylem characterized by abundant starch-containing storage parenchyma, few tracheary elements, and a lack of secretory canals. Druse crystals are sometimes present with vascular parenchyma cells.

- **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* <561>: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Alcohol-Soluble Extractives*, *Method 2* <561>: NLT 14.0%
- **LOSS ON DRYING** <731>: Dry 1.0 g of finely powdered Asian Ginseng at 105° for 2 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 8.0%, determined on 1.0 g of finely powdered Asian Ginseng
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* <561>: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store in a cool, dry place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** <11>
USP Powdered Asian Ginseng Extract RS

Powdered Asian Ginseng

DEFINITION

Powdered Asian Ginseng is Asian Ginseng reduced to a fine or very fine powder. It contains NLT 0.2% of ginsenoside Rg₁ and NLT 0.1% of ginsenoside Rb₁, both calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution: 5 mg/mL each of arbutin and escin, in methanol
Sample solution: 1.0 g of Powdered Asian Ginseng in a 25-mL flask fitted with a reflux condenser. Add 10.0 mL of a mixture of methanol and water (7:3), and heat under reflux for 15 min. Cool, filter, and dilute the filtrate with methanol to 10.0 mL.
Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)
Application volume: 20 μL, as bands
Developing solvent system: The upper layer of a mixture of butyl alcohol, ethyl acetate, and water (10:2.5:5) in an unsaturated chamber
Spray reagent: Dissolve 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid, and add 85 mL of methanol, mix, and carefully add 5 mL of sulfuric acid to this mixture.

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, mark the solvent front, and allow the plate to dry. Spray with *Spray reagent*. Heat the plate at 105°–110° for 10 min, and examine the plate.

System suitability: The *Standard solution* chromatogram shows, in the upper third, a brown zone corresponding to arbutin, and in the lower third, a gray zone corresponding to escin.

Acceptance criteria: The *Sample solution* exhibits violet-gray zones corresponding to ginsenoside Rg₁ in the upper portion and to ginsenoside Re in the middle and in between the zones corresponding to arbutin and escin in the *Standard solution*. A violet-gray zone corresponding to ginsenoside Rb₁ is located at the same R_F value as the gray zone corresponding to escin in the *Standard solution*. Other, less intense bands may be observed between the zones due to ginsenosides Rb₁ and Re, and the zone closest to the origin corresponds to ginsenoside Rc. Other spots may be visible in the lower third of the chromatogram.

- **B.** The retention times of the peaks for ginsenosides Rg₁, Re, Rf, Rb₁, Rc, and Rd in the *Sample solution* chromatogram correspond to those in the *Standard solution*, as obtained in the test for *Content of Ginsenosides Rb₁ and Rg₁*. The ratio of the peak area for ginsenoside Rb₂ to the peak area for ginsenoside Rb₁ is NLT 0.4 (differentiation from American Ginseng).

COMPOSITION

- **CONTENT OF GINSENOSES RB₁ AND RG₁**

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Diluent: Alcohol and water (4:6)

Standard solution: Transfer a quantity of USP Powdered Asian Ginseng Extract RS, equivalent to 2 mg of ginsenoside Rg₁, to a suitable container, and dissolve in 10.0 mL of *Diluent*. [NOTE—The concentrations of ginsenoside Rg₁ and ginsenoside Rb₁ in this solution are not expected to be equal and are determined on the basis of the labeled quantities present in USP Powdered Asian Ginseng Extract RS.]

Sample solution: Transfer about 1.0 g of Powdered Asian Ginseng, accurately weighed, to a 100-mL, round-bottom flask fitted with a reflux condenser. Add 50 mL of a mixture of *Diluent* and a few grains of pumice, and boil on a water bath under reflux for 1 h. Cool, and filter. Wash the flask and the residue with 20 mL of *Diluent*, and pass through the same filter. Combine the filtrates, and evaporate in a rotary evaporator at 50° to dryness. Dissolve the residue in 10.0 mL of *Diluent*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Analytical column: 4.6-mm × 15-cm; 3-μm packing L1

Guard column: 4.6-mm × 2.0-cm; packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the 6 major ginsenosides, in replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of ginsenosides Rb₁ and Rg₁ in the portion of Powdered Asian Ginseng taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of ginsenoside Rg₁ or ginsenoside Rb₁ from the *Sample solution*

r_S = peak response of ginsenoside Rg₁ or ginsenoside Rb₁ from the *Standard solution*

C_S = concentration of ginsenoside Rg₁ or ginsenoside Rb₁ in the *Standard solution* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Powdered Asian Ginseng used to prepare the *Sample solution* (mg)

Acceptance criteria

Ginsenoside Rg₁: NLT 0.2% on the dried basis

Ginsenoside Rb₁: NLT 0.1% on the dried basis

CONTAMINANTS

- **HEAVY METALS, Method III <231>:** NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis <561>:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic microbial count does not exceed 10⁴ cfu/g. The total combined molds and yeasts count does not exceed 10² cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** It meets the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Pale yellowish-brown powder with a slightly aromatic odor. When examined under a microscope, the powder shows the following: traces of cork composed of thin-walled polygonal cells but mainly with phelloderm on the outside; wide cortex of parenchymatous cells with numerous secretory canals arranged in concentric zones; parenchymatous xylem with nonlignified tracheids and slightly lignified vessels with spiral and reticulate thickening, isolated or in small groups; small granules of starch 0.5–1.0 μm in diameter in all of the parenchymatous cells; and occasional cluster crystals of calcium oxalate in the cells of the central region.
- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter <561>:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method 2 <561>:** NLT 14.0%
- **LOSS ON DRYING <731>:** Dry 1.0 g of finely Powdered Asian Ginseng at 105° for 2 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash <561>:** NMT 8.0%, determined on 1.0 g of finely Powdered Asian Ginseng
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash <561>:** NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store in a cool, dry place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.
- **USP REFERENCE STANDARDS** <11>
USP Powdered Asian Ginseng Extract RS

Powdered Asian Ginseng Extract**DEFINITION**

Powdered Asian Ginseng Extract is prepared from Asian Ginseng by maceration, percolation, or both processes performed at room temperature with suitable solvents such as alcohol, methanol, water, or mixtures of these solvents, and by concentrating the fluidextract at temperatures below 50°. The ratio of the starting crude plant material to Powdered Asian Ginseng Extract is between 3:1 and 7:1. It contains NLT 3.0% of ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd combined, calculated on the anhydrous basis. It may contain other added substances.

IDENTIFICATION**A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Extraction column: Use a solid-phase extraction column that contains C18 packing with 55- to 105-μm particle size and a ratio of sorbent mass to column volume of 360 mg/0.85 mL, or equivalent. Condition the column before use by washing with 3 mL of methanol and 8 mL of water.

Standard solution: Transfer about 0.1 g of USP Powdered Asian Ginseng Extract RS to a 5-mL volumetric flask, and proceed as directed for the *Sample solution*, beginning with "Dissolve in water".

Sample solution: About 1.0 g of Powdered Asian Ginseng Extract in a 25-mL volumetric flask. Dissolve in water, sonicating if necessary. Dilute with water to volume. Transfer 4.0 mL of this solution to the *Extraction column*, wash with 10 mL of water, and discard the eluate. Elute the column with 2 mL of methanol. [NOTE—Do not use vacuum, elute manually and slowly.] Collect the eluate in a suitable vial.

Adsorbent: 0.2-mm layer of chromatographic silica gel mixture on a high-performance thin-layer plate

Application volume: 10 μL, as bands

Developing solvent system: Chloroform, methanol, and water (65:35:10). Use the lower phase.

Spray reagent: Alcohol, acetic anhydride, and sulfuric acid (18:1:1)

Analysis

Samples: *Standard solution* and *Sample solution*
Saturate the chamber with *Developing solvent system* for 2 h. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, mark the solvent front, and allow the plate to dry. Spray with *Spray reagent*, and heat in an oven at 105° for 10 min. Immediately examine the plate in white light.

Acceptance criteria: The *Sample solution* exhibits, among other spots, eight brown-violet spots at the *R_f* values of about 0.70, 0.60, 0.50, 0.36, 0.30, 0.28, 0.20, and 0.18, corresponding in color and *R_f* values to those obtained for the *Standard solution*.

- **B.** Add 2 mL of glacial acetic acid to 0.1 g of Powdered Asian Ginseng Extract, warm for 5 min in a hot water bath, and filter. Gently add 0.5 mL of sulfuric acid to 1.0 mL of the filtrate.
Acceptance criteria: A red-brown color develops at the zone of contact.
- **C.** The retention times of the peaks for ginsenosides Rg₁, Re, Rf, Rb₁, Rb₂, Rc, and Rd in the *Sample solution* chro-

matogram correspond to those in the *Standard solution*, as obtained in the test for *Content of Ginsenosides*. The ratio of the peak area of Rb₂ to the peak area of Rb₁ is NLT 0.4 (differentiation from American Ginseng).

COMPOSITION**• CONTENT OF GINSENOSES**

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Diluent: Alcohol and water (4:6)

Standard solution: 24 mg/mL of USP Powdered Asian Ginseng Extract RS in *Diluent*. Dissolve by sonicating for 10 min, mix, and filter.

Sample solution: Proceed as directed for *Standard solution*, except use Powdered Asian Ginseng Extract.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Analytical column: 4.6-mm × 15-cm; 3-μm packing L1

Guard column: 4.6-mm × 2.0-cm; packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the 6 major ginsenosides, in replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the peaks for the ginsenosides by comparison with the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used, and measure the peak areas for the 6 major ginsenosides.

Calculate the percentage of each relevant ginsenoside (Rg₁, Re, Rb₁, Rc, Rb₂, and Rd) in the portion of Powdered Asian Ginseng Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times P$$

r_U = peak area for each relevant ginsenoside from the *Sample solution*

r_S = peak area for each relevant ginsenoside from the *Standard solution*

C_S = concentration of USP Powdered Asian Ginseng Extract RS in the *Standard solution* (mg/mL)

C_U = concentration of Powdered Asian Ginseng Extract in the *Sample solution* (mg/mL)

P = labeled amount, in percentage, of each relevant ginsenoside in the USP Powdered Asian Ginseng Extract RS

Calculate the percentage of ginsenosides by adding the percentages of each relevant ginsenoside.

Acceptance criteria: NLT 3.0% on the anhydrous basis

CONTAMINANTS

- **HEAVY METALS** (231): NMT 30 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 300 cfu/g. The total combined molds and yeasts count does not exceed 100 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

SPECIFIC TESTS

- **WATER DETERMINATION**, *Method I* (921): NMT 7.0%, determined on a 0.15-g specimen
- **ALCOHOL DETERMINATION**, *Method II* (611): NMT 0.25%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Meets the requirements in *Botanical Extracts* (565)
- **LABELING:** Meets the requirements in *Botanical Extracts* (565)
- **USP REFERENCE STANDARDS** (11)
USP Powdered Asian Ginseng Extract RS

Asian Ginseng Tablets

DEFINITION

Asian Ginseng Tablets are prepared from Powdered Asian Ginseng Extract. They contain NLT 90.0% and NMT 110.0% of Powdered Extract, calculated as the sum of ginsenosides Rg₁, Re, Rb₁, Rc, Rb₂, and Rd.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** (201)
Standard solution: 5 mg/mL each of arbutin and escin, in methanol
Sample solution: Transfer the equivalent of 100 mg of Powdered Extract from powdered Tablets to a conical flask, and extract three times, each with a 20-mL portion of a mixture of methanol and water (4:1), in a 55° bath for 30 min, stirring with a magnetic stirrer. Evaporate the combined extracts to dryness in vacuum between 45° and 50°, and dissolve the residue in 10 mL of a mixture of methanol and water (3:2).
Application volume: 20 µL, as bands
Developing solvent system: The upper layer of a mixture of butyl alcohol, ethyl acetate, and water (4:1:2) in an unsaturated chamber
Spray reagent: 0.5 mL of anisaldehyde in 10 mL of glacial acetic acid. Add 85 mL of methanol, carefully add 5 mL of sulfuric acid, and mix.
Analysis
Samples: *Standard solution* and *Sample solution*
Proceed as directed in the chapter. Remove the plate from the developing chamber, and allow it to dry.
Spray with *Spray reagent*. Heat the plate at 105°–110° for 10 min, and examine the plate.
Acceptance criteria: The chromatogram of the *Standard solution* shows, in the upper third, a brown zone corresponding to arbutin and, in the lower third, a gray zone corresponding to escin. Between these two zones, the chromatogram of the *Sample solution* exhibits violet-gray zones corresponding to ginsenoside Rg₁ in the upper portion and to ginsenoside Re in the middle. A violet-gray zone corresponding to ginsenoside Rb₁ is lo-

cated at the same R_F value as the gray zone corresponding to escin in the chromatogram of the *Standard solution*. Other, less intense bands may be observed between the zones due to ginsenosides Rb₁ and Re, and the zone closest to the origin corresponds to ginsenoside Rc. Other spots may be visible in the lower third of the chromatogram.

- **B.** The retention times of the relevant analytes of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Content of Ginsenosides*. The retention time of the peak for ginsenoside R_F of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Ginsenosides*.

STRENGTH

• CONTENT OF GINSENOSES

Diluent: Water and alcohol (3:2)

Solution A: Water

Solution B: Acetonitrile and water (4:1)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	76	24
12	76	24
28	65	35
51.5	56.5	43.5
52.5	0	100
64.5	76	24
77	76	24

Standard solution: 40 mg/mL of USP Powdered Asian Ginseng Extract RS in *Diluent*. Filter.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a quantity of the powder, equivalent to 200 mg of Powdered Extract to a conical flask, and extract three times, each with a 20-mL portion of a mixture of methanol and water (4:1), in a 55° bath for 30 min, stirring with a magnetic stirrer. Evaporate the combined extracts to dryness in a vacuum between 45° and 50°. Dissolve the residue in 5.0 mL of *Diluent*, and filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 203 nm

Column

Guard: 4.6-mm × 2.0-cm; packing L1

Analytical: 4.6-mm × 15-cm; 3-µm packing L1

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Chromatogram similarity: The *Standard solution* chromatogram is similar to the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used.

Relative standard deviation: NMT 2.0%, determined for the sum of the peak areas for the six major ginsenosides, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*
Record the chromatograms, identify the peaks for the ginsenosides by comparison with the Reference Chromatogram provided with the lot of USP Powdered Asian Ginseng Extract RS being used, and measure the peak areas for the six major ginsenosides. Calculate the quantity, in mg, of each relevant ginsenoside (Rg₁, Re, Rb₁, Rc, Rb₂, and Rd) in the portion of Tablets taken:

$$\text{Result} = 0.05 \times (r_u/r_s) \times C_s \times P$$

- r_U = peak areas for each relevant ginsenoside from the *Sample solution*
 r_S = peak areas for each relevant ginsenoside from the *Standard solution*
 C_S = concentration of USP Powdered Asian Ginseng Extract RS in the *Standard solution* (mg/mL)
 P = labeled amount, in percentage, of each relevant ginsenoside in the USP Powdered Asian Ginseng Extract RS lot being used

Calculate the content of total ginsenosides, T , in mg, by adding the amounts of individual ginsenosides.
 Calculate the percentage of Powdered Extract with respect to the label claim:

$$\text{Result} = T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

- T = content of total ginsenosides in the portion of Tablets taken (mg)
 A_{WT} = average weight of Tablets (mg/Tablet)
 W = weight of the portion of Tablets taken (mg)
 L_E = content of total ginsenosides in 100 mg of the Extract used to prepare the Tablets (mg)
 L = amount of Extract per Tablet according to label claim (mg/Tablet)

Acceptance criteria: 90.0%–110.0% of Powdered Extract, calculated as the sum of ginsenosides R_{g1} , R_E , R_{b1} , R_C , R_{b2} , and R_d

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS <2040>:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 1000 cfu/g. Tablets meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. The label also indicates the amount of Powdered Extract, in mg/Tablet, and the content, in mg, of ginsenosides per 100 mg of Powdered Extract.
- **USP REFERENCE STANDARDS <11>**
USP Powdered Asian Ginseng Extract RS

Andrographis

DEFINITION

Andrographis consists of the dried stems and leaves of *Andrographis paniculata* (Burm. f.) Nees (Fam. Acanthaceae). It contains NLT 1.0% of diterpene lactones, calculated on the dried basis as the sum of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide and andrograpanin.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST <201>**
Standard solution 1: Use *Standard solution A*, prepared as directed in the test for *Content of Diterpene Lactones*.
Standard solution 2: Sonicate an amount of USP Powdered Andrographis Extract RS, equivalent to about 15 mg of diterpene lactones, for 10–15 min in 25 mL of methanol, centrifuge, and use the supernatant.

Sample solution: Use *Sample stock solution*, prepared as directed in the test for *Content of Diterpene Lactones*.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μm (TLC plates)

Application volume: 10 μL , as 5–10 mm bands

Developing solvent system: Chloroform, acetone, and toluene (2:2:1)

Spray reagent: A mixture of 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)

Analysis

Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*

Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about 90% of the length of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 5–10 min at 100°, and examine under visible light.

Acceptance criteria: The *Sample solution* exhibits three main grayish blue zones with R_f values of approximately 0.4, 0.6, and 0.8 that correspond in position and color to zones in *Standard solution 2*. *Standard solution 1* exhibits a grayish blue zone due to andrographolide at an R_f of about 0.4. The *Sample solution* exhibits a zone similar in color and R_f value to that due to andrographolide in *Standard solution 1*.

- **B.** The retention time of the main peak of the *Sample solution* obtained in the test for *Content of Diterpene Lactones* corresponds to that of andrographolide in *Standard solution A*. Identify other diterpene lactone peaks in the *Sample solution* by comparison with *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS. The *Sample solution* shows additional peaks corresponding to neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin.

COMPOSITION

• CONTENT OF DITERPENE LACTONES

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Use filtered and degassed acetonitrile.

Standard solution A: Dissolve a weighed quantity of USP Andrographolide RS in methanol to obtain a solution having a known concentration of about 1.0 mg/mL. Transfer 5.0 mL of this solution to a 10-mL volumetric flask, dilute with acetonitrile to volume, and mix.

Standard solution B: Transfer an amount of USP Powdered Andrographis Extract RS, equivalent to about 25 mg of diterpene lactones, to a 50-mL volumetric flask, add 25 mL of methanol, heat gently for 15–20 min, dilute with acetonitrile to volume, and mix. Before injection, pass through a membrane filter of 0.45- μm or finer pore size, discarding the first 5 mL of the filtrate.

Sample stock solution: Transfer about 2.0 g of finely powdered Andrographis to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for 15 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume to 50.0 mL using methanol.

Sample solution: Transfer 25.0 mL of *Sample stock solution* to a 50-mL volumetric flask, dilute with acetonitrile to volume, and mix. Before injection, pass through a membrane filter of 0.45- μm or finer pore size, discarding the first 5 mL of the filtrate.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45

Time (min)	Solution A (%)	Solution B (%)
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 223 nm**Column:** 4.6-mm × 25-cm; 5-μm packing L1**Flow rate:** 1.5 mL/min**Injection size:** 20 μL**System suitability****Samples:** *Standard solution A* and *Standard solution B***Suitability requirements**The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS.**Column efficiency:** NLT 5000 theoretical plates, *Standard solution A***Tailing factor:** NMT 1.5 for the andrographolide peak, *Standard solution A***Relative standard deviation:** NMT 2.0%, determined from the andrographolide peak for replicate injections, *Standard solution A***Resolution:** NLT 5 between the neoandrographolide and 14-deoxy-11,12-didehydroandrographolide peaks, *Standard solution B***Analysis****Samples:** *Standard solution A*, *Standard solution B*, and *Sample solution*Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS being used, identify the retention times of the peaks corresponding to the different diterpene lactones. The approximate relative retention times of the different diterpene lactones are provided in the following table:

Analyte	Relative Retention Time
Andrographolide	1.00
Neoandrographolide	1.16
14-Deoxy-11,12-didehydroandrographolide	1.31
Andrograpanin	1.50

Separately calculate the percentages of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin in the portion of Andrographis taken:

$$\text{Result} = (C_s/W) \times (r_u/r_s) \times 10F$$

- C_s = concentration of USP Andrographolide RS in *Standard solution A* (mg/mL)
 W = weight of Andrographis taken to prepare the *Sample solution* (g)
 r_u = peak response for each diterpene lactone from the *Sample solution*
 r_s = peak response for andrographolide from *Standard solution A*
 F = conversion factor for each analyte (1.00 for andrographolide, 3.90 for neoandrographolide, 1.45 for 14-deoxy-11,12-didehydroandrographolide, and 2.65 for andrograpanin)

Acceptance criteria: NLT 1.0%, on the dried basis, of the sum of the percentages of andrographolide,

neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin

IMPURITIES**Inorganic Impurities**• **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* <561>: NMT 3.0%• **HEAVY METALS**, *Method II* <231>: NMT 20 ppm**Organic Impurities**• **PROCEDURE 1: ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* <561>: NMT 2.0%• **PROCEDURE 2: ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements**SPECIFIC TESTS**• **BOTANIC CHARACTERISTICS**

Macroscopic: Stem is dark green, woody, 2–6 mm in diameter, bearing numerous branches, showing slightly swollen nodes, the upper part is distinctly quadrangular with four bulges in the four corners, and the lower part is somewhat rounded; texture fragile, easily broken; branches quadrangular, often narrowly winged in the upper part. Leaves are simple, opposite, short petiolated or nearly sessile; lamina crumpled and easily broken, when whole, lanceolate or ovate-lanceolate, 2–7 cm long, 1–3 cm wide, with acuminate apex, reticulate venation and cuneate-decurrent base, margin entire or undulate; the upper surface green, the lower surface grayish green; both surfaces are glabrous. Pharmacopeial article consists of dry mixtures of crisp, dark-green broken leaves and quadrangular stems; leaves brittle; stems fracture short, fibrous.

Histology

Transverse section of stems: Epidermal layer showing cells containing round, long-elliptical or clavate calcium carbonate deposits (cystoliths), 1–4 celled nonglandular hairs and multicellular, disk-shaped glandular hairs; collenchyma below the epidermis and in the bulges; endodermis is distinct; vascular bundles surround the parenchyma of the central pith; small acicular crystals of calcium oxalate present in the cortex and pith.

Transverse section of leaves: Subsquare or rectangular upper and lower epidermal cells; lower epidermal cells are relatively smaller; both epidermal layers show cells containing cystoliths, nonglandular hairs and glandular hairs similar to those of the stem; mesophyll composed of 1–2 layers of palisade parenchyma and spongy parenchyma; loosely arranged spongy parenchyma appear across the upper part of the midrib; vascular bundles of midrib are collateral and grooved; cells containing cystoliths appear above the xylem.

- **LOSS ON DRYING** <731>: Dry 1.0 g of finely powdered Andrographis at 105° for 3 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 15%, determined on 1.0 g of finely powdered Andrographis
- **ARTICLES OF BOTANICAL ORIGIN**, *Alcohol-Soluble Extractives*, *Method 2* <561>: NLT 8.0%
- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic bacterial count does not exceed 10⁵ cfu/g; the total combined molds and yeasts count does not exceed 10³ cfu/g; and the bile-tolerant Gram-negative bacteria does not exceed 10³ cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** <11>
USP Andrographolide RS
USP Powdered Andrographis Extract RS

Powdered Andrographis

DEFINITION

Powdered Andrographis is Andrographis reduced to a fine or very fine powder.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST <201>

Standard solution 1: Use *Standard solution A*, prepared as directed in the test for *Content of Diterpene Lactones*.

Standard solution 2: Sonicate an amount of USP Powdered Andrographis Extract RS, equivalent to about 15 mg of diterpene lactones, for 10–15 min in 25 mL of methanol, centrifuge, and use the supernatant.

Sample solution: Use *Sample stock solution*, prepared as directed in the test for *Content of Diterpene Lactones*.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL, as 5–10 mm bands

Developing solvent system: Chloroform, acetone, and toluene (2:2:1)

Spray reagent: A mixture of 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)

Analysis

Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*

Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about 90% of the length of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 5–10 min at 100°, and examine under visible light.

Acceptance criteria: The *Sample solution* exhibits three main grayish blue zones with R_f values of approximately 0.4, 0.6, and 0.8 that correspond in position and color to the main zones of *Standard solution 2*. *Standard solution 1* exhibits a grayish blue zone due to andrographolide at an R_f of about 0.4. The *Sample solution* exhibits a zone similar in color and R_f value to that due to andrographolide in *Standard solution 1*.

- **B.** The retention time of the main peak of the *Sample solution* obtained in the test for *Content of Diterpene Lactones* corresponds to that of andrographolide in *Standard solution A*. Identify other diterpene lactone peaks in the *Sample solution* by comparison with *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS. The *Sample solution* shows additional peaks corresponding to neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin.

COMPOSITION

• CONTENT OF DITERPENE LACTONES

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Use filtered and degassed acetonitrile.

Standard solution A: Dissolve a weighed quantity of USP Andrographolide RS in methanol to obtain a solution having a known concentration of about 1.0 mg/mL. Transfer 5.0 mL of this solution to a 10-mL volumetric flask, dilute with acetonitrile to volume, and mix.

Standard solution B: Transfer an amount of USP Powdered Andrographis Extract RS, equivalent to about

25 mg of diterpene lactones, to a 50-mL volumetric flask, add 25 mL of methanol, heat gently for 15–20 min, dilute with acetonitrile to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

Sample stock solution: Transfer about 2.0 g of Powdered Andrographis to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for 15 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume to 50.0 mL using methanol.

Sample solution: Transfer 25.0 mL of *Sample stock solution* to a 50-mL volumetric flask, dilute with acetonitrile to volume, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size discarding the first 5 mL of the filtrate.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 223 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS.

Column efficiency: NLT 5000 theoretical plates, *Standard solution A*

Tailing factor: NMT 1.5 for the andrographolide peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, determined from the andrographolide peak for replicate injections, *Standard solution A*

Resolution: NLT 5 between the neoandrographolide and 14-deoxy-11,12-didehydroandrographolide peaks, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS being used, identify the retention times of the peaks corresponding to the different diterpene lactones. The approximate relative retention times of the different diterpene lactones are provided in the following table:

Analyte	Relative Retention Time
Andrographolide	1.00
Neoandrographolide	1.16
14-Deoxy-11,12-didehydroandrographolide	1.31
Andrograpanin	1.50

Separately calculate the percentages of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin in the portion of Powdered Andrographis taken:

$$\text{Result} = (C_s/W) \times (r_U/r_s) \times 10F$$

- r_U = peak response for each diterpene lactone from the *Sample solution*
 r_s = peak response for andrographolide from *Standard solution A*
 C_s = concentration of USP Andrographolide RS in *Standard solution A* (mg/mL)
 W = weight of Powdered Andrographis taken to prepare the *Sample solution* (g)
 F = conversion factor for each analyte (1.00 for andrographolide, 3.90 for neoandrographolide, 1.45 for 14-deoxy-11,12-didehydroandrographolide, and 2.65 for andrograpanin)

Acceptance criteria: NLT 1.0%, on the dried basis, of the sum of the percentages of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin

IMPURITIES

Inorganic Impurities

- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** <561>: NMT 3.0%

- **HEAVY METALS, Method II** <231>: NMT 20 ppm

Organic Impurities

- **PROCEDURE: ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** <561>: Meets the requirements

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: It is a grayish brown powder.

Histology

Microscopic examination: It reveals cells of the upper and lower epidermis of the leaves, some cells containing large cystoliths, up to 36 μm in diameter and 180 μm long, with a hilum-shaped scar in the large end; 1–4 celled nonglandular hairs; disk-shaped glandular hairs, 8-celled head and very short stalk; diacytic stomata mostly on the lower epidermis; stem epidermal cells, some cells containing cystoliths, stomata, nonglandular hairs and glandular hairs similar to those of the leaves; thin-walled parenchyma cells; collenchyma cells; acicular phloem fibers; tracheids; vessels, with spiral and scalariform thickening.

- **LOSS ON DRYING** <731>: Dry 1.0 g of Powdered Andrographis at 105° for 3 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 15%, determined on 1.0 g of Powdered Andrographis
- **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method 2** <561>: NLT 8.0%
- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic bacterial count does not exceed 10^5 cfu/g; the total combined molds and yeasts count does not exceed 10^3 cfu/g; and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.

• USP REFERENCE STANDARDS <11>

- USP Andrographolide RS
- USP Powdered Andrographis Extract RS

Powdered Andrographis Extract

DEFINITION

Powdered Andrographis Extract is prepared from Andrographis by extraction with methanol or alcohol. The ratio of plant material to extract is between 15:1 and 10:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of diterpene lactones, calculated on the dried basis as the sum of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin. The content of 14-deoxy-11,12-didehydroandrographolide is NMT 15% of the total diterpene lactones. It may contain suitable added substances as carriers.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST <201>

Standard solution 1: Use *Standard solution A*, prepared as directed in the test for *Content of Diterpene Lactones*.

Standard solution 2: Sonicate for 10–15 min a quantity of USP Powdered Andrographis Extract, equivalent to about 15 mg of diterpene lactones, in 25 mL of methanol. Centrifuge, and use the supernatant.

Sample solution: Sonicate for 10–15 min a quantity of Powdered Andrographis Extract, equivalent to about 15 mg of diterpene lactones, in 25 mL of methanol. Centrifuge, and use the supernatant.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μm (TLC plates)

Application volume: 10 μL , as 5–10 mm bands

Developing solvent system: Chloroform, acetone, and toluene (2:2:1)

Spray reagent: A mixture containing 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)

Analysis

Samples: *Standard solution 1*, *Standard solution 2*, and *Sample solution*

Use a saturated chamber. Develop until the solvent front has moved up about 90% of the plate. Remove the plate from the chamber. Dry, and spray with *Spray reagent*. Heat for 5–10 min at 100°, and examine under visible light.

Acceptance criteria: The *Sample solution* exhibits three main grayish blue zones with R_f values of approximately 0.4, 0.6, and 0.8 that correspond in position and color to zones in *Standard solution 2*. *Standard solution 1* exhibits a grayish blue zone due to andrographolide at an R_f of about 0.4. The *Sample solution* exhibits a zone similar in color and R_f value to that due to andrographolide in *Standard solution 1*.

- **B.** The *Sample solution* in the test for *Content of Diterpene Lactones* shows a main peak at a retention time corresponding to that of andrographolide in *Standard solution A*. Identify other diterpene lactone peaks in the *Sample solution* by comparison with *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS. The *Sample solution* shows additional peaks corresponding to neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin.

COMPOSITION

• CONTENT OF DITERPENE LACTONES

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Use filtered and degassed acetonitrile.

Standard solution A: Dissolve a weighed quantity of USP Andrographolide RS in methanol to obtain a solution having a known concentration of about 1.0 mg/mL. Transfer 5.0 mL of this solution to a 10-mL volumetric flask, dilute with acetonitrile to volume, and mix.

Standard solution B: Transfer an amount of USP Powdered Andrographis Extract RS, equivalent to about 25 mg of diterpene lactones, to a 50-mL volumetric flask, add 25 mL of methanol, heat gently for 15–20 min, dilute with acetonitrile to volume, and mix. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first 5 mL of the filtrate.

Sample solution: Transfer a weighed amount of Powdered Andrographis Extract, equivalent to about 25 mg of diterpene lactones, to a 50-mL volumetric flask, add 25 mL of methanol, heat gently for 15–20 min, dilute with acetonitrile to volume, and mix. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first 5 mL of the filtrate.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 223 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 1.5 mL/min

Injection size: 20 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements
The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS.

Column efficiency: NLT 5000 theoretical plates, *Standard solution A*

Tailing factor: NMT 1.5 for the andrographolide peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, determined from the andrographolide peak for replicate injections, *Standard solution A*

Resolution: NLT 5 between the neoandrographolide and 14-deoxy-11,12-didehydroandrographolide peaks, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Andrographis Extract RS being used, identify the retention times of the peaks corresponding to different diterpene lactones. The approximate relative retention times of the different diterpene lactones are provided in the following table:

Analyte	Relative Retention Time
Andrographolide	1.00
Neoandrographolide	1.16

Analyte	Relative Retention Time
14-Deoxy-11,12-didehydroandrographolide	1.31
Andrograpanin	1.50

Separately calculate the percentages of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin in the portion of Powdered Andrographis Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times 5F$$

r_U = peak response for each diterpene lactone from the *Sample solution*

r_S = peak response for andrographolide from *Standard solution A*

C_S = concentration of USP Andrographolide RS in *Standard solution A* (mg/mL)

W = weight of Powdered Andrographis Extract taken to prepare the *Sample solution* (g)

F = conversion factor for each analyte (1.00 for andrographolide, 3.90 for neoandrographolide, 1.45 for 14-deoxy-11,12-didehydroandrographolide, and 2.65 for andrograpanin)

Acceptance criteria: 90.0%–110.0%, on the dried basis, of the labeled amount of diterpene lactones calculated as the sum of the percentages of andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, and andrograpanin

IMPURITIES

Inorganic Impurities

• **HEAVY METALS**, *Method II* ⟨231⟩: NMT 20 ppm

Organic Impurities

• **PROCEDURE: ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* ⟨561⟩: Meets the requirements

SPECIFIC TESTS

• **LOSS ON DRYING** ⟨731⟩: Dry 2.0 g at 105° for 3 h: it loses NMT 5.0% of its weight.

• **MICROBIAL ENUMERATION TESTS** ⟨2021⟩: The total aerobic microbial count does not exceed 10⁴ cfu/g. The total combined yeasts and molds count does not exceed 10³ cfu/g.

• **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** ⟨2022⟩: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. It meets other labeling requirements under *Botanical Extracts* ⟨565⟩.

• **USP REFERENCE STANDARDS** ⟨11⟩

USP Andrographolide RS

USP Powdered Andrographis Extract RS

Arginine—see *Arginine General Monographs*

Arginine Hydrochloride—see *Arginine Hydrochloride General Monographs*

Arginine Capsules

DEFINITION

Arginine Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of arginine or arginine hydrochloride in an amount equivalent to arginine ($C_6H_{14}N_4O_2$).

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)

Standard solution: 1.5 mg/mL of USP L-Arginine RS or USP Arginine Hydrochloride RS in water

Sample solution: Weigh the content of NLT 20 Capsules, mix, and transfer a portion of the content, equivalent to about 150 mg of arginine, to a 100-mL volumetric flask, add 80 mL of water, sonicate for 15 min, dilute with water to volume, mix, and filter.

Application volume: 5 μ L

Developing solvent system: Isopropyl alcohol and ammonium hydroxide (7:3)

Spray reagent: 2 mg/mL of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (95:5)

Analysis: Proceed as directed for *Chromatography* (621), *Thin-Layer Chromatography*. Dry the plate at 100°–105° until the ammonia disappears completely. Spray with the *Spray reagent*, and heat at 100°–105° for about 15 min. Examine the plate under white light.

Acceptance criteria: The principal spot from the *Sample solution* corresponds in appearance and R_f value to that of the *Standard solution*.

- **B.** The retention time of the major peak of the *Sample solution* corresponds to that from the *Standard solution*, as obtained in the *Strength*.

STRENGTH

• PROCEDURE

Buffer: 6.9 mg/mL of monobasic sodium phosphate in water. Adjust with phosphoric acid to a pH of 3.5.

Solution A: 0.5 mg/mL of 1-octanesulfonic acid sodium salt in *Buffer*

Mobile phase: *Solution A* and acetonitrile (95:5)

Standard solution: 1.5 mg/mL of USP L-Arginine RS or USP Arginine Hydrochloride RS in *Buffer*

Sample solution: Weigh the content of NLT 20 Capsules, mix, and transfer a portion of the content, equivalent to about 150 mg of arginine, to a 100-mL volumetric flask, add 80 mL of *Buffer*, sonicate for 15 min, dilute with *Buffer* to volume, mix, and filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 215 nm

Column: 4.6-mm \times 25-cm; packing L7

Flow rate: 0.8 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 1500 theoretical plates

Relative standard deviation: NMT 2.0% from the arginine peak, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of arginine in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak response from the *Sample solution*
- r_S = peak response from the *Standard solution*
- C_S = concentration of the *Standard solution* (mg/mL)
- C_U = nominal concentration of Arginine in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amount of arginine ($C_6H_{14}N_4O_2$)

PERFORMANCE TESTS

• DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS

(2040): Meet the requirements for *Dissolution*

Medium: 0.1 N hydrochloric acid; 900 mL

Apparatus 2: 100 rpm

Time: 60 min

Standard solution: Proceed as directed in the *Procedure* for *Strength*.

Sample solution: Sample per *Disintegration and Dissolution of Dietary Supplements* (2040). Dilute with *Medium* to a concentration similar to that of the *Standard solution*.

Analysis: Determine the amount of arginine dissolved in the *Procedure* for *Strength*, making any necessary modifications.

Tolerances: NLT 75% of the labeled amount of arginine ($C_6H_{14}N_4O_2$) is dissolved.

- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** (2091): Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the form of arginine that is used and the equivalent amount of arginine.
- **USP REFERENCE STANDARDS** (11)
USP L-Arginine RS
USP Arginine Hydrochloride RS

Arginine Tablets

DEFINITION

Arginine Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of arginine or arginine hydrochloride in an amount equivalent to arginine ($C_6H_{14}N_4O_2$).

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)

Standard solution: 1.5 mg/mL of USP L-Arginine RS or USP Arginine Hydrochloride RS in water

Sample solution: Weigh and finely powder NLT 20 Tablets, mix, and transfer a portion of the powder, equivalent to about 150 mg of arginine, to a 100-mL volumetric flask, add 80 mL of water, sonicate for 15 min, dilute with water to volume, mix, and filter.

Application volume: 5 μ L

Developing solvent system: Isopropyl alcohol and ammonium hydroxide (7:3)

Spray reagent: 2 mg/mL of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (95:5)

Analysis: Proceed as directed for *Chromatography* (621), *Thin-Layer Chromatography*. Dry the plate at 100°–105° until the ammonia disappears completely. Spray with the *Spray reagent*, and heat at 100°–105° for about 15 min. Examine the plate under white light.

Acceptance criteria: The principal spot from the *Sample solution* corresponds in appearance and R_f value to that from the *Standard solution*.

- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in *Strength*.

STRENGTH

• PROCEDURE

Buffer: 6.9 mg/mL of monobasic sodium phosphate in water. Adjust with phosphoric acid to a pH of 3.5.

Solution A: 0.5 mg/mL of 1-octanesulfonic acid sodium salt in *Buffer*

Mobile phase: *Solution A* and acetonitrile (95:5)

Standard solution: 1.5 mg/mL of USP L-Arginine RS or USP Arginine Hydrochloride RS in *Buffer*

Sample solution: Weigh and finely powder NLT 20 Tablets, mix, and transfer a portion of the powder, equivalent to about 150 mg of arginine, to a 100-mL volumetric flask, add 80 mL of *Buffer*, sonicate for 15 min, dilute with *Buffer* to volume, mix, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 215 nm

Column: 4.6-mm × 25-cm; packing L7

Flow rate: 0.8 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 1500 theoretical plates

Relative standard deviation: NMT 2.0% from the arginine peak, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of arginine in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of the *Standard solution* (mg/mL)

C_U = nominal concentration of Arginine in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amount of arginine ($C_6H_{14}N_4O_2$)

PERFORMANCE TESTS

• DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS

<2040>: Meet the requirements for *Dissolution*

Medium: 0.1 N hydrochloric acid; 900 mL

Apparatus 2: 100 rpm

Time: 60 min

Standard solution: Proceed as directed in the *Procedure* for *Strength*.

Sample solution: Sample per *Disintegration and Dissolution of Dietary Supplements* <2040>. Dilute with *Medium* to a concentration similar to that of the *Standard solution*.

Analysis: Determine the amounts of arginine dissolved in the *Procedure* for *Strength*, making any necessary modifications.

Tolerances: NLT 75% of the labeled amount of arginine ($C_6H_{14}N_4O_2$) is dissolved.

• WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>: Meet the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **LABELING:** The label states the form of arginine that is used and the equivalent amount of arginine.

• **USP REFERENCE STANDARDS** <11>
USP L-Arginine RS
USP Arginine Hydrochloride RS

Ascorbic Acid—see *Ascorbic Acid General Monographs*

Ascorbic Acid Oral Solution—see *Ascorbic Acid Oral Solution General Monographs*

Ascorbic Acid Tablets—see *Ascorbic Acid Tablets General Monographs*

Ashwagandha Root

DEFINITION

Ashwagandha Root is the dried mature roots of *Withania somnifera* (L.) Dunal (Fam. Solanaceae). It contains NLT 0.3% of withanolides, calculated on the dried basis as the sum of withanolide aglycones, calculated as withanolide A, and withanolide glycosides, calculated as withanoside IV.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST <201>

Standard solution: About 200 mg of USP Powdered Ashwagandha Root Extract RS in 10 mL of methanol. Heat gently for 10–15 min, centrifuge, and use the supernatant. [NOTE—Save the remaining volume of the supernatant for use in the test for *Content of Withanolides*.]

Sample solution: Transfer about 5.0 g of Ashwagandha Root, finely powdered, to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for 10–15 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum to about 40 mL, and adjust the volume with methanol to 50.0 mL. [NOTE—Save the remaining volume of the *Sample solution* for use in the test for *Content of Withanolides*.]

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 25 µL

Developing solvent system: A mixture of ethyl acetate, toluene, and acetic acid (45:55:3)

Spray reagent: Mix 0.5 mL of anisaldehyde, 10 mL of glacial acetic acid, 85 mL of methanol, and 5 mL of concentrated sulfuric acid in the order given.

Analysis

Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands to a suitable plate (see *Chromatography* <621>). Use a saturated chamber. Develop until the solvent front has moved up about 90% of the length of the plate. Dry the plate, spray with *Spray reagent*, heat for 5–10 min at 100°, and examine under visible light.

Acceptance criteria: The *Sample solution* exhibits five main grayish-blue bands with R_f values of approximately 0.12, 0.29, 0.47, 0.67, and 0.73 that are similar in position and color to the main bands from the *Standard solution*. Other less intense bands are observed for the *Sample solution* and the *Standard solution*.

- **B.** The *Sample solution* in the test for *Content of Withanolides* shows main peaks at retention times corresponding to those of withanolide A and withanoside IV in *Standard solution A* and *Standard solution B*, respectively. Identify other withanolide peaks in the *Sample solution* by comparison with *Standard solution C* and the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS being used. The *Sample solution* shows additional peaks corresponding to some of the following withanolides: physagulin D, 27-hydroxy-withanone, withanoside V, withanoside VI, withaferin A, withastramonolide, withanone, and withanolide B.

COMPOSITION**• CONTENT OF WITHANOLIDES**

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, and mix.

Solution B: Filtered and degassed acetonitrile

Standard solution A: Dissolve, using gentle heat, a quantity of USP Withanolide A RS in methanol to obtain a solution having a known concentration of about 0.1 mg/mL.

Standard solution B: Dissolve, using gentle heat, a quantity of USP Withanoside IV RS in methanol to obtain a solution having a known concentration of about 0.1 mg/mL.

Standard solution C: Dilute 5 mL of the *Standard solution* prepared in *Identification test A* with methanol to 10 mL, and mix. Before injection, pass through a membrane filter of 0.45-μm or finer pore size.

Sample solution: Use the *Sample solution* prepared in *Identification test A*. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
30	95	5
40	95	5

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 227 nm

Column: 4.6-mm × 25-cm, end-capped; packing L1

Temperature: 27 ± 1°

Flow rate: 1.5 mL/min

Injection size: 20 μL

System suitability

Samples: *Standard solution A* and *Standard solution C*
Using the chromatogram of *Standard solution C* and the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS being used, identify the retention times of the peaks corresponding to the various withanolide aglycones and glycosides. The approximate relative retention times of the withanolide aglycones and glycosides are provided in the following table.

Analyte	Relative Retention Time
Withanoside IV	0.70
Physagulin D	0.75
27-hydroxywithanone	0.80
Withanoside V	0.89
Withanoside VI	0.89
Withaferin A	0.92
Withastramonolide	0.96
Withanolide A	1.00
Withanone	1.01
Withanolide B	1.14

Suitability requirements

The chromatogram for *Standard solution C* is similar to the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS being used.

Resolution: NLT 1.0 for the withanolide A and withanone peaks, *Standard solution C*; NLT 3.0 between

the withaferin A and coeluting withanoside V and withanoside VI peaks, *Standard solution C*

Tailing factor: NMT 1.5 for the withanolide A peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for replicate injections, withanolide A peak, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Calculate the percentage of withanolide aglycones in the portion of Ashwagandha Root taken:

$$\text{Result} = 5(r_T/r_S)(C_S/W)$$

r_T = sum of the peak responses for withaferin A, withastramonolide, withanolide A, withanone, and withanolide B from the *Sample solution*

r_S = peak response of withanolide A from *Standard solution A*

C_S = concentration of USP Withanolide A RS in *Standard solution A* (mg/mL)

W = weight of Ashwagandha Root taken to prepare the *Sample solution* (g)

Calculate the percentage of withanolide glycosides in the portion of Ashwagandha Root taken:

$$\text{Result} = 5(r_T/r_S)(C_S/W)$$

r_T = sum of the peak responses for withanoside IV, withanoside V, and withanoside VI from the *Sample solution*

r_S = peak response of USP Withanoside IV from *Standard solution B*

C_S = concentration of USP Withanoside IV RS in *Standard solution B* (mg/mL)

W = weight of Ashwagandha Root taken to prepare the *Sample solution* (g)

Acceptance criteria: The sum of the percentages of withanolide aglycones and withanolide glycosides is NLT 0.3%, calculated on the dried basis. [NOTE—Because of inherent variations, some of the withanolides mentioned in this test may be present in minor quantities or may be totally absent. The sample will be deemed compliant if the sum of the total withanolides is NLT 0.3%.]

IMPURITIES**Inorganic Impurities**

• **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** <561>: NMT 1.0%

• **HEAVY METALS, Method II** <231>: NMT 20 ppm

Organic Impurities

• **PROCEDURE 1: ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** <561>: NMT 2.0%

• **PROCEDURE 2: ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** <561>: Meets the requirements

SPECIFIC TESTS**• BOTANIC CHARACTERISTICS**

Macroscopic: Primary roots are not branched and are straight, conical, or fingerlike in shape and variable in thickness with age; some carry a crown, consisting of a number of remains of stem base; the outer surface is buff to grayish-yellow with longitudinal wrinkles; fracture is short and uneven; secondary roots are thin and fibrous.

Histology

Transverse section of roots: It shows a narrow band of yellowish crumpled cork, moderate-size cortex and a wide wood. The cork cells are rectangular, radially flattened, nonlignified, and filled with starch grains and reddish brown content; cork cambium is 2–4 diffused rows of cells; secondary cortex is formed of 20–25 rows of thin-wall parenchymatous cells, filled

with starch grains, and shows occasional microspenoidal crystals of calcium oxalate; phloem consists of sieve tubes, companion cells, and phloem parenchyma; vascular cambium consists of tangentially elongated parenchymatous cells; vessels and tracheids are in radial rows toward the periphery of the wood; medullary rays are uniseriate to 2- to 3-seriate, and are filled with starch grains; scattered vessels in groups are embedded in the parenchyma; vessels have pitted and scalariform thickening, and generally the end walls are perforated; and a few fibers with thick lignified walls are also found scattered in the wood.

- **LOSS ON DRYING** (731): Dry 1.0 g of finely powdered Ashwagandha Root at 105° for 3 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 7.0%, determined on 1.0 g of finely powdered Ashwagandha Root
- **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method 2** (561): NLT 10.0%
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10³ cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
- **ARTICLES OF BOTANICAL ORIGIN, Aflatoxins** (561): Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11)
USP Powdered Ashwagandha Root Extract RS
USP Withanolide A RS
USP Withanoside IV RS

Powdered Ashwagandha Root

DEFINITION

Powdered Ashwagandha Root is Ashwagandha Root reduced to a fine or very fine powder.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** (201)

Standard solution: Heat gently, for 10–15 min, about 200 mg of USP Powdered Ashwagandha Root Extract RS in 10 mL of methanol, centrifuge, and use the supernatant. [NOTE—Save the remaining volume of the supernatant for use in the test for *Content of Withanolides*.]

Sample solution: Transfer about 5.0 g of Powdered Ashwagandha Root to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for 10–15 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum to about 40 mL, and adjust the volume with methanol to 50.0 mL. [NOTE—Save the remaining volume of the *Sample solution* for use in the test for *Content of Withanolides*.]

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 25 µL

Developing solvent system: A mixture of ethyl acetate, toluene, and acetic acid (45:55:3)

Spray reagent: Mix 0.5 mL of anisaldehyde, 10 mL of glacial acetic acid, 85 mL of methanol, and 5 mL of concentrated sulfuric acid in the order mentioned.

Analysis

Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands to a suitable plate (see *Chromatography* (621)). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about 90% of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 5–10 min at 100°, and examine under visible light.

Acceptance criteria: The *Sample solution* exhibits five main grayish-blue bands with R_f values of approximately 0.12, 0.29, 0.47, 0.67, and 0.73 that are similar in position and color to the main bands from the *Standard solution*. Other less intense bands are observed for the *Sample solution* and *Standard solution*.

- **B.** The *Sample solution* from the test for *Content of Withanolides* shows main peaks at retention times corresponding to those of withanolide A and withanoside IV in *Standard solution A* and *Standard solution B*, respectively. Identify other withanolide peaks in the *Sample solution* by comparison with *Standard solution C* and the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS. The *Sample solution* shows additional peaks corresponding to some of the following withanolides: physagulin D, 27-hydroxywithanone, withanoside V, withanoside VI, withaferin A, withastramonolide, withanone, and withanolide B.

COMPOSITION

• CONTENT OF WITHANOLIDES

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, and mix.

Solution B: Filtered and degassed acetonitrile

Standard solution A: Dissolve, using gentle heat, a quantity of USP Withanolide A RS in methanol to obtain a solution having a known concentration of about 0.1 mg/mL.

Standard solution B: Dissolve, using gentle heat, a quantity of USP Withanoside IV RS in methanol to obtain a solution having a known concentration of about 0.1 mg/mL.

Standard solution C: Dilute 5 mL of the *Standard solution* prepared in *Identification test A* with methanol to 10 mL, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Use the *Sample solution* prepared in *Identification test A*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
30	95	5
40	95	5

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 227 nm

Column: 4.6-mm × 25-cm, end-capped; packing L1

Temperature: 27 ± 1°

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution C*
Using the chromatogram of *Standard solution C* and the Reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS, identify the retention times of the peaks corresponding to the different withanolide aglycones and glycosides. The approximate relative retention times of the withanolide aglycones and glycosides are provided in the following table.

Analyte	Relative Retention Time
Withanoside IV	0.70
Physagulin D	0.75
27-hydroxywithanone	0.80
Withanoside V	0.89
Withanoside VI	0.89
Withaferin A	0.92
Withastramonolide	0.96
Withanolide A	1.00
Withanone	1.01
Withanolide B	1.14

Suitability requirements

The chromatogram of *Standard solution C* is similar to the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS.

Resolution: NLT 1.0 for the withanolide A and withanone peaks, and NLT 3.0 between the peak corresponding to withaferin A and the peak corresponding to the coeluting withanoside V and withanoside VI, *Standard solution C*

Tailing factor: NMT 1.5 for the withanolide A peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for replicate injections, withanolide A peak, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Calculate the percentage of withanolide aglycones in the portion of Powdered Ashwagandha Root taken:

$$\text{Result} = 5(r_T/r_S)(C_S/W)$$

r_T = sum of the peak responses for withaferin A, withastramonolide, withanolide A, withanone, and withanolide B in the *Sample solution*

r_S = peak response of withanolide A from *Standard solution A*

C_S = concentration of USP Withanolide A RS in *Standard solution A* (mg/mL)

W = weight of Powdered Ashwagandha Root taken to prepare the *Sample solution* (g)

Calculate the percentage of withanolide glycosides in the portion of Powdered Ashwagandha Root taken:

$$\text{Result} = 5(r_T/r_S)(C_S/W)$$

r_T = sum of the peak responses for withanoside IV, withanoside V, and withanoside VI in the *Sample solution*

r_S = peak response of USP Withanoside IV RS from *Standard solution B*

C_S = concentration of USP Withanoside IV RS in *Standard solution B* (mg/mL)

W = weight of Powdered Ashwagandha Root taken to prepare the *Sample solution* (g)

Acceptance criteria: Add the percentages of withanolide aglycones and withanolide glycosides: NLT 0.3% is found, calculated on the dried basis. [NOTE—Due to inherent variations, some of the withanolides mentioned in the above test may be present in minor quantities or may be totally absent. The sample will be deemed compliant as long as the sum of the total withanolides is NLT 0.3%.]

IMPURITIES

Inorganic Impurities

• **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* <561>: NMT 1.0%

• **HEAVY METALS**, *Method II* <231>: NMT 20 ppm

Organic Impurities

• **PROCEDURE: ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements

SPECIFIC TESTS

BOTANIC CHARACTERISTICS

Macroscopic: It is a dusty white or grey to light brown powder with a characteristic odor and a mucilagenous, bitter, acrid taste.

Histology

Microscopic examination: It shows collapsed cork cells filled with starch grains and reddish-brown content; thin-walled cortex parenchyma cells filled with starch grains and occasional microsphenoidal crystals of calcium oxalate; vessels, with pitted and scalariform thickening, and generally with end walls perforated; a few fibers with thick lignified walls and simple pits; abundant starch grains, mostly simple, sometimes compound, spherical, reniform-oval with central hilum.

• **LOSS ON DRYING** <731>: Dry 1.0 g at 105° for 3 h: it loses NMT 12.0% of its weight.

• **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 7.0%, determined on 1.0 g of Powdered Ashwagandha Root

• **ARTICLES OF BOTANICAL ORIGIN**, *Alcohol-Soluble Extractives*, *Method 2* <561>: NLT 10.0%

• **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10³ cfu/g.

• **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

• **ARTICLES OF BOTANICAL ORIGIN**, *Aflatoxins* <561>: Meets the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.

• **USP REFERENCE STANDARDS** <11>

USP Powdered Ashwagandha Root Extract RS

USP Withanolide A RS

USP Withanoside IV RS

Powdered Ashwagandha Root Extract

DEFINITION

Powdered Ashwagandha Root Extract is prepared from Ashwagandha using methanol, alcohol, water, or mixtures

of these solvents. It contains NLT 2.5% of withanolides, calculated on the dried basis as the sum of withanolide aglycones and withanolide glycosides.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

(201)

Standard solution: Heat gently for 10–15 min about 200 mg of USP Powdered Ashwagandha Extract Root RS in 10 mL of methanol, centrifuge, and use the supernatant. [NOTE—Save the remaining volume of the supernatant for use in the test for *Content of Withanolides*.]

Sample solution: Heat gently for 10–15 min about 200 mg of Powdered Ashwagandha Root Extract in 10 mL of methanol, centrifuge, and use the supernatant.

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 25 μ L

Developing solvent system: A mixture of ethyl acetate, toluene, and acetic acid (45:55:3)

Spray reagent: Mix 0.5 mL of anisaldehyde, 10 mL of glacial acetic acid, 85 mL of methanol, and 5 mL of concentrated sulfuric acid in the order mentioned.

Analysis

Samples: *Standard solution* and *Sample solution*
Apply the *Samples* as bands to a suitable plate (see *Chromatography* (621)). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about 90% of the length of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 5–10 min at 100°, and examine under visible light.

Acceptance criteria: The *Sample solution* exhibits five main grayish-blue bands with R_f values of approximately 0.12, 0.29, 0.47, 0.67, and 0.73 that are similar in position and color to the main bands for the *Standard solution*. Other less intense bands are observed for the *Sample solution* and the *Standard solution*.

- **B.** The *Sample solution* in the test for *Content of Withanolides* shows main peaks at retention times corresponding to those of withanolide A and withanolide IV for *Standard solution A* and *Standard solution B*, respectively. Identify other withanolide peaks in the *Sample solution* by comparison with *Standard solution C* and the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS being used. The *Sample solution* shows additional peaks corresponding to some of the following withanolides: physagulin D, 27-hydroxywithanone, withanoside V, withanoside VI, withaferin A, withastramonolide, withanone, and withanolide B.

COMPOSITION

• CONTENT OF WITHANOLIDES

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, and mix.

Solution B: Filtered and degassed acetonitrile

Standard solution A: Dissolve, using gentle heat, a quantity of USP Withanolide A RS in methanol to obtain a solution having a known concentration of about 0.1 mg/mL.

Standard solution B: Dissolve, using gentle heat, a quantity of USP Withanoside IV RS in methanol to obtain a solution having a known concentration of about 0.1 mg/mL.

Standard solution C: Dilute 5 mL of the *Standard solution* prepared in *Identification test A* with methanol to 10 mL, and mix. Before injection, pass through a membrane filter of 0.45- μ m or finer pore size.

Sample solution: Transfer about 100 mg of Powdered Ashwagandha Root Extract, weighed, to a 10-mL volumetric flask, add about 7 mL of methanol, heat gently

on a water bath for 15–20 min, dilute with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45- μ m or finer pore size, discarding the first few mL of the filtrate.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
30	95	5
40	95	5

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 227 nm

Column: 4.6-mm \times 25-cm, end-capped; packing L1

Temperature: 27 \pm 1°

Flow rate: 1.5 mL/min

Injection size: 20 μ L

System suitability

Samples: *Standard solution A* and *Standard solution C*
Using the chromatogram of *Standard solution C* and the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS being used, identify the retention times of the peaks corresponding to the various withanolide aglycones and glycosides. The approximate relative retention times of the withanolide aglycones and glycosides are provided in the following table.

Analyte	Relative Retention Time
Withanoside IV	0.70
Physagulin D	0.75
27-hydroxywithanone	0.80
Withanoside V	0.89
Withanoside VI	0.89
Withaferin A	0.92
Withastramonolide	0.96
Withanolide A	1.00
Withanone	1.01
Withanolide B	1.14

Suitability requirements

The chromatogram for *Standard solution C* is similar to the reference chromatogram provided with the lot of USP Powdered Ashwagandha Root Extract RS being used.

Resolution: NLT 1.0 for the withanolide A and withanone peaks, and NLT 3.0 between the peak corresponding to withaferin A and the peak corresponding to coeluting withanoside V and withanoside VI, *Standard solution C*

Tailing factor: NMT 1.5 for the withanolide A peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for replicate injections, withanolide A peak, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Calculate the percentage of withanolide aglycones in the portion of Powdered Ashwagandha Root Extract taken:

$$\text{Result} = (r_T/r_S)(C_S/W)$$

- r_T = sum of the peak responses for withaferin A, withastramonolide, withanolide A, withanone, and withanolide B from the *Sample solution*
- r_S = peak response of withanolide A from *Standard solution A*
- C_S = concentration of USP Withanolide A RS in *Standard solution A* (mg/mL)
- W = weight of Powdered Ashwagandha Root Extract taken to prepare the *Sample solution* (g)

Calculate the percentage of withanolide glycosides in the portion of Powdered Ashwagandha Root Extract taken:

$$\text{Result} = (r_T/r_S)(C_S/W)$$

- r_T = sum of the peak responses for withanoside IV, withanoside V and withanoside VI from the *Sample solution*
- r_S = peak response of USP Withanoside IV from *Standard solution B*
- C_S = concentration of USP Withanoside IV RS in *Standard solution B* (mg/mL)
- W = weight of Powdered Ashwagandha Root Extract taken to prepare the *Sample solution* (g)

Acceptance criteria: The sum of the percentages of the withanolide aglycones and withanolide glycosides is NLT 2.5%, calculated on the dried basis. [NOTE—Because of inherent variations, some of the withanolides mentioned in this test may be present in minor quantities or may be totally absent. The sample will be deemed compliant if the sum of the total withanolides is NLT 2.5%.]

IMPURITIES

Inorganic Impurities

- **HEAVY METALS, Method II <231>:** NMT 20 ppm

Organic Impurities

- **PROCEDURE: ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis <561>:** Meets the requirements

SPECIFIC TESTS

- **LOSS ON DRYING <731>:** Dry 2.0 g at 105° for 3 h: it loses NMT 6.0% of its weight.
- **MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic bacterial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts count does not exceed 10³ cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
- **ARTICLES OF BOTANICAL ORIGIN, Aflatoxins <561>:** Meets the requirements
- **BOTANICAL EXTRACTS, Residual Solvents <565>:** Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. It meets other labeling requirements under *Botanical Extracts <565>*.
- **USP REFERENCE STANDARDS <11>**
USP Powdered Ashwagandha Root Extract RS
USP Withanolide A RS
USP Withanoside IV RS

Aspartic Acid—see Aspartic Acid General Monographs

Bacopa

DEFINITION

Bacopa consists of the dried stems and leaves of *Bacopa monnieri* (L.) Pennell (Fam. Scrophulariaceae). It contains NLT 2.5% of triterpene glycosides, calculated on the dried basis as the sum of bacopaside I, bacoside A₃, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C.

IDENTIFICATION

- **A.** Bacopa meets the requirements for *Specific Tests, Botanic Characteristics*.

- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST <201>**

Standard solution: Transfer about 10 mg of USP Powdered Bacopa Extract RS to a 10-mL volumetric flask, and add about 8 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, mix, centrifuge, and use the supernatant.

Sample solution: Use the *Sample solution*, prepared as directed in the test for *Content of Triterpene Glycosides*.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μm (TLC plates)

Application volume: 15 μL, as 5–10 mm bands

Developing solvent system: Ethyl acetate, methanol, and water (7:2:1)

Spray reagent: 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)

Analysis

Samples: *Standard solution* and *Sample solution*
Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography <621>*). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 5–10 min at about 70°, and examine under visible light.

Acceptance criteria: The *Sample solution* exhibits a main dark blue zone due to a mixture of bacoside A₃, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C at an R_F value of approximately 0.6 and a faint pink spot due to bacopaside I at an R_F value of approximately 0.4, both of which correspond in position and color to zones in the chromatogram of the *Standard solution*. Other zones are observed for the *Sample solution* and *Standard solution*.

- **C. HPLC IDENTIFICATION TEST:** The *Sample solution* from the test for *Content of Triterpene Glycosides* shows a main peak at the retention time corresponding to that of bacoside A₃ in the chromatogram of *Standard solution A*. Identify other triterpene glycoside peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS. The *Sample solution* shows additional peaks corresponding to bacopaside I, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C.

COMPOSITION

- **CONTENT OF TRITERPENE GLYCOSIDES**

Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Use filtered and degassed acetonitrile.
Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	70	30
25	60	40
26	70	30
30	70	30

Standard solution A: Sonicate a weighed quantity of USP Bacoside A₃ RS in methanol to obtain a solution with a concentration of about 0.5 mg/mL.

Standard solution B: Transfer about 10 mg of USP Powdered Bacopa Extract RS to a 10-mL volumetric flask, and add about 8 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first 5 mL of the filtrate.

Sample solution: Transfer about 2.5 g of Bacopa, finely powdered, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 25 mL of methanol, reflux on a water bath for 10 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume to 100 mL using methanol. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first 5 mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm × 25-cm; 5-μm, endcapped, base-deactivated packing L1

Column temperature: 27 ± 1°

Flow rate: 1.5 mL/min

Injection size: 20 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used.

Resolution: NLT 1.0 between the bacopaside II and bacoside A₃ peaks, *Standard solution B*

Tailing factor: NMT 1.5 for the bacoside A₃ peak, *Standard solution A*

Relative standard deviation: NMT 2% determined from the bacoside A₃ peak for replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A* and *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS, identify the retention times of the peaks corresponding to different triterpene glycosides. The approximate relative retention times of the different triterpene glycosides are provided in the following table.

Analyte	Relative Retention Time
Bacopaside I	0.73
Bacoside A ₃	1.00
Bacopaside II	1.04
The jujubogenin isomer of bacopasaponin C	1.15
Bacopasaponin C	1.22

Separately calculate the percentages of bacopaside I, bacoside A₃, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C in the portion of Bacopa taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak response for each triterpene glycoside from the *Sample solution*

r_S = peak response for bacoside A₃ from *Standard solution A*

C_S = concentration of USP Bacoside A₃ RS in *Standard solution A* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Bacopa used to prepare the *Sample solution* (mg)

F = conversion factor for each analyte: 1.00 for bacoside A₃, 1.03 for bacopaside I, 0.81 for bacopaside II, 0.99 for the jujubogenin isomer of bacopasaponin C, and 0.75 for bacopasaponin C

Acceptance criteria: Add the percentages of bacopaside I, bacoside A₃, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C: NLT 2.5% is found, calculated on the dried basis.

IMPURITIES

Inorganic Impurities

• **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** <561>: NMT 6.0%

• **HEAVY METALS, Method III** <231>: NMT 20 ppm

Organic Impurities

• **PROCEDURE 1: ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matters** <561>: NMT 2.0%

• **PROCEDURE 2: ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** <561>: Meets the requirements

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Stem is creeping, succulent, glabrous, soft, obtuse-angular; with long internodes, rooting at nodes; devoid of leaves toward the base; branches ascending. Leaves are simple, sessile or short petiolate, opposite, succulent, 1–2 mm thick; oblong-obovate or spatulate, margin entire or rarely dentate, apex rounded, midrib indistinct, 0.6–2.5 cm long, 3–8 mm wide; the upper surface is green, the lower surface is green and dotted. Pharmacopeial article is yellowish in color; consists of dry mixtures of broken leaves and stems, with majority of leaves detached; mild and hay-like odor, and very bitter taste.

Histology

Transverse section of stems: Epidermal layer; a wide cortex composed of thin-wall parenchyma cells and large intercellular spaces; xylem vessels radially arranged, uniseriate medullary rays; pith composed of thin-wall, round or isodiametric cells with distinct intercellular spaces. Resin canals and pericyclic sclereids are absent.

Transverse section of leaves: Shows a more or less isobilateral structure; epidermis with glandular hair and stomata; upper surface has more hairs and less stomata than the lower surface; mesophyll composed of spongy tissue, a few prisms of calcium oxalate, and vascular bundles are present.

• **LOSS ON DRYING** <731>: Dry 1.0 g of finely powdered Bacopa at 105° for 3 h: it loses NMT 12.0% of its weight.

• **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 18%, determined on 1.0 g of finely powdered Bacopa

• **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method 2** <561>: NLT 6.0%

- **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS (2022):** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS (11)**
USP Bacoside A₃ RS
USP Powdered Bacopa Extract RS

Powdered Bacopa

DEFINITION

Powdered Bacopa is Bacopa reduced to a powder or very fine powder. It contains NLT 2.5% of triterpene glycosides, calculated on the dried basis as the sum of bacopaside I, bacoside A₃, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C.

IDENTIFICATION

- **A.** Powdered Bacopa meets the requirements under *Specific Tests, Botanic Characteristics*.

- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)**

Standard solution: Transfer about 10 mg of USP Powdered Bacopa Extract RS to a 10-mL volumetric flask, and add about 8 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, mix, centrifuge, and use the supernatant.

Sample solution: Use the *Sample solution*, prepared as directed in the test for *Content of Triterpene Glycosides*.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μ m (TLC plates)

Application volume: 15 μ L, as 5–10 mm bands

Developing solvent system: Ethyl acetate, methanol, and water (7:2:1)

Spray reagent: 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 5–10 min at about 70°, and examine under visible light.

Acceptance criteria: The *Sample solution* exhibits a main dark blue zone due to mixture of bacoside A₃, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C at an R_F value of approximately 0.6 and a faint pink spot due to bacopaside I at an R_F value of approximately 0.4, both of which correspond in position and color to zones in the chromatogram of the *Standard solution*. Other zones are observed for the *Sample solution* and *Standard solution*.

- **C. HPLC IDENTIFICATION TEST:** The *Sample solution* from the test for *Content of Triterpene Glycosides* shows a main peak at a retention time corresponding to that of bacoside A₃ in the chromatogram of *Standard solution A*. Identify other triterpene glycoside peaks in the *Sample solution* by comparison with the chromatogram of *Standard*

solution B and the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used. The *Sample solution* shows additional peaks corresponding to bacopaside I, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C.

COMPOSITION

• CONTENT OF TRITERPENE GLYCOSIDES

Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Use filtered and degassed acetonitrile.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	70	30
25	60	40
26	70	30
30	70	30

Standard solution A: Sonicate an accurately weighed quantity of USP Bacoside A₃ RS in methanol to obtain a solution having a known concentration of about 0.5 mg/mL.

Standard solution B: Transfer about 10 mg of USP Powdered Bacopa Extract RS to a 10-mL volumetric flask, and add about 8 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45- μ m or finer pore size, discarding the first 5 mL of the filtrate.

Sample solution: Transfer about 2.5 g of Powdered Bacopa, accurately weighed, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 25 mL of methanol, reflux on a water bath for 10 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume to 100 mL using methanol. Before injection, pass through a membrane filter of 0.45- μ m or finer pore size, discarding the first 5 mL of the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm \times 25-cm; 5- μ m, endcapped, base-deactivated packing L1

Column temperature: 27 \pm 1°

Flow rate: 1.5 mL/min

Injection size: 20 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used.

Resolution: NLT 1.0 between the bacopaside II and bacoside A₃ peaks, *Standard solution B*

Tailing factor: NMT 1.5 for the bacoside A₃ peak, *Standard solution A*

Relative standard deviation: NMT 2% determined from the bacoside A₃ peak for replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A* and *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS, identify the retention times of the peaks corresponding to different triterpene glycosides. The ap-

proximate relative retention times of the different triterpene glycosides are provided in the following table.

Analyte	Relative Retention Time
Bacopaside I	0.73
Bacoside A ₃	1.00
Bacopaside II	1.04
The jujubogenin isomer of bacopasaponin C	1.15
Bacopasaponin C	1.22

Separately calculate the percentages of bacopaside I, bacoside A₃, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C in the portion of Powdered Bacopa taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

- r_U = peak response for each triterpene glycoside from the *Sample solution*
 r_S = peak response for bacoside A₃ from *Standard solution A*
 C_S = concentration of USP Bacoside A₃ RS in *Standard solution A* (mg/mL)
 V = final volume of the *Sample solution* (mL)
 W = weight of Powdered Bacopa used to prepare the *Sample solution* (mg)
 F = conversion factor for each analyte: 1.00 for bacoside A₃, 1.03 for bacopaside I, 0.81 for bacopaside II, 0.99 for the jujubogenin isomer of bacopasaponin C, and 0.75 for bacopasaponin C

Acceptance criteria: Add the percentages of bacopaside I, bacoside A₃, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C: NLT 2.5% is found on the dried basis.

IMPURITIES

Inorganic Impurities

- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561): NMT 6.0%
- **HEAVY METALS, Method III** (231): NMT 20 ppm

Organic Impurities

- **PROCEDURE: ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** (561): Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Yellowish in color; mild and hay-like odor, and very bitter taste. Under a microscope, it shows fragments of upper and lower epidermal cells of the leaves in surface view, having sessile glandular trichomes with 4–8 cells and diacytic or anomocytic stomata; upper epidermis has more trichomes and less stomata than the lower epidermis; lower epidermis cells with sinuous anticlinal walls and at places striated cuticle; fragments of epidermal cells of the stem in surface view; parenchyma cells enclosing air cavities and some contain rosette and prismatic crystals of calcium oxalate; fragments of longitudinally cut annular and spiral vessels; fragments of cortical cells of the stem; and crystals of calcium oxalate.
- **LOSS ON DRYING** (731): Dry 1.0 g of Powdered Bacopa at 105° for 3 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 18%, determined on 1.0 g of Powdered Bacopa
- **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method 2** (561): NLT 6.0%
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed

10³ cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10³ cfu/g.

- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11):
 USP Bacoside A₃ RS
 USP Powdered Bacopa Extract RS

Powdered Bacopa Extract

DEFINITION

Powdered Bacopa Extract is prepared from Bacopa by extraction with water, alcohol, methanol, or a mixture of these solvents. The ratio of plant material to extract is between 20:1 and 10:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of triterpene glycosides, calculated on the dried basis as the sum of bacopaside I, bacoside A₃, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C. It may contain suitable added substances as carriers.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: Transfer about 10 mg of USP Powdered Bacopa Extract RS to a 10-mL volumetric flask, and add about 8 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, mix, centrifuge, and use the supernatant.

Sample solution: Sonicate for about 10 min an amount of Powdered Bacopa Extract equivalent to about 40 mg of triterpene glycosides in 10 mL of methanol, centrifuge, and use the supernatant.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μm (TLC plates)

Application volume: 15 μL, as 5–10 mm bands

Developing solvent system: Ethyl acetate, methanol, and water (7:2:1)

Spray reagent: 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 5–10 min at about 70°, and examine under visible light.

Acceptance criteria: The *Sample solution* exhibits a main dark blue zone due to a mixture of bacoside A₃, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C at an R_F value of approximately 0.6 and a faint pink spot due to bacopaside I at an R_F value of approximately 0.4, both of which correspond in position and color to zones in the chromatogram of the *Standard solution*. Other zones are observed for the *Sample solution* and *Standard solution*.

- **B. HPLC IDENTIFICATION TEST:** The *Sample solution* from the test for *Content of Triterpene Glycosides* shows a main peak at a retention time corresponding to that of bacoside A₃ in the chromatogram of *Standard solution A*. Identify other triterpene glycoside peaks in the *Sample solution* by comparison with the chromatogram of *Standard*

solution B and the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used. The *Sample solution* shows additional peaks corresponding to bacopaside I, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C.

COMPOSITION

• CONTENT OF TRITERPENE GLYCOSIDES

Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Use filtered and degassed acetonitrile.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	70	30
25	60	40
26	70	30
30	70	30

Standard solution A: Sonicate a weighed quantity of USP Bacoside A₃ RS in methanol to obtain a solution with a concentration of about 0.5 mg/mL.

Standard solution B: Transfer about 10 mg of USP Powdered Bacopa Extract RS to a 10-mL volumetric flask, and add about 8 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first 5 mL of the filtrate.

Sample solution: Transfer an amount of Powdered Bacopa Extract, equivalent to about 25 mg triterpene glycosides, to a 25-mL volumetric flask, and add 15 mL of methanol. Sonicate and heat gently for 15–20 min, dilute with methanol to volume, and mix. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first 5 mL of the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm × 25-cm; 5-μm, endcapped, base-deactivated packing L1

Column temperature: 27 ± 1°

Flow rate: 1.5 mL/min

Injection size: 20 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements
Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used.

Resolution: NLT 1.0 between the bacopaside II and bacoside A₃ peaks, *Standard solution B*

Tailing factor: NMT 1.5 for the bacoside A₃ peak, *Standard solution A*

Relative standard deviation: NMT 2% determined from the bacoside A₃ peak for replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatograms of *Standard solution A* and *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Bacopa Extract RS being used, identify the retention times of the peaks corresponding to different triterpene glycosides. The approximate relative retention times of the different triterpene glycosides are provided in the following table.

Analyte	Relative Retention Time
Bacopaside I	0.73
Bacoside A ₃	1.00
Bacopaside II	1.04
The jujubogenin isomer of bacopasaponin C	1.15
Bacopasaponin C	1.22

Separately calculate the percentages of bacopaside I, bacoside A₃, bacopaside II, jujubogenin isomer of bacopasaponin C, and bacopasaponin C in the portion of Powdered Bacopa Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response for each triterpene glycoside from the *Sample solution*

r_S = peak response for bacoside A₃ in *Standard solution A*

C_S = concentration of USP Bacoside A₃ RS in *Standard solution A* (mg/mL)

C_U = concentration of Powdered Bacopa Extract in the *Sample solution* (mg/mL)

F = conversion factor for each analyte: 1.00 for bacoside A₃, 1.03 for bacopaside I, 0.81 for bacopaside II, 0.99 for the jujubogenin isomer of bacopasaponin C, and 0.75 for bacopasaponin C

Acceptance criteria: Add the percentages of bacopaside I, bacoside A₃, bacopaside II, the jujubogenin isomer of bacopasaponin C, and bacopasaponin C: NLT 90.0%–NMT 110.0% of the labeled amount of triterpene glycosides is found on the dried basis.

IMPURITIES

Inorganic Impurities

• **HEAVY METALS**, *Method III* (231): NMT 20 ppm

Organic Impurities

• **PROCEDURE: ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements

SPECIFIC TESTS

• **LOSS ON DRYING** (731): Dry 1.0 g of Powdered Bacopa Extract at 105° for 3 h: it loses NMT 5% of its weight.

• **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10⁴ cfu/g. The total combined molds and yeasts count does not exceed 10³ cfu/g.

• **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

• **OTHER REQUIREMENTS:** It meets the requirements of the test for *Residual Solvents* under *Botanical Extracts* (565).

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements under *Botanical Extracts* (565).

• **USP REFERENCE STANDARDS** (11)

USP Bacoside A₃ RS

USP Powdered Bacopa Extract RS

Beta Carotene—see *Beta Carotene General Monographs***Beta Carotene Capsules**—see *Beta Carotene Capsules General Monographs***Beta Carotene Preparation****DEFINITION**

Beta Carotene Preparation is a combination of beta carotene with one or more inert substances. It may be in a solid or a liquid form. It contains NLT 95.0% and NMT 130.0% of the labeled amount of total beta carotene ($C_{40}H_{56}$) on the anhydrous basis. It contains NLT 95.0% of all-*trans*-beta carotene in the total beta carotene content.

IDENTIFICATION

- **A.** **Sample solution:** Transfer 5.0 mL of *Sample stock solution A* or *Sample stock solution B* from the test for *Content of Beta Carotene* into a 100-mL volumetric flask, and dilute with cyclohexane to volume. Pass the solution through a membrane filter of 0.45- μ m pore size.
Analysis: Record the UV-Vis spectrum from 300–600 nm.
Acceptance criteria: The *Sample solution* shows a shoulder at about 427 nm, an absorption maximum at about 455 nm, and another maximum at about 483 nm. The absorbance ratio A_{455}/A_{483} is between 1.14 and 1.18.
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Beta Carotene*.

COMPOSITION• **CONTENT OF BETA CAROTENE**

[NOTE—Use low-actinic glassware.]

Mobile phase: Transfer 50 mg of butylated hydroxytoluene into a 1-L volumetric flask, and dissolve with 20 mL of 2-propanol. Add 0.2 mL of *N*-ethyldiisopropylamine, 25 mL of 0.2% ammonium acetate solution, 455 mL of acetonitrile, and about 450 mL of methanol. Allow the solution to reach to room temperature, and dilute with methanol to volume.

Diluent: 50 μ g/mL of butylated hydroxytoluene in alcohol

System suitability solution: Transfer 20 mg of USP Beta Carotene System Suitability RS to a 50-mL volumetric flask. Add 1 mL of water, 4 mL of tetrahydrofuran, and sonicate for 5 min. Dilute with *Diluent* to volume and sonicate for 5 min. Cool to room temperature, pass the suspension through a membrane filter of 0.45- μ m pore size, and use the clear filtrate.

Standard stock solution: 60 μ g/mL of USP Beta Carotene RS in tetrahydrofuran

Standard solution A: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, add 5.0 mL of tetrahydrofuran, and dilute with *Diluent* to volume. The concentration of the all-*trans*-beta carotene in this solution will be determined by the spectrophotometric procedure using *Standard solution B* as follows.

Standard solution B: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, and dilute with cyclohexane to volume. Prepare in triplicate.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Analytical wavelength: 457 nm

Cell path: 1 cm

Blank: Cyclohexane

Analysis

Sample: *Standard solution B*

Calculate the concentration of total beta carotene (mg/mL) as all-*trans*-beta carotene ($C_{40}H_{56}$) in *Standard solution B*.

$$\text{Result} = A/F$$

A = average absorbance of the three preparations of *Standard solution B*

F = absorptivity of pure all-*trans*-beta carotene in cyclohexane, 250.5

Sample stock solution A (for solid Beta Carotene Preparations): Transfer an amount of Beta Carotene Preparation equivalent to 10 mg of beta carotene to a 250-mL volumetric flask. Add 250 mg of butylated hydroxytoluene, 0.5 mL of alkaline Protease R, and 15 mL of water. Tilt the flask gently to wet the entire contents. Sonicate the solution in an ultrasonic bath at about 50° for 30 min, and swirl at 10 min intervals. Add 100 mL of alcohol to the warm suspension and shake vigorously. Add 135 mL of methylene chloride and shake again. Let the mixture stand in the dark until it reaches room temperature (about 2 h). Dilute with methylene chloride to volume, shake vigorously, and allow solids to settle in the dark.

Sample stock solution B (for liquid Beta Carotene suspensions in oil Preparations): Transfer an amount of Beta Carotene Preparation equivalent to 20 mg of beta carotene to a 250-mL volumetric flask. Add 250 mg of butylated hydroxytoluene, 120 mL of methylene chloride, and 100 mL of alcohol. Shake the flask until the sample is completely dissolved or suspended. Let the mixture stand in the dark until it reaches room temperature (about 2 h). Add methylene chloride to volume and shake again vigorously.

Sample solution: Transfer 5.0 mL of *Sample stock solution A* or *Sample stock solution B* into a 50-mL volumetric flask, and dilute with a mixture of methylene chloride and *Diluent* (1:1) to volume. Pass through a membrane filter of 0.45- μ m pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 448 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L68

Column temperature: 30°

Flow rate: 0.6 mL/min

Injection size: 20 μ L

System suitability

Samples: *System suitability solution* and *Standard solution A*

The approximate relative retention times of the components in the *System suitability solution* are listed in *Table 1*.

Table 1

Name	Relative Retention Time	Relative Response Factor
All- <i>trans</i> -alpha carotene	0.93	1.1
All- <i>trans</i> -beta carotene	1.00	1
9- <i>cis</i> -Beta carotene	1.07	1
13- <i>cis</i> -Beta carotene	1.17	1.2
15- <i>cis</i> -Beta carotene	1.21	1.4

Suitability requirements

Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the

Reference Chromatogram provided with the USP Beta Carotene System Suitability RS being used.

Resolution: NLT 1.5 between beta carotene and alpha carotene and between beta carotene and 9-*cis*-beta carotene, *System suitability solution*

Tailing factor: NMT 2.0 for the beta carotene peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the beta carotene peak from replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*

[NOTE—The relative response factors for 13-*cis*-beta carotene and 15-*cis*-beta carotene are 1.2 and 1.4, respectively.]

Record the chromatograms, and identify the peaks of the relevant analytes of the *Sample solution* by comparing with those of the *System suitability solution*. Measure the peak-area responses. Calculate the percentage total beta carotene in the portion of Preparation taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = [(peak area of all-*trans*-beta carotene) + (peak area of 9-*cis*-beta carotene) + (peak area of 13-*cis*-beta carotene \times 1.2) + (peak area of 15-*cis*-beta carotene \times 1.4) + (sum of peak area of other *cis*-isomers of beta carotene)] in the *Sample solution*

r_S = peak area of all-*trans*-beta carotene in *Standard solution A*

C_S = concentration of all-*trans*-beta carotene in *Standard solution A* as determined by spectrometric procedure (mg/mL)

C_U = nominal concentration of Preparation in the *Sample solution* (mg/mL)

Calculate the percentage of all-*trans*-beta carotene in the portion of Preparation taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of all-*trans*-beta carotene in the *Sample solution*

r_S = peak area of all-*trans*-beta carotene in *Standard solution A*

C_S = concentration of all-*trans*-beta carotene in *Standard solution A* as determined by spectrometric procedure (mg/mL)

C_U = nominal concentration of Preparation in the *Sample solution* (mg/mL)

Acceptance criteria: The Preparation contains 95.0%–130% of the labeled amount of total beta carotene, calculated as (C₄₀H₅₆) on the anhydrous basis, and NLT 95.0% of all-*trans*-beta carotene in the total beta carotene content.

• ALPHA CAROTENE AND OTHER RELATED COMPOUNDS

Mobile phase, System suitability solution, Sample solution, and Chromatographic system: Proceed as directed in the test for *Content of Beta Carotene*.

Analysis

Sample: *Sample solution*

Injection size: 20 μ L

Calculate the percentage of alpha carotene and other individual related compounds relative to total beta carotene in the portion of Preparation taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of alpha carotene or other individual related compounds

r_T = sum of the peak areas of all the peaks

Acceptance criteria

Alpha carotene: NMT 1.0%

Any other individual related compound: NMT 1.0%

Total related compounds (including alpha carotene): NMT 5.0%

IMPURITIES

• **RESIDUE ON IGNITION** (281): NMT 2.0%

• **HEAVY METALS, Method II** (231): NMT 10 ppm

SPECIFIC TESTS

• **WATER DETERMINATION, Method I** (921): NMT 8.0% for solid preparations. NMT 1.0% for liquid preparations.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tightly sealed, light- and oxygen-resistant containers. Store in a cool place.

• **LABELING:** The label states the name and content of any carriers and antioxidants added to the formulation, and the content of total carotenoids as beta carotene.

• **USP REFERENCE STANDARDS** (11)

USP Beta Carotene RS

(*all-E*)-1,1'-(3,7,12,16-Tetramethyl-1,3,5,7,9,11,13,15,17-octadecanonaene-1,18-diyl)bis[2,6,6-trimethylcyclohexene].

USP Beta Carotene System Suitability RS

Beta Glucan

DEFINITION

Beta Glucan is obtained by extraction from the cell wall of fermented and thermally processed Baker's yeast (*Saccharomyces cerevisiae*). It is comprised mainly of β -(1,3)/(1,6) branched glucan polymers. Small amounts of β -(1,6)-glucan and chitin are also expected to be present in the final product. It contains NLT 70% beta glucan, calculated as glucose after enzymatic hydrolysis, on the dried basis.

IDENTIFICATION

• **NUCLEAR MAGNETIC RESONANCE** (761)

Standard solution: Dissolve 10 mg of USP Beta Glucan RS in 0.6 mL of dimethyl sulfoxide-*d*₆, and heat at 100° for 1 h. Then add 0.1 mL of D₂O, mix the solution, and transfer to an NMR tube.

Sample solution: Dissolve 10 mg of Beta Glucan in 0.6 mL of dimethyl sulfoxide-*d*₆, and heat at 100° for 1 h. Then add 0.1 mL of D₂O, mix the solution, and transfer to an NMR tube.

Analysis: Collect ¹H NMR spectra at 80°, and compare individual resonances from the *Sample solution* to those from the *Standard solution*. The major signals associated with Beta Glucan are shown in *Table 1*.

Table 1

¹ H NMR Major Signals	USP Beta Glucan RS
H-1 (1,3)-glucan	4.52, d, J = 7.5 Hz, 1H
H-2, 4, and 5 (1,3-)	3.27–3.33, m, 3H
H-3 and 6b (1,3-)	3.45–3.48, m, 2H
H-6a (1,3-)	3.71, d, J = 11 Hz, 1H
H-1 (1,6)-glucan	4.27, d, J = 7.7 Hz, 1H

Integrate the area under the peaks five times for each sample, and average. Determine the relative percentage of (1,6) linked glucan in the portion of Beta Glucan taken:

$$\text{Result} = \{A/(A + B)\} \times 100$$

A = integration values for the signal at 4.27 ppm, corresponding to H-1 from (1,6) glucan

B = integration values for the signal at 4.52 ppm corresponding to H-1 (1,3) glucan

Acceptance criteria: The ^1H spectrum of the *Sample solution* exhibits a chemical shift pattern with signal locations and relative intensities that correspond to those of the *Standard solution*. In addition, the relative percentage of (1,6) linked glucan is 10%–18% of the total linkages.

COMPOSITION

• CONTENT OF BETA GLUCAN

Buffer A: Dissolve 11.12 g of glacial acetic acid in approximately 900 mL of water. Adjust the solution with 20% sodium hydroxide solution to a pH of 5, and dilute with water to 1000 mL.

Buffer B: Dissolve 69.6 mL of glacial acetic acid in approximately 800 mL of water. Adjust with 20% sodium hydroxide solution to a pH of 3.8, and dilute with water to 1000 mL.

Buffer C: Dissolve 12.12 g of tris(hydroxymethyl)-aminomethane (TRIS), 11.69 g sodium chloride, and 4.16 g of ethylenediaminetetraacetic acid (EDTA) tetrasodium salt dihydrate in approximately 900 mL of water. Adjust with concentrated hydrochloric acid or 20% sodium hydroxide solution to a pH of 7.5, and dilute with water to 1000 mL. [NOTE—*Buffer C* can be stored for 1 year at 2°–8°.]

Buffer D¹: Transfer 45.287 g of dibasic potassium phosphate, 30.382 g of *p*-hydroxybenzoic acid, and 4 g of sodium azide into a 1000-mL volumetric flask, and carefully add 800 mL of water. Mix with a stirring bar and mild heat until completely dissolved. Allow the solution to cool, adjust with 16.7% potassium hydroxide solution to a pH of 7.4, and dilute with water to volume. [NOTE—Store *Buffer D* in an amber bottle with an expiration date of 3 years at 4°.]

Lyticase solution: Prepare the required volume of lyticase from *Arthrobacter luteus*² at a concentration of 10 U/ μL by dissolving the quantity stated by the manufacturer (U/mg) in a solution containing 10% (v/v) *Buffer C*. [NOTE—Unused solution can be stored at NMT –15° with an expiration date of 1 year. Every time a different lot of lyticase is used, the concentration of lyticase solution required needs to be qualified.]

(1,6)-Glucanase solution: 1 U/300 μL solution of lyophilized (1,6)-glucanase³ in *Buffer A*. [NOTE—Solids may not fully dissolve; therefore, this solution should be handled as a homogeneous suspension. The solution is stable for at least 60 days at NMT –15°.]

Polishing enzyme mix: Mix 2000 U of *exo*-beta-glucanase⁴ and 400 U of beta-glucosidase⁵ in 100.0 mL of *Buffer A*. A premix of the enzymes may be used as an alternative⁶. [NOTE—Store on ice during the procedure, and for use in a same-day assay. Unused *Polishing enzyme mix* can be refrozen once at NMT –15° with an expiration date of 2 years.]

Glucose oxidase/oxidase reagent: Dissolve the contents of the Glucose Determination Reagent⁷ in 1 L of water containing 50 mL of *Buffer D*. [NOTE—Store the reagent in an amber bottle, and label with an expiration date of 3 months at a temperature between 2° and 8° or 1 year at NMT –17°. Minimize the time spent at room temperature.]

Sample solution: Transfer 15–20 mg of Beta Glucan into a 16- × 100-mm glass vial. Place the vial in an ice bath. Add a 0.4-mL aliquot of cold potassium hydroxide (1 in 6) while mixing on a vortex mixer to disperse the

powder. Return the vial to the ice bath. Continue cycling through mixing on a vortex mixer and placing the vials in the ice bath for 20 min. The mixture should turn into a homogenous, translucent dispersion.

Standard solution: Proceed as directed for the *Sample solution* except replace Beta Glucan with USP Beta Glucan RS. [NOTE—Prepare the *Sample solution* and *Standard solution* in triplicate. It is critical for the success of the assay that the sample is well dispersed.]

Lyticase digestion: Upon removal of all vials containing the *Sample solution* or the *Standard solution* from the ice bath, add 1.6 mL of *Buffer B* and 600 μL of *Lyticase solution* to each vial. Incubate the mixture at 50° for 12–18 h, and cool to room temperature.

(1,6)-Glucanase digestion: After cooling of all vials, remove a 130- μL aliquot of each vial, and digest further by adding 25 μL of 16.7% potassium hydroxide solution and 300 μL of (1,6)-*Glucanase solution*. Incubate vials at 80° for 15 min, and cool to room temperature.

Beta glucanase/glucosidase digestion: After cooling of all vials, add 390 μL of the *Polishing enzyme mix* to each vial, and incubate the vials at 40° for 1 h. Cool them to room temperature, centrifuge, and transfer 50- μL aliquots (in duplicate) to new vials.

Enzyme blank solution: Prepare enzyme blanks in triplicate by combining all the reagents used during the digestion steps except the *Sample solution* or *Standard solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Vis

Analytical wavelength: 510 nm

Analysis: Dilute the 50- μL aliquots obtained after the *Beta glucanase/glucosidase digestion* with 50 μL of water, and then add 3 mL of *Glucose oxidase/oxidase reagent*. Incubate the vials for 20 min at 40°. Determine the absorbance of each vial of the *Sample solution* or *Standard solution* against the *Enzyme blank solution*. Prepare a standard curve using the absorbance of similarly treated series of glucose standards (0, 0.1, 0.25, 0.5, and 1.0 mg/mL). From the slope of the standard curve and the absorbance of the digested *Sample solution* and *Standard solutions*, determine the concentration, C , in mg/mL, of liberated glucose in the cuvette:

$$\text{Result} = (A_s - A_b) / \text{slope}$$

A_s = average absorbance of the sample or USP Beta Glucan RS

A_b = average absorbance of the *Enzyme blank solution*

Calculate the percentage of beta glucan as glucose in the portion of Beta Glucan taken:

$$\text{Result} = 100 \times C / \{[(WT_s/F1) \times (F2/F3)]/2\}$$

WT_s = original weight of the sample or USP Beta Glucan RS (mg)

$F1$ = total volume in the vial during *Lyticase digestion*, 2.6 mL

$F2$ = volume of the sample or USP Beta Glucan RS transferred to a new vial during (1,6)-*Glucanase digestion*, 0.130 mL

$F3$ = total volume during *Beta glucanase/glucosidase digestion*, 0.845 mL

Acceptance criteria: NLT 70% beta glucan, calculated as glucose after enzymatic hydrolysis, on the dried basis

• CONTENT OF PROTEIN

Sample: 1.0 g of Beta Glucan

Analysis: Proceed as directed in *Nitrogen Determination* (461), and multiply the nitrogen content by 6.25.

¹ This buffer is also available as Bottle #3 of the K-YBGL kit (Megazyme), or Bottle #1 of the GOPOD kit (Megazyme).

² Lyticase from *Arthrobacter luteus*, Sigma L4025, or equivalent.

³ Commercially available as Pustulanase, Cel136, Prokzyme, or equivalent.

⁴ E-EXBGL 200 U/mL, 200 U/bottle, Megazyme, or equivalent.

⁵ 200 U/bottle, Megazyme, or equivalent.

⁶ E-EXBGOS, Megazyme, or equivalent.

⁷ Bottle #4 of K-YBGL kit, or Bottle #2 of GOPOD kit, Megazyme, or equivalent.

Acceptance criteria: NMT 10.0%

• **CONTENT OF FAT**

Sample: 2 g of Beta Glucan, previously dried

Analysis: Transfer the *Sample* to an extraction thimble, and mix with an equivalent quantity of dry, clean sand. Place a fat-free cotton or glass wool plug on top of the thimble. Place the thimble in a continuous-extraction apparatus provided with a tared collection flask. Pour 75 mL of solvent hexane through the sample into the collection flask. Extract at a condensation rate of 5–6 drops/s for 4 h, then at a rate of 2–3 drops/s for the next 16 h. Detach the collection flask, carefully evaporate the solvent, and dry the collection flask and its contents in a drying oven at 100° for 30 min to constant weight. Calculate the percentage of the extract (crude fat) in the portion of Beta Glucan taken.

Acceptance criteria: NMT 20.0%

CONTAMINANTS

• **ELEMENTAL IMPURITIES—PROCEDURES (233)**

Acceptance criteria

Arsenic: NMT 0.5 µg/g

Cadmium: NMT 0.5 µg/g

Lead: NMT 0.5 µg/g

Mercury: NMT 0.1 µg/g

- **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic bacterial count does not exceed 2×10^4 cfu/g, the total combined molds and yeasts count does not exceed 2.5×10^1 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10 cfu/g.

- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• **GLYCOGEN**

Buffer B: Prepare as directed in *Content of Beta Glucan*.

Amyloglucosidase/invertase solution⁸: A mixture of 1630 U/mL of amyloglucosidase and 500 U/mL of invertase in glycerol solution (50% v/v)

Sample: 100 mg

Analysis: Transfer the *Sample* in triplicate into individual 16- × 150-mm glass screw-cap vials. Place the vials in an ice bath, and add to each vial a 2-mL aliquot of cold potassium hydroxide (1 in 6) while mixing on a vortex mixer to disperse the powder. Return the vial to the ice bath. Continue cycling through mixing on a vortex mixer and placing vials in the ice bath for 20 min. The mixture should turn into a homogenous, translucent dispersion. Add 8 mL of *Buffer B*. Mix thoroughly on a vortex mixer, and immediately add 200 µL of *Amyloglucosidase/invertase solution*, and mix again on a vortex mixer. Incubate the mixture at 40° for 30–35 min. Cool to room temperature. Mix again on a vortex mixer, transfer to a suitable centrifuge tube, and centrifuge until a clear supernatant is obtained. Transfer duplicate 50-µL aliquots of supernatant into new vials, and proceed as directed for the *Analysis* in *Content of Beta Glucan*.

Acceptance criteria: NMT 1.0%

• **MANNOSE**

Solution A: 100% purified water

Solution B: 956 mM sodium hydroxide

Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	36.0	64.0
15.0	36.0	64.0

⁸ Alternatively, Bottle #2 of K-YBGL kit (Megazyme, or equivalent) could be used directly.

Table 2 (Continued)

Time (min)	Solution A (%)	Solution B (%)
35.0 (sample injection)	59.4	40.6
80.0	59.4	40.6

[NOTE—The run time typically required is 80 min.]

Internal standard solution: 0.8 mg/mL of USP Inositol RS in water

Sample solution: Weigh 2.0–4.0 mg of Beta Glucan in duplicate into vials with stir bars. Add 500 µL of pure trifluoroacetic acid (TFA), and allow the mixture to form a uniform dispersion by stirring for 1 h at room temperature. Incubate in a 80° water bath for 2 h with stirring, and then cool to room temperature. Add 100 µL of the *Internal standard solution* to each vial, and incubate with stirring in a boiling water bath for 15 min. Cool again to room temperature, then add 1.07 mL of water to each vial, and incubate with stirring in a boiling water bath for 1 h. Cool the solutions to room temperature, and dry overnight on a SpeedVac, or equivalent, at low heat with the cryopumping system off. Dissolve the dried preparation in 2.5 mL of deionized water, and pass through a PTFE syringe filter of 0.2-µm pore size. Dilute with an equal volume of water before injection.

Standard solutions: Prepare a 4-mg/mL solution of USP Dextrose RS and a 80-µg/mL solution of USP Mannose RS. Separately transfer aliquots of these solutions to individual vials (see *Table 3*). Prepare each standard in duplicate, and freeze-dry them. Treat the freeze-dried vials as directed in the *Sample solution*, beginning with “Add 500 µL of pure trifluoroacetic acid”.

Table 3

Standard Identification Number	µL/Vial of 4 mg/mL Glucose	Content of Glucose (µg)	µL/Vial of 80 µg/mL Mannose	Content of Mannose (µg)
0	0	0	0	0
1	100	400	25	2
2	200	800	50	4
3	300	1200	100	8
4	500	2000	200	16
5	1000	4000	400	32

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Electrochemical detector

Detector mode: Pulsed amperometric detection

Detector range: 3000 µC (may be modified if needed)

Working electrode: Gold

Reference electrode: pH, silver–silver chloride

Electrochemical waveform: See *Table 4*.

Table 4

Time (s)	Potential (V)	Integration
0.00	0.10	
0.20	0.10	Start
0.40	0.10	End
0.41	–2.00	
0.42	–2.00	
0.43	0.60	
0.44	–0.10	
0.50	–0.10	

Column: 4-mm × 25-cm; packing L47
Guard column: 4-mm × 5-cm; packing L47
Column temperature: 30°
Flow rate: 0.4 mL/min
Injection size: 10 µL

System suitability

Sample: *Standard Identification #5*

[NOTE—The relative retention times for inositol, mannose, and glucose are 0.68, 0.95 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between mannose and glucose

Analysis

Samples: *Standard solutions* and *Sample solution*

Calculate the ratios of the peak area response of the glucose and mannose to the peak area response of the internal standard from the *Standard solutions*. Make two standard response lines by plotting the peak area response ratio versus the amount (µg) of the glucose and mannose in the *Standard solutions*. Calculate the ratio of the peak area response of the glucose and mannose to the peak area response of the internal standard from the *Sample solution*. From the calculated ratios of peak responses for glucose and mannose and their respective standard response lines, determine the content of glucose, C_G , and mannose, C_M , both in µg, in the *Sample solution*.

Calculate the percentage of mannose in the portion of Beta Glucan taken:

$$\text{Result} = C_M / (C_M + C_G) \times 100$$

C_M = content of mannose in the *Sample solution* from the mannose regression line (µg)

C_G = content of glucose in the *Sample solution* from the glucose regression line (µg)

Acceptance criteria: NMT 1.0% mannose, as a function of total hexose recovered (glucose and mannose)

• **RESIDUE ON IGNITION** (281): NMT 2.5%

• **LOSS ON DRYING** (731)

Sample: 1 g

Analysis: Dry the *Sample* at 105° for 3 h.

Acceptance criteria: NMT 8.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight and light-resistant containers.

• **USP REFERENCE STANDARDS** (11)

USP Beta Glucan RS

USP Dextrose RS

USP Inositol RS

USP Mannose RS

Powdered Bilberry Extract

DEFINITION

Powdered Bilberry Extract is prepared from the ripe fruits of *Vaccinium myrtillus* L. (Fam. Ericaceae) using suitable solvents such as alcohol, methanol, or water or mixtures of these solvents. The ratio of the starting plant material to Powdered Extract is between 153:1 and 76:1. It contains NLT 36.0% of anthocyanosides, calculated as cyanidin-3-O-glucoside chloride, and NMT 1.0% of anthocyanidins, calculated as cyanidin chloride; both are calculated on the anhydrous basis.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 4 mg/mL of USP Powdered Bilberry Extract RS in methanol. Centrifuge, and use the clear supernatant.

Sample solution: 4 mg/mL of Powdered Bilberry Extract in methanol. Centrifuge, and use the clear supernatant.

Adsorbent: Use suitable thin-layer chromatographic plates coated with a layer of cellulose.

Application volume: 10 µL

Developing solvent system A: Glacial acetic acid, hydrochloric acid, and water (15:3:82)

Developing solvent system B: Glacial acetic acid and water (6:4)

Analysis

Samples: *Standard solution* and *Sample solution*

Use a saturated chamber. Develop the chromatograms using *Developing solvent system A*, and dry the plate with the aid of a current of warm air. Develop the chromatograms in the same direction using *Developing solvent system B*. Examine the plate under visible light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits three main red bands with R_f values of approximately 0.55, 0.65, and 0.70 that are similar in position and color to the corresponding main bands in the chromatogram of the *Standard solution*.

- **B.** The retention times of the anthocyanoside peaks in the chromatogram of the *Sample solution* correspond to those in the chromatogram of *Standard solution C*, as obtained in the test for *Content of Anthocyanosides and Anthocyanidins*. The peaks due to delphinidin-3-O-galactoside chloride and delphinidin-3-O-glucoside chloride are the most intense peaks and are of similar intensity, and each is more intense than the peak due to cyanidin-3-O-glucoside chloride. The peaks due to cyanidin-3-O-galactoside chloride, delphinidin-3-O-arabinoside chloride, and cyanidin-3-O-glucoside chloride are of similar intensity. Each of the remaining anthocyanoside peaks is of lower intensity than the peak due to cyanidin-3-O-glucoside chloride.

COMPOSITION

• **CONTENT OF ANTHOCYANOSIDES AND ANTHOCYANIDINS**

Solvent: Methanol and hydrochloric acid (49:1)

Diluent: 85% phosphoric acid and water (1:9)

Solution A: Formic acid and water (1:9)

Solution B: Acetonitrile, methanol, formic acid, and water (45:45:20:80)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	93	7
35	75	25
45	35	65
46	0	100
50	0	100
51	93	7
60	93	7

Standard stock solution A: 0.4 mg/mL of USP Cyanidin-3-O-glucoside Chloride RS in *Solvent*. [NOTE—Dissolve using sonication.]

Standard solution A: 0.08 mg/mL of USP Cyanidin-3-O-glucoside Chloride RS from *Standard stock solution A* in *Diluent*. [NOTE—This solution is stable for 48 h at 4°.]

Standard stock solution B: 0.5 mg/mL of USP Cyanidin Chloride RS in *Solvent*. [NOTE—Dissolve using sonication.]

Standard solution B: 0.01 mg/mL of USP Cyanidin Chloride RS from *Standard stock solution B* in *Diluent*. [NOTE—This solution is stable for 36 h at 4°]

Standard solution C: Transfer 125 mg of USP Powdered Bilberry Extract RS to a 100-mL volumetric flask, add 25 mL of *Solvent*, sonicate to dissolve, and dilute

with *Diluent* to volume. [NOTE—This solution is stable for 48 h at 4°.]

Sample solution: Transfer 125 mg of Powdered Bilberry Extract to a 100-mL volumetric flask, add 25 mL of *Solvent*, sonicate to dissolve, and dilute with *Diluent* to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

[NOTE—Use deactivated silanized HPLC vials.]

Mode: LC

Detector: UV-Vis 535 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Temperature

Refrigerated autosampler: 4°

Column: 30 ± 1°

Flow rate: 1 mL/min

Injection size: 10 μL

System suitability

Samples: *Standard solution A* and *Standard solution C*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution C* is similar to the Reference Chromatogram provided with USP Powdered Bilberry Extract RS.

Resolution: NLT 0.8 between the delphinidin-3-O-arabinoside, malvidin-3-O-galactoside, and petunidin-3-O-arabinoside peaks and NLT 1.0 for other components, *Standard solution C*

Tailing factor range: 0.8–2.0 for the cyanidin-3-O-glucoside chloride peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for cyanidin-3-O-glucoside chloride peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Using the chromatogram of *Standard solution C* and the Reference Chromatogram, identify the retention times of the peaks corresponding to the different anthocyanosides and anthocyanidins. The approximate relative retention times, relative to cyanidin-3-O-glucoside chloride, are provided for the anthocyanosides in *Table 2* and for the anthocyanidins in *Table 3*.

Separately calculate the percentages of each anthocyanoside (see *Table 2*) in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each of the anthocyanosides in the *Sample solution*

r_S = peak response of cyanidin-3-O-glucoside chloride in *Standard solution A*

C_S = concentration of USP Cyanidin-3-O-glucoside Chloride RS in *Standard solution A* (mg/mL)

C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Table 2

Analyte	Relative Retention Time
Delphinidin-3-O-galactoside chloride	0.61
Delphinidin-3-O-glucoside chloride	0.73
Cyanidin-3-O-galactoside chloride	0.84
Delphinidin-3-O-arabinoside chloride	0.86
Cyanidin-3-O-glucoside chloride	1.00
Petunidin-3-O-galactoside chloride	1.08
Cyanidin-3-O-arabinoside chloride	1.11
Petunidin-3-O-glucoside chloride	1.24
Peonidin-3-O-galactoside chloride	1.36
Petunidin-3-O-arabinoside chloride	1.39

Table 2 (Continued)

Analyte	Relative Retention Time
Peonidin-3-O-glucoside chloride	1.55
Malvidin-3-O-galactoside chloride	1.58
Peonidin-3-O-arabinoside chloride	1.67
Malvidin-3-O-glucoside chloride	1.76
Malvidin-3-O-arabinoside chloride	1.91

Separately calculate the percentages of anthocyanidins (see *Table 3*) in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each of the anthocyanidins in the *Sample solution*

r_S = peak response of cyanidin chloride in *Standard solution B*

C_S = concentration of USP Cyanidin Chloride RS in *Standard solution B* (mg/mL)

C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Table 3

Analyte	Relative Retention Time
Delphinidin chloride	1.28
Cyanidin chloride	1.82
Petunidin chloride	2.08
Peonidin chloride	2.27
Malvidin chloride	2.30

Acceptance criteria

Sum of all anthocyanosides: NLT 36.0% on the anhydrous basis

Sum of all anthocyanidins: NMT 1.0% on the anhydrous basis

CONTAMINANTS

• **HEAVY METALS**, *Method II* <231>: NMT 20 ppm

• **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements

• **MICROBIAL ENUMERATION TESTS** (2021)

Total aerobic microbial count: NMT 10⁴ cfu/g

Total combined yeasts and molds count: NMT 10³ cfu/g

• **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• **ACID INSOLUBLE FRACTION**

Sample: 5 g of Powdered Extract finely ground

Analysis: Transfer about 1 g to a 500-mL flask, add 200 mL of 0.1 N hydrochloric acid, and shake vigorously for 2 h. Pass the solution through a previously tared sintered-glass filter, wash the flask with 30 mL of 0.1 N hydrochloric acid, and transfer the washings to the filter. Wash the filter with 30 mL of 0.1 N hydrochloric acid in 5-mL portions. Dry the filter for 3 h at 105°, cool in a desiccator, and weigh. Calculate the percentage of the acid insoluble fraction.

Acceptance criteria: NMT 5%

• **WATER DETERMINATION**, *Method Ia* <921>: NMT 4.5%, determined on 0.5 g

• **RESIDUE ON IGNITION** <281>: NMT 3.0%, determined on 1.0 g

• **BOTANICAL EXTRACTS**, *Residual Solvents* <565>: Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. It meets other labeling requirements under *Botanical Extracts* (565).
- **USP REFERENCE STANDARDS** (11)
 - USP Powdered Bilberry Extract RS
 - USP Cyanidin Chloride RS
 - USP Cyanidin-3-O-glucoside Chloride RS

Biotin—see *Biotin General Monographs*

Black Cohosh

DEFINITION

Black Cohosh consists of the dried rhizome and roots of *Actaea racemosa* L. [*Cimicifuga racemosa* (L.) Nutt.] (*Ranunculaceae*). It is harvested in the summer. It contains NLT 0.4% of triterpene glycosides, calculated as 23-*epi*-26-deoxyactein¹ (C₃₇H₅₆O₁₀) on the dried basis.

IDENTIFICATION• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: 100 mg/mL of USP Powdered Black Cohosh Extract RS in methanol

Standard solution B: 1 mg/mL each of USP Actein RS, USP 23-*epi*-26-Deoxyactein RS, and isoferulic acid in methanol

Sample solution: Transfer 5 g of powdered Black Cohosh to a screw-capped centrifuge tube, add 10 mL of a mixture of alcohol and water (7:3), and heat on a steam bath for 10 min. Centrifuge, and use the clear supernatant.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL

Developing solvent system: Use the upper phase of a mixture of butyl alcohol, glacial acetic acid, and water (5:1:4).

Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85:10:5:0.5). [NOTE—Store in a refrigerator. The reagent is colorless; discard if color appears.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved about 15 cm, and dry the plate with the aid of a current of air.

Acceptance criteria: Examine the plate under UV light at 365 nm. The chromatogram of the *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. In the upper third of the plate, the *Sample solution* exhibits a blue fluorescent zone at the level of the zone due to isoferulic acid of *Standard solution B*. Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight. The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein. The *Sample solution* exhibits several greenish-brown spots in the lower third of the plate and several violet zones above; two of these violet zones occur at *R_f* values similar to those due to actein and 23-*epi*-26-deoxyactein of *Standard solution B*.

• **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Standard solution A: 0.5 mL of *Standard solution A* prepared in *Identification test A*, diluted with methanol to 2 mL

Standard solution B: 1.0 mL of *Standard solution B* prepared in *Identification test A*, diluted with methanol to 5 mL

Sample solution: Transfer 0.5 g of powdered Black Cohosh to a screw-capped tube, add 5 mL of methanol, sonicate for 10 min, and filter into a 10-mL volumetric flask. Wash the residue on the filter paper four times, using 1 mL of methanol for each washing; add the washings to the volumetric flask; and dilute with methanol to volume.

Application volume: 2 µL as an 8-mm band

Developing solvent system: Toluene, ethyl formate, and formic acid (5:3:2)

Spray reagent: Proceed as directed for *Identification test A*.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved about two-thirds of the length of the plate, and dry the plate with the aid of a current of air.

Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight.

Acceptance criteria: The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein at *R_f* values of about 0.5 and 0.4, respectively. The *Sample solution* exhibits zones similar in color and *R_f* values to those due to actein and 23-*epi*-26-deoxyactein of *Standard solution B*.

- **C.** The *Sample solution* exhibits peaks for cimicracemoside A, 26-deoxycimicifugoside, (26S)-actein, 23-*epi*-26-deoxyactein, cimigenol-arabinside, and cimigenol-xyloside at retention times corresponding to these compounds in the *Standard solution*, as obtained in the test for *Content of Triterpene Glycosides*. The ratio of the peak areas of cimigenol-arabinside to cimigenol-xyloside is NLT 0.4 (distinction from *Cimicifuga foetida*).

COMPOSITION• **CONTENT OF TRITERPENE GLYCOSIDES**

Standard solution: Dissolve a quantity of USP Powdered Black Cohosh Extract RS in methanol with shaking for 1 min, and dilute with methanol to obtain a solution having a known concentration of 30 mg/mL. Pass through a membrane filter of 0.45-µm or finer pore size.

23-*epi*-26-Deoxyactein standard solutions: Dissolve USP 23-*epi*-26-Deoxyactein RS in methanol with shaking for 1 min. Dilute quantitatively, and stepwise if necessary, to obtain solutions having known concentrations of 500, 100, 50, 25, and 12.5 µg/mL. Pass through a membrane filter of 0.45-µm or finer pore size.

System suitability solution: 0.1 mg/mL each of USP Actein RS and USP 23-*epi*-26-Deoxyactein RS in methanol

Sample solution: Accurately weigh about 750 mg of ground plant material, and place in a 20-mL polytetrafluoroethylene capped centrifuge tube. Pipet 15 mL of methanol, sonicate for 30 min, centrifuge, and transfer the supernatant to an evaporation flask. Repeat the extraction twice. Evaporate the combined extracts under vacuum at 45°–50°. Dissolve the residue in methanol, and quantitatively transfer to a 10-mL volumetric flask. Dilute with methanol to volume, and pass through a membrane filter of 0.45-µm or finer pore size.

¹ 23-*epi*-26-Deoxyactein is sometimes referred to as 27-deoxyactein.

Solution A: 0.05% trifluoroacetic acid in water

Solution B: Acetonitrile

Mobile phase: See Table 1.

Table 1

Time (min)	Water (%)	Solution A (%)	Solution B (%)
0	0	80	20
8	0	80	20
15	68	0	32
55	36	0	64
65	5	0	95
70	5	0	95
85	0	80	20

Chromatographic system

(See Chromatography <621>, System Suitability.)

Mode: LC

Detector: Evaporative light-scattering

[NOTE—The detector is set up according to the manufacturer's instruction in order to achieve a signal-to-noise ratio of NLT 10 for the 12.5-μg/mL 23-*epi*-26-Deoxyactein standard solution.]

Column: 4.6-mm × 25-cm; 5-μm packing L1

Column temperature: 35°

Flow rate: 1.6 mL/min

Injection size: 20 μL

System suitability

Samples: System suitability solution, Standard solution, and 100-μg/mL 23-*epi*-26-Deoxyactein standard solution

Suitability requirements

Chromatogram similarity: The chromatogram of the Standard solution is similar to the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS being used.

Resolution: NLT 1.0 between the (26S)-actein and the 23-*epi*-26-deoxyactein peaks, System suitability solution

Tailing factor: NMT 2.0 for the 23-*epi*-26-deoxyactein peak, 100-μg/mL 23-*epi*-26-Deoxyactein standard solution

Relative standard deviation: NMT 2.0% of the logarithm of the area response of the 23-*epi*-26-deoxyactein peak in repeated injections, 100-μg/mL 23-*epi*-26-Deoxyactein standard solution

Analysis

Samples: System suitability solution, Standard solution, 23-*epi*-26-Deoxyactein standard solutions, and Sample solution

Using the chromatogram of the Standard solution and the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS, identify the retention times of the peaks corresponding to the triterpene glycosides. The approximate relative retention times of the triterpene glycosides are provided in Table 2.

Table 2

Compound	Relative Retention Time
Cimicifugoside H-1	0.61
Cimiracemoside A	0.78
(26R)-Actein	0.94
26-Deoxycimicifugoside	0.96
(26S)-Actein	0.98
23- <i>epi</i> -26-Deoxyactein	1.00
Acetyl-shengmanol-xyloside	1.03
Cimigenol-arabinoside	1.08
Cimigenol-xyloside (cimicifugoside)	1.13

Table 2 (Continued)

Compound	Relative Retention Time
26-Deoxyactein	1.22
25-Acetyl-cimigenol-arabinoside	1.60
(24S)-25-Acetyl-cimigenol-xyloside	1.64
25-O-Methyl-cimigenol-arabinoside	1.90
25-O-Methyl-cimigenol-xyloside	1.93

Plot the logarithms of the peak area responses versus the logarithms of the concentrations, in μg/mL, of the 23-*epi*-26-Deoxyactein standard solutions, and determine the regression line using a least-squares analysis. The correlation coefficient for the regression line is NLT 0.995. From the graphs so obtained, determine the concentration, C, in μg/mL, of the relevant analyte in the Sample solution. Separately calculate the percentages of cimicifugoside H-1, cimiracemoside A, (26R)-actein, 26-deoxycimicifugoside, (26S)-actein, 23-*epi*-26-deoxyactein, acetyl-shengmanol-xyloside, cimigenol-arabinoside, cimigenol-xyloside (cimicifugoside), 26-deoxyactein, 25-acetyl-cimigenol-arabinoside, (24S)-25-acetyl-cimigenol-xyloside, 25-O-methyl-cimigenol-arabinoside, and 25-O-methyl-cimigenol-xyloside as 23-*epi*-26-deoxyactein (C₃₇H₅₆O₁₀) in the portion of Black Cohosh taken:

$$\text{Result} = (V \times C) / (F \times W) \times 100$$

V = final volume of the Sample solution (mL)

C = concentration of the relevant analyte in the Sample solution (μg/mL)

F = factor to convert mg to μg, 1000 μg/mg

W = weight of Black Cohosh taken to prepare the Sample solution (mg)

Calculate the percentage of triterpene glycosides in the portion of Black Cohosh taken by adding all of the percentages calculated for the individual analytes.

Acceptance criteria: NLT 0.4% on the dried basis

CONTAMINANTS

- **HEAVY METALS <231>:** NMT 10 ppm
- **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis <561>:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS <2021>:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 10⁵ cfu/g; the total combined molds and yeasts count does not exceed 10³ cfu/g; and the bile-tolerant Gram-negative bacteria count does not exceed 10³ cfu/g.

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: The Black Cohosh rhizome is dark brown, longitudinally grooved, rough, strongly knotty, and somewhat curled and irregular. It is 15 cm long and up to 2.5 cm thick. The upper surface is covered with numerous round scars of the earlier stalks; laterally, it is clearly curled, and the lower surface is covered with thin, longitudinally grooved, dark brown, easily breakable roots. The fracture is horny and fibrous. The transverse cut shows a thin outer bark surrounding a ring of numerous pale, narrow wedges of vascular tissue alternating with dark medullary rays and a large central pith. Black Cohosh roots are dark brown, between 1 and 3 mm in diameter, brittle, nearly cylindrical or obtusely quadrangular, and longitudinally wrinkled. The fracture is short. The transverse cut shows a distinct cambium line separating a wide outer bark from a central region composed of three to six wedges of lignified xylem tissue united by their apices and separated by broad nonlignified medullary rays.

Microscopic: In a surface view, suberous epidermal cells are tabular with moderately thickened walls. The parenchymatous cortex is filled with starch. Xylem wedges are lignified and composed of numerous small vessels with bordered pits or reticulately thickened walls, thin-walled fibers, and xylem parenchyma. The parenchyma of the pith is unlignified. Medullary rays are filled with starch granules, which are spherical or polygonal and are mostly simple or two to three compounded but can be up to six compounded. Individual starch granules are between 3 and 15 μm in diameter, each with a somewhat central slit-shaped hilum.

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** <561>: NMT 2.0% of foreign organic matter, and NMT 5.0% of stem bases
- **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method 2** <561>: NLT 8.0%, using a mixture of alcohol and water (1:1) instead of alcohol
- **LOSS ON DRYING** <731>: Dry at 105° for 2 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** <561>: NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a well-closed, light-resistant container. Protect from moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article. Dosage forms prepared with this article should bear the following statement: Discontinue use and consult a healthcare practitioner if you have a liver disorder or develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice.
- **USP REFERENCE STANDARDS** <11>
 - USP Actein RS
 - USP Powdered Black Cohosh Extract RS
 - USP 23-*epi*-26-Deoxyactein RS

Black Cohosh Fluidextract

DEFINITION

Black Cohosh Fluidextract is prepared from Black Cohosh by extraction with hydroalcoholic mixtures or isopropyl-alcohol–water mixtures. Each mL contains the extracted constituents of 1 g of the plant material. It contains NLT 90.0% and NMT 110.0% of the labeled amount of triterpene glycosides, calculated as 23-*epi*-26-deoxyactein ($\text{C}_{37}\text{H}_{56}\text{O}_{10}$).

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
 - Standard solution A:** 100 mg/mL of USP Powdered Black Cohosh Extract RS in methanol
 - Standard solution B:** 1 mg/mL each of USP Actein RS, USP 23-*epi*-26-Deoxyactein RS, and isoferulic acid in methanol
 - Sample solution:** Fluidextract
 - Adsorbent:** Chromatographic silica gel mixture with an average particle size of 10–15 μm (TLC plates)
 - Application volume:** 10 μL
 - Developing solvent system:** Use the upper phase of a mixture of butyl alcohol, glacial acetic acid, and water (5:1:4).
 - Spray reagent:** Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85:10:5:0.5). [NOTE—Store in a refrigerator. The reagent is colorless; discard if color appears.]

Analysis

Samples: Standard solution A, Standard solution B, and Sample solution

Develop the chromatograms until the solvent front has moved about 15 cm, and dry the plate with the aid of a current of air.

Acceptance criteria: Examine the plate under UV light at 365 nm. The Sample solution exhibits main zones similar in position and color to the main zones of Standard solution A. In the upper third of the plate, the Sample solution exhibits a blue fluorescent zone at the level of the zone due to isoferulic acid of Standard solution B. Spray the plate with Spray reagent, heat at 100° for 5 min, and examine in daylight. The Sample solution exhibits main zones similar in position and color to the main zones of Standard solution A. Standard solution B exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein. The Sample solution exhibits several greenish-brown spots in the lower third of the plate and several violet zones above; two of these violet zones occur at R_f values similar to those due to actein and 23-*epi*-26-deoxyactein of Standard solution B.

• B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: 0.5 mL of Standard solution A prepared in Identification test A, diluted with methanol to 2.0 mL

Standard solution B: 1.0 mL of Standard solution B prepared in Identification test A, diluted with methanol to 5.0 mL

Sample solution: Use the Fluidextract, diluting if necessary with a suitable solvent to obtain a concentration of 0.25 mg/mL of triterpene glycosides.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 μm (HPTLC plates)

Application volume: 2 μL as an 8-mm band

Developing solvent system: Toluene, ethyl formate, and formic acid (5:3:2)

Spray reagent: Proceed as directed for Identification test A.

Analysis

Samples: Standard solution A, Standard solution B, and Sample solution

Develop until the solvent front has moved two-thirds of the length of the plate, and dry the plate with the aid of a current of air. Spray the plate with Spray reagent, heat at 100° for 5 min, and examine in daylight.

Acceptance criteria: The Sample solution exhibits main zones similar in position and color to the main zones of Standard solution A. Standard solution B exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein at R_f values of about 0.5 and 0.4, respectively. The Sample solution exhibits zones similar in color and R_f values to those due to actein and 23-*epi*-26-deoxyactein of Standard solution B.

- **C.** The Sample solution exhibits peaks for cimracemoside A, 26-deoxycimicifugoside, (26S)-actein, 23-*epi*-26-deoxyactein, cimigenol-arabinoside, and cimigenol-xyloside at retention times corresponding to those compounds in the Standard solution, as obtained in the test for Content of Triterpene Glycosides. The ratio of the peak areas of cimigenol-arabinoside to cimigenol-xyloside is NLT 0.4 (distinction from *Cimicifuga foetida*).

COMPOSITION

• CONTENT OF TRITERPENE GLYCOSIDES

Standard solution: Dissolve a quantity of USP Powdered Black Cohosh Extract RS in methanol with shaking for 1 min, and dilute with methanol to obtain a solution having a known concentration of 30 mg/mL. Pass through a membrane filter of 0.45- μm or finer pore size.

23-*epi*-26-Deoxyactein standard solutions: Dissolve USP 23-*epi*-26-Deoxyactein RS in methanol with shaking for 1 min. Dilute quantitatively, and stepwise if necessary, to obtain solutions having known concentrations

of 500, 100, 50, 25, and 12.5 µg/mL. Pass through a membrane filter of 0.45-µm or finer pore size.

System suitability solution: 0.1 mg/mL each of USP Actein RS and USP 23-*epi*-26-Deoxyactein RS in methanol

Sample solution: Use the Fluidextract, diluting if necessary with methanol to obtain a concentration of about 0.75 mg/mL of triterpene glycosides. Centrifuge, or pass through a filter of 0.45-µm or finer pore size.

Solution A: 0.05% trifluoroacetic acid in water

Solution B: Acetonitrile

Mobile phase: See Table 1.

Table 1

Time (min)	Water (%)	Solution A (%)	Solution B (%)
0	0	80	20
8	0	80	20
15	68	0	32
55	36	0	64
65	5	0	95
70	5	0	95
85	0	80	20

Chromatographic system

(See Chromatography <621>, System Suitability.)

Mode: LC

Detector: Evaporative light-scattering

[NOTE—The detector is set up according to the manufacturer's instruction in order to achieve a signal-to-noise ratio of NLT 10 for the 12.5-µg/mL 23-*epi*-26-Deoxyactein standard solution.]

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 1.6 mL/min

Injection size: 20 µL

System suitability

Samples: System suitability solution, Standard solution, and 100-µg/mL 23-*epi*-26-Deoxyactein standard solution

Suitability requirements

Chromatogram similarity: The chromatogram of the Standard solution is similar to the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS being used.

Resolution: NLT 1.0 between the (26S)-actein and the 23-*epi*-26-deoxyactein peaks, System suitability solution

Tailing factor: NMT 2.0 for the 23-*epi*-26-deoxyactein peak, 100-µg/mL 23-*epi*-26-Deoxyactein standard solution

Relative standard deviation: NMT 2.0% of the logarithm of the area response of the 23-*epi*-26-deoxyactein peak in repeated injections, 100-µg/mL 23-*epi*-26-Deoxyactein standard solution

Analysis

Samples: System suitability solution, Standard solution, 23-*epi*-26-Deoxyactein standard solutions, and Sample solution

Using the chromatogram of the Standard solution and the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS, identify the retention times of the peaks corresponding to the triterpene glycosides. The approximate relative retention times of the triterpene glycosides are provided in Table 2.

Table 2

Compound	Relative Retention Time
Cimicifugoside H-1	0.61
Cimiracemoside A	0.78

Table 2 (Continued)

Compound	Relative Retention Time
(26R)-Actein	0.94
26-Deoxycimicifugoside	0.96
(26S)-Actein	0.98
23- <i>epi</i> -26-Deoxyactein	1.00
Acetyl-shengmanol-xyloside	1.03
Cimigenol-arabinoside	1.08
Cimigenol-xyloside (cimicifugoside)	1.13
26-Deoxyactein	1.22
25-Acetyl-cimigenol-arabinoside	1.60
(24S)-25-Acetyl-cimigenol-xyloside	1.64
25-O-Methyl-cimigenol-arabinoside	1.90
25-O-Methyl-cimigenol-xyloside	1.93

Plot the logarithms of the peak area responses versus the logarithms of the concentrations, in µg/mL, of the 23-*epi*-26-Deoxyactein standard solutions, and determine the regression line using a least-squares analysis. The correlation coefficient for the regression line is NLT 0.995. From the graphs so obtained, determine the concentration, *C*, in µg/mL, of the relevant analyte in the Sample solution. Separately calculate the concentrations, in µg/mL, of cimicifugoside H-1, cimiracemoside A, (26R)-actein, 26-deoxycimicifugoside, (26S)-actein, 23-*epi*-26-deoxyactein, acetyl-shengmanol-xyloside, cimigenol-arabinoside, cimigenol-xyloside (cimicifugoside), 26-deoxyactein, 25-acetyl-cimigenol-arabinoside, (24S)-25-acetyl-cimigenol-xyloside, 25-O-methyl-cimigenol-arabinoside, and 25-O-methyl-cimigenol-xyloside as 23-*epi*-26-deoxyactein (C₃₇H₅₆O₁₀) in the portion of Fluidextract taken:

$$\text{Result} = (D \times C/V)$$

D = dilution factor for the Sample solution, if applicable: final volume of Sample solution/volume of aliquot of Fluidextract taken (mL/mL)

C = concentration of the relevant analyte in the Sample solution (µg/mL)

V = volume of the Fluidextract taken to prepare the Sample solution (mL)

Calculate the percentage of the labeled amount of triterpene glycosides in the portion of Fluidextract taken:

$$\text{Result} = \Sigma C/L \times 100$$

ΣC = sum of concentrations of the individual triterpene glycosides (mg/mL)

L = labeled concentration of triterpene glycosides of the Fluidextract (mg/mL)

Acceptance criteria: 90.0%–110.0%

CONTAMINANTS

- **HEAVY METALS <231>:** NMT 10 ppm
- **MICROBIAL ENUMERATION TESTS <2021>:** The total bacterial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts count does not exceed 10³ cfu/g.
- **OTHER REQUIREMENTS:** It meets the requirements under Botanical Extracts <565>, Residual Solvents and Pesticide Residues.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store in a cool place.
- **LABELING:** It meets the requirements for Labeling under Botanical Extracts <565>. Label it to indicate the content, in percentage, of triterpene glycosides, calculated as

23-*epi*-26-deoxyactein. Dosage forms prepared with this article should bear the following statement: Discontinue use and consult a healthcare practitioner if you have a liver disorder or develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice.

- **USP REFERENCE STANDARDS** <11>
USP Actein RS
USP Powdered Black Cohosh Extract RS
USP 23-*epi*-26-Deoxyactein RS

Powdered Black Cohosh

DEFINITION

Powdered Black Cohosh is Black Cohosh reduced to a powder or a very fine powder. It contains NLT 0.4% of triterpene glycosides, calculated as 23-*epi*-26-deoxyactein ($C_{37}H_{56}O_{10}$) on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: 100 mg/mL of USP Powdered Black Cohosh Extract RS in methanol

Standard solution B: 1 mg/mL each of USP Actein RS, USP 23-*epi*-26-Deoxyactein RS, and isoferulic acid in methanol

Sample solution: Transfer 5 g of Powdered Black Cohosh to a screw-capped centrifuge tube, add 10 mL of a mixture of alcohol and water (7:3), and heat on a steam bath for 10 min. Centrifuge, and use the clear supernatant.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μ m (TLC plates)

Application volume: 10 μ L

Developing solvent system: Use the upper phase of a mixture of butyl alcohol, glacial acetic acid, and water (5:1:4).

Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85:10:5:0.5). [NOTE—Store in a refrigerator. The reagent is colorless; discard if color appears.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved about 15 cm, and dry the plate with the aid of a current of air.

Acceptance criteria: Examine the plate under UV light at 365 nm. The chromatogram of the *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. In the upper third of the plate, the *Sample solution* exhibits a blue fluorescent zone at the level of the zone due to isoferulic acid of *Standard solution B*. Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight. The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein. The *Sample solution* exhibits several greenish-brown spots in the lower third of the plate and several violet zones above; two of these violet zones occur at R_f values similar to those due to actein and 23-*epi*-26-deoxyactein of *Standard solution B*.

• B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: 0.5 mL of *Standard solution A* prepared in *Identification test A*, diluted with methanol to 2 mL

Standard solution B: 1.0 mL of *Standard solution B* prepared in *Identification test A*, diluted with methanol to 5 mL

Sample solution: Transfer 0.5 g of Powdered Black Cohosh to a screw-capped tube, add 5 mL of methanol, sonicate for 10 min, and filter into a 10-mL volumetric

flask. Wash the residue on the filter paper four times, using 1 mL of methanol for each washing; add the washings to the volumetric flask; and dilute with methanol to volume.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 μ m (HPTLC plates)

Application volume: 2 μ L as an 8-mm band

Developing solvent system: Toluene, ethyl formate, and formic acid (5:3:2)

Spray reagent: Proceed as directed for *Identification test A*.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved about two-thirds of the length of the plate, and dry the plate with the aid of a current of air.

Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight.

Acceptance criteria: The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein at R_f values of about 0.5 and 0.4, respectively. The *Sample solution* exhibits zones similar in color and R_f values to those due to actein and 23-*epi*-26-deoxyactein of *Standard solution B*.

- **C.** The *Sample solution* exhibits peaks for cimracemoside A, 26-deoxycimicifugoside, (26*S*)-actein, 23-*epi*-26-deoxyactein, cimigenol-arabinside, and cimigenol-xyloside at retention times corresponding to those compounds in the *Standard solution*, as obtained in the test for *Content of Triterpene Glycosides*. The ratio of the peak areas of cimigenol-arabinside to cimigenol-xyloside is NLT 0.4 (distinction from *Cimicifuga foetida*).

COMPOSITION

• CONTENT OF TRITERPENE GLYCOSIDES

Standard solution: Dissolve a quantity of USP Powdered Black Cohosh Extract RS in methanol with shaking for 1 min, and dilute with methanol to obtain a solution having a known concentration of 30 mg/mL. Pass through a membrane filter of 0.45- μ m or finer pore size.

23-*epi*-26-Deoxyactein standard solutions: Dissolve USP 23-*epi*-26-Deoxyactein RS in methanol with shaking for 1 min. Dilute quantitatively, and stepwise if necessary, to obtain solutions having known concentrations of 500, 100, 50, 25, and 12.5 μ g/mL. Pass through a membrane filter of 0.45- μ m or finer pore size.

System suitability solution: 0.1 mg/mL each of USP Actein RS and USP 23-*epi*-26-Deoxyactein RS in methanol

Sample solution: Accurately weigh about 750 mg of Powdered Black Cohosh, and place in a 20-mL polytetrafluoroethylene capped centrifuge tube. Pipet 15 mL of methanol, sonicate for 30 min, centrifuge, and transfer the supernatant to an evaporation flask. Repeat the extraction twice. Evaporate the combined extracts under vacuum at 45°–50°. Dissolve the residue in methanol, and quantitatively transfer to a 10-mL volumetric flask. Dilute with methanol to volume, and pass through a membrane filter of 0.45- μ m or finer pore size.

Solution A: 0.05% trifluoroacetic acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1* below.

Table 1

Time (min)	Water (%)	Solution A (%)	Solution B (%)
0	0	80	20
8	0	80	20
15	68	0	32

Table 1 (Continued)

Time (min)	Water (%)	Solution A (%)	Solution B (%)
55	36	0	64
65	5	0	95
70	5	0	95
85	0	80	20

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** Evaporative light-scattering[NOTE—The detector is set up according to the manufacturer's instruction in order to achieve a signal-to-noise ratio of NLT 10 for the 12.5-μg/mL 23-*epi*-26-Deoxyactein standard solution.]**Column:** 4.6-mm × 25-cm; 5-μm packing L1**Column temperature:** 35°**Flow rate:** 1.6 mL/min**Injection size:** 20 μL**System suitability****Samples:** *System suitability solution*, *Standard solution*, and the 100-μg/mL 23-*epi*-26-Deoxyactein standard solution**Suitability requirements****Chromatogram similarity:** The chromatogram of the *Standard solution* is similar to the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS being used.**Resolution:** NLT 1.0 between the (26S)-actein and the 23-*epi*-26-deoxyactein peaks, *System suitability solution***Tailing factor:** NMT 2.0 for the 23-*epi*-26-deoxyactein peak, 100-μg/mL 23-*epi*-26-Deoxyactein standard solution**Relative standard deviation:** NMT 2.0% of the logarithm of the area responses for replicate injections, 100-μg/mL 23-*epi*-26-Deoxyactein standard solution**Analysis****Samples:** *System suitability solution*, *Standard solution*, 23-*epi*-26-Deoxyactein standard solutions, and *Sample solution*Using the chromatogram of the *Standard solution* and the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS, identify the retention times of the peaks corresponding to the triterpene glycosides. The approximate relative retention times of the triterpene glycosides are provided in Table 2.**Table 2**

Name	Relative Retention Time
Cimicifugoside H-1	0.61
Cimiracemoside A	0.78
(26R)-Actein	0.94
26-Deoxycimicifugoside	0.96
(26S)-Actein	0.98
23- <i>epi</i> -26-Deoxyactein	1.00
Acetyl-shengmanol-xyloside	1.03
Cimigenol-arabinoside	1.08
Cimigenol-xyloside (cimicifugoside)	1.13
26-Deoxyactein	1.22
25-Acetyl-cimigenol-arabinoside	1.60
(24S)-25-Acetyl-cimigenol-xyloside	1.64
25-O-Methyl-cimigenol-arabinoside	1.90
25-O-Methyl-cimigenol-xyloside	1.93

Plot the logarithms of the peak area responses versus the logarithms of the concentrations, in μg/mL, of the

23-*epi*-26-Deoxyactein standard solution, and determine the regression line using a least-squares analysis. The correlation coefficient for the regression line is NLT 0.995. From the graphs so obtained, determine the concentration, *C*, in μg/mL, of the relevant analyte in the *Sample solution*. Separately calculate the percentages of cimicifugoside H-1, cimiracemoside A, (26R)-actein, 26-deoxycimicifugoside, (26S)-actein, 23-*epi*-26-deoxyactein, acetyl-shengmanol-xyloside, cimigenol-arabinoside, cimigenol-xyloside (cimicifugoside), 26-deoxyactein, 25-acetyl-cimigenol-arabinoside, (24S)-25-acetyl-cimigenol-xyloside, 25-O-methyl-cimigenol-arabinoside, and 25-O-methyl-cimigenol-xyloside as 23-*epi*-26-deoxyactein (C₃₇H₅₆O₁₀) in the portion of Powdered Black Cohosh taken:

$$\text{Result} = (V \times C)/(F \times W) \times 100$$

V = volume of the *Sample solution* (mL)*C* = concentration of the relevant analyte in the *Sample solution* (μg/mL)*F* = factor to convert mg to μg, 1000 μg/mg*W* = weight of Powdered Black Cohosh taken to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled amount of triterpene glycosides in the portion of Powdered Black Cohosh taken by adding all of the percentages calculated for the individual analytes.

Acceptance criteria: NLT 0.4% on the dried basis**CONTAMINANTS**• **HEAVY METALS** <231>: NMT 10 ppm• **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements• **MICROBIAL ENUMERATION TESTS** <2021>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 10⁵ cfu/g; the total combined molds and yeasts count does not exceed 10³ cfu/g; and the bile-tolerant Gram-negative bacteria count does not exceed 10³ cfu/g.**SPECIFIC TESTS**• **BOTANIC CHARACTERISTICS:** The material is a light to dark brown powder, is odorless or has a slight odor, and has an acid and bitter taste. It shows numerous starch granules with concentric striations, simple or compound. The individual granules are spherical or more or less polygonal and are between 3 and 15 μm in diameter, each with a somewhat central slit-shaped hilum. Vessels with bordered pits occur, as do lignified fibers. Reddish to brown fragments of suberized epidermis with more or less tubular cells occur.• **ARTICLES OF BOTANICAL ORIGIN**, *Alcohol-Soluble Extractives*, *Method 2* <561>: NLT 8.0%, using a mixture of alcohol and water (1:1) instead of alcohol• **LOSS ON DRYING** <731>: Dry a sample at 105° for 2 h: it loses NMT 12.0% of its weight.• **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 10.0%• **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* <561>: NMT 4.0%**ADDITIONAL REQUIREMENTS**• **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers, and protect from moisture.• **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant from which the article was derived. Dosage forms prepared with this article should bear the following statement: Discontinue use and consult a healthcare practitioner if you have a liver disorder or develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice.

- **USP REFERENCE STANDARDS** (11)
USP Actein RS
USP Powdered Black Cohosh Extract RS
USP 23-*epi*-26-Deoxyactein RS

Powdered Black Cohosh Extract

DEFINITION

Powdered Black Cohosh Extract is prepared from Black Cohosh by extraction with hydroalcoholic mixtures or other suitable solvents. It contains NLT 90.0% and NMT 110.0% of the labeled amount of triterpene glycosides, calculated as 23-*epi*-26-deoxyactein ($C_{37}H_{56}O_{10}$) on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: 100 mg/mL of USP Powdered Black Cohosh Extract RS in methanol

Standard solution B: 1 mg/mL each of USP Actein RS, USP 23-*epi*-26-Deoxyactein RS, and isoferulic acid in methanol

Sample solution: Shake a quantity of Powdered Extract, equivalent to 25 mg of triterpene glycosides, in 10 mL of methanol. Allow to stand for 15 min before use.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μ m (TLC plates)

Application volume: 10 μ L

Developing solvent system: Use the upper phase of a mixture of butyl alcohol, glacial acetic acid, and water (5:1:4).

Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85:10:5:0.5). [NOTE—Store in a refrigerator. The reagent is colorless; discard if color appears.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved about 15 cm, and dry the plate with the aid of a current of air.

Acceptance criteria: Examine the plate under UV light at 365 nm. The chromatogram of the *Sample solution* exhibits main zones similar in position and color to the main zones in the chromatogram of *Standard solution A*. In the upper third of the plate, the chromatogram of the *Sample solution* exhibits a blue fluorescent zone at the level of the zone due to isoferulic acid in the chromatogram of *Standard solution B*. Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight. The chromatogram of the *Sample solution* exhibits main zones similar in position and color to the main zones in the chromatogram of *Standard solution A*. The chromatogram of *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein. The chromatogram of the *Sample solution* exhibits several greenish-brown spots in the lower third of the plate and several violet zones above; two of these violet zones occur at R_f values similar to those due to actein and 23-*epi*-26-deoxyactein in the chromatogram of *Standard solution B*.

• B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: 0.5 mL of *Standard solution A* prepared in *Identification test A*, diluted with methanol to 2 mL

Standard solution B: 1.0 mL of *Standard solution B* prepared in *Identification test A*, diluted with methanol to 5 mL

Sample solution: Dilute 1 mL of the *Sample solution* prepared in *Identification test A* with methanol to 10 mL.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 μ m (HPTLC plates)

Application volume: 2 μ L as an 8-mm band

Developing solvent system: Toluene, ethyl formate, and formic acid (5:3:2)

Spray reagent: Proceed as directed for *Identification test A*.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved about two-thirds of the length of the plate, and dry the plate with the aid of a current of air.

Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits main zones similar in position and color to the main zones in the chromatogram of *Standard solution A*. The chromatogram of *Standard solution B* exhibits red-violet zones due to actein and 23-*epi*-26-deoxyactein at R_f values of about 0.5 and 0.4, respectively. The chromatogram of the *Sample solution* exhibits zones similar in color and R_f values to those due to actein and 23-*epi*-26-deoxyactein in the chromatogram of *Standard solution B*.

- **C.** The chromatogram of the *Sample solution* exhibits peaks for cimicracemoside A, 26-deoxycimicifugoside, (26S) actein, 23-*epi*-26-deoxyactein, cimigenol- α -arabinoside, and cimigenol-xyloside at retention times corresponding to those compounds in the chromatogram of the *Standard solution*, as obtained in the test for *Content of Triterpene Glycosides*. The ratio of the peak areas of cimigenol- α -arabinoside to cimigenol-xyloside is NLT 0.4 (distinction from *Cimicifuga foetida*).

COMPOSITION

• CONTENT OF TRITERPENE GLYCOSIDES

Standard solution: Dissolve a quantity of USP Powdered Black Cohosh Extract RS in methanol with shaking for 1 min, and dilute with methanol to obtain a solution having a known concentration of 30 mg/mL. Pass through a membrane filter of 0.45- μ m or finer pore size.

23-*epi*-26-Deoxyactein standard solutions: Dissolve USP 23-*epi*-26-Deoxyactein RS in methanol with shaking for 1 min. Dilute quantitatively, and stepwise if necessary, to obtain solutions having known concentrations of 500, 100, 50, 25, and 12.5 μ g/mL. Pass through a membrane filter of 0.45- μ m or finer pore size.

System suitability solution: 0.1 mg/mL each of USP Actein RS and USP 23-*epi*-26-Deoxyactein RS in methanol

Sample solution: Transfer a quantity of Powdered Extract, equivalent to 7.5 mg of triterpene glycosides, to a 10-mL volumetric flask, add 7 mL of methanol, and sonicate for 30 min. Dilute with methanol to volume. Centrifuge, or pass through a filter of 0.45- μ m or finer pore size.

Solution A: 0.05% trifluoroacetic acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Water (%)	Solution A (%)	Solution B (%)
0	0	80	20
8	0	80	20
15	68	0	32
55	36	0	64
65	5	0	95
70	5	0	95
85	0	80	20

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** Evaporative light-scattering[NOTE—The detector is set up according to the manufacturer's instruction in order to achieve a signal-to-noise ratio of NLT 10 for the 12.5-μg/mL 23-*epi*-26-Deoxyactein standard solution.]**Column:** 4.6-mm × 25-cm; 5-μm packing L1**Column temperature:** 35°**Flow rate:** 1.6 mL/min**Injection size:** 20 μL**System suitability****Samples:** *System suitability solution*, *Standard solution*, and 100-μg/mL 23-*epi*-26-Deoxyactein standard solution**Suitability requirements****Chromatogram similarity:** The chromatogram of the *Standard solution* is similar to the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS being used.**Resolution:** NLT 1.0 between the (26*S*)-actein and the 23-*epi*-26-deoxyactein peaks, *System suitability solution***Tailing factor:** NMT 2.0 for the 23-*epi*-26-deoxyactein peak, 100-μg/mL 23-*epi*-26-Deoxyactein standard solution**Relative standard deviation:** NMT 2.0% of the logarithm of the area response of the 23-*epi*-26-deoxyactein peak in repeated injections, 100-μg/mL 23-*epi*-26-Deoxyactein standard solution**Analysis****Samples:** *System suitability solution*, *Standard solution*, 23-*epi*-26-Deoxyactein standard solutions, and *Sample solution*Using the chromatogram of the *Standard solution* and the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS, identify the retention times of the peaks corresponding to the triterpene glycosides. The approximate relative retention times of the triterpene glycosides are provided in Table 2.**Table 2**

Name	Relative Retention Time
Cimicifugoside H-1	0.61
Cimiracemoside A	0.78
(26 <i>R</i>)-Actein	0.94
26-Deoxycimicifugoside	0.96
(26 <i>S</i>)-Actein	0.98
23- <i>epi</i> -26-Deoxyactein	1.00
Acetyl-shengmanol-xyloside	1.03
Cimigenol-arabinside	1.08
Cimigenol-xyloside (cimicifugoside)	1.13
26-Deoxyactein	1.22
25-Acetyl-cimigenol-arabinside	1.60
(24 <i>S</i>)-25-Acetyl-cimigenol-xyloside	1.64
25-O-Methyl-cimigenol-arabinside	1.90
25-O-Methyl-cimigenol-xyloside	1.93

Plot the logarithms of the peak area responses versus the logarithms of the concentrations, in μg/mL, of the 23-*epi*-26-Deoxyactein standard solutions, and determine the regression line using a least-squares analysis. The correlation coefficient for the regression line is NLT 0.995. From the graphs so obtained, determine the concentration, *C*, in μg/mL, of the relevant analyte in the *Sample solution*. Separately calculate the percentages of cimicifugoside H-1, cimiracemoside A, (26*R*)-actein, 26-deoxycimicifugoside, (26*S*)-actein, 23-*epi*-26-deoxyactein, acetyl-shengmanol-xyloside,

cimigenol-arabinside, cimigenol-xyloside (cimicifugoside), 26-deoxyactein, 25-acetyl-cimigenol-arabinside, (24*S*)-25-acetyl-cimigenol-xyloside, 25-O-methyl-cimigenol-arabinside, and 25-O-methyl-cimigenol-xyloside as 23-*epi*-26-deoxyactein (C₃₇H₅₆O₁₀) in the portion of Extract taken:

$$\text{Result} = (V \times C) / (F \times W) \times 100$$

V = volume of the *Sample solution* (mL)*C* = concentration of the relevant analyte in the *Sample solution* (μg/mL)*F* = factor to convert mg to μg, 1000 μg/mg*W* = weight of the Powdered Extract taken to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled amount of triterpene glycosides in the portion of Extract taken by adding all of the percentages calculated for individual analytes.

Acceptance criteria: 90.0%–110.0% on the dried basis**CONTAMINANTS**

- **HEAVY METALS** <231>: NMT 10 ppm
- **MICROBIAL ENUMERATION TESTS** <2021>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total bacterial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts count does not exceed 10³ cfu/g.
- **OTHER REQUIREMENTS:** It meets the requirements under *Botanical Extracts* <565>, *Pesticide Residues*.

SPECIFIC TESTS

- **LOSS ON DRYING** <731>: NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store in a cool place.
- **LABELING:** It meets the requirements under *Botanical Extracts* <565>. Label it to indicate the content of triterpene glycosides, in percentage, calculated as 23-*epi*-26-deoxyactein. Dosage forms prepared with this article should bear the following statement: "Discontinue use and consult a healthcare practitioner if you have a liver disorder or develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice."
- **USP REFERENCE STANDARDS** <11>
 - USP Actein RS
 - USP Powdered Black Cohosh Extract RS
 - USP 23-*epi*-26-Deoxyactein RS

Black Cohosh Tablets**DEFINITION**

Black Cohosh Tablets contain Powdered Black Cohosh Extract or Black Cohosh Fluidextract. Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of Powdered Extract or Fluidextract, represented by the content of triterpene glycosides, calculated as 23-*epi*-26-deoxyactein (C₃₇H₅₆O₁₀).

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** <201>

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μm (TLC plates)**Sample solution:** 10 mL of the *Sample solution* prepared for *Identification test B*. Evaporate to dryness, and redissolve in 1 mL of methanol.**Standard solution A:** 100 mg/mL of USP Powdered Black Cohosh Extract RS in methanol**Standard solution B:** 1 mg/mL each of USP Actein RS, USP 23-*epi*-26-Deoxyactein RS, and isoferulic acid in methanol

Application volume: 10 μ L

Developing solvent system: Use the upper phase of a mixture of butyl alcohol, glacial acetic acid, and water (5:1:4).

Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85:10:5:0.5)

[NOTE—Store in a refrigerator. The reagent is colorless; discard if color appears.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop until the solvent front has moved 15 cm, and dry the plate with the aid of a current of air. Examine the plate under UV light at a wavelength of 365 nm.

Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight.

Acceptance criteria: The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*. Examined under UV light, the *Sample solution* exhibits a blue fluorescent zone at the level of the zone due to isoferulic acid in *Standard solution B*, in the upper third of the plate. Examined after treatment with *Spray reagent*, the *Sample solution* exhibits several greenish-brown spots in the lower third of the plate and several violet zones above; two of these violet zones occur at R_f values similar to those due to actein and 23-*epi*-26-deoxyactein in *Standard solution B*.

• B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 μ m (HPTLC plates)

Sample solution: Transfer the equivalent of the labeled amount of Powdered Extract or Fluidextract, containing 25 mg of triterpene glycosides from a portion of powdered Tablets, to 25 mL of water; shake to disperse; and sonicate for 10 min. Add 75 mL of methanol, and sonicate for 10 min. Allow to stand for 15 min, and use the clear supernatant.

Standard solution A: Methanol and *Standard solution A* prepared in *Identification test A* (3:1)

Standard solution B: Methanol and *Standard solution B* prepared in *Identification test A* (4:1)

Application volume: 2 μ L as an 8-mm band

Developing solvent system: Toluene, ethyl formate, and formic acid (5:3:2)

Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85:10:5:0.5)

[NOTE—Store in a refrigerator. The reagent is colorless; discard if color appears.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop until the solvent front has moved two-thirds of the length of the plate, and dry the plate with the aid of a current of air. Spray the plate with *Spray reagent*, heat at 100° for 5 min, and examine in daylight.

Acceptance criteria: The *Sample solution* exhibits main zones similar in position and color to the main zones of *Standard solution A*, two of which are red-violet zones at R_f values of 0.5 and 0.4, similar in color and R_f values to those due to actein and 23-*epi*-26-deoxyactein in *Standard solution B*.

- **C.** The *Sample solution* exhibits peaks for cimracemoside A, 26-deoxycimicifugoside, (26S) actein, 23-*epi*-26-deoxyactein, cimigenol-arabinoside, and cimigenol-xyloside at retention times corresponding to those compounds in the *Standard solution*, as obtained in the test for *Content of Triterpene Glycosides*. The ratio of the peak areas of cimigenol-arabinoside to cimigenol-xyloside is NLT 0.4 (distinction from *Cimicifuga foetida*).

STRENGTH

• CONTENT OF TRITERPENE GLYCOSIDES

Solution A: Filtered and degassed 0.05% trifluoroacetic acid in water

Solution B: Filtered and degassed acetonitrile

Mobile phase: See the gradient table below.

Time (min)	Water (%)	Solution A (%)	Solution B (%)
0	0	80	20
8	0	80	20
15	68	0	32
55	36	0	64
65	5	0	95
70	5	0	95
85	0	80	20

System suitability solution: 0.1 mg/mL each of USP Actein RS and USP 23-*epi*-26-Deoxyactein RS in methanol

Standard solution: Dissolve a quantity of USP Powdered Black Cohosh Extract RS in methanol with shaking for 1 min, and dilute with methanol to obtain a solution having a known concentration of 30 mg/mL. Pass through a membrane filter having a 0.45- μ m or finer porosity.

23-*epi*-26-Deoxyactein standard solutions: Dissolve USP 23-*epi*-26-Deoxyactein RS in methanol with shaking for 1 min. Dilute quantitatively, and stepwise if necessary, to obtain solutions having known concentrations of 500, 100, 50, 25, and 12.5 μ g/mL. Pass through a membrane filter having a 0.45- μ m or finer porosity.

Sample solution: Weigh NLT 20 Tablets, and finely powder. Transfer a quantity of the powder, equivalent to 8 mg of triterpene glycosides, to a suitable polytef-capped centrifuge tube. Add 3 mL of water, shake to disperse, and sonicate for 10 min at 60°. Add 3 mL of methanol, and sonicate for 10 min. Centrifuge, and transfer the clear supernatant to a 10-mL volumetric flask. Wash the residue twice with 1.5 mL of a mixture of methanol and water (1:1), and transfer the washings to the volumetric flask. Dilute with a mixture of methanol and water (1:1) to volume, and pass through a membrane filter having a 0.45- μ m or finer porosity.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering

[NOTE—Detector is set up according to the manufacturer's instruction in order to achieve a signal-to-noise ratio of NLT 10 for the 12.5 μ g/mL 23-*epi*-26-Deoxyactein standard solution.]

Column: 4.6-mm \times 25-cm; 5- μ m packing L1

Column temperature: 35°

Flow rate: 1.6 mL/min

Injection size: 20 μ L

System suitability

Samples: *System suitability solution* and 100 μ g/mL of 23-*epi*-26-Deoxyactein standard solution

Suitability requirements

Chromatographic profile: The chromatogram of the *Standard solution* is similar to the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS.

Resolution: NLT 1.0 between the (26S)-actein and the 23-*epi*-26-deoxyactein peaks, *System suitability solution*

Tailing factor: NMT 2.0 for the 23-*epi*-26-deoxyactein peak, 100 μ g/mL 23-*epi*-26-Deoxyactein standard solution

Relative standard deviation: NMT 2.0% for the logarithm of the area responses for replicate injections, 100 μ g/mL 23-*epi*-26-Deoxyactein standard solution

Analysis

Samples: *System suitability solution*, *Standard solution*, 23-*epi*-26-Deoxyactein standard solutions, and *Sample solution*

Using the chromatogram of the *Standard solution* and the Reference Chromatogram provided with the lot of USP Powdered Black Cohosh Extract RS, identify the retention times of the peaks corresponding to the triterpene glycosides. The approximate relative retention times of the triterpene glycosides are provided in the following table.

Name	Relative Retention Time
Cimicifugoside H-1	0.61
Cimiracemoside A	0.78
(26 <i>R</i>)-Actein	0.94
26-Deoxycimicifugoside	0.96
(26 <i>S</i>)-Actein	0.98
23- <i>epi</i> -26-Deoxyactein	1.00
Acetyl-shengmanol-xyloside	1.03
Cimigenol-arabinoside	1.08
Cimigenol-xyloside (cimicifugoside)	1.13
26-Deoxyactein	1.22
25-Acetyl-cimigenol-arabinoside	1.60
(24 <i>S</i>)-25-Acetyl-cimigenol-xyloside	1.64
25-O-Methyl-cimigenol-arabinoside	1.90
25-O-Methyl-cimigenol-xyloside	1.93

Plot the logarithms of the peak area responses versus the logarithms of the concentrations, in $\mu\text{g/mL}$, of the 23-*epi*-26-Deoxyactein *standard solutions*, and determine the regression line using a least-squares analysis. The correlation coefficient for the regression line is NLT 0.995. From the graphs so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of the relevant analyte in the *Sample solution*.

Calculate the quantity, in mg, of triterpene glycosides in the portion of Tablets taken:

$$\text{Result} = C_T/100$$

C_T = sum of the concentrations C , in $\mu\text{g/mL}$, of all the relevant triterpene glycosides, calculated as 23-*epi*-26-deoxyactein

Calculate the percentage of the labeled amount of Extract in the portion of Tablets taken:

$$\text{Result} = (A_{WT}/W) \times (100/L_E) \times (100/L) \times C_{TT}$$

A_{WT} = average weight of Tablets

W = weight of sample

L_E = labeled content, as percentage, of triterpenes in the Extract used to prepare the Tablets

L = labeled amount of Extract per Tablet

C_{TT} = content, in mg, of triterpenes in the sample

Acceptance criteria: 90.0%–110.0% of the labeled amount of Powdered Extract or Fluidextract, represented by the content of triterpene glycosides

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** <2040>: Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS** <2021>: The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **MICROBIAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS** <2022>: Tablets meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. The label also indicates the amount, in mg/Tablet, of Powdered Extract or Fluidextract; the solvents used to prepare the Powdered Extract or Fluidextract; and the ratio of starting crude plant material to Powdered Extract or Fluidextract. Label it to indicate the content, in percentage, of triterpene glycosides as 23-*epi*-26-deoxyactein in the Powdered Extract or Fluidextract used to prepare the Tablets. The label bears the following statement: Discontinue use and consult a healthcare practitioner if you have a liver disorder or develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice.
- **USP REFERENCE STANDARDS** <11>
 - USP Actein RS
 - USP Powdered Black Cohosh Extract RS
 - USP 23-*epi*-26-Deoxyactein RS

Add the following:

▲ Black Pepper

DEFINITION

Black Pepper consists of the dried fully developed unripe fruits of *Piper nigrum* L. (Fam. Piperaceae). It contains NLT 2.5% of piperine, calculated on the dried basis.

IDENTIFICATION

- **A.** Black Pepper meets the requirements under *Specific Tests, Botanic Characteristics*.

• B. THIN-LAYER CHROMATOGRAPHY

Standard solution A: 0.9 mg/mL of USP Piperine RS in methanol

Standard solution B: 2.0 mg/mL of borneol in methanol

Standard solution C: 5 mg/mL of USP Powdered Black Pepper Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Sonicate for 10 min about 0.5 g of Black Pepper, finely powdered, in 5 mL of methanol, centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 μm (HPTLC plates)

Application volume: 15 μL of *Standard solution C*, 7 μL of the *Sample solution*, and 3 μL of the *Standard solution A* and *Standard solution B*, as bands

Developing solvent system: A mixture of hexanes and ethyl acetate (5:3)

Derivatization reagent: A mixture of 17 mL of ice-cooled methanol, 2 mL of acetic acid, 1 mL of sulfuric acid, and 0.1 mL of anisaldehyde mixed in this order.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands to a suitable high performance thin-layer chromatographic plate (see *Chromatography* <621>). Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop the chromatograms until the solvent front has moved up about 7 cm from the lower edge of the plate. Remove the plate from the chamber, dry, and examine under UV light at 254 nm. Derivatize with the *Derivatization reagent*, heat for 5 min at 100°, and examine under visible light.

Acceptance criteria: Under UV 254 nm, the chromatogram of the *Sample solution* exhibits an intense quenching band at R_f of about 0.15 corresponding in R_f to the piperine band in the chromatogram of *Standard solution A*, a quenching band at R_f of about 0.02, and three quenching bands located between R_f of about 0.3 and 0.5. Under visible light and after derivatization, the chromatogram of the *Sample solution* exhibits main bands similar in position and color to the main bands in the chromatogram of *Standard solution C*. These bands include a dark green band of the same color and R_f as the piperine band in *Standard solution A* (R_f of about 0.15), a weak violet band at R_f of about 0.47 below the position of the band due to borneol in *Standard solution B*, and a greenish band in the lower part of the chromatogram at R_f of about 0.07. Other minor bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. No blue bands are detected in the chromatogram of the *Sample solution* at R_f of about 0.10 and 0.58 (distinction from Long Pepper).

• C. HPLC

Analysis: Proceed as directed in the test for *Content of Piperine*.

Acceptance criteria: The chromatogram of the *Sample solution* obtained at 343 nm exhibits a major peak at the retention time corresponding to piperine. Identify other piperamide peaks in the *Sample solution* chromatogram by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used. The *Sample solution* chromatogram shows an additional peak corresponding to piperylene. The chromatogram of the *Sample solution* obtained at 270 nm does not exhibit a peak due to (2*E*, 4*E*)-*N*-isobutyldecadienamide at a relative retention time of 1.14 to the piperine peak (distinction from Long Pepper).

COMPOSITION

• CONTENT OF PIPERINE

Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, and add 0.5 mL of phosphoric acid. Dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile

Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

[NOTE—Proceed under subdued light or using low-actinic glassware.]

Standard solution A: 0.1 mg/mL of USP Piperine RS in methanol

Standard solution B: Sonicate a portion of USP Powdered Black Pepper Extract RS in methanol to obtain a solution having a concentration of about 0.5 mg/mL. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Transfer about 2.0 g of Black Pepper, finely powdered and accurately weighed, to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for about 20 min, allow to

settle, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, concentrate under vacuum, and adjust the volume to 100 mL using methanol. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 343 nm and 270 nm

Column: 4.6-mm × 25-cm; 5-μm, 100 Å packing L1

Flow rate: 1.5 mL/min

Injection volume: 20 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used.

Tailing factor: NMT 1.5 for the piperine peak, *Standard solution A*

Relative standard deviation: NMT 2.5% determined from the piperine peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

[NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used, identify the retention times of the peaks corresponding to piperine and piperylene in the *Sample solution* chromatogram.

Calculate the percentage of piperine in the portion of Black Pepper taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area for piperine from the *Sample solution* chromatogram at 343 nm

r_S = peak area for piperine from the *Standard solution A* chromatogram at 343 nm

C_S = concentration of piperine in *Standard solution A* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Black Pepper used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 2.5% on the dried basis

CONTAMINANTS

• **HEAVY METALS**, *Method III* (231): NMT 20 μg/g

• **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements

• **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10³ cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Fruit is an indehiscent, one-seeded berry, globose, ovoid to oblong, 3.5–6 mm in diameter, hard; surface is greyish-black to brownish-black, rough, with raised reticulate wrinkles, shows remains of sessile stigma on the tip and a basal scar showing point of attachment to the axis; characteristic aromatic odor; characteristic pungent taste; seed white and hollow.

Microscopic

Transverse cut: Circular in outline with corrugated margin; shows outer narrow brownish pericarp; a seed coat encircling the wide central whitish perisperm, hollow in center; a narrow vertically running channel connecting the hollow center of the fruit to a small endosperm adherent to the remains of the stigma; a small embryo is present in the endosperm; a conical short projection at the base showing point of attachment to the axis.

Transverse section: Shows a well differentiated pericarp, testa and perisperm; pericarp consists of a layer of epicarp, a wide mesocarp, and a layer of endocarp; the epicarp layer is covered with thick cuticle and containing a few stomata and small prisms of calcium oxalate; mesocarp composed of 2–3 layers of parenchyma cells showing groups of rectangular to circular lignified sclereides, a broad zone (12–15 layers) of tangentially running parenchyma cells containing starch grains and showing isolated oval oil cells, a broad zone (10–15 layers) of compactly arranged parenchyma cells smaller than those of the outer zone and showing groups of fibrovascular bundles, 1–2 layers of tangentially running oil cells, and 2–3 layers of thick-walled parenchyma cells; endocarp is composed of one layer of three-sided thickened pitted stone cells (beaker-shape cells); testa is composed of one layer of cells filled with brown pigments; perisperm is very wide, composed of cells full of starch grains, some aleurone grains, and oil cells.

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** (561): NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method I** (561): NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN, Water-Soluble Extractives, Method I** (561): NMT 9.0%
- **LOSS ON DRYING** (731)
Sample: 1.0 g of finely powdered Black Pepper
Analysis: Dry at 105° for 2 h.
Acceptance criteria: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561)
Sample: 1.0 g of finely powdered Black Pepper
Acceptance criteria: NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561)
Sample: 1.0 g of finely powdered Black Pepper
Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11)
USP Piperine RS
USP Powdered Black Pepper Extract RS▲ USP36

Add the following:

▲ Powdered Black Pepper

DEFINITION

Powdered Black Pepper is Black Pepper reduced to powder or very fine powder. It contains NLT 2.5% of piperine, calculated on the dried basis.

IDENTIFICATION

- **A.** Powdered Black Pepper meets the requirements under *Specific Tests, Botanic Characteristics*.

- **B. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 0.9 mg/mL of USP Piperine RS in methanol

Standard solution B: 2.0 mg/mL of borneol in methanol

Standard solution C: 5 mg/mL of USP Powdered Black Pepper Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Sonicate for 10 min about 0.5 g of Powdered Black Pepper in 5 mL of methanol, centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 15 µL of *Standard solution C*, 7 µL of the *Sample solution*, and 3 µL of *Standard solution A* and *Standard solution B*, as bands, 8 mm

Developing solvent system: A mixture of hexanes and ethyl acetate (5:3)

Derivatization reagent: A mixture of 17 mL of ice-cooled methanol, 2 mL of acetic acid, 1 mL of sulfuric acid, and 0.1 mL of anisaldehyde mixed in this order.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands to a suitable high performance thin-layer chromatographic plate (see *Chromatography* (621)). Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop the chromatograms until the solvent front has moved up about 7 cm from the lower edge of the plate. Remove the plate from the chamber, dry, and examine under UV light at 254 nm. Derivatize with the *Derivatization reagent*, heat for 5 min at 100°, and examine under visible light.

Acceptance criteria: Under UV 254 nm, the chromatogram of the *Sample solution* exhibits an intense quenching band at R_f of about 0.15 corresponding in R_f to the piperine band in the chromatogram of *Standard solution A*, a quenching band at R_f of about 0.02, and three quenching bands of similar intensity equally spaced located between R_f of about 0.3 and 0.5. Under visible light and after derivatization, the chromatogram of the *Sample solution* exhibits main bands similar in position and color to the main bands in the chromatogram of *Standard solution C*. These bands include a dark green band of the same color and R_f as the piperine band in *Standard solution A* (R_f of about 0.15), a weak violet band at R_f of about 0.47 below the position of the band due to borneol in *Standard solution B*, and a greenish band in the lower part of the chromatogram at R_f of about 0.07. Other minor bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. No blue bands are detected in the chromatogram of the *Sample solution* at R_f of about 0.10 and 0.58 (distinction from Long Pepper).

- **C. HPLC**

Analysis: Proceed as directed in the test for *Content of Piperine*.

Acceptance criteria: The chromatogram of the *Sample solution* obtained at 343 nm exhibits a major peak at the retention time corresponding to piperine. Identify other piperamide peaks in the *Sample solution* chromatogram by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used. The *Sample solution* chromatogram shows an additional peak corresponding to piper-ylene. The chromatogram of the *Sample solution* obtained at 270 nm does not exhibit a peak due to (2E, 4E)-N-isobutyldeca-dienamide at a relative retention time

of 1.14 to the piperine peak (distinction from Long Pepper).

COMPOSITION

• CONTENT OF PIPERINE

Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, and add 0.5 mL of phosphoric acid. Dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile

Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

[NOTE—Proceed under subdued light or using low-actinic glassware.]

Standard solution A: 0.1 mg/mL of USP Piperine RS in methanol

Standard solution B: Sonicate a portion of USP Powdered Black Pepper Extract RS in methanol to obtain a solution having a concentration of about 0.5 mg/mL. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Transfer about 2.0 g of Powdered Black Pepper, accurately weighed, to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for about 20 min, allow to settle, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, concentrate under vacuum, and adjust the volume to 100 mL using methanol. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 343 nm and 270 nm

Column: 4.6-mm × 25-cm; 5-μm, 100 Å packing L1

Flow rate: 1.5 mL/min

Injection volume: 20 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used.

Tailing factor: NMT 1.5 for the piperine peak, *Standard solution A*

Relative standard deviation: NMT 2.5% determined from the piperine peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

[NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used, identify the retention times of the peaks corresponding to piperine and piperidine in the *Sample solution* chromatogram.

Calculate the percentage of piperine in the portion of Powdered Black Pepper taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area for piperine from the *Sample solution* chromatogram at 343 nm

r_S = peak area for piperine from the *Standard solution A* chromatogram at 343 nm

C_S = concentration of piperine in *Standard solution A* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Powdered Black Pepper used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 2.5% on the dried basis

CONTAMINANTS

• **HEAVY METALS, Method III (231):** NMT 20 μg/g

• **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis (561):** Meets the requirements

• **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10³ cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Blackish-grey powder; characteristic aromatic odor; characteristic pungent taste.

Microscopic: Fragments of polygonal epicarp cells, some containing small prisms of calcium oxalate; parenchyma cells containing starch grains; oil cells; lignified sclereides; three-sided thickened pitted stone cells (beaker-shape cells) both in surface and side view; yellowish-brown polygonal cells of the testa; fragments of spiral vessels; parenchyma cells full of starch grains; aleurone grains; oil drops; and starch grains.

• **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method I (561):** NLT 10.0%

• **ARTICLES OF BOTANICAL ORIGIN, Water-Soluble Extractives, Method I (561):** NLT 9.0%

• LOSS ON DRYING (731)

Sample: 1.0 g of Powdered Black Pepper

Analysis: Dry at 105° for 2 h.

Acceptance criteria: NMT 12.0%

• ARTICLES OF BOTANICAL ORIGIN, Total Ash (561)

Sample: 1.0 g of Powdered Black Pepper

Acceptance criteria: NMT 5.0%

• ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash (561)

Sample: 1.0 g of Powdered Black Pepper

Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.

• **USP REFERENCE STANDARDS** (11)

USP Piperine RS

USP Powdered Black Pepper Extract RS▲ USP36

Add the following:

▲ **Powdered Black Pepper Extract**

DEFINITION

Powdered Black Pepper Extract is prepared from Black Pepper using suitable solvents such as ethyl acetate, methanol, or a mixture of these solvents. The ratio of plant material to extract is about 15:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of piperine. It may contain suitable added substances as carriers.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHY**

Standard solution A: 0.9 mg/mL of USP Piperine RS in methanol

Standard solution B: 2.0 mg/mL of borneol in methanol

Standard solution C: 5 mg/mL of USP Powdered Black Pepper Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Sonicate for about 10 min an amount of Powdered Extract, equivalent to about 10 mg of piperine, in 10 mL of methanol, centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 15 µL of *Standard solution C*, 7 µL of the *Sample solution*, and 3 µL of *Standard solution A* and *Standard solution B*, as bands, 8 mm

Developing solvent system: A mixture of hexanes and ethyl acetate (5:3)

Derivatization reagent: A mixture of 17 mL of ice-cooled methanol, 2 mL of acetic acid, 1 mL of sulfuric acid, and 0.1 mL of anisaldehyde mixed in this order.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Apply the *Samples* as bands to a suitable high performance thin-layer chromatographic plate (see *Chromatography* (621)). Use a saturated chamber, and condition the plate to a relative humidity of about 33% using a suitable device. Develop the chromatograms until the solvent front has moved up about 7 cm from the lower edge of the plate. Remove the plate from the chamber, dry, and examine under UV light at 254 nm. Derivatize with the *Derivatization reagent*, heat for 5 min at 100°, and examine under visible light.

Acceptance criteria: Under UV 254 nm, the chromatogram of the *Sample solution* exhibits an intense quenching band at R_f of about 0.15 corresponding in R_f to the piperine band in the chromatogram of *Standard solution A*, a quenching band at R_f of about 0.02, and three quenching bands of similar intensity equally spaced located between R_f of about 0.3 and 0.5. Under visible light and after derivatization, the chromatogram of the *Sample solution* exhibits main bands similar in position and color to the main bands in the chromatogram of *Standard solution C*. These bands include a dark green band of the same color and R_f as the piperine band in

Standard solution A (R_f of about 0.15), a weak violet band at R_f of about 0.47 below the position of the band due to borneol in *Standard solution B*, and a greenish band in the lower part of the chromatogram at R_f of about 0.07. Other minor bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. No blue bands are detected in the chromatogram of the *Sample solution* at R_f of about 0.10 and 0.58 (distinction from Long Pepper).

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Piperine*.

Acceptance criteria: The chromatogram of the *Sample solution* obtained at 343 nm exhibits a major peak at the retention time corresponding to piperine. Identify other piperamide peaks in the *Sample solution* chromatogram by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used. The *Sample solution* chromatogram shows an additional peak corresponding to piperidine. The chromatogram of the *Sample solution* obtained at 270 nm does not exhibit a peak due to (2*E*, 4*E*)-*N*-isobutyldecadienamide at a relative retention time of 1.14 to the piperine peak (distinction from Long Pepper).

COMPOSITION

• **CONTENT OF PIPERINE**

Solution A: Dissolve 0.14 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, and add 0.5 mL of phosphoric acid. Dilute with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	95	5
18	55	45
25	20	80
28	20	80
35	55	45
40	95	5
45	95	5

[NOTE—Proceed under subdued light or using low-actinic glassware.]

Standard solution A: 0.1 mg/mL of USP Piperine RS in methanol

Standard solution B: Sonicate a portion of USP Powdered Black Pepper Extract RS in methanol to obtain a solution having a concentration of about 0.5 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Transfer an accurately weighed amount of Powdered Extract, equivalent to about 25 mg of piperine, to a 25-mL volumetric flask, add 15 mL of methanol, and sonicate for 10 min. Cool to room temperature, dilute with methanol to volume, and mix. Dilute the obtained solution in methanol (1:10). Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 343 nm and 270 nm

Column: 4.6-mm × 25-cm; 5-μm, 100 Å packing L1

Flow rate: 1.5 mL/min

Injection volume: 20 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used.

Tailing factor: NMT 1.5 for the piperine peak, *Standard solution A*

Relative standard deviation: NMT 2.5% determined from the piperine peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

[NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 hours at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Black Pepper Extract RS being used, identify the retention time of the peaks corresponding to piperine and piperidine in the *Sample solution* chromatogram.

Calculate the percentage of piperine in the portion of Powdered Extract taken:

$$P = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for piperine from the *Sample solution* chromatogram at 343 nm

r_S = peak area for piperine from the *Standard solution A* chromatogram at 343 nm

C_S = concentration of piperine in *Standard solution A* (mg/mL)

C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of piperine in the portion of Powdered Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = content of piperine as determined above (%)

L = labeled amount of piperine (%)

Acceptance criteria: 90%–110% on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method III* (231): NMT 20 μg/g
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts count does not exceed 10³ cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• LOSS ON DRYING (731)

Sample: 1.0 g of Powdered Extract

Analysis: Dry at 105° for 2 h.

Acceptance criteria: NMT 7.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from

which the article was derived. It meets other labeling requirements in *Botanical Extracts* (565).

• USP REFERENCE STANDARDS (11)

USP Piperine RS

USP Powdered Black Pepper Extract RS▲ USP36

Boswellia serrata

DEFINITION

Boswellia serrata is the oleogum resin obtained by incision or produced by spontaneous exudation from the stem and branches of *Boswellia serrata* Roxb. (Fam. Burseraceae). It contains NLT 1.0% of the keto derivatives of β-boswellic acid, calculated on the dried basis as the sum of 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)

Standard solution: Treat a quantity of USP *Boswellia serrata* Extract RS with gentle heating in methanol to obtain a solution having a known concentration of 30 mg/mL, cool, centrifuge, and use the supernatant.

Sample solution: Use the *Sample solution*, prepared as directed in the test below for *Content of Keto-Derivatives of β-Boswellic Acids*, and concentrate to 10% of the volume.

Adsorbent: 0.25-mm layer of chromatographic silica gel

Developing solvent system: A mixture of hexane and ethyl acetate (6:4)

Dipping reagent: Prepare a solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh immediately before use.]

Application volume: 10 μL

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)). Use a saturated chamber. Develop until the solvent front has moved up about 90% of the plate. Remove, dry, and examine under UV light at 254 nm. Dip in the *Dipping reagent*, heat for 5–10 min at 100°, and examine under visible light.

Acceptance criteria: Under UV light at 254 nm, the *Sample solution* exhibits two main zones due to 11-keto-β-boswellic acid and 3-acetyl-11-keto-β-boswellic acid at R_f values of about 0.30 and 0.36, respectively, corresponding to zones from the *Standard solution*. Under visible light, the *Sample solution* exhibits two additional zones due to β-boswellic acid and 3-acetyl-β-boswellic acid at R_f values of about 0.49 and 0.58, respectively, corresponding to zones from the *Standard solution*. Other, less intense zones are observed for the *Sample solution* and the *Standard solution*.

- **B.** The 210-nm chromatogram of the *Sample solution*, in the test for *Content of Keto-Derivatives of β-Boswellic Acids*, exhibits peaks for 11-keto-β-boswellic acid, 3-acetyl-11-keto-β-boswellic acid, β-boswellic acid, and 3-acetyl-β-boswellic acid at retention times that correspond to those in the 210-nm chromatogram of *Standard solution B* and the 210-nm Reference Chromatogram provided with the USP *Boswellia serrata* Extract RS.

COMPOSITION

• CONTENT OF KETO-DERIVATIVES OF β-BOSWELLIC ACIDS

Standard solution A: Dissolve a quantity of USP 3-Acetyl-11-keto-β-Boswellic Acid RS in methanol to obtain a solution having a known concentration of 0.1 mg/mL.

Standard solution B: Treat a quantity of USP *Boswellia serrata* Extract RS with gentle heating in methanol to obtain a solution having a known concentration of

10 mg/mL. Before injection, pass through a filter of 0.45- μ m pore size.

Sample solution: Transfer about 2.0 g of crushed *Boswellia serrata* to a round-bottom flask, and reflux in 50 mL of methanol in a water bath for 15 min, stirring with a magnetic stirrer. Repeat till the last extract turns colorless. Evaporate the combined extracts to about 50 mL, transfer to a 100-mL volumetric flask, and dilute with methanol to volume. Before injection, pass through a filter of 0.45- μ m pore size, and discard the first few mL of the filtrate.

Mobile phase: Prepare a filtered and degassed mixture of acetonitrile, water, and glacial acetic acid (900:100:0.1). Make adjustments if necessary.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm \times 25-cm; packing L1

Flow rate: See the gradient table below.

Time (min)	Flow Rate (mL/min)
0	1
5	1.5
10	2
30	2
32	1
45	1

Injection size: 20 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*
[NOTE—The relative retention times for 11-keto- β -boswellic acid and 3-acetyl-11-keto- β -boswellic acid are about 1.0 and 1.4, respectively.]

Suitability requirements: The chromatogram of *Standard solution B* is similar to the 254-nm Reference Chromatogram provided with the USP *Boswellia serrata* Extract RS.

Relative standard deviation: NMT 2.0% of the 3-acetyl-11-keto- β -boswellic acid peak response for replicate injections, *Standard solution A*

Tailing factor: NMT 1.5, 11-keto- β -boswellic acid peak, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution B* and the 254-nm Reference Chromatogram provided with the lot of USP *Boswellia serrata* Extract RS, identify the retention times of the peaks of 11-keto- β -boswellic acid and 3-acetyl-11-keto- β -boswellic acid in the *Sample solution*.

Separately calculate the percentages of the two analytes in the portion of *Boswellia serrata* taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times 10F$$

r_U = peak area of each analyte from the *Sample solution*

r_S = peak area of 3-acetyl-11-keto- β -boswellic acid from *Standard solution A*

C_S = concentration of USP 3-Acetyl-11-keto- β -Boswellic Acid RS in *Standard solution A* (mg/mL)

W = weight of *Boswellia serrata* taken to prepare the *Sample solution* (g)

F = conversion factor for each analyte (0.93 for 11-keto- β -boswellic acid and 1.0 for 3-acetyl-11-keto- β -boswellic acid)

Acceptance criteria: Add the percentages calculated for 11-keto- β -boswellic acid and 3-acetyl-11-keto- β -boswellic acid: NLT 1.0% on the dried basis.

IMPURITIES

Inorganic Impurities

• **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** <561>: NMT 0.5%

• **HEAVY METALS, Method II** <231>: NMT 20 ppm

Organic Impurities

• **PROCEDURE 1: ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** <561>: NMT 2.0%

• **PROCEDURE 2: ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** <561>: Meets the requirements

SPECIFIC TESTS

BOTANIC CHARACTERISTICS

Macroscopic: It occurs as small ovoid tears, sometimes forming agglomerated masses up to 5 cm long and 2 cm thick; whitish to golden yellow; fracture is brittle, and fractured surface is waxy and translucent; characteristic aromatic odor; aromatic, slightly mucilaginous taste.

• **LOSS ON DRYING** <731>: Dry 1.0 g of finely powdered *Boswellia serrata* at 105° for 2 h: it loses NMT 12.0% of its weight.

• **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 2.0%, determined on 2.0 g of finely powdered *Boswellia serrata*.

• **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method 2** <561>: NLT 56%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.

• **LABELING:** The label states the Latin binomial of the species of *Boswellia* from which the oleogum resin was obtained.

• **USP REFERENCE STANDARDS** <11>
USP 3-Acetyl-11-keto- β -Boswellic Acid RS
USP *Boswellia serrata* Extract RS

Boswellia serrata Extract

DEFINITION

Boswellia serrata Extract is prepared from pulverized *Boswellia serrata*, using suitable solvents such as isopropanol, alcohol, methanol, hexanes, or mixtures of these solvents. The ratio of starting plant material to Extract is approximately 6:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of Extract, calculated, on the dried basis, as the sum of 11-keto- β -boswellic acid and 3-acetyl-11-keto- β -boswellic acid; it may contain suitable added substances.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** <201>

Standard solution: Treat a quantity of USP *Boswellia serrata* Extract RS with gentle heating in methanol to obtain a solution having a known concentration of 30 mg/mL, cool, centrifuge, and use the supernatant.

Sample solution: Treat a quantity of Extract with gentle heating in methanol to obtain a solution having a known concentration of 30 mg/mL, cool, centrifuge, and use the supernatant.

Adsorbent: 0.25-mm layer of chromatographic silica gel

Developing solvent system: A mixture of hexane and ethyl acetate (6:4)

Dipping reagent: Prepare a solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh immediately before use.]

Application volume: 10 μ L

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate (see *Chromatography* <621>). Use a saturated chamber. Develop until the solvent front has moved up about 90% of the plate. Remove, dry, and examine under UV light at 254 nm. Dip in the *Dipping reagent*, heat for 5–10 min at 100°, and examine under visible light.

Acceptance criteria: Under UV light at 254 nm, the *Sample solution* exhibits two main zones due to 11-keto- β -boswellic acid and 3-acetyl-11-keto- β -boswellic acid at R_f values of about 0.30 and 0.36, respectively, corresponding to zones from the *Standard solution*. Under visible light, the *Sample solution* exhibits two additional zones due to β -boswellic acid and 3-acetyl- β -boswellic acid at R_f values of about 0.49 and 0.58, respectively, corresponding to zones from the *Standard solution*. Other, less intense zones are observed for the *Sample solution* and the *Standard solution*.

- **B.** The 210-nm chromatogram of the *Sample solution*, in the test for *Content of Keto-Derivatives of β -Boswellic Acids*, exhibits peaks for 11-keto- β -boswellic acid, 3-acetyl-11-keto- β -boswellic acid, β -boswellic acid, and 3-acetyl- β -boswellic acid at retention times that correspond to those in the 210-nm chromatogram of *Standard solution B* and the 210-nm Reference Chromatogram provided with the USP *Boswellia serrata* Extract RS.

COMPOSITION

• CONTENT OF KETO-DERIVATIVES OF β -BOSWELIC ACIDS

Standard solution A: Dissolve a quantity of USP 3-Acetyl-11-keto- β -Boswellic Acid RS in methanol to obtain a solution having a known concentration of 0.1 mg/mL.

Standard solution B: Treat a quantity of USP *Boswellia serrata* Extract RS with gentle heating in methanol to obtain a solution having a known concentration of 10 mg/mL. Before injection, pass through a filter of 0.45- μ m pore size.

Sample solution: Treat a quantity of Extract with gentle heating in methanol to obtain a solution having a known concentration of 10 mg/mL. Before injection, pass through a filter of 0.45- μ m pore size, and discard the first few mL of the filtrate.

Mobile phase: Prepare a filtered and degassed mixture of acetonitrile, water, and glacial acetic acid (900:100:0.1). Make adjustments if necessary.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm \times 25-cm; packing L1

Flow rate: See the gradient table below.

Time (min)	Flow Rate (mL/min)
0	1
5	1.5
10	2
30	2
32	1
45	1

Injection size: 20 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*
[NOTE—The relative retention times for 11-keto- β -boswellic acid and 3-acetyl-11-keto- β -boswellic acid are about 1.0 and 1.4, respectively.]

Suitability requirements: The chromatogram of *Standard solution B* is similar to the 254-nm Reference Chromatogram provided with the USP *Boswellia serrata* Extract RS.

Tailing factor: NMT 1.5, 11-keto- β -boswellic acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0% of the 3-acetyl-11-keto- β -boswellic acid peak response for replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution B* and the 254-nm Reference Chromatogram provided with the lot of USP *Boswellia serrata* Extract RS, identify the retention times of the peaks of 11-keto- β -boswellic acid and 3-acetyl-11-keto- β -boswellic acid in the *Sample solution* chromatogram.

Separately calculate the percentages of 11-keto- β -boswellic acid and 3-acetyl-11-keto- β -boswellic acid in the portion of Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/V/W) \times 100F$$

r_U = peak area of each analyte from the *Sample solution*

r_S = peak area of 3-acetyl-11-keto- β -boswellic acid from *Standard solution A*

C_S = concentration of USP 3-Acetyl-11-keto- β -Boswellic Acid RS in *Standard solution A* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Extract taken to prepare the *Sample solution* (mg)

F = conversion factor for each analyte (0.93 for 11-keto- β -boswellic acid and 1.0 for 3-acetyl-11-keto- β -boswellic acid)

Acceptance criteria: Add the percentages of the two analytes. It contains NLT 90.0% and NMT 110.0% of the labeled amount of Extract, calculated on the dried basis, as the sum of 11-keto- β -boswellic acid and 3-acetyl-11-keto- β -boswellic acid.

IMPURITIES

Inorganic Impurities

- **HEAVY METALS**, *Method II* <231>: NMT 20 ppm

Organic Impurities

- **PROCEDURE: ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements

SPECIFIC TESTS

- **LOSS ON DRYING** <731>: Dry 1.0 g of Extract at 105° for 2 h: it loses NMT 5.0% of its weight.
- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. It meets other labeling requirements under *Botanical Extracts* <565>.
- **USP REFERENCE STANDARDS** <11>
USP 3-Acetyl-11-keto- β -Boswellic Acid RS
USP *Boswellia serrata* Extract RS

Calcium Ascorbate—see *Calcium Ascorbate General Monographs*

Calcium Carbonate—see *Calcium Carbonate General Monographs*

Calcium Carbonate Oral Suspension—see *Calcium Carbonate Oral Suspension General Monographs*

Calcium Carbonate Tablets—see *Calcium Carbonate Tablets General Monographs*

Calcium Citrate—see *Calcium Citrate General Monographs*

Calcium Citrate Tablets

DEFINITION

Calcium Citrate Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of calcium (Ca).

IDENTIFICATION

- A.** The *Sample solution* from the test for *Strength* produces line emissions or absorptions at the characteristic wavelengths for calcium.
- B. IDENTIFICATION TESTS—GENERAL.** *Calcium* (191) and *Citrate* (191)

Analysis: Grind a Tablet to a fine powder in a mortar. Transfer the powder to a centrifuge tube, add 2–5 mL of water, sonicate for 1 min, shake, and centrifuge.

Acceptance criteria: The supernatant meets the requirements of the tests.

STRENGTH

[NOTE—A standard stock solution is commercially available at different calcium concentrations. Necessary volumetric adjustment can be made in the *Standard solution*. Concentrations of the *Standard solution* and the *Sample solution* may be modified to fit the linear or working range of the instrument.]

- CONTENT OF CALCIUM, Procedure 1**

Standard stock solution: Weigh about 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, and dissolve in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 100 mL to obtain a solution having a known concentration of about 4000 µg/mL of calcium.

Standard solution: To a 200-mL volumetric flask add 100 mL of water and 4 mL of nitric acid, and mix thoroughly. Pipet 25.0 mL of the *Standard stock solution* into the volumetric flask, and dilute with water to volume to obtain a solution having a known concentration of about 500 µg/mL of calcium.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a weighed portion of the powdered Tablets, equivalent to about 0.1 g of calcium, to a 50-mL flask. Add 4 mL of nitric acid, and heat the solution to boil gently, during which fuming evolves. Boil the solution for an additional 30 min with constant swirling, during which no fuming should be observed.

Cool the solution to room temperature, quantitatively transfer all of the solution to a 200-mL volumetric flask, dilute with water to volume, mix, and filter.

Instrumental conditions

(See *Plasma Spectrochemistry* (730).)

Mode: ICP-AES

Analytical wavelength: 317.93 nm. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. A typical setting includes radio frequency (RF) power of about 1300 watts, argon torch flow of about 15 L/min, argon auxiliary flow of about 0.2 L/min, and a nebulizer flow rate of about 0.8 L/min.]

Analysis: Determine the emission of the *Standard solution*, the *Sample solution*, and a 2% nitric acid solution as the blank at the wavelength indicated above.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of calcium from the *Sample solution*

r_S = peak response of calcium from the *Standard solution*

C_S = concentration of calcium in the *Standard solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0%

- CONTENT OF CALCIUM, Procedure 2**

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL to obtain a concentration of 400 µg/mL of calcium.

Standard stock solution: 100 µg/mL of calcium from *Calcium standard solution* in 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Sample solution: [NOTE—Finely powder NLT 20 Tablets.]

Transfer an equivalent to 5 Tablets from powdered Tablets to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively, with 0.125 N hydrochloric acid to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Calcium emission line at 422.7 nm

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide-acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and the *Sample solution*. Determine the absorbances of the solutions, using the *Blank*. From a linear regression equation, calculated using the absorbance of the *Standard solutions* versus concentrations, determine the concentration, *C*, in µg/mL of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of calcium in the *Sample solution*

C_U = nominal concentration of calcium in the *Sample solution*

Acceptance criteria: 90.0%–110.0%

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 1000 cfu/g. The total combined yeasts and molds count does not exceed 100 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meet the requirements of the test for absence of *Escherichia coli*

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** (2040): Meet the requirements for *Disintegration*, 15 min
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** (2091): Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** The label states the quantity of calcium in terms of mg/Tablet.

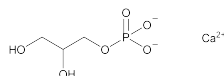
Calcium Glubionate Syrup—see *Calcium Glubionate Syrup General Monographs*

Calcium Gluceptate—see *Calcium Gluceptate General Monographs*

Calcium Gluconate—see *Calcium Gluconate General Monographs*

Calcium Gluconate Tablets—see *Calcium Gluconate Tablets General Monographs*

Calcium Glycerophosphate



C₃H₇CaO₆P 210.14
1,2,3-Propanetriol, mono(dihydrogen phosphate) calcium salt (1:1);
Calcium glycerophosphate [27214-00-2].

DEFINITION

Calcium Glycerophosphate is a mixture, in variable proportions, of calcium (RS)-2,3-dihydroxypropyl phosphate and calcium 2-hydroxy-1-(hydroxymethyl)ethyl phosphate, which may be hydrated. Calcium Glycerophosphate contains NLT 18.6% and NMT 19.4% of calcium (Ca), calculated on the dried basis.

IDENTIFICATION

- **A.**
Analysis: Ignite 0.1 g in a crucible. Take up the residue with 5 mL of nitric acid, heat on a water bath for 1 min, and filter. Mix 1 mL of the filtrate with 2 mL of ammonium molybdate TS.
Acceptance criteria: A yellow color develops.
- **B.**
Analysis: Dissolve 20 mg of the substance being examined in 5 mL of 5 M acetic acid, and add 0.5 mL of potassium ferrocyanide solution (53 mg/mL). The resulting solution remains clear. To the clear solution, add 50 mg of ammonium chloride.
Acceptance criteria: A white crystalline precipitate is produced.

ASSAY

- **PROCEDURE**
Sample: 200 mg
Titrimetric system
(See *Titrimetry* (541).)
Mode: Direct titration
Titrant: 0.1 M edetate disodium VS
Endpoint detection: Colorimetric
Blank: 300 mL of water. Add 6 mL of 10 M sodium hydroxide and 15 mg of calconcarboxylic acid tritrate.
Analysis: Dissolve the *Sample* in 300 mL of water, add 6 mL of 10 M sodium hydroxide and 15 mg of calconcarboxylic acid tritrate. Titrate with *Titrant* until the solution is a distinct blue color.
Calculate the percentage of calcium (Ca) in the portion of Calcium Glycerophosphate taken:

$$\text{Result} = [(V - B) \times M \times F \times 100]/W$$

V = *Sample* titrant volume (mL)
B = *Blank* titrant volume (mL)
M = titrant molarity (mM/mL)
F = equivalency factor, 40.08 mg/mM
W = weight of the *Sample* (mg)

Acceptance criteria: 18.6%–19.4% on the dried basis

IMPURITIES

- **LEAD** (251): NMT 4 ppm
- **IRON** (241)
Standard solution: Dilute 1 volume of *Standard Iron Solution*, prepared as directed in the chapter, with water to 10 volumes (1 µg/mL).
Analysis: Dissolve 0.20 g in 10 mL of water. Add 2 mL of a 20% (w/v) solution of citric acid, 0.1 mL of thioglycolic acid, and mix. Make alkaline with 10 M ammonia, dilute with water to 20 mL, and allow to stand for 5 min. Any pink color produced is not more intense than that obtained by treating 4 mL of the *Standard solution* in the same manner.
Acceptance criteria: NMT 20 ppm
- **HEAVY METALS, Method I** (231): NMT 20 ppm
- **LIMIT OF CHLORIDE**
Standard solution: 8.24 µg/mL of sodium chloride in water
Sample solution: Dissolve 125 mg in a 10-mL mixture of 5 M acetic acid and water (2:8), and dilute with water to 15 mL.
Analysis: To the *Sample solution* add 1 mL of 2 M nitric acid, then add 1 mL of a silver nitrate solution (17 mg/mL), and allow to stand for 5 min protected from light. To 10 mL of the *Standard solution* add 5 mL

of water, 1 mL of 2 M nitric acid, and 1 mL of silver nitrate solution (17 mg/mL), and allow to stand for 5 min protected from light. When viewed against a dark background, the *Sample solution* is not more turbid than the *Standard solution*.

Acceptance criteria: NMT 0.04%

- **LIMIT OF SULFATE**

Standard solution: 36.2 µg/mL of potassium sulfate in water

Sample solution: Use the *Sample solution* prepared as directed in the test for *Appearance of Solution*.

Analysis: To 15 mL of the *Standard solution* add 0.5 mL of 5 M acetic acid and 1 mL of barium chloride solution (250 mg/mL). Repeat, using 15 mL of the *Sample solution*. Allow the solutions to stand for 5 min protected from light. When viewed against a dark background, the *Sample solution* is not more turbid than the *Standard solution*.

Acceptance criteria: NMT 0.2%

- **LIMIT OF ARSENIC**

Standard stock solution: In a 250-mL volumetric flask dissolve 330 mg of arsenic trioxide in 5 mL of 2 N sodium hydroxide, and dilute with water to volume. Transfer 1 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Standard solution: Transfer 1 mL of *Standard stock solution* into a 10-mL volumetric flask, and dilute with water to volume.

Tin(II) chloride solution: Heat 20 g of tin with 85 mL of hydrochloric acid until no more hydrogen is released. Allow to cool. Dilute 1 volume of this solution with 10 volumes of dilute hydrochloric acid (200 mg/mL of hydrochloric acid in water).

Sample solution: Transfer 330 mg of Calcium Glycerophosphate to a 25.0-mL volumetric flask, and dilute with water to volume.

Apparatus: Prepare a 100-mL side-arm conical flask containing a magnetic stirring bar. Attach to the conical flask a ground-glass stopper through which passes a glass tube 20-cm long, with an internal diameter of 5 mm. The lower end of the tube is inside the conical flask and has been drawn to a tip with an internal diameter of 1 mm. An orifice 3 mm in diameter is 15 mm from the tip and at least 3 mm below the lower surface of the stopper. The upper end of the tube has a flat ground surface at a right angle to the axis of the tube. A second glass tube of the same internal diameter and 30 mm long, with a similar flat ground surface, is placed in contact with the ground surface of the first tube and is held in position by a clamp and springs. Into the lower tube, insert 55 mg of loosely packed lead acetate cotton. Place a disk of mercuric bromide paper between the flat surfaces of the tubes.

Analysis: Before placing the tube assembly into the flask, transfer the *Sample solution* to the flask, and add 15.0 mL of hydrochloric acid, 0.1 mL of *Tin(II) chloride solution*, and 5 mL of potassium iodide TS. Allow to stand for 15 min, and add 5 g of activated zinc. Assemble the apparatus immediately, and immerse the flask in a water bath at a temperature such that a uniform evolution of gas is maintained. After not less than 2 h, examine the stain produced on the mercuric bromide paper. Perform the same procedure using 1.0 mL of the *Standard solution*. The stain produced on the mercuric bromide paper by the *Sample solution* is not more intense than that produced by the *Standard solution*.

Acceptance criteria: NMT 3 ppm

- **LIMIT OF PHOSPHATES**

Sulfomolybdic solution: Dissolve with heating 2.5 g of ammonium molybdate in 20 mL of water. Dilute 28 mL of sulfuric acid with 50 mL of water, and cool. Mix the two solutions, and dilute with water to 100 mL.

Tin(II) chloride solution: Prepare as directed in the test for *Limit of Arsenic*.

Standard stock solution: 14.3 µg/mL of monobasic potassium phosphate in water.

Standard solution: Transfer 1.0 mL of *Standard stock solution* to a 100-mL volumetric flask, and dilute with water to volume.

Sample solution: Transfer 2.5 mL of the *Sample solution* from the test for *Appearance of Solution* to a 100-mL volumetric flask, and dilute with water to volume.

Analysis: To 100 mL of the *Sample solution* add 4 mL of *Sulfomolybdic solution*, mix, and add 0.1 mL of *Tin(II) chloride solution*. To 100 mL of the *Standard solution*, add 4 mL of *Sulfomolybdic solution*, mix, and add 0.1 mL of *Tin(II) chloride solution*. Allow the preparations to stand for 10 min, then examine 20 mL of each preparation. Any color produced by the *Sample solution* is not more intense than that produced by the *Standard solution*.

Acceptance criteria: NMT 0.04%

- **CITRIC ACID**

Mercury(II) sulfate solution: 1 g of mercuric oxide in 20 mL of water and 4 mL of sulfuric acid

Analysis: Mix 5 g of Calcium Glycerophosphate with 20 mL of carbon dioxide-free water, and filter. To the filtrate add 0.15 mL of sulfuric acid, and filter. To the filtrate add 5 mL of *Mercury(II) sulfate solution*, and heat to boiling. Add 0.5 mL of 0.2 M potassium permanganate, and heat to boiling.

Acceptance criteria: No precipitate is formed.

- **GLYCEROL AND ALCOHOL-SOLUBLE SUBSTANCES**

Analysis: Mix 1 g with 25 mL of alcohol, and shake for 1 min. Filter, evaporate the filtrate to dryness on a water bath, and dry the residue at 70° for 1 h.

Acceptance criteria: The residue weighs NMT 5 mg (0.5%).

SPECIFIC TESTS

- **APPEARANCE OF SOLUTION**

Opalescent suspension: Dissolve 1 g of hydrazine sulfate in 100 mL of water and allow to stand for 4–6 h. Add 25 mL of this solution to 25 mL of a solution containing 100 mg/mL of methenamine in water, and allow to stand for 24 h.

Primary reference suspension: Dilute 15.0 mL of the *Opalescent suspension* with water to 1000 mL. [NOTE—This suspension is freshly prepared and may be stored for NMT 24 h.]

Reference suspension: *Primary reference suspension* and water (30:70). [NOTE—Shake before using.]

Sample solution: Dissolve 1.5 g at room temperature in 150 mL of carbon dioxide-free water.

Analysis: Compare the opalescence of equal volumes of the *Sample solution* and the *Reference suspension*.

Acceptance criteria: The *Sample solution* is not more opalescent than the *Reference suspension*.

- **ACIDITY OR ALKALINITY**

Analysis: Dissolve 1 g of Calcium Glycerophosphate in 100 mL of water. Add 0.1 mL of 1.0% (w/v) phenolphthalein solution.

Acceptance criteria: NMT 0.5 mL of 0.1 M sodium hydroxide or 0.1 M hydrochloric acid is required to change the color of the indicator.

- **LOSS ON DRYING (731):** Dry a sample at 150° for 4 h: it loses NMT 12.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.

Calcium Lactate—see Calcium Lactate General Monographs

Calcium Lactate Tablets—see *Calcium Lactate Tablets General Monographs*

Calcium Lactobionate—see *Calcium Lactobionate General Monographs*

Calcium Levulinate—see *Calcium Levulinate General Monographs*

Calcium Pantothenate—see *Calcium Pantothenate General Monographs*

Calcium Pantothenate Tablets—see *Calcium Pantothenate Tablets General Monographs*

Calcium Pantothenate, Racemic—see *Racemic Calcium Pantothenate General Monographs*

Anhydrous Dibasic Calcium—see *Anhydrous Dibasic Calcium Phosphate General Monographs*

Dibasic Calcium Phosphate Dihydrate—see *Dibasic Calcium Phosphate Dihydrate General Monographs*

Calcium Phosphate, Dibasic Tablets—see *Dibasic Calcium Phosphate Tablets General Monographs*

Calcium Phosphate, Tribasic—see *Tribasic Calcium Phosphate NF Monographs*

Calcium with Vitamin D Tablets

DEFINITION

Calcium with Vitamin D Tablets contain NLT 90.0% and NMT 125.0% of the labeled amount of calcium (Ca), derived from substances generally recognized as safe, and NLT 90.0% and NMT 165.0% of the labeled amount of Vitamin D, as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O). They contain no other vitamins or minerals for which nutritional value is claimed. They may contain other labeled added substances or additional ingredients in amounts that are unobjectionable.

STRENGTH

- **CALCIUM** [NOTE—A commercially available atomic absorption standard solution for calcium may be used where preparation of a *Calcium standard stock solution* is described in the following assay. Concentrations of the *Standard solutions* and the *Sample stock solution* may be modified to fit the linear or working range of the instrument.]
Lanthanum chloride solution: Dissolve 26.7 g of lanthanum chloride in 0.125 N hydrochloric acid to make 100 mL.
Calcium standard stock solution: Weigh 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, and dissolve in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL to obtain a solution having a concentration of 400 µg/mL of calcium.
Standard stock solution: 100 µg/mL of calcium from a volume of *Calcium standard stock solution* in 0.125 N hydrochloric acid
Standard solutions: Into separate 100-mL volumetric flasks, separately pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, dilute with water to volume, and obtain *Standard solutions* having concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.
Sample stock solution: Weigh and finely powder NLT 20 Tablets. Transfer the equivalent to 500 mg of calcium, in 25 mL of concentrated hydrochloric acid, and heat for 30 min on a steam bath. Cool, dilute with water to 1000 mL, and filter.
Sample solution: Quantitatively dilute a volume of *Sample stock solution* with 0.125 N hydrochloric acid to obtain a concentration of 100 µg/mL of calcium. Transfer 2.0 mL of this solution to a 100-mL volumetric flask, add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume.
Spectrometric conditions
 (See *Spectrophotometry and Light-Scattering* (851).)
Mode: Atomic absorption spectrophotometer
Lamp: Calcium hollow-cathode
Flame: Nitrous oxide–acetylene
Analytical wavelength: Calcium emission line, 422.7 nm
Blank: 1 mL of *Lanthanum chloride solution* per 100 mL of 0.125 N hydrochloric acid
Analysis
Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions, against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of calcium in the *Sample solution*.
 Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

 C = measured concentration of calcium in the *Sample solution* (µg/mL)
 C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)
Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)
- **CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D)**
 [NOTE—Use low-actinic glassware throughout this procedure.]
Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)
Standard solution: 2 µg/mL of USP Ergocalciferol RS or USP Cholecalciferol RS in *n*-hexane
System suitability solution: Heat a volume of *Standard solution* at 60° for 1 h to partially isomerize vitamin D

(ergocalciferol or cholecalciferol) to its corresponding precursor.

Sample solution: Weigh, and grind NLT 20 Tablets. Transfer the equivalent to 20 µg of cholecalciferol or ergocalciferol to a container having a polytetrafluoroethylene-lined screw cap. Add 8 mL of dimethyl sulfoxide and 12 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker with tubes in a water bath maintained at 60°. Centrifuge for 10 min, withdraw the hexane layer by means of a pipet, and transfer to an evaporation flask. Add 12 mL of *n*-hexane to the dimethyl sulfoxide layer, mix on a vortex mixer for 5 min, and again withdraw the hexane layer by means of a pipet, and add to the evaporation flask. Repeat this extraction with three additional 12-mL portions of *n*-hexane, adding the hexane extracts to the evaporation flask. Evaporate the combined hexane extracts in vacuum at room temperature to dryness. Dissolve in and dilute the residue in a volume of *n*-hexane to obtain a concentration of 2 µg/mL.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

- r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
 r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
 C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)
 C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)
 F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

PERFORMANCE TESTS

- DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** <2040>: Meet the requirements for *Dissolution* with respect to calcium

Medium: 0.1 N hydrochloric acid; 900 mL

Apparatus 2: 75 rpm

Time: 30 min

Analysis: Determine the amount of calcium (Ca) dissolved, using the procedure in *Calcium*, making any necessary volumetric adjustments.

Tolerances: NLT 75% of the labeled amount of Ca is dissolved.

- WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements

CONTAMINANTS

- MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 3000 cfu/g, and the total combined molds and yeasts count does not exceed 300 cfu/g.
- MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- LABELING:** The label states that the product is *Calcium with Vitamin D Tablets*. The label states also the quantities of calcium and Vitamin D in terms of metric units/Tablet, and the salt form of calcium and the chemical form of Vitamin D present in the Tablet.
- USP REFERENCE STANDARDS** <11>
 USP Cholecalciferol RS
 USP Ergocalciferol RS

Calcium and Vitamin D with Minerals Tablets

DEFINITION

Calcium and Vitamin D with Minerals Tablets contain Vitamin D as Ergocalciferol (Vitamin D₂) or Cholecalciferol (Vitamin D₃), Calcium, and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: copper, magnesium, manganese, and zinc. Tablets contain NLT 90.0% and NMT 165.0% of the labeled amount of vitamin D, as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O), and NLT 90.0% and NMT 125.0% of the labeled amounts of calcium (Ca), copper (Cu), magnesium (Mg), manganese (Mn), and zinc (Zn). They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

- CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D)**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Ergocalciferol RS or USP Cholecalciferol RS in *n*-hexane

System suitability solution: Heat a volume of *Standard solution* at 60° for 1 h to partially isomerize vitamin D (ergocalciferol or cholecalciferol) to its corresponding precursor.

Sample solution: Weigh NLT 20 Tablets, and grind the Tablets to a fine powder. Transfer the equivalent of 20 µg of cholecalciferol or ergocalciferol to a container having a polytetrafluoroethylene-lined screw cap. Add 8 mL of dimethyl sulfoxide and 12 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker with tubes in a water bath maintained at 60°. Centrifuge for 10 min, withdraw the hexane layer by means of a pipet, and transfer to an evaporation flask. Add 12 mL of *n*-hexane to the dimethyl sulfoxide layer, mix on a vortex mixer for 5 min, and again withdraw the hexane layer by means of a pipet, and add to the evaporation flask. Repeat this extraction with three additional 12-mL portions of *n*-hexane, adding the hexane extracts to the evaporation flask. Evaporate the combined hexane extracts in vacuum at room temperature to dryness. Dissolve in and dilute the residue in a volume of *n*-hexane to obtain a concentration of 2 µg/mL.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 5-μm packing L8

Flow rate: 1 mL/min

Injection size: 100 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the responses for the vitamin D peaks. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak height for cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak height for cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Ergocalciferol RS or USP Cholecalciferol RS in the *Standard solution* (μg/mL)

C_U = nominal concentration of ergocalciferol or cholecalciferol in the *Sample solution* (μg/mL)

F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• **CALCIUM, Method 1**

[NOTE—A commercially available atomic absorption standard solution for calcium may be used where preparation of a *Calcium standard stock solution* is described in the following section. Concentrations of the *Standard solutions* and the *Sample stock solution* may be modified to fit the linear or working range of the instrument.]

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard stock solution: 400 μg/mL of calcium

Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, and dissolve in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 μg/mL of calcium from *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain *Standard solutions* having concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 μg/mL of calcium.

Sample stock solution: Weigh and finely powder NLT 20 Tablets. Mix a portion of the powder equivalent to a nominal amount of 500 mg of calcium with 25 mL of concentrated hydrochloric acid, and heat for 30 min on a steam bath. Cool, dilute with water to 1000 mL, and filter.

Sample solution: Quantitatively dilute a volume of the *Sample stock solution* with 0.125 N hydrochloric acid to obtain a nominal concentration of 100 μg/mL of calcium. Transfer 2.0 mL of this solution to a 100-mL

volumetric flask, add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: Calcium emission line, 422.7 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution*/100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in μg/mL, of calcium, and draw the straight line best fitting the five plotted points.

From the graph, determine the concentration, in μg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (μg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of Calcium (Ca)

• **COPPER, Method 1**

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% (v/v) solution of nitric acid solution, and dilute with a 1% (v/v) solution of nitric acid to 1000 mL. This solution contains 1000 μg/mL of copper.

Standard stock solution: 100 μg/mL of copper from *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 μg/mL of copper.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer the equivalent of 5 mg of copper from powdered Tablets to a porcelain crucible. Heat for 6–12 h in a muffle furnace maintained at 550°, and cool. Add 15 mL of hydrochloric acid, and boil gently on a hot plate or a steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask, rinsing the crucible with portions of 6 N hydrochloric acid. Dilute the contents of the flask with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute the filtrate quantitatively with 0.125 N hydrochloric acid to obtain a concentration of 2 μg/mL of copper.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Copper hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Copper emission line, 324.7 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in μg/mL, of copper, and draw the straight line best fitting the five plotted points.

From the graph, determine the concentration, C , in μg/mL, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of Copper (Cu) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)

C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **MAGNESIUM, Method 1**

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Magnesium standard stock solution: Transfer 1.00 g of magnesium to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution having a known concentration of 1000 µg/mL.

Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.

Sample solution: Finely powder NLT 20 Tablets.

Transfer the equivalent of 200 mg of magnesium to a porcelain crucible. Heat for 6–12 h in a muffle furnace maintained at 550°, and cool. Add 15 mL of hydrochloric acid, and boil gently on a hot plate or a steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask, rinsing the crucible with portions of 6 N hydrochloric acid. Dilute the contents of the flask with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute the filtrate quantitatively with 0.125 N hydrochloric acid to obtain a concentration of 0.4 µg/mL of magnesium, adding 1 mL of *Lanthanum chloride solution*/100 mL of the final volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Magnesium emission line, 285.2 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution*/100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph, determine the concentration, C, in µg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)

C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE, Method 1**

Manganese standard stock solution: Transfer 1.00 g of manganese, weighed, to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of manganese.

Standard stock solution: 50 µg/mL of manganese from *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Sample solution: Finely powder NLT 20 Tablets.

Transfer the equivalent of 9 mg of manganese from powdered Tablets to a porcelain crucible. Heat for 6–12 h in a muffle furnace maintained at 550°, and cool. Add 15 mL of hydrochloric acid, and boil gently on a hot plate or a steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask, rinsing the crucible with portions of 6 N hydrochloric acid. Dilute the contents of the flask with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute the filtrate quantitatively with 0.125 N hydrochloric acid to obtain a concentration of 1 µg/mL of manganese.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Manganese emission line, 279.5 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in mg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)

C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese

• **ZINC, Method 1**

Zinc standard stock solution: 1000 µg/mL of zinc from zinc oxide dissolved in 5 M hydrochloric acid (3.89 mg/mL), and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from *Zinc standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer the equivalent of 40 mg of zinc from powdered Tablets to a porcelain crucible. Heat for 6–12 h in a muffle furnace maintained at 550°, and cool. Add 15 mL of hydrochloric acid, and boil gently on a hot plate or a steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask, rinsing the crucible with portions of 6 N hydrochloric acid. Dilute the contents of the flask with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute the filtrate quantitatively with 0.125 N hydrochloric acid to obtain a concentration of 2 µg/mL of zinc.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption spectrophotometry

Lamp: Zinc hollow-cathode

Flame: Air-acetylene

Analytical wavelength: Zinc emission line, 213.8 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph, determine the concentration, *C*, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• CALCIUM, COPPER, MAGNESIUM, MANGANESE, and ZINC, Method 2

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding one volume of *Stock aqua regia solution* to two volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% (v/v) nitric acid solution and 1000 mg/L of scandium in 5% (v/v) nitric acid solution with *Diluent* (1:1:198), and mix.

Standard stock solution (Ca, Cu, Mg, Mn, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% (v/v) nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% (v/v) nitric acid solution to obtain a solution having final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 500 mg/L of magnesium, 100 mg/L of manganese, and 250 mg/L of zinc.

Standard solutions: Prepare a mixture of *Standard stock solution* in *Diluent* to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion equal to the average Tablet weight to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments,

followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a 5-µm pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Spectrometric conditions

(See *Spectrochemistry* <730>.)

Mode: Inductively coupled plasma spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength.

[NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

[NOTE—Analyze the *System suitability solution* and obtain the response as directed for Analysis.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*. Calculate the percentage of the labeled amount for each mineral:

$$\text{Result} = C \times (V/W) \times F \times (W_T/L) \times 100$$

C = measured concentration of the relevant element in the *Sample solution* (mg/L)

V = volume of the *Sample solution* (L)

W = sample weight (mg)

F = dilution factor of the *Sample solution*

W_T = average Tablet weight (mg)

L = labeled amount of the relevant element (mg/Tablet)

Acceptance criteria: NLT 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), magnesium (Mg), manganese (Mn), and zinc (Zn).

PERFORMANCE TESTS

• DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS <2040>: Meet the requirements for *Dissolution* with respect to calcium

Medium: 0.1 N hydrochloric acid; 900 mL

Apparatus 2: 75 rpm

Time: 30 min

Analysis: Determine the amount of calcium (Ca) dissolved, using the procedure in the assay for *Calcium*, making any necessary volumetric adjustments.

Tolerances: NLT 75% of the labeled amount of Ca is dissolved.

• WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>: Meet the requirements

SPECIFIC TESTS

• MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS <2021>: The total aerobic microbial count does not exceed 3000 cfu/g, and the total combined molds and yeasts count does not exceed 300 cfu/g. Tablets also meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

- **ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS <2022>**: Meet the requirements of the tests for absence of *Salmonella species*, *Escherichia coli*, and *Staphylococcus aureus*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight, light-resistant containers.
- **LABELING**: The label states that the product is *Calcium and Vitamin D with Minerals Tablets*. The label also states the quantities of each mineral and vitamin D/dosage unit, the salt form of the mineral used as the source of each element present, and the chemical form of vitamin D present in the dosage unit.
- **USP REFERENCE STANDARDS <11>**
USP Cholecalciferol RS
USP Ergocalciferol RS

Cat's Claw

DEFINITION

Cat's Claw consists of the inner bark of the stems of *Uncaria tomentosa* (Willd.) DC. (*Rubiaceae*). It contains NLT 0.3% of pentacyclic oxindole alkaloids as isopteropodine, calculated on the dried basis, as the sum of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 100 mg of USP Powdered Cat's Claw Extract RS in 2 mL of methanol. Sonicate for 5 min, shaking occasionally, heat in a water bath at 60° for 15 min, cool, and centrifuge.

Sample solution: 5 g of powdered Cat's Claw in 10 mL of methanol. Sonicate for 5 min, shaking occasionally. Heat the mixture in a water bath at 60° for 15 min, cool, and filter.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 µm (TLC plates)

Application volume: 20 µL, as bands that are 1 cm in length

Developing solvent system: Ethyl acetate and hexane (95:5)

Spray reagent A: Dissolve 0.85 g of basic bismuth nitrate in 10 mL of glacial acetic acid and 40 mL of water by heating. Filter if necessary (Solution A). Dissolve 8 g of potassium iodide in 30 mL of water (Solution B). Mix *Solution A* and *Solution B* (1:1) to obtain a stock solution. Dilute 1 mL of the stock solution with 2 mL of glacial acetic acid and 10 mL of water. [NOTE—Use freshly mixed *Solution A* and *Solution B*.]

Spray reagent B: Use a 10% solution of sodium nitrite in water.

Analysis

Samples: *Standard solution* and *Sample solution*
Develop the chromatogram to a length of NLT 12 cm, and dry the plate in a current of air.

Acceptance criteria: Examine the plate under short UV light. The *Sample solution* chromatogram shows multiple zones that correspond in R_f values to those observed from the *Standard solution* chromatogram. Other zones of varying intensities may be observed in the *Sample solution*. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under daylight. The *Sample solution* chromatogram shows multiple orange-brown zones that correspond in color and R_f values to those observed in the *Standard solution* chromatogram. Other colored zones of varying intensities may be observed in the *Sample solution*.

- **B.** The *Sample solution* exhibits peaks for speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine,

and isopteropodine at retention times that correspond to those in *Standard solution A*, as obtained in the test for *Content of Pentacyclic Oxindole Alkaloids and Limit of Tetracyclic Oxindoles*.

COMPOSITION

• CONTENT OF PENTACYCLIC OXINDOLE ALKALOIDS AND LIMIT OF TETRACYCLIC OXINDOLES

Standard solution A: Dissolve an accurately weighed quantity of USP Powdered Cat's Claw Extract RS in methanol, shaking for 1 min. Dilute with methanol to obtain a solution having a known concentration of about 0.5 mg of the labeled amount of total oxindole alkaloids per mL. Pass through a filter of 0.45-µm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Isopteropodine RS in methanol. Pass through a nylon filter of 0.45-µm or finer pore size.

Sample solution: Accurately weigh approximately 750 mg of ground Cat's Claw, and place in a 10-mL centrifuge tube. Sonicate with 2.5 mL of methanol for 10 min. Centrifuge, and transfer this solution to a 10-mL volumetric flask. Repeat the extraction three additional times combining the extracts in the 10-mL volumetric flask, and dilute with methanol to volume. Transfer about 3 mL of the solution to a test tube containing 300 mg of polyamide powder, and shake for 1 min. Pass through a nylon filter of 0.45-µm or finer pore size, discarding the first part of the filtrate.

Solution A: Prepare a filtered and degassed 10 mM pH 7.0 phosphate buffer by mixing 6 mL of 1 N sodium hydroxide, 10 mL of 1 M monobasic potassium phosphate, and sufficient water to make 1000 mL. Adjust to a pH of 7.0 ± 0.1 by adding more of either solution.

Solution B: Acetonitrile

Solution C: Methanol and glacial acetic acid (99:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	65	35	0
17	65	35	0
25	50	50	0
30	50	50	0
31	0	0	100
36	0	0	100
39	65	35	0
49	65	35	0

Chromatographic system

(See *Chromatography <621>*, *System Suitability*.)

Mode: LC

Detector: UV 245 nm

Column: 4.6-mm × 10-cm; end-capped 3-µm packing L1

Flow rate: 0.75 mL/min

Injection size: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*
Suitability requirements

Chromatogram similarity: The chromatogram obtained using *Standard solution A* is similar to the Reference Chromatogram provided with the USP Powdered Cat's Claw Extract RS.

Tailing factor: NMT 2.0 for the isopteropodine peak, *Standard solution B*

Relative standard deviation: NMT 2.0% for the isopteropodine peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Measure the areas of the analyte peaks. Identify the retention times of the peaks corresponding to speciophylline, uncarine F, mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline, pteropodine, and isopteropodine by comparison of the chromatogram of *Standard solution A* with the Reference Chromatogram provided with the lot of the USP Powdered Cat's Claw Extract RS used.

Separately calculate the percentages of speciophylline, uncarine F, mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline, pteropodine, and isopteropodine, in the portion of Cat's Claw taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

r_u = peak response for each relevant alkaloid from the *Sample solution*

r_s = peak response for isopteropodine from *Standard solution B*

C_s = concentration of USP Iopteropodine RS in *Standard solution B* (mg/mL)

C_u = concentration of Cat's Claw in the *Sample solution* (mg/mL)

Calculate the content of pentacyclic oxindole alkaloids by adding the percentages of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine.

Calculate the content of tetracyclic oxindole alkaloids by adding the individual percentages of rhynchophylline and isorhynchophylline.

Acceptance criteria

Pentacyclic oxindole alkaloids: NLT 0.3%

Tetracyclic oxindole alkaloids: NMT 0.05%

CONTAMINANTS

- **HEAVY METALS**, *Method III* (231): NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 10^5 /g; the total combined molds and yeasts count does not exceed 10^3 /g; and the bile-tolerant Gram-negative bacteria do not exceed 10^3 /g.

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

[NOTE—The pharmacopeial article is constituted only by the stem inner bark of *U. tomentosa* (Willd.) DC. Descriptions of other parts of the plant are given to aid in the collection of the right species. Compliance should be determined using the entire monograph and not only the botanical description.]

Macroscopic: Cat's Claw is a woody vine with a main stem up to 20 cm in diameter and up to 30 m long. The branches are obtusely quadrangular and generally puberulous. Stipules in the buds are densely tomentose in the upper side (different from *U. guianensis* in which the stipules are glabrous) with the hairs, often with curved tips, meshed together and with the longer hairs of the leaf helping to connect the pair of stipules along the margins, but split when older (different from *U. guianensis* in which the stipules separate early in the bud development). Thorns are straight to sickle-shaped, not spirally twisted (different from *U. guianensis*), very pungent and woody, from 8 to 20 mm long and from 3 to 6 mm wide. When recently cut, the color of the inner bark can be whitish gray, yellowish brown, or dark red, with longitudinal fissures and persistent rhytidome. The internal part has a slightly dusty fibrous and laminar texture with a characteristic ferruginous dust and an extremely astringent taste. The terminal branches have a quadrangular section and yellowish green internal medulla.

Microscopic: The periderm with cork (phellem) is constituted by 6–8 rows of cells having walls evenly thickened, a compressed phellogen and a phellogen with 1–7 rows of sclereids. [NOTE—The periderm and phellogen should be absent in the pharmacopeial article.] The secondary cortex with concentric rings of fibers are separated by rings of parenchyma; rings of fibers are frequently interrupted by radial rows of parenchyma cells (predominately 1 cell broad) or narrow medullary rays (few cells broad), forming rectangular bundles of fibers in a regular network. In longitudinal view, the fibers appear with numerous conspicuous pits; calcium oxalate microcrystals (sand-like) are abundant in the parenchyma, but usually absent as large polyhedral crystals or in the form of styloids with bifurcated endings, the latter forms typically present in the parenchyma of *U. guianensis*; a brown substance is dispersed in parenchyma cells; starch is abundant, granules are solitary (circular in outline, up to 10 μ m in diameter) or compound (2–3 components up to 15 μ m in diameter).

- **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* (561): NMT 2.0%
- **LOSS ON DRYING** (731): Dry at 105° for 2 h: it loses NMT 7.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* (561): NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11)
USP Iopteropodine RS
USP Powdered Cat's Claw Extract RS

Powdered Cat's Claw

DEFINITION

Powdered Cat's Claw is Cat's Claw reduced to a powder or very fine powder. It contains NLT 0.3% of pentacyclic oxindole alkaloids as isopteropodine, calculated on the dried basis, as the sum of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 100 mg of USP Powdered Cat's Claw Extract RS in 2 mL of methanol. Sonicate for 5 min, shaking occasionally, heat in a water bath at 60° for 15 min, cool, and centrifuge.

Sample solution: 5 g of Powdered Cat's Claw in 10 mL of methanol. Sonicate for 5 min, shaking occasionally. Heat the mixture in a water bath at 60° for 15 min, cool, and filter.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μ m (TLC plates)

Application volume: 20 μ L, as bands that are 1 cm in length

Developing solvent system: Ethyl acetate and hexane (95:5)

Spray reagent A: Dissolve 0.85 g of basic bismuth nitrate in 10 mL of glacial acetic acid and 40 mL of water by heating. Filter if necessary (Solution A). Dissolve 8 g of potassium iodide in 30 mL of water (Solution B). Mix *Solution A* and *Solution B* (1:1) to obtain a stock solution. Dilute 1 mL of the stock solution with 2 mL of

glacial acetic acid and 10 mL of water. [NOTE—Use freshly mixed *Solution A* and *Solution B*.]

Spray reagent B: Use a 10% solution of sodium nitrite in water.

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatogram to a length of NLT 12 cm, and dry the plate in a current of air.

Acceptance criteria: Examine the plate under short UV light. The *Sample solution* chromatogram shows multiple zones that correspond in R_f values to those observed from the *Standard solution* chromatogram. Other zones of varying intensities may be observed in the *Sample solution*. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under daylight. The *Sample solution* chromatogram shows multiple orange-brown zones that correspond in color and R_f values to those observed in the *Standard solution* chromatogram. Other colored zones of varying intensities may be observed in the *Sample solution*.

- **B.** The *Sample solution* exhibits peaks for speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine at retention times that correspond to those in *Standard solution A*, as obtained in the test for *Content of Pentacyclic Oxindole Alkaloids and Limit of Tetracyclic Oxindoles*.

COMPOSITION

• CONTENT OF PENTACYCLIC OXINDOLE ALKALOIDS AND LIMIT OF TETRACYCLIC OXINDOLES

Standard solution A: Dissolve an accurately weighed quantity of USP Powdered Cat's Claw Extract RS in methanol, shaking for 1 min. Dilute with methanol to obtain a solution having a known concentration of about 0.5 mg of the labeled amount of total oxindole alkaloids per mL. Pass through a filter of 0.45- μ m or finer pore size.

Standard solution B: 0.1 mg/mL of USP Isopteropodine RS in methanol. Pass through a nylon filter of 0.45- μ m or finer pore size.

Sample solution: Accurately weigh approximately 750 mg of Powdered Cat's Claw, and place in a 10-mL centrifuge tube. Sonicate with 2.5 mL of methanol for 10 min. Centrifuge, and transfer this solution to a 10-mL volumetric flask. Repeat the extraction three additional times combining the extracts in the 10-mL volumetric flask, and dilute with methanol to volume. Transfer about 3 mL of the solution to a test tube containing 300 mg of polyamide powder, and shake for 1 min. Pass through a nylon filter of 0.45- μ m or finer pore size, discarding the first part of the filtrate.

Solution A: Prepare a filtered and degassed 10 mM pH 7.0 phosphate buffer by mixing 6 mL of 1 N sodium hydroxide, 10 mL of 1 M monobasic potassium phosphate, and sufficient water to make 1000 mL. Adjust to a pH of 7.0 ± 0.1 by adding more of either solution.

Solution B: Acetonitrile

Solution C: Methanol and glacial acetic acid (99:1)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	65	35	0
17	65	35	0
25	50	50	0
30	50	50	0
31	0	0	100
36	0	0	100
39	65	35	0
49	65	35	0

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 245 nm

Column: 4.6-mm \times 10-cm; end-capped 3- μ m packing L1

Flow rate: 0.75 mL/min

Injection size: 10 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained using *Standard solution A* is similar to the Reference Chromatogram provided with the USP Powdered Cat's Claw Extract RS.

Tailing factor: NMT 2.0 for the isopteropodine peak, *Standard solution B*

Relative standard deviation: NMT 2.0% for the isopteropodine peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Measure the areas of the analyte peaks. Identify the retention times of the peaks corresponding to speciophylline, uncarine F, mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline, pteropodine, and isopteropodine by comparison of the chromatogram of *Standard solution A* with the Reference Chromatogram provided with the lot of the USP Powdered Cat's Claw Extract RS used.

Separately calculate the percentages of speciophylline, uncarine F, mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline, pteropodine, and isopteropodine, as isopteropodine, in the portion of Powdered Cat's Claw taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for each relevant alkaloid from the *Sample solution*

r_S = peak response for isopteropodine from *Standard solution B*

C_S = concentration of USP Isopteropodine RS in *Standard solution B* (mg/mL)

C_U = concentration of Powdered Cat's Claw in the *Sample solution* (mg/mL)

Calculate the content of pentacyclic oxindole alkaloids by adding the percentages of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine.

Calculate the content of tetracyclic oxindole alkaloids by adding the individual percentages of rhynchophylline and isorhynchophylline.

Acceptance criteria

Pentacyclic oxindole alkaloids: NLT 0.3%

Tetracyclic oxindole alkaloids: NMT 0.05%

CONTAMINANTS

- **HEAVY METALS, Method III (231):** NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis (561):** Meets the requirements
- **MICROBIAL ENUMERATION TESTS (2021):** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 10^5 /g; the total combined molds and yeasts count does not exceed 10^3 /g; and the bile-tolerant Gram-negative bacteria do not exceed 10^3 /g.

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Presence of fragments of cork and suberized cells, with cell walls evenly thickened; presence of phelloderm sclereids; fragments of fibers crossed by vascular rays are darkened due to the presence of sand-like calcium oxalate microcrystals;

solitary or two- to three-compound starch grains up to 15 μm in diameter; absence of styloids, typically present in *U. guianensis*

- **LOSS ON DRYING** (731): Dry at 105° for 2 h: it loses NMT 7.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, Total Ash (561): NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN**, Acid-Insoluble Ash (561): NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** (11)
 - USP Isopteropodine RS
 - USP Powdered Cat's Claw Extract RS

Powdered Cat's Claw Extract

DEFINITION

Powdered Cat's Claw Extract is prepared from Cat's Claw by extraction with hydroalcoholic mixtures or other suitable solvents. The ratio of plant material to extract is between 4:1 to 6:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of pentacyclic oxindole alkaloids as isopteropodine, calculated on the dried basis, as the sum of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine. It may contain suitable added substances.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 100 mg of USP Powdered Cat's Claw Extract RS in 2 mL of methanol. Sonicate for 5 min, shaking occasionally, heat in a water bath at 60° for 15 min, cool, and centrifuge.

Sample solution: Shake a quantity of Powdered Extract, equivalent to about 25 mg of the labeled amount of pentacyclic oxindole alkaloids, in 20 mL of methanol. Allow to stand for 15 min before use.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μm (TLC plates)

Application volume: 20 μL , as bands that are 1 cm in length

Developing solvent system: Ethyl acetate and hexane (95:5)

Spray reagent A: Dissolve 0.85 g of basic bismuth nitrate in 10 mL of glacial acetic acid and 40 mL of water by heating. Filter if necessary (Solution A). Dissolve 8 g of potassium iodide in 30 mL of water (Solution B). Mix Solution A and Solution B (1:1) to obtain a stock solution. Dilute 1 mL of the stock solution with 2 mL of glacial acetic acid and 10 mL of water. [NOTE—Use freshly mixed Solution A and Solution B.]

Spray reagent B: Use a 10% solution of sodium nitrite in water.

Analysis

Samples: Standard solution and Sample solution
Develop the chromatogram to a length of NLT 12 cm, and dry the plate in a current of air.

Acceptance criteria: Examine the plate under short UV light. The Sample solution chromatogram shows multiple zones that correspond in R_f values to those observed from the Standard solution chromatogram. Other zones of varying intensities may be observed in the Sample solution. Spray the plate with Spray reagent A followed by Spray reagent B, and examine the plate under daylight. The Sample solution chromatogram shows multiple orange-brown zones that correspond in

color and R_f values to those observed in the Standard solution chromatogram. Other colored zones of varying intensities may be observed in the Sample solution.

- **B.** The chromatogram of the Sample solution exhibits peaks for speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine at retention times that correspond to those of Standard solution A, as obtained in the test for Content of Pentacyclic Oxindole Alkaloids and Limit of Tetracyclic Oxindole Alkaloids. The sum of the peak areas for the tetracyclic oxindole alkaloids rhynchophylline and isorhynchophylline is less than 25% of the total peak areas detected for pentacyclic oxindole alkaloids.

COMPOSITION

• CONTENT OF PENTACYCLIC OXINDOLE ALKALOIDS AND LIMIT OF TETRACYCLIC OXINDOLES

Standard solution A: Dissolve an accurately weighed quantity of USP Powdered Cat's Claw Extract RS in methanol, shaking for 1 min. Dilute with methanol to obtain a solution having a known concentration of about 0.5 mg of the labeled amount of total oxindole alkaloids per mL. Pass through a filter of 0.45- μm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Isopteropodine RS in methanol. Pass through a nylon filter of 0.45- μm or finer pore size.

Sample solution: Transfer an accurately weighed quantity of Powdered Extract, equivalent to about 5 mg of the labeled content of pentacyclic oxindole alkaloids, to a 10-mL centrifuge tube. Add 2.5 mL of methanol, and sonicate for 10 min. Centrifuge, and transfer the supernatant to a 10-mL volumetric flask. Repeat the extraction three additional times combining the extracts in the 10-mL volumetric flask, and dilute with methanol to volume. Transfer about 3 mL of the solution to a test tube containing 300 mg of polyamide powder, and shake for 1 min. Pass through a nylon filter of 0.45- μm or finer pore size, discarding the first part of the filtrate.

Solution A: Prepare a filtered and degassed 10 mM pH 7.0 phosphate buffer by mixing 6 mL of 1 N sodium hydroxide, 10 mL of 1 M monobasic potassium phosphate, and sufficient water to make 1000 mL. Adjust to a pH of 7.0 \pm 0.1 by adding more of either solution.

Solution B: Acetonitrile

Solution C: Methanol and glacial acetic acid (99:1)

Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	65	35	0
17	65	35	0
25	50	50	0
30	50	50	0
31	0	0	100
36	0	0	100
39	65	35	0
49	65	35	0

Chromatographic system

(See Chromatography (621), System Suitability.)

Mode: LC

Detector: UV 245 nm

Column: 4.6-mm \times 10-cm; end-capped 3- μm packing L1

Flow rate: 0.75 mL/min

Injection size: 10 μL

System suitability

Samples: Standard solution A and Standard solution B

Suitability requirements

Chromatogram similarity: The chromatogram obtained using Standard solution A is similar to the Ref-

erence Chromatogram provided with the USP Powdered Cat's Claw Extract RS being used.

Tailing factor: NMT 2.0 for the isopteropodine peak, *Standard solution B*

Relative standard deviation: NMT 2.0% for the isopteropodine peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Measure the areas of the analyte peaks. Identify the retention times of the peaks corresponding to speciophylline, uncarine F, mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline, pteropodine, and isopteropodine by comparison of the chromatogram of *Standard solution A* with the Reference Chromatogram provided with the lot of the USP Powdered Cat's Claw Extract RS used.

Separately calculate the percentages of speciophylline, uncarine F, mitraphylline, isomitraphylline, rhynchophylline, isorhynchophylline, pteropodine, and isopteropodine, as isopteropodine, in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for each relevant alkaloid from the *Sample solution*

r_S = peak response for isopteropodine from *Standard solution B*

C_S = concentration of USP Isopteropodine RS in *Standard solution B* (mg/mL)

C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Calculate the percentage of the labeled amount of pentacyclic oxindole alkaloids in the Powdered Extract:

$$\text{Result} = (\Sigma PA/L) \times 100$$

ΣPA = sum of percentages of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine (%)

L = labeled amount of pentacyclic alkaloids as isopteropodine (%)

Calculate the content of tetracyclic oxindole alkaloids by adding the individual percentages of rhynchophylline and isorhynchophylline.

Acceptance criteria

Pentacyclic oxindole alkaloids: 90.0%–110.0% of the labeled amount on the dried basis

Tetracyclic oxindole alkaloids: NMT 25% of the labeled amount of pentacyclic oxindole alkaloids on the dried basis

CONTAMINANTS

- **HEAVY METALS, Method II (231):** NMT 10 ppm
- **MICROBIAL ENUMERATION TESTS (2021):** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 10^4 /g, and the total combined molds and yeasts count does not exceed 10^3 /g.
- **OTHER REQUIREMENTS:** It meets the requirements for *Botanical Extracts (565)*, *Residual Solvents* and *Pesticide Residues*.

SPECIFIC TESTS

- **LOSS ON DRYING (731):** Dry 1 g at 105° for 2 h: it loses NMT 10.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of pentacyclic oxindole alkaloids, the

extracting solvent or solvent mixture used for preparation, and the ratio of the starting crude plant material to Powdered Extract. It meets the requirements for *Botanical Extracts (565)*, *Labeling*.

• USP REFERENCE STANDARDS (11)

USP Isopteropodine RS

USP Powdered Cat's Claw Extract RS

Cat's Claw Capsules

DEFINITION

Cat's Claw Capsules contain Powdered Cat's Claw Extract.

Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of Powdered Extract, calculated as pentacyclic oxindole alkaloids.

IDENTIFICATION

- The *Sample solution* chromatogram exhibits peaks for speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine at retention times that correspond to those in *Standard solution A*, as obtained in the test for *Content of Pentacyclic Oxindole Alkaloids and Limit of Tetracyclic Oxindole Alkaloids*. The content of tetracyclic oxindole alkaloids, calculated as the sum of rhynchophylline and isorhynchophylline, is NMT 25% of the labeled amount of pentacyclic oxindole alkaloids.

STRENGTH

• CONTENT OF PENTACYCLIC OXINDOLE ALKALOIDS AND LIMIT OF TETRACYCLIC OXINDOLE ALKALOIDS

Solution A: Prepare a 10 mM pH 7.0 phosphate buffer by mixing 1 N sodium hydroxide, 1 M monobasic potassium phosphate, and water (3:5:492), and adjust to a pH of 7.0 ± 0.1 by adding more of either solution.

Solution B: Acetonitrile

Solution C: Methanol and glacial acetic acid (99:1)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	65	35	0
17	65	35	0
25	50	50	0
30	50	50	0
31	0	0	100
36	0	0	100
39	65	35	0
49	65	35	0

Standard solution A: Dissolve an accurately weighed quantity of USP Powdered Cat's Claw Extract RS in methanol, shake for 1 min, and dilute with methanol to obtain a solution having a known concentration of about 0.5 mg/mL of the labeled amount of total oxindole alkaloids. Pass through a filter of 0.45-μm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Isopteropodine RS in methanol. Pass through a nylon filter of 0.45-μm or finer pore size.

Sample solution: Accurately weigh the contents of not fewer than 20 Capsules and pulverize. Transfer an accurately weighed quantity of the powder, equivalent to 20 mg of the labeled amount of pentacyclic oxindole alkaloids, to a 50-mL centrifuge tube. Sonicate with 10 mL of methanol for 10 min. Centrifuge and transfer this solution to a 50-mL volumetric flask. Repeat the above extraction three more times, combining the extracts in the 50-mL volumetric flask, and dilute with methanol to volume. Transfer 3 mL of the solution to a

test tube containing 300 mg of polyamide powder, and shake for 1 min. Pass through a nylon filter of 0.45- μ m or finer pore size, and discard the first part of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 245 nm

Column: 4.6-mm \times 10-cm; endcapped 3- μ m packing L1

Flow rate: 0.75 mL/min

Injection size: 10 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained using *Standard solution A* is similar to the Reference Chromatogram provided with the USP Powdered Cat's Claw Extract RS being used.

Tailing factor: NMT 2.0 for the isopteropodine peak, *Standard solution B*

Relative standard deviation: NMT 2.0% from the isopteropodine peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Measure the areas of the analyte peaks. Identify the retention times of the peaks corresponding to speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, isopteropodine, rhynchophylline, and isorhynchophylline by comparison of the chromatogram of *Standard solution A* with the Reference Chromatogram provided with the lot of the USP Powdered Cat's Claw Extract RS being used.

Calculate the content, in mg, of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine, as isopteropodine, in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times C_S \times V$$

r_U = peak response for each relevant alkaloid from the *Sample solution*

r_S = peak response for isopteropodine from *Standard solution B*

C_S = concentration of USP Isopteropodine RS in *Standard solution B* (mg/mL)

V = final dilution volume of the *Sample solution* (mL)

Calculate the content, in mg, of total pentacyclic oxindole alkaloids (C_C) in the portion of Capsules taken by adding the individual contents of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine. Calculate the percentage of Powdered Cat's Claw Extract with respect to the label claim:

$$\text{Result} = C_C \times (A_{WC}/W) \times (100/L_E) \times (100/L)$$

C_C = content of total pentacyclic oxindole alkaloids in the portion of Capsules taken (mg)

A_{WC} = average weight of Capsules contents (mg/Capsule)

W = weight of the portion of Capsules taken (mg)

L_E = content of total pentacyclic oxindole alkaloids, mg, in 100 mg of the Extract used to prepare the Capsules

L = amount of Extract per Capsule according to label claim (mg/Capsule)

Calculate the percentage of tetracyclic oxindole alkaloids with respect to the content of pentacyclic oxindole alkaloids in the portion of Capsules taken:

$$\text{Result} = (r_T/r_P) \times 100$$

r_T = sum of peak responses for rhynchophylline and isorhynchophylline in the chromatogram of the *Sample solution*

r_P = sum of peak responses for speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine and isopteropodine in the chromatogram of the *Sample solution*

Acceptance criteria: 90.0%–110.0% of the labeled amount of Powdered Extract calculated as pentacyclic oxindole alkaloids; and NMT 25% of tetracyclic oxindole alkaloids with respect to the labeled amount of pentacyclic oxindole alkaloids is found.

PERFORMANCE TESTS

• DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS

<2040>: Meets the requirements for *Disintegration*

• WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>:

Meets the requirements

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Capsules were prepared. If prepared with Extract, the label also indicates the quantity, in mg, of Extract per Capsule and the content, in mg, of pentacyclic oxindole alkaloids per 100 mg of Powdered Extract.

• USP REFERENCE STANDARDS <11>

USP Isopteropodine RS

USP Powdered Cat's Claw Extract RS

Cat's Claw Tablets

DEFINITION

Cat's Claw Tablets contain Powdered Cat's Claw Extract. Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of Powdered Extract, calculated as pentacyclic oxindole alkaloids.

IDENTIFICATION

• The *Sample solution* chromatogram exhibits peaks for speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine at retention times that correspond to those in *Standard solution A*, as obtained in the test for *Content of Pentacyclic Oxindole Alkaloids and Limit of Tetracyclic Oxindole Alkaloids*. The content of tetracyclic oxindole alkaloids, calculated as the sum of rhynchophylline and isorhynchophylline, is NMT 25% of the labeled amount of pentacyclic oxindole alkaloids.

STRENGTH

• CONTENT OF PENTACYCLIC OXINDOLE ALKALOIDS AND LIMIT OF TETRACYCLIC OXINDOLE ALKALOIDS

Solution A: Prepare a 10 mM pH 7.0 phosphate buffer by mixing 1 N sodium hydroxide, 1 M monobasic potassium phosphate, and water (3:5:492), and adjust to a pH of 7.0 ± 0.1 by adding more of either solution.

Solution B: Acetonitrile

Solution C: Methanol and glacial acetic acid (99:1)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	65	35	0
17	65	35	0
25	50	50	0
30	50	50	0
31	0	0	100
36	0	0	100
39	65	35	0
49	65	35	0

Standard solution A: Dissolve an accurately weighed quantity of USP Powdered Cat's Claw Extract RS in methanol, shake for 1 min, and dilute with methanol to obtain a solution having a known concentration of about 0.5 mg/mL of the labeled amount of total oxindole alkaloids. Pass through a filter of 0.45- μ m or finer pore size.

Standard solution B: 0.1 mg/mL of USP Isopteropodine RS in methanol. Pass through a nylon filter of 0.45- μ m or finer pore size.

Sample solution: Accurately weigh not fewer than 20 Tablets and pulverize. Transfer an accurately weighed quantity of the powder, equivalent to 20 mg of the labeled amount of pentacyclic oxindole alkaloids, to a 50-mL centrifuge tube. Sonicate with 10 mL of methanol for 10 min. Centrifuge and transfer this solution to a 50-mL volumetric flask. Repeat the above extraction three more times, combining the extracts in the 50-mL volumetric flask, and dilute with methanol to volume. Transfer 3 mL of the solution to a test tube containing 300 mg of polyamide powder, and shake for 1 min. Pass through a nylon filter of 0.45- μ m or finer pore size, and discard the first part of the filtrate.

Chromatographic system

(See Chromatography <621>, System Suitability.)

Mode: LC

Detector: UV 245 nm

Column: 4.6-mm \times 10-cm; endcapped 3- μ m packing L1

Flow rate: 0.75 mL/min

Injection size: 10 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*
Suitability requirements

Chromatogram similarity: The chromatogram obtained using *Standard solution A* is similar to the Reference Chromatogram provided with the USP Powdered Cat's Claw Extract RS being used.

Tailing factor: NMT 2.0 for the isopteropodine peak, *Standard solution B*

Relative standard deviation: NMT 2.0% from the isopteropodine peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Measure the areas of the analyte peaks. Identify the retention times of the peaks corresponding to speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, isopteropodine, rhynchophylline, and isorhynchophylline by comparison of the chromatogram of *Standard solution A* with the Reference Chromatogram provided with the lot of the USP Powdered Cat's Claw Extract RS being used.

Calculate the content, in mg, of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine,

and isopteropodine, as isopteropodine, in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times C_S \times V$$

r_U = peak response for each relevant alkaloid from the *Sample solution*

r_S = peak response for isopteropodine from *Standard solution B*

C_S = concentration of USP Isopteropodine RS in *Standard solution B* (mg/mL)

V = final dilution volume of the *Sample solution* (mL)

Calculate the content, in mg, of total pentacyclic oxindole alkaloids (C_T) in the portion of Tablets taken by adding the individual contents of speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine, and isopteropodine.

Calculate the percentage of Powdered Cat's Claw Extract with respect to the label claim:

$$\text{Result} = C_T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

C_T = content of total pentacyclic oxindole alkaloids in the portion of Tablets taken (mg)

A_{WT} = average weight of Tablets (mg/Tablet)

W = weight of the portion of Tablets taken (mg)

L_E = content of total pentacyclic oxindole alkaloids, mg, in 100 mg of the Extract used to prepare the Tablets

L = amount of Extract per Tablet according to label claim (mg/Tablet)

Calculate the percentage of tetracyclic oxindole alkaloids with respect to the content of pentacyclic oxindole alkaloids in the portion of Tablets taken:

$$\text{Result} = (r_T/r_P) \times 100$$

r_T = sum of peak responses for rhynchophylline and isorhynchophylline in the chromatogram of the *Sample solution*

r_P = sum of peak responses for speciophylline, uncarine F, mitraphylline, isomitraphylline, pteropodine and isopteropodine in the chromatogram of the *Sample solution*

Acceptance criteria: 90.0%–110.0% of the labeled amount of Powdered Extract calculated as pentacyclic oxindole alkaloids; and NMT 25% of tetracyclic oxindole alkaloids with respect to the labeled amount of pentacyclic oxindole alkaloids is found.

PERFORMANCE TESTS

• DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS

<2040>: Meets the requirements for *Disintegration*

• WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>:

Meets the requirements

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the article from which Tablets were prepared. The label also indicates the quantity of Powdered Extract per Tablet, in mg. Label Tablets to indicate the content, in mg, of pentacyclic oxindole alkaloids per 100 mg of Powdered Extract.

- **USP REFERENCE STANDARDS** <11>
USP Isopteropodine RS
USP Powdered Cat's Claw Extract RS

Centella asiatica

DEFINITION

Centella asiatica consists of the dried aerial parts of *Centella asiatica* (L.) Urb. [Syn: *Hydrocotyle asiatica* L.] (Fam. Apiaceae). It is also known in commerce as gotu kola. It contains NLT 2.0% of triterpene derivatives, calculated on the dried basis.

IDENTIFICATION

- **A.** *Centella asiatica* meets the requirements for *Specific Tests, Botanic Characteristics*.
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution A: 0.5 mg/mL of USP Asiaticoside RS in methanol

Standard solution B: 10 mg/mL of USP Powdered *Centella asiatica* Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: About 0.5 g of *Centella asiatica*, finely powdered, in 5 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates) or with an average particle size of 5 µm (HPTLC plates)

Application volume: 10 µL (TLC plates) or 4 µL (HPTLC plates)

Developing solvent system: A mixture of methylene chloride, methanol, and water (14:6:1)

Spray reagent: A solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* <621>). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 3 min at 120°, and examine under visible light.

Acceptance criteria: The *Sample solution* chromatogram exhibits a violet band in the lower third of the plate due to asiaticoside, corresponding in color and R_f to that in *Standard solution A*; a violet band due to madecassoside at an R_f lower than that of asiaticoside; and two additional violet bands in the upper third of the plate due to asiatic acid and madecassic acid. Bands detected in the *Sample solution* correspond in position and color to bands in *Standard solution B*. Other minor bands may be observed in the *Sample solution* and *Standard solution B*.

- **C. HPLC IDENTIFICATION TEST:** The *Sample solution* chromatogram from the test for *Content of Triterpene Derivatives* shows a peak at the retention time corresponding to that of asiaticoside in *Standard solution A*. Identify other triterpene derivative peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered *Centella asiatica* Extract RS being used. The *Sample solution* shows additional peaks corresponding to madecassoside and asiaticoside B (these two peaks may co-elute), madecassic acid, terminolic acid, and asiatic acid.

COMPOSITION

- **CONTENT OF TRITERPENE DERIVATIVES**

Solution A: Dilute 3 mL of phosphoric acid with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	78	22
65	45	55
66	5	95
75	5	95
76	78	22
85	78	22

Standard solution A: 0.05 mg/mL of USP Asiaticoside RS in methanol

Standard solution B: Sonicate a portion of USP Powdered *Centella asiatica* Extract RS in methanol to obtain a solution with a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Sample stock solution: Transfer about 1.0 g of *Centella asiatica*, finely powdered and accurately weighed, to a Soxhlet apparatus. Add 100 mL of methanol, extract for 8 h, cool, and dilute with methanol to 100 mL. Pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate. [NOTE—Use a thimble of a suitable size such that the volume of methanol used in the Soxhlet extraction is at least twice the volume of the thimble.]

Sample solution: Dilute 5.0 mL of *Sample stock solution* with methanol to 10 mL.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 1.0 mL/min

Injection size: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*
Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered *Centella asiatica* Extract RS being used.

Tailing factor: Between 0.8 and 2.0 for the asiaticoside peak, *Standard solution A*

Resolution: NLT 1.5 between the madecassic acid and terminolic acid peaks, *Standard solution B*

Relative standard deviation: NMT 2.0% determined from the asiaticoside peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 48 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered *Centella asiatica* Extract RS being used, identify the retention times of the peaks corresponding to different triterpene derivatives. The approximate relative retention times of the different triterpene derivatives are provided in the following table.

Analyte	Approximate Relative Retention Time
Madecassoside	0.71
Asiaticoside B	0.72
Asiaticoside	1.00
Madecassic acid	2.40

Analyte	Approximate Relative Retention Time
Terminolic acid	2.44
Asiatic acid	3.12

Separately calculate the percentages of the sum of madecassoside and asiaticoside B (these two peaks may co-elute), asiaticoside, the sum of madecassic acid and terminolic acid, and asiatic acid in the portion of *Centella asiatica* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times F \times 100$$

- r_U = peak response of the triterpene derivative(s) from the *Sample solution*
 r_S = peak response(s) of asiaticoside from *Standard solution A*
 C_S = concentration of USP Asiaticoside RS in *Standard solution A* (mg/mL)
 V = final volume of *Sample stock solution* (mL)
 W = weight of *Centella asiatica* used to prepare *Sample stock solution* (mg)
 D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*
 F = conversion factors for analytes: 1.00 for asiaticoside, 1.017 for the sum of madecassoside and asiaticoside B, 0.526 for the sum of madecassic acid and terminolic acid, and 0.509 for asiatic acid

Acceptance criteria: Add the percentages of different triterpene derivatives: NLT 2.0% on the dried basis.

CONTAMINANTS

- **HEAVY METALS, Method III (231):** NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis (561):** Meets the requirements
- **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined yeasts and molds count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS (2022):** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

BOTANIC CHARACTERISTICS

Macroscopic: Stem is slender, yellowish-brown, with long internodes, rooting at nodes; leaves are grayish-green, simple, alternate or grouped together at the nodes, reniform or oblong-elliptic, have palmate venation, usually with 7 veins, apex obtuse, margin crenate, base cordate, variable in size, 1–4 cm long, 2–4 cm and sometimes up to 7 cm wide, young leaves show a few trichomes on the lower surface and adult leaves are glabrous; petioles are long, grooved, base wider and sheathing; the inflorescence, if present, is a single umbel and consists of 3 flowers, rarely 2 or 4; the flowers are very small (about 2 mm), pentamerous, and have an inferior ovary; the fruit, brownish-gray, orbicular cremocarp, up to 5 mm long, is very flattened laterally and has 7–9 prominent curved ridges. Pharmacopeial article is green to yellowish-green masses composed mostly of leaves and stems fragments; odor slight; taste slightly bitter to sweet.

Histology

Transverse section of stems: Epidermal layer, subrounded or subsquare cells; 2–4 layers of collenchyma cells; 6–8 layers of thin-wall parenchyma cells with intercellular spaces; 6–7 collateral vascular bundles, xylem vessels radially arranged, slightly lignified fiber groups occurring outside the phloem; pith large, composed of thin-wall parenchyma cells; secretory canals, composed of 5–7 secretory cells, observed in cortex and medullary rays

Transverse section of leaves: Upper and lower epidermis; mesophyll composed of parenchyma cells, some contain crystals of calcium oxalate; 2–3 layers of collenchyma present in the midrib region next to both epidermal layers; vascular bundles in the center with xylem on the ventral side and phloem on the dorsal side. Transverse section of petioles has a U shape, showing an upper and a lower epidermis, followed by 2–3 layers of collenchyma next to both epidermal layers; a broad parenchymatous zone, some cells contain crystals of calcium oxalate; 7 vascular bundles forming a U shape in the parenchymatous zone, the two present in the projecting arms being less developed

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter (561):** NMT 7.0%, of which NMT 5.0% is of underground organs and NMT 2% is of other foreign matter
- **LOSS ON DRYING (731):** Dry 1.0 g of finely powdered *Centella asiatica* at 105° for 2 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 12%, determined on 1.0 g of finely powdered *Centella asiatica*
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash (561):** NMT 3.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article. The label states that the article is exempted from the requirements of the *General Notices* with respect to the pregnancy and lactation statement (section 10.40.50, *Labeling Botanical-Containing Products*).
- **USP REFERENCE STANDARDS (11)**
 USP Asiaticoside RS
 USP Powdered *Centella asiatica* Extract RS

Powdered *Centella asiatica*

DEFINITION

Powdered *Centella asiatica* is *Centella asiatica* reduced to a powder or very fine powder. It contains NLT 2.0% of triterpene derivatives, calculated on the dried basis.

IDENTIFICATION

- **A.** Powdered *Centella asiatica* meets the requirements for *Specific Tests, Botanic Characteristics*.
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution A: 0.5 mg/mL of USP Asiaticoside RS in methanol
Standard solution B: 10 mg/mL of USP Powdered *Centella asiatica* Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
Sample solution: About 0.5 g of Powdered *Centella asiatica* in 5 mL of methanol. Sonicate for 10 min, centrifuge, and use the supernatant.
Adsorbent: Chromatographic silica gel with an average particle size of 10–15 μm (TLC plates) or with an average particle size of 5 μm (HPTLC plates)
Application volume: 10 μL (TLC plates) or 4 μL (HPTLC plates)
Developing solvent system: A mixture of methylene chloride, methanol, and water (14:6:1)
Spray reagent: A solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh.]
Analysis
Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 3 min at 120°, and examine under visible light.

Acceptance criteria: The *Sample solution* chromatogram exhibits a violet band in the lower third of the plate due to asiaticoside, corresponding in color and R_f to that in *Standard solution A*; a violet band due to madecassoside at an R_f lower than that of asiaticoside; and two additional violet bands in the upper third of the plate due to asiatic acid and madecassic acid. Bands detected in the *Sample solution* correspond in position and color to bands in *Standard solution B*. Other minor bands may be observed in the *Sample solution* and *Standard solution B*.

- **C. HPLC IDENTIFICATION TEST:** The *Sample solution* chromatogram from the test for *Content of Triterpene Derivatives* shows a peak at the retention time corresponding to that of asiaticoside in *Standard solution A*. Identify other triterpene derivative peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered *Centella asiatica* Extract RS being used. The *Sample solution* shows additional peaks corresponding to madecassoside and asiaticoside B (these two peaks may co-elute), madecassic acid, terminolic acid, and asiatic acid.

COMPOSITION

• CONTENT OF TRITERPENE DERIVATIVES

Solution A: Dilute 3 mL of phosphoric acid with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	78	22
65	45	55
66	5	95
75	5	95
76	78	22
85	78	22

Standard solution A: 0.05 mg/mL of USP Asiaticoside RS in methanol

Standard solution B: Sonicate a portion of USP Powdered *Centella asiatica* Extract RS in methanol to obtain a solution with a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Sample stock solution: Transfer about 1.0 g of Powdered *Centella asiatica*, accurately weighed, to a Soxhlet apparatus. Add 100 mL of methanol, extract for 8 h, cool, and dilute with methanol to 100 mL. Pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate. [NOTE—Use a thimble of a suitable size such that the volume of methanol used in the Soxhlet extraction is at least twice the volume of the thimble.]

Sample solution: Dilute 5.0 mL of *Sample stock solution* with methanol to 10 mL.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 1.0 mL/min

Injection size: 10 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered *Centella asiatica* Extract RS being used.

Tailing factor: Between 0.8 and 2.0 for the asiaticoside peak, *Standard solution A*

Resolution: NLT 1.5 between the madecassic acid and terminolic acid peaks, *Standard solution B*

Relative standard deviation: NMT 2.0% determined from the asiaticoside peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and *Sample solution* are stable for 48 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered *Centella asiatica* Extract RS being used, identify the retention times of the peaks corresponding to different triterpene derivatives. The approximate relative retention times of the different triterpene derivatives are provided in the following table.

Analyte	Approximate Relative Retention Time
Madecassoside	0.71
Asiaticoside B	0.72
Asiaticoside	1.00
Madecassic acid	2.40
Terminolic acid	2.44
Asiatic acid	3.12

Separately calculate the percentages of the sum of madecassoside and asiaticoside B (these two peaks may co-elute), asiaticoside, the sum of madecassic acid and terminolic acid, and asiatic acid in the portion of Powdered *Centella asiatica* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times F \times 100$$

r_U = peak response(s) of the triterpene derivative(s) from the *Sample solution*

r_S = peak response of asiaticoside from *Standard solution A*

C_S = concentration of USP Asiaticoside RS in *Standard solution A* (mg/mL)

V = final volume of *Sample stock solution* (mL)

W = weight of Powdered *Centella asiatica* used to prepare the *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*

F = conversion factors for analytes: 1.00 for asiaticoside, 1.017 for the sum of madecassoside and asiaticoside B, 0.526 for the sum of madecassic acid and terminolic acid, and 0.509 for asiatic acid

Acceptance criteria: Add the percentages of different triterpene derivatives: NLT 2.0% on the dried basis.

CONTAMINANTS

• **HEAVY METALS, Method III (231):** NMT 20 ppm

• **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis (561):** Meets the requirements

- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined yeast and mold count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**: Greenish-gray to greenish-brown in color; odor slight; taste slightly bitter to sweet. Under a microscope, fractions of epidermal cells of the leaves with irregular striated cuticle, showing anisocytic, some paracytic, and rarely anomocytic stomata; epidermal cells of young leaves showing unicellular, occasionally multicellular, non-glandular trichomes; secretory canals composed of 5–7 secretory cells; parenchyma cells, some showing prisms or rosettes of calcium oxalate; bundles of narrow septate fibers from the stem; spiral vessels; fragments of the fruits, layers of wide cells in a parquetry arrangement, annular vessels, parenchyma cells containing simple or compound starch granules.
- **LOSS ON DRYING** (731): Dry 1.0 g of Powdered *Centella asiatica* at 105° for 2 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 12%, determined on 1.0 g of Powdered *Centella asiatica*
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561): NMT 3.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING**: The label states the Latin binomial and, following the official name, the parts of the plant contained in the article. The label states that the article is exempted from the requirements of the *General Notices* with respect to the pregnancy and lactation statement (section 10.40.50, *Labeling Botanical-Containing Products*).
- **USP REFERENCE STANDARDS** (11)
USP Asiaticoside RS
USP Powdered *Centella asiatica* Extract RS

Powdered *Centella asiatica* Extract

DEFINITION

Powdered *Centella asiatica* Extract is prepared from *Centella asiatica* by extraction with alcohol, methanol, acetone, or a mixture of these solvents. The ratio of plant material to extract is between 65:1 and 30:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of triterpene derivatives; the labeled amount of triterpene derivatives is NMT 40%, calculated on the dried basis as the sum of madecassoside, asiaticoside B, asiaticoside, madecassic acid, terminolic acid, and asiatic acid. It may contain suitable added substances as carriers.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution A: 0.5 mg/mL of USP Asiaticoside RS in methanol
Standard solution B: 10 mg/mL of USP Powdered *Centella asiatica* Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
Sample solution: Transfer an amount of Powdered *Centella asiatica* Extract equivalent to about 5 mg of triterpene derivatives to a centrifuge tube. Add 5 mL of methanol, sonicate for 10 min, centrifuge, and use the supernatant.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 μ m (TLC plates) or with an average particle size of 5 μ m (HPTLC plates)

Application volume: 10 μ L (TLC plates) or 4 μ L (HPTLC plates)

Developing solvent system: A mixture of methylene chloride, methanol, and water (14:6:1)

Spray reagent: A solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with the *Spray reagent*, heat for 3 min at 120°, and examine under visible light.

Acceptance criteria: The *Sample solution* chromatogram exhibits a violet band in the lower third of the plate due to asiaticoside, corresponding in color and R_f to that in *Standard solution A*; a violet band due to madecassoside at an R_f lower than that of asiaticoside; and two additional violet bands in the upper third of the plate due to asiatic acid and madecassic acid. Bands detected in the *Sample solution* correspond in position and color to bands in *Standard solution B*. Other minor bands may be observed in the *Sample solution* and *Standard solution B*.

- **B. HPLC IDENTIFICATION TEST**: The *Sample solution* chromatogram from the test for *Content of Triterpene Derivatives* shows a peak at the retention time corresponding to that of asiaticoside in *Standard solution A*. Identify other triterpene derivative peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered *Centella asiatica* Extract RS being used. The *Sample solution* shows additional peaks corresponding to madecassoside and asiaticoside B (these two peaks may co-elute), madecassic acid, terminolic acid, and asiatic acid.

COMPOSITION

• CONTENT OF TRITERPENE DERIVATIVES

Solution A: Dilute 3 mL of phosphoric acid with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	78	22
65	45	55
66	5	95
75	5	95
76	78	22
85	78	22

Standard solution A: 0.1 mg/mL of USP Asiaticoside RS in methanol

Standard solution B: Sonicate a portion of USP Powdered *Centella asiatica* Extract RS in methanol to obtain a solution with a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45- μ m or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Sonicate a portion of Powdered *Centella asiatica* Extract in methanol to obtain a solution with a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45- μ m or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 200 nm**Column:** 4.6-mm × 25-cm; 5-μm packing L1**Flow rate:** 1.0 mL/min**Injection size:** 10 μL**System suitability****Samples:** *Standard solution A* and *Standard solution B***Suitability requirements****Chromatogram similarity:** The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered *Centella asiatica* Extract RS being used.**Tailing factor:** Between 0.8 and 2.0 for the asiaticoside peak, *Standard solution A***Resolution:** NLT 1.5 between the madecassic acid and terminolic acid peaks, *Standard solution B***Relative standard deviation:** NMT 2.0% determined from the asiaticoside peak in repeated injections, *Standard solution A***Analysis****Samples:** *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 48 h at room temperature.]Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered *Centella asiatica* Extract RS being used, identify the retention times of the peaks corresponding to different triterpene derivatives. The approximate relative retention times of the different triterpene derivatives are provided in the following table.

Analyte	Approximate Relative Retention Time
Madecassoside	0.71
Asiaticoside B	0.72
Asiaticoside	1.00
Madecassic acid	2.40
Terminolic acid	2.44
Asiatic acid	3.12

Separately calculate the percentages of the sum of madecassoside and asiaticoside B (these two peaks may co-elute), asiaticoside, the sum of madecassic acid and terminolic acid, and asiatic acid in the portion of Powdered *Centella asiatica* Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

 r_U = peak response(s) of the triterpene derivative(s) from the *Sample solution* r_S = peak response of asiaticoside from *Standard solution A* C_S = concentration of USP Asiaticoside RS in *Standard solution A* (mg/mL) C_U = concentration of Powdered *Centella asiatica* Extract in the *Sample solution* (mg/mL) F = conversion factors for analytes: 1.00 for asiaticoside, 1.017 for the sum of madecassoside and asiaticoside B, 0.526 for the sum of madecassic acid and terminolic acid, and 0.509 for asiatic acid**Acceptance criteria:** Add the percentages of different triterpene derivatives: NLT 90.0% and NMT 110.0% of the labeled amount of triterpene derivatives; the labeled amount of triterpene derivatives is NMT 40%, calculated on the dried basis.**CONTAMINANTS**

- **HEAVY METALS**, *Method III* <231>: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 10^4 cfu per g. The total combined yeast and mold count does not exceed 10^3 cfu per g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **LOSS ON DRYING** <731>: Dry 1.0 g of Powdered *Centella asiatica* Extract at 105° for 2 h: it loses NMT 5% of its weight.
- **OTHER REQUIREMENTS**: Meets the requirements of the test for *Residual Solvents* under *Botanical Extracts* <565>

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING**: The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements under *Botanical Extracts* <565>.
- **USP REFERENCE STANDARDS** <11>
 - USP Asiaticoside RS
 - USP Powdered *Centella asiatica* Extract RS

Centella asiatica Triterpenes**DEFINITION***Centella asiatica* Triterpenes is a fraction enriched in *Centella asiatica* triterpenes derivatives. It is prepared from *Centella asiatica* Extract using suitable solvents or other means. It contains NLT 90.0% of triterpene derivatives, calculated on the anhydrous basis, as the sum of two or more of the following: madecassoside, asiaticoside B, asiaticoside, madecassic acid, terminolic acid, and asiatic acid.**IDENTIFICATION**

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
 - Standard solution A:** 0.5 mg/mL of USP Asiaticoside RS in methanol
 - Standard solution B:** 10 mg/mL of USP Powdered *Centella asiatica* Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
 - Sample solution:** Transfer an amount of *Centella asiatica* Triterpenes, equivalent to about 5 mg of triterpene derivatives, to a centrifuge tube. Add 5 mL of methanol, sonicate for 10 min, centrifuge, and use the supernatant.
 - Adsorbent:** Chromatographic silica gel with an average particle size of 10–15 μm (TLC plates) or with an average particle size of 5 μm (HPTLC plates)
 - Application volume:** 10 μL (TLC plates) or 4 μL (HPTLC plates)
 - Developing solvent system:** A mixture of methylene chloride, methanol, and water (14:6:1)
 - Spray reagent:** A solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh.]

Analysis**Samples:** *Standard solution A*, *Standard solution B*, and *Sample solution*Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* <621>). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the cham-

ber, dry, spray with *Spray reagent*, heat for 3 min at 120°, and examine under visible light.

Acceptance criteria: The *Sample solution* chromatogram exhibits a violet band in the lower third of the plate due to asiaticoside, corresponding in color and R_f to that in *Standard solution A*. The *Sample solution* shows additional bands corresponding to some or all of the following triterpene derivatives: a violet band due to madecassoside at an R_f lower than that of asiaticoside, a violet band in the upper third of the plate due to asiatic acid, and a violet band due to madecassic acid at an R_f lower than that of asiatic acid. Bands detected in the *Sample solution* correspond in position and color to bands in *Standard solution B*. Other minor bands may be observed in the *Sample solution* and *Standard solution B*.

- **B. HPLC IDENTIFICATION TEST:** The *Sample solution* chromatogram from the test for *Content of Triterpene Derivatives* shows a peak at the retention time corresponding to that of asiaticoside in *Standard solution A*. Identify other triterpene derivative peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered *Centella asiatica* Extract RS being used. The *Sample solution* shows additional peaks corresponding to some or all of the following: madecassoside and asiaticoside B (these two peaks may co-elute), madecassic acid, terminolic acid, and asiatic acid.

COMPOSITION

• CONTENT OF TRITERPENE DERIVATIVES

Solution A: Dilute 3 mL of phosphoric acid with water to 1000 mL, mix, filter, and degas.

Solution B: Acetonitrile

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	78	22
65	45	55
66	5	95
75	5	95
76	78	22
85	78	22

Standard solution A: 0.2 mg/mL of USP Asiaticoside RS in methanol

Standard solution B: Sonicate a portion of USP Powdered *Centella asiatica* Extract RS in methanol to obtain a solution with a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: About 1.0 mg/mL of *Centella asiatica* Triterpenes in methanol; sonicate if necessary. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 1.0 mL/min

Injection size: 10 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered *Centella asiatica* Extract RS being used.

Tailing factor: Between 0.8 and 2.0 for the asiaticoside peak, *Standard solution A*

Resolution: NLT 1.5 between the madecassic acid and terminolic acid peaks, *Standard solution B*

Relative standard deviation: NMT 2.0% determined from the asiaticoside peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 48 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered *Centella asiatica* Extract RS being used, identify the retention times of the peaks corresponding to different triterpene derivatives. The approximate relative retention times of the different triterpene derivatives are provided in the following table.

Analyte	Approximate Relative Retention Time
Madecassoside	0.71
Asiaticoside B	0.72
Asiaticoside	1.00
Madecassic acid	2.40
Terminolic acid	2.44
Asiatic acid	3.12

Separately calculate the percentages of the triterpene derivatives in the portion of *Centella asiatica* Triterpenes taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response(s) of the triterpene derivative(s) from the *Sample solution*

r_S = peak response of asiaticoside from *Standard solution A*

C_S = concentration of USP Asiaticoside RS in *Standard solution A* (mg/mL)

C_U = concentration of *Centella asiatica* Triterpenes in the *Sample solution* (mg/mL)

F = conversion factors for analytes: 1.00 for asiaticoside, 1.017 for madecassoside, 1.017 for asiaticoside B, 0.526 for madecassic acid, 0.526 for terminolic acid, and 0.509 for asiatic acid

Acceptance criteria: Add the percentages of different triterpene derivatives: NLT 90.0% on the anhydrous basis.

CONTAMINANTS

- **HEAVY METALS**, *Method III* (231): NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10^3 cfu/g. The total combined yeast and mold count does not exceed 10^2 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Escherichia coli*

SPECIFIC TESTS

- **WATER DETERMINATION**, *Method I* (921): NMT 5%
- **OTHER REQUIREMENTS:** Meets the requirements of the test for *Residual Solvents* under *Botanical Extracts* (565)

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.

- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** <11>
USP Asiaticoside RS
USP Powdered *Centella asiatica* Extract RS

Chamomile

DEFINITION

Chamomile consists of the dried flower heads of *Matricaria recutita* L. (*Matricaria chamomilla* L., *Matricaria chamomilla* L. var. *courrantiana*, *Chamomilla recutita* L.) Rauschert (Fam. Asteraceae alt. Compositae). It contains NLT 0.4% of blue volatile oil, NLT 0.3% of apigenin-7-glucoside, and NLT 0.15% of bisabolol derivatives, calculated as levomenol.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 1.0 mg/mL of borneol, 2.0 mg/mL of bornyl acetate, and 0.4 mg/mL of guaiazulene in toluene

Sample solution: Reduce 1.0 g of Chamomile to a coarse powder, using a porcelain pestle and mortar. Transfer to a 1.5-cm × 15-cm chromatographic column, and tamp lightly with a short length of rubber hose. Rinse the pestle and mortar twice, each time with 10 mL of methylene chloride. Pour the rinsings into the column. Collect the percolate in a flask with a long, narrow neck, and remove the solvent by evaporation on a water bath. Dissolve the residue in 0.5 mL of toluene.

Adsorbent: 0.25-mm layer of chromatographic silica gel

Developing solvent: Chloroform

Spray reagent: Mix anisaldehyde, glacial acetic acid, and methanol (0.5: 10: 85). Then carefully add 5 mL of sulfuric acid to this solution.

Application volume: 10 µL, as 3-mm × 20-mm bands

Analysis

Samples: *Standard solution* and *Sample solution*
Examine the plate under short-wavelength UV light: the *Sample solution* exhibits a number of quenching areas, the largest of which is due to en-yne-dicycloether and has the same R_f value as the band due to bornyl acetate in the *Standard solution*. There is also a band due to matricin near the line of application. Spray the plate evenly with the *Spray reagent*. Examine the plate in daylight while heating at 100°–105° for 5–10 min. The chromatogram obtained from the *Standard solution* shows in the lower third a brownish yellow zone that becomes violet-gray after a few hours and is due to borneol; in the middle a yellowish brown to gray zone due to bornyl acetate; and in the upper third a deep red zone with a blue edge due to guaiazulene.

Acceptance criteria: The *Sample solution* exhibits a blue zone due to matricin near the starting point; several violet-red zones, one of which is due to bisabolol, at R_f values between those of borneol and bornyl acetate; a brownish zone, due to en-yne-dicycloether, at an R_f value corresponding to that of bornyl acetate; red zones, due to terpenes, at R_f values similar to those of guaiazulene; and other zones that appear in the middle and lower parts of the chromatogram.

• B.

Analysis: Dissolve 0.25 g of dimethylaminobenzaldehyde in a mixture of 5 mL of phosphoric acid, 45 mL of acetic acid, and 45 mL of water. Transfer 2.5 mL of this solution and 0.1 mL of the *Sample solution*, prepared as directed for *Identification* test A, to a test tube. Heat on

a water bath for 2 min, and allow to cool. Add 5 mL of solvent hexane, and shake.

Acceptance criteria: The aqueous layer has a distinct greenish blue or blue color.

COMPOSITION

• CONTENT OF APIGENIN-7-GLUCOSIDE

Dilute phosphoric acid: Mix 5.0 mL of phosphoric acid in 50 mL of water. Dilute with water to 100 mL.

Solution A: 0.005 M solution of monobasic potassium phosphate. Adjust with *Dilute phosphoric acid* to a pH of 2.55 ± 0.05 .

Solution B: Acetonitrile and methanol (13:7)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	74	26
3	74	26
22	15	85
27	74	26
30	74	26

Standard solution: 25.0 µg/mL of USP Apigenin-7-glucoside RS and 10.0 µg/mL of 7-methoxycoumarin in methanol and water (1:1)

Sample solution: Transfer 1.0 g of Chamomile to a suitable flask fitted with a reflux condenser and a stirrer. Add 80.0 mL of methanol, and reflux the mixture with stirring for 1 h. Cool the flask to room temperature, pass the extract through a folded filter paper, and collect the filtrate in a 100-mL volumetric flask. Rinse the flask with 3 mL of methanol, pour the methanolic rinsings through the filter paper, and add the filtrate to the volumetric flask. Dilute with methanol to volume, and mix. Transfer 25.0 mL of the filtered solution to a round-bottom flask fitted with a reflux condenser and a stirrer; add 5.0 mL of sodium hydroxide solution, prepared by dissolving 0.4 g of sodium hydroxide in 5.0 mL of water; and reflux the mixture for 25 min. Cool the flask, and adjust the solution with hydrochloric acid to a pH of 5.0–6.2. Quantitatively transfer the solution to a 50-mL volumetric flask, dilute with methanol to volume, and filter, discarding the first 10 mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 335 nm

Column: 4-mm × 12.5-cm; packing L1

Flow rate: 1 mL/min

[NOTE—Make adjustments, if necessary, to obtain relative retention times of 0.63 for apigenin-7-glucoside and 1.0 for 7-methoxycoumarin.]

Injection size: 15 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for apigenin-7-glucoside, 7-methoxycoumarin, apigenin, *trans*-spiroether, and *cis*-spiroether are about 0.63, 1.0, 1.2, 1.6, and 1.8, respectively.]

Suitability requirements

Resolution: NLT 3.5 between apigenin-7-glucoside and 7-methoxycoumarin

Relative standard deviation: NMT 2.0% for apigenin-7-glucoside

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—Allow the *Sample solution* to elute for NLT 6 times the retention time of apigenin-7-glucoside.]

Calculate the percentage of apigenin-7-glucoside in the portion of Chamomile taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

- r_U = peak response of apigenin-7-glucoside from the *Sample solution*
 r_S = peak response of apigenin-7-glucoside from the *Standard solution*
 C_S = concentration of USP Apigenin-7-glucoside RS in the *Standard solution* (mg/mL)
 V = volume of *Sample solution* (mL)
 W = weight of Chamomile taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.3%

• CONTENT OF BISABOLANE DERIVATIVES

Standard solution: 1 mg/mL of USP Levomenol RS in cyclohexane

Sample solution: Transfer the volatile oils obtained in the test for *Articles of Botanical Origin* (561), *Volatile Oil Determination* to a 25-mL volumetric flask, rinse the graduated tube of the apparatus with a small portion of cyclohexane, transfer the rinsing to the 25-mL volumetric flask, add cyclohexane to volume, and mix.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica capillary; coated with a 0.25-μm film of phase G16

Temperature

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	4	230	10

Detector: 250°

Injection port: 220°

Carrier gas: Helium

Flow rate: 1.0 mL/min

Injection size: 1 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 1.8 for levomenol

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Identify the peaks due to levomenol, bisabolol oxide B, bisabolol oxide, and bisabolol oxide A in the *Sample solution*, using the retention time of levomenol in the *Standard solution* and the approximate relative retention times of 0.89, 0.97, and 1.1 for bisabolol oxide B, bisabolol oxide, and bisabolol oxide A, respectively, with reference to the levomenol peak.

Calculate the percentage of bisabolane derivatives in the portion of Chamomile taken:

$$\text{Result} = (r_T/r_S) \times C_S \times (V/W) \times 100$$

- r_T = sum of the peak areas for bisabolol oxide B, bisabolol oxide, levomenol, and bisabolol oxide A from the *Sample solution*
 r_S = levomenol peak area from the *Standard solution*
 C_S = concentration of USP Levomenol RS in the *Standard solution* (mg/mL)
 V = volume of *Sample solution* (mL)

W = weight of Chamomile taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.15%

• ARTICLES OF BOTANICAL ORIGIN, Volatile Oil Determination (561)

Analysis: Proceed as directed, except use 60 g of coarsely powdered Chamomile as the test specimen, a 2-L round-bottom flask, 300 mL of water as distillation liquid, and 0.5 mL of xylene in the graduated tube. Distill for 4 h at a rate of 3–4 mL/min.

Acceptance criteria: NLT 0.4% of blue volatile oil is found. [NOTE—Retain the volatile oils for use in the test for *Content of Bisabolane Derivatives*.]

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS (2021):** The total bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacterial count does not exceed 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

• **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues (561):** Meets the requirements

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Flower head is hemispherical, about 6 mm in diameter, composed of a few ray florets and numerous disk florets (distinction from *Matricaria discoidea*, which has disk florets only), carried on a receptacle surrounded by an involucre. Involucre is green, formed of two to three rows of lanceolate, glabrous, and imbricated bracts with blunt apices and scarious whitish edges. Ray florets, which usually have fallen off, have 10–20 pistils; corolla is ligulate, white, but darkens at a length of 6 mm and a width of about 2 mm, 3-toothed, and traversed by four main veins. Disk florets are yellow, perfect, about 2 mm in length; corolla is tubular with five teeth; five stamens are epipetalous and syngenesious. Receptacle is hollow (distinction from *Chrysanthemum* and *Anthemis* species), hemispherical in the young and conical in the old flower head, 3–10 mm in width, and lacking paleae. Achene is ovoid, and has three to five longitudinal ribs.

Microscopic: Separate the capitulum into its parts, and examine under a microscope. The outer, abaxial epidermis of the involucre bracts shows a scarious margin with a single layer of radially elongated cells and a central part made up of chlorophyll tissue covered by elongated epidermal cells with sinuous lateral walls, stomata, and secretory trichomes. The vascular bundles are surrounded by numerous elongated, pitted sclereids with fairly large lumens. In surface view, ligulate and tubular corollas show isodiametric or elongated cells with more or less wavy walls and a few glandular trichomes. The outer part of the epidermis of the ligulate florets consists of papillary cells with cuticular striations radiating from their tips. In the mesophyll, very small clusters of calcium oxalate are sometimes seen. Four main veins run lengthwise through the entire mesophyll, sometimes accompanied by one or two other veins, which are shorter and run parallel to the main veins. Each of the two main median veins split into two near the tip and, with the lateral veins, anastomose two by two to form three arcs at the three terminal teeth of the ligule. The ovaries, oval to spherical, of both kinds of florets have at their base a sclerous ring consisting of a single row of cells. The epidermis of the ovary is made up of elongated cells with sinuous walls between which secretory trichomes are situated. The ovaries contain numerous, very small clusters of calcium oxalate. In the tubular florets, the low part of each stamen filament is

surrounded by thick-walled cells. The ends of the two stigmata have papillose epidermal cells. The pollen grains have a diameter of about 30 μm and are rounded and triangular, with three germinal pores and a spiny exine.

- **BROKEN FLOWERS:** NMT 25% passes through a No. 25 standard-mesh sieve (see *Particle Size Distribution Estimation by Analytical Sieving* (786)).
- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter (561):** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 13.0%, determined on 1.0 g of powdered Chamomile

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article. This article is exempted from the requirements of the *General Notices* with respect to the pregnancy and lactation statement (section 10.40.50. *Labeling Botanical-Containing Products*).
- **USP REFERENCE STANDARDS (11)**
USP Apigenin-7-glucoside RS
USP Levomenol RS

Chaste Tree

DEFINITION

Chaste Tree consists of the dried ripe fruits of *Vitex agnus-castus* L. (Verbenaceae). It contains NLT 0.05% of agnuside and NLT 0.08% of casticin, calculated on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 100 mg of USP Powdered Chaste Tree Extract RS in 1 mL of methanol. Heat in a water bath at 60° for 10 min. Centrifuge, and use the clear supernatant.

Sample solution: Transfer about 1 g of powdered plant material to a screw-capped centrifuge tube. Add 10 mL of methanol, heat in a water bath at 60° for 10–15 min, cool, and filter.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 μm (TLC plates)

Application volume: 90 μL , *Standard solution*; 60 μL , *Sample solution*; in bands that are 2 cm in length

Developing solvent system: Ethyl acetate, methanol, and water (77:15:8)

Spray reagent: 10 mg/mL of *p*-dimethylaminobenzaldehyde in 1 N hydrochloric acid

Analysis

Samples: *Standard solution* and *Sample solution*
Develop to a length of NLT 12 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and heat for 10 min at 120°.

Acceptance criteria: The *Sample solution* shows the following: a blue zone (at an R_f value of about 0.21) due to the presence of aucubin and that corresponds in color and R_f value to a similar zone for the *Standard solution*; a blue zone (at an R_f value of about 0.44) as a result of the presence of agnuside that corresponds in color and R_f value to a similar zone for the *Standard solution*; and one broad zone, violet in the middle, near the solvent front and that corresponds in color and R_f value to a similar zone for the *Standard solution*. Other colored zones of varying intensities may be observed for the *Sample solution*.

- **B.** In the test for *Content of Casticin*, the chromatogram of the *Sample solution* shows a peak at the retention time

corresponding to the casticin peak in the chromatogram of the *Standard solution*.

COMPOSITION

• CONTENT OF CASTICIN

Standard solution: About 0.05 mg/mL of USP Casticin RS in methanol, with sonication. Pass through a cellulose membrane filter of 0.45- μm or finer pore size.

Sample solution: Place about 1000 mg of ground plant material in a container with a stopper. Extract twice with 40 mL of methanol, using a hand homogenizer at 19,000 rpm for 2 min. Filter each supernatant, and transfer to a 250-mL round-bottom flask. Rinse the residue with methanol, and filter the resulting solution into the flask. Evaporate the combined extract to dryness. Dissolve the residue in methanol, quantitatively transfer to a 20-mL volumetric flask, and dilute with methanol to volume. Pass through a cellulose membrane filter of 0.45- μm or finer pore size.

Solution A: Methanol

Solution B: 5.88 g/L of phosphoric acid in water

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	50	50
0	50	50
13	65	35
18	100	0
23	50	50

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 348 nm

Column: 3.1-mm \times 12.5-cm; 5- μm packing L1

Column temperature: 25°

Flow rate: 1 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the casticin peak

Relative standard deviation: NMT 2.0% for the casticin peak, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of casticin in the portion of Chaste Tree taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of casticin from the *Sample solution*

r_S = peak response of casticin from the *Standard solution*

C_S = concentration of USP Casticin RS in the *Standard solution* (mg/mL)

C_U = concentration of Chaste Tree in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.08% of casticin on the dried basis

• CONTENT OF AGNUSIDE

Solvent: Methanol and water (1:19)

Standard solution: Dissolve a quantity of USP Agnuside RS in *Solvent*, with sonication. Dilute with methanol to obtain a concentration of about 0.125 mg/mL. Pass through a cellulose membrane filter of 0.45- μm or finer pore size.

Sample solution: Place about 1000 mg of ground plant material in a container with a stopper. Extract twice with 40 mL of methanol, using a hand homogenizer at 19,000 rpm for 2 min. Centrifuge, and transfer each

supernatant to a 250-mL round-bottom flask. Rinse the residue with methanol, and filter the resulting solution into the flask. Evaporate the combined extract to dryness, and dissolve the residue in 2 mL of *Solvent*. Quantitatively transfer the solution to a solid-phase extraction cartridge packed with neutral aluminum oxide previously conditioned with 5 mL of *Solvent*. Connect the cartridge to a vacuum pressure not exceeding 300 mbar, and collect the eluate. Rinse the round-bottom flask with 2 mL of *Solvent*, pass this solution through the cartridge, apply the vacuum, and collect the eluate. Rinse the cartridge with 4 mL of *Solvent*, and collect the eluate. Combine the eluates from the cartridge, transfer to a 10-mL volumetric flask, and dilute with *Solvent* to volume.

Solution A: Acetonitrile

Solution B: 5.88 g/L of phosphoric acid in water

Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	7	93
0.6	10	90
5	10	90
7	14	86
13	15	85
13.1	100	0
18	100	0
18.1	7	93
23	7	93

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 258 nm

Column: 3.1-mm × 12.5-cm; 5-μm packing L1

Column temperature: 25°

Flow rate: 1.3 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the agnuside peak

Relative standard deviation: NMT 2.0% for the agnuside peak, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of agnuside in the portion of Chaste Tree taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of agnuside from the *Sample solution*

r_S = peak response of agnuside from the *Standard solution*

C_S = concentration of USP Agnuside RS in the *Standard solution* (mg/mL)

C_U = concentration of Chaste Tree in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.05% of agnuside on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method III* <231>: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** <2021>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 10⁶ cfu/g, the total combined

molds and yeast count does not exceed 10⁴ cfu/g, and the enterobacterial count does not exceed 10³ cfu/g.

SPECIFIC TESTS

• BOTANICAL CHARACTERISTICS

Macroscopic: Mature chaste tree fruits are spherical to ovoid, 2–4 mm in diameter, very hard, usually with a short pedicel. The fruit is reddish brown to black, slightly rough, and covered with glandular hairs. There are four grooves perpendicular to one another, and a slight depression on the apex, more evident on large fruits. The internal appearance of the fruit is yellowish. The internal structure of the fruit includes four compartments, each containing an oblong seed with a high fat content. A group of up to six spongy, light tan, immature fruits may also accompany mature fruits. The fruit is often covered by a tubular, greenish-gray, fine tomentose calyx, which is persistent and has five teeth.

Microscopic: The exocarp is brown and narrow, consisting of parenchymatous cells with thin walls and partially lignified cells with many pitted thickenings on the inside. In surface view, the exocarp shows an epidermis of polygonal cells with irregular thickenings and glandular hairs, each with a short single-celled stalk and a four-celled head containing essential oil. The outer mesocarp consists of several layers of brown, isodiametric parenchyma cells. The inner mesocarp consists of finely pitted sclerenchymatous cells, some with moderately thickened walls, others consisting of isodiametric stone cells with small lumen. The endocarp consists of a layer of small brown sclereid cells. The seeds are small, having large cotyledons surrounded by thin-walled, large parenchymatous cells that have ribbed thickenings. The nutritive tissue and the cells of the germ contain aleuron grains and oil globules. Starch is absent. The outer epidermis of calyx is composed of polygonal cells, covered by abundant unicellular or multicellular curved trichomes. The inner epidermis of calyx is glabrous and composed of rectangular, elongated cells with slightly wavy walls.

- **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* <561>: NMT 3.0%
- **LOSS ON DRYING** <731>: Dry 1 g at 105° for 2 h: it loses NMT 10.0%.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* <561>: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** <11>
 - USP Agnuside RS
 - USP Casticin RS
 - USP Powdered Chaste Tree Extract RS

Powdered Chaste Tree

DEFINITION

Powdered Chaste Tree is Chaste Tree reduced to a powder or a very fine powder. It contains NLT 0.05% of agnuside and NLT 0.08% of casticin, calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 100 mg of USP Powdered Chaste Tree Extract RS in 1 mL of methanol. Heat in a water

bath at 60° for 10 min. Centrifuge, and use the clear supernatant.

Sample solution: Transfer about 1 g of Powdered Chaste Tree to a screw-capped centrifuge tube. Add 10 mL of methanol, heat in a water bath at 60° for 10–15 min, cool, and filter.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)

Application volume: 90 µL, *Standard solution*; 60 µL, *Sample solution*; in bands that are 2 cm in length

Developing solvent system: Ethyl acetate, methanol, and water (77:15:8)

Spray reagent: 10 mg/mL of *p*-dimethylaminobenzaldehyde in 1 N hydrochloric acid

Analysis

Samples: *Standard solution* and *Sample solution*
Develop to a length of NLT 12 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and heat for 10 min at 120°.

Acceptance criteria: The *Sample solution* shows the following: a blue zone (at an R_f value of about 0.21) due to the presence of aucubin and that corresponds in color and R_f value to a similar zone for the *Standard solution*; a blue zone (at an R_f value of about 0.44) as a result of the presence of agnuside that corresponds in color and R_f value to a similar zone for the *Standard solution*; and one broad zone, violet in the middle, near the solvent front and that corresponds in color and R_f value to a similar zone for the *Standard solution*. Other colored zones of varying intensities may be observed for the *Sample solution*.

- **B.** In the test for *Content of Casticin*, the chromatogram of the *Sample solution* shows a peak at the retention time corresponding to the casticin peak in the chromatogram of the *Standard solution*.

COMPOSITION

• CONTENT OF CASTICIN

Standard solution: About 0.05 mg/mL of USP Casticin RS in methanol, with sonication. Pass through a cellulose membrane filter of 0.45-µm or finer pore size.

Sample solution: Place about 1000 mg of Powdered Chaste Tree in a container with a stopper. Extract twice with 40 mL of methanol, using a hand homogenizer at 19,000 rpm for 2 min. Filter each supernatant, and transfer to a 250-mL round-bottom flask. Rinse the residue with methanol, and filter the resulting solution into the flask. Evaporate the combined extract to dryness. Dissolve the residue in methanol, quantitatively transfer to a 20-mL volumetric flask, and dilute with methanol to volume. Pass through a cellulose membrane filter of 0.45-µm or finer pore size.

Solution A: Methanol

Solution B: 5.88 g/L of phosphoric acid in water

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	50	50
0	50	50
13	65	35
18	100	0
23	50	50

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 348 nm

Column: 3.1-mm × 12.5-cm; 5-µm packing L1

Column temperature: 25°

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the casticin peak

Relative standard deviation: NMT 2.0% for the casticin peak, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of casticin in the portion of Powdered Chaste Tree taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of casticin from the *Sample solution*

r_S = peak response of casticin from the *Standard solution*

C_S = concentration of USP Casticin RS in the *Standard solution* (mg/mL)

C_U = concentration of Powdered Chaste Tree in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.08% of casticin on the dried basis

• CONTENT OF AGNUSIDE

Solvent: Methanol and water (1:19)

Standard solution: Dissolve a quantity of USP Agnuside RS in *Solvent*, with sonication. Dilute with methanol to obtain a concentration of about 0.125 mg/mL. Pass through a cellulose membrane filter of 0.45-µm or finer pore size.

Sample solution: Place about 1000 mg of Powdered Chaste Tree in a container with a stopper. Extract twice with 40 mL of methanol, using a hand homogenizer at 19,000 rpm for 2 min. Centrifuge, and transfer each supernatant to a 250-mL round-bottom flask. Rinse the residue with methanol, and filter the resulting solution into the flask. Evaporate the combined extract to dryness, and dissolve the residue in 2 mL of *Solvent*. Quantitatively transfer the solution to a solid-phase extraction cartridge packed with neutral aluminum oxide previously conditioned with 5 mL of *Solvent*. Connect the cartridge to a vacuum pressure not exceeding 300 mbar, and collect the eluate. Rinse the round-bottom flask with 2 mL of *Solvent*, pass this solution through the cartridge, apply the vacuum, and collect the eluate. Rinse the cartridge with 4 mL of *Solvent*, and collect the eluate. Combine the eluates from the cartridge, transfer to a 10-mL volumetric flask, and dilute with *Solvent* to volume.

Solution A: Acetonitrile

Solution B: 5.88 g/L of phosphoric acid in water

Mobile phase: See *Table 2*.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	7	93
0.6	10	90
5	10	90
7	14	86
13	15	85
13.1	100	0
18	100	0
18.1	7	93
23	7	93

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 258 nm**Column:** 3.1-mm × 12.5-cm; 5-μm packing L1**Column temperature:** 25°**Flow rate:** 1.3 mL/min**Injection size:** 10 μL**System suitability****Sample:** *Standard solution***Suitability requirements****Tailing factor:** NMT 2.0 for the agnuside peak**Relative standard deviation:** NMT 2.0% for the agnuside peak, in repeated injections**Analysis****Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of agnuside in the portion of Powdered Chaste Tree taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak response of agnuside from the *Sample solution* r_S = peak response of agnuside from the *Standard solution* C_S = concentration of USP Agnuside RS in the *Standard solution* (mg/mL) C_U = concentration of Powdered Chaste Tree in the *Sample solution* (mg/mL)**Acceptance criteria:** NLT 0.05% of agnuside on the dried basis**CONTAMINANTS**

- **HEAVY METALS**, *Method III* <231>: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** <2021>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 10^6 cfu/g, the total combined molds and yeast count does not exceed 10^4 cfu/g, and the enterobacterial count does not exceed 10^3 cfu/g.

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Powdered Chaste Tree is dark brown, with a musty, slightly aromatic odor, and a taste resembling that of sage. The following characteristics are present: fragments of the calyx with covering and glandular trichomes on the outer side and rectangular, elongated cells with slightly wavy walls on the inner side; fragments of exocarp with trichomes and cells with large pits in the outer wall; thin-walled parenchymatous cells and globules of fixed oil; stone-pitted cells from the mesocarp; ovoid, lignified cells with bands of reticulate thickening from the testa; and endosperm and cotyledons with fixed oil.
- **LOSS ON DRYING** <731>: Dry 1 g at 105° for 2 h: it loses NMT 10.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* <561>: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** <11>
 - USP Agnuside RS
 - USP Casticin RS
 - USP Powdered Chaste Tree Extract RS

Powdered Chaste Tree Extract**DEFINITION**

Powdered Chaste Tree Extract is prepared from Chaste Tree by extraction with hydroalcoholic mixtures or other suitable solvents. It contains NLT 90.0% and NMT 110.0% of the labeled amount of casticin and agnuside, calculated on the dried basis. It may contain suitable added substances.

IDENTIFICATION• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 100 mg of USP Powdered Chaste Tree Extract RS in 1 mL of methanol. Heat in a water bath at 60° for 10 min. Centrifuge, and use the clear supernatant.

Sample solution: Shake a quantity of Extract, equivalent to about 10 mg of the labeled amount of agnuside, in 10 mL of methanol. Heat in a water bath at 60°. Centrifuge or filter before use.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 μm (TLC plates)

Application volume: 90 μL, *Standard solution*; 60 μL, *Sample solution*; in bands that are 2 cm in length

Developing solvent system: Ethyl acetate, methanol, and water (77:15:8)

Spray reagent: 10 mg/mL of *p*-dimethylaminobenzaldehyde in 1 N hydrochloric acid

Analysis

Samples: *Standard solution* and *Sample solution*
Develop the chromatograms to a length of NLT 12 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and heat for 10 min at 120°.

Acceptance criteria: The *Sample solution* shows the following: a blue zone (at an R_f value of about 0.21) due to the presence of aucubin and that corresponds in color and R_f value to a similar zone for the *Standard solution*; a blue zone (at an R_f value of about 0.44) as a result of the presence of agnuside that corresponds in color and R_f value to a similar zone for the *Standard solution*; and one broad zone, violet in the middle, near the solvent front and that corresponds in color and R_f value to a similar zone for the *Standard solution*. Other colored zones of varying intensities may be observed in the *Sample solution*.

- **B.** In the test for *Content of Casticin*, the chromatogram of the *Sample solution* exhibits a peak at the retention time corresponding to casticin.
- **C.** In the test for *Content of Agnuside*, the chromatogram of the *Sample solution* exhibits a peak at the retention time corresponding to agnuside.

COMPOSITION• **CONTENT OF CASTICIN**

Standard solution: About 0.05 mg/mL of USP Casticin RS in methanol, with sonication. Pass through a cellulose membrane filter of 0.45-μm or finer pore size.

Sample solution: Transfer a quantity of Extract, equivalent to about 2.5 mg of the labeled content of casticin, into a 50-mL volumetric flask. Add 25 mL of methanol, and sonicate in a bath at 40° for 10 min, shaking to disperse the solid. Cool to room temperature, and dilute with methanol to volume. Centrifuge or pass through a filter of 0.45-μm or finer pore size.

Solution A: Methanol
Solution B: 5.88 g/L of phosphoric acid in water
Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	50	50
0	50	50
13	65	35
18	100	0
23	50	50

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 348 nm**Column:** 3.1-mm × 12.5-cm; 5-μm packing L1**Column temperature:** 25°**Flow rate:** 1 mL/min**Injection size:** 10 μL**System suitability****Sample:** Standard solution**Suitability requirements****Tailing factor:** NMT 2.0 for the casticin peak**Relative standard deviation:** NMT 2.0% for the casticin peak, in repeated injections**Analysis****Samples:** Standard solution and Sample solutionCalculate the percentage of casticin, P_c , in the portion of Extract taken:

$$P_c = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak response of casticin from the Sample solution r_S = peak response of casticin from the Standard solution C_S = concentration of USP Casticin RS in the Standard solution (mg/mL) C_U = concentration of Extract in the Sample solution (mg/mL)

Calculate the percentage of the labeled amount of casticin in the portion of Extract taken:

$$\text{Result} = (P_c/L) \times 100$$

 P_c = content of casticin as calculated above (%) L = labeled amount of casticin (%)**Acceptance criteria:** 90.0%–110.0% on the dried basis• **CONTENT OF AGNUSIDE****Solvent:** Methanol and water (1:19)**Standard solution:** Dissolve a quantity of USP Agnuside RS in Solvent, with sonication. Dilute with methanol to obtain a concentration of about 0.125 mg/mL. Pass through a cellulose membrane filter of 0.45-μm or finer pore size.**Sample solution:** Transfer an amount of Extract, equivalent to about 6.25 mg of the labeled content of agnuside, into a 50-mL volumetric flask. Add 25 mL of Solvent, and sonicate in a bath at 40° for 10 min, shaking to disperse the solid. Cool to room temperature, and dilute with Solvent to volume. Centrifuge or pass through a filter of 0.45-μm or finer pore size.

Solution A: Acetonitrile
Solution B: 5.88 g/L of phosphoric acid in water
Mobile phase: See Table 2.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	7	93
0.6	10	90
5	10	90
7	14	86
13	15	85
13.1	100	0
18	100	0
18.1	7	93
23	7	93

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 258 nm**Column:** 3.1-mm × 12.5-cm; 5-μm packing L1**Column temperature:** 25°**Flow rate:** 1.3 mL/min**Injection size:** 10 μL**System suitability****Sample:** Standard solution**Suitability requirements****Tailing factor:** NMT 2.0 for the agnuside peak**Relative standard deviation:** NMT 2.0% for the agnuside peak, in repeated injections**Analysis****Samples:** Standard solution and Sample solutionCalculate the percentage of agnuside, P_a , in the portion of Extract taken:

$$P_a = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak response of agnuside from the Sample solution r_S = peak response of agnuside from the Standard solution C_S = concentration of USP Agnuside RS in the Standard solution (mg/mL) C_U = concentration of Extract in the Sample solution (mg/mL)

Calculate the percentage of the labeled amount of agnuside in the portion of Extract taken:

$$\text{Result} = (P_a/L) \times 100$$

 P_a = content of agnuside calculated above (%) L = labeled amount of agnuside (%)**Acceptance criteria:** 90.0%–110.0% on the dried basis**CONTAMINANTS**

- **HEAVY METALS, Method II <231>:** NMT 20 ppm
- **MICROBIAL ENUMERATION TESTS <2021>:** The total bacterial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 1000 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **OTHER REQUIREMENTS:** It meets the requirements for Botanical Extracts <565>, Residual Solvents and Pesticide Residues.

SPECIFIC TESTS

- **LOSS ON DRYING <731>:** NMT 6.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool place, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from

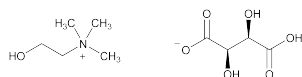
which the article was prepared. The label also indicates the content of casticin and agnuside, the extracting solvent or solvent mixture used for preparation, the ratio of the starting crude plant material to Extract, the percentage of native extract, and the name and quantity of any added substances. It meets the requirements for *Botanical Extracts* <565>, *Labeling*.

- **USP REFERENCE STANDARDS** <11>
USP Agnuside RS
USP Casticin RS
USP Powdered Chaste Tree Extract RS

Cholecalciferol—see *Cholecalciferol General Monographs*

Cholecalciferol Solution—see *Cholecalciferol Solution General Monographs*

Choline Bitartrate



C₉H₁₉NO₇ 253.25
2-Hydroxyethanaminium, -N,N,N-trimethyl-, [R-(R*,R*)]-2,3-dihydroxybutanedioate (1:1);
(2-Hydroxyethyl)trimethylammonium-L-(+)-tartrate salt (1:1) [87-67-2].

DEFINITION

Choline Bitartrate contains NLT 99.0% and NMT 100.5% of choline bitartrate (C₉H₁₉NO₇), calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>

• **B.**

Sample: 1 g

Analysis: Dissolve the *Sample* in 20 mL of water, and add 2 mL of potassium chloride solution (1 in 4).

Acceptance criteria: A white precipitate of potassium bitartrate is formed.

ASSAY

- **PROCEDURE**

Sample: 200 mg

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric

Blank: 50 mL of glacial acetic acid

Analysis: Dissolve the *Sample* in 50 mL of glacial acetic acid and titrate with *Titrant*.

Calculate the percentage of choline bitartrate (C₉H₁₉NO₇) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100] / W$$

V = *Sample* titrant volume (mL)

B = *Blank* titrant volume (mL)

N = titrant normality (mEq/mL)

F = equivalency factor, 253.2 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5% on the anhydrous basis

IMPURITIES

- **RESIDUAL SOLVENTS** <467>: Meet the requirements, except that the limit for 1,4-dioxane is 10 µg/g

- **RESIDUE ON IGNITION** <281>: NMT 0.1%

- **ARSENIC, Method I** <211>

Analysis: Add 30 mL of water and 5 mL of hydrochloric acid to dissolve the sample.

Acceptance criteria: NMT 2 ppm

- **LEAD** <251>

[NOTE—Use methylene chloride in place of chloroform to prepare the *Dithizone Extraction Solution* and *Standard Dithizone Solution*.]

Solution A: Transfer 8.4 g of sodium hydroxide solution (1 in 2) to a plastic bottle, add 100 mL of ammonium hydroxide, and mix.

Standard solution: Transfer 1.0 mL of the *Diluted Standard Lead Solution* to a separatory funnel containing 25.0 mL of water.

Sample solution: Dissolve 3.00 g of Choline Bitartrate in a separatory funnel containing 25.0 mL of water.

Analysis

Samples: *Standard solution* and *Sample solution*
Separately add 6.0 mL of *Ammonium Citrate Solution* and 3.0 mL of *Potassium Cyanide Solution* to the *Standard solution* and the *Sample solution*. Extract each of the resulting solutions three times with 5.0-mL portions of *Dithizone Extraction Solution*, shaking for 60 s and draining off each extract into another separator. Shake the combined dithizone solutions for 30 s with 20.0 mL of nitric acid (1 in 100), and discard the methylene chloride layer. Add 6.0 mL of *Ammonia-Cyanide Solution*, 2 mL of *Solution A*, and 10 mL of *Standard Dithizone Solution*, and shake for 45 s. Allow the phases to separate, and measure the absorbance of the lower layer at 510 nm with a suitable spectrophotometer.

Acceptance criteria: The absorbance of the *Sample solution* is NMT the absorbance of the *Standard solution* (NMT 0.3 ppm).

- **HEAVY METALS, Method II** <231>: NMT 10 ppm

- **LIMIT OF TOTAL AMINES**

Standard solution: 500 µg/mL of trimethylamine hydrochloride

Sample solution: Transfer 10.0 g of Choline Bitartrate to a beaker containing a plastic-coated stirring bar, add 70 mL of sodium hydroxide TS and 130 mL of water, and stir until dissolved.

System suitability stock solution: 10 µg/mL of trimethylamine hydrochloride

System suitability solution: Transfer 10.0 mL of *System suitability stock solution* containing a plastic-coated stirring bar, add 160 mL water and 30.0 mL sodium hydroxide TS, and stir until dissolved.

Electrode system: Use a gas-sensing, ammonia-specific indicating electrode with internal reference connected to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.1 mV (see *pH* <791>).

Standard response line: Mix 30.0 mL of sodium hydroxide TS and 170 mL of water. Add a plastic-coated stirring bar, insert the electrode into the solution, and record the potential, in mV. Continue stirring, and at 5-min intervals, add 0.200, 0.600, 1.00, and 2.00 mL of *Standard solution*, and record the potential after each addition. Plot the logarithms of the cumulative trimethylamine hydrochloride concentrations (0.50, 1.50, 2.50, and 5.00 µg/mL) versus potential, in mV, and determine the slope (S) of the *Standard response line* for the electrode.

System suitability

Sample: *System suitability solution*

Proceed as directed in *Analysis*, except to replace the *Sample solution* with the *System suitability solution* and

in the formula below to replace *W* with *V*, which equals 10 mL.

Suitability requirements: The total change is NLT 10 mV for a 0.4-mL cumulative addition of the *Standard solution*; the amount of trimethylamine hydrochloride found is 8.5–11.5 µg/L.

Analysis

Samples: *Standard solution* and *Sample solution*

Rinse the electrode, insert it into the *Sample solution*, stir, and record the potential, in mV. Add 0.100 mL of the *Standard solution*, and record the potential. Add another 0.100 mL of the *Standard solution*, and record the potential. [NOTE—If the total change after the second addition of the *Standard solution* is less than 10 mV, add a third aliquot of 0.200 mL.]

Calculate the content, in µg/g, of total amines as trimethylamine hydrochloride in the portion of sample taken:

$$\text{Result} = (C_S \times V_A) / [(F - 1) \times W]$$

C_S = concentration of the *Standard solution* (µg/mL)

V_A = total volume of the *Standard solution* added to the *Sample solution* (mL)

W = weight of Choline Bitartrate taken to prepare the *Sample solution* (g)

F = correction factor, calculated by the formula:

$$F = \text{antilog} [(mV_F - mV_0) / S]$$

mV_F = final reading after the additions of the *Standard solution* (mV)

mV₀ = initial reading of the *Sample solution* (mV)

S = slope of the *Standard response line* for the electrode

Acceptance criteria: NMT 10 µg/g

• CHROMATOGRAPHIC PURITY

Buffer solution: 7.1 g/L of anhydrous dibasic sodium phosphate. Adjust with phosphoric acid to a pH of 2.5.

Mobile phase: *Buffer solution* and acetonitrile (7:3)

Standard solution: Transfer an amount, NMT 100 mg, of USP Choline Chloride RS to a 24-mL screw-capped vial, and add 400 mg of 3,5-dinitrobenzoyl chloride and 10 mL of acetonitrile. Cap the vial, heat to 55°, and continue heating for 2 h. Cool to room temperature, and add 5 mL of water. Allow to stand for 5 min. Quantitatively transfer the solution to a 25-mL volumetric flask and dilute with acetonitrile to volume. Dilute a volume of this solution with *Mobile phase* to obtain a concentration of 2.0 µg/mL of USP Choline Chloride RS.

Sample solution: Transfer 500 mg of Choline Bitartrate to a centrifuge tube, add 2.0 mL of water, and swirl to dissolve. Add 0.5 mL of potassium chloride solution (7.5 in 25), centrifuge, and transfer 1.0 mL of the supernatant to a 24-mL screw-capped vial. Dry at 120° for 2 h. Add 400 mg of 3,5-dinitrobenzoyl chloride and 10 mL of acetonitrile. Cap the vial, and heat at 55° for 2 h. Cool to room temperature, add 5 mL of water, and allow to stand for 5 min. Quantitatively transfer this solution to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume. Pipet 2.0 mL of the solution to a 25-mL volumetric flask, and dilute with *Mobile phase* to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 208 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 30°

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Capacity factor (*k'*): NLT 2

Relative standard deviation: NMT 5%, determined from the choline derivative peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each impurity in the portion of Choline Bitartrate taken:

$$\text{Result} = (r_U / r_S) \times (C_S / C_U) \times (M_{r1} / M_{r2}) \times 100$$

r_U = peak response for each impurity, excluding that for the choline derivative and 3,5-dinitrobenzoic acid from the *Sample solution*

r_S = peak response for the choline derivative from the *Standard solution*

C_S = concentration of USP Choline Chloride RS in the *Standard solution* (mg/mL)

C_U = concentration of Choline Bitartrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of choline bitartrate, 253.25

M_{r2} = molecular weight of choline chloride, 139.62

Acceptance criteria

Individual impurities: NMT 0.3%

Total impurity: NMT 2.0%

SPECIFIC TESTS

• OPTICAL ROTATION, *Specific Rotation* <781S>

Sample solution: 400 mg/mL in water

Acceptance criteria: +17.5° to +18.5°

• PH <791>: 3.0–4.0, in a solution (1 in 10)

• WATER DETERMINATION, *Method I* <921>: NMT 0.5%

ADDITIONAL REQUIREMENTS

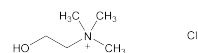
• PACKAGING AND STORAGE: Preserve in well-closed containers.

• USP REFERENCE STANDARDS <11>

USP Choline Bitartrate RS

USP Choline Chloride RS

Choline Chloride



C₅H₁₄ClNO 139.62
(2-Hydroxyethyl)trimethylammonium chloride;
2-Hydroxy-*N,N,N*-trimethylethanaminium chloride [67-48-1].

DEFINITION

Choline Chloride contains NLT 99.0% and NMT 100.5% of choline chloride (C₅H₁₄ClNO), calculated on the anhydrous basis.

IDENTIFICATION

• A. INFRARED ABSORPTION <197K>

• B. IDENTIFICATION TESTS—GENERAL, *Chloride* <191>: A solution (1 in 20) meets the requirements.

ASSAY

• PROCEDURE

Sample: 120 mg

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N silver nitrate VS

Endpoint detection: Potentiometric

Blank: 35 mL of water. Add 3 drops of acetic acid.

Analysis: Dissolve the *Sample* in 35 mL of water and add 3 drops of acetic acid. Titrate with *Titrant*.

Calculate the percentage of choline chloride ($C_5H_{14}ClNO$) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100] / W$$

V = *Sample* titrant volume (mL)

B = *Blank* titrant volume (mL)

N = titrant normality (mEq/mL)

F = equivalency factor, 139.6 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5% on the anhydrous basis

IMPURITIES

- **RESIDUAL SOLVENTS** (467): Meets the requirements, except that the limit for 1,4-dioxane is 10 µg/g

- **RESIDUE ON IGNITION** (281): NMT 0.05%

- **ARSENIC, Method I** (211)

Analysis: Add 30 mL of water and 5 mL of hydrochloric acid to dissolve the sample.

Acceptance criteria: NMT 2 ppm

- **LEAD** (251)

[NOTE—Use methylene chloride in place of chloroform to prepare the *Dithizone Extraction Solution* and *Standard Dithizone Solution*.]

Solution A: Transfer 8.4 g of sodium hydroxide solution (1 in 2) to a plastic bottle, add 100 mL of ammonium hydroxide, and mix.

Standard solution: Transfer 1.0 mL of the *Diluted Standard Lead Solution* to a separatory funnel containing 25.0 mL of water.

Sample solution: Dissolve 3.00 g of Choline Chloride in a separatory funnel containing 25.0 mL of water.

Analysis

Samples: *Standard solution* and *Sample solution*
Separately add 6.0 mL of *Ammonium Citrate Solution* and 3.0 mL of *Potassium Cyanide Solution* to the *Standard solution* and the *Sample solution*. Extract each of the resulting solutions three times with 5.0-mL portions of *Dithizone Extraction Solution*, shaking for 60 s and draining off each extract into another separator. Shake the combined dithizone solutions for 30 s with 20.0 mL of nitric acid (1 in 100), and discard the methylene chloride layer. Add 6.0 mL of *Ammonia–Cyanide Solution*, 2 mL of *Solution A*, and 10 mL of *Standard Dithizone Solution*, and shake for 45 s. Allow the phases to separate, and measure the absorbance of the lower layer at 510 nm with a suitable spectrophotometer.

Acceptance criteria: The absorbance of the *Sample solution* is NMT the absorbance of the *Standard solution* (NMT 0.3 ppm).

- **HEAVY METALS, Method II** (231): NMT 10 ppm

- **LIMIT OF TOTAL AMINES**

Standard solution: 500 µg/mL of trimethylamine hydrochloride in water

Sample solution: Transfer 10.0 g of Choline Chloride to a beaker containing a plastic-coated stirring bar, add 170 mL of water and 30.0 mL of sodium hydroxide TS, and stir until dissolved.

System suitability stock solution: 10 µg/mL of trimethylamine hydrochloride in water

System suitability solution: Transfer 10.0 mL of *System suitability stock solution* to a beaker containing a plastic-coated stirring bar, add 160 mL of water and 30.0 mL of sodium hydroxide TS, and stir until dissolved.

Electrode system: Use a gas-sensing, ammonia-specific indicating electrode with internal reference connected

to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.1 mV (see *pH* (791)).

Standard response line: Mix 30.0 mL of sodium hydroxide TS, and 170 mL of water. Add a plastic-coated stirring bar, insert the electrode into the solution, and record the potential, in mV. Continue stirring, and at 5-min intervals add 0.200, 0.600, 1.00, and 2.00 mL of *Standard solution*, and record the potential after each addition. Plot the logarithms of the cumulative trimethylamine hydrochloride concentrations (0.50, 1.50, 2.50, and 5.00 µg/mL) versus potential, in mV, and determine the slope (*S*) of the *Standard response line* for the electrode.

System suitability

Sample: *System suitability solution*

Proceed as directed in *Analysis*, except to replace the *Sample solution* with the *System suitability solution* and in the formula below to replace *W* with *V*, which equals 10 mL.

Suitability requirements: The total change is NLT 10 mV for a 0.4-mL cumulative addition of the *Standard solution*; the amount of trimethylamine hydrochloride found is 8.5–11.5 µg/L.

Analysis

Samples: *Standard solution* and *Sample solution*
Rinse the electrode, insert it into the *Sample solution*, stir, and record the potential, in mV. Add 0.100 mL of the *Standard solution*, and record the potential. Add another 0.100 mL of the *Standard solution*, and record the potential. [NOTE—If the total change after the second addition of the *Standard solution* is less than 10 mV, add a third aliquot of 0.200 mL.]

Calculate the content, in µg/g, of total amines as trimethylamine hydrochloride in the portion of sample taken:

$$\text{Result} = (C_S \times V_A) / [(F - 1) \times W]$$

C_S = concentration of *Standard solution* (µg/mL)

V_A = total volume of the *Standard solution* added to the *Sample solution* (mL)

W = weight of Choline Chloride taken to prepare the *Sample solution* (g)

F = correction factor, calculated by the formula:

$$F = \text{antilog} [(mV_f - mV_0) / S]$$

mV_f = final reading after the additions of the *Standard solution* (mV)

mV₀ = initial reading of the *Sample solution* (mV)

S = slope of the *Standard response line* for the electrode

Acceptance criteria: NMT 10 µg/g

- **CHROMATOGRAPHIC PURITY**

Buffer solution: 7.1 g/L of anhydrous dibasic sodium phosphate. Adjust with phosphoric acid to a pH of 2.5.

Mobile phase: *Buffer solution* and acetonitrile (7:3)

Standard solution: Transfer an amount, NMT 100 mg, of USP Choline Chloride RS to a 24-mL screw-capped vial, and add 400 mg of 3,5-dinitrobenzoyl chloride and 10 mL of acetonitrile. Cap the vial, heat to 55°, and continue heating for 2 h. Cool to room temperature, and add 5 mL of water. Allow to stand for 5 min. Quantitatively transfer the solution to a 25-mL volumetric flask, and dilute with acetonitrile to volume. Dilute a volume of this solution with *Mobile phase* to obtain a concentration of 2.0 µg/mL of USP Choline Chloride RS.

Sample solution: Transfer 110 mg of Choline Chloride to a 24-mL screw-capped vial. Dry at 120° for 2 h. Add 400 mg of 3,5-dinitrobenzoyl chloride and 10 mL of acetonitrile. Cap the vial, heat to 55°, and continue heating for 2 h. Cool to room temperature, and add 5 mL of water. Allow to stand for 5 min. Quantitatively transfer the solution to a 50-mL volumetric flask, and

dilute with *Mobile phase* to volume. Pipet 2.0 mL of the solution to a 25-mL volumetric flask, and dilute with *Mobile phase* to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 208 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 30°

Flow rate: 1.0 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Capacity factor (k'): NLT 2

Relative standard deviation: NMT 5%, determined from the choline derivative peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each impurity in the portion of Choline Chloride taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak response for each impurity, excluding that for the choline derivative and 3,5-dinitrobenzoic acid from the *Sample solution*
- r_S = peak response for the choline derivative from the *Standard solution*
- C_S = concentration of USP Choline Chloride RS in the *Standard solution* (mg/mL)
- C_U = concentration of Choline Chloride in the *Sample solution* (mg/mL)

Acceptance criteria

Individual impurities: NMT 0.3%

Total impurities: NMT 2.0%

SPECIFIC TESTS

- **PH** <791>: 4.0–7.0, in a solution (1 in 10)
- **WATER DETERMINATION**, *Method I* <921>: NMT 0.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** <11>
USP Choline Chloride RS

Chondroitin Sulfate Sodium

Chondroitin, hydrogen sulfate, sodium salt [9082-07-9].

DEFINITION

Chondroitin Sulfate Sodium is the sodium salt of the sulfated linear glycosaminoglycan obtained from bovine, porcine, or avian cartilages of healthy and domestic animals used for food by humans. Chondroitin Sulfate Sodium consists mostly of the sodium salt of the sulfate ester of *N*-acetylchondrosamine (2-acetamido-2-deoxy-β-D-galactopyranose) and D-glucuronic acid copolymer. These hexoses are alternately linked β-1,4 and β-1,3 in the polymer. Chondrosamine moieties in the prevalent glycosaminoglycan are monosulfated primarily on position 4 and less so on position 6. It contains NLT 90.0% and NMT 105.0% of chondroitin sulfate sodium, calculated on the dried basis.

[NOTE—Chondroitin Sulfate Sodium is extremely hygroscopic once dried. Avoid exposure to the atmosphere, and weigh promptly.]

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>
- **B. IDENTIFICATION TESTS—GENERAL**, *Sodium* <191>: Meets the requirements

Sample solution: 0.5 g in 10 mL of water

COMPOSITION

• CONTENT OF CHONDROITIN SULFATE SODIUM

Standard solutions: 1.5, 1.0, and 0.5 mg/mL of USP Chondroitin Sulfate Sodium RS in water

Sample solution: Transfer 100 mg of dried Chondroitin Sulfate Sodium into a 100-mL volumetric flask, dissolve in 30 mL of water, and dilute with water to volume.

Diluent: Weigh about 297 mg of monobasic potassium phosphate, 492 mg of dibasic potassium phosphate, and 250 mg of polysorbate 80, and transfer into a 1-L beaker. Dissolve in 900 mL of water, and adjust with potassium hydroxide or phosphoric acid to a pH of 7.0 ± 0.2. Dilute with water to 1 L, and mix thoroughly.

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Photometric titration

Titrant: 1 mg/mL of cetylpyridinium chloride in water. Degas before use.

Endpoint detection: Turbidimetric with a photoelectric probe

Analysis: Transfer 5.0 mL of each *Standard solution* and the *Sample solution* to separate titration vessels, and add 25 mL of *Diluent* to each. Stir until a steady reading is obtained with a phototrode either at 420, 550, or 660 nm. Set the instrument to zero in absorbance mode. Titrate with *Titrant* using the phototrode to determine the endpoint turbidimetrically. From a linear regression equation, calculated using the volumes of *Titrant* consumed versus concentrations of the *Standard solutions*, determine the concentration of chondroitin sulfate sodium in the *Sample solution*.

Calculate the percentage of chondroitin sulfate sodium in the portion Chondroitin Sulfate Sodium taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of chondroitin sulfate sodium in the aliquot of the *Sample solution*, obtained from the regression equation (mg/mL)

C_U = concentration of Chondroitin Sulfate Sodium in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–105.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** <281>: 20.0%–30.0% on the dried basis
- **CHLORIDE AND SULFATE**, *Chloride* <221>: NMT 0.50%; a 0.10-g portion shows no more chloride than corresponds to 0.7 mL of 0.020 N hydrochloric acid.
- **CHLORIDE AND SULFATE**, *Sulfate* <221>

Sample solution: Dissolve 200 mg in 40 mL of water.

Add 10 mL of a solution of cetylpyridinium chloride having a concentration of 30 mg/mL, and pass through a filter. Use a 25-mL portion of the filtrate.

Acceptance criteria: NMT 0.24%; the *Sample solution* shows no more sulfate than corresponds to 0.25 mL of 0.020 N sulfuric acid.

• ELECTROPHORETIC PURITY

(See *Electrophoresis* <726>.)

Barium acetate buffer: Dissolve 25.24 g of barium acetate in 900 mL of water. Adjust with acetic acid to a pH of 5.0, and dilute with water to 1000 mL.

Staining reagent: Dissolve 1 g of toluidine blue in 1000 mL of 0.1 M acetic acid.

Standard solution A: 30 mg/mL of USP Chondroitin Sulfate Sodium RS in water

Standard solution B: Dilute 1 mL of *Standard solution A* with water to 50 mL.

Sample solution: 30 mg/mL of Chondroitin Sulfate Sodium in water

Analysis: Fill the chambers of an electrophoresis apparatus suitable for separations on cellulose acetate

membranes¹ (a small submarine gel chamber or one dedicated to membrane media) with *Barium acetate buffer*. Soak a cellulose acetate membrane, 5–6 cm × 12–14 cm, in *Barium acetate buffer* for 10 min, or until evenly wetted, then blot dry between two sheets of absorbent paper. Using an applicator² suitable for electrophoresis, apply equal volumes (0.5 µL) of the *Sample solution*, *Standard solution A*, and *Standard solution B* to the brighter side of the membrane held in position in an appropriate applicator stand or on a separating bridge in the chamber. Ensure that both ends of the membrane are dipped at least 0.5–1.0 cm deep into the buffer chambers. Apply a constant 60 volts (6 mA at the start) for 2 h. [NOTE—Perform the application of solutions and voltage within 5 min because further drying of the blotted paper reduces sensitivity.]

Place the membrane in a plastic staining tray, and with the application side down, float or gently immerse in *Staining reagent* for 5 min. Then stir the solution gently for 1 min. Remove the membrane, and destain in 5% acetic acid until the background clears. Compare the bands.

[NOTE—Document the results by taking a picture within 15 min of completion of destaining.]

Acceptance criteria: The electropherogram from the *Sample solution* exhibits a major band that is identical in position to the band from *Standard solution A*. The band from *Standard solution B* is clearly visible at a mobility similar to the band from *Standard solution A*. Any secondary band in the electropherogram of the *Sample solution* is not more intense than the band from *Standard solution B*. NMT 2% of any individual impurity is found. [NOTE—Document the results by taking a picture within 15 min of completion of destaining.]

• LIMIT OF PROTEIN

Solution A: 20 mg/mL of sodium tartrate dihydrate

Solution B: 10 mg/mL of cupric sulfate

Solution C: 20 mg/mL of anhydrous sodium carbonate in 0.1 M sodium hydroxide

Dilute Folin-Ciocalteu reagent: Dilute Folin-Ciocalteu phenol TS with water (1:5). Prepare immediately before use.

Alkaline cupric tartaric reagent: Mix 1 mL each of *Solution A* and *Solution B*, and to the mixture slowly add 100 mL of *Solution C* with stirring. Use within 24 h, and discard afterward.

Standard solution: 36 µg/mL of bovine serum albumin certified standard in water

Sample solution: Transfer a portion of Chondroitin Sulfate Sodium, equivalent to 60 mg of the dried substance, to a 100-mL volumetric flask, and dissolve in and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Analytical wavelength: 750 nm

Blank: Water

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank*. Add 2.0 mL of freshly prepared *Alkaline cupric tartaric reagent* to test tubes containing 2.0 mL of the *Standard solution*, 2.0 mL of the *Sample solution*, or 2.0 mL of the *Blank*. After 10 min, add 1.0 mL of *Dilute Folin-Ciocalteu reagent* to each test tube, and mix immediately and vigorously. After 30 min, measure the absorbance of the *Standard solution* and *Sample solution* against the *Blank*.

Acceptance criteria: NMT 6.0% on the dried basis; the absorbance of the *Sample solution* is NMT the absorbance of the *Standard solution*.

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** <2021>: The total bacterial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g.
- **MICROBIAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements of the tests for absence of *Salmonella* species, and *Escherichia coli*.
- **HEAVY METALS**, *Method II* <231>: NMT 20 ppm

SPECIFIC TESTS

• CLARITY AND COLOR OF SOLUTION

Sample solution: Transfer 2.5 g of Chondroitin Sulfate Sodium to a 50-mL volumetric flask. Dissolve in and dilute with carbon dioxide-free water to volume, and examine immediately.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Analytical wavelength: 420 nm

Cell: 1 cm

Blank: Carbon dioxide-free water

Analysis: Measure the absorbance of the *Sample solution*.

Acceptance criteria: Its absorbance is NMT 0.35.

- **OPTICAL ROTATION**, *Specific Rotation* <781S>: −20.0° to −30.0°

Sample solution: 30 mg/mL

- **PH** <791>: 5.5–7.5, in a solution (1 in 100)

- **LOSS ON DRYING** <731>: Dry a sample at 105° for 4 h: it loses NMT 10.0% of its weight. [NOTE—Chondroitin Sulfate Sodium is extremely hygroscopic once dried. Avoid exposure to the atmosphere, and weigh promptly.]

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to state the source(s) from which the article was derived, whether bovine, porcine, avian, or a mixture of any of them.
- **USP REFERENCE STANDARDS** <11>
USP Chondroitin Sulfate Sodium RS

Chondroitin Sulfate Sodium Tablets

DEFINITION

Chondroitin Sulfate Sodium Tablets contain NLT 90.0% and NMT 120.0% of the labeled amount of chondroitin sulfate sodium.

[NOTE—Chondroitin Sulfate Sodium is extremely hygroscopic once dried. Avoid exposure to the atmosphere, and weigh promptly.]

IDENTIFICATION

• A. ELECTROPHORESIS <726>

Barium acetate buffer: Dissolve 25.24 g of barium acetate in 900 mL of water. Adjust with acetic acid to a pH of 5.0, and dilute with water to 1000 mL.

Staining reagent: 0.1% (w/v) toluidine blue in 0.1 M acetic acid

Standard solution: Use the *Standard solution* of middle concentration from the *Content of Chondroitin Sulfate Sodium*.

Sample solution: Prepare as directed in the *Content of Chondroitin Sulfate Sodium*.

Analysis: Fill the chambers of an electrophoresis apparatus suitable for separations on cellulose acetate mem-

¹ Suitable cellulose acetate membranes for electrophoresis are available from Malta Chemetron SRL, Milano, Italy (www.maltachemetron.com); Fluka Chemical Corp., Milwaukee, WI; and DiaSys Corp., Waterbury, CT (www.diasys.com).

² Suitable applicators are available from DiaSys Corp., Waterbury, CT (www.diasys.com) and Helena Laboratories, Beaumont, TX (www.helena.com).

branes¹ (a small submarine gel chamber or one dedicated to membrane media) with *Barium acetate buffer*. Soak a cellulose acetate membrane 5–6 cm × 12–14 cm in *Barium acetate buffer* for 10 min, or until evenly wetted, then blot dry between two sheets of absorbent paper. Using an applicator² suitable for electrophoresis, apply equal volumes (0.5 µL) of the *Sample solution* and *Standard solution* to the brighter side of the membrane held in position in an appropriate applicator stand or on a separating bridge in the chamber. Ensure that both ends of the membrane are dipped at least 0.5–1.0-cm deep into the buffer chambers. Apply a constant 60 volts (6 mA at the start) for 2 h. [NOTE—Perform the application of solutions and voltage within 5 min because further drying of the blotted paper reduces sensitivity.]

Place the membrane in a plastic staining tray, and with the application side down, float or gently immerse in *Staining reagent* for 5 min. Then stir the solution gently for 1 min. Remove the membrane, and destain in 5% acetic acid until the background clears.

Acceptance criteria: The principal spot from the *Sample solution* has the same migration as the principal spot from the *Standard solution*. [NOTE—Document the results by taking a picture within 15 min of completion of destaining.]

STRENGTH

• CONTENT OF CHONDROITIN SULFATE SODIUM

Standard solutions: 1.5, 1.0, and 0.5 mg/mL of USP Chondroitin Sulfate Sodium RS in water

Sample solution: Transfer an equivalent to 100 mg of chondroitin sulfate sodium from NLT 20 Tablets, finely powdered, to 60 mL of water, and shake to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, stir or shake for 5 min, dilute with water to 100 mL, and centrifuge or pass through a suitable filter.

Diluent: Weigh about 297 mg of monobasic potassium phosphate, 492 mg of dibasic potassium phosphate, and 250 mg of polysorbate 80, and transfer into a 1-L beaker. Dissolve in approximately 900 mL of water, and adjust with potassium hydroxide or phosphoric acid to a pH of 7.0 ± 0.2. Dilute with water to 1 L, and mix thoroughly.

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Photometric titration

Titrant: 1 mg/mL of cetylpyridinium chloride in water

Endpoint detection: Turbidimetric with photoelectric probe

Analysis: Transfer 5.0 mL of each *Standard solution* and the *Sample solution* to separate titration vessels, and add 25 mL of *Diluent* to each. Stir until a steady reading is obtained with a photoelectric probe either at 420, 550, or 660 nm. Set the instrument to zero in absorbance mode. Titrate with *Titrant* using the photoelectric probe to determine the endpoint turbidimetrically. From a linear regression equation, calculated using the volumes of *Titrant* consumed versus concentrations of the *Standard solutions*, determine the concentration of chondroitin sulfate sodium in the *Sample solution*.

Calculate the percentage of the labeled amount of chondroitin sulfate sodium in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

C_U = nominal concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–120.0% of the label claim

PERFORMANCE TESTS

• DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS

<2040>: Meet the requirements for *Dissolution*

Medium: Water; 900 mL

Apparatus 2: 75 rpm

Time: 60 min

Titrant and Diluent: Prepare as directed as in *Content of Chondroitin Sulfate Sodium*.

Standard solutions: 1.5, 1.0, and 0.5 mg/mL of USP Chondroitin Sulfate Sodium RS in water

Sample solution: Combine equal portions of the solutions withdrawn from 6 dissolution vessels and pass through a suitable filter; use the pooled sample as the test specimen.

Analysis: Transfer 5.0 mL of each *Standard solution*, and an aliquot of the *Sample solution* equivalent to about 5 mg of chondroitin sulfate sodium, to separate titration vessels. Add 25 mL of *Diluent* to each titration vessel. Stir until a steady reading is obtained with a photoelectric probe. Set the instrument to zero in absorbance mode. Titrate with *Titrant* using the photoelectric probe to determine the endpoint turbidimetrically, either at 420, 550, or 660 nm. From a linear regression equation, calculated using the volumes of *Titrant* consumed versus amount, in mg, of chondroitin sulfate sodium from each *Standard solution*, determine the amount, in mg, of chondroitin sulfate sodium in the aliquot of *Sample solution* taken. Calculate the percentage of the labeled amount of chondroitin sulfate sodium dissolved:

$$\text{Result} = (Ws/a) \times (V/L) \times 100$$

Ws = amount of chondroitin sulfate sodium in the aliquot of the *Sample solution* taken (mg)

a = volume of the aliquot of *Sample solution* taken

V = volume of *Medium*, 900 mL

L = label claim of chondroitin sulfate sodium (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of chondroitin sulfate sodium is dissolved.

• WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>: Meet the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.

• **LABELING:** Label it to indicate the species of the source from which the chondroitin used to prepare the Tablets was derived. Label it to state the source(s) of chondroitin sulfate sodium, whether bovine, porcine, avian, or a mixture of any of them. The label states on the front panel the content of chondroitin sulfate sodium on the dried basis.

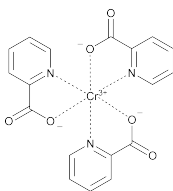
• USP REFERENCE STANDARDS <11>

USP Chondroitin Sulfate Sodium RS

¹ Suitable cellulose acetate membranes for electrophoresis are available from Malta Chemetron SRL, Milano, Italy (www.maltachemetron.com); Fluka Chemical Corp., Milwaukee, WI; and DiaSys Corp., Waterbury, CT (www.diasys.com).

² Suitable applicators are available from DiaSys Corp., Waterbury, CT (www.diasys.com) and Helena Laboratories, Beaumont, TX (www.helena.com).

Chromium Picolinate



$C_{18}H_{12}N_3O_6Cr$ 418.30
Chromium Tripicolinate [14639-25-9].

DEFINITION

Chromium Picolinate contains NLT 98.0% and NMT 102.0% of chromium picolinate ($C_{18}H_{12}N_3O_6Cr$), calculated on the dried basis.

IDENTIFICATION

• A. INFRARED ABSORPTION <197M>

• B.

Sample solution: 4 mg/mL

Analysis: To 5 mL of the *Sample solution* add 1 mL of 5 N sodium hydroxide and 10 drops of 30% hydrogen peroxide, and heat gently for 2 min.

Acceptance criteria: A yellow color develops.

ASSAY

• PROCEDURE

Standard stock solution: 100 µg/mL of chromium. Transfer 0.283 g of potassium dichromate, previously dried at 120° for 4 h, to a 1000-mL volumetric flask, and dilute with water to volume. Store in a polyethylene bottle.

Standard solutions: 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium. Separately transfer 1.0 and 2.0 mL of the *Standard stock solution* to 100-mL volumetric flasks, and transfer 1.5 and 2.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Add 1.0 mL of nitric acid to each flask, and dilute the contents of each flask with water to volume.

Sample solution: Transfer 200 mg of Chromium Picolinate to a 100-mL beaker, and add 25 mL of water. Slowly add 10 mL of nitric acid, and boil for 10 min with constant swirling. Cool the solution, quantitatively transfer to a 500-mL volumetric flask, and dilute with water to volume. Filter a portion of the solution, and transfer 5.0 mL of the filtrate to a 100-mL volumetric flask. Add 1 mL of nitric acid, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 357.9 nm

Lamp: Chromium hollow-cathode

Flame: Air-acetylene

Blank: Diluted nitric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the *Standard solutions* and the *Sample solution*. Plot the absorbances of the *Standard solutions* versus the chromium concentration, in µg/mL, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the chromium concentration, in µg/mL, in the *Sample solution*.

Calculate the percentage of chromium picolinate ($C_{18}H_{12}N_3O_6Cr$) in the portion of Chromium Picolinate taken:

$$\text{Result} = (C_{Cr}/C_U) \times (M_r/A_r) \times 100$$

C_{Cr} = concentration of chromium in the *Sample solution*, obtained from the graph (µg/mL)

C_U = concentration of Chromium Picolinate in the *Sample solution* (µg/mL)

M_r = molecular weight of chromium picolinate, 418.31

A_r = atomic weight of chromium, 51.996

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

• CHLORIDE AND SULFATE, Chloride <221>

Sample solution: Dissolve 30 mg of Chromium Picolinate in 30–40 mL of water, and heat to 70°. Cool overnight, and filter to remove the precipitate.

Analysis: Add 1 mL each of nitric acid and silver nitrate TS, and add sufficient water to make 50 mL. Mix, and allow to stand for 5 min, protected from direct sunlight.

Acceptance criteria: Any turbidity formed is NMT that produced in a similarly treated control solution containing 0.25 mL of 0.002 N hydrochloric acid (NMT 0.06%).

• CHLORIDE AND SULFATE, Sulfate <221>

Sample solution: Dissolve 100 mg of Chromium Picolinate in 30–40 mL of water, and heat to 90°. Cool overnight, and filter to remove the precipitate.

Analysis: Add 1 mL of 3 N hydrochloric acid, 3 mL of barium chloride TS, and sufficient water to make 50 mL. Mix, and allow to stand for 10 min.

Acceptance criteria: Any turbidity formed is NMT that produced in a similarly treated control solution containing 0.2 mL of 0.02 N sulfuric acid (NMT 0.2%).

SPECIFIC TESTS

• **LOSS ON DRYING** <731>: Dry a sample at 105° for 4 h: it loses NMT 4.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **USP REFERENCE STANDARDS** <11>
USP Chromium Picolinate RS

Chromium Picolinate Tablets

DEFINITION

Chromium Picolinate Tablets contain NLT 95.0% and NMT 125.0% of the labeled amount of chromium (Cr).

IDENTIFICATION

• **A.** The *Sample solution* prepared as directed in the test for *Strength* gives a positive test for chromium, determined at 357.9 nm using the *Instrumental conditions* in the test for *Content of Chromium*.

STRENGTH

• CONTENT OF CHROMIUM

Standard stock solution A: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h, in water. Store in a polyethylene bottle.

Standard stock solution B: Transfer 1.0 mL of *Standard stock solution A* to a 100-mL volumetric flask, add 5.0 mL of 6 N hydrochloric acid, and dilute with water to volume to obtain a solution having a concentration of 10 µg/mL of chromium.

Standard solutions: Dilute *Standard stock solution B* with 0.125 N hydrochloric acid to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a porcelain crucible, heat the crucible in a muffle furnace maintained at about 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, inter-

mittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, mix, and filter, discarding the first 5 mL of the filtrate. Dilute this solution with 0.125 N hydrochloric acid to obtain a solution having a concentration of 2.5 µg/mL of chromium.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Chromium hollow-cathode

Flame: Air-acetylene

Analytical wavelength: 357.9 nm (chromium emission line)

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the *Samples*, using the *Blank*. From a linear regression equation, calculated using the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of chromium in the *Sample solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 95.0%–125.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** (2040): Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** (2091): Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.

Clover, Red—see *Red Clover*

Cod Liver Oil—see *Cod Liver Oil General Monographs*

Cod Liver Oil Capsules

DEFINITION

Cod Liver Oil Capsules contain NLT 95.0% and NMT 105.0% of the labeled amount of Cod Liver Oil, where Cod Liver Oil is the partially destearinated fixed oil obtained from fresh livers of *Gadus morrhua* L. and other species of Fam. Gadidae. Cod Liver Oil contains, in each g, NLT 180 µg (600 USP Units) and NMT 750 µg (2500 USP Units) of vitamin A and NLT 1.5 µg (60 USP Units) and NMT 6.25 µg (250 USP Units) of vitamin D.

Cod Liver Oil may be flavored by the addition of NMT 1% of a suitable flavor or a mixture of flavors. A suitable antioxidant may be added.

IDENTIFICATION

• A. PRESENCE OF VITAMIN A

Sample solution: 25 mg/mL of oil contained in the Capsules in chloroform

Analysis: To 1 mL of the *Sample solution* add 10 mL of antimony trichloride TS.

Acceptance criteria: A blue color results immediately.

• B. FATTY ACID PROFILE

Antioxidant solution: 0.05 mg/mL of butylated hydroxytoluene in hexanes

System suitability solution: Prepare a mixture containing equal amounts of methyl palmitate, methyl stearate, methyl arachidate, and methyl behenate in *Antioxidant solution*.

Standard stock solution: 45 mg/mL of USP Cod Liver Oil RS in *Antioxidant solution*

Standard solution: Transfer 2.0 mL of the *Standard stock solution* into a quartz tube, and evaporate with a gentle stream of nitrogen. Add 1.5 mL of a 2% solution of sodium hydroxide in methanol. Cap tightly with a polytetrafluoroethylene-lined cap, mix, and heat in a water bath for 7 min. Cool, add 2 mL of a 120 mg/mL solution of boron trichloride in methanol. Cover with nitrogen, cap tightly, and mix. Heat in a water bath for 30 min. Cool to 40°–50°. Add 1 mL of isooctane, cap, and mix in a vortex mixer or shake vigorously for at least 30 s. Immediately add 5 mL of saturated sodium chloride solution. Cover with nitrogen, cap, and mix in a vortex mixer or shake thoroughly for at least 15 s. Allow the upper layer to become clear, and transfer to a separate tube. Shake the methanol layer once more with 1 mL of isooctane, and combine the isooctane extracts. Wash the combined extracts twice with 1 mL of water, and dry over anhydrous sodium sulfate.

Sample solution: Proceed as directed in the *Standard solution*, except use a weighed quantity of the oil contained in the Capsules.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused silica capillary column coated with a 0.25-µm film of G16

Temperature

Injector: 250°

Detector: 280°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
170	1	225	20

Carrier gas: Helium

Split flow ratio: 1:200

Injection size: 1 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram from the *Standard solution* is similar to the Reference Chromatogram supplied with USP Cod Liver Oil RS. Identify the retention times of the relevant fatty acid methyl esters by comparing the chromatogram of the *Standard solution* with the Reference Chromatogram supplied with USP Cod Liver Oil RS.

Resolution: NLT 1.3 between methyl oleate and methyl *cis*-vaccinate, and that between methyl gadoleate and methyl gondoate is sufficient for purposes of identification and area measurement, *Standard solution*

Theoretical area percentages: 24.4 ± 1 for methyl palmitate, 24.8 ± 1 for methyl stearate, 25.2 ± 1 for methyl arachidate, and 25.6 ± 1 for methyl behenate, *System suitability solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the retention times of the relevant fatty acid methyl esters in the *Sample solution* by comparing the chromatogram of the *Sample solution* with that of the *Standard solution*.

Determine the number of fatty acid methyl ester peaks in the *Sample solution*: The number of fatty acid methyl ester peaks exceeding 0.05% of the total area of fatty acid methyl esters is at least 24, and the 24 largest peaks of the methyl esters account for more than 90% of the total area. (These correspond to the following, in common elution order: 14:0, 15:0, 16:0, 16:1 n-7, 16:4 n-1, 18:0, 18:1 n-9, 18:1 n-7, 18:2 n-6, 18:3 n-3, 18:4 n-3, 20:1 n-11, 20:1 n-9, 20:1 n-7, 20:2 n-6, 20:4 n-6, 20:3 n-3, 20:4 n-3, 20:5 n-3, 22:1 n-11, 22:1 n-9, 21:5 n-3, 22:5 n-3, and 22:6 n-3.) Calculate the area percentage for each fatty acid methyl ester in the portion of Capsules taken:

$$\text{Result} = (r_A/r_B) \times 100$$

r_A = peak area of each individual fatty acid
 r_B = total area from all peaks, except the solvent peak and butylated hydroxytoluene

Acceptance criteria: The *Sample solution* meets the limits described in *Table 2*.

Table 2

Fatty Acid	Shorthand Notation	Lower Limit (area %)	Upper Limit (area %)
Saturated fatty acids			
Myristic acid	14:0	2.0	6.0
Palmitic acid	16:0	7.0	14.0
Stearic acid	18:0	1.0	4.0
Monounsaturated fatty acids			
Palmitoleic acid	16:1 n-7	4.5	11.5
cis-Vaccenic acid	18:1 n-7	2.0	7.0
Oleic acid	18:1 n-9	12.0	21.0
Gadoleic acid	20:1 n-11	1.0	5.5
Gondoic acid	20:1 n-9	5.0	17.0
Erucic acid	22:1 n-9	0	1.5
Cetoleic acid	22:1 n-11	5.0	12.0
Polyunsaturated fatty acids			
Linoleic acid	18:2 n-6	0.5	3.0
α-Linolenic acid	18:3 n-3	0	2.0
Moroctic acid	18:4 n-3	0.5	4.5
Eicosapentaenoic acid	20:5 n-3	7.0	16.0
Docosahexaenoic acid	22:6 n-3	6.0	18.0

STRENGTH

• VITAMIN A

Sample: 500 mg to 1 g of oil contained in the Capsules

Analysis: Proceed as directed under *Vitamin A Assay* (571).

Acceptance criteria: 180 µg (600 USP Units) to 750 µg (2500 USP Units) of vitamin A per g of oil contained in the Capsules

• VITAMIN D

Solution A: *n*-Amyl alcohol and dehydrated hexane (3:997)

Solution B: Acetonitrile, water, and phosphoric acid (96:3.8:0.2)

Butylated hydroxytoluene solution: 10 mg/mL of butylated hydroxytoluene in chromatographic hexane

Aqueous potassium hydroxide solution: 800 mg/mL of potassium hydroxide in freshly boiled water. Mix, and cool. [NOTE—Prepare this solution fresh daily.]

Alcoholic potassium hydroxide solution: Dissolve 3 g of potassium hydroxide in 50 mL of freshly boiled water. Add 10 mL of alcohol, and dilute with freshly boiled water to 100 mL. [NOTE—Prepare this solution fresh daily.]

Ascorbic acid solution: 100 mg/mL of ascorbic acid in water. [NOTE—Prepare this solution fresh daily.]

Internal standard solution: 5 µg/mL of USP

Ergocalciferol RS in alcohol

Standard stock solution: 5 µg/mL of USP

Cholecalciferol RS in alcohol

Standard solution: Transfer 2.0 mL of the *Standard stock solution* and 2.0 mL of the *Internal standard solution* to a round-bottomed flask. Proceed as directed in *Sample solution 1*, beginning with "Add 5 mL of...".

Sample solution 1: Transfer a quantity equivalent to 4.00 g of oil contained in the Capsules to a round-bottomed flask. Add 5 mL of *Ascorbic acid solution*, 100 mL of alcohol, and 10 mL of *Aqueous potassium hydroxide solution*, and mix. Reflux the mixture on a steam bath for 30 min. Add 100 mL of a 10 mg/mL sodium chloride solution. Cool rapidly under running water, and transfer the saponified mixture to a 500-mL separator, rinsing the saponification flask with 75 mL of a 10 mg/mL sodium chloride solution and then with 150 mL of a mixture of ether and hexane (1:1). Shake the combined saponified mixture and rinsings vigorously for 30 s, and allow to stand until both layers are clear. Discard the lower layer. Wash the ether-hexane extracts by shaking vigorously with 50 mL of *Alcoholic potassium hydroxide solution*, and then washing with three 50-mL portions of a 10 mg/mL sodium chloride solution. Pass the upper layer through 5 g of anhydrous sodium sulfate on a fast filter paper into a 250-mL flask suitable for a rotary evaporator. Wash the filter with 10 mL of a mixture of ether and hexane (1:1), and combine with the extract. Evaporate the solvent at reduced pressure at a temperature not exceeding 30°, and fill with nitrogen when the evaporation is complete. Alternatively evaporate the solvent under a gentle stream of nitrogen at a temperature not exceeding 30°. Dissolve the residue in 1.5 mL of *Solution A*. [NOTE—Gentle heating in an ultrasonic bath may be required. A large fraction of the white residue is cholesterol.]

Sample solution 2: To 4.00 g of oil contained in the Capsules add 2.0 mL of *Internal standard solution*, and proceed as directed for *Sample solution 1*, beginning with "Add 5 mL of...".

Clean-up chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Mobile phase: *Solution A*

Column: 25-mm × 4.6-cm stainless steel; packing L10

Injection size: 350 µL

Analysis (clean-up)

Samples: *Standard solution*, *Sample solution 1*, and *Sample solution 2*

Collect separately the eluates from 2 min before until 2 min after the retention time of cholecalciferol in a glass tube containing 1 mL of *Butylated hydroxytoluene solution* and fitted with a hermetic closure. Evaporate each tube under a stream of nitrogen at a temperature not exceeding 30°. Dissolve each residue in 1.5 mL of acetonitrile.

Analytical chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Mobile phase: *Solution B*

Column: 15-mm × 4.6-cm stainless steel; 5-μm packing L1

Injection size: 200 μL

System suitability

Sample: *Standard solution* (after the clean-up)

Suitability requirements

Resolution: NLT 1.4 between cholecalciferol and ergocalciferol

Relative standard deviation: NMT 2.0% for the cholecalciferol peak from replicate injections

Analysis

Samples: *Standard solution, Sample solution 1, and Sample solution 2* (after the clean-up)

Calculate the content of vitamin D, in μg/g, in the portion of oil taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U)$$

R_U = response of cholecalciferol relative to the corrected internal standard in *Sample solution 2*, as calculated below

R_S = response of cholecalciferol relative to the internal standard in the *Standard solution*

C_S = concentration of USP Cholecalciferol RS in the *Standard solution* (μg/mL)

C_U = concentration of oil in *Sample solution 2* (g/mL)

$$R_U = r_{U2}/[r_{S2} - (r_{S1} \times r_{U2}/r_{U1})]$$

r_{U2} = peak response of cholecalciferol from *Sample solution 2*

r_{S2} = peak response of the internal standard from *Sample solution 2*

r_{S1} = peak response of the internal standard from *Sample solution 1*

r_{U1} = peak response of cholecalciferol from *Sample solution 1*

Acceptance criteria: 1.5 μg (60 USP Units) to 6.25 μg (250 USP Units) of vitamin D per g of oil contained in the Capsules

• CONTENT OF COD LIVER OIL

Analysis: Weigh NLT 10 Capsules in a tared weighing bottle. With a sharp blade, or by other appropriate means, carefully open the Capsules, without loss of shell material, and transfer the combined Capsule contents to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of isooctane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the isooctane is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average net weight per Capsule.

Acceptance criteria: 95.0%–105.0% of the labeled amount of cod liver oil

PERFORMANCE TESTS

- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements
- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** <2040>: Meet the requirements for *Rupture Test for Soft Shell Capsules*

SPECIFIC TESTS

- **FATS AND FIXED OILS, Unsaponifiable Matter** <401>: NMT 1.30%
- **FATS AND FIXED OILS, Acid Value** <401>
Sample solution: Mix 15 mL of alcohol with 15 mL of ether, add 5 drops of phenolphthalein TS, and neutralize with 0.1 N sodium hydroxide. Dissolve 2.0 g

of oil contained in the Capsules in the mixture, and boil the oil solution gently under a reflux condenser for 10 min.

Analysis: Cool, and titrate the mixture with 0.1 N sodium hydroxide VS to the production of a pink color that persists after shaking for 30 s.

Acceptance criteria: NMT 1.0 mL of 0.1 N sodium hydroxide is required.

- **FATS AND FIXED OILS, Iodine Value** <401>: 145–180
- **FATS AND FIXED OILS, Saponification Value** <401>: 180–192
[NOTE—If carbon dioxide has been used as a preservative, expose the oil contained in the Capsules in a shallow dish in a vacuum desiccator for 24 h before weighing the specimen for determination of the saponification value.]

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers at room temperature. Protect from light.
- **LABELING:** Vitamin A potency and vitamin D potency, when designated on the label, may be expressed in USP Units per g of oil. The potency may also be expressed in metric units, on the basis that 1 USP Vitamin A Unit = 0.3 μg of all-*trans* retinol and 40 USP Vitamin D Units = 1 μg. Label them to emphasize the need to avoid freezing or exposure to excessive humidity or to a temperature above 40°. Where the content of docosahexaenoic acid and eicosapentaenoic acid is claimed, state the amount in mg per Capsule on the label.
- **USP REFERENCE STANDARDS** <11>
USP Cholecalciferol RS
USP Cod Liver Oil RS
USP Ergocalciferol RS
USP Vitamin A RS

Cohosh, Black—see *Black Cohosh*

Copper Gluconate—see *Copper Gluconate General Monographs*

Co-Q10—see *Ubidecarenone in Dietary Supplements section*

Cranberry Liquid Preparation

DEFINITION

Cranberry Liquid Preparation is a bright red juice derived from the fruits of *Vaccinium macrocarpon* Ait. or *Vaccinium oxycoccos* L. (Fam. Ericaceae). It contains no added substances.

IDENTIFICATION

- **A. HPLC** The retention times of the quinic acid, malic acid, and citric acid peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Content of Organic Acids*.
- **B. ABSENCE OF ADULTERANTS**
Standard solution: 1.0 mg/mL of tartaric acid and 0.1 mg/mL of fumaric acid
Sample solution: Use the Liquid Preparation.
Mobile phase and Chromatographic system: Proceed as directed in the test for *Content of Organic Acids*.

Analysis**Samples:** *Standard solution* and *Sample solution***Injection size:** 20 μ L**Acceptance criteria:** The retention times of the tartaric acid and fumaric acid peaks of the *Standard solution* do not correspond to any of the retention times for peaks observed from the *Sample solution*.**COMPOSITION****• CONTENT OF DEXTROSE AND FRUCTOSE****Mobile phase:** Water**Standard solution:** 6.0 mg/mL of USP Dextrose RS and 2.0 mg/mL of USP Fructose RS in water**Sample solution:** Transfer 1.0 g of sodium carboxylate cation-exchange resin to a 50-mL beaker, add 5 mL of water to make a slurry, and transfer the slurry to a polypropylene automatic pipet fitted with a small plug of silanized glass wool. Quantitatively transfer the slurry to a small chromatographic tube, rinsing the beaker with water and packing the column evenly. Keep the column wet until ready for use. Using a volumetric pipet, transfer 1.0 mL of Liquid Preparation to the column, collect the eluate, and discard it. Pipet 4.0 mL of water onto the top of the column, collect the eluate in a clean vial, and filter if necessary.**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** Refractive index**Columns****Guard:** Packing L19**Analytical:** 7.8-mm \times 30-cm; packing L19**Column temperature:** 85°**Flow rate:** 0.6 mL/min**Injection size:** 20 μ L**System suitability****Sample:** *Standard solution*

[NOTE—The approximate relative retention times for dextrose and fructose are about 0.8 and 1.0, respectively.]

Suitability requirements**Resolution:** NLT 1.8 between the dextrose and fructose peaks**Relative standard deviation:** NMT 2.0%**Analysis****Samples:** *Standard solution* and *Sample solution*

Calculate the percentages of dextrose and fructose in the volume of Liquid Preparation taken:

$$\text{Result} = (r_U/r_S) \times (C_S/V) \times 0.5$$

 r_U = peak response of each appropriate analyte from the *Sample solution* r_S = peak response of each appropriate analyte from the *Standard solution* C_S = concentration of the appropriate USP Reference Standard in the *Standard solution* (mg/mL) V = volume of Liquid Preparation taken for the *Sample solution* (mL)**Acceptance criteria:** NLT 2.4% dextrose and NLT 0.7% fructose**• CONTENT OF ORGANIC ACIDS****Mobile phase:** Transfer 27.2 g of monobasic potassium phosphate to a 1000-mL volumetric flask, and dissolve in 950 mL of water. Adjust with phosphoric acid to a pH of 2.4, and dilute with water to volume.**Standard solution:** 1.0 mg/mL each of USP Citric Acid RS, USP Malic Acid RS, and USP Quinic Acid RS**Sample solution:** Use the filtered Liquid Preparation.**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 214 nm**Columns****Guard:** 5- μ m; packing L1[NOTE—Before use, condition the column with methanol, then with water, and finally with *Mobile phase*.]**Analytical:** 4.6-mm \times 25-cm analytical; packing L1**Flow rate:** 0.6 mL/min**Injection size:** 20 μ L**System suitability****Sample:** *Standard solution*

[NOTE—The approximate relative retention times for quinic acid, malic acid, and citric acid are 0.4, 0.5, and 1.0, respectively.]

Suitability requirements**Resolution:** NLT 2.5 between quinic acid and malic acid**Relative standard deviation:** NMT 2.0%**Analysis****Samples:** *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentages of quinic acid, malic acid, and citric acid in the volume of Liquid Preparation taken:

$$\text{Result} = (r_U/r_S) \times C_S \times 0.1$$

 r_U = peak area of each appropriate analyte from the *Sample solution* r_S = peak area of each appropriate analyte from the *Standard solution* C_S = concentration of the appropriate USP Reference Standard in the *Standard solution* (mg/mL)**Acceptance criteria:** NLT 0.9% each of quinic acid and citric acid; NLT 0.7% of malic acid. The ratio of quinic acid to malic acid is NLT 1.0.**ADULTERANTS****• LIMIT OF SORBITOL AND SUCROSE****Mobile phase and Sample solution:** Prepare as directed in the test for *Content of Dextrose and Fructose*.**Standard solution:** 0.5 mg/mL each of USP Sorbitol RS and USP Sucrose RS**Chromatographic system:** Proceed as directed in the test for *Content of Dextrose and Fructose*.**System suitability****Sample:** *Standard solution*

[NOTE—The relative retention times for sucrose and sorbitol are about 0.4 and 1.0, respectively.]

Suitability requirements**Resolution:** NLT 1.8 between the sucrose and sorbitol peaks**Relative standard deviation:** NMT 2.0%**Analysis****Samples:** *Standard solution* and *Sample solution***Injection size:** 20 μ L

Calculate the percentages of sucrose and sorbitol in the volume of Liquid Preparation taken:

$$\text{Result} = (r_U/r_S) \times (C_S/V) \times 0.5$$

 r_U = peak response of each appropriate analyte from the *Sample solution* r_S = peak response of each appropriate analyte from the *Standard solution* C_S = concentration of the appropriate USP Reference Standard in the *Standard solution* (mg/mL) V = volume of Liquid Preparation taken for the *Sample solution* (mL)**Acceptance criteria:** NMT 0.05% each of sorbitol and sucrose

SPECIFIC TESTS

- **REFRACTIVE INDEX** (831): 1.3435–1.3445
- **PH** (791): 2.5 ± 0.1

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store in a refrigerator.
- **LABELING:** The label states the Latin binomial name and, following the official name, the parts of the plant source from which the article was derived. The label also states that it is for manufacturing purposes only. This article is exempt from the requirements of the *General Notices* with respect to the pregnancy and lactation statement (section 10.40.50, *Labeling Botanical-Containing Products*).
- **USP REFERENCE STANDARDS** (11)
 - USP Citric Acid RS
 - USP Dextrose RS
 - USP Fructose RS
 - USP Malic Acid RS
 - USP Quinic Acid RS
 - USP Sorbitol RS
 - USP Sucrose RS

Cryptocodinium cohnii* Oil*DEFINITION**

Cryptocodinium cohnii Oil is obtained from the fermentation and extraction of algae of the species *Cryptocodinium cohnii* and contains NLT 35.0% (w/w) of docosahexaenoic acid (DHA, C₂₂H₃₂O₂) (C22: 6 n–3), as the only significant polyunsaturated fatty acid present. Suitable antioxidants in appropriate concentration may be added.

IDENTIFICATION

- **LONG-CHAIN UNSATURATED FATTY ACID PROFILE:** Proceed as directed in *Content of DHA*.

Analysis

Samples: *Standard Solution 2* and *Test Solution 1*
Calculate the area percentage for each fatty acid as methyl ester in *Test Solution 1*:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual fatty acid as methyl ester

r_T = sum of all the peak responses, except the solvent and butylated hydroxytoluene peaks

Acceptance criteria: The retention time of the peaks of the docosahexaenoic acid methyl ester and the eicosapentaenoic acid methyl ester from *Test Solution 1* corresponds to that from *Standard Solution 2*, as obtained in the test for *Content of EPA and DHA*. The area percent for the methyl esters of the fatty acids from *Test Solution 1* in the test for *Content of EPA and DHA* meet the requirements for each fatty acid indicated in the table below.

Fatty Acid	Relative Retention Time	Shorthand Notation	Lower Limit (Area, %)	Upper Limit (Area, %)
Linoleic acid	0.52	18:2 n–6	0	1.0
Eicosapentaenoic acid	0.79	20:5 n–3	0	0.1
Docosapentaenoic acid	0.94	22:5 n–6	0	0.1
Docosahexaenoic acid	1.00	22:6 n–3	35.0	47.0

COMPOSITION**• CONTENT OF DHA**

Analysis: Proceed as directed in *Fats and Fixed Oils* (401), *Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA*.

Acceptance criteria: NLT 35.0% (w/w) docosahexaenoic acid (DHA)

IMPURITIES**Inorganic Impurities****• LIMIT OF ARSENIC**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: Transfer 1 g of ultrapure palladium metal into a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution B: Transfer 1 g of ultrapure magnesium nitrate into a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (3:2:5). A volume of 5 μ L provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed under *Arsenic* (211), to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 μ g/mL of arsenic.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 μ g/mL of arsenic.

Sample solution: For preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytetrafluoroethylene liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes. [CAUTION—Wear proper eye protection and protective clothing and gloves.]

Transfer approximately 500 mg of *Cryptocodinium cohnii* Oil, weighed to the nearest 0.1 mg, into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. [NOTE—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the

microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115°, using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min; char the sample at 1000°, using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of *Crypthecodinium cohnii* Oil taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration as obtained above

W = weight of *Crypthecodinium cohnii* Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• LIMIT OF LEAD

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Lead Nitrate Stock Solution*, prepared as directed in *Heavy Metals* (231), to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of lead.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of lead.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon

flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2100°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of lead in the portion of *Crypthecodinium cohnii* Oil taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above

W = weight of *Crypthecodinium cohnii* Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• LIMIT OF CADMIUM

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytef, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytef, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid to volume (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution A: 0.1372 mg/mL of cadmium nitrate

Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before make up to final volume dissolve in a portion of water and nitric acid.]

Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the

Sample solution, and the *Blank*, followed by an injection of 5 μL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in $\mu\text{g}/\text{mL}$, and calculate the regression line best fitting the points.

Determine the concentration, C , in $\mu\text{g}/\text{mL}$, of cadmium in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of cadmium in the *Crypthecodinium cohnii* Oil taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above

W = weight of *Crypthecodinium cohnii* Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 $\mu\text{g}/\text{g}$

- **LIMIT OF MERCURY:** Proceed as directed in *Mercury* <261>, Method IIa, except use a *Standard Mercury Solution* having the equivalent of 0.1 $\mu\text{g}/\text{mL}$ of mercury.

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*, combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution*.

Acceptance criteria: NMT 0.1 $\mu\text{g}/\text{g}$

SPECIFIC TESTS

- **FATS AND FIXED OILS, Anisidine Value** <401>: NMT 20.0
- **FATS AND FIXED OILS, Acid Value (Free Fatty Acids)** <401>: The free fatty acids in 10 g require for neutralization NMT 1.42 mL of 0.1 N sodium hydroxide.
- **FATS AND FIXED OILS, Peroxide Value** <401>: NMT 5.0
- **FATS AND FIXED OILS, Total Oxidation Value (TOTOX)** <401>: NMT 26, calculated as

$$\text{Result} = (2 \times \text{PV}) + \text{AV}$$

PV = peroxide value

AV = anisidine value

- **FATS AND FIXED OILS, Unsaponifiable Matter** <401>: NMT 3.5%
- **SPECIFIC GRAVITY** <841>: 0.91–0.93

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.
- **LABELING:** The label states the content of docosahexaenoic acid in mg/g. It also states the name and concentration of any added antioxidant.
- **USP REFERENCE STANDARDS** <11>
 - USP Docosahexaenoic Acid Ethyl Ester RS
 - USP Eicosapentaenoic Acid Ethyl Ester RS
 - USP Methyl Tricosanoate RS

Crypthecodinium cohnii Oil Capsules

DEFINITION

Crypthecodinium cohnii Oil Capsules are prepared from *Crypthecodinium cohnii* Oil and contain NLT 95.0% and NMT 105.0% of the labeled amount of docosahexaenoic acid (DHA, $\text{C}_{22}\text{H}_{32}\text{O}_2$) ($\text{C}_{22}:6 \text{ n}-3$).

IDENTIFICATION

- **LONG-CHAIN UNSATURATED FATTY ACID PROFILE:** Proceed as directed in *Content of DHA*.

Analysis

Samples: *Standard Solution 2* and *Test solution 1*
Calculate the area percentage for each fatty acid as methyl ester in *Test solution 1*:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual fatty acid as methyl ester

r_T = sum of all the peak responses, except the solvent and butylated hydroxytoluene peaks

Acceptance criteria: The retention time of the peaks of the docosahexaenoic acid methyl ester and the eicosapentaenoic acid methyl ester from *Test solution 1* corresponds to that from *Standard Solution 2*, as obtained in the test for *Content of EPA and DHA*. The area percent for the methyl esters of the fatty acids from *Test solution 1* in the test for *Content of EPA and DHA* meet the requirements for each fatty acid indicated in *Table 1*.

Table 1

Fatty Acid	Relative Retention Time	Shorthand Notation	Lower Limit (Area, %)	Upper Limit (Area, %)
Linoleic acid	0.52	18:2 n-6	0	1.0
Eicosapentaenoic acid	0.79	20:5 n-3	0	0.1
Docosapentaenoic acid	0.94	22:5 n-6	0	0.1
Docosahexaenoic acid	1.00	22:6 n-3	35.0	47.0

STRENGTH

• CONTENT OF DHA

Test solution 1 and Test solution 2: Weigh NLT 10 Capsules in a tared weighing bottle. With a sharp blade or other appropriate means, carefully open the Capsules, without loss of the shell material, and transfer the combined Capsule contents to a 100-mL beaker. Remove any adhering substance from the emptied capsules by washing with several small portions of isooctane. Discard the washings, and allow the empty capsules to dry in a current of dry air until the isooctane is completely evaporated. Weigh the empty capsules in the original tared weighing bottle, and calculate the average fill weight (AFW) of *Crypthecodinium cohnii* oil/Capsule. Proceed with the content of capsules as directed in the *Analysis*.

Analysis: Proceed as directed in *Fats and Fixed Oils* <401>, *Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA*.

Calculate the percentage of the labeled amount of docosahexaenoic acid (DHA) in the Capsules taken:

$$\text{Result} = R \times \text{AFW}/L$$

R = determined percentage of DHA in the portion of oil taken from the Capsules (%)

AFW = average fill weight of the Capsules taken (mg)

L = labeled amount of DHA (mg/Capsule)

Acceptance criteria: NLT 95.0% and NMT 105.0% of the labeled amount of DHA

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** <2040>: Meet the requirements for *Rupture Test for Soft Shell Capsules*

- **WEIGHT VARIATION (2091):** Meet the requirements

IMPURITIES

Inorganic Impurities

- **LIMIT OF ARSENIC**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: Transfer 1 g of ultrapure palladium metal into a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution B: Transfer 1 g of ultrapure magnesium nitrate into a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (3:2:5). A volume of 5 μ L provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed in the test for *Arsenic* (211), to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 μ g/mL of arsenic.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 μ g/mL of arsenic.

Sample solution: For preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytetrafluoroethylene liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes. [CAUTION—Wear proper eye protection and protective clothing and gloves.]

Transfer approximately 500 mg of *Crypthecodinium cohnii* oil from Capsules, weighed to the nearest 0.1 mg, into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature.

[NOTE—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into

25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115°, using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min; char the sample at 1000°, using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 μ L) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 μ L of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in μ g/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in μ g/mL, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration as obtained above

W = weight of Capsules content taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 μ g/g

- **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (2:1:2). A volume of 5 μ L provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Lead Nitrate Stock Solution*, prepared as directed in *Heavy Metals* (231), to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 μ g/mL of lead.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 μ g/mL of lead.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for

10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2100°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of the *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line. Calculate the content of lead in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration as obtained above

W = weight of Capsules content taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• LIMIT OF CADMIUM

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid to volume (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution A: 0.1372 mg/mL of cadmium nitrate

Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before make up to final volume dissolve in a portion of water and nitric acid.]

Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of the *Solution C* for each of the samples, into

the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line. Calculate the content of cadmium in the Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration as obtained above

W = weight of Capsules content taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF MERCURY:** Proceed as directed in *Mercury* (261), *Method IIa*, except use a *Standard Mercury Solution* having the equivalent of 0.1 µg/mL of mercury.

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*, combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution*.

Acceptance criteria: NMT 0.1 µg/g

SPECIFIC TESTS

- **FATS AND FIXED OILS, Anisidine Value (401):** NMT 20.0, determined on the contents of the Capsules
- **FATS AND FIXED OILS, Free Fatty Acids (401):** The free fatty acids in 10 g require for neutralization NMT 1.42 mL of 0.1 N sodium hydroxide.
- **FATS AND FIXED OILS, Peroxide Value (401):** NMT 5.0, determined on the contents of the Capsules
- **FATS AND FIXED OILS, Total Oxidation Value (TOTOX) (401):** NMT 26 (determined on the contents of the Capsules), calculated as

$$\text{Result} = (2 \times \text{PV}) + \text{AV}$$

PV = peroxide value

AV = anisidine value

- **FATS AND FIXED OILS, Unsaponifiable Matter (401):** NMT 3.5%, determined on the contents of the Capsules
- **SPECIFIC GRAVITY (841):** 0.91–0.93, determined on the contents of the Capsules

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.
- **LABELING:** The label states the content of docosahexaenoic acid in mg/Capsule. It also states the name and concentration of any added antioxidant.
- **USP REFERENCE STANDARDS (11)**
 - USP Docosahexaenoic Acid Ethyl Ester RS
 - USP Eicosapentaenoic Acid Ethyl Ester RS
 - USP Methyl Tricosanoate RS

Curcuminoids

DEFINITION

Curcuminoids is a partially purified natural complex of diaryl heptanoid derivatives isolated from Turmeric, *Curcuma longa* L. It contains NLT 95.0% of curcuminoids, calculated on the dried basis, as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin. It contains NLT 70.0% and NMT 80.0% of curcumin, NLT 15.0% and NMT 25.0% of desmethoxycurcumin, and NLT 2.5% and NMT 6.5% of bisdesmethoxycurcumin.

IDENTIFICATION**A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 0.2 mg/mL of USP Curcuminoids RS in acetone

Sample solution: 2 mg/mL of Curcuminoids in acetone

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)

Application volume: 10 µL, as bands

Developing solvent system: Chloroform, methanol, and formic acid (96:4:1)

Analysis

Samples: *Standard solution* and *Sample solution*
Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* <621>, *Thin-Layer Chromatography*). Use saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, and examine under UV light at 365 nm.

Acceptance criteria: The *Sample solution* chromatogram shows yellowish-brown bands due to bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin at R_f values of about 0.4, 0.6, and 0.7, respectively, corresponding in position and color to those obtained from the *Standard solution*.

- B.** The retention times of the peaks for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin of the *Sample solution* chromatogram correspond to those of the *Standard solution* for the appropriate USP Reference Standard, as obtained in the test for *Content of Curcuminoids*.

COMPOSITION**C. CONTENT OF CURCUMINOIDS**

Mobile phase: Tetrahydrofuran and 1 mg/mL of citric acid in water (4:6)

[NOTE—Sonication may be necessary to dissolve the RS in each *Standard solution*; all solutions should be passed through a filter with 0.45-µm pore size before injection. USP Curcumin RS, USP Desmethoxycurcumin, and USP Bisdesmethoxycurcumin RS can also be prepared in one standard solution containing the final concentration specified below for each.]

Standard solution A: 40 µg/mL of USP Curcuminoids RS in *Mobile phase*

Standard solution B: 40 µg/mL of USP Curcumin RS in *Mobile phase*

Standard solution C: 10 µg/mL of USP Desmethoxycurcumin RS in *Mobile phase*

Standard solution D: 2 µg/mL of USP Bisdesmethoxycurcumin RS in *Mobile phase*

Sample solution: Transfer about 20 mg of Curcuminoids to a 50-mL volumetric flask, add about 30 mL of acetone, and sonicate for 30 min. Dilute with acetone to volume and centrifuge. Transfer 5 mL to a 50-mL volumetric flask, dilute with *Mobile phase* to volume, and mix.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 420 nm

Column: 4.6-mm × 20-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution A*

[NOTE—The relative retention times for the curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin peaks are 1.0, 1.2, and 1.4, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the Reference Chromatogram provided with USP Curcuminoids RS.

Resolution: NLT 2.0, between curcumin and desmethoxycurcumin peaks and the desmethoxycurcumin and bisdesmethoxycurcumin peaks

Tailing factor: NMT 1.5 for bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin peaks

Relative standard deviation: NMT 2.0% for desmethoxycurcumin peak, in repeated injections

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*

Calculate the percentage of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in the portion of Curcuminoids taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the *Sample solution*

r_S = peak response for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the appropriate *Standard solution*

C_S = concentration of the appropriate *Standard solution* (mg/mL)

C_U = concentration of Curcuminoids in the *Sample solution* (mg/mL)

Acceptance criteria: Curcuminoids contains NLT 95.0% of curcuminoids, calculated on the dried basis, as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin. It contains NLT 70.0% and NMT 80.0% of curcumin, NLT 15.0% and NMT 25.0% of desmethoxycurcumin, and NLT 2.5% and NMT 6.5% of bisdesmethoxycurcumin.

CONTAMINANTS

- HEAVY METALS, Method III <231>:** NMT 20 ppm
- ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues <561>:** Meets the requirements
- ARTICLES OF BOTANICAL ORIGIN, Test for Aflatoxins <561>:** Meets the requirements
- MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- MICROBIOLOGICAL PROCEDURE FOR ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- MELTING RANGE OR TEMPERATURE, Class I <741>:** 172°–178°
- LOSS ON DRYING <731>:** Dry 1.0 g at 105° for 2 h: it loses NMT 2.0% of its weight.
- ARTICLES OF BOTANICAL ORIGIN, Total Ash <561>:** NMT 1.0%

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in well-closed containers; protect from light and moisture, and store at room temperature.
- LABELING:** The label states the content of curcuminoids and the content of the individual curcuminoids, on the dried basis; and the Latin binomial and the part of the plant used to prepare the article.
- USP REFERENCE STANDARDS <11>**
 - USP Bisdesmethoxycurcumin RS
 - USP Curcumin RS
 - USP Curcuminoids RS
 - USP Desmethoxycurcumin RS

Curcuminoids Capsules

DEFINITION

Curcuminoids Capsules are prepared from Curcuminoids and contain NLT 90.0% and NMT 110.0% of the labeled amount of curcuminoids, calculated as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin.

IDENTIFICATION

A. THIN-LAYER CHROMATOGRAPHY

Standard solution: 0.2 mg/mL of USP Curcuminoids RS in acetone

Sample solution: Weigh and finely powder the contents of NLT 20 Capsules. Transfer a portion of the powder, equivalent to about 10 mg of curcuminoids, to a suitable container, add 5 mL of acetone, shake for 1 min, and sonicate for 10 min. Allow to stand for 15 min before use.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μm (TLC plates)

Application volume: 10 μL , as bands

Developing solvent system: Chloroform, methanol, and formic acid (96:4:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* <621>). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, and examine under UV light at 365 nm.

Acceptance criteria: The *Sample solution* chromatogram shows yellowish-brown bands due to bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin at R_f values of about 0.4, 0.6, and 0.7, respectively, corresponding in position and color to those obtained from the *Standard solution*.

- B.** The retention times of the peaks for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin of the *Sample solution* correspond to those of the *Standard solution* for the appropriate USP Reference Standard, as obtained in the test for *Content of Curcuminoids*.

STRENGTH

CONTENT OF CURCUMINOIDS

Mobile phase: Tetrahydrofuran and 1 mg/mL of citric acid in water (4:6)

[NOTE—Sonication may be necessary to dissolve the RS in each *Standard solution*; all solutions should be passed through a filter with 0.45- μm pore size before injection. USP Curcumin RS, USP Desmethoxycurcumin RS, and USP Bisdesmethoxycurcumin RS can also be prepared in one standard solution containing the final concentration specified below for each.]

Standard solution A: 40 μg /mL of USP Curcuminoids RS in *Mobile phase*

Standard solution B: 40 μg /mL of USP Curcumin RS in *Mobile phase*

Standard solution C: 10 μg /mL of USP Desmethoxycurcumin RS in *Mobile phase*

Standard solution D: 2 μg /mL of USP Bisdesmethoxycurcumin RS in *Mobile phase*

Sample stock solution: Weigh and finely powder the contents of NLT 20 Capsules. Transfer an accurately weighed amount of the powder, equivalent to about 20 mg of curcuminoids, to a 50-mL volumetric flask. Add about 30 mL of acetone, sonicate for 30 min, dilute with acetone to volume, mix, and centrifuge.

Sample solution: Dilute a portion of the *Sample stock solution* 1 in 10 with *Mobile phase*, and mix.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV-Vis 420 nm

Column: 4.6-mm \times 20-cm; 5- μm packing L1

Flow rate: 1 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution A*

[NOTE—The relative retention times for the curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin peaks are about 1.0, 1.2, and 1.4, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram provided with the lot of USP Curcuminoids RS being used.

Resolution: NLT 2.0 between the curcumin and desmethoxycurcumin peaks and the desmethoxycurcumin and bisdesmethoxycurcumin peaks

Tailing factor: NMT 1.5 for the bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin peaks

Relative standard deviation: NMT 2.0% for desmethoxycurcumin peak, in repeated injections

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*

Calculate the quantity, in mg, of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in each Capsule:

$$\text{Result} = (r_U/r_S) \times C_S \times D \times V \times (W_F/W_U)$$

r_U = peak area for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the *Sample solution*

r_S = peak area for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the appropriate *Standard solution*

C_S = concentration of the appropriate *Standard solution* (mg/mL)

D = dilution factor to prepare the *Sample solution* from *Sample stock solution*

V = volume of *Sample stock solution* (mL)

W_F = average fill weight of Capsules (mg)

W_U = weight of content of Capsules taken to prepare the *Sample stock solution* (mg)

Calculate the percentage of the labeled amount of curcuminoids in the Capsule:

$$\text{Result} = (\Sigma Q/L) \times 100$$

ΣQ = sum of the quantities of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in the Capsule (mg)

L = labeled amount of curcuminoids (mg/Capsule)

Acceptance criteria: 90.0%–110.0% of the label claim

PERFORMANCE TESTS

DISINTEGRATION AND DISSOLUTION <2040>

Mode: Dissolution

Medium: Water containing 1% sodium lauryl sulfate; 900 mL

Apparatus 2: 100 rpm

Time: 60 min

Sample solution: Combine 25-mL portions of the solution under test from each of the six dissolution vessels, and mix. Transfer 5 mL to a 25-mL volumetric flask, and dilute with *Mobile phase* to volume.

Analysis: Determine the amount of curcumin ($\text{C}_{21}\text{H}_{20}\text{O}_6$) dissolved by using the method used in *Strength*, making any necessary modifications.

Tolerances: NLT 75% of the content of curcumin ($\text{C}_{21}\text{H}_{20}\text{O}_6$) is dissolved.

- **WEIGHT VARIATION** (2091): Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meet the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protect from light and moisture, and store at room temperature.
- **LABELING:** The label states the content of curcuminoids in mg/Capsule.
- **USP REFERENCE STANDARDS** (11)
 - USP Bisdesmethoxycurcumin RS
 - USP Curcumin RS
 - USP Curcuminoids RS
 - USP Desmethoxycurcumin RS

Curcuminoids Tablets

DEFINITION

Curcuminoids Tablets are prepared from Curcuminoids and contain NLT 90.0% and NMT 110.0% of the labeled amount of curcuminoids, calculated as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHY**

Standard solution: 0.2 mg/mL of USP Curcuminoids RS in acetone

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to about 10 mg of curcuminoids, to a suitable container, add 5 mL of acetone, shake for 1 min, and sonicate for 10 min. Allow to stand for 15 min before use.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10-15 μ m (TLC plates)

Application volume: 10 μ L, as bands

Developing solvent system: Chloroform, methanol, and formic acid (96:4:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, and examine under UV light at 365 nm.

Acceptance criteria: The *Sample solution* chromatogram shows yellowish-brown bands due to bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin at R_f values of about 0.4, 0.6, and 0.7, respectively, corresponding in position and color to those obtained from the *Standard solution*.
- **B.** The retention times of the peaks for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin of the *Sample solution* correspond to those of the *Standard solution* for the appropriate USP Reference Standard, as obtained in the test for *Content of Curcuminoids*.

STRENGTH

- **CONTENT OF CURCUMINOIDS**

Mobile phase: Tetrahydrofuran and 1 mg/mL of citric acid in water (4:6) [NOTE—Sonication may be necessary to dissolve the RS in each *Standard solution*; all solutions should be passed through a filter with 0.45- μ m pore

size before injection. USP Curcumin RS, USP Desmethoxycurcumin RS and USP Bisdesmethoxycurcumin RS can also be prepared in one standard solution containing the final concentration specified below for each.]

Standard solution A: 40 μ g/mL of USP Curcuminoids RS in *Mobile phase*

Standard solution B: 40 μ g/mL of USP Curcumin RS in *Mobile phase*

Standard solution C: 10 μ g/mL of USP Desmethoxycurcumin RS in *Mobile phase*

Standard solution D: 2 μ g/mL of USP Bisdesmethoxycurcumin RS in *Mobile phase*

Sample stock solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed amount of the powder, equivalent to about 20 mg of curcuminoids, to a 50-mL volumetric flask. Add about 30 mL of acetone, sonicate for 30 min, dilute with acetone to volume, mix, and centrifuge.

Sample solution: Dilute a portion of the *Sample stock solution* (1 in 10) with *Mobile phase*, and mix.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV-Vis 420 nm

Column: 4.6-mm \times 20-cm; 5- μ m packing L1

Flow rate: 1 mL/min

Injection size: 20 μ L

System suitability

Sample: *Standard solution A*

[NOTE—The relative retention times for the curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin peaks are about 1.0, 1.2, and 1.4, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram provided with the lot of USP Curcuminoids RS being used.

Resolution: NLT 2.0 between the curcumin and desmethoxycurcumin peaks and the desmethoxycurcumin and bisdesmethoxycurcumin peaks

Tailing factor: NMT 1.5 for the bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin peaks

Relative standard deviation: NMT 2.0% for desmethoxycurcumin peak, in repeated injections

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*

Calculate the quantity, in mg, of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in each Tablet:

$$\text{Result} = (r_U/r_S) \times C_S \times D \times V \times (W_F/W_U)$$

r_U = peak area for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the *Sample solution*

r_S = peak area for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the appropriate *Standard solution*

C_S = concentration of the appropriate *Standard solution* (mg/mL)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*

V = volume of *Sample solution* (mL)

W_F = average weight of Tablets (mg)

W_U = weight of Tablets powder taken to prepare the *Sample stock solution* (mg)

Calculate the percentage of the labeled amount of curcuminoids in the Tablet:

$$\text{Result} = (\Sigma Q/L) \times 100$$

ΣQ = sum of the quantities of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in the Tablet (mg)

L = labeled amount of curcuminoids (mg/Tablet)
Acceptance criteria: 90.0%–110.0% of the label claim

PERFORMANCE TESTS**• DISINTEGRATION AND DISSOLUTION <2040>**

Mode: Dissolution

Medium: Water containing 1% sodium lauryl sulfate; 900 mL

Apparatus 2: 100 rpm

Time: 60 min

Sample solution: Combine 25-mL portions of the solution under test from each of the six dissolution vessels, and mix. Transfer 5 mL to a 25-mL volumetric flask, and dilute with *Mobile phase* to volume.

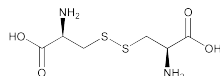
Analysis: Determine the amount of curcumin ($C_{21}H_{20}O_6$) dissolved by using the method used in the *Content of Curcuminoids*, making any necessary modifications.

Tolerances: NLT 75% of the content of curcumin ($C_{21}H_{20}O_6$) is dissolved.

• WEIGHT VARIATION (2091): Meet the requirements**CONTAMINANTS****• MICROBIAL ENUMERATION TESTS <2021>** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.**• ABSENCE OF SPECIFIED MICROORGANISMS <2022>** Meet the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli***ADDITIONAL REQUIREMENTS****• PACKAGING AND STORAGE:** Preserve in well-closed containers, protect from light and moisture, and store at room temperature.**• LABELING:** The label states the content of curcuminoids in mg/Tablet.**• USP REFERENCE STANDARDS <11>**
 USP Bisdesmethoxycurcumin RS
 USP Curcumin RS
 USP Curcuminoids RS
 USP Desmethoxycurcumin RS

Cyanocobalamin—see *Cyanocobalamin General Monographs*

Cysteine Hydrochloride—see *Cysteine Hydrochloride General Monographs*

Cysteine

$C_6H_{12}N_2O_4S_2$ 240.30
 L-Cystine;
 3,3'-Disulfanedibis [(2*R*)-2-aminopropanoic acid] [56-89-3].

DEFINITION

Cystine contains NLT 98.5% and NMT 101.5% of $C_6H_{12}N_2O_4S_2$, as L-Cystine, calculated on the dried basis.

IDENTIFICATION**• A. INFRARED ABSORPTION <197K>****• B. OPTICAL ROTATION, Specific Rotation <781S>** −215 to −225, determined at 20°

Sample solution: 20 mg/mL, in 1 N hydrochloric acid

- C.** The R_f value of the principal spot of the *Sample solution* in the test for *Organic Impurities* corresponds to that of the *Standard solution*.

ASSAY**• PROCEDURE**

Sample solution: Transfer about 0.1 g of Cystine to a glass-stoppered flask, and dissolve in a mixture of 2 mL of dilute sodium hydroxide (1 in 20) and 10 mL of water. Add 10 mL of potassium bromide solution (200 g/L in water), 50.0 mL of 0.1 N potassium bromate VS, and 15 mL of dilute hydrochloric acid (17 in 100). Immediately insert the stopper into the flask, and cool in an ice water bath. Allow to stand protected from light for 10 min.

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Residual titration

Titrant: 0.1 N potassium bromate VS

Back-titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Colorimetric

Equivalency: Each mL of 0.1 N potassium bromate VS is equivalent to 2.403 mg of $C_6H_{12}N_2O_4S_2$ on the dried basis.

Analysis: Add 1.5 g of potassium iodide, and after 1 min, titrate with 0.1 N sodium thiosulfate VS, using starch TS as the indicator. Perform a blank determination, and make any necessary correction.

Acceptance criteria: 98.5%–101.5% on the dried basis

IMPURITIES**Inorganic Impurities****• RESIDUE ON IGNITION <281>** NMT 0.1%**• CHLORIDE AND SULFATE, Chloride <221>** NMT 200 ppm. A 0.7-g portion shows no more chloride than corresponds to 0.40 mL of 0.01 N hydrochloric acid.**• CHLORIDE AND SULFATE, Sulfate <221>** NMT 200 ppm. A 1.2-g portion shows no more sulfate than corresponds to 0.25 mL of 0.020 N sulfuric acid.**• IRON <241>** NMT 10 ppm**• HEAVY METALS, Method I <231>** NMT 10 ppm**Organic Impurities****• PROCEDURE**

Standard solution: Dissolve a quantity of USP Cystine RS in 1 N hydrochloric acid, and dilute with water to obtain a solution having a known concentration of about 0.02 mg/mL.

Sample solution: Dissolve a quantity of Cystine in 1 N hydrochloric acid, and dilute with water to obtain a solution having a known concentration of about 10 mg/mL.

System suitability solution: Dissolve quantities of USP Cystine RS and USP Arginine Hydrochloride RS in 1 N hydrochloric acid, and dilute with water to obtain a solution having a known concentration of about 0.4 mg/mL each.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: A 0.25-mm layer of chromatographic silica gel mixture

Application volume: 5 μ L

Developing solvent system: A mixture of ammonia and 2-propanol (3:7)

Spray reagent: Dissolve 0.2 g of ninhydrin in 100 mL of a mixture of butanol and 2 N acetic acid (95:5).

Analysis

Samples: *Standard solution*, *Sample solution*, and *System suitability solution*

Proceed as directed for *Chromatography* <621>, *Thin-Layer Chromatography*. After air-drying the plate, spray with *Spray reagent*, and heat between 100° and 105° for about 15 min. Examine the plate. The chro-

matogram from the *System suitability solution* exhibits two clearly separated spots.

Acceptance criteria: Any secondary spot from the *Sample solution* is not larger or more intense than the principal spot from the *Standard solution*.

Individual impurities: NMT 0.2%

Total impurities: NMT 2.0%

SPECIFIC TESTS

- **LOSS ON DRYING** (731): Dry a sample at 105° for 3 h: it loses NMT 0.2% of its weight.

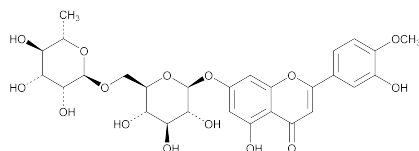
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at a controlled room temperature.
- **USP REFERENCE STANDARDS** (11)
USP Arginine Hydrochloride RS
USP Cystine RS

Dexpanthenol—see *Dexpanthenol General Monographs*

Dexpanthenol Preparation—see *Dexpanthenol Preparation General Monographs*

Diosmin



$C_{28}H_{32}O_{15}$ 608.54

5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-[[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one;

7-[[6-O-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one [520-27-4].

DEFINITION

Diosmin contains NLT 90.0% and NMT 102.0% of diosmin ($C_{28}H_{32}O_{15}$), calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

PROCEDURE

Mobile phase: Methanol, acetonitrile, acetic acid, and water (27:2:6:65)

Standard solution: 1.0 mg/mL of USP Diosmin RS in dimethyl sulfoxide

System suitability solution: 1 mg/mL of USP Diosmin for System Suitability RS in dimethyl sulfoxide

Sample solution: 1.0 mg/mL of Diosmin in dimethyl sulfoxide

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 275 nm

Column: 4.6-mm × 10-cm; 3-μm packing L1

Column temperature: 40°

Flow rate: 1.2 mL/min

Injection size: 10 μL

System suitability

Samples: *System suitability solution* and *Standard solution*.

[NOTE—Allow the run time about 6 times the diosmin retention time. The relative retention times for diosmin, acetoisovanillone, hesperidin, isorhoifolin, linarin, and diosmetin are 1, 0.5, 0.6, 0.8, 2.6, and 4.5, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the Reference Chromatogram provided with the USP Diosmin for System Suitability RS being used.

Resolution: NLT 2.5 between hesperidin and isorhoifolin, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of diosmin ($C_{28}H_{32}O_{15}$), in the portion of Diosmin taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Diosmin RS in the *Standard solution* (mg/mL)

C_U = concentration of Diosmin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–102.0% on the anhydrous basis

IMPURITIES

RESIDUE ON IGNITION (281)

Sample: 1.0 g

Acceptance criteria: NMT 0.2%

HEAVY METALS, Method II (231)

Sample: 2.0 g

Acceptance criteria: NMT 20 ppm

LIMIT OF IODINE

Determine the total content of iodine by potentiometry, using an iodide-selective electrode, after oxygen flask combustion (see *Oxygen Flask Combustion* (471)).

Sample solution: [CAUTION—Observe rigorously the precautions set forth for *Procedure* under *Oxygen Flask Combustion* (471).] Wrap 0.100 g of Diosmin in a piece of free-halide filter paper, and place it in the platinum gauze specimen holder. Introduce into the flask 50.0 mL of a 0.2 g/L solution of hydrazine. Flush the flask with oxygen for 10 min. Ignite the filter paper. Stir the contents of the flask immediately after the end of the combustion to dissolve completely the combustion products. Continue stirring for 1 h.

Standard solution: 33.2 μg/mL of potassium iodide in water, equivalent to 25.4 μg/mL of iodine

Potassium nitrate solution: 200 mg/mL of potassium nitrate in 0.1 M nitric acid

Analysis

Samples: *Sample solution* and *Standard solution*

Transfer 30 mL of *Potassium nitrate solution* to a beaker, immerse the electrodes, and stir for 10 min. The potential (nU_i) must remain stable. Measure the potential (nU_i). Add 1 mL of the *Sample solution*, and measure the potential (nU_2).

Transfer 30 mL of *Potassium nitrate solution* to a beaker, immerse the electrodes, and stir for 10 min. The potential (nS_i) must remain stable. Measure the

potential (nS_1). Add 80 μ L of the *Standard solution*, and measure the potential (nS_2).

Acceptance criteria: NMT 0.1%: The absolute value $|(nU_2) - (nU_1)|$ is not higher than the absolute value $|(nS_2) - (nS_1)|$.

• RELATED COMPOUNDS

Mobile phase, System suitability solution, Sample solution, and Chromatographic system: Proceed as directed in the Assay.

Standard solution: 0.05 mg/mL of USP Diosmin RS in dimethyl sulfoxide

System suitability

Sample: *System suitability solution*. [NOTE—Allow the run time about 6 times that of the diosmin retention time. The relative retention times for diosmin, acetoisovanillone, hesperidin, isorhoifolin, linarin, and diosmetin are 1, 0.5, 0.6, 0.8, 2.6, and 4.5, respectively.]

System suitability requirements

Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the Reference Chromatogram provided with the USP Diosmin for System Suitability RS being used.

Resolution: NLT 2.5 between hesperidin and isorhoifolin, *System suitability solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each impurity in the portion of Diosmin taken: [NOTE—Disregard any impurity less than 0.1%.]

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response for each impurity from the *Sample solution*

r_S = peak response for diosmin from the *Standard solution*

C_S = concentration of USP Diosmin RS in the *Standard solution* (mg/mL)

C_U = concentration of Diosmin in the *Sample solution* (mg/mL)

F = correction factor for each individual impurity (see Table 1)

Acceptance criteria

Total impurities: NMT 10%

Individual impurities: See Table 1.

Total other impurities and acetoisovanillone: NMT 1%

Table 1

Name	Relative Retention Time	Correction Factor (F)	Acceptance Criteria, NMT (%)
Acetoisovanillone ^a	0.5	0.3	1
Hesperidin ^b	0.6	1	5
Isorhoifolin ^c	0.8	1	3
Linarin ^d	2.6	1	3
Diosmetin ^e	4.5	0.5	3

^a 1-(3-Hydroxy-4-methoxyphenyl)ethanone.

^b (2S)-7-[[6-O-(6-Deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4H-1-benzopyran-4-one.

^c 7-[[6-O-(6-Deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-5-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one.

^d 7-[[6-O-(6-Deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-5-hydroxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one.

^e 5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzopyran-4-one.

Table 1 (Continued)

Name	Relative Retention Time	Correction Factor (F)	Acceptance Criteria, NMT (%)
Any other impurity	—	1	1
Total impurities	—	—	10

^a 1-(3-Hydroxy-4-methoxyphenyl)ethanone.

^b (2S)-7-[[6-O-(6-Deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4H-1-benzopyran-4-one.

^c 7-[[6-O-(6-Deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-5-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one.

^d 7-[[6-O-(6-Deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-5-hydroxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one.

^e 5,7-Dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzopyran-4-one.

SPECIFIC TESTS

• **WATER DETERMINATION, Method 1a (921)**

Sample: 0.3 g

Acceptance criteria: NMT 6.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed, tight containers.

• **USP REFERENCE STANDARDS (11)**

USP Diosmin RS

USP Diosmin for System Suitability RS

Echinacea angustifolia

DEFINITION

Echinacea angustifolia consists of the dried rhizome and roots of *Echinacea angustifolia* DC. (Fam. Asteraceae). It is harvested in the fall after 1 or more years of growth. It contains NLT 0.5% of total phenols, calculated on the dried basis as the sum of caffeic acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), chlorogenic acid ($C_{16}H_{18}O_9$), dicaffeoylquinic acids ($C_{25}H_{24}O_{12}$), and echinacoside ($C_{35}H_{46}O_{20}$). It contains NLT 0.075% of dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$) on the dried basis.

IDENTIFICATION

• **A. PRESENCE OF ECHINACOSIDE AND DICAFFEYOYLQUINIC ACIDS (CYNARIN(E))**

Standard solution A: 20 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in methanol

Standard solution B: 1 mg/mL of 1,3-dicaffeoylquinic acid in methanol

Sample solution: Weigh and finely pulverize 10 g of *Echinacea angustifolia*, and transfer about 1 g of the powder to a suitable extraction thimble. Transfer the thimble to a continuous extraction apparatus, and extract with 50 mL of chloroform for 1 h. Retain the chloroform extract for *Identification* test B. Continue the extraction with 50 mL of methanol, and concentrate to a small volume at 40° in vacuum. With the aid of methanol, transfer the extract to a 10-mL volumetric flask, and dilute with methanol to volume.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)

Application volume: 10 μ L

Developing solvent system: Ethyl acetate, formic acid, and water (17:2:1)

Spray reagent A: 10 mg/mL of diphenylborinic acid, ethanolamine ester in methanol

Spray reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and then examine the plate under UV light at 365 nm.

Acceptance criteria: The chromatogram obtained from the *Sample solution* shows a yellowish zone at an R_f value of 0.14 characteristic of echinacoside (absent or only traces present in *Echinacea purpurea*) that corresponds in color and R_f value to that in the chromatogram of *Standard solution A*, and another zone characteristic of 1,3-dicaffeoylquinic acid (absent in *Echinacea pallida* and *Echinacea purpurea*) corresponding in color and R_f value to that in the chromatogram of *Standard solution B*. Other colored zones of varying intensities may be observed in the chromatogram of the *Sample solution*.

• **B. PRESENCE OF ISOBUTYLALKENYLAMIDES**

Standard solution A: Transfer a quantity of USP Powdered *Echinacea angustifolia* Extract RS to a centrifuge tube, and add chloroform to obtain a solution having a known concentration of about 100 mg/mL. Shake by hand to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Standard solution B: 1 mg/mL of β -sitosterol in methanol

Sample solution: Evaporate the chloroform extract retained from preparation of the *Sample solution* in *Identification test A* to dryness at 40° in vacuum. To the residue, add 1 mL of alcohol, and pass through a nylon membrane filter of 0.45- μ m pore size.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)

Application volume: 10 μ L

Developing solvent system: Hexane and ethyl acetate (2:1)

Spray reagent: Prepare a mixture of glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (10:5:0.5) in an ice bath

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Examine the plate under UV light at 254 nm, and then spray the plate with *Spray reagent*, and heat the plate at 100° for 5 min.

Acceptance criteria

Under UV light at 254 nm: The chromatogram obtained from the *Sample solution* shows one main zone at an R_f value of about 0.25 due to 2*E*,4*E*,8*Z*,10*E*-dodecatetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide (absent in *Echinacea pallida*) that corresponds in R_f value to that in the chromatogram of *Standard solution A*.

After treatment with *Spray reagent* and heating: The chromatogram obtained from the *Sample solution* shows a zone due to β -sitosterol that corresponds in R_f value to the principal spot in the chromatogram of *Standard solution B*. Below this spot, there is a zone due to dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and to dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide that corresponds in R_f value to that in the chromatogram of *Standard solution A*; and below this spot, there are several yellowish zones due to α , β -unsaturated isobutylamides (absent in *Echinacea pallida* and mostly violet in *Echinacea purpurea* due to the presence of α , β , γ , δ -unsaturated isobutylamides) that are not visible or are very weak when viewed under UV light at 254 nm.

- **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the echinacoside peak of *Standard solution A*, and the retention time of the peak for 1,3-dicaffeoylquinic acid from the *Sample solution* corresponds to that of *Standard solution A*, all peaks as obtained in the test for *Content of total phenols*.

COMPOSITION

• **CONTENT OF TOTAL PHENOLS**

Solution A: Phosphoric acid (0.1 in 100) in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17	60	40
17.5	90	10
22	90	10

Solvent: Alcohol and water (7:3)

Standard solution A: Dissolve USP Powdered *Echinacea angustifolia* Extract RS in *Solvent*, shaking and heating in a water bath. Dilute with *Solvent* to obtain a solution having a known concentration of 1 mg/mL. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Standard solution B: 40 μ g/mL of USP Chlorogenic Acid RS in *Solvent*

Sample solution: Transfer about 125 mg of finely powdered *Echinacea angustifolia* (capable of passing through a 40-mesh sieve), accurately weighed, to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux, while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter having a 0.45- μ m or finer pore size.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 5 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with the USP Powdered *Echinacea angustifolia* Extract RS.

Resolution: NLT 1.0 between the 1,3-dicaffeoylquinic acid isomer and echinacoside, *Standard solution A*

Capacity factor (k'): NLT 3.0, *Standard solution B*

Tailing factor: NMT 2.0 for the chlorogenic acid peak, *Standard solution B*

Relative standard deviation: NMT 2.5% for the chlorogenic acid peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the relevant analytes in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.

Separately calculate the percentage of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), chlorogenic acid ($C_{16}H_{18}O_9$), dicaffeoylquinic acids ($C_{25}H_{24}O_{12}$), and echinacoside ($C_{35}H_{46}O_{20}$) in the portion of *Echinacea angustifolia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak response for the relevant analyte from the *Sample solution*

- r_s = peak response for chlorogenic acid from *Standard solution B*
 C_s = concentration of USP Chlorogenic Acid RS in *Standard solution B* (mg/mL)
 V = volume of the *Sample solution* (mL)
 W = weight of *Echinacea angustifolia* taken to prepare the *Sample solution* (mg)
 F = response factor: chicoric acid, 0.695; dicaffeoylquinic acids, 0.729; caftaric acid, 0.881; chlorogenic acid, 1.000; and echinacoside, 2.220

Calculate the percentage of total phenols in the portion of *Echinacea angustifolia* taken by adding the individual percentages calculated.

Acceptance criteria: NLT 0.5% of total phenols on the dried basis

• **CONTENT OF DODECATETRAENOIC ACID ISOBUTYLAMIDES**

Mobile phase: Acetonitrile and water (55:45)

Standard solution A: Dissolve, with sonication, USP Powdered *Echinacea angustifolia* Extract RS in methanol, shaking for 10 min, and dilute with methanol to obtain a solution having a concentration of 5 mg/mL. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Standard solution B: 10 μ g/mL of USP 2E,4E-Hexadienoic Acid Isobutylamide RS in methanol

Sample solution: Transfer about 2.5 g, accurately weighed, of finely powdered *Echinacea angustifolia* (capable of passing through a 40-mesh sieve) to a round-bottom flask. Add 80 mL of methanol, and reflux for 30 min. Cool to room temperature, and filter into a 100-mL volumetric flask, using small portions of methanol to rinse the flask and the filter. Dilute with methanol to volume. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L1

Column temperature: 30°

Flow rate: 1.5 mL/min

Injection size: 25 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for alkamides provided with USP Powdered *Echinacea angustifolia* Extract RS.

Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*

Tailing factor: NMT 2.0 for the 2E,4E-hexadienoic acid isobutylamide peak, *Standard solution B*

Relative standard deviation: NMT 2.5% for the 2E, 4E-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks due to 2E,4E,8Z,10E-dodecatetraenoic acid isobutylamide and 2E,4E,8Z,10Z-dodecatetraenoic acid isobutylamide in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks. Calculate the percentage of dodecatetraenoic acid isobutylamides in the portion of *Echinacea angustifolia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

- r_U = sum of the peak responses of the relevant analytes from the *Sample solution*

- r_s = peak response for 2E,4E-hexadienoic acid isobutylamide from *Standard solution B*
 C_s = concentration of USP 2E,4E-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)
 V = volume of the *Sample solution* (mL)
 W = weight of *Echinacea angustifolia* taken to prepare the *Sample solution* (mg)
 F = response factor for 2E,4E-hexadienoic acid isobutylamide, 1.353

Acceptance criteria: NLT 0.075% of dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$) on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method III* (231): NMT 10 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements

SPECIFIC TESTS

• **BOTANIC CHARACTERISTICS**

Macroscopic: The outer surface of the rhizome is pale to yellowish brown, crowned with remains of the aerial stem, and sometimes showing surface annulations up to 15 mm in diameter. The roots are also pale to yellowish brown, cylindrical or slightly tapering, sometimes spirally twisted, longitudinally wrinkled and deeply furrowed, up to 4–10 mm in diameter, and passing imperceptibly into rhizome. The fracture is short when dry and becomes tough and pliable on exposure to air.

Microscopic: The rhizomes and roots in transverse section show a thin outer bark separated from a wide xylem by a distinct cambial line. The cork is composed of several rows of thin-walled cells containing yellowish-brown pigment. The rhizome has a small circular pith, occasional small groups of thick-walled, lignified fibers in the pericycle, and a parenchymatous cortex. The phloem and xylem are composed of narrow strands of vascular tissue separated by wide, nonlignified medullary rays. Xylem vessels are lignified, 25–75 μ m in diameter, usually with reticulate thickening but occasionally with spiral or annular thickening. Sclereids occur singly or in small groups, varying considerably in size and shape from rounded to rectangular to elongated and fiber-like, up to 300 μ m long and 20–40 μ m wide, with intercellular spaces forming schizogenous oleoresin canals that are 80–150 μ m in diameter and contain a dense black deposit. The canals are present outside of the central cylinder only (unlike *Echinacea pallida*, where they are present both inside and outside of the central cylinder). Spherocrystalline masses of inulin occur throughout the parenchymatous tissues. Lignified fibers, 300–800 μ m long, are present in scattered groups, and are usually surrounded by phytomelanin (unlike fibers in *Echinacea pallida*, where they usually occur singly in the periphery of the cortex and are 100–300 μ m long, with phytomelanin often absent).

- **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* (561): NMT 3.0%
- **LOSS ON DRYING** (731): Dry at 105° for 2 h: it loses NMT 10.0%.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* (561): NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in well-closed, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.

- **USP REFERENCE STANDARDS** (11)
USP Chlorogenic Acid RS
USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS
USP Powdered *Echinacea angustifolia* Extract RS

Powdered *Echinacea angustifolia*

DEFINITION

Powdered *Echinacea angustifolia* is *Echinacea angustifolia* reduced to a powder or very fine powder.

IDENTIFICATION

- **A. PRESENCE OF ECHINACOSIDE AND DICAFFELOYQUINIC ACIDS (CYNARIN(E))**

Standard solution A: 20 mg/mL of USP Powdered *Echinacea angustifolia* Extract RS in methanol

Standard solution B: 1 mg/mL of 1,3-dicaffeoylquinic acid in methanol

Sample solution: Transfer about 1 g of Powdered *Echinacea angustifolia* to a suitable extraction thimble. Transfer the thimble to a continuous extraction apparatus, and extract with 50 mL of chloroform for 1 h. Retain the chloroform extract for *Identification test B*. Continue the extraction with 50 mL of methanol, and concentrate to a small volume at 40° in vacuum. With the aid of methanol, transfer the extract to a 10-mL volumetric flask, and dilute with methanol to volume.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)

Application volume: 10 µL

Developing solvent system: Ethyl acetate, formic acid, and water (17:2:1)

Spray reagent A: 10 mg/mL of diphenylborinic acid, ethanalamine ester in methanol

Spray reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under UV light at 365 nm.

Acceptance criteria: The chromatogram obtained from the *Sample solution* shows a yellowish zone at an R_f value of 0.14 characteristic of echinacoside (absent or only traces present in *Echinacea purpurea*) that corresponds in color and R_f value to that in the chromatogram of *Standard solution A*, and another zone characteristic of 1,3-dicaffeoylquinic acid (absent in *Echinacea pallida* and *Echinacea purpurea*) corresponding in color and R_f value to that in the chromatogram of *Standard solution B*. Other colored zones of varying intensities may be observed in the chromatogram of the *Sample solution*.

- **B. PRESENCE OF ISOBUTYLALKENYLAMIDES**

Standard solution A: Transfer a quantity of USP Powdered *Echinacea angustifolia* Extract RS to a centrifuge tube, and add chloroform to obtain a solution of about 100 mg/mL. Shake by hand to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Standard solution B: 1 mg/mL of β -sitosterol in methanol

Sample solution: Evaporate the chloroform extract retained from preparation of the *Sample solution* in *Identification test A* to dryness at 40° in vacuum. To the residue add 1 mL of alcohol, and pass through a nylon membrane filter having a pore size of 0.45 µm.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)

Application volume: 10 µL

Developing solvent system: Hexane and ethyl acetate (2:1)

Spray reagent: Prepare a mixture of glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (10:5:0.5) in an ice bath.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Examine the plate under UV light at 254 nm. Spray the plate with *Spray reagent*, and then heat the plate at 100° for 5 min.

Acceptance criteria

Under UV light: The chromatogram obtained from the *Sample solution* shows one main zone at an R_f value of about 0.25 due to 2*E*,4*E*,8*Z*,10*E*-dodecatetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide (absent in *E. pallida*) that corresponds in R_f value to that in the chromatogram of *Standard solution A*.

After treatment with *Spray reagent* and heating: The chromatogram obtained from the *Sample solution* shows a zone due to β -sitosterol that corresponds in R_f value to the principal spot in the chromatogram of *Standard solution B*. Below this spot, there is a zone due to dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and to dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide that corresponds in R_f value to that in the chromatogram of *Standard solution A*; and below this spot, there are several yellowish zones due to α,β -unsaturated isobutylamides (absent in *Echinacea pallida* and mostly violet in *Echinacea purpurea* due to the presence of $\alpha,\beta,\gamma,\delta$ -unsaturated isobutylamides) that are not visible or are very weak when viewed under UV light at 254 nm.

- **C.** In the test for *Content of Total Phenols* the retention time of the major peak of the *Sample solution* corresponds to that of the echinacoside peak of *Standard solution A*, and a peak is detected at the retention time for 1,3-dicaffeoylquinic acid from the *Sample solution* corresponding to that of *Standard solution A*.

COMPOSITION

- **CONTENT OF TOTAL PHENOLS**

Solution A: Phosphoric acid (0.1 in 100) in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17	60	40
17.5	90	10
22	90	10

Solvent: Alcohol and water (7:3)

Standard solution A: Dissolve USP Powdered *Echinacea angustifolia* Extract RS in *Solvent*, shaking and heating in a water bath. Dilute with *Solvent* to obtain a solution having a known concentration of 1 mg/mL. Pass through a membrane filter having a 0.45-µm or finer pore size.

Standard solution B: 40 µg/mL of USP Chlorogenic Acid RS in *Solvent*

Sample solution: Transfer about 125 mg of Powdered *Echinacea angustifolia* (capable of passing through a 40-mesh sieve), accurately weighed, to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux, while shaking by mechani-

cal means, for 15 min. Centrifuge, or pass through a membrane filter having a 0.45- μ m or finer pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 5 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with the USP Powdered *Echinacea angustifolia* Extract RS.

Resolution: NLT 1.0 between the 1,3-dicaffeoylquinic acid isomer and echinacoside peaks, *Standard solution A*

Capacity factor (k'): NLT 3.0, *Standard solution B*

Tailing factor: NMT 2.0 for the chlorogenic acid peak, *Standard solution B*

Relative standard deviation: NMT 2.5% for the chlorogenic acid peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the relevant analytes in the chromatogram obtained from the *Sample solution* by comparison with the chromatogram obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Separately calculate the percentage of caftaric acid (C₁₃H₁₂O₉), chicoric acid (C₂₂H₁₈O₁₂), chlorogenic acid (C₁₆H₁₈O₉), dicaffeoylquinic acids (C₂₅H₂₄O₁₂), and echinacoside (C₃₅H₄₆O₂₀) in the portion of Powdered *Echinacea angustifolia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak response for the relevant analyte from the *Sample solution*

r_S = peak response for chlorogenic acid from *Standard solution B*

C_S = concentration of USP Chlorogenic Acid RS in *Standard solution B* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered *Echinacea angustifolia* used to prepare the *Sample solution* (mg)

F = response factor: chicoric acid, 0.695; dicaffeoylquinic acids, 0.729; caftaric acid, 0.881; chlorogenic acid, 1.000; and echinacoside, 2.220

Calculate the percentage of total phenols in the portion of Powdered *Echinacea angustifolia* taken by adding the individual percentages calculated.

Acceptance criteria: NLT 0.5% of total phenols on the dried basis

• CONTENT OF DODECATETRAENOIC ACID ISOBUTYLAMIDES

Standard solution A: Dissolve, with sonication, USP Powdered *Echinacea angustifolia* Extract RS in methanol, shaking for 10 min, and dilute with methanol to obtain a solution having a concentration of 5 mg/mL. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Standard solution B: 10 μ g/mL of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in methanol

Sample solution: Transfer about 2.5 g of Powdered *Echinacea angustifolia* (capable of passing through a 40-mesh sieve), accurately weighed, into a round-bottom flask. Add 80 mL of methanol, and reflux for 30 min. Cool to room temperature, and filter into a 100-mL volumetric flask, using small portions of methanol to rinse the flask and the filter. Dilute with

methanol to volume. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Mobile phase: Acetonitrile and water (55:45)

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L1

Column temperature: 30°

Flow rate: 1.5 mL/min

Injection size: 25 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution A* is similar to the Reference Chromatogram for alkamides provided with USP Powdered *Echinacea angustifolia* Extract RS.

Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*

Tailing factor: NMT 2.0 for the 2*E*,4*E*-hexadienoic acid isobutylamide peak, *Standard solution B*

Relative standard deviation: NMT 2.5% for the 2*E*,4*E*-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks due to 2*E*,4*E*,8*Z*,10*E*-dodecatetraenoic acid isobutylamide and 2*E*,4*E*,8*Z*,10*Z*-dodecatetraenoic acid isobutylamide in the chromatogram from the *Sample solution* by comparison with the chromatogram from *Standard solution A*. Measure the areas for the relevant peaks.

Calculate the percentage of dodecatetraenoic acid isobutylamides in the portion of Powdered *Echinacea angustifolia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = sum of the peak responses of the relevant analytes from the *Sample solution*

r_S = peak response of 2*E*,4*E*-hexadienoic acid isobutylamide from the *Standard solution B*

C_S = concentration of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered *Echinacea angustifolia* used to prepare the *Sample solution* (mg)

F = response factor for 2*E*,4*E*-hexadienoic acid isobutylamide, 1.353

Acceptance criteria: NLT 0.075% of dodecatetraenoic acid isobutylamides (C₁₆H₂₅NO) on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method III* <231>: NMT 10 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Powdered *Echinacea angustifolia* is a brown powder with a slight aromatic odor and a sweet taste that quickly becomes bitter, leaving a tingling sensation on the tongue. Under a microscope, the following characteristics are observed: thin-walled polygonal cork cells with red-brown contents; lignified reticulate vessels; abundant stone cells of various shapes; fragments of oleoresin canals with reddish-brown contents; and abundant thin-walled parenchyma with spherocrystalline masses of inulin.
- **LOSS ON DRYING** <731>: Dry a sample at 105° for 2 h: it loses NMT 10.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 7.0%

- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash (561):** NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS (11)**
USP Chlorogenic Acid RS
USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS
USP Powdered *Echinacea angustifolia* Extract RS

Powdered *Echinacea angustifolia* Extract

DEFINITION

Powdered *Echinacea angustifolia* Extract is prepared from *Echinacea angustifolia* roots by extraction with hydroalcoholic mixtures or other suitable solvents. The ratio of the starting crude plant material to Powdered Extract is 2:1–8:1. It contains NLT 4.0% and NMT 5.0% of total phenols, calculated on the dried basis as the sum of caftaric acid (C₁₃H₁₂O₉), chicoric acid (C₂₂H₁₈O₁₂), chlorogenic acid (C₁₆H₁₈O₉), dicaffeoylquinic acids (C₂₅H₂₄O₁₂), and echinacoside (C₃₅H₄₆O₂₀). It contains NLT 0.1% of dodecatetraenoic acid isobutylamides (C₁₆H₂₅NO) on the dried basis.

IDENTIFICATION

• A. PRESENCE OF ISOBUTYLALKENYLAMIDES

Standard solution A: Transfer a quantity of USP Powdered *Echinacea angustifolia* Extract RS to a centrifuge tube, and add chloroform to obtain a solution having a known concentration of about 100 mg/mL. Shake by hand to disperse, sonicate for 5 min, and centrifuge. Use the supernatant.

Standard solution B: 1 mg/mL of β -sitosterol in methanol

Sample solution: 100 mg/mL of Powdered Extract in methanol. Allow to stand for 15 min before use.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long μ m (TLC plates)

Application volume: 10 μ L

Developing solvent system: Hexane and ethyl acetate (2:1)

Spray reagent: Prepare a mixture of glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (10:5:0.5) in an ice bath

Analysis

Samples: Standard solution A, Standard solution B, and Sample solution

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Examine the plate under UV light at 254 nm, and then spray the plate with *Spray reagent*, and heat the plate at 100° for 5 min.

Acceptance criteria

Under UV 254 nm: The chromatogram obtained from the *Sample solution* shows one main zone at an *R_f* value of about 0.25 due to 2*E*,4*E*,8*Z*,10*E*-dodecatetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide (absent in *E. pallida*) that corresponds in *R_f* value to that in the chromatogram of *Standard solution A*.

After treatment with *Spray reagent*: The chromatogram obtained from the *Sample solution* shows a zone due to β -sitosterol that corresponds in *R_f* value to the principal spot in the chromatogram of *Standard solution B*. Below this spot, there is a zone due to dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and to dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide that corresponds in

R_f value to that in the chromatogram of *Standard solution A*; and below this spot, there are several yellowish zones due to α , β -unsaturated isobutylamides (absent in *Echinacea pallida* and mostly violet in *Echinacea purpurea* due to the presence of α , β , γ , δ -unsaturated isobutylamides) that are not visible or are very weak when viewed under UV light at 254 nm.

- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the echinacoside peak of *Standard solution A*, and the retention time of the peak for 1,3-dicaffeoylquinic acid from the *Sample solution* corresponds to that of *Standard solution A*, all peaks as obtained in the test for *Content of total phenols*.

COMPOSITION

• CONTENT OF TOTAL PHENOLS

Solution A: Phosphoric acid (0.1 in 100) in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17	60	40
17.5	90	10
22	90	10

Solvent: Alcohol and water (7:3)

Standard solution A: Dissolve USP Powdered *Echinacea angustifolia* Extract RS in *Solvent*, shaking and heating in a water bath. Dilute with *Solvent* to obtain a solution having a known concentration of 1 mg/mL. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Standard solution B: 40 μ g/mL of USP Chlorogenic Acid RS in *Solvent*

Sample solution: Transfer about 60 mg of Powdered Extract, accurately weighed, to an appropriate round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter having a 0.45- μ m or finer pore size.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 5 μ L

System suitability

Samples: Standard solution A and Standard solution B

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with the USP Powdered *Echinacea angustifolia* Extract RS.

Resolution: NLT 1.0 between the 1,3-dicaffeoylquinic acid isomer and echinacoside peaks, *Standard solution A*

Capacity factor (*k'*): NLT 3.0, *Standard solution B*

Tailing factor: NMT 2.0 for the chlorogenic acid peak, *Standard solution B*

Relative standard deviation: NMT 2.5% for the chlorogenic acid peak in repeated injections, *Standard solution B*

Analysis

Samples: Standard solution A, Standard solution B, and Sample solution

Identify the relevant analytes in the chromatogram obtained from the *Sample solution* by comparison with the chromatogram obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Separately calculate the percentage of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), chlorogenic acid ($C_{16}H_{18}O_9$), dicaffeoylquinic acids ($C_{25}H_{24}O_{12}$), and echinacoside ($C_{35}H_{46}O_{20}$) in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

- r_U = peak response for the relevant analyte from the *Sample solution*
 r_S = peak response for chlorogenic acid from *Standard solution B*
 C_S = concentration of USP Chlorogenic Acid RS in *Standard solution B* (mg/mL)
 C_U = concentration of Powdered *Echinacea angustifolia* Extract in the *Sample solution* (mg/mL)
 F = response factor: chicoric acid, 0.695; dicaffeoylquinic acids, 0.729; caftaric acid, 0.881; chlorogenic acid, 1.000; and echinacoside, 2.220

Calculate the percentage of total phenols in the portion of Powdered Extract taken by adding the individual percentages.

Acceptance criteria: NLT 4.0% and NMT 5.0% of total phenols on the dried basis

• CONTENT OF DODECATETRAENOIC ACID ISOBUTYLAMIDES

Standard solution A: Dissolve USP Powdered *Echinacea angustifolia* Extract RS in methanol, shaking for 1 min, and dilute with methanol to volume to obtain a solution having a known concentration of 1 mg/mL. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Standard solution B: 10 μ g/mL of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in methanol

Sample solution: Transfer about 500 mg of Powdered Extract, accurately weighed, to a 100-mL volumetric flask. Add 80 mL of methanol, and sonicate for 30 min. Dilute with methanol to volume, and pass through a membrane filter having a 0.45- μ m or finer pore size.

Mobile phase: Acetonitrile and water (55:45)

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L1

Column temperature: 30°

Flow rate: 1.5 mL/min

Injection size: 25 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for alkamides provided with USP Powdered *Echinacea angustifolia* Extract RS.

Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*

Tailing factor: NMT 2.0 for the 2*E*,4*E*-hexadienoic acid isobutylamide peak, *Standard solution B*

Relative standard deviation: NMT 2.5% for the 2*E*,4*E*-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks due to 2*E*,4*E*,8*Z*,10*E*-dodecatetraenoic acid isobutylamide and 2*E*,4*E*,8*Z*,10*Z*-dodecatetraenoic acid isobutylamide in the chromatogram from the *Sample solution* by comparison with the chromatogram

obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Calculate the percentage of dodecatetraenoic acid isobutylamides in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

- r_U = sum of the peak responses of the relevant analytes from the *Sample solution*
 r_S = peak response for 2*E*,4*E*-hexadienoic acid isobutylamide from *Standard solution B*
 C_S = concentration of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)
 C_U = concentration of Powdered *Echinacea angustifolia* Extract in the *Sample solution* (mg/mL)
 F = response factor for 2*E*,4*E*-hexadienoic acid isobutylamide, 1.353

Acceptance criteria: NLT 0.1% of dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$) on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method II* <231>: 20 ppm
- **MICROBIAL ENUMERATION TESTS** <2021>: The total bacterial count does not exceed 10^4 cfu/g and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meets the requirements of the tests for absence of *Salmonella* species, and *Escherichia coli*.
- **OTHER REQUIREMENTS:** It meets the requirements for *Botanical Extracts* <565>, *Residual Solvents* and *Pesticide Residues*.

SPECIFIC TESTS

- **LOSS ON DRYING** <731>: Dry 1 g at 105° for 2 h: it loses NMT 5.0%.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, in a cool place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. If standardized by the content of alkamides, label it to indicate the targeted content of dodecatetraenoic acid isobutylamides. The label bears a statement indicating that *Echinacea angustifolia* may cause rare allergic reactions, rashes, or aggravate asthma. It meets the requirements for *Botanical Extracts* <565>, *Labeling*.
- **USP REFERENCE STANDARDS** <11>
 USP Chlorogenic Acid RS
 USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS
 USP Powdered *Echinacea angustifolia* Extract RS

Echinacea pallida

DEFINITION

Echinacea pallida consists of the dried rhizome and roots of *Echinacea pallida* (Nutt.) Nutt. (Fam. Asteraceae). It is harvested in the fall after 3 or more years of growth. It contains NLT 0.5% of total phenols, calculated on the dried basis as the sum of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), chlorogenic acid ($C_{16}H_{18}O_9$), and echinacoside ($C_{35}H_{46}O_{20}$).

IDENTIFICATION

- **A. PRESENCE OF ECHINACOSIDE AND ABSENCE OF DICAFFELOYLQUINIC ACIDS (CYNARIN(E))**

Standard solution A: 10 mg/mL of USP Powdered *Echinacea pallida* Extract RS in methanol

Standard solution B: 1 mg/mL of 1,3-dicaffeoylquinic acid in methanol

Sample solution: Weigh and finely pulverize about 10 g of *Echinacea pallida*, and transfer about 1 g of powder, to a suitable extraction thimble. Transfer the thimble to a continuous extraction apparatus, and extract with 50 mL of chloroform for 1 h. Retain the chloroform extract for *Identification* test B. Continue the extraction with 50 mL of methanol, and concentrate to a small volume at 40° in vacuum. With the aid of methanol, transfer the extract to a 10-mL volumetric flask, and dilute with methanol to volume.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)

Application volume: 10 µL

Developing solvent system: Ethyl acetate, formic acid, and water (17:2:1)

Spray reagent A: 10 mg/mL of diphenylborinic acid, ethanolamine ester in methanol

Spray reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and then examine the plate under UV light at 365 nm.

Acceptance criteria: The chromatogram obtained from the *Sample solution* shows a yellowish zone at an R_f value of 0.14, characteristic of echinacoside (absent or traces in *Echinacea purpurea*), corresponding in color and R_f value to that in the chromatogram of *Standard solution A*, and does not show a zone characteristic of 1,3-dicaffeoylquinic acid (present in *Echinacea angustifolia*) corresponding in color and R_f value to that in the chromatogram of *Standard solution B*. Other colored zones of varying intensities may be observed in the chromatogram of the *Sample solution*.

• B. PRESENCE OF KETOALKENYNES

Standard solution A: 10 mg/mL of USP Powdered *Echinacea pallida* Extract RS in chloroform. Shake for 1 min, and centrifuge. Use the supernatant.

Standard solution B: 1 mg/mL of β -sitosterol in methanol

Sample solution: Evaporate to dryness the chloroform extract retained from preparation of the *Sample solution* in *Identification* test A at 40° in vacuum. To the residue add 1 mL of alcohol, and pass through a nylon membrane filter having a pore size of 0.45 µm.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)

Application volume: 10 µL

Developing solvent system: Toluene and ethyl acetate (7:3)

Spray reagent A: 1% solution of vanillin in alcohol

Spray reagent B: 10% solution of sulfuric acid in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and heat the plate at 120° for 3 min.

Acceptance criteria: The chromatogram obtained from the *Sample solution* shows green, brown, and violet zones above the spot for β -sitosterol (R_f range, 0.6–0.8). These zones (unlike those in *Echinacea angustifolia* and *Echinacea purpurea*) are characteristic of ketoalkenyne, and correspond in R_f value to the zones in the chromatogram obtained from *Standard solution A*.

- **C.** The retention time of the major peak in the *Sample solution* corresponds to that of the echinacoside peak in

Standard solution A, as obtained in the test for *Content of Total Phenols*. The peak area of any peak detected at the locus of 1,3-dicaffeoylquinic acid is NMT 1% of the peak area for the echinacoside peak.

COMPOSITION

• CONTENT OF TOTAL PHENOLS

Solution A: Phosphoric acid (0.1 in 100) in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17	60	40
17.5	90	10
22	90	10

Solvent: Alcohol and water (7:3)

Standard solution A: Dissolve USP Powdered *Echinacea pallida* Extract RS in *Solvent*, by shaking and heating in a water bath. Dilute with *Solvent* to obtain a solution having a known concentration of 1 mg/mL. Pass through a membrane filter having a 0.45-µm or finer pore size.

Standard solution B: 40 µg/mL of USP Chlorogenic Acid RS in *Solvent*. Pass through a membrane filter having a 0.45-µm or finer pore size.

Sample solution: Transfer 125 mg of finely powdered *Echinacea pallida* (capable of passing through a 40-mesh sieve), to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux, while shaking by mechanical means, for 15 min. Centrifuge, or pass through a membrane filter having a 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 5 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained is similar to the Reference Chromatogram for total phenols provided with USP Powdered *Echinacea pallida* Extract RS.

Capacity factor (k'): NLT 3.0 for the chlorogenic acid peak, *Standard solution B*

Tailing factor: NMT 2.0 for the chlorogenic acid peak, *Standard solution B*

Relative standard deviation: NMT 2.5% for the chlorogenic acid peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the relevant analytes in the chromatogram obtained from the *Sample solution* by comparison with the chromatogram obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Separately calculate the percentage of caffeic acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), chlorogenic acid ($C_{16}H_{18}O_9$), and echinacoside ($C_{35}H_{46}O_{20}$) in the portion of *Echinacea pallida* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

- r_U = peak response for the relevant analyte from the *Sample solution*
 r_S = peak response for chlorogenic acid from *Standard solution B*
 C_S = concentration of USP Chlorogenic Acid RS in *Standard solution B* (mg/mL)
 V = volume of the *Sample solution* (mL)
 W = weight of powdered *Echinacea pallida* used to prepare the *Sample solution* (mg)
 F = response factor: chicoric acid, 0.695; caftaric acid, 0.881; chlorogenic acid, 1.000; and echinacoside, 2.220

Calculate the percentage of total phenols in the portion of *Echinacea pallida* taken by adding the individual percentages calculated.

Acceptance criteria: NLT 0.5% of total phenols on the dried basis

CONTAMINANTS

- **HEAVY METALS, Method III (231):** 10 ppm
- **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis (561):** Meets the requirements

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: The outer surface of the rhizome is pale to yellowish-brown, crowned with the remains of the aerial stem, and sometimes shows surface annulations up to 15 mm in diameter. The roots are pale to yellowish-brown, cylindrical or slightly tapering, sometimes spirally twisted, longitudinally wrinkled and deeply furrowed, up to 4–10 mm in diameter, and pass imperceptibly into rhizome. The short fracture, when dry, becomes tough and pliable on exposure to air.

Microscopic: The rhizomes and roots in transverse section show a thin outer bark separated from a wide xylem by a distinct cambial line. The cork is composed of several rows of thin-walled cells containing yellowish-brown pigment. The rhizome has a small circular pith, occasional small groups of thick-walled, lignified fibers in the pericycle, and a parenchymatous cortex. The phloem and xylem are composed of narrow strands of vascular tissue separated by wide, nonlignified medullary rays. Xylem vessels are lignified, 25–75 μm in diameter, usually with reticulate thickening but occasionally with spiral or annular thickening. Sclereids occur singly or in small groups, varying considerably in size and shape from rounded to rectangular to elongated and fiber-like, are up to 300 μm long and 20–40 μm wide, with intercellular spaces forming schizogenous oleoresin canals that are 80–150 μm in diameter and contain a dense black deposit present both inside and outside of the central cylinder (unlike *Echinacea angustifolia*, where the canals are present only outside of the central cylinder). Spherocrystalline masses of inulin occur throughout the parenchymatous tissues. Lignified fibers, present in the periphery of the cortex, are 100–300 μm long and occur singly with phytomelanin often absent (unlike *Echinacea angustifolia*, where the fibers occur scattered in groups, are 300–800 μm long, and are usually surrounded by phytomelanin).

- **ARTICLES OF BOTANICAL ORIGIN, Volatile Oil Determination (561):** 1.0–2.0 mL/100 g
- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter (561):** NMT 3.0%
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash (561):** NMT 4.0%
- **LOSS ON DRYING (731):** Dry a sample at 105° for 2 h: it loses NMT 10.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS (11)**
 USP Chlorogenic Acid RS
 USP Powdered *Echinacea pallida* Extract RS

Powdered *Echinacea pallida*

DEFINITION

Powdered *Echinacea pallida* is *Echinacea pallida* reduced to a powder or very fine powder.

IDENTIFICATION

• A. PRESENCE OF ECHINACOSIDE AND ABSENCE OF DICAFFELOYLQUINIC ACIDS (CYNARIN(E))

Standard solution A: 10 mg/mL of USP Powdered *Echinacea pallida* Extract RS in methanol

Standard solution B: 1 mg/mL of 1,3-dicaffeoylquinic acid in methanol

Sample solution: Transfer 1 g of Powdered *Echinacea pallida* to a suitable extraction thimble. Transfer the thimble to a continuous extraction apparatus, and extract with 50 mL of chloroform for 1 h. Retain the chloroform extract for *Identification* test B. Continue the extraction with 50 mL of methanol, and concentrate to a small volume at 40° in vacuum. With the aid of methanol, transfer the extract to a 10-mL volumetric flask, and dilute with methanol to volume.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture (TLC plates)

Application volume: 10 μL

Developing solvent system: Ethyl acetate, formic acid, and water (17:2:1)

Spray reagent A: 10 mg/mL of diphenylborinic acid, ethanolamine ester in methanol

Spray reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and then examine the plate under UV light at 365 nm.

Acceptance criteria: The chromatogram obtained from the *Sample solution* shows a yellowish zone at an R_f value of 0.14, characteristic of echinacoside (absent or traces in *Echinacea purpurea*), corresponding in color and R_f value to that in the chromatogram of *Standard solution A*, and does not show a zone characteristic of 1,3-dicaffeoylquinic acid (present in *Echinacea angustifolia*) corresponding in color and R_f value to that in the chromatogram of *Standard solution B*. Other colored zones of varying intensities may be observed in the chromatogram of the *Sample solution*.

• B. PRESENCE OF KETOALKENYNES

Standard solution A: 10 mg/mL of USP Powdered *Echinacea pallida* Extract RS in chloroform. Shake for 1 min, and centrifuge. Use the supernatant.

Standard solution B: 1 mg/mL of β -sitosterol in methanol

Sample solution: Evaporate to dryness the chloroform extract retained from preparation of the *Sample solution* in *Identification* test A at 40° in vacuum. To the residue add 1 mL of alcohol, and pass through a nylon membrane filter having a 0.45- μm pore size.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture (TLC plates)

Application volume: 10 μ L

Developing solvent system: Toluene and ethyl acetate (7:3)

Spray reagent A: 1% solution of vanillin in alcohol

Spray reagent B: 10% solution of sulfuric acid in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and heat the plate at 120° for 3 min.

Acceptance criteria: The chromatogram obtained from the *Sample solution* shows green, brown, and violet zones above the spot for β -sitosterol (R_f range 0.6–0.8). These zones (unlike those in *Echinacea angustifolia* and *Echinacea purpurea*) are characteristic of ketoalkenynes, and correspond in R_f value to the zones in the chromatogram obtained from *Standard solution A*.

- **C.** The retention time of the major peak in the *Sample solution* corresponds to that of the echinacoside peak in the *Standard solution A*, as obtained in the test for *Content of Total Phenols*. The peak area of any peak detected at the locus of 1,3-dicaffeoylquinic acid is NMT 1% of the peak area for the echinacoside peak.

COMPOSITION

• CONTENT OF TOTAL PHENOLS

Solution A: Phosphoric acid (0.1 in 100) in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17	60	40
17.5	90	10
22	90	10

Solvent: Alcohol and water (7:3)

Standard solution A: Dissolve USP Powdered *Echinacea pallida* Extract RS in *Solvent*, by shaking and heating in a water bath. Dilute with *Solvent* to obtain a solution having a known concentration of about 1 mg/mL. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Standard solution B: 40 μ g/mL of USP Chlorogenic Acid RS in *Solvent*

Sample solution: Transfer 125 mg of Powdered *Echinacea pallida* (capable of passing through a 40-mesh sieve), to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux, while shaking by mechanical means, for 15 min. Centrifuge, or pass through a membrane filter having a 0.45- μ m or finer pore size.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 5 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with USP Powdered *Echinacea pallida* Extract RS.

Capacity factor (k'): NLT 3.0 for the chlorogenic acid peak, *Standard solution B*

Tailing factor: NMT 2.0 for the chlorogenic acid peak, *Standard solution B*

Relative standard deviation: NMT 2.5% for the chlorogenic acid peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the relevant analytes in the chromatogram obtained from the *Sample solution* by comparison with the chromatogram obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Separately calculate the percentage of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), chlorogenic acid ($C_{16}H_{18}O_9$), and echinacoside ($C_{35}H_{46}O_{20}$) in the portion of Powdered *Echinacea pallida* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak response for the relevant analyte from the *Sample solution*

r_S = peak response for chlorogenic acid from *Standard solution B*

C_S = concentration of USP Chlorogenic Acid RS in *Standard solution B* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Powdered *Echinacea pallida* taken to prepare the *Sample solution* (mg)

F = response factor: chicoric acid, 0.695; caftaric acid, 0.881; chlorogenic acid, 1.000; and echinacoside, 2.220

Calculate the percentage of total phenols in the portion of *Echinacea pallida* taken by adding the individual percentages calculated.

Acceptance criteria: NLT 0.5% of total phenols on the dried basis

CONTAMINANTS

• **HEAVY METALS**, *Method III* (231): 10 ppm

• **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements

SPECIFIC TESTS

• **BOTANICAL CHARACTERISTICS:** Powdered *Echinacea pallida* is a brown powder with a faint aromatic odor and a slightly acid, persistent taste. It turns yellow when mounted in sodium hydroxide solution. Under a microscope, the following characteristics are observed: groups of secretory canals with brown contents, surrounded by parenchymatous cells containing cluster crystals of calcium oxalate; and parenchymatous cells with small starch granules; thick-walled lignified fibers and fragments of reticulate and pitted vessels.

• **ARTICLES OF BOTANICAL ORIGIN**, *Volatile Oil Determination* (561): 1.0–2.0 mL/100 g

• **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 7.0%

• **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* (561): NMT 4.0%

- **LOSS ON DRYING** (731): Dry a sample at 105° for 2 h: it loses NMT 10.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS** (11)
 - USP Chlorogenic Acid RS
 - USP Powdered *Echinacea pallida* Extract RS

Powdered *Echinacea pallida* Extract

DEFINITION

Powdered *Echinacea pallida* Extract is prepared from *Echinacea pallida* roots by extraction with hydroalcoholic mixtures or other suitable solvents. The ratio of the starting crude plant material to Powdered Extract is between 2:1 and 8:1. It contains NLT 4.0% and NMT 5.0% of total phenols, calculated as the sum of caftaric acid (C₁₃H₁₂O₉), chicoric acid (C₂₂H₁₈O₁₂), chlorogenic acid (C₁₆H₁₈O₉), and echinacoside (C₃₅H₄₆O₂₀), on the dried basis.

IDENTIFICATION

- **A. PRESENCE OF ECHNIACOSIDE AND ABSENCE OF DICAFFELOYLQUINIC ACIDS (CYNARIN(E))**

Standard solution A: 100 mg/mL of USP Powdered *Echinacea pallida* Extract RS in methanol

Standard solution B: 1 mg/mL of 1,3-dicaffeoylquinic acid in methanol

Sample solution: Dissolve 1.0 g of Powdered Extract in 10 mL of methanol. Allow to settle before use.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)

Application volume: 10 µL

Developing solvent system: Ethyl acetate, formic acid, and water (17:2:1)

Spray reagent A: 10 mg/mL of diphenylborinic acid, ethanolamine ester in methanol

Spray reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under UV light at 365 nm.

Acceptance criteria: The chromatogram obtained from the *Sample solution* shows a yellowish zone at an *R_f* value of 0.14 characteristic of echinacoside (absent or only traces present in *Echinacea purpurea*) that corresponds in color and *R_f* value to that in the chromatogram of *Standard solution A*, and does not show a zone characteristic of 1,3-dicaffeoylquinic acid (present in *Echinacea angustifolia*) corresponding in color and *R_f* value to that in the chromatogram of *Standard solution B*. Other colored zones of varying intensities may be observed in the chromatogram of the *Sample solution*.

- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the echinacoside peak of *Standard solution A*, as obtained in the test for *Content of Total Phenols*.

COMPOSITION

- **CONTENT OF TOTAL PHENOLS**

Solution A: Phosphoric acid (0.1 in 100)

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17	60	40
17.5	90	10
22	90	10

Solvent: Alcohol and water (7:3)

Standard solution A: Dissolve USP Powdered *Echinacea pallida* Extract RS in *Solvent*, by shaking and heating in a water bath. Dilute with *Solvent* to obtain a solution having a known concentration of 1 mg/mL. Pass through a membrane filter having a 0.45-µm or finer pore size.

Standard solution B: 40 µg/mL of USP Chlorogenic Acid RS in *Solvent*

Sample solution: Transfer about 60 mg of Powdered Extract, accurately weighed, to an appropriate round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter having a 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 5 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with USP Powdered *Echinacea pallida* Extract RS.

Capacity factor (k'): NLT 3.0 for the chlorogenic acid peak, *Standard solution B*

Tailing factor: NMT 2.0 for the chlorogenic acid peak, *Standard solution B*

Relative standard deviation: NMT 2.5% for the chlorogenic acid peak in repeated injections, *Standard solution B*

Analysis

Samples: *Sample solution*, *Standard solution A*, and *Standard solution B*

Identify the relevant analytes in the chromatogram obtained from the *Sample solution* by comparison with the chromatogram obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Separately calculate the percentage of caftaric acid (C₁₃H₁₂O₉), chicoric acid (C₂₂H₁₈O₁₂), chlorogenic acid (C₁₆H₁₈O₉), and echinacoside (C₃₅H₄₆O₂₀) in the portion of Powdered *Echinacea pallida* Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response for the relevant analyte from the *Sample solution*

r_S = peak response for chlorogenic acid from *Standard solution B*

C_S = concentration of USP Chlorogenic Acid RS in *Standard solution B* (mg/mL)

- C_U = concentration of Powdered *Echinacea pallida* Extract in the *Sample solution* (mg/mL)
 F = response factor: chicoric acid, 0.695; caftaric acid, 0.881; chlorogenic acid, 1.000; and echinacoside, 2.220

Calculate the percentage of total phenols in the portion of Powdered *Echinacea pallida* Extract taken by adding the individual percentages calculated.

Acceptance criteria: NLT 4.0% and NMT 5.0% of total phenols on the dried basis

CONTAMINANTS

- **HEAVY METALS** *Method II* (231): NMT 20 ppm
- **MICROBIAL ENUMERATION TESTS** (2021): The total bacterial count does not exceed 10^4 cfu/g and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella* species, and *Escherichia coli*
- **OTHER REQUIREMENTS:** It meets the requirements for *Botanical Extracts* (565), *Residual Solvents* and *Pesticide Residues*.

SPECIFIC TESTS

- **LOSS ON DRYING** (731): Dry 1 g at 105° for 2 h: it loses NMT 5.0%.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store in a cool place.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant from which the article was prepared. The label bears a statement indicating that *Echinacea pallida* may cause rare allergic reactions, rashes, or aggravate asthma. It meets the requirements for *Botanical Extracts* (565), *Labeling*.
- **USP REFERENCE STANDARDS** (11)
 USP Chlorogenic Acid RS
 USP Powdered *Echinacea pallida* Extract RS

Echinacea purpurea Aerial Parts

DEFINITION

Echinacea purpurea Aerial Parts consists of the aerial parts of *Echinacea purpurea* (L.) Moench (Fam. Asteraceae). It is harvested during the flowering stage. It contains NLT 1.0% of the sum of caftaric acid ($C_{13}H_{12}O_9$) and chicoric acid ($C_{22}H_{18}O_{12}$), and NLT 0.01% of dodecatetraenoic acid isobutylamides ($C_{16}H_{25}NO$), calculated on the dried basis.

IDENTIFICATION

- **A. PRESENCE OF CHICORIC ACID AND ABSENCE OF ECHINACOSIDE**
Standard solution: 10 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol
Sample solution: Add 5 mL of diluted alcohol (7:3) to 0.5 g of the powdered plant material, and shake for 1 min. Centrifuge, and use the supernatant.
Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μ m (TLC plates)
Application volume: 10 μ L
Developing solvent system: Ethyl acetate, formic acid, and water (17:2:1)
Spray reagent A: 10 mg/mL of diphenylborinic acid, ethanolamine ester in methanol
Spray reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*
 Develop the chromatograms until the solvent front has moved NLT 18 cm, and dry the plate in a stream of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under UV light at 365 nm.

Acceptance criteria: The chromatogram from the *Sample solution* shows a yellowish-green zone at an R_f value of 0.75 due to chicoric acid and another yellowish-green zone at an R_f value of 0.45 due to caftaric acid, both zones corresponding in color and R_f value to zones in the chromatogram from the *Standard solution*. The chromatogram from the *Sample solution* does not show or shows only traces of a zone at an R_f value of 0.1 due to echinacoside (present in *Echinacea angustifolia* and in *Echinacea pallida*). Other colored zones of varying intensities may be observed in the chromatogram from the *Sample solution*.

- **B.** The retention times for the relevant peaks of the *Sample solution*, mainly due to caftaric acid and chicoric acid, correspond to those of *Standard solution A*, as obtained in the test for *Content of Chicoric Acid and Caftaric Acid*. A peak for echinacoside is not detected or is very weak (difference with *E. pallida* and *E. angustifolia*).
- **C.** The retention times for the relevant peaks of the *Sample solution*, mainly due to dodecatetraenoic isobutyl amides, correspond to those of *Standard solution A*, as obtained in the test for *Content of Dodecatetraenoic Isobutylamides*.

COMPOSITION

- **CONTENT OF CHICORIC ACID AND CAFTARIC ACID**

Solution A: Phosphoric acid (0.1 in 100) in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17.5	60	40
18	90	10
30	90	10

Solvent: Alcohol and water (7:3)

Standard solution A: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in *Solvent*. Dissolve by shaking for 1 min, dilute to volume, and filter through a filter of 0.45- μ m or finer pore size.

Standard solution B: 40 μ g/mL of USP Chlorogenic Acid RS in *Solvent*. Dissolve by shaking for 1 min, dilute to volume, and pass through a filter of 0.45- μ m or finer pore size.

Sample solution: Transfer about 125 mg, accurately weighed, of finely powdered *Echinacea purpurea* Aerial Parts (capable of passing through a 40-mesh sieve), to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux, while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter of 0.45- μ m or finer pore size.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 5 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with USP Powdered *Echinacea purpurea* Extract RS.

Relative standard deviation: NMT 2.0% for the chlorogenic acid peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the relevant analytes in the chromatogram obtained from the *Sample solution* by comparison with the chromatogram obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Separately calculate the percentages of caftaric acid (C₁₃H₁₂O₉) and chicoric acid (C₂₂H₁₈O₁₂) in the portion of *Echinacea purpurea* Aerial Parts taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of chlorogenic acid from *Standard solution B*

C_S = concentration of USP Chlorogenic Acid RS in *Standard solution B* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of *Echinacea purpurea* Aerial Parts taken to prepare the *Sample solution* (mg)

F = response factor: chicoric acid, 0.695; caftaric acid, 0.881; and chlorogenic acid, 1.000

Calculate the percentage of the sum of chicoric acid and caftaric acid in the portion of *Echinacea purpurea* Aerial Parts taken by adding the individual percentages calculated.

Acceptance criteria: NLT 1.0% on the dried basis

• CONTENT OF DODECATETRAENOIC ACID ISOBUTYLAMIDES

Mobile phase: Acetonitrile and water (55:45)

Standard solution A: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Dissolve using sonication and shaking for 10 min. After dilution, pass through a membrane filter of 0.45-μm or finer pore size.

Standard solution B: 10 μg/mL of USP 2E,4E-Hexadienoic Acid Isobutylamide RS in methanol

Sample solution: Transfer about 2.5 g of finely powdered *Echinacea purpurea* Aerial Parts (capable of passing through a 40-mesh sieve), accurately weighed, into a round-bottom flask. Add 80 mL of methanol, and reflux for 30 min. Cool to room temperature, and filter into a 100-mL volumetric flask, using small portions of methanol to rinse the flask and the filter. Dilute with methanol to volume. Pass through a membrane filter of 0.45-μm or finer pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Column temperature: 30°

Flow rate: 1.5 mL/min

Injection size: 25 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the Reference Chromatogram for alkalimides provided with USP Powdered *Echinacea purpurea* Extract RS.

Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*

Tailing factor: NMT 2.0 for 2E,4E-hexadienoic acid isobutylamide, *Standard solution B*

Relative standard deviation: NMT 2.5% for the 2E, 4E-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks of the two isomers of dodecatetraenoic acid isobutylamides in the chromatogram obtained from the *Sample solution* by comparison with the chromatogram obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Calculate the percentage of dodecatetraenoic acid isobutylamides in the portion of *Echinacea purpurea* Aerial Parts taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = sum of the peak areas of the relevant analytes from the *Sample solution*

r_S = peak area of 2E,4E-hexadienoic acid isobutylamide from *Standard solution B*

C_S = concentration of USP 2E,4E-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of *Echinacea purpurea* Aerial Parts taken to prepare the *Sample solution* (mg)

F = response factor to convert 2E,4E-hexadienoic acid isobutylamide into dodecatetraenoic acid isobutylamides, 1.353

Acceptance criteria: NLT 0.01% of dodecatetraenoic acid isobutylamides on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method III* (231): NMT 10 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticides Residues Analysis* (561): Meet the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and the enterobacterial count does not exceed 10³ cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• BOTANICAL CHARACTERISTICS

Macroscopic: The herb is an erect, coarse, rough-hairy perennial, usually up to 90 cm tall, rarely up to 180 cm. The leaves are alternate and simple; the lowermost leaves are slender, long, and petioled, ovate to broadly lanceolate, mostly penta-nerved, acute or acuminate at the apex, abruptly narrowed or rarely cordate at the base, usually sharply dentate, and 7–20 cm long and 2.5–7.5 cm wide; the petioles are mostly winged at the

summit. The upper leaves are narrower, often almost entirely sessile, lanceolate or ovate lanceolate, and usually with 3 veins.

The flower heads are radiate, up to 15 cm across, solitary or few, and long-peduncled, with 12–20 rays, purple, crimson, or rarely pale; the bristle disks are often orange, 3.5–7.5 cm long; the involucre is depressed-hemispheric; the bracts are lanceolate, spreading or appressed, imbricated in 2–4 series, and hairy on the outer surface with ciliate margins; the receptacle is conical, the scales of the receptacle stiff, spinescent, and conspicuously longer than the disc flowers; the chaff is carinate and cuspidate; the achenes are 3–4 mm in length, tetrasided, obpyramidal, and thick; the pappus has a short, dentate crown.

Microscopic

Leaf: The leaf has a thickness of 200–350 μm , with an epidermis 9–13 μm thick, largely without chloroplasts; the stomata are 28–35 μm , abundant on the ventral surface and fewer on the dorsal surface; the mesophyll is clearly divided into palisade parenchyma and sponge parenchyma. The palisade parenchyma is one layer thick, with elongated cells 50–65 μm in length, oriented at right angles to the leaf surface, containing numerous chloroplasts. The sponge parenchyma is 150–250 μm thick, with cells of irregular shape, and has multiple cell layers, few chloroplasts, and large intercellular spaces. The phloem bundles of the lateral veins within the sponge parenchyma are bound by a one-layer sheath of small parenchymous cells, with vascular elements of the midrib surrounded by large-celled parenchyma. The uniseriate trichomes are few in the ventral surface, numerous on the dorsal surface, typically tricelled, occasionally tetra- or pentacelled, 250–500 μm in length, each arising from an epidermal cell; the epidermal cell walls appear with moderate thickening; the vessels are various, scalariform, with variable reticulated width.

Petiole: The parenchyma appear without chloroplasts, in several layers adjacent to a layer of collenchyma; 5–7 phloem bundles of small- to medium-sized vessels are weakly lignified and embedded in the parenchyma in the form of an arc; the wing ribs of the upper surface of the slightly hollowed petiole are marginal.

Inflorescence: The epidermal cells of the ray florets are square, 50 μm , with a transparent, beaded cell wall; various elements of the *Asteraceae* exhibit inflorescence; numerous multicellular jointed trichomes of the involucre bracts are 500–800 μm in length; tangential sections of the paleae with numerous fiber bundles are 10–15 μm in diameter and 100–150 μm in length; cell walls are thin. The epidermis of ray florets is reddish to violet; the epidermal cells from the end of the corolla form rounded papillae; a stigma of papillary cells is present; *Asteraceae* pollen grains are 20–30 μm and spherical with a warty exine.

Calcium oxalate is negative; crystals of inulin and starch granules are rare.

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** (561): NMT 3.0%
- **LOSS ON DRYING** (731): Dry 1 g of the powdered plant material: it loses NMT 12% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 10.0%, determined on 3 g
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561): NMT 2.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in tight, light-resistant containers at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.

• USP REFERENCE STANDARDS (11)

- USP Chlorogenic Acid RS
- USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS
- USP Powdered *Echinacea purpurea* Extract RS

Echinacea purpurea Root

DEFINITION

Echinacea purpurea Root consists of the dried rhizome and roots of *Echinacea purpurea* (L.) Moench (Fam. *Asteraceae*). It is harvested in the fall after 3 or more years of growth. It contains NLT 0.5% of total phenols, calculated on the dried basis as the sum of caftaric acid ($\text{C}_{13}\text{H}_{12}\text{O}_9$), chicoric acid ($\text{C}_{22}\text{H}_{18}\text{O}_{12}$), and chlorogenic acid ($\text{C}_{16}\text{H}_{18}\text{O}_9$). It contains NLT 0.025% of alkamides calculated as decatetraenoic acid isobutylamides ($\text{C}_{16}\text{H}_{25}\text{NO}$).

IDENTIFICATION

• A. PRESENCE OF CHICORIC ACID AND ABSENCE OF ECHINACOSIDE

Standard solution A: 20 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol

Standard solution B: 1 mg/mL of 1,3-dicaffeoylquinic acid in methanol

Sample solution: Transfer 1 g of finely pulverized *Echinacea purpurea* Root to a suitable extraction thimble. Transfer the thimble to a continuous extraction apparatus, and extract with 50 mL of chloroform for 1 h. Retain the chloroform extract for *Identification* test B. Continue the extraction with 50 mL of methanol, and concentrate to a small volume at 40° in vacuum. With the aid of methanol, transfer the extract to a 10-mL volumetric flask, and dilute with methanol to volume.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture (TLC plates)

Application volume: 10 μL

Developing solvent system: Ethyl acetate, formic acid, and water (17:2:1)

Spray reagent A: 10 mg/mL of diphenylborinic acid, ethanolamine ester in methanol

Spray reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 18 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under UV light at 365 nm.

Acceptance criteria: The chromatogram from the *Sample solution* shows a yellowish-green zone at an R_f value of 0.75 due to chicoric acid and another yellowish-green zone at an R_f value of 0.45 due to caftaric acid, both zones corresponding in color and R_f value to zones in the chromatogram from *Standard solution A*. The chromatogram from the *Sample solution* does not show or shows only traces of a zone at an R_f value of 0.1 due to echinacoside (present in *Echinacea angustifolia* and in *Echinacea pallida*), and does not show a zone that corresponds in color and R_f value to the spot for 1,3-dicaffeoylquinic acid (cynarin) (present in *Echinacea angustifolia*) in the chromatogram from *Standard solution B*. Other colored zones of varying intensities may be observed in the chromatogram from the *Sample solution*.

• B. PRESENCE OF ISOBUTYLALKENYLAMIDES

Standard solution A: 100 mg/mL USP *Echinacea purpurea* Extract RS in methanol

Standard solution B: 1 mg/mL of β -sitosterol in methanol

Sample solution: Evaporate the chloroform extract retained from preparation of the *Sample solution* in *Identification* test A to dryness at 40° in vacuum. To the residue add 1 mL of alcohol, and pass through a nylon membrane filter of 0.45-μm pore size.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture (TLC plates)

Application volume: 10 μL

Developing solvent system: Hexane and ethyl acetate (2:1)

Spray reagent: Prepare a mixture of glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (10:5:0.5) in an ice bath.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Examine the plate under UV light at 254 nm, and then spray the plate with *Spray reagent*, and heat the plate at 100° for 5 min. Examine the plate under long-wavelength UV light.

Acceptance criteria

Under UV light at 254 nm: The chromatogram from the *Sample solution* shows one main zone corresponding in *R_f* value to the zone due to dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide in the chromatogram of *Standard solution A*, and below this zone there are several other zones due to α,β,γ,δ-unsaturated isobutylamides.

After treatment with *Spray reagent* and heating: The zone due to dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide turns blue-black, and below this zone there are several other zones due to α,β,γ,δ-unsaturated isobutylamides (not detectable in *Echinacea pallida*) that turn violet (unlike the corresponding zones in the chromatogram of *Echinacea angustifolia* that are mostly yellowish due to α,β-unsaturated isobutylamides). A zone due to β-sitosterol that corresponds in *R_f* value to the principal spot in the chromatogram of *Standard solution B* is also observed.

- **C.** The retention times for the relevant peaks of the *Sample solution*, mainly due to caftaric acid and chicoric acid, correspond to those of *Standard solution A*, as obtained in the test for *Content of Total Phenols*. An echinacoside peak is not detectable or is very weak.

COMPOSITION

• CONTENT OF TOTAL PHENOLS

Solution A: Phosphoric acid (0.1 in 100) in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17.5	60	40
18	90	10
30	90	10

Solvent: Alcohol and water (7:3)

Standard solution A: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in *Solvent*. Dissolve by shaking for 1 min, dilute with *Solvent* to volume, and pass through a membrane filter of 0.45-μm or finer pore size.

Standard solution B: 40 μg/mL of USP Chlorogenic Acid RS in *Solvent*

Sample solution: Transfer 125 mg of finely powdered *Echinacea purpurea* Root (capable of passing through a 40-mesh sieve) to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux, while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter of 0.45-μm or finer pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 5 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with USP Powdered *Echinacea purpurea* Extract RS.

Relative standard deviation: NMT 2% for the chlorogenic acid peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the relevant analytes in the chromatogram obtained from the *Sample solution* by comparison with the chromatogram obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Separately calculate the percentage of caftaric acid (C₁₃H₁₂O₉), chicoric acid (C₂₂H₁₈O₁₂), and chlorogenic acid (C₁₆H₁₈O₉) in the portion of *Echinacea purpurea* Root taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of chlorogenic acid from *Standard solution B*

C_S = concentration of USP Chlorogenic Acid RS in *Standard solution B* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of *Echinacea purpurea* taken to prepare the *Sample solution* (mg)

F = response factor: chicoric acid, 0.695; caftaric acid, 0.881; and chlorogenic acid, 1.000

Calculate the percentage of total phenols in the portion of *Echinacea purpurea* Root taken by adding the individual percentages calculated.

Acceptance criteria: NLT 0.5% of total phenols on the dried basis

• CONTENT OF ALKAMIDES

Mobile phase: Acetonitrile and water (55:45)

Standard solution A: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Dissolve using sonication, and shaking for 10 min. After dilution, pass through a membrane filter having a 0.45-μm or finer pore size.

Standard solution B: 10 μg/mL of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in methanol

Sample solution: Transfer about 2.5 g of finely powdered *Echinacea purpurea* Root (capable of passing through a 40-mesh sieve) into a round-bottom flask. Add 80 mL of methanol, and reflux for 30 min. Cool to room temperature, and filter into a 100-mL volumetric flask, using small portions of methanol to rinse the flask and the filter. Dilute with methanol to volume. Pass through a membrane filter having a 0.45-μm or finer pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC
Detector: UV 254 nm
Column: 4.6-mm × 25-cm; 5-μm packing L1
Column temperature: 30°
Flow rate: 1.5 mL/min
Injection size: 25 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*
Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution A* is similar to the Reference Chromatogram for alkamides provided with USP Powdered *Echinacea purpurea* Extract RS.

Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*

Tailing factor: NMT 2.0 for the 2*E*,4*E*-hexadienoic acid isobutylamide peak, *Standard solution B*

Relative standard deviation: NMT 2.5% for the 2*E*, 4*E*-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks of the 10 major alkamides in the chromatogram obtained from the *Sample solution* by comparison with the chromatogram obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Calculate the percentage of alkamides in the portion of *Echinacea purpurea* Root taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = sum of the peak areas of the relevant analytes from the *Sample solution*

r_S = peak area of 2*E*,4*E*-hexadienoic acid isobutylamide from *Standard solution B*

C_S = concentration of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of *Echinacea purpurea* root taken to prepare the *Sample solution* (mg)

F = response factor to convert 2*E*,4*E*-hexadienoic acid isobutylamide into dodecatetraenoic acid isobutylamide, 1.353

Acceptance criteria: NLT 0.025% on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method III* (231): NMT 10 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *Pesticide Residues* (561): Meets the requirements

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: The roots are cylindrical and irregularly branched. The outer surface is dark brown and longitudinally striated; fractures are short and tough. Transverse sections show a thin periderm and yellowish xylem with distinct rays. In older roots, the pith is spongy, with a brownish center surrounded by yellow.

Microscopic: Rhizomes and roots in transverse section show a thin outer bark separated from a wide xylem by a brown vascular cambium. The cork is composed of several rows of thin-walled cells containing brown pigment. Schizogenous resin canals are present in the cortex. The rhizome contains bast fibers and stone cells. The xylem, with distinct rays, contains tracheary elements composed of reticulated vessels and tracheids (about 80 × 30 μm) with bordered pits and slanted end walls. Vessels and tracheids are surrounded by thick-walled parenchyma and fibers; fibers are elongated with narrow lumens and funnel-shaped ends (20–40 μm wide). Polygonal sclereids (about 50 μm in diameter) are also present. Xylem fibers have minimal or no

phytomelanin deposits (unlike *Echinacea angustifolia* and *Echinacea pallida*). A melanogenic layer is present between adjacent xylem parenchyma cell walls. The rhizome, with pith, is composed of pitted parenchyma cells containing inulin crystals. Starch is minimal to absent, and calcium oxalate crystals are absent.

- **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* (561): NMT 3.0%
- **LOSS ON DRYING** (731): Dry a sample at 105° for 2 h: it loses NMT 10.0%.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* (561): NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in well-closed, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11)
 USP Chlorogenic Acid RS
 USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS
 USP Powdered *Echinacea purpurea* Extract RS

Powdered *Echinacea purpurea*

DEFINITION

Powdered *Echinacea purpurea* is *Echinacea purpurea* Root reduced to a powder or very fine powder.

IDENTIFICATION

- **A. PRESENCE OF CHICORIC ACID AND ABSENCE OF ECHINACOSIDE**

Standard solution A: 20 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol

Standard solution B: 1 mg/mL of 1,3-dicaffeoylquinic acid in methanol

Sample solution: Transfer about 1 g of Powdered *Echinacea purpurea* to a suitable extraction thimble. Transfer the thimble to a continuous extraction apparatus, and extract with 50 mL of chloroform for 1 h. Retain the chloroform extract for *Identification* test B. Continue the extraction with 50 mL of methanol, and concentrate to a small volume at 40° in vacuum. With the aid of methanol, transfer the extract to a 10-mL volumetric flask, and dilute with methanol to volume.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture (TLC plates)

Application volume: 10 μL

Developing solvent system: Ethyl acetate, formic acid, and water (17:2:1)

Spray reagent A: 10 mg/mL of diphenylborinic acid, ethanolamine ester in methanol

Spray reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 18 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under UV light at 365 nm.

Acceptance criteria: The chromatogram from the *Sample solution* shows a yellowish-green zone at an R_f value of 0.75 due to chicoric acid and another yellowish-green zone at an R_f value of 0.45 due to caftaric acid, both zones corresponding in color and R_f value to zones in the chromatogram from *Standard solution A*. The chromatogram from the *Sample solution* does not

show or shows only traces of a zone at an R_f value of 0.1 due to echinacoside (present in *Echinacea angustifolia* and in *Echinacea pallida*), and does not show a zone that corresponds in color and R_f value to the spot for 1,3-dicaffeoylquinic acid (cynarin) (present in *Echinacea angustifolia*) in the chromatogram from *Standard solution B*. Other colored zones of varying intensities may be observed in the chromatogram from the *Sample solution*.

• **B. PRESENCE OF ISOBUTYLALKENYLAMIDES**

Standard solution A: 100 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol

Standard solution B: 1 mg/mL of β -sitosterol in methanol

Sample solution: Evaporate the chloroform extract retained from preparation of the *Sample solution* in *Identification test A* to dryness at 40° in vacuum. To the residue, add 1 mL of alcohol, and pass through a nylon membrane filter having a pore size of 0.45 μ m.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture (TLC plates)

Application volume: 10 μ L

Developing solvent system: Hexane and ethyl acetate (2:1)

Spray reagent: Prepare a mixture of glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (10:5:0.5) in an ice bath.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Examine the plate under UV light at 254 nm, then spray the plate with *Spray reagent*, and heat the plate at 100° for 5 min. Examine the plate under long-wavelength UV light.

Acceptance criteria

Under UV light at 254 nm: The chromatogram from the *Sample solution* shows one main zone corresponding in R_f value to the zone due to dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide and dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide in the chromatogram of *Standard solution A*, and below this zone there are several other zones due to $\alpha,\beta,\gamma,\delta$ -unsaturated isobutylamides.

After treatment with *Spray reagent* and heating: The zone due to dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide and dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide turns blue-black, and below this zone there are several other zones due to $\alpha,\beta,\gamma,\delta$ -unsaturated isobutylamides (not detectable in *Echinacea pallida*) that turn violet (unlike the corresponding zones in the chromatogram of *Echinacea angustifolia* that are mostly yellowish due to α,β -unsaturated isobutylamides). A zone due to β -sitosterol that corresponds in R_f value to the principal spot in the chromatogram of *Standard solution B* is also observed.

- **C.** The retention times for the relevant peaks of the *Sample solution*, mainly due to caftaric acid and chicoric acid, correspond to those of *Standard solution A*, as obtained in the test for *Content of Total Phenols*. An echinacoside peak is not detectable or is very weak.

COMPOSITION

• **CONTENT OF TOTAL PHENOLS**

Solution A: Phosphoric acid (0.1 in 100) in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22

Table 1 (Continued)

Time (min)	Solution A (%)	Solution B (%)
14	60	40
17.5	60	40
18	90	10
30	90	10

Solvent: Alcohol and water (7:3)

Standard solution A: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in *Solvent*. Dissolve by shaking for 1 min. After dilution, pass through a membrane filter of 0.45- μ m or finer pore size.

Standard solution B: 40 μ g/mL of USP Chlorogenic Acid RS in *Solvent*

Sample solution: Transfer about 125 mg of Powdered *Echinacea purpurea* (capable of passing through a 40-mesh sieve), accurately weighed, to a round-bottom flask equipped with a condenser. Add 25.0 mL of *Solvent*, and heat under reflux, while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter of 0.45- μ m or finer pore size.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 5 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the Reference Chromatogram for total phenols provided with USP Powdered *Echinacea purpurea* Extract RS.

Relative standard deviation: NMT 2% for chlorogenic acid peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the relevant analytes in the chromatogram obtained from the *Sample solution* by comparison with the chromatogram obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Separately calculate the percentage of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), and chlorogenic acid ($C_{16}H_{18}O_9$) in the portion of Powdered *Echinacea purpurea* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area for the relevant analyte from the *Sample solution*

r_S = peak area for chlorogenic acid from *Standard solution B*

C_S = concentration of USP Chlorogenic Acid RS in *Standard solution B* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered *Echinacea purpurea* used to prepare the *Sample solution* (mg)

F = response factor: chicoric acid, 0.695; caftaric acid, 0.881; and chlorogenic acid, 1.000

Calculate the percentage of total phenols in the portion of Powdered *Echinacea purpurea* taken by adding the individual percentages calculated.

Acceptance criteria: NLT 0.5% of total phenols on the dried basis

• **CONTENT OF ALKAMIDES**

Mobile phase: Acetonitrile and water (55:45)

Standard solution A: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Dissolve

using sonication and shaking for 10 min. After dilution, pass through a membrane filter having a 0.45- μ m or finer pore size.

Standard solution B: 10 μ g/mL of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in methanol

Sample solution: Transfer about 2.5 g of Powdered *Echinacea purpurea* (capable of passing through a 40-mesh sieve) into a round-bottom flask. Add 80 mL of methanol, and reflux for 30 min. Cool to room temperature, and filter into a 100-mL volumetric flask, using small portions of methanol to rinse the flask and the filter. Dilute with methanol to volume. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L1

Column temperature: 30°

Flow rate: 1.5 mL/min

Injection size: 25 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution A* is similar to the Reference Chromatogram for alkamides provided with USP Powdered *Echinacea purpurea* Extract RS.

Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*

Tailing factor: NMT 2.0 for 2*E*,4*E*-hexadienoic acid isobutylamide peak, *Standard solution B*

Relative standard deviation: NMT 2.5% for the 2*E*, 4*E*-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks of the 10 major alkamides in the chromatogram obtained from the *Sample solution* by comparison with the chromatogram obtained from *Standard solution A*.

Calculate the percentage of alkamides in the portion of Powdered *Echinacea purpurea* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = sum of the peak areas of the relevant analytes from the *Sample solution*

r_S = peak area of 2*E*,4*E*-hexadienoic acid isobutylamide from *Standard solution B*

C_S = concentration of USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered *Echinacea purpurea* used to prepare the *Sample solution* (mg)

F = response factor for 2*E*,4*E*-hexadienoic acid isobutylamide, 1.353

Acceptance criteria: NLT 0.025% on the dried basis

CONTAMINANTS

- **HEAVY METALS, Method III (231):** NMT 10 ppm
- **ARTICLES OF BOTANICAL ORIGIN, General Procedure for Pesticide Residues Analysis (561):** Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Under a microscope, the following characteristics are observed: vessels (80 \times 30 μ m) with slanted end walls and spiral or pitted secondary walls; rectangular cork cells (150 \times 60 μ m) with brown inclusions; rectangular parenchymatous cells (120 \times 30 μ m), some pitted; elongated fiber cells having a narrow lumen with funnel-shaped end (20 to 40 μ m wide); polygonal sclereids; a melanogenic layer of variable

thickness, interspersed between the cell walls of the parenchyma; and lignified sclereids, vessels, and fibers. Starch is present; calcium oxalate and inulin crystals are absent.

- **LOSS ON DRYING (731):** Dry a sample at 105° for 2 h: it loses NMT 10.0%.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash (561):** NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS (11)**
 - USP Chlorogenic Acid RS
 - USP 2*E*,4*E*-Hexadienoic Acid Isobutylamide RS
 - USP Powdered *Echinacea purpurea* Extract RS

Powdered *Echinacea purpurea* Extract

DEFINITION

Powdered *Echinacea purpurea* Extract is prepared from dried *Echinacea purpurea* Root, *Echinacea purpurea* Aerial Parts, or a mixture of them, by extraction with hydroalcoholic mixtures or other suitable solvents. The ratio of the starting crude plant material to Powdered Extract is between 2:1 and 8:1. It contains NLT 4.0% of total phenols, calculated as the sum of caffeic acid (C₁₃H₁₂O₉), chicoric acid (C₂₂H₁₈O₁₂), and chlorogenic acid (C₁₆H₁₈O₉), on the dried basis. It contains NLT 0.025% of dodecatetraenoic acid isobutylamides (C₁₆H₂₅NO), calculated on the dried basis.

IDENTIFICATION

A. PRESENCE OF ISOBUTYLALKENYLAMIDES

Standard solution A: 100 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol

Standard solution B: 1 mg/mL of β -sitosterol in methanol

Sample solution: Transfer about 1 g of Powdered *Echinacea purpurea* Extract to a suitable extraction thimble. Transfer the thimble to a continuous extraction apparatus, and extract with 50 mL of chloroform for 1 h.

Evaporate the chloroform extract to dryness at 40° in vacuum. To the residue add 1 mL of alcohol, and pass through a nylon membrane filter of 0.45- μ m pore size.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μ m (TLC plates)

Application volume: 10 μ L

Developing solvent system: Hexane and ethyl acetate (2:1)

Spray reagent: Prepare a mixture of glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (10:5:0.5) in an ice bath.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved NLT 12 cm, and dry the plate in a current of air. Examine the plate under UV light at 254 nm, and then spray the plate with *Spray reagent*, and heat the plate at 100° for 5 min.

Acceptance criteria

Under UV light at 254 nm: The chromatogram from the *Sample solution* shows one main zone corresponding in R_f value to the zone due to dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide in the chromatogram of *Standard solution A*, and below this zone there are sev-

eral other zones due to $\alpha,\beta,\gamma,\delta$ -unsaturated isobutylamides.

After treatment with *Spray reagent* and heating: The zone due to dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide and dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide turns blue-black, and below this zone there are several other zones due to $\alpha,\beta,\gamma,\delta$ -unsaturated isobutylamides (not detectable in *Echinacea pallida*) that turn violet (unlike the corresponding zones in the chromatogram of *Echinacea angustifolia* that are mostly yellowish due to α,β -unsaturated isobutylamides). A zone due to β -sitosterol that corresponds in R_f value to the principal spot in the chromatogram of *Standard solution B* is also observed.

- **B.** The retention times of the peaks for chicoric and caftaric acids of the *Sample solution* correspond to those of *Standard solution A*, as obtained in the test for *Content of Total Phenols*. An echinacoside peak is not detectable or is very weak.

COMPOSITION

• CONTENT OF TOTAL PHENOLS

Solvent: Alcohol and water (7:3)

Standard solution A: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in *Solvent*. Dissolve by shaking for 1 min. After dilution, pass through a membrane filter having a 0.45- μ m or finer pore size.

Standard solution B: 40 μ g/mL of USP Chlorogenic Acid RS in *Solvent*

Sample solution: Transfer 60 mg of Powdered *Echinacea purpurea* Extract to a round-bottom flask equipped with a condenser. Add 25 mL of *Solvent*, and heat under reflux, while shaking by mechanical means for 15 min. Centrifuge, or pass through a membrane filter of 0.45- μ m or finer pore size.

Solution A: Phosphoric acid (0.1 in 100) in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
13	78	22
14	60	40
17	60	40
17.5	90	10
22	90	10

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 330 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L1

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 5 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the reference chromatogram for total phenols provided with USP Powdered *Echinacea purpurea* Extract RS.

Relative standard deviation: NMT 2% for chlorogenic acid peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the relevant analytes in the chromatogram obtained from the *Sample solution* by comparison with

the chromatogram obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Separately calculate the percentage of caftaric acid ($C_{13}H_{12}O_9$), chicoric acid ($C_{22}H_{18}O_{12}$), and chlorogenic acid ($C_{16}H_{18}O_9$) in the portion of Powdered *Echinacea purpurea* Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response for the relevant analyte from the *Sample solution*

r_S = peak response for chlorogenic acid from *Standard solution B*

C_S = concentration of USP Chlorogenic Acid RS in *Standard solution B* (mg/mL)

C_U = concentration of *Echinacea purpurea* in the *Sample solution* (mg/mL)

F = response factor: chicoric acid, 0.695; caftaric acid, 0.881; and chlorogenic acid, 1.000

Calculate the percentage of total phenols in the portion of Powdered *Echinacea purpurea* Extract taken by adding the individual percentages calculated.

Acceptance criteria: NLT 4.0% on the dried basis

• CONTENT OF DODECATETRAENOIC ACID ISOBUTYLAMIDES

Standard solution A: 5 mg/mL of USP Powdered *Echinacea purpurea* Extract RS in methanol. Dissolve using sonication and shaking for 10 min. After dilution, pass through a membrane filter having a 0.45- μ m or finer pore size.

Standard solution B: 10 μ g/mL of USP 2E,4E-Hexadienoic Acid Isobutylamide RS in methanol

Sample solution: Transfer about 500 mg of Powdered *Echinacea purpurea* Extract, accurately weighed, into a 100-mL volumetric flask. Add 80 mL of methanol, and sonicate for 30 min. Cool to room temperature, and dilute with methanol to volume. Pass through a membrane filter of 0.45- μ m or finer pore size.

Mobile phase: Acetonitrile and water (55:45)

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L1

Column temperature: 30°

Flow rate: 1.5 mL/min

Injection size: 25 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution A* is similar to the reference chromatogram for alkanamides provided with USP Powdered *Echinacea purpurea* Extract RS.

Resolution: NLT 1.0 between dodecatetraenoic acid isobutylamide peaks, *Standard solution A*

Tailing factor: NMT 2.0 for the 2E,4E-hexadienoic acid isobutylamide peak, *Standard solution B*

Relative standard deviation: NMT 2.5% for the 2E, 4E-hexadienoic acid isobutylamide peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks due to 2E,4E,8Z,10E-dodecatetraenoic acid isobutylamide and 2E,4E,8Z,10Z-dodecatetraenoic acid isobutylamide in the chromatogram obtained from the *Sample solution* by comparison with the chromatogram obtained from *Standard solution A*. Measure the areas for the relevant peaks.

Calculate the percentage of dodecatetraenoic acid isobutylamides in the portion of Powdered *Echinacea purpurea* Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

- r_U = sum of the peak responses of the relevant analytes from the *Sample solution*
 r_S = peak response from *Standard solution B*
 C_S = concentration of USP 2E,4E-Hexadienoic Acid Isobutylamide RS in *Standard solution B* (mg/mL)
 C_U = concentration of *Echinacea purpurea* in the *Sample solution* (mg/mL)
 F = response factor to convert 2E,4E-hexadienoic acid isobutylamide into dodecatetraenoic acid isobutylamides, 1.353

Acceptance criteria: NLT 0.025% on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method II* (231): NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total bacterial count does not exceed 10^4 cfu/g. The total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **BOTANICAL EXTRACTS**, *Residual Solvents* (565): Meets the requirements

SPECIFIC TESTS

- **LOSS ON DRYING** (731): Dry 1 g at 105° for 2 h: it loses NMT 5.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, in a cool place.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant from which the article was prepared. If derived from root and aerial parts, indicate the corresponding percentages. Label it to indicate the content of total phenols and dodecatetraenoic isobutylamides. The label bears a statement indicating that *Echinacea purpurea* may cause rare allergic reactions, rashes, or aggravate asthma. It meets the requirements for *Botanical Extracts* (565), *Labeling*.
- **USP REFERENCE STANDARDS** (11)
 USP Chlorogenic Acid RS
 USP Powdered *Echinacea purpurea* Extract RS
 USP 2E,4E-Hexadienoic Acid Isobutylamide RS

trifuge or filter the solution, and use the supernatant or the filtrate.

Adsorbent: Chromatographic silica gel with an average particle size of 5 μ m (HPTLC plates)

Application volume: 10 μ L, as bands

Developing solvent system: Chloroform, methanol, and water (35:15:2)

Spray reagent: Place 18 mL of methanol in a glass flask, and cool in a water-ice-salt bath or in a freezer. To the ice-cold methanol, slowly and carefully add 2 mL of sulfuric acid, and mix well. Allow the mixture to adjust to room temperature.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Before the development of the chromatogram, saturate the chamber for 20 min with *Developing solvent system*. Record the temperature and humidity in the laboratory. If the relative humidity exceeds 50%, condition the plate to about 30% relative humidity, using a suitable device. Develop the plate over a path of 6 cm, dry, and spray with *Spray reagent*. Heat the plate at 100° for 5 min, and examine under visible light and UV light at 365 nm.

Acceptance criteria: Under visible light, the *Sample solution* exhibits two brown bands due to eleutheroside E and eleutheroside B at R_F values of about 0.34 and 0.45, corresponding in color and R_F to the bands exhibited by *Standard solution A* and *Standard solution B*, respectively. The *Sample solution* also exhibits two additional brown bands near the application zone, corresponding in color and R_F values to the bands exhibited by *Standard solution C*. Other bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. Under UV light, the *Sample solution* shows a brown band due to eleutheroside E corresponding in color and R_F to the band exhibited by *Standard solution A*.

- **B. HPLC:** The chromatogram of the *Sample solution* obtained in the test for *Content of Eleutherosides B and E* shows a peak at a retention time corresponding to that of eleutheroside B in the chromatogram of *Standard solution B* and a peak at a retention time corresponding to that of eleutheroside E in the chromatogram of *Standard solution A*.

COMPOSITION

• CONTENT OF ELEUTHEROSIDES B AND E

Solvent: Methanol and water (1:1)

Solution A: Acetonitrile and water (5:95)

Solution B: Acetonitrile and water (60:40)

Mobile phase: See *Table 1*.

Eleuthero

DEFINITION

Eleuthero is the dried rhizome with roots of *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. (Fam. Araliaceae) [*Acanthopanax senticosus* (Rupr. & Maxim.) Harms]. It contains NLT 0.08% of the sum of eleutheroside B and eleutheroside E, calculated on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)

Standard solution A: 1 mg/mL of USP Eleutheroside E RS in methanol

Standard solution B: 1 mg/mL of USP Eleutheroside B RS in methanol

Standard solution C: 0.1 g of USP Powdered Eleuthero Extract RS in 5 mL of aqueous ethanol 50%. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: Transfer about 1 g of finely powdered Eleuthero to a centrifuge tube, add 5 mL of aqueous ethanol 50%, and mix well. Sonicate for 10 min. Cen-

Standard solution A: 0.1 mg/mL of USP Eleutheroside E RS in methanol. Transfer 2.0 mL to a 5-mL volumetric flask, and dilute with *Solvent* to volume.

Standard solution B: 0.1 mg/mL of USP Eleutheroside B RS in methanol. Transfer 2.0 mL to a 5-mL volumetric flask, and dilute with *Solvent* to volume.

Standard solution C: 5.0 mg/mL of USP Powdered Eleuthero Extract RS in *Solvent*. Sonicate for 30 min,

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	97	3
5	97	3
30	60	40
31	5	95
45	5	95
45.1	97	3
60	97	3

cool to room temperature, decant, and pass through a nylon filter of 0.45- μ m or finer pore size.

Sample solution: Transfer about 5.0 g of finely ground Eleuthero, accurately weighed, to a round-bottom flask equipped with a condenser. Add 50 mL of *Solvent*, and heat under reflux for 30 min. Filter the supernatant through cotton wool into a 100-mL volumetric flask. Transfer the cotton wool to the round-bottom flask, and repeat the extraction twice, using 22 mL of *Solvent* for each extraction. Filter through cotton wool into the volumetric flask, wash the residue and the cotton wool with *Solvent*, cool to room temperature, dilute with *Solvent* to volume, and mix. Before injection, pass through a nylon filter of 0.45- μ m or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 220 nm

Column: 4.0-mm \times 25-cm; 5- μ m packing L1

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Samples: *Standard solution B* and *Standard solution C*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution C* is similar to the reference chromatogram provided with the lot of USP Powdered Eleuthero Extract RS being used.

Relative standard deviation: NMT 2.0% determined from the eleutheroside B peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Identify the eleutheroside B and eleutheroside E peaks in the *Sample solution* by comparison with the chromatograms of *Standard solution B* and *Standard solution A*, respectively, and measure the peak responses.

Separately calculate the percentages of eleutheroside B and eleutheroside E in the portion of Eleuthero taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of the relevant analyte from the *Sample solution*

r_S = peak response of eleutheroside E or eleutheroside B from *Standard solution A* or *Standard solution B*, respectively

C_S = concentration of eleutheroside E or eleutheroside B in *Standard solution A* or *Standard solution B*, respectively (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Eleuthero taken to prepare the *Sample solution* (mg)

Acceptance criteria: Add the percentages of eleutheroside B and eleutheroside E: NLT 0.08% on the dried basis.

CONTAMINANTS

- **HEAVY METALS**, *Method III* <231>: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *Pesticide Residues* <561>: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: The rhizome is knotty and of irregular cylindrical form with a diameter of 15–40 mm. The heartwood area is light brown, and the connecting splint wood is pale yellow. The bark is approximately 2 mm thick and is firmly affixed to the xylem. The surface is gray-brown or black-brown, coarse, and longitudinally vallecule and plicate. A broken rhizome is coarse and fibrous, particularly inside the xylem. The fractured surface of the bark shows short, thin fibers. Numerous roots spring from the underside of the rhizome. These roots are 35–150 mm long, cylindrical, and knotty, with a diameter of 3–15 mm. The surface of the roots is gray-brown to black-brown, is smoother than the rhizome, and has longitudinal stripes. A 0.5-mm thin bark is tightly affixed to the pale yellow xylem. A broken root is sparsely fibrous and appears yellowish-gray where the thin epidermis is flaked off.

Histology: The roots have five to seven rows of brown cork cells. Secretory canals with brown contents appear in groups of four or five and are not more than 20 μ m in diameter. Phloem fibers with thick lignified walls occur singly or in small groups; there are cluster crystals of calcium oxalate in the phloem parenchyma. Parenchymatous cells surround the secretory cells, and medullary ray cells contain small starch granules. The xylem shows reticulately thickened and pitted vessels. The rhizome is similar to the roots except for its larger secretory canals, up to 25 μ m in diameter, and the presence of a pith with parenchymatous cells containing starch granules.

- **LOSS ON DRYING** <731>: Dry a sample at 105° to constant weight: it loses NMT 14.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Water-Soluble Extractives*, *Method 2* <561>: NLT 4.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* <561>: NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** <11>
 - USP Eleutheroside B RS
 - β -D-Glucopyranoside, 4-(3-hydroxy-1-propenyl)-2,6-dimethoxyphenyl.
 - $C_{17}H_{24}O_9$ 372.37
 - USP Eleutheroside E RS
 - β -D-Glucopyranoside, (tetrahydro-1H,3H-furo[3,4-c]furan-1,4-diyl)bis(2,6-dimethoxy-4,1-phenylene)bis-.
 - $C_{34}H_{46}O_{18}$ 742.70
 - USP Powdered Eleuthero Extract RS

Powdered Eleuthero

DEFINITION

Powdered Eleuthero is Eleuthero reduced to a powder or very fine powder. It contains NLT 0.08% of the sum of eleutheroside B and eleutheroside E, calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** <201>

Standard solution A: 1 mg/mL of USP Eleutheroside E RS in methanol

Standard solution B: 1 mg/mL of USP Eleutheroside B RS in methanol

Standard solution C: 0.1 g of USP Powdered Eleuthero Extract RS in 5 mL of aqueous ethanol 50%. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: Transfer about 1 g of Powdered Eleuthero to a centrifuge tube, add 5 mL of aqueous ethanol 50%, and mix well. Sonicate for 10 min. Centrifuge or filter the solution, and use the supernatant or the filtrate.

Adsorbent: Chromatographic silica gel with an average particle size of 5 μ m (HPTLC plates)

Application volume: 10 μ L, as bands

Developing solvent system: Chloroform, methanol, and water (35:15:2)

Spray reagent: Place 18 mL of methanol in a glass flask, and cool in a water-ice-salt bath or in a freezer. To the ice-cold methanol, slowly and carefully add 2 mL of sulfuric acid, and mix well. Allow the mixture to adjust to room temperature.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Before the development of the chromatogram, saturate the chamber for 20 min with *Developing solvent system*. Record the temperature and humidity in the laboratory. If the relative humidity exceeds 50%, condition the plate to about 30% relative humidity, using a suitable device. Develop the plate over a path of 6 cm, dry, and spray with *Spray reagent*. Heat the plate at 100° for 5 min, and examine under visible light and UV light at 365 nm.

Acceptance criteria: Under visible light, the *Sample solution* exhibits two brown bands due to eleutheroside E and eleutheroside B at R_F values of about 0.34 and 0.45, corresponding in color and R_F to the bands exhibited by *Standard solution A* and *Standard solution B*, respectively. The *Sample solution* also exhibits two additional brown bands near the application zone, corresponding in color and R_F values to the bands exhibited by *Standard solution C*. Other bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. Under UV light, the *Sample solution* shows a brown band due to eleutheroside E corresponding in color and R_F to the band exhibited by *Standard solution A*.

- **B. HPLC:** The chromatogram of the *Sample solution* obtained in the test for *Content of Eleutherosides B and E* shows a peak at a retention time corresponding to that of eleutheroside B in the chromatogram of *Standard solution B* and a peak at a retention time corresponding to that of eleutheroside E in the chromatogram of *Standard solution A*.

COMPOSITION

• CONTENT OF ELEUTHEROSIDES B AND E

Solvent: Methanol and water (1:1)

Solution A: Acetonitrile and water (5:95)

Solution B: Acetonitrile and water (60:40)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	97	3
5	97	3
30	60	40
31	5	95
45	5	95
45.1	97	3
60	97	3

Standard solution A: 0.1 mg/mL of USP Eleutheroside E RS in methanol. Transfer 2.0 mL to a 5-mL volumetric flask, and dilute with *Solvent* to volume.

Standard solution B: 0.1 mg/mL of USP Eleutheroside B RS in methanol. Transfer 2.0 mL to a 5-mL volumetric flask, and dilute with *Solvent* to volume.

Standard solution C: 5.0 mg/mL of USP Powdered Eleuthero Extract RS in *Solvent*. Sonicate for 30 min, cool to room temperature, decant, and pass through a nylon filter of 0.45- μ m or finer pore size.

Sample solution: Transfer 5.0 g of Powdered Eleuthero, accurately weighed, to a round-bottom flask equipped with a condenser. Add 50 mL of *Solvent*, and heat under reflux for 30 min. Filter the supernatant through cotton wool into a 100-mL volumetric flask. Transfer the cotton wool to the round-bottom flask, and repeat the extraction twice, using 22 mL of *Solvent* for each extraction. Filter through cotton wool into the volumetric flask, wash the residue and the cotton wool with *Solvent*, cool to room temperature, dilute with *Solvent* to volume, and mix. Before injection, pass through a nylon filter of 0.45- μ m or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 220 nm

Column: 4.0-mm \times 25-cm; 5- μ m packing L1

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Samples: *Standard solution B* and *Standard solution C*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution C* is similar to the reference chromatogram provided with the lot of USP Powdered Eleuthero Extract RS being used.

Relative standard deviation: NMT 2.0%, determined from the eleutheroside B peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Identify the eleutheroside B and eleutheroside E peaks in the *Sample solution* by comparison with the chromatograms of *Standard solution B* and *Standard solution A*, respectively, and measure the peak responses.

Separately calculate the percentages of eleutheroside B and eleutheroside E in the portion of Powdered Eleuthero taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of the relevant analyte from the *Sample solution*

r_S = peak response of eleutheroside E or eleutheroside B from *Standard solution A* or *Standard solution B*, respectively

C_S = concentration of eleutheroside E or eleutheroside B in *Standard solution A* or *Standard solution B*, respectively (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered Eleuthero taken to prepare the *Sample solution* (mg)

Acceptance criteria: Add the percentages of eleutheroside B and eleutheroside E: NLT 0.08% on the dried basis.

CONTAMINANTS

• **HEAVY METALS**, *Method III* <231>: NMT 20 ppm

• **ARTICLES OF BOTANICAL ORIGIN**, *Pesticide Residues* <561>: Meets the requirements

• **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3

cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.

- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** The powder is brown with a faint aromatic odor and a slightly acid, persistent taste. Groups of secretory canals with brown contents are surrounded by parenchymatous cells containing cluster crystals of calcium oxalate. The parenchymatous cells show small starch granules, thick-walled lignified fibers, and fragments of reticulate and pitted vessels. It turns bright yellow when mounted in sodium hydroxide solution.
- **LOSS ON DRYING (731):** Dry a sample at 105° to constant weight: it loses NMT 14.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 8.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.
- **USP REFERENCE STANDARDS (11)**
 - USP Eleutheroside B RS
 β -D-Glucopyranoside, 4-(3-hydroxy-1-propenyl)-2,6-dimethoxyphenyl.
 $C_{17}H_{24}O_9$ 372.37
 - USP Eleutheroside E RS
 β -D-Glucopyranoside, (tetrahydro-1*H*,3*H*-furo(3,4-*c*)furan-1,4-diyl)bis(2,6-dimethoxy-4,1-phenylene)bis-.
 $C_{34}H_{46}O_{18}$ 742.70
 - USP Powdered Eleuthero Extract RS

Powdered Eleuthero Extract

DEFINITION

Powdered Eleuthero Extract is prepared from Eleuthero using hydroalcoholic mixtures. The ratio of the starting crude plant material to Powdered Extract is between 13:1 and 25:1. It contains NLT 0.8% of eleutherosides B and E, calculated on the anhydrous basis. It may contain added substances.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)**
 - Standard solution A:** 1 mg/mL of USP Eleutheroside E RS in methanol
 - Standard solution B:** 1 mg/mL of USP Eleutheroside B RS in methanol
 - Standard solution C:** 0.1 g of USP Powdered Eleuthero Extract RS in 5 mL of aqueous ethanol 50%. Sonicate for 10 min, centrifuge, and use the supernatant.
 - Sample solution:** 0.1 g of Powdered Extract in 5 mL of aqueous ethanol 50%. Sonicate for 10 min, centrifuge, and use the supernatant.
 - Adsorbent:** Chromatographic silica gel with an average particle size of 5 μ m (HPTLC plates)
 - Application volume:** 10 μ L, as bands
 - Developing solvent system:** Chloroform, methanol, and water (35:15:2)
 - Spray reagent:** Place 18 mL of methanol in a glass flask, and cool in a water-ice-salt bath or in a freezer. To the ice-cold methanol, slowly and carefully add 2 mL of sulfuric acid, and mix well. Allow the mixture to adjust to room temperature.

Analysis

Samples: Standard solution A, Standard solution B, Standard solution C, and Sample solution
Before the development of the chromatogram, saturate the chamber for 20 min with *Developing solvent system*. Record the temperature and humidity in the laboratory. If the relative humidity exceeds 50%, condition the plate to about 30% relative humidity, using a suitable device. Develop the plate over a path of 6 cm, dry, and spray the plate with *Spray reagent*. Heat the plate at 100° for 5 min, and examine under visible light and UV light at 365 nm.

Acceptance criteria: Under visible light, the *Sample solution* exhibits two brown bands due to eleutheroside E and eleutheroside B at R_F values of about 0.34 and 0.45, corresponding in color and R_F to the bands exhibited by *Standard solution A* and *Standard solution B*, respectively. The *Sample solution* also exhibits two additional brown bands near the application zone, corresponding in color and R_F values to the bands exhibited by *Standard solution C*. Other bands may be observed in the *Sample solution* and *Standard solution C* chromatograms. Under UV light, the *Sample solution* shows a brown band due to eleutheroside E corresponding in color and R_F to the band exhibited by *Standard solution A*.

- **B. HPLC:** The chromatogram of the *Sample solution* obtained in the test for *Content of Eleutherosides B and E* shows a peak at a retention time corresponding to that of eleutheroside B in the chromatogram of *Standard solution B* and a peak at a retention time corresponding to that of eleutheroside E in the chromatogram of *Standard solution A*.

COMPOSITION

• CONTENT OF ELEUTHEROSIDES B AND E

Solvent: Methanol and water (1:1)

Solution A: Acetonitrile and water (5:95)

Solution B: Acetonitrile and water (60:40)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	97	3
5	97	3
30	60	40
31	5	95
45	5	95
45.1	97	3
60	97	3

Standard solution A: 0.1 mg/mL of USP Eleutheroside E RS in methanol. Transfer 2.0 mL to a 5-mL volumetric flask, and dilute with *Solvent* to volume.

Standard solution B: 0.1 mg/mL of USP Eleutheroside B RS in methanol. Transfer 2.0 mL to a 5-mL volumetric flask, and dilute with *Solvent* to volume.

Standard solution C: 5.0 mg/mL of USP Powdered Eleuthero Extract RS in *Solvent*. Sonicate for 30 min, cool to room temperature, and decant. Before injection, pass through a nylon filter of 0.45- μ m or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Transfer 500 mg of Powdered Extract to a 100-mL volumetric flask, add 80 mL of *Solvent*, and sonicate for 30 min. Cool to room temperature, dilute with *Solvent* to volume, and mix. Before injection, pass through a nylon filter of 0.45- μ m or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography (621)*, *System Suitability*.)

Mode: LC

Detector: UV 220 nm

Column: 4.0-mm × 25-cm; 5-μm packing L1

Flow rate: 1 mL/min

Injection size: 10 μL

System suitability

Samples: *Standard solution B* and *Standard solution C*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution C* is similar to the reference chromatogram provided with the lot of USP Powdered Eleuthero Extract RS being used.

Relative standard deviation: NMT 2.0% determined from the eleutheroside B peak in repeated injections, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Identify the eleutheroside B and eleutheroside E peaks in the *Sample solution* by comparison with the chromatograms of *Standard solution B* and *Standard solution A*, respectively, and measure the peak responses. Separately calculate the percentages of eleutheroside B and eleutheroside E in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of the relevant analyte from the *Sample solution*

r_S = peak response of eleutheroside E or eleutheroside B from *Standard solution A* or *Standard solution B*, respectively

C_S = concentration of eleutheroside E or eleutheroside B in *Standard solution A* or *Standard solution B*, respectively (mg/mL)

C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.8% on the anhydrous basis

CONTAMINANTS

- **HEAVY METALS**, *Method II* (231): NMT 20 ppm
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined yeasts and molds count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **WATER DETERMINATION**, *Method Ia* (921): NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 10.0%
- **ALCOHOL DETERMINATION**, *Method II* (611): NMT 0.5%
- **OTHER REQUIREMENTS:** It meets the requirements for *Residual Solvents* and *Pesticide Residues* in *Botanical Extracts* (565).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of eleutherosides, the extracting solvent used for preparation, and the ratio of the starting crude plant material to Powdered Extract. It meets the requirements for *Labeling* in *Botanical Extracts* (565).
- **USP REFERENCE STANDARDS** (11)
 - USP Eleutheroside B RS
 - β-D-Glucopyranoside, 4-(3-hydroxy-1-propenyl)-2,6-dimethoxyphenyl.
 - C₁₇H₂₄O₉ 372.37

USP Eleutheroside E RS

β-D-Glucopyranoside, (tetrahydro-1H,3H-furo(3,4-c)furan-1,4-diyl)bis(2,6-dimethoxy-4,1-phenylene)bis-.
C₃₄H₄₆O₁₈ 742.70

USP Powdered Eleuthero Extract RS

Elm—see *Elm General Monographs*

Ergocalciferol—see *Ergocalciferol General Monographs*

Ergocalciferol Capsules—see *Ergocalciferol Capsules General Monographs*

Ergocalciferol Oral Solution—see *Ergocalciferol Oral Solution General Monographs*

Ergocalciferol Tablets—see *Ergocalciferol Tablets General Monographs*

Ferrous Fumarate—see *Ferrous Fumarate General Monographs*

Ferrous Fumarate Tablets—see *Ferrous Fumarate Tablets General Monographs*

Ferrous Gluconate—see *Ferrous Gluconate General Monographs*

Ferrous Gluconate Capsules—see *Ferrous Gluconate Capsules General Monographs*

Ferrous Gluconate Oral Solution—see *Ferrous Gluconate Oral Solution General Monographs*

Ferrous Gluconate Tablets—see *Ferrous Gluconate Tablets General Monographs*

Ferrous Sulfate—see *Ferrous Sulfate General Monographs*

Ferrous Sulfate Oral Solution—see *Ferrous Sulfate Oral Solution General Monographs*

Ferrous Sulfate Syrup—see *Ferrous Sulfate Syrup General Monographs*

Ferrous Sulfate Tablets—see *Ferrous Sulfate Tablets General Monographs*

Ferrous Sulfate, Dried—see *Dried Ferrous Sulfate General Monographs*

Feverfew

DEFINITION

Feverfew consists of the dried leaves of *Tanacetum parthenium* (L.) Sch. Bip. (Fam. Asteraceae), collected when the plant is in flower.

IDENTIFICATION

- A.** The retention time of the parthenolide peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Parthenolide*.

- B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 1.0 mg/mL of USP Parthenolide RS in methanol

Sample solution: Transfer 1.0 g of finely powdered Feverfew to a suitable flask. Add 20 mL of methanol, heat the flask over a water bath at 60° for 15 min, cool, and filter. Evaporate the filtrate under reduced pressure to dryness, and dissolve the residue in 2.0 mL of methanol.

Adsorbent: 0.5-mm layer of chromatographic silica gel, typically 20 cm long

Application volume: 20 µL

Developing solvent system: Toluene and acetone (85:15)

Spray reagent: 0.5% solution of vanillin in a mixture of sulfuric acid and alcohol (4:1)

Analysis

Samples: *Standard solution* and *Sample solution*
Develop the chromatograms until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the chromatographic chamber, mark the solvent front, and allow it to air-dry. Spray the plate with *Spray reagent*. After 5 min, examine the plate in daylight.

Acceptance criteria: A blue spot in the middle portion of the chromatogram of the *Sample solution* that corresponds in color and R_f value to the principal spot obtained in the chromatogram of the *Standard solution* indicates the presence of parthenolide. The lower one-third of the chromatogram of the *Sample solution* may exhibit two pink spots, and the upper one-third may exhibit one pink spot.

- C. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 0.25 mg/mL of USP Rutin RS in methanol

Sample solution: To 1 g of finely powdered Feverfew, add 10 mL of methanol, and heat on a water bath at 60° for 15 min. Cool, and filter.

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 20 µL

Developing solvent system: Ethyl acetate, anhydrous formic acid, glacial acetic acid, and water (10:1.1:1.1:2.7)

Spray reagent A: 1% solution of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 5% (w/v) solution of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*
Develop the chromatogram until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the chromatographic chamber, and allow it to air-dry. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under UV light at 366 nm.

Acceptance criteria: Relative to the R_f value of the principal spot of the *Standard solution*, the chromatogram of the *Sample solution* exhibits no blue spot at R_f 1.1 (distinction from *Roman chamomile*) but exhibits a green spot at R_f 2.3 (distinction from *Matricaria*), and colored spots at the R_f values indicated are as follows: 1.5 (yellowish orange), 1.65 (yellowish green), 2.0 (greenish blue), and 2.25 (whitish blue).

COMPOSITION

• CONTENT OF PARTHENOLIDE

Mobile phase: Acetonitrile and water (9:11)

Standard solution: 0.04 mg/mL of USP Parthenolide RS in methanol

Sample stock solution: Reduce 100 g of Feverfew to a fine powder. Transfer about 1.0 g of the finely powdered Feverfew, accurately weighed, to a suitable flask. Add 100 mL of methanol, and heat on a water bath at 60° for 10 min. Remove the flask from the water bath, cool, and filter. Rinse the flask with three 5-mL portions of methanol, and filter, adding the rinsings to the filtrate. Transfer the residue left within the filter to the same flask. Add 50 mL of methanol, and continue the rinse procedure as described above. Evaporate the combined filtrates under reduced pressure to dryness, and dissolve the residue in 20.0 mL of methanol.

Sample solution: Transfer 10 mL of the *Sample stock solution* to a 25-mL volumetric flask, and dilute with methanol to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the parthenolide peak

Relative standard deviation: NMT 2.0% for the parthenolide peak in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of parthenolide in the portion of Feverfew taken to prepare the *Sample solution*:

$$\text{Result} = (r_U/r_S) \times [C_S \times (V/W)] \times D \times 100$$

r_U = peak area of the parthenolide peak from the *Sample solution*

r_S = peak area of the parthenolide peak from the *Standard solution*

C_S = concentration of USP Parthenolide RS in the *Standard solution* (mg/mL)

V = final volume of the *Sample stock solution* (mL)

W = weight of powder used to prepare the *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*

Acceptance criteria: NLT 0.2% on the dried basis

CONTAMINANTS

- **HEAVY METALS** (231): NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues** (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022) It meets the requirements of the tests for the absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Yellowish green, petiolate, usually 2–5 cm in length but sometimes up to 10 cm, ovate, deeply divided into 5 or occasionally 7 segments, each with a coarsely crenate margin and obtuse apex; both surfaces downy and the mid-rib prominent on the lower surface

Histology: Upper and lower epidermal cells with wavy anticlinal walls, striated cuticle and anomocytic stomata, more frequent on the lower epidermis; trichomes, more abundant on the lower epidermis, of two types; covering trichomes uniseriate with up to 6 small isodiametric basal cells and elongated, tapering apical cells, often at right angles to the axis of the basal cells; glandular trichomes slightly sunken, composed of a short, biseriate, 2- or 4-celled stalk and a biseriate head of 4 cells, around which the cuticle forms a bladder-like covering

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** (561): NMT 10.0%, including the stalk
- **ARTICLES OF BOTANICAL ORIGIN, Water-Soluble Extractives, Method 2** (561): NLT 15.0%
- **LOSS ON DRYING** (731): Dry 1.0 g of finely powdered Feverfew at 105° for 1 h: it loses NMT 10.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561): NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store in a dry place, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11)
USP Parthenolide RS
USP Rutin RS

Powdered Feverfew

DEFINITION

Powdered Feverfew is Feverfew pulverized to a fine or very fine powder.

IDENTIFICATION

- **A.** The retention time of the parthenolide peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Parthenolide*.
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution: 1.0 mg/mL of USP Parthenolide RS in methanol
Sample solution: Transfer 1.0 g of Powdered Feverfew to a suitable flask. Add 20 mL of methanol, heat the flask over a water bath at 60° for 15 min, cool, and

filter. Evaporate the filtrate under reduced pressure to dryness, and dissolve the residue in 2.0 mL of methanol.

Adsorbent: 0.5-mm layer of chromatographic silica gel, typically 20 cm long

Application volume: 20 μ L

Developing solvent system: Toluene and acetone (85:15)

Spray reagent: 0.5% solution of vanillin in a mixture of sulfuric acid and alcohol (4:1)

Analysis

Samples: *Standard solution* and *Sample solution*
Develop the chromatograms until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the chromatographic chamber, mark the solvent front, and allow it to air-dry. Spray the plate with *Spray reagent*. After 5 min, examine the plate in daylight.

Acceptance criteria: A blue spot in the middle portion of the chromatogram of the *Sample solution* that corresponds in color and R_f value to the principal spot obtained in the chromatogram of the *Standard solution* indicates the presence of parthenolide. The lower one-third of the chromatogram of the *Sample solution* may exhibit two pink spots, and the upper one-third may exhibit one pink spot.

• C. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 0.25 mg/mL of USP Rutin RS in methanol

Sample solution: To 1 g of Powdered Feverfew, add 10 mL of methanol, and heat on a water bath at 60° for 15 min. Cool, and filter.

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 20 μ L

Developing solvent system: Ethyl acetate, anhydrous formic acid, glacial acetic acid, and water (10:1.1:1.1:2.7)

Spray reagent A: 1% solution of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 5% (w/v) solution of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*
Develop the chromatogram until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the chromatographic chamber, and allow it to air-dry. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under UV light at 366 nm.

Acceptance criteria: Relative to the R_f value of the principal spot of the *Standard solution*, the chromatogram of the *Sample solution* exhibits no blue spot at R_f 1.1 (distinction from *Roman chamomile*) but exhibits a green spot at R_f 2.3 (distinction from *Matricaria*), and colored spots at the R_f values indicated are as follows: 1.5 (yellowish orange), 1.65 (yellowish green), 2.0 (greenish blue), and 2.25 (whitish blue).

COMPOSITION

• CONTENT OF PARTHENOLIDE

Mobile phase: Acetonitrile and water (9:11)

Standard solution: 0.04 mg/mL of USP Parthenolide RS in methanol

Sample stock solution: Transfer about 1.0 g of the Powdered Feverfew, accurately weighed, to a suitable flask. Add 100 mL of methanol, and heat on a water bath at 60° for 10 min. Remove the flask from the water bath, cool, and filter. Rinse the flask with three 5-mL portions of methanol, and filter, adding the rinsings to the filtrate. Transfer the residue left within the filter to the same flask. Add 50 mL of methanol, and continue the rinse procedure as described above. Evaporate the combined filtrates under reduced pressure to

dryness, and dissolve the residue in 20.0 mL of methanol.

Sample solution: Transfer 10 mL of the *Standard stock solution* to a 25-mL volumetric flask, and dilute with methanol to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the parthenolide peak

Relative standard deviation: NMT 2.0% for the parthenolide peak in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of parthenolide in the portion of Powdered Feverfew taken to prepare the *Sample solution*:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of the parthenolide peak from the *Sample solution*

r_S = peak area of the parthenolide peak from the *Standard solution*

C_S = concentration of USP Parthenolide RS in the *Standard solution* (mg/mL)

V = final volume of the *Sample stock solution* (mL)

W = weight of Powdered Feverfew used to prepare the *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*

Acceptance criteria: NLT 0.2% on the dried basis

CONTAMINANTS

- **HEAVY METALS** <231>: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues** <561>: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements of the tests for the absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

SPECIFIC TESTS

- **ARTICLES OF BOTANICAL ORIGIN, Water-Soluble Extractives, Method 2** <561>: NLT 15.0%
- **LOSS ON DRYING** <731>: Dry 1.0 g of Powdered Feverfew at 105° for 1 h: it loses NMT 10.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 12.0%
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** <561>: NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.
- **USP REFERENCE STANDARDS** <11>
 - USP Parthenolide RS
 - USP Rutin RS

Fish Oil Containing Omega-3 Acids

DEFINITION

Fish Oil Containing Omega-3 Acids is the purified, winterized, and deodorized fatty oil obtained from fish of the families Engraulidae, Carangidae, Clupeidae, Osmeridae, Scombroideae, and Ammodytidae. The omega-3 acids are defined as the following: alpha-linolenic acid (C18:3 n-3), moroctic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), eicosapentaenoic acid (EPA) (C20:5 n-3), heneicosapentaenoic acid (C21:5 n-3), docosapentaenoic acid (C22:5 n-3), and docosahexaenoic acid (DHA) (C22:6 n-3). It contains NLT 28.0% (w/w) of total omega-3 acids, expressed as free acids, consisting of NLT 13.0% of EPA and NLT 9.0% of DHA. Suitable antioxidants in appropriate concentrations may be added.

IDENTIFICATION

- The retention times of the peaks for eicosapentaenoic acid methyl ester and docosahexaenoic acid methyl ester of *Test Solution 2* in the test for *Content of EPA and DHA* correspond to those for the respective compounds of *Standard Solution 1*. The sum of the area for EPA and DHA methyl esters is NLT 22% of the total detected area for the methyl esters, and no other peak has an area higher than 20% of the total detected area for the methyl esters. The chromatogram of *Test Solution 2* exhibits at least 15 additional peaks at the retention times of the methyl esters of unsaturated fatty acids exhibited in *Standard Solution 2*.

COMPOSITION

• CONTENT OF EPA AND DHA

(See *Fats and Fixed Oils* <401>, *Omega-3 Fatty Acids Determination and Profile*.)

Analysis

Samples: *Standard Solution 1*, *Standard Solution 2*, *Test Solution 1*, and *Test Solution 2*

Identify the retention times of the relevant fatty acid methyl esters peaks by comparing the chromatogram of *Standard Solution 2* with the Reference Chromatogram supplied with the USP Fish Oil RS. Identify the retention time for the internal standard peak in the chromatogram of *Test Solution 2* by comparing with that of *Test Solution 1*.

Calculate the percentage of EPA or DHA in the portion of Fish Oil Containing Omega-3 Acids taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times F \times 100$$

R_S = ratio of peak responses of either EPA or DHA relative to the internal standard in the chromatogram of *Standard Solution 2*

W_S = weight of either USP Docosahexaenoic Acid Ethyl Ester RS or USP Eicosapentaenoic Acid Ethyl Ester RS used to prepare *Standard Solution 1* (mg)

W_U = weight of the Fish Oil Containing Omega-3 Acids taken to prepare *Test Solution 2* (mg)

F = factor to express the content of DHA (0.921) and EPA (0.915) as free fatty acids

R_U = ratio of peak response of either EPA or DHA to the corrected peak response of the internal standard in the chromatogram of *Test Solution 2* calculated as follows:

$$R_U = 1/[(r_{U2}/r_{T2}) - (r_{U1}/r_{T1})]$$

r_{U2} = peak response of the peak at the locus of the internal standard in the chromatogram of *Test Solution 2*

r_{T2} = peak response of EPA or DHA in the chromatogram of *Test Solution 2*

- r_{U1} = peak response of any peak at the locus of the internal standard in the chromatogram of *Test Solution 1*
- r_{T1} = peak response of EPA or DHA in the chromatogram of *Test Solution 1*. [NOTE—If no peak is found at the locus of the internal standard in the chromatogram of *Test Solution 1*, $R_U = r_{T2}/r_{U2}$.]

Acceptance criteria: NLT 13.0% (w/w) of EPA and NLT 9.0% (w/w) of DHA

• CONTENT OF TOTAL OMEGA-3 ACIDS

(See *Fats and Fixed Oils* (401), *Omega-3 Fatty Acids Determination and Profile*.)

Analysis: Proceed as directed in the test for *Content of EPA and DHA*, except to calculate the content of the total omega-3 acids:

$$\text{Result} = \text{EPA} + \text{DHA} + [A_{n-3}(\text{EPA} + \text{DHA})]/(A_{\text{EPA}} + A_{\text{DHA}})$$

EPA = content of EPA from the test for *Content of EPA and DHA* [% (w/w)]

DHA = content of DHA from the test for *Content of EPA and DHA* [% (w/w)]

A_{n-3} = sum of the areas of the peaks corresponding to C18:3 n-3, C18:4 n-3, C20:4 n-3, C21:5 n-3, and C22:5 n-3 methyl esters in the chromatogram of *Test Solution 2*

A_{EPA} = area of the peak corresponding to the EPA methyl ester in the chromatogram of *Test Solution 2*

A_{DHA} = area of the peak corresponding to the DHA methyl ester in the chromatogram of *Test Solution 2*

Acceptance criteria: NLT 28.0% (w/w) of total omega-3 acids, expressed as free acids

CONTAMINANTS

• LIMIT OF ARSENIC

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed first through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

1% Palladium stock solution: Transfer 1 g of ultrapure palladium metal into a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

1% Magnesium nitrate stock solution: Transfer 1 g of ultrapure magnesium nitrate into a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Modifier working solution: 1% Palladium stock solution, 1% Magnesium nitrate stock solution, and 2% nitric acid (3:2:5). A volume of 5 μL provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (5 in 100)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed in the test for *Arsenic* (211), to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 $\mu\text{g}/\text{mL}$ of arsenic.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 $\mu\text{g}/\text{mL}$ of arsenic.

Sample solution: For preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytetrafluoroethylene liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes. [CAUTION—Wear proper eye protection and protective clothing and gloves.] Transfer approximately 500 mg of Fish Oil Containing Omega-3 Acids, weighed to the nearest 0.1 mg, into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. [NOTE—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115° using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min. Char the sample at 1000° using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min. Cool down and purge the air from the furnace for 10 s using a 20° set temperature and an argon flow of 300 mL/min. Atomize at 2400° using a 0-s ramp and a 5-s hold with the argon flow stopped. Clean out at 2600° using a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 μL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by a 5- μL injection of the *Modifier working solution* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in $\mu\text{g}/\text{mL}$, and calculate the regression line best fitting the points. Determine the concentration, C , in $\mu\text{g}/\text{mL}$, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of Fish Oil Containing Omega-3 Acids taken:

$$\text{Result} = (C/W) \times 25$$

W = weight of Fish Oil Containing Omega-3 Acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 $\mu\text{g}/\text{g}$

• LIMIT OF LEAD

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N

nitric acid for 30 min and by rinsing with deionized water.]

10% Monobasic ammonium phosphate solution:

10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

1% Magnesium nitrate solution: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Modifier working solution: 10% Monobasic ammonium phosphate solution, 1% Magnesium nitrate solution, and 2% nitric acid (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (5 in 100)

Standard stock solution: Transfer 10.0 mL of *Lead Nitrate Stock Solution*, prepared as directed in the test for *Heavy Metals* (231), to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask. Add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of lead.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of lead.

Sample solution: Prepare as directed in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120° using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min. Char the sample at 850° using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min. Cool down and purge the air from the furnace for 10 s using a 20° set temperature and an argon flow of 300 mL/min. Atomize at 2100° using a 0-s ramp and a 5-s hold with the argon flow stopped. Clean out at 2600° using a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by a 5-µL injection of *Modifier working solution* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of lead in the portion of Fish Oil Containing Omega-3 Acids taken:

$$\text{Result} = (C/W) \times 25$$

W = weight of Fish Oil Containing Omega-3 Acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT FOR CADMIUM**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

10% Monobasic ammonium phosphate solution:

10 g of ultrapure monobasic ammonium phosphate in

40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

1% Magnesium nitrate solution: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Modifier working solution: 10% Monobasic ammonium phosphate solution, 1% Magnesium nitrate solution, and 2% nitric acid to volume (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (5 in 100)

Standard stock solution A: 0.1372 mg/mL of cadmium nitrate in water

Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before make up to final volume dissolve in a portion of water and nitric acid.]

Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.

Sample solution: Prepare as directed in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120° using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min. Char the sample at 850° using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min. Cool down and purge the air from the furnace for 10 s using a 20° set temperature and an argon flow of 300 mL/min. Atomize at 2400° using a 0-s ramp and a 5-s hold with the argon flow stopped. Clean out at 2600° using a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by a 5-µL injection of *Modifier working solution* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line. Calculate the content of cadmium in the Fish Oil Containing Omega-3 Acids taken:

$$\text{Result} = (C/W) \times 25$$

W = weight of Fish Oil Containing Omega-3 Acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT FOR MERCURY:** Proceed as directed for *Mercury* (261), *Method IIa*, except use a *Standard Mercury Solution* having the equivalent of 0.1 µg/mL of mercury.

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic* combining the 2 duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution*.

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT FOR DIOXINS, FURANS, AND POLYCHLORINATED BIPHENYLS**

Analysis: Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by method No. 1613 revision B of the Environmental Protection Agency. Determine the content of polychlorinated biphenyls (PCBs) by method No. 1668 revision A of the Environmental Protection Agency.

Acceptance criteria: The sum of PCDDs and PCDFs is NMT 2.0 pg/g of WHO toxic equivalents. The sum of PCDDs, PCDFs, and dioxin-like PCBs (polychlorinated biphenyls, non-ortho IUPAC congeners PCB-77, PCB-81, PCB-126, and PCB-169, and mono-ortho IUPAC congeners PCB-105, PCB-114, PCB-118, PCB-123, PCB-156, PCB-157, PCB-167, and PCB-189) is NMT 10.0 pg/g of WHO toxic equivalents.

SPECIFIC TESTS

- **FATS AND FIXED OILS, Acid Value <401>:** NMT 3
- **FATS AND FIXED OILS, Anisidine Value <401>:** NMT 20.0
- **FATS AND FIXED OILS, Peroxide Value <401>:** NMT 5.0
- **FATS AND FIXED OILS, Total Oxidation Value (TOTOX) <401>:** NMT 26, calculated:

$$\text{Result} = (2 \times \text{PV}) + \text{AV}$$

PV = peroxide value

AV = anisidine value

- **FATS AND FIXED OILS, Unsaponifiable Matter <401>:** NMT 1.5%
- **STEARIN:** 10 mL remains clear after cooling at 0° for 3 h
- **ABSORBANCE**
Sample solution: 0.24 mg/mL in isooctane
Acceptance criteria: The absorbance is NMT 0.70, determined at 233 nm.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at controlled room temperature. It may be bottled or otherwise packaged in containers from which air has been expelled by production of a vacuum or by an inert gas.
- **LABELING:** The label states the average content of DHA and EPA in mg/g. It also states the name and concentration of any added antioxidant.
- **USP REFERENCE STANDARDS <11>**
 USP Docosahexaenoic Acid Ethyl Ester RS
 all *cis*-4,7,10,13,16,19-Docosahexaenoic ethyl ester.
 $\text{C}_{24}\text{H}_{36}\text{O}_2$ 356.55
 USP Eicosapentaenoic Acid Ethyl Ester RS
 all *cis*-5,8,11,14,17-Eicosapentaenoic ethyl ester.
 $\text{C}_{22}\text{H}_{34}\text{O}_2$ 330.51
 USP Fish Oil RS
 USP Methyl Tricosanoate RS
 Tricosanoic acid methyl ester.
 $\text{C}_{24}\text{H}_{48}\text{O}_2$ 368.64

Fish Oil Containing Omega-3 Acids Capsules

DEFINITION

Fish Oil Containing Omega-3 Acids Capsules contain NLT 95.0% and NMT 105.0% of the labeled amount of Fish Oil Containing Omega-3 Acids where Fish Oil Containing Omega-3 Acids is the purified, winterized, and deodorized fatty oil obtained from fish of the families Engraulidae, Carangidae, Clupeidae, Osmeridae, Scombroidae, and Ammodytidae. The omega-3 acids are defined as the following: alpha-linolenic acid (C18:3 n-3), morotic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), eicosapentaenoic acid (EPA) (C20:5 n-3), heneicosapentaenoic acid (C21:5 n-3), docosapentaenoic acid (C22:5 n-3), and docosahexaenoic acid (DHA) (C22:6 n-3). It contains NLT 28.0% (w/w) of total omega-3 acids, expressed as free acids, consisting of NLT 13.0% of EPA and NLT 9.0% of DHA. Suitable antioxidants in appropriate concentrations may be added.

IDENTIFICATION

- The oil contained in the Capsules meets the requirements for the following test: The retention times of the peaks for eicosapentaenoic acid methyl ester and docosahexaenoic acid methyl ester obtained in the chromatogram of *Test Solution 2* in the test for *Content of EPA and DHA* correspond to those for the respective compounds in the chromatogram of *Standard Solution 1*. The sum of the area for EPA and DHA methyl esters is NLT 22% of the total detected area for the methyl esters, and no other peak in the chromatogram has an area higher than 20% of the total detected area for the methyl esters. The chromatogram of *Test Solution 2* exhibits at least 15 additional peaks at the retention times of the methyl esters of unsaturated fatty acids exhibited in *Standard Solution 2*.

STRENGTH

- **CONTENT OF FISH OIL:** Weigh NLT 10 Capsules in a tared weighing bottle, carefully open the Capsules, without loss of shell material, and transfer the combined Capsule contents to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of 2,2,4-trimethylpentane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the 2,2,4-trimethylpentane is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average net weight per Capsule.

Acceptance criteria: NLT 95.0% and NMT 105.0% the labeled amount

• CONTENT OF EPA AND DHA

(See *Fats and Fixed Oils <401>*, *Omega-3 Fatty Acids Determination and Profile*.)

Analysis

Samples: *Standard Solution 1*, *Standard Solution 2*, *Test Solution 1*, and *Test Solution 2*

Identify the retention times of the relevant fatty acid methyl esters peaks by comparing the chromatogram of *Standard Solution 2* with the Reference Chromatogram supplied with the USP Fish Oil RS. Identify the retention time for the internal standard peak in the chromatogram of *Test Solution 2* by comparing with that of *Test Solution 1*.

Calculate the percentage of EPA or DHA in the portion of fish oil containing omega-3 acids taken from the Capsules:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times F \times 100$$

R_S = ratio of peak responses of either EPA or DHA relative to the internal standard in the chromatogram of *Standard Solution 2*

W_S = weight of either USP Docosahexaenoic Acid Ethyl Ester RS or USP Eicosapentaenoic Acid Ethyl Ester RS used to prepare *Standard Solution 1* (mg)

W_U = weight of the fish oil containing omega-3 acids taken to prepare *Test Solution 2* (mg)

F = factor to express the content of DHA (0.921) and EPA (0.915) as free fatty acids

R_U = ratio of peak response of either EPA or DHA to the corrected peak response of the internal standard in the chromatogram of *Test Solution 2* calculated as follows:

$$R_U = 1/[(r_{U2}/r_{T2}) - (r_{U1}/r_{T1})]$$

r_{U2} = response of the peak at the locus of the internal standard in the chromatogram of *Test Solution 2*

r_{T2} = peak response of EPA or DHA in the chromatogram of *Test Solution 2*

- r_{UI} = response of any peak at the locus of the internal standard in the chromatogram of *Test Solution 1*
- r_{TI} = peak response of EPA or DHA in the chromatogram of *Test Solution 1*. [NOTE—If no peak is found at the locus of the internal standard in the chromatogram of *Test Solution 1*, $R_U = r_{T2}/r_{U2}$.]

Acceptance criteria: NLT 13.0% (w/w) of EPA and NLT 9.0% (w/w) of DHA

• CONTENT OF TOTAL OMEGA-3 ACIDS

(See *Fats and Fixed Oils* (401), *Omega-3 Fatty Acids Determination and Profile*.)

Analysis: Proceed as directed in the test for *Content of EPA and DHA*. Calculate the percentage of the total omega-3 acids in the portion of fish oil containing omega-3 acids taken from the Capsules:

$$\text{Result} = \text{EPA} + \text{DHA} + [A_{n-3}(\text{EPA} + \text{DHA})]/(A_{\text{EPA}} + A_{\text{DHA}})$$

- EPA = content of EPA from the test for *Content of EPA and DHA* [% (w/w)]
- DHA = content of DHA from the test for *Content of EPA and DHA* [% (w/w)]
- A_{n-3} = sum of the areas of the peaks corresponding to C18:3 n-3, C18:4 n-3, C20:4 n-3, C21:5 n-3, and C22:5 n-3 methyl esters in the chromatogram of *Test Solution 2*
- A_{EPA} = area of the peak corresponding to the EPA methyl ester in the chromatogram of *Test Solution 2*
- A_{DHA} = area of the peak corresponding to the DHA methyl ester in the chromatogram of *Test Solution 2*

Acceptance criteria: NLT 28.0% (w/w) of total omega-3 acids, expressed as free acids

PERFORMANCE TESTS

- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS (2091):** Meet the requirements
- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS (2040):** Meet the requirements for *Rupture Test for Soft Shell Capsules*

CONTAMINANTS

• LIMIT OF ARSENIC

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has first been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

1% Palladium stock solution: Transfer 1 g of ultrapure palladium metal into a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

1% Magnesium nitrate stock solution: Transfer 1 g of ultrapure magnesium nitrate into a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Modifier working solution: 1% Palladium stock solution, 1% Magnesium nitrate stock solution, and 2% nitric acid (3:2:5). A volume of 5 μL provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (5 in 100)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed in the test for *Arsenic* (211), to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 $\mu\text{g}/\text{mL}$ of arsenic.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 $\mu\text{g}/\text{mL}$ of arsenic.

Sample solution: For preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytetrafluoroethylene liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes. [CAUTION—Wear proper eye protection and protective clothing and gloves.] Transfer approximately 500 mg from content of Capsules, weighed to the nearest 0.1 mg, into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. [NOTE—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115° using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min. Char the sample at 1000° using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min. Cool down, and purge the air from the furnace for 10 s using a 20° set temperature and an argon flow of 300 mL/min. Atomize at 2400° using a 0-s ramp and a 5-s hold with the argon flow stopped. Clean out at 2600° using a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 μL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by a 5- μL injection of the *Modifier working solution* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in $\mu\text{g}/\text{mL}$, and calculate the regression line best fitting the points. Determine the concentration, C , in $\mu\text{g}/\text{mL}$, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

W = weight of fish oil containing omega-3 acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has first been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

10% Monobasic ammonium phosphate solution:

10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

1% Magnesium nitrate solution: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Modifier working solution: 10% Monobasic ammonium phosphate solution, 1% Magnesium nitrate solution, and 2% nitric acid (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (5 in 100)

Standard stock solution: Transfer 10.0 mL of *Lead Nitrate Stock Solution*, prepared as directed in the test for *Heavy Metals* (231), to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask. Add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of lead.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of lead.

Sample solution: Prepare as directed in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120° using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min. Char the sample at 850° using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min. Cool down, and purge the air from the furnace for 10 s using a 20° set temperature and an argon flow of 300 mL/min. Atomize at 2100° using a 0-s ramp and a 5-s hold with the argon flow stopped. Clean out at 2600° using a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by a 5-µL injection of *Modifier working solution* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of lead in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

W = weight of fish oil containing omega-3 acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT FOR CADMIUM**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels

before use, use water that has first been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

10% Monobasic ammonium phosphate solution:

10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

1% Magnesium nitrate solution: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Modifier working solution: 10% Monobasic ammonium phosphate solution, 1% Magnesium nitrate solution, and 2% nitric acid to volume (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (5 in 100)

Standard stock solution A: 0.1372 mg/mL of cadmium nitrate in water

Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before make up to final volume dissolve in a portion of water and nitric acid.]

Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.

Sample solution: Prepare as directed in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120° using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min. Char the sample at 850° using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min. Cool down and purge the air from the furnace for 10 s using a 20° set temperature and an argon flow of 300 mL/min. Atomize at 2400° using a 0-s ramp and a 5-s hold with the argon flow stopped. Clean out at 2600° using a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by a 5-µL injection of *Modifier working solution* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line. Calculate the content of cadmium in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

W = weight of fish oil containing omega-3 acids taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF MERCURY:** Proceed as directed for *Mercury* (261), *Method IIa*, except to use a *Standard Mercury Solution* having the equivalent of 0.1 µg/mL of mercury.

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic* combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution*.

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF DIOXINS, FURANS, AND POLYCHLORINATED BIPHENYLS**

Analysis: Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by method No. 1613 revision B of the Environmental Protection Agency. Determine the content of polychlorinated biphenyls (PCBs) by method No. 1668 revision A of the Environmental Protection Agency.

Acceptance criteria: The sum of PCDDs and PCDFs is NMT 2.0 pg/g of WHO toxic equivalents. The sum of PCDDs, PCDFs, and dioxin-like PCBs (polychlorinated biphenyls, non-ortho IUPAC congeners PCB-77, PCB-81, PCB-126, and PCB-169, and mono-ortho IUPAC congeners PCB-105, PCB-114, PCB-118, PCB-123, PCB-156, PCB-157, PCB-167, and PCB-189) is NMT 10.0 pg/g of WHO toxic equivalents.

SPECIFIC TESTS

- **FATS AND FIXED OILS, Acid Value <401>:** NMT 3
- **FATS AND FIXED OILS, Anisidine Value <401>:** NMT 20.0
- **FATS AND FIXED OILS, Peroxide Value <401>:** NMT 5.0
- **FATS AND FIXED OILS, Total Oxidation Value (TOTOX) <401>:** NMT 26, calculated:

$$(2 \times PV) + AV$$

PV = peroxide value

AV = anisidine value

- **FATS AND FIXED OILS, Unsaponifiable Matter <401>:** NMT 1.5%
- **STEARIN:** 10 mL remains clear after cooling at 0° for 3 h
- **ABSORBANCE**
Sample solution: 0.24 mg/mL in isooctane
Acceptance criteria: The absorbance is NMT 0.70, determined at 233 nm.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature. Protect from light.
- **LABELING:** The label states the amount of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in mg/Capsule.
- **USP REFERENCE STANDARDS <11>**
 USP Docosahexaenoic Acid Ethyl Ester RS
 all *cis*-4,7,10,13,16,19-Docosahexaenoic ethyl ester.
 $C_{24}H_{36}O_2$ 356.55
 USP Eicosapentaenoic Acid Ethyl Ester RS
 all *cis*-5,8,11,14,17-Eicosapentaenoic ethyl ester.
 $C_{22}H_{34}O_2$ 330.51
 USP Fish Oil RS
 USP Methyl Tricosanoate RS
 Tricosanoic acid methyl ester.
 $C_{24}H_{48}O_2$ 368.64

Fish Oil Containing Omega-3 Acids Delayed-Release Capsules

DEFINITION

Fish Oil Containing Omega-3 Acids Delayed-Release Capsules are enteric-coated Capsules that contain NLT 95.0% and NMT 105.0% of the labeled amount of Fish Oil Containing Omega-3 Acids where Fish Oil Containing Omega-3 Acids is the purified, winterized, and deodorized fatty oil obtained from fish of the families Engraulidae, Carangidae, Clupeidae, Osmeridae, Scombroideae, and Ammodytidae. The omega-3 acids are defined as the following: alpha-linolenic acid (C18:3 n-3), morotic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), eicosapentaenoic acid (EPA) (C20:5 n-3), heneicosapentaenoic acid (C21:5 n-3), docosapentaenoic acid (C22:5 n-3),

and docosahexaenoic acid (DHA) (C22:6 n-3). It contains NLT 28.0% (w/w) of total omega-3 acids, expressed as free acids, consisting of NLT 13.0% of EPA and NLT 9.0% of DHA. Suitable antioxidants in appropriate concentrations may be added.

IDENTIFICATION

- **A.** The oil contained in the Capsules meets the requirements for the following test: The retention times of the peaks for eicosapentaenoic acid methyl ester and docosahexaenoic acid methyl ester obtained in the chromatogram of *Test Solution 2* in the test for *Content of EPA and DHA* correspond to those for the respective compounds in the chromatogram of *Standard Solution 1*. The sum of the area for EPA and DHA methyl esters is NLT 22% of the total detected area for the methyl esters, and no other peak has an area higher than 20% of the total detected area for the methyl esters. The chromatogram of *Test Solution 2* exhibits at least 15 additional peaks at the retention times of the methyl esters of unsaturated fatty acids exhibited in *Standard Solution 2*.

STRENGTH

- **CONTENT OF FISH OIL:** Weigh NLT 10 Capsules in a tared weighing bottle, carefully open the Capsules, without loss of shell material, and transfer the combined Capsule contents to a 100-mL beaker. Remove any adhering substance from the emptied Capsules by washing with several small portions of 2,2,4-trimethylpentane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the 2,2,4-trimethylpentane is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average net weight per Capsule.

Acceptance criteria: 95.0%–105.0% of the labeled amount

- **CONTENT OF EPA AND DHA**

(See *Fats and Fixed Oils <401>*, *Omega-3 Fatty Acids Determination and Profile*.)

Analysis

Samples: *Standard Solution 1*, *Standard Solution 2*, *Test Solution 1*, and *Test Solution 2*

Identify the retention times of the relevant fatty acid methyl esters peaks by comparing the chromatogram of *Standard Solution 2* with the reference chromatogram supplied with the USP Fish Oil RS. Identify the retention time for the internal standard peak from *Test Solution 2* by comparing with that of *Test Solution 1*. Calculate the percentage of EPA or DHA in the portion of fish oil containing omega-3 acids taken from the Capsules:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times F \times 100$$

R_S = ratio of peak responses of either EPA or DHA relative to the internal standard from *Standard Solution 2*

W_S = weight of either USP Docosahexaenoic Acid Ethyl Ester RS or USP Eicosapentaenoic Acid Ethyl Ester RS used to prepare *Standard Solution 1* (mg)

W_U = weight of the fish oil containing omega-3 acids taken to prepare *Test Solution 2* (mg)

F = factor to express the content of DHA (0.921) and EPA (0.915) as free fatty acids

R_U = ratio of the peak response of either EPA or DHA to the corrected peak response of the internal standard from *Test Solution 2*, calculated as follows:

$$R_U = 1 / [(r_{U2}/r_{T2}) - (r_{U1}/r_{T1})]$$

r_{U2} = response of the peak at the locus of the internal standard from *Test Solution 2*

r_{T2} = peak response of EPA or DHA from *Test Solution 2*

r_{U1} = response of any peak at the locus of the internal standard from *Test Solution 1*

r_{T1} = peak response of EPA or DHA from *Test Solution 1*. [NOTE—If no peak is found at the locus of the internal standard, $R_U = r_{T2}/r_{U2}$.]

Acceptance criteria: NLT 13.0% (w/w) of EPA and NLT 9.0% (w/w) of DHA

• CONTENT OF TOTAL OMEGA-3 ACIDS

(See *Fats and Fixed Oils* <401>, *Omega-3 Fatty Acids Determination and Profile*.)

Analysis: Proceed as directed in the test for *Content of EPA and DHA*.

Calculate the percentage of the total omega-3 acids in the portion of fish oil containing omega-3 acids taken from the Capsules:

$$\text{Result} = \text{EPA} + \text{DHA} + [A_{n-3}(\text{EPA} + \text{DHA})]/(A_{\text{EPA}} + A_{\text{DHA}})$$

EPA = content of EPA from the test for *Content of EPA and DHA* [% (w/w)]

DHA = content of DHA from the test for *Content of EPA and DHA* [% (w/w)]

A_{n-3} = sum of the areas of the peaks corresponding to C18:3 n-3, C18:4 n-3, C20:4 n-3, C21:5 n-3, and C22:5 n-3 methyl esters from *Test Solution 2*

A_{EPA} = area of the peak corresponding to the EPA methyl ester from *Test Solution 2*

A_{DHA} = area of the peak corresponding to the DHA methyl ester from *Test Solution 2*

Acceptance criteria: NLT 28.0% (w/w) of total omega-3 acids, expressed as free acids

PERFORMANCE TESTS

- **WEIGHT VARIATION** (2091): Meet the requirements
- **DISINTEGRATION AND DISSOLUTION** (2040): Meet the requirements for *Disintegration, Delayed-Release (Enteric-Coated) Soft Shell Capsules*

CONTAMINANTS

- **FATS AND FIXED OILS, Trace Metals** <401>: NMT 0.1 ppm each of Pb, Cd, As, and Hg

- **LIMIT OF DIOXINS, FURANS, AND POLYCHLORINATED BIPHENYLS**

Analysis: Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by method No. 1613 revision B of the Environmental Protection Agency. Determine the content of polychlorinated biphenyls (PCBs) by method No. 1668 revision A of the Environmental Protection Agency.

Acceptance criteria: The sum of PCDDs and PCDFs is NMT 2.0 pg/g of WHO toxic equivalents. The sum of PCDDs, PCDFs, and dioxin-like PCBs (polychlorinated biphenyls, non-ortho IUPAC congeners PCB-77, PCB-81, PCB-126, and PCB-169, and mono-ortho IUPAC congeners PCB-105, PCB-114, PCB-118, PCB-123, PCB-156, PCB-157, PCB-167, and PCB-189) is NMT 10.0 pg/g of WHO toxic equivalents.

SPECIFIC TESTS

- **FATS AND FIXED OILS, Acid Value** <401>: NMT 3
- **FATS AND FIXED OILS, Anisidine Value** <401>: NMT 20.0
- **FATS AND FIXED OILS, Peroxide Value** <401>: NMT 5.0
- **FATS AND FIXED OILS, Total Oxidation Value (TOTOX)** <401>: NMT 26, calculated:

$$\text{Result} = (2 \times \text{PV}) + \text{AV}$$

PV = peroxide value

AV = anisidine value

- **FATS AND FIXED OILS, Unsaponifiable Matter** <401>: NMT 1.5%

- **STEARIN:** 10 mL remains clear after cooling at 0° for 3 h.
- **ABSORBANCE**
Sample solution: 0.24 mg/mL in isooctane
Acceptance criteria: NMT 0.70, determined at 233 nm

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature, protected from light.
- **LABELING:** The label states the amount of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in mg/Capsule.
- **USP REFERENCE STANDARDS** <11>
 USP Docosahexaenoic Acid Ethyl Ester RS
 all *cis*-4,7,10,13,16,19-Docosahexaenoic ethyl ester.
 $\text{C}_{24}\text{H}_{36}\text{O}_2$ 356.55
 USP Eicosapentaenoic Acid Ethyl Ester RS
 all *cis*-5,8,11,14,17-Eicosapentaenoic ethyl ester.
 $\text{C}_{22}\text{H}_{34}\text{O}_2$ 330.51
 USP Fish Oil RS
 USP Methyl Tricosanoate RS

Folic Acid—see *Folic Acid General Monographs*

Folic Acid Tablets—see *Folic Acid Tablets General Monographs*

Forskohlii

DEFINITION

Forskohlii consists of the dried roots of *Plectranthus barbatus* Andrews, also known as *Coleus barbatus* (Andrews) Benth. and *Coleus forskohlii* Briq. (Fam. Lamiaceae). It contains NLT 0.4% of forskolin, calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** <201>

Standard solution A: 50 µg/mL of USP Forskolin RS in acetonitrile. Sonicate for about 10 min.

Standard solution B: 5 mg/mL of USP Powdered Forskohlii Extract RS in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant.

Sample stock solution: Use the *Sample solution*, prepared as directed in the test for *Content of Forskolin*.

Sample solution: Dilute 10 mL of the *Sample stock solution* with acetonitrile to 25 mL.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL as 4-mm bands

Developing solvent system: Toluene and ethyl acetate (85:15)

Spray reagent: 5% vanillin in glacial acetic acid and 10% sulfuric acid in water (1:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate (see *Chromatography* <621>). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about 90% of

the plate. Remove the plate from the chamber, dry, spray with the *Spray reagent*, heat for 5–10 min at 105°, and examine under visible light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following: a violet zone due to forskolin at an R_f value of approximately 0.3, corresponding in color and R_f to that in the chromatogram of *Standard solution A*; and a minor violet zone, a pink zone, and a brick red zone at R_f values of approximately 0.1, 0.62, and 0.69, due to isoforskolin, 1,9-dideoxyforskolin and crocetindialdehyde, respectively. Zones detected in the *Sample solution* chromatogram correspond in position and color to zones in the chromatogram of *Standard solution B*. Other minor zones may be observed in the *Sample solution* and *Standard solution B* chromatograms.

- **B.** The chromatogram of the *Sample solution* obtained in the test for *Content of Forskolin* shows a main peak at a retention time corresponding to that of forskolin in the chromatogram of *Standard solution A*. Identify other diterpene peaks in the *Sample solution* chromatogram by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS. The *Sample solution* chromatogram shows an additional peak corresponding to isoforskolin.

COMPOSITION

• CONTENT OF FORSKOLIN

Solution A: Use filtered and degassed acetonitrile.

Solution B: Use filtered and degassed water.

Standard solution A: Sonicate a quantity of USP Forskolin RS in acetonitrile to obtain a solution having a known concentration of about 1.0 mg/mL.

Standard solution B: 5 mg/mL of USP Powdered Forskohlii Extract RS in acetonitrile, sonicate for about 15 min, centrifuge, and use the supernatant. Before injection, pass through a membrane filter of 0.45- μ m or finer pore size.

Sample solution: Transfer about 3.0 g of Forskohlii, finely powdered, to a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of acetonitrile, reflux on a water bath for 20 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume with acetonitrile to 100 mL. Before injection, pass through a membrane filter of 0.45- μ m or finer pore size, discarding the first 5 mL of the filtrate.

System suitability solution: *Standard solution A* and 0.01% toluene in acetonitrile (1:1)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	45	55
25	45	55
28	95	5
35	95	5
36	45	55
45	45	55

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 220 nm

Column: 4.6-mm \times 25-cm; 5- μ m, 100 Å

Column temperature: 30 \pm 2°

Flow rate: 1.8 mL/min

Injection size: 20 μ L

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *System suitability solution*

[NOTE—The approximate relative retention times for isoforskolin and forskolin are 0.51 and 1.00, respectively.]

Suitability requirements: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used.

Resolution: NLT 1.5 between the forskolin and toluene peaks, *System suitability solution*

Relative standard deviation: NMT 2% determined from the forskolin peak for replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS, identify the retention times of the peaks corresponding to isoforskolin and forskolin.

Calculate the percentage of forskolin in the portion of Forskohlii taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of forskolin from the *Sample solution*

r_S = peak response of forskolin from *Standard solution A*

C_S = concentration of USP Forskolin RS in *Standard solution A* (mg/mL)

C_U = concentration of Forskohlii in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.4% on the dried basis

IMPURITIES

Inorganic Impurities

• **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* (561): NMT 2%

• **HEAVY METALS**, *Method III* (231): NMT 20 ppm

Organic Impurities

• **PROCEDURE 1: ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matters* (561): NMT 2.0%

• **PROCEDURE 2: ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Fresh root, pale pinkish yellow, cylindrical to subcylindrical, with tapering ends, 5–12 in length, 1–2 cm in diameter; surface rough, shows lateral rootlets or scars of rootlets and transversely running lenticels. Pharmacopeial article, dark brown; surface rough, irregularly cylindrical, longitudinally wrinkled, showing irregular grooves and prominent ridges; fracture short; cut surface is yellowish white; characteristic and pleasant aromatic odor, and slightly bitter to pungent taste.

Histology

Transverse section of roots: Irregular circular in outline; showing narrow cork, 10–15 rows of tangentially elongated radially arranged cork cells; cortex composed of 10–15 rows of thin-wall parenchyma cells showing sclereids and crystals of calcium oxalate; vascular cambium in the form of a continuous ring; xylem showing narrow rays of vessels, few lignified fibers are present in older roots, 3–8 cell-wide medullary rays, and parenchyma showing few sclereids, oleoresin canals and simple starch grains; pith composed of parenchyma cells in young roots and is replaced by compactly arranged vessels, fibers, and tracheids in mature roots.

- **LOSS ON DRYING** (731): Dry 1.0 g of finely powdered Forskohlii at 105° for 3 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 6%, determined on 1.0 g of finely powdered Forskohlii
- **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method 2** (561): NLT 25.0%
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10³ cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
- **ARTICLES OF BOTANICAL ORIGIN, Test for Aflatoxins** (561): Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11)
USP Forskolin RS
USP Powdered Forskohlii Extract RS

Powdered Forskohlii

DEFINITION

Powdered Forskohlii is Forskohlii reduced to a powder or very fine powder. It contains NLT 0.4% of forskolin, calculated on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)

Standard solution A: 50 µg/mL of USP Forskolin RS in acetonitrile. Sonicate for about 10 min.

Standard solution B: 5 mg/mL of USP Powdered Forskohlii Extract RS in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant.

Sample stock solution: Use the *Sample solution*, prepared as directed in the test for *Content of Forskolin*.

Sample solution: Dilute 10 mL of the *Sample stock solution* with acetonitrile to 25 mL.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL, as 4-mm bands

Developing solvent system: A mixture of toluene and ethyl acetate (85:15)

Spray reagent: A mixture of 5% vanillin in glacial acetic acid and 10% sulfuric acid in water (1:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)). Use saturated chamber. Develop the chromatograms until the solvent front has moved up about 90% of the plate. Remove the plate from the chamber, dry, spray with the *Spray reagent*, heat for 5–10 min at 105°, and examine under visible light.

Acceptance criteria: The *Sample solution* exhibits a violet zone due to forskolin at an *R_f* value of approximately 0.3, corresponding in color and *R_f* to that from *Standard solution A*; and a minor violet zone, a pink zone, and a brick red zone at *R_f* values of approximately 0.1, 0.62, and 0.69, due to isoforskolin, 1,9-dideoxyforskolin, and crocetindialdehyde, respectively. Zones de-

tected from the *Sample solution* correspond in position and color to zones from *Standard solution B*. Other minor zones may be observed from the *Sample solution* and *Standard solution B*.

- **B.** The *Sample solution* from the test for *Content of Forskolin* shows a main peak at a retention time corresponding to that of forskolin from *Standard solution A*. Identify other diterpene peaks in the *Sample solution* by comparison with *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used. The *Sample solution* shows an additional peak corresponding to isoforskolin.

COMPOSITION

• CONTENT OF FORSKOLIN

Solution A: Use filtered and degassed acetonitrile.

Solution B: Use filtered and degassed water.

Standard solution A: Sonicate a quantity of USP Forskolin RS in acetonitrile to obtain a solution having a known concentration of about 1.0 mg/mL.

Standard solution B: 5 mg/mL of USP Powdered Forskohlii Extract RS in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size.

Sample solution: Transfer about 3.0 g of Powdered Forskohlii to a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of acetonitrile, reflux on a water bath for 20 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume with acetonitrile to 100 mL. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

System suitability solution: A mixture of *Standard solution A* and 0.01% toluene in acetonitrile (1:1)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	45	55
25	45	55
28	95	5
35	95	5
36	45	55
45	45	55

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 220 nm

Column: 4.6-mm × 25-cm; 5-µm, 100 Å

Column temperature: 30 ± 2°

Flow rate: 1.8 mL/min

Injection size: 20 µL

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *System suitability solution*

[NOTE—The relative retention times for isoforskolin and forskolin are 0.51 and 1.00, respectively.]

Suitability requirements: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used.

Relative standard deviation: NMT 2% determined from the forskolin peak in repeated injections, *Standard solution A*

Resolution: NLT 1.5 between the forskolin and toluene peaks, *System suitability solution*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used, identify the retention times of the peaks corresponding to isoforskolin and forskolin. Calculate the percentage of forskolin in the portion of Powdered Forskohlii taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak response of forskolin from the *Sample solution*
 r_S = peak response of forskolin from *Standard solution A*
 C_S = concentration of USP Forskolin RS in *Standard solution A* (mg/mL)
 C_U = concentration of Powdered Forskohlii in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.4% is found on the dried basis.

IMPURITIES

Inorganic Impurities

- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561): NMT 2%

- **HEAVY METALS, Method III** (231): NMT 20 ppm

Organic Impurities

- **PROCEDURE: ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** (561): Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Yellowish brown powder; characteristic and pleasant aromatic odor; and slightly bitter to pungent taste. Under a microscope, it shows the presence of parenchyma cells with oleoresin canals, starch grains and prisms of calcium oxalate; oil globules; simple starch grains, cork cells; sclereids; stone cells; pitted vessels; and thin-wall fibers.
- **LOSS ON DRYING** (731): Dry 1.0 g of Powdered Forskohlii at 105° for 3 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 6%, determined on 1.0 g of Powdered Forskohlii
- **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method 2** (561): NLT 25.0%
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10^5 cfu/g; the total combined molds and yeasts count does not exceed 10^3 cfu/g; and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
- **ARTICLES OF BOTANICAL ORIGIN, Test for Aflatoxins** (561): Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11)
 USP Forskolin RS
 USP Powdered Forskohlii Extract RS

Powdered Forskohlii Extract

DEFINITION

Powdered Forskohlii Extract is prepared from Forskohlii using suitable solvents such as methanol, ethyl acetate, hexane

or a mixture of these solvents. The ratio of plant material to extract is between 65:1 and 35:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of forskolin, calculated on the dried basis. It contains suitable added substances as carriers.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)

Standard solution A: 50 µg/mL of USP Forskolin RS in acetonitrile. Sonicate for about 10 min.

Standard solution B: 5 mg/mL of USP Powdered Forskohlii Extract RS in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant.

Sample solution: 5 mg/mL of Powdered Forskohlii Extract in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL, as 4-mm bands

Developing solvent system: A mixture of toluene and ethyl acetate (85:15)

Spray reagent: A mixture of 5% vanillin in glacial acetic acid and 10% sulfuric acid in water (1:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)). Use saturated chamber. Develop the chromatograms until the solvent front has moved up about 90% of the plate. Remove the plate from the chamber, dry, spray with the *Spray reagent*, heat for 5–10 min at 105°, and examine under visible light.

Acceptance criteria: The *Sample solution* exhibits a violet zone due to forskolin at an R_f value of approximately 0.3, corresponding in color and R_f to that from *Standard solution A*; and a minor violet zone, a pink zone, and a brick red zone at R_f values of approximately 0.1, 0.62, and 0.69, due to isoforskolin, 1,9-dideoxyforskolin, and crocetindialdehyde, respectively. Zones detected from the *Sample solution* correspond in position and color to zones from *Standard solution B*. Other minor zones may be observed from the *Sample solution* and *Standard solution B*.

- **B.** The *Sample solution* from the test for *Content of Forskolin* shows a main peak at a retention time corresponding to that of forskolin from *Standard solution A*. Identify other diterpene peaks in the *Sample solution* by comparison with *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used. The *Sample solution* shows an additional peak corresponding to isoforskolin.

COMPOSITION

• CONTENT OF FORSKOLIN

Solution A: Use filtered and degassed acetonitrile.

Solution B: Use filtered and degassed water.

Standard solution A: Sonicate a quantity of USP Forskolin RS in acetonitrile to obtain a solution having a known concentration of about 1.0 mg/mL.

Standard solution B: 5 mg/mL of USP Powdered Forskohlii Extract RS in acetonitrile. Sonicate for about 15 min, centrifuge, and use the supernatant. Before injection, pass through a membrane filter having a 0.45-µm or finer pore size.

Sample solution: Transfer an amount of Powdered Forskohlii Extract equivalent to about 25 mg of forskolin to a 25-mL volumetric flask, and add 15 mL of acetonitrile. Sonicate and heat in a water bath for about 10 min, cool, dilute with acetonitrile to volume, and mix. Before injection, filter through a membrane filter having a 0.45-µm or finer pore size, discarding the first 5 mL of the filtrate.

System suitability solution: A mixture of *Standard solution A* and 0.01% toluene in acetonitrile (1:1)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	45	55
25	45	55
28	95	5
35	95	5
36	45	55
45	45	55

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 220 nm

Column: 4.6-mm × 25-cm; 5-μm, 100 Å

Column temperature: 30 ± 2°

Flow rate: 1.8 mL/min

Injection size: 20 μL

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *System suitability solution*

[NOTE—The relative retention times for isoforskolin and forskolin are 0.51 and 1.00, respectively.]

Suitability requirements: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used.

Relative standard deviation: NMT 2% determined from the forskolin peak in repeated injections, *Standard solution A*

Resolution: NLT 1.5 between the forskolin and toluene peaks, *System suitability solution*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Forskohlii Extract RS being used, identify the retention times of the peaks corresponding to isoforskolin and forskolin.

Calculate the percentage of forskolin in the portion of Powdered Forskohlii Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of forskolin from the *Sample solution*

r_S = peak response of forskolin from *Standard solution A*

C_S = concentration of USP Forskolin RS in *Standard solution A* (mg/mL)

C_U = concentration of Powdered Forskohlii Extract in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 90.0% and NMT 110.0% of the labeled amount of forskolin on the dried basis

IMPURITIES

Inorganic Impurities

• **HEAVY METALS, Method III** <231>: NMT 20 ppm

Organic Impurities

• **PROCEDURE: ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** <561>: Meets the requirements

SPECIFIC TESTS

• **LOSS ON DRYING** <731>: Dry 1.0 g of Powdered Forskohlii Extract at 105° for 3 h: it loses NMT 5.0% of its weight.

• **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 10⁴ cfu/g. The total combined yeasts and molds count does not exceed 10³ cfu/g.

• **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

• **ARTICLES OF BOTANICAL ORIGIN, Test for Aflatoxins** <561>: Meets the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements under *Botanical Extracts* <565>.

• **OTHER REQUIREMENTS:** It meets the requirements of the test for *Residual Solvents* under *Botanical Extracts* <565>.

• **USP REFERENCE STANDARDS** <11>

USP Forskolin RS

USP Powdered Forskohlii Extract RS

Garcinia cambogia

DEFINITION

Garcinia cambogia consists of the dried pericarp of the fruits of *Garcinia gummi-gutta* (L.) N. Robson, also known as *Garcinia cambogia* (Gaertn.) Desr. (Fam. Clusiaceae). It contains NLT 12% of the sum of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone, on the dried basis.

IDENTIFICATION

• **A. *Garcinia cambogia*** meets the requirements under *Specific Tests, Botanic Characteristics*.

• **B. HPLC IDENTIFICATION TEST:** The *Sample solution* chromatogram exhibits a peak for hydroxycitric acid at a retention time corresponding to that of *Standard solution A*, as obtained in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*. The *Sample solution* also exhibits a peak for hydroxycitric acid lactone. The hydroxycitric acid and the hydroxycitric acid lactone peaks are the main peaks in the *Sample solution* chromatogram.

COMPOSITION

• **CONTENT OF (–)-HYDROXYCITRIC ACID AND (–)-HYDROXYCITRIC ACID LACTONE**

Solution A: 30% phosphoric acid in water

Mobile phase: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, adjust with *Solution A* to a pH of 2.5, complete to 1000 mL with water, mix, filter, and degas.

Solvent: A mixture of *Solution A* and water (1:9)

Standard solution A: A solution of USP Calcium (–)-Hydroxycitrate RS equivalent to about 2.5 mg/mL of (–)-hydroxycitric acid in *Solvent*. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Standard solution B: 5 mg/mL of USP Powdered *Garcinia Hydroxycitrate* Extract RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-μm or finer pore size.

Sample solution: Transfer about 5 g of *Garcinia cambogia*, finely powdered and accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of *Solvent*, reflux while stirring on a water bath for 30 min, set aside to settle, and decant the supernatant. Repeat the extraction using four 50-mL portions of water, combine all extracts, cool, filter into a 250-mL volumetric flask, and complete with water to volume. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system(See *Chromatography* (621), *System Suitability*.)**Mode:** LC**Detector:** UV 215 nm**Column:** 4.6-mm × 25-cm; packing L1**Column temperature:** 25 ± 1°**Flow rate:** 1.0 mL/min**Injection size:** 20 µL**System suitability****Samples:** *Standard solution A*, *Standard solution B*, and *Sample solution*

[NOTE—The relative retention times for the hydroxycitric acid lactone and hydroxycitric acid peaks are about 0.9 and 1.0, respectively.]

Suitability requirements**Chromatogram similarity:** The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered *Garcinia Hydroxycitrate Extract RS* being used.**Resolution:** NLT 1.0 between hydroxycitric acid lactone and hydroxycitric acid, *Sample solution***Tailing factor:** NMT 2.0 for the hydroxycitric acid peak, *Standard solution A***Relative standard deviation:** NMT 2.0%, determined from the hydroxycitric acid peak in repeated injections, *Standard solution A***Analysis****Samples:** *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and *Sample solution* are stable for 6 h.]Calculate the percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone in the portion of *Garcinia cambogia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

 r_U = peak area of the relevant analyte from the *Sample solution* r_S = peak area of hydroxycitric acid from *Standard solution A* C_S = concentration of (–)-hydroxycitric acid in *Standard solution A* (mg/mL) V = final volume of the *Sample solution* (mL) W = weight of *Garcinia cambogia* used to prepare the *Sample solution* (mg) F = conversion factor for each analyte: 2.17 for (–)-hydroxycitric acid lactone, and 1.00 for (–)-hydroxycitric acid**Acceptance criteria:** Add the percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone: NLT 12% is found on the dried basis.**IMPURITIES****Inorganic Impurities**• **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561): NMT 2.0%• **HEAVY METALS, Method III** (231): NMT 20 ppm**Organic Impurities**• **PROCEDURE 1: ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** (561): NMT 2.0%• **PROCEDURE 2: ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** (561): Meets the requirements**SPECIFIC TESTS**• **BOTANIC CHARACTERISTICS****Macroscopic:** Fresh fruits are spherical to oval in shape, 4–8 cm in height, 3–6 cm in width, yellowish to pale pinkish when ripe, resemble a miniature pumpkin, with 7–13 deep longitudinal grooves, extending up to a circular elevated base of stigma with blackish tip, situated in the depressed end of the fruit, containing 6–8 seeds surrounded by a succulent aril. Compendial article consists of dried pieces of pericarp, longitudinal,

of variable size and shape, strongly curved inward; leathery; externally rough, irregularly wrinkled, longitudinally grooved; internally smooth, longitudinally faintly striated and ridged. Dark brown to blackish-brown; odor characteristic; taste sour, astringent, and slightly bitter.

Histology**Transverse section of the pericarp:** Shows a layer of epicarp, composed of rectangular to tangentially elongated cells covered with thin cuticle; wide mesocarp, composed of 100–150 rows of parenchyma cells of variable size and shape, the outer rows composed of relatively larger cells with wide intercellular spaces; vascular bundles appear throughout the mesocarp, more toward the inner zone; dark brown gummy exudates, simple and compound starch granules and prisms of calcium oxalate are present in the parenchyma cells throughout the mesocarp.• **LIMIT OF CITRIC ACID****Solvent and Chromatographic system:** Prepare as directed in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*.**Standard solution:** 0.5 mg/mL of USP Citric Acid RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.**Analysis****Sample:** *Standard solution*Calculate the percentage of citric acid in the portion of *Garcinia cambogia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

 r_U = peak area of citric acid from the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone* (mg/mL) r_S = peak area of citric acid from the *Standard solution* C_S = concentration of USP Citric Acid RS in the *Standard solution* (mg/mL) V = final volume of the *Sample solution* (mL) W = weight of *Garcinia cambogia* used to prepare the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone* (mg)**Acceptance criteria:** NMT 2% of citric acid on the dried basis• **LOSS ON DRYING** (731): Dry 2.0 g of finely powdered *Garcinia cambogia* at 105° for 3 h: it loses NMT 12.0% of its weight.• **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561):Determined on 1.0 g of finely powdered *Garcinia cambogia*: NMT 3.0%; and NMT 8.0% if sodium chloride was added as a preservative during collection of the fruits• **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10³ cfu/g.• **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella species* and *Escherichia coli***ADDITIONAL REQUIREMENTS**• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.

- **USP REFERENCE STANDARDS** (11)
 - USP Calcium (–)-Hydroxycitrate RS
 - USP Citric Acid RS
 - USP Powdered Garcinia Hydroxycitrate Extract RS

Powdered *Garcinia cambogia*

DEFINITION

Powdered *Garcinia cambogia* is *Garcinia cambogia* reduced to a powder or very fine powder. It contains NLT 12% of the sum of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone, on the dried basis.

IDENTIFICATION

- **A.** Powdered *Garcinia cambogia* meets the requirements under *Specific Tests, Botanic Characteristics*.
- **B. HPLC IDENTIFICATION TEST:** The *Sample solution* chromatogram exhibits a peak for hydroxycitric acid at a retention time corresponding to that of *Standard solution A*, as obtained in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*. The *Sample solution* also exhibits a peak for hydroxycitric acid lactone. The hydroxycitric acid and the hydroxycitric acid lactone peaks are the main peaks in the *Sample solution* chromatogram.

COMPOSITION

- **CONTENT OF (–)-HYDROXYCITRIC ACID AND (–)-HYDROXYCITRIC ACID LACTONE**

Solution A: 30% phosphoric acid in water

Mobile phase: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, adjust with *Solution A* to a pH of 2.5, complete with water to 1000 mL, mix, filter, and degas.

Solvent: *Solution A* and water (1:9)

Standard solution A: A solution of USP Calcium (–)-Hydroxycitrate RS equivalent to about 2.5 mg/mL of (–)-hydroxycitric acid in *Solvent*. Before injection, pass through a membrane filter of 0.45-μm or finer pore size.

Standard solution B: 5 mg/mL of USP Powdered Garcinia Hydroxycitrate Extract RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of filtrate.

Sample solution: Transfer about 5 g of Powdered *Garcinia cambogia*, accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of *Solvent*, reflux while stirring on a water bath for 30 min, set aside to settle, and decant the supernatant. Repeat the extraction using four 50-mL portions of water, combine all extracts, Cool, filter into a 250-mL volumetric flask, and complete with water to volume. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 215 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 25 ± 1°

Flow rate: 1.0 mL/min

Injection size: 20 μL

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

[NOTE—The relative retention times for the hydroxycitric acid lactone and hydroxycitric acid peaks are about 0.9 and 1.0, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chro-

matogram provided with the lot of USP Powdered Garcinia Hydroxycitrate Extract RS being used.

Resolution: NLT 1.0 between the hydroxycitric acid lactone and hydroxycitric acid peaks, *Sample solution*

Tailing factor: NMT 2.0 for the hydroxycitric acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, determined from the hydroxycitric acid peak for replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

[NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 h.]

Calculate the percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone in the portion of Powdered *Garcinia cambogia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of hydroxycitric acid from *Standard solution A*

C_S = concentration of (–)-hydroxycitric acid in *Standard solution A* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Powdered *Garcinia cambogia* used to prepare the *Sample solution* (mg)

F = conversion factor for each analyte: 2.17 for (–)-hydroxycitric acid lactone, and 1.00 for (–)-hydroxycitric acid

Acceptance criteria: NLT 12% on the dried basis

IMPURITIES

Inorganic Impurities

- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561): NMT 2.0%

- **HEAVY METALS, Method III** (231): NMT 20 ppm

Organic Impurities

- **PROCEDURE: ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** (561): Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Dark brown powder; odor characteristic; taste sour, astringent and slightly bitter. Under a microscope, it shows parenchyma cells containing dark reddish-brown gummy exudates; parenchyma cells containing simple and compound starch granules; prisms of calcium oxalate; and fragments of spiral and annular vessels.

LIMIT OF CITRIC ACID

Solvent: Prepare as directed in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*.

Standard solution: 0.5 mg/mL of USP Citric Acid RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of filtrate.

Analysis

Sample: *Standard solution*

Calculate the percentage of citric acid in the portion of Powdered *Garcinia cambogia* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of citric acid from the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*

r_S = peak area of citric acid from the *Standard solution*

C_S = concentration of USP Citric Acid RS in the *Standard solution* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Powdered *Garcinia cambogia* used to prepare the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone* (mg)

Acceptance criteria: NMT 2% of citric acid, calculated on the dried basis

- **LOSS ON DRYING** (731): Dry 2.0 g of Powdered *Garcinia cambogia* at 105° for 3 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): Determined on 1.0 g of Powdered *Garcinia cambogia*: NMT 3.0%; and NMT 8.0% if sodium chloride was added as a preservative during collection of the fruits
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10³ cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11)
USP Calcium (–)-Hydroxycitrate RS
USP Citric Acid RS
USP Powdered *Garcinia* Hydroxycitrate Extract RS

Powdered *Garcinia* Hydroxycitrate Extract

DEFINITION

Powdered *Garcinia* Hydroxycitrate Extract is prepared from *Garcinia cambogia* or *Garcinia indica* by extraction with water, alcohol, or mixtures of these solvents, followed by stabilization of the (–)-hydroxycitric acid content in the form of a calcium, potassium, magnesium, and/or sodium salt. The ratio of plant material to extract is about 5:1 to 10:1. It contains NLT 40% of (–)-hydroxycitric acid, calculated on the dried basis. It may contain suitable added substances.

IDENTIFICATION

- **A. HPLC IDENTIFICATION TEST:** The *Sample solution* chromatogram exhibits a peak for hydroxycitric acid at a retention time corresponding to that of *Standard solution A*, as obtained in the test for *Content of (–)-Hydroxycitric Acid and Limit of (–)-Hydroxycitric Acid Lactone*.

COMPOSITION

- **CONTENT OF (–)-HYDROXYCITRIC ACID AND LIMIT OF (–)-HYDROXYCITRIC ACID LACTONE**
Solution A: 30% phosphoric acid in water
Mobile phase: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, adjust with *Solution A* to a pH of 2.5, complete to 1000 mL with water, mix, filter, and degas.
Solvent: A mixture of *Solution A* and water (1:9)
Standard solution A: A solution of USP Calcium (–)-Hydroxycitrate RS equivalent to about 2.5 mg/mL of (–)-hydroxycitric acid in *Solvent*. Before injection, pass through a membrane filter of 0.45-μm or finer pore size.
Standard solution B: 5 mg/mL of USP Powdered *Garcinia* Hydroxycitrate Extract RS in *Solvent*. Before in-

jection, pass through a membrane filter of 0.45-μm or finer pore size.

Sample solution: 5 mg/mL of Powdered *Garcinia* Hydroxycitrate Extract in *Solvent*. Before injection, pass through a membrane filter of 0.45-μm or finer pore size.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 215 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 25 ± 1°

Flow rate: 1.0 mL/min

Injection size: 20 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered *Garcinia* Hydroxycitrate Extract RS being used.

Tailing factor: NMT 2.0 for the hydroxycitric acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, determined from the hydroxycitric acid peak, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 h.] Calculate the percentage of (–)-hydroxycitric acid and the limit of (–)-hydroxycitric acid lactone, if present, in the portion of Powdered *Garcinia* Hydroxycitrate Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area for the relevant analyte from the *Sample solution*

r_S = peak area of hydroxycitric acid from *Standard solution A*

C_S = concentration of (–)-hydroxycitric acid in *Standard solution A* (mg/mL)

C_U = concentration of Powdered *Garcinia* Hydroxycitrate Extract in the *Sample solution* (mg/mL)

F = conversion factor for each analyte: 2.17 for (–)-hydroxycitric acid lactone, and 1.00 for (–)-hydroxycitric acid

Acceptance criteria: NLT 40% of (–)-hydroxycitric acid and NMT 8% of (–)-hydroxycitric acid lactone on the dried basis

IMPURITIES

Inorganic Impurities

- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561): NMT 3.0%

- **HEAVY METALS, Method III** (231): NMT 20 ppm

Organic Impurities

- **PROCEDURE: ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues** (561): Meets the requirements

SPECIFIC TESTS

LIMIT OF CITRIC ACID

Solvent: Prepare as directed in the test for *Content of (–)-Hydroxycitric Acid and Limit of (–)-Hydroxycitric Acid Lactone*.

Standard solution: 0.5 mg/mL of USP Citric acid RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-μm or finer pore size.

Analysis

Sample: *Standard solution*

Calculate the percentage of citric acid in the portion of Powdered *Garcinia* Hydroxycitrate Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of citric acid, using the peak area of citric acid from the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and Limit of (–)-Hydroxycitric Acid Lactone*
- r_S = peak area of citric acid from the *Standard solution*
- C_S = concentration of USP Citric Acid RS in the *Standard solution* (mg/mL)
- C_U = concentration of Powdered Garcinia Hydroxycitrate Extract in the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and Limit of (–)-Hydroxycitric Acid Lactone* (mg/mL)

Acceptance criteria: NMT 5% of citric acid on the dried basis

- **IDENTIFICATION TESTS—GENERAL** (191): Test for the presence of calcium, magnesium, potassium, and/or sodium.
- **LOSS ON DRYING** (731): Dry 2.0 g of Powdered Extract at 105° for 3 h: Powdered Extract containing calcium hydroxycitrate loses NMT 5.0% of its weight; Powdered Extract containing other salts loses NMT 9.0% of its weight.
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts count does not exceed 10³ cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **OTHER REQUIREMENTS:** It meets the requirements of the test for *Residual Solvents* under *Botanical Extracts* (565).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. It meets other *Labeling* requirements under *Botanical Extracts* (565).
- **USP REFERENCE STANDARDS** (11)
USP Calcium (–)-Hydroxycitrate RS
USP Citric Acid RS
USP Powdered Garcinia Hydroxycitrate Extract RS

Garcinia indica

DEFINITION

Garcinia indica consists of the dried pericarp of the fruits of *Garcinia indica* (Thouars) Choisy (Fam. Clusiaceae). It contains NLT 12% of the sum of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone, on the dried basis.

IDENTIFICATION

- **A. *Garcinia indica*** meets the requirements under *Specific Tests, Botanic Characteristics*.
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution: 0.5 mg/mL of garcinol in alcohol
Sample solution: Transfer about 2.0 g of *Garcinia indica*, finely powdered, to a Soxhlet apparatus, add 100 mL of alcohol, and extract for 6 h. Filter and concentrate under vacuum to about 10 mL. [NOTE—Use a thimble of suitable size such that the volume of alcohol used in the Soxhlet extraction is at least twice the volume of the thimble.]
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL, as 8-mm bands
Developing solvent system: Toluene, ethyl acetate, and formic acid (4:1:0.5)

Spray reagent: A mixture of 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)

Analysis

Samples: *Standard solution* and *Sample solution*
Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 5–10 min at about 105°, and examine under visible light.

Acceptance criteria: The *Sample solution* chromatogram exhibits a main greenish-grey band due to garcinol at an R_F value of approximately 0.6, which corresponds in position and color to the main band in the chromatogram of the *Standard solution*. The *Sample solution* exhibits the following additional bands: two purple bands, two greenish-grey bands, two blue bands and a purple band at R_F values of approximately 0.31, 0.34, 0.37, 0.47, 0.54, 0.83, and 0.93, respectively. Other bands may be observed for the *Sample solution*.

- **C. HPLC IDENTIFICATION TEST:** The *Sample solution* chromatogram exhibits a peak for hydroxycitric acid at a retention time corresponding to that of *Standard solution A*, as obtained in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*. The *Sample solution* also exhibits a peak for hydroxycitric acid lactone. The hydroxycitric acid and the hydroxycitric acid lactone peaks are the main peaks in the *Sample solution* chromatogram.

COMPOSITION

- **CONTENT OF (–)-HYDROXYCITRIC ACID AND (–)-HYDROXYCITRIC ACID LACTONE**

Solution A: 30% phosphoric acid in water

Mobile phase: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, adjust with *Solution A* to a pH of 2.5, complete with water to 1000 mL, mix, filter, and degas.

Solvent: A mixture of *Solution A* and water (1:9)

Standard solution A: A solution of USP Calcium (–)-Hydroxycitrate RS equivalent to about 4 mg/mL of (–)-hydroxycitric acid in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Standard solution B: 8 mg/mL of USP Powdered Garcinia Hydroxycitrate Extract RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 5 g of *Garcinia indica*, finely powdered and accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of *Solvent*, reflux while stirring on a water bath for 30 min, set aside to settle, and decant the supernatant. Repeat the extraction using four 50-mL portions of water, combine all extracts, cool, filter into a 250-mL volumetric flask, and complete with water to volume. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 215 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 25 ± 1°

Flow rate: 1.0 mL/min

Injection size: 20 µL

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

[NOTE—The relative retention times for the hydroxycitric acid lactone and hydroxycitric acid peaks are about 0.9 and 1.0, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered *Garcinia Hydroxycitrate Extract RS* being used.

Resolution: NLT 1.0 between the hydroxycitric acid lactone and hydroxycitric acid peaks, *Sample solution*

Tailing factor: NMT 2.0 for the hydroxycitric acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, determined from the hydroxycitric acid peak for replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 h.] Calculate the percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone in the portion of *Garcinia indica* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of hydroxycitric acid from *Standard solution A*

C_S = concentration of (–)-hydroxycitric acid in *Standard solution A* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of *Garcinia indica* used to prepare the *Sample solution* (mg)

F = conversion factor for each analyte: 2.17 for (–)-hydroxycitric acid lactone, and 1.00 for (–)-hydroxycitric acid

Acceptance criteria: Add the percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone: NLT 12% is found on the dried basis.

IMPURITIES**Inorganic Impurities**

• **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* (561): NMT 0.5%

• **HEAVY METALS**, *Method III* (231): NMT 20 ppm

Organic Impurities

• **PROCEDURE 1: ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* (561): NMT 2.0%

• **PROCEDURE 2: ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements

SPECIFIC TESTS**• BOTANIC CHARACTERISTICS**

Macroscopic: Fresh fruits are globular in shape, 3–4 cm in diameter, purplish to pinkish orange when ripe, with persistent calyx lobes at the base and flattened radiating sessile stigma at the apex; containing 5–8 seeds surrounded by a succulent aril. Compendial article consists of dried pieces of pericarp, bluish black, of various size and shapes, flattened, flexible; remains of pedicels, calyx, and stigma may be present; odor characteristic; taste sour.

Histology

Transverse section of the pericarp: A layer of epicarp, composed of isodiametric cells, with thin cuticle and stomata; hypodermis consisting of several rows of compactly arranged, tangentially elongated, thick-wall cells, containing dark brown contents, showing narrow irregular elongated cavities; outer mesocarp consisting of loosely arranged, tangentially elongated, parenchyma cells, few are full of starch grains, traversed by narrow bands of collapsed cells and oleoresin ducts; inner mesocarp consisting of collapsed and compactly arranged cells, showing rows of fibrovascular bundles; endocarp is not distinct,

consisting of thin-wall collapsed cells, with dark brown content.

• LIMIT OF CITRIC ACID

Solvent and Chromatographic system: Prepare as directed in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*.

Standard solution: 0.5 mg/mL of USP Citric Acid RS in *Solvent*. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Analysis

Sample: *Standard solution*

Calculate the percentage of citric acid in the portion of *Garcinia indica* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of citric acid from the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*

r_S = peak area of citric acid from the *Standard solution*

C_S = concentration of USP Citric Acid RS in the *Standard solution* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of *Garcinia indica* used to prepare the *Sample solution* in the test for *Content of (–)-Hydroxycitric Acid and Limit of (–)-Hydroxycitric Acid Lactone* (mg)

Acceptance criteria: NMT 2% of citric acid on the dried basis

• **LOSS ON DRYING** (731): Dry 2.0 g of finely powdered *Garcinia indica* at 105° for 3 h: it loses NMT 12.0% of its weight.

• **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): Determined on 1.0 g of finely powdered *Garcinia indica*: NMT 3.0%; and NMT 8.0% if sodium chloride was added as a preservative during collection of the fruits.

• **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10³ cfu/g.

• **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella species* and *Escherichia coli*

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.

• USP REFERENCE STANDARDS (11)

USP Calcium (–)-Hydroxycitrate RS

USP Citric Acid RS

USP Powdered *Garcinia Hydroxycitrate Extract RS*

Powdered *Garcinia indica*

DEFINITION

Powdered *Garcinia indica* is *Garcinia indica* reduced to a fine or very fine powder. It contains NLT 12% of the sum of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone, on the dried basis.

IDENTIFICATION

• **A.** Powdered *Garcinia indica* meets the requirements under *Specific Tests*, *Botanic Characteristics*.

• **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 0.5 mg/mL of garcinol in alcohol

Sample solution: Transfer about 2.0 g of Powdered *Garcinia indica* to a Soxhlet apparatus, add 100 mL of alcohol, and extract for 6 h. Filter and concentrate under vacuum to about 10 mL. [NOTE—Use a thimble of a suitable size such that the volume of alcohol used in the Soxhlet extraction is at least twice the volume of the thimble.]

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL, as 8 mm-bands

Developing solvent system: Toluene, ethyl acetate, and formic acid (4:1:0.5)

Spray reagent: A mixture of 1% vanillin in alcohol and 10% sulfuric acid in alcohol (1:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* <621>). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, spray with *Spray reagent*, heat for 5–10 min at about 105°, and examine under visible light.

Acceptance criteria: The *Sample solution* chromatogram exhibits a main greenish-grey band due to garcinol at an R_f value of approximately 0.6, which corresponds in position and color to the main band in the chromatogram of the *Standard solution*. The *Sample solution* exhibits the following additional bands: two purple bands, two greenish-grey bands, two blue bands, and a purple band at R_f values of approximately 0.31, 0.34, 0.37, 0.47, 0.54, 0.83, and 0.93, respectively. Other bands may be observed for the *Sample solution*.

- **C. HPLC IDENTIFICATION TEST:** The *Sample solution* chromatogram exhibits a peak for hydroxycitric acid at a retention time corresponding to that in the chromatogram of *Standard solution A*, as obtained in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*. The *Sample solution* also exhibits a peak for hydroxycitric acid lactone. The hydroxycitric acid and the hydroxycitric acid lactone peaks are the main peaks in the *Sample solution* chromatogram.

COMPOSITION

• **CONTENT OF (–)-HYDROXYCITRIC ACID AND (–)-HYDROXYCITRIC ACID LACTONE**

Solution A: 30% phosphoric acid in water

Mobile phase: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of water, adjust with *Solution A* to a pH of 2.5, complete with water to 1000 mL, mix, filter, and degas.

Solvent: A mixture of *Solution A* and water (1:9)

Standard solution A: A solution of USP Calcium (–)-Hydroxycitrate RS equivalent to about 4 mg/mL of (–)-hydroxycitric acid in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Standard solution B: 8 mg/mL of USP Powdered *Garcinia Hydroxycitrate Extract RS* in *Solvent*. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 5 g of Powdered *Garcinia indica*, accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of *Solvent*, reflux while stirring on a water bath for 30 min, set aside to settle, and decant the supernatant. Repeat the extraction using four 50-mL portions of water, combine all extracts, cool, filter into a 250-mL volumetric flask, and complete with water to volume. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 215 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 25 ± 1°

Flow rate: 1.0 mL/min

Injection size: 20 µL

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

[NOTE—The relative retention times for the hydroxycitric acid lactone and hydroxycitric acid peaks are about 0.9 and 1.0, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered *Garcinia Hydroxycitrate Extract RS* being used.

Resolution: NLT 1.0 between the hydroxycitric acid lactone and hydroxycitric acid peaks, *Sample solution*

Tailing factor: NMT 2.0 for the hydroxycitric acid peak, *Standard solution A*

Relative standard deviation: NMT 2.0%, determined from the hydroxycitric acid peak for replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 6 h.] Calculate the percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone in the portion of Powdered *Garcinia indica* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of hydroxycitric acid from *Standard solution A*

C_S = concentration of (–)-hydroxycitric acid in *Standard solution A* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Powdered *Garcinia indica* used to prepare the *Sample solution* (mg)

F = conversion factor for each analyte: 2.17 for (–)-hydroxycitric acid lactone, and 1.00 for (–)-hydroxycitric acid

Acceptance criteria: Add the percentages of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone: NLT 12% is found on the dried basis.

IMPURITIES

Inorganic Impurities

• **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** <561>: NMT 0.5%

• **HEAVY METALS, Method III** <231>: NMT 20 ppm

Organic Impurities

• **PROCEDURE: ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** <561>: Meets the requirements

SPECIFIC TESTS

• **BOTANIC CHARACTERISTICS:** Dark brown powder; odor characteristic; taste sour. Under a microscope, it shows cells containing dark brown content; cells containing yellow content, parenchyma cells containing simple and compound starch granules; fragments of epicarp cells containing stomata; and fragments of spiral and annular vessels.

• **LIMIT OF CITRIC ACID**

Solvent and Chromatographic system: Prepare as directed in the test for *Content of (–)-Hydroxycitric Acid and (–)-Hydroxycitric Acid Lactone*.

Standard solution: 0.5 mg/mL of USP Citric Acid RS in *Solvent*. Before injection, pass through a membrane filter of 0.45- μ m or finer pore size, discarding the first few mL of the filtrate.

Analysis

Sample: *Standard solution*

Calculate the percentage of citric acid in the portion of Powdered *Garcinia indica* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of citric acid from the *Sample solution* in the test for *Content of (-)-Hydroxycitric Acid and (-)-Hydroxycitric Acid Lactone*

r_S = peak area of citric acid from the *Standard solution*

C_S = concentration of USP Citric Acid RS in the *Standard solution* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Powdered *Garcinia indica* used to prepare the *Sample solution* in the test for *Content of (-)-Hydroxycitric Acid and (-)-Hydroxycitric Acid Lactone* (mg)

Acceptance criteria: NMT 2% of citric acid on the dried basis

- **LOSS ON DRYING** (731): Dry 2.0 g of Powdered *Garcinia indica* at 105° for 3 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): Determined on 1.0 g of Powdered *Garcinia indica*: NMT 3.0%; and NMT 8.0% if sodium chloride was added as a preservative during collection of the fruits.
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and the bile-tolerant Gram-negative bacteria do not exceed 10³ cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella species* and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11)
USP Calcium (-)-Hydroxycitrate RS
USP Citric Acid RS
USP Powdered *Garcinia Hydroxycitrate Extract* RS

Garlic

DEFINITION

Garlic consists of the fresh or dried compound bulbs of *Allium sativum* L. (Fam. Liliaceae). It contains NLT 0.5% of alliin and NLT 0.2% of γ -glutamyl-(S)-allyl-L-cysteine, calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution A: 0.5 mg/mL of USP L-Methionine RS in a mixture of methanol and water (1:1)
Standard solution B: 0.5 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1)
Sample solution: Cut a freeze-dried garlic bulb into small pieces, transfer 1 g of the cut pieces to an extractor, and extract with two 20-mL portions of a mixture of methanol and water (1:1), combining the extracts.

Concentrate to a small volume (about 5 mL), using a rotary evaporator.

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates).

Application volume: 20 μ L, applied separately as 10-mm bands

Developing solvent system: Butyl alcohol, *n*-propyl alcohol, glacial acetic acid, and water (3:1:1:1)

Spray reagent: 0.2% solution of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (19:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved up about three-fourths of the plate, in a saturated chamber. Remove the plate, and allow the solvent to evaporate. Spray with the *Spray reagent*, heat at 100°–105° for 10 min, and immediately examine the plate.

Acceptance criteria: The chromatogram of the *Sample solution* shows the following orange and pinkish violet zones: a violet zone having an R_f value of about 0.89; a pink zone having an R_f value of about 0.5 and corresponding in color and R_f value to that obtained from the chromatogram of *Standard solution A*; a pinkish zone having an R_f value of about 0.43; a strong orange zone having an R_f value of about 0.38; a pinkish violet zone having an R_f value of about 0.3 and corresponding in color and R_f value to that of the chromatogram of *Standard solution B*; and additional pinkish orange zones situated very close to each other just below the zone attributed to alliin in the chromatogram of *Standard solution B*.

- **B. Sample:** About 10 g of garlic bulbs that have been cut into small pieces

Analysis: Transfer the *Sample* to a suitable flask. Add 10 mL of 1 N sodium hydroxide and 10 mL of water, heat the flask in boiling water for 10 min, cool, and filter. Add a few drops of freshly prepared sodium nitroferrocyanide TS to 2 mL of the filtrate.

Acceptance criteria: The appearance of a red or orange-red color indicates the presence of sulfur-containing compounds in the *Sample*.

- **C. The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the test for Content of Alliin.**
- **D. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Extraction column: 1-cm \times 5-cm solid-phase extraction column; contains styrene-divinylbenzene copolymer packing with a 75- to 150- μ m diameter and a 400- to 600-Å pore size. Condition column before use by washing with 50 mL of methanol and with 50 mL of a mixture of methanol and water (3:7). [NOTE—Do not allow the column to dry.]

Standard solution: 0.2 mg/mL each of USP β -Chlorogenin RS and USP Agigenin RS in methanol

Sample solution: Transfer about 10 g of freshly peeled garlic clove to a 37-mL homogenizing cup, and homogenize with 25 mL of methanol at the highest speed for 1 min. Centrifuge the mixture, and decant the supernatant to a flask. Add 70 mL of water. Transfer to the *Extraction column*, allow to drain, and discard the eluate. Wash the column with 50 mL of a mixture of methanol and water (3:7), allow the solvent mixture to drain, and discard the eluate. Finally, elute the crude saponin fraction on the column with 20 mL of methanol, and collect the eluate. Evaporate the solvent to dryness. Dissolve the residue in 4 mL of a mixture of 8% sulfuric acid and alcohol (1:1), transfer the solution to a screw-capped test tube, and heat on a boiling water bath for 5 h. Cool the test tube, add 20 mL of water, and transfer the solution to a freshly conditioned *Extraction column*, allow to drain, and discard the eluate. Wash the column with 30 mL of a mixture of meth-

anol and water (7:3), and discard the eluate. Finally, elute the column with 50 mL of methanol. Collect the eluate, evaporate it to dryness, and dissolve the residue in 0.5 mL of methanol.

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates).

Application volume: 20 μ L, as 7-mm bands

Developing solvent system: Methylene chloride and methanol (15:2)

Spray reagent: Dissolve 0.5 mL of 4-methoxybenzaldehyde and 0.5 mL of sulfuric acid in sufficient alcohol to make 10 mL.

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved up about three-fourths of the plate, in a saturated chamber. Remove the plate, and allow the solvent to evaporate. Spray the plate with *Spray reagent*, heat the plate at 100°–105° for 5 min, and examine the plate.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits, among several yellowish and grayish green spots, a grayish green spot at an R_f value of about 0.4, corresponding to the grayish green spot due to β -chlorogenin of the *Standard solution*. The chromatogram of the *Sample solution* exhibits no spot at an R_f value of about 0.2, corresponding to agigenin of the *Standard solution*.

COMPOSITION

• CONTENT OF ALLIIN

Allinase inhibitor solution: Dissolve 109 mg of carboxymethoxylamine hemihydrochloride in 100.0 mL of water.

Solution A: Monobasic sodium phosphate 0.045 M in water, adjusted with 0.2 M sodium hydroxide to a pH of 7.1

Buffer: Monobasic sodium phosphate 0.05 M in water, adjusted with 0.2 M sodium hydroxide to a pH of 9.5

Derivatization reagent: Dissolve 140 mg of *o*-phthalaldehyde in 5 mL of methanol, add 100 μ L of *t*-butylthiol, and dilute with *Buffer* to 50 mL. [NOTE—This reagent may occasionally become opaque during preparation. Store at room temperature, and use within 1 week.]

Mobile phase: Acetonitrile, 1,4-dioxane, tetrahydrofuran, and *Solution A* (25: 2.9: 2.2: 69.9)

Standard solution: 0.05 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1)

Sample stock solution: Transfer about 10.0 g of freshly peeled garlic cloves, accurately weighed, to a 110-mL homogenizing cup. Add 70.0 mL of *Allinase inhibitor solution*, and blend at the highest speed for 30 s. Centrifuge, and decant the supernatant into a 100-mL volumetric flask. Mix the remaining solids in the cup with 20 mL of *Allinase inhibitor solution*, centrifuge, and add the supernatant to the volumetric flask. Dilute the contents of the flask with *Allinase inhibitor solution* to volume.

Sample solution: Dilute a portion of the *Sample stock solution* 1 in 10 with a mixture of methanol and water (1:1).

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 337 nm

Column: 4-mm \times 10-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

[NOTE—Alliin exhibits two major peaks representing its diastereomers.]

Relative standard deviation: NMT 2.0% for each of the major peaks, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Using a syringe, transfer 0.1 mL of the *Standard solution* or *Sample solution* to separate septum-capped vials, and add 0.5 mL of the *Derivatization reagent* to each vial. Allow a reaction time of NLT 2 min before injection into the chromatograph. Record the chromatograms, and measure the areas of the alliin diastereomer peaks. Calculate the percentage of alliin in the portion of Garlic taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of alliin from the *Sample solution*

r_S = peak areas of alliin diastereomers from the *Standard solution*

C_S = concentration of USP Alliin RS in the *Standard solution* (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Garlic used to prepare the *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 10

Acceptance criteria: NLT 0.5% on the dried basis

• CONTENT OF γ -GLUTAMYL-(S)-ALLYL-L-CYSTEINE

Solution A: Dissolve 6.80 g of monobasic potassium phosphate in 900 mL of water, and adjust with phosphoric acid to a pH of 2.6. Dilute with water to 1000.0 mL, and mix.

Mobile phase: Methanol and *Solution A* (3:17)

Standard solution: 0.08 mg/mL of USP γ -Glutamyl-(S)-allyl-L-cysteine RS in a mixture of methanol and water (1:1)

Sample solution: Transfer about 10 g of freshly peeled garlic cloves, accurately weighed, to a 110-mL homogenizing cup. Add 80 mL of a mixture of methanol and water (1:1), and homogenize at the highest speed for 1 min. Centrifuge the mixture, and decant the supernatant into a 250-mL volumetric flask. Mix the remaining solids with two 70-mL portions of a mixture of methanol and water (1:1), centrifuge, and transfer the supernatants to the volumetric flask. Dilute the contents of the flask with a mixture of methanol and water (1:1) to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm \times 15-cm; packing L1

Flow rate: 0.8 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0% for the γ -glutamyl-(S)-allyl-L-cysteine peak in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of γ -glutamyl-(S)-allyl-L-cysteine in the portion of Garlic taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response for γ -glutamyl-(S)-allyl-L-cysteine from the *Sample solution*

r_S = peak response for γ -glutamyl-(S)-allyl-L-cysteine from the *Standard solution*

C_S = concentration of USP γ -Glutamyl-(S)-allyl-L-cysteine RS in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Garlic used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.2% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues (561):** Meets the requirements

SPECIFIC TESTS

BOTANICAL CHARACTERISTICS

Macroscopic: Subglobular compound bulbs, 3–5 cm in width, consisting of 8–20 cloves, the whole surrounded by 2–5 layers of white scale leaves attached to a flattened, circular base; cloves ovoid and 3- to 4-sided, summit acute, narrowed into a threadlike portion of fiber base, truncate, each clove covered with a white scale leaf and a pinkish white epidermis, easily separated from the solid portion, consisting of two flaky scale leaves and two yellowish green conduplicate foliage leaves

Microscopic: The protective leaf contains an epidermis enclosing a mesophyll free from chlorophyll. The outer epidermis consists of lignified sclereid cells of thick, pitted walls, elongated, covered with thin cuticle, long fibers up to 500 μm in length and 30 μm in width. The cortical cells are thick-walled, nonlignified, tending to collapse on maturity, isodiametric, and contain purple pigments. The vascular bundles consist of lignified spiral and annular vessels. The storage leaves show an outer epidermis of thin, delicate cells of variable shape, arranged in somewhat irregular rows, 60 μm in length and 30 μm in width. Stomata are present on the outer epidermis only at the extreme tip near the base of the foliage leaves.

The mesophyll consists of swollen storage parenchyma cells filled with fine granular reserve material; scattered in the cortex are 20 laticiferous tubes, 500–1000 μm in length. Two series of vascular bundles consisting of narrow lignified spiral and annular vessels are arranged in the mesophyll.

- **ARTICLES OF BOTANICAL ORIGIN, Water Content (561):** NMT 65.0% for fresh bulbs, and NMT 7.0% for dried bulbs
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash (561):** NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in well-closed containers in a cool, dry place, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS (11)**
 - USP Agigenin RS
 - USP Alliin RS
 - USP β -Chlorogenin RS
 - USP γ -Glutamyl-(S)-allyl-L-cysteine RS
 - USP L-Methionine RS

Powdered Garlic

DEFINITION

Powdered Garlic is produced from Garlic that has been cut, freeze-dried or dried at a temperature not exceeding 65°, and powdered. It contains NLT 0.3% of alliin and NLT 0.1% of γ -glutamyl-(S)-allyl-L-cysteine, calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: 0.5 mg/mL of USP L-Methionine RS in a mixture of methanol and water (1:1)

Standard solution B: 0.5 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1)

Sample solution: Transfer 1 g of the Powdered Garlic to an extractor, and extract with two 20-mL portions of a mixture of methanol and water (1:1), combining the extracts. Concentrate to a small volume (about 5 mL), using a rotary evaporator.

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 20 μL , applied separately as 10-mm bands

Developing solvent system: Butyl alcohol, *n*-propyl alcohol, glacial acetic acid, and water (3:1:1:1)

Spray reagent: 0.2 in 100 solution of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (19:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved up about three-fourths of the plate, in a saturated chamber. Remove the plate, and allow the solvent to evaporate. Spray with *Spray reagent*, heat at 100°–105° for 10 min, and immediately examine the plate.

Acceptance criteria: The chromatogram of the *Sample solution* shows the following orange and pinkish violet zones: a violet zone having an R_f value of about 0.89; a pink zone having an R_f value of about 0.5 and corresponding in color and R_f value to that obtained from the chromatogram of *Standard solution A*; a pinkish zone having an R_f value of about 0.43; a strong orange zone having an R_f value of about 0.38; a pinkish violet zone having an R_f value of about 0.3 and corresponding in color and R_f value to that of the chromatogram of *Standard solution B*; and additional pinkish orange zones situated very close to each other just below the zone attributed to alliin in the chromatogram of *Standard solution B*.

B.

Sample: About 10 g of Powdered Garlic

Analysis: Transfer the *Sample* to a suitable flask. Add 10 mL of 1 N sodium hydroxide and 10 mL of water, heat the flask in boiling water for 10 min, cool, and filter. Add a few drops of freshly prepared sodium nitroferrocyanide TS to 2 mL of the filtrate.

Acceptance criteria: The appearance of a red or orange-red color indicates the presence of sulfur-containing compounds in the *Sample*.

- **C.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Alliin*.

D. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Extraction column: 1-cm \times 5-cm solid-phase extraction column; contains styrene-divinylbenzene copolymer packing with a 75- to 150- μm diameter and a 400- to 600-Å pore size. Condition column before use by washing with 50 mL of methanol and with 50 mL of a mixture of methanol and water (3:7). [NOTE—Do not allow the column to dry.]

Standard solution: 0.2 mg/mL each of USP β -Chlorogenin RS and USP Agigenin RS in methanol

Sample solution: Transfer about 10 g of the Powdered Garlic to a 37-mL homogenizing cup, and homogenize with 25 mL of methanol at the highest speed for 1 min. Centrifuge the mixture, and decant the supernatant to a flask. Add 70 mL of water. Transfer to the *Extraction column*, allow to drain, and discard the eluate. Wash the column with 50 mL of a mixture of methanol and water (3:7), allow the solvent mixture to drain, and discard the eluate. Finally, elute the crude saponin fraction on the column with 20 mL of methanol, and collect the eluate. Evaporate the solvent to dryness. Dissolve the residue in 4 mL of a mixture of 8% sulfuric acid and alcohol (1:1), transfer the solution to a screw-capped test tube, and heat on a boiling water bath for 5 h.

Cool the test tube, add 20 mL of water, and transfer the solution to a freshly conditioned *Extraction column*, allow to drain, and discard the eluate. Wash the column with 30 mL of a mixture of methanol and water (7:3), and discard the eluate. Finally, elute the column with 50 mL of methanol. Collect the eluate, evaporate it to dryness, and dissolve the residue in 0.5 mL of methanol.

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 20 μ L, as 7-mm bands

Developing solvent system: Methylene chloride and methanol (15:2)

Spray reagent: Dissolve 0.5 mL of 4-methoxybenzaldehyde and 0.5 mL of sulfuric acid in sufficient alcohol to make 10 mL.

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved up about three-fourths of the plate, in a saturated chamber. Remove the plate, and allow the solvent to evaporate. Spray the plate with *Spray reagent*, heat the plate at 100°–105° for 5 min, and examine the plate.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits, among several yellowish and grayish green spots, a grayish green spot at an R_f value of about 0.4, corresponding to the grayish green spot due to β -chlorogenin of the *Standard solution*. The chromatogram of the *Sample solution* exhibits no spot at an R_f value of about 0.2, corresponding to agigenin of the *Standard solution*.

COMPOSITION

• CONTENT OF ALLIIN

Alliinase inhibitor solution: Dissolve 109 mg of carboxymethoxylamine hemihydrochloride in 100.0 mL of water.

Solution A: 0.045 M monobasic sodium phosphate in water. Adjust with 0.2 M sodium hydroxide to a pH of 7.1.

Buffer: 0.05 M monobasic sodium phosphate in water. Adjust with 0.2 M sodium hydroxide to a pH of 9.5.

Derivatization reagent: Dissolve 140 mg of *o*-phthalaldehyde in 5 mL of methanol, add 100 μ L of *t*-butylthiol, and dilute with *Buffer* to 50 mL. [NOTE—This reagent may occasionally become opaque during preparation. Store at room temperature, and use within 1 week.]

Mobile phase: Acetonitrile, 1,4-dioxane, tetrahydrofuran, and *Solution A* (25: 2.9: 2.2: 69.9)

Standard solution: 0.05 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1)

Sample stock solution: Transfer about 1.0 g of Powdered Garlic, accurately weighed, to a flask. Add 30.0 mL of *Alliinase inhibitor solution*, and shake vigorously until the powder is fully dispersed. Centrifuge to obtain a clear solution.

Sample solution: Transfer 5.0 mL of the *Sample stock solution* to a 10-mL volumetric flask, and dilute with *Alliinase inhibitor solution* to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 337 nm

Column: 4-mm \times 10-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

[NOTE—Alliin exhibits two major peaks representing its diastereomers.]

Relative standard deviation: NMT 2.0% for each of the major peaks, in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Using a syringe, transfer 0.1 mL of the *Standard solution* or the *Sample solution* to separate septum-capped vials, add 0.5 mL of the *Derivatization reagent* to each vial, and mix. Allow a reaction time of NLT 2 min before injection into the chromatograph. Record the chromatograms, and measure the areas of the alliin diastereomer peaks.

Calculate the percentage of alliin in the portion of Powdered Garlic taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak area of alliin from the *Sample solution*

r_S = sum of the peak areas of alliin diastereomers from the *Standard solution*

C_S = concentration of USP Alliin RS in the *Standard solution* (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Powdered Garlic used to prepare the *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 2

Acceptance criteria: NLT 0.3% on the dried basis

• CONTENT OF γ -GLUTAMYL-(S)-ALLYL-L-CYSTEINE

Solution A: Dissolve 6.80 g of monobasic potassium phosphate in 900 mL of water, and adjust with phosphoric acid to a pH of 2.6. Dilute with water to 1000.0 mL, and mix.

Mobile phase: Methanol and *Solution A* (3:17)

Standard solution: 0.08 mg/mL of USP γ -Glutamyl-(S)-allyl-L-cysteine RS in a mixture of methanol and water (1:1)

Sample solution: Transfer about 1.0 g of Powdered Garlic, accurately weighed, to a 50-mL volumetric flask. Add 30 mL of methanol and water (1:1), and shake vigorously until the powder is fully dispersed. Dilute the contents of the flask with a mixture of methanol and water (1:1) to volume. Centrifuge to obtain a clear solution.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm \times 15-cm; packing L1

Flow rate: 0.8 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0% for the γ -glutamyl-(S)-allyl-L-cysteine peak in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of γ -glutamyl-(S)-allyl-L-cysteine in the portion of Powdered Garlic taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response for γ -glutamyl-(S)-allyl-L-cysteine from the *Sample solution*

r_S = peak response for γ -glutamyl-(S)-allyl-L-cysteine from the *Standard solution*

C_S = concentration of USP γ -Glutamyl-(S)-allyl-L-cysteine RS in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered Garlic used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.1% on the dried basis

CONTAMINANTS

- **HEAVY METALS, Method I (231):** NMT 10 ppm
- **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues (561):** Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Under a microscope, Powdered Garlic shows the following diagnostic characteristics: numerous fragments of parenchyma with large cells containing crystals of calcium oxalate and small triangular or quadrangular intercellular spaces at the corners; spiral vessels accompanied by subquadratic cells; elongated epidermal cells with thick, pitted walls.
- **ABSENCE OF STARCH:** A water slurry of Powdered Garlic shows no blue color when iodine TS is added.
- **LOSS ON DRYING (731):** Dry 1 g of it at 105° for 2 h: it loses NMT 7.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash (561):** NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in well-closed containers in a cool, dry place, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.
- **USP REFERENCE STANDARDS (11)**
 - USP Agigenin RS
 - USP Alliin RS
 - USP β -Chlorogenic RS
 - USP γ -Glutamyl-(S)-allyl-L-cysteine RS
 - USP L-Methionine RS

Powdered Garlic Extract

DEFINITION

Powdered Garlic Extract is prepared from fresh Garlic bulbs by extraction with alcohol. The ratio of the starting crude plant material to Powdered Extract is 9.5:1–13.5:1. It contains NLT 4.0% of alliin ($C_6H_{11}NO_3S$). It may contain added Powdered Garlic or other suitable substances.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
 - Standard solution A:** 0.5 mg/mL of USP L-Methionine RS in a mixture of methanol and water (1:1)
 - Standard solution B:** 0.5 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1)
 - Sample solution:** Transfer a quantity of Powdered Extract, equivalent to about 5 mg of alliin, to a suitable container. Add 40 mL of a mixture of methanol and water (1:1), and shake until the powder is fully dispersed. Centrifuge, and decant the supernatant into a round-bottomed flask. Concentrate to a small volume (about 5 mL) using a rotary evaporator.
 - Adsorbent:** 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates).
 - Application volume:** 20 μ L, applied separately as 10-mm bands
 - Developing solvent system:** Butyl alcohol, *n*-propyl alcohol, glacial acetic acid, and water (3:1:1:1)
 - Spray reagent:** 0.2% solution of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (19:1)
 - Analysis**
 - Samples:** *Standard solutions* and *Sample solution*
- Develop the chromatograms until the solvent front has moved up about three-fourths of the plate, in a saturated chamber. Remove the plate, and allow the sol-

vent to evaporate. Spray with the *Spray reagent*, heat at 100°–105° for 10 min, and immediately examine the plate.

Acceptance criteria: The chromatogram of the *Sample solution* shows the following orange and pinkish violet zones: a violet zone having an R_f value of about 0.89; a pink zone having an R_f value of about 0.5 and corresponding in color and R_f value to that of the chromatogram of *Standard solution A*; a pinkish zone having an R_f value of about 0.43; a strong orange zone having an R_f value of about 0.38; a pinkish violet zone having an R_f value of about 0.3 and corresponding in color and R_f value to that of the chromatogram of *Standard solution B*; and additional pinkish orange zones situated very close to each other just below the zone attributed to alliin in the chromatogram of *Standard solution B*.

- **B.** The retention time of the alliin peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Alliin*.

COMPOSITION

• CONTENT OF ALLIIN

Allinase inhibitor solution: 1.09 mg/mL of carboxymethoxylamine hemihydrochloride

Solution A: Monobasic sodium phosphate 0.045 M in water adjusted with 0.2 M sodium hydroxide to a pH of 7.1

Buffer: Monobasic sodium phosphate 0.05 M in water adjusted with 0.2 M sodium hydroxide to a pH of 9.5

Derivatization reagent: Dissolve 140 mg of *o*-phthalaldehyde in 5 mL of methanol. Add 100 μ L of *t*-butylthiol, dilute with *Buffer* to 50 mL, and mix. [NOTE—This reagent may occasionally become opaque during preparation. Store at room temperature, and use within 1 week.]

Mobile phase: Acetonitrile, 1,4-dioxane, tetrahydrofuran, and *Solution A* (25: 2.9: 2.2: 69.9)

Standard solution: 0.05 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1)

Sample solution: Transfer about 0.10 g of Powdered Extract, accurately weighed, to a 50-mL volumetric flask. Add 30 mL of *Allinase inhibitor solution*, and shake until the Powdered Extract is fully dispersed. Dilute with *Allinase inhibitor solution* to volume. Centrifuge, transfer 5 mL of the clear supernatant to a 10-mL volumetric flask, and dilute with *Allinase inhibitor solution* to volume.

Chromatographic system

(See *Chromatography (621)*, *System Suitability*.)

Mode: LC

Detector: UV 337 nm

Column: 4-mm \times 10-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

[NOTE—Alliin exhibits two major peaks representing its diastereomers.]

Relative standard deviation: NMT 2.0% for each of the major peaks

Analysis

Samples: *Standard solution* and *Sample solution*

Using a volumetric syringe, transfer 0.1 mL of the *Sample solution* or the *Standard solution* to separate septum-capped vials, and add 0.5 mL of the *Derivatization reagent* to each vial. Allow a reaction time of NLT 2 min before injection into the chromatograph. Record the chromatograms, and measure the areas of the alliin diastereomer peaks.

Calculate the percentage of alliin in the portion of Powdered Extract taken:

$$\text{Result} = (r_u/r_s) \times C_s \times (V/W) \times 100$$

- r_U = peak area of alliin from the *Sample solution*
 r_S = peak areas of alliin diastereomers from the *Standard solution*
 C_S = concentration of USP Alliin RS in the *Standard solution* (mg/mL)
 V = volume of the *Sample solution* (mL)
 W = weight of Powdered Garlic Extract used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 4.0% on the dried basis

CONTAMINANTS

- **HEAVY METALS, Method I (231):** NMT 10 ppm
- **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **ALLINASE ACTIVITY**
 Allinase inhibitor solution, Solution A, Buffer, Derivatization reagent, Mobile phase, Standard solution, Chromatographic system, and Analysis: Proceed as directed in the test for *Content of Alliin*.
Sample solution: Incubate 200 mg of Powdered Extract with 20 mL of water at room temperature for 5 min. Immediately after incubation, add 80.0 mL of *Allinase inhibitor solution*, mix, and centrifuge.
Acceptance criteria: The area of the alliin peak of the *Sample solution* is NMT 1% of the area of the alliin peak of the *Standard solution*.
- **ALCOHOL DETERMINATION, Method II (611):** NMT 0.5%
- **ARTICLES OF BOTANICAL ORIGIN, Water Content (561):** NMT 5.0%
- **OTHER REQUIREMENTS:** Meets the requirements under *Botanical Extracts (565)*, *Packaging and Storage* and *Pesticide Residues*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, in a cool place, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of alliin, the extracting solvent or solvent mixture used for preparation, and the ratio of the starting crude plant material to Powdered Extract. It meets the requirements under *Botanical Extracts (565)*, *Labeling*.
- **USP REFERENCE STANDARDS (11)**
 USP Alliin RS
 USP L-Methionine RS

Garlic Fluidextract

DEFINITION

Garlic Fluidextract is prepared as follows. Soak 1000 g of Garlic, whole or sliced, in a volume of a mixture of water and alcohol (between 80:20 and 50:50) sufficient to cover the cloves. Store in a suitable container for a length of time sufficient to extract the constituents, avoiding any contamination, and then filter. Concentrate the filtrate, if necessary, at the lowest possible temperature, and add sufficient water or alcohol to make the product measure 1000 mL. [NOTE—Complete extraction may require 30 days.]

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Extraction column: Use a solid-phase extraction column that contains benzenesulfonylpropyl bonded to silica gel in the hydrogen form having a sorbent mass-to-column volume ratio of 1 g per 6 mL, or equivalent. Condition the column before use by washing with 10 mL of methanol and 10 mL of water. [NOTE—Do not allow the column to dry.]

Standard solution: 0.5 mg/mL of USP S-Allyl-L-cysteine RS in a mixture of methanol and water (1:1)

Sample solution: Mix 1 mL of Fluidextract and 5 mL of water, and transfer to the *Extraction column*. Allow to drain, and discard the eluate. Wash the column with 10 mL of water and 10 mL of methanol, discarding the eluates. Elute the amino acid fraction with 3 mL of ammonium hydroxide solution in methanol (7 in 100), and collect the eluate.

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 5 μ L

Developing solvent system: Ethyl acetate, methanol, acetone, glacial acetic acid, and water (10:4:3:1:3)

Spray reagent: Iodoplatinate TS

Analysis

Samples: *Standard solution* and *Sample solution*
 Develop the chromatograms until the solvent front has moved up about three-fourths of the plate, in a saturated chamber. Remove the plate, and allow the solvent to evaporate. Spray with the *Spray reagent*, and examine the plate.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits, among several yellow spots on the purple plate, a yellow spot at an R_f value of about 0.4 corresponding to that of the yellow spot obtained in the chromatogram of the *Standard solution* (presence of S-allyl-L-cysteine).

COMPOSITION

• CONTENT OF S-ALLYL-L-CYSTEINE

Mobile phase: Transfer 15.8 g of sodium citrate dihydrate to 250 mL of water, and carefully add 10.5 mL of hydrochloric acid. Using a pH meter, adjust with 6 N sodium hydroxide to a pH of 4.0. Dilute with water to 1000 mL, and mix.

Derivatizing reagent: Dissolve 0.8 g of o-phthalaldehyde in 2 mL of 2-mercaptoethanol. Add to a solution containing 24.70 g of boric acid and 22.35 g of potassium hydroxide in 1000 mL of water, and mix.

Reactivating solution: 0.2 N sodium hydroxide. Prepare by dissolving 0.8 g of sodium hydroxide in 100 mL of water.

Standard solution: 0.01 mg/mL of USP S-Allyl-L-cysteine RS in water

Sample solution: Transfer about 2.0 g of Fluidextract, accurately weighed, to a 100-mL volumetric flask, dilute with trichloroacetic acid solution (5 in 100) to volume, and mix. Centrifuge for 5 min, and filter the supernatant.

Chromatographic system

(See *Chromatography (621)*, *System Suitability*.)

Mode: LC

Detector: Fluorometric detector; excitation wavelength of 340 nm and emission wavelength of 455 nm

Column: 4.6-mm \times 12-cm; packing L17

Column temperature: 40°

Injection size: 10 μ L

[NOTE—The *Mobile phase* and the *Reactivating solution* are pumped separately, each at the rate of 0.4 mL/min, by pumps connected to the opposing arms of a tee. The outlet of the tee is connected to the injector and the chromatographic column. The outlet of the column is attached to a tee, the opposing arm of which is attached to a tube from which the *Derivatizing reagent* is constantly pumped through the sys-

tem at a rate of 0.6 mL/min. The outlet of the tee is connected to a 0.5-mm \times 2.0-m postcolumn polytetrafluoroethylene reaction coil maintained at 40°. The outlet of the reaction coil is connected to the detector. The system is programmed to deliver the *Mobile phase* for 10 min, the *Reactivating solution* for the next 6 min, and the *Mobile phase* for the 24 min remaining before the next injection.]

System suitability

Sample: *Standard solution*

Suitability requirements

Capacity factor (k'): 2.5–4.5

Tailing factor: NMT 2.0 for the S-allyl-L-cysteine peak

Relative standard deviation: NMT 2.0% for the S-allyl-L-cysteine peak in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of S-allyl-L-cysteine ($C_6H_{11}SN$) in the portion of Fluidextract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak height of S-allyl-L-cysteine from the *Sample solution*

r_S = peak height of S-allyl-L-cysteine from the *Standard solution*

C_S = concentration of the USP S-Allyl-L-cysteine RS in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Fluidextract used to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.05% on the dried basis

CONTAMINANTS

- **HEAVY METALS, Method II <231>:** NMT 10 ppm
- **MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic bacterial count does not exceed 10^3 cfu/mL, and the total combined molds and yeasts count does not exceed 10^2 cfu/mL.
- **ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **RESIDUE ON EVAPORATION:** Proceed as directed under *Botanical Extracts* <565>; NLT 20% of the Fluidextract portion taken remains as residue.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash <561>:** NMT 3.0%
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash <561>:** NMT 0.2%
- **PH <791>:** 4.5–6.5
- **OTHER REQUIREMENTS:** Meets the requirements under *Botanical Extracts* <565>, *General Pharmacopeial Requirements*, sections for *Packaging and Storage*, *Labeling*, *Pesticide Residues*, and *Alcohol Content* for Fluidextracts

ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS <11>:** USP S-Allyl-L-cysteine RS

Garlic Delayed-Release Tablets

DEFINITION

Garlic Delayed-Release Tablets are prepared from Powdered Garlic or Powdered Garlic Extract and contain NLT 90.0% and NMT 140.0% of the labeled amount of alliin ($C_6H_{11}NO_3S$) and NLT 90.0% and NMT 140.0% of the labeled amount of potential allicin ($C_6H_{10}OS_2$).

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: 0.5 mg/mL of USP L-Methionine RS

Standard solution B: 0.5 mg/mL of USP Alliin RS, in a mixture of methanol and water (1:1)

Sample solution: Transfer an amount of pulverized Tablets, equivalent to 30 mg of alliin, to a 100-mL volumetric flask. Add 70 mL of a mixture of methanol and water (1:1), shake, and centrifuge. Concentrate to a small volume (about 5 mL) using a rotary evaporator.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Application volume: 20 μ L, applied separately as 10-mm bands

Developing solvent system: Butyl alcohol, *n*-propyl alcohol, glacial acetic acid, and water (3:1:1:1)

Spray reagent: 2 mg/mL of ninhydrin, in a mixture of butyl alcohol and 2 N acetic acid (19:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Proceed as directed in the chapter. Spray with the *Spray reagent*, heat at 100°–105° for 10 min, and immediately examine the plate.

Acceptance criteria: The chromatogram of the *Sample solution* shows the following orange and pinkish violet zones: a violet zone having an R_f value of 0.89; a pink zone having an R_f value of 0.5 and corresponding in color and R_f value to that of the chromatogram of *Standard solution A*; a pinkish zone having an R_f value of 0.43; a strong orange zone having an R_f value of 0.38; a pinkish violet zone having an R_f value of 0.3 and corresponding in color and R_f value to that of the chromatogram of *Standard solution B*; and additional pinkish orange zones situated very close to each other, just below the zone attributed to alliin in the chromatogram of *Standard solution B*.

• B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of Alliin*.

Acceptance criteria: The *Sample solution* exhibits a peak for alliin corresponding to one of the diastereoisomer pairs of peaks in the *Standard solution*.

STRENGTH

• CONTENT OF ALLIIN

Alliinase inhibitor solution: Dissolve 109 mg of carboxymethoxylamine hemihydrochloride in 100.0 mL of water.

Solution A: Dissolve 1.24 g of monobasic sodium phosphate in 100 mL of water, adjust with 0.2 M sodium hydroxide to a pH of 7.1, and dilute with water to 200.0 mL.

Buffer: Dissolve 1.38 g of monobasic sodium phosphate in 100 mL of water, adjust with 0.2 M sodium hydroxide to a pH of 9.5, and dilute with water to 200.0 mL.

Derivatization reagent: Dissolve 140 mg of *o*-phthalaldehyde in 5 mL of methanol, add 100 μ L of *t*-butylthiol, and dilute with *Buffer* to 50 mL. [NOTE—This reagent may occasionally become opaque during preparation. Store at room temperature, and use within 1 week.]

Mobile phase: Acetonitrile, 1,4-dioxane, tetrahydrofuran, and *Solution A* (25: 2.9: 2.2: 69.9)

Standard solution: 0.05 mg/mL of USP Alliin RS in a mixture of methanol and water (1:1). Use a syringe to transfer 0.1 mL of this solution to a septum-capped vial, and add 0.5 mL of the *Derivatization reagent*. Allow a reaction time of NLT 2 min before injection into the chromatograph.

Sample solution: Pulverize a counted number of Tablets, equivalent to 50 mg of alliin, with a mortar and pestle. Transfer a quantity of powder equivalent to 5 mg of alliin to a 100-mL volumetric flask. Add 70 mL of *Alliinase inhibitor solution*, and shake for 1 min. Dilute

with *Alliinase inhibitor solution* to volume. Use a volumetric syringe to transfer 0.1 mL of this solution to a septum-capped vial, and add 0.5 mL of the *Derivatization reagent*. Allow a reaction time of NLT 2 min before injection into the chromatograph.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 337 nm

Column: 4-mm × 10-cm; packing L1

Flow rate: 1 mL/min

[NOTE—Alliin exhibits two major peaks, representing its diastereomers.]

Injection size: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0% for each of the major peaks

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—Record the chromatograms, and measure the areas of the responses of the alliin diastereomer peaks.]

Calculate the percentage of alliin in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for alliin from the *Sample solution*

r_S = sum of the peak area for alliin diastereomers from the *Standard solution*

C_S = concentration of USP Alliin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of alliin in the *Sample solution* (µg/mL)

Acceptance criteria: 90%–140.0%

• CONTENT OF POTENTIAL ALLICIN

Alliinase inhibitor solution: Dissolve 109 mg of carboxymethoxylamine hemihydrochloride in 100.0 mL of water.

Crude alliinase solution: Homogenize 5 g of raw garlic cloves with 25 mL of water. Filter, and extract three times with 50 mL of *tert*-butyl methyl ether. Discard the organic phase, and remove the residual solvent from the aqueous phase by rotary evaporation in vacuum for 5 min. Filter, and store frozen in small vials. [NOTE—This solution is stable for 6 months when stored as directed. Thaw at room temperature just before use.]

Mobile phase: Methanol and water (3:2)

Standard stock solution: 50 µg/mL of USP Alliin RS

Standard solution: Transfer 1.0 mL of the *Standard stock solution* to a 5-mL volumetric flask containing 100 µL of *Crude alliinase solution*, and allow to stand for 5 min at room temperature. Dilute with water to volume, and pass through a filter having a 0.45-µm or finer pore size.

Sample solution: Transfer an equivalent to 5 mg of potential alliin, from finely powdered Tablets (NLT 20), to a 200-mL volumetric flask, and add 25 mL of water. Incubate at room temperature for exactly 30 min. Stop the enzymatic reaction by diluting with *Alliinase inhibitor solution* to volume. Centrifuge a portion of this solution, transfer 1.0 mL of the supernatant to a 5-mL volumetric flask, and dilute with water to volume.

Blank solution: 100 µL of *Crude alliinase solution* diluted with water to 1 mL

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 240 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution*, *Sample solution*, and *Blank solution*

[NOTE—The alliin peak is identified by comparing the chromatograms of the *Blank solution* and the *Standard solution*.]

Suitability requirements

Resolution: NLT 2.0 between the alliin peak and the preceding peak at a relative retention time of 0.80 (allyl methyl thiosulfonates), *Sample solution*

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank solution*

Calculate the percentage of potential alliin in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of alliin, corrected by the response of the *Blank solution*, from the *Sample solution*

r_S = peak area of alliin, corrected by the response of the *Blank solution*, from the *Standard solution*

C_S = concentration of USP Alliin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of potential alliin in the *Sample solution* (µg/mL)

M_{r1} = molecular weight of alliin, 162.26

M_{r2} = twice the molecular weight of alliin, 354.42

Acceptance criteria: 90.0%–140.0%

PERFORMANCE TESTS

- **ALLICIN RELEASE:** Proceed as directed in *Dissolution* <711> for *Method A* in *Apparatus 1* and *Apparatus 2*, *Delayed-Release Dosage Forms*. Place a number of Tablets, equivalent to 5 mg of potential alliin, in each vessel.

Apparatus 2: 100 rpm

Time: 60 min for the *Buffer stage*

Crude alliinase solution, Mobile phase, Blank solution, and Chromatographic system: Proceed as directed in the test for *Content of Potential Alliin*.

Standard stock solution: 50 µg/mL of USP Alliin RS

Standard solution: Transfer 1.0 mL of the *Standard stock solution* to a 5-mL volumetric flask containing 100 µL of *Crude alliinase solution*, and allow to stand for 5 min at room temperature. Dilute with water to volume, and pass through a membrane filter having a 0.45-µm or finer pore size.

Sample solution: Transfer 1.0 mL of the solution under test to a test tube containing 50 µL of 0.21 M carboxymethoxylamine hemihydrochloride solution.

[NOTE—The solution must be transferred immediately upon removal from the dissolution vessel to inhibit the alliinase enzyme.]

Injection size: 100 µL

Analysis

[NOTE—Do not perform the alliin determination in the *Acid stage*.]

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of potential alliin released in the *Buffer stage*:

$$\text{Result} = (r_U/r_S) \times (C_S \times D \times V/L) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of alliin from the *Sample solution*

r_S = peak area of alliin from the *Standard solution*

C_S = concentration of USP Alliin RS in the *Standard solution* (µg/mL)

D = dilution factor for the *Sample solution*, 1.050
(1 mL of the *Sample solution* + 50 μ L of 0.21 M carboxymethoxylamine hemihydrochloride solution)

V = volume of final medium, 1000 mL

L = labeled amount of potential allicin (μ g/Tablet)

M_{r1} = molecular weight of allicin, 162.26

M_{r2} = twice the molecular weight of alliin, 354.42

Tolerances: It meets the requirements of *Acceptance Table 4* in *Dissolution* (711). [NOTE— Q is the percentage of the labeled amount of potential allicin released only in the *Buffer stage*.]

- **WEIGHT VARIATION (2091):** Meet the requirements

SPECIFIC TESTS

• ALLIINASE ACTIVITY

Alliinase inhibitor solution, Solution A, Buffer, Derivatization reagent, Mobile phase, Standard solution, and Chromatographic system: Proceed as directed in the test for *Content of Alliin*.

Sample solution: Transfer an equivalent to 5 mg of alliin, from finely powdered Tablets (NLT 20), to a 100-mL volumetric flask, and add 25 mL of water. Incubate at room temperature for exactly 5 min. Stop the enzymatic reaction by diluting with *Alliinase inhibitor solution* to volume. Centrifuge a portion of this solution, and use a volumetric syringe to transfer 0.1 mL of the supernatant to a septum-capped vial. Add 0.5 mL of the *Derivatization reagent*, and allow a reaction time of NLT 2 min before injection into the chromatograph.

Analysis

Samples: *Standard solution* and *Sample solution*
Proceed as directed in the test for *Content of Alliin*.

Acceptance criteria: The area of the alliin peak from the *Sample solution* is NMT 1% of the area of the alliin peak from the *Standard solution*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. Label it to indicate the amount of total alliin, in μ g/Tablet, and the amount of potential allicin, in μ g/Tablet.
- **USP REFERENCE STANDARDS (11)**
USP Alliin RS
USP L-Methionine RS

Ginger

DEFINITION

Ginger is the dried rhizome of *Zingiber officinale* Roscoe (Fam. Zingiberaceae), scraped, partially scraped, or unscraped. It is known in commerce as unbleached ginger.

IDENTIFICATION

- **A.**
Analysis: Pulverize 5 g of Ginger. To 1 g of the pulverized Ginger add 5 mL of dilute acetic acid, prepared by diluting 1 part of glacial acetic acid with 1 part of water, and shake for 15 min. Filter, and add a few drops of ammonium oxalate TS to the filtrate.
Acceptance criteria: NMT a slight turbidity is produced.
- **B.**
Sample: 50 mg of the residue obtained in the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives*
Analysis: Dissolve the *Sample* in 25 mL of water, and extract this solution with two 15-mL portions of ether. Combine the ether extracts, and evaporate in a porcelain dish. To the residue add 5 mL of sulfuric acid solu-

tion (7.5 in 10.0) and 5 mg of vanillin. Allow to stand for 15 min, and add an equal volume of water.

Acceptance criteria: The solution turns azure blue.

• C. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: Proceed as directed for the *Sample solution*, except to use 0.2 g of USP Powdered Ginger RS.

Standard solution B: Use the *System suitability solution*, prepared as directed in the test for *Content of Gingerols and Gingerdiones*.

Sample solution: Pulverize 5 g of Ginger. Transfer 0.2 g of pulverized Ginger to a test tube, add 5 mL of methanol, shake for 30 min, and centrifuge. Apply the supernatant to the plate.

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 20 μ L for the *Sample solution* and *Standard solution A*; 40 μ L for *Standard solution B*

Developing solvent system: Ether and hexanes (7:3)

Spray reagent: 10% sulfuric acid in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Proceed as directed in the chapter. Examine the plate under UV light at 254 nm. Spray the plate with *Spray reagent*, heat at 100°–105° for 10 min, and examine under daylight.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a spot due to gingerols that occurs at an R_f value of 0.2. A spot of shogaols may occur at an R_f value of 0.4, corresponding to those shown in the chromatogram of *Standard solution B*. [NOTE—The chromatograms of the *Sample solution* and *Standard solution A* may exhibit other spots in the upper region and at the origin of the plate.]

COMPOSITION

• CONTENT OF GINGEROLS AND GINGERDIONES

Solution A: Acetonitrile, dilute phosphoric acid (1 in 1000), and methanol (55:44:1)

Solution B: Acetonitrile

Mobile phase: Use *Solution A* for NLT seven times the retention time of capsaicin.

Column washing: After each chromatographic run, wash the column, using *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	0	100
12	0	100
14	100	0
29	100	0

Standard solution: 0.1 mg/mL of USP Capsaicin RS in methanol

System suitability solution: Reconstitute the content of 1 vial of USP Ginger Constituent Mixture RS in 1 mL of the *Standard solution*.

Sample solution: Use the filtrate retained from the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives*.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 282 nm

Column: 4.6-mm \times 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 25 μ L

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for 6-gingerol, capsaicin, and 6-shogaol are about 0.8, 1.0, and 1.9, respectively, *System suitability solution*.]

Suitability requirements

Resolution: NLT 3.0 between the 6-gingerol and capsaicin peaks and NLT 10.0 between the capsaicin and 6-shogaol peaks, *System suitability solution*

Tailing factors: NMT 2.0 for the 6-gingerol, capsaicin, and 6-shogaol peaks, *System suitability solution*

Relative standard deviation: NMT 2.5%, *Standard solution*

Analysis

Samples: *Standard solution*, *Sample solution*, and *System suitability solution*

Calculate the sum of the peak responses due to gingerols and gingerdiones occurring at about the following retention times, relative to 1.0 for capsaicin: 0.8 for 6-gingerol, 1.5 for 8-gingerol A, 2.2 for 8-gingerol B, 2.5 for 6-gingerdiol, 2.6 for 6-gingerdione, 3.4 for 10-gingerol, and 5.2 for 8-gingerdione.

Calculate the percentage of gingerols and gingerdiones in the sample taken:

$$\text{Result} = (r_T/r_S) \times (C_S/W) \times 10$$

r_T = sum of the peak responses for gingerols and gingerdiones from the *Sample solution*

r_S = peak response of capsaicin from the *Standard solution*

C_S = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)

W = weight of Ginger used in the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives* (g)

Acceptance criteria: NLT 0.8%

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues (561):** Meets the requirements
- **MICROBIAL ENUMERATION TESTS (2021):** The total bacterial count does not exceed 10^5 cfu/g; the total combined molds and yeasts count does not exceed 10^3 cfu/g; the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Ginger occurs in horizontal, laterally flattened, sympodially branching pieces. Whole rhizomes are 5–15 cm long, 1.5–6 cm wide, and up to 2 cm thick, sometimes split longitudinally, pale yellowish buff or light brown externally, longitudinally striated, somewhat fibrous; branches flattish, obovate, short, about 2 cm long, each ending with a depressed stem scar; fracture, short with projecting fibers, or sometimes resinous; internally yellowish brown, showing a yellow endodermis separating the narrow cortex from the wide stele, and numerous yellowish points, secretion cells and numerous bigger greyish points, vascular bundles, scattered on the whole surface. The unscrapped rhizome shows in addition an outer layer of dark brown cork. Morphological characteristics of different varieties and forms of Ginger from different geographical areas are listed in *Table 1* of the general information chapter *Supplemental Information for Articles of Botanical Origin* (2030).

Histology: The scrapped rhizome in transverse section shows a cortex composed of multiple layers of parenchyma cells rich in simple, large, flattened, ovoid or sack-shaped starch granules, 5–15 μm wide and 30–60 μm long having an eccentric hilum, some

showing faint transverse striations. The cortex also shows numerous oleoresin cells with a yellow or yellowish-brown content and scattered collateral vascular bundles; a single layer of endodermal cells free from starch; a wide central stele composed of parenchyma cells rich in starch and oleoresin cells similar to those of the cortex, and containing scattered collateral vascular bundles, some enclosed in a sheath of septate nonlignified fibers with wide lumen. In addition to the above, the unscrapped rhizome shows an outer zone of dark brown cork cells.

• LIMIT OF SHOGAOLS

Analysis: From the chromatograms obtained in the test for *Content of Gingerols and Gingerdiones*, calculate the sum of the peak responses due to shogaols, occurring at the following retention times, relative to 1.0 for capsaicin: 1.9 for 6-shogaol, 4.2 for 8-shogaol, and 5.8 for 10-shogaol.

Calculate the percentage of shogaols in the portion taken:

$$\text{Result} = (r_T/r_S) \times (C_S/W) \times 10$$

r_T = sum of the peak responses of shogaols from the *Sample solution*

r_S = peak response of capsaicin from the *Standard solution*

C_S = concentration of USP Capsaicin RS in the *Standard solution*, prepared as directed in the test for *Content of Gingerols and Gingerdiones* (mg/mL)

W = weight of Ginger used in the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives* (g)

Acceptance criteria: NMT 0.18%

- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash (561):** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method 2 (561):** **Analysis:** Collect the filtrate in a 100-mL volumetric flask, and dilute with alcohol to volume. Evaporate 50 mL of the filtrate at a temperature not exceeding 90°. [NOTE—Save the residue for use in *Identification test B* and the remaining volume of the filtrate for the tests for *Limit of Shogaols* and *Content of Gingerols and Gingerdiones*.] **Acceptance criteria:** NLT 4.5% residue
- **ARTICLES OF BOTANICAL ORIGIN, Content of Starch, Method 1 (561):** NLT 42%, *Method 1a* of the *General Procedures* being used
- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter (561):** NMT 1.0%
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN, Volatile Oil Content (561):** NLT 1.8 mL/100 g
- **ARTICLES OF BOTANICAL ORIGIN, Water-Soluble Ash (561):** NLT 1.9%
- **ARTICLES OF BOTANICAL ORIGIN, Water-Soluble Extractives, Method 2 (561):** NLT 10.0%
- **WATER DETERMINATION, Method 1a (921):** NMT 10%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool area.
- **LABELING:** The label states the Latin binominal and, following the official name, the part of the plant contained in the article. This article is exempted from the requirements of the *General Notices* with respect to the pregnancy and lactation statement (section 10.40.50, *Labeling Botanical-Containing Products*).

• **USP REFERENCE STANDARDS** (11)

USP Capsaicin RS
USP Ginger Constituent Mixture RS
USP Powdered Ginger RS

Powdered Ginger

DEFINITION

Powdered Ginger is Ginger reduced to a fine or a very fine powder.

IDENTIFICATION

- **A.**
Analysis: To 1 g of the Powdered Ginger add 5 mL of dilute acetic acid, prepared by diluting 1 part of glacial acetic acid with 1 part of water, and shake for 15 min. Filter, and add a few drops of ammonium oxalate TS to the filtrate.

Acceptance criteria: NMT a slight turbidity is produced.

- **B.**
Sample: 50 mg of the residue obtained in the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives*
Analysis: Dissolve the *Sample* in 25 mL of water, and extract this solution with two 15-mL portions of ether. Combine the ether extracts, and evaporate in a porcelain dish. To the residue add 5 mL of sulfuric acid solution (7.5 in 10.0) and 5 mg of vanillin. Allow to stand for 15 min, and add an equal volume of water.

Acceptance criteria: The solution turns azure blue.

• **C. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: Proceed as directed for the *Sample solution*, except to use 0.2 g of USP Powdered Ginger RS.

Standard solution B: Use the *System suitability solution*, prepared as directed in the test for *Content of Gingerols and Gingerdiones*.

Sample solution: Transfer 0.2 g of Powdered Ginger to a test tube, add 5 mL of methanol, shake for 30 min, and centrifuge. Apply the supernatant to the plate.

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 20 µL for the *Sample solution* and *Standard solution A*; 40 µL for *Standard solution B*

Developing solvent system: Ether and hexanes (7:3)

Spray reagent: 10% sulfuric acid in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Proceed as directed in the chapter. Examine the plate under UV light at 254 nm. Spray the plate with *Spray reagent*, heat at 100°–105° for 10 min, and examine under daylight.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a spot due to gingerols that occurs at an R_f value of 0.2. A spot of shogaols may occur at an R_f value of 0.4, corresponding to those shown in the chromatogram of *Standard solution B*. [NOTE—The chromatograms of the *Sample solution* and *Standard solution A* may exhibit other spots in the upper region and at the origin of the plate.]

COMPOSITION

• **CONTENT OF GINGEROLS AND GINGERDIONES**

Solution A: Acetonitrile, dilute phosphoric acid (1 in 1000), and methanol (55:44:1)

Solution B: Acetonitrile

Mobile phase: Use *Solution A* for NLT seven times the retention time of capsaicin.

Column washing: After each chromatographic run, wash the column, using *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	0	100
12	0	100
14	100	0
29	100	0

Standard solution: 0.1 mg/mL of USP Capsaicin RS in methanol

System suitability solution: Reconstitute the content of 1 vial of USP Ginger Constituent Mixture RS in 1 mL of the *Standard solution*.

Sample solution: Use the filtrate retained from the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives*.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 282 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 25 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for 6-gingerol, capsaicin, and 6-shogaol are about 0.8, 1.0, and 1.9, respectively, *System suitability solution*.]

Suitability requirements

Resolution: NLT 3.0 between the 6-gingerol and capsaicin peaks and NLT 10.0 between the capsaicin and 6-shogaol peaks, *System suitability solution*

Tailing factors: NMT 2.0 for the 6-gingerol, capsaicin, and 6-shogaol peaks, *System suitability solution*

Relative standard deviation: NMT 2.5%, *Standard solution*

Analysis

Samples: *Standard solution*, *Sample solution*, and *System suitability solution*

Calculate the sum of the peak responses due to gingerols and gingerdiones, occurring at about the following retention times, relative to 1.0 for capsaicin: 0.8 for 6-gingerol, 1.5 for 8-gingerol A, 2.2 for 8-gingerol B, 2.5 for 6-gingerdiol, 2.6 for 6-gingerdione, 3.4 for 10-gingerol, and 5.2 for 8-gingerdione.

Calculate the percentage of gingerols and gingerdiones in the sample taken:

$$\text{Result} = (r_T/r_S) \times (C_S/W) \times 10$$

r_T = sum of the peak responses of gingerols and gingerdiones from the *Sample solution*

r_S = peak response of capsaicin from the *Standard solution*

C_S = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)

W = weight of Powdered Ginger used in the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives* (g)

Acceptance criteria: NLT 0.8%

CONTAMINANTS

• **HEAVY METALS** (231): NMT 20 ppm

• **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues** (561): Meets the requirements

• **MICROBIAL ENUMERATION TESTS** (2021): The total bacterial count does not exceed 10^5 cfu/g; the total combined molds and yeasts count does not exceed 10^3 cfu/g; the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.

• **ABSENCE OF MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Under a microscope, Powdered Ginger reveals mainly starch granules and parenchyma cells containing them; simple, large, flattened, ovoid or sack-shaped starch granules, 5–15 μm wide and 30–60 μm long having an eccentric hilum, some showing faint transverse striations; parenchyma cells containing yellow-brown to dark brown resinous substances; groups of large, thin-walled nonlignified septate fibers with wide lumen; portions of septate fibers with attached vessels; large vessels with annular, spiral, or reticulate thickening and often accompanied by parenchyma cells containing brown content; oleoresin in fragments or droplets, staining with iodine TS and potassium iodide TS; and, rarely, fragments of brown cork tissue, usually seen in surface view. Sclerenchymatous cells, trichomes, and calcium oxalate are absent.

- **LIMIT OF SHOGAOLS**

Analysis: From the chromatograms obtained in the test for *Content of Gingerols and Gingerdiones*, calculate the sum of the peak responses due to shogaols, occurring at the following retention times, relative to 1.0 for capsaicin: 1.9 for 6-shogaol, 4.2 for 8-shogaol, and 5.8 for 10-shogaol.

Calculate the percentage of shogaols in the portion of Powdered Ginger taken:

$$\text{Result} = (r_T/r_S) \times (C_S/W) \times 10$$

r_T = sum of the peak responses of shogaols from the *Sample solution*

r_S = peak response of capsaicin from the *Standard solution*

C_S = concentration of USP Capsaicin RS in the *Standard solution*, prepared as directed in the test for *Content of Gingerols and Gingerdiones* (mg/mL)

W = weight of Powdered Ginger used in the test for *Articles of Botanical Origin, Alcohol-Soluble Extractives* (g)

Acceptance criteria: NMT 0.18%

- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash <561>:** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN, Alcohol-Soluble Extractives, Method 2 <561>**
Analysis: Collect the filtrate in a 100-mL volumetric flask, and dilute with alcohol to volume. Evaporate 50 mL of the filtrate at a temperature not exceeding 90°.
Acceptance criteria: NLT 4.5% residue. [NOTE—Save the residue for use in *Identification test B*, and the remaining volume of the filtrate for the tests for *Limit of Shogaols* and *Content of Gingerols and Gingerdiones*.]
- **ARTICLES OF BOTANICAL ORIGIN, Content of Starch, Method 1 <561>:** NLT 42%, *Method 1a* of the *General Procedures* being used
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash <561>:** NMT 8.0%
- **ARTICLES OF BOTANICAL ORIGIN, Volatile Oil Content <561>:** NLT 1.8 mL/100 g
- **ARTICLES OF BOTANICAL ORIGIN, Water-Soluble Ash <561>:** NLT 1.9%
- **ARTICLES OF BOTANICAL ORIGIN, Water-Soluble Extractives, Method 2 <561>:** NLT 10.0%
- **WATER DETERMINATION, Method 1a <921>:** NMT 10%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool area.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived. This article is exempted from the requirements of the *General Notices*

with respect to the pregnancy and lactation statement (section 10.40.50, *Labeling Botanical-Containing Products*).

- **USP REFERENCE STANDARDS <11>**

USP Capsaicin RS

USP Ginger Constituent Mixture RS

USP Powdered Ginger RS

Ginger Capsules

DEFINITION

Ginger Capsules are prepared from Powdered Ginger and contain NLT 90.0% and NMT 110.0% of the labeled amount of gingerols, gingerdiones, and shogaols, and NLT 90.0% of the labeled amount of volatile oil.

IDENTIFICATION

- **A.**

Analysis: Pulverize an amount of the contents of Capsules equivalent to 5 g of ginger. To an amount equivalent to 1 g of ginger add 5 mL of dilute acetic acid, prepared by diluting 1 part of glacial acetic acid with 1 part of water, and shake for 15 min. Filter, and add a few drops of ammonium oxalate TS to the filtrate.

Acceptance criteria: NMT a slight turbidity is produced.

- **B.**

Sample (see *Articles of Botanical Origin <561>, Alcohol-Soluble Extractives, Method 2*): Collect the filtrate in a 100-mL volumetric flask, and dilute with alcohol to volume. Evaporate 50 mL of the filtrate at a temperature not exceeding 90°. Use 50 mg of the residue for the test.

Analysis: Dissolve the *Sample* in 25 mL of water, and extract with two 15-mL portions of ether. Combine the ether extracts, and evaporate in a porcelain dish. To the residue add 5 mL of sulfuric acid solution (7.5 in 10.0) and 5 mg of vanillin. Allow to stand for 15 min, and add an equal volume of water.

Acceptance criteria: The solution turns azure blue.

- **C. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: Proceed as directed for the *Sample solution*, except to use 0.2 g of USP Powdered Ginger RS.

Standard solution B: Use the *System suitability solution*, prepared as directed in the test for *Content of Gingerols, Gingerdiones, and Shogaols*.

Sample solution: Pulverize an amount of the contents of Capsules equivalent to 5 g of ginger. Transfer an amount equivalent to 0.2 g of ginger to a test tube, add 5 mL of methanol, shake for 30 min, and centrifuge. Apply the supernatant to the plate.

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 20 μL for *Standard solution A* and the *Sample solution*; 40 μL for *Standard solution B*

Developing solvent system: Ether and hexanes (7:3)

Spray reagent: 10% sulfuric acid in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Proceed as directed in the chapter. Examine the plate under UV light at 254 nm. Spray the plate with *Spray reagent*, heat at 100°–105° for 10 min, and examine under daylight.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a spot due to gingerols that occurs at an R_f value of 0.2, and a spot of shogaols may occur at an R_f value of 0.4, corresponding to those shown in the chromatogram of *Standard solution B*. [NOTE—The chromatograms of *Standard solution A* and the *Sample solution* may exhibit other spots in the upper region and at the origin of the plate.]

STRENGTH**• CONTENT OF GINGEROLS, GINGERDIONES, AND SHOGAOLS**

Solution A: Acetonitrile, dilute phosphoric acid (1 in 1000), and methanol (55:44:1)

Solution B: Acetonitrile

Mobile phase: Use *Solution A* for NLT seven times the retention time of capsaicin.

Column washing: After each chromatographic run, wash the column, using *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	0	100
12	0	100
14	100	0
29	100	0

Standard solution: 0.1 mg/mL of USP Capsaicin RS in methanol

System suitability solution: Reconstitute the content of 1 vial of USP Ginger Constituent Mixture RS in 1 mL of the *Standard solution*.

Sample solution: Mix and finely powder the contents of NLT 20 Capsules, and transfer an amount equivalent to 2.0 g of powdered ginger to a glass-stoppered conical flask. Add 50 mL of alcohol, insert a stopper into the flask, and macerate for 24 h, shaking frequently during the first 8 h, and then allowing to stand for 16 h. Filter, and use the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 282 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 25 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for 6-gingerol, capsaicin, and 6-shogaol are about 0.8, 1.0, and 1.9, respectively, *System suitability solution*.]

Suitability requirements

Resolution: NLT 3.0 between the 6-gingerol and capsaicin peaks and NLT 10.0 between the capsaicin and 6-shogaol peaks, *System suitability solution*

Tailing factors: NMT 2.0 for the 6-gingerol, capsaicin, and 6-shogaol peaks, *System suitability solution*

Relative standard deviation: NMT 2.5% for the capsaicin peak for replicate injections, *Standard solution*

Analysis

Samples: *Standard solution*, *Sample solution*, and *System suitability solution*

Calculate the sum of the peak responses due to gingerols and gingerdiones occurring at about the following retention times relative to 1.0 for capsaicin: 0.8 for 6-gingerol, 1.5 for 8-gingerol A, 2.2 for 8-gingerol B, 2.5 for 6-gingerdiol, 2.6 for 6-gingerdione, 3.4 for 10-gingerol, and 5.2 for 8-gingerdione.

Calculate the sum of the peak responses due to shogaols, occurring at about the following retention times, relative to 1.0 for capsaicin: 1.9 for 6-shogaol, 4.2 for 8-shogaol, and 5.8 for 10-shogaol.

Calculate the percentage of the labeled amount of gingerols, gingerdiones, and shogaols in the portion of Capsules taken:

$$\text{Result} = (r_T/r_S) \times C_S \times (V/W_U) \times (A_{WC}/L) \times 100$$

r_T = sum of the peak responses for gingerols, gingerdiones, and shogaols from the *Sample solution*

r_S = peak response of capsaicin from the *Standard solution*

C_S = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)

V = final volume of the *Sample solution* (mL)

W_U = weight of the portion of Capsules taken (mg)

A_{WC} = average weight of the Capsule content (mg)

L = labeled amount of gingerols, gingerdiones, and shogaols (mg/Capsule)

Acceptance criteria: 90.0%–110.0% of the labeled amount of gingerols, gingerdiones, and shogaols. Calculate the amount (G_0), in mg, of 6-gingerol in each Capsule taken:

$$G_0 = (r_U/r_S) \times (C_S/W) \times V \times A$$

r_U = peak response of 6-gingerol from the *Sample solution*

r_S = peak response of capsaicin from the *Standard solution*

C_S = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)

W = weight of powdered ginger used in the preparation of the *Sample solution* (g)

V = final volume of the *Sample solution* (mL)

A = average Capsule fill weight (g)

• ARTICLES OF BOTANICAL ORIGIN, Volatile Oil Determination <561>

Sample: Finely powder a quantity of Capsules, equivalent to 100 g of powdered ginger.

Acceptance criteria: NLT 1.4 mL/100 g (NLT 90.0% of the labeled amount of volatile oil)

PERFORMANCE TESTS**• DISINTEGRATION AND DISSOLUTION <2040>:** Meet the requirements for *Dissolution*

Medium: 0.1 N hydrochloric acid; 500 mL

Apparatus 2: 75 rpm

Time: 60 min

[NOTE—In each dissolution vessel, place a number of Capsules equivalent to 20 mg of the labeled amounts of gingerols, gingerdiones, and shogaols.]

Solution A, Solution B, Mobile phase, Column washing, System suitability solution,

Chromatographic system, and System suitability:

Proceed as directed in the test for *Content of Gingerols, Gingerdiones, and Shogaols*.

Standard stock solution: Use the *Standard solution* prepared in the test for *Content of Gingerols, Gingerdiones, and Shogaols*.

Standard solution: 0.025 mg/mL of USP Capsaicin RS from *Standard stock solution* in *Medium*

Sample solution: Transfer an aliquot of solution from each dissolution vial to a suitable vial. Allow to stand for 5 min so that the powder settles into the suspension, or centrifuge to obtain a clear supernatant. Pass through a membrane filter of 0.45-µm or finer pore size.

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—Allow the *Sample solution* to elute for NLT three times the retention time of capsaicin.]

Calculate the quantity, G , in mg, of 6-gingerol dissolved from each Capsule taken:

$$G = (r_U/r_S) \times (C/N) \times V$$

r_U = peak response of 6-gingerol from the *Sample solution*

r_S = peak response of capsaicin from the *Standard solution*

C = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)

N = number of Capsules in each vessel
 V = volume of *Medium*; 500 mL
 Calculate the percentage of the relative amount of 6-gingerol dissolved:

$$\text{Result} = (G/G_0) \times 100$$

G_0 = content of 6-gingerol in each Capsule, as determined in the test for *Content of Gingerols, Gingerdiones, and Shogaols* (mg)

Tolerances: NLT 60% of the content of 6-gingerol ($C_{17}H_{26}O_4$) is dissolved.

- **WEIGHT VARIATION (2091):** Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of gingerols, gingerdiones, and shogaols, in mg per Capsule, and the content of volatile oil, in μL per Capsule. This article is exempted from the requirements of the *General Notices* with respect to the pregnancy and lactation statement (section 10.40.50, *Labeling Botanical-Containing Products*).
- **USP REFERENCE STANDARDS (11)**
 USP Capsaicin RS
 USP Ginger Constituent Mixture RS
 USP Powdered Ginger RS

Ginger Tincture

DEFINITION

Ginger Tincture is prepared as follows.

Ginger	200 g
A mixture of Alcohol and Water (7:3), a sufficient quantity to make	1000 mL

Prepare the Tincture as directed for *Botanical Extracts* (565), *Tinctures, Maceration Process*. It contains NLT 0.10% of gingerols.

IDENTIFICATION

- **THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution A: Transfer 0.2 g of USP Powdered Ginger RS to a test tube, add 5 mL of methanol, shake for 30 min, and centrifuge. Apply the supernatant to the plate.

Standard solution B: Use the *System suitability solution*, prepared as directed in the test for *Content of Gingerols*.

Sample solution: Tincture

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 20 μL for the *Sample solution* and *Standard solution A*; 40 μL for *Standard solution B*

Developing solvent system: Ethyl ether and hexane (7:3)

Spray reagent: 10% sulfuric acid in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber and dry. Examine the plate under UV light at 254 nm. Spray the plate with *Spray reagent*, heat at 100°–105° for 10 min, and examine under daylight.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a spot due to gingerols that occurs at

an R_f value of 0.2, and a spot of shogaols may occur at an R_f value of 0.4, corresponding to the chromatogram of *Standard solution B*. [NOTE—The chromatograms of the *Sample solution* and *Standard solution A* may exhibit other spots in the upper region and at the origin of the plate.]

STRENGTH

- **CONTENT OF GINGEROLS**

Solution A: Acetonitrile, dilute phosphoric acid (1 in 1000), and methanol (55:44:1)

Solution B: Acetonitrile

Mobile phase: Use *Solution A* for NLT seven times the retention time of capsaicin.

Column washing: After each chromatographic run, wash the column, using *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	0	100
12	0	100
14	100	0
29	100	0

Standard solution: 0.1 mg/mL of USP Capsaicin RS in methanol

Sample solution: Tincture

System suitability solution: Reconstitute the content of 1 vial of USP Ginger Constituent Mixture RS in 1 mL of *Standard solution*.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 282 nm

Column: 4.6-mm \times 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 25 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for 6-gingerol, capsaicin, and 6-shogaol are about 0.8, 1.0, and 1.9, respectively, *System suitability solution*.]

Suitability requirements

Resolution: NLT 3.0 between the 6-gingerol and capsaicin peaks and NLT 10.0 between the capsaicin and 6-shogaol peaks, *System suitability solution*

Tailing factors: NMT 2.0 for the 6-gingerol, capsaicin, and 6-shogaol peaks, *System suitability solution*

Relative standard deviation: NMT 2.5%, *Standard solution*

Analysis

Samples: *Standard solution*, *Sample solution*, and *System suitability solution*

Calculate the percentage of gingerols in the portion of Tincture taken:

$$\text{Result} = (r_U/r_S) \times C_S \times 0.1$$

r_U = sum of the peak responses of gingerols from the *Sample solution*

r_S = peak response of capsaicin from the *Standard solution*

C_S = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)

Acceptance criteria: NLT 0.10%

OTHER COMPONENTS

- **ALCOHOL DETERMINATION, Method I (611):** NLT 90.0% and NMT 110.0% of the labeled amount of C_2H_5OH

CONTAMINANTS

- **ARSENIC**, *Method II* (211): NMT 1 ppm
- **HEAVY METALS**, *Method III* (231): NMT 10 µg/g
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts count does not exceed 10³ cfu/g.

SPECIFIC TESTS**LIMIT OF 6-SHOGAOL**

Analysis: Using the chromatograms from the test for *Content of Gingerols*, calculate the percentage of 6-shogaol in the portion of Tincture taken:

$$\text{Result} = (r_U/r_S) \times C_S \times 0.1$$

- r_U = peak response of 6-shogaol from the *Sample solution*
 r_S = peak response of capsaicin from the *Standard solution*
 C_S = concentration of USP Capsaicin RS in the *Standard solution* (mg/mL)

Acceptance criteria: NMT 0.034%

- **LIMIT OF NONVOLATILE RESIDUE:** Evaporate a 10-mL portion in a tared platinum or porcelain dish, and dry at 105° for 6 h: the weight of the residue is 80–120 mg.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 0.5%
- **SPECIFIC GRAVITY** (841): 0.90–0.95
- **OTHER REQUIREMENTS:** Meets the requirements in *Botanical Extracts* (565), *Packaging*, and *Storage*

ADDITIONAL REQUIREMENTS

- **LABELING:** Label it to indicate that it is for manufacturing purposes only, in addition to the information specified in *Botanical Extracts* (565), *Tinctures*, *Labeling*. This article is exempted from the requirements of the *General Notices* with respect to the pregnancy and lactation statement (section 10.40.50. *Labeling Botanical-Containing Products*).
- **USP REFERENCE STANDARDS** (11)
 USP Capsaicin RS
 USP Ginger Constituent Mixture RS
 USP Powdered Ginger RS

Ginkgo

DEFINITION

Ginkgo consists of the dried leaf of *Ginkgo biloba* L. (Fam. Ginkgoaceae). It contains NLT 0.5% of flavonoids, calculated as flavonol glycosides, with a mean molecular mass of 756.7; and NLT 0.1% of terpene lactones, calculated as the sum of bilobalide (C₁₅H₁₈O₈), ginkgolide A (C₂₀H₂₄O₉), ginkgolide B (C₂₀H₂₄O₁₀), and ginkgolide C (C₂₀H₂₄O₁₁), both on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Test for flavonoids

Standard solution: A solution of 0.6 mg/mL of USP Rutin RS, 0.2 mg/mL of USP Chlorogenic Acid RS, and 0.2 mg/mL of USP Quercetin RS in methanol

Sample solution: Transfer 1.0 g of finely powdered Ginkgo to a 50-mL round-bottom flask fitted with a reflux condenser. Add 10 mL of methanol, reflux in a water bath for 10 min, allow to cool to room temperature, and filter. [NOTE—Reserve some of the *Sample solution* for use in the *Test for terpene lactones*.]

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL

Developing solvent system: Ethyl acetate, water, anhydrous formic acid, and glacial acetic acid (100:26:11:11)

Spray reagent 1: 5 mg/mL of 2-aminoethyl diphenylborinate in methanol

Spray reagent 2: 50 mg/mL of polyethylene glycol 400 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Before development of the chromatograms, saturate the chamber for 20 min with *Developing solvent system*. Record temperature and humidity in the laboratory. If the relative humidity exceeds 50%, condition the plate to about 35% relative humidity using a suitable device. Apply the samples separately as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)), and allow the bands to dry. Develop the plate over a path of 6 cm, remove the plate from the chromatographic chamber, and dry in a circulating air oven at 105° for 5 min. Immediately spray the hot plate with *Spray reagent 1*, then with *Spray reagent 2*, dry, and examine under long-wavelength UV light.

Acceptance criteria: The *Standard solution* shows in its lower part with increasing R_f values a yellowish-brown fluorescent zone due to rutin (R_f 0.28), a light blue fluorescent zone due to chlorogenic acid (R_f 0.36), and a yellow fluorescent zone due to quercetin (R_f 0.92). The *Sample solution* shows a yellowish-brown fluorescent zone, a light blue fluorescent zone, and a yellowish-brown fluorescent zone at R_f similar to those of rutin, chlorogenic acid, and quercetin, respectively, in the *Standard solution*. Additional yellowish to yellowish-green zones due to flavonoids detected in the *Sample solution* chromatogram include one zone below the rutin zone, two zones between the rutin and chlorogenic acid zones, and two zones above the chlorogenic acid zone. Other zones may be seen in the *Sample solution* chromatogram.

Test for terpene lactones

Standard solution: Dissolve an amount of USP Ginkgo Terpene Lactones RS in methanol to obtain a solution containing in each mL about 1.0, 0.9, 0.6, 0.7, and 0.2 mg of bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, and ginkgolide J, respectively.

Sample solution: Use the *Sample solution* prepared in the *Test for flavonoids*.

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 5 µL

Developing solvent system: Toluene, ethyl acetate, acetone, and methanol (20: 10: 10: 1.2)

Spray reagent: Acetic anhydride

Analysis

Samples: *Standard solution* and *Sample solution*

Immerse a suitable thin-layer chromatographic plate coated with *Adsorbent* for 2 s in an 8 g/200 mL solution of sodium acetate in methanol. Allow the excess liquid to drip from the plate, dry in a forced-air oven at 70° for 30 min, and cool in a desiccator. Apply the samples separately as bands to the impregnated plate (see *Chromatography* (621)), and allow the spots to air-dry. Before development of the chromatograms, saturate the chamber for 20 min with *Developing solvent system*. Record temperature and humidity in the laboratory. If the relative humidity exceeds 50%, condition the plate to about 35% relative humidity, using a suitable device. Develop the plate over a path of 6 cm, remove the plate from the chromatographic chamber, and dry in cold air. Spray the plate with *Spray reagent*, heat at 180° for 10 min, cool, and examine under short-wavelength UV light.

Acceptance criteria: The *Standard solution* chromatogram shows five distinct quenching zones correspond-

ing to the different ginkgo terpene lactones: ginkgolide C, ginkgolide J, ginkgolide B, ginkgolide A, and bilobalide at R_f values of about 0.13, 0.18, 0.32, 0.38, and 0.45, respectively. The *Sample solution* chromatogram shows a strong quenching zone at the application position, a broad quenching zone near the solvent front, and five distinct quenching zones corresponding to different ginkgo terpene lactones at R_f similar to those detected in the *Standard solution* chromatogram. [NOTE— R_f values may differ from one plate to another due to the impregnation step.]

COMPOSITION

• CONTENT OF FLAVONOL GLYCOSIDES

Extraction solvent: Alcohol, hydrochloric acid, and water (25:4:10)

Mobile phase: Methanol, water, and phosphoric acid (100:100:1)

Standard solution A: 0.02 mg/mL of USP Quercetin RS in methanol

Standard solution B: 0.02 mg/mL of USP Kaempferol RS in methanol

Standard solution C: 0.005 mg/mL of USP Isorhamnetin RS in methanol

Sample solution: Transfer about 1.0 g of Ginkgo, finely powdered, to a 250-mL flask fitted with a reflux condenser. Add 78 mL of *Extraction solvent*, and reflux on a hot water bath for 135 min. [NOTE—The solution will turn deep red. The color of the solution is not a definitive indication of reaction completeness.] Allow to cool to room temperature. Decant to a 100-mL volumetric flask. Add 20 mL of methanol to the 250-mL flask, and sonicate for 30 min. Filter, collect the filtrate in the 100-mL volumetric flask, wash the residue on the filter with methanol, collect the washing in the same 100-mL volumetric flask, dilute with methanol to volume, and mix.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 370 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*

[NOTE—The relative retention times for quercetin, kaempferol, and isorhamnetin are about 1.0, 1.8, and 2.0, respectively; *Standard solution A*, *Standard solution B*, and *Standard solution C*.]

Suitability requirements

Relative standard deviation: NMT 2.0% determined from the quercetin peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Calculate the percentage of each flavonol glycoside in the portion of Ginkgo taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times F \times 10$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of the relevant analyte from *Standard solution A*, *Standard solution B*, or *Standard solution C*

C_S = concentration of the relevant analyte in *Standard solution A*, *Standard solution B*, or *Standard solution C* (mg/mL)

W = weight of Ginkgo taken to prepare the *Sample solution* (g)

F = mean molecular mass factor to convert each analyte into flavonol glycoside with a mean molecular mass of 756.7: 2.504 for quercetin, 2.437 for isorhamnetin, and 2.588 for kaempferol

Calculate the total percentage of flavonol glycosides by adding the individual percentages calculated.

Acceptance criteria: NLT 0.5% of flavonoids, as flavonol glycosides, with a mean molecular mass of 756.7, on the dried basis

• CONTENT OF TERPENE LACTONES

Solvent: Methanol and water (9:1)

Buffer solution: Dissolve 1.19 g of dibasic sodium phosphate and 8.25 g of monobasic potassium phosphate in 1000 mL of water, and adjust to a pH of 5.8.

Diluent: Methanol and water (1:1)

Solution A: Water

Solution B: Methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
23	52	48
28	52	48
30	25	75
35	10	90
40	75	25
50	75	25

Standard solutions: Using the labeled content of the individual terpene lactones, prepare five solutions of the USP Ginkgo Terpene Lactones RS in *Diluent* within the range of 5–500 µg/mL for each of the relevant terpene lactones. Use sonication to dissolve the analytes if necessary. Pass through a filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 2.5 g of Ginkgo, accurately weighed, to a 30-mL glass centrifuge tube with a screw cap and PTFE gasket. Add 10.0 mL of *Solvent*, seal the tube, and mix well on a vortex mixer. Heat in a water bath at 90° for 30 min. Mix the hot suspension on a vortex mixer, and repeat the heating at 90° for 30 min. Cool, centrifuge, transfer the supernatant to a flask, and return the residue to the glass tube. Repeat the extraction two more times, each time using 10.0 mL of *Solvent*. Combine the extracts, allow them to cool to room temperature, and evaporate to dryness under vacuum in a water bath maintained at 50°. Add 10 mL of *Buffer solution* to the residue, and sonicate for 5 min. Quantitatively transfer the solution to a glass chromatographic tube filled with chromatographic siliceous earth capable of holding 20 mL of aqueous phase.¹ Rinse the beaker with two 5-mL portions of *Buffer solution*, and transfer the washings to the column. [NOTE—Do not exceed 20 mL of total aqueous phase or the holding capacity of the chromatographic tube.]

Allow the *Buffer solution* to be absorbed into the column. After 15 min, elute the column with 100 mL of ethyl acetate, collect the ethyl acetate solution, and evaporate to dryness under vacuum in a water bath maintained at 50°. Dissolve the residue in 10.0 mL of *Diluent*.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

¹ Suitable commercially available material is Extrelut® NT 20 from E Merck Science.

Mode: LC

Detector: Evaporative light-scattering detector.

[NOTE—The parameters of the detector are adjusted to achieve the best signal-to-noise ratio, according to manufacturer recommendations.]

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 25 ± 1°

Flow rate: 1 mL/min

Injection volume: 15 µL

System suitability

Samples: *Standard solutions*

Suitability requirements

Chromatogram similarity: The chromatograms from the *Standard solutions* are similar to the reference chromatogram provided with the lot of USP Ginkgo Terpene Lactones RS being used.

Relative standard deviation: NMT 2.0% determined from the bilobalide peak in repeated injections

Correlation coefficient: NLT 0.995 for the regression line as determined in *Analysis*

Analysis

Samples: *Standard solutions* and *Sample solution*

Record the chromatograms, and identify the peaks of the relevant analytes in the chromatograms of the *Standard solutions* by comparison with the reference chromatogram of the USP Ginkgo Terpene Lactones RS lot being used. Measure the areas of the analyte peaks. Plot the logarithms of the relevant peak responses versus the logarithms of concentrations, in mg/mL, of each analyte from the *Standard solutions*, and determine the regression line using a least-squares analysis.

From the graphs, determine the concentration, *C*, in mg/mL, of the relevant analyte in the *Sample solution*.

Separately calculate the percentages of bilobalide (C₁₅H₁₈O₈), ginkgolide A (C₂₀H₂₄O₉), ginkgolide B (C₂₀H₂₄O₁₀), and ginkgolide C (C₂₀H₂₄O₁₁) in the portion of Ginkgo taken:

$$\text{Result} = (C/W) \times 1000$$

C = concentration of the relevant analyte in the *Sample solution* (mg/mL)

W = weight of Ginkgo taken to prepare the *Sample solution* (mg)

Calculate the total percentage of terpene lactones in the portion of Ginkgo taken by adding the percentages calculated for each analyte.

Acceptance criteria: NLT 0.1% of terpene lactones, calculated as the sum of bilobalide, ginkgolide A, ginkgolide B, and ginkgolide C, on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis (561):** Meets the requirements
- **HEAVY METALS, Method III (231):** NMT 20 µg/g
- **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10³ cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

BOTANIC CHARACTERISTICS

Macroscopic: Dried whole, folded, or fragmented leaves, with or without attached petiole, vary from khaki green to greenish brown in color, are often more brown at the apical edge, and darker on the adaxial surface. Lamina are broadly obcordate (fan-shaped), 2–12 cm in width and 2–9.5 cm in length from petiole to apical margin; mostly 1.5–2 times wider than long. The base margins are entire, concave; apical margin sinuate, usually truncate or centrally cleft, and rarely

multiply cleft. The surface is glabrous, with wrinkled appearance due to prominent dichotomous venation appearing parallel and extending from the lamina base to the apical margin. Petioles, similar color to leaf, are channeled on the adaxial surface, and 2–8 cm in length.

Histology

Transverse section of lamina: A thin but marked cuticle occurs over a single layer of epidermal cells on both surfaces. Stomata are present on the lower surface only, with guard cells sunken with respect to adjacent epidermal cells. Palisade elements, elongated, at right angles to the surface and often irregular in appearance, occur just below the upper epidermis. Vascular bundles occur at intervals along the width of the blade, with adjacent cluster crystals of calcium oxalate. Cells of the mesophyll are smaller than the palisade cells, elongated, parallel to the leaf surface, and separated by large intercellular spaces.

Powdered lamina and petiole: Under the microscope, transverse fragments of the leaf display a smooth cuticle, present on both leaf surfaces and staining pinkish orange with sudan III TS. In surface view, cells of the upper epidermis are elongated and wavy-walled, with abundant yellow droplets 2–12 µm in diameter visible in mature and old leaves but not in young leaves. Cells of the lower epidermis are similar in shape but have straighter walls and are interrupted by anisocytic stomata. Numerous lignified elements derived from the lamina and petiole are present, including xylem vessels with annular thickening, tracheids, and vessels with bordered pits. The extent of lignification, particularly in the petiole, increases with age of leaf. Calcium oxalate crystals are numerous, present scattered or associated with vessels, ranging in size from 5–50 µm in young leaves and 15–100 µm in mature leaves. Under crossed polaroids, numerous smaller prism- or tear-shaped shiny features of indeterminate nature may be present. Very occasional, highly elongated, uniseriate, covering trichomes with no obvious cross walls and smooth or warty surfaces may be seen. Mature leaves may show the presence of very rare, polygonal to circular starch granules approximately 20 µm in diameter, with a central hilum and exhibiting a marked Maltese cross under crossed polaroids.

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter (561):** NMT 3.0% of stems and NMT 2.0% of other foreign organic matter
- **LOSS ON DRYING (731)**
Sample: 1.0 g of finely powdered Ginkgo
Analysis: Dry the *Sample* at 105° for 2 h.
Acceptance criteria: NMT 11.0%
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561)**
Sample: 1.0 g of finely powdered Ginkgo
Acceptance criteria: NMT 11.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS (11)**
USP Chlorogenic Acid RS
USP Ginkgo Terpene Lactones RS
USP Isorhamnetin RS
USP Kaempferol RS
USP Quercetin RS
USP Rutin RS

Powdered Ginkgo Extract

DEFINITION

Powdered Ginkgo Extract is prepared from dried and comminuted leaves of Ginkgo extracted with an acetone–water mixture or other suitable solvents. The ratio of the crude plant material to Powdered Extract is between 35:1 and 67:1. It contains NLT 22.0% and NMT 27.0% of flavonoids, calculated as flavonol glycosides, with a mean molecular mass of 756.7, on the dried basis. It contains NLT 5.4% and NMT 12.0% of terpene lactones, consisting of between 2.6% and 5.8% of bilobalide ($C_{15}H_{18}O_8$) and between 2.8% and 6.2% of the sum of ginkgolide A ($C_{20}H_{24}O_9$), ginkgolide B ($C_{20}H_{24}O_{10}$), and ginkgolide C ($C_{20}H_{24}O_{11}$), on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Test for flavanoids

Standard solution: A solution of 0.6 mg/mL of USP Rutin RS, and 0.2 mg/mL each of USP Chlorogenic Acid RS and USP Quercetin RS in methanol

Sample solution: 5 mg/mL of Powdered Extract in a mixture of methanol and water (4:1)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 μ m (HPTLC plates)

Application volume: 5 μ L

Developing solvent system: Ethyl acetate, water, anhydrous formic acid, and glacial acetic acid (100:26:11:11)

Spray reagent 1: 5 mg/mL of 2-aminoethyl diphenylborinate in methanol

Spray reagent 2: 50 mg/mL of polyethylene glycol 400 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*
Before development of the chromatograms, saturate the chamber for 20 min with *Developing solvent system*. Record temperature and humidity in the laboratory. If the relative humidity exceeds 50%, condition the plate to about 35% relative humidity using a suitable device. Apply the samples separately as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)), and allow the bands to dry. Develop the plate over a path of 6 cm, remove the plate from the chromatographic chamber, and dry in a circulating air oven at 105° for 5 min. Immediately spray the hot plate with *Spray reagent 1*, then with *Spray reagent 2*, dry, and examine under long-wavelength UV light.

Acceptance criteria: The *Standard solution* shows in its lower part with increasing R_f values a yellowish-brown fluorescent zone due to rutin (R_f 0.28), a light blue fluorescent zone due to chlorogenic acid (R_f 0.36), and a yellow fluorescent zone due to quercetin (R_f 0.92). The *Sample solution* shows a yellowish-brown fluorescent zone, a light blue fluorescent zone, and a yellowish-brown fluorescent zone at R_f similar to those of rutin, chlorogenic acid, and quercetin, respectively, in the *Standard solution*. Additional yellowish to yellowish-green zones due to flavonoids detected in the *Sample solution* chromatogram include one zone below the rutin zone, two zones between the rutin and chlorogenic acid zones, and two zones above the chlorogenic acid zone. Other zones may be seen in the *Sample solution* chromatogram.

- **B. HPLC:** In the test for *Content of Flavonol Glycosides*, the retention times of the peaks for quercetin, isorhamnetin, and kaempferol of the *Sample solution* correspond to those of the *Standard solution*. In the chromatogram of the *Sample solution*, the ratio of the kaempferol peak to the quercetin peak is NLT 0.7, and the peak for isorhamnetin is NLT 0.1 times the size of the quercetin peak.

COMPOSITION

• CONTENT OF FLAVONOL GLYCOSIDES

Extraction solvent: Alcohol, hydrochloric acid, and water (25:4:10)

Mobile phase: Methanol, water, and phosphoric acid (100:100:1)

Standard solution A: 0.125 mg/mL of USP Quercetin RS in methanol

Standard solution B: 0.125 mg/mL of USP Kaempferol RS in methanol

Standard solution C: 0.03 mg/mL of USP Isorhamnetin RS in methanol

Sample solution: Transfer about 0.3 g of Powdered Extract, accurately weighed, to a 250-mL flask fitted with a reflux condenser. Add 78 mL of *Extraction solvent*, and reflux in a hot water bath for 135 min. [NOTE—The solution will turn deep red. The color of the solution is not a definitive indication of reaction completeness.] Allow to cool to room temperature. Transfer to a 100-mL volumetric flask, dilute with water to volume, and mix.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 370 nm

Column: 4.6-mm \times 25-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 20 μ L

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*

[NOTE—The relative retention times for quercetin, kaempferol, and isorhamnetin are about 1.0, 1.8, and 2.0, respectively; *Standard solution A*, *Standard solution B*, and *Standard solution C*.]

Suitability requirements

Relative standard deviation: NMT 2.0% determined from the quercetin peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Calculate the percentage of each flavonol glycoside in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times F \times 10$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of the relevant analyte from *Standard solution A*, *Standard solution B*, or *Standard solution C*

C_S = concentration of the relevant analyte in *Standard solution A*, *Standard solution B*, or *Standard solution C* (mg/mL)

W = weight of Powdered Extract taken to prepare the *Sample solution* (g)

F = mean molecular mass factor to convert each analyte into flavonol glycoside with a mean molecular mass of 756.7: 2.504 for quercetin, 2.437 for isorhamnetin, and 2.588 for kaempferol

Calculate the total percentage of flavonol glycosides by adding the individual percentages calculated.

Acceptance criteria: 22.0%–27.0% of flavonoids, calculated as flavonol glycosides with a mean molecular mass of 756.7, on the dried basis

• CONTENT OF TERPENE LACTONES

Solvent: Methanol and water (9:1)

Buffer solution: Dissolve 1.19 g of dibasic sodium phosphate and 8.25 g of monobasic potassium phosphate in 1000 mL of water, and adjust to a pH of 5.8.

Diluent: Methanol and water (1:1)
Solution A: Water
Solution B: Methanol
Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
23	52	48
28	52	48
30	25	75
35	10	90
40	75	25
50	75	25

Standard solutions: Using the labeled content of the individual terpene lactones, prepare five solutions of the USP Ginkgo Terpene Lactones RS in *Diluent* within the range of 5–500 µg/mL for each of the relevant terpene lactones. Use sonication to dissolve the analytes if necessary. Pass through a filter of 0.45-µm or finer pore size.

Sample solution: Transfer about 120 mg of Powdered Extract, accurately weighed, to a 25-mL beaker. Add 10 mL of *Buffer solution* to the residue, and sonicate for 5 min. Quantitatively transfer the solution to a glass chromatographic tube filled with chromatographic siliceous earth capable of holding 20 mL of aqueous phase.¹ Rinse the beaker with two 5-mL portions of *Buffer solution*, and transfer the washings to the column. [NOTE—Do not exceed 20 mL of total aqueous phase or the holding capacity of the chromatographic tube.] Allow the *Buffer solution* to be absorbed into the column. After 15 min, elute the column with 100 mL of ethyl acetate, collect the ethyl acetate solution, and evaporate to dryness under vacuum in a water bath maintained at 50°. Dissolve the residue in 20.0 mL of *Diluent*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering. [NOTE—The parameters of the detector are adjusted to achieve the best signal-to-noise ratio, according to manufacturer recommendations.]

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 25 ± 1°

Flow rate: 1 mL/min

Injection volume: 15 µL

System suitability

Samples: *Standard solutions*

Suitability requirements

Chromatogram similarity: The chromatograms obtained from the *Standard solutions* are similar to the reference chromatogram provided with the lot of USP Ginkgo Terpene Lactones RS being used.

Relative standard deviation: NMT 2.0% determined from the bilobalide peak in repeated injections

Correlation coefficient: NLT 0.995 for the regression line as determined in *Analysis*

Analysis

Samples: *Standard solutions* and *Sample solution*

Record the chromatograms, and identify the peaks of the relevant analytes in the chromatogram of the *Standard solution* by comparison with the reference chromatogram of the USP Ginkgo Terpene Lactones RS lot being used. Measure the areas of the analyte peaks. Plot the logarithms of the relevant peak responses versus the logarithms of concentrations, in mg/mL, of

each analyte of the *Standard solutions*, and determine the regression line using a least-squares analysis. From the graphs, determine the concentration, *C*, in mg/mL, of the relevant analyte in the *Sample solution*. Separately calculate the percentages of bilobalide (C₁₅H₁₈O₈), ginkgolide A (C₂₀H₂₄O₉), ginkgolide B (C₂₀H₂₄O₁₀), and ginkgolide C (C₂₀H₂₄O₁₁) in the portion of Powdered Extract taken:

$$\text{Result} = (C/W) \times 2000$$

C = concentration of the relevant analyte in the *Sample solution* (mg/mL)

W = weight of Powdered Extract taken to prepare the *Sample solution* (mg)

Calculate the total percentage of terpene lactones in the portion of Powdered Extract taken by adding the percentages calculated for each analyte.

Acceptance criteria

Total terpene lactones: 5.4%–12.0%

Bilobalide: 2.6%–5.8%

Sum of ginkgolide A, ginkgolide B, and ginkgolide C: 2.8%–6.2%

CONTAMINANTS

• **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues** <561>: Meets the requirements

• **HEAVY METALS, Method II** <231>: NMT 20 µg/g

• **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic bacterial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts count does not exceed 10³ cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

LIMIT OF GINKGOLIC ACIDS

Solution A: 0.01% phosphoric acid in water

Solution B: 0.01% phosphoric acid in acetonitrile

Mobile phase: See Table 2.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	25	75
6	10	90
7	10	90
8	25	75
10	25	75

Standard solution: Dissolve USP Ginkgolic Acids RS in methanol, and dilute, if necessary, with water to obtain a concentration of 0.25 µg/mL of ginkgolic acids, calculated as the sum of the congeners ginkgolic acid C 13:0, ginkgolic acid C 15:1, and ginkgolic acid C 17:1.

Sample solution: Transfer 0.5 g of Powdered Extract to a 10-mL volumetric flask. Add 8 mL of methanol to dissolve, and dilute with water to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.6-mm × 5-cm; base-deactivated packing L7

Column temperature: 35°

Flow rate: 1 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram obtained is similar to the reference chromatogram provided with the lot of USP Ginkgolic Acids RS being used.

¹ Suitable commercially available material is Extrelut® NT 20 from E Merck Science.

Tailing factor: NMT 2.0 for the ginkgolic acid C 15:1 peak

Relative standard deviation: NMT 5.0% for the ginkgolic acid C 15:1 peak in repeated injections

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—Identify the peaks of the relevant analytes by comparison with the reference chromatogram of the USP Ginkgolic Acids RS lot being used. If deterioration of peak shapes is observed, wash the column using a mixture of methanol and water (9:1) for 30 min.]

Calculate the concentration, in µg/g, of each ginkgolic acid in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times P \times 10$$

r_U = peak area for the relevant analyte from the *Sample solution*

r_S = peak area for the relevant analyte from the *Standard solution*

C_S = concentration of USP Ginkgolic Acids RS in the *Standard solution* (mg/mL)

W = weight of Powdered Extract taken to prepare the *Sample solution* (mg)

P = content of the relevant ginkgolic acid in USP Ginkgolic Acids RS (µg/g)

Calculate the total amount of ginkgolic acids by adding the individual contents.

Acceptance criteria: NMT 5 µg/g

• Loss on Drying (731)

Sample: 1.0 g of Powdered Extract

Analysis: Dry the *Sample* at 105° for 2 h.

Acceptance criteria: NMT 5.0%

• OTHER REQUIREMENTS: Meets the requirements for *Residual Solvents* in *Botanical Extracts* (565)

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, protected from moisture, and store at controlled room temperature.

- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of flavonol glycosides and of terpene lactones, the extracting solvent used for preparation, and the ratio of the starting crude plant material to the Powdered Extract.

• USP REFERENCE STANDARDS (11)

USP Chlorogenic Acid RS
USP Ginkgo Terpene Lactones RS
USP Ginkgolic Acids RS
USP Isorhamnetin RS
USP Kaempferol RS
USP Quercetin RS
USP Rutin RS

Ginkgo Capsules

DEFINITION

Ginkgo Capsules are prepared with Powdered Ginkgo Extract and contain, in the labeled amount of Powdered Extract, NLT 22.0% and NMT 27.0% of flavonol glycosides and NLT 5.4% and NMT 12.0% of terpene lactones, calculated as the sum of bilobalide (C₁₅H₁₈O₈), ginkgolide A (C₂₀H₂₄O₉), ginkgolide B (C₂₀H₂₄O₁₀), and ginkgolide C (C₂₀H₂₄O₁₁).

IDENTIFICATION

- **A. HPLC:** In the test for *Content of Flavonol Glycosides*, the retention times of the peaks for quercetin, isorhamnetin, and kaempferol of the *Sample solution* correspond to

those of the *Standard solution*. In the chromatogram of the *Sample solution*, the ratio of the kaempferol peak to the quercetin peak is NLT 0.7, and the peak for isorhamnetin is NLT 0.1 times the size of the quercetin peak.

- **B. HPLC:** The retention times of the peaks for bilobalide, ginkgolide A, ginkgolide B, and ginkgolide C of the *Sample solution* correspond to those of the *Standard solutions*, as obtained in the test for *Content of Terpene Lactones*.

STRENGTH

• CONTENT OF FLAVONOL GLYCOSIDES

Mobile phase: Methanol, water, and phosphoric acid (100:100:1)

Standard solution A: 0.2 mg/mL of USP Quercetin RS in methanol

Standard solution B: 0.2 mg/mL of USP Kaempferol RS in methanol

Standard solution C: 0.05 mg/mL of USP Isorhamnetin RS in methanol

Sample solution: Weigh and finely powder the contents of NLT 20 Capsules. Transfer an accurately weighed quantity of the powder, equivalent to about 50 mg of flavonol glycosides, to a 50-mL volumetric flask. Add 20 mL of methanol, and sonicate for 3 min. Add 20 mL of 1.5 N hydrochloric acid, and sonicate again for 10 min. Allow to cool to room temperature, and dilute with methanol to volume. Centrifuge, and transfer a portion of the clear supernatant to a rubber-capped, low-actinic glass vial. Heat in a steam bath for 25 min, and cool to room temperature in an ice bath.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 370 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*

[NOTE—The relative retention times for quercetin, kaempferol, and isorhamnetin are about 1.0, 1.8, and 2.0, respectively; *Standard solution A*, *Standard solution B*, and *Standard solution C*.]

Suitability requirements

Relative standard deviation: NMT 2.0% determined from the quercetin peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Calculate the quantity, in mg, of each flavonol glycoside in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times C_S \times F \times 50$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of the relevant analyte from *Standard solution A*, *Standard solution B*, or *Standard solution C*

C_S = concentration of the relevant analyte in *Standard solution A*, *Standard solution B*, or *Standard solution C* (mg/mL)

F = mean molecular mass factor to convert each analyte into flavonol glycoside with a mean molecular mass of 756.7: 2.504 for quercetin, 2.437 for isorhamnetin, and 2.588 for kaempferol

Calculate the total quantity, in mg, of flavonol glycosides in the portion of Capsules taken by adding the individual quantities calculated. Calculate the total quantity, in mg, of flavonol glycosides per Capsule and

the percentage of flavonol glycosides in the labeled amount of Powdered Ginkgo Extract.

Acceptance criteria: 22.0%–27.0% of flavonol glycosides

• **CONTENT OF TERPENE LACTONES**

Solvent: Methanol and water (9:1)

Buffer solution: Dissolve 1.19 g of dibasic sodium phosphate and 8.25 g of monobasic potassium phosphate in 1000 mL of water, and adjust to a pH of 5.8.

Diluent: Methanol and water (1:1)

Solution A: Water

Solution B: Methanol

Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
23	52	48
28	52	48
30	25	75
35	10	90
40	75	25
50	75	25

Standard solutions: Using the labeled content of the individual terpene lactones, prepare five solutions of the USP Ginkgo Terpene Lactones RS in *Diluent* within the range of 5–500 µg/mL for each of the relevant terpene lactones. Use sonication to dissolve the analytes if necessary. Pass through a filter of 0.45-µm or finer pore size.

Sample solution: Weigh and finely powder the contents of NLT 20 Capsules. Transfer an accurately weighed quantity of the powder, equivalent to about 120 mg of Powdered Ginkgo Extract, to a 30-mL glass centrifuge tube with a screw cap and PTFE gasket. Add 10.0 mL of *Solvent*, seal the tube, and mix well on a vortex mixer. Heat in a water bath at 90° for 30 min. Mix the hot suspension on a vortex mixer, and repeat the heating at 90° for 30 min. Cool, centrifuge, transfer the supernatant to a flask, and return the residue to the glass tube. Repeat the extraction two more times, each time using 10.0 mL of *Solvent*. Combine the extracts, allow them to cool to room temperature, and evaporate to dryness under vacuum in a water bath maintained at 50°. Add 10 mL of *Buffer solution* to the residue, and sonicate for 5 min. Quantitatively transfer the solution to a glass chromatographic tube filled with chromatographic siliceous earth capable of holding 20 mL of aqueous phase.¹ Rinse the beaker with two 5-mL portions of *Buffer solution*, and transfer the washings to the column. [NOTE—Do not exceed 20 mL of total aqueous phase or the holding capacity of the chromatographic tube.] Allow the *Buffer solution* to be absorbed into the column. After 15 min, elute the column with 100 mL of ethyl acetate, collect the ethyl acetate solution, and evaporate to dryness under vacuum in a water bath maintained at 50°. Dissolve the residue in 20.0 mL of *Diluent*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering. [NOTE—The parameters of the detector are adjusted to achieve the best signal-to-noise ratio, according to manufacturer recommendations.]

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 25 ± 1°

Flow rate: 1 mL/min

Injection volume: 15 µL

System suitability

Samples: *Standard solutions*

Suitability requirements

Chromatogram similarity: The chromatograms from the *Standard solutions* are similar to the reference chromatogram provided with the lot of USP Ginkgo Terpene Lactones RS being used.

Relative standard deviation: NMT 2.0% determined from the bilobalide peak in repeated injections

Correlation coefficient: NLT 0.995 for the regression line as determined in *Analysis*

Analysis

Samples: *Standard solutions* and *Sample solution*

Record the chromatograms, and identify the peaks of the relevant analytes in the chromatogram of the *Standard solutions* by comparison with the reference chromatogram of the USP Ginkgo Terpene Lactones RS lot being used. Measure the areas of the analyte peaks. Plot the logarithms of the relevant peak responses versus the logarithms of concentrations, in mg/mL, of each analyte obtained from the *Standard solutions*, and determine the regression line using a least-squares analysis.

From the graphs, determine the concentration, *C*, in mg/mL, of the relevant analyte in the *Sample solution*. Separately calculate the quantities, in mg, of bilobalide (C₁₅H₁₈O₈), ginkgolide A (C₂₀H₂₄O₉), ginkgolide B (C₂₀H₂₄O₁₀), and ginkgolide C (C₂₀H₂₄O₁₁) in the portion of Capsules taken:

$$\text{Result} = C \times 20$$

C = concentration of the relevant analyte in the *Sample solution* (mg/mL)

Calculate the total quantity of terpene lactones in the portion of Capsules taken by adding the quantities calculated for each analyte. Calculate the total quantity, in mg, of terpene lactones per Capsule and the percentage of terpene lactones in the labeled amount of Powdered Ginkgo Extract.

Acceptance criteria: 5.4%–12.0% of terpene lactones, calculated as the sum of bilobalide, ginkgolide A, ginkgolide B, and ginkgolide C

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** <2040>: Meet the requirements for *Dissolution*

Medium: 0.1 N hydrochloric acid; 500 mL

Apparatus 2: 75 rpm

Time: 45 min

Standard solutions: Proceed as directed in the test for *Content of Terpene Lactones*.

Sample solution: Combine 25-mL portions of the solution under test from each of the six dissolution vessels in a separation funnel. Extract with four 50-mL portions of ethyl acetate. Combine the extracts and evaporate in vacuum to dryness. Dissolve the residue with sonication in 5.0 mL of a mixture of water and methanol (1:1).

Analysis: Proceed as directed in the test for *Content of Terpene Lactones* to determine the concentration, *C*, in mg/mL, of ginkgolide B in the *Sample solution*.

Calculate the percentage of ginkgolide B dissolved:

$$\text{Result} = 5000C/3G$$

C = concentration of ginkgolide B in the *Sample solution* (mg/mL)

G = content of ginkgolide B as determined in the test for *Content of Terpene Lactones* (mg/Capsule)

¹ Suitable commercially available material is Extrelut® NT 20 from E Merck Science.

Tolerances: NLT 75% of the content of ginkgolide B is dissolved.

- **WEIGHT VARIATION** (2091): Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article used to prepare the Capsules. Label the Capsules to indicate the amount, in mg, of Powdered Ginkgo Extract per Capsule.
- **USP REFERENCE STANDARDS** (11)
 - USP Ginkgo Terpene Lactones RS
 - USP Isorhamnetin RS
 - USP Kaempferol RS
 - USP Quercetin RS

Ginkgo Tablets

DEFINITION

Ginkgo Tablets are prepared from Powdered Ginkgo Extract and contain, in the labeled amount of Powdered Extract, NLT 22.0% and NMT 27.0% of flavonol glycosides and NLT 5.4% and NMT 12.0% of terpene lactones, consisting of bilobalide ($C_{15}H_{18}O_8$), ginkgolide A ($C_{20}H_{24}O_9$), ginkgolide B ($C_{20}H_{24}O_{10}$), and ginkgolide C ($C_{20}H_{24}O_{11}$).

IDENTIFICATION

- **A. HPLC:** In the test for *Content of Flavonol Glycosides*, the retention times of the peaks for quercetin, isorhamnetin, and kaempferol of the *Sample solution* correspond to those of the *Standard solution*. In the chromatogram of the *Sample solution*, the ratio of the kaempferol peak to the quercetin peak is NLT 0.7, and the peak for isorhamnetin is NLT 0.1 times the size of the quercetin peak.
- **B. HPLC:** The retention times of the peaks for bilobalide, ginkgolide A, ginkgolide B, and ginkgolide C of the *Sample solution* correspond to those of the *Standard solutions*, as obtained in the test for *Content of Terpene Lactones*.

STRENGTH

• CONTENT OF FLAVONOL GLYCOSIDES

Mobile phase: Methanol, water, and phosphoric acid (100:100:1)

Standard solution A: 0.2 mg/mL of USP Quercetin RS in methanol

Standard solution B: 0.2 mg/mL of USP Kaempferol RS in methanol

Standard solution C: 0.05 mg/mL of USP Isorhamnetin RS in methanol

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed quantity of the powder, equivalent to about 50 mg of flavonol glycosides, to a 50-mL volumetric flask. Add 20 mL of methanol, and sonicate for 3 min. Add 20 mL of 1.5 N hydrochloric acid, and sonicate again for 10 min. Allow to cool to room temperature, and dilute with methanol to volume. Centrifuge, and transfer a portion of the clear supernatant to a rubber-capped, low-actinic glass vial. Heat in a steam bath for 25 min, and cool to room temperature in an ice bath.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 370 nm

Column: 4.6-mm \times 25-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 20 μ L

System suitability

Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*

[NOTE—The relative retention times for quercetin, kaempferol, and isorhamnetin are about 1.0, 1.8, and 2.0, respectively; *Standard solution A*, *Standard solution B*, and *Standard solution C*.]

Suitability requirements

Relative standard deviation: NMT 2.0% determined from the quercetin peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Calculate the quantity, in mg, of each flavonol glycoside in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times C_S \times F \times 50$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of the relevant analyte from *Standard solution A*, *Standard solution B*, or *Standard solution C*

C_S = concentration of the relevant analyte in *Standard solution A*, *Standard solution B*, or *Standard solution C* (mg/mL)

F = mean molecular mass factor to convert each analyte into flavonol glycoside with a mean molecular mass of 756.7: 2.504 for quercetin, 2.437 for isorhamnetin, and 2.588 for kaempferol

Calculate the total quantity, in mg, of flavonol glycosides in the portion of Tablets taken by adding the individual quantities calculated. Calculate the total quantity, in mg, of flavonol glycosides per Tablet and the percentage of flavonol glycosides in the labeled amount of Powdered Ginkgo Extract.

Acceptance criteria: 22.0%–27.0% of flavonol glycosides

• CONTENT OF TERPENE LACTONES

Solvent: Methanol and water (9:1)

Buffer solution: Dissolve 1.19 g of dibasic sodium phosphate and 8.25 g of monobasic potassium phosphate in 1000 mL of water, and adjust to a pH of 5.8.

Diluent: Methanol and water (1:1)

Solution A: Water

Solution B: Methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
23	52	48
28	52	48
30	25	75
35	10	90
40	75	25
50	75	25

Standard solutions: Using the labeled content of the individual terpene lactones, prepare five solutions of the USP Ginkgo Terpene Lactones RS in *Diluent* within the range of 5–500 μ g/mL for each of the relevant terpene

lactones. Use sonication to dissolve the analytes if necessary. Pass through a filter of 0.45- μ m or finer pore size.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed quantity of the powder, equivalent to about 120 mg of Powdered Ginkgo Extract, to a 30-mL glass centrifuge tube with a cap and PTFE gasket. Add 10.0 mL of *Solvent*, seal the tube, and mix well on a vortex mixer. Heat in a water bath at 90° for 30 min. Mix the hot suspension on a vortex mixer, and repeat the heating at 90° for 30 min. Cool, centrifuge, transfer the supernatant to a flask, and return the residue to the glass tube. Repeat the extraction two more times, each time using 10.0 mL of *Solvent*. Combine the extracts, allow them to cool to room temperature, and evaporate to dryness under vacuum in a water bath maintained at 50°. Add 10 mL of *Buffer solution* to the residue, and sonicate for 5 min. Quantitatively transfer the solution to a glass chromatographic tube filled with chromatographic siliceous earth capable of holding 20 mL of aqueous phase.¹ Rinse the beaker with two 5-mL portions of *Buffer solution*, and transfer the washings to the column. [NOTE—Do not exceed 20 mL of total aqueous phase or the holding capacity of the chromatographic tube.] Allow the *Buffer solution* to be absorbed into the column. After 15 min, elute the column with 100 mL of ethyl acetate, collect the ethyl acetate solution, and evaporate to dryness under vacuum in a water bath maintained at 50°. Dissolve the residue in 20.0 mL of *Diluent*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering. [NOTE—The parameters of the detector are adjusted to achieve the best signal-to-noise ratio, according to manufacturer recommendations.]

Column: 4.6-mm \times 25-cm; packing L1

Column temperature: 25 \pm 1°

Flow rate: 1 mL/min

Injection volume: 15 μ L

System suitability

Samples: *Standard solutions*

Suitability requirements

Chromatogram similarity: The chromatograms from the *Standard solutions* are similar to the reference chromatogram provided with the lot of USP Ginkgo Terpene Lactones RS being used.

Relative standard deviation: NMT 2.0% determined from the bilobalide peak in repeated injections

Correlation coefficient: NLT 0.995 for the regression line as determined in *Analysis*

Analysis

Samples: *Standard solutions* and *Sample solution*
Record the chromatograms, and identify the peaks of the relevant analytes in the chromatogram of the *Standard solutions* by comparison with the reference chromatogram of the USP Ginkgo Terpene Lactones RS lot being used. Measure the areas of the analyte peaks. Plot the logarithms of the relevant peak responses versus the logarithms of concentrations, in mg/mL, of each analyte of the *Standard solutions*, and determine the regression line using a least-squares analysis.

From the graphs, determine the concentration, *C*, in mg/mL, of the relevant analyte in the *Sample solution*. Separately calculate the quantities, in mg, of bilobalide (C₁₅H₁₈O₈), ginkgolide A (C₂₀H₂₄O₉), ginkgolide B (C₂₀H₂₄O₁₀), and ginkgolide C (C₂₀H₂₄O₁₁) in the portion of Tablets taken:

$$\text{Result} = C \times 20$$

C = concentration of the relevant analyte in the *Sample solution* (mg/mL)

Calculate the total quantity of terpene lactones in the portion of Tablets taken by adding the quantities calculated for each analyte. Calculate the total quantity, in mg, of terpene lactones per Tablet and the percentage of terpene lactones in the labeled amount of Powdered Ginkgo Extract.

Acceptance criteria: 5.4%–12.0% of terpene lactones, consisting of bilobalide, ginkgolide A, ginkgolide B, and ginkgolide C

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** <2040>: Meet the requirements for *Dissolution*

Medium: 0.1 N hydrochloric acid; 500 mL

Apparatus 2: 75 rpm

Time: 45 min

Standard solutions: Proceed as directed in the test for *Content of Terpene Lactones*.

Sample solution: Combine 25-mL portions of the solution under test from each of the six dissolution vessels in a separation funnel. Extract with four 50-mL portions of ethyl acetate. Combine the extracts, and evaporate in vacuum to dryness. Dissolve the residue with sonication in 5.0 mL of a mixture of water and methanol (1:1).

Analysis: Proceed as directed in the test for *Content of Terpene Lactones* to determine the concentration, *C*, in mg/mL, of ginkgolide B in the *Sample solution*.

Calculate the percentage of ginkgolide B dissolved:

$$\text{Result} = 5000C/3G$$

C = concentration of ginkgolide B in the *Sample solution* (mg/mL)

G = content of ginkgolide B as determined in the test for *Content of Terpene Lactones* (mg/Tablet)

Tolerances: NLT 75% of the content of ginkgolide B is dissolved.

• **WEIGHT VARIATION** <2091>: Meet the requirements

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts count does not exceed 10³ cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the article used to prepare the Tablets. Label the Tablets to indicate the content, in mg, of Powdered Ginkgo Extract per Tablet.

• **USP REFERENCE STANDARDS** <11>
USP Ginkgo Terpene Lactones RS
USP Isorhamnetin RS
USP Kaempferol RS
USP Quercetin RS

Ginseng, American—see *American Ginseng*

Ginseng, Asian—see *Asian Ginseng*

¹ Suitable commercially available material is Extrelut® NT 20 from E Merck Science.

Ginseng, Siberian—see *Eleuthero***Glucosamine and Chondroitin Sulfate Sodium Tablets****DEFINITION**

Glucosamine and Chondroitin Sulfate Sodium Tablets are prepared from either Glucosamine Hydrochloride, Glucosamine Sulfate Sodium Chloride, Glucosamine Sulfate Potassium Chloride, or a mixture of any of them, with Chondroitin Sulfate Sodium. Tablets contain NLT 90.0% and NMT 120.0% of the labeled amounts of chondroitin sulfate sodium and glucosamine ($C_6H_{13}NO_5$).

[NOTE—Chondroitin Sulfate Sodium is extremely hygroscopic once dried. Avoid exposure to atmosphere, and weigh promptly.]

IDENTIFICATION

- A.** The retention time of the major peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Content of Glucosamine*.

- B. ELECTROPHORESIS (726)**

Barium acetate buffer: Dissolve 25.24 g of barium acetate in 900 mL of water. Adjust with acetic acid to a pH of 5.0, and dilute with water to 1000 mL.

Staining reagent: 0.1% (w/v) toluidine blue in 0.1 M acetic acid

Standard solution: Use the *Standard solution* of middle concentration from the test for *Content of Chondroitin Sulfate Sodium*.

Sample solution: Prepare as directed in the test for *Content of Chondroitin Sulfate Sodium*.

Analysis: Fill the chambers of an electrophoresis apparatus suitable for separations on cellulose acetate membranes¹ (a small submarine gel chamber or one dedicated to membrane media) with *Barium acetate buffer*. Soak a cellulose acetate membrane 5–6 cm × 12–14 cm in *Barium acetate buffer* for 10 min, or until evenly wetted, then blot dry between two sheets of absorbent paper. Using an applicator² suitable for electrophoresis, apply equal volumes (0.5 µL) of the *Sample solution* and *Standard solution* to the brighter side of the membrane held in position in an appropriate applicator stand or on a separating bridge in the chamber. Ensure that both ends of the membrane are dipped at least 0.5–1.0 cm deep into the buffer chambers. Apply a constant 60 V (6 mA at the start) for 2 h. [NOTE—Perform the application of solutions and voltage within 5 min because further drying of the blotted paper reduces sensitivity.]

Place the membrane in a plastic staining tray, and with the application side down, float or gently immerse in *Staining reagent* for 5 min. Then stir the solution gently for 1 min. Remove the membrane, and destain in 5% acetic acid until the background clears.

Acceptance criteria: The principal spot of the *Sample solution* has the same migration as the principal spot of the *Standard solution*. [NOTE—Document the results by taking a picture within 15 min of completion of destaining.]

STRENGTH**• CONTENT OF GLUCOSAMINE**

Diluent: Transfer 29 µL of acetic acid and 5 mL of acetonitrile to a 100-mL volumetric flask containing 50 mL of water. Dilute with water to volume.

¹ Suitable cellulose acetate membranes for electrophoresis are available from Malta Chemetron SRL, Milano, Italy (www.maltachemetron.com); Fluka Chemical Corp., Milwaukee, WI; and DiaSys Corp., Waterbury, CT (www.diasys.com).

² Suitable applicators are available from DiaSys Corp., Waterbury, CT (www.diasys.com) and Helena Laboratories, Beaumont, TX (www.helena.com).

Borate buffer: 0.2 M (76.3 g/L of sodium borate in water) adjusted with hydrochloric acid TS to a pH of 9.5

Acetate buffer: 6.80 g/L of sodium acetate trihydrate in water adjusted with dilute acetic acid to a pH of 5.9

Derivatizing reagent: In a 14-mL polypropylene culture tube, dissolve 50 mg of o-phthalaldehyde in 1.25 mL of anhydrous methanol. Add 50 µL of 3-mercaptopropionic acid and 11.2 mL of *Borate buffer*, and mix gently. Allow to stand in the dark for 30 min before use. [NOTE—Reagent strength is maintained by adding 10 µL of 3-mercaptopropionic acid every 2 days. Storage should be in the dark at room temperature, and can be used for NMT 2 weeks.]

Mobile phase: Methanol and *Acetate buffer* (1:9)

Standard solution: 1.0 mg/mL of USP Glucosamine Hydrochloride RS in water. Allow to stand at room temperature for 1 h.

Sample solution: Transfer an equivalent to 25 mg of glucosamine, from finely powdered Tablets (NLT 20), to a 25-mL volumetric flask. Dilute with *Diluent* to volume. Mix on a vortex mixer to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, stir for 5 min with the aid of a magnetic stirrer, and centrifuge.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 340 nm

Column: 3.0-mm × 5-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Samples: Five individual aliquots of the *Standard solution* derivatized as directed in the *Analysis*. Each derivatized aliquot is injected only once.

[NOTE—The relative retention times for the β-anomer and the α-anomer are 1.0 and 1.8, respectively. The retention time for the β-anomer is NLT 4 min.]

Suitability requirements

Relative standard deviation: NMT 2.0% from five replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*
Transfer 100 µL of the *Derivatizing reagent* and 100 µL of the *Standard solution* or *Sample solution* to a vial containing 400 µL of *Borate buffer*. Allow the derivatization to proceed for 1 min. Inject the derivatized solutions immediately after the derivatization reaction. Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of the β-anomer from the derivatized *Sample solution*

r_S = peak response of the β-anomer from the derivatized *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of glucosamine in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of glucosamine, 179.17

M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Acceptance criteria: 90.0%–120.0%

• CONTENT OF CHONDROITIN SULFATE SODIUM

Diluent: Weigh about 297 mg of monobasic potassium phosphate, 492 mg of dibasic potassium phosphate, and 250 mg of polysorbate 80, and transfer into a 1-L beaker. Dissolve in approximately 900 mL of water, and adjust with potassium hydroxide or phosphoric acid to a pH of 7.0 ± 0.2. Dilute with water to 1 L, and mix thoroughly.

Standard solutions: 1.5, 1.0, and 0.5 mg/mL of USP Chondroitin Sulfate Sodium RS in water

Sample solution: Transfer an equivalent to 100 mg of chondroitin sulfate sodium, from finely powdered Tablets (NLT 20), to 60 mL of water. Shake to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, and stir or shake for 5 min. Dilute with water to 100 mL, and centrifuge or pass through a suitable filter.

Titrimetric system

(See *Titrimetry* (541).)

Mode: Photometric titration

Titrant: 1 mg/mL of cetylpyridinium chloride in water. Degass before use.

Endpoint detection: Turbidimetric with a photoelectric probe

Analysis

Samples: *Standard solutions* and *Sample solution*

Transfer 5.0 mL of each *Standard solution* and the *Sample solution* to separate titration vessels. Add 25 mL of *Diluent* to each. Stir until a steady reading is obtained with a photoelectric probe either at 420, 550, or 660 nm. Set the instrument to zero in absorbance mode. Titrate with *Titrant* using the photoelectric probe to determine the endpoint turbidimetrically. From a linear regression equation calculated using the volumes of *Titrant* consumed versus concentrations of the *Standard solutions*, determine the concentration of chondroitin sulfate sodium in the *Sample solution*.

Calculate the percentage of the labeled amount of chondroitin sulfate sodium in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

C_U = nominal concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–120.0%

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS**

(2040): Meet the requirements for *Dissolution*

Medium: Water; 900 mL

Apparatus 2: 75 rpm

Time: 60 min

Determine the percentage of the labeled amount of glucosamine (C₆H₁₃NO₅) dissolved by using the following method.

Standard solution: Prepare as directed in the test for *Content of Glucosamine*. Dilute with a suitable quantity of water, if necessary.

Sample solution: Use the solution under test.

Borate buffer, Acetate buffer, Derivatizing reagent, Mobile phase, and Chromatographic system: Proceed as directed in the test for *Content of Glucosamine*.

Analysis: Proceed as directed in the test for *Content of Glucosamine*.

Calculate the percentage of the labeled amount of glucosamine (C₆H₁₃NO₅) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area from the derivatized *Sample solution*

r_S = peak area from the derivatized *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

V = volume of *Medium*, 900 mL

L = label claim of glucosamine (mg/Tablet)

M_{r1} = molecular weight of glucosamine, 179.17

M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Tolerances: NLT 75% of the labeled amount of glucosamine (C₆H₁₃NO₅) is dissolved.

Determine the percentage of the labeled amount of chondroitin sulfate sodium dissolved by using the following method.

Standard solutions, Titrant, and Diluent: Proceed as directed in the test for *Content of Chondroitin Sulfate Sodium*.

Sample solution: Use the solution under test.

Analysis: Proceed as directed in the test for *Content of Chondroitin Sulfate Sodium*.

Calculate the percentage of the labeled amount of chondroitin sulfate sodium dissolved:

$$\text{Result} = (C \times V/L) \times 100$$

C = determined concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

V = volume of *Medium*, 900 mL

L = label claim of chondroitin sulfate sodium (mg/Tablet)

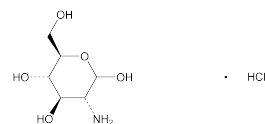
Tolerances: NLT 75% of the labeled amount of chondroitin sulfate sodium is dissolved.

- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS (2091):** Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label indicates the types of glucosamine salts contained in the article and the species source from which the chondroitin was derived. Label it to state the source(s) of chondroitin sulfate sodium, whether bovine, porcine, avian, or a mixture of any of them. The label states on the front panel the content of chondroitin sulfate sodium on the dried basis.
- **USP REFERENCE STANDARDS (11)**
USP Chondroitin Sulfate Sodium RS
USP Glucosamine Hydrochloride RS

Glucosamine Hydrochloride



C₆H₁₃NO₅ · HCl

215.63

D-Glucose, 2-amino-2-deoxy-, hydrochloride;
2-Amino-2-deoxy-β-D-glucopyranose hydrochloride
[66-84-2].

DEFINITION

Glucosamine Hydrochloride contains NLT 98.0% and NMT 102.0% of glucosamine hydrochloride (C₆H₁₃NO₅ · HCl), calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K)**
- **B. IDENTIFICATION TESTS—GENERAL, Chloride (191):** Meets the requirements
- **C.** The retention time of the glucosamine peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the Assay.

ASSAY**• PROCEDURE**

Buffer: In a 1-L volumetric flask, dissolve 3.5 g of dibasic potassium phosphate in water. Add 0.25 mL of ammonium hydroxide, dilute with water to volume, and mix. Adjust with phosphoric acid to a pH of 7.5.

Mobile phase: Acetonitrile and *Buffer* (75:25)

Diluent: Acetonitrile and water (50:50)

Standard solution: 3.8 mg/mL of USP Glucosamine Hydrochloride RS in *Diluent*

Sample solution: 3.8 mg/mL of Glucosamine Hydrochloride in *Diluent*. [NOTE—Shake by mechanical means to aid dissolution.]

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 195 nm

Column: 4.6-mm × 15-cm; 5-μm packing L8

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution*

[NOTE—The peak for the glucosamine moiety elutes at about 10 min. The chromatogram shows a large additional peak near the void volume, due to the chloride ion.]

Suitability requirements

Tailing factor: NMT 2.0 for the glucosamine peak

Efficiency: NLT 1500 theoretical plates

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of glucosamine hydrochloride ($C_6H_{13}NO_5 \cdot HCl$) in the portion of Glucosamine Hydrochloride taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = concentration of Glucosamine Hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%
- **CHLORIDE AND SULFATE**, *Sulfate* (221): A 0.10-g portion shows no more sulfate than corresponds to 0.25 mL of 0.020 N sulfuric acid (NMT 0.24%).
- **ARSENIC**, *Method II* (211): NMT 3 ppm
- **HEAVY METALS**, *Method II* (231): NMT 10 ppm

SPECIFIC TESTS

- **OPTICAL ROTATION**, *Specific Rotation* (781S): +70.0° to +73.0°
Sample solution: 25 mg/mL. Measure the specific rotation 3 h after preparation.
- **pH** (791)
Sample solution: 20 mg/mL
Acceptance criteria: 3.0–5.0
- **LOSS ON DRYING** (731): Dry a sample at 105° for 2 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** (11)
USP Glucosamine Hydrochloride RS

Glucosamine Tablets**DEFINITION**

Glucosamine Tablets are prepared from Glucosamine Hydrochloride, Glucosamine Sulfate Sodium Chloride, Glucosamine Sulfate Potassium Chloride, or a mixture of any of them. Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of glucosamine ($C_6H_{13}NO_5$).

IDENTIFICATION

- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Glucosamine*.
- **B. IDENTIFICATION TESTS—GENERAL**, *Chloride* (191): Meets the requirements
- **C. IDENTIFICATION TESTS—GENERAL**, *Sulfate* (191): Meets the requirements. [NOTE—Only for Tablets labeled as containing glucosamine sodium sulfate or glucosamine potassium sulfate]

STRENGTH**• CONTENT OF GLUCOSAMINE**

Buffer: In a 1-L volumetric flask dissolve 3.5 g of dibasic potassium phosphate in water. Add 0.25 mL of ammonium hydroxide, dilute with water to volume, and mix. Adjust with phosphoric acid to a pH of 7.5.

Mobile phase: Acetonitrile and *Buffer* (75:25)

Diluent: Acetonitrile and water (50:50)

Standard solution: 3.75 mg/mL of USP Glucosamine Hydrochloride RS in *Diluent*

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed portion of the finely powdered material, equivalent to about 312 mg of glucosamine, to a 100-mL volumetric flask. Add 60 mL of *Diluent*, and sonicate for 10 min. Shake by mechanical means for 15 min. Dilute with *Diluent* to volume, and mix. Pass a portion of this solution through a membrane filter of 0.45-μm or finer pore size.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 195 nm

Column: 4.6-mm × 15-cm; 5-μm packing L8

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 10 μL

[NOTE—The peak for glucosamine moiety elutes at about 10 min. The chromatogram shows a large additional peak near the void volume, due to the chloride ion.]

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the glucosamine peak

Column efficiency: NLT 1500 theoretical plates

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of glucosamine from the *Sample solution*

r_S = peak response of glucosamine from the *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of glucosamine in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of glucosamine, 179.17

M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

• DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS

⟨2040⟩: Meet the requirements for *Dissolution*

Medium: Water; 900 mL

Apparatus 2: 75 rpm

Time: 60 min

Standard solution: Dissolve a suitable amount of USP Glucosamine Hydrochloride RS in water to obtain a concentration similar to that expected in the *Sample solution*.

Sample solution: Filtered portion of the solution under test

Buffer: Mix 1.0 mL of phosphoric acid with 2 L of water, and adjust with potassium hydroxide to a pH of 3.0.

Mobile phase: Acetonitrile and *Buffer* (2:3)

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 195 nm

Column: 4.6-mm × 25-cm; packing L7

Flow rate: 0.6 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the glucosamine peak

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

V = volume of *Medium*, 900 mL

L = labeled amount of glucosamine (mg/Tablet)

M_{r1} = molecular weight of glucosamine, 179.17

M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Tolerances: NLT 75% of the labeled amount of glucosamine ($C_6H_{13}NO_5$) is dissolved.

• WEIGHT VARIATION OF DIETARY SUPPLEMENTS ⟨2091⟩: Meet the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **LABELING:** The label indicates the type of glucosamine salt contained in the article.

• **USP REFERENCE STANDARDS ⟨11⟩**

USP Glucosamine Hydrochloride RS

Glucosamine Sulfate Potassium Chloride

$(C_6H_{14}NO_5)_2SO_4 \cdot 2KCl$ 605.52

Bis(D-glucose, 2-amino-2-deoxy-), sulfate potassium chloride complex;

Bis(2-amino-2-deoxy-β-D-glucopyranose) sulfate potassium chloride complex (–,–) [38899-05-7].

DEFINITION

Glucosamine Sulfate Potassium Chloride contains NLT 98.0% and NMT 102.0% of glucosamine sulfate potassium chloride $[(C_6H_{14}NO_5)_2SO_4 \cdot 2KCl]$, calculated on the dried basis.

IDENTIFICATION

• A. INFRARED ABSORPTION ⟨197K⟩

Sample: Transfer 50 mg of Glucosamine Sulfate Potassium Chloride to a centrifuge tube, and dissolve in 2 mL of water. Add 0.5 mL of barium chloride TS, and centrifuge. Collect the supernatant, and evaporate to dryness. Dry the residue at 105° for 2 h.

Acceptance criteria: The IR spectrum of the *Sample* matches that of a similar preparation of USP Glucosamine Hydrochloride RS, except that the addition of barium chloride TS is omitted.

• B. IDENTIFICATION TESTS—GENERAL, Chloride ⟨191⟩ and Potassium ⟨191⟩: Meets the requirements

• C. The retention time of the glucosamine peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

• D. **SULFATE:** In the test for *Content of Sulfate*, after the addition of barium chloride TS a white precipitate is formed.

ASSAY

• PROCEDURE

Buffer: In a 1-L volumetric flask, dissolve 3.5 g of dibasic potassium phosphate in water, add 0.25 mL of ammonium hydroxide, dilute with water to volume, and mix. Adjust with phosphoric acid to a pH of 7.5.

Mobile phase: Acetonitrile and *Buffer* (75:25)

Diluent: Acetonitrile and water (50:50)

Standard solution: 3.8 mg/mL of USP Glucosamine Hydrochloride RS in *Diluent*. Shake for 5 min by mechanical means to completely dissolve.

Sample solution: Transfer 263 mg of Glucosamine Sulfate Potassium Chloride to a 50-mL volumetric flask. Dissolve in 30 mL of *Diluent*, and shake by mechanical means. Dilute with *Diluent* to volume.

Chromatographic system

(See *Chromatography* ⟨621⟩, *System Suitability*.)

Mode: LC

Detector: UV 195 nm

Column: 4.6-mm × 15-cm; 5-µm packing L8

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The peak for the glucosamine moiety elutes at about 10 min. The chromatogram shows additional peaks near the void volume, due to the counter ions.]

Suitability requirements

Tailing factor: NMT 2.0 for the glucosamine peak

Efficiency: NLT 1500 theoretical plates

Relative standard deviation: NMT 2.0%.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of glucosamine sulfate potassium chloride $[(C_6H_{14}NO_5)_2SO_4 \cdot 2KCl]$ in the portion of Glucosamine Sulfate Potassium Chloride taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = concentration of Glucosamine Sulfate Potassium Chloride in the *Sample solution* (mg/mL)

- M_{r1} = molecular weight of glucosamine sulfate potassium chloride, 605.52
 M_{r2} = twice the molecular weight of glucosamine hydrochloride, 431.26

Acceptance criteria: 98.0%–102.0% on the dried basis

OTHER COMPONENTS

• CONTENT OF SULFATE

Sample: 1 g of Glucosamine Sulfate Potassium Chloride
Analysis: Transfer the *Sample* to a 250-mL beaker, and dissolve in 100 mL of water. Add 4 mL of 6 N hydrochloric acid. Heat the solution to boiling, and add, with constant stirring, sufficient boiling barium chloride TS to completely precipitate the sulfate. Add an additional 2 mL of barium chloride TS, and digest on a steam bath for 1 h. Pass the mixture through ashless filter paper. Transfer the residue quantitatively to a new filter, and wash the residue with hot water until no precipitate is obtained when 1 mL of silver nitrate TS is added to 5 mL of washing. Transfer the paper containing the residue to a tared crucible. Char the paper, without burning, and ignite the crucible and its contents to constant weight. Calculate the content of sulfate by multiplying the weight obtained by 0.4116.

Acceptance criteria: 15.5%–16.5%

IMPURITIES

- **RESIDUE ON IGNITION** (281): 26.5%–31.0%
- **SODIUM:** A solution (1 in 10), tested on a platinum wire, does not impart a pronounced yellow color to a nonluminous flame.
- **ARSENIC, Method II** (211): NMT 3 µg/g
- **HEAVY METALS, Method II** (231): NMT 10 ppm

SPECIFIC TESTS

- **OPTICAL ROTATION, Specific Rotation** (781S)
Sample solution: 35 mg/mL. Measure the specific rotation 3 h after preparation.
Acceptance criteria: +47.0° to +53.0°
- **PH** (791)
Sample solution: 20 mg/mL
Acceptance criteria: 3.0–5.0
- **LOSS ON DRYING** (731): Dry a sample at 105° for 2 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** (11)
 USP Glucosamine Hydrochloride RS

Glucosamine Sulfate Sodium Chloride

$(C_6H_{14}NO_5)_2SO_4 \cdot 2NaCl$ 573.31
 Bis(D-glucose, 2-amino-2-deoxy-), sulfate sodium chloride complex;
 Bis(2-amino-2-deoxy-β-D-glucopyranose) sulfate sodium chloride complex (–,–) [38899-05-7].

DEFINITION

Glucosamine Sulfate Sodium Chloride contains NLT 98.0% and NMT 102.0% of glucosamine sulfate sodium chloride $[(C_6H_{14}NO_5)_2SO_4 \cdot 2NaCl]$, calculated on the dried basis.

IDENTIFICATION

• A. INFRARED ABSORPTION (197K)

Sample: Transfer 50 mg of Glucosamine Sulfate Sodium Chloride to a centrifuge tube, and dissolve in 2 mL of water. Add 0.5 mL of barium chloride TS, and centrifuge. Collect the supernatant, and evaporate to dryness. Dry the residue at 105° for 2 h.

Acceptance criteria: The IR spectrum of the *Sample* matches that of a similar preparation of USP Glucosa-

mine Hydrochloride RS, except that the addition of barium chloride TS is omitted.

- **B. IDENTIFICATION TESTS—GENERAL, Chloride** (191) and **Sodium** (191): Meets the requirements
- **C.** The retention time of the glucosamine peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- **D. SULFATE:** In the test for *Content of Sulfate*, after the addition of barium chloride TS a white precipitate is formed.

ASSAY

• PROCEDURE

Buffer: In a 1-L volumetric flask, dissolve 3.5 g of dibasic potassium phosphate in water, add 0.25 mL of ammonium hydroxide, dilute with water to volume, and mix. Adjust with phosphoric acid to a pH of 7.5.

Mobile phase: Acetonitrile and *Buffer* (75:25)

Diluent: Acetonitrile and water (50:50)

Standard solution: 3.8 mg/mL of USP Glucosamine Hydrochloride RS in *Diluent*. Shake for 5 min by mechanical means to completely dissolve.

Sample solution: Transfer 250 mg of Glucosamine Sulfate Sodium Chloride to a 50-mL volumetric flask. Dissolve in 30 mL of *Diluent*, and shake by mechanical means. Dilute with *Diluent* to volume.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 195 nm

Column: 4.6-mm × 15-cm; 5-µm packing L8

Column temperature: 35°

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The peak for the glucosamine moiety elutes at about 10 min. The chromatogram shows additional peaks near the void volume, due to the counter ions.]

Suitability requirements

Tailing factor: NMT 2.0 for the glucosamine peak

Efficiency: NLT 1500 theoretical plates

Relative standard deviation: NMT 2.0%.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of glucosamine sulfate sodium chloride $[(C_6H_{14}NO_5)_2SO_4 \cdot 2NaCl]$ in the portion of Glucosamine Sulfate Sodium Chloride taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = concentration of Glucosamine Sulfate Sodium Chloride in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of glucosamine sulfate sodium chloride, 573.31

M_{r2} = twice the molecular weight of glucosamine hydrochloride, 431.26

Acceptance criteria: 98.0%–102.0% on the dried basis

OTHER COMPONENTS

• CONTENT OF SULFATE

Sample: 1 g of Glucosamine Sulfate Sodium Chloride

Analysis: Transfer the *Sample* to a 250-mL beaker, and dissolve in 100 mL of water. Add 4 mL of 6 N hydrochloric acid. Heat the solution to boiling, and add, with constant stirring, sufficient boiling barium chloride TS to completely precipitate the sulfate. Add an additional 2 mL of barium chloride TS, and digest on a steam bath for 1 h. Pass the mixture through ashless filter paper. Transfer the residue quantitatively to a new

filter, and wash the residue with hot water until no precipitate is obtained when 1 mL of silver nitrate TS is added to 5 mL of washing. Transfer the paper containing the residue to a tared crucible. Char the paper, without burning, and ignite the crucible and its contents to constant weight. Calculate the content of sulfate by multiplying the weight obtained by 0.4116.

Acceptance criteria: 16.3%–17.3%

IMPURITIES

- **RESIDUE ON IGNITION** (281): 22.5%–26.0%
- **ARSENIC**, Method II (211): NMT 3 µg/g
- **POTASSIUM**
Analysis: Acidify 5 mL of a solution (1 in 20) with 6 N acetic acid, and add 5 drops of sodium cobaltinitrite TS.
Acceptance criteria: No precipitate is formed.
- **HEAVY METALS**, Method II (231): NMT 10 ppm

SPECIFIC TESTS

- **OPTICAL ROTATION**, Specific Rotation (781S)
Sample solution: 35 mg/mL. Measure the specific rotation 3 h after preparation.
Acceptance criteria: +50.0° to +55.0°
- **pH** (791)
Sample solution: 20 mg/mL
Acceptance criteria: 3.0–5.0
- **LOSS ON DRYING** (731): Dry a sample at 105° for 2 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** (11)
 USP Glucosamine Hydrochloride RS

Glucosamine and Methylsulfonylmethane Tablets

DEFINITION

Glucosamine and Methylsulfonylmethane Tablets are prepared from either Glucosamine Hydrochloride, Glucosamine Sulfate Sodium Chloride, Glucosamine Sulfate Potassium Chloride, or a mixture of any of them, with Methylsulfonylmethane. Tablets contain NLT 90.0% and NMT 120.0% of the labeled amount of glucosamine ($C_6H_{13}NO_5$) and NLT 90.0% and NMT 110.0% of the labeled amount of methylsulfonylmethane ($C_2H_6O_2S$).

IDENTIFICATION

- **A. PRESENCE OF GLUCOSAMINE:** The retention times of the major peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the *Content of Glucosamine*.
- **B. PRESENCE OF METHYLSULFONYLMETHANE:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Content of Methylsulfonylmethane*.

STRENGTH

• CONTENT OF GLUCOSAMINE

Diluent: Transfer 29 µL of acetic acid and 5 mL of acetonitrile to a 100-mL volumetric flask containing 50 mL of water, and dilute with water to volume.

Borate buffer: 0.2 M (76.3 g/L of sodium borate in water) adjusted with hydrochloric acid TS to a pH of 9.5. [NOTE—Buffer must be stored at room temperature. It must be warmed to dissolve if crystallization occurs.]

Acetate buffer: 6.80 g/L of sodium acetate trihydrate in water adjusted with dilute acetic acid to a pH of 5.9

Derivatizing reagent: In a 14-mL polypropylene culture tube dissolve 50 mg of o-phthalaldehyde in

1.25 mL of anhydrous methanol. Add 50 µL of 3-mercaptopropionic acid and 11.2 mL of *Borate buffer*, and mix gently. Allow to stand in the dark for 30 min before use. [NOTE—Reagent strength is maintained by adding 10 µL of 3-mercaptopropionic acid every 2 days. Storage should be in the dark, at room temperature, and can be used for NMT 2 weeks.]

Mobile phase: Methanol and *Acetate buffer* (1:9)

Standard solution: 1.0 mg/mL of USP Glucosamine Hydrochloride RS in water. Allow to stand at room temperature for 1 h.

Sample solution: Transfer an equivalent to 25 mg of glucosamine from NLT 20 Tablets, finely powdered, to a 25-mL volumetric flask, and dilute with *Diluent* to volume. Mix on a vortex mixer to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, stir for 5 min with the aid of a magnetic stirrer, and centrifuge.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 340 nm

Column: 3.0-mm × 5-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Samples: Five individual aliquots of the *Standard solution* derivatized as directed for *Analysis*. Each derivatized aliquot is injected only once.

[NOTE—The relative retention times for the β-anomer and the α-anomer are 1.0 and 1.8, respectively. The retention time for the β-anomer is NLT 4 min.]

Suitability requirements

Relative standard deviation: NMT 2.0% for five replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*
 Transfer 100 µL of the *Derivatizing reagent* and 100 µL of the *Standard solution* or the *Sample solution* to a vial containing 400 µL of *Borate buffer*. Allow the derivatization to proceed for 1 min. Inject the derivatized solutions immediately after the derivatization reaction. Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of the β-anomer from the derivatized *Sample solution*

r_S = peak response of the β-anomer from the derivatized *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of glucosamine in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of glucosamine, 179.17

M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Acceptance criteria: 90.0%–120.0% of the labeled claim

• CONTENT OF METHYLSULFONYLMETHANE

Diluent: Transfer 950 mL of methanol to a 1-L volumetric flask. Add 0.60 mL of diethylene glycol methyl ether, and dilute with methanol to volume.

Standard solution: 0.4 mg/mL of USP Methylsulfonylmethane RS in *Diluent*. Sonicate at 50° for 1 min, and allow to cool to room temperature.

Sample solution: Finely powder NLT 20 Tablets. Dissolve a portion of the finely powdered material, equivalent to 1 Tablet, in *Diluent*, and sonicate for 15 min at 50°. Allow to cool to room temperature, dilute with *Diluent* to volume, and mix. Quantitatively dilute with *Diluent* to obtain a final concentration of 0.4 mg/mL of methylsulfonylmethane. Transfer 1 mL of the

suspension to a 1.5-mL microcentrifuge tube, and centrifuge for 20 s. Use the supernatant.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm \times 30-m capillary; 5- μ m phase G2 coating

Temperature

Column: 120°

Injector: 250°

Detector: 250°

Carrier gas: Helium

Flow rate: 5 mL/min

Injection size: 1 μ L

Injector type: Split ratio, 2:1

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0% for the peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of methylsulfonylmethane ($C_2H_6O_2S$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from the *Sample solution*

R_S = peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from the *Standard solution*

C_S = concentration of USP Methylsulfonylmethane RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of methylsulfonylmethane in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of the label claim

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** <2040>: Meet the requirements for *Dissolution*

Medium: Water; 900 mL

Apparatus 2: 75 rpm

Time: 60 min

Determine the percentage of glucosamine dissolved as follows.

Standard solution: Prepare as directed in the test for *Content of Glucosamine*. Dilute with a suitable quantity of water, if necessary.

Sample solution: Use the solution under test.

Borate buffer, Acetate buffer, Derivatizing reagent, Mobile phase, Chromatographic system, and

Analysis: Proceed as directed in the test for *Content of Glucosamine*.

Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area obtained from the derivatized *Sample solution*

r_S = peak area obtained from the derivatized *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

V = volume of *Medium*, 900 mL

L = label claim of glucosamine (mg/Tablet)

M_{r1} = molecular weight of glucosamine, 179.17

M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Tolerances: NLT 75% of the labeled amount of glucosamine ($C_6H_{13}NO_5$) is dissolved.

- **WEIGHT VARIATION** (2091): Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label indicates the types of glucosamine salts contained in the article.
- **USP REFERENCE STANDARDS** <11>
 - USP Glucosamine Hydrochloride RS
 - USP Methylsulfonylmethane RS
 - Dimethyl sulfone.
 - $C_2H_6O_2S$ 94.13

Glucosamine, Chondroitin Sulfate Sodium, and Methylsulfonylmethane Tablets

DEFINITION

Glucosamine, Chondroitin Sulfate Sodium, and Methylsulfonylmethane Tablets are prepared from either Glucosamine Hydrochloride, Glucosamine Sulfate Sodium Chloride, Glucosamine Sulfate Potassium Chloride, or a mixture of any of them, with Chondroitin Sulfate Sodium and Methylsulfonylmethane. Tablets contain NLT 90.0% and NMT 120.0% of the labeled amounts of chondroitin sulfate sodium and glucosamine ($C_6H_{13}NO_5$) and NLT 90.0% and NMT 110.0% of the labeled amount of methylsulfonylmethane ($C_2H_6O_2S$).

[NOTE—Chondroitin Sulfate Sodium is extremely hygroscopic once dried. Avoid exposure to atmosphere, and weigh promptly.]

IDENTIFICATION

- **A. PRESENCE OF GLUCOSAMINE:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Glucosamine*.
- **B. PRESENCE OF CHONDROITIN SULFATE** (See *Electrophoresis* <726>.)

Barium acetate buffer: Dissolve 25.24 g of barium acetate in 900 mL of water. Adjust with acetic acid to a pH of 5.0, and dilute with water to 1000 mL.

Staining reagent: 0.1% (w/v) toluidine blue in 0.1 M acetic acid

Standard solution: Use the *Standard solution* of middle concentration from *Content of Chondroitin Sulfate Sodium*.

Sample solution: Prepare as directed in *Content of Chondroitin Sulfate Sodium*.

Analysis: Fill the chambers of an electrophoresis apparatus suitable for separations on cellulose acetate membranes¹ (a small submarine gel chamber or one dedicated to membrane media) with *Barium acetate buffer*. Soak a cellulose acetate membrane 5–6 cm \times 12–14 cm in *Barium acetate buffer* for 10 min, or until evenly wetted, then blot dry between two sheets of absorbent paper. Using an applicator² suitable for electrophoresis, apply equal volumes (0.5 μ L) of the *Sample solution* and *Standard solution* to the brighter side of the membrane held in position in an appropriate applicator stand or on a separating bridge in the chamber. Ensure that both ends of the membrane are dipped at least

¹ Suitable cellulose acetate membranes for electrophoresis are available from Malta Chemetron SRL, Milano, Italy (www.maltachemetron.com); Fluka Chemical Corp., Milwaukee, WI; and DiaSys Corp., Waterbury, CT (www.diasys.com).

² Suitable applicators are available from DiaSys Corp., Waterbury, CT (www.diasys.com) and Helena Laboratories, Beaumont, TX (www.helena.com).

0.5–1.0 cm deep into the buffer chambers. Apply a constant 60 volts (6 mA at the start) for 2 h. [NOTE—Perform the application of solutions and voltage within 5 min because further drying of the blotted paper reduces sensitivity.]

Place the membrane in a plastic staining tray, and with the application side down, float or gently immerse in *Staining reagent* for 5 min. Then stir the solution gently for 1 min. Remove the membrane, and destain in 5% acetic acid until the background clears.

Acceptance criteria: The principal band from the *Sample solution* has the same migration as the principal band from the *Standard solution*. [NOTE—Document the results by taking a picture within 15 min of completion of destaining.]

- **C. PRESENCE OF METHYLSULFONYLMETHANE:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Content of Methylsulfonylmethane*.

STRENGTH

• CONTENT OF GLUCOSAMINE

Diluent: Transfer 29 μ L of acetic acid and 5 mL of acetonitrile to a 100-mL volumetric flask containing 50 mL of water, and dilute with water to volume.

Borate buffer: 0.2 M (76.3 g/L of sodium borate in water) adjusted with hydrochloric acid TS to a pH of 9.5

Acetate buffer: 6.80 g/L of sodium acetate trihydrate in water adjusted with dilute acetic acid to a pH of 5.9.

[NOTE—Buffer must be stored at room temperature and can be warmed to dissolve if crystallization occurs.]

Derivatizing reagent: In a 14-mL polypropylene culture tube, dissolve 50 mg of *o*-phthalaldehyde in 1.25 mL of anhydrous methanol. Add 50 μ L of 3-mercaptopropionic acid and 11.2 mL of *Borate buffer*, and mix gently. Allow to stand in the dark for 30 min before use. [NOTE—Reagent strength is maintained by adding 10 μ L of 3-mercaptopropionic acid every 2 days. Storage should be in the dark, at room temperature, and can be used for NMT 2 weeks.]

Mobile phase: Methanol and *Acetate buffer* (1:9)

Standard solution: 1.0 mg/mL of USP Glucosamine Hydrochloride RS in water. Allow to stand at room temperature for 1 h.

Sample solution: Transfer an equivalent to 25 mg of glucosamine from NLT 20 Tablets, finely powdered, to a 25-mL volumetric flask, and dilute with *Diluent* to volume. Mix on a vortex mixer to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, stir for 5 min with the aid of a magnetic stirrer, and centrifuge.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 340 nm

Column: 3.0-mm \times 5-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Samples: Five individual aliquots of the *Standard solution* derivatized as directed in *Analysis*. Each derivatized aliquot is injected only once.

[NOTE—The relative retention times for the β -anomer and the α -anomer are 1.0 and 1.8, respectively. The retention time for the β -anomer is NLT 4 min.]

Suitability requirements

Relative standard deviation: NMT 2.0% for five replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 100 μ L of the *Derivatizing reagent* and 100 μ L of the *Standard solution* or the *Sample solution* to a vial containing 400 μ L of *Borate buffer*, and allow the deriva-

tization to proceed for 1 min. Inject the derivatized solutions immediately after the derivatization reaction. Calculate the percentage of the labeled amount of glucosamine ($C_6H_{13}NO_5$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak response of the β -anomer from the derivatized *Sample solution*

r_S = peak response of the β -anomer from the derivatized *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of glucosamine in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of glucosamine, 179.17

M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Acceptance criteria: 90.0%–120.0% of the label claim

• CONTENT OF CHONDROITIN SULFATE SODIUM

Standard solutions: 1.5, 1.0, and 0.5 mg/mL of USP Chondroitin Sulfate Sodium RS in water

Sample solution: Transfer an equivalent to 100 mg of chondroitin sulfate sodium from NLT 20 Tablets, finely powdered, to 60 mL of water, and shake to suspend the powder in solution. Sonicate in a 65° water bath for 20 min. Remove from the bath, stir or shake for 5 min, dilute with water to 100 mL, and centrifuge or pass through a suitable filter.

Diluent: Weigh about 297 mg of monobasic potassium phosphate, 492 mg of dibasic potassium phosphate, and 250 mg of polysorbate 80, and transfer into a 1-L beaker. Dissolve in approximately 900 mL of water, and adjust with potassium hydroxide or phosphoric acid to a pH of 7.0 ± 0.2 . Dilute with water to 1 L, and mix thoroughly.

Titrimetric system

(See *Titrimetry* (541).)

Mode: Photometric titration

Titrant: 1 mg/mL of cetylpyridinium chloride in water

Endpoint detection: Turbidimetric with photoelectric probe

Analysis: Transfer 5.0 mL of each *Standard solution* and the *Sample solution* to separate titration vessels, and add 25 mL of *Diluent* to each. Stir until a steady reading is obtained with a photoelectric probe either at 420, 550, or 660 nm. Set the instrument to zero in absorbance mode. Titrate with *Titrant* using the photoelectric probe to determine the endpoint turbidimetrically. From a linear regression equation, calculated using the volumes of *Titrant* consumed versus concentrations of the *Standard solutions*, determine the concentration of chondroitin sulfate sodium in the *Sample solution*. Calculate the percentage of chondroitin sulfate sodium in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

C_U = nominal concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–120.0% of the label claim

• CONTENT OF METHYLSULFONYLMETHANE

Diluent: Transfer 950 mL of methanol to a 1-L volumetric flask. Add 0.60 mL of diethylene glycol methyl ether, and dilute with methanol to volume.

Standard solution: 0.4 mg/mL of USP Methylsulfonylmethane RS in *Diluent*. Sonicate at 50° for 1 min, and allow to cool to room temperature.

Sample solution: Finely powder NLT 20 Tablets. Dissolve a portion of the finely powdered material, equivalent to 1 Tablet, in *Diluent*, and sonicate for 15

min at 50°. Allow to cool to room temperature. Quantitatively dilute with *Diluent* to obtain a final concentration of 0.4 mg/mL of methylsulfonylmethane. Transfer 1 mL of the suspension to a 1.5-mL microcentrifuge tube, and centrifuge for 20 s. Use the supernatant.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m capillary column coated with a 5-μm phase G2

Temperature

Column: 120°

Injector: 250°

Detector: 250°

Carrier gas: Helium

Flow rate: 5 mL/min

Injection size: 1 μL

Injection type: Split ratio, 2:1

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0% for the peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of methylsulfonylmethane (C₂H₆O₂S) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of methylsulfonylmethane to diethylene glycol methyl ether from the *Sample solution*

R_S = peak area ratio of methylsulfonylmethane to diethylene glycol methyl ether from the *Standard solution*

C_S = concentration of USP Methylsulfonylmethane RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of methylsulfonylmethane in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of the label claim

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** <2040>: Meet the requirements for *Dissolution*

Medium: Water; 900 mL

Apparatus 2: 75 rpm

Time: 60 min

Determine the percentage of glucosamine dissolved as follows.

Borate buffer, Acetate buffer, Derivatizing reagent, Mobile phase, Chromatographic system, and

Analysis: Proceed as directed in the test for *Content of Glucosamine*.

Standard solution: Prepare as directed in the test for *Content of Glucosamine*. Dilute with a suitable quantity of water, if necessary.

Sample solution: Use the solution under test.

Calculate the percentage of the labeled amount of glucosamine (C₆H₁₃NO₅) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area obtained from the derivatized *Sample solution*

r_S = peak area obtained from the derivatized *Standard solution*

C_S = concentration of USP Glucosamine Hydrochloride RS in the *Standard solution* (mg/mL)

V = volume of *Medium*, 900 mL

L = label claim of glucosamine (mg/Tablet)

M_{r1} = molecular weight of glucosamine, 179.17

M_{r2} = molecular weight of glucosamine hydrochloride, 215.63

Tolerances: NLT 75% of the labeled amount of glucosamine (C₆H₁₃NO₅) is dissolved.

Determine the percentage of chondroitin sulfate sodium dissolved as follows.

Titration, Diluent, Standard solutions, and Analysis: Proceed as directed in the test for *Content of Chondroitin Sulfate Sodium*.

Sample solution: Use the solution under test.

Calculate the percentage of the labeled amount of chondroitin sulfate sodium dissolved:

$$\text{Result} = (C \times V/L) \times 100$$

C = determined concentration of chondroitin sulfate sodium in the *Sample solution* (mg/mL)

V = volume of *Medium*, 900 mL

L = label claim of chondroitin sulfate sodium (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of chondroitin sulfate sodium is dissolved.

- **WEIGHT VARIATION** (2091): Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

- **LABELING:** The label indicates the types of glucosamine salts contained in the article and the species source from which chondroitin was derived. Label it to state the source(s) of chondroitin sulfate sodium, whether bovine, porcine, avian, or a mixture of any of them. The label states on the front panel the content of chondroitin sulfate sodium on the dried basis.

- **USP REFERENCE STANDARDS** <11>

USP Chondroitin Sulfate Sodium RS

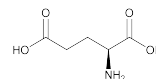
USP Glucosamine Hydrochloride RS

USP Methylsulfonylmethane RS

Dimethyl sulfone.

C₂H₆O₂S 94.13

Glutamic Acid



C₅H₉NO₄

L-Glutamic acid;

S-2-Aminopentanedioic acid [56-86-0].

147.13

DEFINITION

Glutamic Acid contains NLT 98.5% and NMT 101.5% of L-glutamic acid (C₅H₉NO₄), calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>

ASSAY

- **PROCEDURE**

Sample: 140 mg of Glutamic Acid

Blank: Mix 6 mL of formic acid and 50 mL of glacial acetic acid.

Titrimetric system(See *Titrimetry* <541>.)**Mode:** Direct titration**Titrant:** 0.1 N perchloric acid VS**Endpoint detection:** Potentiometric

Analysis: Dissolve the *Sample* in 6 mL of formic acid and 50 mL of glacial acetic acid, and titrate with the *Titrant*. Perform the *Blank* determination. Calculate the percentage of glutamic acid (C₅H₉NO₄) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = volume of *Titrant* consumed by the *Sample* (mL)

V_B = volume of *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 147.1 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: 98.5%–101.5% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** <281>: NMT 0.1%

• **CHLORIDE AND SULFATE**, *Chloride* <221>

Standard solution: 0.40 mL of 0.010 N hydrochloric acid

Sample: 0.7 g of Glutamic Acid

Acceptance criteria: NMT 0.02%

• **CHLORIDE AND SULFATE**, *Sulfate* <221>

Standard solution: 0.25 mL of 0.020 N sulfuric acid

Sample: 1.2 g of Glutamic Acid

Acceptance criteria: NMT 0.02%

• **IRON** <241>: NMT 10 µg/g

• **HEAVY METALS**, *Method 1* <231>: NMT 10 µg/g

RELATED COMPOUNDS

Standard solution: 0.05 mg/mL of USP Glutamic Acid RS in water

Sample solution: 10 mg/mL in a solution of ammonia TS and water (1:1)

System suitability solution: 0.4 mg/mL each of USP Aspartic Acid RS and USP Glutamic Acid RS in water

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 5 µL

Developing solvent system: Butyl alcohol, glacial acetic acid, and water (3:1:1)

Spray reagent: 2 mg/mL of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (95:5)

System suitability

Suitability requirements: The chromatogram from the *System suitability solution* exhibits two clearly separated spots.

Analysis: After air-drying the plate, repeat the development process. After air-drying a second time, spray with *Spray reagent*, and heat between 100° and 105° for about 15 min. Examine the plate under white light.

Acceptance criteria: Any secondary spot of the *Sample solution* is not larger or more intense than the principal spot of the *Standard solution*.

Individual impurities: NMT 0.5%

Total impurities: NMT 2.0%

SPECIFIC TESTS

• **OPTICAL ROTATION**, *Specific Rotation* <781S>

Sample solution: 100 mg/mL in 2 N hydrochloric acid

Analysis: Proceed as directed in the chapter, except measure at 20°.

Acceptance criteria: +31.5° to +32.5°

• **LOSS ON DRYING** <731>: Dry a sample at 105° for 3 h: it loses NMT 0.1% of its weight.

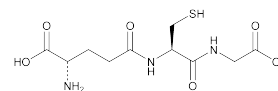
ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.

• **USP REFERENCE STANDARDS** <11>

USP Aspartic Acid RS

USP Glutamic Acid RS

Glutamine—see Glutamine General Monographs**Glutathione**

C₁₀H₁₇N₃O₆S

307.32

Pentanoic acid, 2-amino-5-[(R)-1-(carboxymethylamino)-3-mercapto-1-oxopropan-2-ylamino]-5-oxo-, (S); N-(N-L-γ-Glutamyl-L-cysteinyl)glycine [70-18-8].

DEFINITION

Glutathione contains NLT 98.0% and NMT 101.0% of C₁₀H₁₇N₃O₆S, as glutathione, calculated on the dried basis.

IDENTIFICATION

• **A. INFRARED ABSORPTION** <197K>

• **B. OPTICAL ROTATION**, *Specific Rotation* <781S>

Sample solution: 40 mg/mL in water

Acceptance criteria: −15.5° to −17.5°, at 20°

ASSAY**PROCEDURE**

Sample: 500 mg of glutathione previously dried

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N iodine VS

Endpoint detection: Visual

Blank: 50 mL of metaphosphoric acid (1 in 50)

Analysis: Dissolve the *Sample* in 50 mL of metaphosphoric acid (1 in 50) and titrate with the *Titrant*.

Calculate the percentage of glutathione (C₁₀H₁₇N₃O₆S) in the portion of Glutathione taken:

$$\text{Result} = [(V - B) \times N \times F \times 100]/W$$

V = titrant volume of the *Sample* (mL)

B = titrant volume of the *Blank* (mL)

N = titrant normality (mEq/mL)

F = equivalency factor, 307.32 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 98.0%–101.0% on the dried basis

IMPURITIES**Inorganic Impurities****• AMMONIUM**

Standard solution: 10 µg of ammonium from a diluted ammonium chloride solution

Sample solution: 50 mg of Glutathione

Analysis: Transfer the *Sample solution* and the *Standard solution* to separate 25-mL jars fitted with caps, and dissolve in 1 mL of water. Add 0.30 g of magnesium oxide. Close immediately after placing a piece of silver manganese paper 5-mm square, wetted with a few drops of water, under the caps. Swirl, avoiding projections of liquid, and allow to stand at 40° for 30 min.

Acceptance criteria: If the silver manganese paper shows a gray color, it is not more intense than the standard (NMT 200 ppm).

- **ARSENIC** (211): NMT 2 ppm
- **CHLORIDE AND SULFATE, Chloride** (221): Dissolve 0.7 g with water to make 15 mL. The solution shows no more chloride than corresponds to 0.20 mL of 0.020 N hydrochloric acid (NMT 200 ppm).
- **CHLORIDE AND SULFATE, Sulfate** (221): Dissolve 0.8 g in water to make 15 mL. The solution shows no more sulfate than corresponds to 0.25 mL of 0.020 N sulfuric acid (NMT 300 ppm).
- **HEAVY METALS, Method I** (231): NMT 10 ppm
- **IRON** (241): NMT 10 ppm
- **RESIDUE ON IGNITION** (281): NMT 0.1%

Organic Impurities

PROCEDURE

Mobile phase: 6.8 g/L of potassium dihydrogen phosphate with 2.02 g/L of sodium 1-heptane sulfonate. Adjust with phosphoric acid to a pH of 3.0. Mix 970 mL of this solution with 30 mL of methanol.

System suitability solution: 0.1 mg/mL of USP L-Phenylalanine RS, 0.5 mg/mL of USP Glutathione RS, and 0.5 mg/mL of USP Ascorbic acid RS in *Mobile phase*.

Standard solution: 0.01 mg/mL of USP Glutathione RS in *Mobile phase*. [NOTE—This solution has a concentration equivalent to 2.0% of that of the *Sample solution*.]

Sample solution: 50 mg of glutathione in 100 mL of *Mobile phase*. [NOTE—Allow the solution to stand for 5 min before use.]

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.6-mm × 15-cm; 5-μm packing L1

Column temperature: 30°

Flow rate: Adjust so that the retention time of glutathione is about 5 min.

Injection size: 10 μL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 5.0 between the ascorbic acid and glutathione peaks; and NLT 5.0 between the glutathione and L-phenylalanine peaks

Relative standard deviation: NMT 1.5% for replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of any impurity in the portion of Glutathione taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of any peak from the *Sample solution* other than glutathione

r_S = peak response of the glutathione peak from the *Standard solution*

C_S = concentration of USP Glutathione RS in the *Standard solution* (mg/mL)

C_U = concentration of Glutathione in the *Sample solution* (mg/mL)

Acceptance criteria

Individual impurity: NMT 1.5% for the impurity with the relative retention time of about 4

Total impurities: NMT 2.0%

SPECIFIC TESTS

CLARITY AND COLOR OF SOLUTION

Sample solution: 0.1 g/mL in water

Analysis: Using identical tubes of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm, compare the liquid to be examined with

water, the depth of the layer being 40 mm. Compare the colors in diffused daylight, viewing vertically against a white background.

Acceptance criteria: The solution is clear and colorless.

- **LOSS ON DRYING** (731): NMT 0.5%, 105° for 3 h

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

USP REFERENCE STANDARDS (11)

USP Ascorbic Acid RS

USP Glutathione RS

USP L-Phenylalanine RS

Glycine—see *Glycine General Monographs*

Goldenseal

DEFINITION

Goldenseal consists of the dried roots and rhizomes of *Hydrastis canadensis* L. (Fam. Ranunculaceae). It contains NLT 2.0% of hydrastine ($C_{21}H_{21}NO_6$) and NLT 2.5% of berberine ($C_{20}H_{18}NO_4$), calculated on the dried basis.

IDENTIFICATION

A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 0.5 mg/mL each of USP Berberine Chloride RS and USP Hydrastine RS in methanol

Sample solution: Finely powder the rhizome and the root. Transfer 0.5 g of the powder to a suitable glass vial, and add 0.5 mL of 10% sodium carbonate. Add 5 mL of methanol, and heat for 10 min in a water bath at 60°. Cool to room temperature, filter, and dry under a stream of nitrogen. Add 0.5 mL of methanol to dissolve the residue.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)

Application volume: 10–20 μL, as bands

Developing solvent system: Ethyl acetate, butyl alcohol, formic acid, and water (5:3:1:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved about three-fourths of the length of the plate, in a saturated chamber. Remove the plate, air-dry, and examine under UV light at 365 nm.

Acceptance criteria: The chromatograms show zones having a lemon-yellow fluorescence due to berberine at an R_f value of about 0.53 and a blue-white fluorescence due to hydrastine at an R_f value of about 0.42.

COMPOSITION

CONTENT OF BERBERINE AND HYDRASTINE AND LIMIT OF PALMATINE

Mobile phase: Dissolve 9.93 g of monobasic potassium phosphate in 730 mL of water, add 270 mL of acetonitrile, mix, filter, and degas.

Solvent: A mixture of 0.1 M monobasic potassium phosphate and acetonitrile (60:40)

Standard solution: 0.05 mg/mL each of USP Berberine Chloride RS and USP Hydrastine RS in a mixture of methanol and water (1:1)

System suitability solution: Prepare a solution of palmatine in a mixture of water and methanol (1:1) having a known concentration of about 0.05 mg/mL. Mix equal volumes of this solution and the *Standard solution*.

Sample solution: Finely powder a quantity of Goldenseal, and transfer about 0.12 g, accurately weighed, to a 50-mL volumetric flask. Add 40 mL of *Solvent*, soni-

cate for 5 min, and shake for 10 min. Dilute with *Solvent* to volume, mix, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 235 nm

Column: 4.6-mm × 150-mm; packing L1

Flow rate: 1.8 mL/min

Injection size: 10 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 1.5 between the berberine and palmatine peaks, and NLT 1.5 between the hydrastine and palmatine peaks, *System suitability solution*

Capacity factor: NLT 3.0, determined from the hydrastine and berberine peaks, *Standard solution*

Column efficiency: NLT 5000 theoretical plates determined from the hydrastine and berberine peaks, *Standard solution*

Tailing factor: NMT 2.0 determined from the hydrastine and berberine peaks, *Standard solution*

Relative standard deviation: NMT 2.5% determined from the hydrastine and berberine peaks in repeated injections, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of berberine and hydrastine in the portion of Goldenseal taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of berberine or hydrastine from the *Sample solution*

r_S = peak area of berberine or hydrastine from the *Standard solution*

C_S = concentration of berberine or hydrastine in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of powdered Goldenseal used to prepare the *Sample solution* (mg)

Using the values obtained from the chromatogram of the *Sample solution*, divide the peak area of berberine by the peak area of any peak at the locus for palmatine (if present).

Acceptance criteria: NLT 2.0% of hydrastine ($C_{21}H_{21}NO_6$) and NLT 2.5% of berberine ($C_{20}H_{18}NO_4$), on the dried basis. The ratio of the berberine peak area to any peak area at the locus for palmatine is more than 50:1.

CONTAMINANTS

- **HEAVY METALS,** *Method III* <231>: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN,** *General Method for Pesticide Residues Analysis* <561>: Meets the requirements

SPECIFIC TESTS

BOTANIC CHARACTERISTICS

Macroscopic: The rhizome is knotty, subcylindrical, and occasionally has an aerial stem. It is 1–5 cm in length and 2–10 mm in diameter. Externally, the rhizome is brown to dusky yellowish orange, deeply furrowed, and marked by numerous stem and bud scale scars. Numerous brittle roots arise roughly from the same side of the main axis. Fractures are short and resinous, with a dark yellow to yellowish-brown bark, greenish-yellow margins, and a yellowish-orange center that is waxy in appearance. An interrupted circle of small, radially elongated fibrovascular bundles are also present. The roots are filiform, up to 35 cm in length and 1 mm in diameter, and are either curved or twisted, tangled together, or broken. Fractures are short and brittle, and show an internal color of yellowish orange to greenish yellow.

Histology

Transverse section of rhizome and root: The rhizome has polygonal, yellowish-brown, thin- to slightly thick-walled cork cells. Wedge-shaped vascular bundles are separated by wide medullary rays. Tracheary elements are lignified and have slit-shaped pits. A few large vessels with reticulate thickenings are also present. The parenchyma tissue is composed of polygonal cells with abundant simple or compound starch grains up to 8 µm in diameter. A few irregularly shaped resin cells are present in the cortex and the pith. Masses of granular, orange-brown matter are also present in the parenchyma tissues. The roots have a single layer of irregularly elongated cork cells. The tracheary elements are associated with lignified fibers. Fragments of the epidermis are sometimes present near the base of the rhizome and are composed of cells with thick, lignified, beaded walls.

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** <561>: NMT 2.0%
- **LOSS ON DRYING** <731>: Dry 2 g of finely powdered Goldenseal at 100° for 5 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 9%
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** <561>: NMT 5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store protected from light, moisture, and heat.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** <11>
USP Berberine Chloride RS
USP Hydrastine RS

Powdered Goldenseal

DEFINITION

Powdered Goldenseal is Goldenseal reduced to a fine or very fine powder. It contains NLT 2.0% of hydrastine ($C_{21}H_{21}NO_6$) and NLT 2.5% of berberine ($C_{20}H_{18}NO_4$), calculated on the dried basis.

IDENTIFICATION

A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 0.5 mg/mL each of USP Berberine Chloride RS and USP Hydrastine RS in methanol

Sample solution: Transfer about 0.5 g of Powdered Goldenseal, accurately weighed, to a suitable glass vial, and add 0.5 mL of 10% sodium carbonate. Add 5 mL of methanol, and heat for 10 min in a water bath at 60°. Cool to room temperature, filter, and dry under a stream of nitrogen. Add 0.5 mL of methanol to dissolve the residue.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)

Application volume: 10–20 µL, as bands

Developing solvent system: Ethyl acetate, butyl alcohol, formic acid, and water (5:3:1:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved about three-fourths of the length of the plate, in a saturated chamber. Remove the plate, air-dry, and examine under UV light at 365 nm.

Acceptance criteria: The chromatograms show zones having a lemon-yellow fluorescence due to berberine at an R_f value of about 0.53 and a blue-white fluorescence due to hydrastine at an R_f value of about 0.42.

COMPOSITION**• CONTENT OF BERBERINE AND HYDRASTINE AND LIMIT OF PALMATINE**

Mobile phase: Dissolve 9.93 g of monobasic potassium phosphate in 730 mL of water, add 270 mL of acetonitrile, mix, filter, and degas.

Solvent: A mixture of 0.1 M monobasic potassium and acetonitrile (60:40)

Standard solution: 0.05 mg/mL each of USP Berberine Chloride RS and USP Hydrastine RS in a mixture of methanol and water (1:1)

System suitability solution: Prepare a solution of palmatine in a mixture of water and methanol (1:1) having a known concentration of about 0.05 mg/mL. Mix equal volumes of this solution and the *Standard solution*.

Sample solution: Transfer about 0.12 g of Powdered Goldenseal, accurately weighed, to a 50-mL volumetric flask. Add 40 mL of *Solvent*, sonicate for 5 min, and shake for 10 min. Dilute with *Solvent* to volume, mix, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 235 nm

Column: 4.6-mm × 150-mm; packing L1

Flow rate: 1.8 mL/min

Injection size: 10 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 1.5 between the berberine and palmatine peaks, and NLT 1.5 between the hydrastine and palmatine peaks, *System suitability solution*

Capacity factor: NLT 3.0, determined from the hydrastine and berberine peaks, *Standard solution*

Column efficiency: NLT 5000 theoretical plates determined from the hydrastine and berberine peaks, *Standard solution*

Tailing factor: NMT 2.0 determined from the hydrastine and berberine peaks, *Standard solution*

Relative standard deviation: NMT 2.5% determined from the hydrastine and berberine peaks in repeated injections, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of berberine and hydrastine in the portion of Powdered Goldenseal taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of berberine or hydrastine from the *Sample solution*

r_S = peak area of berberine or hydrastine from the *Standard solution*

C_S = concentration of berberine or hydrastine in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered Goldenseal used to prepare the *Sample solution* (mg)

Using the values from the chromatogram of the *Sample solution*, divide the peak area of berberine by the peak area of any peak at the locus for palmatine (if present).

Acceptance criteria: NLT 2.0% of hydrastine ($C_{21}H_{21}NO_6$) and NLT 2.5% of berberine ($C_{20}H_{18}NO_4$), on the dried basis. The ratio of the berberine peak area to any peak area at the locus for palmatine is more than 50:1.

CONTAMINANTS

- **HEAVY METALS, Method III** <231>: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** <561>: Meets the requirements

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** The powder is dark yellow to moderately greenish yellow with an aromatic odor and bitter taste. Abundant starch granules are present and are either spherical or ovoid; the granules are simple or compounded, with an occasional slit-shaped hilum. Parenchyma cells range from polygonal to round and are filled with starch or brown resin. Tracheary elements with slit-shaped pits and a few large reticulated vessels are present, as are beaded, thick-walled elongated epidermal cells. Thin fragments of cork layer are also present.

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** <561>: NMT 2.0%

- **LOSS ON DRYING** <731>: Dry 2 g of Powdered Goldenseal at 100° for 5 h: it loses NMT 12.0% of its weight.

- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 9%

- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** <561>: NMT 5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store protected from light and moisture.

- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.

- **USP REFERENCE STANDARDS** <11>

USP Berberine Chloride RS

USP Hydrastine RS

Powdered Goldenseal Extract

DEFINITION

Powdered Goldenseal Extract is prepared from the pulverized dried roots and rhizomes of *Hydrastis canadensis* L. (Fam. Ranunculaceae), using suitable solvents. It contains NLT 5% of hydrastine ($C_{21}H_{21}NO_6$) and NLT 10% of the sum of berberine ($C_{20}H_{18}NO_4$) and hydrastine, calculated on the dried basis. The ratio of starting crude plant material to Powdered Extract is 2:1.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 0.5 mg/mL each of USP Berberine Chloride RS and USP Hydrastine RS in methanol

Sample solution: 10 mg/mL of Powdered Extract in a mixture of methanol and water (1:1). Sonicate for 20 min, cool to room temperature, and filter.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)

Application volume: 10–20 µL, as bands

Developing solvent system: Ethyl acetate, butyl alcohol, formic acid, and water (5:3:1:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved about three-fourths of the length of the plate, in a saturated chamber. Remove the plate, air-dry, and examine under UV light at 365 nm.

Acceptance criteria: The chromatograms show zones having a lemon-yellow fluorescence due to berberine at an R_f value of about 0.53 and a blue-white fluorescence due to hydrastine at an R_f value of about 0.42.

COMPOSITION**• CONTENT OF BERBERINE AND HYDRASTINE AND LIMIT OF PALMATINE**

Mobile phase: Dissolve 9.93 g of monobasic potassium phosphate in 730 mL of water, add 270 mL of acetonitrile, mix, filter, and degas.

Standard solution: 0.05 mg/mL each of USP Berberine Chloride RS and USP Hydrastine RS in a mixture of methanol and water (1:1)

System suitability solution: Prepare a solution of palmatine in a mixture of water and methanol (1:1) having a known concentration of about 0.05 mg/mL. Mix equal volumes of this solution and the *Standard solution*.

Sample solution: 2 mg/mL of Powdered Extract in a mixture of methanol and water (1:1). Sonicate for 20 min, cool to room temperature, and filter. [NOTE—The sample to be used in this test should not be subjected to the conditions specified in the test for *Loss on Drying*. A separate sample is used to determine the content on the dried basis.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 235 nm

Column: 4.6-mm × 150-mm; packing L1

Flow rate: 1.8 mL/min

Injection size: 10 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 1.5 between the berberine and palmatine peaks, and NLT 1.5 between the hydrastine and palmatine peaks, *System suitability solution*

Capacity factor: NLT 3.0, determined from the hydrastine and berberine peaks, *Standard solution*

Column efficiency: NLT 5000 theoretical plates determined from the hydrastine and berberine peaks, *Standard solution*

Tailing factor: NMT 2.0 determined from the hydrastine and berberine peaks, *Standard solution*

Relative standard deviation: NMT 2.5% determined from the hydrastine and berberine peaks in repeated injections, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of hydrastine and berberine in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of berberine or hydrastine from the *Sample solution*

r_S = peak area of berberine or hydrastine from the *Standard solution*

C_S = concentration of berberine or hydrastine in the *Standard solution* (mg/mL)

C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Using the values from the chromatogram of the *Sample solution*, divide the peak area of berberine by the peak area of any peak at the locus for palmatine (if present).

Acceptance criteria: NLT 5% of hydrastine and NLT 10% of the sum of hydrastine and berberine, on the dried basis. The ratio of the berberine peak area to any peak area at the locus for palmatine is more than 50:1.

CONTAMINANTS

- **HEAVY METALS**, *Method II* <231>: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 1000 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **LOSS ON DRYING** (731): Dry 1.0 g of Powdered Extract at 105° for 2 h: it loses NMT 5.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store protected from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. Label to indicate the content of hydrastine and berberine, the extracting solvent used for preparation, and the ratio of the starting crude plant material to the Powdered Extract.
- **USP REFERENCE STANDARDS** <11>
 - USP Berberine Chloride RS
 - USP Hydrastine RS

Grape Seeds Oligomeric Proanthocyanidins

DEFINITION

Grape Seeds Oligomeric Proanthocyanidins is a fraction of an extract of the ripe seeds of *Vitis vinifera* L. (Fam. Vitaceae). It contains NLT 75.0% of oligomeric proanthocyanidins, on the anhydrous basis. The extract is prepared using suitable solvents such as alcohol, methanol, acetone, ethyl acetate, water, or mixtures of these solvents, in a ratio of starting plant material to extract between 70:1 and 10:1. The extract is further enriched in oligomeric proanthocyanidins by fractionation with ethyl acetate or by other means.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** <201>

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm and a layer thickness of about 0.2 mm (HPTLC plates)

Standard solution A: Dissolve a quantity of USP Purified Grape Seeds Oligomeric Proanthocyanidins RS in methanol, using sonication, to obtain a solution having a concentration of about 5 mg/mL. Centrifuge if necessary, and use the clear supernatant. [NOTE—Prepare fresh.]

Standard solution B: Dissolve a quantity of USP (+)-Catechin RS in methanol, using sonication, to obtain a solution having a concentration of about 1 mg/mL.

Sample solution: Proceed as directed for *Standard solution A*, except use the Grape Seeds Oligomeric Proanthocyanidins.

Developing solvent system: A mixture of acetone, toluene, and formic acid (15:15:5)

Spray reagent: Dissolve about 100 mg of vanillin in 3-mL of methanol using sonication. Add about 3 mL of hydrochloric acid, dilute with methanol to 10 mL, and carefully mix under cold water. [NOTE—Prepare fresh.]

Application volume: 15 µL, as 5–10-mm bands

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate (see *Chromatography* <621>). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about 90% of the plate. Remove the plate from the chamber, dry, spray with the *Spray reagent*, dry, and examine under visible light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits pink-violet bands, corresponding in color and R_f to those in the chromatogram of *Standard solution A*, at the following approximate R_f values: a pair of bands between 0.20 and 0.23 (trimeric proanthocyanidins), a band at 0.28 (proanthocyanidin-B₂-3'-O-gallate), a band at 0.31 (B-type dimeric proanthocyanidins), and a band at 0.43 ((-)-epicat-

echin-3-O-gallate). The chromatogram of the *Sample solution* may exhibit a pink-violet band at an approximate R_f of 0.49 (residual flavan 3-ol monomers and/or gallic acid) corresponding to the band in the chromatogram of *Standard solution B*. Other pink-violet bands may also be observed.

- **B.** The chromatogram of the *Sample solution* obtained in the test for *Limit of Catechin and Epicatechin* exhibits peaks due to proanthocyanidin dimer B₁, proanthocyanidin dimer B₂, (–)-epicatechin-3-O-gallate, and a broad peak due to other oligomeric proanthocyanidins at retention times corresponding to those in the chromatogram of *Standard solution B*.

COMPOSITION

• CONTENT OF OLIGOMERIC PROANTHOCYANIDINS

Internal standard solution: Prepare a solution of butylated hydroxytoluene in *Mobile phase* containing about 0.3 mg/mL.

Standard solution A: Dissolve a weighed quantity of USP Purified Grape Seeds Oligomeric Proanthocyanidins RS in *Internal standard solution* to obtain a solution having a known concentration of about 1.0 mg/mL.

Standard solution B: Dissolve a portion of USP (+)-Catechin RS in *Internal standard solution* to obtain a solution having a known concentration of about 0.2 mg/mL.

Sample solution: Dissolve a weighed quantity of Grape Seeds Oligomeric Proanthocyanidins in *Internal standard solution* to obtain a solution having a known concentration of about 1.0 mg/mL. Centrifuge, and use the clear supernatant.

Mobile phase: Prepare a filtered and degassed mixture of tetrahydrofuran and an aqueous solution of lithium bromide (about 1 mg/mL) (95:5).

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 7.5-mm × 30-cm; 5-μm, 500-Å, packing L21

Column temperature: 25° ± 1

Flow rate: 1.0 mL/min

Injection size: 10 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements: Measure the responses as determined under *Analysis*.

Relative standard deviation: NMT 2.0% determined from the the peak area ratio of the oligomeric proanthocyanidins to the internal standard in repeated injections, *Standard solution A*

Resolution: NLT 3.0 between the peaks of monomers and the internal standard, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Chromatograph *Standard solution A* and determine the beginning and end of the retention time window for the integration of oligomeric proanthocyanidins, at the points where the response of the main peak is about 0.5% of its maximum. Record the peak area ratio of the oligomeric proanthocyanidins to the internal standard. Chromatograph *Standard solution B* and the *Sample solution* and identify the locus for the monomers. Integrate the areas of the main peaks within the retention time window as determined for *Standard solution A*, excluding the area above the main peak, at the locus identified for the monomers, using a proper integration method.

Calculate the percentage of the oligomeric proanthocyanidins in the portion of the Grape Seeds Oligomeric Proanthocyanidins taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of the oligomeric proanthocyanidins to the internal standard from the *Sample solution*

R_S = peak response ratio of the oligomeric proanthocyanidins to the internal standard from *Standard solution A*

C_S = concentration of USP Purified Grape Seeds Oligomeric Proanthocyanidins RS in *Standard solution A* (mg/mL)

C_U = concentration of Grape Seeds Oligomeric Proanthocyanidins in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 75.0% on the anhydrous basis

IMPURITIES

Inorganic Impurities

- **HEAVY METALS**, *Method II* (231): NMT 10 ppm

Organic Impurities

- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements

SPECIFIC TESTS

• LIMIT OF CATECHIN AND EPICATECHIN

Solution A: Use acetonitrile.

Solution B: Use a 0.3% aqueous solution of 85% phosphoric acid.

Solvent: Prepare a mixture of *Solution A* and *Solution B* (1:9).

Standard solution A: Dissolve, using sonication, a weighed quantity of USP (+)-Catechin RS in *Solvent* to obtain a solution having a known concentration of about 0.5 mg/mL.

Standard solution B: Dissolve, using sonication, a weighed quantity of USP Grape Seeds Oligomeric Proanthocyanidins RS in *Solvent* to obtain a solution having a known concentration of about 5 mg/mL. Centrifuge, and use the clear supernatant.

Sample solution: Proceed as directed for *Standard solution B*, except use the Grape Seeds Oligomeric Proanthocyanidins.

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	10	90
45	20	80
65	60	40
66	10	90
85	10	90

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 278 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 0.7 mL/min

Injection size: 10 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

The chromatogram obtained from *Standard solution B* is similar to the Reference Chromatogram provided with the lot of the USP Grape Seeds Oligomeric Proanthocyanidins RS being used.

Tailing factor: NMT 2.0 for the catechin peak, *Standard solution A*

Relative standard deviation: NMT 2% determined from the catechin peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution A*, *Standard solution B*, and the Reference Chromatogram provided with the lot of USP Grape Seeds Oligomeric Proanthocyanidins RS being used, identify the retention times of the peaks corresponding to (+)-catechin and (–)-epicatechin. The approximate relative retention times of the peaks are 1.0 and 1.43 for (+)-catechin and (–)-epicatechin, respectively. Calculate the sum of the percentages of (+)-catechin and (–)-epicatechin in the portion of the Grape Seeds Oligomeric Proanthocyanidins taken:

$$(r_U/r_S) \times (C \times V/W) \times 100$$

- r_U = sum of the peak responses for (+)-catechin and (–)-epicatechin from the *Sample solution*
 r_S = peak response for (+)-catechin in *Standard solution A*
 C_S = concentration of USP (+)-Catechin RS in *Standard solution A* (mg/mL)
 V = final volume of the *Sample solution* (mL)
 W = weight of Grape Seeds Oligomeric Proanthocyanidins taken to prepare the *Sample solution* (mg)

Acceptance criteria: NMT 19.0% on the anhydrous basis

- MICROBIAL ENUMERATION TESTS (2021):** The total aerobic microbial count does not exceed 10^4 cfu/g. The total combined yeast and mold count does not exceed 10^3 cfu/g.
- ABSENCE OF SPECIFIED MICROORGANISMS (2022):** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- RESIDUE ON IGNITION (281):** NMT 0.5%, determined on 5.0 g
- WATER, Method 1a (921):** NMT 8.0%
- WATER-INSOLUBLE FRACTION**

Analysis: Transfer about 1 g, weighed, to a suitable flask. Add 100 mL of water, and shake vigorously for 15 min. Pass the solution through a previously tared sintered-glass filter, wash the flask with 30 mL of water, and transfer the washings to the filter. Wash the filter with 30 mL of water in 5-mL portions. Dry the filter for 2 h at 105°, cool in a desiccator, and weigh. Calculate the percentage of the water-insoluble fraction.

Acceptance criteria: NMT 2%

- OTHER REQUIREMENTS:** It meets the requirements of the test for *Residual Solvents* under *Botanical Extracts* (565).

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- LABELING:** The label states the Latin binomial and, following the official name, it states "Grape Seeds Oligomeric Proanthocyanidins". It meets other labeling requirements under *Botanical Extracts* (565).
- USP REFERENCE STANDARDS (11)**
 USP (+)-Catechin RS
 USP Grape Seeds Oligomeric Proanthocyanidins RS
 USP Purified Grape Seeds Oligomeric Proanthocyanidins RS

Powdered Decaffeinated Green Tea Extract

DEFINITION

Powdered Decaffeinated Green Tea Extract is prepared from the young, unfermented leaf and leaf buds of *Camellia sinensis* (L.) Kuntze (Fam. Theaceae), also known as *Thea sinensis* L., using suitable solvents such as alcohol, metha-

anol, acetone, or water or mixtures of these solvents; the caffeine has been removed. The ratio of the starting crude plant material to Powdered Extract is 6:1–10:1. It contains NLT 60.0% of polyphenols, calculated as (–)-epigallocatechin-3-O-gallate, NLT 40.0% of (–)-epigallocatechin-3-O-gallate, and NMT 0.1% of caffeine, calculated on the anhydrous basis.

IDENTIFICATION

A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 4 mg/mL of USP Powdered Decaffeinated Green Tea Extract RS in a mixture of alcohol and water (4:1), sonicate for 10 min, and centrifuge. Use the clear supernatant. [NOTE—Prepare fresh. Store below –20°, if storage is needed.]

Sample solution: 4 mg/mL of Powdered Decaffeinated Green Tea Extract in a mixture of alcohol and water (4:1), sonicate for 10 min, and centrifuge. Use the clear supernatant.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Adsorbent: Use a chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates).

Application volume: 1 µL

Developing solvent system: Toluene, acetone, and formic acid (9:9:2)

Immersion reagent: Dissolve 140 mg of fast blue B salt in 10 mL of water, and add 140 mL of methanol and 50 mL of dichloromethane. [NOTE—Prepare weekly, and store at 4° in the dark.]

Analysis

Samples: *Standard solution* and *Sample solution*
 Use an unsaturated chamber, and condition the plate to a relative humidity of 30% using a suitable device. Develop the chromatograms, dry the plate at 100°, dip in the *Immersion reagent*, dry, and immediately examine the plate under visible light. [NOTE—The chromatogram is stable up to 30 min; afterward, the plate's background darkens significantly.]

Acceptance criteria: The chromatogram of the *Sample solution* exhibits main bands similar in position and color to the main bands in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* exhibits four main brownish orange bands with R_f values of approximately 0.38, 0.48, 0.52, and 0.62 corresponding to (–)-epigallocatechin-3-O-gallate, (–)-epigallocatechin, (–)-epicatechin-3-O-gallate, and (–)-epicatechin, respectively. The most intense band is the one for (–)-epigallocatechin-3-O-gallate. The least intense band is the one for (–)-epicatechin.

B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of Polyphenols*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for (–)-epigallocatechin, (+)-catechin, (–)-epicatechin, (–)-epigallocatechin-3-O-gallate, (–)-gallocatechin-3-O-gallate, (–)-epigallocatechin-3-O-(3'-O-methyl)-gallate, and (–)-epicatechin-3-O-gallate at retention times corresponding to those in the chromatogram of *Standard solution B*.

COMPOSITION

C. CONTENT OF POLYPHENOLS

Solution A: Methanol, phosphoric acid 85%, and water (50:3.5:946.5)

Solution B: Acetonitrile and methanol (95:5)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	94	6
20	94	6
50	78	22

Table 1 (Continued)

Time (min)	Solution A (%)	Solution B (%)
70	38	62
75	94	6
90	94	6

Standard solution A: 0.1 mg/mL of USP (–)-Epigallocatechin-3-O-gallate RS in *Solution A*

Standard solution B: 0.4 mg/mL of USP Powdered Decaffeinated Green Tea Extract RS in *Solution A*. Mix, and centrifuge.

Sample solution: 0.4 mg/mL of Powdered Decaffeinated Green Tea Extract in *Solution A*. Mix, and centrifuge.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 278 nm

Column: 4.6-mm 6 25-cm; 5-μm packing L1

Column temperature: 25 ± 1°

Flow rate: 0.8 mL/min

Injection size: 15 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Decaffeinated Green Tea Extract RS being used.

Resolution: NLT 1 between the (–)-epigallocatechin-3-O-gallate peak and the preceding peak, *Standard solution B*

Tailing factor: 0.8–2.0 for the (–)-epigallocatechin-3-O-gallate peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the (–)-epigallocatechin-3-O-gallate peak, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Record the chromatograms, and measure the areas of the analyte peaks. Using the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Decaffeinated Green Tea Extract RS, identify the retention times of the peaks corresponding to the different polyphenols. The approximate relative retention times of the polyphenols are provided in *Table 2*.

Table 2

Analyte	Relative Retention Time
(–)-Epigallocatechin	0.56
(+)-Catechin	0.68
(–)-Epicatechin	0.98
(–)-Epigallocatechin-3-O-gallate	1.00
(–)-Gallocatechin-3-O-gallate	1.09
(–)-Epigallocatechin-3-O-(3'-O-methyl)-gallate	1.19
(–)-Epicatechin-3-O-gallate	1.27

Separately calculate the percentages of (–)-epigallocatechin, (+)-catechin, (–)-epicatechin, (–)-epigallocatechin-3-O-gallate, (–)-gallocatechin-3-O-gallate, (–)-epigallocatechin-3-O-(3'-O-methyl)-gallate, and (–)-epicatechin-3-O-gallate as (–)-epigallocatechin-3-O-gallate in the portion of the Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of each of the polyphenols in the *Sample solution*

r_S = peak area of (–)-epigallocatechin-3-O-gallate in *Standard solution A*

C_S = concentration of USP (–)- Epigallocatechin-3-O-gallate RS in *Standard solution A* (mg/mL)

C_U = concentration of Powdered Decaffeinated Green Tea Extract in the *Sample solution* (mg/mL)

Add the percentages calculated for the individual polyphenols.

Acceptance criteria: NLT 40.0% of (–)-epigallocatechin-3-O-gallate and NLT 60.0% of polyphenols, calculated as (–)-epigallocatechin-3-O-gallate on the anhydrous basis

CONTAMINANTS

• **HEAVY METALS, Method II** <231>: NMT 20 μg/g

• **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues** <561>: Meets the requirements

• **MICROBIAL ENUMERATION TEST** <2021>: The total aerobic microbial count does not exceed 10⁴ cfu/g, and the total combined yeasts and molds count does not exceed 10³ cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

LIMIT OF GALLIC ACID

Solution A, Solution B, Mobile phase, and

Chromatographic system: Proceed as directed in the test for *Content of Polyphenols*.

Standard solution: 0.2 mg/mL of gallic acid in *Solution A*

Sample solution: 20 mg/mL of Powdered Extract in *Solution A*. Mix, and centrifuge.

Analysis

Samples: *Standard solution* and *Sample solution*

Record the chromatograms, and measure the areas of the gallic acid peaks.

Separately calculate the percentages of gallic acid in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of gallic acid in the *Standard solution* (mg/mL)

C_U = concentration of Powdered Decaffeinated Green Tea Extract in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 1.0%

LIMIT OF CAFFEINE

Solution A: Methanol, tetrahydrofuran, phosphoric acid 85%, and water (50:10:3.5:936.5)

Solution B: Acetonitrile, methanol, and phosphoric acid 85% (946.5: 50: 3.5)

Mobile phase: See *Table 3*.

Table 3

Time (min)	Solution A (%)	Solution B (%)
0	100	0
30	100	0
35	0	100
40	0	100
45	100	0
55	100	0

Standard solution: 1 μg/mL of USP Caffeine RS in methanol

Sample solution: 1 mg/mL of Powdered Extract in methanol

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 272 nm

Column: 4.6-mm × 25-cm; 5-μm packing L60¹

Column temperature: 25 ± 1°

Flow rate: 1 mL/min

Injection size: 15 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0% determined from the caffeine peak

Analysis

Samples: *Standard solution* and *Sample solution*

Record the chromatograms, and measure the areas of the caffeine peaks.

Separately calculate the percentages of caffeine in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Caffeine RS in the *Standard solution* (mg/mL)

C_U = concentration of Powdered Decaffeinated Green Tea Extract in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.1%

- **WATER DETERMINATION, Method Ia <921>:** NMT 6.0%, determined on a 0.5 g
- **RESIDUE ON IGNITION <281>:** NMT 0.5%, determined on 1.0 g
- **OTHER REQUIREMENTS:** It meets the requirements of the test for *Residual Solvents* in *Botanical Extracts* <565>, *General Pharmacopeial Requirements*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements in *Botanical Extracts* <565>.
- **USP REFERENCE STANDARDS <11>**
 - USP Caffeine RS
 - USP (–)-Epigallocatechin-3-O-gallate RS
 - USP Powdered Decaffeinated Green Tea Extract RS

Guggul

DEFINITION

Guggul is the oleo-gum-resin obtained by incision or produced by spontaneous exudation from the stem and branches of *Commiphora wightii* (Arnott) Bhandari, also known as *Commiphora mukul* (Hook. ex. Stocks) Engl. or *Balsamodendrum mukul* (Hook.) (Fam. Burseraceae). It contains NLT 1.0% of guggulsterones E and Z, calculated on the dried basis as guggulsterone Z.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 10 mg/mL of USP Purified Guggul Extract RS with heating, in acetonitrile

Sample solution: Transfer about 0.5 g of crushed Guggul to a centrifuge tube. Add 25 mL of acetonitrile, and shake for 1 min. Heat in a water bath for 10–15 min

while shaking, cool, centrifuge, and use the supernatant.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Developing solvent system: A mixture of hexane and ethyl acetate (6:4)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm in length

Application volume: 10 μL

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands to a suitable plate. Use a saturated chamber. Develop until the solvent front has moved about three-fourths the length of the plate, dry the plate, and examine under UV light at 254 nm.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits bands at R_f values of about 0.38 and 0.47, due to guggulsterone E and Z, respectively. Both bands correspond in R_f values to bands from the *Standard solution*.

• **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed in the test for *Content of Guggulsterones E and Z*

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for guggulsterone E and Z at retention times that correspond to those of *Standard solution A*.

COMPOSITION

• **CONTENT OF GUGGULSTERONES E AND Z**

Mobile phase: A mixture of acetonitrile and water (45:55)

Standard solution A: 10 mg/mL of USP Purified Guggul Extract RS with heating, in acetonitrile. Pass the solution through a filter of 0.45-μm pore size before injection.

Standard solution B: 0.1 mg/mL of USP Guggulsterone Z RS in acetonitrile. Pass the solution through a filter of 0.45-μm pore size before injection.

Sample solution: Transfer about 2.0 g of crushed Guggul to a conical flask, and extract four times each with a 50-mL portion of acetonitrile. Shake for 1 min, and reflux in a water bath for 30 min, stirring with a magnetic stirrer. Evaporate the combined extracts to about 50 mL, and transfer to a 100-mL volumetric flask. Dilute with acetonitrile to volume, and pass through a filter of 0.45-μm pore size before injection.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 242 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 2.0 mL/min

Injection size: 20 μL

Column temperature: 27 ± 1°

System suitability

Samples: *Standard solution A* and *Standard solution B* [NOTE—The relative retention times for guggulsterones E and Z are about 0.69 and 1.0, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used.

Resolution: NLT 2.0 between the guggulsterone Z peak and the peak before, *Standard solution A*

Tailing factor: NMT 1.5 for the guggulsterone Z peak, *Standard solution B*

Relative standard deviation: NMT 2.0% for replicate injections for the guggulsterone Z peak, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

¹ Endcapped packing L1 columns can also be used in this test.

Allow *Standard solution A* to elute for NLT two times the retention time of guggulsterone *Z*, as determined in *Standard solution B*. Using the chromatogram of *Standard solution A* and the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used, identify the retention times of the peaks corresponding to guggulsterone *E* and guggulsterone *Z*. Calculate the percentage of guggulsterones *E* and *Z* as guggulsterone *Z* in the portion of Guggul taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

- r_U = sum of the peak responses of guggulsterones *E* and *Z* from the *Sample solution*
 r_S = peak response of guggulsterone *Z* from *Standard solution B*
 C_S = concentration of USP Guggulsterone *Z* RS in *Standard solution B* (mg/mL)
 V = final volume of the *Sample solution*, 100 mL
 W = weight of Guggul taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 1.0% on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method III* (231): NMT 20 µg/g
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Guggul occurs in rounded or irregular conglomerates of tears, of variable sizes, light to dark brown, slightly sticky to touch, with characteristic and aromatic odor, and an aromatic, astringent taste.

- **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* (561): NMT 1%
- **ARTICLES OF BOTANICAL ORIGIN**, *Alcohol-Soluble Extractives*, *Method 2* (561): NLT 33%
- **ETHYL ACETATE SOLUBLE EXTRACTIVES**

Sample: About 5.0 g of coarsely powdered Guggul

Analysis: Transfer the *Sample* to a glass-stoppered, conical flask. Add 25 mL of solvent hexane, insert a stopper into the flask, shake for 1 h, filter, and discard the filtrate. Repeat twice, and dry the residue in a vacuum over phosphorus pentoxide at room temperature for 8 h. Crush the dried material, and extract with four quantities, each of 25 mL, of ethyl acetate, by shaking each time for 1 h at room temperature, followed by filtration through a sintered glass funnel (porosity No. 3). Evaporate the combined filtrates under reduced pressure in a tared flask, dry the residue in a vacuum over phosphorus pentoxide at room temperature for 12 h, and weigh. Determine the percentage of the ethyl acetate soluble extractives calculated from the weight of Guggul taken.

Acceptance criteria: 22%–30%

- **LOSS ON DRYING** (731): Dry 1.0 g at 105° for 2 h: it loses NMT 8.0% of its weight.
- **RESIDUE ON IGNITION** (281): NMT 10.0%, ignited at 800 ± 25°
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* (561): NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial of the species of *Commiphora* from which the oleo-gum-resin was obtained and, following the official name, the part of the plant contained in the article.

- **USP REFERENCE STANDARDS** (11)
 USP Guggulsterone *Z* RS
 USP Purified Guggul Extract RS

Native Guggul Extract

DEFINITION

Native Guggul Extract is prepared from Guggul, using ethyl acetate, alcohol, or methanol. The ratio of starting crude plant material to Native Extract is approximately 9:1. It contains NLT 5.0% of guggulsterones *E* and *Z*, calculated on the anhydrous basis as guggulsterone *Z*. It does not contain added substances.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 10 mg/mL of USP Purified Guggul Extract RS, with heating, in acetonitrile

Sample solution: Homogenize Native Guggul Extract by heating in a water bath at 60°–80°. Prepare a 5-mg/mL solution in acetonitrile with heating.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Developing solvent system: A mixture of hexane and ethyl acetate (6:4)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm in length

Application volume: 10 µL

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands to a suitable plate. Use a saturated chamber. Develop until the solvent front has moved about three-fourths the length of the plate, dry the plate, and examine under UV light at 254 nm.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits bands at R_f values of about 0.38 and 0.47, due to guggulsterone *E* and *Z*, respectively. Both bands correspond in R_f to bands in the chromatogram from the *Standard solution*.

• B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of Guggulsterones E and Z*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for guggulsterones *E* and *Z* at retention times that correspond to those of *Standard solution A*.

COMPOSITION

• CONTENT OF GUGGULSTERONES *E* AND *Z*

Mobile phase: A mixture of acetonitrile and water (45:55)

Standard solution A: 10 mg/mL of USP Purified Guggul Extract RS, with heating, in acetonitrile. Pass the solution through a filter of 0.45-µm pore size before injection.

Standard solution B: 0.1 mg/mL of USP Guggulsterone *Z* RS in acetonitrile. Pass the solution through a filter of 0.45-µm pore size before injection.

Sample solution: Homogenize the Native Guggul Extract by heating in a water bath at 60°–80°. Dissolve a weighed quantity of homogenized Native Extract, with heating, in acetonitrile to obtain a solution having a known concentration of about 0.2 mg/mL of guggulsterones *E* and *Z*. Pass through a filter of 0.45-µm pore size before injection.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 242 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 2.0 mL/min

Injection size: 20 μL

Column temperature: 27 ± 1°

System suitability

Samples: *Standard solution A* and *Standard solution B*
[NOTE—The relative retention times for guggulsterones *E* and *Z* are about 0.69 and 1.0, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used.

Resolution: NLT 2.0 between the guggulsterone *Z* peak and the peak before, *Standard solution A*

Tailing factor: NMT 1.5 for the guggulsterone *Z* peak, *Standard solution B*

Relative standard deviation: NMT 2.0% for the guggulsterone *Z* peak (replicate injections), *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Allow *Standard solution A* to elute for NLT two times the retention time of guggulsterone *Z*, as determined in *Standard solution B*. Using the chromatogram of *Standard solution A* and the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used, identify the retention times of the peaks corresponding to guggulsterone *E* and guggulsterone *Z*. Calculate the percentage of guggulsterones *E* and *Z* as guggulsterone *Z* in the portion of Native Guggul Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of the peak responses of guggulsterones *E* and *Z* from the *Sample solution*

r_S = peak response of guggulsterone *Z* from *Standard solution B*

C_S = concentration of USP Guggulsterone *Z* RS in *Standard solution B* (mg/mL)

V = final volume of *Sample solution* (mL)

W = weight of Native Extract taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 5.0% of guggulsterones *E* and *Z*, calculated as guggulsterone *Z*, on the anhydrous basis

CONTAMINANTS

- **HEAVY METALS**, *Method II* (231): NMT 20 μg/g
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **BOTANICAL EXTRACTS**, *Residual Solvents* (565): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10⁴ cfu/g, and the total combined yeasts and molds count does not exceed 10³ cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **WATER DETERMINATION**, *Method 1a* (921): NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from

which the article was derived. It meets other labeling requirements under *Botanical Extracts* (565).

- **USP REFERENCE STANDARDS** (11)
USP Guggulsterone *Z* RS
USP Purified Guggul Extract RS

Purified Guggul Extract

DEFINITION

Purified Guggul Extract is prepared from Native Guggul Extract by fractionation using aqueous methanol. It contains NLT 90.0% and NMT 110.0% of the labeled amount of guggulsterones *E* and *Z*, calculated as guggulsterones *Z*. It may be a semisolid extract with no added substances or powder extract containing suitable added substances.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 10 mg/mL of USP Purified Guggul Extract RS, with heating, in acetonitrile.

Sample solution: 10 mg/mL in acetonitrile from Purified Guggul Extract, dissolved with heating, cooled, and centrifuged.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Developing solvent system: A mixture of hexane and ethyl acetate (6:4)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm in length

Application volume: 10 μL

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands to a suitable plate. Use a saturated chamber. Develop until the solvent front has moved about three-fourths the length of the plate, dry the plate, and examine under UV light at 254 nm.

Acceptance criteria: The chromatograph of the *Sample solution* exhibits bands at R_f values of about 0.38 and 0.47, due to guggulsterone *E* and *Z*, respectively. Both bands correspond in R_f to bands in the chromatogram from the *Standard solution*.

• B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of Guggulsterones E and Z*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for guggulsterones *E* and *Z* at retention times that correspond to those of *Standard solution A*.

COMPOSITION

• CONTENT OF GUGGULSTERONES E AND Z

Mobile phase: A mixture of acetonitrile and water (45:55)

Standard solution A: 10 mg/mL of USP Purified Guggul Extract RS, with heating, in acetonitrile. Pass the solution through a filter of 0.45-μm pore size before injection.

Standard solution B: 0.1 mg/mL of USP Guggulsterone *Z* RS in acetonitrile. Pass the solution through a filter of 0.45-μm pore size before injection.

Sample solution: Dissolve a weighed quantity of Purified Guggul Extract, with heating, in acetonitrile to obtain a solution having a known concentration of about 0.2 mg/mL of guggulsterones *E* and *Z*. Pass through a filter of 0.45-μm pore size before injection.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC
 Detector: UV 242 nm
 Column: 4.6-mm × 25-cm; 5-μm packing L1
 Flow rate: 2.0 mL/min
 Injection size: 20 μL
 Column temperature: 27 ± 1°

System suitability

Samples: *Standard solution A* and *Standard solution B*
 [NOTE—The relative retention times for guggulsterones *E* and *Z* are about 0.69 and 1.0, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used.

Resolution: NLT 2.0 between the guggulsterone *Z* peak and the peak before, *Standard solution A*

Tailing factor: NMT 1.5 for guggulsterone *Z* peak, *Standard solution B*

Relative standard deviation: NMT 2.0% for replicate injections for the guggulsterone *Z* peak, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Allow *Standard solution A* to elute for NLT two times the retention time of guggulsterone *Z*, as determined in *Standard solution B*. Using the chromatogram of *Standard solution A* and the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used, identify the retention times of the peaks corresponding to guggulsterone *E* and guggulsterone *Z*. Calculate the percentage of guggulsterones *E* and *Z* as guggulsterone *Z* in the portion of Purified Guggul Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = sum of the peak responses of guggulsterones *E* and *Z* from the *Sample solution*

r_S = peak response of guggulsterone *Z* from *Standard solution B*

C_S = concentration of USP Guggulsterone *Z* RS in *Standard solution B* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Purified Guggul Extract taken to prepare the *Sample solution* (mg)

Acceptance criteria: 90.0%–110.0% of the labeled amount of guggulsterones *E* and *Z*, calculated as guggulsterones *Z*

CONTAMINANTS

- **HEAVY METALS, Method II <231>:** NMT 20 μg/g
- **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis <561>:** Meets the requirements
- **BOTANICAL EXTRACTS, Residual Solvents <565>:** Meets the requirements
- **MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic microbial count does not exceed 10⁴ cfu/g, and the total combined yeasts and molds count does not exceed 10³ cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store in a cool place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements in *Botanical Extracts* <565>.

• **USP REFERENCE STANDARDS <11>**

USP Guggulsterone *Z* RS
 USP Purified Guggul Extract RS

Guggul Tablets**DEFINITION**

Guggul Tablets are prepared from Native Guggul Extract or Purified Guggul Extract. Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of Extract, calculated as the sum of guggulsterones *E* and *Z*.

IDENTIFICATION• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 10 mg/mL of USP Purified Guggul Extract RS, with heating, in acetonitrile

Sample solution: Powder and transfer a portion of the Tablets equivalent to 100 mg of Extract to a conical flask. Extract three times, each with 25 mL of acetonitrile, in a 55° water bath for 15 min, stirring with a magnetic stirrer, and filter. Evaporate the combined extracts to dryness in a vacuum between 45° and 50°, dissolve the residue in 10 mL of acetonitrile, centrifuge, and use the clear supernatant.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Developing solvent system: A mixture of hexane and ethyl acetate (6:4)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm in length

Application volume: 10 μL

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands to a suitable plate. Use a saturated chamber. Develop until the solvent front has moved about three-fourths the length of the plate, dry the plate, and examine under UV light at 254 nm.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits bands at R_f values of about 0.38 and 0.47, due to guggulsterone *E* and *Z*, respectively. Both bands correspond in R_f to bands in the chromatogram from the *Standard solution*.

• **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed in the test for *Content of Guggulsterones E and Z*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for guggulsterones *E* and *Z* at retention times that correspond to those of *Standard solution A*.

STRENGTH• **CONTENT OF GUGGULSTERONES E AND Z**

Mobile phase: A mixture of acetonitrile and water (45:55)

Standard solution A: 10 mg/mL of USP Purified Guggul Extract RS, with heating, in acetonitrile. Pass the solution through a filter of 0.45-μm pore size before injection.

Standard solution B: 0.1 mg/mL of USP Guggulsterone *Z* RS in acetonitrile. Pass the solution through a filter of 0.45-μm pore size before injection.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a weighed amount of the powder, equivalent to about 10 mg of guggulsterones *E* and *Z*, to a conical flask, and extract five times, each with a 20-mL portion of acetonitrile, shake for 1 min, and reflux in a water bath for 30 min, stirring with a magnetic stirrer. Evaporate the combined extracts to dryness in a vacuum at 45°–50°. Dissolve the residue in 50.0 mL of acetonitrile, and pass through a filter of 0.45-μm pore size before injection.

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 242 nm**Column:** 4.6-mm × 25-cm; 5-μm packing L1**Flow rate:** 2.0 mL/min**Injection size:** 20 μL**Column temperature:** 27 ± 1°**System suitability****Samples:** *Standard solution A* and *Standard solution B*
[NOTE—The relative retention times for guggulsterones *E* and *Z* are about 0.69 and 1.0, respectively.]**Suitability requirements****Chromatogram similarity:** The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used.**Resolution:** NLT 2.0 between the guggulsterone *Z* peak and the peak before, *Standard solution A***Tailing factor:** NMT 1.5 for the guggulsterone *Z* peak, *Standard solution B***Relative standard deviation:** NMT 2.0% for the guggulsterone *Z* peak (replicate injections), *Standard solution B***Analysis****Samples:** *Standard solution A*, *Standard solution B*, and *Sample solution*Allow *Standard solution A* to elute for NLT two times the retention time of guggulsterone *Z*, as determined in *Standard solution B*. Using the chromatogram of *Standard solution A* and the reference chromatogram provided with the lot of USP Purified Guggul Extract RS being used, identify the retention times of the peaks corresponding to guggulsterone *E* and guggulsterone *Z*. Calculate the content of guggulsterones *E* and *Z* as guggulsterone *Z* in the portion of Tablets taken:

$$C_i = (r_U/r_S) \times C_S \times V$$

 r_U = sum of the peak responses of guggulsterones *E* and *Z* from the *Sample solution* r_S = peak response of guggulsterone *Z* from *Standard solution B* C_S = concentration of USP Guggulsterone *Z* RS in *Standard solution B* (mg/mL) V = final volume of the *Sample solution* (mL)

Calculate the percentage of the labeled amount of Guggul Extract taken:

$$\text{Result} = C_i \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

 C_i = content of guggulsterones *E* and *Z* in the portion of Tablets taken (mg) A_{WT} = average weight of Tablets (mg) W = weight of the powdered Tablets taken (mg) L_E = labeled percentage of the sum of guggulsterones *E* and *Z* in the Extract used to prepare the Tablets L = label claim of Extract (mg/Tablet)**Acceptance criteria:** 90.0%–110.0% of the labeled amount of Extract, calculated as the sum of guggulsterones *E* and *Z***PERFORMANCE TESTS**

- **DISINTEGRATION AND DISSOLUTION** <2040>: It meets the requirement for *Disintegration* only; 30 min, the use of the disk being omitted.
- **WEIGHT VARIATION** (2091): Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 10⁴ cfu/g, and the total combined yeasts and molds count does not exceed 10³ cfu/g.

- **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **RESIDUAL SOLVENTS** <467>: Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binominal and, following the official name, the article from which the Tablets were prepared. The label also indicates the amount of Extract, in mg/Tablet, and the content, in mg, of guggulsterones *E* and *Z* per 100 mg of Extract.
- **USP REFERENCE STANDARDS** <11>
USP Guggulsterone *Z* RS
USP Purified Guggul Extract RS

Hawthorn Leaf with Flower

DEFINITIONHawthorn Leaf with Flower consists of the dried tips of the flower-bearing branches up to 7 cm in length of *Crataegus monogyna* Jacq. emend Lindman. or *Crataegus laevigata* (Poir.) DC., also known as *Crataegus oxyantha* Linné (Fam. Rosaceae). It contains NLT 0.6% of C-glycosylated flavones, expressed as vitexin (C₂₁H₂₀O₁₀), and NLT 0.45% of O-glycosylated flavones, expressed as hyperoside (C₂₁H₂₀O₁₂), calculated on the dried basis.**IDENTIFICATION**

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** <201>

Standard solution: 0.1 mg/mL each of chlorogenic acid, rutin, USP Hyperoside RS, and USP Vitexin RS in methanol. [NOTE—Reserve a portion of this solution for use in *Identification test B*.]**Sample solution:** 0.1 g/mL, prepared as follows. Finely powder 10 g of Hawthorn Leaf with Flower. Transfer 1 g of the powder to a flask, and add 10 mL of methanol. Heat the flask on a water bath maintained at 65° for 5 min, cool, filter, and use the filtrate.**Adsorbent:** 0.50-mm layer of chromatographic silica gel mixture**Developing solvent system:** Ethyl acetate, glacial acetic acid, formic acid, and water (10:1.1:1.1:2.6)**Spray reagent A:** 2-aminoethyl diphenylborinate in methanol (1 in 100)**Spray reagent B:** Polyethylene glycol 4000 in methanol (5 in 100)**Analysis****Samples:** *Standard solution* and *Sample solution*Proceed as directed in the chapter, except to dry the plate at 105°, and spray the plate while still hot with 10 mL of *Spray reagent A* and then with 10 mL of *Spray reagent B*. Allow the plate to air-dry for 30 min, and examine the plate under long-wavelength UV light.**Acceptance criteria:** The chromatogram of the *Standard solution* exhibits an intense orange zone (at R_f value of 0.3) due to rutin; a light blue fluorescent zone (at R_f value of 0.4) due to chlorogenic acid; a yellowish-orange zone (at R_f value of 0.55) due to hyperoside; and a yellowish-green zone (at R_f value of 0.65) due to vitexin. The chromatogram of the *Sample solution*, in addition to the zones due to rutin, chlorogenic acid, hyperoside, and vitexin, exhibits a yellowish-green zone (at R_f value of 0.35) due to vitexin-2-rhamnoside; a light blue fluorescent zone (at R_f value of 0.6) due to spiraeoside; and a light blue fluorescent zone near the solvent front (at R_f value of 0.9) due to caffeic acid. The

chromatogram of the *Sample solution* also exhibits other zones with weaker fluorescence.

• **B. PROCEDURE**

Solution A: Tetrahydrofuran, acetonitrile, and methanol (92.4:3.4:4.2)

Solution B: 0.5% phosphoric acid in water

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	12	88
12	12	88
25	18	82
30	18	82

Standard solution: Use the *Standard solution* reserved from *Identification* test A.

Sample solution: Transfer 3 g of finely powdered Hawthorn Leaf with Flower to a 100-mL round-bottom flask, add 60 mL of a mixture of methanol and water (4:1), and heat on a hot water bath under reflux for 1 h. Cool, filter, and collect the filtrate in a separate flask. Transfer the residue from the filter back to the flask, add 40 mL of a mixture of methanol and water (4:1), and heat on a hot water bath under reflux for 10 min. Cool, filter, and combine the filtrate with the filtrate obtained from the first extraction. Evaporate the solvent from the combined filtrates under vacuum to a volume of 20 mL. Dilute the resulting solution with a mixture of methanol and water (4:1) to 25.0 mL. Filter 5.0 mL of this solution through a freshly conditioned solid-phase extraction column containing 360 mg of packing L1, collect the filtrate in a 10-mL volumetric flask, and dilute with a mixture of methanol and water (4:1) to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 336 nm

Column: 4.0-mm × 10-cm; 5-μm packing L1

Temperature: 25°

Flow rate: 1 mL/min

Injection size: 5 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for chlorogenic acid, vitexin, rutin, and hyperoside are 0.26, 1.0, 1.16, and 1.4, respectively.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—The relative retention times for acetyl vitexin-2''-O-rhamnoside, vitexin, isovitexin, and vitexin-2''-O-rhamnoside are 1.53, 1.0, 0.73, and 0.67, respectively.]

Measure the retention times for the major peaks.

Acceptance criteria: The retention times of the peaks for chlorogenic acid, vitexin, rutin, and hyperoside of the *Sample solution* correspond to those of the *Standard solution*.

COMPOSITION

• **CONTENT OF C-GLYCOSYLATED FLAVONES**

Solution A: 0.5% solution of phosphoric acid in water
Solution B: Tetrahydrofuran, isopropyl alcohol, and acetonitrile (10:8:3)

Mobile phase: *Solution A* and *Solution B* (22:3)

Standard solution: 0.3 mg/mL of USP Vitexin RS in *Solution B*, with heating if necessary

Sample solution: Finely powder 100 g of Hawthorn Leaf with Flower. Transfer about 4 g of the powder, accurately weighed, to a continuous-extraction apparatus fitted with a flask containing 80 mL of methanol, and

extract the test specimen for 5 h. Cool, remove the flask, and evaporate the solvent from the extract under vacuum to 40 mL. Transfer this solution to a 50-mL volumetric flask, and dilute with methanol to volume. Transfer 10.0 mL of the solution to a suitable flask fitted with a reflux condenser, add 4 mL of 25% hydrochloric acid, and heat the flask under reflux on a water bath at 65° for 90 min. Cool, transfer the contents of the flask to a 50-mL volumetric flask, and dilute with methanol to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 336 nm

Column: 4-mm × 10-cm; packing L1

Flow rate: 1 mL/min

Injection size: 5 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 3000 theoretical plates

Tailing factor: 0.8–2

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of C-glycosylated flavones, expressed as vitexin (C₂₁H₂₀O₁₀), in the portion of Hawthorn Leaf with Flower taken:

$$\text{Result} = (C_s/C_u) \times (\Sigma r_u/r_s) \times 100$$

C_s = concentration of USP Vitexin RS in the *Standard solution* (mg/mL)

C_u = concentration of Hawthorn Leaf with Flower in the *Sample solution* (mg/mL)

Σr_u = sum of the peak areas of vitexin and isovitexin, with a relative retention time of about 1.0 and 0.85, respectively, in the chromatogram of the *Sample solution*

r_s = vitexin peak area of the *Standard solution*

Acceptance criteria: NLT 0.6% of C-glycosylated flavones, expressed as vitexin (C₂₁H₂₀O₁₀) on the dried basis

• **CONTENT OF O-GLYCOSYLATED FLAVONES**

Mobile phase: Methanol, phosphoric acid, and water (100:1:100)

Standard solution: 0.05 mg/mL of USP Quercetin RS in methanol

Sample solution: Proceed as directed for the *Sample solution* under *Content of C-Glycosylated Flavones*, except to use 1 mL of 25% hydrochloric acid for 60 min instead of 4 mL of 25% hydrochloric acid for 90 min.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 370 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 3000 theoretical plates

Tailing factor: 0.8–2

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of O-glycosylated flavones, expressed as hyperoside (C₂₁H₂₀O₁₂), in the portion of Hawthorn Leaf with Flower taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times (M_{r1}/M_{r2}) \times 100$$

- r_U = quercetin peak area from the *Sample solution*
 r_S = quercetin peak area from the *Standard solution*
 C_S = concentration of USP Quercetin RS in the *Standard solution* (mg/mL)
 C_U = concentration of Hawthorn Leaf with Flower in the *Sample solution* (mg/mL)
 M_{r1} = molecular weight of hyperoside, 464.38
 M_{r2} = molecular weight of quercetin, 302.24

Acceptance criteria: NLT 0.45% of O-glycosylated flavones, expressed as hyperoside ($C_{21}H_{20}O_{12}$) on the dried basis

IMPURITIES

Inorganic Impurities

- **HEAVY METALS, Method III (231):** NMT 20 ppm

Organic Impurities

- **PROCEDURE 1: ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter (561):** NMT 8.0% of lignified matter
- **PROCEDURE 2: ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues (561):** Meets the requirements

SPECIFIC TESTS

BOTANIC CHARACTERISTICS

Macroscopic: It shows fragments of dark brown, lignified branches, usually from 1 mm to NMT 2.5 mm in diameter, bearing alternate petiolate leaves, with small, often deciduous styles, and bearing numerous white flowers in a corymbose arrangement. The leaves are more or less strongly lobate, and their margins are slightly or very slightly serrate. *C. laevigata* has pinnatilobate to pinnatifid leaves, divided into three, five, or seven obtuse lobes; the leaves of *C. monogyna* are almost pinnatisect with three to five acute lobes. The adaxial surface of the leaf is dark green to brownish-green; the abaxial surface is lighter, greyish-green, and shows a dense network of clearly visible veinlets and slightly prominent principal veins. The leaves of *C. laevigata* and *C. monogyna* are glabrous or bear isolated trichomes. The flowers consist of a brownish-green tubulous calyx, ending in its upper part in five triangular segments, and of five yellowish-white to brownish free petals, rounded to widely oval, shortly unguiculate, and with numerous stamens. The ovary, fused to the tubulous calyx, bears one to three long styles and consists of the same number of carpels, each containing one fertile ovule. *C. monogyna* has one style and one carpel, and *C. laevigata* has two or three styles and carpels.

Microscopic: When reduced to a fine powder and examined under a microscope, the yellowish-green powder shows the following characteristics: unicellular covering trichomes, usually with thick walls and wide lumens, almost straight to somewhat curved, pitted at the base; fragments of leaf epidermis with cells that have sinuous to polygonal walls and large anomocytic stomata surrounded by four to seven subsidiary cells; clusters of parenchymatous cells containing calcium oxalate crystals, usually from 10–20 μ m in length; fragments of petals showing rounded polygonal epidermal cells, strongly papillose, with thick walls, the cuticle of which clearly shows wavy striations; fragments of anthers whose endothecium has an arched and regularly thickened margin; fragments of stems containing collenchymatous cells, vessels and fibers of lignified sclerenchyma, with narrow lumens; numerous rounded to elliptical triangular pollen grains up to 45 μ m in diameter, with free exines and three germinal pores.

- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS (2021):** The total bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 100 cfu/g, and it meets the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

- **LOSS ON DRYING (731):** Dry about 1.0 g of finely powdered Hawthorn Leaf with Flower at 105° for 2 h: it loses NMT 10.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 9.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in a well-closed container, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS (11)**
 USP Hyperoside RS
 USP Quercetin RS
 USP Vitexin RS

Powdered Hawthorn Leaf with Flower

DEFINITION

Powdered Hawthorn Leaf with Flower is Hawthorn Leaf with Flower reduced to a fine or very fine powder.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)**

Standard solution: 0.1 mg/mL each of chlorogenic acid, rutin, USP Hyperoside RS, and USP Vitexin RS in methanol. [NOTE—Reserve a portion of this solution for use in *Identification test B*.]

Sample solution: Transfer about 1 g of Powdered Hawthorn Leaf with Flower to a flask, and add 10 mL of methanol. Heat the flask on a water bath maintained at 65° for 5 min, cool, filter, and use the filtrate.

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Developing solvent system: Ethyl acetate, glacial acetic acid, formic acid, and water (10:1.1:1.1:2.6)

Spray reagent A: 2-Aminoethyl diphenylborinate in methanol (1 in 100)

Spray reagent B: Polyethylene glycol 4000 in methanol (5 in 100)

Analysis

Samples: *Standard solution* and *Sample solution*
 Proceed as directed in the chapter, except to dry the plate at 105°, and spray the plate while still hot with 10 mL of *Spray reagent A* and then with 10 mL of *Spray reagent B*. Allow the plate to air-dry for 30 min, and examine the plate under long-wavelength UV light.

Acceptance criteria: The chromatogram of the *Standard solution* exhibits an intense orange zone (at R_f value of 0.3) due to rutin; a light blue fluorescent zone (at R_f value of 0.4) due to chlorogenic acid; a yellowish-orange zone (at R_f value of 0.55) due to hyperoside; and a yellowish-green zone (at R_f value of 0.65) due to vitexin. The chromatogram of the *Sample solution*, in addition to the zones due to rutin, chlorogenic acid, hyperoside, and vitexin, exhibits a yellowish-green zone (at R_f value of 0.35) due to vitexin-2-rhamnoside; a light blue fluorescent zone (at R_f value of 0.6) due to spiraeoside; and a light blue fluorescent zone near the solvent front (at R_f value of 0.9) due to caffeic acid. The chromatogram of the *Sample solution* also exhibits other zones with weaker fluorescence.

- **B. PROCEDURE**

Solution A: Tetrahydrofuran, acetonitrile, and methanol (92.4:3.4:4.2)

Solution B: 0.5% phosphoric acid in water
Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	12	88
12	12	88
25	18	82
30	18	82

Standard solution: Use the *Standard solution* reserved from *Identification* test A.

Sample solution: Transfer about 3 g of Powdered Hawthorn Leaf with Flower to a 100-mL round-bottom flask, add 60 mL of a mixture of methanol and water (4:1), and heat on a hot water bath under reflux for 1 h. Cool, filter, and collect the filtrate in a separate flask. Transfer the residue from the filter back to the flask, add 40 mL of a mixture of methanol and water (4:1), and heat on a hot water bath under reflux for 10 min. Cool, filter, and combine the filtrate with the filtrate obtained from the first extraction. Evaporate the solvent from the combined filtrates under vacuum to a volume of 20 mL. Dilute the resulting solution with a mixture of methanol and water (4:1) to 25.0 mL. Filter 5.0 mL of this solution through a freshly conditioned solid-phase extraction column containing 360 mg of packing L1, collect the filtrate in a 10-mL volumetric flask, and dilute with a mixture of methanol and water (4:1) to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 336 nm

Column: 4.0-mm × 10-cm; 5-μm packing L1

Temperature: 25°

Flow rate: 1 mL/min

Injection size: 5 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for chlorogenic acid, vitexin, rutin, and hyperoside are 0.26, 1.0, 1.16, and 1.4, respectively.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—The relative retention times for acetyl vitexin-2''-O-rhamnoside, vitexin, isovitexin, and vitexin-2''-O-rhamnoside are 1.53, 1.0, 0.73, and 0.67, respectively.]

Measure the retention times for the major peaks.

Acceptance criteria: The retention times of the peaks for chlorogenic acid, vitexin, rutin, and hyperoside of the *Sample solution* correspond to those of the *Standard solution*.

COMPOSITION

• CONTENT OF C-GLYCOSYLATED FLAVONES

Solution A: 0.5% solution of phosphoric acid in water

Solution B: Tetrahydrofuran, isopropyl alcohol, and acetonitrile (10:8:3)

Mobile phase: *Solution A* and *Solution B* (22:3)

Standard solution: 0.3 mg/mL of USP Vitexin RS in *Solution B*, with heating if necessary

Sample solution: Transfer about 4 g of Powdered Hawthorn Leaf with Flower, accurately weighed, to a continuous-extraction apparatus fitted with a flask containing 80 mL of methanol, and extract the test specimen for 5 h. Cool, remove the flask, and evaporate the solvent from the extract under vacuum to 40 mL. Transfer this solution to a 50-mL volumetric flask, and dilute with methanol to volume. Transfer 10.0 mL of the solution to a suitable flask fitted with a reflux condenser, add 4 mL

of 25% hydrochloric acid, and heat the flask under reflux on a water bath at 65° for 90 min. Cool, transfer the contents of the flask to a 50-mL volumetric flask, and dilute with methanol to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 336 nm

Column: 4-mm × 10-cm; packing L1

Flow rate: 1 mL/min

Injection size: 5 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 3000 theoretical plates

Tailing factor: 0.8–2

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of C-glycosylated flavones, expressed as vitexin (C₂₁H₂₀O₁₀), in the portion of Powdered Hawthorn Leaf with Flower taken:

$$\text{Result} = (\Sigma r_U / r_S) \times (C_S / C_U) \times 100$$

Σr_U = sum of the peak areas of vitexin and isovitexin, with a relative retention time of about 1.0 and 0.85, respectively, from the *Sample solution*

r_S = vitexin peak area from the *Standard solution*

C_S = concentration of USP Vitexin RS in the *Standard solution* (mg/mL)

C_U = concentration of Powdered Hawthorn Leaf with Flower in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.6% of C-glycosylated flavones, expressed as vitexin (C₂₁H₂₀O₁₀)

• CONTENT OF O-GLYCOSYLATED FLAVONES

Mobile phase: Methanol, phosphoric acid, and water (100:1:100)

Standard solution: 0.05 mg/mL of USP Quercetin RS in methanol

Sample solution: Proceed as directed for the *Sample solution* under *Content of C-Glycosylated Flavones*, except to use 1 mL of 25% hydrochloric acid for 60 min instead of 4 mL of 25% hydrochloric acid for 90 min.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 370 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 3000 theoretical plates

Tailing factor: 0.8–2

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of O-glycosylated flavones, expressed as hyperoside (C₂₁H₂₀O₁₂), in the portion of Powdered Hawthorn Leaf with Flower taken:

$$\text{Result} = (r_U / r_S) \times (C_S / C_U) \times (M_{r1} / M_{r2}) \times 100$$

r_U = quercetin peak area from the *Sample solution*

r_S = quercetin peak area from the *Standard solution*

C_S = concentration of USP Quercetin RS in the *Standard solution* (mg/mL)

C_U = concentration of Powdered Hawthorn Leaf with Flower in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of hyperoside, 464.38

M_{r2} = molecular weight of quercetin, 302.24

Acceptance criteria: NLT 0.45% of *O*-glycosylated flavones, expressed as hyperoside ($C_{21}H_{20}O_{12}$)

IMPURITIES

Inorganic Impurities

- **HEAVY METALS**, *Method III* (231): NMT 20 ppm

Organic Impurities

- **PROCEDURE 1: ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* (561): NMT 8.0% of lignified matter
- **PROCEDURE 2: ARTICLES OF BOTANICAL ORIGIN**, *Pesticide Residues* (561): Meets the requirements

SPECIFIC TESTS

BOTANIC CHARACTERISTICS

Microscopic: When examined under a microscope, the yellowish-green powder shows the following characteristics: unicellular covering trichomes, usually with thick walls and wide lumens, almost straight to somewhat curved, pitted at the base; fragments of leaf epidermis with cells that have sinuous to polygonal walls and large anomocytic stomata surrounded by four to seven subsidiary cells; clusters of parenchymatous cells containing calcium oxalate crystals, usually from 10–20 μ m in length; fragments of petals showing rounded polygonal epidermal cells, strongly papillose, with thick walls, the cuticle of which clearly shows wavy striations; fragments of anthers whose endothecium has an arched and regularly thickened margin; fragments of stems containing collenchymatous cells, vessels, and fibers of lignified sclerenchyma, with narrow lumens; and numerous rounded to elliptical triangular pollen grains up to 45 μ m in diameter, with free exines and three germinal pores.

- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS** (2021): The total bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 100 cfu/g, and it meets the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.
- **LOSS ON DRYING** (731): Dry about 1.0 g of Powdered Hawthorn Leaf with Flower at 105° for 2 h: it loses NMT 10.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 9.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in a well-closed container, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant source from which the article was derived.
- **USP REFERENCE STANDARDS** (11)
 - USP Hyperoside RS
 - USP Quercetin RS
 - USP Vitexin RS

Histidine—see *Histidine General Monographs*

Horse Chestnut

DEFINITION

Horse Chestnut consists of the dried seeds of *Aesculus hippocastanum* L. (Fam. Hippocastanaceae). It is harvested in the fall. It contains NLT 3.0% of triterpene glycosides, calculated on the dried basis as escin ($C_{55}H_{86}O_{24}$).

IDENTIFICATION

A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 5 mg/mL of USP Escin RS in methanol

Sample solution: Transfer 1 g of the powdered plant material to a screw-capped centrifuge tube, add 10 mL of a mixture of alcohol and water (7:3), and heat on a steam bath for 10 min. Centrifuge, and use the clear supernatant.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 10 μ L

Developing solvent system: Use the upper phase of a mixture of 1-butanol, glacial acetic acid, and water (5:1:4).

Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85:10:5:0.5)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms to a length of NLT 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, heat the plate at 100° for 5 min, and examine the plate under daylight.

Acceptance criteria: The chromatogram from the *Sample solution* shows a blue-violet zone corresponding to escin, comparable in position and color to the main zone in the chromatogram from the *Standard solution*. Above this zone, the chromatogram of the *Sample solution* shows several narrow, brown to brownish-red zones that are less intense than the zone corresponding to escin.

COMPOSITION

CONTENT OF TRITERPENE GLYCOSIDES

Solvent A: Methanol and water (13:7)

Solvent B: Use the lower phase of a mixture of chloroform, 0.1 N hydrochloric acid, and 1-propanol (5:3:2).

Reagent: Dissolve 75 mg of ferric chloride in 50 mL of glacial acetic acid. Add 50 mL of sulfuric acid, while shaking and cooling. Prepare immediately before use.

Standard solution A: 0.2 mg/mL of USP Escin RS in glacial acetic acid, shaking for 1 min

Standard solution B: 0.4 mg/mL of USP Escin RS in glacial acetic acid, shaking for 1 min

Standard solution C: 0.6 mg/mL of USP Escin RS in glacial acetic acid, shaking for 1 min

Sample solution: Weigh 1 g of ground seeds, and place in a 250-mL round-bottom flask. Add exactly 100 mL of *Solvent A*, and weigh the filled flask with a precision of ± 0.1 g. Attach a condenser to the flask, reflux for 30 min, and allow to cool. Adjust to the initial weight by adding *Solvent A* as needed, and filter. Transfer 30.0 mL of the filtrate to a round-bottom flask, and evaporate the solvents under vacuum. Dissolve the residue with 20 mL of 0.1 N hydrochloric acid, and quantitatively transfer with the aid of two additional 5-mL portions of 0.1 N hydrochloric acid to a 250-mL separation funnel. Add 20 mL of 1-propanol and 50 mL of chloroform, and shake vigorously for 2 min. Separate the chloroform layer, and add *Solvent B* to the upper phase remaining in the separation funnel. Shake vigorously for 2 min, and separate the chloroform layer. Combine the chloroform layers in a round-bottom flask, and evaporate to dryness under vacuum. Evaporate the remaining solvents with the aid of a current of air. Wash the residue with two 10-mL portions of ether, filter, wash the filter with 10 mL of ether, and discard the ether filtrates. After evaporation of the residual ether, add to the residue a 10-mL portion of glacial acetic acid, and pass through the previously used dried filter into a 50-mL volumetric flask. Repeat the addition of glacial acetic acid followed by filtration two additional times, combining the filtrates in the volumetric

flask. Wash the round-bottom flask with small quantities of glacial acetic acid, and filter into the volumetric flask. Dilute with glacial acetic acid to volume.

Instrumental conditions

Mode: Visual

(See *Spectrophotometry and Light-Scattering* (851).)

Wavelength: 540 nm

Blank: Glacial acetic acid

Analysis: Transfer 1 mL each of *Standard solutions A, B, and C, Sample solution*, and *Blank* to separate test tubes with stoppers. Add 4.0 mL of *Reagent* to each tube, cap the tubes, and place them in a water bath at 60° for 25 min, shaking occasionally. Measure the absorbances of the reacted *Sample solution* and the reacted *Standard solutions A, B and C*, and correct for the *Blank*. Plot the absorbances of the reacted *Standard solutions A, B, and C* versus concentrations, in mg/mL of USP Escin RS in the corresponding *Standard solution*. From the graphs determine the concentration, *C*, in mg/mL, of triterpene glycosides as escin ($C_{55}H_{86}O_{24}$) in the *Sample solution*. Calculate the percentage of triterpene glycosides as escin in the portion of Horse Chestnut taken:

$$\text{Result} = (C/W) \times (50/3)$$

C = concentration of triterpene glycosides in the *Sample solution* as obtained above (mg/mL)

W = weight of Horse Chestnut taken to prepare the *Sample solution* (g)

Acceptance criteria: NLT 3.0% of triterpene glycosides, calculated as escin ($C_{55}H_{86}O_{24}$), on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method III* (231): NMT 20 µg/g
- **ARTICLES OF BOTANICAL ORIGIN**, *Pesticide Residues* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10^6 cfu/g, the total combined molds and yeast count does not exceed 10^4 cfu/g, and the enterobacterial count is NMT 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

BOTANIC CHARACTERISTICS

Macroscopic: Horse chestnut seeds are dense and hard, subspherical to oval, slightly flattened, and from 2 to 4 cm in diameter. They have a dark brown seed coat from 1 to 1.5 mm thick, with a large, round, light brown spot (hilum). The seed coat is shiny, but only in fresh condition. The space under the coat is totally filled with the shiny, massive embryo and its large, pale yellow cotyledons lacking endosperm.

Microscopic: The epidermis of the testa in surface view has yellowish-brown cells of fairly uniform size, with the majority of cells rounded to polygonal, and a few that are square to obscurely triangular. The walls of these cells are considerably but rather unevenly thickened, and lack pits. In the sectional view, the cells are columnar, approximately 3–4 times as high as they are wide, with the outer periclinal wall markedly thickened, uneven, and becoming thinner toward the base; beneath the epidermis there are a few layers of small collenchymatously thickened cells with small intercellular spaces; the greater part of the testa consists of larger, loosely packed parenchymatous cells forming a spongy tissue; the walls are variably and unevenly thickened, with intercellular and large circular spaces well marked; the inner layer of the testa is a narrow zone, with ill-defined and thinner-walled cells. All the parenchymatous cells of the testa are darkly pigmented. The embryo has an outer layer of small colorless cells, almost square in sectional view, with outer and side walls thickened. In the surface view, only the irregular

and more or less polygonal lumens are discernible, giving a reticulate, pitted appearance. Cotyledons are moderately thickened and indistinctly pitted, having round to ovoid parenchymatous cells densely filled with starch. Starch granules, mainly simple, are present in two size ranges: from 15 to 30 µm and from 3 to 10 µm. The largest granules vary from circular, ovoid, and bluntly polygonal to pyriform, most of them with a well-marked cleft or stellate hilum, and lacking striations. The smaller starch granules are less variable, spherical to ovoid, with the hilum more often a point. Compound starch granules are found very infrequently.

EXTRACTABLE MATTER

Analysis: Proceed as directed for *Articles of Botanical Origin* (561), *Alcohol-Soluble Extractives, Method 2*, except use a mixture of methanol and water (8:2) instead of alcohol.

Acceptance criteria: NLT 18.0%

ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter

(561): NMT 2.0%

• **LOSS ON DRYING** (731): Dry a sample at 105° for 2 h: it loses NMT 10.0% of its weight.

• **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a well-closed, light-resistant container, protected from moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11)
USP Escin RS

Powdered Horse Chestnut

DEFINITION

Powdered Horse Chestnut is Horse Chestnut reduced to a powder or very fine powder. It contains NLT 3.0% of triterpene glycosides, calculated on the dried basis as escin ($C_{55}H_{86}O_{24}$).

IDENTIFICATION

A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 5 mg/mL of USP Escin RS in methanol

Sample solution: Transfer 1 g of the Powdered Horse Chestnut to a screw-capped centrifuge tube, add 10 mL of a mixture of alcohol and water (7:3), and heat on a steam bath for 10 min. Centrifuge, and use the clear supernatant.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 10 µL

Developing solvent system: Use the upper phase of a mixture of 1-butanol, glacial acetic acid, and water (5:1:4).

Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85: 10: 5: 0.5)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms to a length of NLT 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, heat the plate at 100° for 5 min, and examine the plate under daylight.

Acceptance criteria: The chromatogram from the *Sample solution* shows a blue-violet zone corresponding to escin, comparable in position and color to the main zone in the chromatogram from the *Standard solution*. Above this zone, the chromatogram of the *Sample solu-*

tion shows several narrow, brown to brownish-red zones that are less intense than the zone corresponding to escin.

COMPOSITION

• CONTENT OF TRITERPENE GLYCOSIDES

Solvent A: Methanol and water (13:7)

Solvent B: Use the lower phase of a mixture of chloroform, 0.1 N hydrochloric acid, and 1-propanol (5:3:2).

Reagent: Dissolve 75 mg of ferric chloride in 50 mL of glacial acetic acid. Add 50 mL of sulfuric acid, while shaking and cooling. Prepare immediately before use.

Standard solution A: 0.2 mg/mL of USP Escin RS in glacial acetic acid, shaking for 1 min

Standard solution B: 0.4 mg/mL of USP Escin RS in glacial acetic acid, shaking for 1 min

Standard solution C: 0.6 mg/mL of USP Escin RS in glacial acetic acid, shaking for 1 min

Sample solution: Weigh 1 g of Powdered Horse Chestnut, and place in a 250-mL round-bottom flask. Add exactly 100 mL of *Solvent A*, and weigh the filled flask with a precision of ± 0.1 g. Attach a condenser to the flask, reflux for 30 min, and allow to cool. Adjust to the initial weight by adding *Solvent A* as needed, and filter. Transfer 30.0 mL of the filtrate to a round-bottom flask, and evaporate the solvents under vacuum. Dissolve the residue with 20 mL of 0.1 N hydrochloric acid, and quantitatively transfer with the aid of two additional 5-mL portions of 0.1 N hydrochloric acid to a 250-mL separation funnel. Add 20 mL of 1-propanol and 50 mL of chloroform, and shake vigorously for 2 min. Separate the chloroform layer, and add *Solvent B* to the upper phase remaining in the separation funnel. Shake vigorously for 2 min, and separate the chloroform layer. Combine the chloroform layers in a round-bottom flask, and evaporate to dryness under vacuum. Evaporate the remaining solvents with the aid of a current of air. Wash the residue with two 10-mL portions of ether, filter, wash the filter with 10 mL of ether, and discard the ether filtrates. After evaporation of the residual ether, add to the residue a 10-mL portion of glacial acetic acid, and pass through the previously used dried filter into a 50-mL volumetric flask. Repeat the addition of glacial acetic acid followed by filtration two additional times, combining the filtrates in the volumetric flask. Wash the round-bottom flask with small quantities of glacial acetic acid, and filter into the volumetric flask. Dilute with glacial acetic acid to volume.

Instrumental conditions

Wavelength: 540 nm

Mode: Visible

Blank: Glacial acetic acid

Analysis: Transfer 1 mL each of *Standard solutions A, B, and C*, the *Sample solution*, and the *Blank* to separate test tubes with stoppers. Add 4.0 mL of *Reagent* to each tube, cap the tubes, and place them in a water bath at 60° for 25 min, shaking occasionally. Measure the absorbances of the reacted *Sample solution* and the reacted *Standard solutions A, B, and C*, and correct for the *Blank*. Plot the absorbances obtained from the reacted *Standard solutions A, B, and C*, versus concentrations, in mg/mL of USP Escin RS in the corresponding *Standard solution*. From the graphs so obtained, determine the concentration, *C*, in mg/mL, of triterpene glycosides as escin ($C_{55}H_{86}O_{24}$) in the *Sample solution*.

Calculate the percentage of triterpene glycosides as escin in the portion of Powdered Horse Chestnut taken:

$$\text{Result} = (C/W) \times (50/3)$$

- C* = concentration of triterpene glycosides in the *Sample solution* as obtained above (mg/mL)
W = weight of Powdered Horse Chestnut taken to prepare the *Sample solution* (g)

Acceptance criteria: NLT 3.0% of triterpene glycosides, calculated as escin ($C_{55}H_{86}O_{24}$), on the dried basis

CONTAMINANTS

• **HEAVY METALS**, *Method III* (231): NMT 20 ppm

• **ARTICLES OF BOTANICAL ORIGIN**, *Pesticide Residues* (561): Meets the requirements

• **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10^6 cfu/g, the total combined molds and yeast count does not exceed 10^4 cfu/g, and the enterobacterial count is NMT 10^3 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• **BOTANIC CHARACTERISTICS:** Yellowish-brown powder, odorless, with a somewhat mealy, disagreeably bitter, and lingering taste. It shows numerous, different-sized fatty oil droplets that are free or within the thin-walled, colorless tissue of the cotyledons. Fragments of the testa consist of thick-walled pitted sclerenchymatous cells. The following are also present: pyriform, roundish or reniform larger individual starch granules from 15 to 30 μ m in diameter, smaller individual granules from 3 to 10 μ m, and only a few compounded granules consisting of 2–4 single grains that form rows up to 45 μ m in length. Many of the starch granules have a bistellate or polystellate, but rarely simple, hilum.

• EXTRACTABLE MATTER

Analysis: Proceed as directed for *Articles of Botanical Origin* (561), *Alcohol-Soluble Extractives*, *Method 2*, except use a mixture of methanol and water (8:2) instead of alcohol.

Acceptance criteria: NLT 18.0%

• **LOSS ON DRYING** (731): Dry a sample at 105° for 2 h: it loses NMT 10.0% of its weight.

• **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 4.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers, protected from moisture.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.

• **USP REFERENCE STANDARDS** (11)
USP Escin RS

Powdered Horse Chestnut Extract

DEFINITION

Powdered Horse Chestnut Extract is prepared from Horse Chestnut by extraction with alcohol–water mixtures or methanol–water mixtures. The ratio of starting plant material to extract is between 5:1 and 8:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of triterpene glycosides, calculated on the dried basis as escin ($C_{55}H_{86}O_{24}$). It may contain suitable added substances.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 5 mg/mL of USP Escin RS in methanol

Sample solution: To 10 mL of methanol add a quantity of Powdered Extract equivalent to 25 mg of the labeled amount of triterpene glycosides, and shake. Allow to stand for 15 min before use.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 10 μ L

Developing solvent system: Use the upper phase of a mixture of 1-butanol, glacial acetic acid, and water (5:1:4).

Spray reagent: Methanol, glacial acetic acid, sulfuric acid, and *p*-anisaldehyde (85: 10: 5: 0.5)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms to a length of NLT 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, heat the plate at 100° for 5 min, and examine the plate under daylight.

Acceptance criteria: The chromatogram from the *Sample solution* shows a blue-violet zone corresponding to escin, comparable in position and color to the main zone in the chromatogram from the *Standard solution*. Above this zone, the chromatogram of the *Sample solution* shows several narrow, brown to brownish-red zones that are less intense than the zone corresponding to escin.

COMPOSITION

• CONTENT OF TRITERPENE GLYCOSIDES

Solvent A: Methanol and water (13:7)

Solvent B: Use the lower phase of a mixture of chloroform, 0.1 N hydrochloric acid, and 1-propanol (5:3:2).

Reagent: Dissolve 75 mg of ferric chloride in 50 mL of glacial acetic acid. Add 50 mL of sulfuric acid, while shaking and cooling. Prepare immediately before use.

Standard solution A: 0.2 mg/mL of USP Escin RS in glacial acetic acid, shaking for 1 min

Standard solution B: 0.4 mg/mL of USP Escin RS in glacial acetic acid, shaking for 1 min

Standard solution C: 0.6 mg/mL of USP Escin RS in glacial acetic acid, shaking for 1 min

Sample solution: Transfer a quantity of Powdered Extract, equivalent to 50 mg of the labeled content of triterpene glycosides, into a 50-mL flask. Add 20 mL of 0.1 N hydrochloric acid, and shake for 5 min. Filter into a 250-mL separatory funnel with the aid of two additional 5-mL portions of 0.1 N hydrochloric acid. Add 20 mL of 1-propanol and 50 mL of chloroform, and shake vigorously for 2 min. Separate the chloroform layer, and add *Solvent B* to the upper phase remaining in the separation funnel. Shake vigorously for 2 min, and separate the chloroform layer. Combine the chloroform layers in a round-bottom flask, and evaporate to dryness under vacuum. Evaporate the remaining solvents with the aid of a current of air. Wash the residue with two 10-mL portions of ether, filter, wash the filter with 10 mL of ether, and discard the ether filtrates. After evaporation of the residual ether, add to the residue a 10-mL portion of glacial acetic acid, and pass through the previously used dried filter into a 100-mL volumetric flask. Repeat the addition of glacial acetic acid followed by filtration two additional times, combining the filtrates in the volumetric flask. Wash the round-bottom flask with small quantities of glacial acetic acid, and filter into the volumetric flask. Dilute with glacial acetic acid to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Visible

Wavelength: 540 nm

Blank: Glacial acetic acid

Analysis: Transfer 1 mL each of *Standard solutions A, B, and C*, the *Sample solution*, and the *Blank* to separate test tubes with stoppers. Add 4.0 mL of *Reagent* to each tube, cap the tubes, and place them in a water bath at 60° for 25 min, shaking occasionally. Measure the absorbances of the reacted *Sample solution* and the reacted *Standard solutions A, B, and C*, and correct for the *Blank*. Plot the absorbances of the reacted *Standard solutions A, B, and C* versus concentrations, in mg/mL of USP Escin RS in the corresponding *Standard solution*.

From the graphs determine the concentration, *C*, in mg/mL, of triterpene glycosides as escin ($C_{55}H_{86}O_{24}$) in the *Sample solution*.

Calculate the percentage of the labeled amount of triterpene glycosides in the portion of Powdered Extract taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of triterpene glycosides in the *Sample solution* as obtained above (mg/mL)

C_U = nominal concentration of triterpene glycosides in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amount of triterpene glycosides as escin ($C_{55}H_{86}O_{24}$) on the dried basis

CONTAMINANTS

- **HEAVY METALS, Method II <231>:** NMT 20 μ g/g
- **MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic microbial count does not exceed 10⁴ cfu/g, the total combined molds and yeasts count does not exceed 10² cfu/g, and the count for enterobacteria does not exceed 10³ cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **LOSS ON DRYING <731>:** Dry 1 g at 105° for 2 h: it loses NMT 5.0% of its weight.
- **OTHER REQUIREMENTS:** It meets the requirements in *Botanical Extracts <565>*, *General Pharmacopeial Requirements*, for *Packaging and Storage*, *Residual Solvents*, and *Pesticide Residues* for powdered extracts.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. Store in a cool place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of triterpene glycosides, the extracting solvent or solvent mixture used for preparation, the ratio of the starting crude plant material to Powdered Extract, and the name and content of any added substance. It meets the requirements for labeling in *Botanical Extracts <565>*.
- **USP REFERENCE STANDARDS <11>**
USP Escin RS

Hydroxocobalamin—see *Hydroxocobalamin General Monographs*

Isoleucine—see *Isoleucine General Monographs*

Leucine—see *Leucine General Monographs*

Levocarnitine—see *Levocarnitine General Monographs*

Levocarnitine Oral Solution—see
Levocarnitine Oral Solution General
Monographs

Levocarnitine Tablets—see *Levocarnitine*
Tablets General Monographs

Licorice

DEFINITION

Licorice consists of the roots, rhizomes, and stolons of *Glycyrrhiza glabra* L. or *Glycyrrhiza uralensis* Fish. ex DC. (Fam. Fabaceae). It contains NLT 2.5% of glycyrrhizic acid ($C_{42}H_{62}O_{16}$), calculated on the dried basis.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 5 mg/mL of USP Glycyrrhizic Acid RS in a mixture of alcohol and water (7:3)

Sample solution: 2 g of pulverized Licorice in 10 mL of a mixture of alcohol and water (7:3). Heat by shaking on a water bath for 5 min, cool, and filter.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 2 μ L

Developing solvent system: Butyl alcohol, glacial acetic acid, and water (7:1:2)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatogram in an unsaturated chamber to a length of 10 cm. Examine the plate under 254-nm light.

Acceptance criteria: The chromatograms show a dark purple zone, among other spots, due to glycyrrhizic acid at an R_f value of 0.4.

COMPOSITION

• **CONTENT OF GLYCYRRHIZIC ACID**

Diluent: Alcohol and water (1:1)

Solution A: Diluted acetic acid (1 in 15)

Mobile phase: Acetonitrile and *Solution A* (2:3)

Standard solution: 0.25 mg/mL of USP Glycyrrhizic Acid RS in *Diluent*

Sample solution: Transfer 500 mg of Licorice, reduced to a powder, to a suitable flask. Add 70 mL of *Diluent*, shake for 15 min, centrifuge, and decant the supernatant into a 100-mL volumetric flask. Mix the residue with 25 mL of *Diluent*, shake for 15 min, centrifuge, and add the supernatant to the volumetric flask. Dilute with *Diluent* to volume, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm \times 15-cm; packing L1

Flow rate: 0.6 mL/min

Injection size: 20 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 5000 theoretical plates determined from glycyrrhizic acid

Tailing factor: NMT 2.0 for the glycyrrhizic acid peak

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of glycyrrhizic acid ($C_{42}H_{62}O_{16}$) in the portion of Licorice taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area of glycyrrhizic acid from the *Sample solution*

r_S = peak area of glycyrrhizic acid from the *Standard solution*

C_S = concentration of USP Glycyrrhizic Acid RS in the *Standard solution* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Licorice taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 2.5% on the dried basis

CONTAMINANTS

• **HEAVY METALS**, *Method III* <231>: NMT 30 μ g/g

• **ARTICLES OF BOTANICAL ORIGIN**, *Pesticide Residues* <561>: Meets the requirements

SPECIFIC TESTS

• **BOTANIC CHARACTERISTICS**

Macroscopic: The terrestrial stem is nearly cylindrical, 0.5–3.0 cm in diameter, and over 1 m in length; it is externally dark brown to red-brown and longitudinally wrinkled. It often has lenticels, small buds, and scaly leaves. The transverse section reveals a rather clear border between the phloem and the xylem, and a radial structure that often has radiating splits.

Microscopic: The transverse section reveals several yellow-brown cork layers, and a layer of phelloderm that is 1–3 cells thick. The cortex exhibits medullary rays, and obliterated sieve portions radiate alternately. The phloem exhibits groups of phloem fibers, which are surrounded by crystal cells, with thick but incompletely lignified walls. The vessels are accompanied by xylem fibers, which are surrounded by crystal cells, and by xylem parenchyma cells. The parenchyma cells contain starch grains, and often contain single crystals of calcium oxalate.

• **ARTICLES OF BOTANICAL ORIGIN**, *Alcohol-Soluble Extractives*, *Method 2* <561>: NLT 25.0%

• **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* <561>: NMT 2.0%

• **LOSS ON DRYING** <731>: Dry a sample at 105° for 6 h: it loses NMT 12.0% of its weight.

• **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* <561>: NMT 2.0%

• **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 7.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store in a cool, dry place.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.

• **USP REFERENCE STANDARDS** <11>
USP Glycyrrhizic Acid RS

Powdered Licorice

DEFINITION

Powdered Licorice is Licorice reduced to a fine or very fine powder. It contains NLT 2.5% of glycyrrhizic acid ($C_{42}H_{62}O_{16}$), calculated on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 5 mg/mL of USP Glycyrrhizic Acid RS in a mixture of alcohol and water (7:3)

Sample solution: 2 g of Powdered Licorice in 10 mL of a mixture of alcohol and water (7:3). Heat by shaking on a water bath for 5 min, cool, and filter.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture.

Application volume: 2 μ L

Developing solvent system: Butyl alcohol, glacial acetic acid, and water (7:1:2)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatogram in an unsaturated chamber to a length of 10 cm. Examine the plate under 254-nm light.

Acceptance criteria: The chromatograms show a dark purple zone, among other spots, due to glycyrrhizic acid at an R_f value of 0.4.

COMPOSITION

• CONTENT OF GLYCYRRHIZIC ACID

Diluent: Alcohol and water (1:1)

Solution A: Diluted acetic acid (1 in 15)

Mobile phase: Acetonitrile and *Solution A* (2:3)

Standard solution: 0.25 mg/mL of USP Glycyrrhizic Acid RS in *Diluent*

Sample solution: Transfer 500 mg of Powdered Licorice to a suitable flask. Add 70 mL of *Diluent*, shake for 15 min, centrifuge, and decant the supernatant into a 100-mL volumetric flask. Mix the residue with 25 mL of *Diluent*, shake for 15 min, centrifuge, and add the supernatant to the volumetric flask. Dilute with *Diluent* to volume, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm \times 15-cm; packing L1

Flow rate: 0.6 mL/min

Injection size: 20 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 5000 theoretical plates determined from glycyrrhizic acid

Tailing factor: NMT 2.0 for the glycyrrhizic acid peak

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of glycyrrhizic acid ($C_{42}H_{62}O_{16}$) in the portion of Powdered Licorice taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area for glycyrrhizic acid from the *Sample solution*

r_S = peak area for glycyrrhizic acid from the *Standard solution*

C_S = concentration of USP Glycyrrhizic Acid RS in the *Standard solution* (mg/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Powdered Licorice taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 2.5% on the dried basis

CONTAMINANTS

• **HEAVY METALS**, *Method III* <231>: NMT 30 μ g/g

• **ARTICLES OF BOTANICAL ORIGIN**, *Pesticide Residues* <561>: Meets the requirements

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: It is light yellow-brown and has a slight odor and sweet taste.

Microscopic: Under a microscope, Powdered Licorice reveals parenchyma cells containing starch grains and solitary crystals of calcium oxalate, parenchyma cell fragments, cork tissue, yellow sclerenchymatous fiber bundles that are accompanied by crystal cell rows, and vessels with reticulate and scalariform pits.

• **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* <561>: NMT 2.0%

• **ARTICLES OF BOTANICAL ORIGIN**, *Method II, Alcohol-Soluble Extractives* <561>: NLT 25.0%

• **LOSS ON DRYING** <731>: Dry a sample at 105° for 6 h: it loses NMT 12.0% of its weight.

• **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 7.0%

• **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* <561>: NMT 2.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Store in well-closed containers in a cool, dry place.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.

• **USP REFERENCE STANDARDS** <11>
USP Glycyrrhizic Acid RS

Powdered Licorice Extract

DEFINITION

Powdered Licorice Extract is prepared from comminuted Licorice extracted with water or suitable solvents such as alcohol, water, or mixtures of these solvents. The ratio of the crude plant material to Powdered Extract is between 5:1 and 7:1. It contains NLT 6.0% of glycyrrhizic acid ($C_{42}H_{62}O_{16}$), calculated on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 5 mg/mL of USP Glycyrrhizic Acid RS in a mixture of alcohol and water (7:3)

Sample solution: 60 mg/mL of Powdered Licorice Extract, in a mixture of alcohol and water (1:1)

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 2 μ L

Developing solvent system: Butyl alcohol, glacial acetic acid, and water (7:1:2)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatogram in an unsaturated chamber to a length of 10 cm. Examine the plate under 254-nm light.

Acceptance criteria: The chromatograms show a dark purple zone, among other spots, due to glycyrrhizic acid at an R_f value of 0.4.

COMPOSITION**• CONTENT OF GLYCYRRHIZIC ACID****Diluent:** Alcohol and water (1:1)**Solution A:** Diluted acetic acid (1 in 15)**Mobile phase:** Acetonitrile and *Solution A* (2:3)**Standard solution:** 0.25 mg/mL of USP Glycyrrhizic Acid RS in *Diluent*.**Sample solution:** Transfer 150 mg of Powdered Licorice Extract to a flask. Add 25 mL of *Diluent*, heat at 50° for 30 min, cool, centrifuge, and decant the supernatant into a 100-mL volumetric flask. Mix the residue with 20 mL of *Diluent*, repeat the above procedure, and dilute with *Diluent* to volume.**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 254 nm**Column:** 4.6-mm × 15-cm; packing L1**Flow rate:** 0.6 mL/min**Injection size:** 20 µL**System suitability****Sample:** *Standard solution***Suitability requirements****Column efficiency:** NLT 5000 theoretical plates**Tailing factor:** NMT 2.0**Relative standard deviation:** NMT 2.0%**Analysis****Samples:** *Standard solution* and *Sample solution*Calculate the percentage of glycyrrhizic acid (C₄₂H₆₂O₁₆) in the portion of Powdered Licorice Extract taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

 r_U = peak area for glycyrrhizic acid from the *Sample solution* r_S = peak area for glycyrrhizic acid from the *Standard solution* C_S = concentration of USP Glycyrrhizic Acid RS in the *Standard solution* (mg/mL) V = volume of *Sample solution*, 100 mL W = weight of Powdered Licorice Extract taken to prepare the *Sample solution* (mg)**Acceptance criteria:** NLT 6.0% on the dried basis**CONTAMINANTS****• HEAVY METALS, Method III <231>:** NMT 30 µg/g**• ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues <561>:** Meets the requirements**SPECIFIC TESTS****• LOSS ON DRYING <731>:** Dry a sample at 105° for 6 h: it loses NMT 10.0% of its weight.**• ARTICLES OF BOTANICAL ORIGIN, Total Ash <561>:** NMT 12.0%**ADDITIONAL REQUIREMENTS****• PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.**• LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. The label also indicates the content of glycyrrhizic acid, the extracting solvent or solvent mixture used for preparation, and the ratio of the starting material to final product. The label bears a statement indicating that "Excessive amounts or long-term use of Licorice may cause high blood pressure or low potassium, which have been associated with irregular heartbeat and/or muscle weakness. Licorice may worsen the effects of congestive heart failure, cirrhosis, or kidney failure. Diuretic use may increase the risk. If you are pregnant or nursing a baby, seek the advice of a health professional before using this product." It meets the requirements in *Botanical Extracts* <565>, *Labeling*.**• USP REFERENCE STANDARDS <11>**

USP Glycyrrhizic Acid RS

Licorice Fluidextract—see Licorice Fluidextract NF**Ground Limestone****DEFINITION**Ground Limestone is a fine, white to off-white, microcrystalline powder mainly consisting of calcium carbonate. It is obtained by crushing, grinding, and classifying naturally occurring limestone, benefited by flotation and/or air classification. After drying at 200° for 4 h, it contains NLT 94.0% and NMT 100.5% of calcium carbonate (CaCO₃).**IDENTIFICATION****• A. IDENTIFICATION TESTS—GENERAL, Calcium <191>****Analysis:** Add acetic acid to the Ground Limestone, and boil the resulting solution.**Acceptance criteria:** The solution produces effervescence (presence of carbonate) after the addition of acetic acid. After boiling, it meets the requirements in the chapter.**• B. ACID-INSOLUBLE SUBSTANCES:** It meets the requirements in the test for *Acid-Insoluble Substances*.**ASSAY****• PROCEDURE****Sample:** 200 mg of Ground Limestone, previously dried at 200° for 4 h**Blank:** Proceed as directed in the *Analysis*, except omit the test specimen.**Titrimetric system**(See *Titrimetry* <541>.)**Mode:** Direct titration**Titrant:** 0.05 M edetate disodium VS**Endpoint detection:** Visual**Analysis:** Transfer the *Sample* to a 250-mL beaker. Moisten thoroughly with a few mL of water, and add, dropwise, sufficient 3 N hydrochloric acid to dissolve. Add 100 mL of water, 15 mL of 1 N sodium hydroxide, and 300 mg of hydroxy naphthol blue, and titrate with the *Titrant* until the solution is a distinct blue color. Perform a *Blank* determination.Calculate the percentage of calcium carbonate (CaCO₃) in the *Sample* taken:

$$\text{Result} = \{(V_S - V_B) \times M \times F/W\} \times 100$$

 V_S = *Titrant* volume consumed by the *Sample* (mL) V_B = *Titrant* volume consumed by the *Blank* (mL) M = actual molarity of the *Titrant* (mM/mL) F = equivalency factor, 100.1 mg/mM W = *Sample* weight (mg)**Acceptance criteria:** 94.0%–100.5%**IMPURITIES****• ARSENIC, Method I <211>****Test preparation:** Slowly dissolve 1.0 g in 15 mL of hydrochloric acid, and dilute with water to 55 mL.**Analysis:** Proceed as directed in the chapter, except omit the addition of 20 mL of 7 N sulfuric acid.**Acceptance criteria:** It meets the requirements in the chapter (NMT 3 ppm).**• LEAD <251>****Test preparation:** Mix 1.0 g with 5 mL of water, slowly add 8 mL of 3 N hydrochloric acid, evaporate on a steam bath to dryness, and dissolve the residue in 5 mL of water.

- Acceptance criteria:** NMT 3 ppm
- **HEAVY METALS** (231)
Test preparation: Mix 1.0 g with 5 mL of water, slowly add 8 mL of 3 N hydrochloric acid, and evaporate on a steam bath to dryness. Dissolve the residue in 20 mL of water, filter, and add water to the filtrate to make 25 mL.
Acceptance criteria: NMT 20 ppm
 - **LIMIT OF MAGNESIUM AND ALKALI SALTS**
Sample: 1.0 g
Analysis: Mix the *Sample* with 40 mL of water. Carefully add 5 mL of hydrochloric acid, heat the solution, and boil for 1 min. Rapidly add 40 mL of oxalic acid TS, and stir vigorously until precipitation is well established. Add immediately to the warm mixture 2 drops of methyl red TS and then 6 N ammonium hydroxide, dropwise, until the mixture is just alkaline. Cool to room temperature. Transfer to a 100-mL graduated cylinder, dilute with water to 100 mL, mix, and allow to stand for 4 h or overnight. Filter, and to 50 mL of the clear filtrate in a platinum dish add 0.5 mL of sulfuric acid, and evaporate the mixture on a steam bath to a small volume. Carefully heat over a free flame to dryness, and continue heating to complete decomposition and volatilization of ammonium salts. Ignite the residue to constant weight.
Acceptance criteria: NMT 3.5%. The weight of the residue is NMT 17.5 mg.
 - **LIMIT OF FLUORIDE**
[NOTE—Prepare and store all solutions in plastic containers.]
Buffer solution: 294 mg/mL of sodium citrate dihydrate in water
Standard stock solution: 1.1052 mg/mL of USP Sodium Fluoride RS in water
Standard solution: Transfer 20.0 mL of *Standard stock solution* to a 100-mL volumetric flask containing 50.0 mL of *Buffer solution*, dilute with water to volume, and mix. Each mL of *Standard solution* contains 100 µg of fluoride ion.
Sample solution: Transfer 2.0 g of Ground Limestone to a beaker containing a plastic-coated stirring bar. Add 20 mL of water and 4.0 mL of hydrochloric acid, and stir until dissolved. Add 50.0 mL of *Buffer solution* and sufficient water to make 100 mL.
Electrode system: Use a fluoride-specific ion-indicating electrode and a silver–silver chloride reference electrode connected to a pH meter capable of measuring potentials with a minimum reproducibility of ± 0.2 mV (see *pH* (791)).
Analysis
Samples: *Standard solution* and *Sample solution*
Standard response line: Transfer 50.0 mL of *Buffer solution* and 4.0 mL of hydrochloric acid to a beaker, and add water to make 100 mL. Add a plastic-coated stirring bar, insert the electrodes into the solution, stir for 15 min, and read the potential, in mV. Continue stirring, and at 5-min intervals add 100, 100, 300, and 500 µL of *Standard solution*, reading the potential 5 min after each addition. Plot the logarithms of the cumulative fluoride ion concentrations (0.1, 0.2, 0.5, and 1.0 µg/mL) versus potential, in mV.
Rinse and dry the electrodes, insert them into the *Sample solution*, stir for 5 min, and read the potential, in mV. From the measured potential and the *Standard response line* determine the concentration, *C* (in µg/mL), of fluoride ion in the *Sample solution*. Calculate the content of fluoride in the portion of Ground Limestone taken:

$$\text{Result} = (C \times V)/W$$

C = concentration of fluoride ion, obtained from the *Standard response line*, in the *Sample solution* (µg/mL)

V = volume of the *Sample solution* (mL)
W = weight of Ground Limestone taken to prepare the *Sample solution* (g)
Acceptance criteria: NMT 50 ppm

SPECIFIC TESTS

• ACID-INSOLUBLE SUBSTANCES

Sample: 5.0 g

Analysis: Mix the *Sample* with 25 mL of water. Add 25 mL of hydrochloric acid, dropwise, with agitation, until it ceases to cause effervescence. Add water to make the mixture measure 200 mL, and filter. Wash the insoluble residue with water until the last washing shows no chloride, and ignite and weigh the residue.

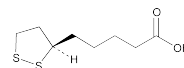
Acceptance criteria: 0.2%–2.5%. The weight of the residue is between 10 and 125 mg.

- **LOSS ON DRYING** (731): Dry a sample at 200° for 4 h: it loses NMT 2.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** (11)
USP Sodium Fluoride RS

Alpha Lipoic Acid



$C_8H_{14}O_2S_2$

206.33

Thiotoxic acid;
1,2-Dithiolane-3-pentanoic acid;
1,2-Dithiolane-3-valeric acid [1077-28-7].

DEFINITION

Alpha Lipoic Acid contains NLT 99.0% and NMT 101.0% of $C_8H_{14}O_2S_2$, calculated on the dried basis.

IDENTIFICATION

- **A.** The retention time of the peak for alpha lipoic acid of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the Assay.
- **B. INFRARED ABSORPTION** (197K)

ASSAY

• PROCEDURE

Buffer solution: 0.68 g/L of monobasic potassium phosphate

Mobile phase: Methanol, *Buffer solution*, and acetonitrile (58:46:9). Adjust with phosphoric acid solution (8.3 in 100) to a pH of 3.0–3.1.

Standard solution: 1.0 mg/mL of USP Alpha Lipoic Acid RS in *Mobile phase*

Sample solution: 1.0 mg/mL of Alpha Lipoic Acid in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 215 nm

Column: 4.6-mm × 250-mm; packing L1

Column temperature: 35°

Flow rate: 1.2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 10,000 theoretical plates

Tailing factor: NMT 2.0 for the alpha lipoic acid peak

Relative standard deviation: NMT 2.0% for alpha lipoic acid

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of alpha lipoic acid ($C_8H_{14}O_2S_2$) in the portion of Alpha Lipoic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
 r_S = peak response from the *Standard solution*
 C_S = concentration of USP Alpha Lipoic Acid RS in the *Standard solution* (mg/mL)
 C_U = concentration of Alpha Lipoic Acid in the *Sample solution* (mg/mL)

Acceptance criteria: 99.0%–101.0% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** (281): Less than 0.1%

• **HEAVY METALS**, *Method II* (231): NMT 10 ppm

• CHROMATOGRAPHIC PURITY, PROCEDURE 1

Buffer solution, *Mobile phase*, *Standard solution*, *Sample solution*, and *Chromatographic system*:
Proceed as directed in the *Assay*.

Diluted standard solution: Dilute the *Standard solution* (1 in 1000) with *Mobile phase*.

System suitability

Sample: *Diluted standard solution*

Suitability requirements

Signal-to-noise ratio: NLT 10

Relative standard deviation: NMT 10.0%

Analysis

Sample: *Sample solution*

Calculate the percentage of each impurity in the portion of Alpha Lipoic Acid taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual impurity from the *Sample solution*

r_T = sum of the responses of all the peaks from the *Sample solution*

Acceptance criteria

Individual impurities: NMT 0.1%

Total impurities: NMT 2.0%

• CHROMATOGRAPHIC PURITY, PROCEDURE 2

[NOTE—Use low-actinic glassware.]

Standard solution A: 40.0 mg/mL of USP Alpha Lipoic Acid RS in dimethylformamide

Standard solution B: 20.0 mg/mL of USP Alpha Lipoic Acid RS in dimethylformamide, prepared from the dilution of *Standard solution A*

Standard solution C: 10.0 mg/mL of USP Alpha Lipoic Acid RS in dimethylformamide, prepared from the dilution of *Standard solution B*

Sample solution: 40.0 mg/mL of Alpha Lipoic Acid in dimethylformamide

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 5 μ L

Developing solvent system: *n*-Propyl alcohol, ethyl acetate, water, and 25% ammonia water (40:40:10:5). Allow the chamber to become saturated for at least 1 h.

Iodine vapor-saturated chamber: Transfer 4 g of iodine crystals to a small watch glass, and place in a chromatographic chamber. Allow the chamber to become saturated for at least 2 h.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

Proceed as directed in the chapter, except to develop until the solvent front has moved 10 cm. Remove the plate, and allow to air-dry until the ammonia disappears completely. Heat at 50° for 20 min, cool the plate, and place in the *Iodine vapor-saturated chamber* until the spots are visible. The R_f value for the alpha lipoic acid spot is 0.25–0.30 and for the polymeric lipoic acid spot is 0.

Acceptance criteria: No spot other than the alpha lipoic acid spot from the *Sample solution* is more intense than the spot at $R_f = 0$ from *Standard solution A*.

SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE** (741): 60.0°–62.0°

• **OPTICAL ROTATION**, *Specific Rotation* (781S)

Sample solution: 50 mg/mL of Alpha Lipoic Acid, in dehydrated alcohol

Acceptance criteria: –1.0° to +1.0°

• **LOSS ON DRYING** (731): Dry a sample in vacuum at 40° for 3 h: it loses NMT 0.2% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** (11)

USP Alpha Lipoic Acid RS

Alpha Lipoic Acid Capsules

DEFINITION

Alpha Lipoic Acid Capsules contain NLT 90.0% and NMT 115.0% of the labeled amount of $C_8H_{14}O_2S_2$.

IDENTIFICATION

• The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Alpha Lipoic Acid*.

STRENGTH

• CONTENT OF ALPHA LIPOIC ACID

Mobile phase: 0.025 M phosphoric acid and acetonitrile (62:38)

Standard solution: 0.05 mg/mL of USP Alpha Lipoic Acid RS in acetonitrile and water (1:1)

Sample solution A (for hard gelatin Capsules): Empty and mix thoroughly the contents of NLT 20 Capsules. Transfer a portion of the powder, equivalent to 100 mg of alpha lipoic acid, to a suitable container. Add 70 mL of a mixture of acetonitrile and water (1:1), and shake for 45 min by mechanical means. Transfer to a 100-mL volumetric flask, dilute with the mixture of acetonitrile and water (1:1) to volume, and filter a portion of this preparation, discarding the first 5 mL of the filtrate. Transfer 5.0 mL of the remaining filtrate to a 100-mL volumetric flask, and dilute with acetonitrile and water (1:1) to volume.

Sample solution B (for soft gelatin Capsules): Using a suitable cutting instrument, open a number of Capsules equivalent to 500 mg of alpha lipoic acid from a counted number of opened Capsules. Transfer the contents and the shells to a suitable container with stopper, add 500.0 mL of a mixture of acetonitrile and water (1:1), and shake for 45 min by mechanical means. Filter a portion of this preparation, discarding the first 5 mL of the filtrate. Transfer 5.0 mL of the remaining filtrate to a 100-mL volumetric flask, and dilute with acetonitrile and water (1:1) to volume.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC
 Detector: UV 220 nm
 Column: 3.9-mm × 30-cm; packing L1
 Flow rate: 1.5 mL/min
 Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 1300 theoretical plates
 Tailing factor: NMT 1.2 for alpha lipoic acid
 Relative standard deviation: NMT 1.0%

Analysis

Samples: *Standard solution* and appropriate *Sample solution*

Calculate the percentage of alpha lipoic acid in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from *Sample solution A* or *Sample solution B*
 r_S = peak response from the *Standard solution*
 C_S = concentration of USP Alpha Lipoic Acid RS in the *Standard solution* (mg/mL)
 C_U = nominal concentration of alpha lipoic acid in *Sample solution A* or *Sample solution B* (mg/mL)

Acceptance criteria: 90.0%–115.0%

PERFORMANCE TESTS**• DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS**

<2040>: Meet the requirements for *Dissolution*

Medium: Water; 900 mL

Apparatus 1 (for hard gelatin Capsules): 100 rpm

Apparatus 2 (for soft gelatin Capsules): 75 rpm

Time: 60 min

Standard solution: 1 mg/mL of USP Alpha Lipoic Acid RS in a mixture of acetonitrile and water (1:1). Dilute with water to obtain a concentration of 0.02 mg/mL.

Sample solution: Withdraw a portion of the solution under test, and filter, discarding the first portion of the filtrate. Transfer an aliquot to a volumetric flask, and dilute with water to volume to obtain a solution having an expected concentration of 0.02 mg/mL of alpha lipoic acid.

Mobile phase and Chromatographic system: Proceed as directed in the test for *Content of Alpha Lipoic Acid*.

Injection size: 50 µL

Analysis

Samples: *Standard solution* and *Sample solution*
 Calculate the percentage of alpha lipoic acid ($C_8H_{14}O_2S_2$) dissolved:

$$\text{Result} = (r_U/r_S) \times (V \times C \times D/L) \times 100$$

r_U = peak area from the *Sample solution*
 r_S = peak area from the *Standard solution*
 V = volume of dissolution Medium, 900 mL
 C = concentration of USP Alpha Lipoic Acid RS in the *Standard solution* (mg/mL)
 D = dilution factor of the sample
 L = label claim of alpha lipoic acid (mg/Capsule)

Tolerances: NLT 70% of the labeled amount of $C_8H_{14}O_2S_2$ is dissolved.

• WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>: Meet the requirements**ADDITIONAL REQUIREMENTS**

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS <11>**
 USP Alpha Lipoic Acid RS

Alpha Lipoic Acid Tablets

DEFINITION

Alpha Lipoic Acid Tablets contain NLT 90.0% and NMT 115.0% of the labeled amount of $C_8H_{14}O_2S_2$.

IDENTIFICATION

- The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Alpha Lipoic Acid*.

STRENGTH**• CONTENT OF ALPHA LIPOIC ACID**

Mobile phase: 0.025 M phosphoric acid and acetonitrile (62:38)

Standard solution: 0.05 mg/mL of USP Alpha Lipoic Acid RS in acetonitrile and water (1:1)

Sample solution: Transfer the equivalent of 100 mg of alpha lipoic acid from NLT 20 finely powdered Tablets to a suitable container. Add 70 mL of a mixture of acetonitrile and water (1:1), and shake for 45 min by mechanical means. Transfer to a 100-mL volumetric flask, dilute with the mixture of acetonitrile and water (1:1) to volume, and filter a portion of this preparation, discarding the first 5 mL of the filtrate. Transfer 5.0 mL of the remaining filtrate to a 100-mL volumetric flask, and dilute with acetonitrile and water (1:1) to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 220 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 1300 theoretical plates

Tailing factor: NMT 1.2 for alpha lipoic acid

Relative standard deviation: NMT 1.0%

Analysis

Samples: *Standard solution* and *Sample solution*
 Calculate the percentage of the labeled amount of alpha lipoic acid ($C_8H_{14}O_2S_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
 r_S = peak response from the *Standard solution*
 C_S = concentration of USP Alpha Lipoic Acid RS in the *Standard solution* (mg/mL)
 C_U = nominal concentration of alpha lipoic acid in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–115.0%

PERFORMANCE TESTS**• DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS**

<2040>: Meet the requirements for *Dissolution*

Medium: Water; 900 mL

Apparatus 2: 75 rpm

Time: 60 min

Standard stock solution: 1 mg/mL of USP Alpha Lipoic Acid RS in a mixture of acetonitrile and water (1:1)

Standard solution: 0.02 mg/mL from the *Standard stock solution* in water

Sample solution: Withdraw a portion of the solution under test, and filter, discarding the first portion of the filtrate. Transfer an aliquot to a volumetric flask, and dilute with water to volume to obtain a solution having an expected concentration of 0.02 mg/mL of alpha lipoic acid.

Mobile phase and Chromatographic system: Proceed as directed in the test for *Content of Alpha Lipoic Acid*.

Injection size: 50 μ L

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of alpha lipoic acid ($C_8H_{14}O_2S_2$) dissolved:

$$\text{Result} = (r_U/r_S) \times (V \times C \times D/L) \times 100$$

r_U = peak area from the *Sample solution*
 r_S = peak area from the *Standard solution*
 V = volume of dissolution *Medium*, 900 mL
 C = concentration of USP Alpha Lipoic Acid RS in the *Standard solution* (mg/mL)
 D = dilution factor of the sample
 L = label claim of alpha lipoic acid (mg/Tablet)

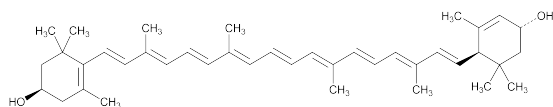
Tolerances: NLT 70% of the labeled amount of alpha lipoic acid ($C_8H_{14}O_2S_2$) is dissolved.

- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>:** Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Tablets that are coated are so labeled.
- **USP REFERENCE STANDARDS <11>**
USP Alpha Lipoic Acid RS

Lutein



$C_{40}H_{56}O_2$ 568.87
 β - ϵ -Carotene-3,3'-diol (3R,3'R,6'R) [127-40-2].

DEFINITION

Lutein is the purified fraction obtained from saponification of the oleoresin of *Tagetes erecta* L. It contains NLT 80.0% of total carotenoids calculated as lutein ($C_{40}H_{56}O_2$). It contains NLT 74.0% of lutein and NMT 8.5% of zeaxanthin, both calculated as lutein ($C_{40}H_{56}O_2$) on the anhydrous basis.

IDENTIFICATION

- **A. ULTRAVIOLET-VISIBLE ABSORPTION <197U>**
Wavelength range: 300–700 nm
Sample solution: Prepare as directed for the *Sample solution* in the test for *Content of Total Carotenoids*.
Acceptance criteria: It meets the requirements in the chapter. The absorbance ratio A_{446}/A_{474} is 1.09–1.14.
- **B.** The retention time for the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Lutein*.

COMPOSITION

- **CONTENT OF TOTAL CAROTENOIDS**
[NOTE—Use low-actinic glassware.]
Diluent: Hexanes, acetone, toluene, and dehydrated alcohol (10:7:7:6)
Sample stock solution: 0.3 mg/mL of Lutein in *Diluent*
Sample solution: 3.0 μ g/mL of Lutein in dehydrated alcohol from dilution of *Sample stock solution*
Instrumental conditions
(See *Spectrophotometry and Light-Scattering <851>*.)

Mode: UV-Vis

Analytical wavelength: 446 nm

Blank: Dehydrated alcohol

Analysis

Sample: *Sample solution*

Calculate the percentage of total carotenoids (T) as lutein ($C_{40}H_{56}O_2$) in the portion of Lutein taken:

$$\text{Result} = A/(F \times C)$$

A = absorbance of the *Sample solution*
 F = coefficient of extinction ($E^{1\%}$) of lutein in alcohol ($100 \text{ mL} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$), 2550
 C = concentration of lutein in the *Sample solution* (g/mL)

Acceptance criteria: NLT 80.0%

- **CONTENT OF LUTEIN**

Mobile phase: Hexane and ethyl acetate (3:1)

Standard solution: 150 μ g/mL of USP Lutein RS in *Mobile phase*

Sample solution: Transfer 1 mL of the *Sample stock solution* from the test for *Content of Total Carotenoids*, and evaporate under a stream of nitrogen to dryness. Add 1 mL of *Mobile phase*, and sonicate to dissolve.

Chromatographic system

(See *Chromatography <621>*, *System Suitability*.)

Mode: LC

Detector: UV-Vis at 446 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L3

Flow rate: 1.5 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution* [NOTE—The relative retention times for lutein and zeaxanthin are about 1.0 and 1.05, respectively.]

Suitability requirements

Resolution: NLT 1.0 between lutein and zeaxanthin

Tailing factor: NMT 2.0

Relative standard deviation: NMT 2.0%

Analysis

Sample: *Sample solution*

Calculate the percentage of the lutein peak as the total detected area in the portion of Lutein taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of lutein
 r_T = sum of the responses of all the peaks

Acceptance criteria: NLT 85%

Calculate the percentage of lutein in the portion of Lutein taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = individual peak response
 r_T = sum of the responses of all the peaks
 T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Acceptance criteria: NLT 74.0% of lutein on the anhydrous basis

- **ZEAXANTHIN AND OTHER RELATED COMPOUNDS**

[NOTE—Use low-actinic glassware.]

Mobile phase, Standard solution, Sample solution, and Chromatographic system: Proceed as directed in *Content of Lutein*.

Analysis

Sample: *Sample solution*

Calculate the percentage of zeaxanthin as the total detected area in the portion of Lutein taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of zeaxanthin
 r_T = sum of the responses of all the peaks

Acceptance criteria: NMT 9.0%

Calculate the percentage of zeaxanthin in the portion of Lutein taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = peak response of zeaxanthin

r_T = sum of the responses of all the peaks

T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Calculate the percentage of other related compounds in the portion of Lutein taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak response of any other peak in the chromatogram (excluding zeaxanthin and lutein)

r_T = sum of the responses of all the peaks

Acceptance criteria: NMT 8.5% of zeaxanthin; NMT 1.0% of any other single related compound on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 2.0%
- **LEAD** (251): NMT 1 ppm
- **HEAVY METALS, Method II** (231): NMT 5 ppm

SPECIFIC TESTS

- **WATER DETERMINATION, Method I** (921): NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in hermetically sealed, light- and oxygen-resistant containers. Store in a cool place.
- **USP REFERENCE STANDARDS** (11)
USP Lutein RS

Lutein Preparation

DEFINITION

Lutein Preparation is a combination of Lutein with one or more inert substances. It may be in a solid or a liquid form. It contains NLT 95.0% and NMT 130.0% of the labeled amount of lutein, calculated as $C_{40}H_{56}O_2$ on the anhydrous basis. It contains NLT 85.0% of lutein and NMT 9.0% of zeaxanthin of the total carotenoid content.

IDENTIFICATION

- **A. ULTRAVIOLET ABSORPTION** (197U)
Analytical wavelength: 300–700 nm
Sample solution: Prepare as directed for the *Sample solution* in the test for *Content of Total Carotenoids*.
Ratio: A_{446}/A_{474} , 1.09–1.14
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Lutein*.

COMPOSITION**• CONTENT OF TOTAL CAROTENOIDS**

Diluent: Hexanes, acetone, toluene, and dehydrated alcohol (10:7:7:6)

Sample stock solution A (for solid lutein preparations labeled as containing gelatin): Transfer an amount of Preparation, equivalent to 3.5 mg of lutein, to a 50-mL centrifuge tube. Add 15 mL of warm water, 60 units of bacterial alkaline protease preparation, and 1 mg of bromelain. Cap and sonicate for 20 min with occasional swirling. Cool to room temperature, and add 20.0 mL of methylene chloride. Shake for 1 min, and centrifuge for 5 min at 2000 rpm. Remove the upper aqueous phase, and add 2–3 g of anhydrous sodium sulfate to the remaining red layer.

Sample stock solution B (for other solid lutein preparations): Transfer an amount of Preparation, equivalent to 1.5 mg of lutein, to a 50-mL centrifuge tube. Add 15 mL of warm water, cap, and sonicate for 30 min with occasional swirling. Cool to room temperature, and add 30.0 mL of ethyl acetate and 2–3 g of sodium chloride. Shake for 1 min, and centrifuge for 5 min at 2000 rpm. Use the upper orange-red layer.

Sample stock solution C (for liquid lutein suspensions in oil): Transfer a weighed amount of Preparation equivalent to 20 mg of lutein to a 100-mL volumetric flask, and dilute with *Diluent* to volume. Add a magnetic bar, and stir for 30 min.

Sample solution: Transfer 1.0 mL of *Sample stock solution A*, or 1.0 mL of *Sample stock solution B*, or 1.0 mL of *Sample stock solution C* into a 100-mL volumetric flask, and dilute with dehydrated alcohol to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Analytical wavelength: 446 nm

Cell path: 1 cm

Blank: Dehydrated alcohol

Analysis

Sample: *Sample solution*

Calculate the percentage of total carotenoids (T) as lutein ($C_{40}H_{56}O_2$) in the Preparation:

$$\text{Result} = (A \times V \times D \times 100)/(F \times W)$$

A = absorbance of the *Sample solution*

F = absorptivity of the lutein in alcohol, 255.0 (mL/mg · cm)

V = volume of organic solvent (20.0 mL for *Sample stock solution A*, 30.0 mL for *Sample stock solution B*, and 100.0 mL for *Sample stock solution C*) used in preparing the *Sample stock solutions*

D = dilution factor used to prepare the *Sample solution* from *Sample stock solutions*

W = weight of Preparation taken to prepare the *Sample stock solutions* (mg)

• CONTENT OF LUTEIN

Diluent: Hexanes, acetone, toluene, and dehydrated alcohol (10:7:7:6)

Mobile phase: Hexane and ethyl acetate (75:25)

Standard solution: 150 µg/mL of USP Lutein RS in *Mobile phase*

Sample solution: Transfer 1.0 mL of *Sample stock solution A*, or 1.0 mL of *Sample stock solution B*, or 2.0 mL of *Sample stock solution C* from the test for *Content of Total Carotenoids* into a suitable vial. Evaporate the solvent to dryness under a stream of nitrogen. Add 1.0 mL of *Mobile phase*, and sonicate to dissolve.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 446 nm

Column: 4.6-mm × 25-cm; 5-µm packing L3

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution* [NOTE—The relative retention times for lutein and zeaxanthin are about 1.0 and 1.05, respectively.]

Suitability requirements

Resolution: NLT 1.0 between lutein and zeaxanthin

Tailing factor: NMT 2

Relative standard deviation: NMT 2.0%

Analysis

Sample: *Sample solution*

Calculate the percentage of lutein relative to total carotenoids in the Preparation taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak response of lutein
 r_T = sum of the responses of all the peaks
 Calculate the percentage of lutein in the Preparation taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = individual peak response of lutein in the *Sample solution*
 r_T = sum of the responses of all the peaks
 T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Acceptance criteria: NLT 85.0% of lutein in the total carotenoid content, and the Preparation contains 95.0%–130.0% of the labeled amount of lutein, calculated as $C_{40}H_{56}O_2$, on the anhydrous basis.

• ZEAXANTHIN AND OTHER RELATED COMPOUNDS

Solvent, Mobile phase, Standard solution, Sample solution, and Chromatographic system: Proceed as directed in the test for *Content of Lutein*.

Analysis

Sample: *Sample solution*

Injection size: 10 μ L

Calculate the percentage of zeaxanthin relative to total carotenoids in the Preparation taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak response of zeaxanthin
 r_T = sum of the responses of all the peaks

Acceptance criteria

Zeaxanthin: NMT 9.0%

Any other single related compound: NMT 1.0%

Total related compounds (including zeaxanthin): NMT 15.0%

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 2.0%
- **LEAD** (251): NMT 1 ppm
- **HEAVY METALS** (231): NMT 10 ppm

SPECIFIC TESTS

- **WATER DETERMINATION, Method I** (921): NMT 10.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tightly sealed, light- and oxygen-resistant containers. Store in a cool place.
- **LABELING:** The label states that this article is not intended for direct administration to humans or to animals.
- **USP REFERENCE STANDARDS** (11)
USP Lutein RS

Lycopene

$C_{40}H_{56}$ 536.88
 [502-65-8].

DEFINITION

Lycopene is a mixture of geometrical isomers of lycopene. It contains NLT 96.0% and NMT 101.0% of lycopene ($C_{40}H_{56}$), calculated on the dried basis.

IDENTIFICATION

- **A. ULTRAVIOLET-VISIBLE ABSORPTION** (197U)
Wavelength range: 300–700 nm
Test solution: Prepare as directed for the *Sample solution* in the test for *Content of Lycopene*.
Acceptance criteria: Meets the requirements in the chapter. The ratio of A_{476}/A_{508} is 1.10–1.14.
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as

obtained in the test for *Content of all-E-Lycopene, 5Z-Lycopene, and Related Compounds*.

COMPOSITION

• CONTENT OF LYCOPENE

Sample stock solution: Transfer 25 mg of Lycopene and 25 mg of butylated hydroxytoluene into a 100-mL volumetric flask. Add 60 mL of methylene chloride, and sonicate to dissolve. Dilute with methylene chloride to volume.

Sample solution: Transfer 2.0 mL of the *Sample stock solution* into a 200-mL volumetric flask, and dilute with cyclohexane to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Analytical wavelength: 476 nm

Blank: Cyclohexane

Analysis

Sample: *Sample solution*

Calculate the percentage of lycopene ($C_{40}H_{56}$) in the portion of Lycopene taken:

$$\text{Result} = A_U/[(a \times C_U)] \times 100$$

A_U = absorbance of the *Sample solution*

a = absorptivity of the pure lycopene in cyclohexane, 331 ($\text{mL} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$)

C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: 96.0%–101.0% on the dried basis

• CONTENT OF ALL-E-LYCOPENE, 5Z-LYCOPENE, AND RELATED COMPOUNDS

Mobile phase: *tert*-Butyl methyl ether, methanol, and tetrahydrofuran (784:665:74)

Standard solution: Transfer a weighed quantity of USP Lycopene RS, equivalent to approximately 5 mg of lycopene, into a 250-mL volumetric flask. Add about 60 units of bacterial alkaline protease preparation or another suitable enzyme, and about 25 mg of butylated hydroxytoluene. Add 2.5 mL of dilute ammonium hydroxide (2 in 100) in water, and mix. Place in an ultrasonic bath at 50° for 10 min, rotate the flask occasionally to avoid having the material stick to the glass surface, and continue until the material is dispersed with no lumps. Add 5 mL of tetrahydrofuran, 40 mL of dehydrated alcohol, and mix. Place in an ultrasonic bath for about 1 min. Cool to room temperature, and dilute with *tert*-butyl methyl ether to volume. Shake vigorously, then allow the precipitate to settle. Filter the supernatant.

Sample stock solution: Transfer 15 mg of Lycopene to a 25-mL volumetric flask, and dissolve in tetrahydrofuran containing 50 mg/L of butylated hydroxytoluene. Dilute with the same solvent to volume.

Sample solution: Pipet 2 mL of the *Sample stock solution* into a 50-mL volumetric flask, and add 8 mL of tetrahydrofuran. Dilute with *tert*-butyl methyl ether to volume.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 472 nm

Column: 4.6-mm \times 25-cm, 5- μ m packing L62; second column connected in series, 4.6-mm \times 25-cm, 3- μ m packing L62. [NOTE—New columns may require conditioning.]

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for all-E-lycopene and 5Z-lycopene peaks are 1.0 and about 1.07, respectively.]

Suitability requirements

Resolution: NLT 1.0 between all-*E*-lycopene and 5*Z*-lycopene peaks

Tailing factor: 0.8–2.0 for the all-*E*-lycopene peak

Relative standard deviation: NMT 2.0% for the all-*E*-lycopene peak

Analysis

Sample: *Sample solution*

Calculate the percentage of all-*E*-lycopene in the portion of Lycopene taken:

$$\text{Result} = (r_E/r_T) \times T$$

r_E = peak response of the all-*E*-lycopene isomer

r_T = sum of the responses of all the peaks

T = percentage of total lycopene isomers obtained in the test for *Content of Lycopene*

Acceptance criteria: NLT 70.0% of all-*E*-lycopene

Calculate the percentage of the 5*Z*-lycopene isomer in the portion of Lycopene taken:

$$\text{Result} = (r_{5Z}/r_T) \times T$$

r_{5Z} = peak response for the 5*Z*-lycopene isomer

r_T = sum of the responses of all the peaks

T = percentage of total lycopene isomers obtained in the test for *Content of Lycopene*

Acceptance criteria: NMT 23.0% of the 5*Z*-lycopene isomer

Calculate the percentage of related compounds in the portion of Lycopene taken:

$$\text{Result} = (r_S/r_T) \times T$$

r_S = sum of the responses of all peaks, except the peak for all-*E*-lycopene and the peak for 5*Z*-lycopene

r_T = sum of the responses of all the peaks

T = percentage of total lycopene isomers obtained in the test for *Content of Lycopene*

Acceptance criteria: NMT 9.0% of other related compounds calculated as lycopene

IMPURITIES

• **RESIDUE ON IGNITION** (281): NMT 0.2%

• **HEAVY METALS**, *Method II* (231): NMT 10 ppm

SPECIFIC TESTS

• **LOSS ON DRYING** (731): Dry a sample in vacuum over phosphorus pentoxide at 40° for 4 h: it loses NMT 0.2% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, under inert gas, and store in a cool place.

• **LABELING:** Label it to indicate whether the article is obtained from natural sources or is prepared synthetically. If obtained from natural sources, label it to indicate the natural source, including its Latin binomial.

• **USP REFERENCE STANDARDS** (11)

USP Lycopene RS

Lycopene Preparation**DEFINITION**

Lycopene Preparation is a combination of Lycopene with one or more inert substances and suitable antioxidants. It may be in a solid or oily liquid form. It contains NLT 95.0% and NMT 120.0% of the labeled amount of lycopene (C₄₀H₅₆), calculated on the anhydrous basis.

IDENTIFICATION

• **A. ULTRAVIOLET-VISIBLE ABSORPTION** (197U)

Wavelength range: 300–700 nm

Test solution: Prepare as directed for the *Sample solution* in the test for *Content of Lycopene*.

Acceptance criteria: Meets the requirements in the chapter. The ratio A₄₇₆/A₅₀₈ is 1.10–1.14 in cyclohexane. The ratio A₄₇₂/A₅₀₄ is 1.09–1.13 in isopropyl alcohol.

COMPOSITION

• **CONTENT OF LYCOPENE**

Procedure for oily preparations

Sample stock solution: Transfer a weighed quantity of oily Preparation containing 25 mg of lycopene to a 100-mL volumetric flask. Add 25 mg of butylated hydroxytoluene and 60 mL of methylene chloride, and sonicate to dissolve. Dilute with methylene chloride to volume.

Sample solution: 2.0 mL of the *Sample stock solution* diluted with cyclohexane to 200.0 mL

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Analytical wavelength: 476 nm

Blank: Cyclohexane

Analysis

Sample: *Sample solution*

Calculate the percentage of the labeled amount of lycopene (C₄₀H₅₆) in the portion of Preparation taken:

$$\text{Result} = A_U/[(a \times C_U)] \times 100$$

A_U = absorbance of the *Sample solution*

a = absorptivity of the pure lycopene in cyclohexane, 331 (mL · mg⁻¹ · cm⁻¹)

C_U = nominal concentration of lycopene in the *Sample solution* (mg/mL)

Acceptance criteria: 95.0%–120.0% on the anhydrous basis

Procedure for solid preparations

Sample stock solution: Transfer a weighed quantity of solid Preparation, equivalent to approximately 5 mg of lycopene, into a 200-mL volumetric flask. Add about 60 units of bacterial alkaline protease preparation, or another suitable enzyme, and about 25 mg of butylated hydroxytoluene. Add 2.5 mL of dilute ammonium hydroxide (2 in 100) in water, and mix. Place in an ultrasonic bath at 50° for 10 min, rotate the flask occasionally to avoid having the material stick to the glass surface, and continue until the material is dispersed with no lumps. Add 5 mL of tetrahydrofuran, 40 mL of dehydrated alcohol, and mix. Place in an ultrasonic bath for about 1 min. Cool to room temperature, and dilute with *tert*-butyl methyl ether to volume. Shake vigorously, then allow to stand until the solid has settled.

Sample solution: 2.0 mL of the *Sample stock solution* diluted with isopropyl alcohol to 25.0 mL

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Analytical wavelength: 472 nm

Blank: Isopropyl alcohol

Analysis

Sample: *Sample solution*

Calculate the percentage of the labeled amount of lycopene in the portion of Preparation taken:

$$\text{Result} = A_U/[(a \times C_U)] \times 100$$

A_U = absorbance of the *Sample solution*

a = absorptivity of the pure lycopene in isopropyl alcohol, 320 (mL · mg⁻¹ · cm⁻¹)

C_U = nominal concentration of lycopene in the *Sample solution* (mg/mL)

Acceptance criteria: 95.0%–120.0% on the anhydrous basis

• **CONTENT OF ALL-*E*-LYCOPENE, 5Z-LYCOPENE, AND RELATED COMPOUNDS**

Mobile phase: *tert*-Butyl methyl ether, methanol, and tetrahydrofuran (784:665:74)

Standard solution: Transfer a weighed quantity of USP Lycopene RS, equivalent to approximately 5 mg of lycopene, into a 250-mL volumetric flask. Add about 60 units of bacterial alkaline protease preparation, or another suitable enzyme, and about 25 mg of butylated hydroxytoluene. Add 2.5 mL of dilute ammonium hydroxide (2 in 100) in water, and mix. Place in an ultrasonic bath at 50° for 10 min, rotate the flask occasionally to avoid having the material stick to the glass surface, and continue until the material is dispersed with no lumps. Add 5 mL of tetrahydrofuran, 40 mL of dehydrated alcohol, and mix. Place in an ultrasonic bath for about 1 min. Cool to room temperature, and dilute with *tert*-butyl methyl ether to volume. Shake vigorously, then allow the precipitate to settle. Filter the supernatant.

Sample solution for oily preparations: Transfer a quantity of oily Preparation, equivalent to 15 mg of lycopene, to a 25-mL volumetric flask, and dissolve in tetrahydrofuran containing 50 mg/L of butylated hydroxytoluene. Dilute with the same solvent to volume. Pipet 2 mL of this solution into a 50-mL volumetric flask, and add 8 mL of tetrahydrofuran. Dilute with *tert*-butyl methyl ether to volume.

Sample solution for solid preparations: Transfer a weighed quantity of solid Preparation, equivalent to approximately 5 mg of lycopene, into a 250-mL volumetric flask. Add about 60 units of bacterial alkaline protease preparation, or another suitable enzyme, and about 25 mg of butylated hydroxytoluene. Add 2.5 mL of dilute ammonium hydroxide (2 in 100) in water, and mix. Place in an ultrasonic bath at 50° for 10 min, rotate the flask occasionally to avoid having the material stick to the glass surface, and continue until the material is dispersed with no lumps. Add 5 mL of tetrahydrofuran, 40 mL of dehydrated alcohol, and mix. Place in an ultrasonic bath for about 1 min. Cool to room temperature, and dilute with *tert*-butyl methyl ether to volume. Shake vigorously, then allow the precipitate to settle. Filter the supernatant.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 472 nm

Column: 4.6-mm × 25-cm, 5-μm packing L62; second column connected in series, 4.6-mm × 25-cm, 3-μm packing L62. [NOTE—New columns may require conditioning.]

Flow rate: 1 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for the all-*E*-lycopene and 5Z-lycopene peaks are 1.0 and about 1.07, respectively.]

Suitability requirements

Resolution: NLT 1.0 between the all-*E*-lycopene and 5Z-lycopene peaks

Tailing factor: 0.8–2.0 for the all-*E*-lycopene peak

Relative standard deviation: NMT 2.0% for the all-*E*-lycopene peak

Analysis

Sample: *Sample solution*

Calculate the percentage of all-*E*-lycopene in the portion of Preparation taken:

$$\text{Result} = (r_E/r_T) \times 100$$

r_E = peak response of the all-*E*-lycopene isomer

r_T = sum of the responses of all the peaks

Acceptance criteria: NLT 65.0% of all-*E*-lycopene
Calculate the percentage of the 5Z-lycopene isomer in the portion of Preparation taken:

$$\text{Result} = (r_{5Z}/r_T) \times 100$$

r_{5Z} = peak response for the 5Z-lycopene isomer

r_T = sum of the responses of all the peaks

Acceptance criteria: NMT 23.0% of the 5Z-lycopene isomer

Calculate the percentage of related compounds in the portion of Preparation taken:

$$\text{Result} = (r_S/r_T) \times 100$$

r_S = sum of the responses of all peaks except the peak for all-*E*-lycopene and the peak for 5Z-lycopene

r_T = sum of the responses of all the peaks

Acceptance criteria: NMT 14% of other related compounds calculated as lycopene

SPECIFIC TESTS

- **WATER DETERMINATION, Method I <921>:** NMT 8.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers under inert gas. Store the oily Preparation in a cool place and the solid Preparation at controlled room temperature.
- **LABELING:** Label it to state the name and content of added antioxidants and inert substances. Label it to indicate whether the article is prepared with lycopene from natural sources or with synthetic lycopene. If prepared with lycopene from natural sources, label it to indicate the natural source, including its Latin binomial.
- **USP REFERENCE STANDARDS <11>**
USP Lycopene RS

Tomato Extract Containing Lycopene

DEFINITION

Tomato Extract Containing Lycopene is an ethyl acetate extract of the natural tomato lipids. It is produced from the pulp of ripe fruits of *Lycopersicon esculentum* Mill. (Fam. Solanaceae), after removing the tomato water-soluble fraction. It contains NLT 95.0% and NMT 105.0% of the labeled amount of lycopene (C₄₀H₅₆). It contains NLT 4.7% and NMT 12.0% of lycopene (C₄₀H₅₆), NLT 0.8% of the combined amount of phytofluene (C₄₀H₆₈) and phytoene (C₄₀H₆₄), NLT 0.2% of beta carotene (C₄₀H₅₆), and NLT 1.0% of tocopherols (C₂₈H₄₈O₂) on the anhydrous basis. Tocopherols may be added as antioxidants.

IDENTIFICATION

- **A. PRESENCE OF LYCOPENE, PHYTOFLUENE, AND PHYTOENE**

Analysis: Proceed as directed in the test for *Content of Other Carotenoids and Tocopherols*.

Acceptance criteria: The retention times of the peaks for lycopene, phytofluene, and phytoene from the *Sample solution* correspond to those from *Standard solution C*.

- **B. RATIO OF ALL-*E*-LYCOPENE AND 5Z-LYCOPENE**

Butylated hydroxytoluene stock solution: Proceed as directed in the test for *Content of Lycopene*.

Mobile phase: 0.05% diisopropylethylamine in *n*-hexane; sonicate for 3–4 min

Sample solution: Proceed as directed in the test for *Content of Lycopene*, except dilute 5 to 100 with *n*-hexane

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV-Vis 472 nm**Column:** Two 4.0-mm × 25-cm columns; 5-μm packing L3 (300 Å pore size), connected in a series**Column temperature:** 22°**Flow rate:** 0.5 mL/min**Injection size:** 10 μL[NOTE—The peak for all-*E*-lycopene elutes in 30–45 min.][NOTE—The relative retention times for all-*E*-lycopene and 5*Z*-lycopene are 1.00 in the range and 1.04–1.10, respectively.]**Analysis****Sample:** *Sample solution*

Measure the areas of the two major peaks, and calculate their area ratio:

$$\text{Result} = r_{U1}/r_{U2}$$

 r_{U1} = peak area of 5*Z*-lycopene r_{U2} = peak area of all-*E*-lycopene**Acceptance criteria:** NMT 0.10 for the area ratio**COMPOSITION****• CONTENT OF LYCOPENE****Butylated hydroxytoluene stock solution:** 5 mg/mL of butylated hydroxytoluene in methylene chloride.

[NOTE—This solution can be stored protected from light for up to 3 months.]

Mobile phase: Acetonitrile, methylene chloride, *n*-hexane, and methanol (850:25:25:100). Add 0.05% of diisopropylethylamine, mix, and sonicate for 3–4 min.**Diluent:** Acetonitrile, methylene chloride, *n*-hexane, butylated hydroxytoluene, and methanol (600:150:100:0.5:150). Add 0.05% of diisopropylethylamine, mix, and sonicate for 3–4 min.**Standard solution A:** Transfer a weighed quantity of USP Lycopene RS, equivalent to approximately 5 mg of lycopene, to a 100-mL volumetric flask. Add about 60 units of bacterial alkaline protease preparation, or another suitable enzyme, and about 25 mg of butylated hydroxytoluene. Add 2.5 mL of dilute ammonium hydroxide (2 in 100) in water, mix, and place in an ultrasonic bath at 50° for 10 min, rotating the flask occasionally to avoid having the material stick to the glass surface. Continue until the material is dispersed with no lumps. Add 5 mL of tetrahydrofuran, and shake until no colored precipitate remains. Add another portion of 2 mL of tetrahydrofuran and 40 mL of *Diluent*, and shake until the mixture is homogeneous. Dilute with *Diluent* to volume, shake vigorously, and allow to stand, if necessary, until the solid has settled. Calculate the exact concentration of this solution by the following method.**Standard solution B:** To 2.0 mL of *Standard solution A* add 10 mL of alcohol and 10 mL of *Butylated hydroxytoluene stock solution*, and dilute with *n*-hexane to 100 mL. Prepare in triplicate.Determine the absorbance of *Standard solution B* at the maximum absorbance at about 472 nm, using a mixture of alcohol, *Butylated hydroxytoluene stock solution*, and *n*-hexane (10:10:80) as the blank. Calculate the concentration of *Standard solution A*, in μg/mL, of lycopene:

$$\text{Result} = (A_x/F) \times 50,000$$

 A_x = average of the absorbance of the three preparations of *Standard solution B* F = absorptivity of pure lycopene in *n*-hexane at 472 nm, 345**Standard solution C:** Transfer a weighed quantity of USP Tomato Extract Containing Lycopene RS, equivalent to about 6 mg of lycopene, to a 100-mL volumetric flask, and dissolve in 1 mL of *Butylated hydroxytoluene stock solution* and 9 mL of methylene chloride, using a sonicator. Dilute with *Diluent* to volume to obtain a solution having a known concentration of about 0.06 mg/mL of lycopene.**Sample stock solution:** Warm the Tomato Extract Containing Lycopene to 50° in a water bath. Mix well with a glass rod or a spatula. Weigh and dissolve a quantity of 1–1.2 g of the sample in 10 mL of *Butylated hydroxytoluene stock solution* and 30 mL of methylene chloride, and sonicate the solution for 1 min to dissolve the sample completely. Cool to room temperature, and dilute with methylene chloride to 100 mL.**Sample solution:** Dilute the *Sample stock solution* with *Diluent* (1 in 10).**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV-Vis 472 nm**Column:** 4.6-mm × 25-cm; 5-μm packing L7**Column temperature:** 39 ± 1°**Flow rate:** 0.7 mL/min**Injection size:** 10 μL**System suitability****Sample:** *Standard solution A*

[NOTE—The retention time for lycopene is about 6 min.]

Suitability requirements**Relative standard deviation:** NMT 1.5% for the lycopene peak area**Analysis****Samples:** *Standard solution A* or *Standard solution C*, and *Sample solution*

Measure the responses of the major lycopene peaks.

Calculate the percentage of lycopene in the portion of Tomato Extract Containing Lycopene taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

 r_U = peak area for lycopene from the *Sample solution* r_S = peak area for lycopene from *Standard solution A* or *Standard solution C* C_S = concentration of lycopene in *Standard solution A* or *Standard solution C* (μg/mL) V = volume of the *Sample stock solution* (mL) W = weight of Tomato Extract Containing Lycopene taken to prepare the *Sample stock solution* (mg) D = dilution factor used to prepare the *Sample solution* from *Sample stock solution***Acceptance criteria:** NLT 95.0%–NMT 105.0% of the labeled amount of lycopene; NLT 4.7%–NMT 12.0% of lycopene (C₄₀H₅₆)**• CONTENT OF OTHER CAROTENOIDS AND TOCOPHEROLS (PHYTOFLUENE, PHYTOENE, BETA CAROTENE, AND TOCOPHEROLS)****Butylated hydroxytoluene stock solution, Diluent, Standard solution A, Standard solution B, Standard solution C, and Sample solution:** Proceed as directed in the test for *Content of Lycopene*.**Mobile phase:** Acetonitrile, methylene chloride, *n*-hexane, and methanol (19:1:1:19). Add 0.05% of diisopropylethylamine, mix, and sonicate for 3–4 min.**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV-Vis; 472 nm for lycopene; 450 nm for beta carotene; 350 nm for phytofluene; and 288 nm for phytoene and tocopherol

Column: 4.6-mm × 25-cm; 5-μm packing L1

Column temperature: 39 ± 1°

Flow rate: 0.6 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution C*

[NOTE—The relative retention times are about 0.6 for the tocopherol isomers, 1.0 for all-*E*-lycopene, 1.5–1.7 for the beta carotene isomers, 1.6–1.8 for the phytofluene isomers, and 1.8–2.2 for phytoene.]

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution C* is similar to the reference chromatogram provided with the lot of USP Tomato Extract Containing Lycopene RS being used.

Relative standard deviation: NMT 2% for all-*E*-lycopene

Analysis

Samples: *Standard solution A* and *Sample solution*
Identify the locus of the peaks for the lycopene isomers, the beta carotene isomers, the phytofluene isomers, and phytoene by comparison with the reference chromatogram provided with the corresponding lot of USP Tomato Extract Containing Lycopene RS. Measure the sum of the peak responses of the lycopene isomers at 472 nm in *Standard solution A*. [NOTE—The lycopene isomers may be resolved in more than one peak in this chromatographic system.] In the *Sample solution*, measure the sum of the peak responses of the beta carotene isomers at 450 nm, the sum of the peak responses of the phytofluene isomers at 350 nm, the peak response of phytoene at 288 nm, and the sum of the peak responses of all tocopherols at 288 nm.

Determine the concentration of *Standard solution A* as directed in the test for *Content of Lycopene*.

Calculate the percentage of beta carotene in the portion of Tomato Extract Containing Lycopene taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times F \times 100$$

r_U = sum of the peak responses for the beta carotene isomers at 450 nm from the *Sample solution*

r_S = peak area for lycopene at 472 nm from *Standard solution A*

C_S = concentration of lycopene in *Standard solution A* (μg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Tomato Extract Containing Lycopene taken to prepare the *Sample stock solution* (mg)

D = dilution factor used to prepare the *Sample solution* from *Sample stock solution*

F = absorptivity ratio of pure lycopene to pure beta carotene, 345/259.2

Calculate the percentage of phytofluene in the portion of Tomato Extract Containing Lycopene taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times F \times 100$$

r_U = sum of the peak responses for phytofluene isomers at 350 nm from the *Sample solution*

r_S = sum of the peak responses for the lycopene isomers at 472 nm from *Standard solution A*

C_S = concentration of lycopene in *Standard solution A* (μg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Tomato Extract Containing Lycopene taken to prepare the *Sample stock solution* (mg)

D = dilution factor used to prepare the *Sample solution* from *Sample stock solution*

F = absorptivity ratio of pure lycopene to pure phytofluene, 345/135

Calculate the percentage of phytoene in the portion of Tomato Extract Containing Lycopene taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times F \times 100$$

r_U = area of the phytoene peak response at 288 nm from the *Sample solution*

r_S = sum of the peak responses for the lycopene isomers at 472 nm from *Standard solution A*

C_S = concentration of lycopene in *Standard solution A* (μg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Tomato Extract Containing Lycopene taken to prepare the *Sample stock solution* (mg)

D = dilution factor used to prepare the *Sample solution* from *Sample stock solution*

F = absorptivity ratio of pure lycopene to pure phytoene, 345/125

Calculate the percentage of tocopherols in the portion of Tomato Extract Containing Lycopene taken to prepare the *Sample solution*:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times F \times 100$$

r_U = sum of the peak responses for all the tocopherol peaks at 288 nm from the *Sample solution*

r_S = sum of the peak responses for the lycopene isomers at 472 nm from *Standard solution A*

C_S = concentration of lycopene in *Standard solution A* (μg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Tomato Extract Containing Lycopene taken to prepare the *Sample stock solution* (mg)

D = dilution factor used to prepare the *Sample solution* from *Sample stock solution*

F = absorptivity ratio of pure lycopene to the average absorptivity for tocopherols, 345/8.5

Acceptance criteria: NLT 0.8% of the combined amount of phytofluene (C₄₀H₆₈) and phytoene (C₄₀H₆₄), NLT 0.2% of beta carotene (C₄₀H₅₆), and NLT 1.0% of tocopherols (C₂₈H₄₈O₂), on the anhydrous basis

CONTAMINANTS

- **HEAVY METALS, Method II (231):** NMT 10 μg/g
- **ARTICLES OF BOTANICAL ORIGIN, Test for Aflatoxins (561):** NMT 4 ng/g of total aflatoxins B1, B2, G1, and G2; NMT 2 ng/g of aflatoxin B1
- **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues (561):** Meets the requirements
- **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic microbial count does not exceed 10³ cfu/g, and the total combined molds and yeasts count does not exceed 2 × 10² cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** It meets the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Pseudomonas aeruginosa*.

SPECIFIC TESTS

CLARITY OF SOLUTION

Analysis: Warm the sample to 50° in a water bath. Mix well with a glass rod or a spatula, and transfer 1 g of the Extract directly into a 100-mL volumetric flask. Add 50 mL of methylene chloride, and sonicate the solution for 1 min to completely dissolve the sample. Bring to room temperature, and dilute with methylene chloride to volume.

Acceptance criteria: The solution is clear: no deposit or turbidity is formed.

ROTATIONAL RHEOMETER METHODS (912)

Analysis: Equilibrate the Tomato Extract Containing Lycopene at 37° in a 30-mL glass vial. Determine the viscosity using a rotational viscometer equipped with a spindle (No. 6) having a cylinder 1.47 cm in diameter

and 0.16 cm high attached to a shaft 0.32 cm in diameter, with a distance of 3.02 cm from the top of the cylinder to the lower tip of the shaft. The spindle is rotating at the appropriate speed and immersion depth to obtain a scale reading of 10%–90% of full scale. Calculate the viscosity, in centipoises, by multiplying the scale reading by the constant for the spindle and speed used.

Acceptance criteria: NMT 5000 centipoises

- **WATER DETERMINATION, Method 1a (921):** NMT 0.8%

- **PARTICLE SIZE DISTRIBUTION**

(See *Optical Microscopy* (776).)

Analysis: Transfer 1 drop to a microscope slide, and spread evenly. Isopropanol may be used as a diluent, if necessary. Examine the slide under a microscope equipped with a calibrated ocular micrometer, using 450× magnification. Scan the slide, and note the size of the individual particles.

Acceptance criteria: NLT 98% of the particles are less than 20 µm in length when measured along the longest axis, NLT 60% of the particles are less than 5 µm, and NLT 40% of the particles are less than 2 µm.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store in a cool place.
- **LABELING:** Label it to state the content of lycopene as a percentage and to state that the material should be heated to 50° and mixed before use. Label it to indicate the Latin binomial and the part of the plant from which the article is derived.
- **USP REFERENCE STANDARDS (11)**
 - USP Lycopene RS
 - USP Tomato Extract Containing Lycopene RS

Lysine Acetate—see *Lysine Acetate General Monographs*

Lysine Hydrochloride—see *Lysine Hydrochloride General Monographs*

Lysine Hydrochloride Tablets

DEFINITION

Lysine Hydrochloride Tablets contain NLT 90.0% and NMT 120.0% of the labeled amount of $C_6H_{14}N_2O_2 \cdot HCl$, as L-lysine hydrochloride.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 0.4 mg/mL of USP L-Lysine Hydrochloride RS in water

Sample solution: A filtered solution in water, equivalent to 0.4 mg/mL of lysine hydrochloride from powdered Tablets

Chromatographic system
(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 10 µL

Developing solvent system: Isopropyl alcohol and ammonium hydroxide (70:30)

Spray reagent: 2 mg/mL of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (95:5)

Analysis

Samples: *Standard solution* and *Sample solution*
Develop the plate, and dry at 100°–105° until the ammonia disappears completely. Spray with *Spray reagent*, and heat at 100°–105° for 15 min. Examine the plate under white light.

Acceptance criteria: The R_f value of the principal spot from the *Sample solution* corresponds to that from the *Standard solution*.

STRENGTH

• **PROCEDURE**

Sample: A portion of the powder from NLT 20 finely powdered Tablets, equivalent to 75 mg of lysine hydrochloride

Blank: Proceed as directed in the *Analysis* without the *Sample*.

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric or visual

Analysis: Dissolve the *Sample* in 5 mL of mercuric acetate TS with gentle heating. Cool, then add 50 mL of glacial acetic acid. Add 3 drops of crystal violet TS when needed. Titrate with *Titrant*. Perform the *Blank* determination.

Calculate the percentage of the labeled amount of L-lysine hydrochloride ($C_6H_{14}N_2O_2 \cdot HCl$) in the portion of *Sample* taken:

$$\text{Result} = \{[(V_s - V_b) \times N \times F]/W\} \times 100$$

V_s = *Titrant* volume consumed by the *Sample* (mL)

V_b = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 91.33 mg/mEq

W = nominal amount of L-lysine hydrochloride in the *Sample* taken (mg)

Acceptance criteria: 90.0–120.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION (2040):** Meet the requirements for *Disintegration*
Time: 20 min
- **WEIGHT VARIATION (2091):** Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.
- **USP REFERENCE STANDARDS (11)**
 - USP L-Lysine Hydrochloride RS

Magnesium Gluconate—see *Magnesium Gluconate General Monographs*

Magnesium Gluconate Tablets—see *Magnesium Gluconate Tablets General Monographs*

Magnesium Oxide Capsules—see *Magnesium Oxide Capsules General Monographs*

Magnesium Oxide Tablets—see Magnesium Oxide Tablets General Monographs

Manganese Gluconate—see Manganese Gluconate General Monographs

Malabar-Nut-Tree, Leaf

DEFINITION

Malabar-Nut-Tree, Leaf, also known in commerce as *vasaka*, consists of the dried leaves of *Justicia adhatoda* L., also known as *Adhatoda vasica* Nees (Fam. Acanthaceae). It contains NLT 0.6% of vasicine, calculated on the dried basis.

IDENTIFICATION

- A.** Malabar-Nut-Tree Leaf meets the requirements in *Specific Tests, Botanic Characteristics*.
- B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution A: 0.5 mg/mL of USP Vasicine RS in methanol. Sonicate to dissolve if necessary.
Standard solution B: 50 mg/mL of USP Powdered Malabar-Nut-Tree, Leaf Extract RS in methanol. Sonicate for about 15 min, centrifuge, and use the supernatant.
Sample solution: Transfer about 2.0 g of Malabar-Nut-Tree, Leaf, finely powdered and accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for 15 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume to 25 mL, using methanol.
 [NOTE—Save the filtrate for use in the test for *Content of Vasicine*.]

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 μm (TLC plates)

Application volume: 10 μL , as 4-mm bands

Developing solvent system: A mixture of ethyl acetate, methanol, and ammonia (8: 2: 0.2)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621), *Thin-Layer Chromatography*). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, and examine under UV at 254 nm.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a quenching zone at an R_f value of approximately 0.35 for vasicine, corresponding to a zone in the chromatogram of *Standard solution A*. The chromatogram of the *Sample solution* also exhibits an additional quenching zone at an R_f value of approximately 0.53 for vasicinone, corresponding to a similar zone in the chromatogram of *Standard solution B*. Other minor zones may be observed in the *Sample solution* and *Standard solution B* chromatograms.

- C. HPLC:** The chromatogram of the *Sample solution* from the test for *Content of Vasicine* shows a main peak at a retention time corresponding to that of vasicine in the chromatogram of *Standard solution A*. Identify other peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used. The *Sample solution* shows an additional peak corresponding to vasicinone.

COMPOSITION

• CONTENT OF VASICINE

Buffer solution: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of water. Add 2.0 mL of phosphoric acid. Dilute with water to 1000 mL, and filter.

Mobile phase: *Buffer solution*, acetonitrile, and tetrahydrofuran (92:5:3)

Standard solution A: 0.1 mg/mL of USP Vasicine RS in methanol. Sonicate to dissolve if necessary.

Standard solution B: 5.0 mg/mL of USP Powdered Malabar-Nut-Tree, Leaf Extract RS in methanol. Sonicate for about 15 min. Before injection, pass through a membrane filter of 0.45- μm or finer pore size.

Sample solution: Dilute the *Sample solution*, prepared as directed in *Identification test A* (1:5), with methanol. Before injection, pass through a membrane filter of 0.45- μm or finer pore size, and discard the first part of the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm \times 25-cm; 5- μm packing L10

Flow rate: 1.0 mL/min

Injection size: 20 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*
 [NOTE—The approximate relative retention times of the vasicine and vasicinone peaks are 1.00 and 1.23, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used.

Resolution: NLT 2.0 between the vasicine and vasicinone peaks, *Standard solution B*

Relative standard deviation: NMT 2.0% determined from the vasicine peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used, identify the retention times of the peaks corresponding to vasicine and vasicinone. Calculate the percentage of vasicine in the portion of Malabar-Nut-Tree, Leaf taken:

$$\text{Result} = (r_u/r_s) \times C_s \times (V/W) \times 100$$

r_u = peak response of vasicine from the *Sample solution*

r_s = peak response of vasicine from *Standard solution A*

C_s = concentration of USP Vasicine RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Malabar-Nut-Tree, Leaf taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.6% on the dried basis

CONTAMINANTS

- HEAVY METALS, Method III (231):** NMT 20 ppm
- ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis (561):** Meets the requirements
- MICROBIAL ENUMERATION TESTS (2021):** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.

- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Leaves, simple, green to dull grayish brown, minutely pubescent, lanceolate to ovate-lanceolate, tapering base and slightly acuminate apex, 10–20 cm long, 3–10 cm broad, petioles 2–8 cm long, and 8–10 pairs of reticulate lateral veins; characteristic odor, and bitter taste. Pharmacopeial article consists of dry, brittle, and greyish brown leaves.

Histology

Transverse section of leaves: Upper epidermal cells uniform in size and sinuous in outline, while lower epidermal cells vary in size and are less wavy; 4–6 layers of collenchymal cells below epidermis in the midrib region; shows two layers of palisade cells with elongated cystoliths, which are absent in epidermal cells (a diagnostic feature that is not seen in the leaves of *Ailanthus excelsa*, an adulterant for Malabar-Nut-Tree, Leaf); globules of oil dispersed in palisade and spongy layers; numerous stomata diacytic or anomocytic; glandular and non-glandular trichomes are visible on both epidermal layers.

- **LOSS ON DRYING (731):** Dry 1.0 g of finely powdered Malabar-Nut-Tree, Leaf at 105° for 3 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 21%, determined on 1.0 g of finely powdered Malabar-Nut-Tree, Leaf
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash (561):** NMT 2%
- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter (561):** NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS (11)**
USP Powdered Malabar-Nut-Tree, Leaf Extract RS
USP Vasicine RS

Powdered Malabar-Nut-Tree, Leaf

DEFINITION

Powdered Malabar-Nut-Tree, Leaf is Malabar-Nut-Tree, Leaf reduced to a powder or very fine powder. It contains NLT 0.6% of vasicine, calculated on the dried basis.

IDENTIFICATION

- **A.** Powdered Malabar-Nut Tree, Leaf meets the requirements in *Specific Tests, Botanic Characteristics*.
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution A: 0.5 mg/mL of USP Vasicine RS in methanol. Sonicate to dissolve if necessary.
Standard solution B: 50 mg/mL of USP Powdered Malabar-Nut-Tree, Leaf Extract RS in methanol. Sonicate for about 15 min, centrifuge, and use the supernatant.
Sample solution: Transfer about 2.0 g of Powdered Malabar-Nut-Tree, Leaf, accurately weighed, to a 250-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of methanol, reflux on a water bath for 15 min, cool to room temperature, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, filter, concentrate under vacuum, and adjust the volume to 25 mL, using methanol.

[NOTE—Save the filtrate for use in the test for *Content of Vasicine*.]

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates)

Application volume: 10 µL, as 4-mm bands

Developing solvent system: A mixture of ethyl acetate, methanol, and ammonia (8: 2: 0.2)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate (see *Chromatography (621)*, *Thin-Layer Chromatography*). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, and examine under UV at 254 nm.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a quenching zone at an R_f value of approximately 0.35 for vasicine, corresponding to a zone in the chromatogram of *Standard solution A*. The chromatogram of the *Sample solution* also exhibits an additional quenching zone at an R_f value of approximately 0.53 for vasicinone, corresponding to a similar zone in the chromatogram of *Standard solution B*. Other minor zones may be observed in the *Sample solution* and *Standard solution B* chromatograms.

- **C. HPLC:** The chromatogram of the *Sample solution* from the test for *Content of Vasicine* shows a main peak at a retention time corresponding to that of vasicine in the chromatogram of *Standard solution A*. Identify other peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used. The *Sample solution* shows an additional peak corresponding to vasicinone.

COMPOSITION

• CONTENT OF VASICINE

Buffer solution: Dissolve 1.36 g of anhydrous potassium dihydrogen orthophosphate in 900 mL of HPLC grade water. Add 2.0 mL of phosphoric acid. Dilute with water to 1000 mL, and filter.

Mobile phase: *Buffer solution*, acetonitrile, and tetrahydrofuran (92:5:3)

Standard solution A: 0.1 mg/mL of USP Vasicine RS in methanol. Sonicate to dissolve if necessary.

Standard solution B: 5.0 mg/mL of USP Powdered Malabar-Nut-Tree, Leaf Extract RS in methanol. Sonicate for about 15 min. Before injection, pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Dilute the *Sample solution*, prepared as directed in *Identification test A (1:5)*, with methanol. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, and discard the first part of the filtrate.

Chromatographic system

(See *Chromatography (621)*, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; 5-µm packing L10

Flow rate: 1.0 mL/min

Injection size: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*
[NOTE—The approximate relative retention times of the vasicine and vasicinone peaks are 1.00 and 1.23, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used.

Resolution: NLT 2.0 between the vasicine and vasicinone peaks, *Standard solution B*

Relative standard deviation: NMT 2.0% determined from the vasicine peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used, identify the retention times of the peaks corresponding to vasicine and vasicinone. Calculate the percentage of vasicine in the portion of Powdered Malabar-Nut-Tree, Leaf taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak response of vasicine from the *Sample solution*

r_S = peak response of vasicine from *Standard solution A*

C_S = concentration of USP Vasicine RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered Malabar-Nut-Tree, Leaf taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.6% on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method III (231)*: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis (561)*: Meets the requirements
- **MICROBIAL ENUMERATION TESTS (2021)**: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS (2022)**: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**: Greenish brown powder; characteristic odor; and bitter taste. Under a microscope, it shows the presence of parenchyma cells with elongated cystoliths; oil globules; epidermal cells with numerous stomata diacytic or anomocytic, glandular and nonglandular, 1- to 3-celled, thin-walled, warty trichomes.
- **LOSS ON DRYING (731)**: Dry 1.0 g of Powdered Malabar-Nut-Tree, Leaf at 105° for 3 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash (561)*: NMT 21%, determined on 1.0 g of Powdered Malabar-Nut-Tree, Leaf
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash (561)*: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING**: The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS (11)**
USP Powdered Malabar-Nut-Tree, Leaf Extract RS
USP Vasicine RS

Powdered Malabar-Nut-Tree, Leaf Extract

DEFINITION

Powdered Malabar-Nut-Tree, Leaf Extract is prepared from Malabar-Nut-Tree, Leaf using suitable solvents such as water, methanol, or a mixture of these solvents. The ratio of plant material to extract is between 8:1 and 5:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of vasicine. It may contain suitable added substances as carriers.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: 0.5 mg/mL of USP Vasicine RS in methanol. Sonicate to dissolve if necessary.

Standard solution B: 50 mg/mL of USP Powdered Malabar-Nut-Tree, Leaf Extract RS in methanol. Sonicate for about 15 min, centrifuge, and use the supernatant.

Sample solution: 50 mg/mL of Powdered Malabar-Nut-Tree, Leaf Extract in methanol. Sonicate for about 15 min, centrifuge, and use the supernatant.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 μm (TLC plates)

Application volume: 10 μL , as 4-mm bands

Developing solvent system: A mixture of ethyl acetate, methanol, and ammonia (8: 2: 0.2)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* as bands to a suitable thin-layer chromatographic plate (see *Chromatography (621)*, *Thin-Layer Chromatography*). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, and examine under UV at 254 nm.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a quenching zone at an RF value of approximately 0.35 for vasicine, corresponding to a zone in the chromatogram of *Standard solution A*. The chromatogram of the *Sample solution* also exhibits an additional quenching zone at an RF value of approximately 0.53 for vasicinone, corresponding to a similar zone in the chromatogram of *Standard solution B*. Other minor zones may be observed in the *Sample solution* and *Standard solution B* chromatograms.

- **B. HPLC:** The chromatogram of the *Sample solution* from the test for *Content of Vasicine* shows a main peak at a retention time corresponding to that of vasicine in the chromatogram of *Standard solution A*. Identify other peaks in the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used. The *Sample solution* shows an additional peak corresponding to vasicinone.

COMPOSITION

• CONTENT OF VASICINE

Buffer solution: Dissolve 1.36 g of anhydrous potassium dihydrogen phosphate in 900 mL of HPLC grade water. Add 2.0 mL of phosphoric acid. Dilute with water to 1000 mL, and filter.

Mobile phase: *Buffer solution*, acetonitrile, and tetrahydrofuran (92:5:3)

Standard solution A: 0.1 mg/mL of USP Vasicine RS in methanol. Sonicate to dissolve if necessary.

Standard solution B: 5.0 mg/mL of USP Powdered Malabar-Nut-Tree, Leaf Extract RS in methanol. Sonicate for about 15 min. Before injection, pass through a membrane filter of 0.45- μm or finer pore size.

Sample solution: 5.0 mg/mL of Powdered Extract in methanol. Sonicate for about 15 min. Before injection,

pass through a membrane filter of 0.45- μ m or finer pore size, and discard the first part of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm \times 25-cm; 5- μ m packing L10

Flow rate: 1.0 mL/min

Injection size: 20 μ L

System suitability

Samples: *Standard solution A* and *Standard solution B*

[NOTE—The approximate relative retention times for vasicine and vasicinone peaks are 1.00 and 1.23, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used.

Resolution: NLT 2.0 between the vasicine and vasicinone peaks, *Standard solution B*

Relative standard deviation: NMT 2.0% determined from the vasicine peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Using the chromatogram of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered Malabar-Nut-Tree, Leaf Extract RS being used, identify the retention times of the peaks corresponding to vasicine and vasicinone. Calculate the percentage of vasicine in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of vasicine from the *Sample solution*

r_S = peak response of vasicine from *Standard solution A*

C_S = concentration of USP Vasicine RS in *Standard solution A* (mg/mL)

C_U = concentration of Powdered Malabar-Nut-Tree, Leaf Extract in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amount of vasicine

CONTAMINANTS

- **HEAVY METALS**, *Method III* <231>: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **LOSS ON DRYING** <731>: Dry 1.0 g of Powdered Extract at 105° for 3 h: it loses NMT 5.0% of its weight.
- **OTHER REQUIREMENTS**: It meets the requirements of the test for *Residual Solvents* in *Botanical Extracts* <565>.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in well-closed containers, protected from light and moisture, and store at controlled room temperature.
- **LABELING**: The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived. It meets other labeling requirements in *Botanical Extracts* <565>.

• USP REFERENCE STANDARDS <11>

USP Powdered Malabar-Nut-Tree, Leaf Extract RS
USP Vasicine RS

Maritime Pine

DEFINITION

Maritime Pine consists of the bark of stems of *Pinus pinaster* Aiton (*Pinus maritima* Poir.) Fam. Pinaceae. It contains NLT 8.0% and NMT 12.0% of procyanidins, calculated on the dried basis.

[NOTE—This article is intended to be used in the preparation of extracts only and is not for direct human consumption.]

IDENTIFICATION

• A. PRESENCE OF PROCYANIDINS

Sample: Pulverize 1 g of the dried Maritime Pine. Use 10 mg.

Analysis: Add the *Sample* to 1 mL of methanol, and add 6 mL of a mixture of butanol and hydrochloric acid (95:5). Heat for 2 min in a water bath.

Acceptance criteria: The solution turns red.

• B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 25 mg/mL of USP Maritime Pine Extract RS in alcohol [NOTE—Retain a portion of this solution for use in *Identification test C*.]

Sample solution: Add 2 g of the powdered dried material to 20 mL of water. Place in a water bath for 20 min, and centrifuge. Extract the supernatant with 40 mL of ethyl acetate. Evaporate the ethyl acetate layer to dryness under a stream of nitrogen, with gentle heating. Dissolve the residue so obtained in 0.25 mL of alcohol.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 5 μ L

Developing solvent system: Ethyl acetate, formic acid, and water (50:5:3)

Spray reagent: Alcohol and phosphoric acid (1:1), containing 1% of vanillin

Analysis

Samples: *Standard solution* and *Sample solution*
Develop the chromatograms, dry the plate with the aid of a current of air, spray the plate with the *Spray reagent*, and heat at 115° for 15 min.

Acceptance criteria: The chromatogram of the *Sample solution* presents three red bands appearing in the middle third of the chromatogram corresponding to two dimeric procyanidins and catechin at the same R_f of similar bands in the *Standard solution*. The chromatogram of the *Sample solution* also exhibits a blue band between the upper band due to upper dimeric procyanidins and the band due to catechin, at the same R_f of a similar band found in the chromatogram of the *Standard solution*.

• C. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: Use the *Standard solution*, prepared as directed for *Identification test B*.

Standard solution B: 1 mg/mL each of ferulic acid and protocatechuic acid

Sample solution: Use the *Sample solution*, prepared as directed for *Identification test B*.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 10 μ L

Developing solvent system: Methylene chloride, methanol, glacial acetic acid, and water (80:15:2:2)

Spray reagent: 5% ferric chloride solution in methanol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatogram, dry the plate at 110°, and examine the plate under short-wavelength and long-wavelength UV light. The chromatograms of *Standard solution A* and *Standard solution B* exhibit bands in the middle third and upper third that correspond to protocatechuic acid and ferulic acid, respectively. Spray the plate with the *Spray reagent*, and heat at 115° for 15 min. The bands due to ferulic acid and protocatechuic acid turn grayish green. Grayish-green bands become visible in the chromatogram of *Standard solution A* above and below protocatechuic acid, indicating the presence of caffeic acid and catechin, respectively.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits bands due to catechin, protocatechuic acid, caffeic acid, and ferulic acid that correspond in color and R_f values to those in the chromatogram of *Standard solution A* and *Standard solution B*.

COMPOSITION

• CONTENT OF PROCYANIDINS

Reagent solution A: Butanol and hydrochloric acid (95:5) [NOTE—Prepare this solution on the day of use.]

Reagent solution B: Dissolve 2 g of ferric ammonium sulfate in a mixture of 100 mL of water and 17.5 mL of hydrochloric acid. [NOTE—This solution can be used within 15 days of preparation.]

Standard solution: 95 µg/mL of procyanidins from USP Maritime Pine Extract RS in methanol

Sample stock solution: Dry crushed Maritime Pine at 110° for 3 h. Place 1.9 g of the crushed material in a 20-mL vial, and add 10 mL of methanol. Crimp the vial, and sonicate for 2 min. Heat in boiling water for 10 min. Cool to room temperature, allow the sediment to settle, and transfer the supernatant to a 100-mL volumetric flask, passing it through a filter having a 0.45-µm pore size. Wash the sediment two times with 10 mL of methanol, and transfer the solution into the same 100-mL volumetric flask, again passing it through a filter having a 0.45-µm pore size. Dilute with methanol to volume.

Sample solution: Dilute the *Sample stock solution* with methanol (1 in 20).

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Vis

Wavelength: 551 nm

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 1.0 mL each of the *Standard solution*, *Sample solution*, and methanol to three separate 10-mL vials. To each vial add 6.0 mL of *Reagent solution A* and 0.25 mL of *Reagent solution B*. Seal the vials with crimp caps. Mix, and heat in a water bath for 40 min. Quickly cool to room temperature in an ice bath. Quantitatively transfer these solutions, with the aid of *Reagent solution A*, to three separate 10-mL volumetric flasks, and dilute with *Reagent solution A* to volume.

Determine the absorbance of the solutions obtained from the *Standard solution* and the *Sample solution*, using the methanol-containing solution as a blank.

Calculate the percentage of total procyanidins in the portion of Maritime Pine taken:

$$\text{Result} = (A_u/A_s) \times C_s \times (V/W) \times D \times P$$

A_u = absorbance of the solution from the *Sample solution*

A_s = absorbance of the solution from the *Standard solution*

C_s = concentration of the USP Maritime Pine Extract RS in the *Standard solution* (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Maritime Pine taken to prepare the *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from *Sample stock solution*, 20

P = percentage of procyanidins in the USP Maritime Pine Extract RS

Acceptance criteria: 8.0%–12.0% on the dried basis

SPECIFIC TESTS

• BOTANICAL CHARACTERISTICS

Macroscopic: Bark pieces are typically 1–3 cm thick.

The inner bark is plane to slightly concave, whitish to light brown, striped longitudinally; shiny and of slightly irregular surface, only a few millimeters thick. Abrupt change to a sequence of hard, convex, nearly parallel layers alternating with smooth, light brown layers. Up to 50 or more layers present, depending on the age of the bark. Outer surface of bark is dark reddish brown composed of irregular scaly patches, with deep V-shaped fissures. Outer surface may also be gray, gray-green, or green-yellow due to presence of lichens.

Microscopic (transverse section of bark): Light inner bark has irregular lateral stripes consisting of 3–5 cell layers of long, slender sieve cells with large pitted horizontal cell walls and large polygonal parenchyma cells containing single, irregular, rounded starch grain, 3–15 mm wide. Lateral stripes are separated from each other by ray parenchyma cells. Ray parenchyma cells are homogeneous in appearance, 1–4 cell layers thick and 4–20 cell layers high, each cell containing single, irregular, rounded starch grain, 3–15 mm wide. Cylindrical parenchyma cells with thin cell walls arranged in vertical rows with calcium oxalate prisms are also present. Outer part of the inner bark contains plate-shaped cells of undifferentiated periderm and older periderm with multiple layers of phellogen. The phellogen grows 3–7 rows of phellum to the exterior and 2–4 rows of small cell phelloderm to the interior. The oldest and outermost part of the bark is composed of lignified sections of phelloderm and phellum cells, 15–35 mm thick, separated by collapsed phellogen. Phelloderm and phellum cells are up to 100 µm wide, square, rectangular, polygonal, or irregularly shaped. The cell walls are colorless. Phelloderm cells are moderately pitted with a reddish-brown content. Phellum cells have a thicker cell wall of strongly pitted, undulated contour, and a yellowish-brown to brownish-red content. Radially between layers of phelloderm and phellum are layers of ray parenchyma cells, 5–8 cells thick, rounded to radially stretched, thin walled, strongly pitted with collapsed cells and dead sieve cells.

• **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** <561>: NMT 5%

• **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 1.5%

• **ARTICLES OF BOTANICAL ORIGIN, Water Content** <561>: NMT 35.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Store at 25°, excursion permitted between 15° and 30°. Preserve in a well-closed container, and protect from moisture and excessive heat.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.

• **USP REFERENCE STANDARDS** <11>
USP Maritime Pine Extract RS

Maritime Pine Extract

DEFINITION

Maritime Pine Extract is prepared from the pulverized Maritime Pine using suitable solvents. It contains NLT 65% and NMT 75% of procyanidins, calculated on the dried basis.

IDENTIFICATION

• A. PRESENCE OF PROCYANIDINS

Sample solution: Dissolve 50 mg of Extract in 6 mL of a mixture of butanol and hydrochloric acid (95:5).

Analysis: Heat for 2 min in a water bath.

Acceptance criteria: The solution turns red.

• B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: 25 mg/mL of USP Maritime Pine Extract RS in methanol

Standard solution B: 1 mg/mL each of ferulic acid and protocatechuic acid in methanol

Sample solution: 25 mg/mL of Extract in methanol

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 5 μ L

Developing solvent system: Methylene chloride, methanol, glacial acetic acid, and water (80:15:2:2)

Spray reagent: 5% ferric chloride solution in methanol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatogram, dry the plate at 110°, and examine the plate under short-wavelength and long-wavelength UV light. The chromatograms of *Standard solution A* and *Standard solution B* exhibit bands in the middle third and upper third that correspond to protocatechuic acid and ferulic acid, respectively.

Spray the plate with the *Spray reagent*, and heat at 115° for 15 min. The bands due to ferulic acid and protocatechuic acid turn grayish green. Grayish-green bands become visible in the chromatogram of *Standard solution A* above and below protocatechuic acid, indicating the presence of caffeic acid and catechin, respectively.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits bands due to catechin, protocatechuic acid, caffeic acid, and ferulic acid that correspond in color and R_f values to those in the chromatogram of *Standard solution A* and *Standard solution B*.

• C. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: Use *Standard solution A*, prepared as directed for *Identification test B*.

Sample solution: Use the *Sample solution*, prepared as directed for *Identification test B*.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 5 μ L

Developing solvent system: Ethyl acetate, formic acid, and water (50:5:3)

Spray reagent: Phosphoric acid and alcohol (1:1), containing 1% of vanillin

Analysis

Samples: *Standard solution* and *Sample solution*

Proceed as directed in the chapter, except to dry the plate with the aid of a current of air, spray the plate with the *Spray reagent*, and heat at 115° for 15 min. Three red bands appear in the middle third of the chromatogram of the *Standard solution* corresponding to two dimeric procyanidins and catechin. The chromatogram of the *Standard solution* also exhibits a blue band

between the upper band due to upper dimeric procyanidins and the band due to catechin.

Acceptance criteria: The chromatogram of the *Sample solution* contains bands that correspond to those found in the chromatogram of the *Standard solution*.

• D. HPLC IDENTIFICATION TEST

Solution A: Methanol

Solution B: 1 mg/mL of phosphoric acid in water

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	8	92
40	34	66
45	2	98
50	2	98
52	8	92
57	8	92

Standard solution: 2 mg/mL of USP Maritime Pine Extract RS in *Solution A*. Pass through a membrane having a 0.45- μ m or finer pore size.

Sample solution: Add 20 mg of Extract to 10 mL of *Solution A*, and sonicate for 10 min to dissolve. Pass through a membrane having a 0.45- μ m or finer pore size, discarding the first 4 mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm \times 15-cm; base-deactivated packing L7, having less than 5- μ m particle size

Column temperature: 40°

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Chromatogram similarity: The chromatogram is similar to the reference chromatogram provided with the lot of USP Maritime Pine Extract RS being used.

Resolution: NLT 3.0 between taxifolin and ferulic acid

Tailing factor: NMT 2.0 for taxifolin

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the peaks for catechin, caffeic acid, taxifolin, and ferulic acid by comparison of the chromatogram of the *Standard solution* with the reference chromatogram.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for catechin, caffeic acid, taxifolin, and ferulic acid at the retention times corresponding to those in the chromatogram of the *Standard solution*.

COMPOSITION

• CONTENT OF PROCYANIDINS

Reagent solution A: Butanol and hydrochloric acid (95:5) [NOTE—Prepare this solution on the day of use.]

Reagent solution B: Dissolve 2 g of ferric ammonium sulfate in a mixture of 100 mL of water and 17.5 mL of hydrochloric acid. [NOTE—This solution can be used within 15 days of preparation.]

Standard solution: 95 μ g/mL of procyanidins from USP Maritime Pine Extract RS in methanol

Sample stock solution: Transfer 250 mg of Extract to a 100-mL volumetric flask. Dissolve with methanol, and dilute with the same solvent to volume.

Sample solution: Dilute the *Sample stock solution* with methanol (1 in 20).

Instrumental conditions

(See *Spectrometry and Light Scattering* <851>.)

Mode: Vis

Wavelength: 551 nm

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 1.0 mL each of the *Standard solution*, *Sample solution*, and methanol to three separate 10-mL vials. To each vial add 6.0 mL of *Reagent solution A* and 0.25 mL of *Reagent solution B*. Seal the vials with crimp caps. Mix, and heat in a water bath for 40 min. Quickly cool to room temperature in an ice bath. Quantitatively transfer these solutions, with the aid of *Reagent solution A*, to three separate 10-mL volumetric flasks, and dilute with *Reagent solution A* to volume.

Determine the absorbance of the solutions obtained from the *Standard solution* and the *Sample solution*, using the methanol-containing solution as a blank.

Calculate the percentage of total procyanidins in the portion of Extract taken:

$$\text{Result} = (A_U/A_S) \times C_S \times (V/W) \times D \times P$$

A_U = absorbance of the solution from the *Sample solution*

A_S = absorbance of the solution from the *Standard solution*

C_S = concentration of the USP Maritime Pine Extract RS in the *Standard solution* (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Maritime Pine Extract taken to prepare the *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from *Sample stock solution*, 20

P = percentage of procyanidines in the USP Maritime Pine Extract RS

Acceptance criteria: 65%–75% on the dried basis

CONTAMINANTS

- **BOTANICAL EXTRACTS**, *Heavy Metals* (565): Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN**, *Pesticide Residue* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

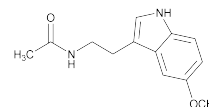
SPECIFIC TESTS

- **LOSS ON DRYING** (731): Dry 1.0 g of Extract for 3 h at 110°: it loses NMT 8.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 0.7%
- **LIMIT OF WATER-INSOLUBLE SUBSTANCES**
Analysis: Weigh 0.50 g of Extract, and stir in 50 mL of water at 20° for 15 min. Pass through a fine sintered glass filter, previously weighed. Dry the filter at 110° for 3 h, cool to room temperature, and weigh the filter. Calculate the amount of water-insoluble material.
Acceptance criteria: NMT 10% of the amount of Extract taken

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at 25°, excursion permitted between 15° and 30°. Protect from light.
- **LABELING:** The label states the Latin binomial and, following the official name of the article, the part of the plant from which the article was prepared, in addition to the information required for *Botanical Extracts* (565), *Labeling*.
- **USP REFERENCE STANDARDS** (11)
USP Maritime Pine Extract RS

Melatonin



$C_{13}H_{16}N_2O_2$

232.28

N-Acetyl-5-methoxytryptamine;

N-(2-(5-Methoxy-1*H*-indol-3-yl)ethyl) acetamide [73-31-4].

DEFINITION

Melatonin contains NLT 98.5% and NMT 101.5% of melatonin ($C_{13}H_{16}N_2O_2$), calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)

- **B. ULTRAVIOLET ABSORPTION** (197U)

Analytical wavelength: 277 nm

Sample solution: 10 µg/mL of Melatonin in isopropyl alcohol

Acceptance criteria: Meets the requirements. Absorptivities, calculated on the dried basis, do not differ by more than 3.0%.

- **C. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed in the *Assay*.

Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*.

ASSAY

- **PROCEDURE**

Buffer: 0.5 g/L of monobasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 3.5, and filter.

Mobile phase: Acetonitrile and *Buffer* (25:75)

System suitability solution: 0.1 mg/mL of USP Melatonin RS and 0.02 mg/mL USP Melatonin Related Compound A RS in *Mobile phase*

Standard solution: 0.1 mg/mL of USP Melatonin RS in *Mobile phase*

Sample solution: 0.1 mg/mL of Melatonin in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 222 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1.0 mL/min

Injection size: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for melatonin related compound A and melatonin are 0.4 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 4 between melatonin and melatonin related compound A, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of melatonin in the portion of Melatonin taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Melatonin RS in the *Standard solution* (mg/mL)

C_U = concentration of Melatonin in the *Sample solution* (mg/mL)

Acceptance criteria: 98.5–101.5% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%
- **CHLORIDE AND SULFATE**, *Chloride* (221)
Standard: 0.10 mL of 0.020 N hydrochloric acid
Sample: 0.36 g of Melatonin
Acceptance criteria: NMT 0.02%
- **HEAVY METALS** (231): NMT 20 µg/g
- **RELATED COMPOUNDS**
Solution A: Acetonitrile
Solution B: Use *Buffer*, prepared as directed in the *Assay*.
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	25	75
7	25	75
15	80	20
18	25	75
25	25	75

Diluent: Mixture of *Solution A* and *Solution B* (25:75)

System suitability solution: 0.1 mg/mL of USP Melatonin RS and 0.02 mg/mL of USP Melatonin Related Compound A RS in *Diluent*

Standard solution: 5 µg/mL of USP Melatonin RS in *Diluent*

Sample solution: 1 mg/mL of Melatonin in *Diluent*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 222 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1.0 mL/min

Injection size: 10 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for melatonin related compound A and melatonin are 0.4 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 4.0 between melatonin and melatonin related compound A

Relative standard deviation: NMT 2.0% for the melatonin peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each individual impurity in the portion of Melatonin taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each individual impurity from the *Sample solution*

r_S = peak response of melatonin from the *Standard solution*

C_S = concentration of USP Melatonin RS in the *Standard solution* (mg/mL)

C_U = concentration of Melatonin in the *Sample solution* (mg/mL)

Acceptance criteria

Individual impurities: NMT 0.1%

Total impurities: NMT 1.0%

SPECIFIC TESTS

• LOSS ON DRYING (731)

Analysis: Dry a sample at 80° in a vacuum for 3 h.

Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.

• USP REFERENCE STANDARDS (11)

USP Melatonin RS

USP Melatonin Related Compound A RS

2-(5-Methoxy-1*H*-indol-3-yl)ethanamine.

$C_{11}H_{14}N_2O$ 190.24

Add the following:

▲ Melatonin Tablets

DEFINITION

Melatonin Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of melatonin ($C_{13}H_{16}N_2O_2$).

IDENTIFICATION

- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in *Strength*.

STRENGTH

• PROCEDURE

Buffer: 0.5 g/L of monobasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 3.5, and filter.

Mobile phase: Acetonitrile and *Buffer* (25:75)

System suitability solution: 0.1 mg/mL of USP Melatonin RS and 0.02 mg/mL of USP Melatonin Related Compound A RS in *Mobile phase*

Standard solution: 0.1 mg/mL of USP Melatonin RS in *Mobile phase*

Sample solution: Filtered portion of the solution in *Mobile phase*, equivalent to 0.1 mg/mL of Melatonin from NLT 20 finely powdered Tablets

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 222 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1.0 mL/min

Injection size: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for melatonin related compound A and melatonin are 0.4 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 4 between melatonin and melatonin related compound A, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of melatonin ($C_{13}H_{16}N_2O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*
 r_S = peak response from the *Standard solution*
 C_S = concentration of USP Melatonin RS in the *Standard solution* (mg/mL)
 C_U = nominal concentration of melatonin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

• DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS

<2040>: Meet the requirements for *Dissolution*

Medium: Water; 500 mL

Apparatus 2: 50 rpm

Time: 30 min

Standard solution: Dissolve a suitable amount of USP Melatonin RS in water to obtain a concentration similar to that expected in the *Sample solution*.

Sample solution: Filtered portion of the solution under test

Analysis: Proceed as directed in *Strength*, making any necessary adjustments.

Calculate the percentage of the labeled amount of melatonin ($C_{13}H_{16}N_2O_2$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/L) \times 100$$

r_U = peak area from the *Sample solution*
 r_S = peak area from the *Standard solution*
 C_S = concentration of USP Melatonin RS in the *Standard solution* (mg/mL)
 V = volume of *Medium*, 500 mL
 L = labeled amount of melatonin (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of melatonin ($C_{13}H_{16}N_2O_2$) is dissolved.

• WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>: Meet the requirements

IMPURITIES

• RELATED COMPOUNDS

Solution A: Acetonitrile

Solution B: Use *Buffer*, prepared as directed in *Strength*.

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	25	75
7	25	75
15	80	20
18	25	75
25	25	75

Diluent: Mixture of *Solution A* and *Solution B* (25:75)

System suitability solution: 0.1 mg/mL of USP Melatonin RS and 0.02 mg/mL of USP Melatonin Related Compound A RS in *Diluent*

Standard solution: 5 µg/mL of USP Melatonin RS in *Diluent*

Sample solution: Filtered solution in *Diluent*, equivalent to 1 mg/mL of melatonin from NLT 20 finely powdered Tablets

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 222 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1.0 mL/min

Injection size: 10 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for melatonin related compound A and melatonin are 0.4 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 4.0 between melatonin and melatonin related compound A

Relative standard deviation: NMT 2.0% for the melatonin peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each individual impurity in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each individual impurity from the *Sample solution*

r_S = peak response of melatonin from the *Standard solution*

C_S = concentration of USP Melatonin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of melatonin in the *Sample solution* (mg/mL)

Acceptance criteria

Individual impurities: NMT 0.1%

Total impurities: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **LABELING:** The label states the quantity of melatonin in mg/Tablet.

• USP REFERENCE STANDARDS <11>

USP Melatonin RS

USP Melatonin Related Compound A RS

2-(5-Methoxy-1*H*-indol-3-yl)ethanamine.

$C_{11}H_{14}N_2O$ 190.24 ▲ *USP36*

Methionine—see Methionine General Monographs

Methylsulfonylmethane



$C_2H_6O_2S$

Dimethyl sulfone;

Sulfonylbismethane [67-71-0].

94.13

DEFINITION

Methylsulfonylmethane contains NLT 98.0% and NMT 102.0% of methylsulfonylmethane ($C_2H_6O_2S$), calculated on the anhydrous basis. The chromatographic purity is NLT 99.8%.

IDENTIFICATION

• **A. INFRARED ABSORPTION** <197K>

• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

• PROCEDURE

Diluent: Transfer 950 mL of methanol to a 1-L volumetric flask. Add 0.60 mL of di(ethylene glycol) methyl ether, and dilute with methanol to volume.

Standard solution: 0.4 mg/mL of USP Methylsulfonylmethane RS in *Diluent*. Sonicate at 50° for 1 min, and allow to cool to room temperature.

Sample solution: 0.4 mg/mL of Methylsulfonylmethane in *Diluent*. Sonicate at 50° for 1 min, and allow to cool to room temperature.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m capillary column coated with a 5-μm phase G2

Temperature

Column: 120°

Injector: 250°

Detector: 250°

Carrier gas: Helium

Flow rate: 5 mL/min

Split ratio: 2:1

Injection size: 1 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0% for the peak response ratio of methylsulfonylmethane to di(ethylene glycol) methyl ether from replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of methylsulfonylmethane (C₂H₆O₂S) in the portion of Methylsulfonylmethane taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of methylsulfonylmethane to di(ethylene glycol) methyl ether from the *Sample solution*

R_S = peak response ratio of methylsulfonylmethane to di(ethylene glycol) methyl ether from the *Standard solution*

C_S = concentration of USP Methylsulfonylmethane RS in the *Standard solution* (mg/mL)

C_U = concentration of Methylsulfonylmethane in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

- **HEAVY METALS, Method I** <231>: NMT 3 μg/g

• **CHROMATOGRAPHIC PURITY AND LIMIT OF DIMETHYL SULFOXIDE**

Standard stock solution: 1.0 mg/mL of USP Dimethyl Sulfoxide RS in methanol

Sensitivity check solution: 2.0 μg/mL from the *Standard stock solution* in methanol

System suitability solution: 0.1 mg/mL of USP Dimethyl Sulfoxide RS and 0.4 mg/mL of USP Methylsulfonylmethane RS in methanol. In a 50-mL volumetric flask dissolve 20 mg of USP Methylsulfonylmethane RS in 5 mL of the *Standard stock solution*, and dilute with methanol to volume.

Sample solution: 2 mg/mL of Methylsulfonylmethane in methanol. Sonicate at 50° for 1 min, and allow to cool to room temperature.

Chromatographic system: Proceed as directed in the *Assay*.

System suitability

Samples: *Sensitivity check solution* and *System suitability solution*

Suitability requirements

Signal-to-noise ratio: NLT 10 for dimethyl sulfoxide peak, *Sensitivity check solution*

Resolution: NLT 2.0 between dimethyl sulfoxide and methylsulfonylmethane, *System suitability solution*

Analysis

Sample: *Sample solution*

Calculate the percentage of each impurity in the portion of Methylsulfonylmethane taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = response of each impurity in the *Sample solution*

r_T = sum of the responses of all of the peaks other than the solvent peak

Acceptance criteria

Individual impurities: NMT 0.1% of dimethyl

sulfoxide; NMT 0.05% of any other individual impurity

Total impurities: NMT 0.2% for all impurities, including dimethyl sulfoxide

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** <741>: 108.5°–110.5°

- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count is NMT 10³ cfu/g or mL, and the total combined molds and yeasts count is NMT 10² cfu/g or mL.

- **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements for absence of *Escherichia coli* in 10 g.

- **WATER DETERMINATION, Method I** <921>: NMT 0.1%.

[NOTE—500 mg of methylsulfonylmethane may be required for this analysis.]

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

- **USP REFERENCE STANDARDS** <11>

USP Dimethyl Sulfoxide RS

USP Methylsulfonylmethane RS

Dimethyl sulfone.

C₂H₆O₂S 94.13

Methylsulfonylmethane Tablets

DEFINITION

Methylsulfonylmethane Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of methylsulfonylmethane (C₂H₆O₂S).

IDENTIFICATION

- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Procedure for Strength*.

STRENGTH

• **PROCEDURE**

Diluent: Transfer 950 mL of methanol to a 1-L volumetric flask. Add 0.60 mL of diethylene glycol methyl ether, and dilute with methanol to volume.

Standard solution: 0.4 mg/mL of USP Methylsulfonylmethane RS in *Diluent*. Sonicate at 50° for 1 min, and allow to cool to room temperature.

Sample solution: Finely powder NLT 20 Tablets. Dissolve a portion of the finely powdered material, equivalent to 1 Tablet, in *Diluent*, and sonicate for 15 min at 50°. Allow to cool to room temperature, dilute with *Diluent* to volume, and mix. Quantitatively dilute with *Diluent* to obtain a final concentration of 0.4 mg/mL of methylsulfonylmethane. Transfer 1 mL of the suspension to a 1.5-mL microcentrifuge tube, and centrifuge for 20 s. Use the supernatant.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m capillary; 5-μm phase G2 coating

Temperature

Column: 120°

Injector: 250°

Detector: 250°

Carrier gas: Helium

Flow rate: 5 mL/min

Injection size: 1 µL

Injector type: Split ratio, 2:1

System suitabilitySample: *Standard solution***Suitability requirements**

Relative standard deviation: NMT 2.0% for the peak response ratio of methylsulfonylmethane to diethylene glycol methyl ether from replicate injections

AnalysisSamples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of methylsulfonylmethane (C₂H₆O₂S) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = internal standard ratio (peak response of methylsulfonylmethane to diethylene glycol methyl ether) from the *Sample solution*

R_S = internal standard ratio (peak response of methylsulfonylmethane to diethylene glycol methyl ether) from the *Standard solution*

C_S = concentration of USP Methylsulfonylmethane RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of methylsulfonylmethane in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** (2040): Meet the requirements for *Disintegration*; 30 min
- **WEIGHT VARIATION** (2091): Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** (11)
USP Methylsulfonylmethane RS
Dimethyl sulfone.
C₂H₆O₂S 94.13

Milk Thistle**DEFINITION**

Milk Thistle consists of the dried ripe fruit of *Silybum marianum* (L.) Gaertn. (Fam. Asteraceae), the pappus having been removed. It contains NLT 2.0% of silymarin, calculated as silybin (C₂₅H₂₂O₁₀), on the dried basis.

IDENTIFICATION• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 1.0 mg/mL of USP Silydianin RS in methanol

Sample solution: Use the *Sample solution*, prepared as directed in the test for *Content of Silymarin*.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Adsorbent: 0.25 mm layer of chromatographic silica gel, typically 20 cm long (TLC plates)

Application volume: 10 µL

Developing solvent system: Freshly prepared mixture of chloroform, acetone, and anhydrous formic acid (75:16.5:8.5)

Spray reagent A: 10-mg/mL solution of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50-mg/mL solution of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved about three-fourths of the plate, and dry it for 30 min in a current of cold air. Spray the plate with *Spray reagent A*, allow to dry, and then spray with *Spray reagent B*. One h later, examine the plate under long-wavelength UV light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits an intense green-blue fluorescent zone at an R_f value of about 0.5 (presence of silybin) and exhibits a gray-blue spot at an R_f value of about 0.4, corresponding to a spot observed in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* may exhibit other colored zones: an intense green-blue zone at an R_f value of about 0.25 (presence of silychristin) and a red-orange zone at an R_f value of about 0.3 (presence of taxifolin).

• **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed for *Content of Silymarin*.

Acceptance criteria: The retention times of the peaks for silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B in the chromatogram of the *Sample solution* correspond to those in the chromatogram of the *Milk thistle standard solution*.

COMPOSITION• **CONTENT OF SILYMARIN**

Solution A: Methanol, phosphoric acid, and water (20: 0.5: 80)

Solution B: Methanol, phosphoric acid, and water (80: 0.5: 20)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	0	0
5	85	15
20	55	45
40	55	45
41	85	15
55	85	15

Milk thistle standard solution: 0.7 mg/mL of USP Powdered Milk Thistle Extract RS in methanol. Sonicate for 20 min to dissolve. Pass through a membrane filter having a 0.45-µm or finer pore size. Dilute 1 in 5 with methanol to obtain a solution of 0.14 mg/mL of USP Powdered Milk Thistle Extract RS.

Silybin standard solutions: 0.20, 0.02, and 0.004 mg/mL of USP Silybin RS in methanol. Pass through a membrane filter having a 0.45-µm or finer pore size.

Sample stock solution: Transfer 10 g of finely powdered Milk Thistle to an extraction thimble, and cover with a small cotton ball. Transfer the thimble to a continuous-extraction apparatus fitted with a 250-mL round-bottom flask containing 150 mL of solvent hexane, and heat the flask on a heating mantle for 4 h. After the extraction, separate the round-bottom flask containing solvent hexane extract from the extraction apparatus, and discard the solvent hexane solution. Remove the adherent solvent hexane from the extraction thimble by drying, and transfer the thimble to an extraction apparatus suitable for hot extraction and fitted with a 250-mL round-bottom flask containing 100 mL of ethyl acetate. [NOTE—Adjust the volume of ethyl acetate, if necessary, to sustain a continuous extraction.] Heat the flask on a heating mantle to allow the solvent to reflux gently. After 8 h, transfer the extract quantita-

tively into a 100-mL volumetric flask, and dilute with methanol to volume.

Sample solution: Dilute the *Sample stock solution* (1 in 25) with methanol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 288 nm

Column: 4.6-mm × 15-cm; 5-μm packing L1

Column temperature: 40°

Flow rate: 1 mL/min

Injection size: 10 μL

System suitability

Sample: *Milk thistle standard solution*

[NOTE—For the relative retention times, see *Table 2*.]

Table 2

	Relative Retention Time
Silychristin	0.68
Silydianin	0.73
Silybin A	1.00
Silybin B	1.05
Dehydrosilybin	1.09
Isosilybin A	1.15
Isosilybin B	1.19

Suitability requirements

Chromatogram similarity: The chromatogram from the *Milk thistle standard solution* is similar to the reference chromatogram provided with the lot of USP Powdered Milk Thistle Extract RS being used.

Resolution: NLT 1.0 between silybin A and silybin B

Tailing factor: 0.8–2.0

Relative standard deviation: NMT 2.0% for the sum of peak responses due to silybin A and silybin B

Analysis

Samples: *Milk thistle standard solution*, each of the *Silybin standard solutions*, and *Sample solution*
Identify the peaks due to silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B by comparison with the chromatogram of the *Milk thistle standard solution*, and measure the peak areas of the relevant peaks. Plot the areas of the sum of silybin A and silybin B peaks versus the concentration of USP Silybin RS in the *Silybin standard solutions*, and obtain a regression line for calibration.

Separately calculate the percentage of each relevant component of silymarin as silibin (C₂₅H₂₂O₁₀) in the portion of Milk Thistle taken:

$$\text{Result} = C \times (V/W) \times D \times 100$$

C = concentration of the relevant component in the *Sample solution* as interpolated from the calibration graph (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of the portion of Milk Thistle taken (mg)

D = dilution factor to prepare the *Sample solution* from *Sample stock solution*, 25

Calculate the content of silymarin, as a percentage, in the portion of Milk Thistle taken by adding the individual percentages.

Acceptance criteria: NLT 2.0% of silymarin, calculated as silybin (C₂₅H₂₂O₁₀), on the dried basis

CONTAMINANTS

• **HEAVY METALS** <231>: NMT 10 μg/g

• **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues** <561>: Meets the requirements

• **MICROBIAL ENUMERATION TESTS** <2021>: The total bacterial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts count does not exceed 10² cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli* and for absence of *Staphylococcus aureus*.

SPECIFIC TESTS

• **BOTANIC CHARACTERISTICS**

Macroscopic: The fruits (achenes) are elongated ovoid, slightly crooked, somewhat flattened, roughly 6–7 mm in length, up to 3 mm in width, and 1.5 mm in thickness, and have a projecting cartilaginous, glossy, yellowish edge on the upper surface and a grooved hilum at the base. The fruit coat is glossy brown-black or mat gray-brown with dark or pale gray streaks; it encloses the straight embryo having two thick, flattened cotyledons that contain fatty oil and aleurone granules.

Microscopic: The fruit wall epidermis consists of almost colorless palisade cells arranged at right angles to the surface. They have greatly thickened outer walls, into which the lumen continues for some distance in the form of a slit. Viewed from above under high magnification, the cells show only a slit-shaped lumen. They have thickened ridges that appear as nodular thickenings of the cell wall when viewed from above. The subepidermal layer of the fruit wall is made up of unlignified thin-walled parenchymal cells and constitutes a pigment layer. Colorless cells and groups of cells alternate with pigment cells, the latter varying in number; this gives the fruit wall its mottled appearance. Next comes the fruit wall tissue, about 8 cell layers thick, with stippled parenchymal cells elongated in the longitudinal axis of the fruit. The cells of the innermost layer of the fruit wall may be collapsed; they contain large cigar-shaped or monoclinic calcium oxalate prisms. The seed coat epidermis is formed from large yellow palisade cells. The cells have a narrow lumen, somewhat expanded at each end of the cell, and the cell walls show conspicuous lamination. The subepidermal cells of the seed coat consist of peculiar stippled cells; their lignified cell membranes have prominent, close-set ridges or thickenings (“net cells”). Next to them is a single layer of cells having tough, somewhat swollen walls and lipophilic contents (endosperm residue). The embryo consists of thin-walled cells that, in addition to small glands, contain clumps of crystals and fat droplets.

• **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** <561>: NMT 2.0%

• **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 8.0%, determined on 1.0 g of finely powdered Milk Thistle

• **LOSS ON DRYING** <731>: Dry 1.0 g of finely powdered Milk Thistle at 105° for 2 h: it loses NMT 8.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.

• **USP REFERENCE STANDARDS** <11>

USP Powdered Milk Thistle Extract RS

USP Silybin RS

USP Silydianin RS

Powdered Milk Thistle

DEFINITION

Powdered Milk Thistle is Milk Thistle reduced to a fine or very fine powder. It contains NLT 2.0% of silymarin, calculated as silybin ($C_{25}H_{22}O_{10}$), on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 1.0 mg/mL of USP Silydianin RS in methanol

Sample solution: Use the *Sample solution*, prepared as directed in the test for *Content of Silymarin*.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long

Application volume: 10 μ L

Developing solvent system: Freshly prepared mixture of chloroform, acetone, and anhydrous formic acid (75:16.5:8.5)

Spray reagent A: 10-mg/mL solution of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50-mg/mL solution of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved about three-fourths of the plate, and dry it for 30 min in a current of cold air. Spray the plate with *Spray reagent A*, allow to dry, and then spray with *Spray reagent B*. One h later, examine the plate under long-wavelength UV light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits an intense green-blue fluorescent zone at an R_f value of about 0.5 (presence of silybin) and exhibits a gray-blue spot at an R_f value of about 0.4, corresponding to a spot observed in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* may exhibit other colored zones: an intense green-blue zone at an R_f value of about 0.25 (presence of silychristin) and a red-orange zone at an R_f value of about 0.3 (presence of taxifolin).

• B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of Silymarin*.

Acceptance criteria: The retention times of the peaks for silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B in the chromatogram of the *Sample solution* correspond to those in the chromatogram of the *Milk thistle standard solution*.

COMPOSITION

• CONTENT OF SILYMARIN

Solution A: Methanol, phosphoric acid, and water (20:0.5:80)

Solution B: Methanol, phosphoric acid, and water (80:0.5:20)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	0	0
5	85	15
20	55	45
40	55	45
41	85	15
55	85	15

Milk thistle standard solution: 0.7 mg/mL of USP Powdered Milk Thistle Extract RS in methanol. Sonicate

for 20 min to dissolve. Pass through a membrane filter having a 0.45- μ m or finer pore size. Dilute 1 in 5 with methanol to obtain a solution of 0.14 mg/mL of USP Powdered Milk Thistle Extract RS.

Silybin standard solutions: 0.20, 0.02, and 0.004 mg/mL of USP Silybin RS in methanol. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Sample stock solution: Transfer 10 g of Powdered Milk Thistle to an extraction thimble, and cover with a small cotton ball. Transfer the thimble to a continuous-extraction apparatus fitted with a 250-mL round-bottom flask containing 150 mL of solvent hexane, and heat the flask on a heating mantle for 4 h. After the extraction, separate the round-bottom flask containing solvent hexane extract from the extraction apparatus, and discard the solvent hexane solution. Remove the adherent solvent hexane from the extraction thimble by drying, and transfer the thimble to an extraction apparatus suitable for hot extraction and fitted with a 250-mL round-bottom flask containing 100 mL of ethyl acetate. [NOTE—Adjust the volume of ethyl acetate, if necessary, to sustain a continuous extraction.] Heat the flask on a heating mantle to allow the solvent to reflux gently. After 8 h, transfer the extract quantitatively into a 100-mL volumetric flask, and dilute with methanol to volume.

Sample solution: Dilute the *Sample stock solution* (1 in 25) with methanol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 288 nm

Column: 4.6-mm \times 15-cm; 5- μ m packing L1

Column temperature: 40°

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Sample: *Milk thistle standard solution*

[NOTE—For the relative retention times, see *Table 2*.]

Table 2

	Relative Retention Time
Silychristin	0.68
Silydianin	0.73
Silybin A	1.00
Silybin B	1.05
Dehydrosilybin	1.09
Isosilybin A	1.15
Isosilybin B	1.19

Suitability requirements

Chromatogram similarity: The chromatogram from the *Milk thistle standard solution* is similar to the reference chromatogram provided with the lot of USP Powdered Milk Thistle Extract RS being used.

Resolution: NLT 1.0 between silybin A and silybin B

Tailing factor: 0.8–2.0

Relative standard deviation: NMT 2.0% for the sum of peak responses due to silybin A and silybin B

Analysis

Samples: *Milk thistle standard solution*, each of the *Silybin standard solutions*, and *Sample solution*

Identify the peaks due to silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B by comparison with the chromatogram of the *Milk thistle standard solution*, and measure the peak areas of the relevant peaks. Plot the areas of the sum of silybin A and silybin B peaks versus the concentration of USP Silybin RS in the *Silybin standard solutions*, and obtain a regression line for calibration.

Separately calculate the percentage of each relevant component of silymarin as silybin ($C_{25}H_{22}O_{10}$) in the portion of Powdered Milk Thistle taken:

$$\text{Result} = C \times (V/W) \times D \times 100$$

- C = concentration of the relevant component in the *Sample solution* as interpolated from the calibration graph (mg/mL)
 V = volume of the *Sample stock solution* (mL)
 W = weight of the portion of Powdered Milk Thistle taken (mg)
 D = dilution factor to prepare the *Sample solution* from *Sample stock solution*, 25

Calculate the content of silymarin, as a percentage, in the portion of Powdered Milk Thistle taken by adding the individual percentages.

Acceptance criteria: NLT 2.0% of silymarin, calculated as silybin ($C_{25}H_{22}O_{10}$), on the dried basis

CONTAMINANTS

- **HEAVY METALS** (231): NMT 20 µg/g
- **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues** (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli* and for absence of *Staphylococcus aureus*.

SPECIFIC TESTS

- **BOTANICAL CHARACTERISTICS:** The powder is characterized by fragments of colorless cells, up to approximately 75 µm in length and 8 µm in width, from the palisade layer of the epidermis of the fruit wall, with their adherent pigment layer (they assume a red color in chloral hydrate preparations), and by gray pieces, viewed from above, with the slitlike lumen produced by the pronounced wall thickening or the nodes on the cell wall formed by the thickened ridges; fragments of the pigment layer viewed from above, with red coloration diffusing out of them in chloral hydrate preparations, pigment cells alternating with colorless parenchymal cells; conspicuously stippled colorless cells through which pigment cells are visible when viewed from above; cigar-shaped or monoclinic calcium oxalate prisms, lying free or in groups of cells; numerous fragments of the lemon yellow palisade cells of the seed coat, up to roughly 150 µm in length, having a very narrow lumen and conspicuously stippled when viewed from above; pale yellowish fragments from the net cell layer together with portions of the embryo consisting of thin-walled cells with small glands and lipophilic substances.
- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** (561): NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 8.0%, determined on 1.0 g of Powdered Milk Thistle
- **LOSS ON DRYING** (731): Dry 1.0 g of Powdered Milk Thistle at 105° for 2 h: it loses NMT 8.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.
- **USP REFERENCE STANDARDS** (11)
 USP Powdered Milk Thistle Extract RS
 USP Silybin RS
 USP Silydianin RS

Powdered Milk Thistle Extract

DEFINITION

Powdered Milk Thistle Extract is prepared from Milk Thistle fruits or seeds by fat removal and subsequent extraction with suitable solvents. It contains NLT 90.0% and NMT 110.0% of the labeled amount of silymarin, calculated as silybin ($C_{25}H_{22}O_{10}$), on the dried basis, consisting of NLT 20.0% and NMT 45.0% for the sum of silydianin and silychristin, NLT 40.0% and NMT 65.0% for the sum of silybin A and silybin B, and NLT 10.0% and NMT 20.0% for the sum of isosilybin A and isosilybin B.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 1.0 mg/mL of USP Silydianin RS in methanol

Sample solution: 10 mg/mL of Powdered Extract in methanol, and allow to stand for 15 min before use.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long

Application volume: 10 µL

Developing solvent system: Freshly prepared mixture of chloroform, acetone, and anhydrous formic acid (75:16.5:8.5)

Spray reagent A: 10-mg/mL solution of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50-mg/mL solution of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved about three-fourths of the plate, and dry it for 30 min in a current of cold air. Spray the plate with *Spray reagent A*, allow to dry, and then spray with *Spray reagent B*. One h later, examine the plate under long-wavelength UV light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits an intense green-blue fluorescent zone at an R_f value of about 0.5 (presence of silybin) and exhibits a gray-blue spot at an R_f value of about 0.4, corresponding to a spot observed in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* may exhibit other colored zones: an intense green-blue zone at an R_f value of about 0.25 (presence of silychristin) and a red-orange zone at an R_f value of about 0.3 (presence of taxifolin).

• B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of Silymarin*.

Acceptance criteria: The retention times of the peaks for silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B in the chromatogram of the *Sample solution* correspond to those in the chromatogram of the *Milk thistle standard solution*.

COMPOSITION

• CONTENT OF SILYMARIN

Solution A: Methanol, phosphoric acid, and water (20:0.5:80)

Solution B: Methanol, phosphoric acid, and water (80:0.5:20)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	0	0
5	85	15
20	55	45

Table 1 (Continued)

Time (min)	Solution A (%)	Solution B (%)
40	55	45
41	85	15
55	85	15

Milk thistle standard solution: 0.7 mg/mL of USP Powdered Milk Thistle Extract RS in methanol. Sonicate for 20 min to dissolve.

Silybin standard solutions: 0.20, 0.02, and 0.004 mg/mL of USP Silybin RS in methanol. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Sample solution: Dissolve a sample of Extract in methanol to obtain a solution equivalent to 0.4 mg/mL of silymarin. Sonicate for 20 min, cool to 20°, and pass through a filter of 0.45- μ m or finer pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 288 nm

Column: 4.6-mm \times 15-cm; 5- μ m packing L1

Column temperature: 40°

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Sample: *Milk thistle standard solution*

[NOTE—For the relative retention times, see *Table 2*.]

Table 2

	Relative Retention Time
Silychristin	0.68
Silydianin	0.73
Silybin A	1.00
Silybin B	1.05
Dehydrosilybin	1.09
Isosilybin A	1.15
Tsosilybin B	1.19

Suitability requirements

Chromatogram similarity: The chromatogram from the *Milk thistle standard solution* is similar to the reference chromatogram provided with the lot of USP Powdered Milk Thistle Extract RS being used.

Resolution: NLT 1.0 between silybin A and silybin B

Tailing factor: 0.8–2.0

Relative standard deviation: NMT 2.0% for the sum of peak responses due to silybin A and silybin B

Analysis

Samples: *Milk thistle standard solution*, each of the *Silybin standard solutions*, and *Sample solution*

Identify the peaks due to silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B by comparison with the chromatogram of the *Milk thistle standard solution*, and measure the peak areas of the relevant peaks. Plot the areas of the sum of silybin A and silybin B peaks versus the concentration of USP Silybin RS in the *Silybin standard solutions*, and obtain a regression line for calibration.

Separately calculate the percentage of each relevant component of silymarin as silybin ($C_{25}H_{22}O_{10}$) in the portion of Powdered Extract taken:

$$\text{Result} = C \times (V/W) \times 100$$

C = concentration of the relevant component in the *Sample solution* as interpolated from the calibration graph (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of the portion of Powdered Extract taken (mg)

Calculate the percentage of the labeled amount of silymarin in the portion of Powdered Extract taken:

$$\text{Result} = \Sigma S/L \times 100$$

ΣS = sum of the percentages of the each relevant component of silymarin

L = labeled amount of silymarin, as a percentage, in the Powdered Extract

Acceptance criteria: 90.0%–110.0% of the labeled amount of silymarin, calculated as silybin ($C_{25}H_{22}O_{10}$), on the dried basis, consisting of: 20.0%–45.0% for the sum of silydianin and silychristin; 40.0%–65.0% for the sum of silybin A and silybin B; and 10.0%–20.0% for the sum of isosilybin A and isosilybin B

CONTAMINANTS

- **HEAVY METALS, Method II <231>:** NMT 20 μ g/g
- **MICROBIAL ENUMERATION TESTS <2021>:** The total bacterial count does not exceed 10^4 /g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the enterobacterial count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.
- **OTHER REQUIREMENTS:** It meets the requirements in *Botanical Extracts* <565> for the tests in sections *Pesticide Residues* and *Residual Solvents*.

SPECIFIC TESTS

- **LOSS ON DRYING <731>:** Dry a sample at 105° for 2 h: it loses NMT 5.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, in a cool place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. It meets the requirements for *Botanical Extracts* <565>, *Labeling*.
- **USP REFERENCE STANDARDS <11>**
USP Powdered Milk Thistle Extract RS
USP Silybin RS
USP Silydianin RS

Milk Thistle Capsules

DEFINITION

Milk Thistle Capsules are prepared from Powdered Milk Thistle Extract. They contain NLT 90.0% and NMT 110.0% of the labeled amount of silymarin as silybin ($C_{25}H_{22}O_{10}$), calculated as the sum of silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution: 1.0 mg/mL of USP Silydianin RS in methanol
Sample solution: Equivalent to 5 mg/mL of silymarin from powdered Capsule contents (finely powder the contents of NLT 20 Capsules) in methanol. Shake for 1 min, and sonicate for 10 min. Allow to stand for 15 min before use.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long

Application volume: 10 μ L

Developing solvent system: Freshly prepared mixture of chloroform, acetone, and anhydrous formic acid (75:16.5:8.5)

Spray reagent A: 10-mg/mL solution of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50-mg/mL solution of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms until the solvent front has moved about three-fourths of the plate, and dry it for 30 min in a current of cold air. Spray the plate with *Spray reagent A*, allow to dry, and then spray with *Spray reagent B*. One h later, examine the plate under long-wavelength UV light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits an intense green-blue fluorescent zone at an R_f value of 0.5 (presence of silybin) and exhibits a gray-blue spot at an R_f value of 0.4, corresponding to a spot observed in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* may exhibit other colored zones: an intense green-blue zone at an R_f value of 0.25 (presence of silychristin) and a red-orange zone at an R_f value of 0.3 (presence of taxifolin).

• B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed for *Content of Silymarin*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B at retention times that correspond to those of the *Milk thistle standard solution*.

STRENGTH

• CONTENT OF SILYMARIN

Solution A: Methanol, phosphoric acid, and water (20:0.5:80)

Solution B: Methanol, phosphoric acid, and water (80:0.5:20)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	0	0
5	85	15
20	55	45
40	55	45
41	85	15
55	85	15

Milk thistle standard solution: 0.7 mg/mL of USP Powdered Milk Thistle Extract RS in methanol. Sonicate for 20 min to dissolve.

Silybin standard solutions: 0.20, 0.02, and 0.004 mg/mL of USP Silybin RS in methanol. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Sample solution: Weigh and finely powder the contents of NLT 20 Capsules. Transfer an accurately weighed quantity of the powder equivalent to 100 mg of silymarin to a 100-mL volumetric flask, add 90 mL of methanol, and sonicate for 20 min with occasional shaking. Cool to 20°, and dilute with methanol to volume. Filter through a membrane with a 0.45- μ m or finer pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 288 nm

Column: 4.6-mm \times 15-cm; 5- μ m packing L1

Column temperature: 40°

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Sample: *Milk thistle standard solution*

[NOTE—For the relative retention times, see *Table 2*.]

Table 2

Name	Relative Retention Time
Silychristin	0.68
Silydianin	0.73
Silybin A	1.00
Silybin B	1.05
Dehydrosilybin	1.09
Isosilybin A	1.15
Isosilybin B	1.19

Suitability requirements

Chromatogram similarity: The chromatogram from the *Milk thistle standard solution* is similar to the reference chromatogram provided with the lot of USP Powdered Milk Thistle Extract RS being used.

Resolution: NLT 1.0 between silybin A and silybin B

Tailing factor: 0.8–2.0

Relative standard deviation: NMT 2.0% for the sum of peak responses due to silybin A and silybin B

Analysis

Samples: *Milk thistle standard solution*, each of the *Silybin standard solutions*, and *Sample solution*

Identify the peaks due to silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B by comparison of the chromatogram of the *Milk thistle standard solution* with the reference chromatogram, and measure the peak areas of the relevant peaks. Plot the areas of the sum of silybin A and silybin B peaks versus the concentration of USP Silybin RS in the *Silybin standard solutions*, and obtain a regression line for calibration.

Separately calculate the content, in mg, of each relevant component of silymarin as silybin ($C_{25}H_{22}O_{10}$) in the portion of contents of Capsules taken:

$$\text{Result} = C \times V$$

C = concentration of the relevant component in the *Sample solution* as interpolated in the calibration graph (mg/mL)

V = volume of the *Sample solution* (mL)

Calculate the content of silymarin, as a percentage, in the portion of Capsules taken:

$$\text{Result} = C_s \times (A_{wc}/W) \times (100/L)$$

C_s = sum of the contents of each relevant component of silymarin in the portion of contents of Capsules taken (mg)

A_{wc} = average weight of the contents of Capsules (mg/Tablet)

W = weight of the portion of content of Capsules taken (mg)

L = labeled amount of silymarin (mg/Capsule)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** <2040>: Meet the requirements for *Dissolution*

Buffer: 6.9 g/L of monobasic sodium phosphate and 1.52 g/L of sodium hydroxide in water. Adjust to a pH of 7.5.

Medium: Buffer containing 2% lauryl sulfate; 900 mL

Apparatus 2: 100 rpm

Time: 45 min

Determine the amount of silymarin as silybin ($C_{25}H_{20}O_{10}$) dissolved by using the method in the test for *Content of Silymarin*, making any necessary modifications.

Tolerances: NLT 75% of the labeled amount of silymarin as silybin ($C_{25}H_{20}O_{10}$) is dissolved.

- **WEIGHT VARIATION (2091):** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS (2021):** The total bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the enterobacterial count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Capsules were prepared. The label also indicates the content of silymarin, in mg/Capsule.
- **USP REFERENCE STANDARDS (11)**
USP Powdered Milk Thistle Extract RS
USP Silybin RS
USP Silydianin RS

Milk Thistle Tablets

DEFINITION

Milk Thistle Tablets are prepared from Powdered Milk Thistle Extract. They contain NLT 90.0% and NMT 110.0% of the labeled amount of silymarin as silybin ($C_{25}H_{20}O_{10}$), calculated as the sum of silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 1.0 mg/mL of USP Silydianin RS in methanol

Sample solution: Equivalent to 5 mg/mL of silymarin from finely powdered Tablets (NLT 20) in methanol. Shake for 1 min, and sonicate for 10 min. Allow to stand for 15 min before use.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel, typically 20 cm long

Application volume: 10 μ L

Developing solvent system: Freshly prepared mixture of chloroform, acetone, and anhydrous formic acid (75:16.5:8.5)

Spray reagent A: 10-mg/mL solution of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50-mg/mL solution of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*
Move the chromatograms until the solvent front has moved about three-fourths of the plate, and dry it for 30 min in a current of cold air. Spray the plate with *Spray reagent A*, allow to dry, and then spray with *Spray reagent B*. One h later, examine the plate under long-wavelength UV light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits an intense green-blue fluorescent zone

at an R_f value of 0.5 (presence of silybin) and exhibits a gray-blue spot at R_f value of 0.4, corresponding to a spot observed in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* may exhibit other colored zones: an intense green-blue zone at an R_f value of 0.25 (presence of silychristin) and a red-orange zone at an R_f value of 0.3 (presence of taxifolin).

- **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed for *Content of Silymarin*.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for silydianin, silychristin, silybin A, silybin B, isosilybin A, and isosilybin B at retention times that correspond to those of the *Milk thistle standard solution*.

STRENGTH

- **CONTENT OF SILYMARIN**

Solution A: Methanol, phosphoric acid, and water (20:0.5:80)

Solution B: Methanol, phosphoric acid, and water (80:0.5:20)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	0	0
5	85	15
20	55	45
40	55	45
41	85	15
55	85	15

Milk thistle standard solution: 0.7 mg/mL of USP Powdered Milk Thistle Extract RS in methanol. Sonicate for 20 min to dissolve.

Silybin standard solutions: 0.20, 0.02, and 0.004 mg/mL of USP Silybin RS in methanol. Pass through a membrane filter having a 0.45- μ m or finer pore size.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed quantity of the powder equivalent to 100 mg of silymarin to a 100-mL volumetric flask, add 90 mL of methanol, and sonicate for 20 min with occasional shaking. Cool to 20°, and dilute with methanol to volume. Filter through a membrane with a 0.45- μ m or finer pore size.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 288 nm

Column: 4.6-mm \times 15-cm; 5- μ m packing L1

Column temperature: 40°

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Sample: *Milk thistle standard solution*

[NOTE—For the relative retention times, see *Table 2*.]

Table 2

Name	Relative Retention Time
Silychristin	0.68
Silydianin	0.73
Silybin A	1.00
Silybin B	1.05
Dehydrosilybin	1.09
Isosilybin A	1.15
Isosilybin B	1.19

Suitability requirements

Chromatogram similarity: The chromatogram from the *Milk thistle standard solution* is similar to the reference chromatogram provided with the lot of USP Powdered Milk Thistle Extract RS being used.

Resolution: NLT 1.0 between silybin A and silybin B

Tailing factor: 0.8–2.0

Relative standard deviation: NMT 2.0% for the sum of peak responses due to silybin A and silybin B

Analysis

Samples: *Milk thistle standard solution*, each of the *Silybin standard solutions*, and *Sample solution*

Identify the peaks due to silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B by comparison of the chromatogram of the *Milk thistle standard solution* with the reference chromatogram, and measure the peak areas of the relevant peaks. Plot the areas of the sum of silybin A and silybin B peaks versus the concentration of USP Silybin RS in the *Silybin standard solutions*, and obtain a regression line for calibration.

Separately calculate the content, in mg, of each relevant component of silymarin as silybin ($C_{25}H_{22}O_{10}$) in the portion of Tablets taken:

$$\text{Result} = C \times V$$

C = concentration of the relevant component in the *Sample solution* as interpolated in the calibration graph (mg/mL)

V = volume of the *Sample solution* (mL)

Calculate the content of silymarin, as a percentage, in the portion of Tablets taken:

$$\text{Result} = C_s \times (A_{WT}/W) \times (100/L)$$

C_s = sum of the contents of each relevant component of silymarin in the portion of Tablets taken (mg)

A_{WT} = average weight of the Tablets (mg/Tablet)

W = weight of the portion of Tablets taken (mg)

L = labeled amount of silymarin (mg/Tablet)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** (2040): Meet the requirements for *Dissolution*

Buffer: 6.9 g/L of monobasic sodium phosphate and 1.52 g/L of sodium hydroxide in water. Adjust to a pH of 7.5.

Medium: Buffer containing 2% lauryl sulfate; 900 mL

Apparatus 2: 100 rpm

Time: 45 min

Determine the amount of silymarin as silybin ($C_{25}H_{22}O_{10}$) dissolved by using the method in the test for *Content of Silymarin*, making any necessary modifications.

Tolerances: NLT 75% of the labeled amount of silymarin as silybin ($C_{25}H_{22}O_{10}$) is dissolved.

- **WEIGHT VARIATION** (2091): Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** (2021): The total bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the enterobacterial count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. The label also indicates the content of silymarin, in mg/Tablet.

- **USP REFERENCE STANDARDS** (11)

USP Powdered Milk Thistle Extract RS

USP Silybin RS

USP Silydianin RS

Minerals Capsules

DEFINITION

Minerals Capsules contain two or more minerals derived from substances generally recognized as safe, furnishing two or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Capsules contain NLT 90.0% and NMT 125.0% of the labeled amounts of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V). They contain no vitamins. They may contain other labeled added substances in amounts that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used. Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a *Standard stock solution* is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the concentrations of the *Standard solutions* and the *Sample solutions* may be modified to fit the linear or working range of the instrument.]

- **CALCIUM, Method 1**

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution* and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Polysorbate 80 solution: Dilute Polysorbate 80 with alcohol (1 in 10)

Sample solution: Transfer 5 Capsules to a 100-mL volumetric flask. [NOTE—For hard gelatin Capsules, weigh NLT 20 Capsules. Open the Capsules, without loss of shell material, and transfer the contents to a suitable container. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and allow the Capsule shells to dry. Weigh the empty Capsule shells, calculate the net weight of the Capsule contents, and transfer a portion of the Capsule contents, equivalent to 5 Capsules, to a 100-mL volumetric flask.] Add 15 mL of water, 10 mL of 6 N hydrochloric acid, and 1 mL of *Polysorbate 80 solution* to the flask. Heat on a hot plate or steam bath, with intermittent swirling, until the Capsules are com-

pletely disintegrated or the contents are dissolved. Boil gently for an additional 15 min. Cool, dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution with 0.125 N hydrochloric acid to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: Calcium emission line at 422.7 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• CHROMIUM, Method 1

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h, in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 1 µg/mL of chromium and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Chromium emission line at 357.9 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• COPPER, Method 1

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% (v/v) solution of nitric acid, and dilute with a 1% (v/v) solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

Standard stock solution: 100 µg/mL of copper from *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 2 µg/mL of copper and to omit the use of the *Lanthanum chloride solution*

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Copper hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Copper emission line at 324.7 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)

C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• FLUORIDE, Method 1

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water contained in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 µg/mL of fluoride from a quantity of sodium fluoride, previously dried at 100° for 4 h and cooled in a desiccator, in water

Intermediate stock solution A: 100 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of 3 M sodium acetate solution, and 25.0 mL of Sodium citrate solution. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 µg/mL of fluoride.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the mixed Capsule contents, equivalent to 200 µg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of 3 M sodium acetate solution, and 25.0 mL of Sodium citrate solution. Dilute with water to volume.

Analysis

Samples: *Standard solutions* and *Sample solution*
To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see pH <791>), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ±0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel-reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer having an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in µg/mL, of the *Standard solutions* versus the potential, in mV. From the standard response curve so obtained and the measured potential of the *Sample solution*, determine the concentration, C, in µg/mL, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• FLUORIDE, Method 2

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution

[NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with one column volume of methanol followed by one column volume of pH 10.0 buffer. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.]

Transfer 10.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and

adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1. Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask. Add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with pH 10.0 buffer to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL container. If necessary, remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 1 mg of fluorine, to a 100-mL volumetric flask. Add 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1, and dilute with water to 100 mL. Proceed as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Conductivity

Guard column: 4.6-mm × 3-cm; packing L17

Analytical column: 7.8-mm × 30-cm; packing L17

Flow rate: 0.5 mL/min

Injection size: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas for fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• IODIDE, Method 1

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the contents, equivalent to 3 mg of iodine, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the

crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.] Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared. Calculate the percentage of the labeled amount of iodine (I) in the portion of Capsules taken:

$$\text{Result} = V \times N_A \times F \times \text{Ime} \times (\text{Aw}/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)
 N_A = actual normality of the sodium thiosulfate solution used (meq/mL)
 F = correction factor to convert mg to μg , 1000 $\mu\text{g}/\text{mg}$
 Ime = milliequivalent of I, 21.16 mg/meq
 Aw = average weight of the Capsules content
 W = weight of the sample of Capsules content taken
 L = labeled amount of iodine ($\mu\text{g}/\text{Capsule}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

- **IODIDE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), Assay for Iodide.

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

- **IRON, Method 1**

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 $\mu\text{g}/\text{mL}$ of iron.

Polysorbate 80 solution: Prepare as directed in the assay for *Calcium, Method 1*.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a nominal concentration of 5 $\mu\text{g}/\text{mL}$ of iron and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Iron emission line at 248.3 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g}/\text{mL}$, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in $\mu\text{g}/\text{mL}$, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* ($\mu\text{g}/\text{mL}$)

C_U = nominal concentration of iron in the *Sample solution* ($\mu\text{g}/\text{mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

- **MAGNESIUM, Method 1**

Lanthanum chloride solution: Prepare as directed in the assay for *Calcium, Method 1*.

Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 $\mu\text{g}/\text{mL}$ of magnesium.

Standard stock solution: 20 $\mu\text{g}/\text{mL}$ of magnesium from *Magnesium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution* and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 $\mu\text{g}/\text{mL}$ of magnesium.

Polysorbate 80 solution: Prepare as directed in the assay for *Calcium, Method 1*.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 0.4 $\mu\text{g}/\text{mL}$ of magnesium.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Magnesium emission line at 285.2 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g}/\text{mL}$, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in $\mu\text{g}/\text{mL}$, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* ($\mu\text{g}/\text{mL}$)

C_U = nominal concentration of magnesium in the *Sample solution* ($\mu\text{g}/\text{mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

- **MANGANESE, Method 1**

Manganese standard stock solution: Transfer 1.00 g of manganese, weighed, to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 $\mu\text{g}/\text{mL}$ of manganese.

Standard stock solution: 50 $\mu\text{g}/\text{mL}$ of manganese from *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 $\mu\text{g}/\text{mL}$ of manganese.

Polysorbate 80 solution: Prepare as directed in the assay for *Calcium, Method 1*.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 1 µg/mL of manganese and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Manganese hollow-cathode

Flame: Air-acetylene

Analytical wavelength: Manganese emission line at 279.5 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)

C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM, Method 1**

Diluent: 20 mg/mL of ammonium chloride in water

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of molybdenum.

Standard stock solution: 100 µg/mL of molybdenum from *Molybdenum standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min. Cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 µg/mL of molybdenum.

Polysorbate 80 solution: Prepare as directed in the assay for *Calcium, Method 1*.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to take a number of Capsules or a portion of Capsule contents, equivalent to 1000 µg of molybdenum, and to make appropriate dilutions to obtain a final concentration of 10 µg/mL of molybdenum, omitting the addition of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Molybdenum hollow-cathode

Flame: Nitrous oxide-acetylene

Analytical wavelength: Molybdenum emission line at 313.3 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the three plotted

points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* (µg/mL)

C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **MOLYBDENUM, Method 2**

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% Stannous chloride solution: Transfer 40 g of stannous chloride to a beaker, add 20 mL of 6.5 N hydrochloric acid, and heat the solution until the stannous chloride is dissolved. Cool, and dilute with water to 100 mL.

Diluted stannous chloride solution: 20% *Stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.

Standard solution: 20 µg/mL of molybdenum from ammonium molybdate, in water

Sample: Remove the contents of a counted number of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Use a quantity of Capsule contents, equivalent to a nominal amount of 40 µg of molybdenum

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 465 nm

Blank: Amyl alcohol

Analysis

Samples: *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to each beaker to complete 50 mL in each beaker. Gently boil the water solutions for a few min. Cool to room temperature. Add 2 drops of methyl orange TS and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of 20% *Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic

layers from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the solution from the *Sample*
 A_S = absorbance of the solution from the *Standard solution*
 V = volume of the *Standard solution* analyzed, 2.0 mL
 C_S = concentration of molybdenum in the *Standard solution* (μg/mL)
 M_U = nominal concentration of molybdenum in the *Sample* (μg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• PHOSPHORUS, Method 1

Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5:100), and mix.

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, then dilute with *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add one drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 μg/mL of phosphorus.

Standard solution: 20 μg/mL of phosphorus from *Phosphorus standard stock solution* diluted with water

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 650 nm

Analysis

Samples: *Standard solution* and *Sample solution*
 To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions, against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
 A_S = absorbance of the *Standard solution*
 C_S = concentration of phosphorus in the *Standard solution* (μg/mL)
 C_U = nominal concentration of phosphorus in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• POTASSIUM

Potassium standard solution: 100 μg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 μg/mL of potassium from *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 μg/mL of potassium.

Polysorbate 80 solution: Prepare as directed in the assay for *Calcium*, Method 1

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium*, Method 1, except to prepare the *Sample solution* to contain 1 μg/mL of potassium and to omit the use of the *Lanthanum chloride solution*

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Potassium emission line at 766.5 nm

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in μg/mL, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in μg/mL, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* (μg/mL)

C_U = nominal concentration of potassium in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• SELENIUM, Method 1

Diluent: Prepare as directed in the assay for *Molybdenum*, Method 1.

Selenium standard solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume, to obtain a concentration of 1000 μg/mL of selenium.

Standard stock solution: 100 μg/mL of selenium from *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to

room temperature, and dilute each with *Diluent* to volume to obtain solutions containing 5.0, 10.0, and 25.0 µg/mL of selenium.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule content, equivalent to 1000 µg of selenium, to 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the sample specimen. Sonicate for 10 min or until the sample specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Selenium emission line at 196.0 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* (µg/mL)

C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• SELENIUM, Method 2

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% Ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, add hydroxylamine hydrochloride, then dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 µg/mL of selenium. Dilute

a volume of the solution with 0.125 N hydrochloric acid, to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10.0 mL of *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule content, equivalent to a nominal amount of 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. [CAUTION—Exercise care at this stage because perchloric acid reaction becomes vigorous.] Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid, and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask. Add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV

Cell: 1 cm

Analytical wavelength: 380 nm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*

Treat the *Sample solution*, the *Standard solution*, and the *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with 50% *Ammonium hydroxide solution* to a pH of 1.1 ± 0.1. Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*. Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbances of the cyclohexane layer from the *Sample solution*

A_S = absorbances of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• ZINC, Method 1

Zinc standard solution: 1000 µg/mL of zinc from zinc oxide dissolved in 5 M hydrochloric acid (3.89 mg/mL),

and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from *Zinc standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Polysorbate 80 solution: Prepare as directed in the assay for *Calcium, Method 1*.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a nominal concentration of 2 µg/mL of zinc and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Zinc hollow-cathode

Flame: Air-acetylene

Analytical wavelength: Zinc emission line at 213.8 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Capsules taken:

$$\text{Result} = (C/C_u) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_u = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

- **BORON, NICKEL, TIN, and VANADIUM, Method 1; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS, and ZINC, Method 2; MOLYBDENUM and SELENIUM, Method 3**

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding one volume of *Stock aqua regia solution* to two volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% (v/v) nitric acid solution and 1000 mg/L of scandium in 5% (v/v) nitric acid solution with *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% (v/v) nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% (v/v) nitric acid solution to obtain a solution with final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/L of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% (v/v) hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% (v/v) hydrochloric acid solution to obtain a solution with final concentrations of about 200 mg/L of boron, and 100 mg/L of chromium, molybdenum, nickel, selenium, tin, and vanadium each.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent*, to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution: Weigh, then transfer 5 Capsules to a 250-mL volumetric flask, and heat gently on a hot plate until the contents begin to release. Cautiously add 25 mL of *Stock aqua regia solution* in 5-mL increments, and swirl. Heat, continue to swirl until the Capsules dissolve into the acid, immediately remove from the heat source, and add 150 mL of water. Cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a 5-µm pore size nylon syringe filter. If necessary, make any further adjustments using the *Diluent*.

Spectrometric conditions

(See *Plasma Spectrochemistry* (730).)

Mode: Inductively coupled plasma spectrometry using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

[NOTE—Analyze the *System suitability solution*, and obtain the response as directed for *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*.

Calculate the percentage of the labeled amount for each mineral taken:

$$\text{Result} = C \times (V/W) \times F \times (T_w/L) \times 100$$

C = measured concentration of the relevant element in the *Sample solution* (mg/L)

V = volume of the *Sample solution* (L)

W = sample weight (mg)

F = dilution factor of the *Sample solution*

T_w = average Capsule weight (mg)

L = labeled amount of the relevant element per Capsule (mg/Capsule)

Acceptance criteria: 90%–125% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); 90%–160% of the labeled amounts of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** <2040>: Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS** <2021>: The total aerobic microbial count does not exceed 3000 cfu/g, and the combined molds and yeasts count does not exceed 300 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS** <2022>: Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states that the product is Minerals Capsules. The label also states the salt form of the mineral used as the source of each element. Where more than one assay method is given for a particular mineral, the labeling states the assay method used only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** <11>
USP Sodium Fluoride RS

Minerals Tablets

DEFINITION

Minerals Tablets contain two or more minerals derived from substances generally recognized as safe, furnishing two or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Tablets contain NLT 90.0% and NMT 125.0% of the labeled amounts of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V). They contain no vitamins. They may contain other labeled added substances in amounts that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used. Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a standard stock solution is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the concentrations of the *Standard solutions* and the *Sample solution* may be modified to fit the linear or working range of the instrument.]

- **CALCIUM, Method 1**

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h. Dissolve in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively with 0.125 N hydrochloric acid, to obtain a nominal concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution*/100 mL of the final volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption spectrophotometry

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: Calcium emission line at 422.7 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution*/100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

- **CHROMIUM, Method 1**

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h, in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Proceed as directed for *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 1 µg/mL of chromium and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption spectrophotometry
Lamp: Chromium hollow-cathode
Flame: Air-acetylene
Analytical wavelength: Chromium emission line at 357.9 nm
Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of chromium in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, Method 1**

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% (v/v) solution of nitric acid, and dilute with a 1% (v/v) solution of nitric acid to 1000 mL. This solution contains 1000 $\mu\text{g/mL}$ of copper.

Standard stock solution: 100 $\mu\text{g/mL}$ of copper from the *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 $\mu\text{g/mL}$ of copper.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 2 $\mu\text{g/mL}$ of copper and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Copper hollow-cathode

Flame: Air-acetylene

Analytical wavelength: Copper emission line at 324.7 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of copper in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **FLUORIDE, Method 1**

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water contained in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 $\mu\text{g/mL}$ of fluoride from a quantity of sodium fluoride, previously dried at 100° for 4 h and cooled in a desiccator, in water

Intermediate stock solution A: 100 $\mu\text{g/mL}$ of fluoride from *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 $\mu\text{g/mL}$ of fluoride from *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of 3 M *sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 $\mu\text{g/mL}$ of fluoride.

Sample solution: Transfer a quantity of the finely powdered Tablets, equivalent to a nominal amount of 200 μg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of 3 M *sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*, and dilute with water to 100 mL.

Analysis

Samples: *Standard solutions* and *Sample solution*
 To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see pH (791)), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ± 0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer having an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in $\mu\text{g/mL}$, of the *Standard solutions* versus the potential, in mV. From the standard response curve so obtained and the measured potential of the *Sample solution*, determine the concentration, C , in $\mu\text{g/mL}$, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of fluorine in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **FLUORIDE, Method 2**

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution

[NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with one column volume of methanol followed by one column volume of *pH 10.0 buffer*. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.]

Transfer 10.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1 . Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask. Add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with *pH 10.0 buffer* to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Finely powder NLT 20 Tablets.

Transfer a portion of powdered Tablets, equivalent to a nominal amount of 1 mg of fluorine, to 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1 , and dilute with water to 100 mL. Proceed as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Conductivity

Guard column: 4.6-mm × 3-cm; packing L17

Analytical column: 7.8-mm × 30-cm; packing L17

Flow rate: 0.5 mL/min

Injection size: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE, Method 1**

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis

Sample: Tablets

Transfer a portion of finely powdered Tablets, equivalent to a nominal amount of 3 mg of iodide, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL

of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.] Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.

Calculate the percentage of the labeled amount of iodine (I) in the portion of Tablets taken:

$$\text{Result} = V \times N_A \times F \times \text{Ime} \times (Aw/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)

N_A = actual normality of the sodium thiosulfate solution used (meq/mL)

F = correction factor to convert mg to µg, 1000 µg/mg

Ime = milliequivalent of I, 21.16 mg/meq

Aw = average weight of the Tablets

W = weight of the portion of Tablets taken

L = labeled amount of iodine (µg/Tablet)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IODIDE, Method 2**

Analysis: Proceed as directed in *Automated Methods of Analysis* <16>, *Assay for Iodide*.

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON, Method 1**

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a nominal concentration of 5 µg/mL of iron and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption spectrophotometry

Lamp: Iron hollow-cathode

Flame: Air-acetylene

Analytical wavelength: Iron emission line at 248.3 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (μg/mL)

C_U = nominal concentration of iron in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM, Method 1**

Lanthanum chloride solution: Prepare as directed in the assay for *Calcium, Method 1*.

Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 μg/mL of magnesium.

Standard stock solution: 20 μg/mL of magnesium from *Magnesium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 μg/mL of magnesium.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a nominal concentration of 0.4 μg/mL of magnesium.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Magnesium emission line at 285.2 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in μg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in μg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (μg/mL)

C_U = nominal concentration of magnesium in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE, Method 1**

Manganese standard stock solution: Transfer 1.00 g of manganese, weighed, to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 μg/mL of manganese.

Standard stock solution: 50 μg/mL of manganese from *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain

solutions with concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 μg/mL of manganese.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 1 μg/mL of manganese and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Manganese emission line at 279.5 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in μg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in μg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (μg/mL)

C_U = nominal concentration of manganese in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM, Method 1**

Diluent: 20 mg/mL of ammonium chloride in water

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 μg/mL of molybdenum.

Standard stock solution: 100 μg/mL of molybdenum from *Molybdenum standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min. Cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 μg/mL of molybdenum.

Sample solution: Transfer a portion of the powder, equivalent to a nominal amount of 1000 μg of molybdenum, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min, or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid, heat until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Quantitatively transfer the contents of the flask to a 100-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Molybdenum hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: Molybdenum emission line at 313.3 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of molybdenum, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of molybdenum in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• MOLYBDENUM, Method 2

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% Stannous chloride solution: Transfer 40 g of stannous chloride to a beaker, add 20 mL of 6.5 N hydrochloric acid, and heat the solution until the stannous chloride is dissolved. Cool, and dilute with water to 100 mL.

Diluted stannous chloride solution: 20% *Stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.

Standard solution: 20 $\mu\text{g/mL}$ of molybdenum from ammonium molybdate, in water

Sample: A portion of finely powdered powder Tablets, equivalent to a nominal amount of 40 μg of molybdenum

Spectrometric conditions

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 465 nm

Blank: Amyl alcohol

Analysis

Samples: *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to complete 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of 20% *Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride*

solution to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layers from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the solution from the Tablets

A_S = absorbance of the solution from the *Standard solution*

V = volume of the *Standard solution* analyzed, 2.0 mL

C_S = concentration of molybdenum in the *Standard solution* ($\mu\text{g/mL}$)

M_U = nominal amount of molybdenum in the *Sample solution* (μg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• PHOSPHORUS, Method 1

Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5:100), and mix.

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, then dilute with *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add one drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 100 $\mu\text{g/mL}$ of phosphorus.

Standard solution: 20 $\mu\text{g/mL}$ of phosphorus from *Phosphorus standard stock solution* diluted with water

Sample solution: [NOTE—Finely powder and weigh a counted number of Tablets.] Transfer a portion of the powder, equivalent to a nominal amount of 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Spectrometric conditions

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 650 nm

Analysis

Samples: *Standard solution* and *Sample solution*

To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

- A_U = absorbance of the *Sample solution*
 A_S = absorbance of the *Standard solution*
 C_S = concentration of phosphorus in the *Standard solution* ($\mu\text{g/mL}$)
 C_U = nominal concentration of phosphorus in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• POTASSIUM

Potassium standard solution: 100 $\mu\text{g/mL}$ of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 $\mu\text{g/mL}$ of potassium from *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 $\mu\text{g/mL}$ of potassium.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a nominal concentration of 1 $\mu\text{g/mL}$ of potassium and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Potassium emission line at 766.5 nm

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of potassium in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• SELENIUM, Method 1

Diluent: Prepare as directed in the assay for *Molybdenum, Method 1*.

Selenium standard solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume, to obtain a concentration of 1000 $\mu\text{g/mL}$ of selenium.

Standard stock solution: 100 $\mu\text{g/mL}$ of selenium from *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to

volume to obtain solutions with concentrations of 5.0, 10.0, and 25.0 $\mu\text{g/mL}$ of selenium.

Sample solution: Transfer a portion of the powder, equivalent to a nominal amount of 1000 μg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the sample specimen. Sonicate for 10 min or until the sample specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Selenium emission line at 196.0 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of selenium in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• SELENIUM, Method 2

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% Ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, add hydroxylamine hydrochloride, and then dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 $\mu\text{g/mL}$ of selenium. Dilute a volume of the solution with 0.125 N hydrochloric

acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10.0 mL of *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Transfer a portion of finely powdered Tablets, equivalent to a nominal amount of 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. **[CAUTION—Exercise care at this stage because the perchloric acid reaction becomes vigorous.]** Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid, and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask, add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV

Cell: 1 cm

Analytical wavelength: 380 nm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*
Treat the *Sample solution*, the *Standard solution*, and the *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with 50% *Ammonium hydroxide solution* to a pH of 1.1 ± 0.1 . Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*. Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the cyclohexane layer from the *Sample solution*

A_S = absorbance of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• ZINC, Method 1

Zinc standard solution: 1000 µg/mL of zinc from zinc oxide dissolved in 5 M hydrochloric acid (3.89 mg/mL), and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from *Zinc standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a nominal concentration of 2 µg/mL of zinc and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Zinc emission line at 213.8 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• BORON, NICKEL, TIN, and VANADIUM, Method 1; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS, and ZINC, Method 2; MOLYBDENUM and SELENIUM, Method 3

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding one volume of *Stock aqua regia solution* to two volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% (v/v) nitric acid solution and 1000 mg/L of scandium in 5% (v/v) nitric acid solution with *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% (v/v) nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% (v/v) nitric acid solution to obtain a solution with final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/mL of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% (v/v) hydrochloric acid solution, pipet the

appropriate amount of element standard solution into a volumetric flask, and dilute with 20% (v/v) hydrochloric acid solution to obtain a solution with final concentrations of about 200 mg/L of boron, and 100 mg/L of chromium, molybdenum, nickel, selenium, tin, and vanadium each.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent* to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution 1 (for Tablets containing minerals found in *Standard stock solution 1* and *Standard stock solution 2*): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a 5- μ m pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Sample solution 2 (for Tablets containing minerals found only in *Standard stock solution 2*): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a 5- μ m pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Sample solution 3 (for Tablets containing minerals found only in *Standard stock solution 1*): Weigh and finely powder NLT 20 Tablets. Transfer a weighed portion, equal to the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently (about 1 h) until fumes cease. Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a 5- μ m pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Spectrometric conditions
(See *Spectrochemistry* <730>.)

Mode: Inductively coupled plasma spectrometry using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

[NOTE—Analyze the *System suitability solution*, and obtain the response as directed for Analysis.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an

inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*.

Calculate the percentage of the labeled amount for each mineral taken:

$$\text{Result} = C \times (V/W) \times F \times (T_w/L) \times 100$$

C = measured concentration of the relevant element in the *Sample solution* (mg/L)
V = volume of the *Sample solution* (L)
W = sample weight (mg)
F = dilution factor of the *Sample solution*
T_w = average Tablet weight (mg)
L = labeled amount of the relevant element/Tablet (mg)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); and 90.0%–160.0% of the labeled amounts of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS <2040>:** Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>:** Meet the requirements

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS <2021>:** The total aerobic microbial count does not exceed 3000 cfu/g, and the combined molds and yeasts count does not exceed 300 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS <2022>:** Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states that the product is Minerals Tablets. The label also states the salt form of the mineral used as the source of each element. Where more than one assay method is given for a particular mineral, the labeling states the assay method used only if *Method 1* is not used.
- **USP REFERENCE STANDARDS <11>**
USP Sodium Fluoride RS

MSM—see *Methylsulfonylmethane in Dietary Supplements section*

Nettle, Stinging—see *Stinging Nettle*

Niacin—see *Niacin General Monographs*

Niacin Tablets—see *Niacin Tablets General Monographs*

Niacinamide—see *Niacinamide General Monographs*

Niacinamide Tablets—see *Niacinamide Tablets General Monographs*

Oleovitamin A and D—see *Oleovitamin A and D General Monographs*

Oleovitamin A and D Capsules—see *Oleovitamin A and D Capsules General Monographs*

Omega-3 Acid Triglycerides

DEFINITION

Omega-3 Acid Triglycerides is a mixture of mono-, di-, and triesters of omega-3 acids with glycerol containing mainly triesters and obtained either by esterification of concentrated and purified omega-3 acids with glycerol or by transesterification of the omega-3 acid ethyl esters with glycerol. The omega-3 acids are from the body oil of fish of the families Engraulidae, Carangidae, Clupeidae, Osmeridae, Salmonidae, and Scombridae and are defined as the following: alpha-linolenic acid (C18:3 n-3), moroctic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), eicosapentaenoic acid (EPA) (C20:5 n-3), heneicosapentaenoic acid (C21:5 n-3), docosapentaenoic acid (C22:5 n-3), and docosahexaenoic acid (DHA) (C22:6 n-3). It contains NLT 58.0% of total omega-3 acids expressed as triglycerides and NLT the labeled amount of EPA and DHA, expressed as the free fatty acids. Suitable antioxidants in appropriate concentrations may be added.

IDENTIFICATION

- A.** The retention times of the peaks for eicosapentaenoic acid methyl ester and docosahexaenoic acid methyl ester of the *Sample solution* in the tests for *Content of EPA and DHA* and *Content of Total Omega-3 Acids* correspond to those for the respective compounds of the *Standard solutions*. If either EPA or DHA is not claimed on the labeling, the peak corresponding to that omega-3 acid does not exceed 15.0% of the total detected area of the *Sample solution* in the test for *Content of EPA and DHA* and *Content of Total Omega-3 Acids*.
- B.** The retention time of the peak corresponding to the triglycerides of the *Sample solution* corresponds to the triglycerides peak of the *System suitability solution* in the test for *Content of Oligomers and Partial Glycerides*.

COMPOSITION

• CONTENT OF EPA AND DHA

Analysis: Proceed as directed in *Fats and Fixed Oils* (401), *Omega-3 Fatty Acids Determination and Profile*.

Acceptance criteria: NLT the labeled amount, expressed as free acids

• CONTENT OF TOTAL OMEGA-3 ACIDS

Analysis: Proceed as directed in *Fats and Fixed Oils* (401), *Omega-3 Fatty Acids Determination and Profile*.

Acceptance criteria: NLT 58.0% of total omega-3 acids, expressed as triglycerides

• CONTENT OF OLIGOMERS AND PARTIAL GLYCERIDES

Mobile phase: Use tetrahydrofuran.

Sample solution: 1.00 mg/mL of Omega-3 Acid Triglycerides in *Mobile phase*

System suitability solution: 0.5 mg/mL of monodocosahexaenoin, 0.3 mg/mL of didocosahexaenoin, and 0.2 mg/mL of tridocosahexaenoin in *Mobile phase*. [NOTE—Suitable grades of monodocosahexaenoin, didocosahexaenoin, and tridocosahexaenoin may be obtained from Nu-Chek Prep.]

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Differential refractometer

Columns: Three 7.8-mm × 30-cm; connected in series; packing L21, 7 μm. Two columns are 50 nm in pore size, and the other is 10 nm, arranged so that the 50-nm pore size columns are closer to the injector.

Flow rate: 0.8 mL/min

Injection size: 40 μL

System suitability

Sample: *System suitability solution*

Suitability requirements

Elution order: Tridocosahexaenoin, didocosahexaenoin, and monodocosahexaenoin

Resolution: NLT 2.0 between monodocosahexaenoin and didocosahexaenoin and NLT 1.0 between didocosahexaenoin and tridocosahexaenoin

Analysis

Sample: *Sample solution*

Measure the areas of the major peaks.

Calculate the percentage of oligomers in the portion of Omega-3 Acid Triglycerides taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = sum of the areas of the peaks with a retention time less than that of the triglyceride peak

r_T = sum of the areas of all peaks in the chromatogram

Calculate the percentage of partial glycerides (mono- and diglycerides) in the portion of Omega-3 Acid Triglycerides taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = sum of the areas of the peaks corresponding to diglycerides and monoglycerides

r_T = sum of the areas of all peaks in the chromatogram

Acceptance criteria: NMT 3.0% of oligomers and NMT 50.0% of partial glycerides

CONTAMINANTS

• LIMIT OF ARSENIC

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: Transfer 1 g of ultrapure palladium metal into a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution B: Transfer 1 g of ultrapure magnesium nitrate into a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature,

transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (3:2:5). A volume of 5 μ L provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed in *Arsenic* <211>, to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 μ g/mL of arsenic.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 μ g/mL of arsenic.

Sample solution: For preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytetrafluoroethylene liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow container. Equip the microwave oven with an exhaust tube to ventilate fumes. **[CAUTION—Wear proper eye protection and protective clothing and gloves.]** Transfer approximately 500 mg of Omega-3 Acid Triglycerides, weighed to the nearest 0.1 mg, into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. **[NOTE—A cool water bath may be used to speed the cooling process.]** Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115°, using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min; char the sample at 1000°, using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold.

Separately inject equal volumes (20 μ L) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 μ L of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in μ g/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in μ g/mL, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of Omega-3 Acid Triglycerides taken:

$$\text{Result} = (C \times V)/W$$

C = concentration of arsenic, as obtained above, in the *Sample solution* (μ g/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Omega-3 Acid Triglycerides taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 μ g/g

• LIMIT OF LEAD

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.**]**

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (2:1:2). A volume of 5 μ L provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Lead Nitrate Stock Solution*, prepared as directed in *Heavy Metals* <231>, to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 μ g/mL of lead.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 μ g/mL of lead.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2100°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold.

Separately inject equal volumes (20 μ L) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 μ L of the *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in μ g/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in μ g/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of lead in the portion of Omega-3 Acid Triglycerides taken:

$$\text{Result} = (C \times V)/W$$

C = concentration of lead, as obtained above, in the *Sample solution* (μ g/mL)

V = final volume of the *Sample solution* (mL)

W = weight of Omega-3 Acid Triglycerides taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF CADMIUM**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid to volume (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution A: 0.1372 mg/mL of cadmium nitrate

Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before make up to final volume, dissolve in a portion of water and nitric acid.]

Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold.

Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of the *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of cadmium in the Omega-3 Acid Triglycerides taken:

$$\text{Result} = (C \times V)/W$$

- C* = concentration of cadmium, as obtained above, in the *Sample solution* (µg/mL)
V = final volume of the *Sample solution* (mL)
W = weight of Omega-3 Acid Triglycerides taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF MERCURY:** Proceed as directed in *Mercury* (261), *Method IIa*, except use a *Standard Mercury Solution* having the equivalent of 0.1 µg/mL of mercury.

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*, combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution*.

Acceptance criteria: NMT 0.1 µg/g

- **LIMIT OF DIOXINS, FURANS, AND POLYCHLORINATED BIPHENYLS (PCBs)**

Analysis: Determine the content of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) by method No. 1613 revision B of the Environmental Protection Agency. Determine the content of polychlorinated biphenyls (PCBs) by method No. 1668 revision A of the Environmental Protection Agency.

Acceptance criteria: The sum of PCDDs and PCDFs is NMT 2.0 pg/g of WHO toxic equivalents. The sum of PCDDs, PCDFs, and dioxin-like PCBs (polychlorinated biphenyls, non-ortho IUPAC congeners PCB-77, PCB-81, PCB-126, and PCB-169, and mono-ortho IUPAC congeners PCB-105, PCB-114, PCB-118, PCB-123, PCB-156, PCB-157, PCB-167, and PCB-189) is NMT 10.0 pg/g of WHO toxic equivalents.

SPECIFIC TESTS

- **FATS AND FIXED OILS, Acid Value (401):** NMT 3
- **FATS AND FIXED OILS, Anisidine Value (401):** NMT 30.0
- **FATS AND FIXED OILS, Peroxide Value (401):** NMT 10.0
- **FATS AND FIXED OILS, Unsaponifiable Matter (401):** NMT 2.0%
- **ABSORBANCE**
Sample solution: 0.24 mg/mL in isooctane
Acceptance criteria: The absorbance is NMT 0.73, determined at 233 nm.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at controlled room temperature. It may be bottled or otherwise packaged in containers from which air has been expelled by production of a vacuum or by an inert gas.
- **LABELING:** The label states the average content of DHA and EPA as free acids, in mg/g, and the total content of omega-3 acids as free acids, in mg/g. It also states the name and concentration of any added antioxidant.
- **USP REFERENCE STANDARDS (11)**
 USP Docosahexaenoic Acid Ethyl Ester RS
 all *cis*-4,7,10,13,16,19-Docosahexaenoic ethyl ester.
 $\text{C}_{24}\text{H}_{36}\text{O}_2$ 356.55
 USP Eicosapentaenoic Acid Ethyl Ester RS
 all *cis*-5,8,11,14,17-Eicosapentaenoic ethyl ester.
 $\text{C}_{22}\text{H}_{34}\text{O}_2$ 330.51
 USP Methyl Tricosanoate RS
 Tricosanoic acid methyl ester.
 $\text{C}_{24}\text{H}_{48}\text{O}_2$ 368.64

Panthenol—see *Panthenol General Monographs*

Phenylalanine—see *Phenylalanine General Monographs*

Phyllanthus amarus

DEFINITION

Phyllanthus amarus consists of the dried aerial parts of *Phyllanthus amarus* Schumach. (Fam. Euphorbiaceae). It contains NLT 0.25% of lignans, calculated as the sum of phyllanthin and hypophyllanthin on the dried basis.

IDENTIFICATION

- **A.** *Phyllanthus amarus* meets the requirements in *Specific Tests for Botanic Characteristics*.

- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)**

Standard solution A: 0.1 mg/mL of USP Phyllanthin RS in methanol

Standard solution B: 10 mg/mL of USP Powdered *Phyllanthus amarus* Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.

Sample solution: Sonicate about 0.5 g of *Phyllanthus amarus*, finely powdered, in 5 mL of methanol for 10 min, centrifuge, and use the supernatant.

Adsorbent: Chromatographic silica gel with an average particle size of 10–15 µm (TLC plates) or with an average particle size of 5 µm (HPTLC plates)

Application volume: 10 µL (TLC plates) or 4 µL (HPTLC plates)

Developing solvent system: Hexane and ethyl acetate (2:1)

Spray reagent: A solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with the *Spray reagent*, heat for 3 min at 120°, and examine under visible light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a blue band in the lower third of the plate due to phyllanthin, corresponding in color and R_f to that in the chromatogram of *Standard solution A*; a violet band due to hypophyllanthin at an R_f higher than that of phyllanthin; a blue band at an R_f higher than that of hypophyllanthin; and an additional violet band in the upper third of the plate. Bands detected in the chromatogram of the *Sample solution* correspond in position and color to bands in the chromatogram of *Standard solution B*. Other minor bands may be observed in the chromatograms of the *Sample solution* and *Standard solution B*.

- **C. HPLC:** The chromatogram of the *Sample solution* obtained in the test for *Content of Lignans* shows a peak at a retention time corresponding to that of phyllanthin in the chromatogram of *Standard solution A*. Identify other lignan peaks in the chromatogram of the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered *Phyllanthus amarus* Extract RS being used. The chromatogram of the *Sample solution* shows an additional peak corresponding to hypophyllanthin.

COMPOSITION

• **CONTENT OF LIGNANS**

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.

Mobile phase: Acetonitrile and *Solution A* (4:6)

Standard solution A: 0.1 mg/mL of USP Phyllanthin RS in methanol

Standard solution B: Sonicate a portion of USP Powdered *Phyllanthus amarus* Extract RS in methanol to obtain a solution having a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Transfer about 3.0 g of *Phyllanthus amarus*, finely powdered and accurately weighed, to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux in a water bath for about 20 min, allow to settle, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, concentrate under vacuum, and adjust the volume to 100 mL with methanol. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 230 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered *Phyllanthus amarus* Extract RS being used.

Resolution: NLT 1.5 between the phyllanthin and hypophyllanthin peaks, *Standard solution B*

Tailing factor: NMT 1.5 for the phyllanthin peak, *Standard solution A*

Relative standard deviation: NMT 2.0% determined from the phyllanthin peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 48 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered *Phyllanthus amarus* Extract RS being used, identify the retention times of the peaks corresponding to phyllanthin and hypophyllanthin.

Separately calculate the percentages of phyllanthin and hypophyllanthin in the portion of *Phyllanthus amarus* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak response of the analyte from the *Sample solution*

r_S = peak response of phyllanthin from *Standard solution A*

C_S = concentration of USP Phyllanthin RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of *Phyllanthus amarus* taken to prepare the *Sample solution* (mg)

F = conversion factors for the analytes: 1.00 for phyllanthin; 0.75 for hypophyllanthin

Acceptance criteria: Add the percentages of phyllanthin and hypophyllanthin: NLT 0.25% is found on the dried basis.

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN,** *General Method for Pesticide Residues Analysis* (561): Meets the requirements

- **HEAVY METALS, Method III (231):** NMT 20 ppm
- **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

BOTANIC CHARACTERISTICS

Macroscopic: Erect annual herb, 10–60-cm high; slender stem, leaves on main stem are reduced to scales; secondary branchlets, short, extend at right angles, each carrying 15–30 leaves; leaves have a green upper surface with raised midrib and a pale green lower surface with prominent midrib and secondary veins, simple, alternate, 3–11 mm long, 1.5–6 mm wide, short petiolate, elliptical-oblong to obovate, apex obtuse and often with small pointed tip, margin entire, base often slightly asymmetric; flowers minute, yellowish, greenish, or whitish, unisexual, axillary on secondary branchlets, 1–2 and sometimes 3 per axil, first 1–2 internodes of each branchlet bear 1–2 male flowers, the rest have male and female flowers; fruits are flattened spherical capsules, straw color, 3-loculed, about 2 mm in diameter; seeds usually 2 per locule, light brown, about 0.9-mm long, triangular with 6–7 longitudinal ribs and many transverse striations on the back. Pharmacopeial article is green to yellowish-green masses composed mostly of leaves, branchlets, and stem fragments; taste bitter.

Histology

Transverse section of stems: Epidermal layer; about 15 layers of cortex cells, thick wall, contain chloroplast, some contain calcium oxalate crystals, inner 7–10 layers are made of thick-wall cells interrupted at regular interval by parenchyma cells; a layer of parenchyma cells containing starch grains; phloem 7–10 layers of thin-wall cells; groups of xylem vessels; pith, multilayer of thin-wall cells, few contain calcium oxalate crystals.

Transverse section of branchlets: The transverse section is round; 6–8 layers of cortex, thick-wall cells, most contain chloroplast and a few calcium oxalate crystals, after 3–4 layers there is a layer of cells containing starch grains, followed by 2–3 layers of fiber cells interrupted by cortex parenchyma; phloem 5–7 layers of thin-wall cells; groups of xylem vessels; pith, multilayer of thin-wall cells, containing chloroplasts.

Transverse section of leaves: Upper and lower epidermis; a single layer of palisade cells, which occupy nearly half of the space between each epidermis; 3–5 layers of parenchyma cells, a few contain crystals of calcium oxalate; vascular bundles are also present.

- **LOSS ON DRYING (731):** Dry 1.0 g of finely powdered *Phyllanthus amarus* at 105° for 2 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 8.0%, determined on 1.0 g of finely powdered *Phyllanthus amarus*
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash (561):** NMT 5.0%, determined on 1.0 g of finely powdered *Phyllanthus amarus*
- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter (561):** NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.

- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS (11)**
USP Phyllanthin RS
USP Powdered *Phyllanthus amarus* Extract RS

Powdered *Phyllanthus amarus*

DEFINITION

Powdered *Phyllanthus amarus* is *Phyllanthus amarus* reduced to a powder or very fine powder. It contains NLT 0.25% of lignans, calculated as the sum of phyllanthin and hypophyllanthin, on the dried basis.

IDENTIFICATION

- **A. Powdered *Phyllanthus amarus*** meets the requirements in *Specific Tests for Botanic Characteristics*.
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)**
Standard solution A: 0.1 mg/mL of USP Phyllanthin RS in methanol
Standard solution B: 10 mg/mL of USP Powdered *Phyllanthus amarus* Extract RS in methanol. Sonicate for about 10 min, centrifuge, and use the supernatant.
Sample solution: Sonicate about 0.5 g of Powdered *Phyllanthus amarus* in 5 mL of methanol for 10 min, centrifuge, and use the supernatant.
Adsorbent: Chromatographic silica gel with an average particle size of 10–15 μm (TLC plates) or with an average particle size of 5 μm (HPTLC plates)
Application volume: 10 μL (TLC plates) or 4 μL (HPTLC plates)
Developing solvent system: Hexane and ethyl acetate (2:1)
Spray reagent: A solution of 10% sulfuric acid in methanol. [NOTE—Prepare fresh.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621)). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the plate. Remove the plate from the chamber, dry, spray with the *Spray reagent*, heat for 3 min at 120°, and examine under visible light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a blue band in the lower third of the plate due to phyllanthin, corresponding in color and R_f to that in the chromatogram of *Standard solution A*; a violet band due to hypophyllanthin at an R_f higher than that of phyllanthin; a blue band at an R_f higher than that of hypophyllanthin; and an additional violet band in the upper third of the plate. Bands detected in the chromatogram of the *Sample solution* correspond in position and color to bands in the chromatogram of *Standard solution B*. Other minor bands may be observed in the chromatograms of the *Sample solution* and *Standard solution B*.

- **C. HPLC:** The chromatogram of the *Sample solution* obtained in the test for *Content of Lignans* shows a peak at a retention time corresponding to that of phyllanthin in the chromatogram of *Standard solution A*. Identify other lignan peaks in the chromatogram of the *Sample solution* by comparison with the chromatogram of *Standard solution B* and the reference chromatogram provided with the lot of USP Powdered *Phyllanthus amarus* Extract RS being used. The chromatogram of the *Sample solution* shows an additional peak corresponding to hypophyllanthin.

COMPOSITION**• CONTENT OF LIGNANS**

Solution A: Dissolve 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, mix, filter, and degas.

Mobile phase: Acetonitrile and *Solution A* (4:6)

Standard solution A: 0.1 mg/mL of USP Phyllanthin RS in methanol

Standard solution B: Sonicate a portion of USP Powdered *Phyllanthus amarus* Extract RS in methanol to obtain a solution having a concentration of about 5.0 mg/mL. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Sample solution: Transfer about 3.0 g of Powdered *Phyllanthus amarus*, accurately weighed, to a 250-mL flask fitted with a reflux condenser. Add 50 mL of methanol, reflux in a water bath for about 20 min, leave to settle, and decant the supernatant. Repeat until the last extract is colorless. Combine the extracts, concentrate under vacuum, and adjust the volume with methanol to 100 mL. Before injection, pass through a membrane filter of 0.45-μm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 230 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 1.5 mL/min

Injection size: 10 μL

System suitability

Samples: *Standard solution A* and *Standard solution B*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution B* is similar to the reference chromatogram provided with the lot of USP Powdered *Phyllanthus amarus* Extract RS being used.

Resolution: NLT 1.5 between the phyllanthin and hypophyllanthin peaks, *Standard solution B*

Tailing factor: NMT 1.5 for the phyllanthin peak, *Standard solution A*

Relative standard deviation: NMT 2.0% determined from the phyllanthin peak in repeated injections, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*. [NOTE—*Standard solution A*, *Standard solution B*, and the *Sample solution* are stable for 48 h at room temperature.]

Using the chromatograms of *Standard solution A*, *Standard solution B*, and the reference chromatogram provided with the lot of USP Powdered *Phyllanthus amarus* Extract RS being used, identify the retention times of the peaks corresponding to phyllanthin and hypophyllanthin.

Separately calculate the percentages of phyllanthin and hypophyllanthin in the portion of Powdered *Phyllanthus amarus* taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak response of the analyte from the *Sample solution*

r_S = peak response of phyllanthin from *Standard solution A*

C_S = concentration of USP Phyllanthin RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered *Phyllanthus amarus* taken to prepare the *Sample solution* (mg)

F = conversion factors for the analytes: 1.00 for phyllanthin; 0.75 for hypophyllanthin

Acceptance criteria: Add the percentages of phyllanthin and hypophyllanthin: NLT 0.25% is found on the dried basis.

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **HEAVY METALS**, *Method III* (231): NMT 20 ppm
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS** Greenish to greenish-brown in color; taste bitter. Under a microscope, fragments of epidermal cells of the leaves with wavy walls, showing anisocytic and paracytic stomata; parenchyma cells, some showing clusters of calcium oxalate crystals; narrow fibers from the stem; pitted vessels of the stem; fragments of the epicarp of the fruits showing anomocytic stomata
- **LOSS ON DRYING** (731): Dry 1.0 g of Powdered *Phyllanthus amarus* at 105° for 2 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 8.0%, determined on 1.0 g of Powdered *Phyllanthus amarus*
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* (561): NMT 5.0%, determined on 1.0 g of Powdered *Phyllanthus amarus*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11)
USP Phyllanthin RS
USP Powdered *Phyllanthus amarus* Extract RS

Phytonadione—see *Phytonadione General Monographs*

Phytonadione Tablets—see *Phytonadione Tablets General Monographs*

Potassium Citrate—see *Potassium Citrate General Monographs*

Potassium Citrate Tablets**DEFINITION**

Potassium Citrate Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of potassium (K).

IDENTIFICATION

- **A.** The *Sample solution* for *Strength* produces line emissions or absorptions at the characteristic wavelengths for potassium.

• **B. IDENTIFICATION TESTS—GENERAL, Citrate (191)**

Analysis: Grind a Tablet to a fine powder in a mortar. Transfer the powder to a centrifuge tube, add 2–5 mL of water, sonicate for 1 min, shake, and centrifuge.

Acceptance criteria: The supernatant meets the requirements for the test.

STRENGTH

• **CONTENT OF POTASSIUM, PROCEDURE 1**

[NOTE—A standard stock solution is commercially available at different potassium concentrations, which may be used for preparation of the *Standard stock solution*. Necessary volumetric adjustment can be made in the *Standard solution*. Concentrations of the *Standard solution* and the *Sample solution* may be modified to fit the linear or working range of the instrument.]

Standard stock solution: Solution of potassium chloride, previously dried at 105° for 2 h, in water containing 1000 mg/L of potassium.

Standard solution: To a 50-mL volumetric flask add 20 mL of water and 1 mL of nitric acid, and mix thoroughly. Pipet 10.0 mL of the *Standard stock solution* into a volumetric flask, and dilute with water to volume to obtain a solution having a known concentration of about 200 µg/mL of potassium.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed portion of the powdered Tablets, equivalent to about 0.1 g of potassium, to a 50-mL flask. Add 10 mL of nitric acid, and heat the solution to a gentle boil, during which fuming evolves. Boil the solution for an additional 30 min with constant swirling, during which time no fuming should be observed. Cool the solution to room temperature. Quantitatively transfer all of the solution to a 500-mL volumetric flask, dilute with water to volume, mix, and filter.

Inductively coupled plasma system

(See *Plasma Spectrochemistry* (730).)

Mode: Atomic emission spectroscopy

Analytical wavelength: 766.49 nm. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. A typical setting includes radio frequency (RF) power of about 1300 watts, argon torch flow of about 15 L/min, argon auxiliary flow of about 0.2 L/min, and a nebulizer flow rate of about 0.8 L/min.]

Blank: 2% nitric acid solution

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank*. Calculate the percentage of the labeled amount of potassium (K) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = response from the *Sample solution*

r_S = response from the *Standard solution*

C_S = concentration of potassium in the *Standard solution* (µg/mL)

C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amount of potassium

• **CONTENT OF POTASSIUM, PROCEDURE 2**

Standard stock solution A: 100 µg/mL of potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution B: 10 µg/mL of potassium from *Standard stock solution A* in 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of *Standard stock solution B* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.

Sample solution: Finely powder NLT 20 Tablets.

Transfer an equivalent to 5 Tablets to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively with 0.125 N hydrochloric acid to obtain a nominal concentration of 2 µg/mL of potassium.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 766.5 nm

Lamp: Potassium hollow-cathode

Flame: Air-acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*. Determine the absorbances of the solutions, using the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of potassium in the *Sample solution* interpolated from the graph (µg/mL)

C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0% of the label claim

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined yeast and mold count does not exceed 10^2 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** Meet the requirement of the test for absence of *Escherichia coli*

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION (2040)**

Medium: Water; 900 mL

Apparatus 2: 75 rpm

Time: 30 min

Analysis: Proceed as directed in *Content of Potassium, Procedure 1* or *Procedure 2*, for *Strength*, making any necessary volumetric adjustments.

Sample solution: If *Content of Potassium, Procedure 1* is used, pipet 10.0 mL of the filtered pooled solution under test to a 50-mL volumetric flask, and dilute with 2% nitric acid solution to 50 mL. If *Content of Potassium, Procedure 2* is used, dilute the filtered pooled solution under test with 0.125 N hydrochloric acid to a concentration falling within the range of the *Standard solutions*.

Calculate the percentage of the labeled amount of potassium (K) dissolved:

$$\text{Result} = (C \times D \times V/L) \times 100$$

C = measured concentration of potassium in the *Sample solution* (mg/mL)

D = dilution factor for the *Sample solution*

V = volume of *Medium*, 900 mL

L = label claim (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of K is dissolved.

- **WEIGHT VARIATION** (2091): Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** The label states the quantity of potassium in terms of mg/Tablet.

Potassium Gluconate—see *Potassium Gluconate General Monographs*

Potassium Gluconate Oral Solution—see *Potassium Gluconate Oral Solution General Monographs*

Potassium Gluconate Tablets—see *Potassium Gluconate Tablets General Monographs*

Proline—see *Proline General Monographs*

Pygeum

DEFINITION

Pygeum consists of the bark of *Prunus africana* (Hook f.) Kalkman (*Pygeum africanum* Hook f.) (Fam. Rosaceae). It contains NLT 9.0% of extractable matter.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: 10 mg/mL of USP Pygeum Extract RS in chloroform

Standard solution B: 1 mg/mL of USP β -Sitosterol RS in chloroform

Sample solution: Transfer 10 g of the powdered plant material to a soxhlet apparatus. Extract with 150 mL of methylene chloride for 4 h. Evaporate the extract under vacuum to dryness. Dissolve the residue with 10 mL of methylene chloride.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 10 μ L

Developing solvent system: Methylene chloride in a saturated chamber

Spray reagent: Sulfuric acid and water (1:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms to a length of NLT 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and heat the plate at 100° for 10 min. Examine the plate under white light.

Acceptance criteria: The chromatogram obtained with the *Sample solution* shows one red-violet zone turning to grayish-brown near the origin that corresponds in color and R_f value to that in the chromatogram of *Standard solution A*; one red-violet zone turning to grayish-

brown at an R_f about 0.08, corresponding in color and R_f value to that in the chromatogram of *Standard solution B*; above these spots a grayish-brown zone may be present, corresponding in color and R_f value to that in the chromatogram of *Standard solution A*. Other colored zones of varying intensities may be observed in the chromatogram of the *Sample solution*.

COMPOSITION

• EXTRACTABLE MATTER

Analysis: Extract 2.00 g of the powdered material in a soxhlet apparatus with 150 mL of alcohol for 6 h. Evaporate the solution to dryness under vacuum, and dry the residue at 105° for 24 h.

Acceptance criteria: NLT 9.0% of extractable matter

CONTAMINANTS

- **HEAVY METALS** (231): NMT 20 μ g/g

- **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues** (561): Meets the requirements

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Bark pieces consist of long fragments of variable dimensions, from only a few cm to 1 m long with a thickness varying from a few mm to 1–2 cm. The color is brown, more or less dark on the external surface; light brown to red-brown on the internal surface. The external part of the bark presents a very dark and fissured rhytidome that in the samples of old trees is fragmented in more or less square plaques of about 1–5 cm. The thickness varies from 1 mm in young plants or branches to 5–8 mm in old plants. The outer surface may also be covered with whitish lichens or thin filamentous moss. The internal bark, under the rhytidome, is clearer and has a more reddish coloration, with a long fibrous break, from reddish to light brown and dark brown in color, often presenting concentric stratification cracks. The internal surface is clearer and presents small wrinkles.

Microscopic: The transverse section of the bark presents a suberized bed having a thickness depending on the age of the plant, consisting of multiple layers of small, square cells with the walls of moderate thickness. It presents a cortical parenchyma of more or less round cells, with a few apparent formations of very thin-walled sclereids, definitely sharp. Often, in the parenchyma, there are groups of cells containing oxalate druses; a few bigger cells with highly thickened walls can also be observed. It shows a liber with phloem zones and presenting medullary rays. The phloemal portions contain groups of fibrous cells with highly thickened walls as well as phloemal and parenchymal elements, sometimes containing druses of oxalate. The medullary rays are of conical shape, larger on the external surface and thinner on the internal side; they can also contain druses of oxalate.

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** (561): NMT 5.0%

- **LOSS ON DRYING** (731): Dry at 60° for 15 h: it loses NMT 10.0% of its weight.

- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 10.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.

- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.

- **USP REFERENCE STANDARDS** (11)

USP Pygeum Extract RS

USP β -Sitosterol RS

Pygeum Extract

DEFINITION

Pygeum Extract is prepared from pulverized Pygeum, using suitable solvents. It contains NLT 90% and NMT 110% of the labeled amount of docosyl ferulate and NLT 90% and NMT 110% of the labeled amount of total sterols as β -sitosterol, calculated on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution A: 15 mg/mL of USP Pygeum Extract RS in chloroform

Standard solution B: 2 mg/mL of USP β -Sitosterol RS in chloroform

Sample solution: Dissolve 150 mg of Extract in 10 mL of chloroform.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 10 μ L

Developing solvent system: Methylene chloride in a saturated chamber

Spray reagent: Sulfuric acid and water (1:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the chromatograms to a length of NLT 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and heat the plate at 100° for 10 min. Examine the plate under white light.

Acceptance criteria: The chromatogram from the *Sample solution* shows one red-violet zone turning to grayish-brown near the origin that corresponds in color and R_f value to that of *Standard solution A*, and one red-violet zone turning to grayish-brown at an R_f of 0.08 corresponding in color and R_f value to that in the chromatogram of *Standard solution B*; above these spots a grayish-brown zone may be present, corresponding in color and R_f value to that of *Standard solution A*; and other colored zones of varying intensities may be observed in the *Sample solution*.

• B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of Docosyl Ferulate*.

Acceptance criteria: The chromatogram of the *Sample solution* presents a peak for docosyl ferulate that corresponds in retention time to the principal peak in the chromatogram of the *Standard solution*.

COMPOSITION

• CONTENT OF STEROLS

Derivatizing solution: Bis(trimethylsilyl)acetamide and trimethylchlorosilane (9:1)

Internal standard solution: 2 mg/mL of 5 α -cholestane in chloroform

System suitability stock solution: 2 mg/mL each of campesterol, stigmasterol, and USP β -Sitosterol RS

System suitability solution: Mix 2.0 mL of the *System suitability stock solution* and 2.0 mL of the *Internal standard solution*, and dilute with chloroform to 10 mL. Evaporate 500 μ L of this solution to dryness using a stream of nitrogen. Dissolve the residue in 80 μ L of *Derivatizing solution* and 20 μ L of pyridine. Allow to stand for NLT 10 min at room temperature.

Standard stock solution: 2.0 mg/mL of USP β -Sitosterol RS in chloroform

Standard solution: Mix 2.0 mL of the *Standard stock solution* and 2.0 mL of the *Internal standard solution*, and dilute with chloroform to 10 mL. Evaporate 500 μ L of this solution to dryness using a stream of nitrogen. Dissolve the residue in 80 μ L of *Derivatizing solution* and

20 μ L of pyridine. Allow to stand for NLT 10 min at room temperature.

Sample solution: Transfer 100 mg of Extract into a 100-mL round-bottom flask. Add 2.0 mL of *Internal standard solution* and 20 mL of diluted hydrochloric acid. Attach a condenser, and reflux in a bath at 100° for 30 min. Cool the solution to room temperature, and adjust by the addition of about 5 mL of 10 N sodium hydroxide to a pH of 8. Extract twice using 50 mL of ether each time, wash the collected organic phases with 50 mL of water, and evaporate the organic phase to dryness under vacuum. Dissolve the residue with 4 mL of chloroform, and transfer to a cartridge containing 500 mg of packing L8¹ that has been conditioned with a 2-column volume of *n*-hexane. Collect the eluate. Elute twice with a 1-column volume of a mixture of chloroform and isopropanol (2:1). Combine the eluates, and evaporate to dryness. Dissolve the residue in 10 mL of chloroform. Evaporate 500 μ L of this solution to dryness under a stream of nitrogen. Dissolve the residue with 80 μ L of *Derivatizing solution* and 20 μ L of pyridine. Allow to stand for NLT 10 min at room temperature.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm \times 30-m capillary; G27 phase coating of 0.25- μ m thickness

Temperature:

Injector: 285°

Detector: 285°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
250	0	250	5
250	5	320	0

Carrier gas: Helium. [NOTE—The carrier gas flow rate should be adjusted to obtain a retention time of about 19 min for β -sitosterol.]

Makeup gas: Helium

Injection size: 2 μ L

Injection type: Split injection system

Split ratio: 1:50

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for 5 α -cholestane, campesterol, stigmasterol, and β -sitosterol are about 0.66, 0.94, 0.96, and 1.00, respectively.]

Suitability requirements

Resolution: NLT 2 between campesterol and stigmasterol

Column efficiency: NLT 150,000 theoretical plates for the 5 α -cholestane peak

Tailing factor: NMT 2.0 for each relevant peak

Analysis

Samples: *Standard solution* and *Sample solution*
Identify the signals corresponding to the relevant analytes by comparison with the chromatograms obtained with the *System suitability solution*.

Separately calculate the individual percentages of campesterol, stigmasterol, and β -sitosterol, as β -sitosterol, respectively, in the portion of Pygeum Extract taken:

$$C_i = (R_i/R_s) \times C_s \times (V/W) \times 100$$

¹ A suitable cartridge is Chromabond NH2, manufactured by Macheray Nagel, or equivalent.

R_U = ratio of the appropriate sterol peak to the internal standard from the *Sample solution*
 R_S = ratio of the β -sitosterol peak to the 5α -cholestane internal standard from the *Standard solution*
 C_S = concentration of β -sitosterol in the *Standard stock solution* (mg/mL)
 V = volume of the *Standard stock solution* taken to prepare the *Standard solution* (mL)
 W = weight of Pygeum Extract taken to prepare the *Sample solution* (mg)
 Calculate the percentage of the labeled amount of total sterols as β -sitosterol:

$$\text{Result} = (\Sigma C_i/L) \times 100$$

C_i = individual percentage of each sterol as calculated above
 L = labeled amount of total sterols in the Pygeum Extract taken

Acceptance criteria: 90%–110% of the labeled amount of total sterols as β -sitosterol on the dried basis

• CONTENT OF DOCOSYL FERULATE

Solution A: Methanol and water (95:5)

Solution B: Acetonitrile

Mobile phase: *Solution A* and *Solution B* (17:3)

Standard solution: Dissolve a quantity of USP Docosyl Ferulate RS in chloroform, and dilute with acetonitrile to obtain a concentration of 0.02 mg/mL. Pass through a filter of 0.45- μ m or finer pore size.

Sample solution: To 250 mg of Extract add 5 mL of chloroform, and dilute with acetonitrile to 25 mL. Pass through a filter of 0.45- μ m or finer pore size, discarding the first 4 mL of filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 323 nm

Column: 4-mm \times 25-cm; packing L7

Column temperature: 25°

Flow rate: 1 mL/min

Injection size: 20 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 1700 theoretical plates for the docosyl ferulate peak

Tailing factor: NMT 2.0 for docosyl ferulate

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of docosyl ferulate (P) in the portion of Extract taken:

$$P = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area for docosyl ferulate from the *Sample solution*

r_S = peak area for docosyl ferulate from the *Standard solution*

C_S = concentration of USP Docosyl Ferulate RS in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Extract taken to prepare the *Sample solution* (mg)

Calculate the percentage of the labeled amount of docosyl ferulate:

$$\text{Result} = (P/L) \times 100$$

P = percent of docosyl ferulate in the portion of Extract taken

L = labeled amount of docosyl ferulate in the Pygeum Extract (%)

Acceptance criteria: 90%–110% of the labeled amount of docosyl ferulate on the dried basis

CONTAMINANTS

- **HEAVY METALS, Method II** <231>: 20 μ g/g
- **ARTICLES OF BOTANICAL ORIGIN, Test for Aflatoxins** <561>: NMT 4 μ g/kg of total aflatoxins B1, B2, G1, and G2; NMT 2 μ g/kg of aflatoxin B1
- **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** <561>: Meets the requirement
- **BOTANICAL EXTRACTS, Preparations** <565>: Meets the requirements in *General Pharmacopeial Requirements, Residual Solvents*
- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **LOSS ON DRYING** <731>: Dry 1.0 g of Extract for 3 h at 110°: it loses NMT 10% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 0.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in tight containers, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. Label the content as a percentage of total sterols as β -sitosterol and the content as a percentage of docosyl ferulate. It also meets the requirements in *Botanical Extracts* <565>, *Labeling*.
- **USP REFERENCE STANDARDS** <11>
 - USP Docosyl Ferulate RS
 - USP Pygeum Extract RS
 - USP β -Sitosterol RS

Pygeum Capsules

DEFINITION

Pygeum Capsules contain Pygeum Extract. Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of Extract, calculated as sterols and docosyl ferulate.

IDENTIFICATION

- **A.** The retention times of the peaks for campesterol, stigmasterol, and β -sitosterol, of the *Sample solution*, correspond to those of the *Standard solution*, as obtained in the test for *Content of Sterols*.
- **B.** The retention time of the peak for docosyl ferulate in the *Sample solution* corresponds to that in the *Standard solution*, as obtained in the test for *Content of Docosyl Ferulate*.

STRENGTH

• CONTENT OF STEROLS

Derivatizing solution: Bis(trimethylsilyl)acetamide and trimethylchlorosilane (9:1)

Internal standard solution: 2 mg/mL of 5α -cholestane in chloroform

System suitability stock solution: 2 mg/mL each of campesterol, stigmasterol, and USP β -Sitosterol RS

System suitability solution: Mix 2.0 mL of the *System suitability stock solution* and 2.0 mL of the *Internal standard solution*, and dilute with chloroform to 10 mL. Evaporate 500 μ L of this solution to dryness using a stream of nitrogen. Dissolve the residue in 80 μ L of *Derivatizing solution* and 20 μ L of pyridine. Allow to stand for NLT 10 min at room temperature.

Standard stock solution: 2.0 mg/mL of USP β -Sitosterol RS in chloroform

Standard solution: Mix 2.0 mL of the *Standard stock solution* and 2.0 mL of the *Internal standard solution*, and dilute with chloroform to 10 mL. Evaporate 500 μ L of this solution to dryness using a stream of nitrogen. Dissolve the residue in 80 μ L of *Derivatizing solution* and 20 μ L of pyridine. Allow to stand for NLT 10 min at room temperature.

Sample solution: Transfer a quantity of Capsules, equivalent to 100 mg of the labeled amount of Extract, into a 100-mL round-bottomed flask. Add 2.0 mL of the *Internal standard solution* and 20 mL of diluted hydrochloric acid. Attach a condenser, and reflux in a bath at 100° for 30 min. Cool the solution to room temperature, and adjust by the addition of about 5 mL of 10 N sodium hydroxide to a pH of 8. Extract twice using 50 mL of ether each time, wash the collected organic phases with 50 mL of water, and evaporate the organic phase to dryness under vacuum. Dissolve the residue with 4 mL of chloroform, and transfer to a cartridge containing 500 mg of packing L8 that has been conditioned with a 2-column volume of *n*-hexane. [NOTE—A suitable cartridge is Chromabond NH2, manufactured by Macherey Nagel, or equivalent.] Collect the eluate. Elute twice with a 1-column volume of a mixture of chloroform and isopropanol (2:1). Combine the eluates, and evaporate to dryness. Dissolve the residue in 10 mL of chloroform. Evaporate 500 μ L of this solution to dryness under a stream of nitrogen. Dissolve the residue with 80 μ L of *Derivatizing solution* and 20 μ L of pyridine. Allow to stand for NLT 10 min at room temperature.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm \times 30-m capillary column coated with a G27 phase of 0.25- μ m thickness

Temperature

Detector: 285°

Injector: 285°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
250	0	250	5
250	5	320	as needed

Carrier gas: Helium. [NOTE—The carrier gas flow rate should be adjusted to obtain a retention time of 19 min for β -sitosterol.]

Makeup gas: Helium

Injection size: 2 μ L

Injection type: Split injection system

Split ratio: 1:50

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for 5 α -cholestane, campesterol, stigmasterol, and β -sitosterol are about 0.66, 0.94, 0.96, and 1.00, respectively.]

Suitability requirements

Resolution: NLT 2 between campesterol and stigmasterol

Column efficiency: NLT 150,000 theoretical plates for the 5 α -cholestane peak

Tailing factor: NMT 2.0 for each relevant peak

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the signals corresponding to the relevant analytes by comparison with the chromatograms obtained with the *System suitability solution*.

Calculate the percentages of the labeled amount of Extract as sterols in the portion of the Capsules taken:

$$\text{Result} = (\Sigma R_U/R_S) \times (C_S \times V/W) \times (A_W \times 100/L_E) \times 100/L$$

ΣR_U = ratio of the sum of peak responses for campesterol, stigmasterol, and β -sitosterol relative to the internal standard from the *Sample solution*

R_S = ratio of the β -sitosterol peak relative to the internal standard from the *Standard solution*

C_S = concentration of β -sitosterol in the *Standard stock solution* (mg/mL)

V = volume of the *Standard stock solution* used to prepare the *Standard solution* (2.0 mL)

W = weight of the sample of Capsules taken to prepare the *Sample solution* (mg)

A_W = average weight of the Capsules contents (mg)

L_E = content of sterols in 100 mg of the Extract used to prepare the Capsules (mg)

L = labeled amount of Extract per Capsule (mg/Capsule)

Acceptance criteria: 90%–110% of the labeled amount of Extract, calculated as sterols

• CONTENT OF DOCOSYL FERULATE

Solution A: Methanol and water (95:5)

Solution B: Acetonitrile

Mobile phase: *Solution A* and *Solution B* (17:3)

Standard solution: Dissolve a quantity of USP Docosyl Ferulate RS in chloroform, and dilute stepwise if necessary, with acetonitrile to obtain a concentration of 0.01 mg/mL. Pass through a membrane filter of 0.45- μ m or finer pore size.

Sample solution: Weigh the contents of NLT 20 Capsules and transfer a quantity of the material, equivalent to 0.2 mg of docosyl ferulate, to a 50-mL beaker. Add 5 mL of chloroform and dissolve in an ultrasonic bath. Transfer to a 20-mL volumetric flask with the aid of NMT 2 mL of chloroform. Dilute with acetonitrile to volume and mix. Pass through a membrane filter of 0.45- μ m or finer pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 323 nm

Column: 4-mm \times 25-cm; packing L7

Column temperature: 25°

Flow rate: 1 mL/min

Injection size: 320 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 1700 theoretical plates for the docosyl ferulate peak

Tailing factor: NMT 2.0 for docosyl ferulate

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the analyte peaks.

Calculate the percentage of the labeled amount of Extract as docosyl ferulate in the portion of the Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/W) \times (A_W \times 100/L_E) \times 100/L$$

r_U = peak response for docosyl ferulate from the *Sample solution*

r_S = peak response for docosyl ferulate from the *Standard solution*

C_S = concentration of USP Docosyl Ferulate RS in the *Standard solution* (mg/mL)

V = final volume of *Sample solution* (20.0 mL)

W = weight of the sample of Capsules taken to prepare the *Sample solution* (mg)

A_W = average weight of the Capsules contents (mg)

- L_E = content of docosyl ferulate in 100 mg of the Extract used to prepare the Capsules (mg)
 L = labeled amount of Extract per Capsule (mg/Capsule)

Acceptance criteria: 90%–110% of the labeled amount of Extract, calculated as docosyl ferulate and sterols (from *Content of Sterols*)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** <2040>: Meet the requirements for *Rupture Test for Soft Shell Capsules*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** <2021>: The total bacterial count does not exceed 1000 cfu/g. The total combined molds and yeasts count does not exceed 1000 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: The Capsules meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Capsules were prepared. The label also indicates the quantity of Extract, in mg/Capsule. Label the Capsules to indicate the quantity of sterols and docosyl ferulate in percentage of the Extract contained in the Capsules.
- **USP REFERENCE STANDARDS** <11>
 USP Docosyl Ferulate RS
 USP Pygeum Extract RS
 USP β -Sitosterol RS

Pyridoxine Hydrochloride—see *Pyridoxine Hydrochloride General Monographs*

Pyridoxine Hydrochloride Tablets—see *Pyridoxine Hydrochloride Tablets General Monographs*

Red Clover

DEFINITION

Red Clover consists of the dried inflorescence of *Trifolium pratense* L. (Fam. Fabaceae). It contains NLT 0.5% of isoflavones, calculated on the dried basis as the sum of daidzein, genistein, formononetin, and biochanin A.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution: Transfer 100 mg of USP Powdered Red Clover Extract RS to a screw-capped centrifuge tube. Add 1 mL of a mixture of alcohol and water (7:3), and heat in a steam bath for 10 min. Centrifuge, and use the clear supernatant.
Sample solution: Transfer 1 g of the powdered plant material to a screw-capped centrifuge tube. Add 10 mL of a mixture of methanol and water (3:2), heat in a steam bath for 10–15 min, cool, and filter.
Chromatographic system
 (See *Chromatography*, <621> *Thin-Layer Chromatography*.)

Adsorbent: 0.25 mm Layer of chromatographic silica gel mixture

Application volume: 20–30 μ L, in bands 2 cm long
Developing solvent system: Ethyl acetate, formic acid, glacial acetic acid, and water (100:11:11:27)

Spray reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*
 Develop the chromatograms to a length of NLT 18 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under UV light at 365 nm.

Acceptance criteria: The *Sample solution* chromatogram exhibits a blue zone, a yellowish-green zone, and a yellowish-orange zone at R_f values of about 0.70, 0.55, and 0.50, respectively, corresponding in color and R_f to zones in the chromatogram of the *Standard solution*. Other colored zones of varying intensities may be observed in the chromatogram from the *Sample solution*.

• B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed for *Content of Isoflavones*. Using the values obtained in the test for *Content of Isoflavones*, calculate the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones:

$$\text{Result} = (B + G)/(D + F)$$

B = percentage of biochanin A

G = percentage of genistein

D = percentage of daidzein

F = percentage of formononetin

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times that correspond to those in the chromatogram of *Standard solution A*, and the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.1 and 10.

COMPOSITION

• CONTENT OF ISOFLAVONES

Solution A: Acetonitrile and water (1:3) containing 0.05% trifluoroacetic acid

Solution B: Acetonitrile containing 0.05% trifluoroacetic acid

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
2.5	87	13
7.5	80	20
7.8	73	27
8.0	55	45
11.0	50	50
13.0	40	60
15.0	26	74
16.0	0	100
18.1	100	0
23.0	100	0

Solvent: Alcohol and water (1:1)

Standard stock solution A: Transfer a quantity of USP Powdered Red Clover Extract RS, equivalent to 30 mg of the labeled content of isoflavones, to a 250-mL volumetric flask. Add 15 mL of dehydrated alcohol,

sonicate until dissolved, and dilute with *Solvent* to volume.

Standard solution A: Evaporate 50 mL of *Standard stock solution A* to dryness under vacuum. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer the resulting solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Centrifuge, or filter through a membrane having a 0.45- μ m or finer pore size.

Standard solution B: 0.1 mg/mL of USP Formononetin RS in a mixture of *n*-propanol and water (1:1). Sonicate, and filter through a membrane having a 0.45- μ m or finer pore size.

Sample stock solution: Transfer about 2.5 g of ground plant material, accurately weighed, into a 120-mL flask with a stopper. Add exactly 100 mL of *Solvent*, close the flask, and shake on an orbital or wrist-action shaker for NLT 12 h.

Sample solution: Evaporate 50 mL of *Sample stock solution* to dryness under vacuum at 40°. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer this solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Filter through a membrane having a 0.45- μ m or finer pore size, discarding the first 4 mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm \times 25-cm; end-capped 5- μ m packing L1

Column temperature: 45°

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution A*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Powdered Red Clover Extract RS being used.

Tailing factor: NMT 2.0 for the formononetin peak
Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* chromatogram by comparison with the chromatogram obtained from *Standard solution A* and the reference chromatogram. Measure the areas of the analyte peaks. Separately calculate the percentages of daidzein, genistein, formononetin, and biochanin A in the portion of Red Clover taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 1/F \times D \times 100$$

r_U = peak response for each relevant isoflavone from the *Sample solution*

r_S = formononetin peak response from the *Standard solution B*

C_S = concentration of USP Formononetin RS in *Standard solution B* (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Red Clover used to prepare the *Sample solution* (mg)

F = conversion factor for each analyte: daidzein, 0.97; genistein, 1.13; formononetin, 1.00; biochanin A, 1.05

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 1

Acceptance criteria: Add the percentages of daidzein, genistein, formononetin, and biochanin A: NLT 0.5% of isoflavones on the dried basis

CONTAMINANTS

- **HEAVY METALS** <231>: NMT 10 μ g/g
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 10⁶ cfu/g, the total combined molds and yeast count does not exceed 10⁴ cfu/g, and the enterobacterial count is NMT 10³ cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Red Clover inflorescences are ovoid with a rounded summit, mostly 12–34 mm in length and width, usually on a very short stalk, shriveled, purplish, and more or less brown from drying, consisting of many papilionaceous flowers, crowded together and clothed at the base with broad, pointed, pale green ciliate stipules with darker veins. The flowers, which may or may not be accompanied by diminutive trifoliate leaves, are up to 15 mm in length and have the following: five green, hairy, subulate calyx teeth, one longer than the other four; petals united into a more or less campanulate tube, somewhat recurved, and colorless with pinkish purple veins; diadelphous stamens; slender style; a faintly aromatic, somewhat tea-like odor; and a sweet, then slightly bitter taste.

Microscopic: Epidermis of calyx composed of polygonal cells with faintly striated cuticle and occasional anomocytic stomata on the outer epidermis only; abundant, uniseriate, covering trichomes with two small, thin-walled basal cells and a thick-walled tapering end cell, up to 1 mm in length with a warty cuticle. Glandular trichomes are also present, particularly on the lower epidermis, each with a one- or two-celled stalk and a large, cylindrical head composed of several cells arranged in two rows. Epidermal cells of the corolla, papillose at the tip, are elongated with slightly wavy walls and a strongly striated cuticle; vascular strands of corolla and calyx are surrounded by a crystal sheath containing prismatic crystals of calcium oxalate. The following are also present: fibrous layer of anthers; subspherical pollen grains, 20–48 μ m in diameter with smooth exine, three distinct pores, and three furrows; upper epidermal cells of leaflets with sinuous and slightly beaded anticlinal walls; lower epidermis with sinuous to wavy walls; anomocytic stomata on both surfaces, but more frequent on the lower surface; abundant covering trichomes on both surfaces and on the margins; and fibrovascular strands surrounded by a crystal sheath containing prismatic crystals of calcium oxalate

- **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* <561>: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Water-Soluble Extractives, Method 2* <561>: NLT 15.0%
- **LOSS ON DRYING** <731>: Dry 1 g at 105° for 2 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* <561>: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a well-closed, light-resistant container, protected from moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.

- **USP REFERENCE STANDARDS** <11>
USP Formononetin RS
USP Powdered Red Clover Extract RS

Powdered Red Clover

DEFINITION

Powdered Red Clover is Red Clover reduced to a powder or very fine powder. It contains NLT 0.5% of isoflavones, calculated on the dried basis as the sum of daidzein, genistein, formononetin, and biochanin A.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: Transfer 100 mg of USP Powdered Red Clover Extract RS to a screw-capped centrifuge tube. Add 1 mL of a mixture of alcohol and water (7:3), and heat in a steam bath for 10 min. Centrifuge, and use the clear supernatant.

Sample solution: Transfer 1 g of Powdered Red Clover to a screw-capped centrifuge tube. Add 10 mL of a mixture of methanol and water (3:2), heat in a steam bath for 10–15 min, cool, and filter.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 20–30 µL, in bands 2 cm long

Developing solvent system: Ethyl acetate, formic acid, glacial acetic acid, and water (100:11:11:27)

Spray reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatograms to a length of NLT 18 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under UV light at 365 nm.

Acceptance criteria: The *Sample solution* chromatogram exhibits a blue zone, a yellowish-green zone, and a yellowish-orange zone at R_f values of about 0.70, 0.55, and 0.50, respectively, corresponding in color and R_f to zones in the chromatogram of the *Standard solution*. Other colored zones of varying intensities may be observed in the chromatogram from the *Sample solution*.

• B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed for the *Content of Isoflavones*.

Using the values obtained in the test for *Content of Isoflavones*, calculate the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones:

$$\text{Result} = (B + G)/(D + F)$$

B = percentage of biochanin A

G = percentage of genistein

D = percentage of daidzein

F = percentage of formononetin

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times that correspond to those in the chromatogram of *Standard solution A*, and the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.1 and 10.

COMPOSITION

• CONTENT OF ISOFLAVONES

Solution A: Acetonitrile and water (1:3) containing 0.05% trifluoroacetic acid

Solution B: Acetonitrile containing 0.05% trifluoroacetic acid

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
2.5	87	13
7.5	80	20
7.8	73	27
8.0	55	45
11.0	50	50
13.0	40	60
15.0	26	74
16.0	0	100
18.1	100	0
23.0	100	0

Solvent: Alcohol and water (1:1)

Standard stock solution A: Transfer a quantity of USP Powdered Red Clover Extract RS, equivalent to 30 mg of the labeled content of isoflavones, to a 250-mL volumetric flask. Add 15 mL of dehydrated alcohol, sonicate until dissolved, and dilute with *Solvent* to volume.

Standard solution A: Evaporate 50 mL of *Standard stock solution A* to dryness under vacuum. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer the resulting solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Centrifuge, or filter through a membrane having a 0.45-µm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Formononetin RS in a mixture of *n*-propanol and water (1:1). Sonicate, and filter through a membrane having a 0.45-µm or finer pore size.

Sample stock solution: Transfer about 2.5 g of Powdered Red Clover, accurately weighed, into a 120-mL flask with a stopper. Add exactly 100 mL of *Solvent*, close the flask, and shake on an orbital or wrist-action shaker for NLT 12 h.

Sample solution: Evaporate 50 mL of *Sample stock solution* to dryness under vacuum at 40°. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer this solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Filter through a membrane having a 0.45-µm or finer pore size, discarding the first 4 mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; end-capped 5-µm packing L1

Column temperature: 45°

Flow rate: 1.0 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution A*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Powdered Red Clover Extract RS being used.

Tailing factor: NMT 2.0 for the formononetin peak
Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* chromatogram by comparison with the chromatogram obtained from *Standard solution A* and the reference chromatogram. Measure the areas of the analyte peaks. Separately calculate the percentages of daidzein, genistein, formononetin, and biochanin A in the portion of Powdered Red Clover taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 1/F \times D \times 100$$

r_U = peak response for each relevant isoflavone from the *Sample solution*

r_S = formononetin peak response from *Standard solution B*

C_S = concentration of USP Formononetin RS in *Standard solution B* (mg/mL)

V = volume of *Sample stock solution* (mL)

W = weight of Powdered Red Clover used to prepare the *Sample solution* (mg)

F = conversion factor for each analyte: daidzein, 0.97; genistein, 1.13; formononetin, 1.00; biochanin A, 1.05

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 1

Acceptance criteria: Add the percentages of daidzein, genistein, formononetin, and biochanin A: NLT 0.5% of isoflavones on the dried basis

CONTAMINANTS

- **HEAVY METALS** <231>: NMT 10 µg/g
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 10^6 cfu/g, the total combined molds and yeast count does not exceed 10^4 cfu/g, and the enterobacterial count is NMT 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** Brownish-red to greenish-red; numerous long, sharp-pointed, unicellular, nonglandular hairs with thick, nonlignified walls, the latter somewhat roughened by slight centrifugal projections; smooth, nearly spheroidal pollen grains from 20–48 µm in diameter; fragments of epidermis with stomata, the latter from 13–23 µm in length; fragments containing sclerenchyma fibers with adherent crystal fibers containing monoclinic prisms of calcium oxalate that are up to 21 µm in length
- **ARTICLES OF BOTANICAL ORIGIN**, *Water-Soluble Extractives, Method 2* <561>: NLT 15.0%
- **LOSS ON DRYING** <731>: Dry 1 g at 105° for 2 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 10.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* <561>: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers, protected from moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was derived.

• USP REFERENCE STANDARDS <11>

USP Formononetin RS
 USP Powdered Red Clover Extract RS

Powdered Red Clover Extract

DEFINITION

Powdered Red Clover Extract is prepared from Red Clover by extraction with hydroalcoholic mixtures or other suitable solvents. The ratio of plant material to extract is 3:1–25:1. It contains NLT 90.0% and NMT 110.0% of the labeled amount of isoflavones, calculated on the dried basis as the sum of daidzein, genistein, formononetin, and biochanin A. It may contain suitable added substances.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: Transfer 100 mg of USP Powdered Red Clover Extract RS to a screw-capped centrifuge tube. Add 1 mL of a mixture of alcohol and water (7:3), and heat in a steam bath for 10 min. Centrifuge, and use the clear supernatant.

Sample solution: Shake a quantity of Powdered Extract, equivalent to 25 mg of the labeled amount of isoflavones, in 20 mL of methanol. Allow to stand for 15 min before use.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture.

Application volume: 20–30 µL, in bands 2 cm long

Developing solvent system: Ethyl acetate, formic acid, glacial acetic acid, and water (100:11:11:27)

Spray reagent A: 10 mg/mL of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50 mg/mL of polyethylene glycol 4000 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*
 Develop the chromatograms to a length of NLT 18 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent A* followed by *Spray reagent B*, and examine the plate under UV light at 365 nm.

Acceptance criteria: The *Sample solution* chromatogram exhibits a blue zone, a yellowish-green zone, and a yellowish-orange zone at R_f values of about 0.70, 0.55, and 0.50, respectively, corresponding in color and R_f to zones in the chromatogram of the *Standard solution*. Other colored zones of varying intensities may be observed in the chromatogram from the *Sample solution*.

• B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of Isoflavones*.

Using the values obtained in the test for *Content of Isoflavones*, calculate the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones:

$$\text{Result} = (B + G)/(D + F)$$

B = percentage of biochanin A

G = percentage of genistein

D = percentage of daidzein

F = percentage of formononetin

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times that correspond to those in the chromatogram of *Standard solution A*. The ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.1 and 10.0.

COMPOSITION**• CONTENT OF ISOFLAVONES**

Solution A: Acetonitrile and water (1:3) containing 0.05% trifluoroacetic acid

Solution B: Acetonitrile containing 0.05% trifluoroacetic acid

Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
2.5	87	13
7.5	80	20
7.8	73	27
8.0	55	45
11.0	50	50
13.0	40	60
15.0	26	74
16.0	0	100
18.1	100	0
23.0	100	0

Solvent: Alcohol and water (1:1)

Standard stock solution A: Transfer a quantity of USP Powdered Red Clover Extract RS, equivalent to 30 mg of the labeled content of isoflavones, to a 250-mL volumetric flask. Add 15 mL of dehydrated alcohol, sonicate until dissolved, and dilute with *Solvent* to volume.

Standard solution A: Evaporate 50 mL of *Standard stock solution A* to dryness under vacuum. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer the resulting solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Centrifuge, or filter through a membrane having a 0.45-μm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Formononetin RS in a mixture of *n*-propanol and water (1:1). Sonicate, and filter through a membrane having a 0.45-μm or finer pore size.

Sample stock solution: Transfer a quantity of Powdered Extract, equivalent to 30 mg of the labeled content of isoflavones, to a 250-mL volumetric flask. Add 15 mL of dehydrated alcohol, sonicate until dissolved, and dilute with *Solvent* to volume.

Sample solution: Evaporate 50.0 mL of *Sample stock solution* to dryness under vacuum. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer the resulting solution with the aid of 15 mL of alcohol to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Centrifuge, or pass through a filter of 0.45-μm or finer pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; end-capped 5-μm packing L1

Column temperature: 45°

Flow rate: 1 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution A*

Suitability requirements

Chromatogram similarity: The chromatogram obtained from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Powdered Red Clover Extract RS being used.

Tailing factor: NMT 2.0 for the formononetin peak

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* chromatogram by comparison with the chromatogram obtained from *Standard solution A* and the reference chromatogram. Measure the areas of the analyte peaks.

Separately calculate the percentage of each isoflavone in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 1/F \times 100$$

r_U = peak response for each relevant isoflavone from *Sample solution*

r_S = peak response for formononetin from *Standard solution B*

C_S = concentration of USP Formononetin RS in *Standard solution B* (mg/mL)

C_U = nominal concentration of isoflavones in the *Sample solution* (mg/mL)

F = conversion factor for each analyte: daidzein, 0.97; genistein, 1.13; formononetin, 1.00; biochanin A, 1.05

Acceptance criteria: 90.0%–110.0% of the labeled amount of isoflavones as the sum of daidzein, genistein, formononetin, and biochanin A, calculated on the dried basis

CONTAMINANTS

• HEAVY METALS, Method II <231>: NMT 10 μg/g

• MICROBIAL ENUMERATION TESTS <2021>: The total aerobic microbial count does not exceed 10⁴ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and the enterobacterial count is NMT 10³ cfu/g.

• ABSENCE OF SPECIFIED MICROORGANISMS <2022>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• LOSS ON DRYING <731>: Dry 1 g at 105° for 2 h: it loses NMT 5.0% of its weight.

• OTHER REQUIREMENTS: It meets the requirements for *Packaging and Storage*, *Residual Solvents*, and *Pesticide Residues* in *Botanical Extracts* <565>.

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in tight, light-resistant containers, in a cool place.

• LABELING: The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of isoflavones, the extracting solvent or solvent mixture used for preparation, and the ratio of the starting crude plant material to Powdered Extract. It meets the requirements for labeling in *Botanical Extracts* <565>.

• USP REFERENCE STANDARDS <11>

USP Formononetin RS

USP Powdered Red Clover Extract RS

Red Clover Tablets**DEFINITION**

Red Clover Tablets contain Powdered Red Clover Extract. Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of Powdered Extract calculated as isoflavones.

IDENTIFICATION**• A. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed for the *Content of Isoflavones*.

Using the values obtained in the test for *Content of Isoflavones*, calculate the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones:

$$\text{Result} = (B + G)/(D + F)$$

B = percentage of biochanin A

G = percentage of genistein

D = percentage of daidzein

F = percentage of formononetin

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times that correspond to those in the chromatogram of *Standard solution A*, and the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.1 and 10.

STRENGTH**• CONTENT OF ISOFLAVONES**

Solution A: Acetonitrile and water (1:3) containing 0.05% trifluoroacetic acid

Solution B: Acetonitrile containing 0.05% trifluoroacetic acid

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
2.5	87	13
7.5	80	20
7.8	73	27
8.0	55	45
11.0	50	50
13.0	40	60
15.0	26	74
16.0	0	100
18.1	100	0
23.0	100	0

Solvent: Alcohol and water (1:1)

Standard stock solution: Transfer a quantity of USP Powdered Red Clover Extract RS, equivalent to 30 mg of the labeled content of isoflavones, to a 250-mL volumetric flask. Add 15 mL of dehydrated alcohol, sonicate until dissolved, and dilute with *Solvent* to volume.

Standard solution A: Evaporate 50 mL of *Standard stock solution* to dryness under vacuum. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer the resulting solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Centrifuge, or filter through a membrane having a 0.45-μm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Formononetin RS in a mixture of *n*-propanol and water (1:1). Sonicate, and filter through a membrane having a 0.45-μm or finer pore size.

Sample stock solution: Weigh NLT 20 Tablets and pulverize. Transfer the equivalent of 40 mg of the labeled amount of isoflavones to a 250-mL volumetric flask. Add 15 mL of water, shake to disperse the

powder, add 15 mL of dehydrated alcohol and 200 mL of *Solvent*, and sonicate for 30 min. If dark particles are present in the bottom of the flask, sonicate again for an additional 10 min or until they disappear. Cool to room temperature, dilute with *Solvent* to volume, and filter.

Sample solution: Transfer 50.0 mL of the resulting solution to a round-bottom flask, and evaporate to dryness under vacuum. Add 15 mL of 2 N hydrochloric acid, and heat in a water bath for 30 min. Quantitatively transfer this solution, with the aid of 15 mL of alcohol, to a 50-mL volumetric flask, and dilute with *Solvent* to volume. Pass 5 mL of the solution through a filter having a 0.45-μm pore size, discarding the first 4 mL of the filtrate. Collect the remaining 1 mL of filtrate for testing.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; end-capped 5-μm packing L1

Column temperature: 45°

Flow rate: 1 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution A*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution A* is similar to the reference chromatogram provided with the lot of USP Powdered Red Clover Extract RS being used.

Tailing factor: NMT 2.0 for the formononetin peak

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Identify the peaks corresponding to daidzein, genistein, formononetin, and biochanin A in the *Sample solution* chromatogram by comparison with the chromatogram obtained from *Standard solution A* and the reference chromatogram. Measure the areas of the analyte peaks. Calculate the content of each isoflavone, in mg, in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times C_S \times V \times 1/F \times D$$

r_U = peak response for each relevant isoflavone from the *Sample solution*

r_S = peak response for formononetin from *Standard solution B*

C_S = concentration of USP Formononetin RS in *Standard solution B* (mg/mL)

V = volume of the *Sample stock solution* (mL)

F = conversion factor for each analyte: daidzein, 0.97; genistein, 1.13; formononetin, 1.00; biochanin A, 1.05

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*, 1

Calculate the percentage of the labeled amount of Powdered Red Clover Extract taken:

$$\text{Result} = C_I \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

C_I = sum of the content of isoflavones in the portion of Tablets taken (mg)

A_{WT} = average weight of Tablets (mg)

W = weight of the powdered Tablets taken (mg)

L_E = labeled percentage of isoflavones in the Extract used to prepare the Tablets

L = label claim of Extract (mg/Tablet)

Acceptance criteria: 90.0%–110.0% as isoflavones

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** (2040): Meet the requirements for disintegration in *Botanical Dosage Forms*
- **WEIGHT VARIATION** (2091): Meet the requirements

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the enterobacterial count is NLT 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which Tablets were prepared. The label also indicates the quantity, in mg, of Powdered Extract per Tablet. Label Tablets to indicate the content, in mg, of isoflavones per 100 mg of Powdered Extract.
- **USP REFERENCE STANDARDS** (11)
USP Formononetin RS
USP Powdered Red Clover Extract RS

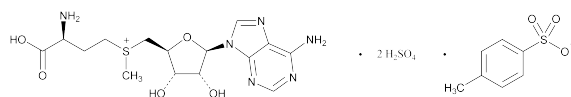
Riboflavin—see *Riboflavin General Monographs*

Riboflavin Tablets—see *Riboflavin Tablets General Monographs*

Riboflavin 5'-Phosphate Sodium—see *Riboflavin 5'-Phosphate Sodium General Monographs*

S-Adenosyl-L-methionine Disulfate Tosylate

Former Title: Ademetionine Disulfate Tosylate



$C_{22}H_{34}N_6O_{16}S_4$ 766.80
S-(Adenosyl)-L-methionine disulfate tosylate;
(3S)-5'-[(3-Amino-3-carboxypropyl)methylsulfonio]-5'-deoxy-adenosine, disulfate-methylbenzenesulfonate
[97540-22-2].

DEFINITION

S-Adenosyl-L-methionine Disulfate Tosylate is the disulfate-tosylate mixed salt of a mixture of diastereoisomers of the S-adenosyl-L-methionine ion. It contains NLT 95.0% and NMT 105.0% of S-adenosyl-L-methionine disulfate tosylate ($C_{22}H_{34}N_6O_{16}S_4$) calculated through the content of S-adenosyl-L-methionine ($C_{15}H_{23}N_6O_5S^+$), calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of S-adenosyl-L-methionine in the *System suitability solution*, as obtained in the *CONTENT OF S-ADENOSYL-L-METHIONINE*.

COMPOSITION

• CONTENT OF S-ADENOSYL-L-METHIONINE

Solution A: 10 mL of glacial acetic acid in 500 mL of water. Add 2.06 g of sodium 1-hexanesulfonate, and dilute with water to 1000 mL.

Mobile phase: Acetonitrile and *Solution A* (15:85)

System suitability solution: 400 µg/mL each of USP S-Adenosyl-L-methionine Disulfate Tosylate RS and USP S-Adenosyl-L-homocysteine RS

Standard solution A: 400 µg/mL of USP S-Adenosyl-L-homocysteine RS

Standard solution B: 200 µg/mL from *Standard solution A*

Standard solution C: 80 µg/mL from *Standard solution A*

Sample solution: 20 mg of S-Adenosyl-L-methionine Disulfate Tosylate in 40 mL of water. Stir for 30 min, then dilute with water to 50.0 mL. Transfer 1.0 mL of the solution to a 1.5-mL microcentrifuge tube, and centrifuge for 1 min. Use a portion of the supernatant.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 260 nm

Column: 4.6-mm × 15-cm; 3-µm packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution B*

[NOTE—The relative retention times for S-adenosyl-L-homocysteine and S-adenosyl-L-methionine disulfate tosylate are about 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between S-adenosyl-L-homocysteine and S-adenosyl-L-methionine

Tailing factor: NMT 1.5, *Standard solution B*

Relative standard deviation: NMT 2.0% for S-adenosyl-L-homocysteine, *Standard solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

[NOTE—Record the chromatograms, and measure the area of the S-adenosyl-L-homocysteine peak in all three *Standard solutions* and the S-adenosyl-L-methionine disulfate tosylate peak in the *Sample solution*.]

Plot a calibration curve of the peak area of the *Standard solutions* versus the corresponding S-adenosyl-L-homocysteine concentration, in mg/mL, and draw the straight line best fitting the three points. From the calibration curve, and using the peak area of S-adenosyl-L-methionine from the chromatogram from the *Sample solution*, determine the concentration, C, in mg/mL, of S-adenosyl-L-methionine as S-adenosyl-L-homocysteine in the *Sample solution*.

Calculate the percentage of $C_{15}H_{23}N_6O_5S^+$ in the portion of S-Adenosyl-L-methionine Disulfate Tosylate taken:

$$\text{Result} = (C/C_U) \times (M_{r1}/M_{r2}) \times 100$$

C = concentration of S-adenosyl-L-methionine as S-adenosyl-L-homocysteine obtained from the linear regression line (mg/mL)

C_U = concentration of S-Adenosyl-L-Methionine Disulfate Tosylate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of S-adenosyl-L-methionine, 399.44

M_{r2} = molecular weight of S-adenosyl-L-homocysteine, 384.41

Acceptance criteria: 49.5%–54.7% on the anhydrous basis, equivalent to 95.0%–105% of S-adenosyl-L-methionine disulfate tosylate on the anhydrous basis

• **CONTENT OF SULFATE**

Mobile phase: 8.0 mM sodium carbonate and 1.0 mM sodium bicarbonate in water

Standard solution: 0.18 mg/mL of potassium sulfate

Sample solution: 0.5 mg/mL of S-Adenosyl-L-methionine Disulfate Tosylate

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Ion detector with suppressed conductivity

Column: 4.0-mm × 25-cm; 7-μm packing L74

Column temperature: 30°

Flow rate: 1 mL/min

Injection size: 25 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 8200 theoretical plates

Tailing factor: NMT 1.5

Relative standard deviation: NMT 1.0%

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—Measure the area of the sulfate peak.]

Calculate the percentage of sulfate (SO₄) in the portion of S-Adenosyl-L-methionine Disulfate Tosylate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of sulfate from the *Sample solution*

r_S = peak response of sulfate from the *Standard solution*

C_S = concentration of sulfate (SO₄) in the *Standard solution* (mg/mL)

C_U = concentration of S-Adenosyl-L-methionine Disulfate Tosylate in the *Sample solution* (mg/mL)

Acceptance criteria: 23.5%–26.5%

IMPURITIES

• **HEAVY METALS,** *Method I* <231>: NMT 20 ppm

SPECIFIC TESTS

• **pH** <791>: 1.0–2.0, in an aqueous solution (1 in 20)

• **WATER DETERMINATION,** *Method Ia* <921>: NMT 3.0%

• **ISOMERIC RATIO**

Buffer: Transfer 4.2 g of citric acid monohydrate and 2.03 g of sodium dihydrogen phosphate dihydrate to a 1-L volumetric flask, and dissolve in and dilute with water to volume.

Mobile phase: 4.0 g of sodium dodecyl sulfate and 440 mL of acetonitrile. Dilute with *Buffer* to 1 L.

Standard solution: 1.0 mg/mL of USP S-Adenosyl-L-methionine Disulfate Tosylate RS

Sample solution: 1.0 mg/mL of S-Adenosyl-L-methionine Disulfate Tosylate

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 1.2 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for R,S-isomers and S,S-isomers are about 0.94 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.0 between the S,S-isomer and the R,S-isomer

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the peaks of the S,S- and R,S-isomers of the *Sample solution* by comparison with the *Standard solution*, and calculate the percentage of the S,S-isomer:

$$\text{Result} = [r_{SS}/(r_{SS} + r_{RS})] \times 100$$

r_{SS} = areas of the peaks corresponding to the S,S-isomer in the *Sample solution*

r_{RS} = areas of the peaks corresponding to the R,S-isomer in the *Sample solution*

Acceptance criteria: NLT 60% and NLT the labeled amount of the S,S-isomer

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store in a refrigerator.
- **LABELING:** Label it to indicate the minimum content of S,S-isomer, as a percentage.
- **USP REFERENCE STANDARDS** <11>
 - USP S-Adenosyl-L-methionine Disulfate Tosylate RS
 - USP S-Adenosyl-L-homocysteine RS

St. John's Wort

DEFINITION

St. John's Wort consists of the dried flowering tops or aerial parts of *Hypericum perforatum* L. (Fam. Hypericaceae), gathered shortly before or during flowering. It contains NLT 0.04% of the combined total of hypericin (C₃₀H₁₆O₈) and pseudohypericin (C₃₀H₁₆O₉) and NLT 0.6% of hyperforin (C₃₅H₅₂O₄), on the dried basis.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 0.5 mg/mL of USP Hyperoside RS in methanol

Sample solution: Finely powder 50 g of St. John's Wort. Shake 10 g in 100 mL of methanol for about 15 min, and filter.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 20 μL

Developing solvent system: Upper layer of a mixture of ethyl acetate, glacial acetic acid, formic acid, and water (10: 1.1: 1.1: 2.6)

Spray reagent A: 10-mg/mL solution of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50-mg/mL solution of polyethylene glycol 400 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands and allow to dry. Develop and allow the plate to air-dry. Spray with *Spray reagent A* and allow the plate to air-dry. Immediately after, spray the plate with *Spray reagent B*, and allow the plate to air-dry. Examine the plate under UV light at 365 nm.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits several zones having a yellowish-orange fluorescence, one of which, appearing at an R_f value of about 0.5, corresponds in R_f value and intensity to a similar zone in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* exhibits also two zones of red fluorescence, one at an R_f value of about 0.85 (presence of hypericin) and the other at an R_f value of about 0.80 (presence of pseudohypericin), and two zones of higher blue fluorescence (pres-

ence of chlorogenic and neochlorogenic acids) located below the yellow to yellowish-orange hyperoside zone.

COMPOSITION

- **CONTENT OF HYPERICIN AND PSEUDOHYPERICIN** [NOTE—Conduct all sample preparations with minimal exposure to subdued light, and use low-actinic glassware to protect solutions from light.]

Solvent: Methanol and acetone (1:1)

Solution A: Phosphoric acid and water (3:997)

Solution B: Acetonitrile

Solution C: Methanol

Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	100	0	0
10	85	15	0
30	70	20	10
40	10	75	15
55	5	80	15
56	100	0	0
66	100	0	0

Standard solution A: 2.5 µg/mL of USP Oxybenzone RS in *Solvent*

Standard solution B: 1 mg/mL of USP Powdered St. John's Wort Extract RS in *Solvent*

Sample solution: Weigh and pulverize 10 g of St. John's Wort. Transfer 1 g to a round-bottom flask equipped with a condenser and protected from light, add 50 mL of *Solvent* and a magnetic stirring bar, and heat at 60° for 2 h while stirring. Cool to room temperature, and pass through filter paper into a 50-mL volumetric flask. Wash the flask and the residue on the filter with *Solvent*, and dilute with the washings to volume. Pass the solution through a polytetrafluoroethylene membrane filter having a 0.45-µm or finer pore size, and use the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm and Vis 588 nm

Columns

Guard: Packing L1

Analytical: 4.6-mm × 25-cm; packing L1

Column temperature: 30°

Flow rate: 1 mL/min

Injection size: 20 µL. [NOTE—First equilibrate the system with 100% *Solution A*.]

System suitability

Samples: *Standard solution A* (record the peak responses at 270 nm), and *Standard solution B* (record the peak responses at 270 nm and 588 nm)

Suitability requirements

Chromatogram similarity: The chromatograms from *Standard solution B* are similar to the respective reference chromatograms provided with the lot of USP Powdered St. John's Wort Extract RS being used.

Column efficiency: NLT 100,000 theoretical plates for oxybenzone, *Standard solution A*

Tailing factor: NMT 1.5 for oxybenzone, *Standard solution A*

Relative standard deviation: NMT 2.0%, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*
Measure the areas of the relevant peaks at 270 nm in the chromatogram of the *Sample solution*.

Separately calculate the percentage of hypericin (C₃₀H₁₆O₈) and pseudohypericin (C₃₀H₁₆O₉) in the portion of St. John's Wort taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of oxybenzone from *Standard solution A*

C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of St. John's Wort taken to prepare the *Sample solution* (mg)

F = relative response factor for the relevant analyte relative to that of oxybenzone, 1.30 for hypericin and 1.24 for pseudohypericin

Calculate the combined total of hypericin and pseudohypericin by adding the corresponding percentages as calculated above.

Acceptance criteria: NLT 0.04% on the dried basis

• CONTENT OF HYPERFORIN

Analysis: Using the chromatograms obtained in the test for *Content of Hypericin and Pseudohypericin*, calculate the percentage of hyperforin (C₃₅H₅₂O₄) in the portion of St. John's Wort taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F \times 100$$

r_U = peak area of hyperforin from the *Sample solution*

r_S = peak area of oxybenzone from *Standard solution A*

C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of St. John's Wort taken to prepare the *Sample solution* (mg)

F = response factor for hyperforin relative to that of oxybenzone, 0.46

Acceptance criteria: NLT 0.6% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total bacterial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts count does not exceed 10² cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli* and for absence of *Staphylococcus aureus*.

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: The two-edged stem is greenish yellow, rounded, and has two ribs running longitudinally on opposite sides. The plant is adversifoliate, its leaves are sessile, ovoid or elongated, up to 3.5 cm in length, smooth-edged, and hairless with translucent perforations. The very numerous yellow, short-stemmed, pentamerous flowers form false umbels shaped like grape clusters. The five lanceolate and black-dotted sepals are about one-half the length of the dark yellow petals, which are shaped like slanted ovals and whose edges are set with dark red glands. The numerous stamens are joined in three to six bundles (usually three). The ovary is surmounted by three styles. Some ovaries are already developed into greenish, elongated, oval triovular capsules with various degrees of maturity. When chopped, the crude plant material is distinguished by numerous yellow to yellowish-brown flower buds and individual petals with dark red glands at the edges. The light green to brown-green leaf

fragments, characterized by plicate marcescence, appear stippled when held up to the light. The greenish yellow or reddish-brown hollow stem fragments are distinguished by two longitudinal edges.

Microscopic: The stems have elongated epidermal cells with straight beaded, anticlinal walls; cuticle smooth; frequent paracytic stomata with two small adjacent epidermal cells; cortex of 5–6 rows of collenchyma; stele with secondary growth consisting of a compacted ring of phloem, with a wide area of lignified xylem and small areas of intraxylary phloem; parenchymatous pith, lignified and pitted in older stems; oil glands may occur in the cortex and phloem.

The upper surface of the leaf has polygonal cells with sinuous, slightly beaded anticlinal walls; cells of lower surface smaller, with anticlinal walls more wavy with frequent paracytic, sometimes anomocytic, stomata; smooth cuticle, thicker on upper surface, straight-walled, elongated epidermal cells of veins occasionally beaded. Dorsiventral, single palisade lamina; large oil glands equal to depth of spongy mesophyll. Midrib containing single, collateral bundle with small area of lignified xylem. Trichomes and calcium oxalate are absent.

The sepal of the flower has characteristics resembling those of the leaf. Petal, narrow, elongated, thin-walled, epidermal cells with straight anticlinal walls on the outer surface and wavy on the inner surface. Stamen, lignified fibrous layer of anther wall; elongated, thin-walled cells of filament with striated cuticle; subprolate pollen grains, about 20 µm in diameter with three pores and a smooth exine. Ovary, small polygonal cells with underlying oil glands; seed testa, brown, thick-walled hexagonal cells.

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** <561>: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 5.0%
- **ARTICLES OF BOTANICAL ORIGIN, Water Content** <561>: NMT 10.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in tight containers, protected from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS** <11>
 - USP Hyperoside RS
 - USP Oxybenzone RS
 - USP Powdered St. John's Wort Extract RS
 - USP Rutin RS

Powdered St. John's Wort

DEFINITION

Powdered St. John's Wort is St. John's Wort reduced to a fine or a very fine powder. It contains NLT 0.6% of hyperforin (C₃₅H₅₂O₄) and NLT 0.04% of hypericin (C₃₀H₁₆O₈) and pseudohypericin (C₃₀H₁₆O₉) combined, on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
 - Standard solution:** 0.5 mg/mL of USP Hyperoside RS in methanol
 - Sample solution:** Shake 10 g of Powdered St. John's Wort in 100 mL of methanol for 15 min, and filter.
 - Chromatographic system**
(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 20 µL

Developing solvent system: Upper layer of a mixture of ethyl acetate, glacial acetic acid, formic acid, and water (10: 1.1: 1.1: 2.6)

Spray reagent A: 10-mg/mL solution of 2-aminoethyl diphenylborinate in methanol

Spray reagent B: 50-mg/mL solution of polyethylene glycol 400 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Samples* as bands and allow to dry. Develop and allow the plate to air-dry. Spray with *Spray reagent A* and allow the plate to air-dry. Immediately after, spray the plate with *Spray reagent B*, and allow the plate to air-dry. Examine the plate under UV light at 365 nm.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits several zones having a yellowish-orange fluorescence, one of which, appearing at an *R_f* value of about 0.5, corresponds in *R_f* value and intensity to a similar zone in the chromatogram of the *Standard solution*. The chromatogram of the *Sample solution* exhibits also two zones of red fluorescence, one at an *R_f* value of about 0.85 (presence of hypericin) and the other at an *R_f* value of about 0.80 (presence of pseudohypericin), and two zones of higher blue fluorescence (presence of chlorogenic and neochlorogenic acids) located below the yellow to yellowish-orange hyperoside zone.

COMPOSITION

- **CONTENT OF HYPERICIN AND PSEUDOHYPERICIN** [NOTE—Conduct all sample preparations with minimal exposure to subdued light, and use low-actinic glassware to protect solutions from light.]

Solvent: Methanol and acetone (1:1)

Solution A: Phosphoric acid and water (3:997)

Solution B: Acetonitrile

Solution C: Methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	100	0	0
10	85	15	0
30	70	20	10
40	10	75	15
55	5	80	15
56	100	0	0
66	100	0	0

Standard solution A: 2.5 µg/mL of USP Oxybenzone RS in *Solvent*

Standard solution B: 1 mg/mL of USP Powdered St. John's Wort Extract RS in *Solvent*

Sample solution: Weigh 10 g of Powdered St. John's Wort. Transfer 1 g to a round-bottom flask equipped with a condenser and protected from light, add 50 mL of *Solvent* and a magnetic stirring bar, and heat at 60° for 2 h while stirring. Cool to room temperature, and pass through filter paper into a 50-mL volumetric flask. Wash the flask and the residue on the filter with *Solvent*, and dilute with the washings to volume. Pass the solution through a polytetrafluoroethylene membrane filter having a 0.45-µm or finer pore size, and use the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm and Vis 588 nm

Columns

Guard: Packing L1

Analytical: 4.6-mm × 25-cm; packing L1

Column temperature: 30°

Flow rate: 1 mL/min

Injection size: 20 µL. [NOTE—First equilibrate the system with 100% Solution A.]

System suitability

Samples: *Standard solution A* (record the peak responses at 270 nm) and *Standard solution B* (record the peak responses at 270 nm and 588 nm)

Suitability requirements

Chromatogram similarity: The chromatograms from *Standard solution B* are similar to the respective reference chromatograms provided with the lot of USP Powdered St. John's Wort Extract RS being used.

Column efficiency: NLT 100,000 theoretical plates for oxybenzone

Tailing factor: NMT 1.5 for oxybenzone

Relative standard deviation: NMT 2.0%, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*
Measure the areas of the relevant peaks at 270 nm in the chromatogram of the *Sample solution*.

Separately calculate the percentage of hypericin (C₃₀H₁₆O₈) and pseudohypericin (C₃₀H₁₆O₉) in the portion of Powdered St. John's Wort taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 1/F \times 100$$

r_U = peak area of the relevant analyte from the *Sample solution*

r_S = peak area of oxybenzone from *Standard solution A*

C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered St. John's Wort taken to prepare the *Sample solution* (mg)

F = relative response factor for the relevant analyte relative to that of oxybenzone, 1.30 for hypericin and 1.24 for pseudohypericin

Acceptance criteria: NLT 0.04% on the dried basis

• CONTENT OF HYPERFORIN

Analysis: Using the chromatograms obtained in the test for *Content of Hypericin and Pseudohypericin*, calculate the percentage of hyperforin (C₃₅H₅₂O₄) in the portion of Powdered St. John's Wort taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 1/F \times 100$$

r_U = peak area of hyperforin from the *Sample solution*

r_S = peak area of oxybenzone from *Standard solution A*

C_S = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Powdered St. John's Wort taken to prepare the *Sample solution* (mg)

F = response factor for hyperforin relative to that of oxybenzone, 0.46

Acceptance criteria: NLT 0.6% on the dried basis

CONTAMINANTS

• **HEAVY METALS** (231): NMT 20 µg/g

• **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis** (561): Meets the requirements

• **MICROBIAL ENUMERATION TESTS** (2021): The total bacterial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts count does not exceed 10² cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli* and for absence of *Staphylococcus aureus*.

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Buff to greenish-brown powder with an aromatic and balsamic odor

Microscopic: Elongated and polygonal epidermal cells with thickened and beaded anticlinal walls, some accompanied by paracytic (occasionally anomocytic) stomata; fragments of leaf and sepal with schizogenous oil and pigment glands; narrow, thin-walled, elongated epidermal cells with straight and wavy anticlinal walls from petal; narrow, lignified vessels with annular or reticulate thickening; tracheids and tracheidal vessels with lignified, pitted thickening; thick-walled lignified fibers with tapering apices and occasional oblique pits; rectangular, lignified, pitted parenchyma; fibrous layer of antheral wall; pollen grains, 20–25 µm in diameter, with smooth exine.

• **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** (561): NMT 2%

• **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 5.0%

• **ARTICLES OF BOTANICAL ORIGIN, Water Content** (561): NMT 10.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Store in tight containers, protected from light and moisture.

• **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant source from which the article was derived.

• **USP REFERENCE STANDARDS** (11)

USP Hyperoside RS

USP Oxybenzone RS

USP Powdered St. John's Wort Extract RS

USP Rutin RS

Powdered St. John's Wort Extract

DEFINITION

Powdered St. John's Wort Extract is prepared from comminuted St. John's Wort extracted with 80% methanol or other suitable solvents. It contains NLT 90.0% and NMT 110.0% of the labeled combined total of hypericin (C₃₀H₁₆O₈) and pseudohypericin (C₃₀H₁₆O₉) and NLT 90.0% and NMT 110.0% of hyperforin (C₃₅H₅₂O₄), on the dried basis.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST:** (presence of hypericin, pseudohypericin, hyperoside, and rutin)

Standard solution: 50 mg/mL of USP Powdered St. John's Wort Extract RS in methanol. Shake well, and use the clear supernatant.

Sample solution: 50 mg/mL of Powdered Extract in methanol. Shake well, and use the clear supernatant.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 10 µL

Developing solvent system: Upper layer of a mixture of ethyl acetate, glacial acetic acid, formic acid, and water (10: 1.1: 1.1: 2.6)

Spray reagent A: 10 mg/mL of diphenylborinic acid, ethanolamine ester in methanol

Spray reagent B: 50 mg/mL of polyethylene glycol 400 in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*
Develop the chromatogram until the solvent front has moved NLT 18 cm, and dry the plate with the aid of a current of air. Spray the plate with *Spray reagent A*, then with *Spray reagent B*, and examine the plate under UV light at 365 nm.

Acceptance criteria: The two red zones due to hypericin and pseudohypericin at R_f values of about 0.85 and 0.80, respectively, in the chromatogram of the *Sample solution*, correspond in color and R_f value to those in the chromatogram of the *Standard solution*; the two yellow zones due to hyperoside and rutin at R_f values of about 0.50 and 0.35, respectively, in the chromatogram of the *Sample solution*, correspond in color and R_f value to those in the chromatogram of the *Standard solution*. Other colored zones of various intensities may be observed in the chromatogram of the *Sample solution*.

• B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST:

(presence of hyperforin)

Standard solution and Sample solution: Proceed as directed in *Identification test A*.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 10 μ L

Developing solvent system: Solvent hexane and ethyl acetate (4:1)

Spray reagent: Prepare a solution containing 0.38 g of ceric ammonium sulfate and 3.8 g of ammonium molybdate in 100 mL of 2 N sulfuric acid.

Analysis

Samples: *Standard solution* and *Sample solution*
Develop the chromatograms in a saturated chamber until the solvent front has moved NLT 18 cm, and dry the plate with the aid of a current of air. Spray the plate with *Spray reagent*, heat the plate at 140° for 15 min, and examine under UV light.

Acceptance criteria: The blue zone due to hyperforin at an R_f value of about 0.54 in the chromatogram of the *Sample solution* corresponds in color and R_f value to that in the chromatogram of the *Standard solution*.

COMPOSITION

• CONTENT OF HYPERICIN AND PSEUDOHYPERICIN

[NOTE—Conduct all sample preparations with minimal exposure to subdued light, and use low-actinic glassware to protect solutions from light.]

Solvent: Methanol and acetone (1:1)

Solution A: Phosphoric acid and water (3:997)

Solution B: Acetonitrile

Solution C: Methanol

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)
0	100	0	0
10	85	15	0
30	70	20	10
40	10	75	15
55	5	80	15
56	100	0	0
66	100	0	0

Standard solution A: 2.5 μ g/mL of USP Oxybenzone RS in *Solvent*

Standard solution B: 1 mg/mL of USP Powdered St. John's Wort Extract RS in *Solvent*

Sample solution: 1 mg/mL of Powdered Extract in a mixture of methanol and water (9:1). Sonicate to dissolve, pass through a polytetrafluoroethylene membrane filter having a 0.45- μ m or finer pore size, and use the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm and Vis 588 nm

Columns

Guard: Packing L1

Analytical: 4.6-mm \times 25-cm; packing L1

Column temperature: 30°

Flow rate: 1 mL/min

Injection size: 20 μ L. [NOTE—First equilibrate the system with 100% *Solution A*.]

System suitability

Samples: *Standard solution A* (record the peak responses at 270 nm) and *Standard solution B* (record the peak responses at 270 and 588 nm)

Suitability requirements

Chromatogram similarity: The chromatograms from *Standard solution B* are similar to the respective reference chromatograms provided with the lot of USP Powdered St. John's Wort Extract RS being used.

Column efficiency: NLT 100,000 theoretical plates for oxybenzone, *Standard solution A*

Tailing factor: NMT 1.5 for oxybenzone, *Standard solution A*

Relative standard deviation: NMT 2.0%, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*
Measure the areas at 270 nm of the relevant peaks in the chromatogram of the *Sample solution*.

Calculate the percentage of the labeled amount of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$) in the portion of Powdered St. John's Wort Extract taken:

$$\text{Result} = (\sum r_{ui}/f_i) \times (1/r_s) \times (C_s/C_u) \times 100$$

$\sum r_{ui}/f_i$ = sum of the peak areas of hypericin and pseudohypericin from the *Sample solution* divided by their respective response factors relative to oxybenzone, 1.30 for hypericin and 1.24 for pseudohypericin

r_s = peak area of oxybenzone from *Standard solution A*

C_s = concentration of USP Oxybenzone RS in *Standard solution A* (mg/mL)

C_u = nominal concentration of the total hypericins content in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0% on the dried basis

• CONTENT OF HYPERFORIN

Analysis: Using the chromatograms obtained in the test for *Content of Hypericin and Pseudohypericin*, calculate the percentage of hyperforin ($C_{35}H_{52}O_4$) in the portion of Powdered St. John's Wort Extract taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 1/F \times 100$$

r_u = peak area of hyperforin from the *Sample solution*

r_s = peak area of oxybenzone from *Standard solution A*

C_s = concentration of USP Oxybenzone RS in the *Standard solution A* (mg/mL)

C_u = nominal concentration of the hyperforin in the *Sample solution* (mg/mL)

F = relative response factor for hyperforin relative to that of oxybenzone, 0.46

Acceptance criteria: 90.0%–110.0% on the dried basis

CONTAMINANTS

- **HEAVY METALS, Method II (231):** NMT 50 µg/g
- **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis (561):** Meets the requirements
- **MICROBIAL ENUMERATION TESTS (2021):** The total bacterial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts does not exceed 10³ cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **WATER DETERMINATION, Method I (921):** NMT 5%
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 7.0%
- **OTHER REQUIREMENTS:** It meets the requirements in *Botanical Extracts (565)*, *Residue on Evaporation* and *Residual Solvents*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from moisture and light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of hypericin, pseudohypericin, and hyperforin; the extracting solvent or solvent mixture used for preparation; and the ratio of the starting crude plant material to Powdered Extract. The label bears a statement indicating that "Rare cases of allergic reactions and photosensitivity have been reported with the use of St. John's Wort. St. John's Wort interacts with numerous medications. Check with your health care provider before using."
- **USP REFERENCE STANDARDS (11)**
 - USP Oxybenzone RS
 - USP Powdered St. John's Wort Extract RS
 - USP Rutin RS

SAMe—see *S-adenosyl-L-methionine Disulfate Tosylate*

Saw Palmetto

DEFINITION

Saw Palmetto consists of partially dried, ripe fruit of *Serenoa repens* (W. Bartram) Small (Fam. Arecaceae) [*Serenoa serotatum* Schult.; *Sabal serrulata* (Michx.) Nutt. ex Schult. & Schult. f.]. It contains NLT 2% (v/w) of volatile oil, NLT 7% of lipophilic extract, and NLT 9.0% of total fatty acids.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)**

[NOTE—Perform the following test under subdued light.]

Reagent solution: 3.7 mg/mL of 4-bromomethyl-7-methoxycoumarin in acetone. Store this solution in a dark place.

Standard solution: 5.0 g of magnesium stearate in a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of ether, 20 mL of 12.5% nitric acid, and 20 mL of water, and heat until solution is complete. Cool, transfer the contents of the flask to a separatory funnel, and withdraw the lower aqueous phase. Extract the ether phase twice, each time using 4 mL of water, separating the aqueous phases. Extract the combined aqueous phases with 15 mL of ether, combining

the ether extracts. Evaporate to dryness, and dry the residue at 105°. Transfer 1 mg of the residue to an amber glass vial fitted with a metal-clamped rubber cap. Add 10 mg of lithium carbonate, 3 µL of tris-[2-(2-methoxyethoxy)ethyl]amine, and 1.0 mg of the *Reagent solution*. Replace the metal-clamped rubber cap, dry at 105° for 2 h, and cool.

Sample solution: 10.0 g of finely powdered Saw Palmetto in a 250-mL round-bottom flask fitted with a reflux condenser. Add 150 mL of alcohol, and heat under a reflux condenser on a steam bath for 1 h. Cool, filter, wash the residue with alcohol, and dilute the combined washings and filtrate with alcohol to 200.0 mL. Transfer 0.6 mL of this solution to a suitable flask, and evaporate to dryness. To the residue add 1.0 mL of the *Reagent solution*. Transfer this solution with the aid of a pipet to an amber glass vial fitted with a metal-clamped rubber cap. Add 3 µL of tris-[2-(2-methoxyethoxy)ethyl]amine and 10 mg of lithium carbonate to the vial. Replace the metal-clamped rubber cap, dry at 105° for 2 h, and cool.

Blank solution: To 10 mg of lithium carbonate in an amber glass vial fitted with a metal-clamped rubber cap, add 3 µL of tris-[2-(2-methoxyethoxy)ethyl]amine and 1.0 mL of the *Reagent solution*. Replace the metal-clamped rubber cap, dry at 105° for 2 h, and cool.

Chromatographic system

(See *Chromatography (621)*, *Thin-Layer Chromatography*.)

Absorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm in length (TLC plates)

Application volume: 2 µL

Developing solvent system: Cyclohexane, ethyl acetate, and acetic acid (70:30:1)

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank solution*

Develop the chromatograms in the solvent system until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the chromatographic chamber, mark the solvent front, and allow the plate to air-dry. Examine the plate under long-wave-length UV light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits at least two zones of blue fluorescence corresponding in *R_F* values to similar zones exhibited in the *Standard solution*. The blue fluorescent zones from the *Sample solution* appear above the blue fluorescent zones exhibited in the *Blank solution*.

COMPOSITION

- **ARTICLES OF BOTANICAL ORIGIN, Volatile Oil Determination (561):** NLT 2% (v/w) of an oil that solidifies to a white solid at room temperature

- **CONTENT OF LIPOPHILIC EXTRACT**

Analysis: Transfer 10 g of pulverized Saw Palmetto to a 250-mL round-bottom flask fitted with a reflux condenser, and add 150 mL of alcohol. Heat the flask on a boiling water bath under reflux for 1 h. Cool, filter, and wash the residue with small portions of alcohol. Collect the filtrate and washings in a 200-mL volumetric flask, and dilute with alcohol to volume. Evaporate 100.0 mL of this solution to dryness in a rotary evaporator under vacuum. Add 40 mL of *n*-hexane to the residue, stir for 5 min, filter, and collect the filtrate in a round-bottom flask. Repeat the above operation of washing with *n*-hexane two more times, and collect all of the filtrates in the same flask. Using a rotary evaporator, evaporate off the organic solvent to dryness. Dry the residue obtained at 105° for 2 h.

Acceptance criteria: The weight of the residue is NLT 0.35 g (NLT 7%).

- **CONTENT OF FATTY ACIDS**

Internal standard solution: 12 mg/mL of nonadecane in hexanes

Standard stock solution: Dissolve quantities of USP Methyl Laurate RS, USP Methyl Oleate RS, USP Methyl Myristate RS, USP Methyl Palmitate RS, USP Methyl Linoleate RS, USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, USP Methyl Palmitoleate RS, USP Methyl Stearate RS, and USP Methyl Linolenate RS in hexanes to obtain concentrations of each methyl ester as given in the *Table 1*.

Table 1

Methyl Ester	Concentration (mg/mL)
Methyl laurate	5
Methyl oleate	5
Methyl myristate	2
Methyl palmitate	2
Methyl linoleate	1
Methyl caproate	0.4
Methyl caprylate	0.4
Methyl caprate	0.4
Methyl palmitoleate	0.4
Methyl stearate	0.4
Methyl linolenate	0.4

Standard solution: Transfer 1.0 mL of *Internal standard solution* to 5.0 mL of the *Standard stock solution*.

Sample solution: Pulverize 50 g of dried Saw Palmetto to a moderately coarse powder. Transfer a quantity of 1 g of the pulverized powder to a 100-mL round-bottom flask fitted with a water-cooled reflux condenser and a magnetic bar. Add 10 mL of 0.5 N methanolic sodium hydroxide solution (20 mg/mL of sodium hydroxide in methanol), and heat the flask with stirring under reflux conditions for 15 min. Pipet 5 mL of a solution of boron trifluoride in methanol (140 mg/mL of boron trifluoride in methanol) through the reflux condenser into the flask, and continue boiling for 2 more min. Add 5.0 mL of hexanes through the condenser, and continue boiling for an additional 1 min. Cool the flask, remove the condenser, add 15 mL of saturated sodium chloride solution, and add 1.0 mL of the *Internal standard solution*. While the solution is still tepid, insert a stopper into the flask, and shake vigorously. Pipet 1.0 mL of the upper hexanes layer into a glass-stoppered test tube containing a small quantity of anhydrous sodium sulfate. Filter the solution, and, if necessary, dilute a volume of the filtrate with hexanes to obtain a known volume. [NOTE—Store this solution in a refrigerator until just before use.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused silica capillary; 0.25-μm film of phase G16 coating

Temperature

Injector: 250°

Detector: 300°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	0	120	3
120	50	220	12

Carrier gas: Helium

Flow rate: 1 mL/min

Injection size: 1 μL

System suitability

Sample: *Standard solution*

[NOTE—See *Table 3* for the relative retention times.]

Table 3

Methyl Ester	Relative Retention Time
Methyl caproate	0.39
Methyl caprylate	0.56
Methyl caprate	0.76
Methyl laurate	0.94
Nonadecane (internal standard)	1.0
Methyl myristate	1.1
Methyl palmitate	1.3
Methyl palmitoleate	1.35
Methyl stearate	1.65
Methyl oleate	1.7
Methyl linoleate	1.8
Methyl linolenate	2.0

Suitability requirements

Resolution: NLT 1.5 between methyl stearate and methyl oleate

Tailing factor: NMT 2.0 for each of the methyl ester peaks

Relative standard deviation: NMT 5.0% for each of the methyl ester peaks

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each fatty acid in the portion of the Saw Palmetto taken:

$$\text{Result} = (R_U/R_S) \times (C_S \times V) \times (1/W) \times (M_{r1}/M_{r2}) \times 100$$

R_U = peak response ratio of the relevant methyl ester to the internal standard obtained from the *Sample solution*

R_S = peak response ratio of the relevant methyl ester to the internal standard obtained from the *Standard solution*

C_S = concentration of the respective methyl ester in the *Standard stock solution* (mg/mL)

V = volume of the *Standard stock solution* used to prepare the *Standard solution* (mL)

W = weight of Saw Palmetto taken to prepare the *Sample solution* (mg)

M_{r1} = molecular weight of the relevant fatty acid

M_{r2} = molecular weight of the methyl ester of the relevant fatty acid

Acceptance criteria: NLT 9.0% for the sum of the percentages of all the fatty acids. See *Table 4* for individual fatty acids.

Table 4

Individual Fatty Acids	Percentages (%)
Oleic acid	NLT 3.0
Lauric acid	NLT 2.0
Myristic acid	NLT 1.2
Palmitic acid	NLT 1.0
Linoleic acid	NLT 0.4
Caproic acid	NLT 0.2
Caprylic acid	NLT 0.2
Capric acid	NLT 0.2
Stearic acid	NLT 0.1

Table 4 (Continued)

Individual Fatty Acids	Percentages (%)
Linolenic acid	NLT 0.05
Palmitoleic acid	NLT 0.01

CONTAMINANTS

- **HEAVY METALS** (231): NMT 10 µg/g
- **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues** (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total bacterial count does not exceed 10⁴ cfu/g, and the total combined molds and yeasts count does not exceed 10² cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the test for absence of *Salmonella* species and *Escherichia coli* and for absence of *Staphylococcus aureus*.

SPECIFIC TESTS**• BOTANIC CHARACTERISTICS**

Macroscopic: Subspherical to ovoid drupes, about 2–3 cm in length and about 1.5 cm thick, dark brown to black with a smooth, dull surface and with large, irregular depressions and ridges caused by shrinkage on drying; remains of style at the summit; base bearing a small depression with the scar of the stalk; epicarp and underlying sarcocarp forming a fragile layer that partially peels off, revealing the hard, pale brown layer of endocarp surrounding the seed. Seed irregularly spherical to ovoid, up to about 1.2 cm in length and 1 cm in width, hard, surface finely pitted and reddish brown with a paler, raised and membranous area over the raphe and micropyle; when cut transversely, it shows a thin testa, a narrow perisperm, and a large area of dense, horny, grayish-white endosperm.

Microscopic: The pulp is covered by a small-celled, thin-walled epidermis and consists chiefly of a very large-celled, mostly thin-walled parenchyma. The outermost layers contain brown substances; farther inside, single cells only with brown contents are scattered in the tissue; occasional large, thick-walled, rather punctate stone cells with a wide lumen are also found. The vascular bundles are accompanied by fibers with cover cells (stigmata) containing siliceous solids attached. The innermost layers of the pulp wall consist of almost completely thickened, rather punctate, quite irregularly shaped stone cells (astrosclereids). The outer layers of the seed coat are large-celled, the cells are coarse-walled; the middle layers are thin-walled, the cells are smaller; the innermost layers are small-celled, flattened. All cells are filled with a brown substance. On the outside, the endosperm exhibits radial elongated, nonpunctate, coarse-walled cells; in the deeper layers, the cells are larger, thick-walled, rather coarse punctate. The middle lamella is fairly recognizable. Aleurone with protein crystalloids is present in the cell contents.

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** (561): NMT 2.0%
- **LOSS ON DRYING** (731): Dry 1.0 g of finely powdered Saw Palmetto at 105° for 2 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 5.0%, determined on 1.0 g of finely powdered Saw Palmetto
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561): NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.

• USP REFERENCE STANDARDS (11)

USP Methyl Caprate RS
 USP Methyl Caproate RS
 USP Methyl Caprylate RS
 USP Methyl Laurate RS
 USP Methyl Linoleate RS
 USP Methyl Linolenate RS
 USP Methyl Myristate RS
 USP Methyl Oleate RS
 USP Methyl Palmitate RS
 USP Methyl Palmitoleate RS
 USP Methyl Stearate RS

Powdered Saw Palmetto**DEFINITION**

Powdered Saw Palmetto is Saw Palmetto reduced to a fine or a very fine powder. It contains NLT 2% (v/w) of volatile oil, NLT 7% of lipophilic extract, and NLT 9.0% of total fatty acids.

IDENTIFICATION**• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

[NOTE—Perform the following test under subdued light.]

Reagent solution: 3.7 mg/mL of 4-bromomethyl-7-methoxycoumarin in acetone. Store this solution in a dark place.

Standard solution: 5.0 g of magnesium stearate in a 100-mL round-bottom flask fitted with a reflux condenser. Add 50 mL of ether, 20 mL of 12.5% nitric acid, and 20 mL of water, and heat until solution is complete. Cool, transfer the contents of the flask to a separatory funnel, and withdraw the lower aqueous phase. Extract the ether phase twice, each time using 4 mL of water, separating the aqueous phases. Extract the combined aqueous phases with 15 mL of ether, combining the ether extracts. Evaporate to dryness, and dry the residue at 105°. Transfer 1 mg of the residue to an amber glass vial fitted with a metal-clamped rubber cap. Add 10 mg of lithium carbonate, 3 µL of tris-[2-(2-methoxyethoxy)ethyl]amine, and 1.0 mg of the *Reagent solution*. Replace the metal-clamped rubber cap, dry at 105° for 2 h, and cool.

Sample solution: 10.0 g of Powdered Saw Palmetto in a 250-mL round-bottom flask fitted with a reflux condenser. Add 150 mL of alcohol, and heat under a reflux condenser on a steam bath for 1 h. Cool, filter, wash the residue with alcohol, and dilute the combined washings and filtrate with alcohol to 200.0 mL. Transfer 0.6 mL of this solution to a suitable flask, and evaporate to dryness. To the residue add 1.0 mL of the *Reagent solution*. Transfer this solution with the aid of a pipet to an amber glass vial fitted with a metal-clamped rubber cap. Add 3 µL of tris-[2-(2-methoxyethoxy)ethyl]amine and 10 mg of lithium carbonate to the vial. Replace the metal-clamped rubber cap, dry at 105° for 2 h, and cool. Use the cooled solution.

Blank solution: To 10 mg of lithium carbonate in an amber glass vial fitted with a metal-clamped rubber cap, add 3 µL of tris-[2-(2-methoxyethoxy)ethyl]amine and 1.0 mL of the *Reagent solution*. Replace the metal-clamped rubber cap, dry at 105° for 2 h, and cool.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Absorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm in length (TLC plates)

Application volume: 2 µL

Developing solvent system: Cyclohexane, ethyl acetate, and acetic acid (70:30:1)

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank solution*

Develop the chromatograms in the solvent system until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the chromatographic chamber, mark the solvent front, and allow the plate to air-dry. Examine the plate under long-wavelength UV light.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits at least two zones of blue fluorescence corresponding in R_F values to similar zones exhibited in the *Standard solution*. The blue fluorescent zones from the *Sample solution* appear above the blue fluorescent zones exhibited in the *Blank solution*.

COMPOSITION

- **ARTICLES OF BOTANICAL ORIGIN, Volatile Oil Determination (561):** NLT 2 mL/100 g of an oil that solidifies to a white solid at room temperature

- **CONTENT OF LIPOPHILIC EXTRACT**

Analysis: Transfer 10 g of Powdered Saw Palmetto to a 250-mL round-bottom flask fitted with a reflux condenser, and add 150 mL of alcohol. Heat the flask on a boiling water bath under reflux for 1 h. Cool, filter, and wash the residue with small portions of alcohol. Collect the filtrate and washings in a 200-mL volumetric flask, and dilute with alcohol to volume. Evaporate 100.0 mL of this solution to dryness in a rotary evaporator under vacuum. Add 40 mL of *n*-hexane to the residue, stir for 5 min, filter, and collect the filtrate in a round-bottom flask. Repeat the above operation of washing with *n*-hexane two more times, and collect all of the filtrates in the same flask. Using a rotary evaporator, evaporate off the organic solvent to dryness. Dry the residue obtained at 105° for 2 h.

Acceptance criteria: The weight of the residue is NLT 0.35 g (NLT 7%).

- **CONTENT OF FATTY ACIDS**

Internal standard solution: 12 mg/mL of nonadecane in hexanes.

Standard stock solution: Dissolve quantities of USP Methyl Laurate RS, USP Methyl Oleate RS, USP Methyl Myristate RS, USP Methyl Palmitate RS, USP Methyl Linoleate RS, USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, USP Methyl Palmitoleate RS, USP Methyl Stearate RS, and USP Methyl Linolenate RS in hexanes to obtain concentrations of each methyl ester as given in the *Table 1*.

Table 1

Methyl Ester	Concentration (mg/mL)
Methyl laurate	5
Methyl oleate	5
Methyl myristate	2
Methyl palmitate	2
Methyl linoleate	1
Methyl caproate	0.4
Methyl caprylate	0.4
Methyl caprate	0.4
Methyl palmitoleate	0.4
Methyl stearate	0.4
Methyl linolenate	0.4

Standard solution: Transfer 1.0 mL of *Internal standard solution* to 5.0 mL of *Standard stock solution*.

Sample solution: Transfer 1 g of Powdered Saw Palmetto to a 100-mL round-bottom flask fitted with a water-cooled reflux condenser and a magnetic bar. Add 10 mL of 0.5 N methanolic sodium hydroxide solution (20 mg/mL of sodium hydroxide in methanol), and heat the flask with stirring under reflux conditions for

15 min. Pipet 5 mL of a solution of boron trifluoride in methanol (140 mg/mL of boron trifluoride in methanol) through the reflux condenser into the flask, and continue boiling for 2 more min. Add 5.0 mL of hexanes through the condenser, and continue boiling for an additional 1 min. Cool the flask, remove the condenser, add 15 mL of saturated sodium chloride solution, and add 1.0 mL of the *Internal standard solution*. While the solution is still tepid, insert a stopper into the flask, and shake vigorously. Pipet 1.0 mL of the upper hexanes layer into a glass-stoppered test tube containing a small quantity of anhydrous sodium sulfate. Filter the solution, and, if necessary, dilute a volume of the filtrate with hexanes to obtain a known volume. [NOTE—Store this solution in a refrigerator until just before use.]

Chromatographic system

(See *Chromatography (621)*, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused silica capillary; 0.25-μm film of phase G16 coating

Temperature

Injector: 250°

Detector: 300°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	0	120	3
120	50	220	12

Carrier gas: Helium

Flow rate: 1 mL/min

Injection size: 1 μL

System suitability

Sample: *Standard solution*

[NOTE—See *Table 3* for the relative retention times.]

Table 3

Methyl Ester	Relative Retention Time
Methyl caproate	0.39
Methyl caprylate	0.56
Methyl caprate	0.76
Methyl laurate	0.94
Nonadecane (internal standard)	1.0
Methyl myristate	1.1
Methyl palmitate	1.3
Methyl palmitoleate	1.35
Methyl stearate	1.65
Methyl oleate	1.7
Methyl linoleate	1.8
Methyl linolenate	2.0

Suitability requirements

Resolution: NLT 1.5 between methyl stearate and methyl oleate

Tailing factor: NMT 2.0 for each of the methyl ester peaks

Relative standard deviation: NMT 5.0% for each of the methyl ester peaks

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each fatty acid in the portion of the Powdered Saw Palmetto taken:

$$\text{Result} = (R_U/R_S) \times (C_S \times V) \times (1/W) \times (M_{r1}/M_{r2}) \times 100$$

- R_U = peak response ratio of the relevant methyl ester to the internal standard obtained from the *Sample solution*
- R_S = peak response ratio of the relevant methyl ester to the internal standard obtained from the *Standard solution*
- C_S = concentration of the respective methyl ester in the *Standard stock solution* (mg/mL)
- V = volume of the *Standard stock solution* used to prepare the *Standard solution* (mL)
- W = weight of Powdered Saw Palmetto taken to prepare the *Sample solution* (mg)
- M_{r1} = molecular weight of the relevant fatty acid
- M_{r2} = molecular weight of the methyl ester of the relevant fatty acid

Acceptance criteria: NLT 9.0% for the sum of the percentages of all the fatty acids. See *Table 4* for individual fatty acids.

Table 4

Individual Fatty Acids	Percentages (%)
Oleic acid	NLT 3.0
Lauric acid	NLT 2.0
Myristic acid	NLT 1.2
Palmitic acid	NLT 1.0
Linoleic acid	NLT 0.4
Caproic acid	NLT 0.2
Caprylic acid	NLT 0.2
Capric acid	NLT 0.2
Stearic acid	NLT 0.1
Linolenic acid	NLT 0.05
Palmitoleic acid	NLT 0.01

CONTAMINANTS

- **HEAVY METALS** (231): NMT 10 µg/g
- **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues** (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the test for absence of *Salmonella* species and *Escherichia coli* and for absence of *Staphylococcus aureus*.

SPECIFIC TESTS

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** (561): NMT 2%
- **LOSS ON DRYING** (731): Dry 1.0 g of Powdered Saw Palmetto at 105° for 2 h: it loses NMT 12.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 5.0%, determined on 1.0 g of Powdered Saw Palmetto
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561): NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.

• USP REFERENCE STANDARDS (11)

USP Methyl Caprate RS
 USP Methyl Caproate RS
 USP Methyl Caprylate RS
 USP Methyl Laurate RS
 USP Methyl Linoleate RS
 USP Methyl Linolenate RS
 USP Methyl Myristate RS
 USP Methyl Oleate RS
 USP Methyl Palmitate RS
 USP Methyl Palmitoleate RS
 USP Methyl Stearate RS

Saw Palmetto Extract

DEFINITION

Saw Palmetto Extract is obtained from comminuted Saw Palmetto by extraction with hydroalcoholic mixtures or solvent hexane, or by supercritical extraction with carbon dioxide. The ratio of starting crude plant material to Extract is from 8.0:1 to 14.3:1. The Extract contains NLT 70.0% and NMT 95.0% of fatty acids and NLT 0.2% and NMT 0.5% of sterols, calculated on the anhydrous basis. The lipophilic Extract contains NLT 0.15% and NMT 0.35% of long-chain alcohols. The hydroalcoholic Extract contains NLT 0.01% and NMT 0.15% of long-chain alcohols. It contains no added substances.

IDENTIFICATION

• A. GAS CHROMATOGRAPHY

Analysis: Proceed as directed in *Content of Fatty Acids*.

Acceptance criteria: The retention times of the 11 major peaks of the *Sample solution* correspond to those in the chromatogram of the *Standard solution*. The ranges for ratios of the concentration of lauric acid to the concentration of the respective fatty acid are in *Table 1*.

Table 1

Fatty Acid	Minimum Ratio	Maximum Ratio
Capric	9.0	16
Caproic	8.5	24
Caprylic	8.5	17.5
Linoleic	5.0	16
Linolenic	31.5	55
Myristic	2.2	2.8
Oleic	0.60	1.15
Palmitic	2.8	3.9
Stearic	14	26

COMPOSITION

• CONTENT OF FATTY ACIDS

Internal standard solution: 12 mg/mL of nonadecane in hexanes

Standard stock solution: Dissolve quantities of USP Methyl Laurate RS, USP Methyl Oleate RS, USP Methyl Myristate RS, USP Methyl Palmitate RS, USP Methyl Linoleate RS, USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, USP Methyl Palmitoleate RS, USP Methyl Stearate RS, and USP Methyl Linolenate RS in hexanes to obtain concentrations of each methyl ester as given in the *Table 2*.

Table 2

Methyl Ester	Concentration (mg/mL)
Methyl laurate	5
Methyl oleate	5
Methyl myristate	2
Methyl palmitate	2
Methyl linoleate	1
Methyl caproate	0.4
Methyl caprylate	0.4
Methyl caprate	0.4
Methyl palmitoleate	0.4
Methyl stearate	0.4
Methyl linolenate	0.4

Standard solution: Transfer 1.0 mL of *Internal standard solution* to 5.0 mL of the *Standard stock solution*.

Sample solution: Transfer 100 mg of Extract to a pressure-proof, screw-capped vial, and add 3.0 mL of a solution of sulfuric acid in methanol (5 in 100). Heat at 100° in an oil bath for 2 h, shaking from time to time. Allow to cool, and add 1.0 mL of *Internal standard solution*, 10.0 mL of water, 1 g of sodium chloride, and 5 mL of hexanes. Shake well, allow the layers to separate completely, and use the hexanes layer. [NOTE—Store in a refrigerator until ready to use.]

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused silica capillary; 0.25-μm film of phase G16 coating

Temperature

Injector: 250°

Detector: 300°

Column: See Table 3.

Table 3

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	0	120	3
120	50	220	12

Carrier gas: Helium

Flow rate: 1 mL/min

Injection size: 1 μL

System suitability

Sample: *Standard solution*

[NOTE—See Table 4 for the relative retention times.]

Table 4

Methyl Ester	Relative Retention Time
Methyl caproate	0.39
Methyl caprylate	0.56
Methyl caprate	0.76
Methyl laurate	0.94
Nonadecane (internal standard)	1.0
Methyl myristate	1.1
Methyl palmitate	1.3
Methyl palmitoleate	1.35
Methyl stearate	1.65
Methyl oleate	1.7
Methyl linoleate	1.8
Methyl linolenate	2.0

Suitability requirements

Resolution: NLT 1.5 between methyl stearate and methyl oleate peaks

Tailing factor: NMT 2.0 for each of the methyl ester peaks

Relative standard deviation: NMT 5.0% for each of the methyl ester peaks

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each fatty acid in the portion of Extract taken:

$$\text{Result} = (R_U/R_S) \times (C_S \times V) \times (1/W) \times (M_{r1}/M_{r2}) \times 100$$

R_U = peak response ratio of the relevant methyl ester to the internal standard from the *Sample solution*

R_S = peak response ratio of the relevant methyl ester to the internal standard from the *Standard solution*

C_S = concentration of the respective methyl ester in the *Standard stock solution* (mg/mL)

V = volume of the *Standard stock solution* used to prepare the *Standard solution* (mL)

W = weight of Extract taken to prepare the *Sample solution* (mg)

M_{r1} = molecular weight of the relevant fatty acid

M_{r2} = molecular weight of the methyl ester of the relevant fatty acid

Acceptance criteria: 70.0%–95.0% for the sum of the percentages of all the fatty acids.

• CONTENT OF LONG-CHAIN ALCOHOLS AND STEROLS

Derivatizing solution A: Bis(trimethylsilyl)acetamide, trimethylsilylimidazole, and trimethylchlorosilane (3:3:2)

Derivatizing solution B: *Derivatizing solution A*, bis(trimethylsilyl)trifluoroacetamide, and pyridine (1:1:1)

Internal standard solution: 10 mg/mL of eicosanol and 5 mg/mL of cholesterol in chloroform

Standard stock solution: 0.75 mg/mL of USP

Hexacosanol RS and 1.4 mg/mL of USP β-Sitosterol RS in chloroform

Standard solution: Mix 5.0 mL of *Standard stock solution* with 1.0 mL of the *Internal standard solution*. Evaporate 0.75 mL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 1.0 mL of *Derivatizing solution B*, and allow to stand for NLT 15 min at room temperature.

Sample solution: Transfer 3.35 g of Extract into a 50-mL round-bottomed flask. Add 1.0 mL of *Internal standard solution*, and evaporate under vacuum at a temperature not exceeding 50°. Add 20 mL of a solution prepared by dissolving 130 g of potassium hydroxide in 200 mL of water in a 1000-mL volumetric flask, and dilute with methanol to volume. Attach a condenser, and reflux in a bath at 100° for 2 h. Quantitatively transfer this solution to a 25-mL volumetric flask, and dilute with water to volume. Transfer a 3-mL portion to a cartridge¹ containing diatomaceous earth capable of holding 3 mL of aqueous phase.

Absorb the solution into the column under vacuum for 20 min until the column is not cold. Elute the analytes from the column with 90 mL of methylene chloride, and evaporate the eluate to dryness. Dissolve the residue in 1.0 mL of *Derivatizing solution B*, and allow to stand for NLT 15 min at room temperature.

System suitability stock solution A: 2 mg/mL each of tetracosanol, octacosanol, USP Hexacosanol RS, and triacontanol in chloroform

System suitability solution A: Mix 5.0 mL of *System suitability stock solution A* with 1.0 mL of *Internal standard solution*. Evaporate 0.75 mL of this solution to dryness using a stream of nitrogen. Dissolve the residue

¹ A suitable cartridge is Extrelut NT3, or an equivalent cartridge.

in 1.0 mL of *Derivatizing solution B*, and allow to stand for NLT 15 min at room temperature.

System suitability stock solution B: 2 mg/mL each of campesterol, stigmasterol, and USP β -Sitosterol RS and 0.37 mg/mL of stigmastanol

System suitability solution B: Mix 5.0 mL of *System suitability stock solution B* with 1.0 mL of the *Internal standard solution*. Evaporate 0.75 mL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 1.0 mL of *Derivatizing solution B*, and allow to stand for NLT 15 min at room temperature.

Chromatographic system

(See *Chromatography* <621>), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.2-mm \times 25-m capillary; 0.33- μ m thickness of phase G1 coating

Temperature

Injector: 325°

Detector: 325°

Column: See *Table 5*.

Table 5

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
200	0	200	3
200	10	300	35

Carrier gas: Helium

Flow rate: 0.5 mL/min

Make up gas flow: 25 mL/min

Injection size: 1 μ L

Injection type: Split ratio, 1:40

System suitability

Samples: *System suitability solution A* and *System suitability solution B*

[NOTE—The relative retention times for tetracosanol, hexacosanol, octacosanol, and triacontanol are 0.89, 1.00, 1.15, and 1.36, respectively, *System suitability solution A*; and the relative retention times for cholesterol, campesterol, stigmasterol, β -sitosterol, and stigmastanol are 0.85, 0.92, 0.95, 1.00, and 1.01, respectively, *System suitability solution B*.]

Suitability requirements

Resolution: NLT 2 between β -sitosterol and stigmastanol peaks, *System suitability solution B*

Column efficiency: NLT 200,000 theoretical plates for the eicosanol peak, *System suitability solution A*; and NLT 150,000 theoretical plates for the cholesterol peak, *System suitability solution B*

Tailing factor: NMT 2.0 for each relevant peak, *System suitability solution A*; and NMT 2.0 for each relevant peak, *System suitability solution B*

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the signals corresponding to the relevant analytes by comparison with the chromatograms obtained with *System suitability solutions A* and *B*. Separately calculate the percentages of tetracosanol, hexacosanol, octacosanol, and triacontanol, respectively, in the portion of Extract taken:

$$\text{Result} = (R_U/R_S) \times (C_S \times V) \times (1/W) \times 100$$

R_U = peak response ratio of the relevant long-chain alcohol to the internal standard from the *Sample solution*

R_S = peak response ratio of hexacosanol to the internal standard from the *Standard solution*

C_S = concentration of hexacosanol in the *Standard stock solution* (mg/mL)

V = volume of the *Standard stock solution* used to prepare the *Standard solution* (mL)

W = weight of the Extract taken to prepare the *Sample solution* (mg)

Calculate the total content of long-chain alcohols as a percentage by adding the individual percentages.

Separately calculate the percentages of campesterol, stigmasterol, β -sitosterol, and stigmastanol, respectively, in the portion of Extract taken:

$$\text{Result} = (R_U/R_S) \times (C_S \times V) \times (1/W) \times 100$$

R_U = peak response ratio of the relevant sterol to the internal standard from the *Sample solution*

R_S = peak response ratio of β -sitosterol to the internal standard from the *Standard solution*

C_S = concentration of β -sitosterol in the *Standard stock solution* (mg/mL)

V = volume of the *Standard stock solution* used to prepare the *Standard solution* (mL)

W = weight of the Extract taken to prepare the *Sample solution* (mg)

Calculate the total content of sterols as a percentage by adding the individual percentages.

Acceptance criteria: 0.2%–0.5% of sterols; the lipophilic Extract contains 0.15%–0.35% of long-chain alcohols; and the hydroalcoholic Extract contains 0.01%–0.15% of long-chain alcohols

CONTAMINANTS

- **HEAVY METALS**, *Method II* <231>: NMT 40 μ g/g
- **BOTANICAL EXTRACTS**, *Pesticide Residues* <565>: Meets the requirements

SPECIFIC TESTS

- **ALCOHOL DETERMINATION**, *Method II* <611> (if present): NMT 1%
- **FATS AND FIXED OILS**, *Iodine Value* <401>: 40–50
- **FATS AND FIXED OILS**, *Saponification Value* <401>: 210–250
- **FATS AND FIXED OILS**, *Unsaponifiable Matter* <401>: 1.8%–3.5%
- **WATER DETERMINATION**, *Method I* <921>: NMT 3% is found in the hydroalcoholic Extract.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Meets the requirements in *Botanical Extracts* <565>, *Packaging and Storage*
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of fatty acids and sterols and the ratio of the starting crude plant material to Extract. It meets the requirements in *Botanical Extracts*, <565> *Labeling*.
- **USP REFERENCE STANDARDS** <11>
 - USP Hexacosanol RS
 - USP Methyl Caprate RS
 - USP Methyl Caproate RS
 - USP Methyl Caprylate RS
 - USP Methyl Laurate RS
 - USP Methyl Linoleate RS
 - USP Methyl Linolenate RS
 - USP Methyl Myristate RS
 - USP Methyl Oleate RS
 - USP Methyl Palmitate RS
 - USP Methyl Palmitoleate RS
 - USP Methyl Stearate RS
 - USP β -Sitosterol RS

Saw Palmetto Capsules

DEFINITION

Saw Palmetto Capsules contain Saw Palmetto Extract. Capsules contain NLT 22.0% and NMT 34.0% of lauric acid in the labeled amount of Saw Palmetto Extract. The ratio of the concentrations of lauric acid to caprylic acid is NLT 8.5 and NMT 17.5. The ratio of the concentrations of lauric acid to myristic acid is NLT 2.2 and NMT 2.8.

IDENTIFICATION

- **A.** The retention times of the peaks for methyl caprate, methyl caproate, methyl caprylate, methyl laurate, methyl linoleate, methyl linolenate, methyl myristate, methyl oleate, methyl palmitate, methyl palmitoleate, and methyl stearate of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Content of Lauric Acid and the Ratios of the Concentrations of Lauric Acid to Caprylic Acid and Lauric Acid to Myristic Acid*.
- **B. PRESENCE OF STEROLS**

Derivatizing stock solution: *N,O*-bis(trimethylsilyl)-acetamide, trimethylsilylimidazole, and trimethylchlorosilane (3:3:2)

Derivatizing solution: *Derivatizing stock solution*, bis(trimethylsilyl)trifluoroacetamide, and pyridine (1:1:1)

Internal standard solution: 10 mg/mL of eicosanol and 5 mg/mL of cholesterol in chloroform

Standard stock solution: 0.75 mg/mL of USP Hexacosanol RS and 1.4 mg/mL of USP β -Sitosterol RS in chloroform

Standard solution: Mix 5.0 mL of *Standard stock solution* with 1.0 mL of the *Internal standard solution*. Evaporate 0.75 mL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 1.0 mL of *Derivatizing solution*, and allow to stand for NLT 15 min at room temperature.

System suitability stock solution A: 2 mg/mL each of tetracosanol, octacosanol, USP Hexacosanol RS, and triacontanol in chloroform

System suitability solution A: Mix 5.0 mL of *System suitability stock solution A* with 1.0 mL of *Internal standard solution*. Evaporate 0.75 mL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 1.0 mL of *Derivatizing solution*, and allow to stand for NLT 15 min at room temperature.

System suitability stock solution B: 2 mg/mL each of campesterol, stigmasterol, and USP β -Sitosterol RS, and 0.37 mg/mL of stigmasterol

System suitability solution B: Mix 5.0 mL of *System suitability stock solution B* with 1.0 mL of *Internal standard solution*. Evaporate 0.75 mL of this solution to dryness using a stream of nitrogen. Dissolve the residue in 1.0 mL of *Derivatizing solution*, and allow to stand for NLT 15 min at room temperature.

β -Cholesterol solution: β -cholesterol in chloroform (1 in 100)

Sample solution

Sample: A number of Capsules, equivalent to 10 g of Saw Palmetto Extract

Open the Capsules, and transfer the shells and contents to a suitable container.

Transfer 5 g of the *Sample* to a 250-mL round-bottom flask, and evaporate in vacuum at a temperature of NMT 50°. Add 50 mL of a solution prepared by dissolving 130 mg/mL of potassium hydroxide in methanol and water (4:1). Attach a condenser, and reflux in a bath at 100° until a clear solution is obtained. Reflux for an additional 10 min, and cool by adding 50 mL of water through the condenser. Transfer to a separation funnel, rinsing the flask with a total of 50 mL of water in small portions. Extract with 80 mL of ether, shaking for 30 s, and repeat twice. [NOTE—If an emulsion forms, it can be eliminated by adding small quantities

of methanol.] Transfer the combined ether layers to a separation funnel, and wash with successive portions of 50 mL of water until a neutral washing is obtained. [NOTE—If an emulsion forms, it can be eliminated by adding small quantities of methanol.] Pass the ether extract through filter paper containing anhydrous sodium sulfate, wash the filter with 30 mL of ether, and evaporate to dryness in vacuum. Dissolve the residue in 2.0 mL of chloroform. Extract the sterols using the following chromatographic system.

Chromatographic extraction system

Mode:

Absorbent: Chromatographic plate coated with 0.25-mm silica gel having an application zone that was previously dipped under 3 cm of a solution prepared by dissolving 13 mg/mL of potassium hydroxide in methanol and water (49:1)

Developing solvent system: Hexanes and ether (7:3)

Application volumes: 200 μ L of chloroform solution containing Capsule residue and 20 μ L of β -Cholesterol solution

After the spots have been applied, allow the plate to dry, and heat it to 100° for 1 h before use. The plate can be stored in a desiccator containing calcium chloride until the time of use. Develop the plates until the solvent front has moved 17–19 cm. Keep the chamber temperature between 15° and 20°. Dry the plate in a current of warm air, then spray with an alkaline solution of 2,7-dichlorofluorescein in alcohol (0.2 in 100). Observe the plate under 366-nm wavelength light, and identify the bands corresponding to the sterols by referring to the β -cholesterol spot. Scrape off these bands and transfer them to a test tube. Add 10 mL of warm chloroform, and shake for 2 min with the aid of several glass beads. Filter the chloroform solution, wash the filter with chloroform, and evaporate the combined filtrate and washings to dryness in vacuum. Dissolve the residue with some drops of anhydrous acetone, and evaporate in vacuum. Dry the residue in an oven at 105° for 15 min. Dissolve the residue in 0.2 mL of *Derivatizing solution*. Use this resulting solution as the *Sample solution* for GC analysis.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode:

Detector: Flame ionization

Column: 0.2-mm \times 25-m capillary, coated with a 0.33- μ m thickness of phase G1

Temperature

Detector: 325°

Injector: 325°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
200	0	200	3
200	10	300	35

Carrier gas: Helium

Flow rate: 0.5 mL/min

Make up gas flow: 25 mL/min

Split ratio: 1:40

Injection size: 1 μ L

Injection type: Split injection system

System suitability

Samples: *System suitability solution A* and *System suitability solution B*

[NOTE—The relative retention times for tetracosanol, octacosanol, hexacosanol, and triacontanol are 0.89, 1.00, 1.15, and 1.36, respectively, *System suitability solution A*; and the relative retention times for cholest-

terol, campesterol, stigmasterol, β -sitosterol, and stigmasterol are 0.85, 0.92, 0.95, 1.00, and 1.01, respectively, *System suitability solution B*.]

Suitability requirements

Resolution: NLT 2 between β -sitosterol and stigmasterol, *System suitability solution B*

Column efficiency: NLT 200,000 theoretical plates for the eicosanol peak, *System suitability solution A*; and NLT 150,000 theoretical plates for the cholesterol peak, *System suitability solution B*

Tailing factor: NMT 2.0 for each relevant peak, *System suitability solution A*; and NMT 2.0 for each relevant peak, *System suitability solution B*

Analysis

Samples: *Standard solution* and *Sample solution*
Identify the signals corresponding to the relevant analytes by comparison with the chromatograms obtained with *System suitability solutions A* and *B*.

Acceptance criteria: The *Sample solution* exhibits peaks for campesterol, β -sitosterol, and stigmasterol, identified by their retention times relative to the β -sitosterol peak in the *Standard solution*.

STRENGTH

• CONTENT OF LAURIC ACID AND THE RATIOS OF THE CONCENTRATIONS OF LAURIC ACID TO CAPRYLIC ACID AND LAURIC ACID TO MYRISTIC ACID

Internal standard solution: 12 mg/mL of nonadecane in hexanes

Standard stock solution: Dissolve quantities of USP Methyl Laurate RS, USP Methyl Oleate RS, USP Methyl Myristate RS, USP Methyl Palmitate RS, USP Methyl Linoleate RS, USP Methyl Caproate RS, USP Methyl Caprylate RS, USP Methyl Caprate RS, USP Methyl Palmitoleate RS, USP Methyl Stearate RS, and USP Methyl Linolenate RS in hexanes to obtain concentration of each methyl ester as given in the table below.

Methyl Ester	Concentration (mg/mL)
Methyl laurate	5
Methyl oleate	5
Methyl myristate	2
Methyl palmitate	2
Methyl linoleate	1
Methyl caproate	0.4
Methyl caprylate	0.4
Methyl caprate	0.4
Methyl palmitoleate	0.4
Methyl stearate	0.4
Methyl linolenate	0.4

Standard solution: Add 1.0 mL of *Internal standard solution* to 5.0 mL of the *Standard stock solution*.

Sample solution: Take a number of Capsules, equivalent to 10 g of Extract, open the Capsules, and transfer the shells and contents to a suitable container. Transfer 100 mg to a pressure-proof screw-capped vial, and add 3.0 mL of a solution of sulfuric acid in methanol (5 in 100). Heat in an oil bath at 100° for 2 h, shaking from time to time. Allow to cool, and add 1.0 mL of *Internal standard solution*, 10.0 mL of water, 1 g of sodium chloride, and 5 mL of hexanes. Shake well, and allow the layers to separate completely. Use the hexanes layer. [NOTE—Store this solution in a refrigerator until use.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm \times 30-m fused silica capillary, coated with a 0.25- μ m film of phase G16

Temperature

Detector: 300°

Injector: 250°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	0	120	3
120	50	220	12

Carrier gas: Helium

Flow rate: 1 mL/min

Injection size: 1 μ L

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for methyl caproate, methyl caprylate, methyl caprate, methyl laurate, nonadecane (internal standard), methyl myristate, methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate, and methyl linolenate are about 0.39, 0.56, 0.76, 0.94, 1.0, 1.1, 1.3, 1.35, 1.65, 1.7, 1.8, and 2.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between the methyl stearate and methyl oleate peaks

Tailing factor: NMT 2.0 for each of the methyl ester peaks

Relative standard deviation: NMT 5.0% for each of the methyl ester peaks

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of lauric acid, myristic acid, and caprylic acid in the labeled amount of Saw Palmetto Extract in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S \times V/W) \times (M_{r1}/M_{r2}) \times A_W/L_E \times 100$$

R_U = ratio of the response of the relevant methyl ester peak and the internal standard peak from the *Sample solution*

R_S = ratio of the response of the relevant methyl ester peak and the internal standard peak from the *Standard solution*

C_S = concentration of the respective methyl ester in the *Standard stock solution* (mg/mL)

V = volume of the *Standard stock solution* taken to prepare the *Standard solution* (5.0 mL)

W = weight of sample used to prepare the *Sample solution* (mg)

M_{r1} = molecular weight of the relevant fatty acid

M_{r2} = molecular weight of the methyl ester of the relevant fatty acid

A_W = average weight of the Capsule contents (mg/Capsule)

L_E = labeled amount of Saw Palmetto Extract per Capsule (mg/Capsule)

Using these percentages, calculate the individual ratios of the concentration of lauric acid to caprylic acid and of lauric acid to myristic acid in the portion of Capsules taken.

Acceptance criteria: 22.0%–34.0% of lauric acid in the labeled amount of Saw Palmetto Extract. The ratio of lauric acid to caprylic acid is 8.5–17.5. The ratio of lauric acid to myristic acid is 2.2–2.8.

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** <2040>: Meet the requirements for *Rupture Test for Soft Shell Capsules*

• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** (2021): The total bacterial count does not exceed 10^4 cfu/g, the total combined molds and yeasts count does not exceed 1000 cfu/g, the coliform count does not exceed 100 cfu/g, and the count for enterobacteria does not exceed 100 cfu/g.
- **MICROBIAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Capsules meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the name of article from which the Capsules were prepared. Label it to indicate the amount of Extract in mg/Capsule.
- **USP REFERENCE STANDARDS** (11)
 - USP Hexacosanol RS
 - USP Methyl Caprate RS
 - USP Methyl Caproate RS
 - USP Methyl Caprylate RS
 - USP Methyl Laurate RS
 - USP Methyl Linoleate RS
 - USP Methyl Linolenate RS
 - USP Methyl Myristate RS
 - USP Methyl Oleate RS
 - USP Methyl Palmitate RS
 - USP Methyl Palmitoleate RS
 - USP Methyl Stearate RS
 - USP β -Sitosterol RS

Schizochytrium Oil

DEFINITION

Schizochytrium Oil is obtained by fermentation and extraction of algae of the genus *Schizochytrium* and contains NLT 30.0% (w/w) of docosahexaenoic acid (DHA, $C_{22}H_{32}O_2$) ($C_{22:6}$ n-3), as the main polyunsaturated fatty acid. Suitable antioxidants in appropriate concentration may be added.

IDENTIFICATION

- **LONG CHAIN UNSATURATED FATTY ACID PROFILE:** Proceed as directed under *Composition, Content of DHA*.

Analysis

Samples: *Standard Solution 2* and *Test Solution 1*
Calculate the area percentage for each fatty acid as methyl ester in *Test Solution 1*:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual fatty acid as methyl ester

r_T = sum of the responses of all the peaks, except the solvent and butylated hydroxytoluene peaks

Acceptance criteria: The retention times of the peaks of the docosahexaenoic acid methyl ester and the eicosapentanoic acid methyl ester of *Test Solution 1* correspond to those of *Standard Solution 2*, as obtained in the test for *Content of EPA and DHA*. The area percentage for the methyl esters of the fatty acids from the chromatogram of *Test Solution 1* in the test for *Content of EPA and DHA* meet the requirements for each fatty acid shown in the table below.

Fatty Acid	Relative Retention Time	Shorthand Notation	Lower Limit (Area %)	Upper Limit (Area %)
Dihomo-gamma-linolenic acid	0.71	20:3 n-6	1.7	2.8
Arachidonic acid	0.73	20:4 n-6	0.6	1.3
Eicosapentanoic acid (EPA)	0.79	20:5 n-3	1.3	3.9
Docosapentanoic acid (DPA n-6)	0.94	22:5 n-6	10.5	16.5
Docosahexanoic acid (DHA)	1.00	22:6 n-3	30.0	40.0

COMPOSITION• **CONTENT OF DHA**

Analysis: Proceed as directed under *Fats and Fixed Oils* (401), *Omega-3 Fatty Acids Determination and Profile*.

Acceptance criteria: NLT 30.0% (w/w) docosahexaenoic acid (DHA)

IMPURITIES**Inorganic Impurities**• **LIMIT OF ARSENIC**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: Transfer 1 g of ultrapure palladium metal to a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (3:2:5). A volume of 5 μ L provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed under *Arsenic* (211), to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 μ g/mL of arsenic.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 μ g/mL of arsenic.

Sample solution: For preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytetrafluoroethylene liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow

container. Equip the microwave oven with an exhaust tube to ventilate fumes. [CAUTION—Wear proper eye protection and protective clothing and gloves.] Transfer approximately 500 mg of Schizochytrium Oil, weighed to the nearest 0.1 mg, to a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. [NOTE—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests to 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115°, using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min; char the sample at 1000°, using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of Schizochytrium Oil taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration as obtained above
W = weight of Schizochytrium Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• LIMIT OF LEAD

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the

solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Lead Nitrate Stock Solution*, prepared as directed under *Heavy Metals* (231), to a 100-mL volumetric flask, add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 µg/mL of lead.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of lead.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2100°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of lead in the portion of Schizochytrium Oil taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above
W = weight of Schizochytrium Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• LIMIT OF CADMIUM

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid to volume (2:1:2). A volume of 5 µL provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution A: 0.1372 mg/mL of cadmium nitrate

Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 µg/mL of cadmium. [NOTE—Before makeup to final volume, dissolve in a portion of water and nitric acid.]

Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 µg/mL of cadmium.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 µL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 µL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in µg/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in µg/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line. Calculate the content of cadmium in the portion of Schizochytrium Oil taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above

W = weight of Schizochytrium Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 µg/g

• LIMIT OF MERCURY

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*, combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution* (see *Mercury* <261>, *Method IIa and Method IIb, Reagents*).

Analysis: Proceed as directed for *Mercury* <261>, *Method IIa and Method IIb*, except use a *Standard Mercury Solution* having the equivalent of 0.1 µg/mL of mercury.

Acceptance criteria: NMT 0.1 µg/g

SPECIFIC TESTS

- **FATS AND FIXED OILS, Anisidine Value** <401>: NMT 20.0
- **FATS AND FIXED OILS, Free Fatty Acids** <401>: The free fatty acids in 10 g require NMT 1.42 mL of 0.1 N sodium hydroxide for neutralization.
- **FATS AND FIXED OILS, Peroxide Value** <401>: NMT 5.0
- **FATS AND FIXED OILS, Total Oxidation Value (TOTOX)** <401>: NMT 26, calculated:

$$\text{Result} = (2 \times \text{PV}) + \text{AV}$$

PV = peroxide value

AV = anisidine value

- **FATS AND FIXED OILS, Unsaponifiable Matter** <401>: NMT 4.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.

- **LABELING:** The label states the content of docosahexaenoic acid in mg/g. It also states the name and concentration of any added antioxidant.
- **USP REFERENCE STANDARDS** <11>
 - USP Docosahexaenoic Acid Ethyl Ester RS
 - USP Eicosapentaenoic Acid Ethyl Ester RS
 - USP Methyl Tricosanoate RS

Schizochytrium Oil Capsules

DEFINITION

Schizochytrium Oil Capsules are prepared from Schizochytrium Oil and contain NLT 95.0% and NMT 105.0% of the labeled amount of docosahexaenoic acid (DHA; C₂₂H₃₂O₂) (C22:6 n-3).

IDENTIFICATION

- **LONG CHAIN UNSATURATED FATTY ACID PROFILE:** Proceed as directed under *Strength, Content of DHA*.

Analysis

Samples: *Standard Solution 2* and *Test Solution 1*
Calculate the area percentage for each fatty acid as methyl ester in *Test Solution 1*:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual fatty acid as methyl ester

r_T = sum of the responses of all the peaks, except the solvent and butylated hydroxytoluene peaks

Acceptance criteria: The retention times of the peaks of the docosahexaenoic acid methyl ester and the eicosapentaenoic acid methyl ester of *Test Solution 1* correspond to those of *Standard Solution 2*, as obtained in the test for *Content of EPA and DHA*. The area percentage for the methyl esters of the fatty acids from the chromatogram of *Test Solution 1* in the test for *Content of EPA and DHA* meet the requirements for each fatty acid shown in the table below.

Fatty Acid	Relative Retention Time	Shorthand Notation	Lower Limit (Area %)	Upper Limit (Area %)
Dihomo-gamma-linolenic acid	0.71	20:3 n-6	1.7	2.8
Arachidonic acid	0.73	20:4 n-6	0.6	1.3
Eicosapentaenoic acid (EPA)	0.79	20:5 n-3	1.3	3.9
Docosapentaenoic acid (DPA n-6)	0.94	22:5 n-6	10.5	16.5
Docosahexaenoic acid (DHA)	1.00	22:6 n-3	30.0	40.0

STRENGTH

• CONTENT OF DHA

Test Solution 1 and Test Solution 2: Weigh NLT 10 Capsules in a tared weighing bottle. With a sharp blade or other appropriate means, carefully open the Capsules, without loss of the shell material, and transfer the combined Capsule contents to a 100-mL beaker. Remove any adhering substance from the emptied

Capsules by washing with several small portions of isooctane. Discard the washings, and allow the empty Capsules to dry in a current of dry air until the isooctane is completely evaporated. Weigh the empty Capsules in the original tared weighing bottle, and calculate the average fill weight (AFW) of schizochytrium oil per Capsule. Proceed with the content of Capsules as directed in the *Analysis*.

Analysis: Proceed as directed in *Fats and Fixed Oils* (401), *Omega-3 Fatty Acids Determination and Profile, Content of EPA and DHA*.

Calculate the percentage of the labeled amount of docosahexaenoic acid (DHA) in the Capsules taken:

$$\text{Result} = R \times \text{AFW}/L$$

R = percentage of DHA in the portion of oil taken from the Capsules
 AFW = average fill weight of the Capsules taken (mg)
 L = the labeled amount of DHA (mg/Capsule)
Acceptance criteria: NLT 95.0% and NMT 105.0% of the labeled amount of DHA

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** (2040): Meet the requirements of the *Rupture Test for Soft Shell Capsules*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** (2091): Meet the requirements

IMPURITIES

Inorganic Impurities

• LIMIT OF ARSENIC

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of arsenic as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: Transfer 1 g of ultrapure palladium metal into a Teflon beaker. Add 20 mL of water and 10 mL of nitric acid, and warm on a hot plate to dissolve. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution B: Transfer 1 g of ultrapure magnesium nitrate into a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it into a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (3:2:5). A volume of 5 μL provides 0.015 mg of palladium and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Standard Arsenic Solution*, prepared as directed in the test for *Arsenic* (211), to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 $\mu\text{g}/\text{mL}$ of arsenic.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 $\mu\text{g}/\text{mL}$ of arsenic.

Sample solution: For preparation of the *Sample solution*, use a microwave oven with a magnetron frequency of 2455 MHz and a selectable output power of 0–950 watts in 1% increments, equipped with advanced composite vessels with 100-mL polytetrafluoroethylene liners. Use rupture membranes to vent vessels should the pressure exceed 125 psi. The vessels fit into a turntable, and each vessel can be vented into an overflow

container. Equip the microwave oven with an exhaust tube to ventilate fumes. [**CAUTION**—Wear proper eye protection and protective clothing and gloves.] Transfer approximately 500 mg of schizochytrium oil from Capsules, weighed to the nearest 0.1 mg, into a Teflon digestion vessel liner. Prepare samples in duplicate. Add 15 mL of nitric acid, and swirl gently. Cover the vessels with lids, leaving the vent fitting off. Predigest overnight under a hood. Place the rupture membrane in the vent fitting, and tighten the lid. Place all vessels on the microwave oven turntable. Connect the vent tubes to the vent trap, and connect the pressure-sensing line to the appropriate vessel. Initiate a two-stage digestion procedure by heating the microwave at 15% power for 15 min, followed by 25% power for 45 min. Remove the turntable of vessels from the oven, and allow the vessels to cool to room temperature. [NOTE—A cool water bath may be used to speed the cooling process.] Vent the vessels when they reach room temperature. Remove the lids, and slowly add 2 mL of 30% hydrogen peroxide to each. Allow the reactions to subside, and seal the vessels. Return the vessels on the turntable to the microwave oven, and heat for an additional 15 min at 30% power. Remove the vessels from the oven, and allow them to cool to room temperature. Transfer the cooled digests into 25-mL volumetric flasks, and dilute with water to volume.

Analysis: Program the graphite furnace as follows. Dry at 115°, using a 1-s ramp, a 65-s hold, and an argon flow of 300 mL/min; char the sample at 1000°, using a 1-s ramp, a 20-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 μL) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 μL of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for arsenic. Determine the peak area at the arsenic emission line at 193.7 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of arsenic, in $\mu\text{g}/\text{mL}$, and calculate the regression line best fitting the points. Determine the concentration, C , in $\mu\text{g}/\text{mL}$, of arsenic in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of arsenic in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration as obtained above
 W = weight of Capsule content taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 $\mu\text{g}/\text{g}$

• LIMIT OF LEAD

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 1 mL of nitric acid and 40 mL of water to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: 1 g of ultrapure magnesium nitrate in a Teflon beaker. Add 40 mL of water and 1 mL of nitric

acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid (2:1:2). A volume of 5 μ L provides 0.2 mg of phosphate plus 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution: Transfer 10.0 mL of *Lead Nitrate Stock Solution*, prepared as directed in the test for *Heavy Metals* <231>, to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, add 50 mL of water and 1 mL of nitric acid, and dilute with water to volume. This solution contains 0.10 μ g/mL of lead.

Standard solutions: Dilute the *Standard stock solution* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 μ g/mL of lead.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2100°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 μ L) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 μ L of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for lead. Determine the peak area at the lead emission line at 283.3 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of lead, in μ g/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in μ g/mL, of lead in each mL of the *Sample solution* by interpolation from the regression line.

Calculate the content of lead in the portion of Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above

W = weight of Capsule content taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 μ g/g

• LIMIT OF CADMIUM

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glass, polytetrafluoroethylene, and plastic vessels before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glass, polytetrafluoroethylene, and plastic vessels before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solution A: 10 g of ultrapure monobasic ammonium phosphate in 40 mL of water and 1 mL of nitric acid to dissolve the phosphate. Dilute with deionized water to 100 mL.

Solution B: Transfer 1 g of ultrapure magnesium nitrate to a Teflon beaker. Add 40 mL of water and 1 mL of nitric acid, and warm on a hot plate to dissolve the solids. Allow the solution to cool to room temperature, transfer it to a 100-mL volumetric flask, and dilute with deionized water to volume.

Solution C: *Solution A*, *Solution B*, and 2% nitric acid to volume (2:1:2). A volume of 5 μ L provides 0.2 mg of phosphate and 0.01 mg of magnesium nitrate.

Blank: Nitric acid and water (1:19)

Standard stock solution A: 0.1372 mg/mL of cadmium nitrate

Standard stock solution B: *Standard stock solution A*, nitric acid, and water (2:1:97). This solution contains 0.10 μ g/mL of cadmium. [NOTE—Before makeup to final volume, dissolve in a portion of water and nitric acid.]

Standard solutions: Dilute *Standard stock solution B* with the *Blank* to obtain concentrations of 0.002, 0.005, 0.010, 0.025, and 0.050 μ g/mL of cadmium.

Sample solution: Prepare as directed for *Sample solution* in the test for *Limit of Arsenic*.

Analysis: Program the graphite furnace as follows. Dry at 120°, using a 1-s ramp, a 55-s hold, and an argon flow of 300 mL/min; char the sample at 850°, using a 1-s ramp, a 30-s hold, and an airflow of 300 mL/min; cool down, and purge the air from the furnace for 10 s, using a 20° set temperature and an argon flow of 300 mL/min; atomize at 2400°, using a 0-s ramp and a 5-s hold with the argon flow stopped; and clean out at 2600° with a 1-s ramp and a 5-s hold. Separately inject equal volumes (20 μ L) of the *Standard solutions*, the *Sample solution*, and the *Blank*, followed by an injection of 5 μ L of *Solution C* for each of the samples, into the graphite tube of a suitable graphite furnace atomic absorption spectrometer equipped with a hollow-cathode lamp for cadmium. Determine the peak area at the cadmium emission line at 228.8 nm, corrected for background absorption. Plot the corrected peak areas of the *Standard solutions* versus their contents of cadmium, in μ g/mL, and calculate the regression line best fitting the points. Determine the concentration, *C*, in μ g/mL, of cadmium in each mL of the *Sample solution* by interpolation from the regression line. Calculate the content of cadmium in the Capsules taken:

$$\text{Result} = (C/W) \times 25$$

C = concentration, as obtained above

W = weight of Capsule content taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1 μ g/g

• LIMIT OF MERCURY

Proceed as directed for *Mercury* <261>, *Method IIa* and *Method IIb*, except use a *Standard Mercury Solution* having the equivalent of 0.1 μ g/mL of mercury.

Sample solution: Prepare as directed for the *Sample solution* in the test for *Limit of Arsenic*, combining the two duplicate cooled digests into 1.0 mL of *Potassium Permanganate Solution*.

Acceptance criteria: NMT 0.1 μ g/g

SPECIFIC TESTS

- **FATS AND FIXED OILS, Anisidine Value <401>:** NMT 20.0, determined on the contents of the Capsules
- **FATS AND FIXED OILS, Free Fatty Acids <401>:** The free fatty acids in 10 g require for neutralization NMT 1.42 mL of 0.1 N sodium hydroxide.
- **FATS AND FIXED OILS, Peroxide Value <401>:** NMT 5.0, determined on the contents of the Capsules
- **FATS AND FIXED OILS, Total Oxidation Value (TOTOX) <401>:** NMT 26 (determined on the contents of the Capsules), calculated as:

$$\text{Result} = (2 \times \text{PV}) + \text{AV}$$

PV = peroxide value

AV = anisidine value

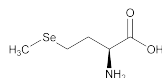
- **FATS AND FIXED OILS, Unsaponifiable Matter <401>:** NMT 4.5%, determined on the contents of the Capsules

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.

- **LABELING:** The label states the content of docosahexaenoic acid in mg/Capsule. It also states the name and concentration of any added antioxidant.
- **USP REFERENCE STANDARDS** (11)
USP Docosahexaenoic Acid Ethyl Ester RS
USP Eicosapentaenoic Acid Ethyl Ester RS
USP Methyl Tricosanoate RS

Selenomethionine



$C_5H_{11}NO_2Se$ 196.11
Butanoic acid, 2-amino-4-(methylseleno)-, (S)-;
(S)-2-Amino-4-(methylselenyl)butyric acid [1464-42-2].

DEFINITION

Selenomethionine contains NLT 97.0% and NMT 103.0% of selenomethionine ($C_5H_{11}NO_2Se$) and contains NLT 39.0% and NMT 41.0% of selenium (Se), calculated on the as-is basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)

ASSAY

• PROCEDURE

Mobile phase: 6.8 g/L of monobasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 2.75 ± 0.25 .

System suitability solution: 0.16 mg/mL of USP Selenomethionine RS and 0.8 mg/mL of USP L-Methionine RS in *Mobile phase*

Standard solution: 0.16 mg/mL of USP Selenomethionine RS in *Mobile phase*

Sample solution: 0.16 mg/mL of Selenomethionine in *Mobile phase* with sonication. Pass through a membrane filter of 0.45- μ m pore size.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 220 nm

Column: 4.6-mm \times 25-cm; packing L1 with polar end-capping

Flow rate: 1 mL/min

Injection size: 20 μ L

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for methionine and selenomethionine are 0.8 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 3.0 between methionine and selenomethionine

Tailing factor: NMT 2.0 for the selenomethionine peak

Relative standard deviation: NMT 2.0% for the selenomethionine peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of selenomethionine ($C_5H_{11}NO_2Se$) in the portion of Selenomethionine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Selenomethionine RS in the *Standard solution* (mg/mL)

C_U = concentration of Selenomethionine in the *Sample solution* (mg/mL)

Acceptance criteria: 97.0%–103.0% on the as-is basis

IMPURITIES

- **HEAVY METALS, Method I** (231): NMT 20 ppm

• LIMIT OF SODIUM

Standard stock solution: 10 μ g/mL of sodium from sodium chloride, previously dried at 105° for 2 h

Standard solutions: 0.2, 0.5, and 1.0 μ g/mL of sodium. Pipet 2.0, 5.0, and 10.0 mL of *Standard stock solution* into separate 100-mL volumetric flasks. To each flask add 2.0 mL of potassium chloride solution (1 in 5) and 1.0 mL of hydrochloric acid, and dilute with water to volume.

Sample solution: Transfer 100 mg of Selenomethionine to a 100-mL volumetric flask, add 2.0 mL of potassium chloride solution (1 in 5) and 1.0 mL of hydrochloric acid, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 589 nm

Lamp: Sodium hollow-cathode

Flame: Air–acetylene

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions. Plot the absorbances of the *Standard solutions* versus their concentrations (μ g/mL of sodium), and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration of sodium, C_{Na} (μ g/mL), in the *Sample solution*.

Calculate the percentage of sodium in the portion of Selenomethionine taken:

$$\text{Result} = (C_{Na}/C_{SA}) \times F \times 100$$

C_{Na} = concentration of sodium in the *Sample solution* (μ g/mL), determined from the regression line

C_{SA} = concentration of Selenomethionine in the *Sample solution* (mg/mL)

F = conversion factor, 0.001 mg/ μ g

Acceptance criteria: NMT 0.1%

• CHROMATOGRAPHIC PURITY

Standard solution A: Transfer 50 mg of USP Selenomethionine RS to a 10-mL volumetric flask, add 2 mL of water, warm to dissolve if necessary, and dilute with methanol to volume.

Standard solution B: Transfer 1.0 mL of *Standard solution A* to a 100-mL volumetric flask, and dilute with methanol to volume.

Sample solution: Transfer 50 mg of Selenomethionine to a 10-mL volumetric flask, add 2 mL of water, warm to dissolve if necessary, and dilute with methanol to volume.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: TLC

Absorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 10 μ L

Developing solvent: Butanol, glacial acetic acid, and water (4:1:1)

Spray reagent: 2 mg/mL of ninhydrin in alcohol

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Proceed as directed for *Chromatography* (621), *Thin-Layer Chromatography*. Allow the spots to dry, and develop the chromatogram in the *Developing solvent* until the solvent front has moved three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Locate the spots on the plate

by spraying with *Spray reagent* and drying it at 110° for 10 min.

Acceptance criteria: NMT 1.0%. The R_f value of the principal spot of the *Sample solution* corresponds to that of *Standard solution A*; and no spot, other than the principal spot of the *Sample solution*, is larger or more intense than the principal spot of *Standard solution B*.

SPECIFIC TESTS

- **OPTICAL ROTATION, Specific Rotation (781S)**
Sample solution: 10 mg/mL in 1 N hydrochloric acid
Acceptance criteria: +17.0° to +19.5°
- **CONTENT OF SELENIUM**
[CAUTION—Selenium is toxic; handle it with care.]
Standard stock solution: Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume. This solution contains 1000 µg/mL of selenium.
Standard solutions: 20, 50, and 100 µg/mL of selenium. Pipet 2.0, 5.0, and 10.0 mL of *Standard stock solution* into separate 100-mL volumetric flasks. Dilute the contents of each flask with water to volume.
Sample solution: 0.125 mg/mL of Selenomethionine in water
Instrumental conditions
 (See *Spectrophotometry and Light-Scattering* (851).)
Mode: Atomic absorption spectrophotometry
Analytical wavelength: 196 nm
Lamp: Selenium hollow-cathode
Flame: Air-acetylene
Blank: Water
Analysis
Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions. Plot the absorbances of the *Standard solutions* versus their concentrations (µg/mL of selenium), and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration of selenium, C_{se} (µg/mL), in the *Sample solution*. Calculate the percentage of selenium in the portion of Selenomethionine taken:

$$\text{Result} = (C_{se}/C_{SA}) \times F \times 100$$

- C_{se} = concentration of selenium in the *Sample solution* (µg/mL), determined from the regression line
 C_{SA} = concentration of Selenomethionine in the *Sample solution* (mg/mL)
 F = conversion factor, 0.001 mg/µg
Acceptance criteria: 39.0%–41.0% on the as-is basis

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS (11)**
 USP L-Methionine RS
 USP Selenomethionine RS

Serine—see *Serine General Monographs*

Siberian Ginseng—see *Eleuthero*

Slippery Elm—see *Elm General Monographs*

Sodium Ascorbate—see *Sodium Ascorbate General Monographs*

Powdered Soy Isoflavones Extract

DEFINITION

Powdered Soy Isoflavones Extract is prepared from the seeds of *Glycine max* Merr. (Fam. Fabaceae) by extraction with water or hydroalcoholic mixtures. It contains NLT 90.0% and NMT 110.0% of the labeled amount of isoflavones, calculated on the dried basis as the sum of daidzin, glycitin, genistin, and one or more of the following isoflavones: malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin, daidzein, glycitein, and genistein.

IDENTIFICATION

- **A. HPLC IDENTIFICATION TEST**
Analysis: Proceed as directed in the test for *Content of Isoflavones*.
Acceptance criteria: The retention times of the daidzin, glycitin, and genistin peaks from the *Sample solution* correspond to those of *Standard solutions A–E*.

COMPOSITION

- **CONTENT OF ISOFLAVONES**
Diluent: Acetonitrile and water (2:3)
Internal standard solution: 2.0 mg/mL USP Apigenin RS in dimethyl sulfoxide. [NOTE—This solution is stable for 6 months when stored in a tightly closed, light-resistant glass container at room temperature.]
System suitability solution 1: Transfer 1 g of USP Defatted Powdered Soy RS to a centrifuge tube, fitted with PTFE or polyethylene-lined screw caps. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane having a 0.45-µm or finer pore size, discarding the first 5 mL of filtrate.
System suitability solution 2: Heat 1 g of USP Defatted Powdered Soy RS in a shallow porcelain dish at 120° for 120 min, and transfer to a centrifuge tube, fitted with PTFE or polyethylene-lined screw caps. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane having a 0.45-µm or finer pore size, discarding the first 5 mL of filtrate.
 [NOTE—*Standard stock solution* and *Standard solutions A–E* are stable for 2 months when stored in a tightly closed, light-resistant glass container at room temperature.]
Standard stock solution: Contains the following in dimethyl sulfoxide: 2.0 mg/mL of USP Daidzin RS, 0.5 mg/mL of USP Glycitin RS, 2.0 mg/mL of USP Genistin RS, 0.2 mg/mL of USP Daidzein RS, 0.2 mg/mL of USP Glycitein RS, and 0.2 mg/mL of USP Genistein RS
Standard solution A: Add 0.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.
Standard solution B: Add 1.0 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution C: Add 1.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution D: Add 2.0 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution E: Add 2.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Sample solution: Transfer a quantity of Powdered Soy Isoflavones Extract, equivalent to NMT 5 mg of isoflavones, to a 30-mL glass centrifuge tube, fitted with a PTFE or polyethylene-lined screw cap. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane having a 0.45- μ m or finer pore size, discarding the first 5 mL of filtrate. [NOTE—Do not use nylon filters. Analyze samples containing significant amounts of acetyl and/or malonyl isoflavones within 4 h of preparation.]

Solution A: 0.05% phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	90	10
60	70	30
60.5	10	90
63.5	10	90
64	90	10
74	90	10

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 260 nm

Column: 3.0-mm \times 25-cm; 5- μ m packing L1

Column temperature: 40°

Flow rate: 0.65 mL/min

Injection size: 5 μ L

[NOTE—*System suitability 1* and *System suitability 2* must both be met.]

System suitability 1

Sample: *Standard solution C*

Suitability requirements

Chromatogram similarity: The chromatogram from *Standard solution C* is similar to the reference chromatogram provided with the lot of USP reference standard being used for the isoflavones mentioned.

Tailing factor: NLT 0.8 and NMT 1.2 for the daidzin peak

Relative standard deviation: NMT 2.0% for the genistin peak

System suitability 2

Samples: *System suitability solution 1* and *System suitability solution 2*

Suitability requirements

Chromatogram similarity: The chromatograms from *System suitability solution 1* and *System suitability solution 2* are similar to those provided with the lot of USP Defatted Powdered Soy RS being used.

Resolution: NLT 1.0 between the acetyl glycitin and malonyl genistin peaks, and NLT 2.0 between any other consecutive pair of isoflavone peaks

Analysis

Samples: *Standard solutions A–E* and *Sample solution*
Measure the peak areas of the analytes and the internal standard. Determine the ratio of the peak areas of each analyte to the internal standard peak area. Plot the ratios of the relevant peak responses versus the concentrations, in mg/mL, of each analyte obtained from the *Standard solutions A–E*, and determine the regression line by least-squares analysis. The correlation coefficient for each of the regression lines is NLT 0.999. From the graphs so obtained, determine the concentration, *C*, in mg/mL, of the relevant analyte in the *Sample solution*. Separately calculate the percentages of daidzin, glycitin, and genistin, and of daidzein, glycitein, and genistein, if present, in the portion of Powdered Soy Isoflavones Extract taken:

$$\text{Result} = (C/W) \times V \times 100$$

C = concentration of each isoflavone as determined above, for the *Sample solution* (mg/mL)

W = weight of Powdered Soy Isoflavones Extract taken to prepare the *Sample solution* (mg)

V = final solution volume of the *Sample solution* (mL)

Identify the peaks of malonyl daidzin, malonyl glycitin, acetyl daidzin, acetyl glycitin, malonyl genistin, and acetyl genistin by comparison with the reference chromatograms provided with USP Defatted Powdered Soy RS and by the difference in peak abundance between *System suitability solution 1* and *System suitability solution 2*. [NOTE—Malonyl derivatives are converted into acetyl derivatives by heat. Malonyl derivatives are more abundant in *System suitability solution 1* and acetyl derivatives are more abundant in *System suitability solution 2*.] From the graphs obtained for daidzin, glycitin, and genistin, determine the corresponding concentration, *C*, in mg/mL, of the malonyl and acetyl derivatives, if present, in the *Sample solution*.

Separately calculate the percentages of malonyl daidzin, acetyl daidzin, malonyl glycitin, acetyl glycitin, malonyl genistin, and acetyl genistin in the portion of Powdered Soy Isoflavones Extract taken:

$$\text{Result} = (C/W) \times V \times F \times 100$$

C = concentration of each isoflavone as determined above, for the *Sample solution* (mg/mL)

W = weight of Powdered Soy Isoflavones Extract taken to prepare the *Sample solution* (mg)

V = final solution volume of the *Sample solution* (mL)

F = conversion factor: malonyl daidzin, 1.207; acetyl daidzin, 1.101; malonyl glycitin, 1.193; acetyl glycitin, 1.094; malonyl genistin, 1.199; and acetyl genistin, 1.097

Calculate the percentage of isoflavones in the portion of the Powdered Soy Isoflavones Extract taken (*P*) by adding the percentages calculated for all analytes present.

Calculate the percentage of the labeled amount of isoflavones in the portion of the Powdered Soy Isoflavones Extract taken:

$$\text{Result} = (P/L) \times 100$$

P = sum of the individual percentages of each isoflavone in the Powdered Soy Isoflavones Extract taken, as calculated above (%)

L = labeled amount of isoflavones in the Powdered Soy Isoflavones Extract (%)

Acceptance criteria: 90.0%–110.0% on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method II* (231): NMT 10 µg/g
- **ARTICLES OF BOTANICAL ORIGIN**, *Test for Aflatoxins* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count is NMT 10⁴ cfu/g, and the total combined molds and yeasts count is NMT 10³ cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **LOSS ON DRYING** (731): Dry 1 g at 130° for 2 h: it loses NMT 7.0% of its weight.
- **OTHER REQUIREMENTS**: It meets the requirements for *Residual Solvents* and *Pesticide Residues* in *Botanical Extracts* (565).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight, light-resistant containers, and store at controlled room temperature.
- **LABELING**: The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. The label also indicates the content of isoflavones. It meets other labeling requirements in *Botanical Extracts* (565).
- **USP REFERENCE STANDARDS** (11)
 - USP Apigenin RS
 - USP Daidzein RS
 - USP Daidzin RS
 - USP Defatted Powdered Soy RS
 - USP Genistein RS
 - USP Genistin RS
 - USP Glycitein RS
 - USP Glycitin RS

Soy Isoflavones Capsules

DEFINITION

Soy Isoflavones Capsules contain Powdered Soy Isoflavones Extract. Capsules contain NLT 90.0% and NMT 110.0% of the labeled amount of Extract, represented by the sum of the content of the isoflavones daidzin, glycitin, genistin, and one or more of the following isoflavones: malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin, daidzein, glycitein, and genistein.

IDENTIFICATION

- The retention times of the daidzin, glycitin, and genistin peaks in the chromatogram of the *Sample solution* correspond to those of *Standard solutions A–E*, as obtained in the test for *Content of Isoflavones*.

STRENGTH

• CONTENT OF ISOFLAVONES

Diluent: Acetonitrile and water (2:3)

Internal standard solution: 2.0 mg/mL of USP Apigenin RS in dimethyl sulfoxide. [NOTE—This solution is stable for 6 months when stored in a tightly closed, light-resistant glass container at room temperature.]

Malonyl/Acetyl isoflavones retention times check solution 1: Heat 1 g of USP Defatted Powdered Soy RS in a shallow porcelain dish at 120° for 120 min, and transfer to a centrifuge tube, fitted with PTFE or polyethylene-lined screw cap. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, mix, and centrifuge. Pass a portion of the supernatant through a hydrophilic pro-

pylene or PVDF membrane of 0.45-µm or finer pore size, discarding the first 5 mL of filtrate.

Malonyl/Acetyl isoflavones retention times check solution 2: Transfer 1 g of USP Defatted Powdered Soy RS to a centrifuge tube, fitted with PTFE or polyethylene-lined screw cap. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, mix, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane of 0.45-µm or finer pore size, discarding the first 5 mL of filtrate.

[NOTE—All *Standard solutions* and *Standard stock solutions* are stable for 2 months when stored in a tightly closed, light-resistant glass container at room temperature.]

Standard stock solution: Contains the following in dimethyl sulfoxide: 2.0 mg/mL of USP Daidzin RS, 0.5 mg/mL of USP Glycitin RS, and 2.0 mg/mL of USP Genistin RS, 0.2 mg/mL of USP Daidzein RS, 0.2 mg/mL of USP Glycitein RS, and 0.2 mg/mL of USP Genistein RS

Standard solution A: Add 0.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution B: Add 1.0 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution C: Add 1.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution D: Add 2.0 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution E: Add 2.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Sample solution

Hard-gelatin Capsules: Weigh and finely powder the content of NLT 20 Capsules. Transfer an accurately weighed quantity of the powder, equivalent to NMT 5 mg of isoflavones, to a suitable glass centrifuge tube, fitted with PTFE or polyethylene-lined screw cap.

Soft-gelatin Capsules: Weigh and homogenize NLT 20 Capsules. Transfer an accurately weighed quantity of the mixture, equivalent to NMT 5 mg of isoflavones, to a suitable glass centrifuge tube, fitted with PTFE or polyethylene-lined screw cap.

Add the following in exact volumes to the centrifuge tube: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, mix, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane of 0.45-µm or finer pore size, discarding the first 5 mL of filtrate. [NOTE—Do not use nylon filters. Analyze samples containing significant amounts of acetyl and/or malonyl isoflavones within 4 h of preparation.]

Solution A: 0.05% phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	90	10
60	70	30
60.5	10	90

Time (min)	Solution A (%)	Solution B (%)
63.5	10	90
64	90	10
74	90	10

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 260 nm**Column:** 3.0-mm × 25-cm; 5-μm packing L1**Column temperature:** 40°**Flow rate:** 0.65 mL/min**Injection size:** 5 μL**System suitability****Samples:** *Standard solution C*, *Malonyl/Acetyl isoflavones retention times check solution 1*, and *Malonyl/Acetyl isoflavones retention times check solution 2***Suitability requirements****Chromatogram similarity:** The chromatograms obtained from *Standard solution C*, *Malonyl/Acetyl isoflavones retention times check solution 1*, and *Malonyl/Acetyl isoflavones retention times check solution 2* are similar to the Reference chromatograms provided with the USP Defatted Powdered Soy RS.**Tailing factor:** NLT 0.8 and NMT 1.2 for the daidzin peak, *Standard solution C***Relative standard deviation:** NMT 2.0%, for the genistin peak, in repeated injections, *Standard solution C***Resolution:** NLT 1.0 between acetyl glycitin and malonyl genistin, and NLT 2.0 between any other consecutive pair of isoflavone peaks, *Malonyl/Acetyl isoflavones retention times check solution 2***Analysis****Samples:** *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, *Standard solution E*, *Malonyl/Acetyl isoflavones retention times check solution 1*, *Malonyl/Acetyl isoflavones retention times check solution 2*, and *Sample solution*

Identify the peaks of daidzin, glycitin, genistin, daidzein, glycitein, and genistein in the chromatograms of *Standard solutions A–E* by comparison with the Reference Standard Chromatogram provided with the appropriate USP Reference Standard used. Measure the peak areas of the analytes and the internal standard peaks. Determine the ratio of the peak areas of each analyte to the internal standard peak areas. Plot the ratios of the relevant peak responses versus the concentrations, in mg/mL, of each analyte obtained from *Standard solutions A–E*, and determine the regression line by least-squares analysis. The correlation coefficient for each of the regression lines is NLT 0.999. From the graphs obtained, determine the concentration, C_o , in mg/mL, of daidzin, glycitin, and genistin, in addition to daidzein, glycitein, and genistein, if present, in the *Sample solution*. Identify the peaks of malonyl daidzin, malonyl glycitin, acetyl daidzin, acetyl glycitin, malonyl genistin, and acetyl genistin in the chromatograms of the *Malonyl/Acetyl isoflavones retention times check solutions* by comparison with the Reference Chromatograms provided with the USP Defatted Powdered Soy RS. From the graphs obtained for daidzin, glycitin and genistin, determine the concentration, C_o , in mg/mL, of the corresponding malonyl and acetyl derivatives, if present, in the *Sample solution*:

$$\text{Result} = C_o \times F$$

C_o = concentration obtained from the relevant graph (mg/mL)

F = conversion factor for each analyte (1.207 for malonyl daidzin, 1.101 for acetyl daidzin, 1.193 for malonyl glycitin, 1.094 for acetyl glycitin, 1.199 for malonyl genistin, and 1.097 for acetyl genistin)

Calculate the quantity, T , in mg, of total isoflavones in the portion of Capsules contents taken:

$$\text{Result} = V \times C_T$$

V = final volume of the *Sample solution* (mL)

C_T = sum of concentrations (C) (mg/mL) of all relevant isoflavones

Calculate the percentage of Powdered Soy Isoflavones Extract with respect to the label claim:

$$\text{Result} = T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

T = content of total isoflavones in the portion of Capsules contents taken (mg)

A_{WT} = average weight of Capsules contents (mg/Capsule)

W = weight of the portion of Capsules contents taken (mg)

L_E = content of total isoflavones, mg, in 100 mg of the Extract used to prepare the Capsules

L = amount of Extract per Capsule according to label claim (mg/Capsule)

Acceptance criteria: NLT 90.0% and NMT 110.0% of the labeled amount of Extract, represented by the sum of the content of the isoflavones daidzin, glycitin, genistin and one or more of the following isoflavones: malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin, daidzein, glycitein, and genistein

PERFORMANCE TESTS

- DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS**

- <2040>:** Meet the requirements for *Disintegration*

- WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>:** Meet the requirements

CONTAMINANTS

- MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic microbial count is NMT 10^4 cfu/g; and the total combined molds and yeasts count is NMT 10^3 cfu/g.

- ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** Capsules meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.

- LABELING:** The label states the Latin binomial and, following the official name, the article from which the Capsules were prepared. Label it to indicate the amount of Extract, in mg, per Capsule. Label it to indicate the percentage of isoflavones in the Extract used to prepare the Capsules.

- USP REFERENCE STANDARDS <11>**

- USP Apigenin RS
- USP Daidzein RS
- USP Daidzin RS
- USP Defatted Powdered Soy RS
- USP Genistein RS
- USP Genistin RS
- USP Glycitein RS
- USP Glycitin RS

Soy Isoflavones Tablets

DEFINITION

Soy Isoflavones Tablets contain Powdered Soy Isoflavones Extract. Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of Extract, represented by the sum of the content of the isoflavones daidzin, glycitin, genistin and one or more of the following isoflavones: malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin, daidzein, glycitein, and genistein.

IDENTIFICATION

- The retention times of the daidzin, glycitin, and genistin peaks in the chromatogram of the *Sample solution* correspond to those of *Standard solutions A–E*, as obtained in the test for *Content of Isoflavones*.

STRENGTH

• CONTENT OF ISOFLAVONES

Diluent: Acetonitrile and water (2:3)

Internal standard solution: 2.0 mg/mL of USP

Apigenin RS in dimethyl sulfoxide. [NOTE—This solution is stable for 6 months when stored in a tightly closed, light-resistant glass container at room temperature.]

Malonyl/Acetyl isoflavones retention times check solution 1: Heat 1 g of USP Defatted Powdered Soy RS in a shallow porcelain dish at 120° for 120 min, and transfer to a centrifuge tube, fitted with PTFE or polyethylene-lined screw cap. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, mix, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane of 0.45-μm or finer pore size, discarding the first 5 mL of filtrate.

Malonyl/Acetyl isoflavones retention times check solution 2: Transfer 1 g of USP Defatted Powdered Soy RS to a centrifuge tube, fitted with PTFE or polyethylene-lined screw cap. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, mix, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane of 0.45-μm or finer pore size, discarding the first 5 mL of filtrate.

[NOTE—All *Standard solutions* and *Standard stock solutions* are stable for 2 months when stored in a tightly closed, light-resistant glass container at room temperature.]

Standard stock solution: Contains the following in dimethyl sulfoxide: 2.0 mg/mL of USP Daidzin RS, 0.5 mg/mL of USP Glycitin RS, 2.0 mg/mL of USP Genistin RS, 0.2 mg/mL of USP Daidzein RS, 0.2 mg/mL of USP Glycitein RS, and 0.2 mg/mL of USP Genistein RS

Standard solution A: Add 0.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution B: Add 1.0 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution C: Add 1.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution D: Add 2.0 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Standard solution E: Add 2.5 mL of *Standard stock solution* and 0.5 mL of *Internal standard solution* to a 25-mL volumetric flask, and dilute with *Diluent* to volume.

Sample solution: Accurately weigh and finely powder NLT 20 Tablets. Transfer an accurately weighed quantity of the powder, equivalent to NMT 5 mg of isoflavones, to a 30-mL glass centrifuge tube, fitted with a PTFE or polyethylene-lined screw cap. Add the following in exact volumes: 0.5 mL of *Internal standard solution*, 10 mL of acetonitrile (swirl to disperse), and 6.0 mL of water. Cap, shake on an orbital or wrist-action shaker for 60 min, add 8.5 mL of water, mix, and centrifuge. Pass a portion of the supernatant through a hydrophilic propylene or PVDF membrane of 0.45-μm or finer pore size, discarding the first 5 mL of filtrate. [NOTE—Do not use nylon filters. Analyze samples containing significant amounts of acetyl and/or malonyl isoflavones within 4 h of preparation.]

Solution A: 0.05% phosphoric acid in water

Solution B: Acetonitrile

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	90	10
60	70	30
60.5	10	90
63.5	10	90
64	90	10
74	90	10

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 260 nm

Column: 3.0-mm × 25-cm; 5-μm packing L1

Column temperature: 40°

Flow rate: 0.65 mL/min

Injection size: 5 μL

System suitability

Samples: *Standard solution C*, *Malonyl/Acetyl isoflavones retention times check solution 1*, and *Malonyl/Acetyl isoflavones retention times check solution 2*

Suitability requirements

Chromatogram similarity: The chromatograms obtained from *Standard solution C*, *Malonyl/Acetyl isoflavones retention times check solution 1*, and *Malonyl/Acetyl isoflavones retention times check solution 2* are similar to the Reference Chromatograms provided with the USP Defatted Powdered Soy RS.

Tailing factor: NLT 0.8 and NMT 1.2 for the daidzin peak, *Standard solution C*

Relative standard deviation: NMT 2.0%, for the genistin peak, in repeated injections, *Standard solution C*

Resolution: NLT 1.0 between acetyl glycitin and malonyl genistin, and NLT 2.0 between any other consecutive pair of isoflavone peaks, *Malonyl/Acetyl isoflavones retention times check solution 2*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, *Standard solution E*, *Malonyl/Acetyl isoflavones retention times check solution 1*, *Malonyl/Acetyl isoflavones retention times check solution 2*, and *Sample solution*

Identify the peaks of daidzin, glycitin, genistin, daidzein, glycitein, and genistein in the chromatograms of *Standard solutions A–E* by comparison with the Reference Standard Chromatogram provided with the appropriate USP Reference Standard used. Measure the peak areas of the analytes and the internal standard peaks. Determine the ratio of the peak areas of each analyte

to the internal standard peak areas. Plot the ratios of the relevant peak responses versus the concentrations, in mg/mL, of each analyte obtained from *Standard solutions A–E*, and determine the regression line by least-squares analysis. The correlation coefficient for each of the regression lines is NLT 0.999. From the graphs obtained, determine the concentration, *C*, in mg/mL, of daidzin, glycitin, and genistin, in addition to daidzein, glycitein, and genistein, if present, in the *Sample solution*. Identify the peaks of malonyl daidzin, malonyl glycitin, acetyl daidzin, acetyl glycitin, malonyl genistin, and acetyl genistin in the chromatograms of the *Malonyl/Acetyl isoflavones retention times check solutions* by comparison with the Reference Chromatograms provided with the USP Defatted Powdered Soy RS. From the graphs obtained for daidzin, glycitin, and genistin, determine the concentration, *C*, in mg/mL, of the corresponding malonyl and acetyl derivatives, if present, in the *Sample solution*:

$$\text{Result} = C_o \times F$$

- C_o* = concentration obtained from the relevant graph (mg/mL)
F = conversion factor for each analyte (1.207 for malonyl daidzin, 1.101 for acetyl daidzin, 1.193 for malonyl glycitin, 1.094 for acetyl glycitin, 1.199 for malonyl genistin, and 1.097 for acetyl genistin)

Calculate the quantity, *T*, in mg, of total isoflavones in the portion of Tablets taken:

$$\text{Result} = V \times C_T$$

- V* = final volume of the *Sample solution* (mL)
C_T = sum of concentrations (*C*) (mg/mL) of all relevant isoflavones

Calculate the percentage of Powdered Soy Isoflavones Extract with respect to the label claim:

$$\text{Result} = T \times (A_{WT}/W) \times (100/L_E) \times (100/L)$$

- T* = content of total isoflavones in the portion of Tablets taken (mg)
A_{WT} = average weight of Tablets (mg/Tablet)
W = weight of the portion of Tablets taken (mg)
L_E = content of total isoflavones, mg, in 100 mg of the Extract used to prepare the Tablets
L = amount of Extract per Tablet according to label claim (mg/Tablet)

Acceptance criteria: NLT 90.0% and NMT 110.0% of the labeled amount of Extract, represented by the sum of the content of the isoflavones daidzin, glycitin, genistin and one or more of the following isoflavones: malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin, daidzein, glycitein, and genistein

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS <2040>:** Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic microbial count is NMT 10⁴ cfu/g; and the total combined molds and yeasts count is NMT 10³ cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** Tablets meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.

- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. Label it to indicate the amount of Extract, in mg, per Tablet. Label it to indicate the content, in percentage, of isoflavones in the Extract used to prepare the Tablets.

- **USP REFERENCE STANDARDS <11>**
 - USP Apigenin RS
 - USP Daidzein RS
 - USP Daidzin RS
 - USP Defatted Powdered Soy RS
 - USP Genistein RS
 - USP Genistin RS
 - USP Glycitein RS
 - USP Glycitin RS

Stinging Nettle

DEFINITION

Stinging Nettle consists of dried roots and rhizomes of *Urtica dioica* L. subsp. *dioica* (Fam. Urticaceae), and may contain *Urtica urens* L., known in commerce as dwarf nettle, as a minor component. It contains NLT 0.8% of total amino acids, NLT 0.05% of β-sitosterol (C₂₉H₅₀O), and NLT 3 μg/g of scopoletin (C₁₀H₈O₄), calculated on the dried basis.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 0.05 mg/mL of USP Scopoletin RS and 0.5 mg/mL of USP β-Sitosterol RS in methanol

Sample solution: Extract 1 g of powder by refluxing with 10 mL of a solution containing toluene, ethyl acetate, and methanol (7:2:1) for 15 min, cool, and filter. Evaporate the filtrate to dryness under reduced pressure at less than 40°, and dissolve the residue in 2 mL of the mixture containing toluene, ethyl acetate, and methanol.

Chromatographic system

(See *Chromatography <621>*, *Thin-Layer Chromatography*.)

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 20 μL for the *Sample solution*; 10 μL for the *Standard solution*

Developing solvent system: Diethyl ether and methanol (9:1)

Spray reagent: 85% phosphoric acid, 10% vanillin in 96% ethanol, and water (4.5: 1: 4.5)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the plate and examine under UV light at 365 nm. Spray the plate with *Spray reagent*, heat between 100° and 105° for 10 min, and examine under daylight.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a violet-red zone corresponding to β-sitosterol at the same *R_f* value as β-sitosterol in the *Standard solution*, weakly visible zones above and below β-sitosterol, and a violet-red zone corresponding to β-sitosterolglucoside.

COMPOSITION

• CONTENT OF β-SITOSTEROL

Derivatizing reagent: A solution containing equal volumes (1:1:1) of BSTFA [N,O-bis(trimethylsilyl)tri-fluoroacetamide], anhydrous pyridine, and a mixture of BSA [N,O-(trimethylsilyl)acetamide], TMSI (N-trimethylsilylimidazole), and TMCS (trimethylchlorosilane) (3:3:2)

Internal standard solution: 10 mg/mL of cholesterol in chloroform

Standard solution: Dissolve 50.0 mg of USP β-sitosterol RS in 2 mL of chloroform, add 1.0 mL of *Internal stan-*

dard solution, and dilute with chloroform to 5 mL. Dry 0.5 mL under reduced pressure, and add 1 mL of *Derivatizing reagent*.

Sample solution: Transfer 50.0 g of finely powdered Stinging Nettle to a Soxhlet apparatus, add chloroform, and extract for 6 h. The volume of chloroform used is at least twice the volume of the thimble with an appropriately sized flask. Dry the solvent under reduced pressure, add 1.0 mL of *Internal standard solution*, and dilute with chloroform to 10 mL. Transfer 0.5 mL of this solution to a 10-mL round-bottom flask, dry the solvent under reduced pressure, and add 0.5 mL of *Derivatizing reagent*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.20-mm × 25-m fused-silica capillary; 0.35-μm film of phase G2 coating

Temperature

Injector: 325°

Detector: 325°

Column: 300°, for 60 min

Carrier gas: Helium

Flow rate: 0.5 mL/min

Injection size: 1 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for each sterol peak

Relative standard deviation: NMT 5.0% determined from each sterol peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of β-sitosterol in the portion of Stinging Nettle taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = peak response ratio of β-sitosterol to the internal standard from the *Sample solution*

R_S = peak response ratio of β-sitosterol to the internal standard from the *Standard solution*

W_S = weight of USP β-sitosterol RS used to prepare the *Standard solution* (mg)

W_U = weight of Stinging Nettle taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.05% on the dried basis

• CONTENT OF SCOPOLETIN

Solution A: Water

Solution B: Methanol

Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
2	60	40
8	60	40
10	0	100
15	0	100
20	75	25
30	75	25

Standard solution: 0.02 μg/mL of USP Scopoletin RS in methanol

Sample stock solution: Extract 160 mg of finely powdered Stinging Nettle in each mL of methanol. Place in an ultrasonic bath for 25 min, and centrifuge.

Sample solution: *Sample stock solution* diluted with methanol 1 in 20

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Fluorescence detector; set at an excitation wavelength of 366 nm and an emission wavelength of 420 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Capacity factor (k'): NLT 5 determined from the scopoletin peak

Tailing factor: NMT 2.0 for the scopoletin peak

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for scopoletin.

Calculate the content of scopoletin (C₁₀H₈O₄), in μg/g, in the portion of Stinging Nettle taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D$$

r_U = peak area of scopoletin from the *Sample solution*

r_S = peak area of scopoletin from the *Standard solution*

C_S = concentration of USP Scopoletin RS in the *Standard solution* (μg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Stinging Nettle taken to prepare the *Sample solution* (g)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*

Acceptance criteria: NLT 3 μg/g on the dried basis

• CONTENT OF TOTAL AMINO ACIDS

Buffer: Mix 5.40 g of anhydrous sodium acetate, 0.3 mL of glacial acetic acid, and water to a final volume of 100 mL. Adjust to pH 5.5.

Reagent solution: Solution containing 1.00 g of ninhydrin, 1.50 g of hydrindantin, and 37.5 mL of propylene glycol. Adjust with *Buffer* to 50.0 mL. [NOTE—Prepare the *Reagent solution* daily.]

Standard solution: 20 μg/mL each of USP Glutamic Acid RS and USP Aspartic Acid RS in water

Sample solution: Finely powder Stinging Nettle, and transfer 1.0 g of it to 80 mL of water. Place in an ultrasonic bath for 25 min, and centrifuge. Transfer the supernatant to a 100-mL volumetric flask, dilute with water to volume, and filter.

Blank: Water

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Visible

Analytical wavelength: 570 nm

Cell: 1 cm

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank*

Transfer 5.0 mL of the *Standard solution*, *Sample solution*, and *Blank* to separate 50-mL volumetric flasks. Add 5.0 mL of *Reagent solution* to each flask. Heat in a boiling water bath for 30 min, cool, and adjust with a mixture of ethanol and water (1:1) to volume.

Calculate the percentage of total amino acids in the portion of Stinging Nettle taken:

$$\text{Result} = (A_U/A_S) \times C_S \times (V/W) \times F \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

C_S = sum of the concentration of USP Glutamic Acid RS and USP Aspartic Acid RS in the *Standard solution* (μg/mL)

V = volume of the *Sample solution*, 100 mL

- W = amount of Stinging Nettle taken to prepare the *Sample solution* (mg)
 F = unit conversion factor, 0.001 mg/μg
 Acceptance criteria: NLT 0.8% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues (561):** Meets the requirements
- **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic microbial count does not exceed 10^6 cfu/g, the total combined molds and yeasts count does not exceed 10^4 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS• **BOTANIC CHARACTERISTICS**

Macroscopic: The rhizome is irregularly bent, about 3–10 mm thick, and light gray-brown on the outside; thin roots spring from the knotty bulges of a lengthwise furrow. A transverse cut of the rhizome shows it is fibrous, light yellowish white, and usually has a small medulla cave. The roots are often very long, usually 0.5–2 mm thick, light yellow-brown on the outside, and contain some deep longitudinal furrows; a transverse cut shows a pale and almost pure-white color.

Microscopic: The transverse section of the rhizome and root shows the following characteristics. The rhizome has a narrow cork composed of brown, thin-walled cells, a few rows of tangentially elongated cortical parenchyma, and a pericyclic region with numerous fibers occurring singly or, more frequently, in small groups. Fibers are much elongated with very thick and lignified walls. Some cells of the pericycle and outer part of secondary phloem contain large globular compound crystals of calcium oxalate. The vascular cambial region is distinct and continuous with narrow radial groups of vascular tissue separated by wide medullary rays. The secondary phloem is mainly parenchymatous with groups of thin-walled sieve tissue. The xylem is dense and completely lignified, containing scattered vessels, isolated or in small groups, associated with moderately thickened xylem parenchyma cells and numerous thicker-walled xylem fibers with slit-shaped pits. Individual vessels have fairly large, closely arranged, bordered pits, while the adjacent parenchyma has simple or bordered pits. Medullary rays indicate alternating areas of lignified and unlignified cells, appearing as tangential bands between the vascular bundles, each composed of 5 or 6 layers of cells; the lignified cells have moderately thickened walls with simple pits. The pith is composed of rounded, unlignified parenchyma, collapsed in the central part to form a cavity. Mature roots show a thin cork, narrow phelloderm, and secondary phloem and xylem with alternating areas of lignified and unlignified parenchyma in the wide medullary rays, similar to that found in the rhizome.

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter (561):** NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 10%
- **LOSS ON DRYING (731)** Dry 1.0 g of Stinging Nettle, finely powdered, at 105° for 2 h: it loses NMT 12.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. Store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.

• **USP REFERENCE STANDARDS (11)**

USP Aspartic Acid RS
 USP Glutamic Acid RS
 USP Scopoletin RS
 USP β -Sitosterol RS

Powdered Stinging Nettle**DEFINITION**

Powdered Stinging Nettle is Stinging Nettle reduced to a fine or very fine powder. It contains NLT 0.8% of total amino acids, NLT 0.05% of β -sitosterol ($C_{29}H_{50}O$), and NLT 3 μg/g of scopoletin ($C_{10}H_8O_4$), calculated on the dried basis.

IDENTIFICATION• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 0.05 mg/mL of USP Scopoletin RS and 0.5 mg/mL of USP β -Sitosterol RS in methanol

Sample solution: Extract 1 g of Powdered Stinging Nettle by refluxing with 10 mL of a solution containing toluene, ethyl acetate, and methanol (7:2:1) for 15 min, cool, and filter. Evaporate the filtrate to dryness under reduced pressure at less than 40°, and dissolve the residue in 2 mL of the mixture containing toluene, ethyl acetate, and methanol.

Chromatographic system

(See *Chromatography (621)*, *Thin-Layer Chromatography*.)

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 20 μL for the *Sample solution*; 10 μL for the *Standard solution*

Developing solvent system: Diethyl ether and methanol (9:1)

Spray reagent: 85% phosphoric acid, 10% vanillin in 96% ethanol, and water (4.5: 1: 4.5)

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the plate and examine under UV light at 365 nm. Spray the plate with *Spray reagent*, heat between 100° and 105° for 10 min, and examine under daylight.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a violet-red zone corresponding to β -sitosterol at the same R_f value as β -sitosterol of the *Standard solution*, weakly visible zones above and below β -sitosterol, and a violet-red zone corresponding to β -sitosterolglucoside.

COMPOSITION• **CONTENT OF β -SITOSTEROL**

Derivatizing reagent: A solution containing equal volumes (1:1:1) of BSTFA [*N,O*-bis(trimethylsilyl)tri-fluoroacetamide], anhydrous pyridine, and a mixture of BSA [*N,O*-(trimethylsilyl)acetamide], TMSI (*N*-trimethylsilylimidazole), and TMCS (trimethylchlorosilane) (3:3:2)

Internal standard solution: 10 mg/mL of cholesterol in chloroform

Standard solution: Dissolve 50.0 mg of USP β -sitosterol RS in 2 mL of chloroform, add 1.0 mL of *Internal standard solution*, and dilute to 5 mL with chloroform. Dry 0.5 mL under reduced pressure, and add 1 mL of *Derivatizing reagent*.

Sample solution: Transfer 50.0 g of Powdered Stinging Nettle to a Soxhlet apparatus, add chloroform, and extract for 6 h. The volume of chloroform used is at least twice the volume of the thimble with an appropriately sized flask. Dry the solvent under reduced pressure, add 1.0 mL of *Internal standard solution*, and dilute with chloroform to 10 mL. Transfer 0.5 mL of this solution to a 10-mL round-bottom flask, dry the solvent under reduced pressure, and add 0.5 mL of *Derivatizing reagent*.

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** GC**Detector:** Flame ionization**Column:** 0.20-mm × 25-m fused-silica capillary; 0.35- μ m film of phase G2 coating**Temperature****Injector:** 325°**Detector:** 325°**Column:** 300°, for 60 min**Carrier gas:** Helium**Flow rate:** 0.5 mL/min**Injection size:** 1 μ L**System suitability****Sample:** *Standard solution***Suitability requirements****Tailing factor:** NMT 2.0 for each sterol peak**Relative standard deviation:** NMT 5.0% determined from each sterol peak**Analysis****Samples:** *Standard solution* and *Sample solution*Calculate the percentage of β -sitosterol in the portion of Powdered Stinging Nettle taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

 R_U = peak response ratio of β -sitosterol to the internal standard from the *Sample solution* R_S = peak response ratio of β -sitosterol to the internal standard from the *Standard solution* W_S = weight of USP β -sitosterol RS used to prepare the *Standard solution* (mg) W_U = weight of Powdered Stinging Nettle taken to prepare the *Sample solution* (mg)**Acceptance criteria:** NLT 0.05% on the dried basis• **CONTENT OF SCOPOLETIN****Solution A:** Water**Solution B:** Methanol**Mobile phase:** See *Table 1*.**Table 1**

Time (min)	Solution A (%)	Solution B (%)
0	75	25
2	60	40
8	60	40
10	0	100
15	0	100
20	75	25
30	75	25

Standard solution: 0.02 μ g/mL of USP Scopoletin RS in methanol**Sample stock solution:** 160 mg of Powdered Stinging Nettle in each mL of methanol. Place in an ultrasonic bath for 25 min, and centrifuge.**Sample solution:** *Sample stock solution* diluted with methanol 1 in 20**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** Fluorescence detector; set at an excitation wavelength of 366 nm and an emission wavelength of 420 nm**Column:** 4.6-mm × 25-cm; packing L1**Flow rate:** 1 mL/min**Injection size:** 10 μ L**System suitability****Sample:** *Standard solution***Suitability requirements****Capacity factor (k'):** NLT 5 determined from the scopoletin peak**Tailing factor:** NMT 2.0 for the scopoletin peak**Relative standard deviation:** NMT 5.0%**Analysis****Samples:** *Standard solution* and *Sample solution*

Measure the peak responses for scopoletin.

Calculate the content of scopoletin ($C_{10}H_8O_4$), in μ g/g, in the portion of Powdered Stinging Nettle taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D$$

 r_U = peak area of scopoletin from the *Sample solution* r_S = peak area of scopoletin from the *Standard solution* C_S = concentration of USP Scopoletin RS in the *Standard solution* (μ g/mL) V = volume of the *Sample stock solution* (mL) W = weight of Powdered Stinging Nettle taken to prepare the *Sample stock solution* (g) D = dilution factor to prepare the *Sample solution* from the *Sample stock solution***Acceptance criteria:** NLT 3 μ g/g on the dried basis• **CONTENT OF TOTAL AMINO ACIDS****Buffer:** Mix 5.40 g of anhydrous sodium acetate, 0.3 mL of glacial acetic acid, and water to a final volume of 100 mL. Adjust to pH 5.5.**Reagent solution:** Solution containing 1.00 g of ninhydrin, 1.50 g of hydrindantin, and 37.5 mL of propylene glycol. Adjust with *Buffer* to 50.0 mL. [NOTE—Prepare the *Reagent solution* daily.]**Standard solution:** 20 μ g/mL each of USP Glutamic Acid RS and USP Aspartic Acid RS, in water**Sample solution:** Transfer 1.0 g of Powdered Stinging Nettle to 80 mL of water. Place in an ultrasonic bath for 25 min, and centrifuge. Transfer the supernatant to a 100-mL volumetric flask, dilute with water to volume, and filter.**Blank:** Water**Instrumental conditions**(See *Spectrophotometry and Light-Scattering* <851>.)**Mode:** Visible**Analytical wavelength:** 570 nm**Cell:** 1 cm**Analysis****Samples:** *Standard solution*, *Sample solution*, and *Blank*Transfer 5.0 mL of the *Standard solution*, *Sample solution*, and *Blank* to separate 50-mL volumetric flasks. Add 5.0 mL of *Reagent solution* to each flask. Heat in a boiling water bath for 30 min, cool, and adjust with a mixture of ethanol and water (1:1) to volume.

Calculate the percentage of total amino acids in the portion of Powdered Stinging Nettle taken:

$$\text{Result} = (A_U/A_S) \times C_S \times (V/W) \times F \times 100$$

 A_U = absorbance from the *Sample solution* A_S = absorbance from the *Standard solution* C_S = sum of the concentration of USP Glutamic Acid RS and USP Aspartic Acid RS in the *Standard solution* (μ g/mL) V = volume of the *Sample solution*, 100 mL W = amount of Powdered Stinging Nettle taken to prepare the *Sample solution* (mg) F = unit conversion factor, 0.001 mg/ μ g**Acceptance criteria:** NLT 0.8% on the dried basis**CONTAMINANTS**• **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues <561>:**

Meets the requirements

• **MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic microbial count does not exceed 10^6 cfu/g, the total combined molds and yeasts count does not exceed 10^4 cfu/g, and the bile-tolerant Gram-negative bacteria count does not exceed 10^3 cfu/g.

- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: The powder is yellowish to brownish gray.

Microscopic: Under a microscope using chloral hydrate solution, it shows several fragments of net and pitted vessels with a strongly varying diameter, usually 50–150 μm . Few, if any, pitted bast fibers are present. Clearly pitted wood fibers, 200–800 μm in length, and thin-walled parenchyma cells, sometimes containing compounded calcium oxalate crystals and occasionally with single crystals and fragments of cork consisting of flat-shaped, thin-walled cells, are also present.

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter (561):** NMT 2.0%

- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 10%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and excessive heat.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS (11)**
 - USP Aspartic Acid RS
 - USP Glutamic Acid RS
 - USP Scopoletin RS
 - USP β -Sitosterol RS

Powdered Stinging Nettle Extract

DEFINITION

Powdered Stinging Nettle Extract is prepared from comminuted Stinging Nettle with 60% alcohol or other suitable solvents. It contains NLT 5.0% of total amino acids, NLT 0.1% of β -sitosterol ($\text{C}_{29}\text{H}_{50}\text{O}$), and NLT 30 $\mu\text{g/g}$ of scopoletin ($\text{C}_{10}\text{H}_8\text{O}_4$). The ratio of the starting crude plant material to Powdered Extract is 10:1.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 0.05 mg/mL of USP Scopoletin RS and 0.5 mg/mL of USP β -Sitosterol RS in methanol

Sample solution: Dissolve 0.6 g of Powdered Extract, in a mixture of toluene, ethyl acetate, and methanol (7:2:1). Filter, and dry under reduced pressure at a temperature below 40°. Dissolve the residue in 2.0 mL of the mixture containing toluene, ethyl acetate, and methanol.

Chromatographic system

(See *Chromatography (621)*, *Thin-Layer Chromatography*.)

Adsorbent: 0.50-mm layer of chromatographic silica gel mixture

Application volume: 20 μL for the *Sample solution*; 10 μL for the *Standard solution*

Developing solvent system: Diethyl ether and methanol (9:1)

Spray reagent: 85% phosphoric acid, 10% vanillin in 96% ethanol, and water (4.5: 1: 4.5)

Analysis

Samples: *Standard solution* and *Sample solution*

Proceed as directed in the chapter. Examine the plates under UV light at 365 nm. Spray the plate with 10 mL of *Spray reagent*, heat between 100° and 105° for 10 min, and examine under daylight.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits a violet-red zone corresponding to β -

sitosterol at the same R_f value as β -sitosterol from the *Standard solution*, weakly visible zones above and below β -sitosterol, and a violet-red zone corresponding to β -sitosterolglucoside.

• B. GAS CHROMATOGRAPHY IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of β -Sitosterol*.

Acceptance criteria: The retention time of β -sitosterol of the *Sample solution* corresponds to that of the *Standard solution*.

• C. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of Scopoletin*.

Acceptance criteria: The retention time of scopoletin of the *Sample solution* corresponds to that of the *Standard solution*.

COMPOSITION

• CONTENT OF β -SITOSTEROL

Derivatizing reagent: A solution containing equal volumes (1:1:1) of BSTFA [*N,O*-bis(trimethylsilyl)tri-fluoroacetamide], anhydrous pyridine, and a mixture of BSA [*N,O*-(trimethylsilyl)acetamide], TMSI (*N*-trimethylsilylimidazole), and TMCS (trimethylchlorosilane) (3:3:2)

Internal standard solution: 10 mg/mL of cholesterol in chloroform

Standard solution: Dissolve 50.0 mg of USP β -Sitosterol RS in 2 mL of chloroform, add 1.0 mL of *Internal standard solution*, and dilute to 5 mL with chloroform. Dry 0.5 mL under reduced pressure, and add 1 mL of *Derivatizing reagent*.

Sample solution: Transfer 20.0 g of Powdered Extract to a Soxhlet apparatus, treat with chloroform, and extract for 6 h. The volume of chloroform used is at least twice the volume of the thimble with an appropriately sized flask. Dry the solvent under reduced pressure, add 1.0 mL of *Internal standard solution*, and dilute with chloroform to 10 mL. Transfer 0.5 mL of this solution to a 10-mL round-bottom flask, dry the solvent under reduced pressure, and add 0.5 mL of *Derivatizing reagent*.

Chromatographic system

(See *Chromatography (621)*, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.20-mm \times 25-m fused-silica capillary; 0.35- μm film of phase G2 coating

Temperature

Injector: 325°

Detector: 325°

Column: 300°, for 60 min

Carrier gas: Helium

Flow rate: 0.5 mL/min

Injection size: 1 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for each sterol peak

Relative standard deviation: NMT 5.0% determined from each sterol peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of β -sitosterol in the portion of Powdered Extract taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = peak response ratio of β -sitosterol to the internal standard from the *Sample solution*

R_S = peak response ratio of β -sitosterol to the internal standard from the *Standard solution*

W_S = weight of USP β -Sitosterol RS used to prepare the *Standard solution* (mg)

W_U = weight of Powdered Extract in the *Sample solution* (mg)

- Acceptance criteria: NLT 0.1%
- **CONTENT OF SCOPOLETIN**
 Solution A: Water
 Solution B: Methanol
 Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	75	25
2	60	40
8	60	40
10	0	100
15	0	100
20	75	25
30	75	25

Standard solution: 0.02 µg/mL of USP Scopoletin RS in methanol

Sample stock solution: 8 mg/mL of Powdered Extract in methanol. Place in an ultrasonic bath for 25 min, and centrifuge.

Sample solution: *Sample stock solution* diluted with methanol 1 in 20

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Fluorescence; set at an excitation wavelength of 366 nm and an emission wavelength of 420 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Capacity factor (k'): NLT 5 determined from the scopoletin peak

Tailing factor: NMT 2.0 for the scopoletin peak

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for scopoletin.

Calculate the content of scopoletin (C₁₀H₈O₄), in µg/g, in the portion of Powdered Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U)$$

r_U = peak area of scopoletin from the *Sample solution*

r_S = peak area of scopoletin from the *Standard solution*

C_S = concentration of USP Scopoletin RS in the *Standard solution* (µg/mL)

C_U = concentration of Powdered Extract in the *Sample solution* (g/mL)

Acceptance criteria: NLT 30 µg/g

• **CONTENT OF TOTAL AMINO ACIDS**

Buffer: Mix 5.40 g of anhydrous sodium acetate, 0.3 mL of glacial acetic acid, and water to a final volume of 100 mL. Adjust to pH 5.5.

Reagent solution: Solution containing 1.00 g of ninhydrin, 1.50 g of hydrindantin, and 37.5 mL of propylene glycol. Adjust with *Buffer* to 50.0 mL. [NOTE—Prepare the *Reagent solution* daily.]

Standard solution: 20 µg/mL each of USP Glutamic Acid RS and USP Aspartic Acid RS in water

Sample solution: Dissolve 50 mg of Powdered Extract in 80 mL of water, shake for 10 min, dilute with water to 100 mL, and filter.

Blank: Water

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Visible

Analytical wavelength: 570 nm

Cell: 1 cm

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank*
 Transfer 5.0 mL of the *Standard solution*, *Sample solution*, and *Blank* to separate 50-mL volumetric flasks. Add 5.0 mL of *Reagent solution* to each flask. Heat in a boiling water bath for 30 min, cool, and adjust with a mixture of ethanol and water (1:1) to volume. Calculate the percentage of total amino acids in the portion of Powdered Extract taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

C_S = sum of the concentration of USP Glutamic Acid RS and USP Aspartic Acid RS in the *Standard solution* (mg/mL)

C_U = concentration of Powdered Extract in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 5.0%

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN, Pesticide Residues** <561>:

Meets the requirements

- **ALCOHOL DETERMINATION, Method II** <611>: NMT 1.0%, if present

- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 10³ cfu/g, and the total combined molds and yeasts count does not exceed 10² cfu/g.

- **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **LOSS ON DRYING** <731>: Dry 1.0 g of Powdered Extract at 105° for 2 h: it loses NMT 8.0% of its weight.

- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 20.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. Store at controlled room temperature.

- **LABELING:** The label states the official name of the article, the Latin binomial, and, following the official name, the part of the plant from which the article was prepared. Label it to indicate the content of total amino acids, β-sitosterol, scopoletin, the extracting solvent used for preparation, and the ratio of the starting crude plant material to Powdered Extract.

- **USP REFERENCE STANDARDS** <11>

USP Aspartic Acid RS

USP Glutamic Acid RS

USP Scopoletin RS

USP β-Sitosterol RS

Thiamine Hydrochloride—see *Thiamine Hydrochloride General Monographs*

Thiamine Hydrochloride Oral Solution—see *Thiamine Hydrochloride Oral Solution General Monographs*

Thiamine Hydrochloride Tablets—see *Thiamine Hydrochloride Tablets General Monographs*

Thiamine Mononitrate—see *Thiamine Mononitrate General Monographs*

Thiamine Mononitrate Oral Solution—see *Thiamine Mononitrate Oral Solution General Monographs*

Threonine—see *Threonine General Monographs*

Tryptophan—see *Tryptophan General Monographs*

Turmeric

DEFINITION

Turmeric is the dried rhizome of *Curcuma longa* L., also known as *C. domestica* Val., (Fam. Zingiberaceae). It is commonly known as Curcuma, Curcum, Haridra, and Indian Saffron. It contains NLT 3.0% of curcuminoids, calculated on the dried basis.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
Standard solution: 0.2 mg/mL of USP Curcuminoids RS in acetone
Sample solution: Pulverize about 5 g of Turmeric. Transfer about 0.2 g of the pulverized sample to a test tube, add 3 mL of acetone, sonicate for 30 min, and centrifuge. Use the supernatant.
Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)
Application volume: 10 µL, as bands
Developing solvent system: Chloroform, methanol, and formic acid (96:4:1)
Analysis
Samples: *Standard solution* and *Sample solution*
 Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621), *Thin Layer Chromatography*). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, and examine in daylight and under UV light at 365 nm.
Acceptance criteria: The *Sample solution* chromatogram shows yellowish-brown bands due to bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin at R_f values of about 0.4, 0.6, and 0.7, respectively, corresponding in position and color to those obtained from the *Standard solution*.
- **B.** The retention times of the peaks for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin of the *Sample solution* chromatogram correspond to those of the *Standard solution* for the appropriate USP Reference Standard, as obtained in the test for *Content of Curcuminoids*.

COMPOSITION

• CONTENT OF CURCUMINOIDS

Mobile phase: Tetrahydrofuran and 1 mg/mL of citric acid in water (4:6)

[NOTE—Sonication may be necessary to dissolve the Reference Standard in each *Standard solution*; all solutions should be passed through a filter of 0.45-µm pore size before injection. USP Curcumin RS, USP Desmethoxycurcumin, and USP Bisdesmethoxycurcumin RS can also be prepared in one standard solution containing the final concentration specified below for each.]

Standard solution A: 40 µg/mL of USP Curcuminoids RS in *Mobile phase*

Standard solution B: 40 µg/mL of USP Curcumin RS in *Mobile phase*

Standard solution C: 10 µg/mL of USP Desmethoxycurcumin RS in *Mobile phase*

Standard solution D: 2 µg/mL of USP Bisdesmethoxycurcumin RS in *Mobile phase*

Sample stock solution: Pulverize about 5.0 g of Turmeric. Transfer about 0.5 g of the pulverized sample to a 50-mL volumetric flask, add 30 mL of acetone, and sonicate for 30 min. Dilute with acetone to volume, mix, and centrifuge.

Sample solution: Transfer 5 mL of the *Sample stock solution* to a 50-mL volumetric flask, dilute with *Mobile phase* to volume, and mix.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 420 nm

Column: 4.6-mm × 20-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution A*

[NOTE—The relative retention times for the curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin peaks are 1.0, 1.2, and 1.4, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the Reference Chromatogram provided with USP Curcuminoids RS.

Resolution: NLT 2.0, between the curcumin and desmethoxycurcumin peaks and the desmethoxycurcumin and bisdesmethoxycurcumin peaks

Tailing factor: NMT 1.5 for bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin peaks

Relative standard deviation: NMT 2.0% for desmethoxycurcumin peak, in repeated injections

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*

Calculate the percentage of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in the portion of Turmeric taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak response for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the *Sample solution*

r_S = peak response for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the appropriate *Standard solution*

C_S = concentration of the appropriate *Standard solution* (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Turmeric used to prepare the *Sample stock solution* (mg)

D = dilution factor to obtain the *Sample solution* from the *Sample stock solution*

Acceptance criteria: NLT 3% as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin on the dried basis

CONTAMINANTS

- **HEAVY METALS**, *Method III* (231): NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* (561): Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN**, *Test for Aflatoxins* (561): Meets the requirements
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

• BOTANIC CHARACTERISTICS

Macroscopic: Turmeric occurs as ovate, oblong, or pear-shaped primary rhizomes, also known as bulb or round turmeric, about 3 cm in diameter and 4–5 cm long, and showing transverse annular leaf scars, and as cylindrical, sometimes short-branched secondary rhizomes, also known as finger or long turmeric, about 1 cm in diameter and 2–7 cm long, and showing scars of lateral branches. The cured and dried turmeric of commerce is bright yellow to dull yellow in appearance, with a rough or polished surface, and a characteristic aromatic odor. The texture is hard and uneasily broken, and the fracture is smooth and finely granular. Internally it is orange-yellow to orange, showing a cortex separated from a central cylinder by a distinct endodermis.

Histology

Transverse section of rhizome: It shows a row of thin-walled, flattened epidermal cells; a few layers of thin-walled, brick-shaped parenchyma cells of the cork; a broad cortex consisting of multiple layers of thin-walled parenchyma cells showing scattered vascular bundles; a thin layer of oblong cells of the endodermis; pericycle consisting of one to two rows of parenchyma cells; and a pith consisting of parenchyma cells showing scattered vascular bundles, most of them forming discontinuous rings near the endodermis and fewer inward. The vascular bundles are of the collateral type; the vessels have mainly spiral thickening, and a few have reticulate and annular thickening. Scattered throughout the parenchyma of the pith and cortex are oleoresin cells containing oil and scattered particles of an orange-yellow pigment, and prisms of calcium oxalate, which are usually obscured due to the bright yellow color of the pigment content. The parenchyma cells are full of starch granules, 15–30 μ m in size, and flat or disk shaped. Bast fibers are absent.

- **ARTICLES OF BOTANICAL ORIGIN**, *Volatile Oil Determination* (561): NLT 3.0 mL/100 g
- **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* (561): NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Alcohol-Soluble Extractives*, *Method 2* (561): NLT 100 mg/g
- **ARTICLES OF BOTANICAL ORIGIN**, *Water-Soluble Extractives*, *Method 2* (561): NLT 9.0%
- **WATER DETERMINATION**, *Method 1a* (921): NLT 10%
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* (561): NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Protect from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** (11)
USP Bisdesmethoxycurcumin RS
USP Curcumin RS
USP Curcuminoids RS
USP Desmethoxycurcumin RS

Powdered Turmeric

DEFINITION

Powdered Turmeric is Turmeric reduced to a fine or very fine powder.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST

Standard solution: 0.2 mg/mL of USP Curcuminoids RS in acetone

Sample solution: Transfer about 0.2 g of Powdered Turmeric to a test tube, add 3 mL of acetone, sonicate for 30 min, and centrifuge. Use the supernatant.

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)

Application volume: 10 μ L, as bands

Developing solvent system: Chloroform, methanol, and formic acid (96:4:1)

Analysis

Samples: *Standard solution* and *Sample solution*
Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* (621), *Thin Layer Chromatography*). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, and examine in daylight and under UV light at 365 nm.

Acceptance criteria: The *Sample solution* chromatogram shows yellowish-brown bands due to bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin at R_f values of about 0.4, 0.6, and 0.7, respectively, corresponding in position and color to those obtained from the *Standard solution*.

- **B.** The retention times of the peaks for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin of the *Sample solution* chromatogram correspond to those of the *Standard solution* for the appropriate USP Reference Standard, as obtained in the test for *Content of Curcuminoids*.

COMPOSITION

• CONTENT OF CURCUMINOIDS

Mobile phase: Tetrahydrofuran and 1 mg/mL of citric acid in water (4:6)

[NOTE—Sonication may be necessary to dissolve the Reference Standard in each *Standard solution*; all solutions should be passed through a filter of 0.45- μ m pore size before injection. USP Curcumin RS, USP Desmethoxycurcumin, and USP Bisdesmethoxycurcumin RS can also be prepared in one standard solution containing the final concentration specified below for each.]

Standard solution A: 40 μ g/mL of USP Curcuminoids RS in *Mobile phase*

Standard solution B: 40 μ g/mL of USP Curcumin RS in *Mobile phase*

Standard solution C: 10 μ g/mL of USP Desmethoxycurcumin RS in *Mobile phase*

Standard solution D: 2 μ g/mL of USP Bisdesmethoxycurcumin RS in *Mobile phase*

Sample stock solution: Transfer about 0.5 g of Powdered Turmeric, accurately weighed, to a 50-mL volumetric flask, add 30 mL of acetone, and sonicate for 30 min. Dilute with acetone to volume, mix, and centrifuge.

Sample solution: Transfer 5 mL of the *Sample stock solution* to a 50 mL volumetric flask. Dilute with *Mobile phase* to volume, and mix.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV-Vis 420 nm

Column: 4.6-mm × 20-cm; 5-μm packing L1

Flow rate: 1 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution A*

[NOTE—The relative retention times for the curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin peaks are 1.0, 1.2, and 1.4, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the Reference Chromatogram provided with USP Curcuminoids RS.

Resolution: NLT 2.0, between curcumin and desmethoxycurcumin peaks and the desmethoxycurcumin and bisdesmethoxycurcumin peaks

Tailing factor: NMT 1.5 for bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin peaks

Relative standard deviation: NMT 2.0% for desmethoxycurcumin peak, in repeated injections

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*

Calculate the percentage of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in the portion of Powdered Turmeric taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times D \times 100$$

r_U = peak response for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the *Sample solution*

r_S = peak response for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the appropriate *Standard solution*

C_S = concentration of the appropriate *Standard solution* (mg/mL)

V = volume of the *Sample stock solution* (mL)

W = weight of Powdered Turmeric used to prepare the *Sample stock solution* (mg)

D = dilution factor to prepare the *Sample solution* from the *Sample stock solution*

Acceptance criteria: NLT 3% as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin

CONTAMINANTS

- **HEAVY METALS**, *Method III* <231>: NMT 20 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements
- **ARTICLES OF BOTANICAL ORIGIN**, *Test for Aflatoxins* <561>: Meets the requirements
- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic bacterial count does not exceed 10^5 cfu/g, the total combined molds and yeasts count does not exceed 10^3 cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed 10^3 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS:** It is deep yellow in color, with a characteristic aromatic odor. Under a microscope, Powdered Turmeric reveals thin-walled parenchyma cells containing starch granules, 15–30 μm in size, flat or disk shaped, gelatinized or ungelatinized; oil cells full of oil and scattered particles of orange-yellow pigments; prisms of calcium oxalate, usually obscured due to the bright yellow color of the pigment content, detected as bright orange prisms under a polarizing microscope; fragments of spiral vessels and a few reticulate and annular vessels; fragments of epidermal and cork cells; starch granules; scattered unicellular nonglandular trichomes; and the absence of bast fibers.
- **ARTICLES OF BOTANICAL ORIGIN**, *Volatile Oil Determination* <561>: NLT 3.0 mL/100 g
- **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* <561>: NMT 2.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Alcohol-Soluble Extractives*, *Method 2* <561>: NLT 100 mg/g
- **ARTICLES OF BOTANICAL ORIGIN**, *Water-Soluble Extractives*, *Method 2* <561>: NLT 9.0%
- **WATER DETERMINATION**, *Method 1a* <921>: NLT 10%
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 7.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* <561>: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Protect from light and moisture, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.
- **USP REFERENCE STANDARDS** <11>
 - USP Bisdesmethoxycurcumin RS
 - USP Curcumin RS
 - USP Curcuminoids RS
 - USP Desmethoxycurcumin RS

Powdered Turmeric Extract

DEFINITION

Powdered Turmeric Extract is prepared from the pulverized rhizomes of *Curcuma longa* L. (Fam. Zingiberaceae), using acetone, methanol, or other suitable solvents. It contains NLT 20% of total curcuminoids, calculated on the dried basis. It may contain other added substances.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**
 - Standard solution:** 0.2 mg/mL of USP Curcuminoids RS in acetone
 - Sample solution:** 10 mg/mL of Powdered Turmeric Extract in acetone
 - Adsorbent:** 0.25-mm layer of chromatographic silica gel mixture, typically 20 cm long (TLC plates)
 - Application volume:** 10 μL, as bands
 - Developing solvent system:** Chloroform, methanol, and formic acid (96:4:1)
 - Analysis**
 - Samples:** *Standard solution* and *Sample solution*
 - Apply the samples as bands to a suitable thin-layer chromatographic plate (see *Chromatography* <621>, *Thin Layer Chromatography*). Use a saturated chamber. Develop the chromatograms until the solvent front has moved up about three-fourths of the length of the plate. Remove the plate from the chamber, dry, and examine in daylight and under UV light at 365 nm.
 - Acceptance criteria:** The *Sample solution* chromatogram shows yellowish-brown bands due to bisdesmethoxycurcumin and curcumin.

oxycurcumin, desmethoxycurcumin, and curcumin at R_f values of about 0.4, 0.6, and 0.7, respectively, corresponding in position and color to those obtained from the *Standard solution*.

- **B.** The retention times of the peaks for curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin of the *Sample solution* chromatogram correspond to those of the *Standard solution* for the appropriate USP Reference Standard, as obtained in the test for *Content of Curcuminoids*.

COMPOSITION

• CONTENT OF CURCUMINOIDS

Mobile phase: Tetrahydrofuran and 1 mg/mL of citric acid in water (4:6)

[NOTE—Sonication may be necessary to dissolve the Reference Standard in each *Standard solution*; all solutions should be passed through a filter of 0.45- μ m pore size before injection. USP Curcumin RS, USP Desmethoxycurcumin, and USP Bisdesmethoxycurcumin RS can also be prepared in one standard solution containing the final concentration specified below for each.]

Standard solution A: 40 μ g/mL of USP Curcuminoids RS in *Mobile phase*

Standard solution B: 40 μ g/mL of USP Curcumin RS in *Mobile phase*

Standard solution C: 10 μ g/mL of USP Desmethoxycurcumin RS in *Mobile phase*

Standard solution D: 2 μ g/mL of USP Bisdesmethoxycurcumin RS in *Mobile phase*

Sample solution: Transfer about 100 mg of Powdered Turmeric Extract, accurately weighed, to a 50-mL volumetric flask, add 30 mL of acetone, and sonicate for 30 min. Dilute with acetone to volume and centrifuge. Transfer 5 mL to a 50-mL volumetric flask, dilute with *Mobile phase* to volume, and mix.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 420 nm

Column: 4.6-mm \times 20-cm; 5- μ m packing L1

Flow rate: 1 mL/min

Injection size: 20 μ L

System suitability

Sample: *Standard solution A*

[NOTE—The relative retention times for the curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin peaks are 1.0, 1.2, and 1.4, respectively.]

Suitability requirements

Chromatogram similarity: The chromatogram of *Standard solution A* is similar to the Reference Chromatogram provided with USP Curcuminoids RS.

Resolution: NLT 2.0, between curcumin and desmethoxycurcumin peaks and the desmethoxycurcumin and bisdesmethoxycurcumin peaks

Tailing factor: NMT 1.5 for bisdesmethoxycurcumin, desmethoxycurcumin, and curcumin peaks

Relative standard deviation: NMT 2.0% for desmethoxycurcumin peak, in repeated injections

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*

Calculate the percentage of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin in the portion of Powdered Turmeric Extract taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

r_u = peak response for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the *Sample solution*

r_s = peak response for curcumin, desmethoxycurcumin, or bisdesmethoxycurcumin from the appropriate *Standard solution*

C_s = concentration of the appropriate *Standard solution* (mg/mL)

C_u = concentration of Powdered Turmeric Extract in the *Sample solution* (mg/mL)

Add the percentages due to curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin.

Acceptance criteria: NLT 20% on the dried basis

CONTAMINANTS

• **HEAVY METALS**, *Method III* <231>: NMT 20 ppm

• **ARTICLES OF BOTANICAL ORIGIN**, *General Method for Pesticide Residues Analysis* <561>: Meets the requirements

• **ARTICLES OF BOTANICAL ORIGIN**, *Test for Aflatoxins* <561>: Meets the requirements

• **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic bacterial count does not exceed 10^4 cfu/g, and the total combined molds and yeasts count does not exceed 10^3 cfu/g.

• **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

• **BOTANICAL EXTRACTS**, *Residual Solvents* <565>: Meets the requirements

SPECIFIC TESTS

• **LOSS ON DRYING** <731>: Dry 1.0 g at 105° for 2 h: it loses NMT 7.0% of its weight.

ADDITIONAL REQUIREMENTS

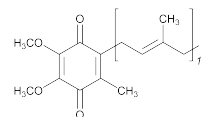
• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Protect from light and moisture, and store at controlled room temperature.

• **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant from which the article was prepared. It meets other labeling requirements under *Botanical Extracts* <565>.

• **USP REFERENCE STANDARDS** <11>
USP Bisdesmethoxycurcumin RS
USP Curcumin RS
USP Curcuminoids RS
USP Desmethoxycurcumin RS

Tyrosine—see Tyrosine General Monographs

Ubidecarenone



$C_{59}H_{90}O_4$ 863.34
2,5-Cyclohexadiene-1,4-dione, 2-[(2E,6E,10E,14E,18E,22E,26E,30E,34E)-3,7,11,15,19,23,27,31,35,39-decamethyl-2,6,10,14,18,22,26,30,34,38-tetracontadecaenyl]-5,6-dimethoxy-3-methyl;
2-[(all-E)-3,7,11,15,19,23,27,31,35,39-Decamethyl-2,6,10,14,18,22,26,30,34,38-tetracontadecaenyl]-5,6-dimethoxy-3-methyl-p-benzoquinone [303-98-0].

DEFINITION

Ubidecarenone (Coenzyme Q₁₀) contains NLT 98.0% and NMT 101.0% of ubidecarenone ($C_{59}H_{90}O_4$), calculated on the anhydrous basis.

IDENTIFICATION• **A. INFRARED ABSORPTION** <197K>• **B.**

Analysis: Dissolve 50 mg of Ubidecarenone in 1 mL of ethyl ether, and add 10 mL of dehydrated alcohol. To 2 mL of this solution add 3 mL of dehydrated alcohol and 2 mL of dimethyl malonate. Add 1 mL of potassium hydroxide solution (1 in 5) dropwise.

Acceptance criteria: A blue color appears.

ASSAY• **PROCEDURE**

Mobile phase: Methanol and dehydrated alcohol (65:35)

System suitability solution: 0.5 mg/mL each of USP Ubidecarenone RS and USP Ubidecarenone Related Compound A RS in dehydrated alcohol. Heat at 50° for 2 min, if necessary, for complete dissolution.

Standard solution: 1.0 mg/mL of USP Ubidecarenone RS in dehydrated alcohol. Heat at 50° for 2 min, if necessary, for complete dissolution.

Sample solution: 1.0 mg/mL of Ubidecarenone in dehydrated alcohol. Heat at 50° for 2 min, if necessary, for complete dissolution.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 275 nm

Column: 4.6-mm × 15-cm; packing L1

Column temperature: 35°

Flow rate: Adjust to obtain a retention time of about 11 min for ubidecarenone.

Injection size: 5 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for ubidecarenone related compound A and ubidecarenone are about 0.75 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 4 between ubidecarenone related compound A and ubidecarenone

Relative standard deviation: NMT 0.8% for ubidecarenone

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of ubidecarenone (C₅₉H₉₀O₄) in the portion of Ubidecarenone taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Ubidecarenone RS in the *Standard solution* (mg/mL)

C_U = concentration of Ubidecarenone in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–101.0% on the anhydrous basis

IMPURITIES• **RESIDUE ON IGNITION** <281>: NMT 0.1%• **HEAVY METALS, Method II** <231>: NMT 20 ppm• **CHROMATOGRAPHIC PURITY**

Procedure 1: Coenzymes Q₇, Q₈, Q₉, Q₁₁, and Related Impurities

Mobile phase, System suitability solution, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the Assay.

Analysis

Sample: *Sample solution*

Calculate the percentage of impurities in the portion of Ubidecarenone taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times 100$$

r_{T1} = sum of all peak responses, other than that for ubidecarenone

r_{T2} = sum of all peak responses

Acceptance criteria: NMT 1.0%

Procedure 2: Ubidecarenone (2Z)-Isomer and Related Impurities

Mobile phase: *n*-Hexane and ethyl acetate (97:3)

System suitability solution: 1 mg/mL of USP

Ubidecarenone for System Suitability RS in *n*-hexane

Sample solution: 1 mg/mL of Ubidecarenone in *n*-hexane

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 275 nm

Column: 4.6-mm × 25-cm; packing L3

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for ubidecarenone (2Z)-isomer and ubidecarenone are about 0.85 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between the ubidecarenone (2Z)-isomer and ubidecarenone

Analysis

Sample: *Sample solution*

Calculate the percentage of impurities in the portion of Ubidecarenone taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times 100$$

r_{T1} = sum of all peak responses, other than that for ubidecarenone

r_{T2} = sum of all peak responses

Acceptance criteria: NMT 1.0%

Total impurities: NMT 1.5%, obtained from *Chromatographic Purity Procedures 1 and 2*

SPECIFIC TESTS• **WATER DETERMINATION, Method I** <921>: NMT 0.2%**ADDITIONAL REQUIREMENTS**• **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.• **USP REFERENCE STANDARDS** <11>

USP Ubidecarenone RS

USP Ubidecarenone Related Compound A RS

[coenzyme Q₉]

USP Ubidecarenone for System Suitability RS

Ubidecarenone Capsules**DEFINITION**

Ubidecarenone Capsules contain NLT 90.0% and NMT 115.0% of the labeled amount of ubidecarenone (C₅₉H₉₀O₄).

IDENTIFICATION• **A.** The retention time of the major peak of either *Sample solution 1* or *Sample solution 2* corresponds to that of the *Standard solution*, as obtained in the *Procedure for Strength*.**STRENGTH**• **PROCEDURE**

[NOTE—Conduct this test promptly with minimum exposure to actinic light.]

Solvent: *n*-Hexane and dehydrated alcohol (5:2)

Mobile phase: Acetonitrile, tetrahydrofuran, and water (55:40:5)

Standard stock solution: 1.0 mg/mL of USP Ubidecarenone RS in *Solvent*

Standard solution: 40 µg/mL in dehydrated alcohol, from the *Standard stock solution*

System suitability stock solution: 1.0 mg/mL of USP Ubidecarenone Related Compound A RS in *Solvent*. Dilute a portion of this solution with dehydrated alcohol to obtain a concentration of 40 µg/mL.

System suitability solution: *Standard solution* and *System suitability stock solution* (1:1)

Sample solution 1 (for soft gelatin Capsules): Open a number of Capsules equivalent to 200 mg of ubidecarenone, quantitatively transfer the shells and contents to a container, add 100 mL of *Solvent*, and shake by mechanical means for 30 min. Using small portions of *Solvent*, quantitatively transfer this mixture to a 200-mL volumetric flask, and dilute with *Solvent* to volume. Centrifuge a portion of this solution, transfer 1.0 mL of the supernatant to a 25-mL volumetric flask, add 2.5 mL of a 0.1% solution of anhydrous ferric chloride in alcohol, and dilute with alcohol to volume.

Sample solution 2 (for hard gelatin Capsules): Empty and thoroughly mix the contents of NLT 20 Capsules. Transfer a portion of the powder, equivalent to 100 mg of ubidecarenone, to a 100-mL volumetric flask, add 60 mL of *Solvent*, and shake by mechanical means for 30 min. Dilute with *Solvent* to volume. Centrifuge a portion of this solution, transfer 1.0 mL of the supernatant to a 25-mL volumetric flask, add 2.5 mL of a 0.1% solution of anhydrous ferric chloride in alcohol, and dilute with alcohol to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 8-mm × 10-cm; packing L1

Flow rate: 2.5 mL/min

Injection size: 15 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 2.5 between ubidecarenone and ubidecarenone related compound A, *System suitability solution*

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0% for ubidecarenone, *Standard solution*

Analysis

Samples: *Sample solution 1* or *Sample solution 2*, and *Standard solution*

Calculate the percentage of the labeled amount of ubidecarenone (C₅₉H₉₀O₄) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of ubidecarenone from *Sample solution 1* or *Sample solution 2*

r_S = peak area of ubidecarenone from the *Standard solution*

C_S = concentration of USP Ubidecarenone RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of ubidecarenone in *Sample solution 1* or *Sample solution 2* (mg/mL)

Acceptance criteria: 90.0%–115.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** <2040>: Meet the requirements of the test for *Disintegration*, except where the product is labeled to contain a water-soluble form of ubidecarenone. Capsules labeled to contain a water-soluble form of ubidecarenone meet the requirements for the test for *Dissolution*, as follows.

Medium: Water; 500 mL

Apparatus 2: 75 rpm

Time: 60 min

Standard solution: Dissolve 25 mg of USP Ubidecarenone RS in 1 mL of ethyl ether, and dilute with alcohol to obtain a concentration of 2.5 µg/mL. [NOTE—Use a freshly prepared solution only.]

Sample solution: Dilute with alcohol a volume of the solution under test, previously passed through a suitable filter of 0.45-µm pore size, to obtain a concentration of 2.5 µg/mL of ubidecarenone.

Mobile phase and Chromatographic system: Proceed as directed in the *Procedure* for *Strength*, except for *Injection size*.

Injection size: 100 µL

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of ubidecarenone (C₅₉H₉₀O₄) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V \times D/L) \times 100$$

r_U = peak area of ubidecarenone from the *Sample solution*

r_S = peak area of ubidecarenone from the *Standard solution*

C_S = concentration of USP Ubidecarenone RS in the *Standard solution* (mg/mL)

V = volume of *Medium*, 500 mL

D = dilution factor for the *Sample solution*

L = label claim (mg/Capsule)

Tolerances: NLT 75% of the labeled amount of ubidecarenone (C₅₉H₉₀O₄) is dissolved.

SPECIFIC TESTS

- **WEIGHT VARIATION** <2091>: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** Where the product contains a water-soluble form of ubidecarenone, this is so stated on the label.
- **USP REFERENCE STANDARDS** <11>
 - USP Ubidecarenone RS
 - USP Ubidecarenone Related Compound A RS
 - Coenzyme Q₉.

Ubidecarenone Tablets

DEFINITION

Ubidecarenone Tablets contain NLT 90.0% and NMT 115.0% of the labeled amount of ubidecarenone (C₅₉H₉₀O₄).

IDENTIFICATION

- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Procedure* for *Strength*.

STRENGTH

- **PROCEDURE** [NOTE—Conduct this test promptly with minimum exposure to actinic light.]

Solvent: *n*-Hexane and dehydrated alcohol (5:2)

Mobile phase: Acetonitrile, tetrahydrofuran, and water (11:8:1)

Standard stock solution: 1.0 mg/mL of USP Ubidecarenone RS in *Solvent*

Standard solution: 40 µg/mL from *Standard stock solution* in dehydrated alcohol

System suitability stock solution: 1.0 mg/mL of USP Ubidecarenone Related Compound A RS in *Solvent*. Dilute a portion of this solution with dehydrated alcohol to obtain a concentration of 40 µg/mL.

System suitability solution: *Standard solution* and *System suitability stock solution* (1:1)

Sample stock solution: Weigh and finely powder NLT 20 Tablets. Transfer a quantity of powder, equivalent to about 100 mg of ubidecarenone, to a 100-mL volumetric flask, add 60 mL of *Solvent*, and shake by mechanical means for 30 min. Dilute with *Solvent* to volume, and mix. Centrifuge a portion of this solution, transfer 1.0 mL of the supernatant to a 25-mL volumetric flask, and add 2.5 mL of a 0.1% solution of anhydrous ferric chloride in alcohol. Dilute with alcohol to volume, and mix.

Sample solution: Centrifuge a portion of *Sample stock solution*, transfer 1.0 mL of the supernatant to a 25-mL volumetric flask, add 2.5 mL of a 0.1% solution of anhydrous ferric chloride in alcohol, and dilute with alcohol to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 8-mm × 10-cm; packing L1

Flow rate: 2.5 mL/min

Injection size: 15 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 2.5 between ubidecarenone and ubidecarenone related compound A, *System suitability solution*

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0% for ubidecarenone, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of ubidecarenone ($C_{59}H_{90}O_4$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of ubidecarenone from the *Sample solution*

r_S = peak area of ubidecarenone from the *Standard solution*

C_S = concentration of USP Ubidecarenone RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of ubidecarenone in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–115.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** <2040>: Meet the requirements of the test for *Disintegration*, except where the product is labeled to contain a water-soluble form of ubidecarenone. Tablets labeled to contain a water-soluble form of ubidecarenone meet the requirements for the test for *Dissolution*, as follows.

Medium: Water; 500 mL

Apparatus 2: 75 rpm

Time: 60 min

Standard solution: Dissolve 25 mg of USP Ubidecarenone RS in 1 mL of ethyl ether, and dilute with alcohol to obtain a concentration of 2.5 µg/mL. [NOTE—Use a freshly prepared solution only.]

Sample solution: Dilute with alcohol a volume of the solution under test, previously passed through a suitable filter of 0.45-µm pore size, to obtain a concentration of 2.5 µg/mL of ubidecarenone.

Mobile phase and Chromatographic system: Proceed as directed in the *Procedure for Strength*, except for *Injection size*.

Injection size: 100 µL

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of ubidecarenone ($C_{59}H_{90}O_4$) dissolved:

$$\text{Result} = (r_U/r_S) \times (C_S \times V \times D/L) \times 100$$

r_U = peak area of ubidecarenone from the *Sample solution*

r_S = peak area of ubidecarenone from the *Standard solution*

C_S = concentration of USP Ubidecarenone RS in the *Standard solution* (mg/mL)

V = volume of *Medium*, 500 mL

D = dilution factor for the *Sample solution*

L = label claim (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of ubidecarenone ($C_{59}H_{90}O_4$) is dissolved.

SPECIFIC TESTS

- **WEIGHT VARIATION** <2091>: Meet the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** Where the product contains a water-soluble form of ubidecarenone, this is so stated on the label.
- **USP REFERENCE STANDARDS** <11>
USP Ubidecarenone RS
USP Ubidecarenone Related Compound A RS
Coenzyme Q₉.

Valerian

DEFINITION

Valerian consists of the subterranean parts of *Valeriana officinalis* L. (Fam. Valerianaceae) including the rhizome, roots, and stolons. It contains NLT 0.5% of volatile oil and NLT 0.05% of valerenic acid ($C_{15}H_{22}O_2$), calculated on the dried basis.

IDENTIFICATION

- **A. COLOR REACTION**

Sample solution: 0.2 g of freshly powdered Valerian in 5 mL of methylene chloride. Shake several times, and allow to stand for 5 min. Filter, wash the filter with 2 mL of methylene chloride, and combine the filtrate and washings in one container. Heat the combined filtrate and washings on a water bath for the minimum time required to evaporate the solvent, and dissolve the residue in 0.2 mL of methylene chloride.

Analysis: To 0.1 mL of the *Sample solution* add 3 mL of a mixture of equal volumes of glacial acetic acid and 25% hydrochloric acid, and shake several times.

Acceptance criteria: A blue color develops within 15 min.

- **B. HPLC IDENTIFICATION TEST**

Analysis: Proceed as directed in the test for *Content of Valerenic Acid*.

Acceptance criteria: The *Sample solution* chromatogram exhibits a peak for valerenic acid at a retention time that corresponds to that of the *Standard solution*.

COMPOSITION

- **CONTENT OF VALERENIC ACID**

Mobile phase: A (4:1) mixture of methanol and dilute phosphoric acid (1 in 200)

Standard solution: 0.05 mg/mL of USP Valerenic Acid RS in 70% alcohol

Sample solution: To 2 g of Valerian, reduced to a powder, add 40.0 mL of 70% alcohol. Shake by mechanical

means for 2 h at room temperature. Centrifuge, and use the clear extract.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 225 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the valerenic acid peak

Relative standard deviation: NMT 2.0% for the valerenic acid peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of valerenic acid (C₁₅H₂₂O₂) in the portion of Valerian taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Valerenic Acid RS in the *Standard solution* (mg/mL)

V = volume of the *Sample solution*, 40 mL

W = weight of Valerian taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.05% on the dried basis

ARTICLES OF BOTANICAL ORIGIN, Volatile Oil Determination <561>

Sample: 100 g, freshly and coarsely comminuted

Acceptance criteria: NLT 0.5% on the dried basis

CONTAMINANTS

- **ARTICLES OF BOTANICAL ORIGIN, General Method for Pesticide Residues Analysis <561>**: Meets the requirements
- **MICROBIAL ENUMERATION TESTS <2021>**: The total bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and bile-tolerant Gram-negative bacteria does not exceed 10³ cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS <2022>**: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

SPECIFIC TESTS

BOTANIC CHARACTERISTICS

Macroscopic: Rhizome erect, entire or usually cut longitudinally, up to 5 cm in length and up to 3 cm in diameter, yellowish gray to pale grayish brown, base elongated or compressed, covered by and merging with numerous roots; apex usually bearing a cup-shaped scar from aerial parts, stem bases rarely present; in longitudinal section, pith exhibiting a central cavity traversed by septa. Roots numerous, slender, almost cylindrical, and of the same color as the rhizome, about 10 cm in length and up to 3 mm in diameter; a few filiform, fragile secondary roots; fracture short. Stolon pale yellowish gray, showing prominent nodes separated by longitudinally striated internodes each from 2 to 5 cm in length; fracture fibrous.

Microscopic: Root, with piliferous layer, of papillose cells, some being developed into root hairs; exodermis, or a single layer of quadrangular to polygonal cells with suberized walls and containing globules of volatile oil; outer cortex of two to four layers of resin-containing cells with thin or collenchymatous, sometimes suberized walls; inner cortex of numerous layers of polygonal to rounded cells filled with starch. Numerous starch granules, mostly compound of 2 to 6 components, spheroidal, plano-convex, up to 30 µm in diameter; or simple, from 8 to 12 µm, occasionally up to 20 µm in

diameter, with a central hilum. Endodermis consisting of a single layer of suberized, tangentially elongated cells, pericycle continuous, starch-filled; parenchyma surrounding the phloem zone; cambium frequently indistinct; vascular bundles forming an interrupted ring surrounding the starch-filled cells. Rhizome in transverse section, different from the root, its structure being complicated by the presence of numerous vascular bundles from root and stolon; epidermis and exodermis partially replaced by poorly developed periderm; central pith wide, including cavities of various sizes, the larger ones being separated by plates of partially sclerified tissue.

ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter <561>

EXTRACTABLE MATTER

Sample: 2 g, carefully dried at 40° and coarsely powdered

Analysis: Mix the *Sample* with 20 mL of 70% alcohol, and allow to stand for 2 h, shaking frequently. Filter, evaporate 5 mL of the filtrate on a water bath to dryness, and dry the residue at 105°.

Acceptance criteria: NLT 20%. The weight of the dried residue is NLT 100 mg.

ARTICLES OF BOTANICAL ORIGIN, Water Content <561>

NMT 12.0%

ARTICLES OF BOTANICAL ORIGIN, Total Ash <561>

NMT 12.0%

ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash <561>

NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, store at room temperature, and protect from light and moisture.
- **LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant contained in the article.
- **USP REFERENCE STANDARDS <11>**
USP Valerenic Acid RS

Powdered Valerian

DEFINITION

Powdered Valerian is Valerian reduced to a fine or a very fine powder. It contains no calcium oxalate crystals and no foreign starch granules. It contains NLT 0.3% of volatile oil and NLT 0.04% of valerenic acid (C₁₅H₂₂O₂).

IDENTIFICATION

A. COLOR REACTION

Sample solution: 0.2 g of Powdered Valerian in 5 mL of methylene chloride. Shake several times, and allow to stand for 5 min. Filter, wash the filter with 2 mL of methylene chloride, and combine the filtrate and washings in one container. Heat the combined filtrate and washings on a water bath for the minimum time required to evaporate the solvent, and dissolve the residue in 0.2 mL of methylene chloride.

Analysis: To 0.1 mL of the *Sample solution* add 3 mL of a mixture of equal volumes of glacial acetic acid and 25% hydrochloric acid, and shake several times.

Acceptance criteria: A blue color develops within 15 min.

B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of Valerenic Acid*.

Acceptance criteria: The *Sample solution* chromatogram exhibits a peak for valerenic acid at a retention time that corresponds to that of the *Standard solution*.

COMPOSITION**• CONTENT OF VALERENIC ACID**

Mobile phase: A (4:1) mixture of methanol and dilute phosphoric acid (1 in 200)

Standard solution: 0.05 mg/mL of USP Valerenic Acid RS in 70% alcohol

Sample solution: To 2 g of Powdered Valerian add 40.0 mL of 70% alcohol. Shake by mechanical means for 2 h at room temperature. Centrifuge, and use the clear extract.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 225 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the valerenic acid peak

Relative standard deviation: NMT 2.0% for the valerenic acid peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of valerenic acid (C₁₅H₂₂O₂) in the portion of Powdered Valerian taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Valerenic Acid RS in the *Standard solution* (mg/mL)

V = volume of the *Sample solution*, 40 mL

W = weight of Powdered Valerian taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 0.04% on the dried basis

• ARTICLES OF BOTANICAL ORIGIN, Volatile Oil Determination <561>

Sample: 100 g

Acceptance criteria: NLT 0.3%

CONTAMINANTS**• HEAVY METALS <231>:** 50 µg/g**• ARTICLES OF BOTANICAL ORIGIN, General Method for**

Pesticide Residues Analysis <561>: Meets the requirements

• MICROBIAL ENUMERATION TESTS <2021>: The total bacterial count does not exceed 10⁵ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, and bile-tolerant Gram-negative bacteria does not exceed 10³ cfu/g.**• ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.**SPECIFIC TESTS****• BOTANIC CHARACTERISTICS**

Microscopic: Numerous fragments of parenchyma cells containing globules of volatile oil and starch granules; fragments of scalariform and reticulate thickened vessels and tracheids and strongly lignified narrow fibers; fragments of periderm and of piliferous layer with root hairs; numerous starch granules, mostly compound of 2 to 6 components, spheroidal, plano-convex, up to 30 µm in diameter; or simple, from 8 to 12 µm, occasionally up to 20 µm in diameter, with a central hilum.

• ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter <561>: NMT 2.0%**• EXTRACTABLE MATTER**

Sample: 2 g, carefully dried at 40°

Analysis: Mix the *Sample* with 20 mL of 70% alcohol, and allow to stand for 2 h, shaking frequently. Filter,

evaporate 5 mL of the filtrate on a water bath to dryness, and dry the residue at 105°.

Acceptance criteria: NLT 20%. The weight of the dried residue is NLT 100 mg.

• WATER, Method 1a <921>: NMT 5.0%**• ARTICLES OF BOTANICAL ORIGIN, Total Ash <561>:** NMT 12.0%**• ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash <561>:** NMT 5.0%**ADDITIONAL REQUIREMENTS****• PACKAGING AND STORAGE:** Preserve in well-closed containers, store at room temperature, and protect from light and moisture.**• LABELING:** The label states the Latin binomial and, following the official name, the parts of the plant from which the article was derived.**• USP REFERENCE STANDARDS <11>**
USP Valerenic Acid RS

Powdered Valerian Extract

DEFINITION

Powdered Valerian Extract is prepared from comminuted Valerian and with 70% alcohol or other suitable solvents. It contains NLT 0.3% of valerenic acid (C₁₅H₂₂O₂). The ratio of the starting crude plant material to the Extract is between 4:1 and 7:1.

IDENTIFICATION**• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 0.5 mg/mL each of USP Fluorescein RS and USP Valerenic Acid RS, in methanol

Sample solution: Dissolve 0.2 g of Extract in 2 mL of water, add 3 mL of a 10% aqueous solution of potassium hydroxide, and extract this mixture with two 5-mL portions of methylene chloride. Discard the organic phase, heat the aqueous phase on a water bath at 40° for 10 min, cool, acidify with 7% hydrochloric acid, and extract this solution with two 5-mL portions of methylene chloride. Dry the organic phase over anhydrous sodium sulfate, and filter. Evaporate the filtrate to dryness, and dissolve the residue in 1.0 mL of methylene chloride.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.5-mm layer of chromatographic silica gel mixture

Application volume

Standard solution: 10 µL

Sample solution: 20 µL

Developing solvent system: Solvent hexane, ethyl acetate, and glacial acetic acid (65: 35: 0.5)

Spray reagent: Mix 0.5 mL of anisaldehyde with 10 mL of glacial acetic acid, 85 mL of methanol, and 5 mL of sulfuric acid, added in the sequence specified.

Analysis

Samples: *Standard solution* and *Sample solution*

Spray the plate with *Spray reagent*. Heat the plate in an oven at 105° for 10 min, and examine the plate under white light.

Acceptance criteria: The *Standard solution* chromatogram shows a violet zone due to valerenic acid at an R_f value of 0.4, and a yellow zone due to fluorescein at an R_f value of 0.1. The *Sample solution* chromatogram shows a violet zone due to valerenic acid at an R_f value of 0.4, and a blue-violet zone due to hydroxyvalerenic acid at an R_f value of 0.12, just above the yellow zone in the *Standard solution*. The chromatogram of the *Sample solution* may show other colored zones at R_f values lower than those of valerenic acid.

• B. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of Valerenic Acid*.

Acceptance criteria: The *Sample solution* chromatogram exhibits a peak at a retention time that corresponds to that of valerenic acid in the *Standard solution*.

COMPOSITION

• CONTENT OF VALERENIC ACID

Mobile phase: Methanol and water (77:27). Add 0.5 mL of phosphoric acid to each 100 mL of the mixture.

Standard solution: 0.024 mg/mL of USP Valerenic Acid RS in methanol

Sample solution: Transfer a quantity of the Extract, nominally equivalent to 0.6 mg of valerenic acid, to a 25-mL volumetric flask, and add 15 mL of methanol. Stir for 10 min, dilute with methanol to volume, mix, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 225 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 30°

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Capacity factor (k'): NLT 5 determined from the valerenic acid peak

Tailing factor: NMT 2.0 for valerenic acid

Relative standard deviation: NMT 2.0% for the valerenic acid peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of valerenic acid (C₁₅H₂₂O₂) in the portion of the Extract taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Valerenic Acid RS in the *Standard solution* (mg/mL)

C_U = concentration of the Extract in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 0.3%

CONTAMINANTS

• ARTICLES OF BOTANICAL ORIGIN, *Pesticide Residues* <561>:

Meets the requirements

• ALCOHOL DETERMINATION, *Method II* <611>: NMT 2.0%, if present

• MICROBIAL ENUMERATION TESTS <2021>: The total bacterial count does not exceed 10⁴ cfu/g, the total combined molds and yeasts count does not exceed 10³ cfu/g, the coliform count does not exceed 10³ cfu/g, and the *Enterobacteriaceae* count does not exceed 10³ cfu/g.

• ABSENCE OF SPECIFIED MICROORGANISMS <2022>: It meets the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

SPECIFIC TESTS

• LOSS ON DRYING <731>: Dry 1.0 g of the Extract at 105° for 2 h: it loses NMT 9.0% of its weight.

• ARTICLES OF BOTANICAL ORIGIN, *Total Ash* <561>: NMT 7.0%

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in tight containers, store at controlled room temperature, and protect from moisture and light.

• LABELING: The label states the official name of the article and states also the Latin binomial and the part of the

plant from which the article was prepared. Label it to indicate the content of valerenic acid, the extracting solvent used for preparation, and the ratio of the starting crude plant material to the Extract.

• USP REFERENCE STANDARDS <11>

USP Fluorescein RS

USP Valerenic Acid RS

Valerian Tablets

DEFINITION

Valerian Tablets contain Powdered Valerian Extract. Tablets contain NLT 90.0% and NMT 120.0% of the labeled amount of Powdered Valerian Extract, calculated as valerenic acid (C₁₅H₂₂O₂).

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST <201>

Standard solution: 0.5 mg/mL each of USP Fluorescein RS and USP Valerenic Acid RS in methanol

Sample solution: Transfer an equivalent to 100 mg of Powdered Valerian Extract from finely powdered Tablets (NLT 10) to a suitable flask. Add 5 mL of water and 3 mL of a 10% aqueous solution of potassium hydroxide, extract this mixture with two 5-mL portions of methylene chloride, and discard the organic phase. Heat the aqueous phase in a water bath at 40° for 10 min, cool, acidify with 7% hydrochloric acid, and extract this solution with two 5-mL portions of methylene chloride. Dry the organic phase over anhydrous sodium sulfate, filter, evaporate the filtrate to dryness, and dissolve the residue in 1.0 mL of methylene chloride.

Adsorbent: 0.5-mm layer of chromatographic silica gel mixture

Application volume

Standard solution: 10 µL

Sample solution: 20 µL, in a 2-cm band

Developing solvent system: Solvent hexane, ethyl acetate, and glacial acetic acid (65:35:0.5)

Spray reagent: Mix 0.5 mL of anisaldehyde with 10 mL of glacial acetic acid, 85 mL of methanol, and 5 mL of sulfuric acid, added in the sequence specified.

Analysis

Samples: *Standard solution* and *Sample solution*

Spray the plate with *Spray reagent*. Heat the plate in an oven at 105° for 10 min, and examine the plate under white light.

Acceptance criteria: The *Standard solution* chromatogram shows a violet zone due to valerenic acid at an R_F value of 0.4, and a yellow zone due to fluorescein at an R_F value of 0.1. The *Sample solution* chromatogram shows a violet zone due to valerenic acid at an R_F value of 0.4, and a blue-violet zone due to hydroxyvalerenic acid at an R_F value of 0.12, just above the yellow zone in the *Standard solution*. The *Sample solution* chromatogram may show other colored zones at R_F values lower than those of valerenic acid.

• B. The retention time of the valerenic acid peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Valerenic Acid*.

STRENGTH

• CONTENT OF VALERENIC ACID

Mobile phase: Methanol and water (77:27). Add 0.5 mL of phosphoric acid to each 100 mL of the mixture.

System suitability solution: 24 µg/mL of USP Valerenic Acid RS in methanol

Standard solution: 3.5 µg/mL of USP Valerenic Acid RS in methanol

Sample solution: Weigh NLT 20 Tablets, and pulverize with a mortar and pestle. Transfer a portion of the powder, nominally equivalent to 0.09 mg of valerenic acid, to a suitable flask. Add 25.0 mL of methanol, shake to disperse the powder, sonicate for 10 min, and centrifuge. Use the clear supernatant.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 225 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 30°

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Capacity factor, k': NLT 5, determined from valerenic acid

Tailing factor: NMT 2.0 for valerenic acid

Relative standard deviation: NMT 2.0% for valerenic acid

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of Extract as valerenic acid in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S \times V/W) \times (100/L_E) \times (A_W \times 100/L)$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Valerenic Acid RS in the *Standard solution* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of the sample of powdered Tablets used to prepare the *Sample solution* (mg)

A_W = average weight of the Tablets (mg/Tablet)

L_E = labeled amount of valerenic acid in 100 mg of the Extract used to prepare the Tablets (mg)

L = labeled amount of Extract per Tablet (mg/Tablet)

Acceptance criteria: 90.0%–120.0%

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** <2040>: Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements

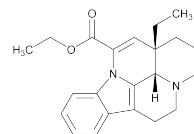
CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 10,000 cfu/g, and the total combined molds and yeasts count does not exceed 1000 cfu/g.
- **MICROBIAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meet the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the article from which the Tablets were prepared. The label also indicates the quantity, in mg, of Powdered Valerian Extract per Tablet and the content, in mg, of valerenic acid per 100 mg of Powdered Valerian Extract.
- **USP REFERENCE STANDARDS** <11>
 - USP Fluorescein RS
 - USP Valerenic Acid RS

Vinpocetine



$C_{22}H_{26}N_2O_2$ 350.45
Eburnamenine-14-carboxylic acid, ethyl ester, (3 α ,16 α)-;
Ethyl apovincamin-22-oate [42971-09-5].

DEFINITION

Vinpocetine contains NLT 98.5% and NMT 101.5% of $C_{22}H_{26}N_2O_2$, calculated on the dried basis.

IDENTIFICATION

• A. INFRARED ABSORPTION <197K>

- **B.** The retention time of the main peak of the *Sample solution* corresponds to that of the principal peak for the *Stock standard solution*, as obtained in the test for *Organic Impurities*.

ASSAY

• TITRIMETRY

(See *Titrimetry* <541>.)

Sample: 300 mg of Vinpocetine in 50 mL of a mixture of acetic anhydride and acetic acid (1:1)

Titrimetric system

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric

Analysis

Perform a blank determination, and make any necessary correction. Calculate the percentage of $C_{22}H_{26}N_2O_2$ in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100]/W$$

V = volume of titrant consumed by the *Sample* (mL)

B = volume of titrant consumed by the blank (mL)

N = normality of the titrant (mEq/mL)

F = equivalency factor, 350.5 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: 98.5%–101.5% on the dried basis

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** <281>: NMT 0.1%
- **HEAVY METALS, Method II** <231>: 10 ppm

Organic Impurities

• PROCEDURE

Ammonium acetate solution: 15.4 g/L ammonium acetate in water

Mobile phase: Acetonitrile and *Ammonium acetate solution* (55:45)

Sample solution: 1.00 mg/mL of Vinpocetine in *Mobile phase*

Stock standard solution: 0.02 mg/mL of USP Vinpocetine RS in *Mobile phase*

Standard solution 1: 0.12 mg/mL of USP Vinpocetine Related Compound A RS and 0.10 mg/mL each of USP Vinpocetine Related Compound B RS, USP Vinpocetine Related Compound C RS, and USP Vinpocetine Related Compound D RS in *Mobile phase*

Standard solution 2: Dilute 1.0 mL of *Stock standard solution* and 1.0 mL of *Standard solution 1* with *Mobile phase* to 20.0 mL.

Chromatographic system

(See *Chromatography* <621>, *System suitability*.)

Valine—see *Valine General Monographs*

Mode: LC

Detector: 280 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 1.0 mL/min

Chromatograph the *Stock standard solution* and *Standard solution 2*, and identify the vinpocetine peak and peaks due to the related compounds listed in *Impurity Table 1*.

Injection size: 15 μL (duplicate equal volumes)

System suitability

Sample: *Standard solution 2*

Suitability requirements

Resolution: NLT 2.0 between vinpocetine related compound B and vinpocetine related compound D

Analysis

Samples: *Sample solution* and *Standard solution 2*

Record the chromatograms for up to a minimum of three times the retention time of vinpocetine. Disregard any peak with an area less than 0.5 times the area of the peak due to vinpocetine in *Standard solution 2*. Calculate the percentage of vinpocetine related compounds A, B, C, and D in the portion of Vinpocetine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak response of each of the known impurities from the *Sample solution*
 r_S = peak response of the corresponding Standard for each of the known impurities from *Standard solution 2*
 C_S = concentration of the corresponding USP Reference Standard for each of the known impurities in *Standard solution 2* (mg/mL)
 C_U = concentration of Vinpocetine in the *Sample solution* (mg/mL)

Calculate the percentage of any unspecified individual impurity, as vinpocetine, in the portion of Vinpocetine taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak response of each of the unspecified impurities from the *Sample solution*
 r_S = peak response of vinpocetine from *Standard solution 2*
 C_S = concentration of USP Vinpocetine RS in *Standard solution 2* (mg/mL)
 C_U = concentration of Vinpocetine in the *Sample solution* (mg/mL)

Acceptance criteria: See *Impurity Table 1*.

Impurity Table 1

Name	Relative Retention Time	Acceptance Criteria, NMT (%)
Vinpocetine	1.00	—
Vinpocetine related compound A ^a	0.40	0.6
Vinpocetine related compound B ^b	0.75	0.5

^a Ethyl (1*2RS*,13*aSR*,13*bSR*)-13*a*-ethyl-12-hydroxy-2,3,5,6,12,13,13*a*,13*b*-octahydro-1*H*-indolo[3,2,1-*de*]pyrido[3,2,1-*ij*][1,5]naphthyridine-12-carboxylate (ethyl vincamine).

^b Methyl (13*aS*,13*bS*)-13*a*-ethyl-9-methoxy-2,3,5,6,13*a*,13*b*-hexahydro-1*H*-indolo[3,2,1-*de*]pyrido[3,2,1-*ij*][1,5]naphthyridine-12-carboxylate (apovincamine).

Impurity Table 1 (Continued)

Name	Relative Retention Time	Acceptance Criteria, NMT (%)
Vinpocetine related compound C ^c	0.83	0.3
Vinpocetine related compound D ^d	0.68	0.5
Unspecified individual impurity	—	0.1
Total impurities	—	1.0

^c Ethyl (13*aS*,13*bS*)-13*a*-ethyl-10-methoxy-2,3,5,6,13*a*,13*b*-hexahydro-1*H*-indolo[3,2,1-*de*]pyrido[3,2,1-*ij*][1,5]naphthyridine-12-carboxylate (methoxyvinpocetine).

^d Ethyl (12*RS*,13*aRS*,13*bRS*)-13*a*-ethyl-2,3,5,6,12,13,13*a*,13*b*-octahydro-1*H*-indolo[3,2,1-*de*]pyrido[3,2,1-*ij*][1,5]naphthyridine-12-carboxylate (dihydrovinpocetine).

SPECIFIC TESTS

- LOSS ON DRYING (731):** Dry a sample in a vacuum at 100° for 3 h: NMT 0.5%.
- OPTICAL ROTATION, Specific rotation (781S):** From +127.0° to +134.0°, determined at 20°.

Sample solution: 10 mg/mL in dimethylformamide

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at controlled room temperature.
- USP REFERENCE STANDARDS (11)**
 - USP Vinpocetine RS
 - USP Vinpocetine Related Compound A RS
 - USP Vinpocetine Related Compound B RS
 - USP Vinpocetine Related Compound C RS
 - USP Vinpocetine Related Compound D RS

Vitamin A—see *Vitamin A General Monographs*

Vitamin A Capsules—see *Vitamin A Capsules General Monographs*

Vitamin A Oral Liquid Preparation—see *Vitamin A Oral Liquid Preparation General Monographs*

Vitamin A Tablets—see *Vitamin A Tablets General Monographs*

Vitamin B₆ Tablets—see *Pyridoxine Hydrochloride Tablets General Monographs*

Vitamin C Tablets—see *Ascorbic Acid Tablets General Monographs*

Vitamin E—see *Vitamin E General Monographs*

Vitamin E Preparation—see *Vitamin E Preparation General Monographs*

Vitamin E Capsules—see *Vitamin E Capsules General Monographs*

Vitamin E Polyethylene Glycol Succinate—see *Vitamin E Polyethylene Glycol Succinate NF*

Oil-Soluble Vitamins Capsules

DEFINITION

Oil-Soluble Vitamins Capsules contain two or more of the following oil-soluble vitamins: Vitamin A, Vitamin D as Ergocalciferol (Vitamin D₂) or Cholecalciferol (Vitamin D₃), Vitamin E, Phytonadione (Vitamin K₁), and Beta Carotene. Capsules contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A (C₂₀H₃₀O) as retinol or esters of retinol in the form of retinyl acetate (C₂₂H₃₂O₂) or retinyl palmitate (C₃₆H₆₀O₂); vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O); vitamin E as alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅); phytonadione (C₃₁H₄₆O₂); and beta carotene (C₄₀H₅₆).

Oil-Soluble Vitamins Capsules contain no other vitamins or any minerals. They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

• VITAMIN A, Method 1

[NOTE—Where the use of a vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in *n*-hexane

System suitability stock solution: 15 µg/mL of retinyl palmitate in *n*-hexane

System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, equivalent to 5 Capsules, to a container having a polytetrafluoroethylene-lined screw cap. [NOTE—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent

to 5 Capsules, to a container having a polytetrafluoroethylene-lined screw cap.] Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [NOTE—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Dilute a volume of this solution with *n*-hexane to obtain a solution with a concentration of 15 µg/mL of vitamin A as retinol (C₂₀H₃₀O).

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection size: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area for all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution*. For products containing vitamin A acetate or vitamin A palmitate, calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₂H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)

• VITAMIN A, Method 2

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution: Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Mobile phase: *n*-Hexane and ethyl acetate (99.7:0.3)

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in 2,2,4-trimethylpentane

System suitability stock solution: 15 µg/mL of retinyl palmitate in 2,2,4-trimethylpentane

System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Using a sharp blade if necessary, carefully open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 30 µg of the labeled amount of cholecalciferol or ergocalciferol (vitamin D), to a container with a polytetrafluoroethylene-lined screw cap. If vitamin D is not present in the formulation, use a portion, equivalent to 90 mg of the labeled amount of vitamin E. If vitamin E is not present in the formulation, use a portion, equivalent to 2.5 mg of the labeled amount of vitamin A, as retinol. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate-pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of 3 *N* methanolic sulfuric acid solution, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. [NOTE—The supernatant is used for the determination of vitamin A, and also vitamin D and vitamin E, if present in the formulation.] If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1.5 mL/min

Injection size: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 8.0 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area of all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution*.

Calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₂H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration. The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)

• VITAMIN A, Method 3

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Extraction solvent: *n*-Hexane and methylene chloride (3:1)

Potassium hydroxide solution: 800 mg/mL of potassium hydroxide in water. [NOTE—Cautiously add the potassium hydroxide to the water. Mix, and cool.]

Diluent: 10 mg/mL of pyrogallol in alcohol

Mobile phase: *n*-Hexane and isopropyl alcohol (92:8)

Standard stock solution: 30 µg/mL of retinyl acetate from USP Vitamin A RS in *Diluent*. [NOTE—This solution may be stored in a refrigerator for 1 week.]

Standard solution: Dilute a volume of *Standard stock solution* with *Diluent* to obtain a concentration of 1 µg/mL of retinyl acetate from USP Vitamin A RS. Transfer 10.0 mL of this solution to a stoppered 125-mL flask, and add 5 mL of water, 5 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. Withdraw a portion of the organic layer for injection into the chromatograph. This *Standard solution* contains 0.34 µg/mL of retinol.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 1.5 mg of retinyl acetate, to a stoppered 125-mL flask. Add 5 mL of water, 15 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s or longer, if necessary, for complete extraction. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. [NOTE—Do not shake, because an emulsion may form.] Withdraw a portion of the organic layer, and dilute quantitatively, and stepwise if necessary, with *Extraction solvent*, to obtain a concentration of 0.34 µg/mL of retinol.

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 335 nm**Column:** 6.2-mm × 8-cm; packing L3**Column temperature:** 40°**Flow rate:** 4 mL/min**Injection size:** 50 µL**System suitability****Samples:** *Standard solution*[NOTE—The relative retention times for 13-*cis*-retinol and all-*trans*-retinol are about 0.92 and 1.0, respectively.]**Suitability requirements****Relative standard deviation:** NMT 5.0%**Analysis****Samples:** *Standard solution* and *Sample solution*Measure the peak areas for all-*trans*-retinol and 13-*cis*-retinol. Calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Capsules taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times (C_S/C_U) \times F \times 100$$

 r_{T1} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Sample solution* r_{T2} = sum of the areas of all-*trans*-retinol and 13-*cis*-retinol peaks from the *Standard solution* C_S = concentration of retinyl acetate (C₂₃H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (µg/mL) C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O) in the *Sample solution* (µg/mL) F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872**Acceptance criteria:** 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)• **CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D),***Method 1*

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)**Standard solution:** 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane**System suitability solution:** Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.**Sample solution:** Proceed as directed for the *Sample solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution retained as specified in the directions for the *Sample solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, if necessary, in vacuum at room temperature to obtain a solution with a concentration of 2 µg/mL of cholecalciferol or ergocalciferol.**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 265 nm**Column:** 4.6-mm × 15-cm; 3-µm packing L8**Flow rate:** 1 mL/min**Injection size:** 100 µL**System suitability****Samples:** *Standard solution* and *System suitability solution***Suitability requirements****Resolution:** NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution***Relative standard deviation:** NMT 3.0%, *Standard solution***Analysis****Samples:** *Standard solution* and *Sample solution*Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

 r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution* r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution* C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL) C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL) F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09**Acceptance criteria:** 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)• **CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D),***Method 2*

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution, Sodium ascorbate-pyrogallol solution, Lecithin solution, and Sample solution: Proceed as directed in *Vitamin A, Method 2*.**Mobile phase:** *n*-Hexane and tertiary butyl alcohol (98.75:1.25)**Standard solution:** 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in 2,2,4-trimethylpentane**System suitability solution:** Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 265 nm**Column:** 4.6-mm × 25-cm; 5-µm packing L24**Flow rate:** 1 mL/min**Injection size:** 40 µL**System suitability****Samples:** *Standard solution* and *System suitability solution***Suitability requirements****Resolution:** NLT 4.0 between the vitamin D form present and its corresponding precursor, *System suitability solution***Relative standard deviation:** NMT 3.0%, *Standard solution***Analysis****Samples:** *Standard solution* and *Sample solution*Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution* r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_s = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($\text{C}_{27}\text{H}_{44}\text{O}$) or ergocalciferol ($\text{C}_{28}\text{H}_{44}\text{O}$)

• **CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D),**

Method 3

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluted acetic acid: Glacial acetic acid solution (1 in 10) in water

Phenolphthalein solution: 10 mg/mL of phenolphthalein in alcohol

Potassium hydroxide solution: Slowly dissolve 14 g of potassium hydroxide in a mixture of 31 mL of dehydrated alcohol and 5 mL of water. Prepare fresh daily.

Extraction solvent: Methylene chloride and isopropyl alcohol (99.8:0.2)

Mobile phase: Acetonitrile and methanol (91:9)

Standard stock solution: 0.2 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in dehydrated alcohol. [NOTE—Prepare fresh every 4 weeks. Store in a freezer.]

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* by initially washing the column with 4.0 mL of a mixture of methylene chloride and isopropyl alcohol (4:1), followed by 5.0 mL of *Extraction solvent*. Do not allow the column to dry.]

Dilute a volume of the *Standard stock solution* with dehydrated alcohol to obtain a concentration of 5 $\mu\text{g/mL}$ of USP Cholecalciferol RS or USP Ergocalciferol RS. Prepare this solution fresh daily. Transfer 2.0 mL of this solution to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase extraction column containing silica packing with a sorbent mass-to-column volume ratio of 500 mg to 2.8 mL or equivalent, rinse the round-bottom flask with 1.0 mL of *Extraction solvent*, and transfer to the column. Elute the column with 2.0 mL of *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of *Extraction*

solvent, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 3*, through “calculate the net weight of the Capsule contents.” Transfer a portion of the Capsule contents, equivalent to 10 μg of ergocalciferol or cholecalciferol, to a stoppered 125-mL flask, and proceed as directed for the *Standard solution*, beginning with “Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*”.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm \times 25-cm; 5- μm packing L1

Column temperature: 27°

Flow rate: 0.7 mL/min

Injection size: 15 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 4.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($\text{C}_{27}\text{H}_{44}\text{O}$) or ergocalciferol ($\text{C}_{28}\text{H}_{44}\text{O}$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($\text{C}_{27}\text{H}_{44}\text{O}$) or ergocalciferol ($\text{C}_{28}\text{H}_{44}\text{O}$)

• **VITAMIN E, Method 1**

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water

Mobile phase: Methanol and *Solution A* (19:1)

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

System suitability solution: Prepare a 0.65-mg/mL solution of USP Ergocalciferol RS in methanol. Transfer 1.0 mL of this solution to a 100-mL volumetric flask containing 100 mg of USP Alpha Tocopheryl Acetate RS. Dissolve in 30 mL of methanol, with the aid of sonication if necessary, and dilute with methanol to volume. Store this solution in a refrigerator.

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution retained as specified in the directions for the *Sample solution* in *Vitamin A, Method 1* to a suitable container, and evaporate if necessary, in vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume, to

obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-μm packing L1

Flow rate: 2 mL/min

Injection size: 100 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for ergocalciferol and alpha tocopheryl acetate are about 0.5 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 12 between ergocalciferol and alpha tocopheryl acetate, *System suitability solution*

Tailing factor: Between 0.8 and 1.2, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• VITAMIN E, Method 2

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 240 mL of methanol with 10 mL of water followed by 0.5 mL of 50% phosphoric acid, and dilute with acetonitrile to 1000 mL.

System suitability solution: 2 mg/mL each of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, and USP Alpha Tocopheryl Acid Succinate RS in methanol

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

3 N methanolic sulfuric acid solution: Cautiously mix sulfuric acid and methanol (9 in 100) in a 100-mL volumetric flask. [NOTE—Dissolve in a portion of methanol, cool, and then dilute to final volume.]

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask. Add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 2*, through “calculate the net weight of the Capsule contents.” Transfer a portion of the Capsule contents, equivalent to 55 mg of vitamin E, to a container having a polytetrafluoroethylene-lined screw cap. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of 3 N *methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant layer. Transfer a volume of the supernatant 2,2,4-trimethylpentane layer to a suitable volumetric flask, the volume of the specimen withdrawn from the 2,2,4-trimethylpentane layer and the size of the volumetric flask being such that the final concentration of the *Sample solution* is equivalent to that of the *Standard solution*. Evaporate nearly to dryness, add several mL of methanol, and evaporate the remaining 2,2,4-trimethylpentane. Dilute with methanol to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 1.5 mL/min

Injection size: 25 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for alpha tocopheryl acid succinate, alpha tocopherol, and alpha tocopheryl acetate are about 0.6, 0.8, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 4.0 between alpha tocopheryl acid succinate and alpha tocopherol and NLT 3.0 between alpha tocopherol and alpha tocopheryl acetate, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane and the dilution factor to exchange the solvent from 2,2,4-trimethylpentane to methanol to calculate the nominal concentration.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **VITAMIN E, Method 3**

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile and ethyl acetate (1:1)

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in methanol

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 3* through “calculate the net weight of the Capsule contents”. Transfer a portion of the Capsule contents, equivalent to 8.0 mg of alpha tocopherol, to a glass-stoppered conical flask. Add 25.0 mL of water, 25.0 mL of dehydrated alcohol, and 3.5 g of potassium hydroxide pellets. Shake for 1 h in a water bath maintained at 55°, cool, and transfer with the aid of a minimum volume of water to a 125-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 60 s, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and add the rinsing to the hexane solution in the flask. Place the flask in a water bath maintained at 50°, and evaporate the hexane solution with the aid of a rotary evaporator to dryness. Immediately add 25.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 40°

Flow rate: 3 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for alpha tocopherol from the *Sample solution*

r_S = peak area for alpha tocopherol from the *Standard solution*

C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)

C_U = nominal concentration of alpha tocopherol the *Sample solution* (mg/mL)

[NOTE—Calculate the content of alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) by dividing the content, in mg/Capsule of

vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), by the factor 0.91 or 0.81, respectively.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl succinate ($C_{33}H_{54}O_5$)

• **PHYTONADIONE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP

Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP

Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution retained as specified in the directions for the *Sample solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, if necessary, in vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for alpha tocopheryl acetate and phytonadione are about 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas. Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of phytonadione from the *Sample solution*

r_S = peak area of phytonadione from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **BETA CAROTENE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Potassium hydroxide solution: Dissolve 58.8 g of potassium hydroxide in 50 mL of water.

Iodine solution: Transfer 10 mg of iodine to a 100-mL volumetric flask. Dissolve in cyclohexane, and dilute with cyclohexane to volume. Dilute 10 mL of this

solution with cyclohexane to 100 mL. [NOTE—Prepare this solution fresh daily.]

Sample solution A (for preparations containing beta carotene in oil solutions): Proceed as directed in *Vitamin A, Method 1*, except use cyclohexane instead of *n*-hexane as the extraction solvent, and dilute the filtered extracts with cyclohexane to obtain a concentration of 2 µg/mL of beta carotene.

Sample solution B (for preparations containing beta carotene in dry powder): Remove the contents of NLT 20 Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature. Add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower aqueous layer into the saponification flask. Add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume. Add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Vis

Analytical wavelength: 452 nm

Blank: Cyclohexane

Analysis

Sample: *Sample solution A* or *Sample solution B*

Determine the absorbance against the *Blank*. Calculate the percentage of the labeled amount of beta carotene ($C_{40}H_{56}$) in the portion of Capsules taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of *Sample solution A* or *Sample solution B*

F = absorptivity of beta carotene at 452 nm, 223

C_U = nominal concentration of beta carotene in *Sample solution A* or *Sample solution B* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

PERFORMANCE TESTS

• DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS

(2040): Meet the requirements

• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** (2091): Meet the requirements

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 3000 cfu/g, and the total combined molds and yeasts count does not exceed 300 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **LABELING:**¹ Label the Capsules to state that the product is Oil-Soluble Vitamins Capsules. The label also states the quantity of each vitamin/dosage unit and, where necessary, the chemical form in which it is present. Where the product contains vitamin E, the label also indicates whether it is the *d*- or *dl*- form. Where more than one assay method is given for a particular vitamin, the labeling states the assay method used only if *Method 1* is not used.

• USP REFERENCE STANDARDS (11)

USP Alpha Tocopherol RS

USP Alpha Tocopheryl Acetate RS

USP Alpha Tocopheryl Acid Succinate RS

USP Cholecalciferol RS

9,10-Secocholesta-5,7,10(19)-trien-3-ol, (3 β ,5Z,7E)-.

Cholecalciferol.

$C_{27}H_{44}O$ 384.64

USP Ergocalciferol RS

9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol, (3 β ,5Z,7E,22E)-.

Ergocalciferol.

$C_{28}H_{44}O$ 396.65

USP Phytonadione RS

1,4-Naphthalenedione, 2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)-, [R-[R*,R*-(E)]]-.

Phylloquinone.

$C_{31}H_{46}O_2$ 450.70

USP Vitamin A RS

Add the following:

▲ Oil-Soluble Vitamins Oral Solution

DEFINITION

Oil-Soluble Vitamins Oral Solution contains two or more of the following oil-soluble vitamins: Vitamin A, as retinol or esters of retinol in the form of retinyl acetate or retinyl palmitate; Vitamin D, as ergocalciferol (Vitamin D₂) or cholecalciferol (Vitamin D₃); Vitamin E, as alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate; Phytonadione (Vitamin K₁); and beta carotene. It contains NLT 90.0% and NMT 150.0% of the labeled amounts of vitamin A, as retinol equivalent ($C_{20}H_{30}O$); vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E, as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl

¹ USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$).

STRENGTH

• VITAMIN A

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution 1: 13 µg/mL of retinol from USP Retinyl Acetate RS in *n*-hexane

Standard solution 2: 13 µg/mL of retinol from USP Retinyl Palmitate RS in *n*-hexane

System suitability solution: Mix equal volumes of *Standard solution 1* and *Standard solution 2*.

Sample solution: Equivalent to 13 µg/mL of retinol from an accurately measured volume of Oral Solution in *n*-hexane

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; packing L8

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution 1* or *Standard solution 2* and *Sample solution*

Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the appropriate *Standard solution*

C_S = concentration of retinol in the appropriate *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of vitamin A as retinol equivalent ($C_{20}H_{30}O$)

• VITAMIN D

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample stock solution: Equivalent to 20 µg/mL of cholecalciferol or ergocalciferol from an accurately measured volume of Oral Solution in *n*-hexane

Sample solution: Transfer 5.0 mL of the *Sample stock solution* to a container having a polytetrafluoroethylene-lined screw cap and heat, with constant shaking, for 1 h in a water bath maintained at 60° to obtain a solution containing vitamin D (cholecalciferol or ergocalciferol) and its corresponding precursor. Cool, and dilute with *n*-hexane to obtain a solution containing 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection volume: 100 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of pre-vitamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–150.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• VITAMIN E

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution in water (1 in 100)

Mobile phase: Methanol and *Solution A* (19:1)

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Equivalent to 2.0 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate from an accurately measured volume of Oral Solution in methanol

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 50 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 1.5

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_s = peak area of the relevant vitamin E form from the *Standard solution*

C_s = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of vitamin E

• PHYTONADIONE (VITAMIN K₁)

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP

Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP

Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Equivalent to 20 µg/mL of phytonadione from an accurately measured volume of Oral Solution in methanol

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 50 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of phytonadione (C₃₁H₄₆O₂) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_s) \times (C_s/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_s = peak area from the *Standard solution*

C_s = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of phytonadione (C₃₁H₄₆O₂)

• BETA CAROTENE

[NOTE—Use low-actinic glassware.]

Mobile phase: Transfer 50 mg of butylated hydroxytoluene into a 1-L volumetric flask, and dissolve with 20 mL of 2-propanol. Add 0.2 mL of N-ethyl-diisopropylamine, 25 mL of 0.2% ammonium acetate solution, 455 mL of acetonitrile, and about 450 mL of methanol. Allow the solution to reach room temperature, and dilute with methanol to volume.

Diluent: 50 µg/mL of butylated hydroxytoluene in alcohol

System suitability solution: Transfer 20 mg of USP Beta Carotene System Suitability RS to a 50-mL volumetric flask. Add 1 mL of water, 4 mL of tetrahydrofuran, and sonicate for 5 min. Dilute with *Diluent* to volume and

sonicate for 5 min. Cool to room temperature, pass the suspension through a membrane filter of 0.45-µm pore size, and use the clear filtrate.

Standard stock solution: 60 µg/mL of USP Beta Carotene RS in tetrahydrofuran

Standard solution A: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, add 5.0 mL of tetrahydrofuran, and dilute with *Diluent* to volume.

Determine the concentration of *Standard solution A* from the concentration of *Standard solution B* as described below.

Standard solution B: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, and dilute with cyclohexane to volume. Prepare in triplicate.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Analytical wavelength: 457 nm

Cell path: 1 cm

Blank: Cyclohexane

Analysis

Sample: *Standard solution B*

Calculate the concentration of total beta carotene (mg/mL) as all-*trans*-beta carotene (C₄₀H₅₆) in *Standard solution B*. [NOTE—The concentration of *Standard solution B* equals the concentration of *Standard solution A*.]

$$\text{Result} = A/F$$

A = average absorbance of the three preparations of *Standard solution B*

F = absorptivity of pure all-*trans*-beta carotene in cyclohexane, 250

Sample stock solution: Transfer an accurately measured volume of Oral Solution equivalent to 20 mg of beta carotene to a 250-mL volumetric flask. Add 250 mg of butylated hydroxytoluene, 120 mL of methylene chloride, and 100 mL of alcohol. Shake the flask until the sample is completely dissolved or suspended. Let the mixture stand in the dark until it reaches room temperature (about 2 h). Add methylene chloride to volume, and shake again vigorously.

Sample solution: Dilute a volume of the *Sample stock solution* with a mixture of methylene chloride and *Diluent* (1:1) to obtain the final concentration of beta carotene of 3 µg/mL. Pass through a membrane filter of 0.45-µm pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV-Vis 448 nm

Column: 4.6-mm × 25-cm; 5-µm packing L68

Column temperature: 30°

Flow rate: 0.6 mL/min

Injection volume: 20 µL

System suitability

Samples: *System suitability solution* and *Standard solution A*

[NOTE—The approximate relative retention times of the components in the *System suitability solution* are listed in *Table 1*.]

Table 1

Name	Relative Retention Time	Relative Response Factor
All- <i>trans</i> -alpha carotene	0.93	1.1
All- <i>trans</i> -beta carotene	1.00	1
9- <i>cis</i> -Beta carotene	1.07	1
13- <i>cis</i> -Beta carotene	1.17	1.2
15- <i>cis</i> -Beta carotene	1.21	1.4

Suitability requirements

Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the reference chromatogram provided with the lot of USP Beta Carotene System Suitability RS being used.

Resolution: NLT 1.5 between beta carotene and alpha carotene and between beta carotene and 9-*cis*-beta carotene, *System suitability solution*

Tailing factor: NMT 2.0 for the beta carotene peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the beta carotene peak from replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*
Calculate the percentage of all-*trans*-beta carotene in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of all-*trans*-beta carotene from the *Sample solution*

r_S = peak area of all-*trans*-beta carotene from *Standard solution A*

C_S = concentration of all-*trans*-beta carotene in *Standard solution A* as determined by the spectrometric procedure

C_U = nominal concentration of beta carotene in the *Sample solution*

Acceptance criteria: 90.0%–150.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

OTHER COMPONENTS

- **ALCOHOL DETERMINATION, Method I <611>** (if present): 90.0%–120.0% of the labeled amount of C_2H_5OH

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS <2021>**: The total aerobic microbial count does not exceed 3×10^3 cfu/mL, and the combined molds and yeasts count does not exceed 3×10^2 cfu/mL.
- **ABSENCE OF SPECIFIED MICROORGANISMS <2022>**: Meets the requirements of the tests for absence of *Salmonella* species, and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, under an inert gas or with a minimum of headspace.
- **LABELING:**¹ The label states that the product is Oil-Soluble Vitamins Oral Solution. The label states the quantity of each vitamin present in a given volume of Oral Solution and, where necessary, the chemical form in which a vitamin is present. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*- form.

¹ USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *d*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *d*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

• USP REFERENCE STANDARDS <11>

USP Alpha Tocopherol RS
USP Alpha Tocopheryl Acetate RS
USP Alpha Tocopheryl Acid Succinate RS
USP Beta Carotene RS
USP Beta Carotene System Suitability RS
USP Cholecalciferol RS
USP Ergocalciferol RS
USP Phytonadione RS
USP Retinyl Acetate RS
USP Retinyl Palmitate RS▲^{USP36}

Oil-Soluble Vitamins Tablets**DEFINITION**

Oil-Soluble Vitamins Tablets contain two or more of the following oil-soluble vitamins: Vitamin A, Vitamin D as Ergocalciferol (Vitamin D₂) or Cholecalciferol (Vitamin D₃), Vitamin E, Phytonadione (Vitamin K₁), and Beta Carotene. Tablets contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A ($C_{20}H_{30}O$) as retinol or esters of retinol in the form of retinyl acetate ($C_{22}H_{32}O_2$) or retinyl palmitate ($C_{36}H_{46}O_2$); vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$).

Oil-Soluble Vitamins Tablets contain no other vitamins or any minerals. They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if Method 1 is not used.]

• VITAMIN A, Method 1

[NOTE—Where the use of a vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in *n*-hexane

System suitability stock solution: 15 µg/mL of retinyl palmitate in *n*-hexane

System suitability solution: Mix equal volumes of *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a container having a polytetrafluoroethylene-lined screw cap. Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [NOTE—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Dilute a 10-mL volume of this solution with *n*-hexane to obtain a solu-

tion with a concentration of 15 µg/mL of vitamin A as retinol (C₂₀H₃₀O).

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection size: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area for all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate in the chromatogram of the *Sample solution*. For products containing vitamin A acetate or vitamin A palmitate, calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O) in the portion of the Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₂H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)

• VITAMIN A, Method 2

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution: Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Mobile phase: *n*-Hexane and ethyl acetate (99.7:0.3)

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in 2,2,4-trimethylpentane

System suitability stock solution: 15 µg/mL of retinyl palmitate in 2,2,4-trimethylpentane

System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E, when present in the formulation.] Finely powder NLT 20 Tablets. If vitamin D is present in the formulation,

transfer a portion of the powder, equivalent to 30 µg of the labeled amount of cholecalciferol or ergocalciferol, to a container having a polytetrafluoroethylene-lined screw cap. If vitamin D is not present in the formulation, use a portion of the powder, equivalent to 90 mg of the labeled amount of vitamin E (as alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl hemisuccinate). If vitamin E is not present in the formulation, use a portion of the powder, equivalent to 2.5 mg of the labeled amount of retinyl acetate or retinyl palmitate. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of 3 N methanolic sulfuric acid solution, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. [NOTE—The supernatant is used for the determination of vitamin A, and also vitamin D and vitamin E, if present in the formulation.] If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1.5 mL/min

Injection size: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 8.0 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area of all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution*.

Calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₂H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in the USP Vitamin A RS, to retinol, 0.872

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration. The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)

• VITAMIN A, Method 3

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use

the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Extraction solvent: *n*-Hexane and methylene chloride (3:1)

Potassium hydroxide solution: 800 mg/mL of potassium hydroxide in water. [NOTE—Cautiously add potassium hydroxide in water. Mix, and cool.]

Diluent: 10 mg/mL of pyrogallol in alcohol

Mobile phase: *n*-Hexane and isopropyl alcohol (92:8)

Standard stock solution: 30 µg/mL of retinyl acetate from USP Vitamin A RS in *Diluent*. [NOTE—This solution may be stored in a refrigerator for 1 week.]

Standard solution: Dilute a volume of *Standard stock solution* with *Diluent* to obtain a concentration of 1 µg/mL of USP Vitamin A RS. Transfer 10.0 mL of this solution to a stoppered 125-mL flask, and add 5 mL of water, 5 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. Withdraw a portion of the organic layer for injection into the chromatograph. This *Standard solution* contains 0.34 µg/mL of retinol.

Sample solution: Finely powder a counted number of Tablets. Transfer a portion of the powder, equivalent to 1.5 mg of retinyl acetate, to a stoppered 125-mL flask. Add 5 mL of water, 15 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s or longer, if necessary, for complete extraction. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. [NOTE—Do not shake, because an emulsion may form.] Withdraw a portion of the organic layer, and dilute with *Extraction solvent* to obtain a concentration of 0.34 µg/mL of retinol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*).

Mode: LC

Detector: UV 335 nm

Column: 6.2-mm × 8-cm; packing L3

Column temperature: 40°

Flow rate: 4 mL/min

Injection size: 50 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for 13-*cis*-retinol and all-*trans*-retinol are 0.92 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for all-*trans*-retinol and 13-*cis*-retinol. Calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Tablets taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times (C_S/C_U) \times F \times 100$$

r_{T1} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Sample solution*

r_{T2} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₃H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)

• CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D),

Method 1

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate, if necessary, in vacuum at room temperature to obtain a concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*).

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection size: 100 µL

System suitability

Sample: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D),

Method 2

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution, Sodium ascorbate–pyrogallol solution, Lecithin solution, and Sample solution: Proceed as directed in *Vitamin A, Method 2*.

Mobile phase: *n*-Hexane and tertiary butyl alcohol (98.75:1.25)

Standard solution: 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in 2,2,4-trimethylpentane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1 mL/min

Injection size: 40 µL

System suitability

Sample: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D), Method 3

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluted acetic acid: Glacial acetic acid solution (1 in 10) in water

Phenolphthalein solution: 10 mg/mL of phenolphthalein in alcohol

Potassium hydroxide solution: Slowly dissolve 14 g of potassium hydroxide in a mixture of 31 mL of dehydrated alcohol and 5 mL of water. Prepare fresh daily.

Extraction solvent: Methylene chloride and isopropyl alcohol (99.8:0.2)

Mobile phase: Acetonitrile and methanol (91:9)

Standard stock solution: 0.2 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in dehydrated alcohol. [NOTE—Prepare fresh every 4 weeks. Store in a freezer.]

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* by initially washing the column with 4.0 mL of a mixture of methylene chloride and isopropyl alcohol (4:1), followed by 5.0 mL of *Extraction solvent*. Do not allow the column to dry.]

Dilute a volume of *Standard stock solution* with dehydrated alcohol to obtain a concentration of 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS. Prepare this solution fresh daily. Transfer 2.0 mL of this solution to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL, round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase extraction column containing silica packing with a sorbent mass-to-column volume ratio of 500 mg to 2.8 mL or equivalent, rinse the round-bottom flask with 1.0 mL of *Extraction solvent*, and transfer to the column. Elute the column with 2.0 mL of *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

Sample solution: Finely powder NLT 20 Tablets.

Transfer a portion of the powder, equivalent to 10 µg of cholecalciferol or ergocalciferol, to the stoppered 125-mL flask, and proceed as directed for the *Standard solution*, beginning with "Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*".

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 27°

Flow rate: 0.7 mL/min

Injection size: 15 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 4.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol

(C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
 r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
 C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (μg/mL)
 C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• VITAMIN E, Method 1

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water

Mobile phase: Methanol and *Solution A* (19:1)

System suitability solution: Prepare a 0.65-mg/mL solution of USP Ergocalciferol RS in methanol. Transfer 1.0 mL of this solution to a 100-mL volumetric flask containing 100 mg of USP Alpha Tocopheryl Acetate RS. Dissolve in 30 mL of methanol, with the aid of sonication if necessary, and dilute with methanol to volume. Store this solution in a refrigerator.

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-μm packing L1

Flow rate: 2 mL/min

Injection size: 100 μL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for ergocalciferol and alpha tocopheryl acetate are about 0.5 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 12 between ergocalciferol and alpha tocopheryl acetate, *System suitability solution*

Tailing factor: Between 0.8 and 1.2, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
 Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅)

• VITAMIN E, Method 2

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 240 mL of methanol with 10 mL of water followed by 0.5 mL of 50% phosphoric acid, and dilute with acetonitrile to 1000 mL.

System suitability solution: 2 mg/mL each of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, and USP Alpha Tocopheryl Acid Succinate RS in methanol

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Proceed as directed for *Sample solution* in *Vitamin A, Method 2*. Transfer a volume of the supernatant 2,2,4-trimethylpentane to a suitable volumetric flask, the volume of the specimen withdrawn from the 2,2,4-trimethylpentane and the size of the volumetric flask being such that the final concentration of the *Sample solution* is equivalent to that of the *Standard solution*. Evaporate nearly to dryness, add several mL of methanol, and evaporate the remaining 2,2,4-trimethylpentane. Dilute with methanol to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 1.5 mL/min

Injection size: 25 μL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for alpha tocopheryl acid succinate, alpha tocopherol, and alpha tocopheryl acetate are about 0.6, 0.8, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 4.0 between alpha tocopheryl acid succinate and alpha tocopherol; NLT 3.0 between alpha tocopherol and alpha tocopheryl acetate, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
 Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

- r_s = peak area of the relevant vitamin E form from the *Standard solution*
 C_s = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)
 C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane and the dilution factor to exchange the solvent from 2,2,4-trimethylpentane to methanol to calculate the nominal concentration.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **VITAMIN E, Method 3**

Diluent: Acetonitrile and ethyl acetate (1:1)

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in methanol

Sample solution: Finely powder NLT 20 Tablets.

Transfer a portion of the powder, equivalent to 8 mg of alpha tocopherol, to a 125-mL flask fitted with a ground-glass joint. Add 25.0 mL of water, 25.0 mL of dehydrated alcohol, and 3.5 g of potassium hydroxide pellets. Shake for 1 h in a water bath maintained at 55°. Cool, and transfer with the aid of a minimum volume of water to a 125-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 60 s, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and add the rinsing to the hexane solution in the flask. Place the flask in a water bath maintained at 50°, and evaporate the hexane solution with the aid of a rotary evaporator to dryness. Immediately add 25.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 40°

Flow rate: 3 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of alpha tocopherol from the *Sample solution*

r_S = peak area of alpha tocopherol from the *Standard solution*

C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin E as alpha tocopherol in the *Sample solution* (mg/mL)

[NOTE—Calculate the content of alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) by dividing the content, in mg/Tablet of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), by the factor 0.91 or 0.81, respectively.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **PHYTONADIONE, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP

Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP

Phytonadione RS from *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Transfer NLT 20 mL of the solution retained as specified in the directions for *Sample solution* in *Vitamin A, Method 1* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for alpha tocopheryl acetate and phytonadione are 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for phytonadione from the *Sample solution*

r_S = peak area for phytonadione from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **PHYTONADIONE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solvent: Methanol and isopropyl alcohol (19:1)

Mobile phase: Mix 800 mL of methanol, 200 mL of methylene chloride, 0.1 mL of glacial acetic acid, 1.36 g of zinc chloride, and 0.41 g of sodium acetate.

Internal standard solution: 5 µg/mL of menaquinone 4 (vitamin K₂) in *Solvent*. [NOTE—A concentrated stock solution of menaquinone 4 (100 µg/mL) can be stored for 2 months in a refrigerator.]

Standard stock solution: 5 µg/mL of USP Phytonadione RS, prepared by dissolving in methylene chloride with the aid of sonication, and diluting with *Solvent* to final volume

Standard solution: Transfer 1.0 mL of the *Standard stock solution* and 1.0 mL of the *Internal standard solution* to a suitable flask and dilute with *Solvent* to 5 mL. Pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Finely powder NLT 20 Tablets. To a centrifuge tube fitted with a cap transfer an amount of powder, not exceeding 800 mg and equivalent to an amount of phytonadione not exceeding 50 µg. Add 4 mL of water. Insert the stopper, and mix using a vortex mixer until the sample is dispersed. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 1 min while the preparation is still hot. Add 8 mL of alcohol, and swirl the contents to mix. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 2 min while the preparation is still hot. Cool to room temperature. Add a volume of *Internal standard solution*, equivalent to 1.0 mL per each 5 µg of the expected amount of phytonadione in the aliquot taken. Add 20.0 mL of petroleum ether, and cap the tube tightly. Shake or mix using a vortex mixer for 15 min to thoroughly mix the contents. Centrifuge to separate the two layers. Transfer a volume of the top layer of petroleum ether, equivalent to 5–50 µg of the nominal amount of phytonadione, to an appropriate flask. Place the flask in a water bath at 35°–45°, and evaporate the solvent under a stream of nitrogen until an oily residue is left. Dissolve the residue in a volume of *Solvent* to obtain a concentration of 1 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Fluorometric detector set at 320 nm for excitation and 420 nm for emission

Column: 4.6-mm × 25-cm; 5-µm, end-capped packing L1, and a postcolumn reactor constituted with a 4.6-mm × 3-cm PEEK column tightly packed with zinc powder. [NOTE—Prepare the postcolumn reactor daily, or as necessary, to meet the system suitability requirements.]

Flow rate: 1 mL/min

Injection size: 25 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for the internal standard and phytonadione are 1.0 and 1.4, respectively.]

Suitability requirements

Column efficiency: NLT 2500 theoretical plates for the phytonadione peak

Tailing factor: NMT 1.5 for the phytonadione peak

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of phytonadione (C₃₁H₄₆O₂) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of phytonadione to that of the internal standard from the *Sample solution*

R_S = peak response ratio of phytonadione to that of the internal standard from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of labeled amount of phytonadione (C₃₁H₄₆O₂)

• **BETA CAROTENE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Potassium hydroxide solution: Dissolve 58.8 g of potassium hydroxide in 50 mL of water.

Iodine solution: 0.01 mg/mL of iodine in cyclohexane.

[NOTE—Prepare this solution fresh daily]

Sample solution: Weigh NLT 20 Tablets. Grind the Tablets to a fine powder, and transfer a quantity of the powder, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature, add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower aqueous layer into the saponification flask, add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume, add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Vis

Analytical wavelength: 452 nm

Blank: Cyclohexane

Analysis

Sample: *Sample solution*

Determine the absorbance against the *Blank*. Calculate the percentage of the labeled amount of beta carotene (C₄₀H₅₆) in the portion of Tablets taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of the *Sample solution*
 F = absorptivity of beta carotene at 452 nm, 223
 C_U = nominal concentration of beta carotene in the
Sample solution (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** (2040): Meet the requirements for *Disintegration*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** (2091): Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 3000 cfu/g, and the combined molds and yeasts count does not exceed 300 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:**¹ Label the Tablets to state that the product is Oil-Soluble Vitamins Tablets. The label also states the quantity of each vitamin/dosage unit and where necessary the chemical form in which it is present. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*-form. Where more than one assay method is given for a particular vitamin, the labeling states the assay method used only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** (11)
 - USP Alpha Tocopherol RS
 - USP Alpha Tocopheryl Acetate RS
 - USP Alpha Tocopheryl Acid Succinate RS
 - USP Cholecalciferol RS
 - 9,10-Secocholesta-5,7,10(19)-trien-3-ol, (3 β ,5Z,7E)-. Cholecalciferol.
 $C_{27}H_{44}O$ 384.64
 - USP Ergocalciferol RS
 - 9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol, (3 β ,5Z,7E,22E)-. Ergocalciferol.
 $C_{28}H_{44}O$ 396.65
 - USP Phytonadione RS
 - 1,4-Naphthalenedione, 2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)-, [R-[R*,R*-(E)]]-. Phylloquinone.
 $C_{31}H_{46}O_2$ 450.70
 - USP Vitamin A RS

¹ USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 μ g of all-*trans*-retinol (vitamin A alcohol) or 0.344 μ g of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 μ g of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 μ g of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 μ g of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 μ g of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

Add the following:

▲ Oil-Soluble Vitamins with Minerals Capsules

DEFINITION

Oil-Soluble Vitamins with Minerals Capsules contain two or more of the following oil-soluble vitamins: Vitamin A, as retinol or esters of retinol in the form of retinyl acetate or retinyl palmitate; Vitamin D as ergocalciferol (Vitamin D₂) or cholecalciferol (Vitamin D₃); Vitamin E, as alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate; Phytonadione (Vitamin K₁); beta carotene; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Capsules contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A, as retinol equivalent ($C_{20}H_{30}O$); vitamin D, as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$); vitamin E, as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$); NLT 90.0% and NMT 125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

• VITAMIN A

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution 1: 13 μ g/mL of retinol from USP Retinyl Acetate RS in *n*-hexane

Standard solution 2: 13 μ g/mL of retinol from USP Retinyl Palmitate RS in *n*-hexane

System suitability solution: Mix equal volumes of *Standard solution 1* and *Standard solution 2*.

Sample solution: Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, equivalent to 5 Capsules, to a container having a polytetrafluoroethylene-lined screw cap. [NOTE—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent to 5 Capsules, to a container having a polytetrafluoroethylene-lined screw cap.] Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min in a water bath maintained at 60°. Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Further dilute this solution with *n*-hexane to obtain a solution with a concentration of 13 μ g/mL of vitamin A as retinol ($C_{20}H_{30}O$).

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; packing L8

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution 1* or *Standard solution 2* and *Sample solution*

Calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the appropriate *Standard solution*

C_S = concentration of retinol in the appropriate *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A as retinol equivalent (C₂₀H₃₀O)

• **VITAMIN D**

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample solution: Transfer NLT 20 mL of a solution prepared as directed for the *Sample solution* in *Vitamin A* to a suitable container, and concentrate, if necessary, in vacuum at room temperature to obtain a solution with an expected concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection volume: 100 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of pre-vitamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• **VITAMIN E**

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water

Mobile phase: Methanol and *Solution A* (19:1)

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 50 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 1.5

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E

• **PHYTONADIONE (VITAMIN K₁)**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP

Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution in Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 50 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of phytonadione (C₃₁H₄₆O₂) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione (C₃₁H₄₆O₂)

• BETA CAROTENE

[NOTE—Use low-actinic glassware.]

Mobile phase: Transfer 50 mg of butylated hydroxytoluene into a 1-L volumetric flask, and dissolve with 20 mL of 2-propanol. Add 0.2 mL of N-ethyldiisopropylamine, 25 mL of 0.2% ammonium acetate solution, 455 mL of acetonitrile, and about 450 mL of methanol. Allow the solution to reach to room temperature, and dilute with methanol to volume.

Diluent: 50 µg/mL of butylated hydroxytoluene in alcohol

System suitability solution: Transfer 20 mg of USP Beta Carotene System Suitability RS to a 50-mL volumetric flask. Add 1 mL of water, 4 mL of tetrahydrofuran, and sonicate for 5 min. Dilute with *Diluent* to volume, and sonicate for 5 min. Cool to room temperature, pass the suspension through a membrane filter of 0.45-µm pore size, and use the clear filtrate.

Standard stock solution: 60 µg/mL of USP Beta Carotene RS in tetrahydrofuran

Standard solution A: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, add 5.0 mL of tetrahydrofuran, and dilute with *Diluent* to volume.

Determine the concentration of *Standard solution A* from the concentration of *Standard solution B* as described below.

Standard solution B: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, and dilute with cyclohexane to volume. Prepare in triplicate.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Analytical wavelength: 457 nm

Cell path: 1 cm

Blank: Cyclohexane

Analysis

Sample: *Standard solution B*

Calculate the concentration of total beta carotene (mg/mL) as all-*trans*-beta carotene (C₄₀H₅₆) in *Standard solution B*. [NOTE—The concentration of *Standard solution B* equals the concentration of *Standard solution A*.]

$$\text{Result} = A/F$$

A = average absorbance of the three preparations of *Standard solution B*

F = absorptivity of pure all-*trans*-beta carotene in cyclohexane, 250

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution in Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Dissolve the residue in a mixture of methylene chloride and *Diluent* (1:1), and dilute with the same mixture to obtain a concentration of 3 µg/mL of beta carotene. Pass through a membrane filter of 0.45-µm pore size if necessary.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV-Vis 448 nm

Column: 4.6-mm × 25-cm; 5-µm packing L68

Column temperature: 30°

Flow rate: 0.6 mL/min

Injection volume: 20 µL

System suitability

Samples: *System suitability solution* and *Standard solution A*

[NOTE—The approximate relative retention times of the components in the *System suitability solution* are listed in *Table 1*.]

Table 1

Name	Relative Retention Time	Relative Response Factor
All- <i>trans</i> -alpha carotene	0.93	1.1
All- <i>trans</i> -beta carotene	1.00	1
9- <i>cis</i> -Beta carotene	1.07	1
13- <i>cis</i> -Beta carotene	1.17	1.2
15- <i>cis</i> -Beta carotene	1.21	1.4

Suitability requirements

Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the reference chromatogram provided with the lot of USP Beta Carotene System Suitability RS being used.

Resolution: NLT 1.5 between beta carotene and alpha carotene and between beta carotene and 9-*cis*-beta carotene, *System suitability solution*

Tailing factor: NMT 2.0 for the beta carotene peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the beta carotene peak from replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*

Calculate the percentage of all-*trans*-beta carotene in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_u = peak area of all-*trans*-beta carotene from the *Sample solution*
 r_s = peak area of all-*trans*-beta carotene from *Standard solution A*
 C_s = concentration of all-*trans*-beta carotene in *Standard solution A* as determined by spectrometric procedure
 C_u = nominal concentration of beta carotene in the *Sample solution*

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

• **CALCIUM, Method 1**

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from the *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Polysorbate 80 solution: Polysorbate 80 and alcohol (1:10)

Sample solution: Transfer 5 Capsules to a 100-mL volumetric flask. [NOTE—For hard gelatin Capsules, weigh NLT 20 Capsules. Open the Capsules, without loss of shell material, and transfer the contents to a suitable container. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and allow the Capsule shells to dry. Weigh the empty Capsule shells, calculate the net weight of the Capsule contents, and transfer a portion of the Capsule contents, equivalent to 5 Capsules, to a 100-mL volumetric flask.] Add 15 mL of water, 10 mL of 6 N hydrochloric acid, and 1 mL of *Polysorbate 80 solution* to the flask. Heat on a hot plate or steam bath, with intermittent swirling, until the Capsules are completely disintegrated or the contents are dissolved. Boil gently for an additional 15 min. Cool, dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution with 0.125 N hydrochloric acid, to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Calcium emission line at 422.7 nm

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in mg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Capsules taken:

$$\text{Result} = (C/C_u) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_u = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, Method 1**

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 2.5 µg/mL of chromium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Chromium emission line at 357.9 nm

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Capsules taken:

$$\text{Result} = (C/C_u) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

C_u = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, Method 1**

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% solution of nitric acid, and dilute with a 1% solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

Standard stock solution: 100 µg/mL of copper from the *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 2 µg/mL of copper and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Copper emission line at 324.7 nm

Lamp: Copper hollow-cathode
Flame: Air-acetylene
Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (C_u) in the portion of Capsules taken:

$$\text{Result} = (C/C_u) \times 100$$

C = measured concentration of copper in the *Sample solution* ($\mu\text{g/mL}$)

C_u = nominal concentration of copper in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (C_u)

• FLUORIDE, Method 1

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water contained in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 $\mu\text{g/mL}$ of fluoride from a quantity of sodium fluoride in water, previously dried at 100° for 4 h and cooled in a desiccator

Intermediate stock solution A: 100 $\mu\text{g/mL}$ of fluoride from the *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 $\mu\text{g/mL}$ of fluoride from the *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of 3 M *sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 $\mu\text{g/mL}$ of fluoride.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the mixed Capsule contents, equivalent to 200 μg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of 3 M *sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute with water to volume.

Analysis

Samples: *Standard solutions* and *Sample solution*
 To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see pH (791)), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ± 0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer having an

insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in $\mu\text{g/mL}$, of the *Standard solutions* versus potential, in mV. From the standard response curve so obtained and the measured potential of the *Sample solution*, determine the concentration, C , in $\mu\text{g/mL}$, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (C/C_u) \times 100$$

C = measured concentration of fluoride in the *Sample solution* ($\mu\text{g/mL}$)

C_u = nominal concentration of fluoride in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• FLUORIDE, Method 2

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 $\mu\text{g/mL}$ of USP Sodium Fluoride RS in water. This solution contains 100 $\mu\text{g/mL}$ of fluoride.

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with one column volume of methanol followed by one column volume of *pH 10.0 buffer*. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.] Transfer 10.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1 . Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask, add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with *pH 10.0 buffer* to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL container. If necessary, remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 1 mg of fluorine, to a 100-mL volumetric flask. Add 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1 , and dilute with water to 100 mL. Proceed as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Conductivity

Columns

Guard: 4.6-mm × 3-cm; packing L17

Analytical: 7.8-mm × 30-cm; packing L17

Flow rate: 0.5 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE, Method 1**

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis

Sample: Capsules

Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the contents, equivalent to 3 mg of iodine, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.]

Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.

Calculate the percentage of the labeled amount of iodine (I) in the portion of Capsules taken:

$$\text{Result} = V \times N_A \times F \times I_{me} \times (A_w/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)

N_A = actual normality of the sodium thiosulfate solution used

F = correction factor to convert mg to µg, 1000 µg/mL

I_{me} = milliequivalent of I, 21.16 mg/meq

A_w = average weight of the Capsules content

W = weight of the sample of Capsules content taken

L = labeled amount of iodine (µg/Capsule)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IODIDE, Method 2**

Analysis: Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Iodide*.

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON, Method 1**

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain a concentration of 5 µg/mL of iron and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Iron emission line at 248.3 nm

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (µg/mL)

C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM, Method 1**

Lanthanum chloride solution: Prepare as directed in *Calcium, Method 1*.

Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a known concentration of 1000 µg/mL of magnesium.

Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 0.4 µg/mL of magnesium.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Magnesium emission line at 285.2 nm

Lamp: Magnesium hollow-cathode

Flame: Air-acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)

C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE, Method 1**

Manganese standard stock solution: Transfer 1.00 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of manganese.

Standard stock solution: 50 µg/mL of manganese from the *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 1 µg/mL of manganese and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Manganese emission line at 279.5 nm

Lamp: Manganese hollow-cathode

Flame: Air-acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)

C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM, Method 1**

Diluent: 20 mg/mL of ammonium chloride in water
Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of molybdenum.

Standard stock solution: 100 µg/mL of molybdenum from the *Molybdenum standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 µg/mL of molybdenum.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except take a number of Capsules or a portion of Capsule contents equivalent to 1000 µg of molybdenum and make appropriate dilutions to obtain a final concentration of 10 µg/mL of molybdenum, omitting the addition of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Molybdenum emission line at 313.3 nm

Lamp: Molybdenum hollow-cathode

Flame: Nitrous oxide-acetylene

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* (µg/mL)

C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **MOLYBDENUM, Method 2**

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% Stannous chloride solution: Transfer 40 mg of stannous chloride to a beaker, add 20 mL of 6.5 N hydrochloric acid solution, and heat the solution until the stannous chloride is dissolved. Cool, and dilute with water to 100 mL.

Diluted stannous chloride solution: 20% *Stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.

Standard solution: 20 µg/mL of molybdenum in water

Sample: Remove the contents of a counted number of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Use a quantity of the Capsule contents, equivalent to 40 µg of molybdenum.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Analytical wavelength: 465 nm

Cell: 1 cm

Blank: Amyl alcohol

Analysis

Samples: *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to complete 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of 20% *Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layer from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the *Sample*

A_S = absorbance of the *Standard solution*

V = volume of the *Standard solution* analyzed, 2.0 mL

C_S = concentration of molybdenum in the *Standard solution* (µg/mL)

M_U = nominal amount of molybdenum in the *Sample* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• PHOSPHORUS, Method 1

Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5: 100), and mix.

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, and then dilute with the *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add one drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of phosphorus.

Standard solution: 20 µg/mL of phosphorus from the *Phosphorus standard stock solution* diluted with water

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Vis

Analytical wavelength: 650 nm

Cell: 1 cm

Analysis

Samples: *Standard solution* and *Sample solution*

To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

C_S = concentration of phosphorus in the *Standard solution* (µg/mL)

C_U = nominal concentration of phosphorus in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• POTASSIUM

Potassium standard solution: 100 µg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 µg/mL of potassium from the *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.

Polysorbate 80 solution: Prepare as directed in *Calcium*, Method 1.

Sample solution: Proceed as directed in *Calcium*, Method 1, except prepare the *Sample solution* to contain 1.5 µg/mL of potassium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry
Analytical wavelength: Potassium emission line at 766.5 nm
Lamp: Potassium hollow-cathode
Flame: Air–acetylene
Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (*K*) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* (µg/mL)

C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (*K*)

• SELENIUM, Method 1

Diluent: Prepare as directed in *Molybdenum, Method 1*.

Selenium standard solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 µg/mL of selenium.

Standard stock solution: 100 µg/mL of selenium from the *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions with concentrations of 5.0, 10.0, and 25.0 µg/mL of selenium.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 1000 µg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry
Analytical wavelength: Selenium emission line at 196.0 nm

Lamp: Selenium hollow-cathode
Flame: Air–acetylene
Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (*Se*) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* (µg/mL)

C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (*Se*)

• SELENIUM, Method 2

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% Ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, then add hydroxylamine hydrochloride, and dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 µg/mL of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10 mL of the *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid.

[CAUTION—Exercise care at this stage, because perchloric acid reaction becomes vigorous.] Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid, and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask. Add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot

plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV

Analytical wavelength: 380 nm

Cell: 1 cm

Blank: 1 mL of perchloric acid and 1 mL of Hydrochloric acid solution diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*

Treat the *Sample solution*, the *Standard solution*, and the *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with 50% *Ammonium hydroxide solution* to a pH of 1.1 ± 0.1 . Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbances of the cyclohexane layer from the *Sample solution*

A_S = absorbances of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• ZINC, Method 1

Zinc standard solution: 1000 µg/mL of zinc from zinc oxide in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from *Zinc standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Polysorbate 80 solution: Prepare as directed in *Calcium, Method 1*.

Sample solution: Proceed as directed in *Calcium, Method 1*, except prepare the *Sample solution* to contain 2 µg/mL of zinc and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Zinc emission line at 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air-acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• BORON, NICKEL, TIN, and VANADIUM, Method 1; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS, and ZINC, Method 2; MOLYBDENUM and SELENIUM, Method 3

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding one volume of *Stock aqua regia solution* to two volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% nitric acid solution, 1000 mg/L of scandium in 5% nitric acid solution, and *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% nitric acid solution to obtain a solution having final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/L of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% hydrochloric acid solution to obtain a solution having final concentrations of about 200 mg/L of boron, and 100 mg/L each of chromium, molybdenum, nickel, selenium, tin, and vanadium.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent*, to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution: Weigh, then transfer 5 Capsules to a 250-mL volumetric flask, and heat gently on a hot plate until the contents begin to release. Cautiously add 25 mL of *Stock aqua regia solution* in 5-mL increments, and swirl. Heat, continue to swirl until the Capsules dissolve into the acid, immediately remove from the heat source, and add 150 mL of water. Cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube, using a 5-µm pore size nylon syringe

filter. If necessary, make any further adjustments using the *Diluent*.

Instrumental conditions

(See *Plasma Spectrochemistry* <730>.)

Mode: Inductively coupled plasma spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

[NOTE—Analyze the *System suitability solution*, and obtain the response as directed in the *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*.

Calculate the percentage of the labeled amount for each mineral taken:

$$\text{Result} = C \times (V/W) \times F \times (C_w/L) \times 100$$

C = measured concentration of the *Sample solution* (mg/L)

V = volume of the *Sample solution* (L)

W = sample weight (mg)

F = dilution factor of the *Sample solution*

C_w = average Capsule weight (mg)

L = labeled amount per Capsule (mg)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); and 90.0%–160.0% of the labeled amounts of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION** <2040>: Meet the requirements for *Dissolution*

• **WEIGHT VARIATION** (2091): Meet the requirements

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **LABELING:**¹ The label states that the product is Oil-Soluble Vitamins with Minerals Capsules. The label also states the quantity of each vitamin and mineral per dosage unit and, where necessary, the chemical form in which a vitamin is present and also states the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*- form. Where more than one assay method is given for a particular vitamin or mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.

• USP REFERENCE STANDARDS <11>

USP Alpha Tocopherol RS

USP Alpha Tocopheryl Acetate RS

USP Alpha Tocopheryl Acid Succinate RS

USP Beta Carotene RS

USP Beta Carotene System Suitability RS

USP Cholecalciferol RS

USP Ergocalciferol RS

USP Phytonadione RS

USP Retinyl Palmitate RS

USP Sodium Fluoride RS

USP Vitamin A RS▲ USP36

Add the following:

▲ Oil-Soluble Vitamins with Minerals Oral Solution

DEFINITION

Oil-Soluble Vitamins with Minerals Oral Solution contains two or more of the following oil-soluble vitamins: Vitamin A, as retinol or esters of retinol in the form of retinyl acetate or retinyl palmitate; Vitamin D as ergocalciferol (Vitamin D₂) or cholecalciferol (Vitamin D₃); Vitamin E, as alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate; Phytonadione (Vitamin K₁); beta carotene; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: chromium, fluorine, iodine, iron, magnesium, manganese, molybdenum, and zinc. It contains NLT 90.0% and NMT 150.0% of the labeled amounts of vitamin A, as retinol equivalent (C₂₀H₃₀O); vitamin D, as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O); vitamin E, as alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₂), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅); phytonadione (C₃₁H₄₆O₂); and beta carotene (C₄₀H₅₆); NLT 90.0% and NMT 160.0% of the labeled amounts of chromium (Cr), fluorine (F), iodine (I), and molybdenum (Mo); and NLT 90.0% and NMT 125.0% of the labeled amounts of iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn).

¹ USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

STRENGTH**• VITAMIN A**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution 1: 13 µg/mL of retinol from USP Retinyl Acetate RS in *n*-hexane

Standard solution 2: 13 µg/mL of retinol from USP Retinyl Palmitate RS in *n*-hexane

System suitability solution: Mix equal volumes of *Standard solution 1* and *Standard solution 2*.

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 3.25 mg of retinol, to a separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 100 mL of *n*-hexane, insert the stopper, and shake for 1 min. Allow the layers to separate, drain the aqueous layer into another separatory funnel, and repeat the extraction with 100 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL volumetric flask. Rinse the funnel and sodium sulfate with *n*-hexane, and add the rinsing to the hexane solution in the flask. Dilute the extracts in the volumetric flask with *n*-hexane to volume to obtain a solution with a concentration of 13 µg/mL of vitamin A as retinol (C₂₀H₃₀O).

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; packing L8

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution 1* or *Standard solution 2* and *Sample solution*

Calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the appropriate *Standard solution*

C_S = concentration of retinol in the appropriate *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of vitamin A as retinol equivalent (C₂₀H₃₀O)

• VITAMIN D

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample stock solution: Using an accurately measured volume of Oral Solution, equivalent to 5.0 mg of cholecalciferol or ergocalciferol, proceed as directed for the *Sample solution* in *Vitamin A* to obtain a solution with a concentration of 20 µg/mL of cholecalciferol or ergocalciferol in *n*-hexane.

Sample solution: Transfer 5.0 mL of the *Sample stock solution* to a container having a polytetrafluoroethylene-lined screw cap. Heat, with constant shaking, for 1 h in a water bath maintained at 60° to obtain a solution containing vitamin D (cholecalciferol or ergocalciferol) and its corresponding precursor. Cool, and dilute with *n*-hexane to obtain a solution containing 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection volume: 100 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of pre-vitamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–150.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• VITAMIN E

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water

Mobile phase: Methanol and *Solution A* (19:1)

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC
Detector: UV 291 nm
Column: 4.6-mm × 15-cm; 5-μm packing L1
Flow rate: 1 mL/min
Injection volume: 50 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 1.5

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of vitamin E

• **PHYTONADIONE (VITAMIN K₁)**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 μg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 μg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 μg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 20 μg/mL of phytonadione.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; 5-μm packing L1

Flow rate: 1 mL/min

Injection volume: 50 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (μg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **BETA CAROTENE**

[NOTE—Use low-actinic glassware.]

Mobile phase: Transfer 50 mg of butylated hydroxytoluene into a 1-L volumetric flask, and dissolve with 20 mL of 2-propanol. Add 0.2 mL of *N*-ethyl-diisopropylamine, 25 mL of 0.2% ammonium acetate solution, 455 mL of acetonitrile, and about 450 mL of methanol. Allow the solution to reach room temperature, and dilute with methanol to volume.

Diluent: 50 μg/mL of butylated hydroxytoluene in alcohol

System suitability solution: Transfer 20 mg of USP Beta Carotene System Suitability RS to a 50-mL volumetric flask. Add 1 mL of water, 4 mL of tetrahydrofuran, and sonicate for 5 min. Dilute with *Diluent* to volume, and sonicate for 5 min. Cool to room temperature, pass the suspension through a membrane filter of 0.45-μm pore size, and use the clear filtrate.

Standard stock solution: 60 μg/mL of USP Beta Carotene RS in tetrahydrofuran

Standard solution A: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, add 5.0 mL of tetrahydrofuran, and dilute with *Diluent* to volume.

Determine the concentration of *Standard solution A* from the concentration of *Standard solution B* as described below.

Standard solution B: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, and dilute with cyclohexane to volume. Prepare in triplicate.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Analytical wavelength: 457 nm

Cell path: 1 cm

Blank: Cyclohexane

Analysis

Sample: *Standard solution B*

Calculate the concentration of total beta carotene (mg/mL) as all-*trans*-beta carotene ($C_{40}H_{56}$) in *Standard solution B*. [NOTE—The concentration of *Standard solution B* equals the concentration of *Standard solution A*.]

$$\text{Result} = A/F$$

A = average absorbance of the three preparations of *Standard solution B*

F = absorptivity of pure all-*trans*-beta carotene in cyclohexane, 250

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Dissolve the residue in a mixture of methylene chloride and *Diluent* (1:1), and dilute with the same mixture to obtain a concentration of 3 μg/mL of beta carotene. Pass through a membrane filter of 0.45-μm pore size if necessary.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC
Detector: UV-Vis 448 nm
Column: 4.6-mm × 25-cm; 5-μm packing L68
Column temperature: 30°
Flow rate: 0.6 mL/min
Injection volume: 20 μL

System suitability

Samples: *System suitability solution* and *Standard solution A*

[NOTE—The approximate relative retention times of the components in the *System suitability solution* are listed in Table 1.]

Table 1

Name	Relative Retention Time	Relative Response Factor
All-trans-alpha carotene	0.93	1.1
All-trans-beta carotene	1.00	1
9-cis-Beta carotene	1.07	1
13-cis-Beta carotene	1.17	1.2
15-cis-Beta carotene	1.21	1.4

Suitability requirements

Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the reference chromatogram provided with the lot of USP Beta Carotene System Suitability RS being used.

Resolution: NLT 1.5 between beta carotene and alpha carotene and between beta carotene and 9-cis-beta carotene, *System suitability solution*

Tailing factor: NMT 2.0 for the beta carotene peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the beta carotene peak from replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*

Calculate the percentage of all-trans-beta carotene in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of all-trans-beta carotene from the *Sample solution*

r_S = peak area of all-trans-beta carotene from *Standard solution A*

C_S = concentration of all-trans-beta carotene in *Standard solution A* as determined by spectrometric procedure

C_U = nominal concentration of beta carotene in the *Sample solution*

Acceptance criteria: 90.0%–150.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

• CHROMIUM

Chromium standard solution: 1000 μg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.

Standard stock solution: 10 μg/mL of chromium from the *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 μg/mL of chromium.

Sample solution: Dilute an accurately measured volume of Oral Solution to obtain a solution equivalent to 2.5 μg/mL of chromium in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Lamp: Chromium hollow-cathode

Flame: Air-acetylene

Analytical wavelength: 357.9 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in μg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, C , in μg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of chromium in the *Standard solution* (μg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• FLUORIDE

[NOTE—Use plastic containers throughout this procedure.]

Ascorbic acid solution: 70 mg/mL of ascorbic acid in water

Mobile phase: Alcohol, water, and 1 N sulfuric acid (50:449:1)

Standard stock solution: 220 μg/mL of USP Sodium Fluoride RS in water. This solution contains 100 μg/mL of fluoride.

Standard solution: Transfer 5.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add 2 mL of *Ascorbic acid solution*, 10 mL of alcohol, and about 70 mL of water, and mix. Adjust with 1 N sodium hydroxide to a pH of 4.25 ± 0.05 . Dilute with water to volume, and mix to obtain 5 μg/mL of fluoride solution.

Sample solution: Transfer an accurately measured volume of the Oral Solution, equivalent to 0.5 mg of fluoride, to a 100-mL volumetric flask. Add 1 drop of hydrochloric acid, 10 mL of alcohol, and about 75 mL of water, and mix. Adjust with 1 N sodium hydroxide to a pH of 4.25 ± 0.05 . Dilute with water to volume.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Conductivity

Columns

Guard: 4.6-mm × 3-cm; packing L17

Analytical: 7.8-mm × 30-cm; packing L17

Flow rate: 0.6 mL/min

Injection volume: 100 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of fluoride from the *Sample solution*
 r_S = peak area of fluoride from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (μg/mL)

C_U = nominal concentration of fluorine in the
Sample solution ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE, Method 1**

Mobile phase: Dissolve 5.15 g of tetrabutylammonium bromide in 320 mL of acetonitrile. Dilute with water to 2000 mL.

System suitability solution: Transfer 0.13 g of potassium iodide and 0.5 g of potassium iodate to a 100-mL volumetric flask. Dissolve in *Mobile phase*, using sonication if necessary, dilute with *Mobile phase* to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, dilute with *Mobile phase* to volume, and mix. Transfer 25.0 mL of this solution to a 100-mL volumetric flask, and dilute with *Mobile phase* to volume.

Standard stock solution: 1.3 mg/mL of potassium iodide in *Mobile phase*. This solution has a concentration of 1 mg/mL of iodide.

Standard solution: 2.5 $\mu\text{g/mL}$ of iodide from the *Standard stock solution* in *Mobile phase*

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain a solution with a concentration of 2.5 $\mu\text{g/mL}$ of iodine in *Mobile phase*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 225 nm

Column: 4.6-mm \times 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection volume: 30 μL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for iodate and iodide are about 0.32 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.5 between iodate and iodide, *System suitability solution*

Relative standard deviation: NMT 2.0% for the iodide peak, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of iodine (I) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of iodide from the *Sample solution*

r_S = peak area of iodide from the *Standard solution*

C_S = concentration of iodide in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of iodine in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IODIDE, Method 2**

Analysis: Proceed as directed for *Automated Methods of Analysis* (16), *Assay for Iodide*.

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON**

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 6 N hydrochloric acid, and dilute with water to volume.

Standard solutions: To separate 100-mL volumetric flasks, transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 $\mu\text{g/mL}$ of iron.

Sample solution: 6 $\mu\text{g/mL}$ of iron from the Oral Solution in 0.125 N hydrochloric acid

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption spectrophotometer

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 248.3 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in $\mu\text{g/mL}$, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of iron in the *Sample solution* from the graph ($\mu\text{g/mL}$)

C_U = nominal concentration of iron in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM**

Magnesium standard solution: Transfer 1.00 g of magnesium ribbon to a 1000-mL volumetric flask. Dissolve in 50 mL of 6 N hydrochloric acid, and dilute with water to volume.

Standard stock solution: 20 $\mu\text{g/mL}$ of magnesium from *Magnesium standard solution* in 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 7.5, 10.0, 12.5, and 15.0 mL of *Standard stock solution*. Dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 $\mu\text{g/mL}$ of magnesium.

Sample solution: 2.5 $\mu\text{g/mL}$ of magnesium from the Oral Solution in 0.125 N hydrochloric acid

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption spectrophotometer

Analytical wavelength: 285.2 nm

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in $\mu\text{g/mL}$, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of magnesium in the *Sample solution* from the graph ($\mu\text{g/mL}$)

C_U = nominal concentration of magnesium in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE**

Manganese standard solution: Transfer 1.0 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, and dilute with 6 N hydrochloric acid to volume.

Standard stock solution: 50 µg/mL of manganese from the *Manganese standard stock solution* in 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions having known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain 1.5 µg/mL of manganese in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Analytical wavelength: 279.5 nm

Lamp: Manganese hollow-cathode

Flame: Air-acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of manganese in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• MOLYBDENUM

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume.

Standard stock solution: 100 µg/mL of molybdenum from the *Molybdenum standard solution* in water

Standard solutions: To separate 100-mL volumetric flasks transfer 0.5, 1.0, 1.5, 2.0, and 3.0 mL of the *Standard stock solution*. Add 0.125 N hydrochloric acid to volume, and mix to obtain the solutions having known concentrations of about 0.5, 1.0, 1.5, 2.0, and 3.0 µg/mL of molybdenum.

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain 1.5 µg/mL of molybdenum from the Oral Solution in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Analytical wavelength: 313.3 nm

Lamp: Molybdenum hollow-cathode

Flame: Nitrous oxide-acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of molybdenum in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• ZINC

Zinc standard solution: Transfer 311 mg of zinc oxide to a 250-mL volumetric flask, and add 80 mL of 6 N hydrochloric acid, warming if necessary to dissolve. Cool, dilute with water to volume, and mix to obtain a solution having a known concentration of 1000 µg/mL of zinc.

Standard stock solution: 50 µg/mL of zinc from the *Zinc standard solution* in 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Dilute an accurately measured volume of Oral Solution to obtain 1.5 µg/mL of zinc in 0.125 N hydrochloric acid.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Analytical wavelength: 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air-acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of zinc in the *Sample solution* from the graph (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

OTHER COMPONENTS

- **ALCOHOL DETERMINATION, Method I (611)** (if present): 90.0%–120.0% of the labeled amount of C₂H₅OH

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic microbial count does not exceed 3×10^3 cfu/mL, and the combined molds and yeasts count does not exceed 3×10^2 cfu/mL.
- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, under an inert gas or with a minimum of headspace.

- **LABELING:**¹ The label states that the product is Oil-Soluble Vitamins with Minerals Oral Solution. The label states the quantity of each vitamin and mineral in a given volume of the Oral Solution and, where necessary, the chemical form in which a vitamin is present, and states also the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*-form. Where more than one assay method is given for a particular mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.

- **USP REFERENCE STANDARDS** (11)

USP Alpha Tocopherol RS
 USP Alpha Tocopheryl Acetate RS
 USP Alpha Tocopheryl Acid Succinate RS
 USP Beta Carotene RS
 USP Beta Carotene System Suitability RS
 USP Cholecalciferol RS
 USP Ergocalciferol RS
 USP Phytonadione RS
 USP Retinyl Acetate RS
 USP Retinyl Palmitate RS
 USP Sodium Fluoride RS▲ USP36

Add the following:

▲ Oil-Soluble Vitamins with Minerals Tablets

DEFINITION

Oil-Soluble Vitamins with Minerals Tablets contain two or more of the following oil-soluble vitamins: Vitamin A, as retinol or esters of retinol in the form of retinyl acetate or retinyl palmitate; Vitamin D as ergocalciferol (Vitamin D₂) or cholecalciferol (Vitamin D₃); Vitamin E, as alpha tocopherol, alpha tocopheryl acetate or alpha tocopheryl acid succinate; Phytonadione (Vitamin K₁); beta carotene; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Tablets contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A, as retinol equivalent (C₂₀H₃₀O); vitamin D, as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O); vitamin E, as alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅); phytonadione (C₃₁H₄₆O₂); and beta carotene (C₄₀H₅₆); NLT 90.0% and NMT 125.0% of the labeled amounts of calcium (Ca), copper (Cu), iron (Fe), manganese (Mn), magnesium (Mg), phosphorus (P), potassium

¹ USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued. However, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

(K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

• VITAMIN A

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution 1: 13 µg/mL of retinol from USP Retinyl Acetate RS in *n*-hexane

Standard solution 2: 13 µg/mL of retinol from USP Retinyl Palmitate RS in *n*-hexane

System suitability solution: Mix equal volumes of *Standard solution 1* and *Standard solution 2*.

Sample solution: Finely powder NLT 20 Tablets. To a portion of the powder, equivalent to 5 Tablets, add 15 mL of water, and sonicate for 5 min. Add 15 mL of *n*-hexane, and shake for 15 min in a water bath maintained at 60°. Add 10 mL of dimethyl sulfoxide, and shake for an additional period of 30 min in a water bath maintained at 60°. Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Further dilute this solution with *n*-hexane to obtain a solution with a concentration equivalent to 13 µg/mL of retinol (C₂₀H₃₀O).

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; packing L8

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution 1* or *Standard solution 2* and *Sample solution*

Calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the appropriate *Standard solution*

C_S = concentration of retinol in the appropriate *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol, in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A as retinol equivalent (C₂₀H₃₀O)

• VITAMIN D

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)
Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A* to a suitable container, and evaporate, if necessary, in vacuum at room temperature to obtain a concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection volume: 100 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of pre-vitamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• VITAMIN E

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution in water (1 in 100)

Mobile phase: Methanol and *Solution A* (19:1)

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 50 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 1.5

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E

• PHYTONADIONE (VITAMIN K₁)

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from the *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection volume: 50 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*
 r_S = peak area from the *Standard solution*
 C_S = concentration of USP Phytonadione RS in the *Standard solution* ($\mu\text{g/mL}$)
 C_U = nominal concentration of phytonadione in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **BETA CAROTENE**

[NOTE—Use low-actinic glassware.]

Mobile phase: Transfer 50 mg of butylated hydroxytoluene into a 1-L volumetric flask, and dissolve with 20 mL of 2-propanol. Add 0.2 mL of *N*-ethyl-diisopropylamine, 25 mL of 0.2% ammonium acetate solution, 455 mL of acetonitrile, and about 450 mL of methanol. Allow the solution to reach room temperature, and dilute with methanol to volume.

Diluent: 50 $\mu\text{g/mL}$ of butylated hydroxytoluene in alcohol

System suitability solution: Transfer 20 mg of USP Beta Carotene System Suitability RS to a 50-mL volumetric flask. Add 1 mL of water, 4 mL of tetrahydrofuran, and sonicate for 5 min. Dilute with *Diluent* to volume, and sonicate for 5 min. Cool to room temperature, pass the suspension through a membrane filter of 0.45- μm pore size, and use the clear filtrate.

Standard stock solution: 60 $\mu\text{g/mL}$ of USP Beta Carotene RS in tetrahydrofuran

Standard solution A: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, add 5.0 mL of tetrahydrofuran, and dilute with *Diluent* to volume.

Determine the concentration of *Standard solution A* from the concentration of *Standard solution B* as follows.

Standard solution B: Transfer 5.0 mL of the *Standard stock solution* into a 100-mL volumetric flask, and dilute with cyclohexane to volume. Prepare in triplicate.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Analytical wavelength: 457 nm

Cell path: 1 cm

Blank: Cyclohexane

Analysis

Sample: *Standard solution B*

Calculate the concentration of total beta carotene (mg/mL) as all-*trans*-beta carotene ($C_{40}H_{56}$) in *Standard solution B*. [NOTE—The concentration of *Standard solution B* equals the concentration of *Standard solution A*.]

$$\text{Result} = A/F$$

A = average absorbance of the three preparations of *Standard solution B*

F = absorptivity of pure all-*trans*-beta carotene in cyclohexane, 250

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A* to a suitable container, and evaporate in vacuum at room temperature to dryness. Dissolve the residue in a mixture of methylene chloride and *Diluent* (1:1), and dilute with the same mixture to obtain a concentration of 3 $\mu\text{g/mL}$ of beta carotene. Pass through a membrane filter of 0.45- μm pore size, if necessary.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV-Vis 448 nm

Column: 4.6-mm \times 25-cm; 5- μm packing L68

Column temperature: 30°

Flow rate: 0.6 mL/min

Injection volume: 20 μL

System suitability

Samples: *System suitability solution* and *Standard solution A*

The approximate relative retention times of the components in the *System suitability solution* are listed in *Table 1*.

Table 1

Name	Relative Retention Time	Relative Response Factor
All- <i>trans</i> -alpha carotene	0.93	1.1
All- <i>trans</i> -beta carotene	1.00	1
9- <i>cis</i> -Beta carotene	1.07	1
13- <i>cis</i> -Beta carotene	1.17	1.2
15- <i>cis</i> -Beta carotene	1.21	1.4

Suitability requirements

Chromatogram similarity: The chromatogram from the *System suitability solution* is similar to the reference chromatogram provided with the USP Beta Carotene System Suitability RS being used.

Resolution: NLT 1.5 between beta carotene and alpha carotene and between beta carotene and 9-*cis*-beta carotene, *System suitability solution*

Tailing factor: NMT 2.0 for the beta carotene peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for the beta carotene peak from replicate injections, *Standard solution A*

Analysis

Samples: *Standard solution A* and *Sample solution*

Calculate the percentage of all-*trans*-beta carotene in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of all-*trans*-beta carotene in the *Sample solution*

r_S = peak area of all-*trans*-beta carotene in *Standard solution A*

C_S = concentration of all-*trans*-beta carotene in *Standard solution A* as determined by spectrometric procedure

C_U = nominal concentration of beta carotene in the *Sample solution*

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

• **CALCIUM, Method 1**

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 $\mu\text{g/mL}$ of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 $\mu\text{g/mL}$ of calcium from the *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 $\mu\text{g/mL}$ of calcium.

Sample solution: Finely powder NLT 20 Tablets.

Transfer a portion of the powder, equivalent to 5 Tablets, to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively, with 0.125 N hydrochloric acid, to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Calcium emission line at 422.7 nm

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions, against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• CHROMIUM, Method 1

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from the *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Proceed as directed for *Calcium, Method 1*, except prepare the *Sample solution* to contain 2.5 µg/mL of chromium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Chromium emission line at 357.9 nm

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• COPPER, Method 1

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% solution of nitric acid, and dilute with a 1% solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

Standard stock solution: 100 µg/mL of copper from the *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Proceed as directed for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 2 µg/mL of copper and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Copper emission line at 324.7 nm

Lamp: Copper hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)

C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• FLUORIDE, Method 1

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water contained in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water

to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 µg/mL of fluoride from a quantity of sodium fluoride in water, previously dried at 100° for 4 h and cooled in a desiccator

Intermediate stock solution A: 100 µg/mL of fluoride from the *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 µg/mL of fluoride from the *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of 3 M sodium acetate solution, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 µg/mL of fluoride.

Sample solution: Transfer a quantity of the finely powdered Tablets, equivalent to 200 µg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of 3 M sodium acetate solution, and 25.0 mL of *Sodium citrate solution*, and dilute with water to volume.

Analysis

Samples: *Standard solutions* and *Sample solution*
To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see pH (791)), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ±0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer having an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in µg/mL, of the *Standard solutions* versus potential, in mV. From the standard response curve so obtained and the measured potential of the *Sample solution*, determine the concentration, *C*, in µg/mL, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (*F*) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (*F*)

• FLUORIDE, Method 2

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with one column volume of methanol followed by one column volume of pH 10.0 buffer. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.] Transfer 10.0 mL of *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1. Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask, add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with pH 10.0 buffer to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Finely powder NLT 20 Tablets.

Transfer a portion of powdered Tablets, equivalent to 1 mg of fluorine, in 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1, and dilute with water to 100 mL. Proceed as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Conductivity

Column

Guard: 4.6-mm × 3-cm; packing L17

Analytical: 7.8-mm × 30-cm; packing L17

Flow rate: 0.5 mL/min

Injection volume: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for fluoride. Calculate the percentage of the labeled amount of fluorine (*F*) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (*F*)

• IODIDE, Method 1

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis

Sample: Tablets

Transfer an amount of finely powdered Tablets, equivalent to 3 mg of iodide, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon

subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.]

Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.

Calculate the percentage of the labeled amount of iodine (I) in the portion of Tablets taken:

$$\text{Result} = V \times N_A \times F \times I_{me} \times (A_w/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)

N_A = actual normality of the sodium thiosulfate solution used

F = correction factor to convert mg to μg , 1000 $\mu\text{g}/\text{mg}$

I_{me} = milliequivalent of I, 21.16 mg/meq

A_w = average weight of the Tablets

W = weight of the portion of Tablets taken

L = labeled amount of iodine ($\mu\text{g}/\text{Tablet}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

- **IODIDE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Iodide*.

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

- **IRON, Method 1**

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of the *Iron standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 $\mu\text{g}/\text{mL}$ of iron.

Sample solution: Proceed as directed for *Calcium, Method 1*, except prepare the *Sample solution* to contain a concentration of 5 $\mu\text{g}/\text{mL}$ of iron and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Iron emission line at 248.3 nm

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g}/\text{mL}$, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g}/\text{mL}$, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Tablets taken:

$$\text{Result} = (C/C_i) \times 100$$

C = measured concentration of iron in the *Sample solution* ($\mu\text{g}/\text{mL}$)

C_i = nominal concentration of iron in the *Sample solution* ($\mu\text{g}/\text{mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

- **MAGNESIUM, Method 1**

Lanthanum chloride solution: Prepare as directed in *Calcium, Method 1*.

Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a known concentration of 1000 $\mu\text{g}/\text{mL}$ of magnesium.

Standard stock solution: 20 $\mu\text{g}/\text{mL}$ of magnesium from the *Magnesium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 $\mu\text{g}/\text{mL}$ of magnesium.

Sample solution: Proceed as directed for *Calcium, Method 1*, except prepare the *Sample solution* to contain a concentration of 0.4 $\mu\text{g}/\text{mL}$ of magnesium.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Magnesium emission line at 285.2 nm

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g}/\text{mL}$, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g}/\text{mL}$, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Tablets taken:

$$\text{Result} = (C/C_i) \times 100$$

C = measured concentration of magnesium in the *Sample solution* ($\mu\text{g}/\text{mL}$)

C_i = nominal concentration of magnesium in the *Sample solution* ($\mu\text{g}/\text{mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

- **MANGANESE, Method 1**

Manganese standard stock solution: Transfer 1.00 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 $\mu\text{g}/\text{mL}$ of manganese.

Standard stock solution: 50 $\mu\text{g}/\text{mL}$ of manganese from the *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 $\mu\text{g}/\text{mL}$ of manganese.

Sample solution: Proceed as directed for *Calcium, Method 1*, except prepare the *Sample solution* to contain a concentration of 1 $\mu\text{g}/\text{mL}$ of manganese and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions(See *Spectrophotometry and Light-Scattering* (851).)**Mode:** Atomic absorption spectrophotometry**Analytical wavelength:** Manganese emission line at 279.5 nm**Lamp:** Manganese hollow-cathode**Flame:** Air-acetylene**Blank:** 0.125 N hydrochloric acid**Analysis****Samples:** *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of manganese in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM, Method 1****Diluent:** 20 mg/mL of ammonium chloride in water

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 $\mu\text{g/mL}$ of molybdenum.

Standard stock solution: 100 $\mu\text{g/mL}$ of molybdenum from the *Molybdenum standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 $\mu\text{g/mL}$ of molybdenum.

Sample solution: Transfer a portion of the powder, equivalent to 1000 μg of molybdenum, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min, or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid, heat until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Quantitatively transfer the contents of the flask to a 100-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions(See *Spectrophotometry and Light-Scattering* (851).)**Mode:** Atomic absorption spectrophotometry**Analytical wavelength:** Molybdenum emission line at 313.3 nm**Lamp:** Molybdenum hollow-cathode**Flame:** Nitrous oxide-acetylene**Blank:** *Diluent* and perchloric acid (20:1)**Analysis****Samples:** *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of molybdenum, and draw the straight line best fitting the three plotted

points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of molybdenum in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **MOLYBDENUM, Method 2**

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% Stannous chloride solution: Transfer 40 mg of stannous chloride to a beaker, add 20 mL of 6.5 N hydrochloric acid solution, and heat the solution until the stannous chloride is dissolved. Cool, and dilute with water to 100 mL.

Diluted stannous chloride solution: 20% *Stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.

Standard solution: 20 $\mu\text{g/mL}$ of molybdenum in water

Sample: A portion of finely powdered Tablets, equivalent to 40 μg of molybdenum

Instrumental conditions(See *Spectrophotometry and Light-Scattering* (851).)**Mode:** UV-Vis**Analytical wavelength:** 465 nm**Cell:** 1 cm**Blank:** Amyl alcohol**Analysis****Samples:** *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to complete 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of 20% *Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layer from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the *Sample*
 A_S = absorbance of the *Standard solution*
 V = volume of the *Standard solution* analyzed, 2.0 mL
 C_S = concentration of molybdenum in the *Standard solution* (μg/mL)
 M_U = nominal amount of molybdenum in the *Sample* (μg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• PHOSPHORUS, Method 1

Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5:100), and mix.

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, and then dilute with *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add one drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 μg/mL of phosphorus.

Standard solution: 20 μg/mL of phosphorus from the *Phosphorus standard stock solution* diluted with water

Sample solution: [NOTE—Finely powder and weigh a counted number of Tablets.] Transfer a portion of the powder, equivalent to 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Vis

Analytical wavelength: 650 nm

Cell: 1 cm

Analysis

Samples: *Standard solution* and *Sample solution*
 To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*
 A_S = absorbance of the *Standard solution*
 C_S = concentration of phosphorus in the *Standard solution* (μg/mL)
 C_U = nominal concentration of phosphorus in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• POTASSIUM

Potassium standard solution: 100 μg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 μg/mL of potassium from the *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 μg/mL of potassium.

Sample solution: Proceed as directed for *Calcium, Method 1*, except prepare the *Sample solution* to contain a concentration of 1.5 μg/mL of potassium and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Potassium emission line at 766.5 nm

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in μg/mL, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in μg/mL, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* (μg/mL)

C_U = nominal concentration of potassium in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• SELENIUM, Method 1

Diluent: Prepare as directed in *Molybdenum, Method 1*.

Selenium standard solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 μg/mL of selenium.

Standard stock solution: 100 μg/mL of selenium from the *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions with concentrations of 5.0, 10.0, and 25.0 μg/mL of selenium.

Sample solution: Transfer a portion of the powder, equivalent to 1000 μg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat

the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Selenium emission line at 196.0 nm

Lamp: Selenium hollow-cathode

Flame: Air-acetylene

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* (µg/mL)

C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• SELENIUM, Method 2

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% Ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, then add hydroxylamine hydrochloride, and dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 µg/mL of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10 mL of the *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Transfer a portion of finely powdered Tablets, equivalent to 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. [CAUTION—Exercise care at this stage, because the perchloric acid reaction becomes vigorous.]

Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask, add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV

Analytical wavelength: 380 nm

Cell: 1 cm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*

Treat the *Sample solution*, the *Standard solution*, and the *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with 50% *Ammonium hydroxide solution* to a pH of 1.1 ± 0.1. Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbances of the cyclohexane layer from the *Sample solution*

A_S = absorbances of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• ZINC, Method 1

Zinc standard solution: 1000 µg/mL of zinc from zinc oxide in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from the *Zinc standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Proceed as directed for the *Sample solution* in *Calcium, Method 1*, except prepare the *Sample solution* to contain a concentration of 2 µg/mL of zinc and omit the use of the *Lanthanum chloride solution*.

Instrumental conditions(See *Spectrophotometry and Light-Scattering* (851).)**Mode:** Atomic absorption spectrophotometry**Analytical wavelength:** Zinc emission line at 213.8 nm**Lamp:** Zinc hollow-cathode**Flame:** Air-acetylene**Blank:** 0.125 N hydrochloric acid**Analysis****Samples:** *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of zinc in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

- **BORON, NICKEL, TIN, AND VANADIUM, Method 1; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS, AND ZINC, Method 2; MOLYBDENUM AND SELENIUM, Method 3**

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding one volume of *Stock aqua regia solution* to two volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% nitric acid solution, 1000 mg/L of scandium in 5% nitric acid solution, and *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% nitric acid solution to obtain a solution having final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/L of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% hydrochloric acid solution to obtain a solution having final concentrations of about 200 mg/L of boron, and 100 mg/L each of chromium, molybdenum, nickel, selenium, tin, and vanadium.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent* to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution 1 (for Tablets containing minerals found in *Standard stock solution 1* and *Standard stock solution 2*): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add

25 mL of *Stock aqua regia solution* in 5-mL increments followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a nylon syringe filter of 5- μm pore size. If necessary, make any further dilutions using the *Diluent*.

Sample solution 2 (for Tablets containing minerals found only in *Standard stock solution 2*): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a nylon syringe filter of 5- μm pore size. If necessary, make any further dilutions using the *Diluent*.

Sample solution 3 (for Tablets containing minerals found only in *Standard stock solution 1*): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently (about 1 h) until fumes cease. Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a nylon syringe filter of 5- μm pore size. If necessary, make any further dilutions using the *Diluent*.

Instrumental conditions(See *Plasma Spectrochemistry* (730).)

Mode: Inductively coupled plasma spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

[NOTE—Analyze the *System suitability solution*, and obtain the response as directed in the *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis**Samples:** *Standard solutions* and *Sample solution*

Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, C , in mg/L, for each mineral of interest in the *Sample solution*.

Calculate the percentage of the labeled amount for each mineral taken:

$$\text{Result} = C \times (V/W) \times F \times (C_W/L) \times 100$$

C = measured concentration of the relevant element in the *Sample solution* (mg/L)

V = volume of the *Sample solution* (L)

W = sample weight (mg)

F = dilution factor of the *Sample solution*

C_w = average weight (mg/Tablet)

L = label claim (mg/Tablet)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); and 90.0%–160.0% of the labeled amounts of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION** (2040): Meet the requirements for *Dissolution*
- **WEIGHT VARIATION** (2091): Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 3×10^3 cfu/g, and the combined molds and yeasts count does not exceed 3×10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:**¹ The label states that the product is Oil-Soluble Vitamins with Minerals Tablets. The label also states the quantity of each vitamin and mineral per dosage unit and where necessary the chemical form in which a vitamin is present and also states the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*-form. Where more than one assay method is given for a particular mineral, the labeling states with which assay method the product complies only if *Method 7* is not used.
- **USP REFERENCE STANDARDS** (11)
 - USP Alpha Tocopherol RS
 - USP Alpha Tocopheryl Acetate RS
 - USP Alpha Tocopheryl Acid Succinate RS
 - USP Cholecalciferol RS
 - USP Ergocalciferol RS
 - USP Phytonadione RS
 - USP Retinyl Acetate RS
 - USP Retinyl Palmitate RS
 - USP Sodium Fluoride RS▲ USP36

¹ USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

Oil- and Water-Soluble Vitamins Capsules

DEFINITION

Oil- and Water-Soluble Vitamins Capsules contain one or more of the following oil-soluble vitamins: Vitamin A, Vitamin D as Ergocalciferol (Vitamin D₂) or Cholecalciferol (Vitamin D₃), Vitamin E, Phytonadione (Vitamin K₁), and Beta Carotene; and one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, Dexpanthenol or Panthenol, Pantothenic Acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate. Capsules contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A (C₂₀H₃₀O) as retinol or esters of retinol in the form of retinyl acetate (C₂₂H₃₂O₂) or retinyl palmitate (C₃₆H₆₀O₂); vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O); vitamin E as alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅); phytonadione (C₃₁H₄₆O₂); and beta carotene (C₄₀H₅₆); and NLT 90.0% and NMT 150.0% of the labeled amounts of ascorbic acid (C₆H₈O₆) or its salts as calcium ascorbate (C₁₂H₁₄CaO₁₂ · 2H₂O) or sodium ascorbate (C₆H₇NaO₆), biotin (C₁₀H₁₆N₂O₃S), cyanocobalamin (C₆₃H₈₈CoN₁₄O₁₄P), folic acid (C₁₉H₁₉N₇O₆), niacin (C₆H₅NO₂) or niacinamide (C₆H₆N₂O), dexpanthenol (C₉H₁₉NO₄) or panthenol (C₉H₁₉NO₄), calcium pantothenate (C₁₈H₃₂CaN₂O₁₀), pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl), riboflavin (C₁₇H₂₀N₄O₆), and thiamine (C₁₂H₁₇ClN₄OS) as thiamine hydrochloride or thiamine mononitrate.

They do not contain any minerals. They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

• VITAMIN A, *Method 1*

[NOTE—Where the use of a vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-acid glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in *n*-hexane

System suitability stock solution: 15 µg/mL of retinyl palmitate in *n*-hexane

System suitability solution: Mix equal volumes of *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, equivalent to 5 Capsules, to a container having a polytetrafluoroethylene-lined screw cap. [NOTE—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent to 5 Capsules, to a container having a polytetrafluoroethylene-lined screw cap.] Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [NOTE—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide-

ide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Dilute a volume of this solution with *n*-hexane to obtain a solution with a concentration of 15 µg/mL of vitamin A as retinol (C₂₀H₃₀O).

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection size: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate obtained from the *Standard solution* and the peak area for all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate in the chromatogram of the *Sample solution*. For products containing vitamin A acetate or vitamin A palmitate, calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₂H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O), in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)

• VITAMIN A, Method 2

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution: Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Mobile phase: *n*-Hexane and ethyl acetate (99.7:0.3)

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in 2,2,4-trimethylpentane

System suitability stock solution: 15 µg/mL of retinyl palmitate in 2,2,4-trimethylpentane

System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution*

to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Using a sharp blade if necessary, carefully open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 30 µg of the labeled amount of cholecalciferol or ergocalciferol (vitamin D), to a container with a polytetrafluoroethylene-lined screw cap. If vitamin D is not present in the formulation, use a portion, equivalent to 90 mg of the labeled amount of vitamin E. If vitamin E is not present in the formulation, use a portion, equivalent to 2.5 mg of the labeled amount of vitamin A, as retinol. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of 3 N methanolic sulfuric acid solution, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. [NOTE—The supernatant is used for the determination of vitamin A, and also vitamin D and vitamin E, if present in the formulation.] If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1.5 mL/min

Injection size: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 8.0 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area of all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution*.

Calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₂H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O), in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration. The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **VITAMIN A, Method 3**

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Extraction solvent: *n*-Hexane and methylene chloride (3:1)

Potassium hydroxide solution: 800 mg/mL of potassium hydroxide in water. [NOTE—Cautiously add potassium hydroxide in water. Mix, and cool.]

Diluent: 10 mg/mL of pyrogallol in alcohol

Mobile phase: *n*-Hexane and isopropyl alcohol (92:8)

Standard stock solution: 30 µg/mL of retinyl acetate from USP Vitamin A RS in *Diluent*. [NOTE—This solution may be stored in a refrigerator for 1 week.]

Standard solution: Dilute a volume of *Standard stock solution* with *Diluent* to obtain a concentration of 1 µg/mL of retinyl acetate from USP Vitamin A RS. Transfer 10.0 mL of this solution to a stoppered 125-mL flask, and add 5 mL of water, 5 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at $60 \pm 5^\circ$, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. Withdraw a portion of the organic layer for injection into the chromatograph. This *Standard solution* contains 0.34 µg/mL of retinol.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 1.5 mg of retinyl acetate, to a stoppered 125-mL flask. Add 5 mL of water, 15 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at $60 \pm 5^\circ$, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s or longer, if necessary, for complete extraction. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. [NOTE—Do not shake, because an emulsion may form.] Withdraw a portion of the organic layer, and dilute quantitatively, and stepwise if necessary, with *Extraction solvent*, to obtain a concentration of 0.34 µg/mL of retinol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 335 nm

Column: 6.2-mm × 8-cm; packing L3

Column temperature: 40°

Flow rate: 4 mL/min

Injection size: 50 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for 13-*cis*-retinol and all-*trans*-retinol are about 0.92 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for all-*trans*-retinol and 13-*cis*-retinol. Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Capsules taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times (C_S/C_U) \times F \times 100$$

r_{T1} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Sample solution*

r_{T2} = sum of the areas of all-*trans*-retinol and 13-*cis*-retinol peaks from the *Standard solution*

C_S = concentration of retinyl acetate ($C_{23}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D),**

Method 1

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate, if necessary, in a vacuum at room temperature to obtain a solution to obtain a concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis**Samples:** *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

- r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
 r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
 C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* ($\mu\text{g/mL}$)
 C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* ($\mu\text{g/mL}$)
 F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D),**

Method 2

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution, Sodium ascorbate–pyrogallol solution, Lecithin solution, and Sample solution: Proceed as directed in *Vitamin A*, *Method 2*.

Mobile phase: *n*-Hexane and tertiary butyl alcohol (98.75:1.25)

Standard solution: 1 $\mu\text{g/mL}$ of USP Cholecalciferol RS or USP Ergocalciferol RS in 2,2,4-trimethylpentane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm \times 25-cm; 5- μm packing L24

Flow rate: 1 mL/min

Injection size: 40 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis**Samples:** *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
 r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
 C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D),**

Method 3

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluted acetic acid: Glacial acetic acid solution (1 in 10) in water

Phenolphthalein solution: 10 mg/mL of phenolphthalein in alcohol

Potassium hydroxide solution: Slowly dissolve 14 g of potassium hydroxide in a mixture of 31 mL of dehydrated alcohol and 5 mL of water. Prepare fresh daily.

Extraction solvent: Methylene chloride and isopropyl alcohol (99.8:0.2)

Mobile phase: Acetonitrile and methanol (91:9)

Standard stock solution: 0.2 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in dehydrated alcohol. [NOTE—Prepare fresh every 4 weeks. Store in a freezer.]

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* by initially washing the column with 4.0 mL of a mixture of methylene chloride and isopropyl alcohol (4:1), followed by 5.0 mL of *Extraction solvent*. Do not allow the column to dry.] Dilute a volume of *Standard stock solution* with dehydrated alcohol to obtain a concentration of 5 $\mu\text{g/mL}$ of USP Cholecalciferol RS or USP Ergocalciferol RS. Prepare this solution fresh daily. Transfer 2.0 mL of this solution to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase extraction column containing silica packing with a sorbent mass-to-column volume ratio of 500 mg to 2.8 mL or equivalent, rinse the round-bottom flask with 1.0 mL of *Extraction solvent*, and transfer to the column. Elute the column with 2.0 mL of *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen.

Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 3*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 10 µg of ergocalciferol or cholecalciferol, to a stoppered 125-mL flask, and proceed as directed for the *Standard solution*, beginning with "Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*".

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 27°

Flow rate: 0.7 mL/min

Injection size: 15 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 4.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• VITAMIN E, Method 1

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water

Mobile phase: Methanol and *Solution A* (19:1)

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

System suitability solution: Prepare a 0.65-mg/mL solution of USP Ergocalciferol RS in methanol. Transfer 1.0 mL of this solution to a 100-mL volumetric flask containing 100 mg of USP Alpha Tocopheryl Acetate RS. Dissolve in 30 mL of methanol, with the aid of sonication if necessary, and dilute with methanol to volume. Store this solution in a refrigerator.

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate if necessary, in a vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume, to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for ergocalciferol and alpha tocopheryl acetate are about 0.5 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 12 between ergocalciferol and alpha tocopheryl acetate, *System suitability solution*

Tailing factor: Between 0.8 and 1.2, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅)

• VITAMIN E, Method 2

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 240 mL of methanol with 10 mL of water, followed by 0.5 mL of 50% phosphoric acid, and dilute with acetonitrile to 1000 mL.

System suitability solution: 2 mg/mL each of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, and USP Alpha Tocopheryl Acid Succinate RS in methanol

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

3 N methanolic sulfuric acid solution: Cautiously mix sulfuric acid and methanol (9 in 100) in a 100-mL volumetric flask. [NOTE—Dissolve in a portion of methanol, cool, and then dilute to final volume.]

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask. Add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 2*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 55 mg of vitamin E, to a container having a polytetrafluoroethylene-lined screw cap. Add

0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate-pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of 3 *N methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant layer. Transfer a volume of the supernatant 2,2,4-trimethylpentane layer to a suitable volumetric flask, the volume of the specimen withdrawn from the 2,2,4-trimethylpentane layer and the size of the volumetric flask being such that the final concentration of the *Sample solution* is equivalent to that of the *Standard solution*. Evaporate nearly to dryness, add several mL of methanol, and evaporate the remaining 2,2,4-trimethylpentane. Dilute with methanol to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 1.5 mL/min

Injection size: 25 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for alpha tocopheryl acid succinate, alpha tocopherol, and alpha tocopheryl acetate are about 0.6, 0.8, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 4.0 between alpha tocopheryl acid succinate and alpha tocopherol and NLT 3.0 between alpha tocopherol and alpha tocopheryl acetate, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane and the dilution factor to exchange the solvent from 2,2,4-trimethylpentane to methanol to calculate the nominal concentration.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• VITAMIN E, Method 3

Diluent: Acetonitrile and ethyl acetate (1:1)

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in methanol

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 3*, through “calculate the net weight of the Capsule contents.” Transfer a portion of the Capsule contents, equivalent to 8.0 mg of alpha tocopherol, to a glass-stoppered conical flask. Add 25.0 mL of water, 25.0 mL of dehydrated alcohol, and 3.5 g of potassium hydroxide pellets. Shake for 1 h in a water bath maintained at 55°. Cool, and transfer with the aid of a minimum volume of water to a 125-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 60 s, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and add the rinsing to the hexane solution in the flask. Place the flask in a water bath maintained at 50°, and evaporate the hexane solution with the aid of a rotary evaporator to dryness. Immediately add 25.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 40°

Flow rate: 3 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of alpha tocopherol from the *Sample solution*

r_S = peak area of alpha tocopherol from the *Standard solution*

C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin E as alpha tocopherol in the *Sample solution* (mg/mL)

[NOTE—Calculate the content of alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) by dividing the content, in mg/Capsule of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), by the factor 0.91 or 0.81, respectively.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• PHYTONADIONE

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP

Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Transfer NLT 20 mL of the solution retained as specified in the directions for the *Sample solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, if necessary, in a vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for alpha tocopheryl acetate and phytonadione are about 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of phytonadione (C₃₁H₄₆O₂) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for phytonadione from the *Sample solution*

r_S = peak area for phytonadione from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione (C₃₁H₄₆O₂)

• BETA CAROTENE

[NOTE—Use low-actinic glassware throughout this procedure.]

Potassium hydroxide solution: Dissolve 58.8 g of potassium hydroxide in 50 mL of water.

Iodine solution: Transfer 10 mg of iodine to a 100-mL volumetric flask. Dissolve in cyclohexane, and dilute with cyclohexane to volume. Dilute 10 mL of this solution with cyclohexane to 100 mL. [NOTE—Prepare this solution fresh daily.]

Sample solution A (for preparations containing beta carotene in oil solutions): Proceed as directed in *Vitamin A, Method 1*, except use cyclohexane instead of *n*-hexane as the extraction solvent, and dilute the filtered extracts with cyclohexane, to obtain a concentration of 2 µg/mL of beta carotene.

Sample solution B (for preparations containing beta carotene in dry powder): Remove the contents of NLT

20 Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature. Add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower aqueous layer into the saponification flask. Add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume. Add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Vis

Analytical wavelength: 452 nm

Blank: Cyclohexane

Analysis

Samples: *Sample solution A* or *Sample solution B*

Determine the absorbance against the *Blank*. Calculate the percentage of the labeled amount of beta carotene (C₄₀H₅₆) in the portion of Capsules taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of *Sample solution A* or *Sample solution B*

F = absorptivity of beta carotene at 452 nm, 223

C_U = nominal concentration of beta carotene in *Sample solution A* or *Sample solution B* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene (C₄₀H₅₆)

• ASCORBIC ACID, Method 1

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 100 mg of ascorbic acid, to a 200-mL volumetric flask, and add 75 mL of metaphosphoric–acetic acids TS. Insert a stopper into the flask, and shake by mechanical means for 30 min. Dilute with water to volume. Transfer a portion of the solution to a centrifuge tube, and centrifuge until a clear supernatant is obtained. Pipet 4.0 mL of this solution into a 50-mL conical flask, and add 5 mL of metaphosphoric–acetic acids TS.

Analysis: Titrate with standard dichlorophenol–indophenol solution VS to a rose-pink color that persists

for at least 5 s. Correct for the volume of dichlorophenol-indophenol solution consumed by a mixture of 5.5 mL of metaphosphoric-acetic acids TS and 15 mL of water. From the ascorbic acid equivalent of the standard dichlorophenol-indophenol solution, calculate the content of ascorbic acid in each Capsule.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **ASCORBIC ACID, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), Assay for Ascorbic Acid.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **CALCIUM ASCORBATE, Method 1:** Proceed as directed in *Ascorbic Acid, Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

- **CALCIUM ASCORBATE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), Assay for Ascorbic Acid.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

- **SODIUM ASCORBATE, Method 1:** Proceed as directed in *Ascorbic Acid, Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **SODIUM ASCORBATE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), Assay for Ascorbic Acid.

Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **BIOTIN, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-µm packing L7

Flow rate: 1.2 mL/min

Injection size: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the responses for the biotin peaks. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

- **BIOTIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by dilution of the *Standard stock solution* with water on the day of the assay

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution having a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine-guanine-uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin-thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin-thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution* To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of *Standard* and *sample* tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to

within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of *Standard* during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength of 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the *Standard*, calculate the response from the sum of the duplicate values of the transmittance (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances (Σ_u) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of lowest and highest points plotted for the *Standard*. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of biotin that was assumed to be present in the portion of Capsules taken
Calculate the percentage of the labeled amount of biotin in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$) in the portion of Capsules taken (μg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$)

• **CYANOCOBALAMIN, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

¹ ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 µg/mL of USP Cyanocobalamin RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

Standard solution: 1 µg/mL of USP Cyanocobalamin RS from *Standard stock solution* diluted with water

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 100 µg of cyanocobalamin, to a 250-mL flask. Add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 0.5 mL/min

Injection size: 200 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for cyanocobalamin.

Calculate the percentage of the labeled amount of cyanocobalamin (C₆₃H₈₈CoN₁₄O₁₄P) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Cyanocobalamin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin (C₆₃H₈₈CoN₁₄O₁₄P)

• CYANOCOBALAMIN, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of USP Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of the *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each g of Capsule contents taken, 25 mL of an aqueous extracting solution prepared just before use to contain 12.9 mg/mL of dibasic sodium phosphate, 11.0 mg/mL of anhydrous citric acid, and 10 mg/mL of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of

the clear solution with water to obtain a final solution containing vitamin B₁₂ activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Calcium Pantothenate, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed in *Calcium Pantothenate, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions to the resulting solution. Add 100 mL of water, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of between 5.5 and 6.0, and add Purified Water to make 250 mL.

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N Hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is

removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.² [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.]

Incubate for 16–24 h at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL of the *Culture medium* each. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension so obtained is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the

spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minute amounts of vitamin B₁₂ activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm \times 150-mm test tubes, and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within $\pm 0.5^\circ$, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating its contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5%, or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances (Σ_u) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to

²Pure cultures of *Lactobacillus leichmannii* may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209.

obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of cyanocobalamin in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of cyanocobalamin that was assumed to be present in the portion of Capsules taken

Calculate the percentage of the labeled amount of cyanocobalamin in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of Capsules taken (μg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Vitamin B₁₂ Activity in Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• **FOLIC ACID, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation. [NOTE—The methanol and water content may be varied (between 1% and 3%).]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8, bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of USP Folic Acid RS in *Internal standard solution*

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer an amount of Capsule contents to a suitable centrifuge tube, and add a volume of *Internal standard solution* to obtain a concentration of 0.016 mg/mL of folic acid. Shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm \times 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 15 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*

R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of folic acid in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$)

• **FOLIC ACID, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: 60 $\mu\text{g/mL}$ of ammonium hydroxide

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 60 $\mu\text{g/mL}$ of USP Folic Acid RS in *Diluent*. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of *Standard stock solution* with 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed for the *Sample solution* in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm \times 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 5 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of folic acid from the *Sample solution*
 r_S = peak area of folic acid from the *Standard solution*
 C_S = concentration of USP Folic Acid RS in the *Standard solution* ($\mu\text{g/mL}$)
 C_U = nominal concentration of folic acid in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$)

• DEXPANTHENOL OR PANTHENOL

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 $\mu\text{g/mL}$ of USP Dexpantenol RS, or 1600 $\mu\text{g/mL}$ of USP Racemic Panthenol RS in water. Store in a refrigerator, protected from light, and use within 30 days.

Standard solution: On the day of the assay, prepare a dilution of 1.2 $\mu\text{g/mL}$ of dexpantenol or 2.4 $\mu\text{g/mL}$ of panthenol from *Standard stock solution* diluted with water.

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents as completely as possible to a beaker. Remove any contents adhering to the empty Capsule shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Dissolve a portion of the Capsule contents, equivalent to 1.2 mg of dexpantenol or 2.4 mg of panthenol, in 100.0 mL of water. Quantitatively dilute a portion of this solution with water to obtain a concentration of 1.2 $\mu\text{g/mL}$ of dexpantenol or 2.4 $\mu\text{g/mL}$ of panthenol.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10° . Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to $75 \pm 5^\circ$, and add hydrochloric acid solution (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10° .

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool. Add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 $\mu\text{g/mL}$ of riboflavin, 10 $\mu\text{g/mL}$ of thiamine hydrochloride, and 0.04 $\mu\text{g/mL}$ of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 $\mu\text{g/mL}$ of p-aminobenzoic acid, 50 $\mu\text{g/mL}$ of niacin, and 40 $\mu\text{g/mL}$ of pyridoxine hydrochloride in neutral 25% alcohol. Store in a refrigerator.

Salt solution A: 50 mg/mL of monobasic potassium phosphate and 50 mg/mL of dibasic potassium phosphate in water. Add 10 drops of hydrochloric acid per L of solution. Store under toluene.

Salt solution B: 20 mg/mL of magnesium sulfate, 1 mg/mL of sodium chloride, 1 mg/mL of ferrous sulfate, and 1 mg/mL of manganese sulfate in water. Add 10 drops of hydrochloric acid per L of the solution. Store under toluene.

Pyridoxal–calcium pantothenate solution: 200 $\mu\text{g/mL}$ of pyridoxal hydrochloride and 1.875 $\mu\text{g/mL}$ of calcium pantothenate in 10% alcohol. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: 50 mg/mL of polysorbate 40 and 0.5 mg/mL of oleic acid in 20% alcohol. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal–calcium pantothenate solution	5 mL
Polysorbate 40–oleic acid solution	5 mL

Dissolve anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL.

Double-strength modified pantothenate medium:

Prepare as directed in *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH of 6.5–6.6, and dilute with water to 1000 mL. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*³ on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of sterile *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with sterile *Modified pantothenate medium*. Resuspend the cells in sufficient sterile *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to sterile glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature,

³ ATCC No. 8042 is suitable.

mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes and in the same order add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*.

Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes, and in the same order, add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature NLT 80°, such as by steaming at atmospheric pressure in a sterilizer for 5–10 min. Cool, and determine the percentage transmittance of the suspensions, in cells of equal pathlength, on a suitable spectrophotometer, at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percentage of transmittance, for each set of tubes of the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency of each tube containing portions of the *Sample solution*. Divide the potency of each tube by the amount of the *Sample solution* added to it, to obtain the individual responses. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the material taken for assay, by multiplying the mean response by the appropriate dilution factor. Calculate the percentage of the labeled amount of dexpantenol or panthenol in the portion of Capsules taken:

$$\text{Result} = (P/N) \times 100$$

P = potency of dexpantenol or panthenol in the portion taken (mg)

N = nominal amount of dexpantenol or panthenol in the portion taken (mg)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpantenol or panthenol ($\text{C}_9\text{H}_{19}\text{NO}_4$)

• **CALCIUM PANTOTHENATE, Method 1**

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” To a centrifuge tube, transfer an amount of mixed Capsule contents and a volume of *Internal standard solution* to obtain a concentration of 0.6 mg/mL in the *Sample solution*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$)

• **CALCIUM PANTOTHENATE, Method 2**

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume, to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in the *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (16.66 mg/mL), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution having approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g

of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: Add 5.0 mL of water containing 0.2 µg of calcium pantothenate to each of a series of test tubes containing 5.0 mL of *Basal medium stock solution*. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture of Lactobacillus*

plantarum to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances for each level of the *Sample solution* (Σ_u). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in mg, of calcium pantothenate in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate that was assumed to be present in the portion of Capsules taken
Calculate the percentage of calcium pantothenate in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Capsules taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies *M* is NMT 0.08, their mean, *M*, is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of *M* that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$)

• CALCIUM PANTOTHENATE, Method 3

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from *Standard stock solution* diluted with water

Sample solution: Proceed as directed for the *Sample solution* in *Ascorbic Acid, Method 1*, through “calculate the net weight of the Capsule contents.” Transfer a portion of the Capsule contents, equivalent to 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse the Capsules contents. Dilute with water to volume, mix, and filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 3.9-mm × 30-cm; 5-µm packing L1

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 25 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for calcium pantothenate.

Calculate the percentage of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$)

• NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 1

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for niacinamide, pyridoxine, riboflavin, and thiamine are about 0.3, 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin or niacinamide, pyridoxine, riboflavin, and thiamine. Calculate the percentage of the labeled amount of niacinamide ($\text{C}_6\text{H}_6\text{N}_2\text{O}$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*

r_S = peak area of niacinamide from the *Standard solution*

C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

For formulations containing niacin:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*

r_S = peak area of niacin from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

r_S = peak area of the corresponding vitamin from the *Standard solution*

C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.

Extraction solvent: *Solution A* and methanol (3:1)

Mobile phase: 0.1 M Sodium acetate solution (13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]

Standard stock solution: 1 mg/mL of USP Niacin RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask. Dilute with *Extraction solvent* to volume.

Sample solution: [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine, and riboflavin, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a beaker. Remove any contents adhering to the shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 2 mg of riboflavin, to a 200-mL volumetric flask. If riboflavin is not present in the formulation, use a

portion, equivalent to 2 mg of pyridoxine. If pyridoxine is not present in the formulation, use a portion, equivalent to 20 mg of niacin or niacinamide. Add 100.0 mL of *Extraction solvent*, and mix for 20 min, using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

[NOTE—If necessary, flush the column with methanol between injections.]

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

• **NIACINAMIDE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, Standard stock solution, Standard solution, Sample solution, and

Chromatographic system: Using USP Niacinamide RS in place of USP Niacin RS, proceed as directed in *Niacin, Method 2*.

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacinamide. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, and Sample

solution: Prepare as directed in *Niacin, Method 2*.

Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

Standard solution: 20 µg/mL of USP Pyridoxine Hydrochloride RS from *Standard stock solution* diluted with *Extraction solvent*

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent and Sample solution: Prepare as directed in *Niacin, Method 2*.

Solution A: 6.8 g/L of sodium acetate in water

Mobile phase: Prepare a mixture of *Solution A* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.

Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.

Standard solution: Dilute 5.0 mL of *Standard stock solution* with *Extraction solvent* to 25.0 mL.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for riboflavin. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **THIAMINE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: 1.88 g/L of sodium 1-hexanesulfonate in 0.1% phosphoric acid

Mobile phase: *Solution A* and acetonitrile (46:9)

Standard stock solution: 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid

Standard solution: 0.02 mg/mL of USP Thiamine Hydrochloride RS from *Standard stock solution* diluted with 0.2 N hydrochloric acid

Sample solution: Proceed as directed for the *Sample solution* in *Ascorbic Acid, Method 1*, through “calculate the net weight of the Capsule contents.” Mix a portion of the Capsule contents with a volume of 0.2 N hydrochloric acid to obtain a concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas for the major peaks. For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 3**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in the *Reagent*, with heating if necessary

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed for the *Sample solution* in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1), and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the peaks. Calculate the percentage of the labeled amount of niacin (C₆H₅NO₂) or niacinamide (C₆H₆N₂O) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*

r_S = peak area of niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl), riboflavin (C₁₇H₂₀N₄O₆), and thiamine hydrochloride (C₁₂H₁₇ClN₄OS · HCl) (for products containing thiamine hydrochloride) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

r_S = peak area of the corresponding vitamin from the *Standard solution*

C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate (C₁₂H₁₇N₅O₄S) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin (C₆H₅NO₂) or niacinamide (C₆H₆N₂O), pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl), riboflavin (C₁₇H₂₀N₄O₆), and thiamine as thiamine hydrochloride (C₁₂H₁₇ClN₄OS · HCl) or thiamine mononitrate (C₁₂H₁₇N₅O₄S)

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS**

<2040>: Meet the requirements

• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 3000 cfu/g and the combined molds and yeasts count does not exceed 300 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **LABELING:**⁴ The label states that the product is Oil- and Water-Soluble Vitamins Capsules. The label also states the quantity of each vitamin per dosage unit and, where necessary, the chemical form in which it is present. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*- form. Where more than one assay method is given for a particular vitamin, the labeling states with which assay method the product complies only if *Method 1* is not used.

⁴ USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *d*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *d*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

• **USP REFERENCE STANDARDS** <11>

- USP Alpha Tocopherol RS
 USP Alpha Tocopheryl Acetate RS
 USP Alpha Tocopheryl Acid Succinate RS
 USP Biotin RS
 1*H*-Thieno3,4-*dimidazole*-4-pentanoic acid, hexahydro-2-oxo-, 3*aS*-(3*aα*,4*β*,6*aα*)-, (3*aS*,4*S*,6*aR*)-Hexahydro-2-oxo-1*H*-thieno3,4-*dimidazole*-4-valeric acid.
 $C_{10}H_{16}N_2O_3S$ 244.31
 USP Calcium Pantothenate RS
 β-Alanine, *N*-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-, calcium salt (2:1), (*R*)-.
 Calcium D-pantothenate (1:2).
 $C_{18}H_{32}CaN_2O_{10}$ 476.53
 USP Cholecalciferol RS
 9,10-Secocholesta-5,7,10(19)-trien-3-ol, (3*β*,5*Z*,7*E*)-. Cholecalciferol.
 $C_{27}H_{44}O$ 384.64
 USP Cyanocobalamin RS
 Vitamin B₁₂.
 $C_{63}H_{88}CoN_{14}O_{14}P$ 1355.37
 USP Dexpanthenol RS
 Butanamide, 2,4-dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethyl-, (*R*)-.
D-(+)-2,4-Dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethylbutyramide.
 $C_9H_{19}NO_4$ 205.25
 USP Ergocalciferol RS
 9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol, (3*β*,5*Z*,7*E*,22*E*)-. Ergocalciferol.
 $C_{28}H_{44}O$ 396.65
 USP Folic Acid RS
 L-Glutamic acid, *N*-[4-[(2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-. Folic acid.
N-[*p*-[(2-Amino-4-hydroxy-6-pteridinyl)methyl]amino]-benzoyl]-L-glutamic acid.
 $C_{19}H_{19}N_7O_6$ 441.40
 USP Niacin RS
 3-Pyridinecarboxylic acid. Nicotinic acid.
 $C_6H_5NO_2$ 123.11
 USP Niacinamide RS
 3-Pyridinecarboxamide. Nicotinamide.
 $C_6H_6N_2O$ 122.12
 USP Phytonadione RS
 1,4-Naphthalenedione, 2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)-, [*R*-(*R*^{*},*R*^{*}-(*E*))]-. Phylloquinone.
 $C_{31}H_{46}O_2$ 450.70
 USP Pyridoxine Hydrochloride RS
 3,4-Pyridinedimethanol, 5-hydroxy-6-methyl-, hydrochloride. Pyridoxol hydrochloride.
 $C_8H_{11}NO_3 \cdot HCl$ 205.64
 USP Riboflavin RS
 Riboflavine.
 $C_{17}H_{20}N_4O_6$ 376.36
 USP Thiamine Hydrochloride RS
 Thiazolium, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methyl-, chloride, monohydrochloride. Thiamine monohydrochloride.
 $C_{12}H_{17}ClN_4OS$ 337.27
 USP Vitamin A RS

Oil- and Water-Soluble Vitamins Oral Solution

DEFINITION

Oil- and Water-Soluble Vitamins Oral Solution contains one or more of the following oil-soluble vitamins: Vitamin A, Vitamin D as Ergocalciferol (Vitamin D₂) or Cholecalciferol (Vitamin D₃), and Vitamin E; one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Cyanocobalamin, Niacin or Niacinamide, Dexpanthenol or Panthenol, Pantothenic Acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin or Riboflavin-5'-Phosphate Sodium, and Thiamine Hydrochloride or Thiamine Mononitrate. It contains NLT 90.0% and NMT 250.0% of the labeled amounts of vitamin A ($C_{20}H_{30}O$) as retinol or esters of retinol in the form of retinyl acetate ($C_{27}H_{32}O_2$) or retinyl palmitate ($C_{36}H_{60}O_2$), vitamin D as ergocalciferol ($C_{28}H_{44}O$) or cholecalciferol ($C_{27}H_{44}O$), vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) or alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$), ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 150.0% of the labeled amounts of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), dexpanthenol ($C_9H_{19}NO_4$) or panthenol ($C_9H_{19}NO_4$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), and riboflavin ($C_{17}H_{20}N_4O_6$) or riboflavin-5'-phosphate sodium ($C_{17}H_{20}N_4NaO_9P$); and NLT 90.0% and NMT 450.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$).

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

• **VITAMIN A**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Tetrahydrofuran and acetonitrile (1:1)

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)

Standard solution: 0.33 mg/mL of retinol ($C_{20}H_{30}O$) from USP Vitamin A RS in *Diluent*. [NOTE—USP Vitamin A RS is retinyl acetate. Use it to analyze Oral Solution that contains vitamin A as retinol, retinyl acetate, or retinyl palmitate.]

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 3.3 mg of retinol, to a 500-mL separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 150 mL of solvent hexane, insert the stopper, and shake for 1 min. Add another 150 mL of solvent hexane, insert the stopper, shake, and allow the layers to separate. Discard the aqueous layer, and filter the solvent hexane extract through anhydrous sodium sulfate into a 500-mL, round-bottom flask. Evaporate the solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 65°. Immediately add 10.0 mL of *Diluent*, swirl to dissolve the residue, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 50-cm (prepared from two concatenated 4.6-mm × 25-cm columns); packing L1

Column temperature: 40°

Flow rate: 1.5 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of retinol or retinyl ester from the *Sample solution*

r_S = peak area of retinyl acetate from the *Standard solution*

C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$), in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–250.0% of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$)

• CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D)

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluent and Mobile phase: Prepare as directed for *Vitamin A*.

Standard solution: 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *Diluent*

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 50 µg of cholecalciferol or ergocalciferol, to a 500-mL separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 150 mL of solvent hexane, insert the stopper, and shake for 1 min. Add another 150 mL of solvent hexane, insert the stopper, shake, and allow the layers to separate. Discard the aqueous layer. Drain the solvent hexane extract through anhydrous sodium sulfate into a 500-mL, round-bottom flask. Evaporate the solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 65°. Immediately add 10.0 mL of *Diluent*, swirl to dissolve the residue, and filter.

Chromatographic system: Prepare as directed in *Vitamin A*.

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak areas of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak areas of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of previtamin D present in the formulation, 1.09

Acceptance criteria: 90.0%–250.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• VITAMIN E

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile and ethyl acetate (1:1)

Potassium hydroxide solution: Transfer 90 g of potassium hydroxide pellets to a 100-mL volumetric flask containing 60 mL of water. Mix to dissolve, cool, and dilute with water to volume.

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in *Diluent*

Sample solution: Transfer an amount of Oral Solution equivalent to 1.5 mg of alpha tocopherol to a 125-mL conical flask fitted with a ground-glass joint, and add 25.0 mL of dehydrated alcohol. Attach a reflux condenser, and reflux in a boiling water bath for 1 min. Cautiously add 3 mL of *Potassium hydroxide solution* through the condenser, and continue to reflux for 30 min. Remove the flask from the bath, and rinse the condenser with about 15 mL of water. Cool, and transfer with a minimum volume of water to a 250-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsings to the separatory funnel. Insert the stopper, shake vigorously for 1 min, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with *n*-hexane, and add the rinsing to the hexane solution in the flask. Evaporate the hexane solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 50°. Immediately add 5.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 40°

Flow rate: 3.0 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of alpha tocopherol from the *Sample solution*

r_S = peak area of alpha tocopherol from the *Standard solution*

C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin E, as alpha tocopherol, in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–250.0% of the labeled amount of vitamin E.

- **ASCORBIC ACID, Method 1:** Proceed as directed for *Automated Methods of Analysis* (16), *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–250.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **ASCORBIC ACID, Method 2**

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 80 mg of ascorbic acid, to a conical flask. Add 50 mL of water, 100 mL of 0.1 N sulfuric acid VS, and 15.0 mL of 0.1 N iodine VS. Stir the contents for 30 s, and add 5 mL of starch TS. Immediately titrate with 0.1 N sodium thiosulfate VS to the disappearance of the color. Each mL of 0.1 N iodine is equivalent to 8.806 mg of ascorbic acid ($C_6H_8O_6$).

Acceptance criteria: 90.0%–250.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **CALCIUM ASCORBATE, Method 1:** Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–250.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

- **CALCIUM ASCORBATE, Method 2:** Proceed as directed for *Ascorbic Acid, Method 2*. Each mL of 0.1 N iodine is equivalent to 10.66 mg of $C_{12}H_{14}CaO_{12} \cdot 2H_2O$.

Acceptance criteria: 90.0%–250.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

- **SODIUM ASCORBATE, Method 1:** Proceed as directed for *Automated Methods of Analysis* (16), *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–250.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **SODIUM ASCORBATE, Method 2:** Proceed as directed for *Ascorbic Acid, Method 2*. Each mL of 0.1 N iodine is equivalent to 9.905 mg of $C_6H_7NaO_6$.

Acceptance criteria: 90.0%–250.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **CYANOCOBALAMIN**

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of cyanocobalamin from USP Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described for *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Transfer an accurately measured volume of Oral Solution, assumed to contain 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each mL of the Oral Solution taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B12 activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve

the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol. Store in a refrigerator.

Vitamin solution A: 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in *Table 1*, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions in the resulting solution. Add 100 mL of water, and dissolve dextrose, sodium acetate, and ascorbic acid. Filter, if necessary, add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of 5.5–6.0, and add Purified Water to make 250 mL.

Table 1

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N Hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g

Table 1 (Continued)

Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter-aid in the supernatant, and filter, with the aid of reduced pressure, through a layer of the filter-aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium

[NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.]

Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*. [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.]

Incubate for 16–24 h at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Transfer cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL of the *Culture medium* each. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. The cell suspension

so obtained is the *Inoculum*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.]

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis: Because of the high sensitivity of the test organism to minimum amounts of vitamin B₁₂ activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes, and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes, add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within $\pm 0.5^\circ$, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating the contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points. Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances (Σ_u) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls

within the range of the lowest and highest points plotted for the standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, x , for each dosage level. Average the values of x for each of three or more dosage levels to obtain \bar{x} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$), in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of cyanocobalamin assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of cyanocobalamin in the portion of the Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of Oral Solution taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *Vitamin B₁₂ Activity*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–450.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• **CALCIUM PANTOTHENATE, Method 1**

Mobile phase: 0.2 M monobasic sodium phosphate and methanol (97:3). Adjust with 1.7 M phosphoric acid to a pH of 3.2 ± 0.1 .

Standard solution: 80 $\mu\text{g}/\text{mL}$ of USP Calcium Pantothenate RS in *Mobile phase*

System suitability solution: 80 $\mu\text{g}/\text{mL}$ of USP Racemic Panthenol RS in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

Sample solution: Equivalent to 80 $\mu\text{g}/\text{mL}$ of calcium pantothenate from Oral Solution, in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.0-mm \times 10-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 1.5 between panthenol and calcium pantothenate, *System suitability solution*

Tailing factor: NMT 2.0 for both the calcium pantothenate and the panthenol peaks, *Standard solution*

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas for calcium pantothenate. Calculate the percentage of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$) in the portion of the Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of calcium pantothenate from the *Sample solution*

r_S = peak area of calcium pantothenate from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$)

• **CALCIUM PANTOTHENATE, Method 2**

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 $\mu\text{g}/\text{mL}$ of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 $\mu\text{g}/\text{mL}$ of calcium pantothenate, the exact concentration being such that the responses obtained as directed for *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a measured volume of this solution quantitatively, and stepwise if necessary, with water to obtain a solution having about the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine-guanine-uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin-thiamine hydrochloride-biotin solution: 20 $\mu\text{g}/\text{mL}$ of riboflavin, 10 $\mu\text{g}/\text{mL}$ of thiamine hydrochloride, and 0.04 $\mu\text{g}/\text{mL}$ of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution: 10 $\mu\text{g}/\text{mL}$ of p-aminobenzoic acid, 50 $\mu\text{g}/\text{mL}$ of niacin, and 40 $\mu\text{g}/\text{mL}$ of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to Table 2, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 2

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL
Riboflavin-thiamine hydrochloride-biotin solution	5 mL
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water; add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar; and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*, incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution*, add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis: To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes, add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool,

add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube. With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points. Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances (Σ_u) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, x , for each dosage level. Average the values of x for each of three or more dosage levels to obtain \bar{x} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in mg, of USP Calcium Pantothenate RS corresponding to the calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Oral Solution (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **DEXPANTHENOL OR PANTHENOL, Method 1**

Mobile phase and Chromatographic system: Proceed as directed in *Calcium Pantothenate, Method 1*.

Standard solution: 80 µg/mL of USP Dexpantenol RS or USP Racemic Panthenol RS in *Mobile phase*. [NOTE—Use USP Dexpantenol RS to analyze Oral Solution that contains dexpantenol and USP Racemic Panthenol RS to analyze Oral Solution that contains panthenol.]

System suitability solution: 80 µg/mL of USP Calcium Pantothenate RS in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

Sample solution: Equivalent to 80 µg/mL of dexpantenol or panthenol from the Oral Solution in *Mobile phase*

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the areas for panthenol. Calculate the percentage of the labeled amount of dexpantenol or panthenol ($C_9H_{19}NO_4$) in the portion of the Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of dexpantenol or panthenol from the *Sample solution*

r_S = peak area of dexpantenol or panthenol from the *Standard solution*

C_S = concentration of USP Dexpantenol RS or USP Racemic Panthenol RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of dexpantenol or panthenol in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpantenol or panthenol ($C_9H_{19}NO_4$)

• **DEXPANTHENOL OR PANTHENOL, Method 2**

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 µg/mL of USP Dexpantenol RS or 1600 µg/mL of USP Racemic Panthenol RS in water. Store in a refrigerator, protected from light, and use within 30 days. [NOTE—Use USP Dexpantenol RS to analyze Oral Solution that contains dexpantenol and USP Racemic Panthenol RS to analyze Oral Solution that contains panthenol.]

Standard solution: On the day of the assay, prepare 1.2 µg/mL of dexpantenol or 2.4 µg/mL of racemic panthenol from the *Standard stock solution* with water.

Sample solution: 1.2 µg/mL of dexpantenol or 2.4 µg/mL of panthenol from the Oral Solution in water

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700 to 800 mL of water, heat to $75 \pm 5^\circ$, and add 6 M hydrochloric acid solution dropwise, with stirring, until the solids are dissolved.

Cool, add water to make 1000 mL, and mix. Store under toluene in a cool place at a temperature not below 10°.

Adenine-guanine-uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, add water to make 200 mL, and mix. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin-thiamine hydrochloride-biotin solution: Prepare a solution of riboflavin, thiamine hydrochloride, and biotin in 0.02 N acetic acid containing 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution: Prepare a solution in neutral 25% alcohol containing 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride. Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Pyridoxal-calcium pantothenate solution: Dissolve 40 mg of pyridoxal hydrochloride and 375 µg of calcium pantothenate in 10% alcohol to make 200 mL, and mix. Store in a refrigerator, and use within 30 days.

Polysorbate 40-oleic acid solution: Dissolve 25 g of polysorbate 40 and 0.25 g of oleic acid in 20% alcohol to make 500 mL, and mix. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium: Dissolve anhydrous dextrose and sodium acetate in the solutions previously mixed according to *Table 3*, adjust with 1 N sodium hydroxide to a pH of 6.8, dilute with water to 250 mL, and mix.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL
Riboflavin-thiamine hydrochloride-biotin solution	5 mL
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal-calcium pantothenate solution	5 mL
Polysorbate 40-oleic acid solution	5 mL

Double-strength modified pantothenate medium:

Prepare as directed under *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar in 800 mL of water, with the aid of heat. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH between 6.5 and 6.6, dilute with water to 1000 mL, and mix. Add 10-mL portions of the solution to culture tubes, place caps on the

tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*¹ on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with *Modified pantothenate medium*. Resuspend the cells in sufficient *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes, and in the same order, add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*.

Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes, and in the same order, add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube, and mix. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature not below 80°, such as by steaming at atmospheric pressure in a sterilizer for 5 to 10 min. Cool, and concomitantly determine the percentage transmittance of the suspensions, in cells of equal pathlength, on a suitable spectrophotometer, at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percent transmittance, for each set of tubes of the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency of each tube containing portions of the *Sample solution*. Divide the potency of each tube by the amount of the *Sample solution* added to it, to obtain the individual responses. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the material taken for assay, by multiplying the mean response by the appropriate dilution factor. Calculate the percentage of the labeled amount of dexpanthenol or panthenol in the portion of Oral Solution taken:

$$\text{Result} = (P/N) \times 100$$

P = calculated potency of dexpanthenol or panthenol in the portion of Oral Solution taken (mg)
N = nominal amount of dexpanthenol or panthenol in the portion of Oral Solution taken (mg)

¹ ATCC No. 8042 is suitable.

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($\text{C}_9\text{H}_{19}\text{NO}_4$)

• NIACIN or NIACINAMIDE

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: 25 mg/mL of edetate disodium in water

Mobile phase: Mix 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol, and dilute with 0.008 M sodium 1-hexanesulfonate to 2000 mL.

Standard solution: 0.10 mg/mL of USP Niacin RS or USP Niacinamide RS in *Diluent*. [NOTE—Use USP Niacin RS for Oral Solution that contains niacin and USP Niacinamide RS for Oral Solution that contains niacinamide.]

Sample solution: Dilute an accurately measured volume of Oral Solution with *Diluent* to obtain a solution with a concentration of 0.1 mg/mL of niacin or niacinamide.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Flow rate: 2 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of niacin ($\text{C}_6\text{H}_5\text{NO}_2$) or niacinamide ($\text{C}_6\text{H}_6\text{N}_2\text{O}$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*

r_S = peak area of niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% the labeled amount of niacin ($\text{C}_6\text{H}_5\text{NO}_2$) or niacinamide ($\text{C}_6\text{H}_6\text{N}_2\text{O}$)

• PYRIDOXINE HYDROCHLORIDE

Diluent, Mobile phase, and Chromatographic system: Proceed as directed in the assay for *Niacin* or *Niacinamide*.

Standard solution: Equivalent to 24 µg/mL of USP Pyridoxine Hydrochloride RS in *Diluent*

Sample solution: Equivalent to 24 µg/mL of Pyridoxine Hydrochloride from Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($\text{C}_8\text{H}_{11}\text{NO}_3 \cdot \text{HCl}$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine hydrochloride from the *Sample solution*

r_S = peak area of pyridoxine hydrochloride from the *Standard solution*

C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($\text{C}_8\text{H}_{11}\text{NO}_3 \cdot \text{HCl}$)

• **RIBOFLAVIN-5'-PHOSPHATE SODIUM, Method 1**

[NOTE—Riboflavin-5'-phosphate sodium is quantitated against USP Riboflavin RS in this procedure. In the chromatogram of the *Sample solution*, the riboflavin-5'-phosphate peak is the only peak measured for calculation.]

Diluent, Mobile phase, and Chromatographic system: Proceed as directed in the assay for *Niacin* or *Niacinamide*.

Standard solution: 8 µg/mL of USP Riboflavin RS in *Diluent*, by heating if necessary.

Sample solution: Equivalent to 8 µg/mL of riboflavin from Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—The relative retention times for riboflavin-5'-phosphate and riboflavin are about 0.18 and 1.0, respectively.]

Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of riboflavin-5'-phosphate from the *Sample solution*

r_S = peak area of riboflavin from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

F = factor for converting the response obtained for riboflavin-5'-phosphate to riboflavin, 1.493. [NOTE—Riboflavin phosphate sodium is a mixture of isomeric monophosphates and diphosphates containing an average amount of 67% of riboflavin-5'-monophosphate, which separates in this chromatographic system. The factor 1.493 assumes 67% of riboflavin-5'-monophosphate.]

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **RIBOFLAVIN OR RIBOFLAVIN-5'-PHOSPHATE SODIUM, Method 2**
[NOTE—Use low-actinic glassware throughout this procedure.]

Solvent blank: 1 N hydrochloric acid, 2.5 M sodium acetate, and water (1:2:97)

Standard stock solution: Transfer 16 mg of USP Riboflavin RS to a 100-mL volumetric flask, dissolve in 1.0 mL of 1 N hydrochloric acid and 2.0 mL of 2.5 M sodium acetate, and dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Standard solution: Transfer 5.0 mL of the *Standard stock solution* to a 500-mL volumetric flask, add 5.0 mL of 1 N hydrochloric acid, 10.0 mL of 2.5 M sodium acetate, and dilute with water to volume.

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 0.8 mg of riboflavin to a 100-mL volumetric flask, and dilute with water to volume. Transfer 10.0 mL of the resulting solution to a 500-mL volumetric flask, add 5.0 mL of 1 N hydrochloric acid, 10.0 mL of 2.5 M sodium acetate, and dilute with water to volume.

Spectrometric conditions

Mode: Fluorescence

Analytical wavelength

Excitation: 440 nm

Emission: 530 nm

Blank: *Solvent blank*

Analysis

Samples: *Standard solution* and *Sample solution*
Determine the maximum fluorescence intensities, I_S and I_U , of the *Standard solution* and the *Sample solution*, respectively. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Oral Solution taken:

$$\text{Result} = (I_U/I_S) \times (C_S/C_U) \times 100$$

I_U = fluorescence intensity from the *Sample solution*

I_S = fluorescence intensity from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **THIAMINE**

Diluent, Mobile phase, and Chromatographic system: Proceed as directed for *Niacin* or *Niacinamide*.

Standard solution: 24 µg/mL of USP Thiamine Hydrochloride RS in *Diluent*

Sample solution: Equivalent to 24 µg/mL of thiamine hydrochloride from Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the areas for the major peaks. Calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine hydrochloride or thiamine mononitrate in the *Sample solution* (mg/mL)

F = 1 (for products containing thiamine hydrochloride) and 0.97 (for products containing thiamine mononitrate)

Acceptance criteria: 90.0%–250.0% of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

OTHER COMPONENTS

- **ALCOHOL DETERMINATION, Method I <611>** (if present): 90.0%–120.0% of the labeled amount of C_2H_5OH

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS <2021>**: The total aerobic microbial count does not exceed 3000 cfu/mL, and the combined molds and yeasts count does not exceed 300 cfu/mL.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS <2022>**: Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, under an inert gas or with a minimum of headspace.

- **LABELING:**² The label states that the product is Oil- and Water-Soluble Vitamins Oral Solution. The label states the quantity of each vitamin present in a given volume of Oral Solution and, where necessary, the chemical form in which a vitamin is present. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*-form. Where the product is labeled to contain panthenol, the label states the equivalent content of dexpanthenol. Where more than one assay method is given for a particular vitamin, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** (11)
 - USP Alpha Tocopherol RS
 - USP Calcium Pantothenate RS
 - β -Alanine, *N*-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-, calcium salt (2:1), (R)-. $C_{18}H_{32}CaN_2O_{10}$ 476.53
 - USP Cholecalciferol RS
 - 9,10-Secocholesta-5,7,10(19)-trien-3-ol, (3 β ,5Z,7E)-. $C_{27}H_{44}O$ 384.64
 - USP Cyanocobalamin RS
 - Vitamin B₁₂. $C_{63}H_{88}CoN_{14}O_{14}P$ 1355.37
 - USP Dexpanthenol RS
 - D-(+)-2,4-Dihydroxy-*N*-(3-hydroxypropyl)-3-3-dimethylbutyramide. $C_9H_{19}NO_4$ 205.25
 - USP Ergocalciferol RS
 - 9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol, (3 β ,5Z,7E,22E)-. $C_{28}H_{44}O$ 396.65
 - USP Niacin RS
 - 3-Pyridinecarboxylic acid. $C_6H_5NO_2$ 123.11
 - USP Niacinamide RS
 - 3-Pyridinecarboxamide. $C_6H_6N_2O$ 122.12
 - USP Pyridoxine Hydrochloride RS
 - 3,4-Pyridinedimethanol, 5-hydroxy-6-methyl-, hydrochloride. $C_8H_{11}NO_3 \cdot HCl$ 205.64
 - USP Racemic Panthenol RS
 - USP Riboflavin RS
 - Riboflavin. $C_{17}H_{20}N_4O_6$ 376.36
 - USP Thiamine Hydrochloride RS
 - Thiazolium, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methyl-, chloride, monohydrochloride. $C_{12}H_{17}ClN_4OS \cdot HCl$ 337.27
 - USP Vitamin A RS
 - 3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl) 2,4,6,8-nonatetraen-1-ol acetate (vitamin A acetate).

² USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 μ g of all-*trans*-retinol (vitamin A alcohol) or 0.344 μ g of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 μ g of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 μ g of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 μ g of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 μ g of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

Oil- and Water-Soluble Vitamins Tablets

DEFINITION

Oil- and Water-Soluble Vitamins Tablets contain one or more of the following oil-soluble vitamins: Vitamin A, Vitamin D as Ergocalciferol (Vitamin D₂) or Cholecalciferol (Vitamin D₃), Vitamin E, Phytonadione (Vitamin K₁), and Beta Carotene, and one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, Pantothenic Acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate. Tablets contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A ($C_{20}H_{30}O$) as retinol or esters of retinol in the form of retinyl acetate ($C_{22}H_{32}O_2$) or retinyl palmitate ($C_{36}H_{60}O_2$), vitamin D as ergocalciferol ($C_{28}H_{44}O$) or cholecalciferol ($C_{27}H_{44}O$); vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$); phytonadione ($C_{31}H_{46}O_2$); and beta carotene ($C_{40}H_{56}$); and NLT 90.0% and NMT 150.0% of the labeled amounts of ascorbic acid ($C_6H_8O_6$) or its salts, as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$); biotin ($C_{10}H_{16}N_2O_3S$); cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$); folic acid ($C_{19}H_{19}N_7O_6$); niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$); calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$); pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$); riboflavin ($C_{17}H_{20}N_4O_6$); and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate.

They do not contain any minerals. They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

[NOTE—Where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

• VITAMIN A, *Method 1*

[NOTE—Where the use of a vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-acid glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution: 15 μ g/mL of retinyl acetate from USP Vitamin A RS in *n*-hexane

System suitability stock solution: 15 μ g/mL of retinyl palmitate in *n*-hexane

System suitability solution: Mix equal volumes of *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 μ g/mL each of retinyl acetate and retinyl palmitate.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a container having a polytetrafluoroethylene-lined screw cap. Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [NOTE—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Dilute a 10-mL volume of this solution with *n*-hexane to obtain a solu-

tion with a concentration of 15 µg/mL of vitamin A as retinol (C₂₀H₃₀O).

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection size: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area for all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution*. For products containing vitamin A acetate or vitamin A palmitate, calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₂H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A as retinol (C₂₀H₃₀O) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)

• VITAMIN A, Method 2

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution: Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Mobile phase: *n*-Hexane and ethyl acetate (99.7:0.3)

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in 2,2,4-trimethylpentane

System suitability stock solution: 15 µg/mL of retinyl palmitate in 2,2,4-trimethylpentane

System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E, when present in the formulation.] Finely powder NLT 20 Tablets. If vitamin D is present in the formulation, transfer a portion of the powder, equivalent to 30 µg of

the labeled amount of cholecalciferol or ergocalciferol, to a container having a polytetrafluoroethylene-lined screw cap. If vitamin D is not present in the formulation, use a portion of the powder, equivalent to 90 mg of the labeled amount of vitamin E (as alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl hemisuccinate). If vitamin E is not present in the formulation, use a portion of the powder, equivalent to 2.5 mg of the labeled amount of retinyl acetate or retinyl palmitate. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of 3 N methanolic sulfuric acid solution, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. [NOTE—The supernatant is used for the determination of vitamin A, and also vitamin D and vitamin E, if present in the formulation.] If necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1.5 mL/min

Injection size: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 8.0 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area of all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution*.

Calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₂H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in the USP Vitamin A RS, to retinol, 0.872

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration. The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)

• VITAMIN A, Method 3

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP

Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Extraction solvent: *n*-Hexane and methylene chloride (3:1)

Potassium hydroxide solution: 800 mg/mL of potassium hydroxide in water. [NOTE—Cautiously add the potassium hydroxide to the water. Mix, and cool.]

Diluent: 10 mg/mL of pyrogallol in alcohol

Mobile phase: *n*-Hexane and isopropyl alcohol (92:8)

Standard stock solution: 30 µg/mL of retinyl acetate from USP Vitamin A RS in *Diluent*. [NOTE—This solution may be stored in a refrigerator for 1 week.]

Standard solution: Dilute a volume of *Standard stock solution* with *Diluent* to obtain a concentration of 1 µg/mL of USP Vitamin A RS. Transfer 10.0 mL of this solution to a stoppered 125-mL flask, and add 5 mL of water, 5 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at $60 \pm 5^\circ$, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. Withdraw a portion of the organic layer for injection into the chromatograph. This *Standard solution* contains 0.34 µg/mL of retinol.

Sample solution: Finely powder a counted number of Tablets. Transfer a portion of the powder, equivalent to 1.5 mg of retinyl acetate, to a stoppered 125-mL flask. Add 5 mL of water, 15 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at $60 \pm 5^\circ$, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s or longer, if necessary, for complete extraction. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. [NOTE—Do not shake, because an emulsion may form.] Withdraw a portion of the organic layer, and dilute with *Extraction solvent* to obtain a concentration of 0.34 µg/mL of retinol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*).

Mode: LC

Detector: UV 335 nm

Column: 6.2-mm \times 8-cm; packing L3

Column temperature: 40°

Flow rate: 4 mL/min

Injection size: 50 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for 13-*cis*-retinol and all-*trans*-retinol are 0.92 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for all-*trans*-retinol and 13-*cis*-retinol. Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Tablets taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times (C_S/C_U) \times F \times 100$$

r_{T1} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Sample solution*

r_{T2} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Standard solution*

C_S = concentration of retinyl acetate ($C_{23}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D),

Method 1

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate, if necessary, in vacuum at room temperature to obtain a concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*).

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm \times 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D),

Method 2

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution, Sodium ascorbate-pyrogallol solution, Lecithin solution, and

Sample solution: Proceed as directed in *Vitamin A, Method 2*.

Mobile phase: *n*-Hexane and tertiary butyl alcohol (98.75:1.25)

Standard solution: 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in 2,2,4-trimethylpentane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1 mL/min

Injection size: 40 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D), Method 3

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluted acetic acid: Glacial acetic acid solution (1 in 10) in water

Phenolphthalein solution: 10 mg/mL of phenolphthalein in alcohol

Potassium hydroxide solution: Slowly dissolve 14 g of potassium hydroxide in a mixture of 31 mL of dehydrated alcohol and 5 mL of water. Prepare fresh daily.

Extraction solvent: Methylene chloride and isopropyl alcohol (99.8:0.2)

Mobile phase: Acetonitrile and methanol (91:9)

Standard stock solution: 0.2 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in dehydrated alcohol. [NOTE—Prepare fresh every 4 weeks. Store in a freezer.]

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* by initially washing the

column with 4.0 mL of a mixture of methylene chloride and isopropyl alcohol (4:1), followed by 5.0 mL of *Extraction solvent*. Do not allow the column to dry.]

Dilute a volume of *Standard stock solution* with dehydrated alcohol to obtain a concentration of 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS. Prepare this solution fresh daily. Transfer 2.0 mL of this solution to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL, round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase extraction column containing silica packing with a sorbent mass to column volume ratio of 500 mg to 2.8 mL or equivalent, rinse the round-bottom flask with 1.0 mL of *Extraction solvent*, and transfer to the column. Elute the column with 2.0 mL of *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 10 µg of cholecalciferol or ergocalciferol, to the stoppered 125-mL flask, and proceed as directed for the *Standard solution*, beginning with "Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*".

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 27°

Flow rate: 0.7 mL/min

Injection size: 15 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 4.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
 r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
 C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* ($\mu\text{g/mL}$)
 C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($\text{C}_{27}\text{H}_{44}\text{O}$) or ergocalciferol ($\text{C}_{28}\text{H}_{44}\text{O}$)

• VITAMIN E, Method 1

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water

Mobile phase: Methanol and *Solution A* (19:1)

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

System suitability solution: Prepare a 0.65-mg/mL solution of USP Ergocalciferol RS in methanol. Transfer 1.0 mL of this solution to a 100-mL volumetric flask containing 100 mg of USP Alpha Tocopheryl Acetate RS. Dissolve in 30 mL of methanol, with the aid of sonication if necessary, and dilute with methanol to volume. Store this solution in a refrigerator.

Sample solution: Proceed as directed for the *Sample solution* in *Vitamin A, Method 1*. Transfer NLT 20 mL of this solution to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm \times 10-cm; 5- μm packing L1

Flow rate: 2 mL/min

Injection size: 100 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for ergocalciferol and alpha tocopheryl acetate are about 0.5 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 12 between ergocalciferol and alpha tocopheryl acetate, *System suitability solution*

Tailing factor: Between 0.8 and 1.2, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol ($\text{C}_{29}\text{H}_{50}\text{O}_2$), alpha tocopheryl acetate ($\text{C}_{31}\text{H}_{52}\text{O}_3$), or alpha tocopheryl acid succinate ($\text{C}_{33}\text{H}_{54}\text{O}_5$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of the relevant vitamin E form from the *Sample solution*
 r_S = peak area of the relevant vitamin E form from the *Standard solution*

- C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)
 C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($\text{C}_{29}\text{H}_{50}\text{O}_2$), alpha tocopheryl acetate ($\text{C}_{31}\text{H}_{52}\text{O}_3$), or alpha tocopheryl acid succinate ($\text{C}_{33}\text{H}_{54}\text{O}_5$)

• VITAMIN E, Method 2

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 240 mL of methanol with 10 mL of water followed by 0.5 mL of 50% phosphoric acid, and dilute with acetonitrile to 1000 mL.

System suitability solution: 2 mg/mL each of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, and USP Alpha Tocopheryl Acid Succinate RS in methanol

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Proceed as directed for *Sample solution* in *Vitamin A, Method 2*. Transfer a volume of the supernatant 2,2,4-trimethylpentane to a suitable volumetric flask, the volume of the specimen withdrawn from the 2,2,4-trimethylpentane and the size of the volumetric flask being such that the final concentration of the *Sample solution* is equivalent to that of the *Standard solution*. Evaporate nearly to dryness, add several mL of methanol, and evaporate the remaining 2,2,4-trimethylpentane. Dilute with methanol to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm \times 25-cm; 5- μm packing L1

Flow rate: 1.5 mL/min

Injection size: 25 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for alpha tocopheryl acid succinate, alpha tocopherol, and alpha tocopheryl acetate are about 0.6, 0.8, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 4.0 between alpha tocopheryl acid succinate and alpha tocopherol; NLT 3.0 between alpha tocopherol and alpha tocopheryl acetate, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol ($\text{C}_{29}\text{H}_{50}\text{O}_2$), alpha tocopheryl acetate ($\text{C}_{31}\text{H}_{52}\text{O}_3$), or alpha tocopheryl acid succinate ($\text{C}_{33}\text{H}_{54}\text{O}_5$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of the relevant vitamin E form from the *Sample solution*
 r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_s = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane and the dilution factor to exchange the solvent from 2,2,4-trimethylpentane to methanol to calculate the nominal concentration.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **VITAMIN E, Method 3**

Diluent: Acetonitrile and ethyl acetate (1:1)

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in methanol

Sample solution: Finely powder NLT 20 Tablets.

Transfer a portion of the powder, equivalent to 8 mg of alpha tocopherol, to a 125-mL flask fitted with a ground-glass joint. Add 25.0 mL of water, 25.0 mL of dehydrated alcohol, and 3.5 g of potassium hydroxide pellets. Shake for 1 h in a water bath maintained at 55°. Cool, and transfer with the aid of a minimum volume of water to a 125-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 60 s, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and add the rinsing to the hexane solution in the flask. Place the flask in a water bath maintained at 50°, and evaporate the hexane solution with the aid of a rotary evaporator to dryness. Immediately add 25.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 40°

Flow rate: 3 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of alpha tocopherol from the *Sample solution*

r_S = peak area of alpha tocopherol from the *Standard solution*

C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin E as alpha tocopherol in the *Sample solution* (mg/mL)

[NOTE—Calculate the content of alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) by dividing the content, in mg/Tablet of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), by the factor 0.91 or 0.81, respectively.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **PHYTONADIONE, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP

Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS

from the *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha

Tocopheryl Acetate RS and 20 µg/mL of USP

Phytonadione RS from the *Standard stock solution*

diluted with methanol. [NOTE—Dissolve USP Alpha

Tocopheryl Acetate RS in a portion of methanol, add

the *Standard stock solution*, and then dilute with

methanol to volume.]

Sample solution: Transfer NLT 20 mL of the solution retained as specified in the directions for *Sample solution* in *Vitamin A, Method 1* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for alpha tocopheryl acetate and phytonadione are 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of phytonadione from the *Sample solution*

r_S = peak area of phytonadione from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• **PHYTONADIONE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solvent: Methanol and isopropyl alcohol (19:1)

Mobile phase: Mix 800 mL of methanol, 200 mL of methylene chloride, 0.1 mL of glacial acetic acid, 1.36 g of zinc chloride, and 0.41 g of sodium acetate.

Internal standard solution: 5 µg/mL of menaquinone 4 (vitamin K₂) in *Solvent*. [NOTE—A concentrated stock solution of menaquinone 4 (100 µg/mL) can be stored for 2 months in a refrigerator.]

Standard stock solution: 5 µg/mL of USP Phytonadione RS, prepared by dissolving in methylene chloride with the aid of sonication, and diluting with *Solvent* to final volume.

Standard solution: Transfer 1.0 mL of the *Standard stock solution* and 1.0 mL of the *Internal standard solution* to a suitable flask, and dilute with *Solvent* to 5 mL. Pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Finely powder NLT 20 Tablets. To a centrifuge tube fitted with a cap transfer an amount of powder, not exceeding 800 mg and equivalent to an amount of phytonadione not exceeding 50 µg. Add 4 mL of water. Insert the stopper, and mix using a vortex mixer until the sample is dispersed. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 1 min while the preparation is still hot. Add 8 mL of alcohol, and swirl the contents to mix. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 2 min while the preparation is still hot. Cool to room temperature. Add a volume of *Internal standard solution*, equivalent to 1.0 mL for each 5 µg of the expected amount of phytonadione in the aliquot taken. Add 20.0 mL of petroleum ether, and cap the tube tightly. Shake or mix using a vortex mixer for 15 min to thoroughly mix the contents. Centrifuge to separate the two layers. Transfer a volume of the top layer of petroleum ether, equivalent to 5–50 µg of the nominal amount of phytonadione, to an appropriate flask. Place the flask in a water bath at 35°–45°, and evaporate the solvent under a stream of nitrogen until an oily residue is left. Dissolve the residue in a volume of *Solvent* to obtain a concentration of 1 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Fluorometric detector set at 320 nm for excitation and 420 nm for emission

Column: 4.6-mm × 25-cm; 5-µm, end-capped packing L1, and a postcolumn reactor constituted with a 4.6-mm × 3-cm PEEK column tightly packed with zinc powder. [NOTE—Prepare the postcolumn reactor daily, or as necessary, to meet the system suitability requirements.]

Flow rate: 1 mL/min

Injection size: 25 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for the internal standard and phytonadione are 1.0 and 1.4, respectively.]

Suitability requirements

Column efficiency: NLT 2500 theoretical plates for the phytonadione peak

Tailing factor: NMT 1.5 for the phytonadione peak

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of phytonadione (C₃₁H₄₆O₂) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of phytonadione to that of the internal standard from the *Sample solution*

R_S = peak response ratio of phytonadione to that of the internal standard from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of labeled amount of phytonadione (C₃₁H₄₆O₂)

• BETA CAROTENE

[NOTE—Use low-actinic glassware throughout this procedure.]

Potassium hydroxide solution: Dissolve 58.8 g of potassium hydroxide in 50 mL of water.

Iodine solution: 0.01 mg/mL of iodine in cyclohexane. [NOTE—Prepare this solution fresh daily.]

Sample solution: Weigh NLT 20 Tablets. Grind the Tablets to a fine powder, and transfer a quantity of the powder, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature, add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower aqueous layer into the saponification flask, add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume, add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Vis

Analytical wavelength: 452 nm

Blank: Cyclohexane

Analysis

Sample: *Sample solution*

Determine the absorbance against the *Blank*. Calculate the percentage of the labeled amount of beta carotene (C₄₀H₅₆) in the portion of Tablets taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of the *Sample solution*

F = absorptivity of beta carotene at 452 nm, 223

C_U = nominal concentration of beta carotene in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene (C₄₀H₅₆)

• ASCORBIC ACID, Method 1

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent of 100 mg of ascorbic acid, to a 200-mL volumetric flask, and add 75 mL of metaphosphoric-acetic acids TS. Insert a

stopper into the flask, and shake by mechanical means for 30 min. Dilute with water to volume. Transfer a portion of the solution to a centrifuge tube, and centrifuge until a clear supernatant is obtained. Pipet 4.0 mL of this solution into a 50-mL conical flask, and add 5 mL of metaphosphoric-acetic acids TS.

Analysis: Titrate with standard dichlorophenol-indophenol solution VS to a rose-pink color that persists for at least 5 s. Correct for the volume of dichlorophenol-indophenol solution consumed by a mixture of 5.5 mL of metaphosphoric-acetic acids TS and 15 mL of water. From the ascorbic acid equivalent of the standard dichlorophenol-indophenol solution, calculate the content of ascorbic acid in each Tablet.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **ASCORBIC ACID, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), Assay for Ascorbic Acid.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **CALCIUM ASCORBATE, Method 1:** Proceed as directed in *Ascorbic Acid, Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

- **CALCIUM ASCORBATE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), Assay for Ascorbic Acid.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

- **SODIUM ASCORBATE, Method 1:** Proceed as directed in *Ascorbic Acid, Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **SODIUM ASCORBATE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), Assay for Ascorbic Acid.

Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **BIOTIN, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Finely powder NLT 20 Tablets.

Transfer a portion of the powder, equivalent to 1 mg of biotin, to a 200-mL volumetric flask, add 3 mL of dimethyl sulfoxide, and swirl to wet. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-µm packing L7

Flow rate: 1.2 mL/min

Injection size: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*

r_S = peak area of biotin from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• Biotin, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by dilution of the *Standard stock solution* with water on the day of the assay

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate, quantitatively and stepwise if necessary, with water to obtain a solution with a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine-guanine-uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin-thiamine hydrochloride solution:

20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add

5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to Table 1, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin-thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an inoculum comparable to a fresh culture.]

Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of culture medium. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*
To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool,

and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength from 540 to 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances for each level of the *Sample solution* (Σ_u). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of biotin in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of biotin that was assumed to be present in the portion of Tablets taken
Calculate the percentage of the labeled amount of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$) in the portion of the Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin in the portion of the Tablets taken (μg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (11), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$)

¹ ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

• **BIOTIN, Method 3**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 800 mL of water and 100 mL of triethylamine to a 1000-mL volumetric flask. Add 80 mL of 85% phosphoric acid, and dilute with water to volume.

Mobile phase: Transfer 80 mL of acetonitrile and 10 mL of *Solution A* to a 1000-mL volumetric flask. Dilute with water to volume.

Standard solution: 0.6 µg/mL of USP Biotin RS in water. [NOTE—A portion of the *Standard solution* will be used to determine the percent recovery of biotin from the *Solid-phase extraction* procedure.]

Sample solution: Finely powder NLT 20 Tablets. Transfer an amount of powdered Tablets to a volumetric flask to obtain a concentration of 0.6 µg/mL of biotin. Add water up to 80% of the flask capacity, sonicate for 30–40 min, with occasional mixing, to dissolve. Dilute with water to volume, and filter. Adjust the pH of the solution with either dilute acetic acid or 0.1 N sodium hydroxide to 6.0–7.0.

Solid-phase extraction: [NOTE—Condition the extraction column specified in this procedure in the following manner. Wash the column with a 2-mL portion of methanol. Equilibrate with a 2-mL portion of water.] Separately pipet 5.0 mL of the *Sample solution* and *Standard solution* into freshly conditioned solid-phase extraction columns consisting of a mixed-mode packing with a sorbent-mass of 60 mg. [NOTE—The mixed-mode packing consists of anion-exchange and reversed-phase sorbents. The reverse-phase component is a copolymer of *N*-vinylpyrrolidone and divinylbenzene. The anion exchange moiety is a trialkylamino group.²] Wash the column with 10 mL of 30% (v/v) methanol in water. Apply an appropriate volume (4.9 mL) of 30% (v/v) methanol in 0.1 N hydrochloric acid to the column. Collect the eluate in a 5-mL volumetric flask containing 100 µL of 40% (w/v) sodium acetate in water, and dilute with 30% (v/v) methanol in 0.1 N hydrochloric acid to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and a portion of the *Standard solution* that has undergone *Solid-phase extraction*

Suitability requirements

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution* and the *Standard solution* that has undergone *Solid-phase extraction*

Recovery: 95%–100%, *Standard solution* that has undergone *Solid-phase extraction*

Analysis

Samples: *Standard solution* and *Sample solution* that have both undergone *Solid-phase extraction*. Measure the peak area of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*

r_S = peak area of biotin from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **CYANOCOBALAMIN, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 µg/mL of USP

Cyanocobalamin RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

Standard solution: 1 µg/mL of USP Cyanocobalamin RS from the *Standard stock solution* diluted with water

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to 100 µg of cyanocobalamin, to a 250-mL flask. Quantitatively add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 0.5 mL/min

Injection size: 200 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area of cyanocobalamin. Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cyanocobalamin from the *Sample solution*

r_S = peak area of cyanocobalamin from the *Standard solution*

C_S = concentration of USP Cyanocobalamin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **CYANOCOBALAMIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of USP

Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Finely powder NLT 20 Tablets.

Transfer a portion of the powdered Tablets, equivalent to 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each g of powdered Tablets taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B₁₂ activity

² A suitable cartridge is the Waters, Oasis MAX Vac RC cartridge, particle size 30 µm, part 186000371.

equivalent to the nominal activity of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Biotin, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed in *Biotin, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in *Table 2*, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions to the resulting solution. Add 100 mL of water, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH between 5.5 and 6.0, and add *Purified Water* to make 250 mL.

Table 2

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter-aid in the

supernatant, and pass, with the aid of reduced pressure, through a layer of the filter-aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.³ [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.]

Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the micro-organism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Transfer cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL of the *Culture medium* each. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. The cell suspension so obtained is the *Inoculum*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.]

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the

³ Pure cultures of *Lactobacillus leichmannii* may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209.

spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minute amounts of vitamin B₁₂ activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes, and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5° for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating its contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances for each level of the *Sample solution* (Σ_u). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the

Sample solution to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of cyanocobalamin in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of cyanocobalamin that was assumed to be present in the portion of Tablets taken

Calculate the percentage of the labeled amount of cyanocobalamin in the portion of Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of the Tablets taken (μg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Vitamin B₁₂ Activity in Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• Folic Acid, Method 1

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use.

[NOTE—The methanol and water content may be varied (between 1% and 3%) by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation.]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask, and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8, bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of USP Folic Acid RS in *Internal standard solution*

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of powder, equivalent to 0.4 mg of folic acid, to a 50-mL amber-colored centrifuge tube. Add 25.0 mL of *Internal standard solution*, shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 15 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*

R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• **FOLIC ACID, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: Dissolve 7.5 g of edetate disodium, with stirring, in 500 mL of water containing 10 mL of ammonium hydroxide.

Diluent: 60 µg/mL of ammonium hydroxide

Mobile phase: Transfer 0.4 mL of triethylamine, 15 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 60 µg/mL of USP Folic Acid RS in *Diluent*. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of *Standard stock solution* with 10.0 mL of methanol and 35.0 mL of *Reagent*, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Transfer a portion of finely powdered Tablets, equivalent to 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of folic acid from the *Sample solution*

r_S = peak area of folic acid from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• **CALCIUM PANTOTHENATE, Method 1**

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to 15 mg of calcium pantothenate, to a centrifuge tube. Add 25.0 mL of the *Internal standard solution*, and shake vigorously for 10 min. Centrifuge, filter, and use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, Method 2**

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume, to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in the *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within

the linear portion of the log-concentration response curve.

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution having approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine-guanine-uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin-thiamine hydrochloride-biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to *Table 3*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL
Riboflavin-thiamine hydrochloride-biotin solution	5 mL
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 mL

Table 3 (Continued)

Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water; add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar; and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*⁴, incubating for 16–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*
To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within $\pm 0.5^\circ$ until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube. With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining

⁴ ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points. Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances for each level of the *Sample solution* (Σ_u). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in mg, of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate that was assumed to be present in the portion of the Tablets taken

Calculate the percentage of the labeled amount of calcium pantothenate in the portion of Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Tablets taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, Method 3**

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 μ g/mL of USP Calcium Pantothenate RS from the *Standard stock solution* diluted with water

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse. Dilute with water to volume, mix, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 3.9-mm \times 30-cm; 5- μ m packing L1

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 25 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of calcium pantothenate.

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of calcium pantothenate from the *Sample solution*

r_S = peak area of calcium pantothenate from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **NIACIN or NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS, to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate.

[NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm \times 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for niacinamide, pyridoxine, riboflavin, and thiamine are about 0.3, 0.5, 0.8, and 1.0, respectively.]

Suitability requirements**Relative standard deviation:** NMT 3.0%**Analysis****Samples:** *Standard solution* and *Sample solution*Measure the peak areas for niacin or niacinamide, pyridoxine, riboflavin, and thiamine. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak area of niacinamide from the *Sample solution* r_S = peak area of niacinamide from the *Standard solution* C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL) C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

For formulations containing niacin:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak area of niacin from the *Sample solution* r_S = peak area of niacin from the *Standard solution* C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL) C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak area of the corresponding vitamin from the *Sample solution* r_S = peak area of the corresponding vitamin from the *Standard solution* C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL) C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

 r_U = peak area of thiamine from the *Sample solution* r_S = peak area of thiamine from the *Standard solution* C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL) C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL) M_{r1} = molecular weight of thiamine mononitrate, 327.36 M_{r2} = molecular weight of thiamine hydrochloride, 337.27**Acceptance criteria:** 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$) or niacin ($C_6H_5NO_2$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)• **NIACIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.**Extraction solvent:** *Solution A* and methanol (3:1)**Mobile phase:** 0.1 M sodium acetate solution(13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]**Standard stock solution:** 1 mg/mL of USP Niacin RS in *Extraction solvent***Standard solution:** Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.**Sample solution:** [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine, and riboflavin, when present in the formulation.] Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 2 mg of riboflavin, to a 200-mL volumetric flask. If riboflavin is not present in the formulation, transfer a portion of the powder, equivalent to 2 mg of pyridoxine. If pyridoxine is not present in the formulation, transfer a portion of the powder, equivalent to 20 mg of niacin or niacinamide. Add 100.0 mL of *Extraction solvent*, and mix for 20 min, using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 254 nm**Column:** 4.6-mm × 25-cm; packing L1**Flow rate:** 1 mL/min**Injection size:** 20 µL**System suitability****Sample:** *Standard solution***Suitability requirements****Relative standard deviation:** NMT 3.0%

[NOTE—If necessary, flush the column with methanol between injections.]

Analysis**Samples:** *Standard solution* and *Sample solution*Measure the peak areas of niacin. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak area of niacin from the *Sample solution* r_S = peak area of niacin from the *Standard solution* C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL) C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)**Acceptance criteria:** 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)• **NIACINAMIDE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A, Extraction solvent, Mobile phase, Standard stock solution, Standard solution, Sample solution, System suitability, and Chromatographic system: Using USP Niacinamide RS in place of USP Niacin RS, proceed as directed in *Niacin, Method 2*.**Analysis****Samples:** *Standard solution* and *Sample solution*Measure the peak areas of niacinamide. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak area of niacinamide from the *Sample solution* r_S = peak area of niacinamide from the *Standard solution*

C_s = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_u = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, and Sample solution: Prepare as directed in *Niacin, Method 2*.

Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas of pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

r_u = peak area of pyridoxine from the *Sample solution*

r_s = peak area of pyridoxine from the *Standard solution*

C_s = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_u = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent and Sample solution: Prepare as directed in *Niacin, Method 2*.

Solution B: 6.8 mg/mL of sodium acetate in water

Mobile phase: Prepare a mixture of *Solution B* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.

Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.

Standard solution: Dilute 5.0 mL of *Standard stock solution* with *Extraction solvent* to 25.0 mL.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas of riboflavin. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Tablets taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

r_u = peak area of riboflavin from the *Sample solution*

r_s = peak area of riboflavin from the *Standard solution*

C_s = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_u = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **THIAMINE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: 1.88 mg/mL of sodium 1-hexanesulfonate in 0.1% phosphoric acid

Mobile phase: *Solution A* and acetonitrile (46:9)

Standard stock solution: 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid

Standard solution: 0.02 mg/mL of USP Thiamine Hydrochloride RS from *Standard stock solution* diluted with 0.2 N hydrochloric acid

Sample solution: Weigh and finely powder NLT 20 Tablets. Mix a portion of the powder with a volume of 0.2 N hydrochloric acid to obtain a concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the areas for the major peaks. For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

r_u = peak area of thiamine from the *Sample solution*

r_s = peak area of thiamine from the *Standard solution*

C_s = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_u = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

- r_U = peak area of thiamine from the *Sample solution*
 r_S = peak area of thiamine from the *Standard solution*
 C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
 C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
 M_{r1} = molecular weight of thiamine mononitrate, 327.36
 M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN or NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 3**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24

mg/mL of USP Thiamine Hydrochloride RS in the *Reagent*, with heating if necessary

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2.0 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin or niacinamide.

Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*

r_S = peak area of niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

r_S = peak area of the corresponding vitamin from the *Standard solution*

C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$) or niacin ($C_6H_5NO_2$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS**

<2040>: Meet the requirements for *Dissolution*.

• **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements.

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 3000 cfu/g, and the combined molds and yeasts count does not exceed 300 cfu/g.

• **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

- **LABELING:**⁵ The label states that the product is Oil- and Water-Soluble Vitamins Tablets. The label also states the quantity of each vitamin per dosage unit and, where necessary, the chemical form in which it is present. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*- form. Where more than one assay method is given for a particular vitamin, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** (11)
 - USP Alpha Tocopherol RS
 - USP Alpha Tocopheryl Acetate RS
 - USP Alpha Tocopheryl Acid Succinate RS
 - USP Biotin RS
 - 1*H*-Thieno[3,4-*d*]imidazole-4-pentanoic acid, hexahydro-2-oxo-, 3*aS*-[(3*aα*,4*β*,6*aα*)]-
 - (3*aS*,4*S*,6*aR*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-valeric acid.
 - C₁₀H₁₆N₂O₃S 244.31
 - USP Calcium Pantothenate RS
 - β-Alanine, *N*-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-, calcium salt (2:1), (*R*)-.
 - Calcium D-pantothenate (1:2).
 - C₁₈H₃₂CaN₂O₁₀ 476.53
 - USP Cholecalciferol RS
 - 9,10-Secocholesta-5,7,10(19)-trien-3-ol, (3*β*,5*Z*,7*E*)-.
 - Cholecalciferol.
 - C₂₇H₄₄O 384.64
 - USP Cyanocobalamin RS
 - Vitamin B₁₂.
 - C₆₃H₈₈CoN₁₄O₁₄P 1355.37
 - USP Ergocalciferol RS
 - 9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol, (3*β*,5*Z*,7*E*,22*E*)-.
 - Ergocalciferol.
 - C₂₈H₄₄O 396.65
 - USP Folic Acid RS
 - L-Glutamic acid, *N*-[4-[(2-amino-1,4-dihydro-4-oxo-6-pteridiny)methyl]amino]benzoyl]-.
 - Folic acid.
 - N*-[*p*-[(2-Amino-4-hydroxy-6-pteridiny)methyl]amino]-benzoyl]-L-glutamic acid.
 - C₁₉H₁₉N₇O₆ 441.40
 - USP Niacin RS
 - 3-Pyridinecarboxylic acid.
 - Nicotinic acid.
 - C₆H₅NO₂ 123.11
 - USP Niacinamide RS
 - 3-Pyridinecarboxamide.
 - Nicotinamide.
 - C₆H₆N₂O 122.12
 - USP Phytonadione RS
 - 1,4-Naphthalenedione, 2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)-, [*R*-(*R**,*R**-(*E*))]-.
 - Phylloquinone.
 - C₃₁H₄₆O₂ 450.70

⁵ USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 μg of all-*trans*-retinol (vitamin A alcohol) or 0.344 μg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 μg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 μg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 μg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 μg of ergocalciferol or cholecalciferol; and 1 mg of *d*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *d*-alpha tocopherol = 0.74, 1 mg of *d*-alpha tocopheryl acetate = 0.67, and 1 mg of *d*-alpha tocopheryl acid succinate = 0.60.

USP Pyridoxine Hydrochloride RS
 3,4-Pyridinedimethanol, 5-hydroxy-6-methyl-, hydrochloride.
 Pyridoxol hydrochloride.
 C₈H₁₁NO₃ · HCl 205.64
 USP Riboflavin RS
 Riboflavine.
 C₁₇H₂₀N₄O₆ 376.36
 USP Thiamine Hydrochloride RS
 Thiazolium, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methyl-, chloride, monohydrochloride.
 Thiamine monohydrochloride.
 C₁₂H₁₇ClN₄OS · HCl 337.27
 USP Vitamin A RS

Oil- and Water-Soluble Vitamins with Minerals Capsules

DEFINITION

Oil- and Water-Soluble Vitamins with Minerals Capsules contain one or more of the following oil-soluble vitamins: Vitamin A, Vitamin D as Ergocalciferol (Vitamin D₂) or Cholecalciferol (Vitamin D₃), Vitamin E, Phytonadione (Vitamin K₁), and Beta Carotene; one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, Dexpantenol or Panthenol, Pantothenic Acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate; and one mineral or more, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc, derived from substances generally recognized as safe. Capsules contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A (C₂₀H₃₀O) as retinol or esters of retinol in the form of retinyl acetate (C₂₂H₃₂O₂) or retinyl palmitate (C₃₆H₆₀O₂), vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O), vitamin E as alpha tocopherol (C₂₉H₅₀O₂) or alpha tocopheryl acetate (C₃₁H₅₂O₃) or alpha tocopheryl acid succinate (C₃₃H₅₄O₅), phytonadione (C₃₁H₄₆O₂), and beta carotene (C₄₀H₅₆); NLT 90.0% and NMT 150.0% of the labeled amounts of ascorbic acid (C₆H₈O₆) or its salts as calcium ascorbate (C₁₂H₁₄CaO₁₂ · 2H₂O) or sodium ascorbate (C₆H₇NaO₆), biotin (C₁₀H₁₆N₂O₃S), cyanocobalamin (C₆₃H₈₈CoN₁₄O₁₄P), folic acid (C₁₉H₁₉N₇O₆), niacin (C₆H₅NO₂) or niacinamide (C₆H₆N₂O), dexpantenol (C₉H₁₉NO₄) or panthenol (C₉H₁₉NO₄), calcium pantothenate (C₁₈H₃₂CaN₂O₁₀), pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl), riboflavin (C₁₇H₂₀N₄O₆), and thiamine (C₁₂H₁₇ClN₄OS) as thiamine hydrochloride or thiamine mononitrate; and NLT 90.0% and NMT 125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, compliance may be determined by following any one of the specified

methods, the method used being stated in the labeling only if *Method 1* is not used.]

• **VITAMIN A, Method 1**

[NOTE—Where the use of a vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in *n*-hexane

System suitability stock solution: 15 µg/mL of retinyl palmitate in *n*-hexane

System suitability solution: Mix equal volumes of *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: Transfer the contents of NLT 20 Capsules to a suitable container, mix, and weigh. Transfer a portion of the mixture, equivalent to 5 Capsules, to a container having a polytetrafluoroethylene-lined screw cap. [NOTE—For hard gelatin Capsules, remove, as completely as possible, the contents of NLT 20 Capsules by cutting open the Capsule shells, transferring the shells and their contents to a suitable container, and triturating to a homogeneous mass. Transfer a portion of the mass, equivalent to 5 Capsules, to a container having a polytetrafluoroethylene-lined screw cap.] Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [NOTE—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Dilute a volume of this solution with *n*-hexane to obtain a solution with a concentration of 15 µg/mL of vitamin A as retinol (C₂₀H₃₀O).

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection size: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate obtained from the *Standard solution* and the peak area for all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate in the chromatogram of the *Sample solution*. For products containing vitamin A acetate or vitamin A palmitate, calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₂H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)

• **VITAMIN A, Method 2**

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution: Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Mobile phase: *n*-Hexane and ethyl acetate (99.7:0.3)

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in 2,2,4-trimethylpentane

System suitability stock solution: 15 µg/mL of retinyl palmitate in 2,2,4-trimethylpentane

System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Using a sharp blade if necessary, carefully open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 30 µg of the labeled amount of cholecalciferol or ergocalciferol (vitamin D), to a container with a polytetrafluoroethylene-lined screw cap. If vitamin D is not present in the formulation, use a portion, equivalent to 90 mg of the labeled amount of vitamin E. If vitamin E is not present in the formulation, use a portion, equivalent to 2.5 mg of the labeled amount of vitamin A, as retinol. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of 3 N *methanolic sulfuric acid solution*, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. [NOTE—The supernatant is used for the determination of vitamin A, and also vitamin D and vitamin E, if present in the formulation.] If

necessary, quantitatively dilute a volume of the supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 25-cm; 5-μm packing L24

Flow rate: 1.5 mL/min

Injection size: 40 μL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 8.0 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area of all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution*.

Calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*

r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₂H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (μg/mL)

C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O), in the *Sample solution* (μg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration. The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)

• VITAMIN A, Method 3

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Extraction solvent: *n*-Hexane and methylene chloride (3:1)

Potassium hydroxide solution: 800 mg/mL of potassium hydroxide in water. [NOTE—Cautiously add potassium hydroxide in water. Mix, and cool.]

Diluent: 10 mg/mL of pyrogallol in alcohol

Mobile phase: *n*-Hexane and isopropyl alcohol (92:8)

Standard stock solution: 30 μg/mL of retinyl acetate from USP Vitamin A RS in *Diluent*. [NOTE—This solution may be stored in a refrigerator for 1 week.]

Standard solution: Dilute a volume of *Standard stock solution* with *Diluent* to obtain a concentration of 1 μg/mL of retinyl acetate from USP Vitamin A RS. Transfer 10.0 mL of this solution to a stoppered 125-mL flask, and add 5 mL of water, 5 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. Withdraw a

portion of the organic layer for injection into the chromatograph. This *Standard solution* contains 0.34 μg/mL of retinol.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 1.5 mg of retinyl acetate, to a stoppered 125-mL flask. Add 5 mL of water, 15 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s or longer, if necessary, for complete extraction. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. [NOTE—Do not shake, because an emulsion may form.] Withdraw a portion of the organic layer, and dilute quantitatively, and stepwise if necessary, with *Extraction solvent*, to obtain a concentration of 0.34 μg/mL of retinol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 335 nm

Column: 6.2-mm × 8-cm; packing L3

Column temperature: 40°

Flow rate: 4 mL/min

Injection size: 50 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for 13-*cis*-retinol and all-*trans*-retinol are about 0.92 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas for all-*trans*-retinol and 13-*cis*-retinol. Calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Capsules taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times (C_S/C_U) \times F \times 100$$

r_{T1} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Sample solution*

r_{T2} = sum of the areas of all-*trans*-retinol and 13-*cis*-retinol peaks from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₃H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (μg/mL)

C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O), in the *Sample solution* (μg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)

• CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D),

Method 1

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 μg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample solution: Transfer NLT 20 mL of a solution prepared as directed for *Sample solution* in *Vitamin A, Method 1* to a suitable container, and concentrate, if necessary, in vacuum at room temperature to obtain a solution with an expected concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection size: 100 µL

System suitability

Sample: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D), Method 2

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution, Sodium ascorbate–pyrogallol solution, Lecithin solution, and Sample solution: Proceed as directed for *Vitamin A, Method 2*.

Mobile phase: *n*-Hexane and tertiary butyl alcohol (98.75:1.25)

Standard solution: 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in 2,2,4-trimethylpentane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1 mL/min

Injection size: 40 µL

System suitability

Sample: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D), Method 3

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluted acetic acid: Glacial acetic acid solution (1 in 10), in water

Phenolphthalein solution: 10 mg/mL of phenolphthalein in alcohol

Potassium hydroxide solution: Slowly dissolve 14 g of potassium hydroxide in a mixture of 31 mL of dehydrated alcohol and 5 mL of water. Prepare fresh daily.

Extraction solvent: Methylene chloride and isopropyl alcohol (99.8:0.2)

Mobile phase: Acetonitrile and methanol (91:9)

Standard stock solution: 0.2 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in dehydrated alcohol. [NOTE—Prepare fresh every 4 weeks. Store in a freezer.]

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* by initially washing the column with 4.0 mL of a mixture of methylene chloride and isopropyl alcohol (4:1), followed by 5.0 mL of *Extraction solvent*. Do not allow the column to dry.] Dilute a volume of *Standard stock solution* with dehydrated alcohol to obtain a concentration of 5 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS. Prepare this solution fresh daily. Transfer 2.0 mL of this solution to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask

with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to stand for 10 min until the layers separate. Drain and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase extraction column containing silica packing with a sorbent mass-to-column volume ratio of 500 mg to 2.8 mL or equivalent, rinse the round-bottom flask with 1.0 mL of *Extraction solvent*, and transfer to the column. Elute the column with 2.0 mL of *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

Sample solution: Proceed as directed for the *Sample solution* for *Vitamin A, Method 3*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 10 µg of ergocalciferol or cholecalciferol, to a stoppered 125-mL flask, and proceed as directed for the *Standard solution*, beginning with "Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*".

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Column temperature: 27°

Flow rate: 0.7 mL/min

Injection size: 15 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 4.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• VITAMIN E, Method 1

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water

Mobile phase: Methanol and *Solution A* (19:1)

System suitability solution: Prepare a 0.65-mg/mL solution of USP Ergocalciferol RS in methanol. Transfer 1.0 mL of this solution to a 100-mL volumetric flask containing 100 mg of USP Alpha Tocopheryl Acetate RS. Dissolve in 30 mL of methanol, with the aid of sonication if necessary, and dilute with methanol to volume. Store this solution in a refrigerator.

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, in vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume, to obtain a solution with an expected concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2 mL/min

Injection size: 100 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for ergocalciferol and alpha tocopheryl acetate are about 0.5 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 12 between ergocalciferol and alpha tocopheryl acetate, *System suitability solution*

Tailing factor: Between 0.8 and 1.2, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅)

• **VITAMIN E, Method 2**

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 240 mL of methanol with 10 mL of water, followed by 0.5 mL of 50% phosphoric acid, and dilute with acetonitrile to 1000 mL.

System suitability solution: 2 mg/mL each of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, and USP Alpha Tocopheryl Acid Succinate RS in methanol

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

3 N methanolic sulfuric acid solution: Cautiously mix sulfuric acid and methanol (9 in 100) in a 100-mL volumetric flask. [NOTE—Dissolve in a portion of methanol, cool, and then dilute to final volume.]

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask. Add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Sample solution: Proceed as directed for *Vitamin A, Method 2*, through “calculate the net weight of the Capsule contents.” Transfer a portion of the Capsule contents, equivalent to 55 mg of vitamin E, to a container having a polytetrafluoroethylene screw cap. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of 3 N methanolic sulfuric acid solution, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant layer. Transfer a volume of the supernatant 2,2,4-trimethylpentane layer to a suitable volumetric flask, the volume of the specimen withdrawn from the 2,2,4-trimethylpentane layer and the size of the volumetric flask being such that the final concentration of the *Sample solution* is equivalent to that of the *Standard solution*. Evaporate nearly to dryness, add several mL of methanol, and evaporate the remaining 2,2,4-trimethylpentane. Dilute with methanol to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 1.5 mL/min

Injection size: 25 μL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for alpha tocopheryl acid succinate, alpha tocopherol, and alpha tocopheryl acetate are about 0.6, 0.8, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 4.0 between alpha tocopheryl acid succinate and alpha tocopherol and NLT 3.0 between

alpha tocopherol and alpha tocopheryl acetate,
System suitability solution

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane and the dilution factor to exchange the solvent from 2,2,4-trimethylpentane to methanol to calculate the nominal concentration.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• **VITAMIN E, Method 3**

Diluent: Acetonitrile and ethyl acetate (1:1)

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in methanol

Sample solution: Proceed as directed for *Vitamin A, Method 3*, through “calculate the net weight of the Capsule contents.” Transfer a portion of the Capsule contents, equivalent to an expected amount of 8.0 mg of alpha tocopherol, to a glass-stoppered conical flask. Add 25.0 mL of water, 25.0 mL of dehydrated alcohol, and 3.5 g of potassium hydroxide pellets. Shake for 1 h in a water bath maintained at 55°. Cool, and transfer with the aid of a minimum volume of water to a 125-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 60 s, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and add the rinsing to the hexane solution in the flask. Place the flask in a water bath maintained at 50°, and evaporate the hexane solution with the aid of a rotary evaporator to dryness. Immediately add 25.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC
Detector: UV 291 nm
Column: 4.6-mm × 25-cm; packing L1
Column temperature: 40°
Flow rate: 3 mL/min
Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E, as alpha tocopherol (C₂₉H₅₀O₂), in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of alpha tocopherol from the *Sample solution*

r_S = peak area of alpha tocopherol from the *Standard solution*

C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin E, as alpha tocopherol in the *Sample solution* (mg/mL)

[NOTE—Calculate the alpha tocopherol equivalent (C₂₉H₅₀O₂) of alpha tocopheryl acetate (C₃₁H₅₂O₃) or alpha tocopheryl acid succinate (C₃₃H₅₄O₅) by multiplying their contents by the factors 0.91 or 0.81, respectively.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E

• **PHYTONADIONE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP

Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with methanol

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for the *Sample solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, if necessary, in vacuum at room temperature to dryness. Transfer the contents of the flask to a suitable volumetric flask with the aid of methanol, and dilute with methanol to volume to obtain a solution with an expected concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2.0 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for alpha tocopheryl acetate and phytonadione are about 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of phytonadione (C₃₁H₄₆O₂) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione (C₃₁H₄₆O₂)

• **BETA CAROTENE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Potassium hydroxide solution: Dissolve 58.8 g of potassium hydroxide in 50 mL of water.

Iodine solution: Transfer 10 mg of iodine to a 100-mL volumetric flask. Dissolve in cyclohexane, and dilute with cyclohexane to volume. Dilute 10 mL of this solution with cyclohexane to 100 mL. [NOTE—Prepare this solution fresh daily.]

Sample solution A (for preparations containing beta carotene in oil solutions): Proceed as directed for *Vitamin A, Method 1*, except use cyclohexane instead of *n*-hexane as the extraction solvent, and dilute the filtered extracts with cyclohexane, to obtain a concentration of 2 µg/mL of beta carotene.

Sample solution B (for preparations containing beta carotene in dry powder): Remove the contents of NLT 20 Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature. Add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower aqueous layer into the saponification flask. Add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume. Add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Vis

Analytical wavelength: 452 nm

Blank: Cyclohexane

Analysis

Sample: *Sample solution*

Determine the absorbance against the *Blank*. Calculate the percentage of the labeled amount of beta carotene ($C_{40}H_{56}$) in the portion of Capsules taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of the *Sample solution*

F = absorptivity of beta carotene at 452 nm, 223

C_U = nominal concentration of beta carotene in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene ($C_{40}H_{56}$)

• **ASCORBIC ACID, Method 1**

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 100 mg of ascorbic acid, to a 200-mL volumetric flask, and add 75 mL of metaphosphoric–acetic acids TS. Insert a stopper into the flask, and shake by mechanical means for 30 min. Dilute with water to volume. Transfer a portion of the solution to a centrifuge tube, and centrifuge until a clear supernatant is obtained. Pipet 4.0 mL of this solution into a 50-mL conical flask, and add 5 mL of metaphosphoric–acetic acids TS.

Analysis: Titrate with standard dichlorophenol–indophenol solution VS to a rose-pink color that persists for at least 5 s. Correct for the volume of dichlorophenol–indophenol solution consumed by a mixture of 5.5 mL of metaphosphoric–acetic acids TS and 15 mL of water. From the ascorbic acid equivalent of the standard dichlorophenol–indophenol solution, calculate the content of ascorbic acid in each Capsule.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

• **ASCORBIC ACID, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

• **CALCIUM ASCORBATE, Method 1:** Proceed as directed for *Ascorbic Acid, Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

• **CALCIUM ASCORBATE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

• **SODIUM ASCORBATE, Method 1:** Proceed as directed for *Ascorbic Acid, Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

• **SODIUM ASCORBATE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

• **BIOTIN, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-µm packing L7

Flow rate: 1.2 mL/min

Injection size: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the responses for the biotin peaks. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by dilution of the *Standard stock solution* with water on the day of the assay.

Sample solution: Proceed as directed for *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution having a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment

with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine-guanine-uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin-thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to Table 1, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin-thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a

refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture* of *Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*
To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of *Standard* and *sample* tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of *Standard* during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength from 540 to 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the *Standard*, calculate the response from the sum of the duplicate values of the transmittance (Σ_S) as the difference, $y = 2.00 - \Sigma_S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_U$, adding together the two transmittances for each level of the *Sample solution* (Σ_U). Read from the standard curve the logarithm of the volume of the *Standard solution*

¹ ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

corresponding to each of those values of γ that fall within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of biotin that was assumed to be present in the portion of the Capsules taken

Calculate the percentage of the labeled amount of biotin in the portion of the Capsules taken

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$) in the portion of Capsules taken (μg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$)

• **CYANOCOBALAMIN, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 $\mu\text{g}/\text{mL}$ of USP Cyanocobalamin RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

Standard solution: 1 $\mu\text{g}/\text{mL}$ of USP Cyanocobalamin RS from *Standard stock solution* diluted with water

Sample solution: Proceed as directed for *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 100 μg of cyanocobalamin, to a 250-mL flask. Add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the clear filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm \times 15-cm; 5- μm packing L1

Flow rate: 0.5 mL/min

Injection size: 200 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for cyanocobalamin.

Calculate the percentage of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Cyanocobalamin RS in the *Standard solution* ($\mu\text{g}/\text{mL}$)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* ($\mu\text{g}/\text{mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• **CYANOCOBALAMIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 $\mu\text{g}/\text{mL}$ of USP Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of the *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Proceed as directed for *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 1.0 μg of cyanocobalamin, to an appropriate vessel containing, for each g of Capsule contents taken, 25 mL of an aqueous extracting solution prepared just before use to contain 12.9 mg/mL of dibasic sodium phosphate, 11.0 mg/mL of anhydrous citric acid, and 10 mg/mL of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B₁₂ activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed for *Calcium Pantothenate, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed for *Calcium Pantothenate, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 μg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as

directed in the labeling, it yields a medium comparable to that obtained from the formula given herein. Add the ingredients in the order listed in *Table 2*, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions to the resulting solution. Add 100 mL of water, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of between 5.5 and 6.0, and add Purified Water to make 250 mL.

Table 2

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N Hydrochloric acid	10 mL
Adenine-guanine-uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.² [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.]

Incubate for 16–24 h at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL of the *Culture medium* each. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. The cell suspension so obtained is the *Inoculum*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.]

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minute amounts of vitamin B₁₂ activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm \times 150-mm test tubes, and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within $\pm 0.5^\circ$, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating its

² Pure cultures of *Lactobacillus leichmannii* may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209.

contents, and read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (ΣS) as the difference, $y = 2.00 - \Sigma S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points. Calculate the response, $y = 2.00 - \Sigma U$, adding together the two transmittances for each level of the *Sample solution* (ΣU). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of cyanocobalamin in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of cyanocobalamin that was assumed to be present in each mg in the portion of Capsules taken

Calculate the percentage of the labeled amount of biotin in the portion of the Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$) in the portion of Capsules taken (μg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Vitamin B₁₂ Activity in Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• **Folic Acid, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1200 mL, and filter. Recheck the pH before use by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation. [NOTE—The methanol and water content may be varied (between 1% and 3%).]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8, bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of USP Folic Acid RS in *Internal standard solution*

Sample solution: Proceed as directed for *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer an amount of Capsule contents to a suitable centrifuge tube, and add a volume of *Internal standard solution* to obtain a concentration of 0.016 mg/mL of folic acid. Shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm \times 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 15 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*

R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of folic acid in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$)

• **Folic Acid, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: Dissolve 7.5 g of edetate disodium, with stirring, in 500 mL of water containing 10 mL of ammonium hydroxide.

Diluent: 60 µg/mL of ammonium hydroxide

Mobile phase: Transfer 0.4 mL of triethylamine, 15 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 60 µg/mL of USP Folic Acid RS in *Diluent*. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of *Standard stock solution* with 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed for the *Sample solution* for *Ascorbic Acid, Method 1*, through "calculate the net weight of the Capsule contents." Transfer a portion of the Capsule contents, equivalent to 0.3 mg of folic acid to a 125-mL stoppered flask. Add 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of folic acid from the *Sample solution*

r_S = peak area of folic acid from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• DEXPANTHENOL or PANTHENOL

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 µg/mL of USP Dexpantenol RS or 1600 µg/mL of USP Racemic Panthenol RS in water. Store in a refrigerator, protected from light, and use within 30 days.

Standard solution: On the day of the assay, prepare a dilution of 1.2 µg/mL of dexpantenol or 2.4 µg/mL of panthenol from *Standard stock solution* diluted with water.

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents as completely as possible to a beaker. Remove any contents adhering to the empty Capsule shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until

the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Dissolve a portion of the Capsule contents, equivalent to 1.2 mg of dexpantenol or 2.4 mg of panthenol, in 100.0 mL of water. Quantitatively dilute a portion of this solution with water to obtain a concentration of 1.2 µg/mL of dexpantenol or

2.4 µg/mL of panthenol.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to $75 \pm 5^\circ$, and add hydrochloric acid solution (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool. Add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in neutral 25% alcohol. Store in a refrigerator.

Salt solution A: 50 mg/mL of monobasic potassium phosphate and 50 mg/mL of dibasic potassium phosphate in water. Add 10 drops of hydrochloric acid/L of solution. Store under toluene.

Salt solution B: 20 mg/mL of magnesium sulfate, 1 mg/mL of sodium chloride, 1 mg/mL of ferrous sulfate, and 1 mg/mL of manganese sulfate in water. Add 10 drops of hydrochloric acid/L of the solution. Store under toluene.

Pyridoxal–calcium pantothenate solution: 200 µg/mL of pyridoxal hydrochloride and 1.875 µg/mL of calcium pantothenate in 10% alcohol. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: 50 mg/mL of polysorbate 40 and 0.5 mg/mL of oleic acid in 20% alcohol. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium: Dissolve anhydrous dextrose and sodium acetate in the solutions previously mixed according to *Table 3*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g

Table 3 (Continued)

Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
<i>p</i> -Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal–calcium pantothenate solution	5 mL
Polysorbate 40–oleic acid solution	5 mL

Double-strength modified pantothenate medium:

Prepare as directed in *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH of 6.5–6.6, and dilute with water to 1000 mL. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*³ on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with *Modified pantothenate medium*. Resuspend the cells in sufficient *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes and in the same order, add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*.

Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes, and in the same order, add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature NLT 80°, such as by steaming at atmospheric pressure in a sterilizer for 5–10 min. Cool, and determine the percentage transmittance of the suspensions, in cells of equal pathlength, on a suitable spectrophotometer, at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percentage of transmittance, for each set of tubes of

the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency, of each tube containing portions of the *Sample solution*. Divide the potency of each tube by the amount of the *Sample solution* added to it, to obtain the individual responses. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the material taken, by multiplying the mean response by the appropriate dilution factor. Calculate the percentage of the labeled amount of dexpanthenol or panthenol in the portion of Capsules taken:

$$\text{Result} = (P/N) \times 100$$

P = potency of dexpanthenol or panthenol in the portion of Capsules taken (mg)
N = nominal amount of dexpanthenol or panthenol in the portion of Capsules taken (mg)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol (C₉H₁₉NO₄)

• **CALCIUM PANTOTHENATE, Method 1**

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

Sample solution: Proceed as directed for *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” To a centrifuge tube, transfer an amount of mixed Capsule contents and a volume of *Internal standard solution* to obtain a concentration of 0.6 mg/mL in the *Sample solution*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate (C₁₈H₃₂CaN₂O₁₀) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate (C₁₈H₃₂CaN₂O₁₀)

³ ATCC No. 8042 is suitable.

• **CALCIUM PANTOTHENATE, Method 2**

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume, to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in the *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Proceed as directed for *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to an amount of 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution having approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to *Table 4*, and adjust with

1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 4

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: Add 5.0 mL of water containing 0.2 µg of calcium pantothenate to each of a series of test tubes containing 5.0 mL of *Basal medium stock solution*. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the of *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of culture medium. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*
To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$ until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance from 540 to 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances for each level of the *Sample solution* (Σ_u). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in mg, of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate that was assumed to be present in the portion of the Capsules taken

Calculate the percentage of calcium pantothenate in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of the Capsules taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (11), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• CALCIUM PANTOTHENATE, *Method 3*

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from *Standard stock solution* diluted with water

Sample solution: Proceed as directed for the *Sample solution for Ascorbic Acid, Method 1*, through “calculate the net weight of the Capsule contents.” Transfer a portion of the Capsule contents, equivalent to 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse the Capsule contents. Dilute with water to volume, mix, and filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 3.9-mm × 30-cm; 5-µm packing L1

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 25 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for calcium pantothenate.

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

r_u = peak area from the *Sample solution*

r_s = peak area from the *Standard solution*

C_s = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_u = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, *Method 1*

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS, to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution: Proceed as directed for *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to

room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for niacinamide, pyridoxine, riboflavin, and thiamine are about 0.3, 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin or niacinamide, pyridoxine, riboflavin, and thiamine. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*

r_S = peak area of niacinamide from the *Standard solution*

C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

For formulations containing niacin:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*

r_S = peak area of niacin from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

r_S = peak area of the corresponding vitamin from the *Standard solution*

C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$) or niacin ($C_6H_5NO_2$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• NIACIN, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.

Extraction solvent: *Solution A* and methanol (3:1)

Mobile phase: 0.1 M sodium acetate solution (13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]

Standard stock solution: 1 mg/mL of USP Niacin RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask. Dilute with *Extraction solvent* to volume.

Sample solution: [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine, and riboflavin, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a beaker. Remove any contents adhering to the shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 2 mg of riboflavin, to a 200-mL volumetric flask. If riboflavin is not present in the formulation, use a portion, equivalent to a nominal amount of 2 mg of pyridoxine. If pyridoxine is not present in the formulation, use a portion, equivalent to 20 mg of niacin or niacinamide. Add 100.0 mL of *Extraction solvent*, and mix for 20 min, using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

[NOTE—If necessary, flush the column with methanol between injections.]

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

• **NIACINAMIDE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A, Extraction solvent, Mobile phase, Standard stock solution, Standard solution, Sample solution, Chromatographic system, and System suitability solution: Using USP Niacinamide RS in place of USP Niacin RS, proceed as directed for *Niacin, Method 2*.

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas for niacinamide. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, and Sample solution: Prepare as directed for *Niacin, Method 2*.

Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

Standard solution: 20 µg/mL of USP Pyridoxine Hydrochloride RS from *Standard stock solution* diluted with *Extraction solvent*

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas for pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent and Sample solution: Prepare as directed for *Niacin, Method 2*.

Solution A: 6.8 g/L of sodium acetate in water

Mobile phase: Prepare a mixture of *Solution A* and methanol (1:3:7). Add 2 mL of triethylamine per L of the

mixture, and adjust with glacial acetic acid to a pH of 5.2.

Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.

Standard solution: Dilute 5.0 mL of *Standard stock solution* with *Extraction solvent* to 25.0 mL.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas for riboflavin. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **THIAMINE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: 1.88 g/L of sodium 1-hexanesulfonate in 0.1% phosphoric acid

Mobile phase: *Solution A* and acetonitrile (46:9)

Standard stock solution: 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid

Standard solution: 0.02 mg/mL of USP Thiamine Hydrochloride RS from *Standard stock solution* diluted with 0.2 N hydrochloric acid

Sample solution: Proceed as directed for the *Sample solution* for *Ascorbic Acid, Method 1*, through “calculate the net weight of the Capsule contents.” Mix a portion of the Capsule contents with a volume of 0.2 N hydrochloric acid to obtain a concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the areas for the major peaks. For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine

hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 3**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in the *Reagent*, with heating if necessary

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed for the *Sample solution for Ascorbic Acid, Method 1*, through “calculate the net weight of the Capsule contents.” Transfer a portion of the Capsule contents, equivalent to 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1), and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2.0 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the peaks. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*

r_S = peak area of niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$) and thiamine ($C_{12}H_{17}ClN_4OS$) (for products containing thiamine hydrochloride) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

r_S = peak area of the corresponding vitamin from the *Standard solution*

C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$) or niacin ($C_6H_5NO_2$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

[NOTE—Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a *Standard stock solution* is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the *Standard solutions* and the

Sample solution may be diluted quantitatively with the solvent specified, if necessary, to yield solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

• **CALCIUM, Method 1**

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Polysorbate 80 solution: Polysorbate 80 and alcohol (1:10)

Sample solution: Transfer 5 Capsules to a 100-mL volumetric flask. [NOTE—For hard gelatin Capsules, weigh NLT 20 Capsules. Open the Capsules, without loss of shell material, and transfer the contents to a suitable container. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and allow the Capsule shells to dry. Weigh the empty Capsule shells, calculate the net weight of the Capsule contents, and transfer a portion of the Capsule contents, equivalent to 5 Capsules, to a 100-mL volumetric flask.] Add 15 mL of water, 10 mL of 6 N hydrochloric acid, and 1 mL of *Polysorbate 80 solution* to the flask. Heat on a hot plate or steam bath, with intermittent swirling, until the Capsules are completely disintegrated or the contents are dissolved. Boil gently for an additional 15 min. Cool, dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution with 0.125 N hydrochloric acid, to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Calcium emission line at 422.7 nm

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide-acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in

mg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Capsules taken:

$$\text{Result} = (C/C_u) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_u = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, Method 1**

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Proceed as directed for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 1 µg/mL of chromium and to omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Chromium emission line at 357.9 nm

Lamp: Chromium hollow-cathode

Flame: Air-acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in

µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Capsules taken:

$$\text{Result} = (C/C_u) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

C_u = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, Method 1**

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% solution of nitric acid, and dilute with a 1% solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

Standard stock solution: 100 µg/mL of copper from *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Proceed as directed for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 2 µg/mL of copper and to omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Copper emission line at 324.7 nm

Lamp: Copper hollow-cathode

Flame: Air-acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions*

versus the concentration, in $\mu\text{g/mL}$, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of copper in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• FLUORIDE, Method 1

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water contained in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 $\mu\text{g/mL}$ of fluoride from a quantity of sodium fluoride, previously dried at 100° for 4 h and cooled in a desiccator, in water

Intermediate stock solution A: 100 $\mu\text{g/mL}$ of fluoride from *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 $\mu\text{g/mL}$ of fluoride from *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of 3 M *sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 $\mu\text{g/mL}$ of fluoride.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the mixed Capsule contents, equivalent to 200 μg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of 3 M *sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute with water to volume.

Analysis

Samples: *Standard solutions* and *Sample solution*
To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see pH <791>), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ± 0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer having an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in $\mu\text{g/mL}$, of the *Standard solutions* versus potential, in

mV. From the standard response curve so obtained and the measured potential of the *Sample solution*, determine the concentration, C , in mg/mL , of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of fluorine in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• FLUORIDE, Method 2

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 $\mu\text{g/mL}$ of USP Sodium Fluoride RS in water. This solution contains 100 $\mu\text{g/mL}$ of fluoride.

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with one column volume of methanol followed by one column volume of pH 10.0 buffer. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.] Transfer 10.0 mL of *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1 . Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask, add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with pH 10.0 buffer to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL container. If necessary, remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 1 mg of fluorine, to a 100-mL volumetric flask. Add 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1 , and dilute with water to 100 mL. Proceed as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See Chromatography <621>, System Suitability.)

Mode: LC

Detector: Conductivity detector

Guard column: 4.6-mm × 3-cm; packing L17

Analytical column: 7.8-mm × 30-cm; packing L17

Flow rate: 0.5 mL/min

Injection size: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Sample solution* and *Standard solution*

Measure the peak areas for fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE, Method 1**

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis

Sample: Capsules

Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the contents, equivalent to 3 mg of iodine, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.]

Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared. Calculate the percentage of the labeled amount of iodine (I) in the portion of Capsules taken:

$$\text{Result} = V \times N_A \times F \times \text{Ime} \times (Aw/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)

N_A = actual normality of the sodium thiosulfate solution used

F = correction factor to convert mg to µg (1000 µg/mL)

Ime = milliequivalent of I (21.16 mg/meq)

Aw = average weight of the Capsules content

W = weight of the sample of Capsules content taken

L = labeled amount of iodine (µg/Capsule)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IODIDE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Iodide*.

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IRON, Method 1**

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Polysorbate 80 solution: Prepare as directed for *Calcium, Method 1*.

Sample solution: Proceed as directed for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a concentration of 5 µg/mL of iron and to omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Iron emission line at 248.3 nm

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (µg/mL)

C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM, Method 1**

Lanthanum chloride solution: Prepare as directed for *Calcium, Method 1*.

Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a known concentration of 1000 µg/mL of magnesium.

Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.

Polysorbate 80 solution: Prepare as directed for *Calcium, Method 1*.

Sample solution: Proceed as directed for *Calcium, Method 1*, except prepare the *Sample solution* to contain 0.4 µg/mL of magnesium.

Instrumental conditions(See *Spectrophotometry and Light-Scattering* (851).)**Mode:** Atomic absorption spectrophotometry**Analytical wavelength:** Magnesium emission line at 285.2 nm**Lamp:** Magnesium hollow-cathode**Flame:** Air-acetylene**Blank:** 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL**Analysis****Samples:** *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of magnesium in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

- MANGANESE, Method 1**

Manganese standard stock solution: Transfer 1.00 g of manganese, to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 $\mu\text{g/mL}$ of manganese.

Standard stock solution: 50 $\mu\text{g/mL}$ of manganese from *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 $\mu\text{g/mL}$ of manganese.

Polysorbate 80 solution: Prepare as directed for *Calcium, Method 1*.

Sample solution: Proceed as directed for *Calcium, Method 1*, except prepare the *Sample solution* to contain to 1 $\mu\text{g/mL}$ of manganese and to omit the use of the *Lanthanum chloride solution*.

Instrumental conditions(See *Spectrophotometry and Light-Scattering* (851).)**Mode:** Atomic absorption spectrophotometry**Analytical wavelength:** Manganese emission line at 279.5 nm**Lamp:** Manganese hollow-cathode**Flame:** Air-acetylene**Blank:** 0.125 N hydrochloric acid**Analysis**

Samples: *Standard solutions* and the *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of manganese in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

- MOLYBDENUM, Method 1**

Diluent: 20 mg/mL of ammonium chloride in water

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 $\mu\text{g/mL}$ of molybdenum.

Standard stock solution: 100 $\mu\text{g/mL}$ of molybdenum from *Molybdenum standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min. Cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 $\mu\text{g/mL}$ of molybdenum.

Polysorbate 80 solution: Prepare as directed for *Calcium, Method 1*.

Sample solution: Proceed as directed for *Calcium, Method 1*, except take a number of Capsules or a portion of Capsule contents equivalent to 1000 μg of molybdenum and make appropriate dilutions to obtain a final concentration of 10 $\mu\text{g/mL}$ of molybdenum, omitting the addition of the *Lanthanum chloride solution*.

Instrumental conditions(See *Spectrophotometry and Light-Scattering* (851).)**Mode:** Atomic absorption spectrophotometry**Analytical wavelength:** Molybdenum emission line at 313.3 nm**Lamp:** Molybdenum hollow-cathode**Flame:** Nitrous oxide-acetylene**Blank:** *Diluent* and perchloric acid (20:1)**Analysis**

Samples: *Standard solutions* and the *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of molybdenum, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of molybdenum in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

- MOLYBDENUM, Method 2**

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% Stannous chloride solution: Transfer 40 mg of stannous chloride to a beaker, add 20 mL of 6.5 N hydrochloric acid solution, and heat the solution until the stannous chloride is dissolved. Cool and dilute with water to 100 mL.

Diluted stannous chloride solution: 20% *stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.

Standard solution: 20 µg/mL of molybdenum in water
Sample: Remove the contents of a counted number of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Use a quantity of the Capsule contents, equivalent to 40 µg of molybdenum.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Analytical wavelength: 465 nm

Cell: 1 cm

Blank: Amyl alcohol

Analysis

Samples: *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to complete 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of 20% *Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layer from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the *Sample*

A_S = absorbance of the *Standard solution*

V = volume of the *Standard solution* analyzed, 2.0 mL

C_S = concentration of molybdenum in the *Standard solution* (µg/mL)

M_U = nominal amount of molybdenum in the *Sample* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **PHOSPHORUS, Method 1**

Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5:100), and mix.

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, and then dilute with *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add one drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of phosphorus.

Standard solution: 20 µg/mL of phosphorus from *Phosphorus standard stock solution* diluted with water

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Vis

Analytical wavelength: 650 nm

Cell: 1 cm

Analysis

Samples: *Standard solution* and *Sample solution*
 To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

C_S = concentration of phosphorus in the *Standard solution* (µg/mL)

C_U = nominal concentration of phosphorus in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• **POTASSIUM**

Potassium standard solution: 100 µg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 µg/mL of potassium from *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.

Polysorbate 80 solution: Prepare as directed for *Calcium, Method 1*.

Sample solution: Proceed as directed for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 1 µg/mL of potassium and to omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Potassium emission line at 766.5 nm

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Blank: Water

Analysis

Samples: *Standard solutions* and the *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of potassium in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• SELENIUM, Method 1

Diluent: Prepare as directed for *Molybdenum, Method 1*.

Selenium standard solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 $\mu\text{g/mL}$ of selenium.

Standard stock solution: 100 $\mu\text{g/mL}$ of selenium from *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions with concentrations of 5.0, 10.0, and 25.0 $\mu\text{g/mL}$ of selenium.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 1000 μg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the sample specimen. Sonicate for 10 min or until the sample specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Selenium emission line at 196.0 nm

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and the *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions*

versus the concentration, in $\mu\text{g/mL}$, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of selenium in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• SELENIUM, Method 2

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, then add hydroxylamine hydrochloride, and dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 $\mu\text{g/mL}$ of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 $\mu\text{g/mL}$ of selenium.

Standard solution: Transfer 10 mL of the *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 20 μg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid.

[CAUTION—Exercise care at this stage, because perchloric acid reaction becomes vigorous.]

Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid, and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask. Add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV

Analytical wavelength: 380 nm

Cell: 1 cm

Blank: 1 mL of perchloric acid and 1 mL of Hydrochloric acid solution diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*

Treat the *Sample solution*, the *Standard solution*, and the *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with 50% *Ammonium hydroxide solution* to a pH of 1.1 ± 0.1 . Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*. Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbances of the cyclohexane layer from the *Sample solution*

A_S = absorbances of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• ZINC, Method 1

Zinc standard solution: 1000 µg/mL of zinc from zinc oxide in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solutions: 50 µg/mL of zinc from *Zinc standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Polysorbate 80 solution: Prepare as directed for *Calcium*, Method 1.

Sample solution: Proceed as directed for *Calcium*, Method 1, except to prepare the *Sample solution* to contain 2 µg/mL of zinc and to omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Zinc emission line at 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and the *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points.

From the graph so obtained, determine the concentration, C , in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• BORON, NICKEL, TIN, AND VANADIUM, Method 1; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS, and ZINC, Method 2; MOLYBDENUM AND SELENIUM, Method 3

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding one volume of *Stock aqua regia solution* to two volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% nitric acid solution, 1000 mg/L of scandium in 5% nitric acid solution, and *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% nitric acid solution to obtain a solution having final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/L of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% hydrochloric acid solution to obtain a solution having final concentrations of about 200 mg/L of boron, and 100 mg/L each of chromium, molybdenum, nickel, selenium, tin, and vanadium.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent*, to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution: Weigh, then transfer 5 Capsules to a 250-mL volumetric flask, and heat gently on a hot plate until the contents begin to release. Cautiously add 25 mL of *Stock aqua regia solution* in 5-mL increments, and swirl. Heat, continue to swirl until the Capsules dissolve into the acid, immediately remove from the heat source, and add 150 mL of water. Cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube, using a 5-µm pore size nylon syringe filter. If necessary, make any further adjustments using the *Diluent*.

Instrumental conditions

(See *Plasma Spectrochemistry* (730).)

Mode: Inductively coupled plasma spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be

demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

[NOTE—Analyze the *System suitability solution*, and obtain the response as directed in the *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*. Calculate the percentage of the labeled amount for each mineral taken:

$$\text{Result} = C \times (V/W) \times F \times (C_w/L) \times 100$$

C = measured concentration of the *Sample solution* (mg/L)

V = volume of the *Sample solution* (L)

W = sample weight (mg)

F = dilution factor of the *Sample solution*

C_w = average Capsule weight (mg)

L = labeled amount per Capsule (mg)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); and 90.0%–160.0% of the labeled amounts of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** <2040>: Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 3000 cfu/g, and the combined molds and yeasts count does not exceed 300 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

- **LABELING:**⁴ The label states that the product is Oil- and Water-Soluble Vitamins with Minerals Capsules. The label also states the quantity of each vitamin and mineral per dosage unit and, where necessary, the chemical form in which a vitamin is present and also states the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*- form. Where more than one assay method is given for a particular vitamin or mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.

• USP REFERENCE STANDARDS <11>

USP Biotin RS
USP Calcium Pantothenate RS
USP Cholecalciferol RS
USP Cyanocobalamin RS
USP Dexpanthenol RS
USP Ergocalciferol RS
USP Folic Acid RS
USP Niacin RS
USP Niacinamide RS
USP Phytonadione RS
USP Pyridoxine Hydrochloride RS
USP Riboflavin RS
USP Sodium Fluoride RS
USP Thiamine Hydrochloride RS
USP Alpha Tocopherol RS
USP Alpha Tocopheryl Acetate RS
USP Alpha Tocopheryl Acid Succinate RS
USP Vitamin A RS

Oil- and Water-Soluble Vitamins with Minerals Oral Solution

DEFINITION

Oil- and Water-Soluble Vitamins with Minerals Oral Solution contains one or more of the following oil-soluble vitamins: Vitamin A, Vitamin D as Ergocalciferol (Vitamin D₂) or Cholecalciferol (Vitamin D₃), and Vitamin E; one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Niacin or Niacinamide, Dexpanthenol or Panthenol, Pantothenic Acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin or Riboflavin-5'-Phosphate Sodium, and Thiamine Hydrochloride or Thiamine Mononitrate; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: chromium, fluorine, iodine, iron, magnesium, manganese, molybdenum, and zinc. It contains NLT 90.0% and NMT 200.0% of the labeled amounts of vitamin A (C₂₀H₃₀O) as retinol or esters of retinol in the form of retinyl acetate (C₂₂H₃₂O₂) or retinyl palmitate (C₃₆H₆₀O₂), vitamin D as

⁴ USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 µg of all-*trans*-retinol (vitamin A alcohol) or 0.344 µg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 µg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 µg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 µg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 µg of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

ergocalciferol ($C_{28}H_{44}O$) or cholecalciferol ($C_{27}H_{44}O$), vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) or alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$), ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 150.0% of the labeled amounts of biotin ($C_{10}H_{16}N_2O_3S$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), dexpantenol ($C_9H_{19}NO_4$) or panthenol ($C_9H_{19}NO_4$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$) or riboflavin-5'-phosphate sodium ($C_{17}H_{20}N_4NaO_9P$); NLT 90.0% and NMT 450.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$); NLT 90.0% and NMT 160.0% of the labeled amounts of chromium (Cr), fluorine (F), iodine (I), and molybdenum (Mo); and NLT 90.0% and NMT 125.0% of the labeled amounts of iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn).

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

• VITAMIN A

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Tetrahydrofuran and acetonitrile (1:1)

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)

Standard solution: 0.33 mg/mL of retinol ($C_{20}H_{30}O$) from USP Vitamin A RS in *Diluent*. [NOTE—USP Vitamin A RS is retinyl acetate. Use it to analyze Oral Solution that contains vitamin A as retinol, retinyl acetate, or retinyl palmitate.]

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 3.3 mg of retinol, to a 500-mL separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 150 mL of solvent hexane, insert the stopper, and shake for 1 min. Add another 150 mL of solvent hexane, insert the stopper, shake, and allow the layers to separate. Discard the aqueous layer, and filter the solvent hexane extract through anhydrous sodium sulfate into a 500-mL, round-bottom flask. Evaporate the solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 65°. Immediately add 10.0 mL of *Diluent*, swirl to dissolve the residue, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm \times 50-cm (prepared from two concatenated 4.6-mm \times 25-cm columns); packing L1

Column temperature: 40°

Flow rate: 1.5 mL/min

Injection size: 20 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of retinol or retinyl ester from the *Sample solution*

r_S = peak area of retinyl acetate from the *Standard solution*

C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) in the *Standard solution* (μ g/mL)

C_U = nominal concentration of Vitamin A as retinol ($C_{20}H_{30}O$) in the *Sample solution* (μ g/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–200.0% of the labeled amount of vitamin A as retinol ($C_{20}H_{30}O$)

• CHOLECALCIFEROL OR ERGOCALCIFEROL (VITAMIN D)

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluent and Mobile phase: Prepare as directed for *Vitamin A*.

Standard solution: 5 μ g/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *Diluent*

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 50 μ g of cholecalciferol or ergocalciferol, to a 500-mL separatory funnel containing 10 mL of water and 20 mL of dehydrated alcohol. Add 150.0 mL of solvent hexane, insert the stopper, and shake for 1 min. Add another 150 mL of solvent hexane, insert the stopper, shake, and allow the layers to separate. Discard the aqueous layer. Drain the solvent hexane extract through anhydrous sodium sulfate into a 500-mL, round-bottom flask. Evaporate the solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 65°. Immediately add 10.0 mL of *Diluent*, swirl to dissolve the residue, and filter.

Chromatographic system: Prepare as directed for *Vitamin A*.

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas of vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak areas of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak areas of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (μ g/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (μ g/mL)

F = correction factor to account for the average amount of previtamin D present in the formulation, 1.09

Acceptance criteria: 90.0%–200.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• VITAMIN E

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile and ethyl acetate (1:1)

Potassium hydroxide solution: Transfer 90 g of potassium hydroxide pellets to a 100-mL volumetric flask containing 60 mL of water. Mix to dissolve, cool, and dilute with water to volume.

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in *Diluent*

Sample solution: Transfer an amount of Oral Solution, equivalent to 1.5 mg of alpha tocopherol, to a 125-mL conical flask fitted with a ground-glass joint, and add 25.0 mL of dehydrated alcohol. Attach a reflux condenser, and reflux in a boiling water bath for 1 min. Cautiously add 3 mL of *Potassium hydroxide solution* through the condenser, and continue to reflux for 30 min. Remove the flask from the bath, and rinse the condenser with about 15 mL of water. Cool, and transfer with a minimum volume of water to a 250-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsings to the separatory funnel. Insert the stopper, shake vigorously for 1 min, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL, round-bottom flask. Rinse the funnel and sodium sulfate with *n*-hexane, and add the rinsing to the hexane solution in the flask. Evaporate the hexane solution to dryness with the aid of a rotary evaporator over a water bath maintained at about 50°. Immediately add 5.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 40°

Flow rate: 3.0 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of alpha tocopherol from the *Sample solution*

r_S = peak area of alpha tocopherol from the *Standard solution*

C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)

C_U = nominal concentration of Vitamin E as alpha tocopherol in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–200.0% of the labeled amount of vitamin E.

- **ASCORBIC ACID, Method 1:** Proceed as directed for *Automated Methods of Analysis* <16>, *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–200.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **ASCORBIC ACID, Method 2**

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 80 mg of ascorbic acid, to a conical flask. Add 50 mL of water, 100 mL of 0.1 N sulfuric acid VS, and 15.0 mL of 0.1 N iodine VS. Stir the contents for 30 s, and add 5 mL of starch TS.

Analysis: Immediately titrate with 0.1 N sodium thiosulfate VS to the disappearance of the color. Each mL of 0.1 N iodine is equivalent to 8.806 mg of ascorbic acid ($C_6H_8O_6$).

Acceptance criteria: 90.0%–200.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **CALCIUM ASCORBATE, Method 1:** Proceed as directed for *Automated Methods of Analysis* <16>, *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–200.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

- **CALCIUM ASCORBATE, Method 2:** Proceed as directed for *Ascorbic Acid, Method 2*. Each mL of 0.1 N iodine is equivalent to 10.66 mg of $C_{12}H_{14}CaO_{12} \cdot 2H_2O$.

Acceptance criteria: 90.0%–200.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

- **SODIUM ASCORBATE, Method 1:** Proceed as directed for *Automated Methods of Analysis* <16>, *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–200.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **SODIUM ASCORBATE, Method 2:** Proceed as directed for *Ascorbic Acid, Method 2*. Each mL of 0.1 N iodine is equivalent to 9.905 mg of $C_6H_7NaO_6$.

Acceptance criteria: 90.0%–200.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **BIOTIN, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: On the day of the assay, prepare 0.1 ng/mL of biotin from the *Standard stock solution* in water.

Sample solution: Dilute an accurately measured portion of the Oral Solution with water to an assumed concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine-guanine-uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid, cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin-thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: 50 mg/mL of monobasic potassium phosphate and 50 mg/mL of dibasic potassium phosphate in water. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: 20 g of magnesium sulfate, 1 mg/mL of sodium chloride, 1 mg/mL of ferrous sulfate, and 1 mg/mL of manganese sulfate in water. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to *Table 1*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin-thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water; add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar; and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ± 0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution*, add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ± 0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis: To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes, add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in

a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ± 0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength from 540 to 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points. Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances (Σ_u) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of lowest and highest points plotted for the standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, x, for each dosage level. Average the values of x for each of three or more dosage levels to obtain x, which equals the log-relative potency, M', of the *Sample solution*. Determine the quantity, in ng, of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of ng of biotin assumed to be present in the portion of Oral solution taken

Calculate the percentage of the labeled amount of biotin in the portion of Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin, in ng, in the portion of Oral Solution taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not

¹ ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 800 mL of water and 100 mL of triethylamine to a 1000-mL volumetric flask. Add 80 mL of 85% phosphoric acid, and dilute with water to volume.

Mobile phase: Transfer 80 mL of acetonitrile and 10 mL of *Solution A* to a 1000-mL volumetric flask. Dilute with water to volume.

Standard solution: 0.6 µg/mL of USP Biotin RS in water. [NOTE—A portion of the *Standard solution* will be used to determine the percent recovery of biotin from the *Solid-phase extraction* procedure.]

Sample solution: 0.6 µg/mL of biotin, from the Oral Solution in water. Adjust the solution with either dilute acetic acid or 0.1 N sodium hydroxide to a pH between 6.0 and 7.0.

Solid-phase extraction: [NOTE—Condition the extraction column specified in this procedure in the following manner. Wash the column with a 2-mL portion of methanol. Equilibrate with a 2-mL portion of water.] Separately pipet 5.0 mL each of the *Sample solution* and *Standard solution* into freshly conditioned solid-phase extraction columns consisting of a mixed-mode packing with a sorbent-mass of 60 mg. [NOTE—The mixed-mode packing consists of anion-exchange and reversed-phase sorbents. The reverse-phase component is a copolymer of *N*-vinylpyrrolidone and divinylbenzene. The anion exchange moiety is a trialkylamino group.²] Wash the column with 10 mL of 30% (v/v) methanol in water. Apply an appropriate volume (4.9 mL) of 30% (v/v) methanol in 0.1 N hydrochloric acid to the column. Collect the eluate in a 5-mL volumetric flask, containing 100 µL of 40% (w/v) sodium acetate in water, and dilute with 30% (v/v) methanol in 0.1 N hydrochloric acid to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and a portion of the *Standard solution* that has undergone *Solid-phase extraction*

Suitability requirements

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution* and the *Standard solution* that has undergone *Solid-phase extraction*

Recovery: 95%–100%, *Standard solution* that has undergone *Solid-phase extraction*

Analysis

Samples: *Standard solution* and *Sample solution* that have both undergone *Solid-phase extraction*
Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*

r_S = peak area of biotin from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **CYANOCOBALAMIN**

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of cyanocobalamin from USP Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described for *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Transfer an accurately measured volume of Oral Solution, assumed to contain 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each mL of the Oral Solution taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B12 activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Biotin, Method 1*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed for *Biotin, Method 1*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in *Table 2*, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions

² A suitable cartridge is the Waters, Oasis MAX Vac RC cartridge, particle size 30 µm, part 186000371.

in the resulting solution. Add 100 mL of water, and dissolve dextrose, sodium acetate, and ascorbic acid. Filter, if necessary, add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of 5.5–6.0, and add Purified Water to make 250 mL.

Table 2

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N Hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter-aid in the supernatant, and filter, with the aid of reduced pressure, through a layer of the filter-aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium

[NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.]

Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.³ [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.]

Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the

microorganism can be increased by daily or twice-daily transfer of the stab culture to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Transfer cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL of the *Culture medium* each. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ± 0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of *Suspension medium*, and combine. Using *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. The cell suspension so obtained is the *Inoculum*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.]

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis: Because of the high sensitivity of the test organism to minimum amounts of vitamin B₁₂ activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes, and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes, add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ± 0.5° for 16–24 h.

Terminate growth by heating to NLT 80° for 5 min. Cool to room temperature. After agitating its contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence

³Pure culture of *Lactobacillus leichmannii* (listed as *Lactobacillus delbrueckii*) may be obtained as No. 7030 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances (Σ_u) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the Standard solution corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, x , for each dosage level. Average the values of x for each of three or more dosage levels to obtain \bar{x} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$), in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of cyanocobalamin assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of cyanocobalamin in the portion of the Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of Oral Solution taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *Vitamin B₁₂ Activity*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–450.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• CALCIUM PANTOTHENATE, Method 1

Mobile phase: Methanol and 0.2 M monobasic sodium phosphate (3:97). Adjust with 1.7 M phosphoric acid to a pH of 3.2 ± 0.1 .

Standard solution: 80 $\mu\text{g}/\text{mL}$ of USP Calcium Pantothenate RS in *Mobile phase*

System suitability solution: 80 $\mu\text{g}/\text{mL}$ of USP Racemic Panthenol RS in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

Sample solution: Equivalent to 80 $\mu\text{g}/\text{mL}$ of calcium pantothenate from the Oral Solution in *Mobile phase*

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.0-mm \times 10-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 1.5 between panthenol and calcium pantothenate, *System suitability solution*

Tailing factor: NMT 2.0 for both the calcium pantothenate and the panthenol peaks, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of calcium pantothenate.

Calculate the percentage of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of calcium pantothenate from the *Sample solution*

r_S = peak area of calcium pantothenate from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$)

• CALCIUM PANTOTHENATE, Method 2

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 $\mu\text{g}/\text{mL}$ of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 $\mu\text{g}/\text{mL}$ of calcium pantothenate, the exact concentration being such that the responses obtained as directed for *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Transfer an accurately measured volume of Oral Solution equivalent to 50 mg of calcium pantothenate to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a measured volume of this solution quantitatively, and stepwise if necessary, with water to obtain a solution having about the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine-guanine-uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin-thiamine hydrochloride-biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to Table 3, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL
Riboflavin-thiamine hydrochloride-biotin solution	5 mL
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water; add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar; and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*¹, incubating for 16–24 h at a temperature between 30° and 37° held constant to within ± 0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution*, add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ± 0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis: To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes, add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ± 0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances (Σ_u) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, x , for each dosage level. Average the values of x for each of three or more dosage levels to obtain \bar{x} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in mg, of USP Calcium Pantothenate RS corresponding to the calcium

pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Oral Solution (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **DEXPANTHENOL OR PANTHENOL, Method 1**

Mobile phase and Chromatographic system: Proceed as directed in the assay for *Calcium Pantothenate, Method 1*.

Standard solution: 80 µg/mL of USP Dexpanthenol RS or USP Racemic Panthenol RS in *Mobile phase*. [NOTE—Use USP Dexpanthenol RS to analyze Oral Solution that contains dexpanthenol and USP Racemic Panthenol RS to analyze Oral Solution that contains panthenol.]

System suitability solution: 80 µg/mL of USP Calcium Pantothenate RS in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

Sample solution: Equivalent to 80 µg/mL of dexpanthenol or panthenol from the Oral Solution in *Mobile phase*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of panthenol. Calculate the percentage of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of dexpanthenol or panthenol from the *Sample solution*

r_S = peak area of dexpanthenol or panthenol from the *Standard solution*

C_S = concentration of USP Dexpanthenol RS or USP Racemic Panthenol RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of panthenol or dexpanthenol in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($C_9H_{19}NO_4$)

• **DEXPANTHENOL OR PANTHENOL, Method 2**

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 µg/mL of USP

Dexpanthenol RS or 1600 µg/mL of USP Racemic Panthenol RS in water. Store in a refrigerator, protected from light, and use within 30 days. [NOTE—Use USP Dexpanthenol RS to analyze Oral Solution that contains dexpanthenol and USP Racemic Panthenol RS to analyze Oral Solution that contains panthenol.]

Standard solution: On the day of the assay, prepare 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of racemic panthenol from the *Standard stock solution* with water.

Sample solution: 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of panthenol from the Oral Solution in water

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of dilute hydrochloric acid (1 in 2), and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to $75 \pm 5^\circ$, and add 6 M hydrochloric acid solution dropwise, with stirring, until the solids are dissolved. Cool, add water to make 1000 mL, and mix. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, add water to make 200 mL, and mix. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: Prepare a solution of riboflavin, thiamine hydrochloride, and biotin in 0.02 N acetic acid containing 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: Prepare a solution in neutral 25% alcohol containing 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride. Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Pyridoxal–calcium pantothenate solution: Dissolve 40 mg of pyridoxal hydrochloride and 375 µg of calcium pantothenate in 10% alcohol to make 200 mL, and mix. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: Dissolve 25 g of polysorbate 40 and 0.25 g of oleic acid in 20% alcohol to make 500 mL, and mix. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium: Dissolve anhydrous dextrose and sodium acetate in the solutions previously mixed according to *Table 4*, adjust with 1 N sodium hydroxide to a pH of 6.8, dilute with water to 250 mL, and mix.

Table 4

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL
Riboflavin-thiamine hydrochloride-biotin solution	5 mL
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal-calcium pantothenate solution	5 mL
Polysorbate 40-oleic acid solution	5 mL

Double-strength modified pantothenate medium:

Prepare as directed under *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar in 800 mL of water, with the aid of heat. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH between 6.5 and 6.6, dilute with water to 1000 mL, and mix. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*⁴ on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of sterile *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with sterile *Modified pantothenate medium*. Resuspend the cells in sufficient sterile *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to sterile glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL.

[NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes, and in the same order, add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*.

Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes, and in the same order, add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube, and mix. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature not below 80°, such as by steaming at atmospheric pressure in a sterilizer for 5 to 10 min. Cool, and concomitantly determine the percentage transmittance of the suspensions, in cells of

equal pathlength, on a suitable spectrophotometer, at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percent transmittance, for each set of tubes of the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency, of each tube containing portions of the *Sample solution*. Divide the potency of each tube by the amount of the *Sample solution* added to it, to obtain the individual responses. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the Oral Solution taken for assay, by multiplying the mean response by the appropriate dilution factor. Calculate the percentage of the labeled amount of dexpanthenol or panthenol in the portion of Oral Solution taken:

$$\text{Result} = (P/N) \times 100$$

- P = calculated potency of dexpanthenol or panthenol in the portion of Oral Solution taken (mg)
 N = nominal amount of dexpanthenol or panthenol in the portion of Oral Solution taken (mg)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol (C₉H₁₉NO₄)

• **NIACIN or NIACINAMIDE**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: 25 mg/mL of edetate disodium in water

Mobile phase: Mix 350 mL of methanol, 15.0 mL of glacial acetic acid, 0.4 mL of triethylamine, and dilute with 0.008 M sodium 1-hexanesulfonate to 2000 mL.

Standard solution: 0.10 mg/mL of USP Niacin RS or USP Niacinamide RS in *Diluent*. [NOTE—Use USP Niacin RS for Oral Solution that contains niacin and USP Niacinamide RS for Oral Solution that contains niacinamide.]

Sample solution: Dilute an accurately measured volume of Oral Solution with *Diluent* to obtain a solution with a concentration of 0.1 mg/mL of niacin or niacinamide.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Flow rate: 2 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of niacin (C₆H₅NO₂) or niacinamide (C₆H₆N₂O) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of niacin or niacinamide from the *Sample solution*
 r_S = peak area of niacin or niacinamide from the *Standard solution*
 C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

⁴ ATCC No. 8042 is suitable.

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE**

Diluent, Mobile phase, and Chromatographic system: Proceed as directed for *Niacin* or *Niacinamide*.

Standard solution: 24 µg/mL of USP Pyridoxine Hydrochloride RS in *Diluent*

Sample solution: Equivalent to 24 µg/mL of Pyridoxine Hydrochloride from Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine hydrochloride from the *Sample solution*

r_S = peak area of pyridoxine hydrochloride from the *Standard solution*

C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN-5'-PHOSPHATE SODIUM, Method 1**

[NOTE—Riboflavin-5'-phosphate sodium is quantitated against USP Riboflavin RS in this procedure. In the chromatogram of the *Sample solution*, the riboflavin-5'-phosphate peak is the only peak measured for calculation.]

Diluent, Mobile phase, and Chromatographic system: Proceed as directed for *Niacin* or *Niacinamide*.

Standard solution: 8 µg/mL of USP Riboflavin RS in *Diluent*, by heating if necessary.

Sample solution: Equivalent to 8 µg/mL of Riboflavin from the Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*
[NOTE—The relative retention times for riboflavin-5'-phosphate and riboflavin are about 0.18 and 1.0, respectively.]

Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of riboflavin-5'-phosphate from the *Sample solution*

r_S = peak area of riboflavin from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

F = factor for converting the response obtained for riboflavin-5'-phosphate to riboflavin, 1.493 [NOTE—Riboflavin phosphate sodium is a mixture of isomeric monophosphates and diphosphates containing an average amount of 67% of riboflavin-5'-monophosphate, which separates in this chromatographic system. The factor 1.493 assumes 67% of riboflavin-5'-monophosphate.]

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **RIBOFLAVIN OR RIBOFLAVIN-5'-PHOSPHATE SODIUM, Method 2**
[NOTE—Use low-actinic glassware throughout this procedure.]

Solvent blank: 1 N hydrochloric acid, 2.5 M sodium acetate, and water (1:2:97)

Standard stock solution: Transfer 0.16 mg/mL of USP Riboflavin RS in 1 N hydrochloric acid, 2.5 M sodium acetate, and water (1:2:97). Mix the resulting solution and water (1:9).

Standard solution: Transfer 5.0 mL of the *Standard stock solution* to a 500-mL volumetric flask, add 5.0 mL of 1 N hydrochloric acid, 10.0 mL of 2.5 M sodium acetate, and dilute with water to volume.

Sample solution: Transfer an accurately measured volume of Oral Solution, equivalent to 0.8 mg of riboflavin to a 100-mL volumetric flask, and dilute with water to volume. Transfer 10.0 mL of the resulting solution to a 500-mL volumetric flask, add 5.0 mL of 1 N hydrochloric acid, 10.0 mL of 2.5 M sodium acetate, and dilute with water to volume.

Spectrometric conditions

Mode: Fluorescence

Analytical wavelength

Excitation: 440 nm

Emission: 530 nm

Blank: *Solvent blank*

Analysis

Samples: *Standard solution* and *Sample solution*
Determine the maximum fluorescence intensities, I_S and I_U , of the *Standard solution* and the *Sample solution*, respectively. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Oral Solution taken:

$$\text{Result} = (I_U/I_S) \times (C_S/C_U) \times 100$$

I_U = fluorescence intensity from the *Sample solution*

I_S = fluorescence intensity from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **THIAMINE**

Diluent, Mobile phase, and Chromatographic system: Proceed as directed for *Niacin* or *Niacinamide*.

Standard solution: 24 µg/mL of USP Thiamine Hydrochloride RS in *Diluent*

Sample solution: Equivalent to 24 µg/mL of thiamine hydrochloride or thiamine mononitrate from Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the areas of the major peaks. Calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine hydrochloride or thiamine mononitrate in the *Sample solution* (mg/mL)

F = 1 (for products containing thiamine hydrochloride) and 0.97 (for products containing thiamine mononitrate)

Acceptance criteria: 90.0%–200.0% of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

[NOTE—Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a standard stock solution is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the *Standard solutions* and the *Sample solution* may be diluted quantitatively with the solvent specified, if necessary, to yield solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

• CHROMIUM

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 mL and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 mL and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Dilute an accurately measured volume of Oral Solution to obtain a solution equivalent to 1 µg/mL of chromium in 0.125 N hydrochloric acid.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 357.9 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions, against the blank. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*. Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of chromium in the *Standard solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution*, (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• FLUORIDE

[NOTE—Use plastic containers throughout this procedure.]

Ascorbic acid solution: 70 mg/mL of ascorbic acid in water

Mobile phase: Alcohol, water, and 1 N sulfuric acid (50:449:1)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 100-mL volumetric flask. Add 2 mL of *Ascorbic acid solution*, 10 mL of alcohol, and about 70 mL of water, and mix. Adjust with 1 N sodium hydroxide to a pH of 4.25 ± 0.05. Dilute with water to volume, and mix to obtain 5 µg/mL of fluoride solution.

Sample solution: Transfer an accurately measured volume of the Oral Solution, equivalent to 0.5 mg of fluoride, to a 100-mL volumetric flask. Add 1 drop of hydrochloric acid, 10 mL of alcohol, and about 75 mL

of water, and mix. Adjust with 1 N sodium hydroxide to a pH of 4.25 ± 0.05. Dilute with water to volume.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Conductivity

Guard column: 4.6-mm × 3-cm; packing L17

Analytical column: 7.8-mm × 30-cm; packing L17

Flow rate: 0.6 mL/min

Injection size: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of fluoride from the *Sample solution*

r_S = peak area of fluoride from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• IODIDE, Method 1

Mobile phase: Dissolve 5.15 g of tetrabutylammonium bromide in 320 mL of acetonitrile. Dilute with water to 2000 mL.

Standard stock solution: 1.3 mg/mL of potassium iodide in *Mobile phase*. This solution has a concentration of 1 mg/mL of iodide.

Standard solution: 2.5 µg/mL of iodide from the *Standard stock solution* in *Mobile phase*

System suitability solution: Transfer 0.13 g of potassium iodide and 0.5 g of potassium iodate to a 100-mL volumetric flask. Dissolve in *Mobile phase*, using sonication if necessary, dilute with *Mobile phase* to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, dilute with *Mobile phase* to volume, and mix. Transfer 25.0 mL of this solution to a 100-mL volumetric flask, and dilute with *Mobile phase* to volume.

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain a solution with a concentration of 2.5 µg/mL of iodine in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 225 nm

Column: 4.6-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 30 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for iodate and iodide are about 0.32 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.5 between iodate and iodide, *System suitability solution*

Relative standard deviation: NMT 2.0% for the iodide peak, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of iodine (I) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of iodide from the *Sample solution*
 r_S = peak area of iodide from the *Standard solution*
 C_S = concentration of iodide in the *Standard solution* ($\mu\text{g/mL}$)
 C_U = nominal concentration of iodine in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

- **IODIDE, Method 2:** Proceed as directed for *Automated Methods of Analysis* (16), Assay for Iodide.

- **IRON**

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask, dissolve in 6 N hydrochloric acid, and dilute with water to volume.

Standard solutions: To separate 100-mL volumetric flasks, transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 $\mu\text{g/mL}$ of iron.

Sample solution: 6 $\mu\text{g/mL}$ of iron from the Oral Solution in 0.125 N hydrochloric acid

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 248.3 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions, against the blank. Plot the absorbances of the *Standard solutions* versus concentration, in $\mu\text{g/mL}$, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of iron in the *Sample solution*. Calculate the percentage of the labeled amount of iron (Fe) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of iron in the *Sample solution* from the graph ($\mu\text{g/mL}$)

C_U = nominal concentration of iron in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

- **MAGNESIUM**

Magnesium standard solution: Transfer 1.00 g of magnesium ribbon to a 1000-mL volumetric flask. Dissolve in 50 mL of 6 N hydrochloric acid, and dilute with water to volume.

Standard stock solution: 20 $\mu\text{g/mL}$ of magnesium from *Magnesium standard solution* in 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 7.5, 10.0, 12.5, and 15.0 mL of *Standard stock solution*. Dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 $\mu\text{g/mL}$ of magnesium.

Sample solution: 2.5 $\mu\text{g/mL}$ of magnesium from the Oral Solution in 0.125 N hydrochloric acid

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 285.2 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions, against the blank. Plot the absorbances of the *Standard solutions* versus concentration, in $\mu\text{g/mL}$, of magnesium, and

draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of magnesium in the *Sample solution*. Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of magnesium in the *Sample solution* from the graph ($\mu\text{g/mL}$)

C_U = nominal concentration of magnesium in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

- **MANGANESE**

Manganese standard solution: Transfer 1.0 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, and dilute with 6 N hydrochloric acid to volume.

Standard stock solution: 50 $\mu\text{g/mL}$ of manganese from the *Manganese standard stock solution* in 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions having known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 $\mu\text{g/mL}$ of manganese.

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain 1.5 $\mu\text{g/mL}$ of manganese in 0.125 N hydrochloric acid.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 279.5 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions, against the blank. Plot the absorbances of the *Standard solutions* versus concentration, in $\mu\text{g/mL}$, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of manganese in the *Sample solution*. Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of manganese in the *Sample solution* from the graph ($\mu\text{g/mL}$)

C_U = nominal concentration of manganese in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

- **MOLYBDENUM**

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume.

Standard stock solution: 100 $\mu\text{g/mL}$ of molybdenum from the *Molybdenum standard solution* in water

Standard solutions: To separate 100-mL volumetric flasks transfer 0.5, 1.0, 1.5, 2.0 and 3.0 mL of the *Standard stock solution*. Add 0.125 N hydrochloric acid to volume, and mix to obtain the solutions having known concentrations of about 0.5, 1.0, 1.5, 2.0, and 3.0 $\mu\text{g/mL}$ of molybdenum.

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain 1 $\mu\text{g/mL}$ of

molybdenum from the Oral Solution in 0.125 N hydrochloric acid.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Lamp: Molybdenum hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: 313.3 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions, against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in $\mu\text{g/mL}$, of molybdenum, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of molybdenum in the *Sample solution*. Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of molybdenum in the *Sample solution* from the graph ($\mu\text{g/mL}$)

C_U = nominal concentration of molybdenum in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• ZINC

Zinc standard solution: Transfer 311 mg of zinc oxide to a 250-mL volumetric flask, and add 80 mL of 6 N hydrochloric acid, warming if necessary to dissolve. Cool, dilute with water to volume, and mix to obtain a solution having a known concentration of 1000 $\mu\text{g/mL}$ of zinc.

Standard stock solution: 50 $\mu\text{g/mL}$ of zinc from the *Zinc standard solution* in 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 $\mu\text{g/mL}$ of zinc.

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain 1 $\mu\text{g/mL}$ of zinc in 0.125 N hydrochloric acid.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 213.8 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions, against the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in $\mu\text{g/mL}$, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of zinc in the *Sample solution*. Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of zinc in the *Sample solution* from the graph ($\mu\text{g/mL}$)

C_U = nominal concentration of zinc in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

OTHER COMPONENTS

- **ALCOHOL DETERMINATION, Method I (611)** (if present): 90.0%–120.0% of the labeled amount of $\text{C}_2\text{H}_5\text{OH}$

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic microbial count does not exceed 3000 cfu/mL, and the combined molds and yeasts count does not exceed 300 cfu/mL.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS (2022):** Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, under an inert gas or with a minimum of headspace.
- **LABELING:**⁵ The label states that the product is Oil- and Water-Soluble Vitamins with Minerals Oral Solution. The label states the quantity of each vitamin and mineral in a given volume of the Oral Solution and, where necessary, the chemical form in which a vitamin is present, and states also the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it is the *d*- or *dl*- form. Where the product is labeled to contain panthenol, the label states the equivalent content of dexpanthenol. Where more than one assay method is given for a particular vitamin or mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS (11)**
 - USP Alpha Tocopherol RS
 - 2*H*-1-Benzopyran-6-ol, 3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-.
 - $\text{C}_{29}\text{H}_{50}\text{O}_2$ 430.70
 - USP Biotin RS
 - 1*H*-Thieno[3,4-*d*]imidazole-4-pentanoic acid, hexahydro-2-oxo-, 3*aS*-(3*a*,4*β*,6*a*)-.
 - $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$ 244.31
 - USP Calcium Pantothenate RS
 - β -Alanine, *N*-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-, calcium salt (2:1), (*R*)-.
 - $\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$ 476.53
 - USP Cholecalciferol RS
 - 9,10-Secocholesta-5,7,10(19)-trien-3-ol, (3*β*,5*Z*,7*E*)-.
 - $\text{C}_{27}\text{H}_{44}\text{O}$ 384.64
 - USP Cyanocobalamin RS
 - Vitamin B₁₂.
 - $\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$ 1355.37
 - USP Dexpanthenol RS
 - Butanamide, 2,4-dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethyl-, (*R*)-.
 - $\text{C}_9\text{H}_{19}\text{NO}_4$ 205.25
 - USP Ergocalciferol RS
 - 9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol, (3*β*,5*Z*,7*E*,22*E*)-.

⁵ USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 μg of all-*trans*-retinol (vitamin A alcohol) or 0.344 μg of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 μg of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 μg of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 μg of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 μg of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

$C_{28}H_{44}O$ 396.65
 USP Niacin RS
 3-Pyridinecarboxylic acid.
 $C_6H_5NO_2$ 123.11
 USP Niacinamide RS
 3-Pyridinecarboxamide.
 $C_6H_6N_2O$ 122.12
 USP Pyridoxine Hydrochloride RS
 3,4-Pyridinedimethanol, 5-hydroxy-6-methyl-, hydrochloride.
 $C_8H_{11}NO_3 \cdot HCl$ 205.64
 USP Racemic Panthenol RS
 Butanamide, 2,4-dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethyl-,(±)-.
 $C_9H_{19}NO_4$ 205.25
 USP Riboflavin RS
 Riboflavine.
 $C_{17}H_{20}N_4O_6$ 376.36
 USP Sodium Fluoride RS
 Sodium fluoride.
 NaF 41.99
 USP Thiamine Hydrochloride RS
 Thiazolium, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methyl-, chloride, monohydrochloride.
 $C_{12}H_{17}ClN_4OS \cdot HCl$ 337.27
 USP Vitamin A RS
 3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl) 2,4,6,8-nonatetraen-1-ol acetate (vitamin A acetate)

Oil- and Water-Soluble Vitamins with Minerals Tablets

DEFINITION

Oil- and Water-Soluble Vitamins with Minerals Tablets contain one or more of the following oil-soluble vitamins: Vitamin A, Vitamin D as Ergocalciferol (Vitamin D₂) or Cholecalciferol (Vitamin D₃), Vitamin E, Phytonadione (Vitamin K₁), and Beta Carotene; one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, Pantothenic Acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Tablets contain NLT 90.0% and NMT 165.0% of the labeled amounts of vitamin A ($C_{20}H_{30}O$) as retinol or esters of retinol in the form of retinyl acetate ($C_{22}H_{32}O_2$) or retinyl palmitate ($C_{36}H_{60}O_2$), vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$), vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) or alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$), phytonadione ($C_{31}H_{46}O_2$), and beta carotene ($C_{40}H_{56}$); NLT 90.0% and NMT 150.0% of the labeled amounts of ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$), biotin ($C_{10}H_{16}N_2O_3S$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 125.0% of the labeled amounts of calcium (Ca), copper (Cu), iron (Fe), manganese (Mn), magnesium (Mg), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of

the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

• VITAMIN A, Method 1

[NOTE—Where the use of a vitamin A ester (retinyl acetate or retinyl palmitate) is specified in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane

Standard solution: 15 µg/mL of retinyl acetate from USP Vitamin A RS in *n*-hexane

System suitability stock solution: 15 µg/mL of retinyl palmitate in *n*-hexane

System suitability solution: Mix equal volumes of *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 µg/mL each of retinyl acetate and retinyl palmitate.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a container having a polytetrafluoroethylene-lined screw cap. Add 10 mL of dimethyl sulfoxide and 15 mL of *n*-hexane, and shake for 45 min on a wrist-action shaker in a water bath maintained at 60°. [NOTE—Set up the wrist-action shaker to ensure that the contents of the container are mixed vigorously and thoroughly.] Centrifuge at 3000 rpm for 10 min, and transfer the hexane layer by means of a pipet to a 100-mL volumetric flask. Add 15 mL of *n*-hexane to the dimethyl sulfoxide layer, shake thoroughly for 5 min, and transfer the hexane layer by means of a pipet to the 100-mL volumetric flask. Repeat this extraction with three additional 15-mL portions of *n*-hexane. Dilute the extracts in the volumetric flask with *n*-hexane to volume. Dilute a 10-mL volume of this solution with *n*-hexane to obtain a solution with a concentration of 15 µg/mL of vitamin A as retinol ($C_{20}H_{30}O$).

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection size: 40 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 10 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
 Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area for all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate in the chromatogram of the *Sample solution*. For products containing vitamin A acetate or vitamin A palmitate, calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$) in the portion of the Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

- r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
 r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*
 C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* ($\mu\text{g/mL}$)
 C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$) in the *Sample solution* ($\mu\text{g/mL}$)
 F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

[NOTE—The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **VITAMIN A, Method 2**

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution: Cautiously add 9 mL of sulfuric acid to 80 mL of methanol in a 100-mL volumetric flask. Cool, and dilute with methanol to volume.

Sodium ascorbate–pyrogallol solution: Transfer 10 g of sodium ascorbate and 5 g of pyrogallol to a 100-mL volumetric flask, and add sufficient water to dissolve. Add 1.7 mL of sulfuric acid, and dilute with water to volume.

Lecithin solution: 5 mg/mL of lecithin in 2,2,4-trimethylpentane

Mobile phase: *n*-Hexane and ethyl acetate (99.7:0.3)

Standard solution: 15 $\mu\text{g/mL}$ of retinyl acetate from USP Vitamin A RS in 2,2,4-trimethylpentane

System suitability stock solution: 15 $\mu\text{g/mL}$ of retinyl palmitate in 2,2,4-trimethylpentane

System suitability solution: Mix equal volumes of the *System suitability stock solution* and the *Standard solution* to obtain concentrations of 7.5 $\mu\text{g/mL}$ each of retinyl acetate and retinyl palmitate.

Sample solution: [NOTE—This preparation is suitable for the determination of vitamin A, vitamin D, and vitamin E, when present in the formulation.] Finely powder NLT 20 Tablets. If vitamin D is present in the formulation, transfer a portion of the powder, equivalent to 30 μg of the labeled amount of cholecalciferol or ergocalciferol, to a container having a polytetrafluoroethylene-lined screw cap. If vitamin D is not present in the formulation, use a portion of the powder, equivalent to 90 mg of the labeled amount of vitamin E (as α -tocopherol, α -tocopheryl acetate, or α -tocopheryl hemisuccinate). If vitamin E is not present in the formulation, use a portion of the powder, equivalent to 2.5 mg of the labeled amount of retinyl acetate or retinyl palmitate. Add 0.5 g of sodium bicarbonate, 1.5 mL of *Lecithin solution*, and 12.5 mL of 2,2,4-trimethylpentane, and disperse on a vortex mixer. Add 6 mL of *Sodium ascorbate–pyrogallol solution*, shake slowly, and allow the solution to degas. Continue shaking until the evolution of gas has ceased, and then shake for an additional 12 min. Add 6 mL of dimethyl sulfoxide, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 6 mL of 3 N methanolic sulfuric acid solution, mix on a vortex mixer to form a suspension, and shake for 12 min. Add 12.5 mL of 2,2,4-trimethylpentane, mix on a vortex mixer to form a suspension, and shake for 10 min. Centrifuge for 10 min to break up the emulsion and to clarify the supernatant. [NOTE—The supernatant is used for the determination of vitamin A, and also vitamin D and vitamin E, if present in the formulation.] If necessary, quantitatively dilute a volume of the

supernatant with 2,2,4-trimethylpentane to obtain a concentration close to that of the *Standard solution*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 325 nm

Column: 4.6-mm \times 25-cm; 5- μm packing L24

Flow rate: 1.5 mL/min

Injection size: 40 μL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 8.0 between all-*trans*-retinyl acetate and all-*trans*-retinyl palmitate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak area for all-*trans*-retinyl acetate from the *Standard solution* and the peak area of all-*trans*-retinyl acetate or all-*trans*-retinyl palmitate from the *Sample solution*.

Calculate the percentage of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$), in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of the all-*trans*-retinyl ester from the *Sample solution*
 r_S = peak area of the all-*trans*-retinyl ester from the *Standard solution*

C_S = concentration of retinyl acetate ($C_{22}H_{32}O_2$) from USP Vitamin A RS in the *Standard solution* ($\mu\text{g/mL}$)
 C_U = nominal concentration of vitamin A, as retinol ($C_{20}H_{30}O$) in the *Sample solution* ($\mu\text{g/mL}$)

F = factor used to convert retinyl acetate, the ester form present in the USP Vitamin A RS, to retinol, 0.872
 [NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration. The molar responses of retinyl acetate and retinyl palmitate are equivalent.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol ($C_{20}H_{30}O$)

• **VITAMIN A, Method 3**

[NOTE—Where a vitamin A ester (retinyl acetate or retinyl palmitate) is indicated in the following procedure, use the chemical form present in the formulation. USP Vitamin A RS is retinyl acetate. It is to be used where USP Vitamin A RS is specified. Use low-actinic glassware throughout this procedure.]

Extraction solvent: *n*-Hexane and methylene chloride (3:1)

Potassium hydroxide solution: 800 mg/mL of potassium hydroxide in water. [NOTE—Cautiously add potassium hydroxide in water. Mix, and cool.]

Diluent: 10 mg/mL of pyrogallol in alcohol

Mobile phase: *n*-Hexane and isopropyl alcohol (92:8)

Standard stock solution: 30 $\mu\text{g/mL}$ of retinyl acetate from USP Vitamin A RS in *Diluent*. [NOTE—This solution may be stored in a refrigerator for 1 week.]

Standard solution: Dilute a volume of *Standard stock solution* with *Diluent* to obtain a concentration of 1 $\mu\text{g/mL}$ of USP Vitamin A RS. Transfer 10.0 mL of this solution to a stoppered 125-mL flask, and add 5 mL of water, 5 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at $60 \pm 5^\circ$, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. Withdraw a portion of the organic layer

for injection into the chromatograph. This *Standard solution* contains 0.34 µg/mL of retinol.

Sample solution: Finely powder a counted number of Tablets. Transfer a portion of the powder, equivalent to 1.5 mg of retinyl acetate, to a stoppered 125-mL flask. Add 5 mL of water, 15 mL of *Diluent*, and 3 mL of *Potassium hydroxide solution*. Insert the stopper tightly, shake for 15 min over a water bath maintained at 60 ± 5°, and cool to room temperature. Add 7 mL of water and 25.0 mL of *Extraction solvent*. Insert the stopper tightly, and shake vigorously for 60 s or longer, if necessary, for complete extraction. Rinse the sides of the flask with 60 mL of water, and allow to stand for 10 min until the layers separate. [NOTE—Do not shake, because an emulsion may form.] Withdraw a portion of the organic layer, and dilute with *Extraction solvent* to obtain a concentration of 0.34 µg/mL of retinol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 335 nm

Column: 6.2-mm × 8-cm; packing L3

Column temperature: 40°

Flow rate: 4 mL/min

Injection size: 50 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for 13-*cis*-retinol and all-*trans*-retinol are 0.92 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for all-*trans*-retinol and 13-*cis*-retinol. Calculate the percentage of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O), in the portion of Tablets taken:

$$\text{Result} = (r_{T1}/r_{T2}) \times (C_S/C_U) \times F \times 100$$

r_{T1} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Sample solution*

r_{T2} = sum of the areas of the all-*trans*-retinol and 13-*cis*-retinol peaks from the *Standard solution*

C_S = concentration of retinyl acetate (C₂₃H₃₂O₂) from USP Vitamin A RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of vitamin A, as retinol (C₂₀H₃₀O) in the *Sample solution* (µg/mL)

F = factor used to convert retinyl acetate, the ester form present in USP Vitamin A RS, to retinol, 0.872

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin A, as retinol (C₂₀H₃₀O)

• CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D), Method 1

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: *n*-Hexane and isopropyl alcohol (99:1)

Standard solution: 2 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in *n*-hexane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for *Sample solution* in *Vitamin A, Method 1* to a suitable container, and evaporate, if necessary, in vacuum at room temperature to obtain a concentration of 2 µg/mL of cholecalciferol or ergocalciferol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 15-cm; 3-µm packing L8

Flow rate: 1 mL/min

Injection size: 100 µL

System suitability

Sample: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 10 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*

r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*

C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* (µg/mL)

F = correction factor to account for the average amount of previtamin D present in the *Sample solution*, 1.09

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol (C₂₇H₄₄O) or ergocalciferol (C₂₈H₄₄O)

• CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D), Method 2

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

3 N methanolic sulfuric acid solution, Sodium ascorbate-pyrogallol solution, Lecithin solution, and Sample solution: Proceed as directed in *Vitamin A, Method 2*.

Mobile phase: *n*-Hexane and tertiary butyl alcohol (98.75:1.25)

Standard solution: 1 µg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in 2,2,4-trimethylpentane

System suitability solution: Heat a volume of the *Standard solution* at 60° for 1 h to partially isomerize vitamin D (cholecalciferol or ergocalciferol) to its corresponding precursor.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm × 25-cm; 5-µm packing L24

Flow rate: 1 mL/min

Injection size: 40 µL

System suitability

Sample: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between the vitamin D form present and its corresponding precursor, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis**Samples:** *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
 r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
 C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* ($\mu\text{g/mL}$)
 C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* ($\mu\text{g/mL}$)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane to calculate the nominal concentration.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **CHOLECALCIFEROL or ERGOCALCIFEROL (VITAMIN D),**

Method 3

[NOTE—Where vitamin D (cholecalciferol or ergocalciferol) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Diluted acetic acid: Glacial acetic acid solution (1 in 10) in water

Phenolphthalein solution: 10 mg/mL of phenolphthalein in alcohol

Potassium hydroxide solution: Slowly dissolve 14 g of potassium hydroxide in a mixture of 31 mL of dehydrated alcohol and 5 mL of water. Prepare fresh daily.

Extraction solvent: Methylene chloride and isopropyl alcohol (99.8:0.2)

Mobile phase: Acetonitrile and methanol (91:9)

Standard stock solution: 0.2 mg/mL of USP Cholecalciferol RS or USP Ergocalciferol RS in dehydrated alcohol. [NOTE—Prepare fresh every 4 weeks. Store in a freezer.]

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* by initially washing the column with 4.0 mL of a mixture of methylene chloride and isopropyl alcohol (4:1), followed by 5.0 mL of *Extraction solvent*. Do not allow the column to dry.] Dilute a volume of *Standard stock solution* with dehydrated alcohol to obtain a concentration of 5 $\mu\text{g/mL}$ of USP Cholecalciferol RS or USP Ergocalciferol RS. Prepare this solution fresh daily. Transfer 2.0 mL of this solution to a stoppered 125-mL flask. Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*, insert the stopper, and shake for 30 min in a water bath maintained at 60°. Allow to cool to room temperature, and transfer the contents of the flask to a 250-mL separatory funnel. Add 15.0 mL of water to the flask, insert the stopper, shake vigorously, and transfer this solution to the separatory funnel. Rinse the flask with 60 mL of *n*-hexane, and transfer the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 90 s, and allow to stand for 15 min until the layers separate. Drain and discard the aqueous layer. Add 15.0 mL of water to the hexane layer in the separatory funnel, insert the stopper, and shake vigorously. Allow to stand for 10 min until the layers separate, and discard the aqueous layer. Add 1 drop of *Phenolphthalein solution* and 15.0 mL of water to the separatory funnel. Add *Diluted acetic acid* dropwise, with shaking, until the washing is neutral. Allow to

stand for 10 min until the layers separate. Drain and discard the aqueous layer. Filter the hexane layer through anhydrous sodium sulfate supported by a small pledget of cotton into a 100-mL, round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and collect the rinsings in the same flask. Evaporate the hexane in the flask on a rotary evaporator at 50° to dryness. Immediately add 2.0 mL of *Extraction solvent* to dissolve the residue. Transfer this solution to a freshly conditioned solid-phase extraction column containing silica packing with a sorbent mass to column volume ratio of 500 mg to 2.8 mL or equivalent, rinse the round-bottom flask with 1.0 mL of *Extraction solvent*, and transfer to the column. Elute the column with 2.0 mL of *Extraction solvent*, and discard this fraction. Elute the column with 7.0 mL of *Extraction solvent*, and collect the eluate in a suitable flask. Place the flask in a warm water bath maintained at 42°, and evaporate the solvent with the aid of a stream of nitrogen. Immediately add 2.0 mL of acetonitrile to the residue, and use the solution for injection into the chromatograph.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 10 μg of cholecalciferol or ergocalciferol, to the stoppered 125-mL flask, and proceed as directed for the *Standard solution*, beginning with "Add 15.0 mL of water and 15.0 mL of *Potassium hydroxide solution*".

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 265 nm

Column: 4.6-mm \times 25-cm; 5- μm packing L1

Column temperature: 27°

Flow rate: 0.7 mL/min

Injection size: 15 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 4.0%

Analysis**Samples:** *Standard solution* and *Sample solution*

Measure the peak areas for vitamin D. Calculate the percentage of the labeled amount of cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of cholecalciferol or ergocalciferol from the *Sample solution*
 r_S = peak area of cholecalciferol or ergocalciferol from the *Standard solution*
 C_S = concentration of USP Cholecalciferol RS or USP Ergocalciferol RS in the *Standard solution* ($\mu\text{g/mL}$)
 C_U = nominal concentration of cholecalciferol or ergocalciferol in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin D as cholecalciferol ($C_{27}H_{44}O$) or ergocalciferol ($C_{28}H_{44}O$)

• **VITAMIN E, Method 1**

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Solution A: Phosphoric acid solution (1 in 100) in water

Mobile phase: Methanol and *Solution A* (19:1)

System suitability solution: Prepare a 0.65-mg/mL solution of USP Ergocalciferol RS in methanol. Transfer 1.0 mL of this solution to a 100-mL volumetric flask containing 100 mg of USP Alpha Tocopheryl Acetate RS.

Dissolve in 30 mL of methanol, with the aid of sonication if necessary, and dilute with methanol to volume. Store this solution in a refrigerator.

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for *Sample solution in Vitamin A, Method 1* to a suitable container, and evaporate in vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 2 mg/mL of alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-μm packing L1

Flow rate: 2 mL/min

Injection size: 100 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for ergocalciferol and alpha tocopheryl acetate are about 0.5 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 12 between ergocalciferol and alpha tocopheryl acetate, *System suitability solution*

Tailing factor: Between 0.8 and 1.2, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅)

• VITAMIN E, Method 2

[NOTE—Where vitamin E (alpha tocopherol, alpha tocopheryl acetate, or alpha tocopheryl acid succinate) is specified in the following procedure, use the chemical form present in the formulation and the relevant USP Reference Standard. Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 240 mL of methanol with 10 mL of water followed by 0.5 mL of 50% phosphoric acid, and dilute with acetonitrile to 1000 mL.

System suitability solution: 2 mg/mL each of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, and USP Alpha Tocopheryl Acid Succinate RS in methanol

Standard solution: 2 mg/mL of USP Alpha Tocopherol RS, USP Alpha Tocopheryl Acetate RS, or USP Alpha Tocopheryl Acid Succinate RS in methanol

Sample solution: Proceed as directed for *Sample solution in Vitamin A, Method 2*. Transfer a volume of the supernatant 2,2,4-trimethylpentane to a suitable volumetric flask, the volume of the specimen withdrawn from the 2,2,4-trimethylpentane and the size of the volumetric flask being such that the final concentration of the *Sample solution* is equivalent to that of the *Standard solution*. Evaporate nearly to dryness, add several mL of methanol, and evaporate the remaining 2,2,4-trimethylpentane. Dilute with methanol to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 1.5 mL/min

Injection size: 25 μL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for alpha tocopheryl acid succinate, alpha tocopherol, and alpha tocopheryl acetate are about 0.6, 0.8, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 4.0 between alpha tocopheryl acid succinate and alpha tocopherol; NLT 3.0 between alpha tocopherol and alpha tocopheryl acetate, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas. Calculate the percentage of the labeled amount of alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the relevant vitamin E form from the *Sample solution*

r_S = peak area of the relevant vitamin E form from the *Standard solution*

C_S = concentration of the corresponding USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding form of vitamin E in the *Sample solution* (mg/mL)

[NOTE—Account for the initial extraction volume of 26.5 mL of 2,2,4-trimethylpentane and the dilution factor to exchange the solvent from 2,2,4-trimethylpentane to methanol to calculate the nominal concentration.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol (C₂₉H₅₀O₂), alpha tocopheryl acetate (C₃₁H₅₂O₃), or alpha tocopheryl acid succinate (C₃₃H₅₄O₅)

• VITAMIN E, Method 3

Diluent: Acetonitrile and ethyl acetate (1:1)

Mobile phase: Methanol, acetonitrile, and *n*-hexane (46.5:46.5:7.0)

Standard solution: 0.3 mg/mL of USP Alpha Tocopherol RS in methanol

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 8 mg of alpha tocopherol, to a 125-mL flask fitted with a ground-glass joint. Add 25.0 mL of water, 25.0 mL of dehydrated alcohol, and 3.5 g of potassium hydroxide

pellets. Shake for 1 h in a water bath maintained at 55°. Cool, and transfer with the aid of a minimum volume of water to a 125-mL separatory funnel. Rinse the flask with 50 mL of *n*-hexane, and add the rinsing to the separatory funnel. Insert the stopper, shake vigorously for 60 s, and allow the layers to separate. Drain the aqueous layer into a second 250-mL separatory funnel, and repeat the extraction with 50 mL of *n*-hexane. Discard the aqueous layer, and combine the hexane extracts. Wash the combined extracts with 25 mL of water, allow the layers to separate, and discard the aqueous layer. Add 3 drops of glacial acetic acid, and repeat the washing procedure two more times. Filter the washed hexane layer through anhydrous sodium sulfate into a 250-mL round-bottom flask. Rinse the funnel and sodium sulfate with a few mL of *n*-hexane, and add the rinsing to the hexane solution in the flask. Place the flask in a water bath maintained at 50°, and evaporate the hexane solution with the aid of a rotary evaporator to dryness. Immediately add 25.0 mL of *Diluent*, and swirl to dissolve the residue.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 291 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 40°

Flow rate: 3 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of alpha tocopherol from the *Sample solution*

r_S = peak area of alpha tocopherol from the *Standard solution*

C_S = concentration of alpha tocopherol in the *Standard solution* (mg/mL)

C_U = nominal concentration of vitamin E as alpha tocopherol in the *Sample solution* (mg/mL)

[NOTE—Calculate the nominal amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), by multiplying the content of alpha tocopheryl acetate ($C_{31}H_{52}O_3$) or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$), in mg/tablet, by the factors 0.91 or 0.81, respectively.]

Acceptance criteria: 90.0%–165.0% of the labeled amount of vitamin E as alpha tocopherol ($C_{29}H_{50}O_2$), alpha tocopheryl acetate ($C_{31}H_{52}O_3$), or alpha tocopheryl acid succinate ($C_{33}H_{54}O_5$)

• PHYTONADIONE, *Method 1*

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (19:1)

Standard stock solution: 200 µg/mL of USP

Phytonadione RS in methanol. Dissolve with the aid of sonication if necessary.

Standard solution: 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with methanol.

System suitability solution: 0.65 mg/mL of USP Alpha Tocopheryl Acetate RS and 20 µg/mL of USP Phytonadione RS from *Standard stock solution* diluted with methanol. [NOTE—Dissolve USP Alpha Tocopheryl Acetate RS in a portion of methanol, add the *Standard stock solution*, and then dilute with methanol to volume.]

Sample solution: Transfer NLT 20 mL of the solution prepared as directed for *Sample solution* in *Vitamin A, Method 1* to a suitable container, and evaporate in a vacuum at room temperature to dryness. Transfer the residue with the aid of methanol to a suitable volumetric flask, and dilute with methanol to volume to obtain a concentration of 20 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 8-mm × 10-cm; 5-µm packing L1

Flow rate: 2 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for alpha tocopheryl acetate and phytonadione are 0.68 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 5 between alpha tocopheryl acetate and phytonadione, *System suitability solution*

Relative standard deviation: NMT 3.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas. Calculate the percentage of the labeled amount of phytonadione ($C_{31}H_{46}O_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of phytonadione ($C_{31}H_{46}O_2$)

• PHYTONADIONE, *Method 2*

[NOTE—Use low-actinic glassware throughout this procedure.]

Solvent: Methanol and isopropyl alcohol (19:1)

Mobile phase: Mix 800 mL of methanol, 200 mL of methylene chloride, 0.1 mL of glacial acetic acid, 1.36 g of zinc chloride, and 0.41 g of sodium acetate.

Internal standard solution: 5 µg/mL of menaquinone 4 (vitamin K₂) in *Solvent*. [NOTE—A concentrated stock solution of menaquinone 4 (100 µg/mL) can be stored for 2 months in a refrigerator.]

Standard stock solution: 5 µg/mL of USP

Phytonadione RS, prepared by dissolving in methylene chloride with the aid of sonication, and diluting with *Solvent* to final volume.

Standard solution: Transfer 1.0 mL of the *Standard stock solution* and 1.0 mL of the *Internal standard solution* to a suitable flask and dilute with *Solvent* to 5 mL. Pass through a membrane filter of 0.45-µm or finer pore size.

Sample solution: Finely powder NLT 20 Tablets. To a centrifuge tube fitted with a cap transfer an amount of powder, not exceeding 800 mg and equivalent to an amount of phytonadione not exceeding 50 µg. Add 4 mL of water. Insert the stopper, and mix using a vortex mixer until the sample is dispersed. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 1 min while the preparation is still hot. Add 8 mL of alcohol, and swirl the contents to mix. Place the tube in a water bath at 60° for 5 min. Remove from the bath, and again shake or mix using a vortex mixer for 2 min while the preparation is still hot. Cool to room temperature. Add a volume of *Internal standard solution*,

equivalent to 1.0 mL per each 5 µg of the expected amount of phytonadione in the aliquot taken. Add 20.0 mL of petroleum ether, and cap the tube tightly. Shake or mix using a vortex mixer for 15 min to thoroughly mix the contents. Centrifuge to separate the two layers. Transfer a volume of the top layer of petroleum ether, equivalent to 5–50 µg of the nominal amount of phytonadione, to an appropriate flask. Place the flask in a water bath at 35°–45°, and evaporate the solvent under a stream of nitrogen until an oily residue is left. Dissolve the residue in a volume of *Solvent* to obtain a concentration of 1 µg/mL of phytonadione.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Fluorometric detector set at 320 nm for excitation and 420 nm for emission

Column: 4.6-mm × 25-cm; 5-µm, end-capped packing L1, and a postcolumn reactor constituted with a 4.6-mm × 3-cm PEEK column tightly packed with zinc powder. [NOTE—Prepare the postcolumn reactor daily, or as necessary, to meet the system suitability requirements.]

Flow rate: 1 mL/min

Injection size: 25 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for the internal standard and phytonadione are 1.0 and 1.4, respectively.]

Suitability requirements

Column efficiency: NLT 2500 theoretical plates for the phytonadione peak

Tailing factor: NMT 1.5 for the phytonadione peak

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of phytonadione (C₃₁H₄₆O₂) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of phytonadione to that of the internal standard from the *Sample solution*

R_S = peak response ratio of phytonadione to that of the internal standard from the *Standard solution*

C_S = concentration of USP Phytonadione RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of phytonadione in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–165.0% of labeled amount of phytonadione (C₃₁H₄₆O₂)

• BETA CAROTENE

[NOTE—Use low-actinic glassware throughout this procedure.]

Potassium hydroxide solution: Dissolve 58.8 g potassium hydroxide in 50 mL of water.

Iodine solution: 0.01 mg/mL of iodine in cyclohexane. [NOTE—Prepare this solution fresh daily.]

Sample solution: Weigh NLT 20 Tablets. Grind the Tablets to a fine powder, and transfer a quantity of the powder, equivalent to 2 mg of beta carotene, to a 500-mL saponification flask. Add 100 mL of alcohol, 6 mL of *Potassium hydroxide solution*, and a magnetic stirring bar. Attach an air condenser to the flask, and heat under reflux for 45 min with constant stirring. Cool to room temperature, add 170 mL of solvent hexane, and stir for 30 min. Quantitatively transfer the contents of the flask to a 500-mL separatory funnel with portions of solvent hexane. Allow the layers to separate for 5–10 min, and transfer the upper organic layer to a 500-mL volumetric flask. Transfer the lower

aqueous layer into the saponification flask, add 170 mL of solvent hexane, and stir for an additional 20 min. Quantitatively transfer the contents of the saponification flask to the separatory funnel with the aid of portions of solvent hexane. Allow the layers to separate for 10 min. Drain the lower aqueous layer, and discard. Transfer the organic layer to the volumetric flask containing the previously collected organic layer. Rinse the separatory funnel with small portions of solvent hexane, and transfer the washings to the volumetric flask. Dilute the hexane extracts with solvent hexane to volume, add 3 g of anhydrous sodium sulfate, shake, and allow to settle. Quantitatively transfer a volume of this solution, equivalent to 100 µg of beta carotene, to a 50-mL volumetric flask. Evaporate under a stream of nitrogen to dryness, and immediately add cyclohexane. Add 2 mL of *Iodine solution*, and heat for 15 min in a water bath maintained at 65°. Cool rapidly, and dilute with cyclohexane to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Vis

Analytical wavelength: 452 nm

Blank: Cyclohexane

Analysis

Sample: *Sample solution*

Determine the absorbance against the *Blank*. Calculate the percentage of the labeled amount of beta carotene (C₄₀H₅₆) in the portion of Tablets taken:

$$\text{Result} = (A_U/F) \times (100/C_U)$$

A_U = absorbance of the *Sample solution*

F = absorptivity of beta carotene at 452 nm, 223

C_U = nominal concentration of beta carotene in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–165.0% of the labeled amount of beta carotene (C₄₀H₅₆)

• ASCORBIC ACID, Method 1

Sample solution: Finely powder NLT 20 Tablets.

Transfer a portion of the powder, equivalent of 100 mg of ascorbic acid, to a 200-mL volumetric flask, and add 75 mL of metaphosphoric–acetic acids TS. Insert a stopper into the flask, and shake by mechanical means for 30 min. Dilute with water to volume. Transfer a portion of the solution to a centrifuge tube, and centrifuge until a clear supernatant is obtained. Pipet 4.0 mL of this solution into a 50-mL conical flask, and add 5 mL of metaphosphoric–acetic acids TS.

Analysis: Titrate with standard dichlorophenol–indophenol solution VS to a rose-pink color that persists for at least 5 s. Correct for the volume of dichlorophenol–indophenol solution consumed by a mixture of 5.5 mL of metaphosphoric–acetic acids TS and 15 mL of water. From the ascorbic acid equivalent of the standard dichlorophenol–indophenol solution, calculate the content of ascorbic acid in each Tablet.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid (C₆H₈O₆)

• ASCORBIC ACID, Method 2: Proceed as directed in *Automated Methods of Analysis* <16>, *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid (C₆H₈O₆)

• CALCIUM ASCORBATE, Method 1: Proceed as directed in *Ascorbic Acid, Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate (C₁₂H₁₄CaO₁₂ · 2H₂O)

• CALCIUM ASCORBATE, Method 2: Proceed as directed in *Automated Methods of Analysis* <16>, *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate (C₁₂H₁₄CaO₁₂ · 2H₂O)

- **SODIUM ASCORBATE, Method 1:** Proceed as directed in *Ascorbic Acid, Method 1*.
Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)
- **SODIUM ASCORBATE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Ascorbic Acid*.
Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)
- **BIOTIN, Method 1**
[NOTE—Use low-actinic glassware throughout this procedure.]
Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, 1 mL of phosphoric acid, and dilute with water to 1000 mL.
Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide
Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water
Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 1 mg of biotin, to a 200-mL volumetric flask, add 3 mL of dimethyl sulfoxide, and swirl to wet. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.
Chromatographic system
(See *Chromatography* (621), *System Suitability*.)
Mode: LC
Detector: UV 200 nm
Column: 4.6-mm × 15-cm; 3-µm packing L7
Flow rate: 1.2 mL/min
Injection size: 100 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 3.0%
Analysis
Samples: *Standard solution* and *Sample solution*
Measure the responses for the biotin peaks. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak response from the *Sample solution*
 r_S = peak response from the *Standard solution*
 C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)
 C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

- Acceptance criteria:** 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)
- **BIOTIN, Method 2**
[NOTE—Use low-actinic glassware throughout this procedure.]
Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.
Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.
Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by dilution of the *Standard stock solution* with water on the day of the assay.
Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate, quantitatively and stepwise if necessary, with water to obtain a solution with a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to *Table 1*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add

10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an inoculum comparable to a fresh culture.] Make a transfer of cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of culture medium. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*
To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength from 540 to 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (ΣS) as the difference, $y = 2.00 - \Sigma S$. Plot this response on the ordinate of cross-

section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma U$, adding together the two transmittances for each level of the *Sample solution* (ΣU). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of biotin in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of biotin that was assumed to be present in the portion of the Tablets taken

Calculate the percentage of the labeled amount of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$) in the portion of the Tablets taken

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin in the portion of the Tablets taken (μg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$)

• BIOTIN, Method 3

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 800 mL of water and 100 mL of triethylamine to a 1000-mL volumetric flask. Add 80 mL of 85% phosphoric acid, and dilute with water to volume.

Mobile phase: Transfer 80 mL of acetonitrile and 10 mL of *Solution A* to a 1000-mL volumetric flask. Dilute with water to volume.

Standard solution: 0.6 $\mu\text{g}/\text{mL}$ of USP Biotin RS in water. [NOTE—A portion of the *Standard solution* will be used to determine the percent recovery of biotin from the *Solid-phase extraction* procedure.]

Sample solution: Finely powder NLT 20 Tablets.

Transfer an amount of powdered Tablets to a volumetric flask to obtain a concentration of 0.6 $\mu\text{g}/\text{mL}$ of biotin. Add water up to 80% of the flask capacity, sonicate for 30–40 min, with occasional mixing, to dissolve. Dilute with water to volume, and filter. Adjust the pH of the solution with either dilute acetic acid or 0.1 N sodium hydroxide to 6.0–7.0.

Solid-phase extraction: [NOTE—Condition the extraction column specified in this procedure in the following manner. Wash the column with a 2-mL portion of methanol. Equilibrate with a 2-mL portion of water.] Separately pipet 5.0 mL of the *Sample solution* and *Standard solution* into freshly conditioned solid-phase extraction columns consisting of a mixed-mode packing

¹ ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

with a sorbent-mass of 60 mg. [NOTE—The mixed-mode packing consists of anion-exchange and reversed-phase sorbents. The reverse-phase component is a polymer of copolymer *N*-vinylpyrrolidone and divinylbenzene. The anion exchange moiety is a trialkylamino group.²] Wash the column with 10 mL of 30% methanol in water. Apply an appropriate volume (4.9 mL) of 30% methanol in 0.1 N hydrochloric acid to the column. Collect the eluate in a 5-mL volumetric flask, containing 100 µL of 40% (w/v) sodium acetate in water, and dilute with 30% methanol in 0.1 N hydrochloric acid to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 100 µL

System suitability

Samples: *Standard solution* and portion of *Standard solution* that has undergone *Solid-phase extraction*

Suitability requirements

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution* and the *Standard solution* that has undergone *Solid-phase extraction*

Recovery: 95%–100%, *Standard solution* that has undergone *Solid-phase extraction*

Analysis

Samples: *Standard solution* and *Sample solution* that have both undergone *Solid-phase extraction*

Measure the responses for the biotin peak. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• CYANOCOBALAMIN, Method 1

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 µg/mL of USP Cyanocobalamin RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

Standard solution: 1 µg/mL of USP Cyanocobalamin RS from *Standard stock solution* diluted with water

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to 100 µg of cyanocobalamin, to a 250-mL flask. Quantitatively add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

² A suitable cartridge is the Waters, Oasis MAX Vac RC cartridge, particle size 30 µm, part 186000371.

Mode: LC

Detector: 550 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 0.5 mL/min

Injection size: 200 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for cyanocobalamin.

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Cyanocobalamin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• CYANOCOBALAMIN, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of USP

Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Finely powder NLT 20 Tablets.

Transfer a portion of the powdered Tablets, equivalent to 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each g of powdered Tablets taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B₁₂ activity equivalent to the nominal activity of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Biotin, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed in *Biotin, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL,

and add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in Table 2, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions to the resulting solution. Add 100 mL of water, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of between 5.5 and 6.0, and add *Purified Water* to make 250 mL.

Table 2

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of

water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.³ [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.]

Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Make a transfer of cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL of the *Culture medium* each. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. The cell suspension so obtained is the *Inoculum*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.]

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*
Because of the high sensitivity of the test organism to minute amounts of vitamin B₁₂ activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes, and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

³ Pure cultures of *Lactobacillus leichmannii* may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within $\pm 0.5^\circ$, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating its contents, and read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances for each level of the *Sample solution* (Σ_u). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of cyanocobalamin in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of cyanocobalamin that was assumed to be present in the portion of Tablets taken

Calculate the percentage of the labeled amount of cyanocobalamin in the portion of the Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of the Tablets taken (μg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Vitamin B₁₂ Activity in Design and*

Analysis of Biological Assays (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• Folic Acid, Method 1

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use.

[NOTE—The methanol and water content may be varied (between 1% and 3%) by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation.]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask, and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8, bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of USP Folic Acid RS in *Internal standard solution*

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of powder, equivalent to 0.4 mg of folic acid, to a 50-mL amber-colored centrifuge tube. Add 25.0 mL of *Internal standard solution*, shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm \times 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 15 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*

R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of folic acid in the
Sample solution ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$)

• **FOLIC ACID, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: Dissolve 7.5 g of edetate disodium, with stirring, in 500 mL of water containing 10 mL of ammonium hydroxide.

Diluent: 60 $\mu\text{g/mL}$ of ammonium hydroxide

Mobile phase: Transfer 0.4 mL of triethylamine, 15 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 60 $\mu\text{g/mL}$ of USP Folic Acid RS in Diluent. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of Standard stock solution with 10.0 mL of methanol and 35.0 mL of Reagent, and shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Transfer a portion of finely powdered Tablets, equivalent to 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of methanol and 35.0 mL of Reagent. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See Chromatography <621>, System Suitability.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm \times 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 5 μL

System suitability

Sample: Standard solution

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: Standard solution and Sample solution
Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of folic acid from the Sample solution

r_S = peak area of folic acid from the Standard solution

C_S = concentration of USP Folic Acid RS in the Standard solution ($\mu\text{g/mL}$)

C_U = nominal concentration of folic acid in the Sample solution ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$)

• **CALCIUM PANTOTHENATE, Method 1**

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in Internal standard solution

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 15 mg of calcium pantothenate, to a centrifuge tube. Add 25.0 mL of the Internal standard solution, and shake vigorously for 10 min. Centrifuge, filter, and use the clear filtrate.

Chromatographic system

(See Chromatography <621>, System Suitability.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm \times 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 10 μL

System suitability

Sample: Standard solution

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: Standard solution and Sample solution

Measure the peak responses for calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the Sample solution

R_S = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the Standard solution

C_S = concentration of USP Calcium Pantothenate RS in the Standard solution (mg/mL)

C_U = nominal concentration of calcium pantothenate in the Sample solution (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$)

• **CALCIUM PANTOTHENATE, Method 2**

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume, to obtain a concentration of 50 $\mu\text{g/mL}$ of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of Standard stock solution with water to obtain a concentration of 0.01–0.04 $\mu\text{g/mL}$ of calcium pantothenate, the exact concentration being such that the responses obtained as directed in the Analysis, 2.0 and 4.0 mL of the Standard solution being used, are within the linear portion of the log-concentration response curve.

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution having approximately the same concentration as that of the Standard solution.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 \pm 0.1, and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to

70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to Table 3, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water; add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar; and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*⁴ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Make a transfer of cells from the *Stock culture* of *Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*
To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of *Standard* and *sample* tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of *Standard* during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the *Standard*, calculate the response from the sum of the duplicate values of the transmittance (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points. Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances for each level of the *Sample solution* (Σ_u). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the *Standard*. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in mg, of calcium

⁴ ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate that was assumed to be present in the portion of the Tablets taken

Calculate the percentage of the labeled amount of calcium pantothenate in the portion of the Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of the Tablets taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE**, *Method 3*

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 μ g/mL of USP Calcium Pantothenate RS from *Standard stock solution* diluted with water

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse. Dilute with water to volume, mix, and filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 3.9-mm \times 30-cm; 5- μ m packing L1

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 25 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for calcium pantothenate.

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **NIACIN or NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE**, *Method 1*

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS, to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm \times 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for niacinamide, pyridoxine, riboflavin, and thiamine are about 0.3, 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin or niacinamide, pyridoxine, riboflavin, and thiamine. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*

r_S = peak area of niacinamide from the *Standard solution*

C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

For formulations containing niacin:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*

r_S = peak area of niacin from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of the corresponding vitamin from the *Sample solution*
 r_S = peak area of the corresponding vitamin from the *Standard solution*
 C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)
 C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

- r_U = peak area of thiamine from the *Sample solution*
 r_S = peak area of thiamine from the *Standard solution*
 C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
 C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
 M_{r1} = molecular weight of thiamine mononitrate, 327.36
 M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$) or niacin ($C_6H_5NO_2$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.

Extraction solvent: *Solution A* and methanol (3:1)

Mobile phase: 0.1 M sodium acetate solution (13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]

Standard stock solution: 1 mg/mL of USP Niacin RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.

Sample solution: [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine, and riboflavin, when present in the formulation.] Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 2 mg of riboflavin, to a 200-mL volumetric flask. If riboflavin is not present in the formulation, transfer a portion of the powder, equivalent to 2 mg of pyridoxine. If pyridoxine is not present in the formulation, transfer a portion of the powder, equivalent to 20 mg of niacin or niacinamide. Add 100.0 mL of *Extraction solvent*, and mix for 20 min, using a wrist-action shaker. Immerse the flask in a water

bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

[NOTE—If necessary, flush the column with methanol between injections.]

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area from the *Sample solution*
 r_S = peak area from the *Standard solution*
 C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)
 C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

• **NIACINAMIDE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A, Extraction solvent, Mobile phase, Standard stock solution, Standard solution, Sample solution, Chromatographic system, and System suitability: Using USP Niacinamide RS in place of USP Niacin RS, proceed as directed for *Niacin, Method 2*.

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacinamide. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area from the *Sample solution*
 r_S = peak area from the *Standard solution*
 C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)
 C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, and Sample

solution: Prepare as directed for *Niacin, Method 2*.

Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent and Sample solution: Prepare as directed for *Niacin, Method 2*.

Solution A: 6.8 mg/mL of sodium acetate in water

Mobile phase: Prepare a mixture of *Solution A* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.

Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.

Standard solution: Dilute 5.0 mL of *Standard stock solution* with *Extraction solvent* to 25.0 mL.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for riboflavin. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **THIAMINE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: 1.88 g/L of sodium 1-hexanesulfonate in 0.1% phosphoric acid

Mobile phase: *Solution A* and acetonitrile (46:9)

Standard stock solution: 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid

Standard solution: 0.02 mg/mL of USP Thiamine Hydrochloride RS from *Standard stock solution* diluted with 0.2 N hydrochloric acid

Sample solution: Weigh and finely powder NLT 20 Tablets. Mix a portion of the Tablets' powder with a volume of 0.2 N hydrochloric acid to obtain a concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas for the major peaks. For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN or NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 3**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in the *Reagent*, with heating if necessary

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2.0 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the peaks. Calculate the percentage of the labeled amount of niacin (C₆H₅NO₂) or niacinamide (C₆H₆N₂O) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*

r_S = peak area of niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl), riboflavin (C₁₇H₂₀N₄O₆), and thiamine hydrochloride (C₁₂H₁₇ClN₄OS · HCl) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

r_S = peak area of the corresponding vitamin from the *Standard solution*

C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine

mononitrate (C₁₂H₁₇N₅O₄S) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide (C₆H₆N₂O) or niacin (C₆H₅NO₂), pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl), riboflavin (C₁₇H₂₀N₄O₆), and thiamine as thiamine hydrochloride (C₁₂H₁₇ClN₄OS · HCl) or thiamine mononitrate (C₁₂H₁₇N₅O₄S)

[NOTE—Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a *Standard stock solution* is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the *Standard solutions* and the *Sample solution* may be diluted quantitatively with the solvent specified, if necessary, to yield solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

• CALCIUM, Method 1

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively, with 0.125 N hydrochloric acid, to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Calcium emission line at 422.7 nm

Lamp: Calcium hollow-cathode
Flame: Nitrous oxide–acetylene
Blank: 0.125 N hydrochloric acid containing 1 mL of Lanthanum chloride solution per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions, against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, Method 1**

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Proceed as directed for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 1 µg/mL of chromium and to omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Chromium emission line at 357.9 nm

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, Method 1**

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% solution of nitric acid, and dilute with a 1% solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

Standard stock solution: 100 µg/mL of copper from *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Proceed as directed for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 2 µg/mL of copper and to omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Copper emission line at 324.7 nm

Lamp: Copper hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)

C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **FLUORIDE, Method 1**

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water contained in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 µg/mL of fluoride from a quantity of sodium fluoride, previously dried at 100° for 4 h and cooled in a desiccator, in water

Intermediate stock solution A: 100 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of 3 M *sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 µg/mL of fluoride.

Sample solution: Transfer a quantity of the finely powdered Tablets, equivalent to 200 µg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of 3 M *sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*, and dilute with water to volume.

Analysis

Samples: *Standard solutions* and *Sample solution*

To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see *pH* <791>), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ± 0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer having an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.]

Plot the logarithms of fluoride concentrations, in $\mu\text{g/mL}$, of the *Standard solutions* versus potential, in mV. From the standard response curve so obtained and the measured potential of the *Sample solution*, determine the concentration, C , in $\mu\text{g/mL}$, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of fluorine in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **FLUORIDE, Method 2**

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 $\mu\text{g/mL}$ of USP Sodium Fluoride RS in water. This solution contains 100 $\mu\text{g/mL}$ of fluoride.

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with one column volume of methanol followed by one column volume of *pH 10.0 buffer*. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.] Transfer 10.0 mL of *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ± 0.1 . Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask, add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with *pH 10.0 buffer* to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonfylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of powdered Tablets, equivalent to 1 mg of fluorine, in 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1 , and dilute with water to 100 mL. Proceed as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Conductivity

Guard column: 4.6-mm \times 3-cm; packing L17

Analytical column: 7.8-mm \times 30-cm; packing L17

Flow rate: 0.5 mL/min

Injection size: 100 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of fluorine in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE, Method 1**

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis

Sample: Tablets

Transfer an amount of finely powdered Tablets, equivalent to 3 mg of iodide, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500° , and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.] Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20° . Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared. Calculate the percentage of the labeled amount of iodine (I) in the portion of Tablets taken:

$$\text{Result} = V \times N_A \times F \times Ime \times (Aw/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)

N_A = actual normality of the sodium thiosulfate solution used

F = correction factor to convert mg to μg (1000 $\mu\text{g/mL}$)

Ime = milliequivalent of I (21.16 mg/meq)

Aw = average weight of the Tablets

W = weight of the portion of Tablets taken

L = labeled amount of iodine ($\mu\text{g/Tablet}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

- **IODIDE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Iodide*.

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

- **IRON, Method 1**

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Sample solution: Proceed as directed for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a concentration of 5 µg/mL of iron and to omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Iron emission line at 248.3 nm

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (µg/mL)

C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

- **MAGNESIUM, Method 1**

Lanthanum chloride solution: Prepare as directed in *Calcium, Method 1*.

Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a known concentration of 1000 µg/mL of magnesium.

Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.

Sample solution: Proceed as directed for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a concentration of 0.4 µg/mL of magnesium.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Magnesium emission line at 285.2 nm

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)

C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the label claim

- **MANGANESE, Method 1**

Manganese standard stock solution: Transfer 1.00 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of manganese.

Standard stock solution: 50 µg/mL of manganese from *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Sample solution: Proceed as directed for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a concentration of 1 µg/mL of manganese and to omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Manganese emission line at 279.5 nm

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)

C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

- **MOLYBDENUM, Method 1**

Diluent: 20 mg/mL of ammonium chloride in water

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary.

Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of molybdenum.

Standard stock solution: 100 µg/mL of molybdenum from *Molybdenum standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 µg/mL of molybdenum.

Sample solution: Transfer a portion of the powder, equivalent to 1000 µg of molybdenum, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min, or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid, heat until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Quantitatively transfer the contents of the flask to a 100-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Molybdenum emission line at 313.3 nm

Lamp: Molybdenum hollow-cathode

Flame: Nitrous oxide-acetylene

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* (µg/mL)

C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• MOLYBDENUM, Method 2

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% stannous chloride solution: Transfer 40 mg of stannous chloride to a beaker, add 20 mL of 6.5 N hydrochloric acid solution, and heat the solution until the stannous chloride is dissolved. Cool and dilute with water to 100 mL.

Diluted stannous chloride solution: 20% *stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.

Standard solution: 20 µg/mL of molybdenum in water

Sample: A portion of finely powdered Tablets, equivalent to 40 µg of molybdenum

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Vis

Analytical wavelength: 465 nm

Cell: 1 cm

Blank: Amyl alcohol

Analysis

Samples: *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to complete 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of 20% *Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layer from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the *Sample*

A_S = absorbance of the *Standard solution*

V = volume of the *Standard solution* analyzed, 2.0 mL

C_S = concentration of molybdenum in the *Standard solution* (µg/mL)

M_U = nominal amount of molybdenum in the *Sample* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• PHOSPHORUS, Method 1

Sulfuric acid solution: Cautionously add sulfuric acid to water (37.5:100), and mix.

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, and then dilute with *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add one drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to

volume, and mix to obtain a solution with a concentration of 1000 µg/mL of phosphorus.

Standard solution: 20 µg/mL of phosphorus from *Phosphorus standard stock solution* diluted with water

Sample solution: [NOTE—Finely powder and weigh a counted number of Tablets.] Transfer a portion of the powder, equivalent to 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Vis

Analytical wavelength: 650 nm

Cell: 1 cm

Analysis

Samples: *Standard solution* and *Sample solution*

To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

C_S = concentration of phosphorus in the *Standard solution* (µg/mL)

C_U = nominal concentration of phosphorus in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• POTASSIUM

Potassium standard solution: 100 µg/mL of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 µg/mL of potassium from *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of potassium.

Sample solution: Proceed as directed for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a concentration of 1 µg/mL of potassium and to omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Potassium emission line at 766.5 nm

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the

concentration, C , in µg/mL, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* (µg/mL)

C_U = nominal concentration of potassium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• SELENIUM, Method 1

Diluent: Prepare as directed in *Molybdenum, Method 1*.

Selenium standard solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 µg/mL of selenium.

Standard stock solution: 100 µg/mL of selenium from *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions with concentrations of 5.0, 10.0, and 25.0 µg/mL of selenium.

Sample solution: Transfer a portion of the powder, equivalent to 1000 µg of selenium, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Selenium emission line at 196.0 nm

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* (µg/mL)

C_U = nominal concentration of selenium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **SELENIUM, Method 2**

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, then add hydroxylamine hydrochloride, and dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 µg/mL of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10 mL of the *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Transfer a portion of finely powdered Tablets, equivalent to 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. [CAUTION—Exercise care at this stage, because the perchloric acid reaction becomes vigorous.]

Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask, add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV

Analytical wavelength: 380 nm

Cell: 1 cm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*
Treat the *Sample solution*, the *Standard solution*, and the *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with 50% *Ammonium hydroxide solution* to a pH of 1.1 ± 0.1. Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels.

Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*. Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbances of the cyclohexane layer from the *Sample solution*

A_S = absorbances of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• **ZINC, Method 1**

Zinc standard solution: 1000 µg/mL of zinc from zinc oxide in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from *Zinc standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Proceed as directed for the *Sample solution* in *Calcium, Method 1*, except to prepare the *Sample solution* to contain a concentration of 2 µg/mL of zinc and to omit the use of the *Lanthanum chloride solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Zinc emission line at 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

- **BORON, NICKEL, TIN, and VANADIUM**, *Method 1*; **CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS, and ZINC**, *Method 2*; **MOLYBDENUM and SELENIUM**, *Method 3*

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding one volume of *Stock aqua regia solution* to two volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% nitric acid solution, 1000 mg/L of scandium in 5% nitric acid solution, and *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% nitric acid solution to obtain a solution having final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/L of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% hydrochloric acid solution to obtain a solution having final concentrations of about 200 mg/L of boron, and 100 mg/L each of chromium, molybdenum, nickel, selenium, tin, and vanadium.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent* to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution 1 (for Tablets containing minerals found in *Standard stock solution 1* and *Standard stock solution 2*): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a 5- μ m pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Sample solution 2 (for Tablets containing minerals found only in *Standard stock solution 2*): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a 5- μ m pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Sample solution 3 (for Tablets containing minerals found only in *Standard stock solution 1*): Weigh and

finely powder NLT 20 Tablets. Transfer a portion, equal to the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently (about 1 h) until fumes cease. Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a 5- μ m pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Instrumental conditions

(See *Plasma Spectrochemistry* <730>.)

Mode: Inductively coupled plasma spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

[NOTE—Analyze the *System suitability solution*, and obtain the response as directed in the *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*. Calculate the percentage of the labeled amount for each mineral taken:

$$\text{Result} = C \times (V/W) \times F \times (C_w/L) \times 100$$

C = measured concentration of the relevant element in the *Sample solution* (mg/L)

V = volume of the *Sample solution* (L)

W = sample weight (mg)

F = dilution factor of the *Sample solution*

C_w = average Tablet weight (mg)

L = labeled amount per Tablet (mg)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); and 90.0%–160.0% of the labeled amounts of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** <2040>: Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS** <2021>: The total aerobic microbial count does not exceed 3000 cfu/g, and the combined molds and yeasts count does not exceed 300 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** <2022>: Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:**⁵ The label states that the product is Oil- and Water-Soluble Vitamins with Minerals Tablets. The label also states the quantity of each vitamin and mineral per dosage unit and where necessary the chemical form in which a vitamin is present and also states the salt form of the mineral used as the source of each element. Where the product contains vitamin E, the label indicates whether it is the D- or DL- form. Where more than one assay method is given for a particular vitamin, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** (11)
 - USP Alpha Tocopherol RS
 - USP Alpha Tocopheryl Acetate RS
 - USP Alpha Tocopheryl Acid Succinate RS
 - USP Biotin RS
 - USP Calcium Pantothenate RS
 - USP Cholecalciferol RS
 - USP Cyanocobalamin RS
 - USP Ergocalciferol RS
 - USP Folic Acid RS
 - USP Niacin RS
 - USP Niacinamide RS
 - USP Phytonadione RS
 - USP Pyridoxine Hydrochloride RS
 - USP Riboflavin RS
 - USP Sodium Fluoride RS
 - USP Thiamine Hydrochloride RS
 - USP Vitamin A RS

Water-Soluble Vitamins Capsules

DEFINITION

Water-Soluble Vitamins Capsules contain two or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Dexpantenol or Panthenol, Pantothenic Acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Niacin or Niacinamide, Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate. Capsules contain NLT 90.0% and NMT 150.0% of the labeled amounts of ascorbic acid ($C_6H_8O_6$), biotin ($C_{10}H_{16}N_2O_3S$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), dexpantenol ($C_9H_{19}NO_4$) or panthenol ($C_9H_{19}NO_4$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate.

⁵ USP Units of activity for vitamins, where such exist or formerly existed, are equivalent to the corresponding international units, where such formerly existed. The USP Unit for Vitamin E has been discontinued. International units (IU) for vitamins also have been discontinued; however, the use of IU on the labels of vitamin products continues. Where articles are labeled in terms of Units in addition to the required labeling, the relationship of the USP Units or IU to mass is as follows. One USP Vitamin A Unit = 0.3 μ g of all-*trans*-retinol (vitamin A alcohol) or 0.344 μ g of all-*trans*-retinyl acetate (vitamin A acetate) or 0.55 μ g of all-*trans*-retinyl palmitate (vitamin A palmitate), and 1 μ g of retinol (3.3 USP Vitamin A Units) = 1 retinol equivalent (RE); 1 IU of beta carotene = 0.6 μ g of all-*trans*-beta carotene; 1 USP Vitamin D Unit = 0.025 μ g of ergocalciferol or cholecalciferol; and 1 mg of *dl*-alpha tocopherol = 1.1 former USP Vitamin E Units, 1 mg of *dl*-alpha tocopheryl acetate = 1 former USP Vitamin E Unit, 1 mg of *dl*-alpha tocopheryl acid succinate = 0.89 former USP Vitamin E Unit, 1 mg of *d*-alpha tocopherol = 1.49 former USP Vitamin E Units, and 1 mg of *d*-alpha tocopheryl acetate = 1.36 former USP Vitamin E Units, 1 mg of *d*-alpha tocopheryl acid succinate = 1.21 former USP Vitamin E Units. In terms of *d*-alpha tocopherol equivalents, 1 mg of *d*-alpha tocopheryl acetate = 0.91, 1 mg of *d*-alpha tocopheryl acid succinate = 0.81, 1 mg of *dl*-alpha tocopherol = 0.74, 1 mg of *dl*-alpha tocopheryl acetate = 0.67, and 1 mg of *dl*-alpha tocopheryl acid succinate = 0.60.

They do not contain any form of Beta Carotene or Vitamin A, D, E, or K. They do not contain any minerals for which nutritional value is claimed. They may contain other labeled added substances in quantities that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

- **ASCORBIC ACID, Method 1**

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 100 mg of ascorbic acid, to a 200-mL volumetric flask, and add 75 mL of metaphosphoric-acetic acids TS. Insert a stopper into the flask, and shake by mechanical means for 30 min. Dilute with water to volume. Transfer a portion of the solution to a centrifuge tube, and centrifuge until a clear supernatant is obtained. Pipet 4.0 mL of this solution into a 50-mL conical flask, and add 5 mL of metaphosphoric-acetic acids TS.

Analysis: Titrate with standard dichlorophenol-indophenol solution VS to a rose-pink color that persists for at least 5 s. Correct for the volume of dichlorophenol-indophenol solution consumed by a mixture of 5.5 mL of metaphosphoric-acetic acids TS and 15 mL of water. From the ascorbic acid equivalent of the standard dichlorophenol-indophenol solution, calculate the content of ascorbic acid in each Capsule.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **ASCORBIC ACID, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), Assay for Ascorbic Acid.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **CALCIUM ASCORBATE, Method 1:** Proceed as directed in *Ascorbic Acid, Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

- **CALCIUM ASCORBATE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), Assay for Ascorbic Acid.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

- **SODIUM ASCORBATE, Method 1:** Proceed as directed in *Ascorbic Acid, Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **SODIUM ASCORBATE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), Assay for Ascorbic Acid.

Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **BIOTIN, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 μ g/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight

per Capsule.” Transfer a portion of the Capsule contents, equivalent to a nominal amount of 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-μm packing L7

Flow rate: 1.2 mL/min

Injection size: 100 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of biotin. Calculate the percentage of the labeled amount of biotin (C₁₀H₁₆N₂O₃S) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of biotin from the *Sample solution*

r_S = peak area of biotin from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (μg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin (C₁₀H₁₆N₂O₃S)

• BIOTIN, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used, provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 μg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by dilution of the *Standard stock solution* with water on the day of the assay.

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 100 μg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution having a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved.

Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 μg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 μg/mL of riboflavin and 10 μg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 μg/mL of p-aminobenzoic acid, 50 μg/mL of niacin, and 40 μg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution: Dissolve anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to *Table 1*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 1

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

¹ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*
To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of *Standard* and *sample* tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$ until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of *Standard* during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength from 540 to 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the *Standard*, calculate the response from the sum of the duplicate values of the transmittance (ΣS) as the difference, $y = 2.00 - \Sigma S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response $y = 2.00 - \Sigma U$, adding together the two transmittances (ΣU) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of lowest and highest points plotted for the *Standard*. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, x , for each dosage level. Average the values of x for each of three or more dosage levels to obtain \bar{x} , which equals the log-relative potency, M' , of

the *Sample solution*. Determine the quantity, in μg , of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of biotin assumed to be present in the portion of Capsules taken

Calculate the percentage of the labeled amount of biotin in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$) in the portion of Capsules taken (μg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$)

• CYANOCOBALAMIN, Method 1

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 $\mu\text{g}/\text{mL}$ of USP

Cyanocobalamin RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

Standard solution: 1 $\mu\text{g}/\text{mL}$ of USP Cyanocobalamin RS from the *Standard stock solution* diluted with water

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without the loss of shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Transfer a portion of the Capsule contents, equivalent to 100 μg of cyanocobalamin, to a 250-mL flask. Add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the clear filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm \times 15-cm; 5- μm packing L1

Flow rate: 0.5 mL/min

Injection size: 200 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas of cyanocobalamin. Calculate the percentage of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of cyanocobalamin from the *Sample solution*

r_S = peak area of cyanocobalamin from the *Standard solution*

- C_s = concentration of USP Cyanocobalamin RS in the *Standard solution* ($\mu\text{g/mL}$)
 C_u = nominal concentration of cyanocobalamin in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• **CYANOCOBALAMIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 $\mu\text{g/mL}$ of USP Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of the *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 1.0 μg of cyanocobalamin, to an appropriate vessel containing, for each g of Capsule contents taken, 25 mL of an aqueous extracting solution prepared just before use to contain 12.9 mg/mL of dibasic sodium phosphate, 11.0 mg/mL of anhydrous citric acid, and 10 mg/mL of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B₁₂ activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Calcium Pantothenate, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed in *Calcium Pantothenate, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 μg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used, provided that, when constituted as

directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed in *Table 2*, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions to the resulting solution. Add 100 mL of water, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH between 5.5 and 6.0, and add Purified Water to make 250 mL.

Table 2

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N Hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter-aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter-aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used, provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.² [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.]

Incubate for 16–24 h at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$. Store in a refrigerator.

²Pure cultures of *Lactobacillus leichmannii* may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture* of *Lactobacillus leichmannii* to two sterile tubes containing 10 mL of the *Culture medium* each. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$. Under aseptic conditions, centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension, using sterile *Basal medium stock solution*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.] The cell suspension so obtained is the *Inoculum*.

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water, with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*
Because of the high sensitivity of the test organism to minute amounts of vitamin B₁₂ activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm \times 150-mm test tubes and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within $\pm 0.5^\circ$, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating its contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains

constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances (Σ_u) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, x , for each dosage level. Average the values of x for each of three or more dosage levels to obtain \bar{x} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of cyanocobalamin in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of cyanocobalamin that was assumed to be present in the portion of Capsules taken

Calculate the percentage of the labeled amount of cyanocobalamin in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of Capsules taken (μg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Vitamin B₁₂ Activity in Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• FOLIC ACID, Method 1

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or

ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation. [NOTE—The methanol and water content may be varied (between 1% and 3%).]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask, and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8, bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of USP Folic Acid RS in *Internal standard solution*

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer an amount of Capsule contents to a suitable centrifuge tube, and add a volume of *Internal standard solution* to obtain a nominal concentration of 0.016 mg/mL of folic acid. Shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 15 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*

R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• Folic Acid, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Diluent: 60 µg/mL of ammonium hydroxide

Standard stock solution: 60 µg/mL of USP Folic Acid RS in *Diluent*. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of *Standard stock solution* with 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Shake for 15 min

in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed for the *Sample solution* in *Ascorbic Acid, Method 1*, through “calculate the net average weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of folic acid from the *Sample solution*

r_S = peak area of folic acid from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• DEXPANTHENOL or PANTHENOL

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used, provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 µg/mL of USP Dexpantenol RS or 1600 µg/mL of USP Racemic Panthenol RS in water. Store in a refrigerator, protected from light, and use within 30 days.

Standard solution: On the day of the assay, prepare a dilution of 1.2 µg/mL of dexpantenol or 2.4 µg/mL of panthenol from *Standard stock solution* diluted with water.

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents as completely as possible to a beaker. Remove any contents adhering to the empty Capsule shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight per Capsule. Dissolve a portion of the Capsule contents, equivalent to 1.2 mg of dexpantenol or 2.4 mg of panthenol, in 100.0 mL of water. Quantitatively dilute a portion of this solution with water to obtain a concentration of 1.2 µg/mL of dexpantenol or 2.4 µg/mL of panthenol.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to $75 \pm 5^\circ$, and add hydrochloric acid solution (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine-guanine-uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin-thiamine hydrochloride-biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in neutral 25% alcohol. Store in a refrigerator.

Salt solution A: 50 mg/mL of monobasic potassium phosphate and 50 mg/mL of dibasic potassium phosphate in water. Add 10 drops of hydrochloric acid per L of solution. Store under toluene.

Salt solution B: 20 mg/mL of magnesium sulfate, 1 mg/mL of sodium chloride, 1 mg/mL of ferrous sulfate, and 1 mg/mL of manganese sulfate in water. Add 10 drops of hydrochloric acid per L of the solution. Store under toluene.

Pyridoxal-calcium pantothenate solution: 200 µg/mL of pyridoxal hydrochloride and 1.875 µg/mL of calcium pantothenate in 10% alcohol. Store in a refrigerator, and use within 30 days.

Polysorbate 40-oleic acid solution: 50 mg/mL of polysorbate 40 and 0.5 mg/mL of oleic acid in 20% alcohol. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium: Dissolve anhydrous dextrose and sodium acetate in the solutions previously mixed according to Table 3, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Double-strength modified pantothenate medium:

Prepare as directed in *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH of 6.5–6.6, and dilute with water to 1000 mL. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*³ on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of sterile *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with sterile *Modified pantothenate medium*. Resuspend the cells in enough sterile *Modified pantothenate medium* that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to sterile glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes and in the same order, add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*.

Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes, and in the same order, add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature NLT 80°, such as by steaming at atmospheric pressure in a sterilizer for 5–10 min. Cool, and determine the percentage transmittance of the suspensions, in cells of equal path length, on a suitable spectrophotometer, at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percentage of transmittance, for each set of tubes of the standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency of each tube containing portions of the *Sample solution*. To obtain the individual responses, divide the potency of each tube by the amount of the *Sample solution* added to it. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the material taken for assay by multiplying the mean response by the appropriate dilution factor.

Table 3

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL
Riboflavin-thiamine hydrochloride-biotin solution	5 mL
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal-calcium pantothenate solution	5 mL
Polysorbate 40-oleic acid solution	5 mL

³ATCC No. 8042 is suitable.

Calculate the percentage of the labeled amount of dexpantenol or panthenol in the portion of Capsules taken:

$$\text{Result} = (P/N) \times 100$$

P = potency of dexpantenol or panthenol in the portion of Capsules taken (mg)

N = nominal amount of dexpantenol or panthenol in the portion of Capsules taken (mg)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpantenol or panthenol ($\text{C}_9\text{H}_{19}\text{NO}_4$)

• **CALCIUM PANTOTHENATE, Method 1**

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” To a centrifuge tube, transfer an amount of mixed Capsule contents and a volume of *Internal standard solution* to obtain a nominal concentration of 0.6 mg/mL in the *Sample solution*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm \times 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak area ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{CaN}_2\text{O}_{10}$)

• **CALCIUM PANTOTHENATE, Method 2**

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 μg /mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 μg /mL of calcium panto-

thenate, the exact concentration being such that the responses obtained as directed in the *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to a nominal amount of 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution having approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine-guanine-uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin-thiamine hydrochloride-biotin solution: 20 μg /mL of riboflavin, 10 μg /mL of thiamine hydrochloride, and 0.04 μg /mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution:** 10 μg /mL of *p*-aminobenzoic acid, 50 μg /mL of niacin, and 40 μg /mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution: Dissolve anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed according to *Table 4*, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Table 4

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL

Table 4 (Continued)

Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: Add 5.0 mL of water containing 0.2 µg of calcium pantothenate to each of a series of test tubes containing 5.0 mL of *Basal medium stock solution*. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Transfer cells from the of *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of *Standard* and *sample* tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5°, until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of *Standard* during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining

tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the *Standard*, calculate the response from the sum of the duplicate values of the transmittance (Σ_s) as the difference $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response $y = 2.00 - \Sigma_u$, adding together the two transmittances for each level of the *Sample solution* (Σ_u). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the *Standard*. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, x , for each dosage level. Average the values of x for each of three or more dosage levels to obtain \bar{x} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in mg, of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate assumed to be present in the portion of Capsules taken

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Capsules taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• CALCIUM PANTOTHENATE, Method 3

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from the *Standard stock solution* diluted with water

Sample solution: Proceed as directed for the *Sample solution* in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to a nominal amount of 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse the Capsule contents. Dilute with water to volume, mix, and filter.

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 205 nm**Column:** 3.9-mm × 30-cm; 5-μm packing L1**Column temperature:** 50°**Flow rate:** 2 mL/min**Injection size:** 25 μL**System suitability****Sample:** *Standard solution***Suitability requirements****Relative standard deviation:** NMT 3.0%**Analysis****Samples:** *Standard solution* and *Sample solution*

Measure the peak areas of calcium pantothenate.

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak area of calcium pantothenate from the *Sample solution* r_S = peak area of calcium pantothenate from the *Standard solution* C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL) C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)**Acceptance criteria:** 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)• **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL**Diluent:** Acetonitrile, glacial acetic acid, and water (5:1:94)**Standard solution:** [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or on a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.**Sample solution:** Proceed as directed in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix on a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 280 nm**Column:** 3.9-mm × 30-cm; packing L1**Flow rate:** 1 mL/min**Injection size:** 10 μL**System suitability****Sample:** *Standard solution*

[NOTE—The relative retention times for niacinamide, pyridoxine, riboflavin, and thiamine are about 0.3, 0.5, 0.8, and 1.0, respectively.]

Suitability requirements**Relative standard deviation:** NMT 3.0%**Analysis****Samples:** *Standard solution* and *Sample solution*Measure the peak areas for niacin or niacinamide, pyridoxine, riboflavin, and thiamine. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak area of niacinamide from the *Sample solution* r_S = peak area of niacinamide from the *Standard solution* C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL) C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

For formulations containing niacin:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak area of niacin from the *Sample solution* r_S = peak area of niacin from the *Standard solution* C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL) C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak area of the corresponding vitamin from the *Sample solution* r_S = peak area of the corresponding vitamin from the *Standard solution* C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL) C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

 r_U = peak area of thiamine from the *Sample solution* r_S = peak area of thiamine from the *Standard solution* C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL) C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL) M_{r1} = molecular weight of thiamine mononitrate, 327.36 M_{r2} = molecular weight of thiamine hydrochloride, 337.27**Acceptance criteria:** 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$) or niacin ($C_6H_5NO_2$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin

(C₁₇H₂₀N₄O₆), and thiamine as thiamine hydrochloride (C₁₂H₁₇ClN₄OS · HCl) or thiamine mononitrate (C₁₂H₁₇N₅O₄S)

• **NIACIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.

Extraction solvent: *Solution A* and methanol (3:1)

Mobile phase: 0.1 M sodium acetate solution (13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]

Standard stock solution: 1 mg/mL of USP Niacin RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask. Dilute with *Extraction solvent* to volume.

Sample solution: [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine, and riboflavin, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a beaker. Remove any contents adhering to the shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 2 mg of riboflavin, to a 200-mL volumetric flask. If riboflavin is not present in the formulation, use a portion equivalent to a nominal amount of 2 mg of pyridoxine. If pyridoxine is not present in the formulation, use a portion equivalent to a nominal amount of 20 mg of niacin or niacinamide. Add 100.0 mL of *Extraction solvent*, and mix for 20 min, using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

[NOTE—If necessary, flush the column with methanol between injections.]

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of niacin. Calculate the percentage of the labeled amount of niacin (C₆H₅NO₂) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*

r_S = peak area of niacin from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin (C₆H₅NO₂)

• **NIACINAMIDE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, Standard stock solution, Standard solution, Sample solution, and Chromatographic system: Using USP Niacinamide RS in place of USP Niacin RS, proceed as directed for *Niacin, Method 2*.

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas of niacinamide. Calculate the percentage of the labeled amount of niacinamide (C₆H₆N₂O) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*

r_S = peak area of niacinamide from the *Standard solution*

C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide (C₆H₆N₂O)

• **PYRIDOXINE HYDROCHLORIDE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, and Sample solution: Prepare as directed for *Niacin, Method 2*.

Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

Standard solution: 20 µg/mL of USP Pyridoxine Hydrochloride RS from the *Standard stock solution* diluted with *Extraction solvent*

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas of pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of pyridoxine from the *Sample solution*

r_S = peak area of pyridoxine from the *Standard solution*

C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl)

• **RIBOFLAVIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent and Sample solution: Prepare as directed for *Niacin, Method 2*.

Solution B: 6.8 mg/mL of sodium acetate in water

Mobile phase: Prepare a mixture of *Solution B* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.

Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.

Standard solution: Dilute 5.0 mL of *Standard stock solution* with *Extraction solvent* to 25.0 mL.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas of riboflavin. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of riboflavin from the *Sample solution*

r_S = peak area of riboflavin from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• THIAMINE, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: 1.88 mg/mL of sodium 1-hexanesulfonate in 0.1% phosphoric acid

Mobile phase: *Solution A* and acetonitrile (46:9)

Standard stock solution: 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid

Standard solution: 0.02 mg/mL of USP Thiamine Hydrochloride RS from the *Standard stock solution* diluted with 0.2 N hydrochloric acid

Sample solution: Proceed as directed for the *Sample solution* in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Mix a portion of the Capsule contents with a volume of 0.2 N hydrochloric acid to obtain a nominal concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Sample solution* and *Standard solution*
Measure the peak areas of the major peaks. For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine

hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 3

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in the *Reagent*, with heating if necessary

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed for the *Sample solution* in *Ascorbic Acid, Method 1*, through “calculate the average net weight per Capsule.” Transfer a portion of the Capsule contents, equivalent to 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1), and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas of niacin or niacinamide.

Calculate the percentage of the labeled amount of niacin (C₆H₅NO₂) or niacinamide (C₆H₆N₂O) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*

r_S = peak area of niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl), riboflavin (C₁₇H₂₀N₄O₆) and thiamine hydrochloride (C₁₂H₁₇ClN₄OS · HCl) (for products containing thiamine hydrochloride) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

r_S = peak area of the corresponding vitamin from the *Standard solution*

C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate (C₁₂H₁₇N₅O₄S) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide (C₆H₆N₂O) or niacin (C₆H₅NO₂), pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl), riboflavin (C₁₇H₂₀N₄O₆), and thiamine as thiamine hydrochloride (C₁₂H₁₇ClN₄OS · HCl) or thiamine mononitrate (C₁₂H₁₇N₅O₄S)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS <2040>:** Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS <2091>:** Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS <2021>:** The total aerobic microbial count does not exceed 3000 cfu/g, and the combined molds and yeasts count does not exceed 300 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS <2022>:** Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states that the product is Water-Soluble Vitamins Capsules. The label also states the quantity of each vitamin in terms of metric units per dosage unit and where necessary the salt form in which it is present. Where more than one assay method is given for a particular vitamin, the labeling states which assay method is used only if *Method 1* is not used. Where products are labeled to contain panthenol, the label states the equivalent content of dexpantenol.
- **USP REFERENCE STANDARDS <11>**
 - USP Biotin RS
(3a*S*,4*S*,6a*R*)-Hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-valeric acid.
C₁₀H₁₆N₂O₃S 244.31
 - USP Calcium Pantothenate RS
Calcium D-pantothenate (1:2).
C₁₈H₃₂CaN₂O₁₀ 476.53
 - USP Cyanocobalamin RS
Vitamin B₁₂.
C₆₃H₈₈CoN₁₄O₁₄P 1355.37
 - USP Dexpantenol RS
D-(+)-2,4-Dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethylbutyramide.
C₉H₁₉NO₄ 205.25
 - USP Folic Acid RS
L-Glutamic acid, *N*-[4-[(2-amino-1,4-dihydro-4-oxo-6-pteridyl)methyl]amino]benzoyl]-.
C₁₉H₁₉N₇O₆ 441.40
 - USP Niacin RS
Nicotinic acid.
C₆H₅NO₂ 123.11
 - USP Niacinamide RS
Nicotinamide.
C₆H₆N₂O 122.12
 - USP Racemic Panthenol RS
Butanamide, 2,4-dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethyl-, (±)-.
C₉H₁₉NO₄ 205.25
 - USP Pyridoxine Hydrochloride RS
Pyridoxol hydrochloride.
C₈H₁₁NO₃ · HCl 205.64
 - USP Riboflavin RS
Riboflavine.
C₁₇H₂₀N₄O₆ 376.36
 - USP Thiamine Hydrochloride RS
Thiamine monohydrochloride.
C₁₂H₁₇ClN₄OS · HCl 337.27

Water-Soluble Vitamins Tablets

DEFINITION

Water-Soluble Vitamins Tablets contain two or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Sodium Ascorbate or Calcium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, Pantothenic Acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate. Tablets contain NLT 90.0% and NMT 150.0% of the

labeled amounts of ascorbic acid ($C_6H_8O_6$) or its equivalent as sodium ascorbate or calcium ascorbate, biotin ($C_{10}H_{16}N_2O_3S$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate.

They do not contain any form of Beta Carotene or Vitamin A, D, E, or K. They do not contain any minerals for which nutritional value is claimed. They may contain other labeled added substances in quantities that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

• ASCORBIC ACID, Method 1

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 100 mg of ascorbic acid, to a 200-mL volumetric flask, and add 75 mL of metaphosphoric-acetic acids TS. Insert a stopper into the flask, and shake by mechanical means for 30 min. Dilute with water to volume. Transfer a portion of the solution to a centrifuge tube, and centrifuge until a clear supernatant is obtained. Pipet 4.0 mL of this solution into a 50-mL conical flask, and add 5 mL of metaphosphoric-acetic acids TS.

Analysis: Titrate with standard dichlorophenol-indophenol solution VS to a rose-pink color that persists for at least 5 s. Correct for the volume of dichlorophenol-indophenol solution consumed by a mixture of 5.5 mL of metaphosphoric-acetic acids TS and 15 mL of water. From the ascorbic acid equivalent of the standard dichlorophenol-indophenol solution, calculate the content of ascorbic acid in each Tablet.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

• ASCORBIC ACID, Method 2: Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

• CALCIUM ASCORBATE, Method 1: Proceed as directed in *Ascorbic Acid, Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

• CALCIUM ASCORBATE, Method 2: Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

• SODIUM ASCORBATE, Method 1: Proceed as directed in *Ascorbic Acid, Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

• SODIUM ASCORBATE, Method 2: Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

• BIOTIN, Method 1

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 1 mg of biotin, to a 200-mL volumetric flask, add 3 mL of dimethyl sulfoxide, and swirl to wet. Place the flask in a water

bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-µm packing L7

Flow rate: 1.2 mL/min

Injection size: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the responses for the biotin peaks. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• BIOTIN, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 µg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: 0.1 ng/mL of USP Biotin RS in water, prepared by dilution of the *Standard stock solution* with water on the day of the assay

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to 100 µg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with 50% alcohol to volume, and filter. Dilute a volume of the filtrate, quantitatively, and stepwise if necessary, with water to obtain a solution with a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine-guanine-uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 µg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution:

20 µg/mL of riboflavin and 10 µg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture* of *Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of *Standard* and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of *Standard* during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength of 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the *Standard*, calculate the response from the sum of the duplicate values of the transmittance (ΣS) as the difference, $y = 2.00 - \Sigma S$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma U$, adding together the two transmittances for each level of the *Sample solution* (ΣU). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of lowest and highest points plotted for the *Standard*. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in µg, of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of µg of biotin that was assumed to be present in the portion of Tablets taken

¹ ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of biotin in the portion of Tablets taken (μg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, Method 3**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 800 mL of water and 100 mL of triethylamine to a 1000-mL volumetric flask. Add 80 mL of 85% phosphoric acid, and dilute with water to volume.

Mobile phase: Transfer 80 mL of acetonitrile and 10 mL of *Solution A* to a 1000-mL volumetric flask. Dilute with water to volume.

Standard solution: 0.6 $\mu\text{g/mL}$ of USP Biotin RS in water. [NOTE—A portion of the *Standard solution* will be used to determine the percent recovery of biotin from the *Solid-phase extraction* procedure.]

Sample solution: Finely powder NLT 20 Tablets. Transfer an amount of powdered Tablets to a volumetric flask to obtain a concentration of 0.6 $\mu\text{g/mL}$ of biotin. Add water up to 80% of the flask capacity, and sonicate for 30–40 min, with occasional mixing, to dissolve. Dilute with water to volume, and filter. Adjust the pH of the solution with either diluted acetic acid or 0.1 N sodium hydroxide to 6.0–7.0.

Solid-phase extraction [NOTE—Condition the extraction column specified in this procedure in the following manner. Wash the column with a 2-mL portion of methanol. Equilibrate with a 2-mL portion of water.]

Separately pipet 5.0 mL of the *Sample solution* and *Standard solution* into freshly conditioned solid-phase extraction columns consisting of a mixed-mode packing with a sorbent-mass of 60 mg. [NOTE—The mixed-mode packing consists of anion-exchange and reversed-phase sorbents. The reverse-phase component is a copolymer of *N*-vinylpyrrolidone and divinylbenzene. The anion exchange moiety is a trialkylamino group.²]

Wash the column with 10 mL of 30% (v/v) methanol in water. Apply an appropriate volume (4.9 mL) of 30% (v/v) methanol in 0.1 N hydrochloric acid to the column. Collect the eluate in a 5-mL volumetric flask, containing 100 μL of 40% (w/v) sodium acetate in water, and dilute with 30% (v/v) methanol in 0.1 N hydrochloric acid to volume.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm \times 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 100 μL

System suitability

Samples: *Standard solution* and a portion of *Standard solution* that has undergone *Solid-phase extraction*

Suitability requirements

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution* and the *Standard solution* that has undergone *Solid-phase extraction*

Recovery: 95%–100%, *Standard solution* that has undergone *Solid-phase extraction*

Analysis

Samples: *Standard solution* and *Sample solution* that have both undergone *Solid-phase extraction*

Measure the responses for the biotin peak. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of biotin in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **CYANOCOBALAMIN, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 $\mu\text{g/mL}$ of USP Cyanocobalamin RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

Standard solution: 1 $\mu\text{g/mL}$ of USP Cyanocobalamin RS from *Standard stock solution*, diluted with water

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to 100 μg of cyanocobalamin, to a 250-mL flask. Quantitatively add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm \times 15-cm; 5- μm packing L1

Flow rate: 0.5 mL/min

Injection size: 200 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for cyanocobalamin.

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Cyanocobalamin RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• **CYANOCOBALAMIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 $\mu\text{g/mL}$ of USP

Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described in the

² A suitable cartridge is the Waters, Oasis MAX Vac RC cartridge, particle size 30 μm , part 186000371.

Analysis, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Finely powder NLT 20 Tablets.

Transfer a portion of the powdered Tablets, equivalent to 1.0 µg of cyanocobalamin, to an appropriate vessel containing, for each g of powdered Tablets taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B₁₂ activity equivalent to the nominal activity of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in *Biotin, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Prepare as directed in *Biotin, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions to the resulting solution. Add 100 mL of water, and dissolve dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH between 5.5 and 6.0, and add *Purified Water* to make 250 mL.

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N Hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL

Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.³ [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.] Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Make a transfer of cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL of the *Culture medium* each. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL

³ Pure cultures of *Lactobacillus leichmannii* (listed as *Lactobacillus delbrueckii*) may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209 (www.atcc.org).

of *Suspension medium*, and combine. Using *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. The cell suspension so obtained is the *Inoculum*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.]

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*
Because of the high sensitivity of the test organism to minute amounts of vitamin B₁₂ activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes, and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5°, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating its contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5%, or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the

logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances for each level of the *Sample solution* (Σ_u). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of cyanocobalamin that was assumed to be present in the portion of Tablets taken

Calculate the percentage of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$) in the portion of Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of Tablets taken (μg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Vitamin B₁₂ Activity in Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• Folic Acid, Method 1

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use.

[NOTE—The methanol and water content may be varied (between 1% and 3%) by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation.]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask, and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8,

bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of USP Folic Acid RS in *Internal standard solution*

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of powder, equivalent to 0.4 mg of folic acid, to a 50-mL amber-colored centrifuge tube. Add 25.0 mL of *Internal standard solution*, shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 15 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*

R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• FOLIC ACID, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: 60 µg/mL of ammonium hydroxide

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 60 µg/mL of USP Folic Acid RS in *Diluent*. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of *Standard stock solution* with 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Transfer a portion of finely powdered Tablets, equivalent to 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of folic acid from the *Sample solution*

r_S = peak area of folic acid from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• CALCIUM PANTOTHENATE, Method 1

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium

Pantothenate RS in *Internal standard solution*

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to 15 mg of calcium pantothenate, to a centrifuge tube. Add 25.0 mL of the *Internal standard solution*, and shake vigorously for 10 min. Centrifuge, filter, and use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, Method 2**

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume, to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed in the *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution having approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water; add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar; and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*⁴ incubating for 16–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Make a transfer of cells from the *Stock culture* of *Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solution* and *Sample solution*
To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of *Standard* and *sample* tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within $\pm 0.5^\circ$ until, following 16–24 h of incubation, there has been no substantial increase in turbidity in

⁴ ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Place the container in a spectrophotometer that has been set at a specific wavelength between 540 and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances for each level of the *Sample solution* (Σ_u). Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in mg, of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate that was assumed to be present in the portion of Tablets taken

Calculate the percentage of the labeled amount of calcium pantothenate in the portion of Tablets taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Tablets taken (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, Method 3**

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from *Standard stock solution* diluted with water

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse. Dilute with water to volume, mix, and filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 3.9-mm × 30-cm; 5-µm packing L1

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 25 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for calcium pantothenate.

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

r_u = peak area from the *Sample solution*

r_s = peak area from the *Standard solution*

C_s = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_u = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **NIACIN or NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution: Finely powder NLT 30 Tablets. Transfer a portion of the powder, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution,

cool to room temperature, and use the clear filtrate.
[NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for niacinamide, pyridoxine, riboflavin, and thiamine are about 0.3, 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin or niacinamide, pyridoxine, riboflavin, and thiamine. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*

r_S = peak area of niacinamide from the *Standard solution*

C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

For formulations containing niacin:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*

r_S = peak area of niacin from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

r_S = peak area of the corresponding vitamin from the *Standard solution*

C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$) or niacin ($C_6H_5NO_2$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• NIACIN, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.

Extraction solvent: *Solution A* and methanol (3:1)

Mobile phase: 0.1 M Sodium acetate solution (13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]

Standard stock solution: 1 mg/mL of USP Niacin RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.

Sample solution: [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine, and riboflavin, when present in the formulation.] Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 2 mg of riboflavin, to a 200-mL volumetric flask. If riboflavin is not present in the formulation, transfer a portion of the powder, equivalent to 2 mg of pyridoxine. If pyridoxine is not present in the formulation, transfer a portion of the powder, equivalent to 20 mg of niacin or niacinamide. Add 100.0 mL of *Extraction solvent*, and mix for 20 min, using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

[NOTE—If necessary, flush the column with methanol between injections.]

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

• NIACINAMIDE, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A, Extraction solvent, Mobile phase, Standard stock solution, Standard solution, Sample solution, Chromatographic system, and System suitability: Using USP Niacinamide RS in place of USP Niacin RS, proceed as directed in *Niacin, Method 2*.

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas for niacinamide. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*
 r_S = peak area from the *Standard solution*
 C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)
 C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

• PYRIDOXINE HYDROCHLORIDE, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, and Sample solution: Prepare as directed in *Niacin, Method 2*.

Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas for pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*
 r_S = peak area from the *Standard solution*
 C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)
 C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• RIBOFLAVIN, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent and Sample solution: Prepare as directed in *Niacin, Method 2*.

Solution A: 6.8 mg/mL of sodium acetate in water

Mobile phase: Prepare a mixture of *Solution A* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.

Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a

cold water bath to room temperature, and dilute with *Extraction solvent* to volume.

Standard solution: Dilute 5.0 mL of *Standard stock solution* with *Extraction solvent* to 25.0 mL.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the peak areas for riboflavin. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*
 r_S = peak area from the *Standard solution*
 C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)
 C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• THIAMINE, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: 1.88 mg/mL of sodium 1-hexanesulfonate in 0.1% phosphoric acid

Mobile phase: *Solution A* and acetonitrile (46:9)

Standard stock solution: 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid

Standard solution: 0.02 mg/mL of USP Thiamine Hydrochloride RS from *Standard stock solution* diluted with 0.2 N hydrochloric acid

Sample solution: Weigh and finely powder NLT 20 Tablets. Mix a portion of the powdered Tablets with a volume of 0.2 N hydrochloric acid to obtain a concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the areas for the major peaks. For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of thiamine from the *Sample solution*
 r_S = peak area of thiamine from the *Standard solution*

- C_s = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
 C_u = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Tablets taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times (M_{r1}/M_{r2}) \times 100$$

- r_u = peak area of thiamine from the *Sample solution*
 r_s = peak area of thiamine from the *Standard solution*
 C_s = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
 C_u = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
 M_{r1} = molecular weight of thiamine mononitrate, 327.36
 M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN or NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 3**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in the *Reagent*, with heating if necessary

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2.0 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the peaks. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$)

or niacinamide ($C_6H_6N_2O$) in the portion of Tablets taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

- r_u = peak area of niacin or niacinamide from the *Sample solution*
 r_s = peak area of niacin or niacinamide from the *Standard solution*
 C_s = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)
 C_u = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

- r_u = peak area of the corresponding vitamin from the *Sample solution*
 r_s = peak area of the corresponding vitamin from the *Standard solution*
 C_s = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)
 C_u = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Tablets taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times (M_{r1}/M_{r2}) \times 100$$

- r_u = peak area of thiamine from the *Sample solution*
 r_s = peak area of thiamine from the *Standard solution*
 C_s = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
 C_u = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
 M_{r1} = molecular weight of thiamine mononitrate, 327.36
 M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** <2040>: Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements

CONTAMINANTS

- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS** <2021>: The total aerobic microbial count does not exceed 3000 cfu/g, and the combined molds and yeasts count does not exceed 300 cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS** <2022>: Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

- **LABELING:** The label states that the product is Water-Soluble Vitamins Tablets. The label also states the quantity of each vitamin in terms of metric units per dosage unit, and where necessary, the salt form in which it is present. Where more than one assay method is given for a particular vitamin, the labeling states which assay method is used only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** <11>
 - USP Biotin RS
 - USP Calcium Pantothenate RS
 - USP Cyanocobalamin RS
 - USP Folic Acid RS
 - USP Niacin RS
 - USP Niacinamide RS
 - USP Pyridoxine Hydrochloride RS
 - USP Riboflavin RS
 - USP Thiamine Hydrochloride RS

Water-Soluble Vitamins with Minerals Capsules

DEFINITION

Water-Soluble Vitamins with Minerals Capsules contain one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Dexpantenol or Panthenol, Pantothenic Acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Niacin or Niacinamide, Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate; and one mineral or more, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc, derived from substances generally recognized as safe. Capsules contain NLT 90.0% and NMT 150.0% of the labeled quantities of ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$), biotin ($C_{10}H_{16}N_2O_3S$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), dexpantenol ($C_9H_{19}NO_4$) or panthenol ($C_9H_{19}NO_4$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 125.0% of the labeled quantities of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and NLT 90.0% and NMT 160.0% of the labeled quantities of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

They do not contain any form of Beta Carotene or Vitamin A, D, E, or K. They may contain other labeled added substances that are generally recognized as safe, in quantities that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

- **ASCORBIC ACID, Method 1**
Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without the loss of

shell material, and transfer the contents to a 100-mL beaker. Remove any contents adhering to the empty shells by washing, if necessary, with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight/Capsule. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 100 mg of ascorbic acid, to a 200-mL volumetric flask, and add 75 mL of metaphosphoric-acetic acids TS. Insert a stopper into the flask, and shake by mechanical means for 30 min. Dilute with water to volume. Transfer a portion of the solution to a centrifuge tube, and centrifuge until a clear supernatant is obtained. Pipet 4.0 mL of this solution into a 50-mL conical flask, and add 5 mL of metaphosphoric-acetic acids TS.

Analysis: Titrate with standard dichlorophenol-indophenol solution VS to a rose-pink color that persists for at least 5 s. Correct for the volume of dichlorophenol-indophenol solution consumed by a mixture of 5.5 mL of metaphosphoric-acetic acids TS and 15 mL of water. From the ascorbic acid equivalent of the standard dichlorophenol-indophenol solution, calculate the content of ascorbic acid in each Capsule.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **ASCORBIC ACID, Method 2:** Proceed as directed in *Automated Methods of Analysis* <16>, Assay for Ascorbic Acid.
Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)
- **CALCIUM ASCORBATE, Method 1:** Proceed as directed in the assay for Ascorbic Acid, Method 1.
Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)
- **CALCIUM ASCORBATE, Method 2:** Proceed as directed in *Automated Methods of Analysis* <16>, Assay for Ascorbic Acid.
Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)
- **SODIUM ASCORBATE, Method 1:** Proceed as directed in the assay for Ascorbic Acid, Method 1.
Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)
- **SODIUM ASCORBATE, Method 2:** Proceed as directed in *Automated Methods of Analysis* <16>, Assay for Ascorbic Acid.
Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **BIOTIN, Method 1**
 [NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS, prepared by diluting the *Standard stock solution* with water

Sample solution: Proceed as directed in the assay for Ascorbic Acid, Method 1, through “calculate the average net weight/Capsule”. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 1 mg of biotin, to a 200-mL volumetric flask. Add 3 mL of dimethyl sulfoxide, and swirl to wet the contents. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 15-cm; 3-μm packing L7

Flow rate: 1.2 mL/min

Injection size: 100 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the responses for the biotin peaks. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* (μg/mL)

C_U = nominal concentration of biotin in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• **BIOTIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 μg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.

Standard solution: On the day of the assay, dilute the *Standard stock solution* with water to a concentration of 0.1 ng/mL of USP Biotin RS.

Sample solution: Proceed as directed in the assay for *Ascorbic Acid, Method 1*, through “calculate the average net weight/Capsule”. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 100 μg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with diluted alcohol to volume, and filter. Dilute a volume of the filtrate quantitatively, and stepwise if necessary, with water to obtain a solution with a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water. Heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a refrigerator at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 μg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution:

20 μg/mL of riboflavin and 10 μg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 μg/mL of p-aminobenzoic acid, 50 μg/mL of niacin, and 40 μg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to test tubes, close or cover the tubes, sterilize in an autoclave at 121°, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: Add 0.5 ng of biotin in 5.0 mL of water to each tube containing 5.0 mL of *Basal medium stock solution*. Plug the tubes with cotton, sterilize in an autoclave at 121°, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture* of *Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. The cell suspension so obtained is the *Inoculum*.

Analysis: To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

¹ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$ until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution*/tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points. Calculate the response, y , adding together the two transmittances for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of USP Biotin RS corresponding to the biotin in each mg of the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of biotin that was assumed to be present in each mg of the portion of the Capsules taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations.

From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$)

• **CYANOCOBALAMIN, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 $\mu\text{g/mL}$ of USP

Cyanocobalamin RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

Standard solution: 1 $\mu\text{g/mL}$ of USP Cyanocobalamin RS from *Standard stock solution* diluted with water

Sample solution: Proceed as directed in the assay for *Ascorbic Acid, Method 1*, through “calculate the average net weight/Capsule.” Transfer a portion of the Capsule contents, equivalent to 100 μg of cyanocobalamin, to a 250-mL flask. Add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the clear filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm \times 15-cm; 5- μm packing L1

Flow rate: 0.5 mL/min

Injection size: 200 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for cyanocobalamin.

Calculate the percentage of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Cyanocobalamin RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• **CYANOCOBALAMIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard cyanocobalamin stock solution: 1.0 $\mu\text{g/mL}$ of USP Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard cyanocobalamin stock solution* with water to a measured volume such that after the incubation period as described in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of the *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Proceed as directed in the assay for *Ascorbic Acid, Method 1*, through “calculate the average net weight/Capsule.” Transfer a portion of the Capsule contents, equivalent to 1.0 μg of cyanocobalamin, to an appropriate vessel containing, for each g of Capsule contents taken, 25 mL of an aqueous extracting solution prepared just before use to contain 12.9 mg/mL of dibasic sodium phosphate, 11.0 mg/mL of anhydrous citric acid, and 10 mg/mL of sodium metabisulfite. Autoclave the mixture at 121° for 10 min.

Allow any undissolved particles of the extract to settle, and filter or centrifuge, if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B₁₂ activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in the assay for *Calcium Pantothenate, Method 2*.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine-guanine-uracil solution: Prepare as directed in the assay for *Calcium Pantothenate, Method 2*.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL. Add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N glacial acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions in the resulting solution. Add 100 mL of water, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of between 5.5 and 6.0, and add Purified Water to make 250 mL.

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N Hydrochloric acid	10 mL
Adenine-guanine-uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave (exhaust line temperature), and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.² [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.]

Incubate for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Make a transfer of cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL of the *Culture medium* each. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within ±0.5°. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using *Basal medium stock solution*. The cell suspension so obtained is the *Inoculum*.

[NOTE—This dilution may be altered, when necessary, to obtain the desired test response.]

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically,

²Pure cultures of *Lactobacillus leichmannii* may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209.

using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis: Because of the high sensitivity of the test organism to minute amounts of vitamin B₁₂ activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm × 150-mm test tubes, and other necessary glassware. To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within ±0.5°, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating its contents, place the container in a spectrophotometer that has been set at a wavelength of 530 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y , adding together the two transmittances for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of USP Cyanocobalamin RS corresponding to the cyanocobalamin in each mg of the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of cyanocobalamin that was assumed to be present in each mg of the portion of the Capsules taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin (C₆₃H₈₈CoN₁₄O₁₄P)

• Folic Acid, Method 1

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation. [NOTE—The methanol and water content may be varied (between 1% and 3%).]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask, and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8, bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of USP Folic Acid RS in *Internal standard solution*

Sample solution: Proceed as directed in the assay for *Ascorbic Acid, Method 1*, through "calculate the average net weight/Capsule." Transfer an amount of Capsule contents to a suitable centrifuge tube, and add a volume of *Internal standard solution* to obtain a nominal concentration of 0.016 mg/mL of folic acid. Shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 15 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*

R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• FOLIC ACID, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: Dissolve 7.5 g of edetate disodium, with stirring, in 500 mL of water containing 10 mL of ammonium hydroxide.

Diluent: 60 µg/mL of ammonium hydroxide

Mobile phase: Transfer 0.4 mL of triethylamine, 15 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 60 µg/mL of USP Folic Acid RS in *Diluent*. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of *Standard stock solution* with 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Ascorbic Acid, Method 1* through "calculate the average net weight/Capsule." Transfer a portion of the Capsule contents, equivalent to a nominal amount of 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of folic acid from the *Sample solution*

r_S = peak area of folic acid from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• DEXPANTHENOL OR PANTHENOL

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 µg/mL of USP

Dexpantenol RS in water. Store in a refrigerator, protected from light, and use within 30 days.

Standard solution: On the day of the assay, prepare a dilution of 1.2 µg/mL of dexpantenol from *Standard stock solution* diluted with water.

Sample solution: Weigh NLT 30 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents as completely as possible to a beaker. Remove any contents adhering to the empty Capsule shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air until the odor of ether is no longer perceptible. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the average net weight/Capsule. Dissolve a portion of the Capsule contents, nominally equivalent to 1.2 mg of dexpantenol or 2.4 mg of panthenol, in 100.0 mL of water. Quantitatively dilute a portion of this solution with water to obtain a nominal concentration of 1.2 µg/mL of dexpantenol or 2.4 µg/mL of panthenol.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to

75 ± 5°, and add hydrochloric acid solution (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool. Add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in neutral 25% alcohol. Store in a refrigerator.

Salt solution A: 50 mg/mL of monobasic potassium phosphate and 50 mg/mL of dibasic potassium phosphate in water. Add 10 drops of hydrochloric acid/L of solution. Store under toluene.

Salt solution B: 20 mg/mL of magnesium sulfate, 1 mg/mL of sodium chloride, 1 mg/mL of ferrous sulfate, and 1 mg/mL of manganese sulfate in water. Add 10 drops of hydrochloric acid/L of the solution. Store under toluene.

Pyridoxal–calcium pantothenate solution: 200 µg/mL of pyridoxal hydrochloride and 1.875 µg/mL of calcium pantothenate in 10% alcohol. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: 50 mg/mL of polysorbate 40 and 0.5 mg/mL of oleic acid in 20% alcohol. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL
Pyridoxal–calcium pantothenate solution	5 mL
Polysorbate 40–oleic acid solution	5 mL

Dissolve anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL.

Double-strength modified pantothenate medium:

Prepare as directed for the *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH of 6.5–6.6, and dilute with water to 1000 mL. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*³ on a slant of

this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with *Modified pantothenate medium*. Resuspend the cells in sufficient *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes, and in the same order, add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*.

Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes, and in the same order, add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature NLT 80°, such as by steaming at atmospheric pressure in a sterilizer for 5–10 min. Cool, and determine the percentage transmittance of the suspensions, in cells of equal pathlength, on a suitable spectrophotometer, at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percentage of transmittance, for each set of tubes of the standard curve against the Standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency, in terms of dexpantenol, of each tube containing portions of the *Sample solution*. Divide the potency of each tube by the amount of the *Sample solution* added to it, to obtain the individual responses. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the material taken for assay, in terms of dexpantenol, by multiplying the mean response by the appropriate dilution factor.

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpantenol (C₉H₁₉NO₄) or panthenol (C₉H₁₉NO₄)

• CALCIUM PANTOTHENATE, Method 1

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of p-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

Sample solution: Proceed as directed in the assay for *Ascorbic Acid, Method 1*, through “calculate the average net weight/Capsule.” Transfer the equivalent to 0.6 mg/mL of calcium pantothenate, from mixed Capsule contents, in *Internal standard solution*, and

³ATCC No. 8042 is suitable.

shake vigorously for 10 min. Centrifuge, filter, and use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• CALCIUM PANTOTHENATE, *Method 2*

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide while protected from absorption of moisture during the weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed for *Analysis*, 2.0–4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Proceed as directed in the assay for *Ascorbic Acid, Method 1*, through “calculate the average net weight/Capsule.” Transfer a portion of the Capsule contents, equivalent to a nominal amount of 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (16.66 mg/mL), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution having approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment

with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

***p*-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution:** 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
<i>p</i> -Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121°, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution* add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121°, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture of Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. The cell suspension so obtained is the *Inoculum*.

Analysis: To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of *Standard* and *sample* tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool. Add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within $\pm 0.5^\circ$ until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of *Standard* during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Place the container in a spectrophotometer that has been set at a specific wavelength between 540 and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the *Standard*, calculate the response from the sum of the duplicate values of the transmittance (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution*/tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y , adding together the two transmittances for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the *Standard*. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative

potency, M' , of the *Sample solution*. Determine the quantity, in mg, of USP Calcium Pantothenate RS corresponding to the calcium pantothenate in each mg of the portion of Capsules taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate that was assumed to be present in each mg of the portion of the Capsules taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• CALCIUM PANTOTHENATE, Method 3

Buffer solution: Dissolve 10.0 g of monobasic potassium phosphate in 2000 mL of water, and adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 μ g/mL of USP Calcium Pantothenate RS from *Standard stock solution* diluted with water

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Ascorbic Acid*, Method 1 through “calculate the average net weight/Capsule.” Transfer a portion of the Capsule contents, equivalent to 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse the Capsules’ contents. Dilute with water to volume, mix, and filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 3.9-mm \times 30-cm; 5- μ m packing L1

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 25 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for calcium pantothenate.

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing Niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution: Proceed as directed in the assay for *Ascorbic Acid, Method 1*, through “calculate the average net weight/Capsule.” Transfer a portion of the Capsule contents, equivalent to 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for niacinamide, pyridoxine, riboflavin, and thiamine are about 0.3, 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin or niacinamide, pyridoxine, riboflavin, and thiamine. Calculate the percentage of the labeled amount of niacinamide (C₆H₆N₂O) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*

r_S = peak area of niacinamide from the *Standard solution*

C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

For formulations containing niacin:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*

r_S = peak area of niacin from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl), riboflavin (C₁₇H₂₀N₄O₆), and thiamine hydrochloride (C₁₂H₁₇ClN₄OS · HCl) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

r_S = peak area of the corresponding vitamin from the *Standard solution*

C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate (C₁₂H₁₇N₅O₄S) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide (C₆H₆N₂O), niacin (C₆H₅NO₂), pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl), riboflavin (C₁₇H₂₀N₄O₆), and thiamine as thiamine hydrochloride (C₁₂H₁₇ClN₄OS · HCl) or thiamine mononitrate (C₁₂H₁₇N₅O₄S)

• **NIACIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.

Extraction solvent: *Solution A* and methanol (3:1)

Mobile phase: 0.1 M sodium acetate solution (13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]

Standard stock solution: 1 mg/mL of USP Niacin RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask. Dilute with *Extraction solvent* to volume.

Sample solution: [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine hydrochloride, and riboflavin, when present in the formulation.] Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a beaker. Remove any contents adhering to the shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to 0.02 mg/mL of riboflavin. If riboflavin is not present in the formulation, use a portion equivalent to 0.02 mg/mL of pyridoxine. If pyridoxine is not present in the formulation, use a

portion equivalent to 0.2 mg/mL of niacin or niacinamide in *Extraction solvent*, and mix for 20 min, using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

[NOTE—If necessary, flush the column with methanol between injections.]

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin. Calculate the percentage of the labeled amount of niacin (C₆H₅NO₂) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin (C₆H₅NO₂)

• NIACINAMIDE, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, Standard stock solution, Standard solution, Sample solution, and Chromatographic system: Using USP Niacinamide RS in place of USP Niacin RS, proceed as directed in the assay for *Niacin*, Method 2.

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacinamide. Calculate the percentage of the labeled amount of niacinamide (C₆H₆N₂O) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*

r_S = peak area of niacinamide from the *Standard solution*

C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide (C₆H₆N₂O)

• PYRIDOXINE HYDROCHLORIDE, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent and Mobile phase: Prepare as directed in the assay for *Niacin*, Method 2.

Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

Standard solution: 20 µg/mL of USP Pyridoxine Hydrochloride RS from *Standard stock solution* diluted with *Extraction solvent*

Sample solution: Prepare as directed for the *Sample solution* in the assay for *Niacin*, Method 2.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl)

• RIBOFLAVIN, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent: Prepare as directed in the assay for *Niacin*, Method 2.

Solution A: 6.8 mg/mL of sodium acetate in water

Mobile phase: Prepare a mixture of *Solution A* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.

Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.

Standard solution: Dilute 5.0 mL of *Standard stock solution* with *Extraction solvent* to 25.0 mL.

Sample solution: Prepare as directed for the *Sample solution* in the assay for *Niacin*, Method 2.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for riboflavin. Calculate the percentage of the labeled amount of riboflavin (C₁₇H₂₀N₄O₆) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin (C₁₇H₂₀N₄O₆)

• **THIAMINE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: 1.88 mg/mL of sodium 1-hexanesulfonate in 0.1% phosphoric acid

Mobile phase: *Solution A* and acetonitrile (46:9)

Standard stock solution: 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid

Standard solution: 0.02 mg/mL of USP Thiamine Hydrochloride RS from *Standard stock solution* diluted with 0.2 N hydrochloric acid

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Ascorbic Acid, Method 1*, through “calculate the average net weight/Capsule.” Mix a portion of the Capsule contents with a volume of 0.2 N hydrochloric acid to obtain a nominal concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the areas for the major peaks. For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN OR NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 3**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in *Reagent*, with heating if necessary

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Ascorbic Acid, Method 1*, through “calculate the average net weight/Capsule.” Transfer a portion of the Capsule contents, equivalent to a nominal amount of 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1), and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Measure the areas of the peak responses. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding analyte from the *Sample solution*

r_S = peak area of the corresponding analyte from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) (for products containing thiamine hydrochloride) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding analyte from the *Sample solution*

- r_s = peak area of the corresponding analyte from the *Standard solution*
 C_s = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)
 C_U = nominal concentration of the corresponding analyte in the *Sample solution* (mg/mL)
 For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_s) \times (C_s/C_U) \times (M_{r1}/M_{r2}) \times 100$$

- r_U = peak area of thiamine from the *Sample solution*
 r_s = peak area of thiamine from the *Standard solution*
 C_s = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)
 C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)
 M_{r1} = molecular weight of thiamine mononitrate, 327.36
 M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$) or niacin ($C_6H_5NO_2$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamin hydrochloride ($C_{12}H_{17}ClN_4OS$) or thiamin mononitrate ($C_{12}H_{17}N_5O_4S$) [NOTE—Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a *Standard stock solution* is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the *Standard solutions* and the *Sample solution* may be diluted quantitatively with the solvent specified, if necessary, to yield solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

• **CALCIUM, Method 1**

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccators for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Polysorbate 80 solution: Dilute Polysorbate 80 with alcohol (1 in 10).

Sample solution: Transfer 5 Capsules to a 100-mL volumetric flask. [NOTE—For hard gelatin Capsules, weigh NLT 20 Capsules. Open the Capsules, without loss of shell material, and transfer the contents to a suitable container. Remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and allow the Capsule shells to dry. Weigh the empty Capsule shells, calculate the net weight of the Capsule contents, and transfer a portion of the Capsule contents, equivalent to 5 Capsules, to a 100-mL volumetric flask.] Add 15 mL of water, 10 mL of 6 N hydrochloric acid, and 1 mL of *Polysorbate 80 solution* to the flask. Heat on a hot plate

or steam bath, with intermittent swirling, until the Capsules are completely disintegrated or the contents are dissolved. Boil gently for an additional 15 min. Cool, dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution with 0.125 N hydrochloric acid to obtain a concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrometry

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: Calcium emission line at 422.7 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, Method 1**

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 mL and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 1 µg/mL of chromium and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrometry

Lamp: Chromium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Chromium emission line at 357.9 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (Cr) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (μg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (Cr)

• **COPPER, Method 1**

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% (v/v) solution of nitric acid, and dilute with a 1% (v/v) solution of nitric acid to 1000 mL. This solution contains 1000 μg/mL of copper.

Standard stock solution: 100 μg/mL of copper from *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 μg/mL of copper.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 2 μg/mL of copper and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrometry

Lamp: Copper hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Copper emission line at 324.7 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in μg/mL, of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in μg/mL, of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (Cu) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (μg/mL)

C_U = nominal concentration of copper in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (Cu)

• **FLUORIDE, Method 1**

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: 408 mg/mL of sodium acetate in water. Adjust with a few drops of acetic acid to a pH of 7.0. [NOTE—Dissolve in a portion of water and allow the solution to equilibrate to room temperature, then dilute with water to volume and adjust the pH.]

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 μg/mL of fluoride from a quantity of sodium fluoride, previously dried at 100° for 4 h and cooled in a desiccator in water

Intermediate stock solution A: 100 μg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 μg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of 3 M *sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 μg/mL of fluoride.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the mixed Capsule contents, equivalent to 200 mg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of 3 M *sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute with water to volume.

Analysis

Samples: *Standard solutions* and *Sample solution*
To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see pH (791)), in mV, of the *Standard solutions* and the *Sample solution*, with a pH meter capable of a minimum reproducibility of ±0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer having an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.] Plot the logarithms of fluoride concentrations, in μg/mL, of the *Standard solutions* versus the potential, in mV. From the standard response curve and the measured potential of the *Sample solution*, determine the concentration, C, in μg/mL, of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* (μg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (μg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **FLUORIDE, Method 2**

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 μg/mL of USP Sodium Fluoride RS in water. This solution contains 100 μg/mL of fluoride.

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with one column volume of methanol followed by one column volume of pH 10.0 buffer. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.] Transfer 10.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ±

0.1. Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask. Add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with pH 10.0 buffer to volume. Elute a portion of this solution through a 3-mL, solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Weigh NLT 20 Capsules in a tared weighing bottle. Open the Capsules, without loss of shell material, and transfer the contents to a 100-mL container. If necessary, remove any contents adhering to the empty shells by washing with several portions of ether. Discard the washings, and dry the Capsule shells with the aid of a current of dry air. Weigh the empty Capsule shells in the tared weighing bottle, and calculate the net weight of the Capsule contents. Transfer a portion of the Capsule contents, equivalent to a nominal amount of 1 mg of fluorine, to a 100-mL volumetric flask. Add 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ± 0.1 , and dilute with water to 100 mL. Proceed as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Conductivity detector

Guard column: 4.6-mm \times 3-cm; packing L17

Analytical column: 7.8-mm \times 30-cm; packing L17

Flow rate: 0.5 mL/min

Injection size: 100 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Capsules taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (μ g/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (μ g/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• IODIDE, Method 1

Bromine water: Bromine and water (1:5). Shake. Allow to stand for 30 min, and use the supernatant.

Analysis: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the contents, equivalent to 3 mg of iodine, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a

higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.]

Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.

Calculate the percentage of the labeled amount of iodine (I) in the portion of Capsules taken:

$$\text{Result} = V \times N_A \times F \times \text{Ime} \times (Aw/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)

N_A = actual normality of the sodium thiosulfate solution used (meq/mL)

F = correction factor to convert mg to μ g, 1000 μ g/mg

Ime = milliequivalent of I (21.16 mg/meq)

Aw = average weight of the Capsules' contents

W = weight of the sample of Capsules' contents taken

L = labeled amount of iodine (μ g/Capsule)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• IODIDE, Method 2: Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Iodide*.

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• IRON, Method 1

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 μ g/mL of iron.

Polysorbate 80 solution: Prepare as directed in the assay for *Calcium*, *Method 1*.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium*, *Method 1*, except to prepare the *Sample solution* to contain a nominal concentration of 5 μ g/mL of iron and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption spectrometry

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Iron emission line at 248.3 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in μ g/mL, of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in μ g/mL, of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* (µg/mL)

C_U = nominal concentration of iron in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• **MAGNESIUM, Method 1**

Lanthanum chloride solution: Prepare as directed in the assay for *Calcium, Method 1*

Magnesium standard solution: 1000 µg/mL of magnesium from magnesium ribbon, in 6 N hydrochloric acid and water (1:19)

Standard stock solution: 20 µg/mL of magnesium from *Magnesium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 µg/mL of magnesium.

Polysorbate 80 solution: Prepare as directed in the assay for *Calcium, Method 1*.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 0.4 µg/mL of magnesium.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrometry

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Magnesium emission line at 285.2 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* (µg/mL)

C_U = nominal concentration of magnesium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• **MANGANESE, Method 1**

Manganese standard stock solution: 1000 µg/mL of manganese in 6 N hydrochloric acid and nitric acid (49:1). [NOTE—Dissolve the manganese in nitric acid and dilute with 6 N hydrochloric acid to final volume.]

Standard stock solution: 50 µg/mL of manganese from *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL of manganese.

Polysorbate 80 solution: Prepare as directed in the assay for *Calcium, Method 1*.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to

prepare the *Sample solution* to contain 1 µg/mL of manganese and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrometry

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Manganese emission line at 279.5 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of manganese in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* (µg/mL)

C_U = nominal concentration of manganese in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• **MOLYBDENUM, Method 1**

Diluent: 20 mg/mL of ammonium chloride in water

Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of molybdenum.

Standard stock solution: 100 µg/mL of molybdenum from *Molybdenum standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min. Cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 µg/mL of molybdenum.

Polysorbate 80 solution: Prepare as directed in the assay for *Calcium, Method 1*.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to take a number of Capsules or a portion of Capsule contents nominally equivalent to 1000 µg of molybdenum and make appropriate dilutions to obtain a final concentration of 10 µg/mL of molybdenum, omitting the addition of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrometry

Lamp: Molybdenum hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: Molybdenum emission line at 313.3 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of molybdenum, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C, in µg/mL, of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of molybdenum in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **MOLYBDENUM, Method 2**

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% Stannous chloride solution: 400 mg/mL of stannous chloride in 6.5 N hydrochloric acid solution and water (1:4). [NOTE—Dissolve first in 6.5 N hydrochloric acid, and heat the solution until the stannous chloride is dissolved. Then, dilute with water to volume.]

Diluted stannous chloride solution: 20% *Stannous chloride solution* and water (1:24). Prepare this solution fresh at the time of use.

Standard solution: 20 $\mu\text{g/mL}$ of molybdenum from ammonium molybdate in water

Sample: Remove the contents of a counted number of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to a nominal amount of 40 mg of molybdenum, to a 200-mL beaker.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 465 nm

Blank: Amyl alcohol

Analysis

Samples: *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to complete 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature. Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of 20% *Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnel for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layers from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of

the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Sample solution*

V = volume of the *Standard solution* analyzed, 2.0 mL

C_S = concentration of molybdenum in the *Standard solution* ($\mu\text{g/mL}$)

M_U = nominal amount of molybdenum in the *Sample* (μg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• **PHOSPHORUS, Method 1**

Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5:100), and mix

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, then dilute with *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add one drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 $\mu\text{g/mL}$ of phosphorus.

Standard solution: 20 $\mu\text{g/mL}$ of phosphorus from *Phosphorus standard stock solution* diluted with water

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, nominally equivalent to 100 mg of phosphorus in 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 650 nm

Analysis

Samples: *Standard solution* and *Sample solution*

To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions, against the *Blank*.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

C_S = concentration of phosphorus in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of phosphorus in the
Sample solution ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of phosphorus (P)

• POTASSIUM

Potassium standard solution: 100 $\mu\text{g/mL}$ of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 $\mu\text{g/mL}$ of potassium from *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 $\mu\text{g/mL}$ of potassium.

Polysorbate 80 solution: Prepare as directed in the assay for *Calcium, Method 1*.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1* except to prepare the *Sample solution* to contain 1 $\mu\text{g/mL}$ of potassium and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Potassium emission line at 766.5 nm

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the
Sample solution ($\mu\text{g/mL}$)

C_U = nominal concentration of potassium in the
Sample solution ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• SELENIUM, Method 1

Diluent: Prepare as directed in the assay for *Molybdenum, Method 1*.

Selenium standard solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness. Add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 $\mu\text{g/mL}$ of selenium.

Standard stock solution: 100 $\mu\text{g/mL}$ of selenium from *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions containing 5.0, 10.0, and 25.0 $\mu\text{g/mL}$ of selenium.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to 1000 μg of selenium in 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.]

Carefully swirl the flask to disperse the sample specimen. Sonicate for 10 min or until the sample specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Selenium emission line at 196.0 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the
Sample solution ($\mu\text{g/mL}$)

C_U = nominal concentration of selenium in the
Sample solution ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• SELENIUM, Method 2

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% Ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water. [NOTE—Dissolve edetate disodium in a portion of water first, add hydroxylamine hydrochloride, then dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 $\mu\text{g/mL}$ of selenium. Dilute a volume of the solution with 0.125 N hydrochloric

acid, to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10.0 mL of *Standard stock solution* to a glass stoppered flask. Add 1 mL of perchloric acid, and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Remove the contents of Capsules by cutting open the Capsules. Mix, and determine the weight of the contents. Transfer a quantity of the Capsule contents, equivalent to a nominal amount of 20 µg of selenium to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. [CAUTION—Exercise care at this stage because perchloric acid reaction becomes vigorous.] Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid, and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask. Add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV

Cell: 1 cm

Analytical wavelength: 380 nm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*
Treat the *Sample solution*, the *Standard solution*, and the *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with 50% *Ammonium hydroxide solution* to a pH of 1.1 ± 0.1 . Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*. Calculate the percentage of the labeled amount of selenium (Se) in the portion of Capsules taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the cyclohexane layer from the *Sample solution*

A_S = absorbance of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (mg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• ZINC, Method 1

Zinc standard solution: 1000 µg/mL of zinc from zinc oxide dissolved in 5 M hydrochloric acid (3.89 mg/mL),

and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from *Zinc standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Polysorbate 80 solution: Prepare as directed in the assay for *Calcium*, Method 1.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium*, Method 1, except to prepare the *Sample solution* to contain 2 µg/mL of zinc and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Zinc emission line at 213.8 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Capsules taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• BORON, NICKEL, TIN, and VANADIUM, Method 1; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS, and ZINC, Method 2; MOLYBDENUM and SELENIUM, Method 3

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding one volume of *Stock aqua regia solution* to two volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% (v/v) nitric acid solution and 1000 mg/L of scandium in 5% (v/v) nitric acid solution with *Diluent*, (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% (v/v) nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% (v/v) nitric acid solution to obtain a solution with final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/L of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% (v/v) hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% (v/v) hydrochloric acid solution to obtain a solution with final concentrations of about 200 mg/L of boron, and 100 mg/L of chromium, molybdenum, nickel, selenium, tin, and vanadium each.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent*, to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution: Weigh, then transfer 5 Capsules to a 250-mL volumetric flask, and heat gently on a hot plate until the contents begin to release. Cautiously add 25 mL of *Stock aqua regia solution* in 5-mL increments, and swirl. Heat, continue to swirl until the Capsules dissolve into the acid, immediately remove from the heat source, and add 150 mL of water. Cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube, using a 5- μ m pore size nylon syringe filter. If necessary, make any further adjustments using *Diluent*.

Spectrometric conditions

(See *Spectrochemistry* <730>.)

Mode: Inductively coupled plasma spectrometry, using a spectrometer, set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

Sample: *System suitability solution*

[NOTE—Analyze the *System suitability solution* and obtain the response as directed for *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an *Inductively coupled plasma system* using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L, of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*. Calculate the percentage of the labeled amount for each mineral:

$$\text{Result} = C \times (V/W) \times F \times (C_w/L) \times 100$$

- C* = measured concentration of the relevant element in the *Sample solution* (mg/L)
V = volume of the *Sample solution* (L)
W = sample weight (mg)
F = dilution factor of the *Sample solution*
C_w = average Capsule weight (mg)
L = labeled amount of the relevant element/Capsule (mg/Capsule)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc (Zn); and 90.0%–160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V)

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** <2040>: Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS** <2021>: The total aerobic microbial count does not exceed 3000 cfu/g, and the combined molds and yeasts count does not exceed 300 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS** <2022>: Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states that the product is Water-Soluble Vitamins with Minerals Capsules. The label also states the quantity of each vitamin and mineral in terms of metric units per dosage unit and where necessary the chemical form in which a vitamin is present and also states the salt form of the mineral used as the source of each element. Where more than one assay method is given for a particular vitamin or mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS** <11>
 - USP Biotin RS
 - USP Calcium Pantothenate RS
 - USP Cyanocobalamin RS
 - USP Dexpanthenol RS
 - USP Folic Acid RS
 - USP Niacin RS
 - USP Niacinamide RS
 - USP Pyridoxine Hydrochloride RS
 - USP Riboflavin RS
 - USP Sodium Fluoride RS
 - USP Thiamine Hydrochloride RS

Water-Soluble Vitamins with Minerals Oral Solution

DEFINITION

Water-Soluble Vitamins with Minerals Oral Solution contains one or more of the following water-soluble vitamins: Cyanocobalamin, Niacin or Niacinamide, Dexpanthenol or Panthenol, Pantothenic Acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin or Riboflavin-5'-Phosphate Sodium, and Thiamine Hydrochloride or Thiamine Mononitrate; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: iodine, iron, magnesium, manganese, and zinc. It contains NLT 90.0% and NMT 450.0% of the labeled amount of cyanocobalamin (C₆₃H₈₈CoN₁₄O₁₄P); NLT 90.0% and NMT 250.0% of the labeled amount of thiamine (C₁₂H₁₇ClN₄O₅) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 150.0% of the labeled amounts of calcium pantothenate (C₁₈H₃₂CaN₂O₁₀), dexpanthenol (C₉H₁₉NO₄) or panthenol (C₉H₁₉NO₄), niacin (C₆H₅NO₂) or niacinamide (C₆H₆N₂O), pyridoxine hydrochloride (C₈H₁₁NO₃ · HCl), and riboflavin (C₁₇H₂₀N₄O₆) or riboflavin-5'-phosphate sodium (C₁₇H₂₀N₄NaO₉P); NLT 90.0% and NMT 160.0% of the labeled amount of iodine (I); and NLT 90.0% and NMT 125.0% of the labeled amounts of iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn).

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

• CYANOCOBALAMIN

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard stock solution: 1.0 µg/mL of cyanocobalamin from USP Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard stock solution* with water to a measured volume such that after the incubation period as described for *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 ng/mL and 0.04 ng/mL of *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Transfer to an appropriate vessel an accurately measured volume of Oral Solution assumed to contain 1.0 µg of cyanocobalamin for each mL of the Oral Solution taken and 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B₁₂ activity approximately equivalent to that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 µg of biotin, and 20 mg of niacin in 0.02 N acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions. To the resulting solution add 100 mL of water, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary, add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of 5.5–6.0, and add Purified Water to make 250 mL.

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N Hydrochloric acid	10 mL
Adenine–guanine–uracil solution	5 mL
Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter-aid in the supernatant, and filter, with the aid of reduced pressure, through a layer of the filter-aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium

[NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.]

Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium* add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution in test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave, and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by

stab transfer of a pure culture of *Lactobacillus leichmannii*. [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.]

Incubate for 16–24 h at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum

[NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Make a transfer of cells from the *Stock culture* of *Lactobacillus leichmannii* to two sterile tubes each containing 10 mL of the *Culture medium*. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile *Suspension medium*, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10 mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using sterile *Basal medium stock solution*. The cell suspension so obtained is the *Inoculum*.

[NOTE—This dilution may be altered, when necessary, to obtain the desired test response.]

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device. Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis: Because of the high sensitivity of the test organism to minimum amounts of vitamin B₁₂ activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm \times 150-mm test tubes, and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes, add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at

a temperature between 30° and 40°, held constant to within $\pm 0.5^\circ$, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating its contents, read the transmittance at 530 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5% or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances (Σ_u) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the Standard solution corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$), in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = μg of cyanocobalamin assumed to be present in the portion of Oral Solution taken

Calculate the percentage of the labeled amount of cyanocobalamin in the portion of the Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of cyanocobalamin in the portion of Oral Solution taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *Vitamin B₁₂ Activity*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–450.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• **CALCIUM PANTOTHENATE, Method 1**

Mobile phase: Methanol and 0.2 M monobasic sodium phosphate (3:97). Adjust with 1.7 M phosphoric acid to a pH of 3.2 ± 0.1 .

Standard solution: 80 µg/mL of USP Calcium Pantothenate RS in *Mobile phase*

System suitability solution: 80 µg/mL of USP Racemic Panthenol RS in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

Sample solution: Equivalent to 80 µg/mL of calcium pantothenate from the Oral Solution, in *Mobile phase*

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4-mm × 10-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Resolution: NLT 1.5 between panthenol and calcium pantothenate, *System suitability solution*

Tailing factor: NMT 2.0 for both the calcium pantothenate and the panthenol peaks, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for calcium pantothenate from the *Sample solution*

r_S = peak area for calcium pantothenate from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• CALCIUM PANTOTHENATE, Method 2

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide and protected from absorption of moisture while weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed for *Analysis*, 2.0 and 4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Transfer an accurately measured volume of Oral Solution equivalent to 50 mg of calcium pantothenate to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), dilute with water to volume, and filter. Dilute a measured volume of this solution quantitatively, and stepwise if necessary, with water to obtain a solution having about the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve

the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine-tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a refrigerator at a temperature not below 10°.

Adenine-guanine-uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin-thiamine hydrochloride-biotin solution: 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution: 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL
Riboflavin-thiamine hydrochloride-biotin solution	5 mL
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water; add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar; and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121° for 15 min, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*, incubating for 1–24 h at a temperature between 30° and 37° held constant to within $\pm 0.5^\circ$. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution*, add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121° for 15 min, and cool.

Inoculum

[NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Make a transfer of cells from the *Stock culture* of *Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis: To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes, add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Read the transmittance between 540 and 660 nm when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ_s) as the difference, $y = 2.00 - \Sigma_s$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, $y = 2.00 - \Sigma_u$, adding together the two transmittances (Σ_u) for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three

or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in mg, of USP Calcium Pantothenate RS corresponding to the calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = mg of calcium pantothenate assumed to be present in the portion of Oral Solution taken
Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Oral Solution taken:

$$\text{Result} = [(\text{antilog } M)/N] \times 100$$

N = nominal amount of calcium pantothenate in the portion of Oral Solution (mg)

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• DEXPANTHENOL OR PANTHENOL, Method 1

Mobile phase and Chromatographic system: Proceed as directed in the assay for *Calcium Pantothenate*, Method 1.

Standard solution: 80 µg/mL of USP Dexpantenol RS or USP Racemic Panthenol RS in *Mobile phase*. [NOTE—Use USP Dexpantenol RS to analyze Oral Solution that contains dexpantenol and use USP Racemic Panthenol RS to analyze Oral Solution that contains panthenol.]

System suitability solution: 80 µg/mL of USP Calcium Pantothenate RS in *Mobile phase*. Mix the resulting solution and *Standard solution* (1:1).

Sample solution: Equivalent to 80 µg/mL of dexpantenol or panthenol from Oral Solution in *Mobile phase*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of the labeled amount of dexpantenol or panthenol ($C_9H_{19}NO_4$) in the portion of Oral Solution taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

r_u = peak area for dexpantenol or panthenol from the *Sample solution*

r_s = peak area for dexpantenol or panthenol from the *Standard solution*

C_s = concentration of USP Dexpantenol RS or USP Racemic Panthenol RS in the *Standard solution* (mg/mL)

C_u = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpantenol or panthenol ($C_9H_{19}NO_4$)

• DEXPANTHENOL OR PANTHENOL, Method 2

[NOTE—The following procedure is applicable also to the determination of the dextrorotatory component of racemic panthenol in preparations containing panthenol.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 800 µg/mL of USP

Dexpanthenol RS or 1600 µg/mL of USP Racemic Panthenol RS in water. Store in a refrigerator, protected from light, and use within 30 days. [NOTE—Use USP Dexpanthenol RS to analyze Oral Solution that contains dexpanthenol and use USP Racemic Panthenol RS to analyze Oral Solution that contains panthenol.]

Standard solution: On the day of the assay, prepare 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of racemic panthenol from *Standard stock solution* with water.

Sample solution: 1.2 µg/mL of dexpanthenol or 2.4 µg/mL of panthenol from Oral Solution in water

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in about 500 mL of water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1 , and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in a solution of 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700 to 800 mL of water, heat to $75 \pm 5^\circ$, and add 6 M hydrochloric acid dropwise, with stirring, until the solids are dissolved. Cool, add water to make 1000 mL, and mix. Store under toluene in a refrigerator at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, add water to make 200 mL, and mix. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution: Prepare a solution of riboflavin, thiamine hydrochloride, and biotin in 0.02 N acetic acid containing 20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: Prepare a solution in neutral 25% alcohol containing 10 µg/mL of p-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride. Store in a refrigerator.

Salt solution 1: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Salt solution 2: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Pyridoxal–calcium pantothenate solution: Dissolve 40 mg of pyridoxal hydrochloride and 375 µg of calcium pantothenate in 10% alcohol to make 200 mL, and mix. Store in a refrigerator, and use within 30 days.

Polysorbate 40–oleic acid solution: Dissolve 25 g of polysorbate 40 and 0.25 g of oleic acid in 20% alcohol to make 500 mL, and mix. Store in a refrigerator, and use within 30 days.

Modified pantothenate medium

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g

Adenine–guanine–uracil solution	5 mL
Riboflavin–thiamine hydrochloride–biotin solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution 1	5 mL
Salt solution 2	5 mL
Pyridoxal–calcium pantothenate solution	5 mL
Polysorbate 40–oleic acid solution	5 mL

Dissolve anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Finally, dilute with water to 250 mL, and mix.

Double-strength modified pantothenate medium:

Prepare as directed under *Modified pantothenate medium*, but make the final dilution to 125 mL instead of 250 mL. Prepare fresh.

Stock culture of *Pediococcus acidilactici*: Dissolve in 800 mL of water, with the aid of heat, 6.0 g of peptone, 4.0 g of pancreatic digest of casein, 3.0 g of yeast extract, 1.5 g of beef extract, 1.0 g of dextrose, and 15.0 g of agar. Adjust with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid to a pH between 6.5 and 6.6, dilute with water to 1000 mL, and mix. Add 10-mL portions of the solution to culture tubes, place caps on the tubes, and sterilize in an autoclave at 121° for 15 min. Cool on a slant, and store in a refrigerator. Prepare a stock culture of *Pediococcus acidilactici*¹ on a slant of this medium. Incubate at 35° for 20–24 h, and store in a refrigerator. Maintain the stock culture by monthly transfer onto fresh slants.

Inoculum: Inoculate three 250-mL portions of *Modified pantothenate medium* from a stock culture slant, and incubate at 35° for 20–24 h. Centrifuge the suspension from the combined portions, and wash the cells with *Modified pantothenate medium*. Resuspend the cells in sufficient *Modified pantothenate medium* so that a 1-in-50 dilution, when tested in a 13-mm diameter test tube, gives 80% light transmission at 530 nm. Transfer 1.2-mL portions of this stock suspension to glass ampuls, seal, freeze in liquid nitrogen, and store in a freezer. On the day of the assay, allow the ampuls to reach room temperature, mix the contents, and dilute 1 mL of thawed culture with sterile saline TS to 150 mL. [NOTE—This dilution may be altered when necessary to obtain the desired test response.]

Analysis: Prepare in triplicate a series of eight culture tubes by adding the following quantities of water to the tubes within a set: 5.0, 4.5, 4.0, 3.5, 3.0, 2.0, 1.0, and 0.0 mL. To these same tubes, and in the same order, add 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*.

Prepare in duplicate a series of five culture tubes by adding the following quantities of water to the tubes within a set: 4.0, 3.5, 3.0, 2.0, and 1.0 mL. To these same tubes, and in the same order, add 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*.

Add 5.0 mL of *Double-strength modified pantothenate medium* to each tube, and mix. Cover the tubes with metal caps, and sterilize in an autoclave at 121° for 5 min. Cool to room temperature in a chilled water bath, and inoculate each tube with 0.5 mL of the *Inoculum*. Allow to incubate at 37° for 16 h. Terminate growth by heating to a temperature not below 80°, such as by steaming at atmospheric pressure in a sterilizer for 5 to 10 min. Cool, and concomitantly determine the percentage transmittance of the suspensions, in cells of equal path length, on a suitable spectrophotometer, at a wavelength of 530 nm.

Calculation: Draw a dose-response curve on arithmetic graph paper by plotting the average response, in percent transmittance, for each set of tubes of the

¹ATCC No. 8042 is suitable.

standard curve against the standard level concentrations. The curve is drawn by connecting each adjacent pair of points with a straight line. From this standard curve, determine by interpolation the potency, in terms of dexpanthenol, of each tube containing portions of the *Sample solution*. Divide the potency of each tube by the amount of the *Sample solution* added to it, to obtain the individual responses. Calculate the mean response by averaging the individual responses that vary from their mean by NMT 15%, using NLT half the total number of tubes. Calculate the potency of the portion of the material taken for assay, by multiplying the mean response by the appropriate dilution factor. Calculate the percentage of the labeled amount of dexpanthenol or panthenol in the portion of Oral Solution taken:

$$\text{Result} = (P/N) \times 100$$

P = calculated potency of dexpanthenol or panthenol in the portion of Oral Solution taken (mg)

N = nominal amount of dexpanthenol or panthenol in the portion of Oral Solution taken (mg)

Acceptance criteria: 90.0%–150.0% of the labeled amount of dexpanthenol or panthenol ($\text{C}_9\text{H}_{19}\text{NO}_4$)

• NIACIN OR NIACINAMIDE

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: 25 mg/mL of edetate disodium in water

Mobile phase: Methanol, glacial acetic acid, triethylamine, and 0.008 M sodium 1-hexanesulfonate (350:15:0.4:1634.6)

Standard solution: 0.10 mg/mL of USP Niacin RS or USP Niacinamide RS in *Diluent*

[NOTE—Use USP Niacin RS for Oral Solution that contains niacin and use USP Niacinamide RS for Oral Solution that contains niacinamide.]

Sample solution: Dilute an accurately measured volume of Oral Solution with *Diluent* to obtain a solution with a concentration of 0.1 mg/mL of niacin or niacinamide.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm \times 25-cm; packing L7

Flow rate: 2 mL/min

Injection size: 5 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of niacin ($\text{C}_6\text{H}_5\text{NO}_2$) or niacinamide ($\text{C}_6\text{H}_6\text{N}_2\text{O}$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for niacin or niacinamide from the *Sample solution*

r_S = peak area for niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($\text{C}_6\text{H}_5\text{NO}_2$) or niacinamide ($\text{C}_6\text{H}_6\text{N}_2\text{O}$)

• PYRIDOXINE HYDROCHLORIDE

Diluent, Mobile phase, and Chromatographic system:
Proceed as directed in the assay for *Niacin* or *Niacinamide*.

Standard solution: Equivalent to 24 μ g/mL of USP Pyridoxine Hydrochloride RS in *Diluent*

Sample solution: Equivalent to 24 μ g/mL of pyridoxine hydrochloride from Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($\text{C}_8\text{H}_{11}\text{NO}_3 \cdot \text{HCl}$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for pyridoxine hydrochloride from the *Sample solution*

r_S = peak area for pyridoxine hydrochloride from the *Standard solution*

C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($\text{C}_8\text{H}_{11}\text{NO}_3 \cdot \text{HCl}$)

• RIBOFLAVIN-5'-PHOSPHATE SODIUM, Method 1

[NOTE—Riboflavin-5'-phosphate sodium is quantitated against USP Riboflavin RS in this procedure. In the chromatogram of the *Sample solution*, the riboflavin-5'-phosphate peak is the only peak measured for calculation.]

Diluent, Mobile phase, and Chromatographic system:
Proceed as directed in the assay for *Niacin* or *Niacinamide*.

Standard solution: Equivalent to 8 μ g/mL of USP Riboflavin RS in *Diluent*, by heating if necessary

Sample solution: Equivalent to 8 μ g/mL of riboflavin from the Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*
[NOTE—The relative retention times for riboflavin-5'-phosphate and riboflavin are about 0.18 and 1.0, respectively.]

Calculate the percentage of the labeled amount of riboflavin ($\text{C}_{17}\text{H}_{20}\text{N}_4\text{O}_6$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak area for riboflavin-5'-phosphate from the *Sample solution*

r_S = peak area for riboflavin from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

F = factor for converting the response obtained for riboflavin-5'-phosphate to riboflavin, 1.493 [NOTE—Riboflavin Phosphate Sodium is a mixture of isomeric monophosphates and diphosphates containing an average amount of 67% of riboflavin-5'-monophosphate, which separates in this chromatographic system. The factor 1.493 assumes 67% of riboflavin-5'-monophosphate.]

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($\text{C}_{17}\text{H}_{20}\text{N}_4\text{O}_6$)

- **RIBOFLAVIN OR RIBOFLAVIN-5'-PHOSPHATE SODIUM, Method 2**
[NOTE—Use low-actinic glassware throughout this procedure.]

Solvent blank: 1 N hydrochloric acid, 2.5 M sodium acetate, and water (1:2:97)

Riboflavin stock solution: 0.16 mg/mL of USP Riboflavin RS in 1 N hydrochloric acid, 2.5 M sodium acetate, and water (1:2:97). Mix the resulting solution and water (1:1:9).

Standard solution: *Riboflavin stock solution*, 1 N hydrochloric acid, 2.5 M sodium acetate, and water (1:1:2:96)

Sample solution: Transfer equivalent to 0.8 mg of riboflavin from the Oral Solution to a 100-mL volumetric flask, and dilute with water to volume. Mix the resulting solution, 1 N hydrochloric acid, 2.5 M sodium acetate, and water (2:1:2:95).

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851)).

Mode: Fluorescence

Analytical wavelength

Excitation: 440 nm

Emission: 530 nm

Blank: *Solvent blank*

Analysis

Samples: *Standard solution* and *Sample solution*
Determine the maximum fluorescence intensities, I_S and I_U , of the *Standard solution* and the *Sample solution*, respectively. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Oral Solution taken:

$$\text{Result} = (I_U/I_S) \times (C_S/C_U) \times 100$$

I_U = fluorescence value from the *Sample solution*

I_S = fluorescence value from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

- **THIAMINE**

Diluent, Mobile phase, and Chromatographic system:
Proceed as directed in the assay for *Niacin* or *Niacinamide*.

Standard solution: 24 µg/mL of USP Thiamine Hydrochloride RS in *Diluent*

Sample solution: Equivalent to 24 µg/mL of thiamine hydrochloride from the Oral Solution in *Diluent*

Analysis

Samples: *Standard solution* and *Sample solution*
For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for thiamine from the *Sample solution*

r_S = peak area for thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–250.0% of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$)

- **IODIDE, Method 1**

Mobile phase: Dissolve 5.15 g of tetrabutylammonium bromide in 320 mL of acetonitrile. Dilute with water to 2000 mL.

Standard stock solution: 1.3 mg/mL of potassium iodide in *Mobile phase*. This solution has a concentration of 1 mg/mL of iodide.

Standard solution: 2.5 µg/mL of iodide from *Standard stock solution* in *Mobile phase*

System suitability solution: Transfer 0.13 g of potassium iodide and 0.5 g of potassium iodate to a 100-mL volumetric flask. Dissolve in *Mobile phase*, using sonication if necessary, dilute with *Mobile phase* to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, dilute with *Mobile phase* to volume, and mix. Transfer 25.0 mL of this solution to a 100-mL volumetric flask, and dilute with *Mobile phase* to volume.

Sample solution: Dilute an accurately measured volume of Oral Solution to obtain a solution having a concentration of 2.5 µg/mL of iodine in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 225 nm

Column: 4.6-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 30 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for iodate and iodide are about 0.32 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.5 between iodate and iodide, *System suitability solution*

Relative standard deviation: NMT 2.0% for the iodide peak, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of the labeled amount of iodine (I) in the portion of Oral Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area for iodide from the *Sample solution*

r_S = peak area for iodide from the *Standard solution*

C_S = concentration of iodide in the *Standard solution* (µg/mL)

C_U = nominal concentration of iodine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

- **IODIDE, Method 2:** Proceed as directed under *Automated Methods of Analysis* (16), *Assay for Iodide*.

- **IRON**

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask, dissolve in 6 N hydrochloric acid, and dilute with water to volume.

Standard solutions: To separate 100-mL volumetric flasks, transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Sample solution: 6 µg/mL of iron from Oral Solution in 0.125 N hydrochloric acid

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Lamp: Iron hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 248.3 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions, using the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of iron, and draw the

straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of iron in the *Sample solution*. Calculate the percentage of the labeled amount of iron (Fe) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of iron in the *Sample solution* from the graph ($\mu\text{g/mL}$)

C_U = nominal concentration of iron in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• MAGNESIUM

Magnesium standard solution: Transfer 1.00 g of magnesium ribbon to a 1000-mL volumetric flask. Dissolve in 50 mL of 6 N hydrochloric acid, and dilute with water to volume.

Standard stock solution: 20 $\mu\text{g/mL}$ of magnesium from *Magnesium standard solution* in 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 7.5, 10.0, 12.5, and 15.0 mL of *Standard stock solution*. Dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 $\mu\text{g/mL}$ of magnesium.

Sample solution: 2.5 $\mu\text{g/mL}$ of magnesium from Oral Solution in 0.125 N hydrochloric acid

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 285.2 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions, using the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in $\mu\text{g/mL}$, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of magnesium in the *Sample solution*. Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of magnesium in the *Sample solution* from the graph ($\mu\text{g/mL}$)

C_U = nominal concentration of magnesium in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• MANGANESE

Manganese standard stock solution: Transfer 1.0 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, and dilute with 6 N hydrochloric acid to volume.

Standard stock solution: 50 $\mu\text{g/mL}$ of manganese from *Manganese standard stock solution* in 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions having known concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 $\mu\text{g/mL}$ of manganese.

Sample solution: Dilute an accurately measured volume of the Oral Solution to obtain a solution having a concentration of 1.5 $\mu\text{g/mL}$ of manganese in 0.125 N hydrochloric acid

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 279.5 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions, using the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in $\mu\text{g/mL}$, of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of manganese in the *Sample solution*. Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of manganese in the *Sample solution* from the graph ($\mu\text{g/mL}$)

C_U = nominal concentration of manganese in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% labeled amount of manganese (Mn)

• ZINC

Zinc standard solution: Transfer 311 mg of zinc oxide to a 250-mL volumetric flask, and add 80 mL of 6 N hydrochloric acid, warming if necessary to dissolve. Cool, dilute with water to volume, and mix to obtain a solution having a known concentration of 1000 μg of zinc/mL.

Standard stock solution: 50 $\mu\text{g/mL}$ of zinc from *Zinc standard solution* in 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 $\mu\text{g/mL}$ of zinc.

Sample solution: Dilute an accurately measured volume of Oral Solution to obtain a solution having a concentration of 1 $\mu\text{g/mL}$ of zinc in 0.125 N hydrochloric acid

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Analytical wavelength: 213.8 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the solutions, using the *Blank*. Plot the absorbances of the *Standard solutions* versus concentration, in $\mu\text{g/mL}$, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of zinc in the *Sample solution*. Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Oral Solution taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of zinc in the *Sample solution* from the graph ($\mu\text{g/mL}$)

C_U = nominal concentration of zinc in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

OTHER COMPONENTS

- **ALCOHOL DETERMINATION**, *Method 1* (611) (if present): 90.0%–120.0% of the labeled amount of C_2H_5OH

CONTAMINANTS

- **MICROBIAL ENUMERATION TEST—NUTRITIONAL AND DIETARY SUPPLEMENTS (2021)**: The total aerobic microbial count does not exceed 3000 cfu/mL, and the combined molds and yeasts count does not exceed 300 cfu/mL.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS (2022)**: Meets the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight, light-resistant containers, under an inert gas or with a minimum of headspace.
- **LABELING**: The label states that the product is Water-Soluble Vitamins with Minerals Oral Solution. The label states the quantity of each vitamin and mineral present in terms of metric units in a given volume of the Oral Solution and, where necessary, the chemical form in which a vitamin is present, and also states the salt form of the mineral used as the source of each element. Where products are labeled to contain panthenol, the label states the equivalent content of dexpanthenol. Where more than one assay method is given for a particular vitamin or mineral, the labeling states with which assay method the product complies only if *Method 1* is not used.
- **USP REFERENCE STANDARDS (11)**
 - USP Calcium Pantothenate RS
 - USP Cyanocobalamin RS
 - USP Dexpanthenol RS
 - USP Niacin RS
 - USP Niacinamide RS
 - USP Pyridoxine Hydrochloride RS
 - USP Racemic Panthenol RS
 - USP Riboflavin RS
 - USP Thiamine Hydrochloride RS

Water-Soluble Vitamins with Minerals Tablets

DEFINITION

Water-Soluble Vitamins with Minerals Tablets contain one or more of the following water-soluble vitamins: Ascorbic Acid or its equivalent as Calcium Ascorbate or Sodium Ascorbate, Biotin, Cyanocobalamin, Folic Acid, Niacin or Niacinamide, Pantothenic Acid (as Calcium Pantothenate or Racemic Calcium Pantothenate), Pyridoxine Hydrochloride, Riboflavin, and Thiamine Hydrochloride or Thiamine Mononitrate; and one or more minerals derived from substances generally recognized as safe, furnishing one or more of the following elements in ionizable form: boron, calcium, chromium, copper, fluorine, iodine, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, selenium, tin, vanadium, and zinc. Tablets contain NLT 90.0% and NMT 150.0% of the labeled amounts of ascorbic acid ($C_6H_8O_6$) or its salts as calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$) or sodium ascorbate ($C_6H_7NaO_6$), biotin ($C_{10}H_{16}N_2O_3S$), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$), folic acid ($C_{19}H_{19}N_7O_6$), calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$), niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine ($C_{12}H_{17}ClN_4OS$) as thiamine hydrochloride or thiamine mononitrate; NLT 90.0% and NMT 125.0% of the labeled amounts of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), potassium (K), and zinc

(Zn); and NLT 90.0% and NMT 160.0% of the labeled amounts of boron (B), chromium (Cr), fluorine (F), iodine (I), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

They do not contain any form of Beta Carotene or Vitamin A, D, E, or K. They may contain other labeled added substances that are generally recognized as safe, in amounts that are unobjectionable.

STRENGTH

[NOTE—In the following assays, where more than one assay method is given for an individual ingredient, the requirements may be met by following any one of the specified methods, the method used being stated in the labeling only if *Method 1* is not used.]

- **ASCORBIC ACID**, *Method 1*

[NOTE—Finely powder NLT 20 Tablets.]

Sample solution: Transfer a portion of powdered Tablets, equivalent to a nominal amount of 100 mg of ascorbic acid, to a 200-mL volumetric flask, and add 75 mL of metaphosphoric–acetic acids TS. Insert a stopper into the flask, and shake by mechanical means for 30 min. Dilute with water to volume. Transfer a portion of the solution to a centrifuge tube, and centrifuge until a clear supernatant is obtained. Pipet 4.0 mL of this solution into a 50-mL conical flask, and add 5 mL of metaphosphoric–acetic acids TS.

Analysis: Titrate with standard dichlorophenol–indophenol solution VS to a rose-pink color that persists for at least 5 s. Correct for the volume of dichlorophenol–indophenol solution consumed by a mixture of 5.5 mL of metaphosphoric–acetic acids TS and 15 mL of water. From the ascorbic acid equivalent of the standard dichlorophenol–indophenol solution, calculate the content of ascorbic acid in each Tablet.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **ASCORBIC ACID**, *Method 2:* Proceed as directed in *Automated Methods of Analysis (16)*, *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of ascorbic acid ($C_6H_8O_6$)

- **CALCIUM ASCORBATE**, *Method 1:* Proceed as directed in the assay for *Ascorbic Acid*, *Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

- **CALCIUM ASCORBATE**, *Method 2:* Proceed as directed in *Automated Methods of Analysis (16)*, *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium ascorbate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$)

- **SODIUM ASCORBATE**, *Method 1:* Proceed as directed in the assay for *Ascorbic Acid*, *Method 1*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **SODIUM ASCORBATE**, *Method 2:* Proceed as directed in *Automated Methods of Analysis (16)*, *Assay for Ascorbic Acid*.

Acceptance criteria: 90.0%–150.0% of the labeled amount of sodium ascorbate ($C_6H_7NaO_6$)

- **BIOTIN**, *Method 1*

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Mix 85 mL of acetonitrile, 1 g of sodium perchlorate, and 1 mL of phosphoric acid, and dilute with water to 1000 mL.

Standard stock solution: 0.333 mg/mL of USP Biotin RS in dimethyl sulfoxide

Standard solution: 5 µg/mL of USP Biotin RS prepared by diluting the *Standard stock solution* in water

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of powder, equivalent to a nominal amount of 1 mg of biotin, to a 200-mL volumetric flask, add 3 mL of dimethyl sulfoxide, and swirl to wet. Place the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with water to volume, and filter.

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 200 nm**Column:** 4.6-mm × 15-cm; 3-μm packing L7**Flow rate:** 1.2 mL/min**Injection size:** 100 μL**System suitability****Sample:** *Standard solution***Suitability requirements****Relative standard deviation:** NMT 3.0%**Analysis****Samples:** *Standard solution* and *Sample solution*

Measure the responses for the biotin peaks. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

 r_U = peak response from the *Sample solution* r_S = peak response from the *Standard solution* C_S = concentration of USP Biotin RS in the *Standard solution* (μg/mL) C_U = nominal concentration of biotin in the *Sample solution* (μg/mL)**Acceptance criteria:** 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)• **BIOTIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Dehydrated mixtures yielding formulations similar to the media described herein may be used provided that, when constituted as directed, they have growth-promoting properties equal to or superior to those obtained with the media prepared as described herein.

Standard stock solution: 50 μg/mL of USP Biotin RS in 50% alcohol. Store this solution in a refrigerator.**Standard solution:** On the day of the assay, dilute the *Standard stock solution* with water to a concentration of 0.1 ng/mL of USP Biotin RS.**Sample solution:** Finely powder NLT 30 Tablets.

Transfer a the portion of powder, equivalent to a nominal amount of 100 μg of biotin, to a 200-mL volumetric flask. Add 3 mL of 50% alcohol, and swirl to wet the contents. Heat the flask in a water bath at 60°–70° for 5 min. Sonicate for 5 min, dilute with diluted alcohol to volume, and filter. Dilute a volume of the filtrate, quantitatively, and stepwise if necessary, with water to obtain a solution with a concentration of 0.1 ng/mL.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine in 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Calcium pantothenate solution: 10 μg/mL of calcium pantothenate in 50% alcohol. Store in a refrigerator.

Riboflavin–thiamine hydrochloride solution: 20 μg/mL of riboflavin and 10 μg/mL of thiamine hydrochloride in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 μg/mL of p-aminobenzoic acid, 50 μg/mL of niacin, and 40 μg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid, and mix. Store under toluene.

Basal medium stock solution

Acid-hydrolyzed casein solution	25 mL
Cystine–tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine–guanine–uracil solution	5 mL
Calcium pantothenate solution	5 mL
Riboflavin–thiamine hydrochloride solution	5 mL
p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water. Add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121°, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*,¹ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution*, add 5.0 mL of water containing 0.5 ng of biotin. Plug the tubes with cotton, sterilize in an autoclave at 121°, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture* of *Lactobacillus plantarum* to a sterile tube containing 10 mL of culture medium. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

¹ ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

Analysis

Samples: *Standard solution* and *Sample solution*

To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes, add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37° held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of Standard during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to a spectrophotometer cell. Place the cell in a spectrophotometer that has been set at a specific wavelength of 540–660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittance (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y , adding together the two transmittances for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of USP Biotin RS corresponding to the biotin in each mg of the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of biotin that was assumed to be present in each mg in the portion of the Tablets taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$)

• **BIOTIN, Method 3**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 800 mL of water and 100 mL of triethylamine to a 1000-mL volumetric flask. Add 80 mL of 85% phosphoric acid, and dilute with water to volume.

Mobile phase: Transfer 80 mL of acetonitrile and 10 mL of *Solution A* to a 1000-mL volumetric flask. Dilute with water to volume.

Standard solution: 0.6 $\mu\text{g}/\text{mL}$ of USP Biotin RS in water. [NOTE—A portion of the *Standard solution* will be used to determine the percent recovery of biotin from the *Solid-phase extraction* procedure.]

Sample solution: Finely powder NLT 20 Tablets. Transfer an amount of powdered Tablets to a volumetric flask to obtain a nominal concentration of 0.6 $\mu\text{g}/\text{mL}$ of biotin. Add water up to 80% of the flask capacity, sonicate for 30–40 min with occasional mixing to dissolve, dilute with water to volume, and filter. Adjust the pH of the solution with either diluted acetic acid or 0.1 N sodium hydroxide to between 6.0 and 7.0.

Solid-phase extraction

[NOTE—Condition the extraction column specified in this procedure in the following manner. Wash the column with a 2-mL portion of methanol. Equilibrate with a 2-mL portion of water.]

Separately pipet 5.0 mL of the *Sample solution* and *Standard solution* into freshly conditioned solid-phase extraction columns consisting of a mixed-mode packing with a sorbent-mass of 60 mg. [NOTE—The mixed-mode packing consists of anion-exchange and reversed-phase sorbents. The reverse-phase component is a polymer of copolymer *N*-vinylpyrrolidone and divinylbenzene. The anion exchange moiety is a trialkylamino group.²]

Wash the column with 10 mL of 30% methanol in water. Apply an appropriate volume (4.9 mL) of 30% methanol in 0.1 N hydrochloric acid to the column. Collect the eluate in a 5-mL volumetric flask, containing 100 μL of 40% (w/v) sodium acetate in water, and dilute with 30% methanol in 0.1 N hydrochloric acid to volume.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm \times 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 100 μL

System suitability

Samples: *Standard solution* and a portion of the *Standard solution* that has undergone *Solid-phase extraction*

Suitability requirements

Tailing factor: NMT 1.5, *Standard solution*

Relative standard deviation: NMT 2.0%, *Standard solution* and the *Standard solution* that has undergone *Solid-phase extraction*

² A suitable cartridge is the Waters, Oasis MAX Vac RC cartridge, particle size 30 μm , part 186000371.

Recovery: 95%–100%, *Standard solution* that has undergone *Solid-phase extraction*

Analysis

Samples: *Standard solution* and *Sample solution* that have both undergone *Solid-phase extraction*

Measure the responses for the biotin peak. Calculate the percentage of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Biotin RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of biotin in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of biotin ($C_{10}H_{16}N_2O_3S$)

• CYANOCOBALAMIN, Method 1

[NOTE—Use low-actinic glassware throughout this procedure.]

Mobile phase: Methanol and water (7:13)

Standard stock solution: 10 $\mu\text{g/mL}$ of USP Cyanocobalamin RS in water. [NOTE—Store this stock solution in a dark place, and discard after 1 week.]

Standard solution: 1 $\mu\text{g/mL}$ of USP Cyanocobalamin RS from the *Standard stock solution*, diluted with water

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to a nominal amount of 100 μg of cyanocobalamin, to a 250-mL flask. Quantitatively add 100.0 mL of water, and carefully extract for 2 min. Filter 10 mL of the extract, and use the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: 550 nm

Column: 4.6-mm \times 15-cm; 5- μm packing L1

Flow rate: 0.5 mL/min

Injection size: 200 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for cyanocobalamin.

Calculate the percentage of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of cyanocobalamin from the *Sample solution*

r_S = peak response of cyanocobalamin from the *Standard solution*

C_S = concentration of USP Cyanocobalamin RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of cyanocobalamin in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$)

• CYANOCOBALAMIN, Method 2

[NOTE—Use low-actinic glassware throughout this procedure.]

Standard cyanocobalamin stock solution: 1.0 $\mu\text{g/mL}$ of USP Cyanocobalamin RS in 25% alcohol. Store in a refrigerator.

Standard solution: Dilute a suitable volume of *Standard cyanocobalamin stock solution* with water to a measured volume such that after the incubation period as directed in the *Analysis*, the difference in transmittance between the inoculated blank and the 5.0-mL level of

the *Standard solution* is NLT that which corresponds to a difference of 1.25 mg in dried cell weight. This concentration usually falls between 0.01 and 0.04 ng/mL of *Standard solution*. Prepare this solution fresh for each assay.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powdered Tablets, equivalent to a nominal amount of 1.0 μg of cyanocobalamin, to an appropriate vessel containing, for each g of powdered Tablets taken, 25 mL of an aqueous extracting solution prepared just before use to contain, in each 100 mL, 1.29 g of dibasic sodium phosphate, 1.1 g of anhydrous citric acid, and 1.0 g of sodium metabisulfite. Autoclave the mixture at 121° for 10 min. Allow any undissolved particles of the extract to settle, and filter or centrifuge if necessary. Dilute an aliquot of the clear solution with water to obtain a final solution containing vitamin B₁₂ activity equivalent to the nominal activity of the *Standard solution*.

Acid-hydrolyzed casein solution: Prepare as directed in the assay for *Calcium Pantothenate*, Method 2.

Asparagine solution: Dissolve 2.0 g of L-asparagine in water to make 200 mL. Store under toluene in a refrigerator.

Adenine-guanine-uracil solution: Prepare as directed in the assay for *Calcium Pantothenate*, Method 2.

Xanthine solution: Suspend 0.20 g of xanthine in 30–40 mL of water, heat to 70°, add 6.0 mL of 6 N ammonium hydroxide, and stir until the solid is dissolved. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Salt solution A: Dissolve 10 g of monobasic potassium phosphate and 10 g of dibasic potassium phosphate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Salt solution B: Dissolve 4.0 g of magnesium sulfate, 0.20 g of sodium chloride, 0.20 g of ferrous sulfate, and 0.20 g of manganese sulfate in water to make 200 mL, and add 2 drops of hydrochloric acid. Store this solution under toluene.

Polysorbate 80 solution: Dissolve 20 g of polysorbate 80 in alcohol to make 200 mL. Store in a refrigerator.

Vitamin solution A: Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 100 μg of biotin, and 20 mg of niacin in 0.02 N glacial acetic acid to make 400 mL. Store under toluene, protected from light, in a refrigerator.

Vitamin solution B: Dissolve 20 mg of *p*-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in a mixture of water and neutralized alcohol (3:1) to make 400 mL. Store, protected from light, in a refrigerator.

Basal medium stock solution: Prepare the medium according to the following formula and directions. A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium comparable to that obtained from the formula given herein.

Add the ingredients in the order listed, carefully dissolving cystine and tryptophan in the hydrochloric acid before adding the next eight solutions in the resulting solution. Add 100 mL of water, and dissolve the dextrose, sodium acetate, and ascorbic acid. Filter, if necessary. Add the *Polysorbate 80 solution*, adjust with 1 N sodium hydroxide to a pH of between 5.5 and 6.0, and add Purified Water to make 250 mL.

L-Cystine	0.1 g
L-Tryptophan	0.05 g
1 N hydrochloric acid	10 mL
Adenine-guanine-uracil solution	5 mL

Xanthine solution	5 mL
Vitamin solution A	10 mL
Vitamin solution B	10 mL
Salt solution A	5 mL
Salt solution B	5 mL
Asparagine solution	5 mL
Acid-hydrolyzed casein solution	25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Ascorbic acid	1 g
Polysorbate 80 solution	5 mL

Tomato juice preparation: Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend 5 g/L of analytical filter aid in the supernatant, and pass, with the aid of reduced pressure, through a layer of the filter aid. Repeat, if necessary, until a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

Culture medium: [NOTE—A dehydrated mixture containing the same ingredients may be used provided that, when constituted as directed in the labeling, it yields a medium equivalent to that obtained from the formula given herein.] Dissolve 0.75 g of yeast extract, 0.75 g of dried peptone, 1.0 g of anhydrous dextrose, and 0.20 g of monobasic potassium phosphate in 60–70 mL of water. Add 10 mL of *Tomato juice preparation* and 1 mL of *Polysorbate 80 solution*. Adjust with 1 N sodium hydroxide to a pH of 6.8, and add water to make 100 mL. Place 10-mL portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121° for 15 min. Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Suspension medium: Dilute a measured volume of *Basal medium stock solution* with an equal volume of water. Place 10-mL portions of the diluted medium in test tubes. Sterilize, and cool as directed for *Culture medium*.

Stock culture of *Lactobacillus leichmannii*: To 100 mL of *Culture medium*, add 1.0–1.5 g of agar, and heat the mixture on a steam bath, with stirring, until the agar dissolves. Place 10-mL portions of the hot solution into test tubes, cover the tubes, sterilize at 121° for 15 min in an autoclave (exhaust line temperature), and allow the tubes to cool in an upright position. Inoculate three or more of the tubes by stab transfer of a pure culture of *Lactobacillus leichmannii*.³ [NOTE—Before first using a fresh culture in this assay, make NLT 10 successive transfers of the culture in a 2-week period.] Incubate for 16–24 h at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$. Store in a refrigerator.

Prepare fresh stab cultures at least three times each week, and do not use them for preparing the *Inoculum* if more than 4 days old. The activity of the microorganism can be increased by daily or twice-daily transfer of the stab culture, to the point where definite turbidity in the liquid *Inoculum* can be observed 2–4 h after inoculation. A slow-growing culture seldom gives a suitable response curve and may lead to erratic results.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus leichmannii* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.] Make a transfer of cells from the *Stock culture of Lactobacillus leichmannii* to two sterile tubes containing 10 mL of the *Culture medium* each. Incubate these cultures for 16–24 h at a temperature between 30° and 40° held constant to within $\pm 0.5^\circ$. Under aseptic conditions centrifuge the cultures, and decant the supernatant. Suspend the cells from the culture in 5 mL of sterile *Suspension medium*, and combine. Using sterile

Suspension medium, adjust the volume so that a 1-in-20 dilution in saline TS produces 70% transmittance when read on a suitable spectrophotometer that has been set at a wavelength of 530 nm, equipped with a 10-mm cell, and read against saline TS set at 100% transmittance. Prepare a 1-in-400 dilution of the adjusted suspension using *Basal medium stock solution*. The cell suspension so obtained is the *Inoculum*. [NOTE—This dilution may be altered, when necessary, to obtain the desired test response.]

Calibration of spectrophotometer: Check the wavelength of the spectrophotometer periodically, using a standard wavelength cell or other suitable device.

Before reading any tests, calibrate the spectrophotometer for 0% and 100% transmittance, using water and with the wavelength set at 530 nm.

Analysis

Samples: *Standard solution* and *Sample solution*

Because of the high sensitivity of the test organism to minute amounts of vitamin B₁₂ activity and to traces of many cleansing agents, cleanse meticulously by suitable means, followed preferably by heating at 250° for 2 h, using hard-glass 20-mm \times 150-mm test tubes, and other necessary glassware.

To separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each of these tubes and to four similar empty tubes, add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, 1.0, 1.5, 2.0, 3.0, and 4.0 mL of the *Sample solution*. To each tube add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of Standard and sample tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order. Cover the tubes to prevent bacterial contamination, and sterilize in an autoclave at 121° for 5 min, arranging to reach this temperature in NMT 10 min by preheating the autoclave if necessary. Cool as rapidly as possible to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, because packing the tubes too closely in the autoclave or overloading it may cause variation in the heating rate.

Aseptically add 0.5 mL of *Inoculum* to each tube so prepared, except two of the four containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 40°, held constant to within $\pm 0.5^\circ$, for 16–24 h.

Terminate growth by heating to a temperature NLT 80° for 5 min. Cool to room temperature. After agitating its contents, place the container in a spectrophotometer that has been set at a wavelength of 530 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of the inoculated blank. If the difference is greater than 5%, or if there is evidence of contamination with a foreign microorganism, disregard the results of the assay.

With the transmittance set at 100% for the uninoculated blank, read the transmittance of each of the remaining tubes. Disregard the results of the assay if the slope of the standard curve indicates a problem with sensitivity.

Calculation: Prepare a standard concentration-response curve by the following procedure. Test for and replace any aberrant individual transmittances. For each level of the Standard, calculate the response from the sum of the duplicate values of the transmittances

³ Pure cultures of *Lactobacillus leichmannii* may be obtained as No. 7830 from ATCC, 10801 University Blvd., Manassas, VA 20110-2209.

(Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points. Calculate the response, y , adding together the two transmittances for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that falls within the range of the lowest and highest points plotted for the Standard. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in μg , of USP Cyanocobalamin RS corresponding to the cyanocobalamin in each mg of the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of μg of cyanocobalamin that was assumed to be present in each mg in the portion of Tablets taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *Confidence Intervals for Individual Assays*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}$)

• **Folic Acid, Method 1**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent A: 25% solution of tetrabutylammonium hydroxide in methanol

Reagent B: Transfer 5.0 g of pentetic acid to a 50-mL volumetric flask. Using sonication if necessary, dissolve in and dilute with 1 N sodium hydroxide to volume.

Mobile phase: 2 g of monobasic potassium phosphate in 650 mL of water. Add 12.0 mL of *Reagent A*, 7.0 mL of 3 N phosphoric acid, and 240 mL of methanol. Cool to room temperature, adjust with phosphoric acid or ammonia TS to a pH of 7.0, dilute with water to 1000 mL, and filter. Recheck the pH before use.

[NOTE—The methanol and water content may be varied (between 1% and 3%) by adding water or methanol to the prepared *Mobile phase* to obtain baseline separation of folic acid and the internal standard. The pH may be increased up to 7.15 to obtain better separation.]

Internal standard solution: Transfer 40 mg of methylparaben to a 1000-mL volumetric flask, and add 220 mL of methanol to dissolve. Dissolve 2.0 g of monobasic potassium phosphate in 300 mL of water in a separate beaker, quantitatively transfer this solution to the flask containing the methylparaben solution, and add an additional 300 mL of water. Add 19 mL of *Reagent A*, 7 mL of 3 N phosphoric acid, and 30 mL of *Reagent B*. Adjust with ammonia TS to a pH of 9.8,

bubble nitrogen through the solution for 30 min, dilute with water to volume, and mix.

Standard solution: 0.016 mg/mL of USP Folic Acid RS in *Internal standard solution*

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to a nominal amount of 0.4 mg of folic acid, to a 50-mL amber-colored centrifuge tube. Add 25.0 mL of *Internal standard solution*, shake by mechanical means for 10 min, and centrifuge. Filter a portion of the clear supernatant, and use the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm \times 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 15 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for folic acid and methylparaben are about 0.8 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for folic acid and methylparaben. Calculate the percentage of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio of folic acid to methylparaben from the *Sample solution*

R_S = peak area ratio of folic acid to methylparaben from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of folic acid in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6$)

• **Folic Acid, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: Dissolve 7.5 g of edetate disodium, with stirring, in 500 mL of water containing 10 mL of ammonium hydroxide.

Diluent: 60 $\mu\text{g/mL}$ of ammonium hydroxide

Mobile phase: Transfer 0.4 mL of triethylamine, 15 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask, and dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 60 $\mu\text{g/mL}$ of USP Folic Acid RS in *Diluent*. Prepare this solution fresh daily.

Standard solution: Mix 5.0 mL of *Standard stock solution* with 10.0 mL of methanol and 35.0 mL of *Reagent*, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Transfer a portion of finely powdered Tablets, equivalent to a nominal amount of 0.3 mg of folic acid, to a 125-mL stoppered flask. Add 10.0 mL of methanol and 35.0 mL of *Reagent*. Shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the major peaks. Calculate the percentage of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Folic Acid RS in the *Standard solution* (µg/mL)

C_U = nominal concentration of folic acid in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of folic acid ($C_{19}H_{19}N_7O_6$)

• **CALCIUM PANTOTHENATE, Method 1**

Mobile phase: Phosphoric acid and water (1:1000)

Internal standard solution: 80 mg of *p*-hydroxybenzoic acid in 3 mL of alcohol. Add 50 mL of water and 7.1 g of dibasic sodium phosphate, and dilute with water to 1000 mL. Adjust with phosphoric acid to a pH of 6.7.

Standard solution: 0.6 mg/mL of USP Calcium Pantothenate RS in *Internal standard solution*

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to a nominal amount of 15 mg of calcium pantothenate, to a centrifuge tube. Add 25.0 mL of the *Internal standard solution*, and shake vigorously for 10 min. Centrifuge, filter, and use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 3.9-mm × 15-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for calcium pantothenate and *p*-hydroxybenzoic acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak responses for calcium pantothenate and the internal standard. Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Sample solution*

R_S = peak response ratio of calcium pantothenate to *p*-hydroxybenzoic acid from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• **CALCIUM PANTOTHENATE, Method 2**

Standard stock solution: Dissolve 50 mg of USP Calcium Pantothenate RS, previously dried and stored in the dark over phosphorus pentoxide while protected from absorption of moisture during the weighing, in 500 mL of water in a 1000-mL volumetric flask. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (1 in 60), and dilute with water to volume, to obtain a concentration of 50 µg/mL of USP Calcium Pantothenate RS. Store under toluene in a refrigerator.

Standard solution: On the day of the assay, dilute a volume of *Standard stock solution* with water to obtain a concentration of 0.01–0.04 µg/mL of calcium pantothenate, the exact concentration being such that the responses obtained as directed for *Analysis*, 2.0–4.0 mL of the *Standard solution* being used, are within the linear portion of the log-concentration response curve.

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder, equivalent to a nominal amount of 50 mg of calcium pantothenate, to a 1000-mL volumetric flask containing 500 mL of water. Add 10 mL of 0.2 N acetic acid and 100 mL of sodium acetate solution (16.66 mg/mL), dilute with water to volume, and filter. Dilute a volume of this solution to obtain a solution having approximately the same concentration as that of the *Standard solution*.

Acid-hydrolyzed casein solution: Mix 100 g of vitamin-free casein with 500 mL of 6 N hydrochloric acid, and reflux the mixture for 8–12 h. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution with 1 N sodium hydroxide to a pH of 3.5 ± 0.1, and add water to make 1000 mL. Add 20 g of activated charcoal, stir for 1 h, and filter. Repeat the treatment with activated charcoal. Store under toluene in a cool place at a temperature not below 10°. Filter the solution if a precipitate forms during storage.

Cystine–tryptophan solution: Suspend 4.0 g of L-cystine and 1.0 g of L-tryptophan (or 2.0 g of D,L-tryptophan) in 700–800 mL of water, heat to 70°–80°, and add dilute hydrochloric acid (1 in 2) dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 mL. Store under toluene in a cool place at a temperature not below 10°.

Adenine–guanine–uracil solution: Dissolve 200 mg each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 mL of 4 N hydrochloric acid. Cool, and add water to make 200 mL. Store under toluene in a refrigerator.

Polysorbate 80 solution: 100 mg/mL of polysorbate 80 in alcohol

Riboflavin–thiamine hydrochloride–biotin solution:

20 µg/mL of riboflavin, 10 µg/mL of thiamine hydrochloride, and 0.04 µg/mL of biotin in 0.02 N acetic acid. Store under toluene, protected from light, in a refrigerator.

p-Aminobenzoic acid–niacin–pyridoxine hydrochloride solution: 10 µg/mL of *p*-aminobenzoic acid, 50 µg/mL of niacin, and 40 µg/mL of pyridoxine hydrochloride in a mixture of neutralized alcohol and water (1:3). Store in a refrigerator.

Salt solution A: Dissolve 25 g of monobasic potassium phosphate and 25 g of dibasic potassium phosphate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Salt solution B: Dissolve 10 g of magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of ferrous sulfate, and 0.5 g of manganese sulfate in water to make 500 mL. Add 5 drops of hydrochloric acid. Store under toluene.

Basal medium stock solution

Acid-hydrolyzed casein solution	25 mL
Cystine-tryptophan solution	25 mL
Polysorbate 80 solution	0.25 mL
Dextrose, anhydrous	10 g
Sodium acetate, anhydrous	5 g
Adenine-guanine-uracil solution	5 mL
Riboflavin-thiamine hydrochloride-biotin solution	5 mL
p-Aminobenzoic acid-niacin-pyridoxine hydrochloride solution	5 mL
Salt solution A	5 mL
Salt solution B	5 mL

Dissolve the anhydrous dextrose and anhydrous sodium acetate in the solutions previously mixed, and adjust with 1 N sodium hydroxide to a pH of 6.8. Dilute with water to 250 mL.

Stock culture of *Lactobacillus plantarum*: Dissolve 2.0 g of yeast extract in 100 mL of water; add 500 mg of anhydrous dextrose, 500 mg of anhydrous sodium acetate, and 1.5 g of agar; and heat the mixture on a steam bath, with stirring, until the agar dissolves. Add 10-mL portions of the hot solution to the test tubes, close or cover the tubes, sterilize in an autoclave at 121°, and allow the tubes to cool in an upright position. Prepare stab cultures in three or more of the tubes, using a pure culture of *Lactobacillus plantarum*⁴ incubating for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. Store in a refrigerator. Prepare a fresh stab of the stock culture every week, and do not use for *Inoculum* if the culture is more than 1 week old.

Culture medium: To each of a series of test tubes containing 5.0 mL of *Basal medium stock solution*, add 5.0 mL of water containing 0.2 µg of calcium pantothenate. Plug the tubes with cotton, sterilize in an autoclave at 121°, and cool.

Inoculum: [NOTE—A frozen suspension of *Lactobacillus plantarum* may be used as the stock culture, provided it yields an *Inoculum* comparable to a fresh culture.]

Make a transfer of cells from the *Stock culture* of *Lactobacillus plantarum* to a sterile tube containing 10 mL of *Culture medium*. Incubate this culture for 16–24 h at a temperature between 30° and 37° held constant to within ±0.5°. The cell suspension so obtained is the *Inoculum*.

Analysis

Samples: *Standard solutions* and *Sample solution*
To similar separate test tubes add, in duplicate, 1.0 and/or 1.5, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard solution*. To each tube and to four similar empty tubes add 5.0 mL of *Basal medium stock solution* and sufficient water to make 10 mL.

To similar separate test tubes add, in duplicate, volumes of the *Sample solution* corresponding to three or more of the levels specified for the *Standard solution*, including the levels of 2.0, 3.0, and 4.0 mL. To each tube add 5.0 mL of the *Basal medium stock solution* and sufficient water to make 10 mL. Place one complete set of *Standard* and *sample* tubes together in one tube rack and the duplicate set in a second rack or section of a rack, preferably in random order. Cover the tubes of both series to prevent contamination, and sterilize in an autoclave at 121° for 5 min. Cool, and add 1 drop of *Inoculum* to each tube, except two of the four tubes containing no *Standard solution* (the uninoculated blanks). Incubate the tubes at a temperature between 30° and 37°, held constant to within ±0.5° until, following 16–24 h of incubation, there has been no substantial increase in turbidity in

the tubes containing the highest level of *Standard* during a 2-h period.

Determine the transmittance of the tubes in the following manner. Mix the contents of each tube, and transfer to an optical container if necessary. Place the container in a spectrophotometer that has been set at a specific wavelength between 540 and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the galvanometer reading remains constant for 30 s or more. Allow approximately the same time interval for the reading on each tube.

With the transmittance set at 1.00 for the uninoculated blank, read the transmittance of the inoculated blank. With the transmittance set at 1.00 for the inoculated blank, read the transmittance for each of the remaining tubes. If there is evidence of contamination with a foreign microorganism, disregard the result of the assay.

Calculation: Prepare a standard concentration-response curve as follows. For each level of the *Standard*, calculate the response from the sum of the duplicate values of the transmittance (Σ) as the difference, $y = 2.00 - \Sigma$. Plot this response on the ordinate of cross-section paper against the logarithm of the mL of *Standard solution* per tube on the abscissa, using for the ordinate either an arithmetic or a logarithmic scale, whichever gives the better approximation to a straight line. Draw the straight line or smooth curve that best fits the plotted points.

Calculate the response, y , adding together the two transmittances for each level of the *Sample solution*. Read from the standard curve the logarithm of the volume of the *Standard solution* corresponding to each of those values of y that fall within the range of the lowest and highest points plotted for the *Standard*. Subtract from each logarithm so obtained the logarithm of the volume, in mL, of the *Sample solution* to obtain the difference, X , for each dosage level. Average the values of X for each of three or more dosage levels to obtain \bar{X} , which equals the log-relative potency, M' , of the *Sample solution*. Determine the quantity, in mg, of USP Calcium Pantothenate RS corresponding to the calcium pantothenate in each mg of the portion of Tablets taken:

$$\text{antilog } M = \text{antilog } (M' + \log R)$$

R = number of mg of calcium pantothenate that was assumed to be present in each mg in the portion of Tablets taken

Replication: Repeat the entire determination at least once, using separately prepared *Sample solutions*. If the difference between the two log-potencies M is NMT 0.08, their mean, \bar{M} , is the assayed log-potency of the test material (see *Design and Analysis of Biological Assays* (111), *The Confidence Interval and Limits of Potency*). If the two determinations differ by more than 0.08, conduct one or more additional determinations. From the mean of two or more values of M that do not differ by more than 0.15, compute the mean potency of the preparation under assay.

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

- CALCIUM PANTOTHENATE, Method 3**

Buffer solution: 5.0 mg/mL of monobasic potassium phosphate in water. Adjust with phosphoric acid to a pH of 3.5.

Mobile phase: Methanol and *Buffer solution* (1:9)

Standard stock solution: 0.25 mg/mL of USP Calcium Pantothenate RS in water. Prepare fresh every 4 weeks. Store in a refrigerator.

Standard solution: 40 µg/mL of USP Calcium Pantothenate RS from *Standard stock solution* diluted with water

⁴ ATCC No. 8014 is suitable. This strain was formerly known as *Lactobacillus arabinosus* 17-5.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 10 mg of calcium pantothenate, to a 250-mL volumetric flask. Add 10 mL of methanol, and swirl the flask to disperse. Dilute with water to volume, mix, and filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 3.9-mm × 30-cm; 5-μm packing L1

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 25 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for calcium pantothenate.

Calculate the percentage of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Calcium Pantothenate RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of calcium pantothenate in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of calcium pantothenate ($C_{18}H_{32}CaN_2O_{10}$)

• NIACIN or NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 1

[NOTE—Use low-actinic glassware throughout this procedure.]

Diluent: Acetonitrile, glacial acetic acid, and water (5:1:94)

Mobile phase: A mixture of methanol, glacial acetic acid, and water (27:1:73) containing 140 mg of sodium 1-hexanesulfonate per 100 mL

Standard solution: [NOTE—Use USP Niacin RS in place of USP Niacinamide RS for formulations containing niacin.] Transfer 80 mg of USP Niacinamide RS, 20 mg of USP Pyridoxine Hydrochloride RS, 20 mg of USP Riboflavin RS, and 20 mg of USP Thiamine Hydrochloride RS to a 200-mL volumetric flask, and add 180 mL of *Diluent*. Immerse the flask in a hot water bath maintained at 65°–70° for 10 min with regular shaking, or using a vortex mixer, until all the solid materials are dissolved. Chill rapidly in a cold water bath for 10 min to room temperature, and dilute with *Diluent* to volume.

Sample solution: Finely powder NLT 30 Tablets.

Transfer a portion of the powder equivalent a nominal amount of 10 mg of niacinamide and 2.5 mg each of pyridoxine hydrochloride, riboflavin, and thiamine hydrochloride, to a 50-mL centrifuge tube. Add 25.0 mL of *Diluent*, and mix using a vortex mixer for 30 s to completely suspend the powder. Immerse the centrifuge tube in a hot water bath maintained at 65°–70°, heat for 5 min, and mix on a vortex mixer for 30 s. Return the tube to the hot water bath, heat for another 5 min, and mix on a vortex mixer for 30 s. Filter a portion of the solution, cool to room temperature, and use the clear filtrate. [NOTE—Use the filtrate within 3 h of filtration.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 3.9-mm × 30-cm; packing L1

Flow rate: 1 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution*. [NOTE—Record the peak areas.]

[NOTE—The relative retention times for niacinamide, pyridoxine, riboflavin, and thiamine are about 0.3, 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin or niacinamide, pyridoxine, riboflavin, and thiamine. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacinamide from the *Sample solution*

r_S = peak area of niacinamide from the *Standard solution*

C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

For formulations containing niacin:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin from the *Sample solution*

r_S = peak area of niacin from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), of riboflavin ($C_{17}H_{20}N_4O_6$), and of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

r_S = peak area of the corresponding vitamin from the *Standard solution*

C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$), niacin ($C_6H_5NO_2$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: Transfer 1 mL of glacial acetic acid and 2.5 g of edetate disodium to a 100-mL volumetric flask. Dissolve in and dilute with water to volume.

Extraction solvent: *Solution A* and methanol (3:1)

Mobile phase: 0.1 M sodium acetate solution (13.6 mg/mL of sodium acetate in water). Adjust with acetic acid to a pH of 5.4. [NOTE—A small amount of methanol (up to 1%) may be added to the *Mobile phase* to improve resolution.]

Standard stock solution: 1 mg/mL of USP Niacin RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.

Sample solution: [NOTE—This preparation is suitable for the determination of niacin or niacinamide, pyridoxine, and riboflavin, when present in the formulation.] Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 2 mg of riboflavin, to a 200-mL volumetric flask. If riboflavin is not present in the formulation, transfer a portion of the powder, equivalent to a nominal amount of 2 mg of pyridoxine. If pyridoxine is not present in the formulation, transfer a portion of the powder, equivalent to a nominal amount of 20 mg of niacin or niacinamide. Add 100.0 mL of *Extraction solvent*, and mix for 20 min, using a wrist-action shaker. Immerse the flask in a water bath maintained at 70°–75°, and heat for 20 min. Mix on a vortex mixer for 30 s, cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

[NOTE—If necessary, flush the column with methanol between injections.]

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacin. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Niacin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$)

• **NIACINAMIDE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, Standard stock solution, Standard solution, Sample solution, and Chromatographic system: Using USP Niacinamide RS

in place of USP Niacin RS, proceed as directed in the assay for *Niacin, Method 2*.

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for niacinamide. Calculate the percentage of the labeled amount of niacinamide ($C_6H_6N_2O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacinamide in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacinamide ($C_6H_6N_2O$)

• **PYRIDOXINE HYDROCHLORIDE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent, Mobile phase, and Sample solution: Prepare as directed in the assay for *Niacin, Method 2*.

Standard stock solution: 0.1 mg/mL of USP Pyridoxine Hydrochloride RS in *Extraction solvent*

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a 25-mL volumetric flask, and dilute with *Extraction solvent* to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for pyridoxine. Calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Pyridoxine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of pyridoxine hydrochloride in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$)

• **RIBOFLAVIN, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Extraction solvent: Prepare as directed in the assay for *Niacin, Method 2*.

Solution A: 6.8 g/L of sodium acetate in water

Mobile phase: Prepare a mixture of *Solution A* and methanol (13:7). Add 2 mL of triethylamine per L of the mixture, and adjust with glacial acetic acid to a pH of 5.2.

Standard stock solution: Transfer 20 mg of USP Riboflavin RS to a 200-mL volumetric flask, and add 180 mL of *Extraction solvent*. Immerse the flask for 5 min in a water bath maintained at 65°–75°. Mix well, and repeat if necessary until dissolved. Chill rapidly in a cold water bath to room temperature, and dilute with *Extraction solvent* to volume.

Standard solution: Dilute 5.0 mL of *Standard stock solution* with *Extraction solvent* to 25.0 mL.

Sample solution: Prepare as directed for the *Sample solution* in the assay for *Niacin*, *Method 2*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for riboflavin. Calculate the percentage of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of USP Riboflavin RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of riboflavin in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–150.0% of the labeled amount of riboflavin ($C_{17}H_{20}N_4O_6$)

• **THIAMINE, Method 2**

[NOTE—Use low-actinic glassware throughout this procedure.]

Solution A: 1.88 mg/mL of sodium 1-hexanesulfonate in 0.1% phosphoric acid

Mobile phase: *Solution A* and acetonitrile (46:9)

Standard stock solution: 0.1 mg/mL of USP Thiamine Hydrochloride RS in 0.2 N hydrochloric acid

Standard solution: 0.02 mg/mL of USP Thiamine Hydrochloride RS from *Standard stock solution* diluted with 0.2 N hydrochloric acid

Sample solution: Weigh and finely powder NLT 20 Tablets. Mix a portion of the powdered Tablets with a volume of 0.2 N hydrochloric acid to obtain a nominal concentration of 0.02 mg/mL of thiamine. Shake for 15 min with a wrist-action shaker, and heat to boiling for 30 min. Cool to room temperature, and filter. Use the clear filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; packing L1

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas for the major peaks. For products containing thiamine hydrochloride, calculate the percentage of the labeled amount of thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine hydrochloride in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

• **NIACIN or NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, and THIAMINE, Method 3**

[NOTE—Use low-actinic glassware throughout this procedure.]

Reagent: 25 mg/mL of edetate disodium in water

Mobile phase: Transfer 0.4 mL of triethylamine, 15.0 mL of glacial acetic acid, and 350 mL of methanol to a 2000-mL volumetric flask. Dilute with 0.008 M sodium 1-hexanesulfonate to volume.

Standard stock solution: 1.5 mg/mL of USP Niacin RS or USP Niacinamide RS, 0.24 mg/mL of USP Pyridoxine Hydrochloride RS, 0.08 mg/mL of USP Riboflavin RS, and 0.24 mg/mL of USP Thiamine Hydrochloride RS in *Reagent*, with heating if necessary

Standard solution: Transfer 5.0 mL of *Standard stock solution* to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to a nominal amount of 7.5 mg of niacin or niacinamide, 1.2 mg of pyridoxine hydrochloride, 0.4 mg of riboflavin, and 1.2 mg of thiamine hydrochloride, to a stoppered 125-mL flask. Add 10.0 mL of a mixture of methanol and glacial acetic acid (9:1) and 30.0 mL of a mixture of methanol and ethylene glycol (1:1). Insert the stopper, shake for 15 min in a water bath maintained at 60°, and cool. Filter, discarding the first few mL of the filtrate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 270 nm

Column: 4.6-mm × 25-cm; packing L7

Column temperature: 50°

Flow rate: 2 mL/min

Injection size: 5 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the areas of the peaks. Calculate the percentage of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of niacin or niacinamide from the *Sample solution*

r_S = peak area of niacin or niacinamide from the *Standard solution*

C_S = concentration of USP Niacin RS or USP Niacinamide RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of niacin or niacinamide in the *Sample solution* (mg/mL)

Separately calculate the percentage of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area of the corresponding vitamin from the *Sample solution*

r_S = peak area of the corresponding vitamin from the *Standard solution*

C_S = concentration of the relevant USP Reference Standard in the *Standard solution* (mg/mL)

C_U = nominal concentration of the corresponding vitamin in the *Sample solution* (mg/mL)

For products containing thiamine mononitrate, calculate the percentage of the labeled amount of thiamine mononitrate ($C_{12}H_{17}N_5O_4S$) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

r_U = peak area of thiamine from the *Sample solution*

r_S = peak area of thiamine from the *Standard solution*

C_S = concentration of USP Thiamine Hydrochloride RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of thiamine mononitrate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of thiamine mononitrate, 327.36

M_{r2} = molecular weight of thiamine hydrochloride, 337.27

Acceptance criteria: 90.0%–150.0% of the labeled amount of niacin ($C_6H_5NO_2$) or niacinamide ($C_6H_6N_2O$), pyridoxine hydrochloride ($C_8H_{11}NO_3 \cdot HCl$), riboflavin ($C_{17}H_{20}N_4O_6$), and thiamine as thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) or thiamine mononitrate ($C_{12}H_{17}N_5O_4S$)

[NOTE—Commercially available atomic absorption standard solutions for the minerals, where applicable, may be used where preparation of a *Standard stock solution* is described in the following assays. Use deionized water where water is specified. Where atomic absorption spectrophotometry is specified in the assay, the *Standard solutions* and the *Sample*

solution may be diluted quantitatively with the solvent specified, if necessary, to yield solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

• **CALCIUM, Method 1**

Lanthanum chloride solution: 267 mg/mL of lanthanum chloride heptahydrate in 0.125 N hydrochloric acid

Calcium standard solution: 400 µg/mL of calcium. Dissolve 1.001 g of calcium carbonate, previously dried at 300° for 3 h and cooled in a desiccator for 2 h, in 25 mL of 1 N hydrochloric acid. Boil to expel carbon dioxide, and dilute with water to 1000 mL.

Standard stock solution: 100 µg/mL of calcium from *Calcium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Into separate 100-mL volumetric flasks pipet 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution*, and dilute with water to volume to obtain concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 µg/mL of calcium.

Sample solution: Finely powder NLT 20 Tablets. Transfer a portion of the powder, equivalent to 5 Tablets, to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution, quantitatively, with 0.125 N hydrochloric acid to obtain a nominal concentration of 2 µg/mL of calcium, adding 1 mL of *Lanthanum chloride solution* per 100 mL of the final volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Calcium hollow-cathode

Flame: Nitrous oxide–acetylene

Analytical wavelength: Calcium emission line at 422.7 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg per mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL of calcium in the *Sample solution*.

Calculate the percentage of the labeled amount of calcium (Ca) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of calcium in the *Sample solution* (µg/mL)

C_U = nominal concentration of calcium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca)

• **CHROMIUM, Method 1**

Chromium standard solution: 1000 µg/mL of chromium from potassium dichromate, previously dried at 120° for 4 h, in water. Store in a polyethylene bottle.

Standard stock solution: 10 µg/mL of chromium from *Chromium standard solution* diluted with 6 N hydrochloric acid and water (1 in 20)

Standard solutions: Transfer 10.0 and 20.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks, and transfer 15.0 and 20.0 mL of the *Standard stock solution* to separate 50-mL volumetric flasks. Dilute the contents of each of the four flasks with 0.125 N hydrochloric acid to volume to obtain concentrations of 1.0, 2.0, 3.0, and 4.0 µg/mL of chromium.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 1 µg/mL of chromium and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Chromium hollow-cathode

Flame: Air-acetylene

Analytical wavelength: Chromium emission line at 357.9 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of chromium, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL of chromium in the *Sample solution*.

Calculate the percentage of the labeled amount of chromium (*Cr*) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of chromium in the *Sample solution* (µg/mL)

C_U = nominal concentration of chromium in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of chromium (*Cr*)

• **COPPER, Method 1**

Copper standard solution: Dissolve 1.00 g of copper foil in a minimum volume of a 50% (v/v) solution of nitric acid, and dilute with a 1% (v/v) solution of nitric acid to 1000 mL. This solution contains 1000 µg/mL of copper.

Standard stock solution: 100 µg/mL of copper from *Copper standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 200-mL volumetric flasks transfer 1.0, 2.0, 4.0, 6.0, and 8.0 mL of the *Standard stock solution*. Dilute with water to volume to obtain concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 µg/mL of copper.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain 2 µg/mL of copper and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Copper hollow-cathode

Flame: Air-acetylene

Analytical wavelength: Copper emission line at 324.7 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions, using the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration in µg/mL of copper, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the

concentration, *C*, in µg/mL of copper in the *Sample solution*.

Calculate the percentage of the labeled amount of copper (*Cu*) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of copper in the *Sample solution* (µg/mL)

C_U = nominal concentration of copper in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of copper (*Cu*)

• **FLUORIDE, Method 1**

[NOTE—Store all solutions in plastic containers.]

3 M sodium acetate solution: Dissolve 408 g of sodium acetate in 600 mL of water in a 1000-mL volumetric flask. Allow the solution to equilibrate to room temperature, and dilute with water to volume. Adjust with a few drops of acetic acid to a pH of 7.0.

Sodium citrate solution: Dissolve 222 g of sodium citrate in 250 mL of water in a 1000-mL volumetric flask. Add 28 mL of perchloric acid, and dilute with water to volume.

Fluoride standard stock solution: 500 µg/mL of fluoride from a quantity of sodium fluoride, previously dried at 100° for 4 h and cooled in a desiccator, in water

Intermediate stock solution A: 100 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Intermediate stock solution B: 10 µg/mL of fluoride from *Fluoride standard stock solution* diluted with water

Standard solutions: To five separate 100-mL volumetric flasks transfer 3.0, 5.0, and 10.0 mL of *Intermediate stock solution B* and 5.0 and 10.0 mL of *Intermediate stock solution A*. To each flask add 10.0 mL of 1 N hydrochloric acid, 25 mL of 3 M *sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 0.3, 0.5, 1.0, 5.0, and 10.0 µg/mL of fluoride.

Sample solution: Transfer a quantity of the finely powdered Tablets, equivalent to a nominal amount of 200 µg of fluoride, to a 100-mL volumetric flask. Add 10.0 mL of 1 N hydrochloric acid, 25.0 mL of 3 M *sodium acetate solution*, and 25.0 mL of *Sodium citrate solution*, and dilute with water to 100 mL.

Analysis

Samples: *Standard solutions* and *Sample solution*

To separate plastic beakers, each containing a plastic-coated stirring bar, transfer 50.0 mL each of the *Standard solutions* and the *Sample solution*. Measure the potentials (see *pH* (791)), in mV, of the *Standard solutions* and the *Sample solution* with a pH meter capable of a minimum reproducibility of ±0.2 mV and equipped with a fluoride-specific ion-indicating electrode and a calomel reference electrode. [NOTE—When taking measurements, immerse the electrodes in the solution, stir on a magnetic stirrer having an insulated top until equilibrium is attained (1–2 min), and record the potential. Rinse and dry the electrodes between measurements, taking care to avoid damaging the crystal of the specific-ion electrode.] Plot the logarithms of fluoride concentrations, in µg/mL, of the *Standard solutions* versus the potential in mV. From the standard response curve so obtained and the measured potential of the *Sample solution*, determine the concentration, *C*, in µg/mL of fluoride in the *Sample solution*.

Calculate the percentage of the labeled amount of fluorine (*F*) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of fluoride in the *Sample solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **FLUORIDE, Method 2**

[NOTE—Use plastic containers and deionized water throughout this procedure.]

pH 10.0 buffer: Add 214 mL of 0.1 N sodium hydroxide to 1000 mL of 0.05 M sodium bicarbonate.

Mobile phase: Alcohol, 0.1 N sulfuric acid, and water (20:5:175)

Standard stock solution: 220 µg/mL of USP Sodium Fluoride RS in water. This solution contains 100 µg/mL of fluoride.

Standard solution: [NOTE—Condition the solid-phase extraction column specified for use in the *Standard solution* and the *Sample solution* in the following manner. Using a vacuum at a pressure not exceeding 5 mm of mercury, wash the column with one column volume of methanol followed by one column volume of *pH 10.0 buffer*. Do not allow the column top to dry. If the top of the column becomes dry, recondition the column.] Transfer 10.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add 75 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.4 ±0.1. Dilute with water to volume. Filter, discarding the first 15 mL of the filtrate. Transfer 25.0 mL of the filtrate to a 50-mL volumetric flask, add 15.0 mL of water, and adjust with 0.1 N sodium hydroxide to a pH of 10.0. Dilute with *pH 10.0 buffer* to volume. Elute a portion of this solution through a 3-mL solid-phase extraction column containing L1 packing that is connected through an adaptor to a second solid-phase extraction column containing sulfonylpropyl strong cation-exchange packing. Discard the first 3 mL of the eluate, and collect the rest of the eluate in a suitable flask for injection into the chromatograph.

Sample solution: Finely powder NLT 20 Tablets.

Transfer a portion of powdered Tablets, equivalent to a nominal amount of 1 mg of fluorine, to 15 mL of water, and shake vigorously. Rinse the sides of the flask with 15 mL of water, and allow to stand for 10 min. Dilute with water to 85 mL, adjust with 1 N sodium hydroxide to a pH of 10.4 ±0.1, and dilute with water to 100 mL. Proceed as directed for the *Standard solution*, beginning with "Filter, discarding the first 15 mL of the filtrate."

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Conductivity

Guard column: 4.6-mm × 3-cm; packing L17

Analytical column: 7.8-mm × 30-cm; packing L17

Flow rate: 0.5 mL/min

Injection size: 100 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for fluoride. Calculate the percentage of the labeled amount of fluorine (F) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak area from the *Sample solution*

r_S = peak area from the *Standard solution*

C_S = concentration of fluoride in the *Standard solution* (µg/mL)

C_U = nominal concentration of fluorine in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of fluorine (F)

• **IODIDE, Method 1**

Bromine water: To 20 mL of bromine in a glass-stoppered bottle add 100 mL of water. Insert the stopper into the bottle, and shake. Allow to stand for 30 min, and use the supernatant.

Analysis

Sample: Tablets

Transfer a portion of finely powdered Tablets, equivalent to a nominal amount of 3 mg of iodide, to a nickel crucible. Add 5 g of sodium carbonate, 5 mL of 50% (w/v) sodium hydroxide solution, and 10 mL of alcohol, taking care that the entire specimen is moistened. Heat the crucible on a steam bath to evaporate the alcohol, then dry the crucible at 100° for 30 min to prevent spattering upon subsequent heating. Transfer the crucible with its contents to a furnace heated to 500°, and heat the crucible for 15 min. [NOTE—Heating at 500° is necessary to carbonize any organic matter present; a higher temperature may be used, if necessary, to ensure complete carbonization of all organic matter.] Cool the crucible, add 25 mL of water, cover the crucible with a watchglass, and boil gently for 10 min. Filter the solution, and wash the crucible with boiling water, collecting the filtrate and washings in a beaker. Add phosphoric acid until the solution is neutral to methyl orange, then add 1 mL excess of phosphoric acid. Add excess of *Bromine water*, and boil the solution gently until colorless and then for 5 min longer. Add a few crystals of salicylic acid, and cool the solution to 20°. Add 1 mL of phosphoric acid and 0.5 g of potassium iodide, and titrate the liberated iodine with 0.005 N sodium thiosulfate VS, adding starch TS when the liberated iodine color has nearly disappeared.

Calculate the percentage of the labeled amount of iodine (I) in the portion of Tablets taken:

$$\text{Result} = V \times N_A \times F \times \text{Ime} \times (Aw/W) \times (100/L)$$

V = volume of sodium thiosulfate consumed (mL)

N_A = actual normality of the sodium thiosulfate solution used (meq/mL)

F = correction factor to convert mg to µg, 1000 µg/mg

Ime = milliequivalent of I (21 mg/meq)

Aw = average weight of the Tablets

W = weight of the portion of Tablets taken

L = labeled amount of iodine (µg/Tablet)

Acceptance criteria: 90.0%–160.0% of the labeled amount of iodine (I)

• **IODIDE, Method 2:** Proceed as directed in *Automated Methods of Analysis* (16), *Assay for Iodide*.

Acceptance criteria: 90%–160% of the labeled amount of iodine (I)

• **IRON, Method 1**

Iron standard stock solution: Transfer 100 mg of iron powder to a 1000-mL volumetric flask. Dissolve in 25 mL of 6 N hydrochloric acid, dilute with water to volume, and mix.

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 4.0, 5.0, 6.0, and 8.0 mL of *Iron standard stock solution*. Dilute the contents of each flask with water to volume to obtain concentrations of 2.0, 4.0, 5.0, 6.0, and 8.0 µg/mL of iron.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a nominal concentration of 5 µg/mL of iron and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption spectrophotometry
Lamp: Iron hollow-cathode
Flame: Air–acetylene
Analytical wavelength: Iron emission line at 248.3 nm
Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$ of iron, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$ of iron in the *Sample solution*.

Calculate the percentage of the labeled amount of iron (Fe) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of iron in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of iron in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of iron (Fe)

• MAGNESIUM, Method 1

Lanthanum chloride solution: Prepare as directed in the assay for *Calcium, Method 1*.

Magnesium standard solution: Transfer 1.0 g of magnesium ribbon to a 1000-mL volumetric flask, dissolve in 50 mL of 6 N hydrochloric acid, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 μg of magnesium/mL.

Standard stock solution: 20 $\mu\text{g/mL}$ of magnesium from *Magnesium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 2.5, and 3.0 mL of *Standard stock solution*. To each flask add 1.0 mL of *Lanthanum chloride solution* and dilute with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.2, 0.3, 0.4, 0.5, and 0.6 $\mu\text{g/mL}$ of magnesium.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a nominal concentration of 0.4 $\mu\text{g/mL}$ of magnesium.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Magnesium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Magnesium emission line at 285.2 nm

Blank: 0.125 N hydrochloric acid containing 1 mL of *Lanthanum chloride solution* per 100 mL

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of magnesium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$ of magnesium in the *Sample solution*.

Calculate the percentage of the labeled amount of magnesium (Mg) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of magnesium in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of magnesium in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of magnesium (Mg)

• MANGANESE, Method 1

Manganese standard stock solution: Transfer a weighed amount of 1.00 g of manganese to a 1000-mL volumetric flask. Dissolve in 20 mL of nitric acid, dilute with 6 N hydrochloric acid to volume, and mix to obtain a solution with a concentration of 1000 $\mu\text{g/mL}$ of manganese.

Standard stock solution: 50 $\mu\text{g/mL}$ of manganese from *Manganese standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: To separate 100-mL volumetric flasks transfer 1.0, 1.5, 2.0, 3.0, and 4.0 mL of *Standard stock solution*. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions with concentrations of 0.5, 0.75, 1.0, 1.5, and 2.0 $\mu\text{g/mL}$ of manganese.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a nominal concentration of 1 $\mu\text{g/mL}$ of manganese and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Manganese hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Manganese emission line at 279.5 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*
 Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$ of manganese, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of manganese (Mn) in the *Sample solution*.

Calculate the percentage of the labeled amount of manganese (Mn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of manganese in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of manganese in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of manganese (Mn)

• MOLYBDENUM, Method 1

Diluent: 20 mg/mL of ammonium chloride in water
Molybdenum standard solution: Transfer 1.0 g of molybdenum wire to a 1000-mL volumetric flask, and dissolve in 50 mL of nitric acid, warming if necessary. Dilute with water to volume, and mix to obtain a solution with a concentration of 1000 $\mu\text{g/mL}$ of molybdenum.

Standard stock solution: 100 $\mu\text{g/mL}$ of molybdenum from *Molybdenum standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 2.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solution in each flask for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain concentrations of 5.0, 10.0, and 25.0 $\mu\text{g/mL}$ of molybdenum.

Sample solution: Transfer a portion of the powder, equivalent to a nominal amount of 1000 μg of molybdenum, to a suitable flask, and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min, or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature.

Carefully add 8 mL of perchloric acid, heat until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until no fumes are present. Cool to room temperature. Quantitatively transfer the contents of the flask to a 100-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Molybdenum hollow-cathode

Flame: Nitrous oxide-acetylene

Analytical wavelength: Molybdenum emission line at 313.3 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL of molybdenum, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL of molybdenum in the *Sample solution*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of molybdenum in the *Sample solution* (µg/mL)

C_U = nominal concentration of molybdenum in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• MOLYBDENUM, Method 2

Sodium fluoride solution: Add 200 mL of water to 10 g of sodium fluoride, stir until the solution is saturated, and filter. Store in a polyethylene bottle.

Ferrous sulfate solution: 4.98 mg/mL of ferrous sulfate in water

Potassium thiocyanate solution: 200 mg/mL of potassium thiocyanate in water

20% Stannous chloride solution: Transfer 40 g of stannous chloride to a beaker, add 20 mL 6.5 N hydrochloric acid, and heat the solution until the stannous chloride is dissolved. Cool and dilute with water to 100 mL.

Diluted stannous chloride solution: 20% *Stannous chloride solution* diluted with water (1 in 25). Prepare this solution fresh at the time of use.

Standard solution: 20 µg/mL of molybdenum in water.

Sample: A portion of finely powdered Tablets, equivalent to a nominal amount of 40 µg of molybdenum

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 465 nm

Blank: Amyl alcohol

Analysis

Samples: *Standard solution* and *Sample*

Transfer the *Sample* and 2.0 mL of the *Standard solution* to separate 200-mL beakers. Add 20 mL of nitric acid to the each beaker. Cover each beaker with a watchglass, and boil slowly on a hot plate for 45 min. Cool to room temperature. Add 6 mL of perchloric acid, cover the beakers with a watchglass, and continue the heating until digestion is complete, as indicated when the liquid becomes colorless or pale yellow. Evaporate the solutions in the beakers to dryness. Rinse the sides of the beakers and the watchglasses with water, and add more water to

complete 50 mL in each beaker. Gently boil the water solution for a few min. Cool to room temperature.

Add 2 drops of methyl orange TS, and neutralize with ammonium hydroxide. Add 8.2 mL of hydrochloric acid. Quantitatively transfer the contents of the beakers to separate 100-mL volumetric flasks, rinse the beakers with water, transfer the rinsings to the corresponding volumetric flasks, and dilute with water to volume. Transfer 50.0 mL of each solution to separatory funnels. To each separatory funnel add 1.0 mL of *Sodium fluoride solution*, 0.5 mL of *Ferrous sulfate solution*, 4.0 mL of *Potassium thiocyanate solution*, 1.5 mL of 20% *Stannous chloride solution*, and 15.0 mL of amyl alcohol, and shake the separatory funnels for 1 min. Allow the layers to separate, and discard the aqueous layers. Add 25 mL of *Diluted stannous chloride solution* to each separatory funnel, and shake gently for 15 s. Allow the layers to separate, and discard the aqueous layers. Transfer the organic layers from each separatory funnel to a centrifuge tube, and centrifuge at 2000 rpm for 10 min. Determine the absorbances of the organic phases obtained from the *Standard solution* and the *Sample*, and correct with the *Blank*.

Calculate the percentage of the labeled amount of molybdenum (Mo) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S)/M_U] \times 100$$

A_U = absorbance of the solution from Tablets

A_S = absorbance of solution from the *Standard solution*

C_S = concentration of molybdenum in the *Standard solution* (µg/mL)

V = volume of the *Standard solution* analyzed, 2.0 mL

M_U = nominal amount of molybdenum in the *Sample* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of molybdenum (Mo)

• PHOSPHORUS, Method 1

Sulfuric acid solution: Cautiously add sulfuric acid to water (37.5:100), and mix.

Ammonium molybdate solution: 50 mg/mL of ammonium molybdate in *Sulfuric acid solution* and water (2:3). [NOTE—Dissolve in water first, and then dilute with *Sulfuric acid solution* to volume.]

Hydroquinone solution: 5 mg/mL of hydroquinone in water. Add one drop of sulfuric acid per 100 mL of solution.

Sodium bisulfite solution: 200 mg/mL of sodium bisulfite in water

Phosphorus standard stock solution: Weigh 4.395 g of monobasic potassium phosphate, previously dried at 105° for 2 h and stored in a desiccator, and transfer to a 1000-mL volumetric flask. Dissolve in water, add 6 mL of sulfuric acid as a preservative, dilute with water to volume, and mix to obtain a solution with a concentration of 1000 µg/mL of phosphorus.

Standard solution: 20 µg/mL of phosphorus from *Phosphorus standard stock solution* diluted with water

Sample solution: [NOTE—Finely powder and weigh a counted number of Tablets.] Transfer a portion of the powder, equivalent to a nominal amount of 100 mg of phosphorus, to 25 mL of nitric acid, and digest on a hot plate for 30 min. Add 15 mL of hydrochloric acid, and continue the digestion to the cessation of brown fumes. Cool, and transfer the contents of the flask to a 500-mL volumetric flask with the aid of small portions of water. Dilute with water to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with water to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Cell: 1 cm

Analytical wavelength: 650 nm

Analysis

Samples: *Standard solution* and *Sample solution*

To three separate 25-mL volumetric flasks transfer 5.0 mL each of the *Standard solution*, the *Sample solution*, and water to provide the blank. To each of the three flasks add 1.0 mL each of *Ammonium molybdate solution*, *Hydroquinone solution*, and *Sodium bisulfite solution*, and swirl to mix. Dilute the contents of each flask with water to volume, and allow the flasks to stand for 30 min. Determine the absorbances of the solutions against the blank.

Calculate the percentage of the labeled amount of phosphorus (P) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

C_S = concentration of phosphorus in the *Standard solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of phosphorus in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90%–125% of the labeled amount of phosphorus (P)

• POTASSIUM

Potassium standard solution: 100 $\mu\text{g/mL}$ of potassium from potassium chloride, previously dried at 105° for 2 h, in water

Standard stock solution: 10 $\mu\text{g/mL}$ of potassium from *Potassium standard solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 5.0, 10.0, 15.0, 20.0, and 25.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 $\mu\text{g/mL}$ of potassium.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a nominal amount of 1 $\mu\text{g/mL}$ of potassium and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Potassium emission line at 766.5 nm

Blank: Water

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of potassium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$ of potassium in the *Sample solution*.

Calculate the percentage of the labeled amount of potassium (K) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of potassium in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of potassium in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–125.0% of the labeled amount of potassium (K)

• SELENIUM, Method 1

Diluent: Prepare as directed in the assay for *Molybdenum, Method 1*.

Selenium standard solution: [**CAUTION**—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a concentration of 1000 $\mu\text{g/mL}$ of selenium.

Standard stock solution: 100 $\mu\text{g/mL}$ of selenium from *Selenium standard solution* diluted with water

Standard solutions: To separate 100-mL volumetric flasks transfer 5.0, 10.0, and 25.0 mL of the *Standard stock solution*, and add 5.0 mL of perchloric acid to each flask. Gently boil the solutions for 15 min, cool to room temperature, and dilute each with *Diluent* to volume to obtain solutions with concentrations of 5.0, 10.0, and 25.0 $\mu\text{g/mL}$ of selenium.

Sample solution: Transfer a portion of powder, equivalent to a nominal amount of 1000 μg of selenium, to a suitable flask and add 12 mL of nitric acid. [NOTE—The volume of nitric acid may be varied to ensure that the powder is uniformly dispersed.] Carefully swirl the flask to disperse the test specimen. Sonicate for 10 min or until the test specimen is completely dissolved. Gently boil the solution for 15 min, and cool to room temperature. Carefully add 8 mL of perchloric acid to the flask, heat the flask until perchloric acid fumes appear, and swirl the flask to dissipate the fumes. Repeat the heating and swirling until the fumes appear again. Cool to room temperature. Transfer the contents of the flask to a 50-mL volumetric flask with the aid of the *Diluent*, and dilute with *Diluent* to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Selenium hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Selenium emission line at 196.0 nm

Blank: *Diluent* and perchloric acid (20:1)

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$ of selenium, and draw the straight line best fitting the three plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$ of selenium in the *Sample solution*.

Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of selenium in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of selenium in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• SELENIUM, Method 2

Hydrochloric acid solution: Hydrochloric acid diluted with water (1 in 10)

50% Ammonium hydroxide solution: Ammonium hydroxide diluted with water (1 in 2)

Reagent A: 9 mg/mL of edetate disodium and 25 mg/mL of hydroxylamine hydrochloride in water.

[NOTE—Dissolve edetate disodium in a portion of water first, add hydroxylamine hydrochloride, and then dilute with water to volume.]

Reagent B: Transfer 200 mg of 2,3-diaminonaphthalene to a 250-mL separatory funnel, and add 200 mL of 0.1 N hydrochloric acid. Wash the solution with three 40-mL portions of cyclohexane, and discard the cyclohexane layer. Filter the solution into a brown bottle, and cover the solution with a 1-cm layer of cyclohexane. This solution is stable for 1 week if stored in a refrigerator.

Standard stock solution: [CAUTION—Selenium is toxic; handle it with care.] Dissolve 1 g of metallic selenium in a minimum volume of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and evaporation to dryness three times. Dissolve the residue in 3 N hydrochloric acid, transfer to a 1000-mL volumetric flask, and dilute with 3 N hydrochloric acid to volume to obtain a solution with a concentration of 1000 µg/mL of selenium. Dilute a volume of the solution with 0.125 N hydrochloric acid to obtain a concentration of 2.0 µg/mL of selenium.

Standard solution: Transfer 10.0 mL of the *Standard stock solution* to a glass-stoppered flask. Add 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution*, and dilute with water to 20 mL.

Sample solution: Transfer a portion of finely powdered Tablets, equivalent to a nominal amount of 20 µg of selenium, to a suitable flask. Add 10 mL of nitric acid, and warm gently on a hot plate. Continue heating until the initial nitric acid reaction has subsided, then add 3 mL of perchloric acid. [CAUTION—Exercise care at this stage because the perchloric acid reaction becomes vigorous.] Continue heating on the hot plate until the appearance of white fumes of perchloric acid or until the digest begins to darken. Add 0.5 mL of nitric acid and resume heating, adding additional amounts of nitric acid if further darkening occurs. Digest for 10 min after the first appearance of perchloric acid fumes or until the digest becomes colorless. Cool the flask, add 2.5 mL of *Hydrochloric acid solution*, and return the flask to the hot plate to expel residual nitric acid. Heat the mixture for 3 min after it begins to boil. Cool the flask to room temperature, and dilute with water to 20 mL.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV

Cell: 1 cm

Analytical wavelength: 380 nm

Blank: 1 mL of perchloric acid and 1 mL of *Hydrochloric acid solution* diluted with water to 20 mL

Analysis

Samples: *Standard solution* and *Sample solution*

Treat the *Sample solution*, the *Standard solution*, and the *Blank* as follows. Add 5 mL of *Reagent A* to each flask, and swirl gently to mix. Adjust the solution in each flask with 50% *Ammonium hydroxide solution* to a pH of 1.1 ± 0.1. Add 5 mL of *Reagent B* to each flask, and swirl gently to mix. Place the flasks in a water bath maintained at 50°, and equilibrate for 30 min, taking care that the flasks are covered to protect them from light. Cool to room temperature, and transfer the contents of each flask to separate separatory funnels. Transfer 10.0 mL of cyclohexane to each separatory funnel, and extract vigorously for 1 min. Discard the aqueous layer. Transfer the cyclohexane layer to a centrifuge tube, and centrifuge at 1000 rpm for 1 min to remove any remaining water. Determine the absorbances of the solutions obtained from the *Samples* against the solution obtained from the *Blank*. Calculate the percentage of the labeled amount of selenium (Se) in the portion of Tablets taken:

$$\text{Result} = (A_U/A_S) \times [(V \times C_S) / M_U] \times 100$$

A_U = absorbance of the cyclohexane layer from the *Sample solution*

A_S = absorbance of the cyclohexane layer from the *Standard solution*

V = volume of the *Standard stock solution* used to prepare the *Standard solution*, 10 mL

C_S = concentration of selenium in the *Standard stock solution* (µg/mL)

M_U = nominal amount of selenium in the *Sample solution* (µg)

Acceptance criteria: 90.0%–160.0% of the labeled amount of selenium (Se)

• ZINC, Method 1

Zinc standard solution: 1000 µg/mL of zinc from zinc oxide dissolved in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [NOTE—Dissolve 5 M hydrochloric acid by warming if necessary, cool, and then dilute to final volume.]

Standard stock solution: 50 µg/mL of zinc from *Zinc standard stock solution* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of the *Standard stock solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Proceed as directed for the *Sample solution* in the assay for *Calcium, Method 1*, except to prepare the *Sample solution* to contain a nominal concentration of 2 µg/mL of zinc and to omit the use of the *Lanthanum chloride solution*.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Analytical wavelength: Zinc emission line at 213.8 nm

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration in µg/mL of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = measured concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–125.0% of the labeled amount of zinc (Zn)

• BORON, NICKEL, TIN, and VANADIUM, Method 1; CALCIUM, CHROMIUM, COPPER, IRON, MAGNESIUM, MANGANESE, PHOSPHORUS, and ZINC, Method 2; MOLYBDENUM and SELENIUM, Method 3

Stock aqua regia solution: Prepare a mixture of hydrochloric acid and nitric acid (3:1) by adding the nitric acid to the hydrochloric acid. [NOTE—Periodically vent the solution in an appropriate fume hood.]

Diluent: Prepare a mixture of *Stock aqua regia solution* and water (1:9) by adding one volume of *Stock aqua regia solution* to two volumes of water. Dilute with additional water to volume, and mix well.

System suitability solution: Prepare a mixture of 1000 mg/L of yttrium in 5% (v/v) nitric acid solution

and 1000 mg/L of scandium in 5% (v/v) nitric acid solution with *Diluent* (1:1:198), and mix.

Standard stock solution 1 (Ca, Cu, Fe, Mg, Mn, P, and Zn): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 5% (v/v) nitric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 5% (v/v) nitric acid solution to obtain a solution with final concentrations of about 1000 mg/L of calcium, 100 mg/L of copper, 250 mg/L of iron, 500 mg/L of magnesium, 100 mg/L of manganese, 800 mg/L of phosphorus, and 250 mg/mL of zinc.

Standard stock solution 2 (B, Cr, Mo, Ni, Se, Sn, and V): [NOTE—It is only necessary to include the minerals of interest in the solution.] Using commercially available element standard (single- or multi-element) solutions in 20% (v/v) hydrochloric acid solution, pipet the appropriate amount of element standard solution into a volumetric flask, and dilute with 20% (v/v) hydrochloric acid solution to obtain a solution with final concentrations of about 200 mg/L of boron, and 100 mg/L each of chromium, molybdenum, nickel, selenium, tin, and vanadium.

Standard solutions: Prepare a mixture of *Standard stock solution 1* and *Standard stock solution 2*, as required, in *Diluent* to prepare a six-point calibration curve to bracket the concentration range of each mineral of interest.

Sample solution 1 (for Tablets containing minerals found in *Standard stock solution 1* and *Standard stock solution 2*): Weigh and finely powder NLT than 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube, using a 5- μ m pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Sample solution 2 (for Tablets containing minerals found only in *Standard stock solution 2*): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to 3.5 times the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently until fumes cease (about 1 h). [NOTE—If the sample contains selenium, digest for NMT 15 min.] Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube using a 5- μ m pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.]

Sample solution 3 (for Tablets containing minerals found only in *Standard stock solution 1*): Weigh and finely powder NLT 20 Tablets. Transfer a portion, equal to the average Tablet weight, to a 250-mL volumetric flask. Slowly add 25 mL of *Stock aqua regia solution* in 5-mL increments, followed by mixing. [NOTE—If the sample contains a carbonate, bubbling will occur. Wait until bubbling ends to proceed.] Bring the solution to a boil on a hot plate. Continue to heat gently (about 1 h) until fumes cease. Remove from heat, cool, and dilute with water to volume. Filter about 30 mL into a centrifuge tube, using a 5- μ m pore size nylon syringe filter. If necessary, make any further dilutions using the *Diluent*.

Spectrometric conditions

(See *Plasma Spectrochemistry* <730>.)

Mode: Inductively coupled plasma emission spectrometry, using a spectrometer set to measure the emission of each mineral of interest at about the corresponding wavelength. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. The wavelengths selected should be demonstrated experimentally to provide sufficient specificity, sensitivity, linearity, accuracy, and precision.]

System suitability

[NOTE—Analyze the *System suitability solution*, and obtain the response as directed for *Analysis*.]

Suitability requirements

Relative standard deviation: NMT 2.0%.

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the emission of each mineral of interest in the *Standard solutions* and *Sample solution* with an inductively coupled plasma system using the *Diluent* as the blank. Plot the emission of the *Standard solutions* versus the concentration, in mg/L of the minerals of interest, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, *C*, in mg/L, for each mineral of interest in the *Sample solution*.

Calculate the percentage of the labeled amount for each mineral:

$$\text{Result} = C \times (V/W) \times F \times (C_w/L) \times 100$$

C = measured concentration of the relevant element in the *Sample solution* (mg/L)
V = volume of the *Sample solution* (L)
W = sample weight (mg)
F = dilution factor of the *Sample solution*
C_w = average Tablet weight (mg)
L = labeled amount/Tablet (mg)

Acceptance criteria: 90.0%–125.0% of the labeled amount of calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), phosphorus (P), and zinc (Zn); and 90.0%–160.0% of the labeled amounts of boron (B), chromium (Cr), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), and vanadium (V).

PERFORMANCE TESTS

- **DISINTEGRATION AND DISSOLUTION OF DIETARY SUPPLEMENTS** <2040>: Meet the requirements for *Dissolution*
- **WEIGHT VARIATION OF DIETARY SUPPLEMENTS** <2091>: Meet the requirements

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS—NUTRITIONAL AND DIETARY SUPPLEMENTS** <2021>: The total aerobic microbial count does not exceed 3000 cfu/g, and the combined molds and yeasts count does not exceed 300 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS** <2022>: Meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, and *Staphylococcus aureus*

ADDITIONAL REQUIREMENTS

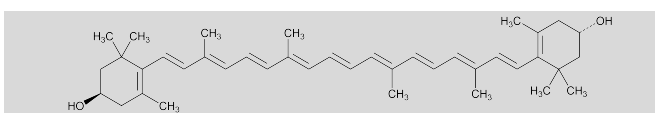
- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states that the product is Water-Soluble Vitamins with Minerals Tablets. The label also states the quantity of each vitamin and mineral in terms of metric units per dosage unit and, where necessary, the chemical form in which a vitamin is present, and also states the salt form of the mineral used as the source of each element. Where more than one assay method is given for a particular vitamin, the labeling states which assay method is used only if *Method 1* is not used.

• **USP REFERENCE STANDARDS** (11)

USP Biotin RS
 USP Calcium Pantothenate RS
 USP Cyanocobalamin RS
 USP Folic Acid RS
 USP Niacin RS
 USP Niacinamide RS
 USP Pyridoxine Hydrochloride RS
 USP Riboflavin RS
 USP Sodium Fluoride RS
 USP Thiamine Hydrochloride RS

Add the following:

▲ **meso-Zeaxanthin**



$C_{40}H_{56}O_2$ 568.88
 β,β -Carotene-3,3'-diol (3R,3'S)-;
 (3R,3'S meso)-Zeaxanthin [31272-50-1].

DEFINITION

meso-Zeaxanthin consists chiefly of the 3R,3'S-isomer of zeaxanthin. It contains NLT 80.0% of total carotenoids calculated as zeaxanthin ($C_{40}H_{56}O_2$) and NLT 74.0% of zeaxanthin ($C_{40}H_{56}O_2$), on the anhydrous basis.

IDENTIFICATION

- **A.**
Sample solution: Use the *Sample solution* of the test for *Content of Total Carotenoids*.
Analysis: Record the UV-Vis spectrum from 300 to 600 nm.
Acceptance criteria: The *Sample solution* shows a shoulder at about 427 nm, an absorption maximum at about 453 nm, and another maximum at about 480 nm.
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Zeaxanthin*.
- **C.** The retention time of the major peak of the *Sample solution* corresponds to that of (3R,3'S meso)-zeaxanthin from the *Standard solution*, as obtained in the test for *Stereoisomeric Composition*.

COMPOSITION

• **CONTENT OF TOTAL CAROTENOIDS**

[NOTE—Use low-actinic glassware.]

Sample stock solution: Transfer 25.0 mg of sample to a 100-mL volumetric flask, add 20 mL of chloroform, and place the flask in an ultrasonic bath at 30° for 2–5 min to obtain a clear solution. Dilute with cyclohexane to volume to obtain a solution containing 250 μ g/mL.

Sample solution: 2.5 μ g/mL of the *Sample stock solution* in cyclohexane

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Analytical wavelength: 453 nm

Cell path: 1 cm

Blank: Cyclohexane

Analysis

Sample: *Sample solution*

Calculate the percentage of total carotenoids as zeaxanthin ($C_{40}H_{56}O_2$):

$$\text{Result} = A/(C \times F)$$

A = absorbance of the *Sample solution*
C = concentration of the *Sample solution* (g/mL)
F = coefficient of extinction ($E^{1\%}$) of zeaxanthin in cyclohexane (100 mL \cdot g⁻¹ \cdot cm⁻¹), 2540

Acceptance criteria: NLT 80.0% of total carotenoids (*T*) as zeaxanthin ($C_{40}H_{56}O_2$) on the anhydrous basis

• **CONTENT OF ZEAXANTHIN**

[NOTE—Use low-actinic glassware.]

Mobile phase: Hexane and ethyl acetate (75:25)

Standard solution: 150 μ g/mL of USP *meso*-Zeaxanthin RS prepared as follows: dissolve 15.0 mg of USP *meso*-Zeaxanthin RS in 10 mL of chloroform, swirling briefly, and dilute with *Mobile phase* to 100 mL.

Sample solution: Transfer 15.0 mg of sample to a 100-mL volumetric flask, add 10 mL of chloroform, and place the flask in an ultrasonic bath at 30° for 2–5 min to obtain a clear solution. Dilute with *Mobile phase* to volume.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: HPLC

Detector: 453 nm

Column: 4.6-mm \times 25-cm; 3- μ m packing L3

Column temperature: 25°

Flow rate: 1.5 mL/min

Injection size: 10 μ L

System suitability

Sample: *Standard solution*

[NOTE—The approximate relative retention times are 0.95 and 1.0 for lutein and zeaxanthin, respectively.]

Suitability requirements

Resolution: NLT 1.0 between zeaxanthin and lutein

Tailing factor: NMT 2.0

Relative standard deviation: NMT 2.0%

Analysis: Inject the *Sample solution*, and measure the peak areas. [NOTE—The peak area of zeaxanthin is NLT 90.0% of the total peak areas.]

Calculate the percentage of zeaxanthin ($C_{40}H_{56}O_2$) in the sample taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = peak area of zeaxanthin

r_T = sum of the areas of all the peaks

T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Acceptance criteria: NLT 74.0% of $C_{40}H_{56}O_2$ on the anhydrous basis

• **LUTEIN AND OTHER RELATED COMPOUNDS**

[NOTE—Use low-actinic glassware.]

Mobile phase, Standard solution, Sample solution, and Chromatographic system: Proceed as directed in the test for *Content of Zeaxanthin*.

Analysis

Sample: *Sample solution*

[NOTE—The peak area of lutein is NMT 9.0% of the total peak areas.]

Calculate the percentage of lutein in the portion of *meso*-Zeaxanthin taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = individual peak area of lutein

r_T = sum of the areas of all the peaks

T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Calculate the percentage of other related compounds in the portion of *meso*-Zeaxanthin taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak area of any other peak (excluding zeaxanthin and lutein)

r_T = sum of the areas of all the peaks

Acceptance criteria**Lutein:** NMT 8.5%**Other related compounds:** NMT 1.0% of any other individual related compound**• STEREOISOMERIC COMPOSITION****Mobile phase:** Hexane, alcohol, and isopropanol (80:5:5)**Standard solution:** 0.1 mg/mL of USP *meso*-Zeaxanthin RS in alcohol and hexane (1:1). Dissolve in 50% of the final volume with alcohol in an ultrasonic bath at 60° for 2–5 min, cool the flask, and dilute with hexane to volume. Pass through a 0.45-μm pore size membrane filter.**Sample solution:** Weigh 10 mg of *meso*-Zeaxanthin into a 100-mL volumetric flask, add 50 mL of alcohol, and place the flask in an ultrasonic bath at 60° for 2–5 min to dissolve. Cool the flask, and dilute with hexane to volume. Pass the solution through a 0.45-μm pore size membrane filter.**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** HPLC**Detector:** 453 nm**Column:** 4.6-mm × 25-cm; 5-μm packing L51**Column temperature:** 35°**Flow rate:** 0.5 mL/min**Injection size:** 20 μL**System suitability****Sample:** *Standard solution*[NOTE—The approximate relative retention times for (3*S*,3'*S*)-zeaxanthin, (3*R*,3'*S* *meso*)-zeaxanthin, (3*R*,3'*R*)-zeaxanthin, and (3*R*,3'*R*,6'*R*)-lutein are 0.94, 1.00, 1.06, and 1.11, respectively.]**Suitability requirements****Resolution:** The resolution between each pair of peaks due to (3*S*,3'*S*)-zeaxanthin, (3*R*,3'*S* *meso*)-zeaxanthin, (3*R*,3'*R*)-zeaxanthin, and (3*R*,3'*R*,6'*R*)-lutein is NLT 1.5.**Chromatogram similarity:** The chromatogram from the *Standard solution* is similar to the reference chromatogram provided with the lot of USP *meso*-Zeaxanthin RS being used.**Analysis****Samples:** *Standard solution* and *Sample solution*
Identify the peaks of the relevant analytes from the *Standard solution* by comparison with the reference chromatogram provided with the USP Reference Standard being used. Measure the peak areas.Calculate the percentages of (3*S*,3'*S*)-zeaxanthin, (3*R*,3'*S* *meso*)-zeaxanthin, and (3*R*,3'*R*)-zeaxanthin:

$$\text{Result} = (r_U/r_S) \times 100$$

 r_U = peak area of the corresponding analyte r_S = total peak area**Acceptance criteria**(3*R*,3'*S* *meso*)-Zeaxanthin: NLT 85.0%(3*R*,3'*R*)-Zeaxanthin: NMT 15.0%(3*S*,3'*S*)-Zeaxanthin: NMT 1.0%**IMPURITIES****Inorganic Impurities****• LEAD** <251>: NMT 1 ppm**• RESIDUE ON IGNITION** <281>: NMT 1.0%**SPECIFIC TESTS****• WATER DETERMINATION, Method I** <921>: NMT 1.0%**ADDITIONAL REQUIREMENTS****• PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.**• USP REFERENCE STANDARDS** <11>USP *meso*-Zeaxanthin RSβ,β-Carotene-3,3'-diol (3*R*,3'*S*)-;(3*R*,3'*S* *meso*)-zeaxanthin.C₄₀H₅₆O₂ 568.88▲ USP36**Add the following:****▲ *meso*-Zeaxanthin Preparation****DEFINITION***meso*-Zeaxanthin Preparation is a combination of *meso*-Zeaxanthin with one or more inert substances. It may be in a solid or a liquid form. It contains NLT 95.0% and NMT 130.0% of the labeled amount of total carotenoids, calculated as zeaxanthin (C₄₀H₅₆O₂) on the anhydrous basis. It contains NLT 85.0% of zeaxanthin and NMT 9.0% of lutein of the total carotenoid content.**IDENTIFICATION****• A.****Sample solution:** Prepare as directed for *Sample solution A* or *Sample solution B* in the test for *Content of Total Carotenoids*.**Analysis:** Record the UV-Vis spectrum from 300 to 600 nm.**Acceptance criteria:** The *Sample solution* shows a shoulder at about 427 nm, an absorption maximum at about 453 nm, and another maximum at about 480 nm.**• B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Zeaxanthin*.**• C.** The retention time of the major peak of the *Sample solution* corresponds to that of (3*R*,3'*S* *meso*)-zeaxanthin from the *Standard solution*, as obtained in the test for *Stereoisomeric Composition*.**COMPOSITION****• CONTENT OF TOTAL CAROTENOIDS****Sample stock solution A** (for solid *meso*-zeaxanthin preparations): Transfer an amount of *meso*-Zeaxanthin Preparation equivalent to 7.5 mg of zeaxanthin to a 100-mL low-actinic volumetric flask, add 5 mL of water, and sonicate at 60° for 5 min. Add 50 mL of alcohol, and dilute with methylene chloride to volume. Shake thoroughly to obtain a fine dispersion, transfer 10 mL of this dispersion to a centrifuge tube, and centrifuge. Discard the foremost 2 mL of the supernatant. Use the clear solution.**Sample solution A:** Transfer 1.0 mL of *Sample stock solution A* to a 50-mL low-actinic flask, and dilute with a mixture of cyclohexane and alcohol (9:1) to volume.**Sample stock solution B** (for liquid *meso*-zeaxanthin suspensions in oil): Transfer an amount of *meso*-Zeaxanthin Preparation equivalent to 7.5 mg of *meso*-zeaxanthin to a 100-mL low-actinic volumetric flask, add 20 mL of chloroform, and sonicate for 5 min. Cool the solution to room temperature, and dilute with cyclohexane to volume. Shake thoroughly to obtain a fine dispersion, transfer 10 mL of this dispersion to a centrifuge tube, and centrifuge.**Sample solution B:** Transfer 1.0 mL of *Sample stock solution B* to a 50-mL low-actinic flask, and dilute with cyclohexane to volume.**Instrumental conditions**(See *Spectrophotometry and Light-Scattering* <851>.)**Analytical wavelength:** 453 nm**Cell path:** 1 cm**Blank:** Cyclohexane**Analysis****Sample:** *Sample solution*Calculate the percentage of total carotenoids (*T*) as *meso*-zeaxanthin (C₄₀H₅₆O₂) in the portion of *meso*-Zeaxanthin Preparation taken:

$$\text{Result} = A/(F \times C)$$

A = absorbance of the *Sample solution*
 F = coefficient of extinction ($E^{1\%}$) of zeaxanthin in cyclohexane ($100 \text{ mL} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$), 2540
 C = concentration of *meso*-Zeaxanthin Preparation in *Sample solution A* or *Sample solution B* (g/mL)
 Calculate the percentage of the labeled amount of total carotenoids:

$$\text{Result} = (T/L) \times 100$$

T = percentage of total carotenoids found as calculated above
 L = labeled amount of total carotenoids
Acceptance criteria: 95.0%–130.0% of the labeled amount of total carotenoids calculated as zeaxanthin ($\text{C}_{40}\text{H}_{56}\text{O}_2$) on the anhydrous basis

• CONTENT OF ZEAAXANTHIN

[NOTE—Use low-actinic glassware.]

Mobile phase: Hexane and ethyl acetate (75:25)

Standard solution: 75 $\mu\text{g/mL}$ of USP *meso*-Zeaxanthin RS in *Mobile phase*

Sample solution: Use *Sample stock solution A* or *Sample stock solution B* from the test for *Content of Total Carotenoids*. Pass through a 0.45- μm pore size membrane filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 453 nm

Column: 4.6-mm \times 25-cm; 3- μm packing L3

Flow rate: 1.5 mL/min

Injection size: 10 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for zeaxanthin and lutein are about 1.0 and 0.95, respectively.]

Suitability requirements

Resolution: NLT 1.0 between zeaxanthin and lutein

Tailing factor: NMT 2.0

Relative standard deviation: NMT 2.0%

Analysis

Sample: *Sample solution*

Calculate the percentage of zeaxanthin relative to total carotenoids in the portion of *meso*-Zeaxanthin Preparation taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak area of zeaxanthin

r_T = sum of the areas of all the peaks

Calculate the percentage of zeaxanthin in the portion of *meso*-Zeaxanthin Preparation taken:

$$\text{Result} = (r_U/r_T) \times T$$

r_U = individual peak area of *meso*-zeaxanthin in the *Sample solution*

r_T = sum of the areas of all the peaks

T = percentage of total carotenoids as determined in the test for *Content of Total Carotenoids*

Acceptance criteria: NLT 85.0% of zeaxanthin in the total carotenoid content

• LUTEIN AND OTHER RELATED COMPOUNDS

Mobile phase, Standard solution, Sample solution, and Chromatographic system: Proceed as directed in the test for *Content of Zeaxanthin*.

Analysis

Sample: *Sample solution*

Injection size: 10 μL

Calculate the percentage of lutein relative to total carotenoids in the portion of *meso*-Zeaxanthin Preparation taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak area of lutein

r_T = sum of the areas of all the peaks

Acceptance criteria

Lutein: NMT 9.0%

Any other single related compound: NMT 1.0%

Total related compounds (including lutein): NMT 15.0%

• STEREOISOMERIC COMPOSITION

Mobile phase: Hexane, alcohol, and isopropanol (80:5:5)

Standard solution: 0.075 mg/mL of USP *meso*-Zeaxanthin RS in alcohol and hexane (1:1). Dissolve in 50% of the final volume with alcohol in an ultrasonic bath at 60° for 2–5 min, cool the flask, and dilute with hexane to volume. Pass through a 0.45- μm pore size membrane filter.

Sample solution: Use *Sample stock solution A* or *Sample stock solution B* from the test for *Content of Total Carotenoids*. Pass through a 0.45- μm pore size membrane filter.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: HPLC

Detector: 453 nm

Column: 4.6-mm \times 25-cm; 5- μm packing L51

Column temperature: 35°

Flow rate: 0.5 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution*

[NOTE—The approximate relative retention times for (3*S*,3'*S*)-zeaxanthin, (3*R*,3'*S*-*meso*)-zeaxanthin, (3*R*,3'*R*)-zeaxanthin, and (3*R*,3'*R*,6'*R*)-lutein are 0.94, 1.00, 1.06, and 1.11, respectively.]

Suitability requirements

Resolution: The resolution between each pair of peaks due to (3*S*,3'*S*)-zeaxanthin, (3*R*,3'*S*-*meso*)-zeaxanthin, (3*R*,3'*R*)-zeaxanthin, and (3*R*,3'*R*,6'*R*)-lutein is NLT 1.5.

Chromatogram similarity: The chromatogram from the *Standard solution* is similar to the reference chromatogram provided with the lot of USP *meso*-Zeaxanthin RS being used.

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the peaks of the relevant analytes in the chromatogram of the *Standard solution* by comparison with the reference chromatogram provided with the USP Reference Standard being used. Measure the peak areas.

Calculate the percentage of (3*S*,3'*S*)-zeaxanthin, (3*R*,3'*S*-*meso*)-zeaxanthin, and (3*R*,3'*R*)-zeaxanthin:

$$\text{Result} = (r_U/r_S) \times 100$$

r_U = peak area of the corresponding analyte

r_S = total peak area

Acceptance criteria

(3*R*,3'*S*-*meso*)-Zeaxanthin: NLT 85.0%

(3*R*,3'*R*)-Zeaxanthin: NMT 15.0%

(3*S*,3'*S*)-Zeaxanthin: NMT 1.0%

IMPURITIES

Inorganic Impurities

• **RESIDUE ON IGNITION** <281>: NMT 2.0%

• **HEAVY METALS**, *Method II* <231>: NMT 10 ppm

SPECIFIC TESTS

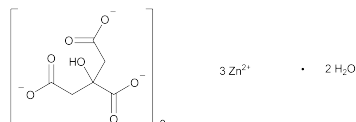
• **WATER DETERMINATION**, *Method I* <921>: NMT 8.0% for solid preparations. NMT 1.0% for liquid preparations.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tightly sealed, light- and oxygen-resistant containers. Store in a cool place.

- **LABELING:** The label states the name and content of any carriers and antioxidants added to the formulation, and the content of total carotenoids as zeaxanthin.
- **USP REFERENCE STANDARDS** (11)
USP *meso*-Zeaxanthin RS
 β,β -Carotene-3,3'-diol (3*R*,3'*S*)-;
(3*R*,3'*S* *meso*)-zeaxanthin.
 $C_{40}H_{56}O_2$ 568.88 ▲ USP36

Zinc Citrate



$C_{12}H_{10}O_{14}Zn_3 \cdot 2H_2O$ 610.36
2-Hydroxy-1,2,3-propanetricarboxylic acid zinc salt, dihydrate [5990-32-9].
Anhydrous [546-46-3].

DEFINITION

Zinc Citrate contains NLT 31.3% of zinc (Zn), calculated on the dried basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Zinc** (191): A solution (1 in 10) meets the requirements.
- **B. IDENTIFICATION TESTS—GENERAL, Citrate** (191): A solution (1 in 10) meets the requirements.

ASSAY

• PROCEDURE

Sample: 350 mg of Zinc Citrate, previously dried at 105° for 2 h

Blank: 60 mL of water

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.05 M edetate disodium VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 60 mL of water. Add 10 mL of ammonia–ammonium chloride buffer TS and 0.1 mL of eriochrome black TS. Titrate with the *Titrant* to a blue endpoint. Perform a blank determination. Calculate the percentage of zinc (Zn) in the portion of Zinc Citrate taken:

$$\text{Result} = [(V - B) \times M \times F \times 100] / W$$

V = sample titrant volume (mL)

B = blank titrant volume (mL)

M = titrant molarity (mM/mL)

F = equivalency factor, 65.4 mg/mM

W = sample weight (mg)

Acceptance criteria: NLT 31.3% on the dried basis

IMPURITIES

- **CHLORIDE AND SULFATE, Chloride** (221): A 1.0-g portion shows no more chloride than corresponds to 0.7 mL of 0.020 N hydrochloric acid (NMT 0.05%).
- **CHLORIDE AND SULFATE, Sulfate** (221): A 1.8-g portion shows no more sulfate than corresponds to 0.5 mL of 0.020 N sulfuric acid (NMT 0.05%).
- **LIMIT OF ARSENIC, CADMIUM, AND LEAD**
Arsenic standard solution: 1.0 µg/mL in 1% nitric acid, prepared from an arsenic standard solution (10 mg/L)

Cadmium standard solution: 1.0 µg/mL in 1% nitric acid, prepared from a cadmium standard solution (10 mg/L)

Lead standard solution: 1.0 µg/mL in 1% nitric acid, prepared from a lead standard solution (10 mg/L)

Multi-element standard solution: 10 µg/L of lead, 5 µg/L of cadmium, and 3 µg/L of arsenic in 1% nitric acid, prepared from the *Lead standard solution*, *Cadmium standard solution*, and *Arsenic standard solution*, respectively

Sample solution: 2 mg/mL of Zinc Citrate in 1% nitric acid

Instrumental conditions

(See *Plasma Spectrochemistry* (730).)

Mode: ICP-MS

Radio frequency: 1350 Watts

Nebulizer flow rate: 0.9 L/min

[NOTE—The radio frequency and nebulizer flow rate settings may be developed and optimized based on the manufacturer's recommendation.]

Detection atomic masses: As, Cd, and Pb

Blank: 1% nitric acid solution

Analysis

Samples: *Multi-element standard solution*, *Sample solution*, and *Blank*

Determine the responses of the *Multi-element standard solution*, *Sample solution*, and *Blank* at the masses indicated above.

Calculate the content of each element, in µg/g, in the portion of Zinc Citrate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U)$$

r_U = peak response of the corresponding element from the *Sample solution*

r_S = peak response of the corresponding element from the *Multi-element standard solution*

C_S = concentration of the corresponding element in the *Multi-element standard solution* (µg/L)

C_U = concentration of Zinc Citrate in the *Sample solution* (g/L)

Acceptance criteria

Arsenic: NMT 3 µg/g

Cadmium: NMT 5 µg/g

Lead: NMT 10 µg/g

SPECIFIC TESTS

- **LOSS ON DRYING** (731): Dry a sample at 105° for 2 h: it loses NMT 1.0% of its weight.
- **MICROBIAL ENUMERATION TESTS** (2021): The total aerobic microbial count does not exceed 10³ cfu/g. The total combined yeasts and molds count does not exceed 10² cfu/g.
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): It meets the requirements of the test for absence of *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Zinc Citrate Tablets

DEFINITION

Zinc Citrate Tablets contain NLT 90.0% and NMT 110.0% of the labeled amount of zinc (Zn).

IDENTIFICATION

- **A.** The *Sample solution* for *Strength* produces line emissions or absorptions at the characteristic wavelengths for zinc.

• **B. IDENTIFICATION TESTS—GENERAL, Citrate (191)**

Sample solution: Transfer a quantity of powdered Tablets, equivalent to about 15 mg of zinc, to a centrifuge tube. Add 2–5 mL of water, sonicate for 1 min, shake, and centrifuge.

Acceptance criteria: Meets the requirements

STRENGTH

• **CONTENT OF ZINC**

Method 1

[NOTE—A standard stock solution is commercially available at different zinc concentrations, which may be used for preparation of the *Standard stock solution*. Necessary volumetric adjustment can be made in the *Standard solution*. Concentrations of the *Standard solution* and the *Sample solution* may be modified to fit the linear or working range of the instrument.]

Standard stock solution: Dissolve 625 mg of zinc oxide, weighed, and previously ignited to constant weight, in 10 mL of nitric acid, and add water to make 500.0 mL. This solution contains 1000 mg/L of zinc.

Standard solution: To a 500-mL volumetric flask add 200 mL of water and 10 mL of nitric acid, and mix thoroughly. Pipette 10.0 mL of the *Standard stock solution* into the volumetric flask, and dilute with water to volume to obtain a solution having a known concentration of about 20 mg/L of zinc.

Sample solution: Weigh and finely powder NLT 20 Tablets. Transfer weighed portion of the powdered Tablets, equivalent to about 0.1 g of zinc, to a 50-mL flask. Add 10 mL of nitric acid, and heat the solution on a hot plate to boil gently, during which process fuming evolves. Boil the solution for an additional 30 min with constant swirling, during which no fuming should be observed. Cool the solution to room temperature, quantitatively transfer all of the solution to a 500-mL volumetric flask, dilute with water to volume, and mix. Pipette 25.0 mL of this solution into a 250-mL volumetric flask, add 5 mL of nitric acid, dilute with water to volume, mix, and filter.

Inductively coupled plasma system

(See *Plasma Spectrochemistry* (730).)

Mode: Atomic emission spectroscopy

Analytical wavelength: 206.20 nm. [NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. A typical setting includes radio frequency (RF) power of about 1300 watts, argon torch flow of about 15 L/min, argon auxiliary flow of about 0.2 L/min, and a nebulizer flow rate of about 0.8 L/min.]

Blank: 2% nitric acid solution

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank*

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = response from the *Sample solution*

r_S = response from the *Standard solution*

C_S = concentration of the *Standard solution* (mg/L)

C_U = nominal concentration of zinc in the *Sample solution* (mg/L)

Acceptance criteria: 90.0%–110.0%

Method 2

Standard stock solution A: 1000 µg/mL of zinc from zinc oxide in 5 M hydrochloric acid (3.89 mg/mL) and diluted with water to final volume. [NOTE—Dissolve in 5 M hydrochloric acid by warming, if necessary. Cool, and then dilute to final volume.]

Standard stock solution B: 50 µg/mL of zinc from *Standard stock solution A* diluted with 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution B* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Finely powder NLT 20 tablets. Transfer an equivalent to 5 tablets to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively, with 0.125 N hydrochloric acid to obtain a nominal concentration of 2 µg/mL of zinc.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Tablets taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of zinc in the *Sample solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0%

PERFORMANCE TESTS

• **DISINTEGRATION AND DISSOLUTION (2040)**

Medium: Water; 900 mL

Apparatus 2: 75 rpm

Time: 60 min

Analysis: Proceed as directed in *Method 1* or *Method 2* for *Strength*, making any necessary volumetric adjustments.

Sample solution: If *Method 1* is used, pipet 10.0 mL of the filtered pooled solution under test to a 50-mL volumetric flask, and dilute with 2% nitric acid solution to 50 mL. If *Method 2* is used, dilute the filtered pooled solution under test with 0.125 N hydrochloric acid to a concentration falling within the range of the *Standard solutions*.

Calculate the percentage of the labeled amount of zinc (Zn) dissolved:

$$\text{Result} = C \times (V_M/a) \times (D/L) \times 100$$

C = concentration of zinc in the *Sample solution* (mg/L)

V_M = volume of *Medium*, 900 mL

a = aliquot of solution under test (mL)

D = dilution factor to prepare the *Sample solution* from the aliquot taken

L = label claim (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of zinc is dissolved.

- **WEIGHT VARIATION (2091):** Meet the requirements

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined yeast and mold count does not exceed 10^2 cfu/g.
- **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** It meets the requirements of the test for absence of *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** The label states the quantity of zinc in terms of mg/Tablet.

Zinc and Vitamin C Lozenges

DEFINITION

Zinc and Vitamin C Lozenges contain NLT 90.0% and NMT 110.0% of the labeled amount of zinc (Zn) derived from substances generally recognized as safe and furnishing an ionizable form of zinc; and NLT 90.0% and NMT 120.0% of the labeled amount of vitamin C, as ascorbic acid ($C_6H_8O_6$), sodium ascorbate ($C_6H_7NaO_6$), or calcium ascorbate dihydrate ($C_{12}H_{14}CaO_{12} \cdot 2H_2O$). It contains no other vitamins or minerals for which nutritional value is claimed. It may contain other labeled added substances or additional ingredients in amounts that are unobjectionable.

IDENTIFICATION

- **A.**
Analysis: Proceed as directed in *Strength for Content of Zinc*.
Acceptance criteria: The *Sample solution* produces line emissions or absorptions at the characteristic wavelengths for zinc.
- **B.**
Analysis: Triturate a quantity of finely powdered Lozenges with sufficient alcohol to obtain a solution containing the equivalent of 20 mg/mL of ascorbic acid, sodium ascorbate, or calcium ascorbate dihydrate, and filter. Add 1 mL of 0.1 N hydrochloric acid to 4 mL of the filtrate from Lozenges containing sodium ascorbate or calcium ascorbate.
Acceptance criteria: A portion of the filtrate reduces alkaline cupric tartrate TS slowly at room temperature but more readily upon heating.

STRENGTH

• CONTENT OF ZINC

Procedure 1

[NOTE—A standard stock solution is commercially available at different zinc concentrations, which may be used for preparation of *Standard stock solution*. Necessary volumetric adjustment can be made in the *Standard solution*. Concentrations of the *Standard solution* and the *Sample solution* may be modified to fit the linear or working range of the instrument.]

Standard stock solution: Dissolve 625 mg of zinc oxide, weighed, and previously ignited to constant weight, in 10 mL of nitric acid, and add water to make 500.0 mL. This solution contains 1000 µg/mL of zinc.

Standard solution: To a 500-mL volumetric flask add 200 mL of water and 10 mL of nitric acid, and mix thoroughly. Pipet 10.0 mL of the *Standard stock solution* into the volumetric flask, and dilute with water to

volume to obtain a solution having a known concentration of about 20 µg/mL of zinc.

Sample solution: Weigh and finely powder NLT 20 Lozenges. Transfer an accurately weighed portion of the powdered Lozenges, equivalent to about 0.1 g of zinc, to a 50-mL flask. Add 10 mL of nitric acid, and heat the solution on a hot plate to boil gently, during which process fuming evolves. Boil the solution for an additional 30 min with constant swirling, during which no fuming should be observed. Cool the solution to room temperature, quantitatively transfer all of the solution to a 500-mL volumetric flask, dilute with water to volume, and mix. Pipet 25.0 mL of this solution into a 250-mL volumetric flask, add 5 mL of nitric acid, dilute with water to volume, mix, and filter.

Inductively coupled plasma system

(See *Plasma Spectrochemistry* (730).)

Mode: Atomic emission spectroscopy

Analytical wavelength: 206.20 nm

[NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation. A typical setting includes radio frequency (RF) power of about 1300 watts, argon torch flow of about 15 L/min, argon auxiliary flow of about 0.2 L/min, and a nebulizer flow rate of about 0.8 L/min.]

Blank: 2% nitric acid solution

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank*

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Lozenges taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = response from the *Sample solution*

r_S = response from the *Standard solution*

C_S = concentration of zinc in the *Standard solution* (µg/mL)

C_U = nominal concentration of zinc in the *Sample solution* (µg/mL)

Acceptance criteria: 90.0%–110.0% of the labeled amount of zinc

Procedure 2

Standard stock solution A: Dissolve zinc oxide in 5 M hydrochloric acid with warming, if necessary, to obtain a solution with a concentration of 3.89 mg/mL. Dilute with water to obtain a solution with a concentration of 1000 µg/mL of zinc.

Standard stock solution B: 50 µg/mL of zinc from

Standard stock solution A in 0.125 N hydrochloric acid

Standard solutions: Transfer 1.0, 2.0, 3.0, 4.0, and 5.0 mL of *Standard stock solution B* to separate 100-mL volumetric flasks. Dilute the contents of each flask with 0.125 N hydrochloric acid to volume to obtain solutions containing 0.5, 1.0, 1.5, 2.0, and 2.5 µg/mL of zinc.

Sample solution: Finely powder NLT 20 Lozenges.

Transfer an equivalent to 5 Lozenges to a porcelain crucible. Heat the crucible in a muffle furnace maintained at 550° for 6–12 h, and cool. Add 60 mL of hydrochloric acid, and boil gently on a hot plate or steam bath for 30 min, intermittently rinsing the inner surface of the crucible with 6 N hydrochloric acid. Cool, and quantitatively transfer the contents of the crucible to a 100-mL volumetric flask. Rinse the crucible with small portions of 6 N hydrochloric acid, and add the rinsings to the flask. Dilute with water to volume, and filter, discarding the first 5 mL of the filtrate. Dilute this solution quantitatively with 0.125 N hydrochloric acid to obtain a nominal concentration of 2 µg/mL of zinc.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 213.8 nm

Lamp: Zinc hollow-cathode

Flame: Air–acetylene

Blank: 0.125 N hydrochloric acid

Analysis

Samples: *Standard solutions* and *Sample solution*

Determine the absorbances of the solutions against the *Blank*. Plot the absorbances of the *Standard solutions* versus the concentration, in $\mu\text{g/mL}$, of zinc, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in $\mu\text{g/mL}$, of zinc in the *Sample solution*.

Calculate the percentage of the labeled amount of zinc (Zn) in the portion of Lozenges taken:

$$\text{Result} = (C/C_U) \times 100$$

C = determined concentration of zinc in the *Sample solution* ($\mu\text{g/mL}$)

C_U = nominal concentration of zinc in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 90.0%–110.0% of the label claim

• CONTENT OF VITAMIN C

Sample solution: Transfer NLT 20 Lozenges to a 1000-mL volumetric flask containing 250 mL of metaphosphoric–acetic acids TS. Insert the stopper in the flask, and shake by mechanical means for 30 min or until the lozenges have disintegrated completely. Dilute with water to volume. Transfer a portion of the solution to a centrifuge tube, and centrifuge until a clear supernatant is obtained. Quantitatively dilute the clear supernatant with water, if necessary, to obtain a solution containing 0.5 mg/mL of ascorbic acid.

Blank: A mixture of 5.5 mL of metaphosphoric–acetic acids TS and 15 mL of water

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: Standard dichlorophenol–indophenol VS

Endpoint detection: Visual, a rose-pink color that persists for at least 5 s

Analysis: Transfer a volume of the *Sample solution*, equivalent to 2 mg of ascorbic acid, into a 50-mL conical flask. Add 5 mL of metaphosphoric–acetic acids TS, and titrate with *Titrant*. Correct for the volume of the *Titrant* consumed by the *Blank*.

Calculate the percentage of the labeled amount of ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$) in the portion of the Lozenges taken:

$$\text{Result} = \{[(V_S - V_B) \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample solution* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

F = ascorbic acid equivalent of the *Titrant* (mg/mL)

W = nominal weight of ascorbic acid taken for *Analysis* (mg)

Acceptance criteria: 90.0%–120.0% of the labeled amount

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS (2021):** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined yeasts and molds count does not exceed 10^2 cfu/g.

• **ABSENCE OF SPECIFIED MICROORGANISMS (2022):** It meets the requirements of the test for absence of *Escherichia coli*.

PERFORMANCE TESTS

• DISINTEGRATION AND DISSOLUTION (2040)

Medium: 0.1 N hydrochloric acid; 900 mL

Apparatus 2: 75 rpm

Time: 60 min

Analysis: Determine the amount of zinc (Zn) and vitamin C dissolved, using the procedures in *Strength for Content of Zinc* and *Content of Vitamin C*, making any necessary volumetric adjustments. [NOTE—Proceed without delay in the vitamin C determination.]

Calculate the percentage of the labeled amount of zinc (Zn) dissolved:

$$\text{Result} = C \times (V_M/a) \times (D/L) \times 100$$

C = measured concentration of Zinc in the *Sample solution* (mg/mL)

V_M = volume of *Medium*, 900 mL

a = aliquot of solution under test taken (mL)

D = dilution factor to prepare the *Sample solution* from the aliquot taken

L = labeled amount of zinc (mg/Tablet)

Calculate the percentage of the labeled amount of vitamin C, as ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$), dissolved:

$$\text{Result} = (V_S - V_B) \times F \times [(V_M/a)/L] \times 100$$

V_S = *Titrant* volume consumed by the *Sample solution* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

F = concentration of the *Titrant* in terms of the equivalent of ascorbic acid (mg/mL)

V_M = volume of *Medium*, 900 mL

a = volume of the aliquot taken for *Analysis*

L = labeled amount of ascorbic acid (mg/Tablet)

Tolerances: NLT 75% of the labeled amount of zinc (Zn) and vitamin C is dissolved.

• **WEIGHT VARIATION (2091):** Meet the requirements

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **LABELING:** The label states the quantity of zinc and vitamin C as ascorbic acid in mg per Lozenge, and the salt form of zinc and the chemical form of vitamin C present in the Lozenges.

Zinc Gluconate—see Zinc Gluconate General Monographs

NF 31

**THE NATIONAL
FORMULARY**

Official from May 1, 2013

THIRTY-FIRST EDITION

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Admissions

Articles Admitted to *NF 31* by Supplement

First Supplement (August 1, 2012)

Betadex Sulfobutyl Ether Sodium

Second Supplement (December 1, 2012)

Ferrosoferric Oxide
Polyoxyl Stearate
Pregelatinized Hydroxypropyl Corn Starch

Pregelatinized Hydroxypropyl Pea Starch
Pregelatinized Hydroxypropyl Potato Starch

New Articles Appearing in *NF 31* That Were Not Included in *NF 30* Including Supplements

[NOTE—The articles included in this list are noted in the book with the following symbols ▲ ▲ *NF31*. This applies to new articles as well as sections of existing items that have been revised.]

New Articles Appearing in *NF 31*

Caprylic Acid
Diethyl Sebacate

Glyceryl Tristearate

ANNOTATED LIST

General Notices, Monographs, General Chapters, Reagents, and Tables Affected by Changes Appearing in NF 31

Page citations refer to the pages of NF 31. Note—In the list below, if a section is new or if a subsection is added to or deleted from an existing section, it is labeled as such in parentheses after the section or subsection name. Items on this list that appear without the designation “new,” “added,” or “deleted” are items in which changes have been made to existing official text.

Excipients

Emulsifying and/or Solubilizing Agent

Caprylic Acid, 1860

Glyceryl Tristearate, 1860

Flavors and Perfumes

Diethyl Sebacate, 1861

Tablet and/or Capsule Lubricant

Glyceryl Tristearate, 1863

Monographs (NF 31)

Benzaldehyde, 1896

CHEMICAL INFORMATION

DEFINITION

IDENTIFICATION

Infrared Absorption, Test A (added)

ASSAY

Procedure

IMPURITIES

Limit of Ethylbenzene, Cyclohexylmethanol, Benzyl Alcohol, and Benzoic Acid (added)

SPECIFIC TESTS

Specific Gravity (deleted), *Refractive Index* (deleted), and *Water Determination, Method I* (added)

ADDITIONAL REQUIREMENTS

Packaging and Storage (added) and *USP Reference Standards* (added)

Caprylic Acid, 1921 (new)

Caprylocaproyl Polyoxylglycerides, 1922

IMPURITIES

Limit of Free Ethylene Oxide and Dioxane

SPECIFIC TESTS

Fats and Fixed Oils, Hydroxyl Value and Fats and Fixed Oils, Saponification Value

ADDITIONAL REQUIREMENTS

Labeling

Diethyl Sebacate, 1994 (new)

Glyceryl Tristearate, 2033 (new)

Myristic Acid, 2104

CHEMICAL INFORMATION

IDENTIFICATION

Infrared Absorption, Test A (added) and *Test B* (added)

IMPURITIES

Limit of Mineral Acids (added)

SPECIFIC TESTS

Fats and Fixed Oils, Saponification Value (deleted) and *Fats and Fixed Oils, Peroxide Value* (added)

Oleic Acid, 2111

DEFINITION

IDENTIFICATION

Test B (added)

ASSAY

Procedure (added)

SPECIFIC TESTS

Specific Gravity (deleted), *Content of Fatty Acids* (added), *Fats and Fixed Oils, Iodine Value, Fats and Fixed Oils, Peroxide Value* (added), *Water Determination, Method I* (added), and *Neutral Fat or Mineral Oil* (deleted)

ADDITIONAL REQUIREMENTS

Packaging and Storage

Sucrose, 2254

Excipients

USP and NF Excipients, Listed by Category

In the following reference table, the grouping of excipients by functional category is intended to summarize the most typically identified purpose that these excipients serve in drug product formulations. The list of substances included in each category is not comprehensive. The statement of category is intended neither to limit in any way the choice or use of the substance nor to indicate that it has no other utility.

Acidifying Agent

Acetic Acid
Acetic Acid, Glacial
Citric Acid, Anhydrous
Citric Acid Monohydrate
Fumaric Acid
Hydrochloric Acid
Hydrochloric Acid, Diluted
Malic Acid
Nitric Acid
Phosphoric Acid
Phosphoric Acid, Diluted
Propionic Acid
Sulfuric Acid
Tartaric Acid

Aerosol Propellant

Butane
Dichlorodifluoromethane
Dichlorotetrafluoroethane
Isobutane
Propane
Trichloromonofluoromethane

Air Displacement

Carbon Dioxide
Nitrogen

Alcohol Denaturant

Denatonium Benzoate
Methyl Isobutyl Ketone
Sucrose Octaacetate

Alkalizing Agent

Ammonia Solution, Strong
Ammonium Carbonate
Diethanolamine
Potassium Hydroxide
Sodium Bicarbonate
Sodium Borate
Sodium Carbonate
Sodium Hydroxide
Trolamine

Anticaking Agent (See *Glidant*)

Antifoaming Agent

Dimethicone
Myristic Acid
Palmitic Acid
Simethicone

Antimicrobial Preservative

Benzalkonium Chloride
Benzalkonium Chloride Solution

Benzethonium Chloride
Benzoic Acid
Benzyl Alcohol
Butylparaben
Calcium Propionate
Cetrimonium Bromide
Cetylpyridinium Chloride
Chlorobutanol
Chlorocresol
Cresol
Dehydroacetic Acid
Erythorbic Acid
Ethylparaben
Methylparaben
Methylparaben Sodium
Phenol
Phenoxyethanol
Phenylethyl Alcohol
Phenylmercuric Acetate
Phenylmercuric Nitrate
Potassium Benzoate
Potassium Sorbate
Propylparaben
Propylparaben Sodium
Sodium Benzoate
Sodium Dehydroacetate
Sodium Propionate
Sorbic Acid
Thimerosal
Thymol

Antioxidant

Ascorbic Acid
Ascorbyl Palmitate
Butylated Hydroxyanisole
Butylated Hydroxytoluene
Erythorbic Acid
Hypophosphorous Acid
Lactobionic Acid
Monothioglycerol
Potassium Metabisulfite
Propyl Gallate
Racemethionine
Sodium Bisulfite
Sodium Formaldehyde Sulfoxylate
Sodium Metabisulfite
Sodium Sulfite
Sodium Thiosulfate
Stannous Chloride
Sulfur Dioxide
Tocopherols Excipient

Buffering Agent

Acetic Acid
Adipic Acid
Ammonium Carbonate
Ammonium Phosphate
Boric Acid
Citric Acid, Anhydrous

Citric Acid Monohydrate
 Alpha-Lactalbumin
 Lactic Acid
 Phosphoric Acid
 Potassium Citrate
 Potassium Metaphosphate
 Potassium Phosphate, Dibasic
 Potassium Phosphate, Monobasic
 Racemethionine
 Sodium Acetate
 Sodium Citrate
 Sodium Lactate Solution
 Sodium Phosphate, Dibasic
 Sodium Phosphate, Monobasic
 Succinic Acid

Bulking Agent for Freeze-Drying

Creatinine
 Alpha-Lactalbumin
 Mannitol
 Polydextrose
 Polydextrose, Hydrogenated
 Pullulan
 Trehalose

Capsule Lubricant (See *Tablet and/or Capsule Lubricant*)**Chelating Agent**

Edetate Calcium Disodium
 Edetate Disodium
 Edetic Acid

Coating Agent

Amino Methacrylate Copolymer
 Ammonio Methacrylate Copolymer
 Ammonio Methacrylate Copolymer Dispersion
 Carboxymethylcellulose Sodium
 Carboxymethylcellulose Sodium, Enzymatically-Hydrolyzed
 Cellaburate
 Cellacefate (formerly Cellulose Acetate Phthalate)
 Cellulose Acetate
 Cellulose Acetate Phthalate (see Cellacefate)
 Chitosan
 Coconut Oil
 Coconut Oil, Hydrogenated
 Copovidone
 Corn Syrup Solids
 Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
 Ethylcellulose
 Ethylcellulose Aqueous Dispersion
 Ethylcellulose Dispersion Type B
 Ethylene Glycol and Vinyl Alcohol Graft Copolymer
 Gelatin
 Glaze, Pharmaceutical
 Hydroxypropyl Cellulose
 Hydroxypropyl Methylcellulose (see Hypromellose)
 Hydroxypropyl Methylcellulose Phthalate (see Hypromellose Phthalate)
 Hypromellose (formerly Hydroxypropyl Methylcellulose)
 Hypromellose Acetate Succinate
 Hypromellose Phthalate (formerly Hydroxypropyl Methylcellulose Phthalate)
 Alpha-Lactalbumin
 Maltodextrin
 Methacrylic Acid and Ethyl Acrylate Copolymer
 Methacrylic Acid and Ethyl Acrylate Copolymer, Partially-Neutralized
 Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion
 Methacrylic Acid and Methyl Methacrylate Copolymer
 Methacrylic Acid Copolymer
 Methacrylic Acid Copolymer Dispersion
 Methylcellulose
 Palm Kernel Oil
 Palm Oil
 Palm Oil, Hydrogenated

Polydextrose, Hydrogenated
 Polyethylene Glycol
 Polyvinyl Acetate
 Polyvinyl Acetate Dispersion
 Polyvinyl Acetate Phthalate
 Pullulan
 Rapeseed Oil, Fully Hydrogenated
 Rapeseed Oil, Superglycerinated Fully Hydrogenated
 Shellac
 Starch, Pregelatinized Modified
 Sucrose
 Titanium Dioxide
 Wax, Carnauba
 Wax, Microcrystalline
 Zein

Color

Caramel
 Ferric Oxide, red, yellow, or blends
 Ferrosoferric Oxide

Coloring Agent

Aluminum Oxide

Complexing Agent

Betadex Sulfobutyl Ether Sodium
 Edetate Calcium Disodium
 Edetate Disodium
 Edetic Acid
 Alpha-Lactalbumin
 Oxyquinoline Sulfate

Desiccant

Calcium Chloride
 Calcium Sulfate
 Polyvinyl Acetate
 Silicon Dioxide

Emollient

Alkyl (C12-15) Benzoate
 Oleyl Oleate
 Polydecene, Hydrogenated
 Soybean Oil, Hydrogenated

Change to read:**Emulsifying and/or Solubilizing Agent**

Acacia
 ▲ Caprylic Acid▲ NF31
 Carbomer Copolymer
 Carbomer Interpolymer
 Cholesterol
 Coconut Oil
 Cyclodextrin, Gamma
 Desoxycholic Acid
 Diethanolamine (Adjunct)
 Diethylene Glycol Stearates
 Ethylene Glycol Stearates
 Glyceryl Distearate
 Glyceryl Monolinoleate
 Glyceryl Monooleate
 Glyceryl Monostearate
 ▲ Glyceryl Tristearate▲ NF31
 Alpha-Lactalbumin
 Lanolin Alcohols
 Lecithin
 Mono- and Di-glycerides
 Monoethanolamine (Adjunct)
 Oleic Acid (Adjunct)
 Oleyl Alcohol (Stabilizer)
 Oleyl Oleate
 Palm Kernel Oil
 Palm Oil
 Poloxamer
 Polyglyceryl 3 Diisostearate
 Polyglyceryl Dioleate
 Polyoxyethylene 50 Stearate
 Polyoxyl 10 Oleyl Ether

Polyoxyl 20 Cetostearyl Ether
 Polyoxyl 35 Castor Oil
 Polyoxyl 40 Hydrogenated Castor Oil
 Polyoxyl 40 Stearate
 Polyoxyl Lauryl Ether
 Polyoxyl Stearyl Ether
 Polysorbate 20
 Polysorbate 40
 Polysorbate 60
 Polysorbate 80
 Polyoxyl Stearate
 Propylene Glycol Dicaprylate/Dicaprate
 Propylene Glycol Monocaprylate
 Propylene Glycol Monostearate
 Rapeseed Oil, Superglycerinated Fully Hydrogenated
 Sodium Cetostearyl Sulfate
 Sodium Lauryl Sulfate
 Sodium Stearate
 Sorbitan Monolaurate
 Sorbitan Monooleate
 Sorbitan Monopalmitate
 Sorbitan Monostearate
 Sorbitan Sesquioleate
 Sorbitan Trioleate
 Stannous Chloride
 Stearic Acid
 Sucrose Stearate
 Trolamine
 Wax, Emulsifying

Film-Forming Agent

Ammonio Methacrylate Copolymer
 Chitosan
 Ethylcellulose Dispersion Type B
 Methacrylic Acid and Ethyl Acrylate Copolymer
 Methacrylic Acid and Ethyl Acrylate Copolymer, Partially-Neutralized
 Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion
 Methacrylic Acid and Methyl Methacrylate Copolymer

Filtering Aid

Cellulose, Powdered
 Siliceous Earth, Purified

Change to read:**Flavors and Perfumes**

Almond Oil
 Anethole
 Benzaldehyde
 ▲ Diethyl Sebacate▲^{NF31}
 Ethyl Acetate
 Ethyl Vanillin
 L-Glutamic Acid, Hydrochloride
 Lactitol
 Maltol
 Menthol
 Methyl Salicylate
 Monosodium Glutamate
 Peppermint
 Peppermint Oil
 Peppermint Spirit
 Racemethionine
 Rose Oil
 Rose Water, Stronger
 Thymol
 Vanillin

Glidant and/or Anticaking Agent

Calcium Silicate
 Magnesium Silicate
 Silica, Hydrophobic Colloidal
 Silicon Dioxide, Colloidal
 Talc

Humectant

Corn Syrup Solids
 Erythritol
 Glycerin
 Hexylene Glycol
 Inositol
 Maltitol
 Polydextrose
 Polydextrose, Hydrogenated
 Propylene Glycol
 Sorbitol
 Sorbitol Sorbitan Solution
 Starch Hydrolysate, Hydrogenated
 Tagatose

Ointment Base

Caprylocaproyl Polyoxylglycerides
 Diethylene Glycol Monoethyl Ether
 Lanolin
 Lauroyl Polyoxylglycerides
 Linoleoyl Polyoxylglycerides
 Ointment, Hydrophilic
 Ointment, White
 Ointment, Yellow
 Oleoyl Polyoxylglycerides
 Petrolatum
 Petrolatum, Hydrophilic
 Petrolatum, White
 Polydecene, Hydrogenated
 Polyethylene Glycol Monomethyl Ether
 Polyglyceryl 3 Diisostearate
 Rose Water Ointment
 Squalane
 Stearoyl Polyoxylglycerides
 Vegetable Oil, Hydrogenated, Type II

Plasticizer

Acetyltributyl Citrate
 Acetyltriethyl Citrate
 Castor Oil
 Diacetylated Monoglycerides
 Dibutyl Sebacate
 Diethyl Phthalate
 Glycerin
 Polyethylene Glycol
 Polyethylene Glycol Monomethyl Ether
 Propylene Glycol
 Pullulan
 Sorbitol Sorbitan Solution
 Triacetin
 Tributyl Citrate
 Triethyl Citrate

Polymer Membrane

Amino Methacrylate Copolymer
 Ammonio Methacrylate Copolymer
 Ammonio Methacrylate Copolymer Dispersion
 Cellulaburate
 Cellulose Acetate
 Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
 Pullulan

Sequestering Agent

Betadex (formerly Beta Cyclodextrin)
 Betadex Sulfobutyl Ether Sodium
 Cyclodextrin, Beta (see Betadex)
 Cyclodextrin, Gamma
 Hydroxypropyl Betadex
 Pullulan
 Sodium Tartrate

Solvent

Acetone
 Alcohol
 Alcohol, Diluted
 Amylene Hydrate
 Benzyl Benzoate
 Butyl Alcohol

- Canola Oil
- Caprylocaproyl Polyoxylglycerides
- Corn Oil
- Cottonseed Oil
- Diethylene Glycol Monoethyl Ether
- Ethyl Acetate
- Glycerin
- Hexylene Glycol
- Isopropyl Alcohol
- Lauroyl Polyoxylglycerides
- Linoleoyl Polyoxylglycerides
- Methyl Alcohol
- Methyl Isobutyl Ketone
- Methylene Chloride
- Methylpyrrolidone
- Mineral Oil
- Oleoyl Polyoxylglycerides
- Peanut Oil
- Polydecene, Hydrogenated
- Polyethylene Glycol
- Polyethylene Glycol Monomethyl Ether
- Propylene Glycol
- Sesame Oil
- Stearoyl Polyoxylglycerides
- Water, Purified
- Water for Injection
- Water for Injection, Sterile
- Water for Irrigation, Sterile
- Sorbent**
 - Cellulose, Powdered
 - Charcoal, Activated
 - Siliceous Earth, Purified
- Sorbent, Carbon Dioxide**
 - Barium Hydroxide Lime
 - Soda Lime
- Stiffening Agent**
 - Castor Oil, Hydrogenated
 - Cetostearyl Alcohol
 - Cetyl Alcohol
 - Cetyl Esters Wax
 - Cetyl Palmitate
 - Hard Fat
 - Alpha-Lactalbumin
 - Paraffin
 - Paraffin, Synthetic
 - Rapeseed Oil, Fully Hydrogenated
 - Rapeseed Oil, Superglycerinated Fully Hydrogenated
 - Stearyl Alcohol
 - Wax, Emulsifying
 - Wax, White
 - Wax, Yellow
- Suppository Base**
 - Cocoa Butter
 - Hard Fat
 - Polyethylene Glycol
- Suspending and/or Viscosity-Increasing Agent**
 - Acacia
 - Agar
 - Alamic Acid
 - Alginic Acid
 - Aluminum Monostearate
 - Attapulgate, Activated
 - Attapulgate, Colloidal Activated
 - Bentonite
 - Bentonite, Purified
 - Bentonite Magma
 - Carbomer 910
 - Carbomer 934
 - Carbomer 934P
 - Carbomer 940
 - Carbomer 941
 - Carbomer 1342
 - Carbomer Copolymer
 - Carbomer Homopolymer
 - Carbomer Interpolymer
 - Carboxymethylcellulose Calcium
 - Carboxymethylcellulose Sodium
 - Carboxymethylcellulose Sodium 12
 - Carboxymethylcellulose Sodium, Enzymatically-Hydrolyzed
 - Carmellose
 - Carrageenan
 - Cellulose, Microcrystalline, and Carboxymethylcellulose Sodium
 - Chitosan
 - Corn Syrup
 - Corn Syrup Solids
 - Dextrin
 - Gelatin
 - Gellan Gum
 - Guar Gum
 - Hydroxyethyl Cellulose
 - Hydroxypropyl Cellulose
 - Hydroxypropyl Methylcellulose (see Hypromellose)
 - Hypromellose (formerly Hydroxypropyl Methylcellulose)
 - Alpha-Lactalbumin
 - Magnesium Aluminum Silicate
 - Maltodextrin
 - Methylcellulose
 - Pectin
 - Polydextrose, Hydrogenated
 - Polyethylene Oxide
 - Polyvinyl Alcohol
 - Povidone
 - Propylene Glycol Alginate
 - Pullulan
 - Silica, Hydrophobic Colloidal
 - Silicon Dioxide
 - Silicon Dioxide, Colloidal
 - Sodium Alginate
 - Starch, Corn
 - Starch, Hydroxypropyl Corn
 - Starch, Pregelatinized Hydroxypropyl Corn
 - Starch, Pea
 - Starch, Hydroxypropyl Pea
 - Starch, Pregelatinized Hydroxypropyl Pea
 - Starch, Potato
 - Starch, Hydroxypropyl Potato
 - Starch, Pregelatinized Hydroxypropyl Potato
 - Starch, Tapioca
 - Starch, Wheat
 - Sucrose Palmitate
 - Tragacanth
 - Xanthan Gum
- Sweetening Agent**
 - Acesulfame Potassium
 - Aspartame
 - Aspartame Acesulfame
 - Corn Syrup
 - Corn Syrup, High Fructose
 - Corn Syrup Solids
 - Dextrates
 - Dextrose
 - Dextrose Excipient
 - Erythritol
 - Fructose
 - Galactose
 - Maltitol
 - Maltose
 - Mannitol
 - Saccharin
 - Saccharin Calcium
 - Saccharin Sodium
 - Sorbitol
 - Sorbitol Solution
 - Starch Hydrolysate, Hydrogenated
 - Sucralose
 - Sucrose

Sugar, Compressible
 Sugar, Confectioner's
 Syrup
 Tagatose
 Trehalose

Tablet Binder

Acacia
 Alginic Acid
 Amino Methacrylate Copolymer
 Ammonio Methacrylate Copolymer
 Ammonio Methacrylate Copolymer Dispersion
 Carbomer Copolymer
 Carbomer Homopolymer
 Carbomer Interpolymer
 Carboxymethylcellulose Sodium
 Cellulose, Microcrystalline
 Cellulose, Silicified Microcrystalline
 Coconut Oil, Hydrogenated
 Copovidone
 Corn Syrup
 Corn Syrup Solids
 Dextrin
 Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion
 Ethylcellulose
 Ethylene Glycol and Vinyl Alcohol Graft Copolymer
 Gelatin
 Glucose, Liquid
 Guar Gum
 Hydroxypropyl Cellulose, Low-Substituted
 Hydroxypropyl Methylcellulose (see Hypromellose)
 Hypromellose (formerly Hydroxypropyl Methylcellulose)
 Hypromellose Acetate Succinate
 Alpha-Lactalbumin
 Maltodextrin
 Maltose
 Methylcellulose
 Palm Oil, Hydrogenated
 Polydextrose, Hydrogenated
 Polyethylene Oxide
 Polyvinyl Acetate
 Povidone
 Pullulan
 Starch, Corn
 Starch, Hydroxypropyl Corn
 Starch, Pregelatinized Hydroxypropyl Corn
 Starch, Pea
 Starch, Hydroxypropyl Pea
 Starch, Pregelatinized Hydroxypropyl Pea
 Starch, Potato
 Starch, Hydroxypropyl Potato
 Starch, Pregelatinized Hydroxypropyl Potato
 Starch, Pregelatinized
 Starch, Pregelatinized Modified
 Starch, Tapioca
 Starch, Wheat
 Starch Hydrolysate, Hydrogenated
 Syrup
 Trehalose

Tablet and/or Capsule Diluent

Calcium Carbonate
 Calcium Phosphate, Dibasic
 Calcium Phosphate, Tribasic
 Calcium Sulfate
 Cellulose, Microcrystalline
 Cellulose, Silicified Microcrystalline
 Cellulose, Powdered
 Corn Syrup
 Corn Syrup Solids
 Dextrates
 Dextrin
 Dextrose Excipient
 Fructose
 Kaolin

Alpha-Lactalbumin
 Lactitol
 Lactose, Anhydrous
 Lactose, Monohydrate
 Maltitol
 Maltodextrin
 Maltose
 Mannitol
 Propylene Glycol Monocaprylate
 Pullulan
 Sorbitol
 Starch
 Starch, Corn
 Starch, Hydroxypropyl Corn
 Starch, Pregelatinized Hydroxypropyl Corn
 Starch, Pea
 Starch, Hydroxypropyl Pea
 Starch, Pregelatinized Hydroxypropyl Pea
 Starch, Potato
 Starch, Hydroxypropyl Potato
 Starch, Pregelatinized Hydroxypropyl Potato
 Starch, Pregelatinized
 Starch, Pregelatinized Modified
 Starch, Tapioca
 Starch, Wheat
 Starch Hydrolysate, Hydrogenated
 Sucrose
 Sugar, Compressible
 Sugar, Confectioner's
 Trehalose

Change to read:**Tablet and/or Capsule Lubricant**

Behenoyl Polyoxylglycerides
 Calcium Stearate
 Coconut Oil, Hydrogenated
 Glyceryl Behenate
 ▲ Glyceryl Tristearate ▲ NF31
 Magnesium Stearate
 Mineral Oil, Light
 Palm Oil, Hydrogenated
 Polyethylene Glycol
 Polyoxyl 10 Oleyl Ether
 Polyoxyl 15 Hydroxystearate
 Polyoxyl 20 Cetostearyl Ether
 Polyoxyl 35 Castor Oil
 Polyoxyl 40 Hydrogenated Castor Oil
 Polyoxyl 40 Stearate
 Polysorbate 20
 Polysorbate 40
 Polysorbate 60
 Polysorbate 80
 Sodium Lauryl Sulfate
 Sodium Stearyl Fumarate
 Sorbitan Monolaurate
 Sorbitan Monooleate
 Sorbitan Monopalmitate
 Sorbitan Monostearate
 Sorbitan Sesquioleate
 Sorbitan Trioleate
 Starch
 Stearic Acid
 Stearic Acid, Purified
 Sucrose Stearate
 Talc
 Vegetable Oil, Hydrogenated, Type I
 Zinc Stearate

Tablet Disintegrant

Alginic Acid
 Cellulose, Microcrystalline
 Cellulose, Silicified Microcrystalline
 Croscarmellose Sodium
 Crospovidone

Hydroxypropyl Cellulose, Low-Substituted
 Maltose
 Polacrilin Potassium
 Pullulan
 Sodium Starch Glycolate
 Starch
 Starch, Corn
 Starch, Hydroxypropyl Corn
 Starch, Pregelatinized Hydroxypropyl Corn
 Starch, Pea
 Starch, Hydroxypropyl Pea
 Starch, Pregelatinized Hydroxypropyl Pea
 Starch, Potato
 Starch, Hydroxypropyl Potato
 Starch, Pregelatinized Hydroxypropyl Potato
 Starch, Pregelatinized
 Starch, Pregelatinized Modified
 Starch, Tapioca
 Starch, Wheat
 Trehalose

Tonicity Agent

Corn Syrup
 Corn Syrup Solids
 Dextrose
 Glycerin
 Mannitol
 Potassium Chloride
 Sodium Chloride

Vehicle

FLAVORED AND/OR SWEETENED

Aromatic Elixir
 Benzaldehyde Elixir, Compound
 Corn Syrup Solids
 Dextrose
 Ethyl Maltol
 Peppermint Water
 Sorbitol Solution
 Syrup
 Trehalose

OLEAGINOUS

Alkyl (C12-15) Benzoate
 Almond Oil
 Canola Oil
 Corn Oil
 Cottonseed Oil
 Ethyl Oleate
 Isopropyl Myristate
 Isopropyl Palmitate
 Mineral Oil
 Mineral Oil, Light
 Octyldodecanol
 Olive Oil
 Peanut Oil

Polydecene, Hydrogenated
 Polyoxyl 15 Hydroxystearate
 Safflower Oil
 Sesame Oil
 Soybean Oil
 Squalane

SOLID CARRIER

Chitosan
 Corn Syrup Solids
 Alpha-Lactalbumin
 Propylene Glycol Dicaprylate/Dicaprate
 Propylene Glycol Monocaprylate
 Sugar Spheres

STERILE

rAlbumin Human
 Sodium Chloride Injection, Bacteriostatic
 Water for Injection, Bacteriostatic

Viscosity-Increasing Agent (See *Suspending and/or Viscosity-Increasing Agent*)**Water-Repelling Agent**

Cyclomethicone
 Dimethicone
 Simethicone

Wetting and/or Solubilizing Agent

Benzalkonium Chloride
 Benzethonium Chloride
 Betadex Sulfobutyl Ether Sodium
 Cetylpyridinium Chloride
 Docusate Sodium
 Nonoxynol 9
 Octoxynol 9
 Poloxamer
 Polyoxyl 10 Oleyl Ether
 Polyoxyl 15 Hydroxystearate
 Polyoxyl 20 Cetostearyl Ether
 Polyoxyl 35 Castor Oil
 Polyoxyl 40 Hydrogenated Castor Oil
 Polyoxyl 40 Stearate
 Polyoxyl Stearate
 Polysorbate 20
 Polysorbate 40
 Polysorbate 60
 Polysorbate 80
 Pullulan
 Sodium Lauryl Sulfate
 Sorbitan Monolaurate
 Sorbitan Monooleate
 Sorbitan Monopalmitate
 Sorbitan Monostearate
 Sorbitan Sesquioleate
 Sorbitan Trioleate
 Tyloxapol

Official Monographs for NF 31

Acacia

DEFINITION

Acacia is the dried gummy exudate from the stems and branches of *Acacia senegal* (L.) Willd. or of other related African species of *Acacia* (Fam. Leguminosae).

IDENTIFICATION

- **A.**
Analysis: To 10 mL of a cold solution (1 in 50) add 0.2 mL of diluted lead subacetate TS.
Acceptance criteria: A flocculent, or curdy, white precipitate is formed immediately.

IMPURITIES

- **ARSENIC**, *Method II* <211>: NMT 3 ppm
- **LEAD** <251>: NMT 10 ppm
- **HEAVY METALS**, *Method II* <231>: NMT 40 ppm

SPECIFIC TESTS

- **BOTANIC CHARACTERISTICS**
Acacia: Spheroidal tears up to 32 mm in diameter or in angular fragments of white to yellowish white color. It is translucent or somewhat opaque from the presence of numerous minute fissures; very brittle, the fractured surface glassy and occasionally iridescent. It is almost odorless and produces a mucilaginous sensation on the tongue.
Flake Acacia: White to yellowish white, thin flakes, appearing under the microscope as colorless, striated fragments
Powdered Acacia: White to yellowish white, angular microscopic fragments with only traces of starch or vegetable tissues present
Granular Acacia: White to pale yellowish white, fine granules. Under the microscope it appears as colorless, glassy, irregularly angular fragments up to 100 µm in thickness, some of which exhibit parallel linear streaks.
Spray-dried Acacia: White to off-white compacted microscopic fragments or whole spheres
- **MICROBIAL ENUMERATION TESTS** <61> and **TESTS FOR SPECIFIED MICROORGANISMS** <62>: Meets the requirements of the tests for absence of *Salmonella* species
- **WATER DETERMINATION**, *Method III (Gravimetric)* <921>
Analysis: For unground Acacia, crush in a mortar until it passes through a No. 40 sieve, and mix the ground material before weighing the test specimen. Dry a sample at 105° for 5 h.
Acceptance criteria: NMT 15.0% of its weight
- **ARTICLES OF BOTANICAL ORIGIN**, *Acid-Insoluble Ash* <561>: NMT 0.5%
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 4.0%
- **INSOLUBLE RESIDUE**
Sample solution: Dissolve 5.0 g of powdered or finely ground Acacia in 100 mL of water. Add 10 mL of 3 N hydrochloric acid.
Analysis: Boil the *Sample solution* gently for 15 min. Pass by suction, while hot, through a tared filtering crucible. Wash thoroughly with hot water, dry at 105° for 1 h, and weigh.

Acceptance criteria: The weight of the residue thus obtained does not exceed 50 mg.

- **STARCH OR DEXTRIN**
Sample solution: A solution (1 in 50)
Analysis: Boil the *Sample solution* cool, and add iodine TS.
Acceptance criteria: No bluish or reddish color is produced.
- **SOLUBILITY AND REACTION**
Sample: 1 g
Analysis: Dissolve the *Sample* in 2 mL of water.
Acceptance criteria: The resulting solution flows readily and is acid to litmus.
- **TANNIN-BEARING GUMS**
Sample solution: A solution (1 in 50)
Analysis: To 10 mL of the *Sample solution* add 0.1 mL of ferric chloride TS.
Acceptance criteria: No blackish coloration or blackish precipitate is produced.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Acacia Syrup

DEFINITION

Prepare Acacia Syrup as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* <795>).

Acacia, granular or powdered	100 g
Sodium Benzoate	1 g
Vanilla Tincture	5 mL
Sucrose	800 g
Purified Water, a sufficient quantity to make	1000 mL

Mix *Acacia*, *Sodium Benzoate*, and *Sucrose*. Add 425 mL of *Purified Water*, and mix. Heat the mixture on a steam bath until dissolved. When cool, remove the scum, add *Vanilla Tincture* and sufficient *Purified Water* to make the product measure 1000 mL, and strain, if necessary.

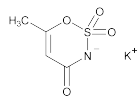
SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** <61> and **TESTS FOR SPECIFIED MICROORGANISMS** <62>: Meets the requirements of the test for absence of *Salmonella* species

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight containers, and prevent exposure to excessive heat.
- **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Acesulfame Potassium



$C_4H_4NO_4SK$ 201.24
6-Methyl-1,2,3-oxathiazine-4(3H)-one-2,2-dioxide potassium salt;
3,4-Dihydro-6-methyl-1,2,3-oxathiazine-4-one-2,2-dioxide potassium salt [55589-62-3].

DEFINITION

Acesulfame Potassium contains NLT 99.0% and NMT 101.0% of $C_4H_4NO_4SK$, calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B. IDENTIFICATION TESTS—GENERAL**, Potassium (191)
Sample solution: 100 mg/mL
Acceptance criteria: Meets the requirements

ASSAY

PROCEDURE

Sample: 150 mg

Titrimetric system

(See *Titrimetry* (541))

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Blank: 50 mL of glacial acetic acid

Endpoint detection: Potentiometric

Analysis: Dissolve the Sample in 50 mL of glacial acetic acid. Titrate with 0.1 N perchloric acid VS. Perform a blank determination.

Calculate the percentage of acesulfame potassium ($C_4H_4NO_4SK$) in the Sample:

$$\text{Result} = [(V - B) \times N \times F \times 100] / W$$

V = titrant volume consumed by the Sample (mL)

B = titrant volume consumed by the Blank (mL)

N = titrant actual normality (mEq/mL)

F = equivalency factor, 201.2 mg/mEq

W = weight of Sample (mg)

Acceptance criteria: 99.0%–101.0% on the dried basis

IMPURITIES

LIMIT OF FLUORIDE

[NOTE—Use plasticware throughout this test.]

Solution A: Dissolve 210 g of citric acid monohydrate in 400 mL of water. Adjust with concentrated ammonia to a pH of 7.0, and dilute with water to 1000 mL.

Solution B: 132 mg/mL of dibasic ammonium phosphate

Solution C: To a suspension of 292 g of edetic acid in 500 mL of water, add 200 mL of ammonium hydroxide, adjust with ammonium hydroxide to a pH between 6 and 7, and dilute with water to make 1000 mL.

Buffer solution: Mix equal volumes of Solution A, Solution B, and Solution C, and adjust with ammonium hydroxide to a pH of 7.5.

Standard stock solution: Weigh 0.442 g of sodium fluoride, previously dried at 300° for 12 h, into a 1-L volumetric flask, and dilute with water to volume. Store the solution in a closed plastic container. Immediately before use, pipet 5 mL of this solution into a 100-mL volumetric flask, and dilute with water to volume. Each mL of this solution contains 10 µg of fluoride ion.

Standard solution A: Mix 0.5 mL of Standard stock solution and 15.0 mL of Buffer solution, and dilute with water to 50 mL.

Standard solution B: Mix 1.0 mL of Standard stock solution and 15.0 mL of Buffer solution, and dilute with water to 50 mL.

Standard solution C: Mix 1.5 mL of Standard stock solution and 15.0 mL of Buffer solution, and dilute with water to 50 mL.

Standard solution D: Mix 3.0 mL of Standard stock solution and 15.0 mL of Buffer solution, and dilute with water to 50 mL.

Sample solution: To a 50-mL volumetric flask add 3 g of Acesulfame Potassium. Dissolve in water, add 15.0 mL of Buffer solution, and dilute with water to volume.

Analysis

Samples: Standard solution A, Standard solution B, Standard solution C, Standard solution D, and Sample solution

Concomitantly measure the potential (see *Titrimetry* (541)), in mV, of the Standard solutions and the Sample solution, with a suitable pH meter equipped with a fluoride-specific ion electrode and a silver-silver chloride reference electrode. When taking the measurements, transfer the solution to a 25-mL beaker, and immerse the electrodes. Insert a polytetrafluoroethylene-coated stirring bar into the beaker, place the beaker on a magnetic stirrer having an insulated top, and allow to stir until equilibrium is attained (1–2 min). Rinse, and dry the electrodes between measurements, taking care not to scratch the crystal in the fluoride-specific ion electrode. Measure the potential of each Standard solution, and plot the fluoride concentration, in µg/mL, versus the potential, in mV, on semilogarithmic paper. Measure the potential of the Sample solution, and determine the fluoride concentration from the standard curve, in µg/mL.

Calculate the content, in ppm, of fluoride in the portion of Acesulfame Potassium taken:

$$\text{Result} = (V \times C / W)$$

V = volume of the Sample solution (mL)

C = concentration of fluoride in the Sample solution, from the standard curve (mg/mL)

W = weight of Acesulfame Potassium taken to prepare the Sample solution (g)

Acceptance criteria: NMT 3 ppm

HEAVY METALS, Method I (231): NMT 10 ppm

CHROMATOGRAPHIC PURITY

Solution A: 3.3 mg/mL of tetrabutylammonium hydrogen sulfate

Mobile phase: Acetonitrile and Solution A (2:3)

System suitability solution: 2 µg/mL each of USP Acesulfame Potassium RS and ethylparaben

Standard solution: 0.2 µg/mL of USP Acesulfame Potassium RS

Sample solution: 10 mg/mL

Chromatographic system

(See *Chromatography* (621), System Suitability.)

Mode: LC

Detector: UV 227 nm

Column: 4.6-mm × 25-cm; 5-µm packing L1

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: System suitability solution

Suitability requirements

Resolution: NLT 2 between acesulfame potassium and ethylparaben

Analysis

Samples: Standard solution and Sample solution

Record the chromatograms for a run time NLT 3 times the retention time of the acesulfame potassium peak, and measure the area responses of the peaks.

Acceptance criteria: The response of any peak at a retention time other than that of acesulfame potassium

from the *Sample solution* does not exceed the response of the acesulfame potassium peak from the *Standard solution* (0.002%).

SPECIFIC TESTS

• ACIDITY OR ALKALINITY

Sample solution: 4.0 g in 20 mL of carbon dioxide-free water

Analysis: Add 0.1 mL of bromothymol blue TS. If the solution is yellow, titrate with 0.01 N sodium hydroxide to produce a blue color. If the solution is blue, titrate with 0.01 N hydrochloric acid to produce a yellow color.

Acceptance criteria: NMT 0.2 mL of 0.01 N sodium hydroxide or NMT 0.2 mL of 0.01 N hydrochloric acid is required.

- **LOSS ON DRYING** (731): Dry a sample at 105° for 3 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a well-closed container, and protect from light. Store at room temperature.
- **USP REFERENCE STANDARDS** (11)
USP Acesulfame Potassium RS

Acetic Acid

Acetic acid;
Acetic acid [64-19-7].

DEFINITION

Acetic Acid is a solution containing NLT 36.0% and NMT 37.0%, by weight, of C₂H₄O₂.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Acetate** (191): Meets the requirements

ASSAY

• PROCEDURE

Analysis: Place 6 mL in a tared, glass-stoppered flask, and weigh. Add 40 mL of water, then add phenolphthalein TS. Titrate with 1 N sodium hydroxide VS. Each mL of 1 N sodium hydroxide is equivalent to 60.05 mg of C₂H₄O₂.

Acceptance criteria: 36.0%–37.0%

IMPURITIES

• NONVOLATILE RESIDUE

Analysis: Evaporate 20 mL in a tared porcelain dish on a steam bath, and dry at 105° for 1 h.

Acceptance criteria: The weight of the residue does not exceed 1.0 mg (0.005%).

• CHLORIDE

Sample solution: Acetic acid (1 in 10) in water

Analysis: To 10 mL of the *Sample solution* add 5 drops of silver nitrate TS.

Acceptance criteria: No opalescence is produced.

• SULFATE

Sample solution: Acetic acid (1 in 10) in water

Analysis: To 10 mL of the *Sample solution* add 5 drops of barium chloride TS.

Acceptance criteria: No turbidity is produced.

• HEAVY METALS (231)

Sample solution: To the residue obtained in the test for *Nonvolatile Residue* add 8 mL of 0.1 N hydrochloric acid, warm gently until completely dissolved, and dilute with water to 100 mL. Use 10 mL of this solution.

Acceptance criteria: NMT 10 ppm

• READILY OXIDIZABLE SUBSTANCES

Analysis: Dilute 4.0 mL in a glass-stoppered vessel with 20 mL of water, and add 0.30 mL of 0.10 N potassium permanganate.

Acceptance criteria: The pink color is not changed to brown at once, and the liquid does not become entirely brown or free from a pink tint in less than 30 s.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Diluted Acetic Acid

DEFINITION

Diluted Acetic Acid is a solution containing, in each 100 mL, NLT 5.7 g and NMT 6.3 g of acetic acid (C₂H₄O₂).

Prepare Diluted Acetic Acid as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* (795)).

Acetic Acid	158 mL
Purified Water, a sufficient quantity to make	1000 mL

Mix the ingredients.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Acetate** (191): Meets the requirements

ASSAY

• PROCEDURE

Sample: 25 mL

Analysis: To the *Sample* add 15 mL of carbon dioxide-free water. Add phenolphthalein TS, and titrate with 1 N sodium hydroxide VS. Each mL of 1 N sodium hydroxide is equivalent to 60.05 mg of acetic acid (C₂H₄O₂).

Acceptance criteria: 5.7–6.3 g of acetic acid per 100 mL of Diluted Acetic Acid

IMPURITIES

• HEAVY METALS, Method I (231)

Test preparation: Evaporate 5 mL in a porcelain dish on a steam bath to dryness. Warm the residue with 2 mL of 1 N acetic acid, and dilute with water to 50 mL. Dilute 20 mL of this solution with water to 25 mL.

Acceptance criteria: NMT 10 ppm

• LIMIT OF CHLORIDE

Sample solution: A solution of Diluted Acetic Acid in water (6 in 10)

Analysis: Add 5 drops of silver nitrate TS to 10 mL of the *Sample solution*.

Acceptance criteria: No opalescence is found.

• LIMIT OF SULFATE

Sample solution: A solution of Diluted Acetic Acid in water (6 in 10)

Analysis: Add 5 drops of barium chloride TS to 10 mL of the *Sample solution*.

Acceptance criteria: No turbidity is produced.

• LIMIT OF NONVOLATILE RESIDUE

Sample: 20 mL

Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath, and dry it at 105° for 1 h.

Acceptance criteria: The weight of the residue does not exceed 1.0 mg (NMT 0.005%).

SPECIFIC TESTS

• READILY OXIDIZABLE SUBSTANCES

Sample: 20 mL in a glass-stoppered flask

Analysis: Add 0.30 mL of 0.10 N potassium permanganate to the *Sample*.

Acceptance criteria: The pink color is not changed to brown immediately, and the liquid does not become entirely brown or free from a pink tint in less than 30 s.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Acetic Acid, Glacial—see *Glacial Acetic Acid General Monographs*

Acetone



C₃H₆O 58.08
2-Propanone;
Acetone [67-64-1].

DEFINITION

Acetone contains NLT 99.0% of C₃H₆O, calculated on the anhydrous basis.

[**CAUTION**—Acetone is very flammable. Do not use where it may be ignited.]

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197F)
- **B.** The retention time of the *Sample* corresponds to that of USP Acetone RS, as obtained in the Assay.

ASSAY

PROCEDURE

Sample: Acetone

System suitability solution: Dilute 1.0 mL of USP Methyl Alcohol RS and 1.0 mL of USP Acetone RS with tetrahydrofuran to 50 mL.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica capillary; 1.8-μm of phase G43

Temperature

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	0	40	5
40	20	240	—

Injector: 200°

Detector: 280°

Carrier gas: Helium

Flow rate: 35 cm/s (linear velocity)

Split ratio: 400:1

Injection volume: 1 μL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for methyl alcohol, acetone, and tetrahydrofuran are about 0.6, 1.0, and 1.9, respectively.]

Suitability requirements

Resolution: NLT 15 between the methyl alcohol and acetone peaks

Analysis

Sample: *Sample*

Calculate the percentage of acetone (C₃H₆O) in the portion of Acetone taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area due to the acetone peak in the *Sample*

r_T = sum of the areas of all the peaks in the *Sample*

[NOTE—No separate correction is applied for water content, because water does not respond to the flame-ionization detector.]

Acceptance criteria: NLT 99.0% on the anhydrous basis

SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): NMT 0.789

- **NONVOLATILE RESIDUE:** Evaporate 50 mL in a tared porcelain dish on a steam bath, and dry at 105° for 1 h. **Acceptance criteria:** The weight of the residue does not exceed 2 mg (0.004%).

WATER

Sample: Acetone

Standard solution: Transfer 0.50 mL of water to a dry 100-mL volumetric flask, dilute with dehydrated isopropyl alcohol to volume, and mix.

Blank: Dehydrated isopropyl alcohol

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Thermal conductivity

Column: 0.32-mm × 50-m capillary; 5.0-μm layer of support S2

Temperature

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	25	190	—

Injector: 250°

Detector: 250°

Carrier gas: Helium

Flow rate: 11 mL/min

Split ratio: 4.5:1

Injection size: 1.0 μL

Analysis

Samples: Acetone, *Standard solution*, and *Blank*

[NOTE—Identify the peaks based on their relative retention times, which are 1.0 for water and about 1.9 for isopropyl alcohol.]

Acceptance criteria: The area of the water peak for Acetone is NMT that from the *Standard solution*, corrected for the area of the water peak from the *Blank* (0.5%).

READILY OXIDIZABLE SUBSTANCES

Analysis: Mix 20 mL with 0.10 mL of 0.10 N potassium permanganate in a glass-stoppered bottle.

Acceptance criteria: The permanganate color of the mixture does not completely disappear within 15 min.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, remote from fire.

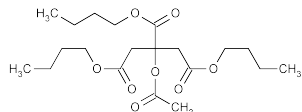
- **USP REFERENCE STANDARDS** (11)

USP Acetone RS

USP Methyl Alcohol RS

Acetylcysteine—see Acetylcysteine General Monographs

Acetyltributyl Citrate

C₂₀H₃₄O₈

402.48

DEFINITION

Acetyltributyl Citrate contains NLT 99.0% of C₂₀H₃₄O₈, calculated on the anhydrous basis.

IDENTIFICATION

- A. INFRARED ABSORPTION** (197F)
- B.** The retention time of the *Sample solution* corresponds to that of a similar preparation of USP Acetyltriethyl Citrate RS, as obtained in the Assay.

ASSAY

PROCEDURE

System suitability solution: 30 mg/mL each of USP Acetyltributyl Citrate RS and USP Tributyl Citrate RS in toluene

Sample solution: 30 mg/mL of Acetyltributyl Citrate in toluene

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m, bonded with a 0.5-μm layer of phase G42

Temperature

Injector: 240°

Detector: 280°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	—	80	0
80	20	230	15

Flow rate: 1.9 mL/min

Carrier gas: Helium

Injection type: Split, 30:1

Injection size: 1 μL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for tributyl citrate and acetyl tributyl citrate are 0.9 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between tributyl citrate and acetyl tributyl citrate

Relative standard deviation: NMT 2.0% determined from both the tributyl citrate and acetyl tributyl citrate peaks, based on area percent calculation

Analysis

Sample: *Sample solution*

[NOTE—Measure all of the peak areas, excluding the solvent peak.]

Calculate the percentage of C₂₀H₃₄O₈ in the portion of Acetyltributyl Citrate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of the *Sample solution*

r_T = sum of all the peak areas

Acceptance criteria: NLT 99.0% on the anhydrous basis

IMPURITIES

Inorganic Impurities

- HEAVY METALS**, *Method II* (231): NMT 10 ppm

SPECIFIC TESTS

- SPECIFIC GRAVITY** (841): 1.045–1.055
- REFRACTIVE INDEX** (831): 1.4410–1.4425
- ACIDITY**

Neutralized isopropyl alcohol: To a suitable quantity of isopropyl alcohol add 2–3 drops of bromothymol blue TS and just sufficient 0.10 N sodium hydroxide dropwise to produce a faint blue color. [NOTE—Prepare *Neutralized isopropyl alcohol* just before use.]

Sample solution: 32.0 g of Acetyltributyl Citrate in 30 mL of *Neutralized isopropyl alcohol*

Analysis: Add bromothymol blue TS. Titrate with 0.10 N sodium hydroxide to a faint blue endpoint.

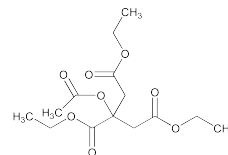
Acceptance criteria: NMT 1.0 mL of 0.10 N sodium hydroxide is required.

- WATER DETERMINATION**, *Method I* (921): NMT 0.25%

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight containers.
- USP REFERENCE STANDARDS** (11)
 - USP Acetyltributyl Citrate RS
 - USP Tributyl Citrate RS

Acetyltriethyl Citrate

C₁₄H₂₂O₈

318.32

DEFINITION

Acetyltriethyl Citrate contains NLT 99.0% of acetyltriethyl citrate (C₁₄H₂₂O₈), calculated on the anhydrous basis.

IDENTIFICATION

- A. INFRARED ABSORPTION** (197F)
- B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *System suitability solution*, as obtained in the Assay.

ASSAY

PROCEDURE

System suitability solution: 30 mg/mL each of USP Acetyltriethyl Citrate RS and USP Triethyl Citrate RS in toluene

Sample solution: 30 mg/mL in toluene

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC, equipped with an on-column, temperature-programmable injector

Detector: Flame ionization

Column: 0.32-mm × 30-m, bonded with a 0.5-μm layer of phase G42

Temperatures

Injector: See Table 1.

Detector: 275°

Column: See Table 2.

Table 1

Start Temperature (°)	Ramp (°)	End Temperature (°)	Hold Time (min)
85	—	85	0.5
85	20	225	10

Table 2

Start Temperature (°)	Ramp (°)	End Temperature (°)	Hold Time (min)
80	—	80	0.5
80	20	220	10

Flow rate: 2.3 mL/min

Carrier gas: Helium

Injection volume: 1 µL

System suitability

Sample: System suitability solution

[NOTE—The relative retention times of triethyl citrate and acetyltriethyl citrate are 0.9 and 1.0, respectively.]

Suitability requirements**Resolution:** NLT 1.5 between triethyl citrate and acetyltriethyl citrate**Relative standard deviation:** NMT 2.0% determined from both the triethyl citrate and acetyltriethyl citrate peaks**Analysis**

Sample: Sample solution

Calculate the percentage of acetyltriethyl citrate (C₁₄H₂₂O₈) in the portion of sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

 r_U = peak area of acetyltriethyl citrate from the Sample solution r_T = sum of all the peaks excluding the solvent peak**Acceptance criteria:** NLT 99.0% on the anhydrous basis**IMPURITIES**

- **HEAVY METALS, Method II (231):** NMT 10 µg/g

SPECIFIC TESTS

- **SPECIFIC GRAVITY (841):** 1.135–1.139

- **REFRACTIVE INDEX (831):** 1.432–1.441

• **ACIDITY**

Sample: 32.0 g

Analysis: Dissolve the Sample in 30 mL of isopropyl alcohol, previously neutralized to bromothymol blue. Add bromothymol blue TS, and titrate with 0.10 N sodium hydroxide to a faint blue endpoint.**Acceptance criteria:** NMT 1.0 mL of 0.10 N sodium hydroxide is required.

- **WATER DETERMINATION, Method I (921):** NMT 0.3%

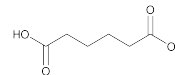
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

- **USP REFERENCE STANDARDS (11)**

USP Acetyltriethyl Citrate RS

USP Triethyl Citrate RS

Activated Charcoal—see Activated Charcoal General Monographs**Adipic Acid**C₆H₁₀O₄

Hexanedioic acid;

1,4-Butanedicarboxylic acid [124-04-9].

146.14

DEFINITIONAdipic Acid contains NLT 99.0% and NMT 101.0% of C₆H₁₀O₄, calculated on the dried basis.**IDENTIFICATION**

- **A. INFRARED ABSORPTION (197K)**

ASSAY• **PROCEDURE**

Sample: 60 mg

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N sodium hydroxide VS

Blank: 50.0 mL of water

Endpoint detection: Colorimetric

Analysis: Dissolve the Sample in 50 mL of water. Add 0.2 mL of phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS to a permanent pale pink endpoint. Perform a blank determination. Calculate the percentage of adipic acid (C₆H₁₀O₄) in the Sample taken:

$$\text{Result} = [(V - B) \times N \times F \times 100]/W$$

 V = titrant volume consumed by the Sample (mL) B = titrant volume consumed by the Blank (mL) N = titrant actual normality (mEq/mL) F = equivalency factor, 73.1 mg/mEq W = weight of the Sample (mg)**Acceptance criteria:** 99.0%–101.0% on the dried basis**IMPURITIES**

- **RESIDUE ON IGNITION (281):** NMT 0.1%

• **LIMIT OF NITRATES****Standard stock solution:** 1.63 mg/mL of potassium nitrate**Standard solution:** Dilute 1 mL of the Standard stock solution with water to 10 mL. Dilute 1 mL of this solution with water to 50 mL to obtain a solution containing 2 µg/mL of nitrate.**Sample solution:** Transfer 5 g of Adipic Acid to a 50-mL volumetric flask. Dissolve in water, with heating, and dilute with water to volume. Allow to cool and crystallize, then pass through a sintered-glass filter. Wash the filter with water, and collect the filtrate and washings until a volume of 50 mL is obtained. [NOTE—This solution is also to be used for Chloride, Sulfate, Iron, and Heavy Metals.]**Control:** 2 mg/L of potassium permanganate**Analysis:** Transfer 1.0 mL of the Sample solution, 1.5 mL of the Standard solution, and 1 mL of water (blank) to three separate flasks. To each flask add 2 mL of concentrated ammonia, 0.5 mL of 10 mg/mL manganese sulfate, and 1 mL of 10 mg/mL sulfanilamide, and dilute each solution with water to 20 mL. Add 100 mg of zinc powder to each of the three flasks, and cool in an ice bath for 30 min, shaking the solutions periodically. Separately filter 10 mL of

each solution, cool in an ice bath, and then add 2.5 mL of hydrochloric acid and 1 mL of 10 mg/mL of naphthylethylenediamine dihydrochloride. Allow the solutions to stand at room temperature for 15 min.

System suitability: The test is invalid if the concomitantly prepared blank solution is darker than the *Control*.

Acceptance criteria: The color of the solution containing the *Sample solution* is not darker than the concomitantly prepared solution containing the *Standard solution* (NMT 30 ppm).

- **HEAVY METALS, Method I (231):** NMT 10 ppm

- **CHLORIDE AND SULFATE, Chloride (221)**

Sample: A 5-mL portion of the *Sample solution* from *Limit of Nitrates*

Analysis: Proceed as directed in the chapter.

Acceptance criteria: The *Sample* shows no more chloride than a corresponding 0.14-mL portion of 0.020 N hydrochloric acid (NMT 0.02%).

- **CHLORIDE AND SULFATE, Sulfate (221)**

Sample: A 5-mL portion of the *Sample solution* from *Limit of Nitrates*

Analysis: Proceed as directed in the chapter.

Acceptance criteria: The *Sample* shows no more sulfate than a corresponding 0.26-mL portion of 0.020 N sulfuric acid (NMT 0.05%).

- **IRON (241)**

Sample: A 10-mL portion of the *Sample solution* from *Limit of Nitrates*

Analysis: Proceed as directed in the chapter.

Acceptance criteria: NMT 10 ppm

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE (741):** 151°–154°
- **LOSS ON DRYING (731):** Dry a sample at 105° to constant weight; it loses NMT 0.2% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a tight containers. No storage requirements specified.
- **USP REFERENCE STANDARDS (11)**
USP Adipic Acid RS

Agar

[9002-18-0].

DEFINITION

Agar is the dried, hydrophilic, colloidal substance consisting of the polysaccharides extracted from *Gelidium cartilagineum* (Linné) Gaillon (Fam. Gelidiaceae), *Gracilaria confervoides* (Linné) Greville (Fam. Sphaerococcaceae), and related red algae (Class Rhodophyceae).

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K)**
- **B.** Iodine TS colors some of the fragments of Agar bluish black, with some areas reddish to violet.
- **C.**
Analysis: Boil a sample with 65 times its weight of water for 10 min, with constant stirring, and subsequently adjust with hot water to a concentration of 1.5%, by weight.
Acceptance criteria: Agar forms a clear liquid that congeals at 30°–39° to form a firm resilient gel, which does not liquefy below 80°.

IMPURITIES

Inorganic Impurities

- **ARSENIC, Method II (211):** NMT 3 ppm
- **LEAD (251):** NMT 10 ppm
- **HEAVY METALS, Method II (231):** NMT 40 ppm

- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash (561):** NMT 0.5%, on a dry-weight basis

Organic Impurities

- **PROCEDURE 1: LIMIT OF GELATIN**

Sample solution: Dissolve 1 g of sample in 100 mL of boiling water. Allow to cool to about 50°.

Analysis: To 5 mL of the *Sample solution* add 2–3 drops of a mixture of 0.2 M potassium dichromate solution and 3 N hydrochloric acid (4:1).

Acceptance criteria: No yellow precipitate is formed.

- **PROCEDURE 2: LIMIT OF FOREIGN STARCH**

Sample solution: Boil 0.10 g in 100 mL of water.

Acceptance criteria: The *Sample solution* does not, upon cooling, produce a blue color upon the addition of iodine TS.

- **PROCEDURE 3: LIMIT OF FOREIGN INSOLUBLE MATTER**

Sample dispersion: Add sufficient water to 7.5 g of sample to make 500 g, boil for 15 min, and readjust to the original 500 g.

Analysis: To 100 g of the uniformly mixed *Sample dispersion* add hot water to make 200 mL. Heat almost to boiling, filter while hot through a tared filtering crucible. Rinse the container with several portions of hot water, and pass these rinsings through the crucible. Dry the crucible and its contents at 105° to a constant weight.

Acceptance criteria: NMT 15 mg (1.0%) remains in the crucible.

- **PROCEDURE 4: ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter (561):** NMT 1.0%

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 10³ cfu/g, and the total combined molds and yeasts count does not exceed 10² cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

- **WATER DETERMINATION, Method III (921)**

Analysis: If necessary, cut a sample into pieces from a 2- to 5-mm square, and dry at 105° for 5 h.

Acceptance criteria: The sample loses NMT 20.0% of its weight.

- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 6.5%, on a dry-weight basis

- **WATER ABSORPTION**

Sample: 5.0 g

Analysis: Place the *Sample* in a 100-mL graduated cylinder, fill to the mark with water, mix, and allow to stand at 25° for 24 h. Pour the contents of the cylinder through moistened glass wool, allowing the water to drain into a second 100-mL graduated cylinder.

Acceptance criteria: NMT 75 mL of water is obtained.

- **BOTANIC CHARACTERISTICS**

Agar: Usually occurs in bundles consisting of thin, membranous, agglutinated strips or in cut, flaked, or granulated forms. It may be colored weak yellowish orange, yellowish gray to pale yellow, or colorless. It is tough when damp, brittle when dry.

Histology: When mounted in water, Agar appears granular and somewhat filamentous; a few fragments of the spicules of sponges and a few frustules of diatoms may be present. In Japanese Agar, the frustules of *Arachnoidiscus ehrenbergii* Baillon often occur, being disk-shaped and 100–300 µm in diameter.

Powdered agar: White to yellowish white or pale yellow; in chloral hydrate TS, its fragments are transparent, more or less granular, striated, and angular, and occasionally they contain frustules of diatoms.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.
- **USP REFERENCE STANDARDS (11)**
USP Agar RS

Albumin Human—see Albumin Human General Monographs

rAlbumin Human

DAHKSEVAHR FKDLGEENFK ALVLIQAFQY LQOCPFEDHV KLVNEVTEFA
KTCVADESSE NCKSLHTLF GDLCTVATL RETYGEADG CAKQEPERNE
CFLOHKDDNP NLRILVRPEV DVMCTAFHON EETFLKKYLY EIAARRHPFY
APELFFAKR YKAATFCCQ AADKAACLLP KLDELDEGK ASSAKQRLKQ
ASLQKFGERA FKAWAVARLS QRFPAEFAE YSKLYDTLTK VHTCCGHDG
LECADDRADIL AKYICENQDS ISSKLKECCQ KPLLEKSHCI AEVENDEMPA
DLPSLAADFV ESKDVCKNYA EAKDVFLGMF LYEYARRHPD YSVLLLRLLA
KTYETTLKQ CAAADPEHCY AKVDFEKLPL VEEPONLIQO NCLEFEOLGE
YKFNALLVR YTKKVPQVST PTLVEVSRL GKVGSCKCKH PEAKRMPQAE
DYLSSVLLNQL EVLHEKTPVS DRVTKCCTES LVNRRPCFSA LEVDETYVPK
EFNAETFTFH ADICLTSEKE RQIKKQALV ELVKHKPKAT KEOLKAVMDD
FAAFVEKCKX ADDKETCFAE EGKKLVAASQ AALGL

C₂₉₃₆H₄₆₂₄N₇₈₆O₈₈₉S₄₁ 66,438 Da

DEFINITION

Recombinant Albumin Human (rAlbumin Human or rHA) is produced by recombinant DNA expression in *Saccharomyces cerevisiae*. Structural equivalence (primary, secondary, and tertiary) between rHA and human serum albumin (HSA) has been demonstrated. It consists of three domains composed of 585 amino acids containing a single tryptophan (Trp₂₁₄), one free thiol (Cys₃₄), and 17 disulfide bridges. It is presented as a sterile and nonpyrogenic aqueous liquid consisting of a 10% (w/v) or 20% (w/v) solution in Water for Injection. No human- or animal-derived raw material is involved in its manufacture. It contains NLT 95% and NMT 105% of the labeled amount and NLT 99% of its total protein is albumin. It contains no added antimicrobial agents, but it may contain appropriate stabilizing agents. The presence of process-related impurities, host cell DNA, and host cell proteins is process specific; suitable limits should be determined by appropriately validated methods. However, the limit for host cell proteins should be NMT 0.15 µg/g.

IDENTIFICATION

A. PEPTIDE MAPPING

[NOTE—See *Biotechnology-Derived Articles—Peptide Mapping* (1055) for guidance.]
Tris buffer: 0.1 M tris(hydroxymethyl)aminomethane. Adjust with hydrochloric acid to a pH of 8.0.
Dilute Tris buffer: Tris buffer and water (50:50)
Solution A: Trifluoroacetic acid and water (1:1000)
Solution B: To 350 mL of acetonitrile add 150 mL of water and 425 µL of trifluoroacetic acid.
Dithiothreitol solution: 0.1 M dithiothreitol
Iodoacetamide solution: 0.1 M iodoacetamide in Tris buffer
Trypsin solution: 1 mg/mL of trypsin in 10 mM hydrochloric acid
EDTA solution: 0.1 M ethylenediaminetetraacetic acid (EDTA) in water
Diluent: To 5.76 g of guanidine hydrochloride add 5 mL of Dilute Tris buffer and 200 µL of EDTA solution. Dilute with Dilute Tris buffer to a final volume of 10 mL.
Standard solution: Add 20 µL of USP rAlbumin Human RS to 80 µL of Diluent. Add 5 µL of Dithiothreitol solution, and incubate at 37° for 75 min. Add 10 µL of Iodoacetamide solution, and incubate for an additional 75 min at 37° in the dark. Add 100 µL of Dilute Tris buffer, 400 µL of water, and 10 µL of Trypsin solution, and incubate at 37° with shaking for 24 h. Centrifuge, and dilute a portion of the supernatant in Solution A (50:50).

Sample solution: 50 mg/mL of rAlbumin Human in water. To 20 µL of this solution add 80 µL of Diluent. Add 5 µL of Dithiothreitol solution, and incubate at 37° for 75 min. Add 10 µL of Iodoacetamide solution, and incubate for an additional 75 min at 37° in the dark. Add 100 µL of Dilute Tris buffer, 400 µL of water, and 10 µL of Trypsin solution, and incubate at 37° with shaking for 24 h. Pulse centrifuge, and dilute a portion of the supernatant in Solution A (50:50).
Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)	Flow Rate (mL/min)
0	95	5	0.5
5	95	5	0.5
75	60	40	0.5
100	40	60	0.5
104	0	100	0.5
108	0	100	0.5
109	95	5	1.0
115	95	5	1.0
116	95	5	0.5
120	95	5	0.5

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC
Detector: UV 214 nm
Column: 4.6-mm × 25-cm; 5-µm packing L1
Column temperature: 35°
Flow rate: See Table 1.
Injection volume: 100 µL

Analysis

Samples: Standard solution and Sample solution
Acceptance criteria: The peptide map chromatographic profiles of the Sample solution are similar to those of the Standard solution.

B. ELECTROSPRAY MASS SPECTROMETRY

Solution A: Trifluoroacetic acid and water (1:1000)
Solution B: To 140 mL of acetonitrile add 60 mL of water and 180 µL of trifluoroacetic acid.
Solution C: Acetonitrile and water (50:50)
Solution D: To 5 mL of Solution C add 10 µL of formic acid.
System suitability solution: Dissolve 2 mg of horse heart myoglobin in 589 µL of Water for Injection. Dilute 25 µL of this solution with 475 µL of Solution D.
Sample solution: 10 mg/mL of rAlbumin Human in water
Mobile phase: See Table 2.

Table 2

Time (min)	Solution A (%)	Solution B (%)
0	95	5
5	95	5
10	0	100
15	0	100

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC
Detector: UV 280 nm
Column: 2.1-mm × 3-cm; desalting cartridge, equilibrated with Solution C¹

¹ A suitable reverse-phase desalting column is available from Perkin Elmer (No. 0711-0056).

Flow rate: 0.2 mL/min

Injection volume: 20 µL of the *Sample solution*

Analysis: Desalt the *Sample solution*, and collect the eluate. Ensure that a single protein peak elutes.

Spectrometric system

(See *Mass Spectrometry* (736).)

Mode: LC/MS (using infusion pump)

[NOTE—The infusion system flow rate can be adjusted as needed. To assist in nebulization, the infusion system can contain a sheathing gas fluid.]

Mobile phase: *Solution C*

Detector: Electrospray in the positive ion mode

Injection volume: 50 µL of desalted *Sample solution*

System suitability

Sample: *System suitability solution*

Suitability requirements

Peak position: A single peak in the 16,949–16,953 Da range is found.

Analysis: Obtain and transform the spectrogram for the desalted *Sample solution*.

Acceptance criteria: The mass is within 20 Da of the theoretical mass.

ASSAY

• **ALBUMIN CONTENT**

Stock sample buffer:² Mix 4 mL of 0.5 M Tris hydrochloride pH 8.6, 0.5 mL of 0.1% bromophenol blue, 2.0 mL of glycerol, and dilute with water to 1000 mL.

Diluted sample buffer: *Stock sample buffer* and water (1:1)

Native stock running buffer:³ 29 mg/mL of Tris base and 144 mg/mL of glycerol

Running buffer: *Native stock running buffer* and water (1:9)

Gel-staining solution: A suitable Coomassie G-250-based solution⁴

Native PAGE gel: Prepare a 14% Tris-Glycine gel.⁵

Sample solution: 4 mg/mL of rAlbumin Human in water. Dilute this solution with *Stock sample buffer* to 2 mg/mL.

Calibration solutions: Dilute the *Sample solution* quantitatively, and stepwise if necessary, with *Diluted sample buffer* to 100, 20, 15, 10, 5, 2, and 1 µg/mL of rAlbumin Human.

Electrophoretic system

(See *Electrophoresis* (726).)

Run buffer: *Running buffer*

Voltage: 125 V

Amperage: 35 mA

Wattage: 5.0 W

Run time: Approximately 2 h

Loading volume: 10 µL

Analysis

Samples: *Sample solution* and *Calibration solutions*

Gel loading scheme

Lane 1: 1 µg/mL *Calibration solution*

Lane 2: 2 µg/mL *Calibration solution*

Lane 3: 5 µg/mL *Calibration solution*

Lane 4: 10 µg/mL *Calibration solution*

Lane 5: 15 µg/mL *Calibration solution*

Lane 6: 20 µg/mL *Calibration solution*

Lane 7: *Diluted sample buffer*

Lane 8: *Sample solution*

Lane 9: *Sample solution*

Lane 10: *Diluted sample buffer*

Gel staining: Place the gel in 100 mL of water, and shake gently with circumgyration for about 30 min. Pour approximately 50 mL of *Gel-staining solution* into a staining container. Place the gel into the staining container, and allow the stain to completely cover

the gel. Place the staining container on an orbital shaker, and stain the gel for 120 min with gentle shaking.

Destaining: Drain the *Gel-staining solution*, and add 100 mL of water to the container to cover the gel. Place the container on an orbital shaker, and shake at low speed for about 60 min. Change the water, and repeat for a total of two washes.

Gel scan procedure: Set up a gel scanner according to the manufacturer's instructions. Place the gel in the detector, and obtain a single image of all 10 lanes of the gel.

Data analysis: Perform image analysis of *Lanes 1–6* to generate a linear calibration curve. Determine the linear regression equation of the standards by the least-squares method, with standard concentrations, in ng, as the dependent variable (*x*), and the sample band intensity (optical density) as the independent variable (*y*). Record the linear regression equation and the correlation coefficient, *r*. A suitable system is one that yields a line having an *r*² of NLT 0.990.

Examine *Lanes 8* and *9* (the *Sample solution* lanes) for the presence of bands below the main albumin band. If bands are present below the main albumin band in either or both lanes, quantify the relative amount, in ng, of protein present in each band against the calibration curve. Convert the quantified value to a contaminant level in percentage by dividing the quantified value by a factor of 200.

Calculate the purity of the *Sample solution*:

$$\text{Result} = 100 - C_i$$

C_i = mean of the percentages of contaminant levels found in *Lanes 8* and *9* (all the bands other than the albumin band), disregarding any band due to the *Diluted sample buffer*

Acceptance criteria: *Sample solution* purity is NLT 99.0%. [NOTE—The main albumin band is not quantitated. See the test for *Total Protein*.]

• **TOTAL PROTEIN**

Sodium chloride solution: 0.15 M sodium chloride in water

Copper sulfate solution: 60 mg/mL of copper sulfate pentahydrate and 600 mg/mL of potassium sulfate⁶ in sulfuric acid low in nitrogen

Sample solution: Dilute 0.5 g of rAlbumin Human with 2.5 mL of *Sodium chloride solution* (equivalent to about 3.3 mg/mL of total protein).

Blank: 33.3 mg/mL of glycine in *Sodium chloride solution*

Analysis: To 3.0 mL of the *Sample solution* and the *Blank*, in suitable distillation tubes, add 5 mL of *Copper sulfate solution*. Incubate at 420° for a minimum of 2 h, or until the residues appear white. When the solutions are cool, transfer the residues quantitatively with a minimum quantity of water to a micro-Kjeldahl flask, and determine the residues, using *Nitrogen Determination* (461), *Method II*. Multiply the result, corrected for the *Blank* and for the specific gravity of the *Sample solution*, by 6.25 to calculate the quantity of protein.

Acceptance criteria: 95%–105% of the quantity of protein stated on the label

OTHER COMPONENTS

• **SODIUM CONTENT**

Diluent: 1.0 mg/mL of cesium chloride in water

Standard solutions: Prepare 0.5, 1.00, 1.50, and 2.00 mg/mL of sodium chloride in *Diluent*.

Sample solution: 80 µg/mL of rAlbumin Human in *Diluent*

² Available from Invitrogen (No. LC2673).

³ Available from Invitrogen (No. LC2672).

⁴ A suitable Coomassie stain is available from Pierce (No. 24890 or No. 24592).

⁵ Alternatively, a precast 14% Tris-Glycine gel is available from Invitrogen (No. EC6485).

⁶ Copper sulfate pentahydrate and potassium sulfate tablets (each tablet with 1.5 g of K₂SO₄ + 0.15 g of CuSO₄ · 5H₂O) are available from Foss (No. 15270054).

Apparatus**Mode:** Atomic absorption**Emission wavelength:** 589 nm**Analysis:** [NOTE—Use peak area measurements for quantitation.]**Samples:** *Diluent* (as blank), *Standard solutions*, and *Sample solution*

Introduce a blank solution (*Diluent*) into the atomic generator, and adjust the instrument reading to zero. Determinations are made by comparison with the *Standard solutions* of known concentration. If the *Sample solution* emission exceeds that of the *Standard solutions* with the highest concentration, dilute the *Sample solution* with *Diluent*. Introduce the most concentrated *Standard solution* into the instrument, and adjust the sensitivity to obtain a suitable reading. Introduce the *Sample solution* and *Standard solutions* into the instrument at least three times, and record the steady reading. Rinse the apparatus with blank solution each time, and ascertain that the reading returns to its initial blank value. Plot the mean of the readings obtained for the *Standard solutions* against their respective sodium concentrations. From the standard curve, calculate the sodium concentration content in the *Sample solution*, and adjust for the specific gravity of the rAlbumin Human (see *Total Protein*).

Acceptance criteria: 120–160 mM sodium**IMPURITIES****• LIMIT OF HIGH MOLECULAR WEIGHT PROTEINS****Solution A:** 200 mg/mL of sodium azide**Buffer:** Dissolve 54.2 g of dibasic sodium phosphate dihydrate, 30.0 g of monobasic sodium phosphate dihydrate, and 284.0 g of anhydrous sodium sulfate in 1600 mL of water. Add 50 mL of *Solution A*, and dilute with water to 2000 mL.**Mobile phase:** *Buffer* and water (10:90)**Sample solution:** 40 mg/mL of rAlbumin Human Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** UV 280 nm**Column:** 7.8-mm × 30-cm; 5-μm packing L59**Flow rate:** 1.0 mL/min**Injection volume:** 50 μL. [NOTE—The peak due to high molecular weight impurities, such as the polymer of albumin, appears in the void volume of the chromatogram.]**Analysis****Sample:** *Sample solution*

Calculate the percentage of albumin polymer in the sample:

$$\text{Result} = (r_U/r_T) \times 100$$

 r_U = peak response of albumin polymer r_T = sum of all rAlbumin Human related peak responses**Acceptance criteria****Individual impurities:** NMT 1.0%**SPECIFIC TESTS****• PH <791>****Sample solution:** 1% (w/v) protein solution diluted with 0.9% (w/v) sodium chloride**Acceptance criteria:** 6.4–7.4**• STERILITY TESTS <71>:** Meets the requirements**• BACTERIAL ENDOTOXINS TEST <85>:** NMT 0.5 USP Endotoxin Unit/mL of rAlbumin Human**ADDITIONAL REQUIREMENTS****• PACKAGING AND STORAGE:** Preserve in tight glass containers, and store at 2°–8°. Do not freeze.**• LABELING:** Label to indicate that the material is of recombinant DNA origin.**• USP REFERENCE STANDARDS <11>**

USP rAlbumin Human RS

USP Endotoxin RS

Alcohol—see *Alcohol General Monographs***Diluted Alcohol****DEFINITION**

Diluted Alcohol is a mixture of Alcohol and water containing NLT 41.0% and NMT 42.0% by weight, corresponding to NLT 48.4% and NMT 49.5% by volume, at 15.56°, of C₂H₅OH.

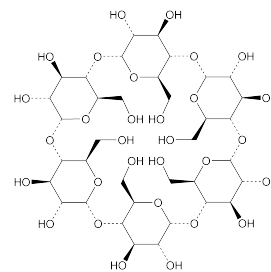
Diluted Alcohol may be prepared as follows.

Alcohol	500 mL
Purified Water	500 mL

Measure the *Alcohol* and the *Purified Water* separately at the same temperature, and mix. If the water and the *Alcohol* and the resulting mixture are measured at 25°, the volume of the mixture will be 970 mL.

SPECIFIC TESTS**• SPECIFIC GRAVITY <841>:** 0.935–0.937 at 15.56°, indicating 41.0%–42.0% by weight, or between 48.4% and 49.5% by volume, of C₂H₅OH**• OTHER REQUIREMENTS:** In other respects, it meets the requirements in *Alcohol*, allowance being made for the difference in alcohol concentration.**ADDITIONAL REQUIREMENTS****• PACKAGING AND STORAGE:** Preserve in tight containers, remote from fire.**• USP REFERENCE STANDARDS <11>**

USP Alcohol RS

Alfadex(C₆H₁₀O₅)₆

Alpha cyclodextrin [10016-20-3].

972.84

DEFINITION

Alfadex is composed of six alpha-(1-4) linked D-glucopyranosyl units. It contains NLT 98.0% and NMT 101.0% of alfadex (C₆H₁₀O₅)₆, calculated on the anhydrous basis.

IDENTIFICATION

- A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- B.** It meets the requirements of the test for *Optical Rotation* <781S>, *Specific Rotation*.

• **C.**

Sample: 0.2 g

Analysis: Mix the *Sample* with 2 mL of iodine TS, warm in a water bath to dissolve, and allow to stand at room temperature.

Acceptance criteria: A yellow-brown precipitate is formed.

ASSAY

• **PROCEDURE**

Mobile phase: Methanol and water (7:93)

System suitability solution: 0.5 mg/mL of USP Alpha Cyclodextrin RS, 0.5 mg/mL of USP Beta Cyclodextrin RS, and 0.5 mg/mL of USP Gamma Cyclodextrin RS

Standard solution: 1.0 mg/mL of USP Alpha Cyclodextrin RS

Sample stock solution: Transfer 250 mg of Alfadex to a 25-mL volumetric flask, and dissolve in water with the aid of heat. Cool, and dilute with water to volume.

Sample solution: 1.0 mg/mL of Alfadex, diluted from the *Sample stock solution*

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 4.6-mm × 15-cm; 5-μm packing L1

Temperature

Column: 30°

Detector: 40°

Flow rate: 1.5 mL/min

Injection volume: 50 μL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for gamma cyclodextrin, alpha cyclodextrin, and beta cyclodextrin are 0.8, 1.0, and 2.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between the gamma cyclodextrin and alpha cyclodextrin peaks

Tailing factor: 0.8–2.0 for the three cyclodextrins

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of alfadex ($C_6H_{10}O_5$)₆ in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of alpha cyclodextrin from the *Sample solution*

r_S = peak response of alpha cyclodextrin from the *Standard solution*

C_S = concentration of alpha cyclodextrin in the *Standard solution* (mg/mL)

C_U = concentration of alpha cyclodextrin in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–101.0% on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION** <281>

Sample: 1.0 g

Acceptance criteria: NMT 0.1%

• **HEAVY METALS, Method II** <231>: NMT 10 μg/g

• **LIMIT OF LIGHT-ABSORBING SUBSTANCES**

Sample solution: 10 mg/mL of Alfadex, calculated on the anhydrous basis, in water that has been previously boiled and cooled to room temperature. Pass through a filter of 0.2-μm pore size.

Analysis: Determine the absorbance of the *Sample solution* in a 1-cm cell with a suitable spectrophotometer, after correcting for the blank.

Acceptance criteria: Between 230 and 350 nm, the absorbance is NMT 0.10; and between 350 and 750 nm, the absorbance is NMT 0.05.

• **LIMIT OF BETADEx, GAMMA CYCLODEXTRIN, AND OTHER RELATED SUBSTANCES**

System suitability solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.

Standard solution: *System suitability solution* and water (1:9)

Sample solution: Use the *Sample stock solution* prepared as directed in the *Assay*.

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria

Beta cyclodextrin: The area of any peak corresponding to beta cyclodextrin is NMT 0.5 times the area of the corresponding peak from the *Standard solution* (0.25%).

Gamma cyclodextrin: The area of any peak corresponding to gamma cyclodextrin is NMT 0.5 times the area of the corresponding peak from the *Standard solution* (0.25%).

Other related substances: The sum of all of the peak areas, excluding the alpha cyclodextrin, beta cyclodextrin, and gamma cyclodextrin is NMT the area of the peak due to alpha cyclodextrin from the *Standard solution* (0.5%).

• **LIMIT OF REDUCING SUGARS**

Cupric solution: 150 mg/mL of cupric sulfate

Tartrate solution: 25 mg/mL of anhydrous sodium carbonate, 25 mg/mL of potassium sodium tartrate, 20 mg/mL of sodium bicarbonate, and 200 mg/mL of anhydrous sodium sulfate

Cupric-tartrate solution: Immediately before use, mix *Cupric solution* with *Tartrate solution* (1:25).

Ammonium molybdate reagent: Mix 10 mL of disodium arsenate solution (6 in 100), 50 mL of a solution of ammonium molybdate (1 in 10), and 90 mL of diluted sulfuric acid, and dilute with water to 200 mL.

Sample solution: 1.0 g of Alfadex, calculated on the anhydrous basis, in 100 mL of water that has been previously boiled and cooled to room temperature. To 1 mL of this solution add 1 mL of *Cupric-tartrate solution*. Heat on a water bath for 10 min, then cool to room temperature. Add 10 mL of *Ammonium molybdate reagent*, and allow to stand for 15 min.

Standard stock solution: 20 mg/L of USP Dextrose RS

Standard solution: Prepare as directed for the *Sample solution*, at the same time, except use 1 mL of *Standard stock solution* in place of 1 mL of 10 mg/mL of Alfadex solution.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Vis

Analytical wavelength: 740 nm

Blank: Water

Analysis

Samples: *Sample solution* and *Standard solution*
Measure the *Samples* at the analytical wavelengths against the *Blank*.

Acceptance criteria: The absorbance of the *Sample solution* is NMT that of the *Standard solution* (0.2%).

SPECIFIC TESTS

• **CLARITY OF SOLUTION**

Sample: 10 mg/mL in previously boiled and cooled water

Acceptance criteria: The resulting solution is clear.

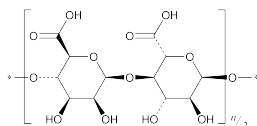
• **MICROBIAL ENUMERATION TESTS** <61> and **TESTS FOR SPECIFIED MICROORGANISMS** <62>: The total aerobic bacterial count does not exceed 10^3 cfu/g, the total combined molds and yeasts count does not exceed 10^2 cfu/g, and it meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

- **OPTICAL ROTATION, Specific Rotation (781S)**
Sample solution: 10 mg/mL
Acceptance criteria: +147° to +152°, determined at 20°
- **pH (791)**
Diluent: 224 g/L of potassium chloride
Sample solution: 30 mL of its aqueous solution (1 in 100) and 1 mL of *Diluent*
Acceptance criteria: 5.0–8.0
- **WATER DETERMINATION, Method I (921):** NMT 11.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **USP REFERENCE STANDARDS (11)**
 USP Alpha Cyclodextrin RS
 USP Beta Cyclodextrin RS
 USP Dextrose RS
 USP Gamma Cyclodextrin RS

Alginate Acid



(C₆H₈O₆)_n

Alginate acid [9005-32-7].

DEFINITION

Alginate Acid is a hydrophilic colloidal carbohydrate extracted with dilute alkali from various species of brown seaweeds (Phaeophyceae).

IDENTIFICATION

- **A.**
Analysis: To 5 mL of a 1-in-150 solution in 0.1 N sodium hydroxide add 1 mL of calcium chloride TS.
Acceptance criteria: A voluminous, gelatinous precipitate is formed.
- **B.**
Analysis: To 5 mL of a 1-in-150 solution in 0.1 N sodium hydroxide add 1 mL of 4 N sulfuric acid.
Acceptance criteria: A heavy, gelatinous precipitate is formed.
- **C.**
Analysis: To 5 mg in a test tube add 5 mL of water, 1 mL of a freshly prepared 1-in-100 solution of 1,3-naphthalenediol in alcohol, and 5 mL of hydrochloric acid. Heat the mixture to boiling, boil gently for 3 min, then cool to 15°. Transfer the contents of the test tube to a 30-mL separator with the aid of 5 mL of water, and extract with 15 mL of isopropyl ether.
Acceptance criteria: The isopropyl ether extract exhibits a deeper purplish hue than that from a blank, similarly prepared.

IMPURITIES

- **ARSENIC, Method II (211):** NMT 3 ppm
- **LEAD (251)**
Standard solution: 5 mL of *Diluted Standard Lead Solution*
Test preparation: Add 1.0 g to 20 mL of nitric acid in a 250-mL conical flask, mix, and heat carefully until the Alginate Acid is dissolved. Continue heating until the volume is reduced to 7 mL. Cool rapidly to room temperature, transfer to a 100-mL volumetric flask, and dilute with water to volume.

Analysis: Use 50.0 mL of the *Test preparation*, and proceed as directed in the chapter, using 15 mL of ammonium citrate solution, 3 mL of potassium cyanide solution, and 0.5 mL of hydroxylamine hydrochloride solution. After the first dithizone extraction, wash the combined chloroform layers with 5 mL of water, discarding the water layer and continuing in the usual manner by extracting with 20 mL of 0.2 N nitric acid.

Acceptance criteria: NMT 5 µg of lead (corresponding to NMT 10 ppm)

- **HEAVY METALS, Method II (231)**

Analysis: Use a platinum crucible for the ignition, and use nitric acid in place of sulfuric acid to wet the test specimen.

Acceptance criteria: NMT 40 ppm

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 2×10^2 cfu/g, and the tests for *Salmonella* species and *Escherichia coli* are negative.
- **pH (791):** 1.5–3.5, in a 3-in-100 dispersion in water
- **LOSS ON DRYING (731):** Dry a sample at 105° for 4 h: it loses NMT 15.0% of its weight.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561)**
Sample: 4 g of Alginate Acid
Analysis: Proceed as directed in the chapter, carefully igniting the *Sample* in a tared platinum dish, until the residue is thoroughly carbonized (5 min). Then ignite in a muffle furnace at a temperature of $800 \pm 25^\circ$ until the carbon is completely burned off (20–35 min).
Acceptance criteria: NMT 4.0% of ash
- **FATS AND FIXED OILS, Acid Value (401)**

Sample: 1 g of Alginate Acid

Analysis: Suspend the *Sample* in a mixture of 50 mL of water and 30.0 mL of calcium acetate solution (11 in 250). Shake thoroughly, allow the mixture to stand for 1 h, and add phenolphthalein TS. Titrate the liberated acetic acid with 0.1 N sodium hydroxide VS. Perform a blank determination, and calculate the *Acid Value*:

$$\text{Result} = [(V_S - V_B) \times N \times F] / W$$

V_S = volume of 0.1 N sodium hydroxide VS consumed in the titration of the *Sample* (mL)

V_B = volume of 0.1 N sodium hydroxide VS consumed in the titration of the blank (mL)

N = normality of sodium hydroxide VS (mEq/mL)

F = equivalency factor of potassium hydroxide, 56.11 (mg/mEq)

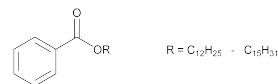
W = weight of Alginate Acid taken (g)

Acceptance criteria: NLT 230 on the dried basis

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Alkyl (C12–15) Benzoate



C₂₀H₃₂O₂

Benzoic acid, C12–15 alkyl ester [68411-27-8].

304.47 (average)

DEFINITION

Alkyl (C12–15) Benzoate consists of esters of a mixture of C12 to C15 primary and branched alcohols and benzoic acid.

IDENTIFICATION

- **A.** Infrared absorption spectrum, obtained by spreading a capillary film of it between sodium chloride plates, exhibits maxima at 2800 cm⁻¹ (broad); 2950 cm⁻¹ (broad); 1730 cm⁻¹ (medium, sharp); 1270 cm⁻¹ (medium, sharp); and 710 cm⁻¹ (medium, sharp).

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.5%

SPECIFIC TESTS

- **WATER DETERMINATION**, *Method I* (921): NMT 0.3%
- **SPECIFIC GRAVITY** (841): 0.915–0.935
- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 0.5
- **REFRACTIVE INDEX** (831): 1.483–1.487 at 20°
- **FATS AND FIXED OILS**, *Saponification Value* (401)
Sample: 2 g of Alkyl (C12–15) Benzoate
Analysis: Transfer the *Sample* to a 200-mL flask. Proceed as directed in the chapter, except use 50.0 mL of 0.5 N alcoholic potassium hydroxide VS instead of 25.0 mL, maintaining reflux for 2 h instead of 30 min, and rinsing the reflux condenser with 25 mL of water after the reflux period.
Acceptance criteria: 169–182
- **ROTATIONAL RHEOMETER METHODS** (912)
Sample: 500 mL
Analysis: Transfer the *Sample* to a 600-mL tall-form beaker, and adjust the temperature to 25 ± 0.1°. Using a suitable rotational viscometer with a spindle having a cylinder 5.63 cm in diameter and 2.25 cm in height attached to a shaft 0.32 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 2.91 cm, and the immersion depth being 6.11 cm (No. 1 spindle), operate the viscometer at 100 rpm for 60 s, accurately timed, and record the scale reading. Convert the scale reading to centipoises by multiplying by the constant for the spindle and speed used.
Acceptance criteria: The average of three viscosities obtained is NMT 100 centipoises.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Almond Oil

Almond Oil [8007-69-0].

DEFINITION

Almond Oil is the refined fixed oil obtained by expression from the kernels of varieties of *Prunus dulcis* (Miller) D.A. Webb (formerly known as *Prunus amygdalus* Batsch) (Fam. Rosaceae), except for *Prunus dulcis* (Miller) D.A. Webb var. *amara* (De Candolle) Focke. It may contain suitable antioxidants.

IDENTIFICATION

- **A.** It meets the requirements in *Specific Tests for Fats and Fixed Oils, Fatty Acid Composition* (401).

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 0.5
- **FATS AND FIXED OILS**, *Fatty Acid Composition* (401): Almond Oil exhibits the following composition profiles of fatty acids.

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<16	0	≤0.1
16	0	4.0–9.0

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
17	0	≤0.2
18	0	≤3.0
20	0	≤0.2
22	0	≤0.2
24	0	≤0.2
16	1	≤0.8
17	1	≤0.2
18	1	62.0–76.0
18	2	20.0–30.0
18	3	≤0.4
20	1	≤0.3
22	1	≤0.1

- **FATS AND FIXED OILS**, *Peroxide Value* (401): NMT 5.0
- **FATS AND FIXED OILS**, *Unsaponifiable Matter* (401): NMT 0.9%
- **SPECIFIC GRAVITY** (841): 0.910–0.915
- **STEROL COMPOSITION**

Separation of the sterols fraction

Reference solution A: 5% (w/v) of cholesterol in chloroform

Developing solvent system: Toluene and acetone (19:1) or hexane and ether (13:7)

Sample solution A: Weigh 5 g of Almond Oil into a 250-mL flask. Add 50 mL of 2 N alcoholic potassium hydroxide, and heat to gentle boiling with continuous vigorous stirring until saponification takes place (the solution becomes clear). Continue heating for an additional 20 min, and add 50 mL of water from the top of the condenser. Cool the flask to approximately 30°. Transfer the contents of the flask to a 500-mL separating funnel with several rinses of water, amounting in all to 50 mL. Add approximately 80 mL of ether, shake vigorously for approximately 30 s, and allow to settle. [NOTE—Any emulsion can be destroyed by adding small quantities of ethyl or methyl alcohol by means of a spray.]

Separate the lower aqueous phase, and collect it into a second separating funnel. Perform two further extractions on the water–alcohol phase in the same way, using 60–70 mL of ether on each occasion. Pool the ether extracts into a single separating funnel, and wash with water, 50 mL at a time, until the wash water is no longer alkaline to phenolphthalein. Dry the ether phase with anhydrous sodium sulfate, and filter on anhydrous sodium sulfate into a previously weighed 250-mL flask, washing the funnel and filter with small quantities of ether. Distill the ether down to a few mL, and bring to dryness under a slight vacuum or in a stream of nitrogen. Completely dry at 100° for approximately 15 min, and then weigh after cooling in a desiccator. Dissolve the unsaponifiables so obtained in chloroform to prepare a solution having a concentration of approximately 5%.

Sample solution B: Treat 5 g of canola oil in the same way as prescribed for Almond Oil in *Sample solution A*, beginning with “Add 50 mL of 2 N alcoholic potassium hydroxide”.

Sample solution C: Treat 5 g of sunflower oil in the same way as prescribed for Almond Oil in *Sample solution A*, beginning with “Add 50 mL of 2 N alcoholic potassium hydroxide”.

Analysis: Precondition the thin-layer chromatographic plate (see *Chromatography* (621)), 20-cm × 20-cm silica gel on polyester with a layer thickness of 200 μm and particle size of 5–17 μm, by immersing completely in the 0.2 N alcoholic potassium hydroxide for 10 s, then allow to dry in a fume cupboard for 2 h, and finally place at 100° for 1 h.

Remove from the validated heating device, and keep the plate in a desiccator until required for use. The plates must be used within 15 days. [NOTE—Thin-layer chromatographic plates without requiring the preconditioning are also commercially available.] Use a separate plate for each *Sample solution*.

Place the *Developing solvent system* in the chamber to a depth of approximately 1 cm. Close the chamber with the appropriate cover, and leave for at least 30 min. Strips of filter paper dipping into the eluant may be placed on the internal surfaces of the chamber. The *Developing solvent system* should be replaced for every test to ensure reproducible elution conditions.

Apply 0.3 mL of *Sample solution A* approximately 2 cm from the lower edge in a streak that is as thin and as uniform as possible. In line with the streak, place 2–3 μ L of *Reference solution A* at one end of the plate. Develop the chromatograms in an equilibrated chamber with a *Developing solvent system* until the solvent front reaches approximately 1 cm from the upper edge of the plate. Remove the plate from the developing chamber, and evaporate the solvent under a current of hot air (avoid excessive heat), or by leaving the plate for a short while under a hood. Spray the plate with a 0.2% alcoholic solution of 2,7-dichlorofluorescein, and examine in UV light at 254 nm.

[NOTE—The plates pretreated with UV indicator are also commercially available and used equivalently.]

In each of the plates, mark the limits of the sterol band identified through being aligned with the stain of *Reference solution A* along the edges of the fluorescence, and additionally include the area of the zones 2–3 mm above and below the visible zones corresponding to *Reference solution A*. Remove the silica gel in the marked area into a filter funnel with a G3 porous septum. Add 10 mL of hot chloroform, mix carefully with the metal spatula, filter under vacuum, and collect the filtrate in the conical flask attached to the filter funnel. Wash the residue in the funnel three times with ether, 10 mL each time, and collect the filtrate in the same flask attached to the funnel. Evaporate the filtrate to a volume of 4–5 mL, transfer the residual solution to a previously weighed 10-mL test tube with a tapering bottom and a sealing stopper, and evaporate to dryness by mild heating in a gentle stream of nitrogen. Dissolve the residue in a few drops of acetone, and evaporate again to dryness. Place at 105° for approximately 10 min, allow to cool in a desiccator, and weigh.

Treat *Sample solution B* and *Sample solution C* the same way as prescribed for *Sample solution A*.

Determination of the sterols

Sample solution D: To the test tube containing the sterol fraction separated from Almond Oil by thin-layer chromatography add a freshly prepared mixture of anhydrous pyridine, hexamethyldisilazane, and chlorotrimethylsilane (9:3:1) in the ratio of 50 μ L for every mg of sterols, avoiding any uptake of moisture.

[NOTE—This reagent is also commercially available and used equivalently.] Stopper the test tube, and shake carefully until the sterols are completely dissolved. Allow it to stand for at least 15 min at ambient temperature, and centrifuge for a few min if necessary. Use the supernatant. The slight opalescence that may form is normal and does not cause an anomaly. However, the formation of a white floc or the appearance of a pink color is indicative of the presence of moisture or deterioration of the reagent. If these occur, repeat the test.

Reference solution E: To 9 parts of the sterols separated from canola oil by thin-layer chromatography add 1 part of cholesterol. Treat the mixture in the same way as prescribed in *Sample solution D*.

Reference solution F: Treat the sterols separated from sunflower oil by thin-layer chromatography in the same way as prescribed in *Sample solution D*.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: Glass or fused-silica capillary column of length 20–30 m, internal diameter 0.25–0.32 mm, coated with a 0.10–0.30- μ m layer of stationary phase G27 or G36

Temperatures

Column: 260 \pm 5°

Detector: 290°

Injection port: 280°

Carrier gas: Either helium with a linear velocity of 20–35 cm/s or hydrogen with a linear velocity of 30–50 cm/s

Injection volume: 1 μ L

Injection type: Split, 50:1 to 100:1

System suitability

Samples: *Reference solution E* and *Reference solution F*

Suitability requirements

The retention time for β -sitosterol is 20 \pm 5 min, and all of the sterols present must be separated.

[NOTE—For peak identification purposes, the chromatogram *Reference solution E* shows four principal peaks corresponding to cholesterol, brassicasterol, campesterol, and β -sitosterol; the chromatogram obtained with *Reference solution F* shows four principal peaks corresponding to campesterol, stigmastanol, β -sitosterol, and Δ 7-stigmastanol. The retention times of the sterols with reference to β -sitosterol are given in *Table 1*.]

Table 1. Relative Retention Times of Sterols for Two Different Columns

Identification	G36 Column	G27 Column
Cholesterol	0.67	0.63
Brassicasterol	0.73	0.71
24-Methylene-cholesterol	0.82	0.80
Campesterol	0.83	0.81
Campestanol	0.85	0.82
Stigmastanol	0.88	0.87
Δ 7-Campesterol	0.93	0.92
Δ 5,23-Stigmastadienol	0.95	0.95
Clerosterol	0.96	0.96
β -Sitosterol	1.00	1.00
Sitostanol	1.02	1.02
Δ 5-Avenasterol	1.03	1.03
Δ 5,24-Stigmastadienol	1.08	1.08
Δ 7-Stigmastanol	1.12	1.12
Δ 7-Avenasterol	1.16	1.16

Analysis

Samples: *Sample solution D*, *Reference solution E*, and *Reference solution F*

Calculate the percentage of each individual sterol in the sterol fraction of Almond Oil taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area due to the sterol component to be determined

r_T = sum of the peak areas due to the components indicated in *Table 1*

Acceptance criteria: Almond Oil exhibits the composition profiles of sterols given in *Table 2*.

Table 2

Component	Percentage (%)
Cholesterol	≤0.7
Brassicasterol	≤0.3
Campesterol	≤5.0
Stigmasterol	≤4.0
β-Sitosterol	73.0–87.0
Δ5-Avenasterol	≥5.0
Δ7-Stigmasterol	≥3.0
Δ7-Avenasterol	≤3.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant, and well-filled containers. No storage requirements specified.
- **LABELING:** Label it to indicate the name and quantity of any added antioxidants.

Aluminum Monostearate

Aluminum, dihydroxy(octadecanoato-O-)-;
Dihydroxy(stearato)aluminum [7047-84-9].

DEFINITION

Aluminum Monostearate is a compound of aluminum with a mixture of solid organic acids obtained from fats, and consists chiefly of variable proportions of aluminum monostearate and aluminum monopalmitate. It contains the equivalent of NLT 14.5% and NMT 16.5% of aluminum monostearate (Al_2O_3), calculated on the dried basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Aluminum (191)**

Sample: 1 g

Analysis: Heat the *Sample* with a mixture of 25 mL of water and 5 mL of hydrochloric acid for 1 h, replacing the water as it evaporates.

Acceptance criteria: Fatty acids are liberated, floating as an oily layer on the surface of the liquid, and the water layer meets the requirements.

- **B. FATS AND FIXED OILS, Solidification Temperature of Fatty Acids (401)**

Sample: 25 g

Analysis: Mix intimately the *Sample* with 100 mL of ether in a 500-mL flask. Add 150 mL of 3 N hydrochloric acid, attach a water-cooled condenser, and heat on a steam bath under reflux for 15 min. Cool, and transfer both layers to a separator with the aid of an additional 100 mL of ether. Shake vigorously, and allow the layers to separate. Remove the water layer, and wash the ether layer with three 30-mL portions of water. Transfer the ether layer to a small beaker, warm on a steam bath until the ether has evaporated and the fatty acids are clear, and dry the acids at 105° for 20 min.

Acceptance criteria: NLT 54°

ASSAY

- **PROCEDURE**

Sample: 5 g

Analysis

Sample: *Sample*

Weigh the *Sample* in a covered platinum crucible that previously has been ignited for 20 min, cooled over anhydrous magnesium perchlorate, and weighed. Heat the open crucible gently, without allowing the *Sample* to burst into flame, and gradually increase the heat until the ash is white. Ignite the ash for 20 min after the organic matter is removed, and cool. Add 15 mL

of water, cover the crucible with a small watch glass, and boil gently for 5 min, using a small stirring rod to break up any large lumps of ash. Decant the solution through ashless filter paper, retaining most of the ash in the crucible. Repeat the extraction with water twice, passing the solutions through the same filter. Transfer the ash to the filter by means of a fine stream of water, and wash the crucible and the residue three times with warm water. Transfer the filter paper and the residue to the crucible, dry, and ignite for 20 min after the filter paper has burned away. Following the ignition period, cover the crucible, cool over anhydrous magnesium perchlorate for 15 min, and weigh the residue of Al_2O_3 rapidly. Repeat the ignition until constant weight is attained, using 20-min ignition periods and 15-min cooling periods. From the weight of the residue remaining in the crucible, calculate the content of Al_2O_3 .

Acceptance criteria: 14.5%–16.5% on the dried basis

IMPURITIES

- **ARSENIC, Method I (211)**

Test preparation: To 3.75 g add 12.5 mL of hydrochloric acid and 0.5 mL of bromine TS, and heat on a steam bath until a transparent layer of melted fatty acid forms. Add 50 mL of water, heat on a hot plate until the volume is about 25 mL, and filter while hot. Cool, dilute the filtrate with water to 50 mL, and to a 10-mL aliquot of this solution add 2.5 mL of hydrochloric acid, then dilute with water to 55 mL.

Acceptance criteria: NMT 4 µg/g; the *Test preparation* meets the requirements, omitting the addition of 20 mL of 7 N sulfuric acid specified for *Procedure*.

- **HEAVY METALS (231)**

Test preparation: To 2 g contained in a 250-mL flask add 20 mL of water and 10 mL of hydrochloric acid. Place a small funnel in the neck of the flask, and boil gently, replacing the water as it evaporates, until the fatty acids separate in a clear layer. Cool rapidly by rotating under a stream of cold water until the fatty acids solidify. Decant through a filter previously washed with 3 N hydrochloric acid, wash until the combined filtrate and washings measure 50 mL, and mix.

Standard solution: To 10 mL of the *Test preparation* add 2 mL of *Standard Lead Solution*, and dilute with water to 20 mL.

Analysis: To 20 mL of the *Test preparation* and the *Standard solution* add 6 N ammonium hydroxide, dropwise, until a permanent turbidity forms. Add 1 N acetic acid until the precipitate just dissolves, then add 2 mL in excess, and add water to make 40 mL. Add 1.2 mL of thioacetamide–glycerin base TS and 2 mL of pH 3.5 *Acetate Buffer*, and allow to stand for 5 min.

Acceptance criteria: NMT 50 µg/g; any brown color produced in the *Test preparation* is not darker than that of the *Standard solution*.

SPECIFIC TESTS

- **LOSS ON DRYING (731)**

Analysis: Dry at 80° for 16 h.

Acceptance criteria: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Aluminum Oxide

Al_2O_3
[1344-28-1].

101.96

DEFINITION

Aluminum Oxide contains NLT 47.0% and NMT 60.0% of Al_2O_3 .

IDENTIFICATION

- IDENTIFICATION TESTS—GENERAL, Aluminum <191>**

Sample: 2.5 g

Analysis: Dissolve the *Sample* in 15 mL of hydrochloric acid on a heated water bath. Dilute with water to 100 mL.

Acceptance criteria: Meets the requirements

ASSAY

- PROCEDURE**

Sample: 800 mg

Analysis: Add the *Sample* to 10 mL of 2 M hydrochloric acid on a heated water bath. Cool, and dilute with water to 50.0 mL. To 10.0 mL of the solution, add dilute ammonia solution (460 mL of strong ammonia solution/L) until a precipitate begins to appear. Add the smallest quantity of 0.6 M hydrochloric acid needed to dissolve the precipitate, and dilute with water to 20 mL. Transfer 20 mL of the *Sample* to a 500-mL conical flask, add 25.0 mL of 0.1 M edetate disodium and 10 mL of a mixture of equal volumes of a 155 g/L solution of ammonium acetate and 2 N acetic acid. Boil for 2 min, then cool. Add 50 mL of ethanol and 3 mL of a freshly prepared 250 mg/L solution of dithizone in alcohol. Titrate (see *Titrimetry* (541)) the excess of edetate sodium with 0.1 M zinc sulfate until the color changes from greenish-blue to reddish-violet. Each mL of 0.1 M edetate sodium is equivalent to 5.098 mg of Al_2O_3 .

Acceptance criteria: 47.0%–60.0% of Al_2O_3

IMPURITIES**Inorganic Impurities**

- ARSENIC, Method I <211>**

Sample solution: Dissolve 2.5 g of Aluminum Oxide in 15 mL of hydrochloric acid on a heated water bath. Dilute with water to 100 mL. Use 30 mL of this solution for the test.

Acceptance criteria: NMT 4 ppm

- HEAVY METALS**

Standard lead solution: Prepare as directed under *Heavy Metals* (231), *Special Reagents*.

Standard solution: On the day of use, dilute 1.0 mL of the *Standard lead solution* with water to 100.0 mL. Each mL of the *Standard solution* contains the equivalent of 1 ppm of lead.

Sample solution: Dissolve 2.5 g of Aluminum Oxide in 15 mL of hydrochloric acid on a heated water bath. Dilute with water to 100 mL. Neutralize 20 mL of the resultant solution with strong ammonia solution, using metanil yellow solution (1 mg/mL in methanol) as an indicator. Filter, if necessary, and dilute with water to 30 mL.

Analysis: To 12 mL of the *Sample solution*, add 2.0 mL of pH 3.5 acetate buffer to 1.2 mL of thioacetamide–glycerin base TS. To 10 mL of the *Standard solution* add 2.0 mL of the *Sample solution*, and add 2.0 mL of pH 3.5 acetate buffer to 1.2 mL of thioacetamide–glycerin base TS. Prepare a blank, using a mixture of 10 mL of water and 2.0 mL of the *Sample solution*. Compared to the blank, the *Standard solution* shows a light brown color.

Acceptance criteria: Any brown color from the *Sample solution* is not darker than that of the *Standard solution* (NMT 60 ppm).

- CHLORIDE AND SULFATE, Chloride <221>**

Chloride solution: 0.824 mg/mL of sodium chloride

Standard solution: On the day of use, dilute 1.0 mL of *Chloride solution* with water to 100 mL. Mix 10 mL of this solution (5 ppm Cl) with 5 mL of water.

Sample solution: Dissolve 0.1 g of Aluminum Oxide with heating in 10 mL of dilute nitric acid and dilute with water to 100 mL. Dilute 5 mL of the solution with water to 15 mL.

Analysis: Proceed as directed.

Acceptance criteria: The *Sample solution* shows no more chloride than the *Standard solution* (NMT 10,000 ppm).

- CHLORIDE AND SULFATE, Sulfate <221>**

Sulfate stock solution: 1.8 mg/mL of potassium sulfate

Sulfate solution: On the day of use, dilute 1.0 mL of *Sulfate stock solution* with water to 100 mL (10 ppm SO_4).

Standard solution: Add 3 mL of a 250 mg/mL solution of barium chloride to 4.5 mL of *Sulfate solution*. Shake and allow to stand for 1 min.

Sample solution: Dissolve 2.5 g of Aluminum Oxide in 15 mL of hydrochloric acid on a heated water bath. Dilute with water to 100 mL. Dilute 4 mL of this solution with water to 100 mL.

Analysis: To two separate glass cylinders, add 2.5 mL of *Standard solution*. Then to cylinder 1, add 15 mL of the *Sample solution* and 0.5 mL of acetic acid. To cylinder 2 add 15 mL of the *Sulfate solution* and 0.5 mL of acetic acid.

Acceptance criteria: After 5 min, any opalescence in cylinder 1 containing the *Sample solution* is not more intense than that of cylinder 2 containing the *Sulfate solution* (NMT 10,000 ppm).

SPECIFIC TESTS

- MICROBIAL ENUMERATION TESTS <61> and TESTS FOR SPECIFIED MICROORGANISMS <62>**: The total aerobic bacterial count is NMT 1000 cfu/g and the total combined molds and yeasts count is NMT 100 cfu/g. It also meets the requirements of the tests for absence of *Escherichia coli* and bile-tolerant Gram-negative bacteria.

- APPEARANCE OF SOLUTION**

Dilute hydrochloric acid: 10 mg/mL

Control solution: [NOTE—Prepare immediately before use.] Mix 9.6 mL of ferric chloride CS, 0.2 mL of cobaltous chloride CS, and 0.2 mL of cupric sulfate CS with *Dilute hydrochloric acid* to make 10 mL, and dilute 1.5 mL of this solution with the *Dilute hydrochloric acid* to 100 mL.

Sample solution: 2.5 g of Aluminum Oxide in 15 mL of hydrochloric acid on a heated water bath. Dilute with water to 100 mL.

Analysis: Make the comparison by viewing 2.0 mL of *Sample solution* and 2.0 mL of *Control solution* downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* (631)).

Acceptance criteria: The *Sample solution* is not more intensely colored than the *Control solution*.

- CLARITY OF SOLUTION**

Hydrazine sulfate solution: 10 mg/mL of hydrazine sulfate. Allow to stand for 4–6 h before use. [**CAUTION**—Hydrazine sulfate is highly toxic. Avoid skin contact.]

Methenamine solution: 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.

Primary opalescent mixture: To the flask containing *Methenamine solution*, add 25.0 mL of *Hydrazine sulfate solution*, mix, and allow to stand for 24 h. [NOTE—This suspension is stable for 2 months. Mix before use, and do not use if it adheres to the container.]

Opalescent standard: Dilute 15.0 mL of the *Primary opalescent mixture* with water to 1000.0 mL. [NOTE—Use this suspension within 24 h after preparation.]

Reference suspension: Transfer 30.0 mL of *Opalescence standard* to a 100-mL volumetric flask, and dilute with water to volume.

Sample solution: Use the *Sample solution* from the test for *Appearance of Solution*.

Analysis: Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer a portion of the *Reference suspension* to a separate matching test tube. Compare the *Sample solution* and the *Reference suspension* in diffused daylight, viewing vertically against a black background (see *Spectrophotometry and Light-Scattering, Visual Comparison* (851)). [NOTE—The *Sample solution* is to be compared to the *Reference suspension* 5 min after preparation of the *Reference suspension*.]

Acceptance criteria: The *Sample solution* is not more opalescent than the *Reference suspension*.

• **ALKALINE IMPURITIES**

Sample: 1.0 g

Analysis: Shake the *Sample* with 20 mL of carbon dioxide-free water for 1 min and filter. To 10 mL of the filtrate add 0.1 mL of phenolphthalein TS.

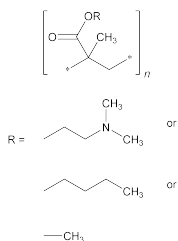
Acceptance criteria: Any pink color disappears with the addition of 0.3 mL of 0.1 M hydrochloric acid.

- **NEUTRALIZING CAPACITY:** Carry out the test at 37°. Disperse 0.5 g of aluminum oxide in 100 mL of water, heat, add 100.0 mL of 0.1 M hydrochloric acid, previously heated, and stir continuously: the pH of the solution after 10, 15, and 20 min is NLT 1.8, 2.3, and 3.0 respectively and is at no time greater than 4.5. Add 10.0 mL of 0.5 M hydrochloric acid, previously heated, stir continuously for 1 h, and titrate with 0.1 M sodium hydroxide to pH 3.5: NMT 35.0 mL of 0.1 M sodium hydroxide is required.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a tight container at a temperature not exceeding 30°.

Amino Methacrylate Copolymer



Ratio: $\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_2 : \text{C}_4\text{H}_9 : \text{CH}_3 = 2:1:1$
 Poly((2-dimethylaminoethyl) methacrylate, butyl methacrylate, methyl methacrylate) (2:1:1);
N,N-Dimethylaminoethyl methacrylate–butyl methacrylate–methyl methacrylate copolymer (2:1:1) [24938-16-7].

DEFINITION

Amino Methacrylate Copolymer is a polymerized copolymer of (2-dimethylaminoethyl) methacrylate, butyl methacrylate, and methyl methacrylate. It contains NLT 20.8% and NMT 25.5% of dimethylaminoethyl groups ($\text{C}_4\text{H}_{10}\text{N}$), calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)

• **B.**

Sample solution: 1 mL of the *Sample solution* in the test for *Viscosity*

Analysis: Pour the *Sample solution* onto a glass plate, and allow the solvent to evaporate.

Acceptance criteria: A clear, colorless film results.

ASSAY

• **PROCEDURE**

Sample: 200 mg

Blank: 4 mL of water and 96 mL of glacial acetic acid

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric

Analysis: Dissolve the *Sample* in a mixture of 4 mL of water and 96 mL of glacial acetic acid. Titrate with the *Titrant* to a potentiometric endpoint. Perform a blank determination.

Calculate the percentage of dimethylaminoethyl groups ($\text{C}_4\text{H}_{10}\text{N}$) in the portion of the sample taken:

$$\text{Result} = \frac{(V_S - V_B) \times N \times F}{W} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 72.1 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: 20.8%–25.5% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%

- **LIMIT OF BUTYL METHACRYLATE AND METHYL METHACRYLATE**

Buffer: 8.9 g/L of anhydrous dibasic sodium phosphate and 8.5 g/L of monobasic potassium phosphate. Adjust with phosphoric acid to a pH of 2.0. This is pH 2.0 phosphate buffer (0.0625 M).

Mobile phase: Methanol and *Buffer* (55:45)

Diluent: Acetonitrile and *Buffer* (40:60)

Standard solution: Dissolve 20 mg of butyl methacrylate and 10 mg of methyl methacrylate in 3 mL of *n*-butanol. Dilute with *Diluent* to 10 mL. Dilute 1.0 mL of this solution with *Diluent* to 250.0 mL, and mix. This solution contains about 8 µg/mL of butyl methacrylate and 4 µg/mL of methyl methacrylate.

Sample solution: 20 mg/mL of Amino Methacrylate Copolymer in *Diluent*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm × 12-cm; packing L1

Flow rate: 2 mL/min

Injection volume: 50 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 10 between butyl methacrylate and methyl methacrylate

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each monomer in the portion of Amino Methacrylate Copolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of each monomer (butyl methacrylate or methyl methacrylate) from the *Sample solution*

r_S = peak response of each monomer (butyl methacrylate or methyl methacrylate) from the *Standard solution*

C_S = concentration of each monomer (butyl methacrylate or methyl methacrylate) in the *Standard solution* (µg/mL)

C_U = concentration of Amino Methacrylate Copolymer in the *Sample solution* (mg/mL)

F = conversion factor, 10^{-3} mg/µg

- Acceptance criteria:** NMT 0.1% for each monomer
- **LIMIT OF 2-DIMETHYLAMINOETHYL METHACRYLATE**
Buffer: 3.4 g/L of monobasic potassium phosphate. This is the monobasic potassium phosphate solution (0.025 M).
Mobile phase: Tetrahydrofuran and *Buffer* (75:25)
Standard solution: 8 µg/mL of 2-dimethylaminoethyl methacrylate in tetrahydrofuran
Sample solution: 20 mg/mL of Amino Methacrylate Copolymer in tetrahydrofuran
Chromatographic system
 (See *Chromatography* <621>, *System Suitability*).
Mode: LC
Detector: UV 215 nm
Column: 4.6-mm × 12-cm; packing L8
Flow rate: 2 mL/min
Injection volume: 50 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
 Calculate the percentage of 2-dimethylaminoethyl methacrylate in the portion of Amino Methacrylate Copolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

- r_U = peak response of 2-dimethylaminoethyl methacrylate from the *Sample solution*
 r_S = peak response of 2-dimethylaminoethyl methacrylate from the *Standard solution*
 C_S = concentration of 2-dimethylaminoethyl methacrylate in the *Standard solution* (µg/mL)
 C_U = concentration of Amino Methacrylate Copolymer in the *Sample solution* (mg/mL)
 F = conversion factor, 10^{-3} mg/µg
Acceptance criteria: NMT 0.1%

SPECIFIC TESTS

• ROTATIONAL RHEOMETER METHODS (912)

Sample solution: Dissolve 12.5 g in a mixture of 35.0 g of acetone and 52.5 g of isopropyl alcohol. [NOTE—Reserve a portion of this solution for the *Color of Solution* test.]

Analysis: Equip a suitable rotational viscometer with an adapter comprising a cylindrical spindle rotating within an accurately machined chamber (or tube).¹ Pipet the *Sample solution* in the specified volume, which is recommended by the instrument manufacturer, into the chamber (or tube), and ensure that the temperature of the test specimen is at $20 \pm 0.1^\circ$. The cylindrical spindle rotates at the value of rpm, which corresponds to a rate of shear of approximately 37 s^{-1} .² Measure the apparent viscosity following the instrument manufacturer's directions.

Acceptance criteria: Viscosity, 3–6 mPa · s

• COLOR OF SOLUTION

Sample solution: Use the *Sample solution* prepared in the test for *Viscosity*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

¹ A commercial device is available from Brookfield as an ultra-low (UL) viscosity adapter. The adapter comprises a 0.4-cm diameter shaft, an accurately machined chamber (or tube) with an internal diameter of 2.8 cm and a depth of 13.5 cm, and a cylindrical spindle 2.5 cm in diameter and 9.1 cm in height.

² For the Brookfield UL adapter, the cylindrical spindle rotates at 30 rpm, which corresponds to a rate of shear of approximately 37 s^{-1} .

Mode: Vis
Analytical wavelength: 420 nm
Cell: 1 cm

Analysis: Determine the absorbance of the *Sample*, using water as the blank.

Acceptance criteria: NMT 0.300

• LOSS ON DRYING (731)

Analysis: Dry a sample at 110° for 3 h.

Acceptance criteria: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at a temperature below 30° .
- **USP REFERENCE STANDARDS** <11>
 USP Amino Methacrylate Copolymer RS

Strong Ammonia Solution

NH₃ 17.03
 Ammonia [7664-41-7].

DEFINITION

Strong Ammonia Solution is a solution of NH₃, containing NLT 27.0% and NMT 31.0% (w/w) of NH₃. On exposure to air, it loses ammonia rapidly.

[**CAUTION**—Use care in handling Strong Ammonia Solution because of the caustic nature of the Solution and the irritating properties of its vapor. Cool the container well before opening, and cover the closure with a cloth or similar material while opening. Do not taste Strong Ammonia Solution, and avoid inhalation of its vapor.]

IDENTIFICATION

• A.

Analysis: Hold a glass rod moistened with hydrochloric acid near the surface of the Solution.

Acceptance criteria: Dense, white fumes are produced.

ASSAY

• PROCEDURE

Sample: Transfer quickly a portion of Strong Ammonia Solution to a stoppered, thick-walled container (a pressure bottle is suitable) to obtain a column height of about 20 cm, insert the stopper, and cool the container and contents to 10° or lower. Accurately weigh a glass-stoppered, 125-mL conical flask containing 35.0 mL of 1 N sulfuric acid VS. Insert a graduated 10-mL measuring pipet into the cooled solution, allow the liquid to rise in the pipet without vacuum, remove the pipet, wipe off adhering liquid, and discard the first mL of the solution permitted to run from the pipet. Hold the pipet just above the surface of the 1 N sulfuric acid VS in the conical flask, and transfer about 2 mL of the solution into the flask. Insert the stopper, and again weigh to obtain the weight of the portion taken of the *Sample*.

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Residual titration

Titrant: 1 N sulfuric acid VS

Back titrant: 1 N sodium hydroxide VS

Blank: Proceed as directed for the *Sample*, omitting the Strong Ammonia Solution

Endpoint detection: Colorimetric

Analysis

Calculate the percentage of NH₃ in the portion of the *Sample* taken:

$$\text{Result} = [(B - V) \times N \times F \times 100]/W$$

B = 1 N sodium hydroxide VS consumed by the *Blank* (mL)

V = 1 N sodium hydroxide VS consumed by the *Sample* (mL)
 N = actual normality of the *Back titrant* (mEq/mL)
 F = equivalency factor, 17.03 mg/mEq
 W = weight of the *Sample* (mg)

Titrate the excess acid with 1 N sodium hydroxide VS, using methyl red TS as the indicator. Perform a blank determination. Each mL of 1 N sulfuric acid is equivalent to 17.03 mg of NH_3 .

Acceptance criteria: 27.0%–31.0%

IMPURITIES

• HEAVY METALS, *Method I* (231)

Test preparation: Evaporate 1.7 mL on a steam bath to dryness, add to the residue 1 mL of 3 N hydrochloric acid, and evaporate to dryness. Dissolve the residue in 2 mL of 1 N acetic acid, and dilute with water to 25 mL.

Analysis: Proceed as directed in the chapter.

Acceptance criteria: NMT 13 ppm

• LIMIT OF NONVOLATILE RESIDUE

Sample: 10 mL

Analysis: Evaporate the *Sample* in a tared platinum or porcelain dish to dryness, and dry at 105° for 1 h.

Acceptance criteria: NMT 5 mg of residue remains (0.05%).

• READILY OXIDIZABLE SUBSTANCES

Sample solution: 4 mL of Strong Ammonia Solution

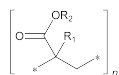
Analysis: Mix the *Sample solution* with 6 mL of water, and add a slight excess of 2 N sulfuric acid and 0.10 mL of 0.1 N potassium permanganate.

Acceptance criteria: The pink color does not completely disappear within 10 min.

ADDITIONAL REQUIREMENTS

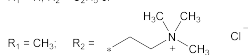
- **PACKAGING AND STORAGE:** Preserve in tight containers, at a temperature not above 25°.

Ammonio Methacrylate Copolymer



$\text{R}_1 = \text{CH}_3$; $\text{R}_2 = \text{CH}_3$ or

$\text{R}_1 = \text{H}$; $\text{R}_2 = \text{C}_2\text{H}_5$ or



Ratio of ethyl acrylate groups to methyl methacrylate groups to trimethylammonioethyl methacrylate groups is about 1: 2: 0.2 as being Type A or 1: 2: 0.1 as being Type B.
 Poly(ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) (1: 2: 0.2);
 Ethyl acrylate–methyl methacrylate–trimethylammonioethyl methacrylate chloride copolymer (1: 2: 0.2);
 Poly(ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) (1: 2: 0.1);
 Ethyl acrylate–methyl methacrylate–trimethylammonioethyl methacrylate chloride copolymer (1: 2: 0.1) [33434-24-1].

DEFINITION

Ammonio Methacrylate Copolymer is a polymerized copolymer of ethyl acrylate, methyl methacrylate, and a low content of methacrylic acid ester with quaternary ammonium groups (trimethylammonioethyl methacrylate chloride). The *Assay* requirements differ for the two types as set forth in the accompanying table.

Ammonio Methacrylate Units, Dried Basis (%)		
Type	Min.	Max.
A	8.85	11.96
B	4.48	6.77

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B.** Meets the requirements of the *Assay*

ASSAY

• PROCEDURE

Sample: 1 g of Ammonio Methacrylate Copolymer Type A, or 2 g of Type B, previously dried

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric

Analysis

Sample: *Sample*

Dissolve the *Sample* in 75 mL of glacial acetic acid at about 50° within 30 min. After the solution cools down to room temperature, add 25 mL of 0.6% cupric acetate in glacial acetic acid. Titrate this solution and a blank, and make any necessary corrections. Each mL of 0.1 N perchloric acid is equivalent to 20.772 mg of ammonio methacrylate ($\text{C}_9\text{H}_{18}\text{ClNO}_2$) units.

Acceptance criteria

Type A: 8.85%–11.96%

Type B: 4.48%–6.77%

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 0.1%
- **HEAVY METALS, *Method II*** (231): NMT 20 ppm

Organic Impurities

• PROCEDURE: LIMIT OF MONOMERS

Sodium perchlorate solution: 35 mg/mL of sodium perchlorate ($\text{NaClO}_4 \cdot \text{H}_2\text{O}$)

Mobile phase: Dilute phosphoric acid with water to obtain a solution with a pH of 2.0. Mix 4 volumes of this solution with 1 volume of methanol.

Standard stock solution: 1.6 mg/mL of ethyl acrylate and 0.2 mg/mL of methyl methacrylate in methanol

Standard solution: Dilute 1 mL of the *Standard stock solution* with methanol to 100 mL. Add 10 mL of this solution to 5 mL of the *Sodium perchlorate solution*. This solution contains about 10.67 $\mu\text{g/mL}$ of ethyl acrylate and 1.33 $\mu\text{g/mL}$ of methyl methacrylate.

Sample solution: Dissolve 5 g of Ammonio Methacrylate Copolymer in methanol, and dilute with the same solvent to 50 mL. Add 5 mL of the *Sodium perchlorate solution* dropwise to 10 mL of this solution while continuously stirring. Remove the precipitated polymer by centrifugation. Use the clear supernatant. Calculate the concentration of Ammonio Methacrylate Copolymer, in mg/mL, as C_u .

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 202 nm

Column: 4.6-mm \times 12-cm; packing L1

Flow rate: 2 mL/min

Injection size: 50 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for ethyl acrylate and methyl methacrylate are 1.00 and 1.14, respectively.]

Suitability requirements

Resolution: NLT 1.0 between the pair of analytes
Relative standard deviation: NMT 5.0% for each analyte

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of ethyl acrylate (or methyl methacrylate) in the portion of Ammonio Methacrylate Copolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

- r_U = peak response of ethyl acrylate (or methyl methacrylate) from the *Sample solution*
 r_S = peak response of ethyl acrylate (or methyl methacrylate) from the *Standard solution*
 C_S = concentration of ethyl acrylate (or methyl methacrylate) in the *Standard solution* (µg/mL)
 C_U = concentration of Ammonio Methacrylate Copolymer in the *Sample solution* (mg/mL)
 F = conversion factor, 10^{-3} mg/µg

Acceptance criteria

Ethyl acrylate: NMT 100 ppm
Methyl methacrylate: NMT 50 ppm

SPECIFIC TESTS

- **LOSS ON DRYING (731):** Dry a sample in a vacuum at 80° for 5 h: it loses NMT 3.0% of its weight.

- **ROTATIONAL RHEOMETER METHODS (912)**

Sample solution: Place 52.5 g of isopropyl alcohol and 35.0 g of acetone in a conical flask with a ground-glass joint. Add a quantity of Ammonio Methacrylate Copolymer, equivalent to 12.5 g of solids on the dried basis, while stirring until the polymer has dissolved completely.

Analysis: Equip a suitable rotational viscometer with an adapter comprising a cylindrical spindle rotating within an accurately machined chamber (or tube).¹ Pipet the *Sample solution* in the specified volume, which is recommended by the instrument manufacturer, into the chamber (or tube), and ensure that the temperature of the test specimen is at $20 \pm 0.1^\circ$. The cylindrical spindle rotates at the value of rpm, which corresponds to a rate of shear of approximately 37 s^{-1} .² Measure the apparent viscosity following the instrument manufacturer's directions.

Acceptance criteria: 1–15 mPa · s

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at a temperature not exceeding 30°.
- **LABELING:** Label it to state whether it is Type A or Type B.
- **USP REFERENCE STANDARDS (11)**
 - USP Ammonio Methacrylate Copolymer, Type A RS Poly(ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) (1: 2: 0.2);
 - Ethyl acrylate–methyl methacrylate–trimethylammonioethyl methacrylate chloride copolymer (1: 2: 0.2).
 - USP Ammonio Methacrylate Copolymer, Type B RS Poly(ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) (1: 2: 0.1);
 - Ethyl acrylate–methyl methacrylate–trimethylammonioethyl methacrylate chloride copolymer (1: 2: 0.1).

¹ A commercial device is available from Brookfield as an ultra-low (UL) viscosity adapter. The adapter comprises a 0.4-cm diameter shaft, an accurately machined chamber (or tube) with an internal diameter of 2.8 cm and a depth of 13.5 cm, and a cylindrical spindle 2.5 cm in diameter and 9.1 cm in height.

² For the Brookfield UL adapter, the cylindrical spindle rotates at 30 rpm, which corresponds to a rate of shear of approximately 37 s^{-1} .

Ammonio Methacrylate Copolymer Dispersion

DEFINITION

Ammonio Methacrylate Copolymer Dispersion is an aqueous dispersion of Ammonio Methacrylate Copolymer Type A or B in water. It may contain suitable antimicrobial preservatives and alkalizing agents. The Assay requirements differ for the two types, as set forth in the accompanying table.

Type	Ammonio Methacrylate Units, Dried Basis (%)	
	Min.	Max.
A	10.18	13.73
B	6.11	8.26

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K)**

Sample: Residue obtained in the test for *Loss on Drying*
Acceptance criteria: Meets the requirements

ASSAY

- **PROCEDURE**

Sample: Dry under vacuum 2 g of Ammonio Methacrylate Copolymer Dispersion Type A, or 4 g of Ammonio Methacrylate Copolymer Dispersion Type B, at 90° for 30 min.

Blank: 75 mL of glacial acetic acid

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric

Analysis: Dissolve the *Sample* in 75 mL of glacial acetic acid at about 50°, within about 30 min. After the solution has cooled down, add 25 mL of 0.6% cupric acetate solution in glacial acetic acid. Titrate this solution with the *Titrant*. Perform a blank determination.

Calculate the percentage of ammonio methacrylate ($\text{C}_9\text{H}_{18}\text{ClNO}_2$) units in the portion of the *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times N \times F/W] \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 207.72 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria

Type A: 10.18%–13.73%

Type B: 6.11%–8.26%

IMPURITIES

- **RESIDUE ON IGNITION (281)**

Analysis: Using mild heating conditions (e.g., steam bath, sand bath, etc.) to avoid loss of material, evaporate the dispersion to dryness before ignition.

Acceptance criteria: NMT 0.5% calculated on the undried dispersion basis

- **LIMIT OF MONOMERS**

Sodium perchlorate solution: 35 mg/mL of sodium perchlorate ($\text{NaClO}_4 \cdot \text{H}_2\text{O}$)

Mobile phase: Dilute phosphoric acid with water to obtain a solution having a pH of 2.0. Mix four volumes of this solution with one volume of methanol, filter, and degas.

Standard stock solution: 1.6 mg/mL of ethyl acrylate and 0.2 mg/mL of methyl methacrylate in methanol

Standard solution: Dilute 1 mL of the *Standard stock solution* with methanol to 100 mL. Add 10 mL of this solution to 5 mL of *Sodium perchlorate solution*.

Sample solution: Dissolve 5 g of Dispersion in methanol, and dilute with the same solvent to 50 mL. Add 5 mL of *Sodium perchlorate solution* dropwise to 10 mL of this solution while continuously stirring. Remove the precipitated polymer by centrifugation. Use the clear supernatant. Calculate the concentration of Dispersion, in mg/mL, as C_U .

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 202 nm

Column: 4.6-mm × 12-cm; packing L1

Flow rate: 2 mL/min

Injection volume: 50 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for ethyl acrylate and methyl methacrylate are 1.00 and 1.14, respectively.]

Suitability requirements

Resolution: NLT 1.0 between the pair of analytes

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of ethyl acrylate and methyl methacrylate in the portion of Dispersion taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of ethyl acrylate or methyl methacrylate from the *Sample solution*

r_S = peak response of ethyl acrylate or methyl methacrylate from the *Standard solution*

C_S = concentration of ethyl acrylate or methyl methacrylate in the *Standard solution* (µg/mL)

C_U = nominal concentration of Dispersion in the *Sample solution* (mg/mL)

F = conversion factor, 10^{-3} mg/µg

Acceptance criteria: NMT 0.002% of methyl methacrylate and NMT 0.008% of ethyl acrylate

SPECIFIC TESTS

• ROTATIONAL RHEOMETER METHODS (912)

Analysis: Use a viscometer equipped with a spindle having a cylinder 1.88 cm in diameter and 6.51 cm high attached to a shaft 0.32 cm in diameter. The distance from the top of the cylinder to the lower tip of the shaft is 0.75 cm, and the immersion depth is 8.15 cm. Adjust the temperature to $20 \pm 0.10^\circ$. With the spindle rotating at 30 rpm, immediately record the scale reading. Multiply the scale reading by the constant for the viscometer spindle and speed used to obtain the viscosity in centipoises.

Acceptance criteria: NMT 100 mPa · s

• LOSS ON DRYING (731)

Analysis: Dry a sample at 110° for 6 h.

Acceptance criteria: 68.5%–71.5%

• COAGULUM CONTENT

Sample: 100 g

Analysis: Weigh a stainless steel sieve having 125-µm openings, and filter the *Sample* through it. Wash the sieve with distilled water until a clear filtrate is obtained, and dry the sieve to constant weight at 105° .

Acceptance criteria: The weight of the residue does not exceed 1000 mg, corresponding to NMT 1%.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, at a temperature not exceeding 25° for Type A and not exceeding 30° for Type B. Protect from freezing.
- **LABELING:** Label it to state whether it is Type A or Type B. Label it to indicate the name and quantity of any added antimicrobial preservative or alkalizing agent.

• USP REFERENCE STANDARDS <11>

USP Ammonio Methacrylate Copolymer, Type A RS

USP Ammonio Methacrylate Copolymer, Type B RS

Ammonium Carbonate

Carbonic acid, monoammonium salt mixture with ammonium carbamate;

Monoammonium carbonate mixture with ammonium carbamate [8000-73-5].

DEFINITION

Ammonium Carbonate consists of ammonium bicarbonate (NH_4HCO_3) and ammonium carbamate ($\text{NH}_2\text{COONH}_4$) in varying proportions. It yields NLT 30.0% and NMT 34.0% of ammonia (NH_3).

IDENTIFICATION

- **A.** When heated, it is volatilized without charring, and the vapor is alkaline to moistened litmus paper. A solution (1 in 20) effervesces with acids.

ASSAY

• PROCEDURE

Sample solution: Place 10 mL of water in a weighing bottle, tare the bottle and its contents, add 2 g of Ammonium Carbonate, and weigh.

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Residual titration

Titrant: 1 N sulfuric acid VS

Back-titrant: 1 N sodium hydroxide VS

Blank: 50.0 mL of 1 N sulfuric acid VS, accurately measured

Endpoint detection: Colorimetric

Analysis: Transfer the *Sample solution* to a 250-mL flask, add 50.0 mL of 1 N sulfuric acid VS, and when the solution has been effected, add methyl orange TS. Titrate the excess acid with 1 N sodium hydroxide VS. Perform a blank determination. Calculate the percentage of ammonia (NH_3) in the sample taken.

$$\text{Result} = [(B - V) \times N \times F \times 100] / W$$

B = 1 N sodium hydroxide VS consumed by the *Blank* (mL)

V = 1 N sodium hydroxide VS consumed by the sample (mL)

N = actual normality of the *Back-titrant* (mEq/mL)

F = equivalency factor, 17.03 mg/mEq

W = weight of the sample (mg)

Acceptance criteria: 30.0%–34.0%

IMPURITIES

- **RESIDUE ON IGNITION** <281>: NMT 0.1%
- **CHLORIDE AND SULFATE, Chloride** <221>: A 2.0-g portion shows no more chloride than corresponds to 0.10 mL of 0.020 N hydrochloric acid (0.0035%).
- **CHLORIDE AND SULFATE, Sulfate** <221>: A 2.0-g portion shows no more sulfate than corresponds to 0.10 mL of 0.020 N sulfuric acid (0.005%).
- **HEAVY METALS, Method I** <231>
Test preparation: Reduce to a coarse powder, and volatilize 2 g on a steam bath. Add 1 mL of 3 N hydrochloric acid, and evaporate to dryness. Dissolve the residue in 2 mL of 1 N acetic acid, and add water to make 25 mL.
Acceptance criteria: NMT 10 ppm

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at a temperature not above 30° .

Ammonium Phosphate

(NH₄)₂HPO₄ 132.06
Phosphoric acid, diammonium salt;
Diammonium phosphate [7783-28-0].

DEFINITION

Ammonium Phosphate contains NLT 96.0% and NMT 102.0% of (NH₄)₂HPO₄.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL**, *Ammonium* <191>: A solution (1 in 20) meets the requirements.
- **B. IDENTIFICATION TESTS—GENERAL**, *Phosphate* <191>: A solution (1 in 20) meets the requirements.

ASSAY

• PROCEDURE

Sample: 600 mg of Ammonium Phosphate
Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N sulfuric acid VS

Endpoint detection: potentiometric

Analysis: Dissolve the *Sample* in 40 mL of water, and titrate with 0.1 N sulfuric acid VS to a pH of 4.6. Each mL of 0.1 N sulfuric acid is equivalent to 13.21 mg of (NH₄)₂HPO₄.

Acceptance criteria: 96.0%–102.0%

IMPURITIES

- **CHLORIDE AND SULFATE**, *Chloride* <221>: A 1.0-g portion shows no more chloride than corresponds to 0.40 mL of 0.020 N hydrochloric acid (0.03%).
- **CHLORIDE AND SULFATE**, *Sulfate* <221>: A 0.20-g portion shows no more sulfate than corresponds to 0.30 mL of 0.020 N sulfuric acid (0.15%).
- **ARSENIC**, *Method I* <211>: NMT 3 ppm
- **HEAVY METALS** <231>
Test preparation: Dissolve 2.0 g in 25 mL of water.
Acceptance criteria: NMT 10 ppm

SPECIFIC TESTS

- **pH** <791>: 7.6–8.2, in a solution (1 in 100)

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Ammonium Sulfate

(NH₄)₂SO₄ 132.14
Ammonium sulfate [7783-20-2].

DEFINITION

Ammonium Sulfate contains NLT 99.0% and NMT 100.5% of (NH₄)₂SO₄.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL**, *Ammonium* <191>: A solution (1 in 20) meets the requirements.
- **B. IDENTIFICATION TESTS—GENERAL**, *Sulfate* <191>: A solution (1 in 20) meets the requirements.

ASSAY

• PROCEDURE

Sample: 2.5 g of Ammonium Sulfate

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Residual titration

Titrant: 1 N sodium hydroxide VS

Back titrant: 1 N sulfuric acid VS

Endpoint detection: Colorimetric

Blank: 50.0 mL of 1 N sodium hydroxide VS, accurately measured

Analysis: Add the *Sample* to a 500-mL conical flask and dissolve in 50 mL of water. Add 50.0 mL of 1 N sodium hydroxide VS, place a filter funnel loosely in the neck of the flask, and boil until ammonia is expelled (about 10–15 min), as determined with litmus paper. Cool, add 0.15 mL of thymol blue TS, and titrate the excess sodium hydroxide with 1 N sulfuric acid VS. Perform a blank determination.

Calculate the percentage of ammonium sulfate [(NH₄)₂SO₄] in the *Sample* taken:

$$\text{Result} = [(B - V) \times N \times F \times 100] / W$$

B = 1 N sulfuric acid VS consumed by the *Blank* (mL)

V = 1 N sulfuric acid VS consumed by the *Sample* (mL)

N = actual normality of the *Back titrant* (mEq/mL)

F = equivalency factor, 66.07 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5%

IMPURITIES

• RESIDUE ON IGNITION <281>

Sample: 20 g

Acceptance criteria: NMT 0.005%

• LIMIT OF INSOLUBLE MATTER

Sample: 20 g

Analysis: Transfer the *Sample* to a covered beaker, and dissolve in 200 mL of water. Heat to boiling, and warm on a steam bath for 1 h. Filter the hot solution through a tared sintered-glass crucible of medium porosity (10–15 μm). Wash the beaker and the filter with hot water, dry the crucible at 105°, cool in a desiccator, and weigh.

Acceptance criteria: NMT 1 mg of insoluble matter is found (0.005%).

• LIMIT OF PHOSPHATE

Standard phosphate solution, Phosphate reagent A, and Phosphate reagent B: Prepare as directed for *Phosphate in Reagents under Reagents, Indicators, and Solutions—General Tests for Reagents*.

Sample: 4.0 g

Control: 0.2 mL of *Standard phosphate solution*

Analysis

[NOTE—The tests for the *Sample* and the *Control* are made preferably in matched color-comparison tubes.] Dissolve the *Sample* in 25 mL of 0.5 N sulfuric acid, add 1 mL each of *Phosphate reagent A* and *Phosphate reagent B*, and allow to stand at room temperature for 2 h. Proceed with the *Control* using the same quantities of the same reagents as in the test for the *Sample*.

Acceptance criteria: Any blue color obtained from the *Sample* should not exceed that produced from the *Control* (NMT 5 ppm).

• CHLORIDE AND SULFATE, *Chloride* <221>

Standard chloride solution: Transfer 165 mg of sodium chloride to a 100-mL volumetric flask. Dissolve in and dilute with water to volume. Transfer 10.0 mL to a 1000-mL volumetric flask, and dilute with water to volume to obtain a solution having a concentration of 10 μg/mL of chloride.

Acceptance criteria: A 2-g portion shows no more chloride than corresponds to 1.0 mL of *Standard chloride solution* (NMT 5 ppm).

Change to read:• **LIMIT OF NITRATE**

Standard nitrate solution and Brucine sulfate solution: Prepare as directed for Nitrate in *Reagents under Reagents, Indicators, and Solutions—General Tests for Reagents*.

Sample solution: Dissolve 1.0 g in 3 mL of water by heating in a boiling water bath, and add *Brucine sulfate solution* to make 50 mL.

Control solution: To 1.0 mL of *Standard nitrate solution* add 1.0 g ammonium sulfate, (ERR 1-Jul-2012) then add *Brucine sulfate solution* to make 50 mL.

Blank: 50 mL of *Brucine sulfate solution*

Analysis: Heat the *Sample solution*, *Control solution*, and *Blank* in a boiling water bath for 15 min with periodic gentle swirling, then cool rapidly in an ice bath to room temperature. Adjust a suitable spectrophotometer with the *Blank* to zero absorbance at 410 nm. Determine the absorbance of the *Sample solution*, note the result, and adjust the instrument with the *Sample solution* to zero absorbance. Determine the absorbance of the *Control solution*.

Acceptance criteria: The absorbance reading for the *Sample solution* does not exceed that for the *Control solution* (NMT 10 ppm).

• **IRON <241>**

Sample solution: Dissolve 2.0 g in 40 mL of water, and add 2 mL of hydrochloric acid.

Acceptance criteria: NMT 5 ppm

SPECIFIC TESTS

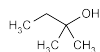
- **MICROBIAL ENUMERATION TESTS <61> and TESTS FOR SPECIFIED MICROORGANISMS <62>:** The total aerobic microbial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 10 cfu/g.

- **PH <791>:** 5.0–6.0 in a solution (1 in 20)

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.

Amylene Hydrate



C₅H₁₂O 88.15
2-Butanol, 2-methyl-;
tert-Pentyl alcohol [75-85-4].

DEFINITION

Amylene Hydrate contains NLT 99.0% and NMT 100.0% of C₅H₁₂O.

IDENTIFICATION

- **A. INFRARED ABSORPTION <197F>**
- **B. PROCEDURE**

Sample solution: 1 in 10

Analysis: To 10 mL of the *Sample solution* quickly add 5 mL of a 1-in-100 solution of vanillin in sulfuric acid.

Acceptance criteria: A violet-red color is produced.

ASSAY• **PROCEDURE**

Sample solution: Amylene Hydrate

Chromatographic system

Mode: GC

Detector: Thermal conductivity

Column: 4-mm × 2-cm glass column; packed with chromatographic support S2 (under typical conditions)

Temperature

Column: 190°

Injector: 200°

Detector block: 200°

Carrier gas: Helium

Flow rate: 50 mL/min

Injection size: 0.4 µL

Analysis

Sample: *Sample solution*

From the area under the curve, calculate the percentage (a/a) of C₅H₁₂O in the Amylene Hydrate taken.

Acceptance criteria: 99.0%–100.0%

IMPURITIES**Inorganic Impurities**• **LIMIT OF NONVOLATILE RESIDUE**

Sample: 10 mL

Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath to a volume of about 1 mL, and allow it to evaporate spontaneously to dryness while protected from dust.

Acceptance criteria: The residue, if any, is colorless, and when dried at 105° for 1 h, weighs NMT 2 mg (0.02%).

• **HEAVY METALS, Method I <231>**

Sample solution: Evaporate 5.0 mL (4 g) on a steam bath to dryness, warm the residue gently with 1 mL of dilute hydrochloric acid (1 in 120), add water to make 25 mL, and filter, if necessary.

Acceptance criteria: NMT 5 ppm

Organic Impurities• **PROCEDURE**

Sample solution: 1 in 20

Analysis: To 10 mL of the *Sample solution* add 0.10 mL of 0.10 N potassium permanganate.

Acceptance criteria: The pink color does not completely disappear within 10 min.

SPECIFIC TESTS

- **SPECIFIC GRAVITY <841>:** 0.803–0.807

- **DISTILLING RANGE, Method I <721>:** It distills completely at between 97° and 103°, a correction factor of 0.037°/mm being applied as necessary.

- **WATER:** The relative retention time of water on the gas chromatographic column used in the *Assay* is approximately 0.2 times that of Amylene Hydrate. From the area under the curve obtained in the *Assay*, calculate the percentage (a/a) of water in the Amylene Hydrate taken: NMT 0.5% is found.

• **ALDEHYDE**

Sample solution: 1 in 20

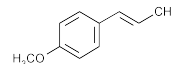
Analysis: To 10 mL of the *Sample solution* add 1 mL of silver-ammonium nitrate TS, and heat the mixture on a water bath at 60° for 10 min.

Acceptance criteria: No darkening occurs.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Anethole



C₁₀H₁₂O
Benzene, 1-methoxy-4-(1-propenyl)-, (*E*)-;
(*E*)-*p*-Propenylanisole [4180-23-8].
Synthetic [104-46-1].

148.20

DEFINITION

Anethole is obtained from Anise Oil and other sources, or it is prepared synthetically.

IMPURITIES

- **HEAVY METALS**, *Method II* (231): NMT 20 ppm

- **LIMIT OF PHENOLS**

Sample: 1 mL

Analysis: Shake the *Sample* with 20 mL of water, and allow the liquids to separate. Pass the water layer through a filter paper previously moistened with water, and to 10 mL of the filtrate add 3 drops of ferric chloride TS.

Acceptance criteria: No purple or purplish color is produced.

- **ALDEHYDES AND KETONES**

Sample: 10 mL

Analysis: Shake the *Sample* with 50 mL of a saturated solution of sodium bisulfite in a graduated cylinder, and allow the mixture to stand for 6 h.

Acceptance criteria: No appreciable diminution in the volume of Anethole occurs, and no crystalline deposit separates.

SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): 0.983–0.988
- **CONGEALING TEMPERATURE** (651): NLT 20°
- **DISTILLING RANGE**, *Method I* (721): 231°–237°, a correction factor of 0.063°/mm being applied as necessary
- **OPTICAL ROTATION**, *Angular Rotation* (781A): –0.15° to +0.15°
- **REFRACTIVE INDEX** (831): 1.557–1.561

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** Label to indicate whether it is of natural sources or is prepared synthetically.

Anise Oil

DEFINITION

Anise Oil is the volatile oil distilled with steam from the dried, ripe fruit of *Pimpinella ansium* L. (Fam. Apiaceae) or from the dried ripe fruit of *Illicium verum* Hook. f. (Fam. Illiciaceae).

[NOTE—If solid material has separated, carefully warm the Anise Oil until it is completely liquefied, and mix before using.]

IMPURITIES

- **HEAVY METALS**, *Method II* (231): NMT 40 ppm

- **LIMIT OF PHENOLS**

Sample solution: Recently distilled Anise Oil in 90% alcohol (1 in 3)

Acceptance criteria: The *Sample solution* is neutral to moistened litmus paper, and develops no blue or brownish color upon the addition of 1 drop of ferric chloride TS to 5 mL of the solution.

SPECIFIC TESTS

- **SOLUBILITY IN 90% ALCOHOL:** One volume dissolves in 3 volumes of 90% alcohol.
- **SPECIFIC GRAVITY** (841): 0.978–0.988
- **CONGEALING TEMPERATURE** (651): NLT 15°
- **OPTICAL ROTATION**, *Angular Rotation* (781A): –2° to +1°
- **REFRACTIVE INDEX** (831): 1.553–1.560 at 20°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers, and avoid exposure to excessive heat.
- **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source

from which the article was derived. The label also states that if solid material has separated, carefully warm the oil until it is completely liquefied, and mix before using.

Arginine—see Arginine General Monographs

Aromatic Elixir

DEFINITION

Prepare Aromatic Elixir as follows.

Suitable essential oil(s)	
Syrup	375 mL
Talc	30 g
Alcohol, Purified Water, each a sufficient quantity to make	1000 mL

Dissolve the oil(s) in *Alcohol* to make 250 mL. To this solution add the *Syrup* in several portions, agitating vigorously after each addition. Then add the required quantity of *Purified Water* in the same manner. Mix *Talc* with the liquid, and pass through a filter, dampened with *Diluted Alcohol*, and return the filtrate until a clear liquid is obtained.

OTHER COMPONENTS

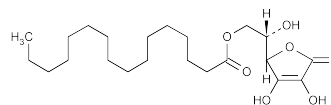
- **ALCOHOL DETERMINATION**, *Method II* (611): 21.0%–23.0% of C₂H₅OH, with acetone being used as the internal standard

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight containers.

Ascorbic Acid—see Ascorbic Acid General Monographs

Ascorbyl Palmitate



C₂₂H₃₈O₇

414.53

L-Ascorbic acid, 6-hexadecanoate;

L-Ascorbic acid 6-palmitate [137-66-6].

DEFINITION

Ascorbyl Palmitate contains NLT 95.0% and NMT 100.5% of C₂₂H₃₈O₇, calculated on the dried basis.

IDENTIFICATION

- **A. PROCEDURE**

Sample solution: 40 mg/mL in alcohol

Analysis: To 25 mL of *Sample solution* add 1 mL of a 1-in-1000 solution of 2,6-dichlorophenol-indophenol sodium in alcohol.

Acceptance criteria: The blue color of the 2,6-dichlorophenol-indophenol sodium solution is discharged immediately.

• **B. INFRARED ABSORPTION** (197K)

ASSAY

• **PROCEDURE**

Sample solution: Dissolve 300 mg of Ascorbyl Palmitate in 50 mL of alcohol, and add 30 mL of water.

Analysis: Immediately titrate the *Sample solution* with 0.1 N iodine VS to a yellow color that persists for NLT 30 s. Each mL of 0.1 N iodine is equivalent to 20.73 mg of C₂₂H₃₈O₇.

Acceptance criteria: 95.0%–100.5% on the dried basis

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 0.1%
- **HEAVY METALS**, *Method II* (231): NMT 10 ppm

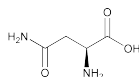
SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** (741): 107°–117°
- **OPTICAL ROTATION**, *Specific Rotation* (781S): +21° to +24°
Sample solution: 100 mg/mL, in methanol
- **LOSS ON DRYING** (731): Dry a sample in vacuum at 60° for 1 h: it loses NMT 2.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, in a cool, dry place.
- **USP REFERENCE STANDARDS** (11)
USP Ascorbyl Palmitate RS

Asparagine



C₄H₈N₂O₃ · H₂O 150.13

C₄H₈N₂O₃ 132.12

L-Asparagine;

L-α-Aminosuccinamic acid, monohydrate [5794-13-8].
Anhydrous [70-47-3].

DEFINITION

Asparagine is anhydrous, or contains one molecule of water of hydration. It contains NLT 98.0% and NMT 101.5% of C₄H₈N₂O₃, as L-asparagine, calculated on the dried basis.

IDENTIFICATION

• **A. INFRARED ABSORPTION** (197K)

[NOTE—Use USP Asparagine Anhydrous RS and USP Asparagine Monohydrate RS for the evaluation of the anhydrous and monohydrate forms of Asparagine, respectively.]

ASSAY

• **PROCEDURE**

Sample: 130 mg

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Blank: 3 mL of formic acid in 50 mL of glacial acetic acid

Endpoint detection: Potentiometrically

Analysis: Dissolve the *Sample* with 3 mL of formic acid in 50 mL of glacial acetic acid. Perform a blank determination.

Calculate the percentage of asparagine (C₄H₈N₂O₃) in the *Sample* taken:

$$[(V - B) \times N \times F \times 100] / W$$

V = volume of *Titrant* consumed by the *Sample* (mL)

B = volume of *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor for asparagine, 132.1 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 98.0%–101.5% on the dried basis

IMPURITIES

• **RESIDUE ON IGNITION** (281)

Sample: 1.0 g

Acceptance criteria: NMT 0.1%

• **LEAD** (251)

Sample: 1 g

Control: 5 mL of *Diluted Standard Lead Solution* (5 µg of Pb)

Acceptance criteria: NMT 5 ppm

• **CHROMATOGRAPHIC PURITY**

Standard solution: 0.05 mg/mL of USP Asparagine

Anhydrous RS or USP Asparagine Monohydrate RS

[NOTE—Use USP Asparagine Anhydrous RS and USP Asparagine Monohydrate RS for the evaluation of the anhydrous and monohydrate forms of Asparagine, respectively.]

Sample solution: 10 mg/mL

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 5 µL

Developing solvent system: Butyl alcohol, glacial acetic acid, and water (3:1:1)

Spray reagent: 2 mg/mL of ninhydrin in butyl alcohol and glacial acetic acid (19:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Proceed as directed in the general test chapter, and then dry the plate at 80° for 30 min. Spray the plate with the *Spray reagent*, heat at 80° for 10 min, and examine under white light.

Acceptance criteria: No secondary spot from the *Sample solution* is larger or more intense than the principal spot from the *Standard solution* (0.5%), and NMT 1.0% of total impurities is found.

SPECIFIC TESTS

• **OPTICAL ROTATION**, *Specific Rotation* (781S)

Sample solution: 10 mg/mL, in 6 N hydrochloric acid

Acceptance criteria: +33.0° to +36.5°, measured at 20°

• **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62):

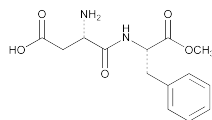
The total aerobic microbial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g.

- **LOSS ON DRYING** (731): Dry a sample at 130° for 3 h: the anhydrous form loses NMT 1.0% of its weight, and the monohydrate loses between 11.5% and 12.5% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers. Store at room temperature.
- **LABELING:** Label it to indicate whether it is anhydrous or the monohydrate.
- **USP REFERENCE STANDARDS** (11)
USP Asparagine Anhydrous RS
USP Asparagine Monohydrate RS

Aspartame



$C_{14}H_{18}N_2O_5$ 294.30
L-Phenylalanine, *N*-L-α-aspartyl-, 1-methyl ester;
3-Amino-*N*-(α-carboxyphenethyl)succinamic acid *N*-methyl
ester [22839-47-0].

DEFINITION

Aspartame contains NLT 98.0% and NMT 102.0% of $C_{14}H_{18}N_2O_5$, calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197M)
[NOTE—Do not dry specimens.]

ASSAY

• PROCEDURE

Sample: 300 mg

Titrimetric system

(See *Titrimetry* (541).)

0.1 N Perchloric acid: Use perchloric acid, tenth-normal (0.1 N) in glacial acetic acid as specified for *Reagents, Indicators, and Solutions—Volumetric Solutions*, but in the standardization, titrate to a green endpoint.

Mode: Direct titration

Titrant: Use 0.1 N Perchloric acid

Blank: 1.5 mL of anhydrous formic acid and 60 mL of glacial acetic acid

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 1.5 mL of anhydrous formic acid, and add 60 mL of glacial acetic acid. Add crystal violet, and immediately titrate with the *Titrant* to a green endpoint. Perform a blank determination.

[NOTE—A blank titration exceeding 0.1 mL may be due to excessive water content, and may cause loss of visual endpoint sensitivity.]

Calculate the percentage of Aspartame ($C_{14}H_{18}N_2O_5$) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100] / W$$

V = volume of the *Titrant* consumed by the *Sample* (mL)

B = volume of the *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 294.3 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.2%
- **HEAVY METALS**, *Method II* (231): NMT 10 ppm
- **LIMIT OF 5-BENZYL-3,6-DIOXO-2-PIPERAZINEACETIC ACID**

Diluent: Methanol and water (1:9)

Mobile phase: Dissolve 5.6 g of monobasic potassium phosphate in 820 mL of water in a 1-L volumetric flask, adjust with phosphoric acid to a pH of 4.3, and dilute with methanol to volume.

Standard solution: 75 µg/mL of USP Aspartame Related Compound A RS in *Diluent*

Sample solution: 5 mg/mL of Aspartame in *Diluent*
[NOTE—Avoid heat and excessive holding times.]

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.6-mm × 25-cm; packing L1

Column temperature: 40°

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0

Relative standard deviation: NMT 4.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of aspartame related compound A in the portion of Aspartame taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of aspartame related

compound A from the *Sample solution*

r_S = peak response of aspartame related

compound A from the *Standard solution*

C_S = concentration of 5-benzyl-3,6-dioxo-2-piperazineacetic acid in the *Standard solution* (mg/mL)

C_U = concentration of Aspartame in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 1.5%

• CHROMATOGRAPHIC PURITY

Diluent, Mobile phase, and Chromatographic system:

Proceed as directed in *Limit of 5-Benzyl-3,6-dioxo-2-piperazineacetic Acid*.

Sample stock solution: Proceed as directed for *Sample solution* under *Limit of 5-Benzyl-3,6-dioxo-2-piperazineacetic Acid*.

Sample solution: 0.1 mg/mL of Aspartame from the *Sample stock solution* in *Diluent*

Analysis

Samples: *Sample stock solution* and *Sample solution*

[NOTE—Continue the elution of *Sample stock solution* for twice the retention time of the aspartame peak.]

Acceptance criteria: The sum of the responses of all the peaks in the chromatogram of the *Sample stock solution*, excluding the 5-benzyl-3,6-dioxo-2-piperazineacetic acid and aspartame peak responses, is not greater than the aspartame peak response of the *Sample solution*, corresponding to NMT 2.0% of chromatographic impurities.

SPECIFIC TESTS

• TRANSMITTANCE

Sample solution: 10 mg/mL of Aspartame in 2 N hydrochloric acid, prepared by means of sonication

Analysis: Determine the transmittance in a 1-cm cell at 430 nm with a suitable spectrophotometer.

Acceptance criteria: Transmittance of NLT 0.95, corresponding to an absorbance of NMT about 0.022

• OPTICAL ROTATION, *Specific Rotation* (781S)

Sample solution: 40 mg/mL in 15 N formic acid

Acceptance criteria: +14.5° to +16.5°, determined at 20° within 30 min after preparation of the *Sample solution*

• LOSS ON DRYING (731):

Dry a sample at 105° for 4 h: it loses NMT 4.5% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• USP REFERENCE STANDARDS (11)

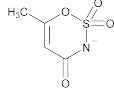
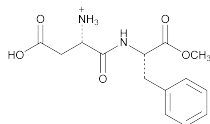
USP Aspartame RS

USP Aspartame Related Compound A RS

5-Benzyl-3,6-dioxo-2-piperazineacetic acid.

$C_{13}H_{14}N_2O_4$ 262.27

Aspartame Acesulfame



$C_{18}H_{23}O_9N_3S$ 457.45
 Aspartame acesulfame salt;
 [2-Carboxy- β -(*N*-b-methoxycarbonyl-2-phenyl)ethyl-carbamoyl]jethanaminium 6-methyl-4-oxo-1,2,3-oxathiazin-3-ide-2,2-dioxide;
 L-Phenylalanine, L- α -aspartyl-2-methyl ester compound with 6-methyl-1,2,3-oxathiazin-4(3*H*)-one 2,2-dioxide (1:1) [106372-55-8].

DEFINITION

Aspartame Acesulfame contains NLT 63.0% and NMT 66.0% of aspartame, calculated on the dried basis. It contains NLT 34.0% and NMT 37.0% of acesulfame, calculated as the acid form on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
 [NOTE—Do not dry specimens.]

ASSAY

• PROCEDURE

Sample: 0.150 g

Analysis: Dissolve the *Sample* in 50 mL of dehydrated alcohol. Titrate with 0.1 N tetrabutylammonium hydroxide in methanol/isopropyl alcohol VS. Potentiometrically determine the volumes of titrant, in mL, at the first equivalence point (V_1) and at the second equivalence point (V_2). Perform a blank titration with 50 mL of dehydrated alcohol, and designate the volume of titrant, in mL, as V_B .

Calculate the percentage of acesulfame (*Result 1*) and aspartame (*Result 2*) in the portion of Aspartame Acesulfame taken:

$$\text{Result 1} = 163N(V_1 - V_B)/10W$$

$$\text{Result 2} = 294N(V_2 - V_1)/10W$$

N = normality of the titrant

W = weight of Aspartame Acesulfame taken (g)

Acceptance criteria: Aspartame, 63.0%–66.0% on the dried basis; acesulfame, 34.0%–37.0% as the acid form on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 1.1%
- **HEAVY METALS**, *Method II* (231): NMT 10 ppm
- **LIMIT OF POTASSIUM**

Potassium stock solution: 19.0 $\mu\text{g/mL}$ of potassium chloride, previously dried at 105° for 2 h, in water. This solution contains 10 μg of potassium/mL.

Standard solution A: Transfer 10.0 mL of *Potassium stock solution* to a 100-mL volumetric flask. Add 2.0 mL of sodium chloride solution (1 in 5) and 1.0 mL of hydrochloric acid, and dilute with water to volume (1.0 $\mu\text{g/mL}$ of potassium).

Standard solution B: Transfer 15.0 mL of *Potassium stock solution* to a 100-mL volumetric flask. Add 2.0 mL of sodium chloride solution (1 in 5) and 1.0 mL of hydrochloric acid, and dilute with water to volume (1.5 $\mu\text{g/mL}$ of potassium).

Standard solution C: Transfer 20.0 mL of *Potassium stock solution* to a 100-mL volumetric flask. Add 2.0 mL of sodium chloride solution (1 in 5) and 1.0 mL of

hydrochloric acid, and dilute with water to volume (2.0 $\mu\text{g/mL}$ of potassium).

Sample stock solution: 3.0 mg/mL of Aspartame Acesulfame

Sample solution: Transfer 10 mL of *Sample stock solution* to a 100-mL volumetric flask. Add 2.0 mL of sodium chloride solution (1 in 5) and 1.0 mL of hydrochloric acid, and dilute with water to volume. Filter the solution.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 766.5 nm (potassium emission line)

Lamp: Potassium hollow-cathode

Flame: Air–acetylene

Blank: Water

Standard curve

Samples: *Standard solution A*, *Standard solution B*, and *Standard solution C*

Plot: Absorbance values versus their corresponding concentration ($\mu\text{g/mL}$) of potassium

Analysis

Sample: *Sample solution*

From the *Standard curve*, determine the concentration of potassium in the *Sample solution*.

Calculate the percentage of potassium in the portion of Aspartame Acesulfame taken:

$$\text{Result} = (C_S/C_U) \times 100$$

C_S = concentration of potassium in the *Sample solution* from the *Standard curve* ($\mu\text{g/mL}$)

C_U = concentration of aspartame acesulfame in the *Sample solution* (g/mL)

Acceptance criteria: NMT 0.5%

• RELATED COMPOUNDS

Diluent: Methanol and water (1:9)

Mobile phase: Prepare a solution by dissolving 5.6 g of monobasic potassium phosphate in 820 mL of water in a 1-L volumetric flask, adjusting with phosphoric acid to a pH of 4.3, diluting with methanol to volume, and mixing.

Standard solution: 25 $\mu\text{g/mL}$ of USP Aspartame Related Compound A RS in *Diluent*

Sample solution: 5 mg/mL in *Diluent*

[NOTE—Avoid heat and excessive holding times.]

Instrumental conditions

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.6-mm \times 25-cm; packing L1

Column temperature: 40°

Flow rate: 1.5 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0

Relative standard deviation: NMT 4.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of aspartame related compound A in the portion of Aspartame Acesulfame taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of aspartame related compound A from the *Sample solution*

r_S = peak response of aspartame related compound A from the *Standard solution*

C_S = concentration of USP Aspartame Related Compound A RS in the *Standard solution* (mg/mL)

C_U = concentration of Aspartame Acesulfame in the Sample solution (mg/mL)

Acceptance criteria: NMT 0.5%

• **CHROMATOGRAPHIC PURITY**

Diluent, Mobile phase, and Instrumental conditions: Proceed as directed in *Related Compounds*.

Acesulfame solution: 2 mg/mL of USP Acesulfame Potassium RS in Diluent

Sample stock solution: Proceed as directed for Sample solution under *Related Compounds*.

Sample solution: 75 µg/mL of Aspartame Acesulfame from Sample stock solution in Diluent

Analysis

Samples: Acesulfame solution, Sample stock solution, and Sample solution

[NOTE—Continue the elution of the Sample stock solution for twice the retention time of the aspartame peak.]

Acceptance criteria: The sum of the responses of all the peaks of the Sample stock solution, excluding the aspartame related compound A, acesulfame, and aspartame peak responses, is not greater than the aspartame peak response from the Sample solution (1.0%).

SPECIFIC TESTS

• **OPTICAL ROTATION, Specific Rotation (781S)**

15 N formic acid: To a 1-L volumetric flask add approximately 300 mL of water, then weigh 719 g of 96% formic acid into this flask, and mix. Cool the solution to 20°. Dilute with water to volume.

Sample solution: 62 mg/mL in 15 N formic acid

Acceptance criteria: +14.5° to +16.5°, determined at 20° within 30 min after preparation of the Sample solution. [NOTE—Divide the calculated specific rotation by 0.646 to correct for the aspartame content in Aspartame Acesulfame.]

• **LOSS ON DRYING (731):** Dry a sample at 105° for 4 h: it loses NMT 0.5% of its weight.

• **TRANSMITTANCE:** The transmittance of a 1-in-100 solution of Aspartame Acesulfame in distilled water, determined in a 1-cm cell at 430 nm with a suitable spectrophotometer, is NLT 0.95.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store in a cool, dry place.

• **USP REFERENCE STANDARDS (11)**

USP Acesulfame Potassium RS
USP Aspartame Acesulfame RS
USP Aspartame Related Compound A RS
5-Benzyl-3,6-dioxo-2-piperazineacetic acid.
 $C_{13}H_{14}N_2O_4$ 262.27

Barium Hydroxide Lime—see *Barium Hydroxide Lime General Monographs*

Behenoyl Polyoxylglycerides

DEFINITION

Behenoyl Polyoxylglycerides is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. The polyethylene glycols used have a mean molecular weight of 400. It is produced by esterification of glycerol and polyethylene glycols with behenic acid.

IDENTIFICATION

• **A. INFRARED ABSORPTION (197K)**

• **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)**

Standard solution: 50 mg/mL of USP Behenoyl Polyoxylglycerides RS in methylene chloride

Sample solution: 50 mg/mL of Behenoyl Polyoxylglycerides in methylene chloride

Chromatographic system

Application volume: 10 µL

Developing solvent system: Ether and hexanes (70:30)

Spray reagent: 0.1-mg/mL solution of rhodamine B in alcohol

Analysis: Proceed as directed in the chapter. Then spray the plate with Spray reagent, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The R_f values of the principal spots of the Sample solution correspond to those of the Standard solution.

• **C.** It meets the requirements in *Specific Tests for Fats and Fixed Oils, Fatty Acid Composition* (401).

IMPURITIES

• **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561)**

Sample solution: 2.0 g

Acceptance criteria: NMT 0.1%

• **HEAVY METALS, Method II (231):** NMT 10 µg/g

• **ALKALINE IMPURITIES**

Sample: 5.0 g

Analysis: Heat the Sample slightly until it melts. Add 10 mL of alcohol and 0.05 mL of bromophenol blue TS, and mix well. While the solution is still warm, titrate with 0.01 N hydrochloric acid VS to change the color to yellow.

Acceptance criteria: NMT 1.0 mL of 0.01 N hydrochloric acid

• **LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE**

[CAUTION—Perform all determinations three times. Ethylene oxide is toxic and flammable. Prepare all solutions in a well-ventilated hood. The operator must protect hands and face by wearing polyethylene protective gloves and an appropriate face mask. Store all solutions in hermetic containers, and refrigerate at a temperature between 4° and 8°.]

Ethylene oxide stock solution: Into a dry, clean test tube that has been cooled in a mixture of 1 part of sodium chloride and 3 parts of crushed ice, introduce a slow current of ethylene oxide gas, allowing condensation onto the inner wall of the test tube. Using a glass syringe, previously cooled to −10°, transfer 300 µL of liquid ethylene oxide, equivalent to about 0.25 g, to 50 mL of polyethylene glycol 200. Determine the absorbed quantity of ethylene oxide by weighing before and after absorption. Dilute with polyethylene glycol 200 to 100.0 mL, and mix. This is the *Ethylene oxide stock solution*.

Transfer 10.0 mL of magnesium chloride solution, prepared by adding 5 g of magnesium chloride to 10 mL of alcohol, to a volumetric flask. Add 20.0 mL of 0.1 M alcoholic hydrochloric acid VS. Insert the stopper, shake to obtain a saturated solution, and allow to equilibrate overnight. Transfer 5.00 mL of *Ethylene oxide stock solution* to the flask, and allow to stand for 30 min. Titrate with 0.1 M alcoholic potassium hydroxide VS, determining the endpoint potentiometrically. Perform a blank titration, using the same quantity of polyethylene glycol 200 instead of *Ethylene oxide stock solution*, and note the difference in volumes required. Each mL of the difference in volumes of 0.1 M alcoholic potassium hydroxide VS consumed is equivalent to 4.404 mg of ethylene oxide. Calculate the concentration of ethylene oxide in the *Ethylene oxide stock solution*.

Ethylene oxide solution: Prepare immediately before use. Quantitatively dilute a volume of *Ethylene oxide stock solution*, measured, with polyethylene glycol 200 to obtain a solution containing about 50 µg/g of ethylene oxide. Dilute 1.0 mL of this solution with water to 5.0 mL to obtain a solution having a known concentration of about 10 µg/mL of ethylene oxide.

Dioxane solution: 500 µg/mL of dioxane

Standard solution 1: Transfer 1.0 g of Behenoyl Polyoxylglycerides to a 10-mL vial. Add 1.0 mL of *N,N*-dimethylacetamide, 0.1 mL of *Ethylene oxide solution*, and 0.1 mL of *Dioxane solution*. Close the vial, and mix to obtain a homogeneous solution. Allow to stand at 90° for 45 min.

Standard solution 2: Transfer 0.1 mL of *Ethylene oxide solution* to a 10-mL vial. Add 0.1 mL of a freshly prepared solution of about 10 mg/L of acetaldehyde, and add 0.1 mL of *Dioxane solution*. Close the vial, and mix to obtain a homogeneous solution.

Sample solution: Transfer 1.0 g of Behenoyl Polyoxylglycerides to a 10-mL vial, and add 1.0 mL of *N,N*-dimethylacetamide and 0.2 mL of water. Close the vial, and mix to obtain a homogeneous solution. Allow to stand at 90° for 45 min.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

[NOTE—Headspace apparatus that automatically transfers a measured amount of headspace may be used.]

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m glass or quartz capillary; 1.0-µm layer of phase G1

Temperatures

Injection port: 150°

Detector: 250°

Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	—
180	30	230	—
230	—	230	5

Carrier gas: Helium

Flow rate: 1 mL/min

Injection volume: 1 mL (the gaseous headspace), using a heated, gas-tight syringe

System suitability

Sample: *Standard solution 2*

Adjust the sensitivity of the system so that the peak heights of the two principal peaks are NLT 15% of the full scale of the recorder.

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are 0.94 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between acetaldehyde and ethylene oxide

Relative standard deviation: NMT 15.0%

Analysis

Samples: *Standard solution 1* and *Sample solution*

Calculate the content of ethylene oxide in the portion of Behenoyl Polyoxylglycerides taken:

$$\text{Result} = C_E \times r_U / [(r_S \times W_U) - (r_U \times W_S)]$$

C_E = concentration of ethylene oxide added in *Standard solution 1* (µg/mL)

r_U = peak response of ethylene oxide from the *Sample solution*

r_S = peak response of ethylene oxide from *Standard solution 1*

W_U = weight of Behenoyl Polyoxylglycerides taken to prepare the *Sample solution* (g)

W_S = weight of Behenoyl Polyoxylglycerides taken to prepare *Standard solution 1* (g)

Calculate the content of dioxane in the portion of Behenoyl Polyoxylglycerides taken:

$$\text{Result} = C_D \times r_U / [5 \times (r_S \times W_U) - (r_U \times W_S)]$$

C_D = concentration of dioxane added to *Standard solution 1* (µg/mL)

r_U = peak response of dioxane from the *Sample solution*

r_S = peak response of dioxane from *Standard solution 1*

W_U = weight of Behenoyl Polyoxylglycerides taken to prepare the *Sample solution* (g)

W_S = weight of Behenoyl Polyoxylglycerides taken to prepare *Standard solution 1* (g)

Acceptance criteria

Ethylene oxide: NMT 1 µg/g

Dioxane: NMT 10 µg/g

• LIMIT OF FREE GLYCEROL

Sample: 1.2 g

Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, and dilute with glacial acetic acid to 100.0 mL.

Potassium iodide solution: 75 mg/mL of potassium iodide

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 M sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 25 mL of methylene chloride, heating if necessary. Cool, and add 100 mL of water and 25.0 mL of *Periodic acetic acid solution*. Shake, and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with *Titrant*. Perform a blank determination, and make any necessary correction. Each mL of *Titrant* is equivalent to 2.3 mg of glycerin.

Acceptance criteria: NMT 3.0%

SPECIFIC TESTS

• FATS AND FIXED OILS, Acid Value (401)

Sample: 2.0 g

Acceptance criteria: NMT 4.0

• FATS AND FIXED OILS, Fatty Acid Composition (401):

Behenoyl Polyoxylglycerides exhibits the composition profile of fatty acids in Table 2.

Table 2

Carbon-Chain Length	No. of Double Bonds	Percentage (%)
12	0	≤1.0
14	0	≤1.0
16	0	≤3.0
18	0	≤5.0
20	0	≤10.0
22	0	≥83.0
22	1	≤3.0
24	0	≤3.0

• FATS AND FIXED OILS, Hydroxyl Value (401)

Sample: 1.0 g

Analysis: Proceed as directed in the chapter. If the volume of 0.5 N sodium hydroxide VS required for the

titration is less than 2 mL, a more dilute titrant may be used.

Acceptance criteria: 30–50

• **FATS AND FIXED OILS, Peroxide Value (401)**

Sample: 2.0 g

Acceptance criteria: NMT 6.0

• **FATS AND FIXED OILS, Saponification Value (401)**

Sample: 2.0 g

Acceptance criteria: 120–140

• **WATER DETERMINATION, Method I (921)**

Sample: 1.0 g

Analysis: Use anhydrous pyridine as the solvent.

Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, protected from heat and moisture. Store at NMT 35°.
- **LABELING:** Label it to indicate the average nominal molecular weight of polyethylene glycols used as part of the official title.
- **USP REFERENCE STANDARDS (11)**
USP Behenoyl Polyoxylglycerides RS

Bentonite

Bentonite [1302-78-9].

DEFINITION

Bentonite is a native, colloidal, hydrated aluminum silicate.

IDENTIFICATION

• **A. X-RAY DIFFRACTION (941)**

Sample A: Add 2 g in small portions to 100 mL of water, with intense agitation. Allow to stand for 12 h to ensure complete hydration. Place 2 mL of the mixture so obtained on a suitable glass slide, and allow to air-dry at room temperature to produce an oriented film. Place the slide in a vacuum desiccator over a free surface of ethylene glycol. Evacuate the desiccator, and close the stopcock so that the ethylene glycol saturates the desiccator chamber. Allow to stand for 12 h.

Sample B: Prepare a random powder specimen of Bentonite.

Analysis

Samples: *Sample A* and *Sample B*

Record the X-Ray diffraction pattern of the samples, and determine the *d* values

Acceptance criteria: The largest peak in the pattern of *Sample A* corresponds to a *d* value between 15.0 and 17.2 Å. The major peak in the region between 1.48 and 1.54 Å from the pattern of *Sample B* is 1.492 and 1.504 Å.

IMPURITIES

• **ARSENIC, Method I (211)**

Test preparation: Transfer 8.0 g to a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1 in 25), mix, and cover with a watch glass. Boil gently, with occasional stirring, for 15 min without allowing excessive foaming. Pass the hot supernatant through a rapid-flow filter paper into a 200-mL volumetric flask, and wash with four 25-mL portions of hot dilute hydrochloric acid (1 in 25), collecting the washings in the volumetric flask. Cool the combined filtrates to room temperature, and add dilute hydrochloric acid (1 in 25) to volume. Transfer 25 mL of this solution to a generator flask, and dilute with water to 35 mL.

Standard preparation: Transfer 5 mL of Standard Arsenic Solution (5 µg) to a generator flask, and dilute with water to 35 mL.

Analysis: Proceed as directed in the chapter, determining the absorbances.

Acceptance criteria: The absorbance due to any red color from the *Test preparation* does not exceed that produced by the *Standard preparation* (NMT 5 ppm).

• **LEAD**

[NOTE—The *Standard solution* and the *Sample solution* may be modified, if necessary, to obtain solutions of suitable concentrations, adaptable to the linear or working range of the instrument. It may be necessary to correct the value obtained for the *Sample solution* for interference from the sample specimen matrix.]

Standard solution: On the day of use, dilute 3.0 mL of *Lead Nitrate Stock Solution* (see *Heavy Metals* (231)) with water to 100 mL. Each mL of the *Standard solution* contains the equivalent of 3 µg of lead.

Sample solution: Transfer 3.75 g to a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1 in 25), stir, and cover with a watch glass. Boil for 15 min, then cool to room temperature, and pass through a rapid-flow filter paper into a 400-mL beaker. Wash the filter with four 25-mL portions of hot water, collecting the washings in the 400-mL beaker. Concentrate the combined extracts by gentle boiling to approximately 20 mL. If a precipitate appears, add 2–3 drops of nitric acid, heat to boiling, and cool to room temperature. Pass the concentrated extracts through a rapid-flow filter paper into a 50-mL volumetric flask. Transfer the remaining contents of the 400-mL beaker through the filter paper and into the flask with water. Dilute with water to volume.

Instrumental conditions

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 283.3 nm

Lamp: Lead hollow cathode with deuterium arc background correction

Flame: Air and acetylene

Analysis: Determine the absorbances of the *Standard solution* and the *Sample solution*.

Acceptance criteria: The absorbance of the *Sample solution* is NMT that of the *Standard solution* (40 ppm).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** Meets the requirements of the test for absence of *Escherichia coli*
- **pH (791):** 9.5–10.5. Disperse 4.0 g in 200 mL of water, mixing vigorously to facilitate wetting.
- **LOSS ON DRYING (731):** Dry a sample at 105° for 2 h: it loses 5.0%–8.0% of its weight.
- **GEL FORMATION:** Mix 6 g with 300 mg of magnesium oxide. Add the mixture, in several divided portions, to 200 mL of water contained in a blender of approximately 500-mL capacity. Blend thoroughly for 5 min at high speed, transfer 100 mL of the mixture to a 100-mL graduated cylinder, and allow to remain undisturbed for 24 h: NMT 2 mL of supernatant appears on the surface.
- **SWELLING POWER:** To 100 mL of water contained in a glass-stoppered cylinder of 100-mL capacity add 2 g in portions, dropping it upon the surface of the water, and allow each portion to settle before adding the next. The mass at the bottom gradually swells until it occupies an apparent volume of NLT 24 mL at the end of a 2-h period.
- **FINESS OF POWDER:** Sprinkle 2 g on 20 mL of water contained in a mortar. Allow to swell, disperse evenly with a pestle, and dilute with water to 100 mL. Pour the suspension through a No. 200 standard sieve, and wash the sieve thoroughly with water. No grit is felt when the fingers are rubbed over the wire mesh of the sieve.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate that absorption of atmospheric moisture should be avoided following the opening of the original package, preferably by storage of the remainder of the contents in a tight container.

Purified Bentonite

DEFINITION

Purified Bentonite is a colloidal montmorillonite that has been processed to remove grit and nonswellable ore components.

IDENTIFICATION

• A. X-RAY DIFFRACTION (941)

Sample A: Add 2 g in small portions to 100 mL of water, with intense agitation. Allow to stand for 12 h to ensure complete hydration. Place 2 mL of the mixture so obtained on a suitable glass slide, and allow to air-dry at room temperature to produce an oriented film. Place the slide in a vacuum desiccator over a free surface of ethylene glycol. Evacuate the desiccator, and close the stopcock so that the ethylene glycol saturates the desiccator chamber. Allow to stand for 12 h.

Sample B: Prepare a random powder specimen of Purified Bentonite.

Analysis

Samples: *Sample A* and *Sample B*

Record the X-ray diffraction pattern of the samples, and determine the *d* values.

Acceptance criteria: The largest peak in the pattern of *Sample A* corresponds to a *d* value between 15.0 and 17.2 Å. The major peak in the region between 1.48 and 1.54 Å from the pattern of *Sample B* is 1.492 and 1.504 Å.

ASSAY

• ALUMINUM CONTENT AND MAGNESIUM CONTENT

[NOTE—The *Standard solutions* and the *Sample solution* may be diluted quantitatively with water, if necessary, to obtain solutions of suitable concentrations adaptable to the linear or working range of the instrument.]

Aluminum content

Aluminum standard stock solution: Dissolve 1.000 g of aluminum in a mixture of 10 mL of hydrochloric acid and 10 mL of water by gentle heating. Transfer the solution to a 1000-mL volumetric flask, and dilute with water to volume. This solution contains the equivalent of 1 mg/mL of aluminum.

Aluminum standard solutions: Transfer 2-, 5-, and 10-mL aliquots of the *Aluminum standard stock solution* to separate 100-mL volumetric flasks containing 200 mg of sodium chloride, and dilute each with water to volume.

Sample stock solution: Transfer 0.200 g of Purified Bentonite to a 25-mL platinum crucible containing 1.0 g of lithium metaborate, and mix. Using a muffle furnace or a suitable burner, heat slowly at first, and ignite at 1000°–1200° for 15 min. Cool, place the crucible in a 100-mL beaker containing 25 mL of dilute nitric acid (50 mg/mL), and add an additional 50 mL of the dilute acid, filling and submerging the upright crucible. Place a polyfluorocarbon-coated magnetic stirring bar into the crucible, and stir gently with a magnetic stirrer to dissolve. Pour the contents into a 250-mL beaker, and remove the crucible. Warm the solution, transfer through a rapid-flow filter paper with the aid of water into a 200-mL volumetric flask, and dilute with water to volume.

Sample solution: Pipet 20 mL of the *Sample stock solution* into a 100-mL volumetric flask. Add 20 mL of sodium chloride solution (10 mg/mL), and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a single-slot burner

Analytical wavelength: 309 nm

Lamp: Aluminum hollow-cathode

Flame: Oxidizing acetylene–air–nitrous oxide

Analysis

Samples: *Aluminum standard solutions* and *Sample solution*

Determine the aluminum content of the purified bentonite in the *Sample solution* from the linear regression equation calculated from the absorbances and concentrations of the *Aluminum standard solutions*.

Magnesium content

Lanthanum solution: Stir 88.30 g of lanthanum chloride (LaCl₃) with 500 mL of 6 N hydrochloric acid to dissolve, transfer with the aid of water to a 1000-mL volumetric flask, and dilute with water to volume.

Magnesium standard stock solution: Place 1.000 g of magnesium in a 250-mL beaker containing 20 mL of water, and carefully add 20 mL of hydrochloric acid, warming, if necessary, to complete the reaction. Transfer the solution to a 1000-mL volumetric flask, and dilute with water to volume. This solution contains the equivalent of 1 mg/mL of magnesium. Transfer 10.0 mL of this solution to a 1000-mL volumetric flask, and dilute with water to volume.

Magnesium standard solutions: Transfer 5-, 10-, 15-, and 20-mL aliquots of the *Magnesium standard stock solution* to separate 100-mL volumetric flasks. To each flask add 20.0 mL of *Lanthanum solution*, and dilute with water to volume.

Sample stock solution: Use the *Sample stock solution* prepared as directed for *Aluminum content*.

Sample solution: Transfer 25 mL of the *Sample stock solution* to a 50-mL volumetric flask, and dilute with water to volume. Transfer 5.0 mL of this solution to a 100-mL volumetric flask, add 20.0 mL of *Lanthanum solution*, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a single-slot burner

Analytical wavelength: 285 nm

Lamp: Magnesium hollow-cathode

Flame: Reducing flame of acetylene–air

Analysis

Samples: *Magnesium standard solutions* and *Sample solution*

Determine the magnesium content of the purified bentonite in the *Sample solution* from the linear regression equation calculated from the absorbances and concentrations of the *Magnesium standard solutions*.

Acceptance criteria: The ratio of the aluminum content to the magnesium content is 3.5–5.5.

IMPURITIES

• ARSENIC, Method I (211)

Standard solution: 5 mL of *Standard Arsenic Solution* (5 µg of As)

Sample solution: Transfer 13.3 g to a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1 in 25), mix, cover with a watch glass, and boil gently, with occasional stirring, for 15 min without allowing excessive foaming. Allow the insoluble material to settle, and decant the hot supernatant through a rapid-flow filter paper into a 200-mL volumetric flask, retaining as much sediment as possible in the beaker. Add 25 mL of hot dilute hydrochloric acid (1 in 25) to the residue in the beaker, stir, heat to boiling, allow the insoluble material to settle, and decant the supernatant through the filter into the 200-mL volumetric flask. Repeat the extraction with four additional 25-mL portions of hot dilute hydrochloric acid (1 in 25), decanting each hot supernatant through the filter into the volumetric flask. At the last extraction, transfer as much of the insoluble material as possible onto the filter. Cool the combined filtrates to

room temperature, and add dilute hydrochloric acid (1 in 25) to volume.

Analysis: Using a 25-mL aliquot of *Sample solution* and the *Standard solution*, treat them as directed in the *Procedure*.

Acceptance criteria: NMT 3 µg/g; the absorbance due to any red color from the *Sample solution* does not exceed that produced by the *Standard solution*.

• LEAD

[NOTE—The *Standard solution* and the *Sample solution* may be modified, if necessary, to obtain solutions of suitable concentrations, adaptable to the linear or working range of the instrument.]

Standard solution: On the day of use, dilute 3.0 mL of *Lead Nitrate Stock Solution* (see *Heavy Metals* (231)) to 100 mL. Each mL of the *Standard solution* contains the equivalent of 3 µg of lead.

Sample solution: Transfer 10.0 g of Purified Bentonite to a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1 in 25), stir, cover with a watch glass, and boil for 15 min. Cool to room temperature, and allow the insoluble matter to settle. Decant the supernatant through a rapid-flow filter paper into a 400-mL beaker. Add 25 mL of hot water to the insoluble matter in the 250-mL beaker, stir, allow the insoluble matter to settle, and decant the supernatant through the filter into the 400-mL beaker. Repeat the extraction with two additional 25-mL portions of water, decanting each supernatant portion through the filter into the 400-mL beaker. Wash the filter with 25 mL of hot water, collecting this filtrate in the 400-mL beaker. Concentrate the combined extracts by gentle boiling to approximately 20 mL. If a precipitate appears, add 2–3 drops of nitric acid, heat to boiling, and cool to room temperature. Filter the concentrated extracts through a rapid-flow filter paper into a 50-mL volumetric flask. Transfer the remaining contents of the 400-mL beaker through the filter paper and into the flask with water. Dilute to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a deuterium arc background correction and a single-slot burner

Analytical wavelength: 284 nm

Lamp: Lead hollow-cathode

Flame: Air-acetylene

Analysis: Determine the absorbances of the *Standard solution* and the *Sample solution*.

Acceptance criteria: 15 µg/g; the absorbance of the *Sample solution* is NMT that of the *Standard solution*.

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): Its total aerobic microbial count does not exceed 1×10^3 cfu/g, and it meets the requirements of the test for absence of *Escherichia coli*.

• pH (791)

Sample suspension: 50 mg/mL

Acceptance criteria: 9.0–10.0

• LOSS ON DRYING (731)

Analysis: Dry at 110° to constant weight.

Acceptance criteria: NMT 8.0%

• VISCOSITY

Analysis: After determining *Loss on Drying* (731), weigh a quantity of Purified Bentonite equivalent to 25.0 g on the dried basis. Over a period of a few s, transfer the undried sample specimen to a suitable 1-L blender jar containing an amount of water, maintained at a temperature of $25 \pm 2^\circ$, that is sufficient to produce a mixture weighing 500 g. Blend for 3 min, accurately timed, at 14,000–15,000 rpm (high speed). [NOTE—Heat generated during blending causes a temperature rise to above 30°.] Transfer the contents of the blender to a 600-mL beaker, allow to stand for 5 min, and adjust, if

necessary, to a temperature of $33 \pm 3^\circ$. Using a suitable rotational viscometer equipped with a spindle having a cylinder 1.87 cm in diameter and 0.69 cm high attached to a shaft 0.32 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 2.54 cm, and the immersion depth being 5.00 cm (No. 2 spindle), operate the viscometer at 60 rpm for 6 min, accurately timed, and record the scale reading.

Acceptance criteria: 40–200 centipoises

• ACID DEMAND

Sample: After determining *Loss on Drying* (731), weigh a quantity of Purified Bentonite equivalent to 5.00 g.

Analysis: Disperse the *Sample* in 500 mL of water with the aid of a suitable blender fitted with a 1-L jar. Using a stopwatch, designate zero time. With constant mixing, add 3.0-mL portions of 0.100 N hydrochloric acid at 5, 65, 125, 185, 245, 305, 365, 425, 485, 545, 605, 665, and 725 s, and add a 1.0-mL portion at 785 s. Determine the pH potentiometrically at 840 s.

Acceptance criteria: NMT 4.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Bentonite Magma

DEFINITION

Prepare Bentonite Magma as follows.

Bentonite	50 g
Purified Water, a sufficient quantity to make	1000 g

Sprinkle the *Bentonite*, in portions, upon 800 g of hot *Purified Water*, allowing each portion to become thoroughly wetted without stirring. Allow it to stand with occasional stirring for 24 h. Stir until a uniform magma is obtained, add *Purified Water* to make 1000 g, and mix.

The Magma may also be prepared by mechanical means such as by use of a blender, as follows. Place 500 g of *Purified Water* in the blender, and while the machine is running, add the *Bentonite*. Add *Purified Water* to make up to 1000 g or up to the operating capacity of the blender. Blend the mixture for 5–10 min, add *Purified Water* to make 1000 g, and mix.

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): It meets the requirements of the tests for absence of *Escherichia coli*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight containers.

Benzaldehyde

Change to read:



C₇H₆O

▲ Benzoic aldehyde;

Phenyl formaldehyde▲ NF31 [100-52-7].

106.12

DEFINITION**Change to read:**

Benzaldehyde contains NLT 98.0% and Δ NMT 102.0% Δ NF31 of benzaldehyde (C₇H₆O).

IDENTIFICATION**Add the following:****▲ • A. INFRARED ABSORPTION (197F) ▲ NF31****ASSAY****Change to read:****• PROCEDURE**

▲ Solution A: Acetonitrile and glacial acetic acid (1000:1, v/v)

Solution B: Glacial acetic acid and water (1:1000, v/v)

Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	18	82
20	60	40
30	60	40

System suitability solution: 0.1 mg/mL of USP Benzoic Acid RS and 0.06 mg/mL of USP Methylparaben RS in acetonitrile

Standard solution: 0.15 mg/mL of USP Benzaldehyde RS in acetonitrile

Sample solution: 0.15 mg/mL of Benzaldehyde in acetonitrile

Chromatographic system

(See Chromatography (621), System Suitability.)

Mode: LC

Detector: UV 235 nm

Detector temperature: 25°

Column: 4.6-mm × 25-cm; 5-μm packing L1

Flow rate: 1.2 mL/min

Injection volume: 10 μL

System suitability

Samples: System suitability solution and Standard solution

Suitability requirements

Resolution: NLT 2.0 between benzoic acid and methylparaben, System suitability solution

Tailing factor: NMT 2.0 for benzoic acid and methylparaben, System suitability solution

Relative standard deviation: NMT 2.0% for benzaldehyde, Standard solution

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of benzaldehyde (C₇H₆O) in the portion of Benzaldehyde taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of benzaldehyde from the Sample solution

r_S = peak response of benzaldehyde from the Standard solution

C_S = concentration of USP Benzaldehyde RS in the Standard solution (mg/mL)

C_U = concentration of Benzaldehyde in the Sample solution (mg/mL)

Acceptance criteria: 98.0%–102.0% Δ NF31

IMPURITIES**• LIMIT OF HYDROCYANIC ACID**

Sample solution: Shake 0.5 mL of Benzaldehyde with 5 mL of water, add 0.5 mL of 1 N sodium hydroxide and 0.1 mL of ferrous sulfate TS, and warm the mixture gently.

Acceptance criteria: Upon the addition of a slight excess of hydrochloric acid, no greenish-blue color or blue precipitate is produced within 15 min.

• LIMIT OF NITROBENZENE

Sample solution: Dissolve 1 mL of Benzaldehyde in 20 mL of alcohol, and mix with 10 mL of water.

Analysis: Add 1-g portions of zinc and 1-mL portions of 2 N sulfuric acid, as needed, to maintain a brisk evolution of hydrogen for 1 h. Filter, evaporate the liquid to 20 mL, and boil 10 mL of the concentrated liquid with 1 drop of potassium dichromate TS.

Acceptance criteria: No purplish color is produced.

• CHLORINATED COMPOUNDS

Analysis: Wind a strip of 20-mesh copper gauze 1.5 cm wide and 5 cm long around the end of a copper wire. Heat the gauze in the nonluminous flame of a Bunsen burner until it glows without coloring the flame green. Permit the gauze to cool, and heat several times until a thick coat of oxide has formed. With a medicine dropper, apply 2 drops of Benzaldehyde to the cooled gauze, ignite, and permit it to burn freely in the air. Again cool the gauze, add 2 more drops of Benzaldehyde, and burn as before. Repeat this process until a total of 6 drops have been added and ignited. Then hold the gauze in the outer edge of the Bunsen flame, adjusted to a height of 4 cm.

Acceptance criteria: Not even a transient green color is imparted to the flame.

Add the following:**▲ • LIMIT OF ETHYLBENZENE, CYCLOHEXYLMETHANOL, BENZYL ALCOHOL, AND BENZOIC ACID**

Sample solution: Neat Benzaldehyde

Standard solution: 0.1% of USP Ethylbenzene RS, 0.1% of USP Cyclohexylmethanol RS, 0.2% of USP Benzoic Acid RS, and 0.2% of USP Benzaldehyde RS in USP Benzyl Alcohol RS

Chromatographic system

(See Chromatography (621), System Suitability.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused silica capillary, coated with a 0.5-μm layer of phase G16

Temperatures

Detector: 310°

Injection port: 200°

Column: See Table 2.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	5	220	35

Carrier gas: Helium
 Flow rate: 1.2 mL/min
 Injection volume: 0.1 µL
 Injection type: Splitless injection
 System suitability
 Sample: Standard solution
 [NOTE—For relative retention times, see Table 3.]

Table 3

Component	Relative Retention Time
Ethylbenzene	0.45
Benzaldehyde	1.00
Cyclohexylmethanol	1.03
Benzyl alcohol	1.45
Benzoic acid	2.04

System suitability requirements

Resolution: NLT 3.0 between benzaldehyde and cyclohexylmethanol

Analysis

Samples: Standard solution and Sample solution
 Calculate the percentage of each impurity in the portion of Benzaldehyde taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each impurity from the Sample solution

r_T = sum of all the peak responses from the Sample solution

Acceptance criteria

Each individual impurity: NMT 1.0%

Total impurities: NMT 2.0%▲ NF31

SPECIFIC TESTS**Delete the following:**

▲ **SPECIFIC GRAVITY** (841): 1.041–1.046 at 25°▲ NF31

Delete the following:

▲ **REFRACTIVE INDEX** (831): 1.544–1.546 at 20°▲ NF31

Add the following:

▲ **WATER DETERMINATION, Method I** (921): NMT 1.5%▲ NF31

ADDITIONAL REQUIREMENTS**Change to read:**

• **PACKAGING AND STORAGE:** Preserve in well-filled, tight, light-resistant containers. ▲ Store at room temperature, and avoid exposure to excessive heat.▲ NF31

Add the following:

▲ **USP REFERENCE STANDARDS** (11)

USP Benzaldehyde RS
 USP Benzoic Acid RS
 USP Benzyl Alcohol RS
 USP Cyclohexylmethanol RS
 USP Ethylbenzene RS
 USP Methylparaben RS▲ NF31

Compound Benzaldehyde Elixir**DEFINITION**

Compound Benzaldehyde Elixir contains 0.05% Benzaldehyde in a suitably flavored and sweetened hydroalcoholic vehicle.

OTHER COMPONENTS

• **ALCOHOL DETERMINATION, Method I** (611): 3.0%–5.0%

ADDITIONAL REQUIREMENTS

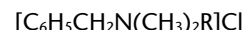
• **PACKAGING AND STORAGE:** Package in tight, light-resistant containers.

Benzalkonium Chloride

Ammonium, alkyldimethyl(phenylmethyl)-, chloride;
 Alkylbenzyltrimethylammonium chloride [8001-54-5].

DEFINITION

Benzalkonium Chloride is a mixture of alkylbenzyltrimethylammonium chlorides of the general formula:



in which R represents a mixture of alkyls, including all or some of the group beginning with *n*-C₈H₁₇ and extending through higher homologs, with *n*-C₁₂H₂₅, *n*-C₁₄H₂₉, and *n*-C₁₆H₃₃ composing the major portion. On the anhydrous basis, the content of the *n*-C₁₂H₂₅ homolog is NLT 40.0%, and the content of the *n*-C₁₄H₂₉ homolog is NLT 20.0% of the total alkylbenzyltrimethylammonium chloride content. The amount of the *n*-C₁₂H₂₅ and *n*-C₁₄H₂₉ homolog components together is NLT 70.0% of the total alkylbenzyltrimethylammonium chloride content. The total alkylbenzyltrimethylammonium chloride content, calculated on the anhydrous basis, with allowance made for the amount of residue on ignition, is NLT 97.0% and NMT 103.0% of [C₆H₅CH₂N(CH₃)₂R]Cl.

IDENTIFICATION**A. PROCEDURE**

Analysis: To 2 mL of a solution (1 in 100) add 1 mL of 2 N nitric acid.

Acceptance criteria: A white precipitate is formed, and it is dissolved after adding 5 mL of alcohol.

B. PROCEDURE

Analysis: Dissolve 200 mg in 1 mL of sulfuric acid, add 100 mg of sodium nitrate, and heat on a steam bath for 5 min. Cool, dilute with water to 10 mL, add 500 mg of zinc dust, and warm for 5 min on a steam bath. To 2 mL of the clear supernatant, add 1 mL of sodium nitrite solution (1 in 20), cool in ice water, and then add 3 mL of a solution of 500 mg of 2-naphthol in 10 mL of 6 N ammonium hydroxide.

Acceptance criteria: An orange-red color is produced.

- **C. IDENTIFICATION TESTS—GENERAL, Chloride** (191): The solution in a mixture of equal volumes of water and alcohol meets the requirements of the tests.
- **D.** The retention times of the major peaks for benzalkonium chloride in the Sample solution correspond to those of the Standard solution, as obtained in the test for Ratio of Alkyl Components.

ASSAY**• RATIO OF ALKYL COMPONENTS**

Solution A: Adjust a 0.1 M solution of sodium acetate with glacial acetic acid to a pH of 5.0.

Mobile phase: Acetonitrile and Solution A (9:11). Acetonitrile and Solution A may be adjusted from (2:3) to (3:2) to meet system suitability requirements.

Standard solution: 4 mg/mL of Benzalkonium Chloride, prepared from USP Benzalkonium Chloride RS and water.

Sample solution: 4 mg/mL of Benzalkonium Chloride Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 3.9-mm × 30-cm; packing L10, or 4.6-mm × 25-cm; 10-μm packing L10

Flow rate: 2 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution*

[NOTE—See the relative retention times in the table below. Relative retention times are provided for information only, and the standard should be used to ensure appropriate peak identification.]

Name	Relative Retention Time
C ₁₀ homolog	0.9
C ₁₂ homolog	1.0
C ₁₄ homolog	1.3
C ₁₆ homolog	1.7

Suitability requirements

Resolution: NLT 1.5 between the C₁₂ and C₁₄ peaks

Relative standard deviation: NMT 2.0% from the C₁₂ peak

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the homolog peaks by comparison of the retention times with those from the *Standard solution*.

Calculate the percentage of each quaternary ammonium homolog taken:

$$\text{Result} = \frac{r_U \times M_r}{\sum (r_U \times M_r)} \times 100$$

r_U = area of the peak due to a given homolog in the chromatogram from the *Sample solution*

M_r = molecular weight of a given homolog. The molecular weights of C₁₀, C₁₂, C₁₄, and C₁₆ homologs are 312, 340, 368, and 396, respectively.

Acceptance criteria: On the anhydrous basis, the content of the *n*-C₁₂H₂₅ homolog is NLT 40.0%, and the content of the *n*-C₁₄H₂₉ homolog is NLT 20.0% of the total alkylbenzyltrimethylammonium chloride content. The amount of the *n*-C₁₂H₂₅ and *n*-C₁₄H₂₉ homolog components together is NLT 70.0% of the total alkylbenzyltrimethylammonium chloride content.

• TOTAL ALKYL BENZYL DIMETHYLAMMONIUM CHLORIDES

Sample: Weigh a quantity of Benzalkonium Chloride equivalent to 500 mg of anhydrous benzalkonium chloride.

Analysis: Transfer the *Sample*, with the aid of 35 mL of water, to a glass-stoppered, 250-mL conical separator containing 25 mL of methylene chloride. Add 10 mL of 0.1 N sodium hydroxide, and 10.0 mL of freshly prepared potassium iodide solution (1 in 20), insert the stopper into the separator, shake, allow the layers to separate, and discard the methylene chloride layer. Wash the aqueous layer with three 10-mL portions of methylene chloride, and discard the washings. Transfer the aqueous layer to a glass-stoppered, 250-mL conical flask, and rinse the separator with three 5-mL portions of water, adding the washings to the flask. Add 40 mL of cold hydrochloric acid to the flask, mix, and titrate with 0.05 M potassium iodate VS until the solution becomes light brown in color. Add 5 mL of methylene

chloride, insert the stopper into the flask, and shake vigorously. Continue the titration, dropwise, with shaking after each addition, until the methylene chloride layer becomes colorless and the aqueous layer is clear yellow. Record the titrant volume, V_t (mL). Perform a blank determination, using 20 mL of water as the sample, and record the titrant volume, V_b (mL). [NOTE— $V_b > V_t$.] The difference between the two titrations represents the amount of potassium iodate equivalent to the weight of benzalkonium chloride in the sample. Each mL of 0.05 M potassium iodate is equivalent to $x/10$ mg of benzalkonium chloride, where x represents the average molecular weight of the sample, derived by summing, for all homologs, the products:

$$\text{Result (x)} = \sum_i [(r_i/r_t) \times M_i]$$

r_U = area of the peak produced by a given homolog in the chromatogram from the *Ratio of Alkyl Components* test

r_T = sum of the peak areas for all homologs in the chromatogram from the *Ratio of Alkyl Components* test

M_r = molecular weight of a given homolog. The molecular weights of the C₁₀, C₁₂, C₁₄, and C₁₆ homologs are 312, 340, 368, and 396, respectively.

Acceptance criteria: 97.0%–103.0% on the anhydrous basis

IMPURITIES

Inorganic Impurities

• **RESIDUE ON IGNITION** (281): NMT 2.0%

Organic Impurities

• PROCEDURE 1: LIMIT OF AMINES AND AMINE SALTS

Sample: 5.0 g of Benzalkonium Chloride

Analysis and Acceptance criteria: Dissolve the *Sample* with heating carefully e.g., on top of a steam bath with water as the steam source in 20 mL of a mixture of methanol and 1 N hydrochloric acid VS (97:3).

[NOTE—However, the mixed solution must not reach the boiling point.] Add 100 mL of isopropyl alcohol. Pass a stream of nitrogen slowly through the solution. Gradually add 12.0 mL of 0.1 N tetrabutylammonium hydroxide VS, while recording the potentiometric titration curve. If the curve shows two inflection points, the volume of titrant added between the two points is NMT 5.0 mL, corresponding to NMT 0.1 mmol/g of amines and amine salts. If the curve shows no point of inflection, the substance being examined does not comply with the test. If the curve shows one point of inflection, repeat the test, but add 3.0 mL of a 25.0 mg/mL solution of dimethyldecylamine in isopropyl alcohol before the titration. If after addition of 12.0 mL of the titrant, the titration curve shows only one point of inflection, the substance being examined does not comply with the test.

• PROCEDURE 2: LIMIT OF BENZYL ALCOHOL, BENZALDEHYDE, AND (CHLOROMETHYL)BENZENE

[NOTE—Prepare the solutions immediately before use.]

Solution A: Dissolve 1.09 g of sodium 1-hexanesulfonate and 6.9 g of monobasic sodium phosphate in water in a 1000-mL volumetric flask, adjust with phosphoric acid to a pH of 3.5, and dilute with water to volume.

Solution B: Methanol

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	80	20
10	80	20

Time (min)	Solution A (%)	Solution B (%)
14	50	50
35	50	50
36	20	80
55	20	80
56	80	20
65	80	20

Standard solution A: 0.25 mg/mL of USP Benzyl Alcohol RS in methanol

Standard solution B: 0.075 mg/mL of USP Benzaldehyde RS in methanol

Standard solution C: 0.025 mg/mL of USP Benzyl Alcohol RS in methanol, prepared from *Standard solution A* and methanol

Sample solution: 50 mg/mL of Benzalkonium Chloride in methanol

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 210 nm for benzyl alcohol and (chloromethyl)benzene; UV 257 nm for benzaldehyde

Column: 4.6-mm × 15-cm analytical column; 5-μm packing L1

Column temperature: 30°

Flow rate: 1.0 mL/min

Injection size: 20 μL

System suitability

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

[NOTE—See the relative retention times in the table below.]

Name	Relative Retention Time
Benzyl alcohol	1.0
Benzaldehyde	1.3
(Chloromethyl)benzene	2.4

Suitability requirements

Relative standard deviation: NMT 5.0% for the benzyl alcohol peak, *Standard solution A*

Signal-to-noise ratio: NLT 10 for the principal peak in the chromatogram, *Standard solution C*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Sample solution*

To calculate the content of (chloromethyl)benzene, multiply the peak area of (chloromethyl)benzene by 1.3. [NOTE—The correction factor is used due to baseline shift.]

Acceptance criteria

Benzyl alcohol: The response of the benzyl alcohol peak from the *Sample solution* is NMT that of the benzyl alcohol peak from the *Standard solution A*, corresponding to NMT 0.5%.

Benzaldehyde: The response of the benzaldehyde peak from the *Sample solution* is NMT that of the benzaldehyde peak from *Standard solution B*, corresponding to NMT 0.15%.

(Chloromethyl)benzene: The response of the (chloromethyl)benzene peak from the *Sample solution* is NMT 0.1 times that of the principal peak in the chromatogram from *Standard solution A*, corresponding to NMT 0.05%.

SPECIFIC TESTS

• ACIDITY OR ALKALINITY

Sample: 0.5 g of Benzalkonium Chloride

Analysis: Dissolve the *Sample* in water, dilute with water to 50 mL, and mix. Add 0.1 mL of bromocresol purple TS.

Acceptance criteria: NMT 0.1 mL of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide is required to change the color of the indicator.

• WATER DETERMINATION, *Method I* <921>: NMT 15.0%

WATER-INSOLUBLE MATTER: A solution (1 in 10) is free from turbidity and insoluble matter.

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in tight containers. No storage requirements specified.

• USP REFERENCE STANDARDS <11>

USP Benzalkonium Chloride RS

USP Benzyl Alcohol RS

USP Benzaldehyde RS

Benzalkonium Chloride Solution

DEFINITION

Benzalkonium Chloride Solution contains NLT 95.0% and NMT 105.0% of the labeled amount of benzalkonium chloride in a solution that has a concentration of 1.0% or more; and NLT 93.0% and NMT 107.0% of the labeled amount in a solution that has a concentration of less than 1.0%. It may contain a suitable coloring agent and may contain NMT 10% of alcohol.

[CAUTION—Mixing Benzalkonium Chloride Solution with ordinary soaps and with anionic detergents may decrease or destroy the bacteriostatic activity of the Solution.]

IDENTIFICATION

• A. PROCEDURE

Analysis: Add 1 mL of 2 N nitric acid to 2 mL of a solution having an equivalent to 10 mg/mL of benzalkonium chloride.

Acceptance criteria: A white precipitate is formed, and it is dissolved after adding 5 mL of alcohol.

• B. IDENTIFICATION TESTS—GENERAL, Chloride <191>: A solution of it in a mixture of equal volumes of water and alcohol meets the requirements.

• C. PROCEDURE

Analysis: Dissolve the residue obtained by evaporating on a steam bath, a volume of Solution equivalent to 200 mg of benzalkonium chloride in 1 mL of sulfuric acid, add 100 mg of sodium nitrate, and heat on a steam bath for 5 min. Cool, dilute with water to 10 mL, add 500 mg of zinc dust, and warm for 5 min on a steam bath. To 2 mL of the clear supernatant add 1 mL of sodium nitrite solution (1 in 20), cool in ice water, then add 3 mL of a solution of 500 mg of 2-naphthol in 10 mL of 6 N ammonium hydroxide.

Acceptance criteria: An orange-red color is produced.

• D. The retention times of the major peaks for benzalkonium chloride in the *Sample solution* correspond to those of the *Standard solution*, as obtained in the test for *Ratio of Alkyl Components*.

ASSAY

• RATIO OF ALKYL COMPONENTS

Solution A: Adjust a 0.1 M solution of sodium acetate with glacial acetic acid to a pH of 5.0.

Mobile phase: Acetonitrile and *Solution A* (9:11). Acetonitrile and *Solution A* may be adjusted from (2:3) to (3:2) to meet system suitability requirements.

Standard solution: 4 mg/mL of benzalkonium chloride, prepared from USP Benzalkonium Chloride RS and water

Sample solution: Transfer a volume of Solution, equivalent to 400 mg of benzalkonium chloride, to a 100-mL volumetric flask, and dilute with water to volume.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 3.9-mm × 30-cm; packing L10, or 4.6-mm × 25-cm; 10-μm packing L10

Flow rate: 2 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention time table below. Relative retention times are provided for information only, and the standard should be used to ensure appropriate peak identification.]

Name	Relative Retention Time
C ₁₀ homolog	0.9
C ₁₂ homolog	1.0
C ₁₄ homolog	1.3
C ₁₆ homolog	1.7

Suitability requirements

Resolution: NLT 1.5 between the C₁₂ and C₁₄ peaks

Relative standard deviation: NMT 2.0% for the C₁₂ peak

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the homolog peaks by comparison of the retention times with those from the *Standard solution*.

Calculate the percentage of each quaternary ammonium homolog taken:

$$\text{Result} = \frac{r_u \times M_r}{\sum (r_i \times M_i)} \times 100$$

r_u = area of the peak due to a given homolog in the chromatogram from the *Sample solution*

M_r = molecular weight of a given homolog. The molecular weights of the C₁₀, C₁₂, C₁₄, and C₁₆ homologs are 312, 340, 368, and 396, respectively.

Acceptance criteria: On the anhydrous basis, the content of the *n*-C₁₂H₂₅ homolog is NLT 40.0%, and the content of the *n*-C₁₄H₂₉ homolog is NLT 20.0% of the total alkylbenzyltrimethylammonium chloride content. The amount of the *n*-C₁₂H₂₅ and *n*-C₁₄H₂₉ homolog components together is NLT 70.0% of the total alkylbenzyltrimethylammonium chloride content.

• **TOTAL ALKYL BENZYL DIMETHYLAMMONIUM CHLORIDES**

Sample solution: Evaporate or dilute with water to 30 mL a volume of Solution, equivalent to 500 mg of benzalkonium chloride.

Analysis: Transfer the *Sample solution* with the aid of a minimum quantity of water to a glass-stoppered, 250-mL conical separator. Transfer 25 mL of methylene chloride. Add 10 mL of 0.1 N sodium hydroxide, and 10.0 mL of freshly prepared potassium iodide solution (1 in 20), insert the stopper in the separator, shake, allow the layers to separate, and discard the methylene chloride layer. Wash the aqueous layer with three 10-mL portions of methylene chloride, and discard the washings. Transfer the aqueous layer to a glass-stoppered, 250-mL conical flask, and rinse the separator with three 5-mL portions of water, adding the washings to the flask. Add 40 mL of cold hydrochloric acid to the flask, mix, and titrate with 0.05 M potassium iodate VS until the solution becomes light brown in color. Add

5 mL of methylene chloride, insert the stopper into the flask, and shake vigorously. Continue the titration, dropwise, with shaking after each addition, until the methylene chloride layer becomes colorless and the aqueous layer is clear yellow. Record the titrant volume, V_t (mL). Perform a blank determination, using 20 mL of water as the sample, and record the titrant volume, V_b (mL). [NOTE— $V_b > V_t$.] The difference between the two titrations represents the amount of potassium iodate equivalent to the weight of benzalkonium chloride in the sample. Each mL of 0.05 M potassium iodate is equivalent to x/10 mg of benzalkonium chloride, where x represents the average molecular weight of the sample, derived by summing, for all homologs, the products:

$$\text{Result (x)} = \sum_i [(r_i/r_t) \times M_i]$$

r_u = area of the peak produced by a given homolog in the chromatogram from the *Ratio of Alkyl Components* test

r_T = sum of the peak areas for all homologs in the chromatogram from the *Ratio of Alkyl Components* test

M_r = molecular weight of a given homolog. The molecular weights of the C₁₀, C₁₂, C₁₄, and C₁₆ homologs are 312, 340, 368, and 396, respectively.

Acceptance criteria

For labeled concentrations of NLT 1%:

95.0%–105.0%

For labeled concentrations less than 1%:

93.0%–107.0%

OTHER COMPONENTS

• **ALCOHOL CONTENT (IF ADDED)**

Internal standard solution: 0.06 mL/mL of tertiary butyl alcohol in water

Alcohol stock solution: 0.03 mL/mL of C₂H₅OH in water, using USP Alcohol RS

Standard solutions: Introduce 5 mL, 10 mL, and 20 mL, respectively, of *Alcohol stock solution* into three separate identical 50-mL volumetric flasks. To each flask, add a 5-mL portion of the *Internal standard solution*. Dilute with water to volume, and mix thoroughly. *Standard solutions* contain alcohol content of 0.003 mL/mL, 0.006 mL/mL, and 0.012 mL/mL, respectively.

Sample solution: Weigh an appropriate amount of Benzalkonium Chloride Solution into a 50-mL volumetric flask, and pipet 5 mL of *Internal standard solution*. Dilute with water to volume, and mix thoroughly to obtain a solution containing an alcohol content of between 0.003 mL/mL and 0.012 mL/mL.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m glass or quartz capillary; 1.4-μm layer of phase G43

Temperature

Detector: 250°

Injection port: 250°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	7
40	30	250	15

Run time: 29 min

Carrier gas: Helium or nitrogen

Flow rate: See the ramped flow program table below.

Initial Flow (mL/min)	Flow Ramp (mL/min ²)	Final Flow (mL/min)	Hold Time at Final Flow (min)
1	—	1	8
1	10	3	21

Injection size: 1 µL

Injection mode: Split 75:1

System suitability

Sample: Standard solution containing alcohol content of 0.006 mL/mL

[NOTE—The relative retention times for alcohol and tertiary butyl alcohol are 0.7 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0, between alcohol and tertiary butyl alcohol

Relative standard deviation: NMT 10%

Analysis

Samples: Standard solutions and Sample solution
Plot the peak response ratios of the alcohol to tertiary butyl alcohol in the Standard solutions versus the content, in mL/mL, of alcohol, and draw the straight line best fitting the plotted points. From the graph obtained, determine the content, C, in mL/mL, of alcohol in the Sample solution. Calculate the percentage of alcohol in the portion of Benzalkonium Chloride Solution (v/v) taken:

$$\text{Result} = V \times (C \times D/W) \times 100$$

V = volume of the Sample solution, 50 mL

D = density of Benzalkonium Chloride Solution (g/mL)

W = weight of Benzalkonium Chloride Solution taken to prepare the Sample solution (g)

Acceptance criteria: If present, between 95.0% and 105.0% of the labeled amount of C₂H₅OH

IMPURITIES

Organic Impurities

• PROCEDURE 1: LIMIT OF AMINES AND AMINE SALTS

Sample: A quantity of Solution, equivalent to 5.0 g of benzalkonium chloride

Analysis and Acceptance criteria: Dissolve the Sample with heating carefully e.g., on top of a steam bath with water as the steam source in 20 mL of a mixture of methanol and 1 N hydrochloric acid VS (97:3).

[NOTE—However, the mixed solution must not reach the boiling point.] Add 100 mL of isopropyl alcohol. Pass a stream of nitrogen slowly through the solution. Gradually add 12.0 mL of 0.1 N tetrabutylammonium hydroxide VS, while recording the potentiometric titration curve. If the curve shows two inflection points, the volume of titrant added between the two points is NMT 5.0 mL, corresponding to NMT 0.1 mmol/g of amines and amine salts. If the curve shows no point of inflection, the substance being examined does not comply with the test. If the curve shows one point of inflection, repeat the test, but add 3.0 mL of a 25.0 mg/mL solution of dimethyldecylamine in isopropyl alcohol before the titration. If after addition of 12.0 mL of the titrant, the titration curve shows only one point of inflection, the substance being examined does not comply with the test.

• PROCEDURE 2: LIMIT OF BENZYL ALCOHOL, BENZALDEHYDE, AND (CHLOROMETHYL)BENZENE

[NOTE—Prepare the solutions immediately before use.]

Solution A: Dissolve 1.09 g of sodium 1-hexanesulfonate and 6.9 g of monobasic sodium phosphate in water in a 1000-mL volumetric flask, adjust with

phosphoric acid to a pH of 3.5, and dilute with water to volume.

Solution B: Methanol

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	80	20
10	80	20
14	50	50
35	50	50
36	20	80
55	20	80
56	80	20
65	80	20

Standard solution A: 0.25 mg/mL of USP Benzyl

Alcohol RS in methanol

Standard solution B: 0.075 mg/mL of USP

Benzaldehyde RS in methanol

Standard solution C: 0.025 mg/mL of USP Benzyl

Alcohol RS in methanol, prepared from Standard solution A and methanol

Sample solution: Determine the density of the Benzalkonium Chloride Solution. Dilute a quantity of the Solution equivalent to 2.5 g of benzalkonium chloride with methanol to 50.0 mL. This solution contains 50 mg/mL of benzalkonium chloride.

Chromatographic system

(See Chromatography <621>, System Suitability.)

Mode: LC

Detector: UV 210 nm for benzyl alcohol and

(chloromethyl)benzene; UV 257 nm for benzaldehyde

Column: 4.6-mm × 15-cm analytical column; 5-µm packing L1

Column temperature: 30°

Flow rate: 1.0 mL/min

Injection size: 20 µL

System suitability

Samples: Standard solution A, Standard solution B,

Standard solution C, and Sample solution

[NOTE—See the relative retention times in the table below.]

Name	Relative Retention Time
Benzyl alcohol	1.0
Benzaldehyde	1.3
(Chloromethyl)benzene	2.4

Suitability requirements

Relative standard deviation: NMT 5.0% for the benzyl alcohol peak, Standard solution A

Signal-to-noise ratio: NLT 10 for the principal peak in the chromatogram, Standard solution C

Analysis

Samples: Standard solution A, Standard solution B, Standard solution C, and Sample solution

To calculate the content of (chloromethyl)benzene, multiply the peak area of (chloromethyl)benzene by 1.3. [NOTE—The correction factor is used due to baseline shift.]

Acceptance criteria

Benzyl alcohol: The response of the benzyl alcohol peak from the Sample solution is NMT that of the benzyl alcohol peak from Standard solution A, corresponding to NMT 0.5%.

Benzaldehyde: The response of the benzaldehyde peak from the Sample solution is NMT that of the benzaldehyde peak from Standard solution B, corresponding to NMT 0.15%.

(Chloromethyl)benzene: The response of the (chloromethyl)benzene peak from the *Sample solution* is NMT 0.1 times that of the principal peak in the chromatogram from *Standard solution A*, corresponding to NMT 0.05%.

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): Solution containing less than 5.0% of benzalkonium chloride meets the requirements of the test for absence of *Pseudomonas aeruginosa*.
- **ACIDITY OR ALKALINITY**
Sample solution: An appropriate quantity of the Solution, equivalent to 500 mg of benzalkonium chloride solid, prepared from Benzalkonium Chloride Solution and carbon dioxide-free water
Analysis: To the *Sample solution* add 0.1 mL of bromocresol purple TS.
Acceptance criteria: NMT 0.1 mL of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide is required to change the color of the indicator.

ADDITIONAL REQUIREMENTS

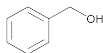
- **PACKAGING AND STORAGE:** Preserve in tight containers, and prevent contact with metals.
- **LABELING:** Label it to indicate the concentration of benzalkonium chloride, and to indicate the name and quantity of coloring agent added. The labeling also indicates the concentration of alcohol added.
- **USP REFERENCE STANDARDS** (11)
 USP Alcohol RS
 USP Benzyl Alcohol RS
 USP Benzaldehyde RS
 USP Benzalkonium Chloride RS

Benzethonium Chloride—see
Benzethonium Chloride General Monographs

Benzoic Acid—see *Benzoic Acid General Monographs*

Benzyl Alcohol

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (♦) to specify this fact.



C₇H₈O 108.14
 Phenylmethanol [100-51-6].

DEFINITION

Benzyl Alcohol contains NLT 98.0% and NMT the equivalent of 100.5% of phenylmethanol (C₇H₈O).

IDENTIFICATION

- **♦A. INFRARED ABSORPTION** (197F): On undried specimen♦

ASSAY

• PROCEDURE

Phenolphthalein solution: Dissolve 0.1 g of phenolphthalein in 80 mL of ethanol (96%), and dilute with

water to 100.0 mL. To test for sensitivity, add 100 mL of carbon dioxide-free water to 0.1 mL of the *Phenolphthalein solution*. The solution is colorless. NMT 0.2 mL of 0.02 M sodium hydroxide is required to change the color to pink.

Sample: 0.900 g

Analysis: To the *Sample* add 15 mL of a freshly prepared mixture of dried pyridine and acetic anhydride (7:1), and heat under a reflux condenser on a boiling water bath for 30 min. Cool, and add 25 mL of water. Using 0.25 mL of *Phenolphthalein solution* as the indicator, titrate with 1 M sodium hydroxide VS. Carry out a blank titration.

Calculate the percentage content of phenylmethanol (C₇H₈O):

$$\text{Result} = 10.81 \times (n_1 - n_2)/m$$

n_1 = amount of 1 M sodium hydroxide used for the blank (mL)

n_2 = amount of 1 M sodium hydroxide used for the sample (mL)

m = amount of sample taken (g)

Acceptance criteria: 98.0%–100.5%

IMPURITIES

- **FATS AND FIXED OILS**, *Peroxide Value* (401): NMT 5

• RESIDUE ON EVAPORATION

Analysis: After ensuring that the Benzyl Alcohol complies with the test for *Fats and Fixed Oils, Peroxide Value*, evaporate 10.0 g to dryness in a tared quartz or porcelain crucible or platinum dish on a hot plate at a temperature not exceeding 200°. Ensure that the Benzyl Alcohol does not boil during evaporation. Dry the residue on the hot plate for 1 h, and allow to cool in a desiccator.

Acceptance criteria: The residue weighs NMT 5 mg, corresponding to NMT 0.05%.

• ORGANIC IMPURITIES, BENZALDEHYDE, AND OTHER RELATED SUBSTANCES

Sample solution: Use the Benzyl Alcohol sample under examination.

Standard solution A: Dissolve 0.100 g of ethylbenzene in 10.0 mL of the *Sample solution*. Dilute 2.0 mL of this solution with the *Sample solution* to 20.0 mL.

Standard solution B: Dissolve 2.000 g of dicyclohexyl in 10.0 mL of the *Sample solution*. Dilute 2.0 mL of this solution with the *Sample solution* to 20.0 mL.

Reference solution A (for use in nonparenteral applications): Dissolve 0.750 g of benzaldehyde and 0.500 g of cyclohexylmethanol in the *Sample solution*, and dilute with the *Sample solution* to 25.0 mL. Add 1.0 mL of this solution to a mixture of 2.0 mL of *Standard solution A* and 3.0 mL of *Standard solution B*, and dilute with the *Sample solution* to 20.0 mL.

Reference solution B (for use in parenteral applications): Dissolve 0.250 g of benzaldehyde and 0.500 g of cyclohexylmethanol in the *Sample solution*, and dilute with the *Sample solution* to 25.0 mL. Add 1.0 mL of this solution to a mixture of 2.0 mL of *Standard solution A* and 2.0 mL of *Standard solution B*, and dilute with the *Sample solution* to 20.0 mL.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Carrier: Helium, chromatography grade

Carrier linear velocity: 25 cm/s, at 50°

Detector temperature: 310°

Column: 30-m × 0.32-mm; 0.5-μm film thickness, G16

Temperature
Injector: 200°
Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	5	220	35

System suitability

Sample: For nonparenteral applications, use *Reference solution A*. For parenteral applications, use *Reference solution B*.

[NOTE—The retention time of benzyl alcohol is about 26 min. The relative retention times for ethylbenzene, dicyclohexyl, benzaldehyde, cyclohexylmethanol, and benzyl alcohol are about 0.28, 0.59, 0.68, 0.71, and 1.0, respectively.]

Injection volume: 0.1 µL without air plug

Suitability requirements

Sensitivity: Adjust the sensitivity of the detector so that the height of the peak due to ethylbenzene is NLT 30% of the full scale of the recorder.

Resolution: NLT 3.0 between the peaks corresponding to benzaldehyde and cyclohexylmethanol

Analysis

Samples: *Sample solution* and *Reference solution A* for nonparenteral applications and *Reference solution B* for parenteral applications

Acceptance criteria (nonparenteral applications): If any peaks are present in the chromatogram obtained with the *Sample solution* that have the same retention times as the peaks due to ethylbenzene and dicyclohexyl, subtract the areas of any such peaks from the peak areas at these retention times in the chromatograms of *Reference solution A* or *Reference solution B* (corrected peak areas of ethylbenzene and dicyclohexyl). Any such peaks in the *Sample solution* should be included in the assessments for the total of other peaks.

In the chromatogram obtained with the *Sample solution*, the area of any peak corresponding to benzaldehyde is NMT the difference between the area of the peak due to benzaldehyde in the chromatogram obtained with *Reference solution A* (0.15%) and the area of the peak due to benzaldehyde in the chromatogram obtained with the *Sample solution*.

In the chromatogram obtained with the *Sample solution*, the area of any peak corresponding to cyclohexylmethanol is NMT the difference between the area of the peak due to cyclohexylmethanol in the chromatogram obtained with *Reference solution A* (0.10%) and the area of the peak due to cyclohexylmethanol in the chromatogram obtained with the *Sample solution*.

In the chromatogram obtained with the *Sample solution*, the sum of the areas of any peak with a relative retention time less than that of benzyl alcohol and apart from the peaks due to benzaldehyde and cyclohexylmethanol is NMT four times the area of ethylbenzene in *Reference solution A*, corrected if necessary as described above (0.04%).

In the chromatogram obtained with the *Sample solution*, the sum of the areas of any peak with a relative retention time greater than that of benzyl alcohol is NMT the area of dicyclohexyl in *Reference solution A*, corrected if necessary as described above (0.3%).

Disregard any peak with an area less than 0.01 times that of the peak due to ethylbenzene in the chromatogram of *Reference solution A*, corrected if necessary as described above.

Acceptance criteria (parenteral applications): If any peaks are present in the chromatogram obtained with

the *Sample solution* that have the same retention times as the peaks due to ethylbenzene and dicyclohexyl, subtract the areas of any such peaks from the peak areas at these retention times in the chromatograms of *Reference solution A* or *Reference solution B* (corrected peak areas of ethylbenzene and dicyclohexyl). Any such peaks in the *Sample solution* should be included in the assessments for the total of other peaks.

In the chromatogram obtained with the *Sample solution*, the area of any peak corresponding to benzaldehyde is NMT the difference between the area of the peak due to benzaldehyde in the chromatogram obtained with *Reference solution B* (0.05%) and the area of the peak due to benzaldehyde in the chromatogram obtained with the *Sample solution*.

In the chromatogram obtained with the *Sample solution*, the area of any peak corresponding to cyclohexylmethanol is NMT the difference between the area of the peak due to cyclohexylmethanol in the chromatogram obtained with *Reference solution B* (0.10%) and the area of the peak due to cyclohexylmethanol in the chromatogram obtained with the *Sample solution*.

In the chromatogram obtained with the *Sample solution*, the sum of the areas of any peak with a relative retention time less than that of benzyl alcohol and apart from the peaks due to benzaldehyde and cyclohexylmethanol is NMT two times the area of ethylbenzene in *Reference solution B*, corrected if necessary as described above (0.02%).

In the chromatogram obtained with the *Sample solution*, the sum of the areas of any peak with a relative retention time greater than that of benzyl alcohol is NMT the area of dicyclohexyl in *Reference solution B*, corrected if necessary as described above (0.2%).

Disregard any peak with an area less than 0.01 times that of the peak due to ethylbenzene in the chromatogram of *Reference solution B*, corrected if necessary as described above.

SPECIFIC TESTS**• ACIDITY**

Phenolphthalein solution: Prepare as directed in the Assay.

Analysis: To 10 mL of Benzyl Alcohol add 10 mL of ethanol (96%) and 1 mL of *Phenolphthalein solution*.

Acceptance criteria: NMT 1 mL of 0.1 M sodium hydroxide is required to change the color of the indicator to pink.

• CLARITY OF SOLUTION

[NOTE—The *Sample solution* is to be compared to *Reference suspension 1* in diffused daylight 5 min after preparation of *Reference suspension 1*.]

Hydrazine solution: Transfer 1.0 g of hydrazine sulfate to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix. Allow to stand 4–6 h before use.

Methenamine solution: Transfer 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.

Primary opalescent suspension: [NOTE—This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.] Transfer 25.0 mL of *Hydrazine solution* to the *Methenamine solution* in the 100-mL glass-stoppered flask. Mix, and allow to stand for 24 h.

Opalescence standard: [NOTE—This suspension should not be used beyond 24 h after preparation.] Transfer 15.0 mL of the *Primary opalescent suspension* to a 1000-mL volumetric flask, dilute with water to volume, and mix.

Reference suspension 1: Transfer 5.0 mL of the *Opalescence standard* to a 100-mL volumetric flask, and dilute with water to volume.

Reference suspension 2: Transfer 10.0 mL of the *Opalescence standard* to a second 100-mL volumetric flask, and dilute with water to volume.

Sample solution: Dissolve 2.0 g of Benzyl Alcohol in 60 mL of water.

Analysis: Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm, to obtain a depth of 40 mm. Similarly transfer portions of *Reference suspension 1*, *Reference suspension 2*, and water to separate matching test tubes. Compare the *Sample solution*, *Reference suspension 1*, *Reference suspension 2*, and water in diffused daylight, viewing vertically against a black background (see *Spectrophotometry and Light-Scattering* (851), *Visual Comparison*). [NOTE—The diffusion of light must be such that *Reference suspension 1* can readily be distinguished from water, and that *Reference suspension 2* can readily be distinguished from *Reference suspension 1*.]

Acceptance criteria: The *Sample solution* shows the same clarity as that of water, or its opalescence is not more pronounced than that of *Reference suspension 1*.♦

♦ **COLOR OF SOLUTION**

Sample solution: Use the *Sample solution* prepared in the test for *Clarity of Solution*.

Analysis: Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm, to obtain a depth of 40 mm. Similarly transfer a portion of water to a separate matching test tube. Compare the color of the *Sample solution* with that of water in diffused daylight, viewing vertically against a white background (see *Spectrophotometry and Light-Scattering* (851), *Visual Comparison*).

Acceptance criteria: The *Sample solution* has the color of water.♦

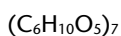
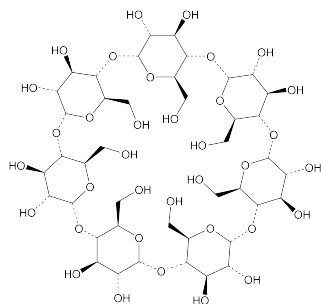
♦ **REFRACTIVE INDEX (831):** 1.538–1.541 at 20°

ADDITIONAL REQUIREMENTS

- ♦ **LABELING:** Where Benzyl Alcohol is intended for use in the manufacture of parenteral applications, it is so labeled.♦
- ♦ **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light.♦
- ♦ **USP REFERENCE STANDARDS**
USP Benzyl Alcohol RS.♦

Benzyl Benzoate—see *Benzyl Benzoate General Monographs*

Betadex



Beta Cyclodextrin [7585-39-9].

DEFINITION

Betadex is a nonreducing cyclic compound composed of seven alpha-(1–4) linked D-glucopyranosyl units. It contains NLT 98.0% and NMT 102.0% of betadex (C₆H₁₀O₅)₇, calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K):** On undried specimen
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- **C.** It meets the requirements of the test for *Optical Rotation*.

• **D.**

Sample: 0.2 g

Analysis: Mix the *Sample* with 2 mL of iodine TS, warm in a water bath to dissolve, and allow to stand at room temperature.

Acceptance criteria: A yellow-brown precipitate is formed.

ASSAY

• **PROCEDURE**

Mobile phase: Methanol and water (7:93)

System suitability solution: 0.5 mg/mL of USP Alpha Cyclodextrin RS, 0.5 mg/mL of USP Beta Cyclodextrin RS, and 0.5 mg/mL of USP Gamma Cyclodextrin RS

Standard solution: 1.0 mg/mL of USP Beta Cyclodextrin RS

Sample stock solution: Transfer 250 mg of Betadex to a 25-mL volumetric flask, and dissolve in water with the aid of heat if necessary. Cool, and dilute with water to volume.

Sample solution: 1 mg/mL of Betadex, prepared from the *Sample stock solution*, diluted with water

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 4.6-mm × 15-cm; 5-μm packing L1

Temperatures

Column: 30°

Detector: 40°

Flow rate: 1.5 mL/min

Injection volume: 50 μL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for gamma cyclodextrin, alpha cyclodextrin, and beta cyclodextrin are 0.4, 0.5, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between the gamma cyclodextrin and alpha cyclodextrin peaks

Tailing factor: 0.8–2.0 for the three cyclodextrins

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of betadex (C₆H₁₀O₅)₇ in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Beta Cyclodextrin RS in the *Standard solution* (mg/mL)

C_U = concentration of Beta Cyclodextrin in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%
- **HEAVY METALS**, *Method II* (231): NMT 5 µg/g
- **LIMIT OF REDUCING SUGARS**
Cupric solution: 150 mg/mL of cupric sulfate
Tartrate solution: 25 mg/mL of anhydrous sodium carbonate, 25 mg/mL of potassium sodium tartrate, 20 mg/mL of sodium bicarbonate, and 20 mg/mL of anhydrous sodium sulfate
Cupric–tartaric solution: Immediately before use, mix 1 part of *Cupric solution* with 25 parts of *Tartrate solution*.

Ammonium molybdate solution: 100 mg/mL of ammonium molybdate

Disodium arsenate solution: 60 mg/mL of disodium arsenate

Ammonium molybdate reagent: Mix 10 mL of *Disodium arsenate solution*, 50 mL of *Ammonium molybdate solution*, and 90 mL of diluted sulfuric acid, and dilute with water to 200 mL.

Sample solution: Transfer 1.0 g of Betadex (as calculated on the anhydrous basis) to a 100-mL volumetric flask. Dissolve in and dilute with water that has been previously boiled and cooled to room temperature to volume, and mix. To 1 mL of this solution add 1 mL of *Cupric–tartaric solution*. Heat on a water bath for 10 min, then cool to room temperature. Add 10 mL of *Ammonium molybdate reagent*, and allow to stand for 15 min.

Standard stock solution: 20 mg/L of USP Dextrose RS

Standard solution: Prepare at the same time as the *Sample solution*. To 1 mL of the *Standard stock solution* add 1 mL of *Cupric–tartaric solution*. Heat on a water bath for 10 min, then cool to room temperature. Add 10 mL of *Ammonium molybdate reagent*, and allow to stand for 15 min.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Vis

Analytical wavelength: 740 nm

Blank: Water

Analysis

Samples: *Sample solution* and *Standard solution*

Acceptance criteria: 0.2%; the absorbance of the *Sample solution* is NMT that of the *Standard solution*.

- **LIMIT OF LIGHT-ABSORBING IMPURITIES**

Sample solution: Transfer 1.0 g of Betadex (as calculated on the anhydrous basis) to a 100-mL volumetric flask. Dissolve in and dilute with water that has been previously boiled and cooled to room temperature to volume. Mix, and pass through a filter of 0.2-µm pore size.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV/Vis

Analytical wavelength: 230–750 nm

Cell: 1 cm

Blank: Water

Analysis

Sample: *Sample solution*

Acceptance criteria

Absorbance:

NMT 0.10, 230–350 nm

NMT 0.05, 350–750 nm

- **RELATED COMPOUNDS**

Mobile phase, System suitability solution, Chromatographic system, and System suitability: Proceed as directed in the Assay.

Standard solution: 0.05 mg/mL each of USP Alpha Cyclodextrin RS, USP Beta Cyclodextrin RS, and USP Gamma Cyclodextrin RS from the *System suitability solution*, diluted with water

Sample solution: Use the *Sample stock solution* from the Assay.

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria

Alpha cyclodextrin: 0.25%; the area of any peak corresponding to alpha cyclodextrin is NMT 0.5 times the area of the corresponding peak from the *Standard solution*.

Gamma cyclodextrin: 0.25%; the area of any peak corresponding to gamma cyclodextrin is NMT 0.5 times the area of the corresponding peak from the *Standard solution*.

Other related substances: 0.5%; the sum of all of the peak areas, excluding the alpha cyclodextrin, beta cyclodextrin, gamma cyclodextrin, and artifact peaks is NMT the area of the peak due to betadex (beta cyclodextrin) from the *Standard solution*.

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 10³ cfu/g, and the total combined molds and yeasts count does not exceed 10² cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

- **OPTICAL ROTATION**, *Specific Rotation* (781S)

Sample solution: 10 mg/mL in water

Acceptance criteria: +160° to +164°, determined at 20°

- **WATER DETERMINATION**, *Method I* (921): NMT 14.0%

- **COLOR AND CLARITY OF SOLUTION**

Sample solution: Dissolve 0.2 g in 20.0 mL of freshly boiled and cooled water.

Acceptance criteria: The resulting solution is clear and colorless.

- **pH** (791)

Sample solution: Add 0.1 mL of a saturated solution of potassium chloride to 10 mL of a 10 mg/mL aqueous solution of Betadex.

Acceptance criteria: 5.0–8.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.

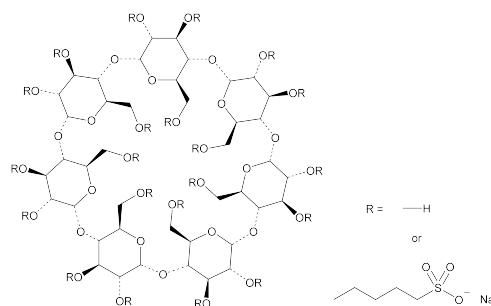
- **USP REFERENCE STANDARDS** (11)

USP Alpha Cyclodextrin RS

USP Beta Cyclodextrin RS

USP Gamma Cyclodextrin RS

USP Dextrose RS

Betadex Sulfobutyl Ether Sodium

$C_{42}H_{70-n}O_{35} \cdot (C_4H_8SO_3Na)_n$ 2163 when $n = 6.5$

Beta cyclodextrin sulfobutyl ethers, sodium salts;

Beta cyclodextrin sulfobutyl ether sodium [182410-00-0].

DEFINITION

Betadex Sulfobutyl Ether Sodium is prepared by alkylation of betadex using 1,4-butane sultone under basic conditions.

The average degree of substitution in betadex is NLT 6.2 and NMT 6.9. It contains NLT 95.0% and NMT 105.0% of $C_{42}H_{70-n}O_{35} \cdot (C_4H_8SO_3Na)_n$ ($n = 6.2-6.9$), calculated on the anhydrous basis.

IDENTIFICATION

- A. INFRARED ABSORPTION** (197K)
- B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- C.** It meets the requirements of the test for *Average Degree of Substitution*.
- D. IDENTIFICATION TESTS—GENERAL, Sodium** (191)

ASSAY

PROCEDURE

Mobile phase: 0.1 M potassium nitrate in a mixture of acetonitrile and water (1:4)

Standard solution: 10 mg/mL of USP Betadex Sulfobutyl Ether Sodium RS in *Mobile phase*

Sample solution: 10 mg/mL of Betadex Sulfobutyl Ether Sodium in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Detector temperature: $35 \pm 2^\circ$

Column: 7.8-mm \times 30-cm analytical column; packing L37. [NOTE—Rinse the column with a solution of acetonitrile and water (1:9) at the completion of the run series.]

Flow rate: 1.0 mL/min

Injection size: 20 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of betadex sulfobutyl ether sodium $[C_{42}H_{70-n}O_{35} \cdot (C_4H_8SO_3Na)_n]$ in the portion of Betadex Sulfobutyl Ether Sodium taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for betadex sulfobutyl ether sodium from the *Sample solution*

r_S = peak response for betadex sulfobutyl ether sodium from the *Standard solution*

C_S = concentration of USP Betadex Sulfobutyl Ether Sodium RS in the *Standard solution* (mg/mL)

C_U = concentration of Betadex Sulfobutyl Ether Sodium in the *Sample solution* (mg/mL)

Acceptance criteria: 95.0%–105.0% on the anhydrous basis

IMPURITIES

- HEAVY METALS, Method II** (231): NMT 5 ppm
- LIMIT OF BETA CYCLODEXTRIN (BETADEX)**
Solution A: 25 mM sodium hydroxide
Solution B: 250 mM sodium hydroxide and 1 M potassium nitrate
Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
4	100	0
5	0	100
10	0	100
11	100	0
20	100	0

Standard solution: 2 μ g/mL of USP Beta Cyclodextrin RS

Sample solution: 2 mg/mL of Betadex Sulfobutyl Ether Sodium

Chromatographic system

(See *Chromatography* (621), *System Suitability* and *Ion Chromatography* (1065).)

Mode: IC

Detector: Pulsed amperometry (amperometric cell with gold working electrode and silver reference electrode)

Column

Guard: 4.0-mm \times 5-cm anion-exchange; packing L61

Analytical: 4.0-mm \times 25-cm anion-exchange; packing L61

Column temperature: $50 \pm 2^\circ$

Flow rate: 1.0 mL/min

Injection size: 20 μ L

Waveform for pulsed amperometric detector: See *Table 2*.

Table 2

Time (s)	Voltage (V)
0.00	0.10
0.30	Start integration
0.50	0.10
0.50	Stop integration
0.51	0.60
0.59	0.60
0.60	−0.60
0.65	−0.60

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of beta cyclodextrin (betadex) in the portion of Betadex Sulfobutyl Ether Sodium taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response for beta cyclodextrin from the *Sample solution*

r_S = peak response for beta cyclodextrin from the *Standard solution*

C_S = concentration of USP Beta Cyclodextrin RS in the *Standard solution* (μ g/mL)

C_U = concentration of Betadex Sulfobutyl Ether Sodium in the *Sample solution* (mg/mL)

F = conversion factor (10^{-3} mg/ μ g)

Acceptance criteria: NMT 0.1%

LIMIT OF 1,4-BUTANE SULTONE

Internal standard solution: 0.25 μ g/mL of diethyl sulfone

Standard stock solution A: 0.5 μ g/mL of 1,4-butane sultone

Standard stock solution B: 1.0 μ g/mL of 1,4-butane sultone

Standard stock solution C: 2.0 μ g/mL of 1,4-butane sultone

Sample stock solution: 250 mg/mL of Betadex Sulfobutyl Ether Sodium in the *Internal standard solution*

Blank solution, and Sample solutions A, B, C, and D: Follow *Table 3* to place the quantities of *Internal standard solution*, each *Standard stock solution*, *Sample stock solution*, water, or methylene chloride in each glass test tube with a stopper. [NOTE—A screw-capped, 10-mL test tube is suitable.] Mix on a vortex mixer each test tube for 30 s, and allow it stand for at least 5 min

or until complete separation of the phase. Extract the organic phase into a GC vial and seal. [NOTE—With great care take the minimum possible amount of aqueous phase.] Added quantities of 1,4-butane sultone in *Sample solutions A, B, C, and D* are 0.5, 1.0, 2.0, and 0 µg, respectively.

Table 3

Sample Name	Solution 1 Added (mL)	Solution 2 Added (mL)	Methylene Chloride Added (mL)
Blank solution	Internal standard solution, 4.0	Water, 1.0	1.0
Sample solution A	Sample stock solution, 4.0	Standard stock solution A, 1.0	1.0
Sample solution B	Sample stock solution, 4.0	Standard stock solution B, 1.0	1.0
Sample solution C	Sample stock solution, 4.0	Standard stock solution C, 1.0	1.0
Sample solution D	Sample stock solution, 4.0	Water, 1.0	1.0

[NOTE—Prepare immediately before use.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 25-m fused-silica capillary column; 0.5-µm layer of phase G46

Temperature

Detector: 270°

Injection port: 200°

Column: See the temperature program in *Table 4*.

Table 4

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	10	200	—
200	35	270	5

Carrier gas: Helium, typically at 12 psi inlet pressure

Injection size: 1.0 µL

Injection type: Splitless injection for 0.5 min, then split at 50 mL/min. [NOTE—The use of an appropriate splitless injection liner is recommended.]

System suitability

Sample: *Sample solution B*

[NOTE—The relative retention times for diethyl sulfone and 1,4-butane sultone are 0.7 and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 10.0%

Analysis

Samples: *Blank solution, Sample solutions A, B, C, and D*

Correct the ratio of peak responses of the 1,4-butane sultone to diethyl sulfone in *Sample solution A, B, C, or D* by subtracting the ratio of peak responses of the 1,4-butane sultone to ethyl sulfone in the *Blank solution*. Plot the corrected ratio of peak response of 1,4-butane sultone to peak response of diethyl sulfone in *Sample solution A, B, C or D*, versus the added quantity, in µg, of 1,4-butane sultone. Extrapolate the line joining the

points on the graph until it meets the quantity axis. The distance between this point and the intersection of the axes represents the quantity of 1,4-butane sultone, *A*, in µg, in the 4-mL portion of *Sample stock solution*. Calculate the content of 1,4-butane sultone in the portion of Betadex Sulfobutyl Ether Sodium taken:

$$\text{Result} = A / (V_{\text{Ext}} \times C_U \times F)$$

A = determined above

*V*_{Ext} = volume of the *Sample stock solution* used in the extraction step, 4.0 mL

*C*_U = concentration of Betadex Sulfobutyl Ether Sodium in the *Sample stock solution* (mg/mL)

F = conversion factor (10⁻³ g/mg)

Acceptance criteria: NMT 0.5 ppm

• LIMIT OF SODIUM CHLORIDE, 4-HYDROXYBUTANE-1-SULFONIC ACID, AND BIS(4-SULFOBUTYL) ETHER DISODIUM

Solution A: 5 mM sodium hydroxide, degas in a closed vessel for 15 min

Solution B: 25 mM sodium hydroxide, degas in a closed vessel for 15 min

Mobile phase: See *Table 5*.

Table 5

Time (min)	Solution A (%)	Solution B (%)
0	100	0
4	100	0
10	70	30
24	70	30
25	100	0
40	100	0

Column wash solution A: 50 mM sodium citrate

Column wash solution B: 150 mM sodium hydroxide

Standard solution: Prepare a solution having known concentrations of 8 µg/mL of USP Sodium Chloride RS, 4 µg/mL of 4-hydroxybutane-1-sulfonic acid, and 4 µg/mL of bis(4-sulfobutyl) ether disodium.

Sample solution: 4 mg/mL of Betadex Sulfobutyl Ether Sodium

Chromatographic system

(See *Chromatography* <621>, *System Suitability* and *Ion Chromatography* <1065>.)

Mode: IC

Detector: Conductivity

Range: 30 µS

Current: 100 mA

Column: [NOTE—At the end of each run, clean the column using *Column wash solution A* at a flow rate of 1 mL/min for 35 min then using *Column wash solution B* at the same flow rate for 35 min.]

Guard: 4.0-mm × 5.0-cm anion-exchange; packing L61

Analytical: 4.0-mm × 25-cm anion-exchange; packing L61

Column temperature: 30°

Suppressor: Micromembrane anion autosuppressor¹ or a suitable chemical suppression system

Suppressant: Autosuppression

Flow rate: 1.0 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

[NOTE—Relative retention times are provided for information only. The relative retention times for 4-hydroxybutane-1-sulfonate ion, chloride ion, and bis(sulfobutyl) ether ion are 1.0, 1.4, and 8.6, respectively.]

¹ Available as Anion Self-Regenerating Suppressor (ASRS) from Dionex Inc., or equivalent.

Suitability requirements**Resolution:** NLT 2.0**Relative standard deviation:** NMT 10.0%**Analysis****Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of sodium chloride, 4-hydroxybutane-1-sulfonic acid, or bis(sulfobutyl) ether disodium in the portion of Betadex Sulfobutyl Ether Sodium taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response for sodium chloride, 4-hydroxybutane-1-sulfonic acid, or bis(sulfobutyl) ether disodium from the *Sample solution*

r_S = peak response for sodium chloride, 4-hydroxybutane-1-sulfonic acid, or bis(sulfobutyl) ether disodium from the *Standard solution*

C_S = concentration of sodium chloride, 4-hydroxybutane-1-sulfonic acid, or bis(sulfobutyl) ether disodium in the *Standard solution* (μg/mL)

C_U = concentration of Betadex Sulfobutyl Ether Sodium in the *Sample solution* (mg/mL)

F = conversion factor (10^{-3} mg/μg)

Acceptance criteria**Sodium chloride:** NMT 0.2%**4-Hydroxybutane-1-sulfonic acid:** NMT 0.09%**Bis(sulfobutyl) ether disodium:** NMT 0.05%**SPECIFIC TESTS**

- **BACTERIAL ENDOTOXINS TEST (85):** The level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Betadex Sulfobutyl Ether Sodium is used can be met. Where the label states that Betadex Sulfobutyl Ether Sodium must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Betadex Sulfobutyl Ether Sodium is used can be met.

- **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 100 cfu/g, and the total combined molds and yeasts count does not exceed 50 cfu/g. It meets the requirements of the test for absence of *Escherichia coli*.

- **CLARITY OF SOLUTION**

Sample solution: 30% (w/v) solution**Analysis:** Examine the *Sample solution* using a light box against white and black backgrounds, and record the presence of any haze, fluorescence, fibers, specks, or other foreign matter.**Acceptance criteria:** The solution is clear, and essentially free from particles of foreign matter.

- **AVERAGE DEGREE OF SUBSTITUTION**

Run electrolyte: 30 mM benzoic acid and adjusted to a pH that is suitable for the instrument used by addition of 100 mM tris(hydroxymethyl) aminomethane buffer. [NOTE—Due to variation between capillaries, a single universally applicable electrolyte pH is not specified. Instead, the optimal pH associated with each individual capillary should be determined according to the instrumental manual.]

Standard solution: 10 mg/mL of USP Betadex Sulfobutyl Ether Sodium RS**Sample solution:** 10 mg/mL of Betadex Sulfobutyl Ether Sodium

Capillary rinsing procedure: Use separate run electrolyte vials for capillary rinse and sample analysis. Perform pre-analysis rinses on a daily basis before each analysis: rinse the capillary with 0.1 N sodium hydroxide for 30 min, with water for NLT 2 h, and with

Run electrolyte for NLT 1 h. Perform pre-injection rinses prior to each injection as follows. Rinse the capillary with 0.1 N sodium hydroxide for NLT 1 min, and with *Run electrolyte* for NLT 3 min. If a new capillary is being used, in addition to the regular rinses described above, a new capillary requires rinsing before its first use. Rinse the new capillary with 1 M sodium hydroxide for 1 h, followed by a 2-h water rinse.

Electrophoretic system(See *Capillary Electrophoresis* (1053).)**Mode:** High-performance CE**Detector:** Inverse UV 200 nm, with a bandwidth of 20 nm. [NOTE—A detection wavelength of 205 nm with a bandwidth of 10 nm may be used as an alternative.]**Column:** 50-μm × 50-cm fused silica column**Column temperature:** 25°**Applied voltage:** 0.00 to +30.00 kV linear ramp over 10 min, then at 30 kV for a further 20 min**Injection size:** Equal volumes at 0.5 psi for 10 s**System suitability****Sample:** *Standard solution*

[NOTE—See Table 6 for the approximate relative migration times for betadex sulfobutyl ether sodium peaks I–X (betadex sulfobutyl ether sodium peaks I, II, III, ..., X, contains beta cyclodextrin molecule with 1, 2, 3, ..., 10 sulfobutyl substituent(s), respectively). The relative migration times are for informational purposes only to aid in peak identification.]

Table 6

Betadex Sulfobutyl Ether Sodium Peaks I–X	Relative Migration Time
I	0.58
II	0.63
III	0.69
IV	0.77
V	0.83
VI	0.91
VII	1.00
VIII	1.10
IX	1.20
X	1.30

Suitability requirements

Resolution: NLT 0.9, between betadex sulfobutyl ether sodium peak IX and betadex sulfobutyl ether sodium peak X

Analysis**Samples:** *Run electrolyte*, water, *Standard solution*, and *Sample solution*

Inject the *Standard solution* and *Sample solution* by applying differential pressure of 0.5 psi, equivalent to 34 mbar, for 10 s, followed by injection of *Run electrolyte* at 0.5 psi for 2 s. [NOTE—Pressure injections should be made with a vial of water or *Run electrolyte* at the outlet end of the capillary.]

Record the electropherograms, and measure the peak responses for the individual betadex sulfobutyl ether sodium peaks (I to X). Calculate the corrected peak area, A_i , for each peak in the electropherogram:

$$\text{Corrected Peak Area } A_i = \frac{\text{Peak Area} \times \text{Effective Capillary Length (cm)}}{\text{Migration Time}}$$

Normalize the corrected peak areas by presenting each as a percentage of the total corrected substitution envelope area:

$$\text{Normalized Area, } NA_i = \frac{A_i}{\sum_{i=1}^n A_i} \times 100$$

n = highest level of substitution

Determine the average degree of substitution:

$$\text{Average Degree of Substitution} = \frac{\sum_{i=1}^n (\text{Level of Substitution for Peak} \times NA_i)}{100}$$

Acceptance criteria: 6.2–6.9 for average degree of substitution

For each of betadex sulfobutyl ether sodium peaks I–X, see limit range (% peak area) in Table 7.

Table 7

Betadex Sulfobutyl Ether Sodium Peaks I–X	Limit Range (% Peak Area)
I	0–0.3
II	0–0.9
III	0.5–5.0
IV	2.0–10.0
V	10.0–20.0
VI	15.0–25.0
VII	20.0–30.0
VIII	10.0–25.0
IX	2.0–12.0
X	0–4.0

- **pH** <791>: 4.0–6.8, in a 30% (w/v) solution in carbon dioxide-free water
- **WATER DETERMINATION**, Method I <921>: NMT 10.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature. Protect from moisture.
- **LABELING:** Label it to indicate its use in the manufacture of injectable dosage forms.
- **USP REFERENCE STANDARDS** <11>
 - USP Beta Cyclodextrin RS
 - USP Betadex Sulfobutyl Ether Sodium RS
 - USP Endotoxin RS
 - USP Sodium Chloride RS

Boric Acid

H₃BO₃ 61.83
Boric acid (H₃BO₃) [10043-35-3].

DEFINITION

Boric Acid contains NLT 99.5% and NMT 100.5% of boric acid (H₃BO₃), calculated on the dried basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL**, Borate <191>: Meets the requirements

ASSAY

PROCEDURE

Sample solution: Dissolve 2 g of Boric Acid in 100 mL of a mixture of glycerin and water (1:1), previously neutralized to phenolphthalein TS.

Analysis: Titrate the Sample solution with 1 N sodium hydroxide VS. Discharge the pink color by the addition of 50 mL of glycerin, neutralized to phenolphthalein TS, and continue the titration until the pink color reappears. Each mL of 1 N sodium hydroxide is equivalent to 61.83 mg of H₃BO₃.

Acceptance criteria: 99.5%–100.5% on the dried basis

IMPURITIES

HEAVY METALS, Method I <231>

Test preparation: Dissolve 1 g in 23 mL of water, and add 2 mL of 1 N acetic acid.

Acceptance criteria: 20 µg/g

SPECIFIC TESTS

LOSS ON DRYING <731>

Analysis: Dry over silica gel for 5 h.

Acceptance criteria: NMT 0.5%

SOLUBILITY IN ALCOHOL

Sample solution: 1 g in 10 mL of boiling alcohol

Acceptance criteria: It dissolves completely.

COMPLETENESS OF SOLUTION

Sample solution: 1 g in 25 mL of water

Acceptance criteria: It produces a clear solution.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Label the container with a warning that it is not for internal use.

Butane

C₄H₁₀ 58.12
n-Butane [106-97-8].

DEFINITION

Butane contains NLT 97.0% of butane (C₄H₁₀).

[**CAUTION**—Butane is highly flammable and explosive.]

IDENTIFICATION

- **A. INFRARED ABSORPTION:** Exhibits maxima, among others, at the following wavelengths, in µm: 3.4 (vs), 6.8 (s), 7.2 (m), and 10.4 (m).

B.

Sample: Use an empty stainless steel cylinder equipped with a stainless steel valve and having a capacity of NLT 200 mL and a pressure rating of 240 psi or more. Dry the cylinder with the valve open at 110° for 2 h, and evacuate the hot cylinder to less than 1 mm of mercury.

Close the valve, cool, and weigh. Connect one end of a charging line tightly to the butane container and the other end loosely to the empty cylinder. Carefully open the butane container, and allow the butane to flush out the charging line through the loose connection. Avoid excessive flushing, which causes moisture to freeze in the charging line and connections. Tighten the fitting on the empty cylinder, and open the empty cylinder valve, allowing the butane to flow into the evacuated cylinder. Continue sampling until the desired amount of butane is obtained, then close the butane container valve, and finally close the sample cylinder valve.

[**CAUTION**—Do not overload the sample cylinder; hydraulic expansion due to temperature change can cause overloaded cylinders to explode.] Weigh the charged sample cylinder, and determine the weight.

Analysis: Determine the vapor pressure of the Sample at 21° by means of a suitable pressure gauge.

Acceptance criteria: 205–235 kPa absolute (30–34 psia)

ASSAY

PROCEDURE

Chromatographic system

(See Chromatography <621>, System Suitability.)

Mode: GC

Detector: Thermal conductivity

Column: 3-mm × 6-m aluminum; packed with 10 weight percent of liquid phase G30 on nonacid-washed support S1C

Column temperature: 33°

Carrier gas: Helium

Flow rate: 50 mL/min

Injection volume: 2 µL

System suitability

Sample: *n*-Butane

Suitability requirements: The peak responses of *n*-butane in the chromatograms from duplicate determinations agree within 1%.

Analysis

Samples: Connect 1 Butane cylinder to the chromatograph through a suitable sampling valve and a flow control valve downstream from the sampling valve.

Flush the liquid specimen through the sampling valve, taking care to avoid entrapment of gas or air in the sampling valve.

Calculate the purity by dividing 100 times the *n*-butane response by the sum of all of the responses.

Acceptance criteria: NLT 97.0%

SPECIFIC TESTS

• HIGH-BOILING RESIDUES

Sample: Use the *Sample* from Identification test B

Analysis: Prepare a cooling coil from copper tubing (about 6-mm outside diameter × about 6.1-m long) to fit into a vacuum-jacketed flask. Immerse the cooling coil in a mixture of dry ice and acetone in a vacuum-jacketed flask, and connect one end of the tubing to the *Sample*. Carefully open the sample cylinder valve, flush the cooling coil with about 50 mL of the *Sample*, and discard this portion of liquefied sample. Continue delivering liquefied sample from the cooling coil, and collect it in a previously chilled 1000-mL sedimentation cone until the cone is filled to the 1000-mL mark. Allow the sample to evaporate, using a warm water bath maintained at about 40° to reduce evaporating time. When all of the liquid has evaporated, rinse the sedimentation cone with two 50-mL portions of pentane, and combine the rinsings in a tared 150-mL evaporating dish. Transfer 100 mL of the pentane solvent to a second tared 150-mL evaporating dish, place both evaporating dishes on a water bath, evaporate to dryness, and heat the dishes in an oven at 100° for 60 min. Cool the dishes in a desiccator, and weigh. Repeat the heating for 15-min periods until successive weighings are within 0.1 mg, and calculate the weight of the residue obtained from the *Sample* as the difference between the weights of the residues in the two evaporating dishes.

Acceptance criteria: NMT 5 µg/mL

• ACIDITY OF RESIDUE

Sample solution: Add 10 mL of water to the residue obtained in the test for *High-Boiling Residues*.

Analysis: Mix the *Sample solution* by swirling for 30 s, add 2 drops of methyl orange TS, insert the stopper in the tube, and shake vigorously.

Acceptance criteria: No pink or red color appears in the aqueous layer.

• LIMIT OF SULFUR COMPOUNDS

Analysis: Carefully open the container valve to produce a moderate flow of gas. Do not direct the gas stream toward the face, but deflect a portion of the stream toward the nose.

Acceptance criteria: The odor is free from the characteristic odor of sulfur compounds.

• WATER DETERMINATION (921)

Sample: 100 g of the *Sample* from Identification test B

Analysis: Proceed as directed in the chapter with the following modifications. (a) Provide the closed-system titrating vessel with an opening through which passes a coarse-porosity gas dispersion tube connected to a sampling cylinder. (b) Dilute the *Reagent* with anhydrous methanol to give a water equivalence factor of 0.2–1.0 mg/mL; age this diluted solution for NLT 16 h

before standardization. (c) Introduce the *Sample* into the titration vessel through the gas dispersion tube at a rate of about 100 mL/min; if necessary, heat the sample cylinder gently to maintain this flow rate.

Acceptance criteria: NMT 0.001%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight cylinders, and prevent exposure to excessive heat.

Butyl Alcohol

DEFINITION

Butyl Alcohol is *n*-butyl alcohol.

IMPURITIES

• BUTYL ETHER

Sample: Butyl Alcohol

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Thermal-conductivity detector

Column: 6-mm × 2-m stainless steel column packed with 25% liquid phase G29 3,3'-thiodipropionitrile on 30- to 40-mesh support S1C

Column temperature: 85°

Carrier gas: Helium

Flow rate: 75 mL/min

Injection size: 10 µL

Analysis

Sample: *Sample*

[NOTE—The retention times for butyl ether, 2-butanol, water, isobutyl alcohol, and butyl alcohol are approximately 6.0, 12.0, 17.0, 18.0, and 25.0 min, respectively.]

Acceptance criteria: The response due to butyl ether is NMT 0.2% of the sum of all of the responses.

SPECIFIC TESTS

- **SPECIFIC GRAVITY (841):** 0.807–0.809
- **DISTILLING RANGE, Method II (721):** Butyl Alcohol distills within a range of 1.5°, including 117.7°.

• ACIDITY

Sample: 74 mL (60 g)

Analysis: Titrate the *Sample* with 0.020 N alcoholic potassium hydroxide, using phenolphthalein TS as the indicator, until a pink color persists for NLT 15 s

Acceptance criteria: NMT 2.5 mL is consumed

• WATER DETERMINATION, Method I (921):

• LIMIT OF NONVOLATILE RESIDUE

Sample: 100 mL

Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath, and dry at 105° for 30 min

Acceptance criteria: The weight of the residue does not exceed 4 mg (0.004%).

• ALDEHYDES

Sample: 10 mL

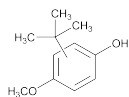
Analysis: To 10 mL of silver-ammonia-nitrate TS in a test tube add the *Sample*, and mix. Allow the mixture to stand in a dark place for 30 min

Acceptance criteria: No color is produced, although a slight precipitate may form at the interface of the two layers.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and prevent exposure to excessive heat.

Butylated Hydroxyanisole



$C_{11}H_{16}O_2$ 180.24
Phenol, (1,1-dimethylethyl)-4-methoxy-;
tert-Butyl-4-methoxyphenol [25013-16-5].

DEFINITION

Butylated Hydroxyanisole contains NLT 98.5% of $C_{11}H_{16}O_2$.

IDENTIFICATION

• A. PROCEDURE

Analysis: To 5 mL of 0.1 mg/mL solution in 72% alcohol add 2 mL of 20 mg/mL sodium borate solution and 1 mL of 0.1 mg/mL solution of 2,6-dichloroquinone-chlorimide in dehydrated alcohol, and mix.

Acceptance criteria: A blue color is produced.

- **B.** The retention times of 3-*tert*-butyl-4-hydroxyanisole and 2-*tert*-butyl-4-hydroxyanisole from the *Sample solution* correspond to those from the *Standard solution*, as obtained in the Assay.

ASSAY

• PROCEDURE

Solution A: 5% acetic acid

Mobile phase: Acetonitrile and *Solution A* (45:55)

Standard solution: 90 µg/mL of USP 3-*tert*-Butyl-4-hydroxyanisole RS and 10 µg/mL of USP 2-*tert*-Butyl-4-hydroxyanisole RS in *Mobile phase*

Sample solution: 100 µg/mL of Butylated Hydroxyanisole in *Mobile phase*

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 290 nm

Column: 4.6-mm × 75-mm; 3.5-µm packing L1

Column temperature: 30°

Flow rate: 1.2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

[NOTE—The retention times of 2-*tert*-butyl-4-hydroxyanisole and 3-*tert*-butyl-4-hydroxyanisole are about 4.2 and 4.6 min, respectively.]

Suitability requirements

Resolution: NLT 1.5 between the 3-*tert*-butyl-4-hydroxyanisole isomer and 2-*tert*-butyl-4-hydroxyanisole isomer peaks

Tailing factor: NMT 1.5

Relative standard deviation: NMT 2.0% for the 3-*tert*-butyl-4-hydroxyanisole isomer and 2-*tert*-butyl-4-hydroxyanisole isomer peaks

Analysis

Samples: *Standard solution* and *Sample solution*

Measure the peak areas for each isomer. Calculate the percentage of each isomer in the portion of Butylated Hydroxyanisole taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

- r_U = peak area of the corresponding isomer from the *Sample solution*
 r_S = peak area of the corresponding isomer from the *Standard solution*
 C_S = concentration of the appropriate USP Reference Standard in the *Standard solution* (µg/mL)
 C_U = concentration of the *Sample solution* (µg/mL)

[NOTE—Calculate the percentage of $C_{11}H_{16}O_2$ in the portion of Butylated Hydroxyanisole taken by adding the quantities of the two isomers.]

Acceptance criteria: NLT 98.5%

IMPURITIES

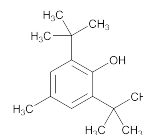
Inorganic Impurities

- **RESIDUE ON IGNITION** <281>: NMT 0.01%, determined on a 10-g specimen
- **HEAVY METALS**, *Method II* <231>: NMT 10 ppm

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** <11>
 USP 2-*tert*-Butyl-4-hydroxyanisole RS
 $C_{11}H_{16}O_2$ 180.25
 USP 3-*tert*-Butyl-4-hydroxyanisole RS
 $C_{11}H_{16}O_2$ 180.25

Butylated Hydroxytoluene



$C_{15}H_{24}O$ 220.35
Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl-;
2,6-Di-*tert*-butyl-*p*-cresol [128-37-0].

DEFINITION

Butylated Hydroxytoluene contains NLT 99.0% of $C_{15}H_{24}O$.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>: On undried sample

IMPURITIES

- **RESIDUE ON IGNITION** <281>

Analysis: Transfer 50 g to a tared crucible, ignite until thoroughly charred, and cool. Moisten the ash with 1 mL of sulfuric acid, and complete the ignition by heating at $800 \pm 25^\circ$ for 15-min periods to constant weight.

Acceptance criteria: NMT 0.002%

- **HEAVY METALS**, *Method II* <231>: NMT 10 ppm

ORGANIC IMPURITIES

Solution A: 50 mg/mL of potassium ferricyanide

Solution B: 105 mg/mL of ferric chloride

Sample solution: 20 mg/mL of Butylated Hydroxytoluene in methanol

Standard solution: 0.1 mg/mL of Butylated Hydroxytoluene from the *Sample solution* diluted with methanol

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Mode: TLC

Absorbent: 0.25-mm layer of chromatographic silica gel mixture

Developing solvent system: Methylene chloride

Application volume: 10 µL

Spray reagent: *Solution A*, *Solution B*, and water (1:2:7), freshly prepared

Analysis

Samples: *Sample solution* and *Standard solution*

Apply the *Samples*, and allow to dry. Place the plate in the chromatographic chamber, and develop until the solvent front has moved three-fourths of the length of the plate. Remove the plate, and allow the plate to dry. Spray the plate with *Spray reagent*.

Acceptance criteria: Any spot from the *Sample solution*, apart from the principal spot, is not more intense than the spot from the *Standard solution* (NMT 0.5%).

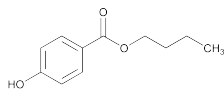
SPECIFIC TESTS

- **CONGEALING TEMPERATURE** (651): NLT 69.2°, corresponding to NLT 99.0% of C₁₅H₂₄O

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** (11)
USP Butylated Hydroxytoluene RS

Butylparaben



C₁₁H₁₄O₃ 194.23
Benzoic acid, 4-hydroxy-, butyl ester;
Butyl *p*-hydroxybenzoate [94-26-8].

DEFINITION

Butylparaben contains NLT 98.0% and NMT 102.0% of butylparaben (C₁₁H₁₄O₃).

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197M)
- **B. MELTING RANGE OR TEMPERATURE** (741): 68°–71°

ASSAY

PROCEDURE

Sample: 1.000 g

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 1 N sulfuric acid VS

Endpoint detection: Visual

Analysis: To the *Sample* add 20.0 mL of 1 N sodium hydroxide VS, and heat at 70° for 1 h. Cool rapidly in an ice bath. Carry out the titration on the solutions at room temperature. Titrate the excess sodium hydroxide with *Titrant*, continuing the titration until the second point of inflection. Perform a blank determination. Each mL of 1 N sodium hydroxide VS is equivalent to 194.2 mg of butylparaben (C₁₁H₁₄O₃).

Acceptance criteria: 98.0%–102.0%

IMPURITIES

- **RESIDUE ON IGNITION** (281)
Sample: 1.0 g
Acceptance criteria: NMT 0.1%

RELATED COMPOUNDS

Adsorbent: 0.25-mm layer of chromatographic octadecylsilylanized silica gel mixture

Sample solution: 10 mg/mL of Butylparaben in acetone

Standard solution A: Transfer 0.5 mL of the *Sample solution* to a 100-mL volumetric flask, and dilute with acetone to volume.

Standard solution B: Dissolve 10 mg of USP Propylparaben RS in 1 mL of the *Sample solution*, and dilute with acetone to 10 mL.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 2 µL

Developing solvent system: Methanol, glacial acetic acid, and water (70:1:30)

Analysis

Samples: *Sample solution* and *Standard solutions*
Develop the chromatogram in the *Developing solvent system* until the solvent front has moved three-fourths of the length of the plate. Examine the plate under short-wavelength UV light, and compare the intensities of any secondary spots observed from the *Sample solution* with that of the principal spot from *Standard solution A*. The test is not valid unless the chromatogram obtained with *Standard solution B* shows two clearly separated principal spots.

Acceptance criteria: NMT 0.5%; the intensity of any individual secondary spot from the *Sample solution* is NMT that of the principal spot obtained from *Standard solution A*.

SPECIFIC TESTS

ACIDITY

Sample solution: To 2 mL of *Sample solution* prepared in the test for *Color of Solution*, add 3 mL of alcohol, 5 mL of carbon dioxide-free water, and 0.1 mL of bromocresol green TS.

Analysis: Titrate with 0.10 N sodium hydroxide.

Acceptance criteria: NMT 0.1 mL is required to produce a blue color.

COLOR OF SOLUTION

Sample solution: 100 mg/mL in alcohol

Comparison solution: Mix 2.4 mL of ferric chloride CS, 1.0 mL of cobaltous chloride CS, and 0.4 mL of cupric sulfate CS with 0.3 N hydrochloric acid to make 10 mL. Dilute 5 mL of this solution with 0.3 N hydrochloric acid to make 100 mL. Prepare and use this solution immediately.

Analysis

Samples: Alcohol, *Sample solution*, and *Comparison solution*

Make the comparison by viewing the solutions downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* (631)).

Acceptance criteria: The *Sample solution* is clear and not more intensely colored than alcohol or the *Comparison solution*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** (11)
USP Butylparaben RS
USP Propylparaben RS

Calcium Carbonate—see *Calcium Carbonate General Monographs*

Calcium Chloride—see *Calcium Chloride General Monographs*

Tribasic Calcium Phosphate

Ca₅(OH)(PO₄)₃

502.31

Calcium hydroxide phosphate;

Calcium hydroxide phosphate [12167-74-7].

DEFINITION

Tribasic Calcium Phosphate consists of a variable mixture of calcium phosphates having the approximate composition 10CaO · 3P₂O₅ · H₂O. It contains NLT 34.0% and NMT 40.0% of calcium (Ca).

IDENTIFICATION

- A.**
Sample solution: Dissolve 100 mg in 5 mL of diluted nitric acid.
Analysis: Warm the *Sample solution*, and add ammonium molybdate TS.
Acceptance criteria: A yellow precipitate is formed.
- B. IDENTIFICATION TESTS—GENERAL, Calcium (191):** Meets the requirements of the flame test

ASSAY**PROCEDURE**

Sample: 150 mg of Tribasic Calcium Phosphate
Blank: Proceed as directed in the *Analysis*, omitting the test specimen.
Titrimetric system
 (See *Titrimetry* (541).)
Mode: Direct titration
Titrant: 0.05 M edetate disodium VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample*, with the aid of gentle heat if necessary, in a mixture of hydrochloric acid and water (5:3), contained in a 250-mL beaker equipped with a magnetic stirrer, and cautiously add 125 mL of water. With constant stirring, add, in the following order, 0.5 mL of triethanolamine, 300 mg of hydroxy naphthol blue, and from the titration buret, about 23 mL of *Titrant*. Add sodium hydroxide solution (45 in 100) until the initial red color changes to clear blue. Continue to add it dropwise until the color changes to violet, and add an additional 0.5 mL. The pH is 12.3–12.5. Continue the titration dropwise with the *Titrant* to the appearance of a clear blue endpoint that persists for NLT 60 s.
 Calculate the percentage of calcium (Ca) in the *Sample* taken:

$$\text{Result} = \left\{ \left[(V_S - V_B) \times M \times F \right] / W \right\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
 V_B = *Titrant* volume consumed by the *Blank* (mL)
 M = actual molarity of the *Titrant* (mM/mL)
 F = equivalency factor, 40.08 mg/mM
 W = *Sample* weight (mg)
Acceptance criteria: 34.0%–40.0%

IMPURITIES

- CHLORIDE AND SULFATE, Chloride (221)**
Standard: 1.0 mL of 0.020 N hydrochloric acid
Sample: 0.500 g of Tribasic Calcium Phosphate
Analysis: Dissolve the *Sample* in 25 mL of 2 N nitric acid, and add 1 mL of silver nitrate TS.
Acceptance criteria: NMT 0.14%
- CHLORIDE AND SULFATE, Sulfate (221)**
Standard: 1.0 mL of 0.020 N sulfuric acid
Sample: 0.500 g of Tribasic Calcium Phosphate
Analysis: Dissolve the *Sample* in the smallest possible amount of 3 N hydrochloric acid. Dilute with water to 100 mL, and filter, if necessary. To 25 mL of the filtrate add 1 mL of barium chloride TS.
Acceptance criteria: NMT 0.8%
- ARSENIC, Method I (211)**
Test preparation: Dissolve 1.0 g in just sufficient 3 N hydrochloric acid.
Acceptance criteria: NMT 3 ppm
- BARIUM**
Sample: 500 mg of Tribasic Calcium Phosphate
Analysis: Mix the *Sample* with 10 mL of water, heat, add hydrochloric acid, dropwise, until solution is effected, and then add 2 drops of the acid in excess. Filter, and add to the filtrate 1 mL of potassium sulfate TS.
Acceptance criteria: No turbidity appears within 15 min.

HEAVY METALS, Method I (231)

Test preparation: Mix 1.3 g with 9 mL of 3 N hydrochloric acid, dilute with water to 50 mL, and heat to boiling. Cool to room temperature, and filter.
 [NOTE—Filter the mixture after the pH adjustment.]

Acceptance criteria: NMT 30 ppm

CARBONATE

Sample: 2 g of Tribasic Calcium Phosphate
Analysis: Mix the *Sample* with 20 mL of water, and add 3 N hydrochloric acid, dropwise, to effect solution.
Acceptance criteria: No effervescence is produced.

ACID-INSOLUBLE SUBSTANCES

Analysis: If an insoluble residue remains in the test for *Carbonate*, boil the solution, filter, wash the residue well with hot water until the last washing is free from chloride, and ignite the residue to constant weight.

Acceptance criteria: The weight of the residue does not exceed 4 mg (NMT 0.2%).

DIBASIC SALT AND CALCIUM OXIDE

Sample: 1.5 g of Tribasic Calcium Phosphate
Blank: 25.0 mL of *Titrant*

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 1 N hydrochloric acid VS

Back-titrant: 0.1 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Dissolve the *Sample* by warming with 25.0 mL of the *Titrant*. Cool, and slowly titrate the excess of *Titrant*, while agitating constantly, with the *Back-titrant* to a pH of 4.0.

Acceptance criteria: 13.0–14.3 mL of 1 N hydrochloric acid is consumed for each g of salt, calculated on the ignited basis.

LIMIT OF FLUORIDE

[NOTE—Prepare and store all solutions in plastic containers.]

Buffer solution: 294 mg/mL of sodium citrate dihydrate in water

Standard stock solution: 1.1052 mg/mL of USP Sodium Fluoride RS in water

Standard solution: Transfer 20.0 mL of *Standard stock solution* to a 100-mL volumetric flask containing 50.0 mL of *Buffer solution*, dilute with water to volume, and mix. Each mL of this solution contains 100 µg of fluoride ion.

Sample solution: Transfer 2.0 g of Tribasic Calcium Phosphate to a beaker containing a plastic-coated stirring bar. Add 20 mL of water and 3.0 mL of hydrochloric acid, and stir until dissolved. Add 50.0 mL of *Buffer solution* and sufficient water to make 100 mL.

Electrode system: Use a fluoride-specific ion-indicating electrode and a silver–silver chloride reference electrode connected to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.2 mV (see pH (791)).

Analysis

Samples: *Standard solution* and *Sample solution*

Standard response line: Transfer 50.0 mL of *Buffer solution* and 3.0 mL of hydrochloric acid to a beaker, and add water to make 100 mL. Add a plastic-coated stirring bar, insert the electrodes into the solution, stir for 15 min, and read the potential, in mV. Continue stirring, and at 5-min intervals add 100, 100, 300, 500, and 500 µL of *Standard solution*, reading the potential 5 min after each addition. Plot the logarithms of the cumulative fluoride ion concentrations (0.1, 0.2, 0.5, 1.0, and 1.5 µg/mL) versus potential, in mV.

Rinse and dry the electrodes, insert them into the *Sample solution*, stir for 5 min, and read the potential, in mV. From the measured potential and the *Standard response line* determine the concentration, C (in µg/mL), of fluoride ion in the *Sample solution*.

Calculate the content of fluoride in the portion of Tribasic Calcium Phosphate taken:

$$\text{Result} = (V \times C)/W$$

- V = volume of the *Sample solution* (mL)
 C = concentration of fluoride ion in the *Sample solution* ($\mu\text{g/mL}$), determined from the *Standard response line*
 W = weight of Tribasic Calcium Phosphate taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 75 ppm

• **LIMIT OF NITRATE**

Sample: 200 mg of Tribasic Calcium Phosphate

Analysis: Mix the *Sample* with 5 mL of water, and add just sufficient hydrochloric acid to effect solution. Dilute with water to 10 mL, add 0.20 mL of indigo carmine TS, then add, with stirring, 10 mL of sulfuric acid.

Acceptance criteria: The blue color persists for NLT 5 min.

• **WATER-SOLUBLE SUBSTANCES**

Sample: 2 g of Tribasic Calcium Phosphate

Analysis: Digest the *Sample* with 100 mL of water on a steam bath for 30 min. Cool, add sufficient water to restore the original volume, stir well, and filter.

Evaporate 50 mL of the filtrate in a tared porcelain dish on a steam bath to dryness, and dry the residue at 120° to constant weight.

Acceptance criteria: The weight of the residue does not exceed 5 mg (NMT 0.5%).

SPECIFIC TESTS

• **LOSS ON IGNITION** (733)

Analysis: Ignite a sample at 800° for 30 min.

Acceptance criteria: NMT 8.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Calcium Propionate

$\text{C}_6\text{H}_{10}\text{CaO}_4$ 186.22
 [4075-81-4].

DEFINITION

Calcium Propionate contains NLT 98.0% and NMT 100.5% of $\text{C}_6\text{H}_{10}\text{CaO}_4$, calculated on the anhydrous basis.

IDENTIFICATION

- **IDENTIFICATION TESTS—GENERAL,** *Calcium* (191): A solution (1 in 20) meets the requirements of the flame test.

ASSAY

• **PROCEDURE**

Sample solution: 4 mg/mL of Calcium Propionate

Analysis: While stirring, preferably with a magnetic stirrer, add 30 mL of 0.05 M disodium EDTA from a 50-mL buret, to 100 mL of *Sample solution*. Add 15 mL of 1 N sodium hydroxide and 300 mg of hydroxy naphthol blue indicator, and continue the titration to a blue endpoint. Each mL of 0.05 M disodium EDTA is equivalent to 9.311 mg of $\text{C}_6\text{H}_{10}\text{CaO}_4$.

Acceptance criteria: 98.0%–100.5% on the anhydrous basis.

IMPURITIES

Inorganic Impurities

• **LIMIT OF FLUORIDE**

Buffer: 294.1 mg/mL of sodium citrate dihydrate

Standard solution: 2.21 mg/mL of USP Sodium Fluoride RS. [NOTE—Store solution in a plastic bottle.] On the day of use, transfer 5.0 mL of the resulting solution

to a 1000-mL volumetric flask, dilute with water to volume, and mix. Each mL of this solution contains 5 μg of fluoride ion.

Electrode system: Use a fluoride-specific, ion-indicating electrode and a silver–silver chloride reference electrode connected to a pH meter capable of measuring potentials with a minimum reproducibility of ± 0.2 mV (see pH (791)).

Standard response line: Transfer 1.0, 2.0, 3.0, 5.0, 10.0, and 15.0 mL of the *Standard solution* into separate 250-mL plastic beakers. Add 50 mL of water, 5 mL of 1 N hydrochloric acid, 10 mL of *Buffer*, and 10 mL of 0.2 M disodium EDTA to each beaker, and mix. Transfer each solution into separate 100-mL volumetric flasks, dilute with water to volume, and mix. Transfer a 50-mL portion of each solution into separate 125-mL plastic beakers, and read the potential, in mV, of each solution using the electrode system. Plot the calibration curve versus potential, in mV, on two-cycle semilogarithmic paper with μg of fluoride/100 mL of solution on the logarithmic scale.¹

Sample: 1.0 g

Analysis: Transfer the *Sample* into a 150-mL glass beaker, add 10 mL of water, and, while stirring continuously, slowly add 20 mL of 1 N hydrochloric acid to dissolve the *Sample*. Boil rapidly for 1 min, then transfer into a 250-mL plastic beaker, and cool rapidly in ice water. Add 15 mL of *Buffer* and 10 mL of 0.2 M disodium EDTA, and mix. Adjust the pH to 5.5 ± 0.1 with 1 N hydrochloric acid or 1 N sodium hydroxide, if necessary. Transfer into a 100-mL volumetric flask, dilute with water to volume, and mix. Transfer a 50-mL portion of this solution into a 125-mL plastic beaker, and record the potential using the electrode system. Determine the concentration of fluoride, in $\mu\text{g/mL}$, in the *Sample*.

Acceptance criteria: NMT 30 ppm

• **LIMIT OF LEAD**

Standard lead solution: Prepare as directed under *Heavy Metals* (231), *Special Reagents*.

Diluted standard lead solutions: On the day of use, transfer 5.0, 10.0, and 20.0 mL of *Standard lead solution* into three separate 100-mL volumetric flasks, add 10 mL of 3 N hydrochloric acid to each, and dilute with water to volume corresponding to 0.5-, 1.0-, and 2.0- $\mu\text{g/mL}$ standards.

25% Sulfuric acid solution: Cautiously add 100 mL of sulfuric acid to 300 mL of water with constant stirring while cooling in an ice bath.

Sample blank: Add 5 mL of 25% *Sulfuric acid solution* into an evaporating dish. Within a hood, place the dish on a steam bath to evaporate most of the water. Place the dish on a burner, and slowly pre-ash the sample by expelling most of the sulfuric acid. Place the dish in a muffle furnace that has been set at 525°, and ash the sample until the residue appears free from carbon. Cool, and cautiously wash down the inside of the evaporation dish with water. Add 5 mL of 1 N hydrochloric acid. Place the dish on a steam bath, and evaporate to dryness. Add 1.0 mL of 3 N hydrochloric acid and approximately 5 mL of water, and heat briefly on a steam bath to dissolve any residue. Transfer the solution quantitatively to a 10-mL volumetric flask, dilute to volume, and mix.

Sample solution: Place 10 g of Calcium Propionate, to the nearest 0.1 mg, into an evaporating dish. Add a sufficient amount of 25% *Sulfuric acid solution*, and distribute the sulfuric acid uniformly through the sample. Proceed as directed under *Sample blank* beginning with "Within a hood".

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

¹A suitable source for semilogarithmic paper is provided at the following web-site: <http://statland.org/GraphPaper/gpaper.html>.

Mode: Atomic absorption
Lamp: Lead electrodeless discharge
Flame: Air-acetylene
Analytical wavelength: 283.3 nm
Slit width: 0.7 nm
Instrument blank: Water

Standard curve

Samples: *Diluted standard lead solutions* and *Sample blank*

Plot: Corrected absorbance values versus their corresponding concentration ($\mu\text{g/mL}$). [NOTE—Determine corrected absorbance values by subtracting the absorbance of the *Sample blank* from the absorbance of the *Diluted standard lead solutions*.]

Analysis

Samples: *Sample solution* and *Sample blank*
 [NOTE—Determine corrected absorbance values by subtracting the absorbance of the *Sample blank* from the absorbance of the *Sample solution*.]

From the *Standard curve*, determine the lead concentration in the *Sample solution*. Calculate the lead content, in ppm, in the portion of Calcium Propionate taken:

$$\text{Result} = C_s/W \times V$$

C_s = concentration of lead from the *Standard curve* ($\mu\text{g/mL}$)

W = weight of the sample taken (g)

V = final volume of the sample (mL)

Acceptance criteria: NMT 2 ppm

- MAGNESIUM** (as MgO)

Magnesium standard solution: Dissolve 50.0 mg of magnesium metal in 1 mL of hydrochloric acid in a 1000-mL volumetric flask, dilute with water to volume, and mix.

Sample solution: Place 400.0 mg of Calcium Propionate, 5 mL of 2.7 N hydrochloric acid, and about 10 mL of water in a small beaker, and dissolve the Calcium Propionate by heating on a hot plate.

Analysis: Evaporate the *Sample solution* to a volume of about 2 mL, and cool. Transfer the residual liquid into a 100-mL volumetric flask, dilute with water to volume, and mix. Dilute 7.5 mL of this solution with water to 20 mL, add 2 mL of 1 N sodium hydroxide and 0.05 mL of a 1:1000 solution of thiazole yellow, mix, allow to stand for 10 min, and shake. Any color produced does not exceed that produced by 1.0 mL of *Magnesium standard solution* in the same volume as that of a control containing 2.5 mL of the *Sample solution* (corresponding to 10 mg of Calcium Propionate) and the quantities of the reagents used in the test.

Acceptance criteria: The solution passes the test (about 0.4%).

SPECIFIC TESTS

- WATER INSOLUBLE SUBSTANCES**

Sample: 10 g

Analysis: Dissolve the *Sample* in 100 mL of hot water, pass through a tared filtering crucible, wash the insoluble residue with hot water, and dry at 105° to constant weight.

Acceptance criteria: NMT 0.2%

- PH** (791): 7.5–10.5, in a solution (1 in 10)

- WATER DETERMINATION, Method I** (921): NMT 5.0%

- LOSS ON DRYING** (731): Dry a sample at 105° for 2 h: it loses NMT 4% of its weight.

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Store in tightly closed containers. No storage requirements specified.

- USP REFERENCE STANDARDS** (11)
 USP Sodium Fluoride RS

Calcium Saccharate—see Calcium Saccharate General Monographs

Calcium Silicate

DEFINITION

Calcium Silicate, crystalline or amorphous, is a compound of calcium oxide and silicon dioxide. It contains NLT 4.0% of calcium oxide and NLT 35.0% of silicon dioxide.

IDENTIFICATION

- A. IDENTIFICATION TESTS—GENERAL, Calcium** (191)

Sample solution: Mix 0.5 g with 10 mL of 3 N hydrochloric acid. Filter, and neutralize the filtrate to litmus paper with 6 N ammonium hydroxide.

Acceptance criteria: The neutralized filtrate meets the requirements.

- B.**

Analysis: Prepare a bead by fusing a few crystals of sodium ammonium phosphate on a platinum loop in the flame of a gas burner. Place the hot, transparent bead in contact with the specimen of Calcium Silicate, and again fuse.

Acceptance criteria: Silica floats about in the bead, producing, upon cooling, an opaque bead having a web-like structure.

ASSAY

- SILICON DIOXIDE**

Sample: Calcium Silicate in the appropriate amount (see Table 1)

Table 1

Sample Weight (mg)	Calcium Oxide Content (%)
400	>25
600	11–25
1000	4–10

Analysis: Transfer the *Sample* to a beaker, add 5 mL of water and 10 mL of perchloric acid, and heat until dense white fumes of perchloric acid are evolved. Cover the beaker with a watch glass, and continue to heat for 2 h. Allow to cool, add 30 mL of water, filter, and wash the precipitate with 200 mL of hot water. [NOTE—Retain the combined filtrate and washings for use in the assay for Calcium Oxide.]

Transfer the filter paper and its contents to a platinum crucible, heat slowly to dryness, then heat sufficiently to char the filter paper. Ignite at about 900° – 1000° to constant weight. Moisten the residue with 5 drops of perchloric acid, add 15 mL of hydrofluoric acid, heat cautiously on a hot plate until all of the acid is driven off, and ignite at a temperature NLT 1000° to constant weight. Cool in a desiccator, and weigh: the loss in weight represents the weight of SiO_2 .

Acceptance criteria: NLT 35.0% of silicon dioxide (SiO_2) and 90.0%–110.0% of the content stated in the labeling, or within the range of percentages stated in the labeling

- CALCIUM OXIDE**

Sample solution: Use the combined filtrate and washings retained from the assay for *Silicon Dioxide*.

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.05 M edetate disodium VS

Endpoint detection: Visual

Analysis: Neutralize the *Sample solution* to litmus with 1 N sodium hydroxide. Add, while stirring, 10 mL of 0.05 M edetate disodium VS from a 50-mL buret. Add

15 mL of 1 N sodium hydroxide and 300 mg of hydroxy naphthol blue, and continue the titration to a blue endpoint.

Calculate *Z*, the percentage of calcium oxide (CaO) in the sample taken:

$$Z = [(V \times M \times F)/W] \times 100$$

V = volume of *Titrant* consumed by the *Sample solution* (mL)

M = actual molarity of the *Titrant* (mmol/mL)

F = equivalency factor, 56.08 mg/mmol

W = weight of the sample (mg)

Calculate the percentage of the labeled amount of calcium oxide (CaO):

$$\text{Result} = (Z/L) \times 100$$

Z = percentage of CaO in the sample taken as calculated above

L = labeled amount of CaO

Acceptance criteria: NLT 4.0% of calcium oxide (CaO) and 90.0%–110.0% of the content stated in the labeling, or within the range of percentages stated in the labeling

IMPURITIES

- **LOSS ON IGNITION** (733): Transfer 1 g to a suitable tared crucible, dry at 105° for 2 h, and ignite at 900° to constant weight: it loses NMT 20.0% of its weight.

- **HEAVY METALS** (231)

Test preparation: Boil 4.0 g with a mixture of 50 mL of water and 10 mL of hydrochloric acid for 20 min, adding water to maintain the volume during the boiling. Add ammonium hydroxide until the mixture is only slightly acid to litmus paper. Filter with the aid of suction, and wash with 15–20 mL of water, combining the washing with the original filtrate. Add 2 drops of phenolphthalein TS, then add a slight excess of 6 N ammonium hydroxide. Discharge the pink color with dilute hydrochloric acid (1 in 100). Dilute with water to 100 mL, and use 25 mL of the solution for the test.

Acceptance criteria: NMT 20 ppm

- **LIMIT OF LEAD**

Palladium matrix modifier: Palladium nitrate (1% Pd)¹

Magnesium matrix modifier: Magnesium nitrate (2% Mg)²

Nitric acid, 65% [7697-37-2]: Use a suitable grade with a content of NLT 65.0%.³

Nitric acid diluent: Transfer 42 mL of *Nitric acid*, 65% to a 100-mL volumetric flask. Dilute with water to volume.

Matrix modifier solution: Transfer 1.0 mL of *Palladium matrix modifier* and 100 µL of *Magnesium matrix modifier* to a 20-mL volumetric flask. Dilute with water to volume, and mix.

Lead standard solution: A solution containing Pb(NO₃)₂ in 0.5 M nitric acid corresponding to 1000 mg of lead/mL⁴

Standard solution: Transfer 100 µL of *Lead standard solution* to a 100-mL volumetric flask. Dilute with water to volume, and mix. This solution contains the equivalent of 1.0 µg/mL of lead.

Calibration solutions

Calibration solution A: Transfer 50 mL of *Nitric acid diluent* to a 100-mL volumetric flask, and dilute with water to volume (0 µg/mL of lead).

Calibration solution B: Transfer 1 mL of *Standard solution* and 50 mL of *Nitric acid diluent* to a 100-mL

volumetric flask, and dilute with water to volume (0.01 µg/mL of lead).

Calibration solution C: Transfer 5 mL of *Standard solution* and 50 mL of *Nitric acid diluent* to a 100-mL volumetric flask, and dilute with water to volume (0.05 µg/mL of lead).

Sample solution: Weigh 2.0 g of Calcium Silicate into a 150-mL beaker. Mix with 50 mL of *Nitric acid diluent*. Cover with a watch glass, and boil for 20 min. Allow to cool. With the aid of a vacuum, pass through a glass filter,⁵ and wash the filter several times with water. Transfer the filtrate into a 100-mL volumetric flask. Dilute with water to volume, and mix.

System suitability solution: Transfer 5 mL of *Standard solution* and 50 mL of *Nitric acid diluent* into a 100-mL volumetric flask. Dilute with water to volume, and mix. This solution contains the equivalent of 0.05 µg of lead/mL.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Graphite furnace atomic absorption spectrophotometer

Analytical wavelength: 283.3 nm (lead emission line)

Lamp: Lead hollow-cathode lamp and an adequate means of Zeeman background correction

Carrier gas: Argon

Under typical conditions, the *Sample solution* and *Calibration solutions* volumes are 20 µL, the volume of the *Matrix modifier solution* is 10 µL, the injection temperature is 20°, and the oven conditions are as follows (see *Table 2*).

[NOTE—These conditions may be optimized for each instrument.]

Table 2

Step	Temperature (°)
Drying 1	110
Drying 2	130
Pyrolysis	950
Read	1800
Clean out	2450

Analysis

Samples: *Calibration solutions*, *Sample solution*, and *System suitability solution*

Plot the absorbance of each *Calibration solution* versus its content of lead, in µg/mL, and draw the best straight line fitting the three points. From this plot, determine the concentration, in µg/mL, of lead in the *Sample solution*: the correlation coefficient is NLT 0.99, and the recovery for the *System suitability solution* is 85%–115%. Calculate the quantity, in ppm, of lead in the *Sample solution*.

$$\text{Result} = (C \times V)/W$$

C = concentration of the *Sample solution* (µg/mL)

V = volume of the *Sample solution* (mL)

W = weight of sample taken (g)

Acceptance criteria: NMT 10 ppm

- **LIMIT OF FLUORIDE**

[NOTE—Store all solutions in polytef containers.]

Buffer solution: 294 mg/mL of sodium citrate

Ionic strength adjustment buffer: Transfer 42 mL of hydrochloric acid, 121 g of tris(hydroxymethyl)aminomethane, and 115 g of sodium tartrate to a 500-mL volumetric flask containing 250 mL of water. Stir to dissolve, and dilute with water to volume.

¹ A suitable grade is available as catalog number RCMMPD10KN-50 from VWR, www.vwr.com.

² Available as catalog number RCMIMG20KN-50, from VWR, www.vwr.com.

³ A suitable grade is available as catalog number 441-2 from EMD Chemicals, www.emdchemicals.com.

⁴ A suitable grade is available as catalog number 1.19776 from EMD Chemicals, www.emdchemicals.com.

⁵ Glass filter: Whatman GF/B glass microfiber filters, 1.0 µm, Whatman no. 1821-090, is suitable. Available as catalog number 28497-492, from VWR, www.vwr.com.

Standard stock solution: 221 µg/mL of USP Sodium Fluoride RS. This stock solution contains 100 µg/mL of fluoride ion.

Sample solution: Transfer 2.0 g of Calcium Silicate to a 100-mL polytetrafluoroethylene beaker containing a magnetic stir bar. Add 20 mL of water and 2.0 mL of hydrochloric acid. Cover with a watch glass, and heat to a vigorous boil for 1 min, stirring continuously. Remove from heat, and cool. Transfer the cooled suspension to a 100-mL polytetrafluoroethylene beaker. Add 25 mL of *Buffer solution*, and adjust with ammonium hydroxide or hydrochloric acid to a pH of 5–6. Add 50 mL of *Ionic strength adjustment buffer* and water to make 100 mL of solution.

Electrode system: Use a fluoride-specific ion-indicating electrode and a suitable reference electrode connected to a pH meter capable of measuring potentials with a reproducibility of ±0.2 mV (see pH <791>).

Standard response line: Obtain a standard response line with four standard solutions containing 0, 0.10, 0.20, and 0.40 µg/mL of fluoride as follows. Add 23 mL of water, 2 mL of hydrochloric acid, and 25 mL of *Buffer solution* to a 100-mL plastic beaker. Adjust with ammonium hydroxide to a pH of 5–6, and add *Ionic strength adjustment buffer* to obtain 100 mL of solution. Insert the electrode into the solution, stir for at least 15 min, and record the potential for the standard solution containing 0 µg/mL of fluoride. When the electrode has stabilized, add 100 µL of the *Standard stock solution* to the beaker, and stir. Allow the electrode to stabilize for 5 min, and measure the potential for the standard solution containing 0.10 µg/mL of fluoride. Similarly add another 100 and 200 µL of the *Standard stock solution* and record the potential for the standard solutions, containing 0.20 µg/mL of fluoride and 0.40 µg/mL of fluoride, respectively. After each addition, continue to stir for 5 min before recording the reading.

Analysis: Insert the calibrated electrode into the *Sample solution*, stir for 5 min, and record the measurement. From the measured potential of the *Sample solution* and the *Standard response line*, determine the concentration, *C*, in µg/mL, of fluoride ion in the *Sample solution*. Calculate the quantity, in ppm, of fluoride in Calcium Silicate:

$$\text{Result} = (C \times V)/W$$

C = concentration of fluoride ion in the *Sample solution* (µg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Calcium Silicate in the *Sample solution* (g)

Acceptance criteria: NMT 50 ppm

SPECIFIC TESTS

- **pH <791>:** 8.4–11.2, determined in a well-mixed aqueous suspension (1 in 20)
- **RATIO OF SILICON DIOXIDE TO CALCIUM OXIDE:** Divide the percentage of silicon dioxide obtained in the test for *Silicon Dioxide* by the percentage of calcium oxide obtained in the test for *Calcium Oxide*.
Acceptance criteria: 0.5–2.0
- **SUM OF CaO, SiO₂, AND LOSS ON IGNITION:** The sum of the percentages obtained in the three tests is NLT 90.0%.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** The labeling states the claimed percentage or range of percentages for the content of calcium oxide and for the content of silicon dioxide.
- **USP REFERENCE STANDARDS <11>**
USP Sodium Fluoride RS

Calcium Stearate

Octadecanoic acid, calcium salt;
Calcium stearate [1592-23-0].

DEFINITION

Calcium Stearate is a compound of calcium with a mixture of solid organic acids obtained from fats. It consists chiefly of variable proportions of calcium stearate and calcium palmitate. It contains the equivalent of NLT 9.0% and NMT 10.5% of calcium oxide (CaO).

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Calcium <191>**

Sample: 1 g

Analysis: Heat the *Sample* with a mixture of 25 mL of water and 5 mL of hydrochloric acid.

Acceptance criteria: Fatty acids are liberated and appear as an oily layer floating on the surface of the liquid. The water layer meets the requirements.

- **B.**

Sample: 25 g

Analysis: Mix the *Sample* with 200 mL of hot water, add 60 mL of 2 N sulfuric acid, and heat the mixture, with frequent stirring, until the separated fatty acid layer is clear. Wash the fatty acids with boiling water until free from sulfate, collect them in a small beaker, and warm on a steam bath until the water has separated and the fatty acids are clear. Allow the acids to cool, pour off the water layer, and melt the acids. Filter into a dry beaker, and dry at 105° for 20 min.

Acceptance criteria: The fatty acids so obtained congeal at a temperature NLT 54° (see *Congeeing Temperature* <651>).

ASSAY

- **PROCEDURE**

Sample: 1.2 g

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.05 M edetate disodium VS

Endpoint detection: Colorimetric

Analysis: Boil the *Sample* with 50 mL of 1 N sulfuric acid for about 3 h using a watch glass cover to avoid splattering, or until the separated fatty acid layer is clear, adding water if necessary, to maintain the original volume. [NOTE—Stirring may be helpful in obtaining a clear layer and decreasing extraction time.] Cool, filter, and wash the filter and the flask thoroughly with water until the last washing is not acid to litmus. Neutralize the filtrate with 1 N sodium hydroxide to litmus. While stirring, preferably with a magnetic stirrer, titrate with 0.05 M edetate disodium VS as follows. Add about 30 mL from a 50-mL buret, then add 15 mL of 1 N sodium hydroxide and 300 mg of hydroxy naphthol blue. Continue the titration to a blue endpoint. Each mL of 0.05 M edetate disodium is equivalent to 2.804 mg of calcium oxide (CaO).

Acceptance criteria: 9.0%–10.5%

IMPURITIES

- **HEAVY METALS <231>**

Test preparation: Place 2.5 g of Calcium Stearate in a porcelain dish, and add 5 mL of a 1-in-4 solution of magnesium nitrate in alcohol. Cover the dish with a 7.5-cm, short-stem funnel so that the stem is straight up. Heat on a hot plate at low heat for 30 min, then heat at medium heat for 30 min, and cool. Remove the funnel, and heat the dish over a suitable burner until most of the carbon is burned off. Cool, add 10 mL of nitric acid, and transfer the solution into a 250-mL beaker. Add 5 mL of 70% perchloric acid, cautiously evaporate to dryness, add 2 mL of hydrochloric acid to

the residue, and wash down the inside of the beaker with water. Evaporate carefully to dryness again, swirling near the dry point to avoid spattering. Repeat the hydrochloric acid treatment, then cool, and dissolve the residue in about 10 mL of water. Add 1 drop of phenolphthalein TS and add sodium hydroxide TS until the solution just turns pink. Then add 3 N hydrochloric acid until the solution becomes colorless. Add 1 mL of 1 N acetic acid and a small amount of charcoal, and pass through filter paper into 50-mL color-comparison tubes. Wash with water, and dilute with water to 40 mL.

Monitor preparation: Place 500 mg of Calcium Stearate in a porcelain dish, and add 5 mL of a 1-in-4 solution of magnesium nitrate in alcohol. Cover the dish with a 7.5-cm, short-stem funnel so that the stem is straight up. Heat on a hot plate at low heat for 30 min, then heat at medium heat for 30 min, and cool. Remove the funnel, add 2 mL of *Standard Lead Solution* (20 µg of Pb), and heat the dish over a suitable burner until most of the carbon is burned off. Cool, add 10 mL of nitric acid, and transfer the solution into a 250-mL beaker. Add 5 mL of 70% perchloric acid, cautiously evaporate to dryness, add 2 mL of hydrochloric acid to the residue, and wash down the inside of the beaker with water. Evaporate carefully to dryness again, swirling near the dry point to avoid spattering. Repeat the hydrochloric acid treatment, then cool, and dissolve the residue in about 10 mL of water. Add 1 drop of phenolphthalein TS and add sodium hydroxide TS until the solution just turns pink. Then add 3 N hydrochloric acid until the solution becomes colorless. Add 1 mL of 1 N acetic acid and a small amount of charcoal, and pass through filter paper into 50-mL color-comparison tubes. Wash with water, and dilute with water to 40 mL.

Analysis: Add 1.2 mL of thioacetamide–glycerin base TS and 2 mL of pH 3.5 acetate buffer to each tube. Allow to stand for 5 min.

Acceptance criteria: The color of the *Test preparation* does not exceed that of the *Monitor preparation* (NMT 10 ppm).

SPECIFIC TESTS

- **LOSS ON DRYING** (731): Dry a sample at 105° to constant weight, using 2-h increments of heating: it loses NMT 4.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Calcium Sulfate

CaSO ₄	136.14
CaSO ₄ · 2H ₂ O	172.17
Sulfuric acid, calcium salt (1:1);	
Calcium sulfate (1:1) [7778-18-9].	
Dihydrate [10101-41-4].	

DEFINITION

Calcium Sulfate is anhydrous or contains two molecules of water of hydration. It contains NLT 98.0% and NMT 101.0% of CaSO₄, calculated on the dried basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Calcium** (191)
Sample solution: Dissolve 200 mg by warming in a mixture of 4 mL of 3 N hydrochloric acid and 16 mL of water.

Acceptance criteria: Meets the requirements

- **B. IDENTIFICATION TESTS—GENERAL, Sulfate** (191)

Sample solution: Dissolve 200 mg by warming in a mixture of 4 mL of 3 N hydrochloric acid and 16 mL of water.

Acceptance criteria: Meets the requirements

ASSAY

- **PROCEDURE**

Sample: Dissolve 300 mg of Calcium Sulfate in 100 mL of water and 4 mL of 3 N hydrochloric acid. Boil, if necessary, to dissolve, and cool before titrating.

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.05 M edetate disodium VS

Blank: 100 mL of water and 4 mL of 3 N hydrochloric acid

Endpoint detection: Visual

Analysis: While stirring, preferably with a magnetic stirrer, add to the *Sample solution*, in the order named, 0.5 mL of triethanolamine, 300 mg of hydroxy naphthol blue, and, from a 50-mL buret, 30 mL of 0.05 M edetate disodium VS. Add sodium hydroxide solution (45 in 100) until the initial red color changes to clear blue, continue to add dropwise until the color changes to violet, and add an additional 0.5 mL. The pH is 12.3–12.5. Continue the titration dropwise with 0.05 M edetate disodium VS to the appearance of a clear-blue endpoint that persists for NLT 60 s.

Perform a blank determination. Calculate the percentage of Calcium Sulfate (CaSO₄) in the *Sample taken*:

$$\text{Result} = [(V - B) \times N \times F \times 100] / W$$

V = volume of titrant consumed by the *Sample* (mL)

B = volume of titrant consumed by the *Blank* (mL)

N = actual normality of the titrant (mEq/mL)

F = equivalency factor, 136.14 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 98.0%–101.0% on the dried basis

IMPURITIES

- **IRON** (241)

Sample solution: Dissolve 100 mg in 8 mL of 3 N hydrochloric acid, and dilute with water to 47 mL.

Acceptance criteria: NMT 100 ppm

- **HEAVY METALS, Method I** (231): NMT 10 ppm

Test preparation: Mix 2.0 g with 20 mL of water, add 25 mL of 3 N hydrochloric acid, and heat to boiling to dissolve the test specimen. Cool, and add ammonium hydroxide to a pH of 7. Filter, evaporate to a volume of about 25 mL, and refilter, if necessary, to obtain a clear solution.

SPECIFIC TESTS

- **LOSS ON DRYING** (731)

Samples: Neat

Analysis: Dry a sample at a temperature NLT 250° to constant weight.

Acceptance criteria: NMT 1.5% for the anhydrous form and NMT 19.0%–23.0% for the dihydrate

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Label it to indicate whether it is anhydrous or the dihydrate.

Anhydrous Dibasic Calcium—see
Anhydrous Dibasic Calcium Phosphate General Monographs

Dibasic Calcium Phosphate Dihydrate—
see *Dibasic Calcium Phosphate Dihydrate General Monographs*

Candelilla Wax

[8006-44-8].

DEFINITION

Candelilla Wax is the purified wax obtained from the leaves of the candelilla plant, *Euphorbia antisiphilitica* Zucc.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197F)
Sample: Use a thin film of melted *Candelilla Wax*.
Acceptance criteria: Meets the requirements
- **B.** It meets the requirements of the test in *Specific Tests for Melting Range or Temperature, Class II* (741).

IMPURITIES

- **LIMIT OF LEAD** (251)
Sample: 3.3 g
Control: 10 µg of lead from a *Diluted Standard Lead Solution* (1 µg/mL of lead)
Analysis: Weigh the *Sample* into a porcelain dish, heat on a hot plate until completely charred, then heat in a muffle furnace at 480° for 8 h or overnight, and cool. Cautiously add 5 mL of nitric acid, evaporate to dryness on a hot plate, then heat again in the muffle furnace for exactly 15 min, and cool. Extract the ash with two 10-mL portions of water, filtering each extract into a separator. Leach any insoluble material on the filter with 6 mL of *Ammonium Citrate Solution*, 2 mL of *Hydroxylamine Hydrochloride Solution*, and 5 mL of water, adding the filtered washings to the separator. Continue as directed in *Lead* (251), *Procedure*, beginning with "Add two drops of phenol red TS", and use 10 mL of *Diluted Standard Lead Solution* for a control.
Acceptance criteria: The color of the *Sample solution* does not exceed that in the control, corresponding to NMT 3 µg/g.
- **HEAVY METALS, Method II** (231): NMT 20 µg/g

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE, Class II** (741): 68.5°–72.5°
- **FATS AND FIXED OILS, Acid Value** (401)
Sample: 3 g
Analysis: Weigh the *Sample* in a 250-mL flask, add 50 mL of a mixture of isopropyl alcohol and toluene (5:4), attach the flask to a reflux condenser, and boil gently until the wax is completely dissolved. Examine the solution: no insoluble particles are present. Remove the flask from the condenser, add 1 mL of phenolphthalein TS, and titrate immediately with 0.5 N alcoholic potassium hydroxide VS to a faint, reddish-yellow color. [NOTE—Do not allow the solution to cool; titrate at a warm temperature after refluxing. After the titration, reserve the solution for use in the test for *Saponification Value*.]
Calculate the acid value as the number of mg of potassium hydroxide required to neutralize the free acids in 1 g of *Candelilla Wax*.

Acceptance criteria: 12–22

- **FATS AND FIXED OILS, Saponification Value** (401)
Sample solution: Use the solution from the test for *Acid Value*.
Analysis: To the *Sample solution* add 15.0 mL of 0.5 N alcoholic potassium hydroxide VS, reflux for 3–4 h, and titrate the excess alkali with 0.5 N hydrochloric acid VS to a yellow-amber color. Perform a blank determination under the same conditions (see *Titrimetry* (541), *Residual Titrations*). The saponification value is the summation of the ester value so obtained and the acid value obtained above.

Acceptance criteria: 43–65

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
- **USP REFERENCE STANDARDS** (11)
USP *Candelilla Wax RS*

Canola Oil

Low erucic acid rapeseed oil;
LEAR oil.

DEFINITION

Canola Oil is the refined fixed oil obtained from the seeds of *Brassica napus* or *Brassica campestris* (Fam. Cruciferae). A suitable antioxidant may be added.

IDENTIFICATION

- **A.** It meets the requirements of the test for *Fats and Fixed Oils* (401), *Fatty Acid Composition*.

IMPURITIES

- **HEAVY METALS, Method II** (231): NMT 10 ppm

SPECIFIC TESTS

- **FATS AND FIXED OILS, Fatty Acid Composition** (401)
Canola Oil exhibits the composition profile of fatty acids in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<14	—	<0.1
14	0	<0.2
16	0	<6.0
16	1	<1.0
18	0	<2.5
18	1	>50
18	2	<40
18	3	<14
20	0	<1.0
20	1	<2.0
22	0	<0.5
22 ^a	1	≤2.0
24	0	<0.2
24	1	<0.2

^a Erucic acid

- **LIMIT OF ERUCIC ACID:** NMT 2.0%, as determined in the test for *Fats and Fixed Oils* (401), *Fatty Acid Composition*

- **SPECIFIC GRAVITY** (841): 0.906–0.920
- **FATS AND FIXED OILS**, Acid Value (401): NMT 6.0
- **FATS AND FIXED OILS**, Iodine Value (401): 110–126
- **FATS AND FIXED OILS**, Peroxide Value (401): NMT 10.0
- **FATS AND FIXED OILS**, Saponification Value (401): 178–193
- **FATS AND FIXED OILS**, Unsaponifiable Matter (401): NMT 1.5%
- **REFRACTIVE INDEX** (831): 1.465–1.467 at 40°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and avoid contact with metals. Fill to the top or flush partially filled containers with nitrogen. No storage requirements specified.
- **LABELING:** Label it to indicate the name and concentration of any added antioxidant.

Add the following:

▲ Caprylic Acid



C₈H₁₆O₂ 144.21
Octanoic acid;
1-Heptanecarboxylic acid [124-07-2].

DEFINITION

Caprylic Acid contains NLT 99.0% of caprylic acid (C₈H₁₆O₂), calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197F)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the Assay.

ASSAY

PROCEDURE

Standard solution: 10 mg/mL of USP Caprylic Acid RS in ethyl acetate

Sample solution: 10 mg/mL of Caprylic Acid in ethyl acetate

System suitability solution: 10 µg/mL of Caprylic Acid in ethyl acetate, prepared from the *Sample solution*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-cm fused silica capillary column bonded with a 0.25-µm layer of phase G25

Temperatures

Detector: 250°

Injection port: 250°

Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	1

Table 1 (Continued)

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	5	220	10

Carrier gas: Helium

Flow rate: 1.5 mL/min

Injection volume: 1 µL

Injection type: Split injection. The split ratio is about 1:100.

System suitability

Samples: *Standard solution* and *System suitability solution*

Suitability requirements

Relative standard deviation: NMT 2.0%, *Standard solution*

Signal-to-noise ratio: NLT 5 for the major peak, *System suitability solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of caprylic acid (C₈H₁₆O₂) in the portion of Caprylic Acid taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

r_u = peak response of caprylic acid from the *Sample solution*

r_s = peak response of caprylic acid from the *Standard solution*

C_s = concentration of USP Caprylic Acid RS in the *Standard solution* (mg/mL)

C_u = concentration of Caprylic Acid in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 99.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%

- **HEAVY METALS** (231)

Sample solution: 12 mL of a 100-mg/mL solution of Caprylic Acid in alcohol

Standard solution 1: 1 mL of *Standard Lead Solution* (10 µg/mL of Pb), prepared as directed in the chapter, and 9 mL of alcohol

Standard solution 2: 10 mL of *Standard solution 1* and 2 mL of *Sample solution*

Blank solution: 10 mL of alcohol and 2 mL of *Sample solution*

Analysis

Samples: *Sample solution*, *Standard solution 2*, and *Blank solution*

To each of the *Samples*, add 2 mL of pH 3.5 *Acetate Buffer* prepared as directed in the chapter. Mix, and add to 1.2 mL of thioacetamide–glycerin base TS. Examine the solutions after 2 min.

System suitability

Samples: *Standard solution 2* and *Blank solution*

Suitability requirements: The *Standard solution* shows a slight brown color compared to the *Blank solution*.

Acceptance criteria: The brown color in the *Sample solution* is not more intense than that in *Standard solution 2*, corresponding to NMT 10 µg/g of heavy metals.

• **LIMIT OF RELATED LINEAR AND BRANCHED ALKYL CARBOXYLIC ACIDS, RELATED ESTERS, CYCLIC ESTERS AND KETONE**

Standard solution, System suitability solution, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the Assay.

Analysis

Sample: *Sample solution*

Calculate the percentage of each impurity in the portion of Caprylic Acid taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual impurity in the *Sample solution*

r_T = sum of all the peaks except the peak due to solvent and peaks below the *Disregard limit* (see Table 2) in the *Sample solution*

Acceptance criteria: See Table 2.

Disregard any peak with an area less than 0.5 times the area of the major peak from the *System suitability solution*.

Table 2

Impurity	Percentage (%)
Each individual	NMT 0.3
Total	NMT 0.5

SPECIFIC TESTS

- **BACTERIAL ENDOTOXINS TEST (85):** The level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Caprylic Acid is used can be met. Where the label states that Caprylic Acid must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Caprylic Acid is used can be met.

- **FATS AND FIXED OILS, Peroxide Value (401):** NMT 10.0
- **WATER DETERMINATION, Method 1a (921):** NMT 0.7%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature.
- **LABELING:** Where Caprylic Acid must be subjected to further processing during the preparation of injectable dosage forms to ensure acceptable levels of bacterial endotoxins, it is so labeled.
- **USP REFERENCE STANDARDS (11)**
USP Caprylic Acid RS
USP Endotoxin RS▲ NF31

Caprylocaproyl Polyoxylglycerides

Former Title: Caprylocaproyl Macrogolglycerides

DEFINITION

Caprylocaproyl Polyoxylglycerides is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. The polyethylene glycols used have a mean molecular weight between 200 and 400. It is produced by partial alcoholysis of medium-chain triglycerides with polyethylene glycol, by esterification of glycerol and polyethylene glycol with caprylic acid and capric acid, or as a mixture of glycerol esters and ethylene oxide condensate with caprylic acid and capric acid. It may contain free polyethylene glycols.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197F)**

- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)**

Standard solution: 50 mg/mL of USP Caprylocaproyl Polyoxylglycerides RS in methylene chloride

Sample solution: 50 mg/mL of Caprylocaproyl Polyoxylglycerides in methylene chloride

Application volume: 50 µL

Developing solvent system: Ether and hexanes (7:3)

Spray reagent: 0.1 mg/mL of rhodamine B in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Proceed as directed in the chapter. Then spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The R_f values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.

- **C.** It meets the requirements of the test for *Fats and Fixed Oils (401)*, *Fatty Acid Composition*.

IMPURITIES

- **HEAVY METALS, Method II (231):** NMT 10 ppm
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 0.1%
- **ALKALINE IMPURITIES**

Sample: 5.0 g of Caprylocaproyl Polyoxylglycerides

Analysis: To the *Sample* add 10 mL of alcohol and 0.05 mL of bromophenol blue TS, and mix well. Titrate with 0.01 N hydrochloric acid VS to change the color to yellow.

Acceptance criteria: NMT 1.0 mL of 0.01 N hydrochloric acid is required.

Change to read:

- **LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE**

▲ **Analysis:** Proceed as directed in *Ethylene Oxide and Dioxane (228)*, *Method I*.

Acceptance criteria

Ethylene oxide: NMT 1 µg/g

Dioxane: NMT 10 µg/g▲ NF31

- **LIMIT OF FREE GLYCEROL**

Sample: 1.20 g

Blank: 25 mL of methylene chloride

Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, and dilute with glacial acetic acid to 100.0 mL.

Potassium iodide solution: 75 mg/mL of potassium iodide

Titrimetric system

(See *Titrimetry (541)*.)

Mode: Direct titration

Titrant: 0.1 M sodium thiosulfate

Analysis: Dissolve the *Sample* in 25 mL of methylene chloride, heating if necessary. Cool, and add 100 mL of water and 25.0 mL of *Periodic acetic acid solution*. Shake, and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with *Titrant*. Perform a blank determination.

Calculate the percentage of free glycerol in the portion of Caprylocaproyl Polyoxylglycerides taken:

$$\text{Result} = [(V_T - V_B) \times N \times E \times F/W] \times 100$$

V_T = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

- E* = equivalency factor for glycerol, 23 mg/mEq
F = unit conversion factor, 10⁻³ g/mg
W = Sample weight taken for the titration (g)
 Acceptance criteria: NMT 5.0%

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Acid Value* <401>: NMT 2.0, determined on a 2.0-g specimen
- **FATS AND FIXED OILS**, *Fatty Acid Composition* <401>: Caprylocaproyl Polyoxylglycerides exhibit the composition profile of fatty acids shown in *Table 1*, as determined in the section *Fatty Acid Composition* under *Fats and Fixed Oils* <401>.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤2.0
8	0	50.0–80.0
10	0	20.0–50.0
12	0	≤3.0
14	0	≤1.0

Change to read:

- **FATS AND FIXED OILS**, *Hydroxyl Value* <401>: The hydroxyl value is within the range specified in *Table 2* for the labeled type, when determined on a 1.0-g specimen.

Table 2

Ethylene Oxide Units per Molecule (Nominal Value)	Type of Polyethylene Glycol	Hydroxyl Value
4	200	80–120
6	300	140–180
8	400	170–205

▲ NF31

- **FATS AND FIXED OILS**, *Iodine Value* <401>: NMT 2.0
- **FATS AND FIXED OILS**, *Peroxide Value* <401>: NMT 6.0, determined on a 2.0-g specimen

Change to read:

- **FATS AND FIXED OILS**, *Saponification Value* <401>: The saponification value is within the range specified in *Table 3* for the labeled type, determined on a 2.0-g specimen.

Table 3

Ethylene Oxide Units per Molecule (Nominal Value)	Type of Polyethylene Glycol	Saponification Value
4	200	265–285
6	300	170–190
8	400	85–105

▲ NF31

- **WATER DETERMINATION**, *Method I* <921>: NMT 1.0%, determined on a 1.0-g specimen. Use as the solvent anhydrous pyridine or a mixture of methylene chloride and anhydrous methanol (7:3).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight containers, protected from light and moisture. Store at controlled room temperature.

Change to read:

- **LABELING**: Label it to indicate the type and the average nominal molecular weight of polyethylene glycol used, ▲ or to indicate the number of ethylene oxide units per molecule (nominal value), ▲ NF31 as part of the official title.
- **USP REFERENCE STANDARDS** <11>
USP Caprylocaproyl Polyoxylglycerides RS

Caramel**DEFINITION**

Caramel is a concentrated solution of the product obtained by heating sugar or glucose until the sweet taste is destroyed and a uniform dark brown mass results, a small amount of alkali or of alkaline carbonate or a trace of mineral acid being added while heating.

[NOTE—Where included in articles for coloring purposes, Caramel complies with the regulations of the FDA concerning color additives (21 CFR 73.85, caramel).]

IMPURITIES

- **ARSENIC**, *Method II* <211>: NMT 3 ppm
- **LEAD** <251>: NMT 10 ppm
- **ASH**: It swells when incinerated, and forms a coke-like charcoal that burns off only after prolonged heating at a high temperature. It yields NMT 8.0% of ash.

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** <61> and **TESTS FOR SPECIFIED MICROORGANISMS** <62>: Meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*
- **SPECIFIC GRAVITY** <841>: NLT 1.30
- **PURITY**: The addition of 0.5 mL of phosphoric acid to 20 mL of a solution (1 in 20) produces no precipitate.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight containers.

Caraway**DEFINITION**

Caraway is the dried, ripe fruit of *Carum carvi* L. (Fam. Apiaceae).

SPECIFIC TESTS**• BOTANIC CHARACTERISTICS****Macroscopic**

Unground caraway: Separate mericarps from about 4 to 7 mm in length, and from 1 to 2 mm in diameter.

The mericarp is oblong, curved, and tapers toward the base and apex, to which half of the stylopodium is attached. Externally, it is dark brown to weak brown and shows five lighter-colored, filiform, primary ribs, between each pair of which a secondary rib occurs on the dorsal surface.

Powdered caraway: Moderate yellowish brown to light olive brown

Microscopic

Unground caraway: The mericarp is nearly equilaterally pentagonal in transverse section and shows a fibrovascular bundle in each primary rib. The epicarp consists of tabular polygonal epidermal cells with thick outer walls possessing a striated cuticle and with occasional stomata. The mesocarp, located between primary rib regions, is composed of collapsed, thin-walled parenchyma normally bearing four dorsal vittae in the intervals and two commissural vittae and sometimes one or

more additional vittae. The endocarp consists of broad, slightly undulate, inner epidermal cross cells that are coherent with the collapsed cells of the spermoderm. The endosperm consists of thick-walled reserve parenchyma containing fixed oil and aleurone grains up to 10 µm in diameter, the latter with embedded rosette aggregates of calcium oxalate up to 4 µm in diameter. An embryo is embedded in the upper end of the endosperm.

Powdered caraway: It shows fragments of the epicarp with striped cuticle, numerous polyhedral endosperm cells containing aleurone grains with embedded rosette aggregates of calcium oxalate, few fragments of slightly lignified fibers and spiral vessels, fragments of cross cells of endocarp, orange to yellow fragments of vittae, and no reticulate parenchyma.

- **ARTICLES OF BOTANICAL ORIGIN, Foreign Organic Matter** (561): NMT 3.0%
- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561): NMT 1.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Preserve against attack by insects.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in the article.

Caraway Oil

DEFINITION

Caraway Oil is the volatile oil distilled from the dried, ripe fruit of *Carum carvi* L. (Fam. Apiaceae). It contains NMT 50.0% of *d*-carvone (C₁₀H₁₄O).

ASSAY

• PROCEDURE

Sample: 10 mL of Caraway Oil

Analysis: Transfer the *Sample* to a cassia flask, and add 50 mL of a saturated solution of sodium sulfite that has been made neutral to 2 drops of phenolphthalein TS by means of a saturated sodium bisulfite solution. Heat the flask in boiling water, and shake it repeatedly, neutralizing the mixture from time to time by adding a few drops of the saturated sodium bisulfite solution. When no coloration appears upon the addition of a few more drops of phenolphthalein TS and heating for 15 min, cool the mixture to room temperature. When the liquids have separated more completely, add a sufficient volume of the saturated sodium bisulfite solution to raise the lower level of the oily layer to the graduated portion of the neck of the flask.

Acceptance criteria: The volume of the residual oily liquid does not exceed 5 mL, indicating the presence of NLT 50.0% of *d*-carvone (C₁₀H₁₄O) in the portion of Oil taken.

IMPURITIES

- **HEAVY METALS, Method II** (231): NMT 40 µg/g

SPECIFIC TESTS

- **SOLUBILITY IN 80% ALCOHOL:** One volume dissolves in 8 volumes of 80% alcohol.
- **SPECIFIC GRAVITY** (841): 0.900–0.910
- **OPTICAL ROTATION, Angular Rotation** (781A): +70° to +80°
- **REFRACTIVE INDEX** (831): 1.484–1.488 at 20°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.

Carbomer 934

DEFINITION

Carbomer 934 is a high molecular weight polymer of acrylic acid cross-linked with allyl ethers of sucrose. Carbomer 934, previously dried under vacuum at 80° for 1 h, contains NLT 56.0% and NMT 68.0% of carboxylic acid (–COOH) groups. The viscosity of a neutralized 0.5% aqueous dispersion of Carbomer 934 is between 30,500 and 39,400 mPa · s.

IDENTIFICATION

• A.

Sample dispersion: 10 mg/mL

Analysis 1: To one portion of the *Sample dispersion* add thymol blue TS.

Acceptance criteria 1: An orange color is produced.

Analysis 2: To another portion of the *Sample dispersion* add cresol red TS.

Acceptance criteria 2: A yellow color is produced.

• B.

Sample dispersion: 10 mg/mL

Analysis: Adjust the *Sample dispersion* with 1 N sodium hydroxide to a pH of 7.5.

Acceptance criteria: A very viscous gel is produced.

ASSAY

• CARBOXYLIC ACID CONTENT

Sample: 400 mg, previously dried

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel–glass

Titrant: 0.25 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continuously at about 1000 rpm, with the stirrer shaft set at the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate potentiometrically with *Titrant*. After each addition of *Titrant* allow 1 min for mixing before recording the pH. Calculate the carboxylic acid content as a percentage of carboxylic acid groups:

$$\text{Result} = [(V \times N/W) \times F] \times 100$$

V = *Titrant* volume consumed (mL)

N = actual normality of the *Titrant* (mEq/mL)

W = *Sample* weight (mg)

F = equivalency factor for the carboxylic acid (–COOH) group, 45.02 mEq/mg

Acceptance criteria: 56.0%–68.0% on the dried basis

IMPURITIES

- **HEAVY METALS, Method II** (231): NMT 20 µg/g

• LIMIT OF BENZENE

Standard solution: 0.2 mg/mL of benzene in methanol. Quantitatively dilute this solution with organic-free water (see *Residual Solvents* (467)) to obtain a solution having a known concentration of about 1.0 µg/mL.

Sample solution: Transfer 20 mg of Carbomer 934 to a 100-mL volumetric flask. Add 75 mL of sodium chloride solution (20 mg/mL), and mix by mechanical means until homogeneous (usually about 30 min). Dilute with sodium chloride solution (20 mg/mL) to volume, and mix until homogeneous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Columns

Guard: 0.53-mm × 5-m silica, deactivated with phenylmethyl siloxane

Analytical: 0.53-mm × 30-m fused silica, coated with 3.0-μm G43 stationary phase

Temperatures

Injection port: 140°

Detector: 260°

Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	Rapidly	240	20

Carrier gas: Helium

Linear velocity: 35 cm/s

Injection volume: 1 μL

Injection type: Splitless

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 15%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of benzene in the portion of Carbomer 934 taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of benzene from the *Sample solution*

r_S = peak response of benzene from the *Standard solution*

C_S = concentration of benzene in the *Standard solution* (μg/mL)

C_U = concentration of Carbomer 934 in the *Sample solution* (μg/mL)

Acceptance criteria: NMT 0.5%

SPECIFIC TESTS

• LOSS ON DRYING (731)

Analysis: Dry a sample under vacuum at 80° for 1 h.

Acceptance criteria: NMT 2.0%

• ROTATIONAL RHEOMETER METHODS (912)

Sample: 2.50 g, dried under vacuum at 80° for 1 h

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel-glass

Titrant: 180 mg/mL of sodium hydroxide

Endpoint detection: pH

Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 10 rpm, with the stirrer shaft set to the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Allow 45–90 s for addition of the *Sample* at a uniform rate, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 10 rpm for 15 min. Remove the stirrer, and place the beaker containing the dispersion in a $25 \pm 0.1^\circ$ water bath for 30 min. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion, and, while stirring at 300 ± 10 rpm, titrate with *Titrant* to a pH of 7.3–7.8 by adding the *Titrant* below the surface. Stir for 2–3 min until neutralization is complete. Then determine the final pH. If the pH is less than 7.3, raise it with additional sodium hydroxide. If it is more than 7.8,

discard the mucilage, and prepare another, using a smaller amount of sodium hydroxide for titration.

Return the neutralized mucilage to the 25° water bath for 1 h. Measure the pH again, and make certain that the mucilage pH is 7.3–7.8. Perform the viscosity determination without delay to avoid slight viscosity changes that occur 75 min after neutralization.

Equip a suitable rotational viscometer with a spindle having a cylinder 1.5 cm in diameter and 0.2 cm high attached to a shaft 0.3 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 3.02 cm.¹ The spindle rotates at 20 rpm at an immersion depth of 4.9 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: 30,500–39,400 mPa · s

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.

• **LABELING:** Label it to indicate that it is not intended for internal use. A carbomer homopolymer manufactured using benzene and complying with the unique requirements of this monograph will be officially titled Carbomer 934 and will not be referred to as Carbomer Homopolymer.

Carbomer 934P

DEFINITION

Carbomer 934P is a high molecular weight polymer of acrylic acid, cross-linked with allyl ethers of sucrose or pentaerythritol. Carbomer 934P, previously dried under vacuum at 80° for 1 h, contains NLT 56.0% and NMT 68.0% of carboxylic acid (–COOH) groups. The viscosity of a neutralized 0.5% aqueous dispersion of Carbomer 934P is between 29,400 and 39,400 mPa · s.

IDENTIFICATION

• A.

Sample dispersion: 10 mg/mL

Analysis 1: To one portion of the *Sample dispersion* add thymol blue TS.

Acceptance criteria 1: An orange color is produced.

Analysis 2: To another portion of the *Sample dispersion* add cresol red TS.

Acceptance criteria 2: A yellow color is produced.

• B.

Sample dispersion: 10 mg/mL

Analysis: Adjust the *Sample dispersion* with 1 N sodium hydroxide to a pH of 7.5.

Acceptance criteria: A very viscous gel is produced.

ASSAY

• CARBOXYLIC ACID CONTENT

Sample: 400 mg, previously dried

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel-glass

Titrant: 0.25 N sodium hydroxide VS.

Endpoint detection: Potentiometric

Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continuously at about 1000 rpm, with the stirrer shaft set at the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate potentiometrically with *Titrant*. After each addition of *Titrant* allow 1 min for mixing before recording the pH.

¹ Available as an RV6 spindle from Brookfield, or the equivalent.

Calculate the carboxylic acid content as a percentage of carboxylic acid groups:

$$\text{Result} = [(V \times N/W) \times F] \times 100$$

V = Titrant volume consumed (mL)
 N = actual normality of the Titrant (mEq/mL)
 W = Sample weight (mg)
 F = equivalency factor for the carboxylic acid (–COOH) group, 45.02 mEq/mg

Acceptance criteria: 56.0%–68.0% on the dried basis

IMPURITIES

• **HEAVY METALS**, Method II (231): NMT 20 µg/g

• **LIMIT OF BENZENE**

Standard solution: 0.2 mg/mL of benzene in methanol. Dilute this solution with organic-free water (see *Residual Solvents* (467)) to obtain a solution having a known concentration of about 1.0 µg/mL.

Sample solution: Transfer 1 g of Carbomer 934P to a 100-mL volumetric flask. Add 75 mL of sodium chloride solution (20 mg/mL), and mix by mechanical means until homogeneous (usually about 30 min). Dilute with sodium chloride solution (20 mg/mL) to volume, and mix until homogeneous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Columns

Guard: 0.53-mm × 5-m silica, deactivated with phenylmethyl siloxane

Analytical: 0.53-mm × 30-m fused silica, coated with 3.0-µm G43 stationary phase

Temperatures

Injection port: 140°

Detector: 260°

Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	Rapidly	240	20

Carrier gas: Helium

Linear velocity: 35 cm/s

Injection volume: 1 µL

Injection type: Splitless

System suitability

Sample: Standard solution

Suitability requirements

Relative standard deviation: NMT 15%

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of benzene in the portion of Carbomer 934P taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of benzene from the Sample solution

r_S = peak response of benzene from the Standard solution

C_S = concentration of benzene in the Standard solution (µg/mL)

C_U = concentration of Carbomer 934P in the Sample solution (µg/mL)

Acceptance criteria: NMT 0.01%

SPECIFIC TESTS

• **LOSS ON DRYING** (731)

Analysis: Dry a sample under vacuum at 80° for 1 h.

Acceptance criteria: NMT 2.0%

• **ROTATIONAL RHEOMETER METHODS** (912)

Sample: 2.50 g, dried under vacuum at 80° for 1 h

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel–glass

Titrant: 180 mg/mL of sodium hydroxide

Endpoint detection: pH

Analysis: Carefully add the Sample to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 10 rpm, with the stirrer shaft set to the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Allow 45–90 s for addition of the Sample at a uniform rate, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 10 rpm for 15 min. Remove the stirrer, and place the beaker containing the dispersion in a $25 \pm 0.1^\circ$ water bath for 30 min. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion, and while stirring at 300 ± 10 rpm, titrate with Titrant to a pH of 7.3–7.8 by adding the Titrant below the surface. Stir for 2–3 min until neutralization is complete. Then determine the final pH. If the pH is less than 7.3, raise it with additional sodium hydroxide. If it is more than 7.8, discard the mucilage, and prepare another, using a smaller amount of sodium hydroxide for titration.

Return the neutralized mucilage to the 25° water bath for 1 h. Measure the pH again, and make certain that the mucilage pH is 7.3–7.8. Perform the viscosity determination without delay to avoid slight viscosity changes that occur 75 min after neutralization.

Equip a suitable rotational viscometer with a spindle having a cylinder 1.5 cm in diameter and 0.2 cm high attached to a shaft 0.3 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 3.02 cm.¹ The spindle rotates at 20 rpm at an immersion depth of 4.9 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: 29,400–39,400 mPa · s

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.

• **LABELING:** A carbomer homopolymer manufactured using benzene and complying with the unique requirements of this monograph will be officially titled Carbomer 934P and will not be referred to as Carbomer Homopolymer.

Carbomer 940

DEFINITION

Carbomer 940 is a high molecular weight polymer of acrylic acid cross-linked with allyl ethers of pentaerythritol. Carbomer 940, previously dried under vacuum at 80° for 1 h, contains NLT 56.0% and NMT 68.0% of carboxylic acid (–COOH) groups. The viscosity of a neutralized 0.5% aqueous dispersion of Carbomer 940 is between 40,000 and 60,000 mPa · s.

¹ Available as an RV6 spindle from Brookfield, or the equivalent.

IDENTIFICATION

- **A.**
Sample dispersion: 10 mg/mL
Analysis 1: To one portion of the *Sample dispersion* add thymol blue TS.
Acceptance criteria 1: An orange color is produced.
Analysis 2: To another portion of the *Sample dispersion* add cresol red TS.
Acceptance criteria 2: A yellow color is produced.
- **B.**
Sample dispersion: 10 mg/mL
Analysis: Adjust the *Sample dispersion* with 1 N sodium hydroxide to a pH of 7.5.
Acceptance criteria: A very viscous gel is produced.

ASSAY• **CARBOXYLIC ACID CONTENT**

Sample: 400 mg, previously dried
Titrimetric system
 (See *Titrimetry* (541).)
Mode: Direct titration
Electrode: Calomel–glass
Titrant: 0.25 N sodium hydroxide VS
Endpoint detection: Potentiometric
Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continuously at about 1000 rpm, with the stirrer shaft set at the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate potentiometrically with *Titrant*. After each addition of *Titrant* allow 1 min for mixing before recording the pH. Calculate the carboxylic acid content as a percentage of carboxylic acid groups:

$$\text{Result} = [(V \times N/W) \times F] \times 100$$

V = *Titrant* volume consumed (mL)
N = actual normality of the *Titrant* (mEq/mL)
W = *Sample* weight (mg)
F = equivalency factor for the carboxylic acid (–COOH) group, 45.02 mEq/mg

Acceptance criteria: 56.0%–68.0% on the dried basis

IMPURITIES

- **HEAVY METALS, Method II (231):** NMT 20 µg/g

• **LIMIT OF BENZENE**

Standard solution: 0.2 mg/mL of benzene in methanol. Dilute this solution with organic-free water (see *Residual Solvents* (467)) to obtain a solution having a known concentration of about 1.0 µg/mL.

Sample solution: Transfer 20 mg of Carbomer 940 to a 100-mL volumetric flask. Add 75 mL of sodium chloride solution (20 mg/mL), and mix by mechanical means until homogeneous (usually about 30 min). Dilute with sodium chloride solution (20 mg/mL) to volume, and mix until homogeneous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Columns

Guard: 0.53-mm × 5-m silica, deactivated with phenylmethyl siloxane

Analytical: 0.53-mm × 30-m fused silica, coated with 3.0-µm G43 stationary phase

Temperatures

Injection port: 140°

Detector: 260°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	Rapidly	240	20

Carrier gas: Helium

Linear velocity: 35 cm/s

Injection volume: 1 µL

Injection type: Splitless

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 15%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of benzene in the portion of Carbomer 940 taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of benzene from the *Sample solution*

r_S = peak response of benzene from the *Standard solution*

C_S = concentration of benzene in the *Standard solution* (µg/mL)

C_U = concentration of Carbomer 940 in the *Sample solution* (µg/mL)

Acceptance criteria: NMT 0.5%

SPECIFIC TESTS• **LOSS ON DRYING (731)**

Analysis: Dry a sample under vacuum at 80° for 1 h.

Acceptance criteria: NMT 2.0%

• **ROTATIONAL RHEOMETER METHODS (912)**

Sample: 2.50 g, dried under vacuum at 80° for 1 h

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel–glass

Titrant: 180 mg/mL of sodium hydroxide

Endpoint detection: pH

Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 10 rpm, with the stirrer shaft set to the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Allow 45–90 s for addition of the *Sample* at a uniform rate, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 10 rpm for 15 min. Remove the stirrer, and place the beaker containing the dispersion in a 25 ± 0.1° water bath for 30 min. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion, and, while stirring at 300 ± 10 rpm, titrate with *Titrant* to a pH of 7.3–7.8 by adding the *Titrant* below the surface. Stir for 2–3 min until neutralization is complete. Then determine the final pH. If the pH is less than 7.3, raise it with additional sodium hydroxide. If it is more than 7.8, discard the mucilage, and prepare another, using a smaller amount of sodium hydroxide for titration. Return the neutralized mucilage to the 25° water bath for 1 h. Measure the pH again, and make certain that the mucilage pH is 7.3–7.8. Perform the viscosity determination without delay to avoid slight viscosity changes that occur 75 min after neutralization.

Equip a suitable rotational viscometer with a spindle having a shaft about 0.3 cm in diameter, the distance from the top of the shaft to the lower tip of the shaft being 5.0 cm.¹ The spindle rotates at 20 rpm at an

¹Available as an RV7 spindle from Brookfield, or the equivalent.

immersion depth of 5.5 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: 40,000–60,000 mPa · s

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** Label to indicate that it is not intended for internal use. A carbomer homopolymer manufactured using benzene and complying with the unique requirements of this monograph will be officially titled Carbomer 940 and will not be referred to as Carbomer Homopolymer.

Carbomer 941

DEFINITION

Carbomer 941 is a high molecular weight polymer of acrylic acid cross-linked with allyl ethers of pentaerythritol. Carbomer 941, previously dried under vacuum at 80° for 1 h, contains NLT 56.0% and NMT 68.0% of carboxylic acid (–COOH) groups. The viscosity of a neutralized 0.5% aqueous dispersion of Carbomer 941 is between 4000 and 11,000 mPa · s.

IDENTIFICATION

- **A.**

Sample dispersion: 10 mg/mL

Analysis 1: To one portion of the *Sample dispersion* add thymol blue TS.

Acceptance criteria 1: An orange color is produced.

Analysis 2: To another portion of the *Sample dispersion* add cresol red TS.

Acceptance criteria 2: A yellow color is produced.
- **B.**

Sample dispersion: 10 mg/mL

Analysis: Adjust the *Sample dispersion* with 1 N sodium hydroxide to a pH of 7.5.

Acceptance criteria: A very viscous gel is produced.

ASSAY

• CARBOXYLIC ACID CONTENT

Sample: 400 mg, previously dried

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel-glass

Titrant: 0.25 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continuously at about 1000 rpm, with the stirrer shaft set at the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate potentiometrically with *Titrant*. After each addition of *Titrant* allow 1 min for mixing before recording the pH. Calculate the carboxylic acid content as a percentage of carboxylic acid groups:

$$\text{Result} = [(V \times N/W) \times F] \times 100$$

V = *Titrant* volume consumed (mL)

N = actual normality of the *Titrant* (mEq/mL)

W = *Sample* weight (mg)

F = equivalency factor for the carboxylic acid (–COOH) group, 45.02 mEq/mg

Acceptance criteria: 56.0%–68.0% on the dried basis

IMPURITIES

- **HEAVY METALS, Method II (231):** NMT 20 µg/g

• LIMIT OF BENZENE

Standard solution: 0.2 mg/mL of benzene in methanol. Dilute this solution with organic-free water (see *Residual Solvents* (467)) to obtain a solution having a known concentration of about 1.0 µg/mL.

Sample solution: Transfer 20 mg of Carbomer 941 to a 100-mL volumetric flask. Add about 75 mL of sodium chloride solution (20 mg/mL), and mix by mechanical means until homogeneous (usually about 30 min). Dilute with sodium chloride solution (20 mg/mL) to volume, and mix until homogenous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Columns

Guard: 0.53-mm × 5-m silica, deactivated with phenylmethyl siloxane

Analytical: 0.53-mm × 30-m fused silica, coated with 3.0-µm G43 stationary phase

Temperatures

Injection port: 140°

Detector: 260°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	Rapidly	240	20

Carrier gas: Helium

Linear velocity: 35 cm/s

Injection volume: 1 µL

Injection type: Splitless

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 15%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of benzene in the portion of Carbomer 941 taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of benzene from the *Sample solution*

r_S = peak response of benzene from the *Standard solution*

C_S = concentration of benzene in the *Standard solution* (µg/mL)

C_U = concentration of Carbomer 941 in the *Sample solution* (µg/mL)

Acceptance criteria: NMT 0.5%

SPECIFIC TESTS

• LOSS ON DRYING (731)

Analysis: Dry a sample under vacuum at 80° for 1 h.

Acceptance criteria: NMT 2.0%

• ROTATIONAL RHEOMETER METHODS (912)

Sample: 2.50 g, dried under vacuum at 80° for 1 h

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel-glass

Titrant: 180 mg/mL of sodium hydroxide

Endpoint detection: pH

Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 10 rpm, with the stirrer shaft set to the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Allow 45–90 s for addition of the *Sample* at a uniform rate, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 10 rpm for 15 min. Remove the stirrer, and place the beaker containing the dispersion in a $25 \pm 0.1^\circ$ water bath for 30 min. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion, and while stirring at 300 ± 10 rpm, titrate with *Titrant* to a pH of 7.3–7.8 by adding the *Titrant* below the surface. Stir for 2–3 min until neutralization is complete. Then determine the final pH. If the pH is less than 7.3, raise it with additional sodium hydroxide. If it is more than 7.8, discard the mucilage, and prepare another, using a smaller amount of sodium hydroxide for titration. Return the neutralized mucilage to the 25° water bath for 1 h. Measure the pH again, and make certain that the mucilage pH is 7.3–7.8. Perform the viscosity determination without delay to avoid slight viscosity changes that occur 75 min after neutralization.

Equip a suitable rotational viscometer with a spindle having a disk about 2.1 cm in diameter and 0.2 cm high, attached to a shaft 0.3 cm in diameter, the distance from the top of the disk to the lower tip of the shaft being 2.7 cm.¹ The spindle rotates at 20 rpm at an immersion depth of 4.9 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: 4,000–11,000 mPa · s

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** Label it to indicate that it is not intended for internal use. A carbomer homopolymer manufactured using benzene and complying with the unique requirements of this monograph will be officially titled Carbomer 941 and will not be referred to as Carbomer Homopolymer.

Carbomer 1342

DEFINITION

Carbomer 1342 is a high molecular weight copolymer of acrylic acid and a long-chain alkyl methacrylate cross-linked with allyl ethers of pentaerythritol. Carbomer 1342, previously dried under vacuum at 80° for 1 h, contains NLT 52.0% and NMT 62.0% of carboxylic acid (–COOH) groups. The viscosity of a neutralized 1.0% aqueous dispersion of Carbomer 1342 is between 9500 and 26,500 centipoises.

IDENTIFICATION

- **A.**

Sample dispersion: 10 mg/mL

Analysis 1: To one portion of the *Sample dispersion* add thymol blue TS.

Acceptance criteria 1: An orange color is produced.

Analysis 2: To another portion of the *Sample dispersion* add cresol red TS.

Acceptance criteria 2: A yellow color is produced.

• B.

Sample dispersion: 10 mg/mL

Analysis: Adjust the *Sample dispersion* with 1 N sodium hydroxide to a pH of 7.5.

Acceptance criteria: A very viscous gel is produced.

ASSAY

• CARBOXYLIC ACID CONTENT

Sample: 400 mg, previously dried

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel-glass

Titrant: 0.25 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continuously at about 1000 rpm, with the stirrer shaft set at the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate potentiometrically with *Titrant*. After each addition of *Titrant*, allow 1 min for mixing before recording the pH. Calculate the carboxylic acid content as a percentage of carboxylic acid groups:

$$\text{Result} = [(V \times N/W) \times F] \times 100$$

V = *Titrant* volume consumed (mL)

N = actual normality of the *Titrant* (mEq/mL)

W = *Sample* weight (mg)

F = equivalency factor for the carboxylic acid (–COOH) group, 45.02 mEq/mg

Acceptance criteria: 52.0%–62.0% on the dried basis

IMPURITIES

• HEAVY METALS, *Method II* (231): NMT 20 µg/g

• LIMIT OF BENZENE

Standard solution: 0.2 mg/mL of benzene in methanol. Dilute this solution with organic-free water (see *Residual Solvents* (467)) to obtain a solution having a known concentration of about 1.0 µg/mL.

Sample solution: Transfer 50 mg of Carbomer 1342 to a 100-mL volumetric flask. Add 75 mL of sodium chloride solution (20 mg/mL), and mix by mechanical means until homogeneous (usually about 30 min). Dilute with sodium chloride solution (20 mg/mL) to volume, and mix until homogeneous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Columns

Guard: 0.53-mm × 5-m silica, deactivated with phenylmethyl siloxane

Analytical: 0.53-mm × 30-m fused silica, coated with 3.0-µm G43 stationary phase

Temperatures

Injection port: 140°

Detector: 260°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	Rapidly	240	20

¹Available as an RV5 spindle from Brookfield, or the equivalent.

Carrier gas: Helium
 Linear velocity: 35 cm/s
 Injection volume: 1 µL
 Injection type: Splitless
 System suitability
 Sample: Standard solution
 Suitability requirements
 Relative standard deviation: NMT 15%

Analysis

Samples: Standard solution and Sample solution
 Calculate the percentage of benzene in the portion of Carbomer 1342 taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of benzene from the *Sample solution*
 r_S = peak response of benzene from the *Standard solution*
 C_S = concentration of benzene in the *Standard solution* (µg/mL)
 C_U = concentration of Carbomer 1342 in the *Sample solution* (µg/mL)

Acceptance criteria: NMT 0.2%

SPECIFIC TESTS• **LOSS ON DRYING (731)**

Analysis: Dry a sample under vacuum at 80° for 1 h.
 Acceptance criteria: NMT 2.0%

• **ROTATIONAL RHEOMETER METHODS (912)**

Sample: 5.00 g, dried under vacuum at 80° for 1 h
 Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel-glass

Titrant: 180 mg/mL of sodium hydroxide

Endpoint detection: pH

Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 10 rpm, with the stirrer shaft set to the side of the beaker at an angle of 60° and with the propeller positioned near the bottom of the beaker. Allow 45–90 s for addition of the sample at a uniform rate, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 10 rpm for 15 min. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion, and while stirring at 300 ± 10 rpm, titrate with *Titrant* to a pH of 7.3–7.8 by adding the *Titrant* below the surface. Stir for 2–3 min until neutralization is complete. Then determine the final pH. If the pH is less than 7.3, raise it with additional sodium hydroxide. If it is more than 7.8, discard the mucilage, and prepare another, using a smaller amount of sodium hydroxide for titration.

Return the neutralized mucilage to the 25° water bath for 1 h. Measure the pH again, and make certain that the mucilage pH is 7.3–7.8. Perform the viscosity determination without delay to avoid slight viscosity changes that occur 75 min after neutralization. Equip a suitable rotational viscometer with a spindle having a cylinder 1.5 cm in diameter and 0.2 cm high attached to a shaft 0.3 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 3.02 cm.¹ The spindle rotates at 20 rpm at an immersion depth of 4.9 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: 9500–26,500 centipoises

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label to indicate that it is not intended for internal use. A carbomer copolymer manufactured using benzene and complying with the unique requirements of

this monograph will be officially titled Carbomer 1342 and will not be referred to as Carbomer Copolymer.

Carbomer Copolymer

DEFINITION

Carbomer Copolymer is a high molecular weight copolymer of acrylic acid and a long-chain alkyl methacrylate cross-linked with allyl ethers of polyalcohols.

[NOTE—The heading of this monograph does not constitute the official title for a Carbomer Copolymer manufactured with the use of benzene. When benzene is used in the manufacturing process, the name will be Carbomer 1342, provided it complies with the existing requirements in the Carbomer 1342 monograph.]

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K):** Exhibits main bands at or near (±5) wavenumbers (cm⁻¹) 1710, 1454, 1414, 1245, 1172, 1115, and 801, with the strongest band at 1710.

• **B.**

Sample: 5 g

Analysis: Add the *Sample* to 500 mL of water, and stir.

Acceptance criteria: A dispersion is formed, with a foam layer that persists after the dispersion is allowed to stand at room temperature for 1 h.

ASSAY• **CONTENT OF CARBOXYLIC ACID**

Sample: 400 mg, previously dried under vacuum at 80° for 1 h

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel-glass

Titrant: 0.25 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continually at 1000 rpm. The stirrer shaft is set at an angle of about 60° and to one side of the beaker, and the propeller is positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate with *Titrant*. After each addition of *Titrant*, allow 1 min for mixing before recording the pH.

Calculate the carboxylic acid content as a percentage of carboxylic acid groups in the portion of Carbomer Copolymer taken:

$$\text{Result} = [(V \times N \times M_r)/W] \times 100$$

V = *Titrant* volume consumed by the *Sample* (mL)

N = actual normality of the *Titrant* (mEq/mL)

M_r = molecular weight of the carboxylic acid (–COOH) group, 45.02

W = *Sample* weight (mg)

Acceptance criteria: 52.0%–62.0%

IMPURITIES

- **HEAVY METALS, Method II (231):** NMT 20 µg/g

• **LIMIT OF ETHYL ACETATE AND CYCLOHEXANE**

[NOTE—This test is required only for those Carbomer Copolymers where the labeling indicates that ethyl acetate or a mixture of ethyl acetate and cyclohexane was used in the polymerization process.]

Standard stock solution: Transfer 5.0 mL of methanol to a 10-mL serum vial, insert a rubber septum, and seal with a metal cap. Add 25.0 µL of ethyl acetate and 20.0 µL of cyclohexane through the septum into the vial, and mix.

Standard solution: Transfer 20.0 mL of methanol to a 30-mL serum vial, insert a rubber septum, and seal with

¹ Available as an RV6 spindle from Brookfield, or the equivalent.

a metal cap. Through the rubber septum add 10 µL of methyl ethyl ketone (internal standard) and 50.0 µL of the *Standard stock solution*, and mix to obtain a solution containing 0.225 mg of ethyl acetate and 0.156 mg of cyclohexane.

Sample solution: Transfer 50 mg of Carbomer Copolymer to a 30-mL serum vial, add 20.0 mL of methanol, insert a rubber septum, and seal with a metal cap. Through the rubber septum add 10 µL of methyl ethyl ketone, and mix.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 2-mm × 3-m; packed with 1% liquid phase G25 on 60- to 80-mesh support S12

Temperatures

Detector: 250°

Injection port: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
115	—	115	4
115	6	175	5

Carrier gas: Helium

Flow rate: 33 mL/min

Injection volume: 2 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for methyl ethyl ketone, ethyl acetate, and cyclohexane are about 0.7, 0.9, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.0 between ethyl acetate and cyclohexane

Relative standard deviation: NMT 2.5%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of ethyl acetate and cyclohexane in the portion of Carbomer Copolymer taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = peak area ratio of the relevant analyte peak to the methyl ethyl ketone peak from the *Sample solution*

R_S = peak area ratio of the relevant analyte peak to the methyl ethyl ketone peak from the *Standard solution*

W_S = weight of ethyl acetate or cyclohexane in the *Standard solution* (mg)

W_U = weight of Carbomer Copolymer taken to prepare the *Sample solution* (mg)

Acceptance criteria

Ethyl acetate: NMT 0.5%

Cyclohexane: NMT 0.3%

• LIMIT OF BENZENE

Benzene solution: 1.0 mg/mL of benzene in dimethyl sulfoxide

Solvent solution: 0.1 µg/mL of *Benzene solution* in organic-free water (see *Residual Solvents* <467>, *Identification, Control, and Quantification of Residual Solvents*)

Sodium chloride solution: 20 mg/mL of sodium chloride

Sample solution: Transfer 50 mg of Carbomer Copolymer to a 10-mL volumetric flask. Add 7.5 mL of *Sodium chloride solution*, and mix by mechanical means

until homogeneous (usually about 30 min). Dilute with *Sodium chloride solution* to volume, and mix until homogeneous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.

Standard solution: Transfer 50 mg of Carbomer Copolymer to a 10-mL volumetric flask. Add 7.5 mL of *Sodium chloride solution*, and mix by mechanical means until homogeneous (usually about 30 min). Add 1.0 mL of the *Solvent solution*, dilute with *Sodium chloride solution* to volume, and mix until homogeneous (usually less than 1 min). This solution contains 0.01 µg/mL of benzene.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC, equipped with a headspace injector

Detector: Flame ionization

Column: 0.53-mm × 30-m fused silica; coated with 3.0-µm G43 stationary phase

Temperatures

Detector: 250°

Injection port: 140°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	10	240	20

Carrier gas: Helium

Flow rate: Linear velocity of 35 cm/s

Split ratio: 5:1

Injection volume: 1 mL (gaseous phase)

[NOTE—The following headspace conditions may be used: a pressurization time of 30 s and a transfer line temperature of 90°.]

Vial temperature: The vials are maintained at 80° for 60 min before headspace injection.

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 15% for three replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 10.0 mL each of the *Standard solution* and the *Sample solution* to separate headspace vials. Close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene, and secure with an aluminum crimped cap. Shake to obtain a homogeneous dispersion. Separately inject the gaseous phase of the *Standard solution* and the *Sample solution*.

Acceptance criteria: The benzene peak response from the *Sample solution* is NMT half of that from the *Standard solution*, corresponding to NMT 2 µg/g.

• LIMIT OF ACRYLIC ACID

Mobile phase: Dissolve 6.80 g of monobasic potassium phosphate in 300 mL of water, dilute with water to 500 mL, and mix. Dilute 100 mL of this solution with water to 1 L, adjust with phosphoric acid to a pH of 3.0 ± 0.1, and mix. Filter and degas.

Standard solution: 25 µg/mL of acrylic acid

Sample solution: Transfer 100 mg of Carbomer Copolymer to a tared serum vial. Add water to obtain 10.0 mL of solution. Cap the vial, and shake by mechanical means for 2 h. Add 2 drops of sodium hydroxide solution (500 mg/mL), and shake by hand for 15 s. Add 1.0 mL of calcium chloride solution (100 mg/mL), and shake until the gel collapses. Centrifuge for 15 min, and use the clear supernatant.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC
 Detector: UV 200 nm
 Column: 8-mm × 10-cm; packing L1
 Flow rate: 1 mL/min
 Injection volume: 10 µL
 System suitability
 Sample: *Standard solution*
 Suitability requirements
 Relative standard deviation: NMT 5%

Analysis

Samples: *Standard solution* and *Sample solution*
 Calculate the percentage of free acrylic acid in the portion of Carbomer Copolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of acrylic acid from the *Sample solution*
 r_S = peak response of acrylic acid from the *Standard solution*
 C_S = concentration of acrylic acid in the *Standard solution* (µg/mL)
 C_U = concentration of Carbomer Copolymer in the *Sample solution* (mg/mL)
 F = unit conversion, 10^{-3} mg/µg
 Acceptance criteria: NMT 0.25%

SPECIFIC TESTS• **LOSS ON DRYING (731)**

Analysis: Dry under vacuum at 80° for 1 h.

Acceptance criteria: NMT 2.0%

• **ROTATIONAL RHEOMETER METHODS (912)**

Sample: 5.00 g of Carbomer Copolymer, previously dried under vacuum at 80° for 1 h

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel-glass

Titrant: 180 mg/mL of sodium hydroxide

Endpoint detection: Potentiometric

Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 50 rpm, with the stirrer shaft set to one side of the beaker at an angle of 60° and the propeller positioned near the bottom of the beaker. The stirrer used is a three-blade, 2-inch-diameter marine impeller. Add the *Sample* at a uniform rate for 45–90 s, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 50 rpm for 15 min.

Remove the stirrer, and place the beaker containing the dispersion in a $25 \pm 0.1^\circ$ water bath for 30 min. Insert a paddle stirrer to a depth necessary to ensure that air is not drawn into the dispersion; and while stirring at 300 ± 25 rpm, titrate to a pH of 7.3–7.8 by adding *Titrant* below the surface. Stir 2–3 min until neutralization is complete. Then determine the final pH. [NOTE—If the pH is below 7.3, raise it with additional sodium hydroxide. If it is above 7.8, discard the mucilage, and prepare another batch, using a smaller amount of sodium hydroxide for titration.]

Return the beaker containing the neutralized mucilage to the $25 \pm 0.1^\circ$ water bath for 1 h. Measure the pH again, making certain that it is between 7.3 and 7.8. Using a rotational viscometer equipped with a suitable spindle at a spindle immersion depth as defined in *Table 3*, perform the viscosity determination without delay to avoid the slight viscosity changes that occur 75 min after neutralization.

The spindle rotates at 20 rpm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: See *Table 4*.

Table 4

Carbomer Copolymer	1% Viscosity Specification (mPa·s)
A	4,500–13,500
B	10,000–29,000
C	25,000–45,000

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, at a temperature not exceeding 45°.
- **LABELING:** If benzene has been used in the manufacturing process, the name of the article will be Carbomer 1342, provided it complies with and is labeled in accordance with the requirements set forth in that monograph. If benzene is not used in the manufacturing process, label it to indicate whether it is Type A, B, or C; and label it to state the measured viscosity, giving the viscosity measurement parameters, the concentration of the solution, and the type of equipment used; the solvent or solvents used in the polymerization process; and the nominal and residual solvent levels for each solvent.

Table 3

Viscosity Ranges (mPa·s)	Spindle No.	A ^a (cm)	B ^b (cm)	C ^c (cm)	D ^d (cm)	E ^e (cm)	Multiplier
100–400	1	5.6	2.2	0.3	2.7	6.1	5
400–1600	2	4.7	0.2	0.3	2.7	4.9	20
1000–4000	3	3.5	0.2	0.3	2.7	4.9	50
2000–8000	4	2.7	0.2	0.3	2.7	4.9	100
4000–16,000	5	2.1	0.2	0.3	2.7	4.9	200
10,000–40,000	6	1.5	0.2	0.3	3.0	4.9	500
40,000–160,000	7	—	—	0.3	—	5.5	2,000

^a Cylinder diameter.

^b Cylinder height.

^c Shaft diameter.

^d Distance from the top of the cylinder to the lower tip of the shaft.

^e Spindle immersion depth.

Carbomer Homopolymer

DEFINITION

Carbomer Homopolymer is a high molecular weight polymer of acrylic acid cross-linked with allyl ethers of polyalcohols. Carbomer Homopolymer, previously dried, contains NLT 56.0% and NMT 68.0% of carboxylic acid (–COOH) groups.

[NOTE—This monograph applies to, but is not limited to, Carbomer 934, Carbomer 934P, Carbomer 940, and Carbomer 941 manufactured without benzene. The heading of this monograph does not constitute the official title for a Carbomer Homopolymer manufactured with the use of benzene. When benzene is used in the manufacturing process, the name of the article will be Carbomer 934, Carbomer 934P, Carbomer 940, or Carbomer 941, whichever is appropriate.]

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K): Exhibits main bands at or near (± 5) wavenumbers (cm^{-1}) 1710, 1454, 1414, 1245, 1172, 1115, and 801, with the strongest band at 1710.
- **B.**
Sample dispersion: 10 mg/mL
Analysis: Adjust the *Sample dispersion* with 1 N sodium hydroxide to a pH of 7.5.
Acceptance criteria: A viscous gel is produced.
- **C.**
Analysis: Add 2 mL of calcium chloride solution (100 mg/mL), while stirring, to 10 mL of the gel obtained from *Identification* test B.
Acceptance criteria: A white precipitate is produced immediately.
- **D.**
Sample dispersion: 10 mg/mL
Analysis 1: Add 0.5 mL of thymol blue TS to 10 mL of the *Sample dispersion*.
Acceptance criteria 1: An orange color is produced.
Analysis 2: To another 10 mL of the *Sample dispersion* add 0.5 mL of cresol red TS.
Acceptance criteria 2: A yellow color is produced.

ASSAY

• CONTENT OF CARBOXYLIC ACID

Sample: 400 mg, previously dried under vacuum at 80° for 1 h

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel–glass

Titrant: 0.25 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continually at 1000 rpm. The stirrer shaft is set at an angle of about 60° and to one side of the beaker, and the propeller is positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate with *Titrant*. After each addition of *Titrant*, allow 1 min for mixing before recording the pH.

Calculate the carboxylic acid content as a percentage of carboxylic acid groups in the portion of Carbomer Homopolymer taken:

$$\text{Result} = [(V \times N \times M_r)/W] \times 100$$

V = *Titrant* volume consumed by the *Sample* (mL)
 N = actual normality of the *Titrant* (mEq/mL)
 M_r = molecular weight of the carboxylic acid (–COOH) group, 45.02
 W = *Sample* weight (mg)

Acceptance criteria: 56.0%–68.0%

IMPURITIES

• RESIDUE ON IGNITION (281)

Sample: 1.0 g

Acceptance criteria: NMT 4.0%

• HEAVY METALS, *Method II* (231): NMT 20 µg/g

• LIMIT OF ETHYL ACETATE AND CYCLOHEXANE

[NOTE—This test is required only for those Carbomer Homopolymers where ethyl acetate or a mixture of ethyl acetate and cyclohexane was used in the polymerization process.]

Standard stock solution: Transfer 5.0 mL of methanol to a 10-mL serum vial, insert a rubber septum, and seal with a metal cap. Add 25.0 µL of ethyl acetate and 20.0 µL of cyclohexane through the septum into the vial, and mix.

Standard solution: Transfer 20.0 mL of methanol to a 30-mL serum vial, insert a rubber septum, and seal with a metal cap. Through the rubber septum add 10 µL of methyl ethyl ketone (internal standard) and 50.0 µL of the *Standard stock solution*, and mix.

Sample solution: Transfer 50 mg of Carbomer Homopolymer to a 30-mL serum vial, add 20.0 mL of methanol, insert a rubber septum, and seal with a metal cap. Through the rubber septum add 10 µL of methyl ethyl ketone, and mix.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 2-mm \times 3-m; packed with 1% liquid phase G25 on 60- to 80-mesh support S12

Temperatures

Detector: 250°

Injection port: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
115	—	115	4
115	6	175	5

Carrier gas: Helium

Flow rate: 33 mL/min

Injection volume: 2 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for methyl ethyl ketone, ethyl acetate, and cyclohexane are about 0.7, 0.9, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.0 between ethyl acetate and cyclohexane

Relative standard deviation: NMT 2.5%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of ethyl acetate and cyclohexane in the portion of Carbomer Homopolymer taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = peak area ratio of the relevant analyte peak to the methyl ethyl ketone peak from the *Sample solution*
 R_S = peak area ratio of the relevant analyte peak to the methyl ethyl ketone peak from the *Standard solution*
 W_S = weight of ethyl acetate or cyclohexane in the *Standard solution* (mg)

W_U = weight of Carbomer Homopolymer taken to prepare the *Sample solution* (mg)

Acceptance criteria

Ethyl acetate: NMT 0.5%

Cyclohexane: NMT 0.3%

• LIMIT OF BENZENE

Benzene solution: 1.0 mg/mL of benzene in dimethyl sulfoxide

Solvent solution: 0.1 µg/mL of *Benzene solution* in organic-free water (see *Residual Solvents* (467), *Identification, Control, and Quantification of Residual Solvents*).

Sodium chloride solution: 20 mg/mL of sodium chloride

Standard solution: Transfer 50 mg of Carbomer Homopolymer to a 10-mL volumetric flask. Add 7.5 mL of *Sodium chloride solution*, and mix by mechanical means until homogeneous (usually about 30 min). Add 1.0 mL of the *Solvent solution*, dilute with *Sodium chloride solution* to volume, and mix until homogeneous (usually less than 1 min). This solution contains 0.01 µg/mL of benzene.

Sample solution: Transfer 50 mg of Carbomer Homopolymer to a 10-mL volumetric flask. Add 7.5 mL of *Sodium chloride solution*, and mix by mechanical means until homogeneous (usually about 30 min). Dilute with *Sodium chloride solution* to volume, and mix until homogeneous (usually less than 1 min). This preparation must be analyzed within 3 h of preparation.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC, equipped with a headspace injector

Detector: Flame ionization

Column: 0.53-mm × 30-m fused silica; coated with a 3.0-µm G43 stationary phase

Temperatures

Detector: 250°

Injection port: 140°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	10	240	20

Carrier gas: Helium

Flow rate: At a linear velocity of 35 cm/s

Injection type: Split ratio, 5:1

Injection volume: 1 mL (gaseous phase)

[NOTE—The following headspace conditions may be used: a pressurization time of 30 s and a transfer line temperature of 90°.]

The vials are maintained at a temperature of 80° for 60 min before headspace injection.

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 15% for three replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 10.0 mL each of the *Standard solution* and *Sample solution* to separate headspace vials. Close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene, and secure with an aluminum crimped cap. Shake to obtain a homogeneous dispersion. Separately inject the gaseous phase of the *Standard solution* and the *Sample solution*.

Acceptance criteria: NMT 2 µg/g; the benzene peak response from the *Sample solution* is NMT half of that from the *Standard solution*.

• LIMIT OF ACRYLIC ACID

Solution A: 1.361 mg/mL of monobasic potassium phosphate

Solution B: Acetonitrile and *Solution A* (1:1)

Mobile phase: See *Table 3*.

Table 3

Time (min)	Solution A (%)	Solution B (%)
0	100	0
8	100	0
9	0	100
20	0	100
21	100	0
30	100	0

Diluent: 25 mg/mL of potassium alum

Standard solution: 12.5 µg/mL of acrylic acid in *Diluent*

Sample solution: Mix 100 mg of Carbomer

Homopolymer with *Diluent*, and add *Diluent* to obtain 20.0 mL of suspension. Heat the suspension at 50° for 20 min with occasional shaking. Then shake the suspension continuously at room temperature for 60 min. Centrifuge, and use the clear supernatant.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm × 12-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of free acrylic acid in the portion of Carbomer Homopolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of acrylic acid from the *Sample solution*

r_S = peak response of acrylic acid from the *Standard solution*

C_S = concentration of acrylic acid in the *Standard solution* (µg/mL)

C_U = concentration of Carbomer Homopolymer in the *Sample solution* (mg/mL)

F = unit conversion, 10^{-3} mg/µg

Acceptance criteria: NMT 0.25%

SPECIFIC TESTS

• LOSS ON DRYING (731)

Analysis: Dry under vacuum at 80° for 1 h.

Acceptance criteria: NMT 2.0%

• ROTATIONAL RHEOMETER METHODS (912)

Sample: 2.50 g of Carbomer Homopolymer, previously dried

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel-glass

Titrant: 180 mg/mL of sodium hydroxide

Endpoint detection: Potentiometric

Analysis: Carefully add the *Sample* to 500 mL of water in a 800-mL beaker, while stirring continuously at 1000 ± 50 rpm. The stirrer shaft is set at an angle of 60° and positioned at one side of the beaker, and the propeller is positioned near the bottom of the beaker. The stirrer used should be a three-blade, 2-inch marine

Table 4

Viscosity Ranges (mPa·s)	Spindle No.	A ^a (cm)	B ^b (cm)	C ^c (cm)	D ^d (cm)	E ^e (cm)	Multiplier
100–400	1	5.6	2.2	0.3	2.7	6.1	5
400–1600	2	4.7	0.2	0.3	2.7	4.9	20
1000–4000	3	3.5	0.2	0.3	2.7	4.9	50
2000–8000	4	2.7	0.2	0.3	2.7	4.9	100
4000–16,000	5	2.1	0.2	0.3	2.7	4.9	200
10,000–40,000	6	1.5	0.2	0.3	3.0	4.9	500
40,000–160,000	7	—	—	0.3	—	5.5	2,000

^a Cylinder diameter.^b Cylinder height.^c Shaft diameter.^d Distance from the top of the cylinder to the lower tip of the shaft.^e Spindle immersion depth.

impeller. Add the *Sample* at a uniform rate over a period of 45–60 s, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 50 rpm for 15 min. [NOTE—Proper dispersion of the Carbomer Homopolymer resin is imperative for accurate viscosity readings.] Remove the stirrer, and allow the beaker containing the dispersion to stand at controlled room temperature for 30 min. Insert a paddle stirrer to a depth necessary to ensure that the air is not drawn into the dispersion, and while stirring at 300 ± 25 rpm, titrate with *Titrant* to a pH of 7.3–7.8. After adding the *Titrant*, stir with a paddle mixer at 300 ± 25 rpm for 2–3 min. [NOTE—After neutralization, care must be taken to avoid excessively high shearing, because aggressive mixing will break the polymer chains and reduce the viscosity reading.] Take the final pH reading with a pH meter to make sure it is between 7.3 and 7.8. [NOTE—If the pH is below 7.3, raise it with additional sodium hydroxide. If the pH is above 7.8, discard the mucilage, and prepare another using a smaller amount of sodium hydroxide for titration.] Place the neutralized mucilage into a water bath maintained at $25 \pm 0.1^\circ$ for 1 h. Measure the pH again, and make certain it is between 7.3 and 7.8. Perform the viscosity determination without delay to avoid the slight viscosity changes that occur 75 min after neutralization.

Equip a suitable rotational viscometer¹ with a suitable spindle, as defined in Table 4.

The spindle rotates at 20 rpm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: See Table 5.

Table 5

Carbomer Homopolymer	Viscosity Specified (mPa·s)
A	4,000–11,000
B	25,000–45,000
C	40,000–60,000

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, at a temperature not exceeding 45° .
- **LABELING:** Label it to indicate whether it is Type A, B, or C; and label it to state the measured viscosity, giving the viscosity measurement parameters, the concentration of the solution, and the type of equipment used; the solvent or solvents used in the polymerization process; and the nominal and residual solvent levels for each solvent.

¹ Available as a Brookfield RV viscometer, or equivalent.

Carbomer Interpolymer

DEFINITION

Carbomer Interpolymer is a carbomer homopolymer or copolymer that contains a block copolymer of polyethylene glycol and a long-chain alkyl acid ester.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K):** Exhibits bands at or near (± 5) wavenumbers (cm^{-1}) 1710, 1454, 1414, 1245, 1172, 1115, and 801, with the strongest band at 1710.
- **B.**
Sample: 2.5 g
Analysis: Without mixing, add the *Sample* to 500 mL of water in an 800-mL beaker, and allow to stand.
Acceptance criteria: The powders wet out in NMT 60 min.
- **C.**
Sample: 5 g
Analysis: Add the *Sample* to 500 mL of water, stir, and adjust with 1 N sodium hydroxide to a pH of 7.5.
Acceptance criteria: A viscous gel is formed.

ASSAY

• CONTENT OF CARBOXYLIC ACID

Sample: 400 mg, previously dried under vacuum at 80° for 1 h

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel–glass

Titrant: 0.25 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Slowly add the *Sample* to 400 mL of water in a 1000-mL beaker, while stirring continually at 1000 rpm. The stirrer shaft is set at an angle of about 60° and to one side of the beaker, and the propeller is positioned near the bottom of the beaker. Continue stirring for 15 min. Reduce the stirring speed, and titrate with *Titrant*. After each addition of *Titrant*, allow 1 min for mixing before recording the pH.

Calculate the carboxylic acid content as a percentage of carboxylic acid groups in the portion of Carbomer Interpolymer taken:

$$\text{Result} = [(V \times N \times M_r)/W] \times 100$$

V = *Titrant* volume consumed by the *Sample* (mL)

N = actual normality of the *Titrant* (mEq/mL)

M_r = molecular weight of the carboxylic acid (–COOH) group, 45.02

W = *Sample* weight (mg)

Acceptance criteria: 52.0%–62.0%

IMPURITIES

• **HEAVY METALS**, *Method II* (231): NMT 20 µg/g

• **LIMIT OF ETHYL ACETATE AND CYCLOHEXANE**

[NOTE—This test is required only for those Carbomer Interpolymers where the labeling indicates that ethyl acetate or a mixture of ethyl acetate and cyclohexane was used in the polymerization process.]

Standard stock solution: Transfer 5.0 mL of methanol to a 10-mL serum vial, insert a rubber septum, and seal with a metal cap. Add 20.0 µL of ethyl acetate and 10.0 µL of cyclohexane through the septum into the vial, and mix.

Standard solution: Transfer 20.0 mL of methanol to a 30-mL serum vial, insert a rubber septum, and seal with a metal cap. Through the rubber septum add 10 µL of methyl ethyl ketone (internal standard) and 50.0 µL of the *Standard stock solution*, and mix to obtain a solution containing 0.18 mg of ethyl acetate and 0.078 mg of cyclohexane.

Sample solution: Transfer 50 mg of Carbomer Interpolymer to a 30-mL serum vial, add 20.0 mL of methanol, insert a rubber septum, and seal with a metal cap. Through the rubber septum add 10 µL of methyl ethyl ketone, and mix.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 2-mm × 3-m; packed with 1% liquid phase G25 on 60- to 80-mesh support S12

Temperatures

Detector: 250°

Injection port: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
115	—	115	4
115	6	175	5

Carrier gas: Helium

Flow rate: 33 mL/min

Injection volume: 2 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for methyl ethyl ketone, ethyl acetate, and cyclohexane are about 0.7, 0.9, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.0 between ethyl acetate and cyclohexane

Relative standard deviation: NMT 2.5%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentages of ethyl acetate and cyclohexane in the portion of Carbomer Interpolymer taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = peak area ratio of the relevant analyte peak to the methyl ethyl ketone peak from the *Sample solution*

R_S = peak area ratio of the relevant analyte peak to the methyl ethyl ketone peak from the *Standard solution*

W_S = weight of ethyl acetate or cyclohexane in the *Standard solution* (mg)

W_U = weight of Carbomer Interpolymer taken to prepare the *Sample solution* (mg)

Acceptance criteria

Ethyl acetate: NMT 0.35%

Cyclohexane: NMT 0.15%

• LIMIT OF BENZENE

Benzene solution: 1.0 mg/mL of benzene in dimethyl sulfoxide

Solvent solution: 0.1 µg/mL of *Benzene solution* in organic-free water (see *Residual Solvents* (467), *Identification, Control, and Quantification of Residual Solvents*)

Sodium chloride solution: 20 mg/mL of sodium chloride

Sample solution: Transfer 50 mg of Carbomer Interpolymer to a 10-mL volumetric flask. Add 7.5 mL of *Sodium chloride solution*, and mix by mechanical means until homogeneous (usually about 30 min). Dilute with *Sodium chloride solution* to volume, and mix until homogeneous (usually less than 1 min). This solution must be analyzed within 3 h of preparation.

Standard solution: Transfer 50 mg of Carbomer Interpolymer to a 10-mL volumetric flask. Add 7.5 mL of *Sodium chloride solution*, and mix by mechanical means until homogeneous (usually about 30 min). Add 1.0 mL of the *Solvent solution*, dilute with *Sodium chloride solution* to volume, and mix until homogeneous (usually less than 1 min). This solution contains 0.01 µg/mL of benzene.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC, equipped with a headspace injector

Detector: Flame ionization

Column: 0.53-mm × 30-m fused silica; coated with 3.0-µm G43 stationary phase

Temperatures

Detector: 250°

Injection port: 140°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	20
40	10	240	20

Carrier gas: Helium

Flow rate: Linear velocity of 35 cm/s

Split ratio: 5:1

Injection volume: 1 mL (gaseous phase)

[NOTE—The following headspace conditions may be used: a pressurization time of 30 s and a transfer line temperature of 90°.]

The vials are maintained at a temperature of 80° for 60 min before headspace injection.

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 15% for three replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 10.0 mL each of the *Standard solution* and *Sample solution* to separate headspace vials. Close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene, and secure with an aluminum crimped cap. Shake to obtain a homogeneous dispersion. Separately inject the gaseous phase of the *Standard solution* and *Sample solution*.

Acceptance criteria: The benzene peak response from the *Sample solution* is NMT half of that from the *Standard solution*, corresponding to NMT 2 µg/g.

Table 3

Viscosity Ranges (mPa·s)	Spindle No.	A ^a (cm)	B ^b (cm)	C ^c (cm)	D ^d (cm)	E ^e (cm)	Multiplier
100–400	1	5.6	2.2	0.3	2.7	6.1	5
400–1600	2	4.7	0.2	0.3	2.7	4.9	20
1000–4000	3	3.5	0.2	0.3	2.7	4.9	50
2000–8000	4	2.7	0.2	0.3	2.7	4.9	100
4000–16,000	5	2.1	0.2	0.3	2.7	4.9	200
10,000–40,000	6	1.5	0.2	0.3	3.0	4.9	500
40,000–160,000	7	—	—	0.3	—	5.5	2,000

^a Cylinder diameter.^b Cylinder height.^c Shaft diameter.^d Distance from the top of the cylinder to the lower tip of the shaft.^e Spindle immersion depth.

• LIMIT OF ACRYLIC ACID

Mobile phase: Dissolve 6.80 g of monobasic potassium phosphate in 300 mL of water, dilute with water to 500 mL, and mix. Dilute 100 mL of this solution with water to 1 L, adjust with phosphoric acid to a pH of 3.0 ± 0.1, and mix. Filter and degas.

Standard solution: 25 µg/mL of acrylic acid

Sample solution: Transfer 100 mg of Carbomer Interpolymer to a tared serum vial. Add water to obtain 10.0 mL of solution. Cap the vial, and shake by mechanical means for 2 h. Add 2 drops of sodium hydroxide solution (500 mg/mL), and shake by hand for 15 s. Add 1.0 mL of calcium chloride solution (100 mg/mL), and shake until the gel collapses. Centrifuge for 15 min, and use the clear supernatant.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 8-mm × 10-cm; packing L1

Flow rate: 1 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5%

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of free acrylic acid in the portion of Carbomer Interpolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times 100$$

r_U = peak response of acrylic acid from the *Sample solution*

r_S = peak response of acrylic acid from the *Standard solution*

C_S = concentration of acrylic acid in the *Standard solution* (µg/mL)

C_U = concentration of Carbomer Interpolymer in the *Sample solution* (mg/mL)

F = unit conversion, 10⁻³ mg/µg

Acceptance criteria: NMT 0.25%

SPECIFIC TESTS

• LOSS ON DRYING (731)

Analysis: Dry under vacuum at 80° for 1 h.

Acceptance criteria: NMT 2.0%

• ROTATIONAL RHEOMETER METHODS (912)

Carbomer Interpolymer A

Sample: 2.50 g of Carbomer Interpolymer, previously dried under vacuum at 80° for 1 h (0.5% aqueous dispersion)

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Calomel-glass

Titrant: 180 mg/mL of sodium hydroxide

Endpoint detection: Potentiometric

Analysis: Carefully add the *Sample* to 500 mL of water in a 1000-mL beaker, while stirring continuously at 1000 ± 50 rpm, with the stirrer shaft set to one side of the beaker at an angle of 60° and the propeller positioned near the bottom of the beaker. The stirrer used is a three-blade, 2-inch-diameter marine impeller. Add the *Sample* at a uniform rate for 45–90 s, being sure that loose aggregates of powder are broken up, and continue stirring at 1000 ± 50 rpm for 15 min. Remove the stirrer, and place the beaker containing the dispersion in a 25 ± 0.1° water bath for 30 min. Insert a paddle stirrer to a depth necessary to ensure that air is not drawn into the dispersion; and while stirring at 300 ± 25 rpm, titrate to a pH of 7.3–7.8 by adding *Titrant* below the surface. Stir 2–3 min until neutralization is complete. Then determine the final pH. [NOTE—If the pH is below 7.3, raise it with additional sodium hydroxide. If it is above 7.8, discard the mucilage, and prepare another batch, using a smaller amount of sodium hydroxide for titration.] Return the beaker containing the neutralized mucilage to the 25 ± 0.1° water bath for 1 h. Measure the mucilage pH again, making certain that it is between 7.3 and 7.8. Using a rotational viscometer equipped with a suitable spindle at a spindle immersion depth as defined in *Table 3*, perform the viscosity determination without delay to avoid the slight viscosity changes that occur 75 min after neutralization.

The spindle rotates at 20 rpm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Carbomer Interpolymer B

Sample: 5.00 g of Carbomer Interpolymer, previously dried under vacuum at 80° for 1 h (1.0% aqueous dispersion)

Analysis: Proceed as directed for *Rotational Rheometer Methods* (912), *Carbomer Interpolymer A*, except adjust the pH of the dispersion to 5.8–6.3 instead of 7.3–7.8.

Carbomer Interpolymer C

Sample: 2.50 g of Carbomer Interpolymer, previously dried under vacuum at 80° for 1 h (0.5% aqueous dispersion)

Analysis: Proceed as directed for *Rotational Rheometer Methods* (912), *Carbomer Interpolymer A*, except adjust the pH of the dispersion to 5.8–6.3 instead of 7.3–7.8.

Acceptance criteria: See *Table 4*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, at a temperature not exceeding 45°.

Table 4

Carbomer Interpolymer	Viscosity Specifications (mPa·s)
A	45,000–65,000
B	47,000–77,000
C	8,500–16,500

- **LABELING:** Label it to indicate whether it is Type A, B, or C. Also label it to state the measured viscosity, giving the viscosity measurement parameters, the concentration of the solution, and the type of equipment used; the solvent or solvents used in the polymerization process; and the nominal and measured residual solvent levels for each solvent.

Carbon Dioxide—see Carbon Dioxide General Monographs

Carboxymethylcellulose Calcium

Cellulose, carboxymethyl ether, calcium salt;
Cellulose carboxymethyl ether calcium salt [9050-04-8].

DEFINITION

Carboxymethylcellulose Calcium is the calcium salt of a polycarboxymethyl ether of cellulose.

IDENTIFICATION

A. PROCEDURE

Sample solution: Shake thoroughly 0.1 g of Carboxymethylcellulose Calcium with 10 mL of water, followed by 2 mL of 1 N sodium hydroxide, and allow to stand for 10 min.

[NOTE—Save the unused portion of the *Sample solution* for use in *Identification* tests B and C.]

Analysis: To 1 mL of the *Sample solution* add water to make 5 mL. To 1 drop of the resulting solution, add 0.5 mL of chromatropic acid TS, and heat in a water bath for 10 min.

Acceptance criteria: A red-purple color develops.

B. PROCEDURE

Analysis: Shake 5 mL of the *Sample solution* prepared in *Identification* test A with 10 mL of acetone.

Acceptance criteria: A white, flocculent precipitate is formed.

C. PROCEDURE

Analysis: Shake 5 mL of the *Sample solution* prepared in *Identification* test A with 1 mL of ferric chloride TS.

Acceptance criteria: A brown, flocculent precipitate is formed.

D. IDENTIFICATION TESTS—GENERAL, Calcium (191):

Analysis: Ignite 1 g to ash, dissolve the residue in 10 mL of water and 5 mL of 6 N acetic acid, and filter, if necessary. Boil the filtrate, cool, and neutralize with 6 N ammonium hydroxide.

Acceptance criteria: Meets the requirements

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION (281):** 10.0%–20.0%; use 1.0 g, previously dried
- **HEAVY METALS, Method II (231):** NMT 20 ppm, adding 1 mL of hydroxylamine hydrochloride solution (1 in 5) to the solution of the residue
- **CHLORIDE AND SULFATE, Chloride (221)**
Sample stock solution: Shake thoroughly 0.80 g with 50 mL of water, dissolve in 10 mL of 1 N sodium hydroxide, and add water to make 100 mL.

[NOTE—Retain a portion of the *Sample stock solution* for use in the test for *Sulfate*.]

Sample solution: Heat 20 mL of the *Sample stock solution* with 10 mL of 2 N nitric acid in a water bath until a flocculent precipitate is formed, cool, centrifuge, and remove the supernatant. Wash the precipitate with three 10-mL portions of water by centrifuging each time, combine the supernatant and the washings, add water to make 100 mL, and mix.

Acceptance criteria: A 25-mL portion of the *Sample solution* shows no more chloride than is contained in 0.20 mL of 0.020 N hydrochloric acid (0.36%).

• CHLORIDE AND SULFATE, Sulfate (221)

Sample solution: Heat 10 mL of the *Sample stock solution* prepared in the test for *Chloride* with 1 mL of hydrochloric acid in a water bath until a flocculent precipitate is formed. Cool, centrifuge, and remove the supernatant. Wash the precipitate with three 10-mL portions of water by centrifuging each time, combine the supernatant and the washings, add water to make 100 mL, and mix.

Acceptance criteria: A 25-mL portion of the *Sample solution* shows no more sulfate than is contained in 0.21 mL of 0.020 N sulfuric acid (1.0%).

SPECIFIC TESTS

- **ALKALINITY:** Shake thoroughly 1.0 g with 50 mL of freshly boiled and cooled water, and add 2 drops of phenolphthalein TS: no red color develops.
- **LOSS ON DRYING (731):** Dry a sample at 105° for 4 h: it loses NMT 10.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Low-Substituted Carboxymethylcellulose Sodium

Cellulose, carboxymethyl ether, sodium salt, low-substituted; Carmellose sodium, low-substituted [9004-32-4].

DEFINITION

Low-Substituted Carboxymethylcellulose Sodium is the sodium salt of a partly *O*-(carboxymethylated) cellulose. It contains NLT 2.0% and NMT 4.5% of sodium (Na), calculated on the dried basis.

IDENTIFICATION

A.

Solution A: 100 mg/mL of sodium hydroxide

Sample: 1 g

Analysis: Shake the *Sample* with 100 mL of *Solution A*.

Acceptance criteria: A suspension is produced.

B.

Sample: 1 g

Analysis: Shake the *Sample* with 50 mL of water. Transfer 1 mL to a test tube, and add 1 mL of water and 1 mL of 1-naphthol TS. Incline the test tube, and add carefully 2 mL of sulfuric acid down the side so that it forms a lower layer.

Acceptance criteria: A reddish-purple color develops at the interface.

- **C.** It meets the requirements in *Impurities* for *Residue on Ignition* (281).

- **D. IDENTIFICATION TESTS—GENERAL, Sodium (191):** The *Test preparation* from the *Impurities, Heavy Metals* test meets the requirements for the pyroantimonate precipitate test.

ASSAY**• CONTENT OF SODIUM**

Analysis: Calculate the percentage of sodium in the Low-Substituted Carboxymethylcellulose Sodium taken:

$$\text{Result} = A \times (M \times M_{r1}/M_{r2})$$

A = percentage obtained from the test for *Residue on Ignition*, determined separately

M = number of moles of sodium per mole of sodium sulfate, 2

M_{r1} = atomic weight of sodium, 22.99

M_{r2} = molecular weight of sodium sulfate, 142.04

Acceptance criteria: 2.0%–4.5%

IMPURITIES**• RESIDUE ON IGNITION (281)**

Sample: 1.0 g

Analysis: Determine using a mixture of sulfuric acid and water (1:1) and an ignition temperature of $600 \pm 50^\circ$.

Acceptance criteria: 6.5%–13.5%

• HEAVY METALS, Method II (231)

Test preparation: To the residue obtained in the test for *Residue on Ignition*, add 1 mL of hydrochloric acid, evaporate on a water bath, and dissolve in 20 mL of water. [NOTE—Use this solution in *Identification* test D.]

Acceptance criteria: NMT 20 µg/g

• LIMIT OF SODIUM CHLORIDE AND SODIUM GLYCOLATE**Sodium Chloride**

Sample: 5 g

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Silver electrode and a mercurous sulfate electrode with a potassium sulfate bridge

Titrant: 0.05 N silver nitrate VS

Endpoint detection: Potentiometric

Analysis: Transfer the *Sample* to a 250-mL conical flask. Add 50 mL of water and 5 mL of 30% hydrogen peroxide, and heat on a water bath for 20 min, stirring occasionally to ensure hydration. Cool, add 100 mL of water and 10 mL of nitric acid. Titrate with *Titrant*.

Calculate the percentage of sodium chloride in the sample taken:

$$\text{Result} = \{[(V \times N \times F)/W] \times [100/(100 - b)]\} \times 100$$

V = volume of silver nitrate (mL)

N = normality of the silver nitrate (mEq/mL)

F = equivalency factor for sodium chloride, 0.05844 g/mEq

b = percentage obtained from the test for *Loss on Drying*, determined separately

W = weight of the sample (g)

Sodium Glycolate

Standard stock solution: Transfer 100 mg of glycolic acid, previously dried overnight in a vacuum desiccator over phosphorus pentoxide, to a 100-mL volumetric flask, and dissolve in and dilute with water to volume.

Standard solution A: Transfer 0.5 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, add 5 mL of glacial acetic acid, then dilute with acetone to volume.

Standard solution B: Transfer 1.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, add 5 mL of glacial acetic acid, then dilute with acetone to volume.

Standard solution C: Transfer 1.5 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, add 5 mL of glacial acetic acid, then dilute with acetone to volume.

Standard solution D: Transfer 2.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water

to make 5 mL, add 5 mL of glacial acetic acid, then dilute with acetone to volume.

Sample solution: Transfer 500 mg of Low-Substituted Carboxymethylcellulose Sodium to a beaker, moisten thoroughly with 5 mL of glacial acetic acid, add 5 mL of water, and stir with a glass rod to ensure proper hydration (about 30 min). Add 80 mL of acetone while stirring, add 2 g of sodium chloride, and stir for several min to ensure the complete precipitation of carboxymethylcellulose. Pass through a fast filter paper, previously wetted with a small amount of acetone, and collect the filtrate in a 100-mL volumetric flask. Rinse the beaker and filter with acetone, and add the washings to the flask. Dilute the filtrate with acetone to volume, and mix. Allow to stand for 24 h without shaking, and use the clear supernatant.

Instrumental conditions

Mode: Vis

Analytical wavelength: 540 nm

Blank: Use acetone solution containing 5% of glacial acetic acid and 5% of water.

Analysis: Transfer 2.0 mL of the *Sample solution* and 2.0 mL of each *Standard solution* to separate 25-mL volumetric flasks. Place the uncovered flasks in a boiling water bath for 20 min, accurately timed, to remove the acetone, remove from the bath, and cool. Add to each flask 5.0 mL of 2,7-dihydroxynaphthalene TS, mix, add an additional 15 mL, and again mix. Cover the mouth of each flask with a small piece of aluminum foil. Place the flasks upright in a boiling water bath for 20 min, then remove from the bath, cool, and dilute with sulfuric acid to volume.

Determine the absorbances of the solutions against the *Blank*, and prepare a standard curve using the absorbances obtained from the solutions prepared from the *Standard solutions*. From the standard curve and the absorbance of the *Sample solution*, determine the concentration, in mg/mL, of glycolic acid in the *Sample solution*, and calculate the percentage of sodium glycolate in the sample taken:

$$\text{Result} = \{[(C \times V)/(W \times F)] \times (M_{r1}/M_{r2}) \times [100/(100 - b)]\} \times 100$$

C = concentration of glycolic acid in the sample, determined from the standard curve (mg/mL)

V = volume of *Sample solution* (mL)

W = weight of sample (g)

F = unit conversion factor, 1000 mg/g

M_{r1} = molecular weight of sodium glycolate, 98.03

M_{r2} = molecular weight of glycolic acid, 76.05

b = percentage obtained from the test for *Loss on Drying*, determined separately

Acceptance criteria: The sum of the percentages from the tests for *Sodium Chloride* and *Sodium Glycolate* is NMT 0.5%.

SPECIFIC TESTS**• PH (791)**

Sample: 1 g

Analysis: Shake the *Sample* with 100 mL of carbon dioxide-free water, centrifuge, and test the suspension.

Acceptance criteria: 6.0–8.5

• LOSS ON DRYING (731)

Sample: 1.0 g

Analysis: Dry the *Sample* at 105° for 3 h.

Acceptance criteria: NMT 10.0%

• WATER-SOLUBLE SUBSTANCES

Sample: 5.0 g

Analysis: Disperse the *Sample* in 400 mL of water, and during the first 30 min, stir for 1 min every 10 min. Allow to stand for 1 h, and centrifuge, if necessary. Decant 100.0 mL of the supernatant onto a fast filter paper in a vacuum filtration funnel, apply a vacuum,

and collect 75.0 mL of the filtrate. Evaporate in a tared platinum or porcelain dish, and dry at 105° for 4 h.

Acceptance criteria: NMT 70%

• **SETTLING VOLUME**

[NOTE—The following test, which can relate to excipient function, may be carried out, depending on the intended use in the formulation. In cases where there are no concerns regarding the settling volume of this article, this test may be omitted. Where the labeling states the settling volume, determine the settling volume as follows.]

Sample: 5.0 g

Analysis: In a 100-mL graduated cylinder, transfer 20 mL of isopropyl alcohol, add the *Sample*, and shake vigorously. Dilute with isopropyl alcohol to 30 mL, then with water to 50 mL, and shake vigorously. Within 15 min, repeat the shaking three times. Allow to stand for 4 h, and determine the volume of the settled mass.

Acceptance criteria: 15.0–35.0 mL

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** When the settling volume is determined, label it to indicate the settling volume value.

Carboxymethylcellulose Sodium—see Carboxymethylcellulose Sodium General Monographs

Carboxymethylcellulose Sodium 12

DEFINITION

Carboxymethylcellulose Sodium 12 is the sodium salt of a polycarboxymethyl ether of cellulose. Its degree of substitution is NLT 1.15 and NMT 1.45, corresponding to a sodium (Na) content of NLT 10.4% and NMT 12.0%, calculated on the dried basis.

IDENTIFICATION

- **A.**
Sample solution: Add 1 g of powdered Carboxymethylcellulose Sodium 12 to 50 mL of water, while stirring to produce a uniform dispersion. Continue the stirring until a clear solution is produced. [NOTE—Save the unused portion of this solution for use in *Identification* tests B and C.]
Analysis: To 1 mL of the *Sample solution*, diluted with an equal volume of water, in a small test tube, add 5 drops of 1-naphthol TS. Incline the test tube, and carefully introduce down the side of the tube 2 mL of sulfuric acid so that it forms a lower layer.
Acceptance criteria: A red-purple color develops at the interface.
- **B.**
Analysis: To 5 mL of the *Sample solution* prepared for *Identification* test A add an equal volume of barium chloride TS.
Acceptance criteria: A fine, white precipitate is formed.
- **C. IDENTIFICATION TESTS—GENERAL, Sodium <191>:** A portion of the *Sample solution* prepared for *Identification* test A meets the requirements.

ASSAY

• **DEGREE OF SUBSTITUTION**

Sample: 200 mg, previously dried at 105° for 3 h

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid in dioxane VS

Endpoint detection: Potentiometric

Electrode system: A pH meter equipped with a standard glass electrode and a calomel electrode modified as follows. Discard the aqueous potassium chloride solution contained in the electrode, rinse, and fill with the supernatant obtained by shaking thoroughly 2 g each of potassium chloride and silver chloride (or silver oxide) with 100 mL of methanol, then add a few crystals of potassium chloride and silver chloride (or silver oxide) to the electrode.

Analysis: Weigh the *Sample*, and transfer to a glass-stoppered, 250-mL conical flask. Add 75 mL of glacial acetic acid, connect the flask to a water-cooled condenser, and reflux gently on a hot plate for 2 h. Cool, and transfer the solution to a 250-mL beaker with the aid of 50 mL of glacial acetic acid. Titrate with *Titrant* while stirring with a magnetic stirrer. Record the amount, in mL, of *Titrant* versus mV (0- to 700-mV range), and continue the titration to a few mL beyond the endpoint. Plot the titration curve, and read the volume of *Titrant* at the inflection point.

Calculate the degree of substitution in the Carboxymethylcellulose Sodium 12 taken:

$$\text{Result} = (M_{r1} \times N \times V) / (G - \Delta M_{r2} \times N \times V)$$

M_{r1} = molecular weight of 1 anhydroglucose unit, 162

N = *Titrant* normality

V = volume of *Titrant* (mL)

G = weight of Carboxymethylcellulose Sodium 12 taken (mg)

ΔM_{r2} = increase in molecular mass of 1 anhydroglucose unit for each sodium carboxymethyl group added, 80

Acceptance criteria: 1.15–1.45, corresponding to 10.4%–12.0% sodium content, on the dried basis

IMPURITIES

- **HEAVY METALS, Method II <231>:** NMT 20 ppm, using 1.0 g of Carboxymethylcellulose Sodium 12 and adding 1 mL of hydroxylamine hydrochloride solution (1 in 5) to the solution of the residue
- **LIMIT OF SODIUM CHLORIDE**
Sample: 5 g
Titrimetric system
(See *Titrimetry* <541>.)
Mode: Direct titration
Titrant: 0.05 N silver nitrate VS
Endpoint detection: Potentiometric
Electrode system: A silver electrode and a mercurous sulfate electrode having a potassium sulfate bridge
Analysis: Transfer the *Sample* to a 250-mL beaker, add 50 mL of water and 5 mL of 30% hydrogen peroxide, and heat on a water bath for 20 min, stirring occasionally to ensure hydration. Cool, add 100 mL of water and 10 mL of nitric acid, and titrate with *Titrant*, determining the endpoint while stirring constantly. Calculate the percentage of NaCl in the sample taken:

$$\text{Result} = (M_r \times V \times N \times F) / [(100 - b) / 100] \times W \times 100$$

M_r = molecular weight of sodium chloride, 58.44

V = volume of *Titrant* (mL)

N = normality of *Titrant*

F = conversion factor, 10⁻³ g/mg

b = percentage obtained from the test for *Loss on Drying*, determined separately

W = *Sample* weight (g)

Acceptance criteria: See *Limit of Sodium Glycolate*.

• **LIMIT OF SODIUM GLYCOLATE**

Standard stock solution: Transfer 100 mg of glycolic acid, previously dried in a desiccator at room

temperature overnight, to a 100-mL volumetric flask. Dissolve in and dilute with water to volume, and mix. [NOTE—Use this solution within 30 days.]

Standard solutions: Into four separate 100-mL volumetric flasks, transfer 1.0-, 2.0-, 3.0-, and 4.0-mL portions of the *Standard stock solution*, respectively. To each flask, add water to make 5 mL, add 5 mL of glacial acetic acid, dilute with acetone to volume, and mix.

Sample solution: Transfer 500 mg to a 100-mL beaker, moisten thoroughly with 5 mL of glacial acetic acid, followed by 5 mL of water, and stir with a glass rod to ensure proper hydration (usually about 15 min). Slowly add 50 mL of acetone, with stirring, then add 1 g of sodium chloride, and stir for several min to ensure complete precipitation of the carboxymethylcellulose. Filter through a soft, open-textured paper, previously wetted with a small amount of acetone, and collect the filtrate in a 100-mL volumetric flask. Use an additional 30 mL of acetone to facilitate the transfer of the solids and to wash the filter cake, then dilute with acetone to volume.

Blank: Prepare a 25-mL blank flask containing 2.0 mL of a solution containing 5% each of glacial acetic acid and water in acetone.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Spectrophotometry

Analytical wavelength: 540 nm

Analysis: Transfer 2.0 mL of the *Sample solution* and 2.0 mL of each *Standard solution* to separate 25-mL volumetric flasks. The 25-mL volumetric flask containing the *Blank* is included in the following tests. Place the uncovered flasks in a boiling water bath for 20 min, accurately timed, to remove the acetone, remove from the bath, and cool. Add to each flask 5.0 mL of 2,7-dihydroxynaphthalene TS, mix, add an additional 15 mL, and again mix. Cover the mouth of each flask with a small piece of aluminum foil. Place the flasks upright in a boiling water bath for 20 min, then remove from the bath, cool, dilute with sulfuric acid to volume, and mix.

Determine the absorbances of each solution against the *Blank*, and prepare a standard curve using the absorbances obtained from the solutions prepared from the *Standard solutions*. From the standard curve and the absorbance of the *Sample solution*, determine the weight (*w*), in mg, of glycolic acid in the *Sample solution*.

Calculate the percentage of sodium glycolate in the specimen taken:

$$\text{Result} = w \times F / [(100 - b)/100] \times W \times 100$$

- w* = weight of glycolic acid in the sample determined from the standard curve (mg)
F = factor converting glycolic acid to sodium glycolate, 1.29
b = percentage of *Loss on Drying*, determined separately
W = weight of the sample taken (mg)

Acceptance criteria: The sum of the percentages from the tests for *Limit of Sodium Chloride* and *Limit of Sodium Glycolate* is NMT 0.5%.

SPECIFIC TESTS

- PH (791):** 6.5–8.5, in a 10-mg/mL solution
- LOSS ON DRYING (731):** Dry a sample at 105° for 3 h: it loses NMT 10.0% of its weight.
- ROTATIONAL RHEOMETER METHODS (912)**

Analysis: Determine the viscosity in a water solution at the concentration stated on the label. Using undried Carboxymethylcellulose Sodium 12, weigh the amount which, on the dried basis, will provide 200 g of solution of the stated concentration. Add the substance in small amounts to 180 mL of stirred water contained in a

tared, wide-mouth bottle, continue stirring rapidly until the powder is well wetted, add sufficient water to make the mixture weigh 200 g, and allow to stand, with occasional stirring, until solution is complete. Adjust the temperature to 25 ± 0.2°, and determine the viscosity, using a rotational type of viscometer, making certain that the system reaches equilibrium before taking the final reading.

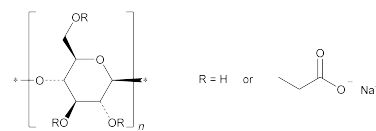
Acceptance criteria: The viscosity of solutions of 2% concentration is NLT 80.0% and NMT 120.0% of that stated on the label; the viscosity of solutions of 1% concentration is NLT 75.0% and NMT 140.0% of that stated on the label or it is between the maximum and minimum values, where stated as a range of viscosities.

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight containers.
- LABELING:** Label it to indicate the nominal viscosity in solutions of stated concentrations of either 1% (w/w) or 2% (w/w). The indicated viscosity may be in the form of a range encompassing 80.0%–120.0% of the nominal viscosity, where the solution concentration is 2% (w/w); or 75.0%–140.0% of the nominal viscosity, where the solution concentration is 1% (w/w).

Carboxymethylcellulose Sodium Paste— see Carboxymethylcellulose Sodium Paste General Monographs

Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium



Sodium salts of polymers containing substituted anhydroglucose units with the general formula:

$[\text{C}_6\text{H}_7\text{O}_2(\text{OH})_x(\text{OCH}_2\text{COONa})_y]_n$
 where *n* is the degree of polymerization;
y is the degree of substitution;
x is between 1.50 and 2.80;
y is between 0.20 and 1.50;
 and *x* + *y* = 3.0

Carboxymethyl cellulose, sodium, partially hydrolyzed enzymatically.

DEFINITION

Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium is the sodium salt of a polycarboxymethyl ether of cellulose, which has been partially hydrolyzed by enzymatic treatment with food grade *Trichoderma reesei* cellulase. Its degree of substitution is NLT 0.20 and NMT 1.50, corresponding to a sodium (Na) content of NLT 2.6% and NMT 12.2%, calculated on the dried basis.

IDENTIFICATION

- A.** Vigorously shake a 0.1% solution of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium: no layer of foam appears.
- B.** To 5 mL of a 0.5% solution of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium add 5 mL of a 5% solution of copper or aluminium sulfate: a precipitate appears.
- C.** Add 0.5 g of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium to 50 mL of water, while stirring, to

produce a uniform dispersion. Continue stirring until a clear solution of 1% is produced. Dilute 1 mL of this solution with 1 mL of water in a small test tube. Add 5 drops of 1-naphthol TS. Incline the tube, and carefully introduce down the side of the tube 2 mL of sulfuric acid, so that it forms a lower layer: a red-purple color develops at the interface.

- **D. IDENTIFICATION TESTS—GENERAL, Sodium (191):** A portion of the 1% solution obtained from *Identification* test C meets the requirements.
- **E.** It meets the requirements of the test for *Viscosity*.

ASSAY

• DEGREE OF SUBSTITUTION

Sample: 2.0 g of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium

Analysis: Ignite a clean and dry quartz crucible with a Bunsen burner, cool to room temperature in a desiccator, and weigh the crucible accurately. Transfer the *Sample* to the crucible. Carefully ignite on a small flame for about 10 min, and make sure that the *Sample* does not burn or excessively glow. Cool, and moisten the residue with 3–5 mL of sulfuric acid. Heat the crucible cautiously until the fuming is complete, and heat further until the *Sample* turns grayish white. Place the crucible in an oven at about 600°, until no black spots are visible. Cool the crucible in a desiccator to room temperature, and weigh. Place the crucible again in an oven at about 600° for 1 h, cool the crucible to room temperature in a desiccator, and weigh. This last step is repeated until a constant weight is achieved.

Calculate the percentage of sodium, P_{Na} , in the portion of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken:

$$\text{Result} = 100 \times N_{Na} \times A_{Na} \times (W_2 - W_1) / \{M_r \times W[(100 - \text{LOD})/100]\}$$

N_{Na} = number of sodium atoms/molecule of sodium sulfate, 2

A_{Na} = atomic weight of sodium, 22.99

W_2 = weight of the crucible with ash residue (g)

W_1 = weight of the crucible (g)

M_r = molecular weight of sodium sulfate, 142.04

W = weight of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken (g)

LOD = Loss on Drying value of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium (%)

Calculate the *Degree of Substitution* (DS):

$$\text{Result} = M_{r1} \times (P_{Na} - P_{NaCl} - P_{NaG}) / \{100 \times A_{Na} - [(M_{r2} - A_H) \times (P_{Na} - P_{NaCl} - P_{NaG})]\}$$

M_{r1} = molar mass of one glucose unit, 162.14

P_{NaCl} = percentage of sodium chloride obtained in the test for *Limit of Sodium Chloride and Sodium Glycolate*

P_{NaG} = percentage of sodium glycolate obtained in the test for *Limit of Sodium Chloride and Sodium Glycolate*

A_{Na} = atomic weight of sodium, 22.99

M_{r2} = molar mass of one sodium carboxymethyl unit (CH_2COONa), 81.03

A_H = atomic weight of hydrogen, 1.01

Acceptance criteria: NLT 0.20 and NMT 1.50 carboxymethyl groups (CH_2COOH)/anhydroglucose unit on the dried basis

IMPURITIES

Inorganic Impurities

• LIMIT OF LEAD

Nitric acid solution: Dilute 10 mL of nitric acid with 20 mL of water. Boil this solution to remove nitrous fumes, and allow it to cool to room temperature.

Lead standard stock solution: Dissolve 1.60 g of lead nitrate ($\text{Pb}(\text{NO}_3)_2$) in about 30 mL of *Nitric acid solution* in a 1000-mL volumetric flask, dilute with water to volume, and mix. At 20°, transfer 10.0 mL of this lead solution to a 500-mL volumetric flask, and dilute with water to volume. The *Lead standard stock solution* contains 20 µg/mL of Pb.

Standard solutions: Transfer 0, 1, 2, 3, 4, and 5 mL of the *Lead standard stock solution* to six identical 100-mL volumetric flasks, and add 50 mL of water to each flask. To each flask, add 8 mL of sulfuric acid and 10 mL of hydrochloric acid, and mix well. After complete dissolution, dilute each flask with water to volume, and mix. Transfer 1 mL of each solution to a separate 50-mL volumetric flask, and dilute with water to a final concentration of 0, 4, 8, 12, 16, and 20 µg/L of Pb, respectively.

Sample solution: Transfer 1 g of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium to a 100-mL beaker, and add 5 mL of 30% hydrogen peroxide. While stirring, add 50 mL of water to the solution, and heat the beaker on a hot plate at 50°, until all solids are dissolved. Quantitatively transfer this solution to a 100-mL volumetric flask, add 300 µL of nitric acid, dilute with water to volume, and mix.

Blank solution: Prepare as directed in the *Sample solution*, but omit the test specimen.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851))

Mode: Graphite furnace atomic absorption spectrophotometer, equipped with a lead hollow-cathode lamp and an adequate means of background correction.

Analytical wavelength: Lead emission line of 283.3 nm

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank solution*

Concomitantly determine the absorbances of the *Samples*. Optimize the instrument program for lead as recommended by the manufacturer. The strongest *Standard solution* is aspirated to optimize the instrument settings to give a full-scale reading on the detector. Correct the area responses of all *Sample solution* and *Standard solutions* for the *Blank solution* area response. Generate the appropriate lead calibration curve, and determine the lead concentration, C , in µg/L, in the *Sample solution*. Calculate the content of lead, in µg, in each g of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken:

$$\text{Result} = (V \times C) / W$$

V = volume of the *Sample solution*, 0.1 L

W = weight of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 3 µg/g of lead.

• LIMIT OF SODIUM CHLORIDE AND SODIUM GLYCOLATE Sodium Chloride

Ferric solution: Dissolve 20 g of ferric ammonium sulfate ($\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$) in a 100-mL volumetric flask containing 80 mL of water, add 0.3 mL of 10 N nitric acid (dilute 600 mL of nitric acid with water to 1000 mL), dilute with water to volume, and mix.

Analysis: Transfer an accurate quantity of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium, equivalent to 5.0 g on the dried basis, to a platinum or porcelain crucible. Heat the test specimen first with a small flame so that the test specimen does not ignite. When the charring is complete, heat further in an electric oven at about 600° for 15 min. After cooling, pulverize the ashes thus obtained and extract several times with water.

Filter the extracts into a 500-mL volumetric flask, add 5 mL of 10 N nitric acid, and dilute with water to volume. Transfer 100 mL of this extract to a suitable flask, add 2.0 mL of 0.2 N silver nitrate and 3 g of potassium nitrate, and mix. [NOTE—Silver chloride precipitate may develop. The potassium nitrate will prevent the silver chloride from interfering with the indication reaction.] After complete dissolution of the potassium nitrate, titrate this mixture with 0.02 N ammonium thiocyanate VS by adding 3 mL of *Ferric solution* as an indicator. Titrate until a red color develops and persists for at least 30 s. Each mL of 0.02 N silver nitrate is equivalent to 1.169 mg of sodium chloride.

Calculate the percentage of sodium chloride, P_{NaCl} , in the portion of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken:

$$\text{Result} = (0.001169 \times D \times V/W) \times 100$$

- D = dilution factor, 5
 V = volume of the 0.02 N silver nitrate consumed (mL)
 W = weight of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken, calculated on the dried basis (g)

Sodium Glycolate

Mobile phase: Prepare a filtered and degassed solution of 0.05% phosphoric acid in water.

Standard stock solution: Transfer 100 mg of glycolic acid, previously dried overnight in a vacuum desiccator over phosphorus pentoxide, and accurately weighed, to a 100-mL volumetric flask, dissolve in and dilute with *Mobile phase* to volume, and mix.

Standard solutions: Transfer 0.5-, 1.0-, 1.5-, 2.0-, and 2.5-mL portions of the *Standard stock solution*, respectively, into five identical 100-mL volumetric flasks. To each flask, dilute with *Mobile phase* to volume, and mix. The *Standard solutions* have concentrations of 5.0, 10.0, 15.0, 20.0, and 25.0 mg/L, respectively.

Sample solution: Transfer an accurate quantity of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium, equivalent to 200 mg on the dried basis, to a flask. Dissolve in and dilute with *Mobile phase* to 20 mL.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 7.8-mm \times 30-cm; packing L22. [NOTE—Two 7.8-mm \times 15-cm columns can be used in place of one 7.8-mm \times 30-cm column, provided that the *System suitability* requirements are met.]

Flow rate: 0.5 mL/min

Injection size: 50 μ L

System suitability

Sample: *Standard solution* with the concentration of 15 mg/L

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solutions* and *Sample solution*

Separately inject equal volumes of the *Standard solutions* and the *Sample solution* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Plot the peak areas from the *Standard solutions* versus the concentration of glycolic acid, in mg/L, in the *Standard solutions*. From the standard curve and the peak area from the *Sample solution*, determine the concentration of glycolic acid, C, in mg/L, in the *Sample solution*.

Calculate the percentage of sodium glycolate, P_{NaG} , in the portion of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken:

$$\text{Result} = \{[V \times (M_{r1}/M_{r2}) \times C]/W\} \times 100$$

- V = volume of the *Sample solution*, 0.02 L
 M_{r1} = molecular weight of sodium glycolate, 98.03
 M_{r2} = molecular weight of glycolic acid, 76.05
 W = weight of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken to prepare the *Sample solution*, calculated on the dried basis (mg)

Acceptance criteria: The sum of the percentages from the tests for sodium chloride and sodium glycolate ($P_{NaCl} + P_{NaG}$) is NMT 0.5%.

Organic Impurities

• PROCEDURE: LIMIT OF RESIDUAL ENZYME

Sample: 20.0 g of the dry Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium

Analysis: Transfer the *Sample* to a 2000-mL beaker. Add 100 mL of sulfuric acid, and mix using a stirring plate, until all particles are wetted. Prevent excessive foam formation during mixing. Add some glass beads to prevent boiling delay, and slowly heat the solution to a temperature not exceeding 150°. Stir for 30 min, and carefully add 5 mL of hydrogen peroxide dropwise at interval steps. After each step, allow the reaction to subside, and ensure that the solution does not contain any particulates. [NOTE—It takes up to approximately 125 mL of hydrogen peroxide.] The temperature is gradually increased to about 300°, until strong fumes of sulfur trioxide appear. Stop the heating. If the solution darkens again during heating, carefully add some drops of hydrogen peroxide until a clear solution is obtained. Allow the solution to cool down, add 10 mL of water, and heat again until strong fumes of sulfur trioxide appear. After the solution becomes clear and is cooled down, flush the sides with water, and transfer the solution quantitatively to a 4000-mL round-bottom flask. Dilute with water to a volume of approximately 500 mL, and place the round-bottom flask under the distillation unit. Pipet 10 mL of 0.1 N sulfuric acid into a 300-mL conical flask, and dilute with water to 100 mL. Place the conical flask at the end of the distillation unit, taking care that the end of the distillation unit is below the surface of the liquid. Through a dropping funnel add 500 mL of a 32% sodium hydroxide solution to the round-bottom flask with heavy stirring. Slowly heat until the solution boils. Boil, and collect the distillate for 20 min. Remove the conical flask, and stop the heating. Flush the inside of the condenser with water into the conical flask. Titrate the solution with 0.1 M sodium hydroxide. Perform a blank determination.

Calculate the percentage of protein in the portion of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken:

$$\text{Result} = \{F \times A_N \times [V_A C_A - (V_{TB} - V_B) \times C_{TB}]/W\} \times 100$$

- F = calculation factor for the theoretical amount of nitrogen present in protein, 6.25
 A_N = atomic weight of nitrogen, 14.01 g/mol
 V_A = volume of 0.1 N sulfuric acid added to the conical flask (mL)
 C_A = exact concentration of sulfuric acid added to the conical flask (N)
 V_{TB} = volume of the sodium hydroxide solution consumed in the titration for the sample determination (mL)
 V_B = volume of the sodium hydroxide solution consumed in the titration for the blank determination (mL)

- C_{TB} = concentration of the sodium hydroxide solution used in the titration (N)
 W = weight of Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium taken (mg)
Acceptance criteria: NMT 0.1% of protein

SPECIFIC TESTS• **ROTATIONAL RHEOMETER METHODS (912)**

Analysis: After determining the *Loss on Drying*, weigh a quantity of undried Enzymatically-Hydrolyzed Carboxymethylcellulose Sodium, equivalent to 60.0 g on the dried basis. In a 400-mL beaker, transfer about 240 mL of water, minus the amount of water in the test specimen. Over a period of seconds, transfer the test specimen to the 400-mL beaker containing water, while slowly and continuously stirring the solution to make the mixture weigh 300 g. When the specimen is well wetted, increase the rate of stirring, taking care to avoid mixing in excess air. Dissolve the test specimen while stirring rapidly. Equilibrate the mixture in a water bath at 25° for 1 h, until all air bubbles dissipate. Stir the solution in the beaker for 5 min at 300 rpm, making sure that no air bubbles are incorporated. Transfer the solution to a 250-mL beaker of 5 cm in internal diameter and about 12 cm in height, for measurement. Determine its viscosity at $25 \pm 0.1^\circ$, using a suitable rotational viscometer with a cylindrical spindle 1.9 cm in diameter and 6.5 cm high, attached to a shaft 0.3 cm in diameter.¹ The spindle rotates at 12 rpm at an immersion depth of 8.1 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: The viscosity is between 200 and 500 mPa · s.

- **PH (791):** 6.0–8.5, in a solution prepared in carbon dioxide-free water (1 in 100)
- **LOSS ON DRYING (731):** Dry it at 105° for 3 h: it loses NMT 12.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store in a cool and dry place.
- **LABELING:** Label it to indicate the viscosity, giving the viscosity measurement parameters, concentration of the solution, and the type of equipment used. The labeling also indicates the value for *Degree of Substitution*.

Cardamom Oil

DEFINITION

Cardamom Oil is the volatile oil distilled from the seed of *Elettaria cardamomum* (L.) Maton var. *cardamomum* (Fam. Zingiberaceae).

IMPURITIES

- **ARSENIC, Method II (211):** NMT 3 µg/g
- **LEAD (251)**
 Diluted standard lead solution: 10 mL
Analysis: Proceed as directed in the chapter.
Acceptance criteria: NMT 10 µg/g
- **HEAVY METALS, Method II (231):** NMT 40 µg/g

SPECIFIC TESTS

- **SPECIFIC GRAVITY (841):** 0.917–0.947
- **OPTICAL ROTATION, Angular Rotation (781A):** +22° to +44°
- **REFRACTIVE INDEX (831):** 1.463–1.466 at 20°
- **SOLUBILITY IN 70% ALCOHOL:** One volume dissolves in 5 volumes of 70% alcohol.

¹ A suitable spindle is available from Brookfield as an LV1 spindle, or the equivalent.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.

Cardamom Seed

DEFINITION

Cardamom Seed is the dried ripe seed of *Elettaria cardamomum* (L.) Maton var. *cardamomum* (Fam. Zingiberaceae).

[NOTE—Cardamom Seed should be recently removed from the capsule.]

SPECIFIC TESTS• **BOTANIC CHARACTERISTICS****Macroscopic**

Unground cardamom seed: Seeds usually appear in agglutinated groups of 2–7 seeds, and as separate seeds surrounded by an adhering membranous aril. The individual seeds are oblong-ovoid or irregularly three- to four-sided, 3–4 mm in length. They are convex on the dorsal side, strongly longitudinally grooved on the ventral side, coarsely tuberculated, and externally pale orange to dark brown. The odor is aromatic. The taste is aromatic, pungent, and slightly bitter.

Powdered cardamom seed: Brown to weak yellow to light olive green

Microscopic

Unground cardamom seed: Sections show a loosely attached membranous aril; a brownish seed coat consisting of an epidermal layer of thick-walled cells, a pigment layer of small cells with red to orange contents, a layer of volatile oil cells with suberized walls and a single layer of radially elongated, strongly lignified stone cells with inner walls heavily thickened, and a minute lumen containing silica; and a large, colorless perisperm surrounding a central, orange to yellow endosperm in which a small, straight embryo is embedded.

Powdered cardamom seed: It consists chiefly of fragments of perisperm, endosperm, embryo, and seed coat. The endosperm and perisperm cells are filled with starch grains 1–4 µm in diameter, or may contain one or more prisms of calcium oxalate 10–25 µm in diameter. The seed coat is characterized by its red- to orange-colored cells, is polygonal in surface view, and is about 20 µm in diameter. Fragments of pericarp tissue with spiral vessels and with accompanying slightly lignified fibers are relatively few.

- **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash (561):** NMT 4.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve against attack by insects.
- **LABELING:** The label states the Latin binomial and the official name.

Compound Cardamom Tincture

DEFINITION

Prepare Compound Cardamom Tincture as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* (795)).

Cardamom Seed, in moderately coarse powder	20 g
Cinnamon, in fine powder	25 g
Caraway, in moderately coarse powder	12 g
Glycerin	50 mL
Diluted Alcohol	950 mL
Diluted Alcohol, a sufficient quantity to make	1000 mL

Macerate the mixed powders in 750 mL of a mixture of 50 mL of *Glycerin* and 950 mL of *Diluted Alcohol* in a container that can be closed, and put it in a warm place. Agitate it frequently during 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter, and when most of the liquid has drained away, wash the residue on the filter by using first the remainder of the mixture of *Glycerin* and *Diluted Alcohol* prepared as directed above, followed by *Diluted Alcohol*, combining the filtrates to produce 1000 mL, and mix. [NOTE—Compound Cardamom Tincture may be colored with one or more colors.]

OTHER COMPONENTS

- **ALCOHOL DETERMINATION**, *Method II* (611): 43.0%–47.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Package in tight, light-resistant containers, and avoid exposure to direct sunlight and excessive heat.

Carmellose

Attributes	EP	JP	USP
Definition	+	+	+
Identification A*	+	+	+
Identification B	+	+	+
Chloride	+	+	+
Sulfate	+	+	+
Loss on Drying	+	+	+
Residue on Ignition	+	+	+

*EP and USP will adopt Carmellose Reference Standard; JP will adopt a Reference spectrum.

Legend: + will adopt and implement; – will not stipulate
Nonharmonized attributes: Heavy Metals and Packaging and Storage (USP)

Reagents and reference materials: Each pharmacopeia will adapt the text to take account of local reference substances and spectra and reagent specifications.

Carboxymethylcellulose [9000-11-7].

DEFINITION

Carmellose is a carboxymethyl ether of cellulose.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B. PH** (791)

Sample solution: 10 mg/mL of Carmellose suspension
Acceptance criteria: 3.5–5.0

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 1.5% on 1 g, calculated on the dried basis
- **CHLORIDES**

Sample solution: Shake well 0.8 g of Carmellose with 50 mL of water, dissolve in 10 mL of sodium hydroxide TS, and add water to make 100 mL. Heat 20 mL of this solution with 10 mL of nitric acid, diluted on a water bath until a flocculent precipitate is produced. Cool, centrifuge, and take out the supernatant. Wash the

precipitate with three 10-mL portions of water by centrifuging each time, combine the supernatant and the washings, and add water to make 100 mL. Take 25 mL of this solution, add 6 mL of nitric acid, diluted, and dilute with water to make 50 mL.

Control solution: 0.40 mL of 0.01 N hydrochloric acid VS and 6 mL of nitric acid, diluted. Add water to make 50 mL.

Analysis: Add 1 mL of silver nitrate TS to the *Sample solution* and to the *Control solution*, mix well, and allow to stand for 5 min protected from direct sunlight. Compare the opalescence developed in both solutions against a black background by viewing downward or transversely.

Acceptance criteria: The turbidity produced in the *Sample solution* is NMT that in the *Control solution* (NMT 0.36%).

• SULFATES

Sample solution: Shake well 0.40 g of Carmellose with 25 mL of water, dissolve in 5 mL of sodium hydroxide TS, and add 20 mL of water. Heat this solution with 2.5 mL of hydrochloric acid in a water bath until a flocculent precipitate is produced. Cool, centrifuge, and take out the supernatant. Wash the precipitate with three 10-mL portions of water by centrifuging each time, combine the supernatant and the washings, and add water to make 100 mL. Filter this solution, discard 5 mL of the first filtrate, take 25 mL of the subsequent filtrate, add 1 mL of hydrochloric acid, and dilute with water to make 50 mL.

Control solution: 1.5 mL of 0.01 N sulfuric acid VS and 1 mL of dilute hydrochloric acid. Add water to make 50 mL.

Analysis: Add 2 mL of barium chloride TS to the *Sample solution* and to the *Control solution*, mix well, and allow to stand for 10 min. Compare the turbidity developed in both solutions against a black background by viewing downward or transversely.

Acceptance criteria: The turbidity produced in the *Sample solution* is NMT that of the *Control solution* (NMT 0.72%).

- **HEAVY METALS**, *Method II* (231): Proceed with 1.0 g of Carmellose and perform the test. Prepare the control solution with 2.0 mL of *Standard Lead Solution*.

Acceptance criteria: NMT 20 ppm.

SPECIFIC TESTS

- **LOSS ON DRYING** (731): Dry 1 g at 105° for 4 h: it loses NMT 8.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight containers.
- **USP REFERENCE STANDARDS** (11)
USP Carmellose RS.

Carrageenan

Carrageenan [9000-07-1].

DEFINITION

Carrageenan is the hydrocolloid obtained by extraction with water or aqueous alkali, from some members of the class Rhodophyceae (red seaweeds). Carrageenan consists chiefly of potassium, sodium, calcium, magnesium, and ammonium sulfate esters of galactose and 3,6-anhydrogalactose copolymers. These hexoses are alternately linked α -1,3 and β -1,4 in the polymer. The prevalent copolymers in the hydrocolloid are designated *kappa*-, *iota*-, and *lambda*-carrageenan. *kappa*-Carrageenan is mostly the alternating polymer of D-galactose-4-sulfate and 3,6-anhydro-D-galactose. *iota*-Carrageenan is similar, except that the 3,6-anhydrogalactose is sulfated at carbon 2. Be-

tween *kappa*-carrageenan and *iota*-carrageenan there is a continuum of intermediate compositions differing in degree of sulfation at carbon 2. In *lambda*-carrageenan, the alternating monomeric units are mostly D-galactose-2-sulfate (1,3-linked) and D-galactose-2,6-disulfate (1,4-linked). The ester sulfate content for Carrageenan ranges from 18% to 40%. In addition, it contains inorganic salts that originate from the seaweed and from the process of recovery from the extract.

Carrageenan is recovered by alcohol precipitation, by drum drying, or by freezing. The alcohols used during recovery and purification are restricted to methanol, alcohol, and isopropyl alcohol. Carrageenan that is recovered by drum-roll drying may contain mono- and di-glycerides or up to 5% of polysorbate 80 used as roll-stripping agents.

IDENTIFICATION

- **A.**
Sample solution: A 20-mg/mL solution prepared by heating a uniform dispersion in a hot water bath to 80°
Analysis: Cool the *Sample solution*.
Acceptance criteria: The *Sample solution* becomes more viscous upon cooling and may form a gel.
- **B.**
Sample solution: Prepare as directed for the *Sample solution* in Identification test A.
Analysis: To 10 mL of the *Sample solution*, while still hot, add 4 drops of a 0.1-g/mL solution of potassium chloride, mix, and cool.
Acceptance criteria: A short-textured ("brittle") gel indicates a carrageenan of a predominantly *kappa* type; a compliant ("elastic") gel indicates a predominantly *iota* type. If the solution does not gel, the carrageenan is of a predominantly *lambda* type.
- **C.**
Analysis: Dilute a portion of the *Sample solution*, prepared as directed in Identification test A, with 4 parts of water, and add 2–3 drops of methylene blue TS.
Acceptance criteria: A blue, stringy precipitate is formed (also positive for furcellaran, a similar colloid).
- **D. INFRARED ABSORPTION**
Sample 1: Disperse 2 g in 200 mL of a 25-mg/mL solution of potassium chloride, and stir for 1 h. Allow to stand for 18 h, stir again for 1 h, and transfer to a centrifuge tube. (If the transfer cannot be made because the dispersion is too viscous, dilute with up to 200 mL of the potassium chloride solution.) Centrifuge at approximately $1000 \times g$ for 15 min.
Remove the clear supernatant, resuspend the residue in 200 mL of a 25-mg/mL solution of potassium chloride, and centrifuge again. Coagulate the combined supernatants by adding 2 volumes of dilute alcohol (9 in 10). (Retain the sediment for use in preparing *Sample 2*.) Recover the coagulum, and wash with 250 mL of the dilute alcohol. Press the excess liquid from the coagulum, and dry it at 60° for 2 h. The material so obtained is the nongelling fraction (*lambda* carrageenan).
Sample 2: Disperse the sediment retained from the preparation of *Sample 1* in 250 mL of cold water, heat at 90° for 10 min, and cool to 60°. Coagulate the mixture, then recover, wash, and dry the coagulum as described above. The material so obtained is the gelling fraction (*kappa*- and *iota*-carrageenan).
Analysis: Prepare a 2-mg/mL solution of each *Sample*, cast films 5 μ m thick (when dry) on a suitable nonsticking surface, and obtain the IR absorption spectrum of each film.
Acceptance criteria: Carrageenan has strong, broad absorption bands, typical of all polysaccharides, in the 1000 to 1100 cm^{-1} region. Absorption maxima are 1065 cm^{-1} and 1020 cm^{-1} for gelling and nongelling types, respectively. Other characteristic absorption bands and their intensities relative to the absorbance at 1050 cm^{-1} are as shown in Table 1.

Table 1

Wave Number (cm^{-1})	Molecular Assignment	Absorbance Relative to 1050 cm^{-1}		
		<i>kappa</i>	<i>iota</i>	<i>lambda</i>
1220–1260	Ester sulfate	0.7–1.2	1.2–1.6	1.4–2.0
928–933	3,6-Anhydro galactose	0.3–0.6	0.2–0.4	0–0.2
840–850	Galactose-4-sulfate	0.3–0.5	0.2–0.4	—
825–830	Galactose-2-sulfate	—	—	0.2–0.4
810–820	Galactose-6-sulfate	—	—	0.1–0.3
800–805	3,6-Anhydro galactose-2-sulfate	0–0.2	0.2–0.4	—

IMPURITIES

- **HEAVY METALS**, Method II (231): NMT 40 ppm
- **ARSENIC** (211): NMT 3 ppm
- **LEAD** (251): NMT 10 ppm

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total bacterial count does not exceed 200 cfu/g, and the tests for *Salmonella* species and *Escherichia coli* are negative.
- **LOSS ON DRYING** (731): Dry a sample at a pressure not exceeding 10 mm of mercury at 70° for 18 h, cool in a desiccator, and weigh: it loses NMT 12.5% of its weight.
- **SOLUBILITY IN WATER**: NMT 30 mL of water is required to dissolve 1 g at a temperature of 80°.
- **ACID-INSOLUBLE MATTER**
Sample: 2 g
Analysis: Transfer the *Sample* to a 250-mL beaker containing 150 mL of water and 1.5 mL of sulfuric acid. Cover with a watch glass, and heat on a steam bath for 6 h, rubbing down the wall of the beaker frequently with a rubber-tipped stirring rod, and replacing any water lost by evaporation. Transfer 500 mg of a suitable filter aid to the beaker, and filter through a tared filtering crucible provided with a 2.4-cm glass fiber filter. Wash the residue several times with hot water, dry at 105° for 3 h, cool in a desiccator, and weigh. The difference between the total weight and the sum of the weights of the filter aid, crucible, and glass fiber filter is the weight of the acid-insoluble matter.
Acceptance criteria: NMT 2.0% of the weight of Carrageenan taken
- **ROTATIONAL RHEOMETER METHODS** (912)
Sample: 7.5 g
Analysis: Transfer the *Sample* to a tared, tall-form, 600-mL beaker, add 450 mL of water, and disperse with agitation for 15 min. Add water to bring the weight to 500 g, and heat in a water bath, with continuous agitation, until a temperature of 80° is reached. Add water to adjust for loss by evaporation, cool to between 76° and 77°, and place in a constant-temperature bath maintained at 75°. Provide a suitable rotational viscometer with a spindle 1.88 cm in diameter and 6.51 cm high, using an immersion depth of 8.10 cm (No. 1 spindle). Allow the spindle to rotate in the solution at 30 rpm for 6 revolutions, then observe the scale reading. Convert the scale reading to centipoises by multiplying by the constant for the spindle and speed used.
Acceptance criteria: At 75° is NLT 5 centipoises
- **ARTICLES OF BOTANICAL ORIGIN**, Total Ash (561): NMT 35.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, preferably in a cool place.

Castor Oil—see Castor Oil General Monographs

Hydrogenated Castor Oil

DEFINITION

Hydrogenated Castor Oil is refined, bleached, hydrogenated, and deodorized Castor Oil, consisting mainly of the triglyceride of hydroxystearic acid.

IMPURITIES

- **HEAVY METALS**, *Method II* (231): NMT 10 µg/g

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE**, *Class II* (741): 85°–88°
- **FATS AND FIXED OILS**, *Iodine Value* (401): NMT 5
- **FATS AND FIXED OILS**, *Saponification Value* (401): 176–182
- **FATS AND FIXED OILS**, *Acid Value (Free Fatty Acids)* (401)

Sample solution: Melt 20 g in a conical flask on a steam bath, add 75 mL of hot alcohol that has previously been neutralized with 0.1 N sodium hydroxide to phenolphthalein TS, swirl, and add 1 mL of phenolphthalein TS.

Analysis: Titrate with 0.1 N sodium hydroxide VS, swirling vigorously, until the solution remains faintly pink after being shaken for 60 s.

Acceptance criteria: NMT 11.0 mL of 0.1 N sodium hydroxide VS

- **FATS AND FIXED OILS**, *Hydroxyl Value* (401)

Sample solution: 2 g in a glass-stoppered, 250-mL conical flask. Add 5.0 mL of a freshly prepared mixture of acetic anhydride and pyridine (1:3), and swirl to mix. Connect the flask to a reflux condenser, and heat on a steam bath for 2 h. Add 10 mL of water through the condenser, swirl to mix, heat on a steam bath for an additional 10 min, and allow to cool to room temperature. Add through the condenser 15 mL of normal butyl alcohol that has previously been neutralized to phenolphthalein, remove the condenser, wash the tip of the condenser and the sides of the flask with an additional 10 mL of neutralized normal butyl alcohol, and add 1 mL of phenolphthalein TS.

Analysis: Titrate with 0.5 N alcoholic potassium hydroxide VS to a faint pink endpoint. Perform a blank determination on a 5.0-mL portion of the acetic anhydride–pyridine mixture. To determine the amount of free acid in the Oil, weigh 10 g into a 250-mL conical flask, add 10 mL of pyridine that has previously been neutralized to phenolphthalein, swirl to mix, add 1 mL of phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS to a faint pink endpoint.

Calculate the hydroxyl value taken:

$$\text{Result} = M_r \times N \times [A + (B \times W/D) - C]/W$$

M_r = molecular weight of potassium hydroxide, 56.1

N = normality of the alcoholic potassium hydroxide solution

A = volume of 0.5 N alcoholic potassium hydroxide consumed by the blank (mL)

B = volume consumed in the free-acid titration (mL)

W = weight of Oil taken (g)

D = weight of Oil used in the free-acid titration (g)

C = volume consumed in the sample titration (mL)

Acceptance criteria: 154–162

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and avoid exposure to excessive heat.

Cellaburate

Cellulose, acetate butanoate;
Cellulose, acetate butyrate;
Acetylbutyrylcellulose;
Cellulose butyrate acetate;
Cellulose acetate butyrate [9004-36-8].

DEFINITION

Cellaburate is a reaction product of cellulose, acetic anhydride or acetic acid, and butyric acid or butyric anhydride. It contains NLT 1.0% and NMT 41.0% of acetyl (C₂H₃O) groups, by weight, and NLT 5.0% and NMT 56.0% of butyryl (C₄H₇O) groups, by weight, calculated on the previously dried, acid-free basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197F)

Sample solution: Dissolve 150 mg in 1 mL of acetone. Evenly cast 1 drop of the solution on a sodium chloride plate. Heat the plate at 105° for 10 min.

Acceptance criteria: Meets the requirements

ASSAY

- **ACETYL AND BUTYRYL CONTENT**

Internal standard solution: 4.6 mg/mL of isovaleric acid in pyridine. Store it in a tightly closed container.

Saponification solution: Place 250 mL of *n*-propyl alcohol in a 500-mL volumetric flask, add 65.5 g of potassium hydroxide, and mix to dissolve. Dilute with *n*-propyl alcohol to volume, and mix.

Acid solution: Place 250 mL of *n*-propyl alcohol in a 500-mL volumetric flask, add 166 mL of hydrochloric acid, and mix. Dilute with *n*-propyl alcohol to volume, and mix.

Standard solution: Transfer 0.20 g of glacial acetic acid and 0.31 g of butyric acid to a 50-mL volumetric flask. Dilute with *Internal standard solution* to volume, and mix.

Sample solution: Transfer 0.15 g of Cellaburate, previously dried at 105° for 1 h, into a 25-mm × 160-mm test tube. Pipet 10 mL of *Internal standard solution* into the test tube, and dissolve by stirring and heating at 110° for 30 min. While stirring, add 5 mL of *Saponification solution* slowly into the tube. Heat at 110° for 10 min. Cool, and add 5 mL of the *Acid solution*. Mix on a vortex mixer, and allow the precipitate to settle.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m fused silica bonded with a 1-µm layer of phase G35

Temperatures

Column: 125°

Injector: 250°

Detector: 250°

Carrier gas: Helium

Flow rate: 8 mL/min

Injection volume: 1 µL

Injection type: Split ratio, 35:1

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for acetic acid, butyric acid, and isovaleric acid are about 0.45, 0.85, and 1.00, respectively.]

Suitability requirements**Tailing factor:** NMT 1.5 for the butyric acid peak**Relative standard deviation:** NMT 3.0%**Calibration****Sample:** *Standard solution***Number of injections:** 3Calculate the average unit weight response, F_{SA} , of acetic acid per 10 mL of the *Internal standard solution*:

$$F_{SA} = (W_{RA}/R_{SA}) \times F_I$$

 W_{RA} = weight of acetic acid in the *Standard solution* (g) R_{SA} = average peak response ratio of acetic acid to isovaleric acid F_I = volume ratio of the *Internal standard solution* in the *Sample solution* to that in the *Standard solution*, 10:50Similarly, calculate the average unit weight response, F_{SB} , of butyric acid per 10 mL of the *Internal standard solution*:

$$F_{SB} = (W_{RB}/R_{SB}) \times F_I$$

 W_{RB} = weight of butyric acid in the *Standard solution* (g) R_{SB} = average peak response ratio of butyric acid to isovaleric acid**Analysis****Sample:** Upper clear solution from the *Sample solution*

Calculate the percentage of acetyl in the portion of Cellaburate taken:

$$\text{Result} = [(M_{r1}/M_{r2}) \times R_{UA} \times F_{SA}]/W_U \times 100$$

 M_{r1} = formula weight of acetyl, 43 M_{r2} = formula weight of acetic acid, 60 R_{UA} = peak response ratio of acetic acid to isovaleric acid in the *Sample solution* W_U = weight of Cellaburate taken to prepare the *Sample solution* (g)

Calculate the percentage of butyryl in the portion of Cellaburate taken:

$$\text{Result} = [(M_{r3}/M_{r4}) \times R_{UB} \times F_{SB}]/W_U \times 100$$

 M_{r3} = formula weight of butyryl, 71 M_{r4} = formula weight of butyric acid, 88 R_{UB} = peak response ratio of butyric acid to isovaleric acid in the *Sample solution***Acceptance criteria:** 1.0%–41.0% of acetyl (C_2H_3O) groups and 5.0%–56.0% of butyryl (C_4H_7O) groups on the previously dried, acid-free basis**IMPURITIES**• **HEAVY METALS**, *Method II* (231): NMT 20 µg/g• **RESIDUE ON IGNITION** (281): NMT 0.1%• **LIMIT OF FREE ACID****Indicator solution:** In a 1-L volumetric flask dissolve 0.675 g of bromocresol purple in 25 mL of 0.10 N sodium hydroxide. Dilute with water to volume, and mix.**Calibration solutions:** Pipet 1, 2, 3, and 4 mL of 0.001 N acetic acid VS into four 100-mL volumetric flasks, respectively. Pipet 4 mL of the *Indicator solution* into each flask and into an empty 100-mL volumetric flask, and dilute each flask with water to volume to obtain solutions containing 0.0, 0.60, 1.20, 1.80, and 2.40 µg/mL of acetic acid.**Control solution:** Place 96 mL of water in a suitable bottle, add a stirring bar, cap the bottle, and stir for 75 min at room temperature. Pipet 4 mL of the *Indicator solution* into the bottle, and mix.**Sample solution:** Transfer 1–2 g of Cellaburate to a bottle, and add 96 mL of water. Add a stirring bar, cap

the bottle, and stir for 75 min at room temperature.

Pipet 4 mL of the *Indicator solution* into the bottle, stir to mix, and allow the solid to settle for 2 min.**Instrumental conditions**(See *Spectrophotometry and Light-Scattering* (851).)**Mode:** Vis**Analytical wavelength:** Determine the maximum absorption of the basic form of bromocresol purple in the *Calibration* at about 589 nm.**Cell:** 1 cm**Blank:** Water**Calibration:** Determine the absorbances of the *Calibration solutions*. The absorbance difference between the 0.0-µg/mL solution and the other solutions adheres to Beer's law over the range stated in the *Calibration solutions*. Plot the absorbance difference versus the concentration of the acetic acid, in µg/mL, on linear graph paper, and draw the straight line best fitting the points, including the origin.**Analysis****Samples:** *Control solution* and *Sample solution*Pass 10 mL of the *Sample solution* through a polytetrafluoroethylene syringe filter that has been presoaked in isopropyl alcohol. Determine the absorbance of the filtered *Sample solution*. In the same manner, determine the absorbance of the *Control solution*.

Calculate the percentage of free acid, as acetic acid, in the portion of Cellaburate taken:

$$\text{Result} = (V \times C_U/W_U)/F \times 100$$

 V = total volume of the *Sample solution*, 100 mL C_U = concentration of free acid, calculated as acetic acid (µg/mL), based on the absorbance difference between the *Control solution* and the *Sample solution* read directly from the calibration plot W_U = weight of Cellaburate taken to prepare the *Sample solution* (g) F = unit conversion, 10^6 µg/g[NOTE—If the C_U value is more than 2.8 µg/mL, reduce the sample size by half in the *Sample solution*, and repeat the determination.]**Acceptance criteria:** NMT 0.1%**SPECIFIC TESTS**• **WATER DETERMINATION**, *Method I* (921): NMT 5.0%, using a mixture of methylene chloride and methanol (2:1) in place of the methanol solvent**ADDITIONAL REQUIREMENTS**

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** The labeling indicates the nominal percentage ranges of the acetyl and butyryl groups.
- **USP REFERENCE STANDARDS** (11)
USP Cellaburate RS

Cellacefate

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (♦) to specify this fact.Cellulose, acetate, 1,2-benzenedicarboxylate;
Cellulose acetate phthalate [9004-38-0].**DEFINITION**Cellacefate is a reaction product of phthalic anhydride and a partial acetate ester of cellulose. It contains NLT 21.5% and NMT 26.0% of acetyl (C_2H_3O) groups and NLT 30.0% and NMT 36.0% of phthalyl (o-carboxybenzoyl) ($C_8H_5O_3$) groups, calculated on the anhydrous, acid-free basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K): Do not dry specimens.

ASSAY• **PHTHALYL CONTENT**

Sample solution: Transfer 1 g to a conical flask, dissolve in 50 mL of a mixture of alcohol and acetone (3:2), and add phenolphthalein TS.

Analysis: Titrate the *Sample solution* with 0.1 N sodium hydroxide VS. Perform a blank determination, and make any necessary correction (see *Titrimetry* (541)).

Calculate the percentage of phthalyl on the acid-free basis:

$$\text{Result} = \{[(1.491 \times A/W) - (1.795 \times B)] / (100 - B)\} \times 100$$

A = volume of 0.1 N sodium hydroxide consumed, corrected for the blank (mL)

W = weight of Cellacefate taken, calculated on the anhydrous basis (g)

B = percentage of acid found in the test for *Limit of Free Acid*

Acceptance criteria: 30.0%–36.0% of phthalyl ($\text{C}_8\text{H}_5\text{O}_3$) on the anhydrous, acid-free basis

• **CONTENT OF ACETYL**

Sample solution: Transfer 100 mg to a glass-stoppered flask, and add 25.0 mL of 0.1 N sodium hydroxide VS. Connect the flask to a reflux condenser, and reflux for 30 min. Cool, and add phenolphthalein TS.

Analysis: Titrate the *Sample solution* with 0.1 N hydrochloric acid VS. Perform a blank determination (see *Titrimetry* (541)).

Calculate the free and combined acids as acetyl:

$$\text{Result} = 0.4305 \times (A/W)$$

A = volume of 0.1 N sodium hydroxide consumed, corrected for the blank (mL)

W = weight of Cellacefate taken, calculated on the anhydrous basis (g)

Calculate the percentage of acetyl on the acid-free basis:

$$\text{Result} = \{[(P - 0.5182 \times B) / (100 - B)] - (0.5772 \times C)\} \times 100$$

P = free and combined acids, as acetyl

B = percentage of acid found in the test for *Limit of Free Acid*

C = percentage of phthalyl found in the test for *Phthalyl Content*

Acceptance criteria: 21.5%–26.0% of acetyl ($\text{C}_2\text{H}_3\text{O}$) on the anhydrous, acid-free basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%
- **HEAVY METALS**, *Method II* (231): NMT 10 µg/g
- **LIMIT OF FREE ACID**

Sample solution: Transfer 3.0 g to a glass-stoppered flask, add 100 mL of dilute methanol (1 in 2), insert the stopper in the flask, and shake for 2 h. Filter, and wash the flask and the filter with two 10-mL portions of the methanol solution, adding the washings to the filtrate.

Analysis: Titrate the combined filtrate and washings from the *Sample solution* with 0.1 N sodium hydroxide VS to a phenolphthalein endpoint. Perform a blank determination on 120 mL of the dilute methanol (1 in 2) (see *Titrimetry* (541)).

Calculate the percentage of free acid, B:

$$\text{Result} = 0.8306 \times A/W$$

A = volume of 0.1 N sodium hydroxide consumed, corrected for the blank (mL)

W = weight of Cellacefate taken, calculated on the anhydrous basis (g)

Acceptance criteria: NMT 3.0%, calculated as phthalic acid

SPECIFIC TESTS• **WATER DETERMINATION**, *Method I* (921)

Sample: 0.5 g

Analysis: Dissolve the *Sample* in a mixture of dehydrated alcohol and methylene chloride (3:2) instead of methanol as the solvent.

Acceptance criteria: NMT 5.0%

• **VISCOSITY—CAPILLARY VISCOMETER METHODS** (911)

Sample: 15 g, calculated on the anhydrous basis

Analysis: Dissolve the *Sample* in 85 g of a mixture of 249 parts of anhydrous acetone and 1 part of water, by weight.

Acceptance criteria: The apparent viscosity (see *Viscosity—Capillary Viscometer Methods* (911), *Method I*) is between 45 and 90 centipoises, determined at $25 \pm 0.2^\circ$.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** (11)
USP Cellacefate RS.

Microcrystalline Cellulose

Cellulose [9004-34-6].

DEFINITION

Microcrystalline Cellulose is purified, partially depolymerized cellulose prepared by treating alpha cellulose, obtained as a pulp from fibrous plant material, with mineral acids.

IDENTIFICATION• **A. PROCEDURE**

Iodinated zinc chloride solution: Dissolve 20 g of zinc chloride and 6.5 g of potassium iodide in 10.5 mL of water. Add 0.5 g of iodine, and shake for 15 min.

Sample: 10 mg

Analysis: Place the *Sample* on a watch glass, and disperse in 2 mL of *Iodinated zinc chloride solution*.

Acceptance criteria: The substance takes on a violet-blue color.

• **B. PROCEDURE**

Sample: 1.3 g of Microcrystalline Cellulose, accurately weighed to 0.1 mg

Analysis: Transfer the *Sample* to a 125-mL conical flask. Add 25.0 mL of water and 25.0 mL of 1.0 M cupriethylenediamine hydroxide solution. Immediately purge the solution with nitrogen, insert the stopper, and shake on a wrist-action shaker, or other suitable mechanical shaker, until completely dissolved. Transfer an appropriate volume of the *Sample solution* to a calibrated number 150 Cannon-Fenske, or equivalent, viscometer. Allow the solution to equilibrate at $25 \pm 0.1^\circ$ for NLT 5 min. Time the flow between the two marks on the viscometer, and record the flow time, t_1 , in s.

Calculate the kinematic viscosity, $(KV)_1$, of the Microcrystalline Cellulose taken:

$$\text{Result} = t_1 \times k_1$$

t_1 = flow time (s)

k_1 = viscometer constant (see *Viscosity—Capillary Viscometer Methods* (911))

Obtain the flow time, t_2 , for 0.5 M cupriethylenediamine hydroxide solutions using a number 100 Cannon-Fenske, or equivalent, viscometer.

Calculate the kinematic viscosity, $(KV)_2$, of the solvent:

$$\text{Result} = t_2 \times k_2$$

t_2 = flow time for 0.5 M cupriethylenediamine hydroxide solutions (s)

k_2 = viscometer constant

Determine the relative viscosity, η_{rel} , of the Microcrystalline Cellulose specimen taken:

$$\text{Result} = (KV)_1 / (KV)_2$$

$(KV)_1$ = kinematic viscosity of the Microcrystalline Cellulose taken

$(KV)_2$ = kinematic viscosity of the solvent

Determine the intrinsic viscosity, $[\eta]_c$, by interpolation, using the *Intrinsic Viscosity Table* in the *Reference Tables* section.

Calculate the degree of polymerization, P:

$$\text{Result} = (95) \times [\eta]_c / W_s \times [(100 - \% \text{LOD}) / 100]$$

$[\eta]_c$ = intrinsic viscosity

W_s = weight of the Microcrystalline Cellulose taken (g)

$\% \text{LOD}$ = value obtained from the test for *Loss on Drying*

Acceptance criteria: The degree of polymerization is not greater than 350.

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 0.1%
- **HEAVY METALS**, *Method II* (231): NMT 10 ppm

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g. It meets the requirements of the tests for absence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* and for the absence of *Escherichia coli* and *Salmonella species*.

- **CONDUCTIVITY**

Sample: 5 g

Analysis: Shake the *Sample* with 40 mL of water for 20 min, and centrifuge. Retain the supernatant for use in the pH test. Using an appropriate conductivity meter that has been standardized with a potassium chloride conductivity calibration standard having a conductivity of 100 $\mu\text{S}/\text{cm}$, measure the conductivity of the supernatant after a stable reading is obtained, and measure the conductivity of the water used to prepare the test specimen.

Acceptance criteria: The conductivity of the supernatant does not exceed the conductivity of the water by more than 75 $\mu\text{S}/\text{cm}$.

- **pH** (791): 5.0–7.5 in the supernatant obtained in the *Conductivity* test
- **LOSS ON DRYING** (731): Dry a sample at 105° for 3 h: it loses NMT 7.0% of its weight, or some other lower percentage, or is within a percentage range, as specified in the labeling.

- **BULK DENSITY**

Analysis: Use a volumeter that has been fitted with a 10-mesh screen. The volumeter is freestanding of the brass or stainless steel cup, which is calibrated to a capacity of 25.0 ± 0.05 mL and has an inside diameter of 30.0 ± 2.0 mm. Weigh the empty cup, position it under the chute, and slowly pour the powder from a height of 5.1 cm (2 in) above the funnel through the volumeter, at a rate suitable to prevent clogging, until the cup overflows. [NOTE—If excessive clogging of the screen occurs, remove the screen.] Level the excess powder, and weigh the filled cup. Calculate the bulk density by dividing the weight of the powder in the cup by the volume of the cup.

Acceptance criteria: The bulk density is within the labeled specification.

- **PARTICLE SIZE DISTRIBUTION**

[NOTE—In cases where there are no functionality-related concerns regarding the particle size distribution of the article, this test may be omitted.]

Where the labeling states the particle size distribution, determine the particle size distribution as directed in *Particle Size Distribution Estimation by Analytical Sieving* (786), or by a suitable validated procedure.

- **WATER-SOLUBLE SUBSTANCES**

Sample: 5.0 g

Analysis: Shake the *Sample* with 80 mL of water for 10 min, and pass with the aid of a vacuum through filter paper (Whatman No. 42 or equivalent) into a vacuum flask. Transfer the filtrate to a tared beaker, evaporate to dryness without charring, dry at 105° for 1 h, cool in a desiccator, and weigh.

Acceptance criteria: The difference between the weight of the residue and the weight obtained from a blank determination does not exceed 12.5 mg (0.25%).

- **ETHER-SOLUBLE SUBSTANCES**

Sample: 10.0 g

Analysis: Place the *Sample* in a chromatographic column having an internal diameter of about 20 mm, and pass 50 mL of peroxide-free ether through the column. Evaporate the eluate to dryness in a previously dried and tared evaporating dish with the aid of a current of air in a fume hood. After all the ether has evaporated, dry the residue at 105° for 30 min, cool in a desiccator, and weigh.

Acceptance criteria: The difference between the weight of the residue and the weight obtained from a blank determination does not exceed 5.0 mg (0.05%).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** The labeling indicates the nominal loss on drying, bulk density, and degree of polymerization values. Degree of polymerization compliance is determined using *Identification* test B. Where the particle size distribution is stated in the labeling, proceed as directed in the test for *Particle Size Distribution*. The labeling indicates with which technique the particle size distribution was determined if a technique other than analytical sieving was used; and the labeling indicates the d_{10} , d_{50} , and d_{90} values and the range for each.

Microcrystalline Cellulose and Carboxymethylcellulose Sodium

DEFINITION

Microcrystalline Cellulose and Carboxymethylcellulose Sodium is a colloid-forming, attrited mixture of Microcrystalline Cellulose and Carboxymethylcellulose Sodium. It contains NLT 75.0% and NMT 125.0% of the labeled amount of carboxymethylcellulose sodium, calculated on the dried basis. The viscosity of its aqueous dispersion of percent by weight stated on the label is NLT 60.0% and NMT 140.0% of that stated on the label in centipoises.

IDENTIFICATION

- **A.**

Sample: 6 g

Analysis: Mix the *Sample* with 300 mL of water in a blender at 18,000 rpm for 5 min.

Acceptance criteria: A white, opaque dispersion is produced that does not settle on standing.
- **B.**

Sample: The dispersion obtained in *Identification* test A

Analysis: Add several drops of the *Sample* to a solution of aluminum chloride (100 mg/mL).

Acceptance criteria: Each drop forms a white, opaque globule that does not disperse on standing.

- **C.**
Sample: The dispersion obtained in *Identification* test A
Analysis: Add 3 mL of iodine TS to the *Sample*.
Acceptance criteria: No blue or purplish-blue color is produced.

ASSAY

• CARBOXYMETHYLCELLULOSE SODIUM

Sample solution: Transfer 2000 mg of Microcrystalline Cellulose and Carboxymethylcellulose Sodium to a glass-stoppered, 250-mL conical flask. Add 75 mL of glacial acetic acid, attach a condenser, and reflux for 2 h. Cool, and transfer the mixture to a 250-mL beaker with the aid of small volumes of glacial acetic acid.

Titrimetric conditions

Mode: Direct titration

Titrant: 0.1 N perchloric acid in dioxane VS

Endpoint detection: Potentiometry

Analysis: Titrate the *Sample solution* and calculate the percentage of carboxymethylcellulose sodium in the sample taken:

$$\text{Result} = [(V_S \times N \times F)/W] \times 100$$

V_S = Titrant volume consumed by the *Sample* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 296.0 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: 75.0%–125.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 5.0%
- **HEAVY METALS**, *Method II* (231): NMT 10 µg/g

SPECIFIC TESTS

• ROTATIONAL RHEOMETER METHODS (912)

Analysis: Determine the amounts of Microcrystalline Cellulose and Carboxymethylcellulose Sodium needed to prepare 600 g of a suitable dispersion, calculated on the dried basis. Transfer an amount of water to a 1000-mL blender bowl. Begin stirring with an 18,000 rpm blender at a reduced speed obtained by adjusting the voltage to 30 volts by means of a suitable transformer, and immediately add the accurately weighed portion of Microcrystalline Cellulose and Carboxymethylcellulose Sodium, taking care to avoid contacting the sides of the bowl with the powder. Continue stirring at this speed for 15 s following the addition of the powder, then increase the transformer setting to 115 volts, and mix for 2 min, accurately timed, at 18,000 rpm. Stop the blender, and lower the appropriate spindle of a suitable rotational viscometer into the dispersion. Thirty s after cessation of mixing, start the viscometer, and determine the viscosity using the appropriate spindle to obtain a scale reading between 10% and 90% of full-scale at a speed of 20 rpm. Determine the scale reading after 30 s of rotation, and calculate the viscosity, in centipoises, by multiplying the scale reading by the constant for the spindle used at 20 rpm.

Acceptance criteria: 60.0%–140.0% of that stated on the label, in centipoises

• PH (791)

Sample solution: The dispersion prepared in the test for *Viscosity*

Acceptance criteria: 6.0–8.0

• LOSS ON DRYING (731)

Analysis: Dry a sample at 105° to constant weight.

Acceptance criteria: NMT 8.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, store in a dry place, and avoid exposure to excessive heat.

- **LABELING:** Label it to indicate the percentage content of carboxymethylcellulose sodium and the viscosity of the dispersion in water of the designated weight percentage composition.

Silicified Microcrystalline Cellulose

DEFINITION

Silicified Microcrystalline Cellulose is composed of intimately associated microcrystalline cellulose and colloidal silicon dioxide particles, derived from aqueous coprocessing prior to drying the material during manufacture. The microcrystalline cellulose component is purified, partially depolymerized cellulose, prepared by treating alpha cellulose, obtained as a pulp from fibrous plant material, with mineral acids. The colloidal silicon dioxide is a submicroscopic fumed silica prepared by the vapor-phase hydrolysis of a silicon compound. The *Residue on Ignition* result indicates the percentage of colloidal silicon dioxide; the remainder is microcrystalline cellulose.

IDENTIFICATION

• A. INFRARED ABSORPTION (197K)

• B.

Sample: 10 mg

Iodinated zinc chloride solution: Dissolve 20 g of zinc chloride and 6.5 g of potassium iodide in 10.5 mL of water. Add 0.5 g of iodine, and shake for 15 min.

Analysis: Place the *Sample* on a watch glass, and disperse in 2 mL of *Iodinated zinc chloride solution*.

Acceptance criteria: The substance takes on a violet-blue color.

• C.

Sample: 5 mg of residue from the test for *Residue on Ignition*

Analysis: Transfer the *Sample* to a platinum crucible, and mix with about 200 mg of anhydrous potassium carbonate. Ignite at a red heat over a burner for about 10 min, and cool. Dissolve the melt in 2 mL of freshly distilled water, warming if necessary, and slowly add 2 mL of ammonium molybdate TS to the solution.

Acceptance criteria: A deep yellow color is produced.

• D. SILICA DISPERSION UNIFORMITY TEST

Conditioned test substance: Pass Silicified Microcrystalline Cellulose through an 850-µm sieve, disperse it into a suitable scale blender,¹ and tumble/mix the test substance for a minimum of 20 min to condition the material in preparation.

Analysis: Assemble a sieve stack composed of the following nested sieves: 60-, 80-, 120-, 200-, 325-, and 400-US mesh, plus pan. Tare each sieve to the nearest 0.1 g. Weigh 200.0 g of the *Conditioned test substance*, and transfer to the top sieve. Agitate the sieve stack on a suitable sieve shaker for 20 min. Separate and record the weight of each sieve, including the *Conditioned test substance* fraction. Determine the *Conditioned test substance* fraction mass by difference. Analyze a test substance from each sieve fraction, using *Residue on Ignition* (281). Obtain the *Residue on Ignition* (ROI) value in percentage, P_i , for each sieve fraction, excluding any fraction weighing less than 0.5 g. Calculate the average percentage of ROI value, P_A , for P_i ($i = 1-6$). Calculate the variance for the sieve fraction, excluding the pan and any fraction weighing less than 0.5 g:

$$\text{Result} = \frac{\sum_{i=1}^n (P_i - P_A)^2}{(n - 1)}$$

¹ Planetary mixer, Turbula T2F mixer, or V-blender.

Acceptance criteria: NMT 0.02

IMPURITIES

- **HEAVY METALS**, *Method II* (231): NMT 10 µg/g

SPECIFIC TESTS

- **RESIDUE ON IGNITION** (281): 1.8%–2.2%
- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **CONDUCTIVITY**
 Sample: 5 g
 Analysis: Shake the *Sample* with 40 mL of water for 20 min, and centrifuge. Retain the supernatant for use in the pH test. Using an appropriate conductivity meter that has been standardized with a potassium chloride conductivity calibration standard having a conductivity of 100 µS/cm, measure the conductivity of the supernatant after a stable reading is obtained, and measure the conductivity of the water used to prepare the test specimen.
 Acceptance criteria: The conductivity of the supernatant does not exceed the conductivity of the water by more than 75 µS/cm.
- **pH** (791): 5.0–7.5 for the supernatant obtained in the Conductivity test
- **LOSS ON DRYING** (731)
 Analysis: Dry at 105° for 3 h.
 Acceptance criteria: NMT 7.0%, within a percentage range, as specified in *Labeling*
- **DEGREE OF POLYMERIZATION**
 Sample: 1.3 g, weighed to 0.1 mg
 Analysis: Transfer the *Sample* to a 125-mL conical flask. Add 25.0 mL of water and 25.0 mL of 1.0 M cupriethylenediamine hydroxide solution. Immediately purge the solution with nitrogen, insert the stopper, and shake on a wrist action shaker or other suitable mechanical shaker until completely dissolved. Transfer an appropriate volume of the solution to a calibrated number 150 Cannon-Fenske, or equivalent, viscometer. Allow the solution to equilibrate at $25 \pm 0.1^\circ$ for NLT 5 min. Time the flow between the two marks on the viscometer. Calculate the kinematic viscosity of the *Sample* taken:

$$v_1 = t_1 \times k_1$$

t_1 = time of flow between the two marks on the viscometer (s)

k_1 = viscometer constant (see *Viscosity—Capillary Viscometer Methods* (911))

Obtain the flow time for a 0.5 M cupriethylenediamine hydroxide solution, using a number 100 Cannon-Fenske, or equivalent, viscometer.

Calculate the kinematic viscosity of the solvent:

$$v_2 = t_2 \times k_2$$

t_2 = time of flow for a 0.5 M cupriethylenediamine hydroxide solution (s)

k_2 = viscometer constant

Determine the relative viscosity of the Silicified Microcrystalline Cellulose taken:

$$\text{Result} = v_1/v_2$$

Calculate the degree of polymerization:

$$\text{Result} = 95 \times [\eta] / \{W_s \times [(100 - ROI)/100] \times [(100 - LOD)/100]\}$$

$[\eta]_c$ = value from interpolation of the relative viscosity, using the *Intrinsic Viscosity Table* in the *Reference Tables* section

W_s = weight of the sample taken (g)

ROI = value from the test for *Residue on Ignition* (%)

LOD = value from the test for *Loss on Drying* (%)

Acceptance criteria: NMT 350

BULK DENSITY

Sample: Silicified Microcrystalline Cellulose powder

Analysis: Use a volumeter that has been fitted with a 10-mesh screen. The volumeter is freestanding of the brass or stainless steel cup, which is calibrated to a capacity of 25.0 ± 0.05 mL and has an inside diameter of 30.0 ± 2.0 mm. Weigh the empty cup, position it under the chute, and slowly pour the powder from a height of 5.1 cm (2 inches) above the funnel through the volumeter, at a rate suitable to prevent clogging, until the cup overflows. [NOTE—If excessive clogging of the screen occurs, remove the screen.] Level the excess powder, and weigh the filled cup. Calculate the bulk density by dividing the weight of the powder in the cup by the volume of the cup.

Acceptance criteria: Within the labeled specification

- **PARTICLE SIZE DISTRIBUTION**: Where the labeling states the particle size distribution, determine the particle size distribution as directed in a suitable validated procedure.

WATER-SOLUBLE SUBSTANCES

Sample: 5.0 g

Analysis: Shake the *Sample* with 80 mL of water for 10 min, and filter with the aid of vacuum through filter paper (Whatman No. 42 or equivalent) into a vacuum flask. Transfer the filtrate to a tared beaker, evaporate to dryness without charring, dry at 105° for 1 h, cool in a desiccator, and weigh.

Acceptance criteria: NMT 0.25%; the difference between the weight of the residue and the weight obtained from a blank determination is NMT 12.5 mg.

ETHER-SOLUBLE SUBSTANCES

Sample: 10.0 g

Analysis: Place the *Sample* in a chromatographic column having an internal diameter of about 20 mm, and pass 50 mL of peroxide-free ether through the column. Evaporate the eluate to dryness in a previously dried and tared evaporating dish with the aid of a current of air in a fume hood. After all the ether has evaporated, dry the residue at 105° for 30 min, cool in a desiccator, and weigh.

Acceptance criteria: NMT 0.05%; the difference between the weight of the residue and the weight obtained from a blank determination is NMT 5.0 mg.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight containers. No storage requirements specified.
- **LABELING**: Label it to indicate the nominal *Loss on Drying*, *Bulk Density*, and *Degree of Polymerization* values. Where the particle size distribution is stated in the labeling, proceed as directed in *Particle Size Distribution*. The labeling indicates the technique with which the particle size distribution was determined, if a technique other than analytical sieving was used. The labeling also indicates the d_{10} , d_{50} , and d_{90} values and the range for each.
- **USP REFERENCE STANDARDS** (11)
 USP Silicified Microcrystalline Cellulose RS

Cellulose, Oxidized—see *Oxidized Cellulose General Monographs*

Cellulose, Oxidized Regenerated—see *Oxidized Regenerated Cellulose General Monographs*

Powdered Cellulose

DEFINITION

Powdered Cellulose is purified, mechanically disintegrated cellulose prepared by processing alpha cellulose obtained as a pulp from fibrous plant materials.

IDENTIFICATION

A. PROCEDURE

Iodinated zinc chloride solution: Dissolve 20 g of zinc chloride and 6.5 g of potassium iodide in 10.5 mL of water. Add 0.5 g of iodine, and shake for 15 min.

Sample: 10 mg

Analysis: Place the *Sample* on a watch glass, and disperse in 2 mL of *Iodinated zinc chloride solution*.

Acceptance criteria: The substance takes on a violet-blue color.

B. PROCEDURE

Sample: 0.25 g of Powdered Cellulose, accurately weighed to 0.1 mg

Analysis: Transfer the *Sample* to a 125-mL conical flask. Add 25.0 mL of water and 25.0 mL of 1.0 M cupriethylenediamine hydroxide solution. Immediately purge the solution with nitrogen, insert the stopper, and shake on a wrist action shaker, or other suitable mechanical shaker, until completely dissolved. Transfer an appropriate volume of the solution to a calibrated number 150 Cannon-Fenske, or equivalent, viscometer. Allow the solution to equilibrate at $25 \pm 0.1^\circ$ for NLT 5 min. Time the flow between the two marks on the viscometer, and record the flow time, t_1 , in s.

Calculate the kinematic viscosity, $(KV)_1$, of the Powdered Cellulose taken:

$$\text{Result} = t_1 \times k_1$$

k_1 = viscometer constant (see *Viscosity—Capillary Viscometer Methods* (911))

Obtain the flow time, t_2 , for a 0.5 M cupriethylenediamine hydroxide solution using a number 100 Cannon-Fenske, or equivalent, viscometer.

Calculate the kinematic viscosity, $(KV)_2$, of the solvent:

$$\text{Result} = t_2 \times k_2$$

k_2 = viscometer constant

Determine the relative viscosity, η_{rel} , of the Powdered Cellulose specimen taken:

$$\text{Result} = (KV)_1 / (KV)_2$$

$(KV)_1$ = kinematic viscosity of the Powdered Cellulose

$(KV)_2$ = kinematic viscosity of the solvent

Determine the intrinsic viscosity, $[\eta]_c$, by interpolation, using the *Intrinsic Viscosity Table* in the *Reference Tables* section.

Calculate the degree of polymerization, P :

$$\text{Result} = 95 \times [\eta]_c / W_s \times [(100 - \% \text{LOD}) / 100]$$

W_s = weight of the Powdered Cellulose taken (g)

$\% \text{LOD}$ = value obtained from the test for *Loss on Drying*

Acceptance criteria: The degree of polymerization is greater than 440.

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 0.3%, calculated on the dried basis
- **HEAVY METALS**, *Method II* (231): NMT 10 ppm

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic

microbial count does not exceed 1000 cfu/g, the total combined molds and yeasts count does not exceed 100 cfu/g, and it meets the requirements of the tests for absence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* and for absence of *Escherichia coli* and *Salmonella* species.

- **pH** (791): The pH of the supernatant is 5.0–7.5. Mix 10 g with 90 mL of water, and allow to stand with occasional stirring for 1 h.
- **LOSS ON DRYING** (731): Dry a sample at 105° for 3 h: it loses NMT 6.5% of its weight.

WATER-SOLUBLE SUBSTANCES

Sample: 6.0 g

Analysis: Mix the *Sample* with 90 mL of recently boiled and cooled water, and allow to stand with occasional stirring for 10 min. Filter, with the aid of vacuum, discard the first 10 mL of the filtrate, and pass the filtrate through the same filter a second time, if necessary, to obtain a clear filtrate. Evaporate a 15.0-mL portion of the filtrate in a tared evaporating dish to dryness without charring, dry at 105° for 1 h, cool in a desiccator, and weigh.

Acceptance criteria: The difference between the weight of the residue and the weight obtained from a blank determination does not exceed 15.0 mg (1.5%).

ETHER-SOLUBLE SUBSTANCES

Sample: 10.0 g

Analysis: Place the *Sample* in a chromatography column having an internal diameter of about 20 mm, and pass 50 mL of peroxide-free ether through the column. Evaporate the eluate to dryness in a previously dried and tared evaporating dish with the aid of a current of air in a fume hood. After all the ether has evaporated, dry the residue at 105° for 30 min, cool in a desiccator, and weigh.

Acceptance criteria: The difference between the weight of the residue and the weight obtained from a blank determination does not exceed 15.0 mg (0.15%).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** The labeling indicates the nominal degree of polymerization value. Degree of polymerization compliance is determined using *Identification test B*.

Cellulose Acetate

Cellulose acetate

Cellulose, diacetate [9035-69-2].

Cellulose, triacetate [9012-09-3].

DEFINITION

Cellulose Acetate is partially or completely acetylated cellulose. It contains NLT 29.0% and NMT 44.8%, by weight, of acetyl (C_2H_3O) groups, calculated on the dried basis. Its acetyl content is NLT 90.0% and NMT 110.0% of that indicated on the label.

IDENTIFICATION

• INFRARED ABSORPTION

Sample solution: Prepare a solution (1 in 10) of Cellulose Acetate, previously dried, in dioxane.

Analysis: Spread 1 drop of the *Sample solution* on a sodium chloride plate, place a second sodium chloride plate over it, and spread the specimen between the plates. Separate the plates, heat them both at 105° for 1 h, and reassemble the dried plates.

Acceptance criteria: The IR absorption spectrum exhibits maxima only at the same wavelengths as that of a similar preparation of USP Cellulose Acetate RS, treated in the same manner.

ASSAY**• CONTENT OF ACETYL**

For Cellulose Acetate labeled to contain NMT 42.0% of acetyl groups

Sample: 2 g of Cellulose Acetate

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Residual titration

Titrant: 1.0 N sodium hydroxide VS

Back-titrant: 1.0 N sulfuric acid VS

Endpoint detection: Colorimetric

Analysis

Transfer the *Sample* to a 500-mL flask. Add 100 mL of acetone and 5–10 mL of water to the flask, insert the stopper into the flask, and stir with a magnetic stirrer until solution is complete. Add 30 mL of *Titrant* to the solution, with constant stirring. A finely divided precipitate of regenerated cellulose, free from lumps, is obtained. Insert the stopper into the flask, and stir with a magnetic stirrer for 30 min. Add 100 mL of water that has been preheated to 80°, washing down the sides of the flask, stir for 2 min, and cool to room temperature. Titrate the excess sodium hydroxide solution with *Back-titrant* to a phenolphthalein endpoint. Treat a blank in the same manner.

Calculate the percentage of acetyl in the portion of Cellulose Acetate taken:

$$\text{Result} = 4.305 \times (B - A)/W$$

B = volume of the *Back-titrant* consumed by the blank (mL)

A = volume of the *Back-titrant* consumed by Cellulose Acetate (mL)

W = weight of Cellulose Acetate taken, calculated on the dried basis (g)

Acceptance criteria: 29.0%–44.8%, by weight, of acetyl ($\text{C}_2\text{H}_3\text{O}$) groups, on the dried basis

For Cellulose Acetate labeled to contain more than 42.0% of acetyl groups

Sample: 2 g of Cellulose Acetate

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Residual titration

Titrant: 0.5 N hydrochloric acid VS

Back-titrant: 0.5 N sodium hydroxide VS

Endpoint detection: Colorimetric

Analysis: Transfer the *Sample* to a 500-mL conical flask. Add 30.0 mL of dimethyl sulfoxide and 100 mL of acetone, and stir for 16 h with the aid of a magnetic stirrer. Pipet 30 mL of 1 N sodium hydroxide VS slowly into the flask, with constant stirring. Insert the stopper into the flask, and stir for 6 min. Allow to stand without stirring for 60 min. Resume stirring, and add 100 mL of water that has been preheated to 80°, washing down the sides of the flask. Stir for 2 min, and cool to room temperature. Add 4–5 drops of phenolphthalein TS, and titrate the excess sodium hydroxide solution with *Titrant*. Add an excess of 0.5 mL of *Titrant*. Stir for 5 min. Allow to stand for 30 min. Titrate with *Back-titrant* to a persistent pink endpoint, using a magnetic stirrer for agitation. Calculate the net number of milliequivalents of sodium hydroxide consumed, and correct this value by use of the average of two blank determinations run concomitantly through the entire procedure. Calculate the percentage of acetyl in the portion of Cellulose Acetate taken:

$$\text{Result} = 4.305 \times n/W$$

n = corrected value of the net number of milliequivalents of sodium hydroxide consumed

W = weight of Cellulose Acetate taken, calculated on the dried basis (g)

Acceptance criteria: 29.0%–44.8%, by weight, of acetyl ($\text{C}_2\text{H}_3\text{O}$) groups, on the dried basis

IMPURITIES**Inorganic Impurities**

• **RESIDUE ON IGNITION** <281>: NMT 0.1%

• **HEAVY METALS**, *Method II* <231>: NMT 10 ppm

Organic Impurities**• PROCEDURE: LIMIT OF FREE ACID**

Sample: 5 g

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.01 N sodium hydroxide VS

Endpoint detection: Colorimetric

Analysis: Transfer the *Sample* to a 250-mL flask. Add 150 mL of freshly boiled, cooled water, insert the stopper into the flask, swirl the suspension gently, and allow it to stand for 3 h. Filter through paper, and wash the flask and the filter with water, adding these washings to the filtrate. Add phenolphthalein TS, and titrate the combined filtrate and washings with the *Titrant*.

Calculate the percentage of free acid in the portion of Cellulose Acetate taken:

$$\text{Result} = 0.06005 \times A/W$$

A = volume of *Titrant* consumed (mL)

W = weight of the Cellulose Acetate taken, calculated on the dried basis (g)

Acceptance criteria: NMT 0.1%, calculated as acetic acid

SPECIFIC TESTS

• **LOSS ON DRYING** <731>: Dry a sample at 105° for 3 h: it loses NMT 5.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **LABELING:** The labeling states the nominal percentage content of acetyl.

• **USP REFERENCE STANDARDS** <11>

USP Cellulose Acetate RS

Cetostearyl Alcohol

DEFINITION

Cetostearyl Alcohol contains NLT 40.0% of stearyl alcohol ($\text{C}_{18}\text{H}_{38}\text{O}$), and the sum of the stearyl alcohol content and the cetyl alcohol ($\text{C}_{16}\text{H}_{34}\text{O}$) content is NLT 90.0%.

IDENTIFICATION

• **A.** The retention times of the major peaks of the *Sample solution* correspond to those of the *System suitability solution*, as obtained in the *Assay*.

ASSAY**• PROCEDURE**

System suitability solution: 5 mg/mL each of USP Cetyl Alcohol RS and USP Stearyl Alcohol RS, in alcohol

Sample solution: 10 mg/mL of Cetostearyl Alcohol, in dehydrated alcohol

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 3-mm × 2-m; 10% liquid phase G2, support S1A

Temperatures

Column: 205°

Injection port: 275°

Detector: 250°

Carrier gas: Helium

Injection volume: 2 µL

System suitability**Sample:** *System suitability solution***Suitability requirements****Resolution:** NLT 4.0 between cetyl alcohol and stearyl alcohol**Relative standard deviation:** NMT 1.5% for the percentages of cetyl alcohol (C₁₆H₃₄O) and stearyl alcohol (C₁₈H₃₈O)**Analysis:** *Sample solution*Calculate the percentages of cetyl alcohol (C₁₆H₃₄O) and stearyl alcohol (C₁₈H₃₈O) in the portion of sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

 r_U = peak area of cetyl alcohol or stearyl alcohol r_T = sum of all the peak areas, except that for the solvent**Acceptance criteria****Stearyl alcohol (C₁₈H₃₈O):** NLT 40.0%**Sum of stearyl alcohol (C₁₈H₃₈O) and cetyl alcohol (C₁₆H₃₄O):** NLT 90.0%**SPECIFIC TESTS**

- **MELTING RANGE OR TEMPERATURE** <741>: 48°–55°

- **FATS AND FIXED OILS, Acid Value** <401>: NMT 2

- **FATS AND FIXED OILS, Hydroxyl Value** <401>

Sample: 2 g**Blank:** Proceed as directed in *Analysis*, excluding the *Sample*.**Titrimetric system****Mode:** Direct titration**Titrant:** 1 N sodium hydroxide VS**Endpoint detection:** Visual

Analysis: Place the *Sample* in a dry, glass-stoppered, 250-mL flask, add 2 mL of pyridine, then add 10 mL of toluene. To the mixture add 10.0 mL of a solution of acetyl chloride prepared by mixing 10 mL of acetyl chloride with 90 mL of toluene. Insert the stopper in the flask, and immerse in a water bath heated at 60°–65° for 20 min. Add 25 mL of water, again insert the stopper in the flask, and shake vigorously for several min to decompose the excess acetyl chloride. Add 0.5 mL of phenolphthalein TS. Titrate with *Titrant* to a permanent pink endpoint, shaking the flask vigorously toward the end of the titration to maintain the contents in an emulsified condition. Perform a blank determination. Calculate the hydroxyl value in the *Sample* taken:

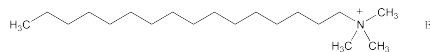
$$\text{Result} = [(V_S - V_B) \times F]/W$$

 V_S = *Titrant* volume consumed by the *Sample* (mL) V_B = *Titrant* volume consumed by the *Blank* (mL) F = calculation factor, 56.1 W = *Sample* weight (g)**Acceptance criteria:** 208–228

- **FATS AND FIXED OILS, Iodine Value** <401>: NMT 4

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** <11>
USP Cetyl Alcohol RS
USP Stearyl Alcohol RS

Cetrimonium BromideC₁₉H₄₂BrN

364.45

Hexadecyltrimethylammonium bromide [57-09-0].

DEFINITIONCetrimonium Bromide contains NLT 96.0% and NMT 101.0% of hexadecyltrimethylammonium bromide, calculated as C₁₉H₄₂BrN, on the dried basis.**IDENTIFICATION**• **A. ULTRAVIOLET ABSORPTION****Sample solution:** 10 mg/mL in alcohol**Analytical wavelength:** 260–280 nm**Cell:** 1 cm**Acceptance criteria:** After correcting for the blank, the absorbance is NMT 0.05.• **B.****Sample solution:** Dissolve 5 mg of Cetrimonium Bromide in 5 mL of phosphate buffer pH 8.0.**Blank:** 5 mL of phosphate buffer pH 8.0**Analysis:** Add a strip of methyl green–iodomercurate paper to the *Sample solution* and *Blank*.**Acceptance criteria:** After 5 min, the *Sample solution* shows a more intense greenish-blue color than the *Blank*.• **C.****Sample:** 2.0 g**Analysis:** Transfer the *Sample* to a 100-mL flask, and dissolve in and dilute with previously boiled and cooled water to volume.**Acceptance criteria:** The solution froths copiously when shaken.• **D. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** <201>**Solution A:** 270 mg/mL of sodium acetate trihydrate**Standard solution:** 20 mg/mL of USP Cetrimonium Bromide RS**Sample solution:** 20 mg/mL of Cetrimonium Bromide**Chromatographic system****Developing solvent system:** Methanol, acetone, and *Solution A* (45:20:35)**Analysis****Samples:** *Standard solution* and *Sample solution*

Proceed as directed in the chapter. Remove the plate from the developing chamber, and dry the plate in a current of hot air. Allow to cool. Expose the plate to iodine vapor, and examine in daylight.

Acceptance criteria: The principal spot of the *Sample solution* is similar in position, color, and size to that of the *Standard solution*.

- **E. IDENTIFICATION TESTS—GENERAL, Bromide** <191>: A solution of it meets the requirements.

ASSAY• **PROCEDURE****Sample solution:** 20 mg/mL of Cetrimonium Bromide**Blank:** Combine 10.0 mL of freshly prepared 50-mg/mL potassium iodide, 20 mL of water, and 40 mL of hydrochloric acid.**Titrimetric system**(See *Titrimetry* <541>.)**Mode:** Residual titration**Titrant:** 0.05 M potassium iodate VS**Analysis****Samples:** *Sample solution* and *Blank*Transfer 25.0 mL of the *Sample solution* to a separatory funnel, and add 25 mL of chloroform, 10 mL of 0.1 N sodium hydroxide VS, and 10.0 mL of a freshly prepared solution of potassium iodide (50 mg/mL). Shake

V_B = Titrant volume consumed by the Blank (mL)

F = factor, 56.1

W = Sample weight (g)

Acceptance criteria: 218–238

- **FATS AND FIXED OILS**, Iodine Value (401): NMT 5

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** (11)
USP Cetyl Alcohol RS
USP Stearyl Alcohol RS

Cetyl Esters Wax

DEFINITION

Cetyl Esters Wax is a mixture consisting primarily of esters of saturated fatty alcohols (C_{14} to C_{18}) and saturated fatty acids (C_{14} to C_{18}).

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE**, Class II (741): 43°–47°
- **FATS AND FIXED OILS**, Acid Value (401): NMT 5
- **FATS AND FIXED OILS**, Iodine Value (401): NMT 1
- **FATS AND FIXED OILS**, Saponification Value (401): 109–120
- **PARAFFIN AND FREE ACIDS**

Sample: 1 g

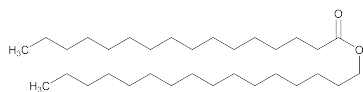
Analysis: Dissolve the Sample in 50 mL of boiling alcohol.

Acceptance criteria: The Sample dissolves completely, and the resulting solution is neutral or acidic to moistened litmus paper.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, in a dry place, and prevent exposure to excessive heat.

Cetyl Palmitate



$C_{32}H_{64}O_2$

Hexadecanoic acid hexadecyl ester;
Cetyl palmitate [540-10-3].

480.85

DEFINITION

Cetyl Palmitate consists of esters of cetyl alcohol and saturated high molecular weight fatty acids, principally palmitic acid.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197F)
Sample: Use a thin film of melted test specimen.
Acceptance criteria: Meets the requirements
- **B.** The retention times of cetyl alcohol and palmitic acid from the Sample solution correspond to those from the System suitability solution, as obtained in the test for Content of Palmitic Acid.

ASSAY

- **CONTENT OF PALMITIC ACID**

System suitability solution: Transfer 20 mg each of cetyl alcohol, stearic acid, palmitic acid, and oleic acid

to a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar, and proceed as directed for Test solution in Fats and Fixed Oils, Fatty Acid Composition (401), beginning with "Add 5.0 mL of a solution prepared by dissolving".

Sample solution: Proceed as directed for Test solution in Fats and Fixed Oils, Fatty Acid Composition (401).

Chromatographic system: Prepare as directed in Fats and Fixed Oils, Fatty Acid Composition (401).

Injection volume: 1 μ L

System suitability

Sample: System suitability solution

[NOTE—The relative retention times for methyl palmitate, cetyl alcohol, methyl stearate, and methyl oleate are about 0.87, 0.96, 0.99, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between the methyl stearate and methyl oleate peaks

Relative standard deviation: NMT 6.0% for the palmitate and stearate peaks; NMT 2.0% for the ratio of the palmitate peak to the stearate peak

Analysis

Samples: System suitability solution and Sample solution
Identify the methyl palmitate peak of the Sample solution by comparing the retention times of the Sample solution peaks with those of the System suitability solution peaks.

Calculate the percentage of palmitic acid in the portion of Cetyl Palmitate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of methyl palmitate

r_T = sum of all the peak areas, excluding the solvent and cetyl alcohol peaks

Acceptance criteria: The palmitate peak is NLT 90% of the total area for all the peaks.

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.05%
- **HEAVY METALS**, Method II (231): NMT 20 μ g/g

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE**, Class II (741): 46°–53°
- **FATS AND FIXED OILS**, Acid Value (401): NMT 1
- **FATS AND FIXED OILS**, Hydroxyl Value (401): NMT 6
- **FATS AND FIXED OILS**, Iodine Value (401): NMT 1
- **FATS AND FIXED OILS**, Saponification Value (401): 110–130
- **LOSS ON DRYING** (731)

Analysis: Dry at 105° for 1 h.

Acceptance criteria: NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers at controlled room temperature, and avoid exposure to excessive heat.
- **USP REFERENCE STANDARDS** (11)
USP Cetyl Palmitate RS

Cetylpyridinium Chloride—see
Cetylpyridinium Chloride General Monographs

Charcoal, Activated—see Activated
Charcoal General Monographs

Cherry Juice

DEFINITION

Cherry Juice is the liquid expressed from the fresh ripe fruit of *Prunus cerasus* L. (Fam. Rosaceae). It contains NLT 1.0% malic acid ($C_4H_6O_5$).

Coarsely crush washed, stemmed, unpitted, sour cherries in a grinder to break the pits but not mash the kernels. Dissolve 0.1% Benzoic Acid in the mixture, and allow to stand at room temperature until a small portion of the filtered Juice, when mixed with one-half of its volume of alcohol, does not become cloudy within 30 min. Press the Juice from the mixture, and filter it.

IDENTIFICATION

• A.

Sample: 5 mL

Analysis: Add lead acetate TS to the *Sample* until the mixture, when filtered, gives no further precipitation with the lead acetate solution. Filter, and to the clear filtrate add 5 mL of a 100-mg/mL sodium oxalate solution to remove the excess lead. Filter, add 5 mL of alkaline cupric tartrate TS to 5 mL of the clear filtrate, and warm.

Acceptance criteria: A red precipitate is formed.

OTHER COMPONENTS

• MALIC ACID

Solution A: 6 N ammonium hydroxide and water (2:98)

Sample: 10.0 mL

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N potassium permanganate VS

Endpoint detection: Visual

Analysis: Place the *Sample* in a 125-mL flask, and add 1 g of calcium carbonate. Heat on a steam bath for 15 min, mixing occasionally, and pass through a filter. Wash the filter with five 5-mL portions of water, and to the combined filtrate and washings add 1 mL of 6 N ammonium hydroxide and 15 mL of ammonium oxalate TS. Heat on a steam bath for 15 min, pass through hardened filter paper, and wash the flask and the filter with five 5-mL portions of *Solution A*. Puncture the filter paper, and wash the precipitate into the same flask by means of hot water, followed by 30 mL of 12 N sulfuric acid. Heat the solution to 80°. Titrate with *Titrant*. Each mL of *Titrant* is equivalent to 6.704 mg of malic acid ($C_4H_6O_5$).

Acceptance criteria: NLT 1.0%

IMPURITIES

• RESIDUE ON IGNITION (281): 0.35%–0.55%

• ARSENIC, Method I (211)

Sample solution: To 10 mL in a Kjeldahl flask add 5 mL of nitric acid and 5 mL of sulfuric acid, and heat the mixture until the volume is reduced to 5 mL and the color becomes brownish or black. Add a small portion of nitric acid, and continue the heating, adding small portions of nitric acid as often as browning recurs, until the organic matter is destroyed and dense, white fumes are liberated. Dilute the solution with about 10 mL of water, add 200 mg of ammonium oxalate, and continue the heating until dense, white fumes again are evolved and the solution is colorless to pale yellow. Cool, cautiously add water to make 20 mL, and mix.

Acceptance criteria: NMT 0.3 µg/g

• LEAD (251)

Test preparation: Add 1.0 mL of Juice to 10 mL of nitric acid in a 250-mL conical flask, and boil for 5–10

min. Cool in an ice bath, and transfer to a separator with the aid of 5 mL of lead-free water.

Analysis: Proceed as directed in the chapter, except use 15 mL of *Ammonium Citrate Solution*, 500 µL of *Hydroxylamine Hydrochloride Solution*, and 3 mL of *Potassium Cyanide Solution*.

Acceptance criteria: NMT 5 µg/mL

SPECIFIC TESTS

• SPECIFIC GRAVITY (841): 1.045–1.075

• REFRACTIVE INDEX (831): NLT 1.350

• PH (791): 3.0–4.0

• LIMIT OF NONVOLATILE RESIDUE

Sample: 5.0 mL

Analysis: Evenly spread the *Sample* over the bottom of a tared half Petri dish, and place on a steam bath for 1 h. Dry in a vacuum desiccator over silica gel for 16 h.

Acceptance criteria: The weight of the residue is NLT 500 mg. NMT 9.5% is found.

• LIMIT OF VOLATILE ACIDS

Sample: 25 mL

Analysis: Distill the *Sample* with steam to obtain 100 mL of distillate. Add phenolphthalein TS, and titrate with 0.10 N sodium hydroxide.

Acceptance criteria: NMT 1.5 mL is required.

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE:

Package in tight, light-resistant containers, and prevent exposure to excessive heat.

• LABELING:

The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Cherry Syrup

DEFINITION

Prepare Cherry Syrup as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* (795)).

Cherry Juice	475 mL
Sucrose	800 g
Alcohol	20 mL
Purified Water, a sufficient quantity to make	1000 mL

Dissolve *Sucrose* in *Cherry Juice* by gently heating on a steam bath, cool, and remove the foam and floating solids. Add *Alcohol* and sufficient *Purified Water* to make 1000 mL, and mix.

OTHER COMPONENTS

• ALCOHOL DETERMINATION, Method I (611): 1.0%–2.0%

ADDITIONAL REQUIREMENTS

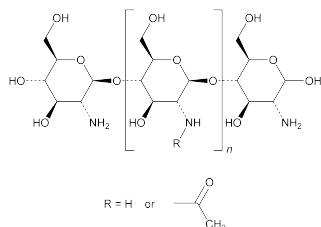
• PACKAGING AND STORAGE:

Package in tight, light-resistant containers, and prevent exposure to excessive heat.

• LABELING:

The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Chitosan



Poly- β -(1,4)-2-amino-2-deoxy-D-glucose [9012-76-4].

DEFINITION

Chitosan is an unbranched binary polysaccharide consisting of *N*-acetyl-D-glucosamine and D-glucosamine units linked in a β (1 \rightarrow 4) manner. Chitosan is obtained by partial deacetylation of chitin, which is extracted from the shells of edible shrimps and crabs suitable for human use. Its degree of deacetylation is NLT 70.0% and NMT 95.0%.

IDENTIFICATION

• A. INFRARED ABSORPTION (197A)

• B.

Sample: 0.2 g of Chitosan powder

Analysis: Add 80 g of water to the *Sample*, and stir briefly to obtain a dispersion. Separately prepare a glycolic acid solution by dissolving 0.1 g of glycolic acid in 20 g of water. Add the solution in one step to the dispersion. Stir the mixture gently at room temperature until it becomes a clear solution. [NOTE—It takes approximately 30–60 min to obtain a clear solution.] Add 5 g of a 0.5% sodium lauryl sulfate aqueous solution to the clear solution.

Acceptance criteria: A gelatinous mass is formed.

ASSAY

• DEGREE OF DEACETYLATION

[NOTE—If tetramethylsilane is not used as the NMR reference, a suitable signal of the solvent itself can be used as a reference.]

Solvent: Deuterated formic acid

NMR reference: Tetramethylsilane

Sample solution: Into a 20-mL scintillation vial with a screw cap, dissolve 5–10 mg of Chitosan in deuterated formic acid, containing 0.5%–1.0% of tetramethylsilane, to obtain 1 mL of solution. Tightly close the vial, and dissolve Chitosan using a magnetic stirrer. To completely dissolve takes it about 48 h; the stirring is stopped when a clear solution with a high viscosity is obtained. Break up any clumps formed during the dissolution process with a spatula, if necessary.

Analysis

Sample: *Sample solution*

Transfer 0.5–1.0 mL of the *Sample solution* to a standard 5-mm NMR spinning tube. Proceed as directed in *Nuclear Magnetic Resonance* (761), *Relative Method of Quantitation*, scanning the region from 0–7 ppm, and using the calculation formulas below. Record as A_1 the average area of the composite band from about 6–3 ppm, representing the seven protons with oxygen neighbors in the sugar ring. Record as A_2 the average area of the signals at about 2 ppm, due to the methyl groups of the acetyl units, with reference to the tetramethylsilane singlet at 0 ppm.

Calculate the percentage of deacetylation degree, by weight, in the Chitosan taken:

$$\text{Result} = \{1 - [(7 \times A_2)/(3 \times A_1)]\} \times 100$$

Acceptance criteria: The degree of deacetylation is NLT 70.0% and NMT 95.0%.

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 1.0%
- **HEAVY METALS**, *Method III* (231): NMT 10 ppm
- **LIMIT OF LEAD, MERCURY, CHROMIUM, NICKEL, CADMIUM, AND ARSENIC**

65% Nitric acid: Use ultratrace nitric acid, 65%–70% HNO_3 , ACS reagent grade.

Internal standard: Transfer 0.2 mL of a solution containing 1000 ppm of yttrium [NOTE—Yttrium ICP standard solutions are commercially available.¹] and 0.2 mL of a solution containing 1000 ppm of lutetium [NOTE—Lutetium ICP standard solutions are commercially available.²] to a 100-mL volumetric flask, add 1 mL of 65% *Nitric acid*, dilute with water to volume, and mix.

Blank standard: Transfer 1.0 mL of the *Internal standard* to a 100-mL volumetric flask, add 1 mL of 65% *Nitric acid*, dilute with water to volume, and mix.

Standard solutions: Transfer 0.1 mL of a solution containing 10 ppm of each of lead, mercury, chromium, nickel, cadmium, and arsenic [NOTE—Multi-element ICP standard solutions are commercially available.³] to a 100-mL volumetric flask, add 1 mL of the *Internal standard* and 1 mL of 65% *Nitric acid*, and dilute with water to volume (*Standard solution* 0.01 ppm). Transfer 0.1 mL of a solution containing 10 ppm of each of lead, mercury, chromium, nickel, cadmium, and arsenic to a 1000-mL volumetric flask, add 10 mL of the *Internal standard* and 10 mL of 65% *Nitric acid*, and dilute with water to volume (*Standard solution* 0.001 ppm).

Sample solution: Transfer 1.0 g of Chitosan to a clean, dry, 100-mL Kjeldahl flask. [NOTE—A 300-mL flask may be used if the reaction foams excessively.] Clamp the flask at an angle of 45°, and add a sufficient quantity of a mixture of 8 mL of sulfuric acid and 10 mL of nitric acid to moisten the substance thoroughly. Warm gently until the reaction commences, allow the reaction to subside, and add portions of the same acid mixture, heating after each addition, until a total of 18 mL of the acid mixture has been added. Increase the amount of heat, and boil gently until the solution darkens. Cool, add 2 mL of nitric acid, and heat again until the solution darkens. Continue the heating, followed by addition of nitric acid until no further darkening occurs, then heat strongly to the production of dense, white fumes. Cool, cautiously add 5 mL of water, boil gently to the production of dense, white fumes, and continue heating until the volume is reduced to a few mL. Cool, cautiously add 5 mL of water, and examine the color of the solution. If the color is yellow, cautiously add 1 mL of 30% hydrogen peroxide, and again evaporate to the production of dense, white fumes and a volume of 2–3 mL. If the solution is still yellow, repeat the addition of 5 mL of water and the peroxide treatment. Cool, dilute cautiously with a few mL of water, rinse into a 20-mL volumetric flask, and dilute with water to volume. Transfer 5.0 mL of digestion solution to a 100-mL volumetric flask, and retain the remaining digestion solution for use in the *Limit of Iron*. Add 1 mL of the *Internal standard* to the 100-mL volumetric flask, dilute with water to volume, and mix.

Blank solution: Prepare the blank digestion solution, following the preparation procedure for the *Sample solution*, but without using Chitosan. Transfer 5.0 mL of blank digestion solution to a 100-mL volumetric flask, and retain the remaining digestion solution for use in the *Limit of Iron*. Add 1 mL of the *Internal standard* to

¹ A suitable yttrium ICP standard is available from LGC Promochem (www.lgcpromochem.com) or from Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany.

² A suitable lutetium ICP standard is available from LGC Promochem (www.lgcpromochem.com) or from Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany.

³ Suitable multi-element ICP standard solutions are available from LGC Promochem (www.lgcpromochem.com) or from Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany.

the 100-mL volumetric flask, dilute with water to volume, and mix.

Instrumental conditions

(See *Plasma Spectrochemistry* <730>.)

Mode: Inductively coupled plasma–mass spectrometer (ICP–MS)

Spectrometer: Quadrupole mass spectrometer

Detector: Ion detector maintained under vacuum

Other requirements: The instrument should read all isotopes for the following elements shown in *Table 1*, for the yttrium internal standard (89 amu) and the lutetium internal standard (175 and 176 amu), and should report the total element contents using the most naturally abundant isotopes.

Table 1

Element	Isotope (amu)
Lead	208
	201
Mercury	202
Chromium	53
	58
Nickel	60
Cadmium	114
Arsenic	75

Analysis

Samples: *Blank standard, Standard solutions, Sample solution, and Blank solution*

Instrument performance must be verified to conform to the manufacturer's specifications for resolution and sensitivity. Before analyzing samples, the instrument must pass a suitable performance check. Perform the evaluation using instrument software such as correction equations for interferences and taking the *Internal standard* into account. Generate the calibration curve using the *Blank standard, Standard solution 0.001 ppm*, and *Standard solution 0.01 ppm*: the linear regression coefficient is NLT 0.999.

Aspirate the *Blank solution* and *Sample solution*, respectively, at least in duplicate, and report the average reading as each element content of the sample. Determine the concentration C_B , in $\mu\text{g/mL}$, of each element in the *Blank solution*, and also determine the concentration C_S , in $\mu\text{g/mL}$, of each element in the *Sample solution* using the calibration curve.

Calculate the quantity, in $\mu\text{g/g}$, of each element in the portion of Chitosan taken:

$$\text{Result} = [(C_S - C_B)/W] \times F \times V$$

F = dilution factor, 4

V = volume of the *Sample solution*, 100 mL

W = weight of the Chitosan taken to prepare the *Sample solution* (g)

Acceptance criteria: See *Table 2*.

Table 2

Element	Acceptance Criteria (ppm)
Lead	NMT 0.5
Mercury	NMT 0.2
Chromium	NMT 1.0
Nickel	NMT 1.0
Cadmium	NMT 0.2
Arsenic	NMT 0.5

• LIMIT OF IRON

65% Nitric acid: Use ultratrace nitric acid, 65%–70% HNO_3 , ACS reagent grade.

Blank standard: Prepared as directed in the *Limit of Lead, Mercury, Chromium, Nickel, Cadmium, and Arsenic*.

Standard stock solution 100 ppm: Immediately before use, dilute an appropriate amount of iron standard⁴ with a solution of 65% *Nitric acid* (1 in 100) to prepare an acidic solution containing the equivalent of 100 $\mu\text{g/mL}$ of iron.

Standard solutions: Separately transfer 0.1 and 0.5 mL of *Standard stock solution 100 ppm* to 100-mL volumetric flasks, dilute with a solution of 65% *Nitric acid* (1 in 100) to volume, and mix. These solutions contain, respectively, 0.1 and 0.5 μg of iron/mL (*Standard solution 0.1 ppm* and *Standard solution 0.5 ppm*).

Sample solution: Transfer 10.0 mL of the digestion solution from the test for *Limit of Lead, Mercury, Chromium, Nickel, Cadmium, and Arsenic* to a 100-mL volumetric flask, dilute with water to volume, and mix.

Blank solution: Transfer 10.0 mL of the blank digestion solution from the test for *Limit of Lead, Mercury, Chromium, Nickel, Cadmium, and Arsenic* to a 100-mL volumetric flask, dilute with water to volume, and mix.

Instrumental conditions

(See *Plasma Spectrochemistry* <730>.)

Mode: Inductively coupled plasma–atomic emission spectrometer (ICP–AES)

Analytical wavelength: 238.040 and 239.562 nm with the settings optimized as directed by the manufacturer

Analysis

Samples: *Blank standard, Standard solutions, Sample solution, and Blank solution*

Instrument performance must be verified to conform to the manufacturer's specifications for resolution and sensitivity. Before analyzing samples, the instrument must pass a suitable performance check. Generate the calibration curve using the *Blank standard, Standard solution 0.1 ppm*, and *Standard solution 0.5 ppm*: the linear regression coefficient is NLT 0.999.

Aspirate the *Blank solution* and *Sample solution*, respectively, at least in duplicate, and report the average reading as the iron content of the sample. Determine the concentration C_B , in $\mu\text{g/mL}$, of iron in the *Blank solution*, and also determine the concentration C_S , in $\mu\text{g/mL}$, of iron in the *Sample solution* using the calibration curve.

Calculate the quantity, in $\mu\text{g/g}$, of iron in the portion of Chitosan taken:

$$\text{Result} = [(C_S - C_B)/W] \times F \times V$$

F = dilution factor, 2

V = volume of the *Sample solution*, 100 mL

W = weight of the Chitosan taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 10 ppm of iron

• LIMIT OF PROTEIN CONTENT

Control solution A: 1.0 mg/mL of bovine serum albumin

Control solution B: 0.1 mg/mL of bovine serum albumin

Sample solution C: Dissolve 100 mg of Chitosan in 4 mL of 100% formic acid, mix, and stir for about 48 h at room temperature using a magnetic stirrer. This solution contains 25 mg/mL of Chitosan in 100% formic acid.

Sample solutions D, E, F: Dilute aliquots of the *Sample solution C* with water to obtain the following solutions with concentrations of 2.5 mg/mL of Chitosan in 10% formic acid, 0.5 mg/mL of Chitosan in 2% formic acid,

⁴ Suitable iron standards are available from LGC Promochem (www.lgcpromochem.com) (Single element standard for ICP, Iron 10,000 $\mu\text{g/mL}$ dilute nitric acid) or from Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany (iron ICP standard, 10000 mg/L in 10% nitric acid).

and 0.25 mg/mL of Chitosan in 1% formic acid, respectively.

Molecular weight standard preparation G: Use a commercially available preparation of apparent molecular weight protein standards of 10,000–190,000 Da dissolved in the loading buffer consisting of 50 mM of tris(hydroxymethyl)aminomethane hydrochloride (pH 6.8), 5 mM of ethylenediaminetetraacetic acid, 10 mM of dithiothreitol [NOTE—A 2%–5% solution of beta-mercaptoethanol can be used to replace dithiothreitol.], 1% (w/v) sodium dodecyl sulfate, and 10% (w/v) glycerol.⁵ [NOTE—A protein ladder containing the following molecular weight standards in 10, 15, 20, 25, 40, 50, 60, 85, 120, and 190 kDa or other appropriate combinations can be used.]

Sample buffer: Transfer 666 mg of tris(hydroxymethyl)aminomethane hydrochloride, 682 mg of tris(hydroxymethyl)aminomethane, 800 mg of lithium dodecyl sulfate, 6 mg of ethylenediaminetetraacetic acid, 4 g of glycerol, 0.75 mL of 1% solution of Coomassie blue G250, and 0.25 mL of 1% solution of phenol red to a 10-mL volumetric flask, add 8 mL of water to the flask, and mix. If necessary, adjust with hydrochloric acid or sodium hydroxide to pH of 7.2. Dilute with water to volume. [NOTE—Store the buffer at 4°. It is stable for 6 months when stored at 4°.] An equivalent commercially available buffer can also be used.⁶

Running buffer: Prepare a solution containing 1 M of tris(hydroxymethyl)aminomethane, 1 M of 2-(4-morpholinyl) ethanesulfonic acid, 20.5 mM ethylenediaminetetraacetic acid, and 69.3 mM dodecyl sodium sulfate in water. If necessary, adjust with hydrochloric acid or sodium hydroxide to pH of 7.3. An equivalent commercially available appropriate SDS running buffer can be used.⁷

Fixing solution: A solution containing 40% ethanol and 10% acetic acid

Sensitizing solution: Transfer 10 mL of a solution mainly containing 10%–20% of 2-(4-morpholinyl) ethanesulfonic acid, 0.1%–1.0% of sodium hydroxide, and 7%–13% of *N,N*-dimethylformamide into a 100-mL volumetric flask, add 30 mL of alcohol, and dilute with water to volume. Alternatively, follow the instructions of a commercially available silver staining kit⁸ to prepare the *Sensitizing solution*, *Staining solution*, and *Developing solution*.

Staining solution: Transfer 1.0 mL of a solution mainly containing 10%–30% of silver nitrate into a 100-mL volumetric flask, and dilute with water to volume.

Developing solution: Transfer 10 mL of a solution mainly containing 10%–30% of sodium carbonate into a 100-mL volumetric flask, add 1 drop of a solution containing 30%–60% of formaldehyde, and dilute with water to volume.

Stopping solution: A solution containing 10%–30% of ethylenediaminetetraacetic acid and 10%–30% of tris(hydroxymethyl)-aminomethane. A *Stopping solution* is commercially available and included in a silver staining kit.

Analysis: Mix 75 µL each of *Sample solutions C, D, E, and F* with 25 µL of the *Sample buffer*, and incubate at 70° for 10 min. In a suitable device for polyacrylamide gel electrophoresis (see *Electrophoresis* (726) and *Biotechnology-Derived Articles—Polyacrylamide Gel Electrophoresis* (1056)) add appropriate volumes of the *Running buffer* in the upper and the lower buffer chambers. Attach a 4%–12% gradient Bis-Tris ready-

made polyacrylamide gel sandwiched between two glass plates, such that the wells for sample application are exposed to the *Running buffer* in the upper buffer chamber.

Separately apply equal volumes (about 20 µL) of each of the treated *Sample solutions C, D, E, and F*, *Control solution A*, and *Control solution B* onto separate lanes; apply equal volumes (about 10 µL) of the *Molecular weight standard preparation G* to both sides of the gel. [NOTE—Do not apply any solution in the outside lanes.] Connect the lower buffer chamber electrode to the positive terminal and the upper buffer chamber electrode to the negative terminal of a suitable power supply unit, and carry out the electrophoresis at a constant voltage of about 100 V for about 100 min.

Remove the gel from the gel assembly. [NOTE—Do not touch the gel with bare hands. Use gloves.]

Place the gel in a clean staining tray of appropriate size. Rinse the gel briefly with water. Fix the gel in 100 mL of *Fixing solution* for 20 min with gentle rotation.

[NOTE—The gel can be stored in the *Fixing solution* overnight.]

Decant the *Fixing solution*, and wash the gel in 30% ethanol for 10 min. Decant the ethanol, and add 100 mL of *Sensitizing solution* to the washed gel in the staining container. Incubate the gel in the *Sensitizing solution* for about 10 min. [NOTE—All incubations should be performed on a rotary shaker rotating at a speed of 1 revolution/s at room temperature.]

Decant the *Sensitizing solution*, and wash the gel in 100 mL of 30% ethanol for 10 min. Wash the gel in 100 mL of water for 10 min. Incubate the gel in 100 mL of *Staining solution* for 15 min. After staining is complete, decant the *Staining solution*, and wash the gel with 100 mL of water for 20–60 s. [NOTE—Washing the gel for more than 1 min will remove the silver ion from the gel resulting in decreased sensitivity.]

Incubate the gel in 100 mL of *Developing solution* for 4–8 min until bands start to appear and the desired band intensity is reached.

Once the appropriate staining intensity is achieved, immediately add 10 mL of *Stopping solution* directly to the gel still immersed in the *Developing solution*. Gently agitate the gel for 10 min. The color changes from pink to colorless indicating that the development has stopped.

Decant the colorless solution, and wash the gel with 100 mL of water for 10 min.

Use a gel imaging system, ideally with a CCD camera, to record the results.

Acceptance criteria: NMT 0.2% of protein

SPECIFIC TESTS

• BACTERIAL ENDOTOXINS TEST (85)

Sample stock solution: Transfer 0.5 g of Chitosan to a 50-mL volumetric flask, add LAL Reagent Water⁹ and 4.6 mL of 1 N hydrochloric acid, dilute with the LAL Reagent Water to volume, and mix well. Incubate this solution in a water bath at 40° for 48 h.

Sample solution: Dilute the *Sample stock solution* to 1:50 with LAL Reagent Water, including dilution 1:2 with a β -glucan blocker.¹⁰

Acceptance criteria: The level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Chitosan is used can be met. Where the label states that Chitosan must be subjected to further processing during the preparation of wound dressing dosage forms, the level of bacterial endotoxins is such that the requirement under the

⁵ A suitable molecular weight standard preparation is available as BenchMark Prestained Protein Ladder from Invitrogen, product number: 10748010.

⁶ A suitable sample buffer is available as 4X NuPAGE LDS sample buffer from Invitrogen, product number: NP0007.

⁷ A suitable running buffer is available as NuPAGE MES SDS Running Buffer from Invitrogen, product number: NP0002.

⁸ A suitable silver staining kit is available as NuPAGE Silver Staining Kit from Invitrogen, product number: LC6070.

⁹ Use Sterile Water for Injection or other water that shows no reaction with the specific LAL Reagent with which it is to be used, at the limit of sensitivity of such reagent.

¹⁰ Available from Cambrex Europe s.p.a., Verviers, Belgium; Charles River, www.criver.com; or all the major LAL manufacturers.

relevant dosage form monograph(s) in which Chitosan is used can be met.

• **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62)

Sample: Prepare a solution (1 in 50).

Acceptance criteria: The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for absence of *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

• **APPARENT AVERAGE MOLECULAR WEIGHT AND MOLECULAR WEIGHT DISTRIBUTION**

[NOTE—This test is applicable to Chitosan of an average molecular weight of NMT 1,000,000 Da. In the following test an apparent average molecular weight is determined.]

Mobile phase: Transfer 12.75 g of sodium nitrate to a 1000-mL volumetric flask containing 800 mL of water, add a suitable amount of formic acid, dilute with water to volume, mix well, and make a concentration of formic acid of 0.5 M. The *Mobile phase* contains 0.15 M sodium nitrate in 0.5 M aqueous formic acid.

System suitability solution: 1.0 mg/mL of ethylene glycol in *Mobile phase*

Standard solutions: Prepare several sets of mixtures, containing ten polyethylene glycol (PEG) standards of different known molecular weight, which are used to cover the molecular weight range from about 200 to 1,100,000 g/mol.¹¹ [NOTE—These standards could be mixtures of polyethylene glycols and polyethylene oxides.] Prepare each set of PEG molecular weight standards to have a known concentration of about 1.0 mg/mL for each standard in *Mobile phase*. Allow the *Standard solutions* to stand at room temperature for at least 8 h. Do not filter before use.

Sample solution: Prepare a solution containing 1.0 mg/mL of Chitosan in *Mobile phase*. Cap, and mix well. Allow the solution to stand at room temperature for at least 12 h. Pass the chitosan solution through a membrane filter of 0.45- μ m pore size, discard an appropriate volume of the initial filtrate, and use the rest of the filtered solution for analysis.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Detector temperature: 35°

Columns: 7.5-mm \times 30-cm analytical column, 10- μ m packing L38; and two 7.5-mm \times 30-cm analytical columns; 17- μ m packing L38

Flow rate: 1.0 mL/min for *System suitability solution*; and 0.5 mL/min for *Standard solutions* and *Sample solution*

Injection size: 20 μ L for *System suitability solution*; and 100 μ L for *Standard solutions* and *Sample solution*

System suitability

Samples: *System suitability solution* and *Standard solution*

Suitability requirements

Plate count: NLT 80% of the value that is certified by the column manufacturer for new columns, determining the plate count for the ethylene glycol peak, *System suitability solution*

Resolution: NLT 1.7 between the PEG standards, *Standard solution*. [NOTE—The resolution between the PEG standard with a molecular weight of 1,000,000 Da and its adjacent PEG standard should be NLT 1.0 if 1.7 cannot be met.]

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine elution peak maxima and corresponding retention volumes (elution volume), V_p , for the ten

PEG standards, corresponding to the specified molecular weight, M_p , at the peak maximum of the standards.

Molecular weight calibration: Analyze each polyethylene glycol standard, and use a suitable gel-permeation chromatography or size-exclusion chromatography (GPC/SEC) software, or an equivalent data handling system, to compute the data and calibration. Plot $\log M_p$ for each standard in the *Standard solutions* versus its retention volume, V_p , in mL, at each standard peak maximum, and construct the best polynomial line fitting the ten points.

Data analysis for sample: [NOTE—Based on the PEG molecular weight calibration curve, calculate the molecular weight and molecular weight distribution of Chitosan using the slice by slice method.]

Analyze the Chitosan sample by identifying retention volumes V_a and V_b corresponding to the beginning and end of the Chitosan chromatogram. The baseline between V_a and V_b is assumed to be linear. [NOTE—Draw a straight line between V_a and V_b .] Data analysis is based on a suitable GPC/SEC computer software or a real-time data acquisition system with either off-line or on-line data processing that is able to provide a means of determining chromatographic peak heights or integrated area segments as prescribed intervals under the SEC chromatogram and handling and reporting the data. The following describes the data processes which can either be computed by the GPC/SEC software or by an equivalent data processing system. Upon acquisition, handle the data under the Chitosan elution peak in discrete segments A_i , integrated area slices, or as digitized chromatogram heights H_i by recording the vertical displacements between the chromatogram trace and the baseline at elution volume, V_i , over designated intervals. A minimum of 40 area segments or heights are required. Obtain the corresponding value of M_i for Chitosan based on its elution volume, V_i , from the molecular weight calibration curve obtained in *Molecular weight calibration*.

Calculate the number-, and weight-average molecular weights, M_n and M_w , in g/mol, respectively, using the following formulas.

$$M_n = \frac{\sum_{i=1}^N A_i}{\sum_{i=1}^N \left(\frac{A_i}{M_i} \right)}$$

$$M_w = \frac{\sum_{i=1}^N (A_i \cdot M_i)}{\sum_{i=1}^N A_i}$$

If the elution volume internal ΔV_i , for instance, $V_2 - V_1 = V_3 - V_2$, etc, is constant; parameters A_i and M_i are the chromatographic peak slice area and Chitosan molecular weight associated with the elution volume, V_i ; and N is the number of data points obtained from the chromatogram between V_a and V_b ($N \geq 40$). [NOTE—If N is sufficiently large, the use of area segments A_i or peak heights H_i will yield equivalent results.]

Calculate the molecular weight distribution or polydispersity for Chitosan using the following expression:

$$\text{Result} = M_w/M_n$$

¹¹ Suitable polyethylene glycol molecular weight standards are available as ReadyCal kits from Polymer Standards Service (PSS), www.polymer.de.

Acceptance criteria: The values of apparent weight-average molecular weight and polydispersity are NLT 85% and NMT 115% of their respective values stated on the label.

- **LOSS ON DRYING** (731): Dry 1.0 g in an oven at 100°–105° for 7 h: it loses NMT 5.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in light-resistant and well-closed containers in a dry place, and store at a temperature below 30°.
- **LABELING:** Label it to indicate its apparent weight-average molecular weight, M_w , and polydispersity (M_w/M_n). Where Chitosan is intended for use in the manufacture of wound dressings, it is so labeled. Where Chitosan must be subjected to further processing during the preparation of wound dressings, it is so labeled. Label to indicate the natural source from which Chitosan is derived.
- **USP REFERENCE STANDARDS** (11)
USP Chitosan RS
USP Endotoxin RS

Chlorobutanol



$C_4H_7Cl_3O$	177.46
$C_4H_7Cl_3O \cdot \frac{1}{2}H_2O$	186.46
2-Propanol, 1,1,1-trichloro-2-methyl-; 1,1,1-Trichloro-2-methyl-2-propanol [57-15-8]. Hemihydrate [6001-64-5].	

DEFINITION

Chlorobutanol is anhydrous or contains NMT one-half molecule of water of hydration. It contains NLT 98.0% and NMT 100.5% of chlorobutanol ($C_4H_7Cl_3O$), calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B.**

Sample solution: 5 mg/mL, freshly prepared solution

Analysis: To 5 mL of the *Sample solution* add 1 mL of 1 N sodium hydroxide, then slowly add 3 mL of iodine TS.

Acceptance criteria: A yellow precipitate of iodoform, recognizable by its odor, appears.

ASSAY

• PROCEDURE

Denatured alcohol: 10% of isopropyl alcohol in alcohol
Potassium hydroxide solution: Prepare fresh just before use. Transfer 58 g of potassium hydroxide to a 1000-mL flask. Add 100 mL of water to dissolve, then cool the solution. Dilute with *Denatured alcohol* to volume.

Sample: 100 mg

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Electrode: Silver-billet combination electrode, consisting of a metallic silver indicator electrode and a double junction reference electrode that allows use of a nonchloride filling solution (such as ammonium nitrate solution) for determining chloride, or equivalent

Titrant: 0.1 N silver nitrate VS

Endpoint detection: Potentiometric

Analysis: Transfer the *Sample* to a glass-stoppered, flat-bottomed boiling flask. Add 50 mL of *Potassium hydrox-*

ide solution, attach the flask to a reflux condenser, and reflux for 1 h. Allow the flask to cool while still attached to the condenser, then add 100 mL of water, using a portion of the water to rinse the condenser and its tip. Add 15 mL of nitric acid while stirring. Titrate with *Titrant*. Perform a blank determination, and make any necessary correction. Each mL of *Titrant* is equivalent to 5.915 mg of chlorobutanol ($C_4H_7Cl_3O$).

Acceptance criteria: 98.0%–100.5% on the anhydrous basis

IMPURITIES

• CHLORIDE

Control solution: 0.50 mL of 0.020 N hydrochloric acid in a mixture of 25 mL of diluted alcohol and 1 mL of nitric acid

Sample solution: 0.50 g of Chlorobutanol in a mixture of 25 mL of diluted alcohol and 1 mL of nitric acid

Analysis: To the *Control solution* and *Sample solution* add 2 mL of silver nitrate TS.

Acceptance criteria: 0.07%; any turbidity produced in the *Sample solution* is NMT that produced in the *Control solution*.

SPECIFIC TESTS

- **WATER DETERMINATION, Method I** (921): NMT 1.0% (anhydrous form) and NMT 6.0% (hydrous form)

• REACTION

Sample: 0.5 g

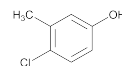
Analysis: Shake the *Sample* thoroughly with 25 mL of water.

Acceptance criteria: The water remains neutral to litmus.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate whether it is anhydrous or hydrous.
- **USP REFERENCE STANDARDS** (11)
USP Chlorobutanol RS

Chlorocresol



C_7H_7ClO	142.58
Phenol, 4-chloro-3-methyl-; 4-Chloro- <i>m</i> -cresol [59-50-7].	

DEFINITION

Chlorocresol contains NLT 99.0% and NMT 101.0% of C_7H_7ClO (4-chloro-3-methylphenol).

IDENTIFICATION

• A.

Sample solution: Dissolve 40 mg of Chlorocresol in 10 mL of water.

Analysis: Add 1 drop of ferric chloride TS to the *Sample solution*.

Acceptance criteria: A blue color develops.

• B.

Sample: 50 mg

Analysis: Transfer the *Sample* to a crucible, add 500 mg of anhydrous sodium carbonate, and mix. Heat the mixture until fused. Cool, add 5 mL of water, and boil. Acidify with 1 mL of nitric acid, filter, and add 1 mL of silver nitrate TS to the filtrate.

Acceptance criteria: A white precipitate is formed.

ASSAY

• PROCEDURE

Sample: 70 mg

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to an iodine flask, add 30 mL of glacial acetic acid, 25.0 mL of 0.1 N bromine VS, 10 mL of potassium bromide solution (150 mg/mL), and 10 mL of hydrochloric acid.

Immediately insert the stopper, and allow to stand for 15 min, protected from light. Quickly add 10 mL of potassium iodide solution (100 mg/mL) and 100 mL of water, taking precautions against the escape of bromine vapor. Immediately insert the stopper, and shake the mixture thoroughly. Remove the stopper, and rinse it and the neck of the flask with a small quantity of water so that the washing flows into the flask. Add 1 mL of chloroform, and shake the mixture thoroughly.

Titrate the liberated iodine with *Titrant*, adding 3 mL of starch TS as the endpoint is approached. Perform a blank determination. Each mL of 0.1 N bromine VS is equivalent to 3.565 mg of C₇H₇ClO (4-chloro-3-methylphenol).

Acceptance criteria: 99.0%–101.0%

SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE** (741): 63°–66°

• **LIMIT OF NONVOLATILE RESIDUE**

Sample: 1.0 g

Analysis: Heat the *Sample* in a tared crucible on a steam bath until it has evaporated, and dry the residue at 105° for 1 h.

Acceptance criteria: NMT 0.1%

• **COMPLETENESS OF SOLUTION**

Sample: 1 g

Analysis: Transfer the *Sample* to a test tube, add 0.4 mL of alcohol, and shake.

Acceptance criteria: The solution is complete.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Chocolate

DEFINITION

Chocolate is a powder prepared from the roasted, cured kernels of the ripe seed of *Theobroma cacao* L. (Fam. Sterculiaceae). Chocolate yields NLT 10.0% and NMT 22.0% of nonvolatile ether-soluble extractive.

COMPOSITION

• **CONTENT OF NONVOLATILE ETHER-SOLUBLE EXTRACTIVE**

Sample: 10 g of Chocolate

Analysis: Extract the *Sample* with anhydrous ethyl ether in a continuous-extraction apparatus for 8 h. Allow the ether solution to evaporate spontaneously in a suitable tared container, dry at 105° for 1 h, and weigh the nonvolatile ether-soluble extractive. [NOTE—Retain the ether-insoluble residue for the test for *Botanic Characteristics*, *Total Ash*, *Acid-Insoluble Ash*, and *Crude Fiber*.]

Acceptance criteria: 10.0%–22.0%

CONTAMINANTS

• **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total bacterial count does

not exceed 5×10^3 cfu/g, and it meets the requirements of the tests for absence of *Escherichia coli*, a 10-g specimen being used.

SPECIFIC TESTS

• **BOTANIC CHARACTERISTICS**

Microscopic: It shows numerous broken parenchyma cells of the cotyledons containing a reddish brown or purplish brown to yellowish orange pigment; numerous starch grains; oil globules; aleurone grains; and occasionally acicular or prismatic crystals of fat. The starch grains are simple and two- to three-compound, the single grains up to 15 μ m in diameter, and they stain slowly with iodine TS. The ether-insoluble residue retained from the *Content of Nonvolatile Ether-Soluble Extractive* shows few or no fragments of cocoa shells and no cereal starch grains.

• **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561)

Sample: Use the ether-insoluble residue of *Content of Nonvolatile Ether-Soluble Extractive*.

Acceptance criteria: NMT 8.0%

• **ARTICLES OF BOTANICAL ORIGIN, Acid-Insoluble Ash** (561)

Sample: Use the ether-insoluble residue of *Content of Nonvolatile Ether-Soluble Extractive*.

Acceptance criteria: NMT 0.4%

• **ARTICLES OF BOTANICAL ORIGIN, Crude Fiber** (561)

Sample: Use the ether-insoluble residue of *Content of Nonvolatile Ether-Soluble Extractive*.

Acceptance criteria: NMT 7.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Chocolate Syrup

DEFINITION

Prepare Chocolate Syrup as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* (795)).

Chocolate	180 g
Sucrose	600 g
Liquid Glucose	180 g
Glycerin	50 mL
Sodium Chloride	2 g
Vanillin	0.2 g
Sodium Benzoate	1 g
Purified Water, a sufficient quantity to make	1000 mL

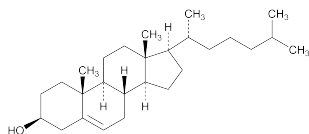
Mix *Chocolate* and *Sucrose*, and to this mixture gradually add a solution of *Liquid Glucose*, *Glycerin*, *Sodium Chloride*, *Vanillin*, and *Sodium Benzoate* in 325 mL of hot *Purified Water*. Bring the entire mixture to a boil, and maintain at boiling temperature for 3 min. Allow to cool to room temperature, and add sufficient *Purified Water* to make the product measure 1000 mL.

[NOTE—Chocolate containing NMT 12% of nonvolatile, ether-soluble extractive ("fat") yields a Syrup having a minimum tendency to separate. "Breakfast Chocolate" contains over 22% of "fat".]

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Package in tight containers, and avoid exposure to excessive heat.

Cholesterol



$C_{27}H_{46}O$ 386.65
 Cholest-5-en-3-ol, (3 β)-;
 Cholest-5-en-3 β -ol [57-88-5].

DEFINITION

Cholesterol is a steroid alcohol used as an emulsifying agent.

IDENTIFICATION

- **A.**
Sample solution: 10 mg in 1 mL of chloroform
Analysis: To the *Sample solution* add 1 mL of sulfuric acid.
Acceptance criteria: The chloroform acquires a blood-red color, and the sulfuric acid shows a green fluorescence.
- **B.**
Sample: 5 mg
Analysis: Dissolve the *Sample* in 2 mL of chloroform, add 1 mL of acetic anhydride, and follow with 1 drop of sulfuric acid.
Acceptance criteria: A pink color is produced, and it rapidly changes to red, then to blue, and finally to a brilliant green.

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** (741): 147°–150°
- **OPTICAL ROTATION, Specific Rotation** (781S)
Sample solution: 20 mg/mL, undried, in dioxane
Acceptance criteria: –34° to –38°
- **ACIDITY**
Sample: 1.0 g
Analysis: Dissolve the *Sample* in 10 mL of ether in a small flask, add 10.0 mL of 0.10 N sodium hydroxide, and shake for about 1 min. Heat gently to expel the ether, and then boil for 5 min. Cool, dilute with 10 mL of water, add phenolphthalein TS, and titrate with 0.10 N sulfuric acid until the pink color just disappears, stirring the solution vigorously throughout the titration. Perform a blank determination (see *Titrimetry* (541), *Residual Titrations*).
Acceptance criteria: The difference between the number of mL of 0.10 N sulfuric acid consumed in the blank and the number of mL consumed in the *Sample* is NMT 0.3 mL.
- **LOSS ON DRYING** (731)
Analysis: Dry under vacuum at 60° for 4 h.
Acceptance criteria: NMT 0.3%
- **SOLUBILITY IN ALCOHOL**
Sample: 500 mg
Analysis: Dissolve the *Sample* in 50 mL of warm alcohol in a stoppered flask or cylinder, and allow to stand at room temperature for 2 h.
Acceptance criteria: No deposit or turbidity is formed.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.

Anhydrous Citric Acid—see *Anhydrous Citric Acid General Monographs*

Citric Acid Monohydrate—see *Citric Acid Monohydrate General Monographs*

Clove Oil

DEFINITION

Clove Oil is the volatile oil distilled with steam from the dried flower buds of *Syzygium aromaticum* (L.) Merr. and L. M. Perry (Fam. Myrtaceae). It contains NLT 85.0% of total phenolic substances, chiefly eugenol ($C_{10}H_{12}O_2$).

ASSAY

PROCEDURE

Sample: 10 mL of Clove Oil

Analysis: Pipet the *Sample* into a suitable cassia flask, the neck of which is graduated from 0–6 mL at intervals of 0.1 mL. Add 75 mL of 1 N potassium hydroxide, and shake the mixture for 5 min. Heat for 10 min in boiling water, shaking the flask at least 3 times during the heating. Remove the flask from the bath, and cool to room temperature. When the liquids have separated completely, add sufficient 1 N potassium hydroxide to raise the lower level of the oily layer so that it is within the graduated portion of the neck. Measure the volume of the oily layer after standing for 18 h.

Acceptance criteria: NMT 1.5 mL, indicating the presence of NLT 85.0%, by volume, of total phenolic substances in the Oil

IMPURITIES

- **HEAVY METALS, Method II** (231): NMT 40 μ g/g
- **LIMIT OF PHENOL**

Sample: 1 mL of Clove Oil

Analysis: Shake the *Sample* with 20 mL of hot water: the water shows not more than a scarcely perceptible acid reaction with blue litmus paper. Cool the mixture, pass the water layer through a wetted filter, and treat the clear filtrate with 1 drop of ferric chloride TS.

Acceptance criteria: The mixture exhibits only a transient, grayish-green color, but not a blue or violet color.

SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): 1.038–1.060
- **OPTICAL ROTATION, Angular Rotation** (781A): It is optically inactive or slightly levorotatory. The angular rotation is not greater than –1.5°.
- **REFRACTIVE INDEX** (831): 1.527–1.535 at 20°
- **SOLUBILITY IN 70% ALCOHOL:** One volume dissolves in 2 volumes of 70% alcohol.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers, and avoid exposure to excessive heat.
- **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Cocoa Butter

DEFINITION

Cocoa Butter is the fat obtained from the seed of *Theobroma cacao* L. (Fam. Sterculiaceae).

SPECIFIC TESTS

• FATTY ACID COMPOSITION

Sample solution: Place 100–150 mg of Cocoa Butter in a 50-mL round-bottom flask, and add 4 mL of 0.5 N sodium hydroxide solution, prepared in methanol. Add a few boiling chips to the flask, connect the round-bottom flask to a condenser, and boil the mixture under total reflux until the fat globules go into solution. Add 5.0 mL of a 2.0 M borontrifluoride in methanol solution to the boiling mixture via the condenser, and continue boiling for 2 min. Add 2–5 mL of chromatographic *n*-heptane to the boiling mixture via the condenser, and boil for another min. Remove the flask from the source of heat, and remove the reflux condenser. Add saturated sodium chloride solution, and swirl the flask gently. Add more of the saturated sodium chloride solution to bring the liquid level into the neck of the round-bottom flask. Transfer 1 mL of the organic layer into a glass-stoppered test tube, add some anhydrous sodium sulfate to remove the last traces of water, and filter. Use the filtrate.

System suitability solution: 1 mg/mL each of methyl stearate and methyl oleate, in *n*-heptane

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 15-m fused-silica capillary; 0.25-μm stationary phase G19 coating

Temperature

Detector: 250°

Injection port: 250°

Column: See Table 1.

[NOTE—The components of interest elute during the temperature program. The final hold at a temperature of 240° serves only to facilitate elution of higher boiling components.]

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
180	10	240	5

Carrier gas: Helium

Linear velocity: 48 cm/s

Injection size: 0.1 μL

Injection type: Split

Split ratio: 60:1

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for stearate and oleate are about 0.97 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between the stearate and oleate peaks

Relative standard deviation: NMT 5.0%

Analysis

Sample: *Sample solution*

Measure the areas for the peaks of the methyl esters of the fatty acids. [NOTE—The relative retention times for palmitate, stearate, oleate, linoleate, linolenate (if present), and arachidate are about 1.0, 1.55, 1.60, 1.72, 1.89, and 2.30, respectively.]

Calculate the percentage of each fatty acid methyl ester in the portion of Cocoa Butter taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = response of each peak

r_T = sum of the responses of all of the peaks

Acceptance criteria: See Table 2.

Table 2

Fatty Acid Methyl Ester	Relative Retention Time	Percentage (%)
Palmitate	1.0	23–30
Stearate	1.55	31–37
Oleate	1.60	31–38
Linoleate	1.72	1.6–4.8
Linolenate (if present)	1.89	0–1.5
Arachidate	2.30	0–1.5

• MELTING RANGE

Analysis: Melt the material to be tested at a temperature of 50°–60°. Place 50 g of the melted material in a beaker, and cool in a water bath at 25°. Stir continuously until it assumes a pasty consistency, taking care to avoid the inclusion of air bubbles. Place the beaker in a water bath maintained at a temperature of 32°–33°. Continue stirring until the specimen reaches the temperature of the water bath and changes to a liquid cream (about 30 min). Pour the contents into another beaker, and allow it to solidify at room temperature for at least 2 h. Press one side of a U-shaped capillary tube, 1.5 mm in diameter and 80 mm in length with a distance of 10 mm between both capillaries, into the solidified specimen. Using a very fine metal rod, push the column down to 10 mm before the bend of the U-tube. Then attach the other arm of the U-tube to a precision thermometer (having 0.1° graduations) by suitable means, with the U-tube bend at the level of the thermometer bulb. Insert the thermometer into a water bath so that the upper edge of the material is at least 20 mm below the surface, and heat as directed under *Melting Range or Temperature* <741>, *Class I* except, within 5° of the expected melting temperature, regulate the rate of the temperature rise so that it does not exceed 0.2°/min.

Acceptance criteria: The slip point (temperature at which the column visibly flows toward the bend in the tube) is 30°–34°. The clear melting point (clarity via magnifying glass) is 31°–35°.

• **REFRACTIVE INDEX** <831>: 1.454–1.459 at 40°

• **FATS AND FIXED OILS, Free Fatty Acids** <401>: The free fatty acids in 10.0 g of it require for neutralization NMT 5.0 mL of 0.10 N sodium hydroxide (1.4% as oleic acid).

• **FATS AND FIXED OILS, Iodine Value** <401>: 33–42

• **FATS AND FIXED OILS, Saponification Value** <401>: 188–198

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Coconut Oil

Coconut oil [8001-31-8].

DEFINITION

Coconut Oil is the refined fixed oil of the seeds of *Cocos nucifera* L. (Fam. Palmae).

IDENTIFICATION

- **A.** It meets the requirements in *Specific Tests for Fats and Fixed Oils, Fatty Acid Composition* (401).

IMPURITIES

- **ARSENIC**, *Method II* (211): NMT 0.5 µg/g

• **ALKALINE IMPURITIES**

Sample: 10 mL of Coconut Oil

Analysis: Mix 10 mL of freshly distilled acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. Neutralize the solution to a green color if necessary with 0.01 N hydrochloric acid or 0.01 N sodium hydroxide. Add the *Sample*, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS to change the color of the upper layer to yellow.

Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid VS is required.

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 0.5, determined on 20.0 g
- **FATS AND FIXED OILS**, *Fatty Acid Composition* (401): Coconut Oil exhibits the composition profile of fatty acids, shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤1.5
8	0	5.0–11.0
10	0	4.0–9.0
12	0	40.0–50.0
14	0	15.0–20.0
16	0	7.0–12.0
18	0	1.5–5.0
20	0	≤0.2
16	1	≤1.0
18	1	4.0–10.0
18	2	1.0–3.0
18	3	≤0.2
20	1	≤0.2

- **FATS AND FIXED OILS**, *Peroxide Value* (401): NMT 5.0
- **FATS AND FIXED OILS**, *Unsaponifiable Matter* (401): NMT 1.0%
- **MELTING RANGE OR TEMPERATURE** (741): 23°–26°
- **WATER DETERMINATION**, *Method I* (921): NMT 0.1%, 50 mL of chloroform being used as the solvent instead of 35–40 mL of methanol

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant, well-filled containers. No storage requirement specified.

Hydrogenated Coconut Oil

Hydrogenated coconut oil [84836-98-6].

DEFINITION

Hydrogenated Coconut Oil is the product obtained by refining and hydrogenating the oil obtained from the seeds of *Cocos nucifera* L. (Fam. Palmae).

IDENTIFICATION

- **A.** It meets the requirements in *Specific Tests for Fats and Fixed Oils, Fatty Acid Composition* (401).
- **B.** It meets the requirements in *Specific Tests for Melting Range or Temperature* (741).

IMPURITIES

- **RESIDUE ON IGNITION** (281)

Sample: 5 g

Acceptance criteria: NMT 0.1%

- **HEAVY METALS**, *Method II* (231): NMT 10 µg/g

• **LIMIT OF NICKEL**

Nickel standard solution: Immediately before use, prepare the equivalent of 0.2 µg/mL of nickel by diluting 10 mL of nickel standard solution TS with water to 500 mL.

Sample solution: Weigh 5.0 g of Hydrogenated Coconut Oil into a previously tared platinum or silica crucible. Cautiously heat the substance, and introduce into it a wick formed from twisted, ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at about 600°. Continue the incineration until a white ash is obtained. After cooling, with the aid of two 2-mL portions of diluted hydrochloric acid, transfer the residue to a 25-mL volumetric flask, add 0.3 mL of nitric acid, and dilute with water to volume.

Standard solutions: Into four separate identical 10-mL volumetric flasks, introduce respectively 0, 1.0, 2.0, and 4.0 mL of *Nickel standard solution*. To each flask add a 2.0-mL portion of the *Sample solution*, and dilute with water to volume to obtain four *Standard solutions* containing an added quantity of nickel of 0, 0.2, 0.4, and 0.8 µg, respectively.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer, equipped with a graphite furnace

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Analysis

Samples: *Standard solutions*

Concomitantly determine the absorbances of the *Standard solutions* at least three times each. Record the average of the steady readings for each of the *Standard solutions*. Plot the absorbances of the *Standard solutions* versus the added quantity, in µg, of nickel. Extrapolate the line joining the points on the graph until it meets the quantity axis. The distance between this point and the intersection of the axes represents the quantity of nickel, in µg, in the 2-mL portion of the *Sample solution*.

Calculate the content of nickel in the portion of the sample taken:

$$\text{Result} = [V \times (A/V_A)]/W$$

V = volume of the *Sample solution*, 25 mL

A = nickel, as determined above

V_A = volume of the *Sample solution* added to the *Standard solutions*, 2 mL

W = weight of Hydrogenated Coconut Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 1 µg/g

• **ALKALINE IMPURITIES**

Sample: 2.0 g

Analysis: Dissolve the *Sample* by gently heating in a mixture of 1.5 mL of alcohol and 3.0 mL of toluene. Add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid VS to a yellow endpoint.

Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid VS is required.

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** (741): 30°–38°
- **FATS AND FIXED OILS**, *Acid Value*, *Method II* (401): NMT 2.0
- **FATS AND FIXED OILS**, *Fatty Acid Composition* (401): Hydrogenated Coconut Oil exhibits the following composition profile of fatty acids in *Table 1*.

Table 1

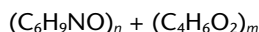
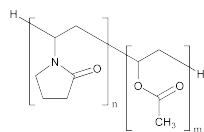
Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤1.5
8	0	5.0–9.0
10	0	4.0–9.0
12	0	44.0–52.0
14	0	15.0–20.0
16	0	8.0–11.0
18	0	8.0–14.0
20	0	≤0.2
>20	0	≤0.5
16	1	≤1.0
18	1	≤1.5
18	2	≤0.5
18	3	≤0.2
20	1	≤0.2

- **FATS AND FIXED OILS, Peroxide Value** <401>: NMT 5.0
- **FATS AND FIXED OILS, Unsaponifiable Matter** <401>: NMT 0.8%
- **LOSS ON DRYING** <731>
Analysis: Dry a sample at 105° for 4 h.
Acceptance criteria: NMT 0.1%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at a temperature not exceeding 55°.

Copovidone



Acetic acid ethenyl ester polymer with 1-ethenyl-2-pyrrolidone;
1-Vinyl-2-pyrrolidone polymer with vinyl acetate [25086-89-9].

DEFINITION

Copovidone is a copolymer of 1-vinyl-2-pyrrolidone and vinyl acetate in the mass proportion of 3:2. The nominal K-value of copovidone as stated in the labeling is NLT 90.0% and NMT 110.0%.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>
- **B.**

Sample solution: 20 mg/mL

Analysis: To 5 mL of the *Sample solution*, add a few drops of iodine TS.

Acceptance criteria: A deep red color is produced.

ASSAY

- **PROCEDURE 1: CONTENT OF COPOLYMERIZED VINYL ACETATE**
Analysis: Determine the saponification value as directed under *Fats and Fixed Oils* <401>, *Saponification Value*. Calculate the percentage of copolymerized vinyl acetate in the portion of Copovidone taken:

$$\text{Result} = 0.1 \times (M_{r1}/M_{r2}) \times S$$

M_{r1} = molecular weight of vinyl acetate, 86.09

M_{r2} = molecular weight of potassium hydroxide, 56.11

S = saponification value

Acceptance criteria: 35.3%–41.4% of the copolymerized vinyl acetate component, calculated on the dried basis

- **PROCEDURE 2: NITROGEN DETERMINATION, Method II** <461>

Analysis: Proceed as directed using 0.1 g of Copovidone. In the procedure, use 5 g of a powdered mixture of potassium sulfate, cupric sulfate, and titanium dioxide (33:1:1) instead of potassium sulfate and cupric sulfate (10:1); omit the use of hydrogen peroxide; and heat until the solution has a clear, yellow-green color and the sides of the flask are free from carbonaceous material. Then heat for a further 45 min; add 20 mL of water, instead of 70 mL, after the second heating; and use bromocresol green–methyl red TS instead of methyl red–methylene blue TS. Titrate the distillate with 0.05 N sulfuric acid VS until the color of the solution changes from green through pale grayish blue to pale grayish red-purple.

Acceptance criteria: 7.0%–8.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** <281>: NMT 0.1%
- **HEAVY METALS** <231>

Sample solution: 100-mg/mL solution of Copovidone in water. [NOTE—Add Copovidone to water in small portions with constant stirring.]

Dilute standard lead solution: *Standard Lead Solution* in water (1 in 5)

Standard solution: *Sample solution* and *Dilute standard lead solution* (1:5)

Blank solution: *Sample solution* and water (1:5)

Analysis: To 12 mL each of the *Sample solution*, *Standard solution*, and *Blank solution*, add 2 mL of pH 3.5 *Acetate Buffer*. Mix and add to 1.2 mL of thioacetamide–glycerin base TS. Mix immediately. Examine the solutions after 2 min. [NOTE—If the result is difficult to judge, filter the solutions through a suitable membrane filter of nominal 0.45-μm pore size. Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.]

System suitability: The reference solution shows a slight brown color compared to the *Blank solution*.

Acceptance criteria: Any brown color in the *Sample solution* is not more intense than that in the *Standard solution* (NMT 20 ppm).

- **LIMIT OF ALDEHYDES**

Solution A: 17.4 mg/mL of monobasic potassium phosphate, adjusted if necessary, with 1 N potassium hydroxide to a pH of 9.0

Solution B: Transfer a quantity of lyophilized aldehyde dehydrogenase equivalent to 70 units to a glass vial, and dissolve in 10.0 mL of water. [NOTE—This solution is stable for 8 h at 4°.]

Solution C: 40 mg of nicotinamide adenine dinucleotide in 10 mL of *Solution A*, in a glass vial. [NOTE—This solution is stable for 4 weeks at 4°.]

Blank solution: Water

Standard solution: Transfer 2 mL of water at 4° to a glass weighing bottle, and weigh. Add 100 mg of freshly distilled acetaldehyde, and weigh. Transfer this solution to a 100-mL volumetric flask. Rinse the weighing bottle with several portions of water at 4°, and transfer each rinsing to the 100-mL volumetric flask. Dilute the solution in the 100-mL volumetric flask with water at 4° to volume. Store at 4° for 20 h. Transfer 1 mL of this solution to a 100-mL volumetric flask, and dilute with *Solution A* to volume.

Sample solution: 10 mg/mL of Copovidone in *Solution A*, in a 100-mL volumetric flask. Insert a stopper into

the flask, heat at 60° for 1 h, and cool to room temperature.

Analysis: Pipet 0.5 mL each of the *Standard solution*, *Sample solution*, and *Blank solution* into separate 1-cm cells. Add 2.5 mL of *Solution A* and 0.2 mL of *Solution C* to each cell. Cover the cells to exclude oxygen. Mix by inversion, and allow to stand for 2–3 min at 22 ± 2°. Determine the absorbances of the solutions at a wavelength of 340 nm. Add 0.05 mL of *Solution B* to each cell. Cover the cells to exclude oxygen. Mix by inversion, and allow to stand for 5 min at 22 ± 2°. Determine the absorbances of the solutions at a wavelength of 340 nm. Calculate the percentage of aldehydes, expressed as acetaldehyde, in the portion of Copovidone taken:

$$\text{Result} = \frac{[(A_{U2} - A_{U1}) - (A_{B2} - A_{B1})] / [(A_{S2} - A_{S1}) - (A_{B2} - A_{B1})]}{(C/W)} \times 10$$

A_{U2} = absorbance of the solution from the *Sample solution*, after the addition of *Solution B*

A_{U1} = absorbance of the solution from the *Sample solution*, before the addition of *Solution B*

A_{B2} = absorbance of the solution from the *Blank solution*, after the addition of *Solution B*

A_{B1} = absorbance of the solution from the *Blank solution*, before the addition of *Solution B*

A_{S2} = absorbance of the solution from the *Standard solution*, after the addition of *Solution B*

A_{S1} = absorbance of the solution from the *Standard solution*, before the addition of *Solution B*

C = concentration of acetaldehyde in the *Standard solution* (mg/mL)

W = weight, calculated on the dried basis, of Copovidone taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.05%

• LIMIT OF HYDRAZINE

Standard solution: 9 µg/mL of salicylaldazine and 10 mg/mL of salicylaldehyde in toluene

Sample solution: Transfer the equivalent of 2.5 g of dried Copovidone to a 50-mL centrifuge tube, add 25 mL of water, and mix to dissolve. Add 500 µL of a 50-mg/mL solution of salicylaldehyde in methanol, adjust the solution with 0.25 N sulfuric acid to a pH of about 2, swirl, and heat in a water bath at 60° for 15 min. Allow to cool, add 2.0 mL of toluene, insert a stopper in the tube, shake vigorously for 2 min, and centrifuge. Use the clear upper toluene layer.

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of dimethylsilanized chromatographic silica gel mixture

Application volume: 10 µL

Developing solvent system: Acetonitrile and water (17:3)

Analysis

Samples: *Standard solution* and *Sample solution*
Proceed as directed in the chapter. Allow the spots to dry, and develop the chromatogram in the *Developing solvent system* until the solvent front has moved about three-fourths of the length of the plate. Locate the spots on the plate by examination under UV light at a wavelength of 365 nm: salicylaldazine appears as a fluorescent spot having an R_f value of about 0.6–0.7, and the fluorescence of any salicylaldazine spot from the *Sample solution* is not more intense than that produced by the spot from the *Standard solution*.

Acceptance criteria: NMT 1 ppm

• LIMIT OF PEROXIDES

Copovidone solution: 40 mg/mL of Copovidone in water calculated on the dried basis

Sample solution: Transfer 25.0 mL of *Copovidone solution* to a 50-mL beaker, and add 2 mL of titanium

trichloride–sulfuric acid TS. Allow to stand for 30 min at room temperature.

Blank solution: Transfer 25.0 mL of *Copovidone solution* to a 50-mL beaker, and add 2 mL of 13% sulfuric acid.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: UV-Vis

Analytical wavelength: 405 nm

Cell: 1 cm

Blank: *Blank solution*

Analysis: Determine the absorbance of the *Sample solution*.

Acceptance criteria: The absorbance is NMT 0.35 (corresponding to NMT 0.04%, expressed as hydrogen peroxide).

• LIMIT OF MONOMERS (1-VINYL-2-PYRROLIDONE, VINYL ACETATE, AND 2-PYRROLIDONE)

Solution A: Water, acetonitrile, and methanol (90:5:5)

Solution B: Water, acetonitrile, and methanol (50:45:5)

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
26	80	20
27	0	100
36	0	100
38	100	0

Standard stock solution: 0.50 mg/mL of 1-vinyl-2-pyrrolidone, 0.50 mg/mL of vinyl acetate, and 3.0 mg/mL of 2-pyrrolidone in methanol

Standard solution: *Standard stock solution* in *Solution A* (1 in 2000)

Sample solution: Dissolve 250 mg of Copovidone in 1 mL of methanol, mix ultrasonically, dilute with water to 10 mL. If necessary, filter to remove undissolved particles.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 205 nm and 235 nm

Column

Guard: 4.0-mm × 2.5-cm; packing L1

Analytical: 4.0-mm × 25-cm; 5-µm packing L1

Column temperature: 30°

Injection size: 10 µL

Flow rate: 1.0 mL/min

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 2.0 between the 2-pyrrolidone and vinyl acetate peaks, and NLT 2.0 between the vinyl acetate and 1-vinyl-2-pyrrolidone peaks. [NOTE—According to the above operating conditions, the order of elution is 2-pyrrolidone, vinyl acetate, and 1-vinyl-2-pyrrolidone.]

Relative standard deviation: NMT 2.0% for each analyte, on replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—After each injection of the *Sample solution* wash the polymeric material of Copovidone from the guard column by passing the *Mobile phase* through the column backwards for 30 min at the same flow rate.]

Calculate the content of 1-vinyl-2-pyrrolidone in the portion of Copovidone taken:

$$\text{Result} = (A_{TA}/A_{SA}) \times (C_{SA}/C_T) \times 100$$

- A_{TA} = 1-vinyl-2-pyrrolidone peak response from the *Sample solution*
 A_{SA} = 1-vinyl-2-pyrrolidone peak response from the *Standard solution*
 C_{SA} = concentration of 1-vinyl-2-pyrrolidone in the *Standard solution* (mg/mL)
 C_T = concentration of Copovidone in the *Sample solution* on the dried basis (mg/mL)

Calculate the content of vinyl acetate in the portion of Copovidone taken:

$$\text{Result} = (A_{TB}/A_{SB}) \times (C_{SB}/C_T) \times 100$$

- A_{TB} = vinyl acetate peak response from the *Sample solution*
 A_{SB} = vinyl acetate peak response from the *Standard solution*
 C_{SB} = concentration of vinyl acetate in the *Standard solution* (mg/mL)
 C_T = concentration of Copovidone in the *Sample solution* on the dried basis (mg/mL)
 Calculate the content of 2-pyrrolidinone in the portion of Copovidone taken:

$$\text{Result} = (A_{TC}/A_{SC}) \times (C_{SC}/C_T) \times 100$$

- A_{TC} = 2-pyrrolidone peak response from the *Sample solution*
 A_{SC} = 2-pyrrolidone peak response from the *Standard solution*
 C_{SC} = concentration of 2-pyrrolidone in the *Standard solution* (mg/mL)
 C_T = concentration of Copovidone in the *Sample solution* on the dried basis (mg/mL)

Acceptance criteria: NMT 0.001% of 1-vinyl-2-pyrrolidone, NMT 0.001% of vinyl acetate, and NMT 0.5% of 2-pyrrolidone

SPECIFIC TESTS

- LOSS ON DRYING** (731): Dry a sample at 105° for 3 h: it loses NMT 5.0% of its weight.

- CLARITY AND COLOR OF SOLUTION**

Sample: 1.0 g

Analysis: Dissolve the *Sample* in 10 mL of water.

Acceptance criteria: The solution is clear or slightly opalescent and colorless to pale yellow or pale red.

- K-VALUE**

Sample solution: Transfer a quantity of undried Copovidone, equivalent to 1.0 g on the dried basis, to a 100-mL volumetric flask, and dissolve in and dilute with water to volume. Allow to stand for 1 h.

Analysis: Determine the viscosity, using a capillary-tube viscometer (see *Viscosity—Capillary Viscometer Methods* (911)), of this solution at 25 ± 0.2°.

Calculate the relative K-value of Copovidone:

$$\text{Result} = \left[\sqrt{300c \log z + (c + 1.5c \log z)^2} + 1.5c \log z - c \right] / (0.15c + 0.003c^2) \times (100/K_U)$$

- c = weight on the dried basis, of the specimen tested in each 100.0 mL of solution (g)
 z = viscosity of the *Sample solution* relative to that of water
 K_U = nominal K-value stated on the label

Acceptance criteria: 90.0%–110.0%

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.

- LABELING:** Label it to indicate its nominal K-value.
- USP REFERENCE STANDARDS** (11)
USP Copovidone RS

Coriander Oil

DEFINITION

Coriander Oil is the volatile oil obtained by steam distillation from the dried ripe fruit of *Coriandrum sativum* L. (Fam. Apiaceae).

IMPURITIES

- HEAVY METALS, Method II** (231): NMT 40 ppm

SPECIFIC TESTS

- SPECIFIC GRAVITY** (841): Between 0.863 and 0.875
- OPTICAL ROTATION, Angular Rotation** (781A): Between +8° and +15°
- REFRACTIVE INDEX** (831): Between 1.462 and 1.472 at 20°
- SOLUBILITY IN 70% ALCOHOL:** One volume dissolves in 3 volumes of 70% alcohol.

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, protect from light, and store at controlled room temperature. Avoid exposure to excessive heat.
- LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.

Corn Oil

[8001-30-7].

DEFINITION

Corn Oil is the refined fixed oil obtained from the embryo of *Zea mays* Linné (Fam. Gramineae).

IDENTIFICATION

- A.** It meets the requirements of the test for *Fats and Fixed Oils* (401), *Fatty Acid Composition*.

IMPURITIES

- ALKALINE IMPURITIES**

Sample: 10 mL of Corn Oil

Analysis: Mix 10 mL of acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. Neutralize the solution to a green color if necessary with 0.01 N hydrochloric acid or 0.01 N sodium hydroxide. Add the *Sample*, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS to change the color of the upper layer to yellow.

Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid is required.

- HEAVY METALS, Method II** (231): NMT 10 ppm

SPECIFIC TESTS

- FATS AND FIXED OILS, Acid Value** (401): NMT 0.2
- FATS AND FIXED OILS, Fatty Acid Composition** (401): Corn Oil exhibits the composition profile of fatty acids in Table 1.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<14	0	≤0.1
14	0	≤0.1
16	0	8.6–16.5

Table 1 (Continued)

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
16	1	≤0.5
18	0	1.0–3.3
18	1	20.0–42.2
18	2	39.4–62.0
18	3	0.5–1.5
20	0	≤0.8
20	1	≤0.5
22	0	≤0.3
22	1	≤0.1
24	0	≤0.4

- **FATS AND FIXED OILS, Peroxide Value** (401): NMT 10.0
- **FATS AND FIXED OILS, Sterol Composition** (401): The sterol fraction of the Corn Oil contains NMT 0.3% of brassicasterol.
- **FATS AND FIXED OILS, Unsaponifiable Matter** (401): NMT 1.5%
- **WATER DETERMINATION, Method I** (921): NMT 0.1%. Use a mixture of equal volumes of decanol and anhydrous methanol as the solvent.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.
- **LABELING:** Where Corn Oil is intended for use in the manufacture of injectable dosage forms, it is so labeled.
- **OTHER REQUIREMENTS:** For Corn Oil intended for use in injectable dosage forms, specified in *Labeling*, the requirements for *Acid Value*, *Peroxide Value*, *Unsaponifiable Matter*, and *Water in Other Vehicles under Injections* (1), *Ingredients* must be met.

Corn Syrup

[8029-43-4].

DEFINITION

Corn Syrup is an aqueous solution of saccharides obtained by partial hydrolysis of edible corn starch by food grade acids and/or enzymes. It contains NLT 20.0% reducing sugar content (dextrose equivalent) expressed as D-glucose, calculated on the dried basis.

IDENTIFICATION

- **A.**
Analysis: Add a few drops of a solution of Syrup (1 in 20) to 5 mL of hot, alkaline cupric tartrate TS.
Acceptance criteria: A copious, red precipitate of cuprous oxide is formed.

ASSAY• **REDUCING SUGARS (DEXTROSE EQUIVALENT)**

Apparatus: Mount a ring support on a ring stand 1–2 inches above a gas burner, and mount a second ring 6–7 inches above the first. Place 6-inch open-wire gauze on the lower ring to support a 250-mL conical flask, and place a 4-inch watch glass with a center hole on the upper ring to deflect heat. Attach a 25-mL buret to the ring stand so that the tip just passes through the watch glass centered above the flask. Place an indirectly lighted white surface behind the assembly for observing the endpoint.

Standard solution: 6 mg/mL of USP Dextrose RS

Sample solution: 10 mg/mL of Corn Syrup

Analysis: Transfer 25.0-mL portions of alkaline cupric tartrate TS to each of two flasks, and boil. Immediately place one flask on the wire gauze of the *Apparatus*, and adjust the burner so that the boiling point is reached in

about 2 min. Titrate with the *Standard solution* to within 0.5 mL of the anticipated endpoint. Heat the flask, with swirling, boil moderately for 2 min, and add 2 drops of methylene blue solution (1 in 100). Immediately add 2 drops of the *Standard solution* from the buret, and bring to a boil. Allow the cuprous oxide to settle slightly, and observe the color of the supernatant. Complete the titration within 1 min by adding the *Standard solution* dropwise, and boil after each addition to the disappearance of the blue color, as determined by viewing against a white background in daylight or under equivalent illumination. If more than 0.5 mL of the titrant is required after the addition of the indicator, repeat the titration, adding the necessary volume of titrant before adding the indicator. Bring the contents of the second flask to a boil, and similarly titrate with the *Sample solution*.

Calculate the percentage of reducing sugars as D-glucose, calculated on the dried basis, in the portion of Corn Syrup taken:

$$\text{Result} = (C_S/C_U) \times (V_S/V_U) \times [1/(0.01 \times A)] \times 100$$

C_S = concentration of USP Dextrose RS in the *Standard solution* (mg/mL)

C_U = concentration of Corn Syrup in the *Sample solution* (mg/mL)

V_S = titrated volume of the *Standard solution* (mL)

V_U = titrated volume of the *Sample solution* (mL)

A = percentage of dry solids in Corn Syrup measured by the refractive index

Acceptance criteria: NLT 20.0% reducing sugar content on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.5%, determined on 20 g of Corn Syrup
- **HEAVY METALS, Method II** (231): NMT 5 ppm
- **LIMIT OF LEAD**

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. For digestion, use acid-cleaned, high-density polyethylene, polypropylene, polytetrafluoroethylene, or quartz tubes. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in borosilicate glass containers. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min and rinsing with deionized water. Store final diluted solutions in acid-cleaned plastic or polytetrafluoroethylene tubes or bottles.]

Modifier solution: 200 mg/mL of magnesium nitrate. Just before use, transfer 1.0 mL of this solution to a 10-mL volumetric flask, and dilute with 5% nitric acid to volume.

Standard stock solution: Transfer 10.0 mL of *Lead Nitrate Stock Solution*, prepared as directed in *Heavy Metals* (231), to a 100-mL volumetric flask, add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, dilute with 5% nitric acid to volume, and mix. This solution contains 0.1 µg/mL of lead.

Standard solutions: Transfer portions of *Standard stock solution* to four suitable containers, and dilute with 5% nitric acid to obtain *Standard solutions* having lead concentrations of 100, 50, 25, and 10 ng/mL, respectively.

Sample solution: [NOTE—Perform this procedure in a fume hood.] Transfer 1.5 g of Corn Syrup to a digestion tube, and add 0.75 mL of nitric acid to the tube. Warm the solution slowly (to avoid spattering) to 90°–95°. Heat until all brown vapors have dissipated and any rust-colored tint has disappeared from the tube (20–30 min). Cool, add 0.5 mL of 50% hydrogen peroxide,

dropwise, to the solution, heat to 90°–95° for 5 min, and cool. Add a second 0.5-mL portion of 50% hydrogen peroxide dropwise to the solution, and heat to 90°–100° until clear (5–10 min). Cool and transfer the solution to a 10-mL volumetric flask. Rinse the digestion tube with 5% nitric acid, add the rinse to the volumetric flask, dilute with 5% nitric acid to volume, and mix.

Standard blank: 5% nitric acid

Sample blank: Transfer 1.5 g of water to a digestion tube, and proceed as directed for the *Sample solution*, beginning with “add 0.75 mL of nitric acid”.

Instrumental conditions

Mode: Graphite furnace atomic absorption with pyrolytically coated graphite tubes and adequate means of background correction

Lamp: A lead hollow-cathode lamp

Analytical wavelength: Lead emission line of 283.3 nm

Furnace program: See *Table 1*. [NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Table 1

Step	Dry	Ash	Purge	Atomize
Temperature (°)	200	750	Cool down, and purge the air from the furnace 20	1800
Ramp time (s)	20	40	—	0
Hold time (s)	30	40	60	10
Argon flow rate (mL/min)	300	300	300	Argon gas flow stopped

Injection volume: 20 µL

Analysis

[NOTE—Use peak area measurements for all quantitations.]

Samples: Add 5 µL of the *Modifier solution* to 20 µL each of the *Standard solutions*, the *Sample solution*, the *Standard blank*, and the *Sample blank*, and mix.

Separately inject equal volumes (about 20 µL) of the *Samples* into the instrument for analysis.

Using the *Standard blank* to set the instrument to zero, determine the integrated absorbances of the *Standard solutions*. Plot the integrated absorbances of the *Standard solutions* versus their contents of lead, in ng/mL, and draw the line best fitting the four points to determine the calibration curve. Similarly determine the integrated absorbances of the *Sample solution* and the *Sample blank*. Correct the absorbance value of the *Sample solution* by subtracting from it the absorbance value obtained from the *Sample blank*.

Calculate the concentration, in ppm (µg/g), of lead in the portion of Corn Syrup taken:

$$\text{Result} = (C_L \times V/W) \times F$$

C_L = concentration of lead in the *Sample solution*, as determined from the calibration curve (ng/mL)

V = volume of the *Sample solution*, 10 mL

W = weight of Corn Syrup taken to prepare the *Sample solution* (g)

F = conversion factor, 10^{-3} µg/ng

Acceptance criteria: 0.5 ppm (µg/g)

• **LIMIT OF SULFUR DIOXIDE**

Starch indicator solution: Mix 10 g of soluble starch with 50 mL of cold water, transfer to 1000 mL of boiling water, stir until completely dissolved, cool, and add 1 g of salicylic acid preservative. [NOTE—Discard this solution after 1 month.]

Sample: 100 g

Blank: 200 mL of water

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.005 N iodine VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 250-mL conical flask, add 100 mL of water, and mix. Cool to 5°–10°. While stirring with a magnetic stirrer, add 10 mL of cold (5°–10°) 1.5 N sodium hydroxide. Stir for an additional 20 s, and add 10 mL of *Starch indicator solution*. Add 10 mL of cold (5°–10°) 2.0 N sulfuric acid, and titrate immediately with *Titrant* until a light blue color persists for 1 min. Perform a blank determination, and make any necessary correction.

Calculate the concentration, in ppm (µg/g), of sulfur dioxide (SO₂) in the *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times N \times F_1/W] \times F_2$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F_1 = equivalency factor, 32.0 mg/mEq

W = *Sample* weight (g)

F_2 = conversion factor, 10^3 µg/mg

Acceptance criteria: NMT 40 ppm (µg/g)

• **ABSENCE OF SOLUBLE STARCH**

Analysis: Dissolve 1 g in 10 mL of water, and add 1 drop of iodine TS.

Acceptance criteria: A yellow color indicates the absence of soluble starch.

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR**

SPECIFIED MICROORGANISMS (62): The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeast count does not exceed 10^2 cfu/g.

• **TOTAL SOLIDS**

Instrumental conditions

(See *Refractive Index* (831).)

Mode: Refractometer equipped with a jacket for water circulation or some other mechanism for maintaining the sample at $20 \pm 0.1^\circ$ or $45 \pm 0.1^\circ$

Before proceeding with measurements, ensure that the sample and the prism have reached the equilibrium temperature and that the instrument has been properly checked and calibrated against a standard provided by the manufacturer.

Analysis: Measure the refractive index of Corn Syrup, and convert the value to approximate percent solids value using *Table 2* and *Table 3*. [NOTE—*Table 2* covers the approximate total solids levels of these products in commerce. If the ash or dextrose equivalent of the sample differs from that of the product in *Table 2*, use *Table 3* for the ash and dextrose equivalent correction.]

Table 2. Reference for Converting the Refractive Index to Approximate Percent Solids

Dextrose Equivalent (DE)	Dry Substance (DS) (%)	Refractive Index at 20°	Refractive Index at 45°	Degrees Baumé at 140° F (60° C) + 1
28 DE Corn syrup—0.3% ash	76.0	1.4888	1.4837	40.98
	77.0	1.4915	1.4864	41.49
	78.0	1.4943	1.4892	42.00
	79.0	1.4971	1.4919	42.51
	80.0	1.4999	1.4947	43.01
34 DE High-maltose corn syrup— 0.3% ash	78.6	1.4933	1.4882	41.99
	79.6	1.4960	1.4909	42.49
	80.6	1.4988	1.4936	42.99
	81.6	1.5015	1.4964	43.49
	82.6	1.5043	1.4992	43.99
36 DE Corn syrup—0.3% ash	78.4	1.4938	1.4887	42.01
	79.4	1.4965	1.4914	42.52
	80.4	1.4993	1.4941	43.02
	81.4	1.5021	1.4969	43.52
	82.4	1.5049	1.4997	44.02
43 DE High-maltose corn syrup— 0.3% ash	78.9	1.4934	1.4883	42.00
	79.9	1.4961	1.4910	42.51
	80.9	1.4988	1.4937	43.01
	81.9	1.5016	1.4964	43.51
	82.9	1.5044	1.4992	44.01
43 DE Corn syrup—0.3% ash	78.7	1.4933	1.4882	42.01
	79.7	1.4960	1.4909	42.51
	80.7	1.4988	1.4936	43.02
	81.7	1.5015	1.4964	43.52
	82.7	1.5043	1.4992	44.01
43 DE (Ion-exchanged) Corn syrup—0.03% ash	78.8	1.4935	1.4884	41.99
	79.8	1.4962	1.4911	42.50
	80.8	1.4990	1.4938	43.00
	81.8	1.5018	1.4966	43.50
	82.8	1.5045	1.4994	43.99
53 DE Corn syrup—0.3% ash	80.5	1.4962	1.4911	42.64
	81.5	1.4989	1.4938	43.14
	82.5	1.5016	1.4965	43.64
	83.5	1.5044	1.4992	44.13
	84.5	1.5072	1.5020	44.63
63 DE Corn syrup—0.3% ash	81.0	1.4955	1.4904	42.53
	82.0	1.4982	1.4931	43.02
	83.0	1.5009	1.4958	43.52
	84.0	1.5037	1.4985	44.01
	85.0	1.5064	1.5012	44.50
63 DE (Ion-exchanged) Corn syrup—0.03% ash	81.3	1.4963	1.4912	42.60
	82.3	1.4990	1.4939	43.10
	83.3	1.5017	1.4965	43.59
	84.3	1.5044	1.4993	44.09
	85.3	1.5072	1.5020	44.58
66 DE Corn syrup—0.3% ash	81.0	1.4949	1.4898	42.36
	82.0	1.4975	1.4924	42.86
	83.0	1.5002	1.4951	43.36
	84.0	1.5029	1.4978	43.85
	85.0	1.5056	1.5005	44.35
95 DE Corn syrup—0.3% ash	69.0	1.4598	1.4550	35.46
	70.0	1.4621	1.4573	35.96
	71.0	1.4644	1.4596	36.46
	72.0	1.4668	1.4619	36.96
	73.0	1.4692	1.4643	37.45
95 DE (Ion-exchanged) Corn syrup—0.03% ash	69.0	1.4597	1.4549	35.39
	70.0	1.4620	1.4572	35.89
	71.0	1.4644	1.4595	36.39
	72.0	1.4667	1.4619	36.89
	73.0	1.4691	1.4642	37.38

Table 3. Ash and Dextrose Equivalent (DE) Corrections for Corn Syrup: Changes in Refractive Index for an Increase in Dry Substance (DS)

Dry Substance (DS) (%)	1% Ash	1 Dextrose Equivalent (DE)
2	0.000000	-0.000001
4	0.000000	-0.000003
6	0.000001	-0.000005
8	0.000002	-0.000007
10	0.000003	-0.000010
12	0.000004	-0.000012
14	0.000006	-0.000015
16	0.000008	-0.000017
18	0.000010	-0.000020
20	0.000013	-0.000023
22	0.000016	-0.000026
24	0.000019	-0.000029
26	0.000022	-0.000033
28	0.000026	-0.000036
30	0.000030	-0.000040
32	0.000034	-0.000044
34	0.000039	-0.000048
36	0.000044	-0.000052
38	0.000049	-0.000057
40	0.000055	-0.000061
42	0.000061	-0.000066
44	0.000068	-0.000071
46	0.000074	-0.000076
48	0.000082	-0.000081
50	0.000089	-0.000087
52	0.000097	-0.000093
54	0.000105	-0.000099
56	0.000114	-0.000105
58	0.000123	-0.000112
60	0.000133	-0.000118
62	0.000143	-0.000125
64	0.000153	-0.000132
66	0.000164	-0.000140
68	0.000175	-0.000147
70	0.000187	-0.000155
72	0.000199	-0.000163
74	0.000212	-0.000172
76	0.000225	-0.000181
78	0.000239	-0.000190
80	0.000253	-0.000199
82	0.000268	-0.000208
84	0.000283	-0.000218

Acceptance criteria: The total solids value is NLT 70.0%.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** Label it to indicate its nominal dextrose equivalent. Label it also to indicate the presence of sulfur dioxide if the residual concentration is greater than 10 ppm (µg/g).
- **USP REFERENCE STANDARDS** <11>
USP Dextrose RS

High Fructose Corn Syrup**DEFINITION**

High Fructose Corn Syrup is a sweet, nutritive saccharide mixture prepared as a clear, aqueous solution from high-dextrose-equivalent corn starch hydrolysate by the partial enzymatic conversion of dextrose to fructose, using an insoluble glucose isomerase enzyme preparation that complies with 21 CFR 184.1372. It is available in two types, 42% and 55%, based on fructose content. High Fructose Corn Syrup 42% contains NLT 97.0% of total saccharides, expressed as a percentage of total solids, of which NLT 92.0% consists of monosaccharides (fructose and dextrose), including NLT 41.5% and NMT 44.8% of fructose, and NMT 8.0% consists of other saccharides. High Fructose Corn Syrup 55% contains NLT 95.0% of total saccharides, expressed as a percentage of total solids, of which NLT 95.0% consists of monosaccharides (fructose and

dextrose), including NLT 54.5% and NMT 56.5% of fructose, and NMT 5.0% consists of other saccharides.

IDENTIFICATION

• A.

Analysis: Add a few drops of a solution (1 in 10) of Syrup to 5 mL of hot, alkaline cupric tartrate TS.

Acceptance criteria: A copious, red precipitate of cuprous oxide is formed (distinction from sucrose).

ASSAY

• PROCEDURE

Mobile phase: Water

Standard solution: Prepare a solution in water containing a total of 10% saccharide solids of USP Dextrose RS, USP Fructose RS, and USP Maltose Monohydrate RS, in which the USP Dextrose RS and USP Fructose RS percentage concentrations are in the same ratio as those in the *Sample solution*, based on the labeled nominal fructose percentage for the Syrup under test.

Calculate the percentage of USP Maltose Monohydrate RS:

$$\text{Result} = 100 - (F + D)$$

F = labeled nominal fructose percentage for the Syrup under test

D = difference between the specified minimum percentage concentration of total monosaccharides for the Syrup and *F*

Sample solution: Dilute a known weight of Syrup, determined from the results of the test for *Total Solids* and on the nominal total saccharides content, with water to a total saccharides concentration of 10% (w/v), and mix.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 30-cm; packing L19

Temperature

Detector: 45°

Column: 85°

Flow rate: 0.6 mL/min

Injection size: 10 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for maltose, dextrose, and fructose are about 0.83, 1.0, and 1.32, respectively.]

Suitability requirements

Resolution: NLT 1.2 between maltose and dextrose

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of fructose and of dextrose in the portion of Syrup taken:

$$\text{Result} = (r_U/r_S) \times C_S \times \{V_A/[W_S \times (0.01 \times S_1) \times (0.01 \times S_2)]\} \times 100$$

r_U = peak area of fructose or dextrose from the *Sample solution*

r_S = peak area of fructose or dextrose from the *Standard solution*

C_S = concentration of USP Fructose RS or USP Dextrose RS in the *Standard solution* (mg/mL)

V_A = volume of the *Sample solution* (mL)

W_S = weight of Syrup taken to prepare the *Sample solution* (mg)

S₁ = percentage of total saccharides in the Syrup as specified on the label

S₂ = percentage of total solids in the Syrup as determined in the test for *Total Solids*

Calculate the percentage of other saccharides, expressed in terms of maltose, in the portion of Syrup taken:

$$\text{Result} = (r_U/r_S) \times C_S \times \{V_A/[W_S \times (0.01 \times S_1) \times (0.01 \times S_2)]\} \times 100$$

r_U = sum of all peak areas from the *Sample solution*, except those of fructose and dextrose

r_S = peak area of maltose from the *Standard solution*

C_S = concentration of USP Maltose Monohydrate RS in the *Standard solution* (mg/mL)

V_A = volume of the *Sample solution* (mL)

W_S = weight of Syrup taken to prepare the *Sample solution* (mg)

S₁ = percentage of total saccharides in the Syrup as specified on the label

S₂ = percentage of total solids in the Syrup as determined in the test for *Total Solids*

Acceptance criteria

For High Fructose Corn Syrup 42%

Total saccharides: NLT 97.0%, expressed as a percentage of total solids. Total saccharides contain monosaccharides and other saccharides as follows.

Monosaccharides: NLT 92.0% (fructose and dextrose)

Fructose: 41.5%–44.8%

Other saccharides: NMT 8.0%

For High Fructose Corn Syrup 55%

Total saccharides: NLT 95.0%, expressed as a percentage of total solids. Total saccharides contain monosaccharides and other saccharides as follows.

Monosaccharides: NLT 95.0% (fructose and dextrose)

Fructose: 54.5%–56.5%

Other saccharides: NMT 5.0%

IMPURITIES

• **RESIDUE ON IGNITION** <281>: NMT 0.05%

• **HEAVY METALS**, *Method II* <231>: NMT 5 ppm, using an ignition temperature of 500°

• LIMIT OF LEAD

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. For digestion, use acid-cleaned, high-density polyethylene, polypropylene, polytet, or quartz tubes. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in borosilicate glass containers. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min and rinsing with deionized water. Store final diluted solutions in acid-cleaned plastic or polytet tubes or bottles.]

Modifier solution: 200 mg/mL of magnesium nitrate. Just before use, transfer 1.0 mL of this solution to a 10-mL volumetric flask, and dilute with 5% nitric acid to volume.

Standard stock solution: Transfer 10.0 mL of *Lead Nitrate Stock Solution*, prepared as directed in *Heavy Metals* <231>, to a 100-mL volumetric flask, add 40 mL of water and 5 mL of nitric acid, and dilute with water to volume. Transfer 1.0 mL of this solution to a second 100-mL volumetric flask, dilute with 5% nitric acid to volume, and mix. This solution contains 0.1 µg/mL of lead.

Standard solutions: Transfer portions of *Standard stock solution* to four suitable containers, and dilute with 5% nitric acid to obtain *Standard solutions* having lead concentrations of 100, 50, 25, and 10 ng/mL, respectively.

Sample solution: [NOTE—Perform this procedure in a fume hood.] Transfer 1.5 g of Syrup to two digestion tubes, labeled “Sample solution” and “Temperature monitor solution”, and add 0.75 mL of nitric acid to

each tube. Warm both solutions slowly to 90°–95° to avoid spattering. Heat until all brown vapors have dissipated and any rust-colored tint is gone from the tube labeled "Sample solution" (20–30 min). Cool, add 0.5 mL of 50% hydrogen peroxide dropwise to both solutions, heat to 90°–95° for 5 min, and cool. Add a second 0.5-mL portion of 50% hydrogen peroxide dropwise to each solution, and heat to 90°–100° for 5–10 min until the tube labeled "Sample solution" is clear. Cool, and transfer the *Sample solution* to a 10-mL volumetric flask. Rinse the tube labeled "Sample solution" with 5% nitric acid, add the rinsing to the volumetric flask, dilute with 5% nitric acid to volume, and mix.

Standard blank: 5% nitric acid

Sample blank: Transfer 1.5 g of water to a digestion tube, and proceed as directed for the *Sample solution*, beginning with "add 0.75 mL of nitric acid".

Instrumental conditions

Mode: Graphite furnace atomic absorption with pyrolytically coated graphite tubes and adequate means of background correction

Lamp: A lead hollow-cathode

Analytical wavelength: Lead emission line of 283.3 nm

Furnace program: See Table 1. [NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Table 1

Step	Dry	Ash	Purge	Atomize
Temperature (°)	200	750	Cool down, and purge the air from the furnace 20	1800
Ramp time (s)	20	40	—	0
Hold time (s)	30	40	60	10
Argon flow rate (mL/min)	300	300	300	Argon gas flow stopped

Injection volume: 20 µL

Analysis

[NOTE—Use peak area measurements for all quantitations.]

Samples: Add 5 µL of the *Modifier solution* to 20 µL each of the *Standard solutions*, the *Sample solution*, the *Standard blank*, and the *Sample blank*, and mix.

Separately inject equal volumes (about 20 µL) of the *Samples* into the instrument for analysis.

Using the *Standard blank* to set the instrument to zero, determine the integrated absorbances of the *Standard solutions*. Plot the integrated absorbances of the *Standard solutions* versus their contents of lead, in ng/mL, and draw the line best fitting the four points to determine the calibration curve. Similarly determine the integrated absorbances of the *Sample solution* and the *Sample blank*. Correct the absorbance value of the *Sample solution* by subtracting from it the absorbance value obtained from the *Sample blank*.

Calculate the concentration of lead, in ppm (µg/g), in the portion of Syrup taken:

$$\text{Result} = (V \times C_L / W) \times F$$

V = volume of the *Sample solution*, 10 mL

C_L = concentration of lead in the *Sample solution*, as determined from the calibration curve (ng/mL)

W = weight of Syrup taken to prepare the *Sample solution* (g)

F = conversion factor, 10^{-3} µg/ng

Acceptance criteria: NMT 0.1 ppm (µg/g)

• LIMIT OF SULFUR DIOXIDE

Starch indicator solution: Mix 10 g of soluble starch with 50 mL of cold water. Transfer to 1000 mL of boiling water, stir until completely dissolved, cool, and add 1 g of salicylic acid preservative. [NOTE—Discard the solution after 1 month.]

Sample: 100 g

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.005 N iodine VS

Blank: 200 mL of water

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 250-mL conical flask, add 100 mL of water, and mix. Cool to 5°–10°. While stirring with a magnetic stirrer, add 10 mL of cold (5°–10°) 1.5 N sodium hydroxide. Stir for an additional 20 s, and add 10 mL of *Starch indicator solution*. Add 10 mL of cold (5°–10°) 2.0 N sulfuric acid, and titrate immediately with *Titrant* until a light blue color persists for 1 min. Perform a blank determination, and make any necessary correction.

Calculate the concentration, in ppm (µg/g), of sulfur dioxide (SO₂) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F_1] / W\} \times F_2$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F_1 = equivalency factor, 32.0 mg/mEq

W = *Sample* weight (g)

F_2 = conversion factor, 10^3 µg/mg

Acceptance criteria: NMT 30 ppm

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeast count does not exceed 10^2 cfu/g.
- **TOTAL SOLIDS:** Determine the refractive index of the Syrup at 20° or 45° (see *Refractive Index* (831)). Use Table 2 for calculating the percentage of dry substance (percentage of total solids on a weight/weight basis).

Table 2

Fructose Content (%)	Dry Substance (DS) (%)	Refractive Index at 20°	Refractive Index at 45°
42	70.5	1.4632	1.4577
—	71.0	1.4643	1.4589
—	72.0	1.4667	1.4612
—	73.0	1.4691	1.4635
55	76.5	1.4774	1.4716
—	77.0	1.4786	1.4728
—	78.0	1.4811	1.4752
—	79.0	1.4835	1.4776

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirement specified.
- **LABELING:** Label it to state, as part of the official title, the nominal percentage of fructose, based on the specified minimum percentage concentration of total saccharides. Label it to indicate the presence of sulfur dioxide if the residual concentration is greater than 10 ppm (µg/g).

- **USP REFERENCE STANDARDS** (11)
USP Dextrose RS
USP Fructose RS
USP Maltose Monohydrate RS

Corn Syrup Solids

DEFINITION

Corn Syrup Solids (Dried Glucose Syrup) is a dried mixture of saccharides obtained by partial hydrolysis of edible corn starch by food grade acids and/or enzymes. It contains NLT 20.0% reducing sugar content (dextrose equivalent) expressed as D-glucose, calculated on the dried basis.

IDENTIFICATION

- **A.**
Sample solution: 50 mg/mL
Analysis: Add a few drops of the *Sample solution* to 5 mL of hot, alkaline cupric tartrate TS.
Acceptance criteria: A copious, red precipitate of cuprous oxide is formed (distinction from sucrose).

ASSAY

• REDUCING SUGARS (DEXTROSE EQUIVALENT)

Apparatus: Mount a ring support on a ring stand 1–2 inches above a gas burner, and mount a second ring 6–7 inches above the first. Place 6-inch open-wire gauze on the lower ring to support a 250-mL conical flask, and place a 4-inch watch glass with a center hole on the upper ring to deflect heat. Attach a 25-mL buret to the ring stand so that the tip just passes through the watch glass centered above the flask. Place an indirectly lighted white surface behind the assembly for observing the endpoint.

Standard solution: 6 mg/mL of USP Dextrose RS

Sample solution: Transfer 4 g of Corn Syrup Solids to a 500-mL volumetric flask, and dilute with water to volume.

Analysis: Transfer 25.0-mL portions of alkaline cupric tartrate TS to each of two flasks, and boil. Immediately place one flask on the wire gauze of the *Apparatus*, and adjust the burner so that the boiling point will be reached in 2 min. Titrate with the *Standard solution* to within 0.5 mL of the anticipated endpoint. Heat the flask with swirling, boil moderately for 2 min, and add 2 drops of a 10-mg/mL methylene blue solution. Immediately add 2 drops of the *Standard solution* from the buret, and bring to a boil. Allow the cuprous oxide to settle slightly, and observe the color of the supernatant. Complete the titration within 1 min by adding the *Standard solution* dropwise, and boiling after each addition to the disappearance of the blue color, as determined by viewing against a white background in daylight or under equivalent illumination. If more than 0.5 mL of the titrant is required after the addition of the indicator, repeat the titration, adding the necessary volume of titrant before adding the indicator. Bring the contents of the second flask to a boil, and similarly titrate with the *Sample solution*.

Calculate the percentage of reducing sugars as D-glucose, calculated on the dried basis, in the portion of Corn Syrup Solids taken:

$$\text{Result} = (C_S/C_U) \times (V_S/V_U) \times [1/(0.01 \times A)] \times 100$$

- C_S = concentration of USP Dextrose RS in the *Standard solution* (mg/mL)
 C_U = concentration of Corn Syrup Solids taken to prepare the *Sample solution* (mg/mL)
 V_S = titrated volumes of the *Standard solution* (mL)

- V_U = titrated volumes of the *Sample solution* (mL)
 A = percentage of dry solids in Corn Syrup Solids, as determined in the test for *Total Solids*

Acceptance criteria: NLT 20.0% reducing sugar content (dextrose equivalent) expressed as D-glucose on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.5%
- **HEAVY METALS**, *Method II* (231): NMT 5 µg/g, using an ignition temperature of 500°

• LIMIT OF SULFUR DIOXIDE

Starch indicator solution: Mix 10 g of soluble starch with 50 mL of cold water. Transfer to 1000 mL of boiling water, and stir until completely dissolved. Cool, and add 1 g of salicylic acid preservative. [NOTE—Discard the solution after 1 month.]

Sample: 78 g

Blank: 200 mL of water

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.005 N iodine VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 250-mL conical flask. Dilute with 122 mL of water, and mix to dissolve. Cool to 5°–10°. While stirring with a magnetic stirrer, add 10 mL of cold 1.5 N sodium hydroxide (5°–10°). Stir for an additional 20 s, and add 10 mL of *Starch indicator solution*. Add 10 mL of cold 2.0 N sulfuric acid (5°–10°), and titrate immediately with *Titrant* until a light blue color persists for 1 min. Perform a blank determination, and make any necessary correction.

Calculate the amount of sulfur dioxide (SO₂) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F_1] \times F_2\} / W$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F_1 = equivalency factor, 32.0 mg/mEq

F_2 = conversion factor, 10³ µg/mg

W = *Sample* weight (g)

Acceptance criteria: NMT 40 µg/g

• LIMIT OF LEAD

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. For digestion, use acid-cleaned, high-density polyethylene, polypropylene, polytetrafluoroethylene, or quartz tubes. Select all reagents to have as low a content of lead as practicable, and store all reagent solutions in borosilicate glass containers. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min and rinsing with deionized water. Store final diluted solutions in acid-cleaned plastic or polytetrafluoroethylene tubes or bottles.]

Modifier solution: 200 mg/mL of magnesium nitrate. Just before use, transfer 1.0 mL of this solution to a 10-mL volumetric flask, and dilute with 5% nitric acid to volume.

Standard solutions: Transfer 10.0 mL of *Lead Nitrate Stock Solution*, prepared as directed in *Heavy Metals* (231), to a 100-mL volumetric flask. Add 40 mL of water and 5 mL of nitric acid, dilute with water to volume, and mix. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, and dilute with 5% nitric acid to volume. This solution contains 0.1 µg/mL of lead. Transfer portions of this solution to four suitable containers, and dilute with 5% nitric acid to obtain *Standard solutions* having lead concentrations of 100, 50, 25, and 10 µg/mL.

Sample solution

[NOTE—Perform this procedure in a fume hood.]

Transfer 1.2 g of Corn Syrup Solids to two digestion tubes labeled sample solution and temperature monitor solution, and add 0.75 mL of nitric acid to each tube. Warm both solutions slowly to 90°–95° to avoid spattering. Heat until all brown vapors have dissipated and any rust-colored tint is gone from the tube labeled *Sample solution* (20–30 min). Cool, then add 0.5 mL of 50% hydrogen peroxide dropwise to both solutions, and heat to between 90° and 95° for 5 min. Cool, then add a second 0.5-mL portion of 50% hydrogen peroxide dropwise to each solution, and heat to 90°–100° until clear (5–10 min). Cool, and transfer the solution labeled *Sample solution* to a 10-mL volumetric flask. Rinse the *Sample solution* digestion tube with 5% nitric acid, add the rinse to the volumetric flask, and dilute with 5% nitric acid to volume.

Standard blank: 5% nitric acid

Sample blank: Transfer 1.5 g of water to a digestion tube, and proceed as directed for the *Sample solution*, beginning with “add 0.75 mL of nitric acid”.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with pyrolytically coated graphite tubes and adequate means of background correction

Analytical wavelength: 283.3 nm

Injection volume: 20 µL

Temperature: Maintain the drying temperature of the furnace at 200° for 30 s after a 20-s ramp time using an argon gas flow of 300 mL/min. Maintain the ashing temperature at 750° for 40 s after a 40-s ramp time using an airflow of about 300 mL/min. Cool down, and purge the air from the furnace for 60 s using a 20° set temperature and an argon gas flow of about 300 mL/min. Maintain the atomization temperature at 1800° for 10 s after a 0-s ramp time with the argon gas flow stopped. [NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Analysis

Samples: *Standard solutions*, *Sample solution*, *Standard blank*, *Sample blank*

Add 5 µL of the *Modifier solution* to 20 µL each of the *Standard solutions*, the *Sample solution*, the *Standard blank*, and the *Sample blank*, and mix. Separately inject each into the atomic absorption spectrophotometer.

Using the *Standard blank* to set the instrument to zero, determine the integrated absorbances of the *Standard solutions*. Plot the integrated absorbances of the *Standard solutions* versus their contents of lead, and draw the line best fitting the four points to determine the calibration curve. Similarly, determine the integrated absorbances of the *Sample solution* and the *Sample blank*. Correct the absorbance value of the *Sample solution* with the *Sample blank*.

Calculate the concentration of lead in the portion of Corn Syrup Solids taken:

$$\text{Result} = (V \times C/W) \times F$$

V = volume of the *Sample solution*, 10 mL

C = concentration of lead in the *Sample solution*, as determined from the calibration curve (ng/mL)

W = weight of Corn Syrup Solids taken to prepare the *Sample solution* (g)

F = conversion factor, 10⁻³ µg/ng

Acceptance criteria: NMT 0.5 µg/g

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 10³ cfu/g, and the total

combined yeasts and molds count does not exceed 10² cfu/g.

- **STARCH**

Sample solution: 100 mg/mL

Analysis: Add 1 drop of iodine TS to 10 mL of the *Sample solution*.

Acceptance criteria: A yellow color indicates the absence of soluble starch.

- **TOTAL SOLIDS**

Analysis: To determine the water content, proceed as directed in *Water Determination* (921), *Method 1a*, except use a weighed amount of Corn Syrup Solids, W_U , for the *Test Preparation*. In *Standardization of the Reagent*, proceed as directed, except use the formula for significant amounts of water (1% or more). [NOTE—Pure methanol can make the detector overly sensitive, particularly at low ppm levels of water, causing it to deflect to dryness and slowly recover with each addition of reagent. This slows down the titration and may allow the system to actually pick up ambient moisture during the resulting long titration. Adding chloroform or a similar nonconducting solvent will retard this sensitivity and can improve the analysis.] In the *Procedure* calculating the water content in the *Test Preparation*, use $W_W = S \times F$.

Calculate the percentage of total solids in the portion of the *Test Preparation* taken:

$$\text{Result} = (W_U - W_W)/W_U \times 100$$

W_U = weight of Corn Syrup Solids for the *Test Preparation* (mg)

W_W = weight of water determined (mg)

Acceptance criteria: NLT 90.0% when the reducing sugar content is NLT 88.0%; NLT 93.0% when the reducing sugar content is 20.0%–88.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tightly closed containers, and store in a cool, dry place.
- **LABELING:** Label it to indicate its nominal dextrose equivalent. Label it also to indicate the presence of sulfur dioxide if the residual concentration is greater than 10 µg/g.
- **USP REFERENCE STANDARDS** (11)
USP Dextrose RS

Cottonseed Oil

[8001-29-4].

DEFINITION

Cottonseed Oil is the refined fixed oil obtained from the seed of cultivated plants of various varieties of *Gossypium hirsutum* Linné or of other species of *Gossypium* (Fam. Malvaceae). It may contain suitable antioxidants.

IDENTIFICATION

- **A.** Cottonseed Oil exhibits the composition profile of fatty acids, as determined in the general test chapter *Fats and Fixed Oils* (401), *Fatty Acid Composition*. See *Table 1*.

Table 1

Carbon-Chain Length	No. of Double Bonds	Percentage (%)
<14	0 or 1	≤0.2
14	0	0.3–1.0
16	0	18.0–26.4
16	1	≤1.2
18	0	2.1–3.3

Table 1 (Continued)

Carbon-Chain Length	No. of Double Bonds	Percentage (%)
18	1	14.0–21.7
18	2	46.7–58.3
18	3	≤1.0
20	0	≤1.0
20	1	≤0.5
22	0	≤0.6
22	1	≤0.5
24	0	≤0.5

IMPURITIES• **ALKALINE IMPURITIES**

Sample: 10 mL of Cottonseed Oil

Analysis: Mix 10 mL of acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. If necessary, neutralize the solution to a green color with 0.01 N hydrochloric acid or 0.01 N sodium hydroxide. Add the *Sample*, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS to change the color of the upper layer to yellow.

Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid is required.

• **HEAVY METALS, Method II <231>:** NMT 10 ppm**SPECIFIC TESTS**

- **FATS AND FIXED OILS, Acid Value <401>:** NMT 0.2
- **FATS AND FIXED OILS, Peroxide Value <401>:** NMT 10.0
- **FATS AND FIXED OILS, Unsaponifiable Matter <401>:** NMT 1.5%
- **WATER DETERMINATION, Method Ic <921>:** NMT 0.1%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.
- **LABELING:** Label it to indicate the name and quantity of any added antioxidants. Where Cottonseed Oil is intended for use in the manufacture of injectable dosage forms, it is so labeled.
- **OTHER REQUIREMENTS:** For Cottonseed Oil intended for use in injectable dosage forms, which is specified in the *Labeling* section, the requirements in the subsection *Other Vehicles* in the section *Ingredients under Injections* <1> must be met.

Hydrogenated Cottonseed Oil**DEFINITION**

Hydrogenated Cottonseed Oil is the product obtained by refining and hydrogenating oil obtained from seeds of cultivated plants of various varieties of *Gossypium hirsutum* L. or of other species of *Gossypium* (Fam. Malvaceae). The product consists mainly of triglycerides of palmitic and stearic acids.

IDENTIFICATION

- **A.** It meets the requirements in *Specific Tests for Fats and Fixed Oils, Fatty Acid Composition* <401>.
- **B.** It meets the requirements in *Specific Tests for Melting Range or Temperature, Class II* <741>.

IMPURITIES• **LIMIT OF NICKEL**

Solution A: Immediately before use, dilute 10 mL of nickel standard solution TS with water to 500 mL. This solution contains the equivalent of 0.2 µg/g of nickel.

Sample solution: Weigh 5.0 g of Hydrogenated Cottonseed Oil into a previously tared platinum or silica crucible. Cautiously heat, and introduce into the sub-

stance a wick formed from twisted ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at 600°. Continue the incineration until white ash is obtained. After cooling, transfer the residue, with the aid of two 2-mL portions of diluted hydrochloric acid, to a 25-mL volumetric flask, add 0.3 mL of nitric acid, and dilute with water to volume.

Standard solutions: Into three identical 10-mL volumetric flasks, introduce respectively 1.0, 2.0, and 4.0 mL of *Solution A*. To each flask add a 2.0-mL portion of the *Sample solution*, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption spectrophotometry, equipped with a graphite furnace

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Analysis: Concomitantly determine the absorbances of the *Sample solution* and each of the *Standard solutions* at least three times each, and record the average of the steady readings. Plot the absorbances of the *Sample solution* and the *Standard solutions* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.

Acceptance criteria: NMT 1 µg/g

• **ALKALINE IMPURITIES**

Sample solution: Dissolve by gentle heating 2.0 g of Hydrogenated Cottonseed Oil in a mixture of 1.5 mL of alcohol and 3.0 mL of toluene.

Analysis: To the *Sample solution* add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid to a yellow endpoint.

Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

- **FATS AND FIXED OILS, Fatty Acid Composition <401>:** Hydrogenated Cottonseed Oil exhibits the composition profile of fatty acids in *Table 1*, as determined in the chapter.

Table 1

Carbon-Chain Length	No. of Double Bonds	Percentage (%)
<14	0	≤0.2
14	0	≤1.0
16	0	19–26
18	0	68–80
20	0	≤1.0
22	0	≤1.0
24	0	≤0.5
18	1	≤4.0
18	2	≤1.0

- **MELTING RANGE OR TEMPERATURE, Class II <741>:** 57°–70°

- **FATS AND FIXED OILS, Acid Value (Free Fatty Acids) <401>**

Sample: 10 g

Analysis: Dissolve the *Sample* in 50 mL of a hot mixture of neutralized alcohol and toluene (1:1), add 0.5 mL of phenolphthalein TS, and titrate with 0.1 N potassium hydroxide VS to produce a permanent, faint pink color.

Acceptance criteria: NMT 0.5

- **FATS AND FIXED OILS, Peroxide Value <401>:** NMT 5.0

- **FATS AND FIXED OILS, Unsaponifiable Matter <401>:** NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirements are specified.

Creatinine

C₄H₇N₃O

113.12

DEFINITION

Creatinine contains NLT 98.5% and NMT 102.0% of C₄H₇N₃O, as Creatinine, calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)

ASSAY

• PROCEDURE

Sample: 40 mg of Creatinine

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric

Indicator electrode: Glass

Reference electrode: Silver–silver chloride

Reference electrode solution: Saturated lithium perchlorate and silver chloride in glacial acetic acid

Analysis: Dissolve the *Sample* in 10 mL of glacial acetic acid. Titrate the *Sample* with 0.1 N perchloric acid VS. Perform a blank determination, and make any necessary correction.

Calculate the percentage of creatinine (C₄H₇N₃O) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100] / W$$

V = volume of the *Titrant* consumed by the *Sample* (mL)

B = volume of the *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 113.12 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 98.5%–102.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.2%
- **HEAVY METALS**, *Method I* (231): 10 ppm

SPECIFIC TESTS

- **LOSS ON DRYING** (731): Dry a sample at 105° for 3 h: it loses NMT 3.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** (11)
USP Creatinine RS

Cresol

C₇H₈O

Phenol, methyl-;
Cresol [1319-77-3].

108.14

DEFINITION

Cresol is a mixture of isomeric cresols obtained from coal tar or from petroleum.

IDENTIFICATION

• A.

Sample solution: A saturated solution

Analysis: To the *Sample solution* add a few drops of ferric chloride TS.

Acceptance criteria: A bluish-violet color is produced.

IMPURITIES

• LIMIT OF PHENOL

Solution A: Bubble air through nitric acid until the acid is colorless, then mix 1 volume of the acid with 4 volumes of water.

Standard solution: Dissolve 1 g of phenol in 100 mL of water, and determine the actual C₆H₆O concentration as follows. Pipet 4 mL of the solution into an iodine flask, add 30.0 mL of 0.1 N bromine VS, then add 5 mL of hydrochloric acid, and immediately insert the stopper. Shake the flask repeatedly for 30 min, and allow to stand for 15 min. Add quickly 5 mL of a 200-mg/mL potassium iodide solution, taking precautions to prevent the escape of bromine vapor, and at once insert the stopper into the flask. Shake thoroughly, remove the stopper, and rinse it and the neck of the flask with a small quantity of water so that the washings flow into the flask. Add 1 mL of chloroform, shake the mixture, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached. Perform a blank determination. Each mL of 0.1 N bromine is equivalent to 1.569 mg of C₆H₆O. Dilute a suitable volume of the solution with water to obtain a concentration of 250 µg/mL of C₆H₆O.

Sample solution: Place 2.5 g of Cresol in a 250-mL volumetric flask, add 10 mL of sodium hydroxide solution (100 mg/mL), and dilute with water to volume. Pipet 5 mL of this solution into a 200-mL volumetric flask, add 45 mL of water and 1 drop of methyl orange TS, neutralize with *Solution A* added dropwise, and then dilute with water to volume.

Analysis: Pipet 5.0 mL of the neutralized *Sample solution* into each of two 20- × 180-mm test tubes, graduated at the 25-mL mark, and pipet 5.0 mL of the *Standard solution* into each of two similar test tubes. To the contents of each tube add 5 mL of Millon's Reagent, allowing it to flow down the inner wall of the tube. Place the tubes simultaneously in a boiling water bath provided with a rack so that the tubes do not touch the bottom of the bath, and maintain the bath at boiling temperature for 30 min, accurately timed. At once remove the tubes from the bath, cool them immediately and thoroughly by placing them in a bath of cold water for NLT 10 min, and add 5 mL of *Solution A* to each tube. Add 3 mL of a 2% formaldehyde solution to one of each pair of tubes, add water to fill all tubes to volume, shake thoroughly, and allow to stand for 16 h, during which time the added formaldehyde imparts a yellow color while the contents of the other two tubes acquire an orange-red color.

Pipet 20 mL from each of the two tubes containing the *Standard solution* into separate 100-mL volumetric flasks, add 5 mL of *Solution A*, and then add water to volume. Transfer the solutions to burets marked *B1* and *B2*, representing, respectively, the solution not treated and the solution treated with formaldehyde.

Pipet 10 mL from each of the two tubes containing the *Sample solution* into separate 50-mL color-comparison tubes marked *N1* and *N2*, representing, respectively, the solution treated with formaldehyde and the solution not treated with formaldehyde.

Add to tube *N1* the orange-red colored solution from buret *B1*, and add to tube *N2* an equal volume of the yellow-colored solution from buret *B2*, until the colors in tubes *N1* and *N2* match when observed in a colorimeter.

Calculate the percentage of phenol (C_6H_6O) in the portion of the sample taken:

$$\text{Result} = V/W \times 5$$

V = volume of the *Standard solution* taken from buret *B1* (mL)

W = weight of Cresol taken (g)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): 1.030–1.038
- **DISTILLING RANGE, Method II** (721): NLT 90% distills between 195° and 205°.
- **HYDROCARBONS**

Sample solution: 1 in 60

Standard solution: To 58 mL of water add 1.5 mL of 0.02 N sulfuric acid and 1 mL of barium chloride solution (100 mg/mL).

Analysis: Compare the turbidity of the *Sample solution* against the *Standard solution* after the *Standard solution* has been shaken and allowed to stand for 5 min.

Acceptance criteria: The *Sample solution* shows no more turbidity than the *Standard solution*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Croscarmellose Sodium

DEFINITION

Croscarmellose Sodium is the sodium salt of a cross-linked, partly *O*-(carboxymethylated) cellulose.

IDENTIFICATION

- **A.** Mix 1 g with 100 mL of methylene blue solution (1 in 250,000), stir the mixture, and allow it to settle. The Croscarmellose Sodium absorbs the methylene blue and settles as a blue, fibrous mass.
- **B.** Mix 1 g with 50 mL of water. Transfer 1 mL of the mixture to a small test tube, and add 1 mL of water and 5 drops of 1-naphthol TS. Incline the test tube, and carefully add 2 mL of sulfuric acid down the side so that it forms a lower layer: a reddish-violet color develops at the interface.
- **C.** A portion of the mixture of Croscarmellose Sodium with water, prepared as directed in *Identification test B*, meets the requirements of the flame test for *Identification Tests—General* (191), *Sodium*.

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): 14.0%–28.0%, calculated on the dried basis. Use 1.0 g for the test, and use sufficient sulfuric acid to moisten the entire residue after the initial charring step, and additional sulfuric acid if an excessive amount of carbonaceous material remains after the initial complete volatilization of white fumes.
- **HEAVY METALS, Method II** (231): 10 ppm
- **SODIUM CHLORIDE** and **SODIUM GLYCOLATE**

Sodium chloride

Sample: 5 g of Croscarmellose Sodium

Analysis: Transfer the *Sample* to a 250-mL beaker.

Add 50 mL of water and 5 mL of 30% hydrogen peroxide, and heat on a steam bath for 20 min, stirring occasionally to ensure hydration. Cool, and add 100 mL of water and 10 mL of nitric acid. Titrate with 0.05 N silver nitrate VS, determining the endpoint potentiometrically, using a silver-based indicator electrode and a double-junction reference electrode containing 10% potassium nitrate filling solution in the outer jacket and a standard filling solution in the in-

ner jacket, and stirring constantly (see *Titrimetry* (541)).

Calculate the percentage of sodium chloride in the specimen taken:

$$\text{Result} = (F \times V \times N)/[(100 - b) \times W]$$

F = equivalence factor for sodium chloride, 584.4

V = volume of the silver nitrate (mL)

N = normality of the silver nitrate

b = percentage of *Loss on Drying*, determined separately

W = weight of the specimen (g)

Sodium glycolate

Sample solution: Transfer 500 mg to a 100-mL beaker. Moisten thoroughly with 5 mL of glacial acetic acid, followed by 5 mL of water, and stir with a glass rod to ensure proper hydration (usually about 15 min). Slowly add 50 mL of acetone while stirring, then add 1 g of sodium chloride, and stir for several min to ensure complete precipitation of the carboxymethylcellulose. Filter through a soft, open-textured paper, previously wetted with a small amount of acetone, and collect the filtrate in a 100-mL volumetric flask. Use an additional 30 mL of acetone to facilitate the transfer of the solids and to wash the filter cake, then dilute with acetone to volume, and mix.

Standard stock solution: Transfer 100 mg of glycolic acid, previously dried in a desiccator at room temperature overnight, to a 100-mL volumetric flask. Dissolve in and dilute with water to volume, and mix.

[NOTE—Use this solution within 30 days.]

Standard solution A: Transfer 1.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, then add 5 mL of glacial acetic acid. Dilute with acetone to volume, and mix.

Standard solution B: Transfer 2.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, then add 5 mL of glacial acetic acid. Dilute with acetone to volume, and mix.

Standard solution C: Transfer 3.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, then add 5 mL of glacial acetic acid. Dilute with acetone to volume, and mix.

Standard solution D: Transfer 4.0 mL of the *Standard stock solution* to a 100-mL volumetric flask. Add water to make 5 mL, then add 5 mL of glacial acetic acid. Dilute with acetone to volume, and mix.

Analysis

Samples: *Sample solution*, *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Standard solution D*

Transfer 2.0 mL of the *Sample solution* and 2.0 mL of each *Standard solution* to separate 25-mL volumetric flasks, and prepare a blank flask containing 2.0 mL of a solution containing 5% each of glacial acetic acid and water in acetone. Place the uncovered flasks in a boiling water bath for 20 min to remove the acetone. Remove from the bath, and cool. Add to each flask 5.0 mL of 2,7-dihydroxynaphthalene TS, mix, add an additional 15 mL, and again mix. Cover the mouth of each flask with a small piece of aluminum foil. Place the flasks upright in a boiling water bath for 20 min, then remove from the bath, cool, dilute with sulfuric acid to volume, and mix.

Determine the absorbance of each solution at 540 nm, with a suitable spectrophotometer, against the blank, and prepare a standard curve using the absorbances obtained from the *Standard solutions*. Calculate the percentage of sodium glycolate in the specimen taken:

$$\text{Result} = (F \times W_1)/[(100 - b) \times W_2]$$

- F = factor converting glycolic acid to sodium glycolate, 12.9
 W_1 = weight of glycolic acid in the specimen (mg), determined from the standard curve and the absorbance of the *Sample solution*
 b = percentage of *Loss on Drying*, determined separately
 W_2 = weight of the specimen taken (g)

Acceptance criteria: The sum of the percentages of sodium chloride and sodium glycolate is NMT 0.5%.

SPECIFIC TESTS

• CONTENT OF WATER-SOLUBLE MATERIAL

Analysis: Disperse 10 g in 800 mL of water, and stir for 1 min every 10 min during the first 30 min. Allow to stand for an additional h, or centrifuge, if necessary. Decant 200 mL of the aqueous slurry onto a rapid-filtering filter paper in a vacuum filtration funnel, apply vacuum, and collect about 150 mL of the filtrate. Pour the filtrate into a tared 250-mL beaker, weigh, and calculate the weight, in g, of the filtrate, W_3 , by difference. Concentrate on a hot plate to a small volume, but not to dryness; dry at 105° for 4 h; again weigh; and calculate the weight, in g, of residue W_1 , by difference.

Calculate the percentage of water-soluble material in the specimen, on the dried basis, taken:

$$\text{Result} = [100 \times W_1 \times (800 + W_2)] / \{W_2 \times W_3 \times [1 - (0.01 \times b)]\}$$

- W_1 = weight of residue by difference (g)
 W_2 = weight of the specimen taken (g)
 W_3 = weight of the filtrate by difference (g)
 b = percentage *Loss on Drying* of the specimen taken

Acceptance criteria: NMT 10.0%

• DEGREE OF SUBSTITUTION

Sample: 1 g

Analysis: Transfer the *Sample* to a glass-stoppered, 500-mL conical flask. Add 300 mL of sodium chloride solution (1 in 10), then add 25.0 mL of 0.1 N sodium hydroxide VS. Insert the stopper, and allow to stand for 5 min with intermittent shaking. Add 5 drops of *m*-cresol purple TS, and from a buret add 15 mL of 0.1 N hydrochloric acid VS. Insert the stopper in the flask, and shake. If the solution is violet, add 0.1 N hydrochloric acid VS in 1-mL portions until the solution becomes yellow, shaking after each addition. Titrate with 0.1 N sodium hydroxide VS to a violet endpoint. Calculate the net number of milliequivalents, M, of base required for the neutralization of 1 g of Croscarmellose Sodium, on the dried basis.

Calculate the degree of acid carboxymethyl substitution, A:

$$\text{Result} = 1150 \times M / [7102 - (412 \times M) - (80 \times C)]$$

- M = milliequivalents
 C = percentage of *Residue on Ignition* of the Croscarmellose Sodium as determined in the test for *Residue on Ignition*

Calculate the degree of sodium carboxymethyl substitution, S:

$$\text{Result} = [162 + (58 \times A)] \times C / [7102 - (80 \times C)]$$

- A = degree of acid carboxymethyl substitution, as determined above
 C = percentage of *Residue on Ignition* of the Croscarmellose Sodium as determined in the test for *Residue on Ignition*

The degree of substitution is the sum of A + S.

Acceptance criteria: The degree of substitution is 0.60–0.85, on the dried basis

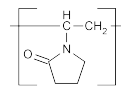
- **LOSS ON DRYING** (731): Dry a sample at 105° for 6 h: it loses NMT 10.0% of its weight.
- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g. It meets the requirements of the tests for absence of *Escherichia coli*.
- **pH** (791): The pH of the dispersion is 5.0–7.0. Mix 1 g with 100 mL of water for 5 min.
- **SETTLING VOLUME**
Analysis: To 75 mL of water in a 100-mL graduated cylinder, add 1.5 g of it in 0.5-g portions, shaking vigorously after each addition. Add water to make 100 mL, shake again until all of the powder is homogeneously distributed, and allow to stand for 4 h. Note the volume of the settled mass.
Acceptance criteria: The volume of the settled mass is 10.0–30.0 mL

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Crospovidone

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (♦) to specify this fact.



(C_6H_9NO)_n
 1-Ethenyl-2-pyrrolidinone homopolymer;
 1-Vinyl-2-pyrrolidinone homopolymer [9003-39-8].

DEFINITION

Crospovidone is a water-insoluble synthetic cross-linked homopolymer of *N*-vinyl-2-pyrrolidinone. It contains NLT 11.0% and NMT 12.8% of nitrogen (N), calculated on the dried basis. Two types of Crospovidone are available, depending on the particle size: Type A and Type B.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K): Previously dried in a vacuum at 105° for 1 h.♦
- **B.**
Sample: 1 g
Analysis: Suspend the *Sample* in 10 mL of water, add 0.1 mL of 0.1 N iodine, and shake for 30 s. Add 1 mL of starch TS, and shake.
Acceptance criteria: No blue color develops.
- **C.** To 10 mL of water add 0.1 g and shake. A suspension is formed, and no clear solution is obtained within 15 min.
- **D.**
Sample: 20 g of the dried substance
Analysis: Clean and dry the analytical sieves used in the analysis by washing the sieves in hot water. Allow to dry overnight in a drying cabinet at 105°. Place the *Sample* in a 1000-mL conical flask, add 500 mL of water, and shake the suspension for 30 min. Pour the suspension through a 63-μm analytical sieve, previously tared, and rinse the sieve with water until the filtrate is clear. Dry the sieve and sample residue at 105° for 5 h in a drying cabinet without circulating air. Cool in a desiccator for 30 min, and weigh.

Calculate the percentage sieving residue fraction of sample particles having a diameter of more than 63 μm :

$$\text{Result} = [(m_1 - m_3) \times 100] / m_2$$

m_1 = mass of the sieve and sample residue, after drying for 5 h (g)

m_3 = mass of the sieve (g)

m_2 = initial mass of the sample, calculated on a dried basis (g)

Acceptance criteria: If the sieving residue fraction is more than 15%, the substance is classified as Type A; if the sieving residue fraction is NMT 15%, the substance is classified as Type B.

ASSAY

• NITROGEN DETERMINATION, Method II (461)

Sample: 0.1 g

Analysis: Proceed as directed, using the *Sample*. In the *Procedure*, omit the use of hydrogen peroxide, and use 5 g of a powdered mixture of potassium sulfate, cupric sulfate, and titanium dioxide (33:1:1), instead of potassium sulfate and cupric sulfate (10:1). Heat until a clear, light green solution is obtained. Heat for an additional 45 min, and proceed as directed for *Procedure*, beginning with "Cautiously add to the digestion mixture 70 mL of water".

Acceptance criteria: 11.0%–12.8% on the dried basis

IMPURITIES

• RESIDUE ON IGNITION (281): NMT 0.1%, determined on 1.0 g

• HEAVY METALS, Method II (231): NMT 10 ppm

• PEROXIDES

Sample suspension A: [NOTE—Use for Type A.] 40 mg/mL in water. To 25 mL of this suspension add 2 mL of titanium trichloride-sulfuric acid TS. Allow to stand for 30 min, and filter.

Sample suspension B: [NOTE—Use for Type B.] 16 mg/mL in water. To 25 mL of this suspension add 2 mL of titanium trichloride-sulfuric acid TS. Allow to stand for 30 min, and filter.

Compensation liquid A: [NOTE—Use for Type A.] 40 mg/mL in water. Filter, take 25 mL, and add 2 mL of a 13% solution of sulfuric acid.

Compensation liquid B: [NOTE—Use for Type B.] 16 mg/mL in water. Filter, take 25 mL, and add 2 mL of a 13% solution of sulfuric acid.

Analysis: Measure the absorbance of the filtrate at 405 nm against the appropriate compensation liquid.

Acceptance criteria: NMT 0.35. For Type A, this corresponds to NMT 400 ppm expressed as H_2O_2 ; for Type B, this corresponds to NMT 1000 ppm expressed as H_2O_2 .

• VINYLPIRROLIDINONE

Mobile phase: Acetonitrile and water (1:9)

Sample solution: 25 mg/mL of suspension in methanol. Shake for 60 min. Leave the bulk to settle, and pass through a filter of 0.2- μm pore size.

Reference stock solution A: 5 μg /mL of vinylpyrrolidinone in methanol

Reference stock solution B: 100 μg /mL of vinylpyrrolidinone and 5 mg/mL of vinyl acetate in methanol

Reference solution A: A 1-in-20 solution of *Reference stock solution A* in *Mobile phase*

Reference solution B: A 1-in-100 solution of *Reference stock solution B* in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV

Analytical wavelength: 235 nm

Precolumn: 4-mm \times 2.5-cm; 5- μm packing L1

Column: 4-mm \times 25-cm; 5- μm packing L1

Column temperature: 40°

Flow rate: 1 mL/min

Injection size: 50 μL . [NOTE—After each injection of the *Sample solution*, wash the precolumn by passing the *Mobile phase* backwards, at the same flow rate as applied in the test, for 30 min.]

System suitability

Samples: *Reference solution A* and *Reference solution B*

Suitability requirements

Resolution: NLT 2.0 between vinylpyrrolidinone and vinyl acetate, *Reference solution B*

Relative standard deviation: NMT 2.0% for 6 injections, *Reference solution A*

Analysis

Samples: *Sample solution* and *Reference solution A*

Record the chromatograms, and measure the responses for the vinylpyrrolidinone peak.

Acceptance criteria: The area of the peak from the *Sample solution* is NMT the area of the principal peak from *Reference solution A* (NMT 10 ppm).

SPECIFIC TESTS

• LOSS ON DRYING (731): Dry 0.5 g at 105° to constant weight: it loses NMT 5.0% of its weight.

• WATER-SOLUBLE SUBSTANCES

Sample: 25.0 g

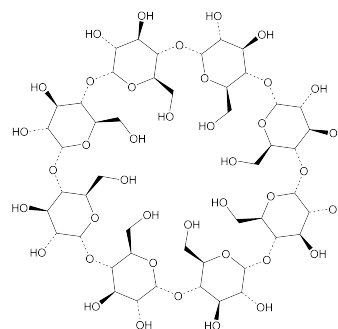
Analysis: Transfer the *Sample* to a 400-mL beaker, add 200 mL of water, and stir on a magnetic stirrer, using a 5-cm stirring bar, for 1 h. Transfer to a 250-mL volumetric flask with the aid of 25 mL of water. Add water to volume. Allow the bulk of the solids to settle. Pass 100 mL of the relatively clear supernatant through a membrane filter of 0.45- μm pore size, protected against clogging by superimposing a membrane filter of 3- μm pore size. While filtering, stir the solution above the filter manually or with a mechanical stirrer, taking care not to physically damage the membrane filter. Transfer 50.0 mL of the clear filtrate to a tared 100-mL beaker, evaporate to dryness, and dry at 110° for 3 h.

Acceptance criteria: The weight of the residue does not exceed 75 mg (1.5%).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** The label states the type (Type A or Type B).
- **USP REFERENCE STANDARDS (11)**
USP Crospovidone RS.

Gamma Cyclodextrin



($\text{C}_6\text{H}_{10}\text{O}_5$)₈
Cyclooctaamylose;
Cyclomaltooctaoase [17465-86-0].

1297.12

DEFINITION

Gamma Cyclodextrin is composed of 8 alpha-(1–4) linked D-glycopyranosyl units. It contains NLT 98.0% and NMT 102.0% of cyclooctaamylose ($C_6H_{10}O_5$)₈, calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B.** The retention time of the major peak from the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- **C.** It meets the requirements of the test for *Specific Rotation*.

ASSAY**PROCEDURE**

Mobile phase: Methanol and water (7:93)

System suitability solution: Prepare an aqueous solution containing 0.5 mg/mL each of USP Alpha Cyclodextrin RS, USP Beta Cyclodextrin RS, and USP Gamma Cyclodextrin RS.

Standard solution: 1.0 mg/mL of USP Gamma Cyclodextrin RS

Sample stock solution: Transfer 250 mg of Gamma Cyclodextrin to a 25-mL volumetric flask, and dissolve in water, with the aid of heat if necessary. Cool, and dilute with water to volume.

Sample solution: 1.0 mg/mL of Gamma Cyclodextrin, prepared from the *Sample stock solution*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 4.6-mm × 15-cm; 5-μm packing L1

Temperature

Detector: 40°

Column: 30°

Flow rate: 1.5 mL/min

Injection size: 50 μL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for gamma cyclodextrin, alfadex, and betadex are 0.8, 1.0, and 1.9, respectively.]

Suitability requirements

Resolution: NLT 1.5 between the gamma cyclodextrin and alfadex peaks

Tailing factors: 0.8–2.0 for the three cyclodextrins

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of gamma cyclodextrin [($C_6H_{10}O_5$)₈] in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of the *Standard solution* (mg/mL)

C_U = concentration of the *Sample solution* (mg/mL) corrected for water found in *Specific Tests*, *Loss on Drying*

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%, determined on a 1.0-g specimen

- **HEAVY METALS**, *Method II* (231): NMT 5 ppm

RELATED COMPOUNDS

Mobile phase, System suitability solution, and Chromatographic system: Proceed as directed in the *Assay*.

Standard solution: Transfer 5.0 mL of *System suitability solution* into a 50-mL volumetric flask, and dilute with water to volume.

Sample solution: Use the *Sample stock solution*, prepared as directed in the *Assay*.

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria: For the *Sample solution*, the areas of any peaks corresponding to alfadex (alpha cyclodextrin) or to betadex (beta cyclodextrin) are not greater than the area of the corresponding peaks in the chromatogram of the *Standard solution* (0.5%); and the sum of the areas of all the peaks, excluding the principal peak, the peaks corresponding to alfadex or to betadex, and artifact peaks, is not greater than the area of the peak corresponding to gamma cyclodextrin in the chromatogram of the *Standard solution* (0.5%).

REDUCING SUBSTANCES

Dextrose standard solution: 10.0 mg/mL of USP Dextrose RS, calculated on the anhydrous basis

Analysis: Transfer a quantity of Gamma Cyclodextrin, equivalent to 1.0 g on the dried basis, to a 500-mL conical flask. Dissolve in 10 mL of water, and add 25 mL of alkaline cupric citrate TS2. Cover the flask with aluminum foil, and boil the solution for 5 min. Cool in an ice bath to room temperature. Add 25 mL of 0.6 N acetic acid, 10 mL of 3 N hydrochloric acid, and 10 mL of 0.1 N iodine solution. [NOTE—The addition of these solutions must be in the order given.]

Titrate the solution with 0.1 N sodium thiosulfate VS, and determine the endpoint potentiometrically. Perform a blank determination (see *Titrimetry* (541), *Residual Titrations*). Calculate the difference in volumes required. Create a calibration curve by similarly titrating 0.25, 0.5, 0.75, and 1.0 mL of *Dextrose standard solution*. Plot the amount, in mg, of dextrose in each titrated *Dextrose standard solution* versus the volume consumed, in mL, of 0.1 N sodium thiosulfate VS in the titration, and draw a straight line through the four points. From the line so obtained and the volume of 0.1 N sodium thiosulfate VS required in the titration of Gamma Cyclodextrin, determine the weight, W , in mg, of the reducing substances as dextrose in the portion of Gamma Cyclodextrin taken.

Calculate the percentage of the reducing substances in the portion of Gamma Cyclodextrin taken:

$$\text{Result} = (W/W_C) \times F \times 100$$

W = weight of the reducing substances as dextrose in the portion of Gamma Cyclodextrin taken (mg)

W_C = weight of Gamma Cyclodextrin taken (g)

F = conversion factor, 10^{-3} g/mg

Acceptance criteria: NMT 0.5%

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g.

COLOR AND CLARITY OF SOLUTION

Sample solution: Transfer a quantity of Gamma Cyclodextrin, equivalent to 2.5 g on the dried basis, into a 25-mL volumetric flask, dissolve in and dilute with water that has been previously boiled and cooled to room temperature to volume, and mix.

Analysis: Determine the absorbance of the *Sample solution* in a 1-cm cell at 420 nm, with a suitable spectrophotometer, after correcting for the blank.

Acceptance criteria: At 420 nm, the absorbance is not greater than 0.20, and the solution is clear.

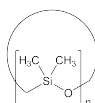
- **LOSS ON DRYING** (731): Dry a sample at 105° for 2 h: it loses NMT 11.0% of its weight.

- **OPTICAL ROTATION, Specific Rotation (781S)**
Sample solution: 10 mg/mL
Analysis: Proceed as directed in the chapter.
Acceptance criteria: +174° to +180°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.
- **USP REFERENCE STANDARDS (11)**
 USP Alpha Cyclodextrin RS
 USP Beta Cyclodextrin RS
 USP Dextrose RS
 USP Gamma Cyclodextrin RS

Cyclomethicone



(C₂H₆OSi)_n
 Cyclopolydimethylsiloxane;
 Cyclomethicone [69430-24-6].

DEFINITION

Cyclomethicone is a fully methylated cyclic siloxane containing repeating units of [-(CH₃)₂SiO-]_n in which *n* is 4, 5, or 6, or a mixture of them. It contains NLT 98.0% of (C₂H₆OSi)_n, calculated as the sum of cyclomethicone 4, cyclomethicone 5, and cyclomethicone 6, and NLT 95.0% and NMT 105.0% of the labeled amount of any one or more of the individual cyclomethicone components.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197S)**
Sample: Use neat liquids.
Acceptance criteria: The IR absorption spectrum exhibits maxima only at the same wavelengths as that of a similar preparation of USP Cyclomethicone 4 RS, USP Cyclomethicone 5 RS, or USP Cyclomethicone 6 RS.

ASSAY

- **PROCEDURE**
Standard solution A: USP Cyclomethicone 4 RS
Standard solution B: USP Cyclomethicone 5 RS
Standard solution C: USP Cyclomethicone 6 RS
Sample solution: Cyclomethicone
Chromatographic system
 (See *Chromatography* (621), *System Suitability*).
Mode: GC
Detector: Thermal conductivity
Column: 3.66-m × 3-mm; packed with 20% liquid phase G1 on 60- to 80-mesh packing S1A
Temperatures
Injection port: 300°
Detector: 350°
Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
125	8	320	—

Carrier gas: Helium
Flow rate: 20 mL/min
Injection volume: 1 µL

Analysis

Samples: Standard solution A, Standard solution B, Standard solution C, and Sample solution
 Calculate the percentage of cyclomethicone 4, cyclomethicone 5, and cyclomethicone 6 by dividing 100 times the response of each peak at the retention time of the corresponding reference standard by the sum of all of the responses in the chromatogram. The percentages obtained from duplicate injections agree to within 1.0%.

Calculate the percentage purity by adding the percentages of cyclomethicone 4, cyclomethicone 5, and cyclomethicone 6.

Acceptance criteria

Sum of cyclomethicone 4, cyclomethicone 5, and cyclomethicone 6: NLT 98.0% of (C₂H₆OSi)_n
Labeled amount: 95.0%–105.0% of the labeled amount of any one or more of the individual cyclomethicone components

IMPURITIES

- **LIMIT OF NONVOLATILE RESIDUE**

Sample: 2.0 g

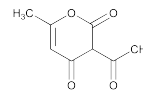
Analysis: Transfer the *Sample* into an open, tared aluminum dish, and evaporate in a circulating air oven at 150° for 2 h. Allow to cool in a desiccator, and weigh.

Acceptance criteria: NMT 3.0 mg, corresponding to NMT 0.15% (w/w)

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to state, as part of the official title, the *n*-value of the Cyclomethicone. Where it is a mixture of 2 or 3 such cyclic siloxanes, the label states the *n*-value and percentage of each in the mixture.
- **USP REFERENCE STANDARDS (11)**
 USP Cyclomethicone 4 RS
 USP Cyclomethicone 5 RS
 USP Cyclomethicone 6 RS

Dehydroacetic Acid



C₈H₈O₄ 168.15
 Keto form: 2*H*-Pyran-2,4(3*H*)-dione, 3-acetyl-6-methyl-; 3-Acetyl-6-methyl-2*H*-pyran-2,4(3*H*)-dione [520-45-6].
 Enol form: 2*H*-Pyran-2-one, 3-acetyl-4-hydroxy-6-methyl-; 3-Acetyl-4-hydroxy-6-methyl-2*H*-pyran-2-one [771-03-9].

DEFINITION

Dehydroacetic Acid contains NLT 98.0% and NMT 100.5% of dehydroacetic acid (C₈H₈O₄), calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K)**

ASSAY

- **PROCEDURE**
Sample: 500 mg
Blank: 75 mL of neutralized alcohol
Titrimetric system
 (See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* into a 250-mL conical flask, dissolve it in 75 mL of neutralized alcohol, and add phenolphthalein TS. Titrate with *Titrant* to a pink endpoint that persists for NLT 30 s. Perform a blank determination.

Calculate the percentage of dehydroacetic acid ($C_8H_8O_4$) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F] / W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 168.2 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: 98.0%–100.5% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** <281>: NMT 0.1%
- **HEAVY METALS**, *Method II* <231>: NMT 10 µg/g

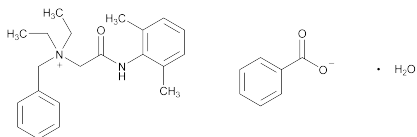
SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE**, *Class I* <741>: 109°–111°
- **LOSS ON DRYING** <731>
Analysis: Dry a sample at 80° for 4 h.
Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
- **USP REFERENCE STANDARDS** <11>
 USP Dehydroacetic Acid RS

Denatonium Benzoate



$C_{28}H_{34}N_2O_3 \cdot H_2O$ 464.60

$C_{28}H_{34}N_2O_3$ 446.59

Benzenemethanaminium, *N*-[2-[(2,6-dimethylphenyl)amino]-2-oxoethyl]-*N,N*-diethyl-, benzoate, monohydrate;
 Benzyldiethyl[(2,6-xylylcarbonyl)methyl] ammonium benzoate monohydrate [86398-53-0].
 Anhydrous [3734-33-6].

DEFINITION

Denatonium Benzoate, dried at 105° for 2 h, contains one molecule of water of hydration or is anhydrous. When dried at 105° for 2 h, it contains NLT 99.5% and NMT 101.0% of denatonium benzoate ($C_{28}H_{34}N_2O_3$).

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>
- **B. ULTRAVIOLET ABSORPTION** <197U>
Analytical wavelength: 263 nm
Sample solution: 100 µg/mL
Medium: Water
Acceptance criteria: Absorptivities, calculated on the dried basis, do not differ by more than 3.0%.
- **C.**
Sample: 150 mg
Analysis: Dissolve the *Sample* in 10 mL of water, and add 15 mL of trinitrophenol TS.

Acceptance criteria: A yellow precipitate is formed.

D.

Sample: 100 mg

Analysis: Dissolve the *Sample* in 10 mL of water, and add 20 mL of 2 N sulfuric acid and 15 mL of ammonium reineckate TS. Mix, filter through a sintered-glass crucible using gentle suction, and wash thoroughly with water. Remove as much water as possible with suction, and then dry in an oven at 105° for 1 h.

Acceptance criteria: The denatonium reineckate so obtained melts at about 170° (see *Melting Range or Temperature* <741>).

ASSAY

PROCEDURE

Sample: 900 mg, previously dried

Blank: 50 mL of glacial acetic acid

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 50 mL of glacial acetic acid, and add 1 drop of crystal violet TS. Titrate with *Titrant* to a green endpoint. Perform a blank determination, and make any necessary correction.

Calculate the percentage of denatonium benzoate ($C_{28}H_{34}N_2O_3$) in the portion of sample taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F] / W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 446.6 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: 99.5%–101.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** <281>: NMT 0.1%
- **CHLORIDE AND SULFATE**, *Chloride* <221>
Standard solution: 0.10 mL of 0.020 N hydrochloric acid
Sample solution: Dissolve 350 mg in 9 mL of water, add 1 mL of nitric acid, and filter.
Analysis: Use 1.0 mL of the *Sample solution*, and proceed as directed in the chapter
Acceptance criteria: 0.2%; the *Sample solution* shows no more chloride than the *Standard solution*.

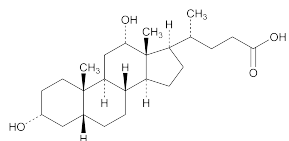
SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** <741>: 163°–170°, on a dried specimen
- **PH** <791>
Sample solution: 30 mg/mL
Acceptance criteria: 6.5–7.5
- **LOSS ON DRYING** <731>
Analysis: Dry at 105° for 2 h.
Acceptance criteria
Monohydrate form: 3.5%–4.5%
Anhydrous form: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate whether it is hydrous or anhydrous.
- **USP REFERENCE STANDARDS** <11>
 USP Denatonium Benzoate RS

Desoxycholic Acid



$C_{24}H_{40}O_4$ 392.57
Desoxycholic acid;
13 α ,12 α -Dihydroxycholan-24-oic acid [83-44-3].

DEFINITION

Desoxycholic Acid contains NLT 98.0% and NMT 102.0% of $C_{24}H_{40}O_4$, calculated on the dried basis.

IDENTIFICATION

- Add 2 drops of benzaldehyde and 3 drops of 75% sulfuric acid to about 10 mg of Desoxycholic Acid, heat at 50° for 5 min, and then add 10 mL of glacial acetic acid. A green color appears. (Cholic acid produces a brown color.)

ASSAY

PROCEDURE

Sample: 500 mg
Analysis: Transfer the *Sample* into a 250-mL Erlenmeyer flask, and add 20 mL of water and 40 mL of alcohol. Cover the flask with a watch glass, heat the mixture gently on a steam bath until the *Sample* is dissolved, and allow the mixture to cool to room temperature. Add a few drops of phenolphthalein TS to the solution, and titrate with 0.1 N sodium hydroxide to a pink endpoint that persists for 15 s. Each mL of 0.1 N sodium hydroxide is equivalent to 39.26 mg of $C_{24}H_{40}O_4$.
Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 0.2%, using 1.0 g
- **LIMIT OF LEAD**

[NOTE—Select reagents having as low a lead content as practicable, and store all solutions in high-density polyethylene containers. Rinse all plastic and glassware thoroughly with warm, 50% nitric acid followed by water.]

Standard lead solution: Prepare as directed under *Heavy Metals* (231), *Special Reagents*.

Standard solutions: [NOTE—Prepare these solutions on the day of use.] Transfer 10.0 and 50.0 mL of the *Standard lead solution* into two separate 100-mL volumetric flasks, add 10 mL of 3 N hydrochloric acid to each, and dilute with water to volume. The third standard, 10.0 μ g/mL, is taken directly from the *Standard lead solution*.

Sample solution: Transfer 10.0 g of Desoxycholic Acid, weighed to the nearest 0.1 mg, into an evaporating dish. Add 5 mL of 25% sulfuric acid (made by adding 25 mL of sulfuric acid to 75 mL of water), and distribute the 25% sulfuric acid solution uniformly. Within a hood, place the dish on a steam bath to evaporate most of the water. Place the dish on a burner, and slowly pre-ash the remaining *Sample solution* by expelling most of the sulfuric acid. Place the dish in a muffle furnace that has been set at 525°, and ash the contents of the dish until the residue appears free from carbon. Cool, and cautiously wash down the inside of the evaporation dish with water. Add 5 mL of 1 N hydrochloric acid. Place the dish on a steam bath, and evaporate to dryness. Add 1.0 mL of 3 N hydrochloric acid and approximately 5 mL of water, and heat briefly on a steam bath to dissolve any residue. Transfer to a 10-mL volumetric flask, dilute with water to volume, and mix.

Sample blank: Prepare by ashing 5 mL of 25% sulfuric acid solution. Cool, and cautiously wash down the inside of the evaporation dish with water. Add 5 mL of 1 N hydrochloric acid. Place the dish on a steam bath, and evaporate to dryness. Add 1.0 mL of 3 N hydrochloric acid and approximately 5 mL of water, and heat briefly on a steam bath to dissolve any residue. Transfer to a 10-mL volumetric flask, dilute with water to volume, and mix.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption

Analytical wavelength: 283.3 nm

Lamp: Lead electrodeless discharge

Flame: Air-acetylene flame

Slit width: 0.7 nm

Instrument blank: Water

Standard curve

Samples: *Standard solutions* and *Sample blank*

Plot: Corrected absorbance values versus their corresponding concentration (μ g/mL). [NOTE—Determine corrected absorbance values by subtracting the absorbance of the *Sample blank* from the absorbance of the *Standard solutions*.]

Analysis

Samples: *Sample solution* and *Sample blank*

[NOTE—Determine corrected absorbance values by subtracting the absorbance of the *Sample blank* from the absorbance of the *Sample solution*.]

From the *Standard curve*, determine the lead concentration in the *Sample solution*. Calculate the lead content, in ppm, in the portion of Desoxycholic Acid taken:

$$\text{Result} = C_s/W \times V$$

C_s = concentration of lead from the *Standard curve* (μ g/mL)

W = weight of the sample taken (g)

V = final volume of the sample (mL)

Acceptance criteria: NMT 4 ppm

SPECIFIC TESTS

- **OPTICAL ROTATION, Specific Rotation** (781S): NLT +55°
Sample solution: 1% (w/v) solution in ethanol
- **MELTING RANGE OR TEMPERATURE, Class I** (741): 172°–175°
- **LOSS ON DRYING** (731): Dry a sample at 140° under vacuum of NMT 5 mm of mercury for 4 h: it loses NMT 1% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.

Dextrates

DEFINITION

Dextrates is a purified mixture of saccharides resulting from the controlled enzymatic hydrolysis of starch. It is either anhydrous or hydrated. Its dextrose equivalent is NLT 93.0% and NMT 99.0%, calculated on the dried basis.

ASSAY

DEXTROSE EQUIVALENT

Standard solution: 10 mg/mL of USP Dextrose RS

Sample solution: 10 mg/mL of Dextrates in hot water

Analysis: Transfer 25.0-mL portions of alkaline cupric tartrate TS to each of two boiling flasks. Bring the contents of one of the flasks to a boil, and titrate with the *Standard solution* to within 0.5 mL of the anticipated endpoint. Again heat the flask, with swirling, boil moderately for 2 min, add 2 drops of a 10-mg/mL methyl-

ene blue solution, immediately add 2 drops of the *Standard solution* from the buret, and again bring to a boil. Allow the cuprous oxide to settle slightly, and observe the color of the supernatant. Complete the titration within 3 min by adding the *Standard solution* dropwise, and boiling after each addition to the disappearance of the blue color, determined by viewing against a white background in daylight or under equivalent illumination. If more than 0.5 mL of the titrant was required after the addition of the indicator, repeat the titration, adding the necessary volume of titrant before adding the indicator. Bring the contents of the second flask to a boil, and similarly titrate with the *Sample solution*. Calculate the percentage of dextrose equivalent taken on the dried basis:

$$\text{Result} = (C_S/C_U) \times (V_S/V_U) \times [1/(1 - 0.01 \times A)] \times 100$$

C_S = concentration of USP Dextrose RS in the *Standard solution* (mg/mL)

C_U = concentration of Dextrates in the *Sample solution* (mg/mL)

V_S = titrant volume consumed by the *Standard solution* (mL)

V_U = titrant volume consumed by the *Sample solution* (mL)

A = loss on drying of Dextrates taken (%)

Acceptance criteria: 93.0%–99.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%
- **HEAVY METALS**, *Method II* (231): NMT 5 µg/g

SPECIFIC TESTS

- **pH** (791)
Sample: 200 mg/mL in carbon dioxide-free water
Acceptance criteria: 3.8–5.8
- **LOSS ON DRYING** (731)
Analysis: Dry at 105° for 16 h in a convection oven.
Acceptance criteria: For the anhydrous form, NMT 2.0%; for the hydrated form, 7.8%–9.2%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store in a cool, dry place.
- **LABELING:** Label it to state whether it is anhydrous or hydrated.
- **USP REFERENCE STANDARDS** (11)
USP Dextrose RS

Dextrin

DEFINITION

Dextrin is starch, or partially hydrolyzed starch, modified by heating in a dry state, with or without acids, alkalies, or pH control agents. During heating, moisture may be added.

IDENTIFICATION

- **A.**
Sample: 1 g
Analysis: Suspend the *Sample* in 20 mL of water, and add a few drops of iodine TS.
Acceptance criteria: A blue to reddish-brown color results.
- **B.** Dextrin is very soluble in boiling water, forming a mucilaginous solution (difference from starch).

ASSAY

• REDUCING SUGARS (DEXTROSE EQUIVALENT)

Sample: A quantity of Dextrin equivalent to 2.0 g on the dried basis

Analysis: To the *Sample* add 100 mL of water, shake for 30 min, dilute with water to 200 mL, accurately measured, and filter. To 10.0 mL of alkaline cupric tartrate TS add 20.0 mL of the filtrate, mix, and heat on a hot plate adjusted to bring the solution to a boil in 3 min. Boil for 2 min, and cool quickly. Add 5 mL of potassium iodide solution (3 in 10) and 10 mL of 2 N sulfuric acid, mix, and titrate immediately with 0.1 N sodium thiosulfate VS, using starch TS, added toward the end of the titration, as an indicator.

Repeat the procedure beginning with "To 10.0 mL of alkaline cupric tartrate TS" using, in place of the filtrate, 20.0 mL of a solution (1 in 1000) of anhydrous dextrose, accurately prepared. Perform a blank titration. Compare ($V_B - V_U$) and ($V_B - V_S$).

$$\text{Result 1} = (V_B - V_U)$$

$$\text{Result 2} = (V_B - V_S)$$

V_B = volume of 0.1 N sodium thiosulfate consumed in the titrations of the blank

V_U = volume of 0.1 N sodium thiosulfate consumed in the titrations of the Dextrin

V_S = volume of 0.1 N sodium thiosulfate consumed in the titrations of the dextrose

Acceptance criteria: ($V_B - V_U$) is NMT ($V_B - V_S$), corresponding to NMT 10%, calculated as dextrose ($C_6H_{12}O_6$).

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.5%
- **CHLORIDE AND SULFATE**, *Chloride* (221)
Sample: 3.0 g
Control: 2.8 mL of 0.020 N hydrochloric acid in 75 mL of water
Analysis: Dissolve the *Sample* in 75 mL of boiling water. Cool, dilute with water to 75 mL, and filter if necessary. To 25 mL of this solution and the *Control*, add 2 mL of nitric acid and 1 mL of silver nitrate TS.
Acceptance criteria: Any turbidity produced is NMT that of the *Control*, corresponding to NMT 0.2% of chloride.
- **HEAVY METALS**, *Method II* (231): NMT 20 ppm
- **LIMIT OF PROTEIN**
Sample: 10 g
Analysis: Proceed as directed in *Nitrogen Determination* (461), using the *Sample* instead of 1 g, 60 mL of sulfuric acid instead of 20 mL, and multiplying the percentage of nitrogen found by 6.25.
Acceptance criteria: NMT 1.0%

SPECIFIC TESTS

- **ACIDITY**
Sample: 10.0 g
Analysis: Add the *Sample* to 100 mL of 70% alcohol, previously neutralized to phenolphthalein. Shake for 1 h, filter, and titrate 50 mL of the filtrate with 0.10 N sodium hydroxide.
Acceptance criteria: NMT 3.0 mL
- **BOTANIC CHARACTERISTICS**
Microscopic: Granules are similar in appearance to the starch from which the Dextrin has been prepared, except that when prepared from corn starch, many of the granules show concentric striations, and when prepared from potato starch, concentric striations are not clearly visible; the hilum is frequently bicleft; and a small proportion of the granules are distorted.
- **LOSS ON DRYING** (731): Dry a sample at a pressure not exceeding 100 mm of mercury at 120° for 4 h: it loses NMT 13.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Dextrose—see *Dextrose General Monographs***Dextrose Excipient****DEFINITION**

Dextrose Excipient is a sugar usually obtained by hydrolysis of starch. It contains one molecule of water of hydration.

IDENTIFICATION

- **A.**
Sample solution: 50 mg/mL
Analysis: Add a few drops of the *Sample solution* to 5 mL of hot alkaline cupric tartrate TS.
Acceptance criteria: A copious red precipitate of cuprous oxide is formed.

IMPURITIES

- **HEAVY METALS** <231>
Test preparation: 4.0 g of Dextrose Excipient in 25 mL of water
Acceptance criteria: NMT 5 ppm
- **RESIDUE ON IGNITION** <281>: NMT 0.1%
- **SOLUBLE STARCH, SULFITES**
Sample solution: 1 g of Dextrose Excipient in 10 mL of water
Analysis: To the *Sample solution* add 1 drop of iodine TS.
Acceptance criteria: The liquid is colored yellow.

SPECIFIC TESTS

- **OPTICAL ROTATION, Specific Rotation** <781S>
Sample solution: 100 mg/mL of Dextrose Excipient in 0.012 N ammonium hydroxide
Acceptance criteria: +52.5° to +53.5°
- **WATER DETERMINATION, Method III** <921>
Analysis: Dry at 105° for 16 h.
Acceptance criteria: 7.5%–9.5%
- **COLOR OF SOLUTION**
Sample solution: Dissolve 25 g of Dextrose Excipient in water to make 50.0 mL.
Control solution: Mix 1.0 mL of cobaltous chloride CS, 3.0 mL of ferric chloride CS, and 2.0 mL of cupric sulfate CS with water to make 10 mL. Dilute 3 mL of this solution with water to 50 mL.
Analysis: Make the comparison by viewing the *Sample solution* and *Control solution* downward in matched color-comparison tubes against a white surface.
Acceptance criteria: The *Sample solution* has no more color than the *Control solution*.
- **ACIDITY**
Sample solution: 100 mg/mL in carbon dioxide-free water
Analysis: Add phenolphthalein TS to 50 mL of the *Sample solution*, and titrate with 0.020 N sodium hydroxide to the production of a distinct pink color.
Acceptance criteria: NMT 0.30 mL
- **CHLORIDE AND SULFATE, Chloride** <221>
Standard solution: 0.50 mL of 0.020 N hydrochloric acid
Sample: 2.0 g
Acceptance criteria: 0.018%; the *Sample* shows no more chloride than the *Standard solution*.
- **CHLORIDE AND SULFATE, Sulfate** <221>
Standard solution: 0.50 mL of 0.020 N sulfuric acid
Sample: 2.0 g
Acceptance criteria: 0.025%; the *Sample* shows no more sulfate than the *Standard solution*.

- **ARSENIC, Method I** <211>: NMT 1 ppm
- **DEXTRIN**
Sample: 1 g of finely powdered Dextrose Excipient
Analysis: Reflux the *Sample* with 20 mL of alcohol.
Acceptance criteria: It dissolves completely.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Label it to indicate that it is not intended for parenteral use.

Diacetylated Monoglycerides**DEFINITION**

Diacetylated Monoglycerides is glycerin esterified with edible fat-forming fatty acids and acetic acid. It may be prepared by the interesterification of edible oils with triacetin in the presence of catalytic agents, followed by molecular distillation, or by the direct acetylation of edible monoglycerides with acetic anhydride without the use of catalyst or molecular distillation.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197F>
Sample: Undried specimen
Acceptance criteria: Meet the requirements

IMPURITIES

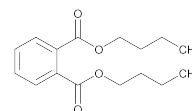
- **RESIDUE ON IGNITION** <281>: NMT 0.1%
- **HEAVY METALS, Method II** <231>: NMT 10 µg/g

SPECIFIC TESTS

- **FATS AND FIXED OILS, Acid Value** <401>: NMT 3
- **FATS AND FIXED OILS, Hydroxyl Value** <401>: NMT 15
- **FATS AND FIXED OILS, Saponification Value** <401>: 365–395

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** <11>
 USP Diacetylated Monoglycerides RS

Diatrizoic Acid—see *Diatrizoic Acid General Monographs***Dibutyl Phthalate**

$C_{16}H_{22}O_4$ 278.34
 1,2-Benzenedicarboxylic acid, dibutyl ester [84-74-2].

DEFINITION

Dibutyl Phthalate contains NLT 99.0% and NMT 101.0% of dibutyl phthalate ($C_{16}H_{22}O_4$).

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197F>: On undried specimen
- **B.** It meets the requirements in *Specific Tests for Refractive Index* <831>.

ASSAY**• PROCEDURE****Sample:** 0.75 g**Titrimetric system**(See *Titrimetry* <541>.)**Mode:** Residual titration**Titrant:** 0.5 N alcoholic potassium hydroxide VS**Back-titrant:** 0.5 N hydrochloric acid VS**Endpoint detection:** Visual

Analysis: Transfer the *Sample* to a flask, add 25.0 mL of *Titrant*, attach a reflux condenser to the flask, and boil in a water bath for 1 h. Add 1 mL of phenolphthalein TS. Titrate immediately with *Back-titrant*. Perform a blank determination. Each mL of *Titrant* is equivalent to 69.59 mg of dibutyl phthalate (C₁₆H₂₂O₄).

Acceptance criteria: 99.0%–101.0%**IMPURITIES****• RESIDUE ON IGNITION** <281>**Sample:** 1.0 g**Acceptance criteria:** NMT 0.1%**• RELATED COMPOUNDS****Internal standard solution:** 3 mg/mL of dibenzyl in methylene chloride**Sample stock solution:** 100 mg/mL of Dibutyl Phthalate in methylene chloride**Sample solution A:** Dilute 5.0 mL of *Sample stock solution* with methylene chloride to 10 mL.**Sample solution B:** Add 5.0 mL of *Sample stock solution*, 1.0 mL of *Internal standard solution*, and dilute with methylene chloride to 10 mL.**Standard solution:** Add 1.0 mL of *Sample solution A*, 10.0 mL of *Internal standard solution*, and dilute with methylene chloride to 100 mL.**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** GC**Detector:** Flame ionization**Column:** 4-mm × 1.5-m; packed with 3% liquid phase G3 on support S1A**Temperatures****Column:** 190°**Injection port:** 225°**Detector:** 225°**Carrier gas:** Nitrogen**Flow rate:** 30 mL/min**Run time:** Three times the retention time of dibutyl phthalate**Injection volume:** 1 µL**System suitability****Samples:** *Sample solution A* and *Standard solution***Suitability requirements**

[NOTE—The elution order is the internal standard peak followed by the dibutyl phthalate peak.]

Resolution: NLT 12 between the internal standard and the dibutyl phthalate peak, *Standard solution*

Additional requirements: Verify that there is no peak with the same retention time as the internal standard in *Sample solution A*. If such a peak is observed, make any necessary correction for factors of dilution, and then determine the area due to the interfering component that must be subtracted from the area of the internal standard peak appearing in the chromatogram recorded for *Sample solution B*.

Analysis**Samples:** *Sample solution B* and *Standard solution*

Calculate the peak area ratio of dibutyl phthalate to the internal standard for the *Standard solution*. Calculate the peak area ratio of the sum of all peaks, excluding the main peak, the solvent peak, and the internal standard peak, to the internal standard peak for *Sample solution B*.

Acceptance criteria: NMT 1.0%; the ratio for *Sample solution B* is NMT the ratio for the *Standard solution*.**SPECIFIC TESTS****• SPECIFIC GRAVITY** <841>: 1.043–1.048 at 20°**• REFRACTIVE INDEX** <831>: 1.490–1.495 at 20°**• ACIDITY****Sample:** 20.0 g

Analysis: Mix the *Sample* with 50 mL of alcohol that previously has been neutralized to a phenolphthalein endpoint. Add 0.2 mL of phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS.

Acceptance criteria: NMT 0.50 mL is required to change the color of the indicator.**• WATER DETERMINATION, Method I** <921>: NMT 0.2%**• APPEARANCE****Sample:** Dibutyl Phthalate (neat)

Standard solution: Prepared immediately before use by mixing 2.4 mL of ferric chloride CS and 0.6 mL of cobaltous chloride CS with dilute hydrochloric acid (10 mg/mL) to make 10 mL, and diluting 5 mL of this solution with dilute hydrochloric acid (10 mg/mL) to make 100 mL.

Analysis: Compare the *Sample* and the *Standard solution* by viewing the substance and the solution downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* <631>).

Acceptance criteria: The *Sample* is clear and not more intensely colored than the *Standard solution*.**ADDITIONAL REQUIREMENTS****• PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.**• USP REFERENCE STANDARDS** <11>

USP Dibutyl Phthalate RS

Dibutyl Sebacate**DEFINITION**

Dibutyl Sebacate consists of esters of *n*-butyl alcohol and saturated dibasic acids, principally sebacic acid. It contains NLT 92.0% of dibutyl sebacate (C₁₈H₃₄O₄).

ASSAY**• PROCEDURE**

System suitability solution: Add 0.2 g of methyl heptadecanoate to 0.3 g of Dibutyl Sebacate, and mix to dissolve.

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** GC**Detector:** Flame ionization**Column:** 2-mm × 1.8-m; packed with 10% liquid phase G41 on 100- to 120-mesh support S1A**Column temperature:** See *Table 1*.**Table 1**

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	—	150	2
150	4	280	30

Carrier gas: Helium**Flow rate:** 30 mL/min**Injection volume:** 1 µL**System suitability****Sample:** *System suitability solution*

[NOTE—The relative retention times for methyl heptadecanoate and dibutyl sebacate are 0.8 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.8 between dibutyl sebacate and methyl heptadecanoate

Relative standard deviation: NMT 2.0%

Analysis

Sample: Dibutyl Sebacate (neat)

Calculate the percentage of dibutyl sebacate ($C_{18}H_{34}O_4$) in the portion of sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for dibutyl sebacate

r_T = sum of the responses of all the peaks

Acceptance criteria: NLT 92.0%

SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): 0.935–0.939 at 20°
- **REFRACTIVE INDEX** (831): 1.429–1.441
- **FATS AND FIXED OILS, Acid Value** (401): NMT 0.1
- **FATS AND FIXED OILS, Saponification Value** (401): 352–360

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Dichlorodifluoromethane

CCl_2F_2

120.91

Methane, dichlorodifluoro-;
Dichlorodifluoromethane [75-71-8].

DEFINITION

Dichlorodifluoromethane contains NLT 99.6% and NMT 100.0% of dichlorodifluoromethane (CCl_2F_2), calculated on the anhydrous basis.

IDENTIFICATION

- **A.** The IR absorption spectrum, determined in a 10-cm cell with sodium chloride windows, at atmospheric pressure, exhibits maxima, among others, at the following wavelengths (μm): 4.33 (m), 4.46 (m), 4.56 (m), 6.29 (m), 7.25 (m), 8.05 (s), 8.63 (s), 9.1 (vs), 10.7 (vs), 10.8 (vs), 11.2 (m), 11.3 (m), 13.1 (w), and 13.9 (w). The stronger maxima are best obtained at pressures less than 10 mm of mercury.

ASSAY• **PROCEDURE**

System suitability solution: Introduce a liquid-phase mixture of dichlorodifluoromethane, dichlorotetrafluoroethane, and trichloromonofluoromethane into an evacuated headspace vial.

Sample solution: Introduce the liquid phase of Dichlorodifluoromethane into an evacuated headspace vial.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 2-mm \times 1.8-m stainless steel; 1% phase G25 on support S12

Temperatures

Injection port: 110°

Detector: 200°

Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	10	170	5

Carrier gas: Helium

Flow rate: 20 mL/min

Headspace sampler: The bath temperature is 100°, the valve/loop temperature is 105°, and the sampling time is 3 s. Make adjustments as necessary to optimize peak areas to record trace-level impurities.

System suitability

Sample: Gas phase headspace of the *System suitability solution*

[NOTE—The relative retention times for dichlorodifluoromethane, dichlorotetrafluoroethane, and trichloromonofluoromethane are about 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between dichlorotetrafluoroethane and trichloromonofluoromethane

Analysis

Sample: Gas phase headspace of the *Sample solution*
Calculate the percentage of dichlorodifluoromethane (CCl_2F_2) in the portion of Dichlorodifluoromethane taken.

Acceptance criteria: 99.6%–100.0% on the anhydrous basis

IMPURITIES• **INORGANIC CHLORIDES**

Sample: 7 g

Analysis: Place 5 mL of anhydrous methanol in a test tube, add 3 drops of a saturated solution of silver nitrate in anhydrous methanol, and shake. Add the *Sample*.

Acceptance criteria: No opalescence or turbidity is produced.

• **CHROMATOGRAPHIC PURITY**

Analysis: In the chromatograms from the *Assay*, identify the dichlorotetrafluoroethane and trichloromonofluoromethane peaks from the relative retention times of those peaks in the chromatogram of the *System suitability solution*.

Acceptance criteria

Sum of the peak areas for dichlorotetrafluoroethane and trichloromonofluoromethane: NMT 0.2% of the total of all peak areas

Sum of the areas of all peaks other than that for dichlorotetrafluoromethane: NMT 0.4% of the total of all peak areas

SPECIFIC TESTS• **BOILING TEMPERATURE**

Analysis: Proceed as directed in *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* (601), *Approximate Boiling Temperature*.

Acceptance criteria: Approximately –30°

• **WATER**

Analysis: Proceed as directed in *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* (601), *Water Content and Water Determination* (921), *Method 1c*.

Acceptance criteria: NMT 0.001%

• **HIGH-BOILING RESIDUES**

Analysis: Proceed as directed in *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* (601), *High-Boiling Residues, Method 1*.

Acceptance criteria: NMT 0.01%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight cylinders, and avoid exposure to excessive heat.

Dichlorotetrafluoroethane



$C_2Cl_2F_4$ 170.92
Ethane, 1,2-dichloro-1,1,2,2-tetrafluoro-;
1,2-Dichlorotetrafluoroethane [76-14-2].

DEFINITION

Dichlorotetrafluoroethane contains NLT 99.6% and NMT 100.0% of dichlorotetrafluoroethane ($C_2Cl_2F_4$), calculated on the anhydrous basis.

IDENTIFICATION

- A.** The IR absorption spectrum, determined in a 10-cm cell with sodium chloride windows, at atmospheric pressure, exhibits maxima, among others, at the following wavelengths (μm): 4.34 (s), 4.48 (m), 5.28 (m), 5.95 (m), 7.36 (s), 9.5 (vs), 10.9 (vs), 11.8 (vs), 13.6 (s), and 14.8 (s). The stronger maxima are best obtained at pressures less than 10 mm of mercury.

ASSAY

PROCEDURE

System suitability solution: Introduce a liquid-phase mixture of dichlorodifluoromethane, dichlorotetrafluoroethane, and trichloromonofluoromethane into an evacuated headspace vial.

Sample solution: Introduce the liquid phase of Dichlorotetrafluoroethane into an evacuated headspace vial.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 2-mm \times 1.8-m stainless steel; 1% phase G25 on support S12

Temperatures

Injection port: 110°

Detector: 200°

Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	10	170	5

Carrier gas: Helium

Flow rate: 20 mL/min

Headspace sampler: The bath temperature is 100°, the valve/loop temperature is 105°, and the sampling time is 3 s. Make adjustments as necessary to optimize peak areas to record trace level impurities.

System suitability

Sample: Gas phase headspace of the *System suitability solution*

[NOTE—The relative retention times for dichlorodifluoromethane, dichlorotetrafluoroethane, and trichloromonofluoromethane are 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between dichlorotetrafluoroethane and trichloromonofluoromethane

Analysis

Sample: Gas phase headspace of the *Sample solution*
Calculate the percentage of dichlorotetrafluoroethane ($C_2Cl_2F_4$) in the portion taken.

Acceptance criteria: 99.6%–100.0% on the anhydrous basis

IMPURITIES

INORGANIC CHLORIDES

Sample: 7 g

Analysis: Place 5 mL of anhydrous methanol in a test tube, add 3 drops of a saturated solution of silver nitrate in anhydrous methanol, and shake. Add the *Sample*.

Acceptance criteria: No opalescence or turbidity is produced.

CHROMATOGRAPHIC PURITY

Analysis: In the chromatograms from the *Assay*, identify the dichlorodifluoromethane and trichloromonofluoromethane peaks from the relative retention times of those peaks in the chromatogram of the *System suitability solution*, as obtained in the *Assay*.

Acceptance criteria

Sum of the peak areas for dichlorodifluoromethane and trichloromonofluoromethane: NMT 0.2% of the total of all peak areas

Sum of the areas of all peaks other than that for dichlorotetrafluoroethane: NMT 0.4% of the total of all peak areas

SPECIFIC TESTS

BOILING TEMPERATURE

Analysis: Proceed as directed in *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* <601>, *Approximate Boiling Temperature*.

Acceptance criteria: Approximately 4°

WATER

Analysis: Proceed as directed in *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* <601>, *Water Content*.

Acceptance criteria: NMT 0.001%

HIGH-BOILING RESIDUES

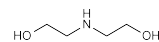
Analysis: Proceed as directed in *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* <601>, *High-Boiling Residues, Method I*.

Acceptance criteria: NMT 0.01%

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight cylinders, and avoid exposure to excessive heat.

Diethanolamine



$C_4H_{11}NO_2$ 105.14
Ethanol, 2,2'-iminobis-;
2,2'-Iminodiethanol [111-42-2].

DEFINITION

Diethanolamine is a mixture of ethanolamines, consisting largely of diethanolamine. It contains NLT 98.5% and NMT 101.0% of ethanolamines, calculated on the anhydrous basis as $NH(C_2H_4OH)_2$.

IDENTIFICATION

- A. INFRARED ABSORPTION** <197F>

ASSAY

PROCEDURE

Sample: 2 g

Blank: 50 mL of water

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.5 N hydrochloric acid VS

Endpoint detection: Visual

Analysis: To the *Sample* add 50 mL of water and bromocresol green TS. Titrate with the *Titrant*. Perform a blank determination.

Calculate the percentage of diethanolamine, expressed as $\text{NH}(\text{C}_2\text{H}_4\text{OH})_2$, in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 105.14 (mg/mEq)

W = *Sample* weight (mg)

Acceptance criteria: 98.5%–101.0% on the anhydrous basis

IMPURITIES

• LIMIT OF TRIETHANOLAMINE

Mixed indicator: 1.5 mg/mL of methyl orange and 0.8 mg/mL of xylene cyanole FF in water

Sample: 20 g

Blank: 100 mL of methanol and 6–8 drops of *Mixed indicator*

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.5 N alcoholic sulfuric acid VS

Endpoint detection: Visual

Analysis: Place 100 mL of methanol and 6–8 drops of *Mixed indicator* in a 500-mL glass-stoppered conical flask, and neutralize with 0.1 N alcoholic sulfuric acid or 0.1 N alcoholic potassium hydroxide. The neutral solution is amber when viewed by transmitted light and is red-brown when viewed by reflected light. Add the *Sample*. Cautiously add 75 mL of acetic anhydride, and swirl to effect complete solution. Cool to room temperature, if necessary, and allow to stand at room temperature for 30 min. Titrate with the *Titrant*. Perform a blank determination.

Calculate the percentage of triethanolamine in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 149.2 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: NMT 1.0% by weight

SPECIFIC TESTS

• **REFRACTIVE INDEX (831):** 1.473–1.476 at 30°

• **WATER DETERMINATION, Method I (921)**

Sample: 20 g

Solvent: A mixture of methanol and glacial acetic acid (40:25)

Acceptance criteria: NMT 0.15%

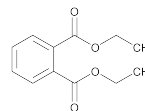
ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **USP REFERENCE STANDARDS (11)**

USP Diethanolamine RS

Diethyl Phthalate



$\text{C}_{12}\text{H}_{14}\text{O}_4$

222.24

1,2-Benzenedicarboxylic acid, diethyl ester;

Diethyl phthalate [84-66-2].

DEFINITION

Diethyl Phthalate contains NLT 98.0% and NMT 102.0% of diethyl phthalate ($\text{C}_{12}\text{H}_{14}\text{O}_4$), calculated on the anhydrous basis. [**CAUTION**—Avoid contact.]

IDENTIFICATION

• **A. INFRARED ABSORPTION (197F):** On undried specimen

ASSAY

• PROCEDURE

Sample: 1.5 g

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 0.5 N alcoholic potassium hydroxide VS

Back-titrant: 0.5 N hydrochloric acid VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a flask, add 50.0 mL of the *Titrant*, and boil with a reflux condenser on a water bath for 1 h. Add 20 mL of water, then add phenolphthalein TS. Titrate the excess potassium hydroxide with the *Back-titrant*. Perform a blank determination. Each mL of 0.5 N potassium hydroxide is equivalent to 55.56 mg of diethyl phthalate ($\text{C}_{12}\text{H}_{14}\text{O}_4$).

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION (281)**

Sample: 10 g

Analysis: Gently heat the *Sample* until the liquid has evaporated, and ignite the residue to constant weight.

Acceptance criteria: NMT 0.02%

SPECIFIC TESTS

• **SPECIFIC GRAVITY (841):** 1.118–1.122 at 20°

• **REFRACTIVE INDEX (831):** 1.500–1.505 at 20°

• **ACIDITY**

Sample: 20.0 g

Analysis: Mix the *Sample* with 50 mL of alcohol that previously has been neutralized to a phenolphthalein endpoint, and titrate with 0.1 N sodium hydroxide VS to a phenolphthalein endpoint.

Acceptance criteria: NMT 0.50 mL is required for neutralization.

• **WATER DETERMINATION, Method I (921):** NMT 0.2%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **USP REFERENCE STANDARDS (11)**

USP Diethyl Phthalate RS

Add the following:

▲ Diethyl Sebacate



$\text{CH}_3\text{CH}_2\text{OOC}(\text{CH}_2)_8\text{COOCH}_2\text{CH}_3$

$\text{C}_{14}\text{H}_{26}\text{O}_4$ 258.35

Decanedioic acid, 1,10-diethyl ester;
Diethyl 1,10-decanedioate [110-40-7].

DEFINITION

Diethyl Sebacate consists of the diester of alcohol and sebacic acid. It contains NLT 98.0% and NMT 100.5% of $\text{C}_{14}\text{H}_{26}\text{O}_4$.

IDENTIFICATION

- A. INFRARED ABSORPTION** (197F)
- B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

PROCEDURE

Internal standard solution: 0.9 mg/mL of methyl heptadecanoate in alcohol

Standard solution: 1.0 mg/mL of USP Diethyl Sebacate RS in the *Internal standard solution*

Sample solution: 1.0 mg/mL of Diethyl Sebacate in the *Internal standard solution*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m fused silica capillary column; 1.5-μm layer of phase G1

Temperatures

Detector: 300°

Injection port: 300°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	—	150	5
150	10	250	5

Carrier gas: Helium

Linear velocity: 50 cm/s

Injection size: 1 μL

Injection type: Split injection. The split ratio is about 3:1.

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for diethyl sebacate and methyl heptadecanoate are 1.0 and 1.2, respectively.]

Suitability requirements

Resolution: NLT 2.0 between diethyl sebacate and methyl heptadecanoate

Relative standard deviation: NMT 2.0%, ratio of the peak response of diethyl sebacate to that of methyl heptadecanoate

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of $\text{C}_{14}\text{H}_{26}\text{O}_4$ in the portion of Diethyl Sebacate taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = ratio of the peak response of diethyl sebacate to that of methyl heptadecanoate from the *Sample solution*

R_S = ratio of the peak response of diethyl sebacate to that of methyl heptadecanoate from the *Standard solution*

C_S = concentration of USP Diethyl Sebacate RS in the *Standard solution* (mg/mL)

C_U = concentration of Diethyl Sebacate in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–100.5%

IMPURITIES

Inorganic Impurities

- RESIDUE ON IGNITION** (281): NMT 0.10%
- HEAVY METALS, Method II** (231): NMT 20 ppm

SPECIFIC TESTS

- SPECIFIC GRAVITY** (841): 0.958–0.968 at 20°
- REFRACTIVE INDEX** (831): 1.435–1.437 at 20°
- FATS AND FIXED OILS, Acid Value** (401): NMT 0.5
- FATS AND FIXED OILS, Iodine Value** (401): NMT 0.5

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool, dry, and well-ventilated place.

- USP REFERENCE STANDARDS** (11)
USP Diethyl Sebacate RS_{NF31}

Diethylene Glycol Monoethyl Ether



$\text{C}_6\text{H}_{14}\text{O}_3$

134.17

DEFINITION

Diethylene Glycol Monoethyl Ether contains NLT 99.0% and NMT 101.0% of $\text{C}_6\text{H}_{14}\text{O}_3$. It is produced by condensation of ethylene oxide and alcohol, followed by distillation.

IDENTIFICATION

- A. INFRARED ABSORPTION** (197F): Potassium bromide plates being used
- B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *System suitability solution*, as obtained in the *Assay*.

ASSAY

PROCEDURE

Sample: Diethylene Glycol Monoethyl Ether (neat)

System suitability solution: 1 mg/mL each of 2-methoxyethanol, 2-ethoxyethanol, ethylene glycol, diethylene glycol, and USP Diethylene Glycol Monoethyl Ether RS in methanol

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica bonded with a 1.0-μm layer of phase G46

Temperature

Injector: 250°

Detector: 275°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	—	120	1
120	12	225	2

Carrier gas: Helium

Flow rate: 2.2 mL/min

Injection size: 0.5 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for 2-methoxyethanol, 2-ethoxyethanol, ethylene glycol, diethylene glycol monoethyl ether, and diethylene glycol are about 0.40, 0.43, 0.50, 0.93, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between 2-ethoxyethanol and ethylene glycol

Relative standard deviation: NMT 2.0%, determined from diethylene glycol monoethyl ether

Analysis

Sample: Diethylene Glycol Monoethyl Ether (neat)

Calculate the percentage of diethylene glycol monoethyl ether (C₆H₁₄O₃) in the portion of Diethylene Glycol Monoethyl Ether taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for diethylene glycol monoethyl ether

r_T = sum of the responses of all the peaks

Acceptance criteria: 99.0%–101.0%

IMPURITIES

Organic Impurities

• **PROCEDURE 1: LIMIT OF FREE ETHYLENE OXIDE**

Acetaldehyde solution: 10 µg/mL of acetaldehyde.

[NOTE—Prepare fresh just before use.]

Ethylene oxide stock solution

[**CAUTION**—Ethylene oxide is toxic and flammable.

Prepare these solutions in a well-ventilated fume hood, using great care. Protect both hands and face by wearing polyethylene protective gloves and an appropriate face mask.]

[NOTE—Before using the polyethylene glycol 200 in this test, remove any volatile components from it by placing 500 mL of the polyethylene glycol 200 in a 1000-mL round-bottom flask, attaching the flask to a rotary evaporator, and evaporating at a temperature of 60° at a pressure of 1.5–2.5 kPa for 6 h.]

Fill a chilled pressure bottle with liquid ethylene oxide, and store in a freezer when not in use. Use a small piece of polyethylene film to protect the liquid from contact with the rubber gasket. Tare a glass-stoppered conical flask, add 50 mL of polyethylene glycol 200, and reweigh the flask. Transfer 5 mL of the liquid ethylene oxide to a 100-mL beaker chilled in a mixture of sodium chloride and wet ice (1:3). Using a gas-tight gas chromatographic syringe that has been previously cooled to –10°, transfer 300 µL (corresponding to about 250 mg) of liquid ethylene oxide to the polyethylene glycol 200, and swirl gently to mix. Replace the stopper, reweigh the flask, and determine the amount of ethylene oxide absorbed by weight difference. Adjust the weight of the mixture with polyethylene glycol 200 to 100.0 g, replace the stopper, and swirl gently to mix. This stock solution contains 2.5 mg/g of ethylene oxide. [NOTE—Prepare this *Ethylene oxide stock solution* fresh just before use, and store in a refrigerator.]

Ethylene oxide standard solution A: Tare a glass-stoppered conical flask, and chill it in a refrigerator. Add 35 mL of polyethylene glycol 200, and reweigh the flask. Use a gas-tight gas chromatographic syringe

that has been chilled in a refrigerator, and transfer 1 g of the chilled *Ethylene oxide stock solution* to the tared, conical flask. Adjust the weight of the solution with polyethylene glycol 200 to 50.0 g, replace the stopper, and swirl gently to mix. Transfer 10 g of this solution to a 50-mL volumetric flask. Add 30 mL of water, and mix. Dilute with water to volume, and mix to obtain a solution containing 10 µg/mL of ethylene oxide.

[NOTE—Prepare this solution fresh just before use, and store in a refrigerator.]

Ethylene oxide standard solution B: Transfer 10.0 mL of *Ethylene oxide standard solution A* to a 50-mL volumetric flask, and dilute with water to volume to obtain a solution containing 2 µg/mL of ethylene oxide. [NOTE—Prepare this solution fresh just before use, and store in a refrigerator.]

System suitability solution: Transfer 0.5 mL of *Ethylene oxide standard solution B* to a 10-mL pressure headspace vial, and add 0.1 mL of *Acetaldehyde solution* and 0.1 mL of water, seal the vial, and mix. Heat the mixture at 70° for 45 min.

Standard solution: Transfer 1 g of Diethylene Glycol Monoethyl Ether to a 10-mL pressure headspace vial, and add 0.5 mL of *Ethylene oxide standard solution B* and 0.5 mL of water. Seal the vial, and mix. Heat the mixture at 70° for 45 min.

Sample solution: Transfer 1 g of Diethylene Glycol Monoethyl Ether to a 10-mL pressure headspace vial, add 1 mL of water, seal the vial, and mix. Heat the mixture at 70° for 45 min.

Chromatographic system

(See *Chromatography* {621}, *System Suitability*.)

[NOTE—The use of a headspace apparatus that automatically transfers a measured amount of headspace is allowed.]

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m glass or quartz capillary bonded with a 1.0-µm layer of phase G1

Temperature

Injector: 150°

Detector: 250°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	—
180	30	230	5

Carrier gas: Helium

Flow rate: 1 mL/min

Injection size: 1 mL

System suitability

Sample: Gaseous headspace of the *System suitability solution*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are about 0.94 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between the acetaldehyde and ethylene oxide peaks

Relative standard deviation: NMT 15%

Analysis

Samples: Gaseous headspace of the *Standard solution* and *Sample solution*

[NOTE—Use a heated, gas-tight gas chromatographic syringe.]

Calculate the amount of ethylene oxide in the portion of Diethylene Glycol Monoethyl Ether taken:

$$\text{Result} = r_U / [(r_S \times W_U) - (r_U \times W_S)]$$

- r_U = ethylene oxide peak area from the *Sample solution*
 r_S = ethylene oxide peak area from the *Standard solution*
 W_U = weight of Diethylene Glycol Monoethyl Ether taken to prepare the *Sample solution* (g)
 W_S = weight of Diethylene Glycol Monoethyl Ether taken to prepare the *Standard solution* (g)

Acceptance criteria: NMT 1 µg/g

PROCEDURE 2: LIMIT OF 2-METHOXYETHANOL, 2-ETHOXYETHANOL, ETHYLENE GLYCOL, AND DIETHYLENE GLYCOL

Sample, System suitability solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.

Analysis: Proceed as directed in the *Assay*.

Calculate the percentage of 2-methoxyethanol in the portion of Diethylene Glycol Monoethyl Ether taken:

$$\text{Result} = (r_U/r_T) \times 100$$

- r_U = peak response for 2-methoxyethanol
 r_T = sum of all the peak responses
 Calculate the percentage of 2-ethoxyethanol in the portion of Diethylene Glycol Monoethyl Ether taken:

$$\text{Result} = (r_U/r_T) \times 100$$

- r_U = peak response for 2-ethoxyethanol
 r_T = sum of all the peak responses
 Calculate the percentage of ethylene glycol in the portion of Diethylene Glycol Monoethyl Ether taken:

$$\text{Result} = (r_U/r_T) \times 100$$

- r_U = peak response for ethylene glycol
 r_T = sum of all the peak responses
 Calculate the percentage of diethylene glycol in the portion of Diethylene Glycol Monoethyl Ether taken:

$$\text{Result} = (r_U/r_T) \times 100$$

- r_U = peak response for diethylene glycol
 r_T = sum of all the peak responses
 Acceptance criteria: See *Impurity Table 1*.

Impurity Table 1

Name	Acceptance Criteria, NMT (ppm)
2-Methoxyethanol	50
2-Ethoxyethanol	160
Ethylene glycol	620
Diethylene glycol	150

SPECIFIC TESTS

- REFRACTIVE INDEX (831):** 1.426–1.428 at 20°
- WATER DETERMINATION, Method I (921):** NMT 0.1%, determined on a 10-g specimen
- FATS AND FIXED OILS, Acid Value (401)**
 [NOTE—This test must be performed promptly after sampling to avoid oxidation of the sample specimen.]
Analysis: Dissolve 30 g of Diethylene Glycol Monoethyl Ether in 30 mL of neutralized alcohol. Add 1 mL of phenolphthalein TS, and titrate with 0.01 N alcoholic potassium hydroxide VS to produce a permanent, faint pink color.
Acceptance criteria: The acid value so obtained is NMT 0.1.
- FATS AND FIXED OILS, Peroxide Value (401):** NMT 8.0, 2 g being used

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight containers under an atmosphere of an inert gas, at a temperature not exceeding 35°.
- LABELING:** Label it to indicate that it is intended for topical or transdermal use only and it is stored under an atmosphere of an inert gas. The material is not to be used for parenterals.
- USP REFERENCE STANDARDS (11)**
 USP Diethylene Glycol Monoethyl Ether RS

Diethylene Glycol Stearates

DEFINITION

Diethylene Glycol Stearates is a mixture of diethylene glycol monoesters and diesters of stearic and palmitic acids. It contains NLT 45.0% of monoesters produced from the condensation of ethylene glycol and stearic acid of vegetable or animal origin.

IDENTIFICATION

- A.** It meets the requirements in *Specific Tests for Melting Range or Temperature, Class II (741)*.
- B.** It meets the requirements in *Specific Tests for Fats and Fixed Oils, Fatty Acid Composition (401)*.

ASSAY

PROCEDURE

Mobile phase: Tetrahydrofuran

Sample solution: 40 mg/mL of Diethylene Glycol Stearates in tetrahydrofuran

Chromatographic system

(See *Chromatography (621)*, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.5-mm × 60-cm; containing 5-µm 100Å packing L21. [NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the 60-cm column, provided that *System suitability* requirements are met.]

Temperatures

Detector: 40°

Column: 40°. [NOTE—The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *Sample solution*

[NOTE—The relative retention times for diesters, monoesters, and diethylene glycol are 0.78, 0.84, and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 2.0% for the monoesters peak

Analysis

Sample: *Sample solution*

Calculate the percentage of free fatty acids, E , in the portion of Diethylene Glycol Stearates taken:

$$E = I_A \times 270/561.1$$

I_A = acid value, determined in *Specific Tests for Fats and Fixed Oils, Acid Value (Free Fatty Acids) (401)*

Calculate the percentage of monoesters in the portion of *Sample* taken:

$$\text{Result} = [r_M/(r_M + r_D)] \times (100 - D - E)$$

r_M = peak response for the monoesters

- r_D = peak response for the diesters
 D = percentage of free diethylene glycol in the portion of Diethylene Glycol Stearates taken, as determined in *Impurities for Limit of Free Diethylene Glycol*

Acceptance criteria: NLT 45.0% of monoesters

IMPURITIES

• LIMIT OF FREE DIETHYLENE GLYCOL

Mobile phase, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.

Standard solution A: 0.5 mg/mL of diethylene glycol in tetrahydrofuran

Standard solution B: 1.0 mg/mL of diethylene glycol in tetrahydrofuran

Standard solution C: 2.0 mg/mL of diethylene glycol in tetrahydrofuran

Standard solution D: 4.0 mg/mL of diethylene glycol in tetrahydrofuran

Analysis

Samples: *Sample solution* and *Standard solutions*

Plot the diethylene glycol peak responses obtained versus the concentration of diethylene glycol in the *Standard solutions*. From the standard curve so obtained, determine the diethylene glycol concentration in the sample.

Calculate the percentage of free diethylene glycol in the portion of sample taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of diethylene glycol, determined from the standard curve (mg/mL)

C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 8.0%

SPECIFIC TESTS

• MELTING RANGE OR TEMPERATURE, *Class II* (741): 43°–50°

• FATS AND FIXED OILS, *Acid Value (Free Fatty Acids)* (401)

Sample: 10.0 g

Acceptance criteria: NMT 4.0

• FATS AND FIXED OILS, *Iodine Value* (401): NMT 3.0

• FATS AND FIXED OILS, *Saponification value* (401)

Sample: 2.0 g

Acceptance criteria: 150–170

• FATS AND FIXED OILS, *Fatty Acid Composition* (401): 40.0%–60.0% of stearic acid, and the sum of palmitic and stearic acids is NLT 90.0%.

• ARTICLES OF BOTANICAL ORIGIN, *Total Ash* (561)

Sample: 1.0 g

Acceptance criteria: NMT 0.1%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements are specified.
- **LABELING:** Label it to indicate that it is intended for topical and vaginal use only.

DEFINITION

Diisopropanolamine is a mixture of isopropanolamines, consisting largely of diisopropanolamine. It contains NLT 98.0% and NMT 102.0% of isopropanolamines, calculated as $\text{NH}(\text{C}_3\text{H}_7\text{OH})_2$ on the anhydrous basis.

IDENTIFICATION

- **A.** The IR absorption spectrum of a thin film exhibits regions of absorption between 2.8 and 4.0 μm , between 6.7 and 7.1 μm , and between 8.5 and 9.4 μm ; and several characteristic peaks, the most pronounced being at about 7.3, 7.5, 8.3, 9.6, 10.4, and 10.7 μm .

ASSAY

• PROCEDURE

Sample: 2 g

Blank: 50 mL of water

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.5 N hydrochloric acid VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 250-mL conical flask. Add 50 mL of water and bromocresol green TS. Titrate with *Titrant*. Perform a blank determination, and make any necessary correction.

Calculate the percentage of isopropanolamines, expressed as $\text{NH}(\text{C}_3\text{H}_7\text{OH})_2$, in the portion of Diisopropanolamine taken:

$$\text{Result} = \{(V_S - V_B) \times N \times F/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 0.1332 g/mEq

W = *Sample* weight (g)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

• LIMIT OF TRIISOPROPANOLAMINE

Indicator solution: 1.5 mg/mL of methyl orange and 0.8 mg/mL of xylene cyanole FF

Sample: 20 g

Blank: 100 mL of methanol

Titrimetric system

(See *Titrimetry* (541).)

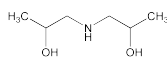
Mode: Direct titration

Titrant: 0.5 N alcoholic sulfuric acid VS

Endpoint detection: Visual

Analysis: To a glass-stoppered, 500-mL conical flask add 100 mL of methanol and 6–8 drops of *Indicator solution*, and neutralize with 0.1 N alcoholic sulfuric acid or 0.1 N alcoholic potassium hydroxide. The neutral solution is amber when viewed by transmitted light and is red-brown when viewed by reflected light. Add the *Sample*, cautiously add 75 mL of acetic anhydride, and swirl to dissolve. Allow to stand at room temperature for 30 min. Cool to room temperature, if necessary. Titrate with *Titrant*. Perform a blank determination, and make any necessary correction.

Diisopropanolamine



$\text{C}_6\text{H}_{15}\text{NO}_2$

2-Propanol, 1,1'-iminobis-;

1,1'-Iminodi-2-propanol [110-97-4].

133.19

Calculate the percentage of triisopropanolamine in the portion of Diisopropanolamine taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = Titrant volume consumed by the *Sample* (mL)

V_B = Titrant volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 0.1914 g/mEq

W = *Sample* weight (g)

Acceptance criteria: NMT 1.0% by weight

SPECIFIC TESTS

• WATER DETERMINATION, Method I (921)

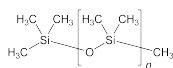
Solvent: A mixture of 5.0 mL of glacial acetic acid and 25 mL of methanol

Acceptance criteria: NMT 0.50%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No specific storage conditions are required.

Dimethicone

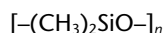


Dimethicone;

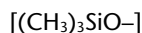
α -(Trimethylsilyl)- ω -methylpoly[oxy(dimethylsilylene)]
[9006-65-9].

DEFINITION

Dimethicone is a mixture of fully methylated linear siloxane polymers containing repeating units of the formula:



stabilized with trimethylsiloxy end-blocking units of the formula:



wherein n has an average value such that the corresponding nominal viscosity is in a discrete range between 20 and 30,000 centistokes. It contains NLT 97.0% and NMT 103.0% of polydimethylsiloxane $([-(\text{CH}_3)_2\text{SiO}-]_n)$.

The requirements for viscosity, specific gravity, refractive index, and loss on heating differ for the several types of Dimethicone, as set forth in *Table 1*.

IDENTIFICATION

- **A.** The IR absorption spectrum of the *Sample* exhibits maxima only at the same wavelengths as that of the *Standard*, obtained as directed in the *Assay*.

ASSAY

• PROCEDURE

Standard: USP Polydimethylsiloxane RS

Sample: Dimethicone

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: IR, with a resolution of 4 cm^{-1} and fitted with an accessory for attenuated total reflectance and a germanium (Ge) sample trough (45° or 60°)

Wavelength range: 4000 to 700 cm^{-1}

Analysis

Samples: *Standard* and *Sample*

Using an empty and clean trough, determine the background spectrum. Fill the trough with the *Sample*, and record the spectrum. Clean the trough, fill it with the *Standard*, and determine the spectrum. Examine the spectra in the range between 1300 and 1200 cm^{-1} , and determine the absorbance of the peak in each spectrum at about 1259 cm^{-1} .

Calculate the percentage of polydimethylsiloxane $([-(\text{CH}_3)_2\text{SiO}-]_n)$ in the Dimethicone taken:

$$\text{Result} = (A_U/A_S) \times (D_S/D_U) \times 100$$

A_U = absorbance of the *Sample*

A_S = absorbance of the *Standard*

D_S = specific gravity of USP Polydimethylsiloxane RS

D_U = specific gravity of Dimethicone

Acceptance criteria: 97.0%–103.0%

IMPURITIES

• HEAVY METALS

Standard solution: To 20 mL of chloroform add 1.0 mL of a freshly prepared 0.002% solution of dithizone in chloroform, 0.5 mL of *Standard Lead Solution* (see *Heavy Metals* (231)), and 0.5 mL of a mixture of 1 volume of ammonia TS and 9 volumes of a 0.2% solution of hydroxylamine hydrochloride.

Sample solution: Mix 1.0 g of Dimethicone with 10 mL of chloroform, and dilute with the same solvent to 20 mL. Add 1.0 mL of a freshly prepared 0.002% solution of dithizone in chloroform, 0.5 mL of water, and 0.5 mL of a mixture of 1 volume of ammonia TS and 9 volumes of a 0.2% solution of hydroxylamine hydrochloride.

Analysis: Immediately shake both solutions vigorously for 1 min.

Acceptance criteria: $5\text{ }\mu\text{g/g}$; any red color in the *Sample solution* is not more intense than that in the *Standard solution*.

SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): See *Table 1*.

- **REFRACTIVE INDEX** (831): See *Table 1*.

• ACIDITY

Sample: 15.0 g

Analysis: Dissolve the *Sample* in a mixture of 15 mL of toluene and 15 mL of butyl alcohol, previously neutralized to bromophenol blue TS, and titrate with 0.050 N alcoholic potassium hydroxide to a bromophenol blue endpoint.

Acceptance criteria: NMT 0.10 mL of 0.050 N alcoholic potassium hydroxide is required.

• LOSS ON HEATING

Sample: 1 g

Analysis: Preheat an open aluminum vessel (60 mm in diameter and 10 mm high) at 150° for 30 min, and allow to cool to room temperature. Tare the vessel, transfer the *Sample* to it, heat at 150° in a circulating air oven for 2 h, and allow to come to room temperature in a desiccator before weighing.

Acceptance criteria: See *Table 1*.

Table 1

Nominal Viscosity (centistokes)	Viscosity (centistokes)		Specific Gravity		Refractive Index		Loss on Heating (%)
	Min.	Max.	Min.	Max.	Min.	Max.	
20	18	22	0.946	0.954	1.3980	1.4020	20.0
50	47.5	52.5	0.955	0.965	1.4005	1.4045	2.0
100	95	105	0.962	0.970	1.4005	1.4045	0.3
200	190	220	0.964	0.972	1.4013	1.4053	0.3
350	332.5	367.5	0.965	0.973	1.4013	1.4053	0.3
500	475	525	0.967	0.975	1.4013	1.4053	0.3
1000	950	1050	0.967	0.975	1.4013	1.4053	0.3
12,500	11,875	13,125	—	—	1.4015	1.4055	2.0
30,000	27,000	33,000	0.969	0.977	1.4010	1.4100	2.0

- **BACTERIAL ENDOTOXINS TEST (85)** (where it is intended for use in coating containers that come in contact with articles for parenteral use)

Sample: 1.0 mL

Analysis: Mix the *Sample* with 4.0 mL of polydimethylsiloxane having a viscosity of 0.65 centistokes, previously tested and shown to be negative for bacterial endotoxins, by mixing on a vortex mixer for 1 min in an extraction tube. Add 10 mL of water, and mix on a vortex mixer for NLT 60 min. Allow the layers to separate, and use the lower aqueous layer as the sample.

Acceptance criteria: It contains NMT 1.0 USP Endotoxin Unit/mL, equivalent to NMT 10 Endotoxin Units/mL of the Dimethicone taken.

- **VISCOSITY—CAPILLARY VISCOMETER METHODS (911) and ROTATIONAL RHEOMETER METHODS (912)**

Analysis: Determine the viscosity of Dimethicone having a nominal viscosity of less than 1000 centistokes at $25 \pm 0.1^\circ$, using a capillary viscometer. Determine the viscosity of Dimethicone having a nominal viscosity of 1000 centistokes or greater at $25 \pm 0.1^\circ$, using a rotational viscometer.

Acceptance criteria: See *Table 1*.

ADDITIONAL REQUIREMENTS

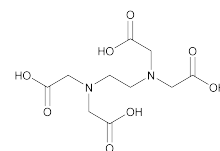
- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate its nominal viscosity value. Dimethicone intended for use in coating containers that come in contact with articles for parenteral use is so labeled.
- **USP REFERENCE STANDARDS (11)**
USP Endotoxin RS
USP Polydimethylsiloxane RS

Docusate Sodium—see *Docusate Sodium General Monographs*

Edetate Calcium Disodium—see *Edetate Calcium Disodium General Monographs*

Edetate Disodium—see *Edetate Disodium General Monographs*

Edetic Acid



$C_{10}H_{16}N_2O_8$ 292.24
Glycine, *N,N'*-1,2-ethanediylbis[*N*-(carboxymethyl)-; (Ethylenedinitrilo)tetraacetic acid [60-00-4].

DEFINITION

Edetic Acid contains NLT 98.0% and NMT 100.5% of edetic acid ($C_{10}H_{16}N_2O_8$).

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K)**

ASSAY

- **PROCEDURE**

Standard: 200 mg of chelometric standard calcium carbonate, previously dried to 110° for 2 h and cooled in a desiccator

Sample solution: Transfer 1.4 g of Edetic Acid to a 100-mL volumetric flask, dissolve in 11 mL of 1 N sodium hydroxide, dilute with water to volume, with cooling if necessary, and mix.

Analysis: Add 10 mL of water to the *Standard*, swirl to form a slurry, and cover the beaker with a watch glass. Without removing the watch glass, add 2 mL of 3 N hydrochloric acid from a pipet, and swirl to dissolve. Wash down the sides of the container, the outer surface of the pipet, and the watch glass with water, and dilute with water to 100 mL. While stirring with a magnetic stirrer, add 30 mL of the *Sample solution* from a 50-mL buret. Add 10 mL of 1 N sodium hydroxide and 300 mg of hydroxy naphthol blue, and continue the titration with the *Sample solution* to a blue endpoint. Calculate the percentage of edetic acid ($C_{10}H_{16}N_2O_8$) in the portion of sample taken:

$$\text{Result} = [W/(V \times C)] \times (M_{r1}/M_{r2}) \times 100$$

W = weight of calcium carbonate (mg)
V = volume of the *Sample solution* consumed in the titration (mL)
C = concentration of the *Sample solution* (mg/mL)
M_{r1} = molecular weight of edetic acid, 292.24
M_{r2} = molecular weight of calcium carbonate, 100.09

Acceptance criteria: 98.0%–100.5%

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.2%
- **HEAVY METALS, Method II** (231): 30 µg/g
- **IRON**

Sample solution: Char 3.0 g of Edetic Acid thoroughly, and heat in an oven at 500° until most of the carbon is consumed. Cool, add 0.15 mL of nitric acid, and heat at 500° until all of the carbon is consumed. Dissolve the residue in 2 mL of a mixture of equal volumes of hydrochloric acid and water, digest in a covered dish on a steam bath for 10 min, remove the cover, and evaporate to dryness. Dissolve the residue in 1 mL of 1 N acetic acid and 20 mL of hot water, digest for 5 min on a steam bath, cool, and dilute with water to 30 mL.

Control solution: Dissolve 43.2 mg of ferric ammonium sulfate in 10 mL of 2 N sulfuric acid, and add water to make 1000 mL. Each mL contains 5 µg of Fe.

Analysis: To 2.0 mL each of *Sample solution* and *Control solution* add 2 mL of hydrochloric acid, and dilute with water to 50 mL. Add 50 mg of ammonium persulfate and 3 mL of ammonium thiocyanate solution (300 mg/mL), mix, and transfer to a color comparison tube.

Acceptance criteria: 0.005%; the color of the *Sample solution* is not deeper than that of the *Control solution*.

• LIMIT OF NITRILOTRIACETIC ACID

Mobile phase: Add 10 mL of 1.0 M tetrabutylammonium hydroxide in methanol to 200 mL of water, and adjust with 1 M phosphoric acid to a pH of 7.5 ± 0.1 . Transfer the solution so obtained to a 1000-mL volumetric flask, add 90 mL of methanol, dilute with water to volume, mix, and pass through a filter of 0.5-µm pore size.

Solution A: 10 mg/mL of cupric nitrate [Cu(NO₃)₂]

Standard stock solution: Transfer 100 mg of nitrilotriacetic acid to a 10-mL volumetric flask, and add 0.5 mL of ammonium hydroxide. Dilute with water to volume.

Standard solution: Transfer 1.0 g of Edetic Acid to a 100-mL volumetric flask, add 300 µL of *Standard stock solution*, and dilute with *Solution A* to volume. Sonicate, if necessary, to completely dissolve.

System suitability solution: Transfer 10 mg of Edetic Acid to a 100-mL volumetric flask, add 100 µL of *Standard stock solution*, and dilute with *Solution A* to volume. Sonicate, if necessary, to completely dissolve.

Sample solution: 10 mg/mL of Edetic Acid in *Solution A*. Sonicate, if necessary, to completely dissolve.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; packing L7

Flow rate: 2 mL/min

Injection volume: 50 µL

System suitability

Samples: *Standard solution* and *System suitability solution*

[NOTE—The relative retention times for nitrilotriacetic acid, copper, and edetate are about 0.35, 0.65, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 3 between the nitrilotriacetic acid and copper peaks, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria: 0.3%; the response of the nitrilotriacetic acid peak of the *Sample solution* does not exceed the difference between the nitrilotriacetic acid

peak responses from the *Standard solution* and the *Sample solution*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** (11)
USP Edetic Acid RS

Egg Phospholipids

DEFINITION

Egg Phospholipids is a mixture of naturally occurring phospholipids obtained from the yolk of hens' eggs that is suitable for use as an emulsifying agent in injectable emulsions. The content of phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, and other related phospholipids is to be reported in the certificate of analysis. It may also contain a suitable stabilizer.

ASSAY

• CONTENT OF PHOSPHOLIPIDS

Solution A: 1341.6 g of *n*-hexane, 334.1 g of 2-propanol, 39.4 g of acetic acid, and 1.45 g of triethylamine (or 2.0 mL triethylamine)

Solution B: 663.5 g of 2-propanol, 140.0 g of water, 15.8 g of acetic acid, and 0.58 g of triethylamine

Solvent: *n*-Hexane, 2-propanol, and water (23:23:4).

[NOTE—To avoid the formation of two phases, mix the 2-propanol and water first, and then add the *n*-hexane.]

Mobile phase: See the gradient table below.

Program Step	Time (min)	Flow (mL/min)	Solution A (%)	Solution B (%)
1	0	1.0	95	5
2	5.0	1.0	80	20
3	8.5	1.0	60	40
4	15.0	1.0	0	100
5	17.5	1.0	0	100
6	17.6	1.0	95	5
7	21.0	1.0	95	5
8	22.0	2.0	95	5
9	27.0	2.0	95	5
10	29.0	1.0	95	5

Standard solutions: Transfer USP Phosphatidylcholine RS, USP Phosphatidylethanolamine RS, and USP Lysophosphatidylcholine RS to separate flasks, dissolve each in *Solvent*, and dilute. *Standard solutions* of five different concentrations are prepared on the basis of the expected content of phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine in the sample. The *Standard solutions* should cover a range of 60% to 140%. Calculate the concentrations of the Standards:

$$\text{Result} = \text{WP/V}$$

W = weight of the Standard (mg)

P = purity of the designated Reference Standard

V = volume of each of the *Standard solutions* (mL)

Sample solution: 100 mg of Egg Phospholipids in a 25-mL volumetric flask. Dissolve in *Solvent*, and dilute. Calculate the concentration, in mg/mL: this value is used as the sample amount.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: evaporative light-scattering detector

Column: 4-mm × 125-mm; 5-μm packing L20

Column temperature: 55°

Injection size: 20 μL

System suitability

Sample: *Standard solutions*

[NOTE—The relative retention times for phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine are 1.00, 0.85, and 1.25, respectively.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: Each of the *Standard solutions* and *Sample solution*

Identify the peaks of the relevant analytes in the chromatogram of the *Sample solution* by comparison with the chromatograms obtained from the *Standard solutions*. Measure the areas of the analyte peaks. Plot the logarithms of the relevant responses versus the logarithms of the concentrations, in mg/mL, of each analyte obtained from the *Standard solutions*, and determine the linear regression line using a least-squares analysis. The correlation coefficient for the linear regression line is NLT 0.995. From the graphs so obtained, determine the concentration, *C*, in mg/mL, of the relevant analyte in the *Sample solution*.

Separately calculate the percentages of phosphatidylethanolamine, phosphatidylcholine, and lysophosphatidylcholine in the portion of Egg Phospholipids taken:

$$\text{Result} = (\text{CV}/\text{W}) \times 100$$

C = concentration of the relevant analyte in the *Sample solution* (mg/mL)

V = volume of the relevant analyte in the *Sample solution* (mL)

W = weight of Egg Phospholipids in the *Sample solution* (mg)

Acceptance criteria: NMT 3.0% of lysophosphatidylcholine

IMPURITIES

Inorganic Impurities

- **HEAVY METALS**, *Method II* (231): NMT 10 ppm

Organic Impurities

- **PROCEDURE: LIMIT OF NONPHOSPHATIDYL LIPIDS**

Solvent: Diethyl ether

Sample solution: 500 mg of Egg Phospholipids, dissolved in 15 mL of *Solvent*, in a 50-mL conical flask

Chromatographic system

(See *Chromatography* (621), *Column Chromatography*.)

Mode: Column

Chromatographic column: Transfer 1000 g of silica gel having a particle size of 0.05–0.2 mm into a container with well-closing screw caps. Add 150 g of water, shake well, and allow to stand for 24 h. Suspend 15 g of prepared adsorbent in 50 mL of *Solvent*, and introduce into a 1- to 2-cm chromatographic column. Drain the *Solvent* through the column to a level of about 1 cm above the silica gel bed.

Analysis

Sample: *Sample solution*

Transfer the *Sample solution* to the *Chromatographic column*. Rinse the column containing the *Sample solution* with two 15-mL portions of *Solvent*, allowing each rinse to pass through the column before adding the next. After rinsing, elute with 105 mL of *Solvent*. Evaporate the eluate (150 mL) in a tared, round-bottom, 250-mL conical flask to dryness, using a suitable rotary evaporator. The volatiles are blown out with a stream of nitrogen, and the residue is dried at 105° for 20 min. The weight of the residue

gives the oil fraction, determined as nonpolar lipids, in Egg Phospholipids.

Calculate the percentage of the nonphosphatidyl lipids taken:

$$\text{Result} = \text{A}/\text{W} \times 100$$

A = weight of the residue (mg)

W = weight of Egg Phospholipids taken in the *Sample solution* (mg)

Acceptance criteria: NMT 7.0%

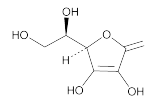
SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 20.0
- **FATS AND FIXED OILS**, *Peroxide Value* (401): NMT 3
- **BACTERIAL ENDOTOXINS TEST** (85): NMT 6 USP Endotoxin Units/g
- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total microbial count does not exceed 100 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **WATER DETERMINATION**, *Method I* (921)
Sample: 2 g in 50 mL of anhydrous methyl alcohol
Acceptance criteria: NMT 6.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve under nitrogen in a sealed container, and store at a temperature of –10° or below.
- **USP REFERENCE STANDARDS** (11)
USP Endotoxin RS
USP Phosphatidylcholine RS
USP Phosphatidylethanolamine RS
USP Lysophosphatidylcholine RS

Erythorbic Acid



$\text{C}_6\text{H}_8\text{O}_6$

o-Araboascorbic acid;

D-Erythro-hex-2-enoic acid delta-lactone;

Isoascorbic acid, D-isoascorbic acid [89-65-6].

176.12

DEFINITION

Erythorbic Acid contains NLT 99.0% and NMT 100.5% of $\text{C}_6\text{H}_8\text{O}_6$, calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)

- **B.**

Sample solution: 20 mg/mL of Erythorbic Acid and water

Analysis: To 2 mL of *Sample solution* add a few drops of sodium nitroferricyanide TS and then add 1 mL of 0.1 N sodium hydroxide.

Acceptance criteria: A transient blue color immediately appears.

- **C.**

Analysis: Dissolve about 15 mg of Erythorbic Acid in 15 mL of trichloroacetic acid (1:20). Add about 200 mg of activated charcoal, and shake the mixture vigorously for 1 min. Pass through a small fluted filter, refilter if necessary to obtain a clear filtrate, agitate the mixture until the pyrrole is dissolved, and heat in a water bath at 50°.

Acceptance criteria: A blue color appears.

ASSAY

PROCEDURE

Sample: 400 mg

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N iodine VS

Endpoint detection: Colorimetric

Analysis: Dissolve the *Sample* in a mixture of 100 mL of recently boiled and cooled water and 25 mL of 2 N sulfuric acid. Add 3 mL of starch TS, and titrate at once with 0.1 N iodine VS. Perform a blank determination. Calculate the percentage of erythorbic acid ($C_6H_8O_6$) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100] / W$$

V = *Titrant* volume consumed by the *Sample* (mL)

B = *Titrant* volume consumed by the *Blank* (mL)

N = *Titrant* actual normality (mEq/mL)

F = equivalency factor, 88.06 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5% on the dried basis

IMPURITIES

RESIDUE ON IGNITION (281): NMT 0.3%

LIMIT OF LEAD

[NOTE—Select reagents having as low a lead content as practicable, and store all solutions in borosilicate glass containers. Rinse all glassware thoroughly with warm 8 N nitric acid followed by deionized water.]

Standard stock solution: Dissolve 160 mg of lead nitrate in 100 mL of water containing 1 mL of nitric acid. Dilute with water to 1000 mL. On the day of use, transfer 10.0 mL of the above solution to a 100-mL volumetric flask, and dilute with water to volume. Each mL of this solution contains the equivalent of about 10 µg of lead.

[NOTE—Prepare the *Standard solutions* on the day of use.]

Standard solution A: 1 µg/mL of lead from the *Standard stock solution*

Standard solution B: 2 µg/mL of lead from the *Standard stock solution*

Standard solution C: 5 µg/mL of lead from the *Standard stock solution*

Sample solution: Transfer a weighed portion of about 10 g of Erythorbic Acid to an evaporating dish. Add 5 mL of 25% sulfuric acid, and distribute the sulfuric acid uniformly through the sample. Within a hood, place the dish on a steam bath to evaporate most of the water. Place the dish on a burner, and slowly pre-ash the sample by expelling most of the sulfuric acid. Place the dish in a muffle furnace at 525°, and ash the sample until the residue appears free from carbon. Cool, and cautiously wash down the inside of the evaporation dish with water. Add 5 mL of 1 N hydrochloric acid. Place the dish on a steam bath, and evaporate to dryness. Add 1.0 mL of 3 N hydrochloric acid and approximately 5 mL of water, and heat briefly on a steam bath to dissolve any residue. Transfer quantitatively to a 10-mL volumetric flask, and dilute with water to volume.

Blank: To an evaporating dish add 5 mL of 25% sulfuric acid, and distribute the sulfuric acid uniformly. Within a hood, place the dish on a steam bath to evaporate most of the water. Place the dish on a burner, and expel most of the sulfuric acid. Then place the dish in a muffle furnace at 525° for about the same amount of time as in the *Sample solution* preparation. Cool, and cautiously wash down the inside of the evaporation dish with water. Add 5 mL of 1 N hydrochloric acid.

Place the dish on a steam bath, and evaporate to dryness. Add 1.0 mL of 3 N hydrochloric acid and approximately 5 mL of water, and heat briefly on a steam bath to dissolve any residue. Transfer quantitatively to a 10-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption

Lamp: Lead electrodeless discharge

Flame: Air–acetylene

Analytical wavelength: 283.3 nm

Slit width: 0.7 nm

Standard curve

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, and *Blank*

Plot: Corrected absorbance values versus their corresponding concentration (µg/mL). [NOTE—Determine the corrected absorbance values by subtracting the absorbance of the *Blank* from the absorbance of the *Samples*.]

Analysis

Sample: *Sample solution*

From the *Standard curve*, determine the lead concentration in the *Sample solution*. Calculate the lead content, in ppm, in the portion of Erythorbic Acid taken:

$$\text{Result} = V \times C_s / W$$

V = volume of the *Sample solution* (mL)

C_s = concentration of lead in the *Sample solution* (µg/mL)

W = weight of Erythorbic Acid in the *Sample solution* (g)

Acceptance criteria: NMT 10 ppm

SPECIFIC TESTS

OPTICAL ROTATION, Specific Rotation (781S): −16.5° to −18.0°

Sample solution: 100 mg/mL in water

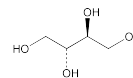
LOSS ON DRYING (731): Dry a sample in a vacuum over silica gel for 3 h: it loses NMT 0.4% of its weight.

ADDITIONAL REQUIREMENTS

PACKAGING AND STORAGE: Preserve in tight, light-resistant containers.

USP REFERENCE STANDARDS (11) USP Erythorbic Acid RS

Erythritol



$C_4H_{10}O_4$

1,2,3,4-Butanetetrol;

Butane 1,2,3,4-tetrol (*meso*-erythritol) [149-32-6].

122.12

DEFINITION

Erythritol is obtained by fermentation of starch enzyme hydrolysate (from starches such as wheat and corn). It is obtained from the fermentation broth of suitable osmophilic yeasts such as *Moniliella pollinis* or *Trichosporonoides megachiliensis*. It contains NLT 96.0% and NMT 102.0% of erythritol ($C_4H_{10}O_4$), calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B. MELTING RANGE OR TEMPERATURE** (741): 119°–123°

ASSAY• **PROCEDURE**

Mobile phase: 0.01% sulfuric acid

System suitability solution: 0.05 mg/mL each of USP Erythritol RS and glycerol

Standard solution: 50 mg/mL of USP Erythritol RS

Sample solution: 50 mg/mL of Erythritol

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 30-cm; packing L17

Column temperature: 70°

Flow rate: 0.8 mL/min

Injection size: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for erythritol and glycerol are about 1.0 and 1.1, respectively.]

Suitability requirements

Resolution: NLT 2.0 between erythritol and glycerol, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—Record chromatograms for over a period of three times the retention time of erythritol.]

Calculate in percentage of erythritol (C₄H₁₀O₄) in the portion of Erythritol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Erythritol RS in the *Standard solution* (mg/mL)

C_U = concentration of Erythritol in the *Sample solution* (mg/mL)

Acceptance criteria: 96.0%–102.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%

• **LIMIT OF LEAD**

Standard lead solution: Prepare as directed under *Heavy Metals* (231), *Special Reagents*.

Sample solution: Dissolve 20.0 g of Erythritol in diluted acetic acid, and dilute with the same medium to 100 mL. Add 2.0 mL of a saturated ammonium pyrrolidinedithiocarbamate solution (10 mg/mL of ammonium pyrrolidinedithiocarbamate) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.

Standard solutions: Prepare as directed for the *Sample solution*, except prepare three solutions by adding 0.5, 1.0, and 1.5 mL of *Standard lead solution* in addition to the 20.0 g of Erythritol.

Blank solution: Prepare as directed for the *Sample solution*, omitting Erythritol.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry, using methyl isobutyl ketone previously treated as described under *Sample solution*, but without the sample added

Analytical wavelength: 283.3 nm

Lamp: Lead hollow-cathode

Flame: Air–acetylene

Analysis

Samples: *Sample solution* and *Standard solutions*

Introduce the *Sample solution* and each of the three *Standard solutions* into the instrument. Record the steady absorbance reading. Plot the absorbance readings against the known concentrations of added lead (in µg), and draw a straight line. Extrapolate the line until it meets the concentration axis, which is equal to the concentration, in ppm, of lead in the sample.

Acceptance criteria: NMT 0.5 ppm

• **RELATED COMPOUNDS**

Mobile phase, System suitability solution, Standard solution, and Sample solution: Proceed as directed in the *Assay*.

Standard solution: Transfer 2.0 mL of the *Standard solution* from the *Assay* to a 100-mL volumetric flask, and dilute with water (1 mg/mL of erythritol).

Chromatographic system: Proceed as directed in the *Assay*, except use an *Injection size* of 20 µL.

Analysis

Samples: *Sample solution* and *Standard solution*

Calculate the percentage of each impurity found:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response of erythritol from the *Standard solution*

C_S = concentration of USP Erythritol RS in the *Standard solution* (mg/mL)

C_U = concentration of Erythritol in the *Sample solution* (mg/mL)

Acceptance criteria

Individual impurities: NMT 2.0%

Total impurities: NMT 2.0%

SPECIFIC TESTS• **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR**

SPECIFIED MICROORGANISMS (62): The total aerobic microbial count using the *Plate Method* is NMT 1000 cfu/g, and the total combined molds and yeasts count is NMT 100 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

- **LOSS ON DRYING** (731): Dry an 8-g sample at 105° for 4 h: it loses NMT 0.2% of its weight.

- **WATER DETERMINATION, Method 1** (921): NMT 0.5%

• **CONDUCTIVITY**

Sample solution: 200 mg/mL in water

Analysis: Using an appropriate conductivity meter, choose a conductivity cell that is appropriate for the properties and conductivity of the solution to be examined. Use a certified reference material,¹ for example, a solution of potassium chloride, that is appropriate for the measurement. The conductivity value of the certified reference material should be near the expected conductivity value of the solution to be examined. After calibrating the apparatus with a certified reference material solution, rinse the conductivity cell several times with water and at least twice with the aqueous solution to be examined. Measure the conductivity of the solution at a temperature of 20° while stirring gently with a magnetic stirrer.

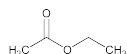
¹ Commercially available conductivity calibration solutions for conductivity meter standardization, standardized by methods traceable to the National Institute of Standards and Technology (NIST), may be used. Solutions prepared according to instructions given in the American Society for Testing and Materials (ASTM) Standard D1125 may be used, provided that the conductivity of the resultant solution is the same as that of the solution prepared from the NIST-certified material.

Acceptance criteria: NMT 20 $\mu\text{S}/\text{cm}$

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.
- **USP REFERENCE STANDARDS** (11)
USP Erythritol RS
meso-Erythritol, 1,2,3,4-butanetetrol.
 $\text{C}_4\text{H}_{10}\text{O}_4$ 122.12

Ethyl Acetate



$\text{C}_4\text{H}_8\text{O}_2$ 88.11
Acetic acid, ethyl ester;
Ethyl acetate [141-78-6].

DEFINITION

Ethyl Acetate contains NLT 99.0% and NMT 100.5% of ethyl acetate ($\text{C}_4\text{H}_8\text{O}_2$).

IDENTIFICATION

- **A.** It is readily volatilized, even at low temperatures, and is flammable. When burned, a yellow flame and acetous odor are produced.

ASSAY

PROCEDURE

Sample: Weigh 1.5 g of Ethyl Acetate in a stoppered weighing bottle.

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 0.5 N sodium hydroxide VS

Back-titrant: 0.5 N hydrochloric acid VS

Blank: 50 mL of 0.5 N sodium hydroxide VS, accurately measured

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a suitable flask. Add 50.0 mL of 0.5 N sodium hydroxide VS, and heat on a steam bath under a reflux condenser for 1 h. Allow to cool, and add phenolphthalein TS. Titrate the excess sodium hydroxide with 0.5 N hydrochloric acid VS. Perform a blank determination.

Calculate the percentage of ethyl acetate ($\text{C}_4\text{H}_8\text{O}_2$) in the *Sample* taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times F] / W\} \times 100$$

V_B = Back-titrant volume consumed by the *Blank* (mL)

V_S = Back-titrant volume consumed by the *Sample* (mL)

N = actual normality of the Back-titrant (mEq/mL)

F = equivalency factor for ethyl acetate, 88.1 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: 99.0%–100.5%

IMPURITIES

LIMIT OF NONVOLATILE RESIDUE

Sample: Ethyl Acetate

Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath, and dry at 105° for 1 h.

Acceptance criteria: NMT 0.02%

LIMIT OF METHYL COMPOUNDS

Sample: 20 mL of Ethyl Acetate

Analysis: Place the *Sample* in a 500-mL separator. Add a solution of 20 g of sodium hydroxide in 50 mL of water, and insert the stopper in the separator. Wrap it

securely in a towel for protection against the heat of the reaction. Shake the mixture vigorously for about 5 min, cautiously opening the stopcock from time to time to permit the escape of air. Continue shaking vigorously until a homogeneous liquid results, then distill, and collect about 25 mL of the distillate. To 0.05 mL of the distillate add 1 drop of dilute phosphoric acid (1 in 20) and 1 drop of potassium permanganate solution (1 in 20). Mix, allow to stand for 1 min, and add sodium bisulfite solution (1 in 20), dropwise, until the permanganate color is discharged. If a brown color remains, add 1 drop of the dilute phosphoric acid. To the colorless solution add 5 mL of freshly prepared chromotropic acid TS, and heat on a steam bath at 60° for 10 min.

Acceptance criteria: No violet color appears.

CHROMATOGRAPHIC PURITY

System suitability solution: Chloroform, ethyl acetate, isobutyl acetate, and *n*-butyl acetate (3:1:1:1)

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 1.8-m \times 4-mm; support S11

Column temperature: See *Table 1*.

Table 1

Initial Temperature (°)	Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
115	—	115	6
115	16	200	15

System suitability

Sample: 0.1 μL of *System suitability solution*

[NOTE—The retention times for chloroform, isobutyl acetate, and *n*-butyl are 0.9, 2.7, and 2.8, respectively, relative to ethyl acetate as 1.0.]

Suitability requirements

Resolution: NLT 1.3 between the chloroform and ethyl acetate peaks; NLT 1.5 between the isobutyl acetate and *n*-butyl acetate peaks

Tailing factor: NMT 1.5 for the ethyl acetate peak

Analysis

Sample: Ethyl Acetate (neat)

Inject about 0.06 μL using a 1- μL syringe.

Acceptance criteria: The area of the ethyl acetate peak is NLT 99.5% of the sum of the areas of all the peaks.

SPECIFIC TESTS

ACIDITY

Sample solution: 2.0 mL of Ethyl Acetate in 10 mL of neutralized alcohol

Analysis: Add 2 drops of phenolphthalein TS to the *Sample solution*. Neutralize with 0.10 N sodium hydroxide.

Acceptance criteria: NMT 0.10 mL of 0.10 N sodium hydroxide is required.

READILY CARBONIZABLE SUBSTANCES TEST (271)

Sample: 2 mL of Ethyl Acetate

Analysis: Add the *Sample* carefully to 10 mL of sulfuric acid to form separate layers.

Acceptance criteria: No dark zone is developed within 15 min.

SPECIFIC GRAVITY (841): 0.894–0.898

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and avoid exposure to excessive heat.

Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion

[9010-88-2].

DEFINITION

Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion is an aqueous dispersion of a copolymer of ethyl acrylate and methyl methacrylate having an average molecular weight of about 800,000. It may contain suitable emulsifying agents.

IDENTIFICATION

• INFRARED ABSORPTION (197)

Analysis: Place 1 drop of Dispersion on a glass plate,¹ and cover the test substance with a water-resistant crystal disk (silver chloride or KRS-5).² Gently press on and then remove the crystal disk. Dry the crystal disk at 80° for approximately 15 min.

Acceptance criteria: The IR absorption spectrum of Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion exhibits maxima corresponding to the same wavelengths as those of a similar preparation of USP Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion RS treated in the same manner.

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION (281):** Using mild heating conditions (e.g., steam bath, sand bath) to avoid loss of material, evaporate the Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion to dryness before ignition: NMT 0.4% residue is obtained, calculated on the undried basis.

Organic Impurities

• PROCEDURE: LIMIT OF MONOMERS

Solution A: 35 mg/mL of sodium perchlorate

Solution B: Dilute phosphoric acid with water to obtain a solution having a pH of 2.0.

Mobile phase: *Solution B* and methanol (4:1)

Standard solution: Prepare a solution in tetrahydrofuran having a concentration of 2 µg/mL each of ethyl acrylate and methyl methacrylate. To 10.0 mL of this solution add 5.0 mL of *Solution A*, and mix. Dilute 5.0 mL of the mixture with water to 10.0 mL, and mix. The solution contains a concentration of 0.67 µg/mL each of ethyl acrylate and methyl methacrylate.

Sample stock solution: 20 mg/mL of Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion in tetrahydrofuran

Sample solution: To 5.0 mL of *Solution A* add 10.0 mL of *Sample stock solution*, dropwise, while stirring continuously. Centrifuge, and filter the clear supernatant. Dilute 5.0 mL of the clear supernatant with water to 10.0 mL, and mix.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 200 nm

Column: 4.6-mm × 12.0-cm; packing L1

Flow rate: 2 mL/min

Injection size: 50 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 2.0 between ethyl acrylate and methyl methacrylate

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each monomer in the portion of Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times D \times F \times 100$$

r_U = peak response of each monomer from the *Sample solution*

r_S = peak response of each monomer from the *Standard solution*

C_S = concentration of each monomer in the *Standard solution* (µg/mL)

C_U = concentration of Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion in the *Sample stock solution* (mg/mL)

D = dilution factor for the preparation of the *Sample solution* from the *Sample stock solution*, 3

F = unit conversion factor, 10^{-3} mg/µg

Total impurities: NMT 0.01% of total monomers

SPECIFIC TESTS

• MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):

The total aerobic microbial count does not exceed 1000 cfu/g, and the total yeasts and molds count does not exceed 100 cfu/g.

• PH (791): 5.5–8.6

• **LOSS ON DRYING (731):** Dry a sample at 110° for 3 h: it loses between 68.5% and 71.5% of its weight.

• **ROTATIONAL RHEOMETER METHODS (912):** Equip a suitable rotational viscometer with an adapter comprising a cylindrical spindle rotating within an accurately machined chamber (or tube).³ Mix the Dispersion, pipet the test specimen in the specified volume, which is recommended by the instrument manufacturer, into the chamber (or tube), and ensure that the temperature of the test specimen is at $20 \pm 0.1^\circ$. The shear rate under the test condition is NLT 1 s^{-1} and NMT 100 s^{-1} .⁴ Measure the apparent viscosity, following the instrument manufacturer's directions.

Acceptance criteria: The viscosity is between 2 and 20 mPa · s.

• COAGULUM

Sample: 100 g of Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion

Analysis: Weigh a stainless steel sieve having 125-µm openings or a suitable single-woven wire cloth with a mesh width of 125 µm, and filter the *Sample* through it. [NOTE—Suitable single-woven wire cloth mesh meets the requirements set in ISO 9044.]

Wash the sieve or the cloth with distilled water until a clear filtrate is obtained, and dry the sieve or the cloth to constant weight at 105°.

Acceptance criteria: The weight of the residue does not exceed 1000 mg (1%).

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store between 5° and 25°, with excursions permitted up to 30°. Do not freeze.

• **LABELING:** Label it to indicate the name and quantity of any added emulsifiers.

• USP REFERENCE STANDARDS (11)

USP Ethyl Acrylate and Methyl Methacrylate Copolymer Dispersion RS

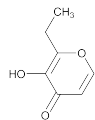
¹A simple glass microscope slide is suitable.

²KRS-5 consists of 42% thallium(I) bromide and 58% thallium(I) iodine by molecular weight. Suitable disks of silver chloride and of KRS-5 are available from www.photonic.saint-gobain.com, www.almazoptics.com, and www.internationalcrystal.net.

³A commercial device is available from Brookfield as an ultra-low (UL) viscosity adapter. The adapter comprises a 0.4-cm diameter shaft, an accurately machined chamber (or tube) with an internal diameter of 2.8 cm and a depth of 13.5 cm, and a cylindrical spindle 2.5 cm in diameter and 9.1 cm in height.

⁴The cylindrical spindle rotates at 30 rpm.

Ethyl Maltol



$C_7H_8O_3$ 140.14
 2-Ethyl-3-hydroxy-4-pyrone;
 2-Ethyl-3-hydroxy-4H-pyran-4-one;
 2-Ethyl pyromeconic acid [4940-11-8].

DEFINITION

Ethyl Maltol contains NLT 99.0% of $C_7H_8O_3$, calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197S): 1:50 solution in chloroform

ASSAY

• PROCEDURE

Standard solution: 10.0 $\mu\text{g/mL}$ of USP Ethyl Maltol RS in 0.1 N hydrochloric acid

Sample solution: 10.0 $\mu\text{g/mL}$ of Ethyl Maltol in 0.1 N hydrochloric acid

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV

Analytical wavelength: 276 nm

Cell: 1 cm

Blank: 0.1 N hydrochloric acid

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of ethyl maltol ($C_7H_8O_3$) in the portion of Ethyl Maltol taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

C_S = concentration of USP Ethyl Maltol RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = concentration of Ethyl Maltol in the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: NLT 99.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.2% at 800° for 15 min

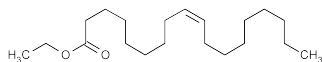
SPECIFIC TESTS

- **WATER DETERMINATION, Method I** (921): NMT 0.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** (11)
 USP Ethyl Maltol RS

Ethyl Oleate



$C_{20}H_{38}O_2$ 310.51

9-Octadecenoic acid, (Z)-, ethyl ester;
 Ethyl oleate [111-62-6].

DEFINITION

Ethyl Oleate consists of esters of ethyl alcohol and high molecular weight fatty acids, principally oleic acid.

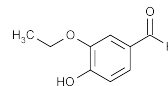
SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): 0.866–0.874 at 20°
- **VISCOSITY—CAPILLARY VISCOMETER METHODS** (911) and **ROTATIONAL RHEOMETER METHODS** (912): NLT 5.15 centipoises
- **FATS AND FIXED OILS, Acid Value** (401): NMT 0.5
- **FATS AND FIXED OILS, Iodine Value** (401): 75–85
- **FATS AND FIXED OILS, Saponification Value** (401): 177–188
- **REFRACTIVE INDEX** (831): 1.443–1.450

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Ethyl Vanillin



$C_9H_{10}O_3$ 166.17
 Benzaldehyde, 3-ethoxy-4-hydroxy-;
 3-Ethoxy-4-hydroxybenzaldehyde [121-32-4].

DEFINITION

Ethyl Vanillin, dried over phosphorus pentoxide for 4 h, contains NLT 98.0% and NMT 101.0% of $C_9H_{10}O_3$.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B. ULTRAVIOLET ABSORPTION** (197U)
Sample solution: 8 $\mu\text{g/mL}$ in methanol
Acceptance criteria: Meets the requirements

ASSAY

• PROCEDURE

Sample: 300 mg of Ethyl Vanillin (previously dried)

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N sodium methoxide VS

Blank: 50 mL of dimethylformamide, accurately measured

Endpoint detection: Visual

Analysis: Transfer the *Sample solution* to a 125-mL conical flask, and dissolve in 50 mL of dimethylformamide. Add thymol blue TS and titrate, using a magnetic stirrer and taking precautions against the absorption of atmospheric carbon dioxide. Perform a blank determination. Calculate the percentage of ethyl vanillin ($C_9H_{10}O_3$) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 166.2 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: 98.0%–101.0% on the previously dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** (741): 76°–78°
- **LOSS ON DRYING** (731): Dry a sample over phosphorus pentoxide for 4 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** (11)
USP Ethyl Vanillin RS

Ethylcellulose

Cellulose, ethyl ether;
Cellulose ethyl ether [9004-57-3].

DEFINITION

Ethylcellulose is a partly O-ethylated cellulose. It contains NLT 44.0% and NMT 51.0% of ethoxy (–OC₂H₅) groups, calculated on the dried basis.

IDENTIFICATION

- **INFRARED ABSORPTION** (197K)

ASSAY

- **PROCEDURE** [NOTE—Hydriodic acid and its reaction byproducts are highly toxic. Perform all steps of the *Sample solution* preparation and the *Standard solution* preparation in a properly functioning hood.]

Internal standard solution: Dilute 120 µL of toluene with o-xylene to 10 mL.

Standard solution: Transfer 100.0 mg of adipic acid, 4.0 mL of the *Internal standard solution*, and 4.0 mL of hydriodic acid into a suitable 10-mL thick-walled reaction vial with a pressure-tight septum closure. Close the vial tightly, and weigh the vial and contents accurately. Afterwards inject 50 µL of iodoethane through the septum with a syringe, weigh the vial again, and calculate the mass of iodoethane added, by difference. Shake well, and allow the layers to separate. Use the upper layer for analysis.

Sample solution: Transfer 50.0 mg of Ethylcellulose, 50 mg of adipic acid, and 2.0 mL of the *Internal standard solution* into a suitable 5-mL thick-walled reaction vial with a pressure-tight septum closure. Cautiously add 2.0 mL of hydriodic acid, immediately close the vial tightly, and weigh the contents and the vial accurately. Shake the vial for 30 s, heat to 125° for 10 min, allow to cool for 2 min, shake again for 30 s, and heat to 125° for 10 min. Afterwards allow to cool for 2 min, and repeat shaking and heating for a third time. Allow the vial to cool for 45 min, and reweigh. If the loss is greater than 10 mg, discard the mixture and prepare another. Use the upper layer for analysis.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 2-mm × 5.0-m stainless steel column packed with 3% G2 on 150–180-µm mesh support S1A

Temperature

Column: 80°

Injector: 200°

Detector: 200°

Carrier gas: Nitrogen

Flow rate: 15 mL/min

Injection volume: 1 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for iodoethane, toluene, and o-xylene are 0.6, 1.0, and 2.3, respectively.]

Suitability requirements

Resolution: NLT 2.0 between iodoethane and toluene

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of ethoxy content of the Ethylcellulose as declared in the labeling:

$$\text{Result} = (451,000/312) \times (R_U \times m_2) / [(R_S \times m_1) \times (100 - d)]$$

R_U = ratio of the iodoethane peak area to the toluene peak area from the *Sample solution*

m_2 = mass of iodoethane used in the *Standard solution* (mg)

R_S = ratio of the iodoethane peak area to the toluene peak area from the *Standard solution*

m_1 = mass of Ethylcellulose used in the *Sample solution* (mg)

d = loss on drying as a percentage

Acceptance criteria: 44.0%–51.0% on the dried basis

IMPURITIES**Inorganic Impurities**

- **RESIDUE ON IGNITION** (281): NMT 0.5%, determined on 1.0 g

- **HEAVY METALS, Method II** (231): NMT 20 ppm

CHLORIDES

Solution A: Nitric acid in water (1 in 5)

Standard stock solution: 0.824 mg/mL of sodium chloride

Standard solution: 8.24 µg/mL of sodium chloride, prepared from the *Standard stock solution*. [NOTE—Prepare immediately before use.]

Sample solution: Disperse 250 mg in 50 mL of water, heat to boiling, and allow to cool, shaking occasionally. Filter, and discard the first 10 mL of the filtrate.

Analysis

Samples: *Standard solution* and *Sample solution*

Separately dilute 10 mL of the *Sample solution* and *Standard solution* with water to 15 mL, add 1 mL of *Solution A*, and pour the mixtures as a single addition into test tubes containing 1 mL of 0.1 N silver nitrate VS. Examine the tubes laterally against a black background.

Acceptance criteria: After standing for 5 min protected from light, any opalescence in the *Sample solution* is not more intense than that in the *Standard solution* (0.1%).

Organic Impurities**• PROCEDURE: ACETALDEHYDE**

Solution A: 0.5 mg/mL of methylbenzothiazolone hydrazone hydrochloride

Solution B: 10 mg/mL of ferric chloride and 10 mg/mL of sulfamic acid

Standard stock solution: 10 mg/mL of acetaldehyde in 2-propanol. [NOTE—Use immediately.]

[NOTE—Prepare the *Standard solution* and *Sample solution* at the same time.]

Standard solution: 3 µg/mL of acetaldehyde from the *Standard stock solution* in water. [NOTE—Use immediately.]

Sample solution: Dissolve 3.0 g of ethylcellulose in 10 mL of water, stir by mechanical means for 1 h,

allow to stand for 24 h, filter, and dilute the filtrate with water to 100.0 mL.

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 5.0 mL of the *Sample solution* and *Standard solution* to separate flasks. To each flask add 5 mL of *Solution A*, and heat in a water bath at 60° for 5 min. Add 2 mL of *Solution B*, and heat again at 60° for 5 min. Cool, and dilute with water to 25.0 mL.

Acceptance criteria: The *Sample solution* is not more intensely colored than the *Standard solution*.

SPECIFIC TESTS

• VISCOSITY—CAPILLARY VISCOMETER METHODS (911)

Solution A: Alcohol and toluene (1:4 w/w)

Sample solution: Shake a quantity of Ethylcellulose, equivalent to 5.00 g of the dried substance, with 95 g of *Solution A* until the substance is dissolved.

Analysis: Determine the viscosity using a capillary viscometer.

Acceptance criteria: The viscosity (mPa · s) determined at 25° is NLT 80.0% and NMT 120.0% of that stated on the label for a nominal viscosity greater than 6 mPa · s; and NLT 75.0% and NMT 140.0% of that stated on the label for a nominal viscosity of NMT 6 mPa · s.

• ACIDITY OR ALKALINITY

Solution A: Dissolve 100 mg of phenolphthalein in 80 mL of alcohol, and dilute with water to 100 mL.

Solution B: Dilute 50 mg of methyl red with 1.86 mL of 0.1 N sodium hydroxide and 50 mL of alcohol, and dilute with water to 100 mL.

Sample solution: To 0.5 g of Ethylcellulose add 25 mL of carbon dioxide-free water, and shake for 15 min. Pass through a sintered-glass filter with a maximum diameter of pores between 16 and 40 µm.

Analysis: To 10 mL of *Sample solution* add 0.1 mL of *Solution A* and 0.5 mL of 0.01 N sodium hydroxide (*Solution C*). To 10 mL of *Sample solution* add 0.1 mL of *Solution B* and 0.5 mL of 0.01 N hydrochloric acid (*Solution D*).

Acceptance criteria: *Solution C* is pink; *Solution D* is red.

• LOSS ON DRYING (731):

Dry a sample at 105° for 2 h: it loses NMT 3.0% of its weight.

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE:

Preserve in well-closed containers.

• LABELING:

Label it to indicate its nominal viscosity in mPa · s for a 5% m/m solution.

• USP REFERENCE STANDARDS (11)

USP Ethylcellulose RS

Analysis: Transfer a few mL of the *Standard solution* and the *Sample solution* to two separate silver chloride plates, and evaporate.

Acceptance criteria: The IR absorption spectrum of the residue in the regions 3600 to 2600 cm⁻¹ and 1500 to 800 cm⁻¹ exhibits maxima only at the same wavenumbers as that of a film of USP Ethylcellulose RS.

• B.

Sample: 2 mL

Analysis: Transfer the *Sample* to a Petri dish, 100 mm in diameter, so that the bottom of the dish is covered uniformly. Place the dish in an oven or on a hot plate to evaporate the water.

Acceptance criteria: A transparent film results.

• C.

Sample solution: Dissolve the film formed in *Identification test B* in 20 mL of chloroform.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 2-mm × 1.8-m; 10% liquid phase G1 on support S1A

Temperatures

Injection port: 250°

Detector: 275°

Column: 220°

Injection volume: 2 µL

Acceptance criteria: The retention time of the major peak following the solvent peak from the sample corresponds to that from a similar solution of USP Cetyl Alcohol RS.

• D.

Indicator solution: To a 150-mL graduated beaker containing 0.7 mL of sulfuric acid and 5 g of anhydrous sodium sulfate slowly add water to the 90-mL mark. Add methylene blue solution (3 in 1000) to the 100-mL mark.

Analysis: To 1 mL of Dispersion in a 100-mL graduated mixing cylinder add 9 mL of water followed by 25 mL of *Indicator solution*. Add 15 mL of chloroform, and shake vigorously. Allow the two phases to separate.

Acceptance criteria: The lower phase is blue, indicating the presence of sodium lauryl sulfate.

ASSAY

• PROCEDURE

Sample solution: Dispersion equivalent to 25 mg of ethylcellulose

Analysis: Determine the ethoxy content, as directed in *Methoxy Determination* (431). Calculate the ethylcellulose content from the ethoxy content found, and calculate the ethoxy content of the ethylcellulose as declared in the labeling. Each mL of 0.1 N sodium thiosulfate is equivalent to 0.7510 mg of (–OC₂H₅).

Acceptance criteria: 90.0%–110.0%

IMPURITIES

• HEAVY METALS, Method II (231):

NMT 10 µg/g

SPECIFIC TESTS

• ROTATIONAL RHEOMETER METHODS (912)

Sample: Neat

Analysis: Use a rotational viscometer equipped with a low-viscosity adapter. Mix the *Sample*, and pipet 20 mL of it into the low-viscosity small sample adapter. Start the viscometer, and take readings after 60, 90, and 120 s at a temperature of 25 ± 2° and at a spindle speed that results in readings of 10%–90% of full-scale. Multiply the average of the three readings by the factor specified for the selected spindle speed to obtain the viscosity in centipoises.

Ethylcellulose Aqueous Dispersion

DEFINITION

Ethylcellulose Aqueous Dispersion is a colloidal dispersion of Ethylcellulose in water. It contains NLT 90.0% and NMT 110.0% of the labeled amount of ethylcellulose. It contains suitable amounts of Cetyl Alcohol and Sodium Lauryl Sulfate, which assist in the formation and stabilization of the dispersion. It may contain suitable antifoaming and antimicrobial agents.

IDENTIFICATION

• A.

Diluent: Toluene and alcohol (80:20)

Standard solution: 250 mg of USP Ethylcellulose RS in 5 mL of *Diluent*

Sample solution: A small quantity of Dispersion in 5 mL of *Diluent*

- Acceptance criteria: NMT 150 centipoises
- **PH** (791): 4.0–7.0
- **LOSS ON DRYING** (731)
Sample: 5 mL
Analysis: Place 10 g of standard 20- to 30-mesh sand, previously dried for at least 30 min at 60°, into a tared Petri dish. Add the *Sample*, and again weigh. Dry at 60° to constant weight.
- Acceptance criteria: NMT 71.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and protect from freezing.
- **LABELING:** The labeling states the ethoxy content of the ethylcellulose and the percentage of ethylcellulose. The labeling also states the names and quantities of any added antifoaming and antimicrobial agents.
- **USP REFERENCE STANDARDS** (11)
USP Cetyl Alcohol RS
USP Ethylcellulose RS

Ethylcellulose Dispersion Type B

DEFINITION

A stabilized dispersion of ethylcellulose in water. It contains NLT 90.0% and NMT 110.0% of the labeled amount of Ethylcellulose. It may contain suitable amounts of plasticizers, stabilizers, and glidants.

IDENTIFICATION

- **A. FILM FORMATION**
Analysis: Transfer an appropriate quantity of Ethylcellulose Dispersion Type B to a clear glass plate, distribute evenly, and place in a laboratory oven at about 60° until dry. [NOTE—It may take less than 60 min.]
Acceptance criteria: A continuous transparent or translucent film is formed.
- **B. INFRARED ABSORPTION**
Analysis: Use the film prepared in *Identification* test A and perform *Infrared Absorption* (197A).
Standard spectrum: Perform *Infrared Absorption* (197A) using USP Ethylcellulose RS.
Acceptance criteria: The IR absorption spectrum of the film so formed in the 3600–2600 cm⁻¹ and 1500–800 cm⁻¹ regions exhibits maxima corresponding to the same wave numbers as the *Standard spectrum*.
- **C.** The retention time of the ethylcellulose peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*. [NOTE—Plasticizer and/or stabilizer peaks may be present in the chromatogram.]

ASSAY

- **PROCEDURE**
Mobile phase: Tetrahydrofuran
Standard solution: Transfer 375 mg of USP Ethylcellulose RS to a 100-mL volumetric flask, and add 70 mL of tetrahydrofuran. Shake by mechanical means until the ethylcellulose is dissolved, and dilute with tetrahydrofuran to volume. The *Standard solution* contains 3.75 mg/mL of USP Ethylcellulose RS.
Sample solution: Add 30 mL of tetrahydrofuran to 1.0 g of Ethylcellulose Dispersion Type B in a 50-mL volumetric flask, and mix the mixture on a suitable shaker for 15 min. Dilute with tetrahydrofuran to volume, and mix. The *Sample solution* contains 20 mg/mL of Ethylcellulose Dispersion Type B in tetrahydrofuran. [NOTE—If Ethylcellulose Dispersion Type B contains inorganic insoluble material, a portion of the *Sample solution* should be centrifuged at 15,800 × g for NLT 15 min.]
Chromatographic system
(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 8.0-mm × 30-cm; 6-μm packing L21

Temperature

Detector: 45°

Column: 45°

Flow rate: 0.5 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 5.0% determined for the ethylcellulose peak

Tailing factor: NMT 2.0 for the ethylcellulose peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of ethylcellulose in the portion of Ethylcellulose Dispersion Type B taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of ethylcellulose from the *Sample solution*

r_S = peak response of ethylcellulose from the *Standard solution*

C_S = concentration of USP Ethylcellulose RS in the *Standard solution* (mg/mL)

C_U = concentration of Ethylcellulose Dispersion Type B in the *Sample solution* (mg/mL)

Acceptance criteria: 90.0%–110.0%

OTHER COMPONENTS

[NOTE—Perform the test for individual plasticizers, stabilizers, or glidants only if they are included in the *Labeling*.]

• CONTENT OF MEDIUM-CHAIN TRIGLYCERIDES

Mobile phase, *Sample solution*, and Chromatographic system: Proceed as directed in the *Assay*.

Standard solution: Transfer 375 mg of USP Ethylcellulose RS, 60 mg of medium-chain triglycerides, and 40 mg of oleic acid to a 100-mL volumetric flask. Add 70 mL of tetrahydrofuran, and shake by mechanical means until the ethylcellulose is dissolved. Dilute with tetrahydrofuran to volume. The *Standard solution* contains 3.75 mg/mL of USP Ethylcellulose RS, 0.6 mg/mL of medium-chain triglycerides, and 0.4 mg/mL of oleic acid. [NOTE—Oleic acid is included in the *Standard solution* to assist with consistent integration between *Standard* and *Sample solutions*.]

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for ethylcellulose, medium-chain triglycerides, and oleic acid are 1.00, 1.18, and 1.25, respectively.]

Suitability requirements

Relative standard deviation: NMT 5.0% determined for the medium-chain triglycerides peak

Resolution: NLT 2.0 between ethylcellulose and medium-chain triglycerides

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of medium-chain triglycerides in the portion of Ethylcellulose Dispersion Type B taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of medium-chain triglycerides from the *Sample solution*

r_S = peak response of medium-chain triglycerides from the *Standard solution*

C_S = concentration of medium-chain triglycerides in the *Standard solution* (mg/mL)

C_U = concentration of Ethylcellulose Dispersion Type B in the *Sample solution* (mg/mL)

Acceptance criteria: The percentage content of medium-chain triglycerides falls within the quantity range indicated by the *Labeling*. The ratio of medium-chain triglycerides to ethylcellulose is less than 0.25.

• **CONTENT OF OLEIC ACID**

Standard solution: 1.68 mg/mL of USP Oleic Acid RS in tetrahydrofuran

Sample solution: Add 15 mL of tetrahydrofuran to 2.0 g of Ethylcellulose Dispersion Type B in a 25-mL volumetric flask, and mix the mixture on a suitable shaker for 15 min. Dilute with tetrahydrofuran to volume, and mix. The *Sample solution* contains 80 mg/mL of Ethylcellulose Dispersion Type B in tetrahydrofuran.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m capillary column; 0.25-μm layer of phase G25 (or G35)

Temperature

Detector: 280°

Injector port: 280°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	—	120	5
120	10	250	20

Carrier gas: Helium

Flow rate: 7.0 mL/min

Injection size: 1.0 μL

Injection type: Splitless

System suitability

Sample: *Standard solution*

[NOTE—The retention time for oleic acid is about 18.5 min.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of oleic acid in the portion of Ethylcellulose Dispersion Type B taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of oleic acid from the *Sample solution*

r_S = peak response of oleic acid from the *Standard solution*

C_S = concentration of USP Oleic Acid RS in the *Standard solution* (mg/mL)

C_U = concentration of Ethylcellulose Dispersion Type B in the *Sample solution* (mg/mL)

Acceptance criteria: The percentage content of oleic acid falls within the quantity range indicated by the *Labeling*. The ratio of oleic acid to ethylcellulose is less than 0.15.

• **CONTENT OF DIBUTYL SEBACATE AND OLEIC ACID**

Standard solution: 0.74 mg/mL of USP Dibutyl Sebacate RS and 0.48 mg/mL of USP Oleic Acid RS in tetrahydrofuran

Sample solution: Add 25 mL of tetrahydrofuran to 1.0 g of Ethylcellulose Dispersion Type B in a 50-mL volumetric flask, and mix the mixture on a suitable shaker for 15 min. Dilute with tetrahydrofuran to volume, and mix. The *Sample solution* contains 20 mg/mL of Ethylcellulose Dispersion Type B in tetrahydrofuran. [NOTE—If Ethylcellulose Dispersion Type B contains inorganic insoluble material, a portion

of the *Sample solution* should be centrifuged at 15,800 × *g* for NLT 15 min.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 15-m capillary column; 0.1-μm layer of phase G25 (or G35)

Temperature

Detector: 280°

Injector port: 280°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	—	150	2
150	10	250	10

Carrier gas: Helium

Flow rate: 5.0 mL/min

Injection size: 0.5 μL

Injection type: Split (ratio about 10:1)

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for dibutyl sebacate and oleic acid are 1.00 and 1.45, respectively.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Resolution: NLT 2.0 between dibutyl sebacate and oleic acid

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each component (dibutyl sebacate or oleic acid) in the portion of Ethylcellulose Dispersion Type B taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each component (dibutyl sebacate or oleic acid) from the *Sample solution*

r_S = peak response of each component (dibutyl sebacate or oleic acid) from the *Standard solution*

C_S = concentration of each component (USP Dibutyl Sebacate RS or USP Oleic Acid RS) in the *Standard solution* (mg/mL)

C_U = concentration of Ethylcellulose Dispersion Type B in the *Sample solution* (mg/mL)

Acceptance criteria

Dibutyl sebacate: The percentage content falls within the quantity range indicated by the *Labeling*. The ratio of dibutyl sebacate to ethylcellulose is less than 0.25.

Oleic acid: The percentage content falls within the quantity range indicated by the *Labeling*. The ratio of oleic acid to ethylcellulose is less than 0.15.

IMPURITIES

Inorganic Impurities

• **RESIDUE ON IGNITION** <281>: NMT 1.95%. [NOTE—Perform this test only if Ethylcellulose Dispersion Type B contains inorganic nonvolatile material.]

Organic Impurities

• **PROCEDURE 1: LIMIT OF GLYCERIN**

[NOTE—Perform this test only if Ethylcellulose Dispersion Type B contains glycerides.]

Standard solution: 0.05 mg/mL of USP Glycerin RS in methanol

Sample solution: Add 15 mL of methanol to 2.0 g of Ethylcellulose Dispersion Type B in a 25-mL volumetric flask, and mix the mixture on a suitable shaker for 15 min. Dilute with methanol to volume, and mix. The

Sample solution contains 80 mg/mL of Ethylcellulose Dispersion Type B in methanol.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m capillary column; 3.0-μm layer of phase G43

Temperature

Detector: 280°

Injector port: 280°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	10	150	2
150	15	240	20

Carrier gas: Helium

Flow rate: 10.0 mL/min

Injection size: 1.0 μL

Injection type: Splitless

System suitability

Sample: *Standard solution*

[NOTE—The retention time for glycerin is about 3.8 min.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Tailing factor: NMT 2.5

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of glycerin in the portion of Ethylcellulose Dispersion Type B taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of glycerin from the *Sample solution*

r_S = peak response of glycerin from the *Standard solution*

C_S = concentration of USP Glycerin RS in the *Standard solution* (mg/mL)

C_U = concentration of Ethylcellulose Dispersion Type B in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.6%

• PROCEDURE 2: LIMIT OF 1-BUTANOL

[NOTE—Perform this test only if Ethylcellulose Dispersion Type B contains butyl esters.]

Standard solution: 0.1 mg/mL of USP 1-Butanol RS in methanol

Sample solution: Add 15 mL of methanol to 2.0 g of Ethylcellulose Dispersion Type B in a 25-mL volumetric flask, and mix the mixture on a suitable shaker for 15 min. Dilute with methanol to volume, and mix. The *Sample solution* contains 80 mg/mL of Ethylcellulose Dispersion Type B in methanol. [NOTE—If Ethylcellulose Dispersion Type B contains inorganic insoluble material, a portion of the *Sample solution* should be centrifuged at $15,800 \times g$ for 30 min.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m capillary column; 1.0-μm layer of phase G16

Temperature

Detector: 250°

Injector port: 250°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
45	—	45	5
45	10	80	—
80	20	220	10

Carrier gas: Helium

Flow rate: 10.0 mL/min

Injection size: 0.5 μL

Injection type: Splitless

System suitability

Sample: *Standard solution*

[NOTE—The retention time for 1-butanol is about 7.8 min.]

Suitability requirements

Relative standard deviation: NMT 5.0%

Tailing factor: NMT 2.0

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of 1-butanol in the portion of Ethylcellulose Dispersion Type B taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of 1-butanol from the *Sample solution*

r_S = peak response of 1-butanol from the *Standard solution*

C_S = concentration of USP 1-Butanol RS in the *Standard solution* (mg/mL)

C_U = concentration of Ethylcellulose Dispersion Type B in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.2%

SPECIFIC TESTS

• pH (791): 9.5–11.5

• TOTAL SOLIDS

Analysis: Place 3 g (4 mm in diameter) of glass beads in an aluminum dish, and weigh. Add 10 g of Ethylcellulose Dispersion Type B, and again weigh. Dry at about 105° for 3 h. Determine the percentage of total solids in Ethylcellulose Dispersion Type B.

Acceptance criteria: 23.0%–26.0%

• ROTATIONAL RHEOMETER METHODS (912)

Sample: 500 mL of Ethylcellulose Dispersion Type B

Analysis: Transfer the *Sample* to a beaker with an inside diameter of 83 mm. Place the beaker in a water bath, cover with a watchglass, allow to equilibrate at $25 \pm 0.1^\circ$, and let air bubbles dissipate. Keep the sample free from entrapped air bubbles and uniform in temperature. Stir the dispersion in the beaker at low agitation speed to ensure homogeneity, making sure that no air bubbles are incorporated. Determine viscosity at $25 \pm 0.1^\circ$, using a suitable rotational viscometer with a spindle having a cylinder 4.7 cm in diameter and 0.2 cm high attached to a shaft 0.3 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 2.7 cm, and the immersion depth being 4.9 cm.¹ Operate the viscometer at 20 rpm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

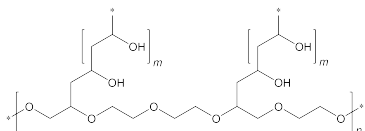
¹ A commercial instrument is available as an RV2 spindle from Brookfield.

Acceptance criteria: 400–1500 mPa · s

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers at a temperature below 25°. Protect from freezing.
- **LABELING:** The labeling states the percentage of ethylcellulose. Label it to indicate the names and quantity ranges of any added plasticizers, stabilizers, and glidants. Label it to indicate whether any fatty acid components, or fatty acid-containing components are derived from animal, vegetable, or synthetic sources.
- **USP REFERENCE STANDARDS** (11)
 - USP 1-Butanol RS
 - 1-Butanol.
 - $C_4H_{10}O$ 74.12
 - USP Dibutyl Sebacate RS
 - Decanedioic acid; Dibutyl ester.
 - $C_{18}H_{34}O_4$ 314.46
 - USP Ethylcellulose RS
 - Cellulose, ethyl ether; Cellulose ethyl ether.
 - USP Glycerin RS
 - 1,2,3-Propanetriol; Glycerol.
 - USP Oleic Acid RS
 - 9-Octadecenoic acid, (Z)-; Oleic acid.
 - $C_{18}H_{34}O_2$ 282.46

Ethylene Glycol and Vinyl Alcohol Graft Copolymer



Polyethylene glycol-graft-polyvinyl alcohol;
Graft-copoly(ethylene glycol-vinyl alcohol) [121786-16-1].

DEFINITION

Ethylene Glycol and Vinyl Alcohol Graft Copolymer is a graft copolymer of ethylene glycol and vinyl alcohol. It consists of about 75% of vinyl alcohol ($-\text{CH}_2\text{CH}(\text{OH})-$) units and 25% of ethylene glycol ($-\text{CH}_2\text{CH}_2\text{O}-$) units. The Copolymer is produced by using polyethylene glycol and vinyl acetate as starting materials. Polyethylene glycol forms the polymer backbone on which polyvinyl acetate is grafted. Hydrolysis of polyvinyl acetate side chains leads to formation of polyvinyl alcohol grafted chains. Ethylene Glycol and Vinyl Alcohol Graft Copolymer may contain glidant to improve flowability.

IDENTIFICATION

• INFRARED ABSORPTION

Sample: 200 mg of Ethylene Glycol and Vinyl Alcohol Graft Copolymer

Analysis: Dissolve the *Sample* in 5–10 mL of water. Spread 1 mL of this solution on a thallium bromide window.¹ Totally moisten the surface area of the window. Dry the window at 110° for approximately 30 min, and allow the window to cool to room temperature.

Acceptance criteria: The IR absorption spectrum of Ethylene Glycol and Vinyl Alcohol Graft Copolymer ex-

¹ Thallium bromide, also known as KRS-5, consists of 42% thallium(I) bromide and 58% thallium(I) iodide by molecular weight. Suitable disks of KRS-5 are available from www.photonic.saint-gobain.com, www.almazoptics.com, and www.internationalcrystal.net.

hibits maxima corresponding to the same wavelengths as those of a similar preparation of USP Ethylene Glycol and Vinyl Alcohol Graft Copolymer RS treated in the same manner.

IMPURITIES

Inorganic Impurities

• RESIDUE ON IGNITION (281)

Sample: 10.0 g

Acceptance criteria: NMT 3.0%

Organic Impurities

• PROCEDURE 1: ETHYLENE OXIDE AND DIOXANE

[**CAUTION**—Ethylene oxide is toxic and flammable. Prepare these solutions in a well-ventilated fume hood, using great care. Protect both hands and face by wearing polyethylene protective gloves and an appropriate face mask. Store all solutions in hermetic containers, and refrigerate at a temperature between 4° and 8°.]

[**NOTE**—Before using polyethylene glycol 200 in this test, remove any volatile components from it by placing 500 mL of polyethylene glycol 200 in a 1000-mL round-bottom flask, and attaching the flask to a rotary evaporator maintained at a temperature of 60° and under a vacuum of 10–20 mm Hg for 6 h.]

Acetaldehyde solution: 10 µg/mL of acetaldehyde.

[**NOTE**—Prepare the *Acetaldehyde solution* immediately before use.]

Ethylene oxide stock solution: Introduce 300 µL (corresponding to 250 mg) of gaseous ethylene oxide to the polyethylene glycol 200. Determine the absorbed mass of ethylene oxide via the weight of the solution before and after the absorption. Dilute the solution with the polyethylene glycol 200 to 100 g. This stock solution contains 2.5 mg/g of ethylene oxide. [**NOTE**—Prepare this stock solution immediately before use, and store in a refrigerator after preparation.]

Ethylene oxide solution: 100 µg/g of ethylene oxide in polyethylene glycol 200 from *Ethylene oxide stock solution*. Transfer 5 g of 100 µg/g ethylene oxide in polyethylene glycol 200 to a 50-mL volumetric flask filled with 30 mL of water. Dilute with water to volume, and mix to obtain a solution containing 10 µg/mL of ethylene oxide. [**NOTE**—Prepare this solution immediately before use, and use the solution directly after preparation.]

Dioxane solution: 500 µg/mL of dioxane

Standard solution A: Transfer 0.1 mL of *Ethylene oxide solution* to a 10-mL pressure headspace vial. [**NOTE**—Other sizes, such as 22-mL, may be used, depending on the operating conditions; however, the same size must be used for *Standard solution A*, *Standard solution B*, and the *Sample solution*.] Add 0.1 mL of *Acetaldehyde solution* and 0.1 mL of *Dioxane solution*, seal the vial, and mix.

Standard solution B: Transfer 1.0 g of Ethylene Glycol and Vinyl Alcohol Graft Copolymer to a 10-mL pressure headspace vial. Add 0.1 mL of *Ethylene oxide solution*, 0.1 mL of *Dioxane solution*, and 1.0 mL of *N,N*-dimethylacetamide, seal the vial, and mix.

Sample solution: Transfer 1.0 g of Ethylene Glycol and Vinyl Alcohol Graft Copolymer to a 10-mL pressure headspace vial. Add 1.0 mL of *N,N*-dimethylacetamide and 0.2 mL of water, seal the vial, and mix.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

[**NOTE**—Use a headspace apparatus that automatically transfers a measured amount of headspace.]

Mode: GC

Detector: Flame ionization

Detector temperature: 250°

Column: 0.25-mm × 30-m glass or quartz capillary column; 1.0-µm layer of phase G1

Column temperature: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (min)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	—
180	30	230	5

Carrier gas: Helium
 Flow rate: 0.8 mL/min
 Injection size: 1 mL (the gaseous headspace)
 Injection type: Split ratio 20:1
 Injection port temperature: 250°
 Headspace sampler
 Equilibration time: 45 min
 Equilibration temperature
 Standard solution A: 70°
 Standard solution B: 90°
 Sample solution: 90°
 Transfer line temperature: 150°
 Pressurization time: 1 min
 Final headspace pressure: 0.7 bar
 Injection time: 12 s

System suitability

Sample: *Standard solution A*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are 0.94 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between acetaldehyde and ethylene oxide

Signal-to-noise: NLT 5 determined from the dioxane peak

Relative standard deviation: NMT 15%

Analysis

Samples: *Standard solution B* and *Sample solution*

Calculate the content of ethylene oxide, in ppm, in the portion of Ethylene Glycol and Vinyl Alcohol Graft Copolymer taken:

$$\text{Result} = A_E \times r_U / [(r_S \times W_U) - (r_U \times W_S)]$$

A_E = quantity of ethylene oxide added to *Standard solution B* (µg)

r_U = ethylene oxide peak response from the *Sample solution*

r_S = ethylene oxide peak response from *Standard solution B*

W_U = weight of Ethylene Glycol and Vinyl Alcohol Graft Copolymer taken to prepare the *Sample solution* (g)

W_S = weight of Ethylene Glycol and Vinyl Alcohol Graft Copolymer taken to prepare *Standard solution B* (g)

Calculate the content of dioxane, in ppm, in the portion of Ethylene Glycol and Vinyl Alcohol Graft Copolymer taken:

$$\text{Result} = A_D \times r_U / [(r_S \times W_U) - (r_U \times W_S)]$$

A_D = quantity of dioxane added to *Standard solution B* (µg)

r_U = dioxane peak response from the *Sample solution*

r_S = dioxane peak response from *Standard solution B*

W_U = weight of Ethylene Glycol and Vinyl Alcohol Graft Copolymer taken to prepare the *Sample solution* (g)

W_S = weight of Ethylene Glycol and Vinyl Alcohol Graft Copolymer taken to prepare *Standard solution B* (g)

Acceptance criteria: NMT 1 ppm of ethylene oxide; NMT 10 ppm of dioxane

• PROCEDURE 2: VINYL ACETATE

Solution A: Acetonitrile, methanol, and water (5:5:90)

Solution B: Acetonitrile, methanol, and water (45:5:50)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
40	85	15
42	0	100
48	0	100
51	100	0

System suitability solution: Transfer 50 mg of vinyl acetate and 50 mg of 1-vinylpyrrolidin-2-one to a 50-mL volumetric flask, add 10 mL of methanol, and sonicate or gently shake the flask to dissolve the materials. Dilute with *Solution A* to volume. Dilute 10 mL of this solution with *Solution A* to 100 mL. Dilute 5 mL of this solution with *Solution A* to 100 mL. The *System suitability solution* contains about 5 µg/mL each of vinyl acetate and 1-vinylpyrrolidin-2-one.

Standard solution: Transfer 50 mg of vinyl acetate to a 100-mL volumetric flask, dissolve in and dilute with methanol to volume, and mix well. Dilute 5.0 mL of the solution with *Solution A* to 100 mL. Dilute 10.0 mL of this solution with *Solution A* to 100 mL. The *Standard solution* contains about 2.5 µg/mL of vinyl acetate. [NOTE—This solution should be analyzed within 1 h when stored at room temperature.]

Sample solution: Transfer 250 mg of Ethylene Glycol and Vinyl Alcohol Graft Copolymer to a 10-mL volumetric flask, and add 1–2 mL of methanol, using an ultrasonic bath if necessary. After cooling to ambient temperature, dilute with water to volume, and mix. Pass through a 0.2-mm membrane filter. [NOTE—This solution should be analyzed within 1 h when stored at room temperature.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.0-mm × 25-cm analytical column; 5-µm packing L1. A 4.0-mm × 3-cm pre-column; 5-µm packing L1 may be used if a matrix effect is observed.

[NOTE—The matrix effect may result in poor reproducibility of the retention times and of the peak shapes.]

Column temperature: 30°

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for vinyl acetate and 1-vinylpyrrolidin-2-one are 1.0 and 1.4, respectively.]

Suitability requirements

Resolution: NLT 5.0 between vinyl acetate and 1-vinylpyrrolidin-2-one

Relative standard deviation: NMT 5.0% determined from the 1-vinylpyrrolidin-2-one peak

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria: The response of the vinyl acetate peak from the *Sample solution* is NMT that of the vinyl acetate peak from the *Standard solution*, corresponding to NMT 100 ppm of vinyl acetate.

• PROCEDURE 3: ACETIC ACID/ACETATE

Mobile phase: 5 mM sulfuric acid

Standard solution: 0.3 mg/mL for each of acetic acid and citric acid in *Mobile phase*

Sample solution: 20 mg/mL of Ethylene Glycol and Vinyl Alcohol Copolymer

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm × 25-cm; 5-μm packing L1

Column clean: After each injection, rinse the column with a mixture of equal volumes of *Mobile phase* and acetonitrile.

Column temperature: Ambient

Flow rate: 1 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for acetic acid and citric acid are 1.0 and 1.3, respectively.]

Suitability requirements

Resolution: NLT 2.0 between acetic acid and citric acid

Relative standard deviation: NMT 5.0% determined from the acetic acid peak

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria: The response of the acetic acid peak from the *Sample solution* is NMT that of the acetic acid peak from the *Standard solution*, corresponding to NMT 1.5% of acetic acid.

SPECIFIC TESTS

- **FATS AND FIXED OILS, Ester Value** <401>: 10–75
 - **LOSS ON DRYING** <731>: Dry 1.0 g of Ethylene Glycol and Vinyl Alcohol Graft Copolymer in a vacuum at 140° for 1 h; it loses NMT 5.0% of its weight.
 - **PH** <791>: 5.0–8.0, in a solution of 20% (w/w) in carbon dioxide-free water
 - **ROTATIONAL RHEOMETER METHODS** <912>: After determining the *Loss on Drying*, weigh a quantity of undried Ethylene Glycol and Vinyl Alcohol Graft Copolymer, equivalent to 100.0 g on the dried basis. Transfer the sample to a short form, 600-mL beaker (internal diameter about 80 mm and height 120 mm), and add water to make the mixture weigh 500 g. Ensure a homogeneous solution by gently stirring at room temperature for 48 h. Afterwards allow the container to stand for 24 h to let the entrapped air dissipate. [NOTE—Ensure that the concentration of this solution is 20% (w/w).] Determine the viscosity of this solution at 23 ± 0.1° using a suitable rotational viscometer with a spindle having a cylinder 4.7 cm in diameter and 0.2 cm high attached to a shaft 0.3 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 2.7 cm, and the immersion depth being 4.9 cm.² Operate the viscometer at 100 rpm. Follow the instrument manufacturer's directions to measure the apparent viscosity.
- Acceptance criteria:** 25–250 mPa · s

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at a temperature below 25°.
- **LABELING:** Label it to indicate the viscosity, giving the viscosity measurement parameters, concentration of the solution, and the type of equipment used. The labeling also indicates the name and quantity of any added glidant.
- **USP REFERENCE STANDARDS** <11>
USP Ethylene Glycol and Vinyl Alcohol Graft Copolymer RS

Ethylene Glycol Stearates

DEFINITION

Ethylene Glycol Stearates is a mixture of ethylene glycol monoesters and diesters of stearic and palmitic acids. It contains NLT 50.0% of monoesters produced from the condensation of ethylene glycol and stearic acid of vegetable or animal origin.

IDENTIFICATION

- **A.** It meets the requirements in *Specific Tests* for *Melting Range or Temperature, Class II* <741>.
- **B.** It meets the requirements in *Specific Tests* for *Fats and Fixed Oils, Fatty Acid Composition* <401>.

ASSAY

• **PROCEDURE**

Mobile phase: Tetrahydrofuran

Sample solution: 40 mg/mL of Ethylene Glycol Stearates in tetrahydrofuran

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.5-mm × 60-cm; containing 5-μm 100Å packing L21. [NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the one 60-cm column, provided that *System suitability* requirements are met.]

Temperatures

Column: 40°. [NOTE—The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]

Detector: 40°

Flow rate: 1 mL/min

Injection volume: 40 μL

System suitability

Sample: *Sample solution*

[NOTE—The relative retention times for diesters, monoesters, and ethylene glycol are 0.76, 0.83, and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 2.0% for the monoesters peak

Analysis

Sample: *Sample solution*

Calculate the percentage of free fatty acids, *E*, in the portion of Ethylene Glycol Stearates taken:

$$E = I_A \times 270/561.1$$

I_A = acid value, determined in *Specific Tests* for *Fats and Fixed Oils, Acid Value (Free Fatty Acids)* <401>

Calculate the percentage of monoesters in the portion of *Sample* taken:

$$\text{Result} = [r_M / (r_M + r_D)](100 - D - E)$$

r_M = peak response for the monoesters

r_D = peak response for the diesters

D = percentage of free ethylene glycol in the portion of Ethylene Glycol Stearates taken, as determined in *Impurities for Limit of Free Ethylene Glycol*

Acceptance criteria: NLT 50.0% of monoesters

IMPURITIES

• **LIMIT OF FREE ETHYLENE GLYCOL**

Mobile phase, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the *Assay*.

² A commercial instrument is available as an RV2 spindle from Brookfield.

Standard solution A: 0.5 mg/mL of ethylene glycol in tetrahydrofuran

Standard solution B: 1.0 mg/mL of ethylene glycol in tetrahydrofuran

Standard solution C: 2.0 mg/mL of ethylene glycol in tetrahydrofuran

Standard solution D: 4.0 mg/mL of ethylene glycol in tetrahydrofuran

Analysis

Samples: *Sample solution* and *Standard solutions*

Plot the ethylene glycol peak responses obtained versus the concentration of ethylene glycol in the *Standard solutions*. From the standard curve so obtained, determine the ethylene glycol concentration in the *Sample solution*.

Calculate the percentage of free ethylene glycol in the portion of sample taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of ethylene glycol, determined from the standard curve (mg/mL)

C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE, Class II (741):** 54°–60°

• **FATS AND FIXED OILS, Acid Value (Free Fatty Acids) (401)**

Sample: 10.0 g

Acceptance criteria: NMT 3.0

• **FATS AND FIXED OILS, Iodine Value (401):** NMT 3.0

• **FATS AND FIXED OILS, Saponification Value (401)**

Sample: 2.0 g

Acceptance criteria: 170–195

• **FATS AND FIXED OILS, Fatty Acid Composition (401):** 40.0%–60.0% of stearic acid, and the sum of palmitic and stearic acids is NLT 90.0%.

• **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561)**

Sample: 1.0 g

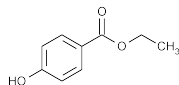
Acceptance criteria: NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.

• **LABELING:** Label it to indicate that it is intended for topical use only.

Ethylparaben



$C_9H_{10}O_3$

Benzoic acid, 4-hydroxy-, ethyl ester;
Ethyl *p*-hydroxybenzoate [120-47-8].

166.17

DEFINITION

Ethylparaben contains NLT 98.0% and NMT 102.0% of $C_9H_{10}O_3$.

IDENTIFICATION

• **A. INFRARED ABSORPTION (197M)**

• **B. MELTING RANGE OR TEMPERATURE (741):** 115°–118°

ASSAY

PROCEDURE

Mobile phase, Sample solution, Standard solution B, and Chromatographic system: Proceed as described in the procedure for *Related Substances*.

System suitability

Sample: *Standard solution B*

Suitability requirements

Relative standard deviation: NMT 0.85% for 6 injections

Analysis

Samples: *Sample solution* and *Standard solution B*

Calculate the percentage of Ethylparaben in the *Sample solution*:

$$\text{Result} = P \times (r_U \times C_S) / (r_S \times C_U)$$

P = labeled purity of USP Ethylparaben RS expressed as a percentage

r_U = peak area of ethylparaben from the *Sample solution*

C_S = concentration of ethylparaben in *Standard solution B*

r_S = peak area of ethylparaben from *Standard solution B*

C_U = concentration of Ethylparaben in the *Sample solution*

Acceptance criteria: 98.0%–102.0%

IMPURITIES

Inorganic Impurities

• **RESIDUE ON IGNITION (281):** NMT 0.1%, determined on 1.0 g

Organic Impurities

PROCEDURE: RELATED SUBSTANCES

Mobile phase: Methanol and a 6.8-g/L solution of potassium dihydrogen phosphate (65:35 v/v)

Sample solution: Dissolve 50.0 mg of Ethylparaben in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.

Standard solution A: 5.0 µg/mL each of *p*-hydroxybenzoic acid, USP Methylparaben RS, and USP Ethylparaben RS in *Mobile phase*

Standard solution B: Dissolve 50.0 mg of USP Ethylparaben RS in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.

Standard solution C: Dilute 1.0 mL of the *Sample solution* with *Mobile phase* to 20.0 mL. Dilute 1.0 mL of this solution with *Mobile phase* to 10.0 mL.

Chromatographic system

(See *Chromatography (621)*, *System Suitability*.)

Mode: LC

Detector: UV 272 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1.3 mL/min

Injection size: 10 µL

Run time: About 4 times the retention time of ethylparaben

System suitability

Sample: *Standard solution A*

[NOTE—The retention time of ethylparaben is about 3.0 min; the relative retention times for *p*-hydroxybenzoic acid, ethylparaben, and methylparaben are about 0.5, 1.0, and 0.8, respectively.]

Suitability requirements

Resolution: NLT 2.0 between the methylparaben and ethylparaben peaks

Analysis

Samples: *Sample solution* and *Standard solution C*

[NOTE—Disregard any limit that is 0.2 times the area of the principal peak in the chromatogram obtained with *Standard solution C* (0.1%).]

Acceptance criteria

***p*-Hydroxybenzoic acid:** The peak area in the *Sample solution*, multiplied by 1.4 to correct for the calculation of content, is NMT the area of the principal peak in *Standard solution C* (0.5%).

Unspecified impurities: The peak area of each impurity in the *Sample solution* is NMT the area of the principal peak in *Standard solution C* (0.5%).
Total impurities: The total peak area for all impurities in the *Sample solution* is NMT twice the area of the principal peak in *Standard solution C* (1.0%).

SPECIFIC TESTS

• COLOR OF SOLUTION

Sample solution: 100 mg/mL in alcohol
Comparison solution: Mix 2.4 mL of ferric chloride CS, 1.0 mL of cobaltous chloride CS, and 0.4 mL of cupric sulfate CS with 0.3 N hydrochloric acid to make 10 mL. Dilute 5 mL of this solution with 0.3 N hydrochloric acid to make 100 mL. [NOTE—Prepare and use this solution immediately.]

Analysis

Samples: Alcohol, *Sample solution*, and *Comparison solution*

Make the comparison by viewing the solutions downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* (631)).

Acceptance criteria: The *Sample solution* is clear and not more intensely colored than alcohol or the *Comparison solution*.

• ACIDITY

Sample solution: To 2 mL of *Sample solution* prepared in the test for *Color of Solution*, add 3 mL of alcohol, 5 mL of carbon dioxide-free water, and 0.1 mL of bromocresol green TS.

Analysis: Titrate with 0.10 N sodium hydroxide.

Acceptance criteria: NMT 0.1 mL is required to produce a blue color.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** (11)
 USP Ethylparaben RS
 USP Methylparaben RS

Hard Fat

DEFINITION

Hard Fat is a mixture of glycerides of saturated fatty acids.

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.05%
- **ALKALINE IMPURITIES**

Sample: 2.0 g

Analysis: Dissolve the *Sample* in a mixture of 1.5 mL of alcohol and 3.0 mL of ether. Add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid to a yellow endpoint.

Acceptance criteria: NMT 0.15 mL of 0.01 N hydrochloric acid

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE, Class II** (741): The melting temperature does not differ by more than 2° from the nominal value given in *Labeling*.
- **FATS AND FIXED OILS, Acid Value** (401): NMT 1.0
- **FATS AND FIXED OILS, Hydroxyl Value** (401): NMT 70
- **FATS AND FIXED OILS, Iodine Value** (401): NMT 7.0
- **FATS AND FIXED OILS, Saponification Value** (401): 215–255
- **FATS AND FIXED OILS, Unsaponifiable Matter** (401): NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers at a temperature that is 5° or more below the melting range stated in the labeling.

- **LABELING:** The labeling includes the nominal melting temperature, which is 27°–44°.

Fennel Oil

DEFINITION

Fennel Oil is the volatile oil distilled with steam from the dried, ripe fruit of *Foeniculum vulgare* Mill. (Fam. Apiaceae).

[NOTE—If solid material has separated, carefully warm the Fennel Oil until it is completely liquefied, and mix before using.]

IMPURITIES

- **HEAVY METALS, Method II** (231): NMT 40 µg/g

SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): 0.953–0.973
- **CONGEALING TEMPERATURE** (651): NLT 3°
- **OPTICAL ROTATION, Angular Rotation** (781A): +12° to +24°
- **REFRACTIVE INDEX** (831): 1.528–1.538 at 20°
- **SOLUBILITY IN 90% ALCOHOL:** 1 volume dissolves in 1 volume of 90% alcohol.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived. The label also states that if solid material has separated, carefully warm Oil until it is completely liquefied, and mix before using.

Ferric Oxide

DEFINITION

Ferric Oxide contains NLT 97.0% and NMT 100.5% of ferric oxide (Fe₂O₃), calculated on the ignited basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Iron** (191)
Sample solution: Dissolve 0.5 g in 50 mL of hydrochloric acid, and dilute with water to 200 mL.
Acceptance criteria: Meets the requirements of the test for *Ferric Salts*

ASSAY

• PROCEDURE

To calculate the percentage of ferric oxide on the ignited basis, ignite about 2 g at 800 ± 25° to constant weight as directed in *Loss on Ignition* (733). [NOTE—Ignited Ferric Oxide is hygroscopic.]

Sample: 1.5 g

Blank: 25 mL of hydrochloric acid

Titrimetric system

Mode: Direct titration

Titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Digest the *Sample* in 25 mL of hydrochloric acid on a water bath until dissolved. Add 10 mL of hydrogen peroxide TS, and evaporate on a water bath almost to dryness to volatilize all hydrogen peroxide. Dissolve the residue by warming with 5 mL of hydrochloric acid; add 25 mL of water; filter into a 250-mL volumetric flask, washing the filter with water; and add water to volume.

Transfer a 50-mL aliquot to a glass-stoppered flask, add 3 g of potassium iodide and 5 mL of hydrochloric acid, and insert the stopper into the flask. Allow the mixture to stand for 15 min, add 50 mL of water, and titrate

the liberated iodine with *Titrant*, using starch TS as the indicator. Perform a blank determination in the same manner.

Calculate the percentage of ferric oxide (Fe_2O_3) in the portion of the sample taken:

$$\text{Result} = \{(V_s - V_b) \times N \times F / W\} \times 100$$

V_s = *Titrant* volume consumed by the *Sample* (mL)
 V_b = *Titrant* volume consumed by the *Blank* (mL)
 N = actual normality of the *Titrant* (mEq/mL)
 F = equivalency factor, 79.85 mg/mEq
 W = *Sample* weight, calculated with a correction for loss on ignition (mg)

Acceptance criteria: 97.0%–100.5% on the ignited basis

IMPURITIES

• MERCURY, Method IIa (261)

Test preparation: Combine 0.67 g of Ferric Oxide and 35 mL of 0.5 N hydrochloric acid. Heat to boiling, and allow to cool.

Analysis: Proceed as directed for *Test preparation* in the chapter, beginning with "Add 2 drops of phenolphthalein".

Acceptance criteria: NMT 3 µg/g

• LIMIT OF ARSENIC

Lead acetate cotton: Immerse absorbent cotton pledgets in a mixture of lead acetate TS and 2 N acetic acid (10:1). Free the cotton pledgets from excess liquid by expression, and allow to air-dry.

Sodium borohydride solution: 30 mg/mL of sodium borohydride in 0.25 N sodium hydroxide. Store in a loosely covered container protected from direct sunlight.

Mercuric bromide paper: Immerse several filter paper disks with a 15-mm diameter in alcoholic mercuric bromide TS, remove the disks from the solution, and allow to dry, protected from light. Store in a glass-stoppered container protected from light.

Arsenic trioxide stock solution: Dissolve 132.0 mg of arsenic trioxide in 2.0 mL of 2 N sodium hydroxide, and dilute with water to 100 mL.

Standard stock solution: On the day of use, dilute 1.0 mL of *Arsenic trioxide stock solution* with water to 1000 mL.

Standard solution: Dilute 1.5 mL of the *Standard stock solution* with hydrochloric acid to 10 mL. This solution contains 0.15 µg/mL of arsenic.

Sample solution: Dissolve 0.5 g of Ferric Oxide in several mL of hydrochloric acid with the aid of heat, and dilute with hydrochloric acid to 10.0 mL.

Apparatus: Prepare a 300-mL, side-arm conical flask containing a magnetic stirring bar. Attach to the conical flask a ground-glass stopper. Pass through the ground-glass stopper a glass tube 20 cm long with an internal diameter of 5 mm. The lower end of this tube is inside the conical flask, and it has been drawn to a tip with an internal diameter of 1 mm. There is an orifice, 2.5 mm in diameter, 15 mm from the tip, and at least 3 mm below the lower surface of the stopper. The upper end of the tube has a flat ground surface at a right angle to the axis of the tube.

A second glass tube, 30 mm long with an internal diameter of 5 mm and with a similar flat ground surface, is placed in contact with the ground surface of the first tube and is held in position by a clamp and springs.

Into the lower tube insert 55 mg of loosely packed *Lead acetate cotton*. Between the flat surfaces of the tubes place a disk of *Mercuric bromide paper*.

Analysis: Before placing the tube assembly into the flask, transfer the *Sample solution* to the flask, and add 5.0 mL of potassium iodide TS and 20 mL of water. Assemble the apparatus immediately, and stir while

slowly adding, over a period of 20 min, 40 mL of *Sodium borohydride solution* through the side arm of the flask. Examine the stain produced on the *Mercuric bromide paper*. Perform the same *Analysis* using the *Standard solution*.

Acceptance criteria: NMT 3 µg/g; the stain produced on the *Mercuric bromide paper* from the *Sample solution* is not more intense than that from the *Standard solution*.

• LIMIT OF LEAD

Lead nitrate stock solution: 1.598 mg/mL of lead nitrate in 0.5 M nitric acid. Prepare and store this solution in glass containers free from soluble lead salts.

Standard stock solution: On the day of use, combine 5.0 mL of *Lead nitrate stock solution* and 10 mL of 1 N hydrochloric acid, and dilute with water to 100 mL.

Standard solution: Transfer 1.0 mL of the *Standard stock solution* to a 100-mL volumetric flask, add 10 mL of 1 N hydrochloric acid, and dilute with water to volume. This solution contains 0.5 µg/mL of lead.

Sample solution: Transfer 2.5 g of Ferric Oxide to a 100-mL, glass-stoppered conical flask. Add 35 mL of 0.1 N hydrochloric acid, and stir for 1 h. Filter, collecting the filtrate in a 50-mL volumetric flask, and dilute with 0.1 N hydrochloric acid to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a flow spoiler

Analytical wavelength: 217.0 nm (lead emission line)

Lamp: Lead hollow-cathode

Flame: Air–acetylene oxidizing

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria: 0.001%; the absorbance of the *Sample solution* does not exceed that of the *Standard solution*.

SPECIFIC TESTS

• WATER-SOLUBLE SUBSTANCES

Sample: 2.0 g

Analysis: Digest the *Sample* in 100 mL of water on a boiling water bath for 2 h, filter, and wash the filter with water. Evaporate the filtrate and washings, and dry the residue at 105° for 1 h.

Acceptance criteria: 1.0%; NMT 20 mg of residue

• ACID-INSOLUBLE SUBSTANCES

Sample: 2.0 g

Analysis: Digest the *Sample* in 25 mL of hydrochloric acid by boiling for 20 min. Add 100 mL of hot water, and filter quantitatively through a tared filtering crucible, with the aid of hot wash water, until the filtrate tests negative for chloride. Dry the crucible and contents at 105° for 1 h.

Acceptance criteria: 0.3%; the residue weighs NMT 6 mg.

• ORGANIC COLORS AND LAKES

Sample: 3.0 g

Analysis: Place 1.0 g of the *Sample* in each of 3 beakers, and add 25 mL of each of the following reagents, one reagent to each beaker: 1-chloronaphthalene, alcohol, and chloroform. Heat the beakers containing alcohol and chloroform just to boiling. Heat the other beaker on a boiling water bath for 15 min, with occasional swirling. Pass the contents of the beakers through retentive, solvent-resistant filter paper. If any of the filtrates shows visible turbidity, centrifuge for 15 min. Record the spectra against respective solvent blanks in 1-cm cells from 350 to 750 nm.

Acceptance criteria: No peak above the noise level with a slope greater than +0.001 absorbance unit/nm is found.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Ferrosoferric Oxide

Fe₃O₄ (FeO · Fe₂O₃) 231.54
 Magnetite;
 Ferrous-ferric oxide;
 Iron (II, III) oxide [1317-61-9].

DEFINITION

Ferrosoferric Oxide contains NLT 97.0% and NMT 100.5% of Fe₂O₃, calculated on the ignited basis.

IDENTIFICATION

- **A.**
Sample solution: Dissolve 0.1 g in 5 mL of hydrochloric acid, and dilute with water to 50 mL.
Analysis 1: Transfer 5 mL of the *Sample solution* to a test tube, and add a few drops of potassium ferrocyanide TS.
Analysis 2: Transfer 5 mL of the *Sample solution* to a test tube, and add a few drops of potassium ferricyanide TS.
Acceptance criteria: For both *Analysis 1* and *Analysis 2*, a blue precipitate (Prussian blue) is formed, which does not dissolve in dilute hydrochloric acid subsequently added. The precipitate dissolves in oxalic acid and sodium or potassium hydroxide.
- **B.** Ferrosoferric Oxide presents as black powder, which is distinguished from ferric oxide exhibiting two basic colors (red and yellow). It is attracted to a magnet.

ASSAY• **PROCEDURE**

To enable the calculation of the percentage of Fe₂O₃ on the ignited basis, ignite about 2 g at 800 ± 25° to constant weight as directed in *Loss on Ignition* (733).
 [NOTE—Ignited Ferrosoferric Oxide is hygroscopic.]
Sample: 1.5 g
Blank: 25 mL of hydrochloric acid
Titrimetric system
 (See *Titrimetry* (541)).
Mode: Direct titration
Titrant: 0.1 N sodium thiosulfate VS
Endpoint detection: Visual
Analysis: Digest the *Sample* in 25 mL of hydrochloric acid on a water bath until dissolved. Add 10 mL of hydrogen peroxide TS, and evaporate on a water bath almost to dryness in order to volatilize all hydrogen peroxide. Dissolve the residue by warming with 5 mL of hydrochloric acid; add 25 mL of water; filter into a 250-mL volumetric flask, washing the filter with water; and add water to volume. Transfer a 50-mL aliquot to a glass-stoppered flask, add 3 g of potassium iodide and 5 mL of hydrochloric acid, and insert the stopper into the flask. Allow the mixture to stand for 15 min, add 50 mL of water, and titrate the liberated iodine with 0.1 N sodium thiosulfate VS, using starch TS as the indicator. Perform a blank determination in the same manner. Calculate the percentage of the labeled amount as ferric oxide (Fe₂O₃) in the portion of the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F] / W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
 V_B = *Titrant* volume consumed by the *Blank* (mL)
 N = actual normality of the *Titrant* (mEq/mL)
 F = equivalency factor, 79.85 mg/mEq
 W = weight of the *Sample*, calculated with a correction for loss on ignition (mg)

Acceptance criteria: 97.0%–100.5% on the ignited basis

IMPURITIES• **LIMIT OF ARSENIC (As)**

[NOTE—Select all reagents to have as low contents of heavy metals as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse all glassware before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solvent: 10% hydrochloric acid in water

Standard stock solution: Prepare a solution containing 2 ppm of arsenic from an Arsenic ICP/AA standard solution in *Solvent*. [NOTE—A commercially available certified standard solution can be used.]

Sample stock solution: In a 100-mL volumetric flask, dissolve 5.0 g of Ferrosoferric Oxide in 50 mL of hydrochloric acid by heating without boiling for about 1 h until the sample is dissolved. Cool down to room temperature. Pass through a filter, if necessary. Dilute with water to volume. Transfer 20.0 mL of the solution to a 100-mL volumetric flask, add 40 mL of ascorbic acid solution (200 mg/mL of ascorbic acid in water) and 20 mL of potassium iodide solution (400 mg/mL of potassium iodide in water), and dilute with water to volume. [NOTE—Reduce arsenic to the lowest possible oxidation state.] The resulting solution contains 10 mg/mL (g/L) of Ferrosoferric Oxide.

Sample solution A: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Dilute with *Solvent* to volume. The resulting solution contains 2 mg/mL (g/L) of Ferrosoferric Oxide.

Sample solution B: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 25 mL of the *Standard stock solution*, and dilute with *Solvent* to volume.

Sample solution C: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 50 mL of the *Standard stock solution*, and dilute with *Solvent* to volume.

Blank: *Solvent*

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Lamp: Arsenic hollow-cathode

Flame: Air-acetylene

Analytical wavelength: 193.696 nm

Analysis

Samples: *Sample solution A*, *Sample solution B*, and *Sample solution C*

[NOTE—To minimize matrix interference, it is recommended that the method of standard additions be used.]

Determine the absorbances of the *Samples* against the *Blank*, and plot the corrected absorbances versus their added content of arsenic (0, 0.5, and 1.0 ppm). Draw the straight line best fitting the three points, and extrapolate the line until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of arsenic, C_A , in ppm (μg/g) in the 2 mg/mL of Ferrosoferric Oxide in *Solvent*.

Calculate the content of arsenic, in μg/g, in the portion of Ferrosoferric Oxide taken:

$$\text{Result} = C_A \times D$$

D = dilution factor, 25

Acceptance criteria: NMT 2 ppm

• **LIMIT OF CADMIUM (Cd) AND CHROMIUM (Cr)**

[NOTE—Select all reagents to have as low contents of heavy metals as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse all glassware before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solvent: 10% hydrochloric acid in water

Standard stock solution: Prepare a solution containing 2 ppm each of cadmium and chromium from an ICP standard solution containing cadmium and chromium in *Solvent*. [NOTE—A commercially available certified standard solution can be used.]

Sample stock solution: In a 100-mL volumetric flask, dissolve 5.0 g of Ferrosoferric Oxide in 50 mL of hydrochloric acid by heating without boiling for about 1 h until the sample is dissolved. Cool down to room temperature. Pass through a filter, if necessary. Dilute with water to volume. Transfer 20.0 mL of the solution to a 100-mL volumetric flask, and add water to volume. The resulting solution contains 10 mg/mL of Ferrosoferric Oxide.

Sample solution A: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Dilute with *Solvent* to volume. The resulting solution contains 2 mg/mL of Ferrosoferric Oxide.

Sample solution B: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 25 mL of the *Standard stock solution*, and dilute with *Solvent* to volume.

Sample solution C: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 50 mL of the *Standard stock solution*, and dilute with *Solvent* to volume.

Blank: *Solvent*

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851) and *Plasma Spectrochemistry* (730).)

Mode: Inductively coupled plasma–atomic emission spectrometer (ICP–AES)

Analytical wavelength

Cadmium: 228.802 nm

Chromium: 267.716 nm

Analysis

Samples: *Sample solution A*, *Sample solution B*, and *Sample solution C*

[NOTE—The operating conditions may be developed and optimized based on the manufacturer's recommendation.][NOTE—To minimize matrix interference, it is recommended that the method of standard additions be used.]

Instrument performance must be verified to conform to the manufacturer's specifications for resolution and sensitivity. Before analyzing samples, the instrument must pass a suitable performance check. Aspirate the *Samples*, at least in duplicate, and calculate the average absorbances for each against the *Blank*. Plot the average absorbances of the *Samples* versus their added content (0, 0.5, and 1.0 ppm) of cadmium (or chromium). Draw the straight line best fitting the three points, and extrapolate the line until it intercepts the concentration axis. The distance between this point and the intersection of the axes represents the concentration of cadmium (or chromium), C_C , in ppm ($\mu\text{g/g}$) in the 2 mg/mL of Ferrosoferric Oxide in *Solvent*.

Calculate the content of cadmium (or chromium), in $\mu\text{g/g}$, in the portion of Ferrosoferric Oxide taken:

$$\text{Result} = C_C \times D$$

D = dilution factor, 25

Acceptance criteria

Cadmium: NMT 1 ppm

Chromium: NMT 100 ppm

• LIMIT OF LEAD (Pb)

[NOTE—Select all reagents to have as low contents of heavy metals as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse all glassware before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solvent: 4% hydrochloric acid and 8% nitric acid in water

Standard solution: Prepare a solution containing 25 ppb of lead in *Solvent*. [NOTE—A commercially available certified standard solution can be used.]

Sample stock solution: Dissolve 1.0 g of Ferrosoferric Oxide in 4 mL of hydrochloric acid and 8 mL of nitric acid in a 100-mL volumetric flask by heating without boiling until the sample is dissolved. Cool down to room temperature. Pass through a filter, if necessary. Dilute with water to volume. The resulting solution in the flask contains 10 mg/mL of Ferrosoferric Oxide.

Sample solution A: Transfer 10 mL of the *Sample stock solution* to a 100-mL volumetric flask, and dilute with *Solvent* to volume. The solution contains 1 mg/mL of Ferrosoferric Oxide.

Sample solution B: Transfer 10 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 4 mL of the *Standard solution*, and dilute with *Solvent* to volume.

Sample solution C: Transfer 10 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 12 mL of the *Standard solution*, and dilute with *Solvent* to volume.

Sample solution D: Transfer 10 mL of the *Sample stock solution* to a 100-mL volumetric flask. Add 20 mL of the *Standard solution*, and dilute with *Solvent* to volume.

Blank: *Solvent*

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with graphite heating device

Analytical wavelength: 283.3 nm

Lamp: Lead hollow-cathode

Analysis

Samples: *Sample solution A*, *Sample solution B*, *Sample solution C*, and *Sample solution D*

[NOTE—To minimize matrix interference, it is recommended that the method of standard additions be used.]

Determine the absorbances of the *Samples* against the *Blank*, and plot the corrected absorbances versus their added content (0, 1, 3, and 5 ppb) of lead. Draw the straight line best fitting the four points, and extrapolate the line until it intercepts the concentration axis. The distance between this point and the intersection of the axes represents the concentration of lead, C_L , in ppm ($\mu\text{g/g}$) in the 1 mg/mL of Ferrosoferric Oxide.

Calculate the content of lead, in $\mu\text{g/g}$, in the portion of Ferrosoferric Oxide taken:

$$\text{Result} = C_L \times D$$

D = dilution factor, 10

Acceptance criteria: NMT 10 ppm

• LIMIT OF MERCURY (Hg) AND NICKEL (Ni)

[NOTE—Select all reagents to have as low contents of heavy metals as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse all glassware before use by soaking in warm 8 N nitric acid for 30 min and by rinsing with deionized water.]

Solvent: 10% hydrochloric acid in water

Standard solution: Prepare a solution containing 2 ppm of mercury (or nickel) from a Mercury (or Nickel) ICP/AA standard solution in *Solvent*. [NOTE—A commercially available certified standard solution can be used.]

Sample stock solution: In a 100-mL volumetric flask, dissolve 5.0 g of Ferrosoferric Oxide in 50 mL of hydrochloric acid by heating without boiling for about 1 h until the sample is dissolved. Cool down to room temperature. Pass through a filter, if necessary. Dilute with water to volume. Transfer 20.0 mL of the solution to a 100-mL volumetric flask, and add water to volume. The resulting solution contains 10 mg/mL of Ferrosoferric Oxide.

Sample solution A: Transfer 20 mL of the *Sample stock solution* to a 100-mL volumetric flask. Dilute with

Solvent to volume. The resulting solution contains 2 mg/mL of Ferrosoferric Oxide.

Sample solution B: Transfer 20 mL of the *Sample solution* to a 100-mL volumetric flask. Add 25 mL of the *Standard solution*, and dilute with *Solvent* to volume.

Sample solution C: Transfer 20 mL of the *Sample solution* to a 100-mL volumetric flask. Add 50 mL of the *Standard solution*, and dilute with *Solvent* to volume.

Blank: *Solvent*

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer

Detection

Mercury: Flameless

Nickel: Air-acetylene flame

Lamp: Mercury hollow-cathode or nickel hollow-cathode

Analytical wavelength

Mercury: 253.7 nm

Nickel: 231.604 nm

Analysis

Samples: *Sample solution A*, *Sample solution B*, and *Sample solution C*

[NOTE—To minimize matrix interference, it is recommended that the method of standard additions be used.]

Determine the absorbances of the *Samples* against the *Blank*, and plot the corrected absorbances versus their content (0, 0.5, and 1.0 ppm) of mercury (or nickel). Draw the straight line best fitting the three points, and extrapolate the line until it intercepts the concentration axis. The distance between this point and the intersection of the axes represents the concentration of mercury (or nickel), C_s , in ppm ($\mu\text{g/g}$) in the 2 mg/mL of Ferrosoferric Oxide in *Solvent*.

Calculate the content of mercury (or nickel), in $\mu\text{g/g}$, in the portion of Ferrosoferric Oxide taken:

$$\text{Result} = C_s \times D$$

D = dilution factor, 25

Acceptance criteria

Mercury: NMT 1 ppm

Nickel: NMT 200 ppm

SPECIFIC TESTS

• WATER-SOLUBLE SUBSTANCES

Sample: 2.0 g

Analysis: Digest the *Sample* in 100 mL of water on a boiling water bath for 2 h, filter, and wash the filter with water. Evaporate the filtrate and washings, and dry the residue at 105° for 1 h.

Acceptance criteria: The weight of the dried residue is NMT 20 mg, corresponding to NMT 1.0%.

• ACID-INSOLUBLE SUBSTANCES

Sample: 2.0 g

Analysis: Digest the *Sample* in 25 mL of hydrochloric acid by boiling for 20 min. Add 100 mL of hot water, and filter quantitatively through a tared filtering¹ crucible, with the aid of hot wash water, until the filtrate tests negative for chloride. Dry the crucible and contents at 105° for 1 h.

Acceptance criteria: The weight of the residue is NMT 6 mg, corresponding to NMT 0.3%.

• ORGANIC COLORS AND LAKES

Analysis: Place 1.0 g in each of 3 beakers, and add 25 mL of each of the following reagents, respectively: 1-chloronaphthalene, alcohol, and chloroform. Heat the beakers containing alcohol and chloroform just to boiling. Heat the other beaker on a boiling water bath for 15 min, with occasional swirling. Pass the contents

of the beakers through retentive, solvent-resistant filter paper.² If any of the filtrates show visible turbidity, centrifuge for 15 min. Record the spectra against respective solvent blanks in 1-cm cells from 350 to 750 nm.

Acceptance criteria: Absorbance in the range of 350–750 nm is NMT 0.01 AU.

• **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.

• **PH** (791): 6–9 in a 10% suspension

• **LOSS ON DRYING** (731)

Analysis: Dry a sample at 105° for 1 h.

Acceptance criteria: NMT 1.0% of its weight

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.

Fructose—see *Fructose General Monographs*

Fumaric Acid



116.07

2-Butenedioic acid, *E*;
Fumaric acid [110-17-8].

DEFINITION

Fumaric Acid contains NLT 99.5% and NMT 100.5% of $\text{C}_4\text{H}_4\text{O}_4$, calculated on the anhydrous basis.

IDENTIFICATION

• **INFRARED ABSORPTION** (197A)

ASSAY

• PROCEDURE

Sample solution: Dissolve 1 g of Fumaric Acid in 50 mL of methanol, and warm gently on a steam bath to effect solution. Cool, and add phenolphthalein TS.

Analysis: Titrate the *Sample solution* with 0.5 N sodium hydroxide VS to the first appearance of a pink color that persists for not less than 30 s. Perform a blank titration (see *Titrimetry* (541)). Each mL of 0.5 N sodium hydroxide is equivalent to 29.02 mg of $\text{C}_4\text{H}_4\text{O}_4$.

Acceptance criteria: 99.5%–100.5% on the anhydrous basis

IMPURITIES

Inorganic Impurities

• **RESIDUE ON IGNITION** (281): NMT 0.1%

• **HEAVY METALS, Method II** (231): NMT 10 ppm

Organic Impurities

• PROCEDURE: LIMIT OF MALEIC ACID

Mobile phase: 0.005 N sulfuric acid

Standard solution: 1 $\mu\text{g/mL}$ of USP Maleic Acid RS in *Mobile phase*

Sample solution: 1 mg/mL of Fumaric Acid in *Mobile phase*

System suitability solution: 10 $\mu\text{g/mL}$ of USP Fumaric Acid RS and 5 $\mu\text{g/mL}$ of USP Maleic Acid RS in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

² A PTFE filter (diameter 25 mm, 0.2- μm pore size), or equivalent.

¹ A glass-filter disc (P4; pore size diameter 9 to 15 μm) can be used, or equivalent.

Mode: LC
Detector: UV 210 nm
Column: 4.6-mm × 22-cm; packing L17
Flow rate: 0.3 mL/min
Injection size: 5 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for maleic acid and fumaric acid are about 0.5 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.5 of the maleic acid and fumaric acid peaks

Relative standard deviation: NMT 2.0% of the maleic acid peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of maleic acid in the total weight of Fumaric Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of maleic acid from the *Sample solution*

r_S = peak response of maleic acid from the *Standard solution*

C_S = concentration of USP Maleic Acid RS in the *Standard solution* (mg/mL)

C_U = nominal concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.1%

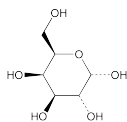
SPECIFIC TESTS

- **WATER DETERMINATION, Method I (921):** 0.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS (11)**
 USP Fumaric Acid RS
 USP Maleic Acid RS

Galactose



$C_6H_{12}O_6$ 180.16
 α -D-Galactopyranose [3646-73-9].

DEFINITION

Galactose is one of the products of the metabolism of lactose, a naturally occurring sugar in dairy products, by the digestive enzyme lactase.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K)**
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)**
Solution A: Methanol and water (60:40)
Standard solution A: 500 µg/mL of USP Galactose RS in *Solution A*
Standard solution B: 500 µg/mL each of USP Galactose RS, USP Dextrose RS, and USP Lactose Monohydrate RS in *Solution A*
Sample solution: Dissolve 10 mg of Galactose in 20 mL of *Solution A*.
Chromatographic system
 (See *Chromatography (621)*, *Thin-Layer Chromatography*.)

Application volume: 2 µL

Developing solvent system: Propanol and water (85:15)

Spray reagent: 0.5 g of thymol in a mixture of alcohol and sulfuric acid (95:5)

System suitability

Sample: *Standard solution B*

Suitability requirements

Resolution: There must be three clearly resolved spots in the chromatogram for *Standard solution B*.

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Develop the plate in an unsaturated tank. After the solvent front has moved over 15 cm, remove the plate from the tank. Dry the plate with warm air, then spray the plate with *Spray reagent*. Heat for 10 min in an oven at 130°.

Acceptance criteria: The R_f of the principal spot of the *Sample solution* corresponds to that of *Standard solution A*.

IMPURITIES

- **RESIDUE ON IGNITION (281):** NMT 0.1%
- **LIMIT OF LEAD**

Diluent: Dilute 12 mL of acetic acid with water to 100 mL. Mix equal parts of this solution and water to prepare the *Diluent*.

Lead standard solution: 16 µg/mL of lead nitrate

Standard solutions: To three identical flasks add 0.5, 1.0, and 1.5 mL of *Lead standard solution*, respectively, and then add to each flask 20.0 g of Galactose. Dilute with *Diluent* to 100 mL. To each flask add 2.0 mL of ammonium pyrrolidinedithiocarbamate solution (10 mg/mL) and 10.0 mL of methyl isobutyl ketone, then shake for 30 s. Protect from light. Allow the layers to separate, and use the methyl isobutyl ketone (upper) layer.

Sample solution: Dissolve 20.0 g of Galactose in 100 mL of *Diluent*. Add 2.0 mL of ammonium pyrrolidinedithiocarbamate solution (10 mg/mL) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from light. Allow the layers to separate, and use the methyl isobutyl ketone (upper) layer.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering (851)*.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 283.3 nm

Lamp: Lead hollow-cathode

Flame: Air-acetylene

Analysis

Samples: *Standard solutions* and *Sample solution*
 Concomitantly determine, at least in triplicate, the absorbances of the *Samples*. Record the average steady readings for each of the *Standard solutions* and the *Sample solution*. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the amount of lead added. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of lead in the *Sample solution*.

Acceptance criteria: NMT 0.5 µg/g

• **BARIUM**

Standard solution: Add 6 mL of water to 5 mL of the *Sample solution* prepared for the *Acidity* test.

Sample solution: Add 5 mL of water and 1 mL of dilute sulfuric acid to 5 mL of the *Sample solution* prepared for the *Acidity* test. Allow to stand for 1 h.

Acceptance criteria: Any opalescence in the *Sample solution* is not more intense than that in the *Standard solution*.

SPECIFIC TESTS**• APPEARANCE OF SOLUTION**

Sample solution: Dissolve, with heating at 50°, 10.0 g of Galactose in 50 mL of carbon dioxide-free water.

Control solution: Prepare immediately before use by mixing 3.0 mL of ferric chloride CS, 3.0 mL of cobaltous chloride CS, and 2.4 mL of cupric sulfate CS with dilute hydrochloric acid (10 mg/mL) to make 10 mL, and diluting 1.5 mL of this solution with the dilute hydrochloric acid to 100 mL.

Analysis: Compare by viewing the *Sample solution* and the *Control solution* downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* <631>).

Acceptance criteria: The *Sample solution* is not more intensely colored than the *Control solution*.

• MICROBIAL ENUMERATION TESTS <61> and TESTS FOR SPECIFIED MICROORGANISMS <62>: The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the test for absence of *Salmonella* species, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.

• OPTICAL ROTATION, Specific Rotation <781S>

Sample solution: Transfer 10.0 g to a 100-mL volumetric flask, and dissolve in 80 mL of water. Add 0.2 mL of ammonia TS, allow to stand for 30 min, then dilute with water to volume.

Analysis: Perform at 20°.

Acceptance criteria: +78.0° to +81.5°

• ACIDITY

Sample solution: Dissolve 10.0 g of Galactose, with heating at 50°, in 40 mL of carbon dioxide-free water. Dilute with carbon dioxide-free water to 50 mL. [NOTE—Use this solution for the *Barium* test.]

Analysis: To 30 mL of the *Sample solution* add 0.3 mL of phenolphthalein TS, and titrate with 0.01 N sodium hydroxide to a pink color.

Acceptance criteria: NMT 1.5 mL of 0.01 N sodium hydroxide is required.

• WATER DETERMINATION, Method I <921>: NMT 1.0%

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in a tight container. No storage requirements specified.

• USP REFERENCE STANDARDS <11>

USP Dextrose RS
USP Galactose RS
USP Lactose Monohydrate RS

Galageenan**DEFINITION**

Galageenan is the hydrocolloid obtained by extraction with water or aqueous alkali from the red seaweed class Rhodophyceae species *Eucheuma gelatinae*. Galageenan consists chiefly of potassium, sodium, calcium, magnesium, and ammonium sulfate esters of galactose and 3,6-anhydrogalactose copolymers. These hexoses are alternately linked as α -1,3 and β -1,4 in the polymer. The ester sulfate content ranges from 8%–18%. In addition, it contains inorganic salts that originate from the seaweed and from the process of recovery from the extract.

Galageenan is recovered by alcohol precipitation or by freezing and pressing.

IDENTIFICATION**• A. FILM FORMATION**

Solution A: A solution (1 in 50), prepared by heating a uniform dispersion in a hot water bath to 80°

Acceptance criteria: *Solution A* becomes more viscous upon cooling and may form a gel.

• B.

Analysis: Dilute a portion of *Solution A* (retained from *Identification test A*) with 4 parts water, and add 2–3 drops of methylene blue TS.

Acceptance criteria: A blue, stringy precipitate is formed.

• C. INFRARED ABSORPTION <197F>

Sample solution: Disperse 2 g of Galageenan in 400 mL of a solution containing 5 g of edetate disodium in 1000 mL of 60% isopropyl alcohol, and stir for 2 h. Filter with the aid of vacuum, and wash the residue with a total of 200 mL of 65% isopropyl alcohol. Finish washing with a total of 100 mL of 80% isopropyl alcohol. Dry the residue for 30 min in a 60° oven, and overnight in a 70° vacuum oven. Break lumps by grinding with a mortar and pestle. Dissolve 15 mg of the alcohol-treated material in 5 mL of water. Heat for 10 min in a water bath. Pipet 2 mL onto a suitable non-sticking surface to produce a 5- μ m-thick film (when dry).

Spectral range: 2000–600 cm^{-1}

Analysis: Subtract the baseline (drawn by connecting the minima in the range of 1500 and 800 cm^{-1}) from the raw spectrum. Record the absorbances for the bands at 1220–1260, 928–933, 840–850, and 800–805 cm^{-1} relative to the absorbance at 1050 cm^{-1} .

Acceptance criteria: The absorbance values so obtained are within the ranges specified in *Table 1*.

Table 1

Wave Number (cm^{-1})	Molecular Assignment	Absorbance Relative to 1050 cm^{-1} Galageenan
1220–1260	Ester sulfate	0.3–0.6
928–933	3,6-Anhydrogalactose	0.3–0.6
840–850	Galactose-4-sulfate	0.1–0.3
800–805	3,6-Anhydrogalactose-2-sulfate	0.0–0.1

ASSAY**• CONTENT OF SULFATE**

Sample: 300 mg

Analysis: Weigh the *Sample* on an ashless filter paper. Fold the paper so as to enclose the *Sample*, and place it in a 500-mL Kjeldahl flask. Add 45 mL of nitric acid, and bring to a boil on a hot plate in a fume hood. Add nitric acid as necessary to keep the sample from evaporating to dryness. Continue boiling until digestion is complete and the volume of nitric acid remaining is about 10 mL. Cool the mixture, and reduce the excess nitric acid by adding formaldehyde TS until the evolution of nitrogen oxide vapors has ceased. Heat this mixture on a hot plate to reduce the volume to about 10 mL. Transfer the mixture to a 150-mL beaker with the aid of several portions of water until the total volume is approximately 100 mL. Add 0.5 mL of hydrochloric acid, and bring to a boil on a hot plate. Add carefully 10 mL of 0.25 M barium chloride, and allow to boil for 1 min. Cover with a watch glass, and allow to stand overnight. Filter the solution through a tared, fine-porosity filtering crucible previously ignited in a muffle furnace at 550° for 30 min and cooled in a desiccator for 30 min. Wash the barium sulfate precipitate so obtained on the crucible several times with boiling water. Place the crucible in an oven, and heat at 100° for 30 min. Transfer the crucible to a muffle furnace, and ignite for 30 min at 550°. Remove the crucible, and cool in a desiccator for 30 min.

Weigh and calculate the percentage of sulfate groups:

$$\text{Result} = (W_B/W_S) \times (M_{r1}/M_{r2}) \times 100$$

- W_B = weight of barium sulfate obtained (mg)
 W_S = weight of Galageenan taken (mg)
 M_{r1} = molecular weight of the sulfate group, 96.02
 M_{r2} = molecular weight of barium sulfate, 233.43
Acceptance criteria: 8%–18% sulfate

IMPURITIES

- **LEAD** (251): NMT 5 ppm
- **HEAVY METALS**, *Method II* (231): NMT 20 ppm
- **ACID-INSOLUBLE MATTER**

Sample: 2 g

Analysis: Transfer the *Sample* to a 250-mL beaker containing 150 mL of water and 1.5 mL of sulfuric acid. Cover with a watch glass, and heat on a steam bath for 6 h, rubbing down the wall of the beaker frequently with a rubber-tipped stirring rod, and replacing any water lost by evaporation. Transfer 500 mg of a suitable filter aid to the beaker, and pass through a tared filtering crucible equipped with a 2.4-cm glass fiber filter. Wash the residue several times with hot water, dry at 105° for 3 h, cool in a desiccator, and weigh. The difference between the total weight and the sum of the weights of the filter aid, crucible, and glass fiber filter is the weight of the acid-insoluble matter.

Acceptance criteria: NMT 2.0% of the weight of Galageenan taken

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 200 cfu/g, the total combined molds and yeasts count does not exceed 20 cfu/g, and it meets the requirements of the tests for absence of *Escherichia coli* and *Salmonella* species.

- **ROTATIONAL RHEOMETER METHODS** (912)

Sample solution: Transfer 7.5 g of Galageenan to a tared, tall-form, 600-mL beaker. Add 450 mL of water, and disperse with agitation for 15 min. Add water to bring the weight to 500 g, and heat in a water bath, with continuous agitation, until a temperature of 80° is reached. Add water to adjust for loss by evaporation, cool to between 76° and 77°, and place in a constant-temperature bath maintained at 75°.

Analysis: Use a suitable rotational viscometer equipped with a spindle having a cylinder 1.88 cm in diameter and 6.51 cm in height, and an immersion depth of 8.10 cm (No. 1 spindle). Allow the spindle to rotate in the solution at 30 rpm for six revolutions, then observe the scale reading. Convert the scale reading to centipoises by multiplying by the constant for the spindle and speed used.

Acceptance criteria: NLT 15 centipoises (at 75°)

- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 35.0%
- **LOSS ON DRYING** (731): Dry a sample at a pressure not exceeding 10 mm of mercury at 70° for 18 h, cool in a desiccator, and weigh: it loses NMT 12.5% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, preferably in a cool place.

Gelatin

DEFINITION

Gelatin is a product obtained by the partial hydrolysis of collagen derived from the skin, white connective tissue, and bones of animals. Gelatin derived from an acid-treated precursor is known as Type A, and Gelatin derived from an alkali-treated precursor is known as Type B. Gelatin, where being used in the manufacture of capsules, or for the coating of tablets, may be colored with a certi-

fied color, may contain NMT 0.15% of sulfur dioxide, and may contain a suitable concentration of sodium lauryl sulfate and suitable antimicrobial agents.

IDENTIFICATION• **A. PROCEDURE**

Sample: 1 g

Analysis: Dissolve the *Sample* in 100 mL of hot water. To this solution add 20 mL of a mixture of 0.2 M potassium dichromate and 3 N hydrochloric acid (4:1).

Acceptance criteria: A yellow precipitate is formed.

• **B. PROCEDURE**

Sample solution: 0.2 mg/mL

Analysis: Heat until hot, and add tannic acid TS.

Acceptance criteria: Turbidity is produced.

OTHER COMPONENTS• **CONTENT OF SULFUR DIOXIDE**

Sample solution: Dissolve 20.0 g in 150 mL of hot water in a flask having a round bottom and a long neck, add 5 mL of phosphoric acid and 1 g of sodium bicarbonate, and at once connect the flask with a condenser. [NOTE—Excessive foaming can be alleviated by the addition of a few drops of a suitable antifoaming agent.]

Analysis: Distill 50 mL, receiving the distillate under the surface of 50 mL of 0.1 N iodine. Acidify the distillate with a few drops of hydrochloric acid, add 2 mL of barium chloride TS, and heat on a steam bath until the liquid is nearly colorless.

Acceptance criteria: The precipitate of barium sulfate, if any, when filtered, washed, and ignited, weighs NMT 3 mg, corresponding to NMT 40 ppm of sulfur dioxide, correction being made for any sulfate that may be present in 50 mL of the 0.1 N iodine.

Gelatin used in the manufacture of capsules or for the coating of tablets yields NMT 109.3 mg of barium sulfate, corresponding to NMT 0.15% of sulfur dioxide.

IMPURITIES**Inorganic Impurities**• **RESIDUE ON IGNITION** (281)

Sample: 5.0 g

Analysis: Incinerate without the use of sulfuric acid, but with the addition of 1.5–2.0 g of paraffin to avoid loss due to swelling, then finish ashing in a muffle furnace at 550° for 15–20 h.

Acceptance criteria: The weight of the residue does not exceed 2.0%.

• **ARSENIC**, *Method I* (211)

Solution A: 5 mg/mL of pepsin in 0.1 N hydrochloric acid

Standard solution: Transfer 3.0 mL of *Standard Arsenic Solution* to an arsine generator flask, and dilute with *Solution A* to 52 mL. Add 3 mL of hydrochloric acid and 4 mL of isopropyl alcohol.

Sample solution: Mix 3.75 g with 40 mL of *Solution A* in an arsine generator flask. Heat cautiously to a temperature between 65° and 70°, and, while maintaining this temperature for 30 min, sonicate the solution for 2 min at each 10-min interval of heating time. Cool, wash down the sides of the generator with *Solution A*, and dilute with *Solution A* to 52 mL. Add 3 mL of hydrochloric acid and 4 mL of isopropyl alcohol.

Analysis: Proceed as directed in the chapter, except omit the addition of 20 mL of 7 N sulfuric acid and 1 mL of isopropyl alcohol to the *Standard solution* and *Sample solution*.

Acceptance criteria: The resulting solution from the *Sample solution* meets the requirements of the test (NMT 0.8 ppm).

• **HEAVY METALS** (231)

Sample: Residue obtained in the test for *Residue on Ignition*

Analysis: To the *Sample* add 2 mL of hydrochloric acid and 0.5 mL of nitric acid, and evaporate on a steam bath to dryness. To the residue add 1 mL of 1 N hydrochloric acid and 15 mL of water, and warm for a few min. Filter and wash with water to make the filtrate measure 100 mL. Dilute 8 mL of the solution with water to 25 mL.

Acceptance criteria: NMT 50 ppm

Organic Impurities

- **PROCEDURE: ODOR AND WATER-INSOLUBLE SUBSTANCES:** A hot solution (1 in 40) is free from any disagreeable odor, and when viewed in a layer 2-cm thick, is only slightly opalescent.

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total bacterial count does not exceed 1000 cfu/g, and the tests for *Salmonella* species and *Escherichia coli* are negative.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers in a dry place.

Gellan Gum

[71010-52-1].

DEFINITION

Gellan Gum is a high-molecular-weight polysaccharide gum produced by a pure-culture fermentation of a carbohydrate with *Pseudomonas elodea*, purified by recovery with isopropyl alcohol, and then dried and milled. It is a heteropolysaccharide comprising a tetrasaccharide repeating unit of one rhamnose, one glucuronic acid, and two glucose units. The glucuronic acid is neutralized to mixed potassium, sodium, calcium, and magnesium salts. It may contain acyl (glyceryl and acetyl) groups as the O-glycosidically linked esters. It yields NLT 3.3% and NMT 6.8% of carbon dioxide, calculated on the dried basis.

IDENTIFICATION

- **A.**
Sample solution: Hydrate 1 g of Gellan Gum in 99 mL of deionized water. Stir the mixture for 2 h, using a motorized stirrer and a propeller-type stirring blade.
Analysis: Draw a small amount of the solution obtained into a wide-bore pipet, and transfer it to a 10% calcium chloride solution. [NOTE—Reserve the remaining portion of this solution for *Identification* test B.]
Acceptance criteria: A tough, wormlike gel will form instantly.
- **B.**
Sample solution: The remaining *Sample solution* from *Identification* test A
Analysis: Add 0.5 g of sodium chloride, heat the solution to 80°, stirring constantly, and hold at 80° for 1 min. Stop heating and stirring the solution, and allow it to cool to room temperature.
Acceptance criteria: A firm gel will form.

ASSAY

- **ALGINATES ASSAY** (311)
Sample: 1.2 g of undried Gellan Gum
Analysis: Proceed as directed for the *Procedure* in the chapter.
Acceptance criteria: 3.3%–6.8% of carbon dioxide on the dried basis

IMPURITIES

- **LEAD** (251)
Test preparation: Prepare as directed, using a 2.0-g portion of Gellan Gum. Use 4 mL of *Diluted Standard Lead Solution* (4 µg of Pb) for the test.
Acceptance criteria: NMT 2.0 µg/g
- **ARSENIC, Method II** (211): NMT 3.0 µg/g
- **LIMIT OF ISOPROPYL ALCOHOL**
Internal standard solution: 1 mg/mL of tertiary butyl alcohol
Standard stock solution: 1 mg/mL of isopropyl alcohol
Standard solution: Pipet 4 mL of the *Standard stock solution* and 4 mL of the *Internal standard solution* into a 100-mL volumetric flask, dilute with water to volume, and mix.
Sample solution: Disperse 1 mL of a suitable antifoam emulsion in 200 mL of water contained in a 1000-mL, round-bottom distilling flask having a 24/40 standard taper ground joint. Add 5 g of Gellan Gum, and shake for 1 h on a wrist-action mechanical shaker. Connect the flask to a fractionating column, and distill about 100 mL, adjusting the heat so that foam does not enter the column. Add by pipet 4 mL of the *Internal standard solution*, and mix.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC
Detector: Flame ionization
Column: 3.2-mm × 1.8-m stainless steel; packed with 80- to 100-mesh surface silanized packing S3, or equivalent
Temperatures
Column: 165°
Detector: 200°
Injection port: 200°
Carrier gas: Helium
Injection volume: 4–5 µL

Analysis

Samples: *Standard solution* and *Sample solution*
 [NOTE—The retention time of tertiary butyl alcohol is 1.5 relative to that of isopropyl alcohol.]
 Calculate the amount of isopropyl alcohol in the portion of Gellan Gum taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of isopropyl alcohol to the internal standard from the *Sample solution*
 R_S = peak response ratio of isopropyl alcohol to the internal standard from the *Standard solution*
 C_S = concentration of isopropyl alcohol in the *Standard solution* (mg/mL)
 C_U = concentration of the *Sample solution* (mg/mL)
Acceptance criteria: NMT 0.075%

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count is NMT 10³ cfu/g, and the total combined molds and yeasts count is NMT 10² cfu/g.
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): 4.0%–14.0% on the dried basis
- **LOSS ON DRYING** (731)
Analysis: Dry at 105° for 2.5 h.
Acceptance criteria: NMT 15.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.

Pharmaceutical Glaze

DEFINITION

Pharmaceutical Glaze is a specially denatured alcoholic solution of Shellac containing between 20.0% and 57.0% of anhydrous shellac and is made with either dehydrated alcohol or alcohol containing 5% water by volume. The solvent is a specially denatured alcohol approved for glaze manufacturing by the Internal Revenue Service. It contains NLT 90.0% and NMT 110.0% of the labeled amount of shellac. It may contain waxes and titanium dioxide as an opaquing agent.

IDENTIFICATION

• A.

Sample: Remaining sample solution retained from the Assay

Analysis: Pour the remainder of the solution in the volumetric flask, retained from the Assay, onto a clean glass plate, and place the plate in a nearly vertical position. After drainage is complete, allow the resulting film to dry in a well-ventilated place at 20° for 1 h, then place the plate in an oven at a temperature of 43° for 16–24 h. Cool, and scrape the film from the plate with a sharp blade, discarding the thick edges. To 50 mg of the *Sample* add a few drops of a mixture of 1 g of ammonium molybdate and 3 mL of sulfuric acid. Retain the remaining film for the tests for *Heavy Metals* and *Rosin*.

Acceptance criteria: A green color is produced, and it becomes lilac on standing for 5 min.

ASSAY

• PROCEDURE

Sample solution A: When testing Glaze that does not contain titanium dioxide, transfer a quantity of Glaze containing 17 g of shellac to a 100-mL volumetric flask, and add alcohol to volume. Pipet 3 mL into a tared dish containing 10 g of washed sand and a small glass rod. Retain the remaining solution in the volumetric flask for *Identification* test A. The tare weight includes the combined weights of the dish, the washed sand, and the glass rod.

Stir until a uniform mixture is obtained, allow the glass rod to remain in the dish, dry at 105° for 1 h in an explosion-proof oven, cool, and weigh.

Sample solution B: When testing Glaze that contains titanium dioxide, transfer a quantity containing 10 g of solids to a beaker, and add 10 mL of alcohol. Filter off the pigment with the aid of a vacuum. Wash the filter with alcohol; transfer the combined filtrate and washing, with the aid of alcohol, to a 200-mL volumetric flask; add alcohol to volume; and mix. Pipet 6 mL into a tared dish containing 10 g of washed sand and a small glass rod. Proceed as directed in *Sample solution A*, beginning with "The tare weight...".

Analysis: The weight of shellac in the quantity of Glaze taken is obtained by subtracting the tare weight from the final weight of the dried dish and contents.

Acceptance criteria: 90.0%–110.0%

OTHER COMPONENTS

• WAX

Sample: Weigh, by difference, a quantity of Glaze containing 10 g of shellac into a 200-mL tall-form beaker.

Analysis: To the *Sample* add with stirring 150 mL of hot water containing 2.5 g of sodium carbonate, immerse the beaker in a boiling water bath, and stir until the solid is dissolved. Cover the beaker with a watch glass, and maintain the heat for 3 h more without agitation. Remove the beaker to a cold water bath. When the wax has floated to the surface, pass the solution through medium-speed quantitative ashless filter paper, transferring the wax to the paper, and wash the filter with

water. Pour 5–10 mL of alcohol onto the filter to facilitate drying. Wrap the paper loosely in a larger piece of filter paper, bind with a piece of fine wire, and dry with the aid of gentle heat. Extract with chloroform in a suitable continuous extraction apparatus for 2 h, using a weighed flask to receive the extracted wax and solvent. Evaporate the solvent, and dry the wax at 105° to constant weight.

Acceptance criteria: It meets the requirements shown in *Table 1*.

Table 1

Type of Shellac	Wax
Orange shellac	NMT 5.5%
Dewaxed orange shellac	NMT 0.2%
Regular bleached shellac	NMT 5.5%
Refined bleached shellac	NMT 0.2%

IMPURITIES

• HEAVY METALS, *Method II* (231)

Test preparation: The film prepared in *Identification* test A

Acceptance criteria: NMT 10 ppm

• ROSIN

Sample: 2 g of the film prepared in *Identification* test A

Analysis: Dissolve the *Sample* by shaking with 10 mL of dehydrated alcohol. Add slowly, with shaking, 50 mL of solvent hexane, wash with two successive 50-mL portions of water, filter the washed alcohol–solvent hexane solution, and evaporate to dryness. To the residue add 2 mL of a mixture of liquefied phenol, dehydrated alcohol, and solvent hexane (1:0.5:2). Stir, and transfer a portion of the solution to the cavity of a color-reaction plate. Fill an adjacent cavity with a mixture of bromine and solvent hexane (1:4), and cover both cavities with an inverted watch glass.

Acceptance criteria: No purple or deep indigo-blue color is produced in or above the liquid containing the residue.

SPECIFIC TESTS

• ACID VALUE

Sample: Weigh, by difference, a quantity of Glaze containing 2 g of shellac.

Analysis: Dissolve the *Sample* in 50 mL of alcohol that has been neutralized to phenolphthalein with 0.1 N sodium hydroxide, add additional phenolphthalein TS if necessary, and titrate with 0.1 N sodium hydroxide VS to a pink endpoint. [NOTE—For Glaze containing orange shellac, titrate slowly, stirring vigorously, until a glass rod dipped into the titrated solution produces a color change when touched to a drop of thymol blue TS on a spot plate.]

Express the acid value in terms of the number of mg of potassium hydroxide required per g of dried shellac.

Acceptance criteria: It meets the requirements shown in *Table 2*.

Table 2

Type of Shellac	Acid Value (on Dried Basis)
Orange shellac	68–76
Dewaxed orange shellac	71–79
Regular bleached shellac	73–89
Refined bleached shellac	75–91

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, lined metal or plastic containers, protected from excessive heat, preferably at a temperature below 25°.

- **LABELING:** Label it to indicate whether the shellac type is bleached or orange and whether it is dewaxed or wax-containing. Label it also to indicate the shellac concentration, the composition of the solvent, and the quantity of titanium dioxide, if present. Where titanium dioxide or waxes are present, the label must state that the Glaze requires mixing before use.

Gluconolactone—see *Gluconolactone* *General Monographs*

Liquid Glucose

[8027-56-3].

DEFINITION

Liquid Glucose is a product obtained by the incomplete hydrolysis of starch. It consists chiefly of dextrose, dextrans, maltose, and water.

IDENTIFICATION

- **A.** It meets the requirements in the *Assay for Reducing Sugars (Dextrose Equivalent)*.

ASSAY

• REDUCING SUGARS (DEXTROSE EQUIVALENT)

Methylene blue solution: 10 mg/mL

Standard solution: 6 mg/mL of USP Dextrose RS

Sample solution: Transfer a quantity of Liquid Glucose, equivalent to about 3.0 g of reducing sugars (dextrose equivalent), to a 500-mL volumetric flask, and dilute with water to volume.

Analysis

Samples: *Standard solution* and *Sample solution*

Transfer 25.0-mL portions of alkaline cupric tartrate TS to each of two boiling flasks. Bring the contents of one flask to boiling within about 2 min while titrating with *Standard solution* to within 0.5 mL of the anticipated endpoint. Boil gently for 2 min. Continue to boil gently, add 2 drops of *Methylene blue solution*, and complete the titration within 1 min by adding the *Standard solution* dropwise or in small increments until the blue color disappears, determined by viewing against a white background in daylight or under equivalent illumination. If more than 0.5 mL of the titrant was required after the addition of the indicator, repeat the titration, adding the necessary volume of titrant before adding the indicator. Bring the contents of the second flask to boiling, and similarly titrate with the *Sample solution*.

Calculate the dextrose equivalent, on the anhydrous basis, taken:

$$\text{Result} = (C_S/C_U) \times (V_S/V_U) \times [1/(1 - 0.01 \times A)] \times 100$$

C_S = concentration of USP Dextrose RS in the *Standard solution* (mg/mL)

C_U = concentration of dextrose equivalent in the *Sample solution* (mg/mL)

V_S = *Titrant volume* consumed by the *Standard solution* (mL)

V_U = *Titrant volume* consumed by the *Sample solution* (mL)

A = percentage of water in the Liquid Glucose taken

Acceptance criteria: 90%–110% of the labeled value on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.5%

- **HEAVY METALS** (231)

Test preparation: Mix 2.0 g of Liquid Glucose with water to make 25 mL.

Acceptance criteria: NMT 10 µg/g

- **SULFITE**

Sample solution: 5 g in 50 mL of water

Analysis: To the *Sample solution* add 0.2 mL of 0.1 N iodine, then add 0.5 mL of starch TS.

Acceptance criteria: A blue color is produced.

- **STARCH**

Sample solution: 5 g in 50 mL of water

Analysis: Boil the *Sample solution* for 1 min, cool, and add 0.2 mL of 0.1 N iodine.

Acceptance criteria: No blue color is produced.

SPECIFIC TESTS

- **ACIDITY**

Sample solution: 5.0 g in 15 mL of water

Analysis: To the *Sample solution* add 5 drops of phenolphthalein TS, and titrate with 0.10 N sodium hydroxide.

Acceptance criteria: NMT 0.60 mL of 0.10 N sodium hydroxide is required to produce a pink color.

- **WATER DETERMINATION, Method 1a** (921)

Sample: 100 mg

Acceptance criteria: NMT 21.0%

ADDITIONAL REQUIREMENTS

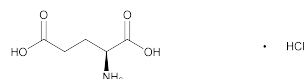
- **PACKAGING AND STORAGE:** Preserve in tightly closed containers. No storage requirements specified.

- **LABELING:** Label it to indicate the natural source of starch. Label it to indicate its nominal dextrose equivalent.

- **USP REFERENCE STANDARDS** (11)

USP Dextrose RS

L-Glutamic Acid, Hydrochloride



$C_5H_9NO_4 \cdot HCl$

183.59

L-2-Aminoglutaric acid, hydrochloride;

2-Aminopentanedioic acid, hydrochloride [138-15-8].

DEFINITION

L-Glutamic Acid, Hydrochloride, contains NLT 98.5% and NMT 101.5% of $C_5H_9NO_4 \cdot HCl$, calculated on the dried basis.

IDENTIFICATION

- **INFRARED ABSORPTION** (197K)

ASSAY

- **PROCEDURE**

Sample: 100 mg of L-Glutamic Acid, Hydrochloride, previously dried

Analysis: Dissolve the *Sample* in 0.5 mL of water, add 15.0 mL of 0.1 N perchloric acid VS, and heat on a water bath for 30 min. After cooling, add 45 mL of glacial acetic acid, and titrate the excess perchloric acid with 0.1 N sodium acetate, determining the endpoint potentiometrically. Perform a blank determination (see *Titrimetry* (541)). Each mL of 0.1 N perchloric acid is equivalent to 18.36 mg of $C_5H_9NO_4 \cdot HCl$.

Acceptance criteria: 98.5%–101.5% on the dried basis

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 0.25%
- **HEAVY METALS**, Method I (231): NMT 5 ppm

SPECIFIC TESTS

- **OPTICAL ROTATION**, Specific Rotation (781S): +25.2° to +25.8°, determined at 20°
Sample solution: 100 mg/mL in 2 N hydrochloric acid
- **LOSS ON DRYING** (731): Dry a sample at 80° for 4 h: it loses NMT 0.5% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Store in well-closed, light-resistant containers.
- **USP REFERENCE STANDARDS** (11)
USP L-Glutamic Acid, Hydrochloride RS

Glutaral Disinfectant Solution

DEFINITION

Glutaral Disinfectant Solution contains NLT 100.0% and NMT 110.0%, by weight, of the labeled amount of glutaraldehyde (C₅H₈O₂).

IDENTIFICATION

- **A. MELTING RANGE OR TEMPERATURE** (741)
2,4-Dinitrophenylhydrazine reagent: To 0.8 g of 2,4-dinitrophenylhydrazine add 4 mL of sulfuric acid, then add 6 mL of water, dropwise, with swirling. When dissolution is essentially complete, add 20 mL of alcohol, and filter. Use the filtrate.
Sample: 5 mL
Analysis: To 20 mL of 2,4-Dinitrophenylhydrazine reagent add the Sample, mix by swirling, and allow to stand for 5 min. Collect the precipitate on a filter, and rinse thoroughly with alcohol. Dissolve the precipitate in 20 mL of hot ethylene dichloride, filter, and cool the filtrate in an ice bath until crystallization occurs. Collect the precipitate on a filter. Redissolve the precipitate by refluxing with 30 mL of acetone, filter, and cool the filtrate in an ice bath until crystallization occurs. Collect the precipitate on a filter.
Acceptance criteria: The 2,4-dinitrophenylhydrazone so obtained melts between 185° and 195°, within a 3° range.

ASSAY

PROCEDURE

Buffer: Dissolve 2.59 g of monobasic potassium phosphate and 6.77 g of anhydrous dibasic sodium phosphate in 500 mL of water in a 1000-mL volumetric flask. Dilute to volume.
Hydroxylamine hydrochloride solution: 70 µg/mL of hydroxylamine hydrochloride in Buffer
Standard solution: 50 µg/mL of glutaraldehyde in water from Glutaral Concentrate
Standard blank solution: Add 10.0 mL of Standard solution and 10.0 mL of Buffer to a 50-mL volumetric flask, and dilute with water to volume.
Sample solution: 50 µg/mL of glutaraldehyde in water from Disinfectant Solution
Sample blank solution: Add 10.0 mL of Sample solution and 10.0 mL of Buffer to a 50-mL volumetric flask, and dilute with water to volume.
Reagent blank solution: Add 10.0 mL of Buffer and 10.0 mL of Hydroxylamine hydrochloride solution to a 50-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

Mode: UV
Analytical wavelength: 238 nm
Blank: Reagent blank solution

Analysis

Samples: Standard solution, Standard blank solution, Sample solution, and Sample blank solution
Transfer 10.0 mL each of the Standard solution and the Sample solution to separate 50-mL volumetric flasks. To each flask add 10.0 mL of Hydroxylamine hydrochloride solution, dilute with water to volume, mix, and allow each flask to stand for 25 min.
Concomitantly determine the absorbances of the Standard solution, Sample solution, Standard blank solution, and Sample blank solution.
Calculate the percentage of the labeled amount of glutaraldehyde (C₅H₈O₂) in the portion of Disinfectant Solution taken:

$$\text{Result} = [(A_U - A_{Ub}) / (A_S - A_{Sb})] \times (C_S / C_U) \times 100$$

A_U = absorbance of the Sample solution
 A_{Ub} = absorbance of the Sample blank solution
 A_S = absorbance of the Standard solution
 A_{Sb} = absorbance of the Standard blank solution
 C_S = concentration of glutaraldehyde in the Standard solution (µg/mL)
 C_U = concentration of the Sample solution (µg/mL)
Acceptance criteria: 100.0%–110.0%

SPECIFIC TESTS

- **PH** (791): 2.7–3.7

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight, light-resistant containers, and avoid exposure to excessive heat.

Glycerin—see Glycerin General Monographs

Glyceryl Behenate

DEFINITION

Glyceryl Behenate is a mixture of glycerides of fatty acids, mainly behenic acid.

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST**

Standard solution: 60 mg/mL of USP Glyceryl Behenate RS in chloroform
Sample solution: 60 mg/mL of Glyceryl Behenate in chloroform
Chromatographic plates: Use suitable thin-layer chromatographic plates (see Chromatography (621)) coated with a 0.25-mm layer of chromatographic silica gel. Predevelop the plates by placing in a chromatographic chamber saturated with ether. Remove the plates from the chamber, allow the ether to evaporate, and immerse the plates in a 2.5% solution of boric acid in alcohol. After about 1 min, withdraw the plates, allow them to dry at ambient temperature, and activate them at 110° for 30 min. Keep the plates in a desiccator.
Chromatographic system
(See Chromatography (621), Thin-Layer Chromatography.)
Mode: TLC
Application volume: 10 µL
Spray reagent: 0.02% solution of dichlorofluorescein in alcohol
Developing solvent system: Chloroform and acetone (96:4)

Analysis

Samples: *Standard solution* and *Sample solution*
Develop in *Developing solvent system* until the solvent front has moved about 12 cm. Remove the plate from the chamber, and allow the solvent to evaporate. Spray the chromatogram with *Spray reagent*. Examine the spots under short-wavelength UV light.

Acceptance criteria: The R_f values of the spots from the *Sample solution* correspond to those from the *Standard solution*.

• **B.**

Standard solution: Dissolve 22 mg of USP Glyceryl Behenate RS in 1 mL of toluene in a screw-capped vial having a polytetrafluoroethylene-lined septum. Add 0.4 mL of 0.2 N methanolic (*m*-trifluoromethylphenyl) trimethylammonium hydroxide. Attach the cap, and mix. Allow the vial to stand at room temperature for NLT 30 min.

Sample solution: Dissolve 22 mg of Glyceryl Behenate in 1 mL of toluene in a screw-capped vial having a polytetrafluoroethylene-lined septum. Add 0.4 mL of 0.2 N methanolic (*m*-trifluoromethylphenyl) trimethylammonium hydroxide. Attach the cap, and mix. Allow the vial to stand at room temperature for NLT 30 min.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 4-mm × 1.8-m, packed with 10% liquid phase G7 on support S1A

Column temperature: 225°

Analysis

Samples: *Standard solution* and *Sample solution*
Introduce a suitable volume into the gas chromatograph, and record the resulting chromatogram.

Acceptance criteria: The retention time of the main peak of the *Sample solution* corresponds to that of the main peak of the *Standard solution*. The ratio of the response of the main peak to the sum of all the responses is NLT 0.83.

IMPURITIES

- **RESIDUE ON IGNITION** <281>: NMT 0.1%
- **HEAVY METALS**, *Method II* <231>: NMT 10 ppm
- **CONTENT OF 1-MONOGLYCERIDES**

Periodic acid solution: Dissolve 5.4 g of periodic acid in 100 mL of water, and add 1900 mL of glacial acetic acid. Store in a light-resistant, glass-stoppered bottle or in a clear glass-stoppered bottle protected from light.

Chloroform: Use chloroform that meets the following test. To each of three 500-mL flasks add 50.0 mL of *Periodic acid solution*. Add 50 mL of chloroform and 10 mL of water to two of the flasks, and add 50 mL of water to the third flask. To each flask add 20 mL of potassium iodide TS, mix gently, and proceed as directed in the *Analysis* below, beginning with "allow to stand for NLT 1 min". The difference between the volumes of 0.1 N sodium thiosulfate required in the titrations with and without the chloroform is NMT 0.5 mL.

Sample: Melt a quantity of Glyceryl Behenate at a temperature not higher than 80°.

Analysis: Transfer 1 g of the *Sample* to a 100-mL beaker, and dissolve in 25 mL of *Chloroform*. Transfer the solution, with the aid of an additional 25 mL of *Chloroform*, to a separator, wash the beaker with 25 mL of water, and add the washing to the separator. Tightly place the stopper in the separator, shake vigorously for 30–60 s, and allow the layers to separate. Add 1–2 mL of glacial acetic acid to break any emulsion formed. Collect the aqueous layer in a glass-stoppered 500-mL conical flask, and extract the nonaqueous layer again, using two 25-mL portions of water. [NOTE—Retain the combined aqueous extracts for the test for *Limit of Free Glycerin*.]

Transfer the nonaqueous layer to a glass-stoppered 500-mL conical flask, and add 50.0 mL of *Periodic acid solution* to this flask and to a blank flask containing 50 mL of *Chloroform* and 10 mL of water, swirling the flasks during the addition. Allow to stand for 30–90 min. To each flask add 20 mL of potassium iodide TS, and allow to stand for 1–5 min before titrating. Add 100 mL of water, and titrate with 0.1 N sodium thiosulfate VS, using a magnetic stirrer to keep the solution mixed, to the disappearance of the brown iodine color. Then add 2 mL of starch TS, and continue the titration to the disappearance of the blue color. [NOTE—If the Glyceryl Behenate titration is less than 0.8 of the blank titration, discard, and repeat using a smaller weight of Glyceryl Behenate.]

Calculate the percentage of 1-monoglycerides as glyceryl monobehenate:

$$\text{Result} = [(B - S) \times F \times N \times 100]/W$$

B = volume of 0.1 N sodium thiosulfate consumed by the blank (mL)

S = volume of 0.1 N sodium thiosulfate consumed by the Glyceryl Behenate (mL)

F = equivalency factor of glyceryl monobehenate, 207.3 mg/mEq

N = normality of the sodium thiosulfate

W = weight of Glyceryl Behenate taken (g)

Acceptance criteria: 12.0%–18.0%

• **LIMIT OF FREE GLYCERIN**

Periodic acid solution and Chloroform: Prepare as directed in the test for *Content of 1-Monoglycerides*.

Analysis: To the combined aqueous extracts obtained as directed in the test for *Content of 1-Monoglycerides* add 50.0 mL of *Periodic acid solution*. Prepare a blank by adding 50.0 mL of *Periodic acid solution* to a glass-stoppered conical flask containing 75 mL of water. Allow to stand for NLT 30 min but NMT 90 min. To each flask add 20 mL of potassium iodide TS, and allow to stand for NLT 1 min but NMT 5 min before titrating. Add 100 mL of water, and titrate with 0.1 N sodium thiosulfate VS, using a magnetic stirrer to keep the solution mixed, to the disappearance of the brown iodine color. Then add 2 mL of starch TS, and continue the titration to the disappearance of the blue color. Calculate the percentage of free glycerin:

$$\text{Result} = [(B - S) \times F \times N \times 100]/W$$

B = volume of 0.1 N sodium thiosulfate consumed by the blank (mL)

S = volume of 0.1 N sodium thiosulfate consumed by the Glyceryl Behenate (mL)

F = equivalency factor of glycerin, 23.0 mg/mEq

N = normality of the sodium thiosulfate

W = weight of Glyceryl Behenate taken (g)

Acceptance criteria: NMT 1.0%

SPECIFIC TESTS• **FATS AND FIXED OILS**, *Acid Value*, *Method I* <401>

Sample: 10 g

Analysis: Suspend the *Sample* in 50 mL of a mixture of alcohol and ether (1:1), which has been neutralized to phenolphthalein with 0.1 N sodium hydroxide, contained in a flask. Connect the flask with a suitable condenser, and warm, with frequent shaking, for 10 min. Continue as directed under *Fats and Fixed Oils* <401>, *Acid Value*, beginning with "Add 1 mL of phenolphthalein TS".

Acceptance criteria: NMT 4

• **FATS AND FIXED OILS**, *Saponification Value* <401>: 145–165• **FATS AND FIXED OILS**, *Iodine Value*, *Method II* <401>: NMT 3

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers at a temperature not higher than 35°.
- **USP REFERENCE STANDARDS** (11)
USP Glyceryl Behenate RS

Glyceryl Distearate

625.01

[1323-83-7].

DEFINITION

Glyceryl Distearate is a mixture of diglycerides, mainly glyceryl distearate, together with variable quantities of monoglycerides and triglycerides. It contains NLT 8.0% and NMT 22.0% of monoglycerides, NLT 40.0% and NMT 60.0% of diglycerides, and NLT 25.0% and NMT 35.0% of triglycerides. It is obtained by partial glycerolysis of vegetable oil that consists mainly of triglycerides of palmitic or stearic acid or by esterification of glycerol with stearic acid. The fatty acids may be of vegetable or animal origin.

IDENTIFICATION

- **A.** It meets the requirements in *Specific Tests for Melting Range or Temperature, Class II* (741).
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** (201)
Standard solution: 50 mg/mL of USP Glyceryl Distearate RS in methylene chloride
Sample solution: 50 mg/mL in methylene chloride
Chromatographic system
Developing solvent system: Ether and hexane (70:30)
Spray reagent: 0.1 mg/mL of rhodamine B in alcohol
Analysis: Proceed as directed in the chapter. Spray with the *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.
Acceptance criteria: The principal spot of the *Sample solution* corresponds in color, size, and R_f value to that of the *Standard solution*.

ASSAY• **PROCEDURE****Mobile phase:** Tetrahydrofuran**Sample solution:** 40 mg/mL of Glyceryl Distearate in tetrahydrofuran**Chromatographic system**(See *Chromatography* (621), *System Suitability*.)**Mode:** LC**Detector:** Refractive index**Column:** 7.5-mm × 60-cm; 5-μm 100-Å packing L21.

[NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the one 60-cm column, provided that *System suitability* requirements are met. The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]

Column temperature: 40°**Flow rate:** 1 mL/min**Injection volume:** 40 μL**System suitability****Sample:** *Sample solution*

[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are 0.75, 0.78, 0.84, and 1.0, respectively.]

Suitability requirements**Resolution:** NLT 1.0 between the diglycerides and monoglycerides peaks**Relative standard deviation:** NMT 2.0%, determined from the monoglycerides peak**Analysis****Sample:** *Sample solution*

Calculate the percentage of monoglycerides, diglycerides, and triglycerides in the portion of Glyceryl Distearate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak response for the monoglycerides, diglycerides, and triglycerides, as appropriate

r_T = sum of the responses for all of the glyceride peaks

Acceptance criteria**Monoglycerides:** 8.0%–22.0%**Diglycerides:** 40.0%–60.0%**Triglycerides:** 25.0%–35.0%**IMPURITIES**• **LIMIT OF NICKEL**

[**CAUTION**—When using closed high-pressure digestion vessels and microwave laboratory equipment, be familiar with the safety and operating instructions given by the manufacturer.]

Magnesium nitrate solution: 5 mg/mL of magnesium nitrate

Standard stock solution: Transfer 5.0 mL of nickel standard solution TS to a 10-mL volumetric flask. Add 0.5 mL of nitric acid and 1.0 mL of 30% hydrogen peroxide, and dilute with water to volume.

Standard solutions: Into four identical 25-mL volumetric flasks, each containing 6 mL of nitric acid, transfer 20, 50, 100, and 150 μL, respectively, of the *Standard stock solution*, and dilute with water to volume. These solutions contain 4, 10, 20, and 30 ng/mL of nickel, respectively.

Sample solution: Transfer 0.1 g of Glyceryl Distearate into a suitable high-pressure resistant digestion vessel (fluoropolymer or quartz), and add 6.0 mL of nitric acid and 2.0 mL of 30% hydrogen peroxide. Place the closed vessel in a laboratory microwave oven, and digest using an appropriate program (for example, 250 W for 10 min; 600 W for 5 min; 400 W for 5 min; and 250 W for 7 min). Allow the digestion vessel to cool before opening. Quantitatively transfer the contents to a 25-mL volumetric flask, and dilute with water to volume.

Blank: Add 6.0 mL of nitric acid and 2.0 mL of 30% hydrogen peroxide to a high-pressure-resistant digestion vessel, and proceed as directed for the *Sample solution*.

Zero solution: Transfer 6.0 mL of nitric acid into a 25-mL volumetric flask. Dilute with water to volume.

Instrumental conditions(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry equipped with a pyrolytically coated tube with a platform

Analytical wavelength: 232 nm**Lamp:** Nickel hollow-cathode

Graphite furnace parameters: Maintain the drying temperature of the furnace at 100° for 10 s after a 10-s ramp, the ashing temperature at 1400° for 10 s after a 20-s ramp, and the atomization temperature at 2500° for 5 s. Use the *Zero solution* to set the instrument to zero.

Analysis

Samples: Into seven separate 25-mL flasks, each containing 5.0 mL of *Magnesium nitrate solution*, transfer respectively 10.0 mL of each of the following: the *Sample solution*, the *Blank*, the four *Standard solutions*, and the *Zero solution*.

Concomitantly determine the absorbances of the *Samples* at least three times each. Record the average of

the steady readings for each of the solutions. [NOTE—If necessary, dilute the *Sample solution* with the *Zero solution* to obtain a reading within the calibrated absorbance range.]

Acceptance criteria: NMT 1 µg/g

• **LIMIT OF FREE GLYCERIN**

Mobile phase, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the Assay.

Standard solutions: 0.2, 0.4, 1.0, and 2.0 mg/mL of glycerin in tetrahydrofuran

Analysis

Samples: *Standard solutions* and *Sample solution*
Plot the glycerin peak responses versus the concentration of glycerin in the *Standard solutions*. From the standard curve so obtained, determine the glycerin concentration in the *Sample solution*. Calculate the percentage of free glycerin in the portion of Glyceryl Distearate taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of glycerin in the *Sample solution* from the standard curve (mg/mL)

C_U = concentration of Glyceryl Distearate in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 1.0%

SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE, Class II (741):** 50°–70°

• **FATS AND FIXED OILS, Acid Value (401)**

Sample: 1.0 g

Analysis: Use a mixture of alcohol and toluene (1:1, v/v) as the solvent and with gentle heating.

Acceptance criteria: NMT 6.0

• **FATS AND FIXED OILS, Fatty Acid Composition (401):** The fatty acid fraction of it contains NLT 40.0% of stearic acid, and the sum of the contents of palmitic and stearic acids is NLT 90.0%.

• **FATS AND FIXED OILS, Iodine Value (401):** NMT 3.0

• **FATS AND FIXED OILS, Saponification Value (401)**

Sample: 2.0 g

Analysis: Carry out the titration with heating.

Acceptance criteria: 165–195

• **WATER DETERMINATION, Method I (921)**

Analysis: Use pyridine in place of methanol in the titration vessel.

Acceptance criteria: NMT 1.0%

• **ARTICLES OF BOTANICAL ORIGIN, Total Ash (561):** NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements are specified.

• **USP REFERENCE STANDARDS (11)**

USP Glyceryl Distearate RS

Glyceryl Monolinoleate

354.5

[26545-74-4].

DEFINITION

Glyceryl Monolinoleate is a mixture of monoglycerides, mainly glyceryl monooleate and glyceryl monolinoleate, together with variable quantities of diglycerides and triglycerides. It is obtained by partial glycerolysis of vegetable oil that consists mainly of triglycerides of linoleic acid. It contains NLT 32.0% and NMT 52.0% of monoglycerides, NLT 40.0% and NMT 55.0% of diglycerides, and NLT 5.0% and NMT 20.0% of triglycerides. A suitable antioxidant may be added.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)**

Standard solution: 50 mg/mL of USP Glyceryl Monolinoleate RS in methylene chloride

Sample solution: 50 mg/mL in methylene chloride

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Developing solvent system: Ether and hexane (70:30)

Spray reagent: 0.1 mg/mL of rhodamine B in alcohol

Analysis: Proceed as directed in the chapter. Spray with the *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The principal spot from the *Sample solution* corresponds in color, size, and R_f value to that of the *Standard solution*.

• **B.** It meets the requirements in *Specific Tests for Fats and Fixed Oils, Iodine Value* (401).

• **C.** It meets the requirements in *Specific Tests for Fats and Fixed Oils, Fatty Acid Composition* (401).

ASSAY

• **PROCEDURE**

Mobile phase: Tetrahydrofuran

Sample solution: 40 mg/mL of Glyceryl Monolinoleate in tetrahydrofuran

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.5-mm × 60-cm; 5-µm 100-Å packing L21

[NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the one 60-cm column, provided that system suitability requirements are met.

The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]

Column temperature: 40°

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *Sample solution*

[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are about 0.76, 0.80, 0.86, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.0 between the diglycerides and monoglycerides peaks

Relative standard deviation: NMT 2.0%, determined from the monoglycerides peak

Analysis

Sample: *Sample solution*

Calculate the percentage of monoglycerides, diglycerides, and triglycerides in the portion of Glyceryl Monolinoleate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = individual peak response for the monoglycerides, diglycerides, and triglycerides, as appropriate

r_T = sum of the responses for all of the glyceride peaks

Acceptance criteria

Monoglycerides: 32.0%–52.0%

Diglycerides: 40.0%–55.0%

Triglycerides: 5.0%–20.0%

IMPURITIES

• **LIMIT OF FREE GLYCERIN**

Mobile phase, Sample solution, Chromatographic system, and System suitability: Proceed as directed in the Assay.

Standard solutions: 0.4, 1.0, 2.0, and 4.0 mg/mL of glycerin in tetrahydrofuran

Analysis

Samples: *Sample solution* and *Standard solutions*
Plot the glycerin peak responses obtained versus the concentration of glycerin in the *Standard solutions*. From the standard curve so obtained, determine the glycerin concentration in the *Sample solution*. Calculate the percentage of free glycerin in the portion of Glyceryl Monolinoleate taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of glycerin in the *Sample solution* from the standard curve (mg/mL)
C_U = concentration of Glyceryl Monolinoleate in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 6.0%

SPECIFIC TESTS

- **FATS AND FIXED OILS, Acid Value <401>**
Sample: 1.0 g
Acceptance criteria: NMT 6.0
- **FATS AND FIXED OILS, Iodine Value <401>**: 100–140
- **FATS AND FIXED OILS, Peroxide Value <401>**
Sample: 2.0 g
Acceptance criteria: NMT 12.0
- **FATS AND FIXED OILS, Saponification Value <401>**
Sample: 2.0 g
Acceptance criteria: 160–180
- **FATS AND FIXED OILS, Fatty Acid Composition <401>**: See Table 1.

Table 1

Carbon-Chain Length	No. of Double Bonds	Percentage (%)
16	0	4.0–20.0
18	0	NMT 6.0
18	1	10.0–35.0
18	2	NLT 50.0
18	3	NMT 2.0
20	0	NMT 1.0
20	1	NMT 1.0

- **WATER DETERMINATION, Method I <921>**
Analysis: Use a mixture of methanol and methylene chloride (1:1) in place of methanol in the titration vessel.
Acceptance criteria: NMT 1.0%
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash <561>**: NMT 0.1%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** The labeling indicates the name and the concentration of any added antioxidant.
- **USP REFERENCE STANDARDS <11>**
USP Glyceryl Monolinoleate RS

Glyceryl Monooleate

Oleic acid, 2,3-dihydroxypropyl ester, (±);
(*RS*)-1-Glyceryl oleate
[25496-72-4]. 356.54

DEFINITION

Glyceryl Monooleate is a mixture of monoglycerides, mainly glyceryl monooleate, together with variable quantities of diglycerides and triglycerides. It is obtained by partial

glycerolysis of vegetable oil that consists mainly of triglycerides of oleic acid, or by esterification of glycerol with oleic acid of vegetable or animal origin. It is defined by the nominal content of monoglycerides. The assay requirements differ as set forth in the accompanying table. A suitable antioxidant may be added.

Nominal Content of Monoglycerides (%)			
	40	60	90
Monoglycerides	32.0–52.0	55.0–65.0	90.0–101.0
Diglycerides	30.0–50.0	15.0–35.0	<10.0
Triglycerides	5.0–20.0	2.0–10.0	<2.0

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST <201>**

Standard solution: 50 mg/mL of USP Glyceryl Monooleate 40% RS or USP Glyceryl Monooleate 90% RS in methylene chloride

Sample solution: 50 mg/mL of Glyceryl Monooleate in methylene chloride

Application volume: 10 µL

Developing solvent system: Ether and hexane (7:3)

Spray reagent: 0.1 mg/mL of rhodamine B in alcohol

Analysis: Proceed as directed in the chapter. Spray with the *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The principal spot of the *Sample solution* corresponds in color, size, and *R_f* value to that of the *Standard solution*.

- **B.** It meets the requirements in *Specific Tests for Fats and Fixed Oils, Iodine Value <401>*.

ASSAY

• **PROCEDURE**

Mobile phase: Tetrahydrofuran

Sample solution: 40 mg/mL of Glyceryl Monooleate in tetrahydrofuran

Chromatographic system

(See *Chromatography <621>*, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.5-mm × 60-cm; 5-µm 100-Å packing L21

[NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the one 60-cm column, provided that the system suitability requirements are met. The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]

Temperature

Column: 40°

Detector: 40°

Flow rate: 1 mL/min

Injection size: 40 µL

System suitability

Sample: *Sample solution*

[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are about 0.76, 0.79, 0.85, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.0 between the diglycerides and monoglycerides

Relative standard deviation: NMT 2.0%, determined from the monoglycerides peak

Analysis

Sample: *Sample solution*

Calculate the percentage of monoglycerides, diglycerides, and triglycerides in the portion of Glyceryl Monooleate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

- r_U = individual peak responses for the monoglycerides, diglycerides, and triglycerides, as appropriate
 r_T = sum of all the glyceride peak responses
Acceptance criteria: See the table in the *Definition*.

IMPURITIES**• LIMIT OF FREE GLYCERIN**

Mobile phase, Sample solution, and Chromatographic system: Proceed as directed in the *Assay*.

Standard solutions: Prepare four solutions by dissolving glycerin in tetrahydrofuran, and diluting each with tetrahydrofuran as necessary, to obtain solutions having known concentrations of 0.4, 1.0, 2.0, and 4.0 mg/mL.

Standard curve

Samples: *Standard solutions*

Plot: Record the chromatograms, and measure the responses for the glycerin peaks. Plot the glycerin peak responses obtained versus the concentration, in mg/mL, of glycerin in the *Standard solutions*.

Analysis

Sample: *Sample solution*

From the *Standard curve*, determine the glycerin concentration, in mg/mL, in the *Sample solution*. Calculate the percentage of free glycerin in the portion of Glyceryl Monooleate taken:

$$\text{Result} = (C_S/C_U) \times 100$$

- C_S = concentration of glycerin in the *Sample solution* from the *Standard curve* (mg/mL)
 C_U = concentration of Glyceryl Monooleate in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 6.0%

SPECIFIC TESTS

- FATS AND FIXED OILS, Acid Value <401>:** NMT 6.0, determined on 1.0 g
- FATS AND FIXED OILS, Iodine Value <401>:** 65.0–95.0
- FATS AND FIXED OILS, Peroxide Value <401>:** NMT 12.0, determined on 2.0 g
- FATS AND FIXED OILS, Saponification Value <401>:** 150–175, determined on 2.0 g
- FATS AND FIXED OILS, Fatty Acid Composition <401>:** Glyceryl Monooleate exhibits the following composition profile of fatty acids (see *Table 1*), determined as directed in the chapter.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage, NMT (%)
16	0	12.0
18	0	6.0
18	1	60.0
18	2	35.0
18	3	2.0
20	0	2.0
20	1	2.0

- WATER DETERMINATION, Method I <921>:** NMT 1.0%, using a mixture of methanol and methylene chloride (1:1) in place of methanol in the titration vessel
- ARTICLES OF BOTANICAL ORIGIN, Total Ash <561>:** NMT 0.1%

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- LABELING:** The labeling indicates the nominal content of monoglycerides and the name and the concentration of any added antioxidant.

- USP REFERENCE STANDARDS <11>**
 USP Glyceryl Monooleate 40% RS
 USP Glyceryl Monooleate 90% RS

Glyceryl Monostearate

Octadecanoic acid, monoester with 1,2,3-propanetriol; Monostearin [31566-31-1].

DEFINITION

Glyceryl Monostearate contains NLT 90.0% of monoglycerides of saturated fatty acids, chiefly glyceryl monostearate ($C_{21}H_{42}O_4$) and glyceryl monopalmitate ($C_{19}H_{38}O_4$). It may contain a suitable antioxidant.

ASSAY**• PROCEDURE**

Mobile phase: Tetrahydrofuran

Sample solution: 8 mg/mL of Glyceryl Monostearate in tetrahydrofuran

Chromatographic system

(See *Chromatography <621>*, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.5-mm \times 60-cm; 5- μ m 100-Å packing L21

Temperature: Column and detector temperatures are maintained at 40°.

[NOTE—Two or three 7.5-mm \times 30-cm L21 columns may be used in place of the one 60-cm column, provided that system suitability requirements are met. The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]

Flow rate: 1 mL/min

Injection size: 40 μ L

System suitability

Sample: *Sample solution*

[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are 0.77, 0.81, 0.86, and 1.0, respectively.]

Suitability requirements

Relative standard deviation: NMT 2.0%, determined from the monoglycerides peak

Analysis

Sample: *Sample solution*

Calculate the percentage of monoglycerides in the portion of Glyceryl Monostearate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of the monoglycerides

r_T = sum of all the glyceride peak responses

Acceptance criteria: NLT 90.0% of monoglycerides of saturated fatty acids, chiefly $C_{21}H_{42}O_4$ and $C_{19}H_{38}O_4$

IMPURITIES**Inorganic Impurities**

- RESIDUE ON IGNITION <281>:** NMT 0.5%
- HEAVY METALS, Method II <231>:** NMT 10 ppm

Organic Impurities**• PROCEDURE: LIMIT OF FREE GLYCERIN**

Propionating reagent: Pyridine and propionic anhydride (1:2)

Internal standard solution: 0.2 mg/mL of tributyrin in chloroform

Standard solution: Transfer 15 mg of glycerin and 50 mg of tributyrin to a glass-stoppered, 25-mL conical flask. Add 3 mL of *Propionating reagent*, and heat at 75° for 30 min. Volatilize the reagents with the aid of a stream of nitrogen at room temperature, and add 12 mL of chloroform. Dilute 1 mL of this mixture with chloroform to 20 mL.

Sample solution: Transfer 50 mg of Glycerol Monostearate to a glass-stoppered, 25-mL conical flask. Add 5 mL of *Internal standard solution* by pipet, and mix to dissolve. Immerse the flask in a water bath, maintained at a temperature between 45° and 50°, and volatilize the chloroform with the aid of a stream of nitrogen. Add 3 mL of *Propionating reagent*, and heat at 75° for 30 min. Volatilize the reagents with the aid of a stream of nitrogen at room temperature, and add 5 mL of chloroform.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization (under typical conditions)

Column: 4-mm × 2.4-m borosilicate glass, packed with 2% liquid phase G16, on 80- to 100-mesh support S1A

Temperature

Injector: 300°

Detector: 310°

Column: The column is maintained isothermally at a temperature between 190° and 200°.

Carrier gas: Helium

Flow rate: 70 mL/min

System suitability

Sample: *Standard solution* (6–10 injections)

Suitability requirements

Resolution: NLT 4.0 between derivatized glycerin and tributyrin

Relative standard deviation: NMT 2.0% of the ratio of their peak areas

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the response factor, F:

$$F = (A_D/A_S) \times (W_S/W_D)$$

A_D = peak area of tributyrin from the *Standard solution*

A_S = peak area of tripropionin from the *Standard solution*

W_S = weight of glycerin in the *Standard solution* (mg)

W_D = weight of tributyrin in the *Standard solution* (mg)

Calculate the percentage of glycerin in the portion of Glycerol Monostearate taken:

$$\text{Result} = (A_U/A_S) \times (W_D/W_U) \times F \times 100$$

A_U = peak area of tripropionin in the *Sample solution*

A_S = peak area of tributyrin in the *Sample solution*

W_D = weight of tributyrin in 5 mL of *Internal standard solution* (mg)

W_U = weight of Glycerol Monostearate in the *Sample solution* (mg)

Acceptance criteria: NMT 1.2%

SPECIFIC TESTS

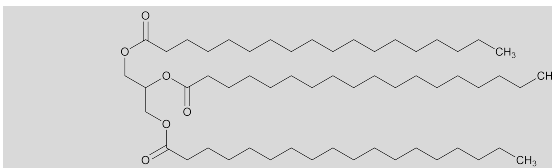
- **MELTING RANGE OR TEMPERATURE, Class I <741>:** Does not melt below 55°
- **FATS AND FIXED OILS, Acid Value <401>:** NMT 6
- **FATS AND FIXED OILS, Hydroxyl Value <401>:** 290–330
- **FATS AND FIXED OILS, Iodine Value <401>:** NMT 3
- **FATS AND FIXED OILS, Saponification Value <401>:** 150–165

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **LABELING:** Label it to indicate the name and quantity of any added antioxidant.

Add the following:

▲ Glycerol Tristearate



$C_{57}H_{110}O_6$ 891.49
Octadecanoic acid, 1,1',1''-(1,2,3-propanetriyl) ester;
Octadecanoic acid, 1,2,3-propanetriyl ester;
Glycerol trioctadecanoate;
Tristearoylglycerol [555-43-1].

DEFINITION

Glycerol Tristearate contains NLT 90.0% of triglycerides of saturated fatty acids, chiefly glycerol tristearate ($C_{57}H_{110}O_6$).

IDENTIFICATION

• FATTY ACID COMPOSITION

Boron trifluoride methanol solution: 140 mg/mL of boron trifluoride in methanol

Saturated sodium chloride solution: Mix 1 part of sodium chloride with 2 parts of water, shake from time to time, and allow to stand. Before use, decant the solution from any undissolved substance and filter, if necessary.

Standard solution 1: 0.5 mg/mL of USP Methyl Myristate RS, 2.0 mg/mL of USP Methyl Stearate RS, and 2.0 mg/mL of USP Methyl Oleate RS in *n*-heptane

Standard solution 2: 0.05 mg/mL of USP Methyl Myristate RS, 0.2 mg/mL of USP Methyl Stearate RS and 0.2 mg/mL of USP Methyl Oleate RS in *n*-heptane, diluted from *Standard solution 1*

Standard solution 3: Dissolve a quantity of an ester mixture¹ containing methyl hexanoate, methyl caprylate, methyl caprate, methyl laurate, methyl myristate, methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate, and methyl arachidate in *n*-heptane to make a solution of about 9.0 mg/mL for methyl stearate and 0.1–0.2 mg/mL for each other methyl ester.

Sample solution: Transfer 100 mg of Glycerol Tristearate to a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 2 mL of a 20-mg/mL solution of sodium hydroxide in methanol, mix, and reflux for about 30 min. Add 2 mL of *Boron trifluoride methanol solution* through the condenser, and reflux for about 30 min. Add 4 mL of *n*-heptane through the condenser, and reflux for 5 min. Cool, remove the condenser, add about 10 mL of *Saturated sodium chloride solution*, and shake. Add a quantity of *Saturated sodium chloride solution* to bring the upper layer up to the neck of the flask, and allow the layers to separate. Collect 2 mL of the *n*-heptane layer (the upper layer); wash with three quantities of water of 2 mL each, and dry the *n*-heptane phase over anhydrous sodium sulfate.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m capillary column, bonded with a 1.0-μm layer of phase G16

¹ Ester mixture can be obtained commercially from Nu-Chek-Prep, Inc., P.O. Box 295, Elysian, MN 56028, or from Sigma-Aldrich; or it can be prepared by mixing a commercially available ester mixture with methyl stearate.

Temperatures
Injection port: 250°
Detector: 250°
Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	20	180	—
180	9	240	12

Carrier gas: Helium
Flow rate: 10 mL/min
Injection volume: 2 µL
Injection type: Split ratio, 4:1
System suitability

Samples: *Standard solution 1* and *Standard solution 2*
[NOTE—The relative retention times for methyl myristate, methyl stearate, and methyl oleate are about 0.70, 1.00, and 1.01, respectively.]

Suitability requirements

Resolution: NLT 1.5 between methyl stearate and methyl oleate, *Standard solution 1*

Signal-to-noise ratio: NLT 5 for methyl myristate, *Standard solution 2*

Analysis

Samples: *Standard solution 3* and *Sample solution*
Measure the areas for the peaks of the methyl esters of the fatty acids. Disregard any peaks with an area less than 0.05% of the total area and any peak due to the solvent. [NOTE—Relative retention times for several methyl esters are summarized in Table 2.]

Table 2

Carbon-Chain Length	Number of Double Bonds	Relative Retention Times
12	0	0.58
14	0	0.70
16	0	0.83
18	0	1.00
18	1	1.01
20	0	1.14

Take the main component of *Standard solution 3* as a reference component, and calculate the calibration factor, F_{FA} , for each fatty acid methyl ester:

$$\text{Result} = [(F_{MC} \times P_{FA1} \times A_{MC}) / (P_{MC} \times A_{FA1})] \times 100$$

F_{MC} = factor for the main component, 1
 P_{FA1} = percentage by weight of the fatty acid methyl ester in *Standard solution 3*
 A_{MC} = peak area of the main component in *Standard solution 3*
 P_{MC} = percentage by weight of the main component of *Standard solution 3*
 A_{FA1} = peak area of the fatty acid methyl ester in *Standard solution 3*

Calculate the percentage of the fatty acid methyl ester by weight in the portion of Glyceryl Tristearate taken:

$$\text{Result} = [(A_{FA2} \times F_{FA}) / A_T] \times 100$$

A_{FA2} = peak area of the fatty acid methyl ester in the *Sample solution*
 A_T = sum of the peak areas of the fatty acid methyl esters in the *Sample solution*

Acceptance criteria: Glyceryl Tristearate exhibits the composition profile of fatty acids shown in Table 3.

Table 3

Carbon-Chain Length	Number of Double Bonds	Percentage (w/w)
6, 8, 10	0	0.0–0.3
12	0	0.0–0.5
14	0	0.0–0.5
16	0	0.0–2.0
16	1	0.0–0.1
18	0	≥97
18	1	0.0–0.5
18	2	0.0–0.5
20	0	0.0–0.5

ASSAY

• **CONTENT OF TRIGLYCERIDES**

Mobile phase: Tetrahydrofuran

System suitability solution: 40 mg/mL of USP Glyceryl Distearate RS in *Mobile phase*

Sample solution: 8 mg/mL of Glyceryl Tristearate in *Mobile phase*

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.5-mm × 60-cm; 3-µm or 5-µm 100-Å packing L21

Temperature

Detector: 40°

Column: 40°

[NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of the one 60-cm column, provided that system suitability requirements are met. The column temperature may be lowered to ambient temperature, although working at 40° provides stable separation conditions and ensures better sample solubility.]

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for triglycerides, diglycerides, monoglycerides, and glycerin are 0.78, 0.81, 0.86, and 1.00, respectively.]

Suitability requirements

Resolution: NLT 1.0 between the diglycerides and the monoglycerides

Relative standard deviation: NMT 2.0%, determined for the monoglycerides peak

Analysis

Samples: *System suitability solution* and *Sample solution*
Calculate the percentage of triglycerides in the portion of Glyceryl Tristearate taken:

$$\text{Result} = (r_U / r_T) \times 100$$

r_U = peak response of the triglycerides in the *Sample solution*

r_T = sum of all the glyceride peak responses from the *Sample solution*

Acceptance criteria: NLT 90.0% of triglycerides

IMPURITIES

• **RESIDUE ON IGNITION** <281>: NMT 0.1%

• **HEAVY METALS, Method II** <231>: NMT 10 ppm

• **ALKALINE IMPURITIES**

Analysis: Prepare a mixture of 2.0 g of Glyceryl Tristearate, 15 mL of alcohol, and 30 mL of ether. Dissolve by gentle heating. Add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid VS until the mixture turns yellow.

Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid is required.

• **LIMIT OF NICKEL**

[CAUTION]—When using closed high-pressure digestion vessels and microwave laboratory equipment, be familiar with the safety and operating instructions given by the manufacturer.]

Magnesium nitrate solution: 10 mg/mL of magnesium nitrate in water

Ammonium dihydrogen phosphate solution: 100 mg/mL of ammonium dihydrogen phosphate in water

Standard stock solution: Transfer 5.0 mL of nickel standard solution TS to a 10-mL volumetric flask, and dilute with water to make a solution containing 5 µg/mL of nickel.

Standard solutions: To four identical 25-mL volumetric flasks, each containing 6 mL of nitric acid, transfer respectively 25, 50, 75, and 100 µL of the *Standard stock solution*. To each flask, add 0.5 mL of *Magnesium nitrate solution* and 0.5 mL of *Ammonium dihydrogen phosphate solution*, and dilute with water to volume. These solutions contain, respectively, 0.005, 0.01, 0.015, and 0.02 µg/mL of nickel.

Sample solution: Transfer 0.25 g of Glyceryl Tristearate to a suitable high-pressure-resistant digestion vessel (fluoropolymer or quartz), and add 6.0 mL of nitric acid and 2.0 mL of 30% hydrogen peroxide. Place the closed vessel in a laboratory microwave oven, and digest using an appropriate program (e.g., 1000 W for 40 min). Allow the digestion vessel to cool before opening. Add 2.0 mL of 30% hydrogen peroxide, and repeat the digestion step. Allow the digestion vessel to cool before opening. Quantitatively transfer the contents to a 25-mL volumetric flask, add 0.5 mL of *Magnesium nitrate solution* and 0.5 mL of *Ammonium dihydrogen phosphate solution*, dilute with water to volume, and mix.

Blank solution: Add 6.0 mL of nitric acid and 2.0 mL of 30% hydrogen peroxide to a high-pressure-resistant digestion vessel, and proceed as directed for the *Sample solution*.

Zero solution: Into a 50.0-mL volumetric flask, introduce 1.0 mL of *Magnesium nitrate solution*, 1.0 mL of *Ammonium dihydrogen phosphate solution*, and 12.0 mL of nitric acid. Dilute with water to volume, and mix.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Maximum absorbance at 232.0 nm

Detection: Graphite furnace

Lamp: Nickel hollow-cathode

Tube: Pyrolytically-coated tube

Other: Background compensation system

Furnace program: See Table 4.

[NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Table 4

Step	Dry	Ash	Cool	Atomize
Temperature (°)	120	1100	800	2600
Ramp time (s)	5	30	5	—
Hold time (s)	35	10	5 (decrease)	7

Analysis

Samples: *Blank solution*, *Standard solutions*, and *Sample solution*

Use the *Zero solution* to set the instrument to zero. Concomitantly determine the absorbances of the

Samples at least three times each. Record the average of the steady readings for each of the solutions. If necessary, dilute the *Sample solution* with the *Zero solution* to obtain a reading within the calibrated absorbance range.

Plot the absorbances versus the concentrations of nickel, in µg/mL, in the *Standard solutions*. On the basis of the calibration curve, determine the concentrations of nickel, in µg/mL, in the *Sample solution* and the *Blank solution* from the corresponding absorptions. Obtain the correct concentration of nickel, C_c , in µg/mL, in the *Sample solution*.

Calculate the content of nickel, in µg/g (ppm), in the portion of Glyceryl Tristearate taken:

[NOTE—If the *Sample solution* is diluted with the *Zero solution*, apply an appropriate correction factor for dilution.]

$$\text{Result} = (C_c \times V)/W$$

C_c = concentration of nickel in the *Sample solution* (µg/mL)

V = volume of the *Sample solution*, 25 mL

W = weight of Glyceryl Tristearate taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 1 µg/g (ppm)

SPECIFIC TESTS

• **MELTING RANGE**, *Class II* (741): 69°–73°

• **FATS AND FIXED OILS**, *Acid Value* (401): NMT 1.0

• **FATS AND FIXED OILS**, *Hydroxyl Value* (401): NMT 5.0

• **FATS AND FIXED OILS**, *Saponification Value* (401): 186–192

• **FATS AND FIXED OILS**, *Peroxide Value* (401): NMT 3

• **FATS AND FIXED OILS**, *Unsaponifiable Matter* (401): NMT 0.5%

• **WATER DETERMINATION**, *Method Ia* (921): NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and store at room temperature. Protect from moisture.

• **USP REFERENCE STANDARDS** (11)

USP Glyceryl Distearate RS

USP Methyl Myristate RS

USP Methyl Oleate RS

USP Methyl Stearate RS▲ NF31

Glycine—see *Glycine General Monographs*

Guar Gum

DEFINITION

Guar Gum is a gum obtained from the ground endosperms of *Cyamopsis tetragonolobus* (Linné) Taub. (Fam. Leguminosae). It consists chiefly of a high molecular weight hydrocolloidal polysaccharide, composed of galactan and mannan units combined through glycosidic linkages, which may be described chemically as a galactomannan.

IDENTIFICATION

• **A.**

Sample: 2 g

Analysis: Place the *Sample* in a 400-mL beaker, and moisten it with 4 mL of isopropyl alcohol. Add 200 mL of cold water with vigorous stirring, and continue stirring until the *Sample* is completely and uniformly dispersed.

Acceptance criteria: An opalescent, viscous solution results.

• **B.**

Analysis: Transfer 100 mL of the solution prepared in *Identification test A* to a 400-mL beaker, heat in a boiling water bath for about 10 min, and cool.

Acceptance criteria: No appreciable increase in viscosity is produced. (Distinction from locust bean gum: see *Reagents, Indicators, and Solutions—Reagents*).

ASSAY• **CONTENT OF GALACTOMANNANS**

Analysis: Subtract from 100.0 the total percentages from the tests for *Articles of Botanical Origin*, *Total Ash*, *Acid-Insoluble Matter*, *Protein*, and *Loss on Drying*.

Acceptance criteria: NLT 66.0%

IMPURITIES• **ARSENIC**, *Method II* (211): NMT 3 ppm• **LEAD** (251)

Analysis: Prepare a *Test Preparation* as directed in the chapter, and use 10 mL of *Diluted Standard Lead Solution* (10 µg of Pb) for the test.

Acceptance criteria: NMT 10 ppm

• **HEAVY METALS**, *Method II* (231): NMT 20 ppm**SPECIFIC TESTS**• **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 1.5%• **ACID-INSOLUBLE MATTER**

Sample: 1.5 g

Analysis: Transfer the *Sample* to a 250-mL beaker containing 150 mL of water and 1.5 mL of sulfuric acid. Cover the beaker with a watch glass, and heat the mixture on a steam bath for 6 h, rubbing down the wall of the beaker frequently with a rubber-tipped stirring rod, and replacing any water lost by evaporation. At the end of the 6-h heating period, add 500 mg of a suitable filter aid, and pass through a suitable tared, ashless filter. Wash the residue several times with hot water, dry the filter and its contents at 105° for 3 h, cool in a desiccator, and weigh. Determine the amount of acid-insoluble matter by subtracting the weight of the filter aid from that of the residue.

Acceptance criteria: NMT 7.0%

• **PROTEIN**

Sample: 1.0 g

Analysis: Transfer the *Sample* to a 500-mL Kjeldahl flask, and proceed as directed in *Nitrogen Determination* (461), *Method I*. Determine the percentage of nitrogen. Calculate the amount of protein by multiplying the percentage of nitrogen by 6.25.

Acceptance criteria: NMT 10.0%

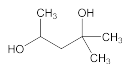
• **STARCH**

Analysis: To a solution (1 in 10) of Guar Gum add a few drops of iodine TS.

Acceptance criteria: No blue color is produced.

• **LOSS ON DRYING** (731): Dry a sample at 105° for 5 h: it loses NMT 15.0% of its weight.**ADDITIONAL REQUIREMENTS**• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Hexylene Glycol



C₆H₁₄O₂
2,4-Pentanediol, 2-methyl-;
2-Methyl-2,4-pentanediol [107-41-5].

118.17

DEFINITION

Hexylene Glycol is 2-methyl-2,4-pentanediol.

IDENTIFICATION• **A. INFRARED ABSORPTION** (197F)

Sample: Undried specimen

Acceptance criteria: Meets the requirements

SPECIFIC TESTS• **SPECIFIC GRAVITY** (841): 0.917–0.923• **REFRACTIVE INDEX** (831): 1.424–1.430• **ACIDITY**

Sample: 10 mL

Analysis: Mix 1 mL of phenolphthalein TS with 50 mL of water, and add 0.1 N sodium hydroxide until the solution remains pink for 30 s. Add the *Sample*, and titrate with 0.10 N sodium hydroxide until the original pink color returns and remains for 30 s.

Acceptance criteria: NMT 0.20 mL of 0.10 N sodium hydroxide is required.

• **WATER DETERMINATION**, *Method I* (921): NMT 0.5%**ADDITIONAL REQUIREMENTS**• **PACKAGING AND STORAGE:** Preserve in tight containers.• **USP REFERENCE STANDARDS** (11)

USP Hexylene Glycol RS

Purified Honey

DEFINITION

Purified Honey is obtained by purification of honey from the comb of the bee, *Apis mellifera* L. and all subspecies of *Apis mellifera*. The honey is extracted by centrifugation, pressure, or other suitable procedures.

IDENTIFICATION• **A.**

Sample solution: 1 in 20 solution

Standard solution: A 7.5 µg/mL solution of USP L-Proline RS

Analysis

Samples: *Standard solution* and *Sample solution*

Place 0.5 mL of the *Sample solution* and the *Standard solution* in separate reaction vials, and add 0.25 mL of formic acid and 1.0 mL of freshly prepared 3% solution of ninhydrin in peroxide-free ethylene glycol monomethyl ether to each vial. Cap the reaction vials tightly, shake well, and place in boiling water for 15 min. Cool for 5 min in a 22° water bath, remove the caps, and add 5.0 mL of 5% aqueous isopropanol solution.

Acceptance criteria: The *Sample solution* shows a purple color similar to, or more intense than that of the *Standard solution*. If necessary, scan the solutions being compared using a suitable spectrophotometer against a water blank. The two solutions each exhibit a maximum at the same wavelength, at about 520 nm. The absorbance of the *Sample solution* should be at least as high as that of the *Standard solution*.

IMPURITIES• **CHLORIDE AND SULFATE**, *Chloride* (221): A 1-g portion shows no more chloride than corresponds to 0.5 mL of 0.020 N hydrochloric acid (0.035%).• **CHLORIDE AND SULFATE**, *Sulfate* (221): A 2-g portion shows no more sulfate than corresponds to 0.5 mL of 0.020 N sulfuric acid (0.024%).• **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 0.3%**SPECIFIC TESTS**• **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total bacterial count does

not exceed 10^3 cfu/g. The total combined molds and yeasts count does not exceed 100 cfu/g.

- **SPECIFIC GRAVITY** (841): 1.400–1.435 at 20°
- **REFRACTIVE INDEX** (831): 1.4900–1.4992 at 20°
- **WATER DETERMINATION, Method I** (921): 15.0%–18.6%
- **MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS** (2022): Where it is intended for use in preparations for infants under one year of age, it meets the requirements of the *Test for Absence of Clostridium Species*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** Label it to indicate that it is not intended for use in preparations for infants under one year of age, unless it meets the requirements of the *Test for Absence of Clostridium Species*.
- **USP REFERENCE STANDARDS** (11)
USP L-Proline RS

Hydrochloric Acid

HCl 36.46
Hydrochloric acid [7647-01-0].

DEFINITION

Hydrochloric Acid contains NLT 36.5% and NMT 38.0%, by weight, of HCl.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Chloride** (191)

ASSAY

• PROCEDURE

Sample: 3 mL

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 1 N sodium hydroxide VS

Blank: 45 mL of water

Endpoint detection: Visual

Analysis: Place the *Sample* in a glass-stoppered flask, previously tared while containing about 20 mL of water, and weigh again to obtain the weight of the substance under assay. Dilute with 25 mL of water, and add methyl red TS. Titrate with 1 N sodium hydroxide VS. Calculate the percentage of hydrochloric acid (HCl) in the *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times N \times F] / W \times 100$$

V_S = volume of *Titrant* consumed by the *Sample* (mL)

V_B = volume of *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 36.46 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 36.5%–38.0%

IMPURITIES

• RESIDUE ON IGNITION (281)

Sample: 20 mL

Analysis: To the *Sample*, add 2 drops of sulfuric acid, evaporate to dryness, and ignite.

Acceptance criteria: NMT 2 mg of residue remains (about 0.008%).

• HEAVY METALS (231)

Sample: 3.4 mL (4 g)

Analysis: Evaporate the *Sample* on a steam bath to dryness, add 2 mL of 1 N acetic acid to the residue, and dilute with water to 25 mL.

Acceptance criteria: NMT 5 ppm

SPECIFIC TESTS

• BROMIDE OR IODIDE

Sample solution: 1 part Hydrochloric Acid to 2 parts water

Analysis: Add 1 mL of chloroform to 10 mL of the *Sample solution*. Cautiously add, dropwise, with constant agitation, chlorine TS that has been diluted with an equal volume of water.

Acceptance criteria: The chloroform remains free from even a transient yellow, orange, or violet color.

• FREE BROMINE OR CHLORINE

Sample solution: 1 part Hydrochloric Acid to 2 parts water

Analysis: Add 1 mL of potassium iodide TS and 1 mL of chloroform to 10 mL of the *Sample solution*, and agitate the mixture.

Acceptance criteria: The chloroform remains free from any violet color for at least 1 min.

• SULFATE

Sample solution: 1 part Hydrochloric Acid to 2 parts water

Analysis: To a mixture of 3 mL of the *Sample solution* and 5 mL of water, add 5 drops of barium chloride TS.

Acceptance criteria: Neither turbidity nor precipitate appears within 1 h.

• SULFITE

Sample solution: 1 part Hydrochloric Acid to 2 parts water

Analysis: Complete the test for *Sulfate*, then add to the liquid 2 drops of 0.1 N iodine.

Acceptance criteria: Neither turbidity nor decolorization of the iodine occurs.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Diluted Hydrochloric Acid

DEFINITION

Diluted Hydrochloric Acid contains, in each 100 mL, NLT 9.5 g and NMT 10.5 g of hydrochloric acid (HCl).

Diluted Hydrochloric Acid may be prepared as follows.

Hydrochloric Acid	226 mL
Purified Water, a sufficient quantity to make	1000 mL

Mix the ingredients.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Chloride** (191)

ASSAY

• PROCEDURE

Sample: 10.0 mL

Blank: 20 mL of water

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 1 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a conical flask, and add about 20 mL of water. Add 3 drops of methyl red TS.

Titrate with *Titrant*. Perform a blank determination.

Calculate the amount of hydrochloric acid (HCl) in the portion of sample taken:

$$\text{Result} = (V_S - V_B) \times N \times F$$

V_S = Titrant volume consumed by the *Sample* (mL)

V_B = Titrant volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 3.646×10^{-2} g/mEq

Acceptance criteria: 9.5–10.5 g of HCl in each 100 mL

IMPURITIES

• RESIDUE ON IGNITION (281)

Sample: 20 mL

Analysis: To the *Sample* add 2 drops of sulfuric acid, evaporate to dryness, and ignite.

Acceptance criteria: NMT 2 mg

• HEAVY METALS (231)

Test preparation: To 3.8 mL (4 g) of Diluted Hydrochloric Acid add 5 mL of water and 1 drop of phenolphthalein TS. Add 6 N ammonium hydroxide until the solution assumes a faint pink color. Add 2 mL of 1 N acetic acid, then add water to make 25 mL.

Acceptance criteria: NMT 5 µg/g

• FREE BROMINE OR CHLORINE

Sample: 10 mL

Analysis: To the *Sample* add 1 mL of potassium iodide TS and 1 mL of chloroform, and agitate the mixture.

Acceptance criteria: The chloroform remains free from any violet coloration for at least 1 min.

• SULFATE

Sample solution: Mix 3 mL of Diluted Hydrochloric Acid and 5 mL of water.

Analysis: To the *Sample solution* add 5 drops of barium chloride TS.

Acceptance criteria: Neither turbidity nor precipitate appears within 1 h.

• SULFITE

Analysis: Complete the test for *Sulfate*, then add to the liquid 2 drops of 0.1 N iodine.

Acceptance criteria: Neither turbidity nor decolorization of the iodine occurs.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Hydroxyethyl Cellulose

Cellulose, 2-hydroxyethyl ether [9004-62-0].

DEFINITION

Hydroxyethyl Cellulose is a partially substituted poly(hydroxyethyl) ether of cellulose. It is available in several grades, varying in viscosity and degree of substitution, and some grades are modified to improve their dispersion in water. It may contain suitable anticaking agents.

IDENTIFICATION

• A.

Sample solution: 1 g in 100 mL of water

Analysis: Stir the *Sample solution*.

Acceptance criteria: It dissolves completely to produce a colloidal solution that remains clear when heated to 60°.

• B.

Sample: 1 mL of the solution from *Identification* test A

Analysis: Place the *Sample* on a glass plate, and allow to evaporate.

Acceptance criteria: A thin, self-sustaining film is formed.

• C.

Sample solution: 1 in 2000

Analysis: To 1 mL of the *Sample solution* add 1 mL of phenol solution (50 mg/mL), then add 5 mL of sulfuric acid, shake, and allow to cool.

Acceptance criteria: The color of the solution becomes orange.

IMPURITIES

- **RESIDUE ON IGNITION (281):** NMT 5.0%

- **LEAD (251):** NMT 10 µg/g

- **HEAVY METALS, Method II (231):** NMT 20 µg/g

SPECIFIC TESTS

• PH (791)

Sample solution: 10 mg/mL

Acceptance criteria: 6.0–8.5

• LOSS ON DRYING (731)

Analysis: Dry at 105° for 3 h.

Acceptance criteria: NMT 10.0%

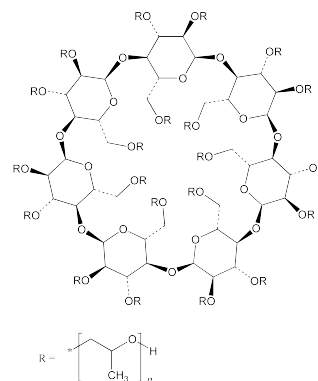
- **VISCOSITY—CAPILLARY VISCOMETER METHODS (911) and ROTATIONAL RHEOMETER METHODS (912):** When determined at the concentration and under the conditions specified in the labeling, its viscosity is NLT 50% and NMT 150% of the labeled viscosity, where stated as a single value, or it is between the maximum and minimum values, where stated as a range of viscosities.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

- **LABELING:** The labeling indicates its viscosity, under specified conditions, in aqueous solution. The indicated viscosity may be in the form of a range encompassing 50%–150% of the average value.

Hydroxypropyl Betadex



$C_{42}H_{70}O_{35}(C_3H_6O)_x$ where $x = 7 \times MS$, MS being molar substitution

Beta cyclodextrin, 2-hydroxypropyl ether [94035-02-6].

DEFINITION

Hydroxypropyl Betadex is a partially substituted poly(hydroxypropyl) ether of Betadex. The number of hydroxypropyl groups per anhydroglucose unit expressed as molar substitution (MS) is NLT 0.40 and NMT 1.50 and is within 10% of the value stated on the label.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K):** The spectrum obtained with Hydroxypropyl Betadex shows the same absorption bands as the spectrum acquired with USP Hydroxypropyl Betadex RS. Due to the difference in the substitution of the substance, the intensity of some absorption bands may vary.

- **B.** It meets the requirements of the test for *Clarity of Solution*.

ASSAY• **MOLAR SUBSTITUTION**

(See *Nuclear Magnetic Resonance* <761>.)

The molar substitution (MS) is calculated from the ratio between the signal from the three protons of the methyl group, contained in the hydroxypropyl functional group, and the signal from the proton attached to the carbon C₁ (glycosidic proton) of the anhydroglucose units.

Use a Fourier-transform nuclear magnetic resonance (NMR) spectrometer having a magnetic field strength of at least 6 Tesla and that is capable of performing quantitative analysis using proton NMR spectroscopy at a temperature of at least 25°.

Sample solution: Mix NLT the equivalent of 10.0 mg of dried Hydroxypropyl Betadex with 0.75 mL of deuterium oxide thoroughly in an NMR tube. Place the tube into an NMR probe.

Analysis: Adjust the spectrometer settings so that a high-resolution proton NMR spectrum can be acquired that will provide quantitative data. Acquire a free induction decay (FID) with at least 8 transients using a spectral window from at least 0–6.2 ppm, with the solvent peak located at 4.8 ppm at 25°. Zero fill the spectrum at least three times, and Fourier transform the FID with no Gaussian line broadening and no more than 0.2 Hz of Lorentzian line broadening.

Determine the peak areas of the doublet from the methyl protons of the hydroxypropyl functional group at 1.2 ppm (*A*₁) and the peak areas from the glycosidic protons, which are located between 5 and 5.4 ppm (*A*₂).

Calculate the MS:

$$\text{Result} = A_1/(3A_2)$$

*A*₁ = area of the methyl group of hydroxypropyl

*A*₂ = area of the glycosidic proton

The degree of substitution is the number of hydroxypropyl groups per molecule of betadex and is obtained by multiplying the MS by 7.

Acceptance criteria: 0.40–1.50 and within 10% of the value stated on the label

IMPURITIES

- **HEAVY METALS,** *Method I* (231): NMT 20 µg/g
- **LIMIT OF BETADEX, PROPYLENE GLYCOL, AND OTHER RELATED SUBSTANCES**

Mobile phase: Water

Standard solution A: 15 mg/mL of USP Beta Cyclodextrin RS and 25 mg/mL of USP Propylene Glycol RS

Standard solution B: 1.0 mL of *Standard solution A* diluted with water to 10.0 mL

Sample solution: Dissolve 2.50 g of Hydroxypropyl Betadex, accurately weighed and calculated on the dried basis, in water with the aid of heat. Cool, and dilute with water to 25.0 mL. The resulting solution is 100 mg/mL of Hydroxypropyl Betadex, calculated on the dried basis, in water.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Differential refractometer

Columns

Guard: Packing L11

Analytical: 3.9-mm × 30-cm; packing L11

Column temperature: 40°

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

[NOTE—The retention time of propylene glycol is about 2.5 min, and the relative retention times with reference to that of propylene glycol for betadex and hydroxypropyl betadex are about 4.2 and about 6, respectively; Hydroxypropyl Betadex elutes as a very wide peak or several peaks.]

Suitability requirements

Resolution: NLT 4 between betadex and propylene glycol, *Standard solution A*

Relative standard deviation: NMT 2.0%, *Standard solution B*

Analysis

Samples: *Standard solution B* and *Sample solution*

Acceptance criteria: Disregard any peaks eluting before propylene glycol and after the hydroxypropyl betadex peak.

Betadex: NMT 1.5%; the area of the betadex peak in the *Sample solution* is NMT the area of the corresponding peak from *Standard solution B*.

Propylene glycol: NMT 2.5%; the area of the propylene glycol peak in the *Sample solution* is NMT the area of the corresponding peak from *Standard solution B*.

Any other single impurity: NMT 0.25%; the area from any other single impurity peak is NMT 0.1 times the area of propylene glycol in the chromatogram of *Standard solution B*.

Total impurities excluding betadex and propylene glycol: NMT 1%; the total area from all impurity peaks, excluding betadex and propylene glycol, is NMT 0.4 times the area of propylene glycol from *Standard solution B*.

Disregard limit: 0.1%; disregard any peaks that are less than 0.04 times the area of propylene glycol from *Standard solution B*.

• **LIMIT OF PROPYLENE OXIDE**

Ether stock solution: Add 75 µL of ether to 30 mL of dimethylacetamide in a 50-mL volumetric flask, dilute with dimethylacetamide to volume, and mix. This solution contains 1.0 mg/mL of ether.

Internal standard solution: Add 30 µL of *Ether stock solution* to 70 mL of dimethylacetamide in a 100-mL volumetric flask, dilute with dimethylacetamide to volume, and mix.

Propylene oxide stock solution

[CAUTION—Propylene oxide is toxic and flammable.

Prepare this solution in a well-ventilated fume hood.]

Add 30 mL of dimethylacetamide into a 50-mL volumetric flask. Weigh the flask and contents accurately, add 60 µL of propylene oxide (cooled in a refrigerator) into the flask with a 100-µL cooled microsyringe, weigh again, and calculate the weight of propylene oxide added, by difference. Dilute with dimethylacetamide to volume, and mix. This solution contains 1.0 mg/mL of propylene oxide.

[NOTE—Propylene oxide is a gas at room temperature. It is usually stored in a lecture-type gas cylinder or small metal pressure bomb. Chill the cylinder in a refrigerator before use. Transfer 5 mL of the liquid propylene oxide to a 100-mL beaker chilled in wet ice. Use a gas-tight syringe that has been chilled in a refrigerator.]

System suitability solution: Add 30 µL of *Ether stock solution* and 20 µL of *Propylene oxide stock solution* to 70 mL of dimethylacetamide in a 100-mL volumetric

flask, dilute with dimethylacetamide to volume, and mix.

Standard stock solutions: Add 7 mL of dimethylacetamide into each of four 10-mL volumetric flasks.

Transfer the following amounts of *Propylene oxide stock solution* into each of the four flasks using a microsyringe, with one amount per flask: 40, 100, 200, and 400 μL . Dilute with dimethylacetamide to volume, and mix. The *Standard stock solutions* contain about 4, 10, 20, and 40 $\mu\text{g}/\text{mL}$ of propylene oxide, respectively.

Standard solutions: Into each of four 10-mL headspace vials, transfer 200 ± 5 mg of Hydroxypropyl Betadex, calculated on the dried basis. Pipet 1.0 mL of the *Internal standard solution* into each vial, and close the vial with septum and cap. Into each of the vials add 10 μL of each of the *Standard stock solutions* using a 10- μL syringe, respectively. Allow each vial to stand, and gently shake until the sample is dissolved. The *Standard solutions* contain, respectively, about 0.04, 0.1, 0.2, and 0.4 $\mu\text{g}/\text{mL}$ of propylene oxide.

Sample solution: Transfer 200 ± 5 mg of Hydroxypropyl Betadex, calculated on the dried basis, into a 10-mL headspace autosampler vial. Pipet 1.0 mL of the *Internal standard solution* into the vial, and close the vial with a septum and cap. Add 10 μL of dimethylacetamide using a 10- μL syringe. Allow the vial to stand, and gently shake until the sample is dissolved.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC with a balanced pressure automatic headspace sampler

Detector: Flame ionization

Column: 0.32-mm \times 10-m fused-silica capillary; coated with a 10- μm layer of stationary phase S3

Temperatures

Injection port: 120°

Detector: 250°

Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	10
50	10	100	10
100	20	220	4

Transfer line: 120°

Carrier gas: Helium

Flow rate: 2 mL/min, corresponding to the linear velocity of 44 cm/s

Injection volume: 1.0 mL

Injection type: Split injection; the split ratio is 1:1.

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for propylene oxide and ether are about 1.0 and 1.3, respectively.]

Suitability requirements

Resolution: NLT 2.0 between ether and propylene oxide

Analysis

Samples: *Standard solutions* and *Sample solution*

Separately place the vials containing the *Standard solutions* and the *Sample solution* in the automated sampler, and start the sequence so that the vial is heated at a temperature of 100° for 30 min before a suitable portion of its headspace is injected into the chromatograph.

Using a 2-mL gas syringe preheated in an oven at 110°, separately inject the headspace from each vial into the chromatograph. Chromatograph the *Standard solutions* and the *Sample solution*, record the chromatograms,

and measure the area ratios of the peak responses of propylene oxide and ether.

Determine, based on a retention time comparison, whether propylene oxide is detected in the *Sample solution*. Plot the area ratios of the peak responses of propylene oxide and ether of the *Sample solution* and the *Standard solutions* versus the content, in μg , of propylene oxide in each vial, as furnished by the *Standard stock solutions*, draw the straight line best fitting the five points, and calculate the correlation coefficient for the line. [NOTE—The *Sample solution* should be plotted as if it had a content of added propylene oxide equivalent to 0 μg .]

A suitable system is one that yields a line having a correlation coefficient of NLT 0.99. Extrapolate the line until it meets the content axis on the negative side. The distance between this point and the intersection of the axes represents the total amount, T_u , in μg , of propylene oxide in the *Sample solution*.

Calculate the percentage of propylene oxide in the portion of sample taken:

$$\text{Result} = (T_u/W) \times 100$$

T_u = total amount of propylene oxide from the graph in the *Sample solution* (μg)

W = weight of Hydroxypropyl Betadex taken to prepare the *Sample solution* (μg)

Acceptance criteria: NMT 0.0001%

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** <61> and **TESTS FOR SPECIFIED MICROORGANISMS** <62>: The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.

• **LOSS ON DRYING** <731>

Sample: 1 g

Analysis: Dry the *Sample* at 120° for 2 h.

Acceptance criteria: NMT 10.0%

• **CLARITY OF SOLUTION**

Sample: 1.0 g

Analysis: Dissolve the *Sample* in 2.0 mL of water, and heat.

Acceptance criteria: The resulting solution is clear and remains transparent after cooling to room temperature.

• **BACTERIAL ENDOTOXINS TEST** <85>: The level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Hydroxypropyl Betadex is used can be met. Where the label states that Hydroxypropyl Betadex must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement under the relevant dosage form monograph(s) in which Hydroxypropyl Betadex is used can be met.

• **STERILITY TESTS** <71>: Where the label states that Hydroxypropyl Betadex is sterile, it meets the requirements for *Sterility Tests* <71> in the relevant dosage form monograph(s) in which Hydroxypropyl Betadex is used.

• **CONDUCTIVITY**

Sample solution: 100 mg/mL of Hydroxypropyl Betadex, calculated on the dried basis in previously boiled and cooled to room temperature water

Apparatus: Use a conductivity meter or resistivity meter that measures the resistance of the column of liquid between the electrodes of the immersed measuring device. The apparatus is supplied with alternating current to avoid the effects of electrode polarization. It is equipped with a temperature compensation device or a precision thermometer.

Standard solutions: Prepare three standard solutions of potassium chloride containing 0.7455, 0.0746, and 0.0149 g, respectively, of potassium chloride per 1000.0 g of solution. These solutions should be

prepared using water, which has been previously boiled and cooled to room temperature and has conductivity that does not exceed $2 \mu\text{S} \cdot \text{cm}^{-1}$. The conductivity and resistivity of these three *Standard solutions* at 20° are given in *Table 2*.

Table 2

Concentration of Solution (g/1000.0 g)	Conductivity ($\mu\text{S} \cdot \text{cm}^{-1}$)	Resistivity ($\Omega \cdot \text{cm}$)
0.7455	1330	752
0.0746	133.0	7519
0.0149	26.6	37,594

Calibration**Samples:** *Standard solutions*

Choose a conductivity cell that is appropriate for the conductivity of the solution to be examined. The higher the expected conductivity, the higher the cell constant that must be chosen. Commonly used conductivity cells have cell constants on the order of 0.1, 1, and 10 cm^{-1} . Use a *Standard solution* of potassium chloride that is appropriate for the measurement. The conductivity value of the *Standard solution* of potassium chloride should be near the expected conductivity value of the *Sample solution*.

Rinse the cell several times with water, which has been previously boiled and cooled to room temperature, and at least twice with the *Standard solution* (potassium chloride solution) used for the determination of the cell constant of the conductivity cell.

Measure the resistance of the conductivity cell using the *Standard solution* (potassium chloride solution) at $20 \pm 0.1^\circ$.

Calculate the constant of the conductivity cell, C (in cm^{-1}):

$$C = R_{KCl} \times K_{KCl}$$

R_{KCl} = measured resistance, expressed in mega-ohms

K_{KCl} = conductivity of the *Standard solution* of potassium chloride used, expressed in $\mu\text{S} \cdot \text{cm}^{-1}$

Calibration acceptance criteria: The measured constant, C , of the conductivity cell must be within 5% of the given value.

Analysis**Sample:** *Sample solution*

Rinse the conductivity cell several times with water, which has been previously boiled and cooled to room temperature, and at least twice with the *Sample solution*. Measure the conductivity of the *Sample solution*, while gently stirring with a magnetic stirrer.

Acceptance criteria: NMT $200 \mu\text{S} \cdot \text{cm}^{-1}$

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.
- **LABELING:** Label it to indicate the molar substitution (MS). Where Hydroxypropyl Betadex is intended for use in the manufacture of injectable dosage forms, it is so labeled. Where Hydroxypropyl Betadex must be subjected to further processing during the preparation of injectable dosage forms to ensure acceptable levels of bacterial endotoxins, it is so labeled. Where Hydroxypropyl Betadex is sterile, it is so labeled.
- **USP REFERENCE STANDARDS (11)**
 - USP Beta Cyclodextrin RS
 - USP Endotoxin RS
 - USP Hydroxypropyl Betadex RS
 - USP Propylene Glycol RS

Hydroxypropyl Cellulose

Cellulose, 2-hydroxypropyl ether [9004-64-2].

DEFINITION

Hydroxypropyl Cellulose is a partially substituted poly(hydroxypropyl) ether of cellulose. It may contain NMT 0.60% of silica or other suitable anticaking agents. When dried at 105° for 1 h, it contains NMT 80.5% of hydroxypropoxy groups.

IDENTIFICATION

- **INFRARED ABSORPTION (197K):** [NOTE—The spectrum may or may not contain a peak at about 1719 cm^{-1} .]

ASSAY• **HYDROXYPROPOXY GROUPS**

Apparatus: The apparatus for hydroxypropoxy group determinations is shown in *Figure 1*.

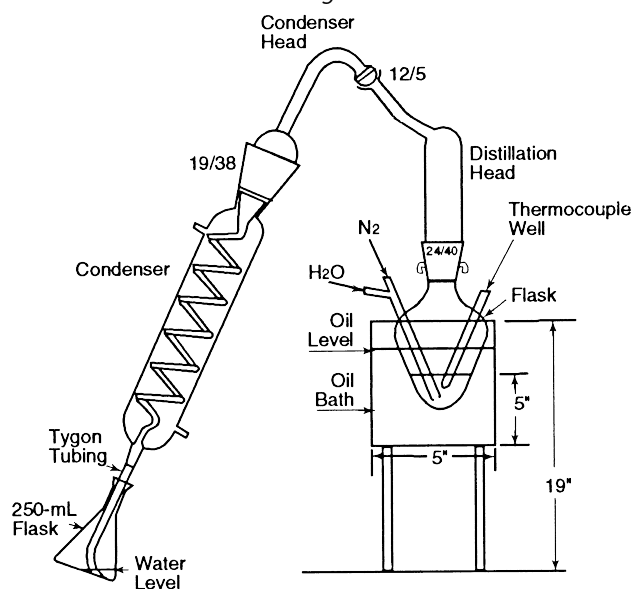


Figure 1. Apparatus for hydroxypropoxy determination.

The boiling or reaction flask, consisting of a 125-mL conical-bottom boiling flask modified to provide a thermocouple (or thermometer) well and an inlet with a 1.0-mm capillary tip for nitrogen and water (see *Figure 2*), is fitted with a distillation head that leads to a condenser. The reaction flask is immersed in an oil bath equipped with an electric heater capable of heating the bath at the desired rate and maintaining the temperature at 155° . The distillate is collected in a flask.

[NOTE—The tube from the condenser to the flask must be below the surface of the liquid in the flask to ensure the capture of all of the acetic acid formed. See *Figure 1*.]

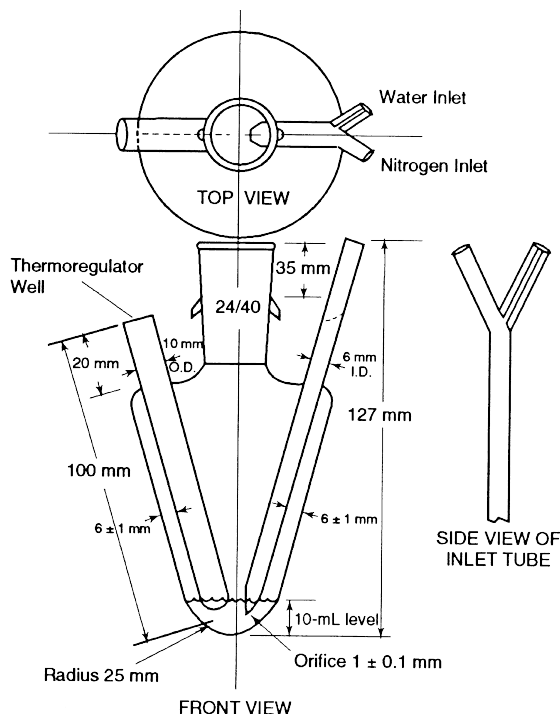


Figure 2. Boiling flask.

Analysis: Transfer 65 mg of Hydroxypropyl Cellulose, previously dried at 105° for 1 h, into the reaction flask. Add 5 mL of water, and swirl gently for 5 min. Add 10 mL of chromium trioxide solution (30 g in 70 mL). Assemble the apparatus as shown in Figures 1 and 2, and immerse the reaction flask in the oil bath slightly above the level of the chromium trioxide solution. Start the condenser cooling water, and pass nitrogen gas through the flask at a rate of about 70–75 mL/min. Raise the temperature of the oil bath to 155° during a 30-min period, and maintain it at this temperature throughout the determination. [NOTE—Too rapid an initial rise in temperature results in high blank determinations.]

Monitor the temperature of the reaction mixture in the reaction flask using a thermocouple or thermometer in a well, as shown in Figures 1 and 2. When a reaction mixture temperature of 102 ± 1° is reached, add water through the water inlet until the reaction mixture temperature drops to 97 ± 1°. Continue this 97° to 102° temperature cycle until 100 mL of distillate has been collected. Detach the condenser from the distillation head, and wash with water, collecting the washings in the flask containing the distillate. Titrate the solution with 0.02 N sodium hydroxide VS to a pH of 7.0 ± 0.1, using an expanded-scale pH meter equipped with glass and calomel electrodes. Record the volume, V, of the 0.02 N sodium hydroxide used, then add 500 mg of sodium bicarbonate and 10 mL of 2 N sulfuric acid. After evolution of carbon dioxide has ceased, add 1 g of potassium iodide, insert the stopper in the flask, shake the mixture, and allow the solution to stand in the dark for 5 min. Titrate the liberated iodine with 0.02 N sodium thiosulfate VS to the sharp disappearance of the yellow iodine color, adding a few drops of starch TS to confirm the endpoint. Record the volume, Y, required. This titration, Y mL, multiplied by the empirical factor, K, appropriate to the particular apparatus and reagents in use (see the calculation below), gives the acid equiv-

alent not caused by acetic acid. The acetic acid equivalent is (V – KY) mL of 0.02 N sodium hydroxide. Obtain the empirical factor, K, for the apparatus by performing a blank determination in which the Hydroxypropyl Cellulose is omitted. The acidity of the blank for a given apparatus and given reagents is in a fixed ratio to the oxidizing equivalent of the distillate in terms of sodium thiosulfate:

$$\text{Result} = (V_B \times N_1) / (Y_B \times N_2)$$

V_B = volume of 0.02 N sodium hydroxide required in the blank run (mL)

N_1 = normality of the 0.02 N sodium hydroxide

Y_B = volume of 0.02 N sodium thiosulfate required in the blank run (mL)

N_2 = normality of the 0.02 N sodium thiosulfate
Calculate the percentage of hydroxypropoxy groups (–OCH₂CHOHCH₃):

$$\text{Result} = (V_A N_1 - K Y_A N_2) \times (0.079/W) \times 100$$

V_A = volume of 0.02 N sodium hydroxide required for titration of the sample (mL)

N_1 = normality of the 0.02 N sodium hydroxide

K = empirical factor

Y_A = volume of 0.02 N sodium thiosulfate required for titration of the sample (mL)

N_2 = normality of the 0.02 N sodium thiosulfate
W = quantity of sample used (g)

Each mL of 0.02 N sodium hydroxide is equivalent to 1.502 mg of hydroxypropoxy groups (–OCH₂CHOHCH₃).

The results obtained as a percentage of hydroxypropoxy content may be converted to terms of average molecular substitution of glucose units by means of the graph in Figure 3.

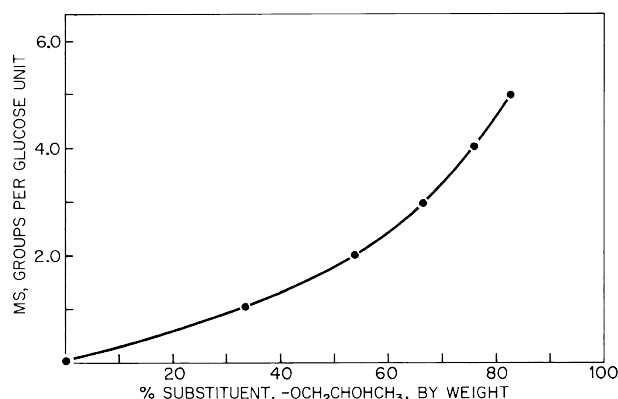


Figure 3. Graph for converting percentage of substitution, by weight, of hydroxypropoxy groups to molecular substitution/glucose unit.

Acceptance criteria: NMT 80.5% of hydroxypropoxy groups

IMPURITIES

Inorganic Impurities

• RESIDUE ON IGNITION (281)

[CAUTION—Perform the mixing and heating of the mixtures containing hydrofluoric acid in a well-ventilated hood.]

Analysis: Proceed as directed for *Residue on Ignition* (281), using a platinum crucible if silica may be present. If more than 0.2% residue is found, and silica is present, moisten the residue with water, and add 5 mL of hydrofluoric acid, in small portions. Evaporate on a steam bath to dryness, and cool. Add 5 mL of hydrofluoric acid and 0.5 mL of sulfuric acid, and

evaporate to dryness. Slowly increase the temperature until all of the acids have been volatilized, and ignite at $1000 \pm 25^\circ$. Cool in a desiccator, and weigh: the difference between the final weight and the weight of the initially ignited portion represents the weight of silica.

Acceptance criteria: The final weight is NMT 0.2% of the weight of the sample taken for the ignition.

- **LEAD** (251): NMT 10 ppm
- **HEAVY METALS**, *Method II* (231): NMT 20 ppm

SPECIFIC TESTS

- **pH** (791): 5.0–8.0, in a solution (1 in 100)
- **LOSS ON DRYING** (731): Dry a sample at 105° for 3 h: it loses NMT 5.0% of its weight.
- **ROTATIONAL RHEOMETER METHODS** (912): Determine the apparent viscosity at the concentration and temperature specified on the label with a suitable rotational viscometer (see *Labeling*).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in well-closed containers.
- **LABELING:** Label it to indicate the viscosity in an aqueous solution of stated concentration and temperature. The indicated viscosity may be in the form of a range encompassing 50%–150% of the average value.
- **USP REFERENCE STANDARDS** (11)
USP Hydroxypropyl Cellulose RS

Low-Substituted Hydroxypropyl Cellulose

DEFINITION

Low-Substituted Hydroxypropyl Cellulose is a low-substituted hydroxypropyl ether of cellulose. When dried at 105° for 1 h, it contains NLT 5.0% and NMT 16.0% of hydroxypropoxy groups ($-\text{OCH}_2\text{CHOHCH}_3$).

IDENTIFICATION

- **A.**
Sample: 20 mg
Analysis: Shake the *Sample* with 2 mL of water, and cautiously add 1 mL of a solution of anthrone in sulfuric acid (350 $\mu\text{g/mL}$).
Acceptance criteria: A blue to greenish-blue color develops at the zone of contact.
- **B.**
Sample: 0.1 g
Analysis: Shake the *Sample* thoroughly with 10 mL of water. Add 1 g of sodium hydroxide, and shake until it becomes homogeneous. Save 5 mL of this solution for *Identification* test C. To 0.1 mL of this solution add 9 mL of 32 N sulfuric acid, and shake. Heat in a water bath for 3 min, accurately timed, and immediately cool in an ice bath. While the mixture is cold, carefully add 0.6 mL of ninhydrin TS. Allow to stand at room temperature.
Acceptance criteria: The red color that appears immediately turns to violet within 100 min.
- **C.**
Sample solution: 5 mL of the solution prepared for *Identification* test B
Analysis: Shake the *Sample solution* with 10 mL of a mixture of acetone and methanol (4:1).
Acceptance criteria: A white, flocculent precipitate is formed.

ASSAY

PROCEDURE

[CAUTION]—Hydriodic acid and its reaction byproducts are highly toxic. Perform all steps of the *Standard solution* and the *Sample solution* in a properly functioning hood.

Specific safety practices to be followed are to be identified to the analyst performing this test.]

Apparatus: For the reaction vial, use a 5-mL pressure-tight serum vial, 50 mm in height, 20 mm in outside diameter, and 13 mm in inside diameter at the mouth. The vial is equipped with a pressure-tight septum having a polytetrafluoroethylene-faced butyl rubber and an air-tight seal using an aluminum crimp or any sealing system that provides sufficient air-tightness. Use a heater having a heating module that has a square-shape aluminum block with holes 20 mm in diameter and 32 mm in depth, into which the reaction vial fits. The heating module is also equipped with a magnetic stirrer capable of mixing the contents of the vial, or use a reciprocal shaker that performs a reciprocating motion of approximately 100 times/min.

Hydriodic acid: Use a reagent having a typical concentration of HI about 57%.

Internal standard solution: 30 mg/mL of *n*-octane in *o*-xylene

Standard solution: Into a suitable serum vial, weigh between 60 and 100 mg of adipic acid, and add 2.0 mL of *Hydriodic acid* and 2.0 mL of *Internal standard solution* into the vial. Close the vial securely with a suitable septum stopper. Weigh the vial and contents, add 15–22 μL of isopropyl iodide through the septum with a syringe, weigh again, and calculate the weight of isopropyl iodide added, by difference. Use the upper layer as the *Standard solution*.

Sample solution: Transfer 0.065 g of dried Low-Substituted Hydroxypropyl Cellulose to a 5-mL thick-walled reaction vial equipped with a pressure-tight septum-type closure, add between 60 and 100 mg of adipic acid, and pipet 2.0 mL of *Internal standard solution* into the vial. Cautiously pipet 2.0 mL of *Hydriodic acid* into the mixture, immediately cap the vial tightly, and weigh. Using the magnetic stirrer equipped in the heating module, or using a reciprocal shaker, mix the contents of the vial continuously, heating and maintaining the temperature of the contents at $130 \pm 2^\circ$ for 60 min. If a reciprocal shaker or magnetic stirrer cannot be used, shake the vial well by hand at 5-min intervals during the initial 30 min of the heating time. Allow the vial to cool, and weigh. If the weight loss is greater than or equal to 0.50% of the contents or there is evidence of a leak, discard the mixture, and prepare another *Sample solution*.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Thermal conductivity or hydrogen flame ionization

Column: 3–4-mm \times 1.8–3-m glass, packed with 20% liquid phase G28 on 100–120 mesh support S1C that is not silanized. [NOTE—Use a column giving well resolved peaks of methyl iodide, isopropyl iodide, and the internal standard in this order.]

Carrier gas: Helium is used for the thermal conductivity detector; helium or nitrogen can be used for the hydrogen flame-ionization detector.

Column temperature: 100°

Flow rate: With the *Standard solution*, adjust the flow rate so that the retention time of the internal standard is about 10 min.

Injection volume: 1–2 μL

Analysis

Samples: Upper layer of the *Standard solution* and the *Sample solution*

Calculate the percentage of hydroxypropoxy ($-\text{OC}_3\text{H}_6\text{OH}$) in the sample taken:

$$\text{Result} = (Q_{Tb}/Q_{Sb}) \times (W_{Sb}/W_U) \times 44.17$$

Q_{Tb} = ratio of the peak area of isopropyl iodide to *n*-octane in the *Sample solution*

- Q_{sb} = ratio of the peak area of isopropyl iodide to *n*-octane in the *Standard solution*
 W_{sb} = weight of isopropyl iodide in the *Standard solution* (mg)
 W_u = weight of Low-Substituted Hydroxypropyl Cellulose calculated on the dried basis, taken for the *Sample solution* (mg)
Acceptance criteria: 5.0%–16.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.5%
- **CHLORIDE AND SULFATE**, *Chloride* (221)
Sample solution: Shake 0.50 g of Low-Substituted Hydroxypropyl Cellulose thoroughly with 30 mL of boiling water, heat on a water bath for 10 min, and filter the supernatant by decantation while hot. Wash the residue thoroughly with 50 mL of boiling water, combine the washings with the filtrate, and add water to make 100 mL after cooling.
Control solution: 0.25 mL of 0.02 N hydrochloric acid
Analysis: Using 10 mL of the *Sample solution* and the *Control solution*, proceed as directed in the chapter, starting with the addition of the nitric acid.
Acceptance criteria: NMT 0.36%; the *Sample* shows no more chloride than the *Control solution*.
- **HEAVY METALS**, *Method II* (231): NMT 10 µg/g

SPECIFIC TESTS

- **LOSS ON DRYING** (731)
Analysis: Dry at 105° for 1 h.
Acceptance criteria: NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Hydroxypropyl Methylcellulose—see Hypromellose General Monographs

Hymetellose

Methylhydroxyethylcellulose;
 Cellulose 2-hydroxyethyl methyl ether [9032-42-2].

DEFINITION

Hymetellose is a partly *O*-(methylated) and *O*-(2-hydroxyethylated) cellulose.

IDENTIFICATION

- **A.**
Sample solution: Use the *Sample solution* prepared in the test for *Color of Solution*.
Analysis: Heat the *Sample solution* in a water bath while stirring.
Acceptance criteria: At a temperature above 50°, the solution becomes cloudy or a flocculent precipitate is formed. The solution becomes clear again on cooling.
- **B.**
Sample solution: 1 mL of the solution from *Identification test A*
Analysis: Transfer the *Sample solution* to a glass plate, and allow the water to evaporate.
Acceptance criteria: A thin film is formed.
- **C.**
Sample solution: 10 mL of the solution from *Identification test A*
Analysis: To the *Sample solution* add 0.3 mL of 2 N acetic acid and 2.5 mL of tannic acid TS.
Acceptance criteria: A yellowish-white, flocculent precipitate is formed that dissolves in ammonia TS.

• **D.**

Sample: 1 g
Diethanolamine–sodium nitroprusside solution: 50 mg/mL of sodium nitroprusside solution adjusted with 1 N hydrochloric acid to a pH of 9.8. Mix 11 mL of this solution with 1 mL of a diethanolamine solution (200 mg/mL) in water.

Analysis: In a test tube about 160 mm long, thoroughly mix the *Sample* with 2 g of finely powdered manganese sulfate. Introduce, to a depth of 2 cm into the upper part of the tube, a strip of filter paper impregnated with a freshly prepared *Diethanolamine–sodium nitroprusside solution*. Insert the tube 8 cm into a silicone-oil bath at 190°–200°. Perform a blank test without the addition of Hymetellose.

Acceptance criteria: The filter paper becomes blue within 10 min.

• **E.**

Sample: 0.2 g
Analysis: Dissolve the *Sample* completely, without heating, in 15 mL of 70% sulfuric acid. Pour the solution while stirring into 100 mL of ice water, and dilute with ice water to 250 mL. Transfer 1 mL of this solution to a test tube, and while cooling in ice water, add dropwise 8 mL of sulfuric acid, and mix thoroughly. Heat in a water bath for exactly 3 min, and immediately cool in ice water. While the mixture is cold, carefully add 0.6 mL of ninhydrin TS, and mix well. Allow to stand at 25°.

Acceptance criteria: A pink color is produced immediately and does not become violet within 100 min.

IMPURITIES

- **RESIDUE ON IGNITION** (281)
Sample: 1.0 g
Acceptance criteria: NMT 1.0%
- **CHLORIDE AND SULFATE**, *Chloride* (221)
Sample solution: Dilute 1.0 mL of the *Sample solution* from the test for *Color of Solution* with water to 20 mL, and add 1 mL of nitric acid and 1 mL of silver nitrate TS. Mix, and allow to stand for 5 min, protected from direct sunlight.
Standard solution: Dilute 0.71 mL of 0.020 N hydrochloric acid to 100 mL. Mix 10 mL of this solution with water to 20 mL, and add 1 mL of nitric acid and 1 mL of silver nitrate TS. Mix, and allow to stand for 5 min, protected from direct sunlight.
Analysis
 (See *Spectrophotometry and Light-Scattering* (851), *Visual Comparison*.)
 Compare the turbidity of the *Sample solution* and *Standard solution*, if any.
Acceptance criteria: 0.5%. Any turbidity produced by the *Sample solution* does not exceed that of the *Standard solution*.
- **HEAVY METALS**, *Method II* (231): NMT 20 µg/g

SPECIFIC TESTS

- **pH** (791)
Sample solution: Use the *Sample solution* from the test for *Color of Solution*.
Acceptance criteria: 5.5–8.0
- **LOSS ON DRYING** (731)
Sample: 1.0 g
Analysis: Dry the *Sample* at 105° to constant weight.
Acceptance criteria: NMT 10.0%
- **ROTATIONAL RHEOMETER METHODS** (912)
Sample: An amount equivalent to 6.0 g of dried Hymetellose
Analysis: While stirring, add the *Sample* to 150 g of carbon dioxide-free water heated to 90°. Stir with a propeller-type stirrer for 10 min, place the flask in a bath of ice water, continue the stirring, and allow to remain in the bath of ice water for 40 min to ensure that dissolution is complete. Adjust the mass of the solution to

300 g, and centrifuge the solution to expel any entrapped air. Adjust the temperature of the solution to $20 \pm 0.1^\circ$, and determine the viscosity using a rotational viscometer with a shear rate of 10/s.

Acceptance criteria: The apparent viscosity is 75%–140% of the value stated on the label.

• **COLOR OF SOLUTION**

Diluent: 27.5 mL of hydrochloric acid in 1000 mL of water

Standard solution: Prepare immediately before use. Mix 2.4 mL of ferric chloride CS and 0.6 mL of cobaltous chloride CS with *Diluent* to make 10 mL, and dilute 5 mL of this solution with *Diluent* to make 100 mL.

Sample solution: While stirring, add a portion equivalent to 1.0 g of dried Hymetellose to 50 g of carbon dioxide-free water heated to 90° . Allow to cool, adjust the weight of the solution to 100 g with carbon dioxide-free water, and stir until dissolution is complete.

Analysis: Make the comparison by viewing the substance and the solution downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* <631>).

Acceptance criteria: The *Sample solution* is not more intensely colored than the *Standard solution*.

• **CLARITY OF SOLUTION**

Hydrazine sulfate solution: 10 mg/mL of hydrazine sulfate. Allow to stand for 4–6 h before use.

[**CAUTION**—Hydrazine sulfate is highly toxic. Avoid skin contact.]

Methenamine solution: Transfer 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.

Primary opalescent mixture: To the flask containing *Methenamine solution* add 25.0 mL of *Hydrazine sulfate solution*, mix, and allow to stand for 24 h. This suspension is stable for 2 months. Mix before use, and do not use if it adheres to the container.

Opalescence standard: Dilute 15.0 mL of *Primary opalescent mixture* with water to 1000.0 mL. Use this suspension within 24 h after preparation.

Reference suspension: Transfer 30.0 mL of *Opalescence standard* to a 100-mL volumetric flask, and dilute with water to volume.

Sample solution: Use the solution from the test for *Color of Solution*.

Analysis: Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer a portion of the *Reference suspension* to a separate matching test tube. Compare the *Sample solution* and the *Reference suspension* in diffused daylight, viewing vertically against a black background (see *Spectrophotometry and Light-Scattering* <851>, *Visual Comparison*) 5 min after preparation of the *Reference suspension*.

Acceptance criteria: The *Sample solution* is not more opalescent than the *Reference suspension*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.
- **LABELING:** Label it to indicate the viscosity of the solution (1 in 50) at 20° .

Hypophosphorous Acid

H₃PO₂ 66.00
Phosphinic acid;
Hypophosphorous acid [6303-21-5].

DEFINITION

Hypophosphorous Acid contains NLT 30.0% and NMT 32.0% of H₃PO₂.

IDENTIFICATION

- **A.** Hypophosphorous Acid yields a white precipitate with mercuric chloride TS. This precipitate becomes gray when an excess of hypophosphite is present.
- **B.** Hypophosphorous Acid acidified with sulfuric acid and warmed with cupric sulfate TS yields a red precipitate.

ASSAY

• **PROCEDURE**

Sample solution: Pour 7 mL of Hypophosphorous Acid into a tared, glass-stoppered flask, and weigh. Dilute with about 25 mL of water, and add phenolphthalein TS.

Analysis: Titrate with 1 N sodium hydroxide VS. Each mL of 1 N sodium hydroxide is equivalent to 66.00 mg of H₃PO₂.

Acceptance criteria: 30.0%–32.0%

IMPURITIES

Inorganic Impurities

• **HEAVY METALS, Method II** <231>

Analysis: Place 0.90 mL (1 g) of Hypophosphorous Acid in a small beaker, and add 3 mL of water. Add 1 mL of nitric acid, and evaporate on a steam bath to about 1 mL. Again add 1 mL of nitric acid, and evaporate on a steam bath. Dissolve the residue in 3 mL of water, add 6 N ammonium hydroxide until the solution is distinctly alkaline to litmus, then boil gently until the odor of ammonia disappears. Add 2 mL of 1 N acetic acid and 15 mL of warm water, filter, and dilute the filtrate with water to 25 mL.

Acceptance criteria: NMT 20 ppm

SPECIFIC TESTS

• **LIMIT OF BARIUM AND OXALATE**

Sample solution: Hypophosphorous Acid and water (1:3)

Analysis 1: Neutralize 30 mL of the *Sample solution* with 6 N ammonium hydroxide: the mixture exhibits little or no precipitation. Filter, acidify 10 mL of the filtrate with hydrochloric acid, and add 2 mL of potassium sulfate TS.

Acceptance criteria 1: No turbidity is produced (barium).

Analysis 2: To a 10-mL portion of the filtrate obtained in *Analysis 1*, add 1 mL of calcium chloride TS.

Acceptance criteria 2: The filtrate shows no turbidity upon the addition of the calcium chloride TS (oxalate).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Hypromellose Acetate Succinate

Hydroxypropyl methylcellulose acetate succinate;
Cellulose, 2-hydroxypropyl methyl ether, acetate hydrogen butanedioate;
Cellulose, 2-hydroxypropyl methyl ether, acetate succinate [71138-97-1].

DEFINITION

Hypromellose Acetate Succinate is a mixture of acetic acid and monosuccinic acid esters of hydroxypropyl methylcellulose. It contains NLT 12.0% and NMT 28.0% of methoxy groups (–OCH₃), NLT 4.0% and NMT 23.0% of hydroxypropoxy groups (–OCH₂CHOHCH₃), NLT 2.0% and NMT 16.0% of acetyl groups (–COCH₃), and NLT 4.0% and NMT 28.0% of succinoyl groups (–COC₂H₄COOH), calculated on the dried basis.

IDENTIFICATION**A. INFRARED ABSORPTION** (197A)

Sample: Neat. Do not dry specimen.

Analysis: Use a Fourier transform IR spectrophotometer fitted with a suitable accessory for single bounce attenuated total reflectance (see *Spectrophotometry and Light-Scattering* (851)) with a diamond or germanium crystal. Acquire a background single-beam spectrum with a clean diamond or germanium crystal sampling plate in place. Place the sample on the diamond or germanium crystal sampling surface with a microspatula or equivalent. For best results, the sample should cover the crystal surface under the pressure point tip. Using the pressure device, apply pressure to the sample, making sure the sample remains centered under the pressure tip. Acquire a single-beam spectrum of the sample, and make the necessary corrections for the background. Release the pressure device, and clear it from the sample area. Wipe the sample off the crystal and pressure device tip, and rinse both with acetone.

Acceptance criteria: The IR spectrum of the *Sample* exhibits maxima only at the same wavelengths as a similarly obtained spectrum of USP Hypromellose Acetate Succinate RS.

ASSAY**• ACETYL AND SUCCINOYL GROUPS**

Phosphoric acid solution: 1.25 M phosphoric acid and water (2:98)

Buffer: 2.72 g/L of monobasic potassium phosphate

Diluent: Adjust the *Buffer* with 1 N sodium hydroxide to a pH of 7.5.

Acetic acid stock solution: Add approximately 20 mL of water to a stoppered, 100-mL volumetric flask, place the flask on a balance, and tare. Transfer 2.0 mL of glacial acetic acid to the flask, and record the weight of the acid added. Fill the flask with water to volume. Transfer 6 mL of the resulting solution into a 100-mL volumetric flask, and dilute with water to volume.

Succinic acid stock solution: 1.3 mg/mL of succinic acid

Mobile phase: Adjust the *Buffer* to a pH of 2.8 by the dropwise addition of 6 M phosphoric acid. Pass through a 0.22- μ m nylon filter.

Standard solution 1: Transfer 4.0 mL of the *Acetic acid stock solution* and 4.0 mL of the *Succinic acid stock solution* to a 25-mL volumetric flask. Dilute with *Mobile phase* to volume, and mix.

Standard solution 2: Prepare as directed for *Standard solution 1*. This solution is prepared as a duplicate.

Sample solution: Weigh 12.4 mg of Hypromellose Acetate Succinate into a glass vial. Transfer 4.0 mL of 1.0 N sodium hydroxide to the vial, and stir the solution for 4 h. Then, add 4.0 mL of 1.25 M phosphoric acid to the same vial to bring the pH of the solution to 3 or less. Invert the test *Sample solution* vial several times to ensure complete mixing, and pass through a filter of 0.22- μ m pore size. Use the clear filtrate.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 215 nm

Column: 4.6-mm \times 15-cm; 5- μ m packing L1

Column temperature: 20°–30°

Flow rate: 1 mL/min

Run time: 15 min

Injection volume: 10 μ L

System suitability

Samples: *Standard solution 1* and *Standard solution 2*

Suitability requirements

Column efficiency: NLT 8000 theoretical plates, determined from the succinic acid peak, *Standard solution 1*

Tailing factor: 0.9–1.5 for the succinic acid peak, *Standard solution 1*

Relative standard deviation: NMT 2.0% for each peak from six replicate injections, *Standard solution 1*

Peak difference: The difference in peak areas between *Standard solution 1* and *Standard solution 2* for both acetic and succinic acids peaks does not exceed 2%.

[NOTE—After each run sequence, the column should be flushed first by 50% water and 50% acetonitrile for 60 min and then by 100% methanol for 60 min. The column should be stored in 100% methanol.]

Analysis

Samples: *Standard solution 1* and *Sample solution*
Calculate the percentage of acetic acid, *A*, in the portion of Hypromellose Acetate Succinate taken:

$$A = (r_{UA}/r_{SA}) \times (C_A/C_U) \times 100$$

r_{UA} = peak response for acetic acid from the *Sample solution*

r_{SA} = peak response for acetic acid from *Standard solution 1*

C_A = concentration of acetic acid in *Standard solution 1* (mg/mL)

C_U = concentration of Hypromellose Acetate Succinate in the *Sample solution* (mg/mL)

Calculate the percentage of acetyl groups ($-\text{COCH}_3$) in the portion of Hypromellose Acetate Succinate taken:

$$\text{Result} = (A - A_{\text{free}}) \times (M_{r1}/M_{r2})$$

A = defined above

A_{free} = percentage of free acetic acid, as determined in the test for *Limit of Free Acetic and Succinic Acids*

M_{r1} = molecular weight of the acetyl group, 43.04

M_{r2} = molecular weight of acetic acid, 60.05

Calculate the percentage of succinic acid, *S*, in the portion of Hypromellose Acetate Succinate taken:

$$S = (r_{US}/r_{SS}) \times (C_S/C_U) \times 100$$

r_{US} = peak response for succinic acid from the *Sample solution*

r_{SS} = peak response for succinic acid from *Standard solution 1*

C_S = concentration of succinic acid in *Standard solution 1* (mg/mL)

C_U = concentration of Hypromellose Acetate Succinate in the *Sample solution* (mg/mL)

Calculate the percentage of succinoyl groups ($-\text{COC}_2\text{H}_4\text{COOH}$) in the portion of Hypromellose Acetate Succinate taken:

$$\text{Result} = (S - S_{\text{free}}) \times (M_{r1}/M_{r2})$$

S = defined above

S_{free} = percentage of free succinic acid, as determined in the test for *Limit of Free Acetic and Succinic Acids*

M_{r1} = molecular weight of the succinoyl group, 101.08

M_{r2} = molecular weight of succinic acid, 118.09

Acceptance criteria

Acetyl groups ($-\text{COCH}_3$): 2.0%–16.0% on the dried basis

Succinoyl groups ($-\text{COC}_2\text{H}_4\text{COOH}$): 4.0%–28.0% on the dried basis

• CONTENT OF METHOXY AND 2-HYDROXYPROPOXY GROUPS

[CAUTION—Hydriodic acid and its reaction byproducts are highly toxic. Perform all steps in the preparation of the *Sample solution* and the *Standard solution* in a properly functioning hood. Specific safety practices to be followed are to be identified to the analyst performing this test.]

Hydriodic acid: Use a reagent having a specific gravity of at least 1.69, equivalent to 55% hydrogen iodide.

Solution A: Methanol and water (10:90)

Solution B: Methanol and water (85:15)

Mobile phase: See Table 1.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	70	30
8	40	60
10	15	85
17	15	85

[NOTE—These gradient elution times are established on an HPLC system with a dwell volume of approximately 2.0 mL. The injection time can be adjusted relative to the start of a run to accommodate the change in dwell volume from one HPLC system to another to achieve the separation described.]

Standard stock solution: Transfer 2 mL of *o*-xylene into a stoppered, 10-mL volumetric flask, place the flask on a balance, and tare. Add 200 µL of methyl iodide, insert the stopper into the flask, and accurately weigh: the weight of methyl iodide is about 350 mg. Tare the flask again, add 34 µL of isopropyl iodide, and weigh the flask: the recorded weight of isopropyl iodide is 50 mg. Dilute with *o*-xylene to volume, and mix.

Standard solution: Transfer 85 mg of adipic acid into an 8-mL vial (or other suitable container), add 2 mL of *Hydriodic acid*, and add 2.0 mL of the *Standard stock solution*. Shake and allow the phases to separate. Carefully transfer approximately 1.5 mL of the *o*-xylene (top) layer to a small vial, making sure that the bottom aqueous layer is not disturbed. Transfer 1.0 mL of the resulting solution to a 10-mL volumetric flask, and dilute with methanol to volume. [NOTE—This solution is stable for 8 h at 5°.]

Sample solution: [CAUTION—Use a cap that has a top safety relief valve, such as a Minniert valve, to prevent accidental explosion of the vial under high pressure when heated.] Weigh 65 mg of Hypromellose Acetate Succinate into a 5-mL reaction vial, and add 2.0 mL of *o*-xylene and about 100 mg of adipic acid. Add 2.0 mL of *Hydriodic acid*, and close the vial tightly with a cap. Weigh the vial before heating, and place the vial into a heating block at 150°. Shake the vial after 5 min and after 30 min of heating. Remove the vial from the heating block after 1 h of heating, and cool. Weigh the vial. If the weight loss is greater than 10 mg, discard the mixture, and prepare another reaction solution. Carefully transfer approximately 1.5 mL of the top *o*-xylene layer into a small glass vial, making sure that the bottom aqueous layer is not disturbed. Transfer 1.0 mL of this solution into a 10-mL volumetric flask, and dilute with methanol to volume. [NOTE—This solution is stable for 8 h at 5°.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Column temperature: 30°

Flow rate: 1 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Column efficiency: NLT 10,000 theoretical plates, determined from the methyl iodide peak

Tailing factor: 0.9–1.5 for the methyl iodide peak

Relative standard deviation: NMT 2.0% for each peak

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of methoxy groups (–OCH₃) in the portion of Hypromellose Acetate Succinate taken:

$$\text{Result} = (r_{UM}/r_{SM}) \times (C_S/C_U) \times (M_{r1}/M_{r2})$$

r_{UM} = peak response for methyl iodide from the *Sample solution*

r_{SM} = peak response for methyl iodide from the *Standard solution*

C_S = concentration of methyl iodide in the *Standard solution* (mg/mL)

C_U = concentration of Hypromellose Acetate Succinate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of the methoxy group, 31.03

M_{r2} = molecular weight of methyl iodide, 141.94
Calculate the percentage of 2-hydroxypropoxy groups (–OCH₂CHOHCH₃) in the portion of Hypromellose Acetate Succinate taken:

$$\text{Result} = (r_{UI}/r_{SI}) \times (C_S/C_U) \times (M_{r1}/M_{r2})$$

r_{UI} = peak response for isopropyl iodide from the *Sample solution*

r_{SI} = peak response for isopropyl iodide from the *Standard solution*

C_S = concentration of isopropyl iodide in the *Standard solution* (mg/mL)

C_U = concentration of Hypromellose Acetate Succinate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of the 2-hydroxypropoxy group, 75.09

M_{r2} = molecular weight of isopropyl iodide, 169.99

Acceptance criteria

Methoxy groups (–OCH₃): 12.0%–28.0% on the dried basis

Hydroxypropoxy groups (–OCH₂CHOHCH₃): 4.0%–23.0% on the dried basis

IMPURITIES

• RESIDUE ON IGNITION <281>

Analysis: Determine at 600 ± 50°.

Acceptance criteria: NMT 0.20%

• HEAVY METALS, Method II <231>: NMT 10 µg/g

• LIMIT OF FREE ACETIC AND SUCCINIC ACIDS

Phosphoric acid solution, Buffer, Diluent, Acetic acid stock solution, Succinic acid stock solution, Mobile phase, Standard solution, and Chromatographic system: Proceed as directed in the *Assay for Acetyl and Succinoyl Groups*.

Sample solution: Weigh 102 mg of Hypromellose Acetate Succinate into a glass vial. Transfer 4.0 mL of *Diluent* to the vial, and stir the content for 2 h. Then, transfer 4.0 mL of the *Phosphoric acid solution* to the same vial to bring the pH of the *Sample solution* to 3 or less. Invert the vial several times to ensure complete mixing, centrifuge, and use the clear supernatant.

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of free acetic acid, A_{free} , in the portion of Hypromellose Acetate Succinate taken:

$$A_{free} = (r_{UA}/r_{SA}) \times (C_A/C_U) \times 100$$

r_{UA} = peak response for acetic acid from the *Sample solution*

r_{SA} = peak response for acetic acid from the *Standard solution*

C_A = concentration of acetic acid in the *Standard solution* (mg/mL)

C_U = concentration of Hypromellose Acetate Succinate in the *Sample solution* (mg/mL)

Calculate the percentage of free succinic acid, S_{free} , in the portion of Hypromellose Acetate Succinate taken:

$$S_{free} = (r_{US}/r_{SS}) \times (C_S/C_U) \times 100$$

r_{US} = peak response for succinic acid from the *Sample solution*

r_{SS} = peak response for succinic acid from the *Standard solution*

C_S = concentration of succinic acid in the *Standard solution* (mg/mL)

C_U = concentration of Hypromellose Acetate Succinate in the *Sample solution* (mg/mL)

Acceptance criteria: The sum of free acetic acid and free succinic acid is NMT 1.0%.

SPECIFIC TESTS

• LOSS ON DRYING (731)

Analysis: Dry at 105° for 1 h.

Acceptance criteria: NMT 5.0%

• VISCOSITY—CAPILLARY VISCOMETER METHODS (911)

Sodium hydroxide solution: Immediately before use, prepare 4.3 mg/mL of sodium hydroxide in carbon dioxide-free water.

Analysis: To 2.00 g of Hypromellose Acetate Succinate, previously dried, add *Sodium hydroxide solution* to make 100.0 g, insert a stopper into the vessel, and dissolve by constant shaking for 30 min. Adjust the temperature of the solution to $20 \pm 0.1^\circ$, and determine the viscosity in a suitable viscometer.

Acceptance criteria: 80%–120% of that stated on the label

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** Label it to indicate its nominal viscosity type.
- **USP REFERENCE STANDARDS (11)**
USP Hypromellose Acetate Succinate RS

Hypromellose Phthalate

DEFINITION

Hypromellose Phthalate is a monophthalic acid ester of hydroxypropyl methylcellulose. It contains methoxy ($-\text{OCH}_3$), hydroxypropoxy ($-\text{OCH}_2\text{CHOHCH}_3$), and phthalyl (*o*-carboxybenzoyl, $\text{C}_8\text{H}_5\text{O}_3$) groups. It contains NLT 21.0% and NMT 35.0% of phthalyl groups, calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K):** Do not dry specimens.

ASSAY

• PHTHALYL CONTENT

Sample: 1 g

Analysis: Transfer the *Sample* to a conical flask, dissolve in 50 mL of a mixture of alcohol, acetone, and water (2:2:1), add phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS. Perform a blank determination (see *Titrimetry* (541)).

Calculate the percentage of phthalyl taken:

$$\text{Result} = [0.01 \times M_{r1} \times (V/W)] - [2 \times (M_{r1}/M_{r2}) \times P]$$

M_{r1} = molecular weight of the phthalyl group, 149.1

V = volume of 0.1 N sodium hydroxide consumed after correction for the blank (mL)

W = weight of Hypromellose Phthalate taken, calculated on the anhydrous basis (g)

M_{r2} = molecular weight of phthalic acid, 166.1

P = percentage of free phthalic acid found as directed in the test for *Limit of Free Phthalic Acid*

Acceptance criteria: 21.0%–35.0% of phthalyl groups on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION (281):** NMT 0.20%

- **CHLORIDE AND SULFATE, Chloride (221)**

Sample solution: Dissolve 1.0 g in 40 mL of 0.2 N sodium hydroxide, add 1 drop of phenolphthalein TS, and add 2 N nitric acid dropwise, with stirring, until the red color is discharged. Add an additional 20 mL of 2 N nitric acid with stirring. Heat on a water bath, with stirring, until the gel-like precipitate formed becomes granular. Cool the mixture, and centrifuge. Separate the liquid phase, and wash the residue with three successive 20-mL portions of water, separating the washings by centrifuging. Dilute the combined liquids with water to 200 mL, mix, and filter.

Standard solution: Treat 0.50 mL of 0.01 N hydrochloric acid with 10 mL of 0.2 N sodium hydroxide, add 7 mL of 2 N nitric acid, and dilute with water to 50 mL.

Acceptance criteria: A 50-mL portion of the *Sample solution* shows no more chloride than the *Standard solution* (0.07%).

- **HEAVY METALS, Method II (231):** NMT 10 µg/g

- **LIMIT OF FREE PHTHALIC ACID**

Mobile phase: Acetonitrile and 0.1 M cyanoacetic acid (15:85)

Standard solution: Transfer 12.5 mg of phthalic acid to a 250-mL volumetric flask, and add 125 mL of acetonitrile. Add 25 mL of water, and dilute with acetonitrile to volume.

Sample solution: Transfer 200 mg of Hypromellose Phthalate to a 100-mL volumetric flask. Add 50 mL of acetonitrile, and sonicate to dissolve partially. Add 10 mL of water, and sonicate to dissolve. Cool to room temperature, and dilute with acetonitrile to volume.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 235 nm

Column: 4.6-mm × 25-cm; packing L1 with a high carbon load

Flow rate: 2 mL/min

Injection volume: 10 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 1.0% for replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of phthalic acid in the portion of Hypromellose Phthalate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of phthalic acid from the *Sample solution*

r_S = peak response of phthalic acid from the *Standard solution*

C_S = concentration of phthalic acid in the *Standard solution* (mg/mL)

C_U = concentration of Hypromellose Phthalate in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 1.0%

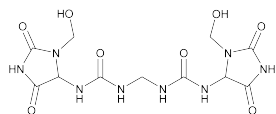
SPECIFIC TESTS

- **WATER DETERMINATION, Method I (921):** NMT 5.0%
- **VISCOSITY—CAPILLARY VISCOMETER METHODS (911)**
Sample solution: Dissolve 10 g, previously dried at 105° for 1 h, in 90 g of a mixture of methanol and methylene chloride (1:1 w/w) by mixing and shaking.
Analysis: Determine the viscosity at 20 ± 0.1°.
Acceptance criteria: 80%–120% of that indicated by the label

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate its viscosity and nominal phthalyl content.
- **USP REFERENCE STANDARDS (11)**
 USP Hypromellose Phthalate RS

Imidurea



$C_{11}H_{16}N_8O_8$ 388.29
N,N'-Methylenebis[*N'*-[3-(hydroxymethyl)-2,5-dioxo-4-imidazolidinyl]urea];
 1,1'-Methylenebis[3-[3-(hydroxymethyl)-2,5-dioxo-4-imidazolidinyl]urea] [39236-46-9].

DEFINITION

Imidurea contains NLT 26.0% and NMT 28.0% of nitrogen (N), calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K)**

OTHER COMPONENTS

• NITROGEN CONTENT

Sample: 150 mg

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N hydrochloric acid VS

Endpoint detection: Visual

Analysis: Place the *Sample* in a 500-mL Kjeldahl flask, and add 8.0 g of anhydrous sodium sulfate, 0.5 g of anhydrous cupric sulfate, 0.1 g of yellow mercuric oxide, and 11 mL of sulfuric acid. Incline the flask at an angle of 45°, and gently heat the mixture, keeping the temperature below the boiling point until frothing has ceased. Increase the heat until the acid boils briskly, and continue the heating until the solution has become clear green in color or practically colorless for 30 min. Allow to cool, cautiously add 225 mL of water, mix the contents of the flask, and again cool. Add cautiously 1.0 g of zinc metal dust, 2.0 g of sodium thiosulfate, and 18.0 g of sodium hydroxide pellets, and without delay connect the flask to a Kjeldahl connecting bulb (trap), previously attached to a condenser, the delivery tube of which dips beneath the surface of 50 mL of boric acid solution (40 mg/mL) in a 300-mL conical flask. Mix the contents of the Kjeldahl flask by gentle rotation, and distill 125 mL into the receiver. Add NLT 3 drops of methyl red–methylene blue TS to the contents of the receiving vessel, and determine the ammonia by titration with *Titrant*. Perform a blank determination, and make any necessary correction.

Calculate the percentage of nitrogen in the portion of Imidurea taken. Each mL of 0.1 N hydrochloric acid is equivalent to 1.401 mg of nitrogen.

Acceptance criteria: 26.0%–28.0% of nitrogen (N) on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION (281):** NMT 3.0%
- **HEAVY METALS, Method II (231):** NMT 10 µg/g

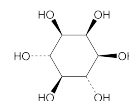
SPECIFIC TESTS

- **PH (791)**
Sample solution: 10 mg/mL
Acceptance criteria: 6.0–7.5
- **LOSS ON DRYING (731)**
Analysis: Dry under vacuum over phosphorus pentoxide for 48 h.
Acceptance criteria: NMT 3.0%
- **COLOR AND CLARITY OF SOLUTION**
Sample: 3.0 g
Analysis: Dissolve the *Sample* in 7.0 mL of water in a test tube.
Acceptance criteria: The solution is clear and colorless.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS (11)**
 USP Imidurea RS

Inositol



$C_6H_{12}O_6$ 180.16
cis-1,2,3,5-*trans*-4,6-Cyclohexanehexol;
myo-Inositol [87-89-8].

DEFINITION

Inositol contains NLT 97.0% and NMT 102.0% of Inositol ($C_6H_{12}O_6$), calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K)**
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

• PROCEDURE

Mobile phase: Water

System suitability solution: 0.05 mg/mL of USP Inositol RS and 0.05 mg/mL of USP Mannitol RS

Standard solution: 50 mg/mL of USP Inositol RS

Sample solution: 50 mg/mL of Inositol

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 30-cm or equivalent; packing L19

Temperature

Column: 85°

Detector: Constant temperature of 30°–35°

Flow rate: 0.5 mL/min

Injection volume: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for inositol and mannitol are 1.0 and 1.3, respectively.]

Suitability requirements

Resolution: NLT 4.0 between inositol and mannitol, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—Record the chromatograms over a period of two times the retention time of inositol, and measure the peak responses.]

Calculate the percentage of Inositol ($C_6H_{12}O_6$) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of inositol from the *Sample solution*

r_S = peak response of inositol from the *Standard solution*

C_S = concentration of USP Inositol RS in the *Standard solution* (mg/mL)

C_U = concentration of Inositol in the *Sample solution* (mg/mL)

Acceptance criteria: 97.0%–102.0% on the anhydrous basis

IMPURITIES• **BARIUM**

Sample solution: Use the *Sample solution* prepared in the test for *Clarity of Solution*. To 10 mL of the *Sample solution* add 1 mL of diluted sulfuric acid.

Acceptance criteria: When examined immediately and after 1 h, any opalescence in the solution is not more intense than that in a mixture of 1 mL of water and 10 mL of the *Sample solution* from the test for *Clarity of Solution*.

• **LIMIT OF LEAD**

Lead nitrate stock solution: Dissolve 159.8 mg of lead nitrate in 100 mL of water to which has been added 1 mL of nitric acid, then dilute with water to 1000 mL. Prepare and store this solution in glass containers free from soluble lead salts.

Standard lead solution: On the day of use, dilute 10.0 mL of the *Lead nitrate stock solution* with water to 100.0 mL. Each mL of the *Standard lead solution* contains the equivalent of 10 µg of lead. A comparison solution prepared on the basis of 100 µL of the *Standard lead solution* per g of substance being tested contains the equivalent of 1 part of lead per million parts of substance being tested.

Sample solution: Dissolve 20.0 g of Inositol in diluted acetic acid, and dilute with diluted acetic acid to 100 mL. Add 2.0 mL of a saturated ammonium pyrrolidinedithiocarbamate solution (containing about 10 g of ammonium pyrrolidinedithiocarbamate per L) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.

Blank solution: Prepare as directed for the *Sample solution*, except omit the use of Inositol.

Standard solutions: Prepare as directed for the *Sample solution*, except prepare three *Standard solutions* by adding 0.5, 1.0, and 1.5 mL, respectively, of the *Standard lead solution* in addition to the 20.0 g of Inositol to be examined.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption

Analytical wavelength: 283.3 nm

Lamp: Lead hollow-cathode

Flame: Air–acetylene

Analysis: Set the atomic absorption spectrometer to zero, using the *Blank solution*. Introduce the *Sample solution* and each of the three *Standard solutions* into the instrument, and record the steady absorbance

reading. Plot the absorbance readings against the known concentrations of added lead (in µg), and draw a straight line. Extrapolate the line until it meets the concentration axis to obtain the concentration, in mg/kg, of lead in the sample.

Acceptance criteria: NMT 0.5 mg/kg

• **ORGANIC IMPURITIES**

Mobile phase, System suitability solution, and Sample solution: Proceed as directed in the *Assay*.

Standard solution: Transfer 2.0 mL of the *Standard solution*, prepared as directed in the *Assay*, to a 100-mL volumetric flask, and dilute with water to volume.

[NOTE—This solution contains 1 mg/mL of inositol.]

Chromatographic system: Proceed as directed in the *Assay*, except use an injection volume of 20 µL.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each impurity in the portion of Inositol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of any impurity from the *Sample solution*

r_S = peak response of inositol from the *Standard solution*

C_S = concentration of USP Inositol RS in the *Standard solution* (mg/mL)

C_U = concentration of Inositol in the *Sample solution* (mg/mL)

Acceptance criteria

Individual impurities: NMT 0.3%

Total impurities: NMT 1.0%. [NOTE—Disregard any impurity peak that is less than 0.05%.]

SPECIFIC TESTS• **CLARITY OF SOLUTION**

[NOTE—The *Sample solution* is to be compared to *Reference suspension A* in diffused daylight 5 min after preparation of *Reference suspension A*.]

Solution A: 10 mg/mL of hydrazine sulfate. Allow to stand for 4–6 h before use.

Solution B: 100 mg/mL of methenamine prepared in a glass-stoppered flask.

Primary opalescent suspension: [NOTE—This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.] *Solution A* and *Solution B* (1:1). Allow to stand for 24 h.

Opalescence standard: Transfer 15.0 mL of the *Primary opalescent suspension* to a 1000-mL volumetric flask, dilute with water to volume. [NOTE—This suspension should not be used beyond 24 h after preparation.]

Reference suspensions

Reference suspension A: Transfer 5.0 mL of the *Opalescence standard* to a 100-mL volumetric flask, and dilute with water to volume.

Reference suspension B: Transfer 10.0 mL of the *Opalescence standard* to a 100-mL volumetric flask, and dilute with water to volume.

Sample solution: 100 mg/mL of Inositol

Analysis: Transfer a sufficient portion of the *Sample solution* to a sample tube of colorless, transparent, neutral glass, with a flat base and an internal diameter of 15–25 mm, to obtain a depth of 40 mm. Similarly transfer portions of *Reference suspension A*, *Reference suspension B*, and water to separate matching sample tubes. Compare the *Sample solution*, *Reference suspension A*, *Reference suspension B*, and water in diffused daylight, viewing vertically against a black background (see *Spectrophotometry and Light-Scattering* <851>, *Visual Comparison*). [NOTE—The diffusion of light must be such that *Reference suspension A* can readily be distinguished from water, and that *Reference suspension*

B can readily be distinguished from *Reference suspension A*.]

Acceptance criteria: The *Sample solution* shows the same clarity as that of water.

• **COLOR OF SOLUTION**

Standard stock solutions: Prepare three solutions, *A*, *B*, and *C*, containing, respectively, the following parts of ferric chloride CS, cobaltous chloride CS, cupric sulfate CS, and diluted hydrochloric acid.

Standard stock solution A: 2.4: 0.6: 0: 7.0

Standard stock solution B: 2.4: 1.0: 0.4: 6.2

Standard stock solution C: 9.6: 0.2: 0.2: 0

Standard solutions: [NOTE—Prepare the *Standard solutions* immediately before use.]

Standard solution A: Transfer 2.5 mL of *Standard stock solution A* to a 100-mL volumetric flask, dilute with diluted hydrochloric acid to volume, and mix.

Standard solution B: Transfer 2.5 mL of *Standard stock solution B* to a 100-mL volumetric flask, dilute with diluted hydrochloric acid to volume, and mix.

Standard solution C: Transfer 0.75 mL of *Standard stock solution C* to a 100-mL volumetric flask, dilute with diluted hydrochloric acid to volume, and mix.

Sample solution: Use the *Sample solution* prepared in *Clarity of Solution*.

Analysis: Transfer a sufficient portion of the *Sample solution* to a sample tube of colorless, transparent, neutral glass, with a flat base and an internal diameter of 15–25 mm, to obtain a depth of 40 mm. Similarly transfer portions of *Standard solution A*, *Standard solution B*, *Standard solution C*, and water to separate matching sample tubes. Compare the *Sample solution*, *Standard solution A*, *Standard solution B*, *Standard solution C*, and water in diffused daylight, viewing vertically against a white background (see *Spectrophotometry and Light-Scattering* <851>, *Visual Comparison*).

Acceptance criteria: The *Sample solution* is not more intensely colored than *Standard solution A*, *Standard solution B*, *Standard solution C*, or water.

• **CONDUCTIVITY**

Sample solution: Transfer 10.0 g of Inositol, weighed and calculated on the dried basis, to a 50-mL volumetric flask, and dissolve in and dilute with water (previously boiled and cooled to room temperature) to volume.

Apparatus: Use a conductivity meter or a resistivity meter that measures the resistance of the column of liquid between the electrodes of the immersed measuring device. The apparatus is supplied with alternating current to avoid the effects of electrode polarization. It is equipped with a temperature compensation device or a precision thermometer.

Reagents: Prepare three *Standard solutions* of potassium chloride containing 0.7455, 0.0746, and 0.0149 g, respectively, of potassium chloride per 1000.0 g of solution. These solutions should be prepared with water that has been previously boiled and cooled to room temperature and whose conductivity does not exceed 2 µS/cm. The conductivity and resistivity of these three solutions at 20° are provided in *Table 1*.

Table 1

Concentration of Solution (g/1000.0 g)	Conductivity (µS/cm)	Resistivity (Ω-cm)
0.7455	1330	752
0.0746	133.0	7519
0.0149	26.6	37,594

Calibration: Choose a conductivity cell that is appropriate for the conductivity of the solution to be examined. The higher the expected conductivity, the higher the cell constant that must be chosen.

Commonly used conductivity cells have cell constants of the order 0.1, 1, and 10 cm⁻¹. Use a standard solution of potassium chloride that is appropriate for the measurement. The conductivity value of the standard solution of potassium chloride should be near the expected conductivity value of the *Sample solution*. Rinse the cell several times with water that has been previously boiled and cooled to room temperature, and rinse at least twice with the potassium chloride solution used for the determination of the cell constant of the conductivity cell. Measure the resistance of the conductivity cell, using the potassium chloride solution at 20 ± 0.1°.

Calculate the constant, in cm⁻¹, of the conductivity cell:

$$\text{Result} = R_{KCl} \times K_{KCl}$$

R_{KCl} = measured resistance, expressed in mega-ohms

K_{KCl} = conductivity of the standard solution of potassium chloride used, expressed in µS/cm. The measured constant of the conductivity cell must be within 5% of the given value.

Analysis: Rinse the conductivity cell several times with water that has been previously boiled and cooled to room temperature, and rinse at least twice with the *Sample solution*. Measure the conductivity of the *Sample solution*, while gently stirring with a magnetic stirrer.

Acceptance criteria: NMT 20 µS/cm

- **WATER DETERMINATION, Method I** <921>: NMT 0.5% determined on a 1.0-g sample

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.
- **USP REFERENCE STANDARDS** <11>
USP Inositol RS
USP Mannitol RS

Isobutane



C₄H₁₀

58.12

DEFINITION

Isobutane contains NLT 95.0% of isobutane (C₄H₁₀).

[**CAUTION**—Isobutane is highly flammable and explosive.]

IDENTIFICATION

- **A. IR ABSORPTION:** Exhibits maxima, among others, at about the following wavelengths (µm): 3.4 (vs), 6.8 (s), 7.2 (m), 8.5 (m), and 10.9 (m)
- **B.** The vapor pressure of a test specimen obtained as directed for *Propellants* (in *Aerosols*, *Nasal Sprays*, *Metered-Dose Inhalers*, and *Dry Powder Inhalers* <601>, *General Sampling Procedures*), and determined at 21° by means of a suitable pressure gauge, is between 303 and 331 kPa absolute (44 and 48 psia).

ASSAY

• **PROCEDURE**

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Thermal conductivity

Column: 3-mm × 6-m aluminum; packed with 10 weight percent of liquid phase G30 on non-acid-washed support S1C

Column temperature: 33°

Carrier gas: Helium

Flow rate: 50 mL/min

Injection volume: 2 µL

System suitability

Sample: Isobutane

Suitability requirements

Sample response comparison: Peak responses for Isobutane from duplicate injections agree within 1%.

Analysis

Sample: Isobutane

Connect 1 Isobutane cylinder to the chromatograph through a suitable sampling valve and a flow control valve downstream from the sampling valve. Flush the liquid specimen through the sampling valve, taking care to avoid entrapment of gas or air in the sampling valve. Calculate the percentage purity of Isobutane:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of isobutane

r_T = sum of all the peak responses

Acceptance criteria: NLT 95.0%

SPECIFIC TESTS

• **WATER:** NMT 0.001%, determined as directed in *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* (601), *Water Content*

• **HIGH-BOILING RESIDUES:** NMT 5 µg/mL, determined as directed in *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* (601), *High-Boiling Residues, Method II*

• **ACIDITY OF RESIDUE**

Sample: Residue from the test for *High-Boiling Residues*

Analysis: Add 10 mL of water to the *Sample*, mix by swirling for about 30 s, add 2 drops of methyl orange TS, insert the stopper in the tube, and shake vigorously.

Acceptance criteria: No pink or red color appears in the aqueous layer.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight cylinders, and prevent exposure to excessive heat.

Isomalt

$C_{12}H_{24}O_{11}$ 344.31

6-O- α -D-Glucopyranosyl-D-sorbitol;
6-O- α -D-Glucopyranosyl-D-glucitol;

$C_{12}H_{24}O_{11} \cdot 2H_2O$ 380.32
1-O- α -D-Glucopyranosyl-D-mannitol dihydrate [64519-82-0].

DEFINITION

Isomalt contains NLT 98.0% and NMT 102.0% of a mixture of 6-O- α -D-glucopyranosyl-D-sorbitol (1,6-GPS) and 1-O- α -D-glucopyranosyl-D-mannitol (1,1-GPM), and neither of the two components is less than 3.0% of the mixture, calculated on the anhydrous basis.

IDENTIFICATION

• **A. THIN-LAYER CHROMATOGRAPHY** (201)

Standard solution: 5 mg/mL of USP Isomalt RS

Sample solution: 5 mg/mL

Chromatographic system

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture containing a fluorescent indicator having optimal intensity at 254 nm

Application volume: 1 µL

Developing solvent system: Ethyl acetate, pyridine, water, acetic acid, and propionic acid (10:10:2:1:1)

Analysis

Samples: *Standard solution* and *Sample solution*

Proceed as directed in the chapter. Thoroughly dry the starting points in warm air. Develop over 10 cm using the *Developing solvent system*, dry the plate in a current of hot air, and dip for 3 s in a 1-mg/mL solution of sodium periodate. Dip the plate for 3 s in a mixture of dehydrated alcohol, sulfuric acid, acetic acid, and anisaldehyde (90:5:1:1). Dry the plate in a current of hot air until colored spots become visible. The background color may be brightened by exposure to warm steam. Examine in daylight.

Acceptance criteria: The principal spots of the *Sample solution* are similar in position and color to those of the *Standard solution*.

- **B.** The retention times of the two principal peaks of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the *Assay*.

ASSAY

PROCEDURE

Mobile phase: Water

Standard solution: 20 mg/mL of USP Isomalt RS

Sample solution: 20 mg/mL of Isomalt

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Guard column: 4.6-mm \times 3-cm; packing L19

Column: 7.8-mm \times 30-cm; packing L19

Column temperature: 80 \pm 1°

Flow rate: 0.5 mL/min

Injection volume: 20 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for 1,1-GPM and 1,6-GPS are about 1.0 and 1.2, respectively.]

Suitability requirements

Resolution: NLT 2.0 between 1,1-GPM and 1,6-GPS

Relative standard deviation: NMT 2.0% for the 1,6-GPS and 1,1-GPM peaks

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of 1,6-GPS in the portion of Isomalt taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of 1,6-GPS from the *Sample solution*

r_S = peak response of 1,6-GPS from the *Standard solution*

C_S = concentration of 1,6-GPS in the *Standard solution*, with calculation based on the declared 1,6-GPS content of USP Isomalt RS (mg/mL)

C_U = concentration of Isomalt in the *Sample solution* (mg/mL)

Calculate the percentage of 1,1-GPM in the portion of Isomalt taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of 1,1-GPM from the *Sample solution*

r_S = peak response of 1,1-GPM from the *Standard solution*

C_S = concentration of 1,1-GPM in the *Standard solution*, with calculation based on the declared 1,1-GPM content of USP Isomalt RS (mg/mL)

C_U = concentration of Isomalt in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% of a mixture of 6-O- α -D-glucopyranosyl-D-sorbitol (1,6-GPS) and 1-O- α -

D-glucopyranosyl-D-mannitol (1,1-GPM), and neither of the two components is less than 3.0%, calculated on the anhydrous basis.

IMPURITIES

- **HEAVY METALS**, *Method I* (231): NMT 10 µg/g
- **LIMIT OF NICKEL**

[NOTE—The purity of the reagents and the water used must be suitable for trace analysis, and the reagents and water must be free of nickel.]

Nickel standard solution: Transfer 1 mL of nickel standard solution TS to a 100-mL volumetric flask, add 1 mL of nitric acid, and dilute with water to volume. This solution contains the equivalent of 0.1 µg/mL of nickel.

Standard solutions: Into seven identical 10-mL volumetric flasks, introduce respectively 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL of *Nickel standard solution* equivalent to 0, 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 µg of nickel. To each flask add a 2.0-mL portion of the *Sample solution*, and dilute with water to volume.

Sample solution: Weigh 8 g of Isomalt into a 50-mL volumetric flask, add 8 mL of water and 3 mL of 65% nitric acid solution, and incubate at 95° for 1 h. Allow the solution to cool to room temperature, add another 3 mL of 65% nitric acid solution, and incubate at 95° until all brown vapors have dissipated (1–1.5 h). Allow the solution to cool to room temperature, carefully add 3 mL of 30% hydrogen peroxide, and keep the solution at 95° until the evolution of gas has ceased (1–2 h). Allow the solution to cool to room temperature. Repeat the procedure two more times, i.e., adding 30% hydrogen peroxide, heating to 95°, and cooling to room temperature. Dilute the resulting solution with water to 50 mL.

Blank: Prepare as directed for the *Sample solution*, except omit the addition of Isomalt.

Blank solutions: Prepare as directed for the *Standard solutions*, except replace 2 mL of the *Sample solution* with 2 mL of the *Blank*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry, using an instrument equipped with a graphite furnace

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Analysis: Concomitantly determine the absorbances of the *Standard solutions* and the *Blank solutions*. Record the average of the steady readings for each of the *Standard solutions* and the *Blank solutions*. Plot the absorbances of the *Standard solutions* versus the quantity of nickel, in µg, in the portion of *Nickel standard solution* added to each *Standard solution* flask. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the amount of nickel (A_T), in µg, in the portion of the *Sample solution* that was added to each of the *Standard solution* flasks. Similarly, plot the absorbances of the *Blank solutions* versus the quantity of nickel, in µg, in the portion of *Nickel standard solution* added to each of the *Blank solutions* flasks to determine the quantity of nickel (A_B) in the portion of *Blank* added to each of the *Blank solutions* flasks.

Calculate the quantity, in µg/g, of nickel in the portion of Isomalt taken:

$$\text{Result} = (A_T - A_B) \times (D/W)$$

A_T = amount of nickel in the portion of the *Sample solution* that was added to each of the *Standard solutions* flasks (µg)

A_B = quantity of nickel in the portion of *Blank* added to each of the *Blank solutions* flasks (µg)

D = volume of the *Sample solution* prepared per volume of the *Sample solution* used for analysis, 50/2

W = weight of Isomalt used to prepare the *Sample solution* (g)

Acceptance criteria: NMT 1 µg/g, calculated on the anhydrous basis

• ORGANIC IMPURITIES

Mobile phase, Chromatographic system, and Sample solution: Proceed as directed in the *Assay*.

Standard solution: 0.1 mg/mL each of USP Sorbitol RS and USP Mannitol RS

System suitability solution: 20 mg/mL of USP Isomalt RS and 0.1 mg/mL each of USP Mannitol RS and USP Sorbitol RS in water

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for 1,1-GPM, 1,6-GPS, mannitol, and sorbitol are about 1.0, 1.2, 1.6, and 2.0, respectively. The typical retention time for 1,1-GPM is about 12.3 min.]

Suitability requirements

Resolution: NLT 2.0 between 1,1-GPM and 1,6-GPS

Analysis

Samples: *Sample solution* and *Standard solution*

Calculate the percentage of mannitol or sorbitol in the portion of Isomalt taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of mannitol or sorbitol from the *Sample solution*

r_S = peak response of mannitol or sorbitol from the *Standard solution*

C_S = concentration of USP Mannitol RS or USP Sorbitol RS in the *Standard solution* (mg/mL)

C_U = concentration of Isomalt in the *Sample solution* (mg/mL)

Calculate the percentage of any unknown impurity in the portion of Isomalt taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of each unknown impurity from the *Sample solution*

r_S = peak response of sorbitol from the *Standard solution*

C_S = concentration of USP Sorbitol RS in the *Standard solution* (mg/mL)

C_U = concentration of Isomalt in the *Sample solution* (mg/mL)

Acceptance criteria: See *Table 1*. [NOTE—Disregard any impurity peak that is less than 0.1%.]

Table 1

Name	Acceptance Criteria, NMT (%)
Mannitol	0.5
Sorbitol	0.5
Any unknown impurity	0.5
Total impurities	2.0

• REDUCING SUGARS

Sample solution: Dissolve 3.3 g in 10 mL of Purified Water with the aid of gentle heat. Cool, and add 20 mL of cupric citrate TS and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 40 mL of dilute acetic acid, 60 mL of water, and 20 mL of 0.025 M iodine VS. With continuous shaking, add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water.

Analysis: After the precipitate has dissolved, titrate the excess iodine with 0.05 N sodium thiosulfate VS, using

1 mL of starch TS, added toward the end of the titration as an indicator.

Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate VS is required, corresponding to NMT 0.3% of reducing sugars, determined on the anhydrous basis as glucose.

SPECIFIC TESTS

• WATER DETERMINATION, Method I (921)

Sample: 0.3 g

Analysis: Add the *Sample* to a mixture of anhydrous methanol and formamide (1:1) at $50 \pm 5^\circ$.

Acceptance criteria: NMT 7%

• CONDUCTIVITY

Sample solution: Dissolve 20 g in carbon dioxide-free water prepared from distilled water, and dilute with the same solvent to 100 mL.

Analysis: Using an appropriate conductivity meter that has been standardized with a potassium chloride conductivity calibration standard, measure the conductivity of the *Sample solution* while gently stirring with a magnetic stirrer.

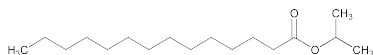
Acceptance criteria: NMT 20 $\mu\text{S}/\text{cm}$

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.
- **LABELING:** Label it to indicate the percentage content of 1,6-GPS and 1,1-GPM.
- **USP REFERENCE STANDARDS (11)**
 - USP Isomalt RS
 - USP Mannitol RS
 - USP Sorbitol RS

Isopropyl Alcohol—see Isopropyl Alcohol General Monographs

Isopropyl Myristate



$\text{C}_{17}\text{H}_{34}\text{O}_2$ 270.45
Tetradecanoic acid, 1-methylethyl ester;
Isopropyl myristate [110-27-0].

DEFINITION

Isopropyl Myristate consists of esters of isopropyl alcohol and saturated high molecular weight fatty acids, principally myristic acid. It contains NLT 90.0% of isopropyl myristate ($\text{C}_{17}\text{H}_{34}\text{O}_2$).

IDENTIFICATION

- **A.** The retention times of the major peaks of the *Sample solution* correspond to those of the *System suitability solution*, as obtained in the *Assay*.

ASSAY

• PROCEDURE

System suitability solution: 4.5 mg/mL of USP Isopropyl Myristate RS and 0.5 mg/mL of USP Isopropyl Palmitate RS in *n*-hexane

Sample solution: 5 mg/mL of Isopropyl Myristate in *n*-hexane

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 4-mm \times 1.8-m; packed with 10% liquid phase G8 on 100- to 120-mesh support S1A

Temperatures

Injection port: 240°

Detector: 280°

Column: See Table 1.

Table 1

Initial Temperature ($^\circ$)	Temperature Ramp ($^\circ/\text{min}$)	Final Temperature ($^\circ$)	Hold Time at Final Temperature (min)
90	2	210	8

Carrier gas: Nitrogen

Flow rate: 45 mL/min

Injection volume: 5 μL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for isopropyl myristate and isopropyl palmitate are 1.0 and 1.3, respectively.]

Suitability requirements

Resolution: NLT 6.0 between the peaks due to isopropyl myristate and isopropyl palmitate

Tailing factor: NMT 2 for the isopropyl palmitate peak

Relative standard deviation: NMT 2.0%

Analysis

Sample: *Sample solution*

Calculate the percentage of isopropyl myristate ($\text{C}_{17}\text{H}_{34}\text{O}_2$) in the portion of Isopropyl Myristate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for isopropyl myristate

r_T = sum of the responses of all the peaks, except the solvent peak

Acceptance criteria: NLT 90.0%

IMPURITIES

- **RESIDUE ON IGNITION (281):** NMT 0.1%

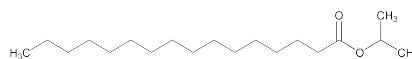
SPECIFIC TESTS

- **SPECIFIC GRAVITY (841):** 0.846–0.854
- **REFRACTIVE INDEX (831):** 1.432–1.436 at 20°
- **FATS AND FIXED OILS, Acid Value (401):** NMT 1
- **FATS AND FIXED OILS, Iodine Value (401):** NMT 1
- **FATS AND FIXED OILS, Saponification Value (401):** 202–212

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS (11)**
 - USP Isopropyl Myristate RS
 - USP Isopropyl Palmitate RS

Isopropyl Palmitate



$\text{C}_{19}\text{H}_{38}\text{O}_2$ 298.50
Hexadecanoic acid, 1-methylethyl ester;
Isopropyl palmitate [142-91-6].

DEFINITION

Isopropyl Palmitate consists of esters of isopropyl alcohol and saturated high molecular weight fatty acids. It contains NLT 90.0% of isopropyl palmitate ($C_{19}H_{38}O_2$).

IDENTIFICATION

- A.** The retention times of the major peaks of the *Sample solution* correspond to those of the *System suitability solution*, as obtained in the *Assay*.

ASSAY**PROCEDURE**

System suitability solution: 4.5 mg/mL of USP Isopropyl Palmitate RS and 0.5 mg/mL of USP Isopropyl Myristate RS in *n*-hexane

Sample solution: 5 mg/mL of Isopropyl Palmitate in *n*-hexane

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 4-mm \times 1.8-m; packed with 10% liquid phase G8 on 100- to 120-mesh support S1A

Temperatures

Injection port: 240°

Detector: 280°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
90	2	210	8

Carrier gas: Nitrogen

Flow rate: 45 mL/min

Injection volume: 5 μ L

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for isopropyl myristate and isopropyl palmitate are 1.0 and 1.3, respectively.]

Suitability requirements

Resolution: NLT 6.0 between the peaks due to isopropyl myristate and isopropyl palmitate

Tailing factor: NMT 2 for the isopropyl palmitate peak

Relative standard deviation: NMT 2.0%

Analysis

Sample: *Sample solution*

Calculate the percentage of isopropyl palmitate ($C_{19}H_{38}O_2$) in the portion of Isopropyl Palmitate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for isopropyl palmitate

r_T = sum of the responses of all the peaks, except the solvent peak

Acceptance criteria: NLT 90.0%

IMPURITIES

- RESIDUE ON IGNITION** <281>: NMT 0.1%

SPECIFIC TESTS

- SPECIFIC GRAVITY** <841>: 0.850–0.855
- REFRACTIVE INDEX** <831>: 1.435–1.438
- FATS AND FIXED OILS, Acid Value** <401>: NMT 1
- FATS AND FIXED OILS, Iodine Value** <401>: NMT 1
- FATS AND FIXED OILS, Saponification Value** <401>: 183–193

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

USP REFERENCE STANDARDS <11>

USP Isopropyl Myristate RS

USP Isopropyl Palmitate RS

Juniper Tar—see *Juniper Tar General Monographs*

Kaolin—see *Kaolin General Monographs*

Alpha-Lactalbumin

$C_{626}H_{958}N_{162}O_{196}S_9$
[9051-29-0].

14178

DEFINITION

Alpha-Lactalbumin is a lyophilized or spray-dried powder of compact globular metalloprotein that may contain a single bound calcium ion and is capable of binding zinc and other metals. Alpha-Lactalbumin is isolated either from bovine milk or from whey, both of which should be from edible sources suitable for human use. All materials derived from bovine sources must originate from countries free of bovine spongiform encephalopathy. It contains alpha-lactalbumin at NLT 90.0% of the labeled total protein content. The remainder consists mostly of beta-lactoglobulin. It may contain suitable stabilizers.

IDENTIFICATION**A. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS**

Gel fixing solution: In a 1-L Pyrex bottle, thoroughly mix 500 mL of water, 400 mL of alcoholic TS, and 100 mL of glacial acetic acid.

Gel staining solution: Prepare a solution of Coomassie blue G-250 having a concentration of 0.25 g/L in a 10.0% (v/v) acetic acid solution.¹ Store at room temperature.

Destaining solution: 10.0% (v/v) acetic acid in water [NOTE—This solution may be stored at room temperature for up to 6 months from the date prepared.]

Sample buffer: Prepare a solution containing 200 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 2% (w/v) sodium dodecyl sulfate (SDS), 40% (v/v) glycerol, and 0.04% (w/v) Coomassie blue G-250. If necessary, adjust with hydrochloric acid or sodium hydroxide to a pH of 6.8.²

Running buffer: Prepare a solution containing 100 mM tris(hydroxymethyl)aminomethane, 100 mM *N*-tris(hydroxymethyl)methylglycine (Tricine), and 0.1% (w/v) SDS in water. If necessary, adjust with hydrochloric acid or sodium hydroxide to a pH of 8.3.³ In a 400-mL beaker, thoroughly mix 35 mL of the solution so obtained (or 10 \times Tris/Tricine/SDS buffer)⁴ with 315 mL of water.

Molecular weight marker: Use a suitable molecular weight marker containing protein bands between 3.5 and 27 kDa.

Molecular weight standard solution: Transfer 16 μ L of the *Sample buffer* into a 0.5-mL microcentrifuge tube. Pipet 4 μ L of the *Molecular weight marker* into the microcentrifuge tube, and mix. Incubate the mixture in the closed microcentrifuge tube for 5 min at 95°. After

¹ A suitable gel staining solution is available from, e.g., Bio-Rad. Coomassie brilliant blue G-250 is available from Bio-Rad, Cat. # 161-0406.

² A suitable sample buffer is available as Tricine sample buffer from Bio-Rad, Cat. # 161-0739.

³ An undiluted suitable running buffer is available as 10 \times Tris/Tricine/SDS buffer from Bio-Rad, Cat. # 161-0744.

⁴ Available as 10 \times Tris/Tricine/SDS buffer from Bio-Rad, Cat. # 161-0744.

incubation, allow the tube to stand for 5 min at room temperature. Centrifuge at 5000 rpm for 1 min.

Alpha-Lactalbumin standard stock solution: 1.0% (w/v) of USP Alpha-Lactalbumin RS in water in a 2-mL centrifuge tube.

Alpha-Lactalbumin standard working solution: Pipet 21 μ L of the *Sample buffer* and 3 μ L of the *Alpha-Lactalbumin standard stock solution* into a 0.5-mL microcentrifuge tube, and mix. Proceed as directed for *Molecular weight standard solution* beginning with "Incubate the mixture".

Sample stock solution: 1.0% (w/v) of Alpha-Lactalbumin in water in a 2-mL centrifuge tube.

Sample solution: Pipet 21 μ L of the *Sample buffer* and 3 μ L of the *Sample stock solution* into a 0.5-mL microcentrifuge tube, and mix. Proceed as directed for *Molecular weight standard solution* beginning with "Incubate the mixture".

SDS-PAGE gel and apparatus set-up: Following the manufacturer's instructions, assemble and fill a 16.5% Tris-Tricine Ready Gel⁵ in the Mini-Protein III Electrophoresis Module,⁶ or in an equivalent module. Add *Running buffer* appropriately to this apparatus.

Analysis

Gel loading: Load 10 μ L of the *Molecular weight standard solution*, 2.5 μ L of the *Alpha-Lactalbumin standard working solution*, and 2.5 μ L of the *Sample solution*, respectively, into the 16.5% Tris-Tricine SDS-PAGE gel. [NOTE—The loaded samples contain approximately 3 μ g of protein based on the sample weight.]

Running the gel: Set the voltage to 100 V, and run at a constant voltage. Run the gel until the tracking dye front is approximately 10 mm from the bottom of the gel (approximately 80–90 min).

Gel fixing: Remove the gel, transfer to a plastic container, and soak in the *Gel fixing solution* for 30 min on a shaking rack. Decant the *Gel fixing solution*. Rinse with water, and decant.

Gel staining: Pour approximately 100 mL of the *Gel staining solution* into the staining container. Place the gel into the staining container, and allow the stain to completely cover the gel. Place the staining container on an appropriate shaker, and stain the gel for 60–90 min with gentle shaking.

Destaining: Drain the *Gel staining solution* into an appropriate waste container, and add 100 mL of *Destaining solution* to the container to cover the gel. Place the container on an appropriate shaker, and shake with gentle agitation for 30 min. Discard the used *Destaining solution*, and repeat destaining as necessary. Repeat rinsing with *Destaining solution* three to four times at 30-min intervals or until the gel is destained to the desired clarity.

Acceptance criteria: The Alpha-Lactalbumin has one major band at 14 kDa, a minor band at 16 kDa, and a molecular weight that is similar to that of USP Alpha-Lactalbumin RS.

- **B.** The retention time of the major peak for alpha-lactalbumin from the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Alpha-Lactalbumin* in the *Assay*.

ASSAY

• CONTENT OF ALPHA-LACTALBUMIN

Mobile phase: Prepare a solution of 0.02 M Tris-HCl, 0.5% SDS, and 0.1 N sodium chloride. Adjust the pH of the solution to 5.95 ± 0.05 . Pass this solution through a filter having a 0.5- μ m or finer porosity, and degas.

Standard solution: 1.0 mg/mL of USP Alpha-Lactalbumin RS in *Mobile phase*. [NOTE—Prepare it immediately before use.]

System suitability solution: 0.5 mg/mL of USP Alpha-Lactalbumin RS and 0.5 mg/mL of beta-lactoglobulin in *Mobile phase*

Sample solution: 1.0 mg/mL of Alpha-Lactalbumin in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 280 nm

Column: 7.8-mm \times 30-cm analytical column, packing L33

[NOTE—Equilibrate the column for approximately 90 min at 0.6 mL/min of *Mobile phase* or until a stable baseline is achieved.]

Flow rate: 0.6 mL/min

Injection size: 20 μ L

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for beta-lactoglobulin and alpha-lactalbumin are 0.91 and 1.00, respectively.]

Suitability requirements

Resolution: NLT 1.65 between beta-lactoglobulin and alpha-lactalbumin

Tailing factor: Not greater than 1.1 for the alpha-lactalbumin peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the purity of Alpha-Lactalbumin as a percentage of total protein:

$$\text{Result} = (r_U/r_S) \times [C_S/(C_U \times P)] \times 100$$

r_U = peak response for alpha-lactalbumin from the *Sample solution*

r_S = peak response for alpha-lactalbumin from the *Standard solution*

C_S = concentration of USP Alpha-Lactalbumin RS in the *Standard solution* (mg/mL)

C_U = concentration of Alpha-Lactalbumin in the *Sample solution* (mg/mL)

P = percentage of total protein content

Acceptance criteria: Content of alpha-lactalbumin is NLT 90.0% of the labeled total protein content.

• TOTAL PROTEIN CONTENT

Sample: 250 mg of Alpha-Lactalbumin

Analysis: Combust the *Sample* in the presence of pure oxygen (99.9%) in an airtight oven at 950° with a suitable nitrogen analyzer. The components such as carbon dioxide, sulfur dioxide, and moisture are absorbed by various in-line chemical filters. All nitrogenous matter is converted into nitrogen in the presence of catalytic converters. The weight percent of nitrogen is measured by a thermal conductivity detector. Blank the system by analyzing a suitable nitrogen blank material, such as powdered cellulose, and obtaining a zero reading. Calibrate and qualify the system by using EDTA. The relative standard deviation for replicate runs is NMT 0.5%. Calculate the weight percent of total protein content in Alpha-Lactalbumin by multiplying the percentage of nitrogen found by 6.23.

Acceptance criteria: Total protein content is NLT 90.0%.

OTHER COMPONENTS

• LIMIT OF BETA-LACTOGLOBULIN

Mobile phase, System suitability solution, Sample solution, Chromatographic system, and System suitability: Prepare as directed in the test for *Content of Alpha-Lactalbumin*.

Standard solution: 1.0 mg/mL of beta lactoglobulin in *Mobile phase*. [NOTE—Prepare it immediately before use.]

⁵ Available from Bio-Rad, Cat. # 161-1107 or 161-1179.

⁶ Available from Bio-Rad, Cat. # 165-3302.

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of beta-lactoglobulin as a percentage of total protein:

$$\text{Result} = (r_U/r_S) \times [C_S/(C_U \times P)] \times 100$$

- r_U = peak response for beta-lactoglobulin from the *Sample solution*
 r_S = peak response for beta-lactoglobulin from the *Standard solution*
 C_S = concentration of beta-lactoglobulin in the *Standard solution* (mg/mL)
 C_U = concentration of Alpha-Lactalbumin in the *Sample solution* (mg/mL)
 P = percentage of total protein content

Acceptance criteria: NMT 6.5%, calculated on the total protein basis

• **CONTENT OF CALCIUM**

Standard stock solution: Dissolve 1.249 g of calcium carbonate in 270 mL of 3 N hydrochloric acid (dilute 250 mL of hydrochloric acid with water to 1000 mL) in a 1000-mL volumetric flask. Dilute with water to volume, and mix. Dilute 50 mL of the solution so obtained to 1000 mL. The *Standard stock solution* contains 25 µg/mL of calcium.⁷

Lanthanum chloride solution: Weigh 11.7 g (± 100 mg) of lanthanum oxide, and transfer to a 1000-mL volumetric flask. Add enough water to wet the powder, and then slowly add 50 mL of hydrochloric acid. [**CAUTION**—Exothermic reaction.] Let the test specimen dissolve, dilute with water to volume, and mix. This solution contains 1% (w/v) of lanthanum and is stable for up to 6 months when stored at room temperature.

Blank solution: 10-fold dilution of *Lanthanum chloride solution*

Working standard solutions: To five identical 25-mL volumetric flasks add 0, 5, 10, 15, and 20 mL, respectively, of *Standard stock solution*. Add 2.5 mL of *Lanthanum chloride solution*, and dilute with water to volume. The *Working standard solutions* contain 0, 5, 10, 15, and 20 µg/mL of calcium, each containing 0.1% (w/v) of lanthanum.

Sample solution: Transfer 1.0 g of Alpha-Lactalbumin to a 100-mL volumetric flask, add 10 mL of *Lanthanum chloride solution*, and dilute with water to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 422.7 nm

Lamp: Calcium hollow-cathode lamp

Flame: Reduced air-acetylene

Analysis

Samples: *Working standard solutions* and *Sample solution*

Concomitantly determine the absorbances of the *Samples* using the *Blank solution*. [NOTE—Optimize flame parameters in accordance with the instrument manufacturer's instructions.]

Plot the absorbances of the *Working standard solutions* versus the concentration, in µg/mL, of calcium, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of calcium in the *Sample solution*.

Calculate the quantity of calcium (Ca), in mg, in each g of Alpha-Lactalbumin taken:

$$\text{Result} = (V \times C)/W \times F \times 100$$

V = volume of the *Sample solution*, 100 mL

C = as determined above

W = weight of Alpha-Lactalbumin taken to prepare the *Sample solution* (g)

F = conversion factor for µg to mg (10^{-3} mg/µg)

Acceptance criteria: NMT 1 mg/g of calcium

IMPURITIES

Inorganic Impurities

• **ASH:** Ignite 1 g of Alpha-Lactalbumin at NMT 550° until free from carbon. Cool in a desiccator, and weigh: NMT 3.5% is found.

• **HEAVY METALS, Method II (231):** NMT 10 ppm

• **LIMIT OF PHOSPHORUS**

Hydrochloric acid solution: Pipet 250 mL of hydrochloric acid into a 1000-mL volumetric flask, dilute with water to volume, and mix.

Molybdovanadate reagent: Dissolve 20 g of ammonium molybdate in 200 mL of water with the aid of heat, and then allow the molybdate solution to cool. Dissolve 1.0 g of ammonium vanadate in 125 mL of water with the aid of heat, cool, and add 160 mL of hydrochloric acid. Gradually add, with stirring, the molybdate solution to the vanadate solution, and dilute with water to 1000 mL.

Phosphorus standard stock solution I: Transfer 8.8 g of monobasic potassium phosphate (KH_2PO_4), previously dried for 2 h at 105°, to a 1000-mL volumetric flask, and add 750 mL of water to dissolve. Dilute with water to volume. This solution contains 2 mg/mL of phosphorus. [NOTE—Store the solution in a refrigerator.]

Phosphorus standard stock solution II: Immediately before use, dilute 50 mL of *Phosphorus standard stock solution I* with water to 1000 mL. [NOTE—Store in a refrigerator.]

Standard solutions: Transfer 0.0 mL, 5.0 mL, 8.0 mL, 10.0 mL, and 15.0 mL of *Phosphorus standard stock solution II*, respectively, to five identical 100-mL volumetric flasks. Proceed as directed in the *Analysis*: after treatment with the *Molybdovanadate reagent*, the resulting final phosphorus concentrations for the *Standard solutions* are 0.0, 5.0, 8.0, 10.0, and 15.0 µg/mL, respectively.

Sample solution: Transfer 4.0 g of Alpha-Lactalbumin to an ashing dish. Dry the test specimen on a hot plate or steam bath. Ignite in a muffle furnace at a maximum temperature of 600° until free of carbon. Cool, add 40 mL of *Hydrochloric acid solution* and several drops of nitric acid, and bring to boil on a hot plate. Cool, transfer to a 100-mL volumetric flask by rinsing the ashing dish with water, dilute with water to volume, and mix. Pipet 20.0 mL of the *Sample solution* into a 100-mL volumetric flask.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Analytical Wavelength: 400 nm

Analysis

Samples: *Standard solutions* and the *Sample solution*
To each of the flasks containing the *Samples*, add 20.0 mL of *Molybdovanadate reagent*, dilute with water to volume, mix, and allow to stand for exactly 10 min for maximum color development. The *Standard solutions* and the *Sample solution* are treated identically. Concomitantly determine the absorbances of each of the *Samples* in 1-cm cells using the *Spectrometric conditions* described above. Use one of the *Standard solutions* with phosphorus concentration at 0.0 µg/mL to zero the spectrophotometer. Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of phosphorus, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of phosphorus in the *Sample solution*.

⁷ A commercially prepared, certified AA standard is available as Calcium AA, ICP standards, 1000 ppm Ca in dilute hydrochloric acid, Cat. # ACA1KH, from RICCA. Make an appropriate dilution to obtain a final concentration of 25 µg/mL of calcium.

Calculate the quantity, in μg , of phosphorus in each g of Alpha-Lactalbumin taken:

$$\text{Result} = (V \times C)/W \times D$$

- V = volume of the *Sample solution*, 100 mL
 C = as determined above
 W = weight of Alpha-Lactalbumin taken to prepare the *Sample solution* (g)
 D = dilution factor, 5

Acceptance criteria: NMT 700 $\mu\text{g/g}$ of phosphorus

Organic Impurities

• PROCEDURE 1: LIMIT OF LIPID (FAT)

Weighing dish preparation: Pre-dry the clean dishes under the same conditions that will be used for final drying after fat extraction. Ensure that all surfaces where weighing dishes will be placed (i.e., hot plate, desiccator, etc.) are clean and free of particulates. At the end of oven drying, place the weighing dishes in a desiccator, and cool to room temperature. Immediately before use, weigh the dishes to the nearest 0.1 mg, and record the weights. Check the balance zero after weighing each dish. Protect the weighed dishes from contamination with extraneous matter.

Analysis

Transfer 0.5 g of Alpha-Lactalbumin to a Mojonnier-style ether extraction flask that has the capacity to hold a volume of 21–23 mL in the lower bulb plus neck at the bottom of the flask. The flask has a smooth, round opening at the top that can be sealed when closed with cork. Add 10 mL of water at a temperature of 40°, and mix. Add 1.5 mL of ammonium hydroxide to the Alpha-Lactalbumin, and mix thoroughly. Add 3 drops of phenolphthalein TS to help sharpen the visual appearance of the interface between the ether and the aqueous layers during extraction. Add 10 mL of alcohol, close with the cork stopper that has been water soaked, and shake the flask for 15 s.

For the first extraction, add 25 mL of ether, replace the cork stopper, and shake the flask very vigorously for approximately 1 min, releasing built-up pressure by loosening the stopper as necessary. Add 25 mL of petroleum ether, replace the cork stopper, and repeat vigorous shaking for about 1 min. Centrifuge the flask at about 600 rpm for NLT 30 s to obtain a clean separation of the aqueous (bright pink) and the ether phases. Decant the ether solution into a suitable weighing dish prepared as directed for *Weighing dish preparation*. When the ether solution is decanted into the dish, be careful not to pour any suspended solids or aqueous phase into the weighing dish. Ether can be evaporated at NMT 100° from the dish while conducting the second extraction.

For the second extraction, add 5 mL of alcohol to the original flask, close with the cork stopper, and shake vigorously for 15 s. Add 15 mL of ether, replace the cork, and shake the flask vigorously for about 1 min. Add 15 mL of petroleum ether, replace the cork stopper, and repeat vigorous shaking for about 1 min. Centrifuge the flask at about 600 rpm for NLT 30 s to obtain a clean separation of the aqueous (bright pink) and the ether phases. If the interface is below the neck of the flask, add water to bring the level about halfway up to the neck. Add water slowly down the inside surface of the flask so that there is minimum disturbance of the interface. Decant the ether solution for the second extraction into the same weighing dish used for the first extraction.

For the third extraction, omit addition of the alcohol and repeat the procedure used for the second extraction. Completely evaporate the solvents in a hood on a hot plate at NMT 100°, and avoid spattering. Dry the extracted fat and the weighing dish to constant weight in a forced air oven at 100° \pm 1° for NLT 30 min or in a vacuum oven at 70°

to 75° at more than 50.8 cm (20 inches) of vacuum for NLT 7 min. Remove the weighing dish from the oven, and place in a desiccator to cool to room temperature. Record the weight of the weighing dish containing the fat.

Run a blank determination using water, and record the weight of any dry residue collected. The reagent blank should be less than 2.0 mg of residue. [NOTE—A negative number is not acceptable.]

Calculate the weight percent of lipid (fat) in the portion of Alpha-Lactalbumin taken:

$$\text{Result} = [(W_2 - W_1) - W_3]/W \times 100$$

- W_1 = weight of empty weighing dish (g)
 W_2 = weight of the weighing dish containing fat (g)
 W_3 = weight of the reagent blank residue (g)
 W = weight of the Alpha-Lactalbumin taken for the fat extraction (g)

Acceptance criteria: NMT 1.0% is found. The difference between duplicate runs is NMT 0.03% fat.

• PROCEDURE 2: LIMIT OF LACTOSE

Carrez I solution: Transfer 3.60 g of potassium ferrocyanide [$\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$] to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

Carrez II solution: Transfer 7.20 g of zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

[NOTE—The following four test reagents are included in a test kit.⁸]

Test reagent 1: About 600 mg of lyophilisate consisting of a mixture of citrate buffer (pH 6.6), nicotinamide adenine dinucleotide (NAD) (35 mg), anhydrous magnesium sulfate, and stabilizers (added if necessary). Dissolve lyophilisate in 7.0 mL of water before use.

Test reagent 2: About 1.7 mL of an enzyme suspension of β -galactosidase (approximately 100 Units).

Test reagent 3: 34 mL of a solution consisting of 0.51 M potassium diphosphate buffer (pH 8.6), and stabilizers (added if necessary).

Test reagent 4: About 1.7 mL of an enzyme suspension of galactose dehydrogenase (about 40 Units).

Sample solution: Transfer 1.0 g of Alpha-Lactalbumin to a 100-mL volumetric flask, add about 60 mL of water, and mix. Add 5 mL of *Carrez I solution*, and mix. Add 5 mL of *Carrez II solution*, and mix. Add 10 mL of 0.1 N sodium hydroxide solution, and mix vigorously. Dilute with water to volume, and mix. Pass through a filter paper, and use the clear filtrate. [NOTE—This procedure breaks emulsions, absorbs some colors, and precipitates proteins.]

Analysis

Label one glass or disposable plastic cuvet as “blank” and the second glass or disposable plastic cuvet as “test”. [NOTE—These two cuvetts should be equivalent.] To each cuvet, pipet 0.20 mL of *Test reagent 1* and 0.05 mL of *Test reagent 2*. Pipet 0.10 mL of the *Sample solution* into the cuvet that is labeled “test”. Mix both cuvetts with their stirrers, and incubate at 20°–25° for 20 min. Pipet 1.00 mL of *Test reagent 3* into each cuvet. Pipet 2.00 mL of water into the cuvet that is labeled “blank” and 1.90 mL of water into the cuvet containing the *Sample solution*. Mix, and incubate at 20°–25° for about 2 min.

Determine the absorbances, A_{51} and A_{61} , at 340 nm, for the *Sample solution* and the blank, respectively. Add

⁸ Available from Boehringer-Mannheim (R-Biopharm, Inc., 7950 Old US 27S, Marshall, MI 49068 USA; Tel: +1-877-789-3033 or +1-269-789-3033; Fax: +1-269-789-3070; www.r-biopharm.com).

0.05 mL of *Test reagent 4* to each cuvet. Mix and incubate at 20°–25° until the reaction has stopped (about 10–15 min). Determine the absorbances A_{S2} and A_{B2} , at 340 nm, again for the *Sample solution* and the blank, respectively. If the reaction has not stopped after 15 min, continue to read the absorbances at 2-min intervals until the absorbance for the *Sample solution* remains constant for two successive measurements.

Calculate the percentage of lactose in the portion of Alpha-Lactalbumin taken:

$$\text{Result} = V_1 \times V_2 \times M_r \times [(A_{S2} - A_{S1}) - (A_{B2} - A_{B1})] / (\epsilon \times L \times V_3 \times W) \times 100$$

- V_1 = volume of the *Sample solution*, 0.1 L
 V_2 = volume of the final sample solution in the cuvet, 3.30 mL
 M_r = molecular weight of lactose monohydrate, 360.32 g/mol
 ϵ = absorption coefficient of nicotinamide adenine dinucleotide reduced form (NADH) at 340 nm, 6300 L · mol⁻¹ · cm⁻¹
 L = light path of the cuvet, 1.0 cm
 V_3 = volume of the *Sample solution* taken into the cuvet, 0.1 mL
 W = weight of Alpha-Lactalbumin taken to prepare the *Sample solution* (g)
Acceptance criteria: NMT 1.0% of lactose

SPECIFIC TESTS

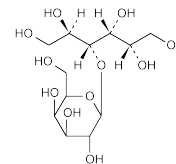
- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic bacterial count does not exceed 1000 cfu/g. The total combined molds and yeasts count does not exceed 100 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **DENATURATION TEMPERATURE**
Sample solution: Prepare a protein dough by mixing 3 g of Alpha-Lactalbumin powder with 2 g of water. Place the dough into a well-sealed sample container.
Analysis: Perform two measurements on the dough sample using a differential scanning calorimeter. Heat to 140°, and scan. Cool rapidly to below room temperature, and rescan. Apply a scan rate of 10°/min. Weigh pans before and after scanning to verify that no moisture loss occurs during the scanning process. Measure and record the denaturation temperatures as peak temperatures. The formation of two peaks indicates the presence of both the apo form and the holo form of Alpha-Lactalbumin.
Acceptance criteria: The denaturation temperature for Alpha-Lactalbumin in the apo form is between 50° and 52°; the denaturation temperature for Alpha-Lactalbumin in the holo form is between 58° and 61°.
- **PH** (791): NMT 7.5, in a solution (1 in 10)
- **LOSS ON DRYING** (731): Dry 1.0–1.5 g in a vacuum oven at 100°, at a pressure of 660 mm of mercury, and with continuous dry air feed for 5 h: it loses NMT 6.5% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at the temperature indicated on the label.
- **LABELING:** Label it to state the protein content, expressed as a total protein percentage. Indicate the type of source material, expressed as bovine milk, whey, or both, used to manufacture the final product. Label it to indicate the storage conditions, the expiration date, and the name and concentration of any added stabilizers.
- **USP REFERENCE STANDARDS** (11)
 USP Alpha-Lactalbumin RS

Lactic Acid—see *Lactic Acid General Monographs*

Lactitol



$C_{12}H_{24}O_{11}$	344.31
$C_{12}H_{24}O_{11} \cdot H_2O$	362.34
$C_{12}H_{24}O_{11} \cdot 2H_2O$	380.35

4-O-β-D-Galactopyranosyl-D-glucitol [585-86-4].
 Monohydrate [81025-04-9].
 Dihydrate [81025-03-8].

DEFINITION

Lactitol contains NLT 98.0% and NMT 101.0% of $C_{12}H_{24}O_{11}$, calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)

ASSAY

PROCEDURE

Mobile phase: Water

Standard solution: 10.0 mg/mL of USP Lactitol RS

Sample solution: 10.0 mg/mL of Lactitol

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 30-cm; packing L34

Column temperature: 85°

Flow rate: 0.7 mL/min

Injection size: 25 µL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 1.0% for lactitol

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of lactitol ($C_{12}H_{24}O_{11}$) in the portion of Lactitol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Lactitol RS in the *Standard solution* (mg/mL)

C_U = concentration of Lactitol in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–101.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.5%

- **HEAVY METALS** (231)

Test preparation: Dissolve 4 g in 25 mL of water.

Acceptance criteria: NMT 5 ppm

- **RELATED COMPOUNDS**

Standard solution: 0.3 mg/mL of USP Lactitol RS

Sample solution: Prepare as directed in the *Assay*.

Chromatographic system: Proceed as directed in the *Assay*.

System suitability**Sample:** *Standard solution*

[NOTE—The relative retention times for lactose, glucose, galactose, lactulitol, lactitol, galactitol, and sorbitol are about 0.53, 0.58, 0.67, 0.72, 1.0, 1.55, and 1.68, respectively.]

Analysis**Samples:** *Standard solution* and *Sample solution*

Calculate the percentages of galactitol, sorbitol, lactulitol, lactose, glucose, and galactose in the portion of Lactitol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of the relevant related compound, if observed, from the *Sample solution*

r_S = peak response of lactitol from the *Standard solution*

C_S = concentration of USP Lactitol RS in the *Standard solution* (mg/mL)

C_U = concentration of Lactitol in the *Sample solution* (mg/mL)

Acceptance criteria: The total of the percentages of all related compounds is NMT 1.5%.

• **REDUCING SUGARS**

Standard solution: Pipet 2 mL of a dextrose solution containing 0.5 mg/mL into a 10-mL conical flask.

Sample solution: Dissolve 500 mg in 2.0 mL of water in a 10-mL conical flask.

Analysis: Concomitantly add 1 mL of alkaline cupric tartrate TS to each solution, heat to boiling, and cool.

Acceptance criteria: NMT 0.2%, calculated as dextrose. The *Sample solution* shows no more turbidity than that produced in the *Standard solution*, in which a reddish brown precipitate forms.

SPECIFIC TESTS

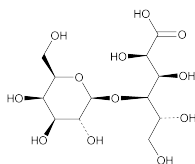
- **WATER DETERMINATION, Method I (921):** For the monohydrate form, 4.5%–5.5%; for the dihydrate form, 9.5%–10.5%; and for the anhydrous form, NMT 0.5%.

ADDITIONAL REQUIREMENTS

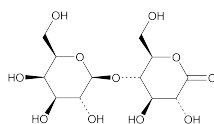
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Label it to indicate whether it is the monohydrate, the dihydrate, or the anhydrous form.
- **USP REFERENCE STANDARDS (11)**
USP Lactitol RS

Lactobionic Acid

$C_{12}H_{22}O_{12}$ (acid form) 358.30
[96-82-2].



$C_{12}H_{20}O_{11}$ (δ -lactone) 340.28
[5965-65-1].



4-O- β -Galactopyranosyl-D-gluconic acid.

DEFINITION

Lactobionic Acid is a mixture in variable proportions of 4-O- β -D-galactopyranosyl-D-gluconic acid and 4-O- β -D-galactopyranosyl-D-glucono-1,5-lactone. It contains NLT 98.0% and NMT 102.0%, on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K):** [NOTE—If the spectra obtained show differences, dissolve the test substance and USP Lactobionic Acid RS separately in water, dry at 105°, and record new spectra using the residues.]

- **B. THIN-LAYER CHROMATOGRAPHY (621)**

Standard solution: 10 mg/mL of USP Lactobionic Acid RS

Sample solution: 10 mg/mL of Lactobionic Acid

Adsorbent: Chromatographic silica gel mixture with an average particle size of 10–15 μ m (TLC plates)

Developing solvent: Methanol, ethyl acetate, ammonium hydroxide, and water (2:1:1:1)

Application volume: 5 μ L

Spray reagent: Slowly add 10 mL of sulfuric acid to about 40 mL of water. Mix, and allow to cool. Dilute with water to 100 mL, and mix. Add 2.5 g of ammonium molybdate and 1 g of ceric sulfate, and shake for 15 min to dissolve.

Analysis: Develop the chromatograms until the solvent front has moved about three-fourths the length of the plate, and allow to dry. Spray the plate with *Spray reagent*, and allow to dry. Repeat two more times, heat at 110° for 15 min, and examine.

Acceptance criteria: The principal spot from the *Sample solution* is similar in position and color to the principal spot from the *Standard solution*.

ASSAY• **PROCEDURE**

Sample: 0.350 g of Lactobionic Acid

Analysis: Dissolve the *Sample* in 50 mL of carbon dioxide-free water, previously heated to 30°. Immediately titrate with 0.1 N sodium hydroxide, and determine the two equivalence points potentiometrically. (See *Titrimetry* (541).)

Each mL of 0.1 N sodium hydroxide consumed to the first equivalency point is equivalent to 35.83 mg of $C_{12}H_{22}O_{12}$ (corresponds to the acid form), and each mL of 0.1 N sodium hydroxide consumed between the first and second equivalency points is equivalent to 34.03 mg of $C_{12}H_{20}O_{11}$ (corresponds to the δ -lactone form).

Calculate the content, expressed as a percentage, of the lactobionic acid as the sum of both results.

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

- **HEAVY METALS (231)**

Thioacetamide reagent: To 0.2 mL of thioacetamide TS add 1 mL of a mixture of 5 mL of water, 15 mL of 1 M sodium hydroxide, and 20 mL of glycerin. Heat in a water bath for 20 s. [NOTE—Prepare immediately before use.]

Lead nitrate stock solution: Prepare as directed for *Special Reagents* in *Heavy Metals* (231).

Standard solution: On the day of use, dilute 2.0 mL of the *Lead nitrate stock solution* (10 ppm Pb) in water to 30 mL.

Sample solution: Dissolve 1 g of Lactobionic Acid in water to 30 mL.

Prepare the filtration apparatus by adapting the barrel of a 50-mL syringe without its piston to a support containing, on the plate, a membrane filter of 3- μ m pore size, and above it a prefilter.

Transfer the *Sample solution* into the syringe barrel, put the piston in place, and then apply an even pressure on it until the whole of the liquid has been filtered. When

opening the support and removing the prefilter, check that the membrane filter remains uncontaminated with impurities. If this is not the case, replace it with another membrane filter, and repeat the operation under the same conditions.

Analysis: To the prefiltrate, add 2 mL of pH 3.5 Acetate Buffer. Mix, and add 1.2 mL of Thioacetamide reagent. Mix immediately, allow to stand for 10 min, and again filter as described above, but inverting the order of the filters, the liquid passing first through the membrane filter before passing through the prefilter. The filtration must be carried out slowly and uniformly by applying moderate and constant pressure to the piston of the syringe. After complete filtration, open the support, remove the membrane filter, and dry using filter paper. In parallel, treat the *Standard solution* in the same manner as the *Sample solution*.

Acceptance criteria: The color of the spot from the *Sample solution* is not more intense than that from the *Standard solution* (NMT 20 ppm).

SPECIFIC TESTS

• WATER DETERMINATION, Method Ia (921)

Sample solution: 0.50 g in a mixture of methanol and formamide (2:1)

Acceptance criteria: NMT 5.0%

• APPEARANCE OF SOLUTION

Sample solution: 120 mg/mL of Lactobionic Acid

Standard stock solution: Pipet 24.0 mL of ferric chloride CS and 6.0 mL of cobaltous chloride CS into a 100-mL volumetric flask. Dilute with 1% (w/v) hydrochloric acid to volume.

Reference solution: Pipet 12.5 mL of the *Standard stock solution* into a 100-mL volumetric flask. Dilute with 1% (w/v) hydrochloric acid to volume.

Acceptance criteria: The *Sample solution* is clear and not more intensely colored than the *Reference solution*.

• OPTICAL ROTATION, Specific Rotation (781S)

Sample solution: 10 mg/mL of Lactobionic Acid. Allow to stand for 24 h.

Acceptance criteria: +23.0° to +29.0° (anhydrous substance)

• REDUCING SUGARS

Sample solution: Dissolve 5.0 g of Lactobionic Acid in 25 mL of water with the aid of gentle heat, and cool.

Analysis: To the *Sample solution* add 20 mL of cupric citrate TS and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 100 mL of a 2.4% solution of glacial acetic acid and 20.0 mL of 0.025 M iodine VS. With continuous shaking, add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water. When the precipitate has dissolved, titrate the excess iodine with 0.05 M sodium thiosulfate VS using 1 mL of starch TS, added toward the end of the titration as an indicator.

Acceptance criteria: NLT 12.8 mL of 0.05 M sodium thiosulfate VS is required, corresponding to NMT 0.2% of reducing sugars, as glucose.

• ARTICLES OF BOTANICAL ORIGIN, Total Ash (561): NMT 0.2%

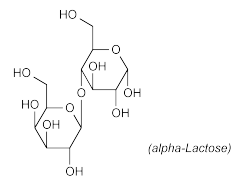
ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in well-closed containers.

• USP REFERENCE STANDARDS (11) USP Lactobionic Acid RS

Anhydrous Lactose

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (♦) to specify this fact.



DEFINITION

Anhydrous Lactose is *O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranose (β-lactose), or a mixture of *O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranose and *O*-β-D-galactopyranosyl-(1→4)-α-D-glucopyranose (α-lactose).

IDENTIFICATION

• A. INFRARED ABSORPTION (197K)

• ♦B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)

Adsorbent: 0.25-mm layer of chromatographic silica gel

Diluent: Methanol and water (3:2)

Standard solution A: 0.5 mg/mL of USP Anhydrous Lactose RS in *Diluent*

Standard solution B: Contains 0.5 mg/mL of USP Dextrose RS, 0.5 mg/mL of USP Anhydrous Lactose RS, 0.5 mg/mL of USP Fructose RS, and 0.5 mg/mL of USP Sucrose RS in *Diluent*

Sample solution: 0.5 mg/mL of Anhydrous Lactose in *Diluent*

Application volume: 2 μL

Developing solvent system: Ethylene dichloride, glacial acetic acid, methanol, and water (10:5:3:2)

Spray reagent: 5 mg/mL of thymol in a mixture of alcohol and sulfuric acid (19:1)

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Allow the spots to dry, and develop the plate in a paper-lined chromatographic chamber equilibrated with the *Developing solvent system* for about 1 h prior to use. Allow the chromatogram to develop until the solvent front has moved about three-quarters of the length of the plate. Remove the plate from the chamber, dry in a current of warm air, and redevelop the plate in fresh *Developing solvent system*. Remove the plate from the chamber, mark the solvent front, and dry the plate in a current of warm air. Spray the plate evenly with *Spray reagent*. Heat the plate at 130° for 10 min.

System suitability: The test is not valid unless *Standard solution B* shows four clearly discernible spots, disregarding any spots at the origin.

Acceptance criteria: The principal spot from the *Sample solution* corresponds in appearance and *R_f* value to that from *Standard solution A*.♦

OTHER COMPONENTS

• ♦CONTENT OF ALPHA AND BETA ANOMERS

Silylation reagent: Dimethyl sulfoxide, pyridine, and trimethylsilylimidazole (19.5: 58.5: 22)

Standard solution: Prepare a mixture of alpha-lactose monohydrate and beta-lactose having an anomeric ratio of about 1:1 based on the labeled anomeric contents of the alpha-lactose monohydrate and the beta-lactose. In-

Introduce 10 mg of this mixture into a vial with a screw cap. Add 4 mL of *Silylation reagent*. Sonicate for 20 min at room temperature. Transfer 400 µL to an injection vial. Add 1 mL of pyridine. Close the vial, and mix well.

Sample solution: Introduce 10 mg of Anhydrous Lactose into a vial with a screw cap. Add 4 mL of *Silylation reagent*. Sonicate for 20 min at room temperature. Transfer 400 µL to an injection vial. Add 1 mL of pyridine. Close the vial, and mix well.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Columns

Precolumn:¹ 0.53-mm × 2-m intermediate polarity deactivated fused silica

Analytical:² 0.25-mm × 15-m G27 on fused silica; film thickness 0.25 µm

Temperatures

Detector: 325°

Injection port: 275° or use cold on-column injection

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	—	80	1
80	35	150	—
150	12	300	2

Carrier gas: Helium

Flow rate: 2.8 mL/min

Injection volume: 0.5 µL

Injection type: Splitless or by cold on-column injection

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 3.0 between the peaks due to alpha-lactose and beta-lactose

Analysis

Sample: *Sample solution*

[NOTE—The relative retention time with reference to beta-lactose is about 0.9 for alpha-lactose (retention time = about 12 min).]

Calculate the percentage content of alpha-lactose:

$$\text{Result} = S_a / (S_a + S_b) \times 100$$

S_a = area of the peak due to alpha-lactose

S_b = area of the peak due to beta-lactose

Calculate the percentage content of beta-lactose:

$$\text{Result} = S_b / (S_a + S_b) \times 100$$

S_a = area of the peak due to alpha-lactose

S_b = area of the peak due to beta-lactose

♦

IMPURITIES

- ♦ **HEAVY METALS**, *Method II* <231>: NMT 5 ppm♦
- ♦ **RESIDUE ON IGNITION** <281>: NMT 0.1%

SPECIFIC TESTS

♦ CLARITY AND COLOR OF SOLUTION

Hydrazine sulfate solution: Dissolve 1.0 g of hydrazine sulfate in water, and dilute to 100.0 mL. Allow to stand for 4–6 h.

Hexamethylenetetramine solution: In a 100-mL ground-glass stoppered flask dissolve 2.5 g of hexamethylenetetramine in 25.0 mL of water.

Primary opalescent suspension: To the *Hexamethylenetetramine solution* in the flask add 25.0 mL of the *Hydrazine sulfate solution*. Mix and allow to stand for 24 h. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.

Standard opalescence: Dilute 15.0 mL of the *Primary opalescent suspension* to 1000.0 mL with water. This suspension is freshly prepared and may be stored for up to 24 h.

Reference suspension: To 5.0 mL of the *Standard opalescence* add 95.0 mL of water. Mix and shake before use.

Reference solution: To 6.0 mL of ferric chloride CS, 2.5 mL of cobaltous chloride CS, and 1.0 mL of cupric sulfate CS add hydrochloric acid (10 g/L HCl) to make 1000 mL.

Sample solution: 1 g in 10 mL of boiling water. Allow to cool.

Instrumental conditions

Mode: Vis

Analytical wavelength: 400 nm

Acceptance criteria: NMT 0.04 for the absorbance divided by the path length in centimeters; and the clarity of the *Sample solution* is the same as that of water or its opalescence is not more pronounced than that of the *Reference suspension*, and it is not more colored than the *Reference solution*.

♦ LOSS ON DRYING <731>

Analysis: Dry a sample at 80° for 2 h.

Acceptance criteria: NMT 0.5%

♦ WATER DETERMINATION, *Method I* <921>

Sample solution: Anhydrous Lactose in a mixture of methanol and formamide (2:1)

Acceptance criteria: NMT 1.0%

♦ MICROBIAL ENUMERATION TESTS <61> and TESTS FOR SPECIFIED MICROORGANISMS <62>

The total aerobic microbial count is NMT 10^2 cfu/g and ♦the total combined molds and yeasts count is NMT 50 cfu/g♦. It meets the requirements of the test for absence of *Escherichia coli*.

♦ PROTEIN AND LIGHT-ABSORBING IMPURITIES

(See *Spectrophotometry and Light-Scattering* <851>.)

Sample solution: 1% solution (w/v)

Instrumental conditions

Mode: UV

Wavelength range: 210–300 nm

Acceptance criteria: NMT 0.25 for the absorbance divided by the path length in centimeters at 210–220 nm; NMT 0.07 for the absorbance divided by the path length in centimeters at 270–300 nm

♦ ACIDITY OR ALKALINITY

Sample solution: Dissolve 6 g by heating in 25 mL of carbon dioxide-free water, cool, and add 0.3 mL of phenolphthalein TS.

Acceptance criteria: The solution is colorless, and NMT 0.4 mL of 0.1 N sodium hydroxide is required to produce a pink or red color.

♦ OPTICAL ROTATION, *Specific Rotation* <781S>

Sample solution: Dissolve 10 g by heating in 80 mL of water to 50°. Allow to cool, and add 0.2 mL of 6 N ammonium hydroxide. Allow to stand for 30 min, and dilute with water to 100 mL.

Acceptance criteria: +54.4° to +55.9°, calculated on the anhydrous basis, at 20°

ADDITIONAL REQUIREMENTS

- ♦ **PACKAGING AND STORAGE:** Preserve in tight containers.
- ♦ **LABELING:** Where the labeling indicates the relative quantities of alpha- and beta-lactose, determine compliance using *Content of Alpha and Beta Anomers*.

¹ Restek Guard column is suitable.

² Varian CP-Sil 8 CB is suitable.

Where the labeling states the particle size distribution, it also indicates the d_{10} , d_{50} , and d_{90} values and the range for each.

- **USP REFERENCE STANDARDS** <11>
USP Dextrose RS
USP Fructose RS
USP Anhydrous Lactose RS
USP Sucrose RS.

Lactose Monohydrate

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (♦) to specify this fact.

DEFINITION

Lactose Monohydrate is the monohydrate of *O*- β -D-galactopyranosyl-(1 \rightarrow 4)- α -D-glucopyranose. [NOTE—Lactose Monohydrate may be modified as to its physical characteristics. It may contain varying proportions of amorphous lactose.]

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>
- ♦ **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** <201>
Diluent: Methanol and water (3:2)
Standard solution A: 0.5 mg/mL of USP Lactose Monohydrate RS in Diluent
Standard solution B: 0.5 mg/mL each of USP Dextrose RS, USP Lactose Monohydrate RS, USP Fructose RS, and USP Sucrose RS in Diluent
Sample solution: 0.5 mg/mL of Lactose Monohydrate in Diluent
Adsorbent: 0.25-mm layer of chromatographic silica gel
Application volume: 2 μ L
Developing solvent system: Ethylene dichloride, glacial acetic acid, methanol, and water (10:5:3:2)
Spray reagent: 5 mg/mL of thymol in a mixture of alcohol and sulfuric acid (19:1)
Analysis
Samples: Standard solution A, Standard solution B, and Sample solution
Allow the spots to dry, and develop the plate in a paper-lined chromatographic chamber equilibrated with Developing solvent system for about 1 h prior to use. Allow the chromatogram to develop until the solvent front has moved about three-quarters of the length of the plate. Remove the plate from the chamber, dry in a current of warm air, and redevelop the plate in fresh Developing solvent system. Remove the plate from the chamber, mark the solvent front, and dry the plate in a current of warm air. Spray the plate evenly with Spray reagent. Heat the plate at 130° for 10 min.
System suitability: The test is not valid unless the chromatogram of Standard solution B shows four clearly discernible spots, disregarding any spots at the origin.
Acceptance criteria: The principal spot from the Sample solution corresponds in appearance and R_f value to that from Standard solution A.

IMPURITIES

- **RESIDUE ON IGNITION** <281>
Analysis: A sample ignited at a temperature of 600 \pm 50°
Acceptance criteria: NMT 0.1%

- ♦ **HEAVY METALS** <231>
Sample solution: 4 g in 20 mL of warm water. Add 1 mL of 0.1 N hydrochloric acid, and dilute with water to 25 mL.
Acceptance criteria: NMT 5 μ g/g.

SPECIFIC TESTS

- **CLARITY AND COLOR OF SOLUTION**
Sample solution: 1 g in 10 mL of boiling water
Analysis: The Sample solution is clear and nearly colorless. Determine the absorbance of this solution at a wavelength of 400 nm.
Acceptance criteria: The absorbance divided by the path length, in cm, is NMT 0.04.
- ♦ **MICROBIAL ENUMERATION TESTS** <61> and **TESTS FOR SPECIFIED MICROORGANISMS** <62>: The total aerobic microbial count does not exceed 1×10^2 cfu/g, the total combined molds and yeasts count does not exceed 5×10^1 cfu/g, and it meets the requirements of the test for absence of *Escherichia coli*.
- **OPTICAL ROTATION, Specific Rotation** <781S>
Sample solution: Dissolve 10 g by heating in 80 mL of water to 50°. Allow to cool, and add 0.2 mL of 6 N ammonium hydroxide. Allow to stand for 30 min, and dilute with water to 100 mL.
Acceptance criteria: +54.4° to +55.9°, calculated on the anhydrous basis, determined at 20°
- **ACIDITY OR ALKALINITY**
Sample solution: Dissolve 6 g by heating in 25 mL of carbon dioxide-free water, cool, and add 0.3 mL of phenolphthalein TS.
Acceptance criteria: The solution is colorless, and NMT 0.4 mL of 0.1 N sodium hydroxide VS is required to produce a pink or red color.
- ♦ **LOSS ON DRYING** <731>
Analysis: Dry a sample at 80° for 2 h.
Acceptance criteria
Monohydrate: NMT 0.5%
Monohydrate, modified: NMT 1.0%.
- **WATER DETERMINATION, Method I** <921>
Sample solution: Prepare a preparation containing Lactose Monohydrate in a mixture of methanol and formamide (2:1).
Acceptance criteria: 4.5%–5.5%
- **PROTEIN AND LIGHT-ABSORBING IMPURITIES** (See Spectrophotometry and Light-Scattering <851>.)
Sample solution: 1% (w/v)
Analysis: Measure the light absorption of the Sample solution in the range of 210–300 nm.
Acceptance criteria: The absorbance divided by the path length, in cm, is NMT 0.25 in the range of 210–220 nm and is NMT 0.07 in the range of 270–300 nm.

ADDITIONAL REQUIREMENTS

- ♦ **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Where the labeling states the particle size distribution, it also indicates the d_{10} , d_{50} , and d_{90} values and the range for each. For modified Lactose Monohydrate, also label it to indicate the method of modification.
- **USP REFERENCE STANDARDS** <11>
USP Dextrose RS
USP Fructose RS
USP Lactose Monohydrate RS
USP Sucrose RS.

Lanolin, Anhydrous—see *Lanolin General Monographs*

Lanolin, Modified—see Modified Lanolin General Monographs

Lanolin Alcohols

[8027-33-6].

DEFINITION

Lanolin Alcohols is a mixture of aliphatic alcohols, triterpenoid alcohols, and sterols, obtained by the hydrolysis of Lanolin. It may contain NMT 0.1% of a suitable antioxidant.

IDENTIFICATION

- **A.**
Sample: 0.5 g
Analysis: Dissolve the *Sample* in 5 mL of chloroform, and add 1 mL of acetic anhydride and 2 drops of sulfuric acid.
Acceptance criteria: A green color is produced.

ASSAY

- **CONTENT OF STEROLS** (as cholesterol)
Sample: 20 g
Analysis: Melt the *Sample* on a water bath, mix, and allow to cool. Dissolve 100 mg in 12 mL of warm (60°) 90% alcohol. Allow to stand for 18 h, pass through a medium-porosity, sintered-glass filter, and wash the residue with two 15-mL portions of 90% alcohol. Combine the filtrate and washings, add 20 mL of a freshly prepared 1-in-100 solution of digitonin in 90% alcohol, and warm to 60°. Allow to cool, pass through a medium-porosity, sintered-glass filter with the aid of gentle vacuum, wash the residue with 10 mL of 90% alcohol, and dry at 105° to constant weight. Each g of residue is equivalent to 0.239 g of cholesterol.
Acceptance criteria: NLT 30.0% of sterols, calculated as cholesterol

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.15%
- **COPPER**
Solution A: 1 mg/mL of sodium diethyldithiocarbamate
Sample: 5.0 g
Control: Add 1 mL of *Solution A* and a few drops of 6 N ammonium hydroxide to 2.5 mL of a 39.3-ppm solution of cupric sulfate. Dilute with water to 50 mL.
Analysis: Heat the *Sample* over a small flame until charred, ignite the residue at 550°, and dissolve the ash in 5 mL of hydrochloric acid, with the aid of heat. Cool, dilute with water, render alkaline with ammonium hydroxide, boil to remove the excess ammonia, add a few drops of bromine TS, boil again, and filter. To the filtrate add 1 mL of *Solution A*, a few drops of 6 N ammonium hydroxide, and sufficient water to bring the volume to 50 mL.
Acceptance criteria: The *Sample* is not darker than the *Control* (5 ppm).

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE**, *Class II* (741): NLT 56°
- **ACIDITY AND ALKALINITY**
Analysis: Boil 10 g with 100 mL of water for 5 min, with frequent stirring. Remove the source of heat, add 0.5 mL of phenolphthalein TS, and stir.
Acceptance criteria: No pink color is produced. Add 0.5 mL of methyl orange TS, and stir: no red color is produced.

- **LOSS ON DRYING** (731)
Analysis: Dry at 105° for 1 h.
Acceptance criteria: NMT 0.5%
- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 2.0.
- **FATS AND FIXED OILS**, *Hydroxyl Value* (401): 120–180
- **FATS AND FIXED OILS**, *Peroxide Value* (401)
Sample: Take wedge-shaped pieces with bases that contain part of the surface.
Analysis: Melt the pieces before carrying out the determination. Before adding the 0.5 mL of saturated potassium iodide solution, cool the solution obtained to room temperature.
Acceptance criteria: NMT 15
- **FATS AND FIXED OILS**, *Saponification Value* (401)
Sample: 5 g of molten Lanolin Alcohols
Analysis: Reflux the *Sample* with the alcoholic potassium hydroxide for 4 h.
Acceptance criteria: NMT 12

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers, and store at controlled room temperature.
- **LABELING:** Label it to indicate the name and quantity of any antioxidant added.

Lauroyl Polyoxylglycerides

DEFINITION

Lauroyl Polyoxylglycerides is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. The polyethylene glycols used have a mean molecular weight between 300 and 1500. The article is produced by partial alcoholysis of saturated oils, mainly containing triglycerides of lauric acid with polyethylene glycols, by esterification of glycerol and polyethylene glycols with fatty acids, or as a mixture of glycerol esters and ethylene oxide condensate with the fatty acids of the hydrogenated oils. It may contain free polyethylene glycols.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** (201)
Standard solution: 50 mg/mL of USP Lauroyl Polyoxylglycerides RS in methylene chloride
Sample solution: 50 mg/mL of Lauroyl Polyoxylglycerides in methylene chloride
Application volume: 10 µL
Developing solvent system: Ether and hexanes (70:30)
Spray reagent: 0.1 mg/mL of rhodamine B in alcohol
Analysis
Samples: *Standard solution* and *Sample solution*
 Proceed as directed in the chapter. Then spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.
Acceptance criteria: The *R_f* values of the principal spots from the *Sample solution* correspond to those from the *Standard solution*.
- **C.** It meets the requirements in *Specific Tests* (see Table 2) for *Fats and Fixed Oils*, *Fatty Acid Composition* (401).

IMPURITIES

- **HEAVY METALS**, *Method II* (231): NMT 10 µg/g
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 0.1%
- **ALKALINE IMPURITIES**
Sample: 5.0 g
Analysis: Heat the *Sample* slightly until the test substance melts, add 10 mL of alcohol and 0.05 mL of bro-

mophenol blue TS, and mix well. While the solution is still warm, titrate with 0.01 N hydrochloric acid VS to change the color to yellow.

Acceptance criteria: NMT 1.0 mL of 0.01 N hydrochloric acid is required.

• **LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE**

[CAUTION—Ethylene oxide is toxic and flammable. Prepare all solutions in a well-ventilated hood. The operator must protect hands and face by wearing polyethylene protective gloves and an appropriate face mask. Store all solutions in hermetic containers, and refrigerate at 4°–8°.]

[NOTE—Perform all determinations three times.]

Magnesium chloride solution: 500 mg/mL of magnesium chloride in alcohol

Ethylene oxide stock solution: Into a dry, clean test tube, cooled in a mixture of sodium chloride and crushed ice (1:3), introduce a slow current of ethylene oxide gas, allowing condensation onto the inner wall of the test tube. Using a glass syringe, previously cooled to –10°, transfer 300 µL of liquid ethylene oxide, equivalent to 0.25 g, to 50 mL of polyethylene glycol 200. Determine the absorbed quantity of ethylene oxide by weighing before and after absorption. Dilute with polyethylene glycol 200 to 100.0 mL.

Standardize this solution by transferring 10.0 mL of *Magnesium chloride solution* and 20.0 mL of 0.1 M alcoholic hydrochloric acid VS to a volumetric flask. Insert the stopper, shake to obtain a saturated solution, and allow to equilibrate overnight. Transfer 5.00 mL of *Ethylene oxide stock solution* to the flask, and allow to stand for 30 min. Titrate with 0.1 M alcoholic potassium hydroxide VS. Perform a blank titration, using the same quantity of polyethylene glycol 200 instead of *Ethylene oxide stock solution*, and note the difference in volumes required. Each mL of the difference in volumes of 0.1 M alcoholic potassium hydroxide VS consumed is equivalent to 4.404 mg of ethylene oxide. Calculate the concentration of ethylene oxide in the *Ethylene oxide stock solution*.

Ethylene oxide solution: Prepare immediately before use. Dilute a volume of *Ethylene oxide stock solution* with polyethylene glycol 200 to obtain a solution containing about 50 µg/g of ethylene oxide. Dilute 1.0 mL of this solution with water to 5.0 mL to obtain a solution having a concentration of 10 µg/mL of ethylene oxide.

Dioxane solution: 0.5 mg/mL of dioxane

Standard solution A: Transfer 1.0 g of Lauroyl Polyoxylglycerides to a 10-mL vial, and add 1.0 mL of *N,N*-dimethylacetamide, 0.1 mL of *Ethylene oxide solution*, and 0.1 mL of *Dioxane solution*. Close the vial, and mix to obtain a homogeneous solution. Allow to stand at 90° for 45 min.

Standard solution B: Transfer 0.1 mL of *Ethylene oxide solution* to a 10-mL vial, add 0.1 mL of a freshly prepared 10 mg/L of acetaldehyde solution, and add 0.1 mL of *Dioxane solution*. Close the vial, and mix to obtain a homogeneous solution.

Sample solution: Transfer 1.0 g of Lauroyl Polyoxylglycerides to a 10-mL vial, and add 1.0 mL of *N,N*-dimethylacetamide and 0.2 mL of water. Close the vial, and mix to obtain a homogeneous solution. Allow to stand at 90° for 45 min.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

[NOTE—Headspace apparatus that automatically transfers a measured amount of headspace may be used.]

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m glass or quartz capillary, bonded with a 1.0-µm layer of phase G1

Temperatures

Injection port: 150°

Detector: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	—
180	30	230	5

Carrier gas: Helium

Flow rate: 1 mL/min

Injection volume: 1 mL

System suitability

Sample: Gaseous phase of *Standard solution B*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are about 0.94 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between acetaldehyde and ethylene oxide

Relative standard deviation: NMT 15.0%

Analysis

Samples: *Standard solution A* and *Sample solution*

Using a heated, gas-tight gas chromatographic syringe, separately inject equal volumes of the gaseous headspace of *Standard solution A* and of the *Sample solution* into the chromatograph, record the chromatograms, and measure the peak responses. **[NOTE—**Adjust the sensitivity of the system so that the heights of the two principal peaks are NLT 15% of the full scale of the recorder.]

Calculate the concentration of ethylene oxide in the sample taken:

$$\text{Result} = \{(C \times r_U) / [(r_S \times M_U) - (r_U \times M_S)]\}$$

C = concentration of ethylene oxide in *Standard solution A* (µg/mL)

r_U = peak response of ethylene oxide from the *Sample solution*

r_S = peak response of ethylene oxide from *Standard solution A*

M_U = quantity of Lauroyl Polyoxylglycerides taken to prepare the *Sample solution* (g)

M_S = quantity of Lauroyl Polyoxylglycerides taken to prepare *Standard solution A* (g)

Calculate the concentration of dioxane in the sample taken:

$$\text{Result} = C_D \times d_U / 5 \times [(d_S \times M_U) - (d_U \times M_S)]$$

C_D = concentration of dioxane in *Standard solution A* (µg/mL)

d_U = peak response of dioxane from the *Sample solution*

d_S = peak response of dioxane from *Standard solution A*

M_U = quantity of Lauroyl Polyoxylglycerides taken to prepare the *Sample solution* (g)

M_S = quantity of Lauroyl Polyoxylglycerides taken to prepare *Standard solution A* (g)

Acceptance criteria

Ethylene oxide: NMT 1 µg/g; the peak area of ethylene oxide from the *Sample solution* is NMT half the corresponding peak area from *Standard solution A*.

Dioxane: NMT 10 µg/g; the peak area of dioxane from the *Sample solution* is NMT half the corresponding peak area from *Standard solution A*.

• **LIMIT OF FREE GLYCEROL**

Sample: 1.2 g

Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, diluting to 100.0 mL with glacial acetic acid.

Potassium iodide solution: 75 mg/mL of potassium iodide

Blank: 25 mL of methylene chloride

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 M sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 25 mL of methylene chloride, heating if necessary. Cool, and add 100 mL of water and 25.0 mL of *Periodic acetic acid solution*. Shake, and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with 0.1 M sodium thiosulfate VS. Perform a blank determination, and make any necessary correction.

Calculate the percentage of glycerol in the sample taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = Titrant volume consumed by the *Sample* (mL)

V_B = Titrant volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 23.0 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS

• **FATS AND FIXED OILS, Acid Value (401)**

Sample: 2.0 g

Acceptance criteria: NMT 2.0

• **FATS AND FIXED OILS, Fatty Acid Composition (401):** Lauroyl Polyoxylglycerides exhibits the composition profile of fatty acids shown in *Table 2*.

Table 2

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
8	0	≤15.0
10	0	≤12.0
12	0	30.0–50.0
14	0	5.0–25.0
16	0	4.0–25.0
18	0	5.0–35.0

• **FATS AND FIXED OILS, Hydroxyl Value (401)**

Sample: 1.0 g

Acceptance criteria: Within the range specified in *Table 3* for the labeled type

Table 3

Type of Polyethylene Glycols	Hydroxyl Value
300	65–85
400	60–80
600	50–70
1500	36–56

• **FATS AND FIXED OILS, Iodine Value (401):** NMT 2.0

• **FATS AND FIXED OILS, Peroxide Value (401)**

Sample: 2.0 g

Acceptance criteria: NMT 6.0

• **FATS AND FIXED OILS, Saponification Value (401)**

Sample: 2.0 g

Acceptance criteria: Within the range specified in *Table 4* for the labeled type

Table 4

Type of Polyethylene Glycols	Saponification Value
300	190–204
400	170–190
600	150–170
1500	79–93

• **WATER DETERMINATION, Method I (921)**

Sample: 1.0 g

Analysis: Instead of using methanol as the solvent, one of two solvent systems can be used: a mixture of methylene chloride and anhydrous methanol (70:30 v/v), or anhydrous pyridine.

Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at controlled room temperature.

• **LABELING:** Label it to indicate the type and the average nominal molecular weight of polyethylene glycols used as part of the official title.

• **USP REFERENCE STANDARDS (11)**

USP Lauroyl Polyoxylglycerides RS

Lecithin

[8002-43-5].

DEFINITION

Lecithin is a complex mixture of acetone-insoluble phosphatides, which consist chiefly of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol, combined with various amounts of other substances such as triglycerides, fatty acids, and carbohydrates, as separated from the crude vegetable oil source. It contains NLT 50.0% of acetone-insoluble matter.

IDENTIFICATION

• **A.**

Sample: 1 g of Lecithin

Analysis: Transfer the *Sample* to a Kjeldahl flask, add 5 g of potassium sulfate, 0.5 g of cupric sulfate, and 20 mL of sulfuric acid. Incline the flask to a 45° angle, heat gently until the effervescence almost ceases, and raise the temperature to boiling. After the contents become a blue, transparent solution, heat for 1–2 h, cool, and add an equal volume of water. To 5 mL of this solution, add 10 mL of ammonium molybdate solution (1 in 5), and heat.

Acceptance criteria: A yellow precipitate is formed.

• **B. PAPER CHROMATOGRAPHY**

Standard solution: Choline chloride solution (1 in 200) using USP Choline Chloride RS

Sample solution: To 0.5 g of Lecithin, add 5 mL of hydrochloric acid solution (1 in 2), heat in a water bath for 2 h, and filter.

Chromatographic system

(See *Chromatography* (621), *Paper Chromatography*.)

Mode: Paper

Developing solvent system: *n*-Butanol, water, and acetic acid (4:2:1)

Application volume: 10 µL

Spray reagent: Dragendorff's TS

Analysis

Samples: *Standard solution* and *Sample solution*

Use a suitable cellulose filter paper for chromatography. Develop the chromatogram over a path of 25 cm, and dry the paper in a current of air. Spray the paper with

Spray reagent to develop a red-orange color, and locate the spots on the paper by examination under daylight.

Acceptance criteria: The R_f value of the principal spot from the *Sample solution* corresponds to that from the *Standard solution*.

ASSAY

• CONTENT OF ACETONE-INSOLUBLE MATTER

Sample: If the substance under test is plastic or semi-solid, soften the Lecithin by warming it briefly at a temperature not exceeding 60°, and then mix. Transfer 2 g to a 40-mL centrifuge tube that previously has been tared along with a stirring rod, cool, and weigh.

Analysis: To the *Sample* add 15.0 mL of acetone, warm carefully in a water bath to melt the test specimen without evaporating the acetone. Stir to help dissolve completely, and place in an ice-water bath for 5 min. Add acetone that has been previously chilled to 0°–5° to the 40-mL mark on the tube, stirring during the addition. Cool in an ice-water bath for 15 min, stir, remove the rod, clarify by centrifuging at about 2000 rpm for 5 min, and decant. Break up the residue with the stirring rod, and refill the centrifuge tube to the 40-mL mark with chilled acetone, while stirring. Cool in an ice-water bath for 15 min, stir, remove the rod, centrifuge, and decant. Break up the residue with the stirring rod. Place the tube in a horizontal position until most of the acetone has evaporated. Mix again, and heat the tube containing the acetone-insoluble residue and the stirring rod at 105° to constant weight.

[**CAUTION**—Acetone is flammable.]

Determine the weight of the residue, and calculate the percentage of acetone-insoluble matter.

Acceptance criteria: NLT 50.0%

IMPURITIES

• **HEAVY METALS**, *Method II* (231): NMT 20 ppm

• **LEAD** (251): NMT 10 ppm

• HEXANE-INSOLUBLE MATTER

Sample: If the substance under test is plastic or semi-solid, soften the Lecithin by warming it at a temperature not exceeding 60°, and then mix. Weigh 10.0 g into a 250-mL conical flask.

Analysis: To the *Sample* add 100 mL of hexanes. Shake until solution is apparently complete or until no more residue seems to be dissolving. Pass through a coarse-porosity filtering funnel that previously has been heated at 105° for 1 h, cooled, and weighed, wash the flask with two 25-mL portions of hexanes, and pour both washings through the funnel. Dry the funnel at 105° for 1 h. [**CAUTION**—Hexane is flammable.] Cool to room temperature, and determine the gain in weight.

Acceptance criteria: NMT 0.3%

SPECIFIC TESTS

• FATS AND FIXED OILS, *Acid Value* (401)

Sample: If the substance under test is plastic or semi-solid, soften the Lecithin by warming it briefly at a temperature not exceeding 60°, and then mix. Transfer 2 g to a 250-mL conical flask.

Analysis: Dissolve the *Sample* in 50 mL of petroleum ether. To this solution add 50 mL of alcohol, previously neutralized to phenolphthalein with 0.1 N sodium hydroxide, and mix. Add phenolphthalein TS. Titrate with 0.1 N sodium hydroxide VS to a pink endpoint that persists for 5 s.

Calculate the amount, in mg, of potassium hydroxide required to neutralize the free acids in 1.0 g of the Lecithin:

$$\text{Result} = (M_R \times N \times V) / W$$

M_R = molecular mass of potassium hydroxide, 56.11

N = normality of the sodium hydroxide VS

V = volume of the sodium hydroxide VS consumed in the titration (mL)

W = weight of Lecithin taken (g)

Acceptance criteria: NMT 36

Change to read:

• PEROXIDE VALUE

Sample: 5 g of Lecithin

Analysis: Transfer the *Sample* into a 250-mL Erlenmeyer flask with a ground-glass stopper, add 35 mL of a mixture of chloroform and glacial acetic acid (2:1), • (ERR 1-May-2012) and mix. Completely dissolve the test specimen while shaking gently. The solution becomes transparent. Completely replace the air in the flask with nitrogen. While purging with nitrogen, add 1 mL of potassium iodide solution (165 mg/mL of potassium iodide), then stop the flow of the nitrogen, and immediately place a stopper in the flask. Shake for 1 min, and allow to stand in a dark place for 5 min. Add 75 mL of water, replace the stopper again, and shake vigorously. Titrate with 0.01 N sodium thiosulfate VS, adding starch TS as the endpoint is approached, and continue the titration until the blue starch color has just disappeared. Perform a blank determination (see *Titrimetry* (541)), and make any necessary correction. Calculate the peroxide value, as milliequivalents of peroxide per 1000 g of Lecithin:

$$\text{Result} = (S \times N / W) \times 1000$$

S = net volume of sodium thiosulfate solution required for titration (mL)

N = normality of the sodium thiosulfate solution

W = weight of Lecithin taken (g)

Acceptance criteria: NMT 10

• WATER DETERMINATION, *Method I* (921): NMT 1.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers. Store at the temperature indicated on the label. Protect from excess heat and moisture.
- **LABELING:** Label it to indicate the storage conditions.
- **USP REFERENCE STANDARDS** (11)
USP Choline Chloride RS

Lemon Oil

DEFINITION

Lemon Oil is the volatile oil obtained by expression, without the aid of heat, from the fresh peel of the fruit of *Citrus x limon* (L.) Osbeck (Fam. Rutaceae), with or without the previous separation of the pulp and the peel. The total aldehyde content, calculated as citral ($C_{10}H_{16}O$), is NLT 2.2% and NMT 3.8% for California-type Lemon Oil, and NLT 3.0% and NMT 5.5% for Italian-type Lemon Oil. [NOTE—Do not use Lemon Oil that has a terebinthine odor.]

ASSAY

• TOTAL ALDEHYDE CONTENT

Reagent solution: Dissolve 4.5 g of hydroxylamine hydrochloride in 13 mL of water. Add 85 mL of tertiary butyl alcohol, mix, and adjust with 0.5 N potassium hydroxide to a pH of 3.4.

Sample: 5 mL

Analysis: Pipet 50 mL of the *Reagent solution* into a conical flask containing the *Sample*. Insert the stopper in the flask, and allow to stand at room temperature for

30 min, with occasional shaking. Titrate the liberated hydrochloric acid with 0.5 N alcoholic potassium hydroxide VS to a pH of 3.4. Each mL of 0.5 N alcoholic potassium hydroxide consumed in the titration is equivalent to 76.12 mg of total aldehydes, calculated as citral (C₁₀H₁₆O).

Acceptance criteria: The total aldehyde content, calculated as citral (C₁₀H₁₆O), is 2.2%–3.8% for California-type Lemon Oil or 3.0%–5.5% for Italian-type Lemon Oil.

IMPURITIES

- **HEAVY METALS, Method II** <231>: NMT 40 µg/g

SPECIFIC TESTS

- **SPECIFIC GRAVITY** <841>: 0.849–0.855
- **OPTICAL ROTATION, Angular Rotation** <781A>: +57° to +65.6°
- **REFRACTIVE INDEX** (831): 1.473–1.476 at 20°
- **ULTRAVIOLET ABSORBANCE**

Sample solution: Dilute 250 mg of Oil to 100 mL with alcohol

Blank: Alcohol

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: UV-Vis

Spectral range: 260–400 nm

Analysis

Samples: *Sample solution* and *Blank*

Record the spectrum in a 1-cm cell, and determine the absorbance at the wavelength of maximum absorbance at about 315 nm using the line drawn tangent to the curves appearing as minima in the spectrum in wavelength regions above and below the maximum wavelength as the baseline.

Acceptance criteria: The absorbance, calculated on the basis of a 250-mg specimen, is NLT 0.20 for California-type Lemon Oil or NLT 0.49 for Italian-type Lemon Oil.

- **FOREIGN OILS:** Place 50 mL of Oil in a four-bulb Ladenburg flask having the following dimensions: the lower or main bulb is about 6 cm in diameter, and the smaller condensing bulbs are about 3.5, 3.0, and 2.5 cm in diameter; the distance from the bottom of the flask to the side-arm is about 20 cm. Distill Oil at a rate of 1 drop/s until the distillate measures 5 mL: the angular rotation of the first 5 mL is NMT 6° less than that of the original Oil. The refractive index at 20° of this same portion is 0.001–0.003 lower than that of the original Oil.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers, and avoid exposure to excessive heat.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived. Label it to also indicate whether it is California-type or Italian-type Lemon Oil. The label indicates that Oil is not to be used if it has a terebinthine odor.

Lemon Tincture

DEFINITION

Lemon Tincture is prepared from lemon peel, which is the outer yellow rind of the fresh, ripe fruit of *Citrus × Limon* Osbeck (Fam. Rutaceae).

Prepare Lemon Tincture as follows.

Lemon Peel	500 g
Alcohol	900 mL
Alcohol, a sufficient quantity to make	1000 mL

Macerate the *Lemon Peel* in 900 mL of *Alcohol* in a closed container, and store in a warm place. Agitate the container frequently for 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter, using talc as the filtering medium, and when most of the liquid has drained away, wash the residue on the filter with a sufficient amount of *Alcohol*, and combine the filtrates so that the preparation is brought to a final volume of 1000 mL.

OTHER COMPONENTS

- **ALCOHOL DETERMINATION, Method I** <611>: 62%–72% of the labeled amount

IMPURITIES

- **HEAVY METALS, Method II** <231>: NMT 40 µg/mL

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight, light-resistant containers, and avoid exposure to direct sunlight and to excessive heat. Store at controlled room temperature.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.

Licorice Fluidextract

DEFINITION

Prepare Licorice Fluidextract as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* <795>). To 1000 g of coarsely ground Licorice, add about 3000 mL of boiling Purified Water, mix, and allow to macerate in a suitable, covered percolator for 2 h. Allow the percolation to proceed at a rate of 1–3 mL/min, gradually adding boiling Purified Water until the Licorice is exhausted. Add enough diluted ammonia solution to the percolate to impart a distinctly ammoniacal odor, and boil the liquid actively under normal atmospheric pressure until it is reduced in volume to about 1500 mL. Filter the liquid, evaporate the filtrate on a steam bath until the residue measures 750 mL, cool, gradually add 250 mL of Alcohol and enough Purified Water to make the product measure 1000 mL, and mix.

OTHER COMPONENTS

- **ALCOHOL CONTENT, Method I** <611>: 20.0%–24.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and avoid exposure to direct sunlight and to excessive heat.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived.

Linoleoyl Polyoxylglycerides

DEFINITION

Linoleoyl Polyoxylglycerides is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. The polyethylene glycols used have a mean molecular weight between 300 and 400. The article is produced by partial alcoholysis of unsaturated oils, mainly containing triglycerides of linoleic acid with polyethylene glycol, by esterification of glycerol and polyethylene glycol with fatty acids, or as a mixture of glycerol esters and ethylene oxide condensate with the fatty acids of the unsaturated oils. It may contain free polyethylene glycols.

IDENTIFICATION• **A. INFRARED ABSORPTION** (197F)• **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** (201)

Standard solution: 50 mg/mL of USP Linoleoyl Polyoxylglycerides RS in methylene chloride

Sample solution: 50 mg/mL of Linoleoyl Polyoxylglycerides in methylene chloride

Application volume: 10 µL

Developing solvent system: Ether and hexanes (70:30)

Spray reagent: 0.1 mg/mL of rhodamine B in alcohol Analysis

Samples: *Standard solution* and *Sample solution*

Proceed as directed in the chapter. Then spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The R_f values of the principal spots from the *Sample solution* correspond to those from the *Standard solution*.

• **C.** It meets the requirements in *Specific Tests* (see *Table 2*) for *Fats and Fixed Oils, Fatty Acid Composition* (401).**IMPURITIES**• **HEAVY METALS, Method II** (231): NMT 10 µg/g• **ARTICLES OF BOTANICAL ORIGIN, Total Ash** (561): NMT 0.1%• **ALKALINE IMPURITIES**

Sample: 5.0 g

Analysis: To the *Sample* add 10 mL of alcohol and 0.05 mL of bromophenol blue TS, and mix well. Titrate with 0.01 N hydrochloric acid VS to change the color to yellow.

Acceptance criteria: NMT 1.0 mL of 0.01 N hydrochloric acid is required.

• **LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE**

[CAUTION—Ethylene oxide is toxic and flammable. Prepare all solutions in a well-ventilated hood. The operator must protect hands and face by wearing polyethylene protective gloves and an appropriate face mask. Store all solutions in hermetic containers, and refrigerate at 4°–8°.]

[NOTE—Perform all determinations three times.]

Magnesium chloride solution: 500 mg/mL of magnesium chloride in alcohol

Ethylene oxide stock solution: Into a dry, clean test tube, cooled in a mixture of sodium chloride and crushed ice (1:3), introduce a slow current of ethylene oxide gas, allowing condensation onto the inner wall of the test tube. Using a glass syringe, previously cooled to –10°, transfer 300 µL of liquid ethylene oxide, equivalent to 0.25 g, to 50 mL of polyethylene glycol 200. Determine the absorbed quantity of ethylene oxide by weighing before and after absorption. Dilute with polyethylene glycol 200 to 100.0 mL.

Standardize this solution by transferring 10.0 mL of *Magnesium chloride solution* and 20.0 mL of 0.1 M alcoholic hydrochloric acid VS to a volumetric flask. Insert the stopper, shake to obtain a saturated solution, and allow to equilibrate overnight. Transfer 5.00 mL of *Ethylene oxide stock solution* to the flask, and allow to stand for 30 min. Titrate with 0.1 M alcoholic potassium hydroxide VS. Perform a blank titration, using the same quantity of polyethylene glycol 200 instead of *Ethylene oxide stock solution*, and note the difference in volumes required. Each mL of the difference in volumes of 0.1 M alcoholic potassium hydroxide VS consumed is equivalent to 4.404 mg of ethylene oxide. Calculate the concentration of ethylene oxide in the *Ethylene oxide stock solution*.

Ethylene oxide solution: Prepare immediately before use. Dilute a volume of *Ethylene oxide stock solution* with polyethylene glycol 200 to obtain a solution containing about 50 µg/g of ethylene oxide. Dilute 1.0 mL of this solution with water to 5.0 mL to obtain a solution hav-

ing a known concentration of 10 µg/mL of ethylene oxide.

Dioxane solution: 0.5 mg/mL of dioxane

Standard solution A: Transfer 1.0 g of Linoleoyl Polyoxylglycerides to a 10-mL vial, and add 1.0 mL of *N,N*-dimethylacetamide, 0.1 mL of *Ethylene oxide solution*, and 0.1 mL of *Dioxane solution*. Close the vial, and mix to obtain a homogeneous solution. Allow to stand at 90° for 45 min.

Standard solution B: Transfer 0.1 mL of *Ethylene oxide solution* to a 10-mL vial, add 0.1 mL of a freshly prepared 10 mg/L of acetaldehyde solution, and add 0.1 mL of *Dioxane solution*. Close the vial, and mix to obtain a homogeneous solution.

Sample solution: Transfer 1.0 g of Linoleoyl Polyoxylglycerides to a 10-mL vial, and add 1.0 mL of *N,N*-dimethylacetamide and 0.2 mL of water. Close the vial, and mix to obtain a homogeneous solution. Allow to stand at 90° for 45 min.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

[NOTE—Headspace apparatus that automatically transfers a measured amount of headspace may be used.]

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m glass or quartz capillary, bonded with a 1.0-µm layer of phase G1

Temperatures

Injection port: 150°

Detector: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	—
180	30	230	5

Carrier gas: Helium

Flow rate: 1 mL/min

Injection volume: 1 mL

System suitability

Sample: Gaseous phase of *Standard solution B*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are about 0.94 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between acetaldehyde and ethylene oxide

Relative standard deviation: NMT 15.0%

Analysis

Samples: *Standard solution A* and *Sample solution*

Using a heated, gas-tight gas chromatographic syringe, separately inject equal volumes of the gaseous headspace of *Standard solution A* and of the *Sample solution* into the chromatograph, record the chromatograms, and measure the peak responses. **[NOTE—Adjust the sensitivity of the system so that the heights of the two principal peaks are NLT 15% of the full scale of the recorder.]**

Calculate the concentration of ethylene oxide in the sample taken:

$$\text{Result} = \{(C \times r_U) / [(r_S \times M_U) - (r_U \times M_S)]\}$$

C = concentration of ethylene oxide in *Standard solution A* (µg/mL)

r_U = peak response of ethylene oxide from the *Sample solution*

r_S = peak response of ethylene oxide from *Standard solution A*

M_U = quantity of Linoleoyl Polyoxylglycerides taken to prepare the *Sample solution* (g)
 M_S = quantity of Linoleoyl Polyoxylglycerides taken to prepare *Standard solution A* (g)
 Calculate the concentration of dioxane in the sample taken:

$$\text{Result} = C_D \times d_U/5 \times [(d_S \times M_U) - (d_U \times M_S)]$$

C_D = concentration of dioxane in *Standard solution A* ($\mu\text{g/mL}$)
 d_U = peak response of dioxane from the *Sample solution*
 d_S = peak response of dioxane from *Standard solution A*
 M_U = quantity of Linoleoyl Polyoxylglycerides taken to prepare the *Sample solution* (g)
 M_S = quantity of Linoleoyl Polyoxylglycerides taken to prepare *Standard solution A* (g)

Acceptance criteria

Ethylene oxide: NMT 1 $\mu\text{g/g}$; the peak area of ethylene oxide from the *Sample solution* is NMT half the corresponding peak area from *Standard solution A*.

Dioxane: NMT 10 $\mu\text{g/g}$; the peak area of dioxane from the *Sample solution* is NMT half the corresponding peak area from *Standard solution A*.

• LIMIT OF FREE GLYCEROL

Sample: 1.2 g

Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, diluting to 100.0 mL with glacial acetic acid.

Potassium iodide solution: 75 mg/mL of potassium iodide

Blank: 25 mL of methylene chloride

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 M sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 25 mL of methylene chloride, heating if necessary. Cool, and add 100 mL of water and 25.0 mL of *Periodic acetic acid solution*. Shake, and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with 0.1 M sodium thiosulfate VS. Perform a blank determination, and make any necessary correction.

Calculate the percentage of glycerol in the sample taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 23.0 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS

• FATS AND FIXED OILS, Acid Value <401>

Sample: 2.0 g

Acceptance criteria: NMT 2.0

• FATS AND FIXED OILS, Fatty Acid Composition <401>

Linoleoyl Polyoxylglycerides exhibits the composition profile of fatty acids shown in *Table 2*.

Table 2

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
16	0	4.0–20.0
18	0	≤6.0
18	1	20.0–35.0

Table 2 (Continued)

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
18	2	50.0–65.0
18	3	≤2.0
20	0	≤1.0
20	1	≤1.0

• FATS AND FIXED OILS, Hydroxyl Value <401>

Sample: 1.0 g

Acceptance criteria: 45–65

• FATS AND FIXED OILS, Iodine Value <401>

• FATS AND FIXED OILS, Peroxide Value <401>

Sample: 2.0 g

Acceptance criteria: NMT 12.0

• FATS AND FIXED OILS, Saponification Value <401>

Sample: 2.0 g

Acceptance criteria: 150–170

• WATER DETERMINATION, Method I <921>

Sample: 1.0 g

Analysis: Instead of using methanol as the solvent, one of two solvent systems can be used: a mixture of methylene chloride and anhydrous methanol (70:30 v/v), or anhydrous pyridine.

Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at controlled room temperature.

• **LABELING:** Label it to indicate the type and the average nominal molecular weight of polyethylene glycol used as part of the official title.

• USP REFERENCE STANDARDS <11>

USP Linoleoyl Polyoxylglycerides RS

Magnesium Aluminometasilicate

DEFINITION

Magnesium Aluminometasilicate is a synthetic material that exists in two forms, Type I-A and Type I-B, having different pH requirements. The required contents for both forms are the same: NLT 29.1% and NMT 35.5% of aluminum oxide (Al_2O_3), NLT 11.4% and NMT 14.0% of magnesium oxide (MgO), and NLT 29.2% and NMT 35.6% of silicon dioxide (SiO_2), calculated on the dried basis.

IDENTIFICATION

• A. IDENTIFICATION TESTS—GENERAL, Aluminum <191>

Sample solution: Transfer 0.5 g of Magnesium Aluminometasilicate to a suitable container, add 5 mL of a sulfuric acid solution (1 in 3), and heat until white fumes are observed. Cool, add 20 mL of water, and filter. Neutralize the filtrate with ammonia TS, and retain for use in *Identification test B*. Collect the precipitate, and dissolve in 3 N hydrochloric acid.

Acceptance criteria: The *Sample solution* meets the requirements.

• B. IDENTIFICATION TESTS—GENERAL, Magnesium <191>

Sample solution: Use the filtrate retained from *Identification test A*.

Acceptance criteria: The *Sample solution* meets the requirements.

• C.

Analysis: Prepare a bead by fusing a few crystals of sodium ammonium phosphate on a platinum loop in the flame of a Bunsen burner. Place the hot, transparent bead in contact with Magnesium Aluminometasilicate, and again fuse.

Acceptance criteria: Silica floats about in the bead producing, upon cooling, an opaque bead with a web-like structure.

ASSAY

• ALUMINUM OXIDE

Edetate disodium titrant: Prepare a solution with a concentration of 18.6 g/L of edetate disodium in water and standardize as follows. Weigh 2 g of aluminum wire, transfer to a 1000-mL volumetric flask, and add 50 mL of a mixture of hydrochloric acid and water (1:1). Swirl the flask to ensure contact of the aluminum and the acid, and allow the reaction to proceed until all of the aluminum has dissolved. Dilute with water to volume. Pipet 10 mL of this solution into a 250-mL beaker and add, in the order named and with continuous stirring, 25.0 mL of *Edetate disodium titrant* and 20 mL of acetic acid–ammonium acetate buffer TS, and boil gently for 5 min. Cool, and add 50 mL of alcohol and 2 mL of dithizone TS. Titrate with 0.05 M zinc sulfate VS to a bright rose-pink color. Perform a blank determination, substituting 10 mL of water for the aluminum solution, and make any necessary correction. Calculate the molarity of the solution taken:

$$\text{Result} = W/(A_r \times V)$$

W = weight of aluminum in the portion of solution taken (mg)

A_r = atomic weight of aluminum, 26.98

V = volume of *Edetate disodium titrant* consumed (mL)

Sample solution: Transfer 1.25 g of Magnesium Aluminometasilicate to a conical flask, add 10 mL of 3 N hydrochloric acid and 50 mL of water, and heat on a water bath for 15 min. To this solution add 8 mL of hydrochloric acid, and heat on a water bath for 10 min. After cooling, transfer the solution to a 250-mL volumetric flask, rinse the conical flask with water, and add the washings to the volumetric flask. Dilute with water to volume. Centrifuge, and use the supernatant as the *Sample solution*. Retain a portion for use in the Assay for *Magnesium Oxide*.

Analysis: Transfer 20.0 mL of the *Sample solution* to a beaker and add 20.0 mL of *Edetate disodium titrant*. To this solution add 15 mL of acetic acid–ammonium acetate buffer TS and 20 mL of water, and boil for 5 min. After cooling, add 50 mL of alcohol and 2 mL of dithizone TS, and titrate with 0.05 M zinc sulfate VS until the color of the solution changes from green-violet to rose-pink. Perform a blank determination. Each mL of 0.05 M *Edetate disodium titrant* is equivalent to 2.5490 mg of Al_2O_3 .

Acceptance criteria: 29.1%–35.5% of aluminum oxide (Al_2O_3) on the dried basis

• MAGNESIUM OXIDE

Sample solution: Use the *Sample solution* prepared for use in the Assay for *Aluminum Oxide*.

Analysis: Transfer 50.0 mL of the *Sample solution* to a suitable container, add 50 mL of water and 25 mL of a triethanolamine solution (1 in 2), and shake well. Add 25 mL of ammonia–ammonium chloride buffer TS and 0.04 g of eriochrome black TS titration as the indicator. Titrate with 0.05 M edetate disodium VS until the red-purple color changes to blue and persists for 30 s. Each mL of 0.05 M edetate disodium VS is equivalent to 2.0152 mg of MgO.

Acceptance criteria: 11.4%–14.0% of magnesium oxide (MgO) on the dried basis

• SILICON DIOXIDE

Sample: 1 g

Analysis: To the *Sample* add 30 mL of 3 N hydrochloric acid, and evaporate on a water bath to dryness. Moisten the residue with hydrochloric acid, and

evaporate again on a water bath to dryness. To the residue add 8 mL of hydrochloric acid and 25 mL of hot water, and stir. Allow to stand, and then decant the supernatant through an ashless filter paper. To the residue in the container add 10 mL of hot water, stir, and decant the supernatant through the filter paper. Wash the residue in the container with three additional 10-mL portions of hot water, stir, and decant as described above. Treat the residue in the container with 50 mL of water, and heat on a water bath for 15 min. Filter, and rinse the residue on the filter paper with hot water until no precipitate is obtained when 1 mL of silver nitrate TS is added to 5 mL of the washing. Transfer the filter paper and its contents to a tared platinum crucible, heat to dryness, incinerate, and continue to heat at $800 \pm 25^\circ$ for 1 h. Cool, and weigh. Moisten the residue with 6 mL of hydrofluoric acid, evaporate to dryness, and ignite for 5 min. Cool, and weigh. The loss in weight represents the weight of silicon dioxide (SiO_2).

Acceptance criteria: 29.2%–35.6% of silicon dioxide (SiO_2) on the dried basis

IMPURITIES

• CHLORIDE AND SULFATE, *Chloride* (221)

Sample: A 20-mL portion of the diluted filtrate retained from the test for *Soluble Salts*

Control: 0.75 mL of 0.020 N hydrochloric acid

Acceptance criteria: NMT 0.053%; the *Sample* shows no more chloride than corresponds to the *Control*.

• CHLORIDE AND SULFATE, *Sulfate* (221)

Sample: A 2-mL portion of the diluted filtrate retained from the test for *Soluble Salts*

Control: 0.5 mL of 0.020 N sulfuric acid

Acceptance criteria: NMT 0.480%; the *Sample* shows no more sulfate than corresponds to the *Control*.

• ARSENIC, *Method I* (211): NMT 3 µg/g

• IRON (241)

Sample solution: To 0.11 g of Magnesium Aluminometasilicate add 8 mL of 2 N nitric acid, boil for 1 min, and cool. Dilute with water to 100 mL, and centrifuge. Dilute 30 mL of the supernatant with water to 45 mL.

Acceptance criteria: NMT 0.03%

• HEAVY METALS, *Method I* (231)

Test preparation: Transfer 2.67 g of Magnesium Aluminometasilicate to a suitable container, add 20 mL of water and 8 mL of hydrochloric acid, and evaporate to dryness on a water bath. To the residue add 5 mL of 1 N acetic acid and 20 mL of water, boil for 2 min, add 0.4 g of hydroxylamine hydrochloride, and heat to boiling. Cool, dilute with water to 100 mL, and filter. Use 25 mL of the filtrate as the *Test preparation*.

Monitor preparation: Transfer another 25 mL of the diluted filtrate to a suitable container, and add 2.0 mL of *Standard Lead Solution*.

Standard solution: Transfer 2 mL of hydrochloric acid to a suitable container, and evaporate to dryness on a water bath. To the residue add 2.0 mL of *Standard Lead Solution* and 0.1 g of hydroxylamine hydrochloride. Dilute with water to 25 mL.

Acceptance criteria: NMT 30 µg/g

SPECIFIC TESTS

• ACID-CONSUMING CAPACITY

Sample solution: Transfer 0.2 g of Magnesium Aluminometasilicate to a glass-stoppered flask, and add 100.0 mL of 0.1 N hydrochloric acid VS. Stopper the flask tightly, shake at $37 \pm 2^\circ$ for 1 h, and filter. Use the filtrate.

Analysis: Transfer 50.0 mL of the *Sample solution* to a beaker, and while stirring, titrate the excess hydrochloric acid with 0.1 N sodium hydroxide VS to attain a pH of 3.5. Perform a blank determination.

Acceptance criteria: NLT 210 mL of 0.1 N hydrochloric acid is consumed per g of Magnesium Aluminometasilicate, calculated on the dried basis.

• **pH** (791)

Sample: 2 g

Analysis: Add 50 mL of water to the *Sample*. While stirring, immerse the pH electrodes in the suspension, and after 2 min, record the pH.

Acceptance criteria

Type I-A: 6.5–8.5

Type I-B: 8.5–10.5

• **LOSS ON DRYING** (731)

Analysis: Dry at 110° for 7 h.

Acceptance criteria: NMT 20.0%

• **SOLUBLE SALTS**

Sample: 10.0 g

Analysis: Transfer the *Sample* to a suitable container, add 150 mL of water, and boil gently for 15 min, with shaking. After cooling, dilute with water to 150 mL, and centrifuge. Dilute 75 mL of the clear filtrate with water to 100 mL, and retain the diluted filtrate for use in the tests for *Alkalinity*, *Chloride*, and *Sulfate*. Evaporate 25 mL of the diluted filtrate on a water bath, and heat at 700° for 2 h.

Acceptance criteria: NMT 0.020 g (NMT 1.6%)

• **ALKALINITY**

Sample: A 20-mL portion of the diluted filtrate retained from the test for *Soluble Salts*

Analysis: Add 2 drops of phenolphthalein TS to the *Sample*.

Acceptance criteria: If a pink color is produced, NMT 0.50 mL of 0.1 N hydrochloric acid is required to discharge it.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and prevent exposure to excessive heat.
- **LABELING:** Label it to indicate whether it is Type I-A or Type I-B.

Magnesium Aluminosilicate

DEFINITION

Magnesium Aluminosilicate is a synthesized material that contains NLT 20.5% and NMT 27.7% of magnesium oxide (MgO), NLT 27.0% and NMT 34.3% of aluminum oxide (Al₂O₃), and NLT 14.4% and NMT 21.7% of silicon dioxide (SiO₂), calculated on the dried basis.

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL, Aluminum** (191)

Sample: 0.5 g

Analysis: Transfer the *Sample* to a suitable container, add 5 mL of a sulfuric acid solution (1 in 3), and heat until white fumes are observed. Cool, add 20 mL of water, and filter. Neutralize the filtrate with ammonia TS, and retain for use in *Identification test B*. Collect the precipitate, and dissolve in 3 N hydrochloric acid.

Acceptance criteria: Meets the requirements

• **B. IDENTIFICATION TESTS—GENERAL, Magnesium** (191)

Sample solution: The filtrate retained from *Identification test A*

Acceptance criteria: Meets the requirements

• **C.**

Analysis: Prepare a bead by fusing a few crystals of sodium ammonium phosphate on a platinum loop in the flame of a Bunsen burner. Place the hot, transparent bead in contact with Magnesium Aluminosilicate, and again fuse.

Acceptance criteria: The silica floats about in the bead, producing, upon cooling, an opaque bead with a web-like structure.

ASSAY

• **ALUMINUM OXIDE**

Edetate disodium titrant: Prepare a solution with a concentration of 18.6 g/L of edetate disodium in water, and standardize as follows. Weigh 2 g of aluminum wire, transfer to a 1000-mL volumetric flask, and add 50 mL of a mixture of hydrochloric acid and water (1:1). Swirl the flask to ensure contact of the aluminum and the acid, and allow the reaction to proceed until all the aluminum has dissolved. Dilute with water to volume. Pipet 10 mL of this solution into a 250-mL beaker, and add, in the order named and with continuous stirring, 25.0 mL of *Edetate disodium titrant* and 20 mL of acetic acid–ammonium acetate buffer TS. Boil gently for 5 min. Cool, and add 50 mL of alcohol and 2 mL of dithizone TS. Titrate with 0.05 M zinc sulfate VS to a bright rose-pink color. Perform a blank determination, substituting 10 mL of water for the aluminum solution, and make any necessary correction.

Calculate the molarity of the solution taken:

$$\text{Result} = W/(A_r \times V)$$

W = weight of aluminum in the portion of solution taken (g)

A_r = atomic weight of aluminum, 26.98 g/mol

V = volume of *Edetate disodium titrant* consumed (mL)

Sample solution: Transfer 1.25 g of Magnesium Aluminosilicate to a conical flask, add 10 mL of 3 N hydrochloric acid and 50 mL of water, and heat on a water bath for 15 min. To this solution add 8 mL of hydrochloric acid, and heat on a water bath for 10 min. After cooling, transfer the solution to a 250-mL volumetric flask, rinse the conical flask with water, and add the washings to the volumetric flask. Dilute with water to volume. Centrifuge, and use the supernatant as the *Sample solution*. [NOTE—Retain a portion of the *Sample solution* for use in the *Assay for Magnesium Oxide*.]

Blank: 10 mL of 3 N hydrochloric acid and 50 mL of water

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: *Edetate disodium titrant*

Back-titrant: 0.05 M zinc sulfate VS

Endpoint detection: Visual

Analysis: Transfer 20.0 mL of the *Sample solution* to a beaker, and add 20.0 mL of *Titrant*. To this solution add 15 mL of acetic acid–ammonium acetate buffer TS and 20 mL of water, and boil for 5 min. After cooling, add 50 mL of alcohol and 2 mL of dithizone TS, and titrate with the *Back-titrant* until the color of the solution changes from green-violet to rose-pink. Perform a blank determination, and make the necessary correction. Each mL of 0.05 M *Edetate disodium titrant* is equivalent to 2.5490 mg of aluminum oxide (Al₂O₃).

Acceptance criteria: 27.0%–34.3% on the dried basis

• **MAGNESIUM OXIDE**

Sample solution: Use the portion retained from the *Sample solution* prepared in the *Assay for Aluminum Oxide*.

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.05 M edetate disodium VS

Endpoint detection: Visual

Analysis: Transfer 50.0 mL of the *Sample solution* to a suitable container, add 50 mL of water and 25 mL of a trolamine solution (500 mg/mL), and shake well. Add 25 mL of ammonia–ammonium chloride buffer TS and 0.04 g of eriochrome black TS trituration as the indicator. Titrate with *Titrant* until the red-purple color changes to blue and persists for 30 s. Each mL of 0.05

M edetate disodium VS is equivalent to 2.0152 mg of magnesium oxide (MgO).

Acceptance criteria: 20.5%–27.7% on the dried basis

• **SILICON DIOXIDE**

Sample: 1 g

Analysis: To the *Sample* add 30 mL of 3 N hydrochloric acid, and evaporate on a water bath to dryness. Moisten the residue with hydrochloric acid, and again evaporate on a water bath to dryness. To the residue add 8 mL of hydrochloric acid and 25 mL of hot water, and stir. Allow to stand, then decant the supernatant through an ashless filter paper. To the residue in the container add 10 mL of hot water, stir, and decant the supernatant through the filter paper. Wash the residue in the container with three additional 10-mL portions of hot water, stir, and decant as described above. Treat the residue in the container with 50 mL of water, and heat on a water bath for 15 min. Filter, and rinse the residue on the filter paper with hot water until no precipitate is obtained when 1 mL of silver nitrate TS is added to 5 mL of the washing. Transfer the filter paper and its contents to a tared platinum crucible, heat to dryness, incinerate, and continue to heat at $800 \pm 25^\circ$ for 1 h. Cool, and weigh. Moisten the residue with 6 mL of hydrofluoric acid, evaporate to dryness, and ignite for 5 min. Cool, and weigh. The loss in weight represents the weight of SiO_2 .

Acceptance criteria: 14.4%–21.7% on the dried basis

IMPURITIES

• **CHLORIDE AND SULFATE, Chloride** (221)

Analysis: A 20-mL portion of the diluted filtrate retained from the test for *Soluble Salts* shows no more chloride than corresponds to 0.75 mL of 0.020 N hydrochloric acid.

Acceptance criteria: NMT 0.053%

• **CHLORIDE AND SULFATE, Sulfate** (221)

Analysis: A 2-mL portion of the diluted filtrate retained from the test for *Soluble Salts* shows no more sulfate than corresponds to 0.5 mL of 0.020 N sulfuric acid.

Acceptance criteria: NMT 0.480%

• **ARSENIC, Method I** (211): NMT 3 µg/g

• **IRON** (241)

Sample: 0.11 g

Analysis: To the *Sample* add 8 mL of 2 N nitric acid, boil for 1 min, and cool. Dilute with water to 100 mL, and centrifuge. Dilute 30 mL of the supernatant with water to 45 mL.

Acceptance criteria: NMT 0.03%

• **HEAVY METALS, Method I** (231)

Standard preparation: Transfer 2 mL of hydrochloric acid to a suitable container, and evaporate to dryness on a water bath. To the residue add 2.0 mL of *Standard Lead Solution* and 0.1 g of hydroxylamine hydrochloride. Dilute with water to 25 mL.

Sample: 2.67 g

Test preparation: Transfer the *Sample* to a suitable container, add 20 mL of water and 8 mL of hydrochloric acid, and evaporate to dryness on a water bath. To the residue add 5 mL of 1 N acetic acid and 20 mL of water. Boil for 2 min, add 0.4 g of hydroxylamine hydrochloride, and heat to boiling. Cool, dilute with water to 100 mL, and filter. Use 25 mL of the filtrate as the *Test preparation*.

Monitor preparation: Transfer 25 mL of the filtrate from the *Test preparation* to a suitable container, and add 2.0 mL of *Standard Lead Solution*.

Acceptance criteria: NMT 30 µg/g

SPECIFIC TESTS

• **ACID-CONSUMING CAPACITY**

Sample solution: Transfer 0.2 g of Magnesium Aluminosilicate to a glass-stoppered flask, and add 100.0 mL of 0.1 N hydrochloric acid VS. Stopper the

flask tightly, shake at $37 \pm 2^\circ$ for 1 h, and filter. Use the filtrate.

Analysis: Transfer 50.0 mL of the filtrate from the *Sample solution* to a beaker, and while stirring, titrate the excess hydrochloric acid with 0.1 N sodium hydroxide VS to a pH of 3.5. Perform a blank determination, and make any necessary corrections.

Acceptance criteria: NLT 250 mL of 0.1 N hydrochloric acid is consumed per g of Magnesium Aluminosilicate, calculated on the dried basis.

• **pH** (791)

Sample: 2 g

Analysis: Add 50 mL of water to the *Sample*. While stirring, immerse the pH electrodes in the suspension, and after 2 min, record the pH.

Acceptance criteria: 8.5–10.5

• **LOSS ON DRYING** (731)

Analysis: Dry at 110° for 7 h.

Acceptance criteria: NMT 20.0%

• **SOLUBLE SALTS**

Sample: 10.0 g

Analysis: Transfer the *Sample* to a suitable container, add 150 mL of water, and boil gently for 15 min, with shaking. After cooling, dilute with water to 150 mL, and centrifuge. Dilute 75 mL of the clear filtrate with water to 100 mL, and retain the diluted filtrate for use in the tests for *Chloride*, *Sulfate*, and *Alkalinity*. Evaporate 25 mL of the diluted filtrate on a water bath, and heat at 700° for 2 h.

Acceptance criteria: NMT 1.6%; the residue weighs NMT 0.020 g.

• **ALKALINITY**

Sample: 20 mL of diluted filtrate retained from the test for *Soluble Salts*

Analysis: Add 2 drops of phenolphthalein TS to the *Sample*, containing 1 g of Magnesium Aluminosilicate.

Acceptance criteria: If a pink color is produced, NMT 0.50 mL of 0.1 N hydrochloric acid is required to discharge it.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and prevent exposure to excessive heat.

Magnesium Aluminum Silicate

DEFINITION

Magnesium Aluminum Silicate is a blend of colloidal montmorillonite and saponite that has been processed to remove grit and nonswellable ore components. The requirements for viscosity and ratio of aluminum content to magnesium content differ for the several types of Magnesium Aluminum Silicate, as set forth in the table below.

Type	Viscosity (cps)		Al content/ Mg content	
	Min.	Max.	Min.	Max.
IA	225	600	0.5	1.2
IB	150	450	0.5	1.2
IC	800	2200	0.5	1.2
IIA	100	300	1.4	2.8

IDENTIFICATION

• **A.**

Sample: 2 g

Analysis 1: Add the *Sample* in small portions to 100 mL of water with intense agitation. Allow to stand for 12 h to ensure complete hydration. Place 2 mL of the resulting mixture on a suitable glass slide, and allow to air-

dry at room temperature to produce an oriented film. Place the slide in a vacuum desiccator over a free surface of ethylene glycol. Evacuate the desiccator, and close the stopcock so that the ethylene glycol saturates the desiccator chamber. Allow to stand for 12 h. Record the X-ray diffraction pattern (see *X-Ray Diffraction* (941)), and calculate the d values.

Acceptance criteria 1: The largest peak corresponds to a d value between 15.0 and 17.2 Å.

Analysis 2: Prepare a random powder specimen of Magnesium Aluminum Silicate, record the X-ray diffraction pattern, and determine the d values in the region between 1.48 and 1.54 Å.

Acceptance criteria 2: Peaks are found at 1.492–1.504 Å and at 1.510–1.540 Å.

ASSAY

• ALUMINUM CONTENT AND MAGNESIUM CONTENT

Aluminum content

Aluminum standard stock solution: Dissolve 1.000 g of aluminum in a mixture of 10 mL of hydrochloric acid and 10 mL of water by gentle heating. Transfer the solution to a 1000-mL volumetric flask, and dilute with water to volume. This solution contains the equivalent of 1 mg/mL of aluminum.

Aluminum standard solutions: Transfer 2-, 5-, and 10-mL aliquots of the *Aluminum standard stock solution* to separate 100-mL volumetric flasks containing 200 mg of sodium chloride, and dilute each with water to volume.

Sample stock solution: Transfer 0.200 g of Magnesium Aluminum Silicate to a 25-mL platinum crucible containing 1.0 g of lithium metaborate, and mix. Using a muffle furnace or a suitable burner, heat slowly at first, and ignite at 1000°–1200° for 15 min. Cool, place the crucible in a 100-mL beaker containing 25 mL of dilute nitric acid (50 mg/mL), and add an additional 50 mL of the dilute acid, filling and submerging the upright crucible. Place a polyfluorocarbon-coated magnetic stirring bar into the crucible, and stir gently with a magnetic stirrer to dissolve. Pour the contents into a 250-mL beaker, and remove the crucible. Warm the solution, transfer through a rapid-flow filter paper with the aid of water into a 200-mL volumetric flask, and dilute with water to volume.

Sample solution: Pipet 20 mL of the *Sample stock solution* into a 100-mL volumetric flask. Add 20 mL of a solution of sodium chloride (10 mg/mL), and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a single-slot burner

Analytical wavelength: 309 nm

Lamp: Aluminum hollow-cathode

Flame: Oxidizing acetylene–air–nitrous oxide

Analysis

Samples: *Aluminum standard solutions* and *Sample solution*

Determine the absorbances of the *Aluminum standard solutions* and the *Sample solution*. From a linear regression equation calculated from the absorbances and concentrations of the *Aluminum standard solutions*, determine the aluminum content of the magnesium aluminum silicate.

Magnesium content

Lanthanum solution: Stir 88.30 g of lanthanum chloride (LaCl_3) with 500 mL of 6 N hydrochloric acid to dissolve, transfer with the aid of water to a 1000-mL volumetric flask, and dilute with water to volume.

Magnesium standard stock solution: Place 1.000 g of magnesium in a 250-mL beaker containing 20 mL of water, and carefully add 20 mL of hydrochloric acid, warming, if necessary, to complete the reaction. Transfer the solution to a 1000-mL volumetric flask, and

dilute with water to volume. This solution contains the equivalent of 1 mg/mL of magnesium. Transfer 10.0 mL of this solution to a 1000-mL volumetric flask, and dilute with water to volume.

Magnesium standard solutions: Transfer 5-, 10-, 15-, and 20-mL aliquots of the *Magnesium standard stock solution* to separate 100-mL volumetric flasks. To each flask, add 20.0 mL of *Lanthanum solution*, and dilute with water to volume.

Sample stock solution: Use the *Sample stock solution* prepared as directed for *Aluminum content*.

Sample solution: Transfer 25 mL of the *Sample stock solution* to a 50-mL volumetric flask, and dilute with water to volume. Transfer 5.0 mL of this solution to a 100-mL volumetric flask, add 20.0 mL of *Lanthanum solution*, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption

Analytical wavelength: 285 nm

Lamp: Magnesium hollow-cathode

Flame: Reducing flame of acetylene–air

Analysis

Samples: *Magnesium standard solutions* and *Sample solution*

Determine the absorbances of the *Sample solution* and the *Magnesium standard solutions*. From a linear regression equation calculated from the absorbances and concentrations of the *Magnesium standard solutions*, determine the magnesium content in the magnesium aluminum silicate.

Ratio of aluminum content to magnesium content

Analysis: Using the results from the *Aluminum content* and the *Magnesium content*, determine the ratio of aluminum content to magnesium content.

Acceptance criteria

Type IA: 0.5–1.2

Type IB: 0.5–1.2

Type IC: 0.5–1.2

Type IIA: 1.4–2.8

IMPURITIES

• ARSENIC, Method I (211)

Standard preparation: Prepare as directed in the chapter.

Control preparation: Transfer 5.0 mL (5 µg of As) of the *Standard preparation* to a 25-mL volumetric flask, and add dilute hydrochloric acid (1:25) to volume.

Test preparation: Transfer 13.3 g of Magnesium Aluminum Silicate to a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1:25), mix, cover with a watch glass, and boil gently, with occasional stirring, for 15 min without allowing excessive foaming. Allow the insoluble material to settle, and decant the hot supernatant through a rapid-flow filter paper into a 200-mL volumetric flask, retaining as much sediment as possible in the beaker. Add 25 mL of hot dilute hydrochloric acid (1:25) to the residue in the beaker, stir, and heat to boiling. Allow the insoluble material to settle, and decant the supernatant through the filter into the 200-mL volumetric flask. Repeat the extraction with four additional 25-mL portions of hot dilute hydrochloric acid (1:25), decanting each hot supernatant through the filter into the volumetric flask. At the last extraction, transfer as much of the insoluble material as possible onto the filter. Cool the combined filtrates to room temperature, add dilute hydrochloric acid (1:25) to volume, and mix. Use 25 mL for the test.

Acceptance criteria: NMT 3 µg/g; the absorbance due to any red color from the *Test preparation* does not exceed that produced by the *Control preparation*.

• LEAD

Standard preparation: On the day of use, dilute 3.0 mL of *Lead Nitrate Stock Solution* (see *Heavy Metals*

(231)) with water to 100 mL. Each mL contains the equivalent of 3 µg of lead.

Sample: 10.0 g

Test preparation: Transfer the *Sample* to a 250-mL beaker containing 100 mL of dilute hydrochloric acid (1:25), stir, cover with a watch glass, and boil for 15 min. Cool to room temperature, and allow the insoluble matter to settle. Decant the supernatant through a rapid-flow filter paper into a 400-mL beaker. Add 25 mL of hot water to the insoluble matter in the 250-mL beaker, and stir. Allow the insoluble matter to settle, and decant the supernatant through the filter into the 400-mL beaker. Repeat the extraction with two additional 25-mL portions of water, decanting each supernatant portion through the filter into the 400-mL beaker. Wash the filter with 25 mL of hot water, collecting this filtrate in the 400-mL beaker. Concentrate the combined extracts by gentle boiling to approximately 20 mL. If a precipitate appears, add 2 to 3 drops of nitric acid, heat to boiling, and cool to room temperature. Filter the concentrated extracts through a rapid-flow filter paper into a 50-mL volumetric flask. Transfer the remaining contents of the 400-mL beaker through the filter paper and into the flask with water. Dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a deuterium arc background correction and a single-slot burner

Analytical wavelength: 284 nm

Lamp: Lead hollow-cathode

Flame: Oxidizing flame of air and acetylene

Acceptance criteria: 15 µg/g; the absorbance of the *Test preparation* is NMT that of the *Standard preparation*.

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): Its total aerobic microbial count does not exceed 10^3 cfu/g, and it meets the requirements of the test for absence of *Escherichia coli*.

- **pH** (791)

Sample suspension: 50 mg/mL

Acceptance criteria: 9.0–10.0

- **LOSS ON DRYING** (731)

Analysis: Dry a sample at 110° to constant weight.

Acceptance criteria: NMT 8.0%

- **VISCOSITY**

Sample solution: After determining the *Loss on Drying*, weigh a quantity of Magnesium Aluminum Silicate, equivalent to 25.0 g on the dried basis. Over a period of a few s, transfer the undried test specimen to a suitable 1-L blender jar containing an amount of water, maintained at a temperature of $25 \pm 2^\circ$, that is sufficient to produce a mixture weighing 500 g. Blend for 3 min, accurately timed, at 14,000–15,000 rpm (high speed). [NOTE—Heat generated during blending causes a temperature rise to above 30° .]

Analysis: Transfer the contents of the blender to a 600-mL beaker, allow to stand for 5 min, and adjust, if necessary, to a temperature of $33 \pm 3^\circ$. Using a suitable rotational viscometer equipped with a spindle as specified below, operate the viscometer at 60 rpm for 6 min, accurately timed, and record the scale reading.

For Type IA, use a spindle having a cylinder 1.87 cm in diameter and 0.69 cm high attached to a shaft 0.32 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 2.54 cm, and the immersion depth being 5.00 cm (No. 2 spindle). If the scale reading is greater than 90% of full scale, repeat the measurement, using a spindle similar to the No. 2 spindle but having the cylinder 1.27 cm in diameter and 0.16 cm high instead (No. 3 spindle).

For Type IC, use a No. 3 spindle. If the scale reading is greater than 90% of full scale, repeat the measurement using a spindle consisting of a cylindrical shaft 0.32 cm in diameter and having an immersion depth of 4.05 cm (No. 4 spindle).

For Types IB and IIA, use a No. 2 spindle.

Acceptance criteria

Type IA: 225–600

Type IB: 150–450

Type IC: 800–2200

Type IIA: 100–300

- **ACID DEMAND**

Sample: After determining the *Loss on Drying*, weigh a quantity of Magnesium Aluminum Silicate equivalent to 5.00 g.

Analysis: Disperse the *Sample* in 500 mL of water with the aid of a suitable blender fitted with a 1-L jar. Using a stopwatch, designate zero time. With constant mixing, add 3.0-mL portions of 0.100 N hydrochloric acid at 5, 65, 125, 185, 245, 305, 365, 425, 485, 545, 605, 665, and 725 s, and add a 1.0-mL portion at 785 s.

Determine the pH potentiometrically at 840 s.

Acceptance criteria: NMT 4.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate its type.

Magnesium Silicate

DEFINITION

Magnesium Silicate is a compound of magnesium oxide and silicon dioxide. It contains NLT 15.0% of magnesium oxide (MgO) and NLT 67.0% of silicon dioxide (SiO₂), calculated on the ignited basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Magnesium** (191)

Sample: 500 mg

Analysis: Mix the *Sample* with 10 mL of 3 N hydrochloric acid. Filter, and neutralize the filtrate to litmus paper with 6 N ammonium hydroxide.

Acceptance criteria: The neutralized filtrate meets the requirements.

- **B.**

Analysis: Prepare a bead by fusing a few crystals of sodium ammonium phosphate on a platinum loop in the flame of a Bunsen burner. Place the hot, transparent bead in contact with Magnesium Silicate, and again fuse.

Acceptance criteria: Silica floats about in the bead, producing, upon cooling, an opaque bead with a web-like structure.

ASSAY

- **MAGNESIUM OXIDE**

Sample: 1.5 g

Titrimetric system

Mode: Residual titration

Titrant: 1 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 50.0 mL of 1 N sulfuric acid VS. Digest on a steam bath for 1 h, cool to room temperature, and add methyl orange TS. Titrate the excess acid in the sample with *Titrant*. Each mL of 1 N sulfuric acid is equivalent to 20.15 mg of MgO.

Acceptance criteria: NLT 15.0% on the ignited basis

- **SILICON DIOXIDE**

Sample: 700 mg

Analysis: Transfer the *Sample* to a small platinum dish. Add 10 mL of 1 N sulfuric acid, and heat on a steam bath to dryness, leaving the dish uncovered. Treat the

residue with 25 mL of water, and digest on a steam bath for 15 min. Decant the supernatant through an ashless filter paper, with the aid of suction, and wash the residue, by decantation, three times with hot water, passing the washings through the filter paper. Finally, transfer the residue to the filter, and wash thoroughly with hot water. Transfer the filter paper and its contents to the platinum dish previously used. Heat to dryness, incinerate, ignite strongly for 30 min, cool, and weigh. Moisten the residue with water, and add 6 mL of hydrofluoric acid and 3 drops of sulfuric acid. Evaporate to dryness, ignite for 5 min, cool, and weigh. The loss in weight represents the weight of SiO_2 .

Acceptance criteria: NLT 67.0% on the ignited basis

IMPURITIES

• FLUORIDE

Indicator solution: 100 mg/mL of lanthanum alizarin complexan mixture in 60% isopropyl alcohol. Filter the solution if it is not clear.

Standard solution: 2.21 $\mu\text{g/mL}$ of sodium fluoride in 0.1 N hydrochloric acid

Sample solution: Prepare a slurry consisting of 5.0 g of Magnesium Silicate and 45 mL of 0.1 N hydrochloric acid. Stir at room temperature for 15 min, and pass through a filter of 0.45- μm pore size into a 50-mL volumetric flask. Wash the filter with five 1-mL portions of 0.1 N hydrochloric acid, collecting the washings in the flask. Dilute with 0.1 N hydrochloric acid to volume.

Instrumental conditions

Mode: Vis

Analytical wavelength: About 620 nm

Cell: 1 cm

Blank: 0.1 N hydrochloric acid, *Indicator solution*, and water (5:5:15)

Analysis: Transfer 5.0 mL of the *Standard solution* and *Sample solution* to separate 25-mL volumetric flasks, add 5.0 mL of *Indicator solution*, dilute with water to volume, and allow to stand for 1 h in diffuse light at ambient temperature. Determine the absorbance of the solutions against the *Blank*.

Acceptance criteria: 10 $\mu\text{g/g}$; the absorbance of the *Sample solution* is NMT than that of the *Standard solution*.

• SOLUBLE SALTS

Sample: 10.0 g

Analysis: Boil the *Sample* with 150 mL of water for 15 min. Cool to room temperature, and allow the mixture to stand for 15 min. Filter with the aid of suction, transfer the filtrate to a 200-mL volumetric flask, and dilute with water to volume. Evaporate 50.0 mL of this solution, representing 2.5 g of the Silicate, in a tared platinum dish to dryness. Ignite gently to constant weight. Retain the remaining diluted solution for the test for *Free Alkali*.

Acceptance criteria: 3.0%; NMT 75.0 mg

• FREE ALKALI

Sample: 20 mL of the retained diluted filtrate prepared in the test for *Soluble Salts*

Analysis: Add 2 drops of phenolphthalein TS to the *Sample*, representing 1 g of Magnesium Silicate.

Acceptance criteria: If a pink color is produced, NMT 2.5 mL of 0.1 N hydrochloric acid is required to discharge it.

• HEAVY METALS (231)

Solution A: Hydrochloric acid in water (1 in 100)

Test preparation: Boil 4.0 g of Magnesium Silicate with a mixture of 50 mL of water and 10 mL of hydrochloric acid for 20 min, and add water to maintain the volume during the boiling. Add ammonium hydroxide until the mixture is only slightly acid to litmus paper. Filter with the aid of suction, and wash with 15–20 mL of water, combining the washings with the original filtrate. Add 2 drops of phenolphthalein TS, then add a slight excess of 6 N ammonium hydroxide. Discharge the pink color

with *Solution A*, then add 8 mL of *Solution A*. Dilute with water to 100 mL, and use 25 mL of the solution for the test.

Acceptance criteria: NMT 20 $\mu\text{g/g}$

• LEAD (251)

Test preparation: Dissolve 1.0 g of Magnesium Silicate in 20 mL of 3 N hydrochloric acid, evaporate on a steam bath to 10 mL, dilute with water to 20 mL, and cool.

Acceptance criteria: NMT 10 $\mu\text{g/g}$

SPECIFIC TESTS

• RATIO OF SiO_2 TO MgO

Analysis: Divide the percentage of SiO_2 obtained in the *Assay for Silicon Dioxide* by the percentage of MgO obtained in the *Assay for Magnesium Oxide*.

Acceptance criteria: 2.50–4.50

• LOSS ON DRYING (731) [NOTE—Retain the dried specimen for the test for *Loss on Ignition*.]

Analysis: Dry at 105° for 2 h.

Acceptance criteria: NMT 15.0%

• LOSS ON IGNITION (733)

Sample: The specimen retained from the test for *Loss on Drying*

Analysis: Ignite the *Sample* at 900°–1000° for 20 min.

Acceptance criteria: NMT 15%, previously dried

• PH (791)

Sample solution: A well-mixed aqueous suspension (1 in 10)

Acceptance criteria: 7.0–10.8

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Magnesium Stearate

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (♦) to specify this fact.

Octadecanoic acid, magnesium salt;
Magnesium stearate [557-04-0].

DEFINITION

Magnesium Stearate is a compound of magnesium with a mixture of solid organic acids, and consists chiefly of variable proportions of magnesium stearate and magnesium palmitate. The fatty acids are derived from edible sources. It contains NLT 4.0% and NMT 5.0% of Mg, calculated on the dried basis.

IDENTIFICATION

• A. IDENTIFICATION TESTS—GENERAL, *Magnesium* (191)

Sample solution: Mix 5.0 g with 50 mL of peroxide-free ether, 20 mL of diluted nitric acid, and 20 mL of water in a round-bottom flask. Connect the flask to a reflux condenser, and reflux until dissolution is complete. Allow to cool, and transfer the contents of the flask to a separator. Shake, allow the layers to separate, and transfer the aqueous layer to a flask. Extract the ether layer with two 4-mL portions of water, and add these aqueous extracts to the main aqueous extract. Wash the aqueous extract with 15 mL of peroxide-free ether, transfer the aqueous extract to a 50-mL volumetric flask, and dilute with water to volume. Retain the unused portion of this solution for the chloride and sulfate impurity tests.

Acceptance criteria: The *Sample solution* meets the requirements.

- **B.** The retention times of the peaks corresponding to stearic acid and palmitic acid of the *Sample solution* correspond to those of the *System suitability solution*, as ob-

tained in the test for *Relative Content of Stearic Acid and Palmitic Acid*.

ASSAY

• PROCEDURE

Buffer: Dissolve 5.4 g of ammonium chloride in water, add 20 mL of ammonium hydroxide, and dilute with water to 100 mL.

Sample: 500 mg

Analysis: To the *Sample* add 50 mL of a mixture of butyl alcohol and dehydrated alcohol (1:1), 5 mL of ammonium hydroxide, 3 mL of *Buffer*, 30.0 mL of 0.1 M edetate disodium VS, and 1 or 2 drops of eriochrome black TS. Heat at 45°–50° until the solution is clear. Cool, and titrate the excess edetate disodium with 0.1 M zinc sulfate VS until the solution color changes from blue to violet (see *Titrimetry* (541)). Perform a blank determination, and make any necessary correction. Each mL of 0.1 M edetate disodium is equivalent to 2.431 mg of Mg.

Acceptance criteria: 4.0%–5.0% on the dried basis

IMPURITIES

- **CHLORIDE AND SULFATE, Chloride (221):** A 10.0-mL portion of the *Sample solution* prepared in *Identification test A* shows no more chloride than corresponds to 1.4 mL of 0.020 N hydrochloric acid (0.1%).
- **CHLORIDE AND SULFATE, Sulfate (221):** A 6.0-mL portion of the *Sample solution* prepared in *Identification test A* shows no more sulfate than corresponds to 3.0 mL of 0.020 N sulfuric acid (1.0%).

• LIMIT OF CADMIUM

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of cadmium, lead, and nickel as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min, and rinse with deionized water.]

Matrix modifier solution: Prepare a solution containing 200 mg/mL of monobasic ammonium phosphate and 10 mg/mL of magnesium nitrate. Alternatively, use an appropriate matrix modifier as recommended by the manufacturer of the graphite furnace atomic absorption (GFAA) spectrophotometer.

Blank: Nitric acid in water (1 in 4)

Standard solution: 0.00825 µg/mL of cadmium nitrate tetrahydrate in *Blank*, corresponding to a known concentration of 0.0030 µg/mL of cadmium

Sample stock solution: Transfer 0.100 g of Magnesium Stearate to a suitable polytetrafluoroethylene (PTFE)-lined acid-digestion bomb, and add 2.5 mL of nitric acid. Close and seal the bomb according to the manufacturer's operating instructions. [CAUTION—When using an acid-digestion bomb, be thoroughly familiar with the safety and operating instructions. Carefully follow the bomb manufacturer's instructions regarding care and maintenance of these acid-digestion bombs. Do not use metal-jacketed bombs or liners that have been used with hydrochloric acid because of contamination from corrosion of the metal jacket by hydrochloric acid.] Heat the bomb in an oven at 170° for 3 h. Air cool the bomb slowly to room temperature as per the bomb manufacturer's instructions. Place the bomb in a hood, and open carefully because corrosive gases may be expelled. Dilute the residue with water to 10.0 mL in a volumetric flask.

Sample solutions: Dilute the *Sample stock solution* with *Blank* (1 in 10). Prepare mixtures of this solution, the *Standard solution*, and the *Blank* with the following proportional compositions, by volume (mL): 1.0/0/1.0, 1.0/0.5/0.5, and 1.0/1.0/0. Add 50 µL of *Matrix modifier solution* to each mixture. These *Sample solutions* con-

tain, respectively, 0, 0.00075, and 0.0015 µg/mL of cadmium from the *Standard solution*. [NOTE—Retain the remaining *Sample stock solution* for use in the tests for *Limit of Lead* and *Limit of Nickel*.]

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry (using a suitable GFAA spectrophotometer equipped with a pyrolytic tube with platform)

Analytical wavelength: Cadmium emission line at 228.8 nm

Lamp: Cadmium hollow-cathode

Temperature: Use the temperature programming recommended for cadmium by the GFAA manufacturer (for examples of temperature parameters for GFAA analysis of cadmium, see *Table 1*).

Table 1

	Drying Stage	Ashing Stage	Atomization Stage
Temperature	110°	600°	1800°
Ramp time	10 s	10 s	0 s
Hold time	20 s	30 s	5 s

Analysis: Use the *Blank* to set the instrument to zero.

Plot the absorbances of the *Sample solutions* versus their contents of cadmium, in µg/mL, as furnished by the *Standard solution*, draw the straight line best fitting the three points, using a linear least-squares fit, and extrapolate the line until it intercepts the concentration axis on the negative side. From the intercept determine the concentration, C, in µg/mL, of cadmium in the *Sample solution* containing 0 mL of the *Standard solution*. Calculate the content, in ppm, of cadmium in the specimen taken:

$$\text{Result} = (C/W) \times F$$

W = weight of Magnesium Stearate taken to prepare the *Sample stock solution* (g)

F = dilution factor for the sample, 200

Alternatively, the GFAA software can be used to calculate the cadmium content of the sample. For either calculation, the correlation coefficient (r) of the standard additions plot must be at least 0.995.

Acceptance criteria: 3 ppm

• LIMIT OF LEAD

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of cadmium, lead, and nickel as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min, and rinse with deionized water.]

Matrix modifier solution: Prepare as directed for *Matrix modifier solution* in *Limit of Cadmium*.

Blank: Prepare as directed for *Blank* in *Limit of Cadmium*.

Standard solution: 0.1598 µg/mL of lead nitrate in *Blank*, corresponding to a known concentration of 0.100 µg/mL of lead. Prepare and store any solutions of lead nitrate in glass containers free from soluble lead salts.

Sample stock solution: Use a portion of the *Sample stock solution* retained from the test for *Limit of Cadmium*.

Sample solutions: Prepare mixtures of the *Sample stock solution*, the *Standard solution*, and the *Blank* with the following proportional compositions, by volume (mL): 1.0/0/1.0, 1.0/0.5/0.5, and 1.0/1.0/0. Add 50 µL of the *Matrix modifier solution* to each mixture. These *Sample*

solutions contain, respectively, 0, 0.025, and 0.05 µg/mL of lead from the *Standard solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry (using a suitable GFAA spectrophotometer equipped with a pyrolytic tube with platform)

Analytical wavelength: Lead emission line at 283.3 nm

Lamp: Lead hollow-cathode

Temperature: Use the temperature programming recommended for lead by the GFAA manufacturer (for examples of temperature parameters for GFAA analysis of lead, see *Table 1*).

Analysis: Use the *Blank* to set the instrument to zero. Plot the absorbances of the *Sample solutions* versus their contents of lead, in µg/mL, as furnished by the *Standard solution*, draw the straight line best fitting the three points, using a linear least-squares fit, and extrapolate the line until it intercepts the concentration axis on the negative side. From the intercept determine the concentration, *C*, in µg/mL, of lead in the *Sample solution* containing 0 mL of the *Standard solution*. Calculate the content, in ppm, of lead in the specimen taken:

$$\text{Result} = (C/W) \times F$$

W = weight of Magnesium Stearate taken to prepare the *Sample stock solution* (g)

F = dilution factor for the sample, 20

Alternatively, the GFAA software can be used to calculate the lead content of the sample. For either calculation, the correlation coefficient (*r*) of the standard additions plot must be at least 0.995.

Acceptance criteria: 10 ppm

• LIMIT OF NICKEL

[NOTE—For the preparation of all aqueous solutions and for the rinsing of glassware before use, use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of cadmium, lead, and nickel as practicable, and store all reagent solutions in containers of borosilicate glass. Cleanse glassware before use by soaking in warm 8 N nitric acid for 30 min, and rinse with deionized water.]

Matrix modifier solution: Prepare as directed for *Matrix modifier solution* in *Limit of Cadmium*.

Blank: Prepare as directed for *Blank* in *Limit of Cadmium*.

Standard solution: 0.2477 µg/mL of nickel nitrate hexahydrate in *Blank*, corresponding to a known concentration of 0.050 µg/mL of nickel

Sample stock solution: Use a portion of the *Sample stock solution* retained from the test for *Limit of Cadmium*.

Sample solutions: Prepare mixtures of the *Sample stock solution*, the *Standard solution*, and the *Blank* with the following proportional compositions, by volume (mL): 1.0/0/1.0, 1.0/0.5/0.5, and 1.0/1.0/0. Add 50 µL of the *Matrix modifier solution* to each mixture. These *Sample solutions* contain, respectively, 0, 0.0125, and 0.025 µg/mL of nickel from the *Standard solution*.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry (using a suitable GFAA spectrophotometer equipped with a pyrolytic tube with platform)

Analytical wavelength: Nickel emission line at 232.0 nm

Lamp: Nickel hollow-cathode

Temperature: Use the temperature programming recommended for nickel by the GFAA manufacturer (for examples of temperature parameters for GFAA analysis of nickel, see *Table 1*).

Analysis: Use the *Blank* to set the instrument to zero.

Plot the absorbances of the *Sample solutions* versus their contents of nickel, in µg/mL, as furnished by the *Standard solution*, draw the straight line best fitting the three points, using a linear least-squares fit, and extrapolate the line until it intercepts the concentration axis on the negative side. From the intercept determine the concentration, *C*, in µg/mL, of nickel in the *Sample solution* containing 0 mL of the *Standard solution*.

Calculate the content, in ppm, of nickel in the specimen taken:

$$\text{Result} = (C/W) \times F$$

W = weight of Magnesium Stearate taken to prepare the *Sample stock solution* (g)

F = dilution factor for the sample, 20

Alternatively, the GFAA software can be used to calculate the nickel content of the sample. For either calculation, the correlation coefficient (*r*) of the standard additions plot must be at least 0.995.

Acceptance criteria: 5 ppm

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 10^3 cfu/g, the total combined molds and yeasts count does not exceed 5×10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

• ACIDITY OR ALKALINITY

Sample solution: To 1.0 g add 20 mL of carbon dioxide-free water, boil on a steam bath for 1 min with continuous shaking, cool, and filter. Add 0.05 mL of bromothymol blue TS to 10 mL of the filtrate.

Acceptance criteria: NMT 0.05 mL of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide is required to change the color of the indicator.

• SPECIFIC SURFACE AREA (846)

[NOTE—In cases where there are no functionality-related concerns regarding the specific surface area of this article, this test may be omitted.]

Where the labeling states the specific surface area, determine the specific surface area value as directed in the chapter in the *P/P₀* range of 0.05–0.15, and using outgassing conditions of 2 h at 40°. If the plot deviates from linearity for *P/P₀* values of 0.05–0.15, then a suitable range of *P/P₀* values should be validated for linearity. In this case, it is necessary to state the range of validated *P/P₀* values, the increment of the *P/P₀* values, and the outgassing conditions used.♦

• **LOSS ON DRYING (731):** Dry a sample at 105° to constant weight: it loses NMT 6.0% of its weight.

• RELATIVE CONTENT OF STEARIC ACID AND PALMITIC ACID

System suitability solution: Transfer 50 mg each of USP Stearic Acid RS and USP Palmitic Acid RS to a small conical flask fitted with a suitable reflux condenser. Add 5.0 mL of a solution prepared by dissolving 14 g of boron trifluoride in methanol to make 100 mL, swirl to mix, and reflux for 10 min until the solids have dissolved. Add 4 mL of chromatographic *n*-heptane through the condenser, and reflux for 10 min. Cool, add 20 mL of saturated sodium chloride solution, shake, and allow the layers to separate. Pass the *n*-heptane layer through 0.1 g of anhydrous sodium sulfate (previously washed with chromatographic *n*-heptane) into a suitable flask. Transfer 1.0 mL of this solution to a 10-mL volumetric flask, and dilute with chromatographic *n*-heptane to volume.

Sample solution: Transfer 100 mg of Magnesium Stearate to a small conical flask fitted with a suitable reflux condenser, and proceed as directed for *System suitability solution*, beginning with "Add 5.0 mL of a solution prepared by dissolving".

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** GC**Detector:** Flame ionization**Column:** 0.32-mm × 30-m fused silica capillary column, bonded with a 0.5-μm layer of phase G16**Temperature****Injector:** 220°**Detector:** 260°**Column:** See *Table 2*.**Table 2**

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	5	240	5

Carrier gas: Helium**Flow rate:** 2.4 mL/min**Injection size:** 1 μL**Injection type:** Splitless injection system**System suitability****Sample:** *System suitability solution*

[NOTE—The relative retention times for methyl palmitate and methyl stearate are about 0.9 and 1.0, respectively.]

Suitability requirements**Resolution:** NLT 5.0 between methyl palmitate and methyl stearate**Relative standard deviation:** NMT 3.0% for the palmitate and stearate peak areas from six replicate injections and NMT 1.0% for the peak area ratio of palmitate to stearate from six replicate injections**Analysis****Sample:** *Sample solution*

Measure the peak areas for all the fatty acid esters in the chromatogram.

Calculate the percentage of stearic acid in the fatty acid fraction of the portion of Magnesium Stearate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

 r_U = peak area due to methyl stearate r_T = sum of the peak areas of all the fatty acid esters

Similarly, calculate the percentage of palmitic acid in the portion of Magnesium Stearate taken.

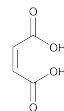
$$\text{Result} = (r_P/r_T) \times 100$$

 r_P = peak area due to methyl palmitate r_T = sum of the areas of all the fatty acid ester peaks**Acceptance criteria:** NLT 40% for the stearate peak.

The sum of the stearate and palmitate peaks is NLT 90% of the total peak areas of all the fatty acids.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **•LABELING:** Where the labeling states the specific surface area, it also indicates which method specified in *Specific Surface Area* <846> is used. Label to indicate that the fatty acids are derived from edible sources.♦
- **USP REFERENCE STANDARDS** <11>
 - USP Palmitic Acid RS
 - USP Stearic Acid RS

Maleic Acid $C_4H_4O_4$

(Z)-Butenedioic acid;

cis-Butenedioic acid [110-16-7].

116.07

DEFINITIONMaleic Acid contains NLT 99.0% and NMT 101.0% of $C_4H_4O_4$, calculated on the anhydrous basis.**IDENTIFICATION**• **A. PROCEDURE****Analysis:** Dissolve 500 mg of Maleic Acid in 10 mL of water.**Acceptance criteria:** The pH of the solution is less than 2.

- **B.** The principal spot from *Sample solution B* corresponds in color, size, and R_f value to that from *Standard solution A*, as obtained in the procedure for *Limit of Fumaric Acid*.

• **C. INFRARED ABSORPTION** <197K>**ASSAY**• **PROCEDURE****Sample solution:** 10 mg/mL of Maleic Acid**Analysis:** Titrate the *Sample solution* with 1 N sodium hydroxide VS, using phenolphthalein TS as the indicator. Each mL of 1 N sodium hydroxide is equivalent to 58.04 mg of $C_4H_4O_4$.**Acceptance criteria:** 99.0%–101.0% on the anhydrous basis**IMPURITIES****Inorganic Impurities**

- **RESIDUE ON IGNITION** <281>: NMT 0.1%, determined on a 1.0-g portion

• **LIMIT OF IRON****Solution A:** Dissolve 9.7 g of potassium thiocyanate in 100 mL of water.**Diluted standard iron solution:** Immediately before use, dilute 1 volume of *Standard Iron Solution*, prepared as directed in *Iron* <241>, with 9 volumes of water.

[NOTE—This solution contains the equivalent of 1 μg/mL of iron.]

Standard solution: Add 6 mL of water to 5 mL of *Diluted standard iron solution*. Add 1 mL of diluted hydrochloric acid and 0.05 mL of bromine TS. After 5 min, remove the excess of bromine with the aid of a current of air, add 3 mL of *Solution A*, and shake well.**Sample solution:** Dissolve 1 g of Maleic Acid in 10 mL of water. Add 2 mL of diluted hydrochloric acid and 0.05 mL of bromine TS. After 5 min, remove the excess of bromine with the aid of a current of air, add 3 mL of *Solution A*, and shake well.**Analysis:** Allow the *Standard solution* and *Sample solution* to stand for 5 min.**Acceptance criteria:** Any red color in the *Sample solution* is not more intense than that in the *Standard solution* (NMT 5 ppm).• **HEAVY METALS, Method II** <231>**Test preparation:** Transfer 1.0 g of Maleic Acid to a quartz crucible, and add 0.5 g of magnesium oxide. Ignite the crucible to dull redness until a homogeneous white or grayish-white mass is obtained. Ignite at 800° for 1 h, cool, and dissolve the residue by adding two 5-mL portions of diluted hydrochloric acid. Add 0.1 mL of phenolphthalein TS, then add ammonium hydroxide until a pink color is obtained. Cool, add glacial acetic acid until the solution is decolorized, then add 0.5 mL

of glacial acetic acid in excess, and dilute with water to 20.0 mL.

Standard solution: To 0.5 g of magnesium oxide add 1.0 mL of *Standard Lead Solution*, and evaporate to dryness at 105° for 1 h. Following the procedure described for preparation of the *Test preparation*, ignite, dissolve in diluted hydrochloric acid, add ammonia and then acetic acid, and dilute with water to 20.0 mL.

Blank: Water and *Test preparation* (10:2)

Analysis: To 12 mL of the *Test preparation* add 2.0 mL of pH 3.5 *Acetate Buffer*, mix, add to 1.2 mL of thioacetamide–glycerin base TS, and mix immediately. To 10 mL of the *Standard solution* add 2.0 mL of the *Test preparation* and 2.0 mL of pH 3.5 *Acetate Buffer*, mix, add 1.2 mL of thioacetamide–glycerin base TS, and mix immediately.

Compared to the *Blank*, the solution from the *Standard solution* shows a light brown color. Dilute each of the solutions from the *Test preparation* and *Standard solution* with water to 50 mL. Allow to stand for 2 min, and view downward over a white surface.

Acceptance criteria: The color of the solution from the *Test preparation* is not darker than that of the solution from the *Standard solution* (NMT 10 ppm).

Organic Impurities

• PROCEDURE: LIMIT OF FUMARIC ACID

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Standard solution A: 2 mg/mL of USP Maleic Acid RS in acetone

Standard solution B: 1.5 mg/mL of USP Fumaric Acid RS in acetone

System suitability solution: Equal volumes of *Standard solution A* and *Standard solution B*

Sample solution A: 100 mg/mL of Maleic Acid in acetone

Sample solution B: 2 mg/mL of Maleic Acid in acetone from *Sample solution A*

Developing solvent system: Heptane, butanol, chloroform, and anhydrous formic acid (44:36:16:16)

Application volume: 10 µL for the *System suitability solution* and 5 µL each for *Standard solution A*, *Standard solution B*, *Sample solution A*, and *Sample solution B*

Analysis

Samples: *Standard solution A*, *Standard solution B*, *System suitability solution*, *Sample solution A*, and *Sample solution B*

Proceed as directed in *Chromatography* (621), *Thin-Layer Chromatography*, using an unsaturated chamber. Dry the plate at 100° for 15 min, and examine the plate under short-wavelength UV light at 254 nm.

Acceptance criteria: The chromatogram from the *System suitability solution* exhibits two clearly separated spots, and any spot corresponding to fumaric acid in the chromatogram from *Sample solution A* does not exceed, in size or intensity, the principal spot in the chromatogram from *Standard solution B* (NMT 1.5% of fumaric acid).

SPECIFIC TESTS

• COLOR AND CLARITY OF SOLUTION

Dilute hydrochloric acid solution: Dilute 27.5 mL of hydrochloric acid with water to 1000 mL.

Reference solution: Mix 2.4 mL of ferric chloride CS and 0.6 mL of cobaltous chloride CS with *Dilute hydrochloric acid solution* to make 10 mL. Dilute 5 mL of this solution with *Dilute hydrochloric acid solution* to make 100 mL.

Sample solution: 100 mg/mL of Maleic Acid

Analysis: Place the *Reference solution* and the *Sample solution* in matched color-comparison tubes, and compare the solutions by viewing them downward against a white surface (see *Color and Achromicity* (631)).

Acceptance criteria: The *Sample solution* is clear and not more intensely colored than the *Reference solution*.

• WATER DETERMINATION, Method I (921): NMT 2.0%

ADDITIONAL REQUIREMENTS

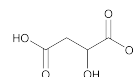
• **PACKAGING AND STORAGE:** Preserve in tight glass containers, protected from light. Store at room temperature.

• **USP REFERENCE STANDARDS** (11)

USP Fumaric Acid RS

USP Maleic Acid RS

Malic Acid



C₄H₆O₅

Hydroxybutanedioic acid, (±)-;

(±)-Malic acid;

(±)-Hydroxysuccinic acid [617-48-1].

134.09

DEFINITION

Malic Acid contains NLT 99.0% and NMT 100.5% of C₄H₆O₅.

IDENTIFICATION

• **A. INFRARED ABSORPTION** (197K): On undried sample

ASSAY

• PROCEDURE

Sample: 2 g

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 1 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 40 mL of recently boiled and cooled water. Add phenolphthalein TS, and titrate to the first appearance of a faint pink color that persists for NLT 30 s. Perform a blank determination. Calculate the percentage of malic acid (C₄H₆O₅) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100] / W$$

V = volume of *Titrant* consumed by the *Sample* (mL)

B = volume of *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 67.04 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5%

IMPURITIES

• **RESIDUE ON IGNITION** (281): NMT 0.1%

• **HEAVY METALS, Method II** (231): NMT 20 ppm

• **FUMARIC AND MALEIC ACIDS**

Mobile phase: 0.01 N sulfuric acid

System suitability solution: 1 mg/mL of Malic Acid, 10 µg/mL of USP Fumaric Acid RS, and 4 µg/mL of USP Maleic Acid RS in *Mobile phase*

Standard solution: 5.0 µg/mL of USP Fumaric Acid RS and 2.0 µg/mL of USP Maleic Acid RS in *Mobile phase*

Sample solution: 1.0 mg/mL of Malic Acid in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 6.5-mm × 30-cm; packing L17

Column temperature: 37 ± 1°

Flow rate: 0.6 mL/min

Injection size: 20 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for maleic acid, malic acid, and fumaric acid are 0.6, 1.0, and 1.5, respectively.]

Suitability requirements

Resolution: NLT 2.5 for the maleic acid and malic acid peaks; NLT 7.0 for the malic acid and fumaric acid peaks

Relative standard deviation: NMT 2.0% for the maleic acid peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of maleic acid and of fumaric acid in the portion of Malic Acid taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of maleic acid or fumaric acid from the *Sample solution*

r_S = peak response of maleic acid or fumaric acid from the *Standard solution*

C_S = concentration of USP Maleic Acid RS or USP Fumaric Acid RS in the *Standard solution* (mg/mL)

C_U = concentration of Malic Acid in the *Sample solution* (mg/mL)

Acceptance criteria

Fumaric acid: NMT 1.0%

Maleic acid: NMT 0.05%

• **WATER-INSOLUBLE SUBSTANCES**

Sample: 25 g

Analysis: Dissolve the *Sample* in 100 mL of water, filter the solution through a tared filtering crucible, wash the filter with hot water, and dry at 100° to constant weight.

Acceptance criteria: The increase in weight is NMT 25 mg (0.1%).

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

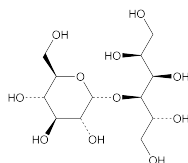
• **USP REFERENCE STANDARDS (11)**

USP Fumaric Acid RS

USP Maleic Acid RS

USP Malic Acid RS

Maltitol



C₁₂H₂₄O₁₁

D-Glucopyranosyl-D-glucitol [585-88-6].

344.31

DEFINITION

Maltitol contains NLT 92.0% and NMT 100.5% of D-maltitol, calculated on the anhydrous basis. The amounts of total sugars, other polyhydric alcohols, and any polyol anhydrides, if detected, are not included in the requirements

or in the calculated amount under *General Notices and Requirements, Other Impurities*.

IDENTIFICATION

• **A. PROCEDURE**

Sample solution: 1 g of Maltitol in 75 mL of water

Analysis: Transfer 3 mL of the *Sample solution* to a 15-cm test tube, and add 3 mL of freshly prepared catechol solution (1 in 10). Add 6 mL of sulfuric acid, mix again, and gently heat the tube in a flame for 30 s.

Acceptance criteria: A deep pink or wine-red color appears.

• **B.** The retention time of the major peak from the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

• **PROCEDURE**

Mobile phase: Water. [NOTE—Degas the *Mobile phase* before use.]

System suitability solution: 4.8 mg/g of USP Maltitol RS and 4.8 mg/g of sorbitol

Standard solution: 10 mg/g of USP Maltitol RS and 1.6 mg/g of sorbitol

Sample solution: Dissolve 0.20 g of Maltitol in water, and dilute with water to 20 g. Record the final solution weight, and mix thoroughly.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 10-cm; packing L34

Temperature

Column: 60 ± 2°

Detector: 35°

Flow rate: 0.5 mL/min

Injection size: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for maltitol and sorbitol are 0.48 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between maltitol and sorbitol, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of maltitol (C₁₂H₂₄O₁₁) in the portion of Maltitol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times [100/(100 - W)] \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Maltitol RS in the *Standard solution* (mg/g)

C_U = concentration of Maltitol in the *Sample solution* (mg/g)

W = percentage obtained in the test for *Water Determination*

Acceptance criteria: 92.0%–100.5% on the anhydrous basis

IMPURITIES

• **LIMIT OF NICKEL**

Sample solution: Dissolve 20.0 g of Maltitol in diluted acetic acid, and dilute with diluted acetic acid to 150 mL.

Blank solution: 150 mL of diluted acetic acid

Standard solutions: Prepare three solutions by adding 0.5, 1.0, and 1.5 mL of nickel standard solution TS to 20.0 g of Maltitol dissolved in diluted acetic acid, and dilute with the same solvent to 150 mL.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Maxima at about 232.0 nm

Lamp: Nickel hollow-cathode

Flame: Air-acetylene

Analysis

Samples: *Standard solutions* and *Sample solution*

To each sample, add 2.0 mL of a saturated ammonium pyrrolidinedithiocarbamate solution (containing 10 g/L of ammonium pyrrolidinedithiocarbamate) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer. Set the instrument to zero using the organic layer from the *Blank solution*. Concomitantly determine the absorbances of the organic layer from the *Samples* at least three times each. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Between each measurement, aspirate the organic layer from the *Blank solution*, and ascertain that the reading returns to zero. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.

Acceptance criteria: NMT 1 ppm

- **REDUCING SUGARS**

Sample: 3.3 g

Analysis: Dissolve the *Sample* in 3 mL of water with the aid of gentle heat. Cool, and add 20.0 mL of cupric citrate TS and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 40 mL of diluted acetic acid, 60 mL of water, and 20.0 mL of 0.05 N iodine VS. With continuous shaking, add 25 mL of hydrochloric acid in water solution (6:94). When the precipitate has dissolved, titrate the excess of iodine with 0.05 N sodium thiosulfate VS using 2 mL of starch TS, added towards the end of the titration, as an indicator.

Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate VS is required, corresponding to NMT 0.3% of reducing sugars, as glucose. [NOTE—The amount determined in this test is not included in the calculated amount under *General Notices and Requirements, Other Impurities*.]

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count using the *Plate Method* does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g.

- **CONDUCTIVITY**

Sample solution: 200 mg/mL

Analysis: Using an appropriate conductivity meter, choose a conductivity cell that is appropriate for the properties and conductivity of the solution to be examined. Use a certified reference material, for example a solution of potassium chloride, that is appropriate for the measurement.¹

The conductivity value of the certified reference material should be near the expected conductivity value of the solution to be examined. After calibrating the apparatus

with a certified reference material solution, rinse the conductivity cell several times with water and at least twice with the aqueous solution to be examined. Measure the conductivity of the *Sample solution* at a temperature of 20°, while gently stirring with a magnetic stirrer.

Acceptance criteria: NMT 20 µS/cm

- **WATER DETERMINATION, Method I** (921): NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.
- **USP REFERENCE STANDARDS** (11)
USP Maltitol RS

Maltitol Solution

DEFINITION

Maltitol Solution is a water solution containing, on the anhydrous basis, NLT 50.0% of D-maltitol (C₁₂H₂₄O₁₁) (w/w) and NMT 8.0% of D-sorbitol (C₆H₁₄O₆) (w/w). The amounts of total sugars, other polyhydric alcohols, and any polyol anhydrides, if detected, are not included in the requirements nor in the calculated amount under *Other Impurities*.

IDENTIFICATION

- **A. PROCEDURE**

Sample: 1.4 g of Maltitol Solution

Analysis: Dissolve the *Sample* in 75 mL of water. Transfer 3 mL of this solution to a 15-cm test tube, add 3 mL of freshly prepared catechol (1 in 10), and mix. Add 6 mL of sulfuric acid, and mix. Gently heat the tube in a flame for about 30 s.

Acceptance criteria: A deep pink or wine-red color appears.

- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

- **C. LIMIT OF DIETHYLENE GLYCOL AND ETHYLENE GLYCOL**

Diluent: Acetone and water (96:4)

Standard stock solution: 0.5 mg/mL of USP Diethylene Glycol RS and 0.5 mg/mL of USP Ethylene Glycol RS in *Diluent*

Internal standard stock solution: 0.5 mg/mL of 1,3-butanediol (internal standard) in *Diluent*

Standard solution: 0.04 mg/mL of USP Diethylene Glycol RS, 0.04 mg/mL of USP Ethylene Glycol RS, and 0.04 mg/mL of 1,3-butanediol, in *Diluent*, prepared from the *Standard stock solution* and *Internal standard stock solution*

Sample solution: Transfer 1.0 g of Maltitol Solution to a 25-mL volumetric flask. Add 1.0 mL of water to the flask, and mix on a vortex mixer for 3 min. Add 2.0 mL of the *Internal standard stock solution* and 5 mL of *Diluent*, and mix on a vortex mixer for 3 min. Add the remaining *Diluent* to the flask to volume in two equal portions. Mix the contents for about 3 min after each addition of *Diluent*. Pass a portion of the supernatant layer through a nylon filter of 0.45-µm pore size. Discard the first 2 mL of the filtrate, and collect the rest of the filtrate for analysis. [NOTE—Acetone is used to precipitate maltitol.]

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 15-m fused-silica capillary column; 0.25-µm layer of phase G46

¹ Commercially available conductivity calibration solutions for conductivity meter standardization, standardized by methods traceable to the National Institute of Standards and Technology (NIST), may be used. Solutions prepared according to instructions given in ASTM Standard D1125 may be used provided the conductivity of the resultant solution is the same as that of the solution prepared from the NIST-certified material.

Temperature**Detector:** 300°**Injection port:** 240°**Column:** See temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	50	300	5

Carrier gas: Helium**Flow rate:** 3.0 mL/min**Injection size:** 1.0 µL**Injection type:** Split injection. The split ratio is about 10:1. [NOTE—A general purpose split/splitless, taper, glass wool, and deactivated liner is used.]**System suitability****Sample:** *Standard solution*

[NOTE—See the relative retention time table below.

Relative retention times are provided for information only, and the standards should be used to ensure appropriate peak identification.]

Name	Relative Retention Time
Ethylene glycol	1.0
1,3-Butanediol (internal standard)	2.2
Diethylene glycol	2.8

Suitability requirements**Resolution:** NLT 15 between ethylene glycol and 1,3-butanediol**Analysis****Samples:** *Standard solution* and *Sample solution*Based on the *Standard solution*, identify the peaks of ethylene glycol, 1,3-butanediol (internal standard), and diethylene glycol. Compare peak area ratios of ethylene glycol to the internal standard and of diethylene glycol to the internal standard in the *Standard solution* and *Sample solution*, respectively.**Acceptance criteria****Diethylene glycol:** The peak area ratio of diethylene glycol to the internal standard in the *Sample solution* is NMT the peak area ratio of diethylene glycol to the internal standard in the *Standard solution*, corresponding to NMT 0.10% of diethylene glycol in Maltitol Solution.**Ethylene glycol:** The peak area ratio of ethylene glycol to the internal standard in the *Sample solution* is NMT the peak area ratio of ethylene glycol to the internal standard in the *Standard solution*, corresponding to NMT 0.10% of ethylene glycol in Maltitol Solution.**ASSAY****• PROCEDURE****Mobile phase:** Water**Standard solution:** 10 mg/g of USP Maltitol RS and 1.6 mg/g of USP Sorbitol RS**Sample solution:** 20 mg/g of Maltitol Solution in water**Chromatographic system**(See *Chromatography* <621>, *System Suitability*.)**Mode:** LC**Detector:** Refractive index**Column:** 7.8-mm × 10-cm; packing L34**Temperature****Column:** 60 ± 2°**Detector:** 35°**Flow rate:** 0.5 mL/min**Injection size:** 10 µL**System suitability****Sample:** *Standard solution*

[NOTE—The relative retention times for maltotriitol, maltitol, and sorbitol are 0.38, 0.48, and 1.0, respectively.]

Suitability requirements**Tailing factor:** NMT 1.2 for maltitol and sorbitol**Relative standard deviation:** NMT 2.0%**Analysis****Samples:** *Standard solution* and *Sample solution*Calculate the percentage, on the anhydrous basis, of C₁₂H₂₄O₁₁ and C₆H₁₄O₆ in the portion taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times [100/(100 - W)] \times 100$$

 r_U = peak response of D-maltitol or D-sorbitol from the *Sample solution* r_S = peak response of D-maltitol or D-sorbitol from the *Standard solution* C_S = concentration of the appropriate USP Reference Standard in the *Standard solution* (mg/g) C_U = concentration of Maltitol Solution in the *Sample solution* (mg/g) W = percentage in the test for *Water Determination***Acceptance criteria:** NLT 50.0% of D-maltitol (w/w) and NMT 8.0% of D-sorbitol (w/w), on the anhydrous basis**IMPURITIES****Inorganic Impurities****• RESIDUE ON IGNITION (281):** NMT 0.1%, calculated on the anhydrous basis, determined on a 2-g portion**• LIMIT OF NICKEL****Solution A:** 10 mg/mL of ammonium pyrrolidine dithiocarbamate**Sample solution:** Dissolve and dilute 20.0 g of Maltitol Solution with diluted acetic acid to 100 mL. Add 2.0 mL of *Solution A* and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.**Standard solutions:** Prepare as directed for the *Sample solution*, except to prepare three solutions by adding 0.5, 1.0, and 1.5 mL of nickel standard solution TS.**Blank solution:** Prepare as directed for the *Sample solution*, except to omit the use of the Maltitol Solution.**Spectrometric conditions**(See *Spectrophotometry and Light-Scattering* <851>.)**Mode:** Atomic absorption spectrophotometry**Analytical wavelength:** 232.0 nm (maximum absorbance)**Lamp:** Nickel hollow-cathode**Flame:** Air-acetylene**Analysis****Samples:** *Sample solution*, *Standard solutions*, and *Blank solution*Set the instrument to zero using the *Blank solution*. Concomitantly determine the absorbances of the *Standard solutions* and the *Sample solution* at least three times each. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Between each measurement, aspirate the *Blank solution*, and ascertain that the reading returns to zero. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.**Acceptance criteria:** NMT 1 ppm, calculated on the anhydrous basis

Organic Impurities**• PROCEDURE: REDUCING SUGARS**

Sample: An amount of Maltitol Solution equivalent to 3.3 g on the anhydrous basis

Analysis: To the *Sample* add 3 mL of water, 20.0 mL of cupric citrate TS, and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 40 mL of diluted acetic acid, 60 mL of water, and 20.0 mL of 0.05 N iodine VS. With continuous shaking, add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water. When the precipitate has dissolved, titrate the excess of iodine with 0.05 N sodium thiosulfate VS using 2 mL of starch TS, added toward the end of the titration, as an indicator.

[NOTE—The amount determined in this test is not included in the calculated amount under *Other Impurities*.]

Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate VS is required, corresponding to NMT 0.3% of reducing sugars, on the anhydrous basis, as glucose.

SPECIFIC TESTS

- MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIC MICROORGANISMS (62):** The total aerobic microbial count using the *Plate Method* is NMT 1000 cfu/mL, and the total combined molds and yeasts count is NMT 100 cfu/mL.
- PH (791):** 5.0–7.5, in a 14% (w/w) solution of Maltitol Solution in carbon dioxide-free water
- WATER DETERMINATION, Method I (921):** NMT 31.5%

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.
- USP REFERENCE STANDARDS (11)**
 - USP Diethylene Glycol RS
 - USP Ethylene Glycol RS
 - USP Maltitol RS
 - USP Sorbitol RS

Maltodextrin

DEFINITION

Maltodextrin is a nonsweet, nutritive saccharide mixture of polymers that consists of D-glucose units, with a Dextrose Equivalent less than 20. It is prepared by the partial hydrolysis of a food grade starch with suitable acids and/or enzymes. It may be physically modified to improve its physical and functional characteristics.

ASSAY**• DEXTROSE EQUIVALENT**

Standard solution: 10 mg/mL of USP Dextrose RS

Sample solution: Transfer 5 g of Maltodextrin with the aid of hot water to a 100-mL volumetric flask, cool, add water to volume, and mix.

Analysis: Transfer 25.0-mL portions of alkaline cupric tartrate TS to each of two boiling flasks. Bring the contents of one flask to boiling within 2 min while titrating with the *Standard solution* to within 0.5 mL of the anticipated endpoint. Boil gently for 2 min. Continue to boil gently, add 2 drops of methylene blue solution (1 in 100), and complete the titration within 1 min by adding the *Standard solution* dropwise or in small increments until the blue color disappears, determined by viewing against a white background in daylight or under equivalent illumination. If more than 0.5 mL of the titrant was required after the addition of the indicator, repeat the titration, adding the necessary volume of titrant before adding the indicator. Bring the contents

of the second flask to boiling, and similarly titrate with the *Sample solution*.

Calculate the Dextrose Equivalent, on the dried basis, in the portion of Maltodextrin taken:

$$\text{Result} = [100/(1 - 0.01 \times A)] \times (C_S/C_U) \times (V_S/V_U)$$

- A* = percentage *Loss on Drying* of the Maltodextrin taken
- C_S* = concentration of USP Dextrose RS in the *Standard solution* (mg/mL)
- C_U* = concentration of Maltodextrin in the *Sample solution* (mg/mL)
- V_S* = titrated volume of the *Standard solution* (mL)
- V_U* = titrated volume of the *Sample solution* (mL)

Acceptance criteria: Less than 20 [NOTE—This is a limit test. For Maltodextrins with lower reducing values, other procedures may give other results.]

IMPURITIES

- RESIDUE ON IGNITION (281):** NMT 0.5%
- HEAVY METALS, Method II (231):** NMT 5 ppm
- LIMIT OF PROTEIN**

Sample: 10 g

Analysis: Transfer the *Sample* to an 800-mL Kjeldahl flask, and add 10 g of anhydrous potassium sulfate or sodium sulfate, 300 mg of copper selenite or mercuric oxide, and 60 mL of sulfuric acid. Gently heat the mixture, keeping the flask inclined at about a 45° angle, and after frothing has ceased, boil briskly until the solution has remained clear for about 1 h. Cool, and very cautiously add about 50 mL of water while swirling to dissipate the resulting heat. Add an additional 150–250 mL of water, mix, and cool again. Cautiously pour 75 mL (or enough to make the mixture strongly alkaline) of sodium hydroxide solution (2 in 5) down the inside of the flask so that it forms a layer under the acid solution, and then add a few pieces of granular zinc. Immediately connect the flask to a distillation apparatus consisting of a Kjeldahl connecting bulb and a condenser, the delivery tube of which extends well beneath the surface of an accurately measured excess of 0.1 N sulfuric acid contained in a 500-mL flask. Gently rotate the contents of the Kjeldahl flask to mix, and distill until all ammonia has passed into the absorbing acid solution (about 250 mL of distillate). To the receiving flask add 0.25 mL of methyl red–methylene blue TS, and titrate the excess acid with 0.1 N sodium hydroxide. Perform a blank determination, substituting pure sucrose or dextrose for the test specimen, and make any necessary correction. Each mL of 0.1 N sulfuric acid consumed is equivalent to 1.401 mg of nitrogen (N).

Calculate the percentage of N in the specimen taken, and then calculate the percentage of protein by multiplying the percentage of N by 6.25.

Acceptance criteria: NMT 0.1%

• LIMIT OF SULFUR DIOXIDE

Hydrogen peroxide solution: Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Just before use, add 3 drops of methyl red TS, and neutralize to a yellow endpoint with 0.01 N sodium hydroxide. Do not exceed the endpoint.

Nitrogen: Use high-purity nitrogen, with a flow regulator that will maintain a flow of 200 ± 10 mL/min. Guard against the presence of oxygen by passing the nitrogen through a scrubber, such as alkaline pyrogallol, prepared as follows. Add 4.5 g of pyrogallol to a gas-washing bottle, purge the bottle with nitrogen for 3 min, and add a solution containing 85 mL of water and 65 g of potassium hydroxide, while maintaining an atmosphere of nitrogen in the bottle.

Apparatus: The apparatus (see *Figure 1*) is designed to effect the selective transfer of sulfur dioxide from the specimen in boiling aqueous hydrochloric acid to the

Hydrogen peroxide solution. The backpressure is limited to the unavoidable pressure due to the height of the *Hydrogen peroxide solution* above the tip of the bubbler, *F*. Keeping the backpressure as low as possible reduces the likelihood that sulfur dioxide will be lost through leaks. Preboil vinyl and silicone tubing. Apply a thin film of stopcock grease to the sealing surfaces of all of the joints except the joint between the separatory funnel and the flask, and clamp the joints to ensure tightness. The separatory funnel, *B*, has a capacity of 100 mL or greater. The inlet adapter, *A*, with a hose connector provides a means of applying headpressure over the solution. [NOTE—A pressure-equalizing dropping funnel is not recommended because condensate, which may contain sulfur dioxide, is deposited in the funnel and the side arm.]

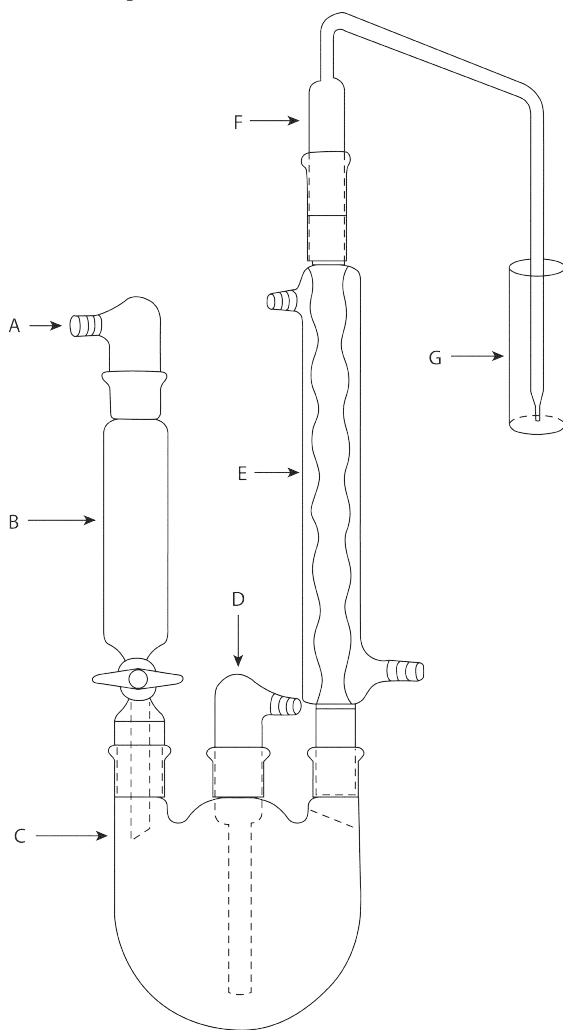


Figure 1. Apparatus for the Sulfur Dioxide Test.

The round-bottomed flask, *C*, is a 1000-mL flask with three 24/40 tapered joints. The gas inlet tube, *D*, is long enough to permit introduction of the nitrogen within 2.5 cm of the bottom of the flask. The Allihn condenser, *E*, has a jacket length of 300 mm. The bubbler, *F*, is fabricated from glass according to the dimensions given in Figure 2. The *Hydrogen peroxide solution* is contained in a vessel, *G*, having an inside diameter of about 2.5 cm and a depth of about 18 cm. Circulate coolant, such as a mixture of water and methanol (4:1) maintained at 5°, to chill the condenser.

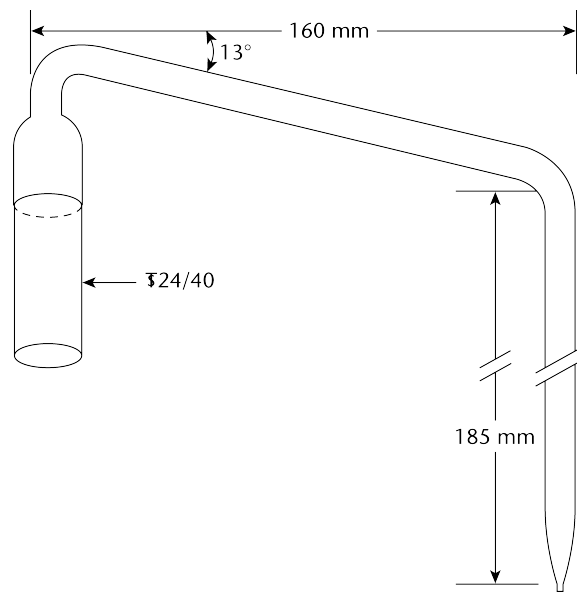


Figure 2. Bubbler (*F*) for the Sulfur Dioxide Apparatus.

Analysis: Position the *Apparatus* in a heating mantle controlled by a power-regulating device. Add 400 mL of water to the flask. Close the stopcock of the separatory funnel, and add 90 mL of 4 N hydrochloric acid to the separatory funnel. Begin the flow of *Nitrogen* at a rate of 200 ± 10 mL/min. Start the condenser coolant flow. Add 30 mL of the *Hydrogen peroxide solution* to vessel *G*. After 15 min, remove the separatory funnel, and transfer a mixture of 50.0 g of Maltodextrin and 100 mL of alcohol solution (5 in 100). Apply stopcock grease to the outer joint of the separatory funnel, return the separatory funnel to the tapered joint flask, and concomitantly resume the nitrogen flow. Apply headpressure above the hydrochloric acid solution in the separatory funnel with a rubber bulb equipped with a valve. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the flask. Continue to maintain sufficient pressure above the hydrochloric acid solution to force it into the flask. [NOTE—The stopcock may be temporarily closed, if necessary, to pump up the pressure.]

To guard against escape of sulfur dioxide (SO_2) into the separatory funnel, close the stopcock before the last few mL of hydrochloric acid drain out. Apply power to the heating mantle sufficient to cause about 85 drops of reflux/min. After refluxing for 1.75 h, remove vessel *G*, add 3 drops of methyl red TS, and titrate the contents with 0.01 N sodium hydroxide VS, using a 10-mL buret with an overflow tube and a hose connection to a carbon dioxide-absorbing tube, to a yellow endpoint that persists for at least 20 s. Perform a blank determination, and make any necessary correction (see *Titrimetry* (541)).

Calculate the quantity, in $\mu\text{g/g}$, of SO_2 in the portion of Maltodextrin taken:

$$\text{Result} = 1000 \times F \times V \times N/W$$

F = milliequivalent weight of sulfur dioxide, 32.03
V = titrant volume consumed (mL)
N = actual normality of the titrant
W = weight of Maltodextrin taken (g)

Acceptance criteria: NMT 40 $\mu\text{g/g}$ (ppm)

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.

- **pH** (791): 4.0–7.0, in a 0.2-g/mL solution in carbon dioxide-free water.
- **Loss on Drying** (731): Dry a sample at 105° for 2 h in a forced-air oven: it loses NMT 6.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, or in well-closed containers, at a temperature not exceeding 30° and a relative humidity not exceeding 50%.
- **USP REFERENCE STANDARDS** (11)
USP Dextrose RS

Maltol

$C_6H_6O_3$ 126.11
3-Hydroxy-2-methyl-4-pyrone [118-71-8].

DEFINITION

Maltol contains NLT 99.0% of maltol ($C_6H_6O_3$), calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B. ULTRAVIOLET ABSORPTION** (197U)
Sample solution: 0.01 mg/mL in 0.1 N hydrochloric acid
Blank: 0.1 N hydrochloric acid

ASSAY• **PROCEDURE**

Standard solution: 0.01 mg/mL of USP Maltol RS in 0.1 N hydrochloric acid
Sample solution: 0.01 mg/mL of Maltol in 0.1 N hydrochloric acid

Instrumental conditions

Mode: UV

Analytical wavelength: Maximum at about 274 nm

Blank: 0.1 N hydrochloric acid

Analysis

Samples: Standard solution and Sample solution

Calculate the percentage of maltol ($C_6H_6O_3$) in the portion of Maltol taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the Sample solution

A_S = absorbance of the Standard solution

C_S = concentration of USP Maltol RS in the Standard solution (mg/mL)

C_U = concentration of the Sample solution (mg/mL)

Acceptance criteria: NLT 99.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.2%, determined on 1.0 g
- **LEAD** (251): NMT 10 ppm
- **HEAVY METALS**, Method II (231): NMT 20 ppm

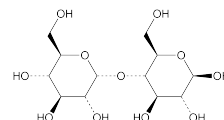
SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE**, Class Ia (741): 160°–164°
- **WATER DETERMINATION**, Method I (921): NMT 0.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. No storage requirements are specified.

- **USP REFERENCE STANDARDS** (11)
USP Maltol RS

Maltose

$C_{12}H_{22}O_{11} \cdot H_2O$ 360.31

$C_{12}H_{22}O_{11}$ 342.30

4-O- α -D-Glucopyranosyl- β -D-glucopyranose [69-79-4].

4-O- α -D-Glucopyranosyl- β -D-glucopyranose monohydrate [6363-53-7].

DEFINITION

Maltose is a sugar. It contains one molecule of water of hydration or is anhydrous. It contains NLT 92.0% of maltose, calculated on the anhydrous basis. The amounts of other sugars, if detected, are not included in the requirements or the calculated amount in *General Notices and Requirements*, 5.60.10 *Other Impurities in USP and NF Articles*.

IDENTIFICATION

- **A.**
Sample solution: 50 mg/mL
Analysis: Add 2–3 drops of the Sample solution to 5 mL of hot alkaline cupric tartrate TS.
Acceptance criteria: A red precipitate is formed.
- **B.** The retention time of the major peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY• **PROCEDURE**

Mobile phase: Water

System suitability solution: 10 mg/g each of maltotriose, maltose, and glucose

Standard solution: Dissolve USP Maltose Monohydrate RS in water to obtain a solution having a concentration of about 10 mg/g. Calculate the exact concentration on the anhydrous basis.

Sample solution: Dissolve 0.10 g of Maltose in water, and dilute with water to 10 g. Record the final solution weight, and mix thoroughly.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm \times 30-cm; packing L58

Temperatures

Column: 80 \pm 2°

Detector: 40°

Flow rate: 0.35 mL/min; adjust so that the resolution between maltotriose and maltose is NLT 1.6.

Injection volume: 20 μ L

System suitability

Samples: System suitability solution and Standard solution

[NOTE—The relative retention times for maltotriose, maltose, and glucose are about 0.9, 1.0, and 1.2, respectively.]

Suitability requirements

Resolution: NLT 1.6 between maltotriose and maltose, System suitability solution

Relative standard deviation: NMT 2.0%, Standard solution

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate, on the anhydrous basis, the percentage of Maltose taken:

$$\text{Result} = [(r_U/r_S) \times (C_S/C_U)] / [(100 - W)/100] \times 100$$

r_U = peak response from the *Sample solution*
 r_S = peak response from the *Standard solution*
 C_S = concentration of USP Maltose Monohydrate RS in the *Standard solution*, on the anhydrous basis (mg/g)
 C_U = concentration of Maltose in the *Sample solution* (mg/g)
 W = percentage of water from the test for *Water Determination*

Acceptance criteria: NLT 92.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** <281>
Sample: 2 g
Acceptance criteria: NMT 0.05%
- **HEAVY METALS**, *Method I* <231>: NMT 5 µg/g

SPECIFIC TESTS

- **DEXTRIN, STARCH, AND SULFITE**
Sample solution: 1.0 g of Maltose in 10 mL of water
Analysis: Add 1 drop of iodine TS to the *Sample solution*.
Acceptance criteria: A yellow color develops. Then add 1 drop of starch TS; a blue color develops.
- **pH** <791>
Sample solution: 100 mg/mL in carbon dioxide-free water
Acceptance criteria
Anhydrous form: 3.7–4.7
Monohydrate form: 4.0–5.5
- **WATER DETERMINATION**, *Method I* <921>
Anhydrous: NMT 1.5%
Monohydrate: 4.5%–6.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
- **USP REFERENCE STANDARDS** <11>
USP Maltose Monohydrate RS

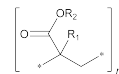
Mannitol—see *Mannitol General Monographs*

Meglumine—see *Meglumine General Monographs*

Menthol—see *Menthol General Monographs*

Methacrylic Acid Copolymer

(Any article currently titled *Methacrylic Acid Copolymer, Type A or Type B*, will be officially titled *Methacrylic Acid and Methyl Methacrylate Copolymer* after December 1, 2015. Any article currently titled *Methacrylic Acid Copolymer, Type C*, will be officially titled *Methacrylic Acid and Ethyl Acrylate Copolymer* after December 1, 2015. After December 1, 2015, the *Methacrylic Acid Copolymer monograph* will no longer be valid.)



Type	R ₁	R ₂	Ratio
A	CH ₃	H or CH ₃	1:1
B	CH ₃	H or CH ₃	1:2
C	CH ₃	H	1:1
or	H	C ₂ H ₅	

Type A or Type B: Poly(methacrylic acid, methyl methacrylate);
Methacrylic acid–methyl methacrylate copolymer [25086-15-1].
Type C: Poly(methacrylic acid, ethyl acrylate);
Methacrylic acid–ethyl acrylate copolymer [25212-88-8].

DEFINITION

Methacrylic Acid Copolymer consists of methacrylic acid and methyl methacrylate monomers arranged in a random distribution or consists of methacrylic acid and ethyl acrylate monomers arranged in a random distribution. It may contain suitable surface-active agents. The *Assay* and *Viscosity* requirements differ for the three types, as described in the table below.

Type	Methacrylic Acid Units, Dried Basis (%)		Viscosity mPa·s	
	Min.	Max.	Min.	Max.
A	46.0	50.6	60	120
B	27.6	30.7	50	200
C	46.0	50.6	100	200

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>
- **B. PROCEDURE:** It meets the requirements of the test for *Assay*.

ASSAY

- **PROCEDURE:**
Sample: 1 g, previously dried
Analysis: Dissolve the *Sample* in 100 mL of neutralized acetone and titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically (see *Titrimetry* <541>). Each mL of 0.1 N sodium hydroxide is equivalent to 8.609 mg of methacrylic acid (C₄H₆O₂) units.
Acceptance criteria
Type A: 46.0%–50.6%
Type B: 27.6%–30.7%
Type C: 46.0%–50.6%

IMPURITIES**Inorganic Impurities**

- **RESIDUE ON IGNITION** <281>: NMT 0.1% for Types A and B; NMT 0.4% for Type C
- **HEAVY METALS**, *Method II* <231>: NMT 20 ppm

Organic Impurities**PROCEDURE: LIMIT OF MONOMERS**

Phosphate buffer: Prepare an aqueous solution containing 17.8 g/L of anhydrous dibasic sodium phosphate and 17.0 g/L of monobasic potassium phosphate. Adjust with phosphoric acid to a pH of 2.0. This buffer has a concentration of 0.125 M.

Sodium perchlorate solution: 35 mg/mL of sodium perchlorate. This solution has a concentration of 0.25 M.

Mobile phase: Add phosphoric acid dropwise to water to obtain a solution having a pH of 2.0. Prepare a mixture of this acidified water and methanol (80:20), and degas.

Standard solution for Type A or Type B: Dissolve 0.05 g of methacrylic acid and 0.05 g of methyl methacrylate in 5 mL of butanol, and add methanol to exactly 100 mL. Transfer 1.0 mL of this solution to a 100-mL volumetric flask. Dilute with methanol to volume. Mix 3.0 mL of this solution with 10.0 mL of *Phosphate buffer*. This solution contains 1.15 µg/mL each of methacrylic acid and methyl methacrylate.

Standard solution for Type C: Dissolve 0.01 g of methacrylic acid and 0.01 g of ethyl acrylate in 5 mL of butanol, and add methanol to exactly 100 mL. Transfer 1.0 mL of this solution to a 100-mL volumetric flask. Dilute with methanol to volume. Mix 5.0 mL of this solution with 5.0 mL of *Sodium perchlorate solution*. This solution contains about 0.5 µg/mL each of methacrylic acid and ethyl acrylate.

Sample solution for Type A or Type B: Transfer 1 g of Methacrylic Acid Copolymer, Type A or Type B, to a 50-mL volumetric flask, dilute with methanol to volume, and mix. Add 3 mL of this solution dropwise, while continuously stirring, to a beaker that contains 10.0 mL of *Phosphate buffer*. Remove the precipitated polymer to obtain a clear supernatant by centrifugation (e.g., NLT 5000 × g for NLT 5 min). Use the clear supernatant.

Sample solution for Type C: Transfer 3 g of Methacrylic Acid Copolymer, Type C, to a 50-mL volumetric flask, dilute with methanol to volume, and mix. Add 5 mL of this solution dropwise, while continuously stirring, to a beaker that contains 5.0 mL of *Sodium perchlorate solution*. Remove the precipitated polymer to obtain a clear supernatant by centrifugation (e.g., NLT 5000 × g for NLT 5 min). Use the clear supernatant.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 202 nm

Column: 4.0-mm × 12.5-cm analytical column; 7-µm packing L1

Flow rate: 2 mL/min

Injection size: 20 µL

For Type A or Type B

System suitability

Sample: *Standard solution for Type A or Type B*

[NOTE—The relative retention times for methacrylic acid and methyl methacrylate are 1.0 and 2.8, respectively.]

Suitability requirements

Resolution: NLT 2.0 between methacrylic acid and methyl methacrylate

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution for Type A or Type B* and *Sample solution for Type A or Type B*

Calculate the percentage of each monomer (methacrylic acid or methyl methacrylate) in the portion of Methacrylic Acid Copolymer Type A or Type B taken:

$$\text{Result} = (r_U/r_S) \times (C/W) \times V_F \times D \times F \times 100$$

r_U = monomer (methacrylic acid or methyl methacrylate) peak response from the *Sample solution for Type A or Type B*

r_S = monomer (methacrylic acid or methyl methacrylate) peak response from the *Standard solution for Type A or Type B*

C = concentration of the monomer (methacrylic acid or methyl methacrylate) in the *Standard solution for Type A or Type B* (µg/mL)

W = weight of Methacrylic Acid Copolymer Type A or Type B taken to prepare the *Sample solution for Type A or Type B* (g)

V_F = final volume of the *Sample solution for Type A or Type B*, 13 mL

D = dilution factor for preparation of the *Sample solution for Type A or Type B*, 16.7

F = conversion factor, 10^{-6} g/µg

Acceptance criteria: NMT 0.05% for the total amount of monomers

For Type C

System suitability

Sample: *Standard solution for Type C*

[NOTE—The relative retention times for methacrylic acid and ethyl acrylate are 1.0 and 2.6, respectively.]

Suitability requirements

Resolution: NLT 2.0 between methacrylic acid and ethyl acrylate

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution for Type C* and *Sample solution for Type C*

Calculate the percentage of each monomer (methacrylic acid or ethyl acrylate) in the portion of Methacrylic Acid Copolymer Type C taken:

$$\text{Result} = (r_U/r_S) \times (C/W) \times V_F \times D \times F \times 100$$

r_U = monomer (methacrylic acid or ethyl acrylate) peak response from the *Sample solution for Type C*

r_S = monomer (methacrylic acid or ethyl acrylate) peak response from the *Standard solution for Type C*

C = concentration of the monomer (methacrylic acid or ethyl acrylate) in the *Standard solution for Type C* (µg/mL)

W = weight of Methacrylic Acid Copolymer Type C taken to prepare the *Sample solution for Type C* (g)

V_F = final volume of the *Sample solution for Type C*, 10 mL

D = dilution factor for preparation of the *Sample solution for Type C*, 10

F = conversion factor, 10^{-6} g/µg

Acceptance criteria: NMT 0.01% for the total amount of monomers

SPECIFIC TESTS

• ROTATIONAL RHEOMETER METHODS (912)

Analysis: Place 254.6 g of isopropyl alcohol and 7.9 g of water in a test flask. Add a quantity of Methacrylic Acid Copolymer, equivalent to 37.5 g of solids on the dried basis, while stirring by means of a magnetic stirrer. Close the flask, and continue stirring until the polymer has dissolved completely. Adjust the temperature to $20 \pm 0.1^\circ$. Equip a rotational viscometer with an accessory.¹ The shear rate under the test condition is NLT 1 s^{-1} and NMT 100 s^{-1} . Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria

Type A: 60–120 mPa · s

Type B: 50–200 mPa · s

Type C: 100–200 mPa · s

• **LOSS ON DRYING (731):** Dry a sample at 110° for 6 h: it loses NMT 5.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. Store at controlled room temperature.

• **LABELING:** Label it to state whether it is Type A, B, or C. The labeling also indicates the name and quantity of any added surface-active agent.

¹ A suitable accessory is available from Brookfield Engineering as the LV1 spindle, a cylindrical spindle 1.9 cm in diameter and 6.5 cm high attached to a shaft 0.3 cm in diameter. The spindle rotates at 30 rpm at an immersion depth of 8.15 cm.

- **USP REFERENCE STANDARDS** <11>
USP Methacrylic Acid Copolymer, Type A RS
USP Methacrylic Acid Copolymer, Type B RS
USP Methacrylic Acid Copolymer, Type C RS

Methacrylic Acid Copolymer Dispersion

(Title for this monograph—not to change until May 1, 2017)

(Prior to May 1, 2017, the current practice of labeling the article of commerce with the name *Methacrylic Acid Copolymer Dispersion* may be continued. Use of the name *Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion* will be permitted as of May 1, 2012, but the use of this name will not be mandatory until May 1, 2017. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)

DEFINITION

Methacrylic Acid Copolymer Dispersion is an aqueous dispersion of Methacrylic Acid and Ethyl Acrylate Copolymer in water. It contains, on the basis of the calculated amount of dry substance in the Dispersion, NLT 46.0% and NMT 50.6% of methacrylic acid units. It may contain suitable surface-active agents.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>: Proceed as directed, except to use the residue obtained in the test for *Loss on Drying* as the sample.
- **B.** It meets the requirements of the Assay.

ASSAY

PROCEDURE

Sample: 2.5 g of the Dispersion

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Dissolve the *Sample* in 100 mL of neutralized acetone. Titrate the solution as directed in *Titrimetric system*. Each mL of 0.1 N sodium hydroxide is equivalent to 8.609 mg of methacrylic acid (C₄H₆O₂) units. Calculate, on the dried basis, the percentage of methacrylic acid units in the portion of Dispersion taken:

$$\text{Result} = [V \times N/W \times (100 - L)] \times 860.9$$

V = volume of titrant consumed (mL)

N = normality of the titrant

W = weight of Dispersion taken (g)

L = percentage of the *Loss on Drying* value for the Dispersion

Acceptance criteria: 46.0%–50.6% based on the calculated amount of dry substance in the Dispersion

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** <281>: Using mild heating conditions (e.g., steam bath, sand bath) to avoid loss of material, evaporate the Dispersion to dryness prior to ignition: NMT 0.2% residue is obtained, calculated on the undried Dispersion basis.
- **HEAVY METALS, Method II** <231>: Using mild heating conditions (e.g., steam bath, sand bath) to avoid loss of material, evaporate the Dispersion to dryness prior to wetting with sulfuric acid and ignition: the color of the solution from the *Test Preparation* is not darker than that of the solution from the *Standard Preparation* (20 ppm).

Organic Impurities

LIMIT OF MONOMERS

Sodium perchlorate solution: Dissolve 3.5 g of sodium perchlorate in 100 mL of water. This solution has a concentration of 0.25 M.

Mobile phase: Add phosphoric acid dropwise to water to obtain a solution having a pH of 2.0. Prepare a mixture of this acidified water and methanol (80:20), and degas.

Standard solution: Dissolve 0.01 g of methacrylic acid and 0.01 g of ethyl acrylate in 5 mL of butanol, and add methanol to make exactly 100 mL. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, and dilute with methanol to volume. Mix 5.0 mL of this solution with 5.0 mL of *Sodium perchlorate solution*, accurately measured. This solution contains about 0.5 µg/mL each of methacrylic acid and ethyl acrylate.

Sample solution: Transfer a quantity of Dispersion, equivalent to 3 g of solids on the dried basis, to a 50-mL volumetric flask, dilute with methanol to volume, and mix. Add 5 mL of this solution dropwise while continuously stirring into a beaker that contains 5.0 mL of *Sodium perchlorate solution*, accurately measured. Remove the precipitated polymer by centrifugation (e.g., NLT 5000 × *g* for NLT 5 min). Use the clear supernatant.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 202 nm

Column: 4.0-mm × 12.5-cm; 7-µm packing L1

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for methacrylic acid and ethyl acrylate are 1.0 and 2.6, respectively.]

Suitability requirements

Resolution: NLT 2.0 between methacrylic acid and ethyl acrylate

Relative standard deviation: NMT 5.0%, determined for each analyte

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each monomer in the solid portion of the Dispersion taken:

$$\text{Result} = (r_U/r_S) \times (C/W) \times V_f \times D \times F \times 100$$

r_U = monomer (methacrylic acid or ethyl acrylate) peak response from the *Sample solution*

r_S = monomer (methacrylic acid or ethyl acrylate) peak response from the *Standard solution*

C = concentration of the monomer (methacrylic acid or ethyl acrylate) in the *Standard solution* (µg/mL)

W = solid weight of the Dispersion, calculated on the dried basis, taken to prepare the *Sample solution* (g)

V_f = final volume of the *Sample solution*, 10 mL

D = dilution factor for preparation of the *Sample solution*, 10

F = conversion factor, 10⁻⁶ g/µg

Acceptance criteria: NMT 0.01% of total monomers, based on the weight of the solid portion of the Dispersion taken

SPECIFIC TESTS

- **COAGULUM CONTENT:** Weigh a stainless steel sieve having 90-µm openings or a suitable single-woven wire cloth with a mesh width of 90 µm, and filter 100 g of the Dispersion through it. [NOTE—Suitable single-woven wire cloth mesh meets the requirements set in ISO 9044.] Wash the sieve or the cloth with distilled water until a clear filtrate is obtained, and dry the sieve or the cloth to

constant weight at 110°: the weight of the residue does not exceed 1000 mg (1%).

- **LOSS ON DRYING** (731): Dry a sample at 110° for 6 h: it loses 68.5%–71.5% of its weight.
 - **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
 - **PH** (791): 2.0–3.0
 - **ROTATIONAL RHEOMETER METHODS** (912): Equip a suitable rotational viscometer with an adapter comprising a cylindrical spindle rotating within an accurately machined chamber (or tube).¹ Mix the Dispersion, pipet the volume of test specimen recommended by the instrument manufacturer into the chamber (or tube), and ensure that the temperature of the test specimen is at $20 \pm 0.1^\circ$. The shear rate under the test condition is NLT 1 s^{-1} and NMT 100 s^{-1} .² Measure the apparent viscosity following the instrument manufacturer's directions.
- Acceptance criteria:** The viscosity is between 2 and 15 mPa · s.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. Store at controlled room temperature. Protect from freezing.
- **LABELING:** The label indicates the name and amount of any substance added as a surface-active agent.
- **USP REFERENCE STANDARDS** (11)
USP Methacrylic Acid and Ethyl Acrylate Copolymer (1:1) RS (USP Methacrylic Acid Copolymer, Type C RS)

Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion

(Title for this monograph—to become official May 1, 2017)

(Prior to May 1, 2017, the current practice of labeling the article of commerce with the name *Methacrylic Acid Copolymer Dispersion* may be continued. Use of the name *Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion* will be permitted as of May 1, 2012, but the use of this name will not be mandatory until May 1, 2017. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)

DEFINITION

Methacrylic Acid and Ethyl Acrylate Copolymer Dispersion is an aqueous dispersion of Methacrylic Acid and Ethyl Acrylate Copolymer. It contains, on the basis of the calculated amount of dry substance in the Dispersion, NLT 46.0% and NMT 50.6% of methacrylic acid units. It may contain suitable surface-active agents.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K): Proceed as directed, except to use the residue obtained in the test for *Loss on Drying* as the sample.
- **B.** It meets the requirements of the Assay.

ASSAY

PROCEDURE

Sample: 2.5 g of the Dispersion
Titrimetric system
(See *Titrimetry* (541).)

¹ A commercial device is available from Brookfield as an ultra-low (UL) viscosity adapter. The adapter comprises a 0.4-cm diameter shaft, an accurately machined chamber (or tube) with an internal diameter of 2.8 cm and a depth of 13.5 cm, and a cylindrical spindle 2.5 cm in diameter and 9.1 cm in height.

² The cylindrical spindle rotates at 30 rpm.

Mode: Direct titration

Titrant: 0.1 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Dissolve the *Sample* in 100 mL of neutralized acetone. Titrate the solution as directed in *Titrimetric system*. Each mL of 0.1 N sodium hydroxide is equivalent to 8.609 mg of methacrylic acid ($\text{C}_4\text{H}_6\text{O}_2$) units. Calculate, on the dried basis, the percentage of methacrylic acid units in the portion of Dispersion taken:

$$\text{Result} = [V \times N/W \times (100 - L)] \times 860.9$$

V = volume of titrant consumed (mL)

N = normality of the titrant

W = weight of Dispersion taken (g)

L = percentage of the *Loss on Drying* value for the Dispersion

Acceptance criteria: 46.0%–50.6% based on the calculated amount of dry substance in the Dispersion

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): Using mild heating conditions (e.g., steam bath, sand bath) to avoid loss of material, evaporate the Dispersion to dryness prior to ignition: NMT 0.2% residue is obtained, calculated on the undried Dispersion basis.
- **HEAVY METALS, Method II** (231): Using mild heating conditions (e.g., steam bath, sand bath) to avoid loss of material, evaporate the Dispersion to dryness prior to wetting with sulfuric acid and ignition: the color of the solution from the *Test Preparation* is not darker than that of the solution from the *Standard Preparation* (20 ppm).

Organic Impurities

LIMIT OF MONOMERS

Sodium perchlorate solution: Dissolve 3.5 g of sodium perchlorate in 100 mL of water. This solution has a concentration of 0.25 M.

Mobile phase: Add phosphoric acid dropwise to water to obtain a solution having a pH of 2.0. Prepare a mixture of this acidified water and methanol (80:20), and degas.

Standard solution: Dissolve 0.01 g of methacrylic acid and 0.01 g of ethyl acrylate in 5 mL of butanol, and add methanol to make exactly 100 mL. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, and dilute with methanol to volume. Mix 5.0 mL of this solution with 5.0 mL of *Sodium perchlorate solution*, accurately measured. This solution contains about 0.5 µg/mL each of methacrylic acid and ethyl acrylate.

Sample solution: Transfer a quantity of Dispersion, equivalent to 3 g of solids on the dried basis, to a 50-mL volumetric flask, dilute with methanol to volume, and mix. Add 5 mL of this solution dropwise while continuously stirring into a beaker that contains 5.0 mL of *Sodium perchlorate solution*, accurately measured. Remove the precipitated polymer by centrifugation (e.g., NLT $5000 \times g$ for NLT 5 min). Use the clear supernatant.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 202 nm

Column: 4.0-mm × 12.5-cm; 7-µm packing L1

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for methacrylic acid and ethyl acrylate are 1.0 and 2.6, respectively.]

Suitability requirements

Resolution: NLT 2.0 between methacrylic acid and ethyl acrylate

Relative standard deviation: NMT 5.0%, determined for each analyte

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each monomer in the solid portion of the Dispersion taken:

$$\text{Result} = (r_U/r_S) \times (C/W) \times V_F \times D \times F \times 100$$

- r_U = monomer (methacrylic acid or ethyl acrylate) peak response from the *Sample solution*
 r_S = monomer (methacrylic acid or ethyl acrylate) peak response from the *Standard solution*
 C = concentration of the monomer (methacrylic acid or ethyl acrylate) in the *Standard solution* ($\mu\text{g/mL}$)
 W = solid weight of the Dispersion, calculated on the dried basis, taken to prepare the *Sample solution* (g)
 V_F = final volume of the *Sample solution*, 10 mL
 D = dilution factor for preparation of the *Sample solution*, 10
 F = conversion factor, 10^{-6} g/ μg

Acceptance criteria: NMT 0.01% of total monomers, based on the weight of the solid portion of the Dispersion taken

SPECIFIC TESTS

- **COAGULUM CONTENT:** Weigh a stainless steel sieve having 90- μm openings or a suitable single-woven wire cloth with a mesh width of 90 μm , and filter 100 g of the Dispersion through it. [NOTE—Suitable single-woven wire cloth mesh meets the requirements set in ISO 9044.] Wash the sieve or the cloth with distilled water until a clear filtrate is obtained, and dry the sieve or the cloth to constant weight at 110°: the weight of the residue does not exceed 1000 mg (1%).
- **LOSS ON DRYING** (731): Dry a sample at 110° for 6 h: it loses 68.5%–71.5% of its weight.
- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.
- **PH** (791): 2.0–3.0
- **ROTATIONAL RHEOMETER METHODS** (912): Equip a suitable rotational viscometer with an adapter comprising a cylindrical spindle rotating within an accurately machined chamber (or tube).¹ Mix the Dispersion, pipet the volume of test specimen recommended by the instrument manufacturer into the chamber (or tube), and ensure that the temperature of the test specimen is at $20 \pm 0.1^\circ$. The shear rate under the test condition is NLT 1 s^{-1} and NMT 100 s^{-1} .² Measure the apparent viscosity following the instrument manufacturer's directions.
Acceptance criteria: The viscosity is between 2 and 15 mPa · s.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. Store at controlled room temperature. Protect from freezing.
- **LABELING:** The label indicates the name and amount of any substance added as a surface-active agent.
- **USP REFERENCE STANDARDS** (11)
 USP Methacrylic Acid and Ethyl Acrylate Copolymer (1:1) RS (USP Methacrylic Acid Copolymer, Type C RS)

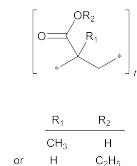
¹ A commercial device is available from Brookfield as an ultra-low (UL) viscosity adapter. The adapter comprises a 0.4-cm diameter shaft, an accurately machined chamber (or tube) with an internal diameter of 2.8 cm and a depth of 13.5 cm, and a cylindrical spindle 2.5 cm in diameter and 9.1 cm in height.

² The cylindrical spindle rotates at 30 rpm.

Methacrylic Acid and Ethyl Acrylate Copolymer

(Title for this new monograph—to become official December 1, 2015)

(Prior to December 1, 2015, the current practice of labeling the article of commerce with the name *Methacrylic Acid Copolymer, Type C*, may be continued. Use of the name *Methacrylic Acid and Ethyl Acrylate Copolymer* will be permitted as of December 1, 2010, but the use of this name will not be mandatory until December 1, 2015. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)



Poly(methacrylic acid, ethyl acrylate);
 Methacrylic acid–ethyl acrylate copolymer [25212-88-8].

DEFINITION

Methacrylic Acid and Ethyl Acrylate Copolymer consists of methacrylic acid and ethyl acrylate monomers arranged in a random distribution. Methacrylic acid units in Methacrylic Acid and Ethyl Acrylate Copolymer are NLT 46.0% and NMT 50.6%, calculated on the dried basis. It may contain suitable surface-active agents.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
 Use USP Methacrylic Acid and Ethyl Acrylate Copolymer (1:1) RS for Methacrylic Acid and Ethyl Acrylate Copolymer having a range of 46.0%–50.6% for methacrylic acid units.
- **B.** It meets the requirements of the Assay.

ASSAY**PROCEDURE**

Sample: 1 g, previously dried

Analysis: Dissolve the *Sample* in 100 mL of neutralized acetone, and titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically (see *Titrimetry* (541)). Each mL of 0.1 N sodium hydroxide is equivalent to 8.609 mg of methacrylic acid ($\text{C}_4\text{H}_6\text{O}_2$) units.

Acceptance criteria: 46.0%–50.6% for Methacrylic Acid and Ethyl Acrylate Copolymer on the dried basis

IMPURITIES**Inorganic Impurities**

- **RESIDUE ON IGNITION** (281): NMT 0.4%
- **HEAVY METALS, Method II** (231): NMT 20 ppm

Organic Impurities

- **PROCEDURE: LIMIT OF METHACRYLIC ACID AND ETHYL ACRYLATE**

Sodium perchlorate solution: 35 mg/mL of sodium perchlorate. This solution has a concentration of 0.25 M.

Mobile phase: Add phosphoric acid dropwise to water to obtain a solution having a pH of 2.0. Prepare a mixture of this acidified water and methanol (80:20) and degas.

Standard solution: Dissolve 0.01 g of methacrylic acid and 0.01 g of ethyl acrylate in 5 mL of butanol and add methanol to exactly 100 mL. Transfer 1.0 mL of this solution to a 100-mL volumetric flask. Dilute with methanol to volume. Mix 5.0 mL of this solution with 5.0 mL of *Sodium perchlorate solution*. This solution contains about 0.5 $\mu\text{g/mL}$ each of methacrylic acid and ethyl acrylate.

IMPURITIES**Inorganic Impurities**

- **RESIDUE ON IGNITION** (281): 2.0%–3.5%
- **HEAVY METALS**, *Method II* (231): NMT 20 ppm

Organic Impurities

- **PROCEDURE: LIMIT OF METHACRYLIC ACID AND ETHYL**

ACRYLATE

Phosphoric acid solution: 0.1% phosphoric acid prepared from phosphoric acid

Mobile phase: Methanol and *Phosphoric acid solution* (3:7)

Standard solution: 1.0 µg/mL each of methacrylic acid and ethyl acrylate in methanol

Sample solution: Transfer 0.5 g of Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer to a 25-mL volumetric flask, and dissolve in 20 mL of methanol. Add *Phosphoric acid solution* dropwise to precipitate the polymer while continuously stirring until the volume of 25 mL is reached. [NOTE—Stir with a magnetic stirrer for 10 min. Any volume deviation caused by the precipitation is negligible for contents in the ppm range. Use a magnetic stirrer of appropriate size to avoid a large variance from the final volume of the *Sample solution*.] As soon as the solid matter has settled, pass the supernatant through a filter of 0.45-µm pore size. [NOTE—Solution that cannot be filtered is centrifuged at $NLT\ 20,000 \times g$ for $NLT\ 30\ min$.] Use the clear supernatant.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.0-mm \times 12.5-cm analytical column, 5-µm packing L1 or 4.6-mm \times 15.0-cm analytical column, 5-µm packing L1

Flow rate: 1.2 mL/min

Injection size: 20 µL

[NOTE—Where appropriate, the volume must be adapted to the sensitivity of the detector.]

[NOTE—Column switching system may be used for extension of column lifetime.]

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for methacrylic acid and ethyl acrylate are 1.0 and 2.2, respectively.]

Suitability requirements

Resolution: $NLT\ 5.0$ between methacrylic acid and ethyl acrylate

Relative standard deviation: $NMT\ 5.0\%$

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each monomer (methacrylic acid or ethyl acrylate) in the portion of Methacrylic Acid and Ethyl Acrylate Copolymer taken:

$$\text{Result} = (r_U/r_S) \times (C_S/W) \times V_F \times F \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of the *Standard solution* (µg/mL)

W = weight of Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer taken to prepare the *Sample solution* (g)

V_F = final volume of the *Sample solution*, 25 mL

F = conversion factor, $10^{-6}\ g/\mu g$

Acceptance criteria: $NMT\ 0.01\%$ for the total amount of monomers

SPECIFIC TESTS

- **LOSS ON DRYING** (731): Dry a sample at 110° for 6 h: it loses $NMT\ 5.0\%$ of its weight.
- **ROTATIONAL RHEOMETER METHODS** (912): Weigh 400 g of water into a short form, 600-mL beaker (internal

diameter about 80 mm and height 120 mm). After determining the *Loss on Drying*, weigh a quantity of undried Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer, equivalent to 100 g on the dried basis. Transfer the sample to the beaker very slowly while effectively stirring (avoid lumps). Ensure that the stirring is very effective at the beginning and that the powder is immersed very slowly at the same time. Once the powder is dispersed and no lumps are visible, gentle stirring is then sufficient. Ensure a colloidal dispersion (milky white liquid) by stirring at room temperature for 3 h and taking care to avoid mixing in excess air. Afterwards allow the container to stand for 1 h, control the temperature to $23 \pm 0.1^\circ$, and let the entrapped air dissipate. [NOTE—Ensure that the concentration of this solution is 20% (w/w).] Determine the viscosity of this solution at $23 \pm 0.1^\circ$ using a suitable rotational viscometer with a cylindrical spindle 1.9 cm in diameter and 6.5 cm high, attached to a shaft 0.3 cm in diameter.¹ The spindle rotates at 50 rpm at an immersion depth of 8.1 cm. Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: 20–100 mPa · s

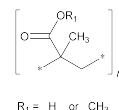
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store at controlled room temperature.
- **LABELING:** Label it to indicate the range of non-neutralized methacrylic acid units. The labeling also indicates the name and quantity of any emulsifier if the content is 0.10% or greater.
- **USP REFERENCE STANDARDS** (11)
USP Partially-Neutralized Methacrylic Acid and Ethyl Acrylate Copolymer (1:1) RS

Methacrylic Acid and Methyl Methacrylate Copolymer

(Title for this new monograph—to become official December 1, 2015)

(Prior to December 1, 2015, the current practice of labeling the article of commerce with the name *Methacrylic Acid Copolymer, Type A or Type B*, whichever is appropriate, may be continued. Use of the name *Methacrylic Acid and Methyl Methacrylate Copolymer* will be permitted as of December 1, 2010, but the use of this name will not be mandatory until December 1, 2015. The 60-month extension will provide the time needed by manufacturers and users to make necessary changes.)



(Ratio of H to CH_3 is either 1:1 or 1:2)

Poly(methacrylic acid, methyl methacrylate);
Methacrylic acid–methyl methacrylate copolymer
[25086-15-1].

DEFINITION

Methacrylic Acid and Methyl Methacrylate Copolymer consists of methacrylic acid and methyl methacrylate monomers arranged in a random distribution. Methacrylic acid units in Methacrylic Acid and Methyl Methacrylate Copolymer are $NLT\ 27.6\%$ and $NMT\ 50.6\%$, calculated on the dried basis. It may contain suitable surface-active agents.

¹ A suitable spindle is available from Brookfield as an LV1 spindle, or the equivalent.

IDENTIFICATION**A. INFRARED ABSORPTION** (197K)

Use USP Methacrylic Acid and Methyl Methacrylate Copolymer (1:1) RS for Methacrylic Acid and Methyl Methacrylate Copolymer, with a range of 46.0%–50.6% for methacrylic acid units.

Use USP Methacrylic Acid and Methyl Methacrylate Copolymer (1:2) RS for Methacrylic Acid and Methyl Methacrylate Copolymer, with a range of 27.6%–30.7% for methacrylic acid units.

B. It meets the requirements of the Assay.**ASSAY****PROCEDURE**

Sample: 1 g, previously dried

Analysis: Dissolve the *Sample* in 100 mL of neutralized acetone, and titrate with 0.1 N sodium hydroxide VS, determining the endpoint potentiometrically (see *Titrimetry* (541)). Each mL of 0.1 N sodium hydroxide is equivalent to 8.609 mg of methacrylic acid ($C_4H_6O_2$) units.

Acceptance criteria

27.6%–30.7% for Methacrylic Acid and Methyl Methacrylate Copolymer (1:2) on the dried basis

46.0%–50.6% for Methacrylic Acid and Methyl Methacrylate Copolymer (1:1) on the dried basis

IMPURITIES**Inorganic Impurities****RESIDUE ON IGNITION** (281): NMT 0.1%**HEAVY METALS, Method II** (231): NMT 20 ppm**Organic Impurities****PROCEDURE: LIMIT OF METHACRYLIC ACID AND METHYL METHACRYLATE**

Phosphate buffer: Prepare an aqueous solution containing 17.8 g/L of anhydrous dibasic sodium phosphate and 17.0 g/L of monobasic potassium phosphate. Adjust with phosphoric acid to a pH of 2.0. This buffer has a concentration of 0.125 M.

Mobile phase: Add phosphoric acid dropwise to water to obtain a solution having a pH of 2.0. Prepare a mixture of this acidified water and methanol (80:20), and degas.

Standard solution: Dissolve 0.05 g of methacrylic acid and 0.05 g of methyl methacrylate in 5 mL of butanol, and add methanol to exactly 100 mL. Transfer 1.0 mL of this solution to a 100-mL volumetric flask. Dilute with methanol to volume. Mix 3.0 mL of this solution with 10.0 mL of *Phosphate buffer*. This solution contains 1.15 µg/mL each of methacrylic acid and methyl methacrylate.

Sample solution: Transfer 1 g of Methacrylic Acid and Methyl Methacrylate Copolymer to a 50-mL volumetric flask, dilute with methanol to volume, and mix. Add 3 mL of this solution dropwise, while continuously stirring, to a beaker that contains 10.0 mL of *Phosphate buffer*. Remove the precipitated polymer to obtain a clear supernatant by centrifugation (e.g., NLT 5000 × g for NLT 5 min). Use the clear supernatant.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 202 nm

Column: 4.0-mm × 12.5-cm analytical column; 7-µm packing L1

Flow rate: 2 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for methacrylic acid and methyl methacrylate are 1.0 and 2.8, respectively.]

Suitability requirements

Resolution: NLT 2.0 between methacrylic acid and methyl methacrylate

Relative standard deviation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each monomer (methacrylic acid or methyl methacrylate) in the portion of Methacrylic Acid and Methyl Methacrylate Copolymer taken:

$$\text{Result} = (r_U/r_S) \times (C/W) \times V_F \times D \times F \times 100$$

r_U = monomer (methacrylic acid or methyl methacrylate) peak response from the *Sample solution*

r_S = monomer (methacrylic acid or methyl methacrylate) peak response from the *Standard solution*

C = concentration of the monomer (methacrylic acid or methyl methacrylate) in the *Standard solution* (µg/mL)

W = weight of Methacrylic Acid and Methyl Methacrylate Copolymer taken to prepare the *Sample solution* (g)

V_F = final volume of the *Sample solution*, 13 mL

D = dilution factor for preparation of the *Sample solution*, 16.7

F = conversion factor, 10^{-6} g/µg

Acceptance criteria: NMT 0.05% for the total amount of monomers

SPECIFIC TESTS**ROTATIONAL RHEOMETER METHODS** (912)

Analysis: Place 254.6 g of isopropyl alcohol and 7.9 g of water in a test flask. Add a quantity of Methacrylic Acid and Methyl Methacrylate Copolymer, equivalent to 37.5 g of solids on the dried basis, while stirring by means of a magnetic stirrer. Close the flask, and continue stirring until the polymer has dissolved completely. Adjust the temperature to $20 \pm 0.1^\circ$. Equip a rotational viscometer with an accessory.¹ The shear rate under the test condition is NLT 1 s^{-1} and NMT 100 s^{-1} . Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria

60–120 mPa · s for Methacrylic Acid and Methyl Methacrylate Copolymer having a range of 46.0%–50.6% for methacrylic acid units

50–200 mPa · s for Methacrylic Acid and Methyl Methacrylate Copolymer having a range of 27.6%–30.7% for methacrylic acid units

LOSS ON DRYING (731): Dry a sample at 110° for 6 h: it loses NMT 5.0% of its weight.**ADDITIONAL REQUIREMENTS**

PACKAGING AND STORAGE: Preserve in tight containers, and store at controlled room temperature.

LABELING: Label it to indicate the range of methacrylic acid units. The labeling also indicates the name and quantity of any added surface-active agent.

USP REFERENCE STANDARDS (11)

USP Methacrylic Acid and Methyl Methacrylate Copolymer (1:1) RS (USP Methacrylic Acid Copolymer, Type A RS)

USP Methacrylic Acid and Methyl Methacrylate Copolymer (1:2) RS (USP Methacrylic Acid Copolymer, Type B RS)

¹ A suitable accessory is available from Brookfield Engineering as the LV1 spindle, a cylindrical spindle 1.9 cm in diameter and 6.5 cm high attached to a shaft 0.3 cm in diameter. The spindle rotates at 30 rpm at an immersion depth of 8.15 cm.

Methyl Alcohol



CH₄O 32.04
Methanol [67-56-1].

DEFINITION

Methyl Alcohol contains NLT 99.5% of CH₃OH.

[CAUTION—Methyl Alcohol is poisonous.]

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197F)
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

• PROCEDURE

System suitability solution: Dilute 1.0 mL of USP Methyl Alcohol RS and 1.0 mL of USP Acetone RS with tetrahydrofuran to 50 mL.

Internal standard solution: 2% (v/v) acetonitrile in tetrahydrofuran

Standard solution: 15.8 mg/mL of USP Methyl Alcohol RS in *Internal standard solution*

Sample solution: 15.8 mg/mL of Methyl Alcohol in *Internal standard solution*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica capillary column, coated with a 1.8-μm layer of phase G43

Temperature

Injector: 200°

Detector: 280°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
40	—	40	5
40	20	240	—

Carrier gas: Helium

Linear velocity: 35 cm/s

Injection type: Split ratio, 20:1

Injection size: 1 μL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for methyl alcohol, acetone, and acetonitrile are 1.0, about 1.6, and about 1.8, respectively.]

Suitability requirements

Resolution: NLT 15 between methyl alcohol and acetone, *System suitability solution*

Tailing factor: NLT 1.5 for methyl alcohol, *System suitability solution*

Relative standard deviation: NMT 2.0% for the ratio of the peak area of methyl alcohol to acetonitrile, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of methyl alcohol (CH₃OH) in the portion of Methyl Alcohol taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak area ratio from the *Sample solution*

R_S = peak area ratio from the *Standard solution*

C_S = concentration of USP Methyl Alcohol RS in the *Standard solution* (mg/mL)

C_U = concentration of Methyl Alcohol in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 99.5%

IMPURITIES

• NONVOLATILE RESIDUE

Sample: 250 mL of Methyl Alcohol

Analysis: Evaporate the *Sample* in a 600-mL beaker on a steam bath, in a well-ventilated hood, until the volume is reduced to about 100 mL. Cool, transfer a portion of the liquid to a suitable, tared 50-mL platinum dish on a steam bath, and evaporate. Repeat the process until all of the liquid has been transferred, and then evaporate to dryness. Dry at 105° for 30 min, cool, and weigh.

Acceptance criteria: The weight of the residue does not exceed 2 mg, corresponding to NMT 0.001% (w/w).

• ACETONE AND ALDEHYDES (as acetone)

Standard solution: Dilute 1.9 mL (1.5 g) of acetone with water to 1000 mL, then dilute 1.0 mL of this solution with water to 100 mL. Dilute 2 mL of the resulting solution with water to 5 mL. The *Standard solution* contains 30 μg of acetone and is freshly prepared.

Sample solution: Dilute 1.25 mL (1 g) of Methyl Alcohol with water to 5 mL.

Analysis: Adjust to and maintain each solution at 20°. Add 5 mL of alkaline mercuric-potassium iodide TS to each of the *Standard solution* and *Sample solution*.

Acceptance criteria: Any turbidity produced in the *Sample solution* is not greater than that produced in the *Standard solution* (NMT 0.003%).

• READILY CARBONIZABLE SUBSTANCES (271)

Sample: 5 mL

Analysis: Cool 5 mL of sulfuric acid, contained in a small conical flask, to 10°, and add the *Sample* dropwise with constant mixing, maintaining the temperature below 20° throughout the test.

Acceptance criteria: No discoloration develops.

• READILY OXIDIZABLE SUBSTANCES

Sample: 20 mL of Methyl Alcohol

Analysis: Cool the *Sample* to 15°, add 0.1 mL of 0.1 N potassium permanganate, and allow to stand at 15°.

Acceptance criteria: The pink color does not completely disappear within 5 min.

SPECIFIC TESTS

• ACIDITY

Sample solution: Mix 25 mL of water with 10 mL of alcohol and 0.5 mL of phenolphthalein TS, and add 0.02 N sodium hydroxide until a slight pink color persists after shaking for 30 s. Taking precautions to avoid absorption of carbon dioxide, add 19 mL (15 g) of Methyl Alcohol.

Analysis: Titrate the *Sample solution* with 0.020 N sodium hydroxide.

Acceptance criteria: NMT 0.45 mL of 0.020 N sodium hydroxide is required to produce a pink color.

• ALKALINITY (as ammonia)

Sample: 28.6 mL (22.6 g) of Methyl Alcohol

Analysis: Mix the *Sample* with 25 mL of water, add 1 drop of methyl red TS, and titrate with 0.020 N sulfuric acid.

Acceptance criteria: NMT 0.20 mL of 0.020 N sulfuric acid is required to produce a pink color (3 ppm).

• WATER DETERMINATION, *Method 1* (921): NMT 0.1%

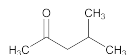
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, remote from heat, sparks, and open flames.

• **USP REFERENCE STANDARDS** <11>

- USP Acetone RS
USP Methyl Alcohol RS

Methyl Isobutyl Ketone



C₆H₁₂O 100.16

2-Pentanone, 4-methyl-;
4-Methyl-2-pentanone [108-10-1].

DEFINITION

Methyl Isobutyl Ketone contains NLT 99.0% of methyl isobutyl ketone (C₆H₁₂O).

IDENTIFICATION

- **A.** The IR absorption spectrum of a thin film of it between sodium chloride crystals exhibits maxima, among others, at the following wavelengths, in μm : 5.81 (vs), 6.80 (m), 7.00 (m), 7.09 (m), 7.29 (vs), 7.72 (m), 8.06 (m), 8.31 (sh), 8.53 (s), and 8.91 (m).

IMPURITIES

• **LIMIT OF NONVOLATILE RESIDUE**

Sample: 50 mL

Analysis: Evaporate the *Sample* in a tared porcelain dish on a steam bath, and dry at 105° for 1 h. Weigh the residue.

Acceptance criteria: NMT 4 mg (0.008%)

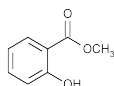
SPECIFIC TESTS

- **SPECIFIC GRAVITY** <841>: NMT 0.799, indicating NLT 99.0% of methyl isobutyl ketone (C₆H₁₂O)
- **DISTILLING RANGE**, *Method I* <721>: Between 114° and 117°, a correction factor of 0.046°/mm Hg being applied as necessary
- **ACIDITY**
Sample: 15.0 mL
Analysis: Mix the *Sample* with 15 mL of neutralized alcohol, add phenolphthalein TS, and titrate with 0.050 N sodium hydroxide.
Acceptance criteria: NMT 0.40 mL is required for neutralization.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Methyl Salicylate



C₈H₈O₃ 152.15

Benzoic acid, 2-hydroxy-, methyl ester;
Methyl salicylate [119-36-8].

DEFINITION

Methyl Salicylate is produced synthetically or is obtained by maceration and subsequent distillation with steam from the leaves of *Gaultheria procumbens* Linné (Fam. Ericaceae) or from the bark of *Betula lenta* Linné (Fam. Betulaceae). It contains NLT 98.0% and NMT 100.5% of methyl salicylate (C₈H₈O₃).

IDENTIFICATION

• **A.**

Sample: 1 drop of Methyl Salicylate

Analysis: Shake the *Sample* with 5 mL of water, and add 1 drop of ferric chloride TS.

Acceptance criteria: The resulting mixture has a deep violet color.

ASSAY

• **PROCEDURE**

Sample: 2 g of Methyl Salicylate

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Residual titration

Titrant: 1 N sodium hydroxide VS

Endpoint detection: Colorimetric

Analysis: Place the *Sample* in a flask, and add 40.0 mL of 1 N sodium hydroxide VS. Boil gently under a reflux condenser for 2 h. Cool, rinse the condenser and the sides of the flask with a few mL of water, and add phenolphthalein TS. Titrate the excess alkali with 1 N sulfuric acid VS. Perform a blank determination. Each mL of 1 N sodium hydroxide corresponds to 152.2 mg of methyl salicylate (C₈H₈O₃).

Acceptance criteria: 98.0%–100.5%

IMPURITIES

- **HEAVY METALS**, *Method II* <231>: NMT 20 ppm

SPECIFIC TESTS

- **SOLUBILITY IN 70% ALCOHOL:** One volume of synthetic Methyl Salicylate dissolves in 7 volumes of 70% alcohol. One volume of natural Methyl Salicylate dissolves in 7 volumes of 70% alcohol, the solution having NMT a slight cloudiness.
- **SPECIFIC GRAVITY** <841>: 1.180–1.185 for the synthetic variety; 1.176–1.182 for the natural variety
- **OPTICAL ROTATION**, *Angular Rotation* <781A>: Synthetic Methyl Salicylate and that from *Betula* are optically inactive. Methyl Salicylate from *Gaultheria* is slightly levorotatory, the angular rotation not exceeding -1.5° in a 100-mm tube.
- **REFRACTIVE INDEX** <831>: 1.535–1.538 at 20°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate whether it was made synthetically or distilled from either of the plants of *Gaultheria procumbens* or *Betula lenta*.

Methylcellulose—see Methylcellulose General Monographs

Methylene Chloride

CH₂Cl₂ 84.93

Methane, dichloro-;
Dichloromethane [75-09-2].

DEFINITION

Methylene Chloride contains NLT 99.0% of methylene chloride (CH₂Cl₂). [**CAUTION**—Perform all steps involving evaporation of methylene chloride in a well-ventilated fume hood.]

IDENTIFICATION

• **A.**

Sample: 5 mL

Analysis: Place the *Sample* into a glass-stoppered, 10-mL conical flask, and shake for several min. Remove

the stopper, quickly withdraw a portion of the vapor into a 50-mL syringe that is not fitted with a needle, and inject the vapor into a suitable evacuated gas cell.
Acceptance criteria: The IR absorption spectrum of the vapor shows strong doublet peaks at 7.8 and 7.9 μm and at 13.2 and 13.4 μm , and relatively few minor peaks.

ASSAY

• PROCEDURE

System suitability solution: Methylene chloride and chloroform (3:7)

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Thermal conductivity (under typical conditions)

Column: 4-mm \times 1.8-m; packed with 15% liquid phase G18 on 30- to 60-mesh S1C unsilanized support

Temperatures

Injection port: 200°

Detector: 250°

Column: 60°

Carrier gas: Helium

Flow rate: 20 mL/min

Injection volume: 1 μL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between methylene chloride and chloroform

Tailing factor: NMT 1.4

Relative standard deviation: The peak response ratio does not exceed 2% for five replicate injections.

Analysis

Sample: Methylene Chloride

Inject the *Sample*, and determine the peak responses by any convenient means. [NOTE—The order of elution is amylenes (5 or 6 peaks), if present, and then methylene chloride.]

Calculate the percentage of methylene chloride (CH_2Cl_2) in the portion of sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of methylene chloride

r_T = sum of all the peak responses

Acceptance criteria: NLT 99.0%

IMPURITIES

• LIMIT OF NONVOLATILE RESIDUE

Sample: 50 g

Analysis: Evaporate the *Sample* in a platinum or porcelain dish on a steam bath, and dry at 105° for 30 min.

Acceptance criteria: NMT 0.002%; NMT 1 mg of residue

• HEAVY METALS, Method I <231>

Test preparation: 15 mL (20 g)

Analysis: Evaporate the *Test preparation* in a glass evaporating dish on a steam bath to dryness. Cool, add 2 mL of hydrochloric acid, and slowly evaporate again on a steam bath to dryness. Dissolve the residue in 1 mL of 1 N acetic acid, and add 24 mL of water.

Acceptance criteria: NMT 1 $\mu\text{g/g}$

SPECIFIC TESTS

• LIMIT OF HYDROGEN CHLORIDE

Sample: 20.0 mL

Analysis: Into each of two glass-stoppered, 50-mL color-comparison cylinders having an internal diameter of 20 mm, place 10 mL of water, 2 drops of phenolphthalein TS, and sufficient 0.010 N sodium hydroxide to produce a pink color that persists after

vigorous shaking for 30 s and is of equal intensity in each cylinder.

[NOTE—In the following step, take special care to avoid contamination with carbon dioxide.]

Into one of the cylinders, place the *Sample* and 0.70 mL of 0.010 N sodium hydroxide, and shake again.

Acceptance criteria: NMT 0.001%; the pink color in the sample cylinder is at least as intense as that in the comparison cylinder, and the color persists for NLT 15 min.

• **SPECIFIC GRAVITY** <841>: 1.318–1.322

• **WATER DETERMINATION, Method I** <921>: NMT 0.02%

• FREE CHLORINE

Sample: 10 mL

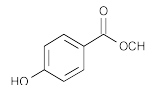
Analysis: To the *Sample* add 10 mL of water and 0.1 mL of potassium iodide TS, shake for 2 min, and allow the liquids to separate.

Acceptance criteria: The lower layer does not show a violet tint.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

Methylparaben



$\text{C}_8\text{H}_8\text{O}_3$

152.15

Benzoic acid, 4-hydroxy-, methyl ester;
Methyl *p*-hydroxybenzoate [99-76-3].

DEFINITION

Methylparaben contains NLT 98.0% and NMT 102.0% of $\text{C}_8\text{H}_8\text{O}_3$.

IDENTIFICATION

• **A. INFRARED ABSORPTION** <197M>

• **B. MELTING RANGE OR TEMPERATURE** <741>: 125°–128°

ASSAY

• PROCEDURE

Mobile phase, Sample solution, Standard solution B, and Chromatographic system: Proceed as described in the procedure for *Related Substances*.

System suitability

Sample: *Standard solution B*

Suitability requirements

Relative standard deviation: NMT 0.85% for 6 injections

Analysis

Samples: *Sample solution* and *Standard solution B*

Calculate the percentage of Methylparaben in the *Sample solution*:

$$\text{Result} = P \times (r_U \times C_S)/(r_S \times C_U)$$

P = labeled purity of USP Methylparaben RS expressed as a percentage

r_U = peak area of methylparaben from the *Sample solution*

C_S = concentration of methylparaben in *Standard solution B*

r_S = peak area of methylparaben from *Standard solution B*

C_U = concentration of Methylparaben in the *Sample solution*

Acceptance criteria: 98.0%–102.0%

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 0.1%, determined on 1.0 g

Organic Impurities

• PROCEDURE: RELATED SUBSTANCES

Mobile phase: Methanol and a 6.8 g/L solution of potassium dihydrogen phosphate (65:35 v/v)

Sample solution: Dissolve 50.0 mg of Methylparaben in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.

Standard solution A: 5.0 µg/mL each of *p*-hydroxybenzoic acid and USP Methylparaben RS in *Mobile phase*

Standard solution B: Dissolve 50.0 mg of USP Methylparaben RS in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.

Standard solution C: Dilute 1.0 mL of the *Sample solution* with *Mobile phase* to 20.0 mL. Dilute 1.0 mL of this solution with *Mobile phase* to 10.0 mL.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 272 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1.3 mL/min

Injection size: 10 µL

Run time: About 5 times the retention time of methylparaben

System suitability

Sample: *Standard solution A*

[NOTE—The retention time of methylparaben is about 2.3 min; the relative retention time for *p*-hydroxybenzoic acid is about 0.6.]

Suitability requirements

Resolution: NLT 2.0 between the *p*-hydroxybenzoic acid and methylparaben peaks

Analysis

Samples: *Sample solution* and *Standard solution C*
[NOTE—Disregard any limit that is 0.2 times the area of the principal peak in the chromatogram obtained with *Standard solution C* (0.1%).]

Acceptance criteria

***p*-Hydroxybenzoic acid:** The peak area in the *Sample solution*, multiplied by 1.4 to correct for the calculation of content, is NMT the area of the principal peak in *Standard solution C* (0.5%).

Unspecified impurities: The peak area of each impurity in the *Sample solution* is NMT the area of the principal peak in *Standard solution C* (0.5%).

Total impurities: The total peak area for all impurities in the *Sample solution* is NMT twice the area of the principal peak in *Standard solution C* (1.0%).

SPECIFIC TESTS

• COLOR OF SOLUTION

Sample solution: 100 mg/mL in alcohol

Comparison solution: Mix 2.4 mL of ferric chloride CS, 1.0 mL of cobaltous chloride CS, and 0.4 mL of cupric sulfate CS with 0.3 N hydrochloric acid to make 10 mL. Dilute 5 mL of this solution with 0.3 N hydrochloric acid to make 100 mL. [NOTE—Prepare and use this solution immediately.]

Analysis

Samples: Alcohol, *Sample solution*, and *Comparison solution*

Make the comparison by viewing the solutions downward in matched color-comparison tubes against a white surface (see *Color and Achromaticity* (631)).

Acceptance criteria: The *Sample solution* is clear and not more intensely colored than alcohol or the *Comparison solution*.

• ACIDITY

Sample solution: To 2 mL of the *Sample solution* prepared in the test for *Color of Solution*, add 3 mL of alcohol, 5 mL of carbon dioxide-free water, and 0.1 mL of bromocresol green TS.

Analysis: Titrate with 0.10 N sodium hydroxide.

Acceptance criteria: NMT 0.1 mL is required to produce a blue color.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

- **USP REFERENCE STANDARDS** (11)
USP Methylparaben RS

Methylparaben Sodium

DEFINITION

Methylparaben Sodium contains NLT 98.5% and NMT 101.5% of methylparaben sodium (C₈H₇NaO₃), calculated on the anhydrous basis.

IDENTIFICATION

• A.

Sample: Dissolve 0.5 g of Methylparaben Sodium in 5 mL of water, acidify with hydrochloric acid, and filter the resulting precipitate. Wash the precipitate with water, and dry it over silica gel for 5 h.

Acceptance criteria: The IR absorption spectrum of a mineral oil dispersion of the *Sample* exhibits maxima only at the same wavelengths as that of a similar preparation of USP Methylparaben RS.

• B.

Sample solution: Ignite 0.3 g of Methylparaben Sodium, cool, and dissolve the residue in about 3 mL of 3 N hydrochloric acid.

Acceptance criteria: A platinum wire dipped in the *Sample solution* imparts an intense, persistent yellow color to a nonluminous flame.

ASSAY

• PROCEDURE

Sample: 100 mg

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Gently reflux the *Sample* with 30 mL of 1 N sodium hydroxide for 30 min. Cool, add 25.0 mL of potassium bromate solution (5.56 mg/mL), 5 mL of potassium bromide solution (125 mg/mL), and 10 mL of hydrochloric acid, and immediately insert the stopper into the flask. Cool, shake for 15 min, and allow to stand for 15 min. Quickly add 15 mL of potassium iodide TS, taking care to avoid the escape of bromine vapor, at once replace the stopper in the flask, and shake vigorously. Rinse the stopper and the neck of the flask with a small quantity of water. Titrate the liberated iodine with *Titrant*, adding 3 mL of starch TS as the endpoint is approached. [NOTE—About 15 mL of *Titrant* is needed.]

Perform a blank determination, and note the difference in volumes required. Each mL of the difference in volume of *Titrant* is equivalent to 2.902 mg of methylparaben sodium (C₈H₇NaO₃).

Acceptance criteria: 98.5%–101.5% on the anhydrous basis

IMPURITIES

- **CHLORIDE AND SULFATE**, *Chloride* <221>
Sample: 0.2 g
Control: 0.10 mL of 0.020 N hydrochloric acid
Acceptance criteria: 0.035%; the *Sample* shows no more chloride than the *Control*.
- **CHLORIDE AND SULFATE**, *Sulfate* <221>
Sample: 0.25 g
Control: 0.30 mL of 0.020 N sulfuric acid
Acceptance criteria: 0.12%; the *Sample* shows no more sulfate than the *Control*.

SPECIFIC TESTS

- **COMPLETENESS OF SOLUTION** <641>
Sample solution: 1 g of Methylparaben Sodium dissolved in water
Acceptance criteria: Meets the requirements
- **PH** <791>
Sample solution: 1 mg/mL
Acceptance criteria: 9.5–10.5
- **WATER DETERMINATION**, *Method I* <921>: NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight containers.
- **USP REFERENCE STANDARDS** <11>
USP Methylparaben RS

MethylpyrrolidoneC₅H₉NO

99.13

1-Methyl-2-pyrrolidinone;
N-Methyl-2-pyrrolidone;
N-Methylpyrrolidone;
1-Methyl-2-Pyrrolidone;
Pyrrolidin, 1-methyl-2-one-;
1-Methylpyrrolidin-2-one;
N-Methyl-γ-butyrolactam;
N-Methyl tetrahydropyrrolone;
1-Methyl-2-oxopyrrolidine;
N-Methyl-1-oxotetramethyleneamine;
2-Methyl-2-azacyclopentanone [872-50-4].

IDENTIFICATION

- **INFRARED ABSORPTION** <197F>

IMPURITIES**Inorganic Impurities**

- **HEAVY METALS** <231>: NMT 10 ppm

Organic Impurities• **PROCEDURE**

Standard solution: To 1 mL of USP Methylpyrrolidone RS, add 1 mL of pyrrolidone, and dilute with methylene chloride to 20 mL.

Sample solution: Methylpyrrolidone (neat)

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica capillary column, 5-μm layer of phase G2

Temperature

Injector: 280°

Detector: 280°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	0
100	3	170	30

Carrier gas: Nitrogen

Linear velocity: 20 cm/s

Split ratio: 100:1

Injection size: 1 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 2.0 between pyrrolidone and methylpyrrolidone

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each impurity, excluding any solvent peaks and peaks NMT 0.02%, in the portion of Methylpyrrolidone taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of each individual impurity from the *Sample solution*

r_T = sum of the responses of all the peaks from the *Sample solution*

Acceptance criteria: NMT 0.1% of any individual impurity; and NMT 0.3% of total impurities

SPECIFIC TESTS• **ALKALINITY**

Bromothymol blue solution: Dissolve 50 mg of bromothymol blue in a mixture of 4 mL of 0.02 M sodium hydroxide and 20 mL of alcohol, and dilute with water to 100 mL.

Sample: Methylpyrrolidone (neat)

Analysis: Add 0.5 mL of *Bromothymol blue solution* as indicator to 50 mL of water, and adjust with 0.02 M potassium hydroxide or 0.02 M hydrochloric acid until a yellow color is obtained. Add 50 mL of the *Sample*. Titrate with 0.02 M hydrochloric acid to the initial coloration.

Acceptance criteria: NMT 8.0 mL of 0.02 M hydrochloric acid is required.

• **CLARITY OF SOLUTION**

[NOTE—The *Sample* is to be compared to the *Reference suspension* in diffused daylight 5 min after preparation of the *Reference suspension*.]

Hydrazine solution: 10 mg/mL of hydrazine sulfate.

[NOTE—Allow to stand 4–6 h before use.]

Methenamine solution: Transfer 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.

Primary opalescent suspension

[NOTE—This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.]

Transfer 25.0 mL of the *Hydrazine solution* to the *Methenamine solution* in the 100-mL glass-stoppered flask.

[NOTE—Allow to stand for 24 h.]

Opalescence standard: Transfer 15.0 mL of the *Primary opalescent suspension* to a 1000-mL volumetric flask, and dilute with water to volume. [NOTE—This suspension should not be used beyond 24 h after preparation.]

Reference suspension: Transfer 5.0 mL of the *Opalescence standard* to a 100-mL volumetric flask, and dilute with water to volume.

Sample: Methylpyrrolidone (neat)

Analysis: Transfer a sufficient portion of the *Sample* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer portions of the *Reference suspension* and water to separate matching test tubes. Compare the *Sample*, *Reference suspension*, and water in diffused daylight, viewing vertically against a black background (see *Spectrophotometry and Light-Scattering* <851>, *Visual Comparison*). [NOTE—The diffusion of light must be such that the *Reference suspension* can readily be distinguished from water.]

Acceptance criteria: The *Sample* shows the same clarity as that of water, or its opalescence is not more pronounced than that of the *Reference suspension*.

• COLOR OF SOLUTION

Sample Methylpyrrolidone (neat)

Analysis: Transfer a sufficient portion of the *Sample* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm, to obtain a depth of 40 mm. Similarly transfer a portion of water to a separate matching test tube. Compare the color of the *Sample* with that of water in diffused daylight, viewing vertically against a white background (see *Spectrophotometry and Light-Scattering* <851>, *Visual Comparison*).

Acceptance criteria: The *Sample* has the color of water.

- **WATER, Method 1c** <921>: NMT 0.1%, determined on 1.0 g

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in light-resistant containers.
- **USP REFERENCE STANDARDS** <11>
USP Methylpyrrolidone RS

Mineral Oil—see *Mineral Oil General Monographs*

Mineral Oil, Rectal—see *Mineral Oil, Rectal General Monographs*

Light Mineral Oil

DEFINITION

Light Mineral Oil is a purified mixture of liquid hydrocarbons obtained from petroleum. It may contain a suitable stabilizer.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197F>
- **B.** It meets the requirements in *Specific Tests for Viscosity—Capillary Viscometer Methods* <911>.

IMPURITIES

• LIMIT OF POLYCYCLIC AROMATIC HYDROCARBONS

Dimethyl sulfoxide: Use spectrophotometric grade dimethyl sulfoxide.

***n*-Hexane:** Use *n*-hexane that has been washed by being shaken previously twice with one-fifth its volume of *Dimethyl sulfoxide*.

Standard solution: 7.0 µg/mL of USP Naphthalene RS in isooctane (2,2,4-trimethylpentane)

Standard blank: 2,2,4-Trimethylpentane

Sample solution: Transfer 25.0 mL of Light Mineral Oil and 25 mL of *n*-Hexane to a 125-mL separator, and mix. [NOTE—Use no lubricants other than water on the stopcock, or use a separator equipped with a suitable polymeric stopcock.]

Add 5.0 mL of *Dimethyl sulfoxide*, and shake the mixture vigorously for 1 min. Allow to stand until the lower layer is clear, transfer the lower layer to another 125-mL separator, add 2 mL of *n*-Hexane, and shake vigorously. Use the lower layer.

Sample blank: *Dimethyl sulfoxide* that has been shaken previously vigorously for 1 min with *n*-Hexane in the ratio of 5 mL of *Dimethyl sulfoxide* to 25 mL of *n*-Hexane

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: UV

Analytical wavelengths

Standard solution: 275 nm

Sample solution: 260–350 nm

Cell: 1 cm

Analysis

Samples: *Standard solution*, *Standard blank*, *Sample solution*, and *Sample blank*

Acceptance criteria: The absorbance at any wavelength in the specified range of the *Sample solution* is NMT one-third of the absorbance of the *Standard solution*.

SPECIFIC TESTS

- **SPECIFIC GRAVITY** <841>: 0.818–0.880

- **VISCOSITY—CAPILLARY VISCOMETER METHODS** <911>:

3.0–34.4 mm² · s⁻¹ for kinematic viscosity, measured with a capillary viscometer at 40 ± 0.1°

- **ACIDITY**

Sample solution: Combine 10 mL of Light Mineral Oil and 20 mL of boiling water, shake vigorously for 1 min, and allow to cool. Remove, and filter the aqueous layer.

Analysis: To 10 mL of the *Sample solution* add 0.1 mL of phenolphthalein TS.

Acceptance criteria: The solution does not produce a pink color. NMT 1.0 mL of 0.01 N sodium hydroxide is required to produce a pink color.

- **READILY CARBONIZABLE SUBSTANCES TEST** <271>

Sample: 5 mL

Standard solution: In a glass-stoppered test tube that previously has been rinsed with hot nitric acid (see *Cleaning Glass Apparatus* <1051>), mix 3 mL of ferric chloride CS, 1.5 mL of cobaltous chloride CS, and 0.5 mL of cupric sulfate CS then overlaid with 5 mL of Light Mineral Oil.

Analysis: Place the *Sample* in a glass-stoppered test tube that previously has been rinsed with hot nitric acid (see *Cleaning Glass Apparatus* <1051>), then rinsed with water, and dried. Add 5 mL of sulfuric acid containing 94.5%–94.9% of H₂SO₄, and heat in a boiling water bath for 10 min. After the test tube has been in the bath for 30 s, remove it quickly, and, while holding the stopper in place, give three vigorous, vertical shakes over an amplitude of about 5 in. Repeat every 30 s. Do not keep the test tube out of the bath longer than 3 s for each shaking period. At the end of 10 min from the time when first placed in the water bath, remove the test tube.

Acceptance criteria: The *Sample* may turn hazy, but it remains colorless, or shows a slight pink or yellow color, and the *Sample* does not become darker than the *Standard solution*.

- **SOLID PARAFFIN**

Sample: Light Mineral Oil that has been dried previously in a beaker at 105° for 2 h and cooled to room temperature in a desiccator over silica gel

Analysis: Fill a tall, cylindrical, standard oil-sample bottle of colorless glass of 120-mL capacity with the *Sample*. Insert the stopper, and immerse the bottle in a mixture of ice and water for 4 h.

Acceptance criteria: The *Sample* is sufficiently clear that a black line 0.5 mm in width, on a white background, held vertically behind the bottle, is clearly visible.

• **LIMIT OF SULFUR COMPOUNDS**

Solution A: Saturated solution of lead(II) oxide in sodium hydroxide (200 mg/mL)

Sample: 4.0 mL

Analysis: Combine the *Sample*, 2 mL of dehydrated alcohol, and 2 drops of *Solution A*, heat at 70° for 10 min with frequent shaking, and cool.

Acceptance criteria: No dark brown color develops.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirements specified.
- **LABELING:** Label it to indicate the name and quantity of any substance added as a stabilizer, and label packages intended for direct use by the public to indicate that it is not intended for internal use.
- **USP REFERENCE STANDARDS** <11>
USP Mineral Oil RS
USP Naphthalene RS

Topical Light Mineral Oil—see *Topical Light Mineral Oil General Monographs*

Mono- and Di-glycerides

DEFINITION

Mono- and Di-glycerides is a mixture of glycerol mono- and di-esters, with minor amounts of tri-esters, of fatty acids from edible oils. It contains NLT 40.0% of monoglycerides. The monoglyceride content is NLT 90.0% and NMT 110.0% of the value indicated in the labeling. It may contain suitable stabilizers.

ASSAY

• **MONOGLYCERIDES**

Mobile phase: Tetrahydrofuran

Sample solution: 40 mg/mL of Mono- and Di-glycerides in tetrahydrofuran

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7-mm × 60-cm; 5-μm packing L21 (100 Å)

[NOTE—Two or three 7.5-mm × 30-cm L21 columns may be used in place of one 60-cm column provided that *System suitability* requirements are met.]

Temperatures

Column: 40°

Detector: 40°

Flow rate: 1 mL/min

Injection volume: 40 μL

System suitability

Sample: *Sample solution*

[NOTE—The order of elution is triglycerides, diglycerides, monoglycerides, and glycerin.]

Suitability requirements

Relative standard deviation: NMT 1.0%, determined from the monoglycerides peak

Analysis

Sample: *Sample solution*

Calculate the percentage of monoglycerides in the portion of Mono- and Di-glycerides taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for monoglycerides

r_T = sum of the responses of all the peaks, except the solvent peak

Acceptance criteria: 90.0%–110.0% of the value indicated in the labeling

IMPURITIES

• **RESIDUE ON IGNITION** <281>: NMT 0.1%

• **ARSENIC**, *Method II* <211>: NMT 3 μg/g

• **HEAVY METALS**, *Method II* <231>: NMT 10 μg/g

• **LIMIT OF FREE GLYCERIN**

Mobile phase, Sample solution, and Chromatographic system: Proceed as directed in the *Assay* for *Monoglycerides*.

Standard solutions: 0.5, 1.0, 2.0, and 4.0 mg/mL of USP Glycerin RS in tetrahydrofuran

Analysis

Samples: *Sample solution* and *Standard solutions*

Measure the responses for the glycerin peaks. Plot the concentration, in mg/mL, of USP Glycerin RS in the *Standard solutions* versus the glycerin peak responses obtained. From the standard curve so obtained, determine the glycerin concentration in the *Sample solution*.

Calculate the percentage of glycerin in the portion of Mono- and Di-glycerides taken:

$$\text{Result} = (C_U/C_S) \times 100$$

C_U = glycerin concentration in the *Sample solution* from the standard curve (mg/mL)

C_S = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 7.0%

SPECIFIC TESTS

• **FATS AND FIXED OILS**, *Acid Value* <401>: NMT 4

• **FATS AND FIXED OILS**, *Hydroxyl Value* <401>: 90.0%–110.0% of the value indicated in the labeling

• **FATS AND FIXED OILS**, *Iodine Value* <401>: 90.0%–110.0% of the value indicated in the labeling. If the value stated in the labeling is less than 10, the iodine value is NMT 10.

• **FATS AND FIXED OILS**, *Saponification Value* <401>: 90.0%–110.0% of the value indicated in the labeling

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

• **LABELING:** The labeling indicates the monoglyceride content, hydroxyl value, iodine value, saponification value, and name and quantity of any stabilizers.

• **USP REFERENCE STANDARDS** <11>

USP Glycerin RS

Monoethanolamine



C₂H₇NO

Ethanol, 2-amino-;

2-Aminoethanol [141-43-5].

61.08

DEFINITION

Monoethanolamine contains NLT 98.0% and NMT 100.5% by weight of monoethanolamine (C₂H₇NO).

IDENTIFICATION• **A. INFRARED ABSORPTION** <197F>**ASSAY**• **PROCEDURE**

Sample solution: Weigh a glass-stoppered weighing bottle containing 25 mL of water. Add 1 g of Monoethanolamine, and reweigh.

Blank: 25 mL of water

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.5 N hydrochloric acid VS

Endpoint detection: Visual

Analysis: Transfer the *Sample solution* to a suitable flask, add a mixed indicator of 5 parts bromocresol green TS and 6 parts methyl red TS for a total of approximately 11 parts of solution. Titrate the *Sample solution* with *Titrant*. Perform a blank determination.

Calculate the percentage of monoethanolamine (C_2H_7NO) in the portion of sample taken:

$$\text{Result} = \{(V_S - V_B) \times N \times F / W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample solution* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 61.08 mg/mEq

W = sample weight (mg)

Acceptance criteria: 98.0%–100.5%

IMPURITIES• **RESIDUE ON IGNITION** <281>: NMT 0.1%**SPECIFIC TESTS**• **SPECIFIC GRAVITY** <841>: 1.013–1.016• **DISTILLING RANGE, Method II** <721>: NLT 95% of it distills between 167° and 173°, a correction factor of 0.052° per mm applied as necessary.**ADDITIONAL REQUIREMENTS**• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.• **USP REFERENCE STANDARDS** <11>
USP Monoethanolamine RS

Monoglyceride Citrate

Citric acid ester of glyceryl monooleate [36291-32-4].

DEFINITION

Monoglyceride Citrate is a mixture of glyceryl monooleate and its citric acid monoester, manufactured by the reaction of glyceryl monooleate with citric acid under controlled conditions. It contains NLT 14.0% and NMT 17.0% of total citric acid, calculated on the anhydrous basis.

IDENTIFICATION• **A.**

Sample: 1 g

Analysis: Reflux the *Sample* with 15 mL of 0.5 N potassium hydroxide solution in dehydrated alcohol for 1 h. Add 15 mL of water, and acidify with diluted hydrochloric acid (about 6 mL). Dissolve any oil drops or solid produced in 5 mL of hexane. Remove the hexane layer, extract again with 5 mL of hexane, and again remove the hexane layer. [NOTE—Keep the resulting aqueous layer for *Identification* tests B and C.]

Acceptance criteria: Oil drops or a white to yellowish-white solid are produced that are soluble in 5 mL of hexane.

• **B. IDENTIFICATION TESTS—GENERAL, Citrate** <191>

Sample: 1 mL of the aqueous layer resulting from *Identification* test A

Analysis: Evaporate the *Sample* in a porcelain dish.

Acceptance criteria: The residue meets the requirements.

• **C.**

Sample: 5 mL of the aqueous layer resulting from *Identification* test A

Analysis: Transfer the *Sample* to a test tube. Add excess calcium hydroxide as a powder, place in boiling water for 5 min, shaking several times, cool, and filter. Transfer one drop of the filtrate into a test tube, and add about 50 mg of potassium hydrogen sulfate. On top of the test tube, place a filter paper moistened with a reagent for acrolein consisting of a mixture of 5% nitroprusside solution in water and 20% piperidine solution in water (1:1). Heat the test tube.

Acceptance criteria: The filter paper turns blue (presence of glycerin). The color changes to light red after addition of sodium hydroxide TS.

ASSAY• **CONTENT OF CITRIC ACID**

Standard solution: 0.23 mg/mL of USP Citric Acid RS

Sample solution: Transfer 150 mg of Monoglyceride Citrate into a saponification flask, add 50 mL of 4% potassium hydroxide solution in dehydrated alcohol, and reflux for 1 h. Acidify the reaction mixture with hydrochloric acid to a pH of 2.8–3.2, transfer into a 400-mL beaker, and evaporate to dryness on a steam bath. Quantitatively transfer the contents of the beaker into a separator, using NMT 50 mL of water, and extract with three 50-mL portions of petroleum ether, discarding the extracts. Transfer the water layer to a 100-mL volumetric flask, and dilute with water to volume.

Blank: Water

Instrumental conditions

Mode: UV-Vis

Analytical wavelength: 450 nm

Cell: 1 cm

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank* Pipet 2.0 mL each of the *Standard solution*, *Sample solution*, and *Blank* into separate 40-mL graduated centrifuge tubes. Add 2 mL of a 1 in 2 sulfuric acid solution and 11 mL of water to each tube. Boil for 3 min, cool, and add 5 mL of bromine TS to each tube. Dilute to 20 mL, allow to stand for 10 min, and centrifuge. Transfer 4.0 mL of the supernatant from each tube into separate 19- × 110-mm test tubes, add 1 mL of water, 0.5 mL of a 1 in 2 sulfuric acid solution, and 0.3 mL of 1 M potassium bromide, and shake. Add 0.3 mL of 1.5 N potassium permanganate, shake, and allow to stand for 2 min. Add 1 mL of a saturated solution of ferrous sulfate, shake, allow to stand for 2 min, and then dilute with water to 10 mL. Add 10.0 mL of *n*-hexane (previously washed with sulfuric acid, followed by a water wash, and then dried over anhydrous sodium sulfate), shake vigorously for 2 min, and centrifuge at low speed for 1 min. Transfer 5.0 mL of the hexane extract into a 20- × 145-mm tube containing 10.0 mL of 4% sodium sulfide solution, and briefly shake vigorously (three oscillations only). Centrifuge the mixture at low speed for 1 min. Immediately determine the absorbance of each aqueous layer from the *Standard solution* and *Sample solution* against the aqueous layer from the *Blank*. Calculate the percentage of citric acid in the portion of Monoglyceride Citrate taken:

$$\text{Result} = (A_U/A_S) \times (V \times C_S/W) \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

V = volume of the *Sample solution* (mL)

C_s = concentration of USP Citric Acid RS in the *Standard solution* (mg/mL)

W = weight of Monoglyceride Citrate taken to prepare the *Sample solution* (mg)

Acceptance criteria: 14.0%–17.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.3%, determined on 1 g
- **HEAVY METALS**, *Method II* (231): NMT 10 ppm

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Acid Value* (401): 70–100
- **FATS AND FIXED OILS**, *Saponification Value* (401): 260–265
- **WATER DETERMINATION**, *Method I* (921): NMT 0.2%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
- **USP REFERENCE STANDARDS** (11)
USP Citric Acid RS

Monosodium Glutamate

DEFINITION

Monosodium Glutamate contains NLT 99.0% and NMT 100.5% of $C_5H_8NNaO_4 \cdot H_2O$.

IDENTIFICATION

- **A.**
Sample solution: 1 in 30
Analysis: To 1 mL of the *Sample solution* add 1 mL of ninhydrin TS and 100 mg of sodium acetate, and heat in a boiling water bath for 10 min.
Acceptance criteria: An intense, violet blue color is formed.
- **B.**
Sample solution: 1 in 10
Analysis: To 10 mL of the *Sample solution* add 5.6 mL of 1 N hydrochloric acid.
Acceptance criteria: A white, crystalline precipitate of glutamic acid is formed on standing. Precipitation is promoted by agitation. When 6 mL of 1 N hydrochloric acid is added to the turbid solution, the glutamic acid dissolves on stirring.
- **C. IDENTIFICATION TESTS—GENERAL**, *Sodium* (191): It meets the requirements of the pyroantimonate precipitate test.

ASSAY

- **PROCEDURE**
Sample: 250 mg
Titrimetric system
(See *Titrimetry* (541).)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Blank: 100 mL of glacial acetic acid with a few drops of water
Endpoint detection: Potentiometric
Analysis: Wet the *Sample* with a few drops of water. Dissolve in 100 mL of glacial acetic acid. Titrate with 0.1 N perchloric acid VS. Perform a blank determination.
Calculate the percentage of monosodium glutamate ($C_5H_8NNaO_4 \cdot H_2O$) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F \times 100] / W$$

V = volume of *Titrant* consumed by the *Sample* (mL)

B = volume of *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 93.56 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5%

IMPURITIES

- **CHLORIDE AND SULFATE**, *Chloride* (221): A 280-mg portion shows no more chloride than corresponds to 1.0 mL of 0.020 N hydrochloric acid (0.25%).
- **LEAD** (251): NMT 10 ppm
- **HEAVY METALS**, *Method II* (231): NMT 20 ppm

SPECIFIC TESTS

• CLARITY AND COLOR OF SOLUTION

Sample solution: 1.0 g in 10 mL of water

Standard solution: To 0.2 mL of a solution of sodium chloride containing 10 µg/mL of chloride ion (Cl), add 20 mL of water, and mix. Then add 1 mL of 5 N nitric acid, 0.2 mL of dextrin solution (1 in 50), and 1 mL of silver nitrate TS, and allow to stand for 15 min.

Analysis: Compare the *Sample solution* with the *Standard solution* (see *Spectrophotometry and Light-Scattering—Visual Comparison* (851)).

Acceptance criteria: The *Sample solution* is colorless and has no more turbidity than the *Standard solution*.

• OPTICAL ROTATION, *Specific Rotation* (781S)

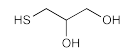
Sample solution: 100 mg/mL in 2 N hydrochloric acid
Acceptance criteria: +24.8° to +25.3°, determined at 20°

- **PH** (791): 6.7–7.2, in a solution (1 in 20)
- **LOSS ON DRYING** (731): Dry a sample at 100° for 5 h: it loses NMT 0.5% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Monothioglycerol



$C_3H_8O_2S$
1,2-Propanediol, 3-mercapto-;
3-Mercapto-1,2-propanediol [96-27-5].

108.16

DEFINITION

Monothioglycerol contains NLT 97.0% and NMT 101.0% of monothioglycerol ($C_3H_8O_2S$), calculated on the anhydrous basis.

ASSAY

- **PROCEDURE**
Sample: 400 mg
Titrimetric system
(See *Titrimetry* (541).)
Mode: Direct titration
Titrant: 0.1 N iodine VS
Endpoint detection: Visual
Analysis: Dissolve the *Sample* in 50 mL of water. Titrate with *Titrant*, adding 3 mL of starch TS as the endpoint is approached. Each mL of *Titrant* is equivalent to 10.82 mg of monothioglycerol ($C_3H_8O_2S$).
Acceptance criteria: 97.0%–101.0% on the anhydrous basis

IMPURITIES

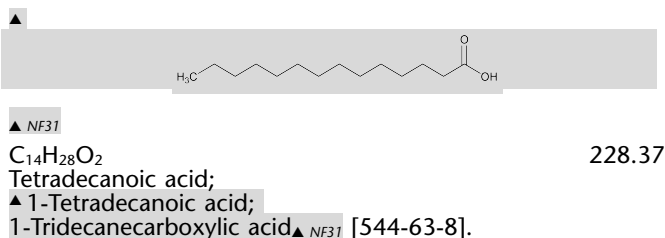
- **RESIDUE ON IGNITION** (281): NMT 0.1%
- **SELENIUM** (291)
 - Test solution: 200 µL
 - Acceptance criteria: 30 µg/g
- **HEAVY METALS, Method II** (231): NMT 20 µg/g

SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): 1.241–1.250
- **REFRACTIVE INDEX** (831): 1.521–1.526
- **PH** (791)
 - Sample solution: 1 in 10
 - Acceptance criteria: 3.5–7.0
- **WATER DETERMINATION, Method II** (921): NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Myristic Acid**Change to read:****DEFINITION**

Myristic Acid is obtained from coconut oil and other fats. It contains NLT 97.0% of myristic acid ($C_{14}H_{28}O_2$).

IDENTIFICATION**Add the following:**

- ▲ • **A. INFRARED ABSORPTION** (197D) or (197K)
 - Sample: Undried specimen
 - Acceptance criteria: Meets the requirements ▲ NF31

Add the following:

- ▲ • **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Fats and Fixed Oils, Fatty Acid Composition* in the *Assay*. ▲ NF31

ASSAY

- **FATS AND FIXED OILS, Fatty Acid Composition** (401)
 - System suitability solution:** Prepare as directed in the chapter, except that only stearic acid and palmitic acid are used.
 - Sample solution:** Prepare as directed for the *Test Solution* in the chapter.
 - Standard solution:** Prepare as directed for the *Sample solution*, using 100 mg of USP Myristic Acid RS instead of the substance to be examined.
 - Chromatographic system:** Prepare as directed in the chapter.
 - Injection size:** 1 µL
 - System suitability**
 (See *Chromatography* (621), *System Suitability*.)

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 1.5 between methyl stearate and methyl palmitate

Analysis

Samples: *Standard solution* and *Sample solution*

Identify the methyl myristate peak from the *Sample solution* by comparing the retention times of the peaks with those from the *Standard solution*. Measure the responses for all the peaks from the *Sample solution*, excluding the solvent peak.

Calculate the percentage of myristic acid ($C_{14}H_{28}O_2$) in the portion of Myristic Acid taken:

$$\text{Result} = (A/B) \times 100$$

A = peak response for methyl myristate from the *Sample solution*

B = sum of all the peak responses in the *Sample solution* except the solvent peak

Acceptance criteria: NLT 97.0%

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%
- **LIMIT OF LEAD**

[NOTE—Select reagents with as low a lead content as practicable, and store all solutions in high-density polyethylene containers. Rinse all plastic and glassware thoroughly with warm 8 N nitric acid followed by deionized water.]

Standard stock solution: Dissolve 160 mg of lead nitrate in 100 mL of water containing 1 mL of nitric acid. Dilute with water to 1000 mL.

Standard solutions: [NOTE—Prepare these solutions on the day of use.] Transfer 10.0 mL of *Standard stock solution* to a 100-mL volumetric flask, and dilute with water to volume. Each mL of this solution contains the equivalent of about 10 µg of lead. Dilute accurately measured volumes of the diluted *Standard stock solution* with water to obtain solutions with known concentrations of 1, 2, and 5 µg/mL of lead.

Sample solution: Transfer 5 g of Myristic Acid to an evaporating dish. Add 5 mL of a 25% sulfuric acid solution, and distribute the sulfuric acid uniformly through the sample. Within a hood, place the dish on a steam bath to evaporate most of the water. Place the dish on a burner, and slowly pre-ash the sample by expelling most of the sulfuric acid. Place the dish in a muffle furnace that has been set at 525°, and ash the sample until the residue appears free from carbon. Prepare a blank by ashing 5 mL of a 25% sulfuric acid solution. Cool, and cautiously wash down the inside of each evaporation dish with water. Treat both the sample and the blank as follows. Add 5 mL of 1 N hydrochloric acid. Place each dish on a steam bath, and evaporate to dryness. To each dish add 1.0 mL of 3 N hydrochloric acid and about 5 mL of water, and heat briefly on a steam bath to dissolve any residue. Transfer each solution quantitatively to a 10-mL volumetric flask, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 283.3 nm at the lead emission line

Lamp: Lead electrodeless discharge

Flame: Air-acetylene with a suitable burner head

Slit width: 0.7 nm

Blank: Water. [NOTE—Perform a blank determination following the manufacturer's operating instructions.]

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank*
 Determine the corrected absorbance values by subtracting the absorbance of the *Blank* from the absorbance of each of the *Standard solutions* and from

the absorbance of the *Sample solution*. Prepare a standard curve by plotting the corrected absorbance values of the *Standard solutions* versus their corresponding concentration, in $\mu\text{g/mL}$. From the calibration curve, determine the lead concentration in the *Sample solution*.

Calculate the lead content, in ppm, in the portion of Myristic Acid taken:

$$\text{Result} = (C/W_s) \times V$$

C = measured concentration of lead in the *Sample solution* from the standard curve ($\mu\text{g/mL}$)

W_s = weight of Myristic Acid taken (g)

V = final volume of the *Sample solution*, 10 mL

Acceptance criteria: NMT 2 ppm

Add the following:

▲ • LIMIT OF MINERAL ACIDS

Sample: 5 g of melted Myristic Acid

Analysis: Shake the *Sample* with an equal volume of hot water for 2 min, cool, and filter.

Acceptance criteria: The filtrate is not reddened by the addition of 1 drop of methyl orange TS.▲ NF31

SPECIFIC TESTS

- **CONGEALING TEMPERATURE** <651>: 48°–55.5°
- **FATS AND FIXED OILS**, Acid Value <401>: 242–249
- **FATS AND FIXED OILS**, Iodine Value <401>: NMT 1.0

Delete the following:

- ▲ • **FATS AND FIXED OILS**, Saponification Value <401>: 242–251▲ NF31

Add the following:

- ▲ • **FATS AND FIXED OILS**, Peroxide Value <401>: NMT 10.0▲ NF31
- **FATS AND FIXED OILS**, Unsaponifiable Matter <401>: NMT 1%
- **WATER DETERMINATION**, Method I <921>: NMT 0.2%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in well-closed containers. No storage requirements specified.
- **USP REFERENCE STANDARDS** <11>
USP Myristic Acid RS

Myristyl Alcohol

DEFINITION

Myristyl Alcohol contains NLT 90.0% of myristyl alcohol ($\text{C}_{14}\text{H}_{30}\text{O}$), the remainder consisting chiefly of related alcohols.

IDENTIFICATION

- **A**. The retention time of the major peak of the *Sample solution* corresponds to that of the *System suitability solution*, as obtained in the Assay.

ASSAY

• PROCEDURE

System suitability solution: 9 mg/mL of USP Myristyl Alcohol RS and 1 mg/mL of USP Cetyl Alcohol RS in alcohol

Sample solution: 10 mg/mL of Myristyl Alcohol in dehydrated alcohol

Chromatographic system

(See Chromatography <621>, System Suitability.)

Mode: GC

Detector: Flame ionization

Column: 3-mm \times 2-m, packed with 10% liquid phase G2 on support S1A

Temperature

Column: 205°

Detector: 250°

Injector port: 275°

Carrier gas: Helium

Injection size: 2 μL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between cetyl alcohol and myristyl alcohol

Relative standard deviation: NMT 1.5%

Analysis

Sample: *Sample solution*

Calculate the percentage of myristyl alcohol ($\text{C}_{14}\text{H}_{30}\text{O}$) in the portion of Myristyl Alcohol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of myristyl alcohol from the *Sample solution*

r_T = sum of the peak areas except the solvent peak from the *Sample solution*

Acceptance criteria: NLT 90.0%

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE**, Class II <741>: 36° to 42°
- **FATS AND FIXED OILS**, Acid Value <401>: NMT 2
- **FATS AND FIXED OILS**, Iodine Value <401>: NMT 1
- **FATS AND FIXED OILS**, Hydroxyl Value <401>

Sample: Place 2 g in a dry, glass-stoppered, 250-mL flask, and add 2 mL of pyridine, followed by 10 mL of toluene. To the mixture add 10.0 mL of a solution of acetyl chloride, prepared by mixing 10 mL of acetyl chloride with 90 mL of toluene. Insert the stopper in the flask, and immerse in a water bath heated at 60° to 65° for 20 min. Add 25 mL of water, again insert the stopper in the flask, and shake vigorously for several minutes to decompose the excess acetyl chloride.

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Residual titration

Titrant: Acetyl chloride

Back titrant: 1 N sodium hydroxide VS

Blank: Proceed as directed for the *Sample*, omitting Myristyl Alcohol.

Endpoint detection: Colorimetric

Analysis: Add 0.5 mL of phenolphthalein TS to the *Sample* and *Blank*. Titrate each to a permanent pink endpoint with 1 N sodium hydroxide VS, shaking the flask vigorously toward the end of the titration to maintain the contents in an emulsified condition.

Calculate the hydroxyl value:

$$\text{Result} = [(V_U - V_B) \times F]/W$$

V_U = volume of 1 N sodium hydroxide consumed by the *Sample* (mL)

V_B = volume of 1 N sodium hydroxide consumed by the *Blank* (mL)

F = equivalent weight of potassium hydroxide, 56.1 mg/mEq

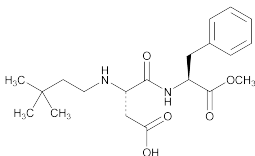
W = weight of the *Sample* (g)

Acceptance criteria: 250–267

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** <11>
USP Cetyl Alcohol RS
USP Myristyl Alcohol RS

Neotame



$C_{20}H_{30}N_2O_5$ 378.46
 L-Phenylalanine, *N*-[*N*-(3,3-dimethylbutyl)-*L*-α-aspartyl]-
 1-methyl ester;
N-[*N*-(3,3-Dimethylbutyl)-*L*-α-aspartyl]-*L*-phenylalanine
 1-methyl ester [165450-17-9].

DEFINITION

Neotame contains NLT 97.0% and NMT 102.0% of neotame ($C_{20}H_{30}N_2O_5$), calculated on the anhydrous basis.

IDENTIFICATION

• A. INFRARED ABSORPTION (197K)

ASSAY

• PROCEDURE

Mobile phase: Dissolve 3.0 g of sodium 1-heptanesulfonate in 740 mL of water in a suitable 1000-mL vessel, and add 3.8 mL of triethylamine. Adjust the resulting solution with phosphoric acid to a pH of 3.5, and dilute with water to 750 mL. Add 250 mL of acetonitrile, and adjust with phosphoric acid to an apparent pH of 3.7.

Standard solution: 1.0 mg/mL of USP Neotame RS in *Mobile phase*

Sample solution: 1.0 mg/mL of Neotame in *Mobile phase*.

[NOTE—This solution is stable for up to 32 h when stored at a temperature of 0°–10°.]

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 210 nm

Column: 4.6-mm × 10-cm; packing L1

Column temperature: 45°

Flow rate: 1.5 mL/min

Injection size: 25 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of neotame ($C_{20}H_{30}N_2O_5$) in the portion of Neotame taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Neotame RS in the *Standard solution* (mg/mL)

C_U = concentration of Neotame in the *Sample solution* (mg/mL)

Acceptance criteria: 97.0%–102.0% on the anhydrous basis

IMPURITIES

• RESIDUE ON IGNITION (281): NMT 0.2%

• LEAD (251)

[NOTE—Use acid-cleaned (mixture of 5% nitric acid and 5% hydrochloric acid followed by rinsing with water) autosampler cups and volumetric glassware to avoid contamination. For the preparation of all aqueous solutions and for the rinsing of glassware before use,

use water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin. Select all reagents to have as low a content of lead as practicable. Store standards and samples in acid-cleaned polyethylene containers.]

Diluent: Transfer 2 mL of lead-free nitric acid into a 1000-mL volumetric flask, dilute with water to volume, and mix.

Standard stock solution: 79.9 mg of lead nitrate in 100 mL of *Diluent* in a 500-mL volumetric flask, and dilute with *Diluent* to volume. Transfer 10.0 mL of the resulting solution into a 100-mL volumetric flask, and dilute with *Diluent* to volume. Each mL of the *Standard stock solution* contains the equivalent of 10 μg of lead.

Standard solution A: Dilute an aliquot of the *Standard stock solution* with *Diluent* to obtain a solution having a concentration of 0.03 μg/mL.

Standard solution B: Dilute an aliquot of the *Standard stock solution* with *Diluent* to obtain a solution having a concentration of 0.015 μg/mL.

Sample solution: Transfer 160 mg of Neotame to a 10-mL volumetric flask. Dissolve in and dilute with *Diluent* to volume.

Blank: *Diluent*

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer with a graphite furnace, pyrolytically coated graphite tubes, a solid pyrolytic graphite platform, and a background compensation system

Analytical wavelength: 283.3 nm

Lamp: Lead hollow-cathode

Purge gas: Argon

Alternate gas: Breathing-quality air

Volume: 15 μL. [NOTE—Optimize the instrument program as recommended by the manufacturer for lead, using a char temperature of 500° and an atomization temperature of 2000°.]

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Sample solution*, and *Blank*

Correct the area responses of the *Sample solution*, *Standard solution A*, and *Standard solution B* for the *Blank* area response. Generate the appropriate lead calibration algorithm, and determine the lead concentration in the *Sample solution*, in μg/mL. Calculate the ppm of lead in the portion of Neotame taken:

$$\text{Result} = (C/W) \times V \times F$$

C = blank-corrected lead concentration in the *Sample solution* (μg/mL)

W = weight of Neotame taken to prepare the *Sample solution* (mg)

V = volume of the *Sample solution*, 10 mL

F = conversion of mg to g

Acceptance criteria: NMT 2 ppm

• RELATED COMPOUNDS

Mobile phase and Chromatographic system: Proceed as directed in the *Assay*.

Standard solution A: 0.03 mg/mL of USP Neotame Related Compound A RS in *Mobile phase*

Standard solution B: Prepare as directed for the *Standard solution* in the *Assay*.

Detector sensitivity solution: Transfer 2 mL of *Standard solution A* to a 50-mL volumetric flask, and dilute with *Mobile phase* to volume.

Sample solution: 2 mg/mL of Neotame in *Mobile phase*. [NOTE—This solution is stable for up to 32 h when stored at a temperature of 0° to 10°.]

System suitability

Samples: *Standard solution A* and *Detector sensitivity solution*

Suitability requirements

Signal-to-noise ratio: NLT 10, *Detector sensitivity solution*

Relative standard deviation: NMT 5.0%, *Standard solution A*

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Calculate the percentage of neotame related compound A in the portion of Neotame taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = neotame related compound A peak response from the *Sample solution*

r_S = neotame related compound A peak response from *Standard solution A*

C_S = concentration of USP Neotame Related Compound A RS in *Standard solution A* (mg/mL)

C_U = concentration of Neotame in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 1.5%

Calculate the percentage of other impurities in the portion of Neotame taken:

$$\text{Result} = (r_T/r_S) \times (C_S/C_U) \times 100$$

r_T = sum of the responses of all impurity peaks (except that of neotame related compound A and the solvent peak, if observed) in the *Sample solution*

r_S = response of the neotame peak in *Standard solution B*

C_S = concentration of USP Neotame RS in *Standard solution B* (mg/mL)

C_U = concentration of Neotame in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 2.0%

SPECIFIC TESTS

- **OPTICAL ROTATION**, *Specific Rotation* (781S): -40.0° to -43.4° , at 20°

Sample solution: 5 mg/mL in water

- **WATER DETERMINATION**, *Method 1c* (921): NMT 5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, store in a dry place, and avoid exposure to excessive heat.
- **USP REFERENCE STANDARDS** (11)
 - USP Neotame RS
 - USP Neotame Related Compound A RS
 - N*-[3,3-Dimethylbutyl]-L- α -aspartyl]-L-phenylalanine.

Nitric Acid

HNO₃ 63.01
Nitric acid [7697-37-2].

DEFINITION

Nitric Acid contains NLT 69.0% and NMT 71.0%, by weight, of nitric acid (HNO₃). [CAUTION—Avoid contact, because Nitric Acid rapidly destroys tissues.]

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL**, *Nitrate* (191): Meets the requirements

ASSAY• **PROCEDURE**

Sample solution: To 2 mL of Nitric Acid in a tared, glass-stoppered conical flask add 25 mL of water. Add methyl red TS.

Analysis: Titrate the *Sample solution* with 1 N sodium hydroxide VS. Each mL of 1 N sodium hydroxide is equivalent to 63.01 mg of HNO₃ (see *Titrimetry* (541)).

Acceptance criteria: 69.0%–71.0%

IMPURITIES• **RESIDUE ON IGNITION** (281)

Sample: 70 mL (100 g)

Analysis: Place the *Sample* in a tared crucible, add 2 drops of sulfuric acid, and evaporate to dryness. Ignite for 15 min.

Acceptance criteria: NMT 0.5 mg (5 ppm)

• **CHLORIDE AND SULFATE**, *Chloride* (221)

Sample: 35 mL (50 g)

Control: 35 μ L of 0.020 N hydrochloric acid

Acceptance criteria: NMT 0.5 ppm; the *Sample* shows no more chloride than corresponds to the *Control*.

• **CHLORIDE AND SULFATE**, *Sulfate* (221)

Sample: 28 mL

Control: 40 μ L of 0.020 N sulfuric acid in an equal volume of solution containing the quantities of reagents used in the analysis

Analysis: Add 10 mg of sodium carbonate to the *Sample*. Evaporate to dryness, dissolve in a mixture of 4 mL of water and 1 mL of dilute hydrochloric acid (50 mg/mL), and filter if necessary. Wash with two 2-mL portions of water, dilute with water to 10 mL, and add 1 mL of barium chloride TS. Observe 10 min after adding the barium chloride.

Acceptance criteria: 1 ppm; any turbidity produced by the *Sample* is not greater than that produced by the *Control*.

• **IRON** (241)

Sample: 35 mL (50 g)

Analysis: Evaporate the *Sample* to dryness, dissolve the residue in 2 mL of hydrochloric acid, and dilute with water to 47 mL.

Acceptance criteria: NMT 0.2 μ g/g

• **HEAVY METALS**, *Method I* (231)

Test preparation: To 70 mL (100 g) of Nitric Acid in a 250-mL beaker add 10 mg of sodium carbonate, and evaporate on a steam bath to dryness. Add 25 mL of water.

Acceptance criteria: NMT 0.2 ppm

SPECIFIC TESTS• **CLARITY AND COLOR OF SOLUTION**

Analysis: Mix it in its original container, and transfer 10 mL to a 20- \times 150-mm test tube. Compare with water in a similar test tube.

Acceptance criteria: The liquids are equally clear and free from suspended matter, and when viewed transversely by transmitted light, exhibit no apparent difference in color.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Nitrogen

N₂ 28.01
Nitrogen [7727-37-9].

DEFINITION

Nitrogen contains NLT 99.0%, by volume, of nitrogen (N₂).

IDENTIFICATION

- **A.** The flame of a burning wood splinter is extinguished when inserted into a test tube filled with Nitrogen. [NOTE—Exercise caution.]

ASSAY• **PROCEDURE**

Sample: Nitrogen

Standard: Oxygen–helium certified standard (see *Reagents, Indicators, and Solutions*)

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Thermal conductivity

Column: 3-m length × 4-mm inside diameter: molecular sieve prepared from a synthetic alkali-metal aluminosilicate capable of absorbing molecules having diameters of up to 0.5 nm and completely separating oxygen from nitrogen

Carrier gas: Helium (99.99%)

Temperature: Thermostatically controlled

Analysis

Samples: *Standard* and *Sample*

Introduce the *Samples* separately into the gas chromatograph by means of a gas sampling valve.

Acceptance criteria: The peak response produced by the *Sample* exhibits a retention time corresponding to that produced by the *Standard* and is equivalent to NMT 1.0% of oxygen when compared to the peak response of the *Standard*, indicating NLT 99.0%, by volume, of N₂.

SPECIFIC TESTS

[NOTE—Reduce the container pressure by means of a regulator. Measure the gases with a gas volume meter downstream from the detector tube to minimize contamination or change of the specimens.]

• **CARBON MONOXIDE**

Sample: 1000 ± 50 mL

Analysis: Pass the *Sample* through a carbon monoxide detector tube (see *Reagents, Indicators, and Solutions*) at the rate specified for the tube.

Acceptance criteria: NMT 10 ppm

• **LIMIT OF OXYGEN**

Analysis: Determined as directed in the *Assay*

Acceptance criteria: NMT 1.0%

• **ODOR**

Analysis: Carefully open the container valve to produce a moderate flow of gas. Do not direct the gas stream toward the face, but deflect a portion of the stream toward the nose.

Acceptance criteria: No appreciable odor is discernible.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in cylinders.

Nitrogen 97 Percent

DEFINITION

Nitrogen 97 Percent is Nitrogen produced from air by physical separation methods. It contains NLT 97.0%, by volume, of nitrogen (N₂).

IDENTIFICATION

- **A.** The flame of a burning wood splinter is extinguished when inserted into a test tube filled with Nitrogen 97 Percent. [NOTE—Exercise caution.]

ASSAY• **PROCEDURE**

Standard: Oxygen–helium certified standard (see *Reagents, Indicators, and Solutions*)

Sample: Nitrogen 97 Percent

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Thermal conductivity

Column: 3-m length × 4-mm inside diameter: molecular sieve prepared from a synthetic alkali-metal aluminosilicate capable of absorbing molecules having diameters of up to 0.5 nm and completely separating oxygen from nitrogen

Carrier gas: Helium (99.99%)

Temperature: Thermostatically controlled

Analysis

Samples: *Standard* and *Sample*

Introduce the *Samples* separately into the gas chromatograph by means of a gas sampling valve.

Acceptance criteria: The peak response produced by the *Sample* exhibits a retention time corresponding to that produced by the *Standard* and is equivalent to NMT 3.0% of oxygen when compared to the peak response of the *Standard*, indicating NLT 97.0%, by volume, of nitrogen (N₂).

IMPURITIES

[NOTE—Reduce the container pressure by means of a regulator. Measure the gases with a gas volume meter downstream from the detector tube to minimize contamination or change of the specimens.]

• **CARBON DIOXIDE**

Sample: 1000 ± 50 mL

Analysis: Pass the *Sample* through a carbon dioxide detector tube (see *Reagents, Indicators, and Solutions*) at the rate specified for the tube.

Acceptance criteria: The indicator change corresponds to NMT 300 ppm

• **CARBON MONOXIDE**

Sample: 1000 ± 50 mL

Analysis: Pass the *Sample* through a carbon monoxide detector tube (see *Reagents, Indicators, and Solutions*) at the rate specified for the tube.

Acceptance criteria: NMT 10 ppm

• **SULFUR DIOXIDE**

Sample: 1000 ± 50 mL

Analysis: Pass the *Sample* through a sulfur dioxide detector tube (see *Reagents, Indicators, and Solutions*) at the rate specified for the tube.

Acceptance criteria: NMT 5 ppm

• **LIMIT OF NITRIC OXIDE AND NITROGEN DIOXIDE**

Sample: 500 ± 50 mL

Analysis: Pass the *Sample* through a nitric oxide–nitrogen dioxide detector tube (see *Reagents, Indicators, and Solutions*) at the rate specified for the tube.

Acceptance criteria: NMT 2.5 ppm

• **LIMIT OF OXYGEN**

Analysis: Determined as directed in the *Assay*

Acceptance criteria: NMT 3.0%

SPECIFIC TESTS• **ODOR**

Analysis: Carefully open the container valve to produce a moderate flow of gas. Do not direct the gas stream toward the face, but deflect a portion of the stream toward the nose.

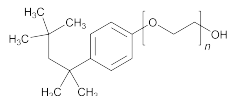
Acceptance criteria: No appreciable odor is discernible.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in cylinders or in a low-pressure collecting tank.
- **LABELING:** Where it is piped directly from the collecting tank to the point of use, label each outlet “Nitrogen 97 Percent”.

Nonoxynol 9—see Nonoxynol 9 General Monographs

Octoxynol 9



Poly(oxy-1,2-ethanediyl), α -(octylphenyl)- ω -hydroxy-; Polyethylene glycol mono(octylphenyl) ether [9002-93-1].

DEFINITION

Octoxynol 9 is an anhydrous liquid mixture consisting chiefly of mono(octylphenyl) ethers of polyethylene glycols, corresponding to: $\text{C}_8\text{H}_{17}\text{C}_6\text{H}_4(\text{OCH}_2\text{CH}_2)_n\text{OH}$ in which the average value of n is about 9.

IDENTIFICATION

- A. INFRARED ABSORPTION (197F)**
Sample: Undried specimen
Acceptance criteria: Meets the requirements

IMPURITIES

- RESIDUE ON IGNITION (281):** NMT 0.4%
- HEAVY METALS (231):** NMT 20 ppm
- LIMIT OF FREE ETHYLENE OXIDE**

Stripped octoxynol 9: Maintain Octoxynol 9 at a temperature of 150° with constant stirring in an open vessel until it no longer displays a peak for ethylene oxide when chromatographed as directed below.

System suitability solution: 10 µg/mL of ethylene oxide and 10 µg/mL of acetaldehyde in *Stripped octoxynol 9*

Standard stock solution: [CAUTION—Ethylene oxide is toxic and flammable. Prepare these solutions in a well-ventilated hood, using great care.] Chill all apparatus and reagents used in the preparation of standards in a refrigerator or freezer before use. Fill a chilled pressure bottle with liquid ethylene oxide from a lecture bottle, and store in a freezer when not in use. Use a small piece of polyethylene film to protect the liquid from contact with the rubber gasket. Transfer about 100 mL of chilled isopropyl alcohol to a 500-mL volumetric flask. Using a chilled graduated cylinder, transfer 25 mL of ethylene oxide to the isopropyl alcohol, and swirl gently to mix. Dilute with additional chilled isopropyl alcohol to volume, replace the stopper, and swirl gently to mix. This stock solution contains about 43.6 mg/mL of ethylene oxide.

Standard solutions: Pipet 25 mL of 0.5 N alcoholic hydrochloric acid, prepared by mixing 45 mL of hydrochloric acid with 1 L of alcohol, into a 500-mL conical flask containing 40 g of magnesium chloride hexahydrate. Shake the mixture to effect saturation. Pipet 10 mL of the *Standard stock solution* into the flask, and add 20 drops of bromocresol green TS. If the solution is not yellow (acid), add an additional volume, accurately measured, of 0.5 N alcoholic hydrochloric acid to give an excess of about 10 mL. Record the total volume of 0.5 N alcoholic hydrochloric acid added. Insert the stopper into the flask, and allow to stand for 30 min. Titrate the excess acid with 0.5 N alcoholic potassium hydroxide VS. Perform a blank titration, using 10.0 mL of isopropyl alcohol instead of *Standard stock solution*, adding the same total volume of 0.5 N alcoholic hydrochloric acid, and note the difference in volumes required. Each mL of the difference in volumes of 0.5 N alcoholic potassium hydroxide consumed is equivalent to 22.02 mg of ethylene oxide. Calculate the concentration, in mg/mL, of ethylene oxide in the *Standard stock*

solution. Standardize daily. Store in a refrigerator. Prepare a 1000-ppm standard by pipeting into a container the calculated volume (about 2 mL) of cold *Standard stock solution* that on the basis of the standardization contains 88.6 mg of ethylene oxide, and adding 87.0 g of *Stripped octoxynol 9*. Prepare 10-, 5-, and 0.5-ppm standards by quantitatively diluting the 1000-ppm standard with additional *Stripped octoxynol 9*.

Standard solution 0.5 ppm: Transfer 5 ± 0.01 g of the *Standard solution* containing 0.5 ppm ethylene oxide to suitable serum vials equipped with pressure-tight septum closures designed to relieve any excessive pressure, and seal.

Standard solution 5 ppm: Transfer 5 ± 0.01 g of the *Standard solution* containing 5 ppm ethylene oxide to suitable serum vials equipped with pressure-tight septum closures designed to relieve any excessive pressure, and seal.

Standard solution 10 ppm: Transfer 5 ± 0.01 g of the *Standard solution* containing 10 ppm ethylene oxide to suitable serum vials equipped with pressure-tight septum closures designed to relieve any excessive pressure, and seal.

Sample solution: Transfer 5 ± 0.01 g of Octoxynol 9 to a serum vial of the same kind as the vials used for the *Standard solutions*.

Chromatographic system

(See *Chromatography (621)*, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 2.1-mm \times 6.4-m nickel; 60- to 80-mesh support S9

Temperatures

Column: 100°

Injection port: 160°

Detector: 200°

Carrier gas: Helium

Flow rate: 30 mL/min

Injection volume: 100 µL

System suitability

Samples: *System suitability solution*, *Standard solution 0.5 ppm*, *Standard solution 5 ppm*, and *Standard solution 10 ppm*

Suitability requirements

Resolution: NLT 1.5 between ethylene oxide and acetaldehyde, *System suitability solution*

Calibration: None of the points used for constructing the *Calibration curve* deviates from the line by more than 10%.

Analysis

Samples: *System suitability solution*, *Standard solution 0.5 ppm*, *Standard solution 5 ppm*, *Standard solution 10 ppm*, and *Sample solution*

Calibration curve: Place the vial containing *Standard solution 10 ppm* in an oven, and heat at 90° for 30 min. Remove the vial from the oven. Using a gas-tight syringe, immediately inject the headspace gas into the chromatograph. Obtain the area for the ethylene oxide peak (retention time approximately 8 min). Raise the temperature of the column to 200° after ethylene oxide elutes to volatilize heavy components. Re-equilibrate the column at 100°. Repeat the foregoing steps, using the vials containing *Standard solution 0.5 ppm* and *Standard solution 5 ppm*. On linear graph paper, plot area versus ppm ethylene oxide for the standards, and draw the best straight line through the points.

Place the vial containing the *Sample solution* in an oven, and heat at 90° for 30 min. Remove the vial from the oven. Immediately inject the headspace gas into the gas chromatograph, and obtain the area for the ethylene oxide peak.

Calculate the concentration of ethylene oxide in the sample, in ppm:

$$\text{Result} = r_U \times S$$

r_U = peak area from the *Sample solution*
 S = slope of the standard curve (ppm/peak area unit)

Acceptance criteria: NMT 5 ppm

• **LIMIT OF DIOXANE**

Apparatus: Assemble a closed-system vacuum distillation apparatus, using glass vacuum stopcocks (A, B, and C), as shown in Figure 1. The concentrator tube (D)¹ is made of borosilicate or quartz (not flint) glass, graduated precisely enough to measure the 0.9 mL or more of distillate collected and marked so that the analyst can dilute accurately to 2.0 mL.

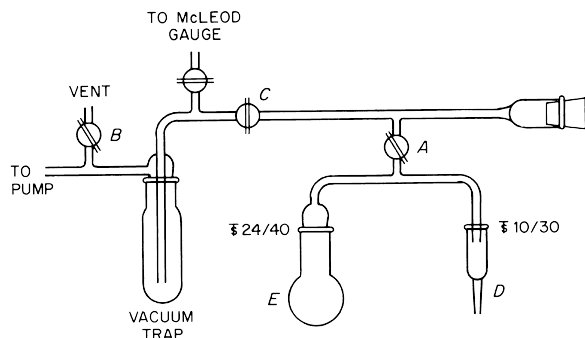


Figure 1. Closed-system vacuum distillation apparatus for dioxane.

Standard solution: 100 µg/mL of dioxane in water. Use a freshly prepared solution.

Sample solution: Transfer 20.0 g to a 50-mL round-bottom flask (E) having a 24/40 ground-glass neck joint. Add 1.0 mL of water. Place a small polytetrafluoroethylene-covered stirring bar in the flask, insert the stopper, and stir to mix. Immerse the flask in an ice bath, and chill for 1 min. Wrap heating tape around the tube connecting the concentrator tube (D) and the round-bottom flask, and apply 10 V to the tape. Apply a light coating of high-vacuum silicone grease to the ground-glass joints, and connect the concentrator tube to the 10/30 joint and the round-bottom flask to the 24/40 joint. Immerse the vacuum trap in a Dewar flask filled with liquid nitrogen, close stopcocks A and B, open stopcock C, and begin evacuating the system with a vacuum pump. Prepare a slurry bath from powdered dry ice and methanol, and raise the bath to the neck of the round-bottom flask. After freezing the contents of the flask for 10 min, and when the vacuum system is operating at a 0.05-mm pressure or lower, open stopcock A for 20 s, then close it. Remove the slurry bath, and allow the flask to warm in air for 1 min. Immerse the flask in a water bath maintained at a temperature of 20°–25°, and after about 5 min warm the water bath to 35°–40° (sufficient to liquefy most specimens) while stirring slowly but constantly with the magnetic bar. Cool the water in the bath by adding ice, and chill for about 2 min. Replace the water bath with the slurry bath, freeze the contents of the round-bottom flask for 10 min, open stopcock A for 20 s, and then close it. Remove the slurry bath, and repeat the heating steps as before, this time reaching a final temperature of 45°–50° or a temperature necessary to melt the specimen completely. If there is any condensation in the tube connecting the round-bottom flask to the concentrator tube, slowly increase the voltage to the heating tape, and heat until the condensation disappears.

Stir with the magnetic stirrer throughout the following steps. Very slowly immerse the concentrator tube in a Dewar flask containing liquid nitrogen.

[CAUTION—When there is liquid distillate in the concentrator tube, immerse the tube in the liquid nitrogen very slowly, or the tube will break.]

Water will begin to distill into the concentrator tube. As ice forms in the concentrator tube, raise the Dewar flask to keep the liquid nitrogen level only slightly below the level of ice in the tube. When water begins to freeze in the neck of the 10/30 joint, or when liquid nitrogen reaches the 2.0-mL graduation mark on the concentrator tube, remove the Dewar flask, and allow the ice to melt without heating. After the ice has melted, check the volume of water that has distilled, and repeat the sequence of chilling and thawing until NLT 0.9 mL of water has been collected. Freeze the tube once again for about 2 min, and release the vacuum first by opening stopcock B, followed by opening stopcock A. Remove the concentrator tube from the apparatus, close it with a greased stopper, and allow the ice to melt without heating. Mix the contents of the concentrator tube by swirling, note the volume of distillate, and dilute with water to 2.0 mL, if necessary.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 2-mm × 1.8-m glass; support S10

Temperatures

Column: 140°

Injection port: 200°

Detector: 250°

Carrier gas: Nitrogen or helium

Flow rate: 35 mL/min

Install an oxygen scrubber between the carrier gas line and the column. Condition the column for 72 h at 230° with 30–40 mL/min carrier flow. [NOTE—Support S10 is oxygen-sensitive. Each time a column is installed, flush with carrier gas for 30–60 min before heating.]

Injection volume: 2–4 µL

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria: NMT 10 µg/g; the height of the peak from the *Sample solution* is NMT that from the *Standard solution*.

SPECIFIC TESTS

• **CLOUD POINT**

Sample: 1.00 g

Analysis: Weigh the *Sample* into a 250-mL beaker, and add 99 g of water. Dissolve completely by careful heating, while stirring at a constant, slow speed with a small-propeller-blade stirrer. Center a thermometer vertically in the solution, and heat rapidly until the entire solution becomes cloudy, then raise the temperature 10°. Remove the source of heat, continue stirring, and record the temperature at which the solution becomes sufficiently clear to permit seeing the entire thermometer bulb plainly.

Acceptance criteria: 63°–69°

• **FATS AND FIXED OILS, Hydroxyl Value** (401): 85–101

• **WATER DETERMINATION, Method I** (921): NMT 0.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

• **USP REFERENCE STANDARDS** (11)

USP Octoxynol 9 RS

¹ A suitable tube is available as Chromaflex concentrator tube, Kontes Glass Co., Vineland, NJ (Catalog No. K42560-0000).

Octyldodecanol

DEFINITION

Octyldodecanol contains NLT 90.0% of 2-octyldodecanol, the remainder consisting chiefly of related alcohols.

IDENTIFICATION

- A.** The retention time of the major peak of the *Sample solution* corresponds to that of the major peak of the *System suitability solution*, as obtained in the *Assay*.

ASSAY

PROCEDURE

System suitability solution: 9 mg/mL of USP Octyldodecanol RS and 1 mg/mL of USP Stearyl Alcohol RS in alcohol

Sample solution: 9 mg/mL of Octyldodecanol in alcohol

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 2-mm × 2-m; packed with 3% liquid phase G2 on support S1A

Temperatures

Injection port: 280°

Detector: 280°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	6	300	—

Carrier gas: Nitrogen

Injection volume: 2 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between octyldodecanol and stearyl alcohol

Relative standard deviation: NMT 1.5%

Analysis

Sample: *Sample solution*

Calculate the percentage of 2-octyldodecanol in the portion of Octyldodecanol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for octyldodecanol from the *Sample solution*

r_T = sum of the responses of all the peaks except the solvent peak

Acceptance criteria: NLT 90.0% of 2-octyldodecanol

SPECIFIC TESTS

- FATS AND FIXED OILS, Acid Value <401>:** NMT 0.5
- FATS AND FIXED OILS, Hydroxyl Value <401>:** 175–190
- FATS AND FIXED OILS, Iodine Value <401>:** NMT 8
- FATS AND FIXED OILS, Saponification Value <401>:** NMT 5

ADDITIONAL REQUIREMENTS

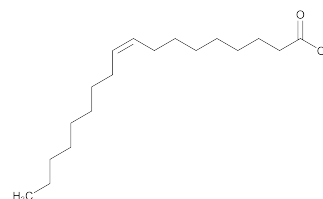
- PACKAGING AND STORAGE:** Preserve in tight containers.
- USP REFERENCE STANDARDS <11>**
 - USP Octyldodecanol RS
 - USP Stearyl Alcohol RS

Ointment, Hydrophilic—see *Hydrophilic Ointment General Monographs*

Ointment, White—see *White Ointment General Monographs*

Ointment, Yellow—see *Yellow Ointment General Monographs*

Oleic Acid



C₁₈H₃₄O₂

9-Octadecenoic acid, (Z)-;
Oleic acid [112-80-1].

282.46

DEFINITION

Change to read:

Oleic Acid is manufactured from fats and oils derived from edible sources, animal or vegetable, and consists chiefly of (Z)-9-octadecenoic acid [CH₃(CH₂)₇CH:CH(CH₂)₇COOH].
▲ It contains NLT 65.0% of (Z)-9-octadecenoic acid [CH₃(CH₂)₇CH:CH(CH₂)₇COOH].▲ NF31 It may contain suitable stabilizers.

[NOTE—Oleic Acid labeled solely for external use is exempt from the requirement that it be prepared from edible sources.]

IDENTIFICATION

- A. INFRARED ABSORPTION <197F>**

Sample: Undried specimen

Acceptance criteria: Meets the requirements

Add the following:

- ▲ **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.▲ NF31

ASSAY

Add the following:

PROCEDURE

Standard solution: 1.7 mg/mL of USP Oleic Acid RS in tetrahydrofuran

Sample solution: 1.7 mg/mL of Oleic Acid in tetrahydrofuran

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm × 30-m capillary; 0.25-µm layer of phase G25 (or G35)

Temperature
 Detector: 280°
 Injection port: 280°
 Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	—	120	5
120	10	250	20

Carrier gas: Helium
 Flow rate: 7.0 mL/min
 Injection volume: 1.0 µL
 Injection type: Splitless
 System suitability
 Sample: Standard solution
 [NOTE—The retention time for oleic acid is about 19.2 min.]

System suitability requirements
 Relative standard deviation: NMT 5.0%

Analysis

Samples: Standard solution and Sample solution
 Calculate the percentage of oleic acid in the portion of the sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for oleic acid from the Sample solution
 r_S = peak response for oleic acid from the Standard solution
 C_S = concentration of USP Oleic Acid RS in the Standard solution (mg/mL)
 C_U = concentration of Oleic Acid in the Sample solution (mg/mL)

Acceptance criteria: NLT 65.0%▲ NF31

IMPURITIES

- **RESIDUE ON IGNITION** <281>
 Sample: 10 mL
 Acceptance criteria: NMT 1 mg (about 0.01%)

SPECIFIC TESTS

Delete the following:

- ▲ • **SPECIFIC GRAVITY** <841>: 0.889–0.895▲ NF31

Add the following:

▲ • **CONTENT OF FATTY ACIDS**

Oleic Acid exhibits the composition profiles of fatty acids shown in Table 2 below, as determined in *Fats and Fixed Oils* <401>, *Fatty Acid Composition*.

Test solution: Prepare as directed in the chapter, but omitting the initial hydrolysis.

Table 2

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
14	0	≤5.0
16	0	≤16.0
16	1	≤8.0
18	0	≤6.0
18	1	≥65.0
18	2	≤18.0

▲ The sum of these fatty acids should be NMT 4.0%.

Table 2 (Continued)

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
18	3	≤4.0
20, 22 ^a	0	≤4.0

^a The sum of these fatty acids should be NMT 4.0%.

▲ NF31

- **CONGEALING TEMPERATURE** <651>: 3°–10° for Oleic Acid derived from animal sources; 10°–16° for Oleic Acid derived from vegetable sources
- **FATS AND FIXED OILS**, Acid Value <401>: 196–204, 2 g being used

Change to read:

- **FATS AND FIXED OILS**, Iodine Value <401>: ▲ 85–105▲ NF31

Add the following:

- ▲ • **FATS AND FIXED OILS**, Peroxide Value <401>: NMT 10.0▲ NF31

Add the following:

- ▲ • **WATER DETERMINATION**, Method I <921>: NMT 0.4%▲ NF31

• **MINERAL ACIDS**

Sample: 5 mL

Analysis: Shake the Sample with an equal volume of water at a temperature of about 25° for 2 min, allow the liquids to separate, and pass the water layer through a paper filter previously moistened with water.

Acceptance criteria: The filtrate is not reddened by the addition of 1 drop of methyl orange TS.

Delete the following:

▲ • **NEUTRAL FAT OR MINERAL OIL**

Sample: 1 mL

Analysis: Boil the Sample with 500 mg of sodium carbonate and 30 mL of water in a 250-mL flask.

Acceptance criteria: The resulting solution, while hot, is clear or, at most, opalescent.▲ NF31

ADDITIONAL REQUIREMENTS

Change to read:

- **PACKAGING AND STORAGE**: ▲ Preserve in well-closed containers, protected from light. Store at room temperature, and avoid exposure to excessive heat.▲ NF31
- **LABELING**: If it is for external use only, the labeling so indicates. Label it to indicate whether it is derived from animal or vegetable sources. Indicate the names and quantity of any added stabilizers.
- **USP REFERENCE STANDARDS** <11>
 USP Oleic Acid RS

Oleoyl Polyoxylglycerides**DEFINITION**

Oleoyl Polyoxylglycerides is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. Polyethylene glycols used have a mean molecular weight between 300 and 400. The article is produced by partial alcoholysis of unsaturated oils, mainly containing triglycerides of oleic acid with polyeth-

ylene glycol, by esterification of glycerol and polyethylene glycol with fatty acids, or as a mixture of glycerol esters and ethylene oxide condensate with the fatty acids of the unsaturated oils. It may contain free polyethylene glycols.

IDENTIFICATION

• A. INFRARED ABSORPTION (197F)

• B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)

Standard solution: 50 mg/mL of USP Oleoyl Polyoxylglycerides RS in methylene chloride

Sample solution: 50 mg/mL of Oleoyl Polyoxylglycerides in methylene chloride

Application volume: 10 µL

Developing solvent system: Ether and hexanes (70:30)

Spray reagent: 0.1 mg/mL of rhodamine B in alcohol

Analysis: *Standard solution* and *Sample solution*

Proceed as directed in the chapter. Then spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The R_f values of the principal spots from the *Sample solution* correspond to those from the *Standard solution*.

• C. It meets the requirements in *Specific Tests* (see *Table 2*) for *Fats and Fixed Oils, Fatty Acid Composition* (401).

IMPURITIES

• HEAVY METALS, *Method II* (231): NMT 10 µg/g

• ALKALINE IMPURITIES

Sample: 5.0 g

Analysis: To the *Sample* add 10 mL of alcohol and 0.05 mL of bromophenol blue TS, and mix well. Titrate with 0.01 N hydrochloric acid VS to change the color to yellow.

Acceptance criteria: NMT 1.0 mL of 0.01 N hydrochloric acid is required.

• LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE

[CAUTION—Ethylene oxide is toxic and flammable. Prepare these solutions in a well-ventilated fume hood, using great care. Protect both hands and face by wearing polyethylene protective gloves and an appropriate face mask. Store all solutions in hermetic containers, and refrigerate at 4°–8°.]

[NOTE—Perform all determinations three times.]

Magnesium chloride solution: 500 mg/mL of magnesium chloride in alcohol

Ethylene oxide stock solution: Into a dry, clean test tube, cooled in a mixture of sodium chloride and crushed ice (1:3), introduce a slow current of ethylene oxide gas, allowing condensation onto the inner wall of the test tube. Using a glass syringe, previously cooled to –10°, transfer 300 µL of liquid ethylene oxide, equivalent to 0.25 g, to 50 mL of polyethylene glycol 200. Determine the absorbed quantity of ethylene oxide by weighing before and after absorption. Dilute with polyethylene glycol 200 to 100.0 mL.

Standardize this solution by transferring 10.0 mL of *Magnesium chloride solution* and 20.0 mL of 0.1 M alcoholic hydrochloric acid VS to a volumetric flask. Insert the stopper, shake to obtain a saturated solution, and allow to equilibrate overnight. Transfer 5.00 mL of *Ethylene oxide stock solution* to the flask, and allow to stand for 30 min. Titrate with 0.1 M alcoholic potassium hydroxide VS. Perform a blank titration, using the same quantity of polyethylene glycol 200 instead of *Ethylene oxide stock solution*, and note the difference in volumes required. Each mL of the difference in volumes of 0.1 M alcoholic potassium hydroxide VS consumed is equivalent to 4.404 mg of ethylene oxide. Calculate the concentration of ethylene oxide in the *Ethylene oxide stock solution*.

Ethylene oxide solution: Prepare immediately before use. Dilute a volume of *Ethylene oxide stock solution* with

polyethylene glycol 200 to obtain a solution containing about 50 µg/g of ethylene oxide. Dilute 1.0 mL of this solution with water to 5.0 mL to obtain a solution having a concentration of 10 µg/mL of ethylene oxide.

Dioxane solution: 0.5 mg/mL of dioxane

Standard solution A: Transfer 1.0 g of Oleoyl Polyoxylglycerides to a 10-mL vial, and add 1.0 mL of *N,N*-dimethylacetamide, 0.1 mL of *Ethylene oxide solution*, and 0.1 mL of *Dioxane solution*. Close the vial, and mix to obtain a homogeneous solution. Allow to stand at 90° for 45 min.

Standard solution B: Transfer 0.1 mL of *Ethylene oxide solution* to a 10-mL vial, add 0.1 mL of a freshly prepared 10 mg/L of acetaldehyde solution, and add 0.1 mL of *Dioxane solution*. Close the vial, and mix to obtain a homogeneous solution.

Sample solution: Transfer 1.0 g of Oleoyl Polyoxylglycerides to a 10-mL vial, and add 1.0 mL of *N,N*-dimethylacetamide and 0.2 mL of water. Close the vial, and mix to obtain a homogeneous solution. Allow to stand at 90° for 45 min.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

[NOTE—Headspace apparatus that automatically transfers a measured amount of headspace may be used.]

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m glass or quartz capillary, bonded with a 1.0-µm layer of phase G1

Temperatures

Injection port: 150°

Detector: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	—
180	30	230	5

Carrier gas: Helium

Flow rate: 1 mL/min

Injection volume: 1 mL

System suitability

Sample: Gaseous phase of *Standard solution B*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are about 0.94 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between acetaldehyde and ethylene oxide

Relative standard deviation: NMT 15.0%

Analysis

Samples: *Standard solution A* and *Sample solution*

Using a heated, gas-tight gas chromatographic syringe, separately inject equal volumes of the gaseous headspace of *Standard solution A* and of the *Sample solution* into the chromatograph, record the chromatograms, and measure the peak responses. [NOTE—Adjust the sensitivity of the system so that the heights of the two principal peaks are NLT 15% of the full scale of the recorder.]

Calculate the concentration of ethylene oxide in the sample taken:

$$\text{Result} = \{(C \times r_U) / [(r_S \times M_U) - (r_U \times M_S)]\}$$

C = concentration of ethylene oxide in *Standard solution A* (µg/mL)

r_U = peak response of ethylene oxide from the *Sample solution*

- r_s = peak response of ethylene oxide from *Standard solution A*
 M_U = quantity of Oleoyl Polyoxylglycerides taken to prepare the *Sample solution* (g)
 M_S = quantity of Oleoyl Polyoxylglycerides taken to prepare *Standard solution A* (g)

Calculate the concentration of dioxane in the sample taken:

$$\text{Result} = C_D \times d_U/5 \times [(d_S \times M_U) - (d_U \times M_S)]$$

- C_D = concentration of dioxane in *Standard solution A* ($\mu\text{g/mL}$)
 d_U = peak response of dioxane from the *Sample solution*
 d_S = peak response of dioxane from *Standard solution A*
 M_U = quantity of Oleoyl Polyoxylglycerides taken to prepare the *Sample solution* (g)
 M_S = quantity of Oleoyl Polyoxylglycerides taken to prepare *Standard solution A* (g)

Acceptance criteria

Ethylene oxide: NMT 1 $\mu\text{g/g}$; the peak area of ethylene oxide from the *Sample solution* is NMT half the corresponding peak area from *Standard solution A*.

Dioxane: NMT 10 $\mu\text{g/g}$; the peak area of dioxane from the *Sample solution* is NMT half the corresponding peak area from *Standard solution A*.

• LIMIT OF FREE GLYCEROL

Sample: 1.2 g

Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, and dilute to 100.0 mL with glacial acetic acid.

Potassium iodide solution: 75 mg/mL of potassium iodide

Blank: 25 mL of methylene chloride

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 M sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 25 mL of methylene chloride, heating if necessary. Cool, and add 100 mL of water and 25.0 mL of *Periodic acetic acid solution*. Shake, and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with 0.1 M sodium thiosulfate VS. Perform a blank determination, and make any necessary correction.

Calculate the percentage of glycerol in the sample taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

- V_S = *Titrant* volume consumed by the *Sample* (mL)
 V_B = *Titrant* volume consumed by the *Blank* (mL)
 N = actual normality of the *Titrant* (mEq/mL)
 F = equivalency factor, 23.0 mg/mEq
 W = *Sample* weight (mg)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS

- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 0.1%
- **FATS AND FIXED OILS, Acid Value** <401>
Sample: 2.0 g
Acceptance criteria: NMT 2.0
- **FATS AND FIXED OILS, Fatty Acid Composition** <401>: Oleoyl Polyoxylglycerides exhibits the composition profile of fatty acids shown in *Table 2*.

Table 2

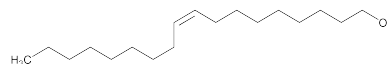
Carbon-Chain Length	Number of Double Bonds	Percentage (%)
16	0	4.0–9.0
18	0	≤ 6.0
18	1	58.0–80.0
18	2	15.0–35.0
18	3	≤ 2.0
20	0	≤ 2.0
20	1	≤ 2.0

- **FATS AND FIXED OILS, Hydroxyl Value** <401>
Sample: 1.0 g
Acceptance criteria: 45–65
- **FATS AND FIXED OILS, Iodine Value** <401>: 75–95
- **FATS AND FIXED OILS, Peroxide Value** <401>
Sample: 2.0 g
Acceptance criteria: NMT 12.0
- **FATS AND FIXED OILS, Saponification Value** <401>
Sample: 2.0 g
Acceptance criteria: 150–170
- **WATER DETERMINATION, Method I** <921>
Sample: 1.0 g
Analysis: Instead of using methanol as the solvent, one of two solvent systems can be used: a mixture of methylene chloride and anhydrous methanol (70:30 v/v), or anhydrous pyridine.
Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at controlled room temperature.
- **LABELING:** Label it to indicate the type and the average nominal molecular weight of polyethylene glycol used as part of the official title.
- **USP REFERENCE STANDARDS** <11>
 USP Oleoyl Polyoxylglycerides RS

Oleyl Alcohol



$\text{C}_{18}\text{H}_{36}\text{O}$
 9-Octadecen-1-ol, (Z)-;
 (Z)-9-Octadecen-1-ol [143-28-2].

268.48

DEFINITION

Oleoyl Alcohol is a mixture of unsaturated and saturated high molecular weight fatty alcohols consisting chiefly of oleyl alcohol ($\text{C}_{18}\text{H}_{36}\text{O}$).

SPECIFIC TESTS

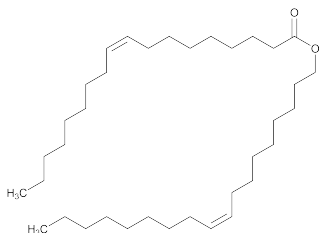
• CLOUD POINT

Analysis: Place 60 g in a 150-mL beaker, heat to 30°, cool, and immerse the beaker in an ice-water bath with the surfaces of the water and the test specimen at the same level. Insert a thermometer, and, using it as a stirring rod, begin stirring rapidly and steadily when the temperature falls below 20°. Keep the thermometer immersed throughout the test, remove and inspect the beaker containing the test specimen at regular intervals, and record the temperature at which the immersed portion of the thermometer, positioned vertically in the center of the beaker, is no longer visible when viewed horizontally through the beaker and test specimen.

- Acceptance criteria: The cloud point is not above 10°.
- **REFRACTIVE INDEX** (831): 1.458–1.460
 - **FATS AND FIXED OILS**, *Acid Value* (401): NMT 1
 - **FATS AND FIXED OILS**, *Hydroxyl Value* (401): 205–215
 - **FATS AND FIXED OILS**, *Iodine Value* (401): 85–95

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers, and store at controlled room temperature.

Oleyl OleateC₃₆H₆₈O₂

532.92

9-Octadecenoic acid, (Z)-, oleyl ester;
Oleyl oleate [3687-45-4].

DEFINITION

Oleyl Oleate consists of esters of oleyl alcohol and high molecular weight fatty acids, principally oleic acid.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197F)

IMPURITIES

- **RESIDUE ON IGNITION** (281)
Sample: 2 g
Acceptance criteria: NMT 0.1%
- **ARSENIC**, *Method II* (211): 2 µg/g
- **HEAVY METALS**, *Method II* (231): NMT 20 µg/g

SPECIFIC TESTS

- **CLARITY OF SOLUTION**
Sample solution: 200 mg/mL in ether
Acceptance criteria: The resulting solution is clear.
- **SPECIFIC GRAVITY** (841): 0.860–0.884 at 20°
- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 3.0
- **FATS AND FIXED OILS**, *Hydroxyl Value* (401): NMT 10
- **FATS AND FIXED OILS**, *Iodine Value* (401): 70–120
- **FATS AND FIXED OILS**, *Saponification Value* (401): 90–125
- **REFRACTIVE INDEX** (831): 1.464–1.468 at 20°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage conditions specified.
- **USP REFERENCE STANDARDS** (11)
USP Oleyl Oleate RS

Olive Oil

[8001-25-0].

DEFINITION

Olive Oil is the refined fixed oil obtained from the ripe fruit of *Olea europaea* Linné (Fam. Oleaceae). It may contain suitable antioxidants.

IDENTIFICATION

- It meets the requirements for *Fats and Fixed Oils*, *Fatty Acid Composition* (401).

IMPURITIES**Inorganic Impurities**

- **HEAVY METALS**, *Method II* (231): NMT 10 ppm
- **ALKALINE IMPURITIES**

Sample: 10 mL of Olive Oil

Analysis: Mix 10 mL of freshly opened acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. Add the Sample, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS to change the color of the upper layer to yellow.

Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Fatty Acid Composition* (401): Olive Oil exhibits the following composition profile of fatty acids.

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<16	0	≤0.1
16	0	7.5–20.0
16	1	≤3.5
18	0	0.5–5.0
18	1	56.0–85.0
18	2	3.5–20.0
18	3	≤1.2
20	0	≤0.7
20	1	≤0.4
22	0	≤0.2
24	0	≤0.2

ABSENCE OF SESAME OIL

Sample: 10 mL of Olive Oil

Analysis: Mix the Sample with a mixture of 0.5 mL of a 0.35% (v/v) solution of furfural in acetic anhydride and 4.5 mL of acetic anhydride, and shake the mixture for about 1 min. Pass through a filter paper previously wetted with acetic anhydride. Add 0.2 mL of sulfuric acid to the filtrate.

Acceptance criteria: No bluish-green color develops.

- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 0.3. [NOTE—Petroleum ether with 100–120° boiling range can be used to replace ether in the test.]
- **FATS AND FIXED OILS**, *Peroxide Value* (401): NMT 10.0.
- **FATS AND FIXED OILS**, *Unsaponifiable Matter* (401): NMT 1.5%. [NOTE—Petroleum ether with 40–60° boiling range can be used to replace ether in the test.]

SPECIFIC ABSORBANCE

Sample solution: Dissolve 1.0 g of Olive Oil in cyclohexane, and dilute with cyclohexane to 100 mL.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Wavelength: 270 nm

Analysis: Determine the UV-Vis absorbance using the *Spectrometric conditions* described above.

Acceptance criteria: The absorbance is NMT 1.20.

- **WATER DETERMINATION**, *Method 1c* (921): NMT 0.1%
- **FATS AND FIXED OILS**, *Sterol Composition* (401): Olive Oil exhibits the following composition profiles of sterols.

Component	Percentage (%)
Cholesterol	≤0.5
Campesterol	≤4.0
Δ7-Stigmastenol	≤0.5
Sum of the contents of Δ5,23-stigmastadienol, clerosterol, β-sitosterol, sitostanol, Δ5-avenasterol, and Δ5,24-stigmastadienol	≥93.0

The content of stigmasterol is not greater than that of campesterol.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant, well-filled containers, and prevent exposure to excessive heat.
- **LABELING:** Label it to indicate the name and quantity of any suitable antioxidants.

Vehicle for Oral Solution

DEFINITION

Prepare Vehicle for Oral Solution as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* <795>).

Sucrose	80 g
Glycerin	5 g
Sorbitol	5 g
Sodium Phosphate, Dibasic	120 mg
Citric Acid	200 mg
Potassium Sorbate	100 mg
Methylparaben	100 mg
Purified Water, a sufficient quantity to make	100 mL

Calculate the quantity of each ingredient required for the total amount to be prepared. Accurately weigh/measure each ingredient. Heat about 30 mL of *Purified Water* to 70°–75°. Add the *Glycerin* and *Methylparaben*, and stir until the *Methylparaben* is dissolved. Add the *Dibasic Sodium Phosphate*, *Citric Acid*, *Potassium Sorbate*, and *Sorbitol*, and mix well. Add the *Sucrose*, and mix until dissolved; remove from the heat, and allow to cool. Add sufficient *Purified Water* to volume, and mix well. Adjust the pH if necessary. Package, and label.

SPECIFIC TESTS

- **pH** <791>: An apparent pH between 4.0 and 5.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in a tight, light-resistant container, and store at controlled room temperature.
- **LABELING:** Label it to indicate that it is for use in compounding oral solutions and suspensions.
- **BEYOND-USE DATE:** NMT 6 months after preparation. A beyond-use date of more than 6 months may be assigned if supporting stability data exist. (See *Stability Criteria and Beyond-Use Dating* in *Pharmaceutical Compounding—Nonsterile Preparations* <795>.)

Vehicle for Oral Solution, Sugar Free

DEFINITION

Prepare Vehicle for Oral Solution, Sugar Free as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* <795>).

Xanthan Gum	50 mg
Glycerin	10 mL
Sorbitol Solution	25 mL
Saccharin Sodium	100 mg
Citric Acid Monohydrate	1.5 g
Sodium Citrate	2 g
Methylparaben	100 mg
Potassium Sorbate	100 mg
Purified Water, a sufficient quantity to make	100 mL

Calculate the quantity of each ingredient required for the total amount to be prepared. Accurately weigh/measure each ingredient. Heat about 60 mL of *Purified Water* to about 70°–75°. Add the *Methylparaben*, and stir until dissolved. Remove from the heat, and add the *Glycerin*, *Sorbitol Solution*, *Saccharin Sodium*, *Citric Acid Monohydrate*, *Sodium Citrate*, *Potassium Sorbate*, and *Xanthan Gum*. Add sufficient *Purified Water* to volume, and mix well. Adjust the pH if necessary. Package, and label.

SPECIFIC TESTS

- **pH** <791>: An apparent pH between 4.0 and 5.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in a tight, light-resistant container, and store at controlled room temperature.
- **LABELING:** Label it to indicate that it is for use in compounding sugar-free oral solutions and suspensions.
- **BEYOND-USE DATE:** NMT 6 months after preparation. A beyond-use date of more than 6 months may be assigned if supporting stability data exist. (See *Stability Criteria and Beyond-Use Dating* in *Pharmaceutical Compounding—Nonsterile Preparations* <795>.)

Vehicle for Oral Suspension

DEFINITION

Prepare Vehicle for Oral Suspension as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* <795>).

Cellulose, Microcrystalline	800 mg
Xanthan Gum	200 mg
Carrageenan	150 mg
Carboxymethylcellulose Sodium (High Viscosity)	25 mg
Citric Acid	250 mg
Sodium Phosphate, Dibasic	120 mg
Simethicone	0.1 mL
Potassium Sorbate	100 mg
Methylparaben	100 mg
Purified Water, a sufficient quantity to make	100 mL

Calculate the quantity of each ingredient required for the total amount to be prepared. Accurately weigh/measure each ingredient. Heat about 90 mL of the *Purified Water* to 70°–75°. Dissolve the *Methylparaben*, followed by the *Citric Acid*, *Dibasic Sodium Phosphate*, and *Potassium Sorbate* in the heated water. Remove from the heat. With constant mixing, slowly sprinkle the *Microcrystalline Cellulose*, *Xanthan Gum*, *Carrageenan*, and *Carboxymethylcellulose Sodium* into the mixture. Continue to stir until fully hydrated, add the *Simethicone*, and mix well. Add sufficient *Purified Water* to volume, and mix well. Adjust the pH if necessary. Package, and label.

SPECIFIC TESTS

- **pH** <791>: An apparent pH between 4.0 and 5.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in a tight, light-resistant container, and store at controlled room temperature.
- **LABELING:** Label it to indicate that it is for use in compounding oral solutions and suspensions.
- **BEYOND-USE DATE:** NMT 6 months after preparation. A beyond-use date of more than 6 months may be assigned if supporting stability data exist. (See *Stability Criteria and Beyond-Use Dating* in *Pharmaceutical Compounding—Nonsterile Preparations* <795>.)

Orange Oil

DEFINITION

Orange Oil is the volatile oil obtained by expression from the fresh peel of the ripe fruit of *Citrus sinensis* (L.) Osbeck (Fam. Rutaceae). The total aldehyde content, calculated as decanal (C₁₀H₂₀O), is NLT 1.2% and NMT 2.5%. It may contain a suitable antioxidant.

[NOTE—Do not use Orange Oil that has a terebinthine odor.]

ASSAY

• TOTAL ALDEHYDE CONTENT

Reagent solution: Dissolve 4.5 g of hydroxylamine hydrochloride in 13 mL of water. Add 85 mL of tertiary butyl alcohol, mix, and adjust with 0.5 N potassium hydroxide to a pH of 3.4.

Sample: 5 mL of Oil

Analysis: Pipet 50 mL of the *Reagent solution* into a conical flask containing the *Sample*. Insert the stopper in the flask, and allow to stand at room temperature for 30 min, with occasional shaking. Titrate the liberated hydrochloric acid with 0.5 N alcoholic potassium hydroxide VS to a pH of 3.4. Each mL of 0.5 N alcoholic potassium hydroxide consumed in the titration is equivalent to 78.13 mg of total aldehydes, calculated as decanal (C₁₀H₂₀O).

Acceptance criteria: 1.2%–2.5%

IMPURITIES

- **HEAVY METALS, Method II <231>:** NMT 40 µg/g

SPECIFIC TESTS

- **SPECIFIC GRAVITY <841>:** 0.842–0.846
- **OPTICAL ROTATION, Angular Rotation <781A>:** +94° to +99°
- **REFRACTIVE INDEX <831>:** 1.472–1.474 at 20°
- **ULTRAVIOLET ABSORBANCE**

Sample solution: 250 mg of Oil in 100 mL of alcohol
Blank: Alcohol

Instrumental conditions

(See *Spectrophotometry and Light-Scattering <851>*.)

Mode: UV-Vis

Wavelength range: 260–400 nm

Analysis: Record the spectrum in 1-cm cell. Determine the absorbance at the wavelength of maximum absorbance at 330 nm, using the line drawn tangent to the curves appearing as minima in the spectrum in wavelength regions above and below the maximum wavelength as the baseline.

Acceptance criteria: The absorbance, calculated on the basis of a 250-mg specimen, is NLT 0.130 for California-type Orange Oil or NLT 0.240 for Florida-type Orange Oil.

• FOREIGN OILS

Analysis: Place 50 mL of Oil in a four-bulb Ladenburg flask having the following dimensions: the lower or main bulb is about 6 cm in diameter, and the smaller condensing bulbs are about 3.5, 3.0, and 2.5 cm in diameter; the distance from the bottom of the flask to the side-arm is about 20 cm. Distill Oil at a rate of 1 drop/s until the distillate measures 5 mL.

Acceptance criteria: The angular rotation of the distillate does not differ from that of the original Oil by more than 2°, and the refractive index at 20° is 0.001–0.002 lower than that of the original Oil.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers, and avoid exposure to excessive heat.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived. Label it also to indicate whether it is California-type or Florida-type Orange Oil.

The label indicates that Oil is not to be used if it has a terebinthine odor.

Compound Orange Spirit

DEFINITION

Compound Orange Spirit contains NLT 25 mL and NMT 30 mL of the mixed oils in 100 mL of Spirit.

Prepare Compound Orange Spirit as follows (see *Pharmaceutical Compounding—Nonsterile Preparations <795>*).

Orange Oil	200 mL
Lemon Oil	50 mL
Coriander Oil	20 mL
Anise Oil	5 mL
Alcohol, a sufficient quantity to make	1000 mL

Mix the oils with sufficient *Alcohol* to make the product measure 1000 mL.

ASSAY

• PROCEDURE

Sample solution: Transfer 2.0 mL of Compound Orange Spirit and 1.0 mL of kerosene to a Babcock bottle, graduated to 8%, and mix.

Analysis: To the *Sample solution* add sufficient saturated calcium chloride solution, acidified with hydrochloric acid, to almost fill the bulb of the bottle. Rotate the bottle vigorously to ensure mixing, then add a sufficient quantity of the calcium chloride solution to bring the separated oil into the neck of the bottle. Centrifuge for 5 min at 1500 rpm, and read the volume of oil in the stem. Subtract five divisions on the volumetric flask for the kerosene added, and multiply the remaining number of divisions by 10.5 to obtain the volume, in mL, of mixed oils in 100 mL of the Compound Orange Spirit.

Acceptance criteria: 25–30 mL

OTHER COMPONENTS

- **ALCOHOL DETERMINATION, Method I <611>:** 65.0%–70.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight containers, protected from light, and store in a cold place.

Sweet Orange Peel Tincture

DEFINITION

Sweet Orange Peel Tincture is prepared from sweet orange peel, which is the outer rind of the non-artificially colored, fresh, ripe fruit of *Citrus sinensis* (L.) Osbeck (Fam. Rutaceae).

Prepare Sweet Orange Peel Tincture as follows.

Sweet Orange Peel	500 g
Alcohol	900 mL
Alcohol, a sufficient quantity to make	1000 mL

Macerate the *Sweet Orange Peel* in 900 mL of *Alcohol* in a container that can be closed, and put in a warm place. Agitate it frequently during 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter, using Talc as the filtering medium, and when most of the liquid has drained away, wash the residue on the filter with a sufficient quantity of *Alcohol*, combining the filtrates, to produce 1000 mL, and mix. [NOTE—Exclude the inner, white portion of the rind.]

OTHER COMPONENTS

- **ALCOHOL DETERMINATION**, *Method I* <611>: 62.0%–72.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Package in tight, light-resistant containers. Avoid exposure to direct sunlight and excessive heat.
- **LABELING**: The label states the Latin binomial and the official name.

Orange Syrup**DEFINITION**

Orange Syrup contains NLT 0.45% and NMT 0.55% of citric acid ($C_6H_8O_7$).

Prepare Orange Syrup as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* <795>).

Sweet Orange Peel Tincture	50 mL
Anhydrous Citric Acid	5 g
Talc	15 g
Sucrose	820 g
Purified Water, a sufficient quantity to make	1000 mL

Triturate *Talc* with *Sweet Orange Peel Tincture* and *Anhydrous Citric Acid*, and gradually add 400 mL of *Purified Water*. Filter, returning the first portions of the filtrate until it becomes clear, and wash the mortar and the filter with sufficient *Purified Water* to make the filtrate measure 450 mL. Dissolve *Sucrose* in this filtrate by agitation, without heating, and add *Purified Water* to make the product measure 1000 mL. Mix, and strain. [NOTE—Do not use Orange Syrup that has a terebinthine odor or taste or shows other indications of deterioration.]

ASSAY• **CITRIC ACID**

Sample: 20 mL

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: To the *Sample* add 20 mL of water and add phenolphthalein TS. Titrate with *Titrant*. Each mL of *Titrant* is equivalent to 6.404 mg of citric acid ($C_6H_8O_7$).

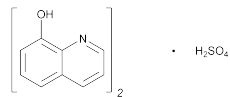
Acceptance criteria: 0.45%–0.55%

OTHER COMPONENTS

- **ALCOHOL DETERMINATION**, *Method I* <611>: 2.0%–5.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Package in tight containers, and store in a cold place.
- **LABELING**: The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived. The label indicates that Syrup is not to be used if it has a terebinthine odor or taste or shows other indications of deterioration.

Oxyquinoline Sulfate

$(C_9H_7NO)_2 \cdot H_2SO_4$

8-Quinolinol sulfate (2:1) (salt) [134-31-6].

388.39

DEFINITION

Oxyquinoline Sulfate is 8-hydroxyquinoline sulfate. It contains NLT 97.0% and NMT 101.0% of oxyquinoline sulfate $[(C_9H_7NO)_2 \cdot H_2SO_4]$, calculated on the anhydrous basis.

IDENTIFICATION• **A. INFRARED ABSORPTION** <197M>

Analysis: On the undried specimen

Acceptance criteria: Meets the requirements

• **B. IDENTIFICATION TESTS—GENERAL**, *Sulfate* <191>

Analysis: 100 mg/mL

Acceptance criteria: Meets the requirements

ASSAY• **PROCEDURE**

Sample: 100 mg

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Residual titration

Titrant: 0.1 N bromine VS

Back-titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to an iodine flask, add 30 mL of glacial acetic acid, 25.0 mL of *Titrant*, 10 mL of potassium bromide solution (150 mg/mL), and 10 mL of hydrochloric acid. Immediately insert the stopper, mix, and allow to stand for 15 min, protected from light. Quickly add 10 mL of potassium iodide solution (100 mg/mL) and 100 mL of water, taking precautions against the escape of bromine vapor. At once insert the stopper, and shake the mixture thoroughly. Remove the stopper, and rinse it and the neck of the flask with a small quantity of water so that the washing flows into the flask. Shake the mixture thoroughly. Titrate the liberated iodine with *Back-titrant*, adding 3 mL of starch TS as the endpoint is approached. Perform a blank determination. Each mL of *Titrant* is equivalent to 4.855 mg of oxyquinoline sulfate $[(C_9H_7NO)_2 \cdot H_2SO_4]$.

Acceptance criteria: 97.0%–101.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** <281>: NMT 0.3%

- **HEAVY METALS**, *Method II* <231>: NMT 20 µg/g

SPECIFIC TESTS

- **WATER DETERMINATION**, *Method I* <921>: 4.0%–6.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** <11>
USP Oxyquinoline Sulfate RS

Palm Oil

Palm oil [8002-75-3].

DEFINITION

Palm Oil is the refined fixed oil obtained from the pulp of the fruit of the oil palm *Elaeis guineensis* Jacq. (Fam. Arecaceae). It may contain suitable antioxidants.

IDENTIFICATION

- **A.** It meets the requirements of the test for *Fats and Fixed Oils, Fatty Acid Composition* (401).
- **B.** It meets the requirements of the test for *Melting Range or Temperature* (741).

IMPURITIES

- **RESIDUE ON IGNITION** (281)
Sample: 5 g of Palm Oil
Acceptance criteria: NMT 0.1%
- **HEAVY METALS, Method II** (231): NMT 10 µg/g
- **ALKALINE IMPURITIES**
Sample: 10 mL of Palm Oil
Analysis: Mix 10 mL of acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. If necessary, neutralize the solution to a green color with 0.01 N hydrochloric acid or 0.01 N sodium hydroxide. Add the Sample, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS until the color of the upper layer changes to yellow.
Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** (741): 30°–40°
- **FATS AND FIXED OILS, Acid Value, Method II** (401): NMT 2.0
- **FATS AND FIXED OILS, Fatty Acid Composition** (401):
Palm Oil exhibits the composition profile of fatty acids as shown in Table 1.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage
≤12	0	≤2.5
14	0	0.5–5.9
16	0	39.0–47.0
18	0	2.0–8.0
16	1	≤0.5
18	1	36.0–44.0
18	2	7.0–12.0
18	3	≤0.5
≥20	0 or 1	≤1.0

- **FATS AND FIXED OILS, Peroxide Value** (401): NMT 5.0
- **FATS AND FIXED OILS, Unsaponifiable Matter** (401): NMT 1.0%
- **WATER DETERMINATION, Method Ic** (921): NMT 0.1%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Do not store above 55°.
- **LABELING:** Label it to indicate the name and quantity of any added antioxidants.

Hydrogenated Palm Oil

$R^1\text{COOCH}_2\text{—CH}(\text{OOCR}^2)\text{—CH}_2\text{OOCR}^3$, where R^1 , R^2 , and R^3 are mainly C_{15} and C_{17} ;
Hydrogenated palm oil [68514-74-9].

DEFINITION

Hydrogenated Palm Oil is the product obtained by refining and hydrogenating the oil obtained from the pulp of the fruit of the oil palm *Elaeis guineensis* Jacq. (Fam. Arecaceae). The product consists mainly of triglycerides of palmitic and stearic acids.

IDENTIFICATION

- **A.** It meets the requirements of the test for *Fats and Fixed Oils, Fatty Acid Composition* (401).
- **B.** It meets the requirements of the test for *Melting Range or Temperature* (741).

IMPURITIES

- **RESIDUE ON IGNITION** (281)
Sample: 5 g
Acceptance criteria: NMT 0.1%
- **HEAVY METALS, Method II** (231): NMT 10 µg/g
- **LIMIT OF NICKEL**
Sample solution: Weigh 5.0 g of Hydrogenated Palm Oil into a previously tared platinum or silica crucible. Cautiously heat the substance, and introduce into it a wick formed from twisted, ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at about 600°. Continue the incineration until a white ash is obtained. After cooling, with the aid of two 2-mL portions of diluted hydrochloric acid, transfer the residue to a 25-mL volumetric flask, add 0.3 mL of nitric acid, and dilute with water to volume.
Nickel standard solution: Immediately before use, dilute 10 mL of nickel standard solution TS with water to 500 mL. This solution contains the equivalent of 0.2 µg/mL of nickel.
Standard solutions: Into four separate identical 10-mL volumetric flasks introduce respectively 0, 1.0, 2.0, and 4.0 mL of Nickel standard solution. To each flask add a 2.0-mL portion of the Sample solution, and dilute with water to volume to obtain four Standard solutions containing added quantities of 0, 0.2, 0.4, and 0.8 µg of nickel, respectively.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a graphite furnace

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Analysis

Samples: Standard solutions
Concomitantly determine the absorbances of the Standard solutions at least three times each. Record the average of the steady readings for each of the Standard solutions. Plot the absorbances of the Standard solutions versus the added quantity, in µg, of nickel. Extrapolate the line joining the points on the graph until it meets the quantity axis. The distance between this point and the intersection of the axes represents the quantity of nickel in the 2-mL portion of the Sample solution added to the Standard solutions.

Calculate the content of nickel in the portion of Hydrogenated Palm Oil taken:

$$\text{Result} = [V \times (A/V_A)]/W$$

- V = volume of the *Sample solution*, 25 mL
 A = quantity of nickel (μg)
 V_A = volume of the *Sample solution* added to the *Standard solutions*, 2 mL
 W = weight of Hydrogenated Palm Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 1 $\mu\text{g/g}$

• ALKALINE IMPURITIES

Sample: A mixture of 2.0 g of Hydrogenated Palm Oil, 1.5 mL of alcohol, and 3.0 mL of toluene

Analysis: Dissolve the *Sample* by gentle heating. Add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid VS until the mixture turns yellow.

Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** (741): 58°–62°
- **FATS AND FIXED OILS, Acid Value, Method II** (401): NMT 2.0
- **FATS AND FIXED OILS, Fatty Acid Composition** (401): Hydrogenated Palm Oil exhibits the composition profile of fatty acids as shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage
≤12	0	≤2.5
14	0	0.5–5.9
16	0	32.0–47.0
18	0	49.0–57.0
20	0	≤1.0
22	0	≤1.0
16	1	≤2.5
18	1	≤2.5
18	2	≤0.5
18	3	≤0.5

- **FATS AND FIXED OILS, Peroxide Value** (401): NMT 5.0
- **FATS AND FIXED OILS, Unsaponifiable Matter** (401): NMT 0.8%
- **LOSS ON DRYING** (731)
Analysis: Dry a sample at 105° for 4 h.
Acceptance criteria: NMT 0.1%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirement specified.

Palm Kernel Oil

Elaeis guineensis seed oil [8023-79-8].

DEFINITION

Palm Kernel Oil is the refined fixed oil obtained from the kernel of the fruit of the oil palm *Elaeis guineensis* Jacq. (Fam. Arecaceae). It may contain suitable antioxidants.

IDENTIFICATION

- **A.** It meets the requirements in *Specific Tests for Fats and Fixed Oils, Fatty Acid Composition* (401).
- **B.** It meets the requirements in *Specific Tests for Melting Range or Temperature* (741).

IMPURITIES

• LIMIT OF LEAD

[NOTE—For this test, use reagent-grade chemicals with as low a lead content as is practicable, as well as high-purity water and gases. Before use in this analysis, rinse all glassware and plasticware twice with diluted nitric acid and twice with diluted hydrochloric acid, and then rinse them thoroughly with Purified Water.]

Hydrogen peroxide–nitric acid solution: 10% hydrogen peroxide and diluted nitric acid (1:1). [NOTE—Use caution.]

Lead nitrate stock solution: Dissolve 159.8 mg of lead nitrate in 100 mL of *Hydrogen peroxide–nitric acid solution*. Dilute with *Hydrogen peroxide–nitric acid solution* to 1000 mL, and mix. Prepare and store this solution in glass containers that are free from lead salts. Each mL of this solution contains the equivalent of 100 μg of lead.

Standard lead solution: On the day of use, dilute 10.0 mL of *Lead nitrate stock solution* with *Hydrogen peroxide–nitric acid solution* to 100.0 mL, and mix. Each mL of *Standard lead solution* contains the equivalent of 10 μg of lead.

Butanol–nitric acid solution: Slowly add 50 mL of nitric acid to approximately 500 mL of butanol in a 1000-mL volumetric flask. Dilute with butanol to volume.

Standard solutions: Into five separate 100-mL volumetric flasks pipet 0.2, 0.5, 1, 2, and 5 mL, respectively, of *Standard lead solution*, and dilute with *Butanol–nitric acid solution* to volume. The *Standard solutions* contain 0.02, 0.05, 0.1, 0.2, and 0.5 $\mu\text{g/mL}$ of lead, respectively.

Sample solution: [CAUTION—Prepare this solution in a fume hood, and wear safety glasses.] Transfer 1.0 g of Oil into a large test tube. Add 1 mL of nitric acid. Place the test tube in a rack in a boiling water bath. As soon as the rusty tint is gone, add 1 mL of 30% hydrogen peroxide dropwise to avoid a vigorous reaction, and wait for bubbles to form. Stir with an acid-washed plastic spatula if necessary. Remove the test tube from the water bath, and allow it to cool. Transfer the solution to a 10-mL volumetric flask, and dilute with *Butanol–nitric acid solution* to volume.

Tungsten solution: Transfer 0.1 g of tungstic acid and 5 g of sodium hydroxide pellets to a 50-mL plastic bottle. Add 5.0 mL of water, and mix. Heat the mixture in a hot water bath until complete solution is achieved. Cool, and store at room temperature.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Graphite furnace atomic absorption spectrophotometry

Analytical wavelength: 283.3 nm lead emission line

Injection size: 20 μL

Lamp: Lead hollow-cathode

Furnace conditioning: Place the graphite tube in the furnace. Inject the *Tungsten solution* into the graphite tube, using an argon flow rate of 300 mL/min. Maintain the drying temperature at 110° for 20 s, the ashing temperature at 700°–900° for 20 s, and with the argon flow stopped, the atomization temperature at 2700° for 10 s; repeat this process once more using a second 20- μL aliquot of the *Tungsten solution*. Clean the quartz windows.

Analysis

Samples: *Standard solutions* and *Sample solution*

[NOTE—The sample injection technique is the most crucial step in controlling the precision of the analysis; the volume of each of the *Standard solutions* and the *Sample solution* must remain constant. Rinse the μL -pipet tip three times with either the *Standard solutions* or the *Sample solution* before injection. Use a fresh pipet tip for each injection, and start the atomization process immediately after injecting the *Sam-*

ples. Between injections, flush the graphite tube of any residual lead by purging at a high temperature recommended by the manufacturer.]

Concomitantly determine the absorbances of the *Samples*.

Atomize equal volumes of the *Standard solutions* and the *Sample solution* with an argon flow rate of 300 mL/min.

Maintain the drying temperature of the furnace at 110° for 30 s after a 20-s ramp time and a 10-s hold time; the ashing temperature at 700° for 42 s after a 20-s ramp time and a 22-s hold time; and the atomization temperature at 2300° for 7 s with the argon flow stopped.

Plot the absorbance of each of the *Standard solutions*, compensated for background correction, versus its content of lead, in µg/mL, and draw the best straight line fitting the five points. From this plot, determine the concentration, *C*, in µg/mL, of lead in the *Sample solution*.

Calculate the quantity, in µg/g, of lead in the portion of Oil taken:

$$\text{Result} = (C/W) \times V$$

C = measured concentration of lead in the *Sample solution* (µg/mL)

W = weight of the Oil taken to prepare the *Sample solution* (g)

V = final volume of the *Sample solution*, 10 mL

Acceptance criteria: NMT 0.1 µg/g of lead

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 2.0
- **FATS AND FIXED OILS**, *Fatty Acid Composition* (401): Palm Kernel Oil exhibits the composition profile of fatty acids shown in Table 1.

Table 1

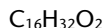
Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤1.5
8	0	3–5
10	0	2.5–6
12	0	40–52
14	0	14–18
16	0	7–10
18	0	1–3
20	0	≤1
16	1	≤1
18	1	11–19
18	2	0.5–4

- **FATS AND FIXED OILS**, *Peroxide Value* (401): NMT 10.0
- **FATS AND FIXED OILS**, *Unsaponifiable Matter* (401): NMT 1.5%
- **MELTING RANGE OR TEMPERATURE** (741): 27°–29°
- **WATER DETERMINATION**, *Method I* (921): NMT 0.1%, 50 mL of chloroform being used instead of 35–40 mL of methanol as the solvent.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Do not store above 45°.
- **LABELING:** Label it to indicate the name and quantity of any added antioxidants.

Palmitic Acid



Hexadecanoic acid [57-10-3].

256.42

DEFINITION

Palmitic Acid is a mixture of solid organic acids obtained from fats or oils of animal or vegetable origin. It contains NLT 92.0% of palmitic acid ($\text{C}_{16}\text{H}_{32}\text{O}_2$) and NMT 6.0% of stearic acid ($\text{C}_{18}\text{H}_{36}\text{O}_2$).

IDENTIFICATION

- **A.** The retention time of the major peak for palmitic acid of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

ASSAY

PROCEDURE

Sample solution: Proceed as directed for *Test solution* in *Fats and Fixed Oils* (401), *Fatty Acid Composition*.

Standard solution: Prepare the *Standard solution* in the same manner as the *Sample solution*, using a mixture of 50 mg of USP Palmitic Acid RS and 50 mg of USP Stearic Acid RS instead of the substance to be examined.

Chromatographic system: Prepare as directed in *Fats and Fixed Oils* (401), *Fatty Acid Composition*.

Injection size: 1 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for methyl palmitate and methyl stearate are 0.9 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 3.0 between methyl stearate and methyl palmitate

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of $\text{C}_{16}\text{H}_{32}\text{O}_2$ in the portion of Palmitic Acid taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for methyl palmitate from the *Sample solution*

r_T = sum of the responses of all the peaks in the chromatogram except the solvent peak

Similarly, calculate the percentage of $\text{C}_{18}\text{H}_{36}\text{O}_2$ in the portion of Palmitic Acid taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for methyl stearate from the *Sample solution*

r_T = sum of the responses of all the peaks in the chromatogram except the solvent peak

Acceptance criteria: NLT 92.0% of palmitic acid ($\text{C}_{16}\text{H}_{32}\text{O}_2$) and NMT 6.0% of stearic acid ($\text{C}_{18}\text{H}_{36}\text{O}_2$)

IMPURITIES

- **HEAVY METALS**, *Method II* (231): NMT 10 ppm

SPECIFIC TESTS

- **COLOR:** Heat a sample of Palmitic Acid to 75°. The resulting liquid is not more intensely colored than a solution prepared by mixing 1.2 mL of ferric chloride CS and 0.3 mL of cobaltous chloride CS with 0.3 N hydrochloric acid to make 10 mL, and diluting 5 mL of this solution with 0.3 N hydrochloric acid to make 100 mL. Make the comparison by viewing the solutions downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* (631)).
- **CONGEALING TEMPERATURE** (651): 60°–66°
- **FATS AND FIXED OILS**, *Acid Value* (401): 216–220, using 1 g
- **FATS AND FIXED OILS**, *Iodine Value* (401): NMT 1. Proceed as directed in *Method I*, except use 35 mL of chloroform.

- **MINERAL ACID**

Analysis: Shake 5 g of melted Palmitic Acid with an equal volume of hot water for 2 min. Cool, and filter.
Acceptance criteria: The filtrate is not reddened by the addition of 1 drop of methyl orange TS.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.
- **LABELING:** Label it to indicate whether it is derived from animal or vegetable sources.
- **USP REFERENCE STANDARDS** <11>
 USP Palmitic Acid RS
 USP Stearic Acid RS

Paraffin

[8002-74-2].

DEFINITION

Paraffin is a purified mixture of solid saturated hydrocarbons obtained from petroleum. It may contain suitable antioxidants.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197>
Sample: Use a thin film of melted specimen.
Analysis: Ensure complete melting to avoid doublet peaks that may be observed at wavenumbers at about 1460 and 730 cm^{-1} .
Acceptance criteria: Meets the requirements
- **B.** It meets the requirements in *Specific Tests for Congealing Temperature* <651>.

IMPURITIES

- **LIMIT OF SULFUR COMPOUNDS**
Sample: 4.0 g
Analysis: To the *Sample* add 2 mL of dehydrated alcohol, and add 2 drops of a clear saturated solution of lead(II) oxide in sodium hydroxide solution (200 mg/mL). Heat the mixture at 70° for 10 min with frequent shaking, and cool.
Acceptance criteria: No dark brown color develops.
- **LIMIT OF POLYCYCLIC AROMATIC HYDROCARBONS**
Dimethyl sulfoxide: Use spectrophotometric grade dimethyl sulfoxide.
Standard solution: 7.0 $\mu\text{g/mL}$ of USP Naphthalene RS in *Dimethyl sulfoxide*. Determine the absorbance of this solution at 278 nm using *Dimethyl sulfoxide* as the blank.
Sample: 0.50 g
Instrumental conditions
 (See *Spectrophotometry and Light-Scattering* <851>.)
Mode: UV
Wavelength range: 260–350 nm
Cell: 1 cm

Analysis: Dissolve the *Sample* in 25 mL of *n*-heptane, place in a 125-mL separator with unlubricated ground-glass parts (stopper, stopcock), and mix. Add 5.0 mL of *Dimethyl sulfoxide*, and shake the mixture vigorously for 1 min. Allow to stand until two clear layers are formed. Transfer the lower layer to another 125-mL separator, add 2 mL of *n*-heptane, and shake the mixture vigorously. Allow to stand until two clear layers are formed. Separate the lower layer, and determine its absorbance using as the blank *Dimethyl sulfoxide* that previously has been shaken vigorously for 1 min with *n*-heptane in the ratio of 5 mL of *Dimethyl sulfoxide* to 25 mL of *n*-heptane.

Acceptance criteria: The absorbance at any wavelength in the specified range is not greater than one-third of the absorbance, at 278 nm, of the *Standard solution*.

SPECIFIC TESTS

- **CONGEALING TEMPERATURE** <651>: 47°–65°
- **ACIDITY**
Sample: 15 g
Analysis: Introduce the *Sample* into a suitable separator, add 30 mL of boiling water, and shake vigorously for about 1 min. Allow to cool, and draw off the separated water. To 10 mL of the filtrated aqueous layer add 0.1 mL of phenolphthalein TS.
Acceptance criteria: The solution does not produce a pink color. NMT 1.0 mL of 0.01 M sodium hydroxide is subsequently required to change the color of the indicator to pink.
- **ALKALINITY**
Sample: 10 mL of the filtrated aqueous layer obtained from the test for *Acidity*
Analysis: To the *Sample* add 0.1 mL of methyl red TS2.
Acceptance criteria: The solution produces a yellow color. NMT 0.5 mL of 0.01 M hydrochloric acid is subsequently required to change the color of the indicator to red.
- **READILY CARBONIZABLE SUBSTANCES** <271>
Standard solution: A mix of 3 mL of ferric chloride CS, 1.5 mL of cobaltous chloride CS, and 0.50 mL of cupric sulfate CS, overlaid with 5 mL of mineral oil
Sample: 5 mL, at a temperature just above the melting point
Analysis: Use a clean, dry, heat-resistant, glass-stoppered test tube, 140 \pm 2 mm in length with an outside diameter between 14.5 and 15.0 mm, and calibrated at the 5- and 10-mL liquid levels. The capacity of the tube with the stopper inserted is between 13.6 and 15.6 mL.¹ Place the *Sample* in the test tube, add 5 mL of sulfuric acid (94.5%–94.9% of H_2SO_4), and heat in a water bath at 70° for 10 min. When 5 min have elapsed, and at each successive min thereafter, remove the tube from the bath, place a finger over the stopper, and give the tube three vigorous vertical shakes over an amplitude of about 12 cm, returning the tube to the bath within 3 s after the time when it was removed therefrom.
Acceptance criteria: At the end of 10 min from the time the tube was placed in the bath, the acid (lower layer) has no more color than the *Standard solution*. If the sulfuric acid remains dispersed in the molten paraffin, the color of the emulsion is not darker than that of the *Standard solution* when shaken vigorously.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in light resistant, well-closed containers, and avoid exposure to excessive heat.
- **LABELING:** Label it to indicate the name and quantity of any antioxidants.
- **USP REFERENCE STANDARDS** <11>
 USP Naphthalene RS
 USP Paraffin RS

Synthetic Paraffin

DEFINITION

Synthetic Paraffin is synthesized by the Fischer-Tropsch process from carbon monoxide and hydrogen, which are catalytically converted to a mixture of paraffin hydrocarbons; the lower molecular weight fractions are removed by distillation, and the residue is hydrogenated and further treated by percolation through activated charcoal. This mixture may be fractionated into its components by a solvent separation method, using a suitable synthetic

¹ A suitable test tube is available from Kimble Kontes. Item number: 34-19426. Description: Nessler Tube. Contact: phone 800-682-6644, fax 856-692-6644, e-mail customglass@kimkon.com.

isoparaffinic petroleum hydrocarbon solvent. It may contain NMT 0.005% of a suitable antioxidant.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197): A thin film of it, cast from a melt onto a cesium bromide plate, exhibits a pair of very strong IR absorption peaks between 2840 cm^{-1} and 3000 cm^{-1} , a pair of moderately strong peaks between 1430 cm^{-1} and 1490 cm^{-1} , a pair of medium peaks between 720 cm^{-1} and 750 cm^{-1} , and only weak peaks at any other wavenumbers.

IMPURITIES

- **HEAVY METALS**, *Method II* (231): NMT 20 ppm

- **LIMIT OF OIL CONTENT**

Analysis: Follow ASTM Method D721-68, "Standard Test Method for Oil Content of Petroleum Waxes" (Reapproved 1987).¹

Acceptance criteria: NMT 0.5%

SPECIFIC TESTS

- **ABSORPTIVITY**

Sample solution: Transfer 50–100 mg to a 100-mL volumetric flask. Dissolve in decahydronaphthalene at 88°, dilute with the same solvent at this temperature to volume, and mix.

Blank: Decahydronaphthalene

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV

Analytical wavelength: 290 nm

Cell: 10 cm (jacketed cells maintained at 88°)

Analysis

Samples: *Sample solution* and *Blank*

Determine the absorbance of the *Sample solution*, and calculate the absorptivity.

Acceptance criteria: NMT 0.01

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** The labeling indicates its congealing temperature, viscosity, and needle penetration range under the specified conditions.

Peanut Oil

[8002-03-7].

DEFINITION

Peanut Oil is the fully-refined (alkali-refined, bleached, and deodorized at 230°–260°) oil obtained from the seed kernels of one or more of the cultivated varieties of *Arachis hypogaea* Linné (Fam. Leguminosae). It may contain suitable antioxidants.

IDENTIFICATION

- It meets the requirements for *Fats and Fixed Oils, Fatty Acid Composition* (401).

IMPURITIES

Inorganic Impurities

- **HEAVY METALS**, *Method II* (231): NMT 10 ppm

- **ALKALINE IMPURITIES**

Sample: 10 mL of Peanut Oil

Analysis: Mix 10 mL of freshly opened acetone and 0.3 mL of water, and add 0.05 mL of bromophenol blue TS. Add the *Sample*, shake, and allow to stand. Titrate with 0.01 N hydrochloric acid VS to change the color of the upper layer to yellow.

¹ Available from the American Society for Testing and Materials, 1916 Race St, Philadelphia, PA 19103.

Acceptance criteria: NMT 0.1 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Fatty Acid Composition* (401): Peanut Oil exhibits the following fatty acid composition profile.

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<14	0	≤0.1
14	0	≤0.2
16	0	7.0–16.0
16	1	≤1.0
18	0	1.3–6.5
18	1	35.0–72.0
18	2	13.0–43.0
18	3	≤0.6
20	0	0.5–3.0
20	1	0.5–2.1
22	0	1.0–5.0
22	1	≤0.5
24	0	0.5–3.0

- **RANCIDITY**

Sample: 1 mL of a solution (1 in 10) of Oil in ether

Analysis: Shake the *Sample* with 1 mL of hydrochloric acid, and add 1 mL of a solution (1 in 1000) of phloroglucinol in ether.

Acceptance criteria: No red or pink color develops.

- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 0.2

- **FATS AND FIXED OILS**, *Peroxide Value* (401): NMT 5.0

- **FATS AND FIXED OILS**, *Unsaponifiable Matter* (401): NMT 1.5%

- **WATER DETERMINATION**, *Method Ic* (921): NMT 0.1%.

- **OTHER REQUIREMENTS:** For Peanut Oil intended for use in injectable dosage forms, which is specified in the *Labeling*, the requirements under *Injections* (1), *Ingredients*, *Other Vehicles*, must be met.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and prevent exposure to excessive heat.
- **LABELING:** Label it to indicate the name and quantity of any added antioxidant. Where Peanut Oil is intended for use in the manufacture of injectable dosage forms, it is so labeled.

Pectin—see *Pectin General Monographs*

Peppermint

DEFINITION

Peppermint consists of the dried leaf and flowering top of *Mentha piperita* L. (Fam. Labiatae).

SPECIFIC TESTS

- **ARTICLES OF BOTANICAL ORIGIN**, *Foreign Organic Matter* (561): NMT 2.0% of stems more than 3 mm in diameter

and other foreign organic matter

- **BOTANIC CHARACTERISTICS**

Unground peppermint: Leaves, slender stems, and flowering tops. The leaves are opposite, usually more or less crumpled, and frequently detached from the stem. The petiole is 4–15 mm in length, slightly pubescent; the blade, when entire, is ovate-oblong to oblong-lanceolate, 1.5–9 cm in length with an acute apex, a narrowed or rounded base, and a sharply serrate margin; light green to dark green in color; its upper surface

is nearly glabrous, its lower surface has a few hairs on the veins and many amber-colored glandular hairs. The stem is quadrangular, 1–3 mm in diameter, glabrous except for a few scattered deflexed hairs, green to dark purple. The flowers occur as a compact, oblong or oval spike of verticillasters, 1–1.5 cm in breadth, rounded at the summit, and in fruit attaining a length of 3–7 cm. The bracts are oblong-lanceolate, 4–7 mm in length; the calyx, tubular-campanulate, equally five-toothed, pubescent, and glandular-punctate, green to dark purple; the corolla is glabrous, light purple, tubular-campanulate, four-cleft, 3 mm in length; stamens, 4, short and equal; style two- or rarely three-cleft at the summit. The nutlets are ellipsoidal, 500 μ m in diameter. Peppermint has an aromatic, characteristic odor and a pungent taste, and produces a cooling sensation in the mouth.

Histology

Leaf: The lamina is dorsiventral. Both the upper and the lower epidermis consist of epidermal cells with wavy, anticlinal walls and stomata, the latter enclosed by a pair of subsidiary cells with a common wall at right angles to the guard cells. Many of the epidermal cells, especially over the veins and midrib, bear nonglandular and glandular hairs. The nonglandular hairs, also numerous along the margin, are uniseriate with longitudinally striate and papillose cuticle, up to 8 cells in length and tapered to a pointed apex. The glandular hairs occur in two types. The larger of these are sunken in depressions of the epidermis and consist of a one- to two-celled stalk and a glandular head of 8 radiating cells beneath the raised cuticle of which volatile oil is secreted. The smaller type of glandular hair consists of a one- to two-celled stalk and a one-celled glandular head containing volatile oil. Beneath the upper epidermis occurs a single layer of palisade parenchyma up to 80 μ m in length and, directly underneath it, spongy parenchyma of 3 or 4 layers of chloroplast-containing cells, through which zone course the fibrovascular tissues of the veins.

Stem: The stem is quadrangular. It shows a layer of epidermis bearing hairs similar to those of the leaf and possessing cuticularized outer convex walls, a narrow cortex of chlorenchyma, a clear endodermis of tangentially elongated, thin-walled cells with colorless contents, a narrow phloem, a cambium, and a xylem broadest in the regions beneath the stem angles and containing narrow wood-wedges separated by xylem rays one cell in width. The wood-wedges consist chiefly of simple pitted and spiral vessels, tracheids, and wood-fibers. Beneath each of the four angles of the stem occurs an elliptic to ovate zone of collenchyma. A large pith composed of thin-walled parenchyma occupies the center.

Powdered peppermint: Green to light olive green. Shows fragments of leaf epidermis with wavy vertical walls and, if from the lower surface of the leaf, with numerous stomata and glandular and nonglandular hairs, the latter especially numerous along the veins; glandular hairs with a one- to two-celled stalk and one- to eight-celled head, usually set in a depression in the leaf and containing volatile oil and frequently yellowish or brownish crystals that are birefringent; nonglandular hairs with thin, papillose walls and frequently with short, longitudinal striations of 1–8 cells and up to 1.4 mm in length, the terminal cell pointed or sometimes globular; fragments of chlorenchyma with vascular tissue, the vessels spiral or with simple pits and but slightly lignified; fragments of collenchyma and of thin-walled, nonlignified fibers associated with parenchyma. The pollen grains are spheroidal and smooth.

Peppermint Oil

DEFINITION

Peppermint Oil is the volatile oil distilled with steam from the fresh overground parts of the flowering plant of *Mentha piperita* Linné (Fam. Labiatae), rectified by distillation and neither partially nor wholly dementholized. It yields NLT 5.0% of esters, calculated as menthyl acetate ($C_{12}H_{22}O_2$), and NLT 50.0% of total menthol ($C_{10}H_{20}O$), free and as esters.

IDENTIFICATION

• A.

Sample: 6 drops of Oil

Analysis: Place the *Sample* in a dry test tube and mix with 5 mL of a 1-in-300 solution of nitric acid in glacial acetic acid, and place the tube in a beaker of boiling water.

Acceptance criteria: Within 5 min the liquid develops a blue color that, on continued heating, deepens and shows a copper-colored fluorescence and then fades, leaving a golden-yellow solution.

ASSAY

• TOTAL ESTERS

Sample: 10 g of Oil

Analysis: Place the *Sample* in a 250-mL conical flask, add 10 mL of neutralized alcohol and 2 drops of phenolphthalein TS, then add, dropwise, 0.1 N sodium hydroxide until a faint pink color appears. Add 25.0 mL of 0.5 N alcoholic potassium hydroxide VS, connect the flask to a reflux condenser, and heat on a boiling water bath for 1 h. Allow the mixture to cool, add 20 mL of water, and add phenolphthalein TS.

Titrate the excess alkali with 0.5 N hydrochloric acid VS. Perform a blank determination, disregarding the 0.1 N sodium hydroxide (see *Titrimetry* <541>, *Residual Titrations*). Each mL of 0.5 N alcoholic potassium hydroxide consumed in the saponification is equivalent to 99.15 mg of total esters calculated as $C_{12}H_{22}O_2$.

Acceptance criteria: NLT 5.0% of esters, calculated as $C_{12}H_{22}O_2$

• TOTAL MENTHOL

Sample: 10 mL of Oil

Analysis: Place the *Sample* in an acetylation flask of 100-mL capacity, and add 10 mL of acetic anhydride and 1 g of anhydrous sodium acetate. Boil the mixture gently for 1 h, accurately timed, cool, disconnect the flask from the condenser, transfer the mixture to a small separator, rinsing the acetylation flask with three 5-mL portions of warm water, and add the rinsings to the separator. When the liquids have completely separated, discard the water layer, and wash the remaining oil with successive portions of sodium carbonate TS, diluted with an equal volume of water, until the last washing is alkaline to phenolphthalein TS. Dry the resulting oil with anhydrous sodium sulfate, and filter. Transfer 5 mL of the dry acetylated oil to a tared, 100-mL conical flask, and weigh. Add 50.0 mL of 0.5 N alcoholic potassium hydroxide VS, connect the flask to a reflux condenser, and boil the mixture on a steam bath for 1 h, accurately timed. Allow the mixture to cool, and add 10 drops of phenolphthalein TS.

Titrate the excess alkali with 0.5 N hydrochloric acid VS. Perform a blank determination (see *Titrimetry* <541>, *Residual Titrations*).

Calculate the percentage of total menthol in the Oil tested:

$$\text{Result} = 7.813 \times A \times (1 - 0.0021 \times E) / (W - 0.021 \times A)$$

A = result obtained by subtracting the number of mL of 0.5 N hydrochloric acid required in the above titration from the number of mL of 0.5 N hydrochloric acid required in the residual titration blank

E = percentage of esters calculated as menthyl acetate ($C_{12}H_{22}O_2$)

W = weight of acetylated Oil taken (g)

Acceptance criteria: NLT 50.0% of $C_{10}H_{20}O$, free and as esters

IMPURITIES

• **HEAVY METALS**, *Method II* (231): NMT 20 ppm

• **LIMIT OF DIMETHYL SULFIDE**

Analysis: Distill 1 mL from 25 mL of Oil, and carefully superimpose the distillate on 5 mL of mercuric chloride TS in a test tube.

Acceptance criteria: A white film does not form at the zone of contact within 1 min.

SPECIFIC TESTS

• **SPECIFIC GRAVITY** (841): 0.896–0.908

• **OPTICAL ROTATION**, *Angular Rotation* (781A): -18° to -32°

• **REFRACTIVE INDEX** (831): 1.459–1.465 at 20°

• **SOLUBILITY IN 70% ALCOHOL:** One volume dissolves in 3 volumes of 70% alcohol, with NMT slight opalescence.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and prevent exposure to excessive heat.

Peppermint Spirit—see *Peppermint Spirit General Monographs*

Peppermint Water

DEFINITION

Peppermint Water is a clear, saturated solution of Peppermint Oil in Purified Water, prepared by one of the processes described in *Pharmaceutical Dosage Forms* (1151), *Solutions, Waters, Aromatic*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers.

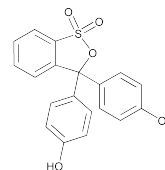
Petrolatum—see *Petrolatum General Monographs*

Petrolatum, Hydrophilic—see *Petrolatum, Hydrophilic General Monographs*

Petrolatum, White—see *Petrolatum, White General Monographs*

Phenol—see *Phenol General Monographs*

Phenolsulfonphthalein



$C_{19}H_{14}O_5S$ 354.38

Phenol red;

Phenol, 4,4'-(3*H*-2,1-benzoxathiol-3-ylidene)bis-, (*S*,*S*-dioxide);

3,3-Bis(4-hydroxyphenyl)-3*H*-2,1-benzoxathiole 1,1-dioxide [143-74-8].

DEFINITION

Phenolsulfonphthalein contains NLT 98.0% and NMT 102.0% of 4,4'-(3*H*-2,1-benzoxathiol-3-ylidene)bis-, (*S*,*S*-dioxide) phenol ($C_{19}H_{14}O_5S$), calculated on the dried basis.

IDENTIFICATION

• **A.**

Sample: 5 mg of Phenolsulfonphthalein

Analysis: Transfer the *Sample* to a 100-mL volumetric flask, dissolve in and dilute with sodium carbonate solution (1 in 100) to volume, and mix. Dilute 5.0 mL of the solution so obtained to 100.0 mL with sodium carbonate solution (1 in 100). Examine between 400 and 630 nm.

Acceptance criteria: The solution exhibits an absorption maximum at 558 nm, and the specific absorbance at the maximum is between 1900 and 2100.

• **B.**

Sample: 10 mg of Phenolsulfonphthalein

Analysis: Dissolve the *Sample* in 2 mL of 1 N sodium hydroxide, and add 8 mL of water. To 5 mL of the solution so obtained add 1 mL of 0.1 N potassium bromide–bromate and 1 mL of diluted hydrochloric acid, shake, and allow to stand for 15 min. Render the solution alkaline with 1 N sodium hydroxide.

Acceptance criteria: An intense violet-blue color is produced.

ASSAY

• **PROCEDURE**

Sample: 0.9 g

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 250-mL volumetric flask, dissolve in 15 mL of 1 N sodium hydroxide, dilute with water to volume, and mix. Transfer 10.0 mL of the solution so obtained to a glass-stoppered flask. Add 25 mL of glacial acetic acid, 20.0 mL of 0.1 N potassium bromate VS, 5 mL of potassium bromide solution (1 in 10), and 5 mL of hydrochloric acid, and immediately insert the stopper into the flask. Allow to stand protected from light for 15 min. Quickly add 10 mL of potassium iodide solution (1 in 10), taking care to avoid the escape of bromine vapor. Immediately insert the stopper into the flask, and shake vigorously. Rinse the stopper and the neck of the flask with a small quantity of water. Titrate the liberated iodine with 0.1 N sodium thiosulfate VS, using starch TS as the indicator. Perform a blank determination, and note the difference in volumes required. Each mL of the difference in volumes of 0.1 N sodium thiosulfate is equivalent to 4.43 mg of phenolsulfonphthalein ($C_{19}H_{14}O_5S$).

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

• RESIDUE ON IGNITION (281)

Sample: 0.5 g

Acceptance criteria: NMT 0.2%

• CHROMATOGRAPHIC PURITY

Sample solution A: 20 mg/mL of Phenolsulfonphthalein in 0.1 N sodium hydroxide

Sample solution B: Transfer 0.5 mL of *Sample solution A* to a 100-mL volumetric flask, dilute with 0.1 N sodium hydroxide to volume, and mix.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 10 µL

Developing solvent system: *tert*-Amyl alcohol, glacial acetic acid, and water (4:1:1)

Analysis

Samples: *Sample solution A* and *Sample solution B*
Allow the plate to air-dry until the solvent has evaporated, and expose the plate to ammonia vapor. Examine the plate under short-wavelength UV light.

Acceptance criteria: NMT 0.5%; NMT one spot, apart from the principal spot, appears in the chromatogram from *Sample solution A*. This spot is not more intense than the spot in the chromatogram from *Sample solution B*.

• INSOLUBLE SUBSTANCES

Sample: 1 g of finely powdered Phenolsulfonphthalein

Analysis: To the *Sample* add a solution of 0.5 g of sodium bicarbonate in 12 mL of water. Allow to stand for 1 h, shaking frequently. Dilute with sufficient water to make 100 mL, and allow to stand for 15 h. Centrifuge at 2000–3000 *g* for 30 min, and decant the supernatant. Wash the residue first with 25 mL of sodium bicarbonate solution (1 in 100), then with 25 mL of water, and dry at 105°.

Acceptance criteria: The weight of the insoluble residue does not exceed 0.5% of the weight of the Phenolsulfonphthalein taken.

SPECIFIC TESTS

• VISUAL TRANSITION INTERVAL

Potassium chloride solution: Dissolve 1.0 g of potassium chloride in 100 mL of water, and adjust with 0.01 N hydrochloric acid or sodium hydroxide to a pH of 6.8.

Sample solution: Dissolve 0.1 g of Phenolsulfonphthalein in 100 mL of alcohol.

Analysis 1: Add 0.15 mL of the *Sample solution* to the *Potassium chloride solution*.

Acceptance criteria 1: The color is yellow with NMT a faint trace of green color.

Analysis 2: Titrate the solution from *Analysis 1* with 0.01 N sodium hydroxide to a pH of 7.0.

Acceptance criteria 2: The color of the solution becomes orange.

Analysis 3: Continue the titration of the solution from *Analysis 2* with 0.01 N sodium hydroxide to a pH of 8.2.

Acceptance criteria 3: The color of the solution becomes red. NMT 0.20 mL of 0.01 N sodium hydroxide is consumed in the entire titration.

• MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):

The total aerobic microbial count does not exceed 10³ cfu/g, and the total combined molds and yeasts count does not exceed 10² cfu/g.

• LOSS ON DRYING (731)

Sample: 1 g of powdered Phenolsulfonphthalein

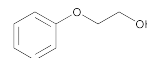
Analysis: Dry the *Sample* at 105° to constant weight.

Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Phenoxyethanol



C₈H₁₀O₂

138.16

2-Phenoxyethanol;

2-Phenoxyethyl alcohol;

Ethylene glycol, 2-monophenyl ether [122-99-6].

DEFINITION

Phenoxyethanol contains NLT 98.0% and NMT 102.0% of phenoxyethanol (C₈H₁₀O₂).

IDENTIFICATION

• **A. INFRARED ABSORPTION (197F):** On an undried specimen

ASSAY

• PROCEDURE

Phenol solution, Standard solution, and Chromatographic system: Prepare as directed in the test for *Organic Impurities*.

Sample stock solution: 5 mg/mL of Phenoxyethanol in isopropyl alcohol

Sample solution: Transfer 500 µL of the *Sample stock solution* to a vial, add 1000 µL of isopropyl alcohol, crimp the vial, and mix on a vortex mixer for 15 s.

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of phenoxyethanol (C₈H₁₀O₂) in the portion of Phenoxyethanol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Phenoxyethanol RS in the *Standard solution* (mg/mL)

C_U = concentration of Phenoxyethanol in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0%

IMPURITIES

• ORGANIC IMPURITIES

Phenol solution: 0.25 mg/mL of phenol in isopropyl alcohol

Standard stock solution: 5 mg/mL of USP Phenoxyethanol RS in the *Phenol solution*

Standard solution: Transfer 500 µL of the *Standard stock solution* to a vial, add 1000 µL of isopropyl alcohol, crimp the vial, and mix on a vortex mixer for 15 s.

Sample solution: Transfer 500 µL of Phenoxyethanol to a tared vial, and determine the weight of Phenoxyethanol taken. Add 1000 µL of isopropyl alcohol, crimp the vial, and mix on a vortex mixer for 15 s.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 10-m capillary coated with a 5-µm film of stationary phase G27

Temperatures

Injection port: 300°

Detector: 300°

Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	8	260	10

Carrier gas: Helium

Injection volume: 1 µL

Injection type: Split injection mode

Split flow rate: 44 mL/min

System suitabilitySample: *Standard solution***Suitability requirements****Resolution:** NLT 10 between the phenol and phenoxyethanol peaks**Relative standard deviation:** NMT 2.0% for the phenoxyethanol peak**Analysis**Samples: *Standard solution* and *Sample solution*

Calculate the percentage of total impurities in the portion of Phenoxyethanol taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

r_u = peak response of all additional peak areas in the *Sample solution*, excluding the main peak, the solvent peak, and the phenol peak

r_s = peak response of phenoxyethanol from the *Standard solution*

C_s = concentration of phenoxyethanol in the *Standard solution* (mg/mL)

C_u = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 1.0%**• LIMIT OF PHENOL**

Phenol solution, Standard solution, Sample solution, and Chromatographic system: Proceed as directed in the test for *Organic Impurities*.

AnalysisSamples: *Standard solution* and *Sample solution*

Calculate the percentage of phenol in the portion of Phenoxyethanol taken:

$$\text{Result} = (r_u/r_s) \times (C_s/C_u) \times 100$$

r_u = peak response of phenol from the *Sample solution*

r_s = peak response of phenol from the *Standard solution*

C_s = concentration of phenol in the *Standard solution* (mg/mL)

C_u = concentration of the *Sample solution* (mg/mL)

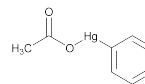
Acceptance criteria: NMT 0.1%**SPECIFIC TESTS**

- SPECIFIC GRAVITY** (841): 1.105–1.110 at 20°

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool, dry place, protected from light.
- USP REFERENCE STANDARDS** (11)
USP Phenoxyethanol RS

Phenylethyl Alcohol—see *Phenylethyl Alcohol General Monographs*

Phenylmercuric AcetateC₈H₈HgO₂

336.74

Mercury, (acetato-O)phenyl-;
(Acetato)phenylmercury [62-38-4].**DEFINITION**

Phenylmercuric Acetate contains NLT 98.0% and NMT 100.5% of phenylmercuric acetate (C₈H₈HgO₂).

IDENTIFICATION**• A.**

Sample: 0.1 g

Analysis: To the *Sample* add 0.5 mL of nitric acid. Warm gently until a dark brown color is produced, and dilute with water to 10 mL.

Acceptance criteria: The characteristic odor of nitrobenzene is evolved.

• B.

Sample: 0.1 g

Analysis: To the *Sample* add 0.5 mL of sulfuric acid and 1 mL of alcohol, and warm.

Acceptance criteria: The characteristic odor of ethyl acetate is evolved.

• C.

Sample solution: Saturated solution in water

Analysis: To 5 mL of the *Sample solution* add a few drops of sodium sulfide TS.

Acceptance criteria: A white precipitate is formed, which turns black when the mixture is boiled and then allowed to stand.

ASSAY**• PROCEDURE**

Sample solution: Transfer 500 mg of Phenylmercuric Acetate to a 100-mL flask. Add 15 mL of water, 5 mL of formic acid, and 1 g of zinc dust, and reflux for 30 min. Cool, filter, and wash the filter paper and the amalgam with water until the washings are no longer acid to litmus. Dissolve the amalgam in 40 mL of 8 N nitric acid. Heat on a steam bath for 3 min, and then add 500 mg of urea and enough potassium permanganate TS to produce a permanent pink color. Cool, decolorize the solution with hydrogen peroxide TS, and add 1 mL of ferric ammonium sulfate TS.

Titrimetric system(See *Titrimetry* (541).)**Mode:** Direct titration**Titrant:** 0.1 N ammonium thiocyanate VS**Endpoint detection:** Visual

Analysis: Titrate with *Titrant*, and each mL of 0.1 N ammonium thiocyanate is equivalent to 16.84 mg of phenylmercuric acetate (C₈H₈HgO₂).

Acceptance criteria: 98.0%–100.5%**IMPURITIES**

- RESIDUE ON IGNITION** (281): NMT 0.2%

• MERCURIC SALTS AND HEAVY METALS

Sample solution: Heat 100 mg of Phenylmercuric Acetate with 15 mL of water, cool, and filter.

Analysis: To the *Sample solution* filtrate add a few drops of sodium sulfide TS.

Acceptance criteria: The resulting precipitate shows no immediate color.

• POLYMERURATED BENZENE COMPOUNDS

Sample solution: Shake 2.0 g of Phenylmercuric Acetate with 100 mL of acetone, and filter.

Analysis: Wash the residue with successive portions of acetone until a total of 50 mL is used, then dry the residue at 105° for 1 h. Weigh the residue.

Acceptance criteria: NMT 1.5%; the weight of residue is NMT 30 mg.

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** (741): 149°–153°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Phenylmercuric Nitrate

Mercury, (nitrate-O)phenyl-;
Nitratophenylmercury [55-68-5].

DEFINITION

Phenylmercuric Nitrate is a mixture of phenylmercuric nitrate and phenylmercuric hydroxide containing NLT 87.0% and NMT 87.9% of phenylmercuric ion ($C_6H_5Hg^+$), and NLT 62.75% and NMT 63.50% of mercury (Hg).

IDENTIFICATION

- **A.**
Sample: 0.1 g
Analysis: To the *Sample* add 3 mL of sulfuric acid.
Acceptance criteria: The mixture becomes yellow, and the characteristic odor of nitrobenzene is evolved.
- **B.**
Sample solution: Saturated solution in water
Analysis: To 5 mL of the *Sample solution* add 1 mL of 3 N hydrochloric acid.
Acceptance criteria: A white precipitate is formed.
- **C.**
Sample solution: Saturated solution in water
Analysis: To 5 mL of the *Sample solution* add 5 mL of ammonium sulfide TS.
Acceptance criteria: There is no reaction in the cold, but upon heating in a boiling water bath for 10 min, a black precipitate is formed.

ASSAY

• PHENYLMERCURIC IONS

Sample: 200 mg
Analysis: Dissolve the *Sample* in 90 mL of water and 10 mL of nitric acid. Add 2 mL of ferric ammonium sulfate TS. Titrate with 0.05 N ammonium thiocyanate VS. Each mL of 0.05 N ammonium thiocyanate is equivalent to 13.88 mg of phenylmercuric ion ($C_6H_5Hg^+$).
Acceptance criteria: 87.0%–87.9% of phenylmercuric ion

• MERCURY

Sample solution: Transfer 400 mg of Phenylmercuric Nitrate to a 100-mL flask. Add 15 mL of water, 5 mL of formic acid, and 1 g of zinc dust, and reflux for 30 min. Cool. Filter, and wash the filter paper and the amalgam with water until the washings are no longer acid to litmus. Dissolve the amalgam in 40 mL of 8 N nitric acid. Heat on a steam bath for 3 min, then add 0.5 g of urea and enough potassium permanganate TS to produce a permanent pink color. Cool. Decolorize the solution with hydrogen peroxide TS, and add 1 mL of ferric ammonium sulfate TS.

Analysis: Titrate with 0.1 N ammonium thiocyanate VS. Each mL of 0.1 N ammonium thiocyanate is equivalent to 10.03 mg of Hg.

Acceptance criteria: 62.75%–63.50% of mercury

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%

SPECIFIC TESTS

• MERCURY IONS

Sample solution: Saturated solution in water

Analysis: To 5 mL of the *Sample solution* add 5 mL of 1 N sodium hydroxide.

Acceptance criteria: No yellow precipitate is formed (mercuric ions), and the solution does not darken (mercurous ions).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.

Phosphoric Acid

H_3PO_4 98.00
Phosphoric acid [7664-38-2].

DEFINITION

Phosphoric Acid contains NLT 85.0% and NMT 88.0%, by weight, of H_3PO_4 .

[**CAUTION**—Avoid contact, because Phosphoric Acid rapidly destroys tissues.]

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Phosphate** (191): When carefully neutralized with 1 N sodium hydroxide, phenolphthalein TS being used as the indicator, it meets the requirements.

ASSAY

• PROCEDURE

Sample: 1 g

Blank: 120 mL of water

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 1 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: Place the *Sample* in a tared, glass-stoppered flask, and dilute it with water to 120 mL. Add 0.5 mL of thymolphthalein TS. Titrate with 1 N sodium hydroxide VS to the first appearance of a blue color. Perform a blank determination.

Calculate the percentage of phosphoric acid (H_3PO_4) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = volume of *Titrant* consumed by the *Sample* (mL)

V_B = volume of *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 49.00 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 85.0%–88.0% by weight

IMPURITIES

- **HEAVY METALS, Method I** (231): NMT 10 ppm

• LIMIT OF NITRATE

Sample solution: Dilute 6 mL of Phosphoric Acid with 14 mL of water.

Analysis: Mix 5 mL of the *Sample solution* with about 0.1 mL of indigo carmine TS, then add 5 mL of sulfuric acid.

Acceptance criteria: The blue color is not discharged within 1 min.

SPECIFIC TESTS

- **CHLORIDE AND SULFATE, Sulfate** <221>
Sample solution: Dilute 6 mL of Phosphoric Acid with 90 mL of water.
Analysis: To the *Sample solution* add 1 mL of barium chloride TS.
Acceptance criteria: No precipitate is formed immediately.
- **ALKALI PHOSPHATES**
Sample: 1 mL
Analysis: Transfer the *Sample* to a graduated cylinder, and add 6 mL of ether and 2 mL of alcohol.
Acceptance criteria: No turbidity is produced.
- **PHOSPHOROUS OR HYPOPHOSPHOROUS ACID**
Sample solution: Dilute 6 mL of Phosphoric Acid with 14 mL of water.
Analysis: Gently warm 5 mL of the *Sample solution*, and add 2 mL of silver nitrate TS.
Acceptance criteria: The mixture does not become brownish.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Diluted Phosphoric Acid

DEFINITION

Diluted Phosphoric Acid contains, in each 100 mL, NLT 9.5 g and NMT 10.5 g of phosphoric acid (H₃PO₄). Diluted Phosphoric Acid may be prepared as follows.

Phosphoric Acid	69 mL
Purified Water, a sufficient quantity to make	1000 mL

Mix the ingredients.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Phosphate** <191>
Sample: 100 mL
Analysis: Carefully neutralize the *Sample* with 1 N sodium hydroxide. Use phenolphthalein TS as the indicator.
Acceptance criteria: Meets the requirements

ASSAY

- **PROCEDURE**
Sample: 10 mL
Blank: 50 mL of water
Titrimetric system
 (See *Titrimetry* <541>.)
Mode: Direct titration
Titrant: 1 N sodium hydroxide VS
Endpoint detection: Visual
Analysis: Dilute the *Sample* with water to 50 mL. Add 0.5 mL of thymolphthalein TS. Titrate with *Titrant* to the first appearance of a blue color. Perform a blank determination.
 Calculate the amount of phosphoric acid (H₃PO₄) in the portion of the sample taken:

$$\text{Result} = (V_S - V_B) \times N \times F$$

V_S = *Titrant* volume consumed by the *Sample* (mL)
 V_B = *Titrant* volume consumed by the *Blank* (mL)
 N = actual normality of the *Titrant* (mEq/mL)
 F = equivalency factor, 4.9×10^{-2} g/mEq

Acceptance criteria: 9.5–10.5 g per 100 mL

IMPURITIES

- **HEAVY METALS, Method I** <231>
Test preparation: Dilute 10 g (9.5 mL) with 10 mL of water, add 6 mL of 1 N sodium hydroxide, and dilute with water to 50 mL. Dilute 20 mL of this solution with water to 25 mL.
Acceptance criteria: NMT 5 µg/g
- **LIMIT OF NITRATE**
Sample: 100 mL
Analysis: To the *Sample* add 0.1 mL of indigo carmine TS, then 5 mL of sulfuric acid.
Acceptance criteria: The blue color is not discharged within 1 min.

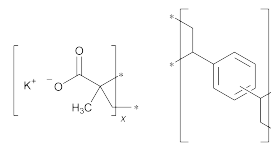
SPECIFIC TESTS

- **ALKALI PHOSPHATES**
Sample: 20 mL
Analysis: Evaporate the *Sample* on a steam bath to a weight of 5 g. Cool, transfer 2 mL to a graduated cylinder, and add 6 mL of ether and 2 mL of alcohol.
Acceptance criteria: No turbidity is produced
- **PHOSPHOROUS OR HYPOPHOSPHOROUS ACID**
Sample: 100 mL
Analysis: Gently warm the *Sample*, and add 2 mL of silver nitrate TS.
Acceptance criteria: The mixture does not become brownish.
- **CHLORIDE AND SULFATE, Sulfate** <221>
Sample: 100 mL
Analysis: To the *Sample* add 1 mL of barium chloride TS.
Acceptance criteria: No precipitate is formed immediately.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Polacrilin Potassium



2-Propenoic acid, 2-methyl-, potassium salt, polymer with diethenylbenzene;
 Potassium methacrylate–divinylbenzene, copolymer [65405-55-2].

DEFINITION

Polacrilin Potassium is the potassium salt of a unifunctional low-cross-linked carboxylic cation-exchange resin prepared from methacrylic acid and divinylbenzene. When previously dried at 105° for 6 h, it contains NLT 20.6% and NMT 25.1% of potassium (K).

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>
- **B. IDENTIFICATION TESTS—GENERAL, Potassium** <191>
Sample solution A: Shake 1 g with 10 mL of water.
Sample solution B: Shake 1 g with 10 mL of 0.1 N hydrochloric acid.
Analysis: Use the aqueous phase from *Sample solution A* and *Sample solution B*.

Acceptance criteria**Sample solution A:** Does not meet the requirements**Sample solution B:** Meets the requirements**ASSAY****• CONTENT OF POTASSIUM****Sodium stock solution:** 14.612 mg/mL of sodium chloride, previously dried at 125° for 30 min. This solution contains 5.76 mg/mL of sodium (Na).**Potassium stock solution:** 745.5 µg/mL of potassium chloride, previously dried at 125° for 30 min. This solution contains 391 µg/mL of potassium (K).**Surfactant solution:** Transfer 5.0 g of a suitable nonionic surfactant to a 250-mL beaker, add 200 mL of water, and stir to dissolve. Transfer this solution to a 500-mL volumetric flask, dilute with water to volume, and mix. [NOTE—To prevent foaming when using this solution, gently run the solution down the sides of the vessel, and use gentle action when mixing.]**Diluted sodium solution:** Transfer 50.0 mL of *Sodium stock solution* and 10.0 mL of *Surfactant solution* to a 100-mL volumetric flask, dilute with water to volume, and mix gently to prevent foaming.**Standard solutions:** To three separate 500-mL volumetric flasks transfer, respectively, 3.0-, 4.0-, and 5.0-mL portions of *Potassium stock solution*. To each flask add 50.0 mL of *Sodium stock solution* and 10.0 mL of *Surfactant solution*, dilute with water to volume, and mix gently to prevent foaming. Each mL of these solutions contains 2.346, 3.128, and 3.910 µg of K, respectively.**Sample solution:** Transfer 1.4 g of Polacrilin Potassium, previously dried, to a 50-mL silica crucible, moisten with 4 mL of sulfuric acid, heat over a small flame until the acid has fumed off, moisten the residue with a few drops of sulfuric acid, and ignite strongly. Allow to cool, transfer, with the aid of water, to a 1000-mL volumetric flask, dilute with water to volume, and mix. Transfer 1.00 mL of this solution to a 100-mL volumetric flask, add 20.0 mL of *Diluted sodium solution*, dilute with water to volume, and mix gently to prevent foaming.**Instrumental conditions**(See *Spectrophotometry and Light-Scattering* (851).)**Mode:** Flame photometry**Analytical wavelength:** 766 nm**Analysis****Samples:** *Standard solutions* and *Sample solution* Concomitantly determine the emittances of the *Standard solutions* and *Sample solution*, adjusting the instrument so that the most concentrated *Standard solution* gives a reading near 100%.Prepare a standard curve by plotting the readings from the *Standard solutions* versus the square root of the potassium concentrations. From the curve, determine the concentration of potassium in the *Sample solution*. Calculate the percentage of potassium in the portion of sample taken:

$$\text{Result} = (C_o/C_u) \times 100$$

 C_o = concentration of the *Sample solution* determined from the standard curve (µg/mL) C_u = concentration of Polacrilin Potassium in the *Sample solution* (µg/mL)**Acceptance criteria:** 20.6%–25.1%**IMPURITIES****• HEAVY METALS, Method III (231):** NMT 20 µg/g**• IRON (241)****Sample:** 0.10 g**Analysis:** Transfer the *Sample* to a suitable crucible, and ignite at a low heat until thoroughly ashed. Add to the carbonized mass 2 mL of nitric acid and 5 drops of sulfuric acid, and heat cautiously until white fumes are no longer evolved. Ignite, preferably in a muffle

furnace, at 500°–600°, until the carbon is completely burned off. Cool, add 4 mL of 6 N hydrochloric acid, cover, digest on a steam bath for 15 min, uncover, and slowly evaporate on a steam bath to dryness. Moisten the residue with 1 drop of hydrochloric acid, add 10 mL of hot water, and digest for 2 min. Dilute with water to 25 mL. Filter, if necessary. Rinse the crucible and the filter with 10 mL of water, combining the filtrate and rinsing in a 50-mL color-comparison tube, add 2 mL of hydrochloric acid, dilute with water to 45 mL, and mix.

Acceptance criteria: NMT 0.01%**• LIMIT OF SODIUM****Sample solution:** Transfer 2 g to a 400-mL borosilicate beaker, add 20 mL of sulfuric acid, cover with a borosilicate watch glass, and heat until charring is complete. While continuing to heat the beaker, add 20 mL of nitric acid dropwise. Continue to heat, and add nitric acid until all of the organic material has been destroyed as indicated by the contents of the beaker turning from brown to a very pale straw-colored or colorless solution. Continue to evaporate the solution, and if it turns brown during the evaporation, add nitric acid dropwise until the brown color disappears. Evaporate just to dryness, cool, and dissolve the residue in 40 mL of water and 10 mL of 6 N hydrochloric acid. Heat to boiling, cool, transfer to a 100-mL volumetric flask, dilute with water to volume, and mix.**Standard solutions:** To three separate 100-mL volumetric flasks add, respectively, 1.00, 2.00, and 3.00 mL of a solution containing 254.2 mg of sodium chloride in 1000 mL of water. Add water to volume, and mix to obtain sodium chloride solutions having concentrations equivalent to 1, 2, and 3 µg/mL of Na, respectively.**Instrumental conditions**(See *Spectrophotometry and Light-Scattering* (851).)**Mode:** Flame photometry**Analytical wavelength:** 589 nm**Analysis****Samples:** *Standard solutions* and *Sample solution* Adjust the instrument so that the emission of the *Standard solution* with a concentration of 3 µg/mL reads close to 100% at 589 nm.Determine the readings of the three *Standard solutions* at 589 nm. Readjust the wavelength setting to 580 nm, and determine the background emission reading for one of these *Standard solutions*.Pipet 5 mL of the *Sample solution* into a 100-mL volumetric flask, add water to volume, and mix.

Observe the emission reading of this solution at 589 nm, using the same instrument settings, then readjust the wavelength setting to 580 nm, and observe the background emission reading.

Subtract the corresponding background readings from the readings of *Standard solutions* and *Sample solution*.Prepare a standard curve by plotting the corrected *Standard solution* readings versus the square root of the sodium concentration. From this standard curve, determine the sodium content in the sample taken.**Acceptance criteria:** NMT 0.20%**SPECIFIC TESTS****• POWDER FINENESS (811)****Sample:** 4 g**Analysis:** Transfer the *Sample* to a No. 100 standard sieve placed on top of a No. 200 standard sieve and pan. Using a soft 2-cm brush, brush the sample lightly across the No. 100 sieve until no more particles pass through. By brushing and tapping, dust off the particles on the underside of the No. 100 sieve into the No. 200 sieve. Obtain the weight of the material retained on the No. 100 sieve. Similarly, determine the weight of material retained by the No. 200 sieve.

Acceptance criteria: NMT 1.0% is retained on the No. 100 sieve, and NMT 30.0% is retained on the No. 200 sieve.

• **LOSS ON DRYING** <731>

Analysis: Dry at 105° for 6 h.

Acceptance criteria: NMT 10.0%

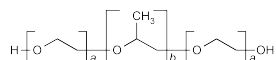
ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** <11>

USP Polacrilin Potassium RS

Poloxamer



$\text{HO}(\text{C}_2\text{H}_4\text{O})_a(\text{C}_3\text{H}_6\text{O})_b(\text{C}_2\text{H}_4\text{O})_a\text{H}$

Oxirane, methyl-, polymer with oxirane;

α -Hydro- ω -hydroxypoly(oxyethylene)_a-poly(oxypropylene)_b-poly(oxyethylene)_a block copolymer, in which a and b have the values shown in the following table:

Poloxamer	a	b
124	12	20
188	80	27
237	64	37
338	141	44
407	101	56

Polyethylene-polypropylene glycol [9003-11-6].

DEFINITION

Poloxamer is a synthetic block copolymer of ethylene oxide and propylene oxide. It is available in several types, conforming to the requirements shown in the following table.

Pol-ox-amer	Physical Form	Average Molecular Weight	Weight (% Oxyethylene)	Unsaturation (mEq/g)
124	Liquid	2090–2360	46.7 ± 1.9	0.020 ± 0.008
188	Solid	7680–9510	81.8 ± 1.9	0.026 ± 0.008
237	Solid	6840–8830	72.4 ± 1.9	0.034 ± 0.008
338	Solid	12,700–17,400	83.1 ± 1.7	0.031 ± 0.008
407	Solid	9840–14,600	73.2 ± 1.7	0.048 ± 0.017

It may contain a suitable antioxidant.

IDENTIFICATION

• **A. INFRARED ABSORPTION** <197F>: Use a thin film of melted specimen if it is a solid. Use USP Poloxamer Liquid RS for Poloxamer 124, and use USP Poloxamer Solid RS for Poloxamer 188, 237, 338, and 407. Because of the differences in the ratios of copolymer composition, the intensity of some absorption bands may vary.

ASSAY

• **AVERAGE MOLECULAR WEIGHT**

Phthalic anhydride–pyridine solution: Dissolve 144 g of phthalic anhydride in freshly opened or freshly distilled pyridine containing less than 0.1% of water, and dilute with pyridine to 1000 mL. Protect from light, and allow to stand overnight. To verify that the *Phthalic anhydride–pyridine solution* has adequate strength, pipet 10 mL into a 250-mL conical flask, add 25 mL of pyridine and 50 mL of water, and after 15 min add 0.5 mL

of a solution of phenolphthalein in pyridine (1 in 100), then titrate with 0.5 N sodium hydroxide VS: it consumes between 37.6 and 40.0 mL of 0.5 N sodium hydroxide.

Analysis: Weigh a suitable quantity, not exceeding 15 g, of Poloxamer, calculated by multiplying the average molecular weight by 0.004, into a glass-stoppered, 250-mL boiling flask. Carefully pipet 25 mL of *Phthalic anhydride–pyridine solution* into the flask, touching the tip of the drained pipet to the protrusion in the flask. Add a few glass beads, and swirl to dissolve the specimen. Pipet 25 mL of *Phthalic anhydride–pyridine solution* into a second, glass-stoppered, conical flask, add a few glass beads, and use as the reagent blank. (An additional 25-mL portion of pyridine may be added to both the test specimen and reagent blank, before refluxing, if necessary to ensure fluidity.) Heat both flasks, fitted with suitable reflux condensers, and allow to reflux for 1 h. Allow to cool, and pour two 10-mL portions of pyridine through each condenser. Remove the flasks from the condensers, add 10 mL of water to each, insert the stoppers, swirl, and allow to stand for 10 min. To each flask add 50.0 mL of 0.66 N sodium hydroxide and 0.5 mL of a solution (1 in 100) of phenolphthalein in pyridine. Titrate with 0.5 N sodium hydroxide VS to a light pink endpoint that persists for NLT 15 s. Calculate the average molecular weight:

$$\text{Result} = 2000 \times W / [(V_8 - V_5) \times N]$$

W = weight of the sample taken (g)

V₈ = volume of 0.5 N sodium hydroxide VS consumed by the blank (mL)

V₅ = volume of 0.5 N sodium hydroxide VS consumed by the residual acid in the test solution (mL)

N = actual normality of the 0.5 N sodium hydroxide VS

Acceptance criteria: See the table in the *Definition*.

• **WEIGHT PERCENT OXYETHYLENE**

Solvent: Use deuterated water or deuteriochloroform.

NMR reference: Use sodium 2,2-dimethyl-2-silapentane-5-sulfonate (for deuterated water) or tetramethylsilane (for deuteriochloroform).

Sample solution: Dissolve 0.1–0.2 g of Poloxamer in deuterated water containing 1% of sodium 2,2-dimethyl-2-silapentane-5-sulfonate to obtain 1 mL of solution, or, if the Poloxamer does not dissolve in water, use deuteriochloroform containing 1% of tetramethylsilane as the solvent.

Instrumental conditions

(See *Nuclear Magnetic Resonance* <761>, *Relative Method of Quantitation*)

Mode: Nuclear magnetic spectrometry

Sample size: 0.5–1.0 mL of the *Sample solution*

Analysis:

Sample: *Sample solution*

Transfer the *Sample solution* to a standard 5-mm NMR spinning tube, and if deuteriochloroform is the solvent, add 1 drop of deuterated water, and shake the tube. Scan the region at 0–5 ppm, and use the calculation formulas specified below. Record as A₁ the average area of the doublet appearing at about 1.08 ppm, representing the methyl groups of the oxypropylene units, and record as A₂ the average area of the composite band at a range of 3.2–3.8 ppm, due to the CH₂O groups of both the oxyethylene and oxypropylene units and also the CHO groups of the oxypropylene units, with reference to the sodium 2,2-dimethyl-2-silapentane-5-sulfonate or tetramethylsilane singlet at 0 ppm.

Calculate the percentage of oxyethylene, by weight, in the Poloxamer taken:

$$\alpha = (A_2/A_1) - 1$$

- A_2 = average area of the composite band at a range of 3.2–3.8 ppm
 A_1 = average area of the doublet appearing at about 1.08 ppm

$$\text{Result} = 3300 \times \alpha / (33 \times \alpha + 58)$$

Acceptance criteria: See the table in the *Definition*.

• **UNSATURATION**

Solution A: Place 50 g of mercuric acetate in a 1000-mL volumetric flask, and dissolve with 900 mL of methanol to which 0.5 mL of glacial acetic acid has been added. Dilute with methanol to volume, and mix. Discard the solution if it is yellow. If it is turbid, filter it. Discard it if it is still turbid. Use fresh reagents if it is necessary to repeat the preparation of the solution. Protect the solution from light by storing it in an amber bottle in the dark.

Sample: 15.0 g

Analysis: Transfer the *Sample* to a 250-mL conical flask. Pipet 50 mL of *Solution A* into the flask, and mix on a magnetic stirrer until solution is complete. Allow to stand for 30 min with occasional swirling. Add 10 g of sodium bromide crystals, and stir on a magnetic stirrer for 2 min. Without delay, add 1 mL of phenolphthalein TS, and titrate the liberated acetic acid with 0.1 N methanolic potassium hydroxide VS. Perform a blank determination. Determine also the initial acidity as follows. Dissolve 15.0 g of Poloxamer in 75 mL of methanol that has been neutralized with methanolic potassium hydroxide to the phenolphthalein endpoint. Add 1 mL of phenolphthalein TS, and titrate with the same 0.1 N methanolic potassium hydroxide VS under a nitrogen sweep.

Calculate the unsaturation, in mEq/g:

$$\text{Result} = (V_U - V_B - V_A) \times N/15$$

- V_U = volume of 0.1 N methanolic potassium hydroxide used for titrating the test specimen (mL)
 V_B = volume of 0.1 N methanolic potassium hydroxide used for titrating the blank (mL)
 V_A = volume of 0.1 N methanolic potassium hydroxide used for titrating the initial acidity (mL)
 N = normality of the titrant

Acceptance criteria: See the table in the *Definition*.

IMPURITIES

- **HEAVY METALS, Method I (231):** NMT 20 ppm
 • **LIMIT OF FREE ETHYLENE OXIDE, PROPYLENE OXIDE, AND 1,4-DIOXANE**

Stripped poloxamer: Place 500 g of Poloxamer 124 into a suitable 3-neck, round-bottom flask equipped with a stirrer, a thermometer, a vacuum outlet, and a heating mantle. Evacuate the flask carefully at room temperature to a pressure of less than 10 mm of mercury, applying the vacuum slowly to avoid excessive foaming due to entrapped gases. After any foaming has subsided, heat the flask to 80° and continue to apply vacuum for 2 h; then cool to room temperature. Shut off the vacuum pump, and introduce nitrogen to bring the flask pressure back to atmospheric pressure. Transfer the *Stripped poloxamer* to a suitable nitrogen-filled container.

Standard solution

[**CAUTION**—Ethylene oxide, propylene oxide, and 1,4-dioxane are toxic and flammable. Prepare these solutions in a well-ventilated fume hood.]

To a tared vial that can be sealed add 50.0 g of *Stripped poloxamer*. Add 60 µL of 1,4-dioxane and 75 µL of propylene oxide from a chilled syringe. Add ethylene oxide, using the following special handling procedure. Ethylene oxide, which is a gas at room temperature, is

usually stored in a lecture-type gas cylinder or a small, metal pressure-bomb. Chill the cylinder in a refrigerator before use. Transfer 5 mL of the liquid ethylene oxide to a 100-mL beaker chilled in wet ice. Using a gas-tight syringe that has been chilled in a refrigerator, transfer 15 µL of the liquid ethylene oxide to the mixture. Immediately seal the vial, and shake on a vortex mixer for at least 30 s. Transfer 0.20 g of this solution to a tared vial that can be sealed, and add *Stripped poloxamer* to obtain a *Standard solution* having a final weight of 50.0 g. Each g of this *Standard solution* contains 1 µg of ethylene oxide, 5 µg of propylene oxide, and 5 µg of 1,4-dioxane. Transfer 1.00 ± 0.01 g of this solution to a 22-mL pressure headspace vial, and add about 0.01 g of butylated hydroxytoluene. Seal with a silicone septum with or without a pressure-relief star spring and with a pressure-relief, aluminum, safety sealing-cap, and crimp the cap closed with a cap-sealing tool.

Sample solution: Transfer 1.00 ± 0.01 g of Poloxamer to a 22-mL pressure headspace vial, and add 0.01 g of butylated hydroxytoluene. Seal, cap, and crimp as directed for the *Standard solution*.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC (equipped with a balanced-pressure automated headspace sampler)

Detector: Flame ionization

Column: 0.32-mm × 50-m fused-silica capillary; 5-µm layer of stationary phase G27 coating

Temperature

Detector: 250°

Injector: 250°

Transfer line: 140°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	10
70	10	240	10

Carrier gas: Helium

Flow rate: 1.6 mL/min

Injection size: Separately place the vials containing the *Standard solution* and the *Sample solution* in the automated sampler, and start the sequence so that the vial is heated at a temperature of 110° for 30 min before a suitable portion of its headspace is injected into the chromatograph.

Autosampler

Needle-withdrawal time: 0.3 min

Pressurization time: 1 min

Injection time: 0.08 min

Vial pressure: 22 psig with the vial vent off

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for ethylene oxide, propylene oxide, and 1,4-dioxane are about 1.0, 1.3, and 3.8, respectively.]

Suitability requirements

Resolution: NLT 2.0 between ethylene oxide and propylene oxide

Relative standard deviation: NMT 15%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the concentrations, in µg/g, of ethylene oxide, propylene oxide, and 1,4-dioxane in the portion of Poloxamer taken:

$$\text{Result} = (r_U/r_S) \times C$$

r_U = peak response from the *Sample solution*
 r_S = peak response from the *Standard solution*
 C = concentration of ethylene oxide, propylene oxide, or 1,4-dioxane in the *Standard solution* ($\mu\text{g/g}$)

Acceptance criteria

Ethylene oxide: NMT 1 $\mu\text{g/g}$
 Propylene oxide: NMT 5 $\mu\text{g/g}$
 1,4-Dioxane: NMT 5 $\mu\text{g/g}$

SPECIFIC TESTS

- PH** (791): 5.0–7.5, in a solution (1 in 40)

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- LABELING:** Label it to state, as part of the official title, the Poloxamer number. Label it to indicate the name and quantity of any antioxidant.
- USP REFERENCE STANDARDS** (11)
 USP Poloxamer Liquid RS
 USP Poloxamer Solid RS

Hydrogenated Polydecene

 $\text{C}_{30\leq n \leq 70}\text{H}_{2n+2}$

1-Decene, homopolymer, hydrogenated [68037-01-4].

DEFINITION

Hydrogenated Polydecene is a mixture of saturated, synthetic hydrocarbons in the range $\text{C}_{30}\text{H}_{62}$ through $\text{C}_{70}\text{H}_{142}$ made from direct oligomerization of 1-decene (C_{10} alpha olefin). The oligomer mixture may be distilled to fractions of a suitable calculated viscosity and hydrogenated to reach saturation, or it may be hydrogenated to reach saturation and then distilled to the desired viscosity. The requirements for specific gravity, viscosity, and content of decene oligomer differ for the various types of Hydrogenated Polydecene, as set forth in the two tables below. Hydrogenated Polydecene may contain a suitable stabilizer.

Specific Gravity and Viscosity

Type	Specific Gravity	Kinematic Viscosity Range, Centistokes (mm^2/s)
I	0.814–0.819	16.0–20.0
II	0.823–0.827	28.0–34.0
III	0.828–0.832	40.0–52.0

Content of Decene Oligomers

Type	$\text{C}_{30}\text{H}_{62}$	$\text{C}_{40}\text{H}_{82}$	$\text{C}_{50}\text{H}_{102}$	$\text{C}_{60}\text{H}_{122}$	$\text{C}_{70}\text{H}_{142}$
I	70–93	5–25	0–5	0–1	0–1
II	13–40	35–70	9–25	0–7	0–2
III	3–15	25–55	25–40	13–28	0–10

IDENTIFICATION

- A.** The chromatogram of the *Sample solution* from the test for *Content of Decene Oligomer* exhibits major peaks for trimers, tetramers, pentamers, hexamers, and possibly heptamers. The decene oligomer content is within the range given in the table *Content of Decene Oligomers* in the *Definition* for the labeled type of Hydrogenated Polydecene.

ASSAY

- CONTENT OF DECENE OLIGOMER**

System suitability solution: 10 mg/mL of hexadecane, 10 mg/mL of squalane, and 1 mg/mL of tetradecane in pentane

Sample solution: Dissolve 0.1 mL of Hydrogenated Polydecene in 10 mL of pentane.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.52-mm \times 16-m fused-silica capillary; coated with 0.1-mm stationary phase G2

Carrier gas: Helium

Flow rate: 10 mL/min

Injection volume: 2 μL

Temperatures

Injection port: 310°

Detector: 320°

Column: See Table 1.

Table 1

Initial Temperature (°)	Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
35	5	50	—
50	12	170	—
170	10	310	18

System suitability

Sample: *System suitability solution*

[NOTE—The retention time for squalene is about 18 min; the relative retention times for tetradecane, hexadecane, and squalene are about 0.5, 0.6, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between tetradecane and hexadecane

Relative standard deviation: NMT 2.0% for each peak

Analysis

Sample: *Sample solution*

Record the chromatogram, and measure the areas for the major peaks.

[NOTE—The tetramer oligomer has a retention time of about 23 min. The trimer, pentamer, hexamer, and heptamer oligomers, if present, have relative retention times of about 0.8, 1.1, 1.3, and 1.4, respectively, relative to the tetramer.]

Calculate the percentage of each oligomer present:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = response of each oligomer

r_T = sum of the responses of all the peaks, excluding the solvent peak

Acceptance criteria: The decene oligomer content is within the limits specified in the table *Content of Decene Oligomers* in the *Definition*.

IMPURITIES

- LIMIT OF NICKEL**

Nickel stock solution: Immediately before use, dilute an appropriate quantity of organometallic standard¹ with kerosene to prepare a solution containing the equivalent of 1.0 $\mu\text{g/mL}$ of nickel.

Standard solutions: Transfer 0.5, 1.0, 2.0, and 4.0 mL of *Nickel stock solution*, respectively, to four identical 10-mL volumetric flasks, dilute the contents of each flask with kerosene to volume, and mix. These *Standard*

¹ Suitable organometallic standards are available from, e.g., Continental Oil Co., Ponca City, OK (Conostan, 100 ppm), or Merck, D-6100 Darmstadt, Germany (metal in standard oil, 1000 ppm).

solutions contain, respectively, 0.05, 0.1, 0.2, and 0.40 µg/mL of nickel. [NOTE—The calibration range, especially the upper limit, can be adjusted for certain instruments, provided that instrument validation and calibration linearity are achieved.]

Sample solution: 0.3 g/mL of Hydrogenated Polydecene in kerosene. [NOTE—If necessary, dilute with an appropriate quantity of kerosene to obtain a reading within the calibrated absorbance range.]

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Graphite furnace atomic absorption spectrophotometer equipped with a deuterium background corrector and a pyrolytically coated tube with platform

Analytical wavelength: 232.0 nm (nickel emission line)

Injection volume: 20 µL

Lamp: Nickel hollow-cathode

Blank: Kerosene

Temperature: See Table 2.

[NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Table 2

Step	Temperature (°)	Hold Time (s)
Drying	80	1
Drying	120	10
Drying	300	20
Ashing	600	20
Ashing	1000	20
Atomization	2500	3
Cleaning	2600	5

Analysis

Samples: *Standard solutions* and *Sample solution*

Place the *Standard solutions* and the *Sample solution* in an oven, setting the temperature at about 60° during the period of determination, and shake these solutions vigorously before analysis. Use micropipettor and pipettor tips to make all injections. [NOTE—Positive displacement pipets can be used when viscosity may become a problem.]

Pretreat the pipettor tip by pipetting and then discarding 20 µL of heptane. The tip must be pretreated before each injection. [NOTE—The film of heptane remaining on the wall of the tip facilitates a reproducible transfer of the oil sample.]

Separately inject the *Standard solutions* and the *Sample solution* into a graphite furnace, and concomitantly determine the integrated absorbances of the *Standard solutions* and the *Sample solution*.

Plot the integrated absorbances of the *Standard solutions* versus concentration, in µg/mL, of nickel, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration of nickel, *C*, in µg/mL, in the *Sample solution*.

Calculate the content of nickel in the Hydrogenated Polydecene taken:

$$\text{Result} = C/C_U$$

C = concentration of nickel obtained from the graph (µg/mL)

C_U = concentration of nickel in the *Sample solution* (g/mL)

Acceptance criteria: NMT 1 µg/g

• **LIMIT OF SHORT-CHAIN HYDROCARBONS**

System suitability solution, Sample solution, Chromatographic system, System suitability, and

Analysis: Proceed as directed in the test for *Content of Decene Oligomer*.

Calculate the percentage of each of the short-chain hydrocarbons present:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of any peak eluting before the trimer but different from the solvent peak

r_T = sum of the responses of all the peaks in the chromatogram, excluding the solvent peak

Acceptance criteria: NMT 2.5% of total short-chain hydrocarbons is found.

SPECIFIC TESTS

• **SPECIFIC GRAVITY (841)**

Analysis: Determine at 20°.

Acceptance criteria: Meets the requirements of the specific gravity range specified in the table *Specific Gravity and Viscosity* in the *Definition* for the labeled type

• **VISCOSITY—CAPILLARY VISCOMETER METHODS (911)**

Analysis: Determine using a capillary viscometer, in a liquid bath maintained at 40.0 ± 0.1°.

Acceptance criteria: Meets the requirements of the viscosity range specified in the table *Specific Gravity and Viscosity* in the *Definition* for the labeled type.

• **READILY CARBONIZABLE SUBSTANCES TEST (271)**

Standard solution: 3 mL of ferric chloride CS, 1.5 mL of cobaltous chloride CS, and 0.5 mL of cupric sulfate CS in a glass-stoppered test tube previously treated to remove organic matter (see *Cleaning Glass Apparatus* (1051)).

Sample: 5 mL

Analysis: Transfer the *Sample* to a glass-stoppered test tube previously treated to remove organic matter (see *Cleaning Glass Apparatus* (1051)), add 5 mL of sulfuric acid, and heat in a boiling water bath for 30 s. Quickly remove the test tube, and, while holding the stopper in place, shake three times in a vertically reciprocating cycle with an amplitude of about 13 cm. Repeat this procedure every 30 s for 10 min. Do not keep the test tube out of the water bath any longer than 3 s for each shaking cycle. Remove the test tube from the water bath, and let it cool for about 20 min to room temperature.

Acceptance criteria: The oil phase of the *Sample* may turn hazy but remains colorless; the interface between the two layers is free from solids; and the acid layer does not become darker than the standard color produced by the *Standard solution*, the *Standard solution* being overlaid with 5 mL of Hydrogenated Polydecene.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements are specified.

• **LABELING:** Label it to indicate, as part of the official title, the Hydrogenated Polydecene type (Type I, Type II, or Type III), and label it to indicate the name and concentration of any added stabilizer.

Polydextrose

[68424-04-4].

DEFINITION

Polydextrose is a randomly branched polymer prepared by melting and subsequent condensation of the ingredients, which consist of approximately 90 parts dextrose, 10 parts sorbitol, and up to 1 part citric acid or 0.1 part phosphoric acid. The 1,6-glycosidic linkage predominates in the polymer but other linkages are present. It contains NLT 90.0% of dextrose polymer units, calculated on the

anhydrous and ash-free basis. It contains small quantities of free dextrose, sorbitol, and 1,6-anhydro-D-glucose (levoglucosan), with traces of citric acid or phosphoric acid.

IDENTIFICATION

- **A.** To 1 drop of a solution (1 in 10), add 4 drops of 5% phenol solution, then rapidly add 15 drops of sulfuric acid TS: a deep yellow to orange color is produced.
- **B.** With vigorous swirling, add 1 mL of acetone to 1 mL of a solution (1 in 10): the solution remains clear.
- **C.** With vigorous swirling, add 2 mL of acetone to the solution obtained in *Identification test B*: a heavy, milky turbidity develops immediately.
- **D.** To 1 mL of a solution (1 in 50), add 4 mL of alkaline cupric citrate TS. Boil vigorously for 2–4 min. Remove from heat, and allow the precipitate (if any) to settle: the supernatant is blue or blue-green.

ASSAY

PROCEDURE

Mobile phase: 0.001 N sulfuric acid. Pass this solution through a filter of 0.5- μ m pore size, and degas.

Standard solution: 4.0 mg/mL of USP Polydextrose RS, calculated on the anhydrous and ash-free basis, in *Mobile phase*

Sample solution: 4.0 mg/mL of Polydextrose, calculated on the anhydrous and ash-free basis, in *Mobile phase*

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index

Detector temperature: $35 \pm 0.1^\circ$

Guard column: 4.6-mm \times 3.0-cm, packing L17

Analytical column: 7.8-mm \times 30-cm, packing L17

Flow rate: 0.6 mL/min

Injection size: 20 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of dextrose polymer units in the portion of Polydextrose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of dextrose polymer units from the *Sample solution*

r_S = peak response of dextrose polymer units from the *Standard solution*

C_S = concentration of USP Polydextrose RS in the *Standard solution* (mg/mL)

C_U = concentration of Polydextrose in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 90.0%

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** <281>: NMT 0.3%

LIMIT OF LEAD

[NOTE—Use reagent-grade chemicals with as low a lead content as is practicable, as well as high-purity water and gases. Before use in this analysis, rinse all glassware and plasticware twice with 10% nitric acid and twice with 10% hydrochloric acid, and then rinse them thoroughly with Purified Water.]

Matrix modifier solution: Prepare a solution in water containing 100.0 mg of dibasic ammonium phosphate per 10 mL of solution.

Lead nitrate stock solution: Dissolve 159.8 mg of lead nitrate in 100 mL of water to which has been added 1 mL of nitric acid, then dilute with water to 1000 mL.

Prepare and store this solution in glass containers free from soluble lead salts.

Standard lead solution: On the day of use, dilute 10.0 mL of *Lead nitrate stock solution* with water to 100.0 mL. Each mL of *Standard lead solution* contains the equivalent of 10 μ g of lead.

Standard solution A: 0.02 μ g/mL of lead, from *Standard lead solution* in water

Standard solution B: 0.05 μ g/mL of lead, from *Standard lead solution* in water

Standard solution C: 0.1 μ g/mL of lead, from *Standard lead solution* in water

Standard solution D: 0.2 μ g/mL of lead, from *Standard lead solution* in water

Standard solution E: 0.5 μ g/mL of lead, from *Standard lead solution* in water

Sample solution: Transfer 1.0 g of Polydextrose, weighed and calculated on the anhydrous and ash-free basis, into a 10-mL volumetric flask, and dissolve in and dilute with water to volume.

Spiked sample solution: Transfer 1.0 g of Polydextrose, weighed and calculated on the anhydrous and ash-free basis, into a 10-mL volumetric flask, and dissolve in water. Add 100 μ L of the *Standard lead solution*, and dilute with water to volume. This solution contains 0.1 μ g/mL of added lead.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Graphite furnace atomic absorption spectrophotometer, equipped with a pyrolytic tube with a platform

Lamp: A lead hollow-cathode lamp, using a slit width of 0.7 mm (set low) and a deuterium arc lamp for background correction

Analytical wavelength: Lead emission line of 283.3 nm

Autosampler

Sample volume: 10 μ L

Alternative volume: 10 μ L of *Matrix modifier solution*

Furnace program: See the temperature program table below.

Step	Dry	Char	Atomize	Clean	Recharge
Temperature ($^\circ$)	130	800	2400	2600	20
Ramp time (s)	20	20	0	1	2
Hold time (s)	40	40	6	5	20
Argon flow rate (mL/min)	300	300	50	300	300

Analysis

Samples: 10 μ L of the *Matrix modifier solution* was added into each 10- μ L aliquot of the five *Standards solutions*, a mixture of 10 μ L of the *Matrix modifier solution* and 10 μ L of the *Sample solution*, and a mixture of 10 μ L of the *Matrix modifier solution* and 10 μ L of the *Spiked sample solution*

Concomitantly determine the absorbances of the *Samples* using the *Spectrometric conditions* described above. Plot the absorbance of each *Standard solution*, compensated for background correction, versus its content of lead, in μ g/mL, and draw the best straight line fitting the five points. From this plot, determine the concentrations, C_T and C_{ST} , in μ g/mL, of lead in the *Sample solution* and the *Spiked sample solution*, respectively.

Calculate the percentage recovery taken:

$$\text{Result} = [(C_{ST} - C_T)/A] \times 100$$

A = quantity of lead added to the *Spiked sample solution*, 0.1 µg/mL
Calculate the content, in µg/g, of lead in the portion of Polydextrose taken:

$$\text{Result} = (C_T/W) \times V$$

W = weight of Polydextrose taken to prepare the *Sample solution* (g)

V = volume of the *Sample solution*, 10 mL

Acceptance criteria: NMT 0.5 µg/g. The recovery is 80%–120%.

Organic Impurities

• PROCEDURE 1: LIMIT OF 5-HYDROXYMETHYLFURFURAL AND RELATED COMPOUNDS

Sample solution: 1.0 g of Polydextrose, weighed and calculated on the anhydrous and ash-free basis, diluted with water to 100 mL.

Analysis: Determine the absorbance of the *Sample solution* in a 1-cm quartz cell at 283 nm, with a suitable spectrophotometer, using water as the blank. Calculate the percentage of 5-hydroxymethylfurfural and related compounds in the Polydextrose taken:

$$\text{Result} = 100 \times (V \times M_r \times A)/(M \times L \times W)$$

V = volume of the *Sample solution*, 0.1 L

M_r = molecular weight of 5-hydroxymethylfurfural, 126 g/mol

A = absorbance of the *Sample solution*

M = molar extinction coefficient of 5-hydroxymethylfurfural at a wavelength of 283 nm, 16,830 L/mol cm

L = path length of the spectrophotometer cell (cm)

W = weight of Polydextrose taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1%

• PROCEDURE 2: LIMIT OF MONOMERS

Mobile phase, Sample solution, and Chromatographic system: Prepare as directed in the *Assay*.

Standard solution: 0.08 mg/mL of each of USP 1,6-Anhydro-D-glucose RS and USP Sorbitol RS, and 0.16 mg/mL of USP Dextrose RS, in *Mobile phase*

System suitability

Sample: *Standard solution*

[NOTE—For relative retention times, see *Table 1* below.]

Table 1

Name	Relative Retention Time
Dextrose (glucose)	0.7
Sorbitol	0.8
An isomer of 1,6-anhydro-D-glucose (D-anhydroglucose furanose form)	0.9
1,6-Anhydro-D-glucose (D-anhydroglucose pyranose form)	1.0

Suitability requirements

Resolution: NLT 1.0

Relative standard derivation: NMT 5.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Use peak response of USP 1,6-Anhydro-D-glucose RS in the *Standard solution* for calculation of percentage of the isomer of 1,6-anhydro-D-glucose in the *Sample solution*.

Calculate the percentage of each monomer in the portion of Polydextrose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of the respective monomer from the *Sample solution*

r_S = peak response of the respective monomer from the *Standard solution*

C_S = concentration of the respective standard monomer in the *Standard solution* (mg/mL)

C_U = concentration of Polydextrose in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 4.0% for 1,6-anhydro-D-glucose, NMT 4.0% for dextrose, and NMT 2.0% for sorbitol. [NOTE—In the case of 1,6-anhydro-D-glucose, the peak areas for the pyranose and furanose forms are combined.]

SPECIFIC TESTS

• MOLECULAR WEIGHT LIMIT

Mobile phase: Dissolve 35.0 g of sodium nitrate and 1.0 g of sodium azide in 100 mL of water. Dilute with water to 4 L. Pass through a filter of 0.45-µm pore size, and degas by applying an aspirator vacuum for 30 min. The resulting *Mobile phase* is 0.1 N sodium nitrate containing 0.025% sodium azide.

Standard solution: Transfer 20 mg each of USP Dextrose RS, stachyose, and 5800-, 23,700-, and 100,000-molecular weight (MW) pullulan standards into a 10-mL volumetric flask. Dissolve in and dilute with *Mobile phase* to volume. Pass through a syringe filter of 0.45-µm pore size into a suitable autosampler vial, and seal.

Sample solution: Transfer 50 mg of Polydextrose into a 10-mL volumetric flask. Dissolve in and dilute with *Mobile phase* to volume. Pass through a syringe filter of 0.45-µm pore size into a suitable autosampler vial, and seal.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index set at a sensitivity of 4×10^{-6} refractive index units full scale and maintained at a temperature of $35 \pm 0.1^\circ$

Column: 7.8-mm × 30-cm; packing L39

Column temperature: 45°

Flow rate: 0.8 mL/min

[NOTE—After installation of a new column, pump *Mobile phase* through the column overnight at a rate of 0.3 mL/min. Before calibration or analysis, increase the flow slowly over a 1-min period to 0.8 mL/min. Continue to pump *Mobile phase* through the column at this flow rate for at least 1 h before the first injection. Check the flow gravimetrically, and adjust it if necessary. Reduce the flow rate to about 0.1 mL/min when the system is not in use.]

Injection size: 50 µL

System suitability

Sample: *Standard solution*

[NOTE—The retention times for each component determined on replicate injections agree within ± 2 s.]

Chromatograph five replicate injections of the *Standard solution*, allowing 15 min between injections, and record the retention times of the components of the *Standard solution*.

Insert the average retention time along with the molecular weight of each component in the *Standard solution* into the calibration table of the molecular weight distribution software. Check the regression results for a cubic fit of the calibration points, and obtain a correlation coefficient, R, for the line.

Suitability requirements

Resolution: Dextrose and stachyose are baseline resolved from one another and from the 5800-MW pullulan standard.

[NOTE—Prominent negative baseline valleys are usually observed between the peaks for the 5800-; 23,700-; and 100,000-MW pullulan standards.]

Correlation coefficient R: NLT 0.9999

Analysis

Samples: *Standard solution* and *Sample solution*

Use the molecular weight distribution software of the data reduction system to generate a molecular weight distribution plot of Polydextrose.

Acceptance criteria: No measurable peak above a molecular weight of 22,000 is found.

- **pH** (791): 2.5–5.0, in a solution (1 in 10)
- **WATER DETERMINATION, Method I** (921): NMT 4.0%. Use a mixture of Hydranal solvent and Hydranal formamide dry (2:1) as a solvent. Perform the titration at 50° in a jacketed beaker.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. Store in a cool and dry place.
- **USP REFERENCE STANDARDS** (11)
 - USP 1,6-Anhydro-D-glucose RS
 - USP Dextrose RS
 - USP Polydextrose RS
 - USP Sorbitol RS

Hydrogenated Polydextrose

DEFINITION

Hydrogenated Polydextrose is obtained by transition metal catalytic hydrogenation of Polydextrose in aqueous solution. It contains NLT 90.0% of dextrose polymer units, calculated on the anhydrous and ash-free basis. The polymer chain end groups are mainly sorbitol-terminated.

IDENTIFICATION

- **A.** To 1 drop of a solution (1 in 10), add 4 drops of 5% phenol solution, then rapidly add 15 drops of sulfuric acid TS: a deep yellow to orange color is produced.
- **B.** With vigorous swirling, add 1 mL of acetone to 1 mL of a solution (1 in 10): the solution remains clear.
- **C.** With vigorous swirling, add 2 mL of acetone to the solution obtained in *Identification test B*: a heavy, milky turbidity develops immediately.
- **D.** To 1 mL of a solution (1 in 50), add 4 mL of alkaline cupric citrate TS. Boil vigorously for 2–4 min. Remove from heat, and allow the precipitate (if any) to settle: the supernatant is blue or blue-green.
- **E.** Meets the requirements for dextrose in *Procedure 2, Limit of Monomers*

ASSAY

PROCEDURE

Mobile phase: 0.001 N sulfuric acid. Pass through a filter of 0.5-μm or finer pore size, and degas.

Standard solution: 4.0 mg/mL of USP Polydextrose RS, calculated on the anhydrous and ash-free basis, in *Mobile phase*

Sample solution: 4.0 mg/mL of Hydrogenated Polydextrose, calculated on the anhydrous and ash-free basis, in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Detector temperature: 35 ± 0.1°

Guard column: 4.6-mm × 3.0-cm; packing L17

Analytical column: 7.8-mm × 30-cm; packing L17

Flow rate: 0.6 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of dextrose polymer units in the Hydrogenated Polydextrose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for dextrose polymer units from the *Sample solution*

r_S = peak response for dextrose polymer units from the *Standard solution*

C_S = concentration of USP Polydextrose RS in the *Standard solution* (mg/mL)

C_U = concentration of Hydrogenated Polydextrose in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 90.0% on the anhydrous and ash-free basis

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 0.3%

LIMIT OF LEAD

[NOTE—Use reagent-grade chemicals with a lead content of as low as possible, as well as high-purity water and gases. Before use in this analysis, rinse all glassware and plasticware twice with 10% nitric acid and twice with 10% hydrochloric acid, and then rinse them thoroughly with Purified Water.]

Matrix modifier solution: 10.0 mg/mL of dibasic ammonium phosphate

Lead nitrate stock solution: Dissolve 159.8 mg of lead nitrate in 100 mL of water to which has been added 1 mL of nitric acid, then dilute with water to 1000 mL. Prepare and store this solution in glass containers free from soluble lead salts.

Standard lead solution: On the day of use, dilute 10.0 mL of *Lead nitrate stock solution* with water to 100.0 mL. Each mL of *Standard lead solution* contains the equivalent of 10 μg of lead.

Standard solution A: 0.02 μg/mL of lead, from *Standard lead solution* in water

Standard solution B: 0.05 μg/mL of lead, from *Standard lead solution* in water

Standard solution C: 0.1 μg/mL of lead, from *Standard lead solution* in water

Standard solution D: 0.2 μg/mL of lead, from *Standard lead solution* in water

Standard solution E: 0.5 μg/mL of lead, from *Standard lead solution* in water

Sample solution: Transfer 1.0 g of Hydrogenated Polydextrose, weighed and calculated on the anhydrous and ash-free basis, into a 10-mL volumetric flask, dissolve in and dilute with water to volume.

Spiked sample solution: Transfer 1.0 g of Hydrogenated Polydextrose, weighed and calculated on the anhydrous and ash-free basis, into a 10-mL volumetric flask, and dissolve in water. Add 100 μL of *Standard lead solution*, and dilute with water to volume. This solution contains 0.1 μg/mL of added lead.

Spectrometric conditions
(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Graphite furnace atomic absorption spectrophotometer, equipped with a pyrolytic tube with a platform

Lamp: A lead hollow-cathode lamp, using a slit width of 0.7 mm (set low) and a deuterium arc lamp for background correction

Analytical wavelength: Lead emission line of 283.3 nm

Autosampler**Sample volume:** 10 µL**Alternative volume:** 10 µL of *Matrix modifier solution***Furnace program:** See the temperature program table below.

Step	Dry	Char	Atomize	Clean	Recharge
Temperature (°)	130	800	2400	2600	20
Ramp time (s)	20	20	0	1	2
Hold time (s)	40	40	6	5	20
Argon flow rate (mL/min)	300	300	50	300	300

Analysis

Samples: 10 µL of the *Matrix modifier solution* was added into each of the 10-µL aliquots of the five *Standard solutions*, a mixture of 10 µL of the *Matrix modifier solution* and 10 µL of the *Sample solution*, and a mixture of 10 µL of the *Matrix modifier solution* and 10 µL of the *Spiked sample solution*

Concomitantly determine the absorbances of the *Samples* using the *Spectrometric conditions* described above. Plot the absorbance of each *Standard solution*, compensated for background correction, versus its content of lead, in µg/mL, and draw the best straight line fitting the five points. From this plot, determine the concentrations, C_T and C_{ST} , in µg/mL, of lead in the *Sample solution* and the *Spiked sample solution*, respectively.

Calculate the percentage recovery taken:

$$\text{Result} = [(C_{ST} - C_T)/A] \times 100$$

A = quantity of lead added to the *Spiked sample solution*, 0.1 µg/mL

Calculate the content, in µg/g, of lead in Hydrogenated Polydextrose taken:

$$\text{Result} = (C_T/W) \times V$$

W = weight of Hydrogenated Polydextrose taken to prepare the *Sample solution* (g)

V = volume of the *Sample solution*, 10 mL

Acceptance criteria: NMT 0.5 µg/g; recovery is 80%–120%

• **LIMIT OF NICKEL**

[NOTE—All glassware used must be soaked in 1% *Nitric acid* for at least 2 h and then rinsed with water.]

1% Nitric acid: Cautiously add 10 mL of nitric acid to a 1000-mL volumetric flask containing about 500 mL of water. Mix, and dilute with water to volume.

Blank solution: Use 1% *Nitric acid*.

Nickel stock standard solution: Immediately before use, dilute an appropriate amount of nickel standard* with 1% *Nitric acid* to prepare a solution containing the equivalent of 10 µg/mL of nickel.

Standard solutions: Into four identical 100-mL volumetric flasks, introduce respectively 1.0, 2.0, 5.0, and 10.0 mL of *Nickel stock standard solution*. Dilute with 1% *Nitric acid* to volume, and mix. These standards contain 0.1, 0.2, 0.5, and 1.0 µg/mL of nickel.

Sample solution: Weigh 5 g of Hydrogenated Polydextrose into a 100-mL volumetric flask. Dissolve in and dilute with 1% *Nitric acid* to volume, and mix.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with an air–acetylene flame

Lamp: Nickel hollow-cathode

Analytical wavelength: 352.0 nm

System suitability

Sample: *Standard solution* of 0.2 µg/mL of nickel

Suitability requirements

Relative standard deviation: NMT 20%

Analysis

Samples: *Standard solutions* and *Sample solution*

Use the *Blank solution* to zero the instrument.

Concomitantly determine the absorbances of the *Samples* at least three times each. Record the average of the steady readings for each of the *Samples*. Clear the nebulizer using the *Blank solution*, and aspirate each of the *Samples* in turn. The standard chosen for reslope should be run every four to five samples. If there is a significant change in its response, reslope and repeat the previous samples.

Plot the absorbances of the *Standard solutions* versus concentration, in µg/mL, of nickel, and draw the straight line best fitting the four plotted points. From the graph so obtained, determine the concentration, C , in µg/mL, of nickel in the *Sample solution*. Calculate the quantity, in µg, of nickel in each g of Hydrogenated Polydextrose taken:

$$\text{Result} = (V \times C)/W$$

V = volume of the *Sample solution*, 100 mL

W = weight of Hydrogenated Polydextrose taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 2 µg/g

Organic Impurities

• **PROCEDURE 1: LIMIT OF 5-HYDROXYMETHYLFURFURAL AND RELATED COMPOUNDS**

Sample solution: 1.0 g of Hydrogenated Polydextrose, weighed and calculated on the anhydrous and ash-free basis, diluted with water to 100 mL

Analysis: Determine the absorbance of the *Sample solution* in a 1-cm quartz cell at 283 nm, with a suitable spectrophotometer, using water as the blank. Calculate the percentage of 5-hydroxymethylfurfural and related compounds in the Hydrogenated Polydextrose taken:

$$\text{Result} = (V \times M_r \times A)/(\epsilon_{283} \times L \times W) \times 100$$

V = volume of the *Sample solution*, 0.1 L

M_r = molecular weight of 5-hydroxymethylfurfural, 126 g/mol

A = absorbance of the *Sample solution*

ϵ_{283} = molar extinction coefficient of 5-hydroxymethylfurfural at a wavelength of 283 nm, 16,830 L · mol⁻¹ · cm⁻¹

L = path length of the spectrophotometer cell (cm)

W = weight of Hydrogenated Polydextrose taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 0.1%

• **PROCEDURE 2: LIMIT OF MONOMERS**

Mobile phase, Sample solution, and Chromatographic system: Prepare as directed in the *Assay*.

Standard solution: 0.08 mg/mL of each for USP 1,6-Anhydro-D-glucose RS and USP Sorbitol RS, and 0.04 mg/mL of USP Dextrose RS, in *Mobile phase*

*Suitable nickel standards are available from e.g., Fisher Scientific, Fair Lawn, NJ (nickel, reference standard solution, 1000 ppm ±1%, certified, application for atomic absorption) or RICCA Chemical Company, Arlington, TX (nickel standard, 1000 ppm Ni, for atomic absorption).

System suitability**Sample:** *Standard solution*

[NOTE—See the relative retention times table below.]

Name	Relative Retention Time
Dextrose (glucose)	0.7
Sorbitol	0.8
An isomer of 1,6-anhydro-D-glucose (D-anhydroglucose furanose form)	0.9
1,6-Anhydro-D-glucose (levoglucosan) (D-anhydroglucose pyranose form)	1.0

Suitability requirements**Resolution:** NLT 1.0**Relative standard derivation:** NMT 5.0%**Analysis****Samples:** *Standard solution* and *Sample solution*

Use the peak response of USP 1,6-Anhydro-D-glucose RS in the *Standard solution* for calculation of the percentage of the isomer of 1,6-anhydro-D-glucose in the *Sample solution*. Calculate the percentage of each monomer in the portion of Hydrogenated Polydextrose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response for the respective monomer from the *Sample solution*

r_S = peak response for the respective monomer from the *Standard solution*

C_S = concentration of the respective standard monomer in the *Standard solution* (mg/mL)

C_U = concentration of Hydrogenated Polydextrose in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 4.0% of 1,6-anhydro-D-glucose, NMT 5.75% for sorbitol and NMT 0.25% for dextrose

[NOTE—In the case of 1,6-anhydro-D-glucose, the peak areas for the pyranose and furanose forms are combined.]

SPECIFIC TESTS**• MOLECULAR WEIGHT LIMIT**

Mobile phase: Dissolve 35.0 g of sodium nitrate and 1.0 g of sodium azide in 100 mL of water. Dilute with water to 4 L. Pass through a filter of 0.45- μ m or finer pore size, and degas by applying an aspirator vacuum for 30 min. The resulting *Mobile phase* is 0.1 N sodium nitrate containing 0.025% sodium azide.

Standard solution: Transfer 20 mg each of USP Dextrose RS, stachyose, and 5800-, 23,700-, and 100,000-molecular weight (MW) pullulan standards into a 10-mL volumetric flask. Dissolve in and dilute with *Mobile phase* to volume. Pass through a syringe filter of 0.45- μ m or finer pore size into a suitable autosampler vial, and seal.

Sample solution: Transfer 50 mg of Hydrogenated Polydextrose into a 10-mL volumetric flask. Dissolve in and dilute with *Mobile phase* to volume. Pass through a syringe filter of 0.45- μ m or finer pore size into a suitable autosampler vial, and seal.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index set at a sensitivity of 4×10^{-6} refractive index units full scale and maintained at a temperature of $35 \pm 0.1^\circ$

Column: 7.8-mm \times 30-cm; packing L39**Column temperature:** 45° **Flow rate:** 0.8 mL/min

[NOTE—After installation of a new column, pump

Mobile phase through the column overnight at a rate

of 0.3 mL/min. Before calibration or analysis, increase the flow slowly over a 1-min period to 0.8 mL/min. Continue to pump *Mobile phase* through the column at this flow rate for at least 1 h before the first injection. Check the flow gravimetrically, and adjust it if necessary. Reduce the flow rate to about 0.1 mL/min when the system is not in use.]

Injection size: 50 μ L**System suitability****Sample:** *Standard solution*

Chromatograph five replicate injections of the *Standard solution*, allowing 15 min between injections, and record the retention times of the components of the *Standard solution*.

Insert the average retention time along with the molecular weight of each component in the *Standard solution* into the calibration table of the molecular weight distribution software. Check the regression results for a cubic fit of the calibration points, and obtain a correlation coefficient, R, for the line.

Suitability requirements

Retention time: The retention times for each component determined on replicate injections agree within ± 2 s.

Resolution: Dextrose and stachyose are baseline resolved from one another and from the 5800-MW pullulan standard.

[NOTE—Prominent negative baseline valleys are usually observed between the peaks for the 5800-, 23,700-, and 100,000-MW pullulan standards.]

Correlation coefficient R: NLT 0.9999**Analysis****Samples:** *Standard solution* and *Sample solution*

Use the molecular weight distribution software of the data reduction system to generate a molecular weight distribution plot of Hydrogenated Polydextrose.

Acceptance criteria: No measurable peak above a molecular weight of 22,000 is found.

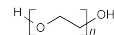
• **pH** <791>: 5.0–7.0, in a solution (1 in 10)

• **WATER DETERMINATION, Method I** <921>: NMT 4.0%. Use a mixture of Hydranal Solvent and Hydranal Formamide dry (2:1) as a solvent. Perform the titration at 50° in a jacketed beaker.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. Store in a cool and dry place.

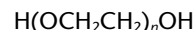
• **USP REFERENCE STANDARDS** <11>
 USP 1,6-Anhydro-D-glucose RS
 USP Dextrose RS
 USP Polydextrose RS
 USP Sorbitol RS

Polyethylene Glycol

Poly(oxy-1,2-ethanediyl), α -hydro- ω -hydroxy-;
 Polyethylene glycol [25322-68-3].

DEFINITION

Polyethylene Glycol is an addition polymer of ethylene oxide and water, represented:



in which n represents the average number of oxyethylene groups. The average molecular weight is NLT 95.0% and NMT 105.0% of the labeled nominal value if the labeled nominal value is less than 1000; it is NLT 90.0% and NMT 110.0% of the labeled nominal value if the labeled

nominal value is between 1000 and 7000; and it is NLT 87.5% and NMT 112.5% of the labeled nominal value if the labeled nominal value is more than 7000. It may contain a suitable antioxidant.

ASSAY

• AVERAGE MOLECULAR WEIGHT

Phthalic anhydride solution: Place 49.0 g of phthalic anhydride into an amber bottle, and dissolve in 300 mL of pyridine from a freshly opened bottle or pyridine that has been freshly distilled over phthalic anhydride. Shake vigorously until completely dissolved. Add 7 g of imidazole, swirl carefully to dissolve, and allow to stand for 16 h before using.

Sample solution for liquid Polyethylene Glycols:

Carefully introduce 25.0 mL of the *Phthalic anhydride solution* into a dry, heat-resistant pressure bottle. Add an amount of the specimen equivalent to its expected average molecular weight divided by 160. Insert the stopper in the bottle, and wrap it securely in a cloth bag.

Sample solution for solid Polyethylene Glycols: Carefully introduce 25.0 mL of *Phthalic anhydride solution* into a dry, heat-resistant pressure bottle. Add an amount of the specimen equivalent to its expected molecular weight divided by 160; however, because of limited solubility, do not use more than 25 g. Add 25 mL of pyridine, from a freshly opened bottle or pyridine that has been freshly distilled over phthalic anhydride. Swirl to dissolve, insert the stopper in the bottle, and wrap it securely in a cloth bag.

Blank: 25.0 mL of *Phthalic anhydride solution* plus any additional pyridine added to the bottle

Analysis: Immerse the bottle in a water bath maintained at a temperature between 96° and 100°, to the same depth as that of the mixture in the bottle. Remove the bottles from the bath after 5 min and, without unwrapping, swirl for 30 s to homogenize. Heat in the water bath for 30 min (60 min for Polyethylene Glycols having molecular weights of 3000 or more), then remove from the bath, and allow it to cool to room temperature. Uncap the bottle carefully to release any pressure, remove from the bag, add 10 mL of water, and swirl thoroughly. Wait 2 min, add 0.5 mL of a solution of phenolphthalein in pyridine (1 in 100), and titrate with 0.5 N sodium hydroxide VS to the first pink color that persists for 15 s. Perform a blank determination.

Calculate the average molecular weight taken:

$$\text{Result} = (2000 \times W) / [(V_B - V_S) \times N]$$

W = weight of the Polyethylene Glycol taken for the *Sample solution* (g)
 V_B = volume of 0.5 N sodium hydroxide consumed by the *Blank* (mL)
 V_S = volume of 0.5 N sodium hydroxide consumed by the specimen (mL)
 N = normality of the sodium hydroxide solution
Acceptance criteria: See *Table 1*.

Table 1

Label Claim (Nominal Value)	Acceptance Criteria
<1000	95.0–105.0%
1000–7000	90.0–110.0%
>7000	87.5–112.5%

IMPURITIES

• RESIDUE ON IGNITION (281)

Sample: 25 g

Analysis: Proceed as directed, using a platinum dish and moistening the residue with 2 mL of sulfuric acid.

Acceptance criteria: NMT 0.1%

• HEAVY METALS (231)

Test preparation: 4.0 g in 5.0 mL of 0.1 N hydrochloric acid. Dilute with water to 25 mL.

Acceptance criteria: NMT 5 ppm

• LIMIT OF FREE ETHYLENE OXIDE AND 1,4-DIOXANE

Stripped polyethylene glycol 400: Into a 5000-mL, 3-neck, round-bottom flask equipped with a stirrer, a gas dispersion tube, and a vacuum outlet, place 3000 g of Polyethylene Glycol 400. At room temperature, evacuate the flask carefully to a pressure of less than 1 mm of mercury, applying the vacuum slowly while observing for excessive foaming due to entrapped gases. After any foaming has subsided and while stirring continuously, sparge with nitrogen, allowing the pressure to rise to 10 mm of mercury. Continue stripping for a minimum of 1 h. Completeness of the stripping procedure should be verified by making a headspace injection of the stripped polyethylene glycol 400. [NOTE—The 10-mm value is a guideline. Deviations from this value affect only the total time required to strip the Polyethylene Glycol 400.]

Shut off the vacuum pump, and bring the flask pressure back to atmospheric pressure while maintaining nitrogen sparging. Remove the gas dispersion tube with the gas still flowing, and then turn off the gas flow. Transfer the *Stripped polyethylene glycol 400* to a suitable nitrogen-filled container.

Standard solution: Transfer 4.90 g of *Stripped polyethylene glycol 400* to a tared 22-mL pressure headspace vial that can be sealed. Add 48 µL of 1,4-dioxane, equivalent to 50 mg of 1,4-dioxane, from a syringe; seal; and cap the vial.

[CAUTION—Ethylene oxide and 1,4-dioxane are toxic and flammable. Prepare these solutions in a well-ventilated fume hood.]

Using the special handling described in the following, complete the preparation. Ethylene oxide is a gas at room temperature. It is usually stored in a lecture-type gas cylinder or small metal pressure bomb. Chill the cylinder in a refrigerator before use. Transfer 5 mL of the liquid ethylene oxide to a 100-mL beaker chilled in wet ice. Using a gas-tight syringe that has been chilled in a refrigerator, transfer 57 µL of the liquid ethylene oxide, equivalent to 50 mg of ethylene oxide, to the mixture contained in the headspace vial, and mix. With the aid of a syringe, transfer 2 mL of this solution to a 5-mL beaker. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, and dilute with *Stripped polyethylene glycol 400* to volume. Transfer 10 mL of this solution to a 100-mL volumetric flask, dilute with *Stripped polyethylene glycol 400* to volume, and mix to obtain a *Standard solution* having known concentrations of 10 µg/g for both ethylene oxide and 1,4-dioxane. Transfer 1.0 mL of the *Standard solution* to a 22-mL pressure headspace vial, seal with a silicone septum with or without a pressure relief star spring and a pressure relief safety aluminum sealing cap, and crimp the cap closed with a cap-sealing tool.

System suitability solution: Transfer 4.90 g of *Stripped polyethylene glycol 400* to a 22-mL pressure headspace vial. Pipet 50 µL of acetaldehyde into the vial. Using the special handling described in *Standard solution*, transfer 50.0 µL of liquid ethylene oxide into the vial. Immediately seal the vial, and shake. Transfer 1.0 mL of this solution to a 100-mL volumetric flask, and dilute with *Stripped polyethylene glycol 400* to volume. Transfer 10.0 mL of this solution to a 100-mL volumetric flask, and dilute with *Stripped polyethylene glycol 400* to volume. Transfer 1.0 mL of this *System suitability solution* to a 22-mL pressure headspace vial, and seal, cap, and crimp as directed for the *Standard solution*.

Sample solution: Transfer 1.0 g of Polyethylene Glycol to a 22-mL pressure headspace vial, and seal, cap, and crimp as directed for the *Standard solution*.

Chromatographic system(See *Chromatography* (621), *System Suitability*.)**Mode:** Headspace GC (balanced pressure automatic headspace sampler)**Detector:** Flame ionization**Column:** 0.32-mm × 50-m fused-silica capillary column containing bonded phase G27 in a 5-μm film thickness**Temperature****Column:** See Table 2.**Table 2**

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	10	250	—

Injector: 85°**Detector:** 250°**Flow rate:** 2.9 mL/min**Carrier gas:** Helium**Injection size:** 1.0 mL of headspace using a 2-mL gas syringe preheated in an oven at 90°**System suitability****Sample:** *System suitability solution*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are about 0.9 and 1.0, respectively.]

Suitability requirements**Resolution:** NLT 1.3 between the acetaldehyde peak and the ethylene oxide peak**Analysis****Samples:** *Standard solution* and *Sample solution*

[NOTE—The relative retention times for ethylene oxide and 1,4-dioxane are about 1.0 and 3.4, respectively.]

Place the vials containing the *Standard solution* and the *Sample solution* into the automated sampler, and heat the vials at a temperature of 80° for 30 min. [NOTE—A headspace apparatus that automatically transfers the measured amount of headspace may be used to perform the injection.]**Acceptance criteria:** The peak areas for ethylene oxide and 1,4-dioxane of the *Sample solution* are not greater than those of the corresponding peaks of the *Standard solution* corresponding to NMT 10 μg/g of ethylene oxide and NMT 10 μg/g of 1,4-dioxane.

- **LIMIT OF ETHYLENE GLYCOL AND DIETHYLENE GLYCOL** (for Polyethylene Glycol having a nominal molecular weight less than 450)

Standard solution: 0.5 mg/mL each of ethylene glycol and of diethylene glycol in water**Sample solution:** 400 mg/mL of Polyethylene Glycol in water**Chromatographic system**(See *Chromatography* (621), *System Suitability*.)**Mode:** GC**Detector:** Flame ionization**Column:** 3-mm × 1.5-m stainless steel; packing of 12% G13 on support S1NS**Temperature****Column:** 140°**Injector port:** 250°**Detector:** 280°**Carrier gas:** Nitrogen or another suitable inert gas**Flow rate:** 50 mL/min**Injection size:** 2.0 μL**Analysis****Samples:** *Standard solution* and *Sample solution*

[NOTE—The elution order is ethylene glycol followed by diethylene glycol.]

Calculate the percentage of ethylene glycol in the portion of Polyethylene Glycol taken:

$$\text{Result} = (r_{U1}/r_{S1}) \times (C_{S1}/C_U) \times 100$$

 r_{U1} = peak height of ethylene glycol from the *Sample solution* r_{S1} = peak height of ethylene glycol from the *Standard solution* C_{S1} = concentration of ethylene glycol in the *Standard solution* (mg/mL) C_U = concentration of Polyethylene Glycol in the *Sample solution* (mg/mL)

Calculate the percentage of diethylene glycol in the portion of Polyethylene Glycol taken:

$$\text{Result} = (r_{U2}/r_{S2}) \times (C_{S2}/C_U) \times 100$$

 r_{U2} = peak height of diethylene glycol from the *Sample solution* r_{S2} = peak height of diethylene glycol from the *Standard solution* C_{S2} = concentration of diethylene glycol in the *Standard solution* (mg/mL) C_U = concentration of Polyethylene Glycol in the *Sample solution* (mg/mL)**Acceptance criteria:** NMT 0.25% of the sum of ethylene glycol and diethylene glycol is found.

- **LIMIT OF ETHYLENE GLYCOL AND DIETHYLENE GLYCOL** (for Polyethylene Glycol having a nominal molecular weight 450 or more but NMT 1000)

Ceric ammonium nitrate solution: 6.25 g of ceric ammonium nitrate in 100 mL of 0.25 N nitric acid. Use within 3 days.**Standard stock solution:** 2.5 mg/mL of diethylene glycol in 1:1 freshly distilled acetonitrile:water**Sample stock solution:** Dissolve 50.0 g of Polyethylene Glycol in 75 mL of diphenyl ether, previously warmed if necessary, just to melt the crystals, in a 250-mL distilling flask. Slowly distill at a pressure of 1–2 mm of mercury, into a receiver graduated to 100 mL in 1-mL subdivisions, until 25 mL of distillate has been collected. Add 20.0 mL of water to the distillate, shake vigorously, and allow the layers to separate. Cool in an ice bath to solidify the diphenyl ether and facilitate its removal. Filter the separated aqueous layer, wash the diphenyl ether with 5.0 mL of ice-cold water, pass the washings through the filter, and collect the filtrate and washings in a 25-mL volumetric flask. Warm to room temperature, and dilute with water to volume, if necessary. Mix this solution with 25.0 mL of freshly distilled acetonitrile in a glass-stoppered, 125-mL conical flask.**Standard solution:** Add 10.0 mL of the *Standard stock solution* to 15.0 mL of *Ceric ammonium nitrate solution*. Within 2–5 min, determine the absorbance of the *Standard solution*.**Sample solution:** Add 10.0 mL of the *Sample stock solution* to 15.0 mL of *Ceric ammonium nitrate solution*. Within 2–5 min, determine the absorbance of the *Sample solution*.**Blank:** Mixture of 15.0 mL of *Ceric ammonium nitrate solution* and 10.0 mL of 1:1 freshly distilled acetonitrile:water**Instrumental conditions**(See *Spectrophotometry and Light-Scattering* (851).)**Mode:** UV-Vis**Cell:** 1 cm**Analytical wavelength:** 450 nm**Analysis****Samples:** *Standard solution* and *Sample solution***Acceptance criteria:** The absorbance of the *Sample solution* does not exceed that of the *Standard solution*, corresponding to NMT 0.25% of combined ethylene glycol and diethylene glycol.

SPECIFIC TESTS• **PH** (791)

Sample solution: 5.0 g of Polyethylene Glycol in 100 mL of carbon dioxide-free water. Add 0.30 mL of saturated potassium chloride solution.

Acceptance criteria: 4.5–7.5

- **COMPLETENESS AND COLOR OF SOLUTION:** A solution of 5 g of Polyethylene Glycol in 50 mL of water is colorless; it is clear for liquid grades and NMT slightly hazy for solid grades.

• **VISCOSITY—CAPILLARY VISCOMETER METHODS (911):**

Determine its viscosity by using a capillary viscometer giving a flow time of NLT 200 s and using a liquid bath maintained at $98.9 \pm 0.3^\circ$ (210° F). The viscosity is within the limits specified in Table 3. For a Polyethylene Glycol not listed in the table, calculate the limits by interpolation.

Table 3

Nominal Average Molecular Weight	Viscosity Range, Centistokes	Nominal Average Molecular Weight	Viscosity Range, Centistokes
200	3.9–4.8	2400	49–65
300	5.4–6.4	2500	51–70
400	6.8–8.0	2600	54–74
500	8.3–9.6	2700	57–78
600	9.9–11.3	2800	60–83
700	11.5–13.0	2900	64–88
800	12.5–14.5	3000	67–93
900	15.0–17.0	3250	73–105
1000	16.0–19.0	3350	76–110
1100	18.0–22.0	3500	87–123
1200	20.0–24.5	3750	99–140
1300	22.0–27.5	4000	110–158
1400	24–30	4250	123–177
1450	25–32	4500	140–200
1500	26–33	4750	155–228
1600	28–36	5000	170–250
1700	31–39	5500	206–315
1800	33–42	6000	250–390
1900	35–45	6500	295–480
2000	38–49	7000	350–590
2100	40–53	7500	405–735
2200	43–56	8000	470–900
2300	46–60	—	—

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to state, as part of the official title, the average nominal molecular weight of the Polyethylene Glycol. Label it to indicate the name and quantity of any added antioxidant.

Polyethylene Glycol Ointment**DEFINITION**

Prepare Polyethylene Glycol Ointment as follows.

Polyethylene Glycol 3350	400 g
Polyethylene Glycol 400	600 g
To make	1000 g

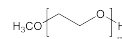
Heat the two ingredients on a water bath to 65° . Allow to cool, and stir until congealed. If a firmer preparation is desired, replace up to 100 g of the *Polyethylene Glycol 400*

with an equal amount of *Polyethylene Glycol 3350*.

[NOTE—If 6%–25% of an aqueous solution is to be incorporated in the Ointment, replace 50 g of the *Polyethylene Glycol 3350* with an equal amount of stearyl alcohol.]

ADDITIONAL REQUIREMENTS

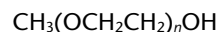
- **PACKAGING AND STORAGE:** Package in well-closed containers.

Polyethylene Glycol Monomethyl Ether

Poly(oxy-1,2-ethanediyl), α -methyl- ω -hydroxy-; Methoxy polyethylene glycol [9004-74-4].

DEFINITION

Polyethylene Glycol Monomethyl Ether is an addition polymer of ethylene oxide and methanol, represented as:



in which n represents the average number of oxyethylene groups. The average molecular weight is NLT 95.0% and NMT 105.0% of the labeled nominal value if the labeled nominal value is below 1000; it is NLT 90.0% and NMT 110.0% of the labeled nominal value if the labeled nominal value is between 1000 and 4750; and it is NLT 87.5% and NMT 112.5% of the labeled nominal value if the labeled nominal value is above 4750.

ASSAY• **AVERAGE MOLECULAR WEIGHT**

Phthalic anhydride solution: Place 49.0 g of phthalic anhydride into an amber bottle, and dissolve in 300 mL of pyridine, either from a freshly opened bottle or freshly distilled over phthalic anhydride. Shake vigorously until completely dissolved. Add 7 g of imidazole, swirl carefully to dissolve, and allow to stand for 16 h before using.

Sample solution for liquid Polyethylene Glycol Monomethyl Ethers: Carefully introduce 25.0 mL of the *Phthalic anhydride solution* into a dry, heat-resistant pressure bottle. Add a weighed amount of the sample, equivalent to its expected average molecular weight divided by 80. Insert the stopper in the bottle, and wrap it securely in a cloth bag.

Sample solution for solid Polyethylene Glycol Monomethyl Ethers: Carefully introduce 25.0 mL of *Phthalic anhydride solution* into a dry, heat-resistant pressure bottle. Add an amount of the sample, equivalent to its expected molecular weight divided by 80; however, because of limited solubility, do not use more than 25 g. Add 25 mL of pyridine, either from a freshly opened bottle or freshly distilled over phthalic anhydride, swirl to dissolve, insert the stopper in the bottle, and wrap it securely in a cloth bag.

Analysis: Immerse the bottle in a water bath maintained at 96° – 100° to the same depth as that of the mixture in the bottle. Remove the bottle from the bath after 5 min, and without unwrapping, swirl for 30 s to homogenize. Heat in the water bath for 30 min (60 min for Polyethylene Glycol Monomethyl Ethers having molecular weights of 3000 or higher), then remove from the bath, and allow to cool to room temperature. Uncap the bottle carefully to release any pressure, remove from the bag, add 10 mL of water, and swirl thoroughly. Wait for 2 min, add 0.5 mL of a solution of phenolphthalein in pyridine (1 in 100). Titrate with 0.5 N sodium hydroxide VS to the first pink color that persists for 15 s, recording the volume, in mL, of 0.5 N

sodium hydroxide required as V_S . Perform a blank determination on 25.0 mL of *Phthalic anhydride solution* plus any additional pyridine added to the bottle, and record the volume, in mL, of 0.5 N sodium hydroxide required as V_B .

Calculate the average molecular weight:

$$\text{Result} = (1000 \times W) / [(V_B - V_S) \times N]$$

W = weight of Polyethylene Glycol Monomethyl Ether taken for the *Sample solution* (g)
 V_B = volume of 0.5 N sodium hydroxide consumed by the blank (mL)
 V_S = volume of 0.5 N sodium hydroxide consumed by the sample (mL)
 N = normality of the sodium hydroxide solution
Acceptance criteria: See *Table 1*.

Table 1

Label Claim (nominal value)	Acceptance Criteria (%)
<1000	95.0–105.0
1000–4750	90.0–110.0
>4750	87.5–112.5

IMPURITIES

• RESIDUE ON IGNITION (281)

Sample: 25 g of Polyethylene Glycol Monomethyl Ether, moistened with 2 mL of sulfuric acid in a platinum dish

Acceptance criteria: NMT 0.1%

• HEAVY METALS (231)

Test preparation: Mix 4.0 g with 5.0 mL of 0.1 N hydrochloric acid, and dilute with water to 25 mL.

Acceptance criteria: NMT 5 ppm

• LIMIT OF ETHYLENE GLYCOL AND DIETHYLENE GLYCOL (for Polyethylene Glycol Monomethyl Ether having a nominal molecular weight of less than 600)

Standard solution: 500 µg/mL of ethylene glycol and 500 µg/mL of diethylene glycol in water

Sample solution: 400 mg/mL of Polyethylene Glycol Monomethyl Ether in water

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 3-mm × 1.0-m; 60- to 80-mesh support S2

Temperatures

Column: 200°

Injection port: 260°

Carrier gas: Nitrogen or another suitable inert gas

Flow rate: 20 mL/min

Injection volume: 1.0 µL

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—The elution order is ethylene glycol, diethylene glycol, and polyethylene glycol monomethyl ether.]

Calculate the percentage of ethylene glycol and diethylene glycol in the portion of Polyethylene Glycol Monomethyl Ether taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak height of ethylene glycol or diethylene glycol from the *Sample solution*

r_S = peak height of ethylene glycol or diethylene glycol from the *Standard solution*

C_S = concentration of ethylene glycol or diethylene glycol in the *Standard solution*

C_U = concentration of Polyethylene Glycol Monomethyl Ether in the *Sample solution*

Acceptance criteria: NMT 0.25% of combined ethylene glycol and diethylene glycol

• LIMIT OF ETHYLENE GLYCOL AND DIETHYLENE GLYCOL (for Polyethylene Glycol Monomethyl Ether having a nominal molecular weight of 600–1500)

Solution A: 62.5 mg/mL of ceric ammonium nitrate in 0.25 N nitric acid. Use within 3 days.

Solution B: Freshly distilled acetonitrile and water (50:50)

Standard solution: 2.5 mg/mL of diethylene glycol in *Solution B*

Sample solution: Dissolve 50.0 g of Polyethylene Glycol Monomethyl Ether in 75 mL of diphenyl ether, previously warmed if necessary, to melt the crystals, in a 250-mL distilling flask. Slowly distill at a pressure of 1–2 mm of mercury into a receiver graduated to 100 mL in 1-mL subdivisions until 25 mL of distillate has been collected. Add 20.0 mL of water to the distillate, shake vigorously, and allow the layers to separate. Cool in an ice bath to solidify the diphenyl ether and facilitate its removal. Filter the separated aqueous layer, wash the diphenyl ether with 5.0 mL of ice-cold water, pass the washings through the filter, and collect the filtrate and washings in a 25-mL volumetric flask. Warm to room temperature, and dilute with water to volume, if necessary. Mix this solution with 25.0 mL of freshly distilled acetonitrile in a glass-stoppered, 125-mL conical flask.

Instrumental conditions

Mode: Vis

Analytical wavelength: About 450 nm

Cell: 1 cm

Blank: *Solution A* and *Solution B* (60:40)

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank*. Transfer 10.0 mL each of the *Standard solution* and the *Sample solution* to separate 50-mL flasks, each containing 15.0 mL of *Solution A*. Within 2–5 min, determine the absorbances of the *Samples*.

Acceptance criteria: The absorbance of the *Sample solution* does not exceed that of the *Standard solution*, corresponding to NMT 0.25% of combined ethylene glycol and diethylene glycol.

• FREE ETHYLENE OXIDE AND 1,4-DIOXANE

Stripped MPEG 350: Into a 5000-mL 4-neck, round-bottom flask, equipped with a stirrer, a thermometer, a gas dispersion tube, a dry ice trap, a vacuum outlet, and a heating mantle, place 3000 g of Polyethylene Glycol Monomethyl Ether 350. At room temperature, evacuate the flask carefully to a pressure of less than 1 mm of mercury, applying the vacuum slowly while observing for excessive foaming due to entrapped gases. After any foaming has subsided, sparge with nitrogen, allowing the pressure to rise to 10 mm of mercury. Heat the flask to 130° while increasing the pressure to 60 mm of mercury. Continue stripping for 4 h, then cool to room temperature. Shut off the vacuum pump, and bring the flask pressure back to atmospheric pressure while maintaining nitrogen sparging. Remove the sparging tube with the gas still flowing, then turn off the gas flow. Transfer the *Stripped MPEG 350* to a suitable nitrogen-filled container.

Standard solutions: [CAUTION—Ethylene oxide and 1,4-dioxane are toxic and flammable. Prepare these solutions in a well-ventilated fume hood.] To a known weight of *Stripped MPEG 350* in a vial that can be sealed add a suitable quantity of 1,4-dioxane. Determine the amount added by weight difference. Using the special handling described in the following, complete the preparation. Ethylene oxide is a gas at room temperature. It is usually stored in a lecture-type gas cylinder or small metal pressure bomb. Chill the cylinder in a refrigerator before use. Transfer 5 mL of the liquid ethylene oxide to a 100-mL beaker chilled in wet ice. Using a gas-tight gas chromatographic syringe that has been chilled in a refrigerator, transfer a suitable amount of the liquid ethylene oxide into the mixture.

Immediately seal the vial, and shake. Determine the amount added by weight difference. By appropriate dilution with *Stripped MPEG 350*, prepare four solutions, covering a range of 5–20 ppm for the two components added to the matrix (e.g., 5, 10, 15, and 20 ppm). Transfer 1.0 mL of each of these solutions to separate 22-mL pressure headspace vials. Seal each with a silicone septum, star spring, and pressure relief safety aluminum sealing cap. Crimp the cap closed with a cap-sealing tool.

Sample solution: Transfer 1 ± 0.01 g of Polyethylene Glycol Monomethyl Ether to a 22-mL pressure headspace vial. Seal, cap, and crimp as directed for the *Standard solutions*.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC (equipped with a balanced pressure automatic headspace sampler)

Detector: Flame ionization

Column: 50-m \times 0.32-mm fused silica; bonded phase G27 in a 5- μ m film thickness

Temperatures

Detector: 250°

Transfer line: 140°

Column: See *Table 2*.

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	10	250	—

Carrier gas: Helium

Flow rate: 0.8 mL/min

Calibration: Place the vials containing the *Standard solutions* in the automated sampler, and start the sequence so that each vial is heated at 110° for 30 min before a suitable portion of its headspace is injected into the chromatograph. Set the automatic sampler for a needle withdrawal time of 0.3 min, a pressurization time of 1 min, an injection time of 0.08 min, and a vial pressure of 22 psig with the vial vent off. Obtain the peak areas for ethylene oxide and 1,4-dioxane, which have relative retention times of 1.0 and 3.1, respectively. Plot the area versus parts per million on linear graph paper, and draw the best straight line through the points. On the two *Calibration* plots, no point digresses from its line by more than 10%.

Analysis: Place the vial containing the *Sample solution* in the automatic sampler, and chromatograph its headspace as done for the *Standard solutions*. Obtain the peak areas of each of the components, and read the concentrations directly from the *Calibration* plots.

Acceptance criteria: NMT 10 ppm of ethylene oxide or 1,4-dioxane

• LIMIT OF 2-METHOXYETHANOL

Stripped MPEG 350 and Sample solution: Prepare as directed in the test for *Free Ethylene Oxide and 1,4-Dioxane*.

Standard solutions: [CAUTION—2-Methoxyethanol is toxic and flammable. Prepare these solutions in a well-ventilated fume hood.] To a known weight of *Stripped MPEG 350* in a vial that can be sealed add a suitable quantity of 2-methoxyethanol. Determine the amount added by weight difference. By appropriate dilution with *Stripped MPEG 350*, prepare four solutions, covering a range of 5–20 ppm (e.g., 5, 10, 15, and 20 ppm). Transfer 1.0 mL of each of these solutions to separate 22-mL pressure headspace vials. Seal each with a silicone septum, star spring, and pressure relief safety aluminum sealing cap. Crimp the cap closed with a cap-sealing tool.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC (equipped with a balanced pressure automatic headspace sampler)

Detector: Flame ionization

Column: 15-m \times 0.53-mm fused silica capillary; bonded phase G16 in a 1- μ m film thickness

Temperatures

Detector: 275°

Transfer line: 140°

Column: See *Table 3*.

Table 3

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	2
70	10	250	—

Carrier gas: Helium

Flow rate: 15 mL/min

Calibration: Place the vials containing the *Standard solutions* in the automated sampler, and start the sequence so that each vial is heated at 100° for 20 min before a suitable portion of its headspace is injected into the chromatograph. Set the automatic sampler for a needle withdrawal time of 0.3 min, a pressurization time of 1 min, an injection time of 0.08 min, and a vial pressure of 22 psig with the vial vent off. Obtain the peak area for 2-methoxyethanol. Plot the area versus ppm on linear graph paper, and draw the best straight line through the points. On the two *Calibration* plots, no point digresses from its line by more than 10%.

Analysis: Place the vial containing the *Sample solution* in the automatic sampler, and chromatograph its headspace as done for the *Standard solutions*. Obtain the peak area, and read the concentration directly from the *Calibration* plot.

Acceptance criteria: NMT 10 ppm

SPECIFIC TESTS

• pH <791>

Sample solution: 5.0 g of Polyethylene Glycol Monomethyl Ether in 100 mL of carbon dioxide-free water. Add 0.30 mL of saturated potassium chloride solution.

Acceptance criteria: 4.5–7.5

• COMPLETENESS AND COLOR OF SOLUTION

Sample solution: 5 g of Polyethylene Glycol Monomethyl Ether in 50 mL of water

Acceptance criteria: The resulting solution is colorless, and is clear for liquid grades and NMT slightly hazy for solid grades.

• VISCOSITY—CAPILLARY VISCOMETER METHODS <911>:

Determine its viscosity, using a capillary viscometer giving a flow time of NLT 200 s and a liquid bath maintained at $98.9 \pm 0.3^\circ$. The viscosity is within the limits specified in *Table 4*. For a Polyethylene Glycol Monomethyl Ether not listed in *Table 4*, calculate the limits by interpolation.

Table 4

Nominal Average Molecular Weight	Viscosity Range (centistokes)	Nominal Average Molecular Weight	Viscosity Range (centistokes)
350	3.5–4.5	2750	50–78
450	4.9–6.0	3000	60–95
550	6.1–7.3	3250	72–113
650	7.9–9.2	3500	85–133
750	9.7–11.1	3750	99–155

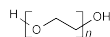
Table 4 (Continued)

Nominal Average Molecular Weight	Viscosity Range (centistokes)	Nominal Average Molecular Weight	Viscosity Range (centistokes)
850	11.5–13.1	4000	114–178
950	13.3–15.2	4250	130–204
1000	13.3–17.3	4500	148–231
1100	15.0–19.7	4750	167–260
1200	16.9–22.1	5000	175–305
1300	18.8–24.6	5500	215–375
1400	20.7–27.1	6000	260–455
1500	23–30	6500	310–545
1600	25–33	7000	365–640
1700	27–35	7500	425–745
1800	29–38	8000	490–860
1900	31–41	8500	560–980
2000	33–44	9000	640–1110
2250	36–54	9500	715–1250
2500	40–64	10000	775–1475

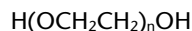
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to state, as part of the official title, the average nominal molecular weight of the Polyethylene Glycol Monomethyl Ether.

Polyethylene Oxide

**DEFINITION**

Polyethylene Oxide is a nonionic homopolymer of ethylene oxide, represented:



in which *n* represents the average number of oxyethylene groups. The number *n* varies from about 2000 to 200,000. It is a white to off-white powder obtainable in several grades, varying in viscosity profile in an aqueous isopropyl alcohol solution. It may contain a suitable antioxidant.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
Sample: Previously dried in a vacuum at room temperature to constant weight
- **B. PROCEDURE**
Viscosity
 [NOTE—Based on *Labeling* information, perform the following test accordingly.]
Analysis: Pass Polyethylene Oxide through a 20-mesh screen. Then transfer Polyethylene Oxide to a 800-mL low-form beaker the amount which is specified in *Table 1* to provide the solution concentration.

Table 1

Polyethylene oxide weight	Sample solution		
	1% Solution	2% Solution	5% Solution
	6 g	12 g	30 g

Add 125 mL of dehydrated isopropyl alcohol to the beaker. Place the stirrer into the beaker with an appropriate glass cover. Stir the polyethylene oxide-isopro-

panol mixture at a rate to ensure that a slurry is formed. Add the prescribed amount of water to the polyethylene oxide-isopropanol slurry, refer to *Table 2*. All solution concentrations are based on the water content of the aqueous isopropyl alcohol solution. Be careful to avoid splashing of the water. Adjust the temperature to near 25° to assist the final solution coming to temperature.

Table 2

Water weight	Sample solution		
	1% Solution	2% Solution	5% Solution
	594 g	588 g	570 g

Ensure that the stirring is very effective at the beginning for about 1 min. Then allow to gently stir for at least 3 h. Ensure that Polyethylene Oxide dissolves in solution, take care to avoid mixing in excess air, and stop the stirring. [NOTE—Ensure a colloidal dispersion by stirring for at least 3 h if added antioxidant or silicon dioxide is not soluble in the system.]

Place a watchglass over the beaker and place in a bath for at least 30 min. When the solution reaches 25 ± 0.1°, determine the viscosity of the *Sample solution* using the viscometer, spindle, and speed indicated on the *Labeling*. [NOTE—A guard may be required as indicated on the *Labeling*.] Follow the instrument manufacturer's directions to measure the apparent viscosity.

Acceptance criteria: Viscosity falls within the viscosity range indicated by the *Labeling*.

IMPURITIES**Inorganic Impurities**

- **HEAVY METALS,** *Method II* (231):NMT 10 ppm
- **SILICON DIOXIDE AND NONSILICON DIOXIDE RESIDUE ON IGNITION**

Sample: 1 g

Analysis: Weigh the *Sample* into a previously ignited, tared 50-mL platinum crucible. Add 4 drops of sulfuric acid. Heat carefully on a hot plate until the specimen is thoroughly charred and fumes no longer are evolved. Ignite the crucible at 700 ± 25° (see *Residue on Ignition* (281)) to constant weight. Wet the residue carefully with 1 mL of water, and slowly add 20 drops of hydrofluoric acid. [CAUTION—Hydrofluoric acid is an extremely hazardous chemical. When handling it, wear a face shield, arm protection, and rubber gloves, and perform the operation in a hood.] Evaporate slowly on a hot plate to dryness, then ignite at 700 ± 25° for 10 min, cool to room temperature in a desiccator, and weigh. Repeat the addition of hydrofluoric acid, evaporation, and ignition, to constant weight.

Calculate the percentage of silicon dioxide residue on ignition from the difference between the net weights before and after the hydrofluoric acid treatment. Calculate the percentage of nonsilicon dioxide residue on ignition from the final net weight.

Acceptance criteria

Silicon dioxide residue on ignition: NMT 3%

Nonsilicon dioxide residue on ignition: NMT 2%

Organic Impurities

- **PROCEDURE: LIMIT OF FREE ETHYLENE OXIDE**

Standard stock solution: [CAUTION—Ethylene oxide is toxic and flammable. Prepare solutions of it in a well-ventilated fume hood.] Prepare the solution using the special handling described below. Ethylene oxide is a gas at room temperature. It is usually stored in a lecture-type gas cylinder or small metal pressure bomb. Chill the cylinder in a refrigerator before use. Transfer 5 mL of the liquid ethylene oxide to a cold, 10-mL serum vial. Seal the vial, and store in a refrigerator. Transfer 40 g of acetone to a tared 50-mL serum vial that is capable of being tightly sealed with a polytetrafluoroethylene-lined septum and a metallic crimp cap. Seal the vial, and weigh

it. Using a gas-tight gas chromatographic syringe that has been chilled in a refrigerator, transfer 60 μL of the liquefied ethylene oxide to the same vial. Weigh the vial, and determine the amount added by weight difference. The solution contains about 1 $\mu\text{g}/\mu\text{L}$ of ethylene oxide. [NOTE—This solution may be kept for 1 week in the crimp-sealed serum vial, stored in a freezer.]

[NOTE—Standard solutions A–D and the Sample solution should be prepared in vials designed for use in the headspace sampling system specified in the *Chromatographic system*.]

Standard solution A: To a tared vial add 1.0 g of Polyethylene Oxide, and seal the vial. Through the septum add 2.0 μL of *Standard stock solution*, heat the vial at 100° for 30 min, and cool to room temperature.

Standard solution B: To a tared vial add 1.0 g of Polyethylene Oxide, and seal the vial. Through the septum add 4.0 μL of *Standard stock solution*, heat the vial at 100° for 30 min, and cool to room temperature.

Standard solution C: To a tared vial add 1.0 g of Polyethylene Oxide, and seal the vial. Through the septum add 6.0 μL of *Standard stock solution*, heat the vial at 100° for 30 min, and cool to room temperature.

Standard solution D: To a tared vial add 1.0 g of Polyethylene Oxide, and seal the vial. Through the septum add 8.0 μL of *Standard stock solution*, heat the vial at 100° for 30 min, and cool to room temperature.

Sample solution: To a tared vial add 1.0 g of Polyethylene Oxide, and seal the vial. Heat the vial at 100° for 30 min, and cool to room temperature.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm \times 10-m capillary column bonded with a 20- μm layer of phase G45

Temperature

Injector: 200°

Detector: 250°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	5
70	10	200	5

Flow rate: 15 mL/min

Injection size: 300 μL of headspace gas

Injection type: Split

Carrier gas: Helium

[NOTE—The makeup gas is also helium, with a split flow rate of 15 mL/min.]

System suitability

Samples: *Standard stock solution* and *Standard solution C*

Suitability requirements

Relative standard deviation: NMT 5%. [NOTE—Multiple vials are prepared for replicate injections.]

Analysis

Samples: *Standard solutions A–D* and *Sample solution*
[NOTE—A headspace apparatus that automatically transfers the measured amount of gaseous headspace may be used to perform the injections.]

Using a gas-tight syringe, separately inject equal volumes (about 300 μL) of the gaseous headspace of each of the *Standard solutions* and the *Sample solution* into the gas chromatograph, record the chromatograms, and measure the areas of the peak responses. Determine by a retention time comparison whether

ethylene oxide is detected in the *Sample solution*. Plot the responses of the *Sample solution* and the *Standard solutions* versus the content, in μg , of ethylene oxide in each vial, as furnished by the *Standard stock solution*. Draw the straight line best fitting the five points, and calculate the correlation coefficient for the line.

[NOTE—The content of ethylene oxide, as furnished by the *Standard stock solution*, is 0 μg in the *Sample solution*.]

A suitable system is one that yields a line having a correlation coefficient of NLT 0.99. Extrapolate the line until it intercepts the content axis on the negative side. From the intercept, determine the total amount, T_U , in μg , of ethylene oxide in the *Sample solution*.

Calculate the percentage of ethylene oxide in the portion of Polyethylene Oxide taken:

$$\text{Result} = (T_U/W) \times 100$$

T_U = total amount of ethylene oxide in the *Sample solution* (μg)

W = weight of Polyethylene Oxide taken to prepare the *Sample solution* (μg)

Acceptance criteria: NMT 0.001%

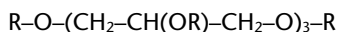
SPECIFIC TESTS

- **LOSS ON DRYING** <731>: Dry 4 g at 105° for 45 min: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirements specified.
- **LABELING:** Label it to indicate the viscosity and acceptable limits, giving the viscosity measurement parameters, concentration of the solution, and the type of equipment used. Label it to indicate the name and quantity of any added antioxidant.
- **USP REFERENCE STANDARDS** <11>
USP Polyethylene Oxide RS

Polyglyceryl Dioleate



$\text{R} = \text{H}$, or $\text{CO}-\text{C}_{17}\text{H}_{33}$

1,2,3-Propanetriol, homotrimer, di[(9Z)-9-octadecenoate]; Triglyceril dioleate;

Polyglyceryl 3 dioleate [9007-48-1].



$\text{R} = \text{H}$, or $\text{CO}-\text{C}_{17}\text{H}_{33}$

1,2,3-Propanetriol, homohexamer, di[(9Z)-9-octadecenoate]; Hexaglyceril dioleate;

Polyglyceryl 6 dioleate [76009-37-5].

DEFINITION

Polyglyceryl Dioleate is a mixture of polyglyceryl diesters of mainly oleic acid, obtained by esterification of polyglycerin and oleic acid. The polyglycerin consists mainly of triglycerin or hexaglycerin.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197F>
- **B.** Meets the requirements of the test for *Content of Fatty Acids*
- **C.** Meets the requirements of the test for *Fats and Fixed Oils* <401>, *Hydroxyl Value*. [NOTE—This test will differentiate for Polyglyceryl 3 Dioleate and Polyglyceryl 6 Dioleate.]

ASSAY**• CONTENT OF FATTY ACIDS**

0.5 N methanolic sodium hydroxide solution: Dissolve 20 g of sodium hydroxide in 50 mL of water, and mix. Cool to room temperature, and add 950 mL of methanol.

Boron trifluoride methanol solution: Dissolve 14 g of boron trifluoride in methanol to make 100 mL, and mix well.¹

Saturated sodium chloride solution: Dissolve about 375 g of sodium chloride in water to make 1000 mL.

Standard solution: Prepare the calibration ester mixture by mixing up each individual ester component (see Table 1 for the component's composition). Dissolve 500 mg of the calibration ester mixture in *n*-heptane, and dilute with *n*-heptane to 50 mL. [NOTE—Commercially available mixtures of fatty acid methyl esters may also be used.]

Table 1

Component in the Calibration Ester Mixture	Composition (%)
USP Methyl Myristate RS (C14:0)	5
USP Methyl Palmitate RS (C16:0)	15
USP Methyl Palmitoleate RS (C16:1)	10
USP Methyl Stearate RS (C18:0)	10
USP Methyl Oleate RS (C18:1)	20
USP Methyl Linoleate RS (C18:2)	15
USP Methyl Linolenate RS (C18:3)	10
Methyl arachidate (C20:0)	10
Methyl gadoleate (C20:1)	5

Sample solution: Introduce 100 mg of Polyglyceryl Dioleate into a 25-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 2 mL of 0.5 N methanolic sodium hydroxide solution, mix, and reflux for about 30 min. Add 2 mL of Boron trifluoride methanol solution through the condenser and reflux for about 30 min. Add 4 mL of *n*-heptane through the condenser, and reflux for 5 min. Cool, remove the condenser, add about 10 mL of Saturated sodium chloride solution, shake, add a quantity of Saturated sodium chloride solution to bring the upper layer up to the neck of the flask, and allow the layers to separate. Collect 2 mL of the *n*-heptane layer (upper layer), wash with three quantities, each at 2 mL of water, and dry the *n*-heptane phase over anhydrous sodium sulfate.

Chromatographic system

(See Chromatography <621>, System Suitability.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica capillary; 0.25-μm layer of phase G16

Temperature

Detector: 250°

Injection port: 240°

Column: See temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	6	250	6

¹ Boron trifluoride-methanol solution (14% in methanol) is also commercially available from Sigma, B-1252, or equivalent quality.

Carrier gas: Nitrogen

Flow rate: 1.0–1.2 mL/min

Injection size: 1 μL

Injection type: Split injection. The split ratio is about 1:80.

System suitability

Sample: Standard solution

[NOTE—See the relative retention time table below.]

Component	Relative Retention Time
Methyl myristate	0.74
Methyl palmitate	1.00
Methyl palmitoleate	1.03
Methyl stearate	1.29
Methyl oleate	1.33
Methyl linoleate	1.37 ^a
Methyl linolenate	1.46 ^b
Methyl arachidate	1.55
Methyl gadoleate	1.58

^a There could be an isomer eluting at a relative retention time of 1.39.

^b There could be two isomers eluting at relative retention times of 1.45 and 1.48.

Suitability requirements

Resolution: NLT 1.5 between the peaks due to methyl stearate and methyl oleate

Relative standard deviation: NMT 6.0% for the palmitate and stearate peak areas

Analysis

Samples: Standard solution and Sample solution

Identify the fatty acid ester peaks of the Sample solution by comparing the retention times of these peaks with those of the Standard solution, and measure the peak areas for all of the fatty acid esters in the Sample solution.

Calculate the percentage of each fatty acid ester component in the test specimen:

$$\text{Result} = (A/B) \times 100$$

A = peak area of each individual fatty acid ester component

B = sum of the peak areas, excluding the solvent peak, of the Sample solution

Acceptance criteria: Polyglyceryl Dioleate exhibits the following composition profile of fatty acids.

Carbon-Chain Length	Number of Double Bonds	Percentage
14	0	≤5.0
16	0	2.0–16.0
16	1	≤8.0
18	0	≤6.0
18	1	65.0–88.0
18	2	5.0–18.0 ^a
18	3	≤4.0 ^a
Sum of fatty acids with C >18	0	≤4.0

^a The content of C18:2 or C18:3 is the content of each fatty acid with its respective isomers.

IMPURITIES**Inorganic Impurities****• RESIDUE ON IGNITION**

Analysis: Heat a silica crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Evenly distribute about 1.0 g of Polyglyceryl Dioleate in the crucible, and weigh. Dry at 100°–105° for 1 h, and ignite in a muffle furnace at 600 ± 25°, until the test

substance is thoroughly charred. Perform the test for *Residue on Ignition* (281) on the residue obtained, starting with "Moisten the sample with a small amount (usually 1 mL) of sulfuric acid."

Acceptance criteria: NMT 1%

- **HEAVY METALS**, *Method II* (231): NMT 10 ppm

SPECIFIC TESTS

• ACID VALUE

Analysis: Accurately weigh (to within 0.1 mg) 5–10 g of Polyglyceryl Dioleate, add 10 mL of alcohol and 3 drops of phenolphthalein TS, and titrate with 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS until the solution remains faintly pink after shaking for 30 s. Proceed as directed in *Fats and Fixed Oils* (401), *Acid Value* to perform the calculation.

Acceptance criteria

Polyglyceryl 3 dioleate: NMT 6

Polyglyceryl 6 dioleate: NMT 6

- **FATS AND FIXED OILS**, *Hydroxyl Value* (401)

Acceptance criteria

Polyglyceryl 3 dioleate: 195–245, determined on a 0.7-g to 1.0-g specimen

Polyglyceryl 6 dioleate: 270–320, determined on 0.5-g to 0.7-g specimen

• IODINE VALUE

Analysis: Accurately weigh 0.15 g of Polyglyceryl Dioleate, transfer to a dry 250-mL flask with a ground-glass stopper, and add 25 mL of methylene chloride. Add 20 mL of the Wijs' solution.² Close the flask, and keep it in the dark for 1 h while shaking frequently. Perform the test in *Fats and Fixed Oils* (401), *Iodine Value*, starting with "Then add, in the order named, 30 mL of potassium iodide TS and 100 mL of water."

Acceptance criteria

Polyglyceryl 3 dioleate: 60–80

Polyglyceryl 6 dioleate: 50–70

- **FATS AND FIXED OILS**, *Peroxide Value* (401): Use 30 mL of a mixture of glacial acetic acid and methylene chloride (3:2) to replace 30 mL of a mixture of glacial acetic acid and chloroform (3:2).

Acceptance criteria

Polyglyceryl 3 dioleate: NMT 12.5

Polyglyceryl 6 dioleate: NMT 12.5

- **FATS AND FIXED OILS**, *Saponification Value* (401):

Determined on 1-g specimen

Acceptance criteria

Polyglyceryl 3 dioleate: 135–155

Polyglyceryl 6 dioleate: 110–140

- **WATER**, *Method I* (921): NMT 1%, determined on a 2.0-g specimen

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, protected from heat and moisture.

- **LABELING:** Label to indicate whether it is Polyglyceryl 3 Dioleate or Polyglyceryl 6 Dioleate.

- **USP REFERENCE STANDARDS** (11)

USP Methyl Linoleate RS

USP Methyl Linolenate RS

USP Methyl Myristate RS

USP Methyl Oleate RS

USP Methyl Palmitate RS

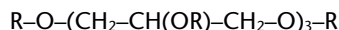
USP Methyl Palmitoleate RS

USP Methyl Stearate RS

USP Polyglyceryl 3 Dioleate RS

USP Polyglyceryl 6 Dioleate RS

Polyglyceryl 3 Diisostearate



R = H, or CO-C₁₇H₃₅-iso

1,2,3-Propanetriol, homopolymer, diisooctadecanoate; Triglyceryl diisostearate [63705-03-3].

DEFINITION

Polyglyceryl 3 Diisostearate is a mixture of polyglyceryl diesters of mainly isostearic acid, obtained by esterification of polyglycerin and isostearic acid. The polyglycerin consists mainly of triglycerin.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197F)

- **B.** It meets the requirements of the test for *Content of Fatty Acids*.

ASSAY

- **CONTENT OF FATTY ACIDS**

0.5 N methanolic sodium hydroxide solution: Dissolve 20 g of sodium hydroxide in 50 mL of water, and mix. Cool to room temperature, and add 950 mL of methanol.

Boron trifluoride-methanol solution: Dissolve 14 g of boron trifluoride in methanol to make 100 mL, and mix well.¹

Saturated sodium chloride solution: Dissolve about 375 g of sodium chloride in water to make 1000 mL.

Standard solution: Prepare the calibration ester mixture by mixing up each individual ester component (see Table 1). Dissolve 500 mg of the calibration ester mixture in *n*-heptane, and dilute with *n*-heptane to 50 mL.

Table 1

Component in the Calibration Ester Mixture	Composition (%) ^a
USP Methyl Myristate RS (C14:0)	7
USP Methyl Palmitate RS (C16:0)	70
USP Methyl Stearate RS (C18:0)	23

^a Composition is proposed according to the relative composition of these three fatty acid groups in Polyglyceryl 3 Diisostearate.

Sample solution: Introduce 100 mg of Polyglyceryl 3 Diisostearate into a 25-mL conical flask, fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 2 mL of 0.5 N methanolic sodium hydroxide solution, mix, and reflux for about 30 min. Add 2 mL of Boron trifluoride-methanol solution through the condenser, and reflux for about 30 min. Add 4 mL of *n*-heptane through the condenser, and reflux for 5 min. Cool, remove the condenser, add about 10 mL of Saturated sodium chloride solution, shake, add a quantity of Saturated sodium chloride solution to bring the upper layer up to the neck of the flask, and allow the layers to separate. Collect 2 mL of *n*-heptane layer (upper layer), wash with three quantities, each of 2 mL of water, and dry the *n*-heptane phase over anhydrous sodium sulfate.

Chromatographic system

(See Chromatography (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica capillary column, 0.25-μm layer of phase G16

¹ Boron trifluoride-methanol solution (14% in methanol) is also commercially available from Sigma, B-1252, or equivalent quality.

² Wijs' reagent RPE for analysis from Carlo Erba Reference 491902; Wijs' solution from www.sigmaaldrich.com, or equivalent quality.

Temperature

Detector: 250°

Injection port: 240°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	6	250	6

Carrier gas: Nitrogen

Flow rate: 1.0–1.2 mL/min

Injection size: 1 µL

Injection type: Split injection. Split ratio is about 1:80.

System suitability

Sample: *Standard solution*

[NOTE—See the relative retention time table below.]

Name	Relative Retention Time
Methyl myristate	1.0
Methyl palmitate	1.4
Methyl stearate	1.8

Suitability requirements

Resolution: NLT 10 between the peaks due to methyl palmitate and methyl stearate

Relative standard deviation: NMT 6.0% for the peak responses for palmitate and stearate

AnalysisSamples: *Standard solution* and *Sample solution*

Identify the fatty acid ester peaks in the chromatogram of the *Sample solution* by comparing the retention times of these peaks with those obtained in the chromatogram of the *Standard solution*, and measure the peak areas for all of the fatty acid ester peaks in the chromatogram obtained from the *Sample solution*.

Calculate the percentage of each fatty acid ester component in the test specimen:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response for each individual fatty acid ester component

r_T = sum of the peak responses, excluding the solvent peak, in the chromatogram obtained from the *Sample solution*

Acceptance criteria

Sum of the contents of the fatty acids eluting between palmitic acid and stearic acid (excluding palmitic acid and stearic acid): NLT 60.0%

Sum of the contents of myristic acid, palmitic acid, and stearic acid: NMT 11.0%

IMPURITIES**Inorganic Impurities****• RESIDUE ON IGNITION**

Analysis: Heat a silica crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Evenly distribute about 1.0 g of Polyglyceryl 3 Diisostearate in the crucible and weigh. Dry at 100°–105° for 1 h, and ignite in a muffle furnace at 600 ± 25°, until the test substance is thoroughly charred. Perform the test for *Residue on Ignition* (281) on the residue obtained, starting with "Moisten the sample with a small amount (usually 1 mL) of sulfuric acid".

Acceptance criteria: NMT 0.5%

• Heavy Metals, Method II (231): NMT 10 ppm**SPECIFIC TESTS****• ACID VALUE**

Analysis: Accurately weigh (to within 0.1 mg) 5–10 g of Polyglyceryl 3 Diisostearate, add 10 mL of alcohol and 3 drops of phenolphthalein TS, and titrate with 0.1 N potassium hydroxide VS or 0.1 N sodium hydroxide VS until the solution remains faintly pink after shaking for 30 s. Follow the procedures for *Fats and Fixed Oils, Acid Value* (401) to perform the calculation.

Acceptance criteria: NMT 3.0

• FATS AND FIXED OILS, Hydroxyl Value (401): 180–230, determined on a 0.25-g specimen**• IODINE VALUE**

Analysis: Accurately weigh 3 g of Polyglyceryl 3 Diisostearate, transfer to a dry 250-mL flask with a ground-glass stopper, and add 25 mL of methylene chloride. Add 20 mL of the Wijs' solution.² Close the flask, and keep it in the dark for 1 h while shaking frequently. Perform the test in *Fats and Fixed Oils (401) Iodine Value*, starting with "Then add, in the order named, 30 mL of potassium iodide TS and 100 mL of water".

Acceptance criteria: NMT 3.0

• FATS AND FIXED OILS, Peroxide Value (401): NMT 6.0. Use 30 mL of a mixture of glacial acetic acid and methylene chloride (3:2) to replace the 30 mL of a mixture of glacial acetic acid and chloroform (3:2).**• FATS AND FIXED OILS, Saponification Value (401):** 128–160**• WATER DETERMINATION, Method I (921):** NMT 0.5%, determined on a 2.0-g specimen**ADDITIONAL REQUIREMENTS****• PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, protected from heat and moisture.**• USP REFERENCE STANDARDS (11)**

USP Methyl Myristate RS

USP Methyl Palmitate RS

USP Methyl Stearate RS

USP Polyglyceryl 3 Diisostearate RS

Polyisobutylene

[9003-27-4].

DEFINITION

Polyisobutylene is a synthetic polymer produced by the low-temperature polymerization of isobutylene in liquid ethylene, methylene chloride, or hexane, using an aluminum-chloride or boron-trifluoride catalyst. It may contain a suitable stabilizer.

IDENTIFICATION**• A. INFRARED ABSORPTION (197F)**

Analysis: Prepare the sample by dissolving it in hot toluene and evaporating on a salt plate.

Acceptance criteria: Meets the requirements

IMPURITIES**• LEAD (251)**

Sample: 3.3 g

Control: 10 mL of *Diluted Standard Lead Solution* (10 µg of lead)

Analysis: Transfer the *Sample* to a porcelain dish, and heat on a hot plate until completely charred. Then heat in a muffle furnace at 480° for 8 h, and cool. Cautiously add 5 mL of nitric acid, evaporate to dryness on a hot

² Wijs' reagent RPE for analysis from Carlo Erba Reference 491902, Wijs' solution from www.sigmaaldrich.com, or equivalent quality.

plate, then heat again in the muffle furnace for exactly 15 min, and cool. Extract the ash with two 10-mL portions of water, filtering the extract into a separator. Leach any insoluble material on the filter with 6 mL of *Ammonium Citrate Solution*, 2 mL of *Hydroxylamine Hydrochloride Solution*, and 5 mL of water, adding the filtered washings to the separator. To the resulting solution and *Control* continue as directed in the chapter for *Procedure*, beginning with "Add 2 drops of phenol red TS".

Acceptance criteria: NMT 3 mg/g; the color generated by the *Sample* does not exceed that generated by the *Control*.

SPECIFIC TESTS

• VISCOSITY—CAPILLARY VISCOMETER METHODS (911)

Solvent: Use isooctane.

Sample solution: Prepare a solution of Polyisobutylene in the *Solvent* having a known concentration as indicated in *Table 1*. The solution must be homogenous before testing. For the Polyisobutylene having a Staudinger Index of 100 and higher, add the *Solvent* to the weighed material, and allow it to stand in an oven at 80° for 12–24 h. [NOTE—A heated mechanical shaker may be used to shorten the dissolution time; it is recommended that a gentle shaker be used to avoid shearing the polymers. Take adequate precautions to prevent evaporation of the *Solvent*.]

Table 1

Staudinger Index ^a	Concentration (g/cm ³)
25–60	0.01
60–100	0.005
100–350	0.002
350–700	0.001

^a The Staudinger Index is equal to 100 times the intrinsic viscosity.

Analysis: Before each measurement, let the solutions be temperature equilibrated for 10 min. Using a suitable Ubbelohde capillary viscometer having dimensions such that the flow time is NLT 200 s, immersed in a controlled temperature bath, measure the flow of the *Solvent* and of the *Sample solution* at 20°. Repeat the *Analysis* three times, and calculate the average. Calculate the reduced viscosity:

$$\eta = (t/t_0 - 1)/C$$

t = average flow time of the *Sample solution* (s)

t_0 = average flow time of the *Solvent* (s)

C = concentration of the *Sample solution* (g/cm³)

Calculate the Staudinger Index:

$$I_0 = \eta/[1 + 0.31(t/t_0 - 1)]$$

Acceptance criteria: It is within the limits specified on the label.

• LOSS ON DRYING (731)

Sample: 5 g

Analysis: Dry for 2 h at 105°.

Acceptance criteria: NMT 0.3%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
- **LABELING:** Label it to indicate the range for intrinsic viscosity or the range for the Staudinger Index, and the name and quantity of any added stabilizer. [NOTE—The Staudinger Index is equal to 100 times the intrinsic viscosity.]

• USP REFERENCE STANDARDS (11)

USP Polyisobutylene RS

Polyoxyl 10 Oleyl Ether

Polyoxy-1,2-ethanediyl, α -(Z)-9-octadecenyl- ω -hydroxy-; Polyethylene glycol monooleyl ether [9004-98-2].

DEFINITION

Polyoxyl 10 Oleyl Ether is a mixture of the mono-oleyl ethers of mixed polyoxyethylene diols, the average polymer length being equivalent to NLT 9.1 and NMT 10.9 oxyethylene units. It may contain suitable stabilizers.

IDENTIFICATION

• A. INFRARED ABSORPTION (197F)

Sample: Use undried specimen.

Acceptance criteria: Meets the requirements

IMPURITIES

• RESIDUE ON IGNITION (281)

Sample: 25 g

Analysis: Weigh the *Sample* into a tared 40-mL porcelain crucible, and heat in contact with air until it ignites spontaneously or can be ignited with a glowing splint. Allow the flame to go out, and place the crucible in a muffle furnace with the door partly open until the carbon is consumed. Close the door, and heat at 700 \pm 100° for 1 h. Cool in a desiccator, weigh, and calculate the percentage of residue. If it exceeds 0.4%, again heat until constant weight is attained.

Acceptance criteria: NMT 0.4%

• HEAVY METALS, *Method II* (231): NMT 20 μ g/g

• FREE POLYETHYLENE GLYCOLS

Sample solution: Transfer 12 g to a 500-mL separator containing 50 mL of ethyl acetate. Add 50 mL of sodium chloride solution (0.29 g/mL), shake vigorously for 2 min, and allow to separate for 15 min. Drain the lower, aqueous phase into a second 500-mL separator, and extract the upper layer with a second 50-mL portion of sodium chloride solution (0.29 g/mL). To the combined aqueous layers add 50 mL of ethyl acetate, shake vigorously for 2 min, and allow to separate as before. Drain the lower, aqueous phase into a third 500-mL separator, and extract with two 50-mL portions of chloroform by shaking for 2 min each time.

Analysis: Evaporate the combined chloroform extracts in a 150-mL beaker on a steam bath, with the aid of a stream of nitrogen, to apparent dryness. Redissolve in 15 mL of chloroform, and transfer to a filter, collecting the filtrate in a 150-mL beaker. Rinse the funnel with several small portions of chloroform, and evaporate the combined filtrate and rinsings, as described above, until no odor of chloroform or ethyl acetate is perceptible. Cool in a desiccator, and weigh.

Acceptance criteria: NMT 7.5%

• FREE ETHYLENE OXIDE

Internal standard solution: 100 mg/mL of *n*-butyl chloride in chlorobenzene. Store in a tightly closed container. Prepare fresh weekly.

Standard stock solution

[**CAUTION**—Ethylene oxide is toxic and flammable. Prepare this solution in a well-ventilated hood, using great care.]

Place 250 mL of chlorobenzene in a glass-stoppered 500-mL conical flask. Bubble ethylene oxide through the chlorobenzene at a moderate rate for 30 min, insert the stopper, and store with protection from heat. Pipet 25 mL of 0.5 N alcoholic hydrochloric acid solution, prepared by mixing 45 mL of hydrochloric acid with 1 L of alcohol, into a 500-mL conical flask containing 40 g of magnesium chloride hexahydrate. Shake the mixture

to effect saturation. Pipet 10 mL of the ethylene oxide solution into the flask, and add 20 drops of bromocresol green TS. If the solution is not yellow (acid) at this point, add an additional volume of 0.5 N alcoholic hydrochloric acid to give an excess of 10 mL. Record the total volume of 0.5 N alcoholic hydrochloric acid added. Insert the stopper in the flask, and allow to stand for 30 min. Titrate the excess acid with 0.5 N alcoholic potassium hydroxide VS. Perform a blank titration, using 10.0 mL of chlorobenzene instead of ethylene oxide solution, adding the same total volume of 0.5 N alcoholic hydrochloric acid, and note the difference in volumes required. Each mL of the difference in volumes of 0.5 N alcoholic potassium hydroxide consumed is equivalent to 22.02 mg of ethylene oxide. Calculate the concentration, in mg/mL, of ethylene oxide in the *Standard stock solution*. Standardize daily.

Standard solution: Transfer 5 g of Polyoxyl 10 Oleyl Ether to a suitable glass bottle of 60-mL capacity, and add 10 mL of chlorobenzene, exactly 50 μ L of *Internal standard solution*, and a volume of *Standard stock solution* containing 0.5 mg of ethylene oxide. Insert a magnetic stirring bar, cap the bottle tightly, and stir until homogeneity is attained.

Sample solution: Transfer 5 g of Polyoxyl 10 Oleyl Ether to a suitable glass bottle of 60-mL capacity, and add 10 mL of chlorobenzene and 50 μ L of *Internal standard solution*. Add a volume of chlorobenzene equal to the volume of the *Standard stock solution* added to prepare the *Standard solution*. Insert a magnetic stirring bar, cap the bottle tightly, and stir until homogeneity is attained.

Interference check solution: Transfer 5 g of Polyoxyl 10 Oleyl Ether to a suitable glass bottle of 60-mL capacity, and add 10 mL of chlorobenzene. Add a volume of chlorobenzene equal to the volume of the *Standard stock solution* used to prepare the *Standard solution*. Insert a magnetic stirring bar, cap the bottle tightly, and stir until homogeneity is attained.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 3-mm (OD) \times 1.8-m stainless steel packed with S3

Temperatures

Injection port: 210°

Detector: 230°

Column: 160°

Carrier gas: Helium

Flow rate: 66 mL/min

Injection volume: 2 μ L

System suitability

Samples: Chlorobenzene, *Internal standard solution*, *Standard stock solution*, and *Interference check solution*

Interference check: Inject a suitable volume of chlorobenzene into the gas chromatograph, and allow the chromatogram to run until the solvent has eluted.

Similarly inject and chromatograph the *Internal standard solution*, the *Standard stock solution*, and the *Interference check solution*. No interfering peaks are observed.

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the weight of ethylene oxide in the portion of sample taken:

$$W_T = (W_E \times W_U \times R_U) / [(W_U \times R_S) - (W_S \times R_U)] \times F$$

W_E = weight of ethylene oxide added to the *Standard solution* (mg)

W_U = weight of Polyoxyl 10 Oleyl Ether used to prepare the *Sample solution* (g)

R_U = peak area ratio of ethylene oxide to the internal standard for the *Sample solution*

R_S = peak area ratio of ethylene oxide to the internal standard for the *Standard solution*
 W_S = weight of Polyoxyl 10 Oleyl Ether used to prepare the *Standard solution* (g)

F = unit conversion, mg to g (10^{-3})

Calculate the percentage of ethylene oxide in the portion of Polyoxyl 10 Oleyl Ether taken:

$$\text{Result} = (W_T / W_U) \times 100$$

W_T and W_U are as defined above.

Acceptance criteria: NMT 0.01%

SPECIFIC TESTS

• **WATER DETERMINATION, Method I** <921>: NMT 3.0%

• **FATS AND FIXED OILS, Acid Value** <401>: NMT 1.0.

• **FATS AND FIXED OILS, Hydroxyl Value** <401>: 75–95

• **FATS AND FIXED OILS, Iodine Value, Method I** <401>

Sample: 550 mg

Analysis: Proceed as directed in the chapter, with the reaction time being extended to 60 min.

Acceptance criteria: 23–40

• **FATS AND FIXED OILS, Saponification Value** <401>: NMT 3

• **AVERAGE POLYMER LENGTH**

Sample solution: If solid material is present, place the Polyoxyl 10 Oleyl Ether in a 60° water bath overnight. Shake vigorously to eliminate any possibility of molecular weight gradients within it. Add 1 mL of the melt to 1 mL of deuterated chloroform in a test tube, and shake the test tube until dissolution is complete. Transfer 0.5 mL to an NMR tube, and add a small amount of tetramethylsilane as an internal reference standard. Cap the tube tightly, and shake thoroughly.

Analysis: Place the tube in an NMR spectrometer that is capable of performing quantitative analysis, and record the NMR spectrum (see *Nuclear Magnetic Resonance* <761>, *Quantitative Applications*). Integrate the areas from 0.4 to 2.35 ppm (A_1), and from 2.35 to 4.9 ppm (A_2).

Calculate the number of oxyethylene units per molecule taken:

$$\text{Result} = [(31A_2 / A_1) - 3] / 4$$

31 = total number of protons in the molecule not activated by either oxygen or a double bond

3 = number of oxygen-activated protons not included in the oxyethylene unit count

4 = number of protons in each oxyethylene unit

Acceptance criteria: 9.1–10.9

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool place.

• **LABELING:** Label to indicate the names and proportions of any added stabilizers.

• **USP REFERENCE STANDARDS** <11>

USP Polyoxyl 10 Oleyl Ether RS

Polyoxyl 15 Hydroxystearate

12-Hydroxyoctadecanoic acid polymer with α -hydro- ω -hydroxypoly(oxy-1,2-ethanediyl);
Polyethylene glycol 15 hydroxystearate [70142-34-6].

DEFINITION

Polyoxyl 15 Hydroxystearate results from the reaction of about 15 moles of ethylene oxide with 1 mole of 12-hydroxystearic acid. The product consists mainly of 12-hydroxystearic acid polyethoxylated at both the carboxyl and the hydroxyl groups with polyethylene glycol. It contains free polyethylene glycols.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197F): If the sample is solid or too viscous for thin film formation, the sample should be gently warmed to achieve a mobile liquid, which may then be used to prepare the thin film.

- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** (201)

Sample solution: To 1.0 g of Polyoxyl 15 Hydroxystearate add 100 mL of a 100-mg/mL solution of potassium hydroxide, and boil under a reflux condenser for 30 min. Acidify the warm solution with 20 mL of hydrochloric acid, and cool to room temperature. Shake the mixture with 50 mL of ether, and allow to stand until a separation of the layers is visible. Separate the clear upper layer, add 5 g of anhydrous sodium sulfate, wait for 30 min, filter, and evaporate to dryness on a water bath. Dissolve 50 mg of the residue in 25 mL of ether.

Standard solution: 2 mg/mL of USP 12-Hydroxystearic Acid RS in methylene chloride

Plate: Octadecylsilyl silica gel for chromatography as the coating substance

Application volume: 2 μ L

Developing solvent system: Acetone, methylene chloride, and glacial acetic acid (50:10:40)

Spray reagent: Prepare a solution of 80 mg/mL of phosphomolybdic acid in 2-propanol.

Analysis: Proceed as directed in the chapter. Develop over two-thirds of the plate, and dry in a current of cold air. Then spray the plate with *Spray reagent*, heat the plate at 120° for 1–2 min, and locate the spots on the plate.

Acceptance criteria: The R_f value and color of the principal spot of the *Sample solution* correspond to those of the *Standard solution*.

- **C.** It meets the requirements of the test for *Free Polyethylene Glycols*.

COMPOSITION

- **FREE POLYETHYLENE GLYCOLS**

Mobile phase: Methanol and water (8:2)

Standard solution A: 1.6 mg/mL of USP Polyethylene Glycol 1000 RS in *Mobile phase*

Standard solution B: 0.8 mg/mL of USP Polyethylene Glycol 1000 RS in *Mobile phase*, diluted from *Standard solution A* in *Mobile phase*

Sample solution: 4.8 mg/mL of Polyoxyl 15 Hydroxystearate in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Columns: 7.8-mm \times 30-cm analytical column; 6- μ m packing L39 and a 12-nm pore size; two 4-mm \times 12.5-cm precolumns; 5- μ m packing L1 and a 10-nm pore size.

Connect both precolumns to the analytical column using a 3-way valve, and switch the *Mobile phase* flow according to the following program. [NOTE—Shown in *Figure 1*, the analysis is started with precolumn 2 and an analytical column in series. After about 114 s, the valves, controlled by the detector program, switch over such that the eluent flows past precolumn 2, and direct to precolumn 1 and the analytical column. The columns are switched when the components to be determined, but not the interfering matrix, are ready to reach the analytical column. Simultaneously, precolumn 2 is washed out in the reverse direction by a second pump to remove the unwanted matrix components.]

Time (s)	Program
0–114	Precolumn 2 and analytical column
115–end	Precolumn 1 and analytical column
115–420	Reverse flow of precolumn 2

Temperature

Column: Room temperature

Detector: Room temperature

Flow rate: 1.1 mL/min

Injection size: 50 μ L

System suitability

Sample: *Standard solution A*

Suitability requirements

Relative standard deviation: NMT 3.0%

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Calculate the percentage of polyethylene glycols in the portion of Polyoxyl 15 Hydroxystearate taken:

$$\text{Result} = 2 \times (C_S/C_U)[r_U/(r_{S1} + 2r_{S2})] \times 100$$

C_S = concentration of USP Polyethylene Glycol 1000 RS in *Standard solution A* (mg/mL)

C_U = concentration of Polyoxyl 15 Hydroxystearate in the *Sample solution* (mg/mL)

r_U = peak response of polyethylene glycol from the *Sample solution*

r_{S1} = peak response of polyethylene glycol 1000 from *Standard solution A*

r_{S2} = peak response of polyethylene glycol 1000 from *Standard solution B*

Acceptance criteria: 27.0%–39.0% of free polyethylene glycols

IMPURITIES**Inorganic Impurities**

- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 0.3%, determined on 1.0 g

- **LIMIT OF NICKEL**

[**CAUTION**—When using closed high-pressure digestion vessels and microwave laboratory equipment, the safety precautions and operating instructions given by the manufacturer must be followed.]

[NOTE—If an alternative apparatus is used, adjustment of the instrument parameters may be necessary.]

Nickel standard stock solution: Dilute nickel standard solution TS two-fold with water. This solution contains the equivalent of 5 μ g/mL of nickel.

Standard solutions: Transfer 25, 50, 75, and 100 μ L of *Nickel standard stock solution* to four identical 25-mL volumetric flasks. To each flask add 0.5 mL of a 10-mg/mL solution of magnesium nitrate, 0.5 mL of a 100-mg/mL solution of monobasic ammonium phosphate, and 6.0 mL of nickel-free nitric acid, dilute with water to volume, and mix well. [NOTE—Content of nickel in the nickel-free nitric acid is NMT 0.005 ppm.] The *Standard solutions* contain 0.005, 0.01, 0.015, and 0.02 μ g/mL of nickel, respectively.

Sample solution: Transfer about 250 mg of Polyoxyl 15 Hydroxystearate to a suitable high-pressure-resistant digestion vessel (fluoropolymer or quartz glass), and add 6.0 mL of nickel-free nitric acid and 2.0 mL of 30% hydrogen peroxide. Place the closed vessel in a laboratory microwave oven, and digest using an appropriate program, e.g., 1000 W for 40 min. Allow the digestion vessel to cool down before opening. Add 2.0 mL of 30% hydrogen peroxide, and repeat the digestion step. Allow the digestion vessel to cool down before opening. Quantitatively transfer to a 25-mL volumetric flask, add 0.5 mL of a 10-mg/mL solution of magnesium nitrate and 0.5 mL of a 100-mg/mL

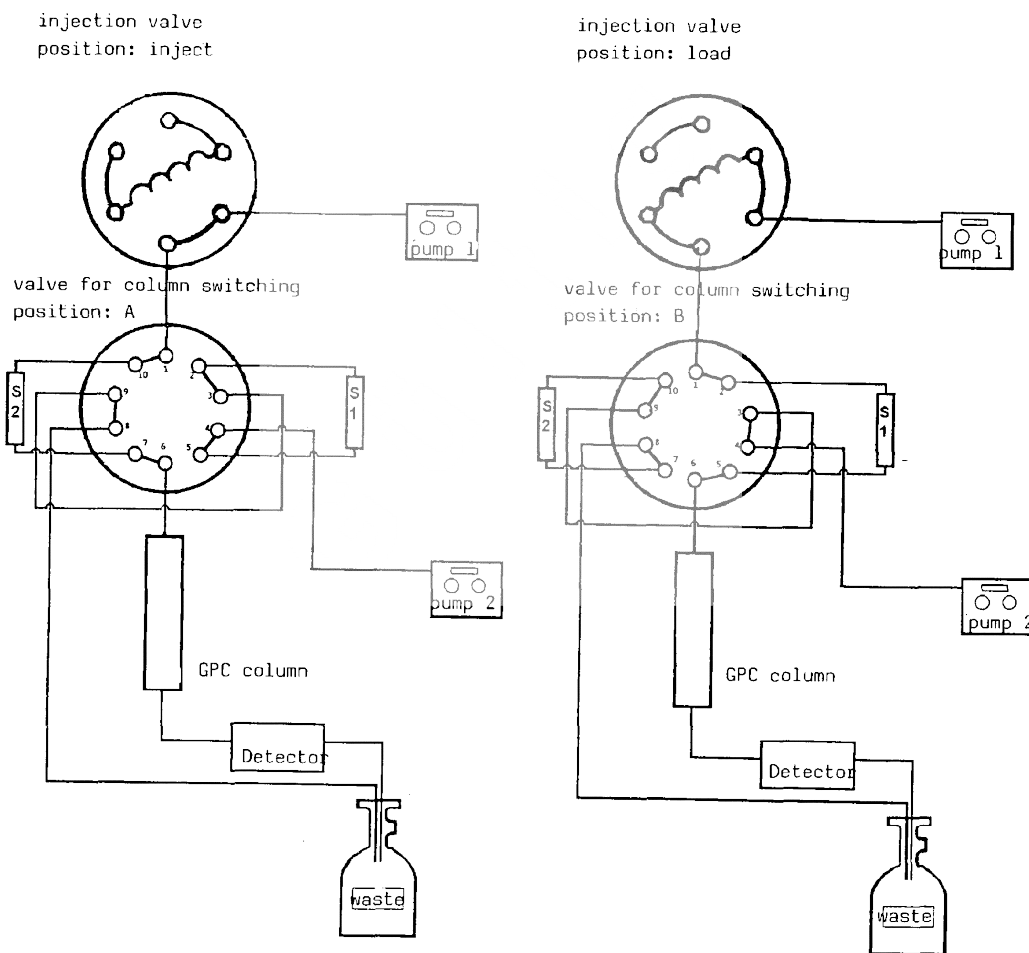


Figure 1. Apparatus

solution of monobasic ammonium phosphate, dilute with water to volume, and mix well.

Blank solution: Place 6.0 mL of nickel-free nitric acid and 2.0 mL of 30% hydrogen peroxide in a suitable high-pressure-resistant digestion vessel. Proceed as directed under *Sample solution*, beginning with "Place the closed vessel in a laboratory microwave oven, and digest using an appropriate program, e.g., 1000 W for 40 min."

Zero solution: In a 50-mL volumetric flask, introduce 1.0 mL of a 10-mg/mL solution of magnesium nitrate, 1.0 mL of a 100-mg/mL solution of monobasic ammonium phosphate, and 12.0 mL of nickel-free nitric acid. Dilute with water to volume, and mix well.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Graphite furnace atomic absorption spectrophotometer equipped with a background compensation system, a coated tube resistant to pyrolysis, and a nickel hollow-cathode lamp.

Analytical wavelength: Nickel emission line of 232.0 nm

Temperature: Maintain the drying temperature of the furnace at 120° for 35 s after a 5-s ramp; maintain the ashing temperature at 1100° for 10 s after a 30-s ramp; maintain the cooling temperature at 800° for 5 s after a 5-s decrease; and maintain the atomization temperature at 2600° for 7 s. [NOTE—The temperature program may be modified to obtain optimum furnace temperatures.]

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank solution*

Concomitantly determine the absorbances of the *Samples* using the *Spectrometric conditions* described above. Use the *Zero solution* to set the instrument to zero. Plot the absorbances of the *Standard solutions* versus the concentration, in µg/mL, of nickel, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration, C_T , in µg/mL, of nickel in the *Sample solution*, and determine the concentration, C_B , in µg/mL, of nickel in the *Blank solution*. If necessary, dilute with the *Zero solution* to obtain a reading within the calibrated absorbance range.

Calculate the quantity, in µg, of nickel in each g of Polyoxyl 15 Hydroxystearate taken:

$$\text{Result} = V \times (C_T - C_B)/W$$

V = volume of the *Sample solution* and the *Blank solution*, 25 mL

W = weight of Polyoxyl 15 Hydroxystearate taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 1 µg/g of nickel

Organic Impurities

• PROCEDURE: LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE

[CAUTION—Ethylene oxide is toxic and flammable. Prepare these solutions in a well-ventilated fume hood, using great care. Protect both hands and face by wearing

polyethylene protective gloves and an appropriate face mask. Store all solutions in hermetic containers, and refrigerate at a temperature between 4° and 8°.] [NOTE—Before using the polyethylene glycol 200 in this test, remove any volatile components from it by placing 500 mL of polyethylene glycol 200 in a 1000-mL round-bottom flask, attaching the flask to a rotary evaporator maintained at a temperature of 60° and under a vacuum of 10–20 mm Hg for 6 h.]

Acetaldehyde solution: 10 µg/mL of acetaldehyde. [NOTE—Prepare the *Acetaldehyde solution* immediately prior to use.]

Ethylene oxide stock solution: Fill a chilled pressure bottle with liquid ethylene oxide, and store in a freezer when not in use. Use a small piece of polyethylene film to protect the liquid from contact with the rubber gasket. Tare a glass-stoppered conical flask, add about 50 mL of polyethylene glycol 200, and reweigh the flask. Transfer about 5 mL of the liquid ethylene oxide to a 100-mL beaker chilled in a mixture of sodium chloride and ice (1:3). Using a gas-tight syringe that has been previously cooled to –10°, transfer about 300 µL (corresponding to about 250 mg) of liquid ethylene oxide to the polyethylene glycol 200, and swirl gently to mix. Replace the stopper, reweigh the flask, and determine the amount of ethylene oxide absorbed by weight difference. Adjust the weight of the mixture with polyethylene glycol 200 to 100.0 g, replace the stopper, and swirl gently to mix. This stock solution contains about 2.5 mg/g of ethylene oxide. [NOTE—Prepare this stock solution immediately prior to use, and store in a refrigerator.]

Ethylene oxide solution: Tare a glass-stoppered conical flask, and chill it in a refrigerator. Add about 35 mL of polyethylene glycol 200, and reweigh the flask. Using a gas-tight gas chromatographic syringe that has been chilled in a refrigerator, transfer about 1 g of the chilled *Ethylene oxide stock solution*, weighed, to the tared, conical flask. Adjust the weight of the solution with polyethylene glycol 200 to 50.0 g, replace the stopper, and swirl gently to mix. Transfer about 10 g of this solution, weighed, to a 50-mL volumetric flask. Add 30 mL of water, and mix. Dilute with water to volume, and mix to obtain a solution containing about 10 µg/mL of ethylene oxide. [NOTE—Prepare this solution immediately prior to use, and use directly after preparation.]

Dioxane solution: 500 µg/mL of dioxane

Standard solution A: Transfer 0.1 mL of *Ethylene oxide solution* to a 10-mL pressure headspace vial. [NOTE—Other sizes may be used depending on the operating conditions, however, the same size must be used for *Standard solution A*, *Standard solution B*, and the *Sample solution*.] Add 0.1 mL of *Acetaldehyde solution* and 0.1 mL of *Dioxane solution*, seal the vial, and mix.

Standard solution B: Transfer about 1.0 g of Polyoxyl 15 Hydroxystearate to another 10-mL pressure headspace vial, add 0.1 mL of *Ethylene oxide solution*, 0.1 mL of *Dioxane solution*, and 1.0 mL of *N,N*-dimethylacetamide. Seal the vial, and mix.

Sample solution: Transfer about 1.0 g of Polyoxyl 15 Hydroxystearate to a 10-mL pressure headspace vial, add 1.0 mL of *N,N*-dimethylacetamide and 0.2 mL of water, seal the vial, and mix.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

[NOTE—The use of a headspace apparatus that automatically transfers a measured amount of headspace is allowed.]

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m glass or quartz capillary; 1.0-µm layer of phase G1

Temperature

Injector port: 150°

Detector: 250°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	—
180	30	230	—
230	—	230	5

Carrier gas: Helium

Linear velocity: 20 cm/s

Injection size: 1 mL (the gaseous headspace)

Injection type: Split ratio 20:1

[NOTE—If the headspace apparatus is used, then an injection time of 12 s and a transfer line temperature of 150° are recommended.]

Headspace sampler: Each vial is heated at a temperature of 90° for 45 min, before a suitable portion of its headspace is injected.

System suitability

Sample: *Standard solution A*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are 0.94 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between acetaldehyde and ethylene oxide

Signal-to-noise ratio: NLT 5, determined from the dioxane peak

Relative standard deviation: NMT 15%

Analysis

Samples: *Standard solution B* and *Sample solution*

Using a heated, gas-tight, gas chromatographic syringe, separately inject equal volumes of the gaseous headspace of the *Samples* into the chromatograph, record the chromatograms, and measure the areas for the major peaks: the mean areas of the ethylene oxide and dioxane peaks from the *Sample solution* are not greater than half the mean areas of the corresponding peaks from *Standard solution B*.

Calculate the content of ethylene oxide, in ppm, in the portion of Polyoxyl 15 Hydroxystearate taken:

$$\text{Result} = (A_E \times r_U) / [(r_S \times W_U) - (r_U \times W_S)]$$

A_E = quantity of ethylene oxide added to *Standard solution B* (µg)

r_U = ethylene oxide peak response from the *Sample solution*

r_S = ethylene oxide peak response from *Standard solution B*

W_U = weight of test substance taken to prepare the *Sample solution* (g)

W_S = weight of test substance taken to prepare *Standard solution B* (g)

Calculate the content of dioxane, in ppm, in the portion of Polyoxyl 15 Hydroxystearate taken:

$$\text{Result} = (A_D \times r_U) / [(r_S \times W_U) - (r_U \times W_S)]$$

A_D = quantity of dioxane added to *Standard solution B* (µg)

r_U = dioxane peak response from the *Sample solution*

r_S = dioxane peak response from *Standard solution B*

W_U = weight of test substance taken to prepare the *Sample solution* (g)

W_S = weight of test substance taken to prepare *Standard solution B* (g)

Acceptance criteria

Ethylene oxide: NMT 1 ppm

Dioxane: NMT 50 ppm

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Acid Value* <401>: NMT 1.0, determined on 2.0 g
- **FATS AND FIXED OILS**, *Hydroxyl Value* <401>: 90–110
- **FATS AND FIXED OILS**, *Iodine Value, Method I* <401>: NMT 2.0
- **FATS AND FIXED OILS**, *Peroxide Value* <401>: NMT 5.0
- **FATS AND FIXED OILS**, *Saponification Value* <401>: 53–63
- **WATER DETERMINATION**, *Method Ia* <921>: NMT 1.0%, determined on 2.0 g

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight containers at a temperature below 25°.
- **USP REFERENCE STANDARDS** <11>
USP 12-Hydroxystearic Acid RS
USP Polyethylene Glycol 1000 RS
USP Polyoxyl 15 Hydroxystearate RS

Polyoxyl 20 Cetostearyl Ether

DEFINITION

Polyoxyl 20 Cetostearyl Ether is a mixture of mono-cetostearyl (mixed hexadecyl and octadecyl) ethers of mixed polyoxyethylene diols, the average polymer length being equivalent to NLT 17.2 and NMT 25.0 oxyethylene units.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197F>: Use undried specimen.

IMPURITIES

- **RESIDUE ON IGNITION** <281>

Sample: 25 g

Analysis: Weigh the *Sample* into a tared 40-mL porcelain crucible, and heat in contact with air until it ignites spontaneously or can be ignited with a glowing splint. Allow the flame to go out, place the crucible in a muffle furnace with the door partly open until the carbon is consumed, close the door, and heat at $700 \pm 100^\circ$ for 1 h. Cool in a desiccator, weigh, and calculate the percentage of residue. If the amount so obtained exceeds 0.4%, heat again until constant weight is attained.

Acceptance criteria: NMT 0.4%

- **HEAVY METALS**, *Method II* <231>: NMT 20 µg/g
- **FREE POLYETHYLENE GLYCOLS**

Sample solution: Transfer 12 g to a 500-mL separator containing 50 mL of ethyl acetate. Add 50 mL of sodium chloride solution (0.29 g/mL), shake vigorously for 2 min, and allow to separate for 15 min. Drain the lower, aqueous phase into a second 500-mL separator, and extract the upper layer with a second 50-mL portion of sodium chloride solution (0.29 g/mL). To the combined aqueous layers, add 50 mL of ethyl acetate, shake vigorously for 2 min, and allow to separate as before. Drain the lower, aqueous phase into a third 500-mL separator, and extract with two 50-mL portions of chloroform by shaking for 2 min each time.

Analysis: Evaporate the combined chloroform extracts in a 150-mL beaker on a steam bath, with the aid of a stream of nitrogen, to apparent dryness. Redissolve in 15 mL of chloroform, and transfer to a filter, collecting the filtrate in a 150-mL beaker. Rinse the funnel with several small portions of chloroform, and evaporate the combined filtrate and rinsings, as described above, until no odor of chloroform or ethyl acetate is perceptible. Cool in a desiccator, and weigh.

Acceptance criteria: NMT 7.5%

• **FREE ETHYLENE OXIDE**

Internal standard solution: 100 mg/mL of *n*-butyl chloride in chlorobenzene. Store in a tightly closed container. Prepare fresh weekly.

Standard stock solution

[**CAUTION**—Ethylene oxide is toxic and flammable. Prepare this solution in a well-ventilated hood, using great care.]

Place 250 mL of chlorobenzene in a glass-stoppered, 500-mL conical flask. Bubble ethylene oxide through the chlorobenzene at a moderate rate for 30 min, insert the stopper, and store with protection from heat. Pipet 25 mL of a 0.5 N alcoholic hydrochloric acid solution, prepared by mixing 45 mL of hydrochloric acid with 1 L of alcohol, into a 500-mL conical flask containing 40 g of magnesium chloride hexahydrate. Shake the mixture to effect saturation. Pipet 10 mL of the ethylene oxide solution into the flask, and add 20 drops of bromocresol green TS. If the solution is not yellow (acid) at this point, add an additional volume of 0.5 N alcoholic hydrochloric acid to give an excess of 10 mL. Record the total volume of 0.5 N alcoholic hydrochloric acid added. Insert the stopper in the flask, and allow to stand for 30 min. Titrate the excess acid with 0.5 N alcoholic potassium hydroxide VS.

Perform a blank titration, using 10.0 mL of chlorobenzene instead of ethylene oxide solution, adding the same total volume of 0.5 N alcoholic hydrochloric acid, and note the difference in volumes required. Each mL of the difference in volumes of 0.5 N alcoholic potassium hydroxide consumed is equivalent to 22.02 mg of ethylene oxide. Calculate the concentration, in mg/mL, of ethylene oxide in the *Standard stock solution*. Standardize daily.

Standard solution: Transfer 5 g of Polyoxyl 20 Cetostearyl Ether to a suitable glass bottle of 60-mL capacity. Add 10 mL of chlorobenzene, exactly 50 µL of *Internal standard solution*, and a volume of *Standard stock solution* containing 0.5 mg of ethylene oxide. Insert a magnetic stirring bar, cap the bottle tightly, and stir until homogeneity is attained.

Sample solution: Transfer 5 g of Polyoxyl 20 Cetostearyl Ether to a suitable glass bottle of 60-mL capacity. Add 10 mL of chlorobenzene and 50 µL of *Internal standard solution*. Add a volume of chlorobenzene equal to the volume of the *Standard stock solution* added to prepare the *Standard solution*. Insert a magnetic stirring bar, cap the bottle tightly, and stir until homogeneity is attained.

Interference check solution: Transfer 5 g of Polyoxyl 20 Cetostearyl Ether to a suitable glass bottle of 60-mL capacity, and add 10 mL of chlorobenzene. Add an additional volume of chlorobenzene equal to the volume of the *Standard stock solution* used to prepare the *Standard solution*. Insert a magnetic stirring bar, cap the bottle tightly, and stir until homogeneity is attained.

Chromatographic system(See *Chromatography* <621>, *System Suitability*.)**Mode:** GC**Detector:** Flame ionization**Column:** 3-mm (OD) × 1.8-m stainless steel packed with S3**Temperatures****Injection port:** 210°**Detector:** 230°**Column:** 160°**Carrier gas:** Helium**Flow rate:** 66 mL/min**Injection volume:** 2 µL**System suitability**

Samples: Chlorobenzene, *Internal standard solution*, *Standard stock solution*, and *Interference check solution*

Interference check: Inject a suitable volume of chlorobenzene, and allow the chromatogram to run until the

solvent has eluted. Similarly inject the *Internal standard solution*, *Standard stock solution*, and *Interference check solution*. No interfering peaks are observed.

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the weight of ethylene oxide in the portion of the sample taken:

$$W_T = (W_E \times W_U \times R_U) / [(W_U \times R_S) - (W_S \times R_U)] \times F$$

W_E = weight of ethylene oxide added to the *Standard solution* (mg)

W_U = weight of Polyoxyl 20 Cetostearyl Ether used to prepare the *Sample solution* (g)

R_U = peak area ratio of ethylene oxide to the internal standard from the *Sample solution*

R_S = peak area ratio of ethylene oxide to the internal standard from the *Standard solution*

W_S = weight of Polyoxyl 20 Cetostearyl Ether used to prepare the *Standard solution* (g)

F = unit conversion, mg to g (10^{-3})

Calculate the percentage of ethylene oxide in the portion of Polyoxyl 20 Cetostearyl Ether taken:

$$\text{Result} = (W_T / W_U) \times 100$$

W_T and W_U are as defined above.

Acceptance criteria: NMT 0.01%

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 0.5
- **FATS AND FIXED OILS**, *Hydroxyl Value* (401): 42–60
- **FATS AND FIXED OILS**, *Saponification Value* (401): NMT 2
- **PH** (791)

Sample solution: 100 mg/mL

Acceptance criteria: 4.5–7.5

- **WATER DETERMINATION**, *Method I* (921): NMT 1.0%

AVERAGE POLYMER LENGTH

Sample solution: Place the Polyoxyl 20 Cetostearyl Ether in a 50° water bath overnight to melt it completely. Shake vigorously to eliminate any possibility of molecular weight gradients within it, and transfer 200 µL to a 5- × 180-mm high-resolution NMR sample tube. Add 200 µL of deuterated chloroform by means of a separate microsyringe. Add 5 drops of tetramethylsilane as an internal reference standard. Cap the tube tightly, and shake thoroughly.

Analysis: Place the tube in the NMR spectrometer, and record the NMR spectrum at an appropriate RF power level and a sweep time of 250 s/500 Hz (see *Nuclear Magnetic Resonance* (761), *Qualitative Applications*). Adjust the spectrum amplitude so that the signal at 1.1 ppm is at least 80% of full scale. Record the integral areas, from 0.4 to 2.35 ppm (A_1), and from 2.35 to 4.9 ppm (A_2), at a sweep time of 50 s/500 Hz at an integral power level such that the integral of the ethylene oxide peak at 3.5 ppm is at least 80% of full chart height. Do not change the power level during the sweep. Record the integral of each peak several times, and calculate the average integral area.

Calculate the number of oxyethylene units per molecule taken:

$$\text{Result} = [32 \times A_2 / (A_1 - 3)] / 4$$

32 = total number of protons in the molecule not activated by oxygen, averaged for the cetyl and stearyl radicals

3 = number of oxygen-activated protons not included in the oxyethylene unit count

4 = number of protons in each oxyethylene unit

Acceptance criteria: 17.2–25.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, in a cool place.

- **USP REFERENCE STANDARDS** (11)
USP Polyoxyl 20 Cetostearyl Ether RS

Polyoxyl 35 Castor Oil

DEFINITION

Polyoxyl 35 Castor Oil contains mainly the tri-ricinoleate ester of ethoxylated glycerol, with smaller amounts of polyethylene glycol ricinoleate and the corresponding free glycols. It results from the reaction of glycerol ricinoleate with 35 moles of ethylene oxide.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197F)

• B.

Sample: 0.1 g

Analysis: Dissolve the *Sample* in 10 mL of alcoholic potassium hydroxide TS, boil for 3 min, and evaporate to dryness. Mix the residue with 5 mL of water.

Acceptance criteria: The residue dissolves, yielding a clear solution. Add a few drops of glacial acetic acid. A white precipitate is formed.

• C.

Sample solution: 1 in 20

Analysis: Add bromine TS dropwise to the *Sample solution*.

Acceptance criteria: The bromine is decolorized.

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.3%
- **HEAVY METALS**, *Method II* (231): NMT 10 µg/g

SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): 1.05–1.06
- **VISCOSITY—CAPILLARY VISCOMETER METHODS** (911): 600–850 centipoises at 25°, using a capillary viscometer
- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 2.0
- **FATS AND FIXED OILS**, *Hydroxyl Value* (401): 65–80
- **FATS AND FIXED OILS**, *Iodine Value* (401): 25–35
- **FATS AND FIXED OILS**, *Saponification Value* (401): 60–75
- **WATER DETERMINATION**, *Method I* (921): NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** (11)
USP Polyoxyl 35 Castor Oil RS

Polyoxyl 40 Hydrogenated Castor Oil

DEFINITION

Polyoxyl 40 Hydrogenated Castor Oil contains mainly the tri-hydroxystearate ester of ethoxylated glycerol, with smaller amounts of polyethylene glycol tri-hydroxystearate and of the corresponding free glycols. It results from the reaction of glycerol tri-hydroxystearate with 40–45 moles of ethylene oxide.

IDENTIFICATION

• A.

Sample: 0.1 g

Analysis: Dissolve the *Sample* in 1 mL of water, add 9 mL of sodium chloride solution (50 mg/mL), and heat in a water bath.

Acceptance criteria: The solution becomes turbid at 70°–85°.

• **B.**

Sample: 0.1 g

Analysis: Dissolve the *Sample* in 10 mL of alcoholic potassium hydroxide TS, boil for 3 min, and evaporate to dryness. Mix the residue with 5 mL of water.

Acceptance criteria: The residue dissolves, yielding a clear solution. Add a few drops of glacial acetic acid. A white precipitate is formed.

IMPURITIES

- **RESIDUE ON IGNITION** <281>: NMT 0.3%
- **HEAVY METALS**, *Method II* <231>: NMT 10 µg/g

SPECIFIC TESTS

- **CONGEALING TEMPERATURE** <651>: 16°–26°
- **FATS AND FIXED OILS**, *Acid Value* <401>: NMT 2.0
- **FATS AND FIXED OILS**, *Hydroxyl Value* <401>: 60–80
- **FATS AND FIXED OILS**, *Iodine Value* <401>: NMT 2.0
- **FATS AND FIXED OILS**, *Saponification Value* <401>: 45–69
- **WATER DETERMINATION**, *Method I* <921>: NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Polyoxyl 40 Stearate

Poly(oxy-1,2-ethanediyl), α -hydro- ω -hydroxy-, octadecanoate;
Polyethylene glycol monostearate [9004-99-3].

DEFINITION

Polyoxyl 40 Stearate is a mixture of the mono-esters and di-esters of Stearic Acid or Purified Stearic Acid with mixed polyoxyethylene diols, the average polymer length being equivalent to 40 oxyethylene units.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197M>
Sample: Undried specimen
Acceptance criteria: Meets the requirements

IMPURITIES

- **HEAVY METALS**, *Method II* <231>: NMT 10 µg/g

SPECIFIC TESTS

- **CONGEALING TEMPERATURE** <651>: 37°–47°
- **FATS AND FIXED OILS**, *Acid Value* <401>: NMT 2
- **FATS AND FIXED OILS**, *Hydroxyl Value* <401>: 25–40
- **FATS AND FIXED OILS**, *Saponification Value* <401>: 25–35
- **WATER DETERMINATION**, *Method I* <921>: NMT 3.0%
- **FREE POLYETHYLENE GLYCOLS**

Solution A: 290 mg/mL of sodium chloride

Sample: 6 g

Analysis: Transfer the *Sample* to a 500-mL separator containing 50 mL of ethyl acetate. Dissolve completely, then add 50 mL of *Solution A*, shake vigorously for 2 min, and allow to separate for 15 min. If separation is incomplete, carefully insert the separator into the well of a steam bath for short time intervals. Repeat this technique as many times as necessary to ensure the complete separation of the two phases. Cool, and drain the lower, aqueous phase into a second 500-mL separator, and extract the upper layer with a second 50-mL portion of *Solution A*.

Repeat the separation as before, including the steam bath technique, to enhance complete separation. To the combined aqueous layers add 50 mL of ethyl acetate, shake vigorously for 2 min, and allow to separate as before. Drain the lower, aqueous phase into a third 500-mL separator, and extract it with two 50-mL portions of chloroform by shaking for 2 min each time. Repeat the steam bath technique to ensure complete separation. Evaporate the combined chloroform extracts

in a 150-mL beaker on a steam bath, with the aid of a stream of nitrogen, to apparent dryness. Redissolve in 15 mL of chloroform, and transfer to a filter, collecting the filtrate in a 150-mL beaker. Rinse the funnel with several small portions of chloroform, and evaporate the combined filtrate and rinsings, as described above, until no odor of chloroform or ethyl acetate is perceptible. Dry under vacuum at 60° for 1 h. Cool in a desiccator, and weigh.

Acceptance criteria: 17%–27%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** <11>
USP Polyoxyl 40 Stearate RS

Polyoxyl Lauryl Ether

$\text{CH}_3(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_n\text{OH}$, $n = 3\text{--}23$
Polyethylene glycol monolauryl ether [9002-92-0].

DEFINITION

Polyoxyl Lauryl Ether is a mixture of the monolauryl ethers of mixed polyethylene glycols, the average polymer length being equivalent to NLT 3 and NMT 23 oxyethylene units (nominal value). It contains various amounts of free lauryl alcohol, and it may contain some free polyethylene glycols.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197F>
Sample: Use a thin film of melted Polyoxyl Lauryl Ether if the material is a solid.
Acceptance criteria: Meets the requirements
- **B. PROCEDURE**
Sample: 0.1 g
Analysis: Dissolve or disperse the *Sample* in 5 mL of alcohol, and add 10 mL of diluted hydrochloric acid, 5 mL of barium chloride TS, and 10 mL of phosphomolybdic acid solution (1 in 10).
Acceptance criteria: A precipitate is formed.
- **C.** It meets the requirements of the test for *Fats and Fixed Oil*, *Hydroxyl Values* <401>.

IMPURITIES**Organic Impurities**

- **PROCEDURE: LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE**
Analysis: Proceed as directed in *Ethylene Oxide and Dioxane*, *Method I* <228>.
Acceptance criteria
Ethylene oxide: NMT 1 µg/g (ppm)
Dioxane: NMT 10 µg/g (ppm)

SPECIFIC TESTS

- **ALKALINITY**
Sample: 2.0 g of Polyoxyl Lauryl Ether
Analysis: Dissolve the *Sample* in a hot mixture of 10 mL of alcohol and 10 mL of water. Add 0.05 mL of bromothymol blue TS, and titrate with 0.1 N hydrochloric acid to a yellow endpoint.
Acceptance criteria: NMT 0.5 mL of 0.1 N hydrochloric acid is required.
- **APPEARANCE OF SOLUTION:** 5.0 g of Polyoxyl Lauryl Ether in 50.0 mL of alcohol. The solution is not more intensely colored than a solution prepared immediately before use by mixing 12.0 mL of ferric chloride CS, 5.0 mL of cobaltous chloride CS, and 2.0 mL of cupric sulfate CS with dilute hydrochloric acid (10 g/L) to make 50.0 mL, and diluting 12.5 mL of this solution with dilute hydrochloric acid (10 g/L) to make 100.0 mL. Make the comparison by viewing the substance and the solution downward in

matched color-comparison tubes against a white surface (see *Color and Achromicity* <631>).

- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 0.2%, determined on 2.0 g
- **FATS AND FIXED OILS, Acid Value** <401>: NMT 1.0, determined on 5.0 g
- **FATS AND FIXED OILS, Hydroxyl Value** <401>: Within the ranges specified in the accompanying table

Oxyethylene Units/Molecule (Nominal Value)	Hydroxyl Value
3	165–185
4	145–165
5	130–140
9	90–100
10	85–95
12	73–83
15	64–74
20–23	40–60

- **FATS AND FIXED OILS, Iodine Value** <401>: NMT 2.0
- **FATS AND FIXED OILS, Saponification Value** <401>: NMT 3.0, determined on 10.0 g
- **WATER DETERMINATION, Method I** <921>: NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool, dry place.
- **LABELING:** Label it to indicate the average nominal number of oxyethylene units.
- **USP REFERENCE STANDARDS** <11>
USP Polyoxyl 4 Lauryl Ether RS
USP Polyoxyl 9 Lauryl Ether RS
USP Polyoxyl 23 Lauryl Ether RS

Polyoxyl Oleate

Polyethylene glycol monooleate [9004-96-0].

DEFINITION

Polyoxyl Oleate is a mixture of the monoesters and diesters of oleic acid and mixed polyethylene glycols. It may be obtained by ethoxylation of oleic acid or by esterification of polyethylene glycols with oleic acid of animal or vegetable origin. It may contain free polyethylene glycol. The average polymer length is equivalent to either 5–6 or 10 oxyethylene units (nominal values). A suitable antioxidant may be added.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197F>
Sample: Undried specimen
Acceptance criteria: Meets the requirements

IMPURITIES

- **LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE**
Analysis: Proceed as directed in *Ethylene Oxide and Dioxane* <228>, Method 1.
Acceptance criteria
Ethylene oxide: NMT 1 µg/g
Dioxane: NMT 10 µg/g

SPECIFIC TESTS

- **FATS AND FIXED OILS, Acid Value** <401>
Sample: 10.0 g
Acceptance criteria: NMT 1.0
- **FATS AND FIXED OILS, Peroxide Value** <401>: NMT 12.0
- **FATS AND FIXED OILS, Hydroxyl Value** <401>: See Table 1.
- **FATS AND FIXED OILS, Iodine Value** <401>: See Table 1.

- **FATS AND FIXED OILS, Saponification Value** <401>: See Table 1.

Table 1

	5–6 Ethylene Oxide Units	10 Ethylene Oxide Units
Hydroxyl value	50–70	65–90
Iodine value	50–60	27–34
Saponification value	105–120	68–85

- **FATS AND FIXED OILS, Fatty Acid Composition** <401>: Polyoxyl Oleate exhibits the composition profile of fatty acids shown in Table 2.

Table 2

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
14	0	≤5.0
16	0	≤16.0
18	0	≤6.0
16	1	≤8.0
18	1	65.0–88.0
18	2	≤18.0
18	3	≤4.0
>18	—	≤4.0

ALKALINITY

Sample solution: 100 mg/mL of Polyoxyl Oleate in alcohol

Analysis: To 2 mL of the *Sample solution* add 0.05 mL of phenol red TS.

Acceptance criteria: The solution is not red.

- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>

Sample: 1.0 g

Acceptance criteria: NMT 0.3%

- **WATER DETERMINATION, Method I** <921>: NMT 2.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool, dry place. Protect from moisture.
- **LABELING:** Label it to indicate the number of ethylene oxide units per molecule (nominal value), and the name and concentration of any added antioxidant.
- **USP REFERENCE STANDARDS** <11>
USP Polyoxyl Oleate RS

Polyoxyl Stearate

R-CO-(OCH₂CH₂)_n-OH

R-CO-(OCH₂CH₂)_n-OOC-R

R = CH₃(CH₂)₁₆ or CH₃(CH₂)₁₄

n = 6–100

Polyethylene glycol stearate;

Polyethylene glycol monostearate;

Poly(oxy-1,2-ethanediyl), α-hydro-ω-hydroxyoctadecanoate [9004-99-3].

DEFINITION

Polyoxyl Stearate is a mixture of monoesters and diesters of mainly stearic (octadecanoic) acid and/or palmitic (hexadecanoic) acid and polyethylene glycols. The fatty acids may be of vegetable, animal, or synthetic origin. Polyoxyl Stearate Type I or Type II differs in its content of stearic acid. It may contain free polyethylene glycols. The aver-

age polymer length is equivalent to 6–100 ethylene oxide units per molecule (nominal value).

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197A)
Sample: Use an undried specimen.
- **B.** It meets the requirements of the test for *Content of Stearic Acid and Palmitic Acid*.

ASSAY

• CONTENT OF STEARIC ACID AND PALMITIC ACID

Polyoxyl Stearate exhibits the composition profiles of fatty acids shown in *Table 1* below, as determined in *Fats and Fixed Oils* (401), *Fatty Acid Composition*.

Table 1

	Content of Stearic Acid and Palmitic Acid
Polyoxyl Stearate Type I	Stearic Acid: 40.0%–60.0%; sum of Palmitic and Stearic acids: NLT 90.0%
Polyoxyl Stearate Type II	Stearic Acid: 90.0%–99.0%; sum of Palmitic and Stearic acids: NLT 96.0%

• CONTENT OF FREE POLYETHYLENE GLYCOLS

[NOTE—This test is for Polyoxyl 40 Stearate only.]

Sample: 6 g of Polyoxyl 40 Stearate

Analysis: Transfer the *Sample* to a 500-mL separator containing 50 mL of ethyl acetate. Dissolve completely, then add 50 mL of sodium chloride solution (29 in 100), shake vigorously for 2 min, and allow to separate for 15 min. If separation is incomplete, carefully insert the separator into the well of a steam bath for short time intervals. Repeat this technique as many times as necessary to ensure the complete separation of the two phases. Cool, and drain the lower, aqueous phase into a second 500-mL separator. Extract the upper layer with a second 50-mL portion of sodium chloride solution (29 in 100). Repeat the separation as before, including the steam bath technique, to facilitate complete separation.

To the combined aqueous layers add 50 mL of ethyl acetate, shake vigorously for 2 min, and allow to separate as before. Drain the lower, aqueous phase into a third 500-mL separator, and extract it with two 50-mL portions of chloroform, shaking for 2 min each time. Repeat the steam bath technique to ensure complete separation.

Evaporate the combined chloroform extracts in a 150-mL beaker on a steam bath, with the aid of a stream of nitrogen, to apparent dryness.

Redissolve in about 15 mL of chloroform, and filter, collecting the filtrate in a weighed 150-mL beaker. Record the weight of the empty 150-mL beaker, W_1 , in g. Rinse the funnel with several small portions of chloroform, and evaporate the combined filtrate and rinsings, as described above, to remove chloroform or ethyl acetate. Dry in vacuum at 60° for 1 h. Cool in a desiccator, and weigh. Record the weight, W_2 , in g.

Calculate the percentage of free polyethylene glycols in Polyoxyl 40 Stearate taken:

$$\text{Result} = [(W_2 - W_1)/W] \times 100$$

W = weight of Polyoxyl 40 Stearate (g)

Acceptance criteria: 17%–27% of free polyethylene glycols for Polyoxyl 40 Stearate only

IMPURITIES

- **HEAVY METALS**, *Method II* (231): NMT 10 ppm
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 0.3%, determined on 1.0 g

• LIMIT OF ETHYLENE OXIDE AND DIOXANE

Analysis: Proceed as directed in *Ethylene Oxide and Dioxane* (228), *Method II*.

Acceptance criteria

Ethylene oxide: 1 ppm

Dioxane: 380 ppm

SPECIFIC TESTS

• ALKALINITY

Phenol red solution: Dissolve 100 mg of phenolsulfonphthalein in a mixture of 2.82 mL of 0.1 M sodium hydroxide and 20 mL of alcohol, and dilute with water to 100 mL.

Sample solution: 2.0 g of Polyoxyl Stearate

Analysis: Dissolve the *Sample* in alcohol and dilute with alcohol to 20 mL. To 2 mL of this solution add 0.05 mL of *Phenol red solution*.

Acceptance criteria: The solution does not turn red.

• FATS AND FIXED OILS, Acid Value (401): NMT 6.0

• FATS AND FIXED OILS, Hydroxyl Value (401): Within the ranges specified in *Table 2*

• FATS AND FIXED OILS, Iodine Value (401): NMT 3.0

• FATS AND FIXED OILS, Peroxide Value (401): NMT 10.0

• FATS AND FIXED OILS, Saponification Value (401): Within the ranges specified in *Table 2*

• MELTING RANGE OR TEMPERATURE (741)

Sample: 10 g

Analysis: Melt the *Sample* at 80°–90°. Introduce a sufficient amount of the *Sample* into the tube by capillary action to form a column of the prescribed height in the tube. Allow to stand at 0° for 2 h.

Acceptance criteria: Within the ranges specified in *Table 2*

Table 2

Ethylene Oxide Units/Molecule (Nominal Value)	Melting Range or Temperature (°)	Hydroxyl Value	Saponification Value
6	26–37	80–110	90–115
8	26–35	80–105	88–100
32	46–50	20–40	30–45
40	Measure Congealing Temperature	25–40	25–35
75	53–59	15–35	8–25
100	48–60	15–30	5–20

• CONGEALING TEMPERATURE (651): 37°–47° for Polyoxyl 40 Stearate only

• WATER DETERMINATION, *Method I* (921): NMT 3.0%

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE:

Preserve in tight containers, and store at room temperature. Protect from light and moisture.

• LABELING:

Label it to indicate the number of ethylene oxide units/molecule (nominal value), and the type of Polyoxyl Stearate. Label it to indicate whether the fatty acids are derived from vegetable, animal, or synthetic sources.

• USP REFERENCE STANDARDS (11)

USP Polyoxyl 6 Stearate RS

USP Polyoxyl 8 Stearate RS

USP Polyoxyl 32 Stearate RS

USP Polyoxyl 40 Stearate RS

USP Polyoxyl 75 Stearate RS

USP Polyoxyl 100 Stearate RS

Polyoxyl Stearyl Ether

Polyethylene glycol monostearyl ether
CH₃(CH₂)₁₇(OCH₂CH₂)_nOH, n = 2–20 [9005-00-9].

DEFINITION

Polyoxyl Stearyl Ether is a mixture of the monostearyl ethers of mixed polyethylene glycols, the average polymer length being equivalent to NLT 2 and NMT 20 oxyethylene units (nominal value). It may contain various amounts of free stearyl alcohol and some free polyethylene glycol.

IDENTIFICATION

- A. INFRARED ABSORPTION** <197F>: Use a thin film of melted Polyoxyl Stearyl Ether.
- B. PROCEDURE**
Sample: 0.1 g
Analysis: Dissolve or disperse the Sample in alcohol. Add 10 mL of diluted hydrochloric acid, 5 mL of barium chloride TS, and 10 mL of phosphomolybdic acid solution (1 in 10).
Acceptance criteria: A precipitate is formed.
- C.** It meets the requirements in the test for *Fats and Fixed Oils, Hydroxyl Value* <401>.

IMPURITIES

Organic Impurities

- PROCEDURE: LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE**
Analysis: Proceed as directed in *Ethylene Oxide and Dioxane* <228>, Method I.
Acceptance criteria
Ethylene oxide: NMT 1 µg/g (ppm)
Dioxane: NMT 10 µg/g (ppm)

SPECIFIC TESTS

- FATS AND FIXED OILS, Acid Value** <401>: NMT 1.0, determined on 5.0 g
- FATS AND FIXED OILS, Hydroxyl Value** <401>: Within the ranges specified in the table below

Oxyethylene Units/ Molecule (Nominal Value)	Hydroxyl Value
2	150–180
10	75–90
20	40–60

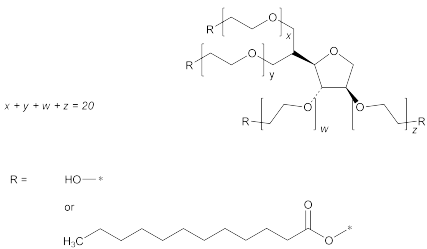
- FATS AND FIXED OILS, Iodine Value** <401>: NMT 2.0
- FATS AND FIXED OILS, Saponification Value** <401>: NMT 3.0, determined on 10.0 g
- ALKALINITY**
Sample: 2.0 g of Polyoxyl Stearyl Ether
Analysis: Dissolve the Sample to a hot mixture of 10 mL of alcohol and 10 mL of water. Add 0.05 mL of bromothymol blue TS. Titrate with 0.1 N hydrochloric acid to a yellow endpoint.
Acceptance criteria: NMT 0.5 mL of 0.1 N hydrochloric acid is required.
- WATER DETERMINATION, Method I** <921>: NMT 3.0%
- APPEARANCE OF SOLUTION:** 5.0 g of Polyoxyl Stearyl Ether in 50.0 mL of alcohol. The solution is not more intensely colored than a solution prepared immediately before use by mixing 12.0 mL of ferric chloride CS, 5.0 mL of cobaltous chloride CS, and 2.0 mL of cupric sulfate CS with dilute hydrochloric acid (10 g/L) to make 50.0 mL, and diluting 12.5 mL of this solution with dilute hydrochloric acid (10 g/L) to make 100.0 mL. Make the comparison

by viewing the substance and the solution downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* <631>).

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cool, dry place.
- LABELING:** Label it to indicate the average nominal number of oxyethylene units.
- USP REFERENCE STANDARDS** <11>
USP Polyoxyl 2 Stearyl Ether RS
USP Polyoxyl 10 Stearyl Ether RS
USP Polyoxyl 20 Stearyl Ether RS

Polysorbate 20



The ratio of the OH group to the (C₁₁H₂₃COO) group is mainly 3:1.
Polyethylene glycol 20 sorbitan ether monolaurate;
Polyoxyethylene 20 sorbitan monododecanoate;
Polyoxyethylene 20 sorbitan monolaurate [9005-64-5].

DEFINITION

Polysorbate 20 is a laurate ester of sorbitol and its anhydrides, copolymerized with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides. The fatty acids may be of vegetable, animal, or synthetic origin.

IDENTIFICATION

- A. INFRARED ABSORPTION** <197F>
- B.** It meets the requirements in the *Assay for Composition of Fatty Acids*.

ASSAY

- COMPOSITION OF FATTY ACIDS**
Polysorbate 20 exhibits the composition profiles of fatty acids shown in Table 1, as determined in *Fats and Fixed Oils* <401>, *Fatty Acid Composition*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤1.0
8	0	≤10.0
10	0	≤10.0
12	0	40.0–60.0
14	0	14.0–25.0
16	0	7.0–15.0
18	0	≤7.0
18	1	≤11.0
18	2	≤3.0

IMPURITIES

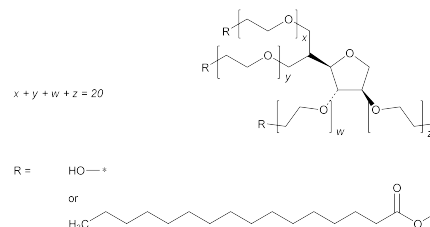
- **RESIDUE ON IGNITION** (281): NMT 0.25%
- **HEAVY METALS**, *Method II* (231): NMT 10 ppm
- **LIMIT OF ETHYLENE OXIDE AND DIOXANE**, *Method II* (228)
Acceptance criteria
Ethylene oxide: NMT 1 ppm
Dioxane: NMT 10 ppm

SPECIFIC TESTS

- **BACTERIAL ENDOTOXINS TEST** (85)
For Polysorbate 20 intended for use in the manufacture of injectable dosage forms: The level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Polysorbate 20 is used can be met. Where the label states that Polysorbate 20 must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Polysorbate 20 is used can be met.
- **FATS AND FIXED OILS**, *Acid Value* (401)
Sample: 10.0 g
Analysis: Transfer the *Sample* to a wide-mouth, 250-mL conical flask, and add 50 mL of neutralized alcohol. Heat on a steam bath nearly to boiling, occasionally shaking thoroughly while heating. Invert a beaker over the mouth of the flask, cool under running water, and add 5 drops of phenolphthalein TS. Titrate with 0.1 N sodium hydroxide VS. Calculate the acid value as directed in the chapter.
Acceptance criteria: NMT 2.0
- **FATS AND FIXED OILS**, *Hydroxyl Value* (401): 96–108
- **FATS AND FIXED OILS**, *Peroxide Value* (401)
Sample: 10.0 g
Saturated potassium iodide solution: Prepare a saturated solution of potassium iodide in carbon dioxide-free water. Make sure the solution remains saturated as indicated by the presence of undissolved crystals.
Analysis: Introduce the *Sample* into a 100-mL beaker, and dissolve with 20 mL of glacial acetic acid. Add 1 mL of *Saturated potassium iodide solution*, mix, and allow to stand for 1 min. Add 50 mL of carbon dioxide-free water and a magnetic stirring bar. Titrate with 0.01 M sodium thiosulfate VS, determining the endpoint potentiometrically (see *Titrimetry* (541)). Perform a blank titration.
Calculate the peroxide value as directed in the chapter.
Acceptance criteria: NMT 10.0
For Polysorbate 20 intended for use in the manufacture of injectable dosage forms: NMT 5.0
- **FATS AND FIXED OILS**, *Saponification Value* (401): 40–50
- **WATER DETERMINATION**, *Method I* (921): NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at room temperature.
- **LABELING:** Label it to indicate whether the fatty acids are derived from animal, vegetable, or synthetic sources. Where Polysorbate 20 is intended for use in the manufacture of injectable dosage forms, it is so labeled.
- **USP REFERENCE STANDARDS** (11)
USP Polysorbate 20 RS

Polysorbate 40

The ratio of OH group to $\text{C}_{15}\text{H}_{31}\text{COO}$ group is mainly 3:1. Polyethylene glycol 20 sorbitan ether monopalmitate; Polyoxyethylene 20 sorbitan monohexadecanoate; Polyoxyethylene 20 sorbitan monopalmitate [9005-66-7].

DEFINITION

Polysorbate 40 is a palmitate ester of sorbitol and its anhydrides copolymerized with about 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides. The fatty acids may be of vegetable, animal, or synthetic origin.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197F)
- **B.** It meets the requirements in the *Assay for Composition of Fatty Acids*.

ASSAY

- **COMPOSITION OF FATTY ACIDS**
Polysorbate 40 exhibits the composition profiles of fatty acids shown in *Table 1*, as determined in *Fats and Fixed Oils* (401), *Fatty Acid Composition*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
16	0	≥92.0

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.25%
- **HEAVY METALS**, *Method II* (231): NMT 10 ppm
- **ETHYLENE OXIDE AND DIOXANE**, *Method II* (228)
Acceptance criteria
Ethylene oxide: NMT 1 ppm
Dioxane: NMT 10 ppm

SPECIFIC TESTS

- **BACTERIAL ENDOTOXINS TEST** (85)
For Polysorbate 40 intended for use in the manufacture of injectable dosage forms: The level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Polysorbate 40 is used can be met. Where the label states that Polysorbate 40 must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Polysorbate 40 is used can be met.
- **FATS AND FIXED OILS**, *Acid Value* (401)
Sample: 10.0 g
Analysis: Transfer the *Sample* to a wide-mouth, 250-mL conical flask, and add 50 mL of neutralized alcohol. Heat on a steam bath nearly to boiling, shaking thoroughly occasionally while heating. Invert a beaker over the mouth of the flask, cool under running water, and add 5 drops of phenolphthalein TS. Titrate with 0.1 N sodium hydroxide VS. Calculate the acid value as directed in the chapter.

Acceptance criteria: NMT 2.0

- **FATS AND FIXED OILS**, *Hydroxyl Value* <401>: 89–105
- **FATS AND FIXED OILS**, *Peroxide Value* <401>

Sample: 10.0 g

Saturated potassium iodide solution: Prepare a saturated solution of potassium iodide in carbon dioxide-free water. Make sure the solution remains saturated as indicated by the presence of undissolved crystals.

Analysis: Introduce the *Sample* into a 100-mL beaker, and dissolve with 20 mL of glacial acetic acid. Add 1 mL of *Saturated potassium iodide solution*, mix, and allow to stand for 1 min. Add 50 mL of carbon dioxide-free water and a magnetic stirring bar. Titrate with 0.01 M sodium thiosulfate VS, determining the endpoint potentiometrically (see *Titrimetry* <541>). Perform a blank titration.

Calculate the peroxide value as directed in the chapter.

Acceptance criteria: NMT 10.0

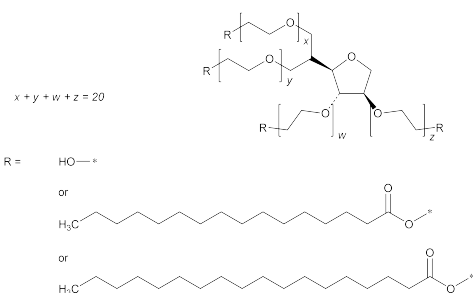
For Polysorbate 40 intended for use in the manufacture of injectable dosage forms: NMT 5.0

- **FATS AND FIXED OILS**, *Saponification Value* <401>: 41–52
- **WATER DETERMINATION**, *Method I* <921>: NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at room temperature.
- **LABELING:** Label it to indicate whether the fatty acids are derived from animal, vegetable, or synthetic sources. Where Polysorbate 40 is intended for use in the manufacture of injectable dosage forms, it is so labeled.
- **USP REFERENCE STANDARDS** <11>
USP Polysorbate 40 RS

Polysorbate 60



The ratio of OH group to the sum of $\text{C}_{15}\text{H}_{31}\text{COO}$ and $\text{C}_{17}\text{H}_{35}\text{COO}$ groups is mainly 3:1.
Polyethylene glycol 20 sorbitan ether monostearate;
Polyoxyethylene 20 sorbitan monooctadecanoate;
Polyoxyethylene 20 sorbitan monostearate [9005-67-8].

DEFINITION

Polysorbate 60 is a mixture of stearate and palmitate esters of sorbitol and its anhydrides copolymerized with about 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides. The fatty acids may be of vegetable, animal, or synthetic origin.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197F>
- **B.** It meets the requirements in the *Assay for Composition of Fatty Acids*.

ASSAY

COMPOSITION OF FATTY ACIDS

Polysorbate 60 exhibits the composition profiles of fatty acids shown in *Table 1*, as determined in *Fats and Fixed Oils* <401>, *Fatty Acid Composition*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
18	0	40.0–60.0
Sum of stearic acid (C18:0) and palmitic acid (C16:0)		≥90.0

IMPURITIES

- **RESIDUE ON IGNITION** <281>: NMT 0.25%
- **HEAVY METALS**, *Method II* <231>: NMT 10 ppm
- **ETHYLENE OXIDE AND DIOXANE**, *Method II* <228>
Acceptance criteria
Ethylene oxide: NMT 1 ppm
Dioxane: NMT 10 ppm

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Acid Value* <401>

Sample: 10.0 g of Polysorbate 60

Analysis: Transfer the *Sample* to a wide-mouth, 250-mL conical flask, and add 50 mL of neutralized alcohol. Heat on a steam bath nearly to boiling, shaking thoroughly occasionally while heating. Invert a beaker over the mouth of the flask, cool under running water, and add 5 drops of phenolphthalein TS. Titrate with 0.1 N sodium hydroxide VS. Calculate the acid value as directed in the chapter.

Acceptance criteria: NMT 2.0

- **FATS AND FIXED OILS**, *Hydroxyl Value* <401>: 81–96
- **FATS AND FIXED OILS**, *Peroxide Value* <401>

Sample: 10.0 g

Saturated potassium iodide solution: Prepare a saturated solution of potassium iodide in carbon dioxide-free water. Make sure the solution remains saturated as indicated by the presence of undissolved crystals.

Analysis: Introduce the *Sample* into a 100-mL beaker, and dissolve with 20 mL of glacial acetic acid. Add 1 mL of *Saturated potassium iodide solution*, mix, and allow to stand for 1 min. Add 50 mL of carbon dioxide-free water and a magnetic stirring bar. Titrate with 0.01 M sodium thiosulfate VS, determining the endpoint potentiometrically (see *Titrimetry* <541>). Perform a blank titration.

Calculate the peroxide value as directed in the chapter.

Acceptance criteria: NMT 10.0

- **FATS AND FIXED OILS**, *Saponification Value* <401>: 45–55
- **WATER DETERMINATION**, *Method I* <921>: NMT 3.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at room temperature.
- **LABELING:** Label to indicate whether the fatty acids are derived from animal, vegetable, or synthetic sources.
- **USP REFERENCE STANDARDS** <11>
USP Polysorbate 60 RS

Polysorbate 80

Attributes	EP	JP	USP
Definition	+	+	+
Identification (Composition of Fatty Acids)	+	+	+
Acid Value	+	+	+
Hydroxyl Value	+	+	+
Peroxide Value	+	+	+
Saponification Value	+	+	+
Composition of Fatty Acids	+	+	+
Ethylene Oxide and Dioxane	+	+	+
Water	+	+	+
Residue on Ignition	+	+	+
Storage	+	+	+

Legend: + will adopt and implement; – will not stipulate
Nonharmonized attributes: Characters, Identification by IR (EP), Heavy Metals (USP)

Each pharmacopeia will adapt the text to take account of local reference substances and spectra and reagent specifications.

Sorbitan, mono-9-octadecenoate, poly(oxy-1,2-ethanediyl) derivs., (Z)-;
 Polyoxyethylene 20 sorbitan monooleate [9005-65-6].

DEFINITION

Polysorbate 80 is a mixture of partial esters of fatty acids, mainly oleic acid, with sorbitol and its anhydrides ethoxylated with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides.

IDENTIFICATION

- It complies with the test for *Composition of Fatty Acids*.

ASSAY

COMPOSITION OF FATTY ACIDS

Diluent: 20 g/L of sodium hydroxide in methanol

Saturated sodium chloride solution: Sodium chloride and water (1:2). Before use, decant the solution from any undissolved substance and filter, if necessary.

Reference solution A: Prepare 0.50 g of the mixture of calibrating substances with the composition described in *Table 1*. Dissolve in heptane, and dilute with heptane to 50.0 mL.

Reference solution B: *Reference solution A* in heptane (1 in 10)

Reference solution C: Prepare 0.50 g of a mixture of fatty acid methyl esters, which corresponds to the composition of the substance to be examined. Dissolve in heptane, and dilute with heptane to 50.0 mL. [NOTE—Commercially available mixtures of fatty acid methyl esters may also be used.]

Sample solution: Dissolve 0.10 g of Polysorbate 80 in 2 mL of *Diluent* in a 25-mL conical flask, and boil under a reflux condenser for 30 min. Add 2.0 mL of 14% boron trifluoride-methanol through the condenser, and boil for 30 min. Add 4 mL of heptane through the condenser, and boil for 5 min. Cool and add 10.0 mL of *Saturated sodium chloride solution*, shake for about 15 s, and add a quantity of *Saturated sodium chloride solution* such that the upper phase is brought into the neck of the flask. Collect 2 mL of the upper phase, wash with three quantities, each of 2 mL, of water, and dry over anhydrous sodium sulfate.

Table 1

Mixture of the Following Substances	Composition (%)
Methyl myristate	5
Methyl palmitate	10
Methyl stearate	15
Methyl arachidate	20
Methyl oleate	20
Methyl eicosenoate	10
Methyl behenate	10
Methyl lignocerate	10

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m G16 on fused silica, film thickness 0.5 µm

Temperature

Injector: 250°

Detector: 250°

Column: See the temperature program table below.

Temperature (°)	Rate (°/min)	Time (min)
80→220	10	—
220	—	40

Carrier gas: Helium

Linear velocity: 50 cm/s

Injection size: 1 µL

System suitability

Samples: *Reference solution A* and *Reference solution B*

Suitability requirements

Resolution: NLT 1.8 between the peaks due to methyl oleate and methyl stearate, *Reference solution A*

Signal-to-noise ratio: NLT 5 for the peak of methyl myristate, *Reference solution B*

Theoretical plates: NLT 30,000 calculated for the peak of methyl stearate, *Reference solution A*

Analysis

Sample: *Sample solution*

Identify the peaks from *Reference solution C*. Calculate the percentage of each component in the *Sample solution*:

$$\text{Result} = A_c/A_T \times 100$$

A_c = peak area for the component of interest

A_T = total area of all peaks related to fatty acids

Acceptance criteria: Myristic acid, NMT 5.0%; palmitic acid, NMT 16.0%; palmitoleic acid, NMT 8.0%; stearic acid, NMT 6.0%; oleic acid, NLT 58.0%; linoleic acid, NMT 18.0%; linolenic acid, NMT 4.0%

IMPURITIES

Inorganic Impurities

- RESIDUE ON IGNITION:** Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Evenly distribute 2.00 g of the substance to be examined in the crucible. Dry at 100° to 105° for 1 h and ignite to constant mass in a muffle furnace at 600 ± 25°, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, take up with hot water, pass through an ashless filter paper, and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness, and ignite to constant mass.

Acceptance criteria: NMT 0.25%

- **HEAVY METALS**, *Method II* (231): NMT 10 ppm.

Organic Impurities

- **PROCEDURE: ETHYLENE OXIDE AND DIOXANE**

Ethylene oxide standard solution: Dilute 0.5 mL of a commercially available solution of ethylene oxide in methylene chloride (50 mg/mL) with water to 50.0 mL. [NOTE—The solution is stable for 3 months, if stored in vials with a teflon-coated, silicon membrane and crimped caps at -20° .] Allow to reach room temperature. Dilute 1.0 mL of this solution with water to 250.0 mL.

Dioxane standard solution: Dioxane in water (v/v) 1 in 20,000

Acetaldehyde standard solution: 0.01 mg/mL of acetaldehyde in water

Standard solution: Dilute 6.0 mL of *Ethylene oxide standard solution* and 2.5 mL of *Dioxane standard solution* with water to 25.0 mL.

Sample solution A: Transfer 1.0 g of Polysorbate 80 to a 10-mL headspace vial. Add 2.0 mL of water, and seal the vial immediately with a teflon-coated, silicon membrane and an aluminum cap.

Sample solution B: Transfer 1.0 g of Polysorbate 80 to a 10-mL headspace vial. Add 2.0 mL of *Standard solution*, and seal the vial immediately with a teflon-coated, silicon membrane and an aluminum cap.

Reference solution: Introduce 2.0 mL of *Acetaldehyde standard solution* and 2.0 mL of *Ethylene oxide standard solution* to a 10-mL headspace vial, and seal the vial immediately with a teflon-coated, silicon membrane and an aluminum cap.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: Headspace GC

Detector: Flame ionization

Column¹: 0.53-mm \times 50-m G27 on fused silica, film thickness 5 μ m

Temperature

Injector: 85°

Detector: 250°

Column: See the temperature program table below.

Temperature ($^{\circ}$)	Rate ($^{\circ}$ /min)	Time (min)
70 \rightarrow 250	10	—
250	—	5

Split ratio: 3:5

Carrier gas: Helium

Flow rate: 4.0 mL/min

Injection size: 1 mL

System suitability

Sample: *Reference solution*

[NOTE—The relative retention times for ethylene oxide, acetaldehyde, and dioxane are 1.0, 0.9, and 1.9, respectively. The retention time for ethylene oxide is about 6.5 min.]

Suitability requirements

Resolution: NLT 2.0 between the peaks due to acetaldehyde and ethylene oxide

Analysis

Samples: *Sample solution A* and *Sample solution B*
Calculate the content of ethylene oxide:

$$\text{Result} = (2 \times C_{EO} \times A_A) / (A_B - A_A)$$

C_{EO} = concentration of ethylene oxide in *Sample solution B* (μ g/mL)

A_A = peak area of ethylene oxide from *Sample solution A*

A_B = peak area of ethylene oxide from *Sample solution B*

Calculate the content of dioxane:

$$\text{Result} = (2 \times D \times C_D \times A_A) / (A_B - A_A)$$

D = density of dioxane, 1.03 g/mL

C_D = concentration of dioxane in *Sample solution B* (μ L/mL)

A_A = peak area of dioxane from *Sample solution A*

A_B = peak area of dioxane from *Sample solution B*

Acceptance criteria: NMT 1 ppm for ethylene oxide; NMT 10 ppm for dioxane

SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): 1.06–1.09

- **VISCOSITY—CAPILLARY VISCOMETER METHODS** (911) and **ROTATIONAL RHEOMETER METHODS** (912): 300–500 centistokes at 25°

Change to read:

- **FATS AND FIXED OILS**, *Acid Value* (401)

Sample solution: Dissolve 5.0 g in 50 mL of a mixture of equal volumes of alcohol and hexane (previously neutralized with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide), using 0.5 mL of **phenolphthalein TS** (ERR 1-Jul-2012) as indicator. If necessary, heat to about 90° to dissolve the substance to be examined.

Analysis: Titrate the *Sample solution* with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide until the pink color persists for at least 15 s. When heating has been applied to aid dissolution, maintain the temperature at about 90° during the titration.

Acceptance criteria: NMT 2.0

Change to read:

- **FATS AND FIXED OILS**, *Hydroxyl Value* (401)

Sample: 2.0 g

Analysis: Introduce the *Sample* into a 150-mL acetylation flask fitted with an air condenser. Add 5.0 mL of *Pyridine–Acetic Anhydride Reagent*, and attach the air condenser. Heat the flask in a water bath for 1 h keeping the level of the water about 2.5 cm above the level of the liquid in the flask. Withdraw the flask, and allow to cool. Add 5 mL of water through the upper end of the condenser. If a cloudiness appears, add sufficient pyridine to clear it, noting the volume added. Shake the flask, and replace in the water bath for 10 min. Withdraw the flask, and allow to cool. Rinse the condenser and the walls of the flask with 5 mL of alcohol, previously neutralized with **phenolphthalein TS** (ERR 1-Jul-2012). Titrate with 0.5 N alcoholic potassium hydroxide using 0.2 mL of **phenolphthalein TS** (ERR 1-Jul-2012) as indicator. Carry out a blank test under the same conditions.

Acceptance criteria: 65–80

- **FATS AND FIXED OILS**, *Peroxide Value* (401)

Sample: 10.0 g

Saturated potassium iodide solution: Prepare a saturated solution of potassium iodide in carbon dioxide-free water. Make sure the solution remains saturated as indicated by the presence of undissolved crystals.

Analysis: Introduce the *Sample* into a 100-mL beaker, and dissolve with 20 mL of glacial acetic acid. Add 1 mL of *Saturated potassium iodide solution*, and allow to stand for 1 min. Add 50 mL of carbon dioxide-free water and a magnetic stirring bar. Titrate with 0.01 M sodium thiosulfate, determining the endpoint potentiometrically. Carry out a blank titration.

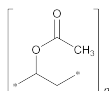
¹ CP-Sil 8 CB is suitable.

- Acceptance criteria:** NMT 10
- **FATS AND FIXED OILS, Saponification Value (401)**
Sample: 4.0 g
Analysis: Introduce the *Sample* into a 250-mL borosilicate glass flask fitted with a reflux condenser. Add 30.0 mL of 0.5 N alcoholic potassium hydroxide and a few glass beads. Attach the condenser, and heat under reflux for 60 min. Add 1 mL of phenolphthalein TS and 50 mL of dehydrated alcohol, and titrate immediately with 0.5 N hydrochloric acid. Carry out a blank test under the same conditions.
Acceptance criteria: 45–55
 - **WATER DETERMINATION, Method I (921):** NMT 3.0%, determined on 1.0 g

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Store in an airtight container, protected from light.

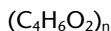
Polyvinyl Acetate



(C₄H₆O₂)_n
 Vinyl acetate homopolymer
 Vinyl acetate resin [9003-20-7].

DEFINITION

Polyvinyl acetate is a thermoplastic polymer, represented by the formula:



in which the value of *n* lies between approximately 100 and 17,000.

IDENTIFICATION

- **A. PROCEDURE**
Sample: 100 mg of Polyvinyl Acetate
Analysis: Dissolve the *Sample* in 2.5 mL of acetone, place two drops on a potassium bromide plate, and dry to evaporate the solvent.
Acceptance criteria: The IR absorption spectrum of polyvinyl acetate exhibits maxima corresponding to the same wavelengths as that of a similar preparation of USP Polyvinyl Acetate RS, treated in the same manner.
- **B. PROCEDURE**
Sample: 0.5 g of Polyvinyl Acetate
Analysis: Saponify the *Sample* in a mixture of 25.0 mL of 0.5 N alcoholic potassium hydroxide and 25.0 mL of water.
Acceptance criteria: The solution so obtained meets the requirements of the tests for *Identification Tests—General* (191), *Acetate*.

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION (281):** NMT 0.1%
- **HEAVY METALS, Method II (231):** NMT 10 ppm
- **RESIDUAL PEROXIDES**
Sample: 0.85 g of Polyvinyl Acetate
Analysis: Place the *Sample* in a borosilicate glass flask with a ground-glass neck. Add 10.0 mL of ethyl acetate, and heat under a reflux condenser with constant agitation. Allow to cool. Replace the air in the container with oxygen-free nitrogen, and add a solution of 1.0 mL of glacial acetic acid and 0.5 g of sodium iodide in 40.0 mL of water. Shake thoroughly, and allow to

stand protected from light for 20 min. Titrate with 0.005 N sodium thiosulfate VS until the yellow color is discharged. Perform a blank titration.

Acceptance criteria: The difference between the titration volumes is not greater than 1.0 mL; and NMT 100 ppm, calculated as hydrogen peroxide, is found.

Organic Impurities

PROCEDURE: LIMIT OF VINYL ACETATE

Standard stock solution: 1.0 mg/mL of Vinyl Acetate in toluene

Standard solutions: 0.1, 0.3, 1, 3, and 10 µg/mL of vinyl acetate in toluene, prepared from *Standard stock solution*

Sample solution: 0.1 g/mL of Polyvinyl Acetate in toluene

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Hydrogen flame ionization

Column: 0.32-mm × 30-m fused-silica capillary column, 5-µm layer of phase G1

Temperature

Detector: 250°

Injector port: 150°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	8
100	20	250	5

Carrier gas: Helium

Flow rate: Adjusted so that the vinyl acetate peak appears after about 7 min

Injection size: 1.0 µL

Injection type: Split ratio is about 8:1.

System suitability

Sample: *Standard solution* containing 1 µg/mL of vinyl acetate in toluene

Suitability requirements

Relative standard deviation: NMT 15%

Analysis

Samples: *Standard solution* and *Sample solution*
 Plot the peak responses of the vinyl acetate in the *Standard solutions* versus the concentration, in µg/mL, of vinyl acetate, and draw the straight line best fitting the five plotted points. From the graph so obtained, determine the concentration, *C*, in µg/mL, of vinyl acetate in the *Sample solution*.

Calculate the quantity, in µg, of vinyl acetate in each g of Polyvinyl Acetate taken:

$$\text{Result} = (C/C_p)$$

C = as determined above

C_p = concentration of the Polyvinyl Acetate in the *Sample solution* (g/mL)

Acceptance criteria: NMT 5 µg/g (5 ppm) of vinyl acetate

SPECIFIC TESTS

FATS AND FIXED OILS, Acid Value (401)

Sample: 10.0 g of Polyvinyl Acetate

Analysis: Transfer the *Sample* to a 250-mL glass-stoppered conical flask, dissolve in 75 mL of ethylene dichloride, add 60 mL of denatured alcoholic TS, and mix. Add 1 mL of phenolphthalein TS, and titrate with 0.02 N alcoholic potassium hydroxide VS until the solution remains faintly pink after shaking for 30 s. Perform a blank determination, and make any necessary correction.

Acceptance criteria: The acid value is NMT 0.5.

• **FATS AND FIXED OILS, Ester Value (401)**

Sample: 0.5 g of Polyvinyl Acetate

Analysis: Saponify the *Sample* in a mixture of 25.0 mL of 0.5 N alcoholic potassium hydroxide VS and 25.0 mL of water. Proceed as directed under *Fats and Fixed Oils* (401), *Saponification Value*, beginning with "Heat the flask on a steam bath".

Acceptance criteria: The ester value, calculated from the *Saponification Value* and the *Acid Value*, is between 615 and 675.

• **LOSS ON DRYING (731):** Dry 1.5 g at 100° for 2 h in a vacuum: it loses NMT 1.0% of its weight.

• **AVERAGE MOLECULAR WEIGHT AND MOLECULAR WEIGHT DISTRIBUTION**

[**CAUTION**—Tetrahydrofuran (THF) is considered to be a carcinogen and embryo-fetal toxic substance. It is also a peroxide former and is flammable. A safe-handling practice must be in place in the laboratory. Carefully review appropriate Material Safety Data Sheets before use.]

Mobile phase: Tetrahydrofuran inhibited with 250 ppm butylated hydroxytoluene. Do not sparge or degas.

Standard solutions: Prepare two sets of mixtures, each set containing five narrow polystyrene standards of different known molecular weights, totaling 10 narrow polystyrene standards covering the molecular weight range from about 600 to 3,000,000 g/mol.¹ Prepare each set of five narrow polystyrene standards to have a known concentration at about 0.05% (w/v) for each standard in *Mobile phase*.

Sample solution: Transfer 0.025 g of polyvinyl acetate to a vial, and add 10 mL of *Mobile phase*. Cap and mix well, using an appropriate laboratory shaker, for 1 h. Pass the polyvinyl acetate solution through a polytetrafluoroethylene filter having a porosity of 0.45 µm, discard an appropriate volume of the initial filtrate, and use the rest of the filtered solution for analysis.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive Index (RI)

Detector temperature: 35°

Columns: Two 10-mm × 50-cm analytical columns; 5-µm packing L73, and a 10-mm × 10-cm, 500-Å guard column; packing L73. [NOTE—The analytical column is suitable for molecular weight ranges from 100 to 10,000,000 g/mol.]

Flow rate: 1.1 mL/min

Injection size: 200 µL²

System suitability

Sample: *Standard solutions*

Suitability requirements

Resolution: NLT 1.7 between the polystyrene standards

Analysis

Samples: *Standard solutions* and *Sample solution*
Separately inject equal volumes of the *Standard solutions* and the *Sample solution* into the chromatograph, record the chromatograms, and determine the elution peak maxima and the corresponding retention volumes for the 10 polystyrene standards.

Universal calibration: Analyze each polystyrene standard, and use a data handling system or a suitable gel permeation chromatography or size exclusion chromatography (GPC/SEC) software to compute the data and calibration. Construct the *Universal calibration curve* as follows, and use it in the section *Data analysis for sample*.

Plot $\log([\eta] \times M_r)$ for each polystyrene standard in the *Standard solutions* versus its retention volume, V , in mL, at each standard peak maximum; and construct the best cubic line fitting the 10 points. In this expression, M_r is the molecular weight, in g/mol, of polystyrene standard; and $[\eta]$ is the intrinsic viscosity of a polymer and is related to polymer molecular weight (M_r), especially viscosity-average molecular weight, M_v , by the following Mark-Houwink equation:

$$[\eta] = K \times M_v^a$$

K = constant for a given polymer/solvent system at a specified temperature

a = constant for a given polymer/solvent system at a specified temperature

For polystyrene in THF at 25°, $K = 0.0128$ mL/g, and $a = 0.712$

For polyvinyl acetate in THF at 25°, $K = 0.025$ mL/g, and $a = 0.63$

Based on the Mark-Houwink equation and the fact that M_r can represent the molecular weight (M_r), the following equation is given:

$$\log([\eta] \times M_r) = \log K + (a + 1) \log(M_r)$$

M_r can be obtained as M_v , a viscosity-average molecular weight of polystyrene standard.

Data analysis for sample: Analyze the polyvinyl acetate sample by identifying retention volumes V_a and V_b , corresponding to the beginning and end of the polyvinyl acetate chromatogram. The baseline between V_a and V_b is assumed to be linear. [NOTE—Draw a straight line between V_a and V_b .] Data analysis is based on a suitable GPC/SEC computer software or a real-time data acquisition system with either offline or online data processing that is able to provide a means of determining chromatographic peak heights or integrated area segments at prescribed intervals under the SEC chromatogram and a means of handling and reporting the data. The following describes the data processes, which can be computed either by the GPC/SEC software or by an equivalent data processing system. Upon acquisition, handle the data under the polyvinyl acetate elution peak in discrete segments A_i , integrated area slices, or as digitized chromatogram heights H_i , by recording the vertical displacements between the chromatogram trace and the baseline at retention volume, V_i , over designated intervals. A minimum of 40 area segments or heights is required. Obtain the corresponding molecular weight value M_i for Polyvinyl Acetate at its retention volume, V_i , from the *Universal calibration curve* obtained in the section *Universal calibration*, since the constants K and a for Polyvinyl Acetate are known and given above.

Calculate the number-, weight-, and viscosity-average molecular weights, M_n , M_w , and M_v , respectively, in g/mol, of polyvinyl acetate, using the following formula:

$$M_n = \frac{\sum_{i=1}^N A_i}{\sum_{i=1}^N \left(\frac{A_i}{M_i} \right)}$$

$$M_w = \frac{\sum_{i=1}^N (A_i \cdot M_i)}{\sum_{i=1}^N A_i}$$

¹ Narrow polystyrene standards are available from polymer laboratories, such as EasiCal, or are available as various individual polystyrene standards.

² A sample loop of 400 µL and a syringe of 250 µL were used in the *Analysis*.

$$M_v = \left[\frac{\sum_{i=1}^N (A_i \cdot M_i^a)}{\sum_{i=1}^N A_i} \right]^{1/a}$$

If the retention volume internal ΔV_i (for instance, $V_2 - V_1 = V_3 - V_2$, etc.) is constant, parameters A_i and M_i are the chromatographic peak slice area and the Polyvinyl Acetate molecular weight associated with the retention volume, V_i ; and N is the number of data points obtained from the chromatogram between V_a and V_b ($N \geq 40$). [NOTE—If N is sufficiently large, the use of area segments A_i or peak heights H_i will yield equivalent results.]

Calculate the molecular weight distribution or polydispersity for Polyvinyl Acetate:

$$\text{Result} = M_w/M_n$$

Acceptance criteria: The values of weight-average molecular weight and polydispersity are, respectively, NLT 85% and NMT 115% of their respective values as stated on the label.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirement specified.
- **LABELING:** Label it to indicate its weight-average molecular weight, M_w , and polydispersity (M_w/M_n).
- **USP REFERENCE STANDARDS** <11>
USP Polyvinyl Acetate RS

Polyvinyl Acetate Dispersion

DEFINITION

Dispersion of polyvinyl acetate in water. It contains 25.0% to 30.0% of polyvinyl acetate. It may contain suitable surface active agents and stabilizers.

IDENTIFICATION

- **A.** Place one drop of Dispersion on a glass plate and allow to dry. A clear and homogeneous film is formed.
- **B. INFRARED ABSORPTION**

Analysis: Place one drop of the Dispersion on a glass plate, and cover the test substance with a water-resistant crystal disk (silver chloride or KRS-5¹). Gently press on, and then remove the crystal disk. Dry the crystal disk in a drying chamber until a homogeneous film is formed.

Acceptance criteria: The IR absorption spectrum of the film so formed exhibits maxima corresponding to the same wavelengths as those of a similar preparation of USP Polyvinyl Acetate Dispersion RS treated in the same manner.

ASSAY

PROCEDURE

Sample: 1.5 g of Dispersion

Analysis: Transfer the *Sample* to a 250-mL borosilicate glass flask fitted with a reflux condenser. Add 25.0 mL of 0.5 M alcoholic potassium hydroxide and a few glass beads. Attach the condenser and heat under reflux for 30 min. Add 1 mL of phenolphthalein TS, and titrate immediately (while still hot) with 0.5 N hydrochloric acid VS. Perform a blank determination under the same conditions (see *Titrimetry* <541>, *Residual Titrations*.)

¹ KRS-5 consists of 42% thallium(I) bromide and 58% thallium(I) iodine by molecular weight. Suitable disks of silver chloride and of KRS-5 are available from www.photonic.saint-gobain.com, www.almazoptics.com, and www.internationalcrystal.net.

Calculate the saponification value, I_s , by the formula:

$$\text{Result} = [M_{r1} \times (V_B - V_T)] \times N/W$$

- M_{r1} = molecular weight of potassium hydroxide, 56.11
 V_B = volume of 0.5 N hydrochloric acid consumed in the blank test (mL)
 V_T = volume of 0.5 N hydrochloric acid consumed in the actual test (mL)
 N = exact normality of the hydrochloric acid
 W = weight of the Dispersion taken for the test (g)
 Calculate the percentage content of polyvinyl acetate using the following formula:

$$\text{Result} = F \times (M_{r2} \times I_s/M_{r1}) \times 100$$

- F = factor converting mg to g, 10^{-3} g/mg
 M_{r2} = molecular weight of vinyl acetate, 86.09
 M_{r1} = molecular weight of potassium hydroxide, 56.11

Acceptance criteria: The content of polyvinyl acetate is 25.0%–30.0%.

OTHER COMPONENTS

Stabilizers or Surface Active Agents

POVIDONE

[NOTE—Perform this test only if the Dispersion contains povidone.]

Sample: 0.25 g

Analysis: Perform nitrogen determination by sulfuric acid digestion on the *Sample* as directed in *Nitrogen Determination* <461>, *Method II*.

Calculate the percentage content of povidone using the following formula:

$$\text{Result} = N/0.126$$

- N = percentage content of nitrogen
 0.126 = percentage content, expressed as a decimal number, of nitrogen in vinylpyrrolidone

Acceptance criteria: The content of povidone is NMT 4.0%.

IMPURITIES

Inorganic Impurities

RESIDUE ON IGNITION <281>

Sample: 1.0 g of Dispersion

Analysis: Heat a silica crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Evenly distribute the *Sample* in the crucible and weigh. Dry the crucible at 100° to 105° for 1 h and ignite in a muffle furnace at 600° ± 25°, until the test substance is thoroughly charred. Continue the experiment as directed under *Residue on Ignition* <281> on the residue obtained, beginning with "Moisten the sample with a small amount (usually 1 mL) of sulfuric acid..."

Acceptance criteria: NMT 0.5%

Organic Impurities

PROCEDURE 1: LIMIT OF VINYL ACETATE

Solution A: Acetonitrile, methanol, and water (5:5:90)

Solution B: Acetonitrile, methanol, and water (45:5:50)

Mobile phase: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
40	85	15
42	0	100
48	0	100
51	100	0

Standard solution: Transfer 50 mg of vinyl acetate to a 100-mL volumetric flask, dissolve in and dilute with

methanol to volume, and mix well. Dilute 5.0 mL of the solution with *Solution A* to 100 mL. Dilute 10.0 mL of this solution with *Solution A* to 100 mL. The *Standard solution* contains about 2.5 µg/mL of vinyl acetate. [NOTE—This solution should be analyzed within 1 h when stored at room temperature.]

System suitability solution: Transfer 50 mg of vinyl acetate and 50 mg of 1-vinylpyrrolidin-2-one to a 50-mL volumetric flask, add 10 mL of methanol, sonicate or gently shake the flask to dissolve the materials. Dilute with *Solution A* to volume. Dilute 10 mL of this solution with *Solution A* to 100 mL. Dilute 5 mL of this solution with *Solution A* to 100 mL. The *System suitability solution* contains about 5 µg/mL each of vinyl acetate and 1-vinylpyrrolidin-2-one.

Sample solution: Transfer 250 mg of the Dispersion to a 10-mL volumetric flask, add about 4 mL of methanol, and sonicate. After cooling to ambient temperature, dilute with water to volume, and mix. Centrifuge at $4000 \times g$ for 10 min, and pass through a 0.2-µm membrane filter. [NOTE—This solution should be analyzed within 1 h when stored at room temperature.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Columns

Analytical column: 4.0-mm \times 25-cm; 5-µm packing L1

Pre-column: 4.0-mm \times 3-cm; 5-µm packing L1 may be used if a matrix effect is observed.

[NOTE—The matrix effect may result in poor reproducibility of the retention times and of the peak shapes.]

Column temperature: 30°

Flow rate: 1 mL/min

Injection size: 10 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for vinyl acetate and 1-vinylpyrrolidin-2-one are 1.0 and 1.2, respectively.]

Suitability requirements

Resolution: NLT 5.0 between vinyl acetate and 1-vinylpyrrolidin-2-one

Relative standard deviation: NMT 5.0% determined from the 1-vinylpyrrolidin-2-one peak

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria: The response of the vinyl acetate peak from the *Sample solution* is NMT that of the vinyl acetate peak from the *Standard solution*, corresponding to NMT 100 ppm of vinyl acetate.

• PROCEDURE 2: LIMIT OF ACETIC ACID/ACETATE

Mobile phase: 5 mM sulfuric acid

Standard solution: 0.3 mg/mL for each of acetic acid and citric acid in *Mobile phase*

Sample solution: Transfer 200 mg of the Dispersion to a 10-mL volumetric flask, add about 8 mL of water, and sonicate for about 10 min. Cool to ambient temperature, and dilute with water to volume.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: UV 205 nm

Column: 4.6-mm \times 25-cm; 5-µm packing L1

Column clean: After each injection, rinse the column with a mixture of equal volumes of *Mobile phase* and acetonitrile.

Column temperature: Ambient

Flow rate: 1 mL/min

Injection size: 20 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for acetic acid and citric acid are 1.0 and 1.2, respectively.]

Suitability requirements

Resolution: NLT 2.0 between acetic acid and citric acid

Relative standard deviation: NMT 5.0% determined from the acetic acid peak

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria: The response of the acetic acid peak from the *Sample solution* is NMT that of the acetic acid peak from the *Standard solution*, corresponding to NMT 1.5% of acetic acid.

SPECIFIC TESTS

• MICROBIAL ENUMERATION TESTS <61> and TESTS FOR SPECIFIED MICROORGANISMS <62>:

The total aerobic microbial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g

• PH <791>:

3.0–5.5

• LOSS ON DRYING <731>:

Dry 1.0 g of the Dispersion at 110° for 5 h: it loses between 68.5% and 71.5% of its weight.

• COAGULUM CONTENT

Sample: 100 g of Dispersion

Analysis: Accurately weigh a stainless steel sieve having 45-µm openings or a suitable single-woven wire cloth with a mesh width of 45 µm, and filter the *Sample* through it. [NOTE—Suitable single-woven wire cloth mesh meets the requirements set in ISO 9044.] Wash the sieve or the cloth with distilled water until a clear filtrate is obtained, and dry the sieve or the cloth to constant weight at 100°–105°.

Acceptance criteria: The weight of the residue does not exceed 500 mg (0.5%).

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers at a temperature below 25°. Protect from freezing.

• **LABELING:** Label it to indicate the names and quantities of any added surface active agents and stabilizers.

• USP REFERENCE STANDARDS <11>

USP Polyvinyl Acetate Dispersion RS

Polyvinyl Acetate Phthalate

DEFINITION

Polyvinyl Acetate Phthalate is a reaction product of phthalic anhydride and a partially hydrolyzed polyvinyl acetate. It contains NLT 55.0% and NMT 62.0% of phthalyl (o-carboxybenzoyl, C₈H₅O₃) groups, calculated on the anhydrous, acid-free basis.

IDENTIFICATION

• **A.** The *Sample solution* in the Assay exhibits a maximum at 277 ± 3 nm.

• B.

Sample: 10 mg

Analysis: Place the *Sample* in a small test tube, add 10 mg of resorcinol, and mix. Add 0.5 mL of sulfuric acid, and heat in a liquid bath at 160° for 3 min. Cool, and pour the solution into a mixture of 25 mL of 1 N sodium hydroxide and 200 mL of water.

Acceptance criteria: The solution shows a vivid green fluorescence.

• **C.**

Sample solution: 100 mg/mL of Polyvinyl Acetate Phthalate in methanol

Analysis: Pour 1 mL of the *Sample solution* onto a clear glass plate.

Acceptance criteria: A film is deposited as the methanol evaporates.

ASSAY• **PHTHALYL CONTENT**

Standard solution: 0.05 mg/mL of phthalic anhydride in alcohol, prepared as follows. Transfer 50 mg of phthalic anhydride to a 1000-mL volumetric flask. Dissolve with heat in 100 mL of alcohol, dilute with alcohol to volume, and mix.

Sample solution: 0.1 mg/mL of Polyvinyl Acetate Phthalate in alcohol, prepared as follows. Transfer 100 mg of Polyvinyl Acetate Phthalate to a 1000-mL volumetric flask, dissolve in alcohol, and dilute with alcohol to volume.

Instrumental conditions

Mode: UV

Cell: 1 cm

Analytical wavelength: 275 nm

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate, on the acid-free basis, the percentage of phthalyl taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

C_S = concentration of phthalic anhydride in the *Standard solution* (mg/mL)

C_U = concentration of Polyvinyl Acetate Phthalate in the *Sample solution* (mg/mL)

Acceptance criteria: 55.0%–62.0% of phthalyl (o-carboxybenzoyl, $C_8H_5O_3$) groups on the anhydrous, acid-free basis

IMPURITIES• **RESIDUE ON IGNITION** (281): NMT 1.0%• **FREE PHTHALIC ACID**

Standard solution: 0.05 mg/mL of phthalic anhydride

Sample solution: 6 mg/mL of polyvinyl acetate in water prepared as follows. Dissolve 1500 mg of Polyvinyl Acetate Phthalate in 50 mL of a mixture of methylene chloride and methanol (4:1). Transfer the solution to a separator with the aid of 75 mL of water, and swirl, taking care not to shake. Add 100 mL of hexanes, shake, and allow the mixture to stand until it separates into two layers. Transfer the water layer to a 250-mL volumetric flask. Add 100 mL of water to the separator, shake, and allow to stand until the layers separate. Transfer the water layer to the same volumetric flask, and dilute with water to volume. If the solution is cloudy, centrifuge a portion until clear.

Instrumental conditions

Mode: UV

Cell: 1 cm

Analytical wavelength: 277 nm

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of free phthalic acid in the portion taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times (M_{r1}/M_{r2}) \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

C_S = concentration of phthalic anhydride in the *Standard solution* (mg/mL)

C_U = concentration of Polyvinyl Acetate Phthalate in the *Sample solution* (mg/mL)

M_{r1} = molecular weight of phthalic acid, 166.13

M_{r2} = molecular weight of phthalic anhydride, 148.12

Acceptance criteria: NMT 0.6% on the anhydrous basis

• **FREE ACID OTHER THAN PHTHALIC**

Sample solution: Proceed as directed for *Sample solution* in *Impurities, Free Phthalic Acid*, but instead of transferring the water extracts to a volumetric flask, transfer them to a 400-mL beaker.

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: Titrate the *Sample solution* with *Titrant* to a phenolphthalein endpoint.

Calculate the volume, V_p , in mL, of 0.1 N sodium hydroxide consumed by the free phthalic acid in the sample taken:

$$V_p = (1/M_r) \times (1/N) \times P \times W$$

M_r = equivalent weight of phthalic acid, 83.065 mg/mEq

N = actual normality of the *Titrant* (mEq/mL)

P = percentage of free phthalic acid, previously determined, in decimal form

W = sample weight of Polyvinyl Acetate Phthalate on the anhydrous basis (mg)

Calculate the percentage of free acid other than phthalic, as acetic acid, in the portion taken:

$$\text{Result} = [(V - V_p) \times M_{r3} \times N]/W \times 100$$

V = total volume of 0.1 N sodium hydroxide used (mL)

M_{r3} = equivalent weight of acetic acid, 60.05 mg/mEq

N = actual normality of the *Titrant*

W = sample weight on the anhydrous basis (mg)

Acceptance criteria: NMT 0.6% on the anhydrous basis

SPECIFIC TESTS• **VISCOSITY—CAPILLARY VISCOMETER METHODS** (911)

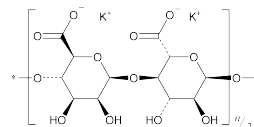
Sample solution: Dissolve a quantity, equivalent to 15 g on the anhydrous basis, in 85 g of methanol.

Analysis: Determine the viscosity of the *Sample solution*, using a capillary viscometer at $25 \pm 0.2^\circ$.

Acceptance criteria: The apparent viscosity is 7–11 mPa · s (centipoises).

• **WATER DETERMINATION, Method I** (921): NMT 5.0%**ADDITIONAL REQUIREMENTS**• **PACKAGING AND STORAGE:** Preserve in tight containers.

Polyvinyl Alcohol—see *Polyvinyl Alcohol General Monographs*

Potassium Alginate

Alginic acid, potassium salt;
Potassium alginate [9005-36-1].

DEFINITION

Potassium Alginate is the purified carbohydrate product extracted from various species of brown seaweeds by the use of dilute alkali. It consists chiefly of the potassium salt of Alginic Acid, a linear glycuronoglycan consisting of β -1,4-linked D-mannuronic acid and L-guluronic acid units in the pyranose ring form. It yields NLT 16.5% and NMT 19.5% of carbon dioxide (CO₂), equivalent to NLT 89.2% and NMT 105.5% of potassium alginate, calculated on the dried basis.

IDENTIFICATION

- **A.**
Analysis: To 5 mL of a 1-in-100 solution in 0.1 N sodium hydroxide add 1 mL of calcium chloride TS.
Acceptance criteria: A voluminous, gelatinous precipitate is formed.
- **B.**
Analysis: To 10 mL of a 1-in-100 solution in 0.1 N sodium hydroxide add 1 mL of 2 N sulfuric acid.
Acceptance criteria: A heavy, gelatinous precipitate is formed.
- **C.**
Analysis: To 5 mg in a test tube add 5 mL of water, 1 mL of a freshly prepared 1-in-100 solution of 1,3-naphthalenediol in alcohol, and 5 mL of hydrochloric acid. Heat the mixture to boiling, boil gently for 3 min, then cool to 15°. Transfer the contents of the test tube to a 30-mL separator with the aid of 5 mL of water, and extract with 15 mL of isopropyl ether.
Acceptance criteria: The isopropyl ether extract exhibits a deeper purplish hue than that from a blank, similarly prepared.
- **D. IDENTIFICATION TESTS—GENERAL, Potassium <191>**
Analysis: Ignite completely 0.2 g at as low a temperature as possible.
Acceptance criteria: A solution of the residue meets the requirements of the tests.

ASSAY

- **ALGINATES ASSAY <311>**: 16.5%–19.5% of CO₂, equivalent to 89.2%–105.5% of potassium alginate on the dried basis

IMPURITIES

- **ARSENIC, Method II <211>**: NMT 1.5 ppm
- **LEAD <251>**
Standard solution: 5 mL of *Diluted standard lead solution*
Test Preparation: Add 1.0 g to 20 mL of nitric acid in a 250-mL conical flask, mix, and heat carefully until the Potassium Alginate is dissolved. Continue the heating until the volume is reduced to 7 mL. Cool rapidly to room temperature, transfer to a 100-mL volumetric flask, and dilute with water to volume. Use a 50-mL sample of the *Test Preparation*.
Analysis: Proceed as directed in the chapter using 15 mL of ammonium citrate solution, 3 mL of potassium cyanide solution, and 0.5 mL of hydroxylamine hydrochloride solution being used for the test. After the first dithizone extractions, wash the combined chloroform layers with 5 mL of water, discarding the water layer and continuing in the usual manner by extracting with 20 mL of 0.2 N nitric acid.
Acceptance criteria: 50.0 mL portion of the *Test preparation* contains NMT 5 µg of lead (corresponding to NMT 10 ppm of lead)
- **HEAVY METALS, Method II <231>**
Analysis: Conduct the ignition in a platinum crucible, and use nitric acid in place of sulfuric acid to wet the sample.

Acceptance criteria: NMT 40 ppm

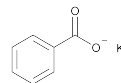
SPECIFIC TESTS

- **ARTICLES OF BOTANICAL ORIGIN, Total Ash <561>**
Analysis: Proceed as directed for *Total Ash* under *Methods of Analysis*, carefully igniting 3 g in a tared platinum dish, until the residue is thoroughly carbonized (5 min), and then igniting in a muffle furnace at a temperature of 800 ± 25° until the carbon is completely burned off (approximately 75 min).
Acceptance criteria: 24.0%–32.0% of ash is found, calculated on the as-is basis.
- **MICROBIAL ENUMERATION TESTS <61> and TESTS FOR SPECIFIED MICROORGANISMS <62>**: The total aerobic microbial count does not exceed 10³ cfu/g, and the total combined molds and yeasts count does not exceed 10² cfu/g.
- **LOSS ON DRYING <731>**: Dry a sample at 105° for 4 h: it loses NMT 15% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Potassium Benzoate



C₇H₅KO₂ 160.21
 Benzoic acid, potassium salt;
 Potassium benzoate [582-25-2].

DEFINITION

Potassium Benzoate contains NLT 99.0% and NMT 100.5% of potassium benzoate (C₇H₅KO₂), calculated on the anhydrous basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Potassium <191> and Benzoate <191>**: Meets the requirements for the flame test for *Potassium*; meets the requirements for *Benzoate*

ASSAY

- **PROCEDURE**
Sample: 600 mg of Potassium Benzoate
Blank: 100 mL of glacial acetic acid
Titrimetric system
 (See *Titrimetry* <541>.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Endpoint detection: Visual
Analysis: Transfer the *Sample* to a 250-mL beaker. Add 100 mL of glacial acetic acid, stir until the *Sample* is completely dissolved, and add 2 drops of crystal violet TS. Titrate with *Titrant*. Perform a blank determination. Calculate the percentage of potassium benzoate (C₇H₅KO₂) in the portion of the *Sample* taken:

$$\text{Result} = \{(V_s - V_b) \times N \times F\} / W \times 100$$

V_s = volume of *Titrant* consumed by the *Sample* (mL)

V_b = volume of *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 160.2 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5% on the anhydrous basis

IMPURITIES• **HEAVY METALS** (231)

Test preparation: Dilute 4.0 g in 40 mL of water. Add, dropwise, with vigorous stirring, 10 mL of 3 N hydrochloric acid, and filter. Use 25 mL of the filtrate.

Acceptance criteria: NMT 10 ppm

SPECIFIC TESTS• **ALKALINITY**

Sample: 2 g

Analysis: Dissolve the *Sample* in 20 mL in hot water, and add 2 drops of phenolphthalein TS.

Acceptance criteria: If a pink color is produced, it is discharged by the addition of 0.20 mL of 0.10 N sulfuric acid.

• **WATER DETERMINATION, Method I** (921): NMT 1.5%**ADDITIONAL REQUIREMENTS**• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Potassium Carbonate—see *Potassium Carbonate General Monographs*

Potassium Chloride—see *Potassium Chloride General Monographs*

Potassium Citrate—see *Potassium Citrate General Monographs*

Potassium Hydroxide

KOH 56.11
Potassium hydroxide [1310-58-3].

DEFINITION

Potassium Hydroxide contains NLT 85.0% of total alkali, calculated as KOH, including NMT 3.5% of K_2CO_3 .

[**CAUTION**—Exercise great care in handling Potassium Hydroxide, because it rapidly destroys tissues.]

IDENTIFICATION• **A. IDENTIFICATION TESTS—GENERAL, Potassium (191):** A solution (1 in 25) meets the requirements.**ASSAY**• **PROCEDURE**

Sample: 1.5 g of Potassium Hydroxide

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 1 N sulfuric acid VS

Endpoint detection: Colorimetric

Analysis: Dissolve the *Sample* in 40 mL of carbon dioxide-free water. Cool the solution to 15°, and add phenolphthalein TS. Titrate with 1 N sulfuric acid VS. At the discharge of the pink color of the indicator, record the volume of acid solution required, then add methyl orange TS, and continue the titration to a persistent pink color. Each mL of 1 N sulfuric acid is equivalent to 56.11 mg of total alkali, calculated as KOH, and each mL of acid consumed in the titration with methyl orange is equivalent to 138.2 mg of K_2CO_3 .

Acceptance criteria: NLT 85.0% of total alkali, calculated as KOH, including NMT 3.5% of K_2CO_3

IMPURITIES• **HEAVY METALS** (231)

Test preparation: 0.67 g of Potassium Hydroxide in a mixture of 5 mL of water and 7 mL of 3 N hydrochloric acid. Heat to boiling, cool, and dilute with water to 25 mL.

Acceptance criteria: NMT 30 ppm

SPECIFIC TESTS• **INSOLUBLE SUBSTANCES**

Sample solution: 1 g of Potassium Hydroxide in 20 mL of water

Acceptance criteria: The *Sample solution* is complete, clear, and colorless.

ADDITIONAL REQUIREMENTS• **PACKAGING AND STORAGE:** Preserve in tight containers.**Potassium Metabisulfite**

$K_2S_2O_5$ 222.32
Disulfurous acid, dipotassium salt;
Dipotassium pyrosulfite [16731-55-8].

DEFINITION

Potassium Metabisulfite contains an amount of $K_2S_2O_5$ equivalent to NLT 51.8% and NMT 57.6% of SO_2 .

IDENTIFICATION• **A. IDENTIFICATION TESTS—GENERAL, Potassium (191) and Sulfite (191):** A solution (1 in 20) meets the requirements.**ASSAY**• **PROCEDURE**

Sample: 250 mg of Potassium Metabisulfite

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 0.1 N iodine VS

Back titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a glass-stoppered conical flask containing 50.0 mL of 0.1 N iodine VS, and swirl to dissolve. Allow to stand for 5 min, protected from light, and then add 1 mL of hydrochloric acid. Titrate the excess iodine with 0.1 N sodium thiosulfate VS, adding 3 mL of starch TS as the endpoint is approached.

Calculate the percentage of SO_2 in the portion of *Sample* taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times F] / W\} \times 100$$

V_B = Back titrant volume consumed by the Blank (mL)

V_S = Back titrant volume consumed by the Sample (mL)

N = Back titrant actual normality (mEq/mL)

F = equivalency factor, 32.03 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 51.8%–57.6% of SO_2

IMPURITIES• **IRON** (241)

Test preparation: Dissolve 1.00 g in 14 mL of dilute hydrochloric acid (2 in 7), and evaporate on a steam bath to dryness. Dissolve the residue in 7 mL of dilute hydrochloric acid (2 in 7), and again evaporate to dryness. Dissolve the resulting residue in a mixture of 2 mL of hydrochloric acid and 20 mL of water. Add 3 drops of bromine TS, and boil to expel the bromine. Cool, and dilute with water to 47 mL.

- Acceptance criteria: NMT 10 ppm
- **HEAVY METALS, Method I (231)**
Test preparation: Dilute 2 g in 20 mL of water, add 5 mL of hydrochloric acid, and evaporate on a steam bath to 1 mL. Dissolve the residue in 25 mL of water.
Acceptance criteria: NMT 10 ppm

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-fitted, tight containers, and avoid exposure to excessive heat.

Potassium Metaphosphate

KPO₃ 118.07
 Metaphosphoric acid (HPO₃), potassium salt;
 Potassium metaphosphate [7790-53-6].

DEFINITION

Potassium Metaphosphate is a straight-chain polyphosphate, having a high degree of polymerization. It contains the equivalent of NLT 59.0% and NMT 61.0% of P₂O₅.

IDENTIFICATION

- **A.**
Sample: 1 g, finely powdered
Analysis: Add the *Sample*, slowly and with vigorous stirring, to 100 mL of sodium chloride solution (20 mg/mL).
Acceptance criteria: A gelatinous mass is formed.
- **B. IDENTIFICATION TESTS—GENERAL, Potassium (191)**
Sample: 0.5 g
Analysis: Boil a mixture of the *Sample*, 10 mL of nitric acid, and 50 mL of water for 30 min, and cool.
Acceptance criteria: The resulting solution meets the requirements of the test.
- **C. IDENTIFICATION TESTS—GENERAL, Phosphate (191)**
Sample: 0.5 g
Analysis: Boil a mixture of the *Sample*, 10 mL of nitric acid, and 50 mL of water for 30 min, and cool.
Acceptance criteria: The resulting solution meets the requirements of the test.

ASSAY

- **PROCEDURE**
Sample: 200 mg
Titrimetric system
 (See *Titrimetry* (541).)
Mode: Residual titration
Titrant: 1 N sodium hydroxide VS
Back-titrant: 1 N sulfuric acid VS
Endpoint detection: Visual
Analysis: Mix the *Sample* with 15 mL of nitric acid and 30 mL of water. Boil for 30 min, cool, and dilute with water to 100 mL. Heat to 60°, add an excess of ammonium molybdate TS, and heat at 50° for 30 min. Filter, and wash the precipitate, first with 0.5 N nitric acid, and then with 10 mg/mL of potassium nitrate until the filtrate is no longer acid to litmus. Add 25 mL of water to the precipitate, dissolve it in 50.0 mL of *Titrant*, and add phenolphthalein TS. Titrate the excess sodium hydroxide with *Back-titrant*. Each mL of *Titrant* is equivalent to 3.086 mg of P₂O₅.
Acceptance criteria: 59.0%–61.0%

IMPURITIES

- **LEAD (251)**
Sample solution: 1 g of Potassium Metaphosphate in 10 mL of 3 N hydrochloric acid
Acceptance criteria: The *Sample solution* contains NMT 5 µg of lead (corresponding to NMT 5 ppm of Pb).

- **HEAVY METALS, Method I (231)**

Sample: Warm 1 g of Potassium Metaphosphate with 10 mL of 3 N hydrochloric acid until no more dissolves. Add 15 mL of water, mix and filter.

Acceptance criteria: NMT 20 ppm

- **LIMIT OF FLUORIDE**

Control: Water

Sample solution: Place 5.0 g of Potassium Metaphosphate, 25 mL of water, 50 mL of perchloric acid, 5 drops of 500 mg/mL silver nitrate, and a few glass beads in a 250-mL distilling flask connected with a condenser and carrying a thermometer and a capillary tube, both of which extend into the liquid. Connect a small dropping funnel, filled with water, or a steam generator to the capillary tube. Support the flask on a distillation shield with a hole that exposes one-third of the bottom of the flask to the flame. Distill into a 250-mL volumetric flask until the temperature reaches 135°. Add water from the funnel or introduce steam through the capillary to maintain the temperature between 135° and 140°. Continue the distillation until 225–240 mL has been collected, then dilute with water to volume.

Analysis: Transfer 50.0 mL of the *Sample solution* to a 100-mL color-comparison tube, and transfer 50.0 mL of the *Control* to a similar tube. Add to each tube 0.1 mL of a filtered solution of sodium alizarin-sulfonate TS and 1 mL of freshly prepared 0.25 mg/mL hydroxylamine hydrochloride. Add, dropwise and with stirring, 0.05 N sodium hydroxide to the tube containing the distillate until its color just matches that of the *Control*, which is faintly pink. Then add to each tube 1.0 mL of 0.1 N hydrochloric acid. From a buret, graduated in 0.05-mL increments, add slowly to the tube containing the distillate, enough 0.25-mg/mL thorium nitrate solution so that, after mixing, the color of the liquid just changes to a faint pink. Note the volume of the solution added, add the same volume to the *Control*, and mix. Then add sodium fluoride TS (10 µg/mL of F) to the *Control* from a buret to make the colors of the two tubes match after dilution to the same volume. Mix, and allow all air bubbles to escape before making the final color comparison. Check the endpoint by adding 1 or 2 drops of sodium fluoride TS to the control. A distinct change in color appears.

Acceptance criteria: 10 µg/g; the volume of sodium fluoride TS required for the *Control* is NMT 1.0 mL.

SPECIFIC TESTS

- **VISCOSITY—CAPILLARY VISCOMETER METHODS (911) and ROTATIONAL RHEOMETER METHODS (912)**

Sample solution: Mix 300 mg with 200 mL of 3.5 mg/mL sodium pyrophosphate, using a magnetic stirrer.

Analysis: Determine the viscosity of the clear solution obtained, or of the liquid phase of the mixture obtained after 30 min of continuous stirring.

Acceptance criteria: 6.5–15 centipoises

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Monobasic Potassium Phosphate

KH₂PO₄
 Phosphoric acid, monopotassium salt;
 Monopotassium phosphate [7778-77-0].

136.09

DEFINITION

Monobasic Potassium Phosphate, dried at 105° for 4 h, contains NLT 98.0% and NMT 100.5% of monobasic potassium phosphate (KH₂PO₄).

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Potassium** <191>
Sample solution: 50 mg/mL
Acceptance criteria: Meets the requirements
- **B. IDENTIFICATION TESTS—GENERAL, Phosphate** <191>
Sample solution: 50 mg/mL
Acceptance criteria: Meets the requirements

ASSAY• **PROCEDURE**

Sample solution: Transfer 5 g of Monobasic Potassium Phosphate, previously dried, to a 250-mL beaker. Add 100 mL of water and 5.0 mL of 1 N hydrochloric acid VS, and stir until the assay specimen is completely dissolved.

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 1 N sodium hydroxide VS

Endpoint detection: Potentiometrically

Analysis: Slowly titrate the excess acid in the *Sample solution*, stirring constantly, with *Titrant* to the inflection point occurring at about pH 4 (*V*_{S1}). Continue the titration with *Titrant* until the inflection point occurring at about pH 8.8 is reached (*V*_{S2}).

Calculate the percentage of monobasic potassium phosphate (KH₂PO₄) in the sample taken:

$$\text{Result} = \{[(V_{S2} - V_{S1}) \times N \times F] / W\} \times 100$$

*V*_{S2} = *Titrant* volume consumed by the *Sample solution* to the second inflection point (mL)

*V*_{S1} = *Titrant* volume consumed by the *Sample solution* to the first inflection point (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 0.1361 g/mEq

W = weight of monobasic potassium phosphate taken to prepare the *Sample solution* (g)

Acceptance criteria: 98.0%–100.5% on the previously dried basis

IMPURITIES

- **ARSENIC, Method I** <211>: 3 µg/g
- **LEAD** <251>
Test preparation: 1 g in 20 mL of water
Acceptance criteria: NMT 5 µg/g
- **HEAVY METALS, Method I** <231>
Test preparation: 40 mg/mL
Acceptance criteria: NMT 20 µg/g
- **LIMIT OF FLUORIDE**

[NOTE—Prepare and store all solutions in plastic containers.]

Buffer solution: 294 mg/mL of sodium citrate dihydrate

Standard stock solution: 1.1052 mg/mL of USP Sodium Fluoride RS

Standard solution: Dilute 20.0 mL of *Standard stock solution* and 50.0 mL of *Buffer solution* with water to 100 mL. Each mL of this solution contains 100 µg of fluoride ion.

Sample solution: Transfer 2.0 g of Monobasic Potassium Phosphate to a beaker containing a plastic-coated stirring bar. Add 20 mL of water and 2.0 mL of hydrochloric acid, and stir until dissolved. Add 50.0 mL of *Buffer solution* and sufficient water to make 100 mL.

Electrode system: Use a fluoride-specific ion-indicating electrode and a silver–silver chloride reference electrode connected to a pH meter capable of measuring potentials with a minimum reproducibility of ±0.2 mV (see *pH* <791>).

Analysis

Standard response line: Transfer 50.0 mL of *Buffer solution* and 2.0 mL of hydrochloric acid to a beaker, and add water to make 100 mL. Add a plastic-coated stirring bar, insert the electrodes into the solution, stir for 15 min, and read the potential, in mV. Continue stirring, and at 5-min intervals add 100, 100, 300, and 500 µL of *Standard solution*, reading the potential 5 min after each addition. Plot the logarithms of the cumulative fluoride ion concentrations (0.1, 0.2, 0.5, and 1.0 µg/mL) versus potential, in mV.

Rinse and dry the electrodes, insert them into the *Sample solution*, stir for 5 min, and read the potential in mV. From the measured potential and the *Standard response line* determine the concentration, *C* (in µg/mL), of fluoride ion in the *Sample solution*.

Calculate the content of fluoride in the portion of Monobasic Potassium Phosphate taken:

$$\text{Result} = (V \times C) / W$$

V = volume of the *Sample solution* (mL)

C = concentration of fluoride ion, determined from the *Standard response line*, in the *Sample solution* (µg/mL)

W = weight of Monobasic Potassium Phosphate taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 10 µg/g

SPECIFIC TESTS• **INSOLUBLE SUBSTANCES**

Sample solution: 10 g in 100 mL of hot water

Analysis: Filter the *Sample solution* through a tared filtering crucible, wash the insoluble residue with hot water, and dry at 105° for 2 h.

Acceptance criteria: NMT 20 mg (0.2%)

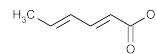
• **LOSS ON DRYING** <731>

Analysis: Dry a sample at 105° for 4 h.

Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** <11>
USP Sodium Fluoride RS

Potassium Sorbate

C₆H₇KO₂ 150.22
2,4-Hexadienoic acid, (*E,E*)-, potassium salt;
2,4-Hexadienoic acid, potassium salt;
Potassium (*E,E*)-sorbate;
Potassium sorbate [590-00-1; 24634-61-5].

DEFINITION

Potassium Sorbate contains NLT 98.0% and NMT 101.0% of C₆H₇KO₂, calculated on the dried basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Potassium** <191>
Sample: Dissolve 1 g of Potassium Sorbate in 10 mL of water.
Acceptance criteria: Meets the requirements
- **B.**
Sample: 0.2 g of Potassium Sorbate
Analysis: Dissolve the *Sample* in 2 mL of water, and add a few drops of bromine TS.

Acceptance criteria: The color is discharged.

ASSAY

PROCEDURE

Sample: 300 mg of Potassium Sorbate

Blank: 40 mL of glacial acetic acid

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 40 mL of glacial acetic acid, warming, if necessary, to dissolve the solution.

Cool to room temperature, and add 1 drop of crystal violet TS. Titrate with *Titrant* to a blue-green endpoint. Perform a blank determination.

Calculate the percentage of potassium sorbate ($C_6H_7KO_2$) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* consumed by the *Sample* (mL)

V_B = *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 150.2 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: 98.0%–101.0% on the dried basis

IMPURITIES

- **HEAVY METALS,** *Method II* <231>: NMT 10 ppm

SPECIFIC TESTS

ACIDITY OR ALKALINITY

Sample solution: 1.1 g of Potassium Sorbate in 20 mL of water

Analysis: Add phenolphthalein TS to the *Sample* solution.

Acceptance criteria: If the solution is colorless, titrate with 0.10 N sodium hydroxide to a pink color that persists for 15 s: NMT 1.1 mL of 0.10 N sodium hydroxide is required. If the solution is pink in color, titrate with 0.10 N hydrochloric acid to discharge the pink color: NMT 0.80 mL of 0.10 N hydrochloric acid is required.

- **LOSS ON DRYING** <731>: Dry a sample at 105° for 3 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light, and avoid exposure to excessive heat.

Povidone—see *Povidone General Monographs*

Propane

C_3H_8 44.10
[74-98-6].

DEFINITION

Propane contains NLT 98.0% of propane (C_3H_8).

[CAUTION]—Propane is highly flammable and explosive.]

IDENTIFICATION

- **A. INFRARED ABSORPTION:** Exhibits maxima, among others, at the following wavelengths, in μm : 3.4 (vs), 6.8 (s), and 7.2 (m).

B.

Sample: Use an empty stainless steel cylinder equipped with a stainless steel valve and having a capacity of NLT 200 mL and a pressure rating of 240 psi or more. Dry the cylinder with the valve open at 110° for 2 h, and evacuate the hot cylinder to less than 1 mm of mercury.

Close the valve, cool, and weigh. Connect one end of a charging line tightly to the propane container and the other end loosely to the empty cylinder. Carefully open the propane container, and allow the propane to flush out the charging line through the loose connection. Avoid excessive flushing, which causes moisture to freeze in the charging line and connections. Tighten the fitting on the empty cylinder, and open the empty cylinder valve, allowing the propane to flow into the evacuated cylinder. Continue sampling until the desired amount of propane is obtained, then close the propane container valve, and finally close the sample cylinder valve. **[CAUTION]**—Do not overload the sample cylinder; hydraulic expansion due to temperature change can cause overloaded cylinders to explode.] Weigh the charged sample cylinder, and determine the the weight.

Analysis: Determine the vapor pressure of the *Sample* at 21° by means of a suitable pressure gauge.

Acceptance criteria: 820–875 kPa absolute (119–127 psia)

ASSAY

PROCEDURE

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Thermal conductivity

Column: 6-m \times 3-mm aluminum; packed with 10 weight percent of liquid phase G30 on nonacid-washed support S1C

Column temperature: 33°

Carrier gas: Helium

Flow rate: 50 mL/min

Injection volume: 2 μL

System suitability

Sample: Propane

Suitability requirements: The peak responses for Propane from duplicate determinations agree within 1%.

Analysis: Connect 1 Propane cylinder to the chromatograph through a suitable sampling valve and a flow control valve downstream from the sampling valve. Flush the liquid specimen through the sampling valve, taking care to avoid entrapment of gas or air in the sampling valve. Calculate the percentage purity by dividing 100 times the propane response by the sum of all of the responses.

Acceptance criteria: NLT 98.0%

SPECIFIC TESTS

HIGH-BOILING RESIDUES

Sample: Use the *Sample* from *Identification* test B.

Analysis: Prepare a cooling coil from copper tubing (about 6-mm outside diameter \times about 6.1-m long) to fit into a vacuum-jacketed flask. Immerse the cooling coil in a mixture of dry ice and acetone in a vacuum-jacketed flask, and connect one end of the tubing to the *Sample*. Carefully open the sample cylinder valve, flush the cooling coil with about 50 mL of the *Sample*, and discard this portion of liquefied sample. Continue delivering liquefied sample from the cooling coil, and collect it in a previously chilled 1000-mL sedimentation cone until the cone is filled to the 1000-mL mark. Allow the sample to evaporate, using a warm water bath maintained at about 40° to reduce evaporating time. When all of the liquid has evaporated, rinse the sedimentation cone with two 50-mL portions of pentane, and combine the rinsings in a tared 150-mL evaporating dish. Transfer 100 mL of the pentane solvent to a

second tared 150-mL evaporating dish, place both evaporating dishes on a water bath, evaporate to dryness, and heat the dishes in an oven at 100° for 60 min. Cool the dishes in a desiccator, and weigh. Repeat the heating for 15-min periods until successive weighings are within 0.1 mg, and calculate the weight of the residue obtained from the *Sample* as the difference between the weights of the residues in the two evaporating dishes.

Acceptance criteria: NMT 5 µg/mL

• **ACIDITY OF RESIDUE**

Sample solution: Add 10 mL of water to the residue obtained in the test for *High-Boiling Residues*, mix by swirling for 30 s, add 2 drops of methyl orange TS, insert the stopper in the tube, and shake vigorously.

Acceptance criteria: No pink or red color appears in the aqueous layer.

• **LIMIT OF SULFUR COMPOUNDS**

Analysis: Carefully open the container valve to produce a moderate flow of gas. Do not direct the gas stream toward the face, but deflect a portion of the stream toward the nose.

Acceptance criteria: The odor is free from the characteristic odor of sulfur compounds.

• **WATER DETERMINATION (921)**

Sample: 100 g of the *Sample* from Identification test B

Analysis: Proceed as directed in the chapter with the following modifications. (a) Provide the closed-system titrating vessel with an opening through which passes a coarse-porosity gas dispersion tube connected to a sampling cylinder. (b) Dilute the *Reagent* with anhydrous methanol to give a water equivalence factor of 0.2–1.0 mg/mL; age this diluted solution for NLT 16 h before standardization. (c) Introduce the *Sample* into the titration vessel through the gas dispersion tube at a rate of about 100 mL/min; if necessary, heat the sample cylinder gently to maintain this flow rate.

Acceptance criteria: NMT 0.001%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight cylinders, and prevent exposure to excessive heat.

Propionic Acid

DEFINITION

Propionic Acid contains NLT 99.5% and NMT 100.5%, by weight, of propionic acid (C₃H₆O₂).

ASSAY

• **PROCEDURE**

Sample: 1.5 g of Propionic Acid

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.5 N sodium hydroxide VS

Endpoint detection: Visual

Analysis: Mix the *Sample* with 100 mL of recently boiled and cooled water in a 250-mL conical flask. Add phenolphthalein TS to the *Sample solution*, and titrate with *Titrant* to the first appearance of a faint pink endpoint that persists for NLT 30 s.

Calculate the percentage of propionic acid (C₃H₆O₂) in the sample taken:

$$\text{Result} = \{[(V_s - V_b) \times N \times F]/W\} \times 100$$

V_s = volume of *Titrant* consumed by the sample (mL)

V_b = volume of *Titrant* consumed by the blank (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 74.08 mg/mEq

W = weight of the sample (mg)

Acceptance criteria: 99.5%–100.5% by weight

IMPURITIES

- **LIMIT OF NONVOLATILE RESIDUE:** Evaporate 20 g of Propionic Acid in a tared dish, and dry at 105° for 1 h: the weight of the residue does not exceed 2.0 mg.

• **HEAVY METALS (231)**

Test preparation: To the residue obtained in the test for *Limit of Nonvolatile Residue* add 8 mL of 0.1 N hydrochloric acid, warm gently until solution is complete, dilute with water to 100 mL, and use 10 mL of the solution.

Acceptance criteria: NMT 10 ppm

• **LIMIT OF ALDEHYDES**

Sample solution: Transfer 10.0 mL of Propionic Acid to a glass-stoppered 250-mL conical flask containing 50 mL of water and 10.0 mL of sodium bisulfite solution (1 in 80), insert the stopper, and shake vigorously. Allow the mixture to stand for 30 min.

Blank: Add 50 mL of water and 10.0 mL of sodium bisulfite solution (1 in 80) to a glass-stoppered 250-mL conical flask, insert the stopper, and shake vigorously. Allow the mixture to stand for 30 min.

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 0.1 N iodine VS

Endpoint detection: Visual

Analysis: Titrate the *Sample solution* and the *Blank* with *Titrant* to the same brownish-yellow endpoint.

Acceptance criteria: The difference between the volume of 0.1 N iodine required for the *Blank* and that required for the *Sample solution* is NMT 1.75 mL.

SPECIFIC TESTS

- **DISTILLING RANGE, Method I (721):** 138.5°–142.5°

- **SPECIFIC GRAVITY (841):** 0.988–0.993

• **READILY OXIDIZABLE SUBSTANCES**

Sample solution: Dissolve 15 g of sodium hydroxide in 50 mL of water. Cool, add 6 mL of bromine, stirring to effect complete solution, and dilute with water to 2000 mL. Transfer 25.0 mL of this solution to a glass-stoppered, 250-mL conical flask containing 100 mL of water, and add 10 mL of sodium acetate solution (1 in 5) and 10.0 mL of Propionic Acid. Allow to stand for 15 min, and add 5 mL of potassium iodide solution (1 in 4) and 10 mL of hydrochloric acid.

Blank: Dissolve 15 g of sodium hydroxide in 50 mL of water. Cool, add 6 mL of bromine, stirring to effect complete solution, and dilute with water to 2000 mL. Transfer 25.0 mL of this solution to a glass-stoppered, 250-mL conical flask containing 100 mL of water, and add 10 mL of sodium acetate solution (1 in 5). Allow to stand for 15 min, and add 5 mL of potassium iodide solution (1 in 4) and 10 mL of hydrochloric acid.

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

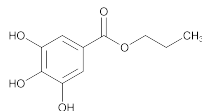
Analysis: Titrate the *Sample solution* and the *Blank* with *Titrant* just to the disappearance of the brown color.

Acceptance criteria: The difference between the volume of 0.1 N sodium thiosulfate required for the *Blank* and that required for the *Sample solution* is NMT 2.2 mL.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Propyl Gallate

C₁₀H₁₂O₅

212.20

Benzoic acid, 3,4,5-trihydroxy-, propyl ester;
Propyl gallate [121-79-9].

DEFINITION

Propyl Gallate contains NLT 98.0% and NMT 102.0% of propyl gallate (C₁₀H₁₂O₅), calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197M>
- **B. ULTRAVIOLET ABSORPTION** <197U>
Sample solution: 10 µg/mL in methanol
Acceptance criteria: Meets the requirements

ASSAY

- **PROCEDURE**
Standard solution: 10 µg/mL of USP Propyl Gallate RS in methanol
Sample solution: 10 µg/mL of Propyl Gallate in methanol
Blank: Methanol
Instrumental conditions
(See *Spectrophotometry and Light-Scattering* <851>.)
Mode: UV
Analytical wavelength: Maximum at about 273 nm
Cell: 1 cm
Analysis
Samples: Standard solution and Sample solution
Calculate the percentage of propyl gallate (C₁₀H₁₂O₅) in the portion of the sample taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the Sample solution

A_S = absorbance of the Standard solution

C_S = concentration of USP Propyl Gallate RS in the Standard solution (µg/mL)

C_U = concentration of the Sample solution (µg/mL)

Acceptance criteria: 98.0%–102.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** <281>: NMT 0.1%
- **HEAVY METALS**, Method II <231>: NMT 10 ppm

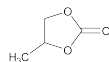
SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** <741>: 146°–150°
- **LOSS ON DRYING** <731>: Dry a sample at 105° for 4 h: it loses NMT 0.5% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight containers, protected from light, and avoid contact with metals.
- **USP REFERENCE STANDARDS** <11>
USP Propyl Gallate RS

Propylene Carbonate

C₄H₆O₃

102.09

4-Methyl-1,3-dioxolan-2-one;

Cyclic propylene carbonate [108-32-7].

DEFINITION

Propylene Carbonate contains NLT 99.0% and NMT 100.5% of propylene carbonate (C₄H₆O₃).

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197F>

ASSAY

• PROCEDURE

Barium hydroxide solution: 75 mg/mL of barium hydroxide (octahydrate). Filter the solution before use.

Sample: 600 mg

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.5 N hydrochloric acid VS

Endpoint detection: Visual

Analysis: Flush a 250-mL iodine flask with nitrogen to expel the air, and insert the stopper in the flask to exclude carbon dioxide. Transfer 50.0 mL of *Barium hydroxide solution* and the *Sample* to the flask, and loosely reinsert the stopper. Moisten the stopper with 3 drops of water, and heat the flask on a steam bath for 10 min. Remove the flask from the steam bath, add 6 drops of phenolphthalein TS, and titrate while hot with *Titrant* until only a trace of pink color remains. Perform a blank determination, using the same *Barium hydroxide solution*. Each mL of 0.5 N hydrochloric acid consumed is equivalent to 25.52 mg of propylene carbonate (C₄H₆O₃).

Acceptance criteria: 99.0%–100.5%

IMPURITIES

- **RESIDUE ON IGNITION** <281>: NMT 0.01%

SPECIFIC TESTS

- **SPECIFIC GRAVITY** <841>: 1.203–1.210 at 20°
- **PH** <791>

Sample solution: Mix 10 mL of propylene carbonate with 0.3 mL of saturated potassium chloride solution in a 100-mL borosilicate volumetric flask. Dilute with carbon dioxide-free water having a pH of 7.0 ± 0.5 to volume.

Analysis: Completely purge the solution by vigorous nitrogen bubbling, and continue the bubbling during the pH measurement. Determine the pH potentiometrically when the reading stabilizes.

Acceptance criteria: 6.0–7.5

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight containers.
- **USP REFERENCE STANDARDS** <11>
USP Propylene Carbonate RS

Propylene Glycol—see *Propylene Glycol*
General Monographs

Propylene Glycol Alginate

DEFINITION

Propylene Glycol Alginate is a propylene glycol ester of alginic acid. Each gram yields NLT 0.16 and NMT 0.20 g of carbon dioxide, calculated on the dried basis.

IDENTIFICATION

• A.

Sample solution: Place 20 mL of the saponified solution obtained in the determination of *Esterified Carboxyl*

Groups in a 250-mL conical flask. Add 50 mL of a solution of periodic acid (1 in 50), swirl, and allow to stand for 30 min. Add 2 g of potassium iodide, titrate with sodium thiosulfate TS to a faint yellow color, dilute the mixture with water to 200 mL, and mix to obtain the *Sample solution* for *Identification* test A and *Identification* test B.

Modified Schiff's reagent: Dissolve 200 mg of rosaniline hydrochloride ($C_{20}H_{20}ClN_3$) in 120 mL of hot water. Cool, add 2 g of sodium bisulfite ($NaHSO_3$), followed by 2 mL of hydrochloric acid, and dilute with water to 200 mL. [NOTE—Store this solution in a brown bottle at 15° or lower.]

Analysis: To 10 mL of the *Sample solution* add 5 mL of hydrochloric acid and 10 mL of *Modified Schiff's reagent*.

Acceptance criteria: A blue to blue-violet color, due to formaldehyde, develops in about 20 min.

• **B.**

Analysis: To 10 mL of the *Sample solution* prepared in *Identification* test A add 1 mL of a saturated solution of piperazine and 0.5 mL of sodium nitroferricyanide TS.

Acceptance criteria: A green color, due to acetaldehyde, develops.

ASSAY

• **CONTENT OF ALGINATE**

Analysis: Proceed as directed for *Procedure* in *Alginates* Assay (311), without preliminary drying of the Propylene Glycol Alginate.

Acceptance criteria: 0.16–0.20 g of carbon dioxide/g of Propylene Glycol Alginate, calculated on the dried basis

OTHER COMPONENTS

• **FREE CARBOXYL GROUPS**

Sample: 1 g

Titrimetric system

Mode: Direct titration

Titrant: 0.1 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Transfer the *Sample* to a 600-mL beaker. Dissolve in 200 mL of water, stirring by mechanical means for NLT 30 min. Titrate with 0.1 N sodium hydroxide VS to a pH of 7.0.

Calculate the weight, in g, of free carboxyl groups in the *Sample* taken:

$$\text{Result} = [(V \times M_r \times N)/W] \times F$$

V = *Titrant* volume consumed (mL)

M_r = mEq of CO_2 , 44 mg/mEq

N = actual normality of the *Titrant* (mEq/mL)

W = *Sample* weight (g)

F = conversion factor, 10^{-3} g/mg

Acceptance criteria: The weight of free carboxyl groups found, calculated on the dried basis, is NMT 35% of the weight of carbon dioxide yielded by an equal weight of specimen in the *Assay*.

• **ESTERIFIED CARBOXYL GROUPS**

Sample solution: The solution obtained in the test for *Free Carboxyl Groups*

Analysis: Transfer the *Sample solution* with the aid of water to a 1000-mL conical flask. Add phenolphthalein TS and 50.0 mL of 0.1 N sodium hydroxide VS, insert a stopper in the flask, mix, and allow to stand for 30 min at ambient temperature. Titrate the excess sodium hydroxide with 0.1 N hydrochloric acid VS to a faint pink endpoint. Transfer the solution with the aid of water to a 600-mL beaker, and complete the titration to a pH of 7.0, determining the endpoint potentiometrically.

Calculate the weight, in g, of esterified carboxyl groups in the weight, W , in g, of the specimen taken:

$$\text{Result} = [(V \times M_r \times N)/W] \times F$$

V = volume of 0.1 N sodium hydroxide consumed (mL)

M_r = mEq of CO_2 , 44 mg/mEq

N = actual normality of 0.1 N sodium hydroxide (mEq/mL)

W = specimen weight (g)

F = conversion factor, 10^{-3} g/mg

Acceptance criteria: The weight of esterified carboxyl groups found, calculated on the dried basis, is 40%–85% of the weight of carbon dioxide yielded by an equal weight of specimen in the *Assay*.

IMPURITIES

• **ARSENIC**, *Method II* (211): 3 ppm

• **HEAVY METALS**, *Method II* (231): NMT 40 ppm, using a platinum crucible for the ignition, and nitric acid being used in place of sulfuric acid to wet the sample specimen

• **LEAD** (251)

Standard solution: 5 mL of *Diluted Standard Lead Solution*

Test preparation: Add 1.0 g to 20 mL of nitric acid in a 250-mL conical flask, mix, and heat carefully until the specimen is dissolved. Continue the heating until the volume is reduced to 7 mL. Cool rapidly to room temperature, transfer to a 100-mL volumetric flask, dilute with water to volume, and mix. Use a 50-mL portion.

Analysis: Proceed as directed in the chapter, using 15 mL of ammonium citrate solution, 3 mL of potassium cyanide solution, and 0.5 mL of hydroxylamine hydrochloride solution for the test. After the first dithizone extractions, wash the combined chloroform layers with 5 mL of water, discarding the water layer and continuing in the usual manner by extracting with 20 mL of 0.2 N nitric acid.

Acceptance criteria: A 50.0-mL portion of this solution contains NMT 5 µg of lead (corresponding to NMT 10 ppm of Pb).

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total bacterial count does not exceed 200 cfu/g, and the tests for *Salmonella* species and *Escherichia coli* are negative.

• **LOSS ON DRYING** (731): Dry a sample at 105° for 4 h: it loses NMT 20.0% of its weight.

• **ASH**

Sample: 3 g

Analysis: Weigh the *Sample* in a tared crucible, and incinerate at $650 \pm 25^\circ$ until free from carbon. Cool in a desiccator, weigh, and determine the weight of the ash.

Acceptance criteria: NMT 10.0% on the dried basis

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Propylene Glycol Dicaprylate/Dicaprate

Decanoic acid, mixed diesters with octanoic acid and propylene glycol [68583-51-7].

DEFINITION

Propylene Glycol Dicaprylate/Dicaprate is a mixture of the propylene glycol mono- and diesters of caprylic acid ($C_8H_{16}O_2$) and capric acid ($C_{10}H_{20}O_2$), the diesters fraction being predominant.

IDENTIFICATION

• **A. INFRARED ABSORPTION** (197F)

• **B. THIN-LAYER CHROMATOGRAPHY IDENTIFICATION TEST** (201)

Standard solution: 50 mg/mL of USP Propylene Glycol Dicaprylate/Dicaprate RS in methylene chloride

Sample solution: 50 mg/mL of Propylene Glycol Dicaprylate/Dicaprate in methylene chloride

Application volume: 10 μ L, as streaks

Developing solvent system: Ether and hexane (7:3)

Spray reagent: 0.1 mg/mL of rhodamine 6G in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatogram over a path of 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The R_f values of the principal spots from the *Sample solution* correspond to those from the *Standard solution*.

- **C.** It meets the requirements of the test for *Fats and Fixed Oils* <401>, *Fatty Acid Composition*.

IMPURITIES

Inorganic Impurities

- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>: NMT 0.1%

ALKALINE IMPURITIES

Sample: 2.0 g of Propylene Glycol Dicaprylate/Dicaprate

Analysis: Dissolve the *Sample* in a mixture of 1.5 mL of alcohol and 3.0 mL of ether. Add 0.05 mL of bromophenol blue TS.

Acceptance criteria: NMT 0.15 mL of 0.01 N hydrochloric acid is required in order to change the color of the indicator to yellow.

SPECIFIC TESTS

- **FATS AND FIXED OILS, Acid Value** <401>: NMT 0.2
- **FATS AND FIXED OILS, Fatty Acid Composition** <401>: Propylene Glycol Dicaprylate/Dicaprate exhibits the composition profile of fatty acids shown in the following table:

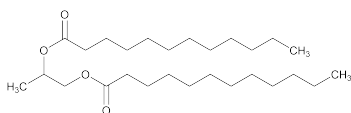
Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤ 2.0
8	0	50.0–80.0
10	0	20.0–50.0
12	0	≤ 3.0
14	0	≤ 1.0

- **FATS AND FIXED OILS, Hydroxyl Value** <401>: NMT 10
- **FATS AND FIXED OILS, Iodine Value** <401>: NMT 1.0
- **FATS AND FIXED OILS, Peroxide Value** <401>: NMT 1.0
- **FATS AND FIXED OILS, Saponification Value** <401>: 320–340
- **FATS AND FIXED OILS, Unsaponifiable Matter** <401>: NMT 0.5%, determined on 5.0 g
- **WATER DETERMINATION, Method Ia** <921>: NMT 0.1%, using a mixture of methanol and methylene chloride (1:1) in place of methanol in the titration vessel

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and protect from moisture. No storage requirements specified.
- **USP REFERENCE STANDARDS** <11>
USP Propylene Glycol Dicaprylate/Dicaprate RS

Propylene Glycol Dilaurate



$C_{27}H_{52}O_4$

Lauric acid, diester with propane-1,2-diol;
Propane-1,2-diyl didodecanoate
[22788-19-8].

440.70

DEFINITION

Propylene Glycol Dilaurate is a mixture of the propylene glycol mono- and diesters of lauric acid. It contains NLT 70.0% of diesters and NMT 30.0% of monoesters.

IDENTIFICATION

A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST <201>

Standard solution: 50 mg/mL of USP Propylene Glycol Dilaurate RS in methylene chloride

Sample solution: 50 mg/mL of Propylene Glycol Dilaurate in methylene chloride

Developing solvent system: Hexane and ether (3:7)

Spray reagent: 0.1 mg/mL of rhodamine 6G in alcohol

Analysis: Apply 200 μ g of the *Standard solution* and *Sample solution*. Develop the chromatogram over a path of 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The R_f values of the principal spots of the *Sample solution* correspond to those of the *Standard solution*.

- **B. FATS AND FIXED OILS, Fatty Acid Composition** <401>:
Meets the requirements

ASSAY

PROCEDURE

Mobile phase: Tetrahydrofuran

Sample solution: 200 mg of Propylene Glycol Dilaurate in 5 mL of tetrahydrofuran

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7-mm \times 60-cm; 5- μ m packing L21 (100Å)

[NOTE—Two 7-mm \times 30-cm L21 columns may be used in place of one 60-cm column, provided system suitability requirements are met.]

Temperature

Column: 40°

Detector: 40°

Flow rate: 1 mL/min

Injection size: 40 μ L

System suitability

Sample: *Sample solution*

[NOTE—Elution order: diesters, monoesters, propylene glycol.]

Suitability requirements

Relative standard deviation: NMT 1.0% is determined from the monoester peak.

Analysis

Sample: *Sample solution*

Calculate the percentage of monoesters or diesters in the portion of Propylene Glycol Dilaurate taken:

$$\text{Result} = (r_U/r_T) \times (100 - D)$$

r_U = peak response for monoesters or diesters

r_T = sum of the responses of the monoester and diester peaks

D = sum of the percentage content of propylene glycol and the percentage content of free fatty acids

Calculate the percentage content of free fatty acids:

$$\text{Result} = (A/561.1) \times 200$$

A = acid value

Acceptance criteria: NLT 70.0% of diesters and NMT 30.0% of monoesters

IMPURITIES**Inorganic Impurities**

- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* (561): NMT 0.1%

Organic Impurities

- **PROCEDURE: LIMIT OF PROPYLENE GLYCOL**

Mobile phase: Proceed as directed in the Assay.

Standard stock solution: 4 mg/mL of USP Propylene Glycol RS in tetrahydrofuran

Standard solutions: Into four 15-mL flasks, introduce respectively 0.25, 0.5, 1.0, and 2.5 mL of *Standard stock solution*, and dilute with tetrahydrofuran to 5.0 mL. In a fifth 15-mL flask, introduce 5.0 mL of *Standard stock solution*.

Sample solution: Use the *Sample solution* from the Assay.

Chromatographic system: Proceed as directed in the Assay.

Analysis

Samples: *Standard solutions* and *Sample solution*

Prepare a standard curve of peak area versus concentration, in mg/mL, of propylene glycol in the *Standard solutions*. Obtain the concentration, *C*, in mg/mL, of propylene glycol in the *Sample solution* from the standard curve.

Acceptance criteria: NMT 2.0% of propylene glycol is found.

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 4
- **FATS AND FIXED OILS**, *Fatty Acid Composition* (401): Propylene Glycol Dilaurate exhibits the following composition profile of fatty acids, determined as directed in the chapter.

Fatty Acids	Carbon-Chain Length	Percentage (%)
Caprylic acid	C8	NMT 0.5
Capric acid	C10	NMT 2.0
Lauric acid	C12	NLT 95.0
Myristic acid	C14	NMT 3.0
Palmitic acid	C16	NMT 1.0

- **FATS AND FIXED OILS**, *Iodine Value* (401): NMT 1
- **FATS AND FIXED OILS**, *Saponification Value* (401): 230–250
- **WATER DETERMINATION**, *Method Ia* (921): NMT 1.0%, using a mixture of methanol and methylene chloride (1:1) in place of methanol in the titration vessel

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and protect from moisture. No storage requirements are specified.
- **USP REFERENCE STANDARDS** (11)
USP Propylene Glycol RS
USP Propylene Glycol Dilaurate RS

Propylene Glycol Monocaprylate

Propylene glycol mono-octanoate;
Octanoic acid, monoester with 1,2-propane diol;
Caprylic acid, monoester with propane-1,2-diol
[31565-12-5].

DEFINITION

Propylene Glycol Monocaprylate is a mixture of the propylene glycol monoesters and diesters of fatty acids composed predominately of caprylic acid. The requirements for monoester and diester content differ for the two types of Propylene Glycol Monocaprylate, as shown in the table below.

	Content of Monoesters (%)		Content of Diesters (%)	
	Min.	Max.	Min.	Max.
Type I	55.0	80.0	20.0	45.0
Type II	90.0	—	—	10.0

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197A)
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** (201)
Standard solution: 50 mg/mL of USP Propylene Glycol Monocaprylate Type I RS or USP Propylene Glycol Monocaprylate Type II RS in methylene chloride
Sample solution: 50 mg/mL in methylene chloride
Chromatographic system
Application volume: 10 µL
Developing solvent system: Ether and hexane (70:30)
Spray reagent: 0.1 mg/mL of rhodamine 6G in alcohol
Analysis: Develop the chromatogram over a path of 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.
Acceptance criteria: The *R_f* values of the principal spots from the *Sample solution* correspond to those from the *Standard solution*.
- **C.** It meets the requirements in *Specific Tests for Fats and Fixed Oils, Fatty Acid Composition* (401).

ASSAY**PROCEDURE**

Mobile phase: Tetrahydrofuran

Sample solution: 40 mg/mL of Propylene Glycol Monocaprylate in tetrahydrofuran

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7-mm × 60-cm; 5-µm packing L21 (100 Å).

[NOTE—Two 7-mm × 30-cm L21 columns may be used in place of one 60-cm column, provided *System suitability* requirements are met.]

Temperatures

Column: 40°

Detector: 40°

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *Sample solution*

[NOTE—The relative retention times with reference to propylene glycol for diesters and monoesters are about 0.85 and 0.90, respectively.]

Suitability requirements

Relative standard deviation: NMT 2.0% for the monoester peak

Analysis

Sample: *Sample solution*

Calculate the percentage of monoesters or diesters in the portion of Propylene Glycol Monocaprylate taken:

$$\text{Result} = (r_u/r_t) \times (100 - D)$$

r_u = peak response for monoesters or diesters

r_t = sum of the peak responses of the monoesters and diesters

D = sum of the percentage content of propylene glycol and the percentage content of free fatty acids

Calculate the percentage content of free fatty acids:

$$\text{Result} = (A/561.1) \times 144$$

A = acid value

Acceptance criteria**Type I**

Monoesters: 55.0%–80.0%

Diesters: 20.0%–45.0%

Type II

Monoesters: NLT 90.0%

Diesters: NMT 10.0%

IMPURITIES

- **HEAVY METALS**, *Method II* <231>: NMT 10 µg/g

- **LIMIT OF PROPYLENE GLYCOL**

Mobile phase, Sample solution, and Chromatographic system: Proceed as directed in the *Assay*.

Standard stock solution: 4 mg/mL of USP Propylene Glycol RS in tetrahydrofuran

Standard solutions: Into four 5-mL volumetric flasks, introduce respectively 0.25, 0.5, 1.0, and 2.5 mL of *Standard stock solution*, and dilute with tetrahydrofuran to volume. In a fifth 5-mL volumetric flask, introduce 5.0 mL of *Standard stock solution*.

Analysis

Samples: *Standard solutions* and *Sample solution*

Prepare a standard curve of peak area versus concentration, in mg/mL, of propylene glycol in the *Standard solutions*. Obtain the concentration of propylene glycol in the *Sample solution* from the standard curve.

Calculate the percentage of free propylene glycol in the portion of Propylene Glycol Monocaprylate taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of propylene glycol in the *Sample solution* from the standard curve

C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria

Propylene glycol monocaprylate (Type I): NMT 1.5%

Propylene glycol monocaprylate (Type II): NMT 1.5%

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Acid Value* <401>: NMT 1.5
- **FATS AND FIXED OILS**, *Fatty Acid Composition* <401>: Propylene Glycol Monocaprylate exhibits the composition profile of fatty acids shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
8	0	≥90.0
10	0	≤3.0
12	0	≤3.0
14	0	≤3.0
16	0	≤1.0

- **FATS AND FIXED OILS**, *Iodine Value* <401>: NMT 1.0
- **FATS AND FIXED OILS**, *Saponification Value* <401>: Propylene glycol monocaprylate (Type I): 285–315
Propylene glycol monocaprylate (Type II): 270–295
- **FATS AND FIXED OILS**, *Peroxide Value* <401>: NMT 6.0
- **WATER DETERMINATION**, *Method Ia* <921>: **Analysis:** Use a mixture of methanol and methylene chloride (1:1) in place of methanol in the titration vessel.
Acceptance criteria: NMT 1.0%
- **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 0.1%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, and protect from moisture. No storage requirements specified.

- **LABELING:** Label it to indicate the type (Type I or Type II).
- **USP REFERENCE STANDARDS** <11>: USP Propylene Glycol RS
USP Propylene Glycol Monocaprylate Type I RS
USP Propylene Glycol Monocaprylate Type II RS

Propylene Glycol Monolaurate

Dodecanoic acid, monoester with 1,2-propanediol;
Lauric acid, monoester with propane-1,2-diol.

DEFINITION

Propylene Glycol Monolaurate is a mixture of the propylene glycol mono- and diesters of lauric acid. The requirements for monoester and diester content differ for the two types of Propylene Glycol Monolaurate, as set forth in the table below.

	Content of Monoesters (%)		Content of Diesters (%)	
	Min.	Max.	Min.	Max.
Type I	45.0	70.0	30.0	55.0
Type II	90.0	—	—	10.0

IDENTIFICATION

- **A. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** <201>

Standard solution: 50 mg/mL of USP Propylene Glycol Monolaurate Type I RS (or USP Propylene Glycol Monolaurate Type II RS) in methylene chloride

Sample solution: 50 mg/mL in methylene chloride

Chromatographic system

(See *Chromatography* <621>, *Thin-Layer Chromatography*.)

Developing solvent system: Ether and hexane (70:30)

Spray reagent: 0.1 mg/mL of rhodamine 6G in alcohol

Analysis: Develop the chromatogram over a path of 15 cm, and dry the plate in a current of air. Spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The *R_f* values of the principal spots from the *Sample solution* correspond to those from the *Standard solution*.

- **B.** It meets the requirements in *Specific Tests for Fats and Fixed Oils*, *Fatty Acid Composition* <401>.

ASSAY

- **PROCEDURE**

Mobile phase: Tetrahydrofuran

Sample solution: 40 mg/mL of Propylene Glycol Monolaurate in tetrahydrofuran

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7-mm × 60-cm; 5-µm packing L21 (100 Å)

[NOTE—Two 7-mm × 30-cm L21 columns may be used in place of one 60-cm column, provided *System suitability* requirements are met.]

Temperatures

Column: 40°

Detector: 40°

Flow rate: 1 mL/min

Injection volume: 40 µL

System suitability

Sample: *Sample solution*

Suitability requirements

[NOTE—The order of elution is diesters, monoesters, and propylene glycol.]

Relative standard deviation: NMT 1.0% for the monoester peak

Analysis

Sample: *Sample solution*

Calculate the percentage of monoesters or diesters in the portion of Propylene Glycol Monolaurate taken:

$$\text{Result} = (r_U/r_T) \times (100 - D)$$

r_U = peak response for monoesters or diesters
 r_T = sum of the peak responses of the monoesters and diesters
 D = sum of the percentage content of propylene glycol and the percentage content of free fatty acids

Calculate the percentage content of free fatty acids in the portion taken:

$$\text{Result} = (A/561.1) \times 200$$

A = acid value

Acceptance criteria

Type I

Monoesters: 45.0%–70.0%

Diesters: 30.0%–55.0%

Type II

Monoesters: NLT 90.0%

Diesters: NMT 10.0%

IMPURITIES

• LIMIT OF PROPYLENE GLYCOL

Mobile phase, Sample solution, and Chromatographic system: Proceed as directed in the *Assay*.

Standard stock solution: 4 mg/mL of USP Propylene Glycol RS in tetrahydrofuran

Standard solutions: Into four 15-mL flasks introduce, respectively, 0.25, 0.5, 1.0, and 2.5 mL of *Standard stock solution*, and dilute with tetrahydrofuran to 5.0 mL. In a fifth 15-mL flask, introduce 5.0 mL of *Standard stock solution*.

Analysis

Samples: *Standard solutions* and *Sample solution*
 Prepare a standard curve of peak area versus concentration, in mg/mL, of propylene glycol in the *Standard solutions*. Obtain the concentration of propylene glycol in the *Sample solution* from the standard curve.

Calculate the percentage of free propylene glycol in the portion of Propylene Glycol Monocaprylate taken:

$$\text{Result} = (C/C_U) \times 100$$

C = concentration of propylene glycol in the *Sample solution* from the standard curve

C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria

Propylene glycol monolaurate (Type I): NMT 5.0%

Propylene glycol monolaurate (Type II): NMT 1.0%

SPECIFIC TESTS

• FATS AND FIXED OILS, Acid Value (401): NMT 4

• FATS AND FIXED OILS, Fatty Acid Composition (401):

Propylene Glycol Monolaurate exhibits the composition profile of fatty acids shown in *Table 1*.

Table 1

Fatty Acids	Carbon-Chain Length	Percentage (%)
Caprylic acid	C8	NMT 0.5
Capric acid	C10	NMT 2.0
Lauric acid	C12	NLT 95.0
Myristic acid	C14	NMT 3.0
Palmitic acid	C16	NMT 1.0

• FATS AND FIXED OILS, Iodine Value (401): NMT 1

• FATS AND FIXED OILS, Saponification Value (401)

Propylene glycol monolaurate (Type I): 210–245

Propylene glycol monolaurate (Type II): 200–230

• WATER DETERMINATION, Method Ia (921)

Analysis: Use a mixture of methanol and methylene chloride (1:1) in place of methanol in the titration vessel.

Acceptance criteria: NMT 1.0%

• ARTICLES OF BOTANICAL ORIGIN, Total Ash (561): NMT 0.1%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and protect from moisture. No storage requirements specified.

• **LABELING:** Label it to indicate the type (Type I or Type II).

• USP REFERENCE STANDARDS (11)

USP Propylene Glycol RS

USP Propylene Glycol Monolaurate Type I RS

USP Propylene Glycol Monolaurate Type II RS

Propylene Glycol Monostearate

Octadecanoic acid, monoester with 1,2-propanediol; 1,2-Propanediol monostearate [1323-39-3].

DEFINITION

Propylene Glycol Monostearate is a mixture of the propylene glycol mono- and di-esters of stearic and palmitic acids. It contains NLT 90.0% of monoesters of saturated fatty acids, chiefly propylene glycol monostearate ($C_{21}H_{42}O_3$) and propylene glycol monopalmitate ($C_{19}H_{38}O_3$).

ASSAY

• PROPYLENE GLYCOL MONOESTERS

Sample: 25 g

Analysis: Place the *Sample* in a 500-mL, round-bottom flask, and add 250 mL of alcohol and 7.5 g of potassium hydroxide. Connect the flask to a suitable condenser, reflux the mixture for 2 h, cool, and transfer to an 800-mL beaker, rinsing the flask with 100 mL of water and combining the rinsing with the mixture in the beaker. Heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol, and continue the evaporation until the odor of alcohol can no longer be detected. Adjust the volume, with hot water, to 250 mL, neutralize with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Heat with stirring until the fatty acid layer separates, and transfer the fatty acids to a 500-mL separator. Wash the fatty acids with four 200-mL portions of hot water, and discard the washings. Dry the fatty acids at 105° for 1 h, cool, and determine the acid value on a 1-g portion, as directed in *Fats and Fixed Oils* (401), *Acid Value* (*Free Fatty Acids*).

Calculate the average molecular weight of the monoesters, in the portion of Propylene Glycol Monostearate taken:

$$M_{rAvg} = (M_{r1}/A) + M_{r2} - M_{r3}$$

M_{r1} = 1000 times the molecular weight of potassium hydroxide, 56,110

A = acid value

M_{r2} = molecular weight of propylene glycol, 76.10

M_{r3} = molecular weight of water, 18.02

Calculate F in the portion taken:

$$F = (M_{r4} \times G)/M_{r5}$$

- M_{r4} = 10 times the molecular weight of potassium hydroxide, 561.1
 G = content, in percentage, of glycerin and propylene glycol in propylene glycol monostearate
 M_{r5} = one-half of the molecular weight of propylene glycol, 38.05
 Calculate the percentage of propylene glycol monoesters:

$$\text{Result} = [(H - F) \times M_{r\text{Avg}}]/M_{r4}$$

- H = hydroxyl value of propylene glycol monostearate

Acceptance criteria: NLT 90.0% of monoesters of saturated fatty acids, chiefly propylene glycol monostearate and propylene glycol monopalmitate

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.5%
- **FREE GLYCERIN AND PROPYLENE GLYCOL**

Periodic acid solution: Dissolve 5.4 g of periodic acid in 100 mL of water, and add 1900 mL of glacial acetic acid. Store in a glass-stoppered bottle, protected from light.

Chloroform: Use chloroform that meets the following additional requirement. To each of three glass-stoppered, 500-mL conical flasks add 50.0 mL of *Periodic acid solution*, then add 50 mL of chloroform and 10 mL of water to two of the flasks and 50 mL of water to the third flask. To each flask add 20 mL of potassium iodide TS, mix gently, and proceed as directed for *Analysis*, beginning with "allow to stand for 1–5 min". The difference between the volumes of 0.1 N sodium thiosulfate required in the titrations with and without the chloroform does not exceed 100 μ L.

Sample solution: Melt the Propylene Glycol Monostearate at a temperature NMT 55°. Transfer a 3-g portion to a 100-mL beaker, and dissolve in 25 mL of *Chloroform*.

Analysis: Transfer the *Sample solution*, with the aid of another 25-mL portion of *Chloroform*, to a separator, wash the beaker with 25 mL of water, and add the washing to the separator. Insert the stopper, shake vigorously for 30–60 s, and allow the layers to separate, adding 1–2 mL of glacial acetic acid, if necessary, to break any emulsion. Transfer the aqueous layer to a glass-stoppered, 500-mL conical flask, wash the chloroform layer with two 25-mL portions of water, combining the washings with the aqueous layer, and discard the chloroform layer. Add, with swirling, 50.0 mL of *Periodic acid solution* to the solution and to another glass-stoppered, 500-mL conical flask containing 75 mL of water to provide the blank. Allow to stand for 30–90 min. To each flask add 20 mL of potassium iodide TS, mix gently, and allow to stand for 1–5 min before titrating. Add 100 mL of water, and titrate with 0.1 N sodium thiosulfate VS until the brown iodine color fades to pale yellow, add 3 mL of starch TS, and continue the titration to the disappearance of the blue color.

Calculate the percentage of free glycerin and propylene glycol, calculated as propylene glycol, in the portion of Propylene Glycol Monostearate taken:

$$\text{Result} = [(V_B - V_S) \times M_r \times N]/W$$

- V_B = volume of sodium thiosulfate consumed by the blank solution (mL)
 V_S = volume of sodium thiosulfate consumed by the *Sample solution* (mL)
 M_r = molecular weight of propylene glycol divided by 20, 3.805
 N = actual normality of the sodium thiosulfate solution

- W = weight of Propylene Glycol Monostearate taken (g)

Acceptance criteria: NMT 1.0% free glycerin and propylene glycol, calculated as propylene glycol

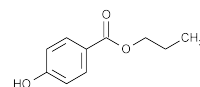
SPECIFIC TESTS

- **CONGEALING TEMPERATURE** (651): NLT 45°
- **FATS AND FIXED OILS**, *Acid Value* (Free Fatty Acids) (401): NMT 4
- **FATS AND FIXED OILS**, *Hydroxyl Value* (401): 160–175
- **FATS AND FIXED OILS**, *Iodine Value* (401): NMT 3
- **FATS AND FIXED OILS**, *Saponification Value* (401): 155–165

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Propylparaben



$C_{10}H_{12}O_3$ 180.20
 Benzoic acid, 4-hydroxy-, propyl ester;
 Propyl *p*-hydroxybenzoate [94-13-3].

DEFINITION

Propylparaben contains NLT 98.0% and NMT 102.0% of $C_{10}H_{12}O_3$.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197M)
- **B. MELTING RANGE OR TEMPERATURE** (741): 96°–99°

ASSAY

PROCEDURE

Mobile phase, Sample solution, Standard solution B, and Chromatographic system: Proceed as described in the procedure for *Related Substances*.

System suitability

Sample: *Standard solution B*

Suitability requirements

Relative standard deviation: NMT 0.85% for 6 injections

Analysis

Samples: *Sample solution* and *Standard solution B*
 Calculate the percentage of Propylparaben in the *Sample solution*:

$$\text{Result} = P \times (r_U \times C_S)/(r_S \times C_U)$$

- P = labeled purity of USP Propylparaben RS expressed as a percentage
 r_U = peak area of propylparaben from the *Sample solution*
 C_S = concentration of propylparaben in *Standard solution B*
 r_S = peak area of propylparaben from *Standard solution B*
 C_U = concentration of Propylparaben in the *Sample solution*

Acceptance criteria: 98.0%–102.0%

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 0.1%, determined on 1.0 g

Organic Impurities**• PROCEDURE: RELATED SUBSTANCES**

Mobile phase: Methanol and a 6.8 g/L solution of potassium dihydrogen phosphate (65:35 v/v)

Sample solution: Dissolve 50.0 mg of Propylparaben in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.

Standard solution A: 5.0 µg/mL each of *p*-hydroxybenzoic acid, USP Ethylparaben RS, and USP Propylparaben RS in *Mobile phase*

Standard solution B: Dissolve 50.0 mg of USP Propylparaben RS in 2.5 mL of methanol, and dilute with *Mobile phase* to 50.0 mL. Dilute 10.0 mL of this solution with *Mobile phase* to 100.0 mL.

Standard solution C: Dilute 1.0 mL of the *Sample solution* with *Mobile phase* to 20.0 mL. Dilute 1.0 mL of this solution with *Mobile phase* to 10.0 mL.

Chromatographic system

(See Chromatography <621>, *System Suitability*.)

Mode: LC

Detector: UV 272 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1

Flow rate: 1.3 mL/min

Injection size: 10 µL

Run time: About 2.5 times the retention time of propylparaben

System suitability

Sample: *Standard solution A*

[NOTE—The retention time of propylparaben is about 4.5 min; the relative retention times for *p*-hydroxybenzoic acid and ethylparaben are about 0.3 and 0.7, respectively.]

Suitability requirements

Resolution: NLT 3.0 between the ethylparaben and propylparaben peaks

Analysis

Samples: *Sample solution* and *Standard solution C*

[NOTE—Disregard any limit that is 0.2 times the area of the principal peak in the chromatogram obtained with *Standard solution C* (0.1%).]

Acceptance criteria

***p*-Hydroxybenzoic acid:** The peak area in the *Sample solution*, multiplied by 1.4 to correct for the calculation of content, is NMT the area of the principal peak in *Standard solution C* (0.5%).

Unspecified impurities: The peak area of each impurity in the *Sample solution* is NMT the area of the principal peak in *Standard solution C* (0.5%).

Total impurities: The total peak area for all impurities in the *Sample solution* is NMT twice the area of the principal peak in *Standard solution C* (1.0%).

SPECIFIC TESTS**• COLOR OF SOLUTION**

Sample solution: 100 mg/mL in alcohol

Comparison solution: Mix 2.4 mL of ferric chloride CS, 1.0 mL of cobaltous chloride CS, and 0.4 mL of cupric sulfate CS with 0.3 N hydrochloric acid to make 10 mL. Dilute 5 mL of this solution with 0.3 N hydrochloric acid to make 100 mL. [NOTE—Prepare and use this solution immediately.]

Analysis

Samples: Alcohol, *Sample solution*, and *Comparison solution*

Make the comparison by viewing the solutions downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* <631>).

Acceptance criteria: The *Sample solution* is clear and not more intensely colored than alcohol or the *Comparison solution*.

• ACIDITY

Sample solution: To 2 mL of *Sample solution* prepared in the test for *Color of Solution*, add 3 mL of alcohol,

5 mL of carbon dioxide-free water, and 0.1 mL of bromocresol green TS.

Analysis: Titrate with 0.10 N sodium hydroxide.

Acceptance criteria: NMT 0.1 mL is required to produce a blue color.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** <11>

USP Ethylparaben RS

USP Propylparaben RS

Propylparaben Sodium

DEFINITION

Propylparaben Sodium contains NLT 98.5% and NMT 101.5% of propylparaben sodium (C₁₀H₁₁NaO₃), calculated on the anhydrous basis.

IDENTIFICATION**• A.**

Standard: 0.5 g of USP Propylparaben RS

Sample: 0.5 g

Analysis: Dissolve the *Sample* in 5 mL of water. Acidify with hydrochloric acid, and filter the resulting precipitate. Wash the precipitate with water, and dry over silica gel for 5 h. Repeat with the *Standard*.

Acceptance criteria: The IR absorption spectrum of a mineral oil dispersion of the *Sample* exhibits maxima only at the same wavelengths as that of a mineral oil dispersion of the *Standard*.

• B.

Sample solution: Ignite 0.3 g of Propylparaben Sodium, cool, and dissolve the residue in 3 mL of 3 N hydrochloric acid.

Acceptance criteria: A platinum wire dipped in the *Sample solution* imparts an intense, persistent yellow color to a nonluminous flame.

ASSAY**• PROCEDURE**

Potassium bromate solution: 5.56 mg/mL

Potassium bromide solution: 125 mg/mL

Sample: 100 mg

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Residual titration

Titrant: Potassium iodide TS

Back-titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Gently reflux 100 mg with 30 mL of 1 N sodium hydroxide for 30 min. Cool; add 25.0 mL of *Potassium bromate solution*, 5 mL of *Potassium bromide solution*, and 10 mL of hydrochloric acid; and immediately insert the stopper into the flask. Cool, shake for 15 min, and allow to stand for 15 min. Quickly add 15 mL of *Titrant*, taking care to avoid the escape of bromine vapor. At once replace the stopper in the flask, and shake vigorously. Rinse the stopper and the neck of the flask with a small quantity of water. Titrate the liberated iodine with *Back-titrant*, adding 3 mL of starch TS as the endpoint is approached. [NOTE—About 15 mL is needed.] Perform a blank determination, and note the difference in volumes required. Each mL of the difference in volume of 0.1 N sodium thiosulfate is equivalent to 3.37 mg of propylparaben sodium (C₁₀H₁₁NaO₃).

Acceptance criteria: 98.5%–101.5% on the anhydrous basis

IMPURITIES

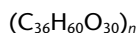
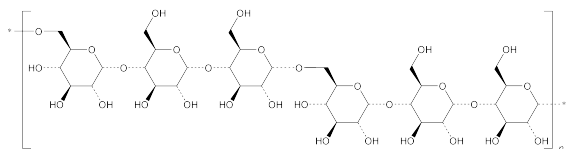
- **CHLORIDE AND SULFATE**, *Chloride* (221)
Standard solution: 0.10 mL of 0.020 N hydrochloric acid
Sample: 0.2 g
Analysis: Proceed as directed in the chapter.
Acceptance criteria: 0.035%; the *Sample* shows no more chloride than the *Standard solution*.
- **CHLORIDE AND SULFATE**, *Sulfate* (221)
Standard solution: 0.30 mL of 0.020 N sulfuric acid
Sample: 0.25 g
Analysis: Proceed as directed in the chapter.
Acceptance criteria: 0.12%; the *Sample* shows no more sulfate than the *Standard solution*.

SPECIFIC TESTS

- **pH** (791)
Sample solution: 1 mg/mL
Acceptance criteria: 9.5–10.5
- **WATER DETERMINATION**, *Method I* (921): NMT 5.0%
- **COMPLETENESS OF SOLUTION** (641)
Sample solution: Dissolve 1 g in water.
Acceptance criteria: Meets the requirements

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight containers.
- **USP REFERENCE STANDARDS** (11)
USP Propylparaben RS

Protamine Sulfate—see *Protamine Sulfate General Monographs***Pullulan**

Poly[6]- α -D-glucopyranosyl-(1 \rightarrow 4)- α -D-glucopyranosyl-(1 \rightarrow 4)- α -D-glucopyranosyl-(1 \rightarrow) [9057-02-7].

DEFINITION

Pullulan is a neutral, simple polysaccharide produced by the growth of *Aureobasidium pullulans*. It has a chain structure of repeated α -1,6-bonds of maltotriose composed of three glucoses in α -1,4-bonds. It may contain some maltotetraosyl units. It contains NLT 90% of glucan, calculated on the dried basis.

IDENTIFICATION

- **A.**
Sample: 10 g
Analysis: Dissolve the *Sample* in 100 mL of water by adding in small portions with stirring.
Acceptance criteria: A viscous solution is produced.
- **B.**
Pullulanase sample solution: 10 units/mL of pullulanase
Sample solution: The viscous solution obtained in *Identification test A*
Analysis: Mix 10 mL of the *Sample solution* with 0.1 mL of *Pullulanase sample solution*, and allow to incubate at 25° for 20 min.

Acceptance criteria: A substantial loss of viscosity is observed.

- **C.**
Sample solution: 20 mg/mL
Analysis: To 10 mL of the *Sample solution* add 2 mL of polyethylene glycol 600.
Acceptance criteria: A white precipitate is formed immediately.

ASSAY

- **CONTENT OF MONOSACCHARIDE, DISACCHARIDE, AND OLIGOSACCHARIDES**

Sample stock solution: 8 mg/mL, on previously dried material

Sample solution: To 1.0 mL of the *Sample stock solution* add 0.1 mL of saturated potassium chloride solution, and shake vigorously with 3 mL of methyl alcohol. Centrifuge, and use the supernatant.

Standard solution: Dilute 1.0 mL of the *Sample stock solution* with water to 50 mL.

Blank: Water

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Vis

Analytical wavelength: 620 nm

Analysis

Samples: *Sample solution*, *Standard solution*, and *Blank*
Transfer 0.2 mL each of the *Standard solution*, *Sample solution*, and *Blank* to a test tube containing 5 mL of a 1-in-500 solution of anthrone in 75% (v/v) sulfuric acid, with the test tube placed in ice water. Mix each tube immediately, and then heat the test tube at 90° for 10 min. Remove the tube, and allow it to cool in cold running water.

Determine the absorbances of the resulting solutions at the specified wavelength.

Determine the percentage of monosaccharide, disaccharide, and oligosaccharides in the portion of the sample taken:

$$\text{Result} = (D_U/D_S) \times (A_U - A_B)/(A_S - A_B) \times 100$$

D_U = dilution factor for the *Sample solution*, 4.1
 D_S = dilution factor for the *Standard solution*, 50
 A_U = absorbance of the *Sample solution*
 A_S = absorbance of the *Standard solution*
 A_B = absorbance of the *Blank*

Acceptance criteria: The total content of monosaccharide, disaccharide, and oligosaccharides is NMT 10.0%, corresponding to NLT 90% of glucan on the dried basis.

IMPURITIES

- **RESIDUE ON IGNITION** (281)
Sample: 2.0 g
Acceptance criteria: NMT 0.3%
- **HEAVY METALS**, *Method II* (231): NMT 5 μ g/g
- **NITROGEN DETERMINATION**, *Method II* (461)
Sample: 3 g, previously dried
Analysis: Proceed as directed in the chapter, replacing the 7 mL of sulfuric acid with 12 mL of sulfuric acid for the decomposition and replacing the 30 mL of sodium hydroxide solution (2 in 5) with 40 mL of a solution of sodium hydroxide (2 in 5).
Acceptance criteria: NMT 0.05%

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 10^2 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g.

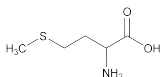
- **pH** <791>
Sample: 1.0 g
Analysis: Dissolve the *Sample* in 10 mL of freshly boiled and cooled water.
Acceptance criteria: 4.5–6.5
- **LOSS ON DRYING** <731>
Analysis: Dry at 90° under vacuum for 6 h.
Acceptance criteria: NMT 6.0%
- **VISCOSITY—CAPILLARY VISCOMETER METHODS** <911>
Sample: Exactly 10.0 g, previously dried
Analysis: Dissolve the *Sample* in water to make exactly 100 g, and perform the test at 30 ± 0.1° using a Ubbelohde-type viscometer.
Acceptance criteria: The kinematic viscosity is 100–180 mm² · s⁻¹.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
- **LABELING:** Label it to indicate the viscosity, giving the type of viscosity parameter, concentration of the solution, and the type of equipment used.

Pumice—see *Pumice General Monographs*

Racemethionine



C₅H₁₁NO₂S 149.21
 Methionine, DL-;
 DL-2-Amino-4-(methylthio)-butyric acid [59-51-8].

DEFINITION

Racemethionine contains NLT 99.0% and NMT 101.0% of C₅H₁₁NO₂S, as DL-methionine, calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>
Sample: Dry the substances at 105°.
Acceptance criteria: Meets the requirements
- **B.** The principal spot from *Sample solution B* is similar in size, color, and position to the principal spot from *Standard solution A*, as obtained in the test for *Organic Impurities, Related Substances*.
- **C. OPTICAL ROTATION, Angular Rotation** <781A>
Sample: 50 mg/mL in 1 M hydrochloric acid
Acceptance criteria: -0.05° to +0.05°
- **D. PROCEDURE**
Analysis: Dissolve 0.1 g of Racemethionine and 0.1 g of glycine in 4.5 mL of dilute sodium hydroxide solution (85 mg/mL). Add 1 mL of sodium nitroferricyanide solution (25 mg/mL). Heat to 40° for 10 min. Allow to cool, and add 2 mL of a mixture of hydrochloric acid and phosphoric acid (90:10).
Acceptance criteria: A deep red color develops.

ASSAY

- **PROCEDURE**
Sample: 140 mg of Racemethionine
Analysis: Dissolve the *Sample* in a mixture of 3 mL of formic acid and 50 mL of glacial acetic acid. Titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically. Perform a blank determination, and make any necessary corrections (see *Titrimetry* <541>).

Each mL of 0.1 N perchloric acid is equivalent to 14.92 mg of C₅H₁₁NO₂S.

Acceptance criteria: 99.0%–101.0% on the dried basis

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** <281>: NMT 0.1%, determined on 1.0 g
- **CHLORIDE AND SULFATE, Chloride** <221>: [NOTE—Prepare the *Sample solution* and the *Standard solution* at the same time.]
Chloride standard solution (5 ppm Cl): 0.824 mg/mL of NaCl. Just before use, dilute 1 mL of this solution with water to 100 mL.
Standard solution: To 10 mL of *Chloride standard solution* add 10 mL of 0.1 N silver nitrate and 25 mL of water, and mix.
Sample solution: Dissolve 0.25 g in 35 mL of water. Add 5 mL of dilute nitric acid and 10 mL of 0.1 N silver nitrate. Allow to stand protected from light for 5 min.
Analysis: Examine the *Sample solution* and *Standard solution* laterally against a black background.
Acceptance criteria: Any opalescence in the *Sample solution* is not more intense than that in the *Standard solution* (200 ppm).
- **CHLORIDE AND SULFATE, Sulfate** <221>: [NOTE—Prepare the *Sample solution* and the *Control solution* at the same time.]
Barium chloride solution: 250 mg/mL
Sulfate standard solution (10 ppm SO₄): 1.81 mg/mL of potassium sulfate in 30% alcohol (v/v). Just before use, dilute 1 mL of this solution with 30% alcohol (v/v) to 100 mL.
Standard solution: Mix 3 mL of the *Barium chloride solution* and 4.5 mL of the *Sulfate standard solution*, and allow to stand for 1 min.
Sample stock solution: 50.0 mg/mL, heated to 60°. Cool to 10°, and filter.
Sample solution: To 2.5 mL of the *Standard solution* add 15 mL of the *Sample stock solution* and 0.5 mL of 5 N acetic acid.
Control solution: To 2.5 mL of the *Standard solution* add 15 mL of the *Sulfate standard solution* and 0.5 mL of 5 N acetic acid.
Analysis
Samples: *Sample solution* and *Control solution*
Acceptance criteria: After 5 min, any opalescence in the *Sample solution* is not more intense than that in the *Control solution* (200 ppm).
- **HEAVY METALS**
Sodium sulfide solution: Dissolve 5 g of sodium sulfide in 10 mL of water. Add 30 mL of glycerin.
Standard lead solution: Prepare as directed for *Special Reagents* in *Heavy Metals* (231).
Standard solution: Transfer 1.0 mL of the *Standard lead solution* to a 10-mL volumetric flask. Add 1 mL of 50% acetic acid and 2 drops of 25% sodium hydroxide, and dilute with water to volume.
Sample solution: Dissolve 5 g of Racemethionine by adding 5 mL of 16% hydrochloric acid and 5 mL of water. Adjust with 25% sodium hydroxide to a pH of 3.0–4.0. Dilute with water to 50 mL. Shake for approximately 15 min, and filter.
Analysis: Add 1 drop of *Sodium sulfide solution* to 10 mL of the *Sample solution*, and add 1 drop of *Sodium sulfide solution* to 10 mL of the *Standard solution*. Let stand for 5 min. View downward over a white surface.
Acceptance criteria: The color of the solution from the *Sample solution* is not darker than that of the solution from the *Standard solution* (NMT 10 ppm).
- **LIMIT OF IRON**
Standard stock solution (125 ppm): Dissolve 1.727 g of ferric ammonium sulfate [FeNH₄(SO₄)₂ · 12H₂O] in water. Add 50 mL of 10% hydrochloric acid, dilute

with water to 1000 mL, and mix. Dilute 1 mL of this solution with water to 40 mL. Pipet 5 mL of this solution into a 200-mL volumetric flask, dilute with water to volume, and mix.

Standard solution: Transfer 2 mL of the *Standard stock solution* to a 25-mL volumetric flask. Add 5 mL of 16% hydrochloric acid, 50 mg of ammonium persulfate, and 3 mL of 30% ammonium thiocyanate, and dilute with water to volume.

Sample solution: Transfer 1 g of Racemethionine to a 25-mL volumetric flask. Add 5 mL of 16% hydrochloric acid, and dissolve. Add 50 mg of ammonium persulfate and 3 mL of 30% ammonium thiocyanate, and dilute with water to volume.

Blank: Transfer 5 mL of 16% hydrochloric acid to a 25-mL volumetric flask. Add 50 mg of ammonium persulfate and 3 mL of 30% ammonium thiocyanate, and dilute with water to volume.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV-Vis

Analytical wavelength: 475 nm

Cell: 1 cm

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank*

Without delay, concomitantly determine the absorbances of each sample, correcting for the *Blank*.

Acceptance criteria: The absorbance of the *Sample solution* is NMT that of the *Standard solution* (NMT 10 ppm).

• LIMIT OF AMMONIUM

Standard solution A: 0.297 mg/mL of USP Ammonium Chloride RS. This solution contains 0.1 mg/mL or 100 ppm of NH_4^+ .

Standard solution B: 0.297 $\mu\text{g/mL}$ of USP Ammonium Chloride RS. This solution contains 0.1 $\mu\text{g/mL}$ or 0.1 ppm of NH_4^+ .

Standard solution C: 2.97 $\mu\text{g/mL}$ of USP Ammonium Chloride RS. This solution contains 1.0 $\mu\text{g/mL}$ or 1 ppm of NH_4^+ .

Standard solution D: 29.7 $\mu\text{g/mL}$ of USP Ammonium Chloride RS. This solution contains 10 $\mu\text{g/mL}$ or 10 ppm of NH_4^+ .

Sample solution: 10 mg/mL of Racemethionine

Electrode system: Use an ammonia-specific,¹ ion-indicating electrode connected to a pH meter capable of measuring potentials (see *pH* (791)).

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C*, *Standard solution D*, and *Sample solution*

Add 100 mL of water to a 150-mL beaker, place the electrode in the beaker, stir, and measure the potential. Add 1 mL of 10 N sodium hydroxide. Stir, and measure the potential after stabilization. [NOTE—It may take about 5 min.] The potential difference must be less than 20 mV.

Add 100.0 mL each of *Standard solutions A*, *B*, *C*, and *D* to four different 150-mL beakers. To each beaker, add 1 mL of 10 N sodium hydroxide. Place the ammonia electrode in the beaker, stir, and concomitantly measure the potential after stabilization. [NOTE—It may take about 5 min.] Draw a calibration curve of the potential, in mV, versus, the quantity of ammonium (NH_4^+), in mg.

Add 100.0 mL of the *Sample solution* to a 150-mL beaker. Add 1 mL of 10 N sodium hydroxide. Adjust

the pH, if necessary, with 10 N sodium hydroxide to a pH of NLT 11. Place the ammonia electrode in the beaker, stir, and measure the potential after stabilization. [NOTE—It may take about 5 min.] Obtain the quantity of NH_4^+ , in mg, in the 100 mL of the *Sample solution* based on the calibration curve.

Calculate the percentage of ammonium (NH_4^+), in the portion of Racemethionine taken:

$$\text{Result} = (C/W) \times F$$

C = quantity of ammonium in the *Sample solution* from the standard curve (mg)

W = weight of Racemethionine taken to prepare the *Sample solution* (mg)

F = conversion factor to $\mu\text{g/g}$ (ppm), 1×10^6

Acceptance criteria: NMT 200 ppm

Organic Impurities

• PROCEDURE: RELATED SUBSTANCES

Standard solution A: 0.40 mg/mL of USP

Racemethionine RS

Standard solution B: 40 $\mu\text{g/mL}$ of USP

Racemethionine RS

Sample solution A: 20 mg/mL of Racemethionine

Sample solution B: 0.40 mg/mL of Racemethionine

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel mixture

Application volume: 5 μL

Developing solvent system: Butyl alcohol, glacial acetic acid, and water (3:1:1)

Spray reagent: 2 mg/mL of ninhydrin in a mixture of butyl alcohol and 2 N acetic acid (95:5)

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Sample solution A*, and *Sample solution B*

Develop over a path of 10 cm using the *Developing solvent system*. After air-drying the plate, spray with *Spray reagent*, and heat between 100° and 105° for 15 min. Examine the plate under white light.

Acceptance criteria: Any spot obtained from *Sample solution A*, apart from the principal spot, is not more intense than the spot obtained from *Standard solution B* (NMT 0.2%).

SPECIFIC TESTS

• **pH (791):** 5.4–6.1, in a 20 mg/mL solution

• **Loss on Drying (731):** Dry a sample at 105° for 3 h: it loses NMT 0.5% of its weight, determined on 1.000 g.

• TRANSMITTANCE

Sample solution: 10% of Racemethionine in 2 N hydrochloric acid, prepared by sonication

Analysis: Determine the transmittance in a 1-cm cell at 430 nm with a suitable spectrophotometer.

Acceptance criteria: Transmittance of NLT 0.98, corresponding to an absorbance of NMT about 0.009

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, protected from light.

• USP REFERENCE STANDARDS (11)

USP Ammonium Chloride RS

USP Racemethionine RS

¹ Orion 95-12 is suitable.

Fully Hydrogenated Rapeseed Oil

Fully hydrogenated rapeseed oil [84681-71-0].

DEFINITION

Fully Hydrogenated Rapeseed Oil is the product obtained by refining and hydrogenating oil obtained from the seeds of *Brassica napus* and *Brassica campestris* (Fam. Cruciferae). The product is a mixture of triglycerides in which the fatty acid composition is a mixture of saturated fatty acids.

IDENTIFICATION

- **A.** It meets the requirements of the test for *Fats and Fixed Oils, Fatty Acid Composition* (401).

IMPURITIES

• ALKALINE IMPURITIES

Sample solution: Prepare a mixture of 2.0 g of Fully Hydrogenated Rapeseed Oil, 1.5 mL of alcohol, and 3.0 mL of toluene. Dissolve by gentle heating.

Analysis: To the *Sample solution* add 0.05 mL of bromo-phenol blue TS, and titrate with 0.01 N hydrochloric acid to a yellow endpoint.

Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid is required.

- **RESIDUE ON IGNITION** (281): NMT 0.5%, when a 5-g sample of Fully Hydrogenated Rapeseed Oil is ignited at an ignition temperature of $800 \pm 25^\circ$
- **HEAVY METALS, Method II** (231): NMT 10 ppm
- **LIMIT OF NICKEL**

Sample solution: Weigh 5.0 g of Fully Hydrogenated Rapeseed Oil into a previously tared platinum or silica crucible. Cautiously heat the substance, and introduce into it a wick formed from twisted ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at about 600° . Continue the incineration until white ash is obtained. After cooling, transfer the residue, with the aid of two 2-mL portions of diluted hydrochloric acid, to a 25-mL volumetric flask. Add 0.3 mL of nitric acid, and dilute with water to volume.

Standard stock solution: 0.2 µg/mL of nickel prepared from nickel standard solution TS and water. Prepare immediately before use.

Standard solutions: Into three identical 10-mL volumetric flasks introduce 1.0, 2.0, and 4.0 mL of *Standard stock solution*, respectively. To each flask add a 2.0-mL portion of the *Sample solution*, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a graphite furnace

Absorbance: 232.0 nm

Lamp: Nickel hollow-cathode

Analysis

Samples: *Sample solution* and *Standard solutions* Concomitantly determine the absorbances of the *Samples* at least three times each. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. [NOTE—The *Sample solution* should be plotted as if it had a content of added nickel equivalent to 0 µg.] Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel, *C*, in µg/mL, in the *Sample solution*.

Calculate the content of nickel in the portion of Fully Hydrogenated Rapeseed Oil taken:

$$\text{Result} = (C/W) \times V$$

C = concentration of nickel in the *Sample solution* (µg/mL)

W = weight of Fully Hydrogenated Rapeseed Oil (g)

V = volume of the *Sample solution*, 25 mL

Acceptance criteria: NMT 1 ppm

- **LIMIT OF ERUCIC ACID:** NMT 1.0%, as determined under *Specific Tests*, in the test *Fats and Fixed Oils, Fatty Acid Composition* (401)

SPECIFIC TESTS

- **FATS AND FIXED OILS, Fatty Acid Composition** (401): Fully Hydrogenated Rapeseed Oil exhibits the fatty acid composition profile shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
14	0	<1.0
16	0	3–5
18	0	38–42
20	0	8–10
22	0	42–50
24	0	1.0–2.0
18	1	≤1.0
18	2	<1.0
20	1	<1.0
22 ^a	1	≤1.0

^a Erucic acid.

- **FATS AND FIXED OILS, Acid Value** (401): NMT 6.0
- **FATS AND FIXED OILS, Iodine Value** (401): NMT 4
- **FATS AND FIXED OILS, Peroxide Value** (401): NMT 2.0
- **FATS AND FIXED OILS, Unsaponifiable Matter** (401): NMT 1.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirements specified.

Superglycerinated Fully Hydrogenated Rapeseed Oil

Superglycerinated fully hydrogenated rapeseed oil.

DEFINITION

Superglycerinated Fully Hydrogenated Rapeseed Oil is the product obtained by refining, hydrogenating, and glycerinating oil obtained from the seeds of *Brassica napus* and *Brassica campestris* (Fam. Cruciferae). The product is a mixture of mono-, di-, and triglycerides, with triglycerides as a minor component.

[NOTE—Use compendial grade glycerin as a starting material.]

IDENTIFICATION

- **A.** It meets the requirements of the test for *Fats and Fixed Oils, Fatty Acid Composition* (401).

COMPOSITION

• CONTENT OF 1-MONOGLYCERIDES

Periodic acid solution: Dissolve 5.4 g of periodic acid in 100 mL of water, add 1900 mL of glacial acetic acid, and mix. Preserve in a light-resistant, glass-stoppered bottle.

Chloroform: Use chloroform that meets the following test. Add 50.0 mL of *Periodic acid solution* to each of three 500-mL flasks. Add 50 mL of chloroform and 10 mL of water to two of the flasks, and add 50 mL of water to the third flask. Add 20 mL of potassium iodide

TS to each flask, mix gently, and continue as directed in the *Analysis*, beginning with "and allow to stand at least 1 min, but no longer than 5 min, before titrating". The difference between the volume of 0.1 N sodium thiosulfate VS required in the titrations with and without the chloroform is not greater than 0.5 mL.

Sample solution: Melt Superglycerinated Fully Hydrogenated Rapeseed Oil at a temperature not higher than 10° above its melting point, and mix thoroughly. Transfer an accurately weighed quantity of it, equivalent to about 150 mg of 1-monoglycerides, to a 100-mL beaker, dissolve in 25 mL of *Chloroform*, and mix.

Analysis: Transfer the *Sample solution*, with the aid of an additional 25 mL of *Chloroform*, to a separator. Wash the beaker with 25 mL of water, and add the washing to the separator. Close the separator tightly with a stopper, shake vigorously for 30–60 s, and allow the layers to separate.

[NOTE—Add 1–2 mL of glacial acetic acid to break emulsions due to the presence of soap.]

Collect the aqueous layer in a 500-mL glass-stoppered Erlenmeyer flask, and again extract the chloroform solution in the separator with two 25-mL portions of water. Retain the combined aqueous extracts, which will be used in the test for *Limit of Free Glycerin*. Transfer the chloroform layer to a 500-mL glass-stoppered Erlenmeyer flask, and add 50.0 mL of *Periodic acid solution* to this flask and to each of two blank flasks containing a mixture of 50 mL of *Chloroform* and 10 mL of water. Swirl the flasks during the addition of *Periodic acid solution*, and allow to stand for at least 30 min, but no longer than 90 min. To each flask, add 20 mL of potassium iodide TS, and allow to stand at least 1 min, but no longer than 5 min, before titrating. Add 100 mL of water, and titrate with 0.1 N sodium thiosulfate VS, using a magnetic stirrer to keep the solution thoroughly mixed, to the disappearance of the brown iodine color. Add 2 mL of starch TS, and continue the titration to the disappearance of the blue color.

Calculate the percentage of 1-monoglycerides in the portion of Superglycerinated Fully Hydrogenated Rapeseed Oil taken:

$$\text{Result} = \{[M_1 \times (V_B - V_S) \times N]/(W \times A)\} \times 100$$

- M_1 = molecular weight of glyceryl monostearate, 358
 V_B = volume of sodium thiosulfate VS consumed in the blank determination (mL)
 V_S = volume of sodium thiosulfate VS required in the titration of the Superglycerinated Fully Hydrogenated Rapeseed Oil (mL)
 N = normality of the sodium thiosulfate VS
 W = weight of the Superglycerinated Fully Hydrogenated Rapeseed Oil taken to prepare the *Sample solution* (mg)
 A = factor number, 2

Acceptance criteria: 90.0%–110.0% of that indicated on the label

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.5%, when a 5-g sample of Superglycerinated Fully Hydrogenated Rapeseed Oil is ignited at an ignition temperature of $800 \pm 25^\circ$
- **HEAVY METALS, Method II** (231): NMT 10 ppm
- **LIMIT OF NICKEL**

Sample solution: Weigh 5.0 g of Superglycerinated Fully Hydrogenated Rapeseed Oil into a previously tared platinum or silica crucible. Cautiously heat the substance, and introduce into it a wick formed from twisted ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at about 600° . Continue the incineration until white ash is obtained. After cooling,

transfer the residue, with the aid of two 2-mL portions of diluted hydrochloric acid, to a 25-mL volumetric flask. Add 0.3 mL of nitric acid, and dilute with water to volume.

Nickel standard solution: Immediately before use, dilute 10 mL of nickel standard solution TS with water to 500 mL. This solution contains the equivalent of 0.2 µg/mL of nickel.

Standard solutions: Into three identical 10-mL volumetric flasks, introduce respectively 1.0, 2.0, and 4.0 mL of *Nickel standard solution*. To each flask, add a 2.0-mL portion of the *Sample solution*, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a graphite furnace

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Analysis

Samples: *Sample solution* and *Standard solutions*
 Concomitantly determine the absorbances at least three times each, at the wavelength of maximum absorbance. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Plot the absorbances of the *Sample solution* and the *Standard solutions* versus the added quantity of nickel.

[NOTE—The *Sample solution* should be plotted as if it had a content of added nickel equivalent to 0 µg.] Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel, C , in µg/mL, in the *Sample solution*.

Calculate the content of nickel in the portion of Superglycerinated Fully Hydrogenated Rapeseed Oil taken:

$$\text{Result} = (V \times C)/W$$

- V = volume of the *Sample solution*, 25 mL
 C = concentration of nickel in the *Sample solution* (µg/mL)
 W = weight of Superglycerinated Fully Hydrogenated Rapeseed Oil taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 1 µg/g (ppm)

- **LIMIT OF ERUCIC ACID:** NMT 1.0%, as determined in the test for *Fats and Fixed Oils* (401), *Fatty Acid Composition*
- **LIMIT OF FREE GLYCERIN**

Periodic acid solution and Chloroform: Prepare as directed in the test for *Content of 1-Monoglycerides*.

Sample solution: Use the combined aqueous extracts obtained as directed in the test for *Content of 1-Monoglycerides*.

Analysis: Transfer 50.0 mL of *Periodic acid solution* to each of two flasks: a 500-mL glass-stoppered Erlenmeyer flask containing the *Sample solution* and a 500-mL glass-stoppered Erlenmeyer blank flask containing 75 mL of water. Continue as directed for *Analysis* in the test for *Content of 1-Monoglycerides*, beginning with "Swirl the flasks during the addition of *Periodic acid solution*, and allow to stand for at least 30 min, but no longer than 90 min".

Calculate the percentage of free glycerin in the portion of Superglycerinated Fully Hydrogenated Rapeseed Oil taken:

$$\text{Result} = \{[M_1 \times (V_B - V_S) \times N]/(W \times A)\} \times 100$$

- M_1 = molecular weight of glycerin, 92
 V_B = volume of sodium thiosulfate VS consumed in the blank determination (mL)

- V_S = volume of sodium thiosulfate VS required in the titration of the Superglycerinated Fully Hydrogenated Rapeseed Oil (mL)
 N = normality of the sodium thiosulfate VS
 W = weight of the Superglycerinated Fully Hydrogenated Rapeseed Oil taken to prepare the *Sample solution* as directed in the test for *Content of 1-Monoglycerides* (mg)
 A = factor number, 4
Acceptance criteria: NMT 1%

SPECIFIC TESTS

- **FATS AND FIXED OILS**, *Acid Value* <401>: NMT 6.0
- **FATS AND FIXED OILS**, *Fatty Acid Composition* <401>: Superglycerinated Fully Hydrogenated Rapeseed Oil exhibits the fatty acid composition profile shown in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
14	0	<1.0
16	0	3–5
18	0	38–42
20	0	8–10
22	0	42–50
24	0	1.0–2.0
18	1	≤1.0
18	2	<1.0
20	1	<1.0
22 ^a	1	≤1.0

^a Erucic acid.

- **FATS AND FIXED OILS**, *Hydroxyl Value* <401>: NLT 90.0% and NMT 110.0% of that indicated on the label
- **FATS AND FIXED OILS**, *Iodine Value* <401>: NMT 4
- **FATS AND FIXED OILS**, *Peroxide Value* <401>: NMT 2.0
- **FATS AND FIXED OILS**, *Unsaponifiable Matter* <401>: NMT 1.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirements specified.
- **LABELING:** Label it to indicate the hydroxyl value and the content of 1-monoglycerides.

Rose Oil**DEFINITION**

Rose Oil is volatile oil distilled with steam from the fresh flowers of *Rosa gallica* L., *Rosa damascena* Miller, *Rosa alba* L., *Rosa centifolia* L., and varieties of these species (Fam. Rosaceae).

SPECIFIC TESTS

- **SPECIFIC GRAVITY** <841>
Analysis: Measure at 30° compared with water at 15°. **Acceptance criteria:** 0.848–0.863
- **OPTICAL ROTATION** <781A>: –1° to –4°
- **REFRACTIVE INDEX** <831>: 1.457–1.463 at 30°
- **SOLUBILITY TEST**
Analysis: 1 mL is miscible with 1 mL of chloroform without turbidity. Add 20 mL of 90% alcohol to this mixture.
Acceptance criteria: The resulting liquid is neutral or acid to moistened litmus paper and, upon standing at 20°, deposits crystals within 5 min.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers.

Rose Water Ointment—see Rose Water Ointment General Monographs**Stronger Rose Water****DEFINITION**

Stronger Rose Water is a saturated solution of the odoriferous principals of the flowers of *Rosa centifolia* L. (Fam. Rosaceae) prepared by distilling the fresh flowers with water and separating the excess volatile oil from the clear, water portion of the distillate. [NOTE—Stronger Rose Water, diluted with an equal volume of purified water, may be supplied when “Rose Water” is required.]

IMPURITIES

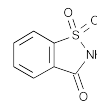
- **HEAVY METALS**, *Method I* <231>
Test preparation: Stronger Rose Water, 1 N acetic acid, and water (10:2:13)
Acceptance criteria: NMT 2 µg/g

SPECIFIC TESTS

- **REACTION:** Neutral or acidic to litmus
- **RESIDUE ON EVAPORATION**
Sample: 100 mL
Analysis: Evaporate the sample on a steam bath, and dry the residue at 105° for 1 h.
Acceptance criteria: NMT 15 mg (0.015%)

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** The odor of Stronger Rose Water is best preserved by allowing a limited access of fresh air to the container.

Saccharin

$C_7H_5NO_3S$ 183.18
 1,2-Benzisothiazol-3(2H)-one, 1,1-dioxide;
 1,2-Benzisothiazolin-3-one 1,1-dioxide [81-07-2].

DEFINITION

Saccharin contains NLT 99.0% and NMT 101.0% of $C_7H_5NO_3S$, calculated on the dried basis.

IDENTIFICATION

- **INFRARED ABSORPTION** <197K>

ASSAY

- **PROCEDURE**
Sample: 500 mg
Analysis: Dissolve the *Sample* in 40 mL of alcohol. Add 40 mL of water and phenolphthalein TS. Titrate with 0.1 N sodium hydroxide. Perform a blank titration, if necessary, and make the appropriate correction. Each mL of 0.1 N sodium hydroxide is equivalent to 18.32 mg of $C_7H_5NO_3S$.

Acceptance criteria: 99.0%–101.0% on the dried basis

IMPURITIES

Inorganic Impurities

• **RESIDUE ON IGNITION** (281): NMT 0.2%. The ignition temperature is $600 \pm 50^\circ$.

• **HEAVY METALS, Method II** (231): NMT 10 ppm

Organic Impurities

• PROCEDURE 1: LIMIT OF TOLUENESULFONAMIDES

Internal standard solution: 0.25 mg/mL of caffeine in methylene chloride

Standard stock solution: 20.0 μ g/mL of USP *o*-Toluenesulfonamide RS and 20.0 μ g/mL of USP *p*-Toluenesulfonamide RS in methylene chloride

Standard solution: Evaporate 5.0 mL of the *Standard stock solution* to dryness in a stream of nitrogen. Dissolve the residue in 1 mL of the *Internal standard solution*.

Sample solution: Suspend 10 g of Saccharin in 20 mL of water, and dissolve using 5–6 mL of 10 N sodium hydroxide. If necessary, adjust the solution with 1 N sodium hydroxide or 1 N hydrochloric acid to a pH of 7–8, and dilute with water to 50 mL. Shake the solution with four quantities each of 50 mL of methylene chloride. Combine the lower layers, dry over anhydrous sodium sulfate, and filter. Wash the filter and the sodium sulfate with 10 mL of methylene chloride. Combine the solution and the washings, and evaporate almost to dryness in a water bath at a temperature not exceeding 40° . Using a small quantity of methylene chloride, quantitatively transfer the residue into a suitable 10-mL tube, evaporate to dryness in a stream of nitrogen, and dissolve the residue in 1.0 mL of the *Internal standard solution*.

Blank solution: Evaporate 200 mL of methylene chloride to dryness in a water bath at a temperature not exceeding 40° . Dissolve the residue in 1 mL of methylene chloride.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.53-mm \times 10-m fused silica column, coated with G3 phase (film thickness 2 μ m)

Temperature

Injector: 250°

Detector: 250°

Column: 180°

Carrier gas: Nitrogen

Flow rate: 10 mL/min

Injection size: 1 μ L

Split ratio: 2:1

System suitability

Samples: *Standard solution* and *Blank solution*

[NOTE—The substances are eluted in the following order: *o*-toluenesulfonamide, *p*-toluenesulfonamide, and caffeine.]

Suitability requirements: No peaks at the retention times for the internal standard, *o*-toluenesulfonamide, or *p*-toluenesulfonamide; *Blank solution*

Resolution: NLT 1.5 between *o*-toluenesulfonamide and *p*-toluenesulfonamide, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Acceptance criteria: If any peaks due to *o*-toluenesulfonamide and *p*-toluenesulfonamide appear in the chromatogram obtained with the *Sample solution*, the ratio of their areas to that of the *Internal standard solution* is NMT the corresponding ratio in the chromatogram obtained with the *Standard solution*.

Individual impurities: See *Impurity Table 1*.

Impurity Table 1

Name	Acceptance Criteria (ppm)
<i>o</i> -Toluenesulfonamide	10
<i>p</i> -Toluenesulfonamide	10

• PROCEDURE 2: LIMIT OF BENZOATE AND SALICYLATE

Sample solution: 10 mL of a hot, saturated solution of saccharin

Analysis: Add ferric chloride TS dropwise to the *Sample solution*.

Acceptance criteria: No precipitate or violet color appears in the liquid.

SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE** (741): 226° – 230°

• **LOSS ON DRYING** (731): Dry a sample at 105° for 2 h: it loses NMT 1.0% of its weight.

• **READILY CARBONIZABLE SUBSTANCES TEST** (271)

Sample solution: 40 mg/mL in sulfuric acid (94.5%–95.5% [w/w] of H_2SO_4), maintained at 48° – 50° for 10 min

Acceptance criteria: The *Sample solution* has no more color than *Matching Fluid A*, when viewed against a white background.

• **CLARITY OF SOLUTION**

[NOTE—The *Sample solution* is to be compared to *Reference suspension A* and to water in diffused daylight 5 min after preparation of *Reference suspension A*.]

Diluent: 200-g/L solution of sodium acetate

Hydrazine solution: 10.0 mg/mL of hydrazine sulfate.

[NOTE—Allow to stand for 4–6 h.]

Methenamine solution: Transfer 2.5 g of methenamine to a 100-mL glass-stoppered flask, add 25.0 mL of water, insert the glass stopper, and mix to dissolve.

Primary opalescent suspension: Transfer 25.0 mL of *Hydrazine solution* to the *Methenamine solution* in the 100-mL glass-stoppered flask. Mix, and allow to stand for 24 h. [NOTE—This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use. Allow the suspension to stand for 24 h.]

Opalescence standard: Dilute 15.0 mL of the *Primary opalescent suspension* with water to 1000 mL. [NOTE—This suspension should not be used beyond 24 h after preparation.]

Reference suspension A: *Opalescence standard* and water (1 in 20)

Reference suspension B: *Opalescence standard* and water (1 in 10)

Sample solution: 200 mg/mL in *Diluent*

Analysis

Samples: *Diluent*, *Reference suspension A*, *Reference suspension B*, *Sample solution*, and water

Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer portions of *Reference suspension A*, *Reference suspension B*, water, and *Diluent* to separate matching test tubes. Compare the solutions in diffused daylight, viewing vertically against a black background (see *Spectrophotometry and Light-Scattering* (851), *Visual Comparison*). [NOTE—The diffusion of light must be such that *Reference suspension A* can readily be distinguished from water, and that *Reference suspension B* can readily be distinguished from *Reference suspension A*.]

Acceptance criteria: The *Sample solution* shows the same clarity as that of water, or *Diluent*, or its opalescence is NMT that of *Reference suspension A*.

• COLOR OF SOLUTION

Diluent A: 200-g/L solution of sodium acetate

Diluent B: 10-g/L solution of hydrochloric acid

Standard stock solution: Ferric chloride CS, cobaltous chloride CS, cupric sulfate CS, and *Diluent B* (3.0:3.0:2.4:1.6)

Standard solution: *Standard stock solution* and *Diluent B* (1 in 100). [NOTE—Prepare the *Standard solution* immediately before use.]

Sample solution: Use the *Sample solution* from the test for *Clarity of Solution*.

Analysis

Samples: *Diluent A*, *Standard solution*, *Sample solution*, and water

Transfer a sufficient portion of the *Sample solution* to a test tube of colorless, transparent, neutral glass with a flat base and an internal diameter of 15–25 mm to obtain a depth of 40 mm. Similarly transfer portions of the *Standard solution*, *Diluent A*, and water to separate, matching test tubes. Compare the solutions in diffused daylight, viewing vertically against a white background (see *Spectrophotometry and Light-Scattering* <851>, *Visual Comparison*).

Acceptance criteria: The *Sample solution* has the appearance of water or *Diluent A*, or is not more intensely colored than the *Standard solution*.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.

• **USP REFERENCE STANDARDS** <11>

USP Saccharin RS

USP *o*-Toluenesulfonamide RS

USP *p*-Toluenesulfonamide RS

Saccharin Calcium—see *Saccharin Calcium General Monographs*

Saccharin Sodium—see *Saccharin Sodium General Monographs*

Safflower Oil—see *Safflower Oil General Monographs*

Sesame Oil

DEFINITION

Sesame Oil is the refined fixed oil obtained from the seed of one or more cultivated varieties of *Sesamum indicum* L. (Fam. Pedaliaceae). It may contain suitable antioxidants.

IDENTIFICATION

• **A.** The peak responses for the eight major triglycerides—LLL, OLL, PLL, OOL, POL, OOO, SOL, and POO—elute between 0 and about 40 min, in the order specified, and at relative retention times of about 0.55, 0.65, 0.69, 0.77, 0.82, 0.93, 0.97, and 1.0, respectively, as obtained in the chromatogram of the *Sample solution* in the test for *Triglyceride Composition*.

ASSAY

• TRIGLYCERIDE COMPOSITION

[NOTE—The fatty acid radicals are designated as linoleic (L), oleic (O), palmitic (P), and stearic (S), and the com-

mon abbreviations for triglycerides used are as follows: trilinolein (LLL), 1,2-dilinoleoyl-3-oleoyl-rac-glycerol (OLL), 1,2-dilinoleoyl-3-palmitoyl-rac-glycerol (PLL), 1,2-dioleoyl-3-linoleoyl-rac-glycerol (OOL), 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol (POL), triolein (OOO), 1-linoleoyl-2-oleoyl-3-stearoyl-rac-glycerol (SOL), and 1,2-dioleoyl-3-palmitoyl-rac-glycerol (POO).]

Mobile phase: Acetonitrile and methylene chloride (60:40)

System suitability solution: 3.0 mg/mL of USP Sesame Oil Related Compound A RS and USP Sesame Oil Related Compound B RS in *Mobile phase*. [NOTE—USP Sesame Oil Related Compound A RS is OLL, and USP Sesame Oil Related Compound B RS is PLL.]

Sample solution: 20 mg/mL of Sesame Oil in *Mobile phase*

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: Two 4.6-mm × 25-cm columns in series; packings L1

Column temperature: 30°

Flow rate: 1.0 mL/min

Injection volume: 20 µL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for OLL and PLL are about 0.93 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.8 between OLL and PLL

Relative standard deviation: NMT 1.5% determined from peak areas; NMT 2.2% determined from the peak area ratio of OLL to PLL

Analysis

[NOTE—The relative retention times for the eight major triglyceride peaks are listed in *Table 1*.]

Sample: *Sample solution*

Calculate the percentage of each of these triglycerides in the portion of sample taken:

$$\text{Result} = (A/B) \times 100$$

A = peak area for each individual triglyceride
B = sum of the areas of all the peaks, excluding the solvent peak

Table 1

Triglyceride	Relative Retention Time	Composition (%)
LLL	0.55	7.0–19.0
OLL	0.65	13.0–30.0
PLL	0.69	5.0–9.0
OOL	0.77	14.0–25.0
POL	0.82	8.0–16.0
OOO	0.93	5.0–14.0
SOL	0.97	2.0–8.0
POO	1.0	2.0–8.0

IMPURITIES

• **HEAVY METALS, Method II** <231>: NMT 10 µg/g

SPECIFIC TESTS

• **SPECIFIC GRAVITY** <841>: 0.916–0.921

• **FATS AND FIXED OILS, Acid Value (Free Fatty Acids)** <401>

Sample: 10 g

Acceptance criteria: NMT 2.0 mL of 0.020 N sodium hydroxide is required for neutralization.

- **FATS AND FIXED OILS**, *Iodine Value* (401): 103–116
- **FATS AND FIXED OILS**, *Saponification Value* (401): 188–195
- **FATS AND FIXED OILS**, *Solidification Temperature of Fatty Acids* (401): 20°–25°
- **FATS AND FIXED OILS**, *Unsaponifiable Matter* (401): NMT 1.5%
- **COTTONSEED OIL**
Sample: 5 mL
Analysis: Mix the *Sample* in a test tube with 5 mL of a mixture of equal volumes of amyl alcohol and a 10-mg/mL solution of sulfur in carbon disulfide. Warm the mixture carefully until the carbon disulfide is expelled, and immerse the tube to one-third of its depth in a boiling saturated solution of sodium chloride.
Acceptance criteria: No reddish color develops within 15 min.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and prevent exposure to excessive heat.
- **LABELING:** Label it to indicate the name and quantity of any added antioxidant.
- **USP REFERENCE STANDARDS** (11)
 USP Sesame Oil Related Compound A RS
 USP Sesame Oil Related Compound B RS

Shellac

DEFINITION

Shellac is obtained by the purification of Lac, the resinuous secretion of the insect *Laccifer lacca* (Kerr) (Fam. Coccidae). Orange Shellac is produced either by a process of filtration in the molten state or by hot solvent process, or both. Orange Shellac may retain most of its wax or be dewaxed, and may contain lesser amounts of the natural color than originally present. Bleached (White) Shellac is prepared by dissolving the Lac in aqueous sodium carbonate, bleaching the solution with sodium hypochlorite, and precipitating the Bleached Shellac with 2 N sulfuric acid. Removal of the wax, by filtration, during the process results in Refined Bleached Shellac. Shellac conforms to the specifications in the table below.

	Acid Value (on dried basis)	Loss on Drying (%)	Wax (%)
Orange Shellac	68–76	NMT 2.0	NMT 5.5
Dewaxed Orange Shellac	71–79	NMT 2.0	NMT 0.2
Regular Bleached Shellac	73–89	NMT 6.0	NMT 5.5
Refined Bleached Shellac	75–91	NMT 6.0	NMT 0.2

IDENTIFICATION

- **A.**
Sample: 50 mg
Analysis: To the *Sample* add a few drops of a mixture of 1 g of ammonium molybdate and 3 mL of sulfuric acid.
Acceptance criteria: A green color is produced, and it becomes lilac on standing for 5 min.

IMPURITIES

- **HEAVY METALS**, *Method II* (231): NMT 10 µg/g

SPECIFIC TESTS

- **LOSS ON DRYING** (731)
Analysis: Dry at 41 ± 2° in a well-ventilated oven for 24 h.

Acceptance criteria: See the table in the *Definition*.

• ACID VALUE

Sample solution: Dissolve 2 g of finely ground Shellac in 50 mL of alcohol that has been neutralized to phenolphthalein with 0.1 N sodium hydroxide.

Analysis: Add additional phenolphthalein TS if necessary, and titrate with 0.1 N sodium hydroxide VS to a pink endpoint. Express the acid value in terms of the number of mg of potassium hydroxide required per g of dried Shellac. [NOTE—For orange Shellac titrate slowly, stirring vigorously, until a glass rod dipped into the titrated solution produces a color change when touched to a drop of thymol blue TS on a spot plate.]

Acceptance criteria: See the table in the *Definition*.

• WAX

Sample: 10 g of finely ground Shellac

Analysis: Transfer the *Sample* and 2.50 g of sodium carbonate to a 200-mL, tall-form beaker. Add 150 mL of hot water, immerse the beaker in a boiling water bath, and stir until the solid is dissolved. Cover the beaker with a watch glass, and maintain the heat for 3 h more without agitation. Remove the beaker to a cold water bath. When the wax has floated to the surface, pass the solution through medium-speed quantitative ashless filter paper, transferring the wax to the paper, and wash the filter with water. Pour 5–10 mL of alcohol onto the filter to facilitate drying. Wrap the paper loosely in a larger piece of filter paper, bind with a piece of fine wire, and dry with the aid of gentle heat. Extract with chloroform in a suitable continuous extraction apparatus for 2 h, using a weighed flask to receive the extracted wax and solvent. Evaporate the solvent, and dry the wax at 105° to constant weight.

Acceptance criteria: See the table in the *Definition*.

• ROSIN

Sample solution: 200 mg/mL in dehydrated alcohol

Analysis: To 10 mL of *Sample solution* add slowly, with shaking, 50 mL of solvent hexane, wash with two successive 50-mL portions of water, filter the washed alcohol–solvent hexane solution, and evaporate to dryness. To the residue add 2 mL of a mixture of liquefied phenol, dehydrated alcohol, and solvent hexane (1:0.5:2). Stir, and transfer a portion of the solution to a cavity of a color-reaction plate. Fill an adjacent cavity with a mixture of bromine and solvent hexane (1:4), and cover both cavities with an inverted watch glass.

Acceptance criteria: No purple or deep indigo-blue color is produced in or above the liquid containing the residue.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, preferably in a cold place.
- **LABELING:** Label it to indicate whether it is bleached or is orange, and whether it is dewaxed or wax-containing.

Dental-Type Silica

DEFINITION

Dental-Type Silica is obtained from sodium silicate solution by destabilizing with acid in such a way as to yield very fine particles. The sum of the Assay values for Silicon Dioxide and Sodium Sulfate is NLT 98.0%.

ASSAY

• SILICON DIOXIDE

Sample: 1 g

Analysis: Ignite the *Sample* at 1000° for 1 h, cool in a desiccator, and weigh. Carefully wet with water, and add 10 mL of hydrofluoric acid, in small increments. Evaporate on a steam bath to dryness, and cool. Add about 10 mL of hydrofluoric acid and about 0.5 mL of

sulfuric acid, and evaporate to dryness. Slowly increase the temperature until all of the acids have been volatilized, and ignite at 1000°. Cool in a desiccator, and weigh. The difference between the final weight and the weight of the initially ignited portion represents the weight of SiO₂.

• **SODIUM SULFATE**

Sample: 1 g

Analysis 1: In a platinum dish, wet the *Sample* with a few drops of water, add 15 mL of perchloric acid, and place the dish on a hot plate. Add 10 mL of hydrofluoric acid. Heat until copious fumes are evolved. Add 5 mL of hydrofluoric acid, and again heat to copious fumes. Add 5 mL of boric acid solution (1 in 25), and heat to fumes. Cool, and transfer the residue to a 400-mL beaker with the aid of 10 mL of hydrochloric acid. Adjust the volume with water to about 300 mL, and bring to boiling on a hot plate. Add 20 mL of hot barium chloride TS. Keep the beaker on the hot plate for 2 h, maintaining the volume above 200 mL. After cooling, transfer the precipitate and solution to a dried, tared crucible with a filter of 0.8-μm pore size. Wash the filter and precipitate 8 times with hot water, dry the crucible at 105° for 1 h, and weigh. The weight, multiplied by 0.6085, is the sodium sulfate content of the amount of specimen taken.

Acceptance criteria 1: NMT 4.0%

Analysis 2: Calculate the sum of the *Assay* values for the silicon dioxide and the sodium sulfate, and calculate the percentage in the Dental-Type Silica taken.

Acceptance criteria 2: NLT 98.0%

SPECIFIC TESTS

• **PH** (791)

Sample solution: 50 mg/mL of slurry

Acceptance criteria: 4.0–8.5

• **LOSS ON DRYING** (731)

Analysis: Dry a sample at 105° for 2 h.

Acceptance criteria: It loses NMT the maximum percentage of its weight as indicated in the labeling.

• **LOSS ON IGNITION** (733)

Sample: 1 g, previously dried

Analysis: Ignite the *Sample* at 1000° for NLT 1 h.

Acceptance criteria: NMT 8.5%

• **CHLORIDE AND SULFATE, Chloride** (221)

Sample solution: Boil 5 g in 50 mL of water under a reflux condenser for 2 h, cool, and filter.

Control: 1.0 mL of 0.020 N hydrochloric acid

Analysis: Use a 7-mL portion of *Sample solution*.

Acceptance criteria: 0.1%; the *Sample solution* shows no more chloride than the *Control*.

• **ARSENIC, Method I** (211)

Sample solution: Transfer 4.0 g of Dental-Type Silica to a platinum dish, add 5 mL of nitric acid and 35 mL of hydrofluoric acid, and evaporate on a steam bath. Cool, add 5 mL of perchloric acid, 10 mL of hydrofluoric acid, and 10 mL of sulfuric acid, and evaporate on a hot plate to the production of heavy fumes. Cool, cautiously transfer to a 100-mL beaker with the aid of a few mL of hydrochloric acid, and evaporate to dryness. Cool, add 5 mL of hydrochloric acid, dilute with water to about 40 mL, and heat to dissolve any residue. Cool, transfer to a 100-mL volumetric flask, and dilute with water to volume.

Analysis: Use a 25.0-mL portion of *Sample solution*.

Acceptance criteria: NMT 3 ppm

• **HEAVY METALS, Method I** (231)

Test preparation: 16.7 mL of the solution prepared in the test for *Arsenic*

Analysis: Transfer the *Test preparation* to a 100-mL beaker, and neutralize to litmus paper with ammonium hydroxide. Adjust with 6 N acetic acid to a pH between 3 and 4. Filter, using medium-speed filter paper, and wash with water until the filtrate and washings measure 40 mL.

Acceptance criteria: NMT 30 ppm

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate the maximum percentage of loss on drying.

Hydrophobic Colloidal Silica

[68611-44-9].

DEFINITION

Hydrophobic Colloidal Silica is prepared by partial alkylation for hydrophobation. It contains NLT 99.0% and NMT 101.0% of SiO₂, calculated on the ignited basis.

IDENTIFICATION

• **A.**

Sample: 25 mg

Analysis: Add the *Sample* to a platinum crucible, and ignite at 900° for 2 h. Using a copper wire, mix the ignited substance with 10 mg of sodium fluoride and a few drops of sulfuric acid to give a thin slurry. Cover the crucible with a thin, transparent plate of plastic under which a drop of water is suspended, and warm gently.

Acceptance criteria: Within a short time, a white ring is rapidly formed around the drop of water.

- **B.** It meets the requirements in *Specific Tests* for *Water-Dispersible Substances*.

ASSAY

• **PROCEDURE**

Sample: The residue obtained in the test for *Loss on Ignition*

Analysis: To the *Sample* add sufficient alcohol to moisten the residue completely, and then add 0.2 mL of sulfuric acid. Add 6 mL of hydrofluoric acid, and evaporate to dryness on a hot plate at about 100°, taking care to avoid loss from sputtering. Wash down the sides of the platinum crucible with 6 mL of hydrofluoric acid, and evaporate to dryness. Ignite at 900° for 2 h, cool in a desiccator, and weigh. The difference between the weight of the residue obtained in the test for *Loss on Ignition* and the weight of the final residue gives the amount of SiO₂ in the quantity of the substance to be examined.

Acceptance criteria: 99.0%–101.0% on the ignited basis

IMPURITIES

• **LOSS ON IGNITION** (733)

Sample: 0.2 g

Analysis: Ignite the *Sample* in a platinum crucible at 900° for 2 h. Cool in a desiccator before weighing.

[NOTE—It is advisable to place the crucible in a cold oven and then heat up the oven.]

Acceptance criteria: NMT 6.0%

• **LIMIT OF LEAD**

Select reagents having as low a lead content as practicable, and store all solutions in containers of borosilicate glass. Rinse all glassware thoroughly with warm, dilute nitric acid (1 in 2) followed by water.

Ammonium acetate buffer solution, pH 3.5: Weigh 25.0 g of ammonium acetate, and dissolve in 25 mL of water. Add 38.0 mL of dilute hydrochloric acid. Adjust the pH, if necessary, with dilute hydrochloric acid or weak ammonia solution (containing 460 mL/L of strong ammonia solution). Dilute with water to 100.0 mL.

Thioacetamide solution: Prepare immediately before use. To 0.2 mL of thioacetamide TS add 1 mL of a mixture of 5 mL of water, 15 mL of 1 N sodium hydroxide,

and 20 mL of 85% glycerol. Heat in a water bath for 20 s.

Sample solution: Suspend 2.5 g of Hydrophobic Colloidal Silica in 30 mL of methanol, stir, and add 30 mL of weak ammonia solution (containing 460 mL/L of strong ammonia solution). With frequent stirring, evaporate on a water bath, and dry the residue in an oven at 140°. When the dried substance is white, break up the mass with a glass rod. Reduce the residue to a powder, and add 15 mL of methanol and 25 mL of 1 N hydrochloric acid. Boil gently for 5 min, stirring frequently with the glass rod. Centrifuge for 20 min, and pass the supernatant through a membrane filter. To the residue in the centrifuge tube add 3 mL of dilute hydrochloric acid and 9 mL of water, and bring to a boil. Centrifuge for 20 min, and pass the supernatant through the same membrane filter. Wash the residue with small quantities of water, combine the filtrates and washings, and dilute with water to 50 mL. To 20 mL of this solution add 50 mg of ascorbic acid and 1 mL of strong ammonia solution. Neutralize with diluted weak ammonia solution (containing 160 mL/L of strong ammonia solution). Dilute with water to 25 mL.

Reference solution: Pipet 10 mL of the *Standard Lead Solution* (see *Heavy Metals* <231>, *Special Reagents*), and mix with 2 mL of the *Sample solution*.

Blank solution: A mixture of 10 mL of water and 2 mL of the *Sample solution*.

Analysis: To the *Sample solution*, *Reference solution*, and *Blank solution* add 2 mL of *Ammonium acetate buffer solution*, pH 3.5.

Mix and add 1.2 mL of *Thioacetamide solution*. Mix immediately. Examine the solutions after 2 min. The test is invalid if the *Reference solution* does not show a slight brown color compared to the *Blank solution*.

Acceptance criteria: 0.0025%; the brown color in the *Sample solution* is not more intense than that in the *Reference solution*. [NOTE—If the result is difficult to judge, pass the solutions through a membrane filter (pore size, 3 µm), and carry out the filtration slowly and uniformly. Compare the spots on the filters obtained with the different solutions.]

• LIMIT OF CHLORIDE

Standard solution: Add 10 mL of 0.15 mM sodium chloride and 5 mL of water. Add 1 mL of dilute nitric acid, and pour into a test tube containing 1 mL of silver nitrate TS.

Sample solution: To 1 g of Hydrophobic Colloidal Silica add 30 mL of methanol and 20 mL of dilute nitric acid. Heat on a water bath for 15 min with frequent stirring. Cool, dilute with water to 50 mL, and filter. Dilute 10 mL of the filtrate with water to 15 mL. Add 1 mL of dilute nitric acid, and pour into a test tube containing 1 mL of silver nitrate TS.

Analysis: Examine the tubes laterally against a black background.

Acceptance criteria: 0.025%; after standing for 5 min protected from light, any opalescence in the *Sample solution* is not more intense than that in the *Standard solution*.

SPECIFIC TESTS

• WATER-DISPERSIBLE SUBSTANCES

Sample: 0.4 g

Analysis: Place the *Sample* in a 500-mL separating funnel, add 100 mL of water, and shake for 1 min. Allow to stand for 1 h. Allow 90 mL of the aqueous phase to run out dropwise without filtration into a suitable dish dried at 140°, and cool in a desiccator. Evaporate to dryness at 140°, starting at a low temperature to avoid splashing. Cool in a desiccator.

Acceptance criteria: 3.0%; the weight of the residue does not exceed 12 mg.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Purified Siliceous Earth

DEFINITION

Purified Siliceous Earth is a form of silica (SiO₂) consisting of the frustules and fragments of diatoms, purified by calcining.

IMPURITIES

• LOSS ON IGNITION <733>

Sample: 1 g, previously dried

Analysis: Ignite the *Sample* at 980 ± 25° for 1 h in a tared platinum or porcelain crucible.

Acceptance criteria: NMT 2.0%

• LEACHABLE ARSENIC

Sample solution: To 10.0 g in a 250-mL beaker add 50 mL of 0.5 N hydrochloric acid, cover with a watch glass, and heat at 70° for 15 min. Cool, and decant through a Whatman No. 3 filter paper into a 100-mL volumetric flask. Wash the slurry with three 10-mL portions of water, preheated to 70°, and dilute with water to volume.

Analysis: A 3.0-mL portion of the *Sample solution* meets the requirements in *Arsenic, Method 1* <211>.

Acceptance criteria: NMT 10 µg/g

• LEACHABLE LEAD

Sample: A 10.0-mL portion of the *Sample solution* prepared in the test for *Leachable Arsenic*

Control: 10 mL of *Diluted Standard Lead Solution* in *Lead* <251>

Analysis: The *Sample* meets the requirements in *Lead* <251>.

Acceptance criteria: NMT 10 µg/g

• LIMIT OF NONSILICEOUS SUBSTANCES

Sample: 200 mg

Analysis: Transfer the *Sample* to a tared platinum crucible, add 5 mL of hydrofluoric acid and 2 drops of dilute sulfuric acid (1 in 2), and evaporate gently to dryness. Cool, add 5 mL of hydrofluoric acid, evaporate again to dryness, and ignite to constant weight.

Acceptance criteria: The weight of the residue is NMT 50 mg.

SPECIFIC TESTS

• LOSS ON DRYING <731>

Analysis: Dry a sample at 105° for 2 h.

Acceptance criteria: NMT 0.5%

• ACID-SOLUBLE SUBSTANCES

Sample: 10.0 g

Analysis: Digest the *Sample* with 50 mL of 0.5 N hydrochloric acid at 70° for 15 min, and filter. Wash the residue, adding the washings to the filtrate to obtain a total volume of 100 mL. Evaporate at 110° in a tared porcelain dish to dryness.

Acceptance criteria: NMT 2.0% (weight of the dried residue is NMT 200 mg)

• WATER-SOLUBLE SUBSTANCES

Sample: 12.5 g

Analysis: Place the *Sample* in a 500-mL conical flask, add 250 mL of water, and shake for 2 h at room temperature. Filter with the aid of vacuum, and again filter if necessary to obtain a clear filtrate. Evaporate in a tared platinum or porcelain dish, and dry at 110°.

Acceptance criteria: NMT 0.2% (weight of the residue is NMT 25 mg)

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Silicon Dioxide

SiO₂ · xH₂O
Anhydrous

60.08

DEFINITION

Silicon Dioxide is obtained by insolubilizing the dissolved silica in sodium silicate solution. Where obtained by the addition of sodium silicate to a mineral acid, the product is termed silica gel. Where obtained by the destabilization of a solution of sodium silicate in such manner as to yield very fine particles, the product is termed precipitated silica. After ignition at 1000° for NLT 1 h, it contains NLT 99.0% of SiO₂.

IDENTIFICATION

• PROCEDURE

Sample: 5 mg

Analysis: Transfer the *Sample* to a platinum crucible, mix with 200 mg of anhydrous potassium carbonate, ignite at a red heat over a burner for 10 min, and cool. Dissolve the melt in 2 mL of recently distilled water, warming if necessary, and slowly add 2 mL of ammonium molybdate TS.

Acceptance criteria: A deep yellow color is produced.

ASSAY

• PROCEDURE

Sample: 1 g

Analysis: Ignite the *Sample* in a tared platinum dish at 1000° for 1 h, cool in a desiccator, and weigh. Carefully wet with water, and add 10 mL of hydrofluoric acid in small increments. Evaporate on a steam bath to dryness, and cool. Add 10 mL of hydrofluoric acid and 0.5 mL of sulfuric acid, and evaporate to dryness. Slowly increase the temperature until all of the acids have been volatilized, and ignite at 1000°. Cool in a desiccator, and weigh. The difference between the final weight and the weight of the initially ignited portion represents the weight of SiO₂.

Acceptance criteria: NLT 99.0% on the previously ignited basis

IMPURITIES

Inorganic Impurities

• LOSS ON IGNITION (733)

Sample: 1 g

Analysis: Ignite the *Sample*, previously dried and weighed, at 1000° for NLT 1 h.

Acceptance criteria: It loses NMT 8.5% of its weight.

• CHLORIDE AND SULFATE, Chloride (221):

Boil 5 g in 50 mL of water under a reflux condenser for 2 h, cool, and filter. A 7-mL portion of the filtrate shows no more chloride than corresponds to 1.0 mL of 0.020 N hydrochloric acid (0.1%).

• CHLORIDE AND SULFATE, Sulfate (221):

A 10-mL portion of the filtrate from the test for *Chloride* shows no more sulfate than corresponds to 5.0 mL of 0.020 N sulfuric acid (0.5%).

• ARSENIC, Method I (211)

Sample solution: Transfer 4.0 g to a platinum dish. Add 5 mL of nitric acid and 35 mL of hydrofluoric acid, and evaporate on a steam bath. Cool. Add 5 mL of perchloric acid, 10 mL of hydrofluoric acid, and 10 mL of sulfuric acid, and evaporate on a hot plate to the production of heavy fumes. Cool. Cautiously transfer to a 100-mL beaker with the aid of a few mL of hydrochloric acid, and evaporate to dryness. Cool. Add 5 mL of hydrochloric acid, dilute with water to 40 mL, and heat to dissolve any residue. Cool. Transfer to a 100-mL volumetric flask, and dilute with water to volume.

Analysis: Use a 25.0-mL portion of the *Sample solution*.

Acceptance criteria: Meets the requirements of the test (NMT 3 ppm)

• HEAVY METALS, Method I (231)

Sample solution: 16.7 mL of the solution prepared for the test for *Arsenic*

Analysis: Transfer the *Sample solution* to a 100-mL beaker, and neutralize to litmus paper with ammonium hydroxide. Adjust with 6 N acetic acid to a pH between 3 and 4. Filter, using medium-speed filter paper, and wash with water until the filtrate and washings measure 40 mL.

Acceptance criteria: NMT 30 ppm

SPECIFIC TESTS

• PH (791):

4–8 in a slurry (1 in 20)

• LOSS ON DRYING (731):

Dry a sample at 145° for 4 h; it loses NMT 5.0% of its weight.

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE:

Preserve in tight containers, protected from moisture.

• LABELING:

Label it to state whether it is silica gel or precipitated silica.

Colloidal Silicon Dioxide

SiO₂

60.08

Silica [7631-86-9].

DEFINITION

Colloidal Silicon Dioxide is a submicroscopic fumed silica prepared by the vapor-phase hydrolysis of a silicon compound. When ignited at 1000° for 2 h, it contains NLT 99.0% and NMT 100.5% of SiO₂.

IDENTIFICATION

• A. PROCEDURE

Analysis: Transfer 5 mg to a platinum crucible, and mix with 200 mg of anhydrous potassium carbonate. Heat the crucible to a red color with the aid of a Bunsen burner for 10 min, and cool. Dissolve the melt in 2 mL of freshly distilled water, warming if necessary, and slowly add 2 mL of ammonium molybdate TS to the solution.

Acceptance criteria: A deep yellow color is produced.

• B. PROCEDURE

[NOTE—Avoid contact with o-tolidine when performing this test, and conduct the test in a well-ventilated hood.]

Analysis: Place 1 drop of the yellow silicomolybdate solution from *Identification* test A on a filter paper, and evaporate the solvent. Add 1 drop of a saturated solution of o-tolidine in glacial acetic acid to reduce the silicomolybdate to molybdenum blue, and place the paper over ammonium hydroxide.

Acceptance criteria: A greenish blue spot is produced.

ASSAY

• PROCEDURE

Sample: 500 mg

Analysis: Ignite the *Sample* in a tared platinum crucible at 1000 ± 25° for 2 h, cool in a desiccator, and weigh. Add 3 drops of sulfuric acid, and add enough alcohol to just moisten the sample completely. Add 15 mL of hydrofluoric acid, and in a well-ventilated hood evaporate on a hot plate to dryness, using medium heat (95°–105°) and taking care that the sample does not spatter as dryness is approached. Heat the crucible to a red color with the aid of a Bunsen burner. Ignite the residue at 1000 ± 25° for 30 min, cool in a desiccator, and weigh. If a residue remains, repeat the *Analysis*, be-

ginning with "Add 15 mL of hydrofluoric acid". The weight lost by the assay specimen, previously ignited at $1000 \pm 25^\circ$, represents the weight of SiO_2 in the portion taken.

Acceptance criteria: 99.0%–100.5% on the previously ignited basis

IMPURITIES

Inorganic Impurities

- **LOSS ON IGNITION (733):** Ignite the portion of Colloidal Silicon Dioxide, retained from the test for *Loss on Drying*, at $1000 \pm 25^\circ$ to constant weight: the previously dried Colloidal Silicon Dioxide loses NMT 2.0% of its weight.
- **ARSENIC, Method 1 (211)**
Sample solution: To 2.5 g add 50 mL of 3 N hydrochloric acid, and reflux for 30 min using a water condenser. Cool, filter with the aid of suction, and transfer the filtrate to a 100-mL volumetric flask. Wash the filter and flask with several portions of hot water, and add the washings to the flask. Cool, and dilute with water to volume.
Analysis: A 15.0-mL portion of *Sample solution*, to which 3 mL of hydrochloric acid has been added, meets the requirements of the test, the addition of the 7 N sulfuric acid being omitted.
Acceptance criteria: NMT 8 ppm

SPECIFIC TESTS

- **PH (791):** 3.5–5.5, in a (1 in 25) dispersion
- **LOSS ON DRYING (731):** Dry in a tared platinum crucible at 105° for 2 h: it loses NMT 2.5% of its weight. Retain the dried specimen in the crucible for the test for *Loss on Ignition*.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Simethicone—see *Simethicone General Monographs*

Simethicone Emulsion—see *Simethicone Emulsion General Monographs*

Soda Lime

DEFINITION

Soda Lime is a mixture of Calcium Hydroxide and Sodium or Potassium Hydroxide or both. It may contain an indicator that is inert toward anesthetic gases such as Ether, Cyclopropane, and Nitrous Oxide and that changes color when the Soda Lime can no longer absorb Carbon Dioxide.

IDENTIFICATION

- **A.**
Analysis: Place a granule on a piece of moistened red litmus paper.
Acceptance criteria: The paper turns blue immediately.
- **B. IDENTIFICATION TESTS—GENERAL, Calcium (191)**
Sample solution: A solution in 6 N acetic acid
Acceptance criteria: Meets the requirements. It also imparts a yellow color to a nonluminous flame that, when viewed through cobalt glass, may show a violet color.

SPECIFIC TESTS

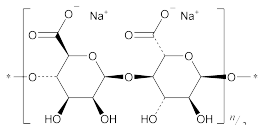
- **LOSS ON DRYING (731)**
Sample: 10 g
Analysis: Dry at 105° for 2 h.
Acceptance criteria: 12.0%–19.0%
- **CARBON DIOXIDE ABSORBENCY**
Analysis: Fill the lower transverse section of a U-shaped drying tube of 15-mm internal diameter and 15-cm height with loosely packed glass wool. In one arm of the tube, place 5 g of anhydrous calcium chloride, and weigh the tube and the contents. In the other arm, place 9.5–10.5 g of Soda Lime, and again weigh. Insert stoppers in the open arms of the tube, and connect the side tube of the arm filled with Soda Lime to a calcium chloride drying tube, which in turn is connected to a suitable source of supply of carbon dioxide. Pass the carbon dioxide through the tube at 75 mL/min for 20 min, accurately timed. Disconnect the tube, cool to room temperature, remove the stoppers, and weigh.
Acceptance criteria: NLT 19.0% increase in weight of the Soda Lime used for the test
- **HARDNESS**
Sample: 200 g
Analysis: Screen the *Sample* on a mechanical sieve shaker (see *Particle Size Distribution Estimation by Analytical Sieving (786)*) having a frequency of oscillation of 285 ± 3 cycles/min, for 3 min, to remove granules both coarser and finer than the labeled particle size. Weigh 50 g of the granules retained on the screen, and place them in a hardness pan that has a diameter of 200 mm and a concave brass bottom 7.9 mm thick at the circumference and 3.2 mm thick at the center, with an inside spherical radius of curvature of 109 cm. Add 15 steel balls of 7.9-mm diameter, and shake on a mechanical sieve shaker for 30 min. Remove the steel balls, brush the contents of the hardness pan onto a sieve of the fine-mesh size designated on the label, shake for 3 min on the mechanical sieve shaker, and weigh.
Acceptance criteria: NLT 75.0% of Soda Lime is retained on the screen.
- **MOISTURE ABSORPTION**
Sample: 10 g
Analysis: Place the *Sample* in a tared 50-mL weighing bottle having a diameter of 50 mm and a height of 30 mm, and weigh. Then place the bottle, with cover removed, for 24 h in a closed container in which the atmosphere is maintained at 85% relative humidity by being in equilibrium with sulfuric acid having a specific gravity of 1.16. Weigh again.
Acceptance criteria: The weight increase is NMT 7.5%.
- **PARTICLE SIZE DISTRIBUTION ESTIMATION BY ANALYTICAL SIEVING, Method I (786)**
Sample: 100 g
Analysis: Screen the *Sample* for 5 min as directed, using a mechanical shaker.
Acceptance criteria: It passes completely through a No. 2 standard-mesh sieve, and NMT 2.0% passes through a No. 40 standard-mesh sieve. NMT 7.0% is retained on the coarse-mesh sieve, and NMT 15.0% passes through the fine-mesh sieve designated on the label.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** If an indicator has been added, the name and color change of such indicator are stated on the container label. The container label also indicates the mesh size in terms of standard-mesh sieve sizes (see *Powder Fineness (811)*).

Sodium Acetate—see Sodium Acetate General Monographs

Sodium Alginate



$(C_6H_7NaO_6)_n$
 Alginic acid, sodium salt;
 Sodium alginate [9005-38-3].

DEFINITION

Sodium Alginate is the purified carbohydrate product extracted from brown seaweeds by the use of dilute alkali. It consists chiefly of the sodium salt of Alginic Acid, a polyuronic acid composed of β -D-mannuronic acid residues linked so that the carboxyl group of each unit is free while the aldehyde group is shielded by a glycosidic linkage. It contains NLT 90.8% and NMT 106.0% of sodium alginate of average equivalent weight 222.00, calculated on the dried basis.

IDENTIFICATION

- **A.**
Sample solution: A solution (1 in 100)
Analysis: To 5 mL of *Sample solution* add 1 mL of calcium chloride TS.
Acceptance criteria: A voluminous, gelatinous precipitate is formed immediately.
- **B.**
Sample solution: A solution (1 in 100)
Analysis: To 10 mL of *Sample solution* add 1 mL of 4 N sulfuric acid.
Acceptance criteria: A heavy, gelatinous precipitate is formed.

ASSAY

- **ALGINATES**
 (See *Alginates Assay* <311>.)
Sample: 250 mg
Analysis: Each mL of 0.2500 N sodium hydroxide consumed is equivalent to 27.75 mg of sodium alginate.
Acceptance criteria: 90.8%–106.0% on the dried basis

IMPURITIES

- **ARSENIC, Method II** <211> NMT 1.5 ppm
- **LEAD** <251>
Standard solution: 5 mL of *Diluted Standard Lead Solution*
Test preparation: Add 1.0 g to 20 mL of nitric acid in a 250-mL conical flask, mix, and heat carefully until the Sodium Alginate is dissolved. Continue heating until the volume is reduced to 7 mL. Cool rapidly to room temperature, transfer to a 100-mL volumetric flask, and dilute with water to volume.
Analysis: Use 50 mL of the *Test preparation*, and proceed as directed in the chapter, using 15 mL of ammonium citrate solution, 3 mL of potassium cyanide solution, and 0.5 mL of hydroxylamine hydrochloride solution. After the first dithizone extraction, wash the combined chloroform layers with 5 mL of water, discarding the water layer and continuing in the usual manner by extracting with 20 mL of 0.2 N nitric acid.
Acceptance criteria: Contains NMT 5 μ g of lead (corresponding to NMT 10 ppm)

- **HEAVY METALS, Method II** <231>
 [NOTE—Conduct the ignition in a platinum crucible, and use nitric acid in place of sulfuric acid to wet the test specimen.]
Acceptance criteria: NMT 40 ppm

SPECIFIC TESTS

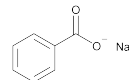
- **MICROBIAL ENUMERATION TESTS** <61> and **TESTS FOR SPECIFIED MICROORGANISMS** <62>: The total bacterial count does not exceed 200 cfu/g, and the tests for *Salmonella* species and *Escherichia coli* are negative.
- **LOSS ON DRYING** <731>: Dry a sample at 105° for 4 h: it loses NMT 15.0% of its weight.
- **ARTICLES FOR BOTANICAL ORIGIN, Total Ash** <561>: Proceed as directed in *Methods of Analysis*, carefully igniting 3 g in a tared platinum dish, until the residue is thoroughly carbonized (5 min), and then igniting in a muffle furnace at a temperature of 800 \pm 25° until the carbon is completely burned off (approximately 75 min).
Acceptance criteria: 18.0%–27.0% of ash on the dried basis

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Sodium Ascorbate—see Sodium Ascorbate General Monographs

Sodium Benzoate



$C_7H_5NaO_2$ 144.10
 Benzoic acid, sodium salt;
 Sodium benzoate [532-32-1].

DEFINITION

Sodium Benzoate contains NLT 99.0% and NMT 100.5% of $C_7H_5NaO_2$, calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197M>: On the undried specimen
- **B. IDENTIFICATION TESTS—GENERAL, Sodium** <191>: Meets the requirements
- **C. IDENTIFICATION TESTS—GENERAL, Benzoate** <191>: Meets the requirements

ASSAY

- **PROCEDURE**
Sample: 600 mg
Titrimetric system
 (See *Titrimetry* <541>.)
Mode: Direct titration
Titrant: 0.1 N perchloric acid VS
Blank: 100 mL of glacial acetic acid
Endpoint detection: Visual
Analysis: To the *Sample* add 100 mL of glacial acetic acid, and stir until completely dissolved. Add 2 drops of crystal violet TS, and titrate with 0.1 N perchloric acid VS to a green endpoint. Perform a blank determination. Calculate the percentage of sodium benzoate ($C_7H_5NaO_2$) in the *Sample* taken:

$$\text{Result} = [(V_s - V_b) \times N \times F] / W \times 100$$

V_s = volume of *Titrant* consumed by the *Sample* (mL)
 V_B = volume of *Titrant* consumed by the *Blank* (mL)
 N = actual normality of the *Titrant* (mEq/mL)
 F = equivalency factor, 144.1 mg/mEq
 W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5% on the anhydrous basis

IMPURITIES

• HEAVY METALS <231>

Test preparation: 4.0 g in 40 mL of water

Analysis: To the *Test preparation* add dropwise with vigorous stirring 10 mL of 3 N hydrochloric acid, and filter. Use 25 mL of the filtrate.

Acceptance criteria: NMT 10 ppm

SPECIFIC TESTS

• WATER DETERMINATION, Method I <921>: NMT 1.5%

• ALKALINITY

Sample solution: 2 g in 20 mL of hot water

Analysis: To the *Sample solution* add 2 drops of phenolphthalein TS.

Acceptance criteria: The pink color produced, if any, is discharged by the addition of 0.20 mL of 0.10 N sulfuric acid.

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in well-closed containers.

• USP REFERENCE STANDARDS <11>

USP Sodium Benzoate RS

Sodium Bicarbonate—see Sodium Bicarbonate General Monographs

Sodium Borate

$\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ 381.37
 $\text{Na}_2\text{B}_4\text{O}_7$ 201.22
 Borax [1303-96-4].
 Anhydrous [1330-43-4].

DEFINITION

Sodium Borate contains an amount of $\text{Na}_2\text{B}_4\text{O}_7$ equivalent to NLT 99.0% and NMT 105.0% of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$.

IDENTIFICATION

• A. IDENTIFICATION TESTS—GENERAL, Sodium <191>

Sample solution: 1 in 20

Acceptance criteria: Meets the requirements

• B. IDENTIFICATION TESTS—GENERAL, Borate <191>

Sample solution: 1 in 20

Acceptance criteria: Meets the requirements

ASSAY

• PROCEDURE

Sample: 3 g of Sodium Borate

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.5 N hydrochloric acid VS

Blank: 50 mL of water

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 50 mL of water, add methyl red TS, and titrate with 0.5 N hydrochloric acid VS. [NOTE—Heating on a steam bath may be required initially to effect solution.]

Calculate the percentage of sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) in the *Sample* taken:

$$\text{Result} = [(V - B) \times N \times F] \times 100/W$$

V = volume of *Titrant* consumed by the *Sample* (mL)

B = volume of *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 190.7 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–105.0%

IMPURITIES

• HEAVY METALS <231>

Test preparation: Dissolve 1 g in 16 mL of water and 6 mL of 1 N hydrochloric acid. Dilute with water to 25 mL.

Acceptance criteria: NMT 20 ppm

• CARBONATE AND BICARBONATE

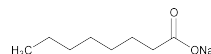
Sample solution: To 5 mL of a solution (1 in 20), contained in a test tube, add 1 mL of 3 N hydrochloric acid.

Acceptance criteria: No effervescence is observed.

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in tight containers.

Sodium Caprylate



$\text{C}_8\text{H}_{15}\text{NaO}_2$
 Sodium octanoate [1984-06-1].

166.19

DEFINITION

Sodium Caprylate contains NLT 99.0% and NMT 101.0% of sodium caprylate ($\text{C}_8\text{H}_{15}\text{NaO}_2$), calculated on the anhydrous basis.

IDENTIFICATION

• A. The retention time of the major peak of *Sample solution* A corresponds to that of the *Standard solution*, as obtained in the test for *Chromatographic Purity in Impurities*.

• B.

Methoxyphenylacetic reagent: Dissolve 2.7 g of methoxyphenylacetic acid in 6 mL of 10% tetramethylammonium hydroxide solution in methanol, and add 20 mL of alcohol. Store in a polyethylene container.

Sample solution: 20 mg

Analysis: Dissolve the *Sample* in 0.5 mL of water, add 1.5 mL of *Methoxyphenylacetic reagent*, and cool in ice water for 30 min. A voluminous, white, crystalline precipitate is formed. Place in water at 20°, and stir for 5 min. The precipitate does not disappear. Add 1 mL of ammonia TS. The precipitate dissolves completely. Add 1 mL of ammonium carbonate solution (160 mg/mL).

Acceptance criteria: No precipitate is formed.

ASSAY

• PROCEDURE

Sample: 150 mg

Blank: Glacial acetic acid

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Potentiometric

Analysis: Transfer the *Sample* to a 125-mL volumetric flask, and dissolve in 50 mL of glacial acetic acid. Titrate with *Titrant*. Perform a blank determination, and make

any necessary correction. Each mL of 0.1 N perchloric acid is equivalent to 16.62 mg of sodium caprylate ($\text{C}_8\text{H}_{15}\text{NaO}_2$).

Acceptance criteria: 99.0%–101.0% on the anhydrous basis

IMPURITIES

• HEAVY METALS, Method II (231)

Test preparation: Dissolve 2.0 g of Sodium Caprylate in 10 mL of glacial acetic acid, and add 10 mL of alcohol.

Standard solution: 1 mL of *Standard Lead Solution* and 9 mL of a mixture of glacial acetic acid and alcohol (1:1)

Analysis: Use 12 mL of the *Test preparation*, and proceed as directed in the chapter.

Acceptance criteria: NMT 5 µg/g

• CHROMATOGRAPHIC PURITY

Standard solution: 1.0 mg/mL of USP Caprylic Acid RS in ethyl acetate

Sample solution A: Dissolve 116 mg of Sodium Caprylate in 5 mL of water, add 1 mL of dilute sulfuric acid (1 in 35), and extract with 10 mL of ethyl acetate. Separate the organic layer, and dry it over anhydrous sodium sulfate.

Sample solution B: Dilute 1.0 mL of *Sample solution A* with ethyl acetate to 100 mL, transfer 5.0 mL of the solution obtained, and dilute with ethyl acetate to 50 mL.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused silica; coated with a 0.25-µm layer of phase G25

Temperatures

Injection port: 250°

Detector: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
100	—	100	1
100	5	220	10

Flow rate: 1.5 mL/min

Carrier gas: Helium

Injection volume: 1 µL

Injection type: Split ratio, 100:1

System suitability

Sample: *Sample solution B*

Suitability requirements

Signal-to-noise ratio: NLT 5

Analysis

Samples: *Standard solution*, *Sample solution A*, and *Sample solution B*

Disregard any peaks with an area less than half of the area of the principal peak from *Sample solution B* and any peak due to the solvent.

Calculate the percentage of each impurity in the portion of Sodium Caprylate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak response of the individual impurity

r_T = sum of all the peak responses

Acceptance criteria

Individual impurities: NMT 0.3%

Total impurities: NMT 0.5%

SPECIFIC TESTS

• APPEARANCE OF SOLUTION

Standard stock solution: Combine 30.0 mL of ferric chloride CS, 30.0 mL of cobaltous chloride CS, and 24.0 mL of cupric sulfate CS, and dilute with 1% (w/v) hydrochloric acid to 100.0 mL.

Standard solution: Dilute 1.0 mL of *Standard stock solution* with 1% (w/v) hydrochloric acid to 100.0 mL.

Sample solution: Dissolve 2.5 g of Sodium Caprylate in 25.0 mL of freshly boiled and cooled water.

Acceptance criteria: The *Sample solution* is clear and colorless, or not more intensely colored than the *Standard solution*.

• PH (791)

Sample solution: Use the *Sample solution* in the test for *Appearance of Solution*.

Acceptance criteria: 8.0–10.5

• WATER DETERMINATION, Method I (921): NMT 3.0%

ADDITIONAL REQUIREMENTS

• USP REFERENCE STANDARDS (11)

USP Caprylic Acid RS

Sodium Carbonate

Na_2CO_3 (anhydrous) 105.99

$\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$ 124.00

Carbonic acid, disodium salt;
Disodium carbonate [497-19-8].
Monohydrate [5968-11-6].

DEFINITION

Sodium Carbonate is anhydrous or contains one molecule of water of hydration. It contains NLT 99.5% and NMT 100.5% of Na_2CO_3 , calculated on the anhydrous basis.

IDENTIFICATION

• **A. IDENTIFICATION TESTS—GENERAL, Sodium (191):** Meets the requirements

• **B. IDENTIFICATION TESTS—GENERAL, Carbonate (191):** Meets the requirements

ASSAY

• PROCEDURE

Sample: 2 g of Sodium Carbonate, previously dried, from the test for *Water Determination*

Blank: 50 mL of water

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 1 N sulfuric acid VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a flask with the aid of 50 mL of water. Add methyl red TS, and titrate with 1 N sulfuric acid VS. Add the acid slowly, with constant stirring, until the solution becomes faintly pink. Heat the solution to boiling, cool, and continue the titration. Heat again to boiling, and titrate further as necessary until the faint pink color is no longer affected by continued boiling.

Calculate the percentage of sodium carbonate (Na_2CO_3) in the *Sample* taken.

$$\text{Result} = [(V - B) \times N \times F \times 100]/W$$

V = Titrant volume consumed by the *Sample* (mL)

B = Titrant volume consumed by the *Blank* (mL)

N = Titrant actual normality (mEq/mL)

F = equivalency factor, 52.99 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 99.5%–100.5% on the anhydrous basis

IMPURITIES

• HEAVY METALS (231)

Test preparation: Dissolve 2.0 g in 10 mL of water.

Analysis: Add 1 drop of phenolphthalein TS to the *Test preparation*, and neutralize the solution with hydrochloric acid, added dropwise. Heat the solution to boiling, and again neutralize by the dropwise addition of hydrochloric acid. Cool, and dilute with water to 25 mL. Proceed as directed in the chapter.

Acceptance criteria: NMT 10 ppm

SPECIFIC TESTS

• WATER DETERMINATION, Method III (921)

Sample: 2 g

Analysis: Dry the *Sample* at 105° for 4 h.

Acceptance criteria: The anhydrous form loses NMT 0.5% of its weight, and the hydrous form loses 12.0%–15.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** Label it to indicate whether it is anhydrous or hydrous.

Sodium Cetostearyl Sulfate

DEFINITION

Sodium Cetostearyl Sulfate is a mixture of sodium cetyl sulfate and sodium stearyl sulfate. It contains NLT 40.0% of sodium cetyl sulfate ($C_{16}H_{33}NaSO_4$), and the sum of the sodium cetyl sulfate content and sodium stearyl sulfate ($C_{18}H_{37}NaSO_4$) content is NLT 90.0% (both contents calculated on the anhydrous basis). It may contain a suitable buffer.

IDENTIFICATION

- **A.** The retention times of the two major peaks of *Sample solution C* correspond to those of the *System suitability solution*, as obtained in the Assay.
- **B. IDENTIFICATION TESTS—GENERAL, Sodium (191):** Meets the requirements of the flame test
- **C.**

Sample solution: 1.0 mg/mL in ethanol

Analysis: Heat 10 mL of *Sample solution* to boiling on a water bath, shaking frequently. Filter immediately, and evaporate to dryness. Dissolve the residue in 7 mL of water, add 3 mL of diluted hydrochloric acid, and evaporate the solution to half its volume. Allow to cool, and filter. To the filtrate add 1 mL of barium chloride solution (60 mg/mL).

Acceptance criteria: A white crystalline precipitate is formed.

ASSAY

• PROCEDURE

System suitability solution: 5 mg/mL each of USP Cetyl Alcohol RS and USP Stearyl Alcohol RS in alcohol

Internal standard solution: 4 mg/mL of 1-heptadecanol in alcohol

Sample solution A: Dissolve 300 mg of Sodium Cetostearyl Sulfate in 50 mL of alcohol, and add 2 mL of the *Internal standard solution* and 48 mL of water. Extract the solution with four 25-mL portions of pentane, adding 10–15 mL of saturated sodium chloride solution, if necessary, to facilitate the separation of the layers. Combine the organic layers, and reserve the hydro-alco-

holic layers for the preparation of *Sample solutions C* and *D*. Wash the organic layer with two 30-mL portions of water, dry over anhydrous sodium sulfate, and filter.

Sample solution B: Dissolve 300 mg of Sodium Cetostearyl Sulfate in 50 mL of alcohol, and add 50 mL of water. Extract the solution with four 25-mL portions of pentane, adding 10–15 mL of saturated sodium chloride solution, if necessary, to facilitate the separation of the layers. Combine the organic layers, wash with two 30-mL portions of water, dry over anhydrous sodium sulfate, and filter.

Sample solution C: Transfer 25 mL of the hydro-alcoholic solution obtained in the preparation of *Sample solution A* to a 200-mL flask that can be fitted with a reflux condenser. Add 20 mL of hydrochloric acid and 10 mL of the *Internal standard solution*, and boil under reflux for 2 h. Allow to cool. Extract with four 20-mL portions of pentane. Wash the combined organic layer with two 20-mL portions of water, dry over anhydrous sodium sulfate, and filter.

Sample solution D: Transfer 25 mL of the hydro-alcoholic solution obtained in the preparation of *Sample solution A* to a 200-mL flask that can be fitted with a reflux condenser. Add 20 mL of hydrochloric acid and 10 mL of alcohol, and boil under reflux for 2 h. Allow to cool. Extract with four 20-mL portions of pentane. Wash the combined organic layer with two 20-mL portions of water, dry over anhydrous sodium sulfate, and filter.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm \times 25-m fused silica capillary; phase G2

Temperatures

Injection port: 250°

Detector: 250°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
150	5	250	Duration of analysis

Injection type: Split ratio; 100:1

Injection volume: 1 μ L

Carrier gas: Nitrogen

Flow rate: 1 mL/min

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between cetyl alcohol and stearyl alcohol

Relative standard deviation: NMT 1.5%

Analysis

Correction for interference: Inject *Sample solution A* and *Sample solution B* into the chromatograph, record the chromatograms, and measure the areas for the major peaks.

If *Sample solution B* shows a peak at the same retention time as the internal standard peak of *Sample solution A*, calculate the ratio:

$$R = S_{CB}/S_I$$

S_{CB} = peak response of cetyl alcohol from *Sample solution B*

S_I = peak response with the same retention time as the internal standard of *Sample solution B*

If R is less than 300, calculate the corrected area, $S_{A(\text{corr})}$, of the peak corresponding to the internal standard of *Sample solution A*:

$$S_{A(\text{corr})} = S_{HA} - (S_I \times S_{CA}/S_{CB})$$

S_{HA} = peak response of the internal standard from *Sample solution A*

S_{CA} = peak response of cetyl alcohol from *Sample solution A*

Inject *Sample solution C* and *Sample solution D* into the chromatograph, record the chromatograms, and measure the areas for the major peaks. Carry out the *Correction for interference* in the same manner as for *Sample solution A*, and calculate the corrected area of the peak corresponding to the internal standard of *Sample solution C*, $S_{C(\text{corr})}$.

Samples: *System suitability solution*, *Sample solution C*, and *Sample solution D*

[NOTE—The substances are eluted in the following order: cetyl alcohol, 1-heptadecanol (internal standard), and stearyl alcohol. Identify the cetyl alcohol and stearyl alcohol peaks in the chromatograms of the *Sample solutions* by comparison with the *System suitability solution*.]

Calculate the percentage of sodium cetyl sulfate ($\text{C}_{16}\text{H}_{33}\text{NaSO}_4$) in the portion of Sodium Cetostearyl Sulfate taken:

$$\text{Result} = (r_C \times W_{CH}) / (S_{C(\text{corr})} \times W_C) \times F \times 100$$

r_C = peak response of cetyl alcohol from *Sample solution C*

W_{CH} = weight of the internal standard added in the preparation of *Sample solution C* (mg)

W_C = weight of Sodium Cetostearyl Sulfate taken to prepare *Sample solution C*, calculated on the anhydrous basis (mg)

F = factor, 1.421

Calculate the percentage of sodium stearyl sulfate ($\text{C}_{18}\text{H}_{37}\text{NaSO}_4$) in the portion of Sodium Cetostearyl Sulfate taken:

$$\text{Result} = B_C \times W_{CH} / (S_{C(\text{corr})} \times W_C) \times F \times 100$$

B_C = peak response of stearyl alcohol from *Sample solution C*

F = factor, 1.377

Acceptance criteria

Sodium cetyl sulfate: NLT 40.0% on the anhydrous basis

Sum of the sodium cetyl sulfate and sodium stearyl sulfate: NLT 90.0% on the anhydrous basis

IMPURITIES

• LIMIT OF SODIUM CHLORIDE AND SODIUM SULFATE

Sodium chloride

Sample: 5 g

Titrimetric system

Mode: Direct titration

Titrant: 0.1 N silver nitrate VS

Endpoint detection: Potentiometric

Analysis: Dissolve the *Sample* in 50 mL of water, and add diluted nitric acid dropwise until the solution is neutral to blue litmus paper. Add 1 mL of potassium chromate TS to the *Sample solution*, and titrate with *Titrant*.

Calculate the percentage of sodium chloride (NaCl) in the Sodium Cetostearyl Sulfate taken:

$$\text{Result} = V \times N / W \times F$$

V = volume of the *Titrant* (mL)

N = actual normality of the *Titrant*

W = weight of the Sodium Cetostearyl Sulfate (g)

F = equivalence factor for sodium chloride, 5.844

Sodium sulfate

Dichloroacetic acid solution: Dilute 67 mL of dichloroacetic acid with water to 300 mL, and neutralize to blue litmus paper using ammonia TS. Cool, add 33 mL of dichloroacetic acid, and dilute with water to 600 mL.

Sample: 0.5 g

Titrimetric system

Mode: Direct titration

Titrant: 0.01 M lead nitrate VS

Endpoint detection: Potentiometric

Analysis: Dissolve the *Sample* in 20 mL of water, warming gently if necessary, and add 1 mL of a solution containing 0.5 g/L of dithizone in acetone. If the solution is red, add 1 N nitric acid dropwise until a bluish-green color is obtained. To the *Sample solution* add 2.0 mL of *Dichloroacetic acid solution* and 80 mL of acetone, and titrate with *Titrant* until a persistent orange-red color is obtained.

Calculate the percentage of sodium sulfate (Na_2SO_4) in the Sodium Cetostearyl Sulfate taken:

$$\text{Result} = V \times M / W \times F$$

V = volume of *Titrant* (mL)

M = actual molarity of *Titrant*

W = weight of the Sodium Cetostearyl Sulfate (g)

F = equivalence factor for sodium sulfate, 14.20

Acceptance criteria: The sum of the percentages of sodium chloride and sodium sulfate is NMT 8.0%.

• LIMIT OF FREE CETOSTEARYL ALCOHOL

Analysis: Examine the chromatogram of *Sample solution A*, obtained as directed in the *Assay*.

Calculate the percentage of free cetostearyl alcohol in the Sodium Cetostearyl Sulfate taken:

$$\text{Result} = 100(r_A + r_B) \times W_{IS} \times (S_{A(\text{corr})} \times W)$$

r_A = peak response of the cetyl alcohol peak from *Sample solution A*

r_B = peak response of stearyl alcohol from *Sample solution A*

W_{IS} = weight of the internal standard added in the preparation of *Sample solution A* (mg)

$S_{A(\text{corr})}$ = corrected peak area corresponding to the internal standard of *Sample solution A* (see *Assay*)

W = weight of Sodium Cetostearyl Sulfate taken to prepare *Sample solution A* (mg)

Acceptance criteria: NMT 4.0%

SPECIFIC TESTS

• ACIDITY OR ALKALINITY

Sample: 500 mg

Analysis: Dissolve the *Sample* by heating in a mixture of 10 mL of water and 15 mL of 90% alcohol. Add 0.1 mL of phenolphthalein TS.

Acceptance criteria: The resulting solution is colorless. Add 0.1 mL of 0.1 N sodium hydroxide, and the resulting solution becomes red.

• WATER DETERMINATION, Method I (921): NMT 1.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

• **LABELING:** Label it to indicate the name and concentration of any added buffer.

• USP REFERENCE STANDARDS (11)

USP Cetyl Alcohol RS

USP Stearyl Alcohol RS

Sodium Chloride—see *Sodium Chloride General Monographs*

Sodium Chloride Injection—see *Sodium Chloride Injection General Monographs*

Sodium Chloride Injection, Bacteriostatic—see *Bacteriostatic Sodium Chloride Injection General Monographs*

Sodium Citrate—see *Sodium Citrate General Monographs*

Sodium Dehydroacetate

$C_8H_7NaO_4$ 190.13
2*H*-Pyran-2,4(3*H*)-dione, 3-acetyl-6-methyl-, monosodium salt [4418-26-2].

DEFINITION

Sodium Dehydroacetate contains NLT 98.0% and NMT 100.5% of sodium dehydroacetate ($C_8H_7NaO_4$), calculated on the anhydrous basis.

IDENTIFICATION

• A. MELTING RANGE OR TEMPERATURE <741>

Sample solution: 150 mg/mL

Analysis: To 10 mL of the *Sample solution* add 5 mL of 3 N hydrochloric acid, collect the crystals by filtration with suction, wash with 10 mL of water, and dry at 80° for 4 h. Determine the melting point as directed in the chapter.

Acceptance criteria: 109°–111°

• B. IDENTIFICATION TESTS—GENERAL, *Sodium* <191>

Sample solution: 1 in 20

Acceptance criteria: Meets the requirements

ASSAY

• PROCEDURE

Sample: 500 mg

Blank: 25 mL of glacial acetic acid containing *p*-naphtholbenzein TS, which has been previously neutralized to a blue color

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 125-mL conical flask, and dissolve it in 25 mL of glacial acetic acid containing *p*-naphtholbenzein TS, which has been previously neutralized to a blue color. Titrate with 0.1 N perchloric acid VS to the original blue color. Perform a blank determination.

Calculate the percentage of dehydroacetate ($C_8H_7NaO_4$) in the *Sample* taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = volume of the *Titrant* consumed by the *Sample* (mL)

V_B = volume of the *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 190.1 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 98.0%–100.5% on the anhydrous basis

IMPURITIES

• **HEAVY METALS**, *Method II* <231>: NMT 10 ppm

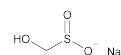
SPECIFIC TESTS

• **WATER DETERMINATION**, *Method I* <921>: 8.5%–10.0%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Sodium Formaldehyde Sulfoxylate



CH_3NaO_3S 118.09

$CH_3NaO_3S \cdot 2H_2O$ 154.11

Methanesulfinic acid, hydroxy-, monosodium salt; Monosodium hydroxymethanesulfinate [149-44-0]. Dihydrate [6035-47-8].

DEFINITION

Sodium Formaldehyde Sulfoxylate contains an amount of sodium formaldehyde sulfoxylate (CH_3NaO_3S) equivalent to NLT 45.5% and NMT 54.5% of SO_2 , calculated on the dried basis. It may contain a suitable stabilizer, such as sodium carbonate.

IDENTIFICATION

• A.

Sample solution: Dissolve 4 g in 10 mL of water in a test tube.

Analysis: To the *Sample solution* add 1 mL of silver–ammonia–nitrate TS.

Acceptance criteria: Metallic silver is produced, either as a finely divided, gray precipitate or as a bright metallic mirror on the inner surface of the tube.

• B.

Sample solution: Dissolve 40 mg of salicylic acid in 5 mL of sulfuric acid, and add 50 mg of Sodium Formaldehyde Sulfoxylate.

Analysis: Warm very gently.

Acceptance criteria: A permanent, deep red color appears.

ASSAY

• PROCEDURE

Sample: 1 g

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N iodine VS. [NOTE—Prepare an adequate amount for both the *Assay* and the test for *Sodium Sulfite*.]

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 50-mL volumetric flask, dissolve in 25 mL of water, and dilute with water to volume. Reserve a portion of this solution for the test for *Sodium Sulfite*. Transfer 4.0 mL of the remaining solution to a conical flask containing 100 mL of water. Titrate with *Titrant*, adding 3 mL of starch TS as the endpoint is approached. Each mL of 0.1 N iodine is equivalent to 1.602 mg of SO_2 .

Acceptance criteria: 45.5%–54.5% of SO_2 on the dried basis

IMPURITIES• **SULFIDE**

Analysis: Dissolve 6 g in 14 mL of water in a test tube, and wet a strip of lead acetate test paper with the clear solution.

Acceptance criteria: No discoloration is evident within 5 min.

• **IRON**

Standard solution: Dissolve 43.2 mg of ferric ammonium sulfate in 10 mL of 2 N sulfuric acid, and add water to make 1000 mL, each mL representing 5 µg of Fe.

Sample solution: Transfer 1.0 g of Sodium Formaldehyde Sulfoxylate to a suitable crucible, and carefully ignite, initially at a low temperature until thoroughly charred, and finally, preferably in a muffle furnace, at 500°–600° until the carbon is all burned off. Cool, dissolve the residue in 2 mL of hydrochloric acid, and dilute with water to 50 mL.

Analysis: To 5.0 mL of the *Standard solution* and 50 mL of the *Sample solution* add 50 mg of ammonium persulfate and 5 mL of ammonium thiocyanate TS, and transfer each to a separate color comparison tube.

Acceptance criteria: 0.0025%; the color of the *Sample* is not deeper than that of the *Standard solution*.

• **SODIUM SULFITE**

Sample solution: 4.0 mL of the solution prepared for the *Assay* in a conical flask containing 100 mL of water

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N iodine VS, prepared in the *Assay*

Endpoint detection: Visual

Analysis: Add 2 mL of formaldehyde TS to the *Sample solution*, and titrate with the *Titrant*, adding 3 mL of starch TS as the endpoint is approached.

Calculate the percentage of sodium sulfite (Na₂SO₃) in the Sodium Formaldehyde Sulfoxylate taken:

$$\text{Result} = (V_2 - V_1) \times (N/W) \times (F \times 1.25)$$

V_2 = volume of 0.1 N iodine VS consumed in the titration performed in the *Assay* (mL)

V_1 = volume of 0.1 N iodine VS consumed in this titration (mL)

N = actual normality of the *Titrant* (mEq/mL)

W = weight of the *Sample* in the *Assay* (g)

F = equivalency weight of sodium sulfite, 63.02 mg/mEq

Acceptance criteria: NMT 5.0% on the dried basis

SPECIFIC TESTS• **PH (791)**

Sample solution: 20 mg/mL

Acceptance criteria: 9.5–10.5

• **LOSS ON DRYING (731)**

Analysis: Dry at 105° for 3 h.

Acceptance criteria: NMT 27.0%

• **ALKALINITY**

Sample solution: 1.0 g of Sodium Formaldehyde Sulfoxylate in 50 mL of water

Analysis: To the *Sample solution* add phenolphthalein TS, and titrate with 0.10 N sulfuric acid.

Acceptance criteria: NMT 3.5 mL is required for neutralization.

• **CLARITY AND COLOR OF SOLUTION**

Sample solution: 1 g of Sodium Formaldehyde Sulfoxylate in 20 mL of water

Analysis: Transfer 10 mL of the *Sample solution* to a 20- × 150-mm test tube. Compare with water in a similar test tube.

Acceptance criteria: The *Sample solution* and the water are equally clear and, when viewed transversely by

transmitted light, exhibit no apparent difference in color.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers, and store at controlled room temperature.

Sodium Hydroxide

NaOH

40.00

Sodium hydroxide [1310-73-2].

DEFINITION

Sodium Hydroxide contains NLT 95.0% and NMT 100.5% of total alkali, calculated as sodium hydroxide (NaOH), including NMT 3.0% of sodium carbonate (Na₂CO₃).

[**CAUTION**—Exercise great care in handling sodium hydroxide, because it rapidly destroys tissues.]

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Sodium (191):** A solution (1 in 25) meets the requirements.

ASSAY• **PROCEDURE**

Sample solution: 1.5 g of Sodium Hydroxide in 40 mL of carbon dioxide-free water. Cool the solution to room temperature.

Blank: 40.0 mL of carbon dioxide-free water

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 1 N sulfuric acid

Endpoint detection: Visual

Analysis: To the *Sample*, add phenolphthalein TS. Titrate with 1 N sulfuric acid VS. At the discharge of the pink color of the indicator, record the volume of *Titrant* (V_{S1}). Add methyl orange TS, and continue the titration until a persistent pink color is produced. Record the volume of *Titrant* (V_{S2}). Perform a blank determination, and make any necessary corrections.

Calculate the percentage of total alkali, calculated as sodium hydroxide (NaOH), in the *Sample* taken:

$$\text{Result} = \{[(V_{S1} - V_B) \times N \times F_1]/W\} \times 100$$

V_{S1} = volume of *Titrant* consumed by the *Sample* to the first endpoint (mL)

V_B = volume of *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F_1 = equivalency factor, 40.00 (mg/mEq)

W = weight of the *Sample* (mg)

Calculate the percentage of sodium carbonate (Na₂CO₃) in the *Sample* taken:

$$\text{Result} = \{[(V_{S2} - V_{S1}) \times N \times F_2]/W\} \times 100$$

V_{S2} = volume of *Titrant* consumed by the *Sample* to the second endpoint (mL)

V_{S1} = volume of *Titrant* consumed by the *Sample* to the first endpoint (mL)

N = actual normality of the *Titrant* (mEq/mL)

F_2 = equivalency factor, 106.0 (mg/mEq)

W = weight of the *Sample* (mg)

Acceptance criteria: 95.0%–100.5% of total alkali; NMT 3.0% of sodium carbonate (Na₂CO₃)

IMPURITIES• **POTASSIUM**

Sample solution: 1 in 20

Analysis: Acidify 5 mL of the *Sample solution* with 6 N acetic acid, then add 5 drops of sodium cobaltinitrite TS.

Acceptance criteria: No precipitate is formed.

• **HEAVY METALS** (231)

Test preparation: Dissolve 0.67 g in a mixture of 5 mL of water and 7 mL of 3 N hydrochloric acid. Heat to boiling, cool, and dilute with water to 25 mL.

Analysis: Proceed as directed in the chapter.

Acceptance criteria: NMT 30 ppm

SPECIFIC TESTS

- **INSOLUBLE SUBSTANCES AND ORGANIC MATTER:** A solution (1 in 20) is complete, clear, and colorless to slightly colored.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Sodium Lauryl Sulfate

Sulfuric acid monododecyl ester sodium salt;
Sodium monododecyl sulfate [151-21-3].

DEFINITION

Sodium Lauryl Sulfate is a mixture of sodium alkyl sulfates consisting chiefly of sodium lauryl sulfate [$\text{CH}_3(\text{CH}_2)_{10}\text{CH}_2\text{OSO}_3\text{Na}$]. The combined content of sodium chloride and sodium sulfate is NMT 8.0%.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Sodium (191):** Ignite 500 mg at 800° until the carbon is consumed. The residue dissolved in 10 mL of water meets the requirements.
- **B. IDENTIFICATION TESTS—GENERAL, Sulfate (191):** A solution (1 in 10), after acidification with hydrochloric acid and gentle boiling for 20 min, meets the requirements.

IMPURITIES**Inorganic Impurities**

- **HEAVY METALS, Method II (231):** 20 ppm
- **SODIUM CHLORIDE**

Sample solution: 100 mg/mL in water

Analysis: Neutralize 50 mL of *Sample solution* with 0.8 N nitric acid, using litmus paper as the indicator. Add 2 mL of potassium chromate TS, and titrate with 0.1 N silver nitrate VS. Each mL of 0.1 N silver nitrate is equivalent to 5.844 mg of NaCl.

SPECIFIC TESTS• **SODIUM SULFATE**

Lead nitrate solution: 33.1 g/L of lead nitrate in water

Analysis: Transfer 1 g of Sodium Lauryl Sulfate, weighed, to a 250-mL beaker. Add 35 mL of water, and warm to dissolve. To the warm solution add 2.0 mL of 1 N nitric acid, mix, and add 50 mL of alcohol. Heat the solution to boiling, and slowly add 10 mL of *Lead nitrate solution*, with stirring. Cover the beaker, simmer for 5 min, and allow to settle. If the supernatant is hazy, allow to stand for 10 min, heat to boiling, and allow to settle. When the solution is almost to the boiling point, decant as much liquid as possible through 9-cm filter paper (Whatman No. 41 or equivalent). Wash four times by decantation, each time using 50 mL of 50% alcohol, and bring the mixture to a boil. Transfer the filter paper to the original beaker, and immediately add 30 mL of water, 20.0 mL of 0.05 M edetate disodium VS, and 1 mL of ammonia-ammonium chloride buffer TS. Warm to dissolve the precipitate, add 0.2 mL of eriochrome black TS, and titrate with 0.05 M

zinc sulfate VS. Each mL of 0.05 M edetate disodium is equivalent to 7.102 mg of Na_2SO_4 .

Acceptance criteria: NMT 8.0% of the combined content of sodium chloride and sodium sulfate

• **ALKALINITY**

Sample solution: Dissolve 1.0 g in 100 mL of water, add phenol red TS, and titrate with 0.10 N hydrochloric acid.

Acceptance criteria: NMT 0.60 mL for neutralization

- **TOTAL ALCOHOLS:** Transfer 5 g to an 800-mL Kjeldahl flask, and add 150 mL of water, 50 mL of hydrochloric acid, and a few boiling chips. Attach a reflux condenser to the Kjeldahl flask, heat carefully to avoid excessive frothing, and boil for 4 h. Cool the flask, rinse the condenser with ether, collecting the ether in the flask, and transfer the contents to a 500-mL separator, rinsing the flask twice with ether and adding the washings to the separator. Extract the solution with two 75-mL portions of ether, evaporate the combined ether extracts in a tared beaker on a steam bath, dry the residue at 105° for 30 min, cool, and weigh.

Acceptance criteria: The residue represents the total alcohols and is NLT 59.0% of the weight of Sodium Lauryl Sulfate taken.

• **UNSULFATED ALCOHOLS**

Sample solution: Dissolve 10 g in 100 mL of water, and add 100 mL of alcohol.

Analysis: Transfer the solution to a separator, and extract with three 50-mL portions of solvent hexane. If an emulsion forms, sodium chloride may be added to promote separation of the two layers. Wash the combined solvent hexane extracts with three 50-mL portions of water, and dry with anhydrous sodium sulfate. Filter the solvent hexane extract into a tared beaker, evaporate on a steam bath until the odor of solvent hexane no longer is perceptible, dry the residue at 105° for 30 min, cool, and weigh.

Acceptance criteria: The weight of the residue is NMT 4.0% of the weight of Sodium Lauryl Sulfate taken.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Sodium Metabisulfite

$\text{Na}_2\text{S}_2\text{O}_5$

190.11

Disulfurous acid, disodium salt;
Disodium pyrosulfite [7681-57-4].

DEFINITION

Sodium Metabisulfite contains an amount of sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) equivalent to NLT 65.0% and NMT 67.4% of SO_2 .

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Sodium (191) and Sulfite (191):** A solution (1 in 20) meets the requirements.

ASSAY• **PROCEDURE**

Sample: 200 mg of Sodium Metabisulfite

Blank: 50.0 mL of 0.1 N iodine VS, accurately measured

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 0.1 N iodine VS

Back-titrant: 0.1 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Add the *Sample* to 50.0 mL of 0.1 N iodine VS in a glass-stoppered conical flask, and swirl to dissolve.

Allow to stand for 5 min, protected from light. Add

1 mL of hydrochloric acid, and titrate the excess iodine with *Back-titrant*, adding 3 mL of starch TS as the endpoint is approached. Perform a blank determination. Calculate the percentage of sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) in the portion of Sodium Metabisulfite taken:

$$\text{Result} = \{[(V_B - V_S) \times N \times F]/W\} \times 100$$

V_B = *Back-titrant* volume consumed by the *Blank* (mL)

V_S = *Back-titrant* volume consumed by the *Sample* (mL)

N = *Back-titrant* normality (mEq/mL)

F = equivalency factor, 32.03 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: 65.0%–67.4% of SO_2

IMPURITIES

• **HEAVY METALS**, *Method I* (231)

Test preparation: 1 g

Analysis: Dissolve the *Test preparation* in 10 mL water. Add 5 mL of hydrochloric acid, evaporate on a steam bath to dryness, and dissolve the residue in 25 mL of water.

Acceptance criteria: NMT 20 ppm

• **LIMIT OF CHLORIDE**

Standard solution: 0.71 mL of 0.020 N hydrochloric acid in 100 mL of water

Sample solution: 1.0 g in 10 mL of water. [NOTE—Pass through a small chloride-free filter, if necessary.] Add 6 mL of 30% hydrogen peroxide. Add 1 N sodium hydroxide until the solution is slightly alkaline to phenolphthalein, and dilute with water to 100 mL.

Analysis

Samples: *Standard solution* and *Sample solution*
Dilute 2.0 mL of the *Samples* with water to 20 mL. Add 1 mL of nitric acid and 1 mL of silver nitrate TS. Allow to stand for 5 min protected from direct sunlight, and compare the turbidity from the *Samples* (see *Spectrophotometry and Light-Scattering* (851), *Visual Comparison*).

Acceptance criteria: Any turbidity produced by the *Sample solution* does not exceed that of the *Standard solution* (0.05%).

• **LIMIT OF THIOSULFATE**

Standard solution: Mix 0.10 mL of 0.10 N sodium thiosulfate with 10 mL of 1 N hydrochloric acid.

Sample solution: Mix 2.2 g with 10 mL of 1 N hydrochloric acid.

Analysis

Samples: *Standard solution* and *Sample solution*
Gently boil the *Samples* for 5 min. Cool, then transfer each solution to a small test tube.

Acceptance criteria: Any turbidity produced by the *Sample solution* does not exceed that of the *Standard solution* (0.05%).

• **IRON** (241)

Test preparation: Dissolve 500 mg of Sodium Metabisulfite in 14 mL of dilute hydrochloric acid (2 in 7), and evaporate on a steam bath to dryness. Dissolve the residue in 7 mL of dilute hydrochloric acid (2 in 7), and again evaporate to dryness. Dissolve the resulting residue in a mixture of 2 mL of hydrochloric acid and 20 mL of water. Add 3 drops of bromine TS, and boil to expel the bromine. Cool, then dilute with water to 47 mL.

Analysis: Proceed as directed in the chapter.

Acceptance criteria: NMT 20 ppm

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-filled, tight containers, and avoid exposure to excessive heat.

Sodium Phosphate, Dibasic—see *Dibasic Sodium Phosphate General Monographs*

Sodium Phosphate, Monobasic—see *Monobasic Sodium Phosphate General Monographs*

Tribasic Sodium Phosphate

Na_3PO_4 (anhydrous)	163.94
Trisodium phosphate, monohydrate	181.96
Phosphoric acid, trisodium salt, dodecahydrate;	
Trisodium phosphate, dodecahydrate	380.13
[10101-89-0].	
Anhydrous [7601-54-9].	

DEFINITION

Tribasic Sodium Phosphate is anhydrous or contains one to twelve molecules of water of hydration. Na_3PO_4 (anhydrous) and $\text{Na}_3\text{PO}_4 \cdot \text{H}_2\text{O}$ (monohydrate) contain NLT 97.0% of Na_3PO_4 , calculated on the ignited basis. $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ (dodecahydrate) contains NLT 92.0% of Na_3PO_4 , calculated on the ignited basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL**, *Sodium* (191) and *Phosphate* (191): A solution (1 in 20) meets the requirements.

ASSAY

• **PROCEDURE**

Sample: 5.5 g of Tribasic Sodium Phosphate, on the anhydrous basis

Blank: 100.0 mL of 1 N hydrochloric acid, accurately measured

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 1 N sodium hydroxide VS

Endpoint detection: Potentiometric

Analysis: Transfer the *Blank* to a 400-mL beaker, and titrate with the *Titrant* to the endpoint at a pH of 7.0. Record as the volume consumed, and designate as A. Transfer the *Sample* to a 400-mL beaker, add 100.0 mL of 1 N hydrochloric acid, and stir until dissolved. Pass a stream of carbon dioxide-free air, in fine bubbles, through the solution for 30 min to expel carbon dioxide, covering the beaker loosely to prevent any loss by spraying. Wash the cover and sides of the beaker with a few mL of water.

Titrate the excess acid potentiometrically with the *Titrant* to the inflection point at a pH of 4. Record the buret reading, and designate as B. Protect the solution from carbon dioxide absorbed from the air, and continue the titration with 1 N sodium hydroxide VS to the inflection point at a pH of 8.8. Record the buret reading, and designate as C.

Calculate the amount of *Titrant* consumed by the *Sample* to the first inflection point, correcting for the *Blank* ($V_1 = A - B$) and the amount of *Titrant* consumed by the *Sample* between the two inflection points ($V_2 = C - B$). If V_1 is equal to or greater than $2V_2$, calculate the amount of Na_3PO_4 in the portion of *Sample* taken:

$$D = V_2 \times N \times F$$

V_2 = volume of *Titrant* consumed between the two inflection points (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 163.9 mg/mEq

If V_1 is less than $2V_2$, calculate the amount of Na_3PO_4 in the portion of *Sample* taken:

$$D = (V_1 - V_2) \times N \times F$$

V_1 = volume of the *Titrant* consumed to the first inflection point, correcting for the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 163.9 mg/mEq

Calculate the percentage of Na_3PO_4 on the ignited basis in the portion of Tribasic Sodium Phosphate taken:

$$\text{Result} = [10/(100 - L)] \times (D/W)$$

L = percentage calculated in the test for *Loss on Ignition* (733)

D = amount of Na_3PO_4 found (mg)

W = weight of the *Sample* (g)

Acceptance criteria: NLT 97.0% of Na_3PO_4 on the ignited basis. $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ (dodecahydrate) contains NLT 92.0% of Na_3PO_4 on the ignited basis.

IMPURITIES

• LOSS ON IGNITION (733)

Sample: 2 g

Analysis: Dry the *Sample* at 110° for 5 h, and then ignite at 800° for 30 min.

Acceptance criteria: The anhydrous form loses NMT 2.0% of its weight, the monohydrate loses 8.0%–11.0% of its weight, and the dodecahydrate loses 45.0%–57.0% of its weight.

• ARSENIC, Method I (211)

Test preparation: Dissolve a portion equivalent to 1.0 g of anhydrous tribasic sodium phosphate in 35 mL of water.

Analysis: Proceed as directed in the chapter.

Acceptance criteria: NMT 3 ppm

• HEAVY METALS, Method I (231)

Test preparation: Dissolve a portion equivalent to 2.0 g of anhydrous tribasic sodium phosphate in 25 mL of water.

Analysis: Proceed as directed in the chapter.

Acceptance criteria: 10 ppm

SPECIFIC TESTS

• INSOLUBLE SUBSTANCES

Sample solution: Dissolve a portion equivalent to 10.0 g of anhydrous tribasic sodium phosphate in 100 mL of hot water.

Analysis: Filter the *Sample solution* through a tared filtering crucible. [NOTE—Do not use glass.] Wash the insoluble residue with hot water, and dry at 105° for 2 h.

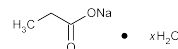
Acceptance criteria: The weight of the residue so obtained does not exceed 20 mg (0.2%).

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.

• **LABELING:** Label it to indicate whether it is anhydrous, the monohydrate, or the dodecahydrate.

Sodium Propionate



$\text{C}_3\text{H}_5\text{NaO}_2 \cdot x\text{H}_2\text{O}$

$\text{C}_3\text{H}_5\text{NaO}_2$

96.06

Propanoic acid, sodium salt, hydrate;
Sodium propionate hydrate [6700-17-0].
Anhydrous [137-40-6].

DEFINITION

Sodium Propionate, dried at 105° for 2 h, contains NLT 99.0% and NMT 100.5% of sodium propionate ($\text{C}_3\text{H}_5\text{NaO}_2$).

IDENTIFICATION

• A. INFRARED ABSORPTION (197K)

Analysis: Perform test on an undried sample.

Acceptance criteria: Meets the requirements

• B. IDENTIFICATION TESTS—GENERAL, Sodium (191)

Sample solution: 1 in 20

Acceptance criteria: Meets the requirements

ASSAY

• PROCEDURE

Sample: 200 mg of Sodium Propionate, previously dried at 105° for 2 h

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Blank: 50 mL of glacial acetic acid

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 50 mL of glacial acetic acid, and add 1 drop of crystal violet TS. Titrate with 0.1 N perchloric acid VS to a green endpoint. Perform a blank determination, and make any necessary correction.

Calculate the percentage of sodium propionate ($\text{C}_3\text{H}_5\text{NaO}_2$) in the *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times N \times F \times 100]/W$$

V_S = volume of the *Titrant* consumed by the *Sample* (mL)

V_B = volume of the *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 96.06 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–100.5% on the dried basis

IMPURITIES

• HEAVY METALS, Method I (231)

Test preparation: Dissolve 2 g of Sodium Propionate in 1 mL of 1 N acetic acid and sufficient water to make 25 mL.

Acceptance criteria: NMT 10 ppm

SPECIFIC TESTS

• WATER DETERMINATION, Method I (921): NMT 1.0%

• ALKALINITY

Sample solution: 2.0 g of Sodium Propionate in 20 mL of water

Analysis: Add phenolphthalein TS to the *Sample solution*.

Acceptance criteria: If a pink color is produced, it is discharged by 0.60 mL of 0.10 N sulfuric acid.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** (11)
USP Sodium Propionate RS

Sodium Starch Glycolate

Starch carboxymethyl ether, sodium salt.

DEFINITION

Sodium Starch Glycolate is the sodium salt of a carboxymethyl ether of starch or of a cross-linked carboxymethyl ether of starch. It may contain NMT 7.0% of Sodium Chloride. The pH and assay requirements for Type A and Type B are set forth in the accompanying table.

Type	pH		% Sodium, Combined as Sodium Starch Glycolate	
	Min.	Max.	Min.	Max.
A	5.5	7.5	2.8	4.2
B	3.0	5.0	2.0	3.4

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B.** A slightly acidified solution is colored blue to violet by the addition of iodine and potassium iodide TS 1.
- **C. PROCEDURE**
Potassium pyroantimonate solution: To 2 g of potassium pyroantimonate add 100 mL of water. Boil the solution for 5 min, cool quickly, and add 10 mL of a solution of potassium hydroxide (3 in 20). Allow to stand for 24 h, and filter.
Analysis: To a 2-mL portion of the *Sample solution* prepared for the test for *Limit of Iron*, add 4 mL of *Potassium pyroantimonate solution*. If necessary, rub the inside of the test tube with a glass rod.
Acceptance criteria: A white, crystalline precipitate is formed.
- **D.** Sodium Starch Glycolate imparts an intense yellow color to a nonluminous flame.

ASSAY**• PROCEDURE**

Sample: 1 g

Analysis: Transfer the *Sample* to a conical flask, add 20 mL of 80% alcohol, stir for 10 min, and filter. Repeat the extraction until the chloride has been completely extracted, as shown by a test with silver nitrate. Dry the insoluble portion at 105° to constant weight, and transfer an accurately weighed portion (700 mg) of the dried 80% alcohol-insoluble portion to a suitable flask. Add 80 mL of glacial acetic acid, heat the mixture under reflux on a boiling water bath for 2 h, cool to room temperature, and titrate with 0.1 N perchloric acid VS, determining the endpoint potentiometrically.

Calculate the percentage of sodium combined in the form of sodium starch glycolate:

$$\text{Result} = 100 \times (22.99) \times V \times N/W$$

V = volume of perchloric acid consumed (mL)

N = normality of the perchloric acid

W = weight of the dried alcohol-insoluble residue taken for the Assay (mg)

Acceptance criteria: 2.8%–4.2% for Type A;
2.0%–3.4% for Type B

OTHER COMPONENTS**• LIMIT OF SODIUM CHLORIDE**

Sample: 500 mg of Sodium Starch Glycolate

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N silver nitrate VS

Endpoint detection: Potentiometric

Indicator electrode: Suitable silver-based

Reference electrode: Double junction electrode containing a 10% potassium nitrate filling solution in the outer jacket and a standard filling solution in the inner jacket

Analysis: Transfer the *Sample* to a beaker, and suspend in 100 mL of water. Add 1 mL of nitric acid. Titrate with the *Titrant*. Each mL of 0.1 N silver nitrate is equivalent to 5.844 mg of sodium chloride.

Acceptance criteria: NMT 7.0%

• LIMIT OF SODIUM GLYCOLATE

[NOTE—Conduct this test without exposure to daylight. Use low-actinic glassware.]

Solution A: 0.1 mg/mL of 2,7-dihydroxynaphthalene in sulfuric acid; allow to stand until decolorized, and use within 2 days.

Standard solution: Transfer 310 mg of glycolic acid, previously dried over phosphorus pentoxide in a desiccator at room temperature overnight, to a 500-mL volumetric flask, and dissolve in and dilute with water to volume. Transfer 5.0 mL of this solution to a 100-mL beaker, add 4 mL of 6 N acetic acid, and allow to stand for about 30 min. Add 50 mL of acetone and 1 g of sodium chloride, mix, and pass through fast filter paper moistened with acetone into a 100-mL volumetric flask. Rinse the beaker and the filter paper with acetone. Combine the filtrate and washings, dilute with acetone to volume, and mix. Allow to stand for 24 h without shaking. Use the clear supernatant as the *Standard solution*.

Sample solution: Transfer 200 mg to a 100-mL beaker. Add 4 mL of 6 N acetic acid and 5 mL of water. Stir until dissolution is complete (about 10 min). Add 50 mL of acetone and 1 g of sodium chloride, and pass through fast filter paper moistened with acetone into a 100-mL volumetric flask. Rinse the beaker and filter paper with acetone. Combine the filtrate and washings, and dilute with acetone to volume. Allow to stand for 24 h without shaking. Use the clear supernatant as the *Sample solution*.

Analysis: Treat the *Sample solution* and the *Standard solution* as follows. Heat 2.0 mL of the solution on a water bath for 20 min to remove the acetone. Cool to room temperature. Add 20.0 mL of *Solution A* to the solution under test, mix, and heat on a water bath for 20 min. Cool under running water, and quantitatively transfer to a 25-mL volumetric flask. Maintain the flask under running water, and dilute with sulfuric acid to volume. Within 10 min, determine the absorbance of the solution at 540 nm with a suitable spectrophotometer, using water as the blank.

Acceptance criteria: The absorbance of the *Sample solution* is NMT that of the *Standard solution* (2.0%).

IMPURITIES**Inorganic Impurities**

- **HEAVY METALS, Method II** (231): 20 ppm

• LIMIT OF IRON

Standard solution: Dissolve 863.4 mg of ferric ammonium sulfate $[\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}]$ in water, add 25 mL of 2 N sulfuric acid, dilute with water to 500.0 mL, and mix. Pipet 10 mL of this solution into a 100-mL volumetric flask, dilute with water to volume, and mix. Pipet 5 mL of this solution into a 100-mL volumetric flask, dilute with water to volume, and mix. This solution contains the equivalent of 1.0 µg/mL of iron.

Sample solution: Place 2.5 g in a silica or platinum crucible, and add 2 mL of 10 N sulfuric acid. Heat on a water bath, then cautiously raise the temperature progressively over an open flame. Ignite, preferably in a muffle furnace, at $600 \pm 25^\circ$. Continue heating until all black particles have disappeared. Cool, add a few drops of 2 N sulfuric acid, and heat and ignite as above. Add a few drops of 2 M ammonium carbonate, evaporate to dryness, and ignite as above. Cool, dissolve the residue in 50 mL of water, and mix.

[NOTE—Reserve a portion of this solution for Identification test C.]

Analysis: Treat the *Sample solution* and the *Standard solution* as follows. Transfer 10 mL of the solution to a suitable beaker, add 2 mL of citric acid solution (1 in 5) and 0.1 mL of thioglycolic acid, and mix. Render the solution alkaline, using litmus paper as an external indicator, by the addition of ammonium hydroxide. Dilute with water to 20 mL, and mix. Allow the solutions to stand for 5 min.

Acceptance criteria: The color of the solution from the *Sample solution* is a shade of pink no deeper than that of the solution from the *Standard solution* (0.002%).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **pH** (791): Disperse 1 g in 30 mL of water. The pH of the resulting suspension is either 5.5–7.5 for Type A or 3.0–5.0 for Type B.
- **LOSS ON DRYING** (731): Dry a sample at 130° for 90 min: it loses NMT 10.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers, preferably protected from wide variations in temperature and humidity, which may cause caking.
- **LABELING:** Label it to indicate the botanical source of the starch from which it was derived, the cross-linking agent (if used), the pH range, and whether it is Type A or Type B.
- **USP REFERENCE STANDARDS** (11)
USP Sodium Starch Glycolate Type A RS
USP Sodium Starch Glycolate Type B RS

Sodium Stearate

Octadecanoic acid, sodium salt;
Sodium stearate [822-16-2].

DEFINITION

Sodium Stearate is a mixture of sodium stearate ($C_{18}H_{35}NaO_2$) and sodium palmitate ($C_{16}H_{31}NaO_2$), which together constitute NLT 90.0% of the total content. The content of $C_{18}H_{35}NaO_2$ is NLT 40.0% of the total. Sodium stearate contains small amounts of the sodium salts of other fatty acids.

IDENTIFICATION

- **A.** When heated, it fuses. At a high temperature it decomposes, emitting flammable vapors and the odor of burning fat, finally leaving a residue that, when moistened with water, is alkaline to litmus paper, effervesces with acids, and colors a nonluminous flame intensely yellow.
- **B.**

Sample: 25 g

Analysis: Dissolve the *Sample* in 300 mL of hot water, add 60 mL of 2 N sulfuric acid, and heat the solution, with frequent stirring, until the separated fatty acid layer is clear. Wash the fatty acids with boiling water

until they are free from sulfate, collect in a small beaker, and warm on a steam bath until the water has settled and the fatty acids are clear. Allow the acids to cool, pour off the water layer, then melt the acids, filter into a dry beaker while hot, and dry at 105° for 20 min.

Acceptance criteria: The solidification temperature of the fatty acids is NLT 54° .

ASSAY

• PROCEDURE

Standard solution: Transfer 50 mg of USP Stearic Acid RS and 50 mg of USP Palmitic Acid RS into a small conical flask fitted with a suitable reflux attachment. Add 5.0 mL of a solution prepared by dissolving 14 g of boron trifluoride in methanol to make 100 mL; swirl and reflux for 15 min or until the solid is dissolved. Cool, transfer the reaction mixture with the aid of 10 mL of chromatographic solvent hexane to a 60-mL separator, and add 10 mL of water and 10 mL of saturated sodium chloride solution. Shake, allow to separate, then drain and discard the lower, aqueous layer. Pass the hexane layer through 6 g of anhydrous sodium sulfate (previously washed with chromatographic solvent hexane) into a suitable flask.

Sample solution: Transfer 100 mg of Sodium Stearate into a small conical flask fitted with a suitable reflux attachment. Add 5.0 mL of a solution prepared by dissolving 14 g of boron trifluoride in methanol to make 100 mL; swirl and reflux for 15 min or until the solid is dissolved. Cool, transfer the reaction mixture with the aid of 10 mL of chromatographic solvent hexane to a 60-mL separator, and add 10 mL of water and 10 mL of saturated sodium chloride solution. Shake, allow to separate, then drain and discard the lower, aqueous layer. Pass the hexane layer through 6 g of anhydrous sodium sulfate (previously washed with chromatographic solvent hexane) into a suitable flask.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 1.5-m \times 3-mm, preferably glass; packed with 15% G4 on support S1A

Carrier gas: Helium, passed through a bed of molecular sieve for drying, if necessary.

Injection volume: 1–2 μ L

Temperatures

Injection port: 210°

Detector: 210°

Column: 165°

System suitability

Samples: *Standard solution* and *Sample solution*

Suitability requirements

Resolution: NLT 2.0 between methyl palmitate and methyl stearate, *Sample solution*

[NOTE—Locate the peaks by comparison with the chromatogram from the *Standard solution*.]

Relative standard deviation: NMT 1.5% for methyl stearate and methyl palmitate for five replicate injections of the *Sample solution*

Analysis

Sample: *Sample solution*

Calculate the percentage of sodium stearate

($C_{18}H_{35}NaO_2$) in the portion of Sodium Stearate taken:

$$\text{Result} = (A/B) \times 100$$

A = peak area due to methyl stearate

B = sum of the peak areas of all the fatty acid esters

Calculate the percentage of sodium palmitate

($C_{16}H_{31}NaO_2$) in the portion of Sodium Stearate taken:

$$\text{Result} = (A/B) \times 100$$

- A = peak area due to methyl palmitate
 B = sum of the peak areas of all the fatty acid esters

Acceptance criteria

Sodium stearate: NLT 40.0%

Sum of sodium stearate and sodium palmitate:
 NLT 90.0%

SPECIFIC TESTS• **ACIDITY**

Sample solution: Heat 50 mL of alcohol to the same temperature, $\pm 5^\circ$, as that attained when the pink endpoint is reached in the titration of the sample specimen. Add 3 drops of phenolphthalein TS and sufficient 0.020 N sodium hydroxide to produce a faint pink color. Add 2.00 g of Sodium Stearate, and dissolve with the aid of a small amount of heat: no pink color is produced.

Analysis: Titrate the solution with 0.020 N sodium hydroxide until a pink color is produced.

Acceptance criteria: 1.00–4.25 mL of 0.020 N sodium hydroxide is required (0.28%–1.2% as stearic acid).

• **LOSS ON DRYING (731)**

Sample: Tare a beaker containing 1 g of washed sand, previously dried at 105° , add 500 mg of Sodium Stearate, and again weigh.

Analysis: To the *Sample* add 10 mL of alcohol and evaporate at 80° to dryness, and dry at 105° for 4 h.

Acceptance criteria: NMT 5.0%

• **FATS AND FIXED OILS, Acid Value of Fatty Acids (401)**

Sample: 1 g of the fatty acids obtained in *Identification* test B

Acceptance criteria: 196–211

• **FATS AND FIXED OILS, Iodine Value of Fatty Acids (401)**

Sample: The fatty acids obtained in *Identification* test B

Acceptance criteria: NMT 4.0

• **ALCOHOL-INSOLUBLE SUBSTANCES**

Sample solution: Reflux 1.0 g with 25 mL of alcohol.

Analysis: It dissolves completely, and the resulting solution is clear or NMT slightly opalescent.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed, light-resistant containers.
- **USP REFERENCE STANDARDS (11)**
 USP Palmitic Acid RS
 USP Stearic Acid RS

Sodium Stearyl Fumarate

DEFINITION

Sodium Stearyl Fumarate contains NLT 99.0% and NMT 101.5% of sodium stearyl fumarate ($C_{22}H_{39}NaO_4$), calculated on the anhydrous basis.

IDENTIFICATION• **INFRARED ABSORPTION (197K)**

Analysis: Perform test on an undried specimen (1 in 300).

Acceptance criteria: Meets the requirements

ASSAY• **PROCEDURE**

Sample: 250 mg

Blank: 10 mL of chloroform

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N perchloric acid VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 50-mL conical flask, mix with 10 mL of chloroform, and add 20 mL of glacial acetic acid to dissolve. Add quinaldine red TS, and titrate with 0.1 N perchloric acid VS.

Calculate the percentage of sodium stearyl fumarate ($C_{22}H_{39}NaO_4$) in the *Sample* taken:

$$\text{Result} = \{(V_s - V_b) \times N \times F / W\} \times 100$$

V_s = volume of *Titrant* consumed by the *Sample* (mL)

V_b = volume of *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 390.5 mg/mEq

W = weight of the *Sample* (mg)

Acceptance criteria: 99.0%–101.5% on the anhydrous basis

OTHER COMPONENTS• **LIMIT OF SODIUM STEARYL MALEATE AND STEARYL ALCOHOL**

Solvent: Chloroform and glacial acetic acid (4:1)

Standard monostearyl maleate stock solution:

1 mg/mL of USP Monostearyl Maleate RS in *Solvent*

Standard monostearyl maleate solution: Pipet 5.0 mL of *Standard monostearyl maleate stock solution*, and dilute with chloroform to 50 mL.

Standard stearyl alcohol stock solution: 1 mg/mL of USP Stearyl Alcohol RS in *Solvent*

Standard stearyl alcohol solution: Pipet 5.0 mL of *Standard stearyl alcohol stock solution*, and dilute with chloroform to 50 mL.

Sample solution: Transfer 200 mg of Sodium Stearyl Fumarate to a small, glass-stoppered conical flask. Add 10.0 mL of *Solvent*. Dissolve by placing the flask in an ultrasonic bath for 10 min.

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel (TLC plates)

Spray reagent: Sulfuric acid and alcohol (1:9).

[NOTE—Add cautiously and with stirring.]

Developing solvent system: Hexanes, toluene, and glacial acetic acid (5:5:1)

Analysis

Samples: *Standard monostearyl maleate solution*, *Standard stearyl alcohol solution*, and *Sample solution*
 Apply 5 μ L of *Standard monostearyl maleate solution* and 10 μ L each of *Standard stearyl alcohol solution* and *Sample solution* to the plate. Immerse the plate in a tank containing a layer of 10 mm of chloroform on the bottom. Allow the solvent front to reach the upper edge of the spots. Withdraw the plate, and dry in a current of cold air. Repeat the immersion, development, and drying. This results in spots having a linear shape. Develop the chromatograph in a saturated chamber containing the *Developing solvent system* until the solvent front has moved 15 cm, and remove the plate from the chamber. Allow to dry for 10 min, and heat in an oven at 90° for 2 min. Allow to cool to room temperature. Replace the plate in the chamber for another 15-cm development, remove the plate, and allow to dry at room temperature for 15 min. Spray the plate with *Spray reagent*. Dry the plate in an oven at 150° for 15 min. Dark spots appear on a light background. Allow to cool. Faint spots at an R_f value of 0.9 may result from traces of distearyl maleate and distearyl fumarate.

Acceptance criteria: The intensity of any spot from the *Sample solution* is not greater than that from the corresponding spot from the *Standard monostearyl maleate solution* and *Standard stearyl alcohol solution*

(NMT 0.25% sodium stearyl maleate, NMT 0.5% stearyl alcohol).

IMPURITIES

- **LEAD** (251): NMT 10 ppm
- **HEAVY METALS**, *Method II* (231): NMT 20 ppm

SPECIFIC TESTS

- **WATER DETERMINATION**, *Method I* (921): NMT 5.0%

Change to read:

- **FATS AND FIXED OILS**, *Saponification Value* (401)

Sample: 450 mg

Ethanol potassium hydroxide: Dissolve 5.5 g of potassium hydroxide in absolute alcohol, heating if necessary to dissolve, and dilute with absolute alcohol to about 1000 mL. Prepare fresh daily, and filter if necessary to remove carbonate.

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 0.1 N hydrochloric acid VS

Blank: 50.0 mL of *Ethanol potassium hydroxide*

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 300-mL conical flask, and add 50.0 mL of *Ethanol potassium hydroxide*, rinsing down the inside of the flask during the addition. Gently reflux the mixture on a steam bath for NLT 2 h, occasionally swirl gently, but avoid splashing the mixture up into the condenser. Rinse the condenser with two 10-mL portions of 70% alcohol, add phenolphthalein TS, and titrate with 0.1 N hydrochloric acid VS to the disappearance of any pink color. Perform a blank determination.

Calculate the *Saponification Value* for Sodium Stearyl Fumarate in the *Sample* taken:

$$\bullet \text{Result} = [(V_B - V_S) \times N \times F] / W$$

V_B = volume of the *Titrant* consumed by the *Blank* (mL)

V_S = volume of the *Titrant* consumed by the *Sample* (mL) • (ERR 1-May-2012)

N = actual normality of the *Titrant* (mEq/mL)

F = molecular weight of potassium hydroxide, 56.11

W = weight of the *Sample* (g)

Acceptance criteria: 142.2–146.0, calculated on the anhydrous basis

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** (11)
 - USP Monostearyl Maleate RS
 - USP Sodium Stearyl Fumarate RS
 - USP Stearyl Alcohol RS

Sodium Sulfite

Na_2SO_3 126.04
[7757-83-7].

DEFINITION

Sodium Sulfite contains NLT 95.0% and NMT 100.5% of sodium sulfite (Na_2SO_3).

IDENTIFICATION

- **A.**

Sample solution: 50 mg/mL of Sodium Sulfite. [NOTE—Reserve portions of the solution so obtained for use in

Identification test B and in the test for *Color and Clarity of Solution*.]

Analysis: Add a drop of phenolphthalein TS.

Acceptance criteria: A pink color is produced.

- **B. IDENTIFICATION TESTS—GENERAL, Sulfate** (191)

Analysis: To 5 mL of the solution from *Identification test A* add 0.5 mL of iodine TS.

Acceptance criteria: The solution is colorless and meets the requirements of the barium chloride test.

- **C. IDENTIFICATION TESTS—GENERAL, Sodium** (191): Meets the requirements of the pyroantimonate precipitate test

ASSAY

PROCEDURE

Sample: 250 mg

Titrimetric system

(See *Titrimetry* (541).)

Mode: Residual titration

Titrant: 0.1 N iodine VS

Back titrant: 0.1 N sodium thiosulfate VS

Blank: 50.0 mL of 0.1 N iodine VS, accurately measured

Endpoint detection: Colorimetric

Analysis: Add the *Sample* to a 500-mL beaker, add 50.0 mL of 0.1 N iodine VS, accurately measured, and shake to dissolve. Add 1 mL of starch TS, and titrate with 0.1 N sodium thiosulfate VS to a clear endpoint. Perform a blank determination, and make any necessary correction. Calculate the percentage of sodium sulfite (Na_2SO_3) in the *Sample* taken:

$$\text{Result} = [(B - V) \times N \times F \times 100] / W$$

B = 0.1 N sodium thiosulfate VS volume consumed by the *Blank*

V = 0.1 N sodium thiosulfate VS volume consumed by the *Sample*

N = actual normality of the *Back titrant* (mEq/mL)

F = equivalency factor, 63.0 mg/mEq

W = weight of *Sample* (mg)

Acceptance criteria: 95.0%–100.5%

IMPURITIES

- **HEAVY METALS**, *Method I* (231)

Sample solution: To 8.0 g of Sodium Sulfite add 25 mL of water. Shake until mostly dissolved, and slowly and carefully add 15 mL of hydrochloric acid. Heat to boiling. Cool, and dilute with water to 100.0 mL. Use a 25-mL portion.

Acceptance criteria: NMT 10 ppm

- **LIMIT OF IRON**

Standard solution: Immediately before use, dilute 1 volume of *Standard Iron Solution*, prepared as directed under *Iron* (241), to 10 mL with water. [NOTE—This solution contains the equivalent of 1 µg/mL of iron.]

Sample solution: 10.0 g of Sodium Sulfite in 25 mL of water. Shake until mostly dissolved, and add 15 mL of hydrochloric acid. Heat to boiling. Cool, and dilute with water to 100.0 mL. Use a 10-mL portion.

Analysis: To the *Standard solution* and the *Sample solution*, separately add 2 mL of a citric acid solution (200 g/L), and then add 0.1 mL of thioglycolic acid. Make alkaline with stronger ammonia water, and dilute with water to 20 mL. Allow to stand for 5 min.

Acceptance criteria: Any pink color in the *Sample solution* is not more intense than that in the *Standard solution* (NMT 10 ppm).

- **LIMIT OF SELENIUM**

[CAUTION—Selenium is toxic; handle with care.]

Selenium standard solution: 100 µg/mL of selenium is prepared as follows. Dissolve 0.1 g of metallic selenium in 2 mL of nitric acid. Evaporate to dryness, add 2 mL of water, and evaporate to dryness. Repeat the addition of water and the evaporation to dryness three more times. Dissolve the residue so obtained in 50 mL of

diluted hydrochloric acid. Transfer to a 1000-mL volumetric flask, and dilute with diluted hydrochloric acid to volume.

Standard solution: To 1.0 g of Sodium Sulfite add 0.2 mL of *Selenium standard solution* and 10 mL of formaldehyde TS, and slowly add 2 mL of hydrochloric acid. Heat in a water bath for 20 min.

Sample solution: To 3.0 g of Sodium Sulfite add 10 mL of formaldehyde TS, and slowly add 2 mL of hydrochloric acid.

Analysis: Heat the *Standard solution* and the *Sample solution* in a water bath for 20 min.

Acceptance criteria: Any pink color in the *Sample solution* is not more intense than that in the *Standard solution* (NMT 10 ppm).

• LIMIT OF THIOSULFATES

Sample solution: 20 mg/mL of Sodium Sulfite

Analysis: To 100 mL of the *Sample solution*, add 10 mL of formaldehyde TS and 10 mL of acetic acid. Allow to stand for 5 min. Add 0.5 mL of starch TS, and titrate with 0.1 N iodine VS. Perform a blank determination (see *Titrimetry* (541), *Residual Titrations*), and note the difference in volumes required.

Acceptance criteria: The difference in volumes is NMT 0.15 mL (NMT 0.1%).

• LIMIT OF ZINC

Zinc standard stock solution: A solution of 1 mL of acetic acid and the amount of zinc sulfate equivalent to 0.440 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 100.0 mL of water. [NOTE—This solution contains the equivalent of 1000 µg/mL of Zn.]

Zinc standard solution: 25 µg/mL of zinc from *Zinc standard stock solution*

Standard solutions: Transfer 1.0-, 2.0-, and 4.0-mL portions of *Zinc standard solution* to separate 100-mL volumetric flasks. Dilute the contents of each flask with water to volume, and mix to obtain solutions having known concentrations of 0.25, 0.5, and 1.0 µg/mL of zinc.

Sample stock solution: 100 mg/mL of Sodium Sulfite is prepared as follows. To 10.0 g of Sodium Sulfite add 25 mL of water. Shake until mostly dissolved, and slowly add 15 mL of hydrochloric acid. Heat to boiling. Cool, and dilute with water to 100.0 mL.

Sample solution: 20.0 mg/mL of Sodium Sulfite from the *Sample stock solution*

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: Zinc emission line at 213.9 nm

Lamp: Zinc hollow-cathode

Flame: Air-acetylene

Analysis

Samples: *Standard solutions* and the *Sample solution*
Plot the absorbances of the *Standard solutions* versus concentration of zinc, in µg/mL, and draw the straight line best fitting the plotted points. From the graph so obtained, determine the concentration of zinc, in µg/mL, in the *Sample solution*.

Acceptance criteria: NMT 25 ppm

SPECIFIC TESTS

• COLOR AND CLARITY OF SOLUTION

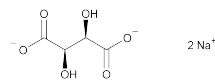
Analysis: Examine the solution prepared for *Identification test A*.

Acceptance criteria: The solution is clear and colorless.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. Store at room temperature.

Sodium Tartrate



$\text{C}_4\text{H}_4\text{Na}_2\text{O}_6 \cdot 2\text{H}_2\text{O}$

230.08

Disodium L-tartrate;

Disodium (+)-2,3-dihydroxybutanedioic acid [868-18-8].

DEFINITION

Sodium Tartrate contains NLT 99.0% and NMT 100.5% of sodium tartrate ($\text{C}_4\text{H}_4\text{Na}_2\text{O}_6$), calculated on the dried basis.

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Sodium (191):** Meets the requirements
- **B. IDENTIFICATION TESTS—GENERAL, Tartrate (191):** Meets the requirements

ASSAY

• PROCEDURE

Sample: 250 mg, previously dried at 150° for 3 h

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N Perchloric acid VS (in glacial acetic acid)

Endpoint detection: Potentiometric

Analysis: Dissolve the *Sample* in 150 mL of glacial acetic acid by stirring and heating to near the boiling point. Cool to room temperature. Titrate with 0.1 N perchloric acid VS (in glacial acetic acid) to a potentiometric endpoint. Perform a blank determination, and make any necessary adjustments. Each mL of 0.1 N perchloric acid is equivalent to 9.703 mg of sodium tartrate ($\text{C}_4\text{H}_4\text{Na}_2\text{O}_6$).

Acceptance criteria: 99.0%–100.5% on the dried basis

IMPURITIES

- **HEAVY METALS, Method I (231):** NMT 20 ppm

SPECIFIC TESTS

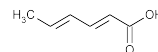
- **pH (791):** 7–9 (1 in 10 solution)
- **LOSS ON DRYING (731):** Dry a sample at 150° for 3 h: it loses 14.0%–17.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a tight container. No storage requirements specified.

Sodium Thiosulfate—see Sodium Thiosulfate General Monographs

Sorbic Acid



$\text{C}_6\text{H}_8\text{O}_2$

112.13

2,4-Hexadienoic acid, (*E,E*)-; 2,4-Hexadienoic acid; (*E,E*)-Sorbic acid; Sorbic acid [110-44-1].

DEFINITION

Sorbic Acid contains NLT 99.0% and NMT 101.0% of $\text{C}_6\text{H}_8\text{O}_2$, calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>
- **B.** A 1-in-400,000 solution in isopropyl alcohol exhibits an absorbance maximum at 254 ± 2 nm.

ASSAY• **PROCEDURE**

Sample solution: Dissolve 250 mg of Sorbic Acid in a mixture of 50 mL of methanol and 25 mL of water that previously has been neutralized with 0.02 N sodium hydroxide. Add phenolphthalein TS.

Analysis: Titrate with 0.1 N sodium hydroxide VS to the first pink color that persists for at least 30 s. Each mL of 0.1 N sodium hydroxide is equivalent to 11.21 mg of $C_6H_8O_2$.

Acceptance criteria: 99.0%–101.0% on the anhydrous basis

IMPURITIES**Inorganic Impurities**

- **RESIDUE ON IGNITION** <281>: NMT 0.2%
- **HEAVY METALS**, *Method II* <231>: 10 ppm

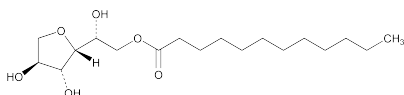
SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** <741>: 132° – 135°
- **WATER DETERMINATION**, *Method I* <921>: NMT 0.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light, and avoid exposure to excessive heat.
- **USP REFERENCE STANDARDS** <11>
USP Sorbic Acid RS

Sorbitan Monolaurate



Sorbitan, esters, monododecanoate;
Sorbitan monolaurate [1338-39-2].

DEFINITION

Sorbitan Monolaurate is a partial ester of lauric acid with Sorbitol and its mono- and dianhydrides. It yields, upon saponification, NLT 55.0% and NMT 63.0% of fatty acids, and NLT 39.0% and NMT 45.0% of polyols (w/w).

IDENTIFICATION

- **A.**
Sample: 1 g of the residue obtained in the Assay for *Fatty Acids*
Acceptance criteria
Fats and Fixed Oils, Acid Value <401>: 260–280
Fats and Fixed Oils, Iodine Value <401>: NMT 5
- **B.**
Standard solution: 33 mg/mL of USP Isosorbide RS, 25 mg/mL of USP 1,4-Sorbitan RS, and 25 mg/mL of sorbitol
Sample solution: 250 mg/mL of the polyols from the Assay for *Polyols*
Chromatographic system
(See *Chromatography* <621>, *Thin-Layer Chromatography*.)
Mode: TLC
Adsorbent: 0.25-mm layer of chromatographic silica gel
Application volume: 2 μ L
Developing solvent system: Acetone and glacial acetic acid (50:1)

Spray reagent: Sulfuric acid and water (50:50)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Standard solution* and *Sample solution*, and allow the spots to dry. Develop the chromatogram until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray evenly with *Spray reagent* until the surface is uniformly wet (do not overspray), and immediately place the sprayed plate on a 200° hot plate in a well-ventilated hood. Char until white fumes of sulfur trioxide cease, and cool.

Acceptance criteria: The R_f values of the spots of the *Sample solution* correspond to those of the spots of the *Standard solution*.

ASSAY• **FATTY ACIDS**

Sample: 10 g

Analysis: Transfer the *Sample* to a 500-mL conical flask. Cautiously add 100 mL of alcohol and 3.0 g of potassium hydroxide, and a few glass beads. Connect a suitable condenser to the flask, reflux the mixture on a hot plate for 2 h, add 100 mL of water, and heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol. Continue the evaporation until the odor of alcohol can no longer be detected, and transfer the saponification mixture, with the aid of 100 mL of hot water, to a 500-mL separator. Using extreme caution, neutralize to litmus paper with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Allow the solution to cool. If salts appear, add sufficient water to produce a clear solution. Cautiously add 100 mL of solvent hexane, shake thoroughly, and withdraw the lower layer into a second 500-mL separator. Similarly extract with two more 100-mL portions of solvent hexane. Extract the combined hexane layers with 50-mL portions of water until neutral to litmus paper. Combine the extracts with the original aqueous phase to use for the Assay for *Polyols*. Evaporate the solvent hexane in a tared beaker on a steam bath nearly to dryness, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the fatty acids.

Acceptance criteria: 55.0%–63.0%

• **POLYOLS**

Sample solution: Aqueous solution retained from the Assay for *Fatty Acids*

Analysis: Neutralize the *Sample solution* with potassium hydroxide solution (1 in 10) to a pH of 7, using a pH meter. Evaporate on a steam bath to a moist residue, extract the polyols from the salts with three 150-mL portions of dehydrated alcohol, boiling the salt residue for 3 min, and crushing it, as necessary, with the flattened end of a stirring rod during each extraction; filtering each extract while hot through a medium-porosity sintered-glass funnel provided with a sheet of retentive filter paper on which a layer of purified siliceous earth has been superimposed; and receiving the filtrates in a 1-L suction flask. Transfer the clear alcoholic polyols solution to a tared beaker, evaporate the alcohol on a steam bath, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the polyols.

Acceptance criteria: 39.0%–45.0% (w/w)

IMPURITIES

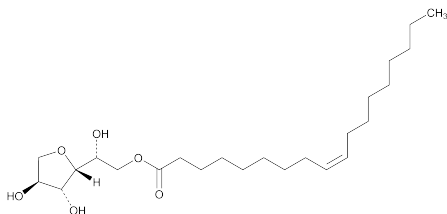
- **RESIDUE ON IGNITION** <281>: NMT 0.5%
- **HEAVY METALS**, *Method II* <231>: NMT 10 μ g/g

SPECIFIC TESTS

- **WATER DETERMINATION**, *Method I* <921>: NMT 1.5%
- **FATS AND FIXED OILS**, *Acid Value* <401>: NMT 8
- **FATS AND FIXED OILS**, *Hydroxyl Value* <401>: 330–358
- **FATS AND FIXED OILS**, *Saponification Value* <401>: 158–170

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** (11)
 - USP Isosorbide RS
 - USP 1,4-Sorbitan RS

Sorbitan Monooleate

Sorbitan, esters, mono(Z)-9-octadecenoate;
Sorbitan monooleate [1338-43-8].

DEFINITION

Sorbitan Monooleate is a partial oleate ester of Sorbitol and its mono- and dianhydrides. It yields, upon saponification, NLT 72.0% and NMT 78.0% of fatty acids, and NLT 25.0% and NMT 31.0% of polyols (w/w).

IDENTIFICATION

- **A.**

Sample: 1 g of the residue obtained in the *Assay for Fatty Acids*

Acceptance criteria

Fats and Fixed Oils, Acid Value (401): 192–204

Fats and Fixed Oils, Iodine Value (401): 75–95
- **B.**

Standard solution: 33 mg/mL of USP Isosorbide RS, 25 mg/mL of USP 1,4-Sorbitan RS, and 25 mg/mL of sorbitol

Sample solution: 250 mg/mL of the polyols, obtained in the *Assay for Polyols*

Chromatographic system
(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 2 μ L

Developing solvent system: Acetone and glacial acetic acid (50:1)

Spray reagent: Sulfuric acid and water (50:50)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Standard solution* and *Sample solution*, and allow the spots to dry. Develop the chromatogram until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray evenly with *Spray reagent* until the surface is uniformly wet (do not overspray), and immediately place the sprayed plate on a 200° hot plate in a well-ventilated hood. Char until white fumes of sulfur trioxide cease, and cool.

Acceptance criteria: The R_f values of the spots from the *Sample solution* correspond to those of the spots from the *Standard solution*.

ASSAY

- **FATTY ACIDS**

Sample: 10 g

Analysis: Transfer the *Sample* to a 500-mL conical flask. Cautiously add 100 mL of alcohol and 3.5 g of potassium hydroxide, and a few glass beads. Connect a suitable condenser to the flask, reflux the mixture on a hot

plate for 2 h, add 100 mL of water, and heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol. Continue the evaporation until the odor of alcohol can no longer be detected, and transfer the saponification mixture, with the aid of 100 mL of hot water, to a 500-mL separator. Using extreme caution, neutralize to litmus paper with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Allow the solution to cool. If salts appear, add sufficient water to produce a clear solution. Cautiously add 100 mL of solvent hexane, shake thoroughly, and withdraw the lower layer into a second 500-mL separator. Similarly extract with two more 100-mL portions of solvent hexane. Extract the combined hexane layers with 50-mL portions of water until neutral to litmus paper. Combine the extracts with the original aqueous phase to use for the *Assay for Polyols*. Evaporate the solvent hexane in a tared beaker on a steam bath nearly to dryness, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the fatty acids.

Acceptance criteria: 72.0%–78.0%

• **POLYOLS**

Sample solution: Aqueous solution retained from the *Assay for Fatty Acids*

Analysis: Neutralize the *Sample solution* with a potassium hydroxide solution (1 in 10) to a pH of 7, using a pH meter. Evaporate on a steam bath to a moist residue, extract the polyols from the salts with three 150-mL portions of dehydrated alcohol, boiling the salt residue for 3 min, and crushing it, as necessary, with the flattened end of a stirring rod during each extraction; filtering each extract, while hot, through a medium-porosity, sintered-glass funnel provided with a sheet of retentive filter paper on which a layer of purified siliceous earth has been superimposed; and receiving the filtrates in a 1-L suction flask. Transfer the clear alcoholic polyols solution to a tared beaker, evaporate the alcohol on a steam bath, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the polyols.

Acceptance criteria: 25.0%–31.0% (w/w)

IMPURITIES

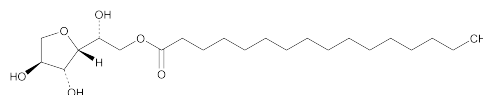
- **RESIDUE ON IGNITION** (281): NMT 0.5%
- **HEAVY METALS, Method II** (231): NMT 10 μ g/g

SPECIFIC TESTS

- **WATER DETERMINATION, Method I** (921): NMT 1.0%
- **FAT AND FIXED OILS, Acid Value** (401): NMT 8
- **FAT AND FIXED OILS, Hydroxyl Value** (401): 190–215
- **FAT AND FIXED OILS, Iodine Value** (401): 62–76
- **FAT AND FIXED OILS, Saponification Value** (401): 145–160

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** (11)
 - USP Isosorbide RS
 - USP 1,4-Sorbitan RS

Sorbitan Monopalmitate

Sorbitan, esters, monohexadecanoate;
Sorbitan monopalmitate [26266-57-9].

DEFINITION

Sorbitan Monopalmitate is a partial ester of palmitic acid with Sorbitol and its mono- and dianhydrides. It yields,

upon saponification, NLT 63.0% and NMT 71.0% of fatty acids, and NLT 32.0% and NMT 38.0% of polyols (w/w).

IDENTIFICATION

- **A.**
Sample: 1 g of the residue obtained in the Assay for Fatty Acids
Acceptance criteria
Fats and Fixed Oils, Acid Value (401): 210–225
Fats and Fixed Oils, Iodine Value (401): NMT 4
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** (201)
Standard solution: 33 mg/mL of USP Isosorbide RS, 25 mg/mL of USP 1,4-Sorbitan RS, and 25 mg/mL of sorbitol
Sample solution: 250 mg/mL of the polyols, obtained in the Assay for Polyols
Chromatographic system
 (See *Chromatography* (621), *Thin-Layer Chromatography*.)
Mode: TLC
Adsorbent: 0.25-mm layer of chromatographic silica gel
Application volume: 2 μ L
Developing solvent system: Acetone and glacial acetic acid (50:1)
Spray reagent: Sulfuric acid and water (50:50)
Analysis
Samples: Standard solution and Sample solution
 Apply the Standard solution and Sample solution, and allow the spots to dry. Develop the chromatogram until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray evenly with *Spray reagent* until the surface is uniformly wet (do not overspray), and immediately place the sprayed plate on a 200° hot plate in a well-ventilated hood. Char until white fumes of sulfur trioxide cease, and cool.
Acceptance criteria: The R_f values of the spots of the Sample solution correspond to those of the spots of the Standard solution.

ASSAY

- **FATTY ACIDS**
Sample: 10 g
Analysis: Transfer the Sample to a 500-mL conical flask. Cautiously add 100 mL of alcohol and 3.0 g of potassium hydroxide. Connect a suitable condenser to the flask, reflux the mixture on a hot plate for 2 h, add 100 mL of water, and heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol. Continue the evaporation until the odor of alcohol can no longer be detected, and transfer the saponification mixture, with the aid of 100 mL of hot water, to a 500-mL separator. Using extreme caution, neutralize to litmus paper with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Allow the solution to cool. If salts appear, add sufficient water to produce a clear solution. Cautiously add 100 mL of solvent hexane, shake thoroughly, and withdraw the lower layer into a second 500-mL separator. Similarly extract with two more 100-mL portions of solvent hexane. Extract the combined hexane layers with 50-mL portions of water until neutral to litmus paper. Combine the extracts with the original aqueous phase to use for the Assay for Polyols. Evaporate the solvent hexane in a tared beaker on a steam bath nearly to dryness, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the fatty acids.
Acceptance criteria: 63.0%–71.0%
- **POLYOLS**
Sample solution: Aqueous solution retained from the Assay for Fatty Acids

Analysis: Neutralize the Sample solution with a potassium hydroxide solution (1 in 10) to a pH of 7, using a pH meter. Evaporate on a steam bath to a moist residue, extract the polyols from the salts with three 150-mL portions of dehydrated alcohol, boiling the salt residue for 3 min, and crushing it, as necessary, with the flattened end of a stirring rod during each extraction; filtering each extract while hot through a medium-porosity, sintered-glass funnel provided with a sheet of retentive filter paper on which a layer of purified siliceous earth has been superimposed; and receiving the filtrates in a 1-L suction flask. Transfer the clear alcoholic polyols solution to a tared beaker, evaporate the alcohol on a steam bath, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the polyols.
Acceptance criteria: 32.0%–38.0% (w/w)

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.5%
- **HEAVY METALS, Method II** (231): NMT 10 μ g/g

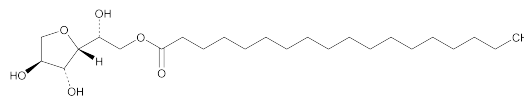
SPECIFIC TESTS

- **WATER DETERMINATION, Method I** (921): NMT 1.5%
- **FATS AND FIXED OILS, Acid Value** (401): NMT 8
- **FATS AND FIXED OILS, Hydroxyl Value** (401): 275–305
- **FATS AND FIXED OILS, Saponification Value** (401): 140–150

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** (11)
 USP Isosorbide RS
 USP 1,4-Sorbitan RS

Sorbitan Monostearate



Sorbitan, esters, mono-octadecanoate;
 Sorbitan Monostearate [1338-41-6].

DEFINITION

Sorbitan Monostearate is a partial ester of Stearic Acid with Sorbitol and its mono- and dianhydrides. It yields, upon saponification, NLT 68.0% and NMT 76.0% of fatty acids, and NLT 27.0% and NMT 34.0% of polyols (w/w).

IDENTIFICATION

- **A.**
Sample: 1 g of the residue obtained in the Assay for Fatty Acids
Acceptance criteria
Fats and Fixed Oils, Acid Value (401): 200–215
Fats and Fixed Oils, Iodine Value (401): NMT 4
- **B.**
Standard solution: 33 mg/mL of USP Isosorbide RS, 25 mg/mL of USP 1,4-Sorbitan RS, and 25 mg/mL of sorbitol
Sample solution: 250 mg/mL of the polyols, obtained in the Assay for Polyols
Chromatographic system
 (See *Chromatography* (621), *Thin-Layer Chromatography*.)
Mode: TLC
Adsorbent: 0.25-mm layer of chromatographic silica gel
Application volume: 2 μ L
Developing solvent system: Acetone and glacial acetic acid (50:1)

Spray reagent: Sulfuric acid and water (50:50)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Standard solution* and *Sample solution*, and allow the spots to dry. Develop the chromatogram until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray evenly with *Spray reagent* until the surface is uniformly wet (do not overspray), and immediately place the sprayed plate on a 200° hot plate in a well-ventilated hood. Char until white fumes of sulfur trioxide cease, and cool.

Acceptance criteria: The R_f values of the spots from the *Sample solution* correspond to those of the spots from the *Standard solution*.

ASSAY

• FATTY ACIDS

Sample: 10 g

Analysis: Transfer the *Sample* to a 500-mL conical flask. Cautiously add 100 mL of alcohol, 3.0 g of potassium hydroxide, and a few glass beads. Connect a suitable condenser to the flask, reflux the mixture on a hot plate for 2 h, add 100 mL of water, and heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol. Continue the evaporation until the odor of alcohol can no longer be detected, and transfer the saponification mixture, with the aid of 100 mL of hot water, to a 500-mL separator. Using extreme caution, neutralize to litmus paper with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Allow the solution to cool. If salts appear, add sufficient water to produce a clear solution. Cautiously add 100 mL of solvent hexane, shake thoroughly, and withdraw the lower layer into a second 500-mL separator. Similarly extract with two more 100-mL portions of solvent hexane. Extract the combined hexane layers with 50-mL portions of water until neutral to litmus paper. Combine the extracts with the original aqueous phase to use for the *Assay for Polyols*. Evaporate the solvent hexane in a tared beaker on a steam bath nearly to dryness, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the fatty acids.

Acceptance criteria: 68.0%–76.0%

• POLYOLS

Sample solution: Aqueous solution retained from the *Assay for Fatty Acids*

Analysis: Neutralize the *Sample solution* with a potassium hydroxide solution (1 in 10) to a pH of 7, using a pH meter. Evaporate on a steam bath to a moist residue, extract the polyols from the salts with three 150-mL portions of dehydrated alcohol, boiling the salt residue for 3 min, and crushing it, as necessary, with the flattened end of a stirring rod, during each extraction; filtering each extract while hot through a medium-porosity, sintered-glass funnel provided with a sheet of retentive filter paper on which a layer of purified siliceous earth has been superimposed; and receiving the filtrates in a 1-L suction flask. Transfer the clear alcoholic polyols solution to a tared beaker, evaporate the alcohol on a steam bath, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the polyols.

Acceptance criteria: 27.0%–34.0% (w/w)

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.5%
- **HEAVY METALS**, *Method II* (231): NMT 10 µg/g

SPECIFIC TESTS

- **WATER DETERMINATION**, *Method I* (921): NMT 1.5%
- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 10
- **FATS AND FIXED OILS**, *Hydroxyl Value* (401): 235–260

- **FATS AND FIXED OILS**, *Saponification Value* (401): 147–157

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers
- **USP REFERENCE STANDARDS** (11)
 - USP Isosorbide RS
 - USP 1,4-Sorbitan RS

Sorbitan Sesquioleate

DEFINITION

Sorbitan Sesquioleate is a partial oleate ester of Sorbitol and its mono- and dianhydrides. It yields, upon saponification, NLT 74.0% and NMT 80.0% of fatty acids, and NLT 22.0% and NMT 28.0% of polyols (w/w).

IDENTIFICATION

• A.

Sample: 1 g of the residue obtained in the *Assay for Fatty Acids*

Acceptance criteria

Fats and Fixed Oils, *Acid Value* (401): 192–204

Fats and Fixed Oils, *Iodine Value* (401): 75–95

• B.

Standard solution: 33 mg/mL of USP Isosorbide RS, 25 mg/mL of USP 1,4-Sorbitan RS, and 25 mg/mL of sorbitol

Sample solution: 250 mg/mL of the polyols, obtained in the *Assay for Polyols*

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Mode: TLC

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 2 µL

Developing solvent system: Acetone and glacial acetic acid (50:1)

Spray reagent: Sulfuric acid and water (50:50)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Standard solution* and *Sample solution*, and allow the spots to dry. Develop the chromatogram until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray evenly with *Spray reagent* until the surface is uniformly wet (do not overspray), and immediately place the sprayed plate on a 200° hot plate in a well-ventilated hood. Char until white fumes of sulfur trioxide cease, and cool.

Acceptance criteria: The R_f values of the spots from the *Sample solution* correspond to those of the spots from the *Standard solution*.

ASSAY

• FATTY ACIDS

Sample: 10 g

Analysis: Transfer the *Sample* to a 500-mL conical flask. Cautiously add 100 mL of alcohol, 3.5 g of potassium hydroxide, and a few glass beads. Connect a suitable condenser to the flask, reflux the mixture on a hot plate for 2 h, add 100 mL of water, and heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol. Continue the evaporation until the odor of alcohol can no longer be detected, and transfer the saponification mixture, with the aid of 100 mL of hot water, to a 500-mL separator. Using extreme caution, neutralize to litmus paper with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Allow the solution to cool. If salts appear, add sufficient

water to produce a clear solution. Cautiously add 100 mL of solvent hexane, shake thoroughly, and withdraw the lower layer into a second 500-mL separator. Similarly extract with two more 100-mL portions of solvent hexane. Extract the combined hexane layers with 50-mL portions of water until neutral to litmus paper. Combine the extracts with the original aqueous phase to use for the *Assay for Polyols*. Evaporate the solvent hexane in a tared beaker on a steam bath nearly to dryness, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the fatty acids.

Acceptance criteria: 74.0%–80.0%

• POLYOLS

Sample solution: Aqueous solution retained from the *Assay for Fatty Acids*

Analysis: Neutralize the *Sample solution* with a potassium hydroxide solution (1 in 10) to a pH of 7, using a pH meter. Evaporate on a steam bath to a moist residue, extract the polyols from the salts with three 150-mL portions of dehydrated alcohol, boiling the salt residue for 3 min, and crushing it, as necessary, with the flattened end of a stirring rod, during each extraction; filtering each extract while hot through a medium-porosity, sintered-glass funnel provided with a sheet of retentive filter paper on which a layer of purified siliceous earth has been superimposed; and receiving the filtrates in a 1-L suction flask. Transfer the clear alcoholic polyols solution to a tared beaker, evaporate the alcohol on a steam bath, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the polyols.

Acceptance criteria: 22.0%–28.0% (w/w)

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 1.4%
- **HEAVY METALS**, *Method II* (231): NMT 10 µg/g

SPECIFIC TESTS

- **WATER DETERMINATION**, *Method I* (921): NMT 1.0%
- **FATS AND FIXED OILS**, *Acid Value* (401): NMT 14
- **FATS AND FIXED OILS**, *Hydroxyl Value* (401): 182–220
- **FATS AND FIXED OILS**, *Iodine Value* (401): 65–75
- **FATS AND FIXED OILS**, *Saponification Value* (401): 143–165

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **USP REFERENCE STANDARDS** (11)
 - USP Isosorbide RS
 - USP 1,4-Sorbitan RS

Sorbitan Trioleate

DEFINITION

Sorbitan Trioleate is the triester of Oleic Acid and Sorbitol and its mono- and dianhydrides. It yields, upon saponification, NLT 85.5% and NMT 90.0% of fatty acids, and NLT 13.0% and NMT 19.0% of polyols (w/w).

IDENTIFICATION

- **A.**
 - Sample:** 1 g of the residue of oleic acid obtained in the *Assay for Fatty Acids*
 - Acceptance criteria**
 - Fats and Fixed Oils*, *Acid Value* (401): 192–204
 - Fats and Fixed Oils*, *Iodine Value* (401): 75–95
- **B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST** (201)
 - Standard solution:** 33 mg/mL of USP Isosorbide RS, 25 mg/mL of USP 1,4-Sorbitan RS, and 25 mg/mL of sorbitol
 - Sample solution:** 250 mg/mL of polyols, obtained in the *Assay for Polyols*

Chromatographic system

(See *Chromatography* (621), *Thin-Layer Chromatography*.)

Adsorbent: 0.25-mm layer of chromatographic silica gel

Application volume: 2 µL

Developing solvent system: Acetone and glacial acetic acid (50:1)

Spray reagent: Sulfuric acid and water (50:50)

Analysis

Samples: *Standard solution* and *Sample solution*

Apply the *Standard solution* and the *Sample solution*, and allow the spots to dry. Develop the chromatogram until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, and allow the solvent to evaporate. Spray evenly with *Spray reagent* until the surface is uniformly wet (do not overspray), and immediately place the sprayed plate on a 200° hot plate in a well-ventilated hood. Char until white fumes of sulfur trioxide cease, and cool.

Acceptance criteria: The R_f values of the spots of the *Sample solution* correspond to those of the spots of the *Standard solution*.

ASSAY

• FATTY ACIDS

Sample: 8.6 g

Analysis: Transfer the *Sample* to a 500-mL conical flask. Cautiously add 100 mL of alcohol and 3.5 g of potassium hydroxide, then add a few glass beads, and mix. Connect a suitable condenser to the flask, reflux the mixture on a hot plate for 2 h, add 100 mL of water, and heat on a steam bath to evaporate the alcohol, adding water occasionally to replace the alcohol. Continue the evaporation until the odor of alcohol can no longer be detected, and transfer the saponification mixture, with the aid of 100 mL of hot water, to a 500-mL separator. Using extreme caution, neutralize to litmus paper with a mixture of equal volumes of sulfuric acid and water, noting the volume used, and add a 10% excess of the dilute acid. Allow the solution to cool. If salts appear, add sufficient water to produce a clear solution. Cautiously add 100 mL of solvent hexane, shake thoroughly, and withdraw the lower layer into a second 500-mL separator. Similarly extract with two more 100-mL portions of solvent hexane. Extract the combined hexane layers with 50-mL portions of water until neutral to litmus paper. Combine the extracts with the original aqueous phase to use for the *Assay for Polyols*. Evaporate the solvent hexane in a tared beaker on a steam bath nearly to dryness, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the fatty acids.

Acceptance criteria: 85.5%–90.0%

• POLYOLS

Sample solution: Aqueous solution of polyols retained from the *Assay for Fatty Acids*

Analysis: Neutralize the *Sample solution* with potassium hydroxide solution (1 in 10) to a pH of 7, using a pH meter. Evaporate on a steam bath to a moist residue. Extract the polyols from the salts with three 150-mL portions of dehydrated alcohol, boiling the salt residue for 3 min, and crushing it, as necessary, with the flattened end of a stirring rod during each extraction. Filter each extract while hot through a medium-porosity, sintered-glass funnel, provided with a sheet of retentive filter paper on which a layer of purified siliceous earth has been superimposed, and receiving the filtrates in a 1-L suction flask. Transfer the clear alcoholic polyols solution to a tared beaker, evaporate the alcohol on a steam bath, dry under vacuum at 60° for 1 h, cool in a desiccator, and weigh the polyols.

Acceptance criteria: 13.0%–19.0% (w/w)

IMPURITIES

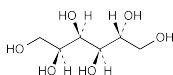
- **RESIDUE ON IGNITION** (281): NMT 0.25%
- **HEAVY METALS**, Method II (231): NMT 10 µg/g

SPECIFIC TESTS

- **WATER DETERMINATION**, Method I (921): NMT 0.7%
- **FATS AND FIXED OILS**, Acid Value (401): NMT 17
- **FATS AND FIXED OILS**, Hydroxyl Value (401): 50–75
- **FATS AND FIXED OILS**, Iodine Value (401): 77–85
- **FATS AND FIXED OILS**, Saponification Value (401): 169–183

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE**: Preserve in tight containers.
- **USP REFERENCE STANDARDS** (11)
 - USP Isosorbide RS
 - USP 1,4-Sorbitan RS
 - C₆H₁₂O₅ 164.16

Sorbitol

C₆H₁₄O₆
D-Glucitol [50-70-4].

182.17

DEFINITION

Sorbitol contains NLT 91.0% and NMT 100.5% of D-sorbitol, calculated on the anhydrous basis. The amounts of total sugars, other polyhydric alcohols, and any hexitol anhydrides, if detected, are not included in the requirements, nor in the calculated amount under *Other Impurities* in *General Notices*.

IDENTIFICATION

- **A.**

Sample solution: 1 g of Sorbitol in 75 mL of water

Analysis: Transfer 3 mL of *Sample solution* to a 15-cm test tube, and add 3 mL of freshly prepared catechol solution (1 in 10), and mix. Add 6 mL of sulfuric acid, then gently heat the tube in a flame for 30 s.

Acceptance criteria: A deep pink or wine-red color appears.
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that from the *Standard solution*, as obtained in the *Assay*.

ASSAY**PROCEDURE**

Mobile phase: Use degassed water.

System suitability solution: Prepare a solution containing 4.8 mg/g of each USP Sorbitol RS and mannitol

Standard solution: 4.8 mg/g of USP Sorbitol RS

Sample solution: Dissolve 0.10 g of Sorbitol in water, and dilute with water to 20 g. Record the final solution weight, and mix thoroughly.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 10-cm; packing L34

Temperature

Column: 50 ± 2°

Detector: 35°

Flow rate: 0.7 mL/min

Injection size: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for mannitol and sorbitol are about 0.6 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between sorbitol and mannitol, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage, on the anhydrous basis, of D-sorbitol in the portion of Sorbitol taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times (100/(100 - W)) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Sorbitol RS in the *Standard solution* (mg/g)

C_U = concentration of Sorbitol in the *Sample solution* (mg/g)

W = percentage obtained in the test for *Water Determination*

Acceptance criteria: 91.0%–100.5% on the anhydrous basis

IMPURITIES**LIMIT OF NICKEL**

Sample solution: Dissolve 20.0 g of Sorbitol in diluted acetic acid, and dilute with diluted acetic acid to 150 mL.

Blank solution: 150 mL of diluted acetic acid

Standard solutions: Prepare three solutions by adding 0.5, 1.0, and 1.5 mL of nickel standard solution TS to 20.0 g of Sorbitol dissolved in diluted acetic acid, and dilute with the same solvent to 150 mL.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Flame: Air–acetylene

Analysis

Samples: *Standard solutions* and *Sample solution*

To each sample add 2.0 mL of a saturated ammonium pyrrolidinedithiocarbamate solution (containing 10 g/L of ammonium pyrrolidinedithiocarbamate) and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer. Set the instrument to zero using the organic layer from the *Blank solution*. Concomitantly determine the absorbances of the organic layer from the *Samples* at least three times each. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Between each measurement, aspirate the organic layer from the *Blank solution*, and ascertain that the reading returns to zero. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.

Acceptance criteria: NMT 1 ppm

- **RESIDUE ON IGNITION** (281): NMT 0.1%, determined on a 1.5-g portion

REDUCING SUGARS

[NOTE—The amount determined in this test is not included in the calculated amount under *Other Impurities* in the *General Notices*.]

Sample solution: Dissolve 3.3 g of Sorbitol in 3 mL of water with the aid of gentle heat. Cool, and add 20.0 mL of cupric citrate TS and a few glass beads. Heat so that boiling begins after 4 min, and maintain

boiling for 3 min. Cool rapidly, and add 40 mL of diluted acetic acid, 60 mL of water, and 20.0 mL of 0.05 N iodine VS. With continuous shaking, add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water.

Analysis: When the precipitate has dissolved, titrate the excess of iodine with 0.05 N sodium thiosulfate VS using 2 mL of starch TS, added toward the end of the titration, as an indicator.

Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate VS is required, corresponding to NMT 0.3% of reducing sugars, as glucose.

- **CHLORIDE AND SULFATE, Chloride** (221) (if labeled for use in preparing parenteral dosage forms)

Sample: 1.5 g

Acceptance criteria: The *Sample* shows no more chloride than corresponds to 0.10 mL of 0.020 N hydrochloric acid (NMT 0.0050%).

- **CHLORIDE AND SULFATE, Sulfate** (221) (if labeled for use in preparing parenteral dosage forms)

Sample: 1.0 g

Acceptance criteria: The *Sample* shows no more sulfate than corresponds to 0.10 mL of 0.020 N sulfuric acid (NMT 0.01%).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62):

The total aerobic count using the *Plate Method* is NMT 1000 cfu/g, and the total combined molds and yeasts count is NMT 100 cfu/g.

- **pH** (791): 3.5–7.0, in a 10% (w/w) solution in carbon dioxide-free water

- **WATER DETERMINATION, Method I** (921): NMT 1.5%

- **CLARITY AND COLOR OF SOLUTION** (if labeled for use in preparing parenteral dosage forms)

Sample: 10.0 g

Analysis: Dissolve the *Sample* in 100.0 mL of carbon dioxide-free water.

Acceptance criteria: The solution is clear and colorless.

- **BACTERIAL ENDOTOXINS TEST** (85) (if labeled for use in preparing parenteral dosage forms): NMT 4 USP Endotoxin Units/g for parenteral dosage forms having a concentration of less than 100 g/L of sorbitol, and NMT 2.5 USP Endotoxin Units/g for parenteral dosage forms having a concentration of 100 g/L or more of sorbitol

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.
- **LABELING:** Sorbitol intended for use in preparing parenteral dosage forms is so labeled.
- **USP REFERENCE STANDARDS** (11)
USP Endotoxin RS
USP Sorbitol RS

Noncrystallizing Sorbitol Solution

DEFINITION

Noncrystallizing Sorbitol Solution is an aqueous solution containing NLT 45.0% of D-sorbitol ($C_6H_{14}O_6$) (w/w). The amounts of total sugars, other polyhydric alcohols, and any hexitol anhydrides, if detected, are not included in the requirements nor in the calculated amount under *General Notices*, 5.60.10. *Other Impurities in USP and NF Articles*.

IDENTIFICATION

- **A. PROCEDURE**

Sample solution: Dissolve 1.4 g of Noncrystallizing Sorbitol Solution in 75 mL of water.

Analysis: Transfer 3 mL of *Sample solution* to a 15-cm test tube. Add 3 mL of freshly prepared catechol solution (1 in 10), and mix. Add 6 mL of sulfuric acid, mix again, and gently heat the tube in a flame for 30 s.

Acceptance criteria: A deep pink or wine-red color appears.

- **B.** The retention time of the major peak from the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.

- **C. LIMIT OF DIETHYLENE GLYCOL AND ETHYLENE GLYCOL**

Diluent: Acetone and water (96:4)

Standard solution: 0.08 mg/mL of USP Diethylene Glycol RS and 0.08 mg/mL of USP Ethylene Glycol RS in *Diluent*.

Sample solution: Transfer 2.0 g of Noncrystallizing Sorbitol Solution to a 25-mL volumetric flask. Add 1.0 mL of *Diluent* to the flask, and mix on a vortex mixer for 3 min. Add the remaining *Diluent* to the flask to volume in three equal portions. Mix on a vortex mixer for about 3 min after each addition of *Diluent*. Pass a portion of the supernatant layer obtained through a 0.45- μ m nylon filter. Discard the first 2 mL of the filtrate, and collect the rest of the filtrate for analysis.

[NOTE—Acetone is used to precipitate sorbitol.]

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm \times 15-m fused-silica capillary column; 0.25- μ m layer of phase G46

Temperature

Detector: 300°

Injector port: 240°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	50	300	5

Carrier gas: Helium

Flow rate: 3.0 mL/min

Injection size: 1.0 μ L

Injection type: Split injection. The split ratio is about 10:1. [NOTE—A split liner, deactivated with glass wool, is used.]

System suitability

Sample: *Standard solution*

[NOTE—Diethylene glycol elutes after ethylene glycol in the chromatogram]

Suitability requirements

Resolution: NLT 30 between ethylene glycol and diethylene glycol

Analysis

Samples: *Standard solution* and *Sample solution*

Based on the *Standard solution*, identify the peaks of ethylene glycol and diethylene glycol. Compare the peak areas of ethylene glycol and diethylene glycol in the *Standard solution* and the *Sample solution*.

Acceptance criteria

Diethylene glycol: The peak area of diethylene glycol in the *Sample solution* is NMT the peak area of diethylene glycol in the *Standard solution*, corresponding to NMT 0.10% of diethylene glycol in Noncrystallizing Sorbitol Solution.

Ethylene glycol: The peak area of ethylene glycol in the *Sample solution* is NMT the peak area of ethylene glycol in the *Standard solution*, corresponding to NMT 0.10% of ethylene glycol in Noncrystallizing Sorbitol Solution.

ASSAY**• PROCEDURE**

Mobile phase: Use degassed water.

System suitability solution: 4.8 mg/g each of mannitol and USP Sorbitol RS

Standard solution: 4.8 mg/g of USP Sorbitol RS

Sample solution: Weigh 0.20 g of Noncrystallizing Sorbitol Solution, and dissolve in and dilute with water to 20 g. Record the final solution weight.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm × 10-cm; packing L34

Temperature

Detector: 35°

Column: 50 ± 2°

Flow rate: 0.7 mL/min

Injection size: 10 µL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for mannitol and sorbitol are about 0.6 and 1.0, respectively, *System suitability solution*.]

Suitability requirements

Resolution: NLT 2.0 between sorbitol and mannitol, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of D-sorbitol (C₆H₁₄O₆) in the portion of Noncrystallizing Sorbitol Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Sorbitol RS in the *Standard solution* (mg/g)

C_U = concentration of Noncrystallizing Sorbitol Solution in the *Sample solution* (mg/g)

Acceptance criteria: NLT 45.0%

IMPURITIES**Inorganic Impurities**

• RESIDUE ON IGNITION <281>: NMT 0.1%, calculated on the anhydrous basis, determined on a 2-g portion

• LIMIT OF NICKEL

Solution A: A saturated ammonium pyrrolidine dithiocarbamate solution (containing 10 mg/mL of ammonium pyrrolidine dithiocarbamate)

Sample solution: Dissolve 20.0 g of Noncrystallizing Sorbitol Solution in diluted acetic acid, and dilute with diluted acetic acid to 100.0 mL. Add 2.0 mL of *Solution A* and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.

Blank solution: Prepare as directed for the *Sample solution*, except omit the use of the Noncrystallizing Sorbitol Solution.

Standard solutions: Prepare as directed for the *Sample solution*, except prepare three solutions by adding 0.5, 1.0, and 1.5 mL of nickel standard solution TS.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 232.0 nm (maximum absorbance)

Lamp: Nickel hollow-cathode

Flame: Air–acetylene

Analysis

Samples: *Standard solutions*, *Sample solution*, and *Blank solution*

Set the instrument to zero using the *Blank solution*. Concomitantly determine the absorbances of the *Standard solutions* and the *Sample solution* at least three times each. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Between each measurement, aspirate the *Blank solution*, and ascertain that the reading returns to zero. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.

Acceptance criteria: NMT 1 µg/g on the anhydrous basis

Organic Impurities**• PROCEDURE: REDUCING SUGARS**

Sample: An amount of Noncrystallizing Sorbitol Solution, equivalent to 3.3 g on the anhydrous basis.

Analysis: To the *Sample*, add 3 mL of water, 20.0 mL of cupric citrate TS, and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 40 mL of diluted acetic acid, 60 mL of water, and 20.0 mL of 0.05 N iodine VS. With continuous shaking, add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water. When the precipitate has dissolved, titrate the excess of iodine with 0.05 N sodium thiosulfate VS using 2 mL of starch TS, added towards the end of the titration, as an indicator.

Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate VS is required corresponding to NMT 0.3% of reducing sugars, on the anhydrous basis, as glucose. The amount determined in this test is not included in the calculated amount under *General Notices*, 5.60.10. *Other Impurities in USP and NF Articles*.

SPECIFIC TESTS

- MICROBIAL ENUMERATION TESTS** <61> and **ABSENCE OF SPECIFIED MICROORGANISMS** <62>: The total aerobic microbial count using the *Plate Method* is NMT 1000 cfu/mL, and the total combined molds and yeasts count is NMT 100 cfu/mL.
- pH** <791>: 5.0–7.5, in a 14% (w/w) solution of Noncrystallizing Sorbitol Solution in carbon dioxide-free water
- WATER DETERMINATION, Method I** <921>: 28.5%–31.5%

ADDITIONAL REQUIREMENTS

- PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements are specified.
- USP REFERENCE STANDARDS** <11>
 - USP Sorbitol RS
 - USP Diethylene Glycol RS
 - USP Ethylene Glycol RS

Sorbitol Sorbitan Solution

Former Title: Anhydriized Liquid Sorbitol

DEFINITION

Sorbitol Sorbitan Solution is a water solution containing, on the anhydrous basis, NLT 25.0% of D-sorbitol (C₆H₁₄O₆) and NLT 15.0% of 1,4-sorbitan (C₆H₁₂O₅). The amounts of total sugars, other polyhydric alcohols, and any other hexitol anhydrides, if detected, are not included in the requirements or in the calculated amount under *General Notices*, 5.60.10. *Other Impurities in USP and NF Articles*.

IDENTIFICATION**A. PROCEDURE**

Sample: 1.4 g of Sorbitol Sorbitan Solution in 75 mL of water

Analysis: Transfer 3 mL of *Sample* to a 15-cm test tube. Add 3 mL of freshly prepared catechol solution (1 in 10), and mix. Add 6 mL of sulfuric acid, mix again, then gently heat the tube in a flame for about 30 s.

Acceptance criteria: A deep pink or wine-red color appears.

- B.** The retention times of the *Sample solution* correspond to those of the *Standard solution*, as obtained in the *Assay*.

C. LIMIT OF DIETHYLENE GLYCOL AND ETHYLENE GLYCOL

Diluent: Acetone and water (96:4)

Standard solution: 0.08 mg/mL of USP Diethylene Glycol RS and 0.08 mg/mL of USP Ethylene Glycol RS in *Diluent*

Sample solution: Transfer 2.0 g of Sorbitol Sorbitan Solution to a 25-mL volumetric flask. Add 1.0 mL of *Diluent* to the flask, and mix on a vortex mixer for about 3 min. Add the remaining *Diluent* to the flask to volume in three equal portions. Mix on a vortex mixer for about 3 min after each addition of *Diluent*. Pass a portion of the supernatant layer obtained through a 0.45- μ m nylon filter. Discard the first 2 mL of the filtrate, and collect the rest of the filtrate for analysis. [NOTE—Acetone is used to precipitate sorbitol.]

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm \times 15-m fused-silica capillary column; 0.25- μ m layer of phase G46

Temperature

Detector: 300°

Injector port: 240°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	50	300	5

Carrier gas: Helium

Flow rate: 3.0 mL/min

Injection size: 1.0 μ L

Injection type: Split injection. The split ratio is about 10:1. [NOTE—A split liner, deactivated with glass wool, is used.]

System suitability

Sample: *Standard solution*

[NOTE—Diethylene glycol elutes after ethylene glycol in the chromatogram.]

Suitability requirements

Resolution: NLT 30 between ethylene glycol and diethylene glycol

Analysis

Samples: *Standard solution* and *Sample solution*
Based on the *Standard solution*, identify the peaks of ethylene glycol and diethylene glycol. Compare peak areas of ethylene glycol and diethylene glycol in the *Standard solution* and the *Sample solution*.

Acceptance criteria

Diethylene glycol: The peak area of diethylene glycol in the *Sample solution* is NMT the peak area of diethylene glycol in the *Standard solution*, corresponding to NMT 0.10% of diethylene glycol in Sorbitol Sorbitan Solution.

Ethylene glycol: The peak area of ethylene glycol in the *Sample solution* is NMT the peak area of ethylene

glycol in the *Standard solution*, corresponding to NMT 0.10% of ethylene glycol in Sorbitol Sorbitan Solution.

ASSAY**PROCEDURE**

Mobile phase: Water

System suitability solution: 10 mg/g of sorbitol, 4 mg/g of 1,4-sorbitan, 4 mg/g of isosorbide, and 1 mg/g of mannitol in water

Standard solution: 10 mg/g of USP Sorbitol RS and 4 mg/g of USP 1,4-Sorbitan RS in water

Sample solution: Dissolve 0.40 g of Sorbitol Sorbitan Solution in water, and dilute with water to about 20 g. Record the weight of the final solution, and mix thoroughly.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm \times 10-cm; packing L34

Temperature

Detector: 35°

Column: 50 \pm 2°

Flow rate: 0.6 mL/min

Injection size: 10 μ L

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for 1,4-sorbitan, isosorbide, mannitol, and sorbitol are about 0.35, 0.43, 0.7, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between 1,4-sorbitan and isosorbide, *System suitability solution*

Relative standard deviation: NMT 2.0% for each analyte, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Separately calculate the percentages, on the anhydrous basis, of 1,4-sorbitan and D-sorbitol in the portion of Sorbitol Sorbitan Solution taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times [100/(100 - W)] \times 100$$

r_U = peak responses of the corresponding analyte from the *Sample solution*

r_S = peak responses of the corresponding analyte from the *Standard solution*

C_S = concentration of the appropriate USP Reference Standard in the *Standard solution* (mg/g)

C_U = concentration of Sorbitol Sorbitan Solution in the *Sample solution* (mg/g)

W = percentage from the test for *Water Determination*

Acceptance criteria: NLT 25.0% of $C_6H_{14}O_6$ and NLT 15.0% of $C_6H_{12}O_5$ on the anhydrous basis

IMPURITIES**Inorganic Impurities**

- RESIDUE ON IGNITION** <281>: NMT 0.20%, calculated on the anhydrous basis on a 2-g portion

LIMIT OF NICKEL

Solution A: A saturated ammonium pyrrolidine dithiocarbamate solution (10 mg/mL of ammonium pyrrolidine dithiocarbamate)

Sample solution: 200 mg/mL of Sorbitol Sorbitan Solution in diluted acetic acid. To 100 mL of this solution add 2.0 mL of *Solution A* and 10.0 mL of methyl isobutyl ketone, and shake for 30 s. Protect from bright light. Allow the two layers to separate, and use the methyl isobutyl ketone layer.

Standard solutions: Prepare as directed for the *Sample solution*, except to prepare three solutions by adding 0.5, 1.0, and 1.5 mL of nickel standard solution TS.

Blank solution: Prepare as directed for the *Sample solution*, except to omit the use of Sorbitol Sorbitan Solution. Quantities should be increased five fold to ensure that a sufficient volume of *Blank solution* is available.

Spectrometric conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometry

Analytical wavelength: 232.0 nm (maximum absorbance)

Lamp: Nickel hollow-cathode

Flame: Air-acetylene

Analysis

Samples: *Blank solution*, *Standard solutions*, and *Sample solution*

Set the instrument to zero, using the *Blank solution*. Concomitantly determine the absorbances of the *Standard solutions* and the *Sample solution* at least three times each. Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution*. Between each measurement, aspirate the *Blank solution*, and ascertain that the reading returns to zero. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.

Acceptance criteria: NMT 1 ppm, calculated on the anhydrous basis

Organic Impurities

• **PROCEDURE: REDUCING SUGARS**

Sample: An amount of Sorbitol Sorbitan Solution equivalent to 3.3 g, on the anhydrous basis.

Analysis: To the *Sample*, add 3 mL of water, 20.0 mL of cupric citrate TS, and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 40 mL of diluted acetic acid, 60 mL of water, and 20.0 mL of 0.05 N iodine VS. With continuous shaking add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water. When the precipitate has dissolved, titrate the excess of iodine with 0.05 N sodium thiosulfate VS, using 2 mL of starch TS, added toward the end of the titration, as an indicator.

Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate VS is required, corresponding to NMT 0.3% of reducing sugars, on the anhydrous basis, as glucose.

[NOTE—The amount determined in this test is not included in the calculated amount under *General Notices*, 5.60.10. *Other Impurities in USP and NF Articles*.]

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED ORGANISMS** (62): The total aerobic microbial count using the *Plate Method* is NMT 1000 cfu/mL. The total combined molds and yeasts count is NMT 100 cfu/mL.
- **pH** (791): 4.0–7.0, in a 14% solution of Sorbitol Sorbitan Solution in carbon dioxide-free water
- **WATER DETERMINATION, Method I** (921): NMT 31.5%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
- **LABELING:** The labeling indicates the percentage content, on the anhydrous basis, of D-sorbitol and 1,4-sorbitan.
- **USP REFERENCE STANDARDS** (11)
 - USP Diethylene Glycol RS
 - USP Ethylene Glycol RS
 - USP 1,4-Sorbitan RS
 - C₆H₁₂O₅ 164.16

USP Sorbitol RS

Sorbitol Solution—see *Sorbitol Solution General Monographs*

Soybean Oil—see *Soybean Oil General Monographs*

Hydrogenated Soybean Oil

[8016-70-4].

DEFINITION

Hydrogenated Soybean Oil is the product obtained by refining, bleaching, hydrogenation, and deodorization of oil obtained from seeds of the soya plant *Glycine max* Merr. (Fabaceae). The product consists mainly of triglycerides of palmitic and stearic acids.

IDENTIFICATION

- **A.** It meets the requirements in *Specific Tests for Fats and Fixed Oils, Fatty Acid Composition* (401).
- **B.** It meets the requirements in *Specific Tests for Melting Range or Temperature, Class II* (741).

IMPURITIES

• **LIMIT OF NICKEL**

Nickel standard solution: Immediately before use, prepare the equivalent of 0.2 µg/g of nickel by diluting 10 mL of nickel standard solution TS with water to 500 mL.

Sample solution: Weigh 5.0 g of Hydrogenated Soybean Oil into a previously tared platinum or silica crucible. Cautiously heat, and introduce into the substance a wick formed from twisted ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at 600°. Continue the incineration until white ash is obtained. After cooling, transfer the residue, with the aid of two 2-mL portions of diluted hydrochloric acid, to a 25-mL volumetric flask, add 0.3 mL of nitric acid, and dilute with water to volume.

Standard solutions: Into three identical 10-mL volumetric flasks introduce 1.0, 2.0, and 4.0 mL of *Nickel standard solution*. To each flask add a 2.0-mL portion of the *Sample solution*, and dilute with water to volume.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption, equipped with a graphite furnace

Analytical wavelength: 232.0 nm

Lamp: Nickel hollow-cathode

Analysis

Samples: *Sample solution* and *Standard solutions*
Determine the absorbances of the *Samples* at least three times each. Record the average of the steady readings for each of the *Samples*. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the added quantity of nickel, and draw the straight line best fitting the three plotted points. Extrapolate the line until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.

Acceptance criteria: NMT 1 µg/g

• **ALKALINE IMPURITIES**

Sample: 2.0 g

Analysis: Dissolve the *Sample* by gently heating in a mixture of 1.5 mL of alcohol and 3.0 mL of toluene. Add 0.05 mL of bromophenol blue TS, and titrate with 0.01 N hydrochloric acid VS to a yellow endpoint.

Acceptance criteria: NMT 0.4 mL of 0.01 N hydrochloric acid VS is required.

SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE**, *Class II* (741): 66°–72°

• **FATS AND FIXED OILS**, *Acid Value* (401)

Sample: 10 g of Hydrogenated Soybean Oil

Analysis: Dissolve the *Sample* in 50 mL of a hot mixture of neutralized alcohol and toluene (1:1). Add 0.5 mL of phenolphthalein TS, and immediately titrate, while still hot, with 0.1 N potassium hydroxide VS to produce a permanent, faint pink color.

Acceptance criteria: NMT 0.5

• **FATS AND FIXED OILS**, *Fatty Acid Composition* (401): Hydrogenated Soybean Oil exhibits the composition profile of fatty acids in *Table 1*.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
<14	0	≤0.1
14	0	≤0.5
16	0	9–16
18	0	79–89
20	0	≤1.0
22	0	≤1.0
18	1	≤4.0
18	2	≤1.0
18	3	≤0.2

• **FATS AND FIXED OILS**, *Peroxide Value* (401): NMT 5.0

• **FATS AND FIXED OILS**, *Unsaponifiable Matter* (401)

Sample: 5.0 g

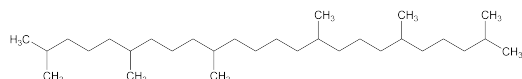
Acceptance criteria: NMT 1.0%

• **WATER DETERMINATION**, *Method I* (921): NMT 0.3%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE**: Preserve in tight, light-resistant containers. No storage requirements are specified.

Squalane



C₃₀H₆₂

422.81

Tetracosane, 2,6,10,15,19,23-hexamethyl-;
2,6,10,15,19,23-Hexamethyltetracosane [111-01-3].

DEFINITION

Squalane is a saturated hydrocarbon obtained by hydrogenation of squalene, an aliphatic triterpene occurring in some fish oils.

IDENTIFICATION

• **A. INFRARED ABSORPTION** (197F)

Sample: Undried specimen

Acceptance criteria: Meets the requirements

IMPURITIES

• **RESIDUE ON IGNITION** (281): NMT 0.5%

• **CHROMATOGRAPHIC PURITY**

Standard solution: 20 mg/mL of USP Squalane RS in *n*-hexane

Sample solution: 20 mg/mL of Squalane in *n*-hexane

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 3-mm × 1.8-m; packed with 3% phase G1 on packing S1A

Temperatures

Detector: 280°

Injection port: 280°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
130	6	270	End

Carrier gas: Nitrogen

Flow rate: 25 mL/min

Injection volume: 2 µL

Analysis

Samples: *Standard solution* and *Sample solution*
Identify the squalane peak in the *Sample solution* by comparison of the retention time with that of the main peak in the *Standard solution*.

Acceptance criteria: The area of the squalane peak in the *Sample solution* is NLT 97% of the sum of the areas of all of the peaks.

SPECIFIC TESTS

• **SPECIFIC GRAVITY** (841): 0.807–0.810 at 20°

• **FATS AND FIXED OILS**, *Acid Value* (401): NMT 0.2

• **FATS AND FIXED OILS**, *Iodine Value* (401): NMT 4

• **FATS AND FIXED OILS**, *Saponification Value* (401): NMT 2

• **REFRACTIVE INDEX** (831): 1.4510–1.4525 at 20°

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE**: Preserve in tight containers.

• **USP REFERENCE STANDARDS** (11)

USP Squalane RS

Stannous Chloride

SnCl₂ · 2H₂O

225.65

Tin chloride (SnCl₂) dihydrate [10025-69-1].

DEFINITION

Stannous Chloride contains NLT 98.0% and NMT 102.0% of SnCl₂ · 2H₂O.

IDENTIFICATION

• **A.**

Sample solution: To 0.40 g of Stannous Chloride add 1 mL of dilute hydrochloric acid solution (236 mL/L of hydrochloric acid), and dilute with water to 20 mL.
[NOTE—Keep a portion for the *Limit of Sulfate* test.]

Analysis: To 1 mL of the *Sample solution* add a mixture of 5 mL of water and 0.05 mL of mercuric chloride TS.

- Acceptance criteria:** A blackish-gray precipitate forms.
- **B.**
Sample: 1.0 g
Analysis 1: Dissolve the *Sample* in 3.0 mL of water. Add 0.5 mL of dilute sodium hydroxide solution (85 mg/mL of sodium hydroxide) to the cloudy solution.
Acceptance criteria 1: A yellowish, flocculent precipitate is formed.
Analysis 2: Add 6.5 mL of water to the solution resulting from *Analysis 1*. To 1.0 mL of the previously shaken suspension add 1.0 mL of sodium hydroxide solution (420 mg/mL of sodium hydroxide).
Acceptance criteria 2: The precipitate dissolves, and the resulting solution is clear and colorless.
 - **C. IDENTIFICATION TESTS—GENERAL, Chloride (191)**
Sample solution: Dissolve 10 mg of Stannous Chloride in 2 mL of 20% nitric acid.
Acceptance criteria: Meets the requirements

ASSAY

• PROCEDURE

Sample: 0.1 g of Stannous Chloride

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N iodine VS

Blank: 50 mL of water

Endpoint detection: Colorimetric

Analysis: Dissolve the *Sample* in 50 mL of water freed from oxygen by purging with carbon dioxide or nitrogen for 15 min prior to the addition. Add 1.5 mL of 0.8 N hydrochloric acid, 5 g of potassium sodium tartrate, 10 g of sodium bicarbonate, and 1 mL of starch TS. Titrate the resulting solution immediately with *Titrant*. Perform a blank determination.

Calculate the percentage of stannous chloride ($\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$) in the *Sample*:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* consumed by the *Sample* (mL)

V_B = *Titrant* consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 112.8 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: 98.0%–102.0%

IMPURITIES

• LIMIT OF SULFATE

Acetic acid solution: Dilute 30 mL of glacial acetic acid with water to 100 mL.

30% alcohol: Dilute 30 mL of alcohol with water to 100 mL.

Potassium sulfate solution 1: 1.8 mg/mL of potassium sulfate in 30% *Alcohol*. Immediately before use, dilute with 30% *Alcohol* to obtain a solution having a known concentration of about 18 µg/mL.

Potassium sulfate solution 2: 1.8 mg/mL of potassium sulfate in water. Immediately before use, dilute with water to obtain a solution having a known concentration of about 18 µg/mL.

Standard solution: Mix 3 mL of barium chloride solution (250 mg/mL) and 4.5 mL of *Potassium sulfate solution 1*. Shake, and let stand for 1 min. To 2.5 mL of this solution add 15 mL of *Potassium sulfate solution 2* and 0.5 mL of *Acetic acid solution*. Allow to stand for 5 min.

Sample solution: Use 15 mL of the solution prepared in *Identification test A*.

Analysis: Mix 3 mL of barium chloride solution (250 mg/mL) and 4.5 mL of *Potassium sulfate solution 1*. Shake, and let stand for 1 min. To 2.5 mL of this solution add the *Sample solution* and 0.5 mL of *Acetic acid solution*. Allow to stand for 5 min.

Acceptance criteria: Any opalescence in the *Sample solution* is not more intense than that in the *Standard solution* (500 ppm).

• LIMIT OF IRON

Standard iron solution: Prepare as directed under *Iron* (241), *Special Reagents*.

Standard solution: Immediately before use, dilute 1 mL of *Standard iron solution* with water to 10 mL. This solution contains the equivalent of 1 µg/mL of iron.

Sample solution: Dilute 5 mL of the *Sample solution* prepared as directed in *Substances Not Precipitated by Thioacetamide* with water to 10 mL.

Analysis: To 10 mL each of the *Standard solution* and the *Sample solution* add 2 mL of dilute citric acid (200 mg/mL) and 0.1 mL of thioglycolic acid, and mix. Make alkaline with a dilute ammonia solution (620 mL/L of ammonium hydroxide). Dilute with water to 20 mL. Allow the treated *Standard solution* and the treated *Sample solution* to stand for 5 min.

Acceptance criteria: Any pink color in the *Sample solution* is not more intense than that in the *Standard solution* (100 ppm).

• LIMIT OF LEAD

Dilute thioacetamide solution: [NOTE—Prepare immediately before use.] To 0.2 mL of thioacetamide TS add 1 mL of a mixture of 5 mL of water, 15 mL of 1 N sodium hydroxide, and 20 mL of 85% glycerin. Heat in a water bath for 20 s.

Standard lead solution: Prepare as directed under *Heavy Metals* (231), *Special Reagents*.

Standard solution: On the day of use, mix 1.0 mL of *Standard lead solution*, 6 mL of water, 3 mL of sodium hydroxide solution (420 mg/mL of sodium hydroxide), and 0.5 mL of *Dilute thioacetamide solution*.

Sample solution: Dissolve 1.0 g of Stannous Chloride in 2 mL of a mixture of nitric acid and hydrochloric acid (1:3). Heat the solution on a water bath until nitrous vapor is no longer evolved. Dissolve the residue in water, and dilute with water to 25 mL. To 5 mL of this solution add 3 mL of sodium hydroxide solution (420 mg/mL of sodium hydroxide) and 2 mL of water. Heat until a clear solution is obtained, and cool. Add 0.5 mL of *Dilute thioacetamide solution*, and allow to stand for 2 min.

Analysis: Compare the *Standard solution* and the *Sample solution*.

Acceptance criteria: Any color in the *Sample solution* is not more intense than that in the *Standard solution* (50 ppm).

SPECIFIC TESTS

• APPEARANCE OF SOLUTION

Standard stock solution: Pipet 30.0 mL of ferric chloride CS, 30.0 mL of cobaltous chloride CS, and 24.0 mL of cupric sulfate CS into a 100-mL volumetric flask. Dilute with 1% (w/v) hydrochloric acid to volume.

Standard solution: Dilute 1.0 mL of the *Standard stock solution* with 1% (w/v) hydrochloric acid to 100 mL.

Sample solution: Dissolve 10.0 g of Stannous Chloride in dilute hydrochloric acid solution, and dilute with dilute hydrochloric acid solution to 20 mL.

Acceptance criteria: The *Sample solution* is clear and colorless, or if not, not more intensely colored than the *Standard solution*.

• SUBSTANCES NOT PRECIPITATED BY THIOACETAMIDE

Sample solution: Dissolve 1.0 g of Stannous Chloride in dilute hydrochloric acid solution, and dilute with the same acid to 30 mL. Heat to boiling. Add 30 mL of thioacetamide TS, and boil for 15 min to produce *Solution A*. Filter 5 mL of *Solution A*, and heat the filtrate to boiling. Add 5 mL of thioacetamide TS, and boil for 15 min. If a precipitate is formed, add the remainder of *Solution A* to the mixture to produce *Solution A1*. Add 10 mL of thioacetamide TS, and boil. Repeat the series of operations from "Filter 5 mL" until a precipitate is no

longer formed on addition of thioacetamide TS to the filtrate obtained from the 5 mL of *Solution A* (*Solution A1*, *Solution A2*, and so on, respectively). If no precipitate is formed, or if no more precipitate is formed, combine the solution obtained with the remainder of *Solution A* (*Solution A1*, *Solution A2*, and so on, respectively), filter, and wash the precipitate with 10 mL of water. Heat the filtrate until the resulting vapor no longer turns a moistened piece of lead acetate test paper blackish-gray. Allow to cool, and dilute with water to 50 mL. [NOTE—Keep a portion for the *Limit of Iron* test.]

Analysis: Evaporate 25 mL of the *Sample solution* to dryness, and ignite at 600°.

Acceptance criteria: The residue weighs NMT 1 mg (0.2%).

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Corn Starch

Attributes	EP	JP	USP
Definition	+	+	+
Identification A	+	+	+
Identification B	+	+	+
Identification C	+	+	+
pH	+	+	+
Loss on Drying	+	+	+
Residue on Ignition	+	+	+
Limit of Iron	+	+	+
Limit of Oxidizing Substances	+	+	+
Limit of Sulfur Dioxide	+	+	+

Legend: + will adopt and implement; – will not stipulate

Nonharmonized attributes: Characters, Microbial Enumeration Tests and Tests for Specified Microorganisms, Labeling, and Packaging and Storage (USP)

Specific local attributes: Foreign matter (EP)

Reagents and reference materials: Each pharmacopeia will adapt the text to take account of local reference materials and reagent specifications.

DEFINITION

Corn Starch consists of the starch granules separated from the mature grain of corn [*Zea mays* Linné (Fam. Gramineae)].

IDENTIFICATION

- **A. PROCEDURE:** Examine under a microscope, using NLT 20× magnification and using a mixture of glycerin and water (1:1) as a mounting agent.
Acceptance criteria: It appears either as angular polyhedral granules of irregular sizes with diameters ranging from 2–23 µm, or as rounded or spheroidal granules of irregular sizes with diameters ranging from 25–35 µm. The central hilum consists of a distinct cavity or two- to five-rayed cleft, and there are no concentric striations. Between orthogonally oriented polarizing plates or prisms, the starch granules show a distinct black cross intersecting at the hilum.
- **B. PROCEDURE**
Sample solution: 20 mg/mL in water
Analysis: Boil for 1 min, and cool.
Acceptance criteria: A thin, cloudy mucilage is formed.
- **C. PROCEDURE**
Sample solution: 1 mL of the mucilage obtained in Identification test B

Analysis: Add 0.05 mL of iodine and potassium iodide TS 2 to the *Sample solution*.

Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION (281):** NMT 0.6%, determined on a 1.0-g sample

LIMIT OF IRON

Standard iron stock solution A: Equivalent to 10 µg/mL of iron prepared as directed under *Iron* (241)

Standard iron stock solution B: 1 µg/mL of iron from *Standard iron stock solution A* in water

[NOTE—Prepare immediately before use.]

Standard iron solution: Transfer 10 mL of *Standard iron stock solution B* to a test tube, and add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.

Sample solution: Shake 1.5 g of Corn Starch with 15 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube, add 2 mL of citric acid solution (2 in 10), and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard iron solution*, corresponding to a limit of 10 ppm of iron.

LIMIT OF SULFUR DIOXIDE

Carbon dioxide: Use carbon dioxide, with a flow regulator that will maintain a flow of 100 ± 10 mL/min.

Bromophenol blue indicator solution: 0.2 mg/mL of bromophenol blue in dilute alcohol. Filter if necessary.

Hydrogen peroxide solution: Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Just before use, add 3 drops of *Bromophenol blue indicator solution*, and neutralize to a violet-blue endpoint with 0.01 N sodium hydroxide. Do not exceed the endpoint.

Apparatus: Figure 1.

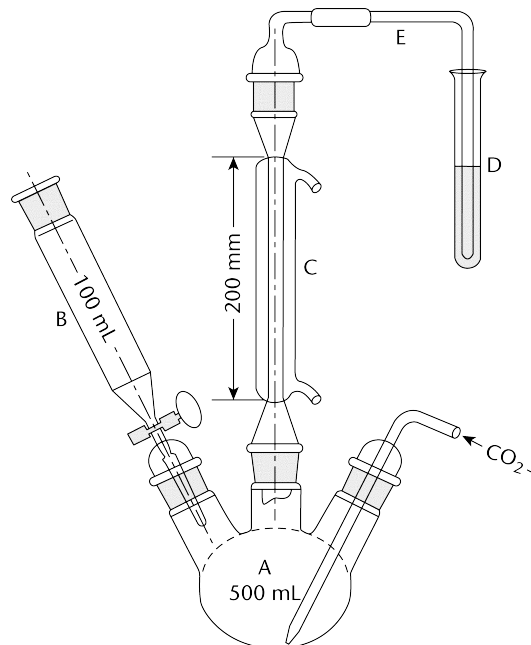


Figure 1

In this test, the sulfur dioxide is released from the sample in a boiling acid medium and is removed by a

stream of carbon dioxide. The separated gas is collected in a dilute hydrogen peroxide solution where the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali. The apparatus consists essentially of a 500-mL three-neck, round-bottom boiling flask, A; separatory funnel, B, having a capacity of 100 mL or greater; a gas inlet tube of sufficient length to permit introduction of the carbon dioxide within 2.5 cm of the bottom of the boiling flask; a reflux condenser, C, having a jacket length of 200 mm, and a delivery tube, E, connecting the upper end of the reflux condenser to the bottom of a receiving test tube, D. Apply a thin film of stopcock grease to the sealing surfaces of all of the joints except the joint between the separatory funnel and the boiling flask, and clamp the joints to ensure tightness.

Sample: 25.0 g of Corn Starch

Analysis: Add 150 mL of water to the boiling flask. Close the stopcock of the separatory funnel, and begin the flow of carbon dioxide at a rate of 100 ± 5 mL/min through the *Apparatus*. Start the condenser coolant flow. Add 10 mL of *Hydrogen peroxide solution* to a receiving test tube. After 15 min, without interrupting the flow of carbon dioxide, remove the separatory funnel from the boiling flask, and transfer the *Sample* into the boiling flask with the aid of 100 mL of water. Apply stopcock grease to the outer joint of the separatory funnel, and replace the separatory funnel in the boiling flask. Close the stopcock of the separatory funnel, and add 80 mL of 2 N hydrochloric acid to the separatory funnel. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the boiling flask, guarding against the escape of sulfur dioxide into the separatory funnel by closing the stopcock before the last few mL of hydrochloric acid drain out. Boil the mixture for 1 h. Remove the receiving test tube, and transfer its contents to a 200-mL wide-necked, conical flask. Rinse the receiving test tube with a small portion of water, add the rinsing to the 200-mL conical flask, and mix. Heat on a water bath for 15 min, and allow to cool.

Add 0.1 mL of *Bromophenol blue indicator solution*, and titrate the contents with 0.1 N sodium hydroxide VS until the color changes from yellow to violet-blue. Perform a blank determination, and make any necessary correction (see *Titrimetry* (541)).

Calculate the content, in ppm, of sulfur dioxide in the *Sample* taken:

$$\text{Result} = 1000 (32.03) \text{ VN/W}$$

32.03 = milliequivalent weight of sulfur dioxide

V = volume of titrant consumed (mL)

N = normality of the titrant

W = weight of the *Sample* (g)

Acceptance criteria: NMT 50 ppm

• **LIMIT OF OXIDIZING SUBSTANCES**

Sample solution: Transfer 4.0 g to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS.

Analysis: Titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch–iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate is required (20 ppm, calculated as H_2O_2).

SPECIFIC TESTS

- **♦MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 1000 cfu/g; the total combined molds and yeasts count does not exceed 100 cfu/g; and it meets the requirements of the test for the absence of *Escherichia coli*. Where it is intended for use in preparing Absorbable Dusting Powder, it also meets the requirements of the tests for absence of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.♦
- **LOSS ON DRYING (731):** Dry 1 g at 130° for 90 min: it loses NMT 15.0% of its weight.
- **pH (791):** 4.0–7.0

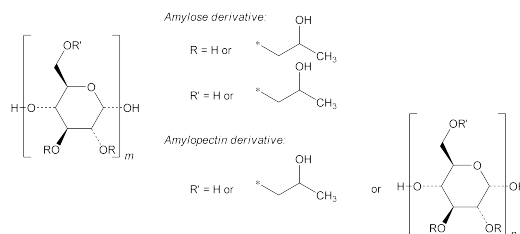
Sample solution: Prepare a slurry by weighing 5.0 g of Corn Starch, transferring to a suitable nonmetallic container, and adding 25.0 mL of freshly boiled and cooled water.

Analysis: Agitate continuously at a moderate rate for 1 min. Stop the agitation, and allow to stand for 15 min. Determine the pH to the nearest 0.1 unit.

ADDITIONAL REQUIREMENTS

- **♦PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.♦
- **♦LABELING:** Where Corn Starch is intended for use in preparing Absorbable Dusting Powder, it is so labeled, and the label states that it must be subjected to further processing during the preparation of Absorbable Dusting Powder.♦

Hydroxypropyl Corn Starch



For the Amylose derivative, *m* is about 300–1000.

DEFINITION

Hydroxypropyl Corn Starch is partially substituted 2-hydroxypropylether obtained from corn starch by a chemical modification of etherification with propylene oxide. In addition, this starch may be partially hydrolyzed using acids or enzymes to obtain thinned starch. It contains NLT 2.0% and NMT 7.0% of hydroxypropyl groups on the dried basis.

IDENTIFICATION

• **A. PROCEDURE**

Analysis: Examine under a microscope, using NLT 20× magnification and a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: It presents either as angular polyhedral granules of irregular sizes with diameters of 2–23 µm, or as rounded or spheroidal granules of irregular sizes with diameters of 25–35 µm. The central hilum consists of a distinct cavity or 2- to 5-rayed cleft, and there are no concentric striations. Between crossed nicol prisms, the Hydroxypropyl Corn Starch granules show a distinct black cross intersecting at the hilum.

• **B. PROCEDURE**

Sample solution: Suspend 1 g of Hydroxypropyl Corn Starch in 50 mL of water, boil for 1 min, and cool.

Acceptance criteria: A translucent or clear mucilage is formed.

• C. PROCEDURE

Analysis: To 1 mL of the *Sample solution* obtained in *Identification test B* add 0.05 mL of iodine and potassium iodide TS 2.

Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

• D. PROCEDURE

Ninhydrin solution: Dissolve 3 g of ninhydrin in 100 mL of a 45.5-g/L solution of sodium metabisulfite.

Diluted sulfuric acid: 98 g/L of H₂SO₄

Sample: 100 mg of Hydroxypropyl Corn Starch

Analysis: Transfer the *Sample* to a 100-mL volumetric flask, and add 12.5 mL of *Diluted sulfuric acid*. Place the flask in a water bath, and heat until the *Sample* is dissolved. Cool, and dilute with water to 100 mL. [**CAUTION**—When sulfuric acid is miscible with water, it produces intense heat.]

Pipet 1 mL of this solution to a glass-stoppered, 25-mL graduated test tube and, with the tube immersed in cold water, add drop-wise 8 mL of sulfuric acid. Mix well, and place the tube in a boiling water bath for exactly 3 min. Immediately transfer the tube to an ice bath until the solution is chilled. Add 0.6 mL of *Ninhydrin solution*, carefully allowing the reagent to run down the walls of the test tube. Immediately shake the tube well, and place it in a water bath at 25° for 100 min. Dilute with sulfuric acid to 25 mL [**CAUTION**—Use sulfuric acid cautiously.], and mix by inverting the tube several times. Do not shake.

Acceptance criteria: A violet color develops within 5 min due to the presence of hydroxypropyl groups (starch ether).

ASSAY

• PROCEDURE FOR HYDROXYPROPYL GROUPS

Deuterium chloride solution: Dilute 1 mL of deuterium chloride (38% w/w) with 5 mL of deuterium oxide.

Internal standard solution: Disperse 50.0 mg of sodium 3-trimethylsilyl-1-propane sulfonate in about 5 g of deuterium oxide, weighed to the nearest 0.1 mg. Store in a sealed bottle.

Sample solution: Disperse 20 g of Hydroxypropyl Corn Starch in 200.0 mL of carbon dioxide-free water at room temperature. Agitate for 15 min, and filter. Repeat the operation two more times. If poor dispersibility or slow filtration is observed, use refrigerated carbon dioxide-free water for the washing operation. Dry the washed starch for NLT 4 h in vacuum at 30 ± 5°. Determine the moisture content (B) on 5 g of the washed and dried starch following the *Loss on Drying* test. Weigh 12.0 mg of the washed and dried starch in a 5-mm NMR tube. Add 0.75 mL of deuterium oxide and 0.1 mL of *Deuterium chloride solution*. Cap the tube, mix, and place it in a boiling water bath until a clear solution is obtained. [NOTE—This may take 3 min to 1 h.] When a clear solution is obtained, allow to cool to room temperature. Dry the exterior of the tube, and weigh to the nearest 0.1 mg. Add 0.05 mL of *Internal standard solution*, and weigh to the nearest 0.1 mg. Determine the mass of the *Internal standard solution* added. Mix thoroughly.

Nuclear magnetic resonance spectrometry

(See *Nuclear Magnetic Resonance* (761), *Quantitative Application*.)

Apparatus: FT-NMR spectrometer at minimum 300 MHz

Acquisition of ¹H NMR spectra: The following parameters may be used.

Sweep width: 8 ppm (about -1.0 to +7 ppm)

Irradiation frequency offset: None

Time domain: NLT 64 K

Pulse width: 90 degree

Pulse delay: 10 s

Dummy scans: 0

Number of scans: 8

Use the CH₃ signal of the internal standard for shift referencing. Set the shift of the peak of the singlet to 0 ppm. Record the FID signal.

Analysis

Samples: *Internal standard solution* and *Sample solution*
Call the integration sub-routine after phase corrections and baseline correction between -0.5 and +6 ppm. Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at +1.2 ppm (A₂), and of the methyl groups at 0 ppm of the internal standard (A₁) without ¹³C-satellites.

Measure the signal coming from the 3 protons of the methyl group in the hydroxypropyl function.

Calculate the content of hydroxypropyl groups as a percentage (w/w, dried basis):

$$\text{Result} = (N \times A_2/A_1) \times (C_i \times W_i/W) \times (M_{r2}/M_{r1}) \times [100/(100 - B)] \times 100$$

N = numerical value representing the 3 methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate), 3

A₂ = area of the methyl groups of hydroxypropyl in Hydroxypropyl Corn Starch

A₁ = area of the methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate)

C_i = concentration of the internal standard in the *Internal standard solution* (mg/g)

W_i = weight of the *Internal standard solution* in the NMR tube (g)

W = weight of the washed and dried Hydroxypropyl Corn Starch in the NMR tube (mg)

M_{r1} = molecular weight of the internal standard, 218.32 g/mol

M_{r2} = molar mass of hydroxypropyl group, 59.09 g/mol

B = moisture content of the washed and dried Hydroxypropyl Corn Starch used in the *Sample solution*, as a percentage (w/w)

Acceptance criteria: The content of hydroxypropyl groups is 2.0%–7.0% on the dried basis.

IMPURITIES

Inorganic Impurities

• **RESIDUE ON IGNITION** (281): NMT 0.6%, determined on a 1.0-g test specimen

• LIMIT OF IRON

Standard iron stock solution: Prepare a solution containing the equivalent of 10 µg/mL of iron, as directed under *Iron* (241).

Diluted standard iron solution: Immediately before use, dilute an accurately measured volume of *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 µg/mL of iron.

Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Sample solution: Shake 1.0 g of Hydroxypropyl Corn Starch with 20 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the

Standard solution, corresponding to a limit of 20 µg/g of iron.

- **LIMIT OF SULFUR DIOXIDE, Method IV (525):** NMT 50 ppm Organic Impurities

- **PROCEDURE 1: LIMIT OF OXIDIZING SUBSTANCES**

Sample: 4.0 g of Hydroxypropyl Corn Starch

Analysis: Transfer the *Sample* to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 µg/g, calculated as H₂O₂).

- **PROCEDURE 2: FOREIGN MATTER**

Sample: 50 mg/mL of Hydroxypropyl Corn Starch in a mixture of glycerin and water (1:1)

Analysis: Examine under a microscope, using NLT 20× magnification and a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: NMT traces of matter other than Hydroxypropyl Corn Starch granules are present.

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 10³ cfu/g, and the total combined molds and yeasts count does not exceed 10² cfu/g. It meets the requirements of the test for the absence of *Escherichia coli*.
- **PH (791)**
Sample solution: Suspend 5.0 g of Hydroxypropyl Corn Starch in 25.0 mL of carbon dioxide-free water, and shake for 60 s. Allow to stand for 15 min.
Acceptance criteria: 4.5–8.0
- **LOSS ON DRYING (731):** Dry about 1 g at 130° for 90 min: it loses NMT 15.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.

Pregelatinized Hydroxypropyl Corn Starch

DEFINITION

Pregelatinized Hydroxypropyl Corn Starch is prepared from Hydroxypropyl Corn Starch by mechanical processing in the presence of water, with or without heat, to rupture all or some of the starch granules, and is subsequently dried. It contains NLT 2.0% and NMT 7.0% of hydroxypropyl groups on the dried basis.

IDENTIFICATION

- **A. TEST FOR PREGELATINIZED STATE**

Sample: 1 g

Analysis: Disperse the *Sample* in 50 mL of water at a temperature NMT 25°. Shake vigorously until lumps completely disperse/solubilize or until lumps disappear. Allow to stand for 20 min.

Acceptance criteria: A translucent or clear mucilage without precipitate is formed.

- **B. TEST FOR STARCH**

Analysis: Disperse 0.5 g in 2 mL of water without heating, and add 0.05 mL of iodine and potassium iodide TS₂.

Acceptance criteria: A reddish-violet or blue color is produced.

- **C. NINHYDRIN TEST**

Ninhydrin solution: Dissolve 3 g of ninhydrin in

100 mL of a 45.5-g/L solution of sodium metabisulfite.

Diluted sulfuric acid: 98 g/L of H₂SO₄

Sample: 100 mg

Analysis: Transfer the *Sample* to a 100-mL volumetric flask, and add 12.5 mL of *Diluted sulfuric acid*. Place the flask in a water bath, and heat until the *Sample* is dissolved. Cool, and dilute with water to 100 mL. [**CAUTION**—When sulfuric acid is miscible with water, it produces intense heat.]

Pipet 1 mL of this solution to a glass-stoppered 25-mL graduated test tube and, with the tube immersed in cold water, add dropwise 8 mL of sulfuric acid. Mix well, and place the tube in a boiling water bath for exactly 3 min. Immediately transfer the tube to an ice bath until the solution is chilled. Add 0.6 mL of *Ninhydrin solution*, carefully allowing the reagent to run down the walls of the test tube. Immediately shake the tube well, and place it in a water bath at 25° for 100 min. Dilute with sulfuric acid to 25 mL. [**CAUTION**—Use sulfuric acid cautiously.] Mix by inverting the tube several times. Do not shake.

Acceptance criteria: A violet color develops within 5 min due to the presence of hydroxypropyl groups (starch ether).

ASSAY

- **ASSAY FOR HYDROXYPROPYL GROUPS**

Deuterium chloride solution: Dilute 1 mL of deuterium chloride (38% w/w) with 5 mL of deuterium oxide.

Internal standard solution: Disperse 50.0 mg of sodium 3-trimethylsilyl-1-propane sulfonate in about 5 g of deuterium oxide, weighed to the nearest 0.1 mg. Store in a sealed bottle.

Sample solution: Determine the moisture content (*B*) of 5 g of Pregelatinized Hydroxypropyl Corn Starch following the *Loss on Drying* test. Weigh 12.0 mg of Pregelatinized Hydroxypropyl Corn Starch in a 5-mm NMR tube. Add 0.75 mL of deuterium oxide and 0.1 mL of *Deuterium chloride solution*. Cap the tube, mix, and place it in a boiling water bath until a clear solution is obtained. [NOTE—This may take from 3 min to 1 h.] When a clear solution is obtained, allow it to cool to room temperature. Dry the exterior of the tube, and weigh to the nearest 0.1 mg. Add 0.05 mL of *Internal standard solution*. Weigh to the nearest 0.1 mg. Determine the mass of the *Internal standard solution* added. Mix thoroughly.

Instrumental conditions

(See *Nuclear Magnetic Resonance (761)*, *Quantitative Applications*.)

Mode: Nuclear magnetic resonance spectrometry

Apparatus: FT-NMR spectrometer at minimum 300 MHz

Acquisition of ¹H NMR spectra: The following parameters may be used:

Sweep width: 8 ppm (about –1.0 to +7 ppm)

Irradiation frequency offset: None

Time domain: NLT 64 K

Pulse width: 90°

Pulse delay: 10 s

Dummy scans: 0

Number of scans: 8

Use the CH₃ signal of the internal standard for shift referencing. Set the shift of the peak of the singlet to 0 ppm. Record the FID signal.

Analysis

Samples: *Internal standard solution* and *Sample solution*
 Call the integration subroutine after phase corrections and baseline correction between -0.5 and +6 ppm. Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at +1.2 ppm (A_2), and of the methyl groups at 0 ppm of the internal standard (A_1) without ^{13}C -satellites. Measure the signal originating from the 3 protons of the methyl group in the hydroxypropyl function. Calculate the content of hydroxypropyl groups as a percentage (w/w, dried basis):

$$\text{Result} = (N \times A_2/A_1) \times (C_i \times W_i/W) \times (M_{r2}/M_{r1}) \times [100/(100 - B)] \times 100$$

- N = numerical value representing the 3 methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate), 3
 A_2 = area of the methyl groups of hydroxypropyl in Pregelatinized Hydroxypropyl Corn Starch
 A_1 = area of the methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate)
 C_i = concentration of the internal standard in the *Internal standard solution* (mg/g)
 W_i = weight of the *Internal standard solution* in the NMR tube (g)
 W = weight of the Pregelatinized Hydroxypropyl Corn Starch in the NMR tube (mg)
 M_{r2} = molar mass of hydroxypropyl groups, 59.09 g/mol
 M_{r1} = molecular weight of the internal standard, 218.32 g/mol
 B = moisture content of the Pregelatinized Hydroxypropyl Corn Starch used in the *Sample solution*, as a percentage (w/w)

Acceptance criteria: 2.0%–7.0% of hydroxypropyl groups on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.6%, determined on a 1.0-g test specimen

- **LIMIT OF IRON**

Standard iron stock solution: Prepare a solution containing the equivalent of 10 $\mu\text{g/mL}$ of iron, as directed under *Iron* (241).

Diluted standard iron solution: Immediately before use, dilute an accurately measured volume of the *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 $\mu\text{g/mL}$ of iron.

Sample solution: Shake the residue obtained from the test for *Residue on Ignition* with 20 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 20 ppm of iron.

- **LIMIT OF SULFUR DIOXIDE, Method IV** (525): NMT 50 ppm

- **LIMIT OF PROPYLENE GLYCOL**

Internal standard solution: 0.5 mg/mL of 1,3-propanediol in anhydrous pyridine

Standard stock solution: 0.5 mg/mL of USP Propylene Glycol RS in *Internal standard solution*

Standard solution: Transfer 0.1 mL of the *Standard stock solution* to a 2-mL vessel with a screw cap fitted with a septum. Add 0.9 mL of anhydrous pyridine, 0.2 mL of hexamethyldisilazane, and 0.1 mL of trimethylchlorosilane. Close, and mix. Allow to stand for 15 min before injection.

Sample stock solution: Transfer 200 mg of Pregelatinized Hydroxypropyl Corn Starch to a 100-mL volumetric flask. Add 1.0 mL of the *Internal standard solution* and 9.0 mL of anhydrous pyridine. Boil under reflux using a water bath for 20 min. Allow to cool to room temperature.

Sample solution: Transfer 1.0 mL of the *Sample stock solution* to a 2-mL vessel with a screw cap fitted with a septum. Add 0.2 mL of hexamethyldisilazane and 0.1 mL of trimethylchlorosilane. Close, and mix. Allow to stand for 15 min before injection.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm \times 30-m fused-silica capillary column; 0.25- μm layer of phase G1

Temperature

Detector: 250°

Injection port: 250°

Column: 70°. [NOTE—The column must be desorbed regularly. Conditions: Program from 70° to 300° at 7°/min, and maintain 10 min at 300°.]

Carrier gas: Helium

Flow rate: 3 mL/min

Injection type: Split ratio of 1:30

Injection size: 1 μL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for the trimethylsilylated derivative of propylene glycol and the trimethylsilylated derivative of 1,3-propanediol are 1.0 and 1.4, respectively.]

Suitability requirements

Resolution: NLT 2.0 between the peaks due to the trimethylsilylated derivative of propylene glycol and the trimethylsilylated derivative of 1,3-propanediol

Analysis

Samples: *Standard solution* and *Sample solution*
 Calculate the percentage of propylene glycol in the portion of Pregelatinized Hydroxypropyl Corn Starch taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = internal standard ratio (peak response of propylene glycol/peak response of 1,3-propanediol) from the *Sample solution*

R_S = internal standard ratio (peak response of propylene glycol/peak response of 1,3-propanediol) from the *Standard solution*

C_S = concentration of USP Propylene Glycol RS in the *Standard solution* (mg/mL)

C_U = concentration of Pregelatinized Hydroxypropyl Corn Starch in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.1%

- **LIMIT OF OXIDIZING SUBSTANCES**

Sample: 4.0 g

Analysis: Transfer the *Sample* to a glass-stoppered 125-mL conical flask, and add 50.0 mL of a mixture of water and methanol (1:1). Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine

color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 ppm, calculated as H₂O₂).

SPECIFIC TESTS

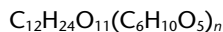
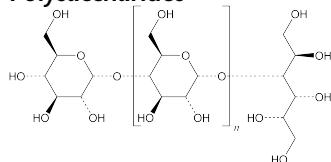
- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 10³ cfu/g, the total combined molds and yeasts count does not exceed 10² cfu/g, and it meets the requirements of the test for the absence of *Escherichia coli*.
- **pH** (791)
Sample solution: Progressively suspend 3.0 g of Pregelatinized Hydroxypropyl Corn Starch in 100.0 mL of carbon dioxide-free water, stirring continuously. Determine the pH when all the solid is wetted.
Acceptance criteria: 4.5–8.0
- **LOSS ON DRYING** (731): Dry about 1 g at 130° for 90 min; it loses NMT 15.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.
- **USP REFERENCE STANDARDS** (11)
USP Propylene Glycol RS

Hydrogenated Starch Hydrolysate

Hydrogenated Polysaccharides



Polyglucitol;
Polyglycitol syrup [68425-17-2].

DEFINITION

Hydrogenated Starch Hydrolysate is a mixture that contains NLT 50% of hydrogenated polysaccharides containing more than 3 D-glucopyranosyl units terminated with a D-glucityl unit, calculated on the anhydrous basis. Other ingredients can include sorbitol, maltitol, and other sugar polyols.

IDENTIFICATION

- **A.** It meets the requirements of the test for *Content of Maltitol and Sorbitol*.
- **B.** It meets the requirements of the test for *Content of Hydrogenated Polysaccharides*.
- **C. LIMIT OF DIETHYLENE GLYCOL AND ETHYLENE GLYCOL**
[NOTE—Perform this test for liquid products of Hydrogenated Starch Hydrolysate.]
Diluent: Acetone and water (96:4)
Standard stock solution: 0.5 mg/mL of USP Diethylene Glycol RS and 0.5 mg/mL of USP Ethylene Glycol RS in *Diluent*
Internal standard stock solution: 0.5 mg/mL of 1,3-butanediol (internal standard) in *Diluent*
Standard solution: 0.04 mg/mL of USP Diethylene Glycol RS, 0.04 mg/mL of USP Ethylene Glycol RS, and 0.04 mg/mL of 1,3-butanediol (internal standard), in *Diluent*, prepared from the *Standard stock solution* and the *Internal standard stock solution*

Sample solution: Transfer 1.0 g of Hydrogenated Starch Hydrolysate to a 25-mL volumetric flask. Add 1.0 mL of water to the flask, and mix on a vortex mixer for 3 min. Add 2.0 mL of the *Internal standard stock solution*, and add the remaining *Diluent* to the flask in three equal portions to volume. Mix the contents for about 3 min after each addition of *Diluent*. Pass a portion of the supernatant layer through a nylon filter of 0.45-µm pore size. Discard the first 2 mL of the filtrate, and collect the rest of the filtrate for analysis. [NOTE—Acetone is used to precipitate sugar alcohols.]

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 15-m fused-silica capillary; 0.25-µm layer of phase G46

Temperature

Detector: 300°

Injection port: 240°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	50	300	5

Carrier gas: Helium

Flow rate: 3.0 mL/min

Injection size: 1.0 µL

Injection type: Split injection. The split ratio is about 10:1. [NOTE—A general-purpose split/splitless, tapered, glass wool, deactivated liner is used.]

System suitability

Sample: *Standard solution*

[NOTE—See *Table 2*. Relative retention times are provided for information only, and the standards should be used to ensure appropriate peak identification.]

Table 2

Name	Relative Retention Time
Ethylene glycol	1.0
1,3-Butanediol (internal standard)	2.0
Diethylene glycol	2.5

Suitability requirements

Resolution: NLT 15 between ethylene glycol and 1,3-butanediol

Analysis

Samples: *Standard solution* and *Sample solution*

Based on the *Standard solution*, identify the peaks of ethylene glycol, 1,3-butanediol (internal standard), and diethylene glycol. Compare peak area ratios of ethylene glycol to the internal standard and of diethylene glycol to the internal standard in the *Standard solution* and *Sample solution*, respectively.

Acceptance criteria

Diethylene glycol: The peak area ratio of diethylene glycol to the internal standard in the *Sample solution* is NMT the peak area ratio of diethylene glycol to the internal standard in the *Standard solution*, corresponding to NMT 0.10% of diethylene glycol in Hydrogenated Starch Hydrolysate.

Ethylene glycol: The peak area ratio of ethylene glycol to the internal standard in the *Sample solution* is NMT the peak area ratio of ethylene glycol to the internal standard in the *Standard solution*, corresponding to

NMT 0.10% of ethylene glycol in Hydrogenated Starch Hydrolysate.

ASSAY

• CONTENT OF MALTITOL AND SORBITOL

Mobile phase: Degassed water

Standard solution: Dissolve accurately weighed quantities of USP Maltose Monohydrate RS, USP Maltitol RS, USP Dextrose RS, and USP Sorbitol RS in water to obtain a solution having known concentrations of about 1.0 mg/mL for each, calculated on the anhydrous basis.

Sample solution: Transfer a quantity of Hydrogenated Starch Hydrolysate, equivalent to 100 mg on the anhydrous basis, to a 100-mL volumetric flask. Dilute with water to volume, and mix. Transfer approximately 10 mL of the solution into a separate container, shake the solution for 30 s, pass through a filter of 0.45-μm or finer pore size into a suitable autosampler vial, and seal.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.7-mm × 30-cm; packing L58

Temperature

Detector: 40°

Column: 80°, controlled within ±2°

Flow rate: 0.3 mL/min

Injection size: 50 μL

System suitability

Sample: *Standard solution*

[NOTE—See *Table 3* for relative retention times.]

Table 3

Name	Relative Retention Time
Maltose	0.81
Maltitol	0.84
Dextrose	0.94
Sorbitol	1.00

[NOTE—Sorbitol is the last peak to elute.]

System suitability requirements

Relative standard deviation: NMT 2.0% for the maltitol peak

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of each component, maltose (P_{M1}), maltitol (P_{M2}), dextrose (P_D), and sorbitol (P_S), in the solid portion of Hydrogenated Starch Hydrolysate taken:

$$\text{Result} = V \times (r_U/r_S) \times (C/W) \times 100$$

V = volume of the *Sample solution*, 100 mL

r_U = peak response for the respective component (maltose, maltitol, dextrose, sorbitol) from the *Sample solution*

r_S = peak response for the respective component (maltose, maltitol, dextrose, sorbitol) from the *Standard solution*

C = concentration of the respective component (maltose, maltitol, dextrose, sorbitol) in the *Standard solution* (mg/mL)

W = weight of Hydrogenated Starch Hydrolysate that was taken to prepare the *Sample solution* (mg), calculated on the anhydrous basis

Acceptance criteria: Less than 50% of total maltitol and sorbitol and less than 1% of total maltose and dextrose, on the anhydrous basis

• CONTENT OF HYDROGENATED POLYSACCHARIDES

Mobile phase, Standard solution, Sample solution, and Chromatographic system: Prepare as directed in the test for *Content of Maltitol and Sorbitol*.

Analysis

Samples: *Standard solution* and *Sample solution*

Inject a volume (about 50 μL) of the *Sample solution*, and measure all the peak areas. The elution pattern includes the higher-molecular-weight hydrogenated polysaccharides containing more than 11 D-glucopyranosyl units, followed by some individual peaks representing hydrogenated polysaccharides containing NMT 10 D-glucopyranosyl units, if this hydrogenated species is present. The higher-molecular-weight hydrogenated polysaccharides containing more than 11 D-glucopyranosyl units can be integrated into one peak; the relative retention time is about 0.29 relative to the peak of sorbitol. The relative retention times for hydrogenated polysaccharides containing NMT 10 D-glucopyranosyl units are given in *Table 4*.

Table 4

Hydrogenated Species for Degree of Polymerization (DP)	Relative Retention Time
Sorbitol (HDP 1)	1.00
Maltitol (HDP 2)	0.84
Maltotriitol (HDP 3)	0.72
Maltotetraitol (HDP 4)	0.64
Maltopentaitol (HDP 5)	0.58
Maltohexaitol (HDP 6)	0.53
Maltoheptaitol (HDP 7)	0.48
Maltooctaitol (HDP 8)	0.45
Maltononaitol (HDP 9)	0.42
Maltodecailol (HDP 10)	0.40

Calculate the percentage of hydrogenated polysaccharides containing more than 3 D-glucopyranosyl units in the solid portion of Hydrogenated Starch Hydrolysate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = sum of peak areas of hydrogenated polysaccharides containing more than 3 D-glucopyranosyl units from the *Sample solution*

r_T = sum of all the peak areas from the *Sample solution*

Acceptance criteria: NLT 50% of hydrogenated polysaccharides containing more than 3 D-glucopyranosyl units, on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION (281):** NMT 0.15%, ignition of a quantity of Hydrogenated Starch Hydrolysate equivalent to 1.0 g of solid, on the anhydrous basis

• LIMIT OF CHLORIDE

Sample solution: Transfer a quantity of Hydrogenated Starch Hydrolysate, equivalent to 25 g on the anhydrous basis, to a beaker, add 100 mL of water, and stir until the Hydrogenated Starch Hydrolysate is completely dissolved.

Analysis: Add 1.0 mL of potassium chromate indicator solution (1 in 20) to the *Sample solution*. Slowly titrate with 0.1 N silver nitrate VS until a reddish-orange color persists.

Calculate the quantity, in μg, of chloride in each g of Hydrogenated Starch Hydrolysate taken:

$$\text{Result} = (F \times M_r \times N \times V)/W$$

F = factor converting mg to μg, 10³ μg/mg

M_r = molar mass of chloride, 35.45

- N* = exact normality of the silver nitrate solution
V = volume of the silver nitrate solution consumed in the titration (mL)
W = weight of Hydrogenated Starch Hydrolysate taken to prepare the *Sample solution* (g)

Acceptance criteria: NMT 50 µg/g (ppm) of chloride

- **CHLORIDE AND SULFATE, Sulfate (221):** 1.0 g of the solid portion of Hydrogenated Starch Hydrolysate shows no more sulfate than corresponds to 0.10 mL of 0.020 N sulfuric acid: NMT 100 µg/g (ppm) of sulfate is found.
- **LIMIT OF NICKEL**

[NOTE—When water is specified as the diluent, use deionized ultra-filtered water.]

Digester solution (aqua regia): Add 360 mL of hydrochloric acid and 240 mL of nitric acid to 1200 mL of water.

Blank solution: Add 40 mL of nitric acid to a 2-L volumetric flask, dilute with water to volume, and mix well.

Internal standard solution: Transfer 2.0 mL of commercially prepared yttrium reference standard solution (1000 ppm) to a 1-L volumetric flask, dilute with *Blank solution* to volume, and mix well. The *Internal standard solution* contains 2 µg/mL of yttrium.

Standard stock solution: [NOTE—Prepare this solution fresh every two months.] Quantitatively dilute an accurately measured volume of a commercially prepared nickel ICP standard (1000 ppm) with *Blank solution* to obtain a solution containing 10 µg/mL of nickel (*Standard stock solution 10 ppm*).

Standard solutions: [NOTE—Prepare these solutions fresh weekly.] Separately pipet 1.0, 2.0, and 4.0 mL of *Standard stock solution*, respectively, into three 200-mL volumetric flasks. Dilute the content in each flask with *Blank solution* to volume, and mix well. These are, respectively, the *Standard nickel solution 50 ppb*, *Standard nickel solution 100 ppb*, and *Standard nickel solution 200 ppb*.

Sample solution: Transfer a quantity of Hydrogenated Starch Hydrolysate, equivalent to 10.0 g on the anhydrous basis, into a 125-mL conical flask. Add 40 mL of *Digester solution*, and place on a hot plate. Heat the solution for about 20 min, being careful to prevent the solution from boiling over. Pull the sample off the hot plate just before it turns a dark caramel color. [NOTE—Do not overburn the sample.] Transfer the flask's contents into a clean, dry, 50-mL volumetric flask with washings of *Blank solution*. Dilute with *Blank solution* to volume. Filter the sample into a 15-mL centrifuge tube, using a 10-mL BD syringe fitted with a syringe filter of 0.45-µm pore size.

Instrumental conditions

(See *Plasma Spectrochemistry* (730).)

Mode: Inductively coupled plasma–optical emission spectroscopy (ICP–OES) configured in an axial optical alignment

Detector: Set a UV detector to scan nickel at 232.005 nm and yttrium at 371.029 nm, and set the sample read time to 10 s minimum and 50 s maximum.

Analysis

Samples: *Blank solution*, *Standard solutions*, and *Sample solution*

Take three replicate scans with the integration set to one point per peak. Set forward power from the RF generator to 1500 watts. The argon plasma feed gas flows at 15 L/min with the auxiliary gas (shear gas) set to flow at 0.5 L/min. Use a gem cone nebulizer with a nebulization gas flow rate of 0.55 L/min. Deliver the sample to the spray chamber with a multichannel peristaltic pump set to deliver sample at a rate of 1.00 mL/min. Add the *Internal standard solution* in-line via a mixing block between the sample probe and the spray chamber. Flush the samples through the system for 30 s at a rate of 4.0 mL/min before analysis. Program a 60-s read delay into the sampling routine to

allow for fluid flow equilibration after the high-speed flush, before the first analytical read of the sample. Between samples, wash the pumping system by flushing the *Blank solution* for 30 s at a rate of 4.0 mL/min.

Instrument performance must be verified to conform to the manufacturer's specifications for resolution and sensitivity. Before analyzing samples, the instrument must pass a suitable performance check.

Generate the calibration curve using the *Blank solution*, *Standard nickel solution 50 ppb*, *Standard nickel solution 100 ppb*, and *Standard nickel solution 200 ppb* as follows. Scan the *Internal standard solution* while running the *Blank solution* to measure the intensity of the yttrium emission. Hold this value constant throughout the remainder of the test. Separately scan the *Blank solution*, *Standard nickel solution 50 ppb*, *Standard nickel solution 100 ppb*, and *Standard nickel solution 200 ppb* for nickel and yttrium. [NOTE—Add the *Internal standard solution* via an in-line mixing chamber.] Normalize the yttrium intensity to the value of the *Internal standard solution*. Apply this normalization factor to the nickel intensity, which is then referred to as the corrected nickel intensity. Construct a calibration curve by plotting the corrected nickel intensity versus the known concentrations, in ng/mL, of the nickel: the linear regression coefficient is NLT 0.999.

Similarly, analyze the *Sample solution* on the ICP. Plot the intensity of the emission of the *Sample solution* on the calibration curve. Obtain the concentration of nickel, *C*, in ng/mL, in the *Sample solution* through the calibration curve.

Calculate the content, in µg/g, of nickel in the solid portion of Hydrogenated Starch Hydrolysate taken:

$$\text{Result} = F \times V \times (C/W)$$

- F* = factor converting ng to µg, 10^{-3} µg/ng
V = volume of the *Sample solution*, 50 mL
C = concentration of nickel in the *Sample solution* (ng/mL)
W = weight of Hydrogenated Starch Hydrolysate calculated on the anhydrous basis (g)

Acceptance criteria: Nickel content is NMT 1 µg/g (ppm).

• LIMIT OF REDUCING SUGARS

Analysis: Dissolve a quantity of Hydrogenated Starch Hydrolysate, equivalent to 1.0 g on the anhydrous basis, in 6 mL of water with the aid of gentle heat, if necessary. Cool, and add 20.0 mL of cupric citrate TS and a few glass beads. Heat so that boiling begins after 4 min, and maintain boiling for 3 min. Cool rapidly, and add 40 mL of diluted acetic acid, 60 mL of water, and 20.0 mL of 0.05 N iodine VS. With continuous shaking, add 25 mL of a mixture of 6 mL of hydrochloric acid and 94 mL of water. When the precipitate has dissolved, titrate the excess of iodine with 0.05 N sodium thiosulfate VS, using 2 mL of starch TS as an indicator, added toward the end of the titration.

Acceptance criteria: NLT 12.8 mL of 0.05 N sodium thiosulfate, corresponding to NMT 1% of reducing sugars

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **PH (791):** 3.0–7.0, in a 20% (w/w) solution in freshly boiled and cooled water

- **WATER DETERMINATION, Method I (921)**
For dried powder product: NMT 6.0%
For liquid product: Within ± 1.5 units of the labeled value

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified for liquid product; protect from moisture for dried powder product.
- **LABELING:** Label it to indicate *Water* content, as the percentage of water, for liquid product.
- **USP REFERENCE STANDARDS (11)**
USP Dextrose RS
USP Diethylene Glycol RS
USP Ethylene Glycol RS
USP Maltitol RS
USP Maltose Monohydrate RS
USP Sorbitol RS

Modified Starch

DEFINITION

Modified Starch is Starch modified by chemical means. Food Starch may be acid-modified, bleached, oxidized, esterified, etherified, or treated enzymatically to change its functional properties (21 CFR 172.892).

IDENTIFICATION

- **A.**
Corn starch: Polygonal, rounded, or spheroidal granules up to 35 μm in diameter and usually having a circular or several-rayed central cleft
Tapioca starch: Spherical granules with one truncated side, typically 5–35 μm in diameter and usually having a circular or several-rayed central cleft
Potato starch: Irregularly shaped, ovoid, or pear-shaped granules, usually 30–100 μm in size but occasionally exceeding 100 μm ; or rounded, 10–35 μm in size. There are occasional compound granules having two to four components. The ovoid and pear-shaped granules have an eccentric hilum, and the rounded granules have an accentric or slightly eccentric hilum. All granules show clearly visible concentric striations.
Wheat starch: Large and small granules, usually 10–60 μm in diameter. The central hilum and striations are visible or barely visible.
- **B.**
Sodium hydroxide solution: 2% (w/w)
Sample: 0.6 g
Analysis: Transfer the *Sample* to a 25-mL glass vial with a plastic cap. Add 9.4 g of water, cap, and shake vigorously to evenly disperse the starch. Add 10 g of the *Sodium hydroxide solution*, cap, and shake vigorously for 1 min to create a smooth mixture. Evaluate within 1 min.
Acceptance criteria: The final solution is translucent to opaque with a fluid consistency. A yellow tint of the final solution is acceptable.
- **C.** A water slurry of the Modified Starch is colored orange-red to deep blue by iodine TS.

IMPURITIES

- **RESIDUE ON IGNITION (281)**
Sample: 2.0 ± 0.1 g
Analysis: Proceed as directed in the chapter.
Acceptance criteria: NMT 1.5%
- **IRON (241)**
Test preparation: Dissolve the residue obtained in the test for *Residue on Ignition* in 8 mL of hydrochloric acid

with the aid of gentle heating. Dilute with water to 100 mL in a volumetric flask, and mix. Dilute 25 mL of this solution with water to 47 ± 1 mL.

Analysis: Proceed as directed in the chapter.

Acceptance criteria: NMT 20 ppm

- **LIMIT OF SULFUR DIOXIDE**

Sample: 20.0 ± 0.1 g

Analysis: Mix the *Sample* with 200 mL of 5% alcohol until a smooth suspension is obtained, and vacuum-filter through paper (Whatman No.1 or equivalent). To 100 mL of the filtrate add 3 mL of starch TS, and titrate with 0.01 N iodine VS to the first permanent blue color.

Acceptance criteria: NMT 1.7 mL of 0.01 N iodine VS is consumed, which corresponds to NMT 50 ppm of sulfur dioxide being found.

- **OXIDIZING SUBSTANCES**

Sample: 4.0 g

Titrimetric system

Mode: Direct titration

Titrant: 0.002 N sodium thiosulfate VS

Blank: 30.0 mL of water, accurately measured

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with *Titrant* to the disappearance of the starch-iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate is equivalent to 34 μg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 12.6 mL of 0.002 N sodium thiosulfate is required (180 ppm, calculated as H_2O_2), which corresponds to NMT 0.018% of oxidizing substances

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined molds and yeasts count does not exceed 10^2 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **pH (791)**
Sample: 20.0 ± 0.1 g
Analysis: Transfer the *Sample* to a suitable nonmetallic container, and add 100 mL of water to obtain a slurry. Stir using a magnetic stirrer at a moderate rate for 5 min, and determine the pH to the nearest 0.1 unit.
Acceptance criteria: 3.0–9.0
- **LOSS ON DRYING (731)**
Analysis: Dry a sample at 120° for 4 h.
Acceptance criteria
Corn starch and Wheat starch: NMT 15.0%
Tapioca starch: NMT 18.0%
Potato starch: NMT 21.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Pea Starch

DEFINITION

Pea Starch is obtained from the seeds of *Pisum sativum* L.

IDENTIFICATION

- **A.** Examined under a microscope using a mixture of equal volumes of glycerin and water, it presents a majority of large elliptical granules, 25-45 μm in size, sometimes irregular, or reniform. It also presents a minority of small rounded granules, 5-8 μm in size. Granules can present cracks or irregularities. Sometimes, granules show barely visible concentric striations. Exceptionally, granules show a slit along the main axis. Between orthogonally oriented polarizing plates or prisms, the granules show a distinct black cross.
- **B.** Suspend 1 g of it in 50 mL of water, boil for 1 min, and cool: a thin, cloudy mucilage is formed.
- **C.** To 1 mL of the mucilage obtained in *Identification test B* add 0.05 mL of iodine and potassium iodide TS 2: an orange-red to dark blue color is produced, which disappears on heating.

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 0.6%, determined on a 1.0-g sample
- **LIMIT OF IRON**

Standard iron stock solution: Prepare a solution containing the equivalent of 10 $\mu\text{g}/\text{mL}$ of iron, as directed under *Iron* (241).

Diluted standard iron solution: Immediately before use, dilute a measured volume of *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 $\mu\text{g}/\text{mL}$ of iron.

Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Sample solution: Shake 1.0 g of Pea Starch with 50 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 50 $\mu\text{g}/\text{g}$ of iron.

- **LIMIT OF SULFUR DIOXIDE**

[NOTE—Perform either *Test 1* or *Test 2*.]

Test 1

Carbon dioxide: Use carbon dioxide with a flow regulator that will maintain a flow of 100 ± 10 mL/min.

Hydrogen peroxide solution: Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Neutralize the solution with 0.01 N sodium hydroxide to a pH of 4.1, determined potentiometrically.

Potassium metabisulfite solution: Transfer 0.87 g of potassium metabisulfite ($\text{K}_2\text{S}_2\text{O}_5$) and 0.2 g of edetate disodium to a 1000-mL volumetric flask. Dilute with water to volume before mixing. [NOTE—Edetate disodium is used to protect the sulfite ion from oxidation.]

Apparatus: In this test, the sulfur dioxide is released from the sample in a boiling acid medium and is removed by a stream of carbon dioxide. The separated gas is collected in a dilute hydrogen peroxide solution where the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali. A suitable apparatus for sulfur dioxide determination is shown in the accompanying diagram (Figure 1).

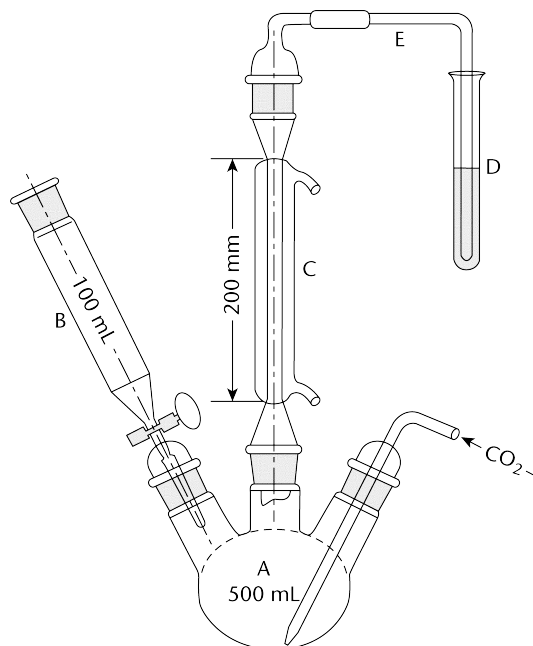


Figure 1

The apparatus consists of a 500-mL three-neck, round-bottom boiling flask, A; a separatory funnel, B, having a capacity of 100 mL or greater; a gas inlet tube of sufficient length to permit introduction of the carbon dioxide within 2.5 cm of the bottom of the boiling flask; a reflux condenser, C, having a jacket length of 200 mm; and a delivery tube, E, connecting the upper end of the reflux condenser to the bottom of a receiving test tube, D. Apply a thin film of stopcock grease to the sealing surfaces of all the joints except the joint between the separatory funnel and the boiling flask, and clamp the joints to ensure tightness.

System suitability test

Test A: Using the *Potassium metabisulfite solution* as the standard, proceed as directed in *Analysis*, except for replacing the 25.0 g of Pea Starch with 20 mL of the *Potassium metabisulfite solution*.

Calculate the content, in $\mu\text{g}/\text{mL}$, of sulfur dioxide in the *Potassium metabisulfite solution* taken:

$$\text{Result} = (F \times \text{MW} \times V \times N) / V_p$$

F = factor for conversion of mg to μg , 1000
MW = milliequivalent weight of sulfur dioxide, 32.03

V = volume of titrant consumed (mL)

N = normality of the titrant

V_p = volume of the *Potassium metabisulfite solution* taken for the test (mL)

Test B: In a 100-mL conical flask, add 20 mL of 0.02 N iodine solution and 5 mL of 2 N hydrochloric acid. Add 1 mL of starch TS, and titrate with *Potassium metabisulfite solution* until the first discoloration is observed.

Calculate the content, in $\mu\text{g}/\text{mL}$, of sulfur dioxide in the *Potassium metabisulfite solution*:

$$\text{Result} = (F \times \text{MW} \times V_i \times N_i) / V_p$$

F = factor for conversion of mg to μg , 1000

MW = milliequivalent weight of sulfur dioxide, 32.03

V_i = the volume of the iodine solution used in the test (mL)

N_i = normality of the iodine solution

V_p = volume of the *Potassium metabisulfite solution* consumed (mL)

The difference between the sulfur dioxide contents obtained from *Test A* and *Test B* is NMT 5% of their mean value. *Test B* shall be performed within 15 min after the completion of *Test A*. [NOTE—This time limit avoids potential variation in the sulfur dioxide content of the *Potassium bisulfite solution* when stored at room temperature.]

Analysis: Add 150 mL of water to the boiling flask (A) (see *Figure 1*). Close the stopcock of the separatory funnel, and begin the flow of carbon dioxide through the apparatus at a rate of 100 ± 5 mL/min. Start the condenser coolant flow. Place 10 mL of *Hydrogen peroxide solution* in the receiving test tube (D). After 15 min, without interrupting the flow of carbon dioxide, remove the separatory funnel (B) from the boiling flask, and transfer 25.0 g of Pea Starch to the boiling flask with the aid of 100 mL of water. Apply stopcock grease to the outer joint of the separatory funnel, and replace the separatory funnel in the boiling flask. Close the stopcock of the separatory funnel, and add 80 mL of 2 N hydrochloric acid to the separatory funnel. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the boiling flask, guarding against the escape of sulfur dioxide into the separatory funnel by closing the stopcock before the last few mL of hydrochloric acid drain out. Boil the mixture for 1 h. Open the stopcock of the funnel, stop the flow of carbon dioxide, discontinue heating the flask, and turn off the cooling water in the condenser. Remove the receiving test tube, and transfer its contents to a 200-mL wide-necked conical flask. Rinse the receiving test tube with a small portion of water, add the rinsing to the 200-mL conical flask, and mix. Heat on a water bath for 15 min, and allow to cool. Titrate the contents with 0.1 N sodium hydroxide VS until the pH reaches 4.1, determined potentiometrically. Perform a blank determination, and make any necessary correction (see *Titrimetry* (541)). Calculate the content, in $\mu\text{g/g}$, of sulfur dioxide in the Pea Starch taken:

$$\text{Result} = (F \times \text{MW} \times V \times N)/W$$

F = factor for conversion of mg to μg , 1000
 MW = milliequivalent weight of sulfur dioxide, 32.03
 V = volume of titrant consumed (mL)
 N = normality of the titrant
 W = weight of the Pea Starch taken (g)

Test 2: Determine the content of sulfur dioxide as directed under *Sulfur Dioxide* (525), *Method I*. Use 200 mL of water as a solvent. Then, to 100 mL of the clear filtrate, add 3 mL of starch TS, and titrate with 0.01 N iodine VS to the first permanent blue color.

Acceptance criteria: NMT 50 $\mu\text{g/g}$ of sulfur dioxide

Organic Impurities

• PROCEDURE 1: LIMIT OF OXIDIZING SUBSTANCES

Sample: 4.0 g of Pea Starch

Analysis: Transfer the *Sample* to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 μg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 $\mu\text{g/g}$, calculated as H_2O_2).

• PROCEDURE 2: FOREIGN MATTER

Analysis: Examine under a microscope, using a mixture of equal volumes of glycerin and water.

Acceptance criteria: NMT traces of matter other than starch granules are present. No starch grains of any other origin are present.

SPECIFIC TESTS

• MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):

The total aerobic microbial count does not exceed 1000 cfu/g, the total combined molds and yeasts count does not exceed 100 cfu/g, and it meets the requirements of the test for the absence of *Escherichia coli*.

• pH (791)

Sample solution: Prepare a slurry by weighing 5.0 g of Pea Starch, transferring to a suitable nonmetallic container, and adding 25.0 mL of freshly boiled and cooled water.

Analysis: Agitate continuously at a moderate rate for 1 min. Stop the agitation, allow to stand for 15 min, and shake again. Determine the pH to the nearest 0.1 unit: the pH is determined potentiometrically.

Acceptance criteria: 5.0–8.0

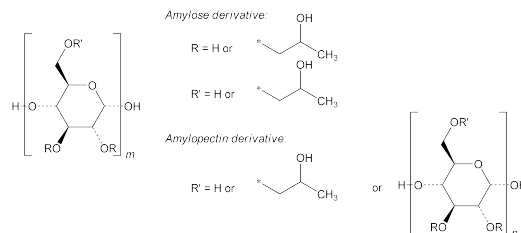
• LOSS ON DRYING (731):

Dry about 1 g at 130° for 90 min: it loses NMT 16.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.

Hydroxypropyl Pea Starch



For the Amylose derivative, *m* is about 300–1000.

DEFINITION

Hydroxypropyl Pea Starch is partially substituted 2-hydroxypropylether obtained from pea starch by a chemical modification of etherification with propylene oxide. In addition, this starch may be partially hydrolyzed using acids or enzymes to obtain thinned starch. It contains NLT 2.0% and NMT 7.0% of hydroxypropyl groups, on the dried basis.

IDENTIFICATION

• A. PROCEDURE

Analysis: Examine under a microscope, using NLT 20 \times magnification and a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: It presents a majority of large elliptical granules 25–45 μm in size, sometimes irregular or reniform. It also presents a minority of small rounded granules 5–8 μm in size. Granules can present cracks or irregularities. Sometimes, granules show barely visible concentric striations. Exceptionally, granules show a slit along the main axis. Between orthogonally oriented polarizing plates or prisms, the granules show a distinct black cross.

• B. PROCEDURE

Sample solution: Suspend 1 g of Hydroxypropyl Pea Starch in 50 mL of water, boil for 1 min, and cool.

Acceptance criteria: A translucent or clear mucilage is formed.

• **C. PROCEDURE**

Analysis: To 1 mL of the *Sample solution* obtained in Identification test B add 0.05 mL of iodine and potassium iodide TS 2.

Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

• **D. PROCEDURE**

Ninhydrin solution: Dissolve 3 g of ninhydrin in 100 mL of a 45.5-g/L solution of sodium metabisulfite.

Diluted sulfuric acid: 98 g/L of H₂SO₄

Sample: 100 mg of Hydroxypropyl Pea Starch

Analysis: Transfer the *Sample* to a 100-mL volumetric flask, and add 12.5 mL of *Diluted sulfuric acid*. Place the flask in a water bath, and heat until the *Sample* is dissolved. Cool, and dilute with water to 100 mL. [**CAUTION**—When sulfuric acid is miscible with water, it produces intense heat.]

Pipet 1 mL of this solution to a glass-stoppered, 25-mL graduated test tube and, with the tube immersed in cold water, add drop-wise 8 mL of sulfuric acid. Mix well, and place the tube in a boiling water bath for exactly 3 min. Immediately transfer the tube to an ice bath until the solution is chilled. Add 0.6 mL of *Ninhydrin solution*, carefully allowing the reagent to run down the walls of the test tube. Immediately shake the tube well, and place it in a water bath at 25° for 100 min. Dilute with sulfuric acid to 25 mL [**CAUTION**—Use sulfuric acid cautiously.], and mix by inverting the tube several times. Do not shake.

Acceptance criteria: A violet color develops within 5 min due to the presence of hydroxypropyl groups (starch ether).

ASSAY

• **PROCEDURE FOR HYDROXYPROPYL GROUPS**

Deuterium chloride solution: Dilute 1 mL of deuterium chloride (38% w/w) with 5 mL of deuterium oxide.

Internal standard solution: Dissolve 50.0 mg of sodium 3-trimethylsilyl-1-propane sulfonate in about 5 g of deuterium oxide, weighed to the nearest 0.1 mg. Store in a sealed bottle.

Sample solution: Disperse 20 g of Hydroxypropyl Pea Starch in 200.0 mL of carbon dioxide-free water at room temperature. Agitate for 15 min, and filter. Repeat the operation two more times. If poor dispersibility or slow filtration is observed, use refrigerated carbon dioxide-free water for the washing operation. Dry the washed starch for NLT 4 h in vacuum at 30 ± 5°. Weigh 12.0 mg of this sample in a 5-mm NMR tube. Add 0.75 mL of deuterium oxide and 0.1 mL of *Deuterium chloride solution*. Cap the tube, mix, and place it in a boiling water bath until a clear solution is obtained. [NOTE—This may take 3 min to 1 h.] When a clear solution is obtained, allow to cool to room temperature. Dry the exterior of the tube, and weigh to the nearest 0.1 mg. Add 0.05 mL of *Internal standard solution*, and weigh to the nearest 0.1 mg. Determine the mass of the *Internal standard solution* added. Mix thoroughly.

Nuclear magnetic resonance spectrometry

(See *Nuclear Magnetic Resonance* (761), *Quantitative Application*.)

Apparatus: FT-NMR spectrometer at minimum 300 MHz

Acquisition of ¹H NMR spectra: The following parameters may be used.

Sweep width: 8 ppm (about -1.0 to +7 ppm)

Irradiation frequency offset: None

Time domain: NLT 64 K

Pulse width: 90 degree

Pulse delay: 10 s

Dummy scans: 0

Number of scans: 8

Use the CH₃ signal of the internal standard for shift referencing. Set the shift of the peak of the singlet to 0 ppm. Record the FID signal.

Analysis

Samples: *Internal standard solution* and *Sample solution*
Call the integration sub-routine after phase corrections and baseline correction between -0.5 and +6 ppm. Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at +1.2 ppm (A₂), and of the methyl groups at 0 ppm of the internal standard (A₁) without ¹³C-satellites.

Measure the signal coming from the 3 protons of the methyl group in the hydroxypropyl function. Calculate the content of hydroxypropyl groups as a percentage (w/w, dried basis):

$$\text{Result} = \left(N \times A_2 / A_1 \right) \times \left(C_i \times W_i / W \right) \times \left(M_{r2} / M_{r1} \right) \times \left[100 / (100 - B) \right] \times 100$$

- N = numerical value representing the 3 methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate), 3
A₂ = area of the methyl groups of hydroxypropyl in Hydroxypropyl Pea Starch
A₁ = area of the methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate)
C_i = concentration of the internal standard in the *Internal standard solution* (mg/g)
W_i = weight of the *Internal standard solution* in the NMR tube (g)
W = weight of the washed and dried Hydroxypropyl Pea Starch in the NMR tube (mg)
M_{r1} = molecular weight of the internal standard, 218.32 g/mol
M_{r2} = molar mass of hydroxypropyl group, 59.09 g/mol
B = moisture content of the washed and dried Hydroxypropyl Pea Starch used in the *Sample solution*, as a percentage (w/w)

Acceptance criteria: The content of hydroxypropyl groups is 2.0%–7.0%.

IMPURITIES

Inorganic Impurities

• **RESIDUE ON IGNITION (281):** NMT 0.6%, determined on a 1.0-g test specimen

• **LIMIT OF IRON**

Standard iron stock solution: Prepare a solution containing the equivalent of 10 µg/mL of iron, as directed under *Iron* (241).

Diluted standard iron solution: Immediately before use, dilute an accurately measured volume of *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 µg/mL of iron.

Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Sample solution: Shake 1.0 g of Hydroxypropyl Pea Starch with 50 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 50 µg/g of iron.

• **LIMIT OF SULFUR DIOXIDE, Method IV (525):** NMT 50 ppm
Organic Impurities

• **PROCEDURE 1: LIMIT OF OXIDIZING SUBSTANCES**

Sample: 4.0 g of Hydroxypropyl Pea Starch

Analysis: Transfer the *Sample* to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 µg/g, calculated as H₂O₂).

• **PROCEDURE 2: FOREIGN MATTER**

Sample: 50 mg/mL of Hydroxypropyl Pea Starch in a mixture of glycerin and water (1:1)

Analysis: Examine under a microscope, using NLT 20× magnification and a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: NMT traces of matter other than Hydroxypropyl Pea Starch granules are present.

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 10³ cfu/g, the total combined molds and yeasts count does not exceed 10² cfu/g, and it meets the requirements of the test for the absence of *Escherichia coli*.

• **PH (791)**

Sample solution: Suspend 5.0 g of Hydroxypropyl Pea Starch in 25.0 mL of carbon dioxide-free water, and shake for 60 s. Allow to stand for 15 min.

Acceptance criteria: 4.5–8.0

- **LOSS ON DRYING (731):** Dry about 1 g at 130° for 90 min: it loses NMT 15.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.

Pregelatinized Hydroxypropyl Pea Starch

DEFINITION

Pregelatinized Hydroxypropyl Pea Starch is prepared from Hydroxypropyl Pea Starch by mechanical processing in the presence of water, with or without heat, to rupture all or some of the starch granules, and is subsequently dried. It contains NLT 2.0% and NMT 7.0% of hydroxypropyl groups on the dried basis.

IDENTIFICATION

• **A. TEST FOR PREGELATINIZED STATE**

Sample: 1 g

Analysis: Disperse the *Sample* in 50 mL of water at a temperature NMT 25°. Shake vigorously until lumps completely disperse/solubilize or until lumps disappear. Allow to stand for 20 min.

Acceptance criteria: A translucent or clear mucilage without precipitate is formed.

• **B. TEST FOR STARCH**

Analysis: Disperse 0.5 g in 2 mL of water without heating, and add 0.05 mL of iodine and potassium iodide TS₂.

Acceptance criteria: A reddish-violet or blue color is produced.

• **C. NINHYDRIN TEST**

Ninhydrin solution: Dissolve 3 g of ninhydrin in 100 mL of a 45.5-g/L solution of sodium metabisulfite.

Diluted sulfuric acid: 98 g/L of H₂SO₄

Sample: 100 mg

Analysis: Transfer the *Sample* to a 100-mL volumetric flask and add 12.5 mL of *Diluted sulfuric acid*. Place the flask in a water bath, and heat until the *Sample* is dissolved. Cool, and dilute with water to 100 mL. [**CAUTION**—When sulfuric acid is miscible with water, it produces intense heat.]

Pipet 1 mL of this solution to a glass-stoppered 25-mL graduated test tube and, with the tube immersed in cold water, add dropwise 8 mL of sulfuric acid. Mix well, and place the tube in a boiling water bath for exactly 3 min. Immediately transfer the tube to an ice bath until the solution is chilled. Add 0.6 mL of *Ninhydrin solution*, carefully allowing the reagent to run down the walls of the test tube. Immediately shake the tube well, and place it in a water bath at 25° for 100 min. Dilute with sulfuric acid to 25 mL. [**CAUTION**—Use sulfuric acid cautiously.] Mix by inverting the tube several times. Do not shake.

Acceptance criteria: A violet color develops within 5 min due to the presence of hydroxypropyl groups (starch ether).

ASSAY

• **ASSAY FOR HYDROXYPROPYL GROUPS**

Deuterium chloride solution: Dilute 1 mL of deuterium chloride (38% w/w) with 5 mL of deuterium oxide.

Internal standard solution: Disperse 50.0 mg of sodium 3-trimethylsilyl-1-propane sulfonate in about 5 g of deuterium oxide, weighed to the nearest 0.1 mg. Store in a sealed bottle.

Sample solution: Determine the moisture content (*B*) on 5 g of Pregelatinized Hydroxypropyl Pea Starch following the *Loss on Drying* test. Weigh 12.0 mg of the Pregelatinized Hydroxypropyl Pea Starch in a 5-mm NMR tube. Add 0.75 mL of deuterium oxide and 0.1 mL of *Deuterium chloride solution*. Cap the tube, mix, and place it in a boiling water bath until a clear solution is obtained. [NOTE—This may take from 3 min to 1 h.] When a clear solution is obtained, allow to cool to room temperature. Dry the exterior of the tube, and weigh to the nearest 0.1 mg. Add 0.05 mL of the *Internal standard solution*. Weigh to the nearest 0.1 mg. Determine the mass of the *Internal standard solution* added. Mix thoroughly.

Instrumental conditions

(See *Nuclear Magnetic Resonance (761)*, *Quantitative Applications*.)

Mode: Nuclear magnetic resonance spectrometry

Apparatus: FT-NMR spectrometer at minimum 300 MHz

Acquisition of ¹H NMR spectra: The following parameters may be used:

Sweep width: 8 ppm (about –1.0 to +7 ppm)

Irradiation frequency offset: None

Time domain: NLT 64 K

Pulse width: 90°

Pulse delay: 10 s

Dummy scans: 0

Number of scans: 8

Use the CH₃ signal of the internal standard for shift referencing. Set the shift of the peak of the singlet to 0 ppm. Record the FID signal.

Analysis

Samples: *Internal standard solution* and *Sample solution*
 Call the integration subroutine after phase corrections and baseline correction between -0.5 and +6 ppm. Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at +1.2 ppm (A_2), and of the methyl groups at 0 ppm of the internal standard (A_1) without ^{13}C -satellites. Measure the signal originating from the 3 protons of the methyl group in the hydroxypropyl function. Calculate the content of hydroxypropyl groups as a percentage (w/w, dried basis):

$$\text{Result} = (N \times A_2/A_1) \times (C_i \times W_i/W) \times (M_{r2}/M_{r1}) \times [100/(100 - B)] \times 100$$

- N = numerical value representing the 3 methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate), 3
 A_2 = area of the methyl groups of hydroxypropyl in Pregelatinized Hydroxypropyl Pea Starch
 A_1 = area of the methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate)
 C_i = concentration of the internal standard in the *Internal standard solution* (mg/g)
 W_i = weight of the *Internal standard solution* in the NMR tube (g)
 W = weight of the Pregelatinized Hydroxypropyl Pea Starch in the NMR tube (mg)
 M_{r2} = molar mass of hydroxypropyl groups, 59.09 g/mol
 M_{r1} = molecular weight of the internal standard, 218.32 g/mol
 B = moisture content of the Pregelatinized Hydroxypropyl Pea Starch used in the *Sample solution*, as a percentage (w/w)

Acceptance criteria: 2.0%–7.0% of hydroxypropyl groups on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.6%, determined on a 1.0-g test specimen

- **LIMIT OF IRON**

Standard iron stock solution: Prepare a solution containing the equivalent of 10 µg/mL of iron, as directed under *Iron* (241).

Diluted standard iron solution: Immediately before use, dilute an accurately measured volume of the *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 µg/mL of iron.

Sample solution: Shake the residue obtained from the test for *Residue on Ignition* with 50 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 50 µg/g (ppm) of iron.

- **LIMIT OF SULFUR DIOXIDE, Method IV** (525): NMT 50 ppm
- **LIMIT OF PROPYLENE GLYCOL**

Internal standard solution: 0.5 mg/mL of 1,3-propanediol in anhydrous pyridine

Standard stock solution: 0.5 mg/mL of USP Propylene Glycol RS in *Internal standard solution*

Standard solution: Transfer 0.1 mL of the *Standard stock solution* to a 2-mL vessel with a screw cap fitted with a septum. Add 0.9 mL of anhydrous pyridine, 0.2 mL of hexamethyldisilazane, and 0.1 mL of trimethylchlorosilane. Close, and mix. Allow to stand for 15 min before injection.

Sample stock solution: Transfer 200 mg of Pregelatinized Hydroxypropyl Pea Starch to a 100-mL volumetric flask. Add 1.0 mL of the *Internal standard solution* and 9.0 mL of anhydrous pyridine. Boil under reflux using a water bath for 20 min. Allow to cool to room temperature.

Sample solution: Transfer 1.0 mL of the *Sample stock solution* to a 2-mL vessel with a screw cap fitted with a septum. Add 0.2 mL of hexamethyldisilazane and 0.1 mL of trimethylchlorosilane. Close, and mix. Allow to stand for 15 min before injection.

Chromatographic system

(See *Chromatography* (621), *System Suitability*).

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica capillary column; 0.25-µm layer of phase G1

Temperature

Detector: 250°

Injection port: 250°

Column: 70°. [NOTE—The column must be desorbed regularly. Conditions: Program from 70° to 300° at 7°/min, and maintain 10 min at 300°.]

Carrier gas: Helium

Flow rate: 3 mL/min

Injection type: Split ratio of 1:30

Injection size: 1 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for the trimethylsilylated derivative of propylene glycol and the trimethylsilylated derivative of 1,3-propanediol are 1.0 and 1.4, respectively.]

Suitability requirements

Resolution: NLT 2.0 between the peaks due to the trimethylsilylated derivative of propylene glycol and the trimethylsilylated derivative of 1,3-propanediol

Analysis

Samples: *Standard solution* and *Sample solution*
 Calculate the percentage of propylene glycol in the portion of Pregelatinized Hydroxypropyl Pea Starch taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = internal standard ratio (peak response of propylene glycol/peak response of 1,3-propanediol) from the *Sample solution*

R_S = internal standard ratio (peak response of propylene glycol/peak response of 1,3-propanediol) from the *Standard solution*

C_S = concentration of USP Propylene Glycol RS in the *Standard solution* (mg/mL)

C_U = concentration of Pregelatinized Hydroxypropyl Pea Starch in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.1%

- **LIMIT OF OXIDIZING SUBSTANCES**

Sample: 4.0 g

Analysis: Transfer the *Sample* to a glass-stoppered 125-mL conical flask, and add 50.0 mL of a mixture of water and methanol (1:1). Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine

color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 ppm, calculated as H₂O₂).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 10³ cfu/g, the total combined molds and yeasts count does not exceed 10² cfu/g, and it meets the requirements of the test for the absence of *Escherichia coli*.
- **pH** (791)
Sample solution: Progressively suspend 3.0 g of Pregelatinized Hydroxypropyl Pea Starch in 100.0 mL of carbon dioxide-free water, stirring continuously. Determine the pH when all the solid is wetted.
Acceptance criteria: 4.5–8.0
- **LOSS ON DRYING** (731): Dry about 1 g at 130° for 90 min: it loses NMT 15.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.
- **USP REFERENCE STANDARDS** (11)
USP Propylene Glycol RS

Potato Starch

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (♦) to specify this fact.

DEFINITION

Potato Starch is obtained from the tuber of *Solanum tuberosum* L.

IDENTIFICATION

A. PROCEDURE

Analysis: Examine under a microscope using a mixture of equal volumes of glycerol and water.

Acceptance criteria: It presents granules, either irregularly shaped, ovoid or pear-shaped, usually 30–100 µm in size but occasionally exceeding 100 µm, or rounded, 10–35 µm in size. There are occasional compound granules having 2–4 components. The ovoid and pear-shaped granules have an eccentric hilum and the rounded granules acentric or slightly eccentric hilum. All granules show clearly visible concentric striations. Between orthogonally oriented polarizing plates or prisms, the granules show a distinct black cross intersecting at the hilum.

B. PROCEDURE

Sample solution: 20 mg/mL in water

Analysis: Boil for 1 min, and cool.

Acceptance criteria: A thick, opalescent mucilage is formed.

C. PROCEDURE

Sample solution: 1 mL of the mucilage obtained in Identification test B

Analysis: Add 0.05 mL of iodine and potassium iodide TS 2 to the *Sample solution*.

Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.6%, determined on a 1.0-g sample
- **LIMIT OF IRON**
Standard iron stock solution A: Equivalent of 10 µg/mL of iron prepared as directed under *Iron* (241)

Standard iron stock solution B: 1 µg/mL of iron from *Standard iron stock solution A* in water [NOTE—Prepare immediately before use.]

Standard iron solution: Transfer 10 mL of *Standard iron stock solution B* to a test tube, and add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.

Sample solution: Shake 1.5 g of Potato Starch with 15 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube, and add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard iron solution*, corresponding to a limit of 10 ppm of iron.

LIMIT OF SULFUR DIOXIDE

Carbon dioxide: Use carbon dioxide, with a flow regulator that will maintain a flow of 100 ± 5 mL/min.

Bromophenol blue indicator solution: 0.2 mg/mL of bromophenol blue in dilute alcohol. Filter if necessary.

Hydrogen peroxide solution: Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Just before use, add three drops of *Bromophenol blue indicator solution*, and neutralize to a violet-blue endpoint with 0.01 N sodium hydroxide. Do not exceed the endpoint.

Apparatus: Figure 1

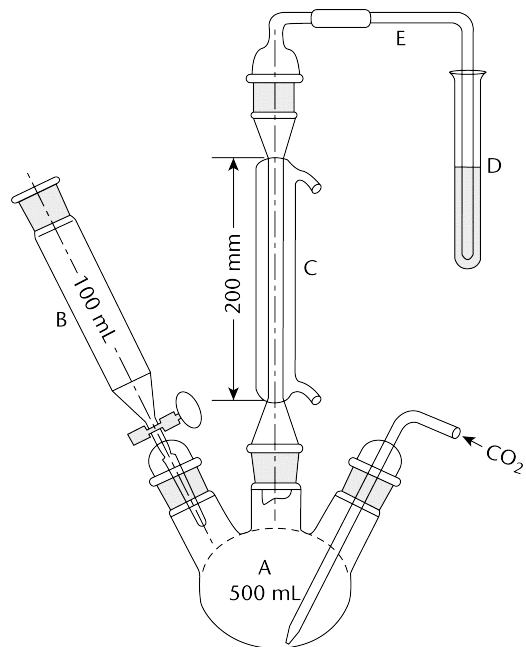


Figure 1

In this test, the sulfur dioxide is released from the sample in a boiling acid medium and is removed by a stream of carbon dioxide. The separated gas is collected in a dilute hydrogen peroxide solution where the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali. The apparatus consists essentially of a 500-mL three-neck, round-bottom boiling flask, A; a separatory funnel, B, having a capacity of 100 mL or greater; a gas inlet tube of sufficient length to permit introduction of the carbon dioxide within 2.5 cm of the bottom of the boiling flask; a reflux condenser, C, having a jacket length of 200 mm; and a delivery tube, E, connecting the upper end of the reflux condenser to the bottom of a receiving test tube, D. Apply a thin

film of stopcock grease to the sealing surfaces of all of the joints except the joint between the separatory funnel and the boiling flask, and clamp the joints to ensure tightness.

Sample: 25.0 g of Potato Starch

Analysis: Add 150 mL of water to the boiling flask.

Close the stopcock of the separatory funnel, and begin the flow of carbon dioxide at a rate of 100 ± 5 mL/min through the *Apparatus*. Start the condenser coolant flow. Add 10 mL of *Hydrogen peroxide solution* to a receiving test tube. After 15 min, without interrupting the flow of carbon dioxide, remove the separatory funnel from the boiling flask, and transfer the *Sample* into the boiling flask with the aid of 100 mL of water. Apply stopcock grease to the outer joint of the separatory funnel, and replace the separatory funnel in the boiling flask. Close the stopcock of the separatory funnel, and add 80 mL of 2 N hydrochloric acid to the separatory funnel. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the boiling flask, guarding against the escape of sulfur dioxide into the separatory funnel by closing the stopcock before the last few mL of hydrochloric acid drain out. Boil the mixture for 1 h. Remove the receiving test tube, and transfer its contents to a 200-mL wide-necked, conical flask. Rinse the receiving test tube with a small portion of water, add the rinsing to the 200-mL conical flask, and mix. Heat on a water bath for 15 min, and allow to cool.

Add 0.1 mL of *Bromophenol blue indicator solution*, and titrate the contents with 0.1 N sodium hydroxide VS until the color changes from yellow to violet-blue. Perform a blank determination, and make any necessary correction (see *Titrimetry* (541)).

Calculate the content, in ppm, of sulfur dioxide in the *Sample* taken:

$$\text{Result} = 1000 \times 32.03 \times (\text{VN}/\text{W})$$

32.03 = milliequivalent weight of sulfur dioxide

V = volume of titrant consumed (mL)

N = normality of the titrant

W = weight of the *Sample* (g)

Acceptance criteria: NMT 50 ppm

• LIMIT OF OXIDIZING SUBSTANCES

Sample solution: Transfer 4.0 g to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS.

Analysis: Titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch–iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate is required (20 ppm, calculated as H_2O_2).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 10^3 cfu/g; the total combined molds and yeasts count does not exceed 10^2 cfu/g; and it meets the requirements of the test for the absence of *Escherichia coli*.

• LOSS ON DRYING (731)

Sample: 1 g

Analysis: Dry the *Sample* at 130° for 90 min.

Acceptance criteria: NMT 20.0%

• pH (791)

Sample solution: Prepare a slurry by weighing 5.0 g of Potato Starch, transferring to a suitable nonmetallic container, and adding 25.0 mL of freshly boiled and cooled water.

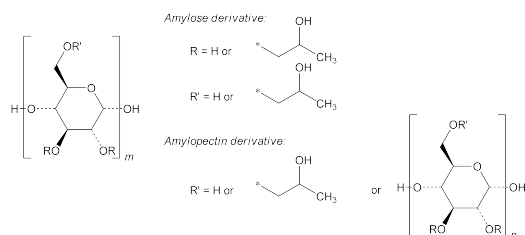
Analysis: Agitate continuously at a moderate rate for 1 min. Stop the agitation, and allow to stand for 15 min. Determine the pH to the nearest 0.1 unit.

Acceptance criteria: 5.0–8.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.♦

Hydroxypropyl Potato Starch



For the Amylose derivative, *m* is about 300–1000.

DEFINITION

Hydroxypropyl Potato Starch is partially substituted 2-hydroxypropylether obtained from potato starch by a chemical modification of etherification with propylene oxide. In addition, this starch may be partially hydrolyzed using acids or enzymes to obtain thinned starch. It contains NLT 2.0% and NMT 7.0% of hydroxypropyl groups, on the dried basis.

IDENTIFICATION

• A. PROCEDURE

Analysis: Examine under a microscope, using NLT 20× magnification and a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: It presents granules, either irregularly shaped, ovoid or pear-shaped, usually 30–100 µm in size, but occasionally exceeding 100 µm, or rounded 10–35 µm in size. There are occasional compound granules having 2–4 components. The ovoid and pear-shaped granules have an eccentric hilum, and the rounded granules have a centric or slightly eccentric hilum. All granules show clearly visible concentric striations. Between crossed nicol prisms, the Hydroxypropyl Potato Starch granules show a distinct black cross intersecting at the hilum.

• B. PROCEDURE

Sample solution: Suspend 1 g of Hydroxypropyl Potato Starch in 50 mL of water, boil for 1 min, and cool.

Acceptance criteria: A translucent or clear mucilage is formed.

• C. PROCEDURE

Analysis: To 1 mL of the *Sample solution* obtained in *Identification test B* add 0.05 mL of iodine and potassium iodide TS 2.

Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

• D. PROCEDURE

Ninhydrin solution: Dissolve 3 g of ninhydrin in 100 mL of a 45.5-g/L solution of sodium metabisulfite.

Diluted sulfuric acid: 98 g/L of H₂SO₄

Sample: 100 mg of Hydroxypropyl Potato Starch

Analysis: Transfer the *Sample* to a 100-mL volumetric flask, and add 12.5 mL of *Diluted sulfuric acid*. Place the flask in a water bath, and heat until the *Sample* is dissolved. Cool, and dilute with water to 100 mL. [**CAUTION**—When sulfuric acid is miscible with water, it produces intense heat.]

Pipet 1 mL of this solution to a glass-stoppered, 25-mL graduated test-tube and, with the tube immersed in cold water, add drop-wise 8 mL of sulfuric acid. Mix well, and place the tube in a boiling water bath for exactly 3 min. Immediately transfer the tube to an ice bath until the solution is chilled. Add 0.6 mL of *Ninhydrin solution*, carefully allowing the reagent to run down the walls of the test tube. Immediately shake the tube well, and place it in a water bath at 25° for 100 min. Dilute with sulfuric acid to 25 mL [**CAUTION**—Use sulfuric acid cautiously.], and mix by inverting the tube several times. Do not shake.

Acceptance criteria: A violet color develops within 5 min due to the presence of hydroxypropyl groups (starch ether).

ASSAY

• PROCEDURE FOR HYDROXYPROPYL GROUPS

Deuterium chloride solution: Dilute 1 mL of deuterium chloride (38% w/w) with 5 mL of deuterium oxide.

Internal standard solution: Dissolve 50.0 mg of sodium 3-trimethylsilyl-1-propane sulfonate in about 5 g of deuterium oxide, weighed to the nearest 0.1 mg. Store in a sealed bottle.

Sample solution: Disperse 20 g of Hydroxypropyl Potato Starch in 200.0 mL of carbon dioxide-free water at room temperature. Agitate for 15 min, and filter. Repeat the operation two more times. If poor dispersibility or slow filtration is observed, use refrigerated carbon dioxide-free water for the washing operation. Dry the washed starch for NLT 4 h in vacuum at 30 ± 5°. Determine the moisture content (B) on 5 g of the washed and dried starch following the *Loss on Drying* test. Weigh 12.0 mg of the washed and dried starch in a 5-mm NMR tube. Add 0.75 mL of deuterium oxide and 0.1 mL of *Deuterium chloride solution*. Cap the tube, mix, and place it in a boiling water bath until a clear solution is obtained. [NOTE—It may take 3 min–1 h.] When a clear solution is obtained, allow to cool to room temperature. Dry the exterior of the tube, and weigh to the nearest 0.1 mg. Add 0.05 mL of *Internal standard solution*, and weigh to the nearest 0.1 mg. Determine the mass of the *Internal standard solution* added. Mix thoroughly.

Nuclear magnetic resonance spectrometry

(See *Nuclear Magnetic Resonance* <761>, *Quantitative Application*.)

Apparatus: FT-NMR spectrometer at minimum 300 MHz

Acquisition of ¹H NMR spectra: The following parameters may be used.

Sweep width: 8 ppm (about –1.0 to +7 ppm)

Irradiation frequency offset: None

Time domain: NLT 64 K

Pulse width: 90 degree

Pulse delay: 10 s

Dummy scans: 0

Number of scans: 8

Use the CH₃ signal of the internal standard for shift referencing. Set the shift of the peak of the singlet to 0 ppm. Record the FID signal.

Analysis

Samples: *Internal standard solution* and *Sample solution*
Call the integration sub-routine after phase corrections and baseline correction between –0.5 and +6 ppm. Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at +1.2 ppm (A₂),

and of the methyl groups at 0 ppm of the internal standard (A₁) without ¹³C-satellites.

Measure the signal coming from the 3 protons of the methyl group in the hydroxypropyl function. Calculate the content of hydroxypropyl groups as a percentage (w/w, dried basis):

$$\text{Result} = \frac{(N \times A_2/A_1) \times (C_i \times W_i/W) \times (M_{r2}/M_{r1}) \times [100/(100 - B)]}{100}$$

- N = numerical value representing the 3 methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate), 3
- A₂ = area of the methyl groups of hydroxypropyl in Hydroxypropyl Potato Starch
- A₁ = area of the methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate)
- C_i = concentration of the internal standard in the *Internal standard solution* (mg/g)
- W_i = weight of the *Internal standard solution* in the NMR tube (g)
- W = weight of the washed and dried Hydroxypropyl Potato Starch in the NMR tube (mg)
- M_{r1} = molecular weight of the internal standard, 218.32 g/mol
- M_{r2} = molar mass of hydroxypropyl group, 59.09 g/mol
- B = moisture content of the washed and dried Hydroxypropyl Potato Starch used in the *Sample solution*, as a percentage (w/w)

Acceptance criteria: The content of hydroxypropyl groups is 2.0%–7.0% on the dried basis.

IMPURITIES

Inorganic Impurities

• **RESIDUE ON IGNITION** <281>: NMT 0.6%, determined on a 1.0-g test specimen

• LIMIT OF IRON

Standard iron stock solution: Prepare a solution containing the equivalent of 10 µg/mL of iron, as directed under *Iron* <241>.

Diluted standard iron solution: Immediately before use, dilute an accurately measured volume of *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 µg/mL of iron.

Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Sample solution: Shake 1.0 g of Hydroxypropyl Potato Starch with 20 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 20 µg/g of iron.

• **LIMIT OF SULFUR DIOXIDE**, *Method IV* <525>: NMT 50 ppm

Organic Impurities

• PROCEDURE 1: LIMIT OF OXIDIZING SUBSTANCES

Sample: 4.0 g of Hydroxypropyl Potato Starch

Analysis: Transfer the *Sample* to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide.

Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 µg/g, calculated as H₂O₂).

• **PROCEDURE 2: FOREIGN MATTER**

Sample: 50 mg/mL of Hydroxypropyl Potato Starch in a mixture of glycerin and water (1:1)

Analysis: Examine under a microscope, using NLT 20× magnification and a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: NMT traces of matter other than Hydroxypropyl Potato Starch granules are present.

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62):

The total aerobic microbial count does not exceed 10³ cfu/g, the total combined molds and yeasts count does not exceed 10² cfu/g, and it meets the requirements of the test for the absence of *Escherichia coli*.

• **pH** (791)

Sample solution: Suspend 5.0 g of Hydroxypropyl Potato Starch in 25.0 mL of carbon dioxide-free water, and shake for 60 s. Allow to stand for 15 min.

Acceptance criteria: 4.5–8.0

• **LOSS ON DRYING** (731): Dry about 1 g at 130° for 90 min: it loses NMT 20.0% of its weight.

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.

Pregelatinized Hydroxypropyl Potato Starch

DEFINITION

Pregelatinized Hydroxypropyl Potato Starch is prepared from Hydroxypropyl Potato Starch by mechanical processing in the presence of water, with or without heat, to rupture all or some of the starch granules, and is subsequently dried. It contains NLT 2.0% and NMT 7.0% of hydroxypropyl groups on the dried basis.

IDENTIFICATION

• **A. TEST FOR PREGELATINIZED STATE**

Sample: 1 g

Analysis: Disperse the *Sample* in 50 mL of water at a temperature NMT 25°. Shake vigorously until lumps completely disperse/solubilize or until lumps disappear. Allow to stand for 20 min.

Acceptance criteria: A translucent or clear mucilage without precipitate is formed.

• **B. TEST FOR STARCH**

Analysis: Disperse 0.5 g in 2 mL of water without heating, and add 0.05 mL of iodine and potassium iodide TS₂.

Acceptance criteria: A reddish-violet or blue color is produced.

• **C. NINHYDRIN TEST**

Ninhydrin solution: Dissolve 3 g of ninhydrin in 100 mL of a 45.5-g/L solution of sodium metabisulfite.

Diluted sulfuric acid: 98 g/L of H₂SO₄

Sample: 100 mg

Analysis: Transfer the *Sample* to a 100-mL volumetric flask, and add 12.5 mL of *Diluted sulfuric acid*. Place the

flask in a water bath, and heat until the *Sample* is dissolved. Cool, and dilute with water to 100 mL. [**CAUTION**—When sulfuric acid is miscible with water, it produces intense heat.]

Pipet 1 mL of this solution to a glass-stoppered 25-mL graduated test tube and, with the tube immersed in cold water, add dropwise 8 mL of sulfuric acid. Mix well, and place the tube in a boiling water bath for exactly 3 min. Immediately transfer the tube to an ice bath until the solution is chilled. Add 0.6 mL of *Ninhydrin solution*, carefully allowing the reagent to run down the walls of the test tube. Immediately shake the tube well, and place it in a water bath at 25° for 100 min. Dilute with sulfuric acid to 25 mL. [**CAUTION**—Use sulfuric acid cautiously.] Mix by inverting the tube several times. Do not shake.

Acceptance criteria: A violet color develops within 5 min due to the presence of hydroxypropyl groups (starch ether).

ASSAY

• **ASSAY FOR HYDROXYPROPYL GROUPS**

Deuterium chloride solution: Dilute 1 mL of deuterium chloride (38% w/w) with 5 mL of deuterium oxide.

Internal standard solution: Disperse 50.0 mg of sodium 3-trimethylsilyl-1-propane sulfonate in about 5 g of deuterium oxide, weighed to the nearest 0.1 mg. Store in a sealed bottle.

Sample solution: Determine the moisture content (*B*) on 5 g of Pregelatinized Hydroxypropyl Potato Starch following the *Loss on Drying* test. Weigh 12.0 mg of the Pregelatinized Hydroxypropyl Potato Starch in a 5-mm NMR tube. Add 0.75 mL of deuterium oxide and 0.1 mL of *Deuterium chloride solution*. Cap the tube, mix, and place it in a boiling water bath until a clear solution is obtained. [NOTE—This may take from 3 min to 1 h.] When a clear solution is obtained, allow it to cool to room temperature. Dry the exterior of the tube, and weigh to the nearest 0.1 mg. Add 0.05 mL of the *Internal standard solution*. Weigh to the nearest 0.1 mg. Determine the mass of the *Internal standard solution* added. Mix thoroughly.

Instrumental conditions

(See *Nuclear Magnetic Resonance* (761), *Quantitative Applications*.)

Mode: Nuclear magnetic resonance spectrometry

Apparatus: FT-NMR spectrometer at minimum 300 MHz

Acquisition of ¹H NMR spectra: The following parameters may be used:

Sweep width: 8 ppm (about –1.0 to +7 ppm)

Irradiation frequency offset: None

Time domain: NLT 64 K

Pulse width: 90°

Pulse delay: 10 s

Dummy scans: 0

Number of scans: 8

Use the CH₃ signal of the internal standard for shift referencing. Set the shift of the peak of the singlet to 0 ppm. Record the FID signal.

Analysis

Samples: *Internal standard solution* and *Sample solution*
Call the integration subroutine after phase corrections and baseline correction between –0.5 and +6 ppm. Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at +1.2 ppm (*A*₂), and of the methyl groups at 0 ppm of the internal standard (*A*₁) without ¹³C-satellites.

Measure the signal originating from the 3 protons of the methyl group in the hydroxypropyl function.

Calculate the content of hydroxypropyl groups as a percentage (w/w, dried basis):

$$\text{Result} = (N \times A_2/A_1) \times (C_1 \times W_1/W) \times (M_{12}/M_{11}) \times [100/(100 - B)] \times 100$$

- N = numerical value representing the 3 methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate), 3
- A_2 = area of the methyl groups of hydroxypropyl in Pregelatinized Hydroxypropyl Potato Starch
- A_1 = area of the methyl groups in the internal standard (sodium 3-trimethylsilyl-1-propane sulfonate)
- C_i = concentration of the internal standard in the *Internal standard solution* (mg/g)
- W_i = weight of the *Internal standard solution* in the NMR tube (g)
- W = weight of the Pregelatinized Hydroxypropyl Potato Starch in the NMR tube (mg)
- M_{r2} = molar mass of hydroxypropyl groups, 59.09 g/mol
- M_{r1} = molecular weight of the internal standard, 218.32 g/mol
- B = moisture content of the Pregelatinized Hydroxypropyl Potato Starch used in the *Sample solution*, as a percentage (w/w)
- Acceptance criteria:** 2.0%–7.0% of hydroxypropyl groups on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.6%, determined on a 1.0-g test specimen

- **LIMIT OF IRON**

Standard iron stock solution: Prepare a solution containing the equivalent of 10 µg/mL of iron, as directed under *Iron* (241).

Diluted standard iron solution: Immediately before use, dilute an accurately measured volume of the *Standard iron stock solution* quantitatively with water to obtain a solution containing the equivalent of 1 µg/mL of iron.

Sample solution: Shake the residue obtained from the test for *Residue on Ignition* with 20 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Standard solution: Transfer 10 mL of the *Diluted standard iron solution* to a test tube. Add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid, and mix. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, dilute with water to 20 mL, and mix.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard solution*, corresponding to a limit of 20 ppm of iron.

- **LIMIT OF SULFUR DIOXIDE**, *Method IV* (525): NMT 50 ppm

- **LIMIT OF PROPYLENE GLYCOL**

Internal standard solution: 0.5 mg/mL of 1,3-propanediol in anhydrous pyridine

Standard stock solution: 0.5 mg/mL of USP Propylene Glycol RS in *Internal standard solution*

Standard solution: Transfer 0.1 mL of the *Standard stock solution* to a 2-mL vessel with a screw cap fitted with a septum. Add 0.9 mL of anhydrous pyridine, 0.2 mL of hexamethyldisilazane, and 0.1 mL of trimethylchlorosilane. Close, and mix. Allow to stand for 15 min before injection.

Sample stock solution: Transfer 200 mg of Pregelatinized Hydroxypropyl Potato Starch to a 100-mL volumetric flask. Add 1.0 mL of the *Internal standard solution* and 9.0 mL of anhydrous pyridine. Boil under reflux using a water bath for 20 min. Allow to cool to room temperature.

Sample solution: Transfer 1.0 mL of the *Sample stock solution* to a 2-mL vessel with a screw cap fitted with a septum. Add 0.2 mL of hexamethyldisilazane and

0.1 mL of trimethylchlorosilane. Close, and mix. Allow to stand for 15 min before injection.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m fused-silica capillary column; 0.25-µm layer of phase G1

Temperature

Detector: 250°

Injection port: 250°

Column: 70°. [NOTE—The column must be desorbed regularly. Conditions: Program from 70° to 300° at 7°/min, and maintain 10 min at 300°.]

Carrier gas: Helium

Flow rate: 3 mL/min

Injection type: Split ratio of 1:30

Injection size: 1 µL

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for the trimethylsilylated derivative of propylene glycol and the trimethylsilylated derivative of 1,3-propanediol are 1.0 and 1.4, respectively.]

Suitability requirements

Resolution: NLT 2.0 between the peaks due to the trimethylsilylated derivative of propylene glycol and the trimethylsilylated derivative of 1,3-propanediol

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of propylene glycol in the portion of Pregelatinized Hydroxypropyl Potato Starch taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = internal standard ratio (peak response of propylene glycol/peak response of 1,3-propanediol) from the *Sample solution*

R_S = internal standard ratio (peak response of propylene glycol/peak response of 1,3-propanediol) from the *Standard solution*

C_S = concentration of USP Propylene Glycol RS in the *Standard solution* (mg/mL)

C_U = concentration of Pregelatinized Hydroxypropyl Potato Starch in the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.1%

- **LIMIT OF OXIDIZING SUBSTANCES**

Sample: 4.0 g

Analysis: Transfer the *Sample* to a glass-stoppered 125-mL conical flask, and add 50.0 mL of a mixture of water and methanol (1:1). Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch-iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (20 ppm, calculated as H₂O₂).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 10³ cfu/g, the total combined molds and yeasts count does not exceed 10² cfu/g, and it meets the requirements of the test for the absence of *Escherichia coli*.

- **pH** <791>
Sample solution: Progressively suspend 3.0 g of Pregelatinized Hydroxypropyl Potato Starch in 100.0 mL of carbon dioxide-free water, stirring continuously. Determine the pH when all the solid is wetted.
Acceptance criteria: 4.5–8.0
- **LOSS ON DRYING** <731>: Dry about 1 g at 130° for 90 min; it loses NMT 20.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. Store at room temperature.
- **USP REFERENCE STANDARDS** <11>
 USP Propylene Glycol RS

Pregelatinized Starch

DEFINITION

Pregelatinized Starch is Starch that has been chemically and/or mechanically processed to rupture all or part of the granules in the presence of water and subsequently dried. Some types of Pregelatinized Starch may be modified to render them compressible and flowable in character.

IDENTIFICATION

- A water slurry of it is colored orange-red to deep blue by iodine TS.

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** <281>: NMT 0.5%, determined on a 2.0-g test specimen
- **IRON** <241>: NMT 20 ppm
Analysis: Dissolve the residue obtained in the test for *Residue on Ignition* in 8 mL of hydrochloric acid with the aid of gentle heating, and dilute with water to 100 mL. Dilute 25 mL of this solution with water to 47 mL.
- **LIMIT OF SULFUR DIOXIDE**
Sample solution: Mix 20 g with 200 mL of a 1-in-5 solution of anhydrous sodium sulfate, and filter.
Analysis: To 100 mL of the clear filtrate add 3 mL of starch TS, and titrate with 0.01 N iodine VS to the first permanent blue color.
Acceptance criteria: NMT 2.7 mL is consumed (80 ppm).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** <61> and **TESTS FOR SPECIFIED MICROORGANISMS** <62>: It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 1000 cfu/g; and the total combined molds and yeasts count does not exceed 100 cfu/g.
- **pH** <791>: 4.5–7.0
 Prepare a slurry by weighing 10.0 ± 0.1 g in 10 mL of alcohol and by diluting with water to 100 mL. Agitate continuously at a moderate rate for 5 min, then cease agitation and immediately potentiometrically determine the pH to the nearest 0.1 unit.
- **LOSS ON DRYING** <731>: Dry a sample at 120° for 4 h: it loses NMT 14.0% of its weight.
- **OXIDIZING SUBSTANCES**
Sample: 5 g
Analysis: To the *Sample* add 20 mL of a mixture of equal volumes of methanol and water, then add 1 mL of 6 N acetic acid, and stir until a homogeneous suspension is obtained. Add 0.5 mL of a freshly prepared, saturated solution of potassium iodide, and allow to stand for 5 min.
Acceptance criteria: No distinct blue, brown, or purple color is observed.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.
- **LABELING:** Label it to indicate the botanical source from which it was derived.

Pregelatinized Modified Starch

DEFINITION

Pregelatinized Modified Starch is Modified Starch that has been chemically or mechanically processed, or both, to rupture all or part of the granules to produce a product that swells in cold water.

IDENTIFICATION

- **A.**
Sample: 0.6 g
Analysis: Transfer the *Sample* to a 25-mL glass vial with a plastic cap. Add 9.4 g of water, cap, and shake vigorously to evenly disperse the starch. Add 10 g of 2% (w/w) NaOH solution, cap, and shake vigorously for 1 min to create a smooth mixture. Evaluate within 1 min.
Acceptance criteria: The final solution is translucent to opaque with a fluid consistency. A yellow tint of the final solution is acceptable.
- **B.** An aqueous dispersion of Pregelatinized Modified Starch is colored orange-red to deep blue by iodine TS.

IMPURITIES

- **RESIDUE ON IGNITION** <281>
Sample: 2.0 ± 0.1 g
Acceptance criteria: NMT 1.5%
- **LIMIT OF SULFUR DIOXIDE**
Sample solution: Mix 20.0 ± 0.1 g of Pregelatinized Modified Starch with 100 mL of 95% alcohol, and stir for several min to completely wet the starch.
Analysis: Slowly add 100 mL of water to the *Sample solution*, and stir until a smooth suspension is obtained. Allow the starch mixture to set undisturbed until most of the starch has settled, and filter the aqueous portion through paper (Whatman No. 1 or equivalent). To 100 mL of the clear filtrate add 100 mL of water. Add 3 mL of starch TS, and titrate with 0.01 N iodine VS to the first permanent blue or purple color.
Acceptance criteria: NMT 1.7 mL of 0.010 N iodine is consumed (NMT 0.005%).

SPECIFIC TESTS

- **pH** <791>
Sample: 10.0 ± 0.1 g
Analysis: Wet the *Sample* with 10 mL of alcohol, then dilute with water to 300 mL to obtain an aqueous dispersion. Stir continuously at a moderate rate for 5 min, and determine the pH to the nearest 0.1 unit.
Acceptance criteria: 3.0–9.0
- **LOSS ON DRYING** <731>
Analysis: Dry at 120° for 4 h.
Acceptance criteria: NMT 15%
- **MICROBIAL ENUMERATION TESTS** <61> and **TESTS FOR SPECIFIED MICROORGANISMS** <62>: The total aerobic microbial count does not exceed 1 × 10³ cfu/g, and the total combined molds and yeasts count does not exceed 1 × 10² cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **IRON** <241>
Sample: The residue obtained in the test for *Residue on Ignition* <281>
Analysis: Dissolve the *Sample* in 8 mL of hydrochloric acid with the aid of gentle heating. Dilute with water to 100 mL in a volumetric flask. Dilute 25 mL of this solution with water to 47 ± 1 mL.

Acceptance criteria: NMT 20 µg/g

• **OXIDIZING SUBSTANCES**

Sample solution: To 5 g of Pregelatinized Modified Starch add 20 mL of a mixture of methanol and water (1:1).

Analysis: To the *Sample solution* add 1 mL of 6 N acetic acid, and stir until a homogeneous suspension is obtained. Add 0.5 mL of a freshly prepared saturated solution of potassium iodide, and allow to stand for 5 min.

Acceptance criteria: No distinct blue, brown, or purple color is observed.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Rice Starch

Attributes	EP	JP	USP
Definition	+	+	+
Identification A	+	+	+
Identification B	+	+	+
Identification C	+	+	+
pH	+	+	+
Iron	+	+	+
Oxidizing Substances	+	+	+
Sulfur Dioxide	+	+	+
Loss on Drying	+	+	+
Sulfated Ash [Residue on Ignition]	+	+	+

Legend: + will adopt and implement; – will not stipulate.

Nonharmonized attributes: Characters, Microbial Enumeration Tests and Tests for Specified Microorganisms, Packaging and Storage

Specific local attributes: Foreign matter (EP)

Reagents and Reference materials: Each pharmacopeia will adapt the text to take account of local reference materials and reagent specifications.

DEFINITION

Rice Starch is obtained from the caryopsis of *Oryza sativa* L.

IDENTIFICATION

• **A. PROCEDURE**

Analysis: Examine under a microscope, using NLT 20x magnification and using a mixture of glycerin and water (1:1) as a mounting agent.

Acceptance criteria: It appears either as angular polyhedral granules of irregular sizes with diameters ranging from 2–23 µm, or as rounded or spheroidal granules of irregular sizes with diameters ranging from 25–35 µm. The central hilum consists of a distinct cavity or two- to five-rayed cleft, and there are no concentric striations. Between orthogonally oriented polarizing plates or prisms, the starch granules show a distinct black cross intersecting at the hilum.

• **B. PROCEDURE**

Sample solution: 20 mg/mL in water

Analysis: Boil for 1 min, and cool.

Acceptance criteria: A thin, cloudy mucilage is formed.

• **C. PROCEDURE**

Sample solution: 1 mL of the mucilage obtained in Identification test B

Analysis: Add 0.05 mL of iodine and potassium iodide TS 2 to the *Sample solution*.

Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION (281):** NMT 0.6%, determined on a 1.0-g sample

• **LIMIT OF IRON**

Standard iron stock solution A: Equivalent to 10 µg/mL of iron prepared as directed under *Iron* (241)

Standard iron stock solution B: 1 µg/mL of iron from *Standard iron stock solution A* in water

[NOTE—Prepare immediately before use.]

Standard iron solution: Transfer 10 mL of *Standard iron stock solution B* to a test tube, and add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.

Sample solution: Shake 1.5 g of Rice Starch with 15 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube, and add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard iron solution*, corresponding to a limit of 10 ppm of iron.

• **LIMIT OF SULFUR DIOXIDE**

Carbon dioxide: Use carbon dioxide, with a flow regulator that will maintain a flow of 100 ± 10 mL/min.

Bromophenol blue indicator solution: 0.2 mg/mL of bromophenol blue in dilute alcohol. Filter if necessary.

Hydrogen peroxide solution: Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Just before use, add 3 drops of *Bromophenol blue indicator solution*, and neutralize to a violet-blue endpoint with 0.01 N sodium hydroxide. Do not exceed the endpoint.

Apparatus: Figure 1.

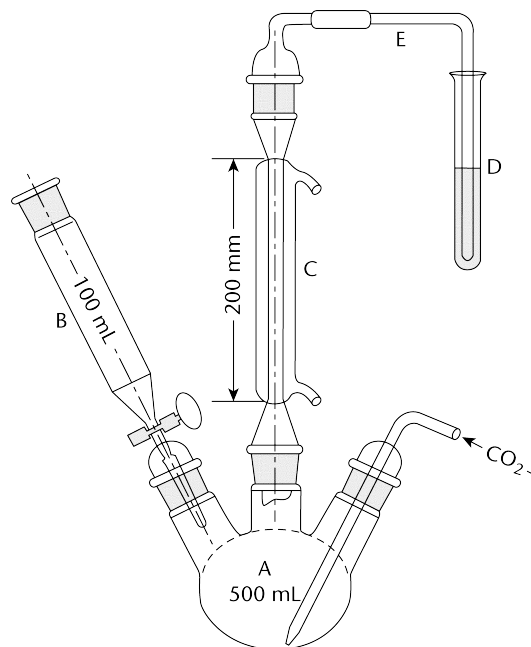


Figure 1

In this test, the sulfur dioxide is released from the sample in a boiling acid medium and is removed by a stream of carbon dioxide. The separated gas is collected in a dilute hydrogen peroxide solution where the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali. The apparatus consists essentially of a 500-mL three-neck, round-bottom boiling

flask, A; a separatory funnel, B, having a capacity of 100 mL or greater; a gas inlet tube of sufficient length to permit introduction of the carbon dioxide within 2.5 cm of the bottom of the boiling flask, a reflux condenser, C, having a jacket length of 200 mm, and a delivery tube, E, connecting the upper end of the reflux condenser to the bottom of a receiving test tube, D. Apply a thin film of stopcock grease to the sealing surfaces of all of the joints except the joint between the separatory funnel and the boiling flask, and clamp the joints to ensure tightness.

Sample: 25.0 g of Rice Starch

Analysis: Add 150 mL of water to the boiling flask. Close the stopcock of the separatory funnel, and begin the flow of carbon dioxide at a rate of 100 ± 5 mL/min through the *Apparatus*. Start the condenser coolant flow. Add 10 mL of *Hydrogen peroxide solution* to a receiving test tube. After 15 min, without interrupting the flow of carbon dioxide, remove the separatory funnel from the boiling flask, and transfer the *Sample* into the boiling flask with the aid of 100 mL of water. Apply stopcock grease to the outer joint of the separatory funnel, and replace the separatory funnel in the boiling flask. Close the stopcock of the separatory funnel, and add 80 mL of 2 N hydrochloric acid to the separatory funnel. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the boiling flask, guarding against the escape of sulfur dioxide into the separatory funnel by closing the stopcock before the last few mL of hydrochloric acid drain out. Boil the mixture for 1 h. Remove the receiving test tube, and transfer its contents to a 200-mL wide-necked, conical flask. Rinse the receiving test tube with a small portion of water, add the rinsing to the 200-mL conical flask, and mix. Heat on a water bath for 15 min, and allow to cool.

Add 0.1 mL of *Bromophenol blue indicator solution*, and titrate the contents with 0.1 N sodium hydroxide VS until the color changes from yellow to violet-blue. Perform a blank determination, and make any necessary correction (see *Titrimetry* (541)).

Calculate the content, in ppm, of sulfur dioxide in the *Sample* taken:

$$\text{Result} = 1000 (32.03) \text{ VN/W}$$

32.03 = milliequivalent weight of sulfur dioxide

V = volume of titrant consumed (mL)

N = normality of the titrant

W = weight of the *Sample* (g)

Acceptance criteria: NMT 50 ppm

• LIMIT OF OXIDIZING SUBSTANCES

Sample solution: Transfer 4.0 g to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS.

Analysis: Titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch–iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate is required (20 ppm, calculated as H₂O₂).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 1000 cfu/g; the total combined molds and yeasts count does not exceed 100

cfu/g; and it meets the requirements of the test for the absence of *Escherichia coli*.♦

- **LOSS ON DRYING (731):** Dry 1 g at 130° for 90 min: it loses NMT 15.0% of its weight.

- **pH (791):** 5.0–8.0

Sample solution: Prepare a slurry by weighing 5.0 g of Rice Starch, transferring to a suitable nonmetallic container, and adding 25.0 mL of freshly boiled and cooled water.

Analysis: Agitate continuously at a moderate rate for 1 min. Stop the agitation, and allow to stand for 15 min. Determine the pH to the nearest 0.1 unit.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.♦

Tapioca Starch

DEFINITION

Tapioca Starch consists of starch granules separated from the tubers of tapioca (cassava) [*Manihot utilissima* Pohl (Fam. Euphorbiaceae)].

IDENTIFICATION

• A.

Analysis: Examine Tapioca Starch under a microscope, using not less than 20× magnification and using glycerin as the mounting agent.

Acceptance criteria: It appears as spherical granules, each having one truncated side, typically having a 5- to 35-µm diameter, and having circular or several-rayed central clefts.

• B.

Sample suspension: 1 g of Tapioca Starch in 50 mL of water

Analysis: Boil the *Sample suspension* for 1 min, and cool.

Acceptance criteria: A thin, cloudy mucilage is formed.

• C.

Sample: The mucilage obtained in *Identification* test B

Analysis: To 1 mL of the *Sample* add 0.05 mL of iodine and potassium iodide TS 2.

Acceptance criteria: An orange-red to dark blue color is produced, which disappears on heating.

IMPURITIES

• RESIDUE ON IGNITION (281)

Sample: 1.0 g

Acceptance criteria: NMT 0.6%

• IRON (241)

Test preparation: Shake 0.75 g of Tapioca Starch with 15 mL of 0.1 N hydrochloric acid, filter, and use 10 mL.

Acceptance criteria: NMT 20 µg/g

• LIMIT OF OXIDIZING SUBSTANCES

Sample: 4.0 g

Blank: 50 mL of water

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.002 N sodium thiosulfate VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Decant into a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide. Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS, and titrate with *Titrant* to the disappearance of the starch–iodide color.

Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate VS is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate VS is required (0.002%).

• **LIMIT OF SULFUR DIOXIDE**

Sample solution: Mix 20 g of Tapioca Starch with 200 mL of water until a smooth suspension is obtained, and filter.

Analysis: To 100 mL of the clear filtrate from the *Sample solution* add 3 mL of starch TS, and titrate with 0.01 N iodine solution VS to the first permanent blue color.

Acceptance criteria: NMT 1.7 mL of 0.01 N iodine solution VS is required (0.005%).

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 10^3 cfu/g, and the total combined yeasts and molds count does not exceed 10^2 cfu/g. Tapioca Starch meets the requirements of the test for absence of *Escherichia coli*.

• **pH** (791)

Sample: 20.0 ± 0.1 g

Analysis: Transfer the *Sample* to a suitable nonmetallic container, and add 100 mL of water to obtain a slurry. Agitate continuously at a moderate rate for 5 min, then stop agitation, and immediately determine the pH.

Acceptance criteria: 4.5–7.0

• **LOSS ON DRYING** (731)

Analysis: Dry at 130° for 90 min.

Acceptance criteria: NMT 16.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.

Topical Starch—see *Topical Starch General Monographs*

Wheat Starch

Portions of the monograph text that are national *USP* text, and are not part of the harmonized text, are marked with symbols (♦) to specify this fact.

DEFINITION

Wheat starch is obtained from the caryopsis of *Triticum aestivum* L. (*T. vulgare* Vill.).

IDENTIFICATION

• **A. PROCEDURE**

Analysis: Examine under a microscope using equal volumes of glycerol and water.

Acceptance criteria: It presents large and small granules, and, very rarely, intermediate sizes. The large granules, usually 10–60 µm in diameter, are discoid or, more rarely, reniform when seen face-on. The central hilum and striations are invisible or barely visible, and the granules sometimes show cracks on the edges. Seen in profile, the granules are elliptical and fusiform and the hilum appears as a slit along the main axis. The small granules, rounded or polyhedral, are 2–10 µm in diameter. Between orthogonally oriented polarizing plates or prisms, the granules show a distinct black cross intersecting at the hilum.

• **B. PROCEDURE**

Sample solution: 20 mg/mL in water

Analysis: Boil for 1 min, and cool.

Acceptance criteria: A thin, cloudy mucilage is formed.

• **C. PROCEDURE**

Sample solution: 1 mL of the mucilage obtained in *Identification test B*

Analysis: Add 0.05 mL of iodine and potassium iodide TS 2 to the *Sample solution*.

Acceptance criteria: An orange-red to dark blue color is produced, which disappears upon heating.

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.6%, determined on a 1.0-g sample

• **LIMIT OF IRON**

Standard iron stock solution A: Equivalent to 10 µg/mL of iron prepared as directed under *Iron* (241)

Standard iron stock solution B: 1 µg/mL of iron from *Standard iron stock solution A* in water [NOTE—Prepare immediately before use.]

Standard iron solution: Transfer 10 mL of *Standard iron stock solution B* to a test tube, and add 2 mL of citric acid solution (2 in 10), and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.

Sample solution: Shake 1.5 g of Wheat Starch with 15 mL of 2 N hydrochloric acid, and filter. Transfer 10 mL of the filtrate to a test tube, and add 2 mL of citric acid solution (2 in 10) and 0.1 mL of thioglycolic acid. Add 10 N ammonium hydroxide until the solution is distinctly alkaline to litmus, and dilute with water to 20 mL.

Acceptance criteria: After 5 min, any pink color in the *Sample solution* is not more intense than that in the *Standard iron solution*, corresponding to a limit of 10 ppm of iron.

• **LIMIT OF SULFUR DIOXIDE**

Carbon dioxide: Use carbon dioxide, with a flow regulator that will maintain a flow of 100 ± 5 mL/min.

Bromophenol blue indicator solution: 0.2 mg/mL of bromophenol blue in dilute alcohol. Filter if necessary.

Hydrogen peroxide solution: Dilute 30% hydrogen peroxide with water to obtain a 3% solution. Just before use, add 3 drops of *Bromophenol blue indicator solution*, and neutralize to a violet-blue endpoint with 0.01 N sodium hydroxide. Do not exceed the endpoint.

Apparatus: *Figure 1*

In this test, the sulfur dioxide is released from the sample in a boiling acid medium and is removed by a stream of carbon dioxide. The separated gas is collected in a dilute hydrogen peroxide solution where the sulfur dioxide is oxidized to sulfuric acid and titrated with standard alkali. The apparatus consists essentially of a 500-mL three-neck, round-bottom boiling flask, A; a separatory funnel, B, having a capacity of 100 mL or greater; a gas inlet tube of sufficient length to permit introduction of the carbon dioxide within 2.5 cm of the bottom of the boiling flask; a reflux condenser, C, having a jacket length of 200 mm; and a delivery tube, E, connecting the upper end of the reflux condenser to the bottom of a receiving test tube, D. Apply a thin film of stopcock grease to the sealing surfaces of all of the joints except the joint between the separatory funnel and the boiling flask, and clamp the joints to ensure tightness.

Sample: 25.0 g of Wheat Starch

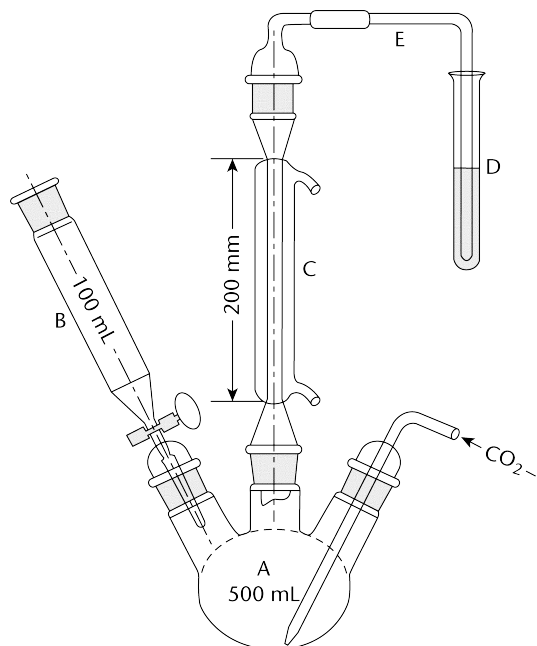


Figure 1

Analysis: Add 150 mL of water to the boiling flask. Close the stopcock of the separatory funnel, and begin the flow of carbon dioxide at a rate of 100 ± 5 mL/min through the Apparatus. Start the condenser coolant flow. Add 10 mL of *Hydrogen peroxide solution* to a receiving test tube. After 15 min, without interrupting the flow of carbon dioxide, remove the separatory funnel from the boiling flask, and transfer the *Sample* into the boiling flask with the aid of 100 mL of water. Apply stopcock grease to the outer joint of the separatory funnel, and replace the separatory funnel in the boiling flask. Close the stopcock of the separatory funnel, and add 80 mL of 2 N hydrochloric acid to the separatory funnel. Open the stopcock of the separatory funnel to permit the hydrochloric acid solution to flow into the boiling flask, guarding against the escape of sulfur dioxide into the separatory funnel by closing the stopcock before the last few mL of hydrochloric acid drain out. Boil the mixture for 1 h. Remove the receiving test tube, and transfer its contents to a 200-mL wide-necked, conical flask. Rinse the receiving test tube with a small portion of water, add the rinsing to the 200-mL conical flask, and mix. Heat on a water bath for 15 min, and allow to cool. Add 0.1 mL of *Bromophenol blue indicator solution*, and titrate the contents with 0.1 N sodium hydroxide VS until the color changes from yellow to violet-blue. Perform a blank determination, and make any necessary correction (see *Titrimetry* (541)). Calculate the content, in ppm, of sulfur dioxide in the *Sample* taken:

$$\text{Result} = 1000 \times 32.03 \times (VN/W)$$

32.03 = milliequivalent weight of sulfur dioxide

V = volume of titrant consumed (mL)

N = normality of the titrant

W = weight of the *Sample* (g)

Acceptance criteria: NMT 50 ppm

• LIMIT OF OXIDIZING SUBSTANCES

Sample solution: Transfer 4.0 g to a glass-stoppered, 125-mL conical flask, and add 50.0 mL of water. Insert the stopper, and swirl for 5 min. Transfer to a glass-stoppered, 50-mL centrifuge tube, and centrifuge to clarify. Transfer 30.0 mL of the clear supernatant to a glass-stoppered, 125-mL conical flask. Add 1 mL of glacial acetic acid and 0.5–1.0 g of potassium iodide.

Insert the stopper, swirl, and allow to stand for 25–30 min in the dark. Add 1 mL of starch TS.

Analysis: Titrate with 0.002 N sodium thiosulfate VS to the disappearance of the starch–iodine color. Perform a blank determination, and make any necessary correction. Each mL of 0.002 N sodium thiosulfate is equivalent to 34 µg of oxidant, calculated as hydrogen peroxide.

Acceptance criteria: NMT 1.4 mL of 0.002 N sodium thiosulfate is required (20 ppm, calculated as H_2O_2).

SPECIFIC TESTS

• **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** The total aerobic microbial count does not exceed 10^3 cfu/g; the total combined molds and yeasts count does not exceed 10^2 cfu/g; and it meets the requirements of the test for the absence of *Escherichia coli*.

• LOSS ON DRYING (731)

Sample: 1 g

Analysis: Dry the *Sample* at 130° for 90 min.

Acceptance criteria: NMT 15.0%

• pH (791)

Sample solution: Prepare a slurry by weighing 5.0 g of Wheat Starch, transferring to a suitable nonmetallic container, and adding 25.0 mL of freshly boiled and cooled water.

Analysis: Agitate continuously at a moderate rate for 1 min. Stop the agitation, and allow to stand for 15 min. Determine the pH to the nearest 0.1 unit.

Acceptance criteria: 4.5–7.0

• TOTAL PROTEIN

Analysis: Weigh 6.0 g of sample containing 2 mg of nitrogen; transfer to a combustion flask; add 4 g of a powdered mixture consisting of 100 g of potassium sulfate, 5 g of cupric sulfate, and 2.5 g of selenium; and add three glass beads. Wash any adhering particles from the neck into the flask with 5 mL of sulfuric acid, allowing it to run down the sides of the flask, and mix the contents by rotation. Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulfuric acid. Heat gradually at first, then increase the temperature until there is vigorous boiling with condensation of sulfuric acid in the neck of the flask; precautions should be taken to prevent the upper part of the flask from becoming overheated. Continue the heating for 30 min, unless otherwise prescribed. Cool, dissolve the solid material by cautiously adding to the mixture 25 mL of water, cool again, and place in a steam distillation apparatus. Add 30 mL of sodium hydroxide solution (42 in 100), and distill immediately by passing steam through the mixture. Collect 40 mL of distillate in 20.0 mL of 0.01 N hydrochloric acid and enough water to cover the tip of the condenser. Toward the end of the distillation, lower the receiver so that the tip of the condenser is above the surface of the acid. Take precautions to prevent any water on the outer surface of the condenser from reaching the contents of the receiver. Titrate the distillate with 0.01 N sodium hydroxide, using methyl purple TS as the indicator (n_1 mL of 0.01 N sodium hydroxide).

Repeat the test using 50 mg of glucose in place of the substance to be examined (n_2 mL of 0.01 N sodium hydroxide).

$$\text{Content of nitrogen} = [0.01401 \times (n_2 - n_1)]/m$$

m = amount of test substance weighed (g)

Acceptance criteria: NMT 0.3% (corresponding to 0.048% N_2 , conversion factor: 6.25)

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers. No storage requirements specified.♦

Stearic Acid

Portions of this monograph that are national *USP* text, and are not part of the harmonized text, are marked with symbols (♦) to specify this fact.

Octadecanoic acid;
Stearic acid [57-11-4].

DEFINITION

Mixture consisting of stearic (octadecanoic) acid ($C_{18}H_{36}O_2$; M_r 284.5) and palmitic (hexadecanoic) acid ($C_{16}H_{32}O_2$; M_r 256.4) obtained from fats or oils of vegetable or animal origin.

Content:

Stearic acid 50	Stearic acid: 40.0%–60.0%. Sum of the contents of stearic acid and palmitic acids: NLT 90.0%.
Stearic acid 70	Stearic acid: 60.0%–80.0%. Sum of the contents of stearic and palmitic acids: NLT 90.0%.
Stearic acid 95	Stearic acid: NLT 90.0%. Sum of the contents of stearic acid and palmitic acids: NLT 96.0%.

[NOTE—Stearic Acid labeled solely for external use is exempt from the requirement that it be prepared from edible sources.]

IDENTIFICATION

- **A.** It meets the requirements of the test for *Freezing Point*.
- **B. ACID VALUE**

Light petroleum: Use a sample that has the following properties: a clear, colorless, liquid without fluorescence; practically insoluble in water; miscible with alcohol; density at 20° about 0.720; distillation range 100°–120°; water content NMT 0.03%.¹

Sample solution: Dissolve 0.5 g of Stearic Acid in 50 mL of a mixture of equal volumes of alcohol and *Light petroleum* previously neutralized with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide, using 0.5 mL of phenolphthalein TS as indicator. If necessary, heat to about 90° to dissolve the substance to be examined.

Analysis: Titrate the *Sample solution* with 0.1 N potassium hydroxide or 0.1 N sodium hydroxide until the pink color persists for at least 15 s. When heating has been applied to aid dissolution, maintain the temperature at about 90° during the titration.

Calculate the acid value of the portion of Stearic Acid taken:

$$\text{Result} = I_A = n/m \times N \times 56.10$$

n = amount of titrant used (mL)

m = amount of Stearic Acid taken to prepare the *Sample solution* (g)

N = normality of the potassium hydroxide solution

56.10 = formula weight of potassium hydroxide

Acceptance criteria: 194–212

- **C.** The retention times of the major peaks from the *Sample solution* correspond to those from the *Standard solution*, as obtained in the *Assay*.

ASSAY

PROCEDURE

Boron trifluoride–methanol solution: 140 g/L of boron trifluoride in methanol

¹ Petroleum ether; boiling range 100°–140°; CAS 64742-49-0 from Fisher Scientific; catalog number AC23302-0025 is suitable.

Standard solution: Prepare as directed in the *Sample solution* using 50 mg of USP Stearic Acid RS and 50 mg of USP Palmitic Acid RS.

Sample solution: Dissolve 100 mg of Stearic Acid in a small conical flask fitted with a suitable reflux attachment with 5 mL of *Boron trifluoride–methanol solution*. Boil under reflux for 10 min. Add 4.0 mL of heptane through the condenser, and boil again under reflux for 10 min. Allow to cool. Add 20 mL of a saturated solution of sodium chloride. Shake, and allow the layers to separate. Remove about 2 mL of the organic layer, and dry it over 0.2 g of anhydrous sodium sulfate. Dilute 1.0 mL of this solution with heptane to 10.0 mL.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 30-m × 0.32-mm fused silica coated with a 0.5-μm layer of stationary phase G16

Temperature

Injector: 220°

Detector: 260°

Column: See *Table 1*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	—	70	2
70	5	240	5

Carrier gas: Helium, passed through a bed of molecular sieve for drying, if necessary

Flow rate: 2.4 mL/min

Injection size: 1 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 5.0 between the methyl palmitate and methyl stearate peaks determined on 6 injections

Relative standard deviation: NMT 3.0% for the methyl stearate and methyl palmitate peaks (from 6 replicate injections of *Sample solution*); NMT 1.0% for the ratio of the peak areas of methyl palmitate to the peak areas of methyl stearate, from 6 replicate injections

Analysis: Calculate the percentage of stearic acid ($C_{18}H_{36}O_2$) in the portion of sample taken:

$$\text{Result} = (A_S/A_T) \times 100$$

A_S = peak area due to methyl stearate

A_T = sum of the peak areas of all the fatty acid esters in the chromatogram

Similarly, calculate the percentage of palmitic acid ($C_{16}H_{32}O_2$) in the portion of sample taken:

$$\text{Result} = (A_P/A_T) \times 100$$

A_P = peak area due to methyl palmitate

A_T = sum of the peak areas of all the fatty acid esters in the chromatogram

Acceptance criteria

For Stearic acid 50: 40.0–60.0% of $C_{18}H_{36}O_2$, and the sum of the stearic acid and palmitic acid is NLT 90.0%.

For Stearic acid 70: 60.0–80.0% of $C_{18}H_{36}O_2$, and the sum of the stearic acid and palmitic acid is NLT 90.0%.

For Stearic acid 95: NLT 90.0% of $C_{18}H_{36}O_2$, and the sum of the stearic acid and palmitic acid is NLT 96.0%.

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 4 mg, determined on a 4-g portion (0.1%).
- **HEAVY METALS, Method II** (231): NMT 10 ppm.

SPECIFIC TESTS

- **FATS AND FIXED OILS, Iodine Value** (401)

Sample: 1 g

Analysis: Proceed as directed in *Method I*, except use 15 mL of chloroform.

Acceptance criteria: See *Table 2*.

Table 2

Type	Iodine Value
Stearic acid 50	NMT 4.0
Stearic acid 70	NMT 4.0
Stearic acid 95	NMT 1.5

- **COLOR OF SOLUTION**

Standard stock solution Y (yellow): 2.4 mL of ferric chloride CS, 0.6 mL of cobaltous chloride CS, and 7.0 mL of hydrochloric acid solution (10 g/L)

Standard stock solution BY (brownish-yellow): 2.4 mL of ferric chloride CS, 1.0 mL of cobaltous chloride CS, 0.4 mL of cupric sulfate CS, and 6.2 mL of hydrochloric acid solution (10 g/L)

Standard solution Y: 2.5 mL of *Standard stock solution Y* and 97.5 mL of hydrochloric acid solution (10 g/L)

Standard solution BY: 2.5 mL of *Standard stock solution BY* and 97.5 mL of hydrochloric acid solution (10 g/L)

Analysis: Heat Stearic Acid to 75°.

Acceptance criteria: The resulting liquid is not more intensely colored than *Standard solution Y* or *Standard solution BY*.

Change to read:

- **ACIDITY**

Analysis: Melt 5.0 g of Stearic Acid, shake for 2 min with 10 mL of hot carbon dioxide-free water, cool slowly, and filter. To the filtrate add 0.05 mL (ERR 1-May-2012) of methyl orange TS.

Acceptance criteria: No red color develops.

- **FREEZING POINT**

Apparatus: Consists of a test tube about 25 mm in diameter and 150 mm long placed inside a test tube about 40 mm in diameter and 160 mm long. The inner tube is closed by a stopper which carries a thermometer about 175 mm long and graduated in 0.2°, fixed so that the bulb is about 15 mm above the bottom of the tube. The stopper has a hole allowing the passage of the stem of a stirrer made from a glass rod or other suitable material formed at one end into a loop of about 18 mm overall diameter at right angles to the rod. The inner tube with its jacket is supported centrally in a 1-L beaker containing a suitable cooling liquid to within 20-mm of the top. A thermometer is supported in the cooling bath. Place in the inner tube sufficient quantity of the liquid or previously melted substance to be examined, to cover the thermometer bulb, and determine the approximate freezing point by cooling rapidly.

Analysis: Place the inner tube in a bath about 5° above the approximate freezing point until all but the last traces of crystals are melted. Fill the beaker with water or a saturated solution of sodium chloride; at a temperature about 5° lower than the expected freezing point, insert the inner tube into the outer tube, ensuring that some seed crystals are present, and stir thoroughly until solidification takes place. Note the highest temperature observed during solidification.

Acceptance criteria: See *Table 3*.

Table 3

Type	Freezing Point (°)
Stearic acid 50	53–59
Stearic acid 70	57–64
Stearic acid 95	64–69

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** If it is for external use only, the labeling so indicates. The label states the type of stearic acid (50, 70, or 95).
- **USP REFERENCE STANDARDS** (11)
USP Palmitic Acid RS
USP Stearic Acid RS

Purified Stearic Acid**DEFINITION**

Purified Stearic Acid is manufactured from fats and oils derived from edible sources and is a mixture of stearic acid (C₁₈H₃₆O₂) and palmitic acid (C₁₆H₃₂O₂), which together constitute NLT 96.0% of the total content. The content of C₁₈H₃₆O₂ is NLT 90.0% of the total.

[NOTE—Purified Stearic Acid labeled solely for external use is exempt from the requirement that it be prepared from edible sources.]

ASSAY

- **PROCEDURE**

Standard solution: Transfer 50 mg of USP Stearic Acid RS and 50 mg of USP Palmitic Acid RS into a small conical flask fitted with a suitable reflux attachment. Add 5.0 mL of a solution prepared by dissolving 14 g of boron trifluoride in methanol to make 100 mL; swirl and reflux for 15 min or until the solid is dissolved. Cool, transfer the reaction mixture with the aid of 10 mL of chromatographic solvent hexane to a 60-mL separator, and add 10 mL of water and 10 mL of saturated sodium chloride solution. Shake, allow to separate, then drain and discard the lower, aqueous layer. Pass the hexane layer through 6 g of anhydrous sodium sulfate (previously washed with chromatographic solvent hexane) into a suitable flask.

Sample solution: Transfer 100 mg of Purified Stearic Acid into a small conical flask fitted with a suitable reflux attachment. Add 5.0 mL of a solution prepared by dissolving 14 g of boron trifluoride in methanol to make 100 mL; swirl and reflux for 15 min or until the solid is dissolved. Cool, transfer the reaction mixture with the aid of 10 mL of chromatographic solvent hexane to a 60-mL separator, and add 10 mL of water and 10 mL of saturated sodium chloride solution. Shake, allow to separate, then drain and discard the lower, aqueous layer. Pass the hexane layer through 6 g of anhydrous sodium sulfate (previously washed with chromatographic solvent hexane) into a suitable flask.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 1.5-m × 3-mm, preferably glass; packing of 15% G4 on S1A

Carrier gas: Helium, passed through a bed of molecular sieve for drying, if necessary

Injection volume: 1–2 μ L

Temperatures

Injection port: 210°

Detector: 210°

Column: 165°

System suitability

Samples: *Standard solution* and *Sample solution*

Suitability requirements

Resolution: NLT 2.0 between methyl palmitate and methyl stearate, *Sample solution*

[NOTE—Locate the peaks by comparison with the chromatogram from the *Standard solution*.]

Relative standard deviation: NMT 1.5% for methyl stearate and methyl palmitate for five replicate injections of the *Sample solution*

Analysis

Sample: *Sample solution*

Calculate the percentage of stearic acid ($C_{18}H_{36}O_2$) in the portion of Purified Stearic Acid taken:

$$\text{Result} = (A/B) \times 100$$

A = peak area due to methyl stearate

B = sum of all the fatty acid ester peaks

Calculate the percentage of palmitic acid ($C_{16}H_{32}O_2$) in the portion of Purified Stearic Acid taken:

$$\text{Result} = (A/B) \times 100$$

A = peak area due to methyl palmitate

B = sum of all the fatty acid ester peaks

Acceptance criteria

Stearic acid: NLT 90.0%

Sum of stearic acid and palmitic acid: NLT 96.0%

IMPURITIES

• RESIDUE ON IGNITION (281)

Sample: 4 g

Acceptance criteria: NMT 4 mg (0.1%)

• HEAVY METALS, Method II (231): NMT 10 μ g/g

SPECIFIC TESTS

• CONGEALING TEMPERATURE (651): 66°–69°

• FATS AND FIXED OILS, Acid Value (401)

Sample: 1 g

Acceptance criteria: 195–200

• FATS AND FIXED OILS, Iodine Value (401)

Analysis: Proceed as directed in *Method I*, except use 35 mL of chloroform.

Acceptance criteria: NMT 1.5

• MINERAL ACID

Sample: 5 g of melted Purified Stearic Acid

Analysis: Shake the *Sample* with an equal volume of hot water for 2 min, cool, and filter.

Acceptance criteria: The filtrate is not reddened by the addition of 1 drop of methyl orange TS.

• NEUTRAL FAT OR PARAFFIN

Sample solution: 1 g of Stearic Acid in 30 mL of anhydrous sodium carbonate solution (16.7 mg/mL)

Analysis: Boil the *Sample solution*.

Acceptance criteria: The resulting solution, while hot, shows NMT a faint opalescence.

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in well-closed containers.

• LABELING: If it is for external use only, the labeling so indicates.

• USP REFERENCE STANDARDS (11)

USP Palmitic Acid RS

USP Stearic Acid RS

Stearoyl Polyoxylglycerides

DEFINITION

Stearoyl Polyoxylglycerides is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols. The polyethylene glycols used have a mean molecular weight between 300 and 4000. It is produced by partial alcoholysis of saturated oils, mainly containing triglycerides of stearic acid, with polyethylene glycol, by esterification of glycerol and polyethylene glycol with fatty acids, or as a mixture of glycerol esters and ethylene oxide condensate with the fatty acids of the hydrogenated oils. The hydroxyl value is NLT 85% and NMT 115% of the labeled nominal value, and the saponification value is NLT 90% and NMT 110% of the labeled nominal value. Stearoyl Polyoxylglycerides may contain free polyethylene glycols.

IDENTIFICATION

• A. INFRARED ABSORPTION (197K)

• B. THIN-LAYER CHROMATOGRAPHIC IDENTIFICATION TEST (201)

Standard solution: 50 mg/mL of USP Stearoyl Polyoxylglycerides in methylene chloride

Sample solution: 50 mg/mL of Stearoyl Polyoxylglycerides in methylene chloride

Chromatographic system

Application volume: 10 μ L

Developing solvent system: Ether and hexanes (70:30)

Spray reagent: 0.1 mg/mL of rhodamine B in alcohol

Analysis

Samples: *Standard solution* and *Sample solution*

Proceed as directed in the chapter. Then spray the plate with *Spray reagent*, and locate the spots on the plate by examination under UV light at a wavelength of 365 nm.

Acceptance criteria: The R_f values of the principal spots from the *Sample solution* correspond to those from the *Standard solution*.

• C. It meets the requirements in *Specific Tests for Fats and Fixed Oils, Fatty Acid Composition* (401).

IMPURITIES

• HEAVY METALS, Method II (231): NMT 10 μ g/g

• ARTICLES OF BOTANICAL ORIGIN, Total Ash (561): NMT 0.2%

• ALKALINE IMPURITIES

Sample: 5.0 g

Analysis: Heat the *Sample* slightly until the test substance melts, add 10 mL of alcohol and 0.05 mL of bromophenol blue TS, and mix well. While the solution is still warm, titrate with 0.01 N hydrochloric acid VS to change the color to yellow.

Acceptance criteria: NMT 1.0 mL of 0.01 N hydrochloric acid VS is required.

• LIMIT OF FREE ETHYLENE OXIDE AND DIOXANE

[CAUTION—Ethylene oxide is toxic and flammable. Prepare all solutions in a well-ventilated hood. The operator must protect hands and face by wearing polyethylene protective gloves and an appropriate face mask. Store all solutions in hermetic containers, and refrigerate at a temperature between 4° and 8°.]

Magnesium chloride solution: 500 mg/mL of magnesium chloride in alcohol

Ethylene oxide stock solution: Into a dry, clean test tube, cooled in a mixture of sodium chloride and crushed ice (1:3), introduce a slow current of ethylene oxide gas, allowing condensation onto the inner wall of the test tube. Using a glass syringe, previously cooled to –10°, transfer 300 μ L of liquid ethylene oxide, equivalent to 0.25 g, to 50 mL of polyethylene glycol 200. Determine the absorbed quantity of ethylene oxide by

weighing before and after absorption. Dilute with polyethylene glycol 200 to 100.0 mL.

Standardize this solution by transferring 10.0 mL of *Magnesium chloride solution* and 20.0 mL of 0.1 M alcoholic hydrochloric acid VS to a volumetric flask. Insert the stopper, shake to obtain a saturated solution, and allow to equilibrate overnight. Transfer 5.00 mL of *Ethylene oxide stock solution* to the flask, and allow to stand for 30 min. Titrate with 0.1 M alcoholic potassium hydroxide VS. Perform a blank titration, using the same quantity of polyethylene glycol 200 instead of *Ethylene oxide stock solution*, and note the difference in volumes required. Each mL of the difference in volumes of 0.1 M alcoholic potassium hydroxide VS consumed is equivalent to 4.404 mg of ethylene oxide. Calculate the concentration of ethylene oxide, in mg/g, in the *Ethylene oxide stock solution*.

Ethylene oxide solution: Prepare immediately before use. Dilute a volume of *Ethylene oxide stock solution* with polyethylene glycol 200 to obtain a solution containing 50 µg of ethylene oxide per g. Dilute 1.0 mL of this solution with water to 5.0 mL to obtain a solution having a concentration of 10 µg/mL of ethylene oxide.

Dioxane solution: 0.5 mg/mL of dioxane

Standard solution A: Transfer 1.0 g of Stearoyl Polyoxylglycerides to a 10-mL vial, and add 1.0 mL of *N,N*-dimethylacetamide, 0.1 mL of *Ethylene oxide solution*, and 0.1 mL of *Dioxane solution*. Close the vial, and mix to obtain a homogenous solution. Allow to stand at 90° for 45 min.

Standard solution B: Transfer 0.1 mL of *Ethylene oxide solution* to a 10-mL vial, add 0.1 mL of a freshly prepared solution of acetaldehyde, containing 10 mg/L of acetaldehyde, and add 0.1 mL of *Dioxane solution*. Close the vial, and mix to obtain a homogenous solution.

Sample solution: Transfer 1.0 g of Stearoyl Polyoxylglycerides to a 10-mL vial, and add 1.0 mL of *N,N*-dimethylacetamide and 0.2 mL of water. Close the vial, and mix to obtain a homogenous solution. Allow to stand at 90° for 45 min.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

[NOTE—Headspace apparatus that automatically transfers a measured amount of headspace may be used.]

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m glass or quartz capillary column bonded with a 1.0-µm layer of phase G1

Temperatures

Injection port: 150°

Detector: 250°

Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
50	—	50	5
50	5	180	—
180	30	230	5

Carrier gas: Helium

Flow rate: 1 mL/min

Injection volume: 1 mL

System suitability

Sample: Gaseous phase of *Standard solution B*

[NOTE—The relative retention times for acetaldehyde and ethylene oxide are 0.94 and 1.0, respectively.]

Suitability requirements

[NOTE—Record the peak responses as directed for *Analysis*, adjusting the sensitivity of the system so that the peak heights of the two principal peaks are NLT 15% of the full scale of the recorder.]

Resolution: NLT 2.0 between acetaldehyde and ethylene oxide

Relative standard deviation: NMT 15.0%

Analysis

Samples: *Standard solution A* and *Sample solution*
Perform all determinations three times. Using a heated, gas-tight gas chromatographic syringe, separately inject the gaseous headspace of *Standard solution A* and of the *Sample solution*.

Calculate the concentration of ethylene oxide in the sample taken:

$$\text{Result} = C \times \{r_U / [(r_S \times C_U) - (r_U \times C_S)]\}$$

C = concentration of ethylene oxide in *Standard solution A* (µg/mL)

r_U = peak response of ethylene oxide from the *Sample solution*

r_S = peak response of ethylene oxide from *Standard solution A*

C_U = concentration of Stearoyl Polyoxylglycerides in *Sample solution* (g/mL)

C_S = concentration of stearyl polyoxylglycerides in *Standard solution A* (g/mL)

Calculate the concentration of dioxane in the sample taken:

$$\text{Result} = C_D \times (d_U/5) \times [(d_S \times C_U) - (d_U \times C_S)]$$

C_D = concentration of dioxane in *Standard solution A* (µg/mL)

d_U = peak response of dioxane from the *Sample solution*

d_S = peak response of dioxane from *Standard solution A*

C_U = concentration of Stearoyl Polyoxylglycerides in *Sample solution* (g/mL)

C_S = concentration of stearyl polyoxylglycerides in *Standard solution A* (g/mL)

Acceptance criteria

Ethylene oxide: NMT 1 µg/g

Dioxane: NMT 10 µg/g

• LIMIT OF FREE GLYCEROL

Sample: 1.20 g

Periodic acetic acid solution: Dissolve 0.446 g of sodium periodate in 2.5 mL of a 25% (v/v) solution of sulfuric acid, diluting to 100.0 mL with glacial acetic acid.

Potassium iodide solution: 75 mg/mL of potassium iodide

Analysis: Dissolve the *Sample* in 25 mL of methylene chloride, heating if necessary. Cool, and add 100 mL of water and 25.0 mL of *Periodic acetic acid solution*. Shake, and allow to stand for 30 min. Add 40 mL of *Potassium iodide solution*, and allow to stand for 1 min. Add 1 mL of starch TS, and titrate the liberated iodine with 0.1 M sodium thiosulfate VS. Perform a blank determination, and make any necessary correction (see *Titrimetry* <541>). Calculate the percentage of glycerol in the sample taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant* volume consumed by the *Sample* (mL)

V_B = *Titrant* volume consumed by the *Blank* (mL)

N = actual normality of the *Titrant* (mEq/mL)

F = equivalency factor, 23.0 mg/mEq

W = *Sample* weight (mg)

Acceptance criteria: NMT 5.0%

SPECIFIC TESTS

• FATS AND FIXED OILS, *Acid Value* <401>

Sample: 2.0 g

Acceptance criteria: NMT 2.0

• FATS AND FIXED OILS, *Fatty Acid Composition* <401>:

Stearoyl Polyoxylglycerides exhibits the following

composition profile of fatty acids, as determined in the chapter (see Table 2).

Table 2

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
12	0	≤5.0
14	0	≤5.0
16	0	40.0–50.0
18	0	48.0–58.0

- **FATS AND FIXED OILS, Hydroxyl Value <401>**
Sample: 1.0 g
Acceptance criteria: 25–56, 85%–115% of the labeled nominal value
- **FATS AND FIXED OILS, Iodine Value <401>**: NMT 2.0
- **FATS AND FIXED OILS, Peroxide Value <401>**
Sample: 2.0 g
Acceptance criteria: NMT 6.0
- **FATS AND FIXED OILS, Saponification Value <401>**
Sample: 2.0 g
Acceptance criteria: 67–112, 90%–110% of the labeled nominal value
- **WATER DETERMINATION, Method I <921>**
Sample: 1.0 g
Solvent: Anhydrous pyridine or a mixture of methylene chloride and anhydrous methanol (70:30)
Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light and moisture. Store at controlled room temperature.
- **LABELING:** Label it to indicate the type and the average nominal molecular weight of polyethylene glycols used as part of the official title. The label also indicates the hydroxyl value and the saponification value.
- **USP REFERENCE STANDARDS <11>**
USP Stearoyl Polyoxylglycerides RS

Stearyl Alcohol



1-Octadecanol;
Octadecan-1-ol [112-92-5].

DEFINITION

Stearyl Alcohol contains NLT 90.0% of stearyl alcohol ($\text{C}_{18}\text{H}_{38}\text{O}$), the remainder consisting chiefly of related alcohols.

IDENTIFICATION

- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *System suitability solution*, as obtained in the *Assay*.

ASSAY

PROCEDURE

System suitability solution: 9 mg/mL of USP Stearyl Alcohol RS and 1 mg/mL of USP Cetyl Alcohol RS in dehydrated alcohol

Sample solution: 10 mg/mL of Stearyl Alcohol in dehydrated alcohol

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 3-mm × 2-m; packed with 10% liquid phase G2 on support S1A

Carrier gas: Helium

Temperatures

Injection port: 275°

Detector: 250°

Column: 205°

Injection volume: 2 µL

System suitability

Sample: *System suitability solution*

Suitability requirements

Resolution: NLT 4.0 between the cetyl alcohol and stearyl alcohol peaks

Relative standard deviation: NMT 1.5% for the area ratio of stearyl alcohol to cetyl alcohol

Analysis

Sample: *Sample solution*

Calculate the percentage of stearyl alcohol ($\text{C}_{18}\text{H}_{38}\text{O}$) in the portion of Stearyl Alcohol taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area for stearyl alcohol from the *Sample solution*

r_T = sum of the areas of all the peaks except the solvent peak from the *Sample solution*

Acceptance criteria: NLT 90.0%

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE <741>**: 55°–60°

- **FATS AND FIXED OILS, Acid Value (Free Fatty Acids) <401>**: NMT 2

- **FATS AND FIXED OILS, Hydroxyl Value <401>**

Sample solution: Add 2 g of Stearyl Alcohol in a dry, glass-stoppered, 250-mL flask, and add 2 mL of pyridine followed by 10 mL of toluene.

Analysis: To the *Sample solution* add 10.0 mL of a solution of acetyl chloride, prepared by mixing 10 mL of acetyl chloride with 90 mL of toluene. Insert the stopper in the flask, and immerse in a water bath heated at 60°–65° for 20 min. Add 25 mL of water, insert the stopper in the flask, and shake vigorously for several min to decompose the excess acetyl chloride. Add 0.5 mL of phenolphthalein TS, and titrate to a permanent pink endpoint with 1 N sodium hydroxide VS, shaking the flask vigorously toward the end of the titration to maintain the contents in an emulsified condition. Perform a blank test with the same quantities of the same reagents and in the same manner.

$$\text{Result} = [(V_S - V_B) \times N \times F]/W$$

V_S = volume of 1 N sodium hydroxide consumed by the sample (mL)

V_B = volume of 1 N sodium hydroxide consumed by the blank test (mL)

N = actual normality of 1 N sodium hydroxide (mEq/mL)

F = molecular weight of potassium hydroxide, 56.11

W = weight of Stearyl Alcohol (g)

Acceptance criteria: 195–220

- **FATS AND FIXED OILS, Iodine Value <401>**: NMT 2

ADDITIONAL REQUIREMENTS

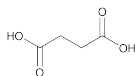
- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

- **USP REFERENCE STANDARDS <11>**

USP Cetyl Alcohol RS

USP Stearyl Alcohol RS

Succinic Acid



C₄H₆O₄ 118.09
Butanedioic acid [110-15-6].

DEFINITION

Succinic Acid contains NLT 99.0% and NMT 100.5% of C₄H₆O₄.

IDENTIFICATION

- **INFRARED ABSORPTION** (197K)

ASSAY

• PROCEDURE

Sample: 250 mg

Analysis: Dissolve in 25 mL of carbon dioxide-free water, add 2 drops of phenolphthalein TS, and titrate with 0.1 N sodium hydroxide VS to the production of a permanent pink color. Each mL of 0.1 N sodium hydroxide is equivalent to 5.905 mg of C₄H₆O₄.

Acceptance criteria: 99.0%–100.5%

IMPURITIES

Inorganic Impurities

- **RESIDUE ON IGNITION** (281): NMT 0.025%
- **HEAVY METALS**, Method I (231): NMT 20 ppm

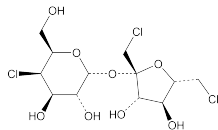
SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** (741): 185.0°–190.0°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a well-closed container. No storage requirements specified.
- **USP REFERENCE STANDARDS** (11)
USP Succinic Acid RS

Sucralose



C₁₂H₁₉Cl₃O₈ 397.63
1,6-Dichloro-1,6-dideoxy-β-D-fructofuranosyl-4-chloro-4-deoxy-α-D-galactopyranoside;
1',4,6'-Trichlorogalactosucrose [56038-13-2].

DEFINITION

Sucralose contains NLT 98.0% and NMT 102.0% of C₁₂H₁₉Cl₃O₈, calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B.** The retention time of the principal peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the Assay.
- **C.** The *R_F* value of the principal spot of the *Sample solution* corresponds to that of *Standard solution A*, as obtained in the test for *Related Compounds*.

ASSAY

• PROCEDURE

Mobile phase: Acetonitrile and water (3:17)

Standard solution: 1 mg/mL of USP Sucralose RS in *Mobile phase*

Sample solution: 1 mg/mL of Sucralose in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 8-mm × 10-cm; packing L1

Flow rate: 1.5 mL/min

Injection size: 20 μL

System suitability

Sample: *Standard solution*

[NOTE—The retention time of sucralose is about 9 min.]

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of sucralose (C₁₂H₁₉Cl₃O₈) in the portion of Sucralose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of the *Sample solution*

r_S = peak response of the *Standard solution*

C_S = concentration of USP Sucralose RS in the *Standard solution* (mg/mL)

C_U = concentration of Sucralose in the *Sample solution* (mg/mL)

Acceptance criteria: 98.0%–102.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.7%
- **HEAVY METALS**, Method II (231): 10 ppm

• LIMIT OF METHANOL

Internal standard solution: 0.1 μL/mL of *n*-propyl alcohol in pyridine

Standard solution: 0.2 μL/mL of methanol in *Internal standard solution*

Sample solution: 0.2 g/mL of Sucralose in *Internal standard solution*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 4-mm × 2-m glass column; packed with 80- to 100-mesh silanized support S6

Temperature

Column: 150°

Detector: 250°

Injector: 200°

Carrier gas: Helium

Flow rate: 20 mL/min

Injection size: 1 μL

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0%

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of methanol in the portion of Sucralose taken:

$$\text{Result} = (R_U/R_S) \times [(C_S/C_U) \times F_1] \times F_2 \times 100$$

R_U = peak response ratio of methanol to *n*-propyl alcohol, from the *Sample solution*

R_S = peak response ratio of methanol to *n*-propyl alcohol, from the *Standard solution*

C_S = concentration of methanol in the *Standard solution* (μL/mL)

C_U = concentration of Sucralose in the *Sample solution* (g/mL)

F_1 = conversion factor from μL to mL

F_2 = specific gravity of methanol, 0.79 g/cm³

Acceptance criteria: NMT 0.1%

• RELATED COMPOUNDS

Adsorbent: 0.20-mm layer of octadecylsilanized chromatographic silica gel. The thin-layer chromatographic plate also has a preadsorbent zone.

Detection reagent: Sulfuric acid in methanol (3 in 20)

Standard solution A: 10.0 mg/mL of USP Sucralose RS in methanol

Standard solution B: 0.5 mL *Standard solution A* diluted to 10.0 mL with methanol

Sample solution: 100.0 mg/mL of Sucralose in methanol

Developing solvent system: Acetonitrile and sodium chloride solution (1 in 20) (3:7)

Application volume: 5 μL

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Proceed as directed under *Chromatography* <621>, *Thin-Layer Chromatography*. Spray the plate with *Detection reagent*. Heat the plate for 10 min at 125°.

Acceptance criteria: The R_f value of the principal spot from the *Sample solution* corresponds to that obtained from *Standard solution A*, and the color of any other single spot from the *Sample solution* is not more intense than that of the principal spot from *Standard solution B* (0.5%).

• LIMIT OF HYDROLYSIS PRODUCTS [NOTE—This test does not require a developing solvent.]

Adsorbent: 0.25-mm layer of chromatographic silica gel

Spray reagent: 12.3 mg/mL of *p*-anisidine and 16.6 mg/mL of phthalic acid in methanol. Store the solution in the dark and refrigerate to prevent discoloration. Discard if the solution becomes discolored. [CAUTION—*p*-Anisidine is toxic if inhaled or if absorbed through the skin.]

Standard solution A: 100 mg/mL of mannitol

Standard solution B: 0.4 mg/mL of fructose and 100 mg/mL of mannitol

Sample solution: 250 mg/mL of Sucralose in methanol

Application volume: 5- μL portions separately applied in 1- μL increments, allowing the plate to dry between applications

Analysis

Samples: *Standard solution* and *Sample solution*
Proceed as directed under *Chromatography* <621>, *Thin-Layer Chromatography*. Spray the plate with *Spray reagent*, and heat the plate at 100 \pm 2° for 15 min. If the spot from *Standard solution A* has darkened, repeat the test, heating for a shorter period of time. Immediately after heating, view the plate against a dark background.

Acceptance criteria: The color of the spot from the *Sample solution* is not more intense than that from *Standard solution B* (0.1%).

SPECIFIC TESTS

• OPTICAL ROTATION, *Specific Rotation* <781S>: +84.0° to +87.5° at 20°

Sample solution: 10 mg/mL of Sucralose

• WATER DETERMINATION, *Method I* <921>: NMT 2.0%

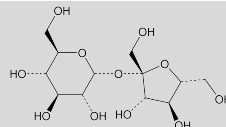
ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in well-closed containers, in a cool, dry place, at a temperature not exceeding 21°.

• USP REFERENCE STANDARDS <11> USP Sucralose RS

Change to read:

Sucrose



$\text{C}_{12}\text{H}_{22}\text{O}_{11}$

342.30

α -D-Glucopyranoside, β -D-fructofuranosyl;
Sucrose [57-50-1].

DEFINITION

Sucrose is a sugar obtained from *Saccharum officinarum* Linné (Fam. Gramineae), *Beta vulgaris* Linné (Fam. Chenopodiaceae), and other sources. It contains no added substances.

IMPURITIES

• RESIDUE ON IGNITION <281>

Sample: 5 g

Acceptance criteria: NMT 0.05%

• CHLORIDE AND SULFATE, *Chloride* <221>

Sample: 2.0 g

Acceptance criteria: 0.0035%; the *Sample* shows no more chloride than corresponds to 0.10 mL of 0.020 N hydrochloric acid.

• CHLORIDE AND SULFATE, *Sulfate* <221>

Sample: 5.0 g

Acceptance criteria: 0.006%; the *Sample* shows no more sulfate than corresponds to 0.30 mL of 0.020 N sulfuric acid.

• CALCIUM

Analysis: To 10 mL of a solution (1 in 10) add 1 mL of ammonium oxalate TS.

Acceptance criteria: The solution remains clear for at least 1 min.

• HEAVY METALS <231>

Sample: 4.0 g

Analysis: Dissolve the *Sample* in 15 mL of water, add 1 mL of 0.12 N hydrochloric acid, and dilute with water to 25 mL.

Acceptance criteria: NMT 5 ppm

SPECIFIC TESTS

• OPTICAL ROTATION, *Specific Rotation* <781S>

Sample solution: Previously dried Sucrose at 105° for 2 h. Prepare a solution of 260 mg/mL of Sucrose in water.

Acceptance criteria: NLT +65.9°

• INVERT SUGAR

Sample solution: 200 mg/mL of Sucrose in water.

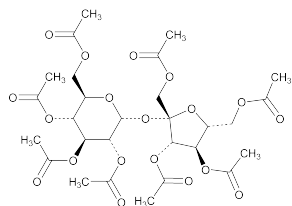
[NOTE—Filter if necessary.]

Analysis: Place 50 mL of the clear liquid in a 250-mL beaker, add 50 mL of alkaline cupric tartrate TS, cover the beaker with a watch glass, and heat the mixture at such a rate that it comes to a boil in approximately 4 min, and boil for 2 min, accurately timed. Add at once 100 mL of cold, recently boiled water, and immediately collect the precipitated cuprous oxide on a tared filtering crucible containing a sintered-glass disk of medium pore size, or suitable equivalent. Thoroughly wash the residue on the filter with hot water, then with 10 mL of alcohol, and finally with 10 mL of ether, and dry at 105° for 1 h.

Acceptance criteria: The weight of the cuprous oxide does not exceed 112 mg.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers. ● (RB 1-Apr-2012)

Sucrose Octaacetate

$C_{28}H_{38}O_{19}$ 678.59
 α -D-Glucopyranoside, 1,3,4,6-tetra-O-acetyl- β -D-fructofuranosyl, tetraacetate;
 Sucrose octaacetate [126-14-7].

DEFINITION

Sucrose Octaacetate contains NLT 98.0% and NMT 100.5% of sucrose octaacetate ($C_{28}H_{38}O_{19}$), calculated on the anhydrous basis.

ASSAY• **PROCEDURE**

Sample: 100 mg in a 500-mL conical flask

Blank: 50 mL of 70% alcohol

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Residual titration

Titrant: 0.1 N sodium hydroxide VS

Back-titrant: 0.1 N sulfuric acid VS

Endpoint detection: Visual

Analysis: Dissolve the *Sample* in 50 mL of 70% alcohol. Neutralize the solution with 0.1 N sodium hydroxide VS, using phenolphthalein TS as the indicator. Add 25.0 mL of 0.1 N sodium hydroxide VS, attach an air condenser to the flask, protect from absorption of carbon dioxide, and reflux on a steam bath for 1 h. Remove from the steam bath, cool quickly, and titrate the excess alkali with 0.1 N sulfuric acid VS, using phenolphthalein TS as the indicator. Perform a blank determination.

Calculate the percentage of sucrose octaacetate ($C_{28}H_{38}O_{19}$) in the portion of Sucrose Octaacetate taken.

Each mL of 0.1 N sodium hydroxide is equivalent to 8.483 mg of $C_{28}H_{38}O_{19}$.

Acceptance criteria: 98.0%–100.5% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** <281>: NMT 0.1%

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** <741>: NLT 78°

• **ACIDITY**

Sample: 1 g

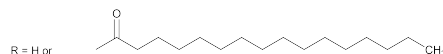
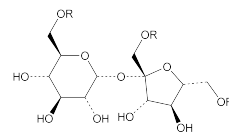
Analysis: Dissolve the *Sample* in 20 mL of neutralized alcohol, and add 2 drops of phenolphthalein TS.

Acceptance criteria: NMT 2 drops of 0.1 N sodium hydroxide are required to produce a red color.

- **WATER DETERMINATION, Method I** <921>: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Sucrose Palmitate

$C_{28}H_{52}O_{12}$ 580.71

$C_{44}H_{82}O_{13}$ 819.11

$C_{60}H_{112}O_{14}$ 1057.52

Sucrose monopalmitate;

Sucrose hexadecanoate [26446-38-8].

DEFINITION

Sucrose Palmitate is a mixture of sucrose monoesters, mainly sucrose monopalmitate, obtained by transesterification of palmitic acid methyl esters of vegetable origin with sucrose. The manufacture of the fatty acid methyl esters includes a distillation step. It contains variable quantities of mono- and diesters as set forth in the following table:

Content of Monoesters (%)	Content of Diesters (%)	Sum of Triesters and Polyesters (%)
NLT 55.0	NMT 40.0	NMT 20.0

IDENTIFICATION

- **A.** It meets the requirements of the *Fatty Acid Composition* test.
- **B.** It meets the requirements of the *Content of Monesters, Diesters, Triesters, and Polyesters*.

ASSAY

- **CONTENT OF MONOESTERS, DIESTERS, TRIESTERS, AND POLYESTERS**

Mobile phase: Tetrahydrofuran

Sample solution: 15 mg/mL of Sucrose Palmitate in tetrahydrofuran

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC, size-exclusion

Detector: Differential refractometer

Column: 7-mm \times 60-cm; packing L21, 100 Å

[NOTE—Two 7-mm \times 30-cm L21 columns may be used in place of one 60-cm column, provided system suitability requirements are met.]

Flow rate: 1.2 mL/min

Injection size: 20 μ L

Analysis

Sample: *Sample solution*

[NOTE—The relative retention time with reference to the monoester peak (retention time is approximately 10 min) is about 0.92 for diesters, and about 0.90 for triesters and polyesters.]

[NOTE—Disregard solvent peaks and peaks having a signal-to-noise ratio less than 10.]

Calculate the percentage of monoesters in the portion of Sucrose Palmitate taken:

$$\text{Result} = A \times (100 - D - S - E)/100$$

- A = percentage of monoesters determined by peak normalization
 D = percentage of free fatty acids, using the following formula:

$$\text{Result} = AV \times 256/561.1$$

- AV = acid value
 S = percentage of free sucrose (see *Free Sucrose in Organic Impurities*)
 E = percentage of water (see *Water Determination, la in Specific Tests*)

Calculate the percentage of diesters in the portion of Sucrose Palmitate taken:

$$\text{Result} = B \times (100 - D - S - E)/100$$

- B = percentage of diesters determined by peak normalization
 D = percentage of free fatty acids (above)
 S = percentage of free sucrose (see *Free Sucrose in Organic Impurities*)
 E = percentage of water (see *Water Determination, la in Specific Tests*)

Calculate the percentage of triesters and polyesters in the portion of Sucrose Palmitate taken:

$$\text{Result} = C \times (100 - D - S - E)/100$$

- C = percentage of triesters and polyesters determined by peak normalization
 D = percentage of free fatty acids (above)
 S = percentage of free sucrose (see *Free Sucrose in Organic Impurities*)
 E = percentage of water (see *Water Determination, la in Specific Tests*)

- **FATTY ACID COMPOSITION:** Sucrose Palmitate exhibits the following composition profiles of fatty acids, as determined under *Fats and Fixed Oils, Fatty Acid Composition* (401).

Fatty Acid	Percentage (%)
Lauric acid	NMT 3.0
Myristic acid	NMT 3.0
Palmitic acid	70.0–85.0
Stearic acid	10.0–25.0
Sum of the contents of palmitic acid and stearic acid	NLT 90.0

IMPURITIES

Inorganic Impurities

- **FATS AND FIXED OILS, Acid Value (401):** NMT 6.0%, determined on a 3-g sample. Use a freshly neutralized mixture of 2-propanol and water (2:1), and gently heat.

Organic Impurities

- **PROCEDURE: FREE SUCROSE**

Solution A: 10 µg/mL of ammonium acetate in acetonitrile

Solution B: 10 µg/mL of ammonium acetate in tetrahydrofuran and water (90:10)

Diluent: Tetrahydrofuran and water (87.5:12.5)

System suitability solution: 500 µg/mL of USP Sucrose RS in *Diluent*

Standard solutions: 0.50, 1.0, 2.0, and 2.5 mg/mL of USP Sucrose RS in *Diluent*

Sample solution: 50 mg/mL of Sucrose Palmitate in *Diluent*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering. [NOTE—If the detector has different setting parameters, adjust the detector settings so that they comply with the *System suitability* requirements.]

Carrier gas: Nitrogen

Detector temperature: 45°

Nebulizer temperature: 40°

Column: 4.6-mm × 0.25-m; packing L8

Injection size: 20 µL

Mobile phase and flow rate: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)	Flow Rate (mL/min)
1	100	0	1.0
8	0	100	1.0
7	0	100	1.0
0.01	0	100	2.5
15.99	0	100	2.5
1	100	0	2.5
3	100	0	1.0

System suitability

Sample: *System suitability solution*

[NOTE—The retention time is about 26 min for sucrose palmitate.]

Suitability requirements

Signal-to-noise ratio: 10:1

Analysis

Samples: *Standard solutions* and *Sample solution*

Prepare a standard curve by plotting the peak response versus concentration of sucrose in the *Standard solutions*. Calculate the amount of free sucrose in the Sucrose Palmitate taken.

Acceptance criteria: NMT 4.0%

SPECIFIC TESTS

- **WATER DETERMINATION, Method la (921):** NMT 4.0% on a 0.20-g sample

- **TOTAL ASH**

Sample: 1.0 g

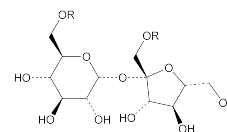
Analysis: Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Transfer the *Sample* into the crucible. Dry at 100°–105° for 1 h and ignite to constant weight in a muffle furnace at 600 ± 25°, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, add hot water, filter through an ashless filter paper, and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness and ignite to constant weight.

Acceptance criteria: NMT 1.5%

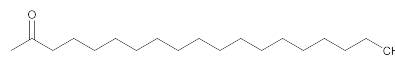
ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in a well-closed container. Protect from humidity and avoid high temperatures.
- **USP REFERENCE STANDARDS (11)**
 USP Sucrose RS

Sucrose Stearate



R = H or



$C_{48}H_{90}O_{13}$ 875.22
 $C_{66}H_{124}O_{14}$ 1141.68
 Sucrose monostearate;
 Sucrose octadecanoate [25168-73-4].

DEFINITION

Sucrose Stearate is a mixture of sucrose esters, mainly sucrose stearate, obtained by transesterification of stearic acid methyl esters derived from vegetable origin with sucrose. The manufacture of the fatty acid methyl esters includes a distillation step. The mono- and diesters requirements differ for the two types of sucrose stearate as set forth in the following table.

	Content of Monoesters (%)	Content of Diesters (%)	Sum of Triesters and Polyesters (%)
Type I	NLT 50.0	NMT 40.0	NMT 25.0
Type II	20.0–45.0	30.0–40.0	NMT 30.0

IDENTIFICATION

- **A.** It meets the requirements of the *Fatty Acid Composition* test.
- **B.** It meets the requirements of *Content of Monoesters, Diesters, Triesters, and Polyesters*.

ASSAY

• **CONTENT OF MONOESTERS, DIESTERS, TRIESTERS, AND POLYESTERS**

Mobile phase: Tetrahydrofuran

Sample solution: 15 mg/mL of Sucrose Stearate in tetrahydrofuran

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC, size-exclusion

Detector: Differential refractometer

Column: 7-mm × 60-cm; packing L21, 100 Å. [NOTE—Two 7-mm × 30-cm L21 columns may be used in place of one 60-cm column, provided system suitability requirements are met.]

Flow rate: 1.2 mL/min

Injection size: 20 µL

Analysis

Sample: *Sample solution*

[NOTE—The relative retention time with reference to the monoester peak (retention time is approximately 10 min) is about 0.92 for diesters, and about 0.90 for triesters and polyesters.]

[NOTE—Disregard solvent peaks and peaks having a signal-to-noise ratio less than 10.]

Calculate the percentage of monoesters in the portion of Sucrose Stearate taken:

$$\text{Result} = A \times (100 - D - S - E)/100$$

- A = percentage of monoesters determined by peak normalization
 D = percentage of free fatty acids, obtained by $AV \times 284.5/561.1$, where AV is the acid value
 S = percentage of free sucrose (see *Free Sucrose* in *Organic Impurities*)
 E = percentage of water (see *Water Determination, Ia* in *Specific Tests*)
 AV = acid value

Calculate the percentage of diesters in the portion of Sucrose Stearate taken:

$$\text{Result} = B \times (100 - D - S - E)/100$$

- B = percentage of diesters determined by peak normalization
 D = percentage of free fatty acids (above)

- S = percentage of free sucrose (see *Free Sucrose* in *Organic Impurities*)
 E = percentage of water (see *Water Determination, Ia* in *Specific Tests*)

Calculate the percentage of triesters and polyesters in the portion of Sucrose Stearate taken:

$$\text{Result} = C \times (100 - D - S - E)/100$$

- C = percentage of triesters and polyesters determined by peak normalization
 D = percentage of free fatty acids (above)
 S = percentage of free sucrose (see *Free Sucrose* in *Organic Impurities*)
 E = percentage of water (see *Water Determination, Ia* in *Specific Tests*)

- **FATTY ACID COMPOSITION:** Sucrose Stearate exhibits the following composition profiles of fatty acids, as determined in *Fats and Fixed Oils* <401>, *Fatty Acid Composition*.

Fatty Acid	Percentage (%)
Lauric acid	NMT 3.0
Myristic acid	NMT 3.0
Palmitic acid	25.0–40.0
Stearic acid	55.0–75.0
Sum of the contents of palmitic acid and stearic acid	NLT 90.0

IMPURITIES**Inorganic Impurities**

- **FATS AND FIXED OILS, Acid Value <401>:** NMT 6, determined on a 3-g sample. Use a freshly neutralized mixture of 2-propanol and water (2:1), and gently heat.

Organic Impurities

• **PROCEDURE: FREE SUCROSE**

Solution A: 10 µg/mL of ammonium acetate in acetonitrile

Solution B: 10 µg/mL of ammonium acetate in tetrahydrofuran and water (90:10)

Diluent: Tetrahydrofuran and water (87.5:12.5)

System suitability solution: 500 µg/mL of USP Sucrose RS in *Diluent*

Standard solutions: 0.50, 1.0, 2.0, and 2.5 mg/mL of USP Sucrose RS in *Diluent*

Sample solution: 50 mg/mL of Sucrose Stearate in *Diluent*

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Evaporative light-scattering. [NOTE—If the detector has different setting parameters, adjust the detector settings so as to comply with the *System Suitability* requirements.]

Carrier gas: Nitrogen

Detector temperature: 45°

Nebulizer temperature: 40°

Column: 4.6-mm × 0.25-m; packing L8

Injection size: 20 µL

Mobile phase and flow rate: See the gradient table below.

Time (min)	Solution A (%)	Solution B (%)	Flow Rate (mL/min)
1	100	0	1.0
8	0	100	1.0
7	0	100	1.0
0.01	0	100	2.5
15.99	0	100	2.5

Time (min)	Solution A (%)	Solution B (%)	Flow Rate (mL/min)
1	100	0	2.5
3	100	0	1.0

System suitability

Sample: *System suitability solution*

[NOTE—The retention time for sucrose stearate is about 26 min.]

Suitability requirements

Signal-to-noise ratio: 10:1

Analysis

Samples: *Standard solutions* and *Sample solution*

Prepare a standard curve by plotting the peak response versus concentration of sucrose in the *Standard solution*. Calculate the quantity of free sucrose in the Sucrose Stearate taken.

Acceptance criteria: NMT 4.0%

SPECIFIC TESTS

- **WATER DETERMINATION, Method 1a (921):** NMT 4.0%, on a 0.20-g sample

• **TOTAL ASH**

Analysis: Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator, and weigh. Transfer a 1.0-g sample into a crucible. Dry at 100°–105° for 1 h and ignite to constant weight in a muffle furnace at 600 ± 25°, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, add hot water, pass through an ashless filter paper, and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness, and ignite to constant weight.

Acceptance criteria: NMT 1.5%

ADDITIONAL REQUIREMENTS

- **LABELING:** Label to indicate whether it is Type I or Type II.
- **PACKAGING AND STORAGE:** Preserve in a well-closed container. Protect from humidity and avoid high temperatures.
- **USP REFERENCE STANDARDS (11)**
USP Sucrose RS

Compressible Sugar**DEFINITION**

Compressible Sugar, previously dried at 105° for 4 h, contains NLT 95.0% and NMT 98.0% of sucrose (C₁₂H₂₂O₁₁). It may contain starch, maltodextrin, or invert sugar, and may contain a suitable lubricant.

IDENTIFICATION

- **A.** The specific rotation of the *Uninverted solution* obtained in the Assay is NLT 62.6°, and the *Acid-inverted solution* obtained in the Assay is levorotatory.

ASSAY• **PROCEDURE**

Sample solution: Transfer 26.0 g of Compressible Sugar, previously dried, to a 100-mL volumetric flask. Add 0.3 mL of a saturated aqueous solution of lead acetate, shake with 90 mL of water, and dilute with water to volume. Distribute evenly on the surface of a sheet of medium-fast filter paper 8 g of chromatographic siliceous earth suitable for column partition chromatography (see *Reagents, Indicators, and Solutions—Reagents*), and filter the solution, with the aid of vacuum, discarding the first 20 mL of the filtrate.

Instrumental conditions

(See *Optical Rotation* (781).)

Mode: Specific rotation

Temperature: 20°

Analysis

Uninverted solution: Pipet 25 mL of the *Sample solution* into a 50-mL volumetric flask. Cool to 20°, and dilute with water to volume at 20°. Maintain a temperature of 20° for 30 min.

Acid-inverted solution: Pipet 25 mL of the *Sample solution* into a 50-mL volumetric flask. Slowly add 6 mL of dilute hydrochloric acid (1 in 2) while rotating it, dilute with water nearly to volume, and mix. Place the flask in a water bath maintained at a temperature of 60°, continuously shake the flask in the bath for 3 min, and allow the flask to remain in the bath for a total of 10 min. Immediately cool to 20° by plunging the flask into a cold bath, and dilute with water to volume at 20°. Maintain the flask at a temperature of 20° for 30 min.

Determine the specific rotation of the *Uninverted solution* and *Acid-inverted solution* at 20°.

Calculate the percentage of sucrose (C₁₂H₂₂O₁₁) in the portion of Compressible Sugar taken:

$$\text{Result} = [(\alpha_o - \alpha_i)/88.3] \times 100$$

α_o = specific rotation of the *Uninverted solution*

α_i = specific rotation of the *Acid-inverted solution*

Acceptance criteria: 95.0%–98.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION (281):** NMT 0.1%

• **CHLORIDE AND SULFATE, Chloride (221)**

Standard solution: 0.40 mL of 0.020 N hydrochloric acid

Sample solution: Transfer 20 g to a 100-mL volumetric flask, add 80 mL of water, shake to dissolve the sucrose, then add water to volume. Separate the solubilized sucrose from any insoluble matter by filtration until the filtrate is sparkling clear, and use the freshly prepared, clear filtrate.

Acceptance criteria: 0.014%; a 10-mL portion of the *Sample solution* shows no more chloride than the *Standard solution*.

• **CHLORIDE AND SULFATE, Sulfate (221)**

Standard solution: 0.50 mL of 0.020 N sulfuric acid

Sample solution: 25 mL of the *Sample solution* obtained from the test for *Chloride and Sulfate, Chloride (221)*

Acceptance criteria: 0.010%; the *Sample solution* shows no more sulfate than the *Standard solution*.

• **IDENTIFICATION TESTS—GENERAL, Calcium (191)**

Sample solution: 5 mL of the *Sample solution* obtained from the test for *Chloride and Sulfate, Chloride (221)*

Analysis: To the *Sample solution* add 1 mL of ammonium oxalate TS.

Acceptance criteria: The solution remains clear for NLT 1 min.

• **HEAVY METALS (231)**

Sample solution: 20 mL of the *Sample solution* obtained from the test for *Chloride and Sulfate, Chloride (221)*

Analysis: To the *Sample solution* add 4 mL of water and 1 mL of 0.1 N hydrochloric acid.

Acceptance criteria: NMT 5 µg/g

SPECIFIC TESTS• **MICROBIAL ENUMERATION TESTS (61) and TESTS FOR SPECIFIED MICROORGANISMS (62):** It meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*.

- **LOSS ON DRYING** (731)
Analysis: Dry at 105° for 4 h.
Acceptance criteria: NMT 1.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Confectioner's Sugar

DEFINITION

Confectioner's Sugar is Sucrose ground together with corn starch to a fine powder. It contains NLT 95.0% of sucrose ($C_{12}H_{22}O_{11}$), calculated on the dried basis.

IDENTIFICATION

- **A.**
Sample solution: Transfer 20 g to a 100-mL volumetric flask, add 80 mL of water, shake to dissolve the sucrose, then add water to volume. Separate the solubilized sucrose from the insoluble starch component by filtration until the filtrate is sparkling clear. Use the insoluble portion for the *Identification* test, and use the freshly prepared, clear filtrate for the tests in *Impurities* for *Chloride and Sulfate*, *Chloride* (221) and *Sulfate* (221), and for *Calcium*; and in *Specific Tests* for *Optical Rotation*, *Specific Rotation* (781).
Analysis: To the water slurry of the insoluble portion add iodine TS.
Acceptance criteria: A reddish-violet to deep blue color is produced.

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.08%
- **CHLORIDE AND SULFATE**, *Chloride* (221)
Standard solution: 0.40 mL of 0.020 N hydrochloric acid
Sample solution: 10 mL of the clear filtrate obtained from *Identification* test A
Acceptance criteria: 0.014%; the *Sample solution* shows no more chloride than the *Standard solution*.
- **CHLORIDE AND SULFATE**, *Sulfate* (221)
Standard solution: 0.30 mL of 0.020 N sulfuric acid
Sample solution: 25 mL of the clear filtrate obtained from *Identification* test A
Acceptance criteria: 0.006%; the *Sample solution* shows no more sulfate than the *Standard solution*.
- **CALCIUM**
Sample solution: 5 mL of the clear filtrate obtained from *Identification* test A
Analysis: To the *Sample solution* add 5 mL of water and 1 mL of ammonium oxalate TS.
Acceptance criteria: The *Sample solution* remains clear for NLT 1 min.
- **HEAVY METALS** (231)
Sample solution: 20 mL of the clear filtrate obtained from *Identification* test A
Analysis: To the *Sample solution* add 4 mL of water and 1 mL of 0.1 N hydrochloric acid.
Acceptance criteria: NMT 5 µg/g

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **LOSS ON DRYING** (731)
Analysis: Dry at 105° for 4 h.
Acceptance criteria: NMT 1.0%
- **OPTICAL ROTATION**, *Specific Rotation* (781)
Sample solution: Clear filtrate obtained from *Identification* test A

Acceptance criteria: NLT +62.6°, corresponding to NLT 95.0% of sucrose ($C_{12}H_{22}O_{11}$), calculated on the dried basis

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Sugar Spheres

DEFINITION

Sugar Spheres contain NLT 62.5% and NMT 91.5% of sucrose ($C_{12}H_{22}O_{11}$), calculated on the dried basis, the remainder consisting chiefly of starch. They consist of approximately spherical particles of a labeled nominal size range. They may contain color additives permitted by the FDA for use in drugs.

IDENTIFICATION

- **A.**
Sample suspension: 1:10
Analysis: Add iodine TS to the *Sample suspension*.
Acceptance criteria: A violet to deep blue color is produced.

ASSAY

- **OPTICAL ROTATION**, *Specific Rotation* (781S)
Sample solution: Transfer 20 g to a 200-mL volumetric flask, add 160 mL of water, shake to dissolve the sucrose, add water to volume, and mix. Pass the solubilized sucrose solution by vacuum filtration through fine filter paper.
Acceptance criteria: +41° to +61°, corresponding to 62.5%–91.5% of sucrose ($C_{12}H_{22}O_{11}$), calculated on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281)
Sample: 2.0 g
Analysis: Ignite at a temperature of 700 ± 25°.
Acceptance criteria: NMT 0.25%
- **HEAVY METALS**, *Method II* (231): 5 µg/g

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count does not exceed 10² cfu/g, and the Spheres meet the requirements of the tests for absence of *Salmonella* species, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*.
- **LOSS ON DRYING** (731)
Analysis: Dry at 105° for 4 h.
Acceptance criteria: NMT 4.0%
- **PARTICLE SIZE DISTRIBUTION ESTIMATION BY ANALYTICAL SIEVING** (786): NLT 90% of it passes the coarser sieve size stated in the labeling; all of it passes the next coarser sieve size listed in *Table 1* of the chapter; and NMT 10% passes the finer sieve size stated in the labeling.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **LABELING:** The label states the nominal particle size range.

Sulfur Dioxide

SO₂ 64.06
Sulfur dioxide [7446-09-5].

DEFINITION

Sulfur Dioxide contains NLT 97.0%, by volume, of sulfur dioxide (SO₂).

[CAUTION—Sulfur Dioxide is poisonous.]

ASSAY

• PROCEDURE

Sample: 100.0 mL of gaseous Sulfur Dioxide

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 0.1 N iodine VS

Endpoint detection: Visual

Analysis: Collect the *Sample* over mercury, and note the temperature of the *Sample* and the pressure upon it. Slowly introduce 50.0 mL of 0.1 N sodium hydroxide into the air space over the mercury, and absorb the *Sample* in the solution by shaking. When absorption is complete, transfer the solution to a 250-mL conical flask, add 3 mL of starch TS, and titrate with *Titrant* until the solution is pale blue in color.

Calculate the percentage of sulfur dioxide (SO₂) at a temperature of 0° and a pressure of 760 mm of mercury in the portion of Sulfur Dioxide taken. Each mL of 0.1 N iodine is equivalent to 1.094 mL of SO₂.

Acceptance criteria: NLT 97.0%, by volume

IMPURITIES

• LIMIT OF NONVOLATILE RESIDUE

Sample: 300 g (209 mL)

Analysis: Transfer the *Sample* to a tared, 250-mL conical flask, and allow the liquid to evaporate spontaneously in a well-ventilated hood. When evaporation appears complete, blow a current of dry, filtered air through the flask until the odor of sulfur dioxide is no longer apparent.

Acceptance criteria: NMT 7.5 mg (0.0025%)

• SULFURIC ACID

Analysis: To the flask containing the residue obtained in the test for *Limit of Nonvolatile Residue* add 25 mL of water previously neutralized to methyl red TS. Swirl the flask, and titrate with 0.10 N sodium hydroxide.

Acceptance criteria: NMT 1.3 mL is required (about 0.002%).

SPECIFIC TESTS

• WATER DETERMINATION, Method I <921>

Sample: 3 g (2.1 mL)

Analysis: Taking precautions to avoid absorption of moisture, transfer the *Sample* to a suitable flask, and add 20 mL of anhydrous pyridine.

Acceptance criteria: NMT 2.0%

ADDITIONAL REQUIREMENTS

• PACKAGING AND STORAGE: Preserve in cylinders.

[NOTE—Sulfur Dioxide is used most in the form of a gas in pharmaceutical applications, and is described herein for such purposes. However, it is usually packaged under pressure, hence the preceding specifications are designed for testing it in liquid form.]

Sulfuric Acid

H₂SO₄ 98.08
Sulfuric acid [7664-93-9].

DEFINITION

Sulfuric Acid contains NLT 95.0% and NMT 98.0%, by weight, of sulfuric acid (H₂SO₄).

[CAUTION—When sulfuric acid is to be mixed with other liquids, always add it to the diluent, and exercise great caution.]

IDENTIFICATION

- **A. IDENTIFICATION TESTS—GENERAL, Sulfate <191>:** Meets the requirements

ASSAY

• PROCEDURE

Sample solution: Place 1 mL of Sulfuric Acid in a weighed, glass-stoppered flask containing 20 mL of water, and weigh again to obtain the weight of the Sulfuric Acid. Dilute with 25 mL of water, and cool.

Titrimetric system

(See *Titrimetry* <541>.)

Mode: Direct titration

Titrant: 1 N sodium hydroxide VS

Blank: 45 mL of water

Endpoint detection: Visual

Analysis: To the *Sample solution* add methyl orange TS, and titrate with 1 N sodium hydroxide VS. Perform a blank determination. Calculate the percentage of sulfuric acid (H₂SO₄) taken:

$$\text{Result} = \{[(V_S - V_B) \times N \times F]/W\} \times 100$$

V_S = *Titrant volume consumed by sulfuric acid in the Sample solution (mL)*

V_B = *Titrant volume consumed by the Blank (mL)*

N = *Titrant actual normality (mEq/mL)*

F = *equivalency factor, 49.04 mg/mEq*

W = *weight of sulfuric acid in the Sample solution (mg)*

Acceptance criteria: 95.0%–98.0%

IMPURITIES

• RESIDUE ON IGNITION <281>

Sample: 22 mL (40 g)

Analysis: Evaporate the *Sample* to dryness, and ignite.

Acceptance criteria: NMT 2 mg of residue remains (0.005%).

- **CHLORIDE AND SULFATE, Chloride <221>:** A dilution of 1.1 mL (2.0 g) in water shows no more chloride than corresponds to 0.15 mL of 0.020 N hydrochloric acid (50 ppm).

• ARSENIC <211>

Test preparation: Add 1.6 mL (3.0 g) to a mixture of nitric acid and water (3:20). Evaporate the solution until dense fumes of sulfur trioxide form. Cool, and cautiously wash the solution into an arsine generating flask with 50 mL of water.

Analysis: Proceed as directed in the chapter, omitting the addition of the 20 mL of 7 N sulfuric acid.

Acceptance criteria: NMT 1 ppm

• HEAVY METALS <231>

Test preparation: Add 2.2 mL (4.0 g) to 10 mg of sodium carbonate dissolved in 10 mL of water. Heat until almost dry. Add 1 mL of nitric acid, and evaporate to dryness. Add 2 mL of 1 N acetic acid to the residue, and dilute with water to 25 mL.

Analysis: Proceed as directed in the chapter.

Acceptance criteria: NMT 5 ppm

SPECIFIC TESTS

• REDUCING SUBSTANCES

Sample: 4.4 mL (8.0 g)

Analysis: Carefully dilute the *Sample* with 50 mL of ice-cold water, keeping the solution cold during the addition. Add 0.10 mL of 0.10 N potassium permanganate.

Acceptance criteria: The solution remains pink for 5 min.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

Sunflower Oil**DEFINITION**

Sunflower Oil is a refined fixed oil obtained from the seeds of the sunflower plant *Helianthus annuus* Linné (Fam. Asteraceae alt. Compositae).

IMPURITIES

- **HEAVY METALS, Method II** (231): NMT 10 ppm

SPECIFIC TESTS

- **FATS AND FIXED OILS, Acid Value (Free Fatty Acids)** (401): NMT 2.5 mL of 0.020 N sodium hydroxide is required for neutralization.

- **FATS AND FIXED OILS, Fatty Acid Composition** (401)

Standard solution: Prepare an ester mixture containing methyl linoleate, methyl oleate, methyl palmitate, methyl stearate, and methyl linolenate (50:35:7:5:3).¹

Sample solution: Transfer 100 mg of Sunflower Oil to a 50-mL conical flask fitted with a suitable water-cooled reflux condenser and a magnetic stir bar. Add 4 mL of 0.5 N methanolic sodium hydroxide solution, and reflux until fat globules disappear (usually 5–10 min). Add 5 mL of a solution prepared by dissolving 14 g of boron trifluoride in methanol to make 100 mL, swirl to mix, and reflux for 2 min. Add 4 mL of chromatographic *n*-heptane through the condenser, and reflux for 1 min. Cool, remove the condenser, add 15 mL of saturated sodium chloride solution, shake, and allow the layers to separate. Pass the *n*-heptane layer through 0.1 g of anhydrous sodium sulfate (previously washed with chromatographic *n*-heptane) into a suitable flask. Transfer 1.0 mL of this solution to a 10-mL volumetric flask, dilute with chromatographic *n*-heptane to volume, and mix.

Chromatographic system

(See Chromatography (621), System Suitability.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 30-m fused-silica capillary column bonded with a 0.25-μm layer of phase G5

Temperature

Injector: 220°

Detector: 250°

Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
120	—	120	2
120	4	240	5

Carrier gas: Hydrogen

Flow rate: 1 mL/min

Injection size: 1 μL

Injection type: Splitless

System suitability

Sample: Standard solution

[NOTE—The relative retention times for methyl palmitate, methyl stearate, and methyl oleate are about 0.87, 0.99, and 1.0, respectively.]

¹Ester mixtures are available commercially from Nu-Chek-Prep, Inc., P.O. Box 295, Elysian, MN 56028. Typical Nu-Chek-Prep ester mixtures useful in this test include Nu-Chek 15A. This mixture may contain other components.

Suitability requirements

Resolution: NLT 1.5 between methyl stearate and methyl oleate

Relative standard deviation: NMT 6.0% peak area responses for the palmitate and stearate peaks for replicate injections; NMT 1.0% peak area response ratio of the palmitate to stearate peaks from these replicate injections

Analysis

Samples: Standard solution and Sample solution

Measure the areas of the five major peaks for the methyl esters of the fatty acids, which elute in the following order: palmitate, stearate, oleate, linoleate, and linolenate.

Calculate the percentage of palmitate, stearate, oleate, linoleate, and linolenate in the portion of Sunflower Oil taken:

$$\text{Result} = (A/B) \times 100$$

A = peak area of palmitate, stearate, oleate, linoleate, or linolenate

B = total area of the five major peaks

Acceptance criteria: See Table 2.

Table 2

	Generic Oil (%)	Mid-Oleic Oil (%)	High-Oleic Oil (%)
Methyl palmitate	3–10	2–9	2–9
Methyl stearate	2–8	2–8	2–8
Methyl oleate	14–24	40–70	70–90
Methyl linoleate	63–73	15–40	5–15
Methyl linolenate	0–3	0–3	0–3

- **FATS AND FIXED OILS, Iodine Value, Method II** (401)

Acceptance criteria

Generic oil: 128–148

Mid-oleic oil: 98–118

High-oleic oil: 78–98

- **FATS AND FIXED OILS, Saponification Value** (401): 180–200

- **FATS AND FIXED OILS, Unsaponifiable Matter** (401): NMT 1.0%

Change to read:

- **LIMIT OF PEROXIDE**

[NOTE—This test must be performed promptly after sampling to avoid oxidation of the test specimen.]

Solvent A: Chloroform and glacial acetic acid (40:60)

Potassium iodide solution: Prepare a saturated solution of potassium iodide in freshly boiled and cooled water, and store protected from light. [NOTE—Discard the solution if it becomes colored upon addition of Solvent A and iodide-free starch TS.] (ERR 1-May-2012)]

Sample: 10 g of Sunflower Oil

Analysis: Transfer the Sample to a conical flask, add 30 mL of Solvent A, and swirl to dissolve. Add 0.5 mL of Potassium iodide solution, swirl for 1.0 min, and add 30 mL of water. Titrate with 0.01 N sodium thiosulfate VS, with vigorous agitation, to a light yellow color. Add 2.0 mL of iodide-free starch TS, and continue the titration until the blue color has disappeared. Perform a blank determination, and make any necessary correction.

Calculate the amount of peroxide, in mEq/kg, in the portion of Sunflower Oil taken:

$$\text{Result} = (V \times N/W) \times F$$

V = volume of sodium thiosulfate used in the titration (mL)

N = normality of sodium thiosulfate VS (mEq/mL)

W = weight of Sunflower Oil taken (g)

F = conversion factor, 1000 g/kg

Acceptance criteria: NMT 10.0 mEq/kg

• **REFRACTIVE INDEX** (831): 1.472–1.474 at 20°

• **SPECIFIC GRAVITY** (841): 0.914–0.924 at 20°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers. No storage requirement specified.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant source from which the article was derived. The label also indicates the name and concentration of any additive.

Suspension Structured Vehicle

DEFINITION

Prepare Suspension Structured Vehicle as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* (795)).

Potassium Sorbate	0.15 g
Xanthan Gum	0.15 g
Citric Acid, Anhydrous	0.15 g
Sucrose	20 g
Purified Water, a sufficient quantity to make	100 mL

Transfer the *Potassium Sorbate* to a suitable beaker, and dissolve in 50 mL of *Purified Water*. Place the beaker on an electric hot plate and stirrer, and add the *Xanthan Gum* into the vortex while slowly stirring. Apply minimal heat, and incorporate the *Citric Acid* and the *Sucrose*. Add a sufficient quantity of *Purified Water* to obtain a final volume of 100 mL, and mix.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight, light-resistant containers. Store at room temperature, and avoid freezing.
- **LABELING:** Label it to state that it must be well shaken before use.
- **BEYOND-USE DATE:** NMT 30 days after the date on which it was compounded

Sugar-Free Suspension Structured Vehicle

DEFINITION

Prepare Sugar-Free Suspension Structured Vehicle as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* (795)).

Xanthan Gum	0.20 g
Saccharin Sodium	0.20 g
Potassium Sorbate	0.15 g
Citric Acid	0.10 g
Sorbitol	2.0 g
Mannitol	2.0 g
Glycerin	2.0 mL
Purified Water, a sufficient quantity to make	100 mL

Transfer 30 mL of *Purified Water* to a beaker, placing it on an electric hot plate and stirrer. Using moderate heat, stir to form a vortex, and slowly sprinkle the *Xanthan Gum* into the vortex. In a separate beaker, dissolve the *Saccharin*

Sodium, Potassium Sorbate, and *Citric Acid* in 50 mL of *Purified Water*. Using moderate heat, incorporate the *Sorbitol*, *Mannitol*, and *Glycerin* into this mixture. Add to this mixture the previously prepared xanthan gum dispersion. Add a sufficient quantity of *Purified Water* to obtain a final volume of 100 mL, and mix.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight, light-resistant containers. Store at room temperature, and avoid freezing.
- **LABELING:** Label it to state that it must be well shaken before use.
- **BEYOND-USE DATE:** NMT 30 days after the date on which it was compounded

Syrup

DEFINITION

Syrup is a solution of *Sucrose* in *Purified Water*. It contains a preservative unless it is used when freshly prepared.

Sucrose	850 g
Purified Water, a sufficient quantity to make	1000 mL

Syrup may be prepared by the use of boiling water or, preferably, without heat, by the following process. Place the *Sucrose* in a suitable percolator, the neck of which is nearly filled with loosely packed cotton, moistened after packing with a few drops of water. Pour carefully 450 mL of *Purified Water* upon the *Sucrose*, and regulate the outflow to a steady drip of percolate. Return the percolate, if necessary, until all of the *Sucrose* has dissolved. Then wash the inside of the percolator and the cotton with sufficient *Purified Water* to bring the volume of the percolate to 1000 mL, and mix.

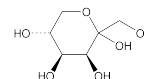
SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): NLT 1.30

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight containers, preferably in a cool place.

Tagatose



C₆H₁₂O₆

D-Tagatose;

D-lyxo-Hexulose [87-81-0].

180.16

DEFINITION

Tagatose is a ketohexose, an epimer of D-fructose inverted at C-4. It is obtained from D-galactose by isomerization under alkaline conditions in the presence of calcium. It contains NLT 98.0% of tagatose (C₆H₁₂O₆), calculated on the dried basis.

IDENTIFICATION

- **A.** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*.
- **B.** It meets the requirements of the test for *Optical Rotation* (781), *Specific Rotation*.

• **C.**

Sample solution: 200 mg/mL of Tagatose

Analysis: Add 3 mL of the *Sample solution* to 5 mL of hot alkaline cupric tartrate TS.

Acceptance criteria: A copious red precipitate of cuprous oxide is formed.

ASSAY• **PROCEDURE**

Mobile phase: 0.05 mg/mL of calcium acetate

Standard solution: 5 mg/mL of USP Tagatose RS. Pass through a filter of 0.2- μ m pore size.

Sample solution: 5 mg/mL of Tagatose, previously dried. Pass through a filter of 0.2- μ m pore size.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC

Detector: Refractive index

Column: 7.8-mm \times 30-cm; 9- μ m packing L19

Column temperature: 85°

Flow rate: 0.6 mL/min

Injection size: 20 μ L

System suitability

Sample: *Standard solution*

Suitability requirements

Relative standard deviation: NMT 2.0% of replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of tagatose (C₆H₁₂O₆) in the portion of Tagatose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response from the *Sample solution*

r_S = peak response from the *Standard solution*

C_S = concentration of USP Tagatose RS in the *Standard solution* (mg/mL)

C_U = concentration of Tagatose in the *Sample solution* (mg/mL)

Acceptance criteria: NLT 98.0% on the dried basis

IMPURITIES• **LIMIT OF LEAD**

Sample solution: 2.5 g of Tagatose dissolved in a mixture of 4 mL of sulfuric acid and 5 mL of hydrochloric acid. Dilute with water to 50 mL.

Standard stock solution A: Dissolve 1.60 g of lead nitrate in diluted nitric acid (10 mL of nitric acid diluted with 20 mL water, boiled to remove nitrous fumes, and cooled), and dilute with water to 1000 mL.

Standard stock solution B: *Standard stock solution A* and water (1:50). [NOTE—This solution contains the equivalent of 20 μ g/mL of lead.]

Standard solutions: To a series of 100-mL volumetric flasks pipet 0, 1, 2, 3, 4, and 5 mL of *Standard stock solution B*, and dilute with water to about 50 mL. Add 8 mL of sulfuric acid and 10 mL of hydrochloric acid to each flask, shake to dissolve, and dilute with water to volume. [NOTE—These solutions contain 0, 0.2, 0.4, 0.6, 0.8, and 1.0 μ g/mL of lead, respectively.]

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Atomic absorption

Analytical wavelength: 283.3 nm

Analysis

Samples: *Standard solutions* and *Sample solution*
Concomitantly determine the absorbances of the *Standard solutions* and the *Sample solution*. Plot the absorbances of the *Standard solutions* versus the concentration of lead. Using this graph, determine the concentration of lead in the *Sample solution*.

Acceptance criteria: NMT 1 ppm

SPECIFIC TESTS• **MICROBIAL ENUMERATION TESTS** <61> and **TESTS FOR SPECIFIED MICROORGANISMS** <62>:

It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total aerobic microbial count does not exceed 1000 cfu/g, and the total combined molds and yeasts count does not exceed 100 cfu/g.

• **MELTING RANGE OR TEMPERATURE**, *Class I* <741>: 133°–144°• **OPTICAL ROTATION**, *Specific Rotation* <781>: –4° to –7°

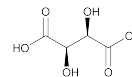
Sample solution: 10 mg/mL

• **LOSS ON DRYING** <731>: Dry a sample at 102° for 2 h: it loses NMT 0.5% of its weight• **ARTICLES OF BOTANICAL ORIGIN**, *Total Ash* <561>: NMT 0.1%, determined on a 1.0-g specimen**ADDITIONAL REQUIREMENTS**• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.• **USP REFERENCE STANDARDS** <11>

USP Tagatose RS

Talc—see *Talc General Monographs*

Tartaric Acid



C₄H₆O₆

150.09

Butanedioic acid, 2,3-dihydroxy-;

Butanedioic acid, 2,3-dihydroxy-, [*R*-(*R**,*R**)]-;

Tartaric acid; L-(+)-Tartaric acid [526-83-0; 87-69-4].

DEFINITION

Tartaric Acid, dried over phosphorus pentoxide for 3 h, contains NLT 99.7% and NMT 100.5% of C₄H₆O₆.

IDENTIFICATION• **A. IDENTIFICATION TESTS—GENERAL**, *Tartrate* <191>: It meets the requirements.• **B. INFRARED ABSORPTION** <197K>**ASSAY**• **PROCEDURE**

Sample: 2 g of Tartaric Acid, previously dried, in a conical flask

Analysis: Dissolve the *Sample* in 40 mL of water, add phenolphthalein TS, and titrate with 1 N sodium hydroxide VS. Each mL of 1 N sodium hydroxide is equivalent to 75.04 mg of C₄H₆O₆.

Acceptance criteria: 99.7%–100.5%

IMPURITIES**Inorganic Impurities**• **RESIDUE ON IGNITION** <281>: NMT 0.1%• **CHLORIDE AND SULFATE**, *Sulfate* <221>

Sample solution: 10 mL of a 1-in-100 solution of Tartaric Acid

Analysis: Add to the *Sample solution* 3 drops of hydrochloric acid and 1 mL of barium chloride TS.

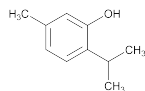
Acceptance criteria: No turbidity is produced.

• **HEAVY METALS**, *Method II* <231>: NMT 10 ppm**SPECIFIC TESTS**• **OPTICAL ROTATION**, *Specific Rotation* <781S>: +12.0° to +13.0°

- Sample solution:** 200 mg/mL in water
- **LOSS ON DRYING** (731): Dry over phosphorus pentoxide for 3 h; it loses NMT 0.5% of its weight.
 - **LIMIT OF OXALATE**
Sample solution: 10 mL of a 1-in-10 solution of Tartaric Acid
Analysis: Nearly neutralize the *Sample solution* with 6 N ammonium hydroxide, and add 10 mL of calcium sulfate TS.
Acceptance criteria: No turbidity is produced.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.
- **USP REFERENCE STANDARDS** (11)
 USP Tartaric Acid RS

Thimerosal—see *Thimerosal General Monographs***Thymol**

$C_{10}H_{14}O$ 150.22
 Phenol, 5-methyl-2-(1-methylethyl)-;
 Thymol;
p-Cymen-3-ol [89-83-8].

DEFINITION

Thymol contains NLT 99.0% and NMT 101.0% of thymol ($C_{10}H_{14}O$).

IDENTIFICATION

- **A. INFRARED ABSORPTION** (197K)
- **B.** It meets the requirements in *Specific Tests for Melting Range or Temperature* (741).

ASSAY• **PROCEDURE**

Sample: 100 mg

Titrimetric system

(See *Titrimetry* (541).)

Mode: Direct titration

Titrant: 0.1 N bromine VS

Endpoint detection: Visual

Analysis: Transfer the *Sample* to a 250-mL iodine flask, and dissolve in 25 mL of 1 N sodium hydroxide. Add 20 mL of hot dilute hydrochloric acid (1 in 2), and immediately titrate with *Titrant* to within 1–2 mL of the calculated endpoint. Warm the solution to between 70° and 80°, add 2 drops of methyl orange TS, and continue the titration slowly, swirling vigorously after each addition. When the color of the methyl orange is bleached, add 2 drops of *Titrant*, shake for 10 s, add 1 drop of methyl orange TS, and shake vigorously. If the solution is red, continue the titration, dropwise and with shaking, until the color is discharged. Repeat the alternate addition of the *Titrant* and methyl orange TS until the red color is discharged after the addition of the methyl orange TS. Each mL of *Titrant* is equivalent to 3.755 mg of thymol ($C_{10}H_{14}O$).

Acceptance criteria: 99.0%–101.0%

IMPURITIES• **LIMIT OF NONVOLATILE RESIDUE**

Sample: 2 g

Analysis: Volatilize the *Sample* on a steam bath, and dry at 105° to constant weight.

Acceptance criteria: NMT 0.05%

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** (741): 48°–51°; but when melted, Thymol remains liquid at a considerably lower temperature.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** (11)
 USP Thymol RS

Titanium Dioxide—see *Titanium Dioxide General Monographs***Tocopherols Excipient****DEFINITION**

Tocopherols Excipient is a vegetable oil solution containing NLT 50.0% of total tocopherols, of which NLT 80.0% consists of varying amounts of beta, gamma, and delta tocopherols.

IDENTIFICATION

- **A.**
Sample solution: 50 mg of Tocopherols Excipient in 10 mL of dehydrated alcohol
Analysis: To the *Sample solution* add with swirling 2 mL of nitric acid, and heat at about 75° for 15 min.
Acceptance criteria: A bright red or orange color develops.
- **B.** The retention time of the third major peak (i.e., the peak occurring just before that of the internal standard) of the *Sample solution* corresponds to that of the *Standard solution*, both relative to that of the internal standard, as obtained in the Assay.

ASSAY• **PROCEDURE**

Solution A: Pyridine and propionic anhydride (2:1)

Internal standard solution: 3 mg/mL of hexadecyl hexadecanoate in *Solution A*

Standard solutions: Using low-actinic glassware, add 12-, 25-, 37-, and 50-mg portions of USP Alpha Tocopherol RS to separate 50-mL conical flasks having 19/38 standard-taper ground-glass necks. Pipet 25 mL of the *Internal standard solution* into each flask, and reflux for 10 min under water-cooled condensers.

Sample solution: Using low-actinic glassware, add 60 mg of Tocopherols Excipient to a 50-mL conical flask similar to the flasks used in preparing the *Standard solutions*. Add 10.0 mL of *Internal standard solution*, and reflux for 10 min under a water-cooled condenser.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 4-mm × 2-m borosilicate glass; packed with 2%–5% liquid phase G2 on 80- to 100-mesh support S1AB using either a glass-lined sample introduction system or on-column injection

Temperatures

Column: 245°–265°, maintained isothermally

Injection port: 10° higher than the *Column* temperature

Detector: 10° higher than the *Column* temperature

Flow rate: Dry carrier gas is adjusted to obtain a hexadecyl hexadecanoate peak 30–32 min after sample introduction. [NOTE—Cure and condition the column as necessary.]

Injection volume: 2–5 μ L

System suitability

Sample: *Sample solution*

[NOTE—The relative retention times for delta tocopheryl propionate, beta plus gamma tocopheryl propionate, and hexadecyl hexadecanoate are about 0.50, 0.63, and 1.00, respectively.]

Suitability requirements

Resolution: Chromatograph a sufficient number of injections to ensure that a resolution of NLT 2.5 between delta tocopheryl propionate and beta plus gamma tocopheryl propionate relative to hexadecyl hexadecanoate is met.

Analysis

Samples: *Standard solutions* and *Sample solution*

Calibration: Chromatograph each *Standard solution*, and calculate the relative response factor, *F*, for each concentration of the *Standard solution* taken:

$$F = (r_s/r_D) \times (C_D/C_s)$$

r_s = peak response of alpha tocopherol in the *Standard solution*

r_D = peak response of hexadecyl hexadecanoate in the *Standard solution*

C_D = concentration of hexadecyl hexadecanoate in the *Standard solution* (mg/mL)

C_s = concentration of USP Alpha Tocopherol RS in the *Standard solution* (mg/mL)

Chromatograph a sufficient number of injections of each *Standard solution* to ensure that *F* is constant within a range of 2.0%. Prepare a relative response factor curve by plotting *F* versus the alpha tocopheryl propionate peak response.

Inject the *Sample solution*, and measure the responses for the four major peaks occurring at relative retention times of approximately 0.50, 0.63, 0.76, and 1.00, and record the values as a_{δ} , $a_{\beta/\gamma}$, a_{α} , and a_D , corresponding to delta tocopheryl propionate, beta plus gamma tocopheryl propionates, alpha tocopheryl propionate, and hexadecyl hexadecanoate, respectively.

Calculate the quantity of each tocopherol form in the Tocopherols Excipient taken:

$$\text{delta tocopherol} = (V \times C_D/F) \times (a_{\delta}/a_D)$$

$$\text{beta plus gamma tocopherols} = (V \times C_D/F) \times (a_{\beta/\gamma}/a_D)$$

$$\text{alpha tocopherol} = (V \times C_D/F) \times (a_{\alpha}/a_D)$$

V = volume of *Internal standard solution* used in the *Sample solution* (mL)

F = obtained from the relative response factor curve (see *Calibration*) for each of the corresponding responses for the delta, beta plus gamma, and alpha tocopheryl propionate peaks produced by the *Sample solution*

[NOTE—The relative response factor for delta tocopheryl propionate and for beta plus gamma tocopheryl propionates has been determined empirically to be the same as for alpha tocopheryl propionate.]

Acceptance criteria: NLT 50.0% of total tocopherols, of which NLT 80.0% consists of varying amounts of beta, gamma, and delta tocopherols

SPECIFIC TESTS• **ACIDITY**

Solution A: Alcohol and ether (50%:50%). Neutralize to phenolphthalein with 0.1 N sodium hydroxide.

Sample solution: Dissolve 1.0 g of Tocopherols Excipient in 25 mL of *Solution A*.

Analysis: To the *Sample solution* add 0.5 mL of phenolphthalein TS, and titrate with 0.10 N sodium hydroxide until the solution remains faintly pink after being shaken for 30 s.

Acceptance criteria: NMT 1.0 mL of 0.10 N sodium hydroxide is required.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. Protect with a blanket of an inert gas.
- **LABELING:** Label it to indicate the content, in mg/g, of total tocopherols and of the sum of beta, gamma, and delta tocopherols.
- **USP REFERENCE STANDARDS** <11>
USP Alpha Tocopherol RS

Tolu Balsam Syrup**DEFINITION**

Prepare Tolu Balsam Syrup as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* <795>).

Tolu Balsam Tincture	50 mL
Magnesium Carbonate	10 g
Sucrose	820 g
Purified Water, a sufficient quantity to make	1000 mL

Add the *Tincture* all at once to the *Magnesium Carbonate* and 60 g of the *Sucrose* in a mortar, and mix. Gradually add 430 mL of *Purified Water* with trituration, and filter. Dissolve the remainder of the *Sucrose* in the clear filtrate with gentle heating, strain the syrup while warm, and add sufficient *Purified Water* through the strainer to make the product measure 1000 mL, and mix.

Tolu Balsam Syrup may also be prepared as follows. Place 760 g of the *Sucrose* in a suitable percolator, the neck of which is nearly filled with loosely packed cotton, moistened after packing with a few drops of water. Pour the filtrate, obtained as directed in the preceding instructions, on the *Sucrose*, and regulate the outflow to a steady drip of percolate. When all of the liquid has run through, return portions of the percolate, if necessary, to dissolve all the *Sucrose*. Then pass enough *Purified Water* through the cotton to make the product measure 1000 mL, and mix.

OTHER COMPONENTS

- **ALCOHOL DETERMINATION, Method II** <611>: 3.0%–5.0%

SPECIFIC TESTS

- **FATS AND FIXED OILS, Acid Value** <401>

Sample solution: 2% of solution

Analysis: Add phenolphthalein TS, and titrate with 0.5 N alcoholic potassium hydroxide VS.

Acceptance criteria: 112–168

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight containers, and store at controlled room temperature.

- **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Tolu Balsam Tincture

DEFINITION

Tolu Balsam Tincture is prepared from Tolu Balsam obtained from *Myroxylon balsamum* (L.) Harms var. *balsamum* (Fam. Fabaceae).

Prepare Tolu Balsam Tincture as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* <795>).

Tolu Balsam	200 g
Alcohol	750 mL
Alcohol, a sufficient quantity to make	1000 mL

Macerate the *Tolu Balsam* with 750 mL of *Alcohol* in a container that can be closed, and put in a warm place. Agitate it frequently during 3 days or until the soluble matter is dissolved. Transfer the mixture to a filter, and when most of the liquid has drained away, wash the residue on the filter with a sufficient quantity of *Alcohol*, combining the filtrates to produce 1000 mL of Tincture, and mix.

OTHER COMPONENTS

- **ALCOHOL DETERMINATION, Method I** <611>: 77.0%–83.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight, light-resistant containers, and store at controlled room temperature. Avoid exposure to direct sunlight and to excessive heat.
- **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Tragacanth

DEFINITION

Tragacanth is the dried gummy exudation from *Astragalus gummifer* Labillardière, or other Asiatic species of *Astragalus* (Fam. Leguminosae).

IDENTIFICATION

- **A.** Add 1 g to 50 mL of water: it swells and forms a smooth, nearly uniform, stiff, opalescent mucilage free from cellular fragments.

IMPURITIES

- **LEAD** <251>: NMT 10 ppm
- **HEAVY METALS, Method II** <231>: NMT 20 ppm

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** <61> and **TESTS FOR SPECIFIED MICROORGANISMS** <62>: It meets the requirements for absence of *Salmonella* species and *Escherichia coli*.

BOTANIC CHARACTERISTICS

Tragacanth: It is flattened, lamellated, frequently curved fragments or straight or spirally twisted linear pieces from 0.5 to 2.5 mm in thickness. It is white to weak yellow in color, translucent, and horny in texture. Its fracture is short. It is rendered more easily pulverizable by heating to 50°. It is odorless.

Histology: Pieces of Tragacanth softened in water and mounted in water or glycerin show numerous lamellae and a few starch grains.

Powdered tragacanth: It is white to yellowish white. When examined in water mounts, it shows numerous

angular fragments of mucilage with circular or irregular lamellae, and occasional starch grains up to 25 µm in diameter, mostly simple, spherical to elliptical, with occasional two- to four-compound grains, a few of the grains being swollen and more or less altered. The powder shows few or no fragments of lignified vegetable tissue (Indian gum).

KARAYA GUM

Sample solution: 1 g in 20 mL of water

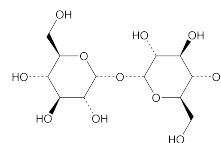
Analysis: Boil the *Sample solution* until a mucilage is formed, add 5 mL of hydrochloric acid, and again boil the mixture for 5 min.

Acceptance criteria: No pink or red color develops.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Trehalose



$C_{12}H_{22}O_{11}$ 342.30

$C_{12}H_{22}O_{11} \cdot 2H_2O$ 378.33

α -D-Glucopyranosyl α -D-glucopyranoside.

Anhydrous [99-20-7].

Dihydrate [6138-23-4].

DEFINITION

Trehalose is a stable, nonreducing disaccharide with two glucose molecules linked in an α,α -1,1 configuration. It is obtained through enzymatic conversion of food-grade starch. It contains NLT 97.0% and NMT 102.0% of $C_{12}H_{22}O_{11}$, calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>

B.

Sample solution: 400 mg/mL of Trehalose

Analysis: Add 0.4 mL of a solution containing 1-naphthol in 95% alcohol (1 in 20) to 1 mL of the *Sample solution*. Gently add 2 mL of sulfuric acid to the solution.

Acceptance criteria: A violet color develops at the interface between the two solutions.

C.

Glycine solution: 40 mg/mL of Glycine

Sample solution: 40 mg/mL of Trehalose

Analysis: Add 1 mL of diluted hydrochloric acid to 2 mL of the *Sample solution*. Allow to stand for 20 min at room temperature. Add 4 mL of sodium hydroxide TS and 2 mL of the *Glycine solution* to the *Sample solution*. Heat the solution for 10 min in boiling water.

Acceptance criteria: A brown color does not develop.

ASSAY

PROCEDURE

Mobile phase: Water

Standard solution: 10 mg/mL of USP Trehalose RS, calculated on the anhydrous basis

Sample solution: 10 mg/mL of Trehalose, calculated on the anhydrous basis

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: LC
Detector: Refractive index
Column: 8-mm × 30-cm; packing L58
Temperature
Detector: 40°
Column: 80°
Flow rate: Adjust so that the retention time of trehalose is about 15 min.
Injection size: 20 µL
System suitability
Sample: *Standard solution*
Suitability requirements
Relative standard deviation: NMT 2.0%
Analysis
Samples: *Standard solution* and *Sample solution*
 Calculate the percentage of trehalose (C₁₂H₂₂O₁₁) in the portion of Trehalose taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of the *Sample solution*
 r_S = peak response of the *Standard solution*
 C_S = concentration of USP Trehalose RS in the *Standard solution* (mg/mL)
 C_U = concentration of Trehalose in the *Sample solution* (mg/mL)

Acceptance criteria: 97.0%–102.0% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.1%, determined on 2.0 g of Trehalose
- **HEAVY METALS, Method I** (231)
Sample: 4.0 g
Monitor preparation: Prepare with 2.5 mL of *Standard Lead Solution*.
Acceptance criteria: NMT 5 ppm
- **RELATED SUBSTANCES**
Mobile phase and Chromatographic system: Prepare as directed in the Assay.
Sample solution: 10 mg/mL of Trehalose
Standard solution: 0.1 mg/mL of *Sample solution*
System suitability solution: Dissolve 2.5 mL of *Sample solution*, 25 mg of maltotriose, and 25 mg of glucose, and dilute with water to 10.0 mL.
System suitability
Sample: *System suitability solution*
 [NOTE—The relative retention times for maltotriose, trehalose, and glucose are about 0.9, 1.0, and 1.2, respectively.]
Suitability requirements
Resolution: NLT 1.5 between trehalose and maltotriose
Relative standard deviation: NMT 2.0% for the trehalose peak

Analysis

Samples: *Sample solution* and *Standard solution*
 Determine the peak areas for all peaks.
Acceptance criteria: For the *Sample solution*, the areas of any peaks corresponding to maltotriose and other polysaccharides and eluting before trehalose are NMT half of the area of the peak corresponding to trehalose in the chromatogram of the *Standard solution* (0.5%). The areas of any peaks corresponding to glucose and eluting after trehalose are NMT half of the area of the peak corresponding to trehalose in the chromatogram of the *Standard solution* (0.5%).

SPECIFIC TESTS

- **COLOR AND CLARITY OF SOLUTION**
Sample solution: 33 g of Trehalose in 67 g of recently boiled water
Analysis: Using a suitable spectrophotometer (see *Spectrophotometry and Light-Scattering* (851)), measure the absorbances of the *Sample solution* at 420 and 720

nm in a 10-cm cuvette. The absorbance of the *Sample solution* at 720 nm is NMT 0.050.
 Determine the absorbance difference:

$$\text{Result} = A_{420} - A_{720}$$

A_{420} = absorbance of the *Sample solution* at 420 nm
 A_{720} = absorbance of the *Sample solution* at 720 nm
Acceptance criteria: The absorbance difference is NMT 0.100.

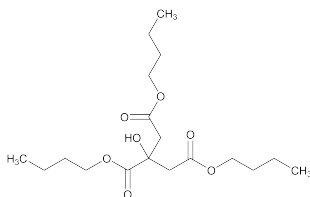
- **OPTICAL ROTATION, Specific Rotation** (781S): +197° to +201° at 20°
Sample solution: 100 mg/mL
- **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total aerobic microbial count is NMT 100 cfu/g, and the total combined molds and yeasts count is NMT 100 cfu/g. It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*.
- **pH** (791)
Sample solution: 100 mg/mL
Acceptance criteria: 4.5–6.5
- **WATER DETERMINATION, Method I** (921): For the anhydrous form, NMT 1.0%; for the dihydrate form, 9.0%–11.0%
Sample: 0.1 g
- **BACTERIAL ENDOTOXINS TEST** (85): If labeled for use in preparing parenteral dosage forms, it also meets the following requirements. The level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Trehalose is used can be met. Where the label states that Trehalose must be subjected to further processing during the preparation of injectable dosage forms, the level of bacterial endotoxins is such that the requirement in the relevant dosage form monograph(s) in which Trehalose is used can be met.
- **CHLORIDE AND SULFATE, Chloride** (221): A 2.0-g sample shows no more chloride than corresponds to 0.70 mL of 0.01 M hydrochloric acid (NMT 0.0125%).
- **CHLORIDE AND SULFATE, Sulfate** (221): A 2.0-g sample shows no more sulfate than corresponds to 0.83 mL of 0.005 M sulfuric acid (NMT 0.0200%).
- **NITROGEN CONTENT, Method I** (461)
Sample: 5.0 g
Analysis: Proceed as directed for *Method I*, increasing the sulfuric acid used for digestion to 30 mL and reducing the sodium hydroxide solution (2 in 5) to 45 mL.
Acceptance criteria: NMT 0.005%
- **SOLUBLE STARCH**
Sample solution: 10% Trehalose (w/v)
Analysis: Add several drops of iodine TS to the *Sample solution*.
Acceptance criteria: No blue color develops.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers. No storage requirements specified.
- **LABELING:** Where Trehalose is intended for use in the manufacture of injectable dosage forms, it is so labeled. Where Trehalose must be subjected to further processing during the preparation of injectable dosage forms to ensure acceptable levels of bacterial endotoxins, it is so labeled.
- **USP REFERENCE STANDARDS** (11)
 USP Endotoxin RS
 USP Glycerin RS
 USP Trehalose RS

Triacetin—see *Triacetin General Monographs*

Tributyl Citrate

C₁₈H₃₂O₇

360.44

DEFINITION

Tributyl Citrate contains NLT 99.0% of tributyl citrate (C₁₈H₃₂O₇), calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197F>
- **B.** The retention time of the major peak of the *Sample solution* corresponds to that of the *System suitability solution*, as obtained in the *Assay*.

ASSAY

• PROCEDURE

System suitability solution: 30 mg/mL each of USP Tributyl Citrate RS and USP Acetyltributyl Citrate RS in toluene

Sample solution: 30 mg/mL of Tributyl Citrate in toluene

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC, equipped with an on-column, temperature-programmable injector

Detector: Flame ionization

Column: 0.32-mm × 30-m, bonded with a 0.5-μm layer of phase G42

Temperatures

Injector: See *Table 1*.

Detector: 275°

Column: See *Table 2*.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
85	—	85	0.5
85	20	225	10

Table 2

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	—	80	0.5
80	20	220	10

Carrier gas: Helium

Flow rate: 2.3 mL/min

Injection volume: 1 μL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for tributyl citrate and acetyltributyl citrate are 0.9 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between tributyl citrate and acetyltributyl citrate

Relative standard deviation: NMT 2.0%, determined from both the tributyl citrate and acetyltributyl citrate peaks

Analysis

Sample: *Sample solution*

Calculate the percentage of tributyl citrate (C₁₈H₃₂O₇) in the portion of sample taken:

$$\text{Result} = (r_U/r_T) \times 100$$

r_U = peak area of tributyl citrate from the *Sample solution*

r_T = sum of all peaks excluding the solvent peak

Acceptance criteria: NLT 99.0% on the anhydrous basis

IMPURITIES

- **HEAVY METALS**, *Method II* <231>: NMT 10 μg/g

SPECIFIC TESTS

- **SPECIFIC GRAVITY** <841>: 1.037–1.045

- **REFRACTIVE INDEX** <831>: 1.443–1.445

• ACIDITY

Sample: 32.0 g

Analysis: Dissolve the *Sample* in 30 mL of isopropyl alcohol, previously neutralized to bromothymol blue. Add bromothymol blue TS, and titrate with 0.10 N sodium hydroxide to a faint blue endpoint.

Acceptance criteria: NMT 1.0 mL of 0.10 N sodium hydroxide is required.

- **WATER DETERMINATION**, *Method I* <921>: NMT 0.2%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

- **USP REFERENCE STANDARDS** <11>

USP Acetyltributyl Citrate RS

USP Tributyl Citrate RS

Trichloromonofluoromethane

CCl₃F

137.37

Methane, trichlorofluoro-

Trichlorofluoromethane [75-69-4].

DEFINITION

Trichloromonofluoromethane contains NLT 99.6% and NMT 100.0% of trichloromonofluoromethane (CCl₃F), calculated on the anhydrous basis.

IDENTIFICATION

- **A.** The IR absorption spectrum, determined in a 10-cm cell with sodium chloride windows, at atmospheric pressure, exhibits maxima, among others, at the following wavelengths, in μm: 4.67 (m), 5.95 (m), 7.28 (s), 8.06 (m), 9.2 (vs), 10.7 (vs), 11.8 (vs), and 13.4 (m). The stronger maxima are best obtained at pressures less than 10 mm of mercury.

ASSAY

• PROCEDURE

System suitability solution: Introduce a liquid-phase mixture of dichlorodifluoromethane, dichlorotetrafluoroethane, and trichloromonofluoromethane into an evacuated headspace vial.

Sample solution: Introduce the liquid phase of Trichloromonofluoromethane into an evacuated headspace vial.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)

Mode: GC
 Detector: Flame ionization
 Column: 2-mm × 1.8-m stainless steel; 1% phase G25 on support S12
 Temperatures
 Injection port: 110°
 Detector: 200°
 Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	10	170	5

Carrier gas: Helium

Flow rate: 20 mL/min

Headspace sampler: The bath temperature is 100°, the valve/loop temperature is 105°, and the sampling time is 3 s. Make adjustments as necessary to optimize peak areas to record trace-level impurities.

System suitability

Sample: Gas phase headspace of the *System suitability solution*

[NOTE—The relative retention times for dichlorodifluoromethane, dichlorotetrafluoroethane, and trichloromonofluoromethane are 0.5, 0.8, and 1.0, respectively.]

Suitability requirements

Resolution: NLT 2.0 between dichlorotetrafluoroethane and trichloromonofluoromethane

Analysis

Sample: Gas phase headspace of the *Sample solution*
 Calculate the percentage of trichloromonofluoromethane (CCl₃F) in the portion of Trichloromonofluoromethane taken.

Acceptance criteria: 99.6%–100.0% on the anhydrous basis

IMPURITIES

• INORGANIC CHLORIDES

Sample: 7 g

Analysis: Place 5 mL of anhydrous methanol in a test tube, add 3 drops of a saturated solution of silver nitrate in anhydrous methanol, shake, and add the *Sample*.

Acceptance criteria: No opalescence or turbidity is produced.

• CHROMATOGRAPHIC PURITY

Analysis: In the chromatogram from the *Assay*, identify the dichlorodifluoromethane and dichlorotetrafluoroethane peaks from relative retention times of those peaks in the chromatogram of the *System suitability solution*.

Acceptance criteria

Sum of the peak areas for dichlorodifluoromethane and dichlorotetrafluoroethane: NMT 0.2% of the total of all peak areas

Sum of the areas of all peaks other than that for trichloromonofluoromethane: NMT 0.4% of the total of all peak areas.

SPECIFIC TESTS

• BOILING TEMPERATURE

Analysis: Determine as directed in *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* (601), *Approximate Boiling Temperature*.

Acceptance criteria: Approximately 24°

• WATER

Analysis: Determine as directed in *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* (601), *Water Content, and Water Determination, Method Ic* (921).

Acceptance criteria: NMT 0.001%

• HIGH-BOILING RESIDUES

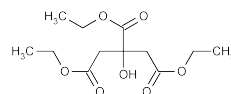
Analysis: Determine as directed in *Aerosols, Nasal Sprays, Metered-Dose Inhalers, and Dry Powder Inhalers* (601), *High-Boiling Residues, Method I*.

Acceptance criteria: NMT 0.01%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in tight cylinders, and avoid exposure to excessive heat.

Triethyl Citrate



C₁₂H₂₀O₇

276.28

DEFINITION

Triethyl Citrate contains NLT 99.0% and NMT 100.5% of C₁₂H₂₀O₇, calculated on the anhydrous basis.

IDENTIFICATION

• A. INFRARED ABSORPTION (197F)

• **B.** The retention time of the major peak of the *Sample solution* corresponds to that of a similar preparation of USP Triethyl Citrate RS, as obtained in the *Assay*.

ASSAY

• PROCEDURE

System suitability solution: 30 mg/mL each of USP Triethyl Citrate RS and USP Acetyltriethyl Citrate RS in toluene

Sample solution: 30 mg/mL of Triethyl Citrate in toluene

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.32-mm × 30-m; 0.5-μm layer of phase G42

Temperature

Injector: 225°

Detector: 275°

Column: See the temperature program table below.

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
80	—	80	0.5
80	20	220	20

Flow rate: 2.3 mL/min

Carrier gas: Helium

Injection type: Split, 30:1

Injection size: 1 μL

System suitability

Sample: *System suitability solution*

[NOTE—The relative retention times for triethyl citrate and acetyltriethyl citrate are 0.9 and 1.0, respectively.]

Suitability requirements

Resolution: NLT 1.5 between triethyl citrate and acetyltriethyl citrate

Relative standard deviation: NMT 2.0% (determined from both the triethyl citrate and acetyltriethyl citrate peaks, based on area percentage calculation)

Analysis**Sample:** *Sample solution*

[NOTE—Measure all of the peak areas, excluding the solvent peak.]

Calculate the percentage of $C_{12}H_{20}O_7$ in the portion of Triethyl Citrate taken:

$$\text{Result} = (r_U/r_T) \times 100$$

 r_U = peak area for triethyl citrate r_T = sum of the area responses of all the peaks**Acceptance criteria:** 99.0%–100.5% on the anhydrous basis**IMPURITIES****Inorganic Impurities**

- **HEAVY METALS**, *Method II* (231): NMT 10 ppm

SPECIFIC TESTS

- **SPECIFIC GRAVITY** (841): 1.135–1.139

- **REFRACTIVE INDEX** (831): 1.439–1.441

- **ACIDITY**

Neutralized isopropyl alcohol: To a suitable quantity of isopropyl alcohol add 2–3 drops of bromothymol blue TS and just sufficient 0.10 N sodium hydroxide dropwise to produce a faint blue color. [NOTE—Prepare *Neutralized isopropyl alcohol* just prior to use.]

Sample solution: 32.0 g of Triethyl Citrate in 30 mL of *Neutralized isopropyl alcohol*

Analysis: Add bromothymol blue TS. Titrate with 0.10 N sodium hydroxide to a faint blue endpoint.

Acceptance criteria: NMT 1.0 mL of 0.10 N sodium hydroxide is required.

- **WATER DETERMINATION**, *Method I* (921): NMT 0.25%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.

- **USP REFERENCE STANDARDS** (11)

USP Acetyltriethyl Citrate RS

USP Triethyl Citrate RS

Medium-Chain Triglycerides

Glycerides, mixed decanoyl and octanoyl; Caprylic and capric triglycerides.

DEFINITION

Medium-Chain Triglycerides consist of a mixture of triglycerides of saturated fatty acids, mainly of caprylic acid ($C_8H_{16}O_2$) and capric acid ($C_{10}H_{20}O_2$). The fatty acids are derived from the oil extracted from the hard, dried fraction of the endosperm of *Cocos nucifera* L. or from the dried endosperm of *Elaeis guineensis* Jacq. They contain NLT 95% of saturated fatty acids with 8 and 10 carbon atoms.

IDENTIFICATION

- **A.** Meet the requirements in *Specific Tests for Fats and Fixed Oils, Saponification Value* (401)
- **B.** Meet the requirements in *Specific Tests for Fats and Fixed Oils, Fatty Acid Composition* (401)

IMPURITIES

- **HEAVY METALS**, *Method II* (231)

[NOTE—Use this test for Medium-Chain Triglycerides intended for use other than in parenteral nutrition.]

Sample solution: Transfer 2.0 g of Medium-Chain Triglycerides to a quartz crucible. Add 0.5 g of magnesium oxide. Ignite the crucible to dull redness until a homogeneous white or grayish-white mass is obtained. Ignite at 800° for 1 h, cool, and dissolve the residue by adding two 5-mL portions of diluted hydrochloric acid. Add 0.1 mL of phenolphthalein TS and then ammonium hy-

droxide until a pink color is obtained. Cool, add glacial acetic acid until the solution is decolorized, then add 0.5 mL in excess, and dilute with water to 20.0 mL.

Standard solution: To 0.5 g of magnesium oxide add 2.0 mL of *Lead Standard Solution*, and evaporate to dryness at 105° for 1 h. Using the same conditions as prescribed for the *Sample solution*, ignite, dissolve in diluted hydrochloric acid, add ammonia and then acetic acid, and dilute with water to 20.0 mL.

Analysis: To 12 mL of the *Sample solution* add 2.0 mL of pH 3.5 *Acetate Buffer* and 1.2 mL of thioacetamide–glycerin base TS. To 10 mL of the *Standard solution* add 2.0 mL of the *Sample solution*, and add 2.0 mL of pH 3.5 *Acetate Buffer* and 1.2 mL of thioacetamide–glycerin base TS. Prepare a blank, using a mixture of 10 mL of water and 2.0 mL of the *Sample solution*. Compared to the blank, the *Standard solution* shows a light brown color. Dilute both the *Sample solution* and the *Standard solution* with water to 50 mL, allow to stand for 2 min, and view downward over a white surface.

Acceptance criteria: NMT 10 µg/g; any brown color of the *Sample solution* is not darker than that of the *Standard solution*.

- **LIMIT OF CHROMIUM**

[NOTE—Use this test for Medium-Chain Triglycerides intended for use in parenteral nutrition.]

Sample stock solution: 500 mg/mL of Medium-Chain Triglycerides in diisobutyl ketone

Sample solution: 200 mg/mL of Medium-Chain Triglycerides in diisobutyl ketone, from *Sample stock solution*

Chromium standard stock solution: 0.283 mg/mL of potassium dichromate in water, using potassium dichromate previously dried at 105° for 4 h

Chromium standard solution: Immediately before use, prepare 0.283 µg/mL of potassium dichromate in water, from the *Chromium standard stock solution*. This solution contains the equivalent of 0.1 µg/mL of chromium.

Standard solutions: Into each of three 10-mL volumetric flasks, transfer 4.0 mL of *Sample stock solution*, add 0.5, 1.0, and 2.0 mL, respectively, of *Chromium standard solution*, and dilute with diisobutyl ketone to volume. These solutions contain 0.005, 0.01, and 0.02 µg/mL of chromium.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a graphite furnace

Analytical wavelength: 357.8 nm

Lamp: Chromium hollow-cathode

Carrier gas: Argon

Analysis

Samples: *Standard solutions* and *Sample solution*
Determine the absorbances of the *Standard solutions* and the *Sample solution* in triplicate, and determine the average of the steady readings for each. Plot the average absorbances of the *Standard solutions* and the *Sample solution* versus the concentration of added chromium. Draw the straight line best fitting the points, and extrapolate the line until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of chromium in the *Sample solution*.

Acceptance criteria: NMT 0.05 µg/g

- **LIMIT OF COPPER**

[NOTE—Use this test for Medium-Chain Triglycerides intended for use in parenteral nutrition.]

Sample stock solution and Sample solution: Proceed as directed in the test for *Limit of Chromium*.

Copper standard stock solution: 0.393 mg/mL of cupric sulfate in water

Copper standard solution: Immediately before use, prepare 0.393 µg/mL of cupric sulfate in water, from the *Copper standard stock solution*. This solution contains the equivalent of 0.1 µg/mL of copper.

Standard solutions: Into each of three 10-mL volumetric flasks, transfer 4.0 mL of *Sample stock solution*. Add 1.0, 2.0, and 4.0 mL, respectively, of *Copper standard solution*, and dilute with diisobutyl ketone to volume. These solutions contain 0.01, 0.02, and 0.04 µg/mL of copper.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a graphite furnace

Analytical wavelength: 324.7 nm

Lamp: Copper hollow-cathode

Carrier gas: Argon

Analysis

Samples: *Standard solutions* and the *Sample solution*
Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution* in triplicate. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the concentration of added copper. Draw the straight line best fitting the points, and extrapolate the line until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of copper in the *Sample solution*.

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF LEAD**

[NOTE—Use this test for Medium-Chain Triglycerides intended for use in parenteral nutrition.]

Sample stock solution and Sample solution: Proceed as directed in the test for *Limit of Chromium*.

Lead standard stock solution: Dissolve 160 mg of lead nitrate in 100 mL of water that contains 1 mL of lead-free nitric acid, and dilute with water to 1000 mL. Pipet 10 mL of this solution into a 100-mL volumetric flask, and dilute with water to volume.

Lead standard solution: Immediately before use, prepare 0.16 µg/mL of lead nitrate from the *Lead standard stock solution*. This solution contains the equivalent of 0.1 µg/mL of lead.

Standard solutions: Into each of three 10-mL volumetric flasks, transfer 4.0 mL of *Sample stock solution*. Add 1.0, 2.0, and 4.0 mL, respectively, of *Lead standard solution*, and dilute with diisobutyl ketone to volume. These solutions contain 0.01, 0.02, and 0.04 µg/mL of lead.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a graphite furnace coated inside with palladium carbide

[NOTE—Calcination is carried out in the presence of oxygen at a temperature below 800°.]

Analytical wavelength: 283.3 nm

Lamp: Lead hollow-cathode

Carrier gas: Argon

Analysis

Samples: *Standard solutions* and the *Sample solution*
Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution* in triplicate. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the concentration of added lead. Draw the straight line best fitting the points, and extrapolate the line until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of lead in the *Sample solution*.

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF NICKEL**

[NOTE—Use this test for Medium-Chain Triglycerides intended for use in parenteral nutrition.]

Sample stock solution and Sample solution: Proceed as directed in the test for *Limit of Chromium*.

Nickel standard solution: Immediately before use, dilute 10 mL of nickel standard solution TS with water to 1000 mL. This solution contains the equivalent of 0.1 µg/g of nickel.

Standard solutions: Into each of three 10-mL volumetric flasks, transfer 4.0 mL of *Sample stock solution*. Add 1.0, 2.0, and 4.0 mL, respectively, of *Nickel standard solution*, and dilute with diisobutyl ketone to volume. These solutions contain 0.01, 0.02, and 0.04 µg/mL of nickel.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a graphite furnace

Analytical wavelength: 232 nm

Lamp: Nickel hollow cathode

Carrier gas: Argon

Analysis

Samples: *Standard solutions* and the *Sample solution*
Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution* in triplicate. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the concentration of added nickel. Draw the straight line best fitting the points, and extrapolate the line until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of nickel in the *Sample solution*.

Acceptance criteria: NMT 0.1 µg/g

• **LIMIT OF TIN**

[NOTE—Use this test for Medium-Chain Triglycerides intended for use in parenteral nutrition.]

Sample stock solution and Sample solution: Proceed as directed in the test for *Limit of Chromium*.

Tin standard stock solution: Dissolve 500 mg of metallic tin (Sn) in a mixture of 5 mL of water and 25 mL of hydrochloric acid, and dilute with water to 1000 mL.

Tin standard solution: Immediately before use, dilute 10 mL of *Tin standard stock solution* with dilute hydrochloric acid (2.5 in 100) to 1000 mL, and then dilute 10 mL of the solution with water to 500 mL. This solution contains the equivalent of 0.1 µg/g of tin.

Standard solutions: Into each of three 10-mL volumetric flasks, transfer 4.0 mL of *Sample stock solution*. Add 1.0, 2.0, and 4.0 mL, respectively, of *Tin standard solution*, and dilute with diisobutyl ketone to volume. These solutions contain 0.01, 0.02, and 0.04 µg/mL of tin.

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* (851).)

Mode: Atomic absorption spectrophotometer equipped with a graphite furnace coated inside with palladium carbide

Analytical wavelength: 286.3 nm

Lamp: Tin hollow-cathode

Carrier gas: Argon

Analysis

Samples: *Standard solutions* and the *Sample solution*
Record the average of the steady readings for each of the *Standard solutions* and the *Sample solution* in triplicate. Plot the absorbances of the *Standard solutions* and the *Sample solution* versus the concentration of added tin. Draw the straight line best fitting the points, and extrapolate the line until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of tin in the *Sample solution*.

Acceptance criteria: NMT 0.1 µg/g

• **ALKALINE IMPURITIES**

Sample solution: Dissolve 2.0 g of Medium-Chain Triglycerides in a mixture of alcohol and ethyl ether (1.5: 3.0).

Analysis: Add 0.05 mL of bromophenol blue TS to the *Sample solution*, and titrate with 0.01 N hydrochloric acid to a yellow endpoint.

Acceptance criteria: NMT 0.15 mL of 0.01 N hydrochloric acid is required.

SPECIFIC TESTS

- **FATS AND FIXED OILS, Unsaponifiable Matter** <401>
Sample: 5.0 g
Acceptance criteria: NMT 0.5%
- **SPECIFIC GRAVITY** <841>: 0.93–0.96 at 20°
- **WATER, Method I** <921>: NMT 0.2%
- **APPEARANCE**
Diluent: Hydrochloric acid and water (2.75%: 97.25%)
Sample: 10 mL
Standard solution: Prepare immediately before use by mixing 2.4 mL of ferric chloride CS and 0.6 mL of cobaltous chloride CS with *Diluent* to make 10.0 mL, and diluting 5.0 mL of the solution with *Diluent* to make 10.0 mL.
Analysis: Compare the *Sample* and the *Standard solution* by viewing them downward in matched color-comparison tubes against a white surface (see *Color and Achromicity* <631>).
Acceptance criteria: The *Sample* is clear and not more intensely colored than the *Standard solution*.
- **FATS AND FIXED OILS, Acid Value (Free Fatty Acids)** <401>: NMT 0.2
- **FATS AND FIXED OILS, Fatty Acid Composition** <401>: The fatty acid fraction of Medium-Chain Triglycerides exhibits the following composition as seen in *Table 1*. Disregard any peak with an area less than 0.05% of the total area.

Table 1

Carbon-Chain Length	Number of Double Bonds	Percentage (%)
6	0	≤2.0
8	0	50.0–80.0
10	0	20.0–50.0
12	0	≤3.0
14	0	≤1.0

- **FATS AND FIXED OILS, Hydroxyl Value** <401>: NMT 10
- **FATS AND FIXED OILS, Iodine Value** <401>: NMT 1.0
- **FATS AND FIXED OILS, Peroxide Value** <401>: NMT 1.0
- **FATS AND FIXED OILS, Saponification Value** <401>
Sample: 1.0 g
Acceptance criteria: 310–360
- **VISCOSITY—CAPILLARY VISCOMETER METHODS** <911>
Analysis: Determine at 20 ± 0.1° with a capillary viscometer.
Acceptance criteria: 25–33 centipoises
- **REFRACTIVE INDEX** <831>: 1.440–1.452 at 20°
- **ARTICLES OF BOTANICAL ORIGIN, Total Ash** <561>
Sample: 2.0 g
Acceptance criteria: NMT 0.1%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, protected from light. Store at temperatures not exceeding 25°.
- **LABELING:** Where it is intended for use in parenteral nutrition, it is so labeled.

Trolamine

C₆H₁₅NO₃ 149.19
Ethanol, 2,2',2''-nitrilotris-;
2,2',2''-Nitrilotriethanol [102-71-6].

DEFINITION

Trolamine is a mixture of alkanolamines consisting largely of triethanolamine [N(C₂H₄OH)₃] containing some diethanolamine [NH(C₂H₄OH)₂] and monoethanolamine [NH₂(C₂H₄OH)]. It contains NLT 99.0% and NMT 107.4%

of alkanolamines, calculated on the anhydrous basis as N(C₂H₄OH)₃.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197F>
- **B.**
Analysis 1: To 1 mL add 0.1 mL of cupric sulfate TS.
Acceptance criteria 1: A deep blue color is produced.
Analysis 2: Add 5 mL of 1 N sodium hydroxide, and concentrate to one-third of the original volume by boiling.
Acceptance criteria 2: The blue color remains.
- **C.**
Analysis: To 1 mL add 0.3 mL of cobaltous chloride TS.
Acceptance criteria: A carmine red color is produced.

ASSAY

- **PROCEDURE**
Sample: 2 g of Trolamine
Analysis: Transfer the *Sample* to a 300-mL conical flask. Add 75 mL of water and 2 drops of methyl red TS, and titrate with 1 N hydrochloric acid VS. Each mL of 1 N hydrochloric acid is equivalent to 149.2 mg of triethanolamine, expressed as N(C₂H₄OH)₃.
Acceptance criteria: 99.0%–107.4% on the anhydrous basis

IMPURITIES

- **RESIDUE ON IGNITION** <281>: NMT 0.05%

SPECIFIC TESTS

- **SPECIFIC GRAVITY** <841>: 1.120–1.128
- **REFRACTIVE INDEX** <831>: 1.481–1.486 at 20°
- **WATER DETERMINATION, Method I** <921>: NMT 0.5%, using a mixture of glacial acetic acid and methanol (1:4) as the solvent

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** <11>
USP Trolamine RS

Tromethamine—see *Tromethamine General Monographs*

Tyloxapol—see *Tyloxapol General Monographs*

Vanilla**DEFINITION**

Vanilla is the cured, full-grown, unripe fruit of *Vanilla planifolia* Andrews, often known in commerce as Mexican, Bourbon, or Madagascar vanilla, or of *Vanilla tahitensis* J. W. Moore, known in commerce as Tahitian vanilla (Fam. Orchidaceae). Vanilla yields NLT 12.0% of anhydrous, diluted alcohol-soluble extractive.

ASSAY

- **CONTENT OF ANHYDROUS, DILUTED ALCOHOL-SOLUBLE EXTRACTIVE**

Sample: 2 g of Vanilla

Analysis: Place the *Sample*, finely cut or coarsely powdered and accurately weighed, in a suitable flask. Add 70 mL of diluted alcohol, shake by mechanical means for 2 h or for 8 h at 30-min intervals, and allow to stand overnight. Decant the liquid into a filter, and

wash the flask and residue with small portions of diluted alcohol, passing the washings through the filter until the filtrate measures 100.0 mL. Mix the filtrate well, evaporate a 50.0-mL portion in a suitable tared container on a steam bath to dryness, and dry the residue at 105° for 4 h. The weight obtained represents the yield of anhydrous, diluted alcohol-soluble extractive from one-half of the portion of Vanilla taken. Calculate the yield for the entire *Sample* taken.

Acceptance criteria: NLT 12.0%

SPECIFIC TESTS

BOTANIC CHARACTERISTICS

Unground Vanilla

Macroscopic: Linear, flattened capsules of 12–35 cm in length and 5–9 mm in width, with an apex terminating in a flat, circular scar and a gradually tapering base that is more or less curved or hooked; or in Tahitian vanilla, broad in the middle and tapering toward either end, the base closely resembling the summit. It is flexible and tough, nearly black, dusky brown to moderate brown externally, longitudinally wrinkled, moist, glossy, and occasionally has efflorescence of acicular or prismatic crystals of vanillin. Internally it is unilocular, with a brownish-black pulp and numerous minute seeds. Occasional capsules are split near the summit into three parts.

Microscopic: The epidermis has a distinct cuticle and occasional stomata. The epidermal cells contain red to brown bodies and occasional prisms of calcium oxalate or crystals of vanillin. It has a collenchyma layer of one or two rows of cells, a thick sarcocarp composed of parenchyma and an interrupted circle of fibrovascular bundles, the latter leptocentric with a few vessels, and an outer circle of fibers with thin, strongly lignified walls and numerous transverse simple pits. The vessels with walls have slit-like pits or spiral thickenings; the parenchyma cells are usually thin-walled and deeply undulate, some thick-walled with oblique, slit-like pits or broad spiral bands, and contain occasional bundles of acicular crystals of calcium oxalate, up to 400 µm in length, or a thin protoplasmic layer enclosing numerous oil globules. It has an endocarp composed of placental and interplacental regions; the placental region consists of six bifid placentas extending into the cavity of the fruit and bears irregularly trianguloid, black to reddish, flattened seeds, up to about 250 µm in diameter, having a deeply reticulate seed coat; the interplacental regions show long, nearly straight hairs more or less matted together by their gummy, resinous secretion.

Powdered Vanilla

Macroscopic: Dusky brown to nearly black

Microscopic: The principal elements of identification are fragments of parenchyma of the sarcocarp with long, oblique, slit-like walls or broad spiral bands, calcium oxalate crystals of acicular outline and up to 400 µm in length, and monoclinic prisms up to 35 µm in length. It has numerous unicellular, nearly straight, glandular hairs, fragments of the seed coat with polygonal stone cells, and slender crystals of vanillin.

TEST FOR VANILLIN

Analysis: Place a few of the crystals, occurring as an efflorescence on the fruit, on a microslide or watch glass, and add 1 drop of phloroglucinol TS and 1 drop of hydrochloric acid.

Acceptance criteria: The solution immediately acquires a red color.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store in a cold place.
- **LABELING:** The label states the Latin binomial and, following the official name, the part of the plant contained in

the article. The commercial variety of Vanilla, whether Mexican, Bourbon, Madagascar, or Tahitian, is also stated on the label. The label states that Vanilla that has become brittle is not to be used.

Vanilla Tincture

DEFINITION

Prepare Vanilla Tincture as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* (795)).

Vanilla, cut into small pieces	100 g
Purified Water	200 mL
Alcohol	207 mL
Sucrose, in coarse granules	200 g
Diluted Alcohol, a sufficient quantity to make	1000 mL

Add *Purified Water* to the comminuted *Vanilla* in a suitable covered container, and macerate for 12 h, preferably in a warm place. Add *Alcohol* to the mixture, mix, and macerate for about 3 days. Transfer the mixture to a percolator containing *Sucrose*, and drain. Pack the drug firmly, and percolate slowly, using *Diluted Alcohol* as the menstruum.

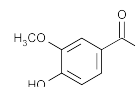
OTHER COMPONENTS

- **ALCOHOL DETERMINATION, Method I (611):** 38.0%–42.0%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Package in tight, light-resistant containers, and avoid exposure to direct sunlight and excessive heat.
- **LABELING:** The label states the Latin binomial name and, following the official name, the part of the plant source from which the article was derived.

Vanillin



C₈H₈O₃
Benzaldehyde, 4-hydroxy-3-methoxy-;
Vanillin [121-33-5].

152.15

DEFINITION

Vanillin contains NLT 97.0% and NMT 103.0% of vanillin (C₈H₈O₃), calculated on the dried basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION (197K)**
- **B. ULTRAVIOLET ABSORPTION (197U)**
Sample solution: 8 µg/mL in methanol
Acceptance criteria: Meets the requirements

ASSAY

- **PROCEDURE**
Standard solution: 8 µg/mL of USP Vanillin RS in methanol
Sample solution: 8 µg/mL of Vanillin in methanol
Blank: Methanol
Instrumental conditions
(See *Spectrophotometry and Light-Scattering* (851).)

Mode: UV

Analytical wavelength: 308 nm

Cell: 1 cm

Analysis

Samples: *Standard solution*, *Sample solution*, and *Blank*
Calculate the percentage of vanillin ($C_8H_8O_3$) in the portion of Vanillin taken:

$$\text{Result} = (A_U/A_S) \times (C_S/C_U) \times 100$$

A_U = absorbance of the *Sample solution*

A_S = absorbance of the *Standard solution*

C_S = concentration of USP Vanillin RS in the *Standard solution* ($\mu\text{g/mL}$)

C_U = concentration of the *Sample solution* ($\mu\text{g/mL}$)

Acceptance criteria: 97.0%–103.0% on the dried basis

IMPURITIES

- **RESIDUE ON IGNITION** (281): NMT 0.05%

SPECIFIC TESTS

- **MELTING RANGE OR TEMPERATURE** (741): 81°–83°
- **LOSS ON DRYING** (731): Dry the sample over silica gel for 4 h: it loses NMT 1.0% of its weight.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers.
- **USP REFERENCE STANDARDS** (11)
USP Vanillin RS

Hydrogenated Vegetable Oil

DEFINITION

Hydrogenated Vegetable Oil is a mixture of triglycerides of fatty acids. The melting range, heavy metals limit, iodine value, and saponification value differ, depending on Type, as described in the table below.

	Type I	Type II
Melting Range or Temperature (741), <i>Class II</i>	57°–85°	20°–50°
Heavy Metals (231), <i>Method II</i>	0.001%	0.001%
Fats and Fixed Oils (401), <i>Iodine Value, Method II</i>	0–5	55–80
Fats and Fixed Oils (401), <i>Saponification Value</i>	175–200	175–200

SPECIFIC TESTS

- **LOSS ON DRYING** (731)
Analysis: Dry a sample at 105° for 4 h.
Acceptance criteria: NMT 0.1%
- **FATS AND FIXED OILS, Acid Value** (401)
Sample: 20 g in a conical flask
Analysis: Melt the *Sample* on a steam bath, add 100 mL of hot alcohol previously neutralized with 0.1 N sodium hydroxide to phenolphthalein TS, swirl, and add 1 mL of phenolphthalein TS. Titrate with 0.10 N sodium hydroxide until the solution remains faintly pink after being shaken for 15 s.
Acceptance criteria: NMT 4.0
- **FATS AND FIXED OILS, Unsaponifiable Matter** (401): NMT 0.8%

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, in a cool place.
- **LABELING:** Label it to state whether it is Type I or Type II.

Vitamin E Polyethylene Glycol Succinate

DEFINITION

Vitamin E Polyethylene Glycol Succinate is a mixture formed by the esterification of *d*-alpha tocopheryl acid succinate and polyethylene glycol. The ester mixture consists primarily of mono-esterified polyethylene glycol and a small amount of di-esterified polyethylene glycol. It contains NLT 25.0% of *d*-alpha tocopherol ($C_{29}H_{50}O_2$).

IDENTIFICATION

• A. GAS CHROMATOGRAPHIC IDENTIFICATION TEST

Analysis: Proceed as directed in the test for *Content of Alpha Tocopherol*.

Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*.

COMPOSITION

• CONTENT OF ALPHA TOCOPHEROL

Solvent: 0.25 mL of phenolphthalein TS in 1 L of alcohol

Internal standard solution: 12 mg/mL of ethyl arachidate in isooctane

Standard solution: Transfer 32.5 mg of USP Alpha Tocopherol RS to a suitable reaction flask. Add 2 mL of pyridine and 0.5 mL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane, and heat the flask at 100° for 10 min. Cool the flask, add 5.0 mL of *Internal standard solution* followed by 20 mL of isooctane, and shake.

Sample solution: Transfer a quantity equivalent to 0.100–0.160 g of Vitamin E Polyethylene Glycol Succinate molten at 60° to a culture tube (about 20 cm long and 2.5 cm in diameter) equipped with a screw cap. Add 40–50 mg of ascorbic acid and a few boiling chips, followed by 20 mL of *Solvent*. [NOTE—Reflux the solution gently without emission of contents.] Place the tube in a heating block set at 100°–150°. When the sample is fully dissolved, add 0.25 g of potassium hydroxide, and continue to reflux for 30 min. Remove the tube from heat, and while contents are still hot, add 1–2 mL of hydrochloric acid dropwise until the pink coloration disappears. [CAUTION—Exothermic reaction. Allow the acid to trickle down the inside of the tube to prevent splashing.] Cool the tube, then wash the sides of the tube with 20 mL of water. Add 5.0 mL of *Internal standard solution*, cap, and shake to ensure thorough mixing. Allow the tube to stand until two distinct layers are formed. Transfer 2.5–3.5 mL of the upper layer into a suitable reaction flask, and add 2.0 mL of pyridine followed by 2.5 mL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane. Heat the flask at 100° for 10 min. Cool, and then add 12 mL of isooctane.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 0.25-mm × 15-m fused-silica capillary; coated with a 0.25- μm film of phase G27

Temperature

Injector: 280°

Detector: 345°

Column: See Table 1.

Table 1

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
260	20	340	1

Carrier gas: Helium
Flow rate: 1.5 mL/min
Injection size: 1 μ L
Injection type: Split ratio, 200:1

System suitability

Sample: *Standard solution*

Suitability requirements

Tailing factor: NMT 2.0 for the alpha tocopherol peak

Relative standard deviation: NMT 2.0% for the ratio of the alpha tocopherol peak area to the internal standard peak area

Analysis

Samples: *Standard solution* and *Sample Solution*

Calculate the percentage of *d*-alpha tocopherol ($C_{29}H_{50}O_2$) in the portion of Vitamin E Polyethylene Glycol Succinate taken:

$$\text{Result} = (R_U/R_S) \times (W_S/W_U) \times 100$$

R_U = internal standard ratio (peak area of alpha tocopherol/peak area of the internal standard) from the *Sample solution*

R_S = internal standard ratio (peak area of alpha tocopherol/peak area of the internal standard) from the *Standard solution*

W_S = weight of USP Alpha Tocopherol RS used to prepare the *Standard solution* (mg)

W_U = weight of Vitamin E Polyethylene Glycol Succinate taken to prepare the *Sample solution* (mg)

Acceptance criteria: NLT 25.0%

SPECIFIC TESTS

• **OPTICAL ROTATION, Specific Rotation (781S)**

[NOTE—This test identifies *d*-alpha tocopherol after saponification.]

Sample solution: Transfer 0.9 g of Vitamin E Polyethylene Glycol Succinate, molten at 60°, to a suitable test tube fitted with a cap, and dissolve in 10.0 mL of alcohol. Place the tube in a heating block set at 100°–105°. [NOTE—Reflux the solution gently without emission of contents.] When the sample is fully dissolved, add 2–3 pellets of sodium hydroxide, and continue to reflux for an additional 30 min. Remove the tube from the heat, and while contents are still hot, neutralize using phenolphthalein as the indicator by slowly adding 10 mL of a mixture of water and hydrochloric acid (1:1) until the pink color disappears.

[**CAUTION**—Exothermic reaction. Allow the acid solution to trickle down the inside of the tube to prevent splashing.] Cool the tube, cap, and shake until contents are well mixed. Add 25.0 mL of heptane, cap, and shake for 1 min to ensure thorough mixing. Allow the tube to stand until two distinct layers are formed.

Transfer the top layer to a clean, dry culture tube, then add 10.0 mL of water to the recovered solution. Cap, shake, and allow the layers to separate. Transfer the upper layer to a clean, dry tube. Add 10.0 mL of potassium ferricyanide solution, prepared by dissolving 2 g of potassium ferricyanide in 10.0 mL of 0.2 M sodium hydroxide, and replace the cap. Shake vigorously for 45 s, and allow the layers to separate for 30 min. If the top heptane layer is clear, proceed with the measurement for specific rotation; if not clear, dry over anhydrous sodium sulfate before proceeding with the test. [NOTE—Use the results of the test for *Content of Alpha Tocopherol* to calculate the specific rotation.]

Acceptance criteria: NLT +24.0°

• **SOLUBILITY IN WATER**

Sample: 20 g of melted Vitamin E Polyethylene Glycol Succinate

Analysis: Place the *Sample* in a glass container on a magnetic stirrer. Immediately add 80 mL of boiling

water while stirring. Allow to cool to room temperature with constant stirring.

Acceptance criteria: The solution becomes clear within 3 h.

• **ACID VALUE**

Sample: 1 g of Vitamin E Polyethylene Glycol Succinate

Analysis: Dissolve the *Sample* in 25 mL of a mixture of alcohol and ether (1:1) that has been neutralized to phenolphthalein with 0.1 N sodium hydroxide. Add 0.5 mL of phenolphthalein TS, and titrate with 0.10 N sodium hydroxide until the solution remains faintly pink after shaking for 30 s.

Acceptance criteria: NMT 0.027 mEq/g, equivalent to NMT 0.27 mL of 0.10 N sodium hydroxide

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers, and store protected from light.
- **LABELING:** The labeling indicates the *d*-alpha tocopherol content, expressed in mg/g.
- **USP REFERENCE STANDARDS** (11)
USP Alpha Tocopherol RS

Water for Injection—see *Water for Injection General Monographs*

Water for Injection, Sterile—see *Sterile Water for Injection General Monographs*

Water for Irrigation, Sterile—see *Sterile Water for Irrigation General Monographs*

Water, Purified—see *Purified Water General Monographs*

Carnauba Wax

DEFINITION

Carnauba Wax is obtained from the leaves of *Copernicia cerifera* Mart. (Fam. Palmae).

IMPURITIES

• **RESIDUE ON IGNITION** (281)

Sample: 2 g

Analysis: Heat the *Sample* in an open porcelain or platinum dish over a flame: it volatilizes without emitting an acrid odor. Ignite.

Acceptance criteria: The weight of the residue is NMT 5 mg, corresponding to NMT 0.25%.

• **HEAVY METALS, Method II** (231): 20 ppm

SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE, Class II** (741): 80°–86°

• **ACID VALUE**

Sample: 3 g

Analysis: Weigh the *Sample* into a 250-mL flask attached to a reflux condenser. Add 50 mL of a mixture of isopropyl alcohol and toluene (5:4), and boil gently until the wax is completely dissolved. Remove the flask from the condenser, add 1 mL of phenolphthalein TS, and immediately titrate with 0.5 N alcoholic potassium

hydroxide VS to a faint, reddish-yellow color. [NOTE—Do not allow the solution to cool. Titrate at warm temperature after refluxing.]

Calculate the acid value as the number of mg of potassium hydroxide required to neutralize the free acids in 1 g of Carnuba Wax.

Acceptance criteria: 2–7

• **FATS AND FIXED OILS, Saponification Value (401)**

Sample: Use the solution from the test for *Acid Value*.

Analysis: To the *Sample* add 15.0 mL of 0.5 N alcoholic potassium hydroxide VS, reflux for 3 h, and titrate the excess alkali with 0.5 N hydrochloric acid VS to a yellow-amber color. Perform a blank determination (see *Titrimetry* (541), *Residual Titrations*).

Calculate the ester value. The saponification value is the sum of the ester value and the acid value.

Acceptance criteria: 78–95

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Emulsifying Wax

DEFINITION

Emulsifying Wax is a waxy solid prepared from Cetostearyl Alcohol containing a polyoxyethylene derivative of a fatty acid ester of sorbitan.

SPECIFIC TESTS

• **MELTING RANGE OR TEMPERATURE (741)**

Sample: Emulsifying Wax

Analysis: Melt a quantity of the *Sample* slowly, while stirring, until it reaches a temperature of 90°–92°. Remove the source of the heat, and allow the molten substance to cool to a temperature of 8°–10° above the expected melting point. Chill the bulb of a suitable thermometer (see *Thermometers* (21)) to 5°, wipe it dry, and while it is still cold, dip it into the molten substance so that the bulb is completely covered. Withdraw it immediately, and hold it vertically away from the heat until the surface dulls. Fix the thermometer securely in a test tube so that the lower point is 15 mm from the bottom of the test tube. Place the test tube in a water bath at 10°–15°, and allow it to remain at that temperature for 30 min. Raise the temperature of the bath at the rate of 2°/min to 30°, then change to a rate of 1°/min, and note the temperature at which the first drop of melted substance leaves the thermometer. Repeat the determination twice on a freshly melted portion of the sample substance. If the variation of three determinations is less than 1°, take the average of the three as the melting point. Otherwise, make two additional determinations, and take the average of the five.

Acceptance criteria: 50°–54°

• **FATS AND FIXED OILS, Hydroxyl Value (401):** 178–192

• **FATS AND FIXED OILS, Iodine Value (401):** NMT 3.5

• **FATS AND FIXED OILS, Saponification Value (401):** NMT 14

• **pH (791)**

Sample dispersion: Heat 3 g of Emulsifying Wax in 100 mL of water to 55°, with stirring, followed by cooling to 25°.

Acceptance criteria: 5.5–7.0

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Microcrystalline Wax

DEFINITION

Microcrystalline Wax is a mixture of straight-chain, branched-chain, and cyclic hydrocarbons, obtained by solvent fractionation of the still bottom fraction of petroleum by suitable dewaxing or deoiling means.

IMPURITIES

• **RESIDUE ON IGNITION (281)**

Sample: 2 g

Analysis: Heat the *Sample* in an open porcelain or platinum dish over a flame.

Acceptance criteria: It volatilizes without emitting an acrid odor and on ignition yields NMT 0.1%.

SPECIFIC TESTS

• **COLOR**

Standard solution: Mix 3.8 mL of ferric chloride CS and 1.2 mL of cobaltous chloride CS in a clear-glass, 16- × 150-mm bacteriological test tube.

Sample solution: Melt 10 g of Microcrystalline Wax on a steam bath, and pour 5 mL of the liquid into a clear-glass, 16- × 150-mm bacteriological test tube.

Analysis: Visually compare the contents of both tubes in reflected light against a white background, holding the tubes directly against the background at such an angle that there is no fluorescence.

Acceptance criteria: The *Sample solution* is not darker than the *Standard solution*.

• **MELTING RANGE OR TEMPERATURE, Class III (741):** 54°–102°

• **CONSISTENCY**

Sample: Microcrystalline Wax

Apparatus: Determine the consistency of the *Sample* by means of a penetrometer fitted with a polished metal needle weighing 2.5 ± 0.05 g and having a truncated symmetric tapered angle of $9^{\circ}0' \pm 15'$. The needle is tapered, with a length of 25.4 mm, and the shaft attached to the needle is 58 mm in length and 3.17 mm in diameter. The plunger that fits into the penetrometer and guides the path of the needle weighs 47.5 ± 0.05 g. An additional weight of 50 ± 0.05 g is added to the top of the plunger to give a total load of 100 g.

Analysis: The *Sample* is cast in a brass cylinder open at both ends. The cylinder has an inside diameter of 25.4 mm and is 31.8 mm in height. Place the cylinder on a brass plate wetted with an equal volume mixture of glycerin and water, and place the plate on two corks. Pour the wax, melted at approximately 17° above its congealing point, into the cylinder. Continue pouring the wax until a convex meniscus is formed above the cylinder. Allow the specimen to cool for 1 h at approximately 24°. Shave excess wax from the top of the cylinder, and remove the plate. With the smooth wax surface in the up position, condition the specimen in a water bath at 25° for 1 h.

Arrange the penetrometer so that the wax specimen is completely immersed in the water bath while penetration is run. Lower the needle until the tip just touches the top surface of the specimen. Release the needle for 5 s, and read the depth of penetration in tenths of millimeters. Perform four determinations, and calculate the average value of the four readings.

Acceptance criteria: 3–100 (0.3–10.0 mm)

• **ORGANIC ACIDS**

Sample solution: 20 g of Microcrystalline Wax in 100 mL of a mixture of neutralized alcohol and water (1:2). Agitate thoroughly, and heat to boiling.

Analysis: To the *Sample solution* add 1 mL of phenolphthalein TS, and titrate rapidly with 0.1 N sodium hydroxide VS, with vigorous agitation, to a sharp pink endpoint in the alcohol-water layer.

Acceptance criteria: NMT 0.4 mL of 0.1 N sodium hydroxide is required.

- **FIXED OILS, FATS, AND ROSIN**

Sample: 10 g

Sample solution: Digest the *Sample* with 50 mL of sodium hydroxide solution (1 in 5) at 100° for 30 min. Separate the water layer, and acidify it with 2 N sulfuric acid.

Acceptance criteria: No oily or solid matter separates.

- **ALKALINITY**

Sample: 35 g

Analysis: Introduce the *Sample* into a 250-mL separator, add 100 mL of boiling water, and shake vigorously for 5 min. Draw off the separated water into a casserole, wash further with two 50-mL portions of boiling water, and add the washings to the casserole. To the pooled washings add 1 drop of phenolphthalein TS, and boil.

Acceptance criteria: The solution does not acquire a pink color.

- **ACIDITY**

Analysis: If the addition of phenolphthalein TS in the test for *Alkalinity* produces no pink color, add 0.1 mL of methyl orange TS.

Acceptance criteria: No red or pink color is produced.

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight containers.
- **LABELING:** Label it to indicate the name and proportion of any added stabilizer.

White Wax

DEFINITION

White Wax is the product of bleaching and purifying Yellow Wax that is obtained from the honeycomb of the bee [*Apis mellifera* L. (Fam. Apidae)] and that meets the requirements of the *Saponification Cloud Test*.

SPECIFIC TESTS

- **SAPONIFICATION CLOUD TEST**

Sample: 3.00 g

Alcoholic potassium hydroxide: Dissolve 40 g of potassium hydroxide in about 900 mL of aldehyde-free alcohol maintained at a temperature not exceeding 15°, and then when solution is complete, warm to room temperature, and add aldehyde-free alcohol to make 1000 mL.

Analysis: Place the *Sample* in a 100-mL round-bottom boiling flask fitted with a ground-glass joint. Add 30 mL of *Alcoholic potassium hydroxide*. Reflux the mixture gently for 2 h. At the end of this period, open the flask, insert a thermometer into the solution, and place the flask in a container of water at a temperature of 80°. Rotate the flask in the bath while both the bath and the solution cool.

Acceptance criteria: The solution shows no cloudiness or globule formation before the temperature reaches 65°.

- **MELTING RANGE OR TEMPERATURE, Class II (741):** 62°–65°

- **FATS OR FATTY ACIDS, JAPAN WAX, ROSIN, and SOAP**

Sample: 1 g

Analysis 1: Boil the *Sample* for 30 min with 35 mL of 3.5 N sodium hydroxide contained in a 100-mL beaker, maintaining the volume of solution by the occasional addition of water, and allow the mixture to cool at room temperature for about 2 h.

Acceptance criteria 1: The wax separates, leaving the liquid clear, turbid, or translucent, but not opaque.

Analysis 2: Filter the cool mixture obtained in *Analysis 1*, and acidify the clear filtrate with hydrochloric acid.

Acceptance criteria 2: The liquid remains clear or shows NMT a slight amount of turbidity or precipitate.

- **FATS AND FIXED OILS, Acid Value (401)**

Sample: 3 g

Analysis: Warm the *Sample* in a 200-mL flask with 25 mL of neutralized dehydrated alcohol until melted, then shake the mixture. Add 1 mL of phenolphthalein TS, and titrate the warm liquid with 0.5 N alcoholic potassium hydroxide VS to produce a permanent, faint pink color. Calculate the acid value as directed in the chapter.

Acceptance criteria: 17–24

- **FATS AND FIXED OILS, Ester Value (401)**

Sample solution: The solution resulting from the determination of *Acid Value*

Analysis: To the *Sample solution* add 25.0 mL of 0.5 N alcoholic potassium hydroxide VS and 50 mL of aldehyde-free alcohol, and reflux the mixture for 4 h. Titrate the excess alkali with 0.5 N hydrochloric acid VS. Perform a blank determination (see *Titrimetry (541)*, *Residual Titrations*). Calculate the ester value as directed in the chapter.

Acceptance criteria: 72–79

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Yellow Wax

DEFINITION

Yellow Wax is the purified wax from the honeycomb of the bee [*Apis mellifera* L. (Fam. Apidae)]. The crude beeswax used to prepare Yellow Wax conforms to the *Saponification Cloud Test*.

SPECIFIC TESTS

- **SAPONIFICATION CLOUD TEST**

Sample: 3.00 g

Alcoholic potassium hydroxide: Dissolve 40 g of potassium hydroxide in about 900 mL of aldehyde-free alcohol maintained at a temperature not exceeding 15°, and then when solution is complete, warm to room temperature, and add aldehyde-free alcohol to make 1000 mL.

Analysis: Place the *Sample* in a 100-mL round-bottom boiling flask fitted with a ground-glass joint. Add 30 mL of *Alcoholic potassium hydroxide*. Reflux the mixture gently for 2 h. At the end of this period, open the flask, insert a thermometer into the solution, and place the flask in a container of water at a temperature of 80°. Rotate the flask in the bath while both the bath and the solution cool.

Acceptance criteria: The solution shows no cloudiness or globule formation before the temperature reaches 65°.

- **MELTING RANGE OR TEMPERATURE, Class II (741):** 62°–65°

- **FATS OR FATTY ACIDS, JAPAN WAX, ROSIN, and SOAP**

Sample: 1 g

Analysis 1: Boil the *Sample* for 30 min with 35 mL of 3.5 N sodium hydroxide contained in a 100-mL beaker, maintaining the volume of solution by the occasional addition of water, and allow the mixture to cool at room temperature for about 2 h.

Acceptance criteria 1: The wax separates, leaving the liquid clear, turbid, or translucent, but not opaque.

Analysis 2: Filter the cool mixture obtained in *Analysis 1*, and acidify the clear filtrate with hydrochloric acid.

Acceptance criteria 2: The liquid remains clear or shows NMT a slight amount of turbidity or precipitate.

- **FATS AND FIXED OILS, Acid Value (Free Fatty Acids) (401)**

Sample: 3 g

Analysis: Warm the *Sample* in a 200-mL flask with 25 mL of neutralized dehydrated alcohol until melted,

then shake the mixture. Add 1 mL of phenolphthalein TS, and titrate the warm liquid with 0.5 N alcoholic potassium hydroxide VS to produce a permanent, faint pink color. Calculate the *Acid Value* as directed in the chapter.

Acceptance criteria: 17–24

• **FATS AND FIXED OILS, *Ester Value* (401)**

Sample solution: The solution resulting from the determination of *Acid Value*

Analysis: To the *Sample solution* add 25.0 mL of 0.5 N alcoholic potassium hydroxide VS and 50 mL of aldehyde-free alcohol, and reflux the mixture for 4 h. Titrate the excess alkali with 0.5 N hydrochloric acid VS. Perform a blank determination (see *Titrimetry* (541), *Residual Titrations*). Calculate the *Ester Value* as directed in the chapter.

Acceptance criteria: 72–79

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Xanthan Gum

DEFINITION

Xanthan Gum is a high molecular weight polysaccharide gum produced by a pure-culture fermentation of a carbohydrate with *Xanthomonas campestris*, then purified by recovery with Isopropyl Alcohol, dried, and milled. It contains D-glucose and D-mannose as the dominant hexose units, along with D-glucuronic acid, and is prepared as the sodium, potassium, or calcium salt. It yields NLT 4.2% and NMT 5.0% of carbon dioxide, calculated on the dried basis, corresponding to NLT 91.0% and NMT 108.0% of Xanthan Gum.

IDENTIFICATION

• **A.**

Sample: Prepare a dry blend of 1.5 g of Xanthan Gum and 1.5 g of locust bean gum.

Control: 3.0 g of Xanthan Gum

Analysis

Samples: *Sample* and *Control*

To two separate 400-mL beakers add 300 mL of water, and heat to 80°. Stir rapidly by mechanical means. Add the *Sample* to one of the beakers and the *Control* to the other beaker at the point of maximum agitation. Stir until the mixtures dissolve, and then continue stirring for 30 min longer. Do not allow the temperature of the mixtures to drop below 60° during the stirring. Discontinue stirring, and allow the mixtures to cool at room temperature for NLT 2 h.

Acceptance criteria: A firm, rubbery gel forms with the *Sample* after the temperature drops below 40°, but no such gel forms with the *Control*.

ASSAY

• **PROCEDURE**

Sample: 1.2 g

Analysis: Proceed as directed in *Alginates Assay* (311).

Acceptance criteria: 4.2%–5.0% of carbon dioxide on the dried basis, corresponding to 91.0%–108.0% of Xanthan Gum

IMPURITIES

- **ARSENIC, *Method II* (211):** NMT 3 µg/g

- **LEAD (251)**

Sample: Prepare a *Test Preparation* as directed in the chapter

Control: Use 5 mL of *Diluted Standard Lead Solution* (5 µg of Pb).

Analysis: Proceed as directed in the chapter.

Acceptance criteria: NMT 5 µg/g

- **HEAVY METALS, *Method II* (231):** NMT 30 µg/g. Use a platinum crucible for the ignition.

- **LIMIT OF ISOPROPYL ALCOHOL**

Internal standard solution: 1 mg/mL of tertiary butyl alcohol

Standard stock solution: 1 mg/mL of isopropyl alcohol

Standard solution: Pipet 4 mL of the *Standard stock solution* and 4 mL of the *Internal standard solution* into a 100-mL volumetric flask, and dilute with water to volume.

Sample solution: Disperse 1 mL of a suitable antifoam emulsion in 200 mL of water contained in a 1000-mL, round-bottom distilling flask having a 24/40 standard taper ground joint. Add 5 g of Xanthan Gum, and shake for 1 h on a wrist-action mechanical shaker. Connect the flask to a fractionating column, and distill 100 mL, adjusting the heat so that foam does not enter the column. Add by pipet 4 mL of the *Internal standard solution*.

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: GC

Detector: Flame ionization

Column: 3.2-mm × 1.8-m stainless steel column packed with 80- to 100-mesh surface-silanized packing S3, or equivalent

Temperatures

Column: 165°

Detector: 200°

Injection port: 200°

Carrier gas: Helium

Injection volume: 4–5 µL

Analysis

Samples: *Standard solution* and *Sample solution*

[NOTE—The retention time of tertiary butyl alcohol is 1.5 relative to that of isopropyl alcohol.]

Calculate the percentage of isopropyl alcohol in the portion of Xanthan Gum taken:

$$\text{Result} = (R_U/R_S) \times (C_S/C_U) \times 100$$

R_U = peak response ratio of isopropyl alcohol to tertiary butyl alcohol from the *Sample solution*

R_S = peak response ratio of isopropyl alcohol to tertiary butyl alcohol from the *Standard solution*

C_S = concentration of isopropyl alcohol in the *Standard solution* (mg/mL)

C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: NMT 0.075%

- **PYRUVIC ACID**

Solution A: 5 mg/mL of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid

Standard stock solution: 90 µg/mL of pyruvic acid

Standard solution: Transfer 10.0 mL of the *Standard stock solution* to a glass-stoppered, 50-mL flask. Add 20.0 mL of 1 N hydrochloric acid, weigh the flask, and reflux for 3 h, taking precautions to prevent loss of vapors. Cool, and add water to make up for any weight loss during refluxing. Transfer 2.0 mL of this solution to a 30-mL separator containing 1.0 mL of *Solution A*. Mix, and allow to stand for 5 min. Extract the mixture with 5 mL of ethyl acetate, and discard the aqueous layer. Extract the hydrazone from the ethyl acetate with three 5-mL portions of sodium carbonate TS, collect the extracts in a 50-mL volumetric flask, and dilute with sodium carbonate TS to volume.

Sample stock solution: 6 mg/mL of Xanthan Gum

Sample solution: Transfer 10.0 mL of the *Sample stock solution* to a glass-stoppered, 50-mL flask. Proceed as directed in the *Standard solution*, beginning with "Add 20.0 mL of 1 N hydrochloric acid..."

Blank: Sodium carbonate TS

Instrumental conditions

(See *Spectrophotometry and Light-Scattering* <851>.)

Mode: Spectrophotometry

Analytical wavelength: 375 nm

Cell: 1 cm

Analysis

Samples: Standard solution, Sample solution, and Blank

Acceptance criteria: The absorbance of the Sample solution is NLT that of the Standard solution, corresponding to NLT 1.5% of pyruvic acid.

SPECIFIC TESTS

- **MICROBIAL ENUMERATION TESTS** <61> and **TESTS FOR SPECIFIED MICROORGANISMS** <62>: It meets the requirements of the tests for *Salmonella* species and *Escherichia coli*.

- **LOSS ON DRYING** <731>

Analysis: Dry at 105° for 2.5 h.

Acceptance criteria: NMT 15.0%

- **ASH**

Sample: Weigh 3 g in a tared crucible.

Analysis: Incinerate the Sample at 650° until free from carbon. Cool the crucible and its contents in a desiccator, and weigh.

Acceptance criteria: The weight of the ash is between 6.5%–16.0%, calculated on the dried basis.

- **ROTATIONAL RHEOMETER METHODS** <912>

Sample: Prepare a dry blend of 3.0 g of Xanthan Gum and 3.0 g of potassium chloride.

Instrumental conditions

Instrument: Rotational viscometer

Spindle cylinder dimensions

Diameter: 1.27 cm

Height: 0.16 cm

Shaft diameter: 0.32 cm

Distance from top of cylinder to lower lip of shaft: 2.54 cm

Immersion depth: 5.00 cm (No. 3 spindle)

Spindle rotation speed: 60 rpm

Analysis: To a 400-mL beaker add 250 mL of water. Add the Sample slowly while stirring at 800 rpm, using a low-pitched, propeller-type stirrer. Add 44 mL of water, rinsing the walls of the beaker. Approximately 10 min after the addition of the Sample to the water, remove the beaker from the propeller-type stirrer, and vigorously stir the solution by hand to ensure that all the particles around the edge of the beaker are in solution. Return the beaker to the stirrer, and agitate at 800 rpm for a total mixing time of 2 h. Adjust the temperature to $24 \pm 1^\circ$, and stir by hand in a vertical motion to eliminate any thixotropic effects or layering. Each hand mixing should be NMT 15–30 s, and the last hand mixing should occur immediately before measuring the viscosity. With the spindle rotating at 60 rpm, immediately observe and record the scale reading. Convert the scale readings to centipoises by multiplying the readings by the constant for the viscometer spindle and speed used.

Acceptance criteria: NLT 600 centipoises at 24°

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in well-closed containers.

Xanthan Gum Solution

DEFINITION

Prepare Xanthan Gum Solution of the designated percentage strength as follows (see *Pharmaceutical Compounding—Nonsterile Preparations* <795>).

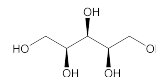
Xanthan Gum (for a 0.1% solution)	100 mg
Xanthan Gum (for 1.0% solution)	1.0 g
Methylparaben	100 mg
Propylparaben	20 mg
Purified Water, a sufficient quantity to make	100 mL

Dissolve a weighed quantity of Propylparaben in Purified Water with heating to 50° and stirring. Cool, and dilute quantitatively, and stepwise if necessary, with Purified Water to obtain 90 mL of solution containing 20 mg of Propylparaben. Heat to 50°, and add the Methylparaben, with stirring, to dissolve. Cool, stir with a blender, slowly sift the Xanthan Gum into the vortex, and continue to blend for 2 min after the Xanthan Gum has been added. Add 10 mL of Purified Water, and blend for 5 min. Allow to stand for 1 h for excess foam to subside, and remove most of the remaining foam by passing the solution through a strainer. Add Purified Water, if necessary, to make the final volume 100 mL, and stir. [NOTE—Depending on the volume needed and the equipment available, adjust the formula proportionately.]

ADDITIONAL REQUIREMENTS

- **PACKAGING AND STORAGE:** Preserve in tight, light-resistant containers, and store at controlled room temperature.
- **LABELING:** Label it to state, as part of the official title, the percentage content of Xanthan Gum.
- **BEYOND-USE DATE:** Six weeks after the day on which it was compounded

Xylitol



C₅H₁₂O₅
Xylitol.

152.15

DEFINITION

Xylitol contains NLT 98.5% and NMT 101.0% of C₅H₁₂O₅, calculated on the anhydrous basis.

IDENTIFICATION

- **A. INFRARED ABSORPTION** <197K>

Sample: Undried

- **B.** The retention time of the xylitol peak of the Sample solution corresponds to that of the Standard solution, as obtained in the Assay.

ASSAY

- **PROCEDURE**

Mobile phase: Acetonitrile and water (20:80)

System suitability solution: 2.5 mg/mL of USP Galactitol RS and 25 mg/mL of USP Xylitol RS in Mobile phase

Standard solution: 25 mg/mL of USP Xylitol RS in Mobile phase

Sample solution: 25 mg/mL of Xylitol in Mobile phase

Chromatographic system

(See *Chromatography* <621>, System Suitability.)

Mode: LC

Detector: UV 192 nm

Column: 8.0-mm × 30-cm; 7-μm packing L34

Column temperature: 80°

Flow rate: 0.5 mL/min

Injection size: 25 μL

System suitability

Sample: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for xylitol and galactitol are about 1.0 and 1.10, respectively.]

Suitability requirements

Resolution: NLT 2.0 between galactitol and xylitol, *System suitability solution*

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of xylitol (C₅H₁₂O₅) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of xylitol from the *Sample solution*

r_S = peak response of xylitol from the *Standard solution*

C_S = concentration of USP Xylitol RS in the *Standard solution* (mg/mL)

C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: 98.5%–101.0% on the anhydrous basis

IMPURITIES

• **RESIDUE ON IGNITION** (281): NMT 0.5%

• **HEAVY METALS** (231): NMT 10 ppm, using 2 g of Xylitol dissolved in 25 mL of water

• REDUCING SUGARS

Sample: 500 mg

Analysis: Dissolve the *Sample* in 2.0 mL of water in a 10-mL conical flask. Into a similar flask, pipet 2 mL of a 0.5 mg/mL dextrose solution. To each flask add 1 mL of alkaline cupric tartrate TS, heat to boiling, and cool.

Acceptance criteria: Any turbidity in the xylitol flask is NMT that in the dextrose flask, in which a reddish-brown precipitate forms (0.2% reducing sugars, as dextrose).

• LIMIT OF OTHER POLYOLS

Mobile phase: Acetonitrile and water (20:80)

System suitability solution: 0.5 mg/mL each of USP L-Arabinitol RS, USP Galactitol RS, USP Mannitol RS, and USP Sorbitol RS, and 100 mg/mL of USP Xylitol RS in *Mobile phase*

Standard solution: 0.5 mg/mL each of USP L-Arabinitol RS, USP Galactitol RS, USP Mannitol RS, and USP Sorbitol RS in *Mobile phase*

Sample solution: 100 mg/mL of Xylitol in *Mobile phase*

Chromatographic system

(See *Chromatography* (621), *System Suitability*.)

Mode: LC

Detector: UV 192 nm

Column: 8.0-mm × 30-cm; 7-μm packing L34

Column temperature: 80°

Flow rate: 0.5 mL/min

Injection size: 25 μL

System suitability

Samples: *System suitability solution* and *Standard solution*

[NOTE—The relative retention times for L-arabinitol, mannitol, xylitol, galactitol, and sorbitol are about 0.76, 0.81, 1.0, 1.12, and 1.22, respectively.]

Suitability requirements

Resolution: NLT 1.5 between all adjacent polyol peaks, *System suitability solution*

Relative standard deviation: NMT 5.0% for the galactitol peak, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*
Calculate the percentage of each polyol (L-arabinitol, galactitol, mannitol, or sorbitol) in the portion of sample taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

r_U = peak response of the individual polyol from the *Sample solution*

r_S = peak response of the individual polyol from the *Standard solution*

C_S = concentration of the individual polyol in the *Standard solution* (mg/mL)

C_U = concentration of the *Sample solution* (mg/mL)

Acceptance criteria: The sum of the polyols is NMT 2.0%, calculated on the anhydrous basis.

SPECIFIC TESTS

• **WATER DETERMINATION**, *Method I* (921): NMT 0.5%

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers.

• **USP REFERENCE STANDARDS** (11)

USP L-Arabinitol RS

USP Galactitol RS

USP Mannitol RS

USP Sorbitol RS

USP Xylitol RS

Xylose—see *Xylose General Monographs*

Zein

[9010-66-6].

DEFINITION

Zein is a prolamine derived from corn [*Zea mays* Linné (Fam. Gramineae)].

IDENTIFICATION

• A.

Sample solution: Dissolve 0.1 g in 10 mL of 0.1 N sodium hydroxide.

Analysis: To the *Sample solution* add a few drops of cupric sulfate TS. Warm in a water bath.

Acceptance criteria: A purple color develops.

• B.

Sample solution: In a test tube add 1 mL of nitric acid to 25 mg of Zein.

Analysis: Agitate the *Sample solution* vigorously.

Acceptance criteria: The solution becomes light yellow. Further addition of about 10 mL of 6 N ammonium hydroxide produces an orange color.

• C. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Solvent: 55% isopropyl alcohol with 2% beta mercaptoethanol

Sample loading buffer:¹ 0.5 M Tris hydrochloride pH 6.8, 20% glycerin, 4% sodium dodecyl sulfate (SDS), and 0.005% bromophenol blue

Gel running buffer stock solution:² 0.25 M Tris base pH 8.6, 1.92 M glycine, and 1.0% SDS

Gel running buffer: *Gel running buffer stock solution* and water (1:9)

¹ Available from Invitrogen as Tris-Glycine SDS Sample Buffer (2X), catalog number LC2676.

² Available from Invitrogen as Tris-Glycine SDS Running Buffer (10X), catalog number LC2675.

Gel staining solution: A suitable Coomassie blue-based solution³

Molecular weight marker: Use a suitable molecular weight marker⁴ containing protein bands at 10–190 kDa. [NOTE—A molecular weight marker with protein bands at 10–100 kDa can also be used.]

Molecular weight standard solution: Dilute the *Molecular weight marker* (1:1) with the *Sample loading buffer*. Incubate the mixture in a closed microcentrifuge tube for 10 min at 95°. After incubation, allow the tube to flash-cool on ice. Place the tube in a microcentrifuge, spin at a top speed for a few seconds, and allow to stop on its own to collect any condensation on the sides and top of the tube.

Sample stock solution: 10 mg/mL of Zein in *Solvent*. Mix on a vortex mixer until the sample is fully dissolved. Centrifuge at 10,000–12,000 rpm in a microcentrifuge with a fixed rotor for 10 min to pellet any undissolved material.

Sample solution: Dilute the *Sample stock solution* (1:1) with the *Sample loading buffer*. Incubate the mixture in a closed microcentrifuge tube for 10 min at 95°. After incubation, allow the tube to flash-cool on ice. Place the tube in a microcentrifuge, spin at a top speed for a few seconds, and allow to stop on its own to collect any condensation on the sides and top of the tube.

Electrophoretic system

(See *Electrophoresis* (726).)

SDS-PAGE gel and apparatus setup: Following the manufacturer's instructions, assemble and fill a precast 16% Tris-Glycine Gel⁵ in an appropriate electrophoresis module.

Running buffer: *Gel running buffer*

Voltage: 100 V

Run time: 2.5 h or until the upper dye front is at the bottom of the gel. [NOTE—The total run time may need to be altered, depending on the molecular weight standards as well on as laboratory equipment variability, because the dye front may co-migrate with or close to the lowest bands of the set.]

Analysis

Samples: *Molecular weight standard solution* and *Sample solution*

Gel loading: Load 25 µL of the *Molecular weight standard solution*. Load a volume of the *Sample solution*, equal to approximately 25 µg of calculated Zein. [NOTE—The actual amount of Zein extracted from the starting material cannot be quantified. Therefore, an estimated amount is derived.]

Gel staining: After electrophoresis, carefully remove the gel from the plates. Rinse the gel three times with water. Stain the gel by following the manufacturer's directions for the stain used.

Destaining: After staining as directed by the manufacturer, destain as directed by the manufacturer.

Gel scan procedure: Set up a gel scanner according to the manufacturer's instructions. Place the gel in the detector, and obtain a single image of all loaded lines of the gel.

Acceptance criteria: Zein has two major bands: the α band is at 21–25 kDa, and the β band is at 17–18 kDa.

³ Available from Invitrogen as SimplyBlue Stain, catalog number LC6065.

⁴ Available from Invitrogen as BenchMark Prestained Protein Ladder, catalog number 1074810.

⁵ Available from Invitrogen, catalog number EC6495. However, these are readily available from several other manufacturers.

IMPURITIES

Inorganic Impurities

• **RESIDUE ON IGNITION** (281): NMT 2.0%, using an ignition temperature of $800 \pm 25^\circ$

• **HEAVY METALS, Method II** (231): NMT 20 ppm

Organic Impurities

• LIMIT OF HEXANE-SOLUBLE MATTER

Sample: 15 g of Zein

Solvent: Alcohol and water (17:3, w/w)

Analysis: Dissolve the *Sample* in 150 mL of *Solvent*. Stir the mixture, using a magnetic stirrer, and heat the solution to 30°. Once the *Sample* is dissolved, transfer the solution to a 500-mL separatory funnel.

Add 60 mL of *n*-hexane. Shake the mixture, and allow the phases to separate. Discharge the bottom layer (alcohol) to a beaker, and transfer the top layer (hexane) to a first 500-mL flask. Weigh the first 500-mL flask, and record the weight. Pour the bottom layer of alcohol back into the separatory funnel. Repeat this step four more times.

After the five 60-mL hexane solutions have been added to the first 500-mL flask, attach it to a rotary evaporator to distill the hexane. Collect the hexane in a second 500-mL flask.

The first 500-mL flask contains a yellow to reddish oil. Record the weight of the flask containing this oil.

Calculate the percentage of hexane-soluble matter in the portion of Zein taken:

$$\text{Result} = [(W_T - W_F)/W] \times 100$$

W_T = weight of the flask (g)

W_F = weight of the first 500-mL flask (g)

W = weight of the *Sample* (g)

Acceptance criteria

For Zein from normal dent corn: NMT 12.5% for hexane-soluble matter

For Zein from waxy corn: NMT 16.0% for hexane-soluble matter

SPECIFIC TESTS

• **LOSS ON DRYING** (731): Dry a sample at 105° for 2 h: it loses NMT 8.0% of its weight.

• **MICROBIAL ENUMERATION TESTS** (61) and **TESTS FOR SPECIFIED MICROORGANISMS** (62): The total bacterial count does not exceed 10^3 cfu/g, and the tests for *Salmonella* species and *Escherichia coli* are negative.

• PROTEIN CONTENT

Analysis: Proceed as directed in *Nitrogen Determination, Method I* (461). Calculate the weight percentage of the protein content in Zein by multiplying the percentage of nitrogen found by 6.25.

Acceptance criteria: 81.9%–100.0% on the dried basis

ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Preserve in well-closed containers, and store at room temperature.

• **LABELING:** Label it to indicate the corn source from which it is derived.

Zinc Stearate—see *Zinc Stearate General Monographs*

Combined Index to USP 36 and NF 31, Volumes 1–3

Page citations refer to the pages of Volumes 1, 2, and 3 of USP 36–NF 31. This index is repeated in its entirety in each volume.

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